Antiproliferative effect of UTP on human arterial and venous smooth muscle cells

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We have investigated the hypothesis that responses associated with proliferation are regulated by extracellular nucleotides such as ATP and UTP in cultured human vascular smooth muscle cells (VSMC) derived from internal mammary artery (IMA) and saphenous vein (SV). Platelet-derived growth factor (PDGF), ATP, and UTP each generated an increase in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in both IMA- and SV-derived cells in the absence of detectable inositol 1,4,5-trisphosphate production. ATP alone had no effect on [\(^3\)H]thymidine incorporation into DNA, but with a submaximal concentration of PDGF it raised [\(^3\)H]thymidine incorporation in SV- but not IMA-derived cells. UTP alone also was without effect on [\(^3\)H]thymidine incorporation or cell number. However, in both SV- and IMA-derived cells, UTP reduced the PDGF-stimulated [\(^3\)H]thymidine response and PDGF-stimulated cell proliferation. This cannot be explained by an inhibitory effect on the p42/p44 mitogen-activated protein kinase (MAPK) cascade, since this response to PDGF was not attenuated by UTP. We conclude that, in human VSMC of both arterial and venous origin, UTP acts as an antiproliferative regulator.

vascular smooth muscle; P2Y receptors; P2 receptors; platelet-derived growth factor; cell proliferation

UNDERSTANDING THE MECHANISMS controlling proliferation of human vascular smooth muscle cells (VSMC) is of considerable importance due to the prevalence of vascular proliferative disease in which intimal smooth muscle cells migrate and proliferate, subsequently occluding the lumen of the blood vessel (26). In coronary artery bypass procedures two vessels are used: internal mammary artery (IMA) and saphenous vein (SV). In a significant number of patients, subsequent problems arise from occlusion of the grafted vessel by this intimal hyperplasia. However, IMA grafts show increased patency over longer periods of time than do SV grafts. Interestingly, it has been shown that the serum-stimulated proliferation of smooth muscle cells from SV explants was greater than that for IMA explants (4).

Intimal proliferation of VSMC is controlled by cell surface receptors, which regulate the cell cycle via mitogenic signaling pathways. These include growth factor receptors, G protein-coupled receptors, and integrins (11, 29). Platelet-derived growth factor (PDGF) has been widely studied as a promoter of VSMC proliferation (36). Yang et al. (36) reported greater proliferative responses to PDGF in VSMC derived from SV explants than in those from IMA explants. Rat VSMC in culture are stimulated to proliferate by both ATP and UTP acting via G protein-coupled P2Y receptors (9, 14, 19, 33). Both PDGF and nucleotides are released from activated platelets. In addition, ATP and UTP are known to be released from endothelial cells (17, 27). Nucleotides acting on P2Y receptors, either alone or in conjunction with PDGF, may play an important role in regulating the smooth muscle of blood vessels in both health and disease (3). Currently, there are five cloned and characterized mammalian P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11) that differ in their agonist profiles. These receptors are each coupled to phospholipase C (PLC) and increases in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). Additional signaling pathways have been studied (2), providing evidence that the ATP-stimulated proliferation of animal VSMC requires activation of p42/p44 mitogen-activated protein kinase (MAPK) pathways (14, 34). Little is known about the regulation of human VSMC by nucleotides.

Studies on rings of human SV and coronary artery showed that ATP could regulate vasoconstriction (12, 25). Furthermore, the responses of human coronary VSMC to ATP and UTP suggested the presence of multiple P2Y receptors (31, 32). Here we investigate the hypothesis that nucleotides regulate proliferative responses in human VSMC, and we report the surprising observation that UTP has an antiproliferative effect.
ANTIPROLIFERATIVE EFFECT OF UTP ON VASCULAR SMOOTH MUSCLE CELLS

METHODS

Isolation and culture of cells. Primary explant cultures were derived from IMA and SV fragments from patients undergoing aortocoronary or peripheral arterial bypass surgery, with the approval of the local Ethical Committee. All comparisons of IMA- and SV-derived cells were made on paired explants from the same patient. Tissue was dissected free of any fat and excess adventitial tissue and then opened along its longitudinal axis, and the endothelial lining was removed by scraping of the luminal surface. Small fragments (~1 mm²) were transferred to a flask containing culture medium (RPMI 1640 with 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin from Gibco). After VSMC outgrowth, a confluent monolayer was obtained and passaged. Cells were used between passages 2 and 6 after 24 h serum free, when they were quiescent and positive for smooth muscle actin immunostaining with monoclonal antibodies.

Inositol 1,4,5-trisphosphate mass measurement. The levels of inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) were measured in cells cultured serum free for 24 h in 96-well plates and used after they had been serum free for 24 h. Cells in 100 μl of serum-free medium were stimulated for 1 h by the addition of 100 μM of agonist at the final concentration. This was followed by incubation for 19 h in serum-free medium and a 4-h incorporation period with [3H]thymidine. DNA was extracted, and [3H]thymidine content was measured by scintillation counting.

Cell proliferation. Cultured cells were seeded at a density of 10⁵ cells/well in 24-well plates and left quiescent for 24 h in serum-free medium before pulse stimulation for 1 h daily with 1 nM PDGF, 300 μM UTP, or both. Cells were stimulated for 2, 4, or 7 days, at which time cell numbers were determined by using a hemacytometer.

Creatine phosphokinase treatment. Regeneration of UTP from UDP during incubation of cells with UTP was achieved by the inclusion of 10 mM creatine phosphate and 20 U/ml creatine phosphokinase (Sigma) for the duration of the 1-h incubation. The UTP was purified by high-performance liquid chromatography (HPLC) immediately before use. HPLC was by anion exchange chromatography (Whatman SAX column). We established that the creatine kinase system was capable of converting UDP to UTP by incubating 100 mM UDP with creatine phosphate and demonstrating by using HPLC that UTP was formed.

Phospho-MAPK Western blots. Cultured cells were close to confluence in six-well plates and were used after they had been made serum free for 24 h. Cells in 100 μl of serum-free medium were stimulated by the addition of 100 μl of agonist at 10-fold final concentration for 5 min. Stimulation was stopped by the addition of lithium chloride, and the cells were extracted into lysis buffer [in mM: 20 Tris-HCl, 125 NaCl, 16.2 NaHCO₃, 3.0 EDTA, and 5.5 glucose, buffered to pH 7.4 with NaOH] containing 10 mM lithium chloride with 10 μl of Serum-free medium were stimulated for 1, 2, 4, or 7 days, at which time cell numbers were determined by using a hemacytometer.

Statistical analysis. Data presented are the means ± SE for three separate experiments performed in triplicate or quadruplicate. Raw data were analyzed with GraphPad Prism (version 3.0) by a one-way analysis of variance (ANOVA) followed by Bonferroni’s posttest to compare selected columns or Dunnett’s posttest to compare all columns with controls. When two groups were compared, a two-tailed Student’s t-test was used.

RESULTS

[Ca²⁺]i and PLC responses. Fura 2-loaded VSMC derived from both IMA and SV responded to ATP and PDGF with increased [Ca²⁺]i, as shown in Fig. 1A and B. UTP similarly stimulated an increase in [Ca²⁺]i in both IMA- (Fig. 1C) and SV-derived cells (data not shown). No consistent difference was seen in the response of cells to UTP or ATP over a number of experiments or in the size of the response of IMA-derived cells compared with that of SV-derived cells. The P2X agonist α,β-methylene-ATP (300 μM) did not raise...
[Ca^{2+}]	extsubscript{i}, and the removal of extracellular Ca^{2+} with EGTA did not significantly attenuate the response to ATP (data not shown). To determine whether the effects of ATP and UTP were consequent to PLC activation and Ins(1,4,5)P	extsubscript{3} generation, we measured nucleotide-induced accumulation of [3H]InsP	extsubscript{x}. As shown in Fig. 2A, PDGF stimulation of IMA-derived cells led to [3H]InsP	extsubscript{x} accumulation. However, stimulation with ATP or UTP alone did not lead to an increase in [3H]InsP	extsubscript{x} accumulation. In addition, in the presence of PDGF, the application of ATP and UTP did not lead to any further increase in [3H]InsP	extsubscript{x} accumulation (Fig. 2A). These responses were the same for SV-derived cells (data not shown). To investigate the possibility that the nucleotides elicit a transient formation of Ins(1,4,5)P	extsubscript{3} that is not detected with the [3H]InsP	extsubscript{x} procedure, we measured the mass of Ins(1,4,5)P	extsubscript{3} formed within seconds of stimulation. As shown in Fig. 2B, no increase in Ins(1,4,5)P	extsubscript{3} level was detected between 10 s and 5 min of stimulation in response to PDGF, despite the modest accumulation of [3H]InsP	extsubscript{x} levels (Fig. 2A). As a positive control for the mass Ins(1,4,5)P	extsubscript{3} procedure, SH-SY5Y neuroblastoma cells were stimulated with carbachol and assayed concurrently with the VSMC; a clear increase in Ins(1,4,5)P	extsubscript{3} accumulation was seen (Fig. 2B, inset).

[3H]thymidine responses. To compare the mitogenic responses of SV- and IMA-derived cells to PDGF, we used [3H]thymidine incorporation into DNA as an index of proliferation. As shown in Fig. 3A, increasing concentrations of PDGF led to increased incorporation of [3H]thymidine. PDGF was significantly more potent in IMA-derived cells than in the paired SV-derived cells (pEC50: −9.22 ± 0.11 for IMA-derived cells and −8.45 ± 0.08 for SV-derived cells; P < 0.001, n = 3 experiments each in quadruplicate). However, the maximal response was greater in SV-derived cells (SV: 4,828 ± 330 dpm/mg protein; IMA: 1,792 ± 121 dpm/mg protein, P < 0.05, n = 3). The effect of passage number on [3H]thymidine incorporation was studied by comparing cells from passages 2–4 in paired IMA- and SV-derived cells from three different patients, with three separate experiments in quadruplicate for each. There was no effect of passage number on the [3H]thymidine incorporation to increasing concentrations of PDGF (data not shown).
To investigate the effect of ATP on proliferative responses, we stimulated cells with 300 μM ATP in the presence and absence of PDGF (1 nM). At this submaximal concentration of PDGF, the \[^{3}H\]thymidine responses of IMA- and SV-derived cells were not substantially different (see Fig. 3, A and B). ATP, either alone or in the presence of PDGF, did not stimulate \[^{3}H\]thymidine incorporation in IMA-derived cells. Similarly, in SV-derived cells, ATP alone had no effect. However, when stimulated with ATP in the presence of PDGF, \[^{3}H\]thymidine incorporation was increased significantly in SV cells but not in IMA cells.

The five cloned and characterized mammalian P2Y receptors have distinct profiles of activation by different nucleotides. A range of nucleotides was therefore tested that would activate each of these receptors: ATP (P2Y2 and P2Y11), UTP (P2Y2 and P2Y4), 2-methylthio-ADP (2-MeS-ADP; P2Y1), and UDP (P2Y6). 2-MeS-ADP had no effect on \[^{3}H\]thymidine incorporation in either IMA- or SV-derived cells whether applied alone or together with 1 nM PDGF (Fig. 4, A and B).

Application of UTP or UDP alone also had no effect on \[^{3}H\]thymidine incorporation, but, surprisingly, both UDP and UTP led to a substantial and significant reduction in the PDGF-stimulated level of \[^{3}H\]thymidine incorporation. This was true for both cell types (Fig. 4). There was no difference in the response to UTP or UDP at 100 μM. The difference in the effect of UTP compared with that of ATP on PDGF-stimulated \[^{3}H\]thymidine incorporation is depicted in Fig. 5A. To determine whether UTP was acting directly, we repeated experiments with UTP purified before use (by high-pressure liquid anion exchange chromatography) and with the inclusion of a creatine phosphokinase-regenerating system to prevent loss of UTP and accumulation of UDP. Figure 5B shows the concentration-response curve under these conditions for the antiproliferative effect of UTP on \[^{3}H\]thymidine incorporation in the presence or absence of PDGF. UTP was effective at concentrations >1 μM, consistent with reports on native P2Y receptors.

Fig. 4. \[^{3}H\]thymidine incorporation into DNA of human VSMC in response to PDGF and various nucleotides in IMA (A) or SV (B) cells, expressed as mean percentages above unstimulated (basal) control values (indicated by the solid line). Data are means ± SE; n = 3 separate experiments, each in quadruplicate. **P < 0.01 compared with unstimulated control. PDGF + ATP was also significantly different from PDGF alone for the SV cells (P < 0.01, by ANOVA with Bonferroni's posttest).

Fig. 3. Stimulated \[^{3}H\]thymidine incorporation into DNA in human VSMC in response to PDGF (1 nM) and ATP (300 μM). A: concentration-response curves to PDGF for SV- and IMA-derived cells. Statistical analysis of data is presented in the text. B: \[^{3}H\]thymidine incorporation (dpm) expressed as a percentage of control values in IMA (left) or SV (right) cells. Data are means ± SE; n = 3 separate experiments, each in quadruplicate. **P < 0.01 compared with unstimulated control. PDGF + ATP was also significantly different from PDGF alone for the SV cells (P < 0.01, by ANOVA with Bonferroni's posttest).
The P2 antagonists suramin and pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), when present at 300 μM for 10 min before and during the 1-h exposure to agonists, had little effect on the responses. Expressed as a percentage of stimulation of [3H]thymidine incorporation by PDGF alone, stimulation by PDGF with 300 μM UTP was 48.8 ± 9.5, PDGF with UTP and suramin was 34.6 ± 5.2, and PDGF with UTP and PPADS was 35.3 ± 2.9 (means ± SE, n = 3 separate experiments each in quadruplicate). These results suggest that the response is not at P2X, P2Y1, or P2Y2 receptors (see DISCUSSION).

The effect of 10% FCS on [3H]thymidine incorporation in IMA and SV VSMC is shown in Fig. 4, A and B. The response was significant but surprisingly smaller than the response to 1 nM PDGF.

Cell proliferation. To measure cell proliferation directly, we counted cells over a number of days in response to repeated stimulation with PDGF in the presence or absence of UTP. Stimulations were daily, over a period of 1 h, at which point the medium was changed for serum-free medium with no agonists present. Table 1 shows results with both SV- and IMA-derived cells pooled across experiments, with stimulation for 2, 4, and 7 days. PDGF stimulation for 2 days alone had no effect, but over 4 or 7 days there was a significant increase in cell number. This increase was greater for SV-derived cells than for IMA-derived cells. UTP alone had no effect on cell number at any time. However, when UTP was present, there was no increase in cell number in response to PDGF. This was true for both cell types. These results show that UTP exerted a powerful antiproliferative effect on both SV- and IMA-derived cells.

Activation of MAPK. Consistent with an earlier report (36), PDGF stimulated phosphorylation of p42/p44 MAPK in both IMA- and SV-derived cells (Fig. 6). Stimulation of SV-derived cells with ATP alone led to an increased phosphorylation of p42/p44 MAPK, but ATP did not stimulate MAPK phosphorylation in IMA-derived cells. When ATP was applied in combination with PDGF, there was no detectable enhancement of p42/p44 MAPK phosphorylation (Fig. 6) over the response to PDGF alone. UTP had no effect on p42/p44 MAPK phosphorylation when added alone to either cell type. In SV-derived cells, but not in IMA-derived cells, UTP enhanced the PDGF-stimulated MAPK phosphorylation.

**Table 1. Stimulation of cell proliferation by PDGF in the presence and absence of UTP**

<table>
<thead>
<tr>
<th></th>
<th>Cell Number, ×10⁶ cells/well</th>
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<tbody>
<tr>
<td></td>
<td>IMA</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
</tr>
<tr>
<td>Control</td>
<td>10.0 ± 0.6</td>
</tr>
<tr>
<td>UTP (300 μM)</td>
<td>8.5 ± 0.1</td>
</tr>
<tr>
<td>PDGF (1 nM)</td>
<td>10.1 ± 1.3</td>
</tr>
<tr>
<td>UTP (300 μM) + PDGF (1 nM)</td>
<td>10.4 ± 0.3</td>
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Cells were stimulated for 1 h on 2, 4, or 7 consecutive days with platelet-derived growth factor (PDGF-BB) or UTP alone or in combination, and cells were counted 24 h after the final stimulation. Values are means ± SE from 3 separate experiments each in triplicate. PDGF stimulations were significantly different from controls at days 4 and 7 (*P < 0.05, †P < 0.01, ‡P < 0.001 by ANOVA with Bonferroni’s posttest), but the response to PDGF + UTP was not significantly different from control at any time. IMA, internal mammary artery; SV, saphenous vein.
enable the identification of these receptors, yet the studies presented do not provide clear evidence for the presence of receptors that respond to ATP and UTP. The proliferation (and also occurs when cells are cultured), the synthetic phenotype, which is necessary for intimal proliferation. It has been proposed that nucleotides such as ATP act in this capacity through their cell surface receptors (3). In the diseased vessel, nucleotide release from platelets (together with PDGF) will be enhanced due to downregulation of the anti-platelet influence of endothelium-derived nitric oxide and prostacyclin. In this model the P2Y receptors on VSMC play a critical role in the pathology of intimal proliferation.

The importance of P2Y receptors in this respect has been suggested by previous studies on animal VSMC (9, 14, 19, 33). In intact rat arterial tissue, ionotropic P2X receptors play a role in the contractile response. In culture, these cells lose their P2X receptors, while a P2Y response is found in both intact tissue and cultured cells (21, 24). A recent quantitative PCR study has shown that the transition from the contractile to the synthetic phenotype, which is necessary for intimal proliferation (and also occurs when cells are cultured), corresponds to a loss of mRNA for P2X1 and an upregulation of mRNA for P2Y1 and P2Y2 receptors (8). This is consistent with the hypothesis that P2Y receptors regulate the proliferative response in the synthetic phenotype.

The increases in [Ca^{2+}]_i reported here in both human venous and arterial smooth muscle cells provide clear evidence for the presence of receptors that respond to ATP and UTP. The studies presented do not enable the identification of these receptors, yet the results indicate that the nucleotides are acting at P2Y receptors. The response to UTP and the lack of effect of α,β-methylene-ATP, together with the response to ATP in the absence of extracellular Ca^{2+}, all argue against the involvement of P2X receptors. The demonstration that UTP stimulated PLC and increased [Ca^{2+}], in rat VSMC (16, 24) led to the proposal of a P2Y receptor sensitive to UTP, both in the original smooth muscle cells and in culture (24). Because all of the cloned P2Y receptors are coupled to the release of intracellular Ca^{2+} via Ins(1,4,5)P_3, the lack of a detectable PLC response reported here was unexpected. However, a similar situation has been reported in other cell types in response to activation of native P2Y receptors (1, 10) and other G protein-coupled receptors (20, 22, 30), in which the [Ca^{2+}], increase is apparently independent of Ins(1,4,5)P_3.

Mitogenic responses to ATP and UTP have been reported in cultured rat VSMC (9, 14, 19, 33). In some cases nucleotides act as progression factors, stimulating the cell cycle in concert with growth factors such as PDGF. This growth factor has been shown to stimulate the progression of quiescent rat aorta cells from G_0 to G_1 but no further (23). ATP acting on P2Y receptors had no effect on the cell cycle of cells in G_0 but could lead to progression from G_1 to S and M phases. These observations indicate that concomitant stimulation of VSMC with growth factors such as PDGF and agonists acting at P2Y receptors leads to proliferation.

The results of our experiments in which IMA and SV cells were stimulated with PDGF are consistent with previous work (29). We report that the intracellular Ca^{2+} response was of a similar magnitude in SV- and IMA-derived cells, while [3H]thymidine incorporation was greater in the SV cells. The larger maximal response to PDGF seen in SV cells occurred with an EC_{50} that was significantly higher than that occurring in the IMA cells; this may reflect a difference in receptor reserve between these two cell types.

ATP did not act as a full mitogen in either SV or IMA cells; no stimulation of [3H]thymidine incorporation was observed in response to this nucleotide alone. However, in SV cells, ATP acted as a progression factor to enhance the PDGF-stimulated [3H]thymidine incorporation into DNA, consistent with the effects described in rat VSMC (23). However, this comitogenic action of ATP was not seen in IMA cells, despite the observation that the intracellular Ca^{2+} response of IMA cells to ATP is similar to that of SV cells. Differences were also seen in the effects of ATP on p42/p44 MAPK phosphorylation in the two cell types. In SV-derived cells ATP stimulated this index of MAPK activation, but this did not occur in IMA-derived cells; interestingly, this correlates with the ability of ATP to act as a comitogen. However, the addition of ATP in the presence of PDGF failed to further elevate MAPK phosphorylation above that of PDGF alone in either cell type. These results are consistent with the suggestion that activation of MAPK is necessary but not sufficient for the mitogenic response (14, 31).
A salient observation here is that ATP exerts a proliferative influence only in SV cells, and this may contribute to the increased intimal proliferation following vascular grafts with SV compared with grafts with IMA. This is consistent with the hypothesis that P2Y receptors on VSMC play a role in the progression of vascular proliferative disorders. 2-MeS-ADP had no proliferative effect, suggesting that P2Y1 receptors (for which this is the most potent agonist) are not involved. ATP and UTP are equally effective at the P2Y2 receptor, and the lack of proliferation in response to UTP suggests that the response is not mediated by this receptor subtype.

The reduction in the PDGF-stimulated [3H]thymidine incorporation and proliferation of both SV and IMA cells by the application of UTP was an unexpected finding. The conclusions we derive from the thymidine incorporation studies are powerfully confirmed by direct measurement of cell numbers. It takes several days for the cell numbers to be reliably increased by PDGF, but wherever this occurs it is essentially ablated by the presence of UTP. Indeed, the antiproliferative effect of UTP is apparently greater when counting cell number than when looking at [3H]thymidine incorporation.

There was one previous report of an antiproliferative response to UTP in endothelium-derived cells (18), although it was also reported that UTP is mitogenic in cardiac vasculature endothelium (28). Here we have shown that UTP can act directly; its antiproliferative action is not dependent on breakdown by ectonucleotidases. This is demonstrated by experiments in which HPLC-purified UTP was used in the presence of a creatine phosphokinase-regenerating system, which will convert any UDP formed during the incubation back into UTP. This system is usually used as an ATP-regenerating system (15), but we have demonstrated that UTP can act as a substrate, although the rate of the kinase reaction is much slower than that with ADP as substrate. These experiments confirm that the antiproliferative P2Y receptor is responsive to UTP. However, the antiproliferative response to UDP observed here may be indirect and dependent on extracellular conversion to UTP. Harden and colleagues (13) showed that the addition of UDP can result in accumulation of extracellular UTP by the action of ectonucleoside diphosphokinase.

Candidates for the receptor responsible for the antiproliferative response to UTP include P2Y2 and P2Y4. Of these, the P2Y4 receptor seems most likely since ATP, which does not elicit an antiproliferative response, is an agonist at P2Y2 receptors but not at human P2Y4 receptors (7). The suramin and PPADS results are consistent with this. Neither compound attenuated the effect of UTP on PDGF-stimulated [3H]thymidine incorporation. Both are effective antagonists at P2X and P2Y1 receptors, suramin is a weak antagonist at P2Y2 receptors, and neither are antagonists at P2Y4 receptors (3, 6).

PDGF-stimulated proliferation was inhibited to a similar degree by UTP in both IMA- and SV-derived cells. This effect was apparently independent of Ca2+, since [Ca2+]i was elevated by both ATP and UTP, but only UTP had an antiproliferative effect. Similarly, the antiproliferative effect showed no correlation with activation of p42/p44 MAPK, since UTP had no effect on PDGF-mediated MAPK phosphorylation in IMA-derived cells. These results rule out changes in [Ca2+]i and p42/p44 MAPK phosphorylation state as the mechanism underlying this antiproliferative effect.

Our initial hypothesis was that nucleotides act on vascular smooth muscle P2Y receptors to enhance proliferation of these cells, thus contributing to intimal proliferation in diseased vessels. The results presented here are consistent with this hypothesis with respect to the action of ATP and indicate that differential activation of SV-derived cells by ATP may contribute to greater intimal proliferation seen with these vessels compared with that seen with IMA. However, our results indicate that the effects of nucleotides are more complex than we initially suggested, since they show that UTP is an antiproliferative regulator of human vascular smooth muscle of both arterial and venous origin.

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