Mechanism and dose-effect of Ginkgolide B on severe acute pancreatitis of rats

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Abstract

AIM: To determine the optimal dosage and mechanism of Ginkgolide B (BN52021) on severe acute pancreatitis (SAP) of rats.

METHODS: Seventy male Wistar rats were randomly divided into seven groups (10 for each group). Sham-operation group (SO), SAP model group (SAP), dimethyl sulfoxide (DMSO) contrast group (DMSO), and groups treated with 2.5 mg/kg BN52021 (BN1), 5 mg/kg BN52021 (BN2), 10 mg/kg BN52021 (BN3), and 20 μg/kg Sandostatin (SS). The SAP model was established in Wistar rats by injecting 5% sodium taurocholate retrogradely into the common bilo-pancreatic duct. The rats of SO, DMSO and BN52021 were injected with 0.9% NaCl, 0.5% DMSO and BN52021 through femoral vein 15 min after the operation. The SS group was injected with Sandostatin subcutaneously. All rats were anesthetized at 6 h after operation, and venous blood was collected to determine the levels of serum amylase and phospholipase A2 (PLA2), and pancreas tissue was harvested and stained.

RESULTS: There was no significant difference between the SAP and DMSO groups in serum amylase level, PLA2, ascites and pathologic score, but significant difference was found in SAP/DMSO groups compared with those in SO group (P < 0.05) and the levels of serum amylase, PLA2, ascites, and pathologic score were lower in the BN1, BN2, BN3 and SS groups than in the SAP and DMSO groups (P < 0.05). However, among BN1, BN2, BN3 and SS groups, BN2 had the best effect in decreasing the levels of serum amylase and PLA2 (P < 0.05). Expression of platelet activating factor (PAF) receptor (PAFR) mRNA and protein showed no significant difference between the SAP and DMSO groups, or among BN1, BN2, BN3 and SS groups, but there was remarkable difference between SAP/DMSO group and SO group (P < 0.05), and expression of PAFR mRNA and protein was higher in the BN1, BN2, BN3 and SS groups than in the SAP and DMSO groups (P < 0.05). PAFR expression was observed in the nucleus and cytoplasm of pancreatic islet cells in Wistar rats by immunohistochemistry.

CONCLUSION: By iv injection, 5 mg/kg of BN52021 is the optimal dosage for SAP rats. BN52021 may inhibit the interaction/binding of PAF with PAFR.

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Key words: Pancreatitis; Ginkgolide B; Dose-effect; Phospholipase A2; Platelet activating factor receptor

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INTRODUCTION
Platelet activating factor (PAF) is a biologically active phospholipid mediator, playing its role by binding to PAF receptor (PAFR) which is a unique G-protein-coupled seven transmembrane receptor, and it activates multiple intracellular signaling pathways. Flickinger et al. revealed specific localization of PAFR in the pancreatic vascular endothelium, but not in other pancreatic cell types. Recent studies demonstrated that PAF played an important role in the pathological progress of severe acute pancreatitis (SAP).

Ginkgolide B (code: BN52021) is one of the four Ginkgolide constituents (Ginkgolide A, B, C and J), which are present in the whole extract of Ginkgo biloba leaves. It has been found that BN52021 possesses activity as antagonists of the action of PAF. It can inhibit PAF-induced cascade effect in inflammatory reactions. For example, it can reduce the portal vein pressure of liver cirrhosis, exhibit an anti-shock effect, and exert protective functions on experimental asthma.

SAP is dangerous to life. It can cause multiple organ system failure (MOSF) and if the patients do not receive effective treatment in time, they may die. But up till now, there has been no effective drug for this disease. Recent studies have shown that BN52021 has best effect on experimental SAP, but there is no report on its optimal dosage, and its mechanism has not been fully understood. Especially as an extract of Ginkgo biloba leaves, it is hypotoxic, and can be used in clinical treatment of SAP. We investigated the optimal dosage of BN52021 and its mechanism on SAP.

MATERIALS AND METHODS
Main reagents and apparatus
BN52021 and sodium taurocholate were purchased from Sigma (St. Louis, MO, USA), amylose kit from Beijing Kemeci Reagent Co. (Beijing, China), trizol and diethyl pyrocarbonate (DEPC) from Invitrogen (Carlsbad, CA, USA), RT kit from MBI Fermentas (Ontario, Canada), DNTPs and RNA enzyme inhibitor from TaKaRa Dalian Co., Ltd. (Dalian, China), DNA Tag enzyme from Promega (Madison, WI, USA), primary antibody of PAF-R rabbit-anti-rat serum and the enhanced chemiluminescence system from Santa Cruz Biotechnology (Santa Cruz, CA, USA), secondary antibody of sheep-anti-rabbit from Beijing Dingguo Biotechnology Co., Ltd. (Beijing, China), β-actin from Beijing Zhongshan Biotechnology Co., Ltd. (Beijing, China), DYY-12 electrophoresis system and electric transfer blot SD from Beijing Liuyi Instrument Factory (Beijing, China), Type-2720 polymerase chain reaction (PCR) apparatus from ABI (Foster, CA, USA), and gel scanning and imaging system and vertical electrophoresis system from Bio-Rad (Hercules, CA, USA).

Primer sequences
PAF-R and β-actin primer series are provided by Invitrogen (Carlsbad, CA, USA): PAF-R: forward primer (F): 5′-CCGCTGTGGATTTCTATT-3′, reverse primer (R): 5′-AGGAGG TGATGAAGATGGG-3′ (377 bp); β-actin: F: 5′-TCC TAGCACCATGAAGATC-3′, R: 5′-AACCCGACGCTCAGTAGACG-3′ (190 bp).

Experimental animal grouping and model preparation
Seventy Wistar male rats (Laboratory Animal Center of PLA, Academy of Military Medical Sciences, Beijing, China), aged 6-8 wk, and weighing 200-220 g (Grade I, Certificate SCXK 2002-001) are used for this study. All rats were maintained in an controlled temperature (22-25°C), humidity (55%-58%), and lighting (12 h light/12 h dark), with free access to tap water and regular chow diet. Rats were divided randomly into 7 groups: Sham-operation group (SO group, n = 10), SAP model group (SAP group, n = 10), DMSO contrast group (DMSO group, n = 10), and treatment groups of 2.5 mg/kg BN52021 (BN1 group, n = 10), 5.0 mg/kg BN52021 (BN2 group, n = 10), 10.0 mg/kg BN52021 (BN3 group, n = 10), and 20.0 μg/kg Sandostatin (SS group, n = 10). All rats were weighed, marked and fasted for 24 h before operation. The rats were anesthetized by abdominal injection of 0.4% pentobarbital sodium (40 mg/kg), and fixed in dorsal decubitus. A 2-cm cut was made at the center of the upper belly, entering the abdominal cavity to look for rat’s duodenum and pancreaticobiliary duct. The hepatic end of the pancreaticobiliary duct was clamped with a non-invasive vascular clip, and pancreaticobiliary duct retrograde cholangiography was performed with an obtuse needle through duodenum seromucosal layer. Then 5% sodium taurocholate (0.1 mL/100 g) was injected in the retrograde direction of pancreaticobiliary duct with a micro-syringe at an injection rate of 0.20 mL/min. After injection, the port of pancreaticobiliary duct entering duodenum was clamped with a non-invasive vascular clip and observed for 10 min. When no active bleeding was confirmed in the abdominal cavity, the abdomen was closed in two layers and the incision was covered with sterile gauze. Same treatment was given to the SO group but without injections into the duct. Fifteen min after the model was completed, the rats in SO and SAP groups were injected with physiological saline (0.9% NaCl) through femoral vein at a dose of 5ml/kg; the rats of DMSO group were injected with DMSO (0.5% DMSO) at a dose of 5ml/kg; the BN1, BN2, and BN3 groups were rapidly injected with BN52021 at doses of 2.5, 5.0 and 10.0 mg/kg, dissolved with 0.5% DMSO; and SS group was injected with Sandostatin subcutaneously at a dose of 20.0 μg/kg.

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Collection and storage of samples
Rats in each group were anesthetized at 6 h after first injection of Sodium taurocholate, because the Ti/2 of BN52021 is 5.23 h[12], venous blood was collected from right atrium and was centrifuged for 10 min at 3000 g/min after a 10-min water bath at 37°C. The supernatant was placed into a sterilized EP tube, and stored in refrigerator at -20°C for serum amylose determination. Portions of pancreatic tissues were placed in liquid nitrogen overnight and frozen in refrigerator at -80°C, meanwhile, another portion of pancreatic tissues was fixed with 40 g/L neutral buffer formaldehyde, embedded in paraffin wax, cut into slices and stained with HE for pathological observation and scoring. Pneumonic tissues were fixed with 40 g/L neutral buffer formaldehyde, embedded in paraffin wax, and cut into slices for immunohistochemical studies.

Determination of serum amylose, PLA2 and pathological observation, scoring of pancreatic tissues
Serum amylose and PLA2 were detected with fully automatic biochemical apparatus, amylose and PLA2 kits. Pancreatic tissue samples were observed pathologically and scored according to the reference[13].

Determination of PAFRmRNA expression by reverse transcription PCR
After total RNA from pancreatic tissue in each group was extracted, its integrity was checked with agarose electrophoresis, and its concentration and purity were analyzed by 2% agarose gel electrophoresis, ethidium bromide staining, and a gel imaging system scanning. The whole mixture was maintained at 42°C for 60 min. Reverse transcription was conducted and reverse transcriptase was deactivated at 95°C for 5 min. The mixture was maintained at 4°C for 5 min and preserved at -20°C. Fifty microliters PCR reaction system consists of 3 μL reverse transcript product, 0.01 μmol DNTPS, 5 μL 10 × PCR buffer, 0.075 mol MgCl2 (Magnesium Chloride), 50 pmol of each PAF-R specific sense strand and anti-sense strand, and 50 pmol of each β-actin sense strand and anti-sense strand. Reaction conditions were as follows: pre-denaturizing at 94°C for 5 min, denaturizing at 94°C for 45 s, annealing at 58°C for 45 s, and extending at 72°C for 45 s, 38 cycles in total; extending at 72°C for 7 min, and preserving at 4°C. The PCR product was included in the PAF-R gene sequence extending at 72°C for 45 s, and extending at 72°C for 7 min, and preserving at 4°C. The PCR product was used in the PAF-R sequence of rat spleen (gi:470384), which was performed by Beijing Boya Biotechnology Co., Ltd. The PCR product was analyzed by 2% agarose gel electrophoresis, ethidium bromide staining, and a gel imaging system scanning. Gray level of PCR product was treated by PAF-R and β-actin was analyzed by the Quantity-One Software, and the change of PAFR mRNA expression was evaluated semi-quantitatively.

RESULTS

Statistical analysis
The experiments were repeated for 3 times and their average values served as the final values; all values were expressed as mean ± SD. Data were processed with SPSS 11.5 Statistical Software, and LSD and Dunnett’s post-hoc method were used for comparison of ANOVA. Statistical significance was established at P < 0.05.

Serum level of amylase and PLA2, quantity of ascites and pathological scores
There were no significant differences in the serum levels of amylase and PLA2, quantity of ascites and pathological scores between SAP and DMSO groups. It was higher in SAP/DMSO group than in SO group (P < 0.05), and lower in BN1, BN2, BN3 and SS group than in SAP/DMSO group (P < 0.05). However, BN2 had the best effect in decreasing the serum level of amylase and PLA2 among BN1, BN2, BN3 and SS groups (P < 0.05) (Table 1, Figure 1).

Expression of PAFR mRNA and its proteins in pancreatic tissues
There was no significant difference in expressions of PAFR mRNA and PAFR protein between SAP and DMSO groups, neither among BN1, BN2, BN3 and SS groups. Expression of PAFR mRNA and its proteins in pancreatic tissues was lower in SAP/DMSO group than in SO group (P < 0.05), and higher in BN1, BN2, BN3 and SS groups than in SAP/DMSO group (P < 0.05) (Table 2, Figures 2 and 3).

Location of PAFR in pancreatic and lung tissues
PAFR was located in pancreatic microvascular endothelial...
cells and bronchia epithelial cells. It was first found in the nuclei of pancreatic islet cells and cytoplasm of Wistar rats (Figure 4).

**DISCUSSION**

Amylase, PLA₂, score of pathologic changes of pancreas and quantity of ascites were reduced after administration of BN52021 at 2.5 mg/kg, and the pancreas PAFR mRNA or protein was protected. PAFR expression was found in the pancreas’ islet, which has not been reported by others up till now. The best effect was shown at 5 mg/kg among BN groups. BN52021 at 5 and 10 mg/kg showed equivalent effects on SAP, and 10 mg/kg was less effective such as in serum amylase. This may be caused by the dose range between 10 and 5 mg/kg.

PLA₂ is pivotal in the pathogenesis of SAP. There are two types of PLA₂: cytosolic phospholipase A₂ (cPLA₂) and secretory PLA₂ (sPLA₂). There is a cross-talk between...
sPLA₂ and cPLA₂. sPLA₂ activates a signaling cascade, including activation of cPLA₂, arachidonic acid release, PAF production, and induction of COX-2[14]. PLA₂ can decompose the membrane to release PAF. It is known that PAF is an initiative factor in the pathogenesis of SAP, and the cascade exacerbates the course of pancreatitis by activating the PLA₂ in membrane. BN52021 antagonizes PAF by restraining the activation of PLA₂ and this is the first report about the relation between BN52021 and PLA₂ in SAP.

Quantity of ascites showed the severity of pathological changes of pancreas. When pancreas is involved in inflammation, hemorrhage and putrescence, it would irritate peritoneum and peritoneal permeability was increased, resulting in the release of ascites[15]. The results indicated that BN52021 can reduce the PLA₂ in plasma and decrease the quantity of ascites, and that BN52021 may interact defi-

Figure 3 Expression of platelet activating factor receptor in pancreatic tissues by immunohistochemistry (HE staining, × 200). A: Sham-operation group; B: Severe acute pancreatitis group; C: DMSO group; D: 2.5 mg/kg BN52021 group; E: 5.0 mg/kg BN52021 group; F: 10 mg/kg BN52021 group; G: 20 μg/kg Sandostatin group.

Figure 4 Location of platelet activating factor receptor in pancreatic and lung tissues in sham-operation group shown by immunohistochemistry (HE staining, × 200). A: Paraffin slice of pancreatic tissues; B: Frozen slice of pancreatic tissues; C: Negative control of pancreatic tissues; D: Positive control of lung tissues.
nearly on other inflammatory factors to reduce peritoneal permeability.

The level or regulation of PAFR mRNA and PAFR protein has not been clearly studied. In our experiments, expression of PAFR in pancreas was up-regulated in the initial 3 h in SAP, but down-regulated after 6 h. The reason for this may be in the first 3 h, pancreas could release more inflammatory factors to induce the up-regulation of PAFR, such as tumor necrosis factor α and interferon-β, but cellular membrane was destroyed completely along with severe damages occurring in the pancreas after 6 h. In addition, protein kinase C excess activation was reported to decrease expression of PAFR, followed by a down-regulation of mRNA. BN52021 protected the membrane so that it maintained the quantity of PAFR. There was significant difference between SAP and BN52021 groups. The ratio ofIOD of PAF to β-actin was gradually improved in 2.5 and 10 mg/kg BN52021 groups, although there was no significant difference among BN52021 groups, and remarkable difference was found among SO group, 5 mg/kg SAP group and 5 mg/kg BN group.

It was firstly reported that PAFR was located in pancreas’ islet, and it is important to study the mechanism of pancreas inflammation. BN52021 exerted its protective effects in patients with diabetes and pancreatitis, but the active mechanism is not clear. When inflammation in pancreas happened, PAFR was up-regulated, and the cells released more PAF, which damaged the pancreas. As BN52021 competitively inhibited the combination of PAF and PAFR and released less PAF, the pancreas was protected. It meant that BN52021 achieved its effects on SAP not only by reducing the quantity of PAFR, but also by inhibiting the combination of PAF and PAFR and maintaining the integrity of the cells of pancreas.

In conclusion, 5 mg/kg BN52021 is the optimal dose in treating SAP rats, which can reduce the amylase and PLAs in serum and the quantity of ascites. It prevents the pancreas from severe damage induced by sodium taurocholate. Compared with Sandostatin, a classical drug for SAP, BN52021 exerts the best effects in treating SAP rats. This paper also showed that BN52021 achieves therapeutic effects in SAP rats not only by reducing the quantity of PAFR, but also by inhibiting the combination of PAF and PAFR and maintaining the integrity of the cells of pancreas. Because of its hypotoxicity as an extract of Ginkgo biloba, it will be of significance in clinical application. More experiments are needed for the extensive application of BN52021 in clinic.

**Research frontiers**

Recent studies demonstrated that PAF played an important role in the pathological progress of severe acute pancreatitis (SAP). It has been found that BN52021 possesses activity as antagonists of the action of PAF. However, there is no report on its optimal dosage. Although BN52021 has shown best effects on treating SAP, its mechanism has not been elucidated in details. This paper investigated the optimal dosage of BN52021 and its mechanism on SAP.

**Innovations and breakthroughs**

Recent reports have highlighted the importance of PAF in SAP and have shown that BN52021 has best effect on experimental SAP. This is the first study to report the optimal dosage of BN52021. The authors discovered that there is PAFR expression in the pancreas’ islet, which has not been reported by others up to now. The in vitro studies suggest that this protein may be the cause of the repression in E-cadherin observed in this cancer.

**Applications**

By understanding how PAF is induced and by blocking its expression, this study may represent a future strategy for SAP treatment with BN52021.

**Terminology**

PAF is a biologically active phospholipid mediator, playing its role by binding to platelet activating factor receptor (PAFR) which is a unique G-protein-coupled seven transmembrane receptor, and it activates multiple intracellular signaling pathways, including the nuclear PAFR. Ginkgolide B (code:BN52021) is one of the four Ginkgolide constituents (Ginkgolide A, B, C and J), which are present in the whole extract of Ginkgo biloba leaves.

**Peer review**

The authors examined the level of serum amylase and PLAs, quantity of ascites, and pathological scores, the expression of PAFR mRNA and its proteins in pancreatic tissues and location of PAFR in pancreatic and lung tissues. It revealed that PAFR expressed in the pancreas’ islet and the best effect on SAP was shown in the 5 mg/kg group. This paper also showed that BN52021 achieves the therapeutic action on SAP rats not only by reducing the quantity of PAFR, but also by inhibiting the combination of PAF and PAFR and maintaining the integrity of the cells of pancreas. The results are interesting and may represent a molecular mechanism of BN52021 on SAP.

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**COMMENTS**

- **Background**
  Platelet activating factor (PAF) is a family of phosphorylcholine esters with diverse potent physiological effects. It is closely associated with the cell membrane and is found in a variety of cells. Numerous cell types and tissues have been shown to synthesize and release PAF upon stimulation and at the same time to exhibit biological responses to it. Flickinger et al found the specific localization of PAF receptor (PAFR) in the pancreatic vascular endothelium, but not in other pancreatic cell types.

- **Research frontiers**
  Recent studies demonstrated that PAF played an important role in the pathological progress of severe acute pancreatitis (SAP). It has been found that BN52021 possesses activity as antagonists of the action of PAF. However, there is no report on its optimal dosage. Although BN52021 has shown best effects on treating SAP, its mechanism has not been elucidated in details. This paper investigated the optimal dosage of BN52021 and its mechanism on SAP.

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  Recent reports have highlighted the importance of PAF in SAP and have shown that BN52021 has best effect on experimental SAP. This is the first study to report the optimal dosage of BN52021. The authors discovered that there is PAFR expression in the pancreas’ islet, which has not been reported by others up to now. The in vitro studies suggest that this protein may be the cause of the repression in E-cadherin observed in this cancer.

- **Applications**
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  The authors examined the level of serum amylase and PLAs, quantity of ascites, and pathological scores, the expression of PAFR mRNA and its proteins in pancreatic tissues and location of PAFR in pancreatic and lung tissues. It revealed that PAFR expressed in the pancreas’ islet and the best effect on SAP was shown in the 5 mg/kg group. This paper also showed that BN52021 achieves the therapeutic action on SAP rats not only by reducing the quantity of PAFR, but also by inhibiting the combination of PAF and PAFR and maintaining the integrity of the cells of pancreas. The results are interesting and may represent a molecular mechanism of BN52021 on SAP.
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