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Effect of cholecystokinin blockade on the recovery of alterations induced by acute pancreatitis in glycoconjugates of rat zymogen granules

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Lectin-binding studies have been performed on rat zymogen granules to investigate alterations in the carbohydrate membrane composition that occur in acute pancreatitis induced by caerulein. The influence of treatment with hydrocortisone for seven days before inducing pancreatitis was also studied. Lectin labeling on zymogen granules was also analyzed seven days after inducing pancreatitis in rats that had previously received a hydrocortisone treatment. During this period L 364,718 (0.1 mg/kg)—specific cholecystokinin (CCK) receptor antagonist—was administered daily to some of the rats, and no treatment was applied to others. Using fluorescein-labelled *T. purpureus* (TP)lectin, a significant decrease in the amount of L-fucose in the granule membrane was observed in rats with caerulein-induced pancreatitis. This effect was directly caused by the pancreatitis and was not influenced by previous hydrocortisone treatment. Seven days later, the density of TP receptors in the granule membrane was similar to the controls both in L-364,718-treated and untreated rats. Therefore, we suggest that endogenous CCK is not an essential factor in the recovery of L-fucose containing glycoconjugates the granule membrane after pancreatitis. Acute pancreatitis did not alter the expression of wheat germ agglutinin (WGA) receptors in the zymogen granule membrane. WGA specifically binds *N*-acetyl glucosamine and sialic acids. L 364,718 administered for seven days after inducing pancreatitis significantly reduced WGA binding, untreated rats showed a normal zymogen granule membrane. Therefore, the blockade of CCK-induced alterations in membrane glycoconjugates enriched in *N*-acetyl glucosamine and sialic acid of newly formed granules after pancreatitis, a finding that could explain the delay in the regression of the disease.

***Keywords*: acute pancreatitis, zymogen granule glycoconjugates, Cholecystokinin-receptor antagonist, rat**

Introduction

Acute pancreatitis induced by supramaximal doses of caerulein causes blockade of exocytosis [1] and structural alterations in the formation and maturation of pancreatic zymogen granules [2–4]. Also, depending on the dose, glucocorticoids reduce pancreatic secretion [5, 6] through a mechanism that partially inhibits exocytosis [7], and their administration increases the enzyme storage in pancreatic acinar cells [8–10]. Therefore, glucocorticoids administered before inducing acute pancreatitis are capable of altering the pathogenesis of the disease [11, 12]. Several agents have been used as potential therapy in acute pancreatitis, among them L 364,718—the most specific cholecystokinin (CCK) receptor antagonist [13]. L 364,718 has demonstrated beneficial effects in rats with caerulein-induced pancreatitis [14], but it delays the recovery of the pancreatic secretory function if the rats are treated with hydrocortisone before inducing pancreatitis [15].

It has been reported that glycoproteins from the zymogen granule membrane are involved in the formation of zymogen granules and in exocytosis [16, 17]. Both processes are disturbed in pancreatitis, a finding that could be related to alterations in the composition of membrane glycoconjugates which have been described in different experimental models of acute pancreatitis [18, 19].

On this basis, the aim of this study was to analyze by flow cytometry the lectin binding to zymogen granules of rats with caerulein-induced pancreatitis treated and not treated previously with hydrocortisone to find out whether alterations in the glycosylation of the membrane are involved in the pathogenesis of pancreatitis. Analyses were also made seven days after inducing pancreatitis in rats treated with L-364,718 and in untreated rats, to deduce if endogenous CCK was necessary for the regeneration of zymogen granules, which would lead to the recovery of the secretory capability of the pancreas.

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Materials and methods

Chemicals

Caerulein, hydrocortisone-2-L-hemisuccinate, bovine serum albumin (BSA), dimethylsulfoxide (DMSO), ethylene glycol-bis-(β-aminoethyl ether)-N,N,N′,N′tetraacetic acid (EGTA), 3-(*N*-morpholino) propanesulfonic acid (MOPS), phenylmethylsulfonyl fluoride (PMSF), 2-(*N*-morpholino) ethanesulfonic acid (MES), Percoll, fluorescein isothiocyanate conjugated with *Tetragonolobus purpureus* lectin (FITC-TP), and wheat-germ agglutinin (FITC-WGA), L-fucose and *N*-acetyl glucosamine were purchased from Sigma Chemical Co. (St. Louis, MO), L-364,718, the cholecystokinin (CCK)–receptor antagonist, was kindly supplied by Merck Sharp and Dohme (Spain).

Animals and treatment

Male Wistar rats about 250 g body weight were used. They were randomly divided into five groups as follows:

Group 1: Control animals that received four 100 µl subcutaneous injections of 0.9% NaCl containing 0.2% BSA at hourly intervals (n=16)

Group 2: Animals that received four 100 µl subcutaneous injections of caerulein (20 µg/kg) in 0.9% NaCl solution containing 0.2% BSA at hourly intervals (n=16)

Group 3: Animals that received 100 µl intramuscular injections of hydrocortisone (10 mg/kg) in 0.9% NaCl solution. This dose was administered daily at 9:00 A.M. for seven days. Afterward, four subcutaneous injections of caerulein were performed as for group 2 (n=12).

In these three groups, the samples were collected 5 h after the last injection.

Group 4: Animals were treated with hydrocortisone and caerulein as for group 3. Five hours after the last caerulein injection 100 µl of subcutaneous injections of a solution of L-364,718 (0.1 mg/kg) in 0.9% NaCl containing 10% DMSO as depot carrier were administered daily for seven days (n=12).

Group 5: Animals were treated with hydrocortisone and caerulein as for group 3. No treatment was applied after caerulein, and on the seventh day, the samples were collected (n=12)

After 18 h fasting the animals were anesthetized with sodium pentobarbital (3mg/kg body weight, i.p.). Blood samples were obtained by cardiac puncture to measure plasma amylase activity and haematocrit. Afterward, the pancreas was quickly removed, freed from fat and lymph nodes. A portion of pancreatic tissue was collected to measure the percentage of fluid, and the rest was used to study the zymogen granules by flow cytometry.

Assays

Amylase activity in plasma was determined according to the method of Noelting and Bernfeld [20]. Pancreatic edema was calculated by drying the pancreas at 90° C over 48 h. The ratio between pancreatic wet weight and dry weight was expressed as percentage of fluid.

Isolation of zymogen granules

Pancreases were put on ice-cold homogenization medium containing 250 mM sucrose, 5 mM MOPS (pH 7.0), 0.1mM $MgSO_4$, and 0.1 mM PMSF at 40 ml/g wet weight pancreas. They were finely minced with scissors and homogenized for 30s in an electric homogenizer (Omni 2000 International) at low speed. Heavy components were removed by centrifugation at low speed. Zymogen granules were isolated according to the method of De Lisle *et al.* [21] by centrifuging at 100,000 *g* for 20 min in a 40% Percoll gradient composed of a homogenization buffer, pH 5.5 (250mM sucrose, 50mM MES, 0.1mM $MgSO_4$, 0.1mM PMSF and 2mM EGTA) mixed with Percoll.

Staining of zymogen granules with FITC-lectins

The staining was carried out as previously described [22–24]. Briefly, FITC-TP and FITC-WGA were individually incubated with zymogen granules at 4° C in the dark for 15 min. The unbound FITC-lectin was removed by centrifugation twice in washing buffer (2 × 1,300 g, 15 min, 4° C). The specificity of the binding of each FITC-lectin to the zymogen granules was assessed by using the same concentration of lectin as a control but in the presence of 0.2 M L-fucose for FITC-TP and *N*-acetyl D-glucosamine for WGA as has been previously reported [22–24].

Flow cytometric analysis of the zymogen granules

All flow cytometry measurements of zymogen granules were performed for at least 10,000 events/test of a pooled pancreas of two animals per experiment. A FACScan flow cytometer (Becton/Dickinson, San Jose, California) equipped with an argon ion laser tuned at 488 nm and 15 m Watts was used. Sample (medium washing)/sheath (FACS flow, Becton/Dickinson) differential pressure was set at low speed to obtain a minimum diameter of the sample part of the flow at the intersection of the laser beam. During the whole measurement process, the zymogen granules remained resuspended in the washing buffer, which is surrounded by the sheath fluid, and no admixture between the two fluids occurs in accordance with FACScan flow cytometer procedure [25]. Calibration of the instrument was performed on a daily basis using CALIBRITE beads (Becton/Dickinson). Fine adjustments were performed using unstained zymogen granules. Each time zymogen granules from controls and each of the other experimental groups were measured in parallel. The Lysis II software program was used for data acquisition and analysis. Results were expressed as the mean value of at least five experiments,

and in each experiment, the mean value per individual zymogen granule was calculated using Lysis II software.

The amount of FITC-lectin bound to individual zymogen granules was measured using molecule equivalent soluble fluorescein (MESF) units. A mixture of five different beads with well-established amounts of FITC (QuickCal beads, Flow Cytometry Standards Corporation) was used to obtain a regression curve that allowed the calculation of the mean MESF content of zymogen granules based on its mean fluorescence. The amount of lectin bound to each zymogen granule was expressed as the number of molecules of lectin calculated from the ratio of the number of MESF obtained per granule and the number of MESF per one molecule of lectin.

Statistical analysis

Results are expressed as means ± SEM. Statistical analysis of the results from the different experimental groups was carried out using the analysis of variance (ANOVA) test followed by the Scheffé test in those cases where significant differences were found by the ANOVA test. In all cases, *P* values lower than .05 were considered to be statistically significant.

Results

As can be seen in Figure 1, all animals with acute pancreatitis induced by caerulein (groups 2 and 3) showed a significant increase in plasma amylase, percentage of fluid in pancreas (edema), and hematocrit. These parameters still remained significantly high after seven days of inducing pancreatitis in rats treated daily during this period with L-364,718 (group 4), while they returned to control values in untreated rats (group 5).

Forward light scatter (FSC) displayed by zymogen granules was measured by flow cytometry. FSC is a parameter directly related to the cross-sectional area [26] and therefore reflects the granule size. The mean values of individual granules in the different groups are shown in Figure 2. There were no significant changes in the granule size of rats with caerulein-induced pancreatitis both in those not treated (group 2) and in those previously treated for seven days with hydrocortisone (group 3). Significantly smaller zymogen granules were observed seven days after inducing acute pancreatitis both in L 364,718-treated rats (group 4) and in those in which no treatment was given after pancreatitis (group 5).

The measurements of the molecules of *T. purpureus* lectin (TP) bound to individual zymogen granules (Figure 3) revealed a significant decrease of TP binding in rats with caerulein-induced pancreatitis. Similar values were found in animals that had been previously treated with hydrocortisone before inducing pancreatitis (group 3) and in untreated rats (group 2). On the seventh day, control values

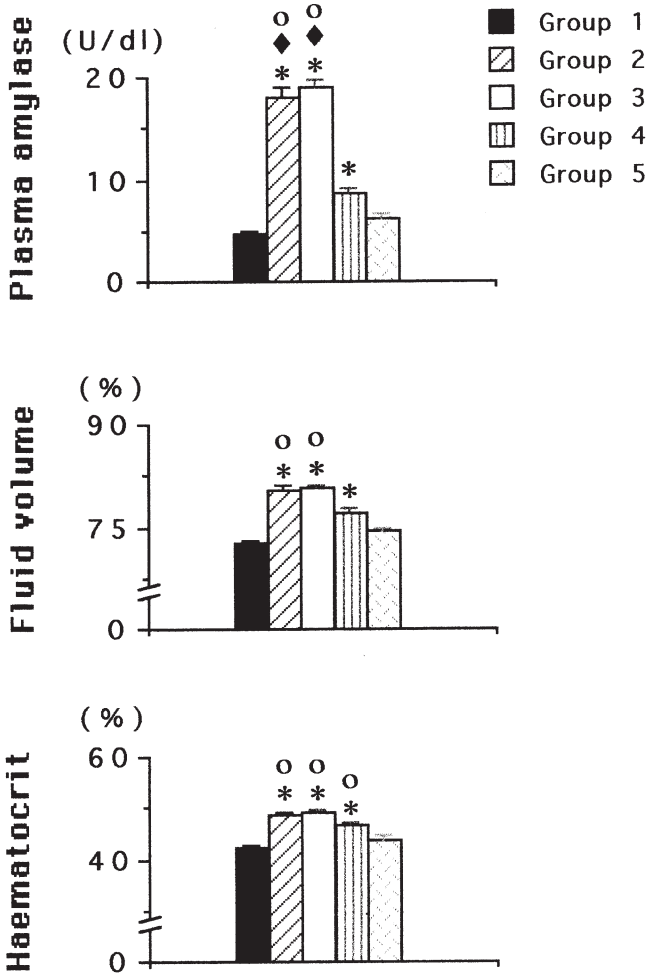

Figure 1. Plasma amylase, percentage of fluid in pancreas and haematocrit in controls (group 1; n=16), caerulein-induced pancreatitis (group 2; n=16), hydrocortisone (10 mg/kg/day) for seven days before pancreatitis (group 3; n=12), L-364,718 (0.1 mg/kg/day) for seven days after hydrocortisone and pancreatitis (group 4; n=10), seven days after hydrocortisone and pancreatitis without treatment (group 5; n=11). Number of animals = n. Values are means ± SEM. Analyses of variance test followed by Scheffé test showed significant differences when compared with group 1 (✻), group 4 (◆), and group 5 (O).

were reached in rats that had not received any treatment after pancreatitis (group 5), whereas a significant decreased TP labeling was observed in rats treated with L-364,718 for seven days after pancreatitis (group 4). However, no significant difference with respect to controls was found in this group of animals when TP binding/FSC ratio was analyzed.

As Figure 4 shows, no significant difference in the amount of WGA molecules bound to zymogen granules were found between controls and rats with pancreatitis (groups 2 and 3). Binding of FITC-WGA significantly decreased in granules of rats that were treated with L 364,718 for seven days after inducing pancreatitis (group 4), and

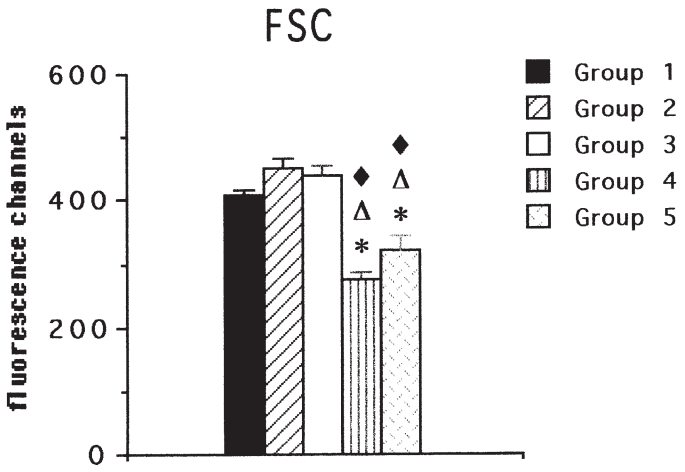

Figure 2. Forward-light scatter of individual pancreatic zymogen granules in controls (group 1; n=8), caerulein-induced pancreatitis (group 2; n=8), hydrocortisone (10 mg/kg/day) for seven days before pancreatitis (group 3; n=6), L 364,718 (0.1 mg/kg/day) for seven days after hydrocortisone and pancreatitis (group 4; n=6), seven days after hydrocortisone and pancreatitis without treatment (group 5; n=6). Number of experiments-n. Values are means ± SEM (arbitrary units scaled from 0 to 10,000). Analyses of variance test followed by Scheffé test showed significant differences when compared with group 1 (✻), group 2 (Δ), and group 3 (◆).

control values were obtained in those rats not treated (group 5).

Discussion

Lectins are useful tools to detect changes in the glycoconjugate composition of cellular and subcellular membrane systems. FITC-lectins have been previously used in flow cytometry to study the membrane of zymogen granules of rats under different physiological, pharmacological, and pathological conditions [19, 22–24]. Flow cytometry has

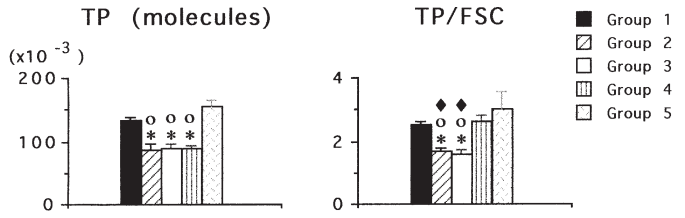

Figure 3. Number of *T. purpureus* (TP) molecules bound to individual granules and TP binding/forward-light scatter ratio in controls (group 1; n=8), caerulein-induced pancreatitis (group 2; n=8), hydrocortisone (10 mg/kg/day) for seven days before pancreatitis (group 3; n=6), L 364,718 (0.1 mg/kg/day) for seven days after hydrocortisone and pancreatitis without treatment (group 5; n=5). Number of experiments-n. Values are means ± SEM. Analyses of variance test followed by Scheffé test showed significant differences when compared with group 1 (✻), group 4 (◆), and group 5 (o)

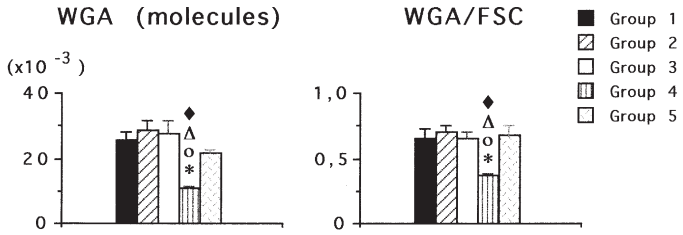

Figure 4. Number of wheat germ agglutinin (WGA) molecules bound to individual granules and WGA binding forward-light scatter ratio in controls (group 1; n=8), caerulein-induced pancreatitis (group 2; n=7), hydrocortisone (10 mg/kg/day) for seven days before pancreatitis (group 3; n=6), L-364,718 (0.1 mg/kg/day) for seven days after hydrocortisone and pancreatitis (group 4; n=6), seven days after hydrocortisone and pancreatitis without treatment (group 5; n=6). Number of experiments-n Values are means ± SEM. Analyses of variance test followed by Scheffé test showed significant differences when compared with group 1 (✻), group 2 (Δ), group 3 (◆), and group 5 (o).

proved to be a sensitive, objective, and quantitative method for the analysis of large numbers of individual granules, which makes it particularly reliable for the assessment of the quantities of lectin bound to each zymogen granule. FITC-lectins can specifically bind to carbohydrates of membrane glycoconjugates located outside or inside zymogen granules as we have previously reported [22, 23].

This study has demonstrated a significant decrease in TP binding to granules of rats with acute pancreatitis. *T. purpureus* lectin specifically binds to L-fucose, a carbohydrate restricted to terminal positions in the oligosaccharide chain in pancreatic glycoconjugates [27, 28], a finding that suggests a correlation between the degree of fucosylation and the maturity of membrane. Saito *et al.* [4] characterized the acinar cell changes that accompany the exposure to supramaximal doses of caerulein in the rat and demonstrated that the transport of newly synthesized proteins through post-Golgi elements was markedly altered. In particular, the maturation of condensing vacuoles into zymogen granules was found to be impaired, and, as result, a large population of vacuoles appears in the acinar cells. The event could be related to the increase in the mean value of granule size (FSC) observed in rats with acute pancreatitis. The two subsets of zymogen granules previously described in rat pancreas [9, 22–24] are not clearly differentiated in rats with acute pancreatitis. We suggest that the vacuoles may not be the result of a single fusion of zymogen granules as has been previously reported [1, 2], since TP binding/FSC ratio is significantly lower in rats with pancreatitis than in controls. Instead, our results indicate that new immature organelles appear in the pancreas of rats with pancreatitis. Garcia-Montero *et al.* [24] demonstrated an increase in TP receptors in zymogen granules of control rats subjected to the same hydrocortisone treatment as that used in this study. However, our results indicate that supramaximal doses of caerulein administered after hydrocortisone treat-

ment (group 3) reduced the TP binding to the same level as it did in rats that had not previously received hydrocortisone (group 2). This finding confirms that the alterations observed in the fucosylation of the granule membrane are directly induced by the pathological process. Our results are consistent with the reports of Willemer *et al.* [18] using *Ulex europeaus* lectin, who also observed a decreased L-fucose content in the membrane of secretory granules and vacuoles from rats with caerulein-induced pancreatitis. The L-fucose amount is also significantly reduced in the granule membrane of rats with acute necrotizing pancreatitis induced by retrograde infusion of taurocholate [19]. Therefore, a relationship may exist between the alterations in zymogen-granule maturation that occur in acute pancreatitis and the deficiency of L-fucose in the membrane glycoconjugates.

GP2 is a glycosyl phosphatidylinositol linked membrane protein [29], which is thought to play a major role in zymogen granule membrane maturation and stability [17]. The decrease in L-fucose observed in rats with pancreatitis could be related to a decrease in GP2. This suggestion is supported by the results obtained by other authors [18] using lectin blotting who observed that a major band of 75 KDa with high L-fucose content was absent in rats with caerulein-induced pancreatitis. This band could correspond to GP2, the most abundant glycoprotein in zymogen-granule membrane, which has a molecular mass close to 75 KDa [29]. Apte *et al.* [30] also demonstrated a significant decrease in the amount of GP2 in the zymogen-granule membrane of rats subjected to chronic ethanol consumption that also leads to pancreatic injury. Future studies should be carried out to find out whether there is a reduction in a specific glycoprotein or in general fucosylation of the granule membrane in acute pancreatitis. In any case, the L-fucose glycoconjugates in the structural membrane is reduced, a change that could influence the development of pancreatitis. Intact membranes seem necessary for the stabilization of the secretory granule content [31].

The experimental model used in this study induces edematous pancreatitis which is reversible. After seven days, the regeneration of the pancreas had occurred, as indicated by a significant decrease in the mean size of zymogen granules observed in groups 4 and 5, since newly formed granules have been shown to be smaller [32]. Although the number of TP molecules bound to zymogen granules was decreased in rats treated with L 364,718, it is noteworthy that the ratio of TP binding to size (FSC) was similar to the appropriate controls. The results suggest that endogenous CCK is associated with the presence of L-fucose in the glycoconjugates of the granule membrane but that it was not essential for the granules to recover a normal density of L-fucose after pancreatitis.

As for the results obtained with WGA labeling, it can be inferred that acute pancreatitis did not induce alterations in the amount of *N*-acetyl D-glucosamine and sialic acids in zymogen granule membrane. WGA specifically binds to those carbohydrates, that are mainly orientated toward the inside of granule [33]. L 364,718 administration for seven days after inducing pancreatitis reduced the total amount of WGA receptors. This result was also previously observed in control rats that received L 364,718 treatment [23]. The effect was not due to the small size of zymogen granules since a significant decrease in WGA labeling/FSC ratio was also observed. Therefore, endogenous CCK is involved in some aspects of glycosylation in the zymogen membrane. It appears to regulate the content of *N*-acetyl D-glucosamine and sialic acid. Parallel changes in pancreatic secretion rate and the amount of WGA receptors in the zymogen-granule membrane have been previously described [23], suggesting that glycoconjugates of the membrane involved in exocytosis contain *N*-acetyl glucosamine and sialic residues. On this basis, the decrease in WGA receptors found in this study could explain why pancreatic secretion did not become reestablished in rats treated with L-364,718 for seven days after inducing pancreatitis but that had received hydrocortisone beforehand as Pescador *et al.* [15] have previously reported. This situation becomes relevant in our experimental conditions, since hydrocortisone administered for seven days before inducing pancreatitis increased the amount of enzymes in individual zymogen granules (ms. in preparation). The accumulation of granules in the pancreas under these conditions could delay the regression of pancreatitis as the continuing high values in plasma amylase, pancreas fluid, and haematocrit suggest.

Acknowledgments

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