Uridine adenosine tetraphosphate acts as a proangiogenic factor in vitro through purinergic P2Y receptors

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Am J Physiol Heart Circ Physiol 311: H299–H309, 2016. First published May 27, 2016; doi:10.1152/ajpheart.00578.2015.—Uridine adenosine tetraphosphate (Up4A), a dinucleotide, exerts vascular influence via purinergic receptors (PR). We investigated the effects of Up4A on angiogenesis and the putative PR involved. Tube formation assay was performed in a three-dimensional system, in which human endothelial cells were cocultured with pericytes with various Up4A concentrations for 5 days. Expression of PR subtypes and angiogenic factors was assessed by quantitative real-time PCR at day 2. In contrast, a significant increase in vascular density in response to Up4A was observed at day 5. Up4A at an optimal concentration of 5 μM promoted total tube length, number of tubes, and number of junctions, all of which were inhibited by the P2Y6R antagonist MRS2578. Higher concentrations of Up4A (10 μM) had no effects on angiogenesis parameters. Up4A increased mRNA level of P2YRs (P2Y2R, P2Y4R, and P2Y6R) but not P2X2R (P2X2R or P2X2R) or P1R (A2AR and A2BR), while Up4A upregulated VEGFA and ANGPT1, but not VEGFR2, ANGPT2, Tie1, and Tie2. In addition, Up4A increased VEGFA protein levels. Transcriptional upregulation of P2YRs by Up4A was inhibited by MRS2578. In conclusion, Up4A is functionally capable of promoting tubule formation in an in vitro coculture system, which is likely mediated by pyrimidine-favored P2YRs but not P2XRs or P1Rs, and involves upregulation of angiogenic factors.

Up4A; purinergic receptors; angiogenesis; P2Y6R; coculture; endothelial cells

NEW & NOTEWORTHY

Novel findings include 1) uridine adenosine tetraphosphate (Up4A) promotes angiogenesis in vitro in a three-dimensional matrix human pericyte-endothelial cell coculture system. 2) Up4A increases expression of P2YRs (P2Y2R, P2Y4R, and P2Y6R) and proangiogenic factors. The proangiogenic properties of Up4A are mediated by activation of pyrimidine-favored P2YRs but not P2X or P1Rs.

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EXTRACELLULAR NUCLEOTIDES such as ATP, ADP, adenosine, UTP, and UDP are increasingly recognized as important regulators of a variety vascular functions, including vascular tone (3), permeability (12), inflammation (6), and angiogenesis (7). All these vascular responses of extracellular nucleotides are initialized by activation of purinergic receptors (PRs) on the plasma membrane (7). PRs have been classified into two subtypes: P1 and P2 receptors, based on their pharmacological properties and molecular structures (10, 27). Four subtypes of P2 receptors (also termed adenosine receptors), all metabotropic, have been cloned, namely A1R, A2AR, A2BR, and A3R (33). The P2 receptors belong to two major families: ionotropic P2X and metabotropic P2Y (1, 5). At least seven P2XRs and eight P2YRs have been cloned to date (4).

Angiogenesis, the formation of new capillaries from preexisting blood vessels, occurs during normal embryonic development and in various physiological and pathological conditions (8). Several in vitro studies have shown that activation of all four subtypes of P1R by adenosine results in angiogenesis via release of angiogenic factors (18). Moreover, injection of an adenosine A2A receptor agonist into diabetic mice has been shown to improve impaired wound healing and increase wound-breaking strength (13). In addition to P1R, activation of not only P2X receptors by ATP (19) but also P2Y receptors by ATP, ADP, or UTP (2, 14, 35) has been reported to influence established angiogenic factors such as vascular endothelial growth factor (VEGF) leading to tubulogenesis, angiogenesis and wound repair. More recently, activation of P2Y6R receptors was shown to play an essential role in postnatal cardiac development (20). Together, these findings suggest that purinergic receptors activated by extracellular nucleotides may be involved in angiogenesis.

Uridine adenosine tetraphosphate (Up4A), endogenously released from endothelial cells and biosynthesized by VEGF receptor type 2 (VEGFR2) (23), has been identified as the first dinucleotide found in living organisms that contains both purine and pyrimidine moieties (25). This suggests that Up4A is capable of activating both P1R and P2R (25, 44, 45). Indeed, early studies regarding the effect of Up4A on vascular tone regulation found that Up4A induces vasoconstriction through P2X2R (25, 46) and P2Y1R (17) but produces vasodilation through A2AR (44, 45), P2X2R, and P2Y6R (44). Recent studies performed at the cellular level showed that Up4A induces human smooth muscle cell proliferation via activation of P2Y6R (16) and is a strong activator of migration in smooth
To date, the effect of UpA on the angiogenic process has not been explored. Here, we investigated the angiogenic potential of UpA. Our data show that UpA stimulation promotes angiogenic tubule formation in a three-dimensional (3D) matrix system in vitro, in which the neovascular growth process is more accurately mimicked by allowing interaction of human endothelial cells with pericytes. Since the biosynthesis of UpA is linked to VEGFR2 in endothelial cells (23), we also investigated the effect of UpA on the expression of well-known angiogenic factors. In addition, we evaluated which putative PRs could be involved in the endothelial angiogenic response to UpA. We found in the present study that UpA mainly affected pyrimidine-favored P2YR (P2Y6R). We subsequently tested the effect of P2Y2R blockade on UpA-mediated angiogenesis and the expression profile of PRs, as well as angiogenic factors.

MATERIALS AND METHODS

Cell culture. Human umbilical vein endothelial cells (HUVECs), human microvascular ECs (HMVECs), human aorta ECs (HAECs), and human carotid arterial endothelial cells (HCATECs) were obtained from Lonza and maintained in EGM2 Bulletkit medium (Lonza) with 100 U/ml penicillin-streptomycin (PS). Human brain vascular pericytes were obtained from SciCell and maintained in DMEM supplemented with 10% FCS and 100 U/ml PS. All cells were used between passages 3 and 6 (9).

HUVECs-GFP, HMVECs-GFP, and pericytes-dsRED were created by transfecting passage 1 HUVECs and pericytes at 60% confluency with lentiviral vectors encoding GFP and dsRED cDNA, respectively, at a multiplicity of infection of 5 in the presence of 6 µg/ml polybrene in EBDM with 0.2% FCS. After 6 h of transduction, the cells were incubated for 72 h in fresh growth medium and were exposed to Puromycin (1-2.5 µg/ml) selection for 10 days (38). HUVECs-GFP, HMVECs-GFP, and pericytes-dsRED batches were stored by cryopreservation before use in experiments.

Collagen-based 3D coculture. Microvessel networks were established by seeding either HUVECs-GFP, HMVECs-GFP, and pericytes-dsRED at a density of 6 x 10^4 HUVECs- or HMVECs- or HAECs- and 1.2 x 10^4 pericytes-dsRED in 50 µl of 2.0 mg/ml type 1 collagen (BD Bioscience), supplemented with stem cell factor, stromal-derived factor-1α, and interleukin-3 (BD-Bioscience), each added at a concentration of 400 ng/µl in the collagen matrix (39). Subsequently, the collagen was allowed to cross link for 1 h at 37°C and 5% CO2. Depending on the test conditions, UpA (0 µM-1 µM-2.5 µM-5 µM-10 µM) and the potent/irreversible P2Y6R antagonist MRS2578 (10 µM) (30, 45) were added per well in EBDM2 + 2%FCS + ascorbic acid + fibroblast growth factor-2. After a 48-h incubation at 37°C and 5% CO2, cells were washed twice with ice cold PBS and harvested for total RNA extraction (9). RNA was isolated using RNeasy kit (Qiagen) and was checked for quality and quantity by spectrophotometer (Nanodrop; ND-1000). Isolated RNA was reverse transcribed into cDNA according to manufacturer’s instruction (Iscript; Bio-Rad). q-PCR reactions were performed using iCycles q5 Detection System (Bio-Rad) according to manufacturer’s instructions. Sequences of the primers that were designed and used for these experiments are described in Table 1. The mRNA expression levels were analyzed and normalized using the housekeeping gene β-actin or GAPDH.

Western blot analysis. HCATECs were seeded at a density of 200,000 cells per well in a precoated (0.1% gelatin) six-well culture plate. HCATECs were treated with UpA (0 µM-5 µM-10 µM) in EBM2 + 2%FCS + ascorbic acid + fibroblast growth factor-2. After a 48-h incubation at 37°C and 5% CO2, cells were washed twice with ice cold PBS and harvested. Protein was extracted in RIPA Buffer (Thermo Scientific) with protease inhibitors (Roche) and centrifuged for 15 min at 12,000 g at 4°C. Total protein content of the extracts was quantified by a bicinchoninic acid protein assay kit (Pierce Biotechnology, Life Technologies). The proteins were separated on 12% SDS gel (30 µg per sample) and transferred onto PVDF membranes (Merck, Millipore, Solna, Sweden). Membranes were blocked with 5% milk for 1 h at room temperature and incubated overnight at 4°C with primary antibodies against VEGFA (1:1,000), ANGPT1 (1:500), and GAPDH (1:2,500). Band densities were analyzed with Image Studio Lite Version 3.1 (LI-COR Bio-sciences), and the data obtained were normalized to GAPDH.

Statistical analysis. Statistical analysis of the 3D coculture data was performed using GraphPad Prism statistical program or Microsoft Excel (Microsoft). Statistical significance was set at minimum with P < 0.05. Data were analyzed by one-way ANOVA, followed by post hoc analysis using Bonferroni’s test. Data are presented as mean ± SE, unless stated otherwise.

RESULTS

UpA promotes vascular tubule formation. To assess the angiogenic potential of UpA, we used an in vitro coculture assay. In this assay, GFP-labeled human endothelial cells and human brain-derived dsRED-labeled pericytes were cocultivated in a 3D collagen gel setting, enabling direct interaction between these two types of vascular cells. Vascular sprouting and initiation of tubule formation was observed after 1 day, with subsequent stabilization of the vascular structures till up to 5 days postseeding. Imaging and quantification of the vascular structures were conducted at 2 and 5 days postseeding (Fig. 1A). Different concentrations of UpA were added to the cocultures at day 0, and the effect was evaluated compared with non-treated samples.

No difference in initial tubule formation was detected between UpA stimulation and control conditions at day 2. In contrast, a significant increase in vascular density in response to UpA was observed at day 5 in HUVEC-pericyte coculture system (Fig. 1, B and C). UpA at a concentration of 2.5 and 5 µM (but not at 10 µM) promoted total tubule length (by ~1.89- and ~2.23-fold), number of tubes (by ~1.71- and ~1.89-fold), as well as number of junctions (by ~2.24- and ~2.80-fold) (Fig. 1C). Similarly, UpA at a concentration of 5 µM significantly promoted angiogenesis in coculture system of HMVECs at day 5 (Fig. 1, D and E). However, an increase in tubule formation was not detected at day 5 if UpA was added at day 2 (data not shown). These findings indicate that UpA is functionally capable of promoting tubule formation, and UpA stimulation during early sprouting in our coculture setup is required to enhance vascular growth.

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Effects of Up4A on expression of purinergic receptors and angiogenic factors. Vascular tone regulation and other biological effects by Up4A are mediated through activation of PRs (31). Next, we evaluated the effects of 24 h of incubation with Up4A on the mRNA expression profile of PRs in HUVECs. Up4A at a concentration of 10 μM significantly increased mRNA levels of P2Y2R, P2Y4R, and P2Y6R (Fig. 2A). In contrast, mRNA levels of A2AR, A2BR, P2X4R, and P2X7R were not affected by Up4A, either at a concentration of 5 or 10 μM, compared with nontreated controls (Fig. 2A).

Further evaluation of Up4A on expression levels of angiogenic factors showed that 10 μM of Up4A significantly increased mRNA levels of VEGFA and ANGPT1, whereas 5 or 10 μM of Up4A failed to increase mRNA levels of VEGFR2, PDGFb, ANGPT2, Tie1, and Tie2 (Fig. 2B). Western blot analysis of protein levels of VEGFA and ANGPT1 revealed that after 48 h of treatment, Up4A at a concentration of 10 μM Up4A significantly increased protein level of VEGFA but not ANGPT1 in HCA Technology (Fig. 2C).

P2Y6R blockade attenuated Up4A-mediated vascular tube formation. There is a lack of selective PR antagonists for many PR subtypes. Since Up4A mainly affected P2YR subtypes in

Fig. 1. Endothelial cells and pericytes coculture in response to different concentrations of uridine adenosine tetraphosphate (Up4A). A: protocol of the coculture assay. GFP-labeled human umbilical vein endothelial cells (HUVECs) and human brain derived dsRED-labeled pericytes are cocultivated in a 3-dimensional (3D) collagen gel setting and monitored at days 2 and 5 postseeding. Up4A was added at day 0 and compared with nontreated controls. B: representative pictures of HUVECs and pericytes coculture in a 3D matrix gel environment treated with 0, 1, 2.5, 5, or 10 μM Up4A. Pictures were taken by fluorescent microscope on days 2 and 5. HUVECs are labeled with GFP (in green); Pericytes are labeled with dsRED (in red). C: quantification of total tubule length, number of tubules and junctions at day 5 of (HUVECs and pericytes) coculture as shown in A. Scale bar = 50 μm. Values are mean ± SE. *P < 0.05 vs. 0 μM; n = 3 individual experiments. D: representative images of human microvascular endothelial cells (HMVECs) and pericytes coculture treated with Up4A alone or in combination with P2Y6R antagonist MRS2578 (MRS, 10 μM). Pictures were taken by fluorescent microscope at day 5. HMVECs are labeled with GFP (in green); Pericytes are labeled with dsRED (in red). E: quantification of fold increase in numbers of junctions, tubules and total tubule length of HMVECs and pericytes cocultures at day 5. HMVECs were treated with 0, 5, or 10 μM Up4A (as indicated in bar graph) with or without P2Y6R antagonist MRS2578 (10 μM). Scale bar = 50 μm. Values are represented as mean ± SE. *P < 0.05; n = 3 individual experiments.
our setting, and MRS2578 is a selective antagonist for P2Y$_6$R that previously has been well-validated by other studies, we used the MRS2578 to investigate the signaling pathway in the Up$_4$A-mediated angiogenic process, using the HUVECs and pericytes coculture setup. Trace amounts of DMSO was used as a solvent for the MRS2578, but did not interfere with the assay, as controls without and with DMSO did not differ in total tubule length (3,357 ± 470 and 3,470 ± 287), number of tubules (200 ± 27 and 213 ± 17), or number of junctions (66 ± 17 and 74 ± 7) in the absence and presence of DMSO, respectively. P2Y$_6$R blockade markedly attenuated Up$_4$A-increased tubule formation at day 5 (Fig. 3A), as evidenced by a significant reduction in total tubule length, number of tubules, as well as number of junctions at a concentration of 1 and
P2Y6R is involved in Up4A-mediated tubule formation and this proangiogenic response that was decreased by P2Y6R inhibition with MRS2578, thus validating our findings with HUVEC assays. Finally, inhibition of P2Y6R prohibited the induction of P2YRs upregulated by Up4A significantly increased VEGFA protein levels.

Antagonism is similar in endothelial cells of different vessel types in response to Up4A stimulation reflects the response in HUVECs (Fig. 4A). As for the angiogenic factors, P2Y6R blockade had no effect on VEGFA, ANGPT1, ANGPT2, Tie1, and Tie2 in HUVECs (Fig. 4B). Furthermore, q-PCR analysis using HAECs revealed that the transcriptional response of P2Y2R, whereas P2Y6R blockade had no effect on VEGFA, ANGPT1, ANGPT2, Tie1, and Tie2 in HUVECs (Fig. 4A). Moreover, P2Y6R blockade decreased Up4A (10 μM)-induced mRNA expression of P2Y2R, whereas P2Y6R blockade had no effect on the expression level of A2A, A2B, and P2Y2R (Fig. 4A).

**DISCUSSION**

The main findings of this study are that 1) Up4A promotes angiogenesis, indicated by vascular tubule formation in our in vitro coculture system. 2) This proangiogenic effect of Up4A was largely attenuated by P2Y2R inhibition. 3) Moreover, Up4A mainly influenced mRNA expression of P2YRs (P2Y2R, P2Y4R, and P2Y6R) but not P2XRs (P2X2R and P2X8R) or ARs (A2A and A2BR), whereas Up4A increased mRNA levels of VEGFA (in HUVECs but not HAECs) and ANGPT1, but not VEGFR2, PDGFRb, ANGPT2, Tie1, and Tie2. 4) Up4A significantly increased VEGFA protein levels. 5) Finally, inhibition of P2Y6R prohibited the induction of P2YRs (P2Y2R and P2Y4R) by Up4A. The implications of these findings are discussed below.

Up4A, endogenously produced from endothelial cells as well as released upon mechanical (shear) stress and chemical challenges (25), is biosynthesized by activation of VEGFR2 (23). The plasma concentrations of Up4A detected in healthy subjects are in the vasoactive range (~4 nM) (25), which can be markedly elevated in diseased condition such as hypertension (~33 nM in juvenile hypertensive patients) (22). Thus Up4A has been shown to regulate vascular tone in various vascular beds (31, 45) and has been implicated in blood pressure regulation (22, 25). Moreover, the effects of Up4A have been shown to induce human vascular smooth muscle cell proliferation (16), rat vascular smooth muscle migration (42), and vascular calcification in rats (37), suggesting a critical role of Up4A in regulation of the homeostasis of blood vessels.

We hypothesize that Up4A can be locally increased to a sufficient level to promote angiogenesis in tissues undergoing, e.g., a hypoxic response or vascular strain (in case of hypertension). Indeed, (local) concentration of other nucleotides such as ATP, ADP, and adenosine with angiogenic potential have been shown to be elevated during stress and injury conditions such as myocardial infarction and hypoxia (7, 18). Based on this assumption, we tested the potential angiogenic effect of Up4A on human endothelial cells. We used HUVECs as the main experimental cell type together with other endothelial cell types including HMVECs, HCA-TECs, and HAECs to reveal a general capacity of Up4A to induce angiogenesis in human endothelial cells of different origins. Indeed, Up4A treatment stimulated vascular sprouting and initiation of tubule formation from 1 day in the coculture setup. This was followed by subsequent stabilization of the vascular structures till up to 5 days postseeding, as evidenced by an increase in vascular density in response to Up4A at relatively low concentrations at day 5 (0–5 μM). Interestingly, increase in tubule formation was not detected at day 5 in HMECs if Up4A was added at day 2, suggesting that Up4A stimulation is required in early sprouting in our coculture setup to induce angiogenic increase. Up4A appears to have a dose optimum at 5 μM, after which the beneficial effects of Up4A on tubule formation are diminished.
angiogenesis. Notably, another dinucleotide Up₄U, with two pyrimidine moieties, has been recently shown to be a potent angiogenic factor in human vascular endothelial cells, inducing migration, proliferation, and tube formation, likely by activation of P2Y2R (24). Similarly to Up₄A, most of the cell responses induced by Up₄U showed a typical dose range effect with limited to no reaction beyond the optimal concentration (24).

Other dinucleotide polyphosphates, such as Ap₄A with two purine moieties, have been reported to be angiogenic inert (29). Since the pyrimidines UTP and UDP preferably activate P2Y2R, P2Y4R, and P2Y6R (33), these different observations for the dinucleotide polyphosphates group regarding angiogenic potential may be explained by the differences in binding properties of the purine and pyrimidine moieties. The four-phosphate groups do not vary between the dinucleotides and thus do not appear to be the decisive factor in compound-receptor signaling in the angiogenic process. In accordance with this concept, it is likely that the purine moieties from Up₄A do not have an effect on angiogenesis in HUVECs or HMVECs, even though Up₄A, with both purine and pyrimidine moieties, is capable of activating both P1Rs and P2Rs (25, 44).

Fig. 2. Expression of purinergic receptors and angiogenic factors in response to Up₄A treatment. The expression of purinergic receptors in response to 24 h of incubation with 0, 5, or 10 μM of Up₄A in HUVECs are shown in A; mRNA levels of A₁AR, A₂BR, P₂X₄R, P₂X₇R, P₂Y₂R, P₂Y₄R, and P₂Y₆R were measured by quantitative (q)-PCR and normalized to β-actin. The expression of angiogenic factors in response to 0, 5 or 10 μM of Up₄A in HUVECs are shown in B; mRNA levels of VEGFA, VEGFR₂, PDGF₅, ANGPT₁, ANGPT₂, Tie1, and Tie2 were detected by q-PCR and normalized to β-actin. The protein expression of angiogenic factors in response to 48 h of incubation with 0, 5, or 10 μM of Up₄A in HCATECs are shown in C. Protein levels of VEGFA and ANGPT1 were detected by Western blot and normalized to GAPDH. Values are mean ± SE. *P < 0.05 vs. all groups except 5 μM; **P < 0.05 vs. all groups except 1 μM; n = 3 individual experiments.

Fig. 3. P2Y₆R antagonist reverses the proangiogenic effects of Up₄A. A: representative pictures of HUVECs and pericytes coculture treated with Up₄A alone or in combination with P2Y₂R antagonist MRS2578 (MRS, 10 μM). Pictures were taken by fluorescent microscope on day 5. HUVECs are labeled with GFP (in green); pericytes are labeled with dsRED (in red). B: quantification of fold increase in total tubule length, numbers of tubules and junctions of HUVECs and pericytes cocultures at day 5. HUVECs were treated with 0, 1, or 5 μM Up₄A with or without P2Y₂R antagonist MRS2578 (10 μM). Scale bar = 50 μm. Values are represented as mean ± SE. *P < 0.05 vs. 0 μM; †P < 0.05 vs. 5 μM; n = 3–5.
45). In line with the concept that UpA mainly exerts proangiogenic actions via its pyrimidine moiety, tubule formation in the coculture setup was significantly attenuated by P2Y6R inhibition. Furthermore, our q-PCR data revealed that UpA upregulated pyrimidine-favored P2YRs (P2Y2R, P2Y4R, and P2Y6R) but not P2XR or P1Rs at mRNA levels. This UpA-induced expression response was in part blunted by P2Y2R inhibition, indicating a feed-forward loop in which UpA activation of P2Y2R amplifies gene expression of P2Y2R. In addition, the observation that the potent and selective P2Y2R antagonist MRS2578 also significantly attenuated P2Y2R expression suggests a possible interaction between P2Y6R and P2Y2R (41). MRS2578 is well known to potently and selectively inhibit P2Y6R-mediated actions in several cell types (21, 26, 30, 32, 34, 43). MRS2578 at 10 μM potently inhibits P2Y6R but has been shown to have very minimal effect on P2Y2R in 1321N astrocytoma cells (30). Future studies are needed to gain more insights into the interaction of PRs.

As shown in the data provided in Figs. 3B and 1C, MRS2578 stimulation alone significantly decreased all angiogenic parameters in both HUVECs and HMVECs coculture systems compared with the 5 μM UpA condition. This is counteracted by stimulation with increasing concentrations of exogenous UpA (5 and 10 μM). We interpret these effects of UpA as an indication that UpA is produced at low level by the HUVECs/ HMVECs or pericytes under normal coculture condition. In Figs. 2A and 4A, we show that stimulation with UpA significantly increases expression level of P2Y2R. Thus we further interpret the data in Figs. 3B and 1C as an indication that in the (10 μM) MRS + 5 μM UpA and (10 μM) MRS + 10 μM UpA conditions, the effect of the blocker is partially reversed by the effect of UpA, which leads to increased P2Y2R expression and receptor bioavailability, thereby counteracting the effect of the MRS2578 blocker. In the condition with only MRS2578 and no exogenous UpA stimulation, the anti-angiogenic effects of MRS2578 would therefore also be more severe.

All together, these findings suggest that P2Y2Rs, particularly P2Y6Rs, are involved in UpA-mediated angiogenic influence in human endothelial cells. Although UpA has been shown to activate not only P2YRs but also P1Rs and P2XR, all of which appear to be involved in the regulation of vascular tone by UpA (31, 44), the concept that only pyrimidine but not purine moieties affect function in HUVECs and HMVECs is further supported by recent studies performed at the cellular level: UpA induces human vascular smooth muscle cell proliferation via activation of P2Y receptors (16) and is a strong inductor of migration in vascular smooth muscle cell obtained from rat thoracic aorta through activation of P2Y2 receptors (42). Moreover, UpA has been shown to influence phenotypic trans-differentiation of rat vascular smooth muscle cells to osteochondrogenic cells, likely via P2Y2 and P2Y6 receptors (37). Further studies are required to validate if pyrimidine moieties and endothelial P2Y2R signaling are indeed a vital requirement for the angiogenic potential for the different compounds of the nucleotide polyphosphates group.

In the present study, UpA also promoted mRNA expression of the proangiogenic genes ANGPT1 and VEGFA. Furthermore, Western blot analysis indicated that VEGFA protein levels were significantly increased in response to UpA stimulation. In contrast, ANGPT1 protein levels were not significantly altered. This poor correlation between ANGPT1 protein and mRNA level could be the result of a shorter half-life of the ANGPT1 protein compared with VEGFA protein or could be associated with differences in posttranscriptional regulation between ANGPT1 and VEGFA protein levels (15). Previous reports demonstrated that purinergic receptor-induced angiogenesis could be mediated by autocrine stimulation of vascular cells by proangiogenic factors (2, 14, 35). Other studies have also indicated that UpA can be biosynthesized by activation of VEGFR2 (23). An autocrine feed-forward loop exists, in which VEGFA signaling via VEGFR2 promotes intracellular transport of VEGFR2 to the plasma membrane, thus amplifying the bioavailability of this receptor tyrosine kinase on the endothelial cell surface (40). This intrinsic pathway may further enhance UpA synthesis and downstream vascular effects. New vascular growth is a complex, multiphasic process, during which both endothelial cells and mural cells (pericytes in the microvasculature and vascular smooth muscle cells in the macrovasculature) undergo activation, sprouting, and neovessel stabilization. VEGFA/VEGFR2 signaling drives the typical initial response of vascular activation and is critical for neo-vascular growth and survival (28). UpA may facilitate its proangiogenic function via upregulation of mRNA and protein levels of VEGFA in endothelial cells.

Methodological considerations. Since UpA mainly affected P2Y2R subtypes in our study, we used a pharmacological blocker of P2Y6R (MRS2578) to investigate the signaling pathway in the UpA-mediated angiogenic process. The potential pitfall of using pharmacological blockers is that while selectivity, i.e., the capability of blocking a particular receptor compared with related receptors, is usually well-defined, specificity, i.e., the potential for discriminating between negative and positive interactions and the cross reactivity with other (unrelated) targets, is less well established (36). However, MRS2578 has been reported to potently inhibit the response to 300 nM UDP in 1321N astrocytoma transfected with the human P2Y2R, with an IC50 value of 37 ± 16 nM (30). At the same time, MRS2578 at 10 μM did not affect the UTP (100 nM)-induced responses of cells expressing human P2Y2R or

Fig. 4. The effect of P2Y2R antagonist on mRNA expression levels of purinergic receptors and angiogenic factors. A: effects of P2Y2R antagonist MRS2578 on purinergic receptor expression responses to UpA at 24 h. HUVECs were treated with 0, 5, or 10 μM UpA in combination with or without the P2Y2R antagonist MRS2578 (10 μM). The relative expression of P2Y2R, P2Y4R, P2Y6R, A2aR, and A3aR were measured by q-PCR followed by β-actin normalization. Values are mean ± SE. *P < 0.05 vs. 0 μM; †P < 0.05 vs. 10 μM UpA with P2Y2R antagonist MRS2578 (10 μM); n = 6–7. B: effects of P2Y2R antagonist MRS2578 (10 μM) on angiogenic factor expression in response to UpA in HUVECs. Similar as A, mRNA levels of ANGPT1, ANGPT2, VEGFA, Tie1 and Tie2 were detected by q-PCR and normalized with β-actin. Values are mean ± SE. *P < 0.05 vs. 0 μM; n = 6–7. C: effects of P2Y2R antagonist MRS2578 (MRS, 10 μM) on P2Y2R and ANGPT1 expression in response to UpA at 24 h in human aorta ECs (HAECS). HAECS were treated with 0.5, or 10 μM UpA with or without the P2Y2R antagonist MRS2578 (10 μM). The relative expression of ANGPT1 and P2Y2R measured by q-PCR followed by β-actin or GAPDH normalization are shown. Values are mean ± SE. *P < 0.05; n = 6.
P2Y₄R nor did it affect the 2-MeSADP (30 nM)-induced responses of cells expressing the P2Y₁R (30). In addition, MRS2578 did not affect the ATP (10 μM)-induced responses of cells expressing the P2Y₁₁R (30).

The effects on tube formation were mainly observed for 5 μM Up₄A, whereas mRNA expression of PRs and angiogenic factors responded mainly to 10 μM Up₄A. Similar to our HUVEC data, our additional experiments performed in HMVECs and HAECs showed a comparable pattern of tube formation predominantly responding to 5 μM Up₄A, whereas mRNA of PRs and angiogenic factors responded mainly to 10 μM Up₄A (Figs. 1 and 4). Thus, the difference in responsive dose between coculture and mRNA expression, was not endothelial cell type specific. This difference in effective dose may be attributed to the difference in our setup between normal (2-dimensional) endothelial cell culture (to yield samples for q-PCR) and our 3D collagen matrix endothelial-pericyte coculture setup. For the 3D coculture, HUVECs/HMVECs and pericytes were before the experiment both lentivirally transfected with GFP and dsRED expression cassettes to obtain stable HUVEC/HMVEC-GFP and pericyte-dsRED cells. These HUVEC/HMVEC-GFP and pericyte-dsRED cells have undergone several passages of selection to obtain a 99% pure marker expressing population. In contrast, for q-PCR analysis, we used HUVECs and HAECs that were not treated with lentivirus and were not selected for several passages on GFP or dsRED marker expression. This difference in treatment of the cells before the experiments could have affected their sensitivity and actual response to Up₄A stimulation (e.g., the GFP cells could have adapted to express higher levels of receptors, or have down-regulated inhibitory pathways that intervene with Up₄A receptor signaling). Furthermore, the coculture assay was conducted in a 3D matrix environment, whereas for q-PCR, the cells were cultured under normal conditions without complex extracellular matrix support. This may also have affected the responsiveness of the vascular cells. For example, interaction of endothelial cells with complex extracellular matrix components such as collagen type 1 fibers via integrin interaction is known to aid activation of endothelial angiogenic sprouting (11). In addition, the phenotypical changes in the 3D coculture assay were the result of transcriptional changes in multiple genes, which, combined together, resulted in the phenotype (tube formation). Therefore, even if transcriptional changes were slightly but nonsignificantly increased in response to 5 μM Up₄A stimulation, combined, this could have still contributed to the acquired phenotype.

Another limitation of our study is that different batches of HUVECs (GFP) and collagen were used for the experiments in Figs. 1 and 3, which may have resulted in the differences observed in tubule formation response to 1 μM Up₄A. However, Up₄A at 5 μM affected the tubule formation in a very similar pattern between these two experiments, indicating that Up₄A at 5 μM may be the optimal concentration for a more stable tubule formation response that surpasses these batch effects. Thus, although the optimal dose and the net amount of increase in angiogenesis varied, these experiments clearly showed that Up₄A could consistently induce a solid proangiogenic response. These findings were further verified by our HMVECs and pericytes coculture assays. We therefore conclude that Up₄A has a strong angiogenic potential, but the optimal dose may vary between endothelial cell populations of different donors. For future clinical use of Up₄A as a therapeutic agent in angiogenic stimulation, establishing the optimal dose range for specific subtypes of patients will be a critical requirement.

Conclusions. We have identified, to our knowledge for the first time, the dinucleotide polyphosphate Up₄A as a novel angiogenic substance, which promotes sprouting and tubule formation in human vascular cells in vitro. This Up₄A proangiogenic function is mediated via P2Y₄R signaling and is associated with upregulation of P2YRs and the proangiogenic factors.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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