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Lack of nitric oxide- and guanosine 3′:5′-cyclic monophosphate-dependent regulation of α-thrombin-induced calcium transient in endothelial cells of spontaneously hypertensive rat hearts

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1 While the expression and/or activity of endothelial nitric oxide synthase (eNOS) has been characterized in spontaneously hypertensive (SHR) and normotensive Wistar Kyoto rat (WKY) hearts, in coronary endothelial cells (ECs) from both strains, the effect of NO on intracellular calcium concentration ($[Ca^{2+}]_i$) is still unknown.

2 Coronary microvascular ECs were isolated from SHR and WKY and characterized. Immunocytochemistry and Western blot analysis showed that eNOS was similarly expressed in ECs from both strains.

3 Measuring $[Ca^{2+}]_i$ by imaging analysis of fura-2-loaded cells, we demonstrated that α-thrombin ($3-180$ U l^{-1}) induced a superimposable dose-dependent calcium transient in ECs from both strains.

4 In WKY ECs, S-nitroso-N-acetyl-DL-penicillamine (SNAP) dose-dependently ($10-100$ μM) and 0.1 μM atrial natriuretic factor (ANF) reduced the maximum and the decay time of α-thrombin-induced calcium transient. The inhibitory effects of SNAP and ANF were prevented by blocking cyclic GMP-dependent protein kinase. Non selective eNOS inhibitors prolonged the decay time of α-thrombin-induced calcium transient, while the selective inducible NOS inhibitor 1400 W was ineffective. SNAP (100 μM) and 0.1 μM ANF increased cyclic GMP content up to 22.9 and 42.3 fold respectively.

5 In SHR ECs, α-thrombin-induced calcium transient was not modified by SNAP, ANF or eNOS inhibition. SNAP (100 μM) and 0.1 μM ANF increased cyclic GMP content up to 9.3 and 51 fold respectively.

6 In WKY ECs, SNAP dose-dependently ($10-100$ μM) reduced also bradykinin-induced calcium transient, while in SHR ECs was ineffective.

7 We concluded that in SHR ECs, the cyclic GMP-dependent regulation of calcium transient is lost.

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Abbreviations: ANF, atrial natriuretic factor; Bk, bradykinin; $[Ca^{2+}]_i$, cytosolic calcium concentration; Cyclic GMP, guanosine 3′:5′-cyclic monophosphate; DiI-ac-LDLs, acetylated LDLs 1,1′-dioctadecyl-3,3,3,3′,3′-tetramethyl indocarbocyanine perchlorate complex; D-PPAK, d-Phe-Pro-Arg chloromethylketone; Δ $[Ca^{2+}]_i$, ($[Ca^{2+}]_i$ at the peak minus $[Ca^{2+}]_i$ resting value); ECs, endothelial cells; eNOS, endothelial nitric oxide synthase (type III); IBMX, isobutylmethylxanthine; iNOS, inducible nitric oxide synthase; L-NMMA, L-N^G-monomethylarginine; L-NOARG, L-N^G-nitroarginine; SHR, spontaneously hypertensive rats; SNAP, S-nitroso-N-acetyl-DL-penicillamine; WKY, Wistar Kyoto rats; 1400 W, N-(3-Aminomethyl)benzylacetamide, dihydrochloride

Introduction

Endothelial cells play a relevant role in the regulation of systemic vascular resistance and therefore several efforts have been made to identify specific endothelial dysfunctions in experimental models of hypertension. Indeed, many reports have focused on endothelial nitric oxide synthase (eNOS, NOS III) characterization in spontaneously hypertensive rats (SHR). Nava *et al*. (1995) report an increase in eNOS activity in coronary endothelial cells from both left and right ventricles of adult SHR (18–25 weeks old) when compared to aged-matched WKY, while no differences are found in 3–4-week-old rats. However, according to Crabos *et al*. (1997), the expression of eNOS identified by immunohistochemistry in coronary arterioles from SHR is decreased when compared to those from WKY. A decrease in the eNOS expression in coronary microvascular endothelial cells isolated from 16-month-old SHR as compared to aged-matched WKY is also reported by Bauersachs *et al*. (1998).

Functionally, a decrease in maximum coronary flow in SHR vs WKY is reported in several studies (Wangler *et al*., 1982; Anderson *et al*., 1989; Clozel *et al*., 1991; Brilla *et al*., 1991; Crabos *et al*., 1997; Bauersachs *et al*., 1998). Moreover, NO fails to antagonize the β-adrenoceptor mediated positive inotropic effect in left ventricular strips isolated from 12–16-week-old SHR, while in WKY left ventricular strips the increase in contractile force induced by isoprenaline is

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prevented by S-nitroso-N-acetyl-DL-penicillamine (SNAP) (Kotchi Kotchi *et al*., 1998).

In endothelial cells isolated from the aorta of 12-week-old SHR the resting cytosolic calcium concentration ($[Ca^{2+}]_i$) is lower than resting $[Ca^{2+}]_i$ of cells isolated from WKY (Wang *et al*., 1995a). Moreover, bradykinin induces a less pronounced increase in $[Ca^{2+}]_i$ than in control WKY, while the $[Ca^{2+}]_i$ increase induced either by angiotensin II or by endothelin is similar in both strains (Wang *et al*., 1995a). Similar results are also reported in endothelial cells isolated from the tail artery. Resting $[Ca^{2+}]_i$ is lower and the calcium increase induced by either bradykinin or endothelin is also reduced in SHR vs control WKY (Wang *et al*., 1995b). According to these authors (Wang *et al*., 1995a,b) this decreased response to agonists could account for the reduced endothelium-dependent relaxation observed in SHR when compared to WKY rats.

NO activates soluble guanylyl cyclase, leading to an increase in guanosine 3′:5′-cyclic monophosphate (cyclic GMP); in different cell types this increase can regulate $[Ca^{2+}]_i$ (Clementi & Meldolesi, 1997). In endothelial cells, NO modulates $[Ca^{2+}]_i$ in an autocrine fashion (Shin *et al*., 1992). Among the agonists used, ATP is able to increase $[Ca^{2+}]_i$ in bovine aortic endothelial cells and preincubations with L-N^G monomethylarginine (L-NMMA), L-N^G-nitroarginine (L-NOARG) or methemoglobin potentiate the ATP-induced $[Ca^{2+}]_i$ increase. In the same study, α-thrombin is also able to increase $[Ca^{2+}]_i$ in endothelial cells (Shin *et al*., 1992).

Studies on the effect of NO donors on bradykinin-stimulated $[Ca^{2+}]_i$ signalling in calf pulmonary artery endothelial cells (passage 14–19) show that several NO-donors (including SNAP) do not modulate $[Ca^{2+}]_i$, while S-morpholinosydnonimine (SIN-1) does (Elliot, 1996). The effect of SIN-1 seems to depend on the formation of peroxynitrite from NO and superoxide radicals, NO and superoxide radicals being contemporaneously produced by SIN-1 (Elliot, 1996).

Moreover, sodium nitroprusside and SNAP can induce an increase in $[Ca^{2+}]_i$ of human umbilical vein cord endothelial cells (HUVEC) independently of guanylyl cyclase activity (Volk *et al*., 1997).

Although many studies have focused on possible NO-related dysfunctions in SHR, no extensive characterization of $[Ca^{2+}]_i$ and its regulation by NO (either physiologically produced or pharmacologically administered) have yet been performed in endothelial cells isolated from SHR. Therefore the aim of our study was to evaluate the effect of NO on the calcium regulation of endothelial cells isolated from the SHR heart and to compare these data with those obtained in endothelial cells isolated from WKY.

Methods

Isolation and culture of microvascular endothelial cells from the heart

This investigation conforms to the rules for the care and use of laboratory animals of the European Community.

Microvascular endothelial cells from hearts were isolated from 2–3-month-old WKY and age-matched SHR according to Piper *et al*. (1990). Briefly, hearts were digested with 0.1% type I collagenase (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), cut and cells were dispersed. After centrifugation ($25 \times g$ for 3 min), the supernatant was maintained under stirring for 30 min at 37°C in the presence of 10 mg/50 ml trypsin (Boehringer Mannheim, Mannheim, Germany). Cells, obtained by centrifugation ($250 \times g$ for 10 min), were resuspended in 15 ml of culture medium (see below) and plated in 7500 mm^2 flasks. After 4 h, cells were washed twice and grown in 10 ml culture medium until confluence (5–6 days). Cells were used for all experiments at the first passage. M199 (Earle Salt, Sigma Chemical Co.) containing 10% foetal calf serum, 10% newborn calf serum (Gibco-BRL, Paisley, U.K.), 250 IU ml^{-1} penicillin G (Sigma Chemical Co.), 0.625 μg ml^{-1} amphotericin (Sigma Chemical Co.) and 250 μg ml^{-1} streptomycin (Sigma Chemical Co.), was used as culture medium.

Immunocytochemical characterization of endothelial cells

Cells plated onto sterile tissue culture chamber slides (Lab-tek, Nunc Inc., Naperville, IL, U.S.A.) were washed twice with phosphate-buffered saline (PBS), dried overnight at room temperature (RT), and fixed in acetone at 4°C for 5 min. Monoclonal antibodies specific for vimentin (V9, Dakopatts, Glostrup, Denmark), α-smooth muscle actin (1A4, Sigma), human desmin (D-33, Dakopatts), pan-cytokeratin (Lu5, Boehringer Mannheim, Mannheim, Germany), and polyclonal antibodies against Von Willebrand factor antigen (Dakopatts) were applied onto cells. Primary antibodies were diluted in a buffer containing 0.1% bovine serum albumin in PBS and incubated for 30 min at room temperature. After further washing, polyclonal antibodies were additionally incubated with monoclonal anti-rabbit antibody (Dakopatts), diluted 1 : 10 in a buffer containing PBS and 10% normal AB human serum for the blockade of non-specific binding for 30 min at room temperature. Cells were washed twice for 5 min each and covered with a polyclonal rabbit anti-mouse antibody (Dakopatts) diluted 1 : 20 in the same buffer described above. After 30 min incubation, cells were rinsed twice in PBS for 5 min and incubated with the alkaline phosphatase anti-alkaline phosphatase immune complex (APAAP) (Dakopatts) diluted 1 : 50 in PBS for 30 min. The chromogenic reaction was developed with new fucsin and naphthol-as-BI-phosphate for 30 min. Negative controls for the immunostaining were obtained either by omission of the primary antibody or incubation with preimmune rabbit immunoglobulins diluted 1 : 400 in PBS/BSA.

Endogenous peroxidase activity was analysed on plated cells, fixed in acetone for 5 min, by incubation with 0.3% H_2O_2:3,3′-diaminobenzidine tetrahydrochloride (Sigma) in PBS for 10–15 min.

Acetylated LDL uptake was performed on confluent cells grown on glass coverslips. Cells were incubated overnight in normal culture medium containing 200 μg ml^{-1} (final concentration) of DiI-ac-LDLs (acetylated LDLs 1,1′-dioctadecyl-3,3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate complex, Biochemical Technologies, Inc., Stoughton, MA, U.S.A.). After washing, cells were fixed (3% formaldehyde) for 20 min at RT. Nuclei were stained by incubation with 1 μg ml^{-1} of bisbenzimide (Hoechst no. 33258, Sigma Chemical Co.) for 2 min. Negative control for the DiI-ac-LDLs uptake was obtained by incubating cells overnight in normal culture medium. Analysis was performed using an inverted microscope (Nikon Diaphot) at two excitation lengths: 550 nm excitation for DiI-ac-LDLs and 360 nm for bisbenzimide.

Nitric oxide synthase determination in endothelial cells

Immunocytochemical characterization Cells were grown until confluence on culture chamber slides and fixed in 10% formalin for 10 min at RT and washed. After pre-incubation for 1 h at RT in PBS (2% BSA) with the addition of 0.1%

Triton-X-100 (TX), the slides were incubated overnight at RT with the primary polyclonal rabbit antibody (Calbiochem Inalco, Milan, Italy) used at a 1 : 100 dilution in PBS. On the following day, they were washed and incubated for 1 h at RT with the secondary antibody (Vector Laboratories, Burlingame, CA, U.S.A.) diluted 1 : 500 in PBS-0.5% BSA and 0.1% TX. After a further wash, incubation with the avidin-biotin-peroxidase complex (Vector kit; 1 : 500 in PBS-BSA, 0.1% TX) followed for 1 h at RT. The primary antibody was finally localized by the diaminobenzidine (DAB)-H_2O_2-peroxidase colour reaction. The reaction was usually completed in 8 min, at the end of which the slides were washed, dehydrated and mounted with coverslips. Analysis was performed by means of a Nikon Labophot-2 microscope. Negative control for the immunostaining was obtained by omission of the primary antibody.

Western blot analysis Confluent endothelial cells from WKY and SHR hearts were washed twice with ice-cold PBS. The cell monolayer was lysed in RIPA buffer (mM composition: Tris-HCl 20, pH 7.4, NaCl 150, 5 EDTA, Na_3VO_4, 1 phenyl methyl sulfonyl fluoride; 1% nonidet P-40, 0.05% [w v^{-1}] aprotinin). Insoluble proteins were discarded by high-speed centrifugation at 4°C. Protein concentration in the supernatant was measured in triplicate (Bradford, 1976). Western blot analysis was carried out essentially as described elsewhere (Marra *et al*., 1997). 75 μg of proteins were separated by 7.5% SDS–PAGE according to Laemmli (1970) and electroblotted on a polyvinyliden-difluoride membrane. The membranes were blocked overnight at 4°C with 2% BSA in 0.1% PBS-Tween, and then sequentially incubated at room temperature with anti-e-NOS polyclonal antibodies (Calbiochem, Inalco, Milan, Italy) and horseradish peroxidase-conjugated secondary antibodies. Detection was carried out using chemiluminescence according to the manufacturer's protocol (Amersham, Arlington Heights, IL, U.S.A.).

Imaging analysis of cytosolic intracellular calcium

Endothelial cells were grown until confluence on round coverslips (diameter 25 mm) and loaded with 5 μM fura-2AM, 0.02% Pluronic F (Poenie *et al*., 1986) (Molecular Probes, Eugene, OR, U.S.A.) for 45 min at room temperature in HEPES/$NaHCO_3$ medium (composition mM: NaCl 140, KCl 2.9, $MgCl_2$ 0.9, NaH_2PO_4 0.5, $NaHCO_3$ 12, glucose 10, HEPES 10, $CaCl_2$ 1, adjusted to pH 7.4 with 1 N NaOH).

Fura-2-loaded endothelial cells were placed on the platform of an inverted fluorescence microscope (Nikon Diaphot, Japan) equipped with a 75 W Xenon lamp. Fluorescence images were collected with an intensified charge-coupled device (CCD) video camera ISIS-M extended camera (Photonic Science, U.K.) at a videorate of 40 ms using an Image Analysis System 'Magiscan' (Applied Imaging, Newcastle, U.K.) equipped with a videotape recorder (Failli *et al*., 1995). At least 20 cells found in an optical field (using 20 × magnification objective) were analysed.

Fluorescence analysis was performed using Imaging Analysis software 'Tardis®' and custom-made software. For quantification of intracellular calcium, following-in-time images obtained at 340 and 380 nm excitation, emission 510 nm (time interval between two following-in-time images: 800 ms) were digitalized by an analogical/digital converter (256 × 256 pixels) and rationed on a pixel-by-pixel basis. In order to decrease cell light exposure which can decrease fura-2 fluorescence, the filter wheeler was stopped in a dark position and a ratio image was obtained every 3 s Calibration curves were performed using ionomycin (Calbiochem) and ethylene-bis (oxyethylenenitrilo)-tetraacetic acid (EGTA, Aldrich-Chemi, Steinheim, Germany) as described (Mason *et al*., 1993) and a dissociation constant for fura-2 of 224 nM. After measuring $[Ca^{2+}]_i$ in resting condition in the absence or presence of either SNAP, ANF or NOS inhibitors, agonists (i.e. α-thrombin or bradykinin) were administered and continuously present during the experiment.

All data were exported in ASCII file format and elaborated for graphic presentation using MicroCal Origin® (2.8 version). A noise/signal ratio of 0.5 was considered as the low detectable limit for calcium transient. L-NMMA, L-NOARG, hirudin, d-Phe-Pro-Arg chloromethylketone (D-PPAK), atrial natriuretic factor (ANF), bradykinin (Bk) and ethylenediaminetetraacetic acid (EDTA) disodium salt, were purchased from Sigma Chemical Co.; α-thrombin was obtained from Boehringer Mannheim; SNAP was purchased from Tocris Cookson Ltd. (Bristol, U.K.). Several experiments were performed in the presence of KT-5823 (Calbiochem), an inhibitor of cyclic GMP-dependent protein kinase (Kase *et al*., 1987). 1400 W (Garvey *et al*., 1997), an inhibitor of the inducible form of NOS (iNOS) was obtained by Calbiochem. All other reagents were of analytical grade. Cell culture plastic supports were purchased from Costar (Corning Costar Co., Costar Italia, Milan, Italy).

Cyclic GMP determination

Confluent endothelial cells were washed twice and preincubated (5 min) with HEPES/$NaHCO_3$ buffer containing 100 μM isobutylmethylxanthine (IBMX, Sigma Chemical Co.); cells were then incubated for 5 min at 37°C either with 20 μl buffer alone (control) or in an equal volume of SNAP (final concentration 100 μM) or ANF (0.1 μM final concentration). Reaction was stopped by adding 2 ml ice-cold trichloroacetic acid (TCA, 10% v v^{-1}); cells were scraped and the reaction mixture was neutralized by extracting TCA with tri-n-octylamine 0.5 M in organic solvent (trichlorotrifluoroethane). The lyophilized aqueous phase was diluted and used for the determination of cyclic GMP using a commercial [^{125}I]-cyclic GMP RIA Kit (Amersham International).

Mathematical and statistical methods

For each experiment, from 20–26 cells were analysed and at least three different cell lines were used. The number of cells analysed is reported as *c* and the number of different lines as *n*. Values are presented as means ± s.e.mean of analysed cells (*c*). The decay time of calcium transient was calculated by fitting the last part of the calcium transient decrease as a monoexponential decay. The constant 'tau' of the mono-exponential fitting was therefore reported as the decay time value (Figure 1).

Statistical comparisons between data groups were performed either using Student's *t*-test (paired and independent as indicated) or Chi-square test; dose-response curves were also analysed by one-way ANOVA. A P value of $\leqslant 0.05$ was considered significant.

Results

Immunocytological characterization of endothelial cells

Extensive cytological characterization of cells obtained according to the experimental procedure (three lines for each

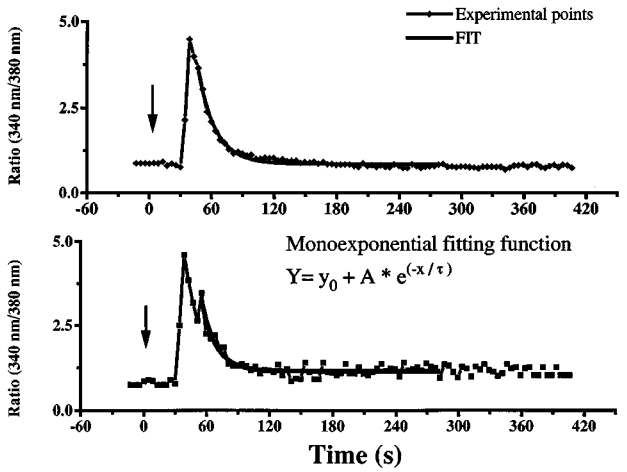

Figure 1 Typical examples of monoexponential fitting of calcium transient decay. When calcium transient showed oscillation, only the last part of the decay was interpolated by monoexponential fitting. According to the fitting function, the 'tau' parameter represents the time necessary for $[Ca^{2+}]_i$ to reach 36.79% of the maximal value. 'Tau' is expressed in seconds and refers to 'decay time'.

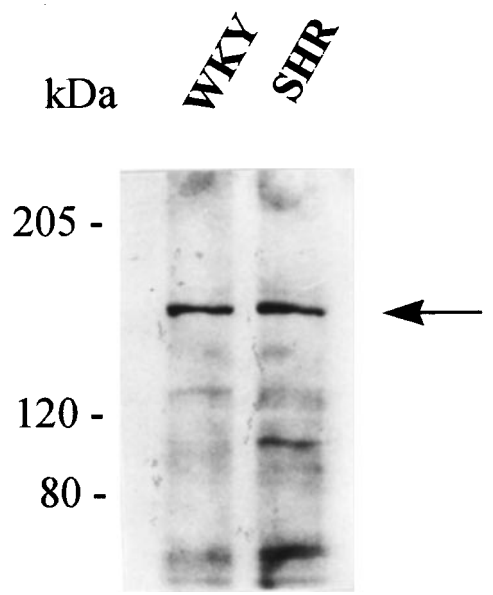

Figure 2 Western blot analysis of eNOS in WKY and SHR endothelial cells; similar results were obtained in two other separate preparations of endothelial cells. The arrow indicates the eNOS band.

strain), showed a negative staining for cytokeratin and endogenous peroxidase and a positive staining for vimentin antibodies, confirming the mesenchymal origin of both SHR and WKY cells. Moreover, cells were negative for desmin and α-smooth muscle actin, while Von Willebrand factor staining was weakly positive. Negative controls obtained either by omission of the primary antibody or incubation with preimmune rabbit immunoglobulins did not show any specific staining. Cells isolated from both WKY and SHR showed an active uptake of acetylated LDL. The dual staining with DiI-ac-LDL and bisbenzimide demonstrated that 97–98% of cells were labelled by DiI-ac-LDL (not shown), whereas both WKY and SHR cells, incubated overnight in the presence of DiI-ac-LDL solvent alone, did not show any significant fluorescence. The percentage of cells positive for vimentin, Von Willebrand factor and DiI-ac-LDL uptake ranged between 95 and 97% and did not vary between WKY and SHR endothelial cells.

Nitric oxide synthase

Both SHR and WKY microvascular endothelial cells were immunopositive for eNOS (not shown). The eNOS immunostaining intensity did not appear different between the SHR and WKY endothelial cells as judged by microscopic examination. We performed Western blot analysis to confirm the similar expression of eNOS in WKY and SHR endothelial cells. Western blotting confirmed that the enzyme was present in similar amounts in endothelial cells isolated from both strains, as indicated by a similar intensity of the band of approximately 140 kDa molecular weight (Figure 2).

Intracellular calcium concentration

Resting condition The $[Ca^{2+}]_i$ in resting condition was 126 ± 7.1 nM ($c = 238$, $n = 10$) in WKY and 135 ± 4.1 nM ($c = 218$, $n = 9$) in SHR endothelial cells.

Effect of α-thrombin The administration of α-thrombin to cell monolayers induced a dose-dependent increase in $[Ca^{2+}]_i$. The non-cumulative dose-response curve of the α-thrombin-induced calcium increase ($\Delta[Ca^{2+}]_i$) performed in WKY and SHR microcoronary endothelial cells is shown in Figure 3. The

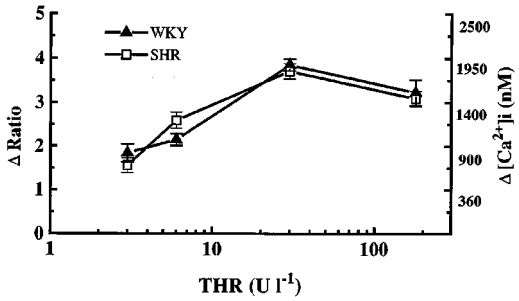

Figure 3 Dose-response curve of α-thrombin in WKY and SHR. Each point is the mean ± s.e.mean of at least 60 cells obtained in three separate experiments. Left axis: Δ fluorescent ratio (340/380 nm); Right axis: $\Delta[Ca^{2+}]_i$ (nM).

maximal effect of α-thrombin-induced $\Delta[Ca^{2+}]_i$ was obtained in both strains using a concentration of 30 U l^{-1}. Therefore this dose was used in all the experiments. Figure 4 shows the effect of 30 U l^{-1} α-thrombin as observed by video-imaging analysis in a representative experiment on WKY endothelial cells. Soon after α-thrombin administration (i.e. 15–18 s), $[Ca^{2+}]_i$ began to increase rapidly reaching the highest level after 24–28 s. Then, $[Ca^{2+}]_i$ decreased more slowly.

In Figures 5 and 6, the effect of 30 U l^{-1} is better analysed in WKY and SHR endothelial cells respectively. α-thrombin induced a short-lasting (i.e. 90–100 s) calcium transient in endothelial cells isolated from both strains. In both strains, the calcium transient was characterized by a rapid increasing phase, followed by a slower decay phase; soon after the decay phase, $[Ca^{2+}]_i$ reached a long-lasting steady-state plateau (i.e. longer than 4 min). At the end of the plateau phase, the $[Ca^{2+}]_i$ value was 253 ± 14.8 nM ($P < 0.001$ vs resting value, paired Student's t-test, $c = 134$, $n = 6$) in WKY-isolated and 290 ± 13.2 nM ($P < 0.001$ vs resting value, paired Student's t-test, $c = 132$, $n = 6$) in SHR endothelial cells. Lag times and times to the peak of the α-thrombin-induced calcium transient were not significantly different between WKY and SHR. Although the rising phase of the calcium transient was similar in endothelial cells from both strains, calcium oscillations

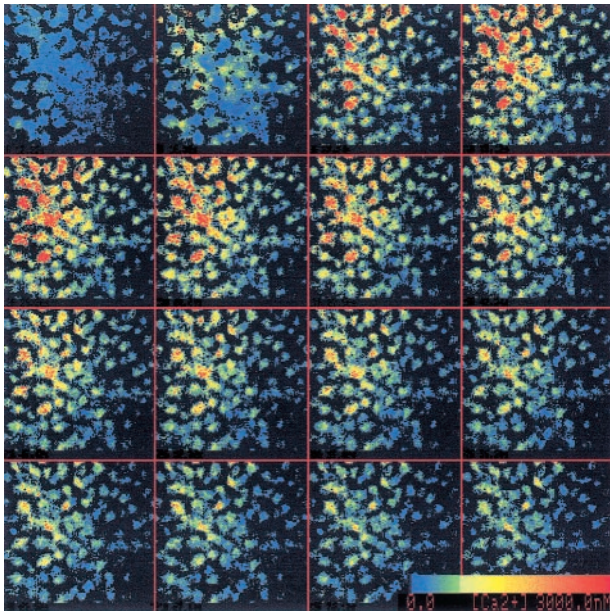

Figure 4 Time sequence of calcium transient in individual fura-2-loaded WKY endothelial cells in response to 30 U l^{-1} α-thrombin (representative experiment). Frames are described left to right and up to down. Frame 1 shows resting $[Ca^{2+}]_i$ before the addition of α-thrombin. Frames 2, 3, 4 and 5 (18, 21, 24 and 27 s after the addition of α-thrombin respectively) illustrate the increase in $[Ca^{2+}]_i$. Frames 6–16 (30–75 s after the addition of α-thrombin respectively) show the decay phase of calcium transient. 340/380 ratio frame are obtained every 3 s and calibrated as described in Methods. $[Ca^{2+}]_i$ (nM) is colour-coded according to the calibration bar.

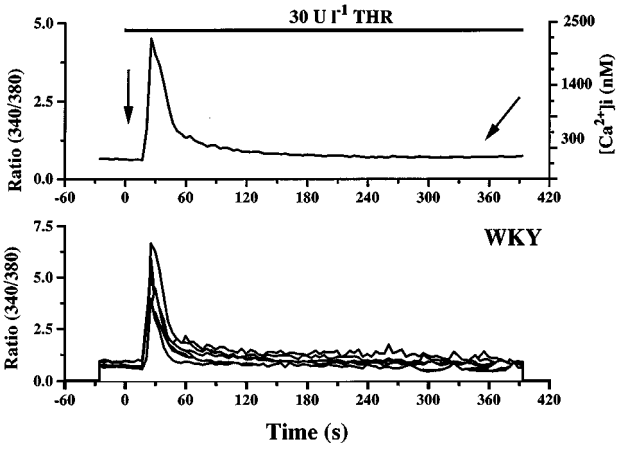

Figure 5 Typical time-course of α-thrombin-induced calcium transient on the fluorescent ratio (340/380 nm, left axis) in WKY endothelial cells. α-thrombin (30 U l^{-1}) was administered at the first arrow (time = 0) and maintained till the end of the experiment. Bottom: each curve is the single response measured in an individual cell. The mean ($c = 26$) time-course is shown in the upper panel. In each point, standard errors (s.e. mean) do not exceed 10% of the value. Upper panel, right axis: intracellular calcium concentration (nM). The arrow indicates the plateau phase of the calcium transient.

superimposed on or shortly after the initial $[Ca^{2+}]_i$ peak were more frequently observed in SHR endothelial cells (45 out of 134 in WKY isolated cells and 61 out of 132 in SHR isolated cells, Chi-square = 3.914, $P < 0.05$). The decay time of the calcium transient was 20.2 ± 0.68 s and 19.0 ± 0.64 s in WKY and SHR endothelial cells respectively. The effect of α-thrombin was abolished by pretreating the cells either with 1 μM D-PPAK or 20 U l^{-1} hirudin, two selective inhibitors of α-thrombin proteolytic activity. However, after washing D-

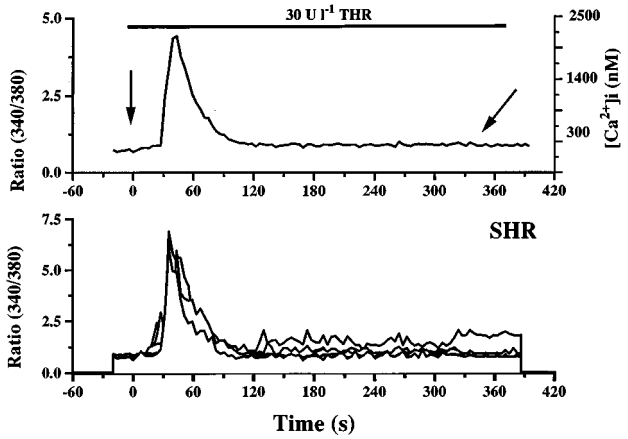

Figure 6 Typical time-course of α-thrombin-induced calcium transient on the fluorescent ratio (340/380 nm, left axis) in SHR endothelial cells. α-thrombin (30 U l^{-1}) was administered at the first arrow (time = 0) and maintained till the end of the experiment. Bottom : each curve is the single response measured in an individual cell. The mean ($c = 25$) time-course is shown in the upper panel. In each point, standard errors (s.e. mean) do not exceed 10% of the value. Upper panel, right axis: intracellular calcium concentration (nM). The arrow indicates the plateau phase of the calcium transient.

PPAK (10 min at a constant flux of 1.5 ml min^{-1}) the effect of α-thrombin was totally restored as shown in Figure 7 for WKY endothelial cells. Similar results were obtained in SHR endothelial cells.

The $\Delta[Ca^{2+}]_i$ increase induced by α-thrombin was decreased in the absence of extracellular calcium (200 μM EDTA in the calcium-free medium); moreover, preincubation with thapsigargin (30 nM) abolished the calcium transient in both WKY and SHR endothelial cells (Figure 8).

Since the α-thrombin response was very similar in both strains, we analysed the effect of exogenously applied SNAP on calcium transient induced by 30 U l^{-1} α-thrombin. In WKY endothelial cells, preincubation (5 min) with the NO-donor SNAP dose-dependently reduced both the peak (Figure 9A, $F = 29.113$, $P < 0.001$, ANOVA) and the decay time of the α-thrombin-induced calcium transient (Figure 9B, $F = 11.700$, $P < 0.001$, ANOVA). Interestingly, preincubation with 100 μM SNAP totally abolished the α-thrombin-induced calcium transient in one-third of analysed cells (26 out of 71 analysed cells). Only cells still presenting the calcium transient were used to calculate the percentage of inhibition, as reported in Figure 9A,B). Neither the $\Delta[Ca^{2+}]_i$, nor the decay time were modified by SNAP in SHR endothelial cells (Figure 9A,B).

In order to test the effect of NO endogenously synthesised by the cells, we performed experiments after 20 min preincubation with NO synthase inhibitors. In WKY endothelial cells, L-NMMA (100 μM) increased the resting calcium (263 ± 20.6 nM, $c = 68$, $n = 3$, $P < 0.001$ independent Student's t-test) and increased the decay time (29.8 ± 1.91 s $P < 0.001$ vs control, $c = 65$, $n = 3$), while it did not influence the peak of α-thrombin-induced calcium transient (not shown). Similar results were obtained with L-NOARG. After 20 min preincubation with 10 μM L-NOARG, the resting calcium was significantly increased as compared to the control value (162 ± 8.1 nM, $c = 140$, $n = 6$, $P < 0.01$ independent Student's t-test) as well as the decay time (32.8 ± 1.12 s, $c = 128$, $n = 6$), while the α-thrombin-induced $\Delta[Ca^{2+}]_i$ was unchanged (not shown). No modifications in either resting calcium, decay time or the α-thrombin-induced $\Delta[Ca^{2+}]_i$ was

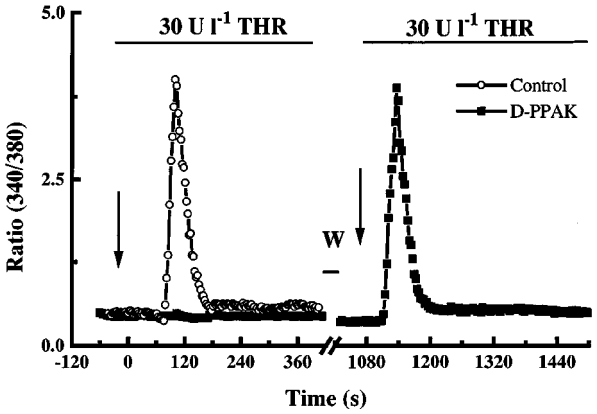

Figure 7 Typical time-course of α-thrombin-induced calcium in control condition and after preincubation with 1 μM D-PPAK in WKY. α-thrombin (30 U l^{-1}) was administered at the first arrow (time = 0) and maintained in the perfusion chamber as indicated. After washing D-PPAK-pretreated cells for 10 min with control solution at a constant flow of 1.5 ml (W), 30 U l^{-1} α-thrombin were re-administered at the second arrow and maintained till the end of the experiment. Curves are the mean of at least 25 single cells. In each point, standard errors (s.e. mean) do not exceed 10% of the value.

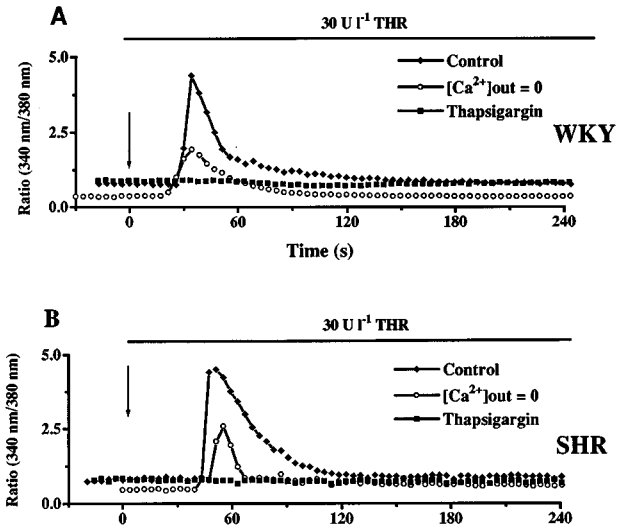

Figure 8 Typical time-course of α-thrombin-induced calcium transient in the absence of extracellular calcium and after preincubation with 30 nM thapsigargin in WKY and SHR endothelial cells. α-thrombin (30 U l^{-1}) was administered at the arrow (time = 0) and maintained in the perfusion chamber till the end of the experiment. Curves are the mean of at least 25 single cells. In each point, standard errors (s.e.mean) do not exceed 10% of the value. (A) WKY endothelial cells; (B) SHR endothelial cells.

observed when cells were preincubated for 30 min with 1 μM 1400 W ($c = 65$, $n = 3$).

In SHR endothelial cells, preincubations with either 100 μM L-NMMA or 1 μM 1400 W were ineffective in modulating all parameters studied (i.e. resting calcium, α-thrombin-induced Δ $[Ca^{2+}]_i$ and decay time).

We also studied the effect of ANF. In WKY endothelial cells, 0.1 μM ANF decreased the $[Ca^{2+}]_i$ peak (865 ± 74.9 nM vs 2081 ± 82.8 nM in control condition, $c = 72$, $n = 3$) and the decay time (8.4 ± 0.82 s, $c = 71$, $n = 3$). In the same experimental conditions, ANF was totally unable to modulate both the $[Ca^{2+}]_i$ peak (1754 ± 141.0 nM vs 2050 ± 90.7 nM in control condition) and the decay time (18.2 ± 0.68 s, $c = 86$, $n = 4$) in SHR endothelial cells. Moreover, in SHR endothelial cells

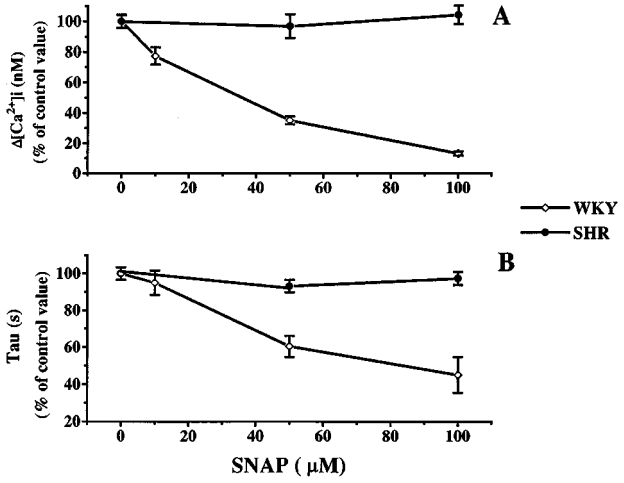

Figure 9 Effect of SNAP on α-thrombin-induced calcium transient (30 U l^{-1}) in WKY and SHR endothelial cells. (A) $\Delta[Ca^{2+}]i$ increase, (B) decay time. Each point is the mean ± s.e. mean of analysed cells obtained in three separate experiments. Values are presented as percentage of control α-thrombin effect. For more details, see text.

SNAP (100 μM) was still unable to modulate α-thrombin-induced calcium transient in experiments performed in the presence of the phosphodiesterase inhibitor IBMX. In control cells $\Delta[Ca^{2+}]_i$ was 1873 ± 82.1 nM ($n = 3$, $c = 74$) and tau was 19.5 ± 0.90 s ($n = 3$, $c = 69$), while in 100 μM SNAP preincubated cells $\Delta[Ca^{2+}]_i$ was 1746 ± 63.0 nM ($n = 3$, $c = 69$) and tau was 18.3 ± 1.05 s ($n = 3$, $c = 65$).

In WKY endothelial cells, we tested the influence of the cyclic GMP-dependent protein kinase inhibitor KT-5823 on α-thrombin-induced calcium transient in control conditions and after preincubation with either 100 μM SNAP or 0.1 μM ANF. After 10 min preincubation with 1 μM KT-5823, resting $[Ca^{2+}]_i$ was not modified, while the decay time of the α-thrombin-induced calcium transient was modestly prolonged (22 ± 0.89 s, $c = 70$, $n = 3$; $P < 0.05$ vs control). In the presence of 1 μM KT-5823, 100 μM SNAP failed to reduce and 0.1 μM ANF only slightly affected the α-thrombin-induced calcium transient. After 10 min preincubation with 1 μM KT-5823, the $\Delta[Ca^{2+}]_i$ was reduced only by an 11 ± 1.6% in the presence of 100 μM SNAP (not significant, $c = 69$, $n = 3$) and 23 ± 2.4% ($P < 0.05$, $c = 66$, $n = 3$) in the presence of 0.1 μM ANF. The tau was not reduced either by SNAP (10 ± 1.5%) or by ANF (9 ± 2%).

Effect of bradykinin Bradykinin (10^{-6} M) induced an increase in $[Ca^{2+}]_i$ very similar in both WKY and SHR endothelial cells. The effects of 10^{-6} M bradykinin on $\Delta[Ca^{2}]_i$ and decay time are summarized in Tables 1 and 2. In WKY endothelial cells, preincubation with SNAP (10–100 μM) dose-dependently reduced the $\Delta[Ca^{2+}]_i$ ($F = 4.895$, $P < 0.02$, ANOVA, Table 1) and, at the higher dose tested, also reduced the decay time of bradykinin-induced calcium transient ($P < 0.01$, Table 2). In SHR endothelial cells, bradykinin-induced calcium transient was not modified by SNAP (Tables 1 and 2).

Cyclic GMP determination

Cyclic GMP determination showed that 100 μM SNAP and 0.1 μM ANF increased cyclic GMP in both WKY and SHR endothelial cells. While the increase in cyclic GMP induced by ANF was very similar in WKY and SHR endothelial cells, the

Table 1 Effect of SNAP on the increase in intracellular calcium ($[Ca^{2+}]_i$) induced by 10^{-6} M bradykinin in WKY and SHR endothelial cells

	$[Ca^{2+}]_i$ (nM) WKY	SHR
10^{-6} M Bk	855±81.5 ($n=3$, $c=79$)	807±61.5 ($n=4$, $c=104$)
10 SNAP + 10^{-6} M Bk	664±89.7* ($n=3$, $c=74$)	N.P.
50 SNAP + 10^{-6} M Bk	571±63.4* ($n=3$, $c=76$)	700±49.7 ($n=4$, $c=98$)
100 SNAP + 10^{-6} M Bk	384±35.6* ($n=3$, $c=69$)	651±52.8 ($n=4$, $c=98$)

*$F=4.895$, $P<0.02$, ANOVA. Values are mean±s.e.mean, number of preparations (n) and analysed cells (c) in brackets. N.P. not performed.

Table 2 Effect of SNAP on the decay time of the bradykinin-induced calcium transient in endothelial cells of WKY and SHR rats

	Decay time (s) WKY	SHR
10^{-6} M Bk	20.4±1.34 ($n=3$, $c=68$)	22.4±0.94 ($n=4$, $c=94$)
10 SNAP + 10^{-6} M Bk	19.1±1.96 ($n=3$, $c=62$)	N.P.
50 SNAP + 10^{-6} M Bk	19.6±1.79 ($n=3$, $c=64$)	20.8±0.76 ($n=4$, $c=84$)
100 SNAP + 10^{-6} M Bk	14.9±1.39** ($n=3$, $c=65$)	20.2±0.77 ($n=4$, $c=87$)

**$P<0.001$ vs control WKY. Values are the mean± s.e.mean, number of preparations (n) and analysed cells (c) in brackets. N.P. not performed.

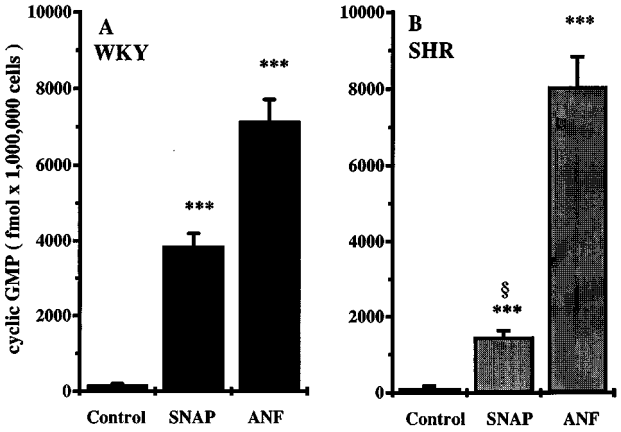

Figure 10 Cyclic GMP content of WKY (A) and SHR (B) endothelial cells after preincubation with buffer (Control) or in the presence of either 100 μM S-nitroso-N-acetylpenicillamine (SNAP) or 0.1 μM atrial natriuretic factor (ANF). Values are the mean±s.e. mean of three independent experiments. ***$P<0.001$ vs control; §$P<0.001$ vs SNAP, WKY.

SNAP-induced increase in cyclic GMP was significantly lower in SHR than in WKY endothelial cells (Figure 10).

Discussion

Isolation and characterization of endothelial cells

According to the isolation method, the cell population obtained both from the heart of WKY and SHR is mainly of microvascular endothelial origin and the percentage of endothelial cells obtained from WKY and SHR does not vary. The microvascular origin of endothelial cells is also confirm by the weak expression of von Willebrand factor (Piper *et al*., 1990).

As demonstrated by immunocytochemistry and Western blotting, the expression of eNOS is also similar in cells isolated from both strains. Although we did not test the activity of eNOS, our data are in agreement with those reported by Nava *et al*. (1995) which show a similar activity of eNOS in freshly-isolated endothelial cells from young WKY and SHR hearts or increased enzymatic activity in cells isolated from adult SHR as compared to age-matched WKY.

Intracellular calcium in WKY and SHR endothelial cells and effect of α-thrombin According to our data, $[Ca^{2+}]_i$ in resting conditions is comparable in WKY and SHR endothelial cells. These data are not in line with those of Wang *et al*. (1995a,b), but our study is performed in microvascular coronary endothelial cells and the different district origin may account for the varying results. As shown, α-thrombin induces a similar dose-dependent increase in $[Ca^{2+}]_i$ both in WKY and SHR endothelial cells. Indeed, at 30 U l^{-1} not only the $\Delta[Ca^{2+}]_i$ increase induced by α-thrombin is similar, but also the decay time of the calcium transient. α-Thrombin receptor was originally cloned in vascular endothelial cells (Vu *et al*., 1991). Thrombin signalling is mediated by G protein-coupled protease-activated receptor (PAR). Thrombin binds and cleaves the amino-terminal exodomain of PAR and the newly formed receptor aminoterminus serves as intramolecular activator of transmembrane signalling. This peculiar mechanism determines the irreversibility of PAR activation. Moreover, PAR is rapidly phosphorylated and uncoupled from signalling after activation (for a review see Coughlin, 1999). The uncoupling of receptor from intracellular signalling probably explains the switch-off of $[Ca^{2+}]_i$ signal that we observed in our experiments in the presence of α-thrombin. Since α-thrombin-induced calcium transient is abolished by hirudin and D-PPAK, α-thrombin activates its own receptor both in WKY and SHR endothelial cells. However, after D-PPAK washing, cell activation by α-thrombin is fully restored.

According to reports in the literature regarding HUVEC (Hallam *et al*., 1988), the α-thrombin-induced calcium transient in both WKY and SHR endothelial cells is dependent on a release from intracellular stores followed by an influx from the extracellular milieu, as demonstrated when comparing experiments performed in the absence of extracellular calcium with those done in its presence. Calcium release from intracellular stores is required in the onset of calcium transient, while calcium influx accounts for late phases of calcium transient and sustained plateau. However, in SHR endothelial cells, the calcium transient obtained in the absence of extracellular calcium is more pronounced than in WKY endothelial cells, suggesting a larger intracellular stored calcium pool in SHR. The intracellular stores are those replenished by a thapsigargin inhibitable mechanism: indeed after preincubation with 30 nM thapsigargin, the calcium transient is absent in both WKY and SHR endothelial cells. According to our data, therefore α-thrombin-induced calcium transient in endothelial cells is initiated by a release from intracellular stores followed by an influx from extracellular milieu, probably dependent on the activation of store-operated calcium channels (SOCC). However, calcium oscillations are more frequently observed in SHR cells compared to WKY, probably indicating an alteration in the storing capability and/or refilling mechanisms. An increase in calcium waves has been

described also in cardiomyocytes isolated from old SHR (Failli *et al*., 1997).

Effect of NO and ANF on α-thrombin-induced calcium transient in WKY endothelial cells Our data show that in WKY endothelial cells, SNAP dose-dependently reduces the calcium peak and abbreviates α-thrombin-induced calcium transient. ANF (0.1 μM) also possesses a similar inhibitory effect, but ANF activity is more pronounced on the decay time than on the calcium rise, as is shown by comparing the ANF values with those obtained with 100 μM SNAP. Both compounds increase cyclic GMP, ANF being more effective than SNAP. The increase in cyclic GMP may account for the inhibition of the α-thrombin-induced calcium transient by both compounds. The data obtained in the presence of the cyclic GMP-dependent protein kinase inhibitor KT-5823 (Kase *et al*., 1987) further support this hypothesis. The inhibitory effect of SNAP and ANF on the decay time is in line with the effectiveness of NO to activate the sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) reported in vascular smooth muscles (Cohen *et al*., 1999). In endothelial cells, SERCA2b and SERCA3 isoforms are both expressed (Mountian *et al*., 1999). The increased activation of SERCA may explain the decrease in decay time obtained in the presence of SNAP and ANF. This mechanism may also explain the inhibition of the calcium peak, since the refilling of internal stores inhibits SOCC (Cohen *et al*., 1999). Moreover, at very high levels, cyclic GMP directly inhibits SOCC (Xu *et al*., 1994). However, other mechanisms than cyclic GMP-dependent protein kinase can also influence the calcium homeostasis in WKY endothelial cells. In fact, in our hands the resting $[Ca^{2+}]_i$ is not influenced by SNAP, ANF or cyclic GMP kinase inhibition. Additional research will clarify these points.

We also demonstrate that eNOS inhibitors (L-NMMA and L-NOARG) increase resting $[Ca^{2+}]_i$. This parameter seems to be regulated by eNOS independently of cyclic GMP mechanisms, since cyclic GMP kinase inhibition does not influence resting $[Ca^{2+}]_i$. Moreover, L-NMMA and L-NOARG both prolong the decay time of the calcium transient, while they do not significantly affect the calcium transient peak. The enzymatic activity of eNOS is calcium dependent (Pollock *et al*., 1991). Therefore, it is feasible that the enzyme will be activated only when $[Ca^{2+}]_i$ is at its highest level during calcium transient and then the endogenous NO produced will reduce mainly the decay time of α-thrombin-induced calcium transient.

Also KT-5823 increases only the decay time, but not the peak of the α-thrombin-induced calcium transient. However, the calcium decay constant of α-thrombin-induced calcium transient is less affected by the cyclic GMP kinase inhibitor than by eNOS inhibition. The ineffectiveness of the selective iNOS inhibitor 1400 W suggests that in our condition iNOS does not play a relevant role in the $[Ca^{2+}]_i$ regulation of endothelial cells.

Effect of NO and ANF on α-thrombin-induced calcium transient in SHR endothelial cells Our data clearly demonstrate that in heart SHR endothelial cells, NO- and cyclic GMP-dependent regulation of α-thrombin-induced calcium transient is lost. Indeed, SNAP and ANF do not antagonise the α-thrombin-induced calcium transient, nor does L-NMMA modify resting $[Ca^{2+}]_i$ or the decay time of the calcium transient. Since the time-course of the α-thrombin-induced calcium transient, and the dose-response curve of the agonist are superimposable in SHR and WKY endothelial cells, the loss of the NO- and cyclic GMP-dependent effect on SHR endothelial cells cannot be ascribed to a difference in the agonist effect. The loss of L-NMMA effect in SHR endothelial cells seems to be unrelated to eNOS expression, since we found a similar expression of the enzyme in WKY and SHR cells. As shown in Figure 10, after preincubation with ANF, cyclic GMP increases to a similar extent in SHR and WKY endothelial cells. Therefore, the loss of NO-dependent regulation of α-thrombin-induced calcium transient should be related to a down stream alteration in the cross-talk between cyclic GMP and $[Ca^{2+}]_i$. The increase in cyclic GMP induced by SNAP is less pronounced in SHR as compared to WKY, implying that soluble guanylyl cyclase can also be down-regulated in SHR. A reduced expression of soluble guanylyl cyclase has been demonstrated in the aorta of old SHR (Bauersaschs *et al*., 1998). Since preincubation of SHR endothelial cells with IBMX does not modify the α-thrombin effect and is unable to restore the inhibitory activity of SNAP, a phosphodiesterase up-regulation is not implicated in the loss of SNAP effect on SHR.

In SHR endothelial cells, different mechanisms may maintain calcium homeostasis and regulate α-thrombin-induced calcium transient, but it is tempting to implicate an altered NO regulation in the generation of calcium oscillations, which we have shown to be increased in SHR isolated cells.

Effect of SNAP on bradykinin-induced calcium transient in WKY and SHR endothelial cells Bradykinin induces a similar calcium transient on WKY and SHR endothelial cells. The data obtained using 10^{-6} M bradykinin as agonist confirm that NO does not regulate intracellular calcium in SHR endothelial cells. Therefore, in SHR endothelial cells the lack of intracellular calcium regulation by NO is independent of the agonist used to activate calcium transient.

In conclusion, our data show for the first time that in SHR endothelial cells $[Ca^{2+}]_i$ is not regulated either by NO or ANF, suggesting a global impairment of cyclic GMP- and NO-dependent calcium regulation.

The lack of NO- cyclic GMP-dependent regulation on $[Ca^{2+}]_i$ can influence the hypertensive and cardiac hypertrophic status of SHR. Pharmacological approaches aimed to restore cyclic GMP-dependent regulation and the identification and up-regulation of alternative regulatory pathways might therefore influence the progression of hypertension.

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