Sarco/Endoplasmic Reticulum Ca\(^{2+}\)-ATPase Gene Transfer Reduces Vascular Smooth Muscle Cell Proliferation and Neointima Formation in the Rat

Larissa Lipskaia, Federica del Monte, Thierry Capiod, Sabrina Yacoubi, Lahouaria Hadri, Michel Hours, Roger J. Hajjar, Anne-Marie Lompré

Abstract—Proliferation of vascular smooth muscle cells (VSMC) is a primary cause of vascular disorders and is associated with major alterations in Ca\(^{2+}\) handling supported by loss of the sarco/endoplasmic reticulum calcium ATPase, SERCA2a. To determine the importance of SERCA2a in neointima formation, we have prevented loss of its expression by adenoviral gene transfer in a model of balloon injury of the rat carotid artery. Two weeks after injury, the intima/media ratio was significantly lower in SERCA2a-infected than in injured noninfected or injured β-galactosidase–infected carotids (0.29±0.04 versus 0.89±0.19 and 0.72±0.14, respectively; P<0.05), and was comparable to that observed in control carotids (0.21±0.03). The pathways leading to proliferation were analyzed in serum-stimulated VSMCs. Forced expression of SERCA2a arrested cell cycle at the G1 phase and prevented apoptosis. SERCA2a inhibits proliferation through inactivation of calcineurin (PP2B) and its target transcription factor NFAT (nuclear factor of activated T-cells) resulting in lowering of cyclin D1 and pRb levels. By using NFAT-competing peptide VIVIT, we showed that NFAT activity is strongly required to promote VSMC proliferation. In conclusion, we provide the first evidence that increasing SERCA2a activity inhibits VSMC proliferation and balloon injury–induced neointima formation. (Circ Res. 2005;97:488-495.)

Key Words: vascular smooth muscle cell proliferation ■ gene transfer ■ SERCA2a ■ calcium signaling ■ nuclear factor of activated T-cells

Cell hyper-proliferation is an important etiology factor of cardiovascular diseases such as primary atherosclerosis, restenosis, and vein-graft disease.\(^1,2\) The neointimal vascular smooth muscle cell (VSMC) proliferation constitutes a primary cause of vascular disorders\(^1\) but, despite increasing knowledge about the cell-cycle regulation of VSMC, the molecular mechanism governing VSMC proliferation remains elusive. Thus, identifying genetic modifiers of VSMC proliferation is a major focus in cardiovascular biology and medicine.\(^3\) VSMCs have the ability to transition between quiescent differentiated and proliferating phenotypes. Acquisition of proliferating phenotype by VSMC is associated with alterations in Ca\(^{2+}\) handling supported by modification of Ca\(^{2+}\) transporter expression.\(^4\)

In quiescent VSMC, the Ca\(^{2+}\) signal consists mainly of localized elementary calcium events. The global increase in Ca\(^{2+}\) concentration is rapidly reduced by calcium pumps, keeping the cytoplasmic Ca\(^{2+}\) concentration low. Spontaneous spark frequency decreases after activation of phosphoinositol-3 kinase (PI3K) and G protein-coupled receptors, probably attributable to inhibition of the ryanodine receptor (RyR).\(^5\) Activation of PI3K and G protein-coupled receptors also results in a sustained increase in cytosolic [Ca\(^{2+}\)] attributable to the generation of repetitive Ca\(^{2+}\) waves,\(^5,6\) inhibition of Ca\(^{2+}\) pump activity,\(^7\) and increased Ca\(^{2+}\) entry.\(^8\) Chronic increases in cytosolic Ca\(^{2+}\) concentration in proliferating VSMC are supported by long-term alterations in the levels of Ca\(^{2+}\)-handling proteins such as loss of RyR and SERCA2a,\(^9\) replacement of L-type voltage-operated Ca\(^{2+}\) channels by T-type voltage-operated Ca\(^{2+}\) channels, loss of the plasma membrane Ca\(^{2+}\) pumps, and upregulation of the transient receptor potential Ca\(^{2+}\) channels.\(^4\)

Any chronic alterations in the spatio-temporal pattern of Ca\(^{2+}\) signals should alter gene expression by activating different kinases and phosphatases, modulating Ca\(^{2+}\)-regulated transcription factors such as NFAT (nuclear factor of activated T lymphocytes).

A sustained increase in cytosolic [Ca\(^{2+}\)] is necessary to activate calcineurin, a Ca\(^{2+}\)/calmodulin-dependent serine/threonine-specific protein phosphatase 2B (PP2B) that de-

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phosphorylates many proteins including NFAT, inducing its translocation into the nucleus.\textsuperscript{10,11} NFAT is exported from the nucleus after phosphorylation by the glycogen synthase kinase, GSK-3β,\textsuperscript{12} or by JNK-2.\textsuperscript{13} This antagonizes the effect of PP2B. PI3K- or Gq/11-coupled receptor agonists, known to be mitogenic factors, induce NFAT-dependent transcriptional activation in vascular myocytes.\textsuperscript{6,7,14–16}

Several previous studies have suggested that SERCA2a is involved in the control of proliferation and growth: transgenic mice with only one allele of the ATP2a2 gene (SERCA2) develop numerous cancers of the upper digestive tract and skin, and cardiac hypertrophy\textsuperscript{17,18}; low levels of SERCA2a are associated with cardiac hypertrophy both in humans and animals\textsuperscript{19–21}; mice in which SERCA2a has been replaced by SERCA2b develop cardiac hypertrophy\textsuperscript{22}; finally, loss of SERCA2a is associated with VSMC proliferation.\textsuperscript{7,9} Similarly, only SERCA2b is present in proliferating BC3H1 muscle cells whereas SERCA1, SERCA2a, and SERCA2b are present in quiescent differentiated BC3H1 muscle cells.\textsuperscript{23}

We hypothesized that increasing the rate of SR Ca\textsuperscript{2+} uptake by restoring SERCA2a expression might inhibit VSMC proliferation and prevent neointima formation induced by injury.

Materials and Methods

Injury of the Rat Carotid Artery and Adenoviral Vector-Mediated Gene Delivery

Animals were treated in accordance with institutional guidelines. The left external carotid artery from adult male Sprague-Dawley rats (Charles River, Mass) weighing 400 to 500 g was injured using a 2F Fogarty embolectomy catheter (Baxter Healthcare Corp) that was introduced into the common carotid artery through the external carotid and inflated to 2 atmospheres 3× 15 s. After both the proximal common and the proximal internal carotid arteries were clamped, viral infusion mixtures containing 1×10\textsuperscript{9} pfu of Ad-SERCA2a/GFP (Ad-S2a), or Ad-βGal/GFP (Ad-βGal)\textsuperscript{24} diluted to a total volume of 50 μL was instilled between the 2 clamps, and the external carotid artery was then ligated. Perfusion was restored through the internal and the common carotid artery after 30 minutes of instillation, and the neck incision was closed. Two weeks after surgery the animals were heparinized and carotids were collected, flushed with saline, included in cryomatrix and frozen at −80°C. Intima-to-media thickness ratio was measured from hematoxylin/eosin stained cross-sections (Figure 1D). Neointima formation in S2a-infected carotids was not different from that of control arteries (0.29±0.03, n=6 and 0.34±0.04, n=6, respectively (NS)). The I/M ratio of S2a-infected carotids whereas there was no sign of neointima formation in S2a-infected carotids. The degree of restenosis was determined by measuring the intima and media thickness and calculating the intima/media (I/M) thickness ratio (Figure 1E). I/M ratios of injured, noninfected, and β-Gal-infected arteries were 0.89±0.19, n=7 and 0.72±0.14, n=6, respectively (NS). The I/M ratio of S2a-infected carotids was not different from that of control arteries (0.29±0.04, n=6 and 0.21±0.03, n=9, respectively; NS), but I/M ratios from both control and S2a-infected arteries were significantly lower than that of injured noninfected or β-Gal-infected carotids (P<0.05). An increase of the adventitial layer was clearly visible as a consequence of injury as already reported.\textsuperscript{25}

Intracellular [Ca\textsuperscript{2+}]i Measurements

Fluorescence images of FURA-2/AM loaded cells were collected at 100 ms or 1.5 s intervals by a Sensicam QE CCD camera (PCO Computer Optics GmbH), digitized, and integrated in real time by an image processor (Metafluor). Results (ΔF/F) are expressed as ratios between 340 and 380 fluorescence signals measured during a response divided by the ratios measured in resting conditions.

Protein Analysis

The sources of primary antibodies and protein analysis protocols are detailed in expanded methods.

Confocal Microscopy

Slides were examined using a Zeiss LSM-150 confocal scanning laser microscope with a Plan Apochromat 63X objective (NA 1.40, oil immersion). All settings were kept constant to allow comparison.

Statistical Analysis

All quantitative data are presented as means of at least 3 independent experiments ±SEM. An unpaired t-test was used to calculate differences between means. Differences were considered significant when P<0.05.

An expanded Materials and Methods is available in the online data supplement at http://circres.ahajournals.org.

Results

SERCA 2a Gene Transfer Prevents Balloon Angioplasty-Associated Restenosis

SERCA2a and SERCA2b are coexpressed in the media of control carotid arteries. SERCA2a is not expressed in the media or intima 2 weeks after injury whereas SERCA2b is still present (Figure 1A). We prevented SERCA2a loss in the injured carotid arteries by infection with Ad-SERCA2a/GFP (Ad-S2a). The same concentration of Ad-βGal/GFP (Ad-βGal) was used as a control. Transfection efficacy was evaluated by using an anti-GFP antibody. Representative SERCA2a, SERCA2b, and GFP immunolabelings are presented in Figure 1B and 1C. Fluorescence of GFP and SERCA are not exactly superimposed for 2 possible reasons: (1) after 14 days the virus and GFP may already be gone whereas SERCA2a is still present because proliferation has been blocked, (2) Ad-S2a could infect not only proliferating cells, but also neighboring cells and prevent loss of SERCA2a in the entire cell population. However, in online Figure IIS, we show that Ad-S2a restores expression only in infected cells. The morphometric analysis was performed on hematoxylin/eosin stained cross-sections (Figure 1D). Neointima formation was observed in injured noninfected and injured β-Gal-infected carotids whereas there was no sign of neointima formation in S2a-infected carotids. The degree of restenosis was determined by measuring the intima and media thickness and calculating the intima/media (I/M) thickness ratio (Figure 1E). I/M ratios of injured, noninfected, and β-Gal-infected arteries were 0.89±0.19, n=7 and 0.72±0.14, n=6, respectively (NS). The I/M ratio of S2a-infected carotids was not different from that of control arteries (0.29±0.04, n=6 and 0.21±0.03, n=9, respectively; NS), but I/M ratios from both control and S2a-infected arteries were significantly lower than that of injured noninfected or β-Gal-infected carotids (P<0.05). An increase of the adventitial layer was clearly visible as a consequence of injury as already reported.\textsuperscript{25}

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Cell Culture

VSMC were isolated from the media of the thoracic aorta from male Wistar rats and cultured as described.\textsuperscript{7} Cells were used between passages 2 to 8. Cells were infected with adenovirus for 48 hours at 1 to 10 pfu/cell. Proliferation was measured by using CellTiter96 Cell Proliferation Assay kit (Promega). Cell cycle was analyzed using a FACS Vantage (Becton-Dickinson) flow cytometer.

For transient transfection and reporter gene assay, cells were infected with Ad-S2a for 48 hours, then cotransfected using FuGene 6 (Roche) with NFAT- promoter-luciferase construct (NFAT-Luc, Stratagene) and CMV-promoter-β-galactosidase construct (pCMV/β, clontech). The luciferase activity was measured by using “the luciferase assay kit” (Promega) and normalized to the β-galactosidase activity. It was expressed as percent of control in relative luciferase units.
SERCA2a Prevents Proliferation-Induced Changes in Phenotype and Apoptosis

Proliferation of VSMC is characterized by decreased expression of smooth muscle myosin heavy chains 1 and 2 (SM1 and SM2) and increased nonmuscle myosin heavy chain B (NM-B). SM1 and SM2 were present in injured S2a-infected and control arteries, but only at low level in injured noninfected or βGal-infected arteries where a high level of NM-B was detected (Figure 2). No apoptosis was detected in the media of S2a-infected arteries, whereas apoptosis was observed in neointima, media and adventitia of injured noninfected or β-Gal-infected arteries (Figure 2).

To decipher the mechanism involved in inhibition of proliferation and apoptosis by SERCA2a we have used cultured rat aortic VSMC. Serum (10%) was used to induce proliferation because neointima formation is promoted by various mitogens released into the serum by the endothelium and blood cells. Freshly dissociated quiescent VSMC display “contractile” phenotype associated with expression of smooth muscle-specific myosins SM1 and SM2 (online...
Both SERCA2a and SERCA2b were expressed in these cells, as described.\(^9\) Proliferating cells did not contain SERCA2a and only low levels of SM1 and SM2, but still expressed NM-B and smooth muscle cell specific markers such as calponin and caldesmon (Figure 1S). Thus, at the time of infection, VSMCs were in the synthetic phenotype. SERCA2a expression was restored by adenovirus infection, but the level of SERCA2b did not change after infection (Figure 3A).

Measuring GFP fluorescence allowed monitoring the efficacy of adenoviral infection that was increased significantly 4 days after infection (online Figure IIS). Thus, experiments were performed on day 4 after infection. At this stage, flow cytometry showed that 60% to 90% of cells were GFP-positive.

Between days 0 and 4, control and Ad-\(\beta\)Gal-infected cell numbers increased 5-fold, whereas the number of Ad-S2a-infected cells increased less (\(P<0.001\)) (Figure 3B). More than 50% of the cells in Ad-S2a-infected cultures were arrested in G\(_1\), whereas most cells in control and Ad-\(\beta\)Gal-infected cultures were in the S phase (Figure 3C). The percentage of apoptotic cells, measured by DNA fragmentation.

Figure 2. SERCA2a prevents proliferation-induced changes in phenotype and apoptosis. Analysis of SM1, SM2, and NM-B myosin heavy chains expression and apoptosis (TUNEL) in control and injured arteries 14 days after angioplasty. Bottom, media position is identified by elastin autofluorescence (green), apoptotic cells are TUNEL-positive (red), and nuclei positions are marked by Hoechst staining (blue). Bar=20 \(\mu\)mol/L.

Figure 3. Expression of SERCA2a in proliferating VSMC blocks cell cycle progression at the G\(_1\) phase. A, Immunoblot of SERCA2 isoforms in freshly dissociated or passaged control and adenovirus-infected VSMC (4 days after infection). 30 \(\mu\)g of total protein extract was loaded on each lane. B, Effect of infection with Ad-S2a or Ad-\(\beta\)Gal on proliferation of VSMC. Cells were infected on day 0 (D0) and cultured for 48 hours in the presence of virus, and for a further 48 hours in virus-free medium supplemented with 10% FCS. The number of cells was determined 4 days (D4) after the beginning of the experiment. Each point represents the mean of 5 (Ad-\(\beta\)Gal) to 12 (Ad-S2a) independent experiments. Values are plotted as the percentage of change with respect to the control on D0. ***\(P<0.001\). C, Flow scan cytfluorimetric analysis of cell cycle progression on day 4 after infection with Ad-S2a or Ad-\(\beta\)Gal. G\(_1\), S, and G\(_2\) estimated phases of cell cycle. The data shown are representative of a typical experiment, repeated at least 3 times.

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tion (TUNEL) in control, Ad-S2a-infected, Ad-βGal-infected cells, and cells treated with staurosporine (1 μg/mL, 4 hours) as positive a control, were 4.3±3.3, 0.8±0.3, 8.6±2.3, and 85±5.2, respectively (average of 3 independent experiments; 50 to 200 cells/experiment). The absence of caspase 9 cleavage (online Figure IIII) and of a sub-G₁ peak in ad-S2a-infected cells also confirmed the absence of apoptosis. Thus, SERCA2a expression inhibits progression into the cell cycle at the G₁ phase and does not induce apoptosis.

SERCA2a Expression Alters Ca²⁺ Patterns

We analyzed the effect of SERCA2a on the kinetics of the Ca²⁺ transient by measuring ATP-induced Ca²⁺ levels in Ad-S2a- or Ad-βGal-infected cells and in noninfected cells (Figure 4). The amplitude of the [Ca²⁺]i increase was dependent on the ATP concentration in both SERCA2a-expressing and control cells. Graded [Ca²⁺]i increases were observed after successive applications of 10 and 100 μmol/L ATP on the same cell in control and SERCA2a-expressing cells in Ca²⁺-free medium. An increase from 10 to 100 μmol/L ATP evoked a 2-fold increase in the amplitude of the Ca²⁺ signals, but the amplitude of the [Ca²⁺]i increase in SERCA2a-expressing VSMC was 6-fold lower than that in control cells after stimulation with either 10 (DF/F: 0.19±0.04, n=6 versus 1.27±0.21, n=17; P<0.05) or 100 μmol/L ATP (0.41±0.13, n=6 versus 2.54±0.32, n=15; P<0.05). However, the responses to 20 μmol/L tBuBHQ were larger in SERCA2a-expressing cells (0.40±0.03, n=5 versus 0.15±0.02, n=5; P<0.01), suggesting increased Ca²⁺ store.

Two features were suggestive of a reduction in the global Ca²⁺ signals in SERCA2a-expressing cells. First, the oscillations observed in controls were totally abolished in SERCA2a-expressing cells (Figure 4). Second, video-imaging showed that Ca²⁺ signals in SERCA2a-expressing cells were restricted to the perinuclear region at 10 μmol/L ATP and slowly spread (5 to 7 s after the onset of the response) to the entire cell at 100 μmol/L ATP, whereas the increases observed at both ATP concentrations in control cells were immediate in the whole cell (see online videos 1 and 2). Thus, the apparent shift in ATP sensitivity not only reflects a decrease in the amplitude of Ca²⁺ signals but also a defect in the propagation of the signal in SERCA2a-expressing cells. Therefore, the fast recapture of the Ca²⁺ released from the intracellular stores on ATP stimulation in SERCA2a-expressing cells greatly reduced Ca²⁺ signals.

SERCA2a Inhibits PP2B

As a sustained increase in cytosolic [Ca²⁺] favors the formation of calmodulin/PP2B complexes, protein extracts were subjected to anti-calmodulin precipitation followed by anti-PP2B specific immunoblot (Figure 5). PP2B did not bind to calmodulin in SERCA2a-expressing cells or when PP2B had been inhibited by cyclosporin A (CsA). PP2B was associated with calmodulin in control cells, in Ad-βGal-infected cells and when SERCA had been inhibited by thapsigargin (Tg). The absence of the complex was not attributable to the absence of either PP2B or calmodulin because the absolute levels of these 2 proteins remained constant, as shown by immunoblot of total extracts.

In the same way we analyzed the activity of the Ca²⁺/calmodulin-dependent kinase II (CaMKII). The amount of calmodulin/CaMKII complexes did not differ between control, Ad S2a-infected, or β-Gal-infected cells but was increased by Tg. Neither the total amount of CaMKII nor the amount of its phosphorylated (autonomously active) form was affected by SERCA2a expression (Figure 5). The maximal CaMKII activity measured in vitro was not different in control, Ad S2a-infected and Ad β-Gal-infected cells: 11.01±0.26; 12.59±0.62; 11.06±0.72 pmoles ATP/min/µg proteins, respectively (NS).

Thus, the activity of SERCA2a is particularly important to maintain PP2B in its inactive form, but has little influence on CaMKII activity.
SERCA2a Controls NFAT Activity

One important role of PP2B is to dephosphorylate and translocate NFAT into the nucleus. Only NFATc3 was detected by RT-PCR in passaged VSMC (not shown). The same isoform has been reported in freshly dissociated cells and in aortic tissue as well as in cerebral arteries. Immunofluorescence demonstrated the presence of NFAT in the cytosol and in the nuclei of proliferating control and Ad-βGal-infected cultures (Figure 6A). NFAT was present mainly in the cytosol of Ad-S2a-infected cells, but inhibition of SERCA activity by Tg (1 μmol/L, 1 hour) induced its translocation to the nucleus. These results were confirmed by immunoblot of cytosolic and nuclear fractions (Figure 6B). Only one band of 115 kDa, corresponding to the predicted apparent mass of NFATc3, was detected and the total amount of NFAT was not altered by infection. Despite a high level of NFAT in the cytosolic fraction of Ad-S2a-infected cells, the nuclear fraction contained little NFAT. The amount of phosphorylated GSK 3β (inactive form) was not altered by SERCA expression or activity. Furthermore, NFAT-binding activity was analyzed by electromobility shift assays (Figure 6C). High NFAT-binding was detected in proliferating control cells. The binding was specific, because it was inhibited by a 50-fold excess of NFAT cold probe. CsA inhibited DNA-protein complex, as expected. We detected low NFAT-binding in Ad-S2a-infected cells, but inhibition of SERCA by Tg restored DNA-protein interaction. The results from NFAT-driven luciferase reporter gene assay also confirmed that Ad-S2a inhibits the transcriptional activity of NFAT (Figure 6D). Tg increased luciferase activity in both control and Ad-S2a-infected cells, but NFAT-driven luciferase activity was lower in Ad-S2a-infected cells. Greater increase in luciferase activity in control can be explained by increased number of transient receptor potential Ca2+ channels in proliferating cells. As expected, CsA completely blocked NFAT promoter activity. These results demonstrate that SERCA2a activity controls NFAT transcriptional activity.

Next, we examined whether SERCA is involved in the regulation of expression or activity of proteins required for passage of G1/S checkpoint and known to be regulated by NFAT. The amounts of cyclin D1 and phosphorylated retinoblastoma protein (pRb) were decreased in SERCA2a-expressing cells as well as in cells treated with CsA (Figure 6E). Finally, we infected proliferating VSMC (in the presence of 10% FCS) with the NFAT-competing peptide, VIVIT. Four days after infection, the number of cells in Ad-VIVIT-infected cultures was significantly less than in controls (24.6 ± 1.6% versus 100 ± 4.7%, P < 0.001) and similar to cells cultured in the presence of 0.5% FCS (22.8 ± 1.7%). These results demonstrate that NFAT transcriptional activity is required for VSMC proliferation.

Discussion

We demonstrate that SERCA2a is a powerful regulator of VSMC proliferation in vivo and in vitro. This conclusion is based on several lines of evidence. First, SERCA2a expression is markedly downregulated in highly proliferating VSMCs in vivo in a model of balloon injury of the rat carotid
artery. Second, SERCA2a is repressed in vitro in serum-induced proliferation and in proliferation induced by PDGF or very low density lipoproteins. Finally, we show here that overexpression of SERCA2a inhibits mitogenic stimuli-mediated-proliferation of VSMC and blocks balloon injury-induced neointimal VSMC proliferation in vivo. These observations suggest that SERCA2a expression and activity are necessary to maintain a nonproliferating state of VSMC and that deregulation of SERCA activity and thus abnormal Ca\(^{2+}\) dynamics results in proliferation.

SERCA2a and SERCA2b are both expressed in VSMC from the carotid artery but the relative amount of each isoform was not determined. We have previously shown by S1 nuclease assay that, in control aorta, the mRNA encoding SERCA2a and SERCA2b represents 30% and 70% of total SERCA2 mRNA respectively. Because Western blot was not possible on injured arteries because of the lack of tissue and immunofluorescence is not quantitative, we conclude that SERCA2a disappears after balloon-injury, but whether SERCA2b increases is not clear. In culture, Western blot (Figure 3A) shows that SERCA2a gene transfer did not affect SERCA2b expression. Balloon injury probably alters expression of other SR Ca\(^{2+}\)-handling proteins, but this has not been analyzed yet.

We have previously shown that induction of VSMC proliferation is associated with prolongation of ATP-induced Ca\(^{2+}\) waves because of a slower rate of Ca\(^{2+}\) clearance. This prolongation of Ca\(^{2+}\) waves or sustained increase in cytosolic Ca\(^{2+}\) level is associated with the activation of the PP2B signaling pathway. Here, we demonstrate that reexpression of SERCA2a in passaged VSMC reduces both the amplitude and propagation of IP3-evoked Ca\(^{2+}\) signals. The decrease in the amplitude of the Ca\(^{2+}\) wave is in agreement with results observed in SERCA2a-overexpressing CHO cells and is consistent with the rapid reuptake of Ca\(^{2+}\) in the endoplasmic reticulum resulting in a higher endoplasmic reticulum Ca\(^{2+}\) level.

We found that reducing cytosolic Ca\(^{2+}\) by means of SERCA2a expression inhibited the activity of PP2B without affecting CaMKII activity. PP2B activity appears to be important for proliferation and hypertrophic growth because: (1) inhibition of PP2B by VIVIT or CsA arrested the proliferation of VSMC (present study and Yellaturu et al\(^{15}\)); (2) PP2B can activate hypertrophic signals both in vitro in neonatal myocytes and in vivo in transgenic mice as well as in the myocardium of patients with pressure overload. Inactivation of PP2B by SERCA2a expression has at least 1 major consequence that is inhibition of NFAT transcriptional activity. SERCA2a does not affect the CaMKII in agreement with the notion that this pathway is more dependent on Ca\(^{2+}\) influx. Yet, we cannot exclude the possibility that other Ca\(^{2+}\)-dependent signaling pathways are altered by SERCA2a. At least 3 members of the NFAT family (NFATc1, NFATc3, and NFATc4) appear to induce proliferation (reviewed by Chen\(^{4}\)). Experiments using mice with targeted disruption of NFATc3 or NFATc3c4 have shown that these isoforms are essential for myogenesis, ventricular myocyte proliferation, and vascular wall assembly. NFAT family members have been implicated in both cardiac hypertrophy and insulin-like growth factor 1-induced skeletal myocyte hypertrophy. The targets of NFAT, responsible for induction of proliferation, have not yet been clearly identified. Neel et al reported that NFATc1 controls cell cycle progression in 3T3-L1 preadipocytes by inducting the cell cycle-related genes encoding cyclin D1, cyclin D2, c-myc, and increasing Rb phosphorylation, all of which are required for passage of the G1/S checkpoint. Here we show that suppression of NFAT activity in VSMC decreases the amount of cyclin D1 and pRb.

Apoptosis has been described in both neointima, media and adventitia with a maximum in neointima and media between 7 and 14 days after surgery. We demonstrate here that apoptosis was present at 7 days in Ad-\(\beta\)Gal-infected arteries and was prevented by infusion of Ad-S2a.

Restoring SERCA2a levels or ablating its inhibitor phospholamban by gene transfer improves function, metabolism, and survival in a rat model of heart failure and in myocytes isolated from patients with end-stage heart failure and clinical trials of SERCA2a and phospholamban gene therapies for heart failure are impending. This study provides new evidence that SERCA2a and its effect on calcium handling also induces a beneficial effect on VSMC proliferation and vascular remodelling. SERCA could be a new target for therapeutic efforts of VSMC disorders.

The carotid injury model used in this study has limitations because it addresses only one part of the whole spectrum of coronary artery disease. However, abnormal regulation and proliferation of vascular smooth muscles at atherosclerotic sites in coronary arteries is an important manifestation of the disease-state.

In conclusion, we provide the first evidence that increasing SERCA2a activity inhibits balloon injury-induced neointima formation. By lowering the cytosolic [Ca\(^{2+}\)], SERCA2a inactivates PP2B and its downstream signaling cascade, resulting in decreased NFAT transcriptional activity.

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EXPANDED MATERIAL AND METHODS

**Material.** All media, sera and antibiotics were purchased from Invitrogen (Cergy Pontoise, France). Cyclosporin A (CsA), thapsigargin (Tg), ATP and 2.5-di-(t-butyl)-1.4-benzo-hydroquinone (tBuBHQ) were from Sigma-Aldrich (France). The following primary antibodies were used: a-NFATc1 (K-18, Santa Cruz Biotechnology), a-pGSK-3β (9916, Cell Signaling), a-SERCA2a and a-SERCA2b were provided by Dr F. Wuytack, university of Leuven, Belgium \(^1\), a-calmodulin (Zymed), a-PP2B (BD Transduction Laboratories), a-SM2 (Ab 683, Abcam), a-NM-B MHC (Ab 684, Abcam), a-SM1 (MCA MH-01, Yamasa, Japan), a-calponin, (C2687, Sigma-Aldrich) a-caldesmon (C4562, Sigma-Aldrich), a-CaMKII (611292, BD Transduction Laboratories), a-pCaMKII (06-881, Upstate), a-PCNA (ab912, Abcam), a-caspase 9 (9506, Cell Signaling) a-pRb(Ser780) (9307, Cell Signaling), a-cyclin D1 (556470, BD Biosciences). AdVIVIT was provided by Dr. Christopher M. Norris (the Sanders-Brown Center on aging, Lexington, KY-USA).

**Injury of the rat carotid artery and adenoviral vector-mediated gene delivery.** Animals were treated in accordance with institutional guidelines. Adult male Sprague-Dawley rats weighing 400 to 500 g were anesthetized with Pentobarbital (50 mg/kg IP). Heparin (35 IU) was administered by IP injection. The left external carotid artery was then exposed and a 2F Fogarty embolectomy catheter (Baxter Healthcare Corp) was introduced into an external carotid arteriotomy incision, advanced to the common carotid artery, and inflated to 2 atmospheres and withdrawn 3 times with rotation. The catheter was then removed, and a dwelling catheter was introduced into the arteriotomy site. After both the proximal common carotid artery and the proximal internal carotid artery were clamped, viral infusion mixtures with 1x10\(^{10}\) pfu of Ad- SERCA2a/GFP (Ad-S2a) or Ad-βGal/GFP (Ad-βGal) \(^{188}\) diluted to a total volume of 50 µL was instilled via the arterial segment between
the 2 clamps, and the external carotid artery was then ligated. Perfusion was restored in the common carotid artery after 30 minutes of instillation, and the neck incision was closed using 4-0 silk sutures. Two weeks after surgery both carotids were collected, flushed with saline, included in cryomatrix and frozen at –80°C.

**Morphometric analysis.** In preliminary experiments, we collected the vessels 3, 7, 14 and 30 days after surgery. Two weeks was used for further studies because both expression of GFP and neointimal proliferation were clearly visible. Neointima thickening was assessed by measuring with a computer-based (Lucia, Nikon) morphometric system, the thickness of the intima and media from haematoxylin-and eosin-stained cross-sections and calculating the intima-to-media ratio. Five measurements were done on each section and 5 to 10 discontinuous sections were analyzed for each vessel. The entire length of the carotid was sectioned either longitudinally or transversally. Expression of GFP was visualized on unfixed sections. The left carotids that did not display GFP were considered as injured non-infected. The sections were fixed in 4% paraformaldehyde or in acetone and used for morphometric analysis or for immunofluorescence, respectively. To assess efficacy of gene transfer, longitudinal sections from each vessel were labeled with FITC labelled a-GFP (Abcam, UK) and anti-SERCA2a antibody followed by TRITC-labelled secondary antibody. Apoptosis was analyzed by TUNEL staining (ApopTag Red, Serologicals Corporation).

**Flow cytometry.** The cell cycle was analyzed by flow cytometry using a FACS Vantage (Becton-Dickinson, Le Pont de Claix, France) flow cytometer. The cells were recovered by centrifugation at 12,000g, 30 sec. Nuclei were released in Galbraith buffer (45 mmol/L magnesium chloride, 30 mmol/L sodium citrate, 20 mmol/L 4-morpholinepropane sulfonate) supplemented with 0.1%(w/v) Triton X-100, pH 7, fixed in formaldehyde (2%), filtered
through a nylon cloth (pore size 25 nm) and stained with propidium iodide (2 g/L). The stained nuclei were excited by an argon laser (Spectra-Physics 2017, Mountain View, CA, USA) tuned at 488 nm and the emission of fluorescence was collected through a 630/22 band pass filter. Analysis was performed on over 10,000 nuclei for each sample. Data were collected with the Cellquest software (Becton Dickinson, Mansfield, USA).

**Intracellular [Ca^{2+}]_{i} Measurements.** Cells were plated on collagen-coated glass delta T culture dishes (Bioptechs, Butler, PA, USA) and cultured with or without viruses as described above. Cells were loaded with 4 µmol/L Fura-2/AM (Molecular Probes) for 45 minutes at 37°C in culture medium, then washed in recording solution containing (mmol/L): NaCl, 116; KCl, 5.6; MgCl₂, 1.2; NaHCO₃, 5; NaH₂PO₄, 1; EGTA, 0.1; HEPES, 20; pH 7.3. ATP (10 to 100 µmol/L) or TbuBHQ (20 µmol/L) were used in the test solution. Cells were continuously superfused with control or test solutions and kept at 37°C using a temperature-controlled dish fixed above a warmed epifluorescence 40 x oil objective (Bioptechs, Butler, PA, USA) and a PTR 200 perfusion temperature regulator (ALA Scientific Instruments, Westbury, NY, USA). The excitation light was supplied by a high pressure 100 W xenon arc lamp and the 340 and 380 nm wavelengths selected by a monochromator (Cairn Research Ltd, Faversham, Kent, UK). Fluorescence images were collected by a Sensicam QE CCD camera (PCO Computer Optics GmbH, Kelheim, Germany), digitized, and integrated in real time by an image processor (Metafluor, Princeton, NJ, USA). Images were collected either at 100 ms or 1.5 s intervals. The fast rate of acquisition was made possible by collecting the 380 nm fluorescence signal only, with the 340 nm fluorescence signal being measured before and after the fast stream. The background fluorescence signals at 340 and 380 nm were collected at the same intervals and subsequently subtracted from the corresponding fluorescent images. Results (ΔF/F) are expressed as ratios between 340 and 380 fluorescence signals measured during a
response divided by the ratios measured in resting conditions, i.e. before the addition of an agent.

Confocal microscopy. Immunocytochemistry was performed on methanol-fixed cells or acetone-fixed sections according to a standard protocol. Proteins were visualized by using either secondary antibodies directly conjugated to Texas Red or by using the biotin/streptavidin-Texas Red conjugated amplification method (Amersham).

Slides were examined with a Zeiss LSM-510 confocal scanning laser microscope equipped with a 25 mW argon laser and a 1 mW helium-neon laser, using a Plan Apochromat 63X objective (NA 1.40, oil immersion). Green fluorescence was observed with a 505-550 nm band-pass emission filter under 488 nm laser illumination. Red fluorescence was observed with a 560 nm long-pass emission filter under 543 nm laser illumination. Pinholes were set at 1.0 Airy units. Stacks of images were collected every 0.4 µm along the z-axis. Three median slices were projected for NFAT samples. All settings were kept constant to allow comparison. For double immunofluorescence, dual excitation using the multitrack mode (images taken sequentially) was achieved using the argon and He/Ne lasers.

Protein analysis. Total cell lysates were prepared according to a standard protocol, cytosolic and nuclear fractions were obtained by hypotonic lysis. Coimmunoprecipitation was performed as described. Proteins were visualized by using the enhanced chemiluminescence detection system (ECL+, Amersham). Electromobility shift assay was performed as described using 32P-labeled double-stranded NFAT probe (Santa Cruz, sc-2577).
RT-PCR analysis. The presence of NFAT isoforms in passaged VSMC was analyzed by RT-PCR as described previously.\(^5,6\).

Statistical analysis. All quantitative data are presented as mean of at least 3 independent experiments \(\pm\) SEM. An unpaired \(t\)-test was used to calculate differences between means. Differences were considered significant when \(P<0.05\).

Online Video Files. Two videos FigS4video1.avi and FigS4video2.avi showing the responses to 10 \(\mu\)M and 100 \(\mu\)M ATP respectively in two smooth muscle cells infected (bottom) or not (top) with Ad-S2a can be viewed at http://circres.ahajournals.org. Frames were collected every 1.5 s and displayed at the rate of 2 frames/second. Color scale represents increases in the ratio between 340 and 380 nm excitation wavelengths with blue indicating a ratio of 1.2 and green a ratio of 2. Scale is set in order to show the small increase in the ratio in infected cells as opposed to the large increase observed in non infected cells.

REFERENCES.


Figure 1S. Pattern of gene expression in freshly isolated and cultured rat aortic VSMC. 

A. Immunoblot of gene expression in freshly isolated rat aortic VSMC (quiescent) and in primaries VSMC cultured for 6 days in the presence of FCS (10%) (proliferating). S2a – Sarco/Endoplasmic Reticulum Ca\(^{2+}\)-ATPase 2a (SERCA2a); S2b - Sarco/Endoplasmic Reticulum Ca\(^{2+}\)-ATPase 2b (SERCA2b); SM1- smooth muscle myosin heavy chain 1; SM2- smooth muscle myosin heavy chain 2; NM B – non-muscular myosin heavy chain B, PCNA – proliferating cell nuclei antigen – marker of proliferation.

B. Immunofluorescence (red) with a-calponin and a-caldesmon in passaged rat aortic VSMC (3rd passage). 2-nd ab- second antibody. Bar =10 µM.
Figure 2S. SERCA 2a expression in growing VSMC is restored by adenovirus infection.

A. Monitoring adenoviral infection by GFP fluorescence (485/530 nm) in cultured VSMC. D0 – day of infection.

B. Analysis of SERCA2 isoforms expression by immunofluorescence at D4 in Ad infected and control VSMC. a, a’, c, c’ - Ad SERCA2a/GFP-infected cells; b, b’, d, d’ – Ad βGal/GFP – infected cells. Bar = 10µM.
Figure 3S. Analysis of apoptosis in Ad-infected cultured VSMC.
Immunoblot of caspase C9 cleavage in Ad infected cells. Only full length form of caspase C9 is observed in control and Ad-S2a infected cells. Induction of apoptosis in VSMC by cycloheximide (CHX, 1 µg/ml, 12h) or by serum deprivation (SD, 4 days) is associated with appearance of cleaved caspase C9 forms - 38 kDa and 17 kDa.