Mitochondrial Reactive Oxygen Species Mediate GPCR-induced TACE/ADAM17-dependent Transforming Growth Factor- α Shedding

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Epidermal growth factor receptor (EGFR) activation by GPCRs regulates many important biological processes. ADAM metalloprotease activity has been implicated as a key step in transactivation, yet the regulatory mechanisms are not fully understood. Here, we investigate the regulation of transforming growth factor- α (TGF- α) shedding by reactive oxygen species (ROS) through the ATP-dependent activation of the P2Y family of GPCRs. We report that ATP stimulates TGF- α proteolysis with concomitant EGFR activation and that this process requires TACE/ADAM17 activity in both murine fibroblasts and CHO cells. ATP-induced TGF- α shedding required calcium and was independent of Src family kinases and PKC and MAPK signaling. Moreover, ATP-induced TGF- α shedding was completely inhibited by scavengers of ROS, whereas calcium-stimulated shedding was partially inhibited by ROS scavenging. Hydrogen peroxide restored TGF- α shedding after calcium chelation. Importantly, we also found that ATP-induced shedding was independent of the cytoplasmic NADPH oxidase complex. Instead, mitochondrial ROS production increased in response to ATP and mitochondrial oxidative complex activity was required to activate TACE-dependent shedding. These results reveal an essential role for mitochondrial ROS in regulating GPCR-induced growth factor shedding.

INTRODUCTION

Epidermal growth factor receptor (EGFR) has long been recognized as a critical component of cellular signal transduction machinery (Holbro and Hynes, 2004). The discovery of the essential role of EGFR in propagating signals generated by G-protein-coupled receptor (GPCR) agonists indicates that EGFR may function as a central signal integration point for stimuli impacting the cell surface (Blobel, 2005; Ohtsu *et al.*, 2006). A wide variety of GPCR agonists, includ-

ing lysophosphatidic acid, phenylephrine, and carbachol, can harness EGFR to promote ERK activation, leading to physiological and pathophysiological responses (Yan *et al.*, 2002; Gschwind *et al.*, 2003; Schafer *et al.*, 2004b; Zhang *et al.*, 2004). GPCR cross-talk was previously thought to consist of entirely intracellular signaling pathways that led to EGFR phosphorylation, independent of ligand binding and dimerization. However, elucidation of a rapid, metalloprotease-dependent growth factor cleavage step leading to EGFR activation (Prenzel *et al.*, 1999) revealed the importance of regulated proteolysis in GPCR-EGFR transactivation. More recent studies have shown the *in vivo* importance of this phenomenon (Zhang *et al.*, 2004; Lautrette *et al.*, 2005).

The seven members of the EGF family of growth factors, amphiregulin (AR), betacellulin, EGF, epigen, epiregulin, heparin-binding EGF (HB-EGF), and transforming growth factor alpha (TGF- α), are all initially synthesized as type-I transmembrane precursors, containing the growth factor moiety in the ectodomain (Lee *et al.*, 2003). Several studies have demonstrated the biological activity of the noncleavable, membrane-anchored precursor molecules (Brachmann *et al.*, 1989; Wong *et al.*, 1989), but the metalloprotease dependence of transactivation, the loss of EGFR signaling in cells treated with metalloprotease inhibitors (Dong *et al.*, 1999; Borrell-Pages *et al.*, 2003), and the convergence of phenotypes of growth factor and protease knockout models indicate that proteolytic cleavage of the growth factors is an important and regulatable step in most contexts (Peschon *et*

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Abbreviations used: ADAM, a disintegrin and metalloprotease; AR, amphiregulin; BIM-I, bisindolylmaleimide I; DPI, diphenyleneiodonium; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GPCR, G-protein-coupled receptor; HB-EGF, heparin-binding growth factor; MAPK, mitogen-activated protein kinase; NAC, N-acetyl-L-cysteine; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RIA, radioimmunoassay; ROS, reactive oxygen species; TACE, tumor necrosis factor- α -converting enzyme; TGF- α , transforming growth factor- α .

al., 1998; Iwamoto *et al.*, 2003; Jackson *et al.*, 2003; Sternlicht *et al.*, 2005).

A variety of *in vitro* and *in vivo* evidence points to the ADAM family member tumor necrosis factor α -converting enzyme (TACE/ADAM17) as the critical convertase for TGF- α , AR, and HB-EGF. ADAMs (a disintegrin and metalloprotease), along with matrix metalloproteases, belong to the metzincin family of zinc-dependent proteases (Blobel, 2005). When mice lacking active TACE were compared with TGF- α , AR, and HB-EGF knockouts, they were found to share epithelial defects with homozygous TGF- α null mice (Peschon *et al.*, 1998), loss of mammary gland branching as in mice lacking AR (Sternlicht *et al.*, 2005), and heart and lung defects with HB-EGF $^{-/-}$ animals (Iwamoto *et al.*, 2003; Jackson *et al.*, 2003). Fibroblasts derived from the TACE-deficient mice were impaired in shedding of TGF- α , HB-EGF, and AR, but shedding could be rescued by transfection of wild-type TACE into the cells (Sunnarborg *et al.*, 2002). TACE also cleaved each growth factor *in vitro* at the physiologically relevant site (Sunnarborg *et al.*, 2002; Hinkle *et al.*, 2004). In cell culture, knockdown of TACE expression can also have an inhibitory effect on growth factor-dependent transactivation by lysophosphatidic acid, angiotensin II, and epoxyeicosatrienoic acid stimulation (Schäfer *et al.*, 2004b; Mifune *et al.*, 2005; Chen *et al.*, 2007). The overlapping phenotypes of mice lacking these growth factors and those lacking TACE/ADAM17, along with the *in vitro* results, support a critical role for the soluble forms of the growth factors and highlight the importance of their proteolysis as a regulatory event.

Despite its presence at a critical signaling juncture, the regulation of ADAM metalloprotease activity is still not fully understood. ADAMs are type I transmembrane proteins that possess an archetypal organization, including the metalloprotease and disintegrin domains, along with a cytoplasmic domain often rich in SH3-binding sites that could potentially regulate ADAM inside-out signaling (Seals and Courtneidge, 2003; Blobel, 2005). Several signaling components have been implicated in GPCR-initiated TACE activation. Src-like nonreceptor tyrosine kinases have long been accepted as intermediates in EGFR transactivation and have been found in association with several ADAMs leading to phosphorylation (Pierce *et al.*, 2001; Seals and Courtneidge, 2003; Luttrell and Luttrell, 2004; Zhang *et al.*, 2006). Elevation of intracellular calcium was also found to stimulate the release of ErbB ligands in an ADAM-dependent manner (Mifune *et al.*, 2005; Sanderson *et al.*, 2005; Horiuchi *et al.*, 2007), whereas protein kinase C (PKC) has long been suspected to play a role in ADAM activation because of the ability of phorbol 12-myristate 13-acetate (PMA) to trigger PKC signaling and to stimulate ectodomain shedding. Mitogen-activated protein kinase (MAPK) proteins have also been implicated both prior and subsequent to EGFR activation (Prenzel *et al.*, 1999; Umata *et al.*, 2001; Gschwind *et al.*, 2003; Fischer *et al.*, 2004). Lastly, reactive oxygen species (ROS) can function as second messengers in response to G-protein signaling (Zhang *et al.*, 2001; Cross and Templeton, 2006). ROS have been shown to be necessary for angiotensin II- and endothelin-1-mediated TACE activation and HB-EGF shedding, possibly through direct modification of a cysteine that coordinates the binding of the inhibitory prodomain (Zhang *et al.*, 2001; Mifune *et al.*, 2005; Chen *et al.*, 2006). The wide variety of possible regulatory mechanisms suggests substrate- or cell type-specific pathways dependent on the stimulant or intended signal effect.

In this study we characterize a ROS-mediated pathway wherein ATP initiates P2Y signaling, stimulating TACE-

dependent TGF- α proteolysis and EGFR phosphorylation. TGF- α shedding is independent of Src, PKC, and MAPK signaling. We report for the first time the requirement for mitochondrially derived ROS, whose production is stimulated by ATP, for regulation of GPCR-induced growth factor shedding. Collectively, these results offer a model that can be utilized to further our understanding of the regulatory mechanisms of TACE-dependent cell surface proteolysis.

MATERIALS AND METHODS

Materials

RC-20 anti-phosphotyrosine antibody, anti-Shc, and anti-flotillin were purchased from BD Transduction Laboratories (San Jose, CA) and anti-phosphotyrosine-EGFR 1068 antibody and anti-EGFR 2232 antibody from Cell Signaling Technology (Danvers, MA). Anti-human TGF- α (MF9) was purchased from Neomarkers (Fremont, CA) and anti-TACE from Exalpha Biologicals (Shirley, MA). Horseradish peroxidase-conjugated secondary antibodies and ATP γ S were from Roche Molecular Biochemicals (Indianapolis, IN). Adenosine and uridine triphosphate were purchased from GE Healthcare (Waukegan, WI). EGF was from Harlan Bioproducts (Indianapolis, IN) and TAPI-2 from Peptides International (Louisville, KY). *N*-Acetyl-L-cysteine, rotenone (NAC), myxothiazol, and AG1478 were from Sigma (St. Louis, MO) and all other chemicals were from Calbiochem (La Jolla, CA).

Cell Lines, Transfections, and Stable Clones

EC-4 (TACE $^{+/+}$) and EC-2 (TACE $\Delta Zn/\Delta Zn$) fibroblasts, provided by Amgen (Thousand Oaks, CA; Reddy *et al.*, 2000), and their derivatives were maintained in DMEM/F-12, 1% FBS with antibiotic. Wild-type and M2 Chinese hamster ovary (CHO) cells and their derivatives were maintained in DMEM, 10% FBS, 1 $\times$ nonessential amino acids and antibiotic. HL-60 cells were maintained in RPMI media, 10% FBS with antibiotic. Plasmid transfections were performed using LipofectAMINE (Invitrogen, Carlsbad, CA). The human EGFR cDNA (Saxon and Lee, 1999) was subcloned into modified pCEP4 (Invitrogen) and stably expressed in all indicated cell lines after selection with 800 μ g/ml hygromycin B (Roche). Alkaline phosphatase-tagged TGF- α (Tokumaru *et al.*, 2000) was kindly sent by Dr. Carl Blobel (Hospital for Special Surgery, New York, NY). Stable expression of AP-TGF- α in wild-type CHO cells was achieved by selecting clones in 500 μ g/ml G418 (Sigma). For stable expression in M2 cells that had already been subjected to G418 selection, AP-TGF- α was subcloned into pcDNA 3.1 hygromycin B vector (Invitrogen) using a PCR-generated fragment (HindIII-capped forward PCR primer 5'-AAGCTTGTCTAGCGGCACCGC-3'; KpnI-capped reverse PCR primer 5'-GGTACCCGTTCTTACAGCAAAAGGC-3') and the TOPO cloning kit (Invitrogen). Stable clones were selected in 800 μ g/ml hygromycin B. For stable expression of pcDNA3 mouse TACE, transfected EC-2 and M2 CHO cells were selected in 100 μ g/ml Zeocin (Reddy *et al.*, 2000).

RNA Interference

Small interfering RNA (siRNA) duplexes against mouse TACE (5'-GGGAUUCUUUCAGCAUUCUUGUCCA-3') from Invitrogen were transiently transfected into EC-4 EGFR cells by adding 6 μ l RNAiMAX (Invitrogen) to 100 nM siRNA per 35-mm plate according to the manufacturer's instructions. A fluorescent-tagged scrambled sequence was used as a control. Cells were harvested 48 h after transfection as described below.

Harvesting, Immunoprecipitation, and Western Blot Analysis

Confluent cells were washed twice in serum-free medium (SFM) and then starved for 4 h at 37°C in SFM. After a 5-min stimulation, conditioned medium was collected, and cells were washed in phosphate-buffered saline (PBS) and lysed in 1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl with 10 μ g/ml leupeptin, 20 μ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 2 μ M sodium orthovanadate, 10 mM sodium fluoride, and 5 mM sodium molybdate (lysis buffer). Protein concentration was determined with a BCA assay kit (Pierce, Rockford, IL). For some experiments media were concentrated using Sep-Pak C-18 reverse-phase cartridges (Waters, Milford, MA; Sandgren *et al.*, 1990), and TGF- α in both lysates and media was measured with a specific radioimmunoassay (RIA; Russell *et al.*, 1993). For immunoprecipitations, equal amounts of protein were incubated for 60 min with primary antibody at 4°C followed by protein G agarose (Invitrogen) at 4°C for 1 h. Beads were washed in 20 mM Tris, pH 7.5, 0.1 M NaCl, 0.1 mM EDTA, and 0.1% Nonidet P-40, separated by SDS-PAGE, and transferred to Immobilon polyvinylidene difluoride (Millipore, Billerica, MA). Membranes were blocked in Tris-buffered saline, 0.1% Tween 20, and 3% BSA or 5% nonfat dry milk. Bands were visualized using Pierce SuperSignal West Pico chemiluminescent system for autoradiography.

Expression of P2Y Receptors by RT-PCR

RNA was extracted from wild-type EC-4 or CHO cells grown in 10-cm dishes using RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was isolated from fresh jejunum tissue with Trizol (Invitrogen) according to the manufacturer's protocol (Troyer *et al.*, 2001). Total RNA was used to prepare cDNA with MMTV Reverse Transcriptase (Invitrogen). PCR amplification used primers for mouse P2Y₂ or P2Y₄, corresponding to analogous regions to yield the same size products and species-specific primers for β -actin as a loading control. Primers for P2Y₂, 5'-ACGGTGTCTGCAAGCTGGTGC-3'; 5'-GTAGAGGGTGCGCGTGACGTGG-3'; P2Y₄, 5'-TACTACTATGCTGCCAGAAACCAC-3'; 5'-AGCAAAGACAGT-CAGACACAGC-3'; mouse β -actin, 5'-GACAACGGATCCGGCATGTG C-3'; 5'-GCGGCAGTGGCC ATCTCCTGC-3'; and hamster β -actin, 5'-ACTCCTACG-TGGGTGACGAG-3'; 5'-AAGGAAGGCTGGAAAAGAGC-3'.

Inositol Phosphate Incorporation

EC-4 cells were seeded into 24-well plates and cultured overnight. The next day, [³H]myo-inositol was added at 0.4 μ Ci/well and incubated overnight. Cells were stimulated for 15 min with vehicle, 100 μ M ATP or 100 μ M UTP as indicated, and [³H]inositol incorporation was assayed as previously described (Hoffmann *et al.*, 1999).

Alkaline Phosphatase Assay

Cells were plated in 24-well dishes at a density of 2.5×10^5 per well for 18 h. Cells were washed twice in SFM and starved in fresh SFM for 4 h at 37°C. Vehicle (DMSO or water) or inhibitors were added directly to cells for 30 min. After inhibition, media were changed to fresh SFM containing inhibitor and agonist for 5 min. Media were then collected and centrifuged for 30 min at $14,000 \times g$ at 4°C. Media aliquots were mixed with an equal volume of 2 $\times$ AP buffer (100 mM glycine, pH 10.4, 1 mM MgCl₂, 1 mM ZnCl₂, and 5.4 mM *p*-nitrophenylphosphate; Sahin *et al.*, 2006) and allowed to develop at room temperature. Absorbance at 405 nm was measured to quantify alkaline phosphatase (AP) activity. To test for possible interference in alkaline phosphatase activity by signaling inhibitors, conditioned media from 150-mm dishes of vehicle or 100 μ M ATP-stimulated CHO-APT cells were divided after harvesting from cells for treatment with the appropriate concentration of each reagent.

Exosome Purification

Exosomes were purified from conditioned media by differential centrifugation, as previously described (Thery *et al.*, 2006). In short, 8×10^7 cells were serum-starved for 4 h and then pretreated with vehicle or 20 μ M TAPI-2 for 30 min. In fresh SFM, cells were stimulated with vehicle or 100 μ M ATP for 5 min, and the media were collected and pooled. To remove live, whole cells the media were subjected to centrifugation at $300 \times g$ for 10 min. An aliquot of supernatant along with the entire pellet, which was collected in lysis buffer, were stored at -80°C. The remaining supernatant was centrifuged again at $2000 \times g$ for 10 min to separate dead cells and $10,000 \times g$ for 30 min to remove cell debris. Aliquots were again collected for analysis. Finally, exosomes were pelleted by centrifugation at $100,000 \times g$ for 60 min. The lysed pellets were prepared for Western blot analysis, and the media aliquots were split and prepared for Western blot analysis and AP assays.

Cell Viability

An equal number of cells was plated in duplicate, 24 h before treatment for each condition. Cells were then serum-starved for 4 h before treatment with the appropriate concentration of reagent for 30 min. After that, cells were washed twice in PBS and then trypsinized and collected. Viable cells were counted on a hemacytometer, and total cells per plate were compared with vehicle-treated cells to determine the effect of each reagent on cell number.

Measurement of Membrane NADPH Activity

CHO or HL-60 cells (1.5×10^6) were stimulated with vehicle, 100 μ M ATP, or 20 μ M PMA for 5 min at 37°C. Media were removed, and cells washed twice in ice-cold PBS. Intact cells were collected from the plates in PBS. Plasma membranes were isolated using the Membrane Protein Extraction Kit as directed by manufacturer (BioVision, Mountain View, CA). Protein concentrations were determined, and 2 μ g of protein was used to measure SOD inhibitable superoxide production by cytochrome C reduction in vitro (Teufelhofer *et al.*, 2003; Dikalov *et al.*, 2007). Briefly, 100 μ M cytochrome C and 100 μ M NADPH with or without 100 U/ml SOD were prepared in HBSS and mixed with plasma membrane proteins in an equal volume in a 96-well plate and incubated at 37°C for the length of the assay. Triplicate samples were assayed. Absorbance at 540, 550, and 560 nm was measured in a SpectraMAX platereader (Molecular Devices, Sunnyvale, CA). The difference in absorbance of 550 nm and the average of 540 and 560 nm was calculated for each well. The average background absorbance (reaction solution without protein) was then subtracted and the difference in SOD-treated and untreated samples was determined. This value was then converted to picomoles of reduced cytochrome C using the extinction coefficient $21.1 \text{ mM}^{-1} \text{ cm}^{-1}$.

Measurement of Mitochondrial ROS

Mouse cells were plated in 24-well dishes at a density of 2.5×10^5 /well and cultured for 18 h. Cells were washed twice in SFM and starved in fresh SFM for 4 h at 37°C. Cells were loaded with 250 nM CM-H₂XROS MitoTracker Red (MTR; Molecular Probes, Eugene, OR) for 15 min at 37°C away from direct light. Media were aspirated, and cells were washed twice in phenol-free, SFM supplemented with 2 mM L-glutamine and left in washing media. Cells were then stimulated for 5 min and excited at 544 nm, and emission was detected at 612 nm on the fluorescent platereader SpectraMax Gemini XS (Molecular Devices). Data are presented as a box-and-whisker plot to exhibit the full range of data points.

Statistical Analysis

The nonparametric Wilcoxon rank-sum test was used for two-group comparisons. $p < 0.05$ was considered statistically significant. Statistical analyses were performed using Prism 5.0 from GraphPad Software (San Diego, CA) and SAS statistical software (version 9.1, SAS Institute, Cary, NC).

RESULTS

To study the regulation of growth factor cleavage induced by GPCR stimulation, we chose TACE/ADAM17 cleavage of proTGF- α as a model system. Stimulation of EC-4–transformed fibroblasts that stably express EGFR (EC-4 EGFR; Figure 1A, bottom panel) with the purinergic receptor agonist ATP led to the phosphorylation of EGFR, as did treatment with exogenous EGF (Figure 1A). ATP transactivation was also able to stimulate phosphorylation of Shc, an adaptor molecule for EGFR (Figure 1B). ATP-induced Shc phosphorylation was dependent on EGFR activity as the EGFR-kinase inhibitor AG1478 was able to block both ATP- and EGF-stimulated Shc activation. Therefore, these cells possess the endogenous components to accomplish GPCR-induced EGFR transactivation and downstream signaling.

Because EC-4 cells constitutively release low levels of TGF- α (Sunnarborg *et al.*, 2002) and fibroblasts often express P2Y receptors, we asked if ATP stimulation would lead to rapid release of soluble TGF- α . ATP stimulates P2Y₂ purinergic receptors with an EC₅₀ of $\sim 25 \mu$ M and the maximal response at 100 μ M (Soltoff *et al.*, 1998). Using a highly specific, TGF- α RIA (Russell *et al.*, 1993), we detected a greater than twofold increase in endogenous TGF- α shedding 5 min after addition of 100 μ M ATP (Figure 1C). The effect of ATP was not dependent on hydrolysis, as indicated by comparable results with the noncleavable ATP analog, ATP γ S. These results indicate that GPCR stimulation via purinergic receptors leads to rapid release of soluble TGF- α , and EGFR transactivation.

We also wanted to identify the purinergic receptor subtype responsible for this shedding event. Purinergic receptors are classified on the basis of their agonist profile. ATP can stimulate members of the P2X and P2Y family of receptors. UTP, in contrast, stimulates only P2Y receptors, and murine P2Y₂ and P2Y₄ receptors are equipotently activated by ATP and UTP (Abbracchio *et al.*, 2006). In mouse EC-4 cells, as shown in Figure 1C, UTP stimulates TGF- α shedding at levels similar to that of ATP, indicating use of P2Y receptors. ATP and UTP also stimulated comparable [³H]inositol incorporation in these cells (Supplemental Figure S1). Semiquantitative reverse transcriptase-PCR on RNA from EC-4 mouse fibroblasts revealed expression of only the P2Y₂ receptor subtype. P2Y₄ receptor was not visible at this low cycle number but was visible in the positive control jejunum tissue sample (Figure 1D). These results, together with the ability of ATP and UTP to stimulate GPCR signaling and TGF- α shedding, identify P2Y₂ as the critical receptor.

The paradigm for GPCR-induced EGFR transactivation requires a metalloprotease-dependent growth factor cleavage step (Prenzel *et al.*, 1999). We verified the dependence on

Figure 1. GPCR stimulation induces EGFR transactivation and TGF- α shedding. Cells were serum-starved for 4 h. Results are representative of three separate experiments. (A) Cells were stimulated with vehicle, 20 ng/ml EGF, or 100 μ M ATP for 5 min. Phosphorylated EGFR (top) was detected in cell lysates by immunoblotting followed by stripping and detection of total EGFR (bottom). (B) EC-4 EGFR cells were inhibited with vehicle or 20 μ M AG1478 for 30 min and then stimulated with vehicle, 20 ng/ml EGF, or 100 μ M ATP for 5 min. Cell lysates were subjected to anti-Shc immunoprecipitation followed by immunoblotting for phosphotyrosine (top, RC-20) and total Shc protein (bottom). (C) EC-4 cells were stimulated for 5 min with vehicle, 100 μ M ATP, 100 μ M ATP γ S, or 100 μ M UTP. Media and lysates were harvested and concentrated as described in *Materials and Methods* and analyzed for TGF- α content

metalloprotease activity in this model system by treating cells with the hydroxamate compound TAPI-2. In the presence of this inhibitor, ATP transactivation of EGFR was abolished, whereas EGF still activated the receptor (Figure 2A). Therefore, purinergic stimulation of EGFR requires metalloprotease activity.

In astrocytoma cells, ATP stimulated the release of amyloid precursor protein through P2Y₂ receptors by activating both ADAM10 and TACE (Camden *et al.*, 2005). In our system, ATP stimulated TGF- α release, and as proTGF- α is preferentially cleaved by TACE over ADAM10 (Le Gall *et al.*, 2009), we hypothesized that TACE was responsible for the ATP-induced TGF- α release and EGFR transactivation that we have observed. To test this hypothesis, we performed siRNA experiments to knock down endogenous TACE in EC-4 EGFR cells. In control transfected cells, both EGF and ATP stimulated phosphorylation of EGFR, whereas in the absence of TACE, ATP-dependent phosphorylation of EGFR was significantly decreased (Figure 2B). These results were confirmed in TACE-deficient EC-2 mouse fibroblasts, which are the complementary cells to wild-type EC-4 cells (Reddy *et al.*, 2000), stably expressing EGFR. Although EGF activated its receptor in wild-type and TACE-deficient cells, ATP was unable to stimulate transactivation of EGFR in the EC-2 cells compared with the wild-type EC-4 cells (Figure 2C). Similar to our previous observations for constitutive shedding (Sunnarborg *et al.*, 2002), induction of TGF- α release by treatment with 100 μ M ATP was abolished in the absence of TACE (Figure 2D). RIA measurements of unstimulated cell lysate indicated comparable TGF- α levels in EC-4 and EC-2 cells. We were also able to restore ATP-induced shedding in EC-2 cells by stably expressing mouse TACE (Figure 2E). Thus, we conclude that TACE is the crucial protease responsible for ATP-induced EGFR transactivation and TGF- α shedding in mouse fibroblasts, though TGF- α may not be exclusively responsible for ATP-induced EGFR activation in this system.

Further investigation into the regulation of TGF- α shedding required an assay system with high-throughput capabilities. Alkaline phosphatase-tagged EGF-like growth factors are an established method of monitoring growth factor cleavage by quantifying AP activity in conditioned media (Tokumaru *et al.*, 2000; Sahin *et al.*, 2004; Tanaka *et al.*, 2004; Horiuchi *et al.*, 2007). To facilitate transfection and culturing, we shifted our cell model to CHO cells, which have been used previously to study the regulation of growth factor shedding (Teixido *et al.*, 1990; Gechtman *et al.*, 1999). Although lacking endogenous EGF-family growth factors, CHO cells express TACE (Borrito *et al.*, 2003; Li and Fan, 2004) and have been reported to respond to purinergic stimulation (Iredale and Hill, 1993). RT-PCR of CHO RNA revealed strong expression of P2Y₂, although P2Y₄ was undetectable even with elevated cycle numbers (Figure 3A and data not shown). Thus, CHO cells should provide a suitable model for further study of GPCR-induced TGF- α shedding.

To test the cells and assay system, we prepared wild-type CHO clones that stably express AP-tagged TGF- α (AP-

by specific RIA. Data are presented as the average amount of TGF- α in conditioned media per milligram of total lysate protein from three separate experiments with at least three replicates. * $p < 0.02$ compared with the control. (D) Total RNA from EC-4 mouse fibroblast cells (Fb) or mouse jejunum tissue (Jej) was subjected to first strand cDNA synthesis with or without reverse transcriptase (RT) and amplified for 28 cycles to detect the expression of P2Y₂ (top) and P2Y₄ (middle) receptors. β -actin was used a loading control (bottom).

Figure 2. The metalloprotease TACE/ADAM17 is required for P2Y₂-stimulated TGF- α shedding and EGFR transactivation. (A) EC-4 EGFR cells were serum-starved for 4 h and pretreated with vehicle or 20 μ M TAPI-2 for 30 min as indicated. Cells were then stimulated with vehicle, 20 ng/ml EGF, or 100 μ M ATP for 5 min. Phosphorylated EGFR (top) was detected in cell lysates by immunoblotting followed by stripping and detection of total EGFR (bottom). Representative results of three separate experiments are shown. (B) EC-4 EGFR cells were transiently transfected with 100 nM siRNA targeting TACE or a scrambled control (Scr). Forty-eight hours after transfection, cells were serum-starved and harvested as described above. Phosphorylated EGFR (top) was detected in cell lysates by immunoblotting followed by stripping and detection of total EGFR (middle) and TACE expression (bottom). Representative results of three separate experiments are shown. (C) EC-4 EGFR or EC-2 (*Tace* Δ Zn/ Δ Zn) EGFR cells were serum-starved and harvested as previously described. Phosphorylated EGFR (top) was detected in cell lysates by immunoblotting followed by stripping and detection of total EGFR (bottom). Representative results of three separate experiments are shown. (D) EC-4 and EC-2 cells or (E) EC-2 and EC-2 cells stably expressing transfected mouse TACE (bottom) were serum-starved for 4 h and then stimulated for 5 min with vehicle or 100 μ M ATP. Media and lysates were harvested and concentrated as described in *Materials and Methods*, and analyzed for TGF- α content by specific RIA. Data are presented

as picograms of TGF- α in conditioned media per milligram of total lysate protein from three separate experiments with at least three replicates. **p* < 0.05 compared with the control; #*p* < 0.05 compared with ATP.

TGF- α) or empty vector (Tokumaru *et al.*, 2000). We next determined the effect of ATP on AP-TGF- α shedding. Analogous to our results in mouse cells, a 5-min stimulation with 100 μ M ATP caused a significant increase of AP activity in the media compared with mock stimulation (Figure 3B). Likewise, 100 μ M UTP was able to stimulate a strong TGF- α shedding response in CHO cells. Metalloprotease dependence was verified for ATP- and UTP-induced shedding by pretreatment of the cells with TAPI-2. When these cells were stimulated with water, ATP, or UTP, shedding was almost completely abrogated (Figure 3B). We next confirmed that

TACE was responsible for AP-TGF- α shedding in CHO cells by using CHO M2 cells that carry inactivating mutations in each endogenous TACE allele (Arribas and Massagué, 1995; Li and Fan, 2004). After ATP treatment, induction of AP activity was negligible in M2-AP-TGF- α cells stably expressing AP-TGF- α , compared with vehicle-treated cells (Figure 3C). When TACE was stably expressed in these cells, constitutive shedding remained low, whereas ATP-induced shedding was partially recovered. These results confirm the presence of a P2Y₂-TACE-TGF- α pathway in CHO cells.

Figure 3. CHO cells demonstrate P2Y₂-TACE-TGF- α shedding. (A) Total RNA from CHO cells was subjected to first strand cDNA synthesis with or without reverse transcriptase (RT) and amplified for 28 cycles to detect the expression of P2Y₂ (top) and P2Y₄ (middle) receptors. β -Actin was used as a loading control (bottom). (B) CHO AP-TGF- α cells were pretreated with vehicle or 20 μ M TAPI-2 for 30 min. Cells were then stimulated for 5 min with vehicle, 100 μ M ATP, or 100 μ M UTP. Media were collected and analyzed for alkaline phosphatase (AP) activity. (C) Mutant M2 CHO AP-TGF- α clones, which lack functional endogenous TACE, or TACE M2 CHO

AP-TGF- α clones, which stably express mouse TACE (bottom), were serum-starved for 4 h. Cells were then stimulated for 5 min with vehicle or 100 μ M ATP. Media were collected and analyzed for AP activity. AP assay results are an average of three separate experiments with at least three replicates and are presented relative to the unstimulated control. **p* < 0.05 compared with control; #*p* < 0.05 compared with uninhibited stimulation.

Figure 4. ATP-stimulated TGF- α shedding requires calcium and ROS. CHO AP-TGF- α cells were serum-starved for 4 h and pretreated with vehicle (DMSO) or the following concentration of inhibitors for 30 min: 50 μ M BAPTA-AM (BAP); 10 μ M PP2; 10 μ M PP3; 20 nM BIM-I; 10 μ M SB202190 (SB2); 10 μ M SB203580 (SB3); 20 μ M PD98059 (PD); and 5 mM NAC. After stimulation with vehicle or 100 μ M ATP for 5 min, media were collected and analyzed for alkaline phosphatase (AP) activity. AP activity is presented relative to the unstimulated control. * p < 0.05 compared with control; # p < 0.05 compared with uninhibited stimulation. All others not significant.

We wanted to confirm that the TGF- α and AP activity we detected in conditioned media resulted from proteolytic cleavage and not a protease-independent secretory event such as exosome release (Stoeck *et al.*, 2006). We therefore isolated exosomes from ATP-stimulated CHO AP-TGF- α -conditioned medium and examined TGF- α levels in this fraction. In ATP-treated cells, AP-TGF- α was detected in supernatants of conditioned media after depletion of each membrane fraction (Supplemental Figure S2A, top panel), including exosomes. The AP activity in conditioned media nonetheless remained strong in the membrane-depleted fractions (Supplemental Figure S2B). AP-TGF- α was also detected in each of the ATP-stimulated membrane fractions (Supplemental Figure S2A, middle panel) including in exosomes as identified by the marker, flotillin (Supplemental Figure S2A, bottom panel). TAPI-2 treatment was able to block release of AP-TGF- α in each case. A small loss of signal at each stage was observed, which likely corresponds to the membrane bound AP-TGF- α removed from the media by removal of the membrane fractions. These results demonstrate that after ATP stimulation the majority of TGF- α released from cells is cleaved and not secreted in membranes.

To determine potential regulatory steps of TACE-dependent ATP-induced TGF- α shedding, we used chemical inhibitors to ask which pathways contributed to the release of TGF- α after ATP stimulation in CHO AP-TGF- α cells. Calcium signaling has been implicated in shedding and EGFR transactivation (Mifune *et al.*, 2005; Horiuchi *et al.*, 2007) and is also a common signaling component of ATP-induced purinergic pathways (Abbraccio *et al.*, 2006); therefore, we wanted to test the effect of calcium on TGF- α shedding in our cell model. Treatment with the calcium specific chelator BAPTA-AM significantly inhibited ATP-dependent TGF- α shedding (Figure 4). We therefore tested whether calcium influx stimulated shedding with a nontoxic concentration of calcium ionophore A23187 (1 μ M, Supplemental Table S1), which led to a significant increase in shedding over control levels, comparable to ATP (Figure 5A). A23187-induced shedding was TACE-dependent, as shedding was not significantly induced in the TACE-deficient M2 AP-TGF- α CHO cells, while being restored by expression of mouse

Figure 5. Calcium regulates ATP-induced, TACE-dependent TGF- α shedding. (A) CHO AP-TGF- α cells were pretreated with vehicle or 50 μ M BAPTA-AM for 30 min. Cells were then stimulated for 5 min with vehicle, 100 μ M ATP, or 1 μ M A23187. (B) Mutant M2 CHO AP-TGF- α clones, which lack functional endogenous TACE, or TACE M2 CHO AP-TGF- α clones, which stably express mouse TACE (bottom panel), were stimulated for 5 min with vehicle or 1 μ M A23187. (C) CHO AP-TGF- α cells were pretreated with water or 500 μ M EGTA for 30 min. Cells were then stimulated for 5 min with vehicle, 100 μ M ATP, or 1 μ M A23187. (D) CHO AP-TGF- α cells were pretreated with water or 500 μ M EGTA for 30 min. Cells were then stimulated for 5 min with vehicle, 1 μ M A23187 or 30 μ M hydrogen peroxide (HP). Media for all experiments were collected and analyzed for alkaline phosphatase (AP) activity. Results are an average of three separate experiments with at least three replicates. AP activity is presented relative to the unstimulated control. * p < 0.05 compared with control; # p < 0.05 compared with uninhibited stimulation.

TACE in these cells (Figure 5B). Similar to ATP, A23187-induced shedding was partially inhibited by BAPTA-AM (Figure 5A). One explanation for the partial inhibition by BAPTA-AM is the possibility that a local influx of extracellular calcium may avoid BAPTA-AM chelation, thus allowing for ATP- or A23187-dependent shedding. To test for a role for extracellular calcium, we used the nonspecific chelator EGTA in aqueous solution to sequester extracellular calcium before stimulation. EGTA strongly inhibited TGF- α shedding by both ATP and A23187 (Figure 5C). Despite a high affinity for calcium, EGTA can also chelate other metal ions such as zinc that could interfere with TACE activity. To rule out this possibility, we used hydrogen peroxide as a calcium-independent stimulator of TGF- α shedding (Shao and Nadel, 2005). Hydrogen peroxide stimulated TGF- α shedding, but was not sensitive to EGTA pretreatment (Figure 5D). These results demonstrate that extracellular calcium is an important signaling component in TACE-mediated ATP-induced TGF- α shedding and indicate that hydrogen peroxide can stimulate TGF- α shedding independently of calcium.

Pathways involving the protein kinase C family and Src-family nonreceptor tyrosine kinases have also been implicated in regulating the shedding of a number of EGF family members, including GPCR-induced shedding (Arribas and Massagué, 1995; Izumi *et al.*, 1998; Pierce *et al.*, 2001; McCole *et al.*, 2002; Nagano *et al.*, 2004; Stoeck *et al.*, 2006). Involvement of PKC proteins can be tested with the inhibitor bisin-

dolylmaleimide-1 (BIM-1), whereas Src family members can be probed with the inhibitor PP2 and the nonfunctional structural homolog PP3. As shown in Figure 4, PP2 had an insignificant effect with results indistinguishable from PP3, indicating no specific contribution of Src and related nonreceptor tyrosine kinase pathways. Treatment with the broad spectrum PKC inhibitor BIM-1 also did not significantly inhibit ATP-stimulated TGF- α shedding. MAPK pathways have also been associated with growth factor shedding after stress or other stimuli (Fan and Derynck, 1999; Gechtman *et al.*, 1999; Umata *et al.*, 2001; Fischer *et al.*, 2004; Zhuang and Schnellmann, 2004). However, we observed no inhibition of TGF- α release when p38 MAPK signaling (SB202190; SB203580) or ERK activity (PD98059) was blocked with inhibitors for each pathway (Figure 4). These results were confirmed by RIA analysis in mouse EC-4 cells (data not shown). To rule out toxicity of inhibitors as a trivial explanation for our results, we tested each inhibitor's effect on cell viability at the concentration used in these experiments. We found that cell numbers remained the same as control-treated cells for all reagents (Supplemental Table S1). Moreover, these signaling inhibitors did not interfere with the ability of the TGF- α antibody to measure TGF- α by RIA (Supplemental Figure S3A), nor did they interfere with the ability to detect AP activity (Supplemental Figure S3B), though BAPTA-AM increased the level of AP activity in some experiments. Therefore, we have established that these reagents have no effect on cell viability or assay function, yet despite the demonstrated importance of these pathways, they are not required for ATP-stimulated TGF- α shedding.

Recent studies of ADAM regulation, and specifically TACE, have focused on the ability of ROS to act as signaling intermediates. Our results indicate that hydrogen peroxide could stimulate TGF- α shedding (Figure 5D), consistent with studies of TACE activation and shedding of both HB-EGF and TGF- α (Zhang *et al.*, 2001; Frank and Eguchi, 2003; Shao and Nadel, 2005). Cell-derived ROS have also been found as signaling intermediates downstream of the angiotensin II receptor and P2Y purinergic receptors (Sauer *et al.*, 2001; Mifune *et al.*, 2005; Pines *et al.*, 2005); thus we hypothesized that cellular ROS may also play a role in ATP-induced TGF- α shedding. We tested this by treating CHO AP-TGF- α cells with the ROS scavenger NAC. Shedding of TGF- α was dramatically reduced in the presence of NAC, pointing to a significant role for ROS in the regulation of TGF- α shedding (Figures 4 and 6A). The importance of cell-derived ROS was confirmed by testing endogenous TGF- α shedding in mouse fibroblasts, where NAC also significantly inhibited ATP-stimulated shedding (Supplemental Figure S4A). To determine if ROS-induced TGF- α shedding was metalloprotease-dependent, we used the inhibitor TAPI-2, which completely blocked hydrogen peroxide-induced TGF- α shedding (Figure 6B). The importance of TACE as a specific metalloprotease was demonstrated by the lack of shedding from M2-AP-TGF- α cells and the restoration of hydrogen peroxide-induced shedding with the expression of mouse TACE (Figure 6C). Thus, ROS signaling appears to be a key intermediate in TACE-mediated TGF- α shedding that is part of a conserved pathway following P2Y receptor activation.

Several sources of ROS exist within nonphagocytic cells, including the cytoplasmic NADPH oxidase, the mitochondrial electron transport chain, nitric oxide synthase, and several other oxidases (Aslan and Freeman, 2002). In most nonphagocytic cells the NADPH oxidase produces the majority of cytoplasmic superoxide, which is then rapidly dismutated into hydrogen peroxide (Brandes and Kreuzer, 2005). Several reports present evidence of a role for the

Figure 6. ROS mediate ATP-induced TGF- α shedding. (A) CHO AP-TGF- α cells were pretreated with vehicle or 5 mM NAC for 30 min. Cells were then stimulated for 5 min with vehicle or 100 μ M ATP. (B) CHO AP-TGF- α cells were pretreated with vehicle or 20 μ M TAPI-2 for 30 min. Cells were then stimulated for 5 min with vehicle, 100 μ M ATP, or 30 μ M hydrogen peroxide (HP). (C) Mutant M2 CHO AP-TGF- α clones, which lack functional endogenous TACE, or TACE M2 CHO AP-TGF- α clones, which stably express mouse TACE (bottom panel), were stimulated for 5 min with vehicle or 30 μ M HP. Media for all experiments were collected and analyzed for alkaline phosphatase activity. Results are an average of three separate experiments with at least three replicates. AP activity is presented relative to the unstimulated control. * $p < 0.05$ compared with control; # $p < 0.05$ compared with uninhibited stimulation.

NADPH oxidase in UV-induced (Singh *et al.*, 2009) and GPCR-induced transactivation of EGFR (Ushio-Fukai *et al.*, 1996; Lemjabbar *et al.*, 2003; Zhang *et al.*, 2004; Shao and Nadel, 2005; Stoeck *et al.*, 2006). To investigate a role for the NADPH oxidase in TGF- α shedding, we tested the effect of apocynin, which blocks formation of the complete NADPH oxidase complex, thus blocking ROS production by this source. CHO AP-TGF- α cells preincubated with 1 mM apocynin and then stimulated with water or ATP showed no effects on TGF- α shedding (Figure 7A). This might be expected since CHO cells have been reported to lack p22phox, a key component of the NADPH oxidase complex (Takeya *et al.*, 2003). To further confirm the inability of CHO cells to produce NADPH oxidase-dependent ROS, we measured the

Figure 7. ATP-induced TGF- α shedding is independent of NADPH oxidase activity. (A) CHO AP-TGF- α cells were pretreated with DMSO or 1 mM apocynin for 30 min. Cells were then stimulated for 5 min with vehicle or 100 μ M ATP. Media were collected and analyzed for alkaline phosphatase (AP) activity. Results are an average of three separate experiments with at least three replicates. AP activity is presented relative to the unstimulated control. Plasma membrane proteins from stimulated CHO (B) and HL-60 (C) cells incubated in cytochrome C reaction buffer were used to measure superoxide production by way of cytochrome C reduction as measured by changes in absorbance at 550 nm in the presence and absence of SOD at the indicated times. Results are an average of three independent experiments. * $p < 0.05$ compared with control.

production of superoxide from isolated membrane proteins of stimulated cells. CHO cells and HL-60 cells, which are human promyelocytic cells that can be stimulated by PMA to form an active NADPH oxidase complex, were stimulated with water, 100 μ M ATP, or 20 μ M PMA for 5 min. Membrane proteins were isolated from these cells and used to measure superoxide production through cytochrome C reduction. In CHO cells, neither ATP nor PMA treatment produced any superoxide, confirming that these cells lack the endogenous machinery to form an active NADPH oxidase complex (Figure 7B). In contrast, in control HL-60 cells, neither water nor ATP was able to stimulate superoxide production, while PMA-treated cells showed a steady increase over time (Figure 7C). Therefore the ROS involved in ATP-stimulated TGF- α shedding in CHO cells must come from another source. We also tested the involvement of the NADPH oxidase complex on endogenous shedding from EC-4 mouse fibroblasts. In these cells, apocynin also had no effect on ATP-stimulated TGF- α shedding at any concentration tested (Supplemental Figure S4B), indicating an NADPH-oxidase independent mechanism of ROS production following P2Y receptor stimulation.

The electron transport chain of the mitochondrion is another major source of cellular ROS. To determine if mitochondrially derived ROS contributes to regulation of TGF- α

Figure 8. Mitochondrially derived ROS are required for ATP-induced TGF- α shedding. (A) CHO AP-TGF- α cells were pretreated with DMSO or 1 μ M myxothiazol for 30 min. Cells were then stimulated for 5 min with vehicle or 100 μ M ATP. (B) CHO AP-TGF- α cells were pretreated with DMSO or 25 μ M rotenone for 30 min. Cells were then stimulated for 5 min with vehicle or 100 μ M ATP. (C) CHO AP-TGF- α cells were pretreated with DMSO, 5 mM NAC, or 1 μ M myxothiazol and 25 μ M rotenone for 30 min. Cells were then stimulated for 5 min with vehicle or 100 μ M ATP. (D) CHO cells were pretreated with vehicle or 1 μ M myxothiazol and 25 μ M rotenone for 30 min. During pretreatment, cells were also loaded with 250 nM MitoTracker Red and treated as described in *Materials and Methods*. Cells were stimulated with vehicle or 100 μ M ATP. Boxes represent the 25th, 50th, and 75th quartiles and error bars the minimum and maximum reading and are presented in this manner to more fully display all the data points. These data represent three separate experiments of at least four replicates relative to the unstimulated control. * $p < 0.03$ compared with the unstimulated control; # $p < 0.001$ compared with the stimulated control. (E) CHO AP-TGF- α cells were serum-starved for 4 h and pretreated with DMSO, 5 mM NAC, 1 μ M myxothiazol, 25 μ M rotenone, or 1 μ M myxothiazol and 25 μ M rotenone for 30 min. Cells were then stimulated for 5 min with vehicle or 1 μ M A23187. Media for all alkaline phosphatase (AP) assays were collected and analyzed for AP activity. All assays are an average of three separate experiments with at least three replicates and presented relative to the unstimulated control samples. * $p < 0.05$ compared with control; # $p < 0.05$ compared with uninhibited stimulation.

shedding, we examined two independent inhibitors of the electron transport chain: rotenone, which inhibits transfer of electrons from iron-sulfur centers to ubiquinone in complex I, and myxothiazol, which inhibits transfer of electrons between cytochrome b and cytochrome c_1 in complex III. Treatment with myxothiazol resulted in a consistent, significant decrease in shedding compared with ATP alone (Figure 8A). The complex I inhibitor rotenone blocked the majority of ATP-stimulated TGF- α shedding (Figure 8B). These inhibitors were individually unable to inhibit shedding to the same extent as the ROS scavenger NAC. However, when

used in tandem, rotenone and myxothiazol reduced shedding as effectively as NAC (Figure 8C). Confirming the relevance of this pathway in endogenous ATP-stimulated shedding of TGF- α , rotenone and myxothiazol in combination also inhibited shedding to the same level as NAC in mouse EC-4 cells (Supplemental Figure S4A). None of these inhibitors had any effect on cell viability or AP enzyme activity (Supplemental Table 1 and Supplemental Figure 3B), ruling out trivial effects on the assay system. These results strongly suggest that ROS produced in combination from mitochondrial complex I and complex III are an essential component of the regulatory pathway for GPCR-induced TGF- α shedding.

We therefore asked whether ATP stimulation increased the levels of mitochondrial ROS, by directly measuring ROS using the CM-H₂XRos MitoTracker Red. This fluorescent dye is specifically sequestered in mitochondria and remains nonfluorescent until oxidized. Cells stimulated with ATP for 5 min displayed a significant, rapid increase in fluorescence intensity over that of control cells (Figure 8D). This increase in ROS-dependent fluorescence was inhibited by pretreatment with the combination of mitochondrial inhibitors rotenone and myxothiazol. Taken together, these results indicate that ATP-stimulated signaling pathways can induce production of ROS from mitochondrial oxidative complexes, which is an essential step in regulating TACE-dependent TGF- α shedding.

Increases in calcium concentration due to ATP activation of purinergic receptors occur rapidly through G-protein coupling to calcium channels (Abbracchio *et al.*, 2006). Calcium is a known effector of mitochondrial function, including increasing production of ROS (Brookes *et al.*, 2004). This suggests that calcium signaling could be an early step in ATP activation of TACE-dependent shedding and could occur upstream of mitochondrial ROS production. This is supported by our results that show that calcium chelation by EGTA was unable to inhibit hydrogen peroxide-dependent TGF- α shedding (Figure 5D). To test whether calcium-induced TGF- α shedding was dependent on mitochondrial ROS we examined ROS inhibitor effects on A23187-stimulated shedding. The ROS scavenger NAC partially inhibited this shedding event (Figure 8E). Mitochondrial inhibitors myxothiazol and rotenone individually and together also partially inhibited A23187-induced shedding as did treatment with both inhibitors. These results further support our hypothesis that ATP-induced calcium mobilization contributes to increases in mitochondrial ROS and activation of TACE-dependent TGF- α shedding.

DISCUSSION

Transactivation of EGFR via GPCR signaling has emerged as an important event for signal integration and amplification in many biologically essential processes and typically requires a metalloprotease-mediated growth factor cleavage event (Blobel, 2005; Ohtsu *et al.*, 2006). This study examines a ROS-dependent transactivation pathway from the P2Y family of GPCRs that utilizes TACE/ADAM17 metalloprotease activity to cleave TGF- α , followed by activation of EGFR. Our goal was to begin to elucidate the signaling events leading to TACE-mediated growth factor cleavage after stimulation with GPCR agonists. We report here the first observation of mitochondrially produced ROS as a critical step in regulated growth factor proteolysis.

Extracellular nucleotides such as ATP are released from many cell types in response to stress and mechanical or biochemical stimulation (Abbracchio *et al.*, 2006) and can

function as a paracrine signal for the P2Y family of purinergic receptors. In this study we identified a P2Y-dependent pathway leading to TGF- α shedding and transactivation of EGFR. Prominent expression of P2Y₂ in mouse cells, along with the lack of detectable P2Y₄ expression in CHO cells, suggests a favored role for the P2Y₂ subtype in this transactivation pathway. P2Y₂ receptors have been previously linked to EGFR transactivation in PC12 cells and rat fibroblasts (Soltoff, 1998; Soltoff *et al.*, 1998), consistent with the ability of these receptors to stimulate proliferation of cells such as epidermal keratinocytes (Greig *et al.*, 2003) and smooth muscle cells (Wilden *et al.*, 1998). P2Y₂ receptors are also important for regulating ion channel function in epithelial cells (Abbracchio *et al.*, 2006). Activation of P2Y₂ can promote mucin release in airway cells, an event that has also been tied to TACE-dependent TGF- α shedding and EGFR transactivation (Yerxa *et al.*, 2002; Shao *et al.*, 2003). Extracellular release of ATP is also a hallmark of epithelial wound healing. In vitro wounding experiments utilize EGFR transactivation by purinergic receptors to initiate migration and healing of epithelial cells (Boucher *et al.*, 2007; Yin *et al.*, 2007). Thus, elucidation of the mechanisms responsible for regulating EGFR transactivation pathways could have important physiological and therapeutic implications.

A recent study showed that ATP stimulation through P2Y₂ receptors led to both ADAM10 and TACE metalloprotease activity toward amyloid precursor protein in astrocytoma cells (Camden *et al.*, 2005). Here, we show that ATP stimulated endogenous TACE-dependent TGF- α shedding in mouse fibroblasts and AP-TGF- α shedding in CHO cells, consistent with previous evidence for TACE as the major constitutive and PMA-stimulated TGF- α sheddase (Peschon *et al.*, 1998; Hinkle *et al.*, 2004; Sahin *et al.*, 2004; Horiuchi *et al.*, 2007). A variety of studies have implicated roles for other ADAM proteases in TGF- α processing under specific conditions (Merlos-Suarez *et al.*, 2001; Hinkle *et al.*, 2003; Schafer *et al.*, 2004b; Horiuchi *et al.*, 2007), including for ADAM10 in the absence of TACE (Le Gall *et al.*, 2009). Although ADAM10 activity may explain the minor residual shedding in both TACE-deficient EC-2 mouse and CHO cells, the significant loss of TGF- α release and of EGFR transactivation associated with TACE deficiency, or knockdown by siRNA, together with restoration of shedding by TACE in EC-2 and CHO M2 cells, indicates that TACE is the predominant TGF- α sheddase in this model.

There is a high degree of correlation between ATP-dependent TGF- α shedding and EGFR transactivation in the mouse fibroblast system that we tested. However, this does not exclude other EGFR ligands from involvement in ATP-induced receptor activation in this system. Other transactivation models have shown TACE-dependent cleavage of AR and HB-EGF to be important for EGFR phosphorylation as well (Hart *et al.*, 2004; Schafer *et al.*, 2004a). It is therefore possible that in the system we tested, additional EGF family members are also shed in response to ATP stimulation and this may contribute to the transactivation that we observed.

In this study, we undertook a further examination of the signaling pathways leading to TACE activity. ROS have recently gained attention as second messengers in GPCR signaling pathways (Forman *et al.*, 2002). P2Y family receptors have been specifically linked to ROS production in eosinophils and prostate tumor cells (Ferrari *et al.*, 2000; Sauer *et al.*, 2001). Cellular ROS have also been implicated in EGFR transactivation initiated via other GPCRs (Wang *et al.*, 2000; Zhang *et al.*, 2004; Stoeck *et al.*, 2006). We used hydrogen peroxide and the ROS scavenger NAC to establish a critical role for ROS in P2Y receptor-dependent TGF- α shed-

ding. These observations are also consistent with other reports that exogenous hydrogen peroxide can stimulate the shedding of TGF- α and other EGF family members such as HB-EGF, AR, and betacellulin (Lemjabbar *et al.*, 2003; Fischer *et al.*, 2004; Kim *et al.*, 2005; Shao and Nadel, 2005; Stoeck *et al.*, 2006).

The cytoplasmic NADPH oxidase complex has been implicated as the main source of cellular ROS in a number of EGFR transactivation pathways. A role for NADPH oxidase-derived ROS has been reported for GPCR agonists ATP, angiotensin II, phenylephrine, endothelin-1, and tobacco smoke (Ushio-Fukai *et al.*, 2001; Lemjabbar *et al.*, 2003; Schafer *et al.*, 2004a; Zhang *et al.*, 2004; Chen *et al.*, 2006; Stoeck *et al.*, 2006; Boots *et al.*, 2009), for UV-induced signaling (Singh *et al.*, 2009), and for the global activator of shedding, PMA (Shao and Nadel, 2005). In addition to inhibitor studies, a role for NADPH oxidase-derived ROS in EGFR transactivation was identified using induced phosphorylation of p47 (Pietri *et al.*, 2005), a key subunit of NADPH oxidase, and also the more stringent knockdown of the NADPH oxidase subunits Duox1 or p47^{phox} (Shao and Nadel, 2005; Chen *et al.*, 2006). ATP signaling through purinergic receptors has also been demonstrated to activate Duox1/Nox in airway epithelial cells (Wesley *et al.*, 2007; Boots *et al.*, 2009). We investigated the involvement of the NADPH oxidase complex in our system using the inhibitor apocynin, which directly blocks complex formation. We observed that at apocynin concentrations ranging from 30 μ M up to 1 mM, ATP-induced TGF- α shedding was unaffected. Furthermore, we found no evidence of an active NADPH oxidase complex in CHO cells, supporting previous findings in the literature (Takeya *et al.*, 2003). These results rule out the NADPH oxidase complex as a component of this pathway.

To further examine the source of ROS for regulation of P2Y-dependent TGF- α shedding, we used specific inhibitors of complex I and complex III of the mitochondrial electron transport chain to gauge the contributions of mitochondrial ROS. The inhibitors myxothiazol and rotenone partially decreased shedding when used individually, but when used together inhibited fully, as did the general ROS scavenger NAC, indicating that the functional ROS generated in this pathway can be entirely attributed to mitochondrial sources. In mouse cells, NAC and the combination of rotenone and myxothiazol also inhibited ATP-stimulated shedding to the same extent, indicating the requirement for mitochondrial ROS in this cell type as well. Mitochondrial ROS have been implicated in aging and cell fate, cell responses to hypoxia and many aspects of cardiovascular biology (Zhang and Guterman, 2007). Hydrogen peroxide-induced EGFR activation can depend on mitochondrial ROS (Chen *et al.*, 2004). Induction of mitochondrial ROS as a downstream signal of EGFR, estrogen, and NMDA receptors reveals a role for mitochondrial ROS as signaling messengers for important cellular receptors (Krieg *et al.*, 2004; Felty *et al.*, 2005; Hao *et al.*, 2006; Lee *et al.*, 2006; Duan *et al.*, 2007). Here, we present evidence for mitochondrial ROS as a signaling intermediate for regulation of P2Y-induced, TACE-dependent growth factor shedding. This novel observation of mitochondrial ROS in regulating shedding reveals another possible mechanism that cells may use to specify certain pathways for responding to various stimuli.

ROS-mediated TACE-dependent shedding may also involve other signaling molecules that have been implicated in EGFR transactivation. Increases in intracellular calcium concentrations are a well-characterized result of GPCR activation (Werry *et al.*, 2003), and this extracellular calcium influx can subsequently stimulate ROS-induced signaling (Sauer *et*

al., 2001; Ushio-Fukai *et al.*, 2001; Mifune *et al.*, 2005; Pines *et al.*, 2005; Watanabe *et al.*, 2006). In our CHO AP-TGF- α cells, TACE-dependent TGF- α shedding was stimulated by the ionophore A23187, and inhibited by calcium chelation with BAPTA-AM and EGTA. Calcium chelation had little to no effect on hydrogen peroxide-stimulated shedding, suggesting that the calcium signal preceded ROS production in our system. This was further supported by the blocking of A23187-induced TGF- α shedding with the mitochondrial inhibitors myxothiazol and rotenone. This may be explained by the previous observation in CHO-BQ1 cells that A23187 induced higher levels of intracellular calcium than ATP treatment (Marcet *et al.*, 2003). The calcium ionophore-induced residual shedding seen in M2 cells and in NAC-treated wild-type CHO cells may indicate a ROS- and TACE-independent shedding event involving ADAM10 such as that shown in mouse *Tace*^{-/-} fibroblasts (Horiuchi *et al.*, 2007; Le Gall *et al.*, 2009). Calcium signaling is a well-known effector of mitochondrial function and mitochondrial ROS production (Brookes *et al.*, 2004). Although the mechanism is still under investigation, the observations here demonstrate a role for calcium signaling upstream of required mitochondrial ROS.

PKC has been widely implicated in regulation of ectodomain shedding based on the well-established ability of phorbol esters to stimulate TACE-dependent TGF- α shedding (Fan *et al.*, 2003; Hinkle *et al.*, 2004; Sahin *et al.*, 2004; Horiuchi *et al.*, 2007). Previous reports implicated PKC isoforms in TACE phosphorylation and activation (Alfa Cisse *et al.*, 2008; Reddy *et al.*, 2008). In our study, treatment of cells with the broad spectrum PKC inhibitor BIM-1 (GF109203X) did not affect ATP-stimulated TGF- α release. Src family kinases have also been implicated in several GPCR-mediated transactivation cascades upstream of growth factor shedding, including the direct association of Src with TACE, that led to TACE phosphorylation and increased shedding of amphiregulin (Zhang *et al.*, 2006) or with P2Y₂ receptors via SH3 motifs (Liu *et al.*, 2004). There is also evidence suggesting ROS may modulate Src function in transactivation schemes (Ushio-Fukai *et al.*, 2001; Zhuang and Schnellmann, 2004). In contrast, Camden *et al.* (2005) demonstrated Src-independent activation of TACE and ADAM10 after ATP stimulation. We also show that ATP-dependent activation of TGF- α shedding is independent of the PP2-sensitive Src family members Src, Fyn, Hck, and Lck. Likewise ERK/MAPK pathways have been implicated in activation of TACE-dependent growth factor shedding by serum, stress, VEGF-A, ATP, and other stimuli (Fan and Derynck, 1999; Gechtman *et al.*, 1999; Umata *et al.*, 2001; Gschwind *et al.*, 2003; Fischer *et al.*, 2004; Zhuang and Schnellmann, 2004; Swendeman *et al.*, 2008; Yin and Yu, 2009) possibly by phosphorylation of the cytoplasmic domain of TACE (Soond *et al.*, 2005). We found no evidence for ERK/MAPK signals playing a regulatory role in TGF- α shedding in our model. This further underscores the selective use of available regulatory pathways for particular substrates or physiological events.

The mechanism of TACE activation remains unclear. However, the large number of agonists and substrates utilizing ROS strongly suggest ROS are common regulators of TACE activity. ROS may cause direct modifications of TACE in response to stimulation. The prodomain of TACE can bind to a cysteine in the metalloprotease active site to inhibit cleavage (Seals and Courtneidge, 2003; Li *et al.*, 2008). ROS have been shown to oxidize the cysteine thiol group thereby

relieving the inhibition of the metalloprotease activity (Zhang *et al.*, 2001). TACE also contains an extracellular cysteine-rich domain including two cysteine residues previously shown to be important in TACE functionality (Li and Fan, 2004) and specifically for hydrogen peroxide-induced, TACE-dependent L-selectin shedding in neutrophils (Wang *et al.*, 2009). These could be potential targets for ROS-dependent modifications. ROS may also indirectly activate TACE by alteration and modification of effector proteins. Demonstrated phosphorylation of TACE by interaction with ERK (Soond *et al.*, 2005), one of several PKC isoforms (Roghani *et al.*, 1999; Alfa Cisse *et al.*, 2008; Reddy *et al.*, 2008), or with phosphoinositide-dependent kinase-1 (Zhang *et al.*, 2006) could all be regulated by ROS modifications of these kinases or by the negative regulation of phosphatases. Modifications by ROS on scaffold proteins that are involved in substrate selection or presentation is another potential regulatory mechanism. The noncatalytic Eve-1 protein is thought to regulate EGF-like growth factor shedding in response to PMA (Tanaka *et al.*, 2004), whereas NRDC enhances shedding of the TACE substrates HB-EGF (Nishi *et al.*, 2006) and TNF- α (Hiraoka *et al.*, 2008). Most recently a tripartite complex between DLG1/SAP97, TACE and proTGF- α was identified, where both knockdown and overexpression of DLG1 led to inhibition of PMA-induced TGF- α shedding (Surena *et al.*, 2009). Lastly, TACE activity regulated by localization in membrane subdomains (Tellier *et al.*, 2006) could be affected by ROS modifications of lipids and membrane components (Zmijewski *et al.*, 2005). By altering the membrane and creating or abolishing subdomains, protein localization complexes and enzyme-substrate interactions could all be influenced. Thus, the ability of ROS to freely diffuse throughout the cell and their high reactivity with proteins and membranes allow many different possible regulatory mechanisms.

In summary, we present evidence for the first time of mitochondrially derived ROS regulation of ATP-stimulated, TACE-dependent shedding of TGF- α . Because TGF- α signals act exclusively through EGFR, this may represent one mechanism to maintain the specificity of biochemical signaling pathways. A recent report examining the existence of multiple transactivating mechanisms in a single cell highlights the need for specific pathways for different transactivation schemes (Rodland *et al.*, 2008). The discovery of mitochondrial ROS as a novel, key regulatory intermediate in TACE-dependent shedding of TGF- α reveals an additional mechanism for regulating the complexity of signaling through common cellular pathways.

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