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Isolation and characterization of coronary endothelial and smooth muscle cells from A₁ adenosine receptor-knockout mice

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Abstract

Mice have been used widely in in vivo and in vitro cardiovascular research. The availability of knockout mice provides further clues to the physiological significance of specific receptor subtypes. Adenosine A₁ receptor (A₁AR)-knockout (A₁KO) mice and their wild-type (A₁WT) controls were employed in this investigation. The heart and aortic arch were carefully removed and retroinfused with enzyme solution (1 mg/ml collagenase type I, 0.5 mg/ml soybean trypsin inhibitor, 3% BSA, and 2% antibiotics) through the aortic arch. The efflux was collected at 30-, 60-, and 90-min intervals. The cells were centrifuged, and the pellets were mixed with medium [medium 199-F-12 medium with 10% FBS and 2% antibiotics (for endothelial cells) and advanced DMEM with 10% FBS, 10% mouse serum, 2% GlutaMax, and 2% antibiotics (for smooth muscle cells)] and plated. Endothelial cells were characterized by a cobblestone appearance and positive staining with acetylated LDL labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate. Smooth muscle cells were characterized by positive staining of smooth muscle α -actin and smooth muscle myosin heavy chain. Homogeneity of the smooth muscle cells was ~91%. Western blot analysis showed expression of smoothelin in the cells from *passages* 3, 7, and 11 in A₁WT and A₁KO mice. Furthermore, the A₁AR was characterized by Western blot analysis using an A₁AR-specific antibody. To our knowledge, this is the first isolation and successful characterization of smooth muscle cells from the mouse coronary system.

Keywords

coronary artery; endothelial cells; smoothelin

Adenosine plays an important role in the regulation of coronary blood flow (CBF). The existence of four known receptor subtypes, A₁, A_{2A}, A_{2B}, and A₃, has been reported in most mammalian vascular beds. It is well established that coronary vasodilation is primarily mediated through A_{2A} receptor activation (13, 19, 20, 26). However, the literature suggests heterogeneity of the adenosine receptor subtypes in the regulation of CBF. We do not fully understand the signaling mechanisms of adenosine receptors in the heart because of organ

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heterogeneity. For example, after acute incubation of porcine coronary artery rings with the protein kinase C activator phorbol 12,13-dibutyrate in the organ bath studies, exposure to the A₁ adenosine receptor (A₁AR) agonist (2S)-N⁶-(2-endorbornyl)adenosine (10⁻⁹ M) reduced endothelin-1-induced contractions by 50%. Subsequently, an upregulation of protein kinase C was thought to occur through the activation of A₁AR coupled to pertussis toxin-sensitive G_i protein (8, 9, 11). However, the production of second messengers and their signaling mechanism through the activation of A₁AR in the regulation of CBF in relation to cell type (endothelial and smooth muscle cells) remain unclear. The availability of A₁AR knockout (A₁KO) mice provided a unique tool to clarify the role of A₁AR in CBF with regard to cell type.

The isolation and use of endothelial and smooth muscle cells from large vessels are well established and have been reported in species such as human and porcine (2, 12, 16). Literature shows isolation of vascular endothelial cells by perfusion of organs or large vessels with digesting enzyme (14, 17). However, no reports of similar methods are available for isolation of mouse coronary smooth muscle cell. Although there is one report of coronary endothelial cell isolation from mouse heart with use of magnetic beads and cloning techniques (12), there is no report of coronary smooth muscle cell isolation from mice.

One of the difficulties of identifying smooth muscle cells is the lack of a consistent smooth muscle cell marker because of sharing of cytoskeletal markers (e.g., smooth muscle α -actin, caldesmon, and vimentin) with other cell types, such as the myofibroblast. Recently, a cytoskeletal protein, smoothelin, was reported only in contractile smooth muscle (24). It has been found that smoothelin would disappear within days in cultured smooth muscle cells (25). However, a recent report demonstrated that smoothelin could be found in cultured porcine smooth muscle cells up to *passage 9* (2).

We present a novel way of isolating endothelial and smooth muscle cells from the mouse coronary system and demonstrate, for the first time, the presence of smoothelin in cultured mouse vascular smooth muscle cells up to *passage 11*.

MATERIALS AND METHODS

Mice

The generation and initial characterization of A₁AR wild-type (A₁WT) and A₁KO mice with a C57BL/6 background have been described previously (18). Heterozygous (+/-) mice were bred to obtain A₁WT (+/+) and A₁KO (-/-) mice. A₁WT and A₁KO mouse pairs were then set up for breeding. For genotyping by PCR, genomic DNA was isolated from tail biopsies. The DNA fragments were detected by 1.5% agarose gel electrophoresis and ethidium bromide staining. Male and female mice, 6–12 wk old, were used in all experiments. The mice were housed in community cages with 12:12-h light-dark cycles and maintained on a standard laboratory mouse diet with access to water. All animal care and experimentation were approved and carried out in accordance with the East Carolina University Institutional Animal Care and Use Committee and in accordance with the principles and guidelines of the National Institutes of Health.

Materials

Culture media and reagents, including medium 199, F-12 nutrition mixture, advanced DMEM, fetal bovine serum (FBS), PBS, trypsin-EDTA, collagenase I, trypsin inhibitor, Hanks' balanced salt solution (HBSS), and antibiotic-antimycotic, were obtained from GIBCO (Carlsbad, CA); mouse serum from Equitech-Bio (Kerrville, TX); the gelatin used

to coat the culture dishes and FITC-conjugated anti- α -smooth muscle actin antibody from Sigma (St. Louis, MO); cloning dishes from Fisher Scientific (Pittsburgh, PA); acetylated LDL labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil-Ac-LDL), rabbit anti-smooth muscle myosin antibodies, and rhodamine-conjugated anti-rabbit IgG secondary antibodies from Biochemical Technologies (Cambridge, MA); goat anti-smoothelin antibodies [smoothelin (c-20) polyclonal antibodies] from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-A₁AR antibody from Alpha Diagnostic (San Antonio, TX); and the mouse fibroblast cell line 3TC from American Type Culture Collection (Manassas, VA).

Cell Isolation

Mice were deeply anesthetized with pentobarbital sodium (25 mg/kg ip). The chest was opened to expose the heart. Ice-cold Krebs-Henseleit (KH) solution (in mM: 118 NaCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 2.5 CaCl₂, and 11 glucose) was poured onto the heart to keep it cold. The heart was excised with an intact aortic arch and immersed in a petri dish filled with ice-cold KH solution. Surrounding fat and connective tissue were removed from the heart. The aortic arch was kept intact during tissue cleaning and dissection. The cleaned heart with intact aorta was transferred to another petri dish with fresh KH solution. A 25-gauge needle filled with HBSS (in mM: 5.0 KCl, 0.3 KH₂PO₄, 138 NaCl, 4.0 NaHCO₃, 0.3 Na₂HPO₄ • 7H₂O, 5.6 D-glucose, and 10.0 HEPES, with 2% antibiotics) was inserted into the aortic lumen opening while the whole heart remained in the ice-cold buffer solution. The opening of the needle was inserted deep into the heart close to the aortic valve. The aorta was tied with the needle as close to the base of the heart as possible (Fig. 1). The infusion pump was started with a 20-ml syringe containing warm HBSS through an intravenous extension set at a rate of 0.1 ml/min. The perfusion fluid was collected in a 15-ml centrifuge tube on ice. The heart was then flushed for 15 min.

HBSS was replaced with warm enzyme solution (1 mg/ml collagenase type I, 0.5 mg/ml soybean trypsin inhibitor, 3% BSA, and 2% antibiotic-antimycotic), which was flushed through the heart at a rate of 0.1 ml/min. Perfusion fluid was collected at 30-, 60-, and 90-min intervals. At 90 min, the heart was cut with scissors, and the apex was opened to flush out the cells that collected inside the ventricle. The fluid was centrifuged at 1,000 rpm for 10 min, the cell-rich pellets were mixed with the one of the media described below, and the cells were planted in 2% gelatin-coated six-well plates and incubated in 5% CO₂-95% O₂ at 37°C. Medium 199-F-12 medium (1:1) with 10% FBS and 2% antibiotics was used for isolated endothelial cells. Advanced DMEM with 10% FBS, 10% mouse serum, and 2% antibiotics was used for isolated smooth muscle cells. The medium was replaced 3 days after cell isolation and then once or twice each week until the cells grew to confluence.

Endothelial cells grew in clusters as primary cultures. If mixed cell types were found, the cells were cloned at this stage as follows: 1) Medium was aspirated from the culture dish and washed once with HBSS. 2) Endothelial cell clusters were marked on the plate under light microscopy, and trypsin-EDTA-soaked cloning disks were placed on top of the marked clusters. 3) After the cloning disks were incubated at 37°C for 10 min, they were transferred to a new six-well plate with fresh culture medium and further incubated at 37°C for 3 days and then removed.

Cell Identification

For characterization purposes, endothelial and smooth muscle cells from porcine coronary arteries were used. Their isolation, culture methods, and characterization have been described previously by our laboratory (10, 15).

Endothelial cell characterization—Endothelial cells were stained with the fluorescent dye Dil-Ac-LDL. Cultured smooth muscle cells were used as negative control.

A cover glass was placed in the culture dish before the cells were planted. As soon as 50% of the cover glass was covered with growing cells, it was placed in a new culture dish. Dil-Ac-LDL (in 10 μ g/ml of serum-containing medium) was added aseptically to the cover glass, and the cells were incubated for 4 h at 37°C. After they were washed several times with fresh serum-free medium, the cells were visualized under fluorescence microscopy. At this stage, the cells could be used for further culture or fixed for staining with smooth muscle α -actin and smooth muscle myosin heavy chain for confocal microscopy (see below).

Smooth muscle cell characterization—The smooth muscle cells were characterized by the following methods.

IMMUNOSTAINING: The smooth muscle cells were identified by the presence of smooth muscle α -actin and smooth muscle myosin heavy chain using monoclonal antibodies. Mouse fibroblast 3TC cells and characterized mouse coronary endothelial cells were used as negative controls.

A cover glass was placed in the culture dish before the cells were planted. As soon as 50% confluence was reached, the cover glass was removed. The cells were then fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 7 min. FITC-conjugated anti-smooth muscle α -actin antibody (1:250 dilution in PBS) was applied after the cells were washed (3 times with PBS for 5–10 min per wash). The cover glass was then incubated at 37°C for 2 h and washed with PBS, and the cells were incubated with rabbit anti-smooth muscle myosin heavy chain antibody (1:300 dilution in PBS containing 3% BSA) at room temperature. The cover glass was washed with PBS, and the cells were further incubated with rhodamine-conjugated anti-rabbit IgG secondary antibody for 2 h at room temperature. After the cover glass was rinsed first in PBS and then in distilled water, it was mounted on a slide and visualized using a confocal microscope (model LSM510, Carl Zeiss).

To examine the purity of the cell culture, smooth muscle cells were stained with the fluorescence-conjugated anti-smooth muscle α -actin and anti-smooth muscle myosin heavy chain antibodies described above and examined using flow cytometry. Briefly, trypsin was used to detach the cells from the culture dish. The trypsinized medium-cell mixture was centrifuged at 1,000 rpm for 10 min, and the cell pellet was reconstituted with 4% paraformaldehyde in PBS and incubated at room temperature for 10 min. After they were washed, the cells were permeabilized by 0.1% Triton X-100 in PBS for 7 min and then stained with anti-smooth muscle α -actin and anti-smooth muscle myosin heavy chain antibodies (see above). After they were stained, the cells were washed with PBS, kept in PBS, and subjected to flow cytometry with a FACScan flow cytometer (BD Biosciences, San Jose, CA).

SMOOTHELIN DETECTION BY WESTERN BLOTTING: We further characterized the smooth muscle cells by Western blot analysis using smooth muscle cell-specific antibody, which detected the presence of smoothelin, as described by others (25). Cultured porcine coronary artery smooth muscle cells (*passage 2*) and fresh mouse aorta were used as positive controls, whereas cultured porcine coronary artery endothelial cells (*passage 2*) were used as negative control.

Fresh aorta tissue from A₁WT mice was homogenized in lysis buffer, and total protein was isolated as described below. Cultured mouse coronary smooth muscle cells were starved with serum-free medium for 24 h and lysed in buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (vol/vol) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 0.1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, and 1 μ g/ml leupeptin. The cells were scraped and transferred to a microcentrifuge tube, sonicated briefly, and centrifuged at 12,000 rpm for 5 min at 4°C. Protein was measured by the Bio-Rad protein assay method based on the Bradford dye assay, with BSA used as standard. An equal amount of protein (40 μ g/lane) was separated on 10% SDS-PAGE. Prestained protein molecular markers (20- to 112-kDa low range) were run in parallel. Proteins were transferred to nitrocellulose membranes and probed with smoothelin antibody (1: 500 dilution) and then incubated with secondary antibody (horseradish peroxidase-conjugated donkey anti-goat IgG at 1:14,000 dilution) for 1 h at 20°C. The membranes were treated with enhanced chemiluminescence (ECL) reagent (Amersham Biosciences) for chemiluminescence detection for 1 min and subsequently exposed to ECL Hyperfilm for 1–2 min.

WESTERN IMMUNOBLOT ANALYSIS OF A₁WT AND A₁KO CORONARY SMOOTH MUSCLE CELLS WITH ANTI-A₁AR ANTIBODY: Brains from A₁WT and A₁KO animals were taken as positive and negative controls, respectively. These brain samples and isolated coronary smooth muscle cells from A₁WT and A₁KO mice were homogenized in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (vol/vol) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 0.1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, and 1 μ g/ml leupeptin as described for porcine coronary smooth muscle cells (22).

For Western immunoblot analysis, protein samples (40 μ g/lane) of A₁WT and A₁KO brain and coronary smooth muscle cells were separated on 10% SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane, probed with anti-A₁AR antibody (1:1,000 dilution) for 2 h at room temperature, and incubated with secondary antibody (goat anti-rabbit IgG-horseradish-conjugated; 1:5,000 dilution) for 1 h at room temperature. The blocking peptide against A₁AR antibody was applied to the membranes as described by Tsutsui et al. (23). The molecular size of the A₁AR-expressed protein bands was determined by comparison with prestained standard proteins in the adjacent lane. For chemiluminescence detection, the membranes were treated with ECL reagent (Amersham) for 1 min and subsequently exposed to ECL Hyperfilm.

RESULTS

Mouse Endothelial Cells

Cell morphology—The coronary endothelial cells were collected as eluate with collagenase. The cells were plated in medium 199-F-12 medium and showed a mixed type of cells in the culture dishes. On the basis of their morphology, shape, and size, the endothelial cells prospered, and other cell types did not survive. Initially, endothelial cells grew in clusters that later grew with a cobblestone appearance and became more uniform endothelial cells (Fig. 2A).

Immunocytochemistry—After endothelial cells were stained with Dil-Ac-LDL, with smooth muscle cells as negative control (Fig. 2, B and D), we observed 100% purity (Fig. 2, A and C). However, a long time (1–2 mo) is usually required for growth of the cells, especially at the primary passage. When the cells were double stained with anti-smooth muscle α -actin and anti-smooth muscle myosin heavy chain fluorescence-conjugated

antibodies, they showed light background stain (Fig. 3, A and D), which clearly indicates that they were not smooth muscle cells.

Mouse Coronary Smooth Muscle Cells

Cell morphology—Mouse coronary smooth muscle cells were identified on the basis of their pattern of growth, morphology, and immunohistological staining and flow cytometry characteristics. Initially, cells started to divide in 5–7 days, especially in areas where they showed as a cluster. When the cells grew to higher density, they formed longitudinal bands of parallel cells and became thinner. This growth pattern was observed in all primary cultures of mouse coronary smooth muscle cells. As soon as cells became confluent, they grew in a “hill-and-valley”-like pattern, as observed by a phase contrast microscope (Fig. 2B). The smooth muscle cells grew to confluency in 10–14 days. We were able to grow these smooth muscle cells up to *passage 11* (Fig. 4).

Immunocytochemistry—The cultured coronary smooth muscle cells stained positive to smooth muscle α -actin and smooth muscle myosin heavy chain, as described by others (1, 5). The staining revealed typical straight, noninterrupted, cable-like fibers running parallel to each other along the long axis of the cells. When the cells became confluent, the cytoplasm of the coronary smooth muscle cells was filled densely with arranged fibers that stained strongly for smooth muscle α -actin and smooth muscle myosin heavy chain (Fig. 3, B and E). To ascertain the purity of coronary smooth muscle cells, we used mouse coronary endothelial cells (Fig. 3, A and D) and a 3TC mouse fibroblast cell line (Fig. 3, C and F), which did not show significant staining, as negative controls. These data demonstrate that our cultured cells were, in fact, smooth muscle cells.

Flow cytometry—To further ascertain the purity of coronary smooth muscle cells, the cells were stained with the same conjugated antibodies and examined using flow cytometry. We found that 97.41% of the cells were positively stained for anti-smooth muscle α -actin antibodies and 91.23% positively stained for anti-smooth muscle myosin heavy chain (Fig. 5). These results demonstrated >91% purity of coronary smooth muscle cells.

Immunochemical Identification of Smoothelin in Mouse Coronary Smooth Muscle Cells From Various Passages

The polyclonal antibody was capable of detecting at least two isoforms of smoothelin: the long (94-kDa) isoform was expressed in all vascularized organs, and the short (59-kDa) isoform was expressed in visceral muscle tissue. Both isoforms of smoothelin were present in porcine and mouse coronary smooth muscle cells in *passage 2*, but not in porcine and mouse coronary endothelial cells (Fig. 6). Further analysis of smoothelin in various passages showed the presence of smoothelin in A₁WT and A₁KO coronary smooth muscle cells up to *passage 11* (Fig. 6, B and C). Smoothelin is also present in fresh aorta from A₁WT mice (Fig. 6A).

The antibody recognized the smoothelin at ~59 and 94 kDa on Western blot analysis in A₁WT and A₁KO mice (Fig. 6A). The specificity of bands from each passage was further confirmed by displacement of the immunoreactive bands with the smoothelin-blocking peptide. Porcine coronary artery smooth muscle cells were used as positive control, whereas A₁KO coronary endothelial cells were utilized as negative control. These results confirm the expression of smoothelin and its presence in the A₁WT and A₁KO mouse coronary smooth muscle cells from *passages 3, 7, and 11* (Fig. 6B).

A₁AR Protein Expression in A₁WT and A₁KO Cells

To confirm the presence and absence of A₁AR in A₁WT and A₁KO cells, the expression of functional A₁AR in the presence and absence of A₁AR-blocking peptide was studied. We used an antibody specific for A₁AR proteins. The antibody recognized a ~36-kDa protein on Western blot analysis in A₁WT and A₁KO coronary artery smooth muscle cells (Fig. 6D). The specificity of the bands from each sample was confirmed by blocking the immunoreactive bands with A₁AR-blocking peptide (Fig. 6D). The A₁AR-enriched brain of A₁WT mice was used as positive control. The densitometric values for brain and coronary smooth muscle cells of A₁KO mice remained undetected in the presence and absence of A₁AR-blocking peptide. These data conclusively demonstrate the existence of functional A₁AR protein expression in A₁WT but not in A₁KO mice.

DISCUSSION

In the present study, we successfully isolated and characterized primary cell cultures of endothelial and smooth muscle cells from the coronary vessels of A₁WT and A₁KO mice. We obtained coronary endothelial and smooth muscle cells that exhibited morphological activity and immunoreactivity consistent with observations of other investigators in other species (2, 12, 16). Similar growth patterns were observed in all primary cell cultures of A₁WT and A₁KO mouse coronary endothelial and smooth muscle cells (Fig. 2). Also, a similar pattern of negative staining was observed for endothelial cells and positive staining was observed for smooth muscle cells immunostained with anti-smooth muscle α -actin and anti-smooth muscle myosin heavy chain antibodies (Fig. 3). The data used to characterize the endothelial cells also clearly show that these cells were indeed endothelial cells (Fig. 2, A and C).

Murine endothelial cells have been isolated and characterized by active uptake of Dil-Ac-LDL or a cell surface marker (4, 12, 14). The isolation of coronary endothelial cells from adult mouse heart was described by Marelli-Berg et al. (12). Briefly, endothelial cells were separated by magnetic beads using antibodies against endothelial cell surface markers (CD31, CD105, and isolectin-B4). Although this method increased the efficiency and specificity of the isolation procedure, the use of trypsin-EDTA solution may have inevitably damaged the markers from some of the endothelial cells and, hence, excluded some population of endothelial cells. Nevertheless, the use of our novel method for isolation and characterization of mouse coronary endothelial cells demonstrates that our isolation procedure is simple and may be used as a future tool for cardiovascular research.

The isolation and characterization of mouse coronary smooth muscle cells showed morphological patterns similar to those observed by others (1, 5). These cells showed positive staining with anti-smooth muscle α -actin and anti-smooth muscle myosin heavy chain antibodies (Fig. 3). These data clearly indicate that the cells were indeed smooth muscle cells.

There are very few reports regarding the isolation of smooth muscle cells from mice, possibly because of the difficulty in sustaining the cells through multiple passages. For instance, in our mouse coronary smooth muscle culture studies, some cells deteriorated after *passage 3*, but the majority of the cells survived and started to proliferate. We studied these cells up to *passage 11*. It is generally accepted that smooth muscle cells can be divided into two major phenotypes: contractile (differentiated) and synthetic (proliferative) (7). Smoothelin is the only protein marker found in contractile smooth muscle cells and has been used as an indicator of the contractile property of smooth muscle (3, 6). Because smoothelin was expressed in all our isolated coronary smooth muscle cells up to *passage 11*, the smooth muscle cells obtained using our methodology for isolation and culture may still have a

contractile property, which requires further studies. Nevertheless, this isolation method may pave the way for future studies of coronary smooth muscle cell receptor signaling mechanisms in various heart conditions. Furthermore, our A₁AR Western blotting experiments demonstrate that A₁AR expression was indeed absent in the cultured coronary smooth muscle cells from A₁KO mice. A previous study from our laboratory also demonstrated that A₁AR are involved in the regulation of vascular tone in A₁WT, but not A₁KO, mice (21). This validation proves that our cultured coronary smooth muscle cells would be a valuable tool to understand the role of A₁AR in the coronary system, including the signaling pathways for adenosine (and other) receptors in a homogenous population of coronary smooth muscle cells.

In summary, the results of this study provide the first evidence for isolation and characterization of coronary endothelial and smooth muscle cells from A₁WT and A₁KO mice, which may prove to be a significant tool for studying coronary disease and the signaling pathways of these cells.

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Fig. 1.
Simplified graphical representation of setup for isolating coronary endothelial and smooth muscle cells from mouse heart.

Fig. 2. Endothelial (A) and smooth muscle (B) cells from the coronary system of a A_1 adenosine receptor (A_1 AR)-knockout (A_1 KO) mouse (*passage 1*). C and D: cells in A and B stained with acetylated LDL labeled with 1,1'-dioctadecyl-3,3,3',3-tetramethylindocarbocyanine perchlorate. Note typical cobblestone arrangement of endothelial cells and "hill-and-valley" appearance of smooth muscle cells.

Fig. 3. Antibody staining of mouse endothelial (*A* and *D*), smooth muscle (*B* and *E*), and fibroblast (*C* and *F*) cells. Green, smooth muscle α -actin; red, smooth muscle myosin heavy chain.

Fig. 4.
Cultured coronary smooth muscle cells from A₁AR wild-type (A₁WT) control mice at passages 5 (A), 7 (B), and 11 (C) and A₁KO mice at passages 5 (D), 7 (E), and 11 (F).

Fig. 5.

Flow cytometry charts of staining of smooth muscle α -actin (A) and smooth muscle myosin heavy chain (B). Purple peaks in A and B represent nonstained control. Pink peak in A represents positive stain of smooth muscle α -actin. Red peak in B represents positive stain of smooth muscle myosin heavy chain. M1, cutoff point for estimating percentage of positive stain. Purity was 97.41% for smooth muscle α -actin-stained cells (A) and 91.23% for smooth muscle myosin heavy chain-stained cells (B).

Fig. 6.

A: typical smoothelin Western blot from porcine coronary smooth muscle cells (a), A₁WT coronary smooth muscle cells at *passage 2* (b), A₁KO coronary smooth muscle cells at *passage 2* (c), A₁WT aorta tissue (d), and porcine coronary artery endothelial cells (e). B: smoothelin Western blot from different passages of A₁WT and A₁KO coronary smooth muscle cells (CSMC): porcine coronary artery smooth muscle cells used as positive control (1), A₁KO coronary endothelial cells used as negative control (2), A₁WT CSMC at *passage 3* (3), A₁KO CSMC at *passage 3* (4), A₁WT CSMC at *passage 7* (5), A₁KO CSMC at *passage 7* (6), A₁WT CSMC at *passage 11* (7), and A₁KO CSMC at *passage 11* (8). C: Western blot samples in B in the presence of smoothelin blocking peptide. D: Western immunoblotting of A₁AR from A₁WT and A₁KO brain and CSMC samples. A ~36-kDa protein band was detected in A₁WT brain and CSMC samples, but not in A₁KO brain. In the presence of A₁AR-blocking peptide, bands of protein from brain and CSMC of A₁WT were completely abolished. Results are shown from 1 experiment that is representative of 3 independent experiments.