Decreases in Substance P and Vasoactive Intestinal Peptide Concentrations in Plasma of Stroke-Prone Spontaneously Hypertensive Rats

Kazuo Mori, M.S.,* Shunji Asakura, B.S.,* Hiroshi Ogawa, Ph.D.,** Sukenari Sasagawa, M.D.,** and Masaharu Takeyama, Ph.D.*

SUMMARY

In order to study alterations of peripheral substance P (SP) and vasoactive intestinal peptide (VIP) in the immunoreactive nervous system in essential hypertension, plasma SP and VIP concentrations in stroke-prone spontaneously hypertensive rats (SHRSP) at 8, 12, 18, 28, 30, 35 and 48 weeks of age and age-matched Wistar-Kyoto rats (WKY) were measured, using enzyme immunoassays (EIAs).

The mean plasma SP concentrations of SHRSP (n=61) and WKY (n=58) were 4.9±1.2 fmol/ml and 6.6±1.9 fmol/ml, respectively. The value of SHRSP was significantly lower than that of WKY (p<0.01). The mean SP concentration of young SHRSP was significantly higher than those of other ages.

The mean plasma VIP concentrations of SHRSP (n=61) and WKY (n=58) were 0.80±0.25 fmol/ml and 1.01±0.32 fmol/ml, respectively. The value of SHRSP was significantly lower than that of WKY (p<0.01).

These decreases in plasma SP and VIP concentrations of SHRSP were observed at all ages. Decreases in the peripheral release of SP and VIP from the endings of SP- and VIP-immunoreactive nerves of SHRSP were seen, and the functional involution of peripheral SP- and VIP-immunoreactive nerves in essential hypertension was suggested. (Jpn Heart J 34: 785-794, 1993)

Key Words: Essential hypertension Substance P VIP SHRSP WKY Neuropeptide Enzyme immunoassay

It is well known that vasomotor tone is mainly regulated by sympathetic vasoconstriction, while some peptide-containing non-adrenergic non-cholinergic nervous systems are considered to be involved in the regulation of blood pressure. The neuropeptides, substance P (SP) and vasoactive intestinal peptide

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Received for publication February 22, 1993.
Accepted July 12, 1993.
(VIP), are widely distributed in the central and peripheral nervous system of mammals.

In the central nervous system, high SP-immunoreactivities have been reported in the substantia nigra, striatum, caudate putamen, nucleus of the tractus solitarius, intermediolateral cell column and dorsal horn of the spinal cord. VIP-immunoreactivities have been reported in the cerebral cortex, suprachiasmatic nucleus, amygdala, striatum, hippocampus, midbrain peri-aqueductal gray and sacral spinal cord.3,4)

Meanwhile, in the peripheral nervous system, SP-immunoreactivities are found in primary sensory afferent nerves.5) VIP-immunoreactivities are found in so-called non-adrenergic non-cholinergic inhibitory nerves.6)-8) Peripheral SP- and VIP-immunoreactive nerves are found beneath or within the skin and epithelium, surrounding glands, within smooth muscle, and around blood vessels. Peripheral SP- and VIP-immunoreactive nerves are found in connection with blood vessels in an innervation-like pattern. SP and VIP are generated and contained in neural cell bodies, and perhaps released from the nerve endings. SP and VIP induce marked vasodilation, and are thought to participate in the regulation of blood pressure. However, the precise roles of peripheral SP- and VIP-immunoreactive nerves in the regulation of blood pressure are still unclear, and the alterations of the SP and VIP levels in essential hypertension have been scarcely investigated.9)-11) Specifically, there are few reports concerning plasma peptide levels. Faulhaber reported decreased plasma SP levels in human essential hypertension.12) However, the plasma peptide levels of human subjects may fluctuate according to the physiological condition. Further investigation of the control of background peptide levels using experimental animal models is necessary.

Spontaneously hypertensive rats (SHR), frequently used as an experimental hypertensive animal model were first developed by Okamoto.13) Spontaneous hypertension of SHR has very similar characteristics to human essential hypertension, and the blood pressure of SHR elevates gradually with advancing age. As shown by several investigators, the hypertensive state of SHR is attributed not to an increase in cardiac output, but to an increase in total vascular resistance.14)-16) The differences of the SP and VIP levels between SHR and normotensive rats are useful. By measuring the plasma SP and VIP concentrations of SHR, alteration of the activities of peripheral SP- and VIP-immunoreactive nerves in essential hypertension can be demonstrated.

In the present study, the plasma SP and VIP concentrations of stroke-prone SHR (SHRSP) and Wistar-Kyoto rats (WKY) were measured in order to investigate the above-stated problems.
**Methods**

**Animals** (Table I): For hypertensive animals, male SHRSP (n=61) 8, 12, 18, 28, 30, 35 and 48 weeks of age were used. For normotensive control animals, age-matched male WKY (n=58) were used. The animals were classified into young (8 weeks), adult (12, 18 weeks), prime (28, 30 weeks) and elder (35, 48 weeks) groups. The physiological data of SHRSP and WKY are shown in Table I.

Separately, in order to preliminarily grasp the background levels of the plasma SP and VIP, the effect of feeding on the plasma SP and VIP concentrations was examined. Male SHRSP (n=10, 212±9 g, 193±4 mmHg, values of weight and blood pressure were significantly different from those of WKY (p<0.01)) and WKY (n=15, 251±24 g, 125±6 mmHg) at the age of 10 weeks were classified into fasting (SHRSP: n=5, WKY: n=7) and feeding groups (SHRSP: n=5, WKY: n=8), respectively. The fasting group underwent blood sampling after overnight fasting, and the feeding group underwent blood sampling without fasting.

**Measurement of blood pressures:** Blood pressure was measured using the tail-pulse pick-up method.

**Collection of blood samples:** After overnight fasting, blood samples (10 ml) were obtained from the abdominal aorta under light ether anesthesia. In the preliminary examination of the effect of the feeding, the feeding group (SHRSP: n=5, WKY: n=8) underwent blood sampling without fasting as mentioned above. The samples were immediately put into chilled tubes containing 500 kallikrein inhibitor units/ml aprotinin and 1.2 mg/ml EDTA.

**Preparation of plasma extract:** Plasma was separated from the blood sample by centrifugation (1670 × g, 4°C, 20 min). The plasma sample (1 ml) was diluted 5-fold with 4% acetic acid (AcOH), and loaded on a reversed-phase C18 cartridge (Sep-Pak C18 cartridge, Waters Co., Inc., Milford, MA, USA). After washing with 4% AcOH, SPs and VIPs were eluted with 70% acetonitrile.

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B. P. = Blood pressure; ** Significantly different from age-matched WKY (p<0.01), mean ±SD.
(MeCN) in 0.5% AcOH, pH 4.0 (2 ml). Eluate was concentrated by spin-vacuum evaporation, lyophilized and stored at -40°C until use. The recoveries of SP and VIP with this extracting procedure were 92±10% and 94±6%, respectively.

**Enzyme immunoassays (EIAs) for SP and VIP:** EIAs for SP and VIP were performed as previously reported. For SP assay, antiserum RA-08-095 (Cambridge Research Biochemicals Ltd., UK) specific to the carboxy-terminal portion of SP was used as the first antibody, and β-D-galactosidase-labeled Tyr8-SP was prepared as the enzyme-labeled antigen. For VIP assay, antiserum 604/001 (UCB-Bioproducts SA, Belgium) specific to the central portion of VIP was used as the first antibody, and β-D-galactosidase-labeled VIP (11-28) was prepared as the enzyme-labeled antigen.

EIAs were performed by the delayed addition method. Separation of bound and free material was performed by the double antibody solid phase method. As the second antibody, goat anti-rabbit IgG (TAGO 4120, TAGO Inc., Burlingame, CA, USA) was used. The detectable minimum amounts of SP and VIP were 0.4 fmol/well and 0.1 fmol/well, respectively.

**HPLC of plasma extracts:** Rat SP and VIP are reported to be identical to human SP and VIP. For the biochemical characterization of SP-immunoreactive substance (SP-IS) and VIP-immunoreactive substance (VIP-IS) in plasma of SHRSP and WKY, plasma extracts from SHRSP and WKY were applied to a reversed-phase C18 column (Cosmosil 5C18-AR, 4.6×150 mm, Nacalai Tesque, Inc., Kyoto, Japan). The column was equilibrated with 0.1% trifluoroacetic acid (TFA). SP-ISs were eluted with a linear gradient of MeCN (15% in 6 min and 15–50% in 35 min) in 0.1% TFA. Synthetic SP and its sulfoxide (SP (O)) were applied to the column under the same conditions. VIPs were eluted with a linear gradient of MeCN (10% in 6 min and 10–45% in 35 min) in 0.1% TFA. Synthetic VIP, its sulfoxide (VIP (O)) and VIP (14-28) were applied to the column under the same conditions. The flow rate was 1 ml/min, and the fraction size was 1 ml. Each fraction was concentrated by spin-vacuum evaporation, and lyophilized. The residue was submitted to EIA.

**Statistical analysis:** All results are expressed as mean±SD. Comparison of mean values was made by the one-way analysis of variance (ANOVA) with Fisher's protected least significant difference used to determine differences between pairs of each group. A P-value of <0.05 was considered significant.

**Results**

**Background levels of plasma SP and VIP (Fig. 1.):** The effects of feeding on the plasma SP and VIP concentrations of SHRSP and WKY at the age of 10
The mean plasma SP concentrations of the SHRSP-fasting group, SHRSP-feeding group, WKY-fasting group and WKY-feeding group were 6.1±1.7 fmol/ml, 8.1±2.0 fmol/ml, 8.6±1.4 fmol/ml and 13.4±5.5 fmol/ml, respectively. The value of the WKY-fasting group was significantly lower than that of the WKY-feeding group (p<0.05). The value of the SHRSP-fasting group was significantly lower than that of the WKY-fasting group (p<0.05), whereas the value of the SHRSP-feeding group was not significantly different from that of the WKY-feeding group.

The mean plasma VIP concentrations of the SHRSP-fasting group, SHRSP-feeding group, WKY-fasting group and WKY-feeding group were 0.80±0.28 fmol/ml, 1.50±0.53 fmol/ml, 1.17±0.28 fmol/ml and 2.43±1.08 fmol/ml, respectively. The value of the SHRSP-fasting group was significantly lower than that of the SHRSP-feeding group (p<0.05). The value of the WKY-fasting group was significantly lower than that of the WKY-feeding group (p<0.05). The value of the SHRSP-fasting group was significantly lower than that of the WKY-feeding group (p<0.05), whereas the value of the SHRSP-feeding group was not significantly different from that of the WKY-feeding group.

The plasma SP and VIP concentrations of the feeding groups were more variable, while those of the fasting group were comparatively stable. Thus, the following examinations were carried out in the fasting state.

**SP concentrations in plasma of rats (Fig. 2):** The SP concentrations in plasma of rats are shown in Fig. 2. The mean SP concentrations of SHRSP and WKY were 4.9±1.2 fmol/ml and 6.6±1.9 fmol/ml, respectively. The value for SHRSP was significantly lower than that for WKY (p<0.01). The mean SP
concentrations of young, adult, prime and elderly SHRSP were 6.4±0.6 fmol/ml, 4.9±0.8 fmol/ml, 4.7±1.2 fmol/ml and 4.6±1.4 fmol/ml, respectively. The value for young SHRSP was significantly higher than those of the other SHRSP groups (p<0.01). The mean SP concentrations for young, adult, prime and elderly WKY were 7.4±0.9 fmol/ml, 6.7±2.1 fmol/ml, 6.5±2.3 fmol/ml and 6.2±1.4 fmol/ml, respectively.

**VIP concentrations in plasma of rats** (Fig. 3): The VIP concentrations in plasma of rats are shown in Figure 3. The mean VIP concentrations of SHRSP and WKY were 0.80±0.25 fmol/ml and 1.01±0.32 fmol/ml, respectively. The value of SHRSP was significantly lower than that of WKY (p<0.01). The mean VIP concentrations of young, adult, prime and elderly SHRSP were 0.82±0.13 fmol/ml, 0.77±0.17 fmol/ml, 0.78±0.21 fmol/ml and 0.85±0.35 fmol/ml, respectively. The mean VIP concentrations of young, adult, prime and elderly WKY were 1.09±0.27 fmol/ml, 1.06±0.33 fmol/ml, 1.03±0.32 fmol/ml and 0.90±0.32 fmol/ml, respectively.

**HPLC of plasma extract** (Fig. 4): HPLC profiles of the plasma extracts from SHRSP and WKY are shown in Fig. 4.

As determined by absorbance at 215 nm, synthetic SP was eluted in the region of 32% MeCN, and synthetic SP(O) was eluted in the region of 28% MeCN. The main SP-ISs in plasma extracts from SHRSP and WKY were eluted equally at the same elution volume of the synthetic SP in the region of 32% MeCN, with minor peaks in the regions of 28% and 38% MeCN. Synthetic VIP was eluted in the region of 30% MeCN. Both synthetic VIP (O) and VIP (14–28)
Fig. 3. VIP concentrations in plasma of rats * p<0.05; ** p<0.01.

Fig. 4. HPLC profiles of plasma extracts from SHRSP and WKY.
A: SP Extracts from plasma (5 ml) were applied. The arrows indicate the elution regions of synthetic SP and SP (O).
B: VIP Extracts from plasma (5 ml) were applied. The arrows indicate the elution regions of synthetic VIP.
were eluted in the region of 24–25% MeCN. The main VIP-ISs in plasma extracts from SHRSP and WKY were eluted equally at the same elution volume of the synthetic VIP in the region of 30% MeCN, with a peak in the region of 24–25% MeCN.

**DISCUSSION**

SP and VIP were detectable in all the plasma samples. For the biochemical characterization of SP-IS and VIP-IS in the plasma of SHRSP and WKY, HPLC was performed. As for SP, the main SP-IS was considered to be composed of SP (1–11). A minor peak in the region of 28% MeCN was considered to be composed of SP (O), whereas another minor peak in the region of 38% MeCN was unidentified. As for VIP, the main VIP-IS was considered to be composed of VIP (1–28). A peak in the region of 24–25% MeCN was considered to be complexly composed of VIP (O) and minor fragments of VIP such as VIP (14–28). Thus, almost all of the peaks in the plasma extracts were considered to be SP- or VIP-derived. The EIAs were considered to be specific.

In the present study, significant decreases in the plasma SP and VIP concentrations of SHRSP were seen. The hypertensive state of SHR is reported to be due to the elevation of vascular resistance. Vascular resistance is modulated by the central and peripheral nervous systems.

SP-immunoreactive cell bodies are variously found in the central nervous system. However, the plasma SP concentration is likely to depend on the peripheral release of SP from the nerve endings. Gamse reported that a major part of circulating SP originated from the intestine. In fact, in the present study, the plasma SP concentrations tended to be increased by feeding. This increase in plasma SP concentration caused by feeding might reflect the excitement of peripheral SP-immunoreactive nerves innervating the digestive organs. Moreover, polypeptides such as SP probably do not cross the tight junction of the blood-brain barrier. Therefore, circulating SP is thought to be mainly derived from the endings of peripheral SP-immunoreactive nerves innervating the digestive and other organs.

In the peripheral nervous system, SP-immunoreactivities are found in primary sensory nerves. The peripheral branches of SP-immunoreactive nerves are found around the walls of blood vessels. SP was reported to be a transmitter of a group of sensory fibers which originate in the regions of baroreceptors and chemoreceptors and terminate in the nucleus of the tractus solitarius.

SP is thought to be released from the endings of these peripheral SP-immunoreactive nerves, and may play an important role in the regulation of blood pressure. SP may be released from the endings of the peripheral branches of SP-
immunoreactive nerves via the axon reflex, and may directly induce vasodilation. The significant decrease in the plasma SP concentration of SHRSP was thought to be the result of a decrease in the peripheral release of SP from the endings of SP-immunoreactive nerves.

Likewise, the significant decrease in the plasma VIP concentration of SHRSP was thought to be the result of a decrease in the peripheral release of VIP, on the basis of the results of the former and present studies. The peripheral branches of VIP-immunoreactive nerves are widely found around the walls of blood vessels. VIP has been considered to be a transmitter of non-adrenergic non-cholinergic inhibitory nerves. VIP may be released from the endings of peripheral VIP-immunoreactive nerves efferently, and may directly induce vasodilation, as does SP.

For SHRSP, the conduction and reflexion of peripheral SP-and VIP-immunoreactive nerves innervating blood vessels may be reduced, and as a result, peripheral release of SP and VIP may be decreased. Therefore, the functional involution of SP-and VIP-immunoreactive nerves of SHRSP was suggested. In addition, the age-related involution of SP-immunoreactive nerves was suggested.

However, whether the involution of SP-and VIP-immunoreactive nerves of SHRSP is the cause or the result of hypertension is still unclear. In the former case, the involution of SP-and VIP-immunoreactive nerves may contribute to the predominance of sympathetic vasoconstriction. In the latter case, the involution of SP-and VIP-immunoreactive nerves may be the result of sclerosing alterations in the walls of blood vessels, as occurs in the chronic hypertensive state.

The alterations of central SP and VIP levels of SHRSP cannot be explained from the present study, although central SP and VIP may indirectly affect the regulation of vasomotor tone.

Further biochemical and immunohistochemical studies are needed.

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