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甘草酸对猪呼吸道冠状病毒的抗病毒作用

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摘　要：利用 ST细胞研究了甘草酸对猪呼吸道冠状病毒(PRCV)体外复制过程中的抑制作用。通过 RT-PCR和 Western blot检测发现甘草酸能有效抑制 PRCV的体外复制而对 ST细胞不引起明显细胞毒作用。抑制效果与甘草酸的剂量成正比关系。PRCV病毒核衣壳蛋白的表达无论在 mRNA水平还是在蛋白质水平均随着甘草酸添加量的增加而降低，提示甘草酸是一种有效的抗 PRCV药物。

关键词：甘草酸；核衣壳蛋白；猪呼吸道冠状病毒

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Antiviral Effect of Glycyrrhizin on Porcine Respiratory Coronavirus (PRCV)

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Abstract: The inhibitory effect of glycyrrhizin (GL) on porcine respiratory coronavirus (PRCV) replication was investigated in vitro in ST cells using RT-PCR and Western blot techniques. GL effectively suppressed PRCV replication without causing apparent cell cytotoxicity in ST cells. The inhibitory effect of GL on PRCV was in a dose-dependent manner. The expression levels of both mRNA and protein of the PRCV nucleocapsid protein in ST cells were impeded by the increasing addition of GL, suggesting it is a potential effective antiviral component against PRCV in vitro.

Key words: glycyrrhizin; nucleocapsid protein; porcine respiratory coronavirus (PRCV)

Introduction

Since the antiviral effect of glycyrrhizin (GL) [20β-carboxyl-11-oxo-30-norolean-12-en-3β-yl-2-O-β-D-glucopyranuronosyl-α-D-glucopyranosiduronic acid] was reported[1], many studies have indicated that GL is active against a variety of viruses including human immunodeficiency virus (HIV)[2], hepatitis A virus[3], hepatitis B virus[4], influenza virus A[5], hepatitis C virus[6,7] and even the recently identified severe acute respiratory syndrome (SARS) coronavirus[8]. Though the inhibition mechanism of GL on different types of virus seems to be different and some of them still remain unclear, its effectiveness suggests that GL may also be applicable to animal viruses.

Porcine respiratory coronavirus (PRCV), a member of the family of coronaviruses, belonging to the order of the Nidovirales, is an enveloped virus containing positive-stranded RNA genome. It is a pathogen causing respiratory tract illness in piglets while no much research has been done on this virus. Recently, the expression of PRCV nucleocapsid protein was performed in our laboratory[9]. In the present study, we investigated the antiviral effect of GL on PRCV.

Materials and Methods

Cell, virus and glycyrrhizin

Swine testis (ST) cells were cultured at 37 ℃ in 5% CO_2 in Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 IU/mL of penicillin and 100 μg/mL of streptomycin. Cell viability was determined by trypan blue exclusion. Porcine respiratory coronavirus was isolated from a piglet farm in Fujian Province. The virus was passaged on ST cells for 35 times as virus stock and the titer of the virus was determined as 10^7, 50% tissue culture infective doses ($TCID_{50}$) per mL.

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Glycyrrhizin (GL) was purchased from Tokyo Kasei Kogyo, Japan with the purity of 99.6%. GL was dissolved in phosphate-buffered saline (PBS, pH 7.4) and adjusted to pH 7.2 with 1 N sodium hydroxide before use. The polyclonal antibody of GST-fused PRCV nucleocapsid protein was prepared in chicken in our laboratory using GST-affinity column purified protein as antigen.

Virus titration

The virus titer was determined by CPE analysis. Briefly, virus infected cell supernatants were harvested 72 h post infection. The supernatants were 10-fold serial diluted and added to 90% confluent ST cells in 96-well culture plates and incubated at 37 ℃ for further 96 h. The infection efficacy was checked by cytopathic effect (CPE) and the 50% tissue culture infective doses ($TCID_{50}$) were calculated.

Cytotoxicity assay

Cytotoxicity was assessed by determining cell viability and media lactate dehydrogenase (LDH) activity. Cell viability was assessed using the trypan blue exclusion technique while the cytoplasmic enzyme lactate dehydrogenase (LDH) was assayed using a cytotoxicity detection kit (Promega, USA) according to the manufacturer's instruction.

Reverse transcription PCR (RT-PCR)

Total cellular RNA was extracted from mock infected or PRCV infected cells post infection using an RNA extraction reagent TRIzol (Life technology, USA) according to the manufacturer's instruction. Two μg of RNA was consequently reverse transcribed to cDNA using an AMV reverse transcription RT kit (Roche, Germany) and Oligo (dT)15 primer. A pair of primers corresponding to PRCV N-protein full-length (1149 bp) was used for PCR amplification using the RT product. Forward: 5′ATGGCCAACCAGGGACAACGTG3′; reverse: 5′ TTAGTTCGTTACCTCGTCAATCAT3′ (GenBank accession No. Z24675). The primers for β-actin of ST cells (partial sequence: 372 bp) were forward: 5′GAGCGGGAGATCGTGCGGGACA3′; reverse: 5′ GATCTTGATCTTCATCGTGCTG3′ (GenBank accession No. Z81198). RT-PCR detected the expression of β-actin as an internal reference. The PCR cycling profile was 94 ℃ for 3 min, [94 ℃ for 30 s, 55 ℃ for 45 s, 72 ℃ for 30 s] for 30 cycles, followed by 72 ℃ for 10 min. The PCR products were run on a 1.2% agarose gel. Each band was quantitated using a densitometer (Vilber Lourmat, France) and the ratio of N-protein mRNA to β-actin mRNA was calculated using the Bio-1D software.

Western blot

Mock infected or PRCV infected ST cells in the presence or absence of GL were harvested and washed with PBS twice prior to the addition of SDS-PAGE buffer in the presence of 5% 2-mercaptoethanol and boiling. SDS-PAGE was carried out on a 12% polyacrylamide gel and was consequently transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in PBS containing 0.05% Tween 20 (PBST) for 1 h and then reacted with anti-PRCV nucleocapsid protein polyclonal antibody for 2 h. After washing, the membrane was allowed to react with horseradish peroxidase labeled rabbit anti-chicken immunoglobulin for 1 h. Following extensive washing, the protein was detected by an enhanced chemiluminescent developing kit (Pierce, USA).

Results

Cytotoxicity of GL

To study the antiviral effect of GL on PRCV, we first evaluated the noncytotoxic concentrations of GL for ST cells. Cells were cultured in the presence of various concentrations of GL for 72 h, cell viability and the cytoplasmic enzyme lactate dehydrogenase (LDH) activity was determined. As LDH is a stable cytoplasmic enzyme present in all cells and is released into the cell culture supernatant once the plasma membrane was damaged. We adopted this convenient and reliable method to analyze cytotoxicity. The result showed that there was no much difference in cell viability between untreated cells and cells treated with GL at the concentration as high as 3 000 μg/mL for 72 h. Only a slight decrease in cell viability was identified when GL concentration reached 4 000 μg/mL (Fig. 1).

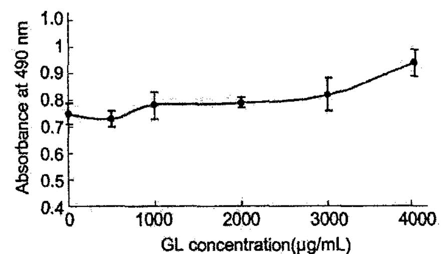

Fig. 1 Cytotoxicity analysis

ST cells (1×10^5 cells/mL) were cultured with different final concentrations of GL for 72 h and the cytotoxicity caused by GL was determined by the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) activity. Results are means ± SE from 5 samples.

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Inhibition of PRCV replication by GL

The antiviral effect of GL on PRCV infectivity in ST cells was first investigated by CPE inhibition assay. PRCV virus was added to ST cells at multiplicity of infection (MOI) of 1.0. After incubation at 37 ℃ for 72 h, the cells were observed under microscope or used for RNA extraction or protein detection. As shown in Fig. 2, compared with control, PRCV infection caused obvious CPE (Fig. 2 b). Addition of GL at lower concentration (20 and 100 μg/mL) (Fig. 2 c and 2 d) did show much effect in the inhibition of CPE. However, when the GL concentration reached 500 and 1,000 μg/mL, the occurrence of CPE was completely inhibited and the cell morphology was similar to control cells that without PRCV virus infection. This result was further confirmed by RT-PCR and Western blot. Compared with the positive control of β-actin, with the increasing addition of GL to the ST cells, the expression of viral nucleocapsid protein mRNA was gradually suppressed. When the GL concentration reached 100 μg/mL, no viral DNA band could be detected, suggesting the replication of PRCV virus was completely inhibited (Fig. 3A). Similar result was also shown in Western blot, where the expression of nucleocapsid protein (42 kDa) was inhibited with the increasing addition of GL and complete inhibition could be identified at the GL concentration of 50 and 100 μg/mL (Fig. 3B). Obviously, RT-PCR is more sensitive than Western blot in the detection of PRCV replication as at GL concentration of 500 μg/mL,

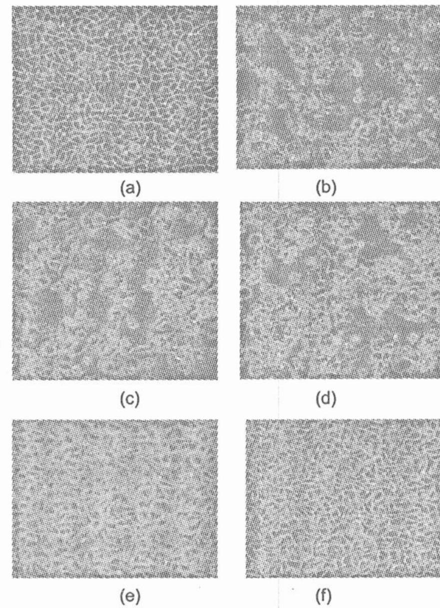

Fig. 2 Effect of GL on PRCV in ST cells

a. Mock infected cells; b. Infected cells without addition of GL; c. Infected cells in the presence of GL (20 μg/mL); d. Infected cells in the presence of GL (100 μg/mL); e. Infected cells in the presence of GL (500 μg/mL); f. Infected cells in the presence of GL (1,000 μg/mL).

Western blot did not show any protein band while RT-PCR still revealed a weak positive band. Most importantly, both methods gave consistent results, indicating the antiviral effect of GL to PRCV is in a dose-dependent manner.

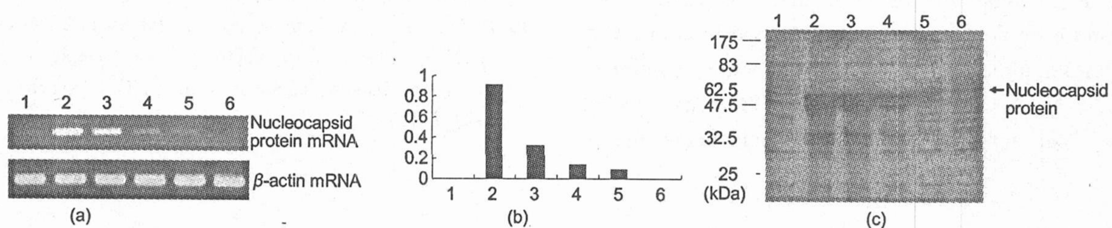

Fig. 3 Dose-dependent inhibition of the replication of PRCV by GL

a. RT-PCR to detect the expression of nuclocapsid protein mRNA with the expression of β-actin as an internal reference; b. Each band in A was quantitated by a densitometer (Vilber Lourmat, France) and the ratio of N-protein mRNA to β-actin mRNA was calculated using the Bio-1D software; c. Western blot to detect the synthesis of nucleocapsid protein. Lane 1, mock infected cells; lane 2-6, PRCV infected cells in the presence of different amounts of GL (0, 20, 100, 500 and 1,000 μg/mL), respectively. The size of molecular marker was labeled on the left.

Discussion

In the present study, we found that GL is an effective antiviral compound against PRCV in ST cells and the effective concentration is lower than the cytotoxic level. This is the first demonstration that GL has the antiviral effect on porcine virus. The antiviral effect of GL against PRCV is dose-dependent.

In order to investigate the antiviral effect of GL, a combination of RT-PCR and Western blot was admitted to

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study the nucleocapsid protein mRNA expression and protein synthesis. Our results showed that RT-PCR is more sensitive than Western blot and both results were consistent while Western blot gave the direct evidence of the formation of mature virus.

To date, many studies have been carried out on the antiviral effect of GL on different viruses, such as HIV[2], Hepatitis A virus[3], Hepatitis B virus[4], influenza virus[5] and SARS coronavirus[8]. The mechanisms of GL in inhibiting the replication of these viruses have also been investigated. The inhibitory effect of GL on HIV was postulated as suppressing the protein kinase C (PKC) activity as the cellular CD4 receptor of HIV-1 particles needs to be phosphorylated by PKC[10]. The suppression of influenza virus by GL was supposed by enhancing the immuno-response of the host (mouse) by inducing the production of interferon-γ, a cytokine that is effective against virus invasion[5]. While the effect against Hepatitis A virus[3] was presumed as affecting the virus penetration to the host cell. The exact mechanism of GL against other viruses, however, remains unclear.

GL is a Chinese medicine commonly used as an anti-inflammatory substance. It has been clinically used as a treatment for chronic hepatitis for more than 20 years in Japan[7]. The more recent report about the effectiveness of GL on the treatment of SARS suggested the importance of GL in the therapeutic use against human viruses. However, the high risk of SARS in experiment greatly affected the study of this virus. On the other hand, as a coronavirus, PRCV is similar in virion structure and even in structural protein composition to SARS and furthermore, PRCV is safe in laboratory handling. Thus, it may be an ideal way to use PRCV as a model virus in the screening of antiviral components and in the study of the antiviral mechanism of GL to coronaviruses in the future.

References

1 Pompei R, Flore O, Marccialis MA, et al. Glycyrrhetinic acid inhibits virus growth and inactivates virus particles. Nature, 1979, 218: 689-690

2 Sasaki H, Takei M, Kobayashi M, et al. Effect of glycyrrhizin, an active component of licorice roots on HIV replication in cultures of peripheral blood mononuclear cells from HIV-seropositive patients. Pathobiology, 2002-2003, 70: 229-236.

3 Crance JM, Leveque F, Biziagos E, et al. Studies on mechanism of action of glycyrrhizin against hepatitis A virus replication in vitro. Antiviral Res, 1994, 23: 63-76.

4 Sato H, Goto W, Yamamura J, et al. Therapeutic basis of glycyrrhizin on chronic hepatitis B. Antiviral Res, 1996, 30: 171-177.

5 Utsunomiya T, Kobayashi M, Pollard RB, et al. Glycyrrhizin, an active component of licorice roots, reduces morbidity and mortality of mice infected with lethal doses of influenza virus. Antimicrob Agents Chemother, 1997, 41: 551-556

6 Van Rossum TGJ, Vulto AG, De Man RA, et al. Glycyrrhizin as a potential treatment for chronic hepatitis A. Aliment Pharmacol Ther, 1998, 12: 199-205.

7 Kumada H. Long-term treatment of chronic hepatitis C with glycyrrhizin [stronger neo-minophagen C (SNMC)] for preventing liver cirrhosis and hepatocellular carcinoma. Oncology, 2002, 62 (suppl.): 94-100.

8 Cinatl J, Morgenstern B, Bauer G, et al. Glycyrrhizin, an active component of liquorice roots, and replication of SARS-associated coronavirus. Lancet, 2003, 361: 2045-2046.

9 Cao MJ, Guo C, Wu GP, et al. Cloning and expression of porcine respiratory coronavirus (PRCV) nucleocapsid protein. J Jimei Univ, 2005, 10: 29-32.

10 Ito M, Nakashima H, Baba M, et al. Inhibitory effect of glycyrrhizin on the in vitro infectivity and cytopathic activity of the human immunodeficiency virus (HIV). Antiviral Res, 1987, 7: 127-137.

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