Effect of uncoupling endothelial nitric oxide synthase on calcium homeostasis in aged porcine endothelial cells

Emeline Perrier1, Marie-Pierre Fournet-Bourguignon1*, Emilie Royere1, Stephanie Molez1, Helene Reure1, Ludovic Lesage1, Willy Gosgnach1, Yves Frapart2, Jean-Luc Boucher2, Nicole Villeneuve1, and Jean-Paul Vilaine1

1Institut de Recherches SERVIER, 11 rue des Moulineaux, 92150 Suresnes, France.; and 2UMR CNRS 8601 Université Paris Descartes, Paris, France

Received 3 April 2008; revised 19 January 2009; accepted 23 January 2009; online publish-ahead-of-print 28 January 2009

Aims The requirement of endothelial NO synthase (NOS3) calcium to produce NO is well described, although the effect of NO on intracellular calcium levels \([\text{Ca}^{2+}]_i\) is still confusing. Therefore, NO and \([\text{Ca}^{2+}]_i\) cross-talk were studied in parallel in endothelial cells possessing a functional or a dysfunctional NO pathway.

Methods and results Dysfunctional porcine endothelial cells were obtained either in vitro by successive passages or in vivo from regenerated endothelium 1 month after coronary angioplasty. Activity of NOS3 was characterized by conversion of arginine to citrulline, BH4 intracellular availability, cGMP, and superoxide anion production. Imaging of the \([\text{Ca}^{2+}]_i\) indicator FURA 2-AM was recorded and sarco/endoplasmic reticulum \([\text{Ca}^{2+}]_i\) ATPase (SERCA) pump activity was analysed by \(^{45}\text{Ca}^{2+}\) uptake into cells. In endothelial cells with a functional NO pathway, NOS3 inhibition increased \([\text{Ca}^{2+}]_i\) and, conversely, an NO donor decreased it. In aged cells with an uncoupled NOS3 as shown by the reduced BH4 level, the increase in superoxide anion and the lower production of cGMP and the decrease in NO bioavailability were linearly correlated with the increase in basal \([\text{Ca}^{2+}]_i\). Moreover, when stimulated by bradykinin, the calcium response was reduced while its decay was slowed down. These effects on the calcium signalling were abolished in calcium-free buffer and were similarly induced by SERCA inhibitors. In aged cells, NO improved the reduced SERCA activity and tended to normalize the agonist calcium response.

Conclusion In control endothelial cells, NO exerts a negative feedback on cytosolic \([\text{Ca}^{2+}]_i\) homeostasis. In aged cells, uncoupled NOS3 produced NO that was insufficient to control the \([\text{Ca}^{2+}]_i\). Consequently, under resting conditions, SERCA activity decreased and \([\text{Ca}^{2+}]_i\) increased. These alterations were reversible as exogenous NO, in a cGMP-independent way, refilled intracellular calcium stores, reduced calcium influx, and improved the agonist-evoked calcium response. Therefore, prevention of the decrease in NO in dysfunctional endothelium would normalize the calcium-dependent functions.

KEYWORDS
Endothelial nitric oxide synthase; Nitric oxide; Tetrahydrobiopterin; \([\text{Ca}^{2+}]_i\) signalling; Ageing

1. Introduction

Endothelial nitric oxide (NO) bioavailability is an important factor as NO induces beneficial effects in vascular pathologies due to its antiatherogenic, anti-inflammatory, antithrombotic, antiproliferative, and antioxidant properties.\(^1\)-\(^5\) Production of NO by NOS3 is a highly regulated and complex phenomenon. NOS3 activity involves many cofactors, among them, the pteridine tetrahydrobiopterin (BH4) emerging as a critical determinant of NO synthesis.\(^6\) Indeed, in the absence of adequate levels of BH4, NOS3 becomes uncoupled from \(\text{L-arginine}\) oxidation and, instead, molecular oxygen is reduced to form superoxide anion \((\text{O}_2^-)\).\(^7\)

Moreover, NO production by NOS3 also requires intracellular \([\text{Ca}^{2+}]_i\) rise that could be induced either by physiological stimuli as shear stress or by endothelial receptor agonists. Calcium mobilization by receptor stimulation triggers a transient increase in \([\text{Ca}^{2+}]_i\), due to the \([\text{Ca}^{2+}]_i\) release from the inositol 1,4,5-triphosphate (IP3)\(^-\) and ryanodine-sensitive stores.\(^8\),\(^9\) The emptying of intracellular calcium stores activates the calcium entry through the plasma membrane.\(^10\),\(^11\) Calcium refilling of the endoplasmic reticulum (ER), compartment which accounts for 75% of endothelial cells intracellular calcium,\(^12\) is mainly achieved by sarco/ER \([\text{Ca}^{2+}]_i\)-transport ATPase pumps (SERCAs) and also depends on a cross-talk between ER and mitochondria.\(^13\),\(^14\) The NOS3 activity is regulated in a \([\text{Ca}^{2+}]_i\)-calmodulin-dependent manner,\(^15\) and agonist-evoked stimulation of NOS3 mainly depends on \([\text{Ca}^{2+}]_i\) entry.\(^16\) On the contrary to the calcium control of NOS3
activity, recent evidences suggested that NO and its second messenger, cGMP, are responsible for control of \( Ca^{2+} \) homeostasis in a highly complex and cell-specific manner in a lot of cells from different tissues and origins. \(^{17,18} \) Indeed, NO potentiated the \( Ca^{2+} \) entry (CCE) in pancreatic acinar cells\(^9\) and, inhibited CCE and refilled the intracellular stores in platelets and in vascular smooth muscle cells (VSMCs).\(^{20,21} \) Conversely, in cardiac myocytes, cGMP, the NO second messenger, inhibited the \( L \)-type \( Ca^{2+} \)-channel current\(^{22} \) and induced oscillatory [\( Ca^{2+} \)] in hepatocytes.\(^{23} \) In endothelial cells, the effects of NO on calcium homeostasis might be mediated through the cGMP-protein kinase G (PKG) pathway.\(^{24,25} \) However, some studies reported that NO was directly able to inhibit CCE by a cGMP-independent suppression of mitochondrial \( Ca^{2+} \) handling.\(^{26,27} \)

Actually, most of the results reported on endothelial cells are conflicting but seem to demonstrate a tight control between NO production and concentration of cytosolic calcium. However, most of these experiments were performed using cells maintained in culture for many passages\(^{28,29} \) or cell lines.\(^{30} \) These cellular models, where many intracellular signalling pathways are altered, could not be relevant for endogenous NO production.\(^{31} \) The present study aimed to assess in detail the highly complex regulation between NO and \([Ca^{2+}]_i\) and cGMP, in models where the NO pathway has already been well documented. Endothelial cells were isolated from pig vessels, known to possess vascular reactivity properties closed to the human arteries.\(^{32} \) Measurements of [\( Ca^{2+} \)]\(_i\) and cGMP were done, in parallel, in control endothelial cells after one passage. These studies were also performed in dysfunctional aged cells, as characterized by their NOS3 activity, cGMP level, \( O_2 \) studies were also performed in dysfunctional aged cells, as in control endothelial cells after one passage. These cultures were trypsinized, divided, and seeded again every week for 1 month. Passage 1 (P1) to Passage 4 (P4). These cultures were trypsinized, and BH4 content, obtained either in vivo or in vitro, inhibited the L-type \( Ca^{2+} \) entry, \( CCE \), by a cGMP-independent mechanism involved in the [\( Ca^{2+} \)]\(_i\) regulation by NO in VSMC, was also compared in these different models. Finally, the beneficial effects of exogenous NO on [\( Ca^{2+} \)]\(_i\) in pathological conditions were pointed out.

2. Methods

2.1 Animals

The experiments were performed in accordance with the recommendations of the French Accreditation of Laboratory Animal Care. Large White pigs of either sex (18–25 kg) were anaesthetized by i.m. injection of a mixture of tiletamine and zolazepam (15 mg/kg).

2.2 Primary cell cultures

Cells were harvested by gently scraping aortic and coronary arteries luminal surface and cultured in Eagle’s Minimal Essential Medium containing 10% foetal calf serum, 5 mM glutamine, penicillin-streptomycin (100 U/mL), 2.5 \( \mu \)g/mL amphotericin, and 125 \( \mu \)g/mL gentamycin. Cultured cells were seeded onto glass 24- or 96-well plates (sensoplates, Greiner) for fluorescence experiments and onto coverslips for confocal microscopy. Cultured cells were seeded onto glass 24- or 96-well plates (sensoplates, Greiner) for fluorescence experiments and onto coverslips for confocal microscopy. Uptake of DIL-acetylated low-density lipoproteins (LDLs) was used to characterize endothelial cells. All control and aged cells were able to uptake DIL-acetylated LDL.

2.3 In vitro model of ageing: successive passages

Cultured endothelial cells from aorta (PAECs) were used from Passage 1 (P1) to Passage 4 (P4). These cultures were trypsinized, divided, and seeded again every week for 1 month.

2.4 In vivo model of ageing: endothelial denudation of coronary artery

The right coronary artery was endothelium denuded using an inflated balloon catheter 1 month before pig sacrifice as already described.\(^{32} \)

2.5 Western blot analysis

Cells were scraped off into lysis buffer containing protease inhibitors. The samples were electrophoresed in a 4–20% SDS–PAGE gel and transferred to a PVDF membrane. After blocking with 5% fat dry milk, the membrane was divided into two parts. The upper part of the membrane was then incubated with 1/1000 polyclonal antibody against NOS3 (Tebu) or with 1/1000 monoclonal antibody against Phospho-Ser1177-NOS3 (BD-Pharmingen) and the lower part was incubated with 1/5000 polyclonal antibody against actin (Tebu). The membranes were then incubated with a peroxidase-conjugated secondary antibody. The bands were visualized by chemiluminescence using a Chemidoc XRS apparatus (Biorad) and quantified with Quantity One® software (Biorad).

2.6 NOS3 activity

Endothelial cells were incubated at 37°C for 45 min under agitation in a buffer containing (in mM): 10 HEPES, 145 NaCl, 5 KCl, 1 MgSO\(_4\), 0.12 Ca\(_{12}\), and 0.15 EGTA with 10 \( \mu \)M Indomethacin, 100 \( \mu \)M IBMX and 10 \( \mu \)M nor-NOHA, arginase inhibitor. Then, 1.7 \( \mu \)M [\( ^{3}H \)]-arginine (2150 TBq/mmol) was added for 5 min and cells were stimulated with 1 \( \mu \)M bradykinin for 10 more minutes. The reaction was stopped by the removal of extracellular medium and addition of NaOH 0.1 M to lyse cells. Each sample was neutralized and filtered before HPLC analysis.\(^{33} \) The NOS activity was expressed as citrulline formed in nanomoles per million cells.

2.7 Intracellular cGMP production

cGMP production was determined under basal condition and after stimulation with different agonists under agitation at 37°C as already described.\(^{35} \) Incubation times were 45 s for bradykinin and 3 min for DETA NONoate. NOS inhibitors N\(^{G}\)-nitro-L-arginine (LNA), N\(^{G}\)-nitro-D-arginine (DNA) and N\(^{G}\)-dimethyl-L-arginine (ADMA) were incubated for 45 min. Results were expressed in fentomoles per million cells.

2.8 Measurement of superoxide formation by spin trapping and electron paramagnetic resonance spectroscopy

The spin trap, 5-tert-butyloxy carbonyl 5-methyl-1-pyrroline N-oxide (BMPO), was synthesized using the procedure described by Zhao et al.\(^{34} \) The endothelial cells were incubated for 1 h in the presence of 50 mM BMPO in phosphate buffer under agitation at 37°C in the absence or presence of SOD (300 U/mL). Extracellular medium was then frozen in liquid nitrogen. Just before analysis, the sample was rapidly transferred into an Aqua X cell (Bruker, Wissembourg, France) fitted in an SHQ 001 cavity on a Bruker EPR Elexsys 500 spectrometer operating at X-band frequency (9.82 GHz) at 20°C.

2.9 Intracellular superoxide anion by flow cytometric analysis

Intracellular superoxide anion was detected through the oxidation of dihydroethidium (DHE) to ethidium.\(^{35} \) Cells were incubated under agitation for 2 h at 37°C with 2 \( \mu \)M DHE in 10 mM HEPES-Tris buffer in the absence or in the presence of PEG-SOD (210 U/mL). Ethidium fluorescence was quantified by flow cytometric analysis on a Beckman Coulter XL with excitation at 488 nm and emission at 610 nm.
2.10 Intracellular BH4 levels

BH4 levels were determined in endothelial cells lysates after oxidation with KI/I2 under acidic and basic conditions using reverse phase HPLC and fluorescent detection. BH4 contents were calculated from the difference in biopterin levels measured in the samples treated under the two different conditions.

2.11 Cytosolic Ca2+ measurements

Endothelial cells were loaded with fura-2 AM for 1 h at 37°C in the presence of pluronic F-127 (0.25%). Cells were imaged at 37°C on an inverted microscope equipped with epi-fluorescence illumination (Leica DMIRB 2). Images were recorded through a cooled charge-coupled device camera (CoolSNAP HQ; Roper Scientific) and controlled by MetaFluor 6.1 Software (Universal Imaging Corp.). Each experiment was expressed as the ratio 340/380 nm excitations at 510 nm emission after subtraction of the background signal.

2.12 Sarco/endoplasmic reticulum Ca2+ ATPase activity followed by 45Ca2+ uptake

Endothelial cells were incubated in buffer A containing (in mM): 10 HEPES, 145 NaCl, 5 KCl, 1 MgSO4, 0.12 CaCl2, and 0.15 EGTA, pH 7.45, at 37°C for 30 min. Buffer was then removed and replaced by buffer A alone or buffer A supplemented with 10 mM oxalate and 3 mM ATP for permeabilized cells (saponin 50 μg/mL, 15 min). In parallel, half of the wells were treated with 10 μM thapsigargin. 10 μCi/mL 45Ca2+ were added during 10 min for permeabilized cells and 2 h for non-permeabilized cells. After washing, the reaction was stopped by cells lysis with NaOH 0.1 N at 4°C. The amount of radioactivity was determined with a scintillation counter (Beckman).

2.13 Statistics

Experimental data are expressed as means ± standard error of the mean (SEM); n refers to the number of primary cultures. Statistical analysis were performed using Student’s t-test for paired observations or analysis of variance (ANOVA) following by Newman-Keuls test. Differences were considered to be significant at *P < 0.05, **P < 0.01, and ***P < 0.001.

3. Results

3.1 NOS3 expression, NOS3 activity, intracellular tetrahydrobiopterin levels, and superoxide anion production

The expression of NOS3 was unchanged (Figure 1A) while its phosphorylation on serine 1177, one of the active sites, was decreased in PAECs P4 in comparison with P1 (Figure 1B). In resting and BK-stimulated conditions, the production of citrulline was reduced in PAECs P4 as compared with P1 by 47 and 67%, respectively (Figure 1C). Tetrahydrobiopterin level was determined in cell lysates. In endothelial cells at P4, the intracellular BH4 level was decreased by 75% in comparison to P1 (Figure 1D). As the BH4 content was reduced, NOS3 should become uncoupled from L-arginine oxidation to produce O2−/C2. Exogenous BH4, 10 μM, for 20 min, was able to improve the basal NOS3 activity while it was unable to modify the BK response (Figure 1G). The extracellular production of O2− was quantified using EPR spectroscopy, the radical adduct formed by the addition of O2− to BMPO while the intracellular production of O2− was followed...
using a cell-permeable fluorophore, the DHE. Superoxide anion levels were increased in PAECs P4 by three times in the extracellular compartment and 36% in the intracellular compartment in comparison to PAECs P1 (Figure 1E and F).

### 3.2 cGMP and $[Ca^{2+}]_i$

An accumulation of intracellular cGMP is generated when NO activates the soluble guanylate cyclase. In resting conditions, LNA, a specific NOS3 inhibitor reduced the basal cGMP content in a concentration-dependent manner (control: 2666 ± 717 vs. LNA 1 mM: 1279 ± 538 fmol/million cells at P1, Figure 2A). In contrast, it increased basal $[Ca^{2+}]_i$, with an effect of 43% at 1 mM [0.306 ± 0.022 vs. 0.214 ± 0.006, F340/F380 ratio in arbitrary unit, A.U.], Figure 2B). Similarly, in the in vivo model of SP, gradual decrease in basal cGMP content in cultures from P1 to P4 (P1: 3131 ± 549 vs. P4: 1428 ± 154 fmol/million cells, Figure 2C) was associated with an increase in $[Ca^{2+}]_i$, (P1: 0.219 ± 0.005, P4: 0.248 ± 0.003 ratio in A.U., Figure 2D). In the in vivo model of regenerated endothelium (RE), the cGMP content was also significantly decreased by 32% in comparison to cells from native endothelium (Figure 2E) and in parallel, cytosolic calcium was increased by 16.5% (Figure 2F). Whatever the model, the decrease in NO bioavailability determined by cGMP content was always linearly correlated with the increase in basal $[Ca^{2+}]_i$, as shown in Figure 2G-I.

In stimulated conditions, bradykinin (1 μM) enhanced four times the cGMP content of endothelial cells (7644 ± 433 vs. control: 1834 ± 109 fmol/million cells at P1, Figure 3A) and induced a fast and transient rise in $[Ca^{2+}]_i$ (0.416 ± 0.014 at the BK peak vs. 0.227 ± 0.003 F340/F380 ratio in A.U, Figure 3B). The increase in cGMP production was significantly inhibited by LNA in a concentration-dependent manner with a maximal inhibition at 10 μM of LNA (1210 ± 142 vs. 7644 ± 433 fmol/million cells, Figure 3A). Calcium peak in response to BK was decreased by 20% while the time course of the second phase was not modified (Figure 3B). In the in vitro model of SP, BK-induced cGMP production gradually decreased from P1 to P4 (P1: 4340 ± 501 vs. P4: 1428 ± 154 fmol/million cells, Figure 3C) while the BK induced peak $[Ca^{2+}]_i$, response was already maximally altered from P2 (Figure 3D). Moreover, in the same conditions, cells from RE produced 31% less cGMP than cells from native endothelium (native: 7804 ± 541 and...
regenerated: 5380 ± 488 fmol/million cells, Figure 3E) and the transient intracellular Ca²⁺ release under stimulation was significantly reduced (BK-induced calcium increase in cells from native endothelium: 86 ± 11% and in cells from RE: 49 ± 9.8%, Figure 3F). Whatever the model, the second phase of the BK-induced calcium response was always more sustained in cells from SP or RE. Exogenous NO donor, DETANONOate, increased the transitory calcium peak in response to BK in aged cells (Figure 5F).

3.3 Effect of NO donor on the [Ca²⁺]i

In resting conditions, DETANONOate reduced the [Ca²⁺]i, (Figure 4A) and suppressed [Ca²⁺]i increase induced by LNA (0.1 μM) in PAECs P1 (Figure 5A). In stimulated conditions, a decrease in the amplitude of the BK-induced Ca²⁺ response was observed after 45 min of incubation with LNA (0.1 μM), while DETANONOate (10 μM) increased it (Figure 5B). An inhibitor of SERCA pump, 2,5-di-(t-butyl)-1,4-hydroquinone (BHQ) at 10 μM (Figure 4B), was able to suppress the NO donor effect on [Ca²⁺]i in resting conditions while an sGC inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (Fig 4C), was not. Moreover, NO donor reversed the effect of LNA and completely normalized the BK-induced Ca²⁺ response (Figure 5B). Similarly, in aged cells, addition of DETANONOate (10 μM) totally normalized the alteration of the basal [Ca²⁺]i in comparison to P1 (Figure 5C and D).

3.4 Specificity of NOS inhibition on [Ca²⁺]i increase

Contrary to LNA, DNA at 0.1 μM, the inactive form of LNA, had no effect on basal cGMP content (control: 3354 ± 482, DNA: 3658 ± 739 fmol/million cells at P1) and on [Ca²⁺]i (control: 0.210 ± 0.009, DNA: 0.213 ± 0.015 F340/F380 ratio in A.U.), indicating that the effects of LNA were the result of its direct effect on NOS3 activity. In contrast, the endogenous NOS3 inhibitor ADMA (10 μM) decreased the cGMP production (control: 3087 ± 347, ADMA 10 μM: 1744 ± 495 fmol/million cells) and, in parallel, increased by 43% the basal [Ca²⁺]i.

3.5 cGMP involvement in the control of [Ca²⁺]i by NO

DETONONate induced a decrease in basal [Ca²⁺]i (23% at 10 μM, Figure 4A). The inhibition of soluble guanylate cyclase by ODQ at 10 μM did not suppress the effect of the NO donor on basal [Ca²⁺]i. (Figure 4C).

3.6 Role of extracellular calcium in the N⁵-nitro-L-arginine-induced [Ca²⁺]i increase

Response to BK was measured in the presence or absence (EDTA 1 mM) of extracellular Ca²⁺ (Figure 6A and B). In the absence of extracellular Ca²⁺, LNA lost its effect on basal [Ca²⁺]i, while Ca²⁺ peak response to BK was always reduced (Figure 6A).
3.7 Effects of sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase inhibition on the [Ca\(^{2+}\)]\(_i\)

Inhibition of the ER calcium-ATPase pumps, SERCA, with thapsigargin (TG, 1 nM) led to an increase in basal [Ca\(^{2+}\)]\(_i\), and a reduced BK-induced [Ca\(^{2+}\)]\(_i\) peak (Figure 6D). In Ca\(^{2+}\)-free buffer, TG 1 nM only reduced BK-induced Ca\(^{2+}\) peak (Figure 6C).

3.8 Effects of nitric oxide on the sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase activity

Maximal SERCA activity was first determined in permeabilized cells in the presence of high ATP concentration (3 mM). An addition of 10 \(\mu\)M DETA NONOate did not modify maximal SERCA activity in cells at P1 while a low BK-induced [Ca\(^{2+}\)] peak (Figure 6D). In Ca\(^{2+}\)-free buffer, TG 1 nM only reduced BK-induced Ca\(^{2+}\) peak (Figure 6C).

4. Discussion

NO production and calcium homeostasis are key determinants for the control of many cell functions. The present study describes the reciprocal regulation occurring between these two factors in endothelial cells.

First, by modulating the NO bioavailability in porcine endothelial cells maintained 8 days in culture (one passage, P1), we have shown that NO induces a negative feedback on cytosolic [Ca\(^{2+}\)]\(_i\). In resting conditions, inhibition of NO production increased [Ca\(^{2+}\)]\(_i\) and, conversely, exogenous NO decreased it. The effect of LNA on [Ca\(^{2+}\)]\(_i\) depends only on NOS3 inhibition since DNA, its inactive form, has no effect. The endogenous NOS3 inhibitor, asymmetric \(N\)-dimethyl-L-arginine (ADMA), exhibited effects similar to LNA at concentrations corresponding to plasma levels measured in different cardiovascular diseases, indicating that this process could be relevant of human pathology.

Similar parallel measurements of NO and [Ca\(^{2+}\)]\(_i\), were performed in porcine models of endothelial dysfunction in order to confirm this hypothesis. As ageing remains the ubiquitous main risk factor for the development of cardiovascular diseases, two models of aged cells were used.

In the first model, ageing of aortic endothelial cells was induced \textit{in vitro} by four SPs in 1 month. In parallel, a decreased phosphorylation of NOS3 on Ser1177, its active site, was found while the expression of the enzyme was unchanged. This led to a reduced basal and stimulated...
production of NO without modification of the NOS3 expression. In basal condition, this reduced bioavailability of NO is certainly the consequence of NOS3 uncoupling as shown by its increased production of $O_2^-\text{and}^2$ instead of NO and by the effect of exogenous BH$_4$, which improved the NO production. In response to BK, the alteration of the NO production could not be modified by the addition of BH$_4$, suggesting that the reduced phosphorylation of NOS3 at Ser1177 could be pivotal in the stimulated NO production. Many studies have shown that BH$_4$ contributed to electron transfer and stabilization of the NOS3 dimer and suggested that when BH$_4$ levels decline, NADPH oxidation and $O_2$ reduction are uncoupled from arginine oxidation and NO formation. In this study, as expected, BH$_4$ content was decreased at P4 in comparison to P1, indicating an uncoupling of NOS3 during ageing. Supplementation with exogenous BH$_4$ was able to improve the NOS3 activity in basal condition while in response to agonist it has no effect.

In the second model, endothelial cells were obtained from regenerated coronary endothelium 1 month after angioplasty. This procedure, equivalent to the human one, induces a complete removal of the endothelial cells. The complete regeneration process requires approximately 1 month, during which cells use their limited ability to divide and become senescent. Their size progressively changes and some of them become flattened and enlarged. Previous studies have shown a close relationship between these phenotypic changes and the appearance of endothelium dysfunction. This dysfunction was characterized by an alteration of NO endothelium-dependent relaxations of injured porcine coronary arteries and by a reduced NO production of regenerated endothelial cells, while NOS3 expression was not modified.

In these two models, representative of human endothelial dysfunction, the decrease in NO bioavailability was always associated with an increase in basal cytosolic [Ca$^{2+}$]. Importantly, a negative linear correlation was established between NO level and [Ca$^{2+}$] in all the tested models, suggesting a tight cross-talk between these two messengers. A similar increase in [Ca$^{2+}$] when NO production is reduced was already described in rat platelets, in red blood cells, and after a chronic inhibition of NOS3 using L-NAME, in endothelial cells of perfused juxtamedullary afferent arteriole. Therefore, in resting conditions, a slight decrease in the NO production led to a dysfunctional calcium release or uptake from the intracellular pools.

Interestingly, a similar co-regulation was described under stimulated conditions. BK, which activates NOS3 and NO production, induced a transitory increase in [Ca$^{2+}$] due to IP$_3$-dependent Ca$^{2+}$ release from the ER followed by a sustained transmembrane Ca$^{2+}$ influx corresponding to the second phase of the response. As expected, this sustained phase was suppressed when cells were incubated in a calcium-free buffer.

Under conditions leading to decreased NO bioavailability and increased basal [Ca$^{2+}$], the amplitude of the calcium response to BK was always reduced. Moreover, a sustained increase in the second phase of the BK response was observed. These effects on the calcium signalling were abolished in a calcium-free buffer. Therefore, the presence of
extracellular calcium is required for the setting up of NOS3 inhibition effects on basal [Ca$^{2+}$]. Increased cytoplasmic calcium may be the result of an abnormal calcium entry due to the depletion of intracellular stores through calcium leak by ryanodine receptors or a defective refilling of the stores by the calcium ATPase pumps. An alteration of the plasma membrane Ca$^{2+}$-ATPase may also be involved.

Thapsigargin (TG), an inhibitor of SERCAs that prevents ER refilling, induced a [Ca$^{2+}$]$_i$ rise in basal conditions, decreased the BK-[Ca$^{2+}$]$_i$ peak, and increased the sustained phase of response involving calcium influx. In absence of extracellular calcium, TG was unable to modify the basal [Ca$^{2+}$]$_i$. Remarkably, the effects of TG on BK response were similar to the effects of the NOS3 inhibitor. Consequently, as in VSMCs, a direct interaction of NO with SERCAs was considered in endothelial cells. Measurements of the uptake of [$^{45}$Ca$^{2+}$] by ER showed that exogenous NO increased the SERCA activity while, conversely, LNA decreased it. Therefore, in native PAEC, endogenous NO may reduce the intracellular calcium levels when necessary by increasing the calcium uptake by SERCAs. Similarly, using BAEC after many passages that displayed no functional NOS3, prolonged incubation with sodium nitroprusside (SNP) enhanced the calcium loading of the ER. In the same way, in cardiomyocytes, SNP activates SERCA2a leading to an increase in Ca$^{2+}$ uptake into the sarcoplasmic reticulum that prevents cytosolic Ca$^{2+}$ overload. This effect was cGMP independent in VSMCs. This might be identical in endothelial cells since exogenous NO induced a similar decrease in cytosolic calcium in the presence of ODQ, a soluble guanylate cyclase inhibitor. The underlying mechanism was not investigated. However, in smooth muscle cells, the activation of SERCA by NO was attributed to a direct S-glutathiolation of the pump by NO.

Interestingly, a reduced [$^{45}$Ca$^{2+}$] uptake was also shown in cells from regenerated compared with native endothelium, indicating that the decrease in [Ca$^{2+}$]$_i$; BK-induced response implied a default in ER calcium refilling. This result could not be attributed to a reduced expression of SERCAs as the maximal calcium uptake determined in permeabilized cells

Figure 6 [Ca$^{2+}$]$_i$ in response to BK in the presence of NOS3 (A and B) and SERCAs (C and D) inhibitors in endothelial cells at P1. Response to BK after 45 min of incubation without or with 0.1 μM LNA or 1 nM TG in the absence (A and C) or presence of extracellular calcium (B and D).
was identical in native and aged cells. Moreover, an irreversible modification of SERCAs, which was described in VSMC from atherosclerotic rabbit aorta, could not be involved as exogenous NO was able to normalize the reduced SERCAs activity in aged endothelial cells. Actually, cells from RE produced more superoxide anion, which can contribute to the alteration of SERCA activity. Altogether, these results suggest that in aged endothelial cells, whatever the model used and, in parallel to the reduced NO production, calcium entry might be increased in response to ER Ca\(^{2+}\) depletion. Different channels have been proposed to be involved in this calcium entry, among them, store-operated calcium channels: receptor-activated or transient receptor potential channels. The decrease in cytosolic calcium could worsen the NOS3 dysfunction. Importantly, this study shows that exogenous NO could normalize the alteration of calcium homeostasis in resting and in stimulated conditions. These cGMP-independent effects mediated by an increase in SERCAs activity induced refilling of intracellular calcium stores, limitation of calcium influx and, consequently, decrease in the cytosolic [Ca\(^{2+}\)]. In these conditions, calcium response to agonist was completely normalized.

In conclusion, these findings are consistent with the hypothesis that NO exerts a negative feedback on cytosolic Ca\(^{2+}\) homeostasis in order to maintain it at physiological levels. As calcium is a crucial regulator of many physiological processes such as enzymatic activities, mediators synthesis, gene expression, cell growth, division, differentiation, cell death, and apoptosis, the control of calcium homeostasis by NO could play a key role against the detrimental effects induced by excessive [Ca\(^{2+}\)], on endothelial cell function. In many cardiovascular diseases, alterations of Ca\(^{2+}\) homeostasis are concomitant with endothelial dysfunction. Thus, modulation of these parameters appears as a promising therapeutic approach.

Conflicts of interest: none declared.

References


