Unaltered Caffeine-induced Relaxation in the Aorta of Stroke-Prone Spontaneously Hypertensive Rats (SHRSP)

Fumiko Sekiguchi, Yoshimasa Miyaake, Takafumi Kashimoto, and Satoru Sunano

Department of Anatomy and Physiology, School of Pharmaceutical Sciences, Kinki University, Higashi-Osaka 577-8502, Japan

Abstract

Caffeine-induced relaxation was studied in aortic segments from Wistar Kyoto rats (WKY) and stroke-prone spontaneously hypertensive rats (SHRSP). Although acetylcholine-induced endothelium-dependent relaxation was impaired in preparations from SHRSP, the relaxation induced by caffeine was identical in both groups. In addition, caffeine-induced relaxation was not affected by removal of the endothelium in either group. The relaxation induced by N6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate (db-cAMP), a membrane-permeable analog of adenosine 3':5'-cyclic monophosphate (cAMP), was identical in both groups. No significant difference was observed in the increase in cAMP content induced by caffeine in the aortic smooth muscle between the groups, although the basal content was significantly higher in preparations from SHRSP. These results suggest that the relaxation induced by caffeine in these preparations is brought about by its direct effect on smooth muscle and that the response of the smooth muscle to caffeine, including cAMP production, is not altered in preparations from SHRSP compared with those from WKY.

Key words: caffeine-induced relaxation, aorta, stroke-prone spontaneously hypertensive rats, adenosine 3':5'-cyclic monophosphate (cAMP)

Introduction

Prolonged application of caffeine causes relaxation of vascular smooth muscle after a transient contraction (Ahn et al., 1988; van der Bent and Beny, 1991; Watanabe et al., 1992). This relaxation has been reported to be mediated, at least in part, by an increase in adenosine 3':5'-cyclic monophosphate (cAMP) concentration in smooth muscle cells (Ahn et al., 1988; Watanabe et al., 1992; Hatano et al., 1995). The increase in cAMP is known to be caused by the inhibitory action of caffeine on phosphodiesterase which degrades cAMP (Butcher and Sutherland, 1962).

Correspondence to: F. Sekiguchi, Department of Anatomy and Physiology, School of Pharmaceutical Sciences, Kinki University, 3-4-1 Kowakae, Higashi-Osaka, Osaka 577-8502, Japan Phone: +81-6-6721-2332, ext. 3827 Fax: +81-6-6730-1394 e-mail: fumiiko@phar.kindai.ac.jp
Caffeine has also been shown to act on endothelial cells and to induce release of Ca\(^{2+}\) from the endoplasmic reticulum (ER) (Rusko et al., 1995; Hatano et al., 1995). This increase in intracellular Ca\(^{2+}\) by caffeine may cause release of endothelium-derived relaxing factor (EDRF) (Zheng et al., 1994; Hutcheson and Griffith, 1997; Mizuno et al., 2000, Kuroiwa-Matsumoto et al., 2000). Hatano et al. (1995) have reported that caffeine can initiate endothelium-dependent relaxation by releasing Ca\(^{2+}\) from the ER. In addition, it has been reported that an increase of cAMP in endothelial cells initiates the release of nitric oxide (NO) in the pulmonary artery (Priest et al., 1999). Thus, there is evidence that caffeine can initiate the relaxation of vascular smooth muscle through both endothelium-dependent and -independent pathways.

In blood vessels of spontaneously hypertensive rats (SHR), endothelium-dependent relaxation induced by various stimulants has been reported to be impaired (Lüscher and Vanhoutte, 1986; Sunano et al., 1989; 1990; 1992). In addition, it has also been reported that the relaxation in response to cAMP was impaired in the aorta of SHR (Cohen and Berkowitz, 1976). Thus it is of interest to examine whether the endothelium-dependent and -independent relaxations induced by caffeine are altered in blood vessels of SHR.

In the present study, endothelium-dependent and -independent relaxations of the aortae induced by caffeine were compared between preparations from normotensive Wistar Kyoto rats (WKY) and stroke-prone SHR (SHRSP) with severe hypertension.

**Methods**

*Animals and measurement of blood pressure*

Male SHRSP and control normotensive WKY of 16 weeks of age were used in the present experiments. These rats were purchased from Shimizu Laboratory Supplies Co. Ltd. (Kyoto, Japan) when 5 weeks old and fed in our animal facility until sacrifice. Rats were handled according to the “Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences” of the Physiological Society of Japan. They were maintained under conditions of constant temperature (22°C) and humidity (50%) and exposed to a 12 hr light-dark cycle. Normal chow (SP, Funabashi, Japan) and tap water were freely available. Blood pressure was measured just before sacrifice using the tail-cuff method. Prior to measurement, the rats were warmed at 40°C for 10 min to obtain a stable blood pressure.

*Preparations and solutions*

The rats were killed by cutting the anterior venae while under CO\(_2\) anesthesia. The thoracic aorta was quickly removed and immersed in a modified Tyrode’s solution as described below. Fat and adherent connective tissue were removed with fine scissors, and the vessel was cut into 1 mm wide ring preparations. In ten preparations, the endothelium was removed by rubbing the inner surface of the lumen gently with a soft rubber band.

The composition of the modified Tyrode’s solution was (mM): NaCl, 137; KCl, 5.4; CaCl\(_2\), 2.0; MgCl\(_2\), 1.0; NaHCO\(_3\), 11.9; NaH\(_2\)PO\(_4\), 0.4; glucose, 5.6; equilibrated with a gas mixture of 95% O\(_2\) and 5% CO\(_2\). The pH of the solution was 7.3 at 37°C. K+-Tyrode’s solution was made by replacing all NaCl in the modified Tyrode’s solution with KCl, and high-K+ Tyrode’s solution
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with 50 mM K⁺ (high-K⁺) was made by mixing the modified Tyrode’s solution and K⁺-Tyrode’s solution at an appropriate ratio.

Mechanical recording

Two tungsten wires (30 μm in diameter) were inserted into the lumen of the preparation, and then one was tied to a plastic holder. This holder was mounted in an organ bath (10 ml) filled with modified Tyrode’s solution at a constant temperature of 37°C. The other tungsten wire was connected to a force transducer (Minebea, Nagano, Japan) so that tension changes could be measured isometrically.

The preparations were equilibrated for at least 60 min and then subjected to two consecutive high-K⁺-induced contractions of 20 min duration separated by an interval of 20 min. These procedures were required to obtain constant results in the following experiments. After these treatments, the preparations were contracted with $5 \times 10^{-7}$ M noradrenaline (NA) in the presence of 26 μM ethylenediaminetetraacetate (EDTA) and, at the peak of contraction, various agents were added to observe relaxation. At the end of the experiments, the preparations were completely relaxed by application of a combination of $10^{-5}$ M verapamil and $10^{-4}$ M papaverine. The relaxations caused by the various agents were measured from the completely relaxed level in the presence of verapamil and papaverine and were expressed as percentages of the NA-induced precontraction.

cAMP measurement

The content of cAMP in the smooth muscle was measured by an immunoassay method. Aortic preparations, from which the endothelium was removed, were incubated in modified Tyrode’s solution for 60 min at 37°C. Preparations for measurement of the basal cAMP content were frozen in liquid nitrogen at −190°C after incubation. The other preparations were then moved to Tyrode’s solution containing NA (5 × 10⁻⁷ M) and EDTA (26 μM) for a further 30 min incubation. After this incubation, preparations for measurement of cAMP content in the presence of NA were frozen in liquid nitrogen. Those to be used to observe the effects of caffeine on the cAMP content were further incubated in Tyrode’s solution containing NA and caffeine (10 mM) for 10 min before being frozen. These frozen preparations were stored at −80°C until assayed. Assay of cAMP was performed using a commercial kit (Amersham Pharmacia UK, Bucskinghamshire, UK) according to the manufacturer’s protocol. Briefly, each sample was homogenized in 6% trichloroacetic acid. The homogenate was centrifuged at 2000 × g at 4°C for 15 min, the supernatant washed 4 times with water-saturated diethyl ether, and the cAMP enzyme immunoassay performed on the aqueous extract. After discarding the upper layer, the remaining aqueous extract was dried under a stream of nitrogen at 60°C, dissolved in 1 N NaOH and used for measurement of protein concentration. cAMP content was expressed as pmol cAMP per mg of protein.

Drugs

Drugs used in the present experiments were: noradrenaline bitartrate (NA, Sigma, St. Louis, MO, USA), acetylcholine hydrochloride (ACh, Wako Chem., Osaka, Japan),
ethylenediaminetetraacetate (EDTA, Wako Chem.), verapamil hydrochloride (Wako Chem.), papaverine hydrochloride (Wako Chem.), N\textsuperscript{6}-nitro-L-arginine (L-NNA, Sigma) methylene blue trihydrate (Wako Chem.) and N\textsuperscript{6},2\textsuperscript{-O}-dibutyryladenosine 3\textsuperscript{\prime}:5\textsuperscript{\prime}-cyclic monophosphate (db-cAMP, Wako Chem.).

Statistics
The values obtained were expressed as the mean ± SE with the number of preparations shown in parentheses. Statistical analyses were performed using the Student’s t-test when the values of two groups were compared and with a one-way analysis of variance (ANOVA) followed by the Bonferroni/Dunn’s post hoc test for differences among more than three groups; \(P\) values smaller than 0.05 were considered to be significant.

Results

Body weight and blood pressure
Body weight and blood pressure are presented in Table 1. SHRSP had significantly lower body weight and significantly higher blood pressure than WKY.

Relaxation of aortae from WKY and SHRSP in response to acetylcholine (ACh)
The application of ACh to preparations precontracted in the presence of NA induced concentration-dependent relaxation (Figs. 1a and 2). The threshold concentration of ACh for relaxation was \(10^{-9}\) M in preparations from both WKY and SHRSP. The relaxation in preparations from SHRSP was significantly impaired in comparison with those from WKY at all concentrations of ACh examined (Fig. 2). The relaxation was completely abolished by removal of the endothelium (Fig. 2).

Effects of N\textsuperscript{6}-nitro-L-arginine (L-NNA) and methylene blue on ACh-induced relaxation
The ACh-induced relaxation of preparations from both WKY and SHRSP was completely blocked by \(10^{-4}\) M L-NNA (Fig. 1b). In preparations from SHRSP, a small elevation of tension instead of a relaxation was observed after application of ACh at concentrations higher than \(10^{-7}\) M in the presence of L-NNA (data not shown). This was not observed in preparations in which the endothelium had been removed.

Methylene blue at concentrations higher than \(10^{-5}\) M blocked ACh-induced relaxation completely in preparations from SHRSP. In preparations from WKY, a slight relaxation was

| Table 1 | Body weight and systolic blood pressure of rats used in the present study |
|---------|---------------------------------------------------------------------------|
|         | n  | Body weight (g) | Systolic blood pressure (mm Hg) |
| WKY     | 46 | 379.9 ± 5.1   | 140.8 ± 1.5  |
| SHRSP   | 42 | 268.6 ± 5.5 **| 239.6 ± 2.3 **|

Asterisks indicate significant differences when compared with values for WKY (**, \(P<0.001\)).
Caffeine relaxation in SHRSP aorta

Caffeine at concentrations higher than $3 \times 10^{-5}$ M induced relaxation of preparations precontracted in the presence of $5 \times 10^{-7}$ M NA (Fig. 4). The development of relaxation was observed at higher concentrations of ACh in the presence of $10^{-5}$ M methylene blue, although the relaxation was completely abolished by $10^{-4}$ M methylene blue (Fig. 3).

### Caffeine-induced relaxation

Caffeine at concentrations higher than $3 \times 10^{-5}$ M induced relaxation of preparations precontracted in the presence of $5 \times 10^{-7}$ M NA (Fig. 4). The development of relaxation was observed at higher concentrations of ACh in the presence of $10^{-5}$ M methylene blue, although the relaxation was completely abolished by $10^{-4}$ M methylene blue (Fig. 3).

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**Fig. 1** Relaxation of endothelium-intact aortae from WKY by acetylcholine (ACh), caffeine and N6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate (db-cAMP). a, ACh-induced relaxation in the absence of L-NNA. b, Abolition of ACh-induced relaxation in the presence of L-NNA. c, Caffeine-induced relaxation in the presence of L-NNA. d, db-cAMP-induced relaxation in the presence of L-NNA. Preparations were contracted by application of noradrenaline (NA, $5 \times 10^{-7}$ M), and each drug was applied cumulatively after the NA contraction reached its peak. Note the differences in the time scales in each trace and the speed of relaxation. Vera and Papa indicate the application of verapamil ($10^{-5}$ M) and papaverine ($10^{-4}$ M) respectively.
markedly slower than that induced by ACh (Figs. 1a and 1c). Removal of the endothelium did not affect the speed or degree of the relaxation by caffeine in preparations from both WKY and SHRSP. No significant difference was observed in the relaxation induced by caffeine between endothelium-intact or -removed preparations from either WKY or SHRSP.

The relaxation induced by caffeine was not affected by L-NNA (10^{-4} M) in the preparations.
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Methylene blue (10^{-5} M) did not affect the caffeine-induced relaxation in preparations from either strain (Fig. 5).

**Effects of db-cAMP**

The application of db-cAMP at concentrations higher than 10^{-5} M induced concentration-dependent relaxation of preparations precontracted in the presence of NA (5 × 10^{-7} M) (Fig. 6). Although the relaxation developed extremely slowly at low concentrations of the drug, at higher
concentrations such as at $10^{-3}$ M, the relaxation developed rapidly and reached the initial tension level observed before application of NA (Fig. 1d).

The relaxation by db-cAMP was not affected by removal of the endothelium, or by application of concentrations of either L-NNA or methylene blue as used in previous experiments.

**Effects of caffeine on cAMP concentration**

The content of cAMP in the aortic smooth muscle in the resting state was significantly greater in preparations from SHRSP than in those from WKY (Fig. 7). The application of NA ($5 \times 10^{-7}$ M) caused no changes in cAMP content in preparations from either strain. Intracellular cAMP level was significantly elevated by the addition of caffeine (10 mM). The degree of elevation by caffeine was slightly greater in the preparations from SHRSP, but the increase was not significant.

**Discussion**

ACh-induced endothelium-dependent relaxation of preparations from SHRSP was impaired compared to that observed in preparations from WKY. As the relaxation induced by ACh was abolished by removal of the endothelium or by application of L-NNA, the release of NO from the endothelium was considered to be the cause of the relaxation (Moore *et al.*, 1990). The relaxation of these preparations was shown to be blocked by methylene blue (Ignarro and Kadowitz, 1985), suggesting that it is mediated by the formation of guanosine 3′:5′-cyclic monophosphate (cGMP) in smooth muscle.

Reduced NO release, decomposition of NO, co-release of endothelium-derived contracting factor (EDCF), and/or reduced formation of cGMP, alone or in combination, may be factors
involved in the impairment of relaxation of these preparations. We observed that the formation of cGMP is not altered in the aorta of SHRSP (Osugi et al., 1990). Thus, alteration of any of the remaining three factors may be responsible for the reduction of the relaxation, although the mechanisms involved were not investigated in the present study.

It is well established that the release of endothelium-derived factors is mediated by an increase in the Ca²⁺ concentration in endothelial cells for both EDRF (Hutcheson and Griffith, 1997; Mizuno et al., 2000) and for endothelium-derived hyperpolarizing factor, EDHF (Chen and Suzuki, 1990). The increase in intracellular Ca²⁺ concentration would be brought about by increased Ca²⁺ influx and/or by the release of Ca²⁺ from the ER. With regard to the latter, caffeine has been reported to cause release of Ca²⁺ from ER of the endothelium of blood vessels (Rusko et al., 1995). The increase in intracellular Ca²⁺ may induce the release of endothelium-derived factors as described above and affect electrical and mechanical activities of smooth muscle. It is also suggested that the increase in cAMP level, which is caused by the inhibition of cAMP phosphodiesterase (Butcher and Sutherland, 1962), induces the release of NO in the pulmonary artery (Priest et al., 1999; Hucks and Ward, 2000).

In the present study, it has been shown that caffeine induced relaxation in the aortae from both WKY and SHRSP. Hatano et al. (1995) have reported similar effects of caffeine in the rat aorta. In another study, evidence has been reported that caffeine causes the release of EDCF (Jino et al., 1994). Thus, it is possible that the relaxation reported in this study is mediated by both EDRF and EDHF, and affected by the amount of EDCF released concomitantly. However, caffeine-induced relaxation was not affected by L-NNA (Moore et al., 1990) and methylene blue (Murad et al., 1978). Moreover, neither the speed nor degree of caffeine-induced relaxation was affected by removal of the endothelium. Thus, we conclude that endothelium-derived factors

Fig. 7 Effects of caffeine on the cAMP content of aortae from WKY and SHRSP. a, cAMP content in the basal state (Basal), in the presence of 5 × 10⁻⁷ M noradrenaline (NA) and in the presence of both noradrenaline and 10 mM caffeine (NA & Caffeine) (see Methods). b, A comparison of the cAMP increase induced by caffeine. It shows the values of difference between NA and NA & Caffeine, which was not significant between WKY and SHRSP.
are not involved in the relaxation induced by caffeine in the aortae from WKY and SHRSP, even though these factors may be released by caffeine.

The relaxation of smooth muscle induced by caffeine may be mediated by increased cAMP (Ahn et al., 1988; Watanabe et al., 1992). In the present study, it has been shown that caffeine caused the increase in cAMP concentration in the smooth muscle from both WKY and SHRSP aortae. In addition, application of the membrane-permeable cAMP analog db-cAMP caused similar relaxation to that induced by caffeine. The slow development of the relaxation induced by db-cAMP may be explained by the slow penetration of the drug through the smooth muscle membrane (Tsujimoto et al., 1986).

The present results have shown that the relaxation induced by both caffeine and db-cAMP was not significantly different between preparations from both WKY and SHRSP. The results agreed with those reported by Asano et al. (1988) but disagreed with those of an earlier report by Cohen and Berkowitz (1976) that the relaxation by db-cAMP in the aorta of conventional SHR was impaired. Relaxation induced by theophylline, which is thought to be mediated by an increase in cAMP, was reported to be weaker in the aorta of SHR than in normotensive controls (Triner et al., 1975). This discrepancy cannot be explained at present, but it should be mentioned that the relaxation induced by sodium nitroprusside, which is mediated by the formation of cGMP as is the case for nitroglycerine, has been reported to be either unaltered or more often potentiated in preparations from SHR and SHRSP (Koga et al., 1989; Osugi et al., 1990; Hayakawa et al., 1993). On the other hand, Cohen and Berkowitz (1976) reported that the relaxation induced by db-cAMP and nitroglycerine was impaired in preparations from SHRSP.

The greater content of cAMP in the aorta of SHRSP than that in WKY at 16 weeks of age agrees with the increased levels of cAMP reported in the aorta of 12 week old SHR (Chatelain et al., 1985). In aged rats, however, it is reported that the cAMP content in SHR was smaller than that in normotensive rats (Amer, 1973). The degree of caffeine-induced increase in cAMP is slightly greater in preparations from SHRSP, although this difference was not significant. Nevertheless, the relaxation caused by caffeine was similar in both preparations. Although the possibility of reduced sensitivity to cAMP has been reported for preparations from SHR (Cohen and Berkowitz, 1976), the relaxation induced by db-cAMP was not different between preparations from WKY and SHRSP. Thus, caffeine-induced cAMP production may be involved in the relaxation of aortic smooth muscle, but a direct action of caffeine on the contractile machinery of smooth muscle (Ozaki et al., 1990) may be the main cause of the relaxation and this action of caffeine may not be significantly different between preparations from both WKY and SHRSP.

In conclusion, caffeine induced the relaxation of aortae from both WKY and SHRSP. Although endothelium-dependent relaxation of the preparations, which was mediated by NO-induced cGMP formation, was impaired in the preparations from SHRSP, the relaxation induced by caffeine was independent of the endothelium and was not significantly different between preparations from WKY and SHRSP. These results indicate that endothelium-derived factors were not involved in the relaxation, but that direct action on the contractile machinery of smooth muscle was the main cause of the relaxation induced by caffeine. This latter action is not altered in the aortae of SHRSP.
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