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

Rossana Ballerio [a], Elisabetta Gianazza [b,c,d], Luciana Mussoni [b], Ingrid Miller [e], Paolo Gelosa [b], Uliano Guerrini [b], Ivano Eberini [b,c,d], Manfred Gemeiner [e], Silvia Belcredito [b,d], Elena Tremoli [a,b], Luigi Sironi [b,d,*]

[a] *Centro Cardiologico "Monzino", IRCCS, Milano, Italy*
[b] *Dipartimento di Scienze Farmacologiche, Università degli Studi di Milano, Italy*
[c] *Gruppo di Studio per la Proteomica e la Struttura delle Proteine, Università degli Studi di Milano, Italy*
[d] *Centro di Eccellenza per le Malattie Neurodegenerative, Università degli Studi di Milano, Italy*
[e] *Institut für Medizinische Chemie, Department für Naturwissenschaften Veterinärmedizinische Universität, Wien, Austria*

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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,

* Corresponding author. Dipartimento di Scienze Farmacologiche, Università degli Studi di Milano, via Balzaretti 9, I-20133 Milano, Italy. Fax: +39 2 50318250.
E-mail address: luigi.sironi@unimi.it (L. Sironi).

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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