Nucleoside Diphosphate Kinase B–Activated Intermediate Conductance Potassium Channels Are Critical for Neointima Formation in Mouse Carotid Arteries

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Objective—Vascular smooth muscle cells (VSMC) proliferation is a hallmark of atherosclerosis and vascular restenosis. The intermediate conductance Ca2+-activated K+ (SK4) channel is required for pathological VSMC proliferation. In T lymphocytes, nucleoside diphosphate kinase B (NDPKB) has been implicated in SK4 channel activation. We thus investigated the role of NDPKB in the regulation of SK4 currents (I\textsubscript{SK4}) in proliferating VSMC and neointima formation.

Approach and Results—Function and expression of SK4 channels in VSMC from injured mouse carotid arteries were assessed by patch-clamping and real-time polymerase chain reaction. I\textsubscript{SK4} was detectable in VSMC from injured but not from uninjured arteries correlating with the occurrence of the proliferative phenotype. Direct application of NDPKB to the membrane of inside-out patches increased I\textsubscript{SK4}, whereas NDPKB did not alter currents in VSMC obtained from injured vessels of SK4-deficient mice. The NDPKB-induced increase in I\textsubscript{SK4} was prevented by protein histidine phosphatase 1, but not an inactive protein histidine phosphatase 1 mutant indicating that I\textsubscript{SK4} is regulated via histidine phosphorylation in proliferating VSMC; moreover, genetic NDPKB ablation reduced I\textsubscript{SK4} by 50% suggesting a constitutive activation of I\textsubscript{SK4} in proliferating VSMC. In line, neointima formation after wire injury of the carotid artery was substantially reduced in mice deficient in SK4 channels or NDPKB.

Conclusions—NDPKB to SK4 signaling is required for neointima formation. Constitutive activation of SK4 by NDPKB in proliferating VSMC suggests that targeting this interaction via, for example, activation of protein histidine phosphatase 1 may provide clinically meaningful effects in vasculoproliferative diseases such as atherosclerosis and post angioplasty restenosis. (Arterioscler Thromb Vasc Biol. 2015;35:1852-1861. DOI: 10.1161/ATVBAHA.115.305881.)

Key Words: atherosclerosis ■ KCa3.1 protein ■ KCNN4 protein ■ neointima formation ■ NM23B nucleoside diphosphate kinase ■ Nme2 protein ■ SK4 protein

Three types of Ca2+-activated potassium channels,1 large (BK), intermediate (SK4), and small (SK3) conductance Ca2+-activated K+ channels, have been detected in blood vessels, with preferential expression of BK in vascular smooth muscle cells (VSMCs) and SKs in endothelial cells.2,3 VSMCs are phenotypically identified and characterized by expression of smooth muscle–specific marker genes, such as α-smooth muscle actin. Unlike other cell types, VSMCs are not terminally differentiated and can alter their gene expression profile in response to physiological as well as pathophysiological stimuli. During vasculoproliferative diseases, such as atherosclerosis and restenosis, smooth muscle cells undergo phenotypic modulation characterized by suppression of smooth muscle marker genes, increased proliferation and migration. Although SK4 channels are primarily expressed in the endothelium contributing to the control of vascular tone and blood pressure,4,5 they belong to the genes, the expression of which is significantly enhanced during the phenotypic change of VSMC from the contractile to the synthetic phenotype. Thus, their expression was found to be upregulated in coronary vessels from patients with coronary artery disease.6 Similarly, in rat carotid artery and pig coronary artery

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injured by balloon-induced overstretch, SK4 channels were functionally detected in proliferating VSMC. In apolipoprotein E–deficient mice, a genetic model of atherosclerosis, SK4 channel expression was increased not only in VSMC but also in macrophages and T lymphocytes that infiltrated atherosclerotic lesions. Most important, in vivo therapy with the selective SK4 inhibitor TRAM-34 (1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole) reduced the development of atherosclerosis in apolipoprotein E–deficient mice, as well as neointimal hyperplasia and stenosis after vascular injury. These data suggest that SK4 channel activity is required for proliferation of VSMC.

SK4 channels are mainly regulated by intracellular Ca$^{2+}$, which binds to the Ca$^{2+}$-binding sites in calmodulin that is constitutively associated with the channel C terminus, thereby increasing channel open probability. Phosphatidylinositol-3-phosphate and its upstream regulator the PI3 kinase isoform C2β have been also linked to SK4 channel activation. In addition, protein kinase A (PKA) and protein kinase C may enhance or reduce SK4 channel activity depending on cell types. Besides regulation through serine/threonine kinases, nucleoside diphosphate kinase type B (NDPKB) directly phosphorylates SK4 channels at histidine 358 (His358), enhancing channel activity. Together with the counteracting histidine phosphatase 1 (PHPT-1), NDPKB forms a local de/phosphorylation teeter-totter, fine-tuning channel activity.

NDPKs are ubiquitously expressed nucleoside 5′-diphosphate (NDP)/nucleoside 5′-triphosphates (NTP)-transphosphorylases. They catalyze the transfer of the γ-phosphate from NTP to NDP by a ping-pong mechanism involving the formation of a high-energy phosphate intermediate on a histidine residue. They are encoded by the NME (nonmetastatic cell) genes, which comprise a family of 10 related genes. Among them, the nonmetastatic cell 1 and 2 proteins, which are frequently named NDPKA and NDPKB, respectively, are responsible for at least 80% of the cellular NDPK activity. They are involved in diverse physiological and pathological processes including proliferation, differentiation, development, and metastasis. However, NDPKB can also act as mammalian protein histidine kinase transferring the phosphate from its high energy phosphate intermediate to histidine residues on other proteins. Besides His358 in SK4 channels, the second well-characterized NDPKB substrate is His266 in the β subunit of heterotrimeric G proteins. Recent evidence from loss of function animal models indicates that the interaction of NDPKB with G proteins as well as with SK4 and TRPV5 (the transient receptor potential cation channel subfamily V member 5) channels are of important physiological relevance. For example, SK4 channel activity was reduced by 50% in T cells of NDPKB-depleted mice and resulted in strongly inhibited cytokine production, pointing to a potential role of NDPKB in inflammation and atherosclerosis. Here, we assessed the role of NDPKB in SK4 channel activation in VSMC and whether NDPKB is required for VSMC proliferation in injured arteries. We found that functional SK4 channels are only detectable in proliferating VSMC isolated from carotid arteries after wire injury and discovered that SK4 channels are constitutively activated by endogenous NDPKB. This activation is obviously required for neointima formation in the injured arteries. Our results demonstrate that NDPKB is an essential component required for the transition of a contractile to a proliferative (synthetic) and proatherosclerotic VSMC phenotype and thus neointima formation.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

## Results

### Functional Expression of SK4 Channels in VSMC From Injured Arteries

To detect the functional role of SK4 channels, whole-cell patch-clamp studies were performed in freshly isolated VSMC from injured and uninjured carotid arteries 2 weeks after operation (Table I in the online-only Data Supplement). To evoke the whole-cell currents, VSMCs were stimulated through the patch pipette by 300 ms pulses from −100 to +80 mV with increments of 10 mV and a holding potential of −50 mV. Mature VSMC from the uninjured artery exhibited outwardly rectifying K+ currents that increased steeply at positive membrane potentials. Application of the selective SK4 channel blocker TRAM-34 had no effect on total current, whereas iberiotoxin, a selective BK channel blocker, inhibited the currents by ≈90% (Figure 1A). These data indicate that the BK current (I_{BK}) is the predominant outward current in mature VSMC from uninjured vessels. At positive membrane potentials, VSMC isolated from the injured artery displayed a smaller total current when compared with VSMC from the uninjured artery. Conversely, at negative membrane potentials total current was larger in VSMC from injured versus uninjured vessels (Figure 1A and 1B). In VSMC from injured vessels, TRAM-34 significantly inhibited the total current (Figure 1B), unmasking the strong contribution of an SK4 current (I_{SK4}). At +80 mV, 100 nmol/L TRAM-34 reduced total current from 111.7±21.3 to 77.1±20.4 pA/pF (n=9; P<0.001). Subsequent application of iberiotoxin further inhibited the current to levels seen in VSMC from uninjured vessels (injured, 27.5±1.8 pA/pF; uninjured, 29.1±1.7 pA/pF). Representative original current recordings are shown as insets in Figure 1. Apamin, a selective blocker for SK1–3 channels, had no significant effect on total current in VSMC from both injured and uninjured vessels (Table I in the online-only Data Supplement). To validate further the identity of TRAM-34–sensitive and iberiotoxin-sensitive currents as I_{SK4} and I_{BK}, respectively, we assessed their Ca$^{2+}$ and voltage-dependent characteristics and compared them to currents from both injured and uninjured vessels.
dependencies. In recordings with Ca\textsuperscript{2+}-free intracellular solution, the total current was reduced by \(\approx 85\%\) and TRAM-34 and iberiotoxin failed to inhibit the residual Ca\textsuperscript{2+}-independent currents (Figure IA in the online-only Data Supplement). To eliminate the influence of the driving force on current amplitude, we converted the TRAM-34–sensitive and iberiotoxin-sensitive currents from density (pA/pF) to conductance (G\textsubscript{m}, nS). Plotting G\textsubscript{m} versus the respective voltages (V\textsubscript{m}) allows

When we dissected the TRAM-34 (ISK4)–sensitive component from the total current, I\textsubscript{m}, was detectable only in VSMC from injured vessels of wild-type (WT) mice (Figure 1D). TRAM-34 failed to inhibit the residual Ca\textsuperscript{2+}-independent current, the total current was reduced by \(\approx 85\%\) and TRAM-34 (100 nmol/L) and TRAM-34 plus iberiotoxin (300 nmol/L) were 279±27.7 pA/pF, 270.7±35.7 pA/pF, \(P<0.05\) vs Ctr and 29.1±1.7 pA/pF (\(P<0.05\) vs TRAM-34), respectively. G and D, I–V curves of iberiotoxin-sensitive (I\textsubscript{bTX}; C) and of TRAM-34-sensitive (I\textsubscript{SK4}; D) currents in VSMC from injured and uninjured vessels. At +80 mV, I\textsubscript{m} was reduced from 241.7±34.5 pA/pF (n=6) to 74.5±13.4 pA/pF (n=6; \(P<0.05\)). I\textsubscript{SK4} was increased from 1.6±1.9 (n=8) pA/pF to 34.7±4.2 pA/pF (n=6; \(P<0.05\)). The amount of mRNA encoding large conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel (BK) was reduced (E), whereas that encoding SK4 channels was strongly increased (F). [Ca\textsuperscript{2+}]=0.5 mmol/L. Data shown in A to D and E and F were analyzed by 1-way repeated measures ANOVA with Dunnett multiple comparisons and unpaired t test, respectively. *\(P<0.05\) vs Ctr (A and B) or uninjured arteries (C–F), n indicates number of cells patched/isolated from number of mice. N indicates number of mice.

Next, we validated the results obtained in VSMC from injured vessels by repeating the experiments shown in Figure 1 in VSMC from SK4−/− mice. TRAM-34 failed to inhibit the total current in VSMC regardless whether they were isolated from injured or uninjured vessels of SK4−/− mice (Figure 2A and 2B). Noteworthy, iberiotoxin exhibited similar effects on the total current in SK4−/− mice as in WT mice, yielding \(\approx 90\%\) inhibition in VSMC from both injured and uninjured vessels. These data clearly indicate that our model recapitulates the transition of a contractile to a proliferative VSMC phenotype in atherosclerosis and restenosis.

**NDPKB Constitutively Activates SK4 Channels in VSMC From Injured Vessels**

To determine whether NDPKB regulates SK4 channels in VSMC from injured vessels, recombinant NDPKB (10 ng/mL) was applied through patch pipette into the cells and I\textsubscript{SK4} was recorded 3 to 5 minutes after the establishment of stable whole-cell currents. Addition of recombinant NDPKB, but not of denatured NDPKB (15 minutes; 95°C), into the patch pipette induced a \(\approx 2\)-fold enhancement of I\textsubscript{SK4} (Figure 3A). Concomitant inclusion of PHPT-1 (30 ng/mL), but not the
The presence of H89 did not influence the stimulatory effect of PHPT-1 on basal I_{SK4} in VSMC from injured vessels with the PKA inhibitor H89 (1 μmol/L) for 30 minutes before applying recombinant NDPKB into the cells. To further proof the specificity of NDPKB as the exclusive regulator of I_{SK4} by NDPKB in VSMC from injured vessels. Current–voltage relationships for the K^+ currents under all these conditions are shown in Figure II in the online-only Data Supplement.

In addition to the direct activation of I_{SK4}, NDPKB strongly influences the adenyl cyclase/cAMP/PKA axis by a direct interaction with heterotrimeric G-protein βγ-dimers. As SK4 channels are also regulated by the cAMP/PKA pathway, we studied whether this pathway is involved in the regulation of I_{SK4} by NDPKB. We treated VSMC from injured vessels with the PKA inhibitor H89 (1 μmol/L) for 30 minutes before applying recombinant NDPKB into the cells. The presence of H89 did not influence the stimulatory effect of NDPKB on I_{SK4} (Figure 3C), suggesting that the effect of NDPKB on I_{SK4} is independent of cAMP/PPKa pathway and likely involves a direct interaction between NDPKB and SK4 channels. To test the latter possibility, we recorded single SK4 currents in inside-out patches excised from VSMC from injured vessels and applied recombinant NDPKB through a superfusion pipette directly to the intracellular side of the membrane. Figure 4A shows the original recordings of a representative experiment. An increase in intracellular Ca^{2+}...
concentration from 0.01 to 0.5 μmol/L enhanced the open probability from 0.007 to 0.58. Ten ng/mL recombinant NDPKB further increased NPo by ≈4-fold (Figure 4B). A subsequent application of the inactive mutant PHPT-1 (H53A) to NDPKB-activated ISK4 in inside-out patches did not significantly influence the effect of NDPKB on NPo, whereas the addition of active PHPT-1 completely reversed the increase in NPo. Most important, the basal single SK4 current activity decreased by ≈80% (0.52±0.22 before versus 0.1±0.03 after PHPT-1; P<0.01) after histidine dephosphorylation, confirming the whole-cell voltage-clamp results illustrated in Figure 3A. Further analysis revealed that NDPKB increased NPo (Figure 4B) without accompanying changes in single channel amplitude (Figure 4C) or single channel conductance (Figure 4D). Collectively, these electrophysiological data suggest that newly expressed SK4 channels in VSMC from injured arteries are directly activated via histidine phosphorylation by constitutively active NDPKB.

Reduced Neointima Formation in SK4−/− and NDPKB−/− Mice

Previous work suggests that ISK4 plays an important role in VSMC proliferation. In accordance, inhibition of SK4 channels reduced neointima formation, atherogenesis, and restenosis.6–8,29 As our results indicate that endogenous NDPKB constitutively activates ISK4 in VSMC from injured vessels, we hypothesized that neointima formation should be reduced in injured arteries of both NDPKB−/− and SK4−/− mice. We therefore histologically assessed injured and uninjured carotid arteries from NDPKB−/− mice by eosin/hematoxylin staining (Figure 5). Four weeks after injury the neointimal area and the ratio of neointimal/medial area were ≈75% to 80% lower in both SK4−/− and NDPKB−/− mice when compared with WT controls. To identify the proliferating type of cells, we stained for the VSMC marker smooth muscle actin and the proliferation marker Ki67 in the neointima of injured vessels from WT mice. As shown in Figure 6A, many of the smooth muscle actin–expressing cells in the neointima are positive for Ki67, indicating proliferating VSMC. We therefore compared Ki67 staining in the neointima of injured arteries of WT and NDPK−/− mice (Figure 6B). A prominent expression of Ki67 was evident in many cells in the neointima from WT mice. In comparison, the number of Ki67-positive cells in the neointima of NDPKB−/− mice was reduced by >50%. To further confirm that a reduced SK4 activity occurs in all proliferating VSMC from NDPKB−/− mice, we isolated VSMC from single mouse aortas and expanded them in cell culture.30 Interestingly, VSMC obtained from the aorta of NDPKB−/− mice grew much
slower than those isolated from WT mice. As shown in Figure III in the online-only Data Supplement, these proliferating VSMCs all express SK4. The resulting \( \frac{\text{SK4}}{\text{Ca}} \) in VSMC obtained from NDPKB−/− mice is however significantly smaller than in VSMC of WT mice. Taken together all these findings support the idea that de novo expression of SK4 channels and their stimulation by constitutively active NDPKB are both required for VSMC proliferation and thus neointima formation in injured arteries.

**Discussion**

In this study, we investigated the regulation of SK4 channels by NDPKB in proliferative VSMC and the contribution of NDPKB to neointimal hyperplasia in injured mouse carotid arteries. We demonstrate that NDPKB constitutively activates SK4 channels in VSMC and that this increased channel activity correlates with VSMC proliferation and neointima formation in injured arteries.

**Comparison With Prior Work**

A striking feature of SMCs is their functional plasticity that is important for regulating blood pressure and controlling vascular growth and repair after injury. In mature healthy arteries, the majority of SMCs exist in a fully differentiated contractile state, which is required for physiological regulation of vascular tone. Vessel wall injury induces a switch from the contractile (quiescent) phenotype to a proliferative (motile or synthetic) phenotype, which is accompanied by characteristic alterations in ion channel function: L-type Ca\(^{2+}\) and BK channels show reduced activity, whereas T-type Ca\(^{2+}\) channels, transient-receptor potential type-C channels and SK4 channels are upregulated. Specifically, in control VSMC, \( \approx 90\% \) of the total K\(^{+}\)Ca current is conducted by IBK with no detectable ISK4. In VSMC from injured vessels, only \( \approx 60\% \) of the total K\(^{+}\)Ca-current was conducted by IBK with a prominent contribution of ISK4. Our present results confirmed the upregulation of SK4 channels, along with a downregulation of BK channels, in VSMC from injured vessels. The functional identity of SK4 channels in VSMC from injured vessels was validated by their sensitivities to the selective SK4 channel blocker TRAM-34 and Ca\(^{2+}\), relative voltage independence, single-channel conductance of expected range (\( \approx 28 \) pS), and absence ISK4 current in SK4−/− mice.

Previous work showed a unique mechanism of phosphorylation-dependent regulation of SK4 channels. Specifically, in vitro reconstitution experiments as well as data from NDPKB−/− mice demonstrated that SK4 channels are dynamically regulated by the phosphorylation and dephosphorylation of His358 by NDPKB and PHPT-1, respectively. Our present data extend these observations to de novo expressed SK4 channels in proliferating VSMC from carotid arteries. The direct interaction between NDPKB and SK4 channels...
resulted in enhanced channel open probability. The maximal (seen with saturating amounts of exogenously added NDPKB) as well as the basal activity (seen with saturating amounts of exogenously added PHPT-1) of the de novo expressed SK4 channels in VSMC from injured arteries of WT and NDPKB-deficient mice was virtually identical. These data indicate that the expression of the SK4-channel protein itself is not regulated by NDPKB. Remarkably, however, the de novo expression of SK4 channels in VSMC is apparently sufficient to allow a constitutive activation by the endogenously expressed NDPKB. In accordance, PHPT-1 application decreased the SK4 channel activity in VSMC obtained from injured arteries of WT mice to the level observed in VSMC obtained from injured arteries of NDPKB−/− mice.

**Potential Significance and Clinical Implications**

In VSMC, BK channels function as a negative feedback regulator for Ca2+ influx, thereby regulating VSMC contraction. Proliferating VSMCs from injured vessels show a reduced contractility which is associated with downregulation of BK channels. The regulation of SK4 channel expression in proliferating VSMC has been linked to many growth factors, for example, fibroblast growth factor, transforming growth factor-β, EGF, epidermal growth factor, and platelet derived growth factor.6,7,34,35 Extracellular-signal–regulated kinase- and activator protein-1–dependent signaling pathways have been implicated in the regulation of SK4 channels by those factors.34,36 The data reported herein indicate that the increase in ISK4 results not only from enhanced channel expression but also from increased channel activity caused by phosphorylation of His358 by endogenous NDPKB. Recent data indicate how these increased ISK4 in proliferating VSMC contribute to the regulation of cell proliferation in atherosclerosis and post angioplasty restenosis. In proliferating VSMC, transient-receptor potential type-C channels (along with T-type Ca2+ channels), which are activated at more negative membrane potentials (20–40 mV more negative than L-type Ca2+ channels), are the main channels responsible for Ca2+ influx.31 Because of their voltage independency, SK4 channels can maintain their opening even at strong negative membrane potentials. An increase in I_{SK4} as reported here,

![Figure 5](http://atvb.ahajournals.org/)

**Figure 5.** Intermediate conductance Ca2+-activated K+ (SK4) channels and nucleoside diphosphate kinase B (NDPKB) contribute to intimal hyperplasia in injured arteries. A to F, Representative cross-sections of uninjured (A and D) and injured carotid arteries (B and C, E, and F) stained with hematoxylin and eosin from wild-type (WT; A, B, D, and E), SK4−/− (C) and NDPKB−/− (F) mice 4 weeks after operation; scale bar 100 μm; dotted lines indicate neointima/media borders. G and H, Mean values of neointimal area (G) and the ratio of neointimal/medial area (H); Data were analyzed by unpaired t test. *P<0.05 vs the respective WT. n indicates number of mice; and N/M, ratio of neointimal/medial area.
will shift the membrane potential to more negative values (hyperpolarization), enhancing the driving force for Ca\(^{2+}\) influx through transient-receptor potential type-C and/or T-type Ca\(^{2+}\) channels and leading to an increase in intracellular Ca\(^{2+}\) concentration. This may trigger the activation of the transcription factor phosphorylated cAMP-response element-binding protein and the subsequent induction of mitogenic immediate early genes such as c-Fos and neuron-derived orphan receptor-1. This potential mechanism is not unique to VSMC, but is also operative in endothelial cells, T-lymphocytes, fibroblasts, and some tumor cell lines.

To the best of our knowledge, we provide the first evidence that NDPKB constitutively activates SK4 channels in proliferating VSMC through its previously described protein histidine kinase activity. Importantly, the neointima thickening in the injured carotid arteries of NDPK-B\(^{-/-}\) mice was reduced to the same extent as in SK4\(^{-/-}\) mice, clearly suggesting that the constitutive activation of SK4 channels by endogenous NDPKB is a key event in VSMC proliferation and neointimal hyperplasia. Most likely, a minimal threshold level for the SK4-mediated K\(^{+}\) current exists in VSMC which is required for cell proliferation. This threshold current is only achieved by activation of SK4 channels by endogenous NDPKB in VSMC. In accordance, the expression of the proliferation marker Ki67 was reduced in the neointima of injured vessels of NDPKB\(^{-/-}\) mice by endogenous NDPKB. Consistent with this notion, CD4-positive T lymphocytes from NDPKB\(^{-/-}\) and SK4\(^{-/-}\) mice show similar profiles of cytokine production and SK4\(^{-/-}\) mice are protected from developing severe colitis in mouse models of inflammatory bowel disease. Therefore, small molecules that inhibit the histidine kinase activity of NDPKB and increase the expression or activity of PHPT-1 might offer new therapeutic options not only for the treatment of post angioplasty restenosis but also for atherosclerosis and other inflammatory diseases. This interpretation is strengthened by the phenotype of the NDPKB\(^{-/-}\) mice. They are born at the expected Mendelian frequency with no obvious defects. Besides a moderate cardiac dysfunction and a beginning vasoregression in the retina at higher age, no impairments have been reported. They have a normal life span and thus even the total absence of NDPKB activity seems to be tolerated.

**Study Limitations**

Here, we did not address the therapeutic potential of specifically targeting the NDPKB/SK4 channel interaction in atherosclerosis. Although this is the first description of the requirement of NDPKB-mediated activation SK4 channels for neointimal hyperplasia, the underlying mechanisms, that is, phosphorylation of His358 in SK4 channels, seem also to occur in other cell types such CD4-positive T lymphocytes. In addition, SK4 channels play an important role in brain function. Therefore, further extensive studies are needed to test whether the specific inhibition of the NDPKB/SK4 channel interaction instead of targeting SK4 channels itself is a viable and a more tissue-specific therapeutic approach for atherosclerosis. Moreover, small molecules specifically inhibiting the catalytic activity of NDPKB or interfering with an interaction of NDPKB with SK4 channels have to be identified. Also, although the role of PHPT-1 as the counteracting phosphatase has been established experimentally, the precise function

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**Figure 6.** Reduced vascular smooth muscle cell proliferation in the neointima of injured arteries of nucleoside diphosphate kinase B (NDPKB\(^{-/-}\)) mice. **A**, Immunohistochemical detection of the proliferation marker Ki67 (green) and \(\alpha\)-smooth muscle actin (SMA, red) in a representative cross-section of injured carotid arteries from wild-type (WT) mice. **B**, Immunohistochemical detection of Ki67 (red) in a representative cross-section of injured carotid arteries from WT (left) and NDPKB\(^{-/-}\) (right) mice. Nuclei were visualized by To-Pro3 staining (blue). A quantification of Ki67-positive cells in the neointima was performed from 8 cross-sections obtained from 2 mice (right). Mean±SD is given. Data were analyzed by unpaired t test. *P<0.05 vs WT.
of this enzyme in a physiological or pathophysiological setting remains enigmatic.\textsuperscript{45} Thus, an evaluation of the therapeutic potential of targeting the NDPK/B5K4 or SK4/PHTP-1 interactions in atherosclerosis needs direct extensive investigations in subsequent studies.

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**Disclosures**

None.

**References**


Vascular smooth muscle cell proliferation and neointima formation are hallmarks of atherosclerosis and vascular restenosis. Herein, our data demonstrate that endogenously expressed nucleoside diphosphate kinase B stimulates Ca\(^{2+}\)-activated potassium channels of intermediate conductance, which are de novo expressed when vascular smooth muscle cells switches from the contractile to a proliferative phenotype. Like the inhibition of this channels by small molecule inhibitors, depletion of nucleoside diphosphate kinase B is able to suppress neointima formation and vascular smooth muscle cell proliferation. Therefore, our data indicate that interference with the histidine kinase activity of nucleoside diphosphate kinase B or inhibition of its interaction with the channels is an attractive, clinically relevant possibility to attenuate neointima formation without touching the nucleoside diphosphate kinase B–independent channel activity possibly required in nontarget tissues.

**Significance**

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35. Peña TL, Chen SH, Konieczny SF, Rane SG. Ras/MEK/ERK Up-regulation of the fibroblast KCa channel FIK is a common mechanism for basic fibroblast growth factor and transforming growth factor-beta suppression of myogenesis. *J Biol Chem*. 2000;275:13677–13682.


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Material and Methods - Online-Only Data Supplement

Mouse Models

SK4\(^{-}\) and NDPKB\(^{-}\) mice were generated as previously described.\(^1\), \(^2\) The study was carried out with wild-type (SV129/C57B16, C57BL/6) and SK4\(^{-}\) (SV129/C57B16,\) as well as NDPKB\(^{-}\) mice (C57BL/6). The study with animals was approved by the Regierungspräsidium Karlsruhe, Germany (approval number 35-9185.81/G-231/10). All experiments have been carried out in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996).

Guide Wire Injury in Carotid Arteries

The carotid artery was injured by the guide wire injury technique, which has been well described previously.\(^3\), \(^4\) Briefly, mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The right carotid artery was exposed through a midline incision on the ventral side of the neck. The bifurcation of the carotid artery was located. Right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were isolated using blunt dissection technique. Distal right ECA was then ligated with a 7-0 silk suture. Following clamping of the right proximal CCA and ICA with microclamps, an arteriotomy was made on the ECA proximal to the silk suture. A curved flexible guide wire (0.36 mm diameter) was introduced into the common carotid artery via the incision hole, and under rotation the wire was passed along the vessel five times. The wire was then removed and proximal ECA was ligated with a 7-0 silk suture proximal to the arteriotomy. Next, the microclamps were released to re-establish the blood flow in CCA and ICA. During the procedure, the left carotid artery was isolated but left intact and was used as uninjured control. Finally, the skin was closed with a 7-0 nylon suture. Carprofen (6mg/kg/day, s.c.) was given to the animals as post-surgery analgesia for 2 to 3 days. Animals were sacrificed at 2 or 4 weeks after surgery. Both right and left CCA (5-6 mm in length proximal to bifurcation) were harvested for patch-clamp recordings, real-time PCR and histological analysis.

Smooth Muscle Cell Isolation

The mouse right (injured) or left (uninjured) carotid artery was dissected and put in cold physiological saline solution (PSS: 130 mM NaCl, 5.9 mM KCl, 2.4 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), 11mM glucose, 10 mM HEPES, pH 7.4 with NaOH). The fat and connecting tissues were removed. The artery was digested at 37\(^\circ\)C for 40 min in Ca\(^{2+}\)-free PSS containing 0.7 mg/ml papain, 1 mg/ml
(1,4)-dithio-D,L-threitol, and 1 mg/ml bovine serum albumin (BSA). Next the buffer was changed for PSS containing 0.05 mM Ca$^{2+}$, 1 mg/ml collagenase type H, 1 mg/ml hyaluronidase, 1 mg/ml BSA, the artery was digested further for about 10 min at 37°C. The digested artery was washed twice with Ca$^{2+}$-free PSS. Single cells were released by gentle trituration of the digested tissue and stored in PSS at room temperature. The experiments were conducted within 6 h of cell isolation.

Aortic smooth muscle cells were isolated from a single mouse aorta exactly as described.\textsuperscript{5} Cells were expanded in Dulbecco’s Modified Eagle Medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were splitted with accutase after reaching 80% of confluence.

**Functional Identification of VSMCs for patch-clamp analysis**

From the literature it is well-known that 1) mature VSMCs display large BK currents without SK channel currents;\textsuperscript{6, 7} 2) most proliferating VSMCs display reduced (still manifest) BK currents with SK4 but not SK1-3 channel currents;\textsuperscript{6} 3) the other non-muscle cells (endothelium, lymphocyte), which may be contained in the isolated cell suspension, demonstrate predominantly SK (SK1-3 and SK4) channel currents without BK current.\textsuperscript{8-10} Therefore we applied TRAM-34, IbTX and apamin sequentially to all the investigated cells. Cells that displayed predominant IbTX-sensitive (BK channel) currents without apamin-sensitive (SK1-3 channel) currents were supposed to be VSMCs and were included in data analysis. It is also known that some proliferating VSMCs have predominant SK4 without BK channel currents.\textsuperscript{6} Those cells were not included in our study.

**Whole-Cell and Single-Channel Recordings**

Standard patch-clamp recording techniques are used to measure currents in the whole-cell or inside-out patch-clamp configuration.\textsuperscript{11} If not otherwise indicated, the free Ca$^{2+}$ concentration ([Ca$^{2+}$]) was adjusted to 0.5 µM as described previously.\textsuperscript{12} Patch electrodes are pulled from borosilicate glass capillaries (MTW 150F; world Precision Instruments, Inc., Sarasota, FL) using a DMZ-Universal Puller (Zeitz-Instrumente Vertriebs GmbH, Martinsried, Germany) and filled with pre-filtered pipette solution (see below). Currents are recorded at room temperature with an EPC-8 amplifier (HEKA Elektronik, Lambrecht, Germany) connected via a 16 bit A/D interface to a pentium IBM clone computer. The signals are low-pass filtered (1 kHz) before 5 kHz digitization. Data acquisition and analysis are performed with an ISO-3 multitasking patch-clamp program (MFK M. Friedrich, Niedernhausen, Germany). Pipette resistance range from 2-3 MΩ for whole cell, 4-5 MΩ
for inside-out recordings. Electrode offset potentials are always zero-adjusted before a Giga-seal is formed. After a Giga-seal is obtained, the membrane under the pipette tip is disrupted by negative pressure and the whole-cell configuration is established. Then the membrane capacitance and series resistance are compensated (60-80%). Whole-cell currents are elicited by applying 300 ms step pulses to potentials ranging from -100 mV to +80 mV in 10 mV increments from a holding potential of -50 mV. Currents are sampled by ISO-3 and saved in the computer for later data analysis. The whole-cell currents were measured at the end of each pulse and normalized to cell capacitance to obtain the current density (pA/pF). The density was then plotted versus the respective voltages, yielding the activation (I/V) curves of channels in the cell. The membrane conductance ($G_m$) was calculated as $G_m = I/(E_m - E_{rev})$, where $I$ is macroscopic current, $E_m$ is the test membrane potential, and $E_{rev}$ is the reversal potential for potassium. $G_m$ values were plotted against test voltages to obtain conductance-voltage (G/V) curves.

Single-channel currents were recorded in inside-out patches excised from VSMCs at a membrane potential of +40 mV. The open and closed level of a channel was determined from an all point amplitude histogram. Channel open-state was defined as 50% of single-channel current level. Leak-subtracted current records were idealized and used to construct the channel open probability (NPo). NPo was calculated by the equation:

$$NPo = \left( \sum_{j=1}^{N} t_j j \right) / T$$

where $Po$ is the single-channel open-state probability, $T$ is the duration of the measurement, $t_j$ is the time spent with $j = 1,2,.. N$ channels open, and $N$ is the maximal number of simultaneous channel openings seen in the patch. NPo calculations were based on 20 second segments of single channel recordings. The NPo averages of 6 to 9 segments (2-3 min) were used for statistical analysis. Single channel conductance was calculated by the equation: $\sigma = i/V$, where $\sigma$ is the single channel conductance, $i$ is the unitary single channel current and $V$ is the holding membrane potential.

Because all the patches have more than one opening channel, the channel open- and closed-time was not analyzed. After establishment of the inside-out configurations, the Ca$^{2+}$-sensitivity of the channels in the patch was checked by superfusing the patch with Ca$^{2+}$-free solutions until the channel opening disappeared, usually within 5 seconds. If the channel opening did not disappear after 2 min (indicating nonSK4 channel openings), the recording was terminated.
In recordings of whole currents, the bath was superfused with PSS. The pipette solution (PS) contained (in mM) 126 KCl, 6 NaCl, 1.2 MgCl$_2$, 5 EGTA, 11 glucose, 1 MgATP, 0.1 Na$_3$GTP, and 10 HEPES adjusted to pH 7.4 with KOH. The free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) was adjusted to 0.5 μM by adding the appropriate amount of CaCl$_2$ as described previously.$^{12}$ In inside-out recordings, the bath is superfused with PS, the pipette is filled with PS without ATP and GTP.

**Recombinant NDPK Isoforms and PHPT-1**

Expression of GST-fusion proteins of NDPKA and NDPKB as well as His$_6$-tagged PHPT-1 and purification from *E. coli* was described in detail before.$^{13}$ Purified recombinant NDPKC was a kind gift of Ioan Lascu, Bordeaux, France.

**Reverse Transcription and Quantitative Real-Time PCR**

Total RNA was isolated from both injured and uninjured mouse carotid arteries using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. cDNA was synthesized with RevertAid First-Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany). The reaction mix consisted of 10 U/μl of reverse transcriptase, 1 U/μl of RNase inhibitor, 1 mM dNTP, 5 μM random primers, and 0.2 μg RNA in 20 μl total volume. The reaction mixture was incubated at 25°C for 10 min and then at 42°C for 60 min. Finally, the mixture was heated at 70°C for 5 min. Real-time PCR was performed by using 2X Taqman Universal PCR mix (Applied Biosystems, Foster City, CA) with commercial primers (BK: Mm00516078_m1; SK4: Mm00464586_m1; Actb: Mm00607939_s1; Applied Biosystems, Foster City, CA) on an ABI7500 Fast system for 20 sec at 95°C, followed by 40 cycles for 3 sec at 95°C and 30 sec at 60°C. Relative amount of target gene in each sample was calculated from duplicate measure of each sample and normalization is performed to get ratio of mean value of the target gene to that of housekeeping gene in corresponding sample.

**Histology**

The left and right carotid arteries were removed, incubated overnight in 4% formalin and embedded in paraffin. Serial sections (6 μm thick) were obtained from proximal to distal, starting 500 μm proximal to the bifurcation. Slides were stained with hematoxylin and eosin. Images were
digitized and analyzed by IM50 1.2 software (Leica, Heidelberg, Germany). The luminal area, the area inside the internal elastic lamina (IEL), and the area encircled by the external elastic lamina (EEL) were measured in each section. The medial area was calculated as the area between the IEL and EEL, the neointimal area was calculated by subtracting the luminal area from the area inside the IEL. Data from thirty adjacent sections (230 to 50 µM proximal to bifurcation) were average for each specimen.

**Immunofluorescence staining**
The carotid arteries were removed and embedded in Tissue-Tek (Sakura) immediately. For staining, 30 micrometer sections were fixed in Roti-Histofix 4% (Carl Roth GmbH, Karlsruhe, Germany) for 15 min, then, washed 3 times with phosphate buffered saline (PBS). After permeabilization in PBS containing 0.25% Triton-100, the sections were incubated in Roti-block solution (Carl Roth GmbH, Karlsruhe, Germany) for 1 h at room temperature. Subsequently, they were subjected to the incubation with the primary antibody solution overnight at 4°C. After washing 3 times with PBS, the samples were incubated with the secondary antibody for 1.5 h at room temperature. For additional staining with anti-α-smooth actin (SMA) antibody, the sections were incubated with a Cy3 coupled anti-SMA antibody (anti-SMA-Cy3). Nuclei were stained with To-Pro-3 iodide (T3605, 1:300, Life technologies, Darmstadt, Germany) for 30 min. After 3 times washing with PBS, the sections were covered with Roti-mount FluorCar mounting medium (Carl Roth GmbH, Karlsruhe, Germany). The primary antibodies used were anti-Ki67 (AB16667 Abcam, 1:200, Cambridge, UK), or anti-SMA-Cy3 (C6198, 1:100, Sigma-Aldrich, Chemie GmbH, Munich, Germany). The secondary antibodies used were swine anti-rabbit-TRITC (R0156, 1:20, DAKOCytomation, Glostrup, Denmark), or swine anti rabbit-FITC (F0205, 1:20, DAKOCytomation, Glostrup, Denmark). Photos were taken with a confocal microscope (Leica TCS SP2 Confocal Microscope, Leica, Wetzlar, Germany).

**Statistical Analysis**
If not otherwise indicated data are shown as mean ± S.E.M and were analyzed using the InStat© program (GraphPad, San Diego, USA). Gaussian distribution of the data was verified by the Kolmogorov-Smirnov test. By analysis of variance (ANOVA) it was decided whether parametric or non-parametric tests were used for further analysis. For parametric multiple comparisons one way ANOVA with Dunnett’s multiple comparisons was performed. For non-parametric multiple
comparisons Kruskal-Wallis test with Dunn's multiple comparisons was used. For repeated measurements, e. g. analysis of current-voltage relationships, parametric one way repeated measures ANOVA with Dunnett's multiple comparisons were applied. Unpaired Student's t test was used for comparison of two independent groups with equal distribution. $p<0.05$ was considered significant.

Material and Methods - References


Supplemental Material
Tables and Figures

Table I. Characterization of isolated cells from mouse carotid artery by morphology and current inhibition by K⁺ channel blockers

Isolated cells were judged morphologically (relaxed, partially contracted or round shaped). Thereafter, apamin, TRAM-34 and IbTX were applied sequentially. Cells that responded to the blocker (inhibition of \( I_m \) by the blocker >5%) were counted as a blocker sensitive cells (response given in % of cells with this morphology). Only partially contracted cells that are sensitive to IbTX (\( I_{BK} \), prominently occurring in SMCs) and insensitive to apamin (\( I_{SK1-3} \), preferentially occurring in endothelial cells, lymphocytes and fibroblasts) are counted as SMCs and have been used for further analysis.

<table>
<thead>
<tr>
<th>Blocker</th>
<th>Uninjured artery</th>
<th></th>
<th></th>
<th>Injured artery</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>relaxed (55%)</td>
<td>SMC partially contracted (30%)</td>
<td>Non-SMC Endothelium/lymphocyte/fibroblast (15%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IbTX</td>
<td>97% (29/30) (^a)</td>
<td>93% (56/60) (^a)</td>
<td>3% (2/58) (^b)</td>
<td>IbTX</td>
<td>94% (33/35) (^b)</td>
<td>74% (48/65) (^b)</td>
</tr>
<tr>
<td>TRAM-34</td>
<td>3% (1/30)</td>
<td>2% (1/60)</td>
<td>93% (54/58)</td>
<td>TRAM-34</td>
<td>17% (6/35)</td>
<td>87% (57/65)</td>
</tr>
<tr>
<td>Apamin</td>
<td>3% (1/30)</td>
<td>3% (2/60)</td>
<td>91% (53/58)</td>
<td>Apamin</td>
<td>3% (1/35)</td>
<td>3% (2/65)</td>
</tr>
</tbody>
</table>

SMC: smooth muscle cell. Numbers in parantheses give number of positive cells / total number of measured cells. Numbers in superscript indicate the number of mice these cells were isolated from.
Figure I: Characteristics of the TRAM-34- and IbTX-sensitive currents. Currents were recorded in VSMC from injured vessels as described in Figure 1. A, I-V curves showing that cells with a Ca\(^{2+}\)-free intracellular solution displayed neither TRAM-34- nor IbTX-sensitive currents. The current densities of \(I_m\) of 7 cells at +80 mV in control condition were: Ctr 16.8 ± 6.7 pA/pF; TRAM-34, 15.7 ± 5.2 pA/pF; TRAM-34 plus IbTX, 17.9 ± 5.8 pA/pF. B, G-V (membrane conductance versus voltage) curves demonstrating that the conductance converted from IbTX-sensitive (BK) but not from TRAM-34-sensitive (SK4) currents increased with the increments of voltages. n=, number of cells patched / isolated from number of mice.
Figure II: I-V curves demonstrating the effects of NDPKB on SK4 channel currents in neointimal VSMCs. Whole current density ($I_m$, pA/pF) was plotted versus voltages ($V_m$) from -100 to +80 mV to obtain the current-voltage relationship curves (I-V curves). TRAM-34 and IbTX were applied to the cells through a perfusion pipette to unmask the component of SK4 and BK channel currents. NDPKB or PHPT-1 was added through the patch-pipette into the cells. In comparison to Fig. 1B in the main article, a larger inhibition by TRAM-34 is evident after addition of NDPKB in VSMC from injured artery (A), indicating an enhancement of SK4 channel currents. The deletion of NDPKB (E) or addition of PHPT-1 into the cells (D) but not of its inactive form (C) abolished this stimulatory effect of NDPKB on the TRAM-34-sensitive current. No TRAM-34 sensitive currents were apparent in VSMCs from uninjured artery of WT mice (B) or from injured artery of SK4-/-mice (F). Addition of NDPKB had no effect on $I_m$ when compared with Fig. 1A and Fig. 2B of the main article. In contrast to NDPKB (A), NDPKA (G) and NDPKC (H) failed to enhance SK4 channel currents. The currents remained similar as in VSMC from injured arteries of WT mice (Fig. 1B, main article). $[Ca^{2+}]_i$=0.5 µM. n=, number of cells patched / isolated from number of mice.
Figure III: Depletion of NDPKB reduced SK4 channel currents in cultured mouse aorta VSMCs. VSMC were isolated from a single aorta of WT and NDPKB⁻/⁻ mice and expanded in culture. Single VSMC were stimulated through the patch pipette by 300 ms pulses from -100 to +80 mV (10 mV steps) with a holding potential of -50 mV. Whole-cell currents (Iₘ) were measured at the end of the pulses. The TRAM-34-sensitive I_SK4 current was plotted versus voltages (Vₘ) to obtain the current-voltage relationship curves (I-V curves). [Ca²⁺]ᵢ = 0.5 μM. Data shown were analyzed by one way repeated measures ANOVA with Dunnett multiple comparison *p<0.05 vs. WT. n=, number of cells patched / obtained from cultures derived of number of mice. Inset: Western blot analysis of NDPKA and NDPKB expression in VSMC using an anti-Nm23 antibody recognizing several isoforms (sc-343, Santa Cruz Biotechnolgy).