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Mechanism of endothelium-dependent relaxation induced by thrombin in the pig coronary artery

Osamu Mizuno [a], Katsuya Hirano [a], Junji Nishimura [a], Chiharu Kubo [b], Hideo Kanaide [a,*]

[a] *Division of Molecular Cardiology, Research Institute of Angiocardiology, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*

[b] *Department of Psychosomatic Medicine, Faculty of Medicine, Kyushu University, Fukuoka 812-8582, Japan*

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Abstract

The mechanism of thrombin-induced endothelium-dependent relaxation was investigated using fura-2 front-surface fluorometry. Thrombin induced an endothelium-dependent relaxation during U46619-induced contractions in pig coronary arterial strips. The relaxation consisted of two components: the early phasic component with a transient decrease in $[Ca^{2+}]_i$ of smooth muscle and the subsequent sustained tonic component without $[Ca^{2+}]_i$ decrease. The phasic relaxation was inhibited by a combination of N^{ω}-nitro-L-arginine and K^+-depolarization, while the tonic component was inhibited by either indomethacin or K^+-depolarization. Thrombin induced a transient $[Ca^{2+}]_i$ increase and nitric oxide (NO) production in pig aortic valvular endothelial cells, which expressed NO synthase as determined by reverse transcription and polymerase chain reaction. Thus, it was concluded that NO and hyperpolarizing factor were involved in the phasic component of thrombin-induced relaxation and that hyperpolarizing factor and prostacyclin were involved in the tonic component.

Keywords: Thrombin; Vasorelaxation; Fura-2; Nitric oxide (NO) synthase, endothelial constitutive; Aortic valvular endothelial cell; Ca^{2+}, cytosolic

1. Introduction

Thrombin plays a central role in homeostasis and acts as a serine protease that converts fibrinogen into fibrin. It is also a potent agonist for many biological responses including platelet aggregation, macrophage chemotaxis, and mitogenesis of lymphocyte, fibroblasts or mesenchymal cells (Shuman, 1986). In the vascular system, thrombin acts as either a vasorelaxant or a vasoconstrictor. Thrombin has been reported to induce the production of endothelium-derived relaxing factor (EDRF), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin in endothelial cells while it also causes endothelium-dependent relaxation (Vanhoutte et al., 1986; Nagao and Vanhoutte, 1992; Lewis and Miller, 1992). It was shown that alterations of the cytosolic calcium concentration, $[Ca^{2+}]_i$, is the primary determinant of the production of EDRF, EDHF and prostacyclin in endothelial cells (Mayer et al., 1989; Chen and Suzuki, 1990; Hallam et al., 1988). The correlation between the $[Ca^{2+}]_i$-transient and the extent of production of each relaxing factor by thrombin is not known. On the other hand, thrombin is a potent and direct vasoconstrictor to smooth muscle cells (Haver and Namm, 1974).

Most of the studies regarding the effect of thrombin on endothelial cells have been performed in cultured endothelial cells. Thrombin increases both the $[Ca^{2+}]_i$ and prostacyclin production in cultured endothelial cells (Jaffe et al., 1987; Hallam et al., 1988). It was suggested, however, that cultured endothelial cells had altered properties as compared with endothelial cells in situ. For example, Del Vecchio and Smith (1981) showed that cultured pulmonary endothelial cells did not express any significant activity of angiotensin-converting enzyme in the growing phase until after cell growth was arrested due to high cell density. The protein composition (Børsum et al., 1982) or membrane permeability (Hennig et al., 1989) of the cultured cells also changed in response to the growth state or culture condition. It thus seems important to elucidate the cellular mechanism of endothelium-dependent relaxation in intact

* Corresponding author. Tel.: +81-92-642-5548; fax: +81-92-642-5552; e-mail: kanaide@molcar.med.kyushu-u.ac.jp

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tissue in situ. In the present study, the effects of thrombin on $[Ca^{2+}]_i$ of smooth muscle and endothelial cells and the mechanism of thrombin-induced endothelium-dependent relaxation in situ were investigated using fura-2 front-surface fluorometry (Kodama et al., 1989; Hirano et al., 1990) for coronary arterial strips (Kuroiwa et al., 1995) and aortic valvular strips (Aoki et al., 1991). The production of nitric oxide, NO, by thrombin and the expression of mRNA of endothelial constitutive NO synthase were also determined in endothelial cells in situ.

2. Materials and methods

2.1. Tissue preparation

For the simultaneous measurement of $[Ca^{2+}]_i$ and tension of smooth muscle, vascular strips with and without an endothelium were prepared from the proximal part of the left circumflex coronary arteries of pig heart obtained at a local slaughterhouse as previously described (Kuroiwa et al., 1993, 1995; Higuchi et al., 1996). Vascular strips measured approximately 1 mm in width $\times$ 5 mm in length $\times$ 0.1 mm in thickness. For the measurement of $[Ca^{2+}]_i$ and NO production of endothelial cells in situ, strips of the pig aortic valve were prepared as previously described (Kuroiwa et al., 1993, 1995; Higuchi et al., 1996). A central part of the valvular leaflet, the corpus arantii, was avoided because this part is the thickest part and not flat enough for optical measurements. Valvular strips for fura-2 fluorometry measured approximately 3 mm in width $\times$ 5 mm in length.

2.2. Simultaneous measurement of $[Ca^{2+}]_i$ in smooth muscle cells and tension of coronary arterial strips with and without endothelial cells

The vascular strips with and without an endothelium were loaded with fura-2-acetoxymethyl ester (fura-2/AM) by incubating them for 3 h at 37°C in gassed (5% CO_2 and 95% O_2) Dulbecco's modified Eagle medium (DMEM) containing 5% fetal bovine serum and 25 μM fura-2/AM as previously described (Kuroiwa et al., 1993). The strips were then washed at least five times to remove any remaining fluorescent dye in the extracellular space, and equilibrated in normal physiological salt solution (PSS) for 1 h at room temperature. The strips were mounted vertically to a force transducer, TB-612T (Nihon Koden, Japan), in a quartz organ bath (37°C) filled with normal PSS gassed with 5% CO_2 and 95% O_2, with the luminal side of the medial layer facing the fiber optics for fluorometry. Changes in the $[Ca^{2+}]_i$ in the vascular strips were monitored as previously described (Kuroiwa et al., 1993), using a front-surface fluorometer, CAM-OF-3, designed in collaboration with the Japan Spectroscopic (Tokyo, Japan). The fluorescence intensities (500 nm) at 340 nm (F340) and 380 nm (F380) excitation and its ratio (R = F340/F380) were continuously monitored. The strips were stimulated with 118-mM K^+ PSS four times during the equilibration period, and the resting tension was adjusted to 200 mg at normal PSS (5.9 mM K^+). The response of each strip to 118-mM K^+-depolarization was then recorded as a control response before starting the experimental protocols. The fluorescence ratio (R = F340/F380) and tension development were expressed as a percentage, assigning the values in normal PSS (5.9 mM K^+) and 118-mM K^+ PSS to be 0% and 100%, respectively. In some experiments where the effects of N^{ω}-nitro-L-arginine (L-NOARG) or indomethacin on thrombin-induced relaxation were examined, the control response to 118-mM K^+-depolarization were recorded in the presence of these regents. The absolute values of $[Ca^{2+}]_i$ were estimated as previously described (Hirano et al., 1990) in separate measurements. The $[Ca^{2+}]_i$ levels at rest (0%) and during 118-mM K^+-depolarization (100%) were 110 ± 5.0 nM and 710 ± 5.0 nM, respectively ($n = 10$). We previously showed evidence that the fura-2/AM fluorescence recorded as above originated exclusively from the smooth muscle cells of the vascular strips despite the presence of the endothelium (Kuroiwa et al., 1993).

2.3. Measurement of $[Ca^{2+}]_i$ in endothelial cells in situ

The endothelial cells in aortic valvular strips were similarly loaded with fura-2/AM as previously described for coronary arterial strips except for the addition of 1 mM probenecid to prevent the leakage of fluorescent dye (Di Virgilio et al., 1989; Kuroiwa et al., 1995) and then were incubated for 90 min with a higher concentration of fura-2/AM (50 μM). After loading with fura-2/AM, the strips were washed at least five times and equilibrated in normal PSS for 1 h at room temperature. The strips were mounted vertically in a quartz organ bath filled with normal PSS gassed with 5% CO_2 and 95% O_2. Using front-surface fluorometry, the measurements were performed at 25°C to prevent the leakage of fluorescent dye (Kuroiwa et al., 1993). The control response to 10 μM ATP was obtained by exposing the strip to ATP for 1 min at least 15 min prior to each experimental measurement (Aoki et al., 1991). The change in the fluorescence ratio ($R = F_{340}/F_{380}$) was expressed as a percentage, assigning the value obtained in normal PSS and with 10 μM ATP to be 0% and 100%, respectively. In separate measurements, the $[Ca^{2+}]_i$ levels of valvular endothelial cells at 0% and 100% were determined to be 70.1 ± 10 nM and 190 ± 30 nM, respectively ($n = 5$).

2.4. Measurement of NO production of the endothelial cells in situ

The free radical NO is rapidly ($\ll 30$ s) oxidized to a stable derivative, nitrite, in a physiological solution (Ro-

bert, 1993). Therefore, the NO production was estimated by measuring the concentration of nitrite in normal PSS with an assay using 2,3-diaminonaphthalene (Misko et al., 1993). The valvular strips (5 mm × 8 mm) were mounted horizontally with pins in a Sylgard chamber (Dow Corning, USA) with 250 μl PSS containing 0.1 mM L-arginine. The application of agonists, ATP and thrombin, was performed by exchanging the whole solution in the chamber. After exposing valvular strips to these agonists for 3 min, 200 μl solution was collected, to which 10 μl of 100 mM EDTA and 20 μl of 0.05 mg/ml 2,3-diaminonaphthalene dissolved in 0.62 M HCl were added. EDTA was added to avoid any possible interference in fluorescence measurement by divalent cation (Damiani and Burini, 1986). The mixture was then incubated at 25°C for 15 min, and the reaction was terminated by the addition of 10 μl of 2.8 M NaOH. The 450 nm fluorescence intensity of the reaction product, 1-(*H*)-naphthotriazole, was measured in a quartz cuvette at 365 nm excitation, using a spectrofluoroscope (Jobin Yvon, France). Standard solutions of sodium nitrite (10 nM–10 μM) were freshly prepared. The fluorescence intensity of standard solutions was determined before each measurement. The level of NO production was expressed as a percentage while assigning the background fluorescence intensity and the peak response obtained with 10 μM ATP to be 0% and 100%, respectively. The basal production of NO was determined by incubating strips in normal PSS for 3 min at rest.

2.5. Culture of pig aortic endothelial cells

Endothelial cells were mechanically obtained from the pig aorta and grown on a 100-mm diameter culture dish (Falcon) in DMEM containing 10% fetal bovine serum and 1% antibiotics as described (Hirano et al., 1993). The cells were trypsinized to dislodge them from the culture dish and then were subcultured. Cells from the primary to 3rd passages were then used to examine the endothelial constitutive NO synthase expression.

2.6. Isolation of total RNA and a reverse transcription and polymerase chain reaction (RT–PCR) analysis of the endothelial constitutive NO synthase mRNA expression

Total RNA was isolated from the endothelium of the aortic side of the aortic valve and cultured endothelial cells from the aorta of primary through 3rd passage, according to the method described by Chomczynki and Sacchi (1987). The endothelial cells of the aortic valve were collected by scraping the aortic side of valvular surface with a scalpel blade. Any possible contaminating genomic DNA was digested with RNase-free DNase (Promega, Madison, WI, USA) in the presence of RNase inhibitor (Toyobo, Osaka, Japan). The method of RT–PCR has already been described (Nishimura et al., 1995; Sakihara et al., 1996). In brief, the first-strand cDNA was synthesized with 200 units Moloney murine leukemia virus reverse transcriptase (BRL, Gaithersburg, USA), 1 μg total RNA, 0.5 mM each dNTPs (dATP, dCTP, dGTP, dTTP), 50 nM RT primer for endothelial constitutive NO synthase, 50 nM RT primer for β-actin, 20 units of RNase inhibitor and 10 mM dithiothreitol (reaction mixture = 20 μl). A 1-μl aliquot of RT product was then subjected to PCR amplification of cDNA of endothelial constitutive NO synthase and β-actin separately. The PCR was performed with *Taq* DNA polymerase (Pharmacia Biotech, Uppsala, Sweden). The cycle number for PCR amplification was selected so as to give the amount of PCR product in a linear range of densitometric scanning (Nishimura et al., 1992). The cycle numbers for endothelial constitutive NO synthase and β-actin were 30 and 25, respectively. The PCR product was separated on 3% agarose gel electrophoresis containing 0.5 μg/ml ethidium bromide, and was recorded on Kodak Tri-X pan 400 and then was quantitated by densitometric scanning of the film with a densitometer CS-9000 (Shimadzu, Japan). The relative level of endothelial constitutive NO synthase expression was expressed as an arbitrary ratio of the density of the endothelial constitutive NO synthase product against that of β-actin product as described (Nishimura et al., 1992).

2.7. Chemicals and solutions

Thrombin (bovine plasma, specific activity 1880 NIH U/mg, 10 nM ≈ 1 U/ml) and probenecid were obtained from Sigma (St. Louis, MO, USA). Sodium salt of ATP was purchased from Boehringer Mannheim (Germany). Fura-2/AM and 2,3-diaminonaphthalene were obtained from Dojindo (Kumamoto, Japan). L-NOARG and indomethacin were purchased from Aldrich Chemical (USA) and Wako (Osaka, Japan), respectively. L-Arginine was obtained from the Ishizu Pharma. (Japan). Fetal bovine serum was purchased from Flow (USA). Dulbecco's modified Eagle's medium and antibiotics were obtained from Gibco (USA). All other chemicals were purchased from Katayama Chemical (Osaka, Japan). Oligonucleotides for primers were synthesized by Sawady Technology (Tokyo, Japan).

The PSS is composed of 123 NaCl, 4.7 KCl, 15.5 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 1.25 $CaCl_2$ and 11.5 D-glucose (in mM).

2.8. Data analysis

All data are the mean ± S.E.M. of the number of experiments. Since one strip obtained from one animal was used for each experiment, the experimental number indicates the number of animals. All values were statistically analyzed by the unpaired Student's *t*-test and an analysis of variance (ANOVA). A P value of less than 0.05 was considered to

be statistically significant. The concentrations for a half-maximal response (EC_{50} or IC_{50}) were determined by the concentration–response curves fitted to a four-parameter logistic model (De Lean et al., 1978). All data were collected at the sampling rate of 17 Hz using a computerized data acquisition system (MacLab, Analog Digital Instruments, Australia: Macintosh, Apple Computer, USA).

3. Results

3.1. Thrombin-induced endothelium-dependent relaxation of the pig coronary arterial strips

The U46619 (a thromboxane A_2 analogue) induced sustained contractions in the pig coronary arterial strips with an endothelium (Fig. 1A). Upon exposure to 100-nM U46619, the $[Ca^{2+}]_i$ and tension of smooth muscle increased rapidly and reached steady state within 10 min ($[Ca^{2+}]_i = 62.5 \pm 2.3\%$, tension $= 101.6 \pm 3.9\%$), ($n = 7$) (Fig. 1B). There was no decrease in the tension during the steady state of U46619-induced contraction, while $[Ca^{2+}]_i$ slightly but significantly decreased ($P < 0.05$). The levels of $[Ca^{2+}]_i$ and tension of the steady state were $55.5 \pm 2.9\%$ and $102.2 \pm 3.3\%$ ($n = 7$) at 30 min after the application of U46619, respectively (Fig. 3, column a).

During the steady state of U46619-induced contraction, 5 U/ml thrombin was applied and a rapid and transient decrease was thus induced in $[Ca^{2+}]_i$ (Fig. 1A). $[Ca^{2+}]_i$ decreased significantly from 67.4 ± 2.7 to $15.9 \pm 4.3\%$ ($P < 0.05$) (Fig. 3, column a, $n = 9$). On the other hand, thrombin induced an early rapid depression of tension (the phasic component of relaxation) with a similar time course to that observed with the decrease in $[Ca^{2+}]_i$, followed by a sustained decrease in tension (the tonic component). The tension decreased significantly from 102.1 ± 3.2 to $31.8 \pm 2.3\%$ ($P < 0.05$) at the phasic component and $91.8 \pm 3.2\%$ ($P < 0.05$) at the tonic component (Fig. 3, column a). Thus, although there was no decrease in $[Ca^{2+}]_i$, a tonic component of relaxation was observed. Although the sustained decrease in tension (the tonic component) was small, it was observed for more than 20 min (Fig. 1A and

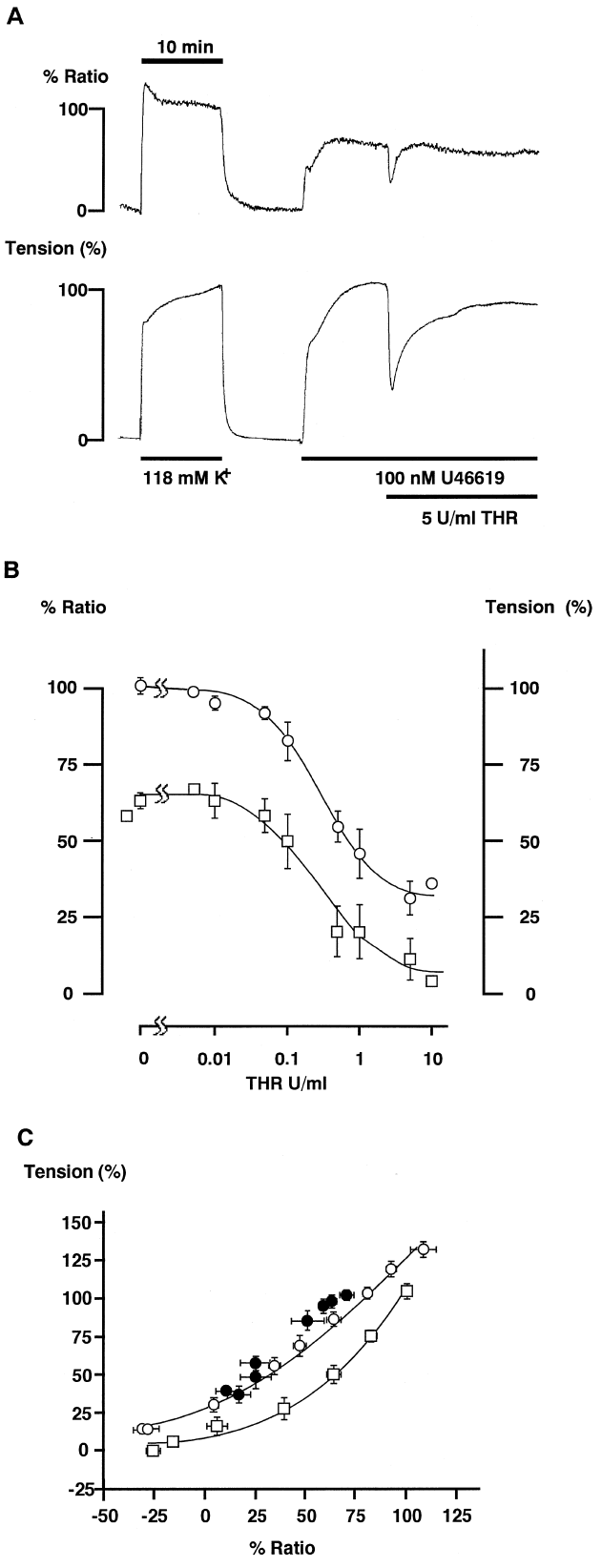

Fig. 1. Thrombin (THR)-induced endothelium-dependent relaxation in the pig coronary arteries. (A) Representative recording showing the effect of THR on $[Ca^{2+}]_i$ and tension in the coronary arterial strips with endothelium precontracted with 100 nM U46619. After recording a control response to 118-mM K^+-depolarization, the strip was precontracted with 100 nM U46619. THR (5 U/ml) was applied 10 min after the application of U46619. (B) The concentration–response curves for the thrombin-induced maximal decrease in $[Ca^{2+}]_i$ (□) and tension (○) during U46619-induced contraction. (C) The $[Ca^{2+}]_i$–tension relationships during the thrombin-induced relaxation. The $[Ca^{2+}]_i$–tension relationships at the phasic component of relaxation induced by various concentrations of thrombin (closed circle) were obtained from the data on the panel (b). The $[Ca^{2+}]_i$–tension curves of the contractions induced by the cumulative application of extracellular Ca^{2+} in the presence of 100 nM U46619 (open circle and line) and 118-mM K^+-depolarization (open square and line) are also shown as references. The data are the mean ± S.E.M. ($n = 6$, $n = 8$, respectively).

Fig. 3, column a). The decreases in $[Ca^{2+}]_i$ and tension were concentration-dependent (Fig. 1B), with IC_{50} for thrombin in inhibition of $[Ca^{2+}]_i$ and tension being 0.22 ± 0.06 U/ml and 0.28 ± 0.03 U/ml, respectively, as determined at the phasic component ($n \geq 4$). Thrombin did not induce relaxation during U46619-induced contraction in the strips without an endothelium. However, 5 U/ml thrombin induced additional small increase in $[Ca^{2+}]_i$ and tension development ($[Ca^{2+}]_i$ from $69.7 \pm 5.7\%$ to $75.0 \pm 9.1\%$; tension from $98.5 \pm 3.1\%$ to $100.8 \pm 4.7\%$, respectively; $n = 7$) in the strips without an endothelium.

The $[Ca^{2+}]_i$–tension relationship during the thrombin-induced relaxation was evaluated by plotting the levels of tension as a function of $[Ca^{2+}]_i$ (Fig. 1C). In this measurement, the $[Ca^{2+}]_i$–tension relationships of the control contraction induced by U46619 were obtained from the levels of $[Ca^{2+}]_i$ and tension induced by the cumulative applications of extracellular Ca^{2+} from 0 mM to 2.5 mM in the presence of 100 nM U46619 (Fig. 1C). This procedure caused stepwise increases in $[Ca^{2+}]_i$ and tension along with stepwise increases in extracellular Ca^{2+} (data not shown). Similarly, the $[Ca^{2+}]_i$–tension relationship of the contraction induced by 118-mM K^+-depolarization was obtained by increasing the concentrations of extracellular Ca^{2+} during 118-mM K^+-depolarization (Fig. 1C). The $[Ca^{2+}]_i$–tension relationships at the phasic component of relaxation induced by various concentrations of thrombin were obtained from data shown in Fig. 1b, which fell on the control $[Ca^{2+}]_i$–tension curve of the U46619-induced contraction (Fig. 1C). During the tonic component of thrombin-induced relaxation, the tension decreased from the level of control contraction induced by U46619 with no decrease in $[Ca^{2+}]_i$, thus indicating that the $[Ca^{2+}]_i$–tension relationships had shifted to the right (data not shown).

To determine the relative contribution of EDRF/NO, prostacyclin and EDHF in thrombin-induced relaxation, the pig coronary arteries were thus pretreated with their specific inhibitors, 100 μM L-NOARG, 10 μM indomethacin and 118-mM K^+-depolarization, respectively. The representative recordings and summaries of at least four different experiments are shown in Figs. 2 and 3, respectively. Treatment with 100 μM L-NOARG alone did not affect the level of $[Ca^{2+}]_i$ and tension at rest. However, the tension induced by 118-mM K^+-depolarization in the presence of 100 μM L-NOARG was $119.6 \pm 6.1\%$ of and significantly larger ($P < 0.05$) than that obtained in the absence of L-NOARG. This, thus, indicated a basal release of NO although it was only to a small extent. During the relaxation induced by 5 U/ml thrombin in the presence of L-NOARG during 100 nM U46619-induced contraction, $[Ca^{2+}]_i$ and tension decreased significantly from $71.1 \pm 6.2\%$ to $38.4 \pm 2.5\%$ ($P < 0.05$) and from $97.6 \pm 5.1\%$ to $65.3 \pm 4.7\%$ ($P < 0.05$) at the phasic component, respectively (Fig. 2A and Fig. 3, column b, $n = 7$). The level of $[Ca^{2+}]_i$ and tension of the tonic component

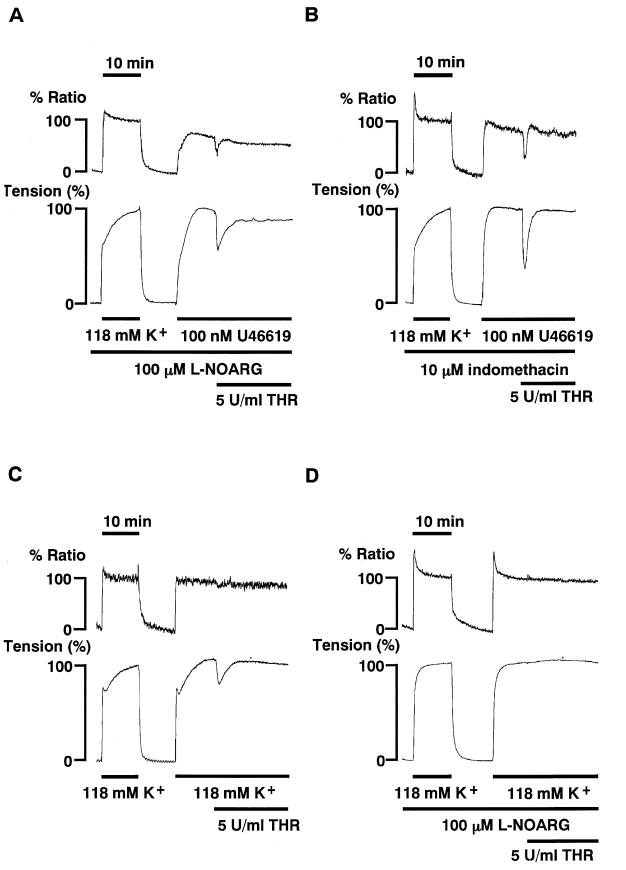

Fig. 2. Effects of N^{ω}-nitro-L-arginine (L-NOARG), indomethacin and membrane depolarization on $[Ca^{2+}]_i$ and tension of the coronary arterial smooth muscle during relaxation induced by thrombin (THR). Representative recordings of at least four independent experiments. THR was applied during U46619-induced contractions in the presence of 100 μM L-NOARG (A) and 10 μM indomethacin (B). Thrombin was applied during contractions induced by 118-mM K^+-depolarization in the absence (C) and the presence of 100 μM L-NOARG (D). The control response to 118-mM K^+-depolarization was recorded at the beginning of each experimental protocol to show the 100% level of $[Ca^{2+}]_i$ and tension.

observed in the presence of L-NOARG was $57.7 \pm 3.9\%$ and $90.9 \pm 3.0\%$, respectively. This level of tension was significantly lower than that obtained during the control contraction induced by U46619 ($P < 0.05$) (Fig. 3, column b). Namely, thrombin induced a transient and subsequent sustained relaxation in the presence of L-NOARG. On the other hand, pretreatment with 10 μM indomethacin had no effect on the decreases in $[Ca^{2+}]_i$ and tension of the phasic component but inhibited the decrease in tension of the tonic component of relaxation induced by 5 U/ml thrombin during 100 nM U46619-induced contraction ($P < 0.05$) (Fig. 2B and Fig. 3, column c, $n = 6$). All recordings showed transient relaxation by thrombin in the presence of indomethacin. There was also no statistically significant decrease in tension at 20 min after the application of thrombin compared with the level of control contraction (Fig. 3, column c). Indomethacin alone had no effect on the tension at rest and the extent of tension development

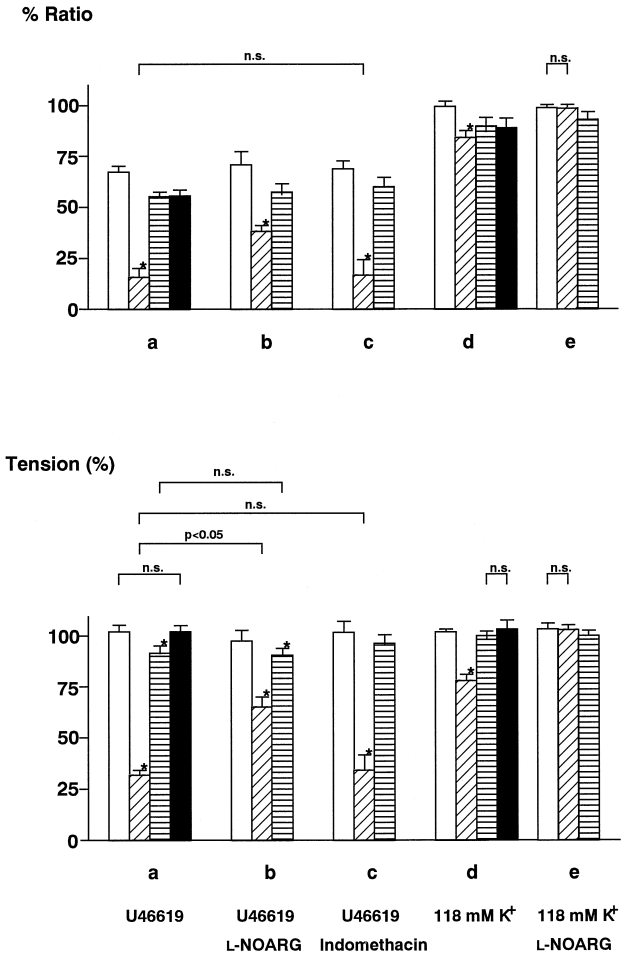

Fig. 3. Summaries of the change in $[Ca^{2+}]_i$ (upper panel) and tension (lower panel) during the thrombin-induced relaxations in different conditions, as shown in Fig. 2. Precontractions were induced by 100 nM U46619 (a, b, c) or 118-mM K^+-depolarization (d, e) in the absence (a, d) or the presence of 100 μM N^{ω}-nitro-L-arginine (L-NOARG) (b, e) or 10 μM indomethacin (c). Open columns, the levels of $[Ca^{2+}]_i$ and tension observed during precontraction just before the application of thrombin. Hatched columns, the levels of $[Ca^{2+}]_i$ and tension observed at the maximum relaxations induced by 5 U/ml thrombin (the phasic component of relaxation). Striped columns, the levels of $[Ca^{2+}]_i$ and tension observed at 20 min after application of 5 U/ml thrombin (the tonic component of relaxation). Filled columns, the levels of $[Ca^{2+}]_i$ and tension of control contraction observed at 30 min after stimulation by 100 nM U46619 and 118-mM K^+-depolarization, respectively. The asterisks at the hatched columns indicate significant differences ($P < 0.05$) from the levels of precontraction just before the application of thrombin. The asterisks at the striped columns indicate significant differences ($P < 0.05$) from the levels of control contraction under each experimental condition; ns: not significant. The data are the mean ± s.e.m. ($n \geq 4$).

induced by 118-mM K^+-depolarization. The thrombin-induced relaxation was next examined during the contraction induced by 118-mM K^+-depolarization, which produced similar force development to that obtained with 100 nM U46619. During the steady state of contraction induced by 118-mM K^+-depolarization, 5 U/ml thrombin caused significant decreases in the $[Ca^{2+}]_i$ and tension. These decreases were transient and there was no sustained component of relaxation. $[Ca^{2+}]_i$ decreased significantly from 99.3 ± 2.6% to 84.1 ± 3.3% ($P < 0.05$) and the tension decreased from 101.9 ± 1.2% to 78.1 ± 3.1% ($P < 0.05$) at the phasic component (Fig. 2C and Fig. 3, column d, $n = 7$). Thus, the extents of decreases in $[Ca^{2+}]_i$ and tension were smaller than those observed during the U46619 precontraction. This thrombin-induced transient relaxation during the contraction induced by 118-mM K^+-depolarization was completely abolished by pretreatment with 100 μM L-NOARG (Fig. 2D and Fig. 3, column e, $n = 6$). Thrombin also induced only a transient decrease in $[Ca^{2+}]_i$ and tension during the precontraction induced by 40-mM K^+-depolarization (data not shown). These results indicated that the EDHF was involved in thrombin-induced relaxation.

3.2. Effects of thrombin on $[Ca^{2+}]_i$ and NO production in endothelial cells in situ

After recording the 100% level of $[Ca^{2+}]_i$ response to 10 μM ATP in normal PSS, thrombin was applied, which induced a rapid and transient increase in $[Ca^{2+}]_i$ in the endothelial cells of aortic valvular strips in a concentration-dependent manner (Fig. 4). At 6 U/ml thrombin in normal PSS, $[Ca^{2+}]_i$ showed a maximum response of 30.1 ± 2.9%, which corresponded to an increase in the absolute $[Ca^{2+}]_i$ level from 70.1 nM to 101 nM. The EC_{50} for an increase in $[Ca^{2+}]_i$ was 0.69 ± 0.05 U/ml.

To determine the concentration dependency of the effect regarding thrombin on NO production, we examined it at three concentration levels: 1 U/ml (a value, about the same as EC_{50} for an increase in $[Ca^{2+}]_i$), 6 U/ml (a value to induce the maximum response of $[Ca^{2+}]_i$ elevation), and 50 U/ml (the maximal concentration of thrombin solution

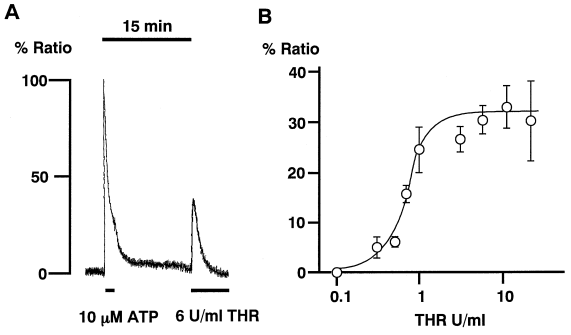

Fig. 4. $[Ca^{2+}]_i$ transients induced by thrombin in pig aortic valvular endothelial cells in situ. (A) Representative recording of changes in $[Ca^{2+}]_i$ induced by 10 μM ATP and 6 U/ml thrombin. (B) The concentration–response curve for thrombin-induced $[Ca^{2+}]_i$ transients of the endothelial cells. The data are the mean ± s.e.m. of four measurements. The fluorescence ratio was expressed as a percentage, thus assigning the levels obtained in normal physiological salt solution and that at the maximum elevation by 10 μM ATP to be 0% and 100%, respectively.

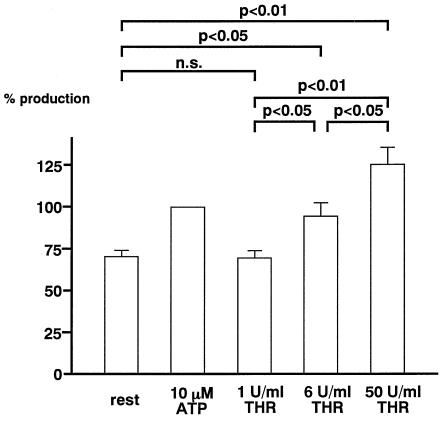

Fig. 5. Nitric-oxide production by pig aortic valvular endothelial cells. The production of nitric oxide was expressed as a percentage, while assigning the levels obtained in the buffer without incubating valvular strips and those obtained in the buffer while exposing the valvular strips to 10 μM ATP for 3 min to be 0% and 100%, respectively. The basal production (rest) was obtained by incubating the valvular strips in normal physiological salt solution (PSS) with no stimulation. The NO production was measured in response to 1, 6 and 50 U/ml thrombin (THR) in normal PSS. The data are the mean ± s.e.m. of four measurements.

obtained using Sigma thrombin preparations). The production of NO by valvular endothelial cells in the normal PSS without stimulation (basal release) was 70.7 ± 3.5% of NO production by 10 μM ATP. Thrombin induced NO production in a concentration-dependent manner at concentrations more than 1 U/ml (Fig. 5). The NO production observed with 1 U/ml thrombin was the same as the basal level of NO production. The NO production obtained with 6 U/ml and 50 U/ml was 94.6 ± 7.8% and 125.3 ± 10.2% of that obtained with 10 μM ATP, respectively. Thus, there was a discrepancy in the concentrations of thrombin required to induce NO production from those required to induce $[Ca^{2+}]_i$ transient of aortic valvular endothelial cells and endothelium-dependent relaxation in vascular strips. It should also be noted that thrombin at concentrations higher than 6 U/ml induced similar or greater NO production but much smaller $[Ca^{2+}]_i$ transient than 10 μM ATP (Figs. 4 and 5).

3.3. Expression of endothelial constitutive type of nitric oxide synthase

Using RT–PCR analysis, the level of expression of endothelial constitutive NO synthase was compared between the endothelial cells of aortic valve in situ and cultured cells. Fig. 6 shows the RT–PCR product separated on 3% agarose gel. Endothelial cells in situ showed a positive expression of endothelial constitutive NO synthase. The primary cultured endothelial cells also showed a positive endothelial constitutive NO synthase expression, but at a decreased level compared with that observed in situ. When the cells were subcultured, the level of expression was lower than that of the primary cultured cells. The ratios of endothelial constitutive NO synthase product to β-actin product determined by densitometric scan of the negative film of five separate measurements were 1.40 ± 0.10 (aortic valve in situ), 0.62 ± 0.04 (primary culture), 0.58 ± 0.06 (2nd passage) and 0.55 ± 0.08 (3rd passage).

4. Discussion

Thrombin has been shown to induce endothelium-dependent relaxation in many types of vascular tissue (Vanhoutte et al., 1986; Glusa and Markward, 1988; Tesfamariam et al., 1993). The EDRF/NO, EDHF and prostacyclin were suggested to be possible mediators of thrombin-induced endothelium-dependent relaxation in vascular smooth muscle (Vanhoutte et al., 1995; Nagao and Vanhoutte, 1992; Lewis and Miller, 1992). In the present study, thrombin induced an endothelium-dependent relaxation in the pig coronary artery. The thrombin-induced relaxation during U46619-induced contraction was composed of two phases: the phasic component was an early rapid depression of tension, and the tonic component was a subsequent sustained decrease in tension. We found the temporal changes in contribution of these three relaxing factors during thrombin-induced relaxation during

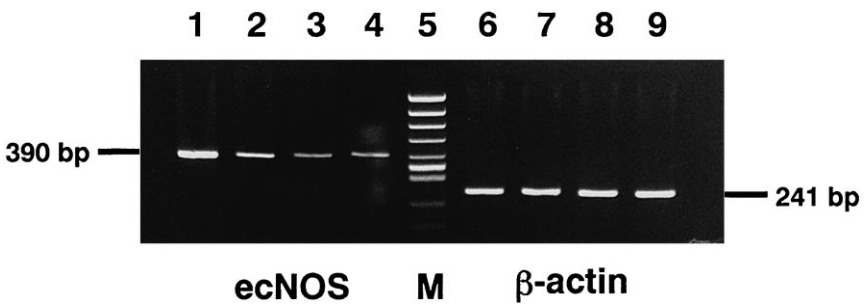

Fig. 6. The RT–PCR analysis of the expression of endothelial constitutive nitric oxide synthase (ecNOS) in endothelial cells of aortic valve and cultured endothelial cells. A representative pattern of 3% agarose gel (containing 0.5 μg/ml ethidium bromide) electrophoresis of RT–PCR products. An amount of 1 μg of total RNA obtained from the endothelial cells of the aortic valve (lanes 1 and 6) and cultured aortic endothelium of primary culture (2 and 7), 2nd passage (3 and 8) and 3rd passage (4 and 9) was analyzed with RT–PCR using a specific primer for ecNOS (lanes 1–4) and β-actin (lanes 6–9). Lane 5, *Hin*cII-digest of ØX174 DNA. The size of the PCR product for ecNOS (390 bp) and β-actin (241 bp) was indicated on the left and right of the gel, respectively.

U46619-induced contraction by examining the effects of inhibitor specific to each factor on the two components of relaxation. It was suggested that EDRF/NO and EDHF were the major mediators of the phasic component of thrombin-induced relaxation and that prostacyclin and/or EDHF were important in the tonic component of relaxation. The EDRF/NO plays, if any, only a minor role in the tonic component.

The phasic component of relaxation was associated with the decreases in $[Ca^{2+}]_i$. On the contrary, it was obvious that the relaxation during the sustained phase was not accompanied by the decrease in $[Ca^{2+}]_i$, suggesting that the decrease in $[Ca^{2+}]_i$-sensitivity of contractile apparatus was the major mechanism of this component of relaxation. During the phasic component of relaxation, there was apparently no difference in the $[Ca^{2+}]_i$–tension relationship from that obtained with the control contraction induced by U46619 (Fig. 1C). It is thus suggested that thrombin induces endothelium-dependent relaxation by decreasing $[Ca^{2+}]_i$-sensitivity of contractile apparatus, as well as $[Ca^{2+}]_i$ in smooth muscle cells. The Ca^{2+}-desensitizing mechanism is important especially in the tonic component of relaxation. It was shown that NO and prostacyclin increased the intracellular cGMP and cAMP, respectively (Vane et al., 1990). It is known that the $[Ca^{2+}]_i$-sensitivity of contraction decreases during the relaxation mediated by cAMP or cGMP (Nishimura and van Breemen, 1989). It is unlikely that EDHF reduces the $[Ca^{2+}]_i$-sensitivity of the contractile apparatus (Ushio-Fukai et al., 1994). Therefore, in the thrombin-induced relaxation, the Ca^{2+}-desensitization of contractile apparatus was mediated by NO and prostacyclin.

There are reports showing that thrombin induced production of not only NO but also prostacyclin in the cultured endothelial cells (Förstermann et al., 1991; Tsukahara et al., 1993; Hirata et al., 1995; Jaffe et al., 1987; Hallam et al., 1988). On the contrary, most studies on the tension measurements showed that indomethacin had no effect or only a slight effect on thrombin-induced endothelium-dependent relaxation, thereby excluding the major contribution of prostacyclin to the relaxation in situ (Glusa and Markward, 1988; Tomita et al., 1990; Tesfamariam et al., 1993). In the present study, it was suggested that prostacyclin was a mediator of the tonic component of relaxation induced by thrombin in the pig coronary artery. The contribution of prostacyclin, however, was small and had little effect on the phasic component. In cultured bovine aortic endothelial cells of eight passages, Parsaee et al. (1992) showed that the level of $[Ca^{2+}]_i$ elevation (350 nM) required to induce prostacyclin production was higher than that required to induce NO production (200 nM), despite the fact that the production of NO and prostacyclin are both dependent on Ca^{2+} in cultured endothelial cells (Jaffe et al., 1987; Hallam et al., 1988; Parsaee et al., 1992). In the present study, thrombin induced small transient increases in $[Ca^{2+}]_i$ in valvular endothelial cells in situ, the level of which was less than 190 nM. Furthermore, endothelial cells in situ may have a preference for NO production or may be less developed in prostacyclin production due to differences in the gene expression pattern compared with cultured cells. Therefore, prostacyclin played only a minor role in thrombin-induced relaxation in pig coronary artery.

Using fluorometry with 2,3-diaminonaphthalene, the present study directly confirmed the NO production both at rest (a basal release) and in response to thrombin in the valvular endothelial cells in situ, as previously reported in cultured endothelial cells (Jaffe et al., 1987; Kruse et al., 1994, 1995; Lerner, 1994; Förstermann et al., 1991; Tsukahara et al., 1993). The finding that the treatment with L-NOARG per se enhanced tension development in pig coronary arterial strips was consistent with a basal release of NO observed in the valvular endothelial cells. As evaluated in Figs. 2 and 3, NO or related compound were involved mainly in the phasic component of the thrombin-induced relaxation but not in the tonic component where prostacyclin or EDHF played a major role. These observations suggested that the production of NO was phasic while the production of prostacyclin or EDHF were tonic. On the other hand, thrombin induced a transient elevation of $[Ca^{2+}]_i$. A correlation might possibly exist between the thrombin-induced elevation of $[Ca^{2+}]_i$ and the NO production. Previous reports have shown endothelial constitutive NO synthase to be a major enzyme involved in NO production in endothelial cells, while the enzymatic activity was shown to be dependent on Ca^{2+} and calmodulin (Mayer et al., 1989; Parsaee et al., 1992; Korenaga et al., 1993; Saito et al., 1996). The Ca^{2+} signalling is therefore an important determinant of NO production in endothelial cells. However, we found that thrombin induced a greater production of NO with a smaller increase in $[Ca^{2+}]_i$ than ATP (Fig. 4A and Fig. 5). This finding suggested that the changes in the Ca^{2+}-sensitivity of process of NO production, as well as changes in $[Ca^{2+}]_i$, might also be important in the regulation of NO production. The Ca^{2+}-sensitivity of NO production varied with the type of stimulation. Phosphorylation of endothelial constitutive NO synthase induced by phorbol ester or at tyrosine residue inhibited enzyme activity in vivo, while phosphorylation by cAMP-dependent kinase and protein kinase C in vitro had no effect (Hirata et al., 1995; Ohara et al., 1995; García-Cardeña et al., 1996). W7, a calmodulin inhibitor, decreased NO release (Saito et al., 1996). Alteration of the state of phosphorylation of endothelial constitutive NO synthase and Ca^{2+}/calmodulin pathway could be a mechanism to modulate Ca^{2+}-sensitivity of enzymatic activity in the NO production induced by thrombin. Thrombin digests its receptor to produce ‘tethered ligand’ (Vu et al., 1991), which induced subsequent signal transduction to activate phospholipase C through a specific G protein and to generate inositol 1,4,5-trisphosphate and diacylglycerol (Brock and Capasso, 1988; Stasek and Garcia, 1992).

Endothelial cells also express a specific receptor for ATP (P_{2Y} and P_{2U}) which also activates phospholipase C (Dubyak and El-Moatassim, 1993). The regulatory mechanism of Ca^{2+}-sensitivity of NO production by thrombin and ATP, however, remains to be elucidated.

There was an apparent difference in EC_{50} of thrombin required to induce relaxation from that required to induce NO production. This apparent difference may have originated from a difference in the condition or sensitivity of measurement, namely the fluorescence measurement of NO vs. the bioassay assessment of EDRF. The detection limit of the fluorescence measurement of NO is reported to be 10 nM (Misko et al., 1993). Myers et al. (1989) measured the NO production of endothelial cells by using a chemiluminescence technique and found that the concentration of NO generated by endothelial cells in response to bradykinin and A23187 was 7–10-fold less than that required to account for the relaxation observed in bioassay preparations. It is still controversial as to whether or not free NO is identical to EDRF, and EDRF has also been suggested to be a compound containing NO or another nitrogen-based precursor of NO, such as *S*-nitroso-L-cystein (Johns, 1991; Rubanyi, 1991), which may not be detected by a fluorescence assay for NO. Thrombin may induce such EDRFs in addition to NO.

We showed that the endothelium of the aortic valve had an active uptake of acetylated LDL (Kuroiwa et al., 1995). In the present study, RT–PCR analysis also revealed an expression of endothelial constitutive NO synthase in the endothelium of aortic valve. These observations thus suggest that the endothelial cells in the aortic valve exhibit characteristics similar to those of arterial endothelial cells. On the other hand, some properties of cultured endothelial cells also differed from arterial endothelial cells in situ even though the former were derived from the latter. The activity of angiotensin-converting enzyme, protein composition or cell permeability also changed with the culture conditions (Del Vecchio and Smith, 1981; Børsum et al., 1982; Hennig et al., 1989). In the present study, 10 μM ATP induced a larger $[Ca^{2+}]_i$ transient than 5 U/ml thrombin in the endothelium in situ. On the other hand, cultured valvular endothelial cells of 3rd–4th passage induced a larger increase in $[Ca^{2+}]_i$ in response to thrombin than ATP (data not shown). In addition, RT–PCR analysis showed the endothelial constitutive NO synthase expression level to decrease in cultured cells. These findings thus emphasize the importance of assessing the function of endothelial cells in situ to examine its physiological significance.

5. Conclusion

In summary, thrombin caused an endothelium-dependent relaxation of the pig coronary artery. The relaxation during U46619-induced contraction was composed of two phases: the early rapid depression of tension associated with a transient decrease in $[Ca^{2+}]_i$ of smooth muscle, and the subsequent sustained small decrease in tension with no decrease in $[Ca^{2+}]_i$. EDRF/NO and EDHF were thus suggested to play a major role in the phasic component of relaxation, while prostacyclin and/or EDHF were important in the tonic component. The mechanisms of the thrombin-induced relaxation was due not only to the decrease in $[Ca^{2+}]_i$ of the smooth muscle but also to the decrease in the Ca^{2+}-sensitivity of the contractile apparatus, especially in the tonic component. EDRF/NO and/or prostacyclin were suggested to be responsible for the decrease in Ca^{2+}-sensitivity of contraction. The present study directly confirmed that thrombin induced both $[Ca^{2+}]_i$ elevation and NO production in the aortic valvular endothelial cells in situ, which were also shown to express endothelial constitutive NO synthase.

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