Supplement Material

Analysis of gene expression by Real time PCR

Total RNA was prepared from wild-type and S1P2R-null SMCs (Qiagen Mini Kit). RNA (1 μg) was reverse transcribed using the MultiScribe reverse transcriptase system (Applied Biosystems). TaqMan (Applied Biosystems) or SYBR green (Quantace) incorporation were utilized to determine gene expression. For RT-PCR using TaqMan probes: 75 ng of cDNA was combined with SensiMix (Quantace), and TaqMan primer probe mix. For RT-PCR using SYBR green, 75 ng of cDNA was combined with a commercially available mix containing polymerase, buffer, nucleotides and internal control (Sensimix, Quantace), SYBR green (Quantace) and primers (Table I). Cycle threshold numbers were determined using an ABI 7500 thermocycler (Applied Biosystems). Specificity of primers was verified by dissociation curves, and gene expression was normalized to expression of 18S RNA or GAPDH, respectively.

GENE	SEQUENCE
S1P1R	F - CTACTCCTTGGTCACGGACTCGAA R - ACTCAGGACAATGATCACCGTCTT probe - 56-FAM/CGCCGCCTGACCTTCCGC/3IABlkFQ
S1P2R	F - AGTGACAAAAGCTGCCGAATG R - GCACGTAGTGCTTAGCATAGAGAGG probe - 56-FAM/TCTGGGTGGCTTGCCCATCCTG/3IABlkFQ
S1P3R	F - ATGTACTTTTTCATCGGCAACTTG R - ACGTCTTCCTGCCGGACATA probe - 56-FAM/TGGCCGGCATAGCATACAAGGTCAAT/3IABlkFQ
18S	F - CGGACAGGATTGACAGATTG R - CAAATCGCTCCACCAACTAA probe - 56-FAM/CACCACCACCC CGGAATCG/3IABlkFQ
SMA	F - TACATGGCGGGGACATTGAA R - CCGATAGAACACGGCATCATCA
CNN1	F - ATGCCCAGACCTGGCTCAAA R - ACTGCAGATGGGCACCAACA
SM22α	F - CAACAAGGGTCCATCCTACGG R - ATCTGGGCGGCCTACATCA
SM-MHC	F - AAGCTGCGGCTAGAGGTCA R - CCCTCCCTTTGATGGCTGAG
myocardin	F - ACACTCCTGGGGTCTGAACA R - GCGGTATTAAGCCTTGGTTAGC
GAPDH	F - GCCCAAGATGCCCTTCAGTG R - CATCCACTGGTGCTGCCAAG

Table I: Primer and probe sequences for Real time PCR

Western blot analysis

Frozen arteries were pulverized using a mortar and pestle. Tissue powder was extracted in lysis buffer (Cytoskeleton). Extracts were cleared by centrifugation prior to determining protein concentration using Precision Red protein detection reagent (Cytoskeleton). SMCs were plated into 6-well plates (100,000 cells/well) and next day, media was changed to serum-free media. After 2-3 days, cells were extracted with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Equal amounts of protein were subjected to SDS-PAGE followed by Western blotting. Blots were probed with primary antibody overnight at 4^oC and then developed with ECL (GE Healthcare). Equal loading of protein was confirmed by re-probing blots for β-actin or β-tubulin.

Chromatin immunoprecipitation (ChIP) assay

Cell Fixation and Lysis: Quiescent or stimulated (1 μmol/L S1P for 15 minutes) SMCs (10^6 cells) were fixed at room temperature by the addition of formaldehyde to the media1 (final concentration: 1.42%). After 45 min, formaldehyde was quenched with 125 mmol/L glycine for 5 minutes. Cells were harvested into ice-cold PBS, washed twice with PBS and lysed in ChIP immunoprecipitation (IP) buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl pH 7.5, 5 mmol/L EDTA, 0.5% v/v NP-40, 1% v/v Triton X-100, 1 mmol/L DTT, 10 μg/mL leupeptin, 10 mmol/L β-glycerophosphate, 10 mmol/L NaF, 0.1 mmol/L Na_3VO_4).

Chromatin Preparation: The nuclear pellet was isolated by centrifugation and resuspended to the equivalent of 2 million cells per 100 μL of IP buffer and chromatin sheared using the Biorupter (a sealed-type sonicating water bath) set to medium intensity with 2 rounds of 15 minute sonication pulses. The remaining insoluble material was removed by centrifugation.

Immunoprecipitation: The supernatant was diluted to the equivalent of 5 million cells/mL with IP buffer. An aliquot of 0.2 mL was combined with antibody (2 μg SRF, 1.5 μg Pol II, 2 μg rabbit IgG or 1.5 μg normal mouse IgG-1κ) and incubated at 4°C in an ultrasonic water bath (Branson) for 15 min. Following removal of non-specific aggregates by centrifugation, 40 μL of a 1:1 slurry of protein A sepharose beads and IP buffer without inhibitors (DTT, leupeptin, β-glycerophosphate, NaF, and Na_3VO_4) was added to samples. The mixture was incubated for 1 hr at 4^0C with constant agitation, before the beads were collected by centrifugation and washed 5 times with IP buffer without inhibitors.

DNA isolation: Beads were mixed with 100 μL of 10% Chelex-100 (w/v in water, Bio-Rad), 20 μg of proteinase K (Qiagen), incubated at 60°C for 20 min in a thermal shaker (Eppendorf), and finally boiled for 10 minutes to elute DNA. The beads were removed by centrifugation and the supernatant subjected to qPCR analysis. See Table II for primer sequences.

REGION	SEQUENCE
promoter CArGs	F - TGGCCACCCAGATTAGAGAGTTT R - CCCACTTCGCTTCCCAAACAA
intronic CArG	F - CGGTGCAGTTCCAAAAGCAG R - CCGCTGTGGTTTGCAACTCTT
+6kB negative control	F - TTCCAGGACCTTTTGCATCC R - CCGGTTAGGGTTCAGTGGTG

Table II: Primer sequences for ChIP assays

S1P2R-null arteries express less SMA compared to wild-type arteries after injury (Figure I). S1P2R-null and wild-type mice underwent ligation injury of the left carotid artery. At the indicated time points, arteries were harvested; right arteries served as controls (0 days). **(A)** Protein was subjected to Western blotting analysis for expression of SMA, SM22α, and β-actin. This experiment was repeated with similar results. **(B)** SMA expression at day 7 after injury was quantified (mean +/- S.E.M. (n=3). *P<0.05

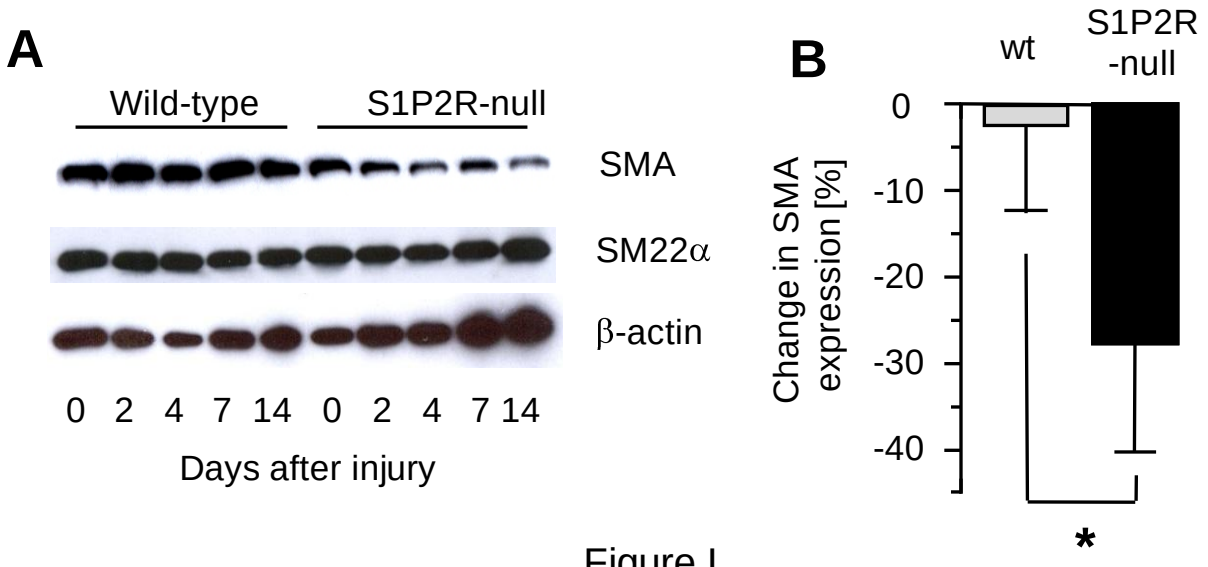

Figure I

Effect of nifedipine, BAPTA-AM and thapsigargin on KCL-induced calcium transients in SMCs (Figure II). As positive control for nifedipine, we confirmed that the drug blocks KCl-induced calcium transients. These control experiments included BAPTA-AM and thapsigargin. As expected, BAPTA-AM completely prevented KCL-mediated calcium transients, and as has been reported previously,[1] thapsigargin was also inhibitory. Quiescent wild-type SMCs were loaded with Fluo4 calcium indicator dye and pretreated with BAPTA-AM, nifedipine and thapsigargin, as described for Figure 4, before stimulation with 60 mmol/L KCl. SMCs without pretreatment served as controls. Changes in intracellular calcium levels were recorded using confocal microscopy. Typical calcium transients are shown for nifedipine **(A)**, BAPTA-AM **(B)** and thapsigargin **(C)**. All experiments have been repeated with a different cell isolate and yielded similar results.

1. Nelson EJ, Li CC, Bangalore R, Benson T, Kass RS, Hinkle PM. Inhibition of L-type calcium-channel activity by thapsigargin and 2,5-t-butylhydroquinone, but not by cyclopiazonic acid. *Biochem J.* 1994;302 (Pt 1):147-154.

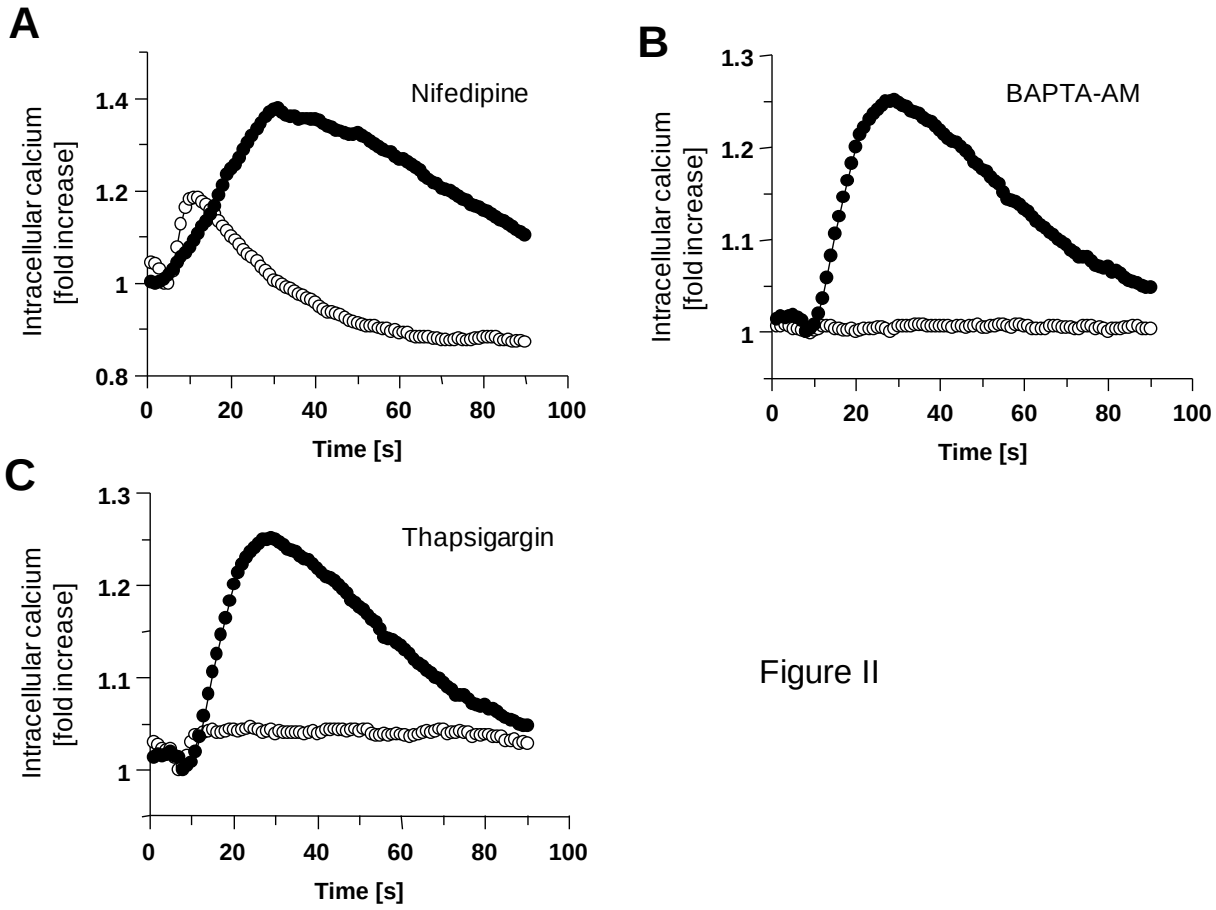
A
Nifedipine
Intracellular calcium
[fold increase]
1.4
1.2
1
0.8
0
20
40
60
80
100
Time [s]
B
BAPTA-AM
Intracellular calcium
[fold increase]
1.3
1.2
1.1
1
0
20
40
60
80
100
Time [s]
C
Thapsigargin
Intracellular calcium
[fold increase]
1.3
1.2
1.1
1
0
20
40
60
80
100
Time [s]

Figure II