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Research Paper

Expression of cyclooxygenase-2 and its pathogenic effects in nonalcoholic fatty liver disease

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Abstract

Objective: To investigate the expression of cyclooxygenase-2 and its pathological effect in the experimental nonalcoholic fatty liver of rats, and to explore its possible mechanism. **Methods:**The rat NAFLD model was established by giving a fat-enriched diet. The blood samples were obtained form abdominal aorta and the levels of serum ALT, AST and IL-1, changes in the hepatic tissue 6-k-PGF1 α TXB2 were measured. The expression level of COX-2 in rats livers were assayed by immunohistochemistry, RT-PCR and Western-blot. **Results:** Light microscope analysis revealed that hepatocytes were injured in the model group and slightly in the treatment group. The levels of serum TXB2 and IL-1 in the fatty liver rats were increased. Compared with the model group, the IL-1 and TXB2 increased significantly($P < 0.05$), on the contrary, compared with the normal group, the hepatic tissue 6-Keto-prostagland decreased significantly in the model group($P < 0.05$), the treatment group also increased but $P > 0.05$. There was no positive expression of COX-2 in hepatic tissue of normal rats. In the model group, there was positive expression of COX-2 antigen and the number of COX positive cells progressively increased at 4, 8, 12 wks. The intensity of expression of COX-2 had significantly increased($P < 0.05$) and the intensity of COX-2 expression in the treated group decreased remarkably compared with the model group($P < 0.05$). The expression of COX-2 mRNA and the level of COX-2 protein were significantly stronger in the liver of model rats compared with normal rats, and significantly weaker in treated rats, than in 8W and 12W model rats($P < 0.05$). **Conclusion:**The increase of COX-2 expression in NAFLD is closely associated with the severity of liver inflammation and damage. COX-2 may play an important role in the progression of rat NAFLD, and the expression of COX-2 mRNA is downregulated by cyclooxygenase-2 inhibitor, which can depress the oxidative stress and control inflammatory response efficiently.

Key words: fatty liver; non-alcoholic; cyclooxygenase-2; interleukin-1; 6-Keto-prostaglandin F1 alpha; thromboxane B2; animal disease model

INTRODUCTION

COX-2 is an induced enzyme, which participates in several pathological processes including inflammatory reaction. It has been identified in previous studies, that over expression of COX-2 is associated with many inflammatory diseases, but the mechanism of COX-2 in Nonalcoholic fatty liver disease(NAFLD) is far from clear. Celecoxib(a specific inhibitor of COX-2) does not interfere with the normal physiological process coherent with COX-1 in tissue, as its selective suppressive intensity to COX-2 is 375 times than that of COX-1[1,2]. The effect of COX-2 in NAFLD and its pathogenic mechanism was studied in this experiment by establishing a rat model of NAFLD and applying the model with Celecoxib.

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MATERIALS AND METHODS

Materials

Forty Male SD rats, weighing 180 to 200 g(Zhongxin company); commercially available pork fat, Orotic Acid and yolk flour; common rat stoyer(Zhongxin company); Trizol kit(Invitrogen company); MMLV first strand cDNA synthesis kit(Invitrogen company); TaqDNA polymerase(Huamei company); RT and fluorescent quantitation PCR kit(Dalian biocompany); Internal

control β-actin(BS0061R, Boaosen company); Celecoxib(Searle company), rabbit anti-rat COX-2 polyclonal antibody(concentration 1:100, Santa company) ; goat anti-rabbit polymer; SABC kit and COX-2 immunohistochemistry kit(Boster Biotechnology); Rat 6-Keto-prostaglandin F1 alpha(6-k-PGF1α) and thromboxane B2(TXB2) radio-immunity kit(Zhongxin Biotechnology); Rat IL-1 ELISA kit (Zhuokang Biotechnology); GD-800 gel image analytical system (UVP company, UK); multifunction microboard test system(BMG company, Germany); TC-150 gene PCR amplifier (MJ Research, USA).

Grouping and modeling of animals

40 Male 8 to 10 week old Sprague-Dawley(SD) rats were bred normally for a week. The animals were randomly divided into two groups: control group(n = 8) NAFLD group(n = 32) and prepared to Peihong Qi[5]. The NAFLD group was randomly divided into three groups: 4w group(n = 8), 8 w group(n = 8) and 12 w group(n = 16). From 12w group, 8 rats were picked out randomly and classified as the Celecoxib treated group. All animals received common food and free water. The NAFLD group was fed with 1 g fat emulsion, containing 5% orotic acid, 10% cholesterol, 20% lard and 1% yolk powder(reaching 1 ml/100 g/time), the Celecoxib treated group was treated with Celecoxib 10 mg/(kg • d) intragastrically 14 days before being sacrificed.

Specimen preparation

The animals were anesthetized with 3% Pentobar-bital and the serum samples were collected and stored in at −70℃, to quantitate the AST, ALT and IL-1. Animals were sacrificed at the end of 4w,8w,12w and collected to calculate the liver index(liver humid weight/body weight). Homogenates of the right liver lobe(100 g) were extracted and vortexed for 2 min and centrifuged at 2 500 r/min for 10 min. The supernatants were stored at −70℃ for the detection of 6-k-PGF1α and TXB2. Liver tissue previously fixed by immersion in formaldehyde saline(10%) solution and embedded by paraffin were performed on 5μm and submitted to hematoxylin-eosin (HE) and immunohistochemistry treatment and then to liver morphology analysis under light microscope.

Detection of ALT, AST, IL-1, 6-k-PGF1 α and TXB2

Alanine aminotransferase(ALT) and aspartate aminotransferase(AST) was detected by GF-D720 biochemistry analyzer, and the activity of IL-1 was detected by ELISA according to the kit procedure, and 6-k-PGF1α、TXB2 was detected by FJ-2008PSγounter.

Immunohistochemical assay for COX-2 expression

For immunohistochemical staining, the paraffin-embedded block was sliced and then deparaffinized. The endogenous peroxidase was blocked by 30 minutes of incubation in 0.3% hydrogen peroxide: methanol, followed by heat treatment for 5 minutes in a microwave oven in a citrate buffer solution to retrieve the antigens. After washing with phosphate-buffered saline(PBS), the tissue sections were pre-blocked for 10 minutes with goat serum, after which rabbit anti-rat COX-2 polyclonal antibody was added overnight at 4℃. After washing with PBS, the sections were incubated with goat anti-rabbit polymer, conjugated with biotin for 30 minutes at room temperature, followed by incubation with streptavidin peroxidase complex for 30 minutes. 3,3-diaminobendizine(DAB) coloration, The slides were counterstained for nuclei using hematoxylin and then dehydration, transparence and mounting. PBS took the place of rabbit anti-rat COX-2 polyclonal antibody as a negative control.

COX-2 mRNA expression

RT-PCR was used to detect the expression of COX-2 in NAFLD. RNA was isolated by using the Trizol reagent. The concentration and purity were detected by ultraviolet spectrophotometer. The RNA was reverse transcribed into cDNA by reverse transcriptase MMLV. The cDNA was amplified by PCR with primers corresponding to COX-2 cDNA. COX-2:Sense 5′-ATGGCTGCAGAGTTGAAAGC-3′ , and antisense 5′-CTCTTACAGCTCAGTTGAACG-3′ . The idio-amplified fragment was 430 bp. In the same RT-PCR reaction, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also amplified with a pair of GAPDH primers as an internal control. GAPDH: sense 5′-AGATCCACAACGGAT,antisense 5′-TCCCTCAA-GATTGTCAGCAA-3′ The product was 308 bp. Amplification condition:2 min of pre-denaturation at 94℃, 30 s of denaturation at 94℃, 35s of renaturation at 60 ℃,30 s of amplification at 72℃. After 30 cycles, a final step of 72℃ for 8 min was implemented. The PCR products were separated on 1.5% agarose gel and 0.5 ×TBE. The banded areas were scanned on UV transilluminator.

COX-2 protein expression in liver tissue

Western Blot was used to detect the expression of COX-2 in liver tissue. The frozen liver tissue was lysed using 1:10 lysis buffer. The protein concentration was measured by Coomassie brilliant blue. A total of 150 μl of protein was loaded on 10% SDS-polyacrylamide gel and separated by electrophoresis. After transfer to nitrocellulose membranes, non-specific sites were blocked with 5% skimmed dried milk for 2 h. Blots were then incubated overnight at 4℃ with rabbit antirat COX-2 polyclonal antibody, and then washed the membrane with TBS. They were then incubated with horseradish peroxidase-conjugated goat anti-rabbit polymer in 5% skimmed dried milk for 2 h, the membrane was washed

with TBS, and visualized by NBT/BCIP kit and the resulting gray scale was analyzed using image analysis.

Statistical analysis

All dada were performed with SPSS12.0 software. Comparisons between all groups were analyzed by one-way ANOVA. Values were expressed as the mean ±SD. The level of statistical significance was set at $P < 0.05$ or $P < 0.01$.

RESULT

Model Evaluation

Three rats died in model group and the biopsy confirmed that gastric mucosa generally eroded and cell infection was found. In the normal group, the liver cells of rats showed array wheel-shaped along the central veins, with clear hepatic lobule structure, well-arranged liver cell cord, normal hepatic sinusoid, with a large, round and central nucleus with no inflammatory cell infiltration and necrosis(***Fig 1***). In the model group(4w, 8w,12w), hepatocytes were larger and swollen with steatosis. Ballooning degeneration, inflammatory infiltration and punctiform necrosis were observed in the lobules and portal area(***Fig 2***), consistent with the diagnostics requirement of NAFLD[4]. In the Celecoxib group, hepatocyte showed mild swelling, ballooning degeneration, while inflammatory infiltration and necrosis were less and the lobule structure was normal (***Fig 3***). The serum AST and ALT incresased significantly($P<0.05$) (***Tab 1***).

The variation of serum IL-1 level

Compared to the control group, the serum IL-1 level increased gradually from the 4w to 12w group. The difference between the model group(at 8w, 12w) and control group was significant($P<0.05$). In the Celecoxib treated group, the serum IL-1 level significantly decreased compared with model group(12w)($P<0.05$)(***Tab 1***).

The 6-k-PGF1α and TXB2 level of liver homogenates

Compared with control group, the 6-k-PGF1α level of model group, decreased significantly($P<0.05$), but in the Celecoxib-treated group, there was no significant difference. Compared with control group, the serum TXB2 level increased significantly in all model groups ($P<0.05$), and compared with model group(8w,12w) the serum TXB2 level in treatment group decreased significantly($P<0.05$)(***Tab 1***).

COX-2 expression in liver tissue

Brown particles of COX-2 polypeptide were stained in the kytoplasm and cell membrane, there was nearly no expression in normal liver cells and low expression in the Disse interspace, and matrix of the portal area and mesenchymocyte(***Fig 4***). In the model group, COX-2 was positively expressed in the liver tissue and COX-2 the distribution of positive particles was widespread in the cytoplasm (mostly observed in hepatocytes around the central vein and hypertrophic Kupffer's cell in portal area). COX-2 expression was significantly increased along with the progression of disease($P=0.028$)(***Fig 5***). After treatment with Celecoxib, the expression of COX-2 decreased significantly compared with model group (***Fig 6***). The gray scale value is shown in ***Tab 2***.

COX-2 mRNA expression in liver tissue

COX-2mRNA expression in liver tissue, integral optical density value and gray-scale value in each group are shown in ***Fig 7*** and ***Tab 2***. COX-2 mRNA expression was observed in liver tissue in the treatment group and model group but was absent in the control group. The result: by calculating the relative expression value of COX-2mRNA PCR products, the expression in model group(4w and 8w) were found similar and higher than that of normal group($P<0.05$). After treatment with Celecoxib, the expression decreased obviously with a significant correlation between the two groups($P<0.05$). In the NAFLD group, the grayscale value of COX-2mRNA/GAPDH increased with the prolongation of time(the F value was 81.9; $P<0.01$) by comparing the three time point of 4w, 8w and 12w, which indicated COX-2 expression increased gradually(***Tab 2***). There was a significant correlation between COX-2 expression and liver inflammation of NAFLD.

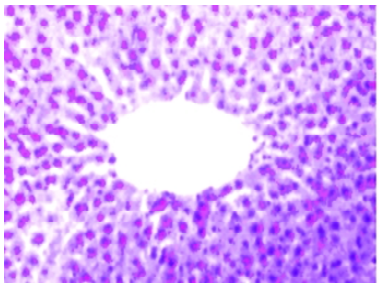
The relative expression value of COX-2 protein

The expression of COX-2 protein was low in the control

Tab 1 **The variation of ALT, AST, IL-1, 6-k-PGF1α TXB2 and the expression in each group** ($n=8, \bar{x} \pm s$)

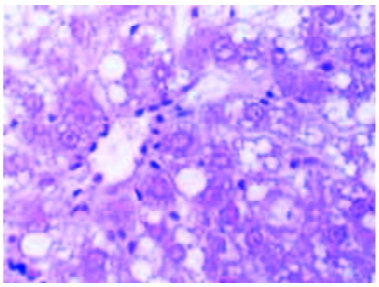
group		ALT(zB/U • L^{-1})	AST(zB/U • L^{-1})	IL-1(pg • ml^{-1})	6-k-PGF1α (nmol • l^{-1})	TXB2(nmol • l^{-1})
Normal control		165.70 ± 35.68	56.200 ± 11.38	87.42 ± 16.77	0.115 ± 0.012	0.047 ± 0.011
Model	4 W	361.75 ± 92.57[a]	192.750 ± 38.75[a]	132.92 ± 22.16[a]	0.092 ± 0.09	0.072 ± 0.019[a]
	8W	569.10 ± 79.34[ab]	305.670 ± 67.03[ab]	218.45 ± 8.56[ab]	0.081 ± 0.006[a]	0.107 ± 0.015[a]
	12W	642.33 ± 104.28[ab]	555.290 ± 92.74[ab]	276.20 ± 20.11[ab]	0.052 ± 0.021[ab]	0.192 ± 0.022[ab]
Celecoxib		334.01 ± 48.49[c]	202.515 ± 40.93[c]	117.29 ± 14.38[c]	0.042 ± 0.004	0.083 ± 0.009[c]

[a]$P<0.05$(vs control group); [b]$P<0.05$(vs model 4w group); [c]$P<0.05$(vs model 8w/12w group)



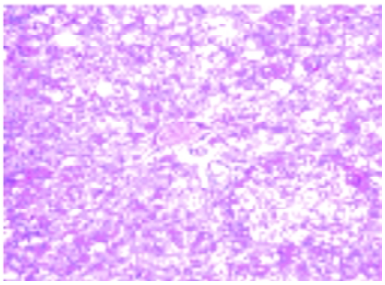
(HE,× 100)

Fig 1 normal rat group, liver sections showed normal liver histology



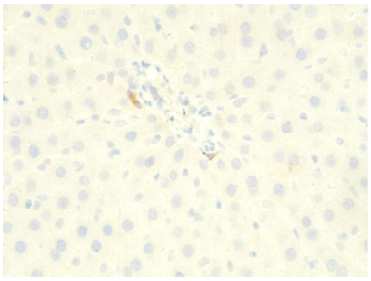
(HE,× 100)

Fig 2 model rat(12w)group showed steatosis, ballooning degeneration, punctiform necrosis, and inflammatory infiltration in lobules and portal area



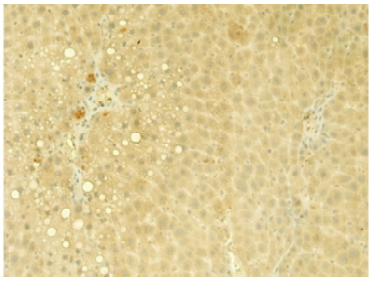
(HE,× 100)

Fig 3 Celecoxib group, showed mild swelling and ballooning degeneration, but less inflammatory infiltration, necrosis, and normal lobule structure.



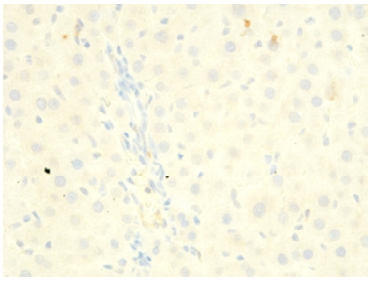
(SABC,× 100)

Fig 4 normal group; nearly no expression in normal liver cells, low expression in Disse interspace, matrix of portal area and mesenchymocyte



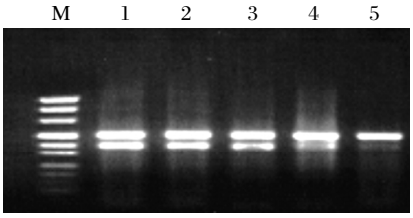
(SABC,× 100)

Fig 5 model group (12w); COX-2 positive particles widespread distribution in the cytoplasm and more in hepatocytes around the central vein and in hypertrophic Kupffer's cell in portal area



(SABC,× 100)

Fig 6 CelceCOXib group; COX-2 positive particle distributed around central vein and portal area



M:marker: 1116 bp、883 bp, 692 bp, 501 bp, 404 bp, 331 bp, 242 bp, 190 bp, 147bp; 1: 12w group; 2: 8w group; 3:4w group; 4:Celecoxib group; 5: control group.

Fig 7 COX-2 mRNA expression in liver tissue

group and high in the model group(8w, 12w) with a significant difference($P < 0.05$). The expression of COX-2 protein decreased obviously after treatment with Celecoxib, comparing with 12w group($P < 0.05$)(***Fig 8***). The COX-2 relative protein expression by BandScan software analysis was as follows(***Tab 2***):

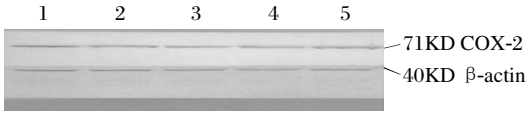


Fig 8 The relative expression value of COX-2 protein in liver tissue:1,12w group;2,8w group;3,4w group; 4,Celecoxib group; 5, control group

Tab 2 **Integral optical density value and grayscale value of COX-2 and relative protein expression in liver tissue**

($n=8$, $\bar{x} \pm s$)

Group	COX-2 mRNA iOD	COX-2mRNA/GAPDH grayscale value	COX-2 relative protein expression	COX-2 mean grayscale value
Normal control	0	0	0	0
Model 4W	0.391 ± 0.085^{a}	0.46 ± 0.03^{a}	0.495 ± 0.108^{a}	80.17 ± 10.22^{a}
8w	0.481 ± 0.353^{ab}	0.67 ± 0.02^{ab}	0.647 ± 0.224^{ab}	99.23 ± 11.78^{ab}
12w	0.612 ± 0.276^{ab}	0.85 ± 0.08^{ab}	0.877 ± 0.075^{ab}	110.52 ± 13.44^{ab}
Celecoxib	0.401 ± 0.054^{c}	0.53 ± 0.03^{c}	0.539 ± 0.187^{c}	82.93 ± 7.63^{c}

vs. control group, $^{a}P < 0.05$; *vs.* model group(4w), $^{b}P < 0.05$; *vs.* model group(12w), $^{c}P < 0.05$.

The correlation analysis between COX-2 and IL-1, 6-k-PGF1α TXB2, 6-k-PGF1α TXB2 by bivariate pearson

There was a positive correlation between the activation of COX-2 and IL-1 TXB2, with a significant difference, and negative correlation with 6-k-PGF1α and 6-k-PGF1α/TXB2(***Tab 3***).

Tab 2 **The correlation analysis of COX-2 and IL-1, PGF1α, TXB2, PGF1α/TXB2 by bivariate pearson**

	r	*p*
IL-1	0.763	0.042
6-k-PGF1α	−0.301	0.234
TXB2	0.821	0.033
6-k-PGF1α/TXB2	−0.432	0.026

DISCUSSION

NAFLD is currently defined as a clinicopathologicl syndrome with fat accumulation and hepatocyte fatty steatosis in the liver, and is a new challenge in the field of liver disease. With the progression of the disease, it includes fatty liver non-alcoholic steatohepatitis (NASH), hepatic fibrosis, hepatic cirrhosis often final stage liver disease[5]. NASH is the key point of poor prognosis in the whole process. Though there is not wide agreement on the most general histological standard of the diagnosis of NASH, it is generally considered that the most frequent injury of NASH is steatosis, mixed scattered polymorphonuclear leukocytes as well as mononuclear cells, hepatocellular ballooning and spotty necrosis[6,7]. We have succeeded in establishing the NAFLD rat model, in which, all the samples included above injuries and met the feature that cell degeneration was typically in zone 3 (around the central vein)[6].

6-k-PGF1α and TXB2 are the metabolites of prostacyclin I2(PGI2) and thromboxane A2(TXA2) produced by arachidonic acid. PGI2 has a function to prevent vessel shrinkage and platelet aggregation. While TXA2 can enhance vessel shrinkage, platelet aggregation and thromboxanes formation, in physical state, they keep balance in vivo and maintain the stabilization of the internal environment[8,9]. In some disease states, on the other hand, this balance can be disturbed. It is helpful for us to understand the mechanism and severity of diseases by calculating their content. Because their half-life is very short, it is very difficult for us to detect them directly. At present, their degradative products, 6-k-PGF1α and TXB2, are tested to reflect their content[10]. Our experiment showed obvious liver injury decreased 6-k-PGF1α level and increased TXB2 level in the model group(8w, 12w). After treatment with Celecoxib, liver injury lessened, 6-k-PGF1α level increased and TXB2 level decreased. The results confirmed that enhanced expression of COX-2 in liver tissue can promote the liver injury of NAFLD by enhancing the synthesis of TXA2 and decreasing PGI2/TXA2 value. Therefore using selective COX-2 inhibitor can protect liver tissue from inflammatory injury via decreasing the synthesis of TXA2.

IL-1 is one of the most common inflammation stimulating factors. In some cell lines, ectogenic IL-1 induces a sustained increase in the expression of the COX-2 mRNA and protein expression and the synthesis of their product, prostaglandin. It was found that this was because it can enhance the stability of COX-2 mRNA[11]. The effect of cytokines(such as IL-1 and LPS) can active the COX-2 translation, mediate leucocyte aggregation, adhesion and infiltration, induce inflammation, capillary endothelium injury, cause increase of microcirculation permeability, tissue hematoma and microcirculation disturbance[10]. Our study showed the IL-1 level in model group was higher obviously than control group and the activation of IL-1 in liver tissue was enhanced with the progression of NAFLD, which is accordance with above theory. In addition, the IL-1 level of liver tissue in treatment group was lower than model group, the underlying mechanism may be that Celecoxib inhibits the activation of COX-2, decreases active oxygen generation in the process of prostaglandin metabolism.

COX-2 is a key enzyme in starting inflammatory reaction, exerts induced expression in vivo, no or mild expression in normal tissue, but in the stimulus of many inflammatory factors the expression can be enhanced and participates in some pathological process, such as inflammatory reaction tumorigenesis[12-14]. In alcoholic liver disease(ALD) COX-2 and its synthesis product participate in hepatic inflammatory fatty change, fibrosis and so on via interfering with the metabolism of fatty saccharide and protein, aggravating hepatic oxidative stress and facilitating the release of some cytokines[15]. Our experiment showed COX-2 expression enhanced hepatic injury, enhanced serum ASL, ALT level in the pristine NAFLD and indicated pristine hepatic chemical inflammatory change can stimulate COX-2 expression. In the treatment group COX-2 expression decreased, so from this we concluded the underlying mechanism may be related to Celecoxib which caused lessened oxidative stress and active oxygen product, therefore influencing COX-2 activity and expression. Our study also found hepatic histomorphology(fatty degeneration in the model and treatment group) which may also be related to its product prostaglandin, and that combined to its receptor caused hepatic lipometabolism[16-20]. In NAFLD, whether the enhanced COX-2 expression is the reason or the result of hepatic injury needs further study.

Taken together, COX-2 expression participates in the genesis and development of NAFLD, using specific COX-2 inhibitor may lessen hepatic injury. We suggest that the hepatocyte toxicity of lipid peroxidation lipopolysaccharide mediated by COX-2 is related to the abnormal hepatic lipometabolism induced by prostaglandin combining to its receptor. Whether selective COX-2 inhibitor can be used to improve hepatic pathological change and reduce the progression of hepatic injury at this time requires further study.

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