An antiangiogenic neurokinin-B/thromboxane A2 regulatory axis

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Establishment of angiogenic circuits that orchestrate blood vessel development and remodeling requires an exquisite balance between the activities of pro- and antiangiogenic factors. However, the logic that permits complex signal integration by vascular endothelium is poorly understood. We demonstrate that a "neuropeptide," neurokinin-B (NK-B), reversibly inhibits endothelial cell vascular network assembly and opposes angiogenesis in the chicken chorioallantoic membrane. Disruption of endogenous NK-B signaling promoted angiogenesis. Mechanistic analyses defined a multicomponent pathway in which NK-B signaling converges upon cellular processes essential for angiogenesis. NK-B−mediated abla-tion of Ca^{2+} oscillations and elevation of 3'−5' cyclic adenosine monophosphate (cAMP) reduced cellular proliferation, migration, and vascular endothelial growth factor receptor expression and induced the antiangiogenic protein calreticulin. Whereas NK-B initiated certain responses, other activities required additional stimuli that increase cAMP. Although NK-B is a neurotransmitter/neuromodulator and NK-B overexpression characterizes the pregnancy-associated disorder preeclampsia, NK-B had not been linked to vascular remodeling. These results establish a conserved mechanism in which NK-B instigates multiple activities that collectively oppose vascular remodeling.

Introduction

The development of new blood vessels from existing vasculature termed angiogenesis is an essential physiological process and is critical for certain pathological disorders including rheumatoid arthritis, diabetic retinopathy, atherosclerosis, psoriasis, and tumor growth/metastasis (Carmeliet and Jain, 2000; Hanahan and Weinberg, 2000; Folkman, 2006). Multiple endogenous factors have been implicated in promoting and suppressing angiogenesis, and a balance between pro- and antiangiogenic activities determines the angiogenic response.

Both intact proteins and proteolytic fragments can be endogenous angiogenesis inhibitors. Although plasminogen, type XVIII collagen (Col XVIII), Col XV, Col IVα1, Col IVα2, Col IVα3, and fibronectin lack antiangiogenic activity, proteolytic cleavage of these proteins yields angiostatin, endostatin, endostatin-like fragment from type XV collagen, arresten,

canstatin, tumstatin, and anastelin, respectively, which are anti-angiogenic (Nyberg et al., 2005). Thrombospondin 1 exemplifies an antiangiogenic protein that functions as an intact protein (Iruela-Arispe et al., 1991; Sund et al., 2005). A protein precursor and proteolytic product can both be antiangiogenic as illustrated by calreticulin and its N-terminal fragment vasostatin (Pike et al., 1998, 1999).

Endogenous angiogenesis inhibitors target endothelium via suppressing cell proliferation and migration, inducing apoptosis, down-regulating proangiogenic factors and signaling pathways, and inducing antiangiogenic factors (Abdollahi et al., 2004; Nyberg et al., 2005; Folkman, 2006). Endogenous angiogenesis inhibitors can antagonize cell surface integrins (Sudhakar et al., 2003; Sund et al., 2005). Novel antiangiogenic mechanisms include impaired tubulin polymerization (Mabjeesh et al., 2003), inhibition of ATP synthase (Moser et al., 2001), and induction of VEGF receptor (VEGFR) proteolysis (Cai et al., 2006).

Our studies to dissect genetic networks initiated by the transcription factor GATA-1 in erythroid cells revealed that GATA-1 activates transcription of the murine preprotachykinin-B gene (*Tac-2*; Pal et al., 2004), which encodes a neurokinin-B

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(NK-B) precursor protein (Kotani et al., 1986). The human orthologue *TAC-3* is highly induced upon ex vivo differentiation of peripheral blood hematopoietic precursors (Pal et al., 2004). *Tac-2* is also expressed by neurons (Kotani et al., 1986), the uterus (Pinto et al., 1999), and syncytiotrophoblasts of the placenta (Page et al., 2000). Biological functions of tachykinins include smooth muscle contraction (Leander et al., 1981), vasodilation (Mills et al., 1974), neurotransmission (Otsuka and Takahashi, 1977), neurogenic inflammation (Levine et al., 1984), and immune system activation (Payan et al., 1983). NK-B activates NK1, NK2, and NK3 G protein−coupled receptors (Shigemoto et al., 1989; Gerard et al., 1990; Fong et al., 1992). As quantitative RT-PCR analysis did not reveal NK receptor expression in erythroid cells, erythroid cell-derived NK-B might act on neighboring cells within the hematopoietic and/or vascular microenvironments. NK receptors are expressed on mouse yolk sac endothelial cells (YSECs) and mouse aortic endothelial cells, and NK-B induces cAMP accumulation in these cells (Pal et al., 2004). NK receptors are also expressed on endothelial cells of rat postcapillary venules (Bowden et al., 1994), human umbilical vein endothelial cells (HUVECs; Greeno et al., 1993;

Brownbill et al., 2003), and bovine corpus luteal endothelial cells (Brylla et al., 2005). These findings led us to hypothesize that NK-B targets endothelial cells, although the ensemble of physiological targets and the molecular and physiological consequences were unclear.

Here, we demonstrate that NK-B blocks endothelial cell motility, represses VEGFR expression, and induces an antiangiogenic protein, all of which collectively oppose vascular network assembly. NK-B activity is greatly potentiated by thromboxane A2 (TXA2) signaling or phosphodiesterase inhibition, providing evidence for an antiangiogenic NK-B/TXA2 axis.

Results

NK-B synergizes with TXA2 signaling to abrogate vascular network assembly, and disruption of endogenous neurokinin signaling is angiogenic

Because endothelial cells express NK receptors (Pal et al., 2004), we tested whether NK-B regulates endothelial cell proliferation, migration, and vascular network assembly. Endothelial cell
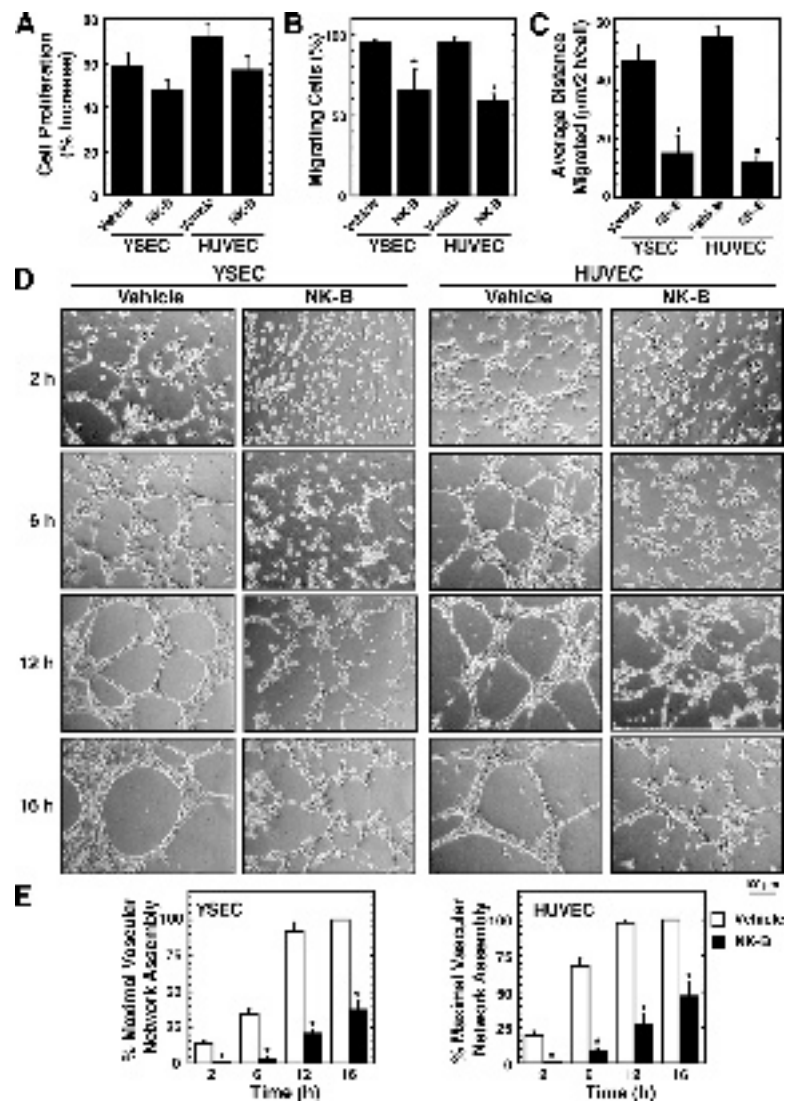
Figure 1. **NK-B suppresses endothelial cell motility and vascular network assembly.** (A) YSECs and HUVECs were cultured in supplement-free medium for 24 h and treated with vehicle or NK-B for 1 h. Cells were incubated with supplemented medium containing vehicle or NK-B for 24 h, and proliferation was quantitated. The graph depicts the percent increase of proliferation relative to cells grown without supplement (mean ± SEM; three independent experiments). (B) YSECs and HUVECs were treated with vehicle or NK-B in supplement-free medium for 1 and 2 h, respectively, and plated on Matrigel with supplemented medium containing the same reagents. Cell migration was monitored for 2 h. Migrating cells were expressed as a percentage of the total cells (four independent experiments). (C) The rate of migrating YSECs and HUVECs analyzed in B was measured using Slidebook 4 software. (D) YSECs and HUVECs were treated with vehicle or NK-B for 1 and 2 h, respectively, in supplement-free medium, plated on Matrigel containing supplemented medium, and incubated for 16 h at 37°C. Representative pictures are shown. (E) The length of tubular structures from three adjacent frames was quantitated from three independent experiments. The length of the structures in vehicle-treated cells at 16 h was designated 100%. Statistical significance was determined relative to the vehicle-treated condition (*, P < 0.05).

growth supplement containing 2% fetal calf serum, FGF2, EGF, hydrocortisone, and heparin (see online supplemental material, available at http://www.jcb.org/cgi/content/full/jcb.200603152/DC1) stimulates YSEC and HUVEC proliferation. NK-B did not inhibit the proliferation response (Fig. 1 A). YSECs and HUVECs assemble extensive vascular networks on Matrigel containing the supplement. Real-time video microscopy revealed that NK-B treatment before culturing cells on Matrigel reduced the number of migrating cells (Fig. 1 B; Video 1, Vehicle; and Video 2, NK-B) and strongly reduced the motility of cells that remained competent to migrate (Fig. 1 C). Importantly, the rate and extent of vascular network assembly were suppressed by NK-B (Fig. 1, D and E). We tested another tachykinin, substance P (SP; Mills et al., 1974), which was reported to promote HUVEC vascular network assembly and angiogenesis (Fan et al., 1993; Ziche et al., 1994; Wiedermann et al., 1996). SP did not affect supplement-induced YSEC and HUVEC network assembly (unpublished data).

A consequence of NK receptor activation is elevation of intracellular cAMP (Nakajima et al., 1992). We demonstrated that NK-B increases cAMP in YSECs in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; Pal et al., 2004). IBMX increased the magnitude of the cAMP induction ∼1.7-fold and converted the transient cAMP induction into a sustained response (Fig. 2 A). As elevated cAMP impairs endothelial cell survival, motility, and angiogenesis (Kim et al., 2000, 2002), we tested whether IBMX potentiates NK-B activity. Although IBMX or NK-B alone did not affect YSEC proliferation (Fig. 1 A), NK-B/IBMX suppressed proliferation (Fig. 2 B). NK-B/IBMX nearly ablated YSEC motility (Fig. 2 C; Video 3, IBMX; and Video 4, NK-B/IBMX, available at http://www.jcb.org/cgi/content/full/jcb.200603152/DC1). Because the motility blockade was prevented by a combination of NK1- and NK3-selective small molecule inhibitors (Fig. 2 C and Video 5, NK-B/IBMX/NK inhibitors), NK receptor interactions appear to mediate the blockade.

Given the importance of proliferation and motility for vascular network organization, we predicted that NK-B/IBMX would severely compromise network assembly. NK-B/IBMX abrogated YSEC network assembly on Matrigel (Fig. 2, D and G). The NK-B/IBMX−mediated induction of cAMP would be expected to activate PKA and downstream effectors. The PKA inhibitor H-89 prevented the NK-B/IBMX−mediated abrogation of vascular network assembly (Fig. 2 G), indicating that PKA is required for NK-B/IBMX actions. Replacement of NK-B/IBMX−containing medium with medium lacking NK-B/IBMX after 20 h restored network assembly by 36 h (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200603152/DC1), indicating the lack of cytotoxicity.

Analogous to the IBMX potentiation of NK-B activity, we reasoned that physiological factors such as TXA2 that activate adenylyl cyclase functionally interact with NK-B. NK-B (Page et al., 2000) and TXA2 are overexpressed in preeclampsia (Walsh, 1985). TXA2 mediates FGF-COX-2−stimulated angiogenesis (Daniel et al., 1999) and inhibits VEGF- and FGF2-induced endothelial cell migration and vascular network formation (Ashton et al., 2004; Ashton and Ware, 2004).

The stable prostaglandin U46619 recapitulates endogenous TXA2 signaling (Rocca et al., 2000). Because IBMX potentiation is associated with sustained and increased magnitude of the cAMP induction, we asked whether U46619 similarly affects cAMP. YSECs and HUVECs express TXA2 receptors (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200603152/DC1). U46619 increased the magnitude of the cAMP induction, without sustaining the response (Fig. 2 E). NK-B and U46619 together (NK-B/U46619; Video 7) abrogated network assembly and motility, whereas U46619 alone (Video 6) had no effect (Fig. 2, F and G). The U46619 activity required TXA2 receptor
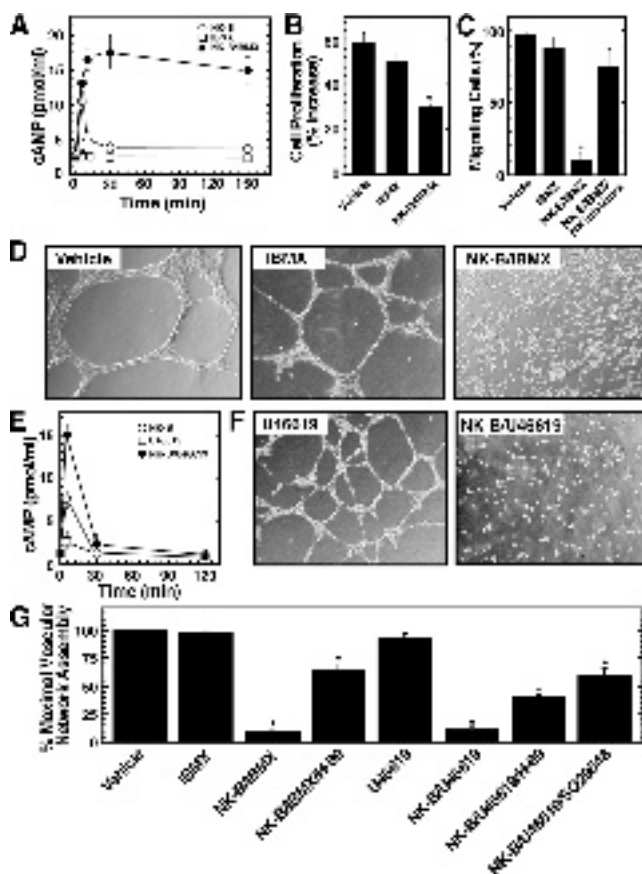
Figure 2. **IBMX and TXA2 potentiate NK-B activity to regulate endothelial cell function.** (A) YSECs were treated with NK-B or NK-B/IBMX in supplement-free medium for the indicated times, and cAMP was assayed (mean ± SEM; three independent experiments). (B) YSECs were cultured in supplement-free medium for 24 h and treated with vehicle, IBMX, or NK-B/IBMX for 1 h. Cells were incubated with supplemented medium containing the same reagents for 24 h, and proliferation was quantitated. The graph depicts the percent increase of proliferation relative to cells grown in supplement-free medium (three independent experiments). (C) YSECs were treated with the indicated reagents in supplement-free medium for 1 h and plated on Matrigel with supplemented medium containing the same reagents, and motility was monitored for 2 h. Migrating cells were expressed as a percentage of total cells (three independent experiments). (D) YSECs were treated with vehicle, IBMX, or NK-B/IBMX for 1 h in supplement-free medium, plated on Matrigel containing supplemented medium, and incubated for 20 h at 37°C. (E) YSECs were treated with NK-B, U46619, or NK-B/U46619 in supplement-free medium. cAMP was quantitated various times thereafter (three independent experiments). (F) YSECs were treated with U46619 or NK-B/U46619 in supplement-free medium for 1 h, and vascular network assembly on Matrigel was assessed. (G) Quantitation of vascular network assembly. Statistical significance was determined relative to the vehicle condition (*, P < 0.05).

Figure 3. **NK-B inhibits vascular remodeling of certain human endothelial cell subtypes.** (A) HUVECs, HAECs, and HMVECs were treated with IBMX or NK-B/IBMX for 2 h in supplement-free medium, plated on Matrigel containing supplemented medium, and incubated for 12 h. (B) Quantitative real-time RT-PCR analysis of NK1, NK2, and NK3 receptor mRNA levels in HUVECs, HAECs, and HMVECs. The relative mRNA levels were normalized by HPRT mRNA levels. The plots depict the mRNA ratios in which the ratios obtained for the -RT condition were designated as 1 (mean ± SEM; three independent experiments). RT, reverse transcriptase. (C) HUVECs, HAECs, HMVECs were treated with IBMX with or without forskolin or NK-B for 1 h. cAMP was quantitated in cell lysates (mean ± SEM; three independent experiments).
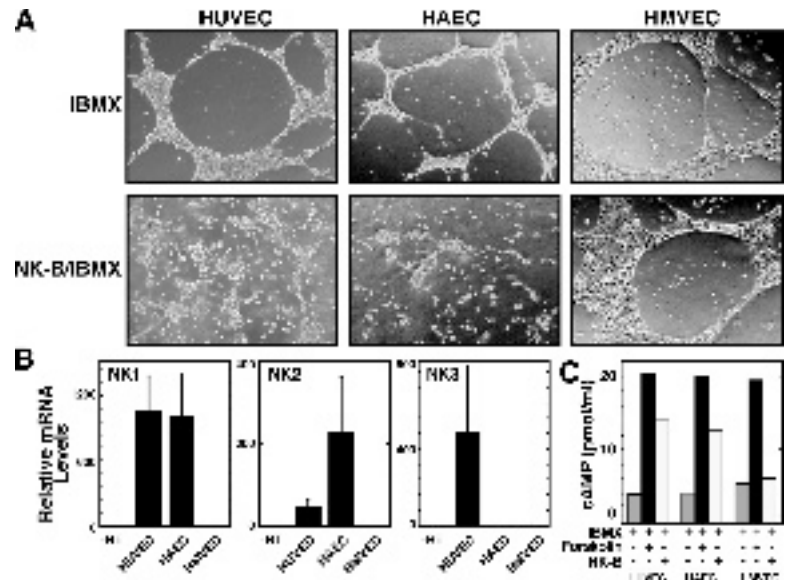
activation, as the selective TXA2 receptor antagonist SQ29548 strongly reduced the NK-B/U46619 activity to block vascular network assembly (Fig. 2 G). Analogous to NK-B/IBMX, H-89 significantly reduced the NK-B/U46619 activity to abrogate network assembly (Fig. 2 G). Thus, TXA2 potentiates NK-B activity to oppose vascular remodeling.

Endothelial cell subtypes have unique gene expression profiles, reflecting their distinct functional roles (Chi et al., 2003; Ho et al., 2003). We tested if NK-B/IBMX regulates the

capacity of human endothelial cells from the umbilical vein (HUVEC), aorta (human aortic endothelial cell [HAEC]), and neonatal dermis microvasculature (human microvascular endothelial cell [HMVEC]) to assemble vascular networks. NK-B/IBMX abrogated HUVEC and HAEC network assembly, whereas HMVEC vascular network assembly was unaffected (Fig. 3 A). The differential responsiveness correlates with NK receptor expression. HUVECs and HAECs, but not HMVECs, express NK receptors (Fig. 3 B). NK-B/IBMX induced cAMP

Figure 4. **NK-B is antiangiogenic and disruption of endogenous neurokinin signaling is angiogenic in the CAM.** (A) Methylcellulose containing vehicle, FGF2 (500 ng)/IBMX (100 µg), FGF2/NK-B (40 µg) with or without IBMX, FGF2/mNK-B/IBMX (mNK-B, inactive mutant of NK-B), or a combination of NK1- (L733060; 5 µM) and NK3- (SB222200; 2 µM) selective inhibitors was applied to the CAM of day 7 chicken embryos. After 48 h, digital images were captured, and representative images are shown (15–20 eggs were analyzed per condition; three to four independent experiments). Bar, 500 mm. (B) The graph depicts the relative microcirculation density. Statistical significance was determined relative to the FGF2/IBMX condition, unless bracketed (*, P < 0.05). (C) Real-time PCR quantitation of NK1 and NK3 mRNA in CAM and chicken embryo (mean ± SEM; three independent experiments). RT, reverse transcriptase.
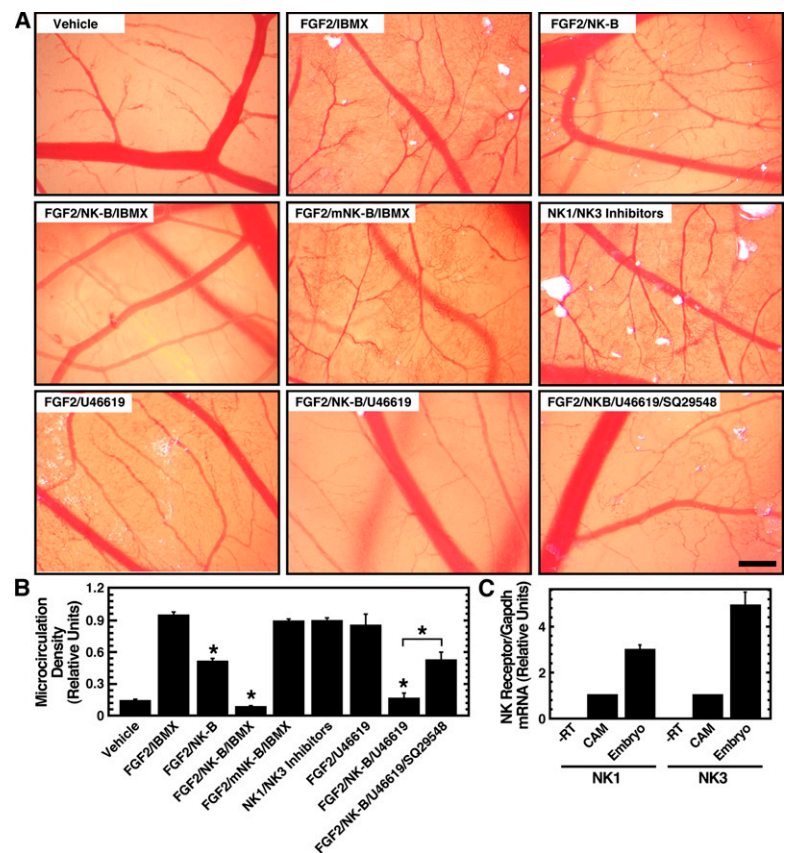
accumulation in HUVECs and HAECs, but not in HMVECs (Fig. 3 C). Direct activation of adenylyl cyclase with forskolin induced cAMP similarly in the three cell types. In contrast to NK-B/IBMX, 8-Br cAMP and forskolin did not affect HUVEC vascular network assembly, whereas NK-B/forskolin and NK-B/8-Br cAMP abrogated network assembly (Fig. S3, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200603152/DC1). These findings indicate that cAMP induction is necessary but insufficient for abrogating network assembly.

Vascular network assembly in vitro reflects the capacity of endothelial cells to form blood vessels, but the milieu that regulates angiogenesis in vivo is considerably more complex due in part to the three dimensionality of the microenvironment (Yancopoulos et al., 2000; Jain, 2003; Rossant and Hirashima, 2003; Stupack and Cheresh, 2004). An alternative assay uses collagen gels to recapitulate a three-dimensional microenvironment. NK-B/IBMX, but not IBMX, abrogated HUVEC vascular network assembly in collagen gels (Fig. S3 C).

To test whether NK-B alone or NK-B/IBMX inhibits angiogenesis in vivo, we asked whether NK-B and NK-B/IBMX oppose FGF2-dependent angiogenesis in the chicken chorioallantoic membrane (CAM) assay. NK-B inhibited FGF2-mediated expansion of the microvasculature by ∼40%, and NK-B/IBMX almost completely blocked angiogenesis (Fig. 4, A and B). A mutant NK-B (mNK-B) inactive in the Matrigel assay (not depicted), in which the methionine residue at position 2 was substituted with the β amino acid analogue β^3-homomethionine, did not influence FGF2-induced angiogenesis with or without IBMX. Thus, the structural integrity of NK-B is crucial for antiangiogenic activity.

If NK-B functions physiologically to oppose proangiogenic factors, blocking endogenous NK-B signaling with NK receptor inhibitors should promote angiogenesis. A combination of NK1- and NK3-selective inhibitors induced a strong angiogenic response equal to FGF2 (Fig. 4, A and B). Based on the inhibitor specificities (Seabrook et al., 1996; Sarau et al., 2000), these data provide evidence that endogenous neurokinin signaling suppresses endogenous angiogenic pathways. Furthermore, NK-B might function physiologically with other factors such as TXA2. Whereas U46619 alone lacked activity in the CAM assay, NK-B/U46619 suppressed angiogenesis, and this activity was antagonized by the TXA2 receptor antagonist SQ29548 (Fig. 4, A and B); SQ29548 was not active in the CAM assay (not depicted). Quantitative RT-PCR confirmed NK1 and NK3 expression in the CAM (Fig. 4 C).

Mechanism of NK-B targeting of endothelium: suppression of intracellular Ca²⁺ oscillations

The NK receptor inhibitor sensitivity of the NK-B/IBMX−induced motility blockade suggested that the blockade requires NK receptor signaling. To genetically assess the role of NK1 and NK3 in vascular network assembly, NK1 and NK3 were knocked down individually via stable expression of short hairpin RNAs. The knockdowns almost completely ablated the respective mRNA (Fig. 5 A). Under conditions in which NK1 or NK3 were knocked down, the ability of NK-B/IBMX to block network assembly was reduced (Fig. 5, B and D). Both NK1 and NK3 are therefore required for NK-B to inhibit network assembly. The role of NK receptors in opposing vascular remodeling was further demonstrated with NK1- and NK3-selective inhibitors, which prevented NK-B/IBMX from abrogating network assembly (Fig. 5, C and E).

Tachykinins induce phosphoinositide hydrolysis yielding metabolites that mobilize intracellular Ca²⁺ (Grandordy et al., 1988; Nakajima et al., 1992). Because dynamic changes in intracellular Ca²⁺ have an important role in regulating motility (Ridley et al., 2003) and differentiation (Spitzer et al., 2000), we investigated whether NK-B alters intracellular Ca²⁺ levels.
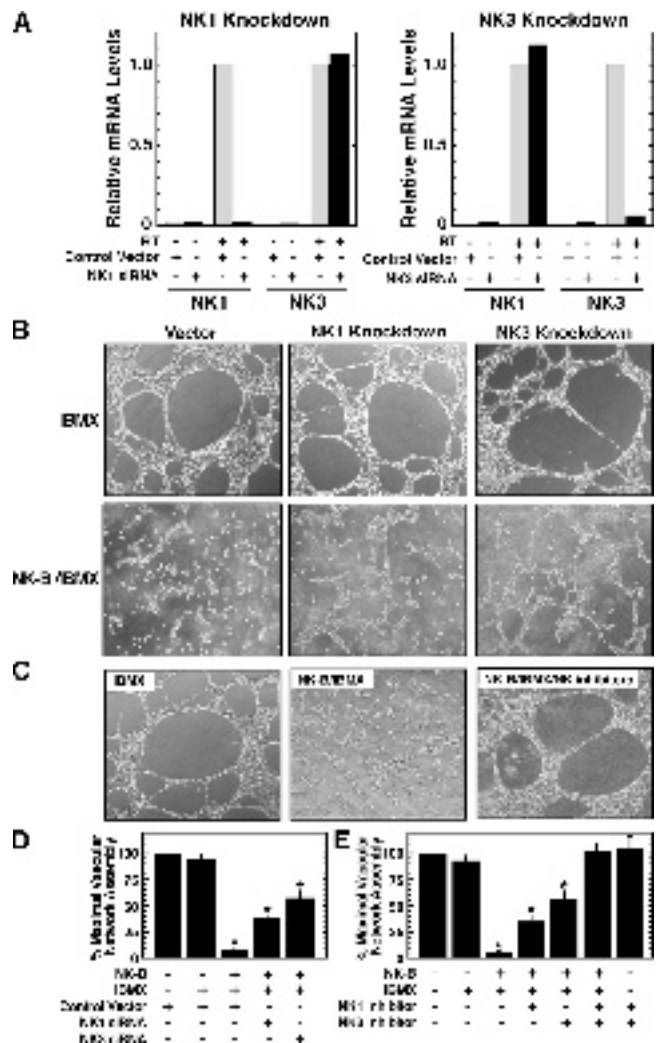

Figure 5. **NK receptor requirement for NK-B−mediated abrogation of vascular network assembly.** (A) Quantitative RT-PCR analysis of NK1 and NK3 mRNA in YSEC clonal lines stably expressing NK1 (left) and NK3 (right) siRNA molecules (RT, reverse transcriptase; two independent experiments). (B) Control and siRNA-expressing YSECs were treated with IBMX or NK-B/IBMX and incubated on Matrigel for 20 h at 37°C. Knocking down either NK1 or NK3 reduced the capacity of NK-B/IBMX to abrogate vascular network assembly. (C) YSECs were treated with IBMX or NK-B/IBMX with or without NK1- (L733060) and NK3- (SB222200) selective inhibitors for 15 min, followed by NK-B/IBMX, and plated on Matrigel to assess vascular network assembly. (D and E) Quantitative analysis of vascular network assembly. Statistical significance was determined in D and E relative to the control vector and untreated conditions, respectively (*, P < 0.05; mean ± SEM; three independent experiments).

The supplement induced Ca^{2+} oscillations in YSECs, which were blocked by NK-B or NK-B/IBMX, but not mNK-B (Fig. 6, A and B). Forskolin did not affect the percentage of cells exhibiting oscillations (Fig. 6 B). NK-B–mediated ablation of Ca^{2+} oscillations is therefore independent of the cAMP response.

If the NK-B−dependent suppression of Ca^{2+} oscillations is functionally important, we reasoned that an independent strategy to ablate Ca^{2+} oscillations should mimic the NK-B activity to oppose vascular network assembly. The Ca^{2+} chelator BAPTA ablated Ca^{2+} oscillations (Fig. 6 C) and significantly reduced network assembly (Fig. 6, D and E), consistent with the notion that suppression of Ca^{2+} oscillations represents a functionally important component of the NK-B mechanism.

Mechanism of NK-B targeting of endothelium: VEGF and VEGFR down-regulation

FGF2 induces VEGF synthesis in certain contexts (Seghezzi et al., 1998), and both YSECs and HUVECs produce more VEGF when cultured in FGF2-containing medium (Fig. 7 A). We tested whether NK-B opposes FGF2-dependent angiogenesis by acting upstream or downstream of FGF2-induced VEGF production. NK-B alone, or in the presence of IBMX or U46619, prevented supplement-induced VEGF production (Fig. 7 A), indicating that NK-B acts upstream of FGF2-induced VEGF

production. Furthermore, blocking Ca^{2+} oscillations with BAPTA also inhibited VEGF production (Fig. 7 A, left), indicating that NK-B−mediated abrogation of Ca^{2+} oscillations is important for reduced VEGF output.

Because VEGF and FGF2 promote vascular network assembly (Yancopoulos et al., 2000), we tested whether NK-B affects expression of Type I (VEGFR1; de Vries et al., 1992) and type II (VEGFR2; Matthews et al., 1991) VEGFRs and FGF receptor 1 (FGFR1; Werner et al., 1992). When starved HUVECs and YSECs were treated with supplement, *VEGFR1* and *VEGFR2* mRNA increased five- and twofold, respectively, by 6 h (Fig. 7 B). NK-B, NK-B/IBMX, and NK-B/U46619 inhibited *VEGFR1* mRNA induction. NK-B/IBMX and NK-B/U46619, but not NK-B alone, inhibited *VEGFR2* expression. Analysis of VEGFR1 and VEGFR2 protein expression validated the mRNA analysis (Fig. 7 C). *VEGFR1* and *VEGFR2* expression were also measured in HUVECs and YSECs plated on Matrigel. *VEGFR1* and *VEGFR2* mRNA increased considerably, and NK-B/IBMX strongly suppressed the induction (Fig. 7 D). *FGFR1* induction in YSECs was not affected by NK-B/IBMX. The supplement did not induce *FGFR1* expression in HUVECs. Because VEGF stimulates endothelial cell proliferation and motility (Leung et al., 1989; Waltenberger et al., 1994; Senger et al., 1996), VEGFR down-regulation should diminish proliferation, migration, and vascular network assembly.
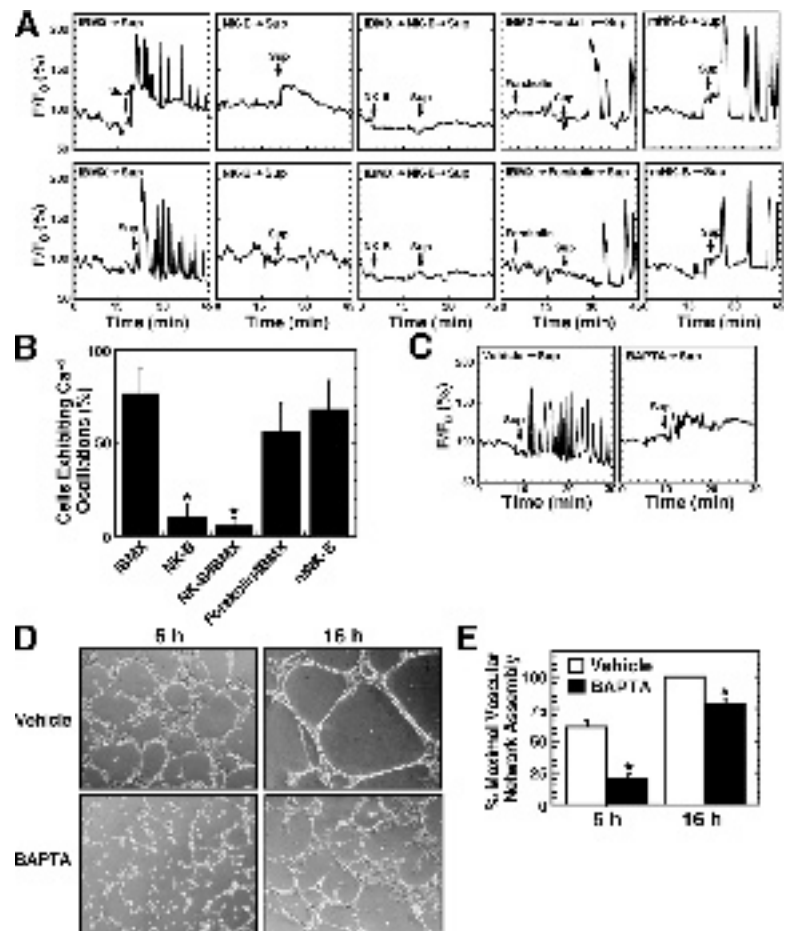

Figure 6. **NK-B ablates Ca^{2+} oscillations.** (A) YSECs were loaded with Fluo-4/AM in supplement-free M200 medium and plated on Matrigel under various conditions. Intracellular Ca^{2+} was monitored every 15 s. The graphs depict Ca^{2+} oscillation patterns, plotted as a percentage of fluorescence intensity at time 0 (F_0), of two representative cells (two independent experiments per condition; Sup, supplemented M200 medium). (B) Quantitation of the percentage of cells exhibiting Ca^{2+} oscillations (mean ± SEM; three independent experiments). (C) YSECs were loaded with Fluo-4/AM with or without 4 μM BAPTA/AM in supplement-free M200 medium and plated on Matrigel in supplement-containing medium with vehicle or BAPTA/AM. Intracellular Ca^{2+} was monitored every 15 s. The graphs depict Ca^{2+} oscillation patterns of representative cells, plotted as a percentage of fluorescence intensity at time 0 (F_0). (D) YSECs were treated with vehicle or 4 μM BAPTA/AM for 1 h in supplement-free medium, plated on Matrigel containing supplemented medium with or without BAPTA, and incubated at 37°C. Vascular network assembly was assessed after 6 and 16 h. (E) Quantitation of vascular network assembly of the experiment described in D (three independent experiments). Statistical significance was determined relative to the vehicle condition (*, $P < 0.05$).
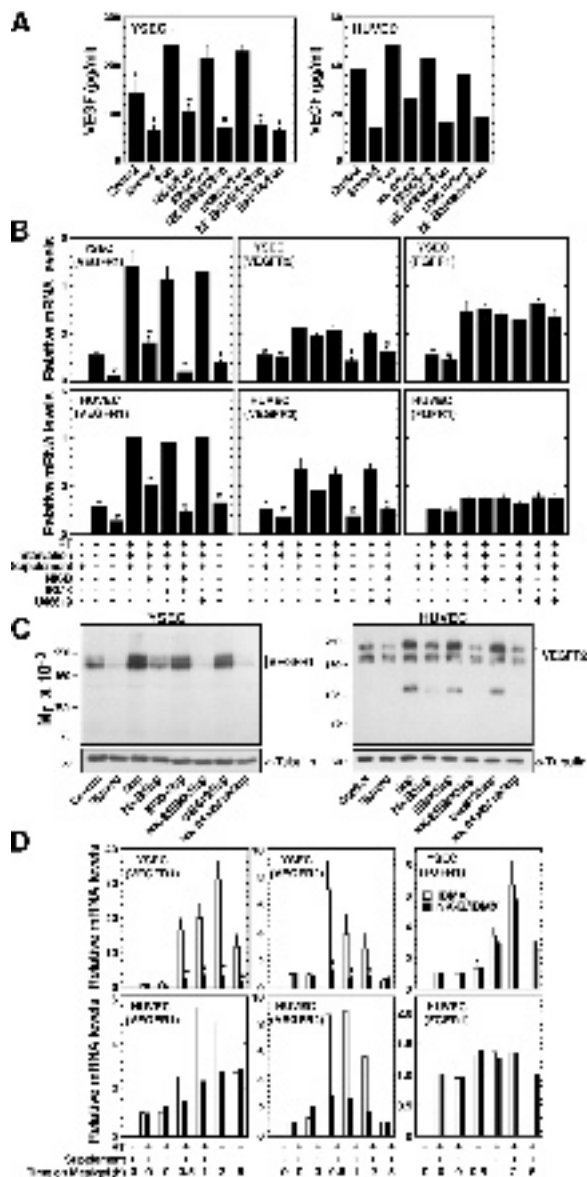
Figure 7. **NK-B down-regulates VEGFRs.** (A) YSECs and HUVECs on tissue culture plates were cultured in medium lacking supplement for 14 h and treated with indicated reagents in supplement-containing medium for 24 h, and VEGF secreted in the culture medium was quantitated by ELISA (two [right] to three [left] independent experiments). Sup, supplemented M200 medium. (A and B) Statistical significance was determined relative to the supplement condition (*, $P < 0.05$; mean ± SEM). (B) YSECs and HUVECs were cultured in medium lacking supplement for 10 h and treated with indicated reagents in supplement-free medium for 2 h. Cells were treated with supplement-containing medium with indicated reagents for 6 h. mRNA was quantitated by real-time RT-PCR. mRNA levels in untreated cells were designated 1 (three independent experiments). (C) Western blot analysis of VEGFR1 and VEGFR2 in YSECs and HUVECs, respectively. (D) YSECs and HUVECs were treated with IBMX or NK-B/IBMX in medium lacking supplement for 1 or 2 h, respectively, and plated on Matrigel containing supplemented medium for 0.5, 1, 2, and 5 h. Transcripts were quantitated by real-time RT-PCR. Transcript levels in untreated cells were designated 1 (two to three independent experiments). RT, reverse transcriptase. Statistical significance was determined relative to the IBMX-treated condition.

As VEGF induces VEGFR1 and VEGFR2 expression (Barleon et al., 1997; Shen et al., 1998) and NK-B suppresses VEGF production (Fig. 7 A), we tested whether NK-B/

IBMX−mediated down-regulation of VEGFR1 and VEGFR2 is counteracted by elevating VEGF164. VEGF164 induced *VEGFR1* and *VEGFR2* mRNA to 30–60% of the control level after 5 or 20 h in YSECs; FGF2 had no effect (Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200603152/DC1). These results support an NK-B action upstream of FGF2-dependent VEGF production. FGF2 was active, as FGF2 induced FGFR1 expression (Fig. S4 A, right). Concomitant with reactivated VEGFR expression, network assembly was partially rescued (Fig. S4 B). These results indicate that a balance between NK-B/IBMX and VEGF signaling dictates the extent of network assembly.

NK-B induces a known antiangiogenic factor

A proteomics screen was conducted to test if NK-B/IBMX regulates the levels of proteins linked to angiogenesis. Two-dimensional SDS-PAGE analysis identified four regulated proteins (Fig. 8 A). MALDI-TOF (matrix-assisted, laser-desorption-ionization/time of flight) mass spectroscopy identified the proteins as β-tubulin (1 and 2), L-lactate dehydrogenase (3), and calreticulin (4), respectively. The Ca^{2+} binding protein calreticulin, (Johnson et al., 2001) which functions as a chaperone and extracellularly as a signaling molecule with thrombospondin (Goicoechea et al., 2002) and integrins (Coppolino et al., 1997), was verified by Western blotting (Fig. 8 B). Whereas NK-B alone and U46619 alone did not induce calreticulin, NK-B/U46619 increased calreticulin approximately twofold. The N-terminal 180 amino acids of calreticulin, termed vasostatin, were purified previously as an antiangiogenic factor (Pike et al., 1998). Both calreticulin and vasostatin are antiangiogenic (Pike et al., 1999; Yao et al., 2000).

To assess whether YSEC and HUVEC vascular network assembly is responsive to calreticulin, calreticulin and vasostatin were overexpressed in *Escherichia coli* as GST fusion proteins, and GST was liberated via site-specific proteolysis (Fig. 8 C). Purified calreticulin and vasostatin impaired network assembly (Fig. 8 D). These results support a model in which NK-B/IBMX−mediated induction of calreticulin, VEGF, VEGFR1, and VEGFR2 down-regulation, reduced cell proliferation, and reduced cell motility constitute a multicomponent antiangiogenic mechanism.

Discussion

Our results demonstrate NK-B targeting of endothelium via a multicomponent mechanism to oppose vascular remodeling. Not only is NK-B antiangiogenic, but disruption of endogenous neurokinin signaling stimulated angiogenesis comparable to FGF2. These results suggest a new mode of vascular regulation in which NK-B functions as an endogenous angiogenesis inhibitor. The TXA2 potentiation of NK-B activity exemplifies how NK-B can interact with other physiological factors to regulate endothelial cell function.

Although a link between NK-B function and vascular regulation had not been established previously, SP can regulate endothelial cell function. SP promotes angiogenesis during acute neurogenic inflammation (Seegers et al., 2003), and expression
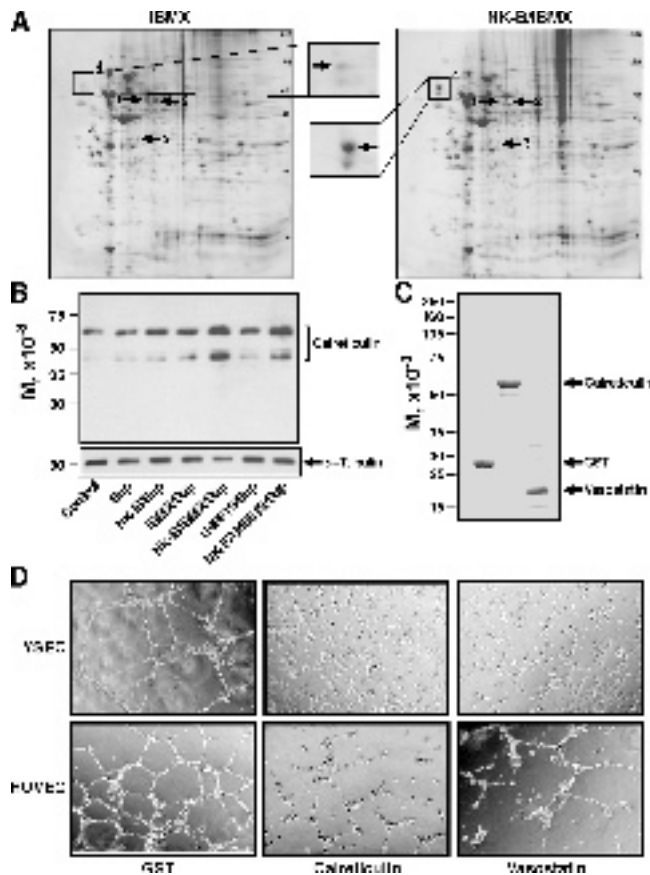
Figure 8. **NK-B induces the antiangiogenic protein calreticulin.** (A) YSECs were treated with IBMX or NK-B/IBMX for 1 h in supplement-free medium and then 10 min in supplemented medium. Whole cell extracts were subjected to two-dimensional gel electrophoresis and stained with Coomassie blue. Representative stained gels are shown, and the inset shows the spot identified as calreticulin. (B) Representative Western blots of calreticulin and α-tubulin in whole cell lysates of YSECs after treatment with indicated reagents for 1 h in supplement-free medium, followed by 10 min in supplemented medium. (C) SDS-PAGE of *E. coli* overexpressed and purified recombinant calreticulin and vasostatin. (D) Calreticulin and vasostatin inhibit YSEC and HUVEC vascular network assembly. Cells were treated with 380 nM GST, 270 nM calreticulin, or 250 nM vasostatin for 1 h in supplement-free medium and plated on Matrigel to assess vascular network assembly.

of the NK receptor preferred by SP, NK1, is up-regulated in endothelial cells upon angiogenesis during chronic airway inflammation in rats (Baluk et al., 1997). These results indicate that NK receptor signaling by endothelial cells mediates vascular regulation in certain contexts, but the underlying mechanisms were not defined.

In addition to regulating angiogenesis, NK receptor modulation alters vascular tone (Page et al., 2001). Synthetic NK receptor agonists induced hypertension in guinea pigs (Roccon et al., 1996), continuous infusion of NK-B in rats increased blood pressure (Page et al., 2000), and NK-B induced vasodilation of TXA2-constricted perfused placental cotyledon (Brownbill et al., 2003). However, mechanisms underlying these activities are unknown.

NK-B resembles certain known endogenous angiogenesis inhibitors (Nyberg et al., 2005) that suppress endothelial cell proliferation and migration. NK-B down-regulated VEGFR1

and VEGFR2 expression, and endostatin down-regulates VEGFR2 transcription approximately twofold (Abdollahi et al., 2004). Both endostatin and TIMP2 inhibit VEGFR2 phosphorylation (Abdollahi et al., 2004). We are unaware of reports in which endogenous angiogenesis inhibitors down-regulate VEGFR1 expression. Pigment epithelium-derived factor inhibits growth factor−induced angiogenesis in microvascular endothelial cells through a mechanism involving cleavage and intracellular translocation of the VEGFR1 transmembrane domain (Cai et al., 2006). Another potentially important component of the NK-B mechanism is calreticulin induction. Other examples of an endogenous angiogenesis inhibitor inducing a distinct angiogenesis inhibitor include endostatin and fibulin 5 up-regulation of thrombospondin 1 and pigment epithelium-derived factor up-regulation of plasminogen kringle 5 (Gao et al., 2002; Abdollahi et al., 2004; Albig and Schiemann, 2004). Besides NK-B, angiogenesis inhibitors have not been reported to induce calreticulin expression.

The molecular steps of the multicomponent mechanism segregate into extracellular (Fig. 9, I), intracellular signaling (Fig. 9, II), and effector (Fig. 9, III) modules. Multiple lines of evidence indicate that NK-B−mediated cAMP induction is required for a subset of the NK-B activities. Inclusion of IBMX with NK-B further reduced YSEC motility (Fig. 2 C) and vascular network assembly (Fig. 2 D) and inhibited *VEGFR2* mRNA induction (Fig. 7 A). Forskolin resembled NK-B in inhibiting *VEGFR1* and *VEGFR2* mRNA induction (Fig. S4 C). H-89 blocked NK-B/IBMX−mediated abrogation of vascular network assembly (Fig. 2 G). Elevated cAMP can impair endothelial cell survival, motility, and angiogenesis (Kim et al., 2002). VEGFR1 and VEGFR2 down-regulation would further suppress proliferation and motility, given the VEGF activity to stimulate proliferation and motility (Leung et al., 1989; Waltenberger et al., 1994; Senger et al., 1996).

Although tachykinins can mobilize intracellular Ca^{2+}, our results show that NK-B blocks growth factor−dependent Ca^{2+} oscillations (Fig. 6 A). As intracellular Ca^{2+} stimulates motility via calpain-dependent and -independent mechanisms (Franco et al., 2004), the suppressive activity of NK-B on Ca^{2+} oscillations should amplify the motility blockade established via cAMP induction. The finding that BAPTA mimics NK-B in ablating Ca^{2+} oscillations and opposing vascular network assembly (Fig. 6, D and E) reinforces the functional significance of the NK-B−mediated abrogation of Ca^{2+} oscillations.

Recombinant calreticulin and vasostatin, which are antiangiogenic in vivo (Pike et al., 1999; Yao et al., 2000), suppressed vascular network assembly but only modestly inhibited motility (unpublished data). Vasostatin inhibits endothelial cell attachment to laminin (Yao et al., 2002). It is attractive to propose that elevated calreticulin/vasostatin synergizes with the NK-B−instigated motility blockade to oppose vascular remodeling. Forskolin did not induce calreticulin (unpublished data), indicating that a distinct NK-B activity mediates calreticulin induction. The Ca^{2+} ionophore ionomycin, which increases intracellular Ca^{2+}, elevated calreticulin expression by approximately twofold (Fig. S4 D), suggesting that reduced intracellular Ca^{2+} does not explain NK-B−mediated calreticulin induction.
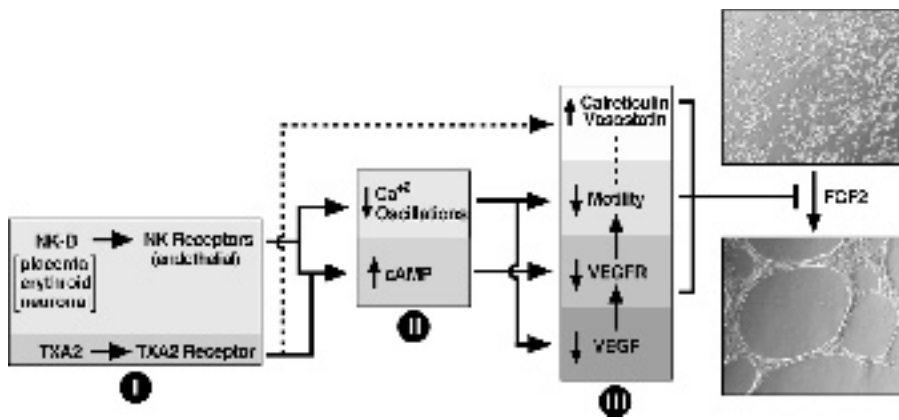
Figure 9. **NK-B/TXA2 regulatory axis.** The model illustrates extracellular signaling (I), intracellular signaling (II), and effector modules (III), each consisting of multiple reactions, which collectively oppose angiogenesis. NK-B, which is expressed in neurons, erythroid cells, and the placenta, signals through NK receptors to increase cAMP and to suppress Ca^{2+} oscillations. TXA2 signaling mimics IBMX in potentiating NK-B−dependent cAMP induction and downstream responses that are sensitive to changes in cAMP levels. The functional consequences include VEGF and VEGFR down-regulation, decreased cell motility, and increased calreticulin synthesis. The dotted line denotes a putative pathway that is not yet supported by experimental evidence.

Because IBMX increased the magnitude and sustained the cAMP induction, whereas U46619 only increased the magnitude of the response, a sustained response is not required for potentiating NK-B activity. Considering that both NK-B and TXA2 are elevated in preeclampsia (Page et al., 2000; Walsh, 2004), it is attractive to propose that NK-B targeting of vascular endothelium and TXA2 potentiation of NK-B activity have important implications for vascular deregulation in preeclampsia. As TXA2 is not likely the sole physiological factor that interacts with NK-B to oppose vascular remodeling in vivo, it will be important to identify additional factors that function combinatorially with NK-B and TXA2. Nevertheless, our demonstration that NK-B directly targets endothelium, that perturbation of endogenous neurokinin signaling is angiogenic, and that TXA2 potentiates NK-B activity and our elucidation of the NK-B signaling circuitry establish an NK-B/TXA2 axis as a new mode of vascular regulation.

Materials and methods

Drugs and chemicals

NK-B, NK1 receptor−selective antagonist L733060 ((2S, 3S)-3-(93,5-bis (trifluoromethyl) phenyl) methoxy)-2-phenylpiperidine hydrochloride), NK3 receptor−selective antagonist SB222200 ((S)-3 methyl-2-phenyl-N-(1-phenylpropyl)-4-quinilinecarboxamide), 8-BrcAMP, forskolin, and H-89 were purchased from Sigma-Aldrich. U46619 and IBMX were purchased from Calbiochem and were solubilized in DMSO. U46619 and H-89 were diluted in ethanol and 50% ethanol, respectively. SQ 29548 (Cayman Chemical) was dissolved in ethanol. The final concentrations of NK-B, IBMX, 8-Br cAMP, forskolin, H-89, U46619, and SQ 29548 in cell culture medium were 100, 100, 100, 10, 10, 1, and 2 μM, respectively. NK-B was maximally effective at 75 μM. The final concentrations of DMSO and ethanol did not exceed 1%. Recombinant bovine FGF2 (provided by A. Rapraeger, University of Wisconsin School of Medicine, Madison, WI) and mouse VEGF164 (R&D Systems) were reconstituted in PBS containing 1% BSA.

Synthesis of mutant NK-B

An NK-B mutant peptide was produced via microwave-assisted solid- phase peptide synthesis (Murray and Gellman, 2005). Fmoc amino acids (Calbiochem-Novabiochem) were activated with HBTU/HOBt in dimethyl-formamide and coupled using microwave irradiation (600 W maximum power, 70°C, ramp 2 min, hold 2 min; MARS multimode microwave [CEM Corporation]). Removal of the Fmoc protecting group was accomplished by treatment with 20% piperidine in dimethylformamide with microwave irradiation (600 W maximum power, 80°C, ramp 2 min, hold 2 min). After cleavage from the solid support (NovaSyn TGR resin; Novabiochem) with trifluoroacetic acid, the crude peptide mixture was purified by reverse phase HPLC and structurally validated by MALDI-TOF mass spectrometry.

Cell lines and cell culture

Mouse YSECs were derived from a hypervascular transgenic mouse expressing the *fps/fes* protooncogene (Lu et al., 1996). YSECs exhibit a normal endothelial phenotype and are not tumorigenic. HUVECs, HAECs, and HMVECs were purchased from Cascade Biologics, Inc. Cells were maintained as described in the online supplemental material (available at http://www.jcb.org/cgi/content/full/jcb.200603152/DC1).

Cell proliferation assay

YSECs and HUVECs were grown in 48-well plates (1.5×10^4 cells/well) with supplement-free M200 medium to allow synchronization of cells in G_1/G_0. After 24 h, supplemented M200 medium containing either vehicle, NK-B, IBMX, or NK-B/IBMX was added and incubated at 37°C for another 24 h. The cell proliferation was quantified using CellTiter 96 AQueous One Solution reagent (Promega) according to the manufacturer's directions. The results from triplicate determinations (mean ± SD) are presented as the percent increase of proliferation compared with nonstimulated growth in supplement-free medium.

Vascular network assembly assay

Vascular network assembly was assessed by measuring the formation of capillary-like structures by endothelial cells on Matrigel (BD Biosciences). Matrigel was diluted 1:1 with supplement-free M200 medium, poured in 24-well plates, and allowed to solidify at 37°C. Subconfluent endothelial cells were harvested and preincubated under different experimental conditions (YSECs, 1 h; HUVECs, HAECs, and HMVECs, 2 h) in growth supplement−free M200 medium in microfuge tubes. An equal volume of supplemented medium containing the indicated reagents was added. Cells were plated on Matrigel (1.5×10^5 cells/well) and incubated at 37°C. Vascular network assembly was measured as a function of time, and digital pictures were captured with an inverted microscope (Axiovert 200M; Carl Zeiss MicroImaging, Inc.; 10× objective, NA 0.25), using the Axiovision 3.1 software (Carl Zeiss MicroImaging, Inc.). To quantitate the vascular network assembly, digital pictures of several adjacent frames were taken, and the lengths of the tubelike structures were measured.

Time-lapse video microscopy

Subconfluent YSECs and HUVECs were pretreated with vehicle, NK-B, IBMX, or IBMX/NK-B for 1 or 2 h, respectively, in supplement-free M200 medium, 5–10 × 10^4 cells were plated per well in a 12-well Matrigel-coated plate, and an equal volume of supplemented medium with or without various reagents was added. NK receptor inhibitors were added 30 min before adding NK-B. Cells were allowed to adhere for 30 min, and time-lapse images were acquired at 37°C with an inverted microscope (TE300; Nikon; 20× objective, NA 0.45), equipped with a CoolSnap fx charge-coupled device camera (Photometrics) and a cube temperature controller (Life Imaging Services). Images were captured with E-See Inovision Software (Inovision) and with Slidebook 4 software (Intelligent Imaging Innovations; one image per minute for 120 min). Cells migrating in a field were quantitated by putting a digital mark in the center of the cell body at time 0 and determining the migration of the cell body away from the digital mark after 2 h. Migrating cells were further validated by Z-stack projection in Image J. The migrating cells were expressed as a percentage of the total cells in each field. The rate of cell migration was quantitated using the Slidebook 4 software. The center of each migrating cell was marked digitally at time 0, and the distance migrated from the digital mark after 2 h

was calculated. For each field, migration of at least 20 cells was analyzed and the mean distance migrated per cell was calculated.

Gene expression analysis
Real-time RT-PCR was conducted as described in the online supplemental material. Real time RT-PCR primers were designed using PRIMER EXPRESS 1.0 (Applied Biosystems) to amplify 50–150-bp amplicons and were based on GenBank or Ensembl sequences. Sequences are provided in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200603152/DC1).

cAMP assay
Cells were cultured in 6-well plates (2×10^5 cells/well), washed with supplement-free M200 medium, and treated with the indicated reagents at 37°C. Reactions were terminated by aspirating medium. Cells were washed with PBS and lysed in 500 μl of 0.1 N HCl containing 0.5% Triton X-100. cAMP was assayed in cell lysates (100 μl) using a direct cAMP enzyme-linked immunosorbent assay (Assay Designs, Inc.).

CAM assay
Fertilized chicken eggs were incubated at 39°C with 90% humidity. On embryonic day 7, a small hole was drilled at the end of the egg that contains the air sac to lower the embryo. A second hole was made above the CAM, and 200 μl of a 1.5% solution of methylcellulose (StemCell Technologies Inc.) containing vehicle, FGF2 (30 pmol)/IBMX (10 nmol), FGF2/NK-B (33 nmol) with or without IBMX, FGF2/mNK-B (33 nmol)/IBMX (mNK-B, inactive mutant of NK-B), a combination of NK1- (L733060, 10 nmol) and NK3- (SB222200, 4 nmol) selective inhibitors, FGF2/U46619 (10 nmol) with or without NK-B, or FGF2/NK- B/U46619 with SQ29548 (40 nmol) was applied to the CAM of day 7 chicken embryos. Vasculature was analyzed after 48 h, and digital images were captured at room temperature with a Wild M3Z dissecting microscope (40×; Leica) attached to a SPOT RT KE charge-coupled device camera, using the software SPOT 3.5.9 (Diagnostic Instruments).

Calcium imaging
Cells were incubated for 30 min in 4 μM fluo-4/AM (Invitrogen) in the presence or absence of 4 μM BAPTA/AM (Invitrogen) in supplement-free M200 medium at 37°C, trypsinized, plated on Matrigel with indicated reagents, and allowed to adhere for 10 min. Time-lapse video microscopy with a FluoView 500 laser scanning confocal microscope (Olympus; 40×, NA 0.8 water-immersion objective) was used to measure intracellular Ca^{2+} as described previously (Robles et al., 2003). An argon laser at 488 nm was used to excite Fluo-4, and the emitted light was detected at wavelengths >510 nm. Images were acquired at 15-s intervals and analyzed using Image J. When indicated, the fluorescence intensity in the region of interest (F) was normalized by dividing fluorescence intensity of the resting cell at time 0 (F_0).

RNA interference
To generate stably expressing siRNAs, oligonucleotides were designed according to the criteria specified by Dharmacon and Oligoengine and cloned into the BglII−HindIII sites of pSUPER Puro (Oligoengine). The NK1 target sequence 5′-CAACAGGACTTACGAGAAA-3′ corresponded to nucleotides 1480–1497 of the mouse NK1 cDNA; the NK3 target sequence 5′-AGATTTCGTGCAGGCTTCA-3′ corresponded to nucleotides 1044–1061 of the mouse NK3 cDNA. The empty vector was used as a negative control. YSECs were transfected with Lipofectin (Invitrogen), and positive clones were selected with 2 μg/ml puromycin.

VEGF ELISA
YSECs and HUVECs were cultured in 6-well plates (3×10^5 cells/well), incubated in supplement-free M200 medium overnight, and treated with vehicle, NK-B, IBMX, NK-B/IBMX, U46619, NK-B/U46619, or BAPTA/AM in supplement-containing medium for 24 h. Secreted VEGF was quantitated with mouse or human VEGF Quantikine ELISA kits (R&D Systems) according to the manufacturer's instructions.

Two-dimensional gel electrophoresis and mass spectrometry
YSECs were treated with IBMX or NK-B/IBMX for 1 h in supplement-free M200 medium, followed by 10 min in supplemented medium. Cells were processed and proteins were analyzed as described in the online supplemental material.

Antibodies and immunoblot analysis
The antibodies used and methodology are described in the online supplemental material.

Recombinant calreticulin and vasostatin production
For construction of the GST-calreticulin and -vasostatin fusion constructs, the coding regions for calreticulin and vasostatin were cloned as C-terminal translational fusions with the GST gene for expression in *E. coli*. Purification of GST-calreticulin was achieved by lysis of the bacteria, followed by sonication, centrifugation, adjusting the pH of supernatants to pH 7.0, and mixing with preequilibrated Glutathione Sepharose 4B (GE Healthcare) in PBS containing 1.0% Triton X-100. After a 30-min incubation, beads were washed, bound protein was cleaved with factor Xa (Novagen), and liberated protein was separated from immobilized GST via centrifugation.

Online supplemental material
Table S1 shows the forward and reverse primers for RT-PCR analysis. Videos 1–7 document YSEC migration on Matrigel after treatment with vehicle, NK-B, IBMX, NK-B/IBMX, NK-B/IBMX + NK1- and NK3-selective inhibitors, U46619, and NK-B/U46619. Fig. S1 shows the reversibility of the NK-B−mediated vascular network assembly blockade. Fig. S2 shows an analysis of TXA2 receptor expression in YSECs and HUVECs. Fig. S3 (A and B) shows that cAMP induction is insufficient to inhibit vascular network assembly, but cAMP potentiates NK-B−mediated inhibition of vascular network assembly. Fig. S3 C shows NK-B/IBMX−mediated abrogation of vascular network assembly in three-dimensional collagen gels. Fig. S4 (A and B) shows VEGF rescue of the NK-B−mediated suppression of VEGFR expression and vascular network assembly. Fig. S4 (C and D) shows that cAMP induction, but not the antiangiogenic proteins calreticulin and vasostatin, down-regulates VEGFR1 and VEGFR2 in YSECs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200603152/DC1.

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