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Regulation of α_1-adrenoceptor on rat hepatocyte apoptosis induced by *D*-galactosamine and lipopolysaccharide[1]

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KEY WORDS adrenergic receptors; apoptosis; lipopolysaccharides; calcium; phospholipases A; tumor necrosis factor; prazosin

ABSTRACT

AIM: To study the regulation of α_1-adrenoceptor on the biochemical changes involved in hepatocyte apoptosis induced by *D*-galactosamine (*D*-GalN) and lipopolysaccharide (LPS). **METHODS**: Prazosin (Pra) 4 $mg \cdot kg^{-1}$ ig was administrated 1 h before ip D-GalN 600 $mg \cdot kg^{-1}$ and LPS 1 $\mu g \cdot kg^{-1}$. The morphological changes of hepatocytes were observed. The concentrations of intracellular free ion calcium ($[Ca^{2+}]_i$), the expression of secretory phospholipase A_2 ($sPLA_2$) and cytosolic phospholipase A_2 ($cPLA_2$) in hepatocytes, and the levels of alanine aminotransferase (ALT), $sPLA_2$, and tumor necrosis factor-α (TNF-α) in serum were all assayed. **RESULTS**: The amounts of apoptotic bodies and apoptotic hepatocytes were reduced by the treatment of Pra ($P < 0.01$). The levels of $[Ca^{2+}]_i$, ALT, $sPLA_2$, and TNF-α were (605 ± 95) $nmol \cdot L^{-1}$, (214 ± 141) $u \cdot L^{-1}$, (11.2 ± 1.1) %, and (314 ± 93) $ng \cdot L^{-1}$, respectively. Pra could decrease all the above biochemical changes to (364 ± 61) $nmol \cdot L^{-1}$, (157 ± 99) $u \cdot L^{-1}$, (7.9 ± 1.6) %, and (156 ± 62) $ng \cdot L^{-1}$ (all $P < 0.05$), respectively. The expression of $cPLA_2$ was reduced by Pra treatment, too, but Pra had no significant effect on the expression of $sPLA_2$. **CONCLUSION**: The apoptosis in rat hepatocytes is regulated by α_1-AR signal transduction pathway including changes in $[Ca^{2+}]_i$, $sPLA_2$ secretion, and $cPLA_2$ expression. TNF-α is also involved in rat hepatocyte apoptotic regulation.

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INTRODUCTION

Apoptosis is a type of cell death that is fundamentally different from necrosis in terms of its morphological appearance, biochemical mechanism, and mode of initiation. Hepatocyte apoptosis exists in many experimental conditions and in various liver diseases, such as alcohol hepatitis and viral hepatitis. The bacterial cell wall constituent lipopolysaccharide (LPS) causes hepatocyte damage and inflammatory responses. *D*-galactosamine (*D*-GalN), which is metabolized in rats only in the liver and leads to DNA transcription block in hepatocytes, enhances the sensitization toward LPS-dependent hepatocyte destruction up to 10 000-fold. Various stimulation factors could induce different cells apoptosis involved in different signal transduction pathway, due to the complicated regulation mechanism of apoptosis. Intracellular free Ca^{2+} is thought to play an important role in hepatocyte damage through activation of many intracellular enzymes, such as endonucleases, protein kinases, etc[1]. Hepatocyte is one of non-excitable cells, on which the membrane Ca^{2+} channels are receptor-operated. α_1-Adrenoceptor (α_1-AR) exists on hepatocyte membranes as calcium-mobilizing receptor[1], and our unpublished studies have proved that α_1-AR agonist can increase hepatocyte $[Ca^{2+}]_i$ including the mobilization of intracellular Ca^{2+} stores and the extracellular Ca^{2+} influx. With the rapid development of research in apoptotic mechanisms, PLA_2 has been found to be correlated to apoptosis[2]. In this paper rat hepatocyte apoptosis model was reproduced with *D*-GalN and LPS, and the effects of α_1-AR antagonist prazosin (Pra) were studied on the rat hepatocyte apoptosis.

MATERIALS AND METHODS

Chemicals LPS (E Coli, O127: B8), collagenase type IV, and fatty acid free bovine serum albumin (FAF BSA), were purchased from Sigma Co. Prazocin·

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HCl was from Beijing Pharmacy Institute, Fura-2/AM was from Shanghai Institute of Cell Biology, Chinese Academy of Sciences, *D*-GalN was provided by Department of Chemistry in our University. [^{3}H]-oleic acid was obtained from Amersham Co. The $sPLA_2$ and $cPLA_2$ polyclonal antibodies were generous gifts from Prof. Marshall LA of Smithkline Beecham Co, USA. All solvents and other reagents were of AR grade.

Animals Wistar rats (♂, weighing 187 g ± 16 g) were supplied by Center of Experimental Animals in our University, and were fasted with free access to water 12 h before the experiment.

Experimental groups Rats were divided into 4 groups. Control group: ig NS 2 mL, and ip 1 mL NS 1 h later; Pra control group: ig Pra 4 $mg \cdot kg^{-1}$, and ip 1 mL NS 1 h later; *D*-GalN + LPS (apoptosis model) group: ig 2 mL NS, and ip *D*-GalN 600 $mg \cdot kg^{-1}$ and LPS 1 $\mu g \cdot kg^{-1}$ 1 h later; Pra + *D*-GalN + LPS group: ig Pra 4 $mg \cdot kg^{-1}$ and 1 h later ip *D*-GalN 600 $mg \cdot kg^{-1}$ and LPS 1 $\mu g \cdot kg^{-1}$. The collections of liver for histopathology and serum samples for estimation of ALT, $sPLA_2$ and TNF-α, and the isolation of hepatocytes *in situ* were all performed 5 h after the above treatments.

Histologic examination After removal of the liver, small portions were immediately fixed in 4 % formalin and embedded in paraplast. Sections of 5 μm thickness were stained with hematoxylin and eosin.

Electron microscopy study Immediately after sacrifice, liver sections were fixed in 2.5 % glutaraldehyde in phosphate buffer 0.1 $mol \cdot L^{-1}$, postfixed in 0.2 % osmium tetroxide in the same buffer, dehydrated, and embedded in Epon 812. Ultrathin sections were stained with lead citrate and uranyl acetate. Electron microscope used was from Japan Hitachi-600. The TUNEL assay was performed according to the kit instructions.

$[Ca^{2+}]_i$ assay The hepatocytes were isolated exactly as described before with collagenase perfusion *in situ* and Percoll purification[3]. $[Ca^{2+}]_i$ was measured with influorescent probe Fura-2[4].

$sPLA_2$ activities assay Serum $sPLA_2$ activities were measured with the colibacillus membrane incooperated with [^{3}H]-oleic acid as the substrate[5], and the activity was expressed as:

Hydrolytic rate =

$$(CPM_{serum} - CPM_{non\text{-}enzymes})/CPM_{total} \times 100\ \%$$

Expression study of $cPLA_2$ and $sPLA_2$ Iced-histological sections of liver were immediately made after sacrificing the animals, were dried by adding acetone for 5 min, were rinsed with PBS, and methanol containing 30 % H_2O_2 was added for 5 min. After washing with PBS, $cPLA_2$ antibody and $sPLA_2$ antibody were added for 1 h at 37 ℃, and the sections were stored at 4 ℃ overnight sequentially. On rinsing with PBS, sheep-anti-rabbit-IgG and sheep-anti-rat-IgG (1:100) were added and samples were incubated at 37 ℃ for 45 min, washed with PBS, and stained with DAB.

Data analysis The results were expressed as $\bar{x} \pm s$, and analyzed with chi-square test and sequence test.

RESULTS

Reproduction of hepatocyte apoptosis model in rat Various dosage combinations of *D*-GalN and LPS were tested. Apoptotic model of rat hepatocyte was stably reproduced at the doses of *D*-GalN 600 $mg \cdot kg^{-1}$ and LPS 1 $\mu g \cdot kg^{-1}$, which was indicated by the formation of apoptotic bodies, DNA fragments, and the condensation of nuclei and chromatins (Tab 1, Fig 1,2,3).

Tab 1. The apoptotic bodies observed by H&E staining and hepatocyte apoptosis by TUNEL test. $n = 6$ rats. $\bar{x} \pm s$/25 oil fields. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* Pra. $^iP < 0.01$ *vs* *D*-GalN + LPS.

group	apoptotic bodies	apoptotic hepatocyte
Control	0.5 ± 0.3	0.5 ± 0.5
Pra	1.0 ± 0.8	0.8 ± 0.5
D-GalN + LPS	$73 \pm 7^{c,f}$	$66 \pm 4^{c,f}$
Pra + *D*-GalN + LPS	$20 \pm 6^{c,f,i}$	$16 \pm 6^{c,f,i}$

Effects on $[Ca^{2+}]_i$ When rat hepatocyte apoptosis was induced by *D*-GalN and LPS, the hepatocyte $[Ca^{2+}]_i$ was higher than that of control ($P < 0.05$). Pra treatment alone did not influence $[Ca^{2+}]_i$ of rat hepatocytes, but hepatocyte $[Ca^{2+}]_i$ of apoptotic model was decreased by Pra ($P < 0.05$) (Tab 2).

Changes in serum enzyme activities and TNF-α levels Pra alone did not change the activities of ALT and $sPLA_2$ and the levels of TNF-α in serum, which were increased in apoptotic model ($P < 0.05$) and reduced by the treatment of Pra ($P < 0.05$) (Tab 2).

Expression of $sPLA_2$ and $cPLA_2$ in hepatocytes Immunohistochemical staining revealed that the expression of $sPLA_2$ in hepatocytes had no difference

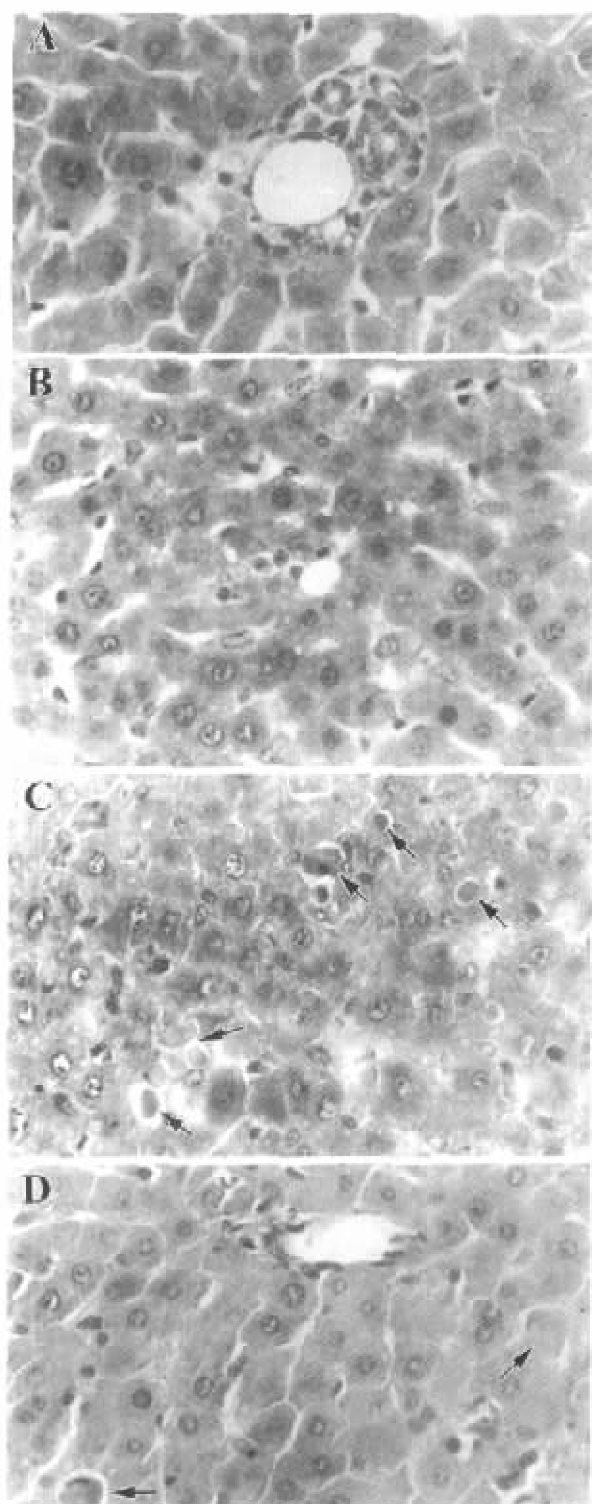

Fig 1. Histopathology of liver damage in rats. A: *control*. *B: Pra only*. *No histological abnormalities* observed. C: *D*-GalN + LPS. Numerous apoptotic bodies and cells with condensed chromatins as signs of hepatocellular apoptosis are shown. D: Pra + *D*-GalN + LPS. Apoptotic bodies decreased prominently. (HE stain, ×400)

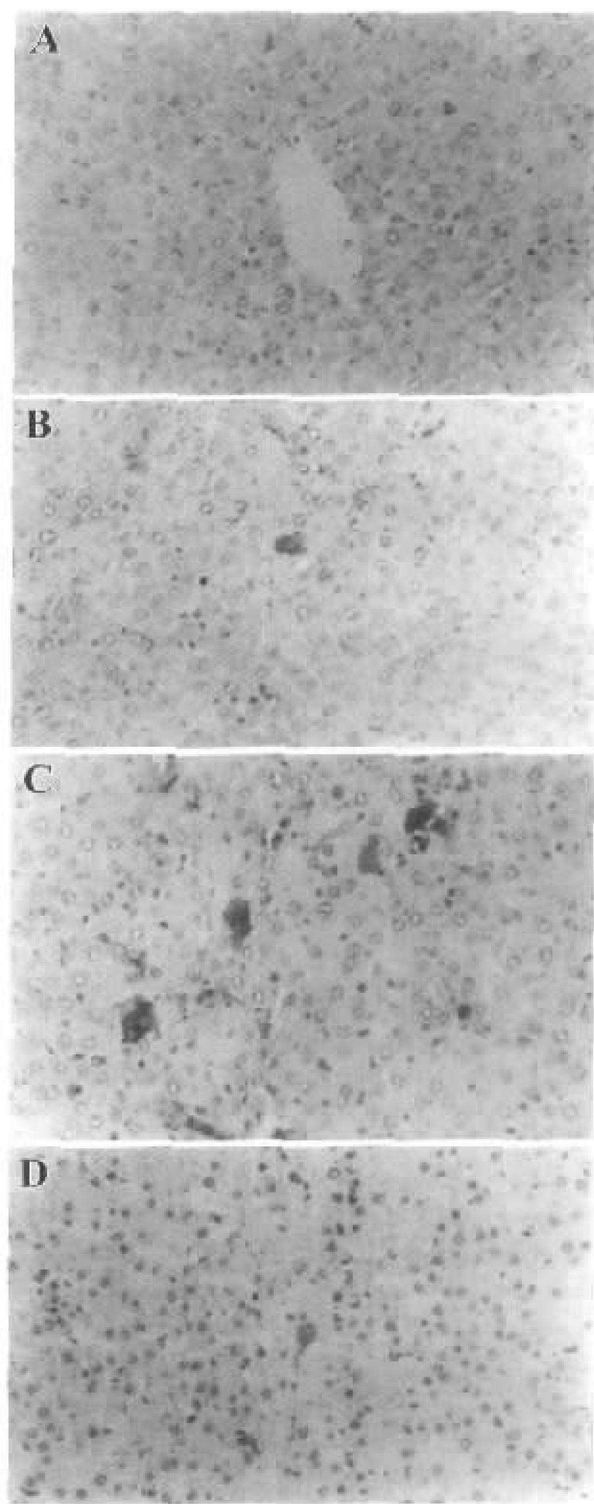

Fig 2. DNA fragmentation detected by the TUNEL *assay of liver in rats*. A: *control*. B: Pra only. No histological abnormalities observed. C: *D*-GalN + LPS. TUNEL-positive hepatocytes are shown. D: Pra + *D*-GalN + LPS. TUNEL-positive hepatocytes decreased prominently. (TUNEL assay, ×400)

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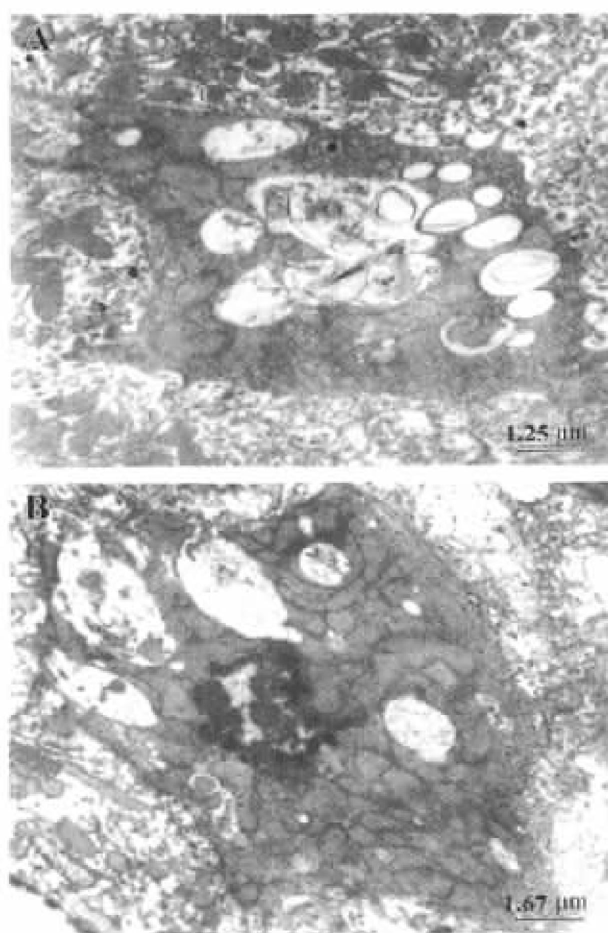

Fig 3. Electron microscopy of rat liver 5 h after treatment with *D*-GalN and LPS. A: apoptotic bodies lying in the extracellular space containing intact mitochondrion. (× 8000) B: Hepatocyte undergoing cytoplasmic condensation coupled with cleavage of nuclei, indicating hepatocyte apoptosis. (× 6000)

between groups of *D*-GalN + LPS and Pra + *D*-GalN + LPS, but compared with control and Pra groups, all of them increased. However, the expression of $cPLA_2$ was markedly induced in hepatocytes of rat treated with *D*-GalN + LPS and inhibited by Pra treatment (Fig 4).

DISCUSSION

Apoptosis can be induced through different signal transduction pathways. In this study, we investigated the effect of α_1-AR on hepatocyte apoptosis. HE staining and TUNEL assay revealed that apoptosis of rat hepatocyte induced by *D*-GalN and LPS can be inhibited by α_1-AR antagonist Pra predominantly, indicating that α_1-AR are involved in the process of hepatocyte apoptosis.

The increase in $[Ca^{2+}]_i$ has been mentioned in many kinds of cell apoptosis[6]. The Ca^{2+} channels operated by α_1-AR, which is an important Ca^{2+}-regulated receptor on the hepatocyte membranes, opened-up. $[Ca^{2+}]_i$ including Ca^{2+} influx and mobilization from Ca^{2+}-store increased in rat hepatocytes[7]. Pra as an α_1-AR antagonist blocked α_1-AR pathways and decreased $[Ca^{2+}]_i$ and apoptotic hepatocytes.

The process of PLA_2 activation is Ca^{2+}-dependent[8]. $sPLA_2$ requires millimole concentration of calcium to exert its activity. In this study, the increased activities of serum $sPLA_2$ by *D*-GalN and LPS were reduced by Pra, but immunohistochemical staining showed that the expression of $sPLA_2$ in hepatocyte was unchanged. The results showed that the increased $sPLA_2$ activities were due to enhanced secretion of $sPLA_2$. $cPLA_2$ plays an important role in lipid-mediated cell signaling, which requires micromole concentration of calcium. Immunohistochemical staining showed that the expression of $cPLA_2$ was increased in apoptotic hepatocytes, and reduced by Pra, accompanying with decrease in $[Ca^{2+}]_i$. These results indicated that one of the regulation mechanism of hepatocyte apoptosis induced by D-GalN and LPS was through α_1-AR signaling pathways and involved changes of intracellular Ca^{2+} levels and $cPLA_2$ expression.

TNF-α alone is sufficient to induce hepatic apoptosis as it is a dominant and terminal mediator, and anti-TNF-IgG can inhibit hepatic apoptosis induced by *D*-GalN and

Tab 2. The changes in ALT, $sPLA_2$, and TNF-α levels in serum and $[Ca^{2+}]_i$ levels in rat hepatocyte. $n = 6$ rats. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control. $^eP < 0.05$, $^fP < 0.01$ *vs* Pra. $^hP < 0.05$ *vs* *D*-GalN + LPS.

group	serum			$[Ca^{2+}]_i$/nmol·L^{-1}
	ALT/u·L^{-1}	$sPLA_2$/%	TNF-α/ng·L^{-1}	
Control	63 ± 28	7.6 ± 1.7	85 ± 24	174 ± 57
Pra	75 ± 18^a	8.8 ± 1.9^a	96 ± 24^a	180 ± 88
D-GalN + LPS	214 ± 141^{cf}	11 ± 1.1^{be}	314 ± 93^{cf}	605 ± 95^{cf}
Pra + *D*-GalN + LPS	157 ± 99^{beh}	7.9 ± 1.6^h	156 ± 62^{beh}	364 ± 61^{beh}

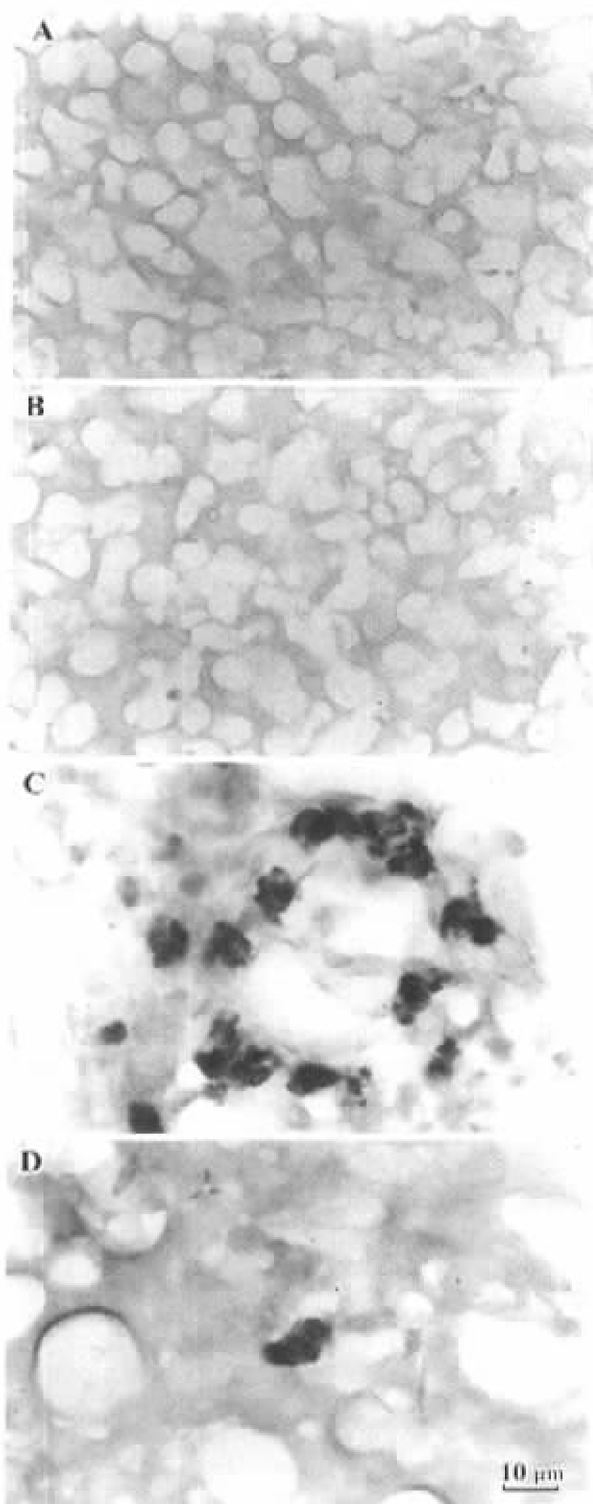

Fig 4. $cPLA_2$ expression in hepatocyte. A: control. B: Pra only. No positive-hepatocytes observed. C: *D*-GalN + LPS. Numerous positive hepatocytes expressing $cPLA_2$ are observed. D: Pra + *D*-GalN + LPS. The positive hepatocytes were observed to decrease. (× 1000)

LPS[9]. In this study, TNF-α was increased in the groups of apoptotic model. TNF-α could enhance apoptosis through elevating the levels of $cPLA_2$ and regulation by noradrenaline through sympathetic nerve system[10,11]. The increased TNF-α was reduced by Pra, which showed that inhibiting the release of TNF-α could reduce hepatocyte apoptosis through α_1-AR signal transduction pathway.

In summary, our results indicate that α_1-AR plays an important role in hepatocyte apoptosis induced by *D*-GalN and LPS, which involve $[Ca^{2+}]_i$, $sPLA_2$, $cPLA_2$, and TNF-α.

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D-GalN 和 LPS 体内诱导大鼠肝细胞凋亡以及 α_1-AR对其调节作用[1]

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关键词 肾上腺素能受体；细胞凋亡；脂多糖类；钙；磷脂酶 A 类；肿瘤坏死因子；哌唑嗪

目的：以 D-GalN 和 LPS 为诱导因子建立大鼠肝细胞凋亡模型，研究此过程中生化机制的改变以及 α_1-AR 的调节作用．**方法**：哌唑嗪(Pra) 4 mg·kg^{-1}灌胃 1 小时后腹腔注射 D-GalN 600 mg·kg^{-1}和 LPS 1 μg·kg^{-1}，5 小时后观察肝细胞形态学变化，检测肝细胞内游离钙离子浓度($[Ca^{2+}]_i$)，$sPLA_2$ 和 $cPLA_2$ 表达，以及血清 ALT，$sPLA_2$和 TNF-α 的水平．**结果**：D-GalN 600 mg·kg^{-1}和 LPS 1 μg·kg^{-1}同时腹腔注射可稳定复制大鼠肝细胞凋亡模型．Pra 可显著降低凋亡小体和凋亡肝细胞数量．注射 D-GalN 和 LPS 5 小时后，$[Ca^{2+}]_i$，血清 ALT 活性，$sPLA_2$以及 TNF-α 水平分别为 (605±95) nmol·L^{-1}，(214±141) u·L^{-1}，(11.2±1.1) %，(314±93) ng·L^{-1}．Pra 可使其分别显著降低至(364±61) nmol·L^{-1}，(157±99) u·L^{-1}，(7.9±1.6) %，(156±62) ng·L^{-1}(均 $P<0.05$)，并同时抑制 $cPLA_2$ 表达．**结论**：通过 α_1-AR 信号传导途径，引起$[Ca^{2+}]_i$、$sPLA_2$ 分泌以及 $cPLA_2$ 表达等改变，调节肝细胞凋亡．TNF-α 也参与调控肝细胞凋亡．

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