



Document heading doi: 10.1016/S1995-7645(14)60349-7

Correlation between expression of 1 α -hydroxylase and hypocalcaemia in rats with severe pancreatitis

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ARTICLE INFO

Article history:

Received 15 February 2015
Received in revised form 20 March 2015
Accepted 15 April 2015
Available online 20 May 2015

Keywords:

Severe pancreatitis
Serum calcium
1, 25 dihydroxy vitamin D3
1-hydroxylase

ABSTRACT

Objective: To investigate the essential biochemical indices like 1 α -hydroxylase and hypocalcaemia in the rats with severe acute pancreatitis and explore the correlation between them. **Methods:** A total of 120 SPF grade Wistar male rats which were in similar physiological status were selected and randomly divided into two groups: sham group (SO group) and severe acute pancreatitis group (SAP group). Then they were divided into 1 h, 3 h, 6 h, and 12 h subgroups according to the killing time. The severe acute pancreatitis model was established by retrograde injection of 5% sodium taurocholate. Serum calcium, serum creatinine, serum urea nitrogen and serum amylase were measured at different time. Serum 1, 25 dihydroxy vitamin D3 level was determined by enzyme linked immunosorbent assay. The expression of 1 α -hydroxylase protein in the kidney tissue was determined with Western blotting and immunohistochemistry to observe its location. The pathologic features of the kidney tissue section was observed under light microscope and submicroscopic structure of the proximal convoluted tubule epithelial cell was observed under transmission electron microscope. **Results:** Compared with the SO group, rats in the SAP group showed continuous pathological injury as time went by. There was significant increase in serum creatinine, serum urea nitrogen and serum amylase in SAP group compared with the SO group 1, 3, 6, 12 hours after the operation ($P < 0.05$). There was significant decrease in serum calcium and 1, 25 dihydroxy vitamin D3 3, 6, 12 hours after the operation ($P < 0.05$). It also showed that the expression of the 1 α -hydroxylase protein in kidney tissues was upregulated at 1 h, 3 h and decreased at 6 h, 12 h compared with the SO group. The serum calcium, 1, 25 dihydroxy vitamin D3 and the expression of the 1 α -hydroxylase protein in kidney tissues of the SAP group showed sustaining decrease. Western blotting showed positive correlation between the 1 α -hydroxylase expression and serum calcium at 3 h, 6 h and 12 h ($r = 0.976$, $P < 0.001$; $r = 0.948$, $P < 0.001$; $r = 0.742$, $P = 0.001$) and also positive correlation between the 1 α -hydroxylase expression and serum 1, 25 dihydroxy vitamin D3 at 1 h, 3 h, 6 h and 12 h ($r = 0.935$, $P < 0.001$; $r = 0.952$, $P < 0.001$; $r = 0.917$, $P < 0.001$; $r = 0.874$, $P < 0.001$). **Conclusions:** At the early stage of the kidney injury, the expression of 1 α -hydroxylase in the kidney tissue is reduced with the progress of the disease and the decrease in its activity has a correlation with the hypocalcaemia.

1. Introduction

Severe pancreatitis is a common type of acute abdominal disease.

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Foundation project: It is supported by Natural Science And Technology Fund of Sichuan Province (sc2812345).

The patient is often in critical condition because of systemic inflammatory response syndrome induced by pancreatitis, one or multiple organ failure or severe metabolic disorder. The common metabolic disorder is hypocalcaemia and the serum calcium can be reduced to 2.0 mmol/L in severe acute pancreatitis[1]. Domestic and foreign scholars put forward various theories, such as increase of free fatty acid, hypoproteinemia, abnormality of parathyroid hormone synthesis and secretion, and increase of calcitonin. But at

present the theories remain controversial, and pathophysiology of hypocalcaemia is not clearly [2,3]. Therefore, this study established the model of rats with severe acute pancreatitis, measured their multiple biochemical indices at early pathological stage, and explored the correlation between the expression of 1-hydroxylase in the kidney tissue and the serum calcium.

2. Materials and methods

2.1. Experimental animals and grouping

A total of 120 SPF grade Wistar male rats aged from 7 to 8 weeks and weighing (284 ± 22) g were selected, which were in similar physiological status, and they were divided into two groups: sham group (SO group) and severe acute pancreatitis group (SAP group). Then every group was divided into 1 h, 3 h, 6 h, 12 h subgroups ($n=15$) according to the killing time.

2.2. Establishment of animal models

All rats were fasted but fed with water 12 h before modeling establishment, which were carried out under sterile condition with preoperative intraperitoneal anesthesia. In SAP group the abdominal cavity was incised from central abdomen approaching the hepatic portal, then the common bile duct was clipped with noninvasive vascular clamp temporarily. The main pancreatic duct was retrograde punctured in the descending part of duodenum with No.4, 5 scalp needles. The model was established by retrograde injection of 5% sodium taurocholate (1 mL/kg) along with pancreatic duct in a constant speed (0.5 mL/min). The model was successfully established when pancreatic hemorrhage and edema appeared 5 min after the main pancreatic duct clipping. Then the vascular clamp was removed and the abdomen was closed. The SO group was treated with the same anesthesia and laparotomy as the SAP group, while the abdomen was closed without treatment after the bowel was flipped. Rats of two groups were treated with infusion (20 mL/kg) after operations.

2.3. Samples collection

After successful modeling, the rats were treated with intraperitoneal anesthesia (with 10% chloral hydrate, 3 mL/kg) at the corresponding time points. Five mL blood was drawn by cardiac puncture with a 5 mL injector inserted into the abdominal cavity through the surgical incision. And the serum was centrifugally separated (2 000 r/min, 15 min). The appropriate size of kidney tissue was removed. One part was fixed with paraformaldehyde, and another was fixed with electron microscope fixative. The remaining part was stored in the

refrigerator at -80°C for the extraction of protein. The rats were killed by air embolism after required materials collection.

2.4. Detection of serum indices

The serum calcium, serum creatinine, serum urea nitrogen and serum amylase were measured by the automatic biochemical analyzer (Olympus Corporation, Japan) after analyzing the serum samples. The serum 1, 25 dihydroxy vitamin D3 level was detected by 1, 25 dihydroxy vitamin D3 enzyme linked immunosorbent assay (ELISA) kit.

2.5. Pathological observation of the kidney

The kidney tissue fixed with paraformaldehyde was embedded in paraffin routinely, sectioned with microtome and stained with HE. The morphological change of the kidney tissue section was observed under the light microscope.

2.6. Expression and location of 1 α -hydroxylase in the kidney

Immunohistochemical SABC staining with a primary antibody of 1:200 and a DAB developing kit for staining was used. Five sections were selected in each group at each time point for positive cell count under the light microscope with a high power field ($\times 400$). The positive rate = number of positive cells/ total number of cells in view, which was the index of the expression of 1-hydroxylase in the kidney and compared between groups. Cytoplasm was dyed brown yellow in view in positive cells. The cells were divided into four groups: negative (-, without positive cells in view), weakly positive (+, the positive rate $\leq 25\%$), positive (++, the positive rate was 25%-50%), strongly positive (+++, the positive rate $\geq 50\%$).

2.7. Determination of the 1 α -hydroxylase expression

Protein was extracted from the pancreas tissue with conventional methods and its concentration was determined by BCA kit (Keygen). The protein was separated with acrylamide gel electrophoresis. The film (200 mA, 70 min) was transferred after the target protein has been completely separated, and sealed overnight at 4°C with 10% skim milk. The primary antibody dilution of 1:2 000 (SANTACRUZ) was added, then it was incubated overnight at 4°C . Horseradish peroxidase-marked secondary antibody dilution of 1:3 000 (SANTACRUZ) was added and incubated at room temperature for 1 h. It was developed avoiding light. Western blotting bands were analyzed using analysis software Image J with reference protein of β -actin. The 1-hydroxylase expression was expressed by gray value of 1-hydroxylase band/ gray value of β -actin band.

2.8. Observation of the submicroscopic structure with the electron microscope

The submicroscopic structure of the proximal convoluted tubule epithelial cell was observed with the transmission electron microscope. And required pictures were collected by software.

2.9. Statistical analysis

The data were analyzed with SPSS15.0 statistical software. Measure data were expressed as mean±sd, and analyzed by analysis of variance and *t* test between groups. The comparison of SO group and SAP group at each time point was analyzed by independent samples *t* test. The experimental data in the SAP group at each time point were compared with analysis of variance. *P* value<0.05 was considered statistically significant different.

3. Results

3.1. Serum indices in rats

The difference of serum creatinine, serum urea nitrogen, serum amylase, serum calcium, and 1, 25 dihydroxy vitamin D3 in the SO group at 1 h, 3 h, 6 h, 12 h was not statistical significant (*P*>0.05). As time passing by in the SAP group, the serum creatinine, serum urea nitrogen and serum amylase were increased gradually (*P*<0.05), while the serum calcium and 1, 25 dihydroxy vitamin D3 were decreased (*P*<0.05) (Table 1).

Compared with the SO group at each time point, there was an increase of the serum creatinine, serum urea nitrogen and serum amylase (*P*<0.05), but decrease of the serum calcium and 1, 25 dihydroxy vitamin D3 (*P*<0.05) in the SAP group (Table 1).

It was shown that with the construction of severe acute pancreatitis rat model and the progress of the disease, rats had a continuous

kidney injury and there was a gradual decrease in serum calcium.

3.2. Pathological changes of the kidney in rats

In the kidney tissue of the SO group, structures of glomerular, renal tubules and renal interstitium were normal. There was no obvious edema or inflammatory cell infiltration. The cell was morphologically intact and had clear boundary. Morphological changes were obvious at each time point in rats of the SAP group. At 1 h mild edema appeared in renal tubular epithelial cell, while there was no obvious pathological changes in glomerular and renal interstitium; at 3 h the renal tubular epithelial cell showed obvious swelling, brush border was obviously damaged, and mild edema appeared in renal interstitium; at 6 h the renal tubular epithelial cell showed a wide range of obvious edema, partially became necrosis, fell off and blocked the lumen, forming tube type; at 12 h a wide range of renal tubular epithelial cells showed necrosis and fell off, and there were a large amount of cell debris and tube types in the lumen. As time passing by, the renal tubular epithelial cell gradually showed edema, inflammation and necrosis, and the injury spread from the epithelial cell to interstitium, with the appearance of tube types.

3.3. Expression and location of 1 α -hydroxylase with immunohistochemistry

The renal tubular epithelial cells in the kidney tissue were dyed brown yellow with blue nucleus after SABC staining with immunohistochemistry. The dyeing of the proximal convoluted tubule was obvious. The degree of dyeing indicated the expression and distribution area of 1 α -hydroxylase. The expression of 1 α -hydroxylase in the SAP group was as follow: strongly positive (+++) at 1h, positive (++) at 3 h, weakly positive (+) at 6 h and 12 h. As time passing by, the expression of 1 α -hydroxylase was decreased gradually (Figure 1).

Table 1

Serum indices in rats of the SO and SAP group at each time point (*n*=15, mean±sd).

Group	Serum creatinine (μ mol/L)	Serum urea nitrogen (mmol/L)	Serum amylase (U/L)	Serum Ca ²⁺ (mmol/L)	1, 25 dihydroxy vitamin D3 (ng/L)
SO group					
1 h	55.9±7.2	5.29±0.69	1 073.6±173.5	2.29±0.09	6.79±0.54
3 h	58.2±5.3	5.33±0.71	1 095.4±181.5	2.28±0.11	6.82±0.63
6 h	56.7±8.2	5.31±0.75	1 087.3±183.2	2.27±0.13	6.75±0.48
12 h	53.5±6.4	5.38±0.68	1 069.7±213.9	2.30±0.11	6.77±0.58
SAP group					
1 h	83.1±11.5 ^{ab}	6.83±0.41 ^{ab}	1 883.5±145.8 ^{ab}	2.28±0.08 ^a	6.92±0.36 ^a
3 h	113.7±12.4 ^{ab}	8.31±0.48 ^{ab}	3 346.2±356.3 ^{ab}	2.13±0.12 ^{ab}	5.74±0.45 ^{ab}
6 h	150.2±13.6 ^{ab}	9.83±0.67 ^{ab}	4 571.7±375.9 ^{ab}	1.79±0.11 ^{ab}	4.39±0.33 ^{ab}
12 h	215.4±17.9 ^{ab}	12.03±0.58 ^{ab}	6 193.3±509.4 ^{ab}	1.08±0.14 ^{ab}	3.01±0.26 ^{ab}

Compared within the SAP group at each time point, ^a*P*<0.05; compared with SO group, ^b*P*<0.05.

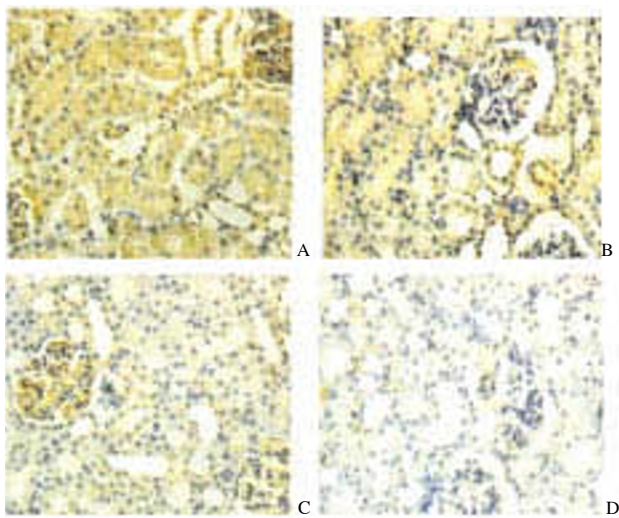


Figure 1. Expression and location of 1-hydroxylase with immunohistochemistry in the kidney tissue of rats.

A: 1 h after modeling, strongly positive (+++); B: 3 h after modeling, positive(++); C: 6 h after modeling, weakly positive(+); D: 12 h after modeling, weakly positive(+).

3.4. Determination of the 1α -hydroxylase expression with western blotting

The protein was extracted from the kidney tissue in rats for western blotting. The bands after developing avoiding light were shown in Figure 2. The expression in the SO group at each time point showed no significant difference ($P>0.05$); as time passing by, the expression in the SAP group was decreased gradually ($P<0.05$). And the 1α -hydroxylase expression in the SAP group was increased significantly at 1 h, 3 h when compared with the SO group ($P<0.05$) (Table 2).

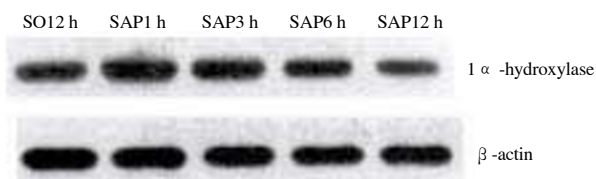


Figure 2. Qualitative detection of 1α -hydroxylase expression with western blotting in the kidney tissue.

3.5. Submicroscopic structure of the renal proximal convoluted tubule epithelial cell

In the SO group, the proximal convoluted tubule epithelial cell

had complete structure, clear boundary and round or oval nucleus. There were large number of mitochondria in the cytoplasm. The microvilli protruded into the lumen. The morphology in the SAP group at each time point was as follow: at 1 h in the proximal convoluted tubule epithelial cell, the morphology of mitochondria was normal and mitochondrial cristae had clear structure; at 3 h in the proximal convoluted tubule epithelial cell, the mitochondria were swelling and the disorder of villus appeared; at 6 h in the proximal convoluted tubule epithelial cell, the mitochondria were swelling significantly and some showed vacuolation; at 12 h in the proximal convoluted tubule epithelial cell, the nuclear morphology changed, mitochondrial swelling was so severe that the mitochondrial cristae couldn't be seen, and lysosomes was increased significantly (Figure 3).

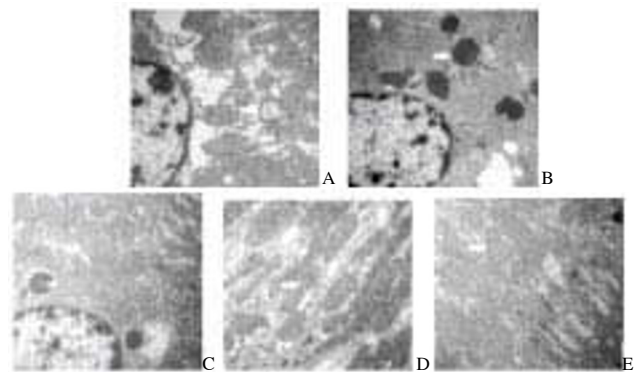


Figure 3. Submicroscopic structure of the renal proximal convoluted tubule epithelial cell under the transmission electron microscope ($\times 10\,000$).

A: In the SO group, the proximal convoluted tubule epithelial cell had complete structure, clear boundary and round or oval nucleus. There were large number of mitochondria in the cytoplasm; B: in the SAP group, at 1 h in the proximal convoluted tubule epithelial cell, the morphology of mitochondria was normal and mitochondrial cristae had clear structure; C: at 3 h in the proximal convoluted tubule epithelial cell, the mitochondria were swelling and the disorder of villus appeared; D: at 6 h in the proximal convoluted tubule epithelial cell, the mitochondria were swelling significantly and some showed vacuolation; E: at 12 h in the proximal convoluted tubule epithelial cell, the nuclear morphology changed, mitochondrial swelling was so severe that the mitochondrial cristae couldn't be seen, and lysosomes increased significantly.

3.6. Analysis of correlation

The 1α -hydroxylase expression and the serum indices of $1,25$ dihydroxy vitamin D₃ and serum calcium of the kidney tissue in the

Table 2

Quantitative analysis of 1α -hydroxylase expression with Western Blotting in the kidney tissue.

Group	1 h	3 h	6 h	12 h
SO group	0.79±0.04	0.83±0.02	0.80±0.03	0.81±0.03
SAP group	1.21±0.03 ^{cd}	0.89±0.04 ^{cd}	0.75±0.05 ^{cd}	0.61±0.04 ^{cd}

Compared within the SAP group at each time point, ^c $P<0.05$, compared between the SAP group and SO group at each time point, ^d $P<0.05$.

SAP group at each time point were entered into the SPSS software before respective correlation analysis. There was positive correlation between the 1-hydroxylase expression in the kidney tissue and serum 1, 25 dihydroxy vitamin D3 at 1 h, 3 h, 6 h, 12 h, which was more than 85% ($r=0.935$, $t=9.506$, $P<0.001$; $r=0.952$, $t=11.214$, $P<0.001$; $r=0.917$, $t=8.289$, $P<0.001$; $r=0.874$, $t=6.485$, $P<0.001$). The 1-hydroxylase expression in the kidney tissue was also strongly positively correlated with the serum calcium ($r=0.976$, $t=16.159$, $P<0.001$; $r=0.948$, $t=10.739$, $P<0.001$; $r=0.742$, $t=3.991$, $P=0.001$). The results showed highly positive correlation between the 1-hydroxylase expression and the serum indices of 1, 25 dihydroxy vitamin D3 and serum calcium of the kidney tissue in the SAP group at each time point.

4. Discussion

Kidney is the common complicatedly injury organ of severe pancreatitis. Study of clinical cases shows that, once pancreatitis are complicated with acute renal failure occurs, the mortality rate of patients can be as high as 80%[4]. The severe acute pancreatitis will activate a lot of trypsin in the body, leading to the production of large amounts of cytokines, inflammatory mediators and vasoactive mediators, as well as the occurrence of immune reaction to some extent in the body. In this process, the patient often will be complicated with hypocalcaemia, and even with nervous system and cardiovascular symptoms, which will aggravate the disease[5]. Many clinical studies have proved that, hypocalcaemia is significantly correlated with the prognosis of pancreatitis. There is a positive correlation between the degree of hypocalcaemia and the progression of the disease. However, the pathophysiological mechanism of hypocalcaemia complicated with severe pancreatitis is still unclear at present. Through the clinical analysis of physiological indices towards these patient, it has been found that both the serum 1, 25 dihydroxy vitamin D3 expression and the 1-hydroxylase expression in the kidney tissue is decreased to a certain degree, suggesting that there may be some mechanisms to regulate and influence each other among them[6]. After a series of clinical cases observation and literature consultation, we constructed severe pancreatitis rat model, and determined the serum indices and the 1-hydroxylase expression of the kidney tissue in rats at each time point, hoping to explore their relationship.

1, 25 dihydroxy vitamin D3 is a kind of open loop steroidal hormone. As an important hormone to regulate serum calcium in the body, it plays a very important role in the metabolism of calcium and bone. It puts the bone calcium into the blood, by means of promoting the absorption of ingested calcium by small intestinal mucosa and increasing the number of osteoclast. It can also promote the reabsorption of calcium by renal tubule in urine. Through these ways

mainly, it keeps the serum calcium in a normal range. In addition, it can also regulate the serum calcium indirectly through the effect on the secretion of parathyroid hormone[7,8]. According to the results of several studies on 1, 25 dihydroxy vitamin D3, after taking 1, 25 dihydroxy vitamin D3, the serum calcium is increased significantly in a short time[9]. In the experimental animal model like rats, after knocking out vitamin D3 receptor gene in rats, the serum calcium decreases, which indicates that a lack of 1, 25 dihydroxy vitamin D3 in rats leads to the regulation disorder of serum calcium, and then results in the decrease of serum calcium. All of these results suggest that the serum calcium in the body is regulated by 1, 25 dihydroxy vitamin D3[10,11].

1-hydroxylase (1-HYD, also called mitochondrial cytochrome P150) is a protease synthesized by mitochondrion of the renal proximal convoluted tubule epithelial cell. Its expression is regulated by CYP1 gene. It plays an important role in the body as the key enzyme in the synthesis of 1, 25 dihydroxy vitamin D3[12]. In normal human body, only in the kidney can we detect that the 1-hydroxylase catalyzes the transformation from 25 dihydroxy vitamin D3 to 1, 25 dihydroxy vitamin D3. The presence of the biochemical reaction and 1-hydroxylase activity can't be detected in other tissues[13]. Therefore, 1-hydroxylase plays an important role in the normal expression of 1-hydroxylase. And a number of experimental studies have also proved that when lacking 1, 25 dihydroxy vitamin D3, an increase of 1-hydroxylase activity can be detected in the body, suggesting that the body compensatorily increases the 1-hydroxylase activity expression[14,15]. This physiological activity is also affected by the serum calcium in the body. Previous experiments have proved that, the serum calcium with excessively high concentration will cause the inhibition of the 1-hydroxylase gene expression, thus leading to a decrease of 1, 25 dihydroxy vitamin D3 concentration[16,17]. All have proved that in the kidney tissue, the 1-hydroxylase expression will directly determine the concentration of serum 1, 25 dihydroxy vitamin D3, and this activity may be influenced by the serum calcium. In this study, the conclusion is also verified by our experimental results, in which the 1-hydroxylase expression in the kidney tissue, the serum 1, 25 dihydroxy vitamin D3 concentration and the serum calcium decreased in rats at each time point in the SAP group. According to statistical analysis, there is a positive correlation among their changes at related time point, and the correlation is high.

In severe acute pancreatitis, necrosis and apoptosis often appear in the renal tubular epithelial cell because of injury and inflammation, resulting in the renal proximal tubule damage and the appearance of tube type in the renal tubular lumen. It has been proved that the injury factors will lead to the disorder of serum calcium[18,19]. In this study, we morphologically observed the pathological changes of the renal tubular epithelial cell with HE staining and the submicroscopic structure changes of the renal proximal convoluted tubule epithelial

cell with electron microscope. Inflammation, necrosis and falling off could be observed, and mitochondria lost normal structure. At the same time, it was shown that the 1-hydroxylase expression in the rat kidney decreased with immunohistochemistry. Considering the decrease of serum calcium, as well as the quantitative analysis results of 1-hydroxylase expression with western blotting, it can be concluded that there is a strongly positive correlation between them.

According to the above results, a conclusion can be drawn that at the early stage of the severe pancreatitis, with the progress of the disease, kidney injury causes the inflammation injury and even necrosis and apoptosis in the proximal convoluted tubule epithelial cell, resulting in a decrease of 1-hydroxylase expression in the kidney tissue, and thus leading to a lack of the synthesis of serum 1, 25 dihydroxy vitamin D3. Several ways in which the 1, 25 dihydroxy vitamin D3 regulates the serum calcium are inhibited, leading to a decrease of the serum calcium and thus the occurrence of hypocalcaemia[20].

Conflict of interest statement

We declare that we have no conflict of interest.

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