Identification of Novel Peptide Safety Markers for Exocrine Pancreatic Toxicity Induced by Cyanohydroxybutene

Jennie L. Walgren,1 Michael D. Mitchell, Laurence O. Whiteley, and David C. Thompson

Pfizer Global Research and Development, Drug Safety Research and Development, Chesterfield, Missouri 63017

Received August 29, 2006; accepted November 29, 2006

Abstract

Historically, serum amylase and lipase levels have been used to indicate pancreas injury; however, these enzyme levels are often not predictive of pathology. In an effort to discover novel biomarkers of pancreatic acinar cell injury, we analyzed serum and pancreas tissue from cyanohydroxybutene (CHB)-treated male IGS rats using proteomics methods. CHB produces an “edematous pancreatitis,” characterized by depletion of zymogen granules, acinar cell apoptosis, and mild to moderate inflammation. Secondary necrosis occurs at higher doses. Rats were treated with 150 mg/kg of CHB and samples were collected at 4, 8, and 24 h. Analyses of serum tryptic digests by surface-enhanced laser desorption-ionization mass spectrometry revealed two novel peptide biomarkers (RA1609 and RT2864) that were predictive of pancreatic damage. Levels of RA1609 decreased, while levels of RT2864 increased by 8 h following CHB treatment. The changes in RA1609 and RT2864 were detected in media from CHB-treated primary rat acini, demonstrating that these peptides are either of pancreatic cell origin or are produced by proteases released from acinar cells. Sequencing revealed that RA1609 is a fragment of rat albumin (accession number P02770, residues 348–360) and RT2864 is a portion of either rat trypsin III (accession number P08426, residues 39–65) or bovine trypsin (accession number P00760, residues 35–61). These two peptides, and possibly other fragments of serum proteins that are digested by pancreatic proteases, may be useful as safety markers for exocrine pancreatic toxicity during drug development or as biomarkers for the diagnosis and/or grading of severity of pancreatic disease.

Key Words: pancreatitis; CHB; SELDI; safety biomarkers.

Acute pancreatitis is an inflammatory condition resulting from chemical or physical insult to the pancreas which induces the release of activated proteases from pancreatic acinar cells. Activity of these proteases leads to autodigestion of the pancreas and surrounding tissues, and release of inflammatory mediators. While most cases of acute pancreatitis are relatively mild and resolve on their own, about 15–25% can progress to more serious complications such as shock and/or multisystem organ failure. The most common causes of acute pancreatitis in humans are alcoholism and gallstones (Vlodov and Tenner, 2001). There are, however, several drugs or drug classes that have been implicated in the development of exocrine pancreatic injury as well. These include azathioprine, estrogens, furosemide, methyldopa, pentamidine, procainamide, sulfonamides, and thiazide diuretics (Scarpelli, 1989; Vlodov and Tenner, 2001). The ability to monitor for pancreatic injury both in drug development and in the clinic has often been a difficult issue due to the poor predictive ability of markers currently available for acute pancreatitis. Due to the diffuse nature of the progression of acute pancreatitis, several markers of this disease are associated with generalized abdominal injury, and many are not detectable in the early stages of the disease. The sensitivity of these markers is therefore often low. In order to search for novel markers of exocrine pancreatic injury which may be more sensitive than available tests, we used the model pancreatic toxicant CHB (1-cyano-2-hydroxy-3-butene).

CHB is a breakdown product of a glucosinolate found in many cruciferous plants (e.g., Brussels sprouts, rapeseed). At low doses, CHB is a selective pancreatic toxicant in the rat, specifically targeting acinar cells (Maher et al., 1991; Wallig et al., 1988, 1989). A single subcutaneous dose (150 mg/kg) of CHB induces pancreatic injury beginning with pancreatic edema, acinar cell vacuolization, and depletion of zymogen granules within 2 h following treatment. By 6 h, acinar cell necrosis can be detected and reaches a peak by 12-h posttreatment. At 18 h the cytoplasm of the acinar cells swells, the cells become vacuolated, and some secondary necrosis occurs, and by 24 h an inflammatory infiltrate is noticeable. By 96-h posttreatment few acinar cells remain, and little regeneration occurs. At 28 days postdosing, the pancreas is mainly composed of fat, collagen, and islet cells (Kelly et al., 1999). It has been reported that CHB can alter pancreatic secretion with disparate effects at different doses, causing an initial decrease and then increase at lower (50 and 100 mg/kg) doses and the opposite pattern (initial increase, then decrease), associated with substantial decreases in secretion at ≥ 2-h postdose, at higher doses (150 and 200 mg/kg) (Maher et al., 1991). CHB has also been shown to increase bile secretion (Maher et al.,...
1991). CHB has an unusual effect on pancreatic glutathione (GSH) levels, producing an initial depletion (approx. 80%) at 2–4 h, followed by a substantial increase in GSH levels (Davis et al., 1993) that remains elevated even 4 days after a single 200 mg/kg dose (Wallig and Jeffrey, 1990). Glutathione-S-transferase (GST), particularly GST2x2 (March et al., 1998), is induced 1.5- to threefold in rats treated with 50–100 mg/kg CHB daily for 7 days (Wallig et al., 1998). The mechanism of pancreatotoxicity and whether the change in GSH levels is related to this toxicity remains unknown.

In this study we used CHB-treated rats as a model for acute exocrine pancreatic injury and profiled serum samples to identify markers of pancreatic injury which would be pancreas specific. To accomplish this, we utilized proteomics techniques including SELDI (surface-enhanced laser desorption-ionization-time of flight) mass spectrometry. SELDI (Ciphergen Biosystems PBSII, Fremont, CA) is a protein analysis instrument similar to matrix-assisted laser desorption-ionization-time of flight except that the chip surfaces in SELDI incorporate various chemistries which bind proteins based on physical characteristics such as hydrophobicity, isoelectric point, or metal-binding affinity. Through profiling of serum digests, we identified two pancreas-specific peptide markers, and also explored the mechanism resulting in the changes in levels of these serum markers through both in vivo and in vitro studies.

MATERIALS AND METHODS

Materials. Male Sprague-Dawley IGS rats were purchased from Charles River Laboratories (Wilmington, MA). CHB was synthesized by Gateway Chemical Technologies (St Louis, MO). Collagenase Type 4 was purchased from Worthington Biochemical (Lakewood, NJ). Ion exchange (Q) columns and ProteinChips were purchased from Ciphergen Biosystems. Gel supplies were from Invitrogen (NuPAGE NOVEX) (Carlsbad, CA). Sequencing grade trypsin was obtained from Roche Diagnostics (Indianapolis, IN). Except where noted, all other chemicals were obtained from Sigma Chemical Company (St Louis, MO) and were of the highest purity available.

In vivo studies. Male Sprague-Dawley IGS rats between 6 and 8 weeks of age were housed in a controlled environment with 12-h light/dark cycle and were provided with water and standard lab chow ad libitum. The animals were fasted 14–16 h prior to dosing. A saline suspension of CHB was administered subcutaneously. Animals were dosed with 150 mg/kg CHB. Based on literature studies with CHB and pilot experiments, 150 mg/kg was found to induce some degree of zymogen depletion and acinar apoptosis in all animals and associated clinical chemistry alterations in at least 50% of rats administered this dose. For animals in all studies, three sections of pancreas from different regions (head, body, and tail) were collected and fixed in formalin for histopathological examination and the remainder of the pancreas was frozen for proteomic analyses. At the scheduled euthanasia, samples of serum were collected from the inferior vena cava of animals anesthetized with CO2. At certain additional time points, blood was collected from the retro-orbital venous plexus or tail vein (studies C and D) of animals anesthetized with CO2. Use of the animals in these studies was reviewed and approved by the Pfizer Institutional Animal Care and Use Committee. The animal care and use program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

Studies A and B were pilot studies aimed at characterizing the pancreatic lesion resulting from a single dose of 150 mg/kg CHB and determining what the best time point(s) were for correlating the clinical chemistry markers, peptide biomarkers, and histopathological response of the animals. Once these time points were evaluated, studies C and D were designed to increase the number of animals examined under the optimum regimen of dosing, blood collection, and euthanasia time points, as well as to look at reversal of the lesion and biomarkers (study C).

Study A consisted of eight vehicle control and eight CHB-treated rats; the study was terminated 24 h after dosing and included an 8-h postdose blood sample. Study B consisted of five animals per group and included three different time points for euthanasia (4-, 8-, or 24-h postdose). In study C, the main group contained 10 vehicle control animals and 20 CHB-treated animals which were euthanized at 24 h and had blood samples drawn at 8 h; this study also contained a reversal group with three vehicle control animals and five CHB-treated animals that were euthanized 8 days postdosing with blood samples drawn at 8- and 24-h postdosing. Finally, study D consisted of 10 vehicle control and 20 CHB-treated animals that were euthanized at 24-h postdose. Blood samples were drawn at 8-h postdose. Please see tabulated details of these individual studies in Table 1.

Serum chemistry. Serum was analyzed on a Hitachi 912 serum chemistry analyzer, using standard clinical chemistry procedures. Sera were analyzed for multiple serum enzymes and metabolic parameters, including total protein, blood urea nitrogen, creatinine, albumin, pancreatic amylase, and lipase levels.

Proteomic analysis of pancreas tissue. Approximately 20 mg of pancreas tissue was homogenized in 75 µl of 1% Triton X-100 in phosphate-buffered saline (PBS) using a Bio101 ThermoSavant Fast Prep system at speed 6 for 10 s. Samples were then spun down at 13,000 × g for 5 min and the supernatants were subjected to fractionation with anion-exchange spin columns from Ciphergen (Q columns, product number C5400-0016). Briefly, spin columns were rehydrated with 800 µl of 20mM Tris pH 9.0 overnight. After removal of equilibration buffer by centrifugation, samples containing equal protein concentrations (in 1% Triton in PBS brought up to 500 µl with Tris pH 9.0) were applied to the spin column and allowed to run through the columns by gravity for ~3 min. Columns were then centrifuged in a VWR Model V microcentrifuge at 56 × g for 1 min. The flow through from this spin was saved as the “nonbinding” fraction. One hundred microliters of 20mM Tris pH 9.0 was applied to column and allowed to sit for 1 min. Columns were then centrifuged at 56 × g for 1 min. Flow through was saved as pH 9 fraction. This procedure was repeated with each of the following buffers for fractions 1.0 pH unit apart: 20mM Tris pH 8.0, 20mM sodium phosphate/20mM sodium citrate pH 7, 6, 5, 4, and 3. Proteins remaining in the column were eluted at the end of the procedure with an organic solution containing 33.3% acetonitrile, 16.7% isopropanol, 0.1% trifluoroacetic acid (TFA).

Q column fractions (nonbinding and pH 3–9 eluted fractions) of the pancreas tissue extract collected from the procedure described above were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% Bis-Tris gel) and proteins were stained with Coomassie Blue. Protein bands with visually large differences in abundance between control and CHB samples were excised and placed in 0.4 ml of 50% methanol, 10% acetic

| TABLE 1 | CHB in In Vivo Studies |
|---|---|---|---|---|
| Study | # of control animals per sacrifice group | # of CHB-treated animals per sacrifice group | Sacrifice time point (postdose) | Additional serum collection time points |
| A | 8 | 8 | 24 h | 8 h |
| B | 5 | 5 | 4, 8, or 24 h | 8 h |
| C | 10 | 20 | 24 h | 8 h |
| D | 3 | 5 | 8 days | 8 and 24 h |
| | 10 | 20 | 24 h | 8 h |

Downloaded from http://toxsci.oxfordjournals.org/ at Pennsylvania State University on March 6, 2014
acid and agitated twice for 15 min each wash. The solution was then replaced with 100mM ammonium bicarbonate (pH 8) and agitated twice for 10 min each wash. The bicarbonate buffer was then removed and samples were incubated with agitation in 50% acetonitrile, 100mM ammonium bicarbonate for 1 h. Samples were next incubated in 100% acetonitrile for 15 min. The acetonitrile was removed and the gel pieces were dried down in a SpeedVac. The gel pieces were rehydrated with 10 µl of a 200 ng/µl solution of trypsin in 25mM ammonium bicarbonate pH 8.0 and were incubated overnight at 37°C.

Identification of proteins from pancreas tissue. SELDI analysis of the tryptic digests from pancreas tissue analysis were performed on a PSBI System (Ciphergen Biosystems). Samples were mixed 1:3 (vol/vol) with the matrix z-cyano-4-hydroxycinnamic acid (CHCA) (50mM) in 50% acetonitrile, 0.5% TFA. The peptide/matrix solutions were placed on a gold chip and allowed to dry. A minimum of 100 laser shots were averaged to produce the mass spectra. The instrument was both internally and externally calibrated with at least three peptide standards from Ciphergen Biosystems. Laser power was optimized for signal intensity and resolution and varied between 160 and 180. Protein identification by peptide mass fingerprinting was accomplished using the software ProFound. Protein identifications were confirmed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis.

Proteomic analyses of serum. Serum was diluted to 1 ng/ml with distilled water. Ten micrograms of each serum sample was then combined with 2 µg of sequencing grade trypsin (Roche) in 25mM ammonium bicarbonate pH 8.0 (100 µl total reaction volume). The serum was digested overnight at 37°C and the resulting digests were then analyzed by SELDI mass spectrometry on either gold, H4, or NP20 and IMAC3 chip surfaces. The H4 chip array utilizes a reverse phase chromatography surface, the NP20 chip surface captures hydrophilic proteins, while the IMAC3 chip surfaces isolates metal-binding proteins. Gold chips were used to capture an overall spectra of peptide mixtures.

For H4 chip analysis, the chip surface was prewashed with acetonitrile, and 1 µl of sample was loaded with 3 µl of 10% acetonitrile in phosphate-buffered saline and incubated for 30 min in a humidity chamber. The chip surface was then washed three times with 3 µl of 10% acetonitrile in phosphate-buffered saline and allowed to air dry. 0.5 µl of 0.83% CHCA was added to each spot and after drying a second application of CHCA was applied.

For NP20 ProteinChip analysis, the chip surface was prewashed with distilled water, and 1 µl of sample was loaded with 1 µl of distilled water and incubated for 30 min in a humidity chamber. The chip surface was then washed twice with 3 µl of distilled water and allowed to air dry. 0.5 µl of 0.83% CHCA was added to each spot and after drying a second application of CHCA was applied.

For IMAC3 ProteinChip analysis, the chip spots were loaded with 100mM copper sulfate twice for 15 min incubations each, and then rinsed with distilled water. The chip surfaces were then rinsed twice with 50mM sodium acetate, pH 4, and again rinsed with distilled water. Two microliters of sample digest was then loaded into 4 µl of 0.1% Triton X-100 in PBS, and the chip was incubated in a humidity chamber at room temperature for 1 h. Following incubation, the chip surfaces were washed three times with 5 µl of 0.1% Triton X-100 in PBS followed by two washes with 5 µl of distilled water. Two applications of 0.5 µl of 0.83% CHCA were applied to each surface and allowed to dry completely. Peptide peaks were analyzed by cluster analysis with the Ciphergen Biomarker Wizard 2.1 software, and peaks that were significantly different in sera from CHB-treated animals were assessed for consistency between studies and correlation with biochemical markers as well as prominence of peak height.

Identification of peptides from serum digests. The SELDI peak at 1609 m/z (RA1609) was sequenced at Ciphergen Biosystems Inc. using a ProteinChip Tandem MS (QSTAR) Interface. The second peptide (RT2864) was identified by LC/MS/MS by the Pfizer Proteomics/Biomarker Discovery Group, St. Louis, MO. Samples were analyzed on a Q-TOF Ultima (quadrupole time of flight) mass spectrometer (Micromass, Beverly, MA).

Acinar cell isolation, culture, and medium analysis. Acinar cells were isolated generally according to the method described in Blinman et al. (2000).

RESULTS

Histopathology

Rats administered a single subcutaneous dose of CHB in saline followed a similar course of pancreatic injury as reported in previous studies; namely, pancreatic edema by 2-h posttreatment, acinar cell apoptosis clearly detectable by 8-h posttreatment, and some single-cell acinar necrosis by 24-h posttreatment (Fig. 1).

In general, animals treated with CHB had diffuse apoptosis of acinar cells and reduced content of zymogen granules in the pancreas. The severity of this change was generally moderate and similar in all animals. Interstitial edema was present in all animals. Multifocal necrosis of acinar cells was noted in several animals. Focal necrosis of fat was noted in the interlobular areas in two of the animals. Minimal to slight interstitial inflammation was noted in all animals. Two animals had a large multilobular area in one of the three sections that was only minimally affected by these changes.

Analysis of Tryptic Digests of Sera

Serum collected from rats 8 h after treatment with CHB or vehicle control was digested with trypsin and analyzed on multiple SELDI chip surfaces, with the most consistent, significant changes identified on NP20 and IMAC-Cu chip surfaces. Samples were all subjected to the same conditions for storage and tryptic digestion. In general, gold and H4 chips were used for first pass screening as these chip surfaces allowed detection of the greatest number of peaks. Peaks were analyzed by cluster analysis with the Ciphergen Biomarker Wizard 2.1 software. The subset of peaks that were found to be significantly different were examined for consistency of change between control and CHB samples, as well as prominence of
peak (peak height). In particular, a prominent peak in the control serum spectra that was optimally detected on the NP20 chip at approximately 1609 m/z was found to be significantly reduced in sera from CHB-treated animals with altered serum chemistry and histopathological evidence of pancreatic lesions (p < 0.007). On the other hand, a peak at approximately 2895 m/z on an H4 chip was significantly increased in sera from those animals with CHB-induced pancreatic damage (p = 0.03). The peptide corresponding to the 2895 m/z peak was later found to be an oxidized form of a peptide of approximately 2864 m/z which displayed optimized binding on an IMAC-Cu chip surface. In all later studies this peptide was analyzed using IMAC-Cu chips. SELDI spectra displaying the peaks of interest are shown in Figure 2; spectra in panel A are from the NP20 chip surface and spectra in panel B are from the IMAC-Cu chip surface. Representative spectra from control and CHB-treated serum samples are shown.

The loss of the 1609 m/z peak and the increase in the 2864 m/z peak were found to be consistent changes in sera from affected versus unaffected animals at 8- and 24-h posttreatment. No changes in peptide levels were detectable at the 4-h time point. The decrease in the 1609 m/z peak and increase in the 2864 m/z peak correlated with changes in serum lipase levels in pilot studies A and B (Fig. 3).

At both 8- and 24-h posttreatment, changes in these peptide markers tracked consistently with changes in serum amylase and lipase levels in the larger studies C and D as well. The mean amylase and lipase levels, as well as the mean peak intensities for the 1609 and 2864 peptide markers from studies C and D are displayed in Table 2. The two peptide markers clearly discriminated between treated and control animals as shown in the graph in Figure 4. In fact, in multiple cases, the peptide markers identified animals with pancreatic injury that showed little to no change in either amylase or lipase, or both (Table 3).

While it is known that there is little pancreatic acinar cell regeneration seen in rats after administration of large doses of CHB (> 100 mg/kg), cellular injury and death declines by
4 days posttreatment and inflammatory infiltration decreases dramatically by 7 days post-CHB exposure (Kelly et al., 1999). In rats that received a single injection of 150 mg/kg CHB and were euthanized 8 days later, histopathological examination revealed repair of pancreatic damage and diffuse fibrosis, with minor chronic active inflammation remaining. The serum peptide markers reflected this recovery period as their levels trended toward normal values (Fig. 5). Figure 5 represents levels of peptide markers in serum collected at the designated time point following CHB administration in three vehicle control animals and five animals treated with 150 mg/kg CHB. Due to the small number of animals in this recovery group, statistical analysis was not performed.

**Detection of Peptide Markers in Primary Acinar Cell Cultures**

To further demonstrate that the peptide markers were either of pancreatic origin or are produced by substances released from acinar cells, we examined media containing rat serum albumin from primary acinar cells which were treated with CHB. The 1609 fragment was present in these samples and was reduced in the presence of CHB. In addition, we detected an increase in peaks at 2864 and 2895 (an oxidized form of 2864) in media from treated cells (Fig. 6). These peaks were not present in media incubated in the absence of cells (data not shown).

**Identification of Peptide Markers**

The SELDI peak at 1609 m/z (RA1609) was sequenced at Ciphergen Biosystems Inc. using a ProteinChip Tandem MS (QSTAR) interface and identified as a fragment of rat albumin (amino acid residues 348–360 of P02770). The second peptide (RT2864) was identified by LC/MS/MS as rat trypsin III, cationic precursor (amino acid residues 39–65 of P08426) and bovine trypsinogen, cationic precursor (amino acid residues 35–61 of P00760) by the Pfizer Proteomics/Biomarker Discovery Group. Samples were analyzed on a Q-TOF Ultima mass spectrometer (Micromass, Beverly, MA). This fragment of rat trypsin III and bovine trypsin differs by only one amino acid.
acid (residue 44 in rat trypsin III and residue 40 in bovine trypsin) which is an alanine in rat trypsin III and a serine in bovine trypsin. Oxidation of the alanine in the rat sequence, resulting in a fragment of identical weight as the bovine sequence, made it particularly difficult to separate signals from these two peptides but evidence for both were found. Rat trypsin III is likely present in the serum samples, and bovine trypsin was added for digestion of the serum, so the presence of both fragments is feasible.

Effect of Pancreatic Proteases and Protease Inhibitors on Peptide Markers

One explanation for the change in peptide levels in serum from rats treated with CHB is the leakage of pancreatic enzymes into the serum, which would alter the digestion patterns of serum proteins in tryptic digests. To test this hypothesis, we exposed control rat serum to various pancreatic proteases, and also incubated control and CHB serum with various protease inhibitors prior to trypsin digestion. Exposure of the serum to 20 ng/µl carboxypeptidase A, chymotrypsin, or elastase resulted in significant decreases in the 1609 m/z peak. The appearance of the 2864 m/z peak was only detected in control serum incubated with carboxypeptidase A and trypsin (data not shown). When control and CHB sera were exposed to specific protease inhibitors (10 µM elastinal, 100 µM N-tosyl-L-phenylalanine chloromethyl ketone [TPCK], 100 µM chymostatin, or 0.1 mg/ml carboxypeptidase inhibitor from potato tuber) during trypsin digestion, only the carboxypeptidase inhibitor prevented the loss of RA1609 and the appearance of RA2864.

### TABLE 2
Changes in Serum Amylase, Lipase, and Peptide Markers in CHB In Vivo Studies C and D

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Amylase mean, U/l ± SEM</th>
<th>Lipase mean, U/l ± SEM</th>
<th>Mean peak height, 1609 (NP20) ± SEM</th>
<th>Mean peak height, 2864 (IMAC) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 h control</td>
<td>1895.4 ± 84.5</td>
<td>5.7 ± 0.5</td>
<td>48.2 ± 2.8</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>8 h CHB</td>
<td>3351.5 ± 337.8</td>
<td>412.7 ± 74.9</td>
<td>6.7 ± 1.5</td>
<td>18.0 ± 2.3</td>
</tr>
<tr>
<td>24 h control</td>
<td>2404.7 ± 182.5</td>
<td>7.4 ± 0.3</td>
<td>48.0 ± 4.2</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>24 h CHB</td>
<td>3326.2 ± 379.8</td>
<td>251.3 ± 41.1</td>
<td>4.3 ± 0.9</td>
<td>19.5 ± 2.0</td>
</tr>
</tbody>
</table>

**Note.** Total histopathology score includes scores from the following diagnoses: acinar apoptosis, zymogen depletion, interstitial edema, interstitial inflammation, acinar necrosis, and interstitial fat necrosis.
Levels of Proteases in Pancreas Tissue

The appearance of pancreatic enzymes in the serum should indicate early release from pancreatic tissue, so to further test this theory we examined ion exchange column (Q column) fractions from homogenized pancreas from control and CHB-treated animals. Elastase 2, carboxypeptidase A1/A2, and chymotrypsin B and trypsin III levels were all shown to decrease in pancreatic tissue from CHB-treated animals (Fig. 8).

DISCUSSION

CHB is a pancreatic toxicant which targets the exocrine pancreas, inducing widespread zymogen release, pancreatic edema and inflammation, and acinar cell necrosis with single doses $\geq 100$ mg/kg in rats (Kelly et al., 1999; Maher et al., 1991; Wallig et al., 1988, 1989). The release of activated proteases from the pancreas, the subsequent autodigestion of the pancreas, and the initiation of inflammation and acinar cell apoptosis/necrosis are hallmark stages of acute pancreatitis (Vlodov and Tenner, 2001). Generally, acute pancreatitis is diagnosed only after the development of significant inflammation which results in the characteristic severe pain, nausea, and vomiting seen clinically. The standard markers of pancreatitis include elevated serum amylase and lipase levels (Treacy et al., 2001). While these markers, in particular lipase which is more pancreas-specific, are fairly good diagnostic indicators once symptomatology is present, they are not very sensitive nor are they indicative of disease severity (Sandberg and Borgström, 2002; Treacy et al., 2001; Vissers et al., 1999). Using CHB-treated rats as a model, we investigated changes in serum proteins that were treatment specific and were correlated with pancreatic lesions and changes in serum lipase levels. The pancreatic lesions detected in our study follow the progression as seen in previous studies with CHB (Kelly et al., 1999); namely, early development of edema followed by apoptosis and secondary necrosis with inflammation (Fig. 1).

Using SELDI mass spectrometry, we profiled serum that was digested with trypsin prior to analysis. We chose to digest the samples due to the greater resolution of peptides on SELDI. We discovered that several peptides were significantly different between control and treated animals (data not shown); however, we chose to focus on two peptide markers, 1609 $\text{m/z}$ and 2864 $\text{m/z}$ (Fig. 2) which correlated with elevations in serum lipase within 24 h following treatment (Fig. 3; Table 2). In our two larger studies (studies C and D) where all of the CHB-treated animals developed some degree of pancreatic injury, the peptide markers clearly delineated the treated and control groups (Fig. 4; Table 3). In a small subset of rats in study C (three control, five CHB-treated) given a 7-day
recovery period, acinar cell injury had diminished and only fibrosis and very mild inflammation remained, and serum biomarkers appeared to track with pancreatic recovery (Fig. 5).

The 1609 m/z peak is a common, highly abundant tryptic fragment of albumin in control serum, and this fragment was not produced from trypsin digestion of serum from CHB-treated animals. It was intriguing that a decrease in this common albumin fragment tracked so consistently with pancreatic toxicity. Knowing that in affected animals CHB should stimulate release of proteases from pancreas, we looked

FIG. 7. Effects of the addition of protease inhibitors on the peptide markers found in trypsin digests of serum from control and CHB-treated rats. Sera from control and CHB-treated rats were digested overnight with trypsin in the presence of various inhibitors including an elastase inhibitor (elastinal, panels A and B), and carboxypeptidase inhibitor (CPI, panels C and D). Samples were analyzed by SELDI on an H4 chip surface. Only CPI prevented both the decrease in the 1609 m/z marker and the increase in the 2864 m/z marker in sera from CHB-treated rats.

FIG. 8. Protease levels decrease in pancreas tissue from CHB-treated rats. Pancreas tissue was frozen, crushed, and hydrated in lysis buffer. Tissue samples (24 h following CHB treatment) were passed over anion-exchange resins (Q columns, Ciphergen) and eluted with buffers of increasing acidity. Proteins were separated by PAGE and stained with Coomassie Blue. Individual bands of interest were excised and identified by LC/MS/MS and SELDI. Evidence for the early release of elastase 2, trypsin III, carboxypeptidase A1/A2, and chymotrypsin B was obtained.
for evidence of protease release from pancreas tissue and activity in serum. We found that the addition of the pancreatic proteases carboxypeptidase A, chymotrypsin, and elastase to control serum during trypsin digestion significantly reduced the level of RA1609 (data not shown). Only the addition of carboxypeptidase A, however, resulted in the appearance of the RT2864 peak (data not shown). Exposing serum from both control and CHB-treated animals to protease inhibitors had similar results; namely, while chymostatin, TPCK, and elastinal did not prevent the loss of RA1609 or the increase in RT2864, exposure of serum from CHB-treated animals to a carboxypeptidase inhibitor produced both changes (Fig. 7; results from elastinal and carboxypeptidase inhibitor incubations shown). So, while we cannot rule out the involvement of multiple proteases in the loss of RA1609, it appears that the main pancreatic enzyme responsible for both the increase in RT2864 and the loss of RA1609 is carboxypeptidase A.

RT2864 was identified as a fragment of cationic trypsin. In order to determine whether the origin of this peptide was pancreatic, and whether we could reproduce the changes in both peptide markers in an in vitro system, we isolated primary pancreatic acinar cells and exposed them to CHB in media containing rat serum albumin. Aliquots of medium were taken at various intervals, and media proteins were digested with trypsin and examined by SELDI. Both the decrease in RA1609 and the increase in the oxidized form of RT2864 (2895 m/z confirmed by sequence comparison; seen in Fig. 8 on an NP20 ProteinChip) were detected in media from primary acinar cells treated with 10 or 100 μM CHB (Fig. 6). Also, the 2864/2895 m/z peak was not found in media incubated in the absence of cells, indicating that RT2864 is either of pancreatic acinar cell origin or results from digestive enzymes released by the acinar cells. We predict that RT2864 is formed from cleavage of cationic trypsin by chymotrypsin and carboxypeptidase A (based on in vitro data) present in serum from CHB-treated animals. The unusual C-terminal amino acid of this peptide is thought to be due to carboxypeptidase cleavage which terminates at the site of the disulfide bond between the cysteines at positions 49 and 65 in rat trypsin III or positions 45 and 61 in bovine cationic trypsin.

In rats pancreatic injury, induced by toxicants such as CHB, is not always predicted by traditional laboratory tests such as serum amylase levels (Wallig et al., 1988). The same is true for humans, as the clinical markers for parenchymal pancreatic damage have historically been poor indicators for both early diagnosis of disease and prediction of disease severity. A difficulty with serum amylase is that it can be indicative of multiple conditions associated with abdominal inflammation (Visser et al., 1999), and in addition is not even found to be elevated in 30% of patients with the most common form of acute pancreatitis, alcoholic pancreatitis (Vlodov and Tenner, 2001). Serum lipase, while being more specific to pancreas and more sensitive than amylase as a marker still only provides ~67% sensitivity (compared to ~45% for serum amylase) (Treacy et al., 2001) and is not predictive of disease severity (Sanberg and Borgström, 2002). While C-reactive protein levels are now often used to determine the severity of pancreatitis, this laboratory value is not valid until 2 days after the onset of symptoms (Sanberg and Borgström, 2002). In recent years the focus of studies aimed at providing earlier, more predictive markers of pancreatitis has been the earliest pathological events in pancreatitis, namely trypsinogen activation and activation of subsequent proteases (Lerch and Gorenliek, 2000). Markers such as trypsinogen activation peptide, serum trypsinogen 2 (Hedström et al., 2001), or complexes containing trypsinogen-2 in urine (Lempinen et al., 2001, 2003), and procarboxypeptidase B in serum (Appelros et al., 1998; Müller et al., 2002) have been evaluated for their ability to diagnose acute pancreatitis as well as predict the severity of the case. For instance, radioimmunoassay of serum carboxypeptidase B activation peptide in a small population of emergency room patients provided 84.6% sensitivity, and 59.4% specificity (Pezzilli et al., 2000).

In conclusion, the two peptide markers identified in this study correlate well with pancreatic injury induced by CHB in rats. These markers are target organ-specific, in that they are either derived from the pancreas or are a result of enzymatic activity of proteases released by the pancreas. The peptide markers appear to be quite sensitive in detecting animals with exocrine pancreatic injury. This is illustrated in studies C and D (Table 3), where at times amylase and lipase changes were modest or absent but one or both of the peptide serum markers changed dramatically in rats with exocrine pancreatic injury. Continuing efforts in our lab will address the question of whether these peptide markers are more sensitive than the standard serum chemistry tests (serum amylase and lipase) in predicting pancreatic injury by conducting a multidose study with CHB. Other important issues to consider are whether these markers are specific to CHB or to exocrine pancreatic damage in general, and whether these markers are useful in species other than rats. In addition to RA1609 and RT2864, other fragments of albumin or serum proteins that are digested by pancreatic proteases may be useful as markers for exocrine pancreatic injury. If these peptide markers prove to be robust in predicting pancreatic injury and severity of the lesion in various species following exposure to different inducers of pancreatitis, they may be useful as safety markers for exocrine pancreatic toxicity during drug development or as biomarkers for the diagnosis and/or grading of severity of pancreatic disease.
REFERENCES


