

Metabolic characteristics of acute necrotizing pancreatitis and chronic pancreatitis

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Abstract. The etiology and pathogenesis of pancreatitis remains unclear. In the presence of pancreatic inflammation, metabolite abnormalities appear before transformation of tissue structure and changes in functions occur. Detection of abnormalities in metabolite levels facilitates a greater understanding of the pathophysiological events and aids in the early diagnosis of the disease. In this study, metabolic profiles from the pancreas of Wistar rats were examined using high-resolution proton magic angle spinning nuclear magnetic resonance (MAS NMR) spectroscopy to investigate the metabolite indicator(s) of acute necrotizing pancreatitis (ANP) and chronic pancreatitis (CP). The animals were divided into three groups: those with histologically confirmed ANP (n=7), those with CP (n=6) and a control group (n=9). The processed NMR spectra were analyzed using principal component analysis (PCA) to extract characteristic metabolites of ANP and CP. Levels of leucine, isoleucine and valine were increased in the ANP group, whereas an opposite trend was observed in the CP group. Increases in phosphocholine, glycerophosphocholine and choline levels, and decreases in fatty acids, lactate, betaine and glycine levels were observed in both the ANP and CP groups. Additionally, the lipid content in the CP group was higher than that observed in the ANP group. An increase in taurine levels was observed only in the CP group. In conclusion, pancreatitis causes a disruption of the metabolism in the pancreas at a molecular level, with increased taurine levels being a potential metabolite indicator for those with CP.

Introduction

Pancreatitis has long been recognized as a painful and potentially fatal condition. The key histopathological features include pancreatic fibrosis, acinar atrophy, inflammation, and

distorted and blocked ducts (1-3). Pancreatitis has been classified into two subtypes, acute pancreatitis (AP) and chronic pancreatitis (CP) (4). As a severe acute pancreatitis, acute necrotizing pancreatitis (ANP) usually results from pancreatic glandular necrosis and accounts for 10-40% of the mortality rate (5-8). Chronic pancreatitis is a condition characterized by progressive and irreversible damage to both exocrine and endocrine components of the gland, eventually resulting in significant exocrine insufficiency and diabetes (9,10). Patients with CP are also at a markedly increased risk of developing pancreatic cancer (11-13).

Despite many years of experimental and clinical investigations, both in animals and in humans, the etiology and pathogenesis of pancreatitis remains unclear (10,14-16). In different countries and regions, due to geographical factors, economic status, living habits and many other variables, both the risk factors and the incidence of pancreatitis demonstrate great variability (10,17). To date, all we know for certain is that the condition results from a multiplicity of factors.

Several difficulties still exist both in the areas of research and treatment. Histological techniques offer visualization of the differences between healthy and affected individuals, but they often necessitate an invasive procedure, which leads to complications. Imaging technologies provide visual information on the focus of disease, but are less helpful in early diagnosis. Genomics locates the gene that promotes pancreatic fibrosis, but gene expression is restricted by environmental factors (18-21). The classification of pancreatitis remains complicated and imprecise (22,23), and a correlation between morphological changes and pancreatic function has yet to be established (24,25). However, as the incidence of pancreatitis is increasing, it is imperative to explore its pathogenesis, so as to aid in the diagnosis and treatment, especially at the early stage of the disease.

In general, in the presence of inflammation of the pancreas, metabolite abnormalities appear before transformation of the tissue structure and changes in functions occur. Detection of abnormalities in metabolite levels facilitates a greater understanding of the pathophysiological events and aids in the early diagnosis of the disease (26). Clinically, serum amylase and serum lipase are widely used as markers of pancreatitis. However, these markers are not sensitive, show low specificity and are primarily used as additional tools (27). Abnormal levels of serum amylase and serum lipase are caused by

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a block in the excretion of pancreatic juice, which causes changes in pancreatic function. As the second largest gland in the body, the pancreas plays a critical role in the metabolism. The metabolic profile of the pancreas demonstrates abnormal characteristics when the organ is affected by certain diseases.

In the present study, we investigated whether it is possible to obtain information that could assist us in our understanding of the pathogenesis of pancreatitis and facilitate our ability to differentiate between ANP and CP at a molecular level, by analyzing pancreatic tissue samples using a metabolic profiling strategy. To clarify the metabolic characteristics of pancreatitis, disease models of ANP and CP were created experimentally, using male Wistar rats. The metabolic profile of the pancreas was investigated using high-resolution proton magic angle spinning nuclear magnetic resonance (^1H MAS NMR) spectroscopy (28-30) of intact tissue, which was obtained from the animals. The processed NMR spectra of the pancreatic tissue were analyzed using principal component analysis (PCA) (31,32) in order to visualize separation among the samples and to extract characteristic metabolites of ANP and CP.

Materials and methods

Animals. A total of 22 male Wistar rats (~2 months old, weighing 200-220 g, obtained from the experimental animal center of the Second Military Medical University, Shanghai, China) were kept under environmentally controlled conditions (temperature, $22\pm 2^\circ\text{C}$; light/dark cycle, 12/12 h; relative humidity, 60-70%). The rats were divided into three groups: the control group ($n=9$), the ANP group ($n=7$) and the CP group ($n=6$). The disease model of CP was induced via the administration of a single dose of DBTC solution (intravenous, 8 mg/kg), which was composed of DBTC (Sigma, St. Louis, MO, USA), alcohol and glycerol. The molar ratio of DBTC, alcohol and glycerol was 1:2:3 (33). An equivalent dose of saline solution was administered to the control group, which was maintained under the same conditions as the CP group. The disease model of ANP was induced via two injections of an L-arginine solution (20%, 2.5 mg/g) with a 1-h interval (34). The study was approved by the local Animal Ethics Committee of the Shanghai Changhai Hospital Ethics Committee, Shanghai, China.

Tissue samples. Twelve hours after the injections of the L-arginine solution, the ANP group mice (~2 months old, weighing 200-220 g) were sacrificed. Two months after injection of the DBTC/saline solution, the CP group and the control group mice (~4 months old, weighing 310-330 g) were sacrificed. The pancreatic tissues were excised from the middle sections in each group, in order to minimize variation due to possible topographical differences. Each tissue sample was cut into two parts: one was placed in formalin for histological tests; the other was transferred into cryo-tubes, labeled with unique identifiers, snap-frozen in liquid nitrogen and stored at -80°C prior to ^1H MAS NMR analysis.

Histology. The tissue samples that had been placed in formalin (4%) were cut, embedded in paraffin and processed routinely. Sections (4 μm) for all samples were stained with

H&E and then examined under a light microscope. Two experienced pathologists reviewed all the sections. In the CP group, the lobular structure was found to be destroyed, the acini had disappeared, collagen fibers had proliferated and an inflammatory cell infiltrate was present. Pancreatic tissue from the ANP group revealed the presence of edema, hemorrhage and yellow-white calcification spots visible with the naked eye. Using light microscopy, it was observed that the acinus structure was destroyed, there were large areas of tissue putrescence, red blood cells appeared in the pancreatic parenchyma and many inflammatory cells had infiltrated the tissue spaces (Fig. 1). No marked pathological changes in the pancreatic tissue were observed in the control group.

MAS NMR experiments. ^1H MAS NMR experiments were recorded using an AVANCE II 600 spectrometer equipped with a 4-mm double resonance ($^1\text{H}/^{13}\text{C}$) MAS probe (^1H frequency at 600.13 MHz; Bruker Biospin, Germany). Tissue samples of ~15 mg were cut and placed into a 4-mm outer diameter zirconia MAS rotor with drops of D_2O as the field lock and spun at 5 kHz. Spectra were acquired at 300.0 K using Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence [relaxation delay (RD)- 90° -(τ - 180° - τ) $_n$ -acquire free induction decay (FID)] with water pre-saturation during the relaxation delay of 2 sec. CPMG pulse sequence was applied as a T2 filter to moderately attenuate signals from the macromolecules with short spin-spin relaxation time. A spin relaxation delay (2 $\pi\tau$) of 180 ms was used. The main parameters used for ^1H MAS NMR experiments were: spectra width (SW) = 15 kHz; data points (TD) = 64 k and number of scans (NS) = 160. Spectral assignments were confirmed by 2-dimensional ^1H - ^1H correlation spectroscopy (COSY) and ^1H - ^1H total correlation spectroscopy (TOCSY) together with information from published data. The stability of the tissue samples was validated by repeating a 1-dimensional NMR experiment after overall acquisition. No biochemical degradation was observed in any of the tissue samples.

Principal component analysis. Spectral data were carefully manually phased and baseline-corrected using Topspin 3.1 software (Bruker Biospin). All free induction decays (FIDs) were multiplied with a 0.5-Hz exponential line broadening prior to Fourier transformation into 128 K points. Each ^1H MAS NMR spectrum was segmented into 170 regions with equal width (0.05 ppm) over the chemical shift region $\delta 0.50$ -9.00 ppm and the signal intensity in each region was integrated using AMIX (version 3.9.5, Bruker Biospin) software. The region $\delta 4.60$ -5.10 ppm was removed in order to minimize the effect of the imperfect baseline caused by the water suppression. Prior to PCA, each integral region was normalized by dividing by the sum of all the utilized integral regions for each spectrum. In order to minimize the effects of the lipids, PCA was again performed for the ^1H NMR spectrum over the range $\delta 2.9$ -9.0 ppm with removal of the region $\delta 4.60$ -5.50 ppm, reducing to 122 regions of equal width (0.05 ppm). PCA was used to calculate a new set of values of uncorrelated variables from linear combinations of the observations of possibly correlated variables within the data. These new uncorrelated variables are called principal components, and the distribution of their values (scores) permits the simple

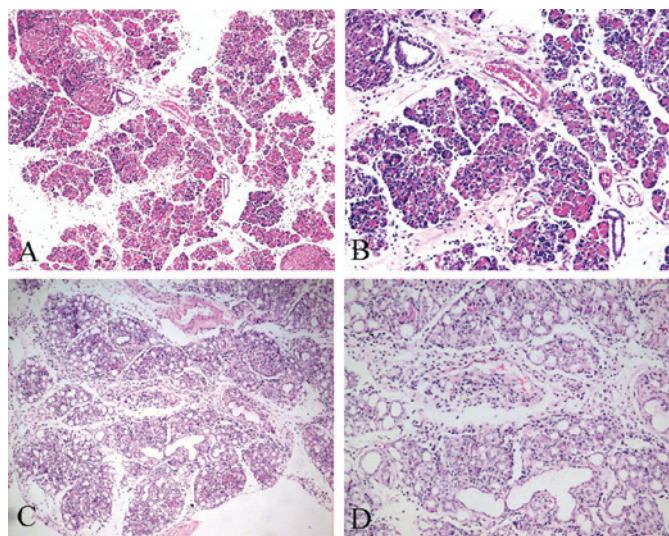


Figure 1. (A and B) Representative photomicrographs of acute necrotizing pancreatitis. (A) H&E, magnification, x100. (B) H&E, magnification, x400. (C and D) Representative photomicrographs of chronic pancreatitis. (C) H&E, magnification, x100. (D) H&E, magnification, x400.

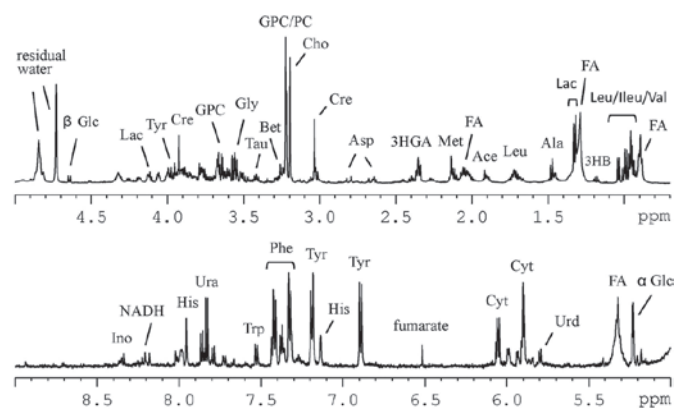


Figure 2. Assignment of proton magic angle spinning nuclear magnetic resonance spectra to pancreatic tissue with ANP. The region of δ 5.00-9.00 ppm is zoomed-in 16 times of that in δ 0.70-5.00 ppm (600 MHz, CPMG pulse sequence, temperature 300.0 K). 3HB, 3-hydroxybutyrate; 3HGA, 3-hydroxyglutaric acid; α -Glc, α glucose; β -Glc, β glucose; Ace, acetate; Ala, alanine; Asp, aspartate; Bet, betaine; Cho, choline; Cre, creatine; Cyt, cytidine; FA, fatty acid; Glc, glucose; Gly, glycine; GPC, glycerophosphocholine; His, histidine; Ileu, iso-leucine; Ino, inosine; Lac, lactate; Leu, leucine; Met, methionine; PC, phosphocholine; Phe, phenylalanine; Tau, taurine; Trp, tryptophan; Tyr, tyrosine; Ura, uracil; Urd, uridine; Val, valine.

visualization of separation or clustering among different samples. The weightings (loadings) that are given to each integral region in calculating the principal components allows for the identification of those spectral regions for the maximum impact to the separation or clustering. Thus, we may obtain information on the biomarkers of ANP and CP by analyzing MAS NMR spectra with PCA.

Results

NMR experiments. Combining 2D NMR experimental results of the present study with published data of related literature (31,35,36), we identified the main resonances in ^1H NMR spectra of pancreatic tissue (Fig. 2). The components, which

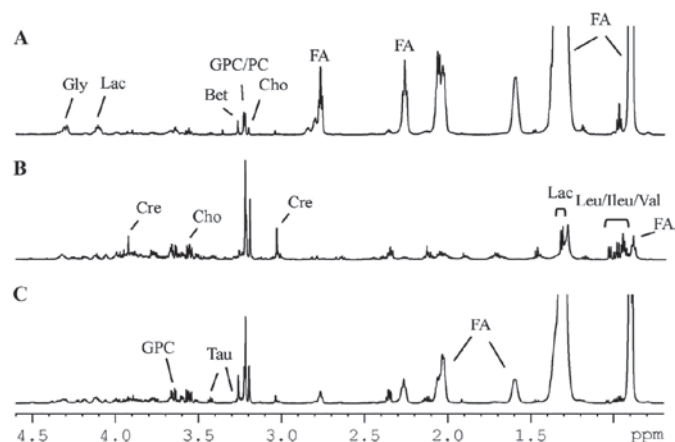


Figure 3. Three typical 600-MHz ^1H MAS NMR CPMG spectra obtained from a rat pancreas; (A) control, (B) acute necrotizing pancreatitis and (C) chronic pancreatitis group.

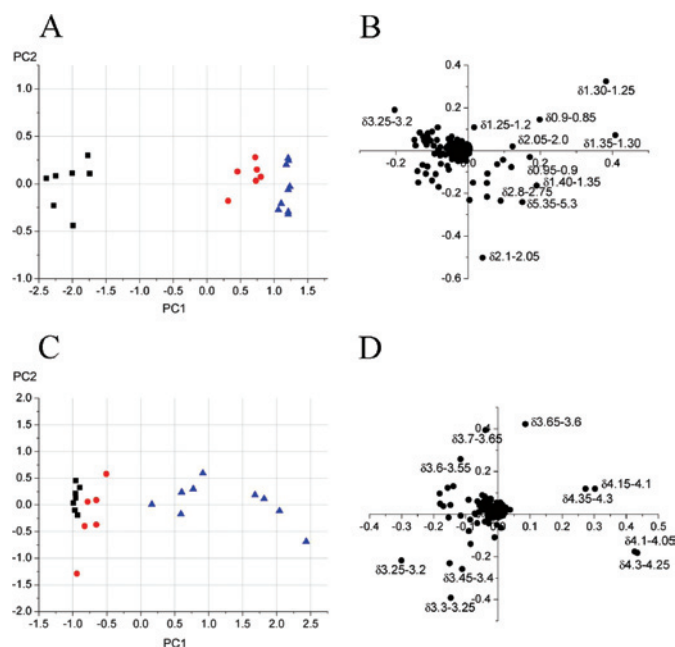


Figure 4. (A and C) Principal component analysis scores plots and (B and D) loadings plots. (A and B) From data of the ^1H CPMG NMR spectra (80.50-9.00 ppm). (C and D) From data of the ^1H CPMG NMR spectra (82.9-9.0 ppm). \blacktriangle Control group; \blacksquare acute necrotizing pancreatitis group; \bullet chronic pancreatitis group.

include leucine (Leu), iso-leucine (Ileu), valine (Val), fatty acids (FA), lactate (Lac), alanine (Ala), leucine (Leu), acetate (Ace), methionine (Met), 3-hydroxyglutaric acid (3HGA), aspartate (Asp), creatine (Cre), choline (Cho), phosphocholine (PC), glycerophosphocholine (GPC), betaine (Bet), taurine (Tau), glycine (Gly), tyrosine (Tyr), β glucose (β -Glc), α glucose (α -Glc), uridine (Urd), cytidine (Cyt), tyrosine (Tyr), histidine (His), phenylalanine (Phe), tryptophan (Trp), uracil (Ura) and inosine (Ino), and the locations, are clearly demonstrated.

Fig. 3 shows representative spectra for ^1H MAS NMR of the control group, the ANP group and the CP group. Compared to Fig. 3A, some peaks in Fig. 3B and C are similar; for example, an increase in GPC/PC and Cho, and a decrease in FA, Lac, Bet and Gly were observed in both the CP and ANP groups,

despite a more marked fluctuation in the CP group. However, the other peaks revealed a distinctively opposite trend, with Leu, Ileu and Val decreasing in the CP group, but increasing in the ANP group. Tau was only increased in the CP group. In addition, the CP group showed higher lipid levels than the ANP group.

Principal component analysis. In the scores plot obtained from the CPMG NMR spectra (δ 0.50–9.00 ppm) (Fig. 4A), each group demonstrated its own individual within-group consistency. The CP group was closer to the control group and further from the ANP group in PC1. The corresponding loadings plot (Fig. 4B) revealed that lipids (δ 0.95–0.85, δ 1.40–1.25, δ 2.10–2.00 and δ 5.35–5.30 ppm), Lac (δ 1.40–1.35 ppm) and GPC/PC (δ 3.25–3.2 ppm) contributed the most to the separation of the three groups in the scores plot (Fig. 4A). In order to decrease the effects of the lipid metabolites, PCA was applied to the CPMG NMR spectra range (δ 2.9–9.00 ppm) with minimizing baseline effects of imperfect water saturation to obtain scores and a loadings plot (Fig. 4C and D). In Fig. 4C, all three groups were clearly separated, with each group demonstrating its own individual within-group similarities. The CP group was closer to the ANP group and further away from the control group in PC1, which is different from the results of Fig. 4A. The corresponding loadings plot (Fig. 4D) revealed that Gly (δ 4.35–4.25 ppm), Lac (δ 4.15–4.05 ppm), Cho (δ 3.60–3.55 ppm) Bet (δ 3.30–3.25 ppm), Tau (δ 3.45–3.40 ppm) and GPC/PC (δ 3.75–3.65 and δ 3.25–3.2 ppm) contributed the most to the separations observed in Fig. 4C.

Discussion

In our study, Gly, Lac, Bet, Cho, GPC/PC, Leu/Ileu/Val and various lipids, all correlated with ANP and CP. ANP demonstrated higher Leu/Ileu/Val and lower Tau and FA levels when compared to CP. These findings demonstrate that pancreatitis causes a clear disruption of the pancreatic metabolism at a molecular level, and suggest that Leu/Ileu/Val, lipids and Tau are all potential metabolic markers for the differentiation of ANP from CP.

With *in vivo* ^1H MRS technology, clinical research has concluded that lipid in chronic focal pancreatitis is significantly less than that observed in pancreatic carcinoma tissues (37). Cancerous tissues generally have high lipid content, but the reverse is not always true for other pancreatic diseases, as tissue lipid content varies widely due to factors such as genetics, aging, gender and the environment. From the ^1H MAS NMR spectra (Fig. 3) we observed that lipid levels, when compared to those of the control group, were slightly decreased in the CP group, whereas they were markedly decreased in the ANP group. The different weighting of lipids brought out the separation of the three groups in the scores plot (Fig. 4A), which could also be certified by the results of the loadings plot (Fig. 4B). This result suggests that the CP group has a more active metabolism than the ANP group. The difference in lipid levels between the CP and the ANP groups showed the characteristic metabolomics that appear as a result of the inflammatory process within the pancreas.

From MRS studies on tissues *in vivo*, the choline signal is primarily observed as a prominent singlet at 3.2 ppm that

includes contributions from free choline, GPC and PC, and is often referred to as ‘total choline’ (38–40). In the present study, we obtained high resolution signals of Cho, PC and GPC at the chemical shifts 3.19, 3.21 and 3.22 ppm, respectively. The ^1H MAS NMR results showed that Cho, PC and GPC were increased in both the ANP and CP groups. The total choline metabolites have been chosen as biomarkers in various carcinoma studies, as they tend to increase as a result of increased membrane concentrations during enhanced proliferation of cancer cells (41,42). As the modest MAS rates (5 kHz) are less than the dipole-dipole interactions of the cell membrane, it is unlikely that membrane-bound Cho, PC and GPC are easily demonstrated (31,43). Hence, Cho, PC and GPC detected in our study were mainly derived from free molecules within the pancreas. The increased Cho, PC and GPC in ANP and CP may result from the blockage of Cho-kinase and PC transferase, or from the consumption of PC through the CDP-Cho pathway. Thus, one may deduce that increased total choline represents a characteristic metabolite in the process of both CP and ANP.

In order to lessen the effects of lipids and to explore the low-molecular-weight metabolites that are relevant to the pathophysiological processes, the regions of 2.9–9.00 ppm in the NMR spectra were analyzed by PCA (Fig. 4C and D). Compared to Fig. 4A, it can be seen that the CP group has migrated towards the ANP group in PC1 (Fig. 4C), which is due to their similar inherent metabolic profiles, apart from a difference in lipid content, and the chemical shift of relevant metabolites could be obtained from the loadings plot (Fig. 4D). The elevation observed in Tau played an important role in the discrimination of ANP from CP. Tau is a free intracellular amino acid that is reported to have a number of biological functions, including osmoregulation and modulation of neurotransmitter action. It is chiefly obtained from food, although it may also be synthesized from other sulfur-containing amino acids (38,44). The metabolic actions of Tau are correlated with the control of serum cholesterol levels, blood sugar and cellular calcium levels (31). Thus, in the process of CP and ANP, normal Tau levels serve to maintain normal serum cholesterol levels, blood sugar and cellular calcium levels. To maintain the serum amylase levels, blood sugar levels are or approach normal levels, and an increase in Tau levels is necessary. Tissue putrescence appears in ANP, and therefore the ability to obtain Tau from food may be restricted, and Tau levels may decrease sharply in ANP.

From Figs. 3 and 4D, we conclude that the decrease in Gly, Lac and Bet in the CP and ANP groups caused the distinct separation in the scores plot (Fig. 4C). Gly is a simple amino acid that acts as an inhibitory neurotransmitter and antioxidant (45). Lac, the product of anaerobic glycolysis, is increased in the presence of hypoxia and ischemia (38). Our results showed that Gly and Lac were markedly decreased in both the ANP and the CP groups, due to their association with a metastatic potential, as has been reported by Sitter *et al* (46). Compared to other low-molecular weight-metabolites, Gly and Lac demonstrated the most noticeable differences between all three groups. Bet donates methyl groups for remethylation of homocysteine to methionine and dimethylglycine, which supports normal liver and pancreatic function, cellular replication and detoxification reactions (31). Therefore, the depletion of Bet metabolites in CP and ANP for the detoxification of the

pancreas is necessary and it is possible that the levels of Bet decrease both in CP and ANP.

Although the number of samples in our study was limited, the potential of ^1H MAS NMR for the investigation of physiological and pathological consequences of pancreatic diseases should not be ignored. The above results clearly demonstrate that the metabolic profiles and characteristics of ANP and CP can be discriminated characteristically without the disturbance of various lipid contents by ^1H MAS NMR coupled with PCA. As metabolite changes observed by ^1H MAS NMR always occur before morphological changes investigated by imaging technologies, the findings will clearly be of benefit to pathological research, early diagnosis and therapeutic evaluation of pancreatic diseases.

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