Serum and tissue glycoconjugates, digoxin and magnesium levels in chronic calcific pancreatitis

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Background: Endogenous or exogenous digoxin can lead to membrane Na⁺,K⁺-ATPase inhibition and hypomagnesemia. Low magnesium levels can lead to increased glycosaminoglycans (GAG) concentration in many organs. Aim: To measure the serum levels of pancreatic GAG and glycoproteins, two major components of the extracellular matrix, in patients with chronic calcific pancreatitis (CCP). Serum levels of magnesium and digoxin were also assessed. Methods: Patients with CCP and age- and sex-matched healthy control subjects (15 each) were studied. Serum GAG, Mg and digoxin levels were measured. RBC membrane Na⁺,K⁺-ATPase activity was also assessed. Pancreatic tissue obtained at autopsy from seven patients with CCP and sex- and age-matched healthy subjects who had died in accidents were also tested for GAG and glycoproteins. Results: Total GAG levels were significantly increased in the serum and pancreas of patients with CCP. This was associated with lower serum Mg levels, increased serum digoxin levels and decreased RBC membrane Na⁺,K⁺-ATPase activity. Conclusion: Exogenous or endogenous digoxin-induced hypomagnesemia and the consequent altered glycoconjugate metabolism may be important in the pathogenesis of CCP. [Indian J Gastroenterol 2001;20:230-233]

Key words: Glycosaminoglycans, glycoproteins, membrane Na⁺,K⁺-ATPase

Data on changes in extracellular matrix components in patients with chronic calcific pancreatitis (CCP) are limited. The available reports suggest an increase in serum levels of procollagen-III-peptide (P-III-P), hyaluronic acid, laminin and extracellular matrix components in patients with acute pancreatitis and an increase in the content of glycosaminoglycans (GAG) in the pancreas of rats with acute pancreatitis.¹ There are reports that the hypothalamus produces an endogenous digoxin-like factor (EDLF), which is an inhibitor of membrane Na⁺,K⁺-ATPase.⁴ Work in our laboratory has identified EDLF as digoxin itself, which is synthesized from acetyl CoA by the isoprenoid pathway.⁵,⁶ Digoxin can also be obtained from exogenous dietary sources.⁷ Digoxin can produce hypomagnesemia, through inhibition of membrane Na⁺,K⁺-ATPase.⁸ We have previously shown that low magnesium levels can lead to increased GAG concentration in many organs.⁹ Magnesium is also known to be involved in the N- and O-glycosylation of proteins and hence affects glycoprotein synthesis.¹⁰

In the present study, we looked at the changes in GAG and glycoproteins in the serum and pancreas of patients with CCP. We also studied changes in the serum concentrations of magnesium and digoxin, and in RBC membrane Na⁺,K⁺-ATPase activity.

Methods
Fifteen consecutive in-patients (aged 20-40 years; 8 men) recently diagnosed to have CCP based on clinical features and ultrasonographic findings were studied. All the patients had presented with abdominal pain and diabetes mellitus of less than one year duration; none had malabsorption or steatorrhea. Their body mass index was less than 18.5 Kg/m². Their renal and liver function tests were normal and they were not receiving any steroi-dal drugs or digoxin at the time of blood collection.

A similar number of age- and sex-matched healthy subjects selected from the general population served as controls. All the controls had normal pancreas at ultrasonography, normal blood sugar levels and normal stool fat. The diet consumed by the patients and the controls was similar, and contained approximately 400 (SD 50) g of carbohydrate, 37.5 (±) g of proteins, 24.5 (±) g of fat and 15 (±) g of dietary fiber (estimated by detergent extraction method). Blood was collected from patients and control subjects after an overnight fast; serum and RBC obtained were used for investigations.

The study was approved by the ethical committee of the Trivandrum Medical College. Informed consent was obtained from all patients and control subjects.

Pancreatic tissue specimens were obtained from 7 patients with CCP at autopsies, which were conducted at the Medical College hospitals at Trivandrum and Thrissur, with the consent of family members. The cause of death in these CCP patients was acute myocardial infarction (3 patients) and diabetic nephropathy with chronic renal failure (4). Control pancreatic tissue samples were obtained from seven sex- and age-matched healthy subjects who had died in accidents.
Analytical procedures

All chemicals used were of analytical grade (E Merck, Germany).

Assay for RBC Na+,K+-ATPase

The assay system contained

- 0.4 mL NaCl-MgSO₄ solution (NaCl 0.25 M, MgSO₄ 0.00138 M), 0.4 mL Na-ATP (0.00125 M) and 0.16 mL Tris-HCl buffer (0.0625 M, pH 8.6).

- 0.4 mL NaCl-MgSO₄-KCl solution (NaCl 0.25 M, MgSO₄ 0.00138 M, KCl 0.00125 M), 0.4 mL Na-ATP (0.00125 M) and 0.16 mL Tris-HCl buffer (0.0625 M, pH 8.6).

Enzyme for the assay was prepared as follows: 1.0 mL RBC suspension was lysed with deionized water, centrifuged at 20,000 x g for 10 minutes at 4°C, and the supernatant discarded. The RBC membrane was washed with deionized water by centrifugation till the supernatant became colorless. The enzyme preparation was then suspended in 1.0 mL Tris-HCl buffer, 0.0625 M (pH 8.6). The system was preincubated at 37°C for 5 minutes. Forty microliter of RBC membrane Na+,K+-ATPase was then added to the assay systems and incubated for 60 minutes at 37°C. The reaction was terminated by the addition of 0.3 mL 30% trichloroacetic acid (TCA) at 0°C. The samples were then centrifuged at 10,000 rpm for 5 minutes at 0-4°C. The inorganic phosphate liberated was estimated in the supernatant.

The activity of the enzyme is the increase in inorganic phosphate liberated (micromoles per hour per mg protein) in the presence of K+ over that in the presence of Na+.

A suspension of RBCs obtained from each patient or control was lysed with deionized water, centrifuged at 20,000 x g at 4°C for 10 min, and the supernatant discarded. The pellet was washed repeatedly with deionized water by centrifugation till the supernatant became colorless. The resulting preparation containing RBC membrane was then suspended in 1.0 mL Tris-HCl buffer, 0.0625 M (pH 8.6) and incubated at 37°C for 5 min. Forty microliter of each of this suspension was then added to two tubes, one containing 0.4 mL NaCl-MgSO₄-KCl solution (NaCl 0.25 M, MgSO₄ 0.00138 M, KCl 0.00125 M), 0.4 mL Na-ATP (0.00125 M) and 0.16 mL Tris-HCl buffer (0.0625 M, pH 8.6) and the other containing all the above reagents except KCl. Both the tubes were incubated at 37°C for 60 min. The reaction was terminated by addition of 0.3 mL 30% TCA at 0°C. The tubes were then centrifuged at 10,000 rpm at 0-4°C for 5 min, and the amount of liberated inorganic phosphate was measured in the supernatant. The increment of inorganic phosphate liberated (nmol/h/mg protein) in the tube containing KCl over that in the tube lacking KCl represents the RBC membrane Na+,K+-ATPase activity.

Assay for endogenous digoxin

Fasting plasma (10 mL) was mixed with 90% ethanol (9 vol/mL plasma). Digoxin (Sigma, St. Louis, USA) in ethanol solution (10-100 ng) was added to the extract. The tubes were centrifuged at 3000 x g for 10 minutes. The residue was extracted twice more with 90% ethanol. The supernatants were combined and shaken twice with equal volumes of petroleum ether (boiling point 40-60°C). The ethanol solution was then evaporated to dryness in a rota vapor at 50°C.

The residue was then dissolved in methanol (0.5 mL) and subjected to TLC separation over silica gel along with pure digoxin standard, using ethyl acetate : methanol : water (85:10:5) as the solvent system. The spot corresponding to digoxin was scraped out and eluted with methanol.

Methanol was removed from the solution in N₂ atmosphere and the residue dissolved in 400 μl solvent system, acetonitrile: 25 mM KH₂PO₄ pH 3.0 (25:75 v/v). 20 μl of the solution was used for HPLC (Supercost ABZ+ plus, 5 mm, 15 cm column, flow rate 2.0 mL/min, detection 220 nm).

Estimation of GAG

Serum (1 vol) was treated with ice-cold ethanol (9 vol) and the precipitate obtained was subjected to papain digestion by the procedure described by Scot. Pancreatic tissue was defatted by Folch's procedure and the dry defatted tissue was subjected to papain digestion. The papain digest, after deproteinizing with trichloroacetic acid (TCA, final concentration 10%), was dialyzed till free of TCA. Total GAG was precipitated from the solution by addition of 4-5 volumes of 95% ethanol (v/v) containing 1-2% potassium acetate (w/v). The precipitate collected by centrifugation was dissolved in a known volume of water. An aliquot was used for the analysis of total GAG by estimating uronic acid content by the method of Bitter and Moir.

Estimation of carbohydrate components of glycoproteins

The carbohydrate components of serum glycoproteins were determined as described by Wagh et al. The protein obtained after precipitation of serum with ethanol (1:9 by volume) or dry defatted pancreas was digested with papain in 0.2 M acetic buffer (pH 7.0) containing 2 mg/mL of cysteine hydrochloride. The papain digest was deproteinized with ethanol (9 vol) and the supernatant evaporated to dryness in vacuum. The residue was dissolved in a known volume of water. Total hexose was determined by the phenol-sulfuric acid method of Dubois et al. Fucose by the method of Dische and Shettles, and sialic acid by the thioribarbituric acid method of Warren. Serum Mg was determined by atomic absorption spectrophotometry.
Table: Mean (SD) concentration of glycosaminoglycans, magnesium and carbohydrate components of glycoproteins and digoxin / RBC Na⁺,K⁺-ATPase activity

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Normal</th>
<th>CCP</th>
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<tbody>
<tr>
<td>Pancreas (n=7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total GAG (mg uronic acid/g dry defatted tissue)</td>
<td>3210 (110)</td>
<td>6940 (250)*</td>
</tr>
<tr>
<td>Glycoproteins (mg/g dry defatted tissue)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexose</td>
<td>186.5 (11.85)</td>
<td>99.4 (6.8)*</td>
</tr>
<tr>
<td>Fucose</td>
<td>28.98 (3.20)</td>
<td>17.88 (2.2)*</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>5.39 (1.04)</td>
<td>2.25 (0.45)*</td>
</tr>
<tr>
<td>Serum (n=15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total GAG (mg/dL)</td>
<td>2.95 (0.760)</td>
<td>24.39 (3.60)*</td>
</tr>
<tr>
<td>Magnesium (mg/dL)</td>
<td>2.20 (0.13)</td>
<td>1.57 (0.09)*</td>
</tr>
<tr>
<td>Digoxin (mg/dL)</td>
<td>12.80 (1.09)</td>
<td>22.02 (1.21)*</td>
</tr>
<tr>
<td>RBC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺,K⁺-ATPase activity (cpg/mg protein) (n=15)</td>
<td>5.04 (0.221)</td>
<td>1.24 (0.130)*</td>
</tr>
<tr>
<td>*p&lt;0.01 as compared to normal</td>
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</table>

Statistical analysis was done using ANOVA.

Results

Concentrations of total GAG in serum and pancreatic tissue were elevated in patients with CCP than in control subjects (Table). The carbohydrate components of glycoproteins (total hexose, fucose and sialic acid) were lower in pancreatic tissue of patients with CCP than in control tissue (Table).

Serum digoxin levels were higher, and RBC membrane Na⁺,K⁺-ATPase activity and serum magnesium were lower, in patients with CCP as compared to those in control subjects (Table).

Discussion

The present study found accumulation of GAG in the pancreas of patients with CCP. Kinare has reported similar results using histochemistry.²⁰ There was also a decrease in the carbohydrate component of glycoproteins in patients with CCP. The decrease in membrane Na⁺,K⁺-ATPase activity observed may be a consequence of increased level of digoxin, which is known to inhibit this enzyme activity.²¹ The decrease in Mg level may be a consequence of inhibition of membrane Na⁺,K⁺-ATPase activity.²² Whether these findings are a consequence of the chronic disease process or a causative factor is difficult to determine; however, since the patients were studied early in the course of CCP, a causative role is possible.

Increase in serum digoxin, a potent inhibitor of membrane Na⁺,K⁺-ATPase, is an important observation. Human hypothalamus is known to synthesize digoxin, which functions as an endogenous regulator of membrane Na⁺,K⁺-ATPase.²³ The inhibition of membrane Na⁺,K⁺-ATPase by digoxin has been reported to cause an increase in intracellular calcium, resulting from increased sodium-calcium exchange, increased entry of calcium via voltage-gated calcium channel and increased release of calcium from intracellular endoplasmic reticulum stores.²⁴ Increase in intracellular calcium displaces magnesium from its binding sites and decreases the functional availability of intracellular magnesium; it also decreases renal tubular magnesium reabsorption and intestinal absorption of magnesium.²⁵ This can bring about decreased mitochondrial ATP formation, which can cause further inhibition of membrane Na⁺,K⁺-ATPase, since ATP-magnesium complex is the actual substrate for this reaction. Increase in intracellular calcium in pancreatic ductular cells may lead to the multifocal calcification in the pancreas noted in CCP.

Mg⁺² deficiency may contribute to accumulation of GAG.²⁶ In the absence of adequate Mg⁺², utilization of glucose by glycolysis (for which Mg⁺² is a cofactor for the kinases) may be decreased and more glucose 6-phosphate may be channeled for producing substrates for GAG synthesis, viz., UDP-uronic acids and UDP-hexosamines. Mg⁺² deficiency can also cause alteration in glycosylation of protein.²⁷ Mg⁺² is needed for the formation of dolichol-1-phosphate from dolichol, which is a rate-limiting step in the formation of N-glycosylated protein. It is also required for the formation of nucleoside diphosphate sugars required for O-glycosylation. The low carbohydrate components of glycoproteins in the pancreas in CCP may also be due to Mg deficiency.

The increase in GAG in the pancreas in CCP is significant from another point of view. GAG are polyanionic molecules that can interact with cations including cationic proteins. Such interaction particularly between heparan sulfate proteoglycan or chondroitin sulfate proteoglycan with protein has been reported.²¹ This interaction makes the proteoglycan-protein complex resistant to catabolism, resulting in its accumulation in the organ,²⁸ a phenomenon that may contribute to the pathogenesis of CCP.

A high intake of dietary fiber has been shown by us to result in increased fecal Mg excretion.²² We have also reported previously that a diet deficient in Mg results in accumulation of GAG in most tissues including the pancreas.²⁷ The diet in the patient and control populations contained the same amount of dietary fiber; but magnesium deficiency was seen only in patients with CCP. Therefore magnesium deficiency may be more a reflection of hyperdigoxinemia than of dietary fiber intake.

Thus, elevated digoxin levels, membrane Na⁺,K⁺-ATPase inhibition and consequent hypomagnesemia may play a role in the pathogenesis of CCP. This leads to tissue mucopolysaccharidosis in the pancreas, leading to CCP. There are no previous reports on the role of hyperdigoxinemia-related magnesium deficiency and altered glycoconjugate metabolism in CCP.

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References

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Received January 19, 2001. Received in final revised form April 12, 2001. Accepted May 14, 2001.