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Gender differences in endothelial function and inflammatory markers along the occurrence of pathological events in stroke-prone rats

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Abstract

Spontaneously hypertensive stroke-prone rats (SHRSP) feature an established model for human cerebrovascular disease. SHRSP, kept on a high-salt permissive diet (JPD), develop hypertension, renal and brain damage. In this report we compared the behavior of female and male SHRSP regarding the main aspects of their pathological condition. Brain abnormalities, detected by magnetic resonance imaging, developed spontaneously in males after 42±3 days, in females after 114±14 days from the start of JPD. Survival was >3-fold longer for females than for males. The development of brain damage was preceded, in both genders, by an inflammatory condition characterized by the accumulation in serum and urine of acute-phase proteins. The increase in thiostatin level was significantly lower and delayed in female in comparison to male SHRSP. During JPD female and male SHRSP developed massive proteinuria, its worsening being significantly slower in females. The alterations of vasculature-bound barriers in kidney and brain were connected with endothelial dysfunction and relative deficiency in nitric oxide (NO). In thoracic aortic rings, basal release of NO was significantly higher in female than in male SHRSP, both if receiving and if not receiving JPD. The gender differences in SHRSP thus appear to be connected to a more efficient control in females of inflammation and of endothelial dysfunction.

Keywords: Stroke-prone rats; Gender differences; Inflammation; Vasculature; Endothelial function

Introduction

The naturally occurring sexual dimorphism has been implicated in risk, progression and recovery from numerous diseases. In fact, women experience lower rates of vascular disease and atherosclerosis-related ischemic stroke than males (Murabito, 1995). These epidemiological findings, although generally attributed to estrogen, remain unclear. In fact, there is no sufficient evidence that hormone replacement therapy is associated with a change in vascular events in postmenopausal women, indicating that other factors may be involved in the natural protection observed in females (Murphy et al., 2004; Bromley et al., 2005).

Usually, only males have been studied in animal models of vascular and neurological diseases. The use of males was justified as a mean of reducing experimental variability caused by female hormone cycling and was based on the assumption that pathological mechanisms or therapeutic effects observed in males would also apply to females. In recent years, it has been recognized that disease conditions and responses to therapy may be different between sexes, and that women must be incorporated into clinical trials (Antony and Berg, 2002). Much remains to be explored in women about vascular diseases,

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and in particular about stroke, such as the effect of age, reproductive status, as well as the definition of gender-specific risk factors.

Stroke-prone spontaneously hypertensive rats (SHRSP) develop spontaneously hypertension and multi-system end-organ damage, particularly at kidney and brain level, and constitute a reliable model for many of the events in human vascular disease (Sironi et al., 2001). A body of experimental evidence indicates for SHRSP a sexual dimorphism in many cardiovascular phenotypes including blood pressure (Clark et al., 1996) and endothelial function (Hamilton et al., 2001). Furthermore, previous data show that, although salt-loaded male and female SHRSP exhibit a similar increase in blood pressure, the average life-span in males is much shorter than that observed in females (Okamoto et al., 1974; Chen et al., 1997). Renal lesions are more frequently observed in male than in female rats and the susceptibility to renal damage is influenced by genetic factors (Gigante et al., 2003). A gender-related difference in response to antihypertensive therapy, in terms of drug-induced NO-dependent relaxation, has been recently reported for the SHRSP strain (Graham et al., 2004). These data support previous observations indicating that NO availability, reduced in males in comparison to females, may be responsible for sex differences in vascular reactivity independently of endothelial NO synthase and of superoxide (McIntyre et al., 1997; Kagota et al., 2001).

Together, the above observations suggested a thorough comparative investigation on the features of male and female SHRSP and specifically of vascular functionality in both genders. Furthermore, because previous studies had indicated that the occurrence of brain damage is invariably preceded, in male SHRSP, by the accumulation of acute-phase proteins (Sironi et al., 2001), we analyzed the proteomes of body fluids in male and female SHRSP for recognizing the occurrence of an inflammatory condition as well as for assessing the integrity with time of vasculature-bound barriers. In addition, because a complex relationship exists between endothelium dysfunction and inflammation, vascular reactivity was investigated in isolated aortic rings from male and female SHRSP.

Materials and methods

Animals and treatments

Procedures involving animals and their care were conducted at Dipartimento di Scienze Farmacologiche dell'Università degli Studi di Milano in conformity with the institution's guidelines, which are in compliance with national (D.L. no. 116, G.U., suppl. 40, 18 Febbraio 1992, Circolare no. 8, G.U., 14 Luglio 1994) and international (EEC Council Directive 86/609, OJL 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996) rules and policies. Spontaneously hypertensive stroke prone rats, SHRSP ($N=50$ males and $N=50$ females) were purchased from Charles River (Calco, Lecco, I). At 6 weeks of age approximately one half of the rats were placed on Japanese diet (JPD; Laboratorio Dr. Piccioni, Gessate, I: 18.7% protein, 0.63% potassium, 0.37% sodium), and received 1% NaCl in drinking water. Twenty-four-hour urine was sampled before the onset of the diet, and every seventh day afterwards; serum was sampled at the beginning of JPD and after brain abnormalities had developed, when also CSF was collected. Once a week, all the rats were housed individually in metabolic cages for 24 h to measure their food and liquid intake and to collect urine. Urinary protein concentration was measured according to Bradford (Bradford, 1976) with bovine albumin as standard. Blood was drawn from the tail vein, and serum was obtained by allowing the blood to clot for 1 h at 37 °C followed by centrifugation for 20 min at 3000 rpm. CSF was drawn at the time of sacrifice from anesthetized animals by cannulation of the cisterna magna with disposable micropipets (Corning Inc. NY, USA). Up to 50 μL was obtained from each rat; specimens contaminated with blood or below 25 μL in volume were discarded. Systolic arterial blood pressure was measured every week in conscious rats by means of tail-cuff plethysmography (PB Recorder 8006, Ugo Basile, Comerio, Italy). All rats underwent weekly magnetic resonance imaging (MRI). MRI assessment was repeated every other day in SHRSP once 24-h proteinuria exceeded 40 mg/day (Blezer et al., 1998), and daily after brain abnormality had been detected by T2W MRI.

Kaplan–Meier analysis as a function of 'time on JPD' and statistics for equality of survival distributions (Log Rank) for 'gender' were carried out with SPSS (SPSS Inc., Chicago, IL, USA).

MRI evaluations

Anesthetized rats were placed in the magnet (4.7T, vertical 15-cm bore) of a Bruker spectrometer (AMX3 with micro-imaging accessory). A 6.4-cm diameter birdcage coil was used for imaging; the field of view (FOV) was 4×4 cm^2. Eight contiguous 2-mm thick T2W slices were acquired caudal to the olfactory bulb; hyperintense areas were interpreted as vasogenic edema. Turbo spin echo (Bruker RARE) was used, with 16 echoes per excitation, 10 ms of inter-echo time, 85 ms equivalent echo time and 4 s repetition time (Guerrini et al., 2002).

Electrophoretic techniques and statistical analysis of the data

2-DE maps were obtained by IPG-DALT (Gianazza, 1998). Sample proteins in biological fluids, reduced with 2% 2-mercaptoethanol, were first resolved according to charge on a non-linear pH 4–10 NL IPG (Gianazza et al., 1985) in the presence of 8 M 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 7.5–17.5% polyacrylamide gradients, two IPG strips mounted on each 160×140 mm^2 SDS slab. Sample loads were 100 μg of urine proteins or 2 μL of serum or 25 μL CSF. Proteins were stained with 0.3% w/v Coomassie, or with silver nitrate (Heukeshoven and Dernick, 1986) for CSF. The protein patterns were scanned with a video camera under the control of NIH Image, and analyzed with the software PDQuest. Data for individual proteins (spots, or spot chains, identified by immunological or physico-chemical means (Haynes et al., 1998; Miller et al., 1998; Miller et al., 1999)) are reported as spot volumes. After Levene's test, differences between groups were evaluated by ANOVA, followed by Bonferroni's or Dunnet's *post hoc* tests.

Ex vivo tests on aortic segments

Experiments were performed on isolated aortic rings from male and female SHRSP. Animals were divided into four groups, as follows: a, untreated males ('standard diet'); b, males receiving JPD and 1% NaCl in drinking water; c, untreated females ('standard diet'); d, female receiving JPD and 1% NaCl in drinking water. When MRI detected brain abnormalities in JPD-rats, the animals were euthanized with CO_2. Thoracic aortas were removed and placed in physiological salt solution, saturated with 95% O_2–5% CO_2. Vascular rings were dissected free of loose connective and adipose tissue and cut into segments of equal length. The segments were suspended on wires in an organ bath filled with Krebs solution and maintained at 37 °C under continuously bubbling with 95% O_2–5% CO_2. Krebs buffer (pH 7.4±0.1) had the following composition: 118.3 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 25 mmol/L $NaHCO_3$ and 11 mmol/L glucose. Indomethacin (10^{-5} M; Chiesi Farmaceutici S.p.A., Parma, Italy) was added to Krebs solutions in order to inhibit prostanoid synthesis. Vascular rings were connected to force transducers (Fort 10, World Precision Instruments, Inc) for isometric tension recording. After one and a half hour stabilization period under a resting tension of 1.5 g, tissues were challenged with KCl (100 mM/L) to check their viability; vessels not responding to KCl were discarded. Vascular

smooth muscle function was determined by cumulative addition of L-phenylephrine (L-Phe; Sigma-Aldrich, St. Louis, MO, USA) (10^{-9}–10^{-5} M) and constriction response was expressed as percentage of KCl response. Baths were washed out and tissues allowed to relax. Rings were then constricted to their individual EC_{80} values for L-Phe and endothelium-dependent relaxation by acetylcholine (Ach; Sigma-Aldrich, St. Louis, MO, USA) (10^{-9}–10^{-5} M) was studied. The baths were again washed out and 30 min later the rings were again constricted to their EC_{80} for L-Phe. If necessary, the dose of L-Phe was adjusted so that the tone was comparable to what previously achieved. A dose–response curve to sodium nitroprusside (10^{-10}–3×10^{-6} M; Sigma-Aldrich, St. Louis, MO, USA) was then built. Alternatively the rings were constricted to the EC_{50} for L-Phe and a dose–response curve to superoxide dismutase (0.1–100 U/mL; Sigma-Aldrich, St. Louis, MO, USA) was constructed. Off-line statistical analysis was performed using the Data Pad window of the Chart software. Data were then imported into Profit software and concentration–response curves were fitted onto a four-parameter logistic equation with the Levenberg–Marquardt algorithm. Results are expressed as means±S.D. Concentration–response curves were compared by analysis of variance (ANOVA). The maximal response (E_{max}) and half-maximal effective concentration (EC_{50}) were calculated from individual curves. The sensitivity to agonists is expressed as the negative logarithm of EC_{50} (pD_2). Statistical analysis was performed using ANOVA followed by appropriate *post hoc* test (Tamhane T2 or Tukey).

Results

Survival rate

The Kaplan–Meier chart in Fig. 1 describes the influence of gender on the survival rate of SHRSP given JPD. All male animals stayed event-free for only 3 weeks of dietary treatment and were dead by 6 weeks while female animals were event-free for 6 weeks and died by 18 weeks (Table 1). Both lag-time and slope of the survival curves are thus statistically different between genders, with mean survival time of 5.25±0.37 weeks for male SHRSP *vs* 12±1.93 weeks for females ($p=0.0003$). This suggests that, in each group, the events that precede overt pathology develop slower in female than in male SHRSP. Blood pressure and NaCl intake were similar in males and females (data not shown); the observed effects are thus independent of these two factors.

Proteomes of the biological fluids

Since in SHRSP proteinuria is predictive for the time of development of brain abnormalities, its changes were monitored over time. Fig. 2 shows the time course of urinary protein loss for males and females given JPD. After a lag time of ca. 3 weeks (20 days) for males, or of approximately 11 weeks (75 days) for females, proteinuria increases steadily in both genders from baseline (m 2±1, f 15±9 mg/day at the beginning of JPD) up to average values close to 300 mg per day (m 285±67, f 261±28 mg/day—no statistical difference between genders). The increase has in both cases a linear course but the difference of the curves in both, slope and intercept (see details in Fig. 2 legend) suggests that, in each animal, the events that precede overt pathology develop slower in female than in male SHRSP.

Fig. 3 shows the two-dimensional pattern of the proteins in serum, urine and CSF from female SHRSP collected after MRI had first detected signs of brain abnormality ('after JPD, *post-stroke*'). To provide an overview of the quantitative changes in protein abundance in the 'after JPD, *post-stroke*' samples of both genders *vs* baseline male and female SHRSP, in the figure the affected proteins are identified by their names, the direction of the change (detailed in Figs. 4 and 5) is rendered by upward and downward arrows and the gender is specified by the standard symbols for masculine and feminine. The number as well as the extent of changes is lower in females than in males.

As brain abnormalities develop much slower in females than in males, a slightly different sampling was planned for serum between males and females. A group of females was kept without JPD ('standard diet') for the same duration as the other females underwent dietary treatment; blood was drawn at the end of the observation period for evaluating in detail the effects of ageing on all serum proteins, and correcting for them. Fig. 4 shows for males (Panel A) and females (Panel B) the statistically significant differences in abundance for affected proteins, after *vs* before the test period. Few of the changes are similar between males and females: in both cases differences are observed for thiostatin, whereas the levels of apolipoprotein A-I, Gc-globulin and α_1-antitrypsin+serine protease inhibitor 3 differ only in males and ceruloplasmin, transferrin and kallikrein-binding protein+α_2-HS-glycoprotein differ only in females. The concentration of a given protein in elderly females given standard diet is usually intermediate between that of young and old females with JPD. However, except for ceruloplasmin, variations are still evident after correction for age, namely, statistically significant differences are evaluated between standard diet ('no permissive dietary treatment') and 'after JPD' groups. For transferrin a trend is also observed for the relative abundance of the alternative forms of the protein, either fully saturated or not saturated with iron. The percent of the holo-form decreases from 19% to 12% and to 11% from 'before JPD' to 'standard diet' and to 'after JPD' groups. The composition of the urinary protein fraction was analyzed in samples collected after JPD, and found to be different between genders. From quantitation of the protein spots on 2DE maps loaded the same amount of urine proteins, Fig. 5 shows that statistically significant differences between males and females

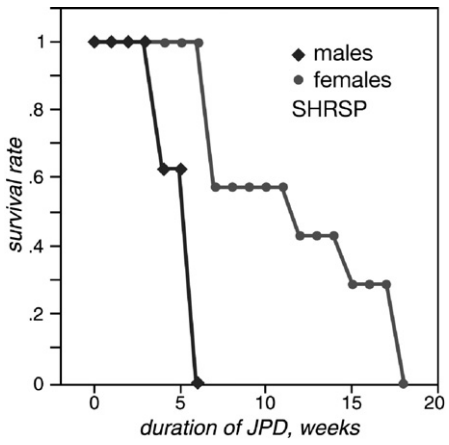

Fig. 1. Kaplan–Meier survival chart for male (◆) and female (●) SHRSP. $N=8$ animals per group.

Table 1
Summary

Delay before appearance of brain abnormalities, days	Gender	
	Male SHRSP	Female SHRSP
From the onset of JPD	42±3	114±14
From proteinuria ≥40 mg/day	9±3	25±4

are observed for the amount of Gc globulin, kallikrein-binding protein+α_2-HS-glycoprotein, α_1-antitrypsin+serine protease inhibitor 3, transferrin and thiostatin. Thiostatin is present in higher amounts in female urine whereas all other proteins are more concentrated in male urine. No statistically significant difference is observed between genders for apolipoprotein A-IV; under the selected experimental conditions albumin spot is saturated and not amenable to quantization.

The features of CSF in *post-stroke* females (Fig. 3C) are alike those of *post-stroke* males (Sironi et al., 2004b). An overall increase of protein concentration is observed. The amount of α-globulins is increased, that of high molecular weight proteins (such as α_1-inhibitor 3) is much increased. Spot trains of two inflammatory markers, thiostatin and haptoglobin, become evident. On the contrary the concentration of a typical protein of neuronal origin, prostaglandin D synthase, is decreased *vs* both, 'before JPD' and 'standard diet' samples.

Vascular function

As shown by the results of proteomic investigation, many of the pathological features in SHRSP are connected with alterations in vasculature-bound barriers, in kidney and brain. Vascular functionality was thus carefully investigated in 'standard diet' and 'after JPD' male and female SHRSP, applying to ex vivo artery specimens (aortic rings) various stimuli, specific for different steps in the signal transduction pathway. The main parameters of their responses are summarized in Table 1.

Contraction elicited by 10^{-1} M KCl was comparable among the four rat groups (standard diet females 1.7±0.3 g, N=6; standard diet males 1.8±0.3 g, N=7; after JPD females 1.8±0.4 g, N=8; after JPD males 1.4±0.5 g, N=10).

Effects induced by phenylephrine (10^{-9}–10^{-5} M) are plotted as dose–response curves in Fig. 6, Panel A. The values computed for curve parameters, E_{max} (in Table 2) and pD_2 (in Table 3) do not significantly differ for any of the experimental groups. This finding suggests that receptor-mediated contraction does not differ between genders and is not influenced by dietary regimen.

In rings pre-contracted with phenylephrine, the concentration–response curves to acetylcholine for 'standard diet' male and 'standard diet' female rats do not significantly differ from one another (Fig. 6, Panel B). JPD impairs endothelial function in both genders: the responses of 'after JPD' animals differ from their 'standard diet' counterparts, E_{max} for the 'after JPD' rats of both genders being significantly lower than in 'standard diet' animals (Table 2). On the contrary, no difference for this parameter is observed between genders for either 'standard diet' or 'after JPD' groups (Table 2) albeit in aortic segments from 'after JPD' female SHRSP relaxation is statistically larger than in 'after JPD' males (Fig. 6, Panel B). No differences are observed for pD_2 among the four experimental groups (Table 3). These data suggest an identical sensitivity to the agonist but an altered plateau response.

The concentration–response curves of nitroprusside, are plotted in Fig. 6, Panel C. The E_{max} values (in Table 3) are comparable in all experimental groups, but the sensitivity is significantly higher in the aorta from 'standard diet' females. The concentration–response curves of superoxide dismutase (SOD) are shown in Fig. 6, Panel D. The responses to SOD significantly differ among the four experimental groups: baseline release of NO increases from 'after JPD' males to 'after JPD' females to 'standard diet' males to 'standard diet' females, and similar levels of relaxation are obtained with SOD concentrations differing approximately 10-fold from one animal group to the other.

Discussion

The major finding of this study is that female SHRSP, when compared to males, show both a longer lag time before the onset of overt disease and a slower worsening of the pathological signs, chiefly brain abnormalities, which together result in a much higher survival rate for female SHRSP.

Changes in the level of serum proteins are compatible both for males and for females with an atypical inflammatory condition, which precedes the appearance of brain abnormalities by several weeks (in males, approximately one half of the time between the start of JPD and the occurrence of stroke; Sironi et al., 2001). The main marker in rats of an acute-phase reaction, thiostatin, sharply increases both in males and in females. Conversely a typical negative acute phase reactant

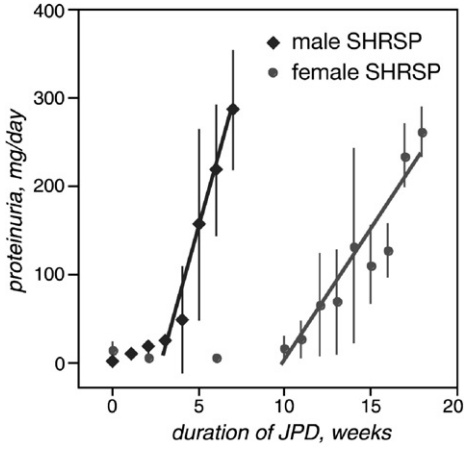

Fig. 2. Time-course of protein loss in urine, for male (◆) and female (●) SHRSP. Average values (±S.D.) are reported as mg proteinuria per 24 h; we fitted the collected data, by linear regression procedures, onto two lines with different m values (lag phase and rise phase). The parameters for the linear regression fitting of data during the rise phase are as follows: for males, (proteinuria, mg/day)=69.21×(time, weeks)−199.75 (mg/day/week), r=0.988; for females, (proteinuria, mg/day)=29.44×(time, weeks)−296.32 (mg/day/week), r=0.945.

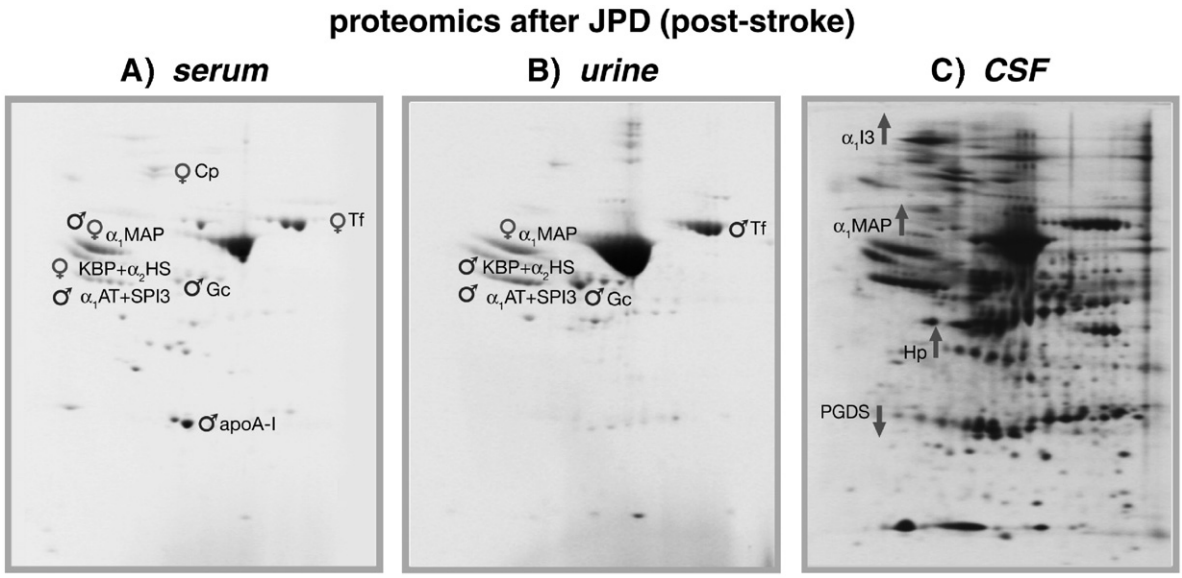

Fig. 3. Two-dimensional maps of biological fluids from post-stroke female SHRSP. (A) Serum. The proteins whose amount is significantly different in post-stroke animals *vs* baseline are identified; standard symbols refer to the gender for which such difference was observed (see Fig. 5). The reference pattern for control female rat serum can be found in Miller et al. (1998) and online at http://linux.farma.unimi.it. For male SHRSP, the pattern of '*post-stroke*' serum can be found in Sironi et al. (2001), and online at http://linux.farma.unimi.it. (B) Urine. The proteins whose amount is significantly different between genders in urine samples from post-stroke animals are identified; standard symbols refer to the gender for which the higher urinary concentration of the protein was observed (see Fig. 6). The pattern of control male urine can be found in Wait et al. (2001), that of *post-stroke* urine in Sironi et al. (2001), and online at http://linux.farma.unimi.it. (C) CSF. The proteins whose amount is either increased or decreased *vs* 'before JPD' and 'standard diet' samples are identified and marked by arrows. The pattern of control CSF can be found in Wait et al. (2001), that of '*post-stroke*' CSF in Sironi et al. (2004b) and online at http://linux.farma.unimi.it.

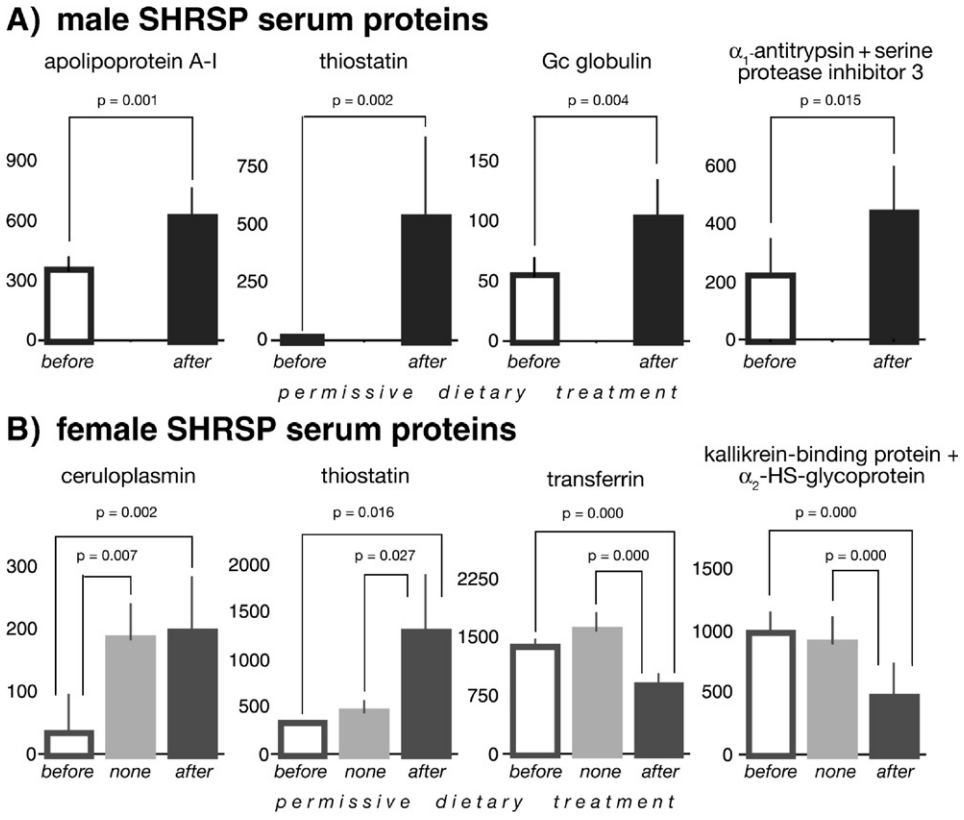

Fig. 4. Statistically significant differences in individual serum protein levels in SHRSP, with or without JPD. Average values (±S.D.) of protein volumes (arbitrary units, from PDQuest output) are reported, together with the associated statistical parameter *p*. (A) Comparison between males before (left, open columns) and after (right, solid columns) JPD. (B) Comparison among females before (left, open columns) and after (right, solid dark columns) JPD and females of the same age as at the end of the treatment but given standard diet.

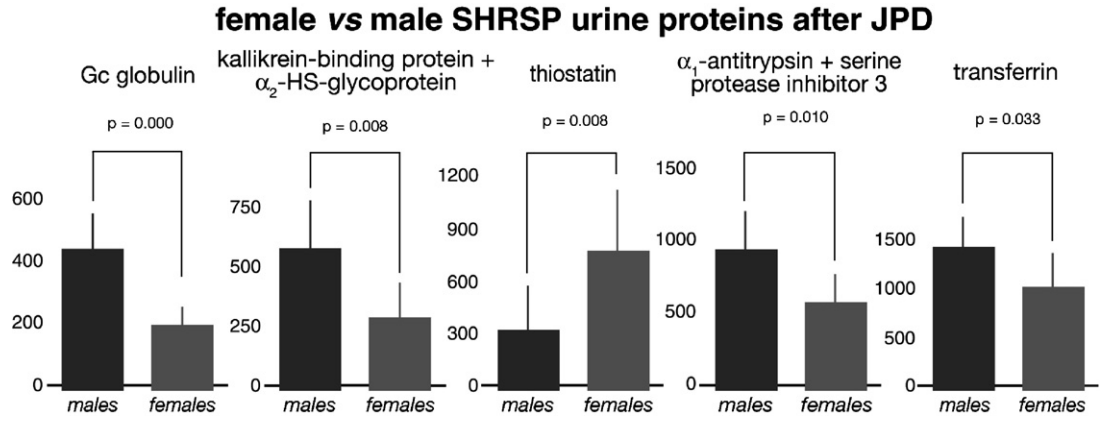

Fig. 5. Statistically significant differences in individual protein excretion between male (left columns) and female (right columns) SHRSP after JPD. Average values (±S.D.) of protein volumes (arbitrary units, from PDQuest output) are reported, together with the associated statistical parameter *p*.

such as α_1-inhibitor 3 and typical positive acute phase reactants such as orosomucoid/α_1-acid glycoprotein and haptoglobin are affected neither in males nor in females. Protein level changes in female SHRSP involve another positive acute phase reactant, ceruloplasmin, and two negative acute phase reactants, transferrin and α_2-HS-glycoprotein (here quantitated together with kallikrein binding protein). In males they involve such positive acute phase reactants as α_1-antitrypsin, serine protease inhibitor 3 and Gc, and apolipoprotein A-I, whose rise is more likely connected with reduced urinary loss than with increased synthesis, as seen in nephrotic syndrome. Since for (control) Sprague–Dawley rats the changes in serum protein levels after experimental inflammation are similar in males and females (Miller et al., 1998), the finding of a differential modulation of serum protein levels during the early stages of the pathology is a further indication of gender differences in SHRSP strain.

The ratios between amount in *post-stroke* urine (Fig. 5) and concentration in *post-stroke* serum (Fig. 4), computed for the various proteins in both genders, are similar for all experimental groups (not shown). On a qualitative basis, the same applies to *post-stroke* CSF *vs post-stroke* serum. Male and female SHRSP

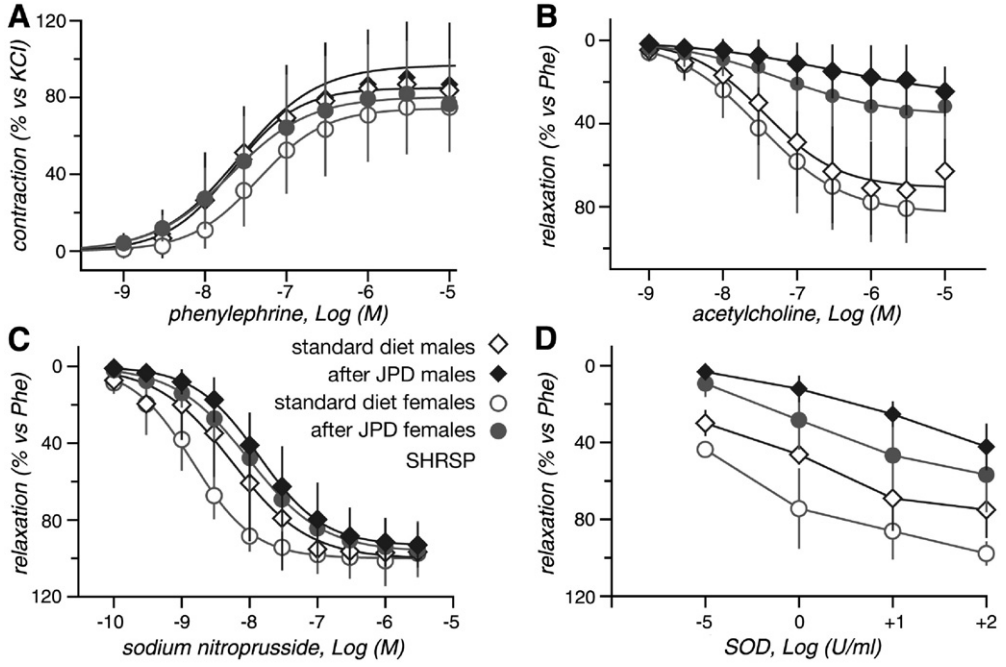

Fig. 6. Cumulative concentration–response curves in thoracic aortic rings from male and female SHRSP with standard diet and with JPD. Panel A=phenylephrine (Phe), Panel B=acetylcholine, Panel C=sodium nitroprusside, Panel D=superoxide dismutase (SOD). Contraction is expressed as percent with respect to KCl effect (mean±S.D.); relaxation is expressed as percent with respect to phenylephrine effect (mean±S.D.). Open diamonds=SHRSP males on standard diet; solid diamonds=SHRSP males on JPD; open circles=SHRSP females on standard diet; solid circles=SHRSP females on JPD. Statistical significance of differences: Panel B: $p<0.001$ JPD males *vs* standard diet males and standard diet females; $p<0.001$ JPD females *vs* standard diet males and standard diet females; $p=0.014$ JPD males *vs* JPD females (Tamhane T2). Panel D: $p=0.044$ standard diet males *vs* standard diet females; $p<0.001$ standard diet males *vs* JPD males; $p=0.018$ standard diet males *vs* JPD females; $p<0.001$ standard diet females *vs* JPD females and JPD males; $p=0.014$ JPD males *vs* JPD females (Tamhane T2).

Table 2
Maximal response (E_{max}) to vasoactive compounds in different experimental groups

Group	L-Phenylephrine	Acetylcholine	Sodium nitroprusside
Standard diet males	82.4±3.6	72.7±22.3	98.6±4.3
After JPD males	91.4±29.9	19.8±19.5 *	96.1±8.3
Standard diet females	77.3±23.7	85.2±14.2	102.9±2.4
After JPD females	83.1±26.6	40.2±20.2 **	97.7±15.3

Data are expressed as mean±standard deviation. All data are presented as percent of KCl or L-Phe pre-contraction.
* $p<0.001$ *vs* control males and control females.
** $p=0.001$ *vs* control females, $p=0.02$ *vs* control males (Tukey).

are thus close to one another as for the alterations in vasculature-bound barriers, exemplified by the increased permeability in kidney glomeruli and at the blood–brain barrier.

A complex relationship exists between vascular dysfunction and inflammation. Various drugs that have been characterized as being able to prevent the accumulation of acute-phase proteins in the body fluids of SHRSP also delay the appearance of overt pathology. Our group has studied in detail the oral administration of various substances at doses ineffective on physiological (e.g. blood pressure) and biochemical parameters (e.g. cholesterol synthesis) but producing an antiinflammatory effect and featuring endothelial protection: valsartan (Sironi et al., 2004a), pentoxyfilline (Banfi et al., 2004) and rosuvastatin (Sironi et al., 2005). The inflammatory reaction occurring in SHRSP before the occurrence of brain abnormalities (Sironi et al., 2001; Sironi et al., 2004b) may exacerbate vascular dysfunction: pharmacologically-induced inflammation increases permeability of the blood–brain barrier through alterations at the tight junctions (Huber et al., 2001). Alterations in vasculature-bound barriers, demonstrated in SHRSP by extensive changes in the 'after JPD' proteomes of urine and CSF, are connected with changes in morphology (Arribas et al., 1997) and function of endothelial cells, which we have investigated with ex vivo pharmacological tests.

The analysis of concentration–response curves for aortic rings from animals of the same age, receiving either standard diet or JPD, demonstrates functional alterations in the vascular segments from SHRSP of both genders having received JPD. The responses to KCl, a substance able to induce a receptor-independent contraction, are comparable in all experimental groups (male and female SHRSP, with standard diet and with JPD), suggesting that vascular smooth muscle contractility is not altered by dietary treatment. Similarly, E_{max} and pD_2 to phenylephrine are not significantly different (Tables 2 and 3), which indicates that the expression of adrenergic receptors and their signal transduction processing do not change either with gender or with diet type.

NO release from male and female SHRSP aortic rings with intact endothelium was evaluated both in the basal state (Fig. 6D) and after acetylcholine challenge (Fig. 6B). The basal release of NO was assessed indirectly by initially inducing moderate active tone and then by observing the effect of SOD in changing the vascular basal tone. SOD is an enzyme catalyzing the catabolism of the superoxide anion, produced in the vasculature, which can scavenge NO and decrease its availability. The complete removal of superoxide anion is a way to evaluate NO production and accumulation in organ baths. All experimental groups differ from one another for their relaxation response to SOD (Fig. 6D). In detail, basal release of NO is greater in females than in males both with 'standard diet' and 'after JPD'. This effect can be ascribed to different NO basal production between vessels from male and female rats. On the other hand, literature data demonstrate a similar eNOS activity in male and female SHRSP (McIntyre et al., 1997). For these reasons, the differences detected in our experimental protocol can be linked not only to differences in basal NO release but also to differences in sensitivity of the soluble guanylate cyclase system between females and males. This observation is in agreement with data obtained by direct administration of NO through sodium nitroprusside addition to precontracted vessels (Fig. 6C). pD_2 (sensitivity) values are significantly greater in 'standard diet' females than in other groups (Table 3). No difference is observed in the endothelium-dependent relaxation evoked by acetylcholine between 'standard diet' females and 'standard diet' males. However, 'after JPD', the vasorelaxation response to acetylcholine is severely impaired in both genders *vs* the 'standard diet' condition; moreover, 'after JPD' some difference between genders is observed, as indicated by the analysis of variance on the overall curve although no changes in the logistic parameters are observed (Fig. 6B). The 'standard diet' data are in agreement with findings in rabbits by Hayashi et al. (1992) who observed no difference in acetylcholine-triggered NO release between male and female (control) animals. They partially contrast, however, with results in SHRSP: McIntyre et al. (1997) reported a more extensive thoracic aorta relaxation (pD_2) in females than in males at 16 weeks of age whereas Dowell et al. (1999) could not detect any difference between 12-week-old animals of either gender. Besides the different sensitivity discussed above, the difference we observe in NO bioactivity between baseline and acetylcholine-stimulated release could also be due to a different half-life of the mediator caused by a differential scavenging and inactivation of NO by free radicals. In fact, cultured aortic endothelial cells from SHRSP have been shown to release higher levels of superoxide radical and lower amounts of NO than WKY controls (Grunfeld et al., 1995). In hypertensive (Dahl) rats the level of free radicals increases after a high-salt diet (Swei et al., 1997). In general, the level of free radicals correlates with occurrence and severity of systemic inflammation (Gunnett et al., 2000; Ridker et al., 2004). Proteomic investigation discussed above shows a difference in acute phase

Table 3
Values of sensitivity expressed as pD_2

Group	L-Phenylephrine	Acetylcholine	Sodium nitroprusside
Standard diet males	7.6±0.1	7.3±0.3	8.3±0.5
After JPD males	7.5±0.4	7.1±0.4	7.8±0.4
Standard diet females	7.3±0.2	7.4±0.4	8.8±0.1 *
After JPD females	7.5±0.3	7.0±0.4	8.0±0.2

Data are expressed as mean±standard deviation.
* $p=0.02$ *vs* control males, JPD males and JPD females.

proteins between males and females both under 'standard diet' and 'after JPD' conditions. Indeed, the baseline release of NO inversely correlates with the level of the acute phase protein thiostatin in SHRSP serum. In NO-dependent relaxation to Ach, the influence of free radicals still appears relevant. The decreased bioactivity of NO in 'after JPD' animals might thus still be linked to inflammatory sequels. When compared to control WKY rats, SHRSP show a higher level of eNOS activity (both as mRNA transcript and as protein) but also a higher level of superoxide (Kerr et al., 1999). Moreover, according to Kagota et al. (2001) soluble guanylate cyclase is down-regulated and less cGMP is produced in SHR receiving a high-salt diet (8% NaCl for 4 weeks); these changes result in a reduced smooth muscle relaxation. The above findings are in agreement with the impairment in the response to acetylcholine and sodium nitroprusside we observe in male and female SHRSP after JPD (Fig. 6B and C), and provide a molecular explanation for its mechanism. In summary, the present investigation demonstrates differences between male and female SHRSP in various parameters, relevant to the genesis of their pathological condition, which may explain the much longer delay in females before the occurrence of brain abnormalities. These include a lower serum level of acute phase proteins, resulting from a milder inflammatory condition, and a less severe proteinuria, associated with better preserved structure and function of kidney. Normal working of glomeruli for a longer time in female than in male SHRSP is just one aspect of normal working for a longer time of their vessels in all districts of the circulatory system. A pathway affected in a gender-dependent way in SHRSP is the release of NO by endothelial cells. Basal release of NO is higher in females than in males of the same age whether or not the rats have received JPD; moreover the molecular NO transduction machinery (soluble guanylate cyclase) seems to have higher sensitivity to NO in females.

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