Pregnenolone Sulphate- and Cholesterol-Regulated TRPM3
Channels Coupled to Vascular Smooth Muscle Secretion
and Contraction

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Rationale: Transient receptor potential melastatin (TRPM)3 is a calcium-permeable ion channel activated by the
neurosteroid pregnenolone sulfate and positively coupled to insulin secretion in β cells. Although vascular
TRPM3 mRNA has been reported, there is no knowledge of TRPM3 protein or its regulation and function in the
vascular system.

Objective: To determine the relevance and regulation of TRPM3 in vascular biology.

Methods and Results: TRPM3 expression was detected at mRNA and protein levels in contractile and proliferating
vascular smooth muscle cells. Calcium entry evoked by pregnenolone sulfate or sphingosine was suppressed by
TRPM3 blocking antibody or knock-down of TRPM3 by RNA interference. Low-level constitutive TRPM3 activity
was also detected. In proliferating cells, channel activity was coupled negatively to interleukin-6 secretion
via a calcium-dependent mechanism. In freshly isolated aorta, TRPM3 positively modulated contractile
responses independently of L-type calcium channels. Concentrations of pregnenolone sulfate required to evoke
responses were higher than the known plasma concentrations of the steroids, leading to a screen for other
stimulators. β-Cyclodextrin was one of few stimulators of TRPM3, revealing the channels to be partially
suppressed by endogenous cholesterol, the precursor of pregnenolone. Elevation of cholesterol further sup-
pressed channel activity and loading with cholesterol to generate foam cells precluded observation of TRPM3
activity.

Conclusions: The data suggest functional relevance of TRPM3 in contractile and proliferating phenotypes of
vascular smooth muscle cells, significance of constitutive channel activity, regulation by cholesterol, and potential
value of pregnenolone sulfate in therapeutic vascular modulation. (Circ Res. 2010;106:1507-1515.)

Key Words: calcium channel ■ transient receptor potential ■ vascular smooth muscle ■ neurosteroid
■ cholesterol ■ interleukin

Mammalian cells contain numerous types of transmem-
brane ion channel that are permeable to Ca$^{2+}$, including many of the 28 mammalian homologs of the
Drosophila melanogaster transient receptor potential
(TRP) channel.1–3 The channels are thought to have struc-
tural similarity to α-subunits of voltage-gated K$^+$ chan-
nels, with intracellular amino and carboxy termini and four
proteins required for coordination of a single ion pore. As
with K$^+$ channels, heteromultimerization confers greater
diversity. However, unlike voltage-gated K$^+$ channels,
membrane depolarization is not the primary trigger for
channel activity. Instead, chemical factors are considered
to be primary stimuli. Details of the chemical sensing
properties are becoming apparent and hold promise for
revealing further complexity and novelty. In addition,
important roles of TRP channels have emerged, including
in sensation and cell survival, but we are far from a full
appreciation of the purposes of these channels and, in some
cases, there is relatively little understanding of TRP family
members – one example being TRPM3.
TRPM3 is a member of the M (melastatin) subtype of TRP channel.\textsuperscript{4–10} It is expressed most obviously in the brain and kidney but wider expression is also apparent. When exogenously overexpressed it forms Ca\textsuperscript{2+}-permeable nonselective cationic channels (ie, channels that are also permeable to Na\textsuperscript{+}). Ion channels form without the need for coexpression with other TRP channels and so TRPM3 seems capable of function as a homomeric channel. Substantial and species-specific splice variation is evident, the biological relevance of which has largely to be determined – intriguingly, one splicing event confers change in ionic selectivity.\textsuperscript{6,7} Activity of heterologously overexpressed TRPM3 channels has been observed to be enhanced by chemical factors including sphingosine, pregnenolone sulfate and dehydroepiandrosterone sulfate (DHEAS).\textsuperscript{5,9} In the mouse, endogenous TRPM3 is activated by pregnenolone sulfate and coupled to insulin secretion in pancreatic β-cells.\textsuperscript{9} In humans the TRPM3 gene is on chromosome 9 (9q21.11-q21.12), relatively close to regions linked to coronary artery disease\textsuperscript{11} and tentatively linked to Kabuki syndrome.\textsuperscript{12} However, no firm genetic linkage to disease has been established and there are no reports on the function or properties of vascular TRPM3 or endogenous human TRPM3.

Even before the discovery of TRP channels it was appreciated that smooth muscle cells contain voltage-gated Ca\textsuperscript{2+} channels and a variety of other Ca\textsuperscript{2+}-permeable channels.\textsuperscript{13} The smooth muscle cell has, therefore, been a focus for investigation of the relevance of TRP channels in mammalian systems.\textsuperscript{13,14} However, although mRNA analysis has indicated expression of the TRPM3 gene in vascular smooth muscle cells (VSMCs),\textsuperscript{14,15} there are no reports on the TRPM3 protein, its regulation, or function in this context.

Smooth muscle cells have crucial roles in all organs of the body. In the physiological setting the cells are most-often in a stable (nonproliferating) contractile phenotype but in development, injury and disease there is modulation to a phenotype characterized by proliferation, motility, increased secretion, and absence of contractility.\textsuperscript{16} The capacity for modulation is fundamentally important for adult life because it enables temporary physiological change, adaptation, and recovery from injury. It also plays pivotal roles in life-threatening vascular diseases. In some instances the remodeling generates potentially lethal neointimal formations following invasive procedures that include percutaneous transluminal angioplasty and coronary artery bypass graft surgery;\textsuperscript{17–19} in the latter case, remodeling is prominent in the saphenous vein, a routine graft.\textsuperscript{17,19,20} TRP channels appear to be particularly important in the remodeling situation.\textsuperscript{21} Here we report on investigation of TRPM3 in proliferating and contractile VSMCs.

**Methods**

**Human VSMCs and Murine Arteries**

Human saphenous vein segments were obtained with ethical approval and proliferating VSMCs were prepared using an explant technique. For contraction studies, 8 week old mice were killed in accordance with the UK Animals Scientific Procedures Act and thoracic aorta was mounted for isometric tension recording in a myograph. For femoral artery studies, 12 week old mice underwent femoral artery injury conducted in accord with accepted standards of humane animal care under a UK Home Office Project License.  

**Cell-Based Assays**

Intracellular Ca\textsuperscript{2+} was detected using fura-2 and measured on a 96-well fluorescence plate reader or a single-well microscope system. Membrane currents were recorded using a planar patch-clamp system in voltage-clamp mode. Secretions were quantified using ELISAs.

**Molecular Biology**

Total RNA was extracted using a Tri-reagent protocol followed by DNase I treatment and reverse transcribed before PCR analysis. Human TRPM3 cDNA was expressed under tetracycline regulation in a HEK 293 cell-line.\textsuperscript{16} For short interfering (si) RNA delivery, cells were suspended in Nucleofector solution, mixed with small interfering (si)RNA and transferred into a cuvette for electroporation.

**Antibodies and Labeling**

Rabbit polyclonal anti-TRPM3 (TM3E3) and anti-TRPC1 (T1E3) antibodies have been described.\textsuperscript{10,20} Antibody labeling of cells and intact vein sections was largely as described previously.\textsuperscript{20} For calcium imaging, cells were preincubated with TM3E3 for 3.5 hours at 37°C. For contraction studies mouse aorta was preincubated with TM3E3 in Hanks’ solution overnight at 4°C.

**Data Analysis**

Averaged data are presented as means±SEM. Data produced in pairs were compared using t tests, where statistical significance is indicated by an asterisk (P<0.05) and no significant difference by NS. Independent experiments were repeated on tissue/cells from at least 3 different patients.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Results**

**Expression of TRPM3 in Contractile and Proliferating VSMCs**

To investigate the relevance of TRPM3 to VSMCs we first focused on saphenous vein obtained from patients undergoing coronary artery bypass operations. TRPM3 mRNA was detected in samples of vein (Figure 1A). Anti-TRPM3 antibody labeled the medial layer, adventitial vessels, and intimal cells (Figure 1B), suggesting that TRPM3 occurs in contractile and preexisting intimal VSMCs. To investigate the relevance to remodeling, veins were organ-cultured for 2
weeks to elicit neointimal hyperplasia (Figure 1C). TRPM3 was detected in the neointimal cells (Figure 1C), showing that TRPM3 is a feature also of human VSMCs proliferating in situ. Further evidence of a link to remodeling came from in vivo studies in the mouse where the femoral artery was injured with a guide wire to evoke VSMC remodeling: TRPM3 was detected in, but not restricted to, remodeling VSMCs and VSMCs of the uninjured (contractile) medial layer (Figure 1D). VSMCs proliferating in planar cell culture also contained TRPM3 mRNA (Figure 1E) and showed anti-TRPM3 antibody labeling (Figure 1F), which was confirmed to reflect TRPM3 protein by specific siRNA knock-down (Figure 1F and Online Figure I). Endothelial TRPM3 was not obvious in the intact vessels labeled with anti-TRPM3 antibody (Figure 1B and 1C); consistent with this observation, cultured endothelial cells showed less TRPM3 mRNA compared with VSMCs (Online Figure II). The data suggest that TRPM3 is expressed in contractile and proliferating VSMCs.

**Pregnenolone Sulfate Responses in Proliferating VSMCs**

To investigate if TRPM3 is functional in VSMCs we made intracellular Ca$^{2+}$ measurements because TRPM3 is Ca$^{2+}$-permeable. The TRPM3 agonist, pregnenolone sulfate, elicited reliable rises in intracellular Ca$^{2+}$ that depended on extracellular Ca$^{2+}$, consistent with activation of Ca$^{2+}$-entry (Figure 2A). Nonspecific TRPM3 inhibitors (gadolinium ions and 2-aminoethoxydiphenylborate) inhibited the responses (Online Figure III), as did anti-TRPM3 blocking antibody (Figure 2B and 2C). Responses to ATP and sphingosine-1-phosphate were unaffected by TM3E3 (Figure 2C and Online Figure IV), consistent with these responses occurring via other mechanisms and confirming specificity of TM3E3. T1E3, a blocking antibody targeted to a different TRP channel (TRPC1), had no effect on pregnenolone sulfate responses (Figure 2C). Further support for the involvement of TRPM3 was the observation that transfection with TRPM3 siRNA inhibited pregnenolone sulfate responses (Figure 2C). Despite investigation of several TRPM3 siRNA molecules (eg, Online Figure I), only partial knockdown of the pregnenolone sulfate response could be achieved (Figure 2C). Whole-cell voltage-clamp recordings from VSMCs showed large membrane currents in response to pregnenolone sulfate. In 27 of 37 recordings the evoked current–voltage relationships (I-V) were relatively linear and reversed polarity near 0 mV (Online Figure V), similar to the currents reported for overexpressed human TRPM3 recorded under similar conditions. However, in the remaining recordings the I-V were outwardly rectifying and did not reverse at 0 mV, suggesting activation of additional undefined ionic mechanisms (Online Figure V). The data suggest that TRPM3 channels are functional in VSMCs.

**Sphingosine Responses in Proliferating VSMCs**

Sphingosine and dihydrosphingosine have also been suggested to be agonists at TRPM3. In VSMCs the compounds elicited Ca$^{2+}$ elevations that were similar to those of HEK293 cells.
293 cells overexpressing TRPM3 (Figure 2C and Online Figure VI).\(^\text{10}\) TM3E3 or TRPM3 siRNA suppressed the VSMC sphingosine responses, again suggesting functional TRPM3 channels (Figure 2C and Online Figure VI).

**Constitutive TRPM3 Activity in VSMCs**

TRPM3 has also been suggested to exhibit constitutive activity.\(^\text{8}\) To investigate this possibility we performed extracellular Ca\(^{2+}\)/H\(^{100}\) add-back experiments in the absence of an exogenous TRPM3 stimulator. The Ca\(^{2+}\)-entry was attenuated by TM3E3 (Figure 2D). Furthermore, electrophysiology recordings revealed that basal ionic current was inhibited by TM3E3 (Figure 2E and 2F); the I-V were mildly outwardly rectifying and reversed polarity near 0 mV (Online Figure VII). The data suggest that endogenous TRPM3 channels of VSMCs have moderate constitutive activity.

**Negative Coupling to Cytokine Secretion**

Based on prior experience with other TRP channels\(^\text{22}\) we hypothesized that TRPM3 might be relevant to VSMC secretion. Impact was found on secretion of the cytokine interleukin-6 (IL-6), which is generated during bypass surgery and stimulates neointimal formation and atherogenesis.\(^\text{23–25}\) Averaged data from VSMCs of 9 patients revealed stimulation of IL-6 secretion following TRPM3 inhibition by TM3E3 (Figure 3A), which is consistent with constitutive TRPM3 activity having tonic inhibitory effect against IL-6. Pregnenolone sulfate suppressed secretion of IL-6 or matrix metalloproteinase-9 but lacked effect on hyaluronan (Figure 3B). To investigate whether Ca\(^{2+}\) entry was the cause of the effect, cells were incubated in 1 mol/L BAPTA-AM to confer intracellular Ca\(^{2+}\) chelation via BAPTA free acid. BAPTA suppressed the inhibitory effect of pregnenolone sulfate and slightly (but not significantly) enhanced IL-6 secretion in the absence of pregnenolone sulfate (Figure 3C). Higher concentrations of BAPTA were not studied because of general suppressive effects on secretion (data not shown). The data suggest that TRPM3 activity is functionally relevant to secretion of factors including IL-6.

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**Figure 2. Functional activity of endogenous TRPM3 in proliferating VSMCs.** Data are based on intracellular Ca\(^{2+}\) measurement (A through D) or whole-cell electrophysiology (E and F) applied to human saphenous vein VSMCs. A, Responses to 25 µmol/L PregS in the presence (+Ca\(^{2+}\)) or absence (0 Ca\(^{2+}\)) of 1.5 mmol/L extracellular Ca\(^{2+}\) (representative of n/N=5/30 for each). B, Example responses to 25 µmol/L PregS after pretreatment with TM3E3 antiserum or its preimmune control. C, Mean data summarizing effects on responses to 25 µmol/L PregS, 20 µmol/L sphingosine (SPH), 100 µmol/L ATP or 1 µmol/L S1P of pretreatments with TM3E3, anti-TRPC1 antiserum (T1E3) or their preimmune controls (n/N=7/76 for TM3E3 and 6/47 for T1E3 tests and controls), or transfection with TRPM3 siRNA compared with scrambled siRNA (n/N=3/18 for each test and control). Each test data set was normalized to its own control. D, Effect of returning 1.5 mmol/L Ca\(^{2+}\) to the extracellular solution following preincubation with TM3E3 or its preimmune serum (n=6 for each; representative of n=5). Data without Ca\(^{2+}\) add-back (0 Ca) are shown for comparison. E, Example spontaneous current at the indicated voltages, showing effects of extracellular TM3E3 and then 75 µmol/L 2-aminoethoxydiphenylborate (2-APB). F, Mean data for the type of experiment shown in E normalized to currents in paired controls (TM3E3 preadsorbed to its antigenic peptide [+pep]).

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**Figure 3. Relationship to secretion from proliferating VSMCs.** A, Absolute interleukin-6 (IL-6) concentration in extracellular medium exposed to TM3E3 (n=9) or TM3E3 preadsorbed to its antigenic peptide (n=7). B, Effects 10 µmol/L PregS on secretion of IL-6, matrix metalloproteinase-9 (MMP9) and hyaluronan (n=8 patients for each condition). C, Effects 1 µmol/L BAPTA-AM (BAPT) on IL-6 secretion and its inhibition by 10 µmol/L PregS (n=7 patients for each condition). All data are from human saphenous vein VSMCs.
Positive Coupling to Contractile Function

For these studies we focused on mouse aorta where physiological contractile responses could be reliably observed. Expression of TRPM3 was confirmed by RT-PCR (Figure 4A). Isometric tension recordings revealed contractile responses to pregnenolone sulfate (≥60 μmol/L) in vessels precontracted with a submaximal concentration of the α-adrenergic agonist, phenylephrine (eg, Figure 4B). Pregnenolone sulfate responses were suppressed by TM3E3 (Figure 4B through 4D), which was validated as an inhibitor of mouse TRPM3 (Online Figure VIII). Responses to phenylephrine were unaffected by TM3E3 (Figure 4D). Pregnenolone sulfate responses occurred despite the presence of 100 nmol/L nicardipine (Figure 4E), which blocked voltage-gated Ca\(^{2+}\) channels as shown by suppression of 60 mmol/L K\(^+\) responses (data not shown). Pregnenolone sulfate failed to evoke contraction in the absence of pretone (Figure 4E). Smooth muscle cells freshly isolated from the aorta exhibited no contractility in response to pregnenolone sulfate that was similar to the current observed through exogenously expressed mouse TRPM3 channels (Online Figure IX). The data suggest the presence of functional native TRPM3 channels in contractile VSMCs which positively modulate contractile responses independently of voltage-gated Ca\(^{2+}\) channels.

Physiological Relevance of Steroid and Sphingolipid Stimulators

Concentration-response curves were generated to determine relevance to plasma concentrations of steroids (Figure 5). Although pregnenolone sulfate activated overexpressed TRPM3 at quite low concentrations, more than 1 μmol/L was required to evoke responses in saphenous vein VSMCs (Figure 5A). Similarly, higher concentrations of DHEAS were required for VSMC responses (Figure 5B and Online Figure X). The differences may be accounted for by different channel expression levels, but they also raise the possibility that TRPM3 in VSMCs does not confer sensitivity to plasma concentrations of pregnenolone sulfate or DHEAS. Sphingolipids are alternative stimulators but, despite the observed effects of sphingosine, it is difficult to generate a case that VSMCs express plasma membrane TRPM3 to sense extracellular sphingosine or related lipids including ceramide and sphingosine-1-phosphate, which do not activate TRPM3. Physiological functions of sphingosine have been suggested but serum concentrations of sphingosine do not reach the micromolar concentrations necessary to activate vascular TRPM3 (Online Figure VI). Without excluding importance of local accumulation of factors, we embarked on a chemical screen to potentially identify other natural activators.

Chemical Screening of TRPM3

176 compounds were selected for screening against HEK 293 cells with (Tet+) and without (Tet-) tetracycline-induced expression of human TRPM3. The majority of compounds were inactive as TRPM3 stimulators, despite inclusion of a large range of endogenous biologically active substances (Figure 6A and Online Figure XI). The only confirmed positive hit was β-cyclodextrin (Figure 6A and Online File I).
Suppression of Constitutive TRPM3 by Membrane Cholesterol

The β-cyclodextrins are not endogenous substances but exogenous carriers or chelators of cholesterol,29 the precursor of pregnenolone. It was striking that β-cyclodextrin stimulated TRPM3 in the absence of another exogenous activator, suggesting that constitutive activity of TRPM3 is normally partially suppressed by endogenous cholesterol of the membrane. In subsequent experiments methyl β-cyclodextrin (mβCD) was used because it has been most commonly used for extraction of endogenous cholesterol in the membrane. Cells were pretreated with mβCD alone or cholesterol delivered with mβCD as a carrier29; cholesterol and mβCD were washed out before measuring Ca2+ signals. To observe constitutive channel activity we measured Ca2+ entry in response to add-back of Ca2+ to Ca2+-free extracellular medium. The signal was inhibited by cholesterol in TRPM3-expressing HEK 293 cells (Figure 6B and 6C). Without TRPM3 expression there was significantly less Ca2+-entry and cholesterol had no effect (Figure 6C). The data support the hypothesis that TRPM3 has constitutive activity that is vulnerable to suppression by cholesterol.

Intermediate Set Point and Further Suppression by Cholesterol Loading

We hypothesized that the channels exist at an intermediate point of modulation, such that elevated cholesterol, a well-established driver of vascular disease,30,31 can act as a negative regulator of TRPM3. The hypothesis was investigated using pregnenolone sulfate as the TRPM3 stimulator to order to maximize clarity of the TRPM3 signal relative to background signals. Pretreatment with mβCD alone potentiated the effect of pregnenolone sulfate on TRPM3-expressing cells (Figure 6D) while having no effect on control cells (Online Figure XII). Exogenous cholesterol suppressed the pregnenolone sulfate response (Figure 6D). Importantly, the control Ca2+ signal (no mβCD or exogenous cholesterol) was intermediate in amplitude, between the mβCD (cholesterol extraction) and mβCD plus exogenous cholesterol (cholesterol loading) signals (Figure 6D). α-Cyclodextrin, which has
a binding cleft too small to accept cholesterol, was without effect (Figure 6E). The data suggest that TRPM3 channels normally exist at an intermediate point in relation to inhibition by cholesterol, with excess cholesterol driving further suppression of channel activity.

Relationship to Cholesterol in VSMCs

In VSMCs pregnenolone sulfate responses were enhanced by cholesterol depletion and suppressed below control amplitude by acute cholesterol loading (Figure 7A). Therefore, the endogenous channels are also at an intermediate set-point and vulnerable to suppression by excess cholesterol. To further investigate the hypothesis we mimicked the pathological cholesterol overload condition by loading VSMCs with cholesterol over 48 hours to generate foam cells (Figure 7B and Online Figure XIII). Pregnenolone sulfate responses were much-reduced in foam VSMCs (Figure 7C). Because the cell density was lower for these cells, we also used microscopy to observe responses; again the foam VSMCs had smaller responses to pregnenolone sulfate (Figure 7D and 7E). The data support the hypothesis that physiological cholesterol of the membrane partially suppresses TRPM3 activity and that excess cholesterol further suppresses the channel activity.

Discussion

Identification of functional TRPM3 in VSMCs adds to an expanding picture of TRP channels as complex and numerous players in VSMC function.13,14 We observed TRPM3 mRNA and protein in contractile and proliferating VSMCs and showed functional activity and relevance to secretion and contraction. Much of the study focused on human cells, providing the first evidence for function of endogenous TRPM3 in this species. Through a chemical screen we found that TRPM3 is resistant to stimulation by many agents, including common vascular agonists, but we identified cholesterol in the membrane conferred ≈50% inhibition of TRPM3 activity, allowing cholesterol loading to confer additional effect. Pregnenolone sulfate sensing by the channels may be useful for therapeutic cardiovascular modulation, but not in conditions of cholesterol loading.

The observed pregnenolone sulfate responses suggest a mechanistic foundation for considering pregnenolone sulfate as a naturally occurring substance for use therapeutically to suppress unwanted vascular inflammation without the adverse effects of glucocorticoids. Effects on proliferating VSMCs occurred at concentrations that have been readily achieved in individuals after oral administration of pregnenolone, which is sulfated in vivo. Other TRPM3-mediated benefits of pregnenolone sulfate would be expected, including enhanced insulin secretion in hyperglycemia.9 Pregnenolone has been described as a fountain of youth, but rigorous clinical trials are needed to determine its true efficacy and safety. We observed contractile effects of pregnenolone sulfate in the aorta but at relatively high concentrations, such that risk of increased vascular tone may only be a potential concern at high doses. Recent studies have suggested that DHEA/DHEAS could be useful to counter unwanted vascular remodeling12,33 but our data do not suggest that such effects relate to vascular TRPM3 channels.

Pregnenolone sulfate was not observed to activate other TRP channels,9 but it is not specific for TRPM3; there is also modulation of NMDA (N-methyl-D-aspartic acid) and GABA-A receptors, for example. NMDA and GABA-A receptors are not known features of VSMCs, but we did make observations that were difficult to explain by TRPM3 alone. Divergence of the I-V shape from that of overexpressed TRPM3 could be accounted for by expression of multiple TRPM3 splice variants or heteromultimerization with other TRP channels, and the transient nature of the pregnenolone sulfate response in VSMCs could be explained by an inactivation mechanism that is lacking in HEK 293 cells. However, observation of
outward ionic current at negative voltages (Online Figure V) could not be explained by a cation-selective channel such as TRPM3.

TRPM3 appears not to be receptor-activated because a large number of G protein–coupled receptor agonists were tested in our chemical screen and none activated TRPM3. Constitutive activity could, however, be critical in TRPM3 and other TRP channels,22 enabling substances such as cholesterol to act in the absence of another agent. There are few reports of effects of cholesterol on TRP channels but an enhancing effect was observed on endogenous channels involving TRPC1, a TRP channel that facilitates neointimal hyperplasia in human saphenous vein.20 Putatively, elevated cholesterol associated with disease and high-fat diets may suppress antiserotenic TRPM3 and enhance proproliferative TRPC1. We emphasize, nevertheless, that cholesterol has wide-ranging effects on membranes and the associated proteins and so effects on TRP channels should be viewed in this broader context.29,30

In summary, the study reveals functional relevance of TRPM3 and pregnenolone sulfate to secretion in proliferating VSMCs and contraction in contractile VSMCs. The pregnenolone precursor cholesterol has been identified as a previously unrecognized TRPM3 modulator, adding to an emerging picture of TRP channel modulation by this key factor and strengthening the case for functional relevance of constitutive TRP channel activity. The data suggest that pregnenolone sulfate should be explored further as a vascular modulator for use therapeutically, particularly in combination with cholesterol-lowering strategies.

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Disclosures

None.

References

What Is Known?

- Twenty-eight mammalian homologs of the transient receptor potential (TRP) ion channel of the fruit fly have been described. The function of most of these channels is to link various slow chemical and physical signals to intracellular calcium pathways.
- TRPM3 is one of the mammalian TRP channels. Its mRNA has been detected in blood vessels.
- TRPM3 is activated by the so-called “fountain of youth” neurosteroids, which decline in abundance with age and correlate with coronary artery disease.

What New Information Does This Article Contribute?

- This work shows that TRPM3 protein exists in smooth muscle cells of human and mouse vascular smooth muscle cells. We provide the first evidence for constitutive activity of endogenous TRPM3 channels, suggesting the possibility of regulation by inhibitors without the need for an activator. Signals were, however, also evoked by known TRPM3 stimulators and were accounted for partly by TRPM3. TRPM3 channel was found to play a role in aortic contraction and the suppression of the secreted proinflammatory interleukin-6. Concentration–response curves suggested pharmacological rather than physiological relevance of neurosteroid effects. From screening 176 compounds, we found that cholesterol is a, negative, modulator of TRPM3. The study shows that TRPM3 is a new ion channel type of the cardiovascular system, which protects against cytokine secretion. Our observations provide mechanistic insights that should encourage consideration of neurosteroids as therapeutic vascular modulators when combined with cholesterol-lowering strategies.
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**Pregnenolone Sulphate- and Cholesterol-Regulated TRPM3 Channels Coupled to Vascular Smooth Muscle Secretion and Contraction**

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**Detailed Methods**

**Human vein and cell culture**
Freshly discarded human saphenous vein segments were obtained anonymously and with informed consent from patients undergoing open heart surgery in the General Infirmary at Leeds. Approval was granted by the Leeds Teaching Hospitals Local Research Ethics Committee. Proliferating vascular smooth muscle cells (VSMCs) were prepared using an explant technique and grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10 % foetal calf serum (FCS), penicillin/streptomycin and L-glutamine at 37 °C in a 5 % CO₂ incubator. Experiments were performed on cells passaged 2-5 times. VSMCs stained positively for smooth muscle α-actin. For culture of endothelial cells (ECs), endothelium was digested using 1 mg/ml Type II collagenase (Worthington Biochemicals, UK) dissolved in Medium 199 (Sigma, UK) (37 °C, 15 min). Cells were resuspended in M199 supplemented with 20 % FCS and 1 % penicillin–streptomycin and including endothelial growth factor (15 μg/ml) and pyruvate (1 μM). Cells were used up to passage 3. Growth of neointima has been described¹. HEK 293 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM)-F12 + Glutamax-1 (Gibco) supplemented with 10 % FCS and penicillin-streptomycin at 37 °C in a 5 % CO₂ incubator.

**Mouse femoral artery injury**
12 week old male C57/BL6 mice were anaesthetized and the left femoral arteries were isolated under aseptic conditions. A small area of the femoral artery was partially transected to allow the passage of a guide wire into the vessel. The guide wire was then removed and the incision closed. A sham procedure (without guide wire passage) was performed on the opposite leg for comparison. At 21 days post injury, mice were anaesthetized and perfused with 4 % paraformaldehyde. Procedures were conducted in accord with accepted standards of humane animal care under UK Home Office Project License 40/2988. Femoral artery bundles were removed and embedded in paraffin wax. For histological staining, sections 10 μm thick were mounted onto slides.

**Physiological mouse aorta**
Eight week old male C57/BL6 mice were killed by CO₂ asphyxiation and cervical dissociation in accordance with Schedule 1 Code of Practice, UK Animals Scientific Procedures Act 1986. The thoracic
aorta was removed and placed in ice-cold Hanks solution. Fat was removed completely by dissection and blood was flushed from the lumen with Hanks solution. Hanks solution contained (mmole/L): NaCl, 137; KCl, 5.4; CaCl2, 0.01; NaH2PO4, 0.34; K2HPO4, 0.44; D-glucose, 8; Heps, 5.

RNA isolation and RT-PCR
Total RNA was extracted using a Tri-reagent protocol followed by DNase I (Ambion) treatment. 1 μg of total RNA was used for reverse transcription (RT) based on oligo-dT primers and AMV RT enzyme. The specificity of PCR was verified by reactions without RT (-RT) and by melt-curve analysis of PCR products. PCR products were electrophoresed on 2 % agarose gels containing ethidium bromide. Sequences of PCR primers are in Supplementary Table I. PCR products were sequenced to confirm identity (Leeds University Sequencing Facility or Lark UK).

Quantitative RT-PCR
Quantitative, real-time, PCR was carried out using a Lightcycler (Roche). Relative abundance of target RNA was normalized to β-actin RNA, which showed no difference between samples. PCR efficiency (E) was 10 \(-\frac{1}{slope}\). Relative abundance of target RNA was calculated from \(\frac{E_{\beta\text{-actin}}^{C_T}}{E_{\text{target}}^{C_T}}\), where PCR cycle crossing-points (Cp) were determined by fit-points methodology.

TRPM3 cDNA expression
Human TRPM3 cDNA (accession number AJ505026) was used for over-expression studies\(^2\). For most experiments we used stable expression of the human TRPM3 in the T-REx expression system (Invitrogen, UK), as previously described\(^2\). The T-REx cells were maintained in the presence of 400 μg/mL zeocin and 5 μg/mL blasticidin S (InvivoGen). To induce TRPM3 expression cells were incubated with 1 μg/mL tetracycline (Sigma, UK) for 24-72 hr prior to experiments. Non-induced cells without addition of tetracycline (Tet-) were used as controls. In some experiments, where specified, we used transient transfection for TRPM3 expression\(^2\): Briefly, wild-type HEK 293 cells were plated onto poly-L-lysine coated coverslips and grown to ~80 % confluence. 1 μg TRPM3-YFP or YFP cDNA was transfected into the cells using FuGENE 6 (Roche, UK). Functional studies were carried out 48 hr after transfection on YFP-fluorescent cells. Also in a small number of experiments we used mouse TRPM3α2 cDNA (accession number AJ544535) in pCAGGSM2-IRESGFP was a gift from Dr SE Philipp\(^3\). 1 μg TRPM3α2 was co-transfected into HEK 293 cells with GFP using FuGENE 6 (Roche, UK). Functional studies were carried out 48 hr after transfection on GFP-fluorescent cells.

Transfection with siRNA
0.5-2 x10\(^6\) VSMCs were centrifuged (100 g) for 10 min, resuspended in Basic Nucleofector solution (Amaxa GmbH, Germany), mixed with 1 μmole/L short interfering (si) RNA and transferred into a cuvette for electroporation (Amaxa). The scrambled control siRNA was Silencer Negative Control #1, a 19 bp scrambled sequence with no significant homology to human gene sequences (Ambion Europe Ltd). Cells were transfected from cuvettes to pre-warmed culture medium and incubated in a 5 % CO\(_2\) incubator at 37 °C. Culture medium was changed after 24 hr and recordings were made after a further 24 hr. Transfection efficiency was about 80 %. TRPM3 siRNA was (sense strand, 5′-3′) CCCAUGUGAUCUGAUUGtt (Ambion). Smart pool TRPM3 siRNA-2 was a mixture of GAGAUGUUGUCCGGCCAUA, GAAGGAUCAUGCCUCUAAG, GACCCAAUGUGAUCGGAU and GAGCGUGGAUAUUCAGUG (Dharmacon).

Intracellular Ca\(^{2+}\) measurement
Cells were pre-incubated with fura-2AM for 1 hr at 37 °C followed by a 0.5 hr wash at room temperature (21±2 °C). Measurements were made at room temperature on a fluorescence microscope (Zeiss, Germany) or a 96-well fluorescence plate reader (FlexStation II\(^\text{384}\), Molecular Devices). The inverted microscope was equipped with a 40x Fluar oil-immersion objective (NA 1.3). Fura-2 dye was excited by light of 340 and 380 nm from a xenon arc lamp, the wavelength of which was selected by a
monochromator (Till photonics, Germany). Emitted light at 510 nm was collected via an emission filter and images captured every 10 s by an Orca-ER digital camera (Hamamatsu, Japan). The same excitation wavelengths were used in the FlexStation, where wells within columns of the 96-well plate were loaded alternately for test and control conditions. For either method, the intracellular calcium (Ca\textsuperscript{2+}) concentration is indicated as the ratio of fura-2 fluorescence (F) emission intensities for 340 nm and 380 nm excitation. The recording solution (standard bath solution, SBS) contained (mmole/L): 130 NaCl, 5 KCl, 8 D-glucose, 10 HEPES, 1.5 CaCl\textsubscript{2} and 1.2 MgCl\textsubscript{2}, titrated to pH 7.4 with NaOH. When 0 Ca\textsuperscript{2+} is indicated, CaCl\textsubscript{2} was omitted and replaced by 0.4 mmole/L EGTA.

**Chemical screen**

Cells were pre-incubated with fluo3 AM for 1 hr at 37 °C followed by a wash at room temperature (21±2 °C). Measurements were made at room temperature on a 96-well fluorescence plate reader (FLIPR, Molecular Devices). Fluo3 dye was excited by light of 485 nm, and emitted light collected at 525 nm. Wells within columns of the 96-well plate were loaded alternately for test and control conditions. The recording solution (standard bath solution, SBS) contained (mmole/L): 130 NaCl, 5 KCl, 8 D-glucose, 10 HEPES, 1.5 CaCl\textsubscript{2} and 1.2 MgCl\textsubscript{2}, titrated to pH 7.4 with NaOH. Response amplitudes were graded to a colour scale, with 1 being the maximal response (dark green) and a lack of response <0.1 (white).

**Cholesterol delivery**

Cells were pre-treated with a cholesterol/mβCD complex (Sigma, UK) at 37 °C for 2 hr during fura-2 loading and washing. Working concentrations were 1 mmole/L cholesterol plus 5 mmole/L mβCD or 0.5 mmole/L cholesterol plus 2.5 mmole/L mβCD. Cells were washed thoroughly with SBS before recordings to avoid the possibility that mβCD might chelate the TRPM3 agonist. For the generation of foam cells, VSMC were plated onto coverslips and incubated with media containing 0.5 mmole/L cholesterol plus 2.5 mmole/L mβCD and 2 % bovine serum albumin for 0-72 hr. To determine cholesterol uptake, cells were washed with 60 % isopropanol before the addition of Oil Red O. Cells were washed with water before stain was extracted by 100 % isopropanol. The optical density of the resulting solution was determined using a spectrophotometer at 500 nm. For Ca\textsuperscript{2+} imaging, cells were cholesterol loaded for 48 hr and washed thoroughly with SBS before use.

**Wire myography**

Vessels were mounted on two 40-μm diameter wires for isometric tension recording in a 410A dual wire myograph system (Danish Myo Technology, Denmark). The wires were separated by 1 mm to provide basal tension and the bath solution was at 37 °C gassed continuously with 95 % O\textsubscript{2} and 5 % CO\textsubscript{2}. The bath solution contained (mmole/L): 125 NaCl, 3.8 KCl, 25 NaHCO\textsubscript{3}, 1.5 MgSO\textsubscript{4}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 8 D-glucose, 1.2 CaCl\textsubscript{2}, 0.02 EDTA.

**Patch-clamp on proliferating VSMCs**

Recordings were made using a planar patch-clamp system (Nanion, Germany) in whole-cell mode\textsuperscript{4}. Prior to recordings, cells were detached from culture flasks with 0.05 % trypsin/EDTA and resuspended at a density of 1x10\textsuperscript{5}–5x10\textsuperscript{5} per ml. The bath (external) solution contained (mmole/L): 145 NaCl, 3 KCl, 10 CsCl, 2 CaCl\textsubscript{2}, 2 MgCl\textsubscript{2}, 10 HEPES and 10 D-glucose; titrated to pH 7.4 with NaOH. The patch pipette (intracellular) solution contained (mmole/L): 40 EGTA, 17 CaCl\textsubscript{2}, 2 MgCl\textsubscript{2}, 8 NaCl, 1 Na\textsubscript{2}ATP, 10 HEPES, 66 L-glutamic acid, 66 Cs\textsubscript{2}O, titrated to pH 7.2 with CsOH (calculated unbound Ca\textsuperscript{2+}, 100 mmole/L). Voltage ramps were applied from -100 mV to +100 mV for 1 s every 10 s from a holding potential of 0 mV. Currents were filtered at 1 kHz and sampled at 3 kHz. Recordings were at room temperature.

**Patch-clamp on contractile VSMCs and HEK 293 cells transiently expressing mouse TRPM3**

For isolation of fresh contractile VSMCs, aortae were flushed slowly with 0.3 % CHAPS in Hanks solution to remove endothelial cells and incubated in Hanks solution containing 0.5 mg/ml collagenase,
0.1 mg/ml protease, 0.13 mg/ml hyaluronidase and 475 Units elastase for 5 min at 37 °C. The adventitia was gently removed, followed by a second incubation in fresh enzyme mix for 30 min at 37 °C. Enzymes were removed and the tissue was incubated in fresh Hanks solution at room temperature for 30 min before being agitated with a modified Pasteur pipette. Myocytes were plated onto coverslips, stored at 4 °C and used within 8 hr. Myocyte or HEK 293 cell recordings were made at room temperature using conventional whole-cell recording. Signals were amplified and sampled using an Axopatch 200B amplifier and pCLAMP 8 software (Molecular Devices, USA). Signals were filtered at 1 kHz and sampled at 2 kHz. Patch pipettes had resistance of 3-5 MΩ. The bath solution contained (mmole/L): 145 NaCl, 3 KCl, 10 CsCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES and 10 D-glucose; titrated to pH 7.4 with NaOH. The patch pipette solution contained (mmole/L): 5 EGTA, 2 MgCl₂, 5 NaCl, 130 KCl, 3 Na₂ATP, 10 HEPES, titrated to pH 7.4 with KOH.

Secretion
IL-6 concentrations were quantified using a PeliKine-compact ELISA kit (Sanquin, Netherlands). MMP-9 was measured using a Quantikine ELISA Kit (R&D Systems, UK). Hyaluronan concentrations were quantified using a Corgenix ELISA kit (USA). Extracellular medium contained TPA (100 nmole/L) to induce tonic submaximal secretion.

Antibodies and labeling
Rabbit polyclonal anti-TRPM3 (TM3E3) and anti-TRPC1 (T1E3) antibodies have been described²⁵. For control experiments TM3E3 antibody was preadsorbed to 10 μM antigenic peptide (ratio 1:1) overnight at 4 °C prior to experiments (+pep). Antibody labeling of cells and intact vein sections was as described¹ except fixation of cells was for 4 min in 3 % paraformaldehyde and incubation with primary antibody was overnight at 4 °C. Tissue samples were incubated with TM3N1 anti-TRPM3 antibody targeted to the peptide CQEKEAAEPEKPTKE⁶ (1:400 dilution) or TM3E3 (1:2000 dilution) overnight at 4 °C. Images were collected using a laser confocal microscope (Zeiss, Germany) and analysed using Image J software (NIH, USA). For calcium imaging, cells were pre-incubated with TM3E3 or preimmune control antisera from the same rabbit at 1:500 dilution in DMEM media and standard bath solution for 3.5 hours at 37 °C. For contraction studies mouse aorta was pre-incubated with TM3E3 at 1:200 dilution or boiled control in Hanks solution overnight at 4 °C prior to recordings.

Data analysis
Averaged data are presented as mean±s.e.mean. Data produced in pairs (test and control) were compared using t tests, where statistical significance is indicated by * (P<0.05) and no significant difference by NS. When there were more than two groups, data sets were first compared using ANOVA. The n is the number of independent experiments, and N is the total number of individual cells, or wells of the 96-well plate. For experiments on saphenous vein, independent experiments were repeated on tissue/cells from at least 3 different patients.

Supplementary Results
There has been concern about whether sphingosine is indeed a TRPM3 activator, based on studies of mouse TRPM3³. While we observe stimulation of human TRPM3 by sphingosine, we also find that sphingosine inhibits activation of TRPM3 by pregnenolone sulphate (Supplementary Fig VI). Our data support the concept of sphingosine as a modulator of TRPM3 but suggest that the polarity of the effect varies depending on the experimental design.

Supplementary References


**Supplementary Table I.** Primers are for human sequences unless indicated.

<table>
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<th>mRNA target indicated by encoded protein</th>
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<tr>
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Supplementary Figure I. Specificity and validation of TRPM3 knock-down by RNAi. (A) Summary of real-time quantitative RT-PCR analysis to determine the abundance of mRNAs encoding the indicated proteins in human VSMCs. Each mRNA was quantified in paired experiments comparing cells transfected with TRPM3 siRNA or scrambled (control) siRNA. The abundance of each mRNA in the TRPM3 siRNA group was normalized to that in its own control group. The data show that TRPM3 mRNA was reduced in abundance by TRPM3 siRNA and that other mRNAs were not affected (n=3). (B) Effect of Smart pool TRPM3 siRNA-2 on the intracellular Ca²⁺ response to 100 μmole/L PregS (N=4 for each).
Supplementary Figure II. Real-time RT-PCR comparison of TRPM3 mRNA abundance in cultured human saphenous vein vascular smooth muscle cells (VSMCs) and endothelial cells (ECs).
Supplementary Figure III. Effects of chemical agents on PregS responses. The data are from intracellular Ca^{2+} measurements. (A) Example comparison of responses to 25 μmole/L PregS in HEK 293 cells induced (Tet+) or not induced (Tet-) to express exogenous human TRPM3. (B) Mean data for amplitudes of responses to 25 μmole/L PregS without (control) or with the presence of 0.1 mmole/L gadolinium ions (Gd^{3+}) or 75 μmole/L 2-aminoethoxydiphenylborate (2-APB) (n/N = 7/23 and 3/28 respectively). DMSO (dimethylsulphoxide) was the solvent and control for 2-APB. Responses are compared for HEK 293 cells expressing exogenous TRPM3 and human VSMCs. (C) Typical microscope-based Ca^{2+} measurement data showing responses to 25 μmole/L PregS in wild-type HEK 293 cells transiently expressing YFP-tagged human TRPM3 or YFP only (representative of n=3 and N=27-48).
Supplementary Figure IV. Functional specificity of anti-TRPM3 antibody TM3E3. The data show example intracellular Ca^{2+} measurements from human VSMCs. Cells were exposed to extracellular 1 µmole/L sphingosine-1-phosphate (S1P, A) or 100 µmole/L adenosine 5’-triphosphate (ATP, B). The responses were at least partly due to intracellular Ca^{2+}-release following activation of G-protein coupled receptors. Incubation with TM3E3 had no effect compared with the preimmune serum control (n/N = 3/17 and 4/22).
Supplementary Figure V. Current-voltage relationships (I-Vs) for PregS-induced currents in human VSMCs. The I-Vs marked ‘initial’ were obtained before bath-application of 25 μmole/L PregS. (A) Typical I-Vs observed in 27 out of 37 recordings. (B) Typical I-Vs observed in 10 out of 37 recordings.
Supplementary Figure VI. Effects of sphingosine or dihydrosphingosine. Data are from intracellular Ca²⁺ measurements from HEK 293 cells (A, B, F) or VSMCs (C, D, E, G). Cells were exposed to 20 μmole/L sphingosine (SPH) or dihydrosphingosine (DHS), ethanol vehicle control (cntrl), or the concentrations of pregnenolone sulphate (PregS) indicated in (F). (A, B) Typical data from HEK 293 cells transiently expressing YFP-tagged human TRPM3 or YFP only (representative of n=3-6 and N=9-29). (D, E) Typical data from VSMCs (representative of n=3-6 and N=9-29). (E) Example responses to 20 μmole/L SPH after pretreatment with TM3E3 antiserum or its preimmune control. (F) Representative experiment for the concentration-dependence of PregS responses in the absence (cntrl) or presence (+SPH) of 20 μmole/L sphingosine (N=2, representative of n=3). HEK 293 cells were induced to express exogenous human TRPM3 (Tet+). (G) Concentration-dependence of Ca²⁺ responses to sphingosine (SPH) in VSMCs normalized to responses to 20 μmole/L SPH (n/N=4/24). (F, G) Fitted curves are Hill equations.
Supplementary Figure VII.

**Supplementary Figure VII.** Typical current-voltage relationship (I-Vs) for constitutive membrane current in a human VSMC, showing the effect of TM3E3 anti-TRPM3 blocking antibody (example from \( n=8 \)).
Supplementary Figure VIII. Inhibition of murine TRPM3 by anti-TRPM3 blocking antibody (TM3E3). The data are from intracellular Ca\textsuperscript{2+} measurements performed on HEK 293 cells transiently transfected with mouse TRPM3\textalpha. (A) Mean time-series data for experiments comparing the 50 µmole/L PregS response in cells preincubated in TM3E3 or its preimmune control (n/N = 6/66 and 6/64). Suppression of the basal Ca\textsuperscript{2+} signal and the PregS response are evident. (B) As for (A) but comparing only the amplitudes of the PregS responses.
Supplementary Figure IX. Pregnenolone sulphate responses in smooth muscle cells freshly-isolated from the mouse aorta compared with HEK 293 cells over-expressing mouse TRPM3. (A) From 5 independent recordings, example ionic currents recorded at +80 and -80 mV from a freshly isolated mouse aorta smooth muscle cell. (B) From 15 independent recordings, example ionic currents recorded at +80 and -80 mV from a HEK 293 cell heterologously expressing mouse TRPM3. Pregnenolone sulphate (PregS, 100 μmole/L) and gadolinium (Gd³⁺, 100 μmole/L) were bath-applied as indicated by horizontal bars. HEK 293 cells not transfected with TRPM3 failed to respond to pregnenolone sulphate (n=13).
Supplementary Figure X. Stimulation by dihydroepiandrosterone sulphate (DHEAS). (A, B) The data show intracellular Ca\textsuperscript{2+} measurement experiments from HEK 293 cells induced to express exogenous human TRPM3 (Tet+) or not (Tet-). (A) In Tet+ cells, a typical paired comparison showing Ca\textsuperscript{2+} responses to 30 μmole/L DHEAS in the presence and absence of extracellular 1.5 mmole/L Ca\textsuperscript{2+} (N=4 for each condition; representative of n=4). (B) Concentration-dependence of Ca\textsuperscript{2+} responses to DHEAS, comparing mean data for Tet+ and Tet- cells (n/N=3/9 for each; estimated EC\textsubscript{50} for Tet+ cells 11.5 μmole/L). Tet+ cell data are the same as shown in Fig. 5B of the main paper. (C) In VSMCs, a typical paired comparison showing Ca\textsuperscript{2+} responses to 100 μmole/L DHEAS in the presence and absence of extracellular 1.5 mmole/L Ca\textsuperscript{2+} (N=4 for each condition; representative of n=4).
### Supplementary Table XI: Decoding of the chemical screen.

Compounds (10 μM/L) were tested in triplicate on a fluorometer containing fluo4-loaded cells that were induced (tet+) or not induced (tet-) to express TRPM3. Responses were normalized to the responses to pregnenolone sulphate (PregS).

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Signal 1</th>
<th>Signal 2</th>
<th>Signal 3</th>
<th>Signal 4</th>
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... (Continued table...)

**Note:**
- Signal values are normalized to PregS.
- Signals represent changes in fluorescence intensity compared to the control.
- The table includes a wide range of chemicals and their respective effects on signal intensities.
Supplementary Figure XII. Stimulation of TRPM3 by methyl-β-cyclodextrin (mβCD). (A) HEK 293 cells were induced to express exogenous human TRPM3 (Tet+). The data summarise paired comparisons of peak and sustained responses to 25 μmole/L PregS in cells pre-incubated for 1 hr with 2.78 mmole/L mβCD or without mβCD (N = 8 for each condition). (B) Typical paired experiment showing lack of effect of 25 μmole/L PregS or 2.78 mmole/L mβCD in non-induced HEK 293 cells (Tet-) (N=8).
Supplementary Figure XIII. Time course for cholesterol uptake by VSMCs during loading with exogenous cholesterol (0.5 mmole/L) and measured by the optical density (OD) of Oil Red O.