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Endothelial dysfunction is induced by inflammatory mediators including multiple G protein–coupled receptor (GPCR) agonists. However, the GPCR signaling pathways that promote endothelial dysfunction are incompletely understood. We previously showed that thrombin promotes endothelial barrier disruption through autophosphorylation and activation of p38 mitogen-activated protein kinase (MAPK) via a non-canonical transforming growth factor-β–activated protein kinase-1–binding protein-1 (TAB1) and TAB2–dependent pathway rather than the canonical three-tiered kinase cascade. Here, we sought to determine whether other GPCR agonists stimulate p38 MAPK activation via this non-canonical pathway in human endothelial cells derived from different vascular beds. Using primary human umbilical vein endothelial cells (HUVECs), HUVEC-derived EA.hy926 cells, and human dermal microvascular endothelial cells (HDMECs), we found that both non-canonical and canonical p38 activation pathways components are expressed in these various endothelial cell types, including TAB3, a structurally-related TAB2 homolog. Moreover, multiple GPCRs agonists, including thrombin, histamine, prostaglandin E_2, and ADP, stimulated robust p38 autophosphorylation, whereas phosphorylation of the upstream MAPKs MAP kinase kinase 3 (MKK3) and MKK6, was virtually undetectable, indicating that non-canonical p38 activation may exist for other GPCRs. Indeed, in EA.hy926 cells, thrombin- and histamine-stimulated p38 activation depended on TAB1–TAB2, whereas in primary HUVECs, both TAB1–TAB2 and TAB1–TAB3 were required for p38 activation. In HDMECs, thrombin-induced p38 activation depended on TAB1–TAB3, but histamine-induced p38 activation required TAB1–TAB2. Moreover, thrombin- and histamine-stimulated interleukin-6 production required both TAB1–TAB2 and TAB1–TAB3 in HUVEC. We conclude that multiple GPCR agonists utilize non-canonical TAB1–TAB2 and TAB1–TAB3–dependent p38 activation to promote endothelial inflammatory responses.

Vascular endothelial dysfunction is caused by inflammatory mediators, many of which signal through G protein–coupled receptors (GPCRs)[4] (1, 2). A single monolayer of endothelial cells lines the lumen of blood vessels and forms a semipermeable barrier that is controlled by the integrity of cell–cell junctions (3). Endothelial dysfunction results in production of inflammatory mediators and disruption of cell–cell junctions, vascular leakage, and tissue edema that contributes to organ failure (4, 5). The activation of RhoA and phosphorylation of myosin light chain (MLC) by MLC kinase are known mediators of thrombin-induced endothelial barrier disruption (6), however, we and others have shown that thrombin-stimulated p38 mitogen-activated protein kinase (MAPK) activation also controls endothelial barrier permeability through a pathway that is independent MLC phosphorylation and presumably RhoA signaling (7, 8). Thus, GPCRs utilize multiple signaling pathways to promote endothelial dysfunction.

The p38 MAPK is a critical mediator of inflammation (9). However, despite enormous efforts, pharmacological inhibitors of p38 that generally target the ATP-binding site have failed to advance in clinical trials due to adverse side effects such as skin and liver toxicity. Thus, a better mechanistic understanding of the activators of p38-driven inflammatory responses is critical for discovery of new strategies to manipulate p38 signaling in various disease settings. All four p38 isoforms (α, β, γ, and δ) are activated by the canonical three-tiered kinase cascade mediated by upstream MAP2Ks and MAP3Ks (10, 11). Interestingly, however, authenticated non-canonical pathways for p38 activation also exist. The direct binding of transforming growth factor-β–activated kinase-1–binding protein-1 (TAB1) to

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[4] The abbreviations used are: GPCR, G protein–coupled receptor; HDMEC, human dermal microvascular endothelial cells; His, histamine; MAPK, mitogen-activated protein kinase; HUVEC, human umbilical vein endothelial cells; IL-6, interleukin 6; MKK, mitogen-activated protein kinase kinase; PAR1, protease-activated receptor-1; PGE_2, prostaglandin E_2; TAB, transforming growth factor-β–activated kinase-1–binding protein; α-Th, thrombin; VEGF, vascular endothelial growth factor; MLC, myosin light chain; NZF, Npl4 zinc finger; NEDD4, neural precursor cell expressed, developmentally down-regulated-4; qPCR, quantitative PCR.

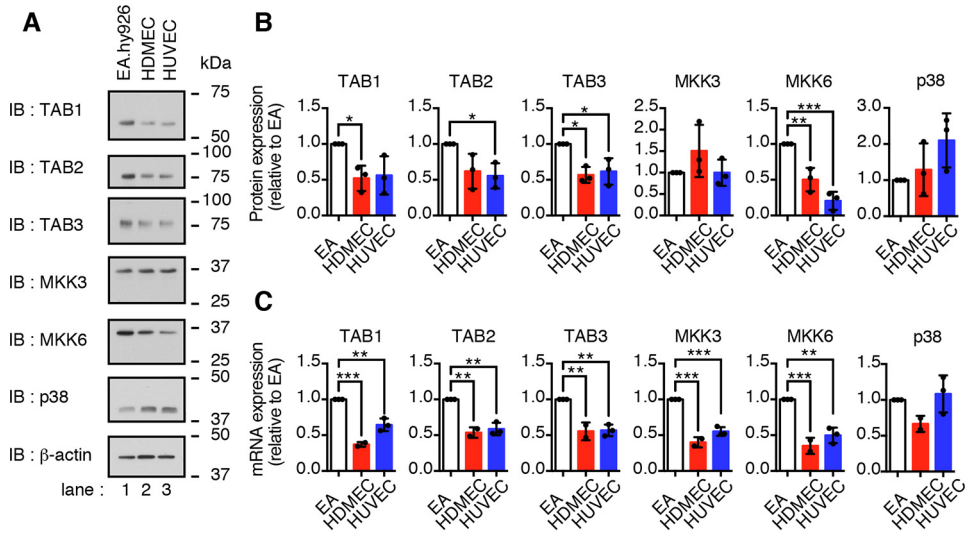

Figure 1. Expression of TAB1, TAB2, and TAB3 and MKK3 and MKK6 in EA.hy926 endothelial cells and primary HUVEC and HDMEC. *A*, human endothelial EA.hy926 cells, HUVEC, and HDMEC were lysed, and equivalent amounts of protein lysates were immunoblotted (*IB*) with the indicated antibodies. Immunoblots shown are representative of three independent experiments. *B*, expression of TAB1, TAB2, TAB3, MKK3, MKK6, and p38 protein in endothelial cell lines were quantified from three independent experiments. The data (mean ± S.D., $n = 3$) are expressed as the fold relative to EA.hy926 cells and were analyzed using a Student's *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *C*, TAB1, TAB2, TAB3, MKK3, MKK6, and p38 mRNA expression in endothelial cells was quantified by qPCR. The data (mean ± S.D., $n = 3$) are representative of three independent experiments, expressed as the fold relative to EA.hy926 mRNA expression and analyzed by one-way analysis of variance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

p38α induces autophosphorylation and autoactivation and bypasses the requirement for upstream MAP2Ks, mitogen-activated protein kinase kinase-3 (MKK3) and MKK6 (12, 13). The physiological relevance of the TAB1–p38α pathway has been demonstrated *in vivo* and shown to function in inflammation, cardiotoxicity, and myocardial ischemia (14–16). A different non-canonical pathway for p38 activation is mediated by ZAP-70 binding, which results in p38α and -β autophosphorylation and activation in immune T cells (17). Although it is presumed that GPCRs activate p38 through the three-tiered kinase cascade there is limited supportive evidence (18). In fact, several studies have shown that GPCRs stimulate p38 MAPK activation through diverse G_s, G_q, and G_{13} signaling pathways (18), however, rarely has the function of MAPK2Ks been directly examined (19).

In previous studies, we showed that activation of protease-activated receptor-1 (PAR1), a GPCR for the coagulant protease thrombin, in endothelial cells promotes p38α activation via a TAB1–dependent pathway and is independent of upstream MAP2Ks, MKK3, and MKK6 (8). We also showed that ubiquitination of activated PAR1 drives recruitment of TAB2, an adaptor protein that binds TAB1 (20) and contains a Npl4 zinc finger (NZF) domain that binds K63-linked ubiquitin (21). The ubiquitin binding capacity of TAB2 and p38α binding determinants for TAB1 are both required for thrombin-stimulated p38 signaling (8). TAB3 is a structurally related homolog of TAB2 that can also bind ubiquitin and mediate inflammatory signaling (22, 23). Ubiquitin-driven p38 signaling induced by thrombin-activated PAR1 further promotes endothelial barrier permeability *in vitro* and p38 activity is required for PAR1-stimulated vascular leakage *in vivo* (8). Thus, PAR1 stimulates p38 inflammatory signaling via a non-canonical TAB1–TAB2–dependent pathway in endothelial cells, however, it is not known if this pathway is broadly applicable to other GPCRs expressed in endothelial cell types derived from different vascular beds.

In this study, we sought to determine whether non-canonical TAB1–dependent p38 activation is induced by other GPCRs in a panel of extensively studied endothelial cell models including human endothelial cells of venous macrovascular origin, human endothelial vein umbilical cells (HUVECs), and HUVEC-derived EA.hy926 cells, and human dermal microvascular endothelial cells (HDMECs). We found that critical components of the canonical and non-canonical p38–activation pathways are expressed in these endothelial cell types, and multiple GPCRs agonists including thrombin, histamine, prostaglandin E_2 (PGE_2), and ADP, stimulated non-canonical p38 autophosphorylation and activation. In addition, whereas all GPCR agonists stimulated robust p38 activation, each displayed a unique requirement for either TAB1–TAB2 or TAB1–TAB3 for p38 activation in distinct endothelial cells types. Thrombin and histamine also stimulated production of the inflammatory mediator interleukin-6 (IL-6) via a TAB1–dependent pathway, suggesting that noncanonical activation of p38 inflammatory signaling is important for multiple GPCR agonists.

Results

TAB1, TAB2, TAB3, MKK3, MKK6, and p38 expression in human cultured endothelial cells

To assess the function of non-canonical *versus* canonical p38 MAPK activation induced by a subset of GPCRs in endothelial cells, we profiled the expression of TAB1, TAB2, TAB3, MKK3, MKK6, and p38 in three extensively studied endothelial cell model systems including primary human HUVECs, EA.hy926 cells derived from HUVEC (24), and primary HDMECs. Components of the p38 canonical (MKK3 and MKK6) and non-canonical (TAB1, TAB2, and TAB3) pathways and p38 MAPK

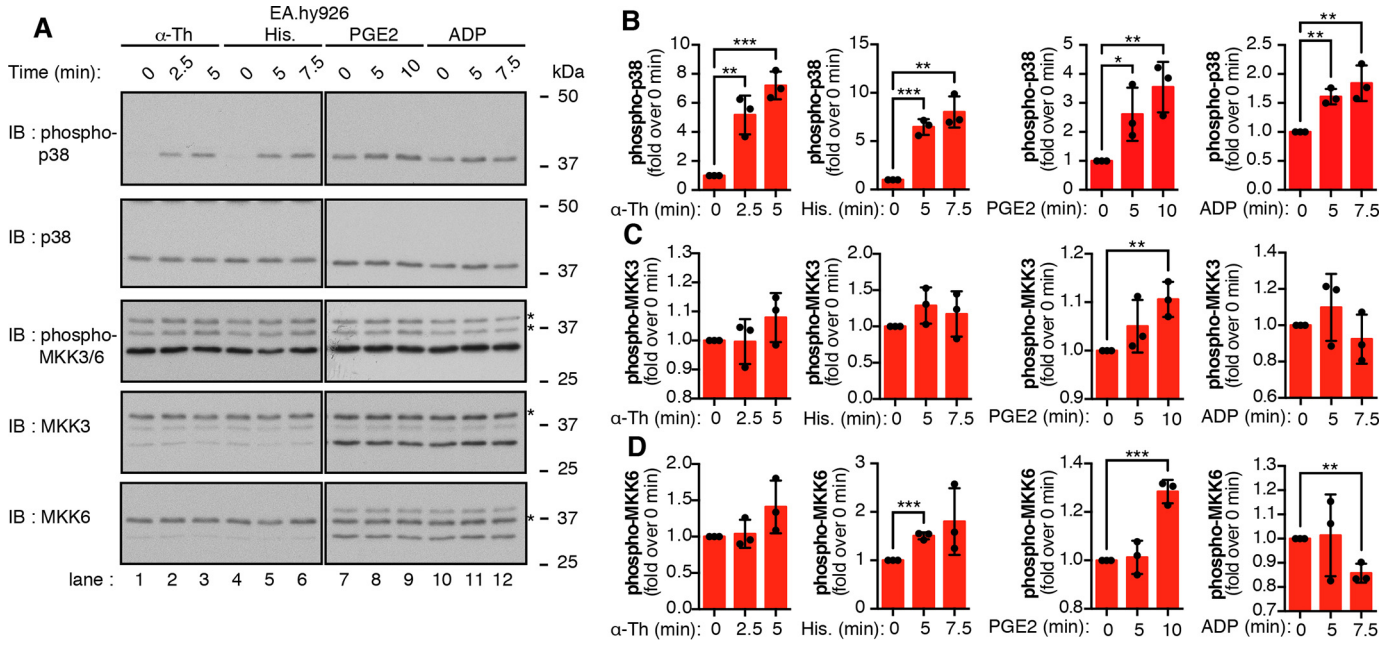

Figure 2. GPCR agonists induce p38 phosphorylation with variable phosphorylation of MKK3 and MKK6 in EA.hy926 endothelial cells. *A*, EA.hy926 cells were stimulated with 10 nM α-thrombin (*α-Th*), 10 μM histamine (*His.*), 10 μM PGE_2, and 10 μM ADP for the indicated times and lysed. Equivalent amounts of cell lysates were immunoblotted (*IB*) for phospho and total p38, MKK3, and MKK6 as indicated. Representative immunoblots are shown, *asterisks* denote MKK3 or MKK6 bands. Quantification of p38 (*B*), MKK3 (*C*), and MKK6 (*D*) phosphorylation induced by agonist was determined. The data (mean ± S.D., $n = 3$) are representative of three independent experiments are expressed as the fold over 0 min and analyzed by Student's *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

were easily detected in HUVEC-derived EA.hy926 cells (Fig. 1*A*, *lane 1*), primary HDMECs (Fig. 1*A*, *lane 2*), and primary HUVECs (Fig. 1*A*, *lane 3*) by immunoblot analysis and real-time quantitative (q) PCR (Fig. 1, *B* and *C*). However, the various cell lines showed differences in expression of the individual components relative to each other. Notably, EA.hy926 cells displayed a significantly higher amount of TAB1 compared with HDMEC, TAB2 compared with HUVEC, and TAB3 and MKK6 compared with both HDMEC and HUVEC based on both immunoblot and quantitative PCR (qPCR) analysis (Fig. 1, *A–C*). In contrast, there was no significant difference in expression of TAB1 in EA.hy926 cells *versus* HUVEC, TAB2 in EA.hy926 *versus* HDMEC, whereas MKK3 and p38 exhibited comparable expression in all endothelial cell lines detected by immunoblotting (Fig. 1, *A* and *B*). Interestingly, however, qPCR analysis of individual mRNA transcripts encoding the p38-activation pathway components revealed a significant higher abundance of TAB1, TAB2, TAB3, MKK3, and MKK6 mRNA transcripts in EA.hy926 cells compared with HDMECs and HUVECs, whereas the levels of p38 MAPK mRNA transcripts were comparable across all endothelial cell types similar to that observed with p38 protein (Fig. 1, *B* and *C*). These findings likely reflect higher transcriptional activity in EA.hy926 cells *versus* HDMECs and HUVECs as well as possibly differences in protein stability of the individual components, which has been clearly demonstrated for TAB1 (8), a critical mediator of non-canonical p38 activation.

GPCR agonists induce significant p38 phosphorylation but limited induction of MKK3/MKK6 phosphorylation in human endothelial cells

To assess the capacity of endothelial GPCRs to activate p38 MAPK signaling via the non-canonical *versus* the canonical MKK3/MKK6 pathway, we first examined the extent of four different agonists that signal through GPCRs to induce phosphorylation of p38 and MKK3/MKK6 in HUVEC-derived EA.hy926 cells. PAR1 is the major GPCR for thrombin in HUVEC and HUVEC-derived EA.hy926 cells (25, 26), whereas histamine signals through H1 and H2 GPCRs in HUVEC and EA.hy926 cells (27, 28). ADP signaling is primarily mediated by the P2Y1 GPCR in HUVEC (29), whereas PGE_2 responses are mediated primarily by the EP4 prostanoid GPCR in endothelial cells (30, 31).

Thrombin induced a robust ~5-fold increase in p38 phosphorylation at 2.5 and 5 min (Fig. 2*A*, *lanes 1–3*, and *B*), with no significant change in the phosphorylation status of MKK3 or MKK6 (Fig. 2, *A*, *lanes 1–3*, *C* and *D*), consistent with our previously published studies (8). Histamine also caused a significant ~5-fold increase in p38 phosphorylation detected at 5 and 7.5 min following agonist incubation (Fig. 2*A*, *lanes 4–6*, and *B*). Although histamine failed to induce a marked change in MKK3 phosphorylation at either time point (Fig. 2, *A*, *lanes 4–6*, and *C*), a significant ~1.5-fold increase in MKK6 phosphorylation was detected after 5 min of agonist stimulation (Fig. 2, *A*, *lanes 4–6*, and *D*). Cells stimulated with PGE_2 also showed an ~2–3–fold increase in p38 phosphorylation at 5 and 10 min (Fig. 2, *A*, *lanes 7–9*, and *B*), and a modest but significant increase in MKK3 and MKK6 phosphorylation at 10 min (Fig. 2, *A*, *lanes 7–9*, *C* and *D*). Incubation with ADP also caused a significant but modest ~1.5-fold increase in p38 phosphorylation (Fig. 2, *A*, *lanes 10–12*, and *B*), with no significant change in MKK3 phosphorylation, whereas a significant decrease in MKK6 phosphorylation was detected (Fig. 2, *A*, *lanes 10–12*, *C* and *D*). These data demonstrate that multiple GPCR agonists consistently stimulate robust p38 phosphorylation in HUVEC-

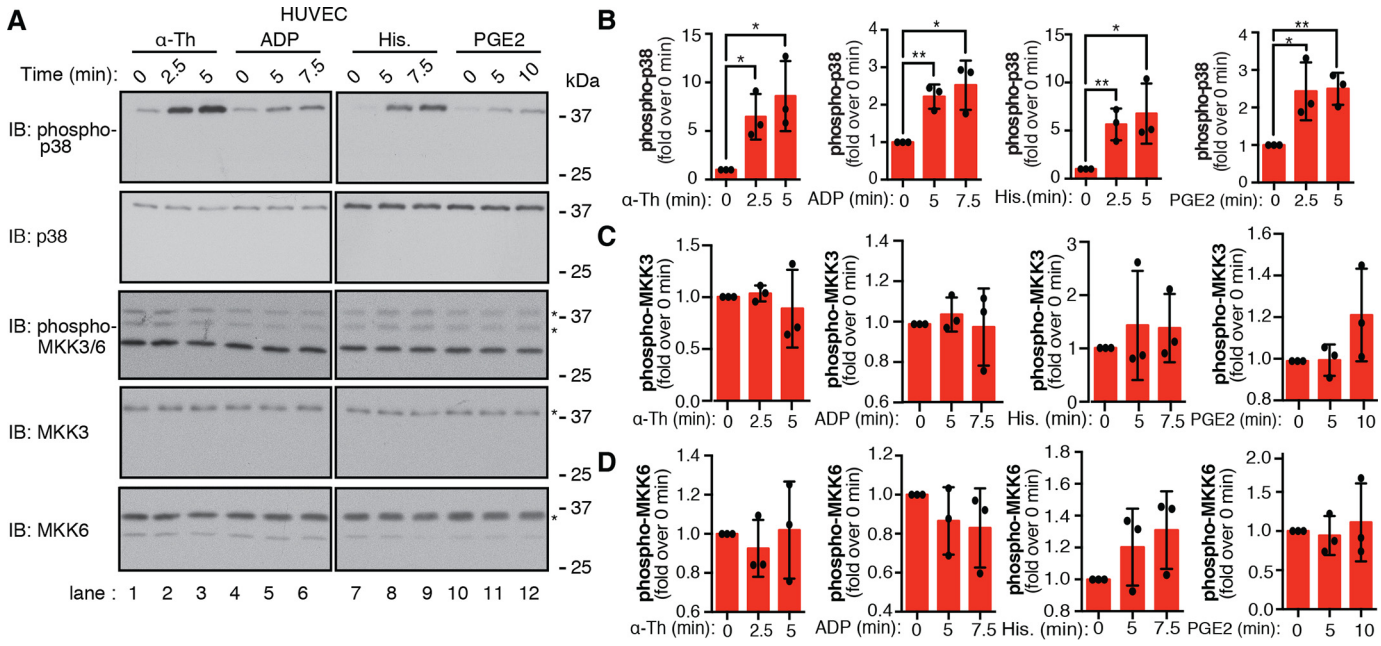

Figure 3. GPCR agonists stimulate phosphorylation of p38 without significant MKK3 or MKK6 phosphorylation in primary HUVECs. *A*, HUVEC were stimulated with 10 nM α-thrombin (*α-Th*), 10 μM ADP, 10 μM histamine (*His.*), and 10 μM PGE_2 for various times and lysed. Equivalent amounts of cell lysates were immunoblotted (*IB*) for p38, MKK3, and MKK6 as indicated. Representative immunoblots are shown, *asterisks* denote MKK3 or MKK6 bands. Quantification of agonist-induced phosphorylation of p38 (*B*), MKK3 (*C*), and MKK6 (*D*) was determined. The data (mean ± S.D., $n = 3$) are representative of three independent experiments expressed as the fold over 0 min and analyzed by Student's *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

derived EA.hy926 cells with modest and varying differences in MKK3 and MKK6 phosphorylation.

We next determined if GPCR agonists induced a similar trend in p38, MKK3, and MKK6 phosphorylation in primary HUVECs. Thrombin stimulated a significant ~7-fold increase in phosphorylation of p38 with no change in the phosphorylation status of MKK3 or MKK6 (Fig. 3, *A*, *lanes 1–3*, *B–D*), like that observed in HUVEC-derived EA.hy926 cells. Similar to thrombin, histamine also caused a significant ~6-fold increase in p38 phosphorylation without affecting the MKK3 or MKK6 phosphorylation (Fig. 3, *A*, *lanes 7–9*, *B–D*). Cells stimulated with ADP and PGE_2 displayed a modest but significant ~2-fold increase in p38 phosphorylation, and no change in MKK3 or MKK6 phosphorylation (Fig. 3, *A*, *lanes 4–6* and *10–12*, *B–D*). Taken together these data suggest that whereas multiple GPCR agonists stimulate activation of p38 MAPK, they fail to consistently induce MKK3 or MKK6 phosphorylation in primary HUVEC and HUVEC-derived EA.hy926 cells, suggesting that p38 activation may occur through non-canonical pathways in different endothelial cell types.

To ensure that phosphorylation of MKK3 and MKK6 is responsive to stimuli in HUVEC and HUVEC-derived EA.hy926 cells, cells were treated with vascular endothelial growth factor (VEGF). VEGF has been previously shown to activate p38 MAPK through an MKK3- and MKK6–dependent pathway in endothelial cells (32). A significant ~10-fold increase in p38 phosphorylation was induced by VEGF at 10 min, which was diminished after 15 min of stimulation in EA.hy926 cells (Fig. 4, *A* and *B*). VEGF-induced p38 phosphorylation coincided with a significant ~1.5-fold increase in phosphorylation of MKK3 and MKK6 in EA.hy926 cells detected at 10 min (Fig. 4, *A*, *C*, and *D*). HUVECs stimulated with VEGF resulted in a similar significant ~12-fold increase in p38 phosphorylation and ~1.5-fold increase in MKK3 and MKK6 phosphorylation after 10 min of stimulation (Fig. 4, *E–H*). These findings indicate that both MKK3 and MKK6 phosphorylation occurs in response to growth factor stimulation in HUVECs and HUVEC-derived EA.hy926 cells. Thus, the failure of certain GPCR agonists to promote MKK3 or MKK6 phosphorylation consistently in various endothelial cell types suggest that p38 activation may not depend exclusively on activation of the upstream MAP2Ks, MKK3 and MKK6.

Multiple GPCR agonists induce p38 autophosphorylation

Activation of p38 MAPK occurs through direct phosphorylation mediated by upstream MAP2Ks, MKK3, and MKK6, or through direct binding of TAB1 to p38α resulting in autophosphorylation and activation. To examine if GPCR-induced p38 phosphorylation is mediated by upstream MAP2Ks or through p38 autophosphorylation, cells were pretreated with the p38 inhibitor SB203580. SB203580 specifically blocks the catalytic activity of p38α and -β isoforms inhibiting autophosphorylation without effecting phosphorylation of p38 mediated by upstream MAP2Ks (12). HUVEC-derived EA.hy926 cells were pretreated with SB203580 and then incubated with thrombin for various times. In control DMSO vehicle-treated cells, thrombin-induced a significant ~2.5-fold increase in p38 activation (Fig. 5*A*, *lanes 1–3*), which was significantly inhibited in endothelial cells pretreated with SB203580 (Fig. 5*A*, *lanes 4–6*). These data are consistent with our previously published results (8). Similarly, pretreatment of endothelial EA.hy926 cells with SB203580 resulted in significant inhibition of histamine-, PGE_2-, and ADP-induced p38 activation measured after various times of agonist incubation compared with control treated

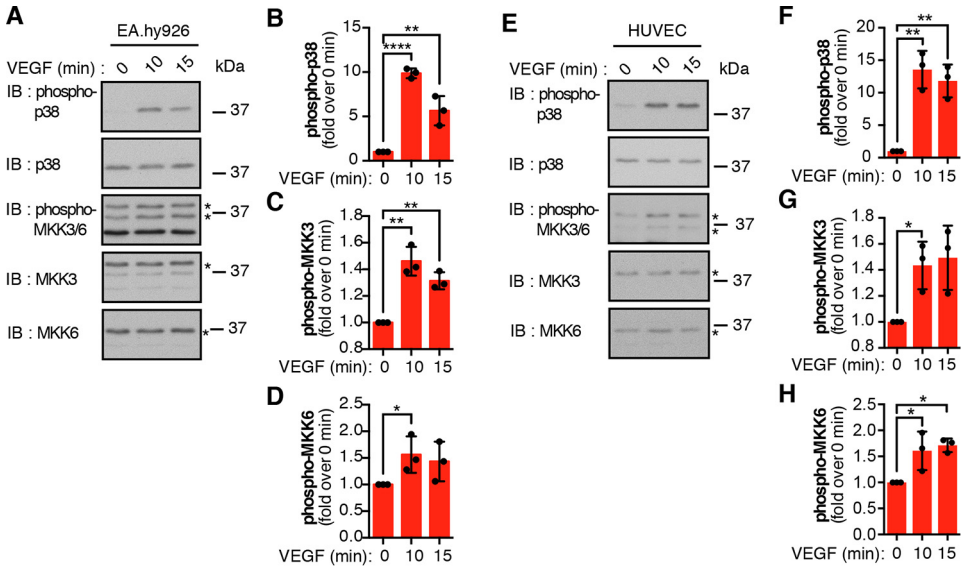

Figure 4. VEGF stimulates robust phosphorylation of p38, MKK3, and MKK6 in HUVEC and HUVEC-derived EA.hy926 endothelial cells. *A*, EA.hy926 cells, and *B*, primary HUVEC were stimulated with 50 μg/ml of VEGF for various times and lysed. Equivalent amounts of cell lysates were immunoblotted (*IB*) as indicated. Immunoblots are representative of three independent experiments, *asterisks* denote MKK3 or MKK6 bands. Quantification of VEGF induced phosphorylation of p38 (*B* and *F*), MKK3 (*C* and *G*), and MKK6 (*D* and *H*) was determined. The data (mean ± S.D., $n = 3$) are expressed as the fold over 0 min and analyzed by Student's *t* test (*, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$).

cells (Fig. 5, *B–D*, *lanes 1–3 versus 4–6*). These findings suggest that p38 activation induced by multiple GPCR agonists occurs through autophosphorylation in EA.hy926 cells.

Next, we determined if GPCR agonists stimulate p38 autophosphorylation in primary HUVEC and HDMEC using the p38 inhibitor SB203580. HUVEC pretreated with SB203580 were stimulated with GPCR agonists for different times and the extent of p38 phosphorylation examined. Thrombin-induced a significant increase in p38 phosphorylation in control cells that was virtually abolished in SB203580-treated cells (Fig. 6*A*, *lanes 1–3 versus lanes 4–6*). Similar to HUVEC-derived EA.hy926 cells, histamine-, ADP-, and PGE_2-induced p38 phosphorylation was significantly inhibited in cells pretreated with the SB203580 p38 inhibitor (Fig. 6, *B–D*, *lanes 1–3 versus* 4–6). However, p38 phosphorylation induced by hyperosmolar NaCl (33) was not altered by SB203580 pretreatment in primary HUVEC (Fig. 6*E*, *lanes 1–3 versus 4–6*). These data are consistent with previous results in endothelial EA.hy926 cells that demonstrated NaCl-stimulated phosphorylation of p38, MKK3, and MKK6 is insensitive to SB203580 treatment (8).

Primary HDMEC were next used to assess the impact of SB203580 on GPCR agonists on p38 autophosphorylation. However, unlike HUVEC, the subtypes of GPCRs that mediate responses to thrombin, histamine, ADP, and PGE_2 in HDMECs are less characterized but likely to be similar (34–36). In contrast to HUVEC-derived EA.hy926 and primary HUVEC, pretreatment of HDMEC with SB203580 caused a significant decrease in basal p38 phosphorylation compared with control cells (Fig. 7, *A–D*, *lanes 1* and *4*), suggesting that detectable basal p38 phosphorylation in HDMEC is mediated predominantly by autophosphorylation. Despite a reduction in basal p38 phosphorylation, pretreatment with the SB203580 p38 inhibitor virtually abolished thrombin, histamine-, PGE_2-, and ADP-induced p38 phosphorylation compared with control treated HDMECs (Fig. 7, *A–D*, *lanes 1–3 versus 4–6*). Collec-

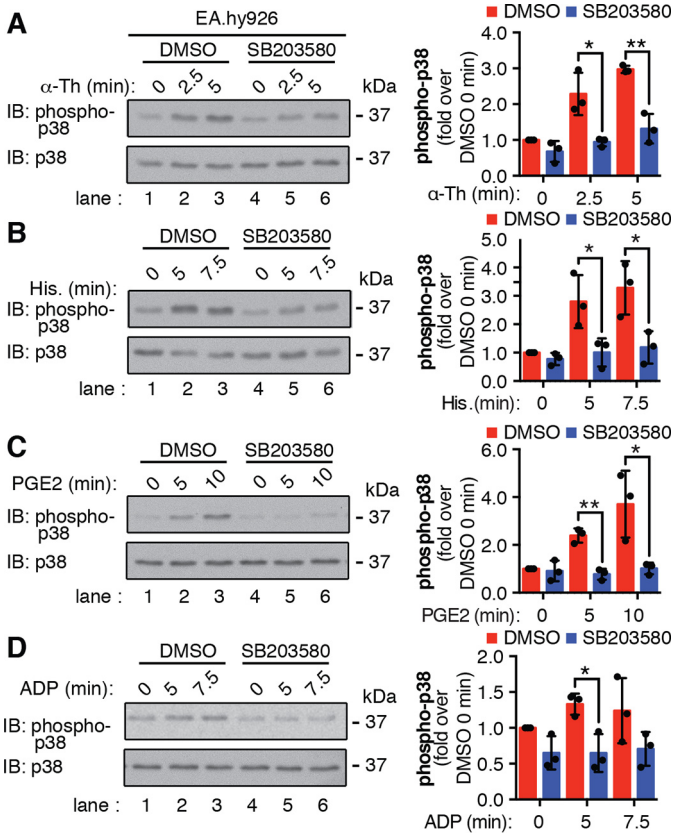

Figure 5. GPCR agonists stimulate p38 autophosphorylation in EA.hy926 endothelial cells. EA.hy926 cells pretreated with 1 μM SB203580 or DMSO vehicle control for 30 min were stimulated with (*A*) 10 nM α-thrombin (*α-Th*), (*B*) 10 μM histamine (*His.*), (*C*) 10 μM PGE_2, or (*D*) 10 μM ADP for various times and lysed. Equivalent amounts of cell lysates were immunoblotted (*IB*) as indicated and representative of three independent experiments. The data (mean ± S.D., $n = 3$) are expressed as the fold over control 0 min and analyzed by Student's *t* test (*, $p < 0.05$; **, $p < 0.01$).

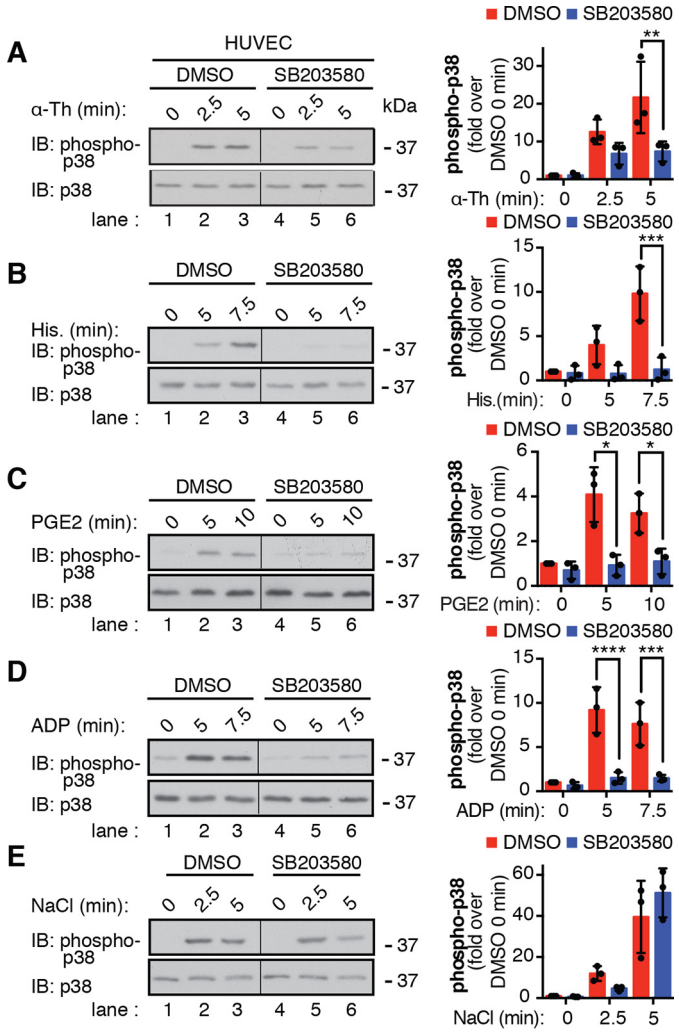

Figure 6. GPCR agonists stimulate p38 autophosphorylation in primary HUVEC. HUVEC pretreated with 1 μM SB203580 or DMSO vehicle for 30 min were stimulated with (*A*) 10 nM α-thrombin (α-*Th*), (*B*) 10 μM histamine (*His.*), (*C*) 10 μM PGE_2, (*D*) 10 μM ADP, or (*E*) 400 mM NaCl for the indicated times and lysed. Equivalent amounts of cell lysates were immunoblotted (*IB*) as indicated and representative immunoblots from three independent experiments are shown. The data (mean ± S.D., $n = 3$) are expressed as the fold over control 0 min and analyzed by Student's *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

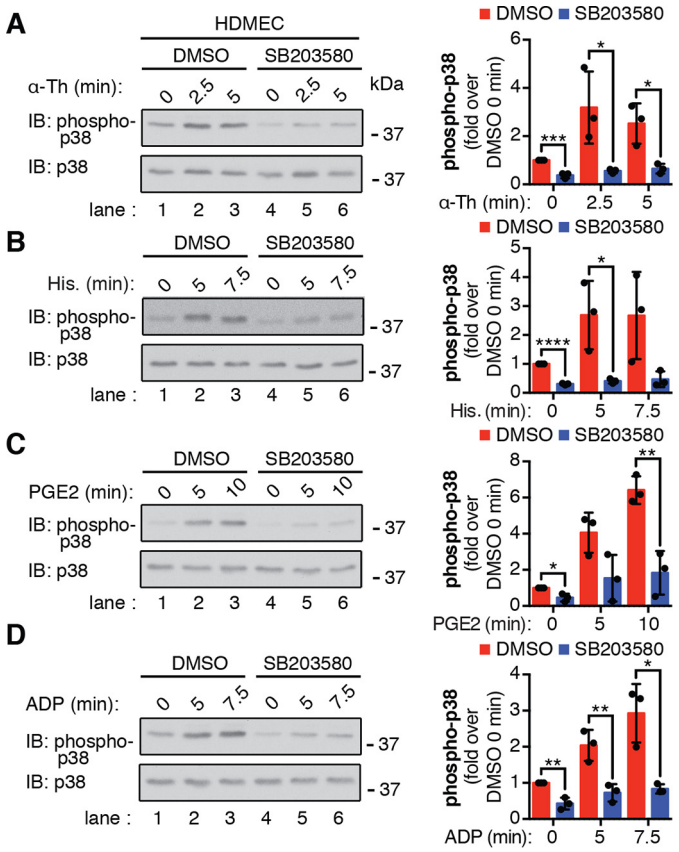

Figure 7. GPCR agonists stimulate p38 autophosphorylation in primary HDMEC. *A*, HDMEC were pretreated with 1 μM SB203580 or DMSO vehicle control for 30 min and then stimulated with (*A*) 10 nM α-thrombin (α-*Th*), (*B*) 10 μM histamine (*His.*), (*C*) 10 μM PGE_2, or (*D*) 10 μM ADP for the indicated times and lysed. Equivalent amounts of cell lysates were immunoblotted (*IB*) as indicated and immunoblots representative of three independent experiments are shown. The data (mean ± S.D., $n = 3$) are expressed as the fold over control 0 min and analyzed by Student's *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

tively, these data suggest that several GPCR agonists induce p38 activation via autophosphorylation independent of upstream MKK3 or MKK6 phosphorylation in multiple endothelial cell lines.

TAB1–TAB2 or TAB1–TAB3 are required for p38 activation induced by multiple GPCR agonists

TAB1 directly binds to p38α inducing autophosphorylation and activation (12). TAB2 associates with TAB1 (20), and TAB3 is a structurally related homolog of TAB2 that can also bind TAB1 (22). TAB1, TAB2, and TAB3 are all expressed in endothelial cells (Fig. 1), however, the relative contribution of TAB1–TAB2 *versus* TAB1–TAB3 to GPCR-induced p38 activation has not been previously determined. We showed that thrombin-stimulated p38 activation requires TAB1 and TAB2 in EA.hy926 cells but whether thrombin or other GPCRs agonists utilize TAB1 and TAB2 or TAB3 for activation of p38 in other endothelial cell types is not known. As expected, co-depletion of TAB1 and TAB2 by siRNA in EA.hy926 cells caused a significant ~40% inhibition of thrombin-induced p38 activation compared with nonspecific siRNA-transfected control cells (Fig. 8*A*, *lanes 1* and *4 versus 2* and *5*), whereas co-depletion of TAB1 and TAB3 by siRNA failed to significantly affect thrombin-induced p38 activation compared with nonspecific siRNA control (Fig. 8*A*, *lanes 1* and *4 versus 3* and *6*). Next, we examined the role of TAB1–TAB2 *versus* TAB1–TAB3 in histamine-induced p38 activation in EA.hy926 cells. Co-depletion of TAB1 and TAB2 by siRNA caused a modest but significant ~35% reduction in p38 activation stimulated by histamine (Fig. 8*B*, *lanes 1* and *4 versus 2* and *5*). Although siRNA-targeted depletion of TAB1 and TAB3 caused an apparent significant reduction in p38 activation, the data were variable and not significant (Fig. 8*B*, *lanes 1* and *4 versus 3* and *6*). These data suggest that multiple GPCR agonists utilize TAB1 and TAB2 to promote non-canonical p38 activation in EA.hy926 cells.

To determine whether thrombin and histamine induced p38 activation via the non-canonical TAB1– dependent pathway in primary endothelial cells we examined HUVECs and HDMECs. In TAB1- and TAB2-deficient HUVEC, thrombin-induced p38 activation was reduced significantly by ~70% compared with

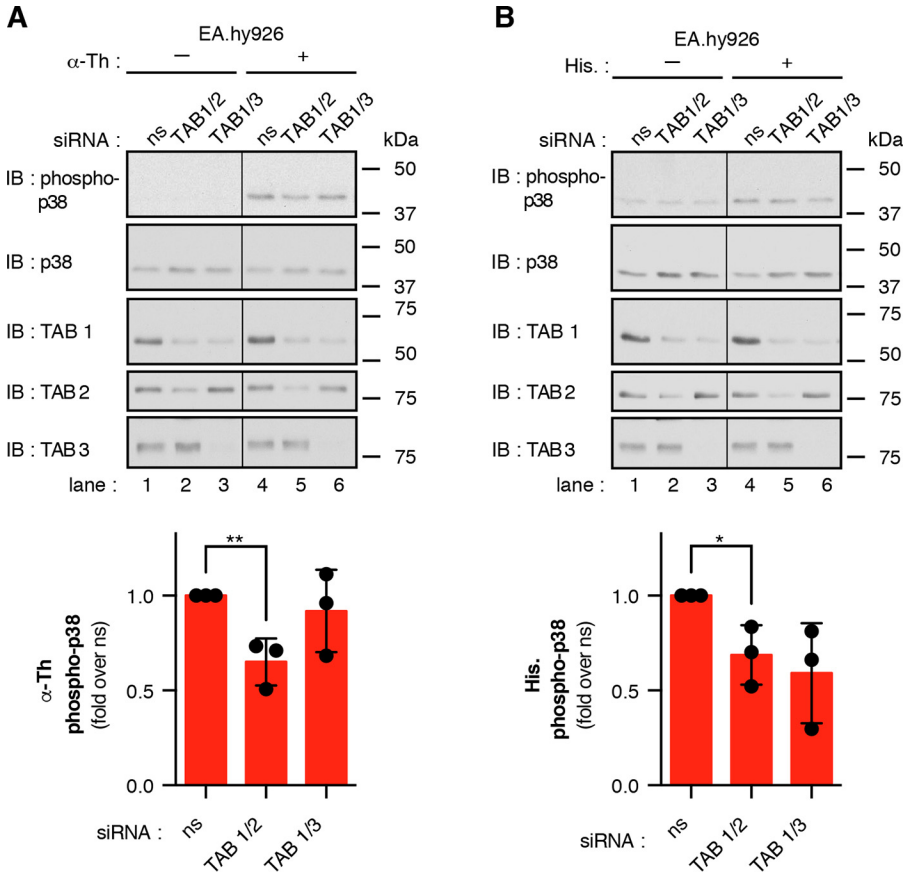

Figure 8. TAB1 and TAB2, but not TAB3 are required for p38 activation induced by a subset of GPCR agonists in EA.hy926 endothelial cells. EA.hy926 cells transfected with nonspecific (*ns*), TAB1 and TAB2 (*TAB1/2*), or TAB1 and TAB3 (*TAB1/3*) siRNAs for 120 h were stimulated with (*A*) 10 nM α-thrombin (*α-Th*) for 5 min or (*B*) 1 μM histamine (*His.*) for 7.5 min and lysed. Equivalent amounts of cell lysates were immunoblotted (*IB*) as indicated and immunoblots representative of three independent experiments are shown. The data (mean ± S.D., $n = 3$) are expressed as the fold over ns siRNA control and analyzed by Student's *t* test (*, $p < 0.05$; **, $p < 0.01$).

nonspecific siRNA-transfected control cells (Fig. 9*A*, *lanes 1* and *4 versus 2* and *5*). In contrast to EA.hy926 cells, co-depletion of TAB1 and TAB3 by siRNA in HUVEC also resulted in a significant ~55% reduction in thrombin-induced p38 activation compared with control cells (Fig. 9*A*, *lanes 1* and *4 versus 3* and *6*). Similar to thrombin, histamine-induced p38 activation was diminished significantly in both TAB1–TAB2 and TAB1–TAB3 co-depleted cells, ~50 and ~60% respectively, compared with control cells (Fig. 9*B*, *lanes 1* and *4 versus 2* and *5* and *3* and *6*), suggesting that TAB3 also makes important contributions to p38 activation induced by GPCRs in endothelial cells.

In contrast to HUVEC and HUVEC-derived EA.hy926 cells, thrombin-induced p38 activation required TAB1–TAB3 and not TAB1–TAB2 in HDMEC (Fig. 10*A*, *lanes 1* and *4 versus 2* and *5*). However, histamine-stimulated p38 activation depended on TAB1–TAB2 and not TAB1–TAB3 in HDMEC like that observed in HUVEC and HUVEC-derived EA.hy926 cells (Fig. 10, *lanes 1* and *4 versus 3* and *6*). Taken together these findings suggest that different GPCR agonists utilize either TAB1–TAB2 or TAB1–TAB3 proteins to stimulate non-canonical p38 activation in primary HDMECs.

Non-canonical TAB1–dependent p38 activation is required for IL-6 production induced by GPCR agonists in endothelial cells

IL-6 is a potent multifunctional cytokine secreted by endothelial cells following stimulation by various GPCRs agonists including thrombin and histamine (37, 38). However, the role and relevance of GPCR-stimulated TAB1–dependent p38 activation in IL-6 production has not been determined and was examined by measuring IL-6 production in HUVECs transfected with siRNAs targeting TAB1 and TAB2 or TAB1 and TAB3. Thrombin induced a ~2-fold increase in IL-6 production that was significantly reduced in endothelial cells co-depleted of TAB1–TAB3 (Fig. 11*A*), consistent with thrombin-induced TAB1–TAB3–dependent activation of p38 in HUVEC (Fig. 9*A*). Despite the apparent decrease in IL-6 production stimulated by thrombin in HUVECs co-depleted of TAB1–TAB2, the results were variable and not significant (Fig. 11*A*). In contrast, co-depletion of either TAB1–TAB2 or TAB1–TAB3 resulted in a marked and significant decrease in IL-6 production stimulated by histamine compared with nonspecific siRNA-transfected control cells (Fig. 11*A*). These findings are consistent with a role for both TAB1–TAB2 and TAB1–TAB3 in histamine-stimulated p38 activation in HUVEC (Fig. 9*B*). Together, these findings indicate that GPCR agonists induce p38 proinflammatory signaling in various endothelial cell types via a non-canonical TAB1–dependent pathway.

Discussion

Vascular endothelial dysfunction is induced by various inflammatory mediators, and many signal through activation of GPCRs. In previous work, we showed that thrombin induced

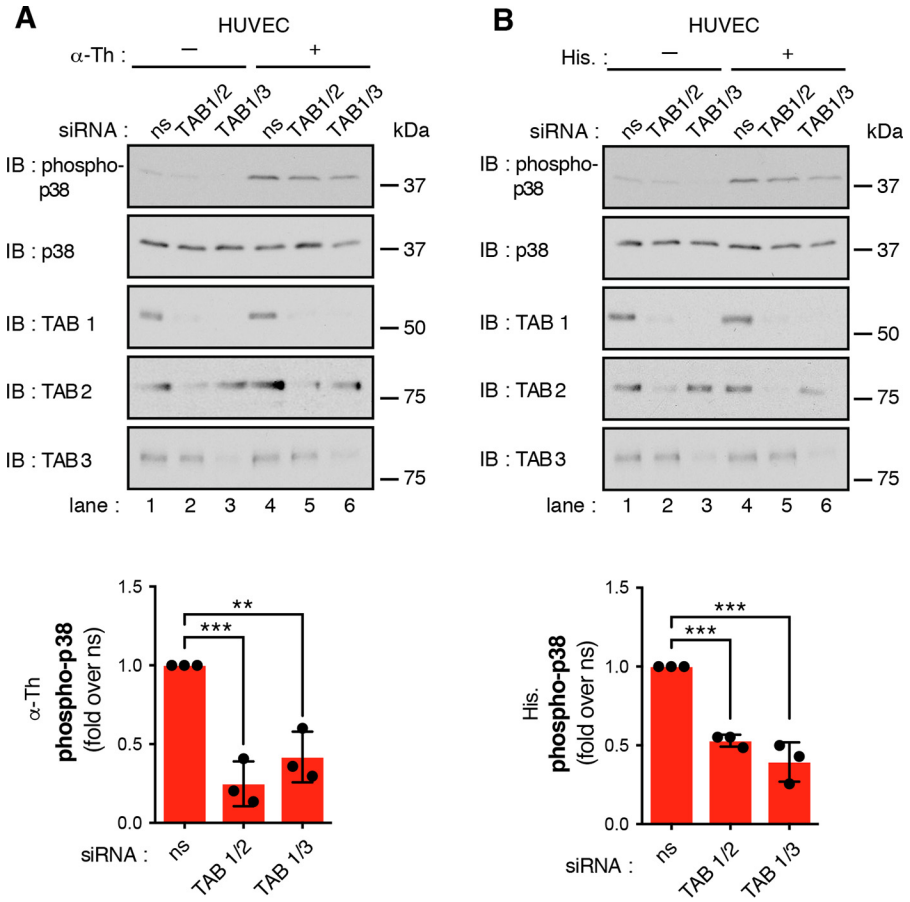

Figure 9. TAB1–TAB2 and TAB1–TAB3 are both required for p38 activation induced by thrombin and histamine in primary HUVEC. HUVEC transfected with nonspecific (*ns*), TAB1 and TAB2 (*TAB1/2*), or TAB1 and TAB3 (*TAB1/3*) siRNAs for 120 h were stimulated with (*A*) 10 nM α-thrombin (*α-Th*) for 5 min or (*B*) 1 μM histamine (*His.*) for 7.5 min and lysed. Equivalent amounts of cell lysates were immunoblotted (*IB*) as indicated and immunoblots shown are representative of three independent experiments. The data (mean ± S.D., $n = 3$) are expressed as the fold over nonspecific siRNA control and analyzed by Student's *t* test (**, $p < 0.01$; ***, $p < 0.001$).

endothelial barrier disruption, a hallmark of inflammation, through non-canonical TAB1–dependent p38 autophosphorylation and activation (8). Ubiquitination of PAR1 was also shown to be critical for initiating the recruitment of TAB2, TAB1, and p38 activation in HUVEC-derived EA.hy926 cells. The $P2Y_1$ receptor was further shown to activate p38 signaling through a ubiquitin–dependent non-canonical TAB1–dependent pathway in HeLa cells (8). However, it is not known if other GPCR agonists promote non-canonical p38 activation and inflammatory signaling in endothelial cells derived from different vascular beds. Here, we report that multiple GPCR agonists induce p38 autophosphorylation and activation via a TAB1–TAB2 and TAB1–TAB3 dependent pathway rather than the three-tiered kinase cascade to promote inflammatory responses in three distinct endothelial cell types.

MAPKs exist as distinct signaling cascades, comprised of three evolutionarily conserved, sequential acting kinases including a MAPK, MAP2K, and MAP3K. MAP3Ks are typically activated by phosphorylation mediated by upstream MAP2Ks. In contrast to canonical MAPK cascades, the p38α isoform can also be autoactivated through its interaction with TAB1 or through phosphorylation facilitated by the tyrosine kinase Zap70 (12, 17, 39). Although MKK3 and MKK6 are the major upstream MAP2Ks for p38α activation in response to cytokines or stress (40, 41), we found that multiple GPCR agonists failed to induce consistent and robust phosphorylation of MKK3 or MKK6 in distinct endothelial cell types compared with VEGF. We also previously showed the siRNA targeted depletion of MKK3 and MKK6 failed to inhibit thrombin-induced p38 activation (8), suggesting that upstream MAP2Ks are not the major pathway for GPCR-stimulated p38α activation. In addition, the p38α- and β-selective inhibitor SB203580 virtually abolished p38 phosphorylation induced by multiple GPCR agonists in endothelial cells, whereas hyperosmolar NaCl-induced p38 phosphorylation remained intact. These data indicate that the catalytic activity of p38 is important of activation induced by GPCRs. Finally, co-depletion of either TAB1–TAB2 or TAB1–TAB3 caused a significant loss of GPCR-induced p38 phosphorylation in primary endothelial cells including HUVECs and HDMECs, as well as HUVEC-derived EA.hy926 cells. Although activation of p38α by the three-tiered kinase cascade is the presumed major pathway for many inflammatory mediators, our data provide compelling evidence that TAB1-mediated p38 autoactivation is the predominate pathway utilized by mammalian GPCRs in human cultured endothelial cells and reveal a new paradigm by which GPCRs stimulate p38 MAPK inflammatory signaling.

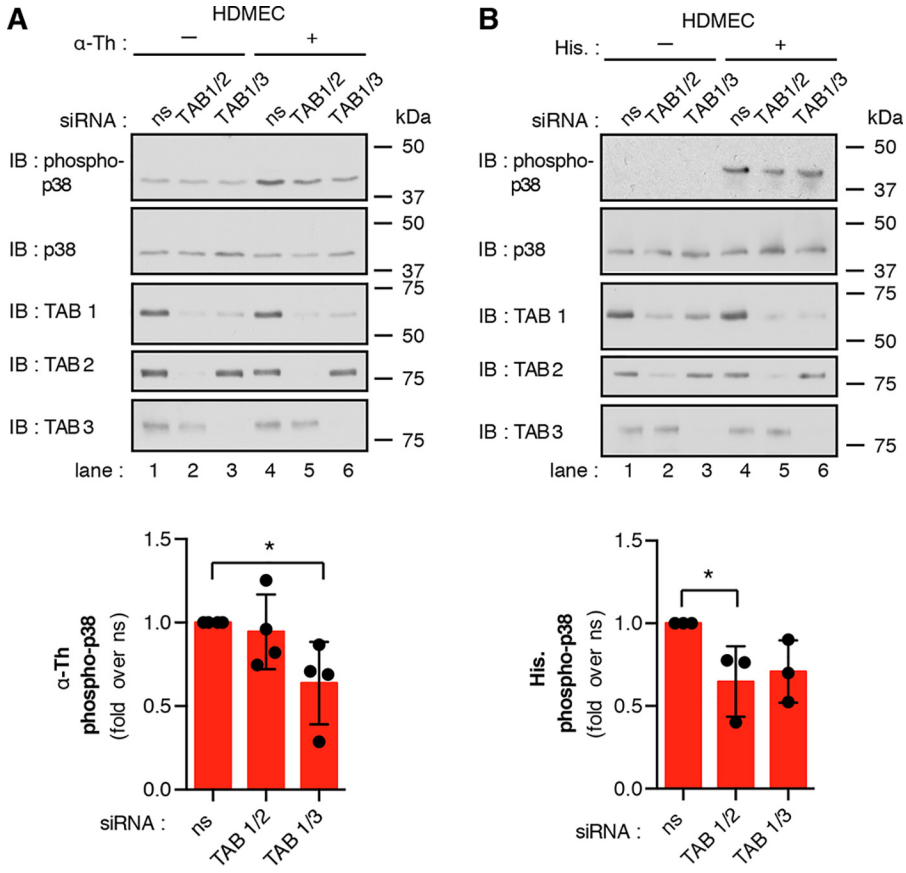

Figure 10. TAB1–TAB3 are required for thrombin-stimulated p38 activation, whereas TAB1–TAB2 are required for histamine-induced p38 activation in primary HDMEC. HDMEC transfected with nonspecific (*ns*), TAB1 and TAB2 (*TAB1/2*), or TAB1 and TAB3 (*TAB1/3*) siRNAs for 120 h were stimulated with (*A*) 10 nM α-thrombin (*α-Th*) for 5 min or (*B*) 1 μM histamine (*His.*) for 7.5 min and lysed. Equivalent amounts of cell lysates were immunoblotted (*IB*) as indicated and immunoblots shown are representative of three independent experiments. The data (mean ± S.D., $n = 3$) are expressed as the fold over nonspecific siRNA control and analyzed by Student's *t* test (*, $p < 0.05$; **, $p < 0.01$).

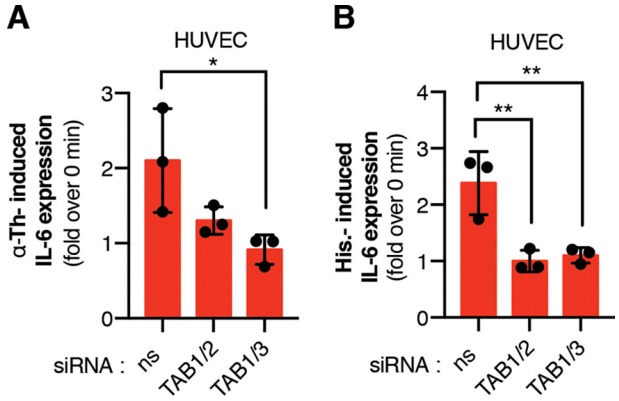

Figure 11. Thrombin-induced IL-6 expression requires TAB1–TAB3, whereas both TAB1–TAB2 and TAB1–TAB3 are required for histamine-induced IL-6 expression in primary HUVEC. HUVEC transfected with nonspecific (*ns*), TAB1 and TAB2 (*TAB1/2*), or TAB1 and TAB3 (*TAB1/3*) siRNAs for 120 h were stimulated with (*A*) 10 nM α-thrombin (*α-Th*) for 5 min or (*B*) 1 μM histamine (*His.*) for 7.5 min and lysed, and total RNA was isolated for qPCR as described above. The data (mean ± S.D., $n = 3$) are representative of three independent experiments expressed as the fold over 0 min control and analyzed by Student's *t* test (*, $p < 0.05$; **, $p < 0.01$).

We previously showed that agonist-induced ubiquitination of PAR1, the GPCR for thrombin, is required for the recruitment of TAB2 and p38 signaling in HUVEC-derived EA.hy926 cells (8). TAB2 is an adaptor protein that binds ubiquitin via its NZF domain, and associates with TAB1 (20, 21). TAB3 is a structurally-related homolog of TAB2, contains an NZF domain, and can also bind ubiquitin (22, 23). In addition to PAR1, the $P2Y_1$ receptor, a GPCR for ADP, also requires agonist-induced receptor ubiquitination and TAB1–TAB2 to stimulate p38 activation in HeLa cells (8). Here, we now show that ADP, histamine, and PGE_2 utilize a TAB1–TAB2 and TAB1–TAB3–dependent pathway for p38 activation. However, it is not known if the endothelial expressed $P2Y_1$, H_1 and H_2 histamine receptors, or the endothelial PGE_2 receptors (EP_2, EP_3, or EP_4) are modified with ubiquitin following agonist stimulation and if receptor ubiquitination is required for either TAB1–TAB2 or TAB1–TAB3–dependent p38 activation. It also remains to be determined if TAB3 is recruited to activated GPCRs via an ubiquitin–dependent process similar to TAB2 and associates with TAB1 to promote p38 activation and necessitates further exploration.

The PAR1 ubiquitin-driven TAB1-mediated mechanism for p38 MAPK activation illustrates a new function for ubiquitin in the regulation of GPCR biology. Our findings now indicate that ubiquitin-driven signaling is likely relevant for at least four different GPCRs from distinct receptor families expressed in endothelial cells. The covalent attachment of ubiquitin to GPCRs is mediated by the sequential actions of E1, E2, and E3 ubiquitin-conjugating enzymes. The neural precursor cell expressed, developmentally down-regulated-4 (NEDD4) family

of nine E3 ligases are primarily responsible for GPCR ubiquitination, currently reported for about 40 different GPCRs (42). However, in most cases it remains unclear how NEDD4 E3 ligase activity is regulated to facilitate GPCR ubiquitination. In recent work, we showed that thrombin activation of PAR1 stimulates c-Src-mediated tyrosine phosphorylation and activation of NEDD4-2 to promote p38 signaling and endothelial barrier disruption (43). Moreover, the $P2Y_1$ also required c-Src and NEDD4-2 tyrosine phosphorylation for p38 activation in endothelial cells (43). However, it remains to be determined whether other endothelial GPCR agonists utilize a conserved c-Src–dependent mechanism to control NEDD4 E3 ligase activity similarly to promote p38 MAPK-induced inflammatory responses in endothelial cells.

In summary, our findings indicate that multiple endothelial GPCR agonists stimulate p38 activation through a TAB1–dependent mechanism rather than the three-tiered kinase cascade in various endothelial cell types. However, the dependence on TAB1–TAB2 *versus* TAB1–TAB3 varies depending on the endothelial cell type and GPCR agonist. The underlying basis for the dependence on TAB1–TAB2 *versus* TAB1–TAB3 is not known and requires further investigation. In addition, the mechanisms by which GPCR-stimulated TAB1-induced p38 MAPK signaling controls various inflammatory responses including induction of cytokine production and endothelial barrier disruption remains poorly understood and warrants further exploration. Finally, *in vivo* studies have documented the relevance of TAB1–p38α activation in various disease settings including myocardial ischemia (13, 16, 44), indicating that TAB1–dependent p38 activation will be likely important for endothelial dysfunction *in vivo*. A recent paper further showed that disruption of TAB1–p38α interaction *in vivo* reduced myocardial ischemic injury (14), suggesting that the development of small molecules targeting the TAB1–p38α interface may provide a specific therapeutic intervention with limited side effects, not otherwise achievable with global p38 inhibition.

Experimental procedures

Reagents and antibodies

α-Thrombin was purchased from Enzyme Research Laboratories Inc. (South Bend, IN). Histamine dihydrochloride (number 3545) and PGE_2 were from Tocris Bio-Techne (Minneapolis, MN), ADP disodium (number 1617-48-6) was from Acros ThermoFisher Scientific (NJ), VEGF was from R&D Systems (Minneapolis, MN). GPCR agonist concentrations were used based on previously published studies: 10 nM α-thrombin (25), 10 μM histamine (28), 10 μM ADP (29), and 10 μM PGE_2 (30, 31). NaCl was used at 400 mM, equivalent to 200 mosmol/kg, shown to stimulate rapid p38 activation (33). The mouse anti-actin antibody (number A5316) was from Sigma. Rabbit anti-TAB1 (number 3226), TAB2 (number 3745), TAB3 (number 14241), p38 (number 8690), phospho-p38 (number 4511), MKK3 (number 5674), MKK6 (number 9264), and phospho-MKK3/MKK6 (number 9236) antibodies were from Cell Signaling Technologies (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit (number 170-6516) and goat anti-mouse (number 170-6515) antibodies were from Bio-Rad Laboratories. TransIT-X2 was purchased from Mirus Bio LLC (Madison, WI). SB203580 was purchased from LC Laboratories (number S340) (Woburn, MA). SYBR Green master mix (number A25741) and TRIzol (number 15596018) were purchased from Thermo Fisher Scientific, Direct-zol™ RNA MiniPrep Plus was purchased from Zymo Research (number R2072) (Irvine, CA), iScript™ gDNA Clear cDNA Synthesis kit purchased from Bio-Rad (number 1725034).

Cell lines

Human umbilical vein endothelial cell-derived EA.hy926 cells were grown and cultured as previously described (24). Primary HUVECs and HDMECs were purchased from Lonza (Basel, Switzerland), grown in human endothelial cell media or human microvascular endothelial cell media per the manufacturer's instructions, expanded and frozen at passage 2. Primary cells were used from these pools for all experiments up to passage 6.

Cell transfections

Cells were seeded on collagen-coated 24-well plates at 1.2×10^5 and grown overnight. Cells were then transfected with siRNA using TransIT-X2 (Mirus) per the manufacturer's instructions. The individual siRNAs used in the study were purchased from Qiagen (Germantown, MD) and include: non-specific (ns) siRNA (5′-CUACGUCCAGGAGCGCACC-3′), TAB1 (5′-CGGCUAUGAUGGCAACCGATT-3′), TAB2 (5-GUCAAUAGCCAGACCUUAATT-3′), and TAB3 (CGGUAUAGUACAAAUCCAATT-3′).

qPCR

The cDNA was generated from mRNA extracted from confluent cell cultures using Direct-zol™ RNA MiniPrep Plus (Zymo), cDNA synthesis was carried out using iScript™ gDNA Clear cDNA Synthesis kit (Bio-Rad). Reverse transcription-qPCRs were performed using iTAQ™ Universal SYBR® Green Supermix (Bio-Rad). The following gene-specific primers were used: TAB1 (forward 5′-TGGAAAGATCAAGCAGGTGG-3′, reverse 5′-GATTGGTTTGGACTTGGCAG-3′); TAB2 (forward 5′-GCGCCTGAAAAGATCAAATTCT-3′, reverse 5′-GGGTTAAAATGTGGTCCTCGG-3′); TAB3 (forward 5′-CTAGAGCGGTTGAAGTCTGAAG-3′, reverse 5′-TCTGGAGTTGTCTGTTCATGC-3′); MKK3 (forward 5′-GTGGTAGAGAAGGTGCGG-3′, reverse 5′-TGTTGATGTCCAGGTCCATG-3′); MKK6 (forward 5′-CCAGACAATTCCAGAGGACATC-3′, reverse 5′-CACATCTTCACTTGACCGAGAG-3′); p38 (forward 5′-TGAAATGACAGGCTACGTGG-3′, reverse 5′-CTTCCAGTCAACAGCTCGG-3′); β-actin (forward 5′-CAAGCAGGAGTATGACGAGTC-3′, reverse 5′-GCCATGCCAATCTCATCTTG-3′); and IL-6 (forward 5′-GGAGACTTGCCTGGTGAAA-3′, reverse 5′-CTGGCTTGTTCCTCACTACTC-3′). The number of cycles until threshold (C_t) was determined using an Eppendorf Mastercycler® RealPlex2 (Hamburg, Germany). To normalize for variation in the total number of cells and the efficiency of the mRNA extraction, the C_t value for β-actin was subtracted from the C_t values for each target. The change in expression for each target

was then determined relative to cells transfected with nonspecific siRNA using the $\Delta\Delta C_T$ method. All experiments were performed in triplicate.

Immunoblotting

EA.hy926 endothelial cells were seeded into 24-well plates, transfected, and grown as described above. Primary HUVECs and HDMECs were seeded into wells pre-coated with 10 μg/cm of rat tail collagen, Type IV. In some experiments, cells were pretreated with 1 μM SB203580, a p38α and β selective inhibitor, for 30 min at 37 °C prior to agonist stimulation. Cells were then lysed, and protein concentrations were determined using a BCA protein assay (ThermoFisher Scientific), diluted in 1× Laemmli sample buffer containing 100 mM DTT and sonicated at 10% amplitude. Equivalent amounts of cell lysates were loaded and resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and incubated with specific antibodies as indicated. The range of protein concentrations loaded for the individual experiments varied between experiments and ranged from 6 to 10 μg. Membranes were developed by chemiluminescence and quantified by densitometry using ImageJ software.

Data analysis

Data were analyzed using Prism 7.0 software (GraphPad Software, La Jolla, CA) and statistical significance determined using Student's *t* test and one-way analysis of variance, as indicted.

Author contributions—N. J. G. and J. T. conceptualization; N. J. G., Y. L., R. N., C. C. R., and H. M.-P. formal analysis; N. J. G. and J. T. supervision; N. J. G., Y. L., R. N., and C. C. R. validation; N. J. G., Y. L., R. N., and H. M.-P. investigation; N. J. G., C. C. R., and J. T. methodology; N. J. G. and J. T. project administration; N. J. G. and J. T. writing-review and editing; Y. L., R. N., C. C. R., and H. M.-P. data curation; Y. L. visualization; J. T. resources; J. T. funding acquisition; J. T. writing-original draft.

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