Association and Cosegregation of Stroke with Impaired Endothelium-dependent Vasorelaxation in Stroke Prone, Spontaneously Hypertensive Rats

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Abstract

While hypertension is a major risk factor for stroke, it is not its sole determinant. Despite similar blood pressures, spontaneously hypertensive rats (SHR) do not share the predisposition to cerebrovascular disease typical of stroke-prone spontaneously hypertensive rats (SHRSP). We investigated vascular function in male SHR and SHRSP as well as in SHRSP/SHR–F_2 hybrid animals. Animals were maintained on the appropriate dietary regimen necessary for the manifestation of stroke. Among the hybrid animals, a group of stroke-prone and a group of stroke-resistant rats were selected. Blood pressure was similar in all groups. Endothelium-independent vascular reactivity tested on isolated rings of thoracic aorta and basilar artery after death showed similar contractile and dilatory responses to serotonin and nitroglycerin, respectively, in all groups. In contrast, endothelium-dependent relaxation, in response to acetylcholine or substance P, was markedly reduced in SHRSP compared with SHR. Similarly, reduced vasodilatory responses were present in aortae of F_2 rats that had suffered a stroke when compared with SHR or F_2 rats resistant to stroke. The observed association and cosegregation of stroke with significant and specific impairment of endothelium-dependent vasorelaxation among SHRSP and stroke-prone F_2 hybrids, respectively, suggest a potential causal role of altered endothelium-dependent vascular relaxation in the pathogenesis of stroke. (*J. Clin. Invest.* 1996. 98:256–261.) Key words: spontaneously hypertensive rats • stroke • high salt diet • genetic hypertension • endothelium

Introduction

Among the world's growing elderly population few illnesses are more dreaded than stroke. With an increasing incidence and a mortality of 30%, stroke carries the threat of death or long term disability and suffering. Elevated blood pressure has long been recognized as one of the most important risk factors; however, there is growing evidence that familial predisposition, or hereditary factors, may play a significant role in the etiology of stroke: stroke has been found to show familial aggregation (1–4), and twin studies have demonstrated a much higher concordance of stroke among monozygotic than dizygotic twins (5, 6). We used an animal model to study whether genetic predisposition to stroke was associated with abnormalities of vascular function, independently of the effects of blood pressure.

The spontaneously hypertensive rat (SHR)[1] strain (7) has been used extensively for the study of genetic hypertension. While elevations of blood pressure and their response to a range of pharmacological agents, as well as the development of hypertensive ventricular hypertrophy mirror observations in human hypertension, these animals, by and large, fail to manifest any of the morbid conditions, such as coronary, peripheral, or cerebrovascular ischemic disease, nephropathy, or ophthalmopathy — "complications" that are prominently associated with the disease in humans. This may be related to the fact that selection for severe hypertension during the establishment with the strain was accompanied, unnoticed at the time, by selection against hypertension-sensitive traits that would have resulted in an intolerable reduction of biological fitness. An exception is the sub-strain of the stroke prone spontaneously hypertensive rat (SHRSP) (8), isolated from still incompletely inbred SHR shortly. The occurrence of stroke in this strain has been recognized as a complex, multifactorial phenomenon in which specific ecogenetic interactions play a decisive role: both elevated blood pressure (9) and a specific dietary regimen (10) (the "Japanese" rat chow, which is higher in sodium, and lower in potassium and protein than Western diets) (11) are essential permissive elements to produce the stroke-prone phenotype. If these conditions are met, SHRSP show an extremely high

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1. *Abbreviations used in this paper:* SHR, spontaneously hypertensive rat; SHRSP, stroke-prone spontaneously hypertensive rats.

rate of stroke which occurs rapidly within a few weeks to months (12–15).

Previous studies comparing SHRSP with normotensive Wistar-Kyoto (WKY) (16–19) rats have suggested that functional or structural abnormalities of the vessel wall may be implicated in the high incidence of stroke in SHRSP. However, differences in blood pressure level between these two strains, a major confounding factor with important effects on vascular biology, have so far precluded any definitive interpretation of these findings.

To gain a better understanding of the possible role of altered vascular functioning in the pathogenesis of stroke, we determined standard parameters of endothelium-dependent and -independent vasomotor responses in aortic preparations from SHRSP and SHR, using an experimental protocol which eliminated blood pressure as a confounding variable. To further minimize the possibility that differences found are due to chance fixation of these properties among the two strains, but without actual pathogenetic importance, we contrasted a group of SHRSP/SHR-intercross F_2 hybrid rats with early stroke with one that was stroke-resistant: only if vascular phenotypes are causally related to the occurrence of stroke would one expect to find cosegregation of the two phenotypes. Our results indicate that reduced endothelium-dependent vasorelaxation may contribute as a genetically determined predisposition to the occurrence of stroke in the SHRSP.

Methods

Animal husbandry. The study was performed in the laboratories of the Federico II University in Naples, Italy. All experimental protocols met the guidelines for animal studies of this institution. SHRSP and SHR animals were obtained from the colonies maintained at the Max Delbrück Center for Molecular Medicine in Berlin-Buch ($SHRSP_{HD}$ and SHR_{HD}). Rats were kept at a constant room temperature of between 22 and 24°C and at regular 12 h day night cycles. They had free access to food and drinking water. Animals were placed on a Japanese style diet (Laboratorio Dott. Piccioni, Gessate, Italy; 18.7% protein, 0.37% sodium, 0.63% potassium) and received 1% NaCl in their drinking water starting at 6 wk of age (13). In our laboratory, the occurrence of cerebrovascular accidents, as documented by unequivocal physical signs (paralysis of at least one limb) and histological evidence, is observed in the SHRSP strain under these experimental conditions between 6 and 12 wk after initiating the dietary regimen described above. Corroborating previous data (13), after 12 wk on the diet, incidence of stroke reaches 100% in SHRSP, while no strokes occur during this time frame among SHR (unpublished observations).

10 male SHRSP and 13 male SHR controls were studied while maintained on regular diet, along with 11 SHRSP and 17 SHR of equal age that had received the stroke-permissive diet for four weeks, but had not manifested any strokes. In addition, four male SHRSP that had suffered strokes, along with an equal number of SHR controls, were studied. 21 male SHRSP/SHR F_2-intercross animals (progeny of brother-sister mated F_1 hybrids form an original F_0 cross between a male SHRSP and a female SHR rat) were selected from a large F_2 cohort ($n = 220$) for early stroke-susceptibility (prior to 14 wk on the dietary regimen; $n = 13$) or stroke-resistance (no stroke within 25 wk of the dietary regimen; $n = 7$). Animals were studied on the day of stroke or after 25 wk of the regimen, respectively. In all animals the aorta was studies, except for 6 SHRSP and 9 SHR in which the basilar artery was used.

Blood pressure measurements. Noninvasive measurements of mean arterial blood pressure (BP) and heart rate were performed weekly using tail-cuff sphygmomanometry (PE-300, Narco Biosystems Inc., Houston, TX) and a multichannel polygraph recorder (Universal Oscillograph, Harvard Instruments, South Natick, MA) in conscious, restrained animals that had been habituated to the procedure.

Histopathology. Six 1.5-mm-thick coronal sections were prepared from the brain of each animal studied, and assessed for the presence (in the 13 F_2 animals with stroke) or absence (all other experimental animals) of cerebral lesions.

Vascular reactivity studies: aortic preparation. Animals were killed by decapitation, and the thoracic aorta was rapidly dissected free of adjacent tissue, excised, and placed into cold Krebs-Ringer bicarbonate solution containing 118.3 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4 \cdot 7H_2$, 1.2 mM $KH2PO_4$, 25 mM $NaHCO_3$, and 5.6 mM glucose. Blood vessels were cleaned of all adherent connective tissue and cut into 4-mm-long rings. Care was taken to avoid any damage to the inner surface of the blood vessel. The functional integrity of endothelium was confirmed by the presence or the absence of an appropriate response to acetylcholine (20).

Vascular rings were suspended in organ chambers which contained 30 ml of Krebs-Ringer solution at 37°C, equilibrated with 5% CO_2, 95%O_2, and connected to force transducers (GM3; Gould Instruments System, Oxford, CA). Changes in isometric force were recorded. Before the actual experiments began, the preparations were progressively stretched and exposed to 30 mM KCl at each level of tension until the optimal point of the length-tension relationship was reached (21). No difference was seen in the optimal basal tension among aortic rings obtained from SHR, SHRSP, or F_2 progeny. After this procedure, the rings were allowed to equilibrate for 60 min. Krebs-Ringer solution was replaced at 20-min intervals.

Endothelium-dependent modulation of vascular tone was then assayed by adding increasing amounts of acetylcholine (Sigma Chemical Co., St. Louis, MO; final concentration 10^{-8}–10^{-5} mM) to the tissue bath; similarly, endothelium-independent vasodilator and vasoconstrictor functions were assayed in the presence of nitroglycerin (Astra-Simes; 10-30 μg/ml) and serotonin (Sigma Chemicals; 10^{-8}–10^{-5} mM).

Vascular reactivity studies: basilar artery. In 6 SHRSP and 9 SHR, basilar arteries were dissected free from the skull and placed into ice-cold Krebs-Ringer solution. Blood vessels were mounted as ring preparation on an isometric myograph (410A; JP Trading, Aarhus, Denmark) by threading onto two stainless steel wires (40 μm diameter). The wires were attached to a force transducer and a micrometer, respectively, as described previously (22, 23). Vessels were warmed to 37°C and allowed to equilibrate for 30 minutes in Krebs-Ringer solution bubbled continuously with 95% O_2 and 5% CO_2. Vessel and wall thickness were measured at 12 sites that were then averaged, using a light microscope with immersion lens (Zeiss) at 600×, which provides a resolution of 0.2 μm. Lower magnification was used for measurements of the distance between the wires and the length of the blood vessel. Resting tension-internal circumference ratio was determined, and vessels were set to a normalized circumference L_1 (reflecting 90% of the internal circumference the vessel would have in vivo if relaxed and exposed to a transmural pressure gradient of 100 mmHg) (22, 23). The normalized internal diameter I_1 was calculated from L_1, and normalized wall and media thickness values were computed under the assumption that cross-sectional area remains constant if the vessel is extended to L_1, and based on measurements of wall and media thickness in the unstretched preparation. Vasoconstrictor properties were assessed in response to serotonin (Sigma Chemicals, 10^{-7}–10^{-5} M); endothelium-independent and -dependent vasorelaxation was tested as the response to nitroprusside (Wyeth-Ayerst, 10^{-10}–10^{-5} M) and to substance P (Sigma Chemical Co.; 10^{-10}–10^{-5} M). Substance P was used because it is considered the most specific tool to investigate the NO pathway and endothelium-dependent vasorelaxation in smaller resistance arteries (24).

Data analysis. All values are presented as means±SEM for each group of animals examined. The contractile responses to serotonin are expressed as percent of the maximal response to serotonin. The absolute level of maximal developed tension did not differ statisti-

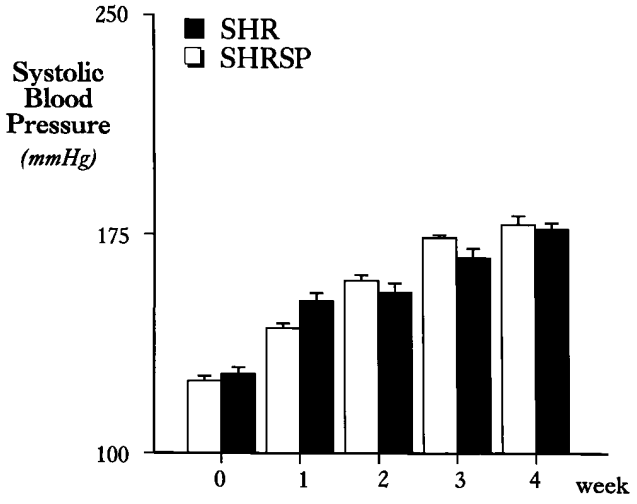

Figure 1. Blood pressure measured by tail plethysmography in SHRSP (*open bars*) and SHR (*solid bars*) during exposure to the stroke-promoting dietary regimen. Bars represent the mean±SEM. No difference was found among the two strains.

cally among different groups of animals. Vascular responses to acetylcholine, substance P, nitroglycerin, and nitroprusside are reported as fractional relaxation from the contraction produced by 10^{-6} M serotonin. Statistical analysis for the comparison of SHR and SHRSP animals was performed by Student *t* test; for repeated measurements, two-way analysis of variance was used, with one between (strain) and one within (dose) factor.

Results

Studies in SHR and SHRSP

Studies in aortic rings. SHRSP and SHR maintained on the permissive diet and on 1% NaCl in the drinking water showed a similar blood pressure profile during the observation period (Fig. 1). After 4 wk of high salt exposure, the contractile responses of aortic rings to serotonin were similar in the two parental strains (Fig. 2, *upper panel*; $F_{1,12} = 4.03$, $P = 0.067$).

Aortic rings from these SHRSP showed vasodilatory responses to nitroglycerin similar in magnitude to that of SHR (Fig. 2, *middle panel*; $F_{1,10} = 0.158$, $P = 0.7$). In contrast, the endothelium-dependent vasodilator response to acetylcholine after sodium exposure was significantly impaired in SHRSP when compared with SHR (Fig. 2, *lower panel*; $F_{1,12} = 38.128$, $P = 0.00005$).

Similar results were obtained in vessels from SHRSP that had experienced a cerebrovascular accident: when compared with vascular rings from age-matched SHR, no differences were observed regarding the response to serotonin and nitroglycerin, while the vasodilatory response to acetylcholine was significantly reduced ($F_{1,5} = 6.936$; $P = 0.019$).

Aortic preparations from both SHRSP and SHR not exposed to high sodium intake showed no inter-strain difference in the responses to either serotonin ($F_{1,22} = 1.07$, $P = 0.43$), nitroglycerin ($F_{1,13} = 0.105$, $P = 0.75$), or acetylcholine ($F_{1,13} = 0.026$, $P = 0.87$).

Studies in basilar artery rings. Similar to the results obtained in aortic rings, the response of basilar artery rings to serotonin was not different in SHRSP as compared with SHR ($F_{1,17} = 0.898$, $P = 0.36$; Fig. 3, *upper panel*). Likewise, endothelium-independent responses to nitroprusside were not different among tissues derived from either strain ($F_{1,12} = 1.265$, $P = 0.28$; Fig. 3, *middle panel*). Endothelium-dependent vasorelaxation, assessed from the response to substance P, however, was significanlty impaired in SHRSP as compared with SHR, analogous to the observation in aortic rings ($F_{1,14} = 6.77$, $P = 0.018$; Fig. 3, *lower panel*).

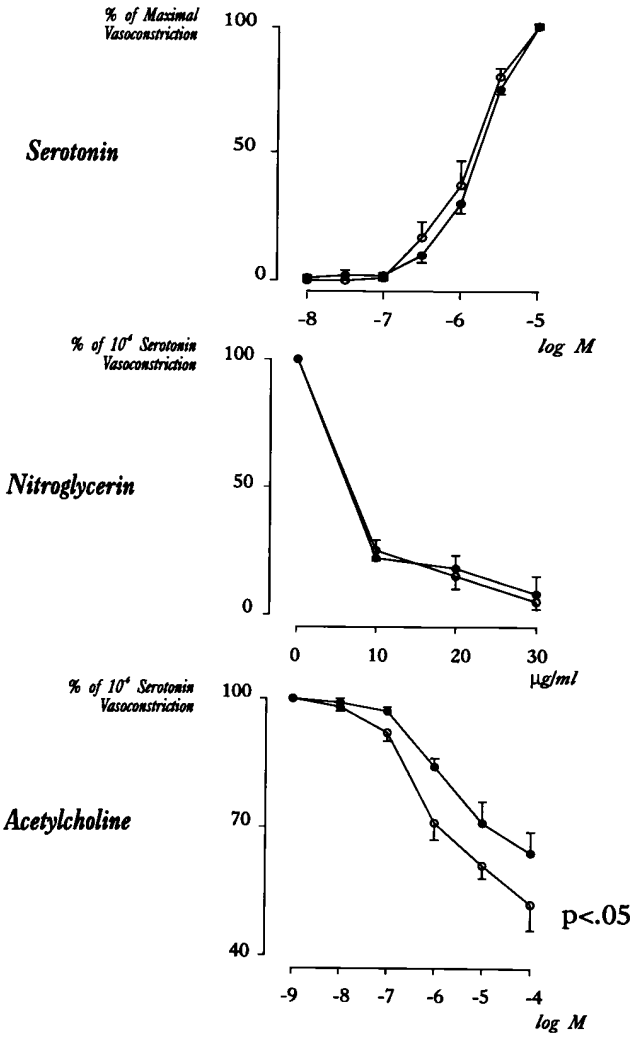

Figure 2. Dose-response curves for serotonin, nitroglycerin, and acetylcholine in aortic rings from SHRSP (*filled symbols*) and SHR (*open symbols*), after 4 wk of permissive dietary regimen. Symbols indicate mean±SEM.

Studies in SHRSP/SHR-F_2 hybrids

13 rats of this group showed the manifestation of stroke between the 8th and the 14th wk of the specific permissive dietary regimen, while the remaining 9 did not present any sign of stroke over a 25-wk observation period. No difference in mean arterial blood pressure (192±1 vs. 191±2 mmHg, respectively; n.s.) was observed between these two groups, using age-matched blood pressure measurements in the non-stroke animals corresponding to the age at stroke in the stroke animals. Absence or presence of stroke was verified in all animals by histopathological examination.

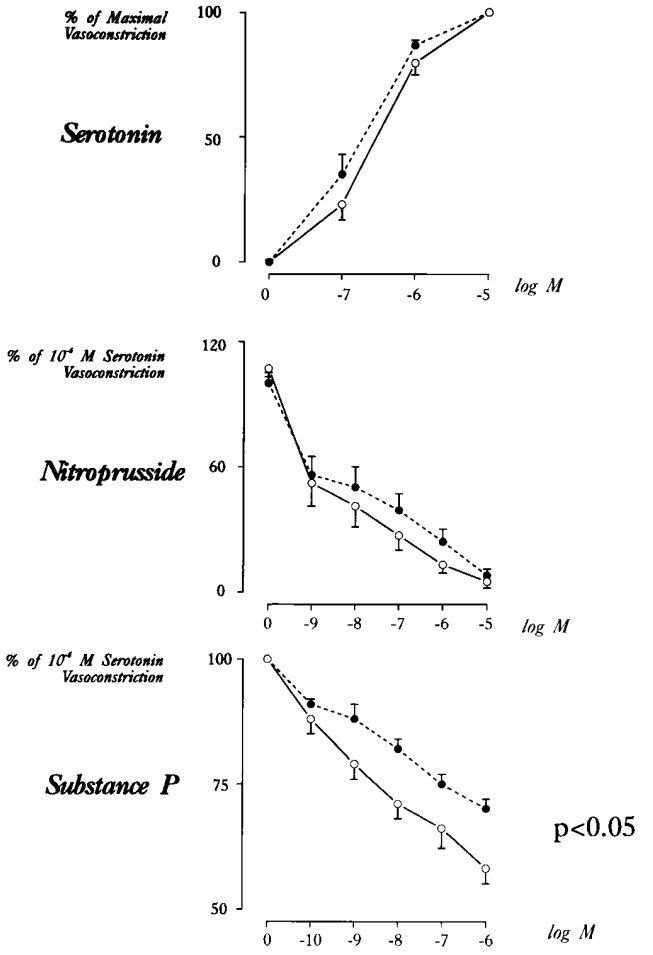

Figure 3. Dose-response curves for serotonin, nitroprusside, and substance P in basilar artery rings from SHRSP (*filled symbols*) and SHR (*open symbols*), after 4 wk of permissive dietary regimen. Symbols indicate mean±SEM.

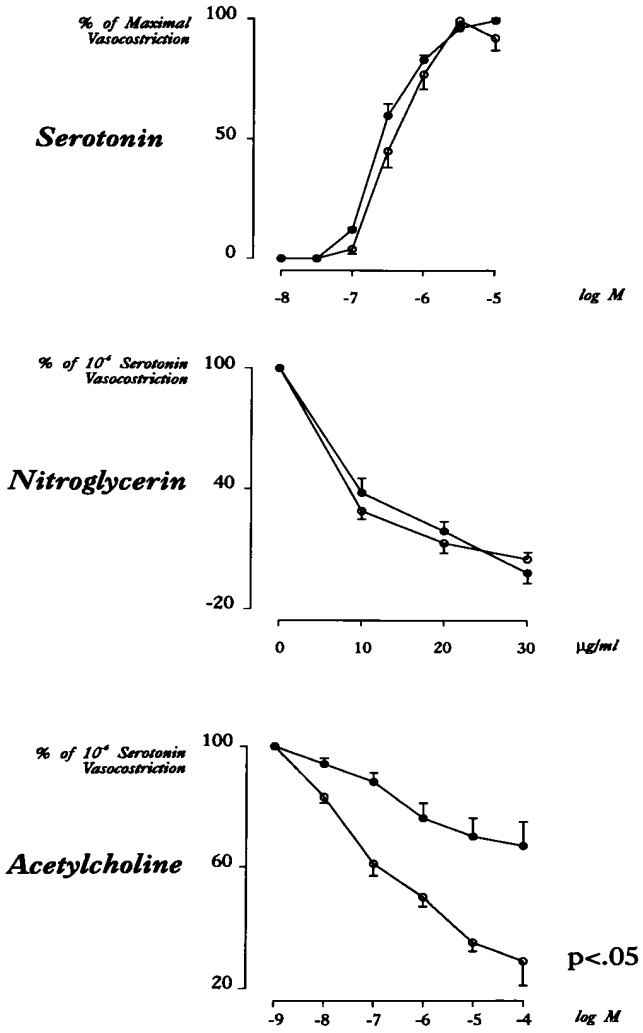

Figure 4. Dose-response curves for serotonin, nitroglycerin, and acetylcholine in aortic rings from SHRSP/SHR F_2 hybrids. Filled symbols indicate mean±SEM for the stroke-prone group, open symbols indicate mean±SEM for the stroke-resistant group.

Aortic rings from both groups showed similar contractile responses to serotonin (Fig. 4, *upper panel;* $F_{1, 17} = 0.898$, $P = 0.36$). Likewise, vasodilatory responses to nitroglycerin were comparable in both F_2 groups (Fig. 4, *middle panel*; $F_{1, 19} = 0.475$, $P = 0.50$). In contrast, endothelium-dependent vasorelaxation in response to acetylcholine was significantly impaired in the aortic rings from animals who had suffered a stroke when compared with those who did not (Fig. 4, *lower panel*; $F_{1, 19} = 17.052$, $P = 0.0006$). Within the group of F_2 animals that had suffered a stroke, no significant correlation between time of stroke and degree of impairment of vasorelaxation was apparent (data not shown).

Discussion

The results of the present study represent, to our knowledge, the first direct evidence that inherited alterations of vascular function may independently of blood pressure contribute to the pathogenesis of stroke. Differences in vascular function among normotensive and hypertensive rat strains are well documented (25–27), but have been difficult to interpret with regard to their primary or secondary nature. Our experimental design, using comparisons among equally hypertensive SHRSP and SHR, allowed us to eliminate blood pressure variance as a confounding variable, while providing the hypertensive levels that are a prerequisite for stroke to occur at all. Thus, the results of our study cannot be explained by possible secondary changes occurring as a consequence of exposure of the blood vessel to elevated pressure, but are likely to represent a primary abnormality that may be linked to the pathogenesis of stroke, a possibility that is further supported by the demonstrated cosegregation of abnormal vascular response and propensity to stroke in the SHRSP/SHR F_2 hybrids studied, and by the association of these abnormalities with high sodium intake, analogous to the stroke phenotype.

The SHRSP is unique among the large number of genetically hypertensive rat models currently used based on the development of cerebrovascular disease which makes it a most intriguing model that mirrors human disease in a number of ways. Similar to the human disease, stroke in the SHRSP appears to be a complex, polygenic, and multifactorial trait. Expression of the morbid phenotype depends on both presence of hypertension and on the interaction with environmental (di-

etary) variables. Lowering of blood pressure to normal levels will greatly reduce or eliminate the occurrence of stroke in both SHRSP and humans, and there is epidemiological data indicating that ethnic groups with particularly low potassium and high sodium intake show an excessively high incidence of cerebrovascular disease. In the SHRSP, potassium supplementation of the "Japanese" diet will eliminate strokes in the absence of any effects on blood pressure (28); similarly, a beneficial effect of dietary potassium intake on the incidence of stroke has been reported in man (29). In addition, in both SHRSP and humans stroke is positively correlated with plasma fibrinogen (30, 31), and negatively with plasma cholesterol (30, 32). Thus, in as much as no animal model will ever ideally resemble a human condition, the SHRSP appears to be an excellent model system for human stroke, and lessons learned from experiments with this strain may well be applicable to the human condition.

All comparative studies among inbred disease and "control" strains are inherently limited by the possibility that observed differences may simply represent the expression of randomly fixed genetic differences among the two strains, which are irrelevant for the phenotype of interest. To address this issue, cosegregation studies are performed in which the demonstration of a continued association between the phenotype of interest and the phenomenon investigated (this may be another phenotype, as in the present study, or a genotype based on DNA markers) provides support for a noncoincidental, and thus potentially causal relationship. Our study in two groups of F_2 intercross animals bred from SHRSP and SHR served this purpose. To gain power, we used the strategy of concentrating on the extremes of the phenotype (33) (very early stroke, or no stroke at all), thus selecting a highly informative subgroup of animals that presumably had inherited either a large or a small dose of stroke = promoting gene(s) from the overall cohort. Our experiments demonstrated indeed cosegregation of impaired endothelium-dependent relaxation with the stroke-prone phenotype, or conversely, of normal vascular reactivity with stroke-resistance, in F_2 intercross hybrids, again in the absence of any difference in blood pressure among the two groups. These results provide strong support against a chance association of altered vasorelaxation with the stroke-prone phenotype. Our findings are commensurate with the observation that antihypertensive treatment does not prevent alterations of vascular function in SHRSP (34) while it is effective in experimentally induced forms of hypertension (26, 27). The finding of decreased vascular reactivity prior to the occurrence of stroke in SHRSP progenitors indicates that reduced vasodilatory responses seen in F_2 animals were not a consequence of the occurrence of stroke, but preceded it; thus, our findings support the possibility that altered vascular reactivity may indeed play a causal role in the pathogenesis of cerebrovascular disease in this model.

The observation that diminished vasodilatory responses were seen only after exposure to the permissive diet mirrors the essential role of this environmental variable for the occurrence of stroke in the SHRSP and provides further support for the speculation that the observed abnormalities in vascular function might be directly related to the pathogenesis of stroke, as has been postulated earlier (18, 29).

The possible relevance of our findings for the pathogenesis of stroke is further emphasized by the finding that abnormal vasodilation is present not only in aortic rings, but also in basilar artery preparations from SHRSP exposed to the permissive diet. While the basilar artery is not a specific predilection site for stroke in the SHRSP, it represents a small resistance vessel that expresses the typical pharmacologic response pattern of cerebral arteries that are directly affected. While these observations are therefore of direct relevance to the occurrence of stroke, the apparent widespread prevalence of abnormal vasodilation, in the absence of other organ pathology, is of interest; it may indicate an interaction between abnormal vascular function and neurohumoral or other influences specific to the brain.

The experimental design of our F_2 study did not provide for comparison of vessels of rats of the same age. While our results therefore cannot address the issue of age, recently published results indicate either no influence of aging on vascular function or impairment at advanced age (35); since in our study compromised vascular function was observed in younger, and normal function in older F_2 animals, age does not appear to be an important confounding factor. As always, the recognized heterogeneity among SHR and SHRSP rats from different colonies also restricts our results presently to the Heidelberg SHRSP and SHR strains, and extrapolations to animals derived from other colonies cannot necessarily be made.

In summary, our results suggest strongly that impaired endothelium-dependent vasodilation in SHRSP is a genetically determined and transmitted feature which is independent of blood pressure, and which cosegregates with the stroke-prone phenotype. This alteration does not occur at the vascular smooth muscle level, since no differences of serotonin-induced vasoconstrictor- or nitroglycerin-stimulated vasodilator-responses were observed among SHRSP and SHR, or among stroke-prone and stroke-resistant F_2 hybrids. Instead, our results suggest that this alteration of vascular function is localized at the endothelial level, as we observed differential acetylcholine- or substance P-induced vasorelaxation in vessels from SHRSP and SHR, and among the two groups of F_2 hybrids. Expression of this functional alteration depends on exposure to permissive dietary factors, appears to be widespread, occurring in both large conduit vessels and in resistance arteries, and is present in cerebral vessels. We believe that our findings represent an important observation in the search for the causes and mechanisms of genetic predisposition to stroke. While currently restricted to the rat model, these studies may eventually provide important insights into the pathogenesis of human cerebrovascular disease.

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