Nucleoside Diphosphate Kinase B Regulates Angiogenesis Through Modulation of Vascular Endothelial Growth Factor Receptor Type 2 and Endothelial Adherens Junction Proteins


Objective—Nucleoside diphosphate kinase B (NDPKB) participates in the activation of heterotrimeric and monomeric G proteins, which are pivotal mediators in angiogenic signaling. The role of NDPKB in angiogenesis has to date not been defined. Therefore, we analyzed the contribution of NDPKB to angiogenesis and its underlying mechanisms in well-characterized in vivo and in vitro models.

Approach and Results—Zebrafish embryos were depleted of NDPKB by morpholino-mediated knockdown. These larvae displayed severe malformations specifically in vessels formed by angiogenesis. NDPKB-deficient (NDPKB−/−) mice were subjected to oxygen-induced retinopathy. In this model, the number of preretinal neovascularizations in NDPKB−/− mice was strongly reduced in comparison with wild-type littermates. In accordance, a delayed blood flow recovery was detected in the NDPKB−/− mice after hindlimb ligation. In in vitro studies, a small interfering RNA–mediated knockdown of NDPKB was performed in human umbilical endothelial cells. NDPKB depletion impaired vascular endothelial growth factor (VEGF)–induced sprouting and hampered the VEGF-induced spatial redistributions of the VEGF receptor type 2 and VE-cadherin at the plasma membrane. Comitantly, NDPKB depletion increased the permeability of the human umbilical endothelial cell monolayer.

Conclusions—This is the first report to show that NDPKB is required for VEGF-induced angiogenesis and contributes to the correct localization of VEGF receptor type 2 and VE-cadherin at the endothelial adherens junctions. Therefore, our data identify NDPKB as a novel molecular target to modulate VEGF-dependent angiogenesis.

Key Word: nucleoside-diphosphate kinase

Angiogenesis, the formation of new capillaries from pre-existing vessels, is a complex process that includes proliferation and migration of endothelial cells, establishment of the basement membrane, and recruitment of periendothelial cells to the walls of the newly formed vessels. Besides physiological angiogenesis, pathological angiogenesis occurs in numerous diseases.1 Angiogenic factors and their receptors are pivotal regulators of angiogenesis. The vascular endothelial growth factor receptor type 2 (VEGFR2) signaling cascade has been extensively investigated, and its importance has been verified, for example, in proliferative diabetic retinopathy and tumor angiogenesis. Therefore, interference with VEGF–VEGFR2 activity and its downstream signaling in angiogenesis, for example, activation of monomeric GTPases of the Rho family, is, therefore, an already used and further sought principal in antiangiogenic therapy.2,3 Also, G protein–coupled receptors contribute to the regulation angiogenesis in endothelial cells.4,5,6 Apparently, G protein–dependent pathways and the VEGF-induced signaling converge downstream on the regulation of RhoGTPases.7,8 Although heterotrimeric G proteins are generally activated by G protein–coupled receptors, the isoform B of the nucleoside diphosphate kinase (NDPKB) activates G protein independently in a complex with G protein βγ dimers by local GTP supply.7 This complex formation is required for accurate G protein function and occurs in signal-transducing caveolae.8,9 Because caveolae play an important role in VEGFR2-dependent signaling in endothelial cells10,11 and a previous publication identified NDPKB as a downstream target in the VEGF–VEGFR2 pathway during endothelial cell differentiation,12 we investigated the role of NDPKB in angiogenesis by using several well-established in vivo and in vitro methods.

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From the Institute of Experimental and Clinical Pharmacology and Toxicology (Y.F., S.G., N.M.W., V.M.B., Y.Q., T.W.), Department of Vascular Biology and Tumor Angiogenesis (J.K.), and the Fifth Medical Clinic (H.-P.H.), Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany; Institute of Neurology (Edinger-Institute), Goethe University, Frankfurt, Germany, (K.D., S.L.); and Division of Nephrology, New York University Langone Medical Center, New York (E.Y.S.).
*These authors contributed equally to this work.

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Correspondence to Thomas Wieland, PhD, Institute of Experimental and Clinical Pharmacology and Toxicology, Medical Faculty Mannheim, Heidelberg University, Maybachstrasse 14, 68169 Mannheim, Germany. E-mail thomas.wieland@medma.uni-heidelberg.de

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**Materials and Methods**

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

**Results**

**NDPKB Depletion in Zebrafish Embryos Causes Impaired Vascular Formation**

To investigate whether NDPKB plays a role in angiogenesis, we assessed the angiogenic response of vessels in several in vivo angiogenesis models with and without NDPKB deficiency. First, we performed a morpholino-mediated knockdown of NDPKB in zebrafish embryos \(^8,9\) and assessed the vessel formation during development using the transgenic zebrafish strain Tg (fli:EGFP). As reported before, \(^8,9\) depletion of NDPKB, but not of NDPKA, caused a severe phenotype including malformations of the vasculature (Figure 1).

At 72 hours postfertilization in the Tg (fli:EGFP) line, control embryos showed a completed formation of intersegmental vessels (ISVs; Figure 1D and 1G; quantification in Figure 1K), which were sprouting from the dorsal aorta and the cardinal vein via angiogenesis. The ISVs were fused together in the majority of the dorsal region of the trunk to form a right and left pair of dorsal longitudinal anastomotic vessels. The depletion of NDPKB caused a dramatic malformation of ISVs. They appeared disrupted and were incapable to form the dorsal longitudinal anastomotic vessels (Figure 1E and 1H; quantification in Figure 1K and 1L). In addition, the NDPKB knockdown embryos were unable to form the parachordal vessels, which run along the horizontal myoseptum and appear by angiogenic growth from secondary sprouts from the posterior cardinal vein.\(^{15}\) Also, the subintestinal veins that provide blood supply to the digestive system were completely missing in the NDPKB-knockdown embryos (Figure 1D and 1E). Besides the vascular abnormalities in the trunk, NDPKB-depleted larvae exhibited brain vessel malformations (Figure I in the online-only Data Supplement). Although the NDPKB\(^{-/-}\) embryos showed a normal formation of the primordial hindbrain channel, the mesencephalic vein, and the middle cerebral vein at 72 hours postfertilization,\(^{14}\) a severe disruption of the central arteries was detected. Interestingly, these central arteries are formed by angiogenesis, whereas the middle cerebral veins arise from vasculogenesis.

To confirm that the observed phenotypes were indeed a result of the loss of NDPKB protein, we performed similar rescue experiments as described before.\(^9\) Restoration of the protein expression level of NDPKB in the zebrafish embryos was achieved by coinjection of a morpholino-insensitive zebrafish NDPKB mRNA together with morpholino-NDPKB (Figure 1J). The rescued embryos exhibited a nearly-complete restoration of the ISV and the dorsal longitudinal anastomotic vessel formation (Figure 1F and 1I; quantification in Figure 1K and 1L). Taken together, our data indicate a severe impairment of angiogenesis because of NDPKB depletion during zebrafish development, whereas vasculogenesis remains obviously unaffected.

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**Figure 1.** Abnormal vessel formation in nucleoside diphosphate kinase B (NDPKB) knockdown zebrafish embryos. A to I, Fluorescent images of Tg (fli:EGFP) zebrafish embryos at 72 h postfertilization (hpf) injected with 4 ng morpholino (MO)-Ctrl as a control (A, D, and G), 4 ng MO-NDPKB to knockdown NDPKB (B, E, and H), and coinjected with 4 ng MO-NDPKB and 0.8 ng morpholino-insensitive NDPKB mRNA to rescue NDPKB in the knockdown embryos (C, F, and I). NDPKB-knockdown embryos showed dramatic abnormal vessel formation of the intersegmental vessels (ISVs, *), the dorsal longitudinal anastomotic vessels (DLAV, ←), and the subintestinal veins (SIV, #), which were rescued by injection of NDPKB mRNA. J, Representative immunoblots of zebrafish lysates prepared at 72 hpf confirmed efficient knockdown of NDPKB protein in embryos injected with MO-NDPKB as well as re-expression of NDPKB protein in embryos coinjected with MO-NDPKB and NDPKB mRNA. β-Actin served as loading control. K and L, Quantification of intact ISV and intact DLAV, respectively. *\(^{\text{P}<0.05}; \text{n}=20\) to 35; scale bar, 50 μm.
Deficiency in NDPKB Delays Blood Flow Recovery in the Hindlimb Angiogenesis Model

Because the zebrafish is primarily a developmental model, we evaluated the impact of NDPKB deficiency on blood flow recovery in the hindlimb angiogenesis model using NDPKB−/− mice.15 As shown in Figure 2A, dark color in the left hindlimb indicates successful ligation of the femoral arteries. Wild-type (WT) mice exhibited the typical blood flow recovery after ligation of the left hindlimb artery, which reached 80% of the preoperation blood flow levels after ≈14 days (Figure 2B). The ligated left hindlimb of NDPKB−/− mice showed a delayed recovery of the blood flow compared with WT mice. A significantly lower blood flow was measured on the third and fourth day after surgery. To analyze the cause of that delay, we harvested the gastrocnemius muscles on the seventh day after ligation and investigated the vascular recovery histologically. The morphology of the gastrocnemius muscles in NDPKB−/− mice was comparable to that of WT littermates. However, the capillary density in the NDPKB−/− mice was significantly reduced in comparison to WT mice (Figure 2C and 2D). The data thus indicate that the delayed vascular recovery in NDPKB−/− mice in the hindlimb model is because of a reduced formation of capillaries by angiogenesis.

NDPKB Deficiency Protects the Retina From Hypoxia-Induced Pathological Retinal Angiogenesis

In the hindlimb model, only pathological angiogenesis can be studied. We, therefore, analyzed the role of NDPKB retinal angiogenesis in mice, in which physiological angiogenesis can also be analyzed. NDPKB−/− mice exhibited a normal vascular development and unaltered morphology of retinal arterioles, venules, and capillaries (Figure II in the online-only Data Supplement). As a model for retinal pathological angiogenesis, oxygen-induced retinopathy was induced in mice by subjecting the mice to hyperoxia between p7 and p12. The area of the avascular zones developed after hyperoxia did not differ between NDPKB−/− and WT littermates (Figure III in the online-only Data Supplement). As expected, preretinal neovascularizations occurred at p17 in WT mice. The amount of these preretinal neovascularizations in NDPKB−/− mice was significantly reduced by ≈70% compared with WT littermates (n=10; P<0.001; Figure 3A and 3B; quantification in Figure 3G). Also in retinal whole mounts, the formation of preretinal neovascularizations was obvious in WT mice but sparsely detectable in NDPKB−/− littermates (Figure 3C and 3D). Furthermore, we assessed the intraretinal vascular regeneration under NDPKB deficiency by measuring the avascular zone at p17. To investigate whether NDPKB deficiency alters the expression of angiogenesis-associated genes, the retinal content in mRNAs encoding such factors were quantified at p17. As expected, the amount of mRNAs coding for VEGF, Ang2, and erythropoietin were significantly elevated in the oxygen-induced retinopathy model (Figure 3G).
There were no differences in the retinal mRNA content for the investigated factors between NDPKB−/− and WT mice (Figure IV in the online-only Data Supplement).

The VEGF–VEGR2 signaling pathway is the predominant driving force in retinal pathological angiogenesis. We, therefore, assessed the expression of VEGFR2 in the hypoxic retinas at p17 by immunohistochemistry (Figure 4). The lectin staining revealed that NDPKB−/− retinas exhibited a decreased number of preretinal neovascularizations. VEGFR2 was strongly expressed in the neovascular tufts in the retinas of WT mice, whereas in NDPKB−/− mice its expression in the neovascularizations was reduced. These results indicate that NDPKB deficiency might interfere with VEGFR2-dependent signaling and, therefore, protects the retina from hypoxia-induced preretinal angiogenesis.

Small Interfering RNA–Mediated Knockdown of NDPKB in Human Umbilical Vein Endothelial Cells Impairs VEGF-Induced Recruitment of VEGFR2

To assess the contribution of NDPKB to the VEGF–VEGFR2 interaction in more detail, we studied VEGFR2 localization in human umbilical vein endothelial cell (HUVEC) after small interfering RNA–mediated NDPKB knockdown. As shown in Figure 5A, the depletion of NDPKB occurred already at 24 hours post-transfection reaching maximal knockdown efficacy of 70% to 80% at 72 to 96 hours after transfection. Therefore, all following measurements were performed at least 72 hours post-transfection. The knockdown was specific as the expression of its closely related isoenzyme NDPKA was not attenuated. Confocal images of control HUVEC showed VEGFR2 clustered in subpopulations in vesicle or endosome-like structures localized from the perinuclear to the submembranous regions. As expected, the VEGFR2 was also localized at the plasma membrane. In accordance with previous data that reported the mobilization of VEGFR2 from an intracellular storage compartment on VEGF stimulation,16 a more prominent, linearized staining of the receptors was detected at the cell membrane in the presence of VEGF (Figure 5B). NDPKB-depleted cells did not show any major difference in the basal distribution of VEGFR2 in comparison with control cells. However, on VEGF stimulation, the reassembly of VEGFR2 at the plasma membrane was disordered (Figure 5B). The receptors appeared scattered and aligned irregularly at the cell plasma membrane. Quantitative assessment by line scans revealed an impairment of the VEGF-induced VEGFR2 recruitment to the plasma membrane in NDPKB-depleted HUVEC (Figure 5C and 5D). As judged by immunoblotting of HUVEC lysates (Figure V in the online-only Data Supplement), the expression of VEGFR2 as well as its phosphorylation in response to VEGF stimulation appeared to be unaltered. Our data thus indicate that NDPKB is required for the shuttling of VEGF2 to the plasma membrane.

Loss of NDPKB Causes an Altered Spatial Rearrangement of Adherens Junction Proteins at the Plasma Membrane and Increases Endothelial Permeability

The endothelial cell adherens junction protein VE-cadherin is sequestered from the plasma membrane together with the
Figure 5. Loss of nucleoside diphosphate kinase B (NDPKB) impairs vascular endothelial growth factor (VEGF)-induced recruitment of the VEGF receptor 2 (VEGFR2) to the plasma membrane of human umbilical endothelial cells (HUVECs). HUVECs were transfected with control small interfering RNA (siRNA) or siRNA against NDPKB. At the indicated time points post-transfection, the expression of NDPKB was monitored by immunoblotting. GAPDH served as loading control (A: left). The specific knockdown of NDPKB was verified at 96 h post-transfection using additionally an antibody primarily directed against NDPK A, which exhibits a weak cross-reactivity against NDPKB (A: right). Here, β-actin served as second independent loading control. VEGFR2 localization in control transfected and NDPKB-knockdown HUVECs was visualized by immunofluorescence and confocal microscopy with and without VEGF stimulation for 10 min at 96 h post-transfection (B). White arrows indicate the cell membrane region. Cropped images of single cells (white boxes in the top panels) are shown in higher magnification in the bottom panels. Pixel densities of line scans were quantified using the Image J software. Representative line scans are shown in C. M1 and M2 denote the plasma membrane of the cell. Quantification of VEGFR2 density at the membrane was obtained from analyzing 15 to 20 cells from each of 3 independent HUVEC isolations (D). ***P<0.001 vs basal control, ###P<0.001 vs VEGF control. The inset shows a representative immunoblot of 1 of the HUVEC isolations with and without NDPKB knockdown. β-Actin served as loading control. Scale bar in B, 20 μm.
activated VEGFR2 into endocytotic vesicles via a crosslink with β-catenin. Because the VEGFR2 can recycle from the early endosome to the plasma membrane, we analyzed the influence of NDPKB depletion on VE-cadherin arrangement at the plasma membrane. The expression of VE-cadherin in NDPKB-depleted HUVEC appeared to be unaltered (Figure V in the online-only Data Supplement). VE-cadherin staining in control transfected cells showed its prototypical localization at the cell membrane, underlining the cell junction. It was, however, also detected in intracellular vesicular structures. On VEGF stimulation, a rearrangement of the adherens junction proteins, VE-cadherin, β-catenin, and p120-catenin (Figure 6A), occurred. As shown in line scan quantification, the VE-cadherin density at the plasma membrane was significantly (Figure 6B) reduced, indicating the disassembly of the adherens junctions. In accordance with this interpretation, VEGF induced a 2-fold increase in EC permeability in comparison to nonstimulated HUVEC (Figure 6C). In NDPKB-knockdown cells, VE-cadherin was still prominently detected at the plasma membrane although additional VE-cadherin staining intensity was present in intracellular vesicles. However, the reorganization of adherens junctions appeared to be partially disorganized displaying no obvious alterations on VEGF stimulation. Interestingly, no VEGF-induced decrease

**Figure 6.** Loss of nucleoside diphosphate kinase B (NDPKB) causes a defective spatial arrangement of VE-cadherin at the membrane and decreases human umbilical endothelial cell (HUVEC) monolayer tightness. HUVECs were transfected with control small interfering RNA (siRNA) or siRNA against NDPKB. VE-cadherin, β-catenin, and p120-catenin localization in control transfected and NDPKB-knockdown HUVECs were visualized with and without vascular endothelial growth factor (VEGF) stimulation for 10 min by immunofluorescence and confocal microscopy at 96 h post-transfection (A). Pixel densities of line scans of VE-cadherin at the plasma membrane were quantified using the Image J software (B). Fifteen to 20 cells from each of 3 independent HUVEC isolations were analyzed. A representative immunoblot from 1 of the HUVEC isolations with and without NDPKB knockdown is shown in the inset. ***P<0.001 vs basal control, ###P<0.001 vs VEGF control. In addition, the permeability of HUVEC monolayers was analyzed by measuring the flux of 70 kDa FITC-Dextran at 96 h post-transfection with and without VEGF stimulation for 10 min (C). Data are derived from 3 independent HUVEC isolations. *P<0.05 vs basal control. Again, a representative immunoblot from 1 of the HUVEC isolations with and without NDPKB knockdown is shown in the inset. Scale bar in A, 20 μm.
in VE-cadherin localization at the plasma membrane could be detected. Concomitantly, the loss of NDPKB caused ≈2-fold increase in endothelial cell permeability without any further alteration by VEGF stimulation. These data clearly indicate a disturbance in endothelial junction formation in HUVEC with NDPKB deficiency. Therefore, NDPKB apparently contributes to the stabilization of adherens junctions and is likely important for the regulation of endothelial cell permeability.

**Small Interfering RNA–Mediated Knockdown of NDPKB in HUVEC Impairs Sprouting Angiogenesis**

The spheroid sprouting angiogenesis of HUVEC is a well-established method for investigating the effects of pro- and antiangiogenic factors in vitro. We, therefore, used this model to investigate the role of NDPKB in sprouting of endothelial cells. As shown in Figure 7, VEGF induced a significant, ≈2-fold increase in the cumulative sprout length of control spheroids at 96 hours post-transfection. The loss of NDPKB did not alter basal sprouting but largely inhibited the formation of sprout induced by VEGF. These results confirm the findings in the zebrafish and the mouse showing that NDPKB is indeed required for VEGF-induced sprouting of endothelial cells.

**Discussion**

In our study, we obtained several lines of evidence indicating that NDPKB, an essential enzyme in nucleoside triphosphate metabolism, is so far an unknown regulator of angiogenesis. First, deficiency in NDPKB results in a decreased angiogenic response in the formation of vessels arising by angiogenesis. Thus, in the basal state, a majority of VEGFR2 do not reside at plasma membrane but in a submembranous vesicular compartment. In accordance with previously published data, we could show that VEGF stimulation induces VEGFR2 depletion of these compartment and an accumulation at the membrane, thereby likely sustaining the angiogenic response to VEGF (see Figure 6). NDPKB depletion did not influence the basal distribution of VEGFR2 in endothelial cells, but inhibited the VEGFR2 trafficking to the plasma membrane on VEGF stimulation, thereby likely hampering sprouting angiogenesis. All our in vivo models are largely VEGF–VEGFR2–dependent angiogenesis models: (1) In the zebrafish, the formation of the ISV as well as the dorsal longitudinal anastomotic vessel was reported to be dependent on VEGFR2 activation. (2) In the mouse hindlimb ischemia model, capillary density was found to be reduced and blood flow recovery to be severely attenuated by interfering with VEGF–VEGFR2 signaling. Similarly, blockade of VEGFR2 suppressed pathological angiogenesis and vascular leakage in the mouse oxygen-induced retinopathy model. Therefore, all our in vivo observations can be explained by the interference in the VEGF–VEGFR2 signaling pathway. In line with this interpretation, a decreased VEGFR2 expression was observed in neovascular tufts in the retinas of NDPKB−/− mice exposed to relative hypoxia (see Figure 4).

A second, apparent difference that we observed in NDPKB-depleted HUVEC was the altered permeability of the EC monolayer and the defect in the rearrangement of adherens junction proteins, namely VE-cadherin, β-catenin, and p120-catenin, on VEGF stimulation. VE-cadherin is associated with VEGFR2 via β-catenin to stabilize the receptor at the cell surface. Thus, the absence of VE-cadherin at the cell junction leads to a more rapid internalization of VEGFR2. On stimulation by VEGF, VE-cadherin dissociates from the receptor and forms complexes with p120- and β-catenin. Both, VEGFR2 as well as VE-cadherin, undergo endocytosis to early endosomes. However, in agreement with the dissociation of VE-cadherin from the activated VEGFR2, VE-cadherin and VEGFR2 do not colocalize within the same endosomal vesicles after internalization. The VEGF-induced internalization of VE-cadherin (see Figure 6B) causes the opening of the adherens junctions and is thus associated with an increase in endothelial permeability (see Figure 6C). Our data indicate that this important mechanism requires NDPKB and is disturbed in its absence. In this context, it is noteworthy that NDPKB knockdown in HUVEC led to a significant decrease of NDPKB not only in the cytosol but even more profound at

**Figure 7.** Small interfering RNA (siRNA)–mediated knockdown of nucleoside diphosphate kinase B (NDPKB) in human umbilical endothelial cells (HUVECs) impairs sprouting angiogenesis. HUVECs were transfected with control siRNA or siRNA against NDPKB. At 72 h after transfection, the cells were transferred into the gel. Thereafter, HUVEC spheroids were cultured in the absence (basal) and presence of vascular endothelial growth factor (VEGF) for other 24 h. Representative spheroids of control and NDPKB-depleted HUVECs with and without VEGF stimulation are shown in A. The cumulative sprout length was averaged for a minimum of 10 spheroids for each condition in an individual independent experiment. Mean±SEM of 6 independent HUVEC isolations are shown in B. *P<0.05 vs basal control. A representative immunoblot from 1 of these HUVEC isolations with and without NDPKB knockdown is shown in the inset. GAPDH served as loading control. Scale bar, 125 μm.
the plasma membrane. Although our data showed an unaltered abundance of the adherens junction proteins VE-cadherin, p120-, and β-catenin at the membranes of NDPKB-depleted HUVECs, the junctions themselves are apparently not formed correctly. Thus, the permeability of the monolayer was largely increased and the VEGF-induced internalization of VE-cadherin was disturbed (see Figure 6). A previous study indicated that the deficiency in VE-cadherin impairs VEGF-induced angiogenesis in mice. Therefore, it is feasible that the deficiency in the correct formation of the adherens junction contributes to the observed reduction in angiogenesis in NDPKB-depleted animals.

We explicitly did not investigate the NDPKB−/− mice as a tumor angiogenesis model because it has been repeatedly reported that tumor cells with high metastatic capability are able to secrete NDPK as well as NDPKA.26,27 The extracellular NDPK converts ADP to ATP, which acts as an agonist at purinergic P2Y receptors and exerts a proangiogenic effect via a Src-dependent transactivation of VEGFR2. Like other nontumor cells, HUVECs are not able to secrete NDPKs and thus neither NDPK proteins nor their enzymatic activity could be detected in supernatants from cultured HUVECs. Thus, all angiogenesis-related effects on NDPKB depletion reported in this study result from the absence of intracellular NDPKB.

**Potential Significance and Implications**

This is the first study showing that NDPKB is a regulator of VEGFR2-dependent angiogenesis. The housekeeping enzyme function in the nucleotide homeostasis of NDPKB-depleted animals is apparently provided by the other NDPK isoforms. Otherwise the animals would die, as, for example, NDPKA/B−/− mice. Nevertheless, we observed an obvious difference between the zebrafish and mouse model. Whereas physiological angiogenesis in NDPKB−/− mice was not impaired, the vascular defects. This apparent difference might be inherent to the comparison of an acute loss model (knockdown) with a genetic knockout model. The investigated NDPKB−/− mice are born and normally live to adulthood without major vascular deficiencies. These animals are, therefore, likely able to compensate for the loss of NDPKB. Only in a pathological situation, when a high intensity of VEGFR2 signaling has to be anticipated as driving force for angiogenesis, this compensation is no longer sufficient and thus the pathological angiogenesis is blunted. Therefore, interfering with NDPKB function likely is an interesting novel strategy for future therapies in which pathological angiogenesis is targeted. In accordance with this hypothesis, the most potent small-molecule inhibitor of the enzymatic activity of NDPKB, ellagic acid,29 exerts antiangiogenic effects via inhibition of the VEGFR2 signaling pathway. Nevertheless, ellagic acid is far from acting specifically on NDPKB. It clearly inhibits the enzymatic activity of other NDPK isoforms and also of other kinases,29,31 and thus its effects might be pleiotropic.

Several possible mechanisms are apparent by which NDPKB likely influences VEGFR2-dependent processes. First, NDPKB was found in a complex with caveolins and thereby promotes caveole formation. The scaffolding domain of caveolin-1 generates a platform for compartmentalization of receptor tyrosine kinases, G protein–coupled receptors, G proteins, and effector proteins in caveolae, thus explaining their important functions in signal transduction, endocytosis, and molecular transport. In endothelial cells, the VEGFR2 resides in caveolae, and angiogenesis in the hindlimb model as well as in colitis model is attenuated by caveolin-1 deficiency. In addition, an increased endothelial permeability has been observed in caveolin-1–knockout mice. Therefore, the alterations observed in NDPKB−/− mice as well as NDPKB-knockdown HUVEC might also be explained by an altered VEGFR2 signaling because of caveole malformation. Second, by its enzymatic activity, NDPKB could locally provide GTP that is required for the activation of G proteins and monomeric GTPases. Recent data showed that the GTPase Rab13, for example, is essential for the transport of vesicles containing RhoA and its GEF Syx to the endocytosed VEGFR2 for the initiation of RhoA activation. Interestingly, Rab13 knockdown in zebrafish embryos caused a similar phenotype as described herein for NDPKB depletion. Another monomeric GTPase that requires NDPK-mediated GTP supply for its proper cellular function is dynamin. It regulates clathrin-dependent and -independent endocytosis as well as tubular membrane fission in a NDPK-dependent manner and directly interacts with NDPK through a C-terminal proline–rich domain. Although this interaction is not specific to NDPKB and can be equally performed by NDPKA,37 we cannot exclude that dynamin 2–dependent VEGFR2 trafficking38 contributes to the observed phenotype. Thus, a more detailed analysis of the underlying mechanisms, for example, whether the catalytic activity of NDPKB or its role in caveole formation is involved in its role in angiogenesis should be performed in future studies.

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**Disclosures**

None.

**References**


Significance

Angiogenesis plays a major role in development, tissue repair, and pathological response to injury processes. Prominent examples for the latter are proliferative eye diseases and malignancy for the former wound healing. Herein, our data demonstrate that nucleoside diphosphate kinase B is relevant for angiogenesis in several disease models and suggests a functional pleiotropic role in tissue homeostasis. The data clearly show that nucleoside diphosphate kinase B is relevant for major cellular events in endothelial cell activation by vascular endothelial growth factor such as adhesions junction opening and vascular endothelial growth factor receptor 2 recruitment. Because the effect of nucleoside diphosphate kinase B depletion is comparable to the effect of established angiogenesis inhibitors, it is conceivable that interference with nucleoside diphosphate kinase B is an attractive, clinically relevant target.
Nucleoside Diphosphate Kinase B Regulates Angiogenesis Through Modulation of Vascular Endothelial Growth Factor Receptor Type 2 and Endothelial Adherens Junction Proteins

Yuxi Feng, Shalini Gross, Nadine M. Wolf, Vicki M. Butenschön, Yi Qiu, Kavi Devraj, Stefan Liebner, Jens Kroll, Edward Y. Skolnik, Hans-Peter Hammes and Thomas Wieland

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Figure I- Abnormal vessel formation in the head of NDPK B knockdown zebrafish embryos.

Fluorescent images of Tg (fli:EGFP) zebrafish embryos at 72 hpf injected with 4 ng MO-Ctrl as a control (A, C), 4 ng MO-NDPK B to knockdown NDPK B (B, D). In the head, zebrafish embryos showed a defective development of the central arteries (*), but not the middle cerebral veins (⇠) after knockdown of NDPK B at 72 hpf.
Figure II. NDPK B deficiency does not impair the physiological angiogenesis in the mouse retina.

Retinal superficial (A, B) and deep (C, D) capillary layers were assessed in retinal whole mounts. There were no differences in the retinal vascular development in the superficial and deep capillary layer between NDPK B⁺/− mice and their WT littermates. White dotted lines: total retinal area. Red lines in A and C indicate superficial and deep capillary layer, respectively. Scale bar in A and C 200 µm.
Figure III. The influence of the relative hyperoxia in WT and NDPK B⁻/⁻ mice in the OIR model at p12.
Avascular zones at p12 in the OIR model were evaluated in retinal whole mounts by lectin staining. The border of the avascular zone is indicated by a white dotted line (A). NDPK B⁻/⁻ and WT mice exhibited similar sizes of the avascular zone areas (B). Scale bar 400 µm.

Figure IV. Relative expression of angiogenesis-associated genes in WT and NDPK B⁻/⁻ mice in the OIR model at p17.
The retinal mRNA content coding for VEGF, Ang1, Ang2, EPO and TNFα was detected by real time PCR. There were no changes in the relative abundance of these gene transcripts in the OIR model at p17.
Figure V. The loss of NDPK B does not alter VEGFR-2 and VE-cadherin expression as well as well VEGFR-2 phosphorylation in HUVECs.

HUVEC were transfected with control siRNA or siRNA against NDPK B. The expression of VEGFR-2 (A,B) and VE-cadherin (B,C) as well as NDPK B knockdown (A) was visualized by immunoblotting. (D) Phosphorylation of VEGFR-2 was induced with 50 ng/ml VEGF for the indicated periods of time and visualized using an anti-phospho-VEGFR-2 antibody. Representative immunoblots are shown in A and D. Pixel densities were quantified from n=3 independent experiments (B–D).
Figure VI. The model of oxygen induced retinopathy.

New-born mice were placed into a chamber with 75% oxygen from p7 to p12, inducing vascular degeneration (avascular zone indicated by * in C at p12). Pre-retinal neovascularizations at p17 can be visualized on paraffin sections stained with PAS (indicated by arrows in B). Vascular morphology in the retina such as avascular zones (* in C), vascular sprouts, capillary density can be assessed in retinal whole mounts stained with lectin labelled with FITC or TRITC. For more details see text. Scale bar in B 20 µm, in C 200 µm.
Material and Methods - online-only Data Supplement

Zebrafish maintenance and manipulation
Zebrafish maintenance and analyses were performed as described previously. The transgenic zebrafish strain Tg (fli:EGFP) strain which expresses EGFP under an endothelial-specific fli-promoter was used. Morpholino (MO)-modified antisense oligonucleotides (Genetools, USA) were directed against the translation start site of zebrafish NDPK B/NDPK-Z1 (5′-GGTGCGCTCGGTCTTAGCAGACATG-3′) and diluted for micro-injection in 0.2M KCl. Zebrafish embryos at the one cell stage were injected with MO-NDPK B (4 ng) or a standard control oligonucleotide at the same concentration. For rescue experiments, a zebrafish morpholino-insensitive NDPK B mRNA containing 5 silent mutations was synthesized from pCS2-NDPK B using the mMESSAGEmMASCHINE system (Ambion). Approximately 0.8 ng/embryo of mRNA were micro-injected into embryos at the one cell stage. Zebrafish embryos at 72 hours post fertilization (hpf) were immobilized in methylcellulose, photographed or analyzed by confocal microscopy. For quantification of the intersegmental vessels, the first 17 ISVs from the anterior part of 20 - 35 embryos (72 hpf) were analyzed. For quantification of the dorsal longitudinal anastomotic vessel, the segments between 17 ISVs from the anterior part of 20-35 embryos (72 hpf) were counted. Immunoblotting was performed according to standard procedures. Embryos at 72 hpf were treated with Deyolking Buffer (55 mM NaCl; 1.8 mM KCl; 1.25 mM NaHCO3) and homogenized in lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% Triton X-100, 150 mM NaCl, 1 mM PMSF and protease inhibitors).

Mice
Animal care and experiments were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement and were approved by the local government. Homozygote NDPK B−/− and wild type littermates with a C57Bl6 background were used in the study. Genotyping was performed as described previously.

Hind limb ischemia
After anesthesia the proximal end of left femoral artery and the distal portion of the saphenous artery were ligated. All side branches were also ligated and the segment of femoral artery was excised. Hind limb blood flow was measured by Doppler examination before, immediately after, and at 3, 7, 10, 14, 21, 28 and 35 days after occlusion. The animals were anesthetized and placed for 15 minutes on 37°C heated pads for the measurement. An LDPI (Moor Instruments) was used to estimate relative blood flow. Ratios of occluded to not occluded values were compared after background subtraction.
**Morphological analysis of hind limb capillaries**
Gastrocnemius muscles of NDPK B knockout mice and their wild type littermates were fixed in 4% paraformaldehyde and embedded in paraffin. Two-micrometer sections were cut and stained with PAS for evaluation of general histology of the muscles, or lectin conjugated with FITC (1:50; Sigma, Germany) for visualization of the vessels in the muscles. 5-6 fields of individual animal were photographed with a digital camera (IM50, Leica, Germany). The capillary density was calculated as the number of capillaries (positively stained with lectin) in relation to the number of muscle fibers.

**Oxygen-Induced Retinopathy (OIR)**
The new-born mice were placed into a chamber with 75% oxygen at postnatal day 7 (p7) for 5 days with their nursing mothers (Fig. S6A). The hyperoxia between p7 and p12 leads to vascular degeneration in the superficial capillary layer causing avascular zones in the center of the retina. At p12, the mice were returned to room air. From p12 to p17, the mice are therefore subjected to a relative hypoxia. At p17, the eyes were enucleated and analyzed for pre-retinal neovascularizations (Fig. S6B), and intra-retinal vascular regeneration in the superficial and deep capillary layers (Supplemental Fig. S6C). The vessels grow from the border of the avascular zones into the hypoxic area causing a reduction in the size of the avascular zones with time.

**Analysis of retinal neovascularization in the OIR model**
Quantitative neovascularization analysis was performed in the p17 eyes from the OIR model as described previously. In brief, eyes embedded in paraffin were cut into 6 µm serial sections through or parallel to the pupil-optic nerve-axis. Then, the sections were stained with Periodic Acid and Schiff’s reagent and hematoxilin. The occurrence of pre-retinal neovascularization nuclei penetrating the inner limiting membrane towards the vitreous was evaluated. Neovascular nuclei were counted in 10 sections around the optic nerve with an interval of 18µm.

**Whole mount retinal immunofluorescence**
To evaluate the vascular morphology such as avascular zones and *de novo* vessel formation in the OIR model, eyes were fixed in 4% formalin at room temperature for 2 h. Thereafter, the retinas were dissected and washed with PBS. The retinas were permeabilized and blocked in a solution containing 1% bovine serum albumin and 0.5% Triton-100. Subsequently, the retinas were incubated with lectin conjugated with FITC or TRITC (1:50, Sigma, Germany) to visualize retinal vessels. Central avascular zones and *de novo* vessel formation in the retina
were quantified by using an analysis program (IX81, Olympus, Hamburg, Germany) or confocal microscopy (Leica, Germany).

**RNA isolation and quantitative PCR**
Retinal RNA was isolated from individual retinas homogenized in 1ml Trizol reagent (Invitrogen, Germany) according to the manufacturer’s instructions. Then, the total RNA was purified utilizing the RNeasy Kit (Qiagen, Germany) with DNase-treatment to completely remove genomic DNA. The mRNA was reversely transcribed using Superscript II RNaseH-Reverse Transcriptase (Invitrogen, Germany) and subjected to Taqman analysis using the Taqman 2xPCR master Mix (Applied Biosystems, Germany). Quantitative PCR was performed in the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Germany). The expression of genes was analysed by the Ct method using 18s ribosomal RNA as endogenous control. All primers with probes were purchased from Applied Biosystems (VEGF: Mm01281449_m1; Angpt1: Mm00456503_m1; Angpt2: Mm00545822_m1; Epo: Mm 01202754_g1; Tnf: Mm00443258_m1; Act b: Mm01205647_g1)

**Isolation and culture of human umbilical vein endothelial cells, transfection with siRNA**
Human umbilical vein endothelial cells (HUVECs) were extracted from the human umbilical vein by dispase digestion and suspended in 10% FCS (Promocell, C 37350) to stop the enzyme activity. Cells suspension were seeded in endothelial cell growth complete medium (ECGM, Promocell) with 10% FCS. Endothelial cells were grown on gelatin-coated culture flasks. The culture flask was placed in a humidified incubator (37°C, 5% CO2) followed by medium exchange after 2 hours. At passage (P) 0, cells were split after they exhibited a cobblestone morphology and reached confluence. Cells were maintained in culture until P3. Cells were seeded and allowed to grow overnight to 70% confluence in a 12-well dish. Thereafter, the cells were transfected using lipofectamine (Invitrogen) according to the manufacturer’s protocol. An amount 100 pmol of a scrambled siRNA (AACUGGUGACUACAAGUCUU, Eurofins MWG operon) or a NDPK B-specific siRNA (AGGUAGUGUAACGCCUUUG) was used. Four hours after transfection, the cells were supplemented with ECGM containing 10% FCS. Analyses were conducted at 48 and 96 h post transfection

**Western Blot analysis**
Cells were scraped off in RIPA buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 mM dithiothreitol, 1% Triton X-100, 1% sodium deoxycholate) and centrifuged for 15 min. The supernatant was analyzed by standard SDS-PAGE. Proteins were electrically transferred
onto nitrocellulose membranes, blocked with Rotiblock (Roth) and incubated with the indicated antibodies, followed by detection with an ECL detection reagent (Thermo Scientific). The following antibodies were used: anti-fish NDPK B (1:200, L-16, Santa Cruz), anti-NDPK A (1:200, C-20, Santa Cruz Biotechnology), anti-NDPK B (1:1000, MC-412, Kamiya), anti-VEGFR-2 antibody (55B11, Cell signaling), anti-VE cadherin antibody (ALX-210-232, Enzo Life Sciences), anti-β actin (1:10,000, A2228, Sigma), anti-GAPDH (1:8000, Meridian).

**Sprouting assay**

HUVECs were suspended in 5 ml ECGM containing 1 ml methocel and 20% FCS. *In vitro* sprouting angiogenesis assays were performed as described. Briefly, spheroids generated for 24 hours with 400 cells were embedded into a 3D collagen-based gel placed in a 48-well plate. They were stimulated under the indicated conditions for 24 h and the cumulative sprout length was measured.

**Immunohistochemistry**

Cells were seeded on gelatin-coated glass cover slips and transfected with siRNA as described. Therafter, the cells were grown overnight in ECGM, washed with PBS, treated without and with VEGF and fixed with 4% formaldehyde for 15 min at room temperature. The cells were then permeabilized with 0.1% Triton X-100 for 3 min and incubated with primary antibodies overnight at 37°C. After washing three times for 5 min with PBS cells were incubated with the secondary antibody for 1 h at 37°C. The cells were washed again with PBS and mounted using mowiol (Calbiochem, Germany). Used primary antibodies: anti-VEGFR-2 antibody (55B11, Cell signaling), anti-VE cadherin antibody (ALX-210-232, Enzo Life Sciences), anti-p120-catenin (sc-23872, Santa Cruz Biotechnology), anti-β-catenin (sc-7199, Santa Cruz Biotechnology). Used secondary antibodies rabbit anti-Dylight 549 (Rockland), polyclonal swine anti-rabbit FITC (F0205, DAKO). Staining intensity was quantified using the Image J software. Pixel densities were measured by drawing a line across the cell, such that the cell membrane and cytoplasmic regions were marked. The highest peaks at the membranes were measured.

**In vitro permeability assay**

Permeability assays were performed on HUVECs as described previously. Briefly, scrambled or NDPK B siRNA-transfected HUVECs (2 days post transfection) were seeded onto 24-well transwell PET inserts at 200,000 cells/cm² pre-coated with 5 µg/cm² fibronectin (Sigma). Upon confluence, the cells were treated with VEGF (50 ng/ml) or vehicle control (0.1% BSA in PBS) in both apical and basal chamber with simultaneous addition of FITC
labelled dextran (70kDa, 10μM) to the top apical chamber. Aliquots from the bottom chamber and the top chamber were obtained after 1 h and read for FITC fluorescence in a plate reader at 490nm (excitation) /520nm (emission) (TECAN M200, Germany). Background fluorescence units from tracer-free culture medium were subtracted from and permeability was expressed as the ratio of bottom chamber fluorescence to the corresponding top chamber fluorescence. The vehicle treated control (scrambled siRNA) condition was set to 100%.

**Statistical analysis**

Data are presented as means ± SEM, unless otherwise stated. The data were analyzed using the GraphPad Prism software (GraphPad Software, La Jolla). Unpaired t-test with Welch’s correction or Analysis of Variance (ANOVA) with appropriate post-tests was used for statistical analysis. A p value <0.05 was considered statistical significant.

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