Acute-Phase Proteins Before Cerebral Ischemia in Stroke-Prone Rats
Identification by Proteomics

Luigi Sironi, PhD; Elena Tremoli, PhD; Ingrid Miller, MS; Uliano Guerrini, PhD; Anna Maria Calvio, MS; Ivano Eberini, MS; Manfred Gemeiner, PhD; Maria Asdente, PhD; Rodolfo Paololetti, MD; Elisabetta Gianazza, PhD

Background and Purpose—A high degree of proteinuria has been reported in stroke-prone spontaneously hypertensive rats (SHRSP). We studied the effect of salt loading on the detailed protein pattern of serum and urine in 3 rat strains: Wistar-Kyoto, spontaneously hypertensive rats, and SHRSP, an inbred animal model for a complex form of cerebrovascular disorder resembling the human disease.

Methods—Rats were given a permissive diet and received 1% NaCl in drinking water. The protein pattern in body fluids was assessed over time by 2-dimensional electrophoretic analysis. Brain alterations were monitored by MRI and histology.

Results—Several proteins were excreted in urine after weeks of treatment and in advance of stroke: transferrin, hemopexin, albumin, α2-HS-glycoprotein, kallikrein-binding protein, α1-antitrypsin, Gc-globulin, and transthyretin. Markers of an inflammatory response, including very high levels of thiostatin, were detected in the serum of SHRSP at least 4 weeks before a stroke occurred.

Conclusions—In SHRSP subjected to salt loading, an atypical inflammatory condition and widespread alterations of vascular permeability developed before the appearance of anomalous features in the brain detected by MRI. Urinary concentrations of each of the excreted serum proteins correlated positively with time before stroke occurred. (Stroke. 2001;32:753-760.)

Key Words: animal models • inflammation • nuclear magnetic resonance • proteome • stroke • rats

The spontaneously hypertensive stroke-prone rat (SHRSP) provides an inbred animal model for a complex form of cerebrovascular pathology resembling, in many aspects, the human disease. In these animals stroke occurs only after high blood pressure has developed; lag time is greatly reduced if rats are exposed to a specific permissive diet, low in potassium and protein and high in sodium (Japanese permissive diet [JPD]). In this model, stroke is not just a consequence of long-standing hypertension but represents the final outcome of a complex interaction between environmental (ie, dietary) and genetic factors. The role of the latter is emphasized by the resistance to stroke of a closely related strain, the spontaneously hypertensive rat (SHR), which remains stroke-free despite a similar degree of hypertension after exposure to JPD.

Recent data show that alterations in structure, regulation, and function of the gene encoding atrial natriuretic peptide (ANP) are significantly linked to cerebral disease in SHRSP. Further reports suggest that molecular variants of the ANP gene represent an independent risk factor for human stroke. These data confirm the resemblance between cerebrovascular accidents in humans and in the SHRSP model.

It has been shown that cerebral edema identified by T2-weighted MRI in salt-loaded SHRSP is preceded by proteinuria. Preliminary experiments performed on urine samples of SHRSP showed that, in addition to albumin, several acute-phase proteins are detectable by means of 2-dimensional electrophoresis. This observation suggested to us that in this animal model inflammation might occur well before the onset of the acute cerebral event. Therefore, this study addressed the issue of a potential contribution of acute inflammation to the occurrence of cerebral damage in salt-loaded SHRSP.

The term proteome refers to proteins expressed by a genome in a given tissue at a given time; its analysis relies on 2-dimensional polyacrylamide gel electrophoresis. The simultaneous characterization and quantification in body fluids of gene products and of their posttranslational modifications...
can suggest which biochemical mechanisms are involved in a pathological situation and may identify disease markers.  

Our group has extensively studied the proteome of rat serum, identifying and quantifying all major proteins under baseline conditions and after a number of experimental treatments.  

In this report the proteome of serum and urine in SHRSP rats exposed to JPD during and after the development of cerebral damage, in comparison with SHR and Wistar-Kyoto rats (WKY), was characterized by 2-dimensional polyacrylamide gel electrophoresis.

### Materials and Methods

#### Animals and Treatments


Male WKY (n=5), SHR (n=5), and SHRSP (n=8) were purchased from Charles River. At 6 weeks of age the animals were placed on JPD (Laboratorio Dr Piccioni; 18.7% protein, 0.63% potassium, 0.37% sodium) and received 1% NaCl in drinking water. Animal treatment lasted up to 49 days. The body weight of the animals, as well as food and liquid intake, was recorded weekly. Serum and 24-hour urine were sampled and arterial blood pressure was monitored before the onset of the diet and every seventh day afterward. Systolic arterial blood pressure was measured in conscious rats by tail-cuff plethysmography (PB Recorder 8006, Ugo Basile), after warming at 37°C.

Blood was drawn from the tail vein at different time intervals (see Results), and serum was obtained by allowing the blood to clot for 1 hour at 37°C followed by centrifugation for 20 minutes at 3000 rpm. Urine was collected by housing rats in individual metabolic cages; its protein concentration was measured according to Bradford15 with bovine albumin as standard.

All rats underwent weekly MRI. MRI assessment was repeated every other day in SHRSP once 24-hour proteinuria exceeded 40 mg/d and daily after brain abnormality had been detected by T2-weighted MRI.

### MRI Evaluations

Rats were anesthetized with 2% isoflurane in 70% N₂/30% O₂, fixed on the animal holder by a rod beneath the teeth, and placed into the

<table>
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<tr>
<th>Protein</th>
<th>Fluid</th>
<th>Curve</th>
<th>Correlation Coefficient</th>
<th>P</th>
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<td>Second order</td>
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<td>&lt;0.0029</td>
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<tr>
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<td>First order</td>
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<td>&lt;0.0001</td>
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<tr>
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<td>First order</td>
<td>0.728</td>
<td>&lt;0.0001</td>
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Abbreviations are as defined in Figure 6.
magnet (4.7 T, vertical 15-cm bore) of a Bruker spectrometer (AMX3 with microimaging accessory). A 6.4-cm-diameter birdcage coil was used for imaging. After a 3-orthogonal plane gradient echo scout, a T2 multislice image was obtained. Sixteen contiguous 1-mm-thick slices were analyzed caudal to the olfactory bulb; field of view was 4×4 cm². Turbo spin-echo sequence was used with 16 echoes per excitation, 10 ms interecho time, 85 ms equivalent echo time, and 4 seconds repetition time. The images were 128×128 points (zero filled to 256×256); 8 images were averaged in 8 minutes and 30 seconds. The occurrence of lesions was identified with presence of areas of high signal intensity on T2-weighted MRI. The extension of the lesions was determined by thresholding the images and interactively drawing outlines of the lesion and of the whole brain; the ratio between the number of pixels in the lesion versus that in the whole brain in each slice gave the percentage of the brain affected by the lesion. The percentage of hyperintense pixels was evaluated in 9 slices: 4 caudal and 4 rostral to the central slice where the primary lesion had been detected.

**Histology**

After the last MRI session, the anesthetized rats were perfused through the left ventricle with 4% paraformaldehyde in 0.1 mmol/L phosphate buffer. The brains were dissected, postfixed overnight in the same solution, cryoprotected by immersion in 30% sucrose-phosphate buffer. The brains were dissected, postfixed overnight in the same solution, cryoprotected by immersion in 30% sucrose-phosphate buffer, and sectioned with a cryotome. Coronal sections, 10 μm thick, were stained with hematoxylin-eosin and/or toluidine blue and examined microscopically.

**Electrophoretic Techniques**

One-dimensional electrophoresis was mainly used to assess the relative concentrations of the major components (transferrin and albumin in serum, α-2u-globulin in urine), and 2-dimensional electrophoresis was mainly used to evaluate with high sensitivity the average- and low-abundance components under conditions in which the signal for albumin was saturated.

One-dimensional electrophoresis was run in the presence of SDS, without sample reduction, in a discontinuous buffer system on polyacrylamide gradients 4% to 20% T. The sample load was 3.75 μg of urine proteins and 0.125 μL of serum per lane. Two-dimensional electrophoresis maps were obtained by the immobilized pH gradient (IPG)–Dalt method. Sample proteins, reduced with 2% 2-mercaptoethanol, were first resolved according to charge on a nonlinear pH 4 to 10 IPG in the presence of 8 mol/L urea and 0.5% carrier ampholytes, with an anode-to-cathode distance of 8 cm. The focused proteins were then fractionated according to size by SDS-PAGE on 7.5% to 17.5% polyacrylamide gradients, with 2 IPG strips mounted on each 160×140-mm² SDS slab. Sample loads were 100 μg of urine proteins or 2 μL of serum. Proteins were stained with 0.3% wt/vol Coomassie or with silver nitrate for some urine samples.

The protein patterns were scanned with a video camera under the control of NIH Image, release 1.61, and analyzed with the software PDQUEST version 5.1 (PDI). Data for individual proteins (spots or spot chains identified by immunological or physicochemical means) are reported as spot volumes as a function of time before or after MRI evidence of stroke.

**Statistical Analysis**

Linear and nonlinear fits were taken as statistically significant for P<0.05. For every fit, R and P values are listed in the Table. Differences between groups were evaluated by ANOVA for repeated measurements, followed by Bonferroni’s post hoc test. For SHRSP this analysis was limited to day 42 from the start of the treatment, the last time point at which all these rats still survived.

**Results**

**Physical Parameters**

The growth of SHRSP was slower than that of SHR and WKY. Between days 28 and 49 of treatment, SHRSP lost 10% of body mass and weighed 55% to 65% of the age-matched controls at the time of euthanasia (Figure 1a).

At baseline, WKY, SHR, and SHRSP had systolic blood pressure of 118±5, 120±10, and 130±8 mm Hg, respectively. Systolic blood pressure of SHR and SHRSP, but not of WKY, increased markedly during JPD and salt loading (Figure 1b).

**Identification of Brain Damage by MRI and Histological Analysis**

As a result of salt loading, all SHRSP developed cerebral lesions, at a median of 42±3 days from the start of treatment. Brain lesions occurred within the gray matter, usually in the frontal or forelimb areas of cortex or in the ventral region of...
From these single foci the lesions then spread throughout the brain. In 3 of 8 rats the damage reached the contralateral hemisphere via the corpus callosum (not shown). Three days after the first evidence of brain damage, the extent of the lesion averaged 12.5 ± 1.6% (SD; n = 8).

Histological evaluation confirmed that the alterations in brain MRI identified areas characterized by tissue thinning due to loss of neuronal cells (Figure 2b). Neither SHR nor WKY developed brain abnormalities detectable with MRI or histological examination throughout the experimental period (not shown).

### Proteomics Data

#### Urine

Between days 0 and 7 of salt loading, protein loss per day (24-hour proteinuria) increased 3- to 4-fold in all experimental groups, but thereafter it changed little for WKY and SHR (Figure 3a). In SHRSP, mean protein loss increased sharply (and linearly) after day 28 of treatment (Figure 3). The delay between the time when proteinuria (evaluated once a week) indicated a protein loss ≥ 40 mg/d and the time when MRI, performed twice a week, identified focal cerebral ischemia was 9 ± 3 days. In 6 of 8 rats, urine protein loss peaked immediately before stroke and declined thereafter.

In SHRSP, not only the total amount of protein in urine but also the qualitative protein composition changed with time, as shown by 1-dimensional electrophoretic analysis through SDS-PAGE (Figure 4). The ratio between 20-kDa components, ie, α-2u-globulin, and 65- to 68-kDa components, mostly albumin, dropped between days 28 and 35 of treatment from approximately 15 (typical for WKY) to approximately 0 (Figure 4a). The largest protein found in SHRSP urine was transferrin (molecular weight [MW], 77 kDa). The ratio of transferrin to 65- to 68-kDa components in urine increased from 0 (typical of WKY) to approximately 0.2, with most of these changes occurring between days 28 and 35 of treatment. After day 35, both ratios in SHRSP differed significantly from those in WKY (and SHR; not shown). The ratio between 77-kDa and 65- to 68-kDa proteins in the serum of rats did not change during the study period (Figure 4b). At day 42, this ratio in urine of SHRSP had approximated that in serum (0.18 ± 0.03) (Figure 4b and 4c) and had even sur-
serum from WKY

urine from WKY

serum from SHR-SP

urine from SHR-SP

Figure 5. Two-dimensional electrophoresis (2-DE) maps of serum (left) and urine (right), from a control WKY (top) and a poststroke SHRSP (bottom). All samples were collected at day 48 from the beginning of the dietary treatment, corresponding to day +3 from stroke for SHRSP (rat 3; refer to Figure 3b). Serum is loaded on a volume basis (2 μL) and proteins on a weight basis (100 μg). α1 indicates α1-antitrypsin; α1I3, α1-inhibitor III; α1M, α1-major acute-phase protein, or haptoglobin; α1HS, α1-HS-glycoprotein; apo, apolipoprotein; C, ceruloplasmin; CRP, C-reactive protein; Gc, Gc-globulin; Hp, haptoglobin; KBP, kallikrein-binding protein, MUP, α2-M, or thiostatin; Tf, transferrin; TTR, transthyretin; and pI, isoelectric point.

passed it (0.20±0.02), so that the bulky proteins were now leaking freely into the urine.

Analysis of the protein pattern in urine by 2-dimensional electrophoresis (Figure 5) confirmed that α2-M-globulin is by far the major protein in male rat urine under baseline conditions. Albumin and IgL were the only serum proteins recognized in the pattern of WKY and SHR at all time points, whereas in SHRSP the proteins of high M lost in urine during treatment were resolved by 2-dimensional electrophoretic analysis as transferrin, albumin, haptoglobin, kallikrein-binding protein, α2-HS-glycoprotein, serine protease inhibitor 3, α1-antitrypsin, Gc-globulin, apolipoprotein A-IV, transthyretin, and the albumin fragments migrating as slanted rows with M, 50 and 30 kDa. Hemopexin is not easily recognized in Coomassie-stained patterns, but its presence was confirmed by immunoblotting (not shown).

Figure 6 shows the best-fit curves for individual SHRSP data of the amounts of major proteins in urine and plasma versus time from stroke. For every protein detected in urine, a positive and highly significant linear correlation (R=0.67, P<0.0001) was observed with time (Table). The intercept of the regression line on the time axis was approximately day −20 from stroke for most proteins in urine but corresponded to −17 for thiostatin (versus −30 for its first appearance in serum) and to −25 for transthyretin.

An extensive preliminary investigation of which gels were heavily loaded and silver-stained for high sensitivity detected no difference in the amounts of minor components among urine from WKY, SHR, and SHRSP until loss of serum proteins began in the latter (not shown).

Discussion

In humans, C-reactive protein is the most sensitive marker of many inflammatory conditions, since its level increases by 1 order of magnitude within a few hours from the noxious stimulus, and several studies have identified C-reactive protein as a strong predictor of myocardial infarction. Specifically, α1-antitrypsin and serine protease inhibitor 3 and thiostatin increase linearly in serum throughout the duration of the treatment. Kallikrein-binding protein and α2-HS-glycoprotein increase at first, then decrease, with α2-HS-glycoprotein, a negative acute-phase protein, almost vanishing at the latest experimental times (Figure 5).

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Phase proteins begin to increase over basal levels soon after the start of the treatment (Figure 6); thiostatin is first detected at least 4 weeks before the occurrence of stroke. Most of the acute-phase proteins found in serum are also detected in urine of SHRSP, and their level increases over time, with maximal values at the date of euthanasia (Figure 6). A time delay with respect to serum that varies from one protein to another is observed. In particular, transthyretin (Mr, 56 kDa) is detected in urine approximately 25 days before the appearance of ischemic brain features; thiostatin is detected only 16 days before. Thus, selectivity of excretion in terms of both size and charge is noticed. Indeed, the higher 77 kDa/65 to 68 kDa ratio measured in urine than in serum (Figure 4) is due to preferential loss of albumin versus other components of similar M, (Figure 5). In addition, in urine samples from SHRSP only the most alkaline isoforms (glycoforms) of transthyretin are observed (Figure 5). α-2u-Globulin is a pheromone-binding lipocalin synthesized in male rat liver under the influence of androgens and readily lost in urine. Its excretion is known to be reduced in animals on a low-protein diet. After 42 days of treatment, SHRSP excrete <7 mg/d α-2u-globulin versus 12 mg/d for both WKY and SHR.

An increase in proteinuria before the occurrence of stroke in susceptible animals (SHRSP) subjected to high sodium intake has been previously reported. No information, however, was available on the protein composition of urine of SHRSP compared with WKY or SHR. Our data detail which proteins are lost in urine, as well as the time course of their excretion, in relationship to their varying concentration in serum.

The changes in the serum levels of several acute-phase proteins, with some of the positive acute-phase proteins being eventually excreted in urine, point to an inflammatory condition developing before stroke. In the present investigation we have not collected evidence on the tissues where the inflammatory status first develops in SHRSP. Data in the literature report extensive kidney abnormalities in this animal model, including thrombotic, proliferative, and necrotic lesions in arterioles and glomeruli, often preceding the occurrence of stroke. In addition, for patients with end-stage renal disease, a complex and mutual interaction between acute-phase inflammatory process and all aspects of kidney disease (uremia, heart failure, malnutrition, anemia) has been proposed. Since subpopulations of individuals with high levels of C-reactive protein and cytokines have been reported among patients both with normal renal function and with chronic renal failure, no conclusion is possible about cause versus consequence. Definitely, genetic background
plays a relevant role in SHRSP because neither organ failure nor acute-phase reaction is observed in the related rat strain SHR under identical environment conditions.

Treatment with angiotensin I–converting enzyme inhibitors and angiotensin II antagonists prevents renal disorders, including proteinuria, as well as the occurrence of brain lesions.\(^{40–43}\) The protective effects of these drugs, however, are independent of alterations in blood pressure, which supports the hypothesis that development of vascular injury requires the concurrent involvement of local factors. In this experimental model the same drugs also exert protective effects against cerebral ischemia, which in turn suggests a potential role in the control of vascular permeability as well as of the development of an inflammatory reaction.

An increasing number of in vivo data suggest that the inflammatory response that follows stroke is involved in the pathogenesis of cerebral ischemia.\(^{44}\) Cytokines, synthesized locally during the development of brain lesions, have been suggested to exacerbate brain ischemic injury by several mechanisms; they activate the synthesis of acute-phase reactants.\(^{45}\) Less is known about the prognostic value of inflammatory markers in this pathological condition. C-reactive protein levels, however, have been reported to be an independent predictor not only of cardiovascular events but also of ischemic brain disease.\(^{46,47}\) Moreover, C-reactive protein concentration within 72 hours of an ischemic stroke is either an independent predictor of survival\(^ {27}\) or an irrelevant parameter.\(^ {37}\) Thus, an inflammatory reaction may also have a pathogenetic role in the occurrence of acute stroke. Experimental models to test this hypothesis, however, are lacking. SHRSP exposed to JPD, in which inflammation develops before the appearance of anomalous features in brain MRI, represent a useful experimental tool to understand how inflammation contributes to ischemic brain injury and to answer the question of whether anti-inflammatory strategies may affect genesis, progression, and outcome of brain damage. On the basis of this growing understanding, new pharmacological approaches should be designed that will enlarge our therapeutic armamentarium for the prevention and/or treatment of brain disease.

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References


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