Recombinant adiponectin inhibits inflammation processes via NF-κB pathway in acute pancreatitis

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ABSTRACT
PURPOSE: Adiponectin is a protein stemming from adipose tissue and having strong anti-inflammatory properties. We aimed to assess the damage diminishing effects of recombinant adiponectin (rAD) through NF-κB in the experimental acute pancreatitis (AP) model.
MATERIALS AND METHODS: Acute pancreatitis was created by applying 50 μg/kg dose of intraperitoneal cerulean. The rats were randomised and divided into 3 groups as control, AP and rAD groups. Moreover, the rats in each group were divided into two sub-groups as 24th and 48th hour subgroups. rAD was injected in the study group intraperitoneally. Tissue and blood samples were taken after 24 and 48 hours. Histopathological assessment and NF-κB activity were investigated in pancreatic tissue.
RESULTS: Serum TNF-α, IL-1β and IL-6 levels were found to be statistically significant in the AP group compared to the rAD group in the 24th and 48th hour (p < 0.05). Similarly, NF-κB activity was also found to be significant in the AP group both in the 24th and 48th hour (p < 0.05). There were significant differences in the AP and the rAD groups histopathologically in terms of edema, inflammation, vacuolisation and necrosis (p < 0.001).
CONCLUSION: rAD has significantly reduced NF-κB activity, cytokine levels and tissue damage (Tab. 1, Fig. 1, Ref. 51).
KEY WORDS: acute pancreatitis, recombinant adiponectin, cytokines, nuclear factor kappa B.

Introduction

Acute pancreatitis is a protean inflammatory disease with a wide range of severity (1)(Lerch, 2013 #343;Lerch, 2013, 23220948). While the majority of its aetiology is composed of gallstones, extreme consumption of alcohol and other factors, underlying reason is not known for 20 % of cases. Although its severity changes in a wide range, in 80 % of patients, the disease limits itself and no serious complications are developed (2). However, pancreatic necrosis and systemic complications develop in 20 % of patients, and mortality rates are between 20–40 % having connection with the severity of the disease (3). The mechanism of acute pancreatitis could not be clarified fully despite increasing frequency of the disease in recent years and many studies conducted in relation to it. The activation of intracellular trypsinogen causes acinar cells to be damaged. The release of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin (IL)-6 and IL-1β activating zymogens in acinar cells in the starting period of the mechanism and starting the activation of vascular endothelium are held responsible for the damage (4, 5). These cytokines increase in direct proportion to the severity of the disease. The fact that inflammatory response was in the centre of pathophysiology caused treatment strategies to focus on the key mechanisms of inflammation. NF-κB inflammation – which is thought to be one of the most important of many inflammatory molecules-activates the transcription of many genes in inflammation and in apoptotic response (6). Today, the focus is on NF-κB in order to describe the physiopathology of severe acute pancreatitis (7–9). The effects of NF-κB on the mechanism of inflammation are displayed by means of cytokines, chemokines, enzymes, immune receptors and adhesion molecules. Activation by immune agents starts the transcription of many inflammatory genes and regulates them. Thus, NF-κB plays an important role in immune and inflammatory responses. Many inflammatory cytokines, especially TNF-α inflammation exerts its effects by using NF-κB pathway (10). Inhibition of NF-κB activation is the most important stage in reducing acute pancreatitis and inflammatory response (10–13).

Adiponectin is an anti-inflammatory protein stemming from adipocyte tissue, and some experimental studies have shown that adiponectin plays a protective role in acute pancreatitis induced by cerulein (14, 15). It has also been reported that there is a negative correlation between the severity of organ failure developing in acute pancreatitis and the levels of serum adiponectin (14). It
was shown that the activation of NF-kB, which was held responsible for the release of proinflammatory cytokines in aortic endothelial cell model, was inhibited by adiponectin (16). This study investigated the damage diminishing effects of recombinant adiponectin through NF-kB in experimental acute pancreatitis model induced by cerulein.

Materials and methods

This study was conducted in the animal laboratory in the Medical School of Gazi University after approval was granted from the Animal Ethical Board of Gazi University School of Medicine. A total of 48 male Wistar albino rats whose weight ranged between 250 and 300 gr were used in this study. The rats were put under laboratory conditions a week before the experiment, and they were kept in darkness for 12 hours and in daylight for 12 hours and were fed on standard rat food.

Study groups

The rats were randomly divided into 3 experimental groups each of which contained 16 rats. The groups of rats were also divided into sub-groups each of which contained 8 rats according to the scarification of the rats in the 24th and 48th hours.

- Group I (n = 16): Control group (24th (n=8) and 48th (n=8) hour subgroup)
- Group II (n = 16): Acute pancreatitis (AP) group (24th (n=8) and 48th (n=8) hour subgroup)
- Group III (n = 16): Recombinant adiponectin (rAD) group (24th (n=8) and 48th (n=8) hour subgroup)

Surgery design

Feeding the rats was stopped 8 hours before the study, but there were no limits in their water drinking. They were given 50 mg/kg hydrochloride (Ketalar®, Eczacibasi, Istanbul, Turkey) and 10 mg/kg Xylazine HCL (Alfazyne® 2%, 20 mg/ml, 30 ml, Alfasan Int.B.V., Netherlands) intraperitoneally, and thus they were anaesthetised. The rats were given choline-poor diet and 0.5% ethanol for 24 hours to generate acute pancreatitis, and then anaesthetised. The rats were given 50 mg/kg hydrochloride (Ketalar®, Eczacibasi, Istanbul, Turkey) and 10 mg/kg Xylazine HCL (Alfazyne® 2%, 20 mg/ml, 30 ml, Alfasan Int.B.V., Netherlands) intraperitoneally, and thus they were anaesthetised. The rats were given choline-poor diet and 0.5% ethanol for 24 hours to generate acute pancreatitis, and then cerulein was injected to them intramuscularly 7 times 50 micrograms/g of recombinant adiponectin through NF-kB in experimental acute pancreatitis model induced by cerulein.

To determine the levels of TNF-α, IL-1β and IL-6, commercial solid phase sandwich enzyme linked immunosorbent assay (ELISA) from Biosource International (Camarillo, CA, USA) was used. TNF-α and IL-1β levels were determined from a standard curve for recombinant TNF-α and IL-1β, and concentrations were expressed as pg/ml. The ELISA detection limit for TNF-α and IL-1β was 3 pg/ml.

Histological examination

Pancreatic tissue samples were buried into paraffin for morphological analysis after they were fixed with formalin, they were dyed with haematoxylin and eosin after section was taken. The preparation was analysed by two experienced pathologists who did not know of the intraperitoneal agent. The pancreatic histological grading was made using a scale ranging from 0 to 4 according to previous study (17).

Nuclear extracts and determination of NF-κB activation

Nuclear extracts were harvested from pancreatic tissue acinar cells by Nuclear Extract kits (Active Motif, Riensart, Belgium) according to the manufacturer’s instructions. Activation of NF-kB was evaluated by determination of the p65 DNA-binding subunit in the nuclear extracts using an ELISA-based assay (18) and commercially available kits (NF-kB p65, Active Motif, Riensart, Belgium) with the technique recommended by the manufacturer.

Determination of serum TNF-α, IL-1β and IL-6 concentrations

Serum TNF-α, IL-1β and IL-6 levels measured in both acute pancreatitis groups and rAD groups at 24th and 48th hour were significantly higher than in the control group (p = 0.002). Serum TNF-α levels at the 24th and 48th hour in rAD group were significantly lower than in the AP group (p = 0.002) (Fig. 1A).

Serum IL-1β levels in the AP group at the 24th and 48th hour were significantly higher than in the control (p = 0.002) and the rAD group (p = 0.002). IL-1β levels at the 24th and 48th hour in the rAD group were lower than in the AP group (p = 0.002). Serum IL-1β levels in the AP and the rAD groups reached the peak level at the 24th hour. Serum IL-1β levels decreased at 48th hour in the AP and rAD groups (Fig. 1B).

Serum IL-6 levels in the AP group at the 24th and 48th hour were significantly higher than in the control (p = 0.002) and the rAD group (p = 0.002). IL-6 levels at the 24th and 48th hour in the rAD group were lower than in the AP group (p = 0.002). Serum IL-6 levels in the AP group reached the peak level at the 48th hour but in rAD group IL-6 levels reached the peak level at the 24th hour (Fig. 1C).
Histopathologic examination of the pancreatic tissue

The histologic severity of pancreatitis was measured with a validated scale based on the degree of edema, inflammatory cell infiltration, haemorrhage, and acinar necrosis. Edema, inflammation, vacuolization and necrosis in AP and rAD groups at 24th and 48th hour were significantly higher than in the sham group (p < 0.001). Edema, inflammation, vacuolization and necrosis were lower at 24th and 48th hour in rAD group when compared to AP group but only edema was statistically significant (p = 0.002) (Tab. 1).

NF-κB expression in the pancreatic tissue and effects of rAD on pancreatitis

The NF-κB p65 protein levels were significantly higher in both AP and rAD groups than in the control group (p = 0.002). NF-κB activity at 24th hour and 48th hour in the rAD group was significantly lower than in the AP group (p = 0.002).

Tab. 1. Histopathological scores of the groups.

|                | Control Group | AP Group          | rAD Group         |
|----------------|---------------|-------------------|-------------------|
|                | 24th hour     | 48th hour         | 24th hour         | 48th hour         |
| Edema          | 0             | 0                 | 3.80±0.25         | 3.70±0.23         |
| Inflammation   | 0             | 0                 | 2.70±0.21         | 2.90±0.20         |
| Hemorrhage     | 0             | 0                 | 1.20±0.17         | 1.20±0.13         |
| Necrosis       | 0             | 0                 | 1.10±0.15         | 1.10±0.13         |

*p = 0.001 versus 24th hour the AP group and *p = 0.002 versus 48th hour AP group. Values are presented as mean ± SD. AP – acute pancreatitis, rAD – recombinant adiponectin
it was down-regulated significantly in the rAD group, compared with AP group (p = 0.002) (Fig. 1D).

Discussion

Previous studies demonstrated that adiponectin has anti-diabetic, anti-atherosclerotic and anti-inflammatory properties in many organs (15, 19–21). Several studies have been conducted so as to understand the mechanisms underlying the anti-inflammatory effect, which is perhaps the most important of these effects of adiponectin (22, 23). Demonstrating the anti-inflammatory effects of especially adiponectin on acute pancreatitis has been hope-inspiring in the treatment of this disease with high rates of mortality and morbidity. In this presented study, we investigated the NF-κB activity in pancreatic tissue and serum levels of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 in experimental cerulein-induced AP in a rat model.

A rise was demonstrated in the levels of pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α (24, 25) and of various chemokines such as IL-8, MCP-1 and macrophage migration inhibitor factors in patients with acute pancreatitis (26, 27). Efforts were made in many models of acute pancreatitis to analyse the roles of IL-6 in the pathophysiology of acute pancreatitis (28, 29). IL-6 levels which were observed to have risen in experimental studies conducted using cerulein induced acute pancreatitis model were also supported by clinical work (7, 30). It was shown in a study conducted with IL-6 knockout mice that the absence of IL-6 increased the local damage in pancreatic tissues when compared with wild-type mice (29). Another study conducted with mice with cerulein and LPS induced pancreatitis demonstrated, in support of these studies, that administering anti-IL-6 antibody to mice suffering from pancreatitis caused reduction in the severity of pancreatitis and in pulmonary inflammation caused by pancreatitis (29). It is thought that the increase in inflammation caused by IL-6 occurs by generating pathological T helper type 17 cells, and triggering MCP-1 and by facilitating the infiltration of inflammatory macrophages to enter the pancreatic tissue. Of other cytokines having an important role in pancreatitis-related inflammation process, IL-1β and TNF-α trigger inflammation cascade by means of mitogen-activated protein kinase (31). IL-1β starts neutrophil infiltration in the location of inflammation, and mediates the release of other pro-inflammatory cytokines into inflammation environment (32, 33). There are also views claiming that IL-1β is autocrine on its own synthesis (32). It was observed in this study that the 48th hour values of IL-1β tended to fall to 24th hour values, but this difference was not found to be significant. Different results can be yielded with the use of greater number of rats in the experiment and by observing the values of IL-1β in longer time periods.

Another pro-inflammatory cytokine, TNF-α, has a central role in the pathogenesis of inflammatory diseases especially in pancreatitis pathogenesis. TNF-α is induced in cells presenting pancreatic antigens and it is thought to cause the start of acinar cell damage (34). The stimulation of pancreatic acinar cells by TNF-α causes direct activation of pancreatic enzymes, and therefore it also causes the activation of cell necrosis and of premature protease.

The source of TNF-α in cerulein induced acute pancreatitis experimental models are in particular pro-inflammatory macrophages, which play critical roles in acinar cell necrosis (35). It was shown that the use of infliximab, which is a monoclonal TNF-α antibody, diminished cerulein induced pancreatic inflammation (35). In this study, as in other studies in the literature, the IL-6, IL-1β and TNF-α levels increased in the 24th hour in the cerulein induced acute pancreatitis group; and the levels reached the peak in the 48th hour. It is clear that the roles pro-inflammatory cytokines play in pancreatitis pathophysiology, which is widely accepted and supported by strong evidence, are also supported by this study.

Adiponectin is a protein with strong anti-inflammatory effects.

These effects are reduced by TNF-α secretion which is a monocyte and macrophage (23). It was shown in previous studies that adiponectin played roles in the down regulation of TNF-α-related inflammatory response and that there were reverse relations between them (36). This effect is bi-directional, that is, primary changes in TNF-α can influence adiponectin concentrations and vice versa. In these studies, TNF-α presses adiponectin secretion and expression in cell cultures (37, 38). It was shown in an experimental study conducted with adiponectin knock-out rats that there were high TNF-α mRNA and TNF-α concentrations in adipose tissues (39). It was also shown that adiponectin inhibits LPS-induced TNF-α gene expression in macrophages strongly (40). In this study, the levels of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β were reduced significantly in the severity of inflammation in the pancreatitis model, which was created with cerulein. Yet, are the effects of adiponectin more remarkable with the severity of inflammation or prolonged inflammatory process cause the activation of pathways, which may inhibit the protective effects of adiponectin? Although no clear answers are given to these questions with the results obtained in this study, it may be thought that there is need to administer additional dose of adiponectin. Experimental studies are needed to explain this situation. Because cerulein, which is an analogue of a cholecystokinin, stimulates pancreatic secretions which is a monocyte vacuolization, acinar cell death, edema formation and the infiltration of inflammatory cells into pancreas are created. This study, which used the system of microscopic scoring to assess the specimen histopathologically, edema, vacuolization, tissue necrosis and inflammatory cell infiltration were found to be significantly higher in rats in which pancreatitis was formed than in the rats of the control group. While improvement was observed in all of these histopathological changes in the group to which adiponectin was administered, only changes in the formation of edema were significant. In consequence, adiponectin administration to the rats in which cerulein-induced acute pancreatitis was formed reduced the histopathological disorder almost to normal levels. The reason for this could be that the severity of pancreatitis formed was mild because the formation of mild oedematous pancreatitis in the cerulein induced acute pancreatitis model is not a perfect model of experimental acute pancreatitis. However, it is frequently used due to being easily applicable and yielding acceptable results. Administering sodium taurocholate to the pancreatic canal as retrograde,
which is another model for experimental acute pancreatitis, causes the formation of severe acute pancreatitis; but is less frequently used since it is invasive (42, 43). We used the cerulein-induced acute pancreatitis model not only because it is less invasive and more frequently used, but also because it forms pancreatitis in the pancreatic acinar cells by activating NF-kB.

Experimental and clinical studies conducted in the last 10 years tried to show the roles of cytokines, chemokines, adhesion molecules in intrapancreatic and systemic circulation and of transcription factors such as NF-kB in acute pancreatitis pathophysiology (44, 45). It was reported that NF-kB acute pancreatitis could be an early and central factor in starting and/or progression of the inflammation in the pathophysiology of pancreatitis (46). The mechanism responsible for intraacinar NF-kB activation could not be clarified fully; but it was shown that pathological calcium signalling, protein C isoforms activation and generation of reactive oxygen products start intraacinar NF-kB inflammatory cascade (47). There are more experimental studies in this respect and there are also clinical studies demonstrating that increased NF-kB activity is present in patients with acute pancreatitis and also studies showing that the severity of acute pancreatitis is diminished by the inhibition of this activity (48). Adiponectin activates NF-kB in endothelial cells, fibroblasts and in hepatocytes (49, 50). Yet, it was reported that adiponectin suppresses LPS-induced (lipopolysaccharide) NF-kB activation in adiposities (51). In this study, NF-kB activity in the 24th hour was inhibited with adiponectin application, but the inhibition did not continue as it was expected in the 48th hour. This might have stemmed from the fact that adiponectin has both stimulatory and inhibitory effects on NF-kB pathway, but further studies are needed to explain when in what situations it is stimulatory and in what inhibitory and by means of which mechanisms.

This study, with the results obtained, supports the idea that adiponectin displays its damage reducing effects on cerulein-induced acute pancreatitis by reducing NF-kB activity and pro-inflammatory cytokine response. Demonstrating the damage reducing effects of adiponectin on the treatment of acute pancreatitis brings hope and inspirationin the developments of therapies for this disease, which has high rates of mortality. However, more clinical and experimental studies are needed so as to understand the effects of adiponectin administration on acute pancreatitis and so as to be able to use it in clinical practice.

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