N-acetylcysteine in acute pancreatitis

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Abstract
Premature trypsinogen activation and production of oxygen free radicals (OFR) are early pathogenic events which occur within acinar cells and trigger acute pancreatitis (AP). OFR exert their harmful effects on various cell components causing lipid peroxidation, disturbances in calcium homeostasis and DNA damage, which lead to increased cell injury and eventually cell death. This review presents the most recent data concerning the effects of N-Acetylcysteine (NAC), in the treatment of AP. NAC is an antioxidant capable of restoring the levels of Glutathione, the most important cellular antioxidant. Studies show the beneficial effects of NAC treatment in preventing OFR production and therefore attenuating oxidative damage. Additionally, NAC treatment has been shown to prevent the increase in cytosolic Ca²⁺ concentration and reduce the accumulation of enzymes in acinar cells during AP. The prevention, by NAC, of these pathological events occurring within acinar would contribute to reducing the severity of AP. NAC is also capable of reducing the activation of transcription factors especially sensitive to the cellular redox state, such as Nuclear factor-κB, signal transducer and activator of transcription-3 and mitogen-activated protein kinase. This leads to a down-regulation of cytokines, adhesion molecules and chemokine expression in various cell types during AP. These findings point to NAC as a powerful therapeutic treatment, attenuating oxidative-stress-induced cell injury and other pathological events at early stages of AP, and potentially contributing to reduction in the severity of disease.

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Key words: Acute pancreatitis; Calcium homeostasis; Cell cycle; Glutathione; Monocyte chemoattractant protein-1; N-acetylcysteine; Oxygen free radicals; Transcription factors

INTRODUCTION
Acute pancreatitis (AP) is an inflammatory disease whose pathophysiology remains poorly understood. It is usually considered to be an autodigestive disease. In addition to premature intracellular protease activation⁹, mechanisms such as oxidative stress have also been shown to be involved in the development of the disease. Since Sanfey et al⁸ suggested the possible role of oxygen free radicals (OFR) in AP, many studies have been carried out in order to investigate the role of oxidative stress in different experimental models of AP. Under healthy conditions, OFR are generated in eukaryotic cells but they are quickly removed by a system of enzymatic and non-enzymatic antioxidants within the cell. If OFR production overwhelms the cellular antioxidant defense systems, oxidative stress develops. This leads to disturbances in cellular homeostasis since these OFR can cause biochemical and functional alterations at different cellular levels, including lipid peroxidation.⁷
protein oxidation and DNA damage, among other toxic effects. Glutathione (GSH) is one of the most important cellular antioxidants and is generally depleted under situations in which OFR production is enhanced, such as in AP. For this reason, the oxidative state of the pancreas is routinely evaluated by indirect measurements on extracts of tissue. Pancreatic GSH levels and the concentration of malondialdehyde, a product of membrane lipid peroxidation, are widely considered as indices of the cellular redox estate. Most recently, analysis carried out by flow cytometry showed the time course of OFR production in individual acinar cells during AP.

A number of antioxidant therapies have been shown, to varying extents, to improve different AP models. These antioxidants include N-Acetylcysteine (NAC), a thiol-containing compound used in the clinic for treatment of congestive and obstructive lung disease, paracetamol intoxication and more recently in the treatment of pulmonary oxygen toxicity and adult respiratory distress syndrome. A wide range of NAC doses and administration protocols have been reported in clinical applications while in experimental studies of AP, NAC dosages varying between 10 to 1000 mg/kg have been administered by infusion or intraperitoneal injections. The extensive use of NAC is due to the multifaceted chemical properties of the cysteinyl thiol of the molecule. This confers antioxidant capacity by interacting directly with OFR and facilitating GSH biosynthesis, since Cys bioavailability appears to be a limiting factor for GSH synthesis, a process that may be induced in the exocrine pancreas under conditions of stress.

### NAC AND INTRACELLULAR EVENTS DURING AP

Besides OFR generation, exocytosis blockade and cytosolic calcium rise, have been considered as the earliest intracellular events that lead to the premature activation of trypsinogen which triggers AP. NAC administration at a dose of 100 mg/kg has been shown to prevent the cytosolic calcium increase found in acinar cells from early pancreatic duct obstruction (PDO) onwards. Increases in intracellular calcium concentration evoked by OFR have been reported in acinar cells, mainly caused by calcium mobilization from intracellular stores and a further partial influx across the plasma membrane. It seems likely that disturbances in calcium homeostasis are due to alterations in the mechanisms responsible for maintaining low intracellular calcium levels as these require ATP, whose pancreatic levels have been found reduced in AP as a result of GSH depletion. Because oxidative stress is avoided at early AP stages by NAC administration, these mechanisms may not be affected. Consequently, normal cytosolic calcium levels are in fact maintained during PDO and cell damage and death is prevented. On the other hand, premature intracellular activation of digestivezymogens is highly dependent on increased cytosolic calcium concentrations. In parallel with an intracellular redistribution of cathepsin B and preceding GSH depletion, intrapancreatic activation of digestive enzymes has been found 15-30 min after inducing AP by supramaximal stimulation with caerulein. On this basis, the occurrence of intracellular zymogen activation under NAC treatment cannot be discounted. However, data strongly suggest that by limiting OFR-evoked increases in acinar cells calcium levels, NAC prevents the progressive autodigestion of the pancreatic tissue which would be initiated inside acinar cells at early stages of AP. As a result, NAC treatment reduces the pancreatic damage induced by PDO AP. Accordingly, oxidative stress is clearly involved in the genesis of the tissue lesions induced in this model of AP.

On the other hand, NAC treatment also seems to prevent cytoskeletal disruption, a structural alteration associated with increased cytosolic calcium concentrations and related to the blockade of enzyme secretion in AP.

Significant decreases in plasma amylase activity as well as the lower quantities of enzymes stored in acinar cells strongly suggest that NAC administration mitigates the exocytosis blockade in AP, in turn reducing the risk of intracellular activation of digestive enzymes.

In summary, OFR neutralization at early AP stages by NAC treatment has been shown to prevent the increase in cytosolic calcium concentrations and reduce the accumulation of enzymes in acinar cells, thus limiting the activation of digestivezymogens inside acinar cells. The prevention of major intracellular pathological mechanisms by NAC administration consequently contributes to ameliorating the severity of AP.

### NAC AND CELL CYCLE

Besides their harmful effects, an increasing body of evidence supports the involvement of OFR in transduction cascades which act as signalling molecules in the regulation of physiological processes such as cell arrest, proliferation, senescence and cell death. The signalling pathways by which OFR regulate cell growth have not been clearly established, although a large family of serine/threonine kinases referred to as mitogen-activated protein kinases (MAPKs), has been identified as a key mediator in this response to stimuli such as cytokines, growth factors, tumour promoters, hormones and oxidants, and is required for cells to overcome the cell cycle checkpoint in the transition from G0/G1 into S-phase. It is widely accepted that the exposure of mammalian cells to a prooxidant environment leads to mitogenic activation. OFR would exert their effect both by direct oxidative modification of signal transduction molecules and indirectly by altering the general redox state of the cell. In this regard, increased OFR generation in acinar cells and subsequent oxidative damage, as well as activated cellular proliferation, have been found at early stages of AP. NAC has been demonstrated to inhibit cell growth and to exert a palliating effect on AP symptoms, when administered at early stages.

In PDO-induced AP, a relationship between the effectiveness of NAC treatment in preventing the generation of OFR and the changes in the cell cycle pattern of pancreatic acinar cells throughout the different AP stages has been reported. The activation of these
pathways is a key regulatory point in the cell cycle and is required for the cell to leave the quiescent state and enter S-phase[27]. It has been suggested that mitogenic stimulation, due both to enhanced plasma cholecystokinin (CCK) concentrations and OFR overproduction in acinar cells following AP, might be inhibited by NAC administration. Both stimuli seem to make use of OFR-dependent MAPK activation in order to achieve the proliferative response. As a result, NAC has been shown to suppress the proliferative response after AP induction and promote cell arrest at early AP stages[33].

Additionally, it has been reported that cells were no longer active in proliferation 24 h after AP induction, probably because they were progressively damaged during the course of this pathology[33]. Pancreatic atrophy was reported 48 h after inducing AP by PDO, and led to increased proportions of G0/G1-phase cells and apoptosis[36]. By contrast, Uruñuela et al[32] reported that cells from NAC-treated PDO rats remained active for DNA synthesis, probably because they were effectively preserved from damage during early stages when different pathophysiological mechanisms are involved in AP. Thus, cells could be freed from the arrest asserted by NAC at earlier AP stages and they may actively enter S-phase, probably in an attempt to restore the normal proliferation pattern. Mitosis might be expected at later stages when it would counteract the depletion of acinar cells during AP[29]. This event could be favoured by CCK, whose plasma levels have been found to be increased for at least 3 d after the onset of gallstone-induced AP[34].

In conclusion, there is evidence that OFR play a critical role in the progression of cell cycle phases in acinar cells. In parallel with the prevention of OFR generation and, therefore, oxidative damage, NAC treatment has been reported to maintain acinar cells in a quiescent state at early AP. These effects may combine in the protection of the cells at early stages of AP and consequently, would retain their ability to proliferate during the course of AP.

**NAC AND OXIDATIVE STRESS-SENSITIVE SIGNALLING PATHWAYS**

OFR also act as mediators of the activation of transcription factors[33]. Nuclear factor-κB (NF-κB) belongs to the Rel family of transcription factors that regulate the expression of genes related to stress, cytokines, and chemokines[34]. This factor is kept silent in the cytoplasm via interaction with inhibitory proteins of the IκB family, that prevent nuclear translocation, and DNA binding of the transcription factor. Phosphorylation and subsequent polyubiquitination and degradation of IκBα are redox-regulated steps[35]. IκBα degradation has been reported to be slowly initiated 6 h after inducing AP and strongly maintained thereafter, leading to NF-κB activation. Although intracellular OFR generation may not be required for NF-κB activation in all cell types[38], in the PDO-induced AP model, NF-κB is activated in response to the oxidative stress developed within acinar cells, thereby resulting in up-regulation of tumour necrosis factor (TNF-α) from 6 h after inducing AP[39]. This notion is supported by the fact that the highest levels of pancreatic GSH depletion and OFR production within acinar cells were reported at 6 h after induction of AP[38]. NAC administration, by increasing the stores of GSH in the pancreas, and preventing the overproduction of OFR in acinar cells, was reported to delay NF-κB activation. Accordingly, acinar cells of rats treated with NAC failed to produce TNF-α during AP. Inhibitory effects on pancreatic mRNA expression of cytokines interleukin-6 and KC has also been reported in response to NAC treatment in caerulein[41] and sodium taurocholate[42] AP models. Because activation of p38-MAPK can occur in response to a variety of stressful stimuli[43] and has been reported in some AP models[44,45], the role of this MAPK as a potential upstream regulator of TNF-α expression in acinar cells during AP has been investigated[40]. Interestingly, it has been suggested that the MAPK pathway may participate in NF-κB activation and, as such, cross-talk between the two pathways may be established[46]. The time course of p38-MAPK phosphorylation during AP induced by PDO showed a peak 6 h after induction of pancreatitis. Thereafter, activation of p38-MAPK was maintained but at lower levels. Phospho-p38-MAPK was significantly attenuated by NAC administration[40]. This study reported maximal p38-MAPK activation at the time of maximal intra-acinar oxidative stress during PDO-induced AP. Given that NF-κB activity was found to be significantly increased immediately afterwards, it is suggested that MAPK is likely to have regulated IκB degradation and consequently the activation of NF-κB. Redox-sensitive pathways may activate MAPKs and NF-κB in a coordinated fashion, suggesting that the activation of MAPKs may be pivotal in “switching on” the cytokine cascade during AP induced by PDO.

The role of oxidative stress in leukocyte recruitment during AP still remains controversial. In vitro studies have shown that OFR function as important messengers for intercellular adhesion molecule (ICAM)-1 expression in endothelial cells, at least in part through the activation of NF-κB[47]. Telek et al[48] have reported a chronological and topographical overproduction of OFR and ICAM-1 upregulation during AP. On this basis, several antioxidants have been used in in vivo and in vitro studies to interfere with the expression of ICAM-1 and divergent results have been reported[49,50]. According to another report[51], no reduction of ICAM-1 was found either in acinar cells or plasma, in rats with PDO-induced AP treated with single doses of NAC[52]. This treatment had previously been proven to be capable of abolishing the overproduction of OFR in acinar cells of rats subjected to this AP model[21]. These results suggest that OFR are not important factors in mediating ICAM-1 expression. Nevertheless, NAC has been shown to reduce the overexpression of CD11b/CD18 in neutrophils, monocytes and pancreatic infiltration[52]. This finding reinforces the notion that ICAM-1 may not be the main molecule involved in the adhesion of leukocytes during PDO-induced AP, since NAC treatment has been shown to significantly protect the pancreas from inflammation.
Although ICAM-1 expression was not abrogated. Moreover, CD11b/CD18 overexpression during AP appears to be triggered by oxidative-dependent mechanisms and it contributes to pancreatic injury by promoting leukocyte infiltration[53].

In summary, ICAM-1 is upregulated at different locations, including pancreatic acinar cells, from early stages of AP by oxidant-nondependent mechanisms but other molecules seem to be required in the recruitment of leukocytes. On the other hand, there is evidence of a correlation between the infiltration of neutrophils within the pancreas and the overexpression in leukocytes of the β2-integrin, CD11b/CD18, an event which was sensitive to antioxidant treatment. Multiple pathways may contribute to the sequestration of leukocytes within damaged tissue during AP.

Blocking treatments of chemokine action have been shown to reduce the severity of AP[53]. Therefore, a complete understanding of the mechanisms involved in chemokine expression in individual cell types would be of great interest in the control of both local and systemic inflammatory response in AP. Chemokine expression and the involvement of oxidative stress in pancreatic acinar cells has recently been reported in in vitro and in vivo studies. It has been demonstrated that pancreatitis-associated ascitic fluid (PAAF) promotes the overexpression of monocyte chemotactic protein-1 (MCP-1) in pancreatic acinar cells mediated by p38-MAPK NF-κB and signal transducer and activator of transcription (STAT)-3 activation and NAC treatment has been shown to reduce this acinar expression of MCP-1[54]. In response to PAAF, acinar cells develop oxidative stress, which has been demonstrated to activate MAPK[55] and NF-κB[56]. On the other hand, the inhibition of phosphatases induced by reactive oxygen species allows the Janus kinase-induced STAT phosphorylation required for STAT activation[55]. Accordingly, the anti-inflammatory action of NAC may be explained by preventing the redox-activation of p38-MAPK and NF-κB and significantly reducing the STAT3 activity.

Overexpression of MCP-1 and cytokine-induced neutrophil chemoattractant (CINC) in acinar cells was reported during PDO- and NaTc-induced AP, mediated by p38-MAPK, NF-κB and STAT3 activation[56]. In this study, the inhibition of the transcriptional MCP-1 and CINC upregulation in acinar cells by NAC treatment occurred in mild AP, which led to a reduction of the infiltration of leukocytes in the pancreas. Nonetheless, NAC has been shown to be unable to reduce the oxidative stress developed within the pancreas of rats with severe AP[56]. This effect can be explained by the fact that, related to the severity of the AP, acinar cells develop intense oxidative stress with the subsequent OFR overproduction in acinar cells. OFR, in addition to acting as second messengers to enhance the production of inflammatory factors, are powerful chemoattractants[57]. As a result, an intense leukocyte recruitment occurs in the pancreas of rats with NaTc-induced AP, which in turns leads to an increase in the production of OFR molecules by infiltrated leukocytes. The overproduction of OFR from different pancreatic cell sources overwhelms the antioxidant capability of NAC treatment in severe AP. As a result, NAC has been shown to fail to hinder the redox-sensitive chemokine expression and its downstream signal pathways. These findings suggest that damaged acinar cells contribute to chemokine production from early stages of AP by activating oxidant dependent signalling mechanisms. Only in mild AP, with lower oxidative stress, were these down-regulated by NAC administration. By contrast, NAC treatment has been shown to exert no inhibitory effect on the overexpression of chemokines found in pancreatic tissue from 3 h after inducing AP. This result suggests that in addition to acinar cells, other cell types resident in the pancreas with the capability of producing chemokines, such as endothelial vascular cells[58], stellate cells[59] and infiltrated leukocytes[60] are also producing MCP-1 and CINC by mechanisms resistant to the antioxidant treatment.

Furthermore, NAC treatment has not been shown to reduce the high MCP-1 and CINC concentrations found in plasma of rats with PDO- and NaTc-induced AP[56]. These increased systemic chemokine levels could be the result of the outpouring of chemokines from the pancreas and from inflammatory cells. Since a similar chemokine profile in response to NAC treatment was found in pancreas and plasma, it is suggested that cell sources other than acini must be contributing to increasing the circulating levels of MCP-1 and CINC[60].

In summary, different results indicate that oxidative stress may trigger the overexpression of MCP-1 and CINC in acinar cells of rats with AP by activating MAPK, NF-κB and STAT3, as oxidant-sensitive downstream signalling pathways. The antioxidant capability of NAC has only been shown to avoid the acinar chemokine expression in mild AP and it was ineffective in severe AP. In addition, NAC failed to prevent the chemokine increase in pancreatic tissue and plasma, even in mild AP, suggesting that additional stimuli, such as cytokines, activate molecular pathways leading to upregulation of chemokines in non-acinar cell sources.

Given the pathogenic role of the oxidative stress in AP, more basic research on the potential therapeutic effects of individual of combined antioxidant products will be necessary.

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