Proteomic analysis of changes in protein expression in serum from animals exposed to paraquat

YONG-SIK KIM¹*, HANA JUNG¹*, HYO-WOOK GIL², SAE-YONG HONG² and HO-YEON SONG¹

¹Department of Microbiology, College of Medicine, Soon Chun Hyang University, Cheonan, Chungnam 330-090; ²Department of Internal Medicine, Soon Chun Hyang Cheonan Hospital, Cheonan, Republic of Korea

Received May 30, 2012; Accepted August 13, 2012

DOI: 10.3892/ijmm.2012.1143

Abstract. Paraquat (PQ) poisoning remains a major public health concern in many countries. Extensive research has focused on finding early diagnostic biomarkers of acute PQ poisoning. In order to investigate the characterization of diagnostic biomarkers in PQ poisoning, we utilized proteomic analysis using serum from rats exposed to PQ, and we identified 8 differentially expressed proteins from over 500 protein spots. The expression of apolipoprotein E (ApoE), preprohaptoglobin (Pphg), a precursor of haptoglobin (Hp), and complement component 3 (C3) proteins was greatly induced by PQ exposure while the expression of fibrinogen γ-chain (FGG) and Ac-158 was dramatically reduced. To further investigate the possibility of ApoE, Pphg and FGG as useful diagnostic biomarkers of PQ poisoning, western blot and qRT-PCR analyses were conducted using cell lines as well as rat and human sera. The expression levels of ApoE, Hp and FGG were significantly altered in the presence of PQ in both rat and human serum suggesting that these proteins may be appropriate candidate molecular biomarkers for the early diagnosis of acute PQ intoxication.

Introduction

Paraquat (trade name Gramoxone®; 1,1’-dimethyl-4,4’-bipyridinium dichloride; PQ) is a potent herbicide widely used throughout the world (1). PQ intoxication by accidental and intentional ingestion has been a public health concern for several decades due to the severe morbidity and mortality rates in both developing and developed countries. A variety of clinical approaches for treatment of PQ intoxication have been employed, but highly effective treatment methods have yet to be defined.

The mechanism of PQ poisoning is based on excessive generation of reactive oxygen species (ROS). PQ is reduced by NADPH and then conveys electrons to molecular oxygen generating ROS. This can disrupt the balance of intracellular redox cycling and lead to cellular toxicity (1,2). Ingested PQ directly targets the lungs, kidneys, and liver tissues, causing severe primary cellular damage. PQ toxicity can lead to acute lung phenomena including infiltration of the lungs by inflammatory cells, alveolar hemorrhage, increased collagen deposition and sequential development of mortal lung fibrosis (1,3). Clinical features of PQ ingestion depend on the quantity ingested and the time elapsed from ingestion. Upon ingestion, PQ is rapidly absorbed, distributed to various tissues, and within 12-24 h most of the absorbed PQ is secreted through urine (4,5). Within a few days, patients may develop severe lung damage such as fibrosis, the main cause of mortality with PQ toxicity. Prompt diagnosis and prognosis of pathophysiological progression is crucial for survival. There is, however, a lack of effective diagnostic methods for PQ intoxication due to considerable variations between patients such as age, gender, susceptibility, time elapsed from PQ exposure, and amount of PQ ingested.

Proteomic analysis is one of the most widely used techniques for defining functional proteins and crosstalk between proteins and DNA (6-8). Furthermore, proteomics is a powerful research tool for the identification of biomarkers and therapeutic targets for toxicology, pharmacology, cancer biology, and other biomedical research (8). In pulmonary-related disease research, proteomics is a highly effective method for identifying diagnostic and prognostic biomarkers using various biomaterials such as cell lines, lung bronchoalveolar lavage fluid (BALF), and tissues from animals (9,10). However, blood samples have not been considered useful for proteomic analysis despite the fact that blood possesses a highly-enriched information source due to the absence of genomic information (11). Recent developments of advanced proteomic techniques have resulted in the recognition of blood as an important source of information for various biomedical studies such as toxicology (8).

In the present study, we employed a conventional proteomics approach using rat serum to investigate and identify diagnostic biomarkers in PQ poisoning. 2D-PAGE and
MALDI-TOF analysis revealed PQ treatment altered protein expression. The expression of apolipoprotein E (ApoE), complement component 3 (C3), and proprohaptoglobin (Pphg), a precursor of haptoglobin (Hp), were greatly induced while the expression of fibrinogen γ-chain (FGG) and Acl-581, a precursor of fibrinogen β-chain (FGB), were reduced. Furthermore, alteration of the protein expression of ApoE, Hp and FGG was confirmed in PQ-exposed cell lines and sera from patients. Therefore, our data suggest that Apo E, Hp and FGG may beneficial diagnostic biomarkers for the early detection of acute PQ intoxication.

Materials and methods

Chemicals and reagents. Paraquat dichloride (1,1'-dimethyl-4,4'-bipyridinium dichloride) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The stock solution of PQ was made using distilled water. Stock solutions were then diluted in 1×PBS to the desired concentrations prior to treatment of animals. Stock solutions were diluted in cell culture media for the treatment of cells.

Cell culture. Macrophage, Raw264.7, and lung adenocarcinoma, A549, cell lines were obtained from the Korean Cell Line Bank (Seoul, Korea) and maintained in DMEM and DMEM/HF-12 medium, respectively, supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) antibiotic/antimycotic cocktail (100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B; Invitrogen, Carlsbad, CA) at 37°C under a humidified atmosphere containing 5% CO₂.

Animals. Six five-week-old male CD(SD)IGS rats were purchased from Orient Bio, Inc. (Seongnam, Korea) and acclimated for 7 days prior to the experiment. Rats were housed with free access to standard rodent food in compliance with the standards set forth by Soon Chun Hyang’s Institutional Animal Care and Use Committee.

Ethics statement. Serum samples from patients intoxicated with PQ were obtained at the department of internal medicine, Soon Chun Hyang University Cheonan Hospital, and the use of patient serum in this study was approved by Soon Chun Hyang University Cheonan Hospital's institutional review board (IRB approval no. 2011-85).

Proteomics analysis. Serum samples were collected from rats following the guidelines for care and use of laboratory animals approved by Soon Chun Hyang’s Institutional Animal Care and Use Committee. Untreated normal blood was initially collected one day prior to PQ treatment (noted as -1 in the text and figures) from the rat tail vein. Blood samples from the tail vein were then collected at 6 h following intraperitoneal injection of PQ (noted as 1). At Day 3, a final blood collection was performed. Serum samples were immediately collected via 1,500 g centrifugation at room temperature, and samples were stored at 4°C.

For 2D-PAGE separation, 200 µg of serum was used for isoelectric focusing (IEF) using a multiphor II electrophoresis kit following the manufacturer’s instructions. For 2D-PAGE, strips were equilibrated in equilibration buffer (50 mM Tris-Cl, pH 6.8 containing 6 M urea, 2% SDS and 30% glycerol) for 10 min, and the equilibrated strips were inserted and run on 20×24 cm (10-16% gradient) SDS-PAGE gels. 2D gels were then stained with modified silver staining according to Oakley et al (12) for image analysis. Quantitative analysis of visualized images was executed using PDQuest (version 7.0; Bio-Rad, Hercules, CA, USA) software following the manufacturer’s instructions. We selected the spots with >2-fold alteration, normalized by total valid spot intensity. In order to characterize the selected spots, the in-gel digestion was performed using porcine trypsin (Promega; Madison, WI, USA) with modified methods as previously described (13). Digested gel pieces were sequentially washed with 50% acetonitrile to remove chemicals and dye, and were then dehydrated. Spots were rehydrated with trypsin (8-10 ng/µl in 50 mM ammonium bicarbonate, pH 8.7), and incubated for 8-10 h at 37°C.

To identify proteins, MALDI-TOF/TOF analysis was conducted. In brief, each spot was verified and analyzed using the TOF/TOF™ ion optics installed in Applied Biosystems 4700 proteomics analyzer (Applied Biosystems; Carlsbad, CA, USA). Both MS and MS/MS data were acquired with an Nd:YAG laser with 200 Hz repetition rate, and up to 4,000 shots were accumulated for each spectrum. Operation MS/MS mode was 2 keV collision energy. Air was used as the collision gas with nominally single collision conditions. For these analyses, a resolution of 100 was applied although the prec ursor resolution selection was 200. Both MS and MS/MS data were acquired using the default instrument calibration without applying internal or external calibration. Sequence tag searches were performed with the program MASCOT (http://www.matrixscience.com).

Quantitative RT-PCR (qRT-PCR). Cells were treated with 166 nM PQ for 24 h and harvested. Total-RNA was purified and converted to cDNA using a first strand cDNA synthesis kit (Intron; Daejeon, Korea). qRT-PCR was performed with human ApoE primer set (forward, 5'-GAGCTGAGCAGCTCCTCCC TGAGAC-3' and reverse, 5'-GAGCTGAGCAGCTCCTCCC TG-3'). As an internal expression control, a GAPDH primer set (forward, 5'-TCCCATCACCATCTTCCA-3' and reverse, 5'-CA TCACGCCACAGTTCAG-3') was used. Amplicon sizes were 158 and 380 bp, respectively.

Western blot analysis. In order to measure the Hp and ApoE protein expression from rat and human patient serum, serum albumin was depleted using an albumin depletion kit (Millipore; Billerica, MA, USA) according to the manufacturer’s instructions. In brief, 25 µl of serum was pre-diluted with 275 µl of 1×PBS and diluted serum was incubated with pre-washed albumin magnetic beads for 6 h at room temperature. Following incubation, supernatant was isolated and collected for further experiments. Serum protein concentration was measured using the Pierce BCA protein assay kit (Thermo Scientific; Rockford, IL, USA) and mixed with 2XSDS-PAGE sample buffer (Sigma-Aldrich). Subsequently, 35 µg serum samples were separated on gradient SDS-PAGE gel and transferred to PVDF membranes. Although we measured the concentration of proteins, gels were stained with the MemCode™ Reversible Protein stain kit (Thermo Scientific) to check the concentra-
tion of serum proteins after gel running on the SDS-PAGE (Fig. 2), and by transferring to PVDF membranes for antibody incubation. Anti-Hp and anti-ApoE antibodies were diluted at 1:1,000 and 1:5,000, respectively.

**Results**

Differentially expressed proteins in serum from rats exposed to PQ. It is generally difficult to obtain normal serum (as a control) from PQ-intoxicated patients since patients are hospitalized after PQ ingestion, be it intentional or accidental ingestion. Therefore, we mimicked the conditions of PQ poisoning using a rat animal model. Six rats were acclimated at least 7 days prior to the experiments. Normal serum was then collected from the tail following sedation with ether (Fig. 1). Rats were weighed and their general physical condition were checked. After administrating 30 mg/kg (166 nM) PQ via intraperitoneal injection, body weight was found to be slightly reduced but recovered to normal by Day 3 (data not shown).

To determine the alteration of functional protein expression in acute PQ-exposed rats, we performed proteomic analysis using 200 µg serum samples at 1 day prior to PQ treatment and 1-3 days post-PQ exposure from 2 individual mice.

Representative 2D-PAGE image showed that >500 spots clearly altered their expression (Fig. 2). For further characterization of each spot, 8 spots which showed >2-fold alteration were collected and MALDI-TOF MS/MS analysis was performed. We identified 8 proteins of interest including inflammatory-related C3, FGG, antioxidant related Pphg, ApoE and Ac1-581 (FGB) via the MASCOT program governed by the National Resource for the Mass Spectrometric Analysis of Biological Macromolecules and the Matrix Science Company (Table I). The MASCOT search program revealed that the mass fingerprint of spot 104 was a 45% polypeptide match with ApoE protein. The expression of ApoE was significantly upregulated with PQ exposure (Fig. 3A and B). Two individual spots, 4103 and 4104, were covered with 2 and 4% C3, respectively, and the expression of C3 was significantly upregulated in PQ-exposed rat serum in a time-dependent manner (Fig. 3C and D). Of note, 2 spots, 1103 and 4105, were matched with antioxidant-related Pphg with 10 and 16% peptide coverage, respectively, and the expression was dramatically induced by PQ treatment (Fig. 3E and F).

The expression of other proteins was found to be down-regulated in PQ-exposed rat serum. Spots 1305 and 1306 were

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Sample</th>
<th>GeneBank no.</th>
<th>MASCOT search results file name (ms/ms)c</th>
<th>Identified proteins</th>
<th>Score</th>
<th>Cov (%)</th>
<th>Changed pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>104</td>
<td>gi</td>
<td>37805241</td>
<td>104 msms.htm</td>
<td>Apolipoprotein E</td>
<td>236</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>1305</td>
<td>gi</td>
<td>61098186</td>
<td>1305 msms.htm</td>
<td>Fibrinogen γ-chain</td>
<td>233</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>1306</td>
<td>gi</td>
<td>61098186</td>
<td>1306 msms.htm</td>
<td>Fibrinogen γ-chain</td>
<td>121</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>4103</td>
<td>gi</td>
<td>116597</td>
<td>4103 msms.htm</td>
<td>Complement C3</td>
<td>121</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>4104</td>
<td>gi</td>
<td>116597</td>
<td>4104 msms.htm</td>
<td>Complement C3</td>
<td>205</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>8406</td>
<td>gi</td>
<td>32527707</td>
<td>8406 msms.htm</td>
<td>Ac1-581 (Fibrinogen β-chain precursor)</td>
<td>182</td>
<td>45</td>
</tr>
<tr>
<td>7</td>
<td>1103</td>
<td>gi</td>
<td>204657</td>
<td>1103 msms.htm</td>
<td>Preprohaptoglobin</td>
<td>44</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>4105</td>
<td>gi</td>
<td>204657</td>
<td>4105 msms.htm</td>
<td>Preprohaptoglobin</td>
<td>67</td>
<td>16</td>
</tr>
</tbody>
</table>

**Figure 1.** Schematic experimental design. Rats were acclimated for 7 days. Blood samples were collected at the indicated days. White arrow indicates the intraperitoneal injection of 30 mg/kg (166 nM) PQ at Day 1. Gray arrows indicate blood collection at Day -1, 1 and 3. B indicates blood collection day.

**Figure 2.** Representative 2DE gel of rat serum proteins which were altered during PQ poisoning. PQ-induced alteration of protein expression was visualized by modified silver staining (12). Arrowheads indicate that the change in protein expression was >2-fold when compared with normalized protein expression to the total intensity of valid spots. Numbered arrows indicate proteins identified using MALDI-TOF MS/MS analysis.
Figure 3. Determination and quantitative analysis of upregulated proteins during PQ poisoning. Upregulated protein expression with PQ treatment was observed. The expression of (A and B) apolipoprotein E, (C and D) complement C3, and (E and F) preprohaptoglobin (Pphg) were shown to be upregulated with PQ treatment. S#1 and S#2 indicate 2 different sources of serum in this experiment. Asterisk denotes statistically significant using a paired Student's t-test with p<0.05.

Figure 4. Determination and quantitative analysis of downregulated proteins during PQ poisoning. In order to present data on downregulated protein expression in PQ-exposed serum, (A and B) fibrinogen γ-chain (FGG), and (C and D) Acl-158 are shown. S#1 and S#2 indicate the 2 different sera used in this experiment. Asterisk denotes statistically significant using a paired Student's t-test with p<0.05.
covered with 18 and 20% FGG polypeptide, respectively, and the expression of FGG was drastically downregulated by PQ exposure in a time-dependent manner (Fig. 4A and B). Markedly, Ac1-581 (spot 8406) which is the preproprotein precursor of FGB was identified, with 45% coverage, using the MASCOT search engine, and found to be downregulated by PQ exposure in a time-dependent manner (Fig. 4C and D).

Verification of the expression of APOE, Hp and FGG in PQ-exposed cell lines, rat and patient sera. To verify the change in mRNA and protein expression with PQ treatment, in vitro analysis was performed. In order to measure the change in mRNA expression, the Raw264.7 cell line was treated with 166 nM PQ for the indicated times (0, 1 and 3 days). Total-RNA was then collected and cDNA was synthesized using 1 µg of total-RNA. qRT-PCR was performed with the ApoE gene specific primer set (described in Materials and methods). ApoE mRNA was drastically upregulated at 6 and 12 h with PQ treatment (Fig. 5A). However, the expression was downregulated at 24 h. In addition, we investigated the
expression of the Hp and ApoE proteins in the PQ-treated Raw264.7 and A549 cell lines (Fig. 5B). As we expected, the expression of the Hp and ApoE proteins was upregulated at 6 and 12 h in Raw264.7 cells and A549 cells after PQ treatment, respectively (Fig. 5B).

In order to investigate any possible implication for clinical relevance, we measured the expression of both ApoE and Hp proteins in rat and human serum. In Fig. 6A, the Hp-α, -β proteins, and ApoE were dramatically induced by PQ treatment in a time-dependent manner. Hp-β proteins were also prominently induced in both resuscitated and deceased PQ patients compared to healthy individuals (Fig. 6B). By contrast, expression of FGG was downregulated in PQ patients.

Discussion

Due to the high rate of mortality with poisoning, prompt prognosis and diagnosis of acute PQ poisoning is an absolute requirement for survival. Serum uric acid and the acute-phase response gene pentraxin 3 (PTX3) were recently characterized and highlighted as putative biomarkers for PQ poisoning in human serum (14,15). However, the protocols for diagnosis, prognosis, and treatment of PQ-poisoned patients are limited. These limitations led us to investigate the existence of potentially more effective diagnostic biomarkers from PQ-treated rat serum using conventional proteomics analysis. Although there was concern of protein spots being masked by high-abundance serum proteins such as albumin, immunoglobulin, and complement on 2D-PAGE images, we were able to perform proteomic analysis with total serum to identify putative biomarkers. More than 500 altered protein spots were gained, and we identified 8 differentially expressed proteins. The expression of ApoE, C3 and Pphg, a precursor of haptoglobin (Hp), were induced by PQ treatment while the FGG and Ac1-581 (FGB) proteins were downregulated. The altered protein expressions were further verified by qRT-PCR and western blot analysis. In addition, we detected the induction of ApoE and Hp mRNA expression in the A549 lung carcinoma cell line. This data suggested that these proteins may be beneficial candidate markers for the diagnosis of acute PQ poisoning.

Haptoglobin is an acute-phase response glycoprotein and it plays an antioxidative role due to its binding activity to hemoglobin (16). Haptoglobin is also a good diagnostic marker of lung cancer and is involved in angiogenesis and cell migration (17,18). Li et al. (20) also demonstrated that Hp is upregulated in acute lung damage after pulmonary embolism (19). A human study demonstrated that serum Hp concentration was significantly increased from approximately 824.37 to 2063 mg/l in patients with pulmonary embolism and deep vein thrombosis (PE/DVT) (20). Based on these results, studies have suggested that increasing concentrations of serum Hp may attenuate lung injury that occurs following PE. Karthik et al. (21) demonstrated that the expression of an Hp precursor, Pphg, was greatly altered in the alloxan-induced diabetes model. In addition, the expression of Pphg and Hp in patient urine was dramatically increased in the presence of passive Heymann nephritis and suggested that Pphg and Hp are appropriate candidates for therapeutic targeting and potentially novel biomarkers in membranous nephropathy (MN) and high altitude pulmonary edema (22,23). We identified Pphg via proteomic analysis and observed that its expression was dramatically induced in PQ-treated rat serum. Prior to treatment with PQ, Pphg expression was almost undetectable but expression was significantly induced at Days 1 and 3 post-PQ administration. Furthermore, the expression of Hp-α and Hp-β was detected in PQ-treated rat and human serum. In accordance with previous studies, we speculated that the induction of Pphg and Hp expression was a response to PQ treatment, therefore, Pphg and Hp serum levels may be effective diagnostic biomarkers for PQ poisoning.

Accumulating evidence from animal studies has revealed that ApoE is a key protein in atherosclerosis via its role in inflammation, control of cholesterol, and blood pressure (24). A previous study demonstrated that ApoE can interact with ApoE in a mechanism by which inflammation affects atherosclerotic progression (25). Cigliano et al. (25) suggested that the interaction between Hp and ApoE represents a novel link between the acute phase of inflammation and ApoE function. In addition, the ApoE knockout mice, a lack of the antioxidant enzyme, and glutathione peroxidase-1 (GPx1), accelerate diabetes-associated atherosclerosis through induction of inflammation and fibrosis (19). However, Cuthbert et al. (26) demonstrated that the level of expression of the major protein component of high-density lipoprotein (HDL), apolipoprotein A-I (ApoA-I) but not ApoB and E, is regulated by PQ exposure in the HepG2 cell line. In this study, we observed that the expression of the ApoE protein was dramatically induced by PQ treatment. Based on our observation, we speculated that early acute PQ exposure may induce ApoE which leads to the regulation of the acute inflammatory response and antioxidant-related genes. The dynamic expression among the Apo family of proteins requires further investigation in order to better understand the regulatory network between PQ and the early immune response.

Although fibrinogen γ-chain (FGG) is known to be a cofactor in blood clots, FGG and fibrinogen β-chain (FGB) are also known as biomarkers for the detection of occurrence and progression of coronary artery disease (CAD) in ApoE−/− mice (27). FGG is also involved in aging through the regulation of oxidative stress and inflammation (28). In this study, FGG was dramatically downregulated by PQ exposure. To date, there has been no data regarding the role of FGG in PQ intoxication. Therefore, we speculated that the antagonistic expression between ApoE and FGG is in direct response to acute PQ poisoning.

It has been well established that PQ induces ROS formation, and, consequently, development of acute lung injury such as lung fibrosis via induction of the NF-κB regulated pro-inflammatory pathway (1). Complements have been involved in early immune reaction but complement component 3 (C3) levels in serum were not altered with long-term PQ exposure in Balb/c mice (29). Sun et al. (30) demonstrated that blocking the complement pathway ameliorates acute lung injury (ALI) induced by PQ treatment. The treatment of complement C3 inhibitor, CR2-Crry, has been shown to particularly effective in reducing PQ-induced inflammation, pathology, and mortality (30). Data suggest that C3 may play an important role in acute, early inflammatory reactions induced by PQ. In the present study, PQ induced expression of C3 which was shown to activate the acute pro-inflammatory response and
subsequent inflammation. Therefore, we propose that the detection of C3 expression in serum is a beneficial diagnostic tool for PQ-induced acute lung inflammation.

In conclusion, based on our proteomics profiling data we suggest that Hp, ApoE, C3 and FGG in PQ-exposed serum might be appropriate diagnostic biomarkers for the early detection and diagnosis of acute PQ poisoning.

Acknowledgements

This study was partially supported by a grant from the Rural Development Administration, Republic of Korea (project no. PJ008246).

References