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Local Delivery of the K_{Ca}3.1 Blocker, TRAM-34, Prevents Acute Angioplasty-Induced Coronary Smooth Muscle Phenotypic Modulation and Limits Stenosis

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Abstract

Objective—We previously demonstrated that upregulation of intermediate-conductance Ca^{2+}-activated K^+ channels (K_{Ca}3.1) is necessary for mitogen-induced phenotypic modulation in isolated porcine coronary smooth muscle cells (SMCs). The objective of the present study was to determine the role of K_{Ca}3.1 in the regulation of coronary SMC phenotypic modulation in vivo using a swine model of postangioplasty restenosis.

Methods and Results—Balloon angioplasty was performed on coronary arteries of swine using either noncoated or balloons coated with the specific K_{Ca}3.1 blocker TRAM-34. Expression of K_{Ca}3.1, c-jun, c-fos, repressor element -1 silencing transcription factor (REST), smooth muscle myosin heavy chain (SMMHC), and myocardin was measured using qRT-PCR in isolated medial cells 2 hours and 2 days postangioplasty. K_{Ca}3.1, c-jun, and c-fos mRNA levels were increased 2 hours postangioplasty, whereas REST expression decreased. SMMHC expression was unchanged at 2 hours, but decreased 2 days postangioplasty. Use of TRAM-34 coated balloons prevented K_{Ca}3.1 upregulation and REST downregulation at 2 hours, SMMHC and myocardin downregulation at 2 days, and attenuated subsequent restenosis 14 and 28 days postangioplasty. Immunohistochemical analysis demonstrated corresponding changes at the protein level.

Conclusion—Blockade of K_{Ca}3.1 by delivery of TRAM-34 via balloon catheter prevented smooth muscle phenotypic modulation and limited subsequent restenosis.

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Disclosures
None.

Keywords

K_{Ca}3.1; TRAM-34; coronary smooth muscle; balloon angioplasty; phenotypic modulation

Coronary smooth muscle cells (SMCs) are phenotypically identified and characterized by expression of smooth muscle specific marker genes, such as smooth muscle alpha actin (SMαA), smooth muscle myosin heavy chain (SMMHC), and smoothelin-B. Unlike other cell types, SMCs are not terminally differentiated and can alter their gene expression profile after both physiological and pathophysiological stimuli, a phenomenon defined as phenotypic modulation.[1] During vasculoproliferative diseases, such as atherosclerosis and restenosis, smooth muscle cells undergo phenotypic modulation characterized by suppression of smooth muscle marker genes, increased proliferation, extracellular matrix synthesis, and migration.[2–5] We previously demonstrated that upregulation of intermediate-conductance Ca^{2+}-activated K^+ channels (K_{Ca}3.1, IKCa1, encoded by the gene KCNN4), whose expression is regulated by both AP-1 (c-jun/c-fos)[6] and repressor element -1 silencing transcription factor (REST),[7] is necessary for mitogen-induced suppression of smooth muscle specific marker genes, as well as SMC migration in porcine coronary SMCs in vitro.[6] Relevance to coronary disease was demonstrated by upregulation of K_{Ca}3.1 and decreased SMMHC in proliferating coronary SMCs in an in vivo swine model of early atherosclerosis.[6] The therapeutic potential of K_{Ca}3.1 inhibitors was alluded to by the report that systemic delivery of TRAM-34 for 1 to 6 weeks decreased neointimal formation by ≈40% in the rat carotid injury model.[8] Although this demonstrated a critical role for K_{Ca}3.1 in neointimal hyperplasia, it did not address the role of K_{Ca}3.1 in SMC phenotypic modulation per se. Additionally, the rat carotid injury model differs in etiology from coronary postangioplasty restenosis in humans in both vessel type (carotid versus coronary) and injury (lack of a medial tear).[8–11] In contrast, the porcine coronary overstretch injury model, widely recognized as the most appropriate model for studying postangioplasty restenosis,[11,12] produces a medial injury and development of a smooth muscle-rich neointima nearly identical to what is seen in humans.[9–12] The purpose of the present study was to investigate the role of K_{Ca}3.1 in the regulation of coronary SMC phenotypic modulation in a swine model of postangioplasty restenosis. Furthermore, we chose to deliver TRAM-34,[13,14] a specific K_{Ca}3.1 channel blocker,[15] to the coronary vessel wall via coated balloon catheter, to allow site-specific delivery and potentially avoid complications of systemic TRAM-34 administration.

Materials and Methods

A detailed description of all methods is provided in the supplemental materials (available online at http://atvb.ahajournals.org).

Coronary Balloon Angioplasty

Angioplasty was performed on the left circumflex (LCX) and left anterior descending (LAD) coronary arteries of castrated male swine (27 to 47 kg; 6 to 8 months old). Animal protocols were approved by the University of Missouri Animal Care and Use Committee in accordance with the “Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training.” Either the LCX or LAD was injured with a noncoated balloon, whereas the remaining artery was injured with a TRAM-34 coated balloon (see supplemental Figure I). After angioplasty, swine recovered for either 2 hours (n=5), 2 days (n=5), 14 days (n=5), or 28 days (n=5). At the time of sacrifice, injured and noninjured segments of the LCX and LAD were isolated and either quickly frozen in liquid nitrogen (LN_2) for subsequent laser capture microdissection and qRT-PCR or placed in paraformaldehyde for subsequent immunohistochemistry.

Balloon Coating

Balloons were inflated to 6 atm (nominal pressure), dipped in TRAM-34 (20 mg/mL in acetone) for 10 seconds, and dried for 1 minute. This cycle was repeated 3 times and followed by a final 5-minute drying time. The balloon was then carefully deflated and immediately guided to the coronary artery for balloon injury.

Laser Capture Microdissection and qRT-PCR

Laser capture microdissection of medial cells and qRT-PCR was performed as previously described.[6,16] Target gene expression was normalized to 18S using the $2^{-\Delta\Delta CT}$ method.[17]

Detection of TRAM-34 in the Vessel Wall

Using high-performance liquid chromatography (HPLC)-MS, TRAM-34 was quantified by its base peak of 277 m/z (2-chlorotrityl fragment) and concentrations calculated with a 5-point calibration curve. The related compound TRAM-46 (base peak of 261 m/z, 2-fluorotrityl fragment) was used as an internal standard.

Statistics

All data are presented as mean±SE. One-way ANOVA was used for all group comparisons with posthoc comparisons where appropriate. Significance was defined as $P<0.05$.

Results

$K_{Ca}3.1$ Is Necessary for Smooth Muscle Phenotypic Modulation Postangioplasty

qRT-PCR was used to measure mRNA in coronary medial cells isolated by laser capture microdissection (Figure 1). Two hours postangioplasty (Figure 1 black bars), balloon injury increased $K_{Ca}3.1$ mRNA by ≈23 fold (Figure 1a) and decreased REST, a transcription factor which suppresses $K_{Ca}3.1$,[7,18] by ≈5 fold (Figure 1b). TRAM-34 delivered via balloon catheter (TRAM) blocked the increase in $K_{Ca}3.1$ mRNA (Figure 1a) and the decrease in REST mRNA (Figure 1b) 2 hours postangioplasty. SMMHC mRNA was unchanged 2 hours after injury (Figure 1c). Two days postangioplasty (Figure 1 gray bars), $K_{Ca}3.1$ mRNA remained elevated (Figure 1a) while REST mRNA remained suppressed (Figure 1b), however the changes in mRNA were not as robust as 2 hours postangioplasty. In contrast to 2 hours postangioplasty, balloon injury suppressed SMMHC mRNA 2 days postangioplasty (Figure 1c). Importantly, TRAM-34 delivered via balloon catheter blocked the increase in $K_{Ca}3.1$ mRNA (Figure 1a), the suppression of REST (Figure 1b), and the loss of SMMHC (Figure 1c) mRNA two days postangioplasty. These data demonstrate that after balloon injury, changes in coronary smooth muscle $K_{Ca}3.1$ and REST precede alterations in SMMHC mRNA expression. Furthermore, TRAM-34 prevented postangioplasty-induced changes in $K_{Ca}3.1$, REST, and SMMHC, demonstrating that blockade of $K_{Ca}3.1$ can prevent phenotypic modulation in vivo.

Balloon injury also increased the AP-1 components, c-jun (Figure 1d) and c-fos (Figure 1e), 2 hours postangioplasty, consistent with previous findings in vitro demonstrating the role of AP-1 in regulating $K_{Ca}3.1$ expression in smooth muscle cells and T cells.[6,19] Interestingly, TRAM-34 failed to prevent the upregulation of either c-jun or c-fos 2 hours postangioplasty (Figure 1d and 1e). Lastly, we observed a transient increase in myocardin mRNA 2 hours postangioplasty, before a decrease 2 days postangioplasty (Figure 1f), both of which were blocked by TRAM-34. Myocardin has previously been demonstrated to regulate SMMHC expression[6,20–25] in vitro. Therefore, decreased myocardin as well as SMMHC mRNA 2 days postangioplasty are consistent with previous data demonstrating the role of myocardin in regulating SMMHC expression.[20]

Delivery of TRAM-34 Into the Vessel Wall

To validate TRAM-34 transfer to the coronary wall, we measured TRAM-34 in arteries harvested 2 hours, 2 days, and 14 days postangioplasty. TRAM-34 was detectable 2 hours (8.61 ±3.97 ng/mg; n=2) as well as 2 days (0.52±0.28 ng/mg; n=2) postangioplasty in coronary arterial segments injured with TRAM-34 coated balloons. By 14 days postangioplasty, only 0.7% of TRAM-34 detected 2 hours postangioplasty remained in the vessel wall (0.06 ng/mg; n=1). TRAM-34 was not detectable in plasma at any point. However, low levels were detected both 2 hours and 2 days postangioplasty in the liver (0.15 and 0.12 ng/mg, respectively), and noninjured right coronary artery (0.45 and 0.12 ng/mg, respectively) demonstrating that low levels of TRAM-34 were available systemically and accumulated in nontarget tissues, consistent with the highly lipophilic nature of TRAM-34.

Immunohistochemistry 2 Hours and 2 Days Postangioplasty

Balloon angioplasty increased $K_{Ca}3.1$ protein expression (pink; Figure 2a black arrowheads), and suppressed SMMHC protein expression (brown; Figure 2b white arrowheads) near the medial tear (m=media). Therefore all comparative images in Figure 3 and 4 were taken at the edge of the medial tear. Representative images demonstrate that compared to noninjured (Control), balloon injury (Injured) increased $K_{Ca}3.1$ positive staining (Figure 3, top) both 2 hours and 2 days postangioplasty, an effect which was blocked by TRAM-34 coated balloons (TRAM). Immunohistochemical analysis verified an ≈8-fold and an ≈12-fold increase in $K_{Ca}3.1$ protein at 2 hours and 2 days postangioplasty, respectively; an effect completely prevented by TRAM (Figure 5a). Consistent with SMMHC mRNA, representative images stained for SMMHC protein demonstrated that balloon injury reduced SMMHC levels near the medial tear 2 days after angioplasty, which was also prevented by the TRAM-34–coated balloon (Figure 3, bottom), whereas SMMHC staining was unaltered 2 hours postangioplasty. Immunohistochemical analysis demonstrated a 70% decrease in SMMHC protein 2 days postangioplasty, which was completely prevented by TRAM (Figure 5b). Double labeling with Ki-67 (blue, a marker of proliferating cells) and $K_{Ca}3.1$ (Figure 4 pink, top), indicated that whereas all Ki-67–positive cells were also $K_{Ca}3.1$-positive, the converse was not true, ie, not all $K_{Ca}3.1$-positive cells were proliferating.

Consistent with previous reports in vascular smooth muscle,[7] we observed REST localized to the nucleus of coronary smooth muscle (Figure 4). In uninjured control coronary arteries, REST was ubiquitiously expressed in medial SMCs (100% REST-positive nuclei; Figure 4 and Figure 5c). Balloon injury significantly reduced the number of REST-positive cells by ≈50% 2 hours and ≈40% 2 days postangioplasty, which was blocked by TRAM-34 coated balloons (Figure 4 and Figure 5c).

TRAM-34–Coated Balloons Reduced Restenosis

Coating balloons with TRAM-34 reduced the normalized intimal to medial thickness ratio (IMT/RI) compared to noncoated balloons within the same animal. Representative histological sections of segments injured with noncoated (Figure 6a) and TRAM-34–coated (Figure 6b) balloons taken 28 days postangioplasty demonstrate that vessels injured with TRAM-34–coated balloons had reduced neointima. Individual injury responses for TRAM-34 versus uncoated balloons are given in supplemental Figure II. Summary data demonstrate that coating balloons with TRAM-34 reduced postangioplasty restenosis by ≈38% and ≈22% at 14 and 28 days, respectively (Figure 6c), similar to what was previously published in the rat carotid injury model.[8] Acetone (vehicle)-coated balloons had similar IMT/RI (14.61±3.4) to the noncoated balloons (15.59±2.7).

Discussion

Recent studies have illustrated that the regulation of smooth muscle–specific marker gene expression, and thus SMC phenotypic modulation, is largely dependent on ion channel activity[6,24]; aka, excitation-transcription coupling.[26,27] This study investigated the role of $K_{Ca}3.1$ and its specific blocker TRAM-34 on excitation-transcription coupling in porcine coronary SMC phenotypic modulation after balloon angioplasty. We also tested a new delivery system for TRAM-34 via coated balloon catheters demonstrating that TRAM-34 was retained in the vessel wall, effectively prevented injury induced decreases in SMMHC gene expression, and reduced subsequent restenosis.

For these studies, we chose the porcine overstretch injury model because it is the closest nonprimate model to human postangioplasty restenosis.[11,12] Not only do swine have similar coronary vessel anatomy (see supplemental Figure I) and medial thickness, but the neointima that develops in response to injury is nearly identical to what is seen in humans.[10–12,28–30] Furthermore, it allows use of standard balloons, catheters, and other equipment designed for human coronary angioplasty. Therefore, the results obtained using the porcine model of postangioplasty restenosis provide the best translational validation for human coronary artery disease and restenosis treatments.

This study is the first to investigate the role of $K_{Ca}3.1$ and smooth muscle phenotypic modulation during coronary postangioplasty restenosis. We demonstrated that balloon injury robustly increased $K_{Ca}3.1$ and decreased REST expression 2 hours postangioplasty (Figure 1a and 1b). Others have also demonstrated suppression of REST expression concurrent with increased $K_{Ca}3.1$ expression.[7] Interestingly, the present study demonstrated that TRAM-34 delivered via balloon catheter not only blocked injury-induced $K_{Ca}3.1$ upregulation, but also the suppression of REST. It has previously been shown that delivery of the silencing transcription factor, REST, to proliferating cells represses $K_{Ca}3.1$ expression,[7] demonstrating that REST regulates $K_{Ca}3.1$ expression.[18] Our data suggests that expression of REST, in turn, is regulated by $K_{Ca}3.1$ channel activity, as inhibition of $K_{Ca}3.1$ channel activity by TRAM-34 prevented the downregulation of REST postangioplasty. It is plausible that the initial hyperpolarization or consequent potentiation of passive calcium entry attributable to $K_{Ca}3.1$ activity drives REST downregulation; however, this remains speculation, in part, because of the lack of information on regulation of REST expression.[31]

Suppression of REST by $K_{Ca}3.1$ channel activity is, however, consistent with a positive, feed forward mechanism for upregulation of $K_{Ca}3.1$ expression by $K_{Ca}3.1$ activity previously observed in coronary smooth muscle cells in culture.[6] We provide further support for this model in the present study in vivo, where TRAM-34 also blocked upregulation of $K_{Ca}3.1$ mRNA postangioplasty.

A common downstream mechanism for increased $K_{Ca}3.1$ expression is likely via AP-1. In vitro PDGF-BB induced upregulation of $K_{Ca}3.1$ mRNA, as well as binding of c-jun to the 5′ AP-1 binding site of the $K_{Ca}3.1$ promoter were blocked by TRAM-34.[6] Both c-jun and c-fos are components of the *cis*-binding element, AP-1,[32] and the $K_{Ca}3.1$ promoter contains several AP-1 binding sites.[19,33] In the present study in vivo, both c-jun and c-fos were increased 2 hours postangioplasty, in association with the increase in $K_{Ca}3.1$ expression. However, although TRAM-34 blocked injury-induced upregulation of $K_{Ca}3.1$ mRNA, it did not prevent increases in c-fos or c-jun mRNA. The reason for this apparent dissociation of c-fos/c-jun from $K_{Ca}3.1$ is not certain. However, our previous observations that TRAM-34 prevented PDGF-BB–induced enrichment of the $K_{Ca}3.1$ promoter with c-jun and acetylated histone-4 (H4Ac) indicate that TRAM-34 may prevent activation of the $K_{Ca}3.1$ promoter at the epigenetic level,

ie, by preventing histone aceytlation and promoter availablity, rather than preventing increases in c-fos/c-jun mRNA. Further investigation will be required to fully delineate this mechanism.

A primary goal of the present study was to determine whether $K_{Ca}3.1$ regulates coronary smooth muscle phenotypic modulation in an in vivo model of postangioplasty restenosis. To this end we clearly demonstrated that blockade of $K_{Ca}3.1$ could prevent postangioplasty phenotypic modulation of coronary SMCs, as indicated by SMMHC gene expression. Balloon injury decreased SMMHC gene expression 2 days postangioplasty, which was associated with a simultaneous decrease in myocardin expression. This result was expected because of previously published data demonstrating the role of myocardin in regulating SMMHC transcription,[20–26] and is consistent with reports in the mouse carotid injury model where myocardin expression was not significantly decreased until 3 days after injury.[34] Interestingly, the decrease in myocardin mRNA seen at 2 days postangioplasty was preceded by a transient increase 2 hours postangioplasty, a time when SMMHC mRNA is unchanged. This "uncoupling" of myocardin from SMMHC expression has been reported previously and proposed to result from the opposing interaction of myocardin with repressor *trans*-acting factors. For example, Doi et al observed an elevation in HERP1 and myocardin in cultured SMCs as well as in neointimal cells after rat aortic balloon injury, and overexpression of HERP1 prevented myocardin-induced smooth muscle gene expression.[35] Furthermore, NOTCH signaling suppresses myocardin-induced smooth muscle gene expression[36] by targeting HERP1.[35] Therefore, it is plausible that repressor *trans*-acting factors, eg, HERP1, were also increased 2 hours postangioplasty, and prevented myocardin-induced SMMHC expression.

We also examined protein levels associated with coronary balloon angioplasty to determine whether they were consistent with changes in mRNA. Alterations in medial $K_{Ca}3.1$, REST, and SMMHC protein as assessed by immunohistochemistry were consistent with mRNA levels determined by qRT-PCR. Additionally, it is important to note that although many of the cells near the medial tear of injured vessels stained positive for $K_{Ca}3.1$ (Figure 3, top), not all of those cells were positive for Ki-67, a marker of proliferation. Thus, not all cells expressing $K_{Ca}3.1$ are concurrently proliferating, although the majority of proliferating medial cells express $K_{Ca}3.1$. These data are consistent with a model whereby coronary SMCs upregulate $K_{Ca}3.1$ before entering the cell cycle, evidenced by inhibition of SMC proliferation by TRAM-34.[8]

Although the focus of the present study was to determine the role of $K_{Ca}3.1$ in early gene expression changes postangioplasty, we also determined whether acute, localized balloon catheter delivery of TRAM-34 was effective at inhibiting subsequent neointimal lesion formation. Local balloon catheter delivery of TRAM-34 inhibited neointimal formation by 38% at 14 days and 22% at 28 days postangioplasty, similar to the 40% reduction in intimal thickening reported with daily systemic administration of TRAM-34 in a rat carotid injury model.[8] Thus, the present study provides translational validation by demonstrating similar effects in the swine model of postangioplasty coronary restenosis, the industry standard for preclinical coronary intervention. Although the development of drug eluting stents has revolutionized interventional cardiology and effectively eliminated early restenosis, recent evidence has demonstrated that patients with drug-eluting stents have higher rates of late restenosis as well as late thrombosis,[37] leading some[38] cardiologists to advocate use of bare metal stents or balloon angioplasty coupled with alternative drug delivery methods.[37,39] Sheller et al demonstrated that paclitaxel delivered via coated balloon catheter significantly reduced restenosis,[39] prompting a call to pursue drug delivery by coated balloon catheter.[37, 38] The present study is the first to report that catheter-based delivery of TRAM-34,[14] a specific $K_{Ca}3.1$ channel blocker,[13,15] prevents phenotypic modulation of SMCs immediately after

coronary angioplasty and limits subsequent restenosis, thus implicating $K_{Ca}3.1$ as a therapeutic target in coronary vasculoproliferative disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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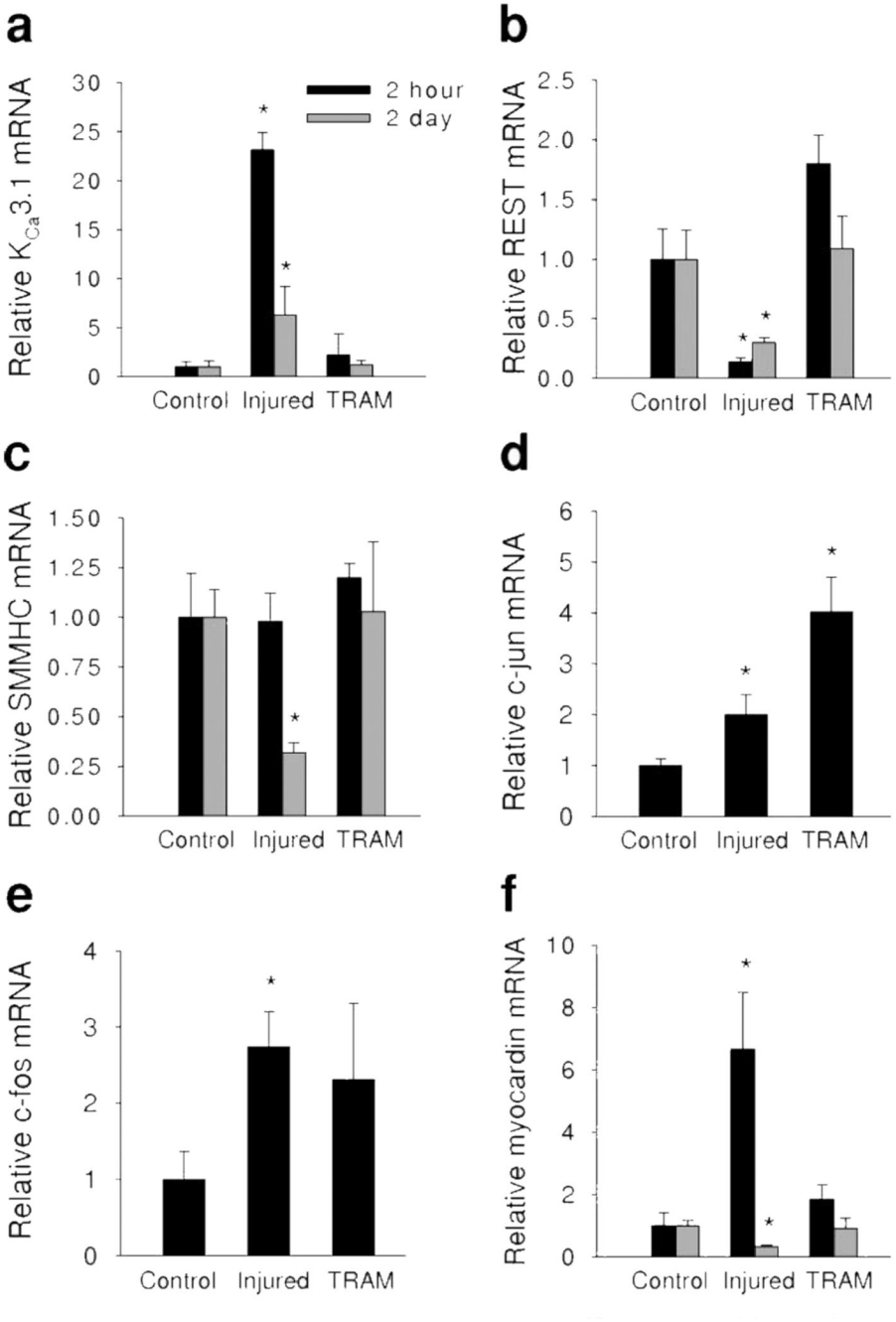

Figure 1. TRAM-34 prevents angioplasty-induced phenotypic modulation

Relative mRNA of K_{Ca}3.1 (a), REST (b), SMMHC (c), c-jun (d), c-fos (e), and myocardin (f) 2 hours (black bars) and 2 days (gray bars) postangioplasty in non-injured (Control), balloon injured (Injured), and TRAM-34–coated balloon injured (TRAM) coronary media. *P<0.05 vs corresponding control (n=4 to 5 per group).

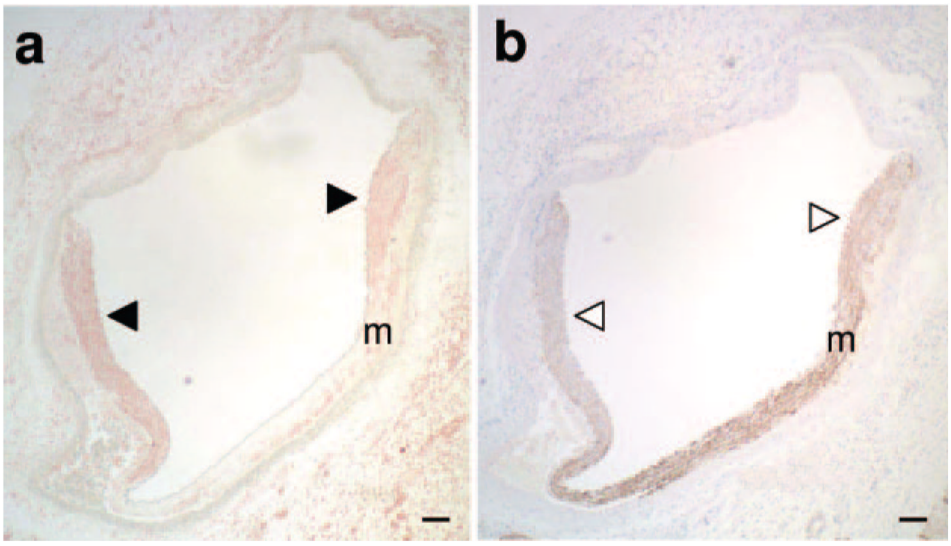

Figure 2. K_{Ca}3.1 and SMMHC protein 2 days postangioplasty

Representative coronary sections isolated 2 days postangioplasty stained for K_{Ca}3.1 (a, HRP, pink) or SMMHC (b, DAB, brown). K_{Ca}3.1 staining was more intense (a, black arrowheads), whereas SMMHC staining was more diffuse (b white arrowheads) near the medial tear. Horizontal bar=100 μm.

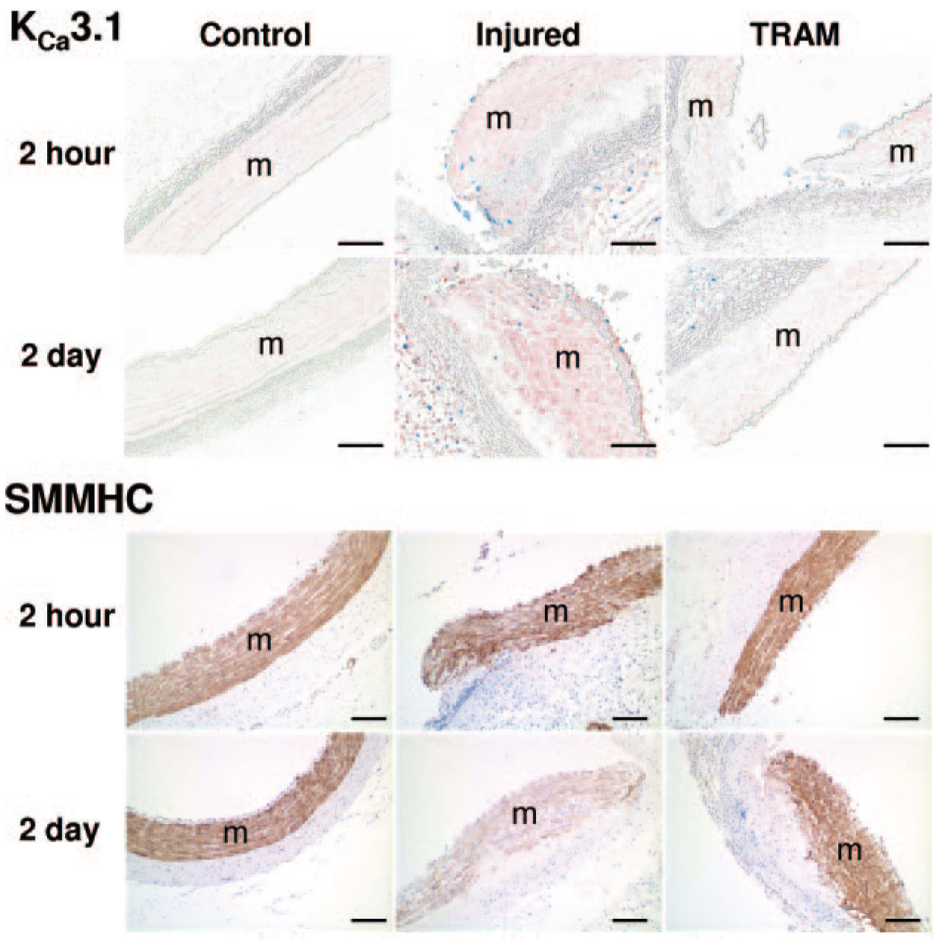

Figure 3. $K_{Ca}3.1$ and SMMHC histology 2 hours and 2 days postangioplasty

Representative cross-sections (8 μm; 4 to 5 per group) of control, injured, and TRAM-34–coated balloon injured (TRAM) LCX and LAD 2 hours and 2 days postangioplasty exposed to antibodies against $K_{Ca}3.1$ (1:600, pink, top), Ki-67 (1:200, blue, top), and SMMHC (1:800, brown, bottom). Horizontal bar=100 μm.

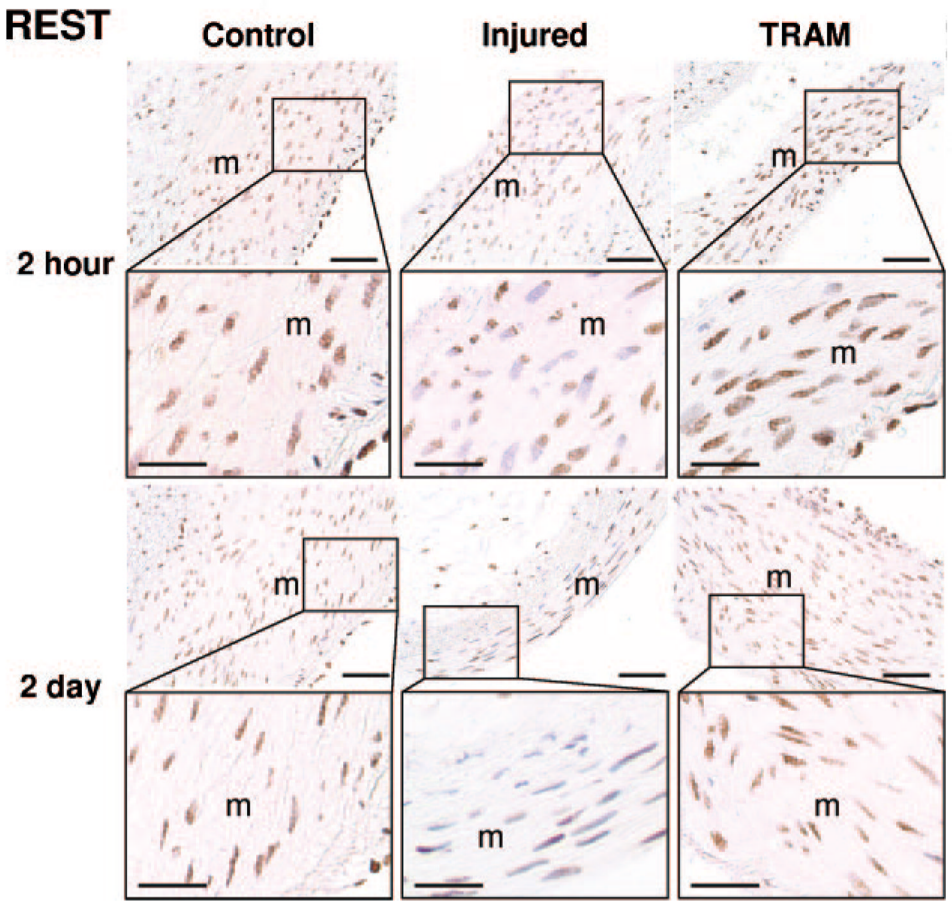

Figure 4. REST histology 2 hours and 2 days postangioplasty

Representative cross-sections (8 μm; 4 to 5 per group) of control, injured, and TRAM-34–coated balloon injured (TRAM) LCX and LAD 2 hours and 2 days postangioplasty exposed to anti-REST (1:50,000, brown), and counterstained with hematoxylin (blue nuclei were considered REST negative). Horizontal bar=50 μm (25 μm in insets).

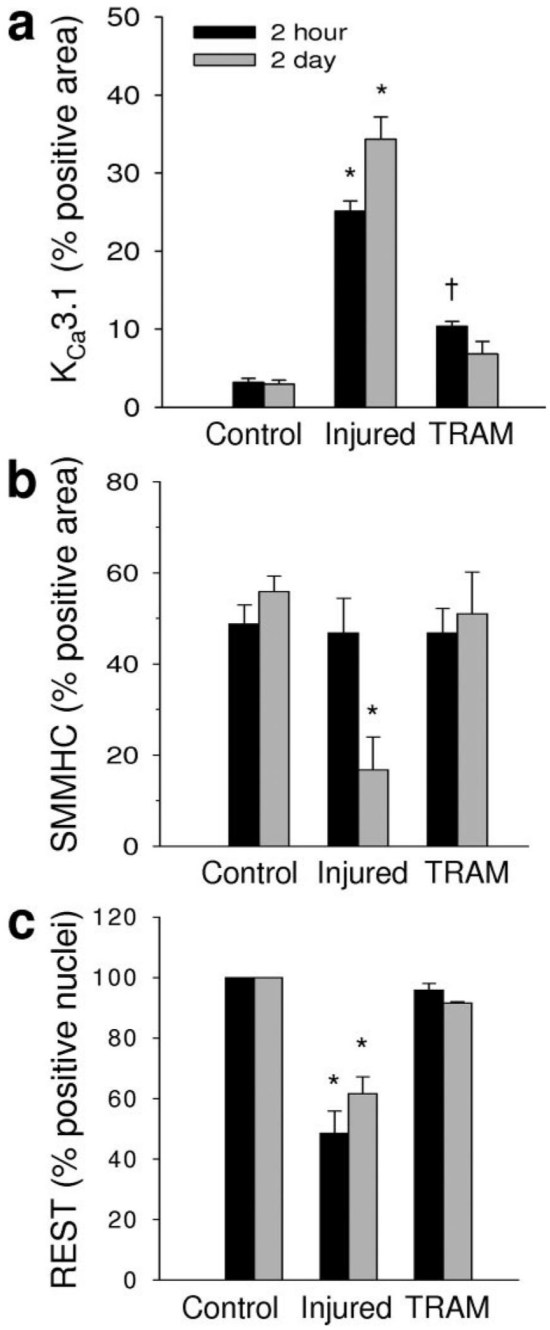

Figure 5. Histological analysis of K_{Ca}3.1, SMMHC, and REST

K_{Ca}3.1 (a), SMMHC (b), and REST (c) staining were quantified using Image Pro Plus. Injury-induced changes in K_{Ca}3.1, SMMHC, and REST staining were blocked by TRAM-34 (see Figure 3 and Figure 4 for representative images). *P<0.05 vs respective control and †P<0.05 vs injured and control (n=4 to 5 per group).

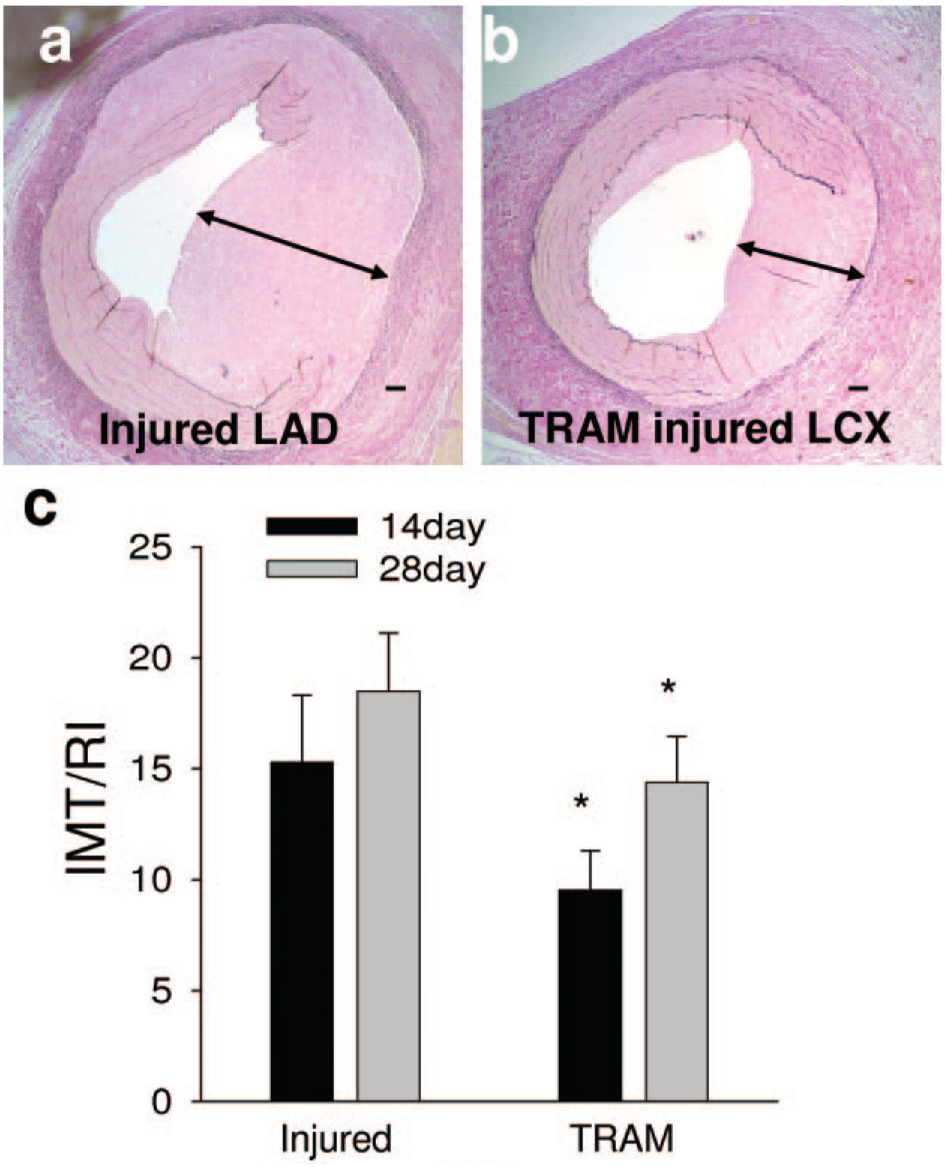

Figure 6. TRAM-34 prevents restenosis 14 and 28 days postangioplasty

Representative coronary arteries injured with a noncoated (a) or TRAM-34 coated balloon (b) 28 days postangioplasty and stained with Verhoff van Giesen stain (VVG). TRAM-34 reduced normalized intimal to medial thickness ratio (IMT/RI) at both 14 (38%) and 28 (22%) days postangioplasty (c). *P<0.05 vs injured segment taken from the same animal (paired t test; n=5). Horizontal bar=100 μm.