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Proteomic identification of haptoglobin as a stroke plasma biomarker in spontaneously hypertensive stroke-prone rats

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ABSTRACT

Aims: We investigated changes in the expression of plasma proteins in spontaneously hypertensive stroke-prone rats (SHRSP) to identify stroke biomarkers.
Main Methods and Key findings: The present analysis using surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) demonstrated that three peaks at mass/charge ratios (m/z) of 9330, 9480 and 9700 decreased in intensity during the development and progression of hypertensive stroke in SHRSPs, but not in age-matched control SHR and Wistar rats. Administration of verapamil, an L-type calcium channel blocker which was effective for hypertension in SHRSP rats, prevented the decrease in plasma protein expression. A candidate biomarker protein (m/z 9330) was identified using LC-MS/MS as haptoglobin (Hp). Immunoblotting with anti-Hp antibody demonstrated the decreased expression of both Hpα and Hpβ chains in SHRSP. In contrast, *haptoglobin* mRNA expression in the liver of SHRSPs slightly increased as compared with control rats.
Significance: These findings suggest that Hp is a biomarker candidate for discriminating pathogenic alterations of stroke.

Introduction

Hypertension is the major risk factor for various kinds of vascular events and subsequent organ damage such as stroke, cardiac hypertrophy, nephrosclerosis and atherosclerosis (Hollander, 1976). The stroke-prone spontaneously hypertensive rat (SHRSP) is a widely used experimental model of malignant hypertension and has a high incidence (95%) of cerebrovascular disease (Yamori et al., 1984; Yamori, 1991; Yang et al., 2002). Previous studies indicate that, in SHRSP, the development of brain damage is invariably preceded by the development of an inflammatory condition characterized by the accumulation of acute-phase proteins in serum and urine (Sironi et al., 2001). However, the pathogenic mechanism has not been completely defined, largely resulting from the difficulty of timing the occurrence of brain damage, locating it, and defining the initial events. Therefore, the identification of biomarkers for diagnosing pathogenic alterations will contribute to improving treatments for the early stages of disease.

ProteinChip® technology (surface-enhanced laser desorption/ionization time of flight mass spectrometry, SELDI-TOF-MS) has recently been developed to facilitate protein profiling of biological mixtures and to discover biomarkers to diagnose various diseases (Adam et al., 2002; Issaq et al., 2002; Kiga et al., 2005; Matsumoto et al., in press; Ogawa et al., 2007; Shiwa et al., 2003; Wulfkuhle et al., 2001; Zhang et al., 2004). SELDI-TOF-MS is analytic innovative technology that can be employed for proteomic analysis to rapidly search for and identify multiple differentially expressed proteins and molecular-related ions (Adam et al., 2002; Cazares et al., 2002; Liggett et al., 2004; Petricoin et al., 2002; Kozak et al., 2003; Wadsworth et al., 2004; Wilson et al., 2004).

The objective of this study is to identify plasma protein biomarkers associated with the progression or development of stroke in SHRSP using ProteinChip array. We found that plasma haptoglobin dramatically decreased in SHRSPs suffering a stroke.

Materials and methods

Animals and treatments

Seven-week-old male Wistar rats ($n=8$), SHR ($n=8$) and SHRSP ($n=10$) were purchased from Japan SLC (Hamamatsu, Japan) and kept in an automatically controlled room (temperature ~23 °C and humidity ~60%) with a conventional dark/light cycle. The rats were maintained under specific pathogen-free conditions and used according to institutional guidelines.

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Systolic blood pressure in the conscious state at room temperature was measured by a tail cuff method using a Model MK2000 BP monitor for rats and mice (Muromachi Kikai, Tokyo, Japan) according to the manufacture's instructions. Body weight was also measured weekly. Rats in the verapamil groups received standard chow containing 0.1% (wt/wt) verapamil from 9 weeks of age.

SELDI protein profiling

Plasma samples were centrifuged at 800 ×*g* for 10 min to remove insoluble debris and stored at −80 °C until used in the SELDI profiling study. Samples were thawed and diluted (1:10 v/v in 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% ampholyte).

The ProteinChip Arrays were assembled into a deep-well-type Bioprocessor (Ciphergen Biosystems, Fremont, CA) as part of the Laboratory Automation Workstation Biomek® 2000 (Beckman Coulter, Fullerton, CA). Prior to sample loading, CM10 arrays were equilibrated with 150 µl of buffer (100 mM sodium acetate, pH 4.0) in each well and then pre-washed two times for 5 min on a shaker at room temperature. When the p*I* value was measured, CM10 arrays were equilibrated with 150 µl of buffer (100 mM sodium acetate pH 4.0 and 5.0, 50 mM HEPES pH 5.5, 6.0, 6.5, and 7.0, 50 mM Tris–HCl pH 8.0 and 9.0) in each well. For plasma protein binding to the arrays, 90 µl of buffer (100 mM sodium acetate, pH 4.0) and 10 µl of diluted sample were added to each well, and then incubated for 30 min on a shaker at room temperature. The arrays were washed three times with 150 µl of buffer for 5 min on a shaker. After rinsing two times with 200 µl of deionized water, the arrays were removed from the Bioprocessor and air-dried. One microliter of 50% solution of the energy-adsorbing molecule (EAM), sinapinic acid (SPA) (Ciphergen Biosystems), was applied two times onto each array.

The ProteinChip Arrays were analyzed using a ProteinChip Biology System Reader (Model PBS-IIc; Ciphergen Biosystems). The protein masses were calibrated externally using purified peptide and protein standards (Ciphergen Biosystems). A mass range of *m*/*z* 3000–10,000 was selected for analysis because this range contained the majority of the resolved protein/peptides.

Data analysis

Spectra were analyzed with ProteinChip Software (Version 3.2.0; Ciphergen Biosystems). In order to use the intensities as indicators of the relative abundance of the supposed peptide in the sample, baselines must be subtracted and the intensities normalized. Normalization was performed by total ion current normalization function following the software instructions. Biomarker Wizard (Ciphergen Biosystems) was then used to identify corresponding peaks in each spectrum within 0.3% of the mass. Signal-to-noise ratio was set to 2 for the first and second passes. We used the Mann–Whitney *U* test for nonparametric data sets to compare the peak intensities of the protein profiling results from different groups.

Identification of candidate proteins

Ion exchange fractionation was undertaken on a CM HyperD F Spin Column (Ciphergen Biosystems) pre-equilibrated with 100 mM sodium acetate, pH 4.5. Plasma was diluted at a ratio of 1:10 in urea denaturing buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% ampholyte) and incubated for 20 min on ice. Samples were then diluted 1:5 in buffer (100 mM sodium acetate, pH 4.5) and applied to the column. Bound proteins were eluted using the buffer with a stepwise increase in NaCl (100 and 200 mM). Eluted fractions were pooled and concentrated by membrane fractionation (cut-off 3 kDa) (Sartorius). SELDI-TOF-MS was used to monitor each fraction for ions of interest.

The peak was identified using SDS-PAGE coupled with mass spectrometry analysis (performed by APRO Life Science Institute, Inc., Tokushima, Japan). Proteins were separated under reducing conditions on 12.5% SDS-PAGE gels (9 cm × 9 cm) and visualized by colloidal Coomassie Brilliant Blue R 250 staining according to standard protocols. The separated proteins were transferred electrophoretically onto a PVDF membrane (Millipore). Membrane pieces containing the proteins of interest were excised and peptide sequences of *m*/*z* 9330 determined from N-terminal amino acid sequence analysis.

Western blot analysis

Proteins were separated under reducing conditions on 12.5% SDS-PAGE gels. For Western blot analysis, SDS-PAGE-separated proteins were transferred electrophoretically onto a PVDF membrane (Millipore). The membrane was treated with BlockAce (Dainippon Pharmaceutical Co. Ltd., Suita, Japan) overnight at 4 °C.

Primary rabbit anti-rat haptoglobin antibodies (Life Diagnostics, Inc.) and secondary anti-rabbit HRP conjugates were diluted 1:1000, and visualized with the ECL system (Amersham Biosciences).

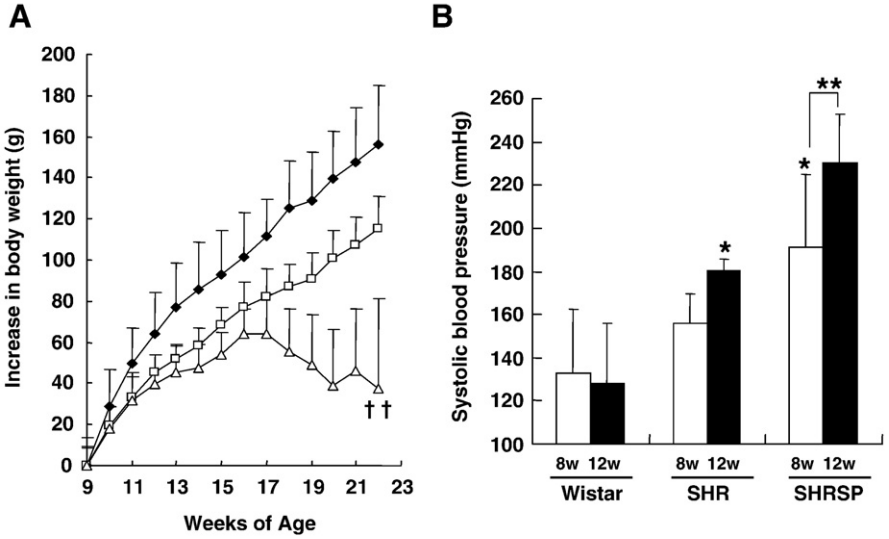

Fig. 1. Changes in body weight and blood pressure. (A) Changes in body weight of Wistar (closed diamond, $n=8$), SHR (open triangle, $n=8$), and SHRSP (open square, $n=10$) rats are shown. Two SHRSP rats died at the age of 21–22 weeks. (B) Blood pressure at 8 weeks of age (open bar), 12 weeks of age (closed bar) is shown. Data are expressed as the group-averaged mean ± SD. *$P<0.05$ vs. age-matched Wistar rats. **$P<0.01$.