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Factors involved in the time course of response to acetylcholine in mesenteric arteries from spontaneously hypertensive rats

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Abstract

The time course of the response to prolonged application of acetylcholine in mesenteric arteries from stroke-prone spontaneously hypertensive rats (SHRSP) and Wistar Kyoto rats (WKY) was compared. Only a relaxing response, which was blocked by N^{ω}-nitro-L-arginine (L-NOARG), was observed after the prolonged application of a low concentration of acetylcholine (10^{-8} M) in both preparations; the response was impaired in SHRSP preparations. Prolonged application of a high concentration of acetylcholine (10^{-5} M) induced a second contractile response after a first relaxing response in SHRSP preparations under basal conditions and in WKY preparations in the presence of L-NOARG. This contractile response was attenuated by indomethacin. In the presence of a combination of apamin and charybdotoxin, the relaxing response to the high concentration of acetylcholine was reduced and a contractile response, which was abolished by indomethacin, appeared. In the presence of all of these blockers, a contractile response, which was blocked by cyclo(D-α-aspartyl-L-propyl-D-valyl-L-leucyl-D-tryptophyl) (BQ-123), was observed in preparations from WKY but not in preparations from SHRSP. Results indicate that prolonged application of acetylcholine in rat mesenteric arteries induces the release of endothelium-derived relaxing, contracting, hyperpolarizing factors and endothelin-1, and that the mode of action differs between preparations from WKY and SHRSP.

Keywords: Mesenteric artery; Stroke-prone spontaneously hypertensive rat (SHRSP); (EDNO) Endothelium-derived nitric oxide ; EDCF (endothelium-derived contracting factor); EDHF (endothelium-derived hyperpolarizing factor); Time course

1. Introduction

The vascular endothelium releases a number of factors that affect the contraction and relaxation of vascular smooth muscle (Pearson and Vanhoutte, 1993; Vanhoutte, 1989; Furchgott and Vanhoutte, 1989). These factors are classified into three groups, i.e. endothelium-derived relaxing (EDRF), contracting (EDCF) and hyperpolarizing factors (EDHF). The influence of these factors on the contraction and relaxation of smooth muscle varies among different types of blood vessels (Pearson and Vanhoutte, 1993; Vanhoutte et al., 1986; Vanhoutte, 1989; Nagao et al., 1992).

The endothelium-dependent responses of resistance arteries, especially the relaxing response, have been shown to be impaired in the blood vessels of hypertensive animals, including spontaneously hypertensive rats (SHR) and stroke-prone SHR (SHRSP) (Watt and Thurston, 1989; Diederich et al., 1990; Fujii et al., 1992; Bennett et al., 1996; Sunano et al., 1999). Various causes of these impairments, such as reduced release of EDRF (Grunfeld et al., 1995; Hirata et al., 1996), increased release of EDCF (Lüscher and Vanhoutte, 1986; Watt and Thurston, 1989; Diederich et al., 1990) and reduced release of EDHF (Fujii et al., 1992, 1993; Sunano et al., 1999), have been proposed. The magnitude of changes in the release of these factors may influence the magnitude of the impairment of endothelium-dependent relaxation of blood vessels.

In studies of the endothelium-dependent responses of blood vessels, most experiments were performed by cumulative application of agonists that stimulate the endothelium. However, the involvement of factors may vary depending on the time after application of the agonists, as suggested by the time course experiments in other blood vessels (Rubanyi et al., 1985, 1987; Osugi et al., 1990; Ito et al., 1991; Fiscus et al., 1992; Parkington et al., 1993).

In the present study, we examined the differences in the time course of responses to various endothelium-derived

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factors between mesenteric resistance arteries from normotensive Wistar Kyoto rats (WKY) and SHRSP by observing the effects of inhibitors of these factors.

2. Materials and methods

2.1. Experimental animals

SHRSP and WKY were used in the present study at the age of 16 weeks. These rats were originally obtained from Dr. Okamoto who generated these strains (Okamoto et al., 1974) and were bred in our animal facility. All animal experiments were performed in accordance with European guidelines and were approved by the ethics committee of our university. Animals were given free access to normal rat chow (SP, Funabashi, Japan) and tap water in our animal facility with a controlled room temperature of 22 °C, a humidity of 60% and a 12-h light–dark cycle. Blood pressure was measured by the tail-cuff method. Prior to the measurement, rats were warmed at 40 °C for 10 min to facilitate precise measurement of blood pressure.

2.2. Preparations

The rats were killed by exsanguination from the vena cava under anesthesia with CO_2 gas. The mesenterium was excised from the abdomen and the second branch of the superior mesenteric artery was dissected in modified Tyrode's solution as described below. In the present study, ring preparations with a width of 1.5 mm were used. In about 10 preparations, the endothelium was removed by perfusing the inner surface of the lumen with modified Tyrode's solution containing 0.3% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) for 2.5 min.

2.3. Solutions

The incubation medium used was a modified Tyrode's solution of the following composition (mM): NaCl, 137; KCl, 5.4; $CaCl_2$, 2.0; $MgCl_2$, 1.0; $NaHCO_3$, 11.9; NaH_2PO_4, 0.4; and glucose, 5.6; equilibrated with a mixture of 95% O_2 and 5% CO_2. The modified Tyrode's solution was kept at 37 °C, and the pH at this temperature was 7.3. K-Tyrode's solution was made by replacing all NaCl with KCl, and solutions with elevated K^+ were made by mixing the Tyrode's and K-Tyrode's solutions at appropriate ratios.

2.4. Measurement of tension

The contraction and relaxation of the preparations were measured using an apparatus similar to that reported by Mulvany and Halpern (1977). The preparations were mounted on the apparatus filled with modified Tyrode's solution with two tungsten wires 50 μm in diameter under a stretching tension of 1 mN. Changes in tension were measured with a tension transducer (Shinkoh, Nagano, Japan). Changes in force are expressed in newtons (N) calculated from tension changes (g), taking 1 kg × 9.8 $m/s^2 = 1$ N.

The preparations were equilibrated in modified Tyrode's solution for at least 60 min and then subjected to two successive high-K^+ (50 mM)-induced contractions of 5 min in duration with an interval of 10 min. This procedure was required to initiate a precontraction of constant amplitude. After 30 min of equilibration, precontraction was initiated by application of 5×10^{-6} M noradrenaline, in the presence of calcium disodium ethylenediaminetetraacetic acid (EDTA, 26 μM), and after maximum precontraction was obtained, acetylcholine was added. The concentration of noradrenaline was chosen because this concentration of the drug induced a sustained contraction with an amplitude between 65–90% of the high-K^+-induced maximum contraction under the respective conditions. Although the amplitude of precontraction varied, the concentration of the drug was fixed, so that the influence of a changing concentration could be avoided. In the time course experiment, acetylcholine was applied at 10^{-8} or 10^{-5} M. These concentrations of acetylcholine were determined from the results of a concentration–response experiment, which showed relaxation and contractile responses in all preparations from SHRSP under control conditions. The responses to acetylcholine at low and high concentrations did not vary greatly among rats. The preparations from WKY showed only the relaxation response regardless of the concentration of the drug, as shown in Figs. 1 and 2. At the end of the experiments, preparations were relaxed completely by adding 10^{-5} M verapamil and 10^{-4} M papaverine, and all tension changes were measured from this relaxed level.

In the present experiments, the involvement of endothelium-derived nitric oxide (EDNO), endothelium-derived vasoconstrictor prostanoids (EDCFs), endothelin and EDHF in the response to acetylcholine was studied by using specific inhibitors of synthesis or under conditions that inhibited the action of the specific factor. Briefly, to study the involvement of EDNO, 10^{-4} M N^{ω}-nitro-L-arginine (L-NOARG) was present throughout the experiment, since the relaxation induced by EDRF in this preparation is mainly thought to be due to endothelium-derived nitric oxide (Sunano et al., 1999). To study the involvement of EDCFs, the experiments were performed in the presence of indomethacin (10^{-5} M), since EDCFs, especially those involved in the impairment of acetylcholine-induced relaxation of the preparations from SHRSP, can be blocked by indomethacin (Sunano et al., 1999). The involvement of EDHF was studied by elevating the external K^+ concentration or by applying a combination of apamin (5×10^{-6} M) and charybdotoxin (10^{-7} M), although these procedures did not block the production of EDHF

itself. In the experiments with an elevated K^+ concentration, the concentration of noradrenaline, which was used to initiate precontraction, was reduced so that a precontraction of the same amplitude as that observed with 5×10^{-6} M noradrenaline in normal Tyrode's solution containing 5.4 mM K^+ could be obtained. The effect of endothelin was examined by application of cyclo (D-α-aspartyl-L-propyl-D-valyl-L-leucyl-D-tryptophyl) (BQ-123).

2.5. Drugs

Drugs used in the present study were: 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS, Sigma, St. Louis, MO, USA), noradrenaline bitartrate salt (Sigma), acetylcholine hydrochloride (Wako, Osaka, Japan), N^{ω}-nitro-L-arginine (L-NOARG, Sigma), indomethacin (Sigma), charybdotoxin (Peptide Inst., Osaka, Japan), apamin (Sigma), cyclo (D-α-aspartyl-L-propyl-D-valyl-L-leucyl-D-tryptophyl) (BQ-123, Funakoshi, Tokyo, Japan), verapamil hydrochloride (Wako), papaverine hydrochloride (Wako) and calcium disodium ethylenediaminetetraacetate (EDTA, Dojindo, Kumamoto, Japan). Stock solutions of indomethacin (10^{-3} M) were made by dissolving the drug in distilled water containing 5×10^{-3} M Na_2CO_3. CHAPS was dissolved in the modified Tyrode's solution to obtain a final concentration of 0.3% (4.9×10^{-3} M). Other drugs were dissolved in distilled water as stock solutions and added during the experiment to 10 ml of incubation medium. The final volume of these stock solutions was less than 100 μl, which did not influence the responses of the preparation.

2.6. Statistics

The obtained values are expressed as means ± SEM. The values of body weight and systolic blood pressure were analyzed by Student's *t*-test. Concentration–response and time course curves were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni/Dunn's post hoc test. *P* values less than 0.05 were considered significant.

3. Results

3.1. Body weight and blood pressure of rats

Body weight of WKY and SHRSP at the age of 16 weeks was 364.3 ± 3.5 g ($n = 35$) and 262.9 ± 4.9 g ($n = 35$), respectively. The difference between the body weight of WKY and SHRSP was significant ($P < 0.001$).

Systolic blood pressure of WKY and SHRSP was 134.0 ± 0.9 mm Hg ($n = 35$) and 237.0 ± 2.0 mm Hg ($n = 35$), respectively. The blood pressure of SHRSP was significantly higher than that of WKY ($P < 0.001$).

3.2. Concentration–response curve for acetylcholine in noradrenaline-precontracted preparations

In the mesenteric artery from WKY, which had been contracted in the presence of 5×10^{-6} M noradrenaline, the application of acetylcholine induced concentration-dependent relaxation. The maximum relaxation was observed at 10^{-4} M, and the magnitude of the relaxation was 95.9 ± 0.9% ($n = 12$) of that of the precontraction. An increase in the concentration of acetylcholine induced no further changes in the magnitude of relaxation (Fig. 1). In the mesenteric artery from SHRSP, low concentrations of acetylcholine induced relaxation of noradrenaline-precontracted preparations in a concentration-dependent manner. The maximum relaxation (62.5 ± 4.0%, $n = 12$) was observed at 3×10^{-7} M, and acetylcholine concentrations higher than this induced an increase in tension (con-

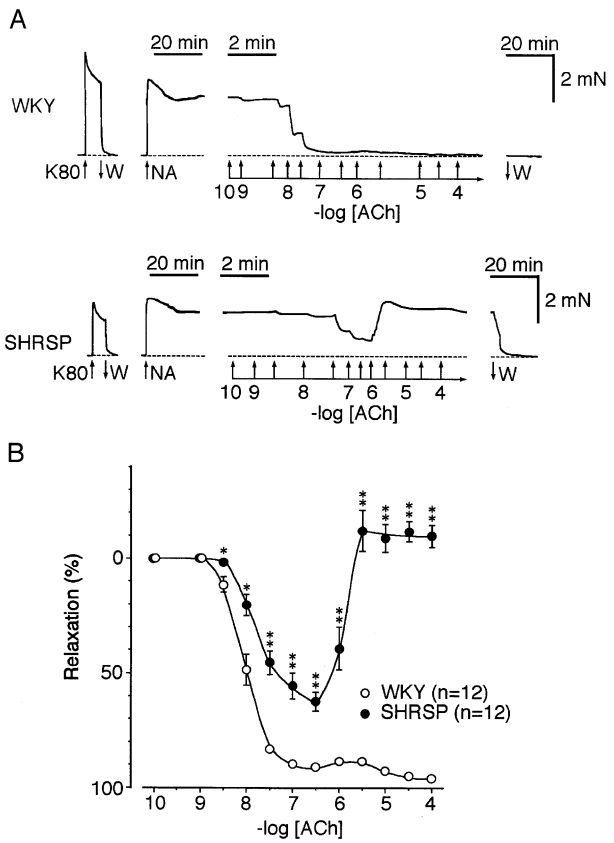

Fig. 1. (A) Response to acetylcholine (ACh) in the mesenteric arteries from WKY (upper) and SHRSP (lower). The concentration of acetylcholine was increased cumulatively from 10^{-10} to 10^{-4} M, as indicated. K80, NA and W indicate application of the solution containing 80 mM K^+, 5×10^{-6} M noradrenaline and wash-out with normal Tyrode's solution, respectively. (B) Concentration–response curves for the action of acetylcholine in the preparations from WKY and SHRSP. Values are expressed as percentages of the precontraction induced by noradrenaline (5×10^{-6} M), so that plus and minus values indicate relaxation and contraction, respectively. Asterisks indicate significant differences from the values obtained in the preparations from WKY (*, $P < 0.05$. **, $P < 0.001$).

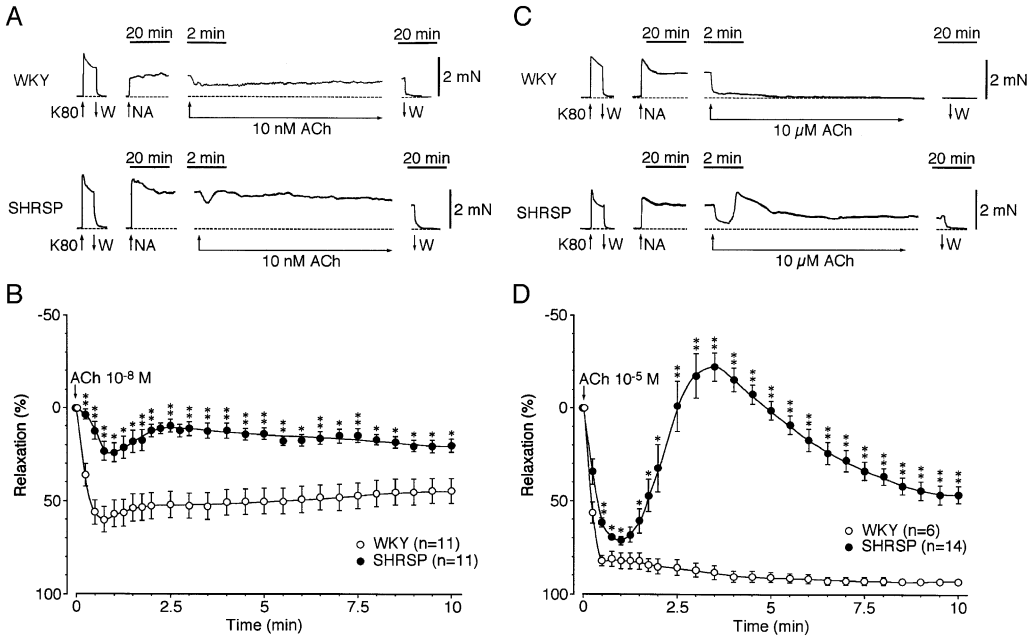

Fig. 2. Time course of the responses to acetylcholine in preparations from WKY and SHRSP. Acetylcholine was applied at time 0 and time-dependent changes in the responses are expressed as percentages of the precontraction induced by noradrenaline (5×10^{-6} M), so that plus and minus values indicate relaxation and contraction, respectively. (A) Typical traces of the responses to a low concentration of acetylcholine in the preparations from WKY (upper) and SHRSP (lower). 10^{-8} M (10 nM) acetylcholine was applied at the arrow. Other points are the same as those in Fig. 1. (B) Time course of the response to a low concentration of acetylcholine (10^{-8} M). (C) Typical traces of the responses to a high concentration of acetylcholine in the preparations from WKY (upper) and SHRSP (lower). 10^{-5} M (10 μM) acetylcholine was applied at the arrow. Other points are the same as those in Fig. 1. (D) Time course of the response to a high concentration of acetylcholine (10^{-5} M). Asterisks indicate significant differences from respective values obtained with the preparations from WKY (*, $P < 0.05$. **, $P < 0.001$).

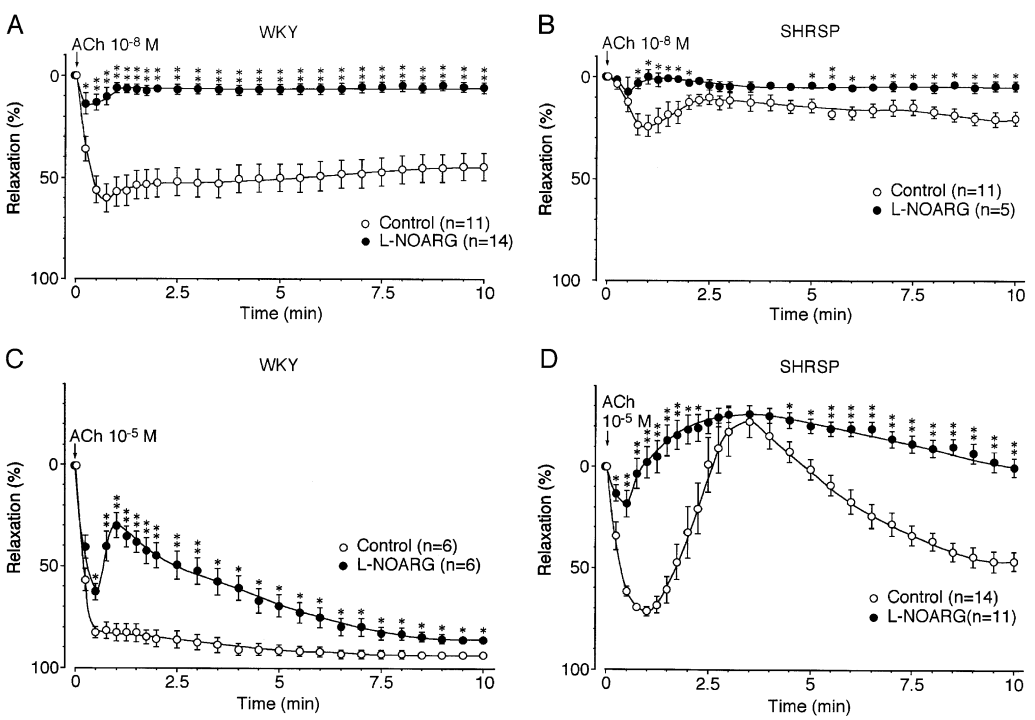

Fig. 3. Effects of L-NOARG on the time course of the responses to acetylcholine. L-NOARG (10^{-4} M) was applied 10 min prior to (A and B) precontraction. Responses to a low concentration of acetylcholine (10^{-8} M) of the preparations from WKY and SHRSP, (C and D) respectively. Responses to a high concentration of acetylcholine (10^{-5} M) of the preparations from WKY and SHRSP, respectively. Asterisks indicate significant differences from respective values obtained in the absence of L-NOARG (*, $P < 0.05$. **, $P < 0.001$). Other conditions were the same as those described in Fig. 2.

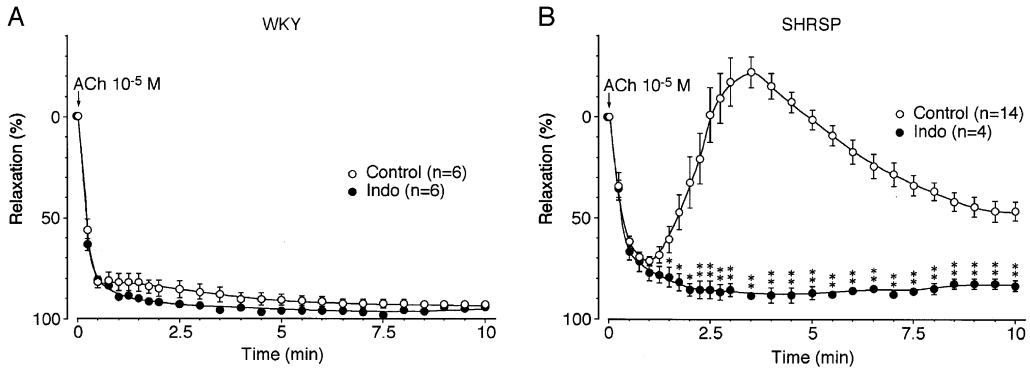

Fig. 4. Effects of indomethacin on the time course of the response to a high concentration of acetylcholine. (A) Preparation from WKY. (B) Preparation from SHRSP. Acetylcholine was applied at 10^{-5} M. Indomethacin (Indo, 10^{-5} M) was applied 30 min prior to precontraction. Asterisks indicate significant differences in respective values obtained in the absence of indomethacin (*, $P < 0.05$. **, $P < 0.001$). Other conditions were the same as those described in Fig. 2.

traction). Thus, the tension at an acetylcholine concentration of 10^{-5} M was $108.8 \pm 5.9\%$ ($n = 12$) of that of the precontraction (Fig. 1). The rebound contraction seen at higher concentrations of acetylcholine in the preparations from SHRSP was abolished in the presence of indomethacin (10^{-5} M), whereas no obvious effect of indomethacin was observed in the preparations from WKY (data not shown).

These responses (relaxation and contraction) of mesenteric arteries of both strains were abolished by the removal of the endothelium; i.e. in preparations that had been perfused with modified Tyrode's solution containing CHAPS (0.3%).

3.3. Time course of the response to acetylcholine

The responses to prolonged application of acetylcholine, especially those of preparations from SHRSP, differed markedly at low and high concentrations of the drug. In the following experiments, we examined the time course of the responses to low (10^{-8} M, low-acetylcholine) and high (10^{-5} M, high-acetylcholine) concentrations of acetylcholine.

In the preparations from WKY, acetylcholine induced a sustained tonic relaxation at both concentrations (Fig. 2). In the preparations from SHRSP, however, the low acetylcholine concentration induced a tonic sustained relaxation but the amplitude of the relaxation was markedly reduced (Fig. 2(A) and (B)). Marked differences in the response were observed when the high acetylcholine concentration was applied to the preparations from SHRSP; i.e. a triphasic response consisting of initial relaxation followed by a second rebound contractile response, and then a third relaxation response (Fig. 2(C) and (D)). Thus, the relaxations in response to both low and high acetylcholine concentrations were markedly reduced in the preparations from SHRSP as compared with those from WKY.

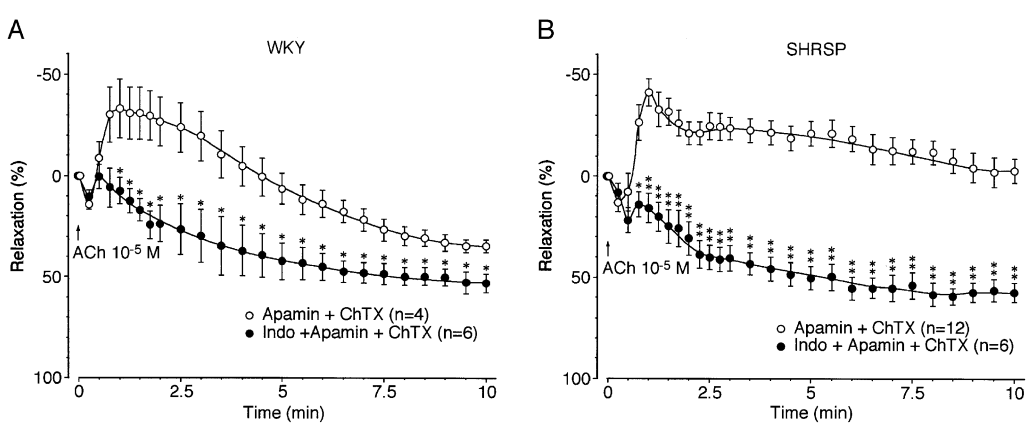

Fig. 5. Effects of apamin plus charybdotoxin on the response to acetylcholine. Acetylcholine was applied at 10^{-5} M in this experiment. (A) Preparation from WKY. (B) Preparation from SHRSP. Apamin (5×10^{-6} M) and charybdotoxin (ChTX, 10^{-7} M) were applied 30 min prior to precontraction. Asterisks indicate significant differences between respective values obtained in the absence of apamin and charybdotoxin (*, $P < 0.05$. **, $P < 0.001$). Other conditions were the same as those described in Fig. 2.

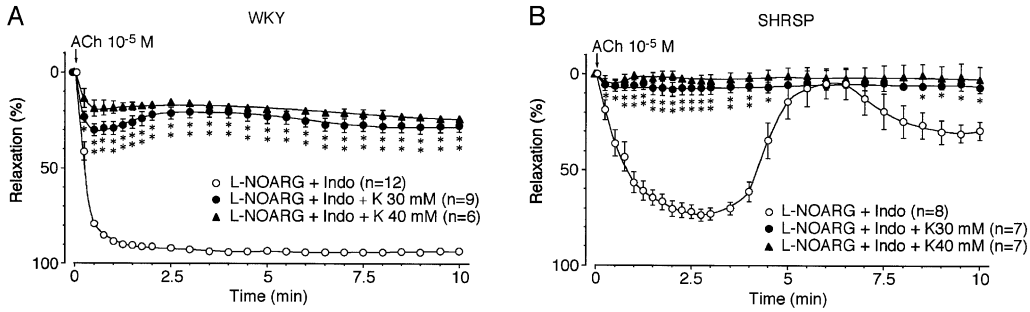

Fig. 6. Effects of increasing K^+ concentrations in the incubation medium on the time course of the response to acetylcholine in the presence of L-NOARG and indomethacin (Indo). (A) Preparation from WKY. (B) Preparation from SHRSP. Acetylcholine was applied at 10^{-5} M in this experiment. The concentration of noradrenaline was adjusted to induce the same amplitude of precontraction, and was 5×10^{-6} M in controls (in the presence of 5.4 mM K^+), 2×10^{-6} M in the presence of 30 mM K^+ and 10^{-6} M in the presence of 30 mM K^+. Asterisks indicate significant differences from respective values obtained in the presence of a normal concentration of K^+ (L-NOARG + Indo) (*, $P < 0.05$. **, $P < 0.001$). Other conditions were the same as those described in Fig. 2.

Again, in the endothelium-removed (CHAPS-treated) preparations from both WKY and SHRSP, acetylcholine at both 10^{-8} and 10^{-5} M showed no effect.

3.4. Effects of drugs on the time course of the response to acetylcholine

In the time course experiment, it was shown that the relaxation in response to acetylcholine was reduced in the presence of 10^{-4} M L-NOARG (Fig. 3). The response to the low acetylcholine concentration was abolished, leaving the first small transient relaxation in the preparations from both WKY and SHRSP (Fig. 3(A) and (B)). When the high acetylcholine concentration was applied, a second rebound contraction was observed in the preparations from WKY (Fig. 3(C)). This rebound contraction was abolished by the application of indomethacin (10^{-5} M), as described below. In the preparations from SHRSP, the relaxation response was markedly attenuated and the response became a sustained contraction (Fig. 3(D)).

Indomethacin showed no obvious effect on the acetylcholine-induced relaxation in the preparations from WKY (Fig. 4(A)). In the preparations from SHRSP, the second contractile response to the high acetylcholine concentration was abolished in the presence of indomethacin (10^{-5} M) and only the sustained relaxation was observed (Fig. 4(B)).

Apamin (5×10^{-6} M) plus charybdotoxin (10^{-7} M) reversed the relaxation into a contraction in the early phase of the response to the high acetylcholine concentration. This response was followed by a slow relaxation in the preparations from WKY (Fig. 5(A)). In the preparations from SHRSP, the contraction in response to acetylcholine in the presence of both drugs was greater, and was sustained for more than 10 min, although a small tension decline was observed (Fig. 5(B)). The tension development induced by acetylcholine observed in the presence of apamin plus charybdotoxin was markedly attenuated by indomethacin (Fig. 5(A) and (B)).

The sustained relaxation in response to the high acetylcholine concentration in the presence of L-NOARG and indomethacin was abolished by increasing the K^+ concentration in Tyrode's solution in both preparations (Fig. 6). Fig. 6 also shows that the contraction observed in the

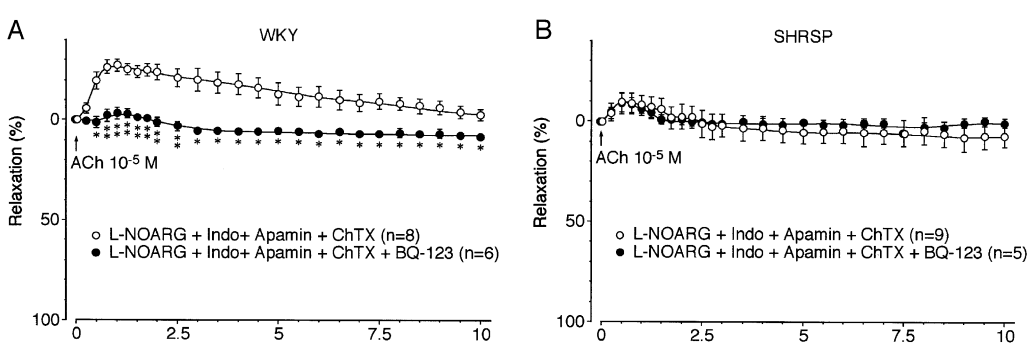

Fig. 7. Effects of BQ-123 on the time course of the response to acetylcholine in the presence of L-NOARG, indomethacin (Indo) and apamin plus charybdotoxin (ChTX). Response to 10^{-5} M acetylcholine. BQ-123 was applied at 10^{-5} M 30 min prior to precontraction. Asterisks indicate significant differences from respective values obtained in the absence of BQ-123 (*, $P < 0.05$. **, $P < 0.001$). Other conditions were the same as those described in Fig. 2.

preparations from WKY in the presence of L-NOARG (Fig. 3(A)) was abolished by indomethacin.

In the presence of L-NOARG, indomethacin and a combination of apamin and charybdotoxin, the high acetylcholine concentration still caused contraction in the preparations from WKY. This contraction was blocked by the application of BQ-123 (Fig. 7(A)). The contraction induced by the high acetylcholine concentration under these conditions was negligible in the preparations from SHRSP, and so BQ-123 showed no obvious effect (Fig.7(B)). BQ-123 at this concentration did not alter the basal tension or the contraction induced by noradrenaline in either preparation.

4. Discussion

The concentration–response experiment showed that the endothelium-dependent responses to acetylcholine were different in preparations from WKY and SHRSP, as we reported previously (Sunano et al., 1999). The impaired relaxation at low acetylcholine concentrations and the rebound contraction at high acetylcholine concentrations in preparations from SHRSP may be explained by a reduced release of EDRF (NO) (Grunfeld et al., 1995; Hirata et al., 1996) and EDHF (Fujii et al., 1992, 1993; Sunano et al., 1999), and/or increased release of EDCF (Lüscher and Vanhoutte, 1986; Watt and Thurston, 1989; Diederich et al., 1990). Although considerable time passed after the previous application of lower concentrations of acetylcholine in the cumulative concentration–response experiments, time-dependent changes in the response to the drug were not taken into consideration. Time-dependent changes in the effects, especially when a high acetylcholine concentration was applied, have been observed (Rubanyi et al., 1985, 1987; Ito et al., 1991; Osugi et al., 1990; Fiscus et al., 1992; Parkington et al., 1993). Thus, it is considered that differences, not only in the sensitivity to acetylcholine but also in the time course of the release of the each factor, should be taken into consideration when differences in endothelium-dependent responses between WKY and SHRSP blood vessels are discussed.

Although the concentration–response experiment showed that high acetylcholine concentrations induced only a contractile response in the preparations from SHRSP, as has been reported previously (Watt and Thurston, 1989; Diederich et al., 1990) and as we also reported recently (Sunano et al., 1999), it was shown in the present time course experiment that the high acetylcholine concentration induced both relaxation and contraction in preparations from SHRSP, depending on the time after application. A similar result was reported earlier for the aortae of SHR (Ito et al., 1991), indicating that acetylcholine induces the release of both relaxing (EDRF and EDHF) and contracting factors (EDCFs). In the preparations from WKY, the release of EDCFs, if any, may be so small under control conditions that it cannot be detected mechanically.

The relaxation induced by the low acetylcholine concentration in preparations from both WKY and SHRSP appears to be mediated mainly by EDNO, since it was blocked by L-NOARG, an inhibitor of NO synthesis (Ishii et al., 1990; Moore et al., 1990). The remaining part of the relaxation, especially that induced by the high acetylcholine concentration in the presence of L-NOARG, may be explained by the action of EDHF, since it disappeared in the presence of elevated extracellular K^+ concentrations, where the hyperpolarization of smooth muscle would be blocked (Fujii et al., 1992; Chen and Suzuki, 1989; Chen et al., 1989). Thus, the smaller relaxation in the presence of L-NOARG in the preparations from SHRSP in response to prolonged application of acetylcholine indicates that the release of EDHF was reduced in these preparations, as described below.

The second contraction phase of the response to the high acetylcholine concentration observed in the preparations from SHRSP could be mediated by EDCFs, which is thought to be a product of the arachidonic acid cascade, since it was blocked by an inhibitor of the cyclooxygenase pathway of the arachidonic acid cascade, indomethacin (Mizuno et al., 1982). The inhibition of the second contractile response by indomethacin has also been demonstrated in the aortae of SHR (Ito et al., 1991). Similarly, the second contraction phase in the preparations from WKY observed in the presence of L-NOARG could also be mediated by the EDCFs. We have reported a similar result in a concentration–response experiment showing that the contraction of preparations observed at higher acetylcholine concentrations in the presence of L-NOARG was blocked by indomethacin (Sunano et al., 1999). The inhibition of EDNO release may initiate EDCFs release or remove the depression of the contraction induced by EDCFs; that is, EDNO inhibits the release or the action of EDCFs. In the aortae of SHR, it has been reported that NO inactivates EDCFs (Auch-Schewlk et al., 1992). Since EDCFs released from the aortae of SHR have been shown to be a product of the cyclooxygenase pathway of the arachidonic acid cascade (Lüscher and Vanhoutte, 1986), a similar interaction may occur in the mesenteric artery of SHRSP. In the present experiment, the release of EDNO in the preparations from SHRSP was not markedly altered or rather increased when the release of EDCFs was reduced, since the relaxation in the presence of indomethacin was not greatly reduced.

The relaxation induced by acetylcholine in the preparations from SHRSP observed in the time course experiment was potentiated by indomethacin, as has previously been reported in concentration–response experiments with preparations from SHR and SHRSP (Lüscher and Vanhoutte, 1986; Watt and Thurston, 1989; Sunano et al., 1999). These observations indicate the involvement of EDCFs in the impairment of relaxation in response to

acetylcholine in the preparations from SHRSP. Larger amounts of EDCFs would be released in the preparations from SHRSP than in those from WKY, especially by high acetylcholine concentrations under normal conditions, since the drug induced the second contractile phase, in addition to impairing the relaxation in these preparations. The present results also indicate that both the impairment of relaxation and the second contractile phase of the response to acetylcholine, observed in the time course experiments, were brought about by an increased release of EDCFs, since the second phase disappeared and a relaxation of a similar magnitude to that of preparations from WKY was observed in preparations from SHRSP. In the preparations from WKY, little or no amounts of EDCFs were released in response to acetylcholine under normal conditions, where NO release was not blocked.

It has been reported that the relaxation in response to EDHF in the rat hepatic artery (Zygmunt and Högestätt, 1996) and guinea pig basilar artery (Petersson et al., 1997) could be blocked by a combination of apamin and charybdotoxin, which have been shown to block small and large conductance Ca^{2+}-activated K^+ channels, respectively. The acetylcholine-induced hyperpolarization of the rat mesenteric artery was also blocked by a combination of these agents as described above (Chen and Cheung, 1997). We recently observed in a concentration–response experiment that the relaxation of the rat mesenteric artery, in response to acetylcholine in the presence of L-NOARG and indomethacin, was also blocked by the application of a combination of apamin and charybdotoxin (Sunano et al., 1999). In the present time course experiment, all phases of the relaxation, in response to acetylcholine in both preparations from WKY and SHRSP, were reduced markedly in the presence of a combination of apamin and charybdotoxin, indicating that the relaxation in response to acetylcholine was mediated mainly by EDHF. Although it has been reported that EDNO induces hyperpolarization of the smooth muscle of guinea pig uterine artery (Tare et al., 1990), available reports concerning the rat mesenteric artery agree in that acetylcholine-induced endothelium-dependent hyperpolarization of the smooth muscle is unaffected by the NO synthase inhibitor, L-NOARG (Ghisdal et al., 1999; Chen and Cheung, 1997; Fukao et al., 1997; Bauersaches et al., 1996; Fujii et al., 1992, 1993). Thus, the involvement of EDNO in the relaxation induced by acetylcholine via hyperpolarization may be excluded. The relaxation induced by acetylcholine, especially that of the third phase of the response to acetylcholine observed in the preparations from WKY (Fig. 5), is also partly mediated by EDNO, since it was completely blocked by the addition of L-NOARG (Fig. 7(A)). The observation that the effect of the combination of apamin and charybdotoxin was smaller in the preparations from SHRSP, as compared with that in the preparations from WKY, indicated that the release of EDHF was reduced in the preparations from SHRSP. In support of this, Fujii et al. (1992, 1993) reported that the endothelium-dependent hyperpolarization induced by acetylcholine was reduced in preparations from SHR as compared with those from WKY.

The results of the time course experiments, showing that there is a time lag in the release or the action of EDNO, EDCFs and EDHF, should be emphasized. Both EDNO and EDHF induced a sustained relaxation, although they were involved in the first rapid and subsequent sustained relaxation differently. EDCFs are released with a delay relative to the release of EDNO and EDHF and the release is transient, as indicated by the results of the time course experiments for the second transient contraction in SHRSP preparations and L-NOARG-treated WKY preparations (the second contractile response).

In the present experiments with prolonged application of acetylcholine, it was clearly shown that the response to one factor was affected by other factors; such interactions among factors have been suggested previously by Auch-Schewlk et al. (1992) and Bauersaches et al. (1996). Differences in the interactions between mesenteric arteries from normotensive and hypertensive rats have also been suggested (Randall and March, 1998). However, further detailed studies are required to elucidate the interactions among these factors.

The acetylcholine-induced contraction of preparations observed in the presence of L-NOARG, indomethacin and a combination of apamin and charybdotoxin may be mediated by the release of endothelin from the endothelium, since no contraction was observed in endothelium-denuded preparations or in the presence of BQ-123 (Ihara et al., 1992). The smaller contraction induced by endothelin in the preparations from SHRSP was in agreement with results reported previously (Touyz et al., 1995).

In conclusion, the results of time course experiments with the mesenteric arteries of rats showed that the release of endothelium-derived factors (EDNO, EDCFs and EDHF) is dependent not only on the concentration but also on the time after application of acetylcholine. In the preparations from SHRSP, the release of EDNO was not markedly altered and the release of EDCFs was increased, while that of EDHF was reduced. In addition, there were interactions between these factors and the mode of the interactions was altered in the preparations from SHRSP.

References

Auch-Schewlk, W., Katusic, Z.S., Vanhoutte, P.M., 1992. Nitric oxide inactivates endothelium-derived contracting factor in the rat aorta. Hypertension 19, 442–445.

Bauersaches, J., Popp, R., Hecher, M., Sauer, E., Fleming, I., Busse, R., 1996. Nitric oxide attenuates the release of endothelium-derived hyperpolarizing factor. Circulation 94, 3341–3347.

Bennett, M.A., Hillier, C., Thurston, H., 1996. Endothelium-dependent relaxation in resistance arteries from spontaneously hypertensive rats: effect of long-term treatment with perindopril, quinapril, hydralazine or amlodipine. J. Hypertens. 14, 389–397.

Chen, G., Cheung, D.W., 1997. Effect of K^+-channel blockers on

ACh-induced hyperpolarization and relaxation in mesenteric arteries. Am. J. Physiol. 272, H2306–H2312 (Heart Circ. Physiol., 41).

Chen, G., Suzuki, H., 1989. Some electrical properties of the endothelium-dependent hyperpolarization recorded from rat arterial smooth muscle cells. J. Physiol. 410, 91–106.

Chen, G., Hashitani, H., Suzuki, H., 1989. Endothelium-dependent relaxation and hyperpolarization of canine coronary artery smooth muscles in relation to the electrogenic Na–K pump. Br. J. Pharmacol. 98, 950–956.

Diederich, D., Yang, Z., Bühler, F.R., Lüscher, T.F., 1990. Impaired endothelium-dependent relaxations in hypertensive resistance arteries involve cyclooxygenase pathway. Am. J. Physiol. 258, H445–H451.

Fiscus, R.R., Gross, D.R., Hao, H., Wang, X., Arden, W.A., Maley, R.H., Salley, R.K., 1992. N^{ω}-nitro-L-arginine blocks the second phase but not the first phase of the endothelium-dependent relaxations induced by substance P in isolated rings of pig carotid artery. J. Cardiovasc. Pharmacol. 20, S105–S108.

Fujii, K., Tominaga, M., Ohmori, S., Kobayashi, K., Koga, T., Takata, Y., Fujishima, M., 1992. Decreased endothelium-dependent hyperpolarization to acetylcholine in smooth muscle of the mesenteric artery of spontaneously hypertensive rats. Circ. Res. 70, 660–669.

Fujii, K., Ohmori, S., Tominaga, M., Abe, I., Takata, Y., Ohya, Y., Kobayashi, K., Fujishima, M., 1993. Age-related changes in endothelium-dependent hyperpolarization in the rat mesenteric artery. Am. J. Physiol. 265, H509–H516 (Heart Circ. Physiol., 34).

Fukao, M., Hattori, Y., Kannno, M., Sakuma, I., Kitabatake, A., 1997. Evidence against a role of cytochrome *P*450-derived arachidonic acid metabolites in endothelium-dependent hyperpolarization by acetylcholine in rat isolated mesenteric artery. Br. J. Pharmacol. 120, 439–446.

Furchgott, R.F., Vanhoutte, P.M., 1989. Endothelium-derived relaxing and contracting factor. FASEB J. 3, 2007–22018.

Ghisdal, P., Godfraind, T., Morel, N., 1999. Effect of nitro-L-arginine on electrical and mechanical responses to acetylcholine in the superior mesenteric artery from stroke-prone hypertensive rat. Br. J. Pharmacol. 128, 1513–1523.

Grunfeld, S., Hamilton, C.A., Mesaros, S., McClain, S.W., Dominiczac, A.F., Bohr, D.F., Malinski, T., 1995. Role of superoxide in the depressed nitric oxide production by the endothelium of genetically hypertensive rats. Hypertension 26, 854–857.

Hirata, Y., Hayakawa, H., Kakoki, M., Tojo, A., Suzuki, E., Kimura, K., Goto, A., Kikuchi, K., Nagano, T., Hirobe, M., Omata, M., 1996. Nitric oxide release from kidneys of hypertensive rats treated with imidapril. Hypertension 27, 672–678.

Ihara, M., Noguchi, K., Saeki, T., Fukuroda, T., Tsuchida, S., Kimura, S., Fukami, T., Ishikawa, K., Nishikibe, M., Yano, M., 1992. Biological profiles of highly potent novel endothelin antagonists selective for the ET_A receptor. Life Sci. 50, 247–255.

Ishii, K., Chang Jr., B., Kerwin, J.F., Huang, Z.-J., Murad, F., 1990. N^{ω}-nitro-L-arginine: a potent inhibitor of endothelium-derived relaxing factor formation. Eur. J. Pharmacol. 176, 219–223.

Ito, T., Kato, T., Iwama, Y., Muramatsu, M., Shimizu, K., Asano, H., Okumura, K., Hashimoto, H., Satake, T., 1991. Prostaglandin H_2 as an endothelium-derived contracting factor and its interaction with endothelium-derived nitric oxide. J. Hypertens. 9, 729–736.

Lüscher, T.F., Vanhoutte, P.M., 1986. Endothelium-dependent contractions to acetylcholine in the aorta of the spontaneously hypertensive rat. Hypertension 8, 344–348.

Mizuno, K., Yamamoto, S., Lands, W.E.M., 1982. Effects of non-steroidal anti-inflammatory drugs on fatty acid cyclooxygenase and prostaglandin hydroperoxidase activities. Prostaglandins 23, 743–757.

Moore, P.K., al-Swayeh, O.A., Chong, N.W.S., Evans, R.A., Gibson, A., 1990. L-N^{G}-nitro arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation in vitro. Br. J. Pharmacol. 99, 408–412.

Mulvany, M.J., Halpern, W., 1977. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. Circ. Res. 41, 19–26.

Nagao, T., Illiano, S., Vanhoutte, P.M., 1992. Heterogeneous distribution of endothelium-dependent relaxations resistant to N^{G}-nitro-L-arginine in rats. Am. J. Physiol. 263, H1090–H1094.

Okamoto, K., Yamori, Y., Nagaoka, A., 1974. Establishment of the stroke-prone spontaneously hypertensive rat (SHR). Circ. Res. 34–35, 143–153 (Suppl.).

Osugi, S., Shimamura, K., Sunano, S., 1990. Decreased modulation by endothelium of noradrenaline-induced contractions in aorta from stroke-prone spontaneously hypertensive rats. Arch. Int. Pharmacodyn. Ther. 305, 86–99.

Parkington, H.C., Tare, M., Tonta, M.A., Coleman, H.A., 1993. Stretch revealed three components in the hyperpolarization of guinea pig coronary artery in response to acetylcholine. J. Physiol. 465, 459–476.

Pearson, P.J., Vanhoutte, P.M., 1993. Vasodilator and vasoconstrictor substances produced by the endothelium. Rev. Physiol., Biochem. Pharmacol. 122, 1–67.

Petersson, J., Zygmunt, P.M., Högestätt, E.D., 1997. Characterization of the potassium channels involved in EDHF-mediated relaxation in cerebral arteries. Br. J. Pharmacol. 120, 1344–1350.

Randall, M.D., March, J.E., 1998. Characterization of endothelium-dependent relaxations in mesenteries from transgenic hypertensive rats. Eur. J. Pharmacol. 358, 31–40.

Rubanyi, G.M., Lorenz, R.R., Vanhoutte, P.M., 1985. Bioassay of endothelium-derived relaxing factor(s): inactivation by catecholamines. Am. J. Physiol. 249, H95–H101.

Rubanyi, G.M., Mckinney, M., Vanhoutte, P.M., 1987. Biphasic release of endothelium-derived relaxing factor(s) by acetylcholine from perfused canine femoral arteries. Characterization of muscarinic receptors. J. Pharmacol. Exp. Ther. 240, 802–808.

Sunano, S., Watanabe, H., Tanaka, S., Sekiguchi, F., Shimamura, K., 1999. Endothelium-derived relaxing, contracting and hyperpolarizing factors of mesenteric arteries of hypertensive and normotensive rats. Br. J. Pharmacol. 126, 709–716.

Tare, M., Parkington, H.C., Coleman, H.A., Neild, G.J., Dusting, G.J., 1990. Hyperpolarization and relaxation of arterial smooth muscle caused by nitric oxide derived from endothelium. Nature 346, 69–71.

Touyz, R.M., Lariviére, R., Schiffrin, E.L., 1995. Endothelin receptor subtypes in mesenteric vascular smooth muscle cells of spontaneously hypertensive rats. Can. J. Physiol. Pharmacol. 73, 1262–1273.

Vanhoutte, P.M., 1989. Endothelium and control of vascular function. State of the art lecture. Hypertension 13, 658–667.

Vanhoutte, P.M., Rubanyi, G.M., Miller, V.M., Houston, D.S., 1986. Modulation of vascular smooth muscle contraction by the endothelium. Annu. Rev. Physiol. 48, 307–320.

Watt, P.A.C., Thurston, H., 1989. Endothelium-dependent relaxation in resistance vessels from the spontaneously hypertensive rats. J. Hypertens. 7, 661–666.

Zygmunt, P.M., Högestätt, E.D., 1996. Role of potassium channels in endothelium-dependent relaxation resistant to nitroarginine in the rat hepatic artery. Br. J. Pharmacol. 117, 1600–1606.