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The Tie-2 ligand Angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies

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The angiopoietins Ang-1 and Ang-2 have been identified as ligands with opposing functions of the receptor tyrosine kinase Tie-2 regulating endothelial cell survival and vascular maturation. Ang-1 acts in a paracrine agonistic manner, whereas Ang-2 appears to act primarily as an autocrine antagonistic regulator. To shed further light on the complexity of autocrine/paracrine agonistic/antagonistic functions of the angiopoietin/Tie-2 system, we have studied Ang-2 synthesis and secretion in different populations of wild-type and retrovirally Ang-2–transduced endothelial cells. Endogenous and overexpressed endothelial cell Ang-2 is expressed in a characteristic granular pattern indicative of a cytoplasmic storage granule. Light and electron microscopic double staining revealed Ang-2 colocalization with von Willebrand factor, identifying Ang-2 as a Weibel-Palade body molecule. Costaining with P-selectin showed that storage of Ang-2 and P-selectin in Weibel-Palade bodies is mutually exclusive. Stored Ang-2 has a long half-life of more than 18 hours and can be secreted within minutes of stimulation (eg, by phorbol 12-myristate 13-acetate [PMA], thrombin, and histamine). Collectively, the identification of Ang-2 as a stored, rapidly available molecule in endothelial cells strongly suggests functions of the angiopoietin/Tie-2 system beyond the established roles during angiogenesis likely to be involved in rapid vascular homeostatic reactions such as inflammation and coagulation. (Blood. 2004;103: 4150-4156)

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Introduction

The angiopoietins Ang-1 and Ang-2 have been identified as ligands of the endothelial receptor tyrosine kinase Tie-2.[1-3] Ang-1–mediated activation of Tie-2 regulates endothelial cell survival and blood vessel maturation.[4] Ang-1 exerts a vessel-sealing effect,[5] acts anti-inflammatory,[6-8] and protects against cardiac allograft arteriosclerosis.[9] Low-level constitutive Tie-2 activation may be required in the adult to maintain the mature quiescent phenotype of the resting vascular endothelium.[10] In turn, Ang-2 acts in blood vessels primarily as a functional antagonist of Ang-1/Tie-2 by binding the receptor without inducing signal transduction.[2,11,12] The opposing effects of Ang-1 and Ang-2 support a model of constitutive Ang-1/Tie-2 interactions controlling vascular homeostasis as a default pathway[10] and Ang-2 acting as a dynamically regulated antagonizing cytokine.[11,13,14]

Expression profiling studies have identified endothelial cells as the primary source of Ang-2 and a dramatic transcriptional regulation of Ang-2 production upon endothelial cell activation.[12,15-20] The expression of Ang-2 in endothelial cells suggests that Ang-2 may act in an autocrine manner to control endothelial cell quiescence and responsiveness.

Loss of the Ang-2 gene and function is compatible with life as evidenced by the observation that Ang-2–deficient mice are born apparently normal.[12] The functionally unaffected blood vascular system of Ang-2–deficient mice has only minor abnormalities (eg, perturbed vessel regression phenotype of the eye's hyaloid blood vessels). Yet, depending on the genetic background of the mice, a significant fraction of newborn mice develops a lethal chylous ascites within the first 14 days as a consequence of a mechanistically hitherto unexplained lymphatic phenotype.[12] In contrast to the mild phenotype of Ang-2–deficient mice, mice transgenically overexpressing Ang-2 have an embryonic lethal phenotype that essentially copies the Ang-1 and the Tie-2 null phenotypes.[2-4] The similarity of the Ang-1 loss-of-function phenotype with the Ang-2 gain-of-function phenotype strongly supports the antagonistic concept of Ang-1 and Ang-2 function. Yet, the embryonic lethal phenotype of systemically Ang-2–overexpressing mice also demonstrates that Ang-2 is a potentially dangerous molecule whose dosage and spatiotemporal appearance must be tightly regulated. The Ang-2 dosage concept is also supported by the observation that local overexpression of Ang-2 in the heart is compatible with life,[21] whereas strong overexpression of Ang-2 with systemic dissemination in a large organ such as the skin leads to an embryonic lethal phenotype similar to the systemic overexpression of Ang-2.[22] Similarly, an activating Tie-2 mutation causes venous malformations that are composed of dilated endothelial channels covered by a variable amount of smooth muscle cells demonstrating that a precise balance of Tie-2 signals is critical.[23] These considerations in combination with the strong transcriptional regulation and the

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preferential endothelial cell production of Ang-2 prompted us to study endothelial cell Ang-2 production in detail. We hypothesized that endothelial cell Ang-2 production and presentation must be tightly regulated to facilitate its function as a dynamically regulated autocrine antagonist of the Ang-1/Tie-2 axis. Using cytochemical and biochemical experimental approaches to study endogenous and retrovirally overexpressed endothelial Ang-2 production, we made the surprising finding that Ang-2 is stored in Weibel-Palade bodies and that it can be made rapidly available on challenge.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVECs), human umbilical artery endothelial cells (HUAECs) and smooth muscle cells (HUASMCs), human aortic endothelial cells (HAoECs), human dermal microvascular endothelial cells (HDMVECs), and human venous saphenous endothelial cells (HSaVECs) were obtained form PromoCell (Heidelberg, Germany). HUVECs, HSaVECs, and HUAECs were grown in endothelial cell growth medium (EGM-2; PromoCell) supplemented with 5% fetal calf serum (FCS). HDMVECs and HAoECs were grown in medium for microvascular endothelial cells (PromoCell) supplemented with 10% FCS and HUASMCs in medium no. 2 for smooth muscle cells (PromoCell). HEK ampho 293 cells were obtained from Clontech (Heidelberg, Germany) and grown in high-glucose Dulbecco modified Eagle medium (DMEM; Invitrogen, Karlsruhe, Germany).

Expression vectors and retroviral transduction

Retroviral transfer of Ang-1 and Ang-2 cDNA was achieved using a modified pantropic retroviral expression system (Clontech; modified and kindly provided by Dr Ralph Graeser, Freiburg, Germany). Briefly, myc-tagged human Ang-1 and Ang-2 cDNAs[24] were cloned into pLIB-IRES-EGFPneo. The constructs were stably transfected into HEK ampho 293 cells and clones selected using 0.5 μg/mL G418 (PAA Laboratories, Pasching, Germany). For generation of pantropic retroviruses, clones were transfected with pVSVG (Clontech) according to the manufacturer's instructions. Endothelial cells and smooth muscle cells (6×10^4 each) were transduced in 2 cycles with 2 mL retrovirus containing supernatant from the HEK ampho cells for 6 hours each. Transduction efficacy ranged from 20% to 60% for the Ang-1 and Ang-2 constructs and between 85% and 100% for eGFPneo as monitored by fluorescence-activated cell sorting (FACS) analysis of enhanced green fluorescent protein (eGFP) expression.

RT-PCR and semiquantitative PCR

Ang-1 and Ang-2 expression in endothelial cells and other cell types was analyzed by reverse transcription–polymerase chain reaction (RT-PCR) using 0.5 μg total cellular RNA for reverse transcription. Ang-1 and Ang-2 and the expression of the housekeeping gene *TBP* were analyzed using the following primers: Ang-2 forward: 5′-cagttcttcaaaagaagcaacatg-3′; Ang-2 reverse: 5′-atggatccggatgtttagaaatctgctggtcg-3′; Ang-1 forward: 5′-tatcggccgcgctttcaaaaatctaaaggtcga-3′; Ang-1 reverse: 5′-atggatccgagcctattcacagtatgacagatt-3′; TBP forward: 5′-atggatcagaacaacagcctg-3′; TBP reverse: 5′-ccctgtgttgcctgctggga-3′.

Antibodies and immunofluorescence microscopy

Expression of Ang-1 and Ang-2 was detected either by staining for the myc-tag (clone 9E10; Biomol, Plymouth Meeting, PA) or by using soluble (s) Tie-2-Fc.[24] Endogenous Ang-2 was also detected with a goat polyclonal antibody (R&D Systems, Wiesbaden, Germany). Antihuman von Willebrand factor (VWF) polyclonal antibody was purchased from Dako (Hamburg, Germany) and the antihuman P-selectin antibody was from R&D Systems. Secondary fluorochrome-coupled antibodies were purchased from Dianova (Hamburg, Germany) and Molecular Probes (Eugene, OR). For immunofluorescence studies, cells were fixed and permeabilized with methanol for 5 minutes and blocked with the corresponding serum. Blocked cells were exposed to the primary reagents for 60 minutes at room temperature, extensively washed, and incubated for 30 minutes with fluorochrome-coupled secondary antibodies. Samples were analyzed with an Olympus IX50 fluorescence microscope (Olympus, Leinfelden-Echterdingen, Germany). At least 50 cells from at least 3 independent experiments were analyzed for each experimental condition.

Electron microscopy

Retrovirally Ang-2–transduced HUVECs were collected, pelleted, and fixed for 30 minutes in 4% formaldehyde. Pellets were embedded for ultrathin sectioning and slides were prepared with an ultramicrotome (Ultracut E; Leica Microsystems, Bensheim, Germany) on Formvar-coated nickel grids. The grids were kept floating on drops of filtered solutions during all subsequent incubation steps. After washing with phosphate-buffered saline (PBS)/0.05 M glycine, unspecific binding was blocked with 2% FCS in PBS containing 2% bovine serum albumin (BSA) and 0.2% fish gelatin for 30 minutes at room temperature. The primary antibody was applied (anti-myc 1 μg/mL in blocking solution, anti-VWF [Dako Diagnostics] 1:5000 in blocking solution) overnight at 4°C. After rinsing with PBS, secondary gold-conjugated antibodies (Plano, Wetzlar, Germany) were applied (6-nm gold particle-coupled goat antirabbit IgG 1:25 and 10-nm gold particle-coupled goat antimouse IgG 1:25) for 1 hour at room temperature. After rinsing with PBS, sections were postfixed with 2% glutaraldehyde for 10 minutes and stained with 0.5% OsO_4. After staining with 2% uranyl acetate for 3 minutes, the sections were finally adsorption-stained with 0.003% lead citrate in 2% polyvinyl alcohol. After air drying, sections were analyzed under a Philips EM 301 transmission electron microscope (Philips, Eindhoven, The Netherlands).

Ang-2 release, synthesis, and stability studies

For regulated Ang-2 secretion, endothelial cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA), 50 μM histamine, 1 U/mL thrombin, 25 ng/mL tumor necrosis factor α (TNF-α), 2.5 μM ionomycin, 2 μM thapsigargin, 1 μM nifedipine (all from Sigma Aldrich, Taufkirchen, Germany). Cells were starved in endothelial cell basal medium supplemented with 10% FCS (EBM-2; PromoCell) 12 hours prior to the induction of Ang-2 release and short-term release experiments were performed in 5% FCS (PMA, ionomycin, tapsigargin, nifedipine, TNF-α) or under serum-free conditions (histamine, thrombin). Secretion of Ang-2 and VWF was monitored biochemically as well as by immunofluorescence microscopy. For biochemical analysis of Ang-2 secretion, supernatants were collected at the indicated time points and myc-tagged Ang-2 was immunoprecipitated with an anti-myc antibody (clone 9E10; Biomol). Samples were resolved on a 10% sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE), blotted, and probed with the anti-myc antibody.

For Ang-2 synthesis experiments, endothelial cells were stimulated with PMA for 1 hour to fully liberate all intracellular Ang-2 pools. The medium was changed and Ang-2 synthesis was monitored over time by immunofluorescence. Concomitantly, cells were harvested and Ang-2 mRNA production assessed by RT-PCR.

Ang-2 stability was analyzed by incubating cells in the presence of 2.5 μg/mL actinomycin D (Sigma Aldrich).

Results

Ang-2 is expressed in endothelial cells in a distinct granular pattern

Endothelial cells have been reported to be the primary source for Ang-2, suggesting that it may act as an autocrine regulator of Ang-1/Tie-2 signaling.[12,15-20] We have verified by RT-PCR analysis that all analyzed cultured human endothelial cell populations

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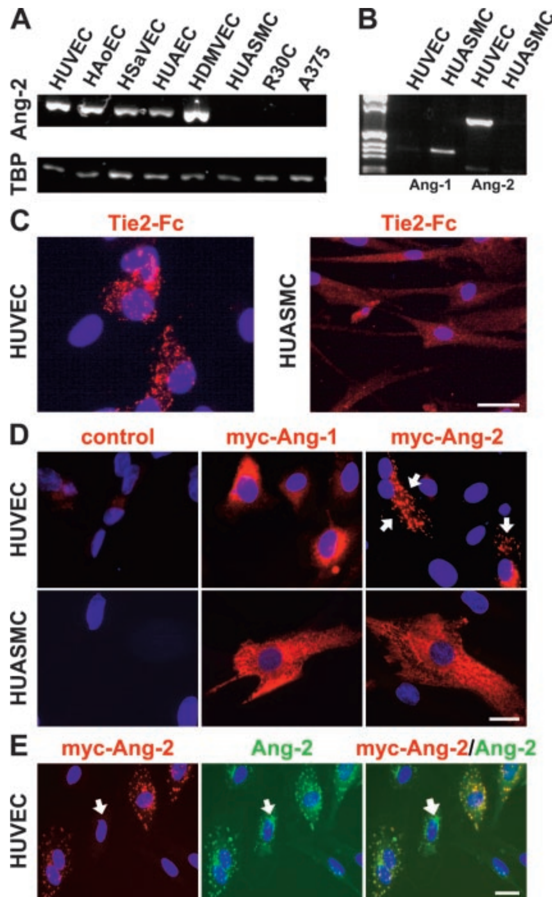

Figure 1. Distinct granular expression of Ang-2 in endothelial cells. (A) RT-PCR analysis identifying Ang-2 expression in HUVECs, HAoECs, HSaVECs, HUAECs, and HDMVECs, but not in HUASMCs and the mammary and melanoma tumor cell lines R30C and A375 (TBP, control RT-PCR for the TATA box-binding protein). (B) RT-PCR analysis of endothelial cell and smooth muscle cell Ang-1 and Ang-2 production identified endothelial cells as a source of Ang-2 and smooth muscle cells a as source of Ang-1 (lanes 2 and 3 are Ang-1 RT-PCRs; lanes 4 and 5 are Ang-2 RT-PCRs). (C) Soluble Tie-2-Fc receptor bodies identify both Ang-1 and Ang-2. Tie-2-Fc stains permeabilized endothelial cells in a distinct granular manner and permeabilized smooth muscle cells with a uniform cytoplasmic pattern. (D) Retrovirally overexpressed Ang-2 is localized in endothelial cells in a characteristic granular pattern (arrows) and in smooth muscle cells with a uniform cytoplasmic distribution. In contrast, Ang-1 displays a uniform cytoplasmic distribution pattern in endothelial cells and smooth muscle cells. (E) Colocalization of endogenous and retrovirally overexpressed Ang-2. Nontransfected cells only express endogenous Ang-2 (arrows). Scale bar = 20 μm (C-E)

express detectable Ang-2 (HUVECs, HAoECs, HSaVECs, HUAECs, HDMVECs; Figure 1). In contrast, cultured HUASMCs, esophageal epithelial cells (KOP), striated muscle cells (KMU), primary fibroblasts, and monocytic cells (U937) do not express Ang-2 (Figure 1 and data not shown). Similarly, of 10 analyzed arbitrarily selected tumor cell lines, only one cell line (MDBK) expressed detectable levels of Ang-2 mRNA (Figure 1 and data not shown). These data confirm and extend the endothelial cell selective expression of Ang-2 and support the concept that Ang-2 acts as an autocrine regulator of the Ang-1/Tie-2 ligand/receptor interaction.

To shed further light on endothelial cell Ang-2 production and presentation, we traced angiopoietin protein expression by probing cultured cells with sTie-2-Fc receptor body–based fluorescence microscopy. Permeabilized HUVECs bind sTie-2-Fc in a characteristic granular pattern that is distinctly different from the uniform cytoplasmic binding pattern of HUASMCs (Figure 1C). The sTie-2-Fc receptor bodies bind both Ang-1 and Ang-2. We consequently verified by RT-PCR that sTie-2-Fc binding of endothelial cells reflects Ang-2 production, whereas smooth muscle cell binding of sTie-2-Fc reflects Ang-1 production (Figure 1B). Based on these findings, we examined Ang-1 and Ang-2 production in endothelial cell and smooth muscle cell populations that were retrovirally transduced to overexpress full-length myc-tagged Ang-1 and Ang-2. Myc staining of Ang-2 in HUVECs identified the same granular pattern as was observed in nontransfected cells with sTie-2-Fc staining (Figure 1D). HUASMCs express myc-Ang-2 in a uniform cytoplasmic pattern. Similarly, myc-Ang-1 is expressed in a uniform cytoplasmic pattern in both HUVECs and HUASMCs (Figure 1D). Double staining of endogenously expressed Ang-2 and overexpressed myc-Ang-2 in HUVECs identified the colocalization of endogenous and myc-tagged Ang-2 in a distinct granular pattern (Figure 1E).

Endothelial cell-derived Ang-2 is stored in Weibel-Palade bodies

The characteristic granular pattern of transfected and endogenous Ang-2 in endothelial cells prompted us to speculate that endothelial cell Ang-2 is stored in Weibel-Palade bodies, the primary endothelial storage granule of the procoagulant VWF.[25-27] Double staining of VWF and endogenous Ang-2 (detected with anti-Ang-2) in HUVECs revealed the colocalization of Ang-2 and VWF and

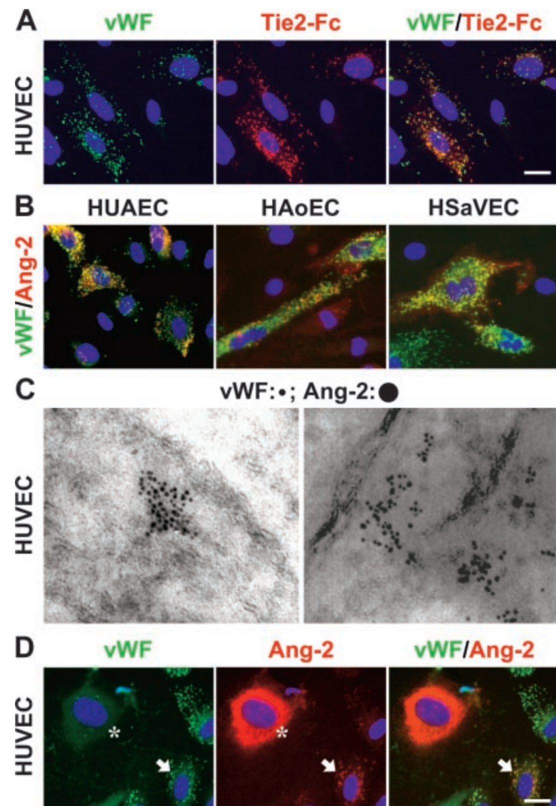

Figure 2. Endothelial cell Ang-2 is localized in Weibel-Palade bodies. (A) Colocalization of endogenous Ang-2 (detected by Tie-2-Fc) and VWF in HUVECs. (B) Colocalization of Ang-2 and VWF in HUAECs, HAoECs, and HSaVECs. (C) Ultrastructural double immunoelectron microscopy of Ang-2 (large 10-nm particles) and VWF (small 6-nm particles) demonstrating colocalization of Ang-2 and VWF. The poor Weibel-Palade body morphology is due to the fixation procedure that imposes limitations on the postembedding immunocytochemistry. (D) VWF is required for Ang-2 storage in Weibel-Palade bodies (arrows). Cells overexpressing Ang-2 but not VWF express Ang-2 in a uniform cytoplasmic pattern (asterisks). Scale bar in panels A and D is 20 μm (panel B set to the same scale as panel A); in panel C, the original magnification is × 45 000.

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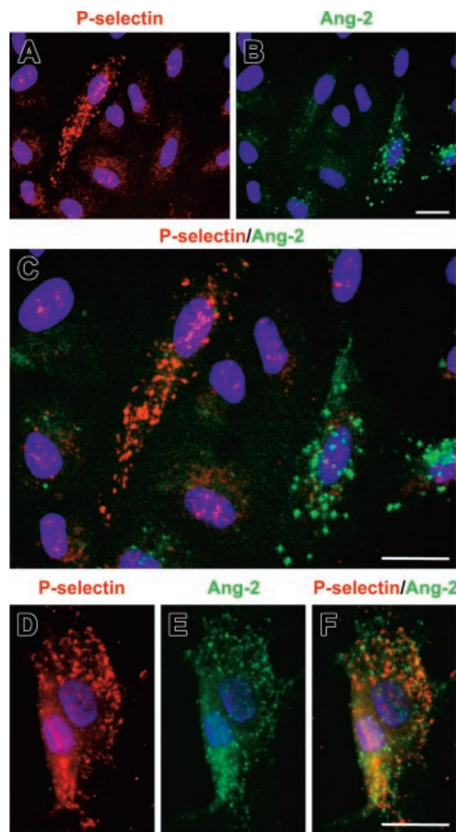

Figure 3. Exclusion of Ang-2 and P-selectin storage in endothelial cell Weibel-Palade bodies. P-selectin (A) and Ang-2 (B) are both expressed in a distinct granular pattern in endothelial cells. Yet, their expression does not overlap (C). (D-F) Granular storage of P-selectin and Ang-2 is not just mutually exclusive in different cells, but also in Weibel-Palade bodies of the same cell (panel D, P-selectin; panel E, Ang-2; and panel F, double staining of P-selectin and Ang-2 with nonoverlapping expression in red and green granules). Scale bar is 20 μm.

identified Ang-2 as a Weibel-Palade body molecule (Figure 2A). Similarly, HUAECs, HAoECs, and HSaVECs were all found to store Ang-2 in Weibel-Palade bodies (Figure 2B), demonstrating that Ang-2 storage in Weibel-Palade bodies is not restricted to cultured HUVECs but rather occurs in all Ang-2–producing endothelial cell populations. In contrast, α granules, the VWF storage granule of platelets, do not contain stored Ang-2 (data not shown). Extending and confirming these immunofluorescence colocalization experiments, endothelial cell VWF and myc-Ang-2 were also found to be colocalized by double-labeling immunogold cytochemistry. The VWF and myc postembedding immunocytochemistry is not compatible with glutaraldehyde fixation, resulting in poor ultrastructural preservation of Weibel-Palade body morphology. Yet, the use of 2 differently sized gold particles (6 nm and 10 nm) confirmed the colocalization of VWF and Ang-2 at the ultrastructural level (Figure 2C). VWF is the primary constituent of Weibel-Palade bodies and expression of VWF in certain nonendothelial cells has been shown to induce the formation of Weibel-Palade bodies.[25,26] Correspondingly, VWF is required for the trafficking of Ang-2 into Weibel-Palade bodies as evidenced by the observation that Ang-2 is expressed in a uniform cytoplasmic pattern in endothelial cells in the absence of VWF (Figure 2D).

Weibel-Palade body storage of Ang-2 and P-selectin are mutually exclusive

Beyond VWF itself, P-selectin is the functionally best characterized Weibel-Palade body molecule.[28,29] We consequently examined if P-selectin and Ang-2 are colocalized in endothelial cell Weibel-Palade bodies. Surprisingly, P-selectin and Ang-2 storage in Weibel-Palade bodies are mutually exclusive (Figure 3) even though P-selectin colocalizes with VWF (data not shown). We identified P-selectin–positive cells and Ang-2–positive cells (Figure 3A-C) and Ang-2 and P-selectin were found to be stored in separate granules even when both molecules are expressed in the same cell (Figure 3D-F). In fact, high-level retroviral overexpression of Ang-2 is able to render P-selectin granular expression completely undetectable (data not shown), suggesting that endothelial cell trafficking of Ang-2 and P-selectin is controlled by different mechanisms.

Ang-2 stored in Weibel-Palade bodies is rapidly released upon stimulation

The detection of Ang-2 in Weibel-Palade bodies suggested that Ang-2 is a stored molecule that may become rapidly available upon release. We consequently stimulated myc-Ang-2–overexpressing HUVECs with secretagogues of VWF release and traced the disappearance of the intracellularly stored Ang-2 pool and the accumulation in the supernatant of stimulated cells. Ang-2 becomes detectable in the supernatant of endothelial cells stimulated for 5 minutes with the protein kinase C (PKC)–activating phorbol ester PMA, accumulating to maximum concentrations in the supernatant within 20 minutes (Figure 4A). Tracing of intracellular VWF and Ang-2 upon PMA stimulation revealed that VWF and Ang-2 export

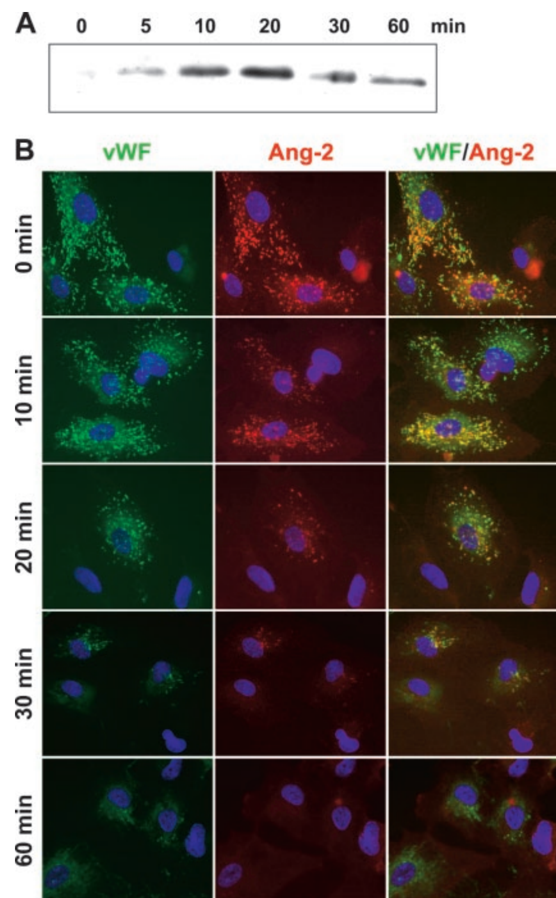

Figure 4. Temporal pattern of Ang-2 secretion. (A) Stimulation of Ang-2 release by PMA leads to detection of Ang-2 in the supernatant of stimulated cells within 5 minutes and reaches maximum concentration after 20 minutes. (B) PMA-induced secretion of Ang-2 parallels the temporal pattern of VWF secretion. The decline in the intensity of Ang-2 and VWF staining runs in parallel. Both Ang-2 and VWF become essentially undetectable after 30 minutes of PMA stimulation. Original magnification, × 40.

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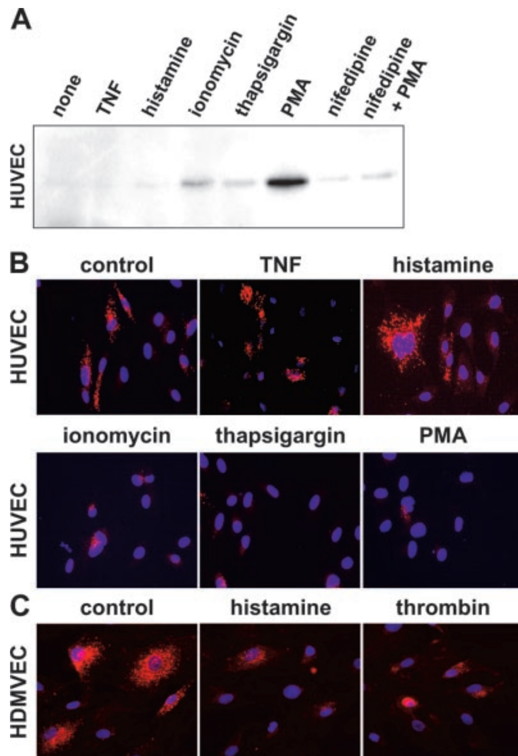

Figure 5. Stimulated release of Ang-2 stored in Weibel-Palade bodies. (A) Western blot analysis of myc-tagged Ang-2 in the supernatant of retrovirally transduced HUVECs identifies ionomycin, thapsigargin, and PMA as inducers of Ang-2 release. The Ca^{2+} channel blocker nifedipine inhibits the PMA-induced release of Ang-2. (B) Cytochemical analysis demonstrates ionomycin, thapsigargin, and PMA as regulators of Ang-2 release in HUVECs. (C) Stimulation of HDMVECs with histamine or thrombin induces Ang-2 release. Original magnification × 20 (B-C).

follows the same temporal kinetic. Weak VWF/Ang-2 colocalization is still detectable after 20 minutes and becomes essentially undetectable after 30 minutes of stimulation (Figure 4B).

PMA proved to be the strongest stimulator of endothelial cell Ang-2 release (Figure 5A-B). Similarly, the Ca^{2+} ionophore ionomycin and the intracellular Ca^{2+} store-discharging ionophore thapsigargin are able to induce the release of Ang-2 into the supernatant of stimulated HUVECs (Figure 5A-B). In fact, the Ca^{2+} channel blocker nifedipine inhibits PMA-induced Ang-2 release, indicating that calcium flow is the primary regulator of Ang-2 release as was previously demonstrated for VWF[27,30] (Figure 5A).

Stimulation of HUVECs with histamine, TNF-α, Ang-1, vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), transforming growth factor β (TGF-β), $CoCl_2$ (hypoxia mimicry), or high glucose does not induce Ang-2 release (Figure 5A-B and data not shown). Yet, stimulation of HDMVECs with either histamine or thrombin or HUVECs with thrombin induces the rapid release of Ang-2 associated with a characteristic perinuclear accumulation of Ang-2 prior to release of intracellular storage granules[31] (Figure 5C).

Intracellular Ang-2 stores rapidly recover upon release and have a long half-life

Ang-2 has been characterized as a transcriptionally strongly regulated cytokine.[12,15-20] We therefore assessed the kinetics of intracellular storage granule recovery upon complete release of all storage compartments, which was accomplished by a 1-hour PMA stimulation. Intracellular storage granules of Ang-2 are detectable within 6 hours of PMA removal and recovery of the storage granules in endothelial cells is detectable within 16 hours (Figure 6A). Reappearance of intracellular Ang-2 is associated with transcriptional up-regulation of Ang-2 mRNA (Figure 6B). These experiments demonstrated the rapid recovery of intracellular Ang-2 pools upon challenge. Conversely, we initiated experiments aimed at determining the half-life of stored Ang-2. Granular Ang-2 is still prominently detectable after 16 hours of transcriptional blockade with actinomycin D (Figure 6C), indicating a long half-life of stored Ang-2 (longer exposure to actinomycin D led to detectable cytotoxicity).

Discussion

The functional consequences of angiopoietin/Tie-2 signaling have been well established through genetic loss-of-function and gain-of-function experiments.[2,4,11,32] Ang-1 acts as the Tie-2–stimulating cytokine, thereby positively controlling endothelial cell survival and vessel maturation, which is associated with the quiescent nonproliferating endothelial cell phenotype.[5,33] The functions of Ang-2 appear to be more complex and Ang-2 can probably act in a context-dependent manner as agonist and antagonist of Tie-2 signaling.[11,32] The Tie-2 antagonistic model of Ang-2 is compatible with most of the genetic experiments including the observation that the phenotype of Ang-1– and Tie-2–deficient mice largely copies the phenotype of transgenic Ang-2–overexpressing mice.[2,4,34] As such, Ang-2 acts as a blood vessel-destabilizing cytokine, thereby facilitating the functions of other vascular cytokines such as VEGF.[2,14,17,21] The net outcome of Ang-2 function is therefore contextual, that is, facilitating angiogenesis in the presence of proangiogenic activity and initiating vessel regression in the absence of proangiogenic activity.[11,13] Recent tumor experiments are compatible with this model.[15,18,35] In contrast to the well-established antagonistic roles of Ang-2, agonistic functions are less well established. Ang-2 has been reported to induce Tie-2 phosphorylation[36] and sprouting of endothelial cells in culture[14] upon long-term stimulation. In vivo, Ang-2 is likely to exert an agonistic effect on lymphangiogenesis even though the mechanistic basis of the lymphangiogenic phenotype of Ang-2–deficient mice is poorly understood.[12]

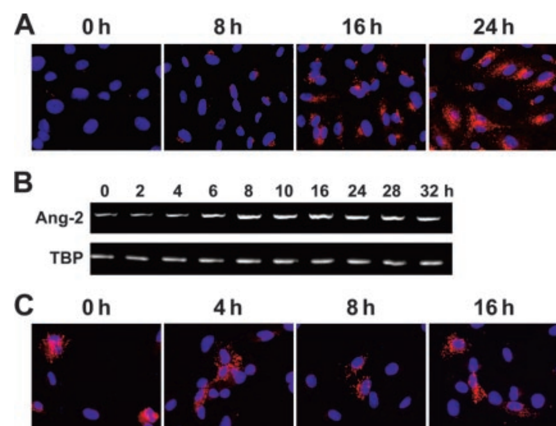

Figure 6. Recovery and half-life of Weibel-Palade body-stored endothelial cell Ang-2. (A) Ang-2–overexpressing HUVECs were stimulated with PMA for 1 hour after which the medium was changed and the recovery of intracellular Ang-2 monitored over time by Ang-2 immunocytochemistry. The recovery of the depleted Ang-2 stores is detectable within 8 hours. The monolayer is uniformly Ang-2^{+} after 24 hours. (B) The recovery of intracellular Ang-2 stores is paralleled by a transcriptional up-regulation of Ang-2 mRNA, which is detectable within 4 to 6 hours on PMA challenge. (C) Ang-2 has a long half-life period. Ang-2 is still detectable after 16 hours of actinomycin D challenge in cultured HUVECs. Original magnification, × 20 (A-C).

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The mechanistic understanding of Ang-2 function is furthermore complicated by the fact that Ang-2 is almost exclusively produced by endothelial cells.[12,15-20] As such, it acts as an autocrine regulator of endothelial cell functions. We therefore initiated experiments aimed at studying autocrine endothelial cell production and presentation. During the course of these experiments, we made the surprising finding that Ang-2 is not only produced by endothelial cells, but also stored in cytoplasmic granules of endothelial cells. A systematic analysis of this observation revealed that (1) endothelial cell-derived Ang-2 is stored in Weibel-Palade bodies, (2) stored Ang-2 is rapidly exported upon stimulation (eg, by PMA, thrombin, and histamine), (3) Ang-2 and P-selectin storage in Weibel-Palade bodies are mutually exclusive, and (4) Ang-2 stored in Weibel-Palade bodies has a long half-life (> 16 hours) and is rapidly regenerated upon challenge.

Given the established functions of Ang-2 in negatively controlling vascular maturation, stability, and quiescence, the identification of Ang-2 as a stored molecule in endothelial cell Weibel-Palade bodies may have broad implications beyond the field of angiogenesis research. Only few molecules have been identified in Weibel-Palade bodies and they are all involved in controlling rapid endothelial cell responses. The primary structural constituent of Weibel-Palade bodies is VWF, which plays a primary role in controlling vascular hemostasis.[25,27,37] In fact, expression of VWF in nonendothelial cells has been shown to induce the formation of Weibel-Palade bodies.[26] The experiments of this study demonstrate that Ang-2 is not just colocalizing with VWF, but that the export of Ang-2 is coregulated with VWF. Thus, the export of VWF parallels the export of Ang-2 and vice versa. It is therefore likely that the 2 molecules are functionally related and the export of Ang-2 during vascular hemostatic processes involving VWF release is likely to be of functional relevance. Likewise, VWF-deficient mice have been reported to have a localized reduction of atherosclerosis.[38] Given that VWF is required for Ang-2 gating into Weibel-Palade bodies, VWF-deficient mice will also exhibit a perturbed Ang-2 turnover as much as the VWF-deficient mice exhibit a perturbed P-selectin turnover.[39] It therefore deserves examination if the reported antiatherosclerotic phenotype of VWF-deficient mice is in fact solely due to the lack of VWF or also resulting as a consequence of a perturbed Ang-2 turnover.

The other hitherto identified Weibel-Palade body molecules are P-selectin,[40,41] CD63/tetraspanin,[42] tissue plasminogen activator (tPA),[43] and interleukin 8 (IL-8).[44] The functional consequences and pathophysiologic relevance of CD63/tetraspanin, tPA, and IL-8 Weibel-Palade body storage have not been analyzed. Beyond VWF itself, P-selectin is the functionally best characterized Weibel-Palade body molecule playing a critical, rate-limiting role for physiologic leukocyte rolling.[45,46] Both, P-selectin and Ang-2 are gated to Weibel-Palade bodies. Yet, we surprisingly found that they do not colocalize and rather appear to be mutually exclusive in Weibel-Palade bodies. This may suggest the presence of different types of Weibel-Palade bodies. Alternatively, P-selectin has been reported to be rapidly internalized following surface expression to be gated for reuse to Weibel-Palade bodies[47] and it may therefore be possible that recycled P-selectin ends up in different Weibel-Palade bodies than newly produced Ang-2. Differential Ang-2 and P-selectin localization may not only be related to Weibel-Palade body turnover but may also have functional consequences and deserves further molecular characterization.

Molecules stored in Weibel-Palade bodies control rapid vascular responses related to coagulation and inflammation. In contrast, vascular morphogenetic programs are primarily considered slow, transcriptionally driven processes. The identification of Ang-2 as a stored, rapidly available molecule of endothelial cells therefore puts Ang-2 conceptually in a totally different biologic context and raises a number of critical questions of the functional consequences of Ang-2 stored in Weibel-Palade bodies. The cellular basis of Ang-2 function has not well been defined so far. Yet, the genetic experiments have established the role of Ang-2 in negatively regulating vascular maturation and endothelial cell quiescence. Likewise, Ang-1 has been shown to act as a paracrine-acting quiescence-mediating anti-inflammatory cytokine.[4,7,8] It is, for example, not widely appreciated that the quiescent organ vasculature is not uniformly responsive to inflammatory activation by TNF-α. High-dose TNF-α–isolated limb perfusion therapies in humans with in-transit melanoma or sarcoma metastases have demonstrated the preferential responsiveness of the destabilized angiogenically activated tumor vasculature toward TNF-α and the nonresponsiveness of the quiescent limb vasculature toward TNF-α.[48] One of the most prominent differences between the quiescent organ vasculature and the angiogenically activated tumor vasculature is the dramatic up-regulation of Ang-2 following angiogenic activation.[15,17-19] This parallels the low-level constitutive expression of P-selectin with storage in Weibel-Palade bodies and its strong transcriptional up-regulation during inflammation. Similarly, Ang-2 could in the quiescent vasculature play a role as a surveillance and responsiveness-regulating molecule and secondarily be transcriptionally regulated at sites of endothelial activation. High-resolution immunolocalization experiments would be key to further validate this hypothesis and to identify regions in the vasculature that are controlled by constitutive autocrine Ang-2 function. These are difficult to pursue given the limited availability of sensitive and specific molecular probes. Likewise, the intense transcriptional activation of P-selectin is well documented in the literature. Yet, there are only few reports of the low-level constitutive expression of P-selectin in the quiescent vasculature even though it is well established that P-selectin controls physiologic leukocyte rolling. To overcome these technical limitations, we have traced Ang-2 expression in the quiescent endothelium of adult heterozygous and null Ang-2 mice carrying the *lacZ* marker gene in the Ang-2 locus.[12] These experiments identified a heterogeneous microvascular staining pattern in different organs including brain, lung, kidney, intestine, liver, spleen, and thymus. These experiments do not formally demonstrate Ang-2 immunolocalization and storage in the adult, but provide strong indirect evidence for the presence of Ang-2 in specialized regions of the quiescent vasculature.

In summary, we have identified the primarily antagonistic, vessel-destabilizing Tie-2 ligand Ang-2 as a molecule stored in the Weibel-Palade body that may become rapidly available upon stimulation. The findings put Ang-2 in a novel biologic context as a potential regulator and modifier of rapid vascular responses. They thereby open a new direction of research to mechanistically and functionally study the angiopoietins not just as regulators of angiogenesis and vessel maturation, but rather as gatekeepers of vascular homeostasis.

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