Dexamethasone mediates protection against acute pancreatitis via upregulation of pancreatitis-associated proteins

Emad Kandil, Yin-Yao Lin, Martin H Bluth, Hong Zhang, Gabriel Levi, Michael E Zenilman

Abstact

**AIM:** To examine the influence of dexamethasone on pancreatitis-associated protein (PAP) gene expression using both in vitro and in vivo models of acute pancreatitis and to study how PAP gene expression correlates with severity of pancreatitis.

**METHODS:** In vitro, IL-6 stimulated pancreas acinar AR42J cells were cultured with increasing concentrations of dexamethasone and assayed for PAP expression (RT-PCR). In vivo, pancreatitis was induced in rats by retrograde injection of 40 g/L taurocholate into the pancreatic duct. Animals were pretreated with dexamethasone (2 mg/kg) daily or saline for 4 d. Pancreata and serum were harvested after 24 h and gene expression levels of PAP I, II and III were measured by RT-PCR. Severity of pancreatitis was based on serum amylase, pancreatic wet weight, and histopathological score.

**RESULTS:** In vitro, dexamethasone and IL-6 induced a marked transcription of PAP I, II and III genes in AR42J cells at 24 h ($P < 0.05$ for all comparisons). In vivo, pancreas mRNA levels of PAP I, II or III increased by 2.6-fold, 1.9-fold, and 1.3-fold respectively after dexamethasone treatment, compared with saline treated animals. Serum amylase levels and edema were significantly lower in the dexamethasone group compared with the saline group. Histopathologic evaluation revealed less inflammation and necrosis in pancreata obtained from dexamethasone treated animals ($P < 0.05$).

**CONCLUSION:** Dexamethasone significantly decreases the severity of pancreatitis. The protective mechanism of dexamethasone may be via upregulating PAP gene expression during injury.

Key words: Pancreatitis; Pancreatitis-associated protein; Pancreatitis-associated protein; Dexamethasone

INTRODUCTION

Acute pancreatitis is an acute inflammatory response to pancreatic injury and induces important changes in the expression of a number of genes in the pancreas. Among these, the most profound change is that of the pancreatitis-associated protein (PAP) family, the expression of which is very low in the normal pancreas and becomes strongly overexpressed after even mild pancreatic inflammation. Multiple functions have been ascribed to PAP. It has been shown to be antibacterial, anti-apoptotic, and mitogenic in vitro, and sequence analysis of PAP reveals the presence of a carbohydrate recognition domain in the protein, suggesting that PAP might act as a carbohydrate-binding lectin and can aggregate bacteria in suspension. Therefore, PAP may function as an endogenous anti-bacterial agent and be protective against infectious complications of pancreatitis, which can otherwise lead to severe disease with a high mortality. In addition, PAP expression is upregulated by free radicals or cytokines, and such upregulation confers cellular resistance to apoptosis. Previous results from our laboratory also showed that reg III (PAP) isolated from cow is mitogenic for pancreatic-derived cells, thus implicating PAP in the proliferative response of pancreas to injury. An anti-inflammatory effect of PAP has been found which protects the lung from leukocyte-induced injury. In addition, we recently demonstrated that antisense knockdown of PAP gene expression exacerbates the severity of pancreatitis, suggesting a protective function of PAP in acute pancreatitis.
treatment remains largely supportive. Corticosteroids have been shown to be beneficial in treatment of acute pancreatitis\(^1\)\(^-\)\(^9\). Although it is thought that steroid administration exerts an anti-inflammatory effect of the inflamed pancreas, the mechanism of how this occurs remains unclear. We postulate that during acute pancreatitis, the pancreas turns on a defense mechanism that includes expression of PAP and other stress proteins that enable the survival of pancreas under conditions of acute stress and that corticosteroid treatment augments the PAP response.

In the present study, the influences of dexamethasone on PAP Ⅰ, Ⅱ and Ⅲ gene expression using both in vitro cellular analysis and an in vivo model of acute pancreatitis were examined and correlated with severity of pancreatitis. It is our hypothesis that dexamethasone mediates protection against acute pancreatitis via PAP gene induction.

### MATERIALS AND METHODS

#### Materials

IL-6 (1-10 MU/L) stimulated pancreas acinar AR42J cells were cultured with dexamethasone (100 nmol/L) and assayed for PAP expression. Sprague Dawley rats obtained from Harlan Sprague Dawley (Indianapolis, IN) and weighing 175-200 g at onset of studies served as subjects. They were fed standard laboratory chow, given water ad libitum, and randomly assigned to control or experimental groups.

#### Pancreatitis induction and dexamethasone treatment

Pancreatitis was induced in rats by retrograde injection of 40 g/L sodium taurocholate (NaT) (Sigma, St. Louis, MO) into the pancreatic duct as previously described\(^{20\text{-}23}\). Briefly, under pentobarbital (Abbott Laboratories, North Chicago, IL) anesthesia (50 mg/kg ip), a midline incision was performed. The common bile duct was identified and randomly assigned to control or experimental groups. They were fed standard laboratory chow, given water ad libitum, and randomly assigned to control or experimental groups.

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#### Biochemical analysis and evaluation of pancreatic morphology

**Serum amylase activity (U/L) was measured** using 4,6-ethylidene (G\(\text{I}\))-, \(\beta\)-nitrophenyl (G\(\text{II}\))-D-maltoheptaoside as the substrate\(^{29}\). The extent of pancreas edema was quantitated by the ratio of pancreas wet weight over rat's total (mg/g) body weight\(^{29}\). For morphologic analysis, 5-µm-thick paraffin sections of pancreas samples were stained with HE. Ten randomly chosen microscopic fields were examined for each tissue sample, and inflammation as well as necrosis, were scored as follows: none = 0; mild = 1; moderate = 2; and severe = 3\(^{24}\).

#### Analysis of PAP gene expression

**PAP Ⅰ, Ⅱ and Ⅲ levels** were measured, in vitro and in vivo, by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR), based on the fluorogenic 5’-nuclease assays previously described\(^{23-26}\). The assay, which confers very high specificity, was carried out using a GeneAmp 5700 sequence-detection system (Applied Biosystems, Foster City, CA), with β-actin as an internal control to standardize the amount of sample RNA added to a reaction. Primers and probes were designed using Primer Express software (PE Biosystems). Sequences for all primers and probes used in these analyses are listed in Table 1.

**Table 1** Taqman primers and probes for real-time quantitative PCR

| Gene (Accession No.) | Primer/Probe sequences (Forward/Reverse/Probe) | Position (nt No.) | Size (bp) |
|----------------------|-----------------------------------------------|------------------|----------|
| PAP Ⅰ (NM_053289)   | F\(^5\)-AAATACCTCTGCAAGCATTG-3\(^3\)            | 153-171          | 67       |
|                      | R\(^5\)-GGCATACGGATAGCCATGCGG-3\(^3\)           | 219-198          |          |
|                      | P\(^5\)-FAM-TGGCGCAAGGCCTGCCATG-TAMRA-3\(^3\)  | 177-196          |          |
| PAP Ⅱ (L10229)      | F\(^5\)-CCAGAAGGGCAGTGCCTCTA-3\(^3\)           | 240-259          | 67       |
|                      | R\(^5\)-GGCATACGGAAGGATAGCCATGCGG-3\(^3\)       | 306-283          |          |
|                      | P\(^5\)-FAM-AGGCAAGGGCCTGCCATG-TAMRA-3\(^3\)   | 261-280          |          |
| PAP Ⅲ (L20869)      | F\(^5\)-TGTCGCACTTCAAGGTATG-3\(^3\)            | 121-140          | 64       |
|                      | R\(^5\)-GGCATACGGAAGGATAGCCATGCGG-3\(^3\)       | 184-162          |          |
|                      | P\(^5\)-FAM-AGGCAAGGGCCTGCCATG-TAMRA-3\(^3\)   | 143-159          |          |
| β-actin              | F\(^5\)-TTCAACACCCCAGCCATGT-3\(^3\)            | 379-397          | 68       |
|                      | R\(^5\)-GTGTAAGGCCGAGCCGATAC-3\(^3\)            | 446-425          |          |
|                      | P\(^5\)-FAM-CGTAAGCCCTACGGTCGGTAC-3\(^3\)      | 399-422          |          |

All primers and probes and other reagents for real-time quantitative PCR were purchased from Applied Biosystems. One hundred ng of total RNA was used to set up 25-µL real-time quantitative PCRs that consisted of 1 X TaqMan Universal PCR Master Mix, 500 nmol/L forward and reverse primers, and 200 nmol/L TaqMan probe. PCR amplification was carried out with the following temperature profile: 30 min at 48°C; 10 min at 95°C;
and 40 cycles of 15 s at 95°C and 1 min at 60°C. Assays were performed in triplicate. Data were analyzed with the relative standard curve method[27]. Standard curves of the genes of interest and β-actin were prepared with three 1:2 dilutions (four points, eightfold range) of total RNA from one of the samples that was expected to have the highest amount of mRNA for the gene of interest. For each reaction tube, the amount of target or internal reference was determined from the standard curves. The mean amount of each sample was calculated from the triplicate data and was normalized by division by the mean quantity of β-actin RNA for the same sample. The mean and SD of each treated group were calculated from the normalized value for each rat in that group.

**Statistical analysis**

Values for results were expressed as means ± SD obtained from multiple determinations in 3 or more separate experiments. P values computed were two-tailed, and P < 0.05 was considered statistically significant (Student’s t-test, ANOVA with Tukey post hoc correction).

**RESULTS**

When AR42J cells which were cultured in the presence of IL-6 were exposed to increasing amounts of dexamethasone, increased gene expression of PAP Ⅰ, Ⅱ, and Ⅲ was observed at 24 h (P < 0.05 when compared with controls) (Figures 1 and 2).

Rats which were treated with dexamethasone prior to pancreatitis induction demonstrated upregulation of pancreas mRNA levels of PAP Ⅰ, Ⅱ or Ⅲ when compared with saline treated controls (PAP Ⅰ: 2.6-fold, PAP Ⅱ: 1.9-fold and PAP Ⅲ: 1.3-fold respectively) (P < 0.05 for PAP Ⅰ and PAP Ⅱ) (Figure 3).

Furthermore, serum amylase levels and edema were significantly lower in the dexamethasone group compared with the saline group (Figure 4A and B) and histopathologic evaluation revealed less inflammation and necrosis in pancreata obtained from dexamethasone treated rats when compared with controls (Figure 4C) (P < 0.05).

**DISCUSSION**

In the current study, the influence of dexamethasone on PAP gene expression using both in vitro cellular analysis and an in vivo model of acute pancreatitis was investigated. Our results suggest that dexamethasone has an anti-
inflammatory effect in acute pancreatitis via upregulating PAP gene expression, which is inversely correlated with local pancreatic inflammation such as pancreatic edema and neutrophil infiltration. Additionally, recent data from our laboratory demonstrates a protective role for PAP at the protein level since administration of anti-PAP antibodies worsened pancreatitis severity in vivo [38,29]. PAP is a member of the family of secretory proteins expressed in the gastrointestinal tract and was originally isolated from the pancreatic juice of rats with acute pancreatitis [3]. Currently, three PAP genes have been characterized in human [8] and rat [6,30,31] mRNA for PAP isoforms (PAP I, II, and III) are expressed in the pancreas and small intestine among other tissues [30,32]. Results of PAP gene regulation studies suggest that PAP is an acute phase stress protein secreted from pancreas. PAP protein, which is not detectable in the healthy pancreas, is significantly expressed six hours after induction and reaches maximal expression after 48 h [31,32]. PAP accounts for about 5% of the secretory proteins during acute pancreatitis and can be detected in blood within 48 h of induction of acute pancreatitis [29]. We have previously reported a direct relationship between the severity of pancreatitis and serum levels of PAP [32]. We have also demonstrated that antisense mediated gene knockdown of PAP expression correlated with worsening pancreatitis in vivo [30]. We postulate that PAP proteins serve a protective role in acute pancreatitis and that the protective effect may be via the downregulation of acute phase cytokine gene expression in the peripheral immune system [6].

In the present study we showed that AR42J, a rat acinar cell line can be induced and used as a model to express all three PAP isoforms (PAP I, II and III) when treated with combination of IL-6 and dexamethasone. Although previous in vitro studies revealed that induction of PAP I gene expression could be obtained via dexamethasone treatment [3], here we demonstrate that all PAP isoforms are upregulated when cultured with increasing amount of dexamethasone and this effect is potentiated with addition of IL-6. Although many cytokines, including IL-6 are upregulated in many inflammatory states including pancreatitis [26,37], it is possible that IL-6, in conjunction with other mediators, contributes to PAP upregulation and disease resolution in vivo. This is likely since PAP/Reg genes possess IL-6 and glucocorticoid response elements [30,34]. We have previously demonstrated that antisense knockdown of PAP correlates with disease progression in vivo [30] and that more recently, plasmid and linear based siRNA gene knockdown of one PAP isoform impacts the expression of other PAP isoforms [39], suggesting that expression of PAP isoforms is contingent on one another rather than being redundant genes of common ancestry. Sequence comparisons of PAP isoforms demonstrate that they are closely related [39,40], although differing in their expression pattern and contain a consensus sequence coding for a bioactive protein component common to all three PAP isoforms (GGWEWSN) [41], which is able to knockdown gene expression of PAP I, PAP II, and PAP III. It is our
hypothesis that this highly homologous sequence common to all three isoforms may harbor important genetic information and may encode a bioactive fragment common to all PAP isoforms. To this end, Bodeker and colleagues have shown that PAP I interacts with PAP II, PAP III and lithostatin (RegIα) as well as itself to form homo/heterodimers [42], suggesting that PAP proteins may provide overlapping function for other members of the Reg protein family.

To evaluate the role of dexamethasone-induced upregulation of PAP expression in limiting the severity of pancreatitis, dexamethasone was administered before pancreatitis induction with NaT, an experimental model of necrotizing acute pancreatitis [43]. It is well known that corticosteroids are immunomodulatory and influence a number of factors involved in the process of tissue inflammation and edema [11,14]. There is evidence that endogenous glucocorticoids may protect acinar cells in acute pancreatitis by decreasing their sensitivity to the induction of cell death [23]. The adenocortical function is stimulated during acute pancreatitis and it has been suggested that the secretion of endogenous glucocorticoids may play an important role in mitigating the progress of this disease, probably by inhibiting cytokine production [45-48]. Additionally, hydrocortisone therapy was shown to be effective and beneficial at a dose of 4-25 mg/kg given 30 min before inducing acute pancreatitis, depending on the experimental system [11,14]. Furthermore, amelioration of acute pancreatitis by glucocorticoid treatment is related to the dose and time factor to achieve optimal therapeutic results [13]. The present study indicated that dexamethasone significantly improved both the local pancreatic inflammatory response as well as systemic inflammatory parameters and correlated with upregulation of PAP gene expression. Similarly, studies by Paszt et al [40], have also demonstrated a reduction in serum amylase and pancreatic weight/body weight ratios in pancreatic rats after treatment with dexamethasone. The beneficial effects of dexamethasone treatment may be directly related to PAP upregulation, possibly through utilization of a glucocorticoid response element found in the PAP/Reg gene family [23]. PAP III gene expression did not differ between dexamethasone and control groups at 24 h. It could be that PAP III responds to corticosteroid treatment at earlier or later time points and may interact with other PAP isoforms and affect their expression, as has been observed with PAP I [23].

In conclusion, the present study demonstrates a protective function of dexamethasone in acute pancreatitis which may be via upregulation of PAP gene expression during injury. In clinical practice, it is well known that the development of severe acute pancreatitis leads to multiple organ failure. Based on the present results, dexamethasone therapy may have the potential to help prevent the progression of acute pancreatitis. Dexamethasone treatment reduces edema, leukocyte infiltration and fat necrosis in the pancreas which is likely due to the upregulation of PAP. Future studies need to generate recombinant PAP and bioactive PAP peptides and explore their protective role against pancreatitis.

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Emad Kandil and Yin-Yao Lin contributed equally to this manuscript.

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