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PROTEASE MEDIATED HUMAN SMOOTH MUSCLE CELL PROLIFERATION BY UROKINASE REQUIRES EPIDERMAL GROWTH FACTOR RECEPTOR TRANSACTIVATION BY TRIPLE MEMBRANE SIGNALING

Enrico A. Duru, PhD[1], **Yuyang Fu, MD, PhD**[1], and **Mark G. Davies, MD, PhD**[1,2]

[1]Vascular Biology and Therapeutics Program, Houston Methodist Research Institute, Houston, Texas

[2]Department of Cardiovascular Surgery, Houston Methodist DeBakey Heart & Vascular Center, Houston Methodist Hospital, Houston, Texas

Abstract

Background—Urokinase [uPA] modulates cellular and extracellular matrix responses within the microenvironment of the vessel wall and has been shown to activate the epidermal growth factor receptor [EGFR]. This study examines the role of the protease domain of uPA during EGFR activation in human vascular smooth muscle cells [VSMC].

Methods—Human coronary VSMC were cultured *in vitro*. Assays of cell proliferation and EGFR phosphorylation were examined in response to the C-terminal fragment of uPA [CTF] in the presence and absence of the plasmin, metalloprotease [MMP] and A Disintegrin and Metalloproteinase [ADAM] inhibitors, heparin-bound epidermal growth factor [HB-EGF], and EGFR inhibitors, and small interfering RNA [siRNA] to EGFR and ADAMs.

Results—CTF produced a dose-dependent increase in DNA synthesis and cell proliferation in human VSMC, which was blocked in a dose-dependent manner by both plasmin inhibitors and the EGFR inhibitor, AG1478. CTF induced time-dependent EGFR phosphorylation, which was

Correspondence: Mark G. Davies, MD, PhD, MBA, Houston Methodist DeBakey Heart & Vascular Center, Department of Cardiovascular Surgery, Houston Methodist Hospital, 6550 Fannin - Smith Tower - Suite 1401, Houston, Texas 77030.

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CONTRIBUTIONS:
Enrico A. Duru: concept and design, performance of the work, analysis and/or interpretation of data; critical writing or revising the intellectual content; and final approval of the version to be published
Yuyang Fu: concept and design, performance of the work, analysis and/or interpretation of data; critical writing or revising the intellectual content; and final approval of the version to be published
Mark G. Davies: concept and design, analysis and/or interpretation of data; critical writing or revising the intellectual content; and final approval of the version to be published

blocked by inhibitors of plasmin and MMP activity. The presence of uPAR was not required. Inhibition of ADAM-10 and -12, and of HB-EGF blocked EGFR activation in response to CTF. CTF-mediated activation of EGFR was mediated through Gβγ, src and NAD[P]H oxidase.

Conclusions—In human coronary VSMC, uPA induces uPAR-independent, domain-dependent smooth muscle cell proliferation through transactivation of EGFR by a plasmin-mediated, ADAM-induced, HB-EGF-dependent process, which is mediated by the intracellular pathways involving Gαi, Gβγ, src and NAD[P]H oxidase.

Keywords

uPA; protease domain; epidermal growth factor receptor; proliferation; cell signaling; human coronary smooth muscle cell

INTRODUCTION

Vessel remodeling occurs in response to flow and injury through an integrative program of cell proliferation, migration and extracellular matrix modulation (1). The proliferation and migration of vascular smooth muscle cells [VSMC] involves the complex regulation of proteases, integrins and extracellular molecules. Urokinase plasminogen activator [uPA] is a serine protease that is the primary plasminogen activator involved in tissue remodeling processes. Elevated uPA and decreased plasminogen activator inhibitor-1 [PAI-1] levels are predictors for angiographic coronary restenosis (2). After injury to the rat carotid artery, there is a time-dependent enhanced expression of plasminogen activators and increased plasminogen activator activity on smooth muscle cells within the vessel wall compared to the uninjured state (3). Overexpression of uPA or a deficiency of PAI-1 in transgenic mice has been shown to promote neointimal formation in response to transmural electrical injury. A lack of uPA or plasminogen leads to a reduction in neointimal hyperplasia (4, 5). uPA binds to the cell membrane through a GPI-tailed receptor, uPAR.

uPA has been shown to induce domain-dependent cell proliferation and migration, with the former due to its carboxyterminal, protease-containing domain [CTF] and the latter due to its aminoterminal, growth factor-binding domain (6). The biological data from the transgenic murine models suggests that uPA has a significant role in stimulating proliferation, and that it is plasmin dependent (7, 8). Using mutants of single chain uPA [sc-uPA], others have shown that cell proliferation is dependent on the mutant sc-uPA molecules containing CTF (9–12). CTF contains a protease domain and leads to plasmin generation, while plasmin inhibition reduces DNA synthesis. Plasmin will induce VSMC proliferation and inhibition of plasmin's protease activity inhibits VSMC proliferation (6). Plasmin can activate metalloproteinases [MMP], which, in turn, may lead to epidermal growth factor receptor [EGFR] activation (5, 13, 14). We have recently demonstrated that uPA induces smooth muscle cell proliferation through ERK1/2 and $p38^{MAPK}$-mediated pathways and is dependent on EGFR transactivation (13). CTF, through its protease domain or through generation of plasmin, may also activate G protein-coupled protease activated receptors on the cell membrane (15, 16).

Receptor transactivation is the pathway whereby activation of a given G protein-coupled receptor [GPCR] activates a heterologous receptor-linked tyrosine kinase [RTK] (17). At present, the triple membrane passing signaling [TMPS] mechanism of GPCR-induced EGFR activation is a widely accepted model of RTK transactivation. In this model, there is a sequence of three transmembrane signaling events: GPCR activation, followed by MMP activation and subsequent activation of EGFR by the tethered ligand heparin binding-EGF [HB-EGF] or other latent ligands of EGFR. Activation of the MMP step has been shown to involve one or more of the following signaling molecules: src, calcium, Pyk2 and protein kinase C [PKC]. This study tests the hypothesis that the protease domain of uPA induces cell proliferation through the triple membrane passing signaling mechanism in human VSMC, leading to transactivation of EGFR, and that this is independent of uPAR.

METHODS

Experimental Design

Human coronary arterial VSMCs were cultured in vitro [passage 3–6; Clontech, Mountain View, CA]. Assays of cellular DNA synthesis and proliferation, EGFR phosphorylation, and MAPK activation were examined in response to CTF in the presence and absence of a general protease inhibitor [1, 10-phenanthrolene], plasmin inhibitors [ε-aminocaproic acid and aprotinin], a matrix metalloproteinase [MMP] inhibitor [GM6001], ADAM inhibitors [TAPI-0 and TAPI-1], HB-EGF inhibitor [CRM197], HB-EGF antibodies and an EGFR inhibitor [AG1478]. Additional experiments were performed with small interfering RNA [siRNA] to EGFR. We further examined intracellular pathways leading to EGFR phosphorylation with specific inhibitors: pertussis toxin [PTx] and GP-2A for Gαi and Gαq, respectively; beta-adrenergic receptor kinase 1 (carboxy terminus) [βARK_{CT}] for Gβγ; PP2 for src; H7 and GF109203X for PKC; BAPTA-AM, a chelator of intracellular calcium; N-acetylcysteine [NAC] as a free radical scavenger; and diphenyleneiodium [DPI] and apocynin as NAD[P]H inhibitors [Table 1].

Materials

CTF was purchased from American Diagnostica, Inc. [Greenwich, CT]. EGF was purchased from Sigma Chemical Co. [St. Louis, MO]. AG1478 and CRM197 were purchased from Calbiochem [La Jolla, CA]. GM6001 was purchased from Chemicon International, Inc. [Temecula, CA]. CRM197, TAPI-0 and TAPI-1 were purchased from Biomol [Hamburg, Germany]. The anti–HB-EGF antibody was purchased from R&D Systems, Inc. [Minneapolis, MN]. The anti-EGFR antibody [151-IgG] developed by Dr. Ann Hubbard was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa [Department of Biological Sciences, Iowa City, IA]. Pertussis toxin, GP2A, AG1478, PP3, PP2, H7, GF109203X, BAPTA-AM, NAC, apocynin and DPI were purchased from Calbiochem [La Jolla, CA]. Peroxidase-conjugated anti-rabbit IgG antibody [raised in goat] was purchased from Jackson ImmunoResearch Laboratories, Inc. [West Grove, PA]. Peroxidase-conjugated anti-mouse IgG antibody [raised in goat] was purchased from Biorad Laboratories [Hercules, CA]. Phospho- EGFR [Y1068], Total EGFR antibodies were obtained from Cell Signaling Technology, Inc. [Beverley, MA]. Dulbecco’s

modified Eagle minimal essential medium [DMEM] and Dulbecco's phosphate-buffered saline were purchased from Mediatech [Herndon, VA].

[^{3}H] Thymidine Incorporation

VSMC were allowed to reach ~70% confluence as judged by microscopic examination. The cells were then placed in DMEM for 2 days to induce a quiescent state in the cells. The experiments were initiated by adding [^{3}H]-thymidine [1 μCi/mL; ICN, Irvine, CA] with and without pharmacological inhibitors, and stimulating the cells with CTF [10 nM]. Incorporation of [^{3}H]-thymidine into acid-precipitable material was measured after a 24-hour incubation period [air plus 5% CO_2 at 37°C]. Stimulation was expressed as fold increa se over starved cells [DMEM only], and the effects of inhibitors were expressed as percentage of the stimulated control.

Cell Proliferation

Culture wells [24-well cluster dishes, Falcon] were inoculated with 3 mL growth medium containing 5×10^4 VSMC and allowed to attach overnight. Cell proliferation in response to CTF [10 nM] with and without pharmacological inhibitors was determined. Cell counts were determined on days 1, 3, 5, and 7. Cells from each well were removed by trypsinization and the resultant suspension counted using a hemocytometer [Hausser Scientific, Horsham, PA]. Proliferation was expressed as fold increase over unstimulated cells [DMEM only].

Western Blotting

VSMC were allowed to grow to 80% confluence and starved for 48 hours. Cells were then stimulated with CTF or EGF alone and in the presence of pharmacological and peptide inhibitors; cells were harvested at time points from 0 to 30 minutes. Western blotting was performed as previously described (18).

siRNA Transfection

Pre-designed HPLC-purified siRNA for gene knockdown for EGFR or control siRNA was purchased from Santa Cruz Biotechnology [Santa Cruz, CA]. siRNA sequences for ADAMs were as follows: ADAM9, AAUCACUGUGGAGACAUUUGCdTdT and AAACUUCC AGUGUGUAGAUGCdTdT; ADAM10, AAUGAAGAGGGACACUUCCCUdTdT and AAGUUGCCUCCUCCUAAACCAdTdT; and ADAM12 AACCUCGCUGCAAAGAAUGUGdTdT and AAGACCUUGATACGACUGCUGdTdT. VSMC of 50% confluence in 60-mm plates were starved overnight in 4 mL Opti-MEM reduced serum medium [Gibco]. siRNA was transfected using Lipofectamine 2000 from Invitrogen, Inc. [Carlsbad, CA] following the product protocol. Briefly, 22 μL of Lipofectamine 2000 was first incubated in a total volume of 250 μL of Opti-MEM for 5 minutes at room temperature. This was then added to 250 μL of Opti-MEM containing 440 pmoles of siRNA. The solution was mixed gently and incubated for 20 minutes at room temperature, after which it was added to the starved plates. The medium can was changed after 4–6 hours of incubation. The cells were used between 24–72 hours after transfection for EGFR assays. Scrambled siRNA served as a control. Using the methodologies described, we conducted concentration-dependent experiments with siRNA against EGFR and

demonstrated a concentration-dependent decrease in protein expression that was specific for the protein targeted without altering the expression of the other proteins.

Adenoviral Infection

Adenoviral vectors were constructed by Welgen, Inc. [Worcester, MA] using purified plasmid encoding βARK_{CT}, obtained from the Guthrie cDNA Resource Center [Missouri University of Science and Technology, Rolla, MO]. VSMC were plated at 70% confluence in 100-mm dishes and allowed to grow overnight. Recombinant adenovirus was then added at the appropriate concentrations [βARK_{CT}; 50 MOI] in a reduced volume of media [1.5 – 2 mL]. After 48-hour incubation, the media was changed and cells grown for an additional 24 hours. The cells were then used for EGFR assays. Empty vector served as control.

Data and Statistical Analysis

All data are presented as the mean ± standard error of the mean [s.e.m.] and statistical differences between groups were tested with a Kruskal-Wallis nonparametric test with post hoc Dunn's multiple comparison correction, where appropriate. A p-value less than 0.05 was regarded as significant. Non-significant p-values were expressed as p=ns.

RESULTS

Cell proliferation

CTF produces an increase in DNA synthesis and cell proliferation of the VSMC. Responses to CTF were blocked by the EGFR kinase inhibitor, AG1478, in a dose-dependent manner, suggesting that EGFR is involved in these responses [Figure 1A and 1B]. DNA synthesis in response to EGF and HB-EGF were also inhibited by the presence of AG1478 [Figure 1A].

EGFR activation

Given the findings that CTF induced DNA synthesis and cell proliferation in an EGFR-dependent manner, we examined the activation of EGFR by CTF. CTF induced time-dependent EGFR phosphorylation [Figure 2A], which was inhibited by AG1478 in a concentration-dependent manner [Figure 2B]. Activation of EGFR by EGF and HB-EGF was also inhibited by AG1478 [Figure 2C and 2D]. Incubation with siRNA to EGFR also inhibited the response to CTF, EGF and HB-EGF in the cells [Figure 2E and 2F]. Activation of EGFR was confirmed by immunoprecipitation of EGFR and immunoblotting for phosphotyrosine. Removal of uPAR by phosphatidylinositol-specific phospholipase C did not alter the proliferative response [Figure 2G].

Extracellular Pathway leading to EGFR activation

Protease activity is involved in EGFR transactivation. We examined EGFR phosphorylation in the presence of a general protease inhibitor and inhibitors of plasmin and metalloproteinases. EGFR phosphorylation by CTF was blocked by the general protease inhibitor, 1–10 phenanthrolene, and by inhibitors of plasmin activation, EACA and aprotinin [Figure 3A and 3B]. The metalloproteinase inhibitor, GM6001, also blocked EGFR activation by CTF [Figure 3A and 3B]. These inhibitors have no effect on EGF-mediated

EGFR phosphorylation [Figure 3C]. The non specific ADAM inhibitors TAPI-0 and TAPI-1 also reduced EGFR activation in response to CTF but did not interrupt EGF activation of EGFR [Figure 3B, 3C and 3D] (19). As CTF-stimulated phosphorylation of EGFR is inhibited by MMP and ADAM inhibitors, this suggests that EGFR activation by CTF requires MMP cleavage of a tethered ligand such as HB-EGF. Pre-incubation with CRM197, an inhibitor of HB-EGF, was able to inhibit EGFR phosphorylation in response to CTF [Figure 3E and 3F]. We confirmed this finding through the use of HB-EGF-blocking antibodies [Figure 3E and 3F]. We have already demonstrated that EGF is capable of inducing EGFR phosphorylation in the presence of each of these inhibitors (19). Incubation with antibodies against the EGFR receptor prevented both CTF and EGF-mediated EGFR phosphorylation [Figure 3E and 3F]. The protease inhibitors did not affect the activation of EGFR by HB-EGF but those inhibitors that target HB-EGF did inhibit the responses appropriately [Figure 3]. These findings suggest that EGFR phosphorylation is dependent on the protease activity and subsequent MMP-mediated shedding of HB-EGF. A likely target of these intermediate kinases is the ADAM [A Disintegrin And Metalloproteinase Domain] family of MMPs, which are transmembrane molecules. VSMC express ADAM-9, ADAM-10 and ADAM-12 (20). We examined their contribution to EGFR activation by the use of siRNA to ADAM-9, ADAM-10 and ADAM-12. siRNA reduced ADAM-9, -10, -12 proteins levels by >50% (20). Incubation with siRNA against ADAM -10 and ADAM-12 significantly reduced EGFR activation in response to CTF. Scrambled siRNA and siRNA against ADAM-9 had no effect [Figure 3H]. There was no effect on EGF and HB-EGF activation of EGFR [Figure 3H].

Intracellular Pathway leading to EGFR activation

An alternative method for activation of surface MMPs and subsequent release of a tethered ligand may be through G protein-coupled receptor activation. These receptors are coupled to Gαi/ Gαq and Gβγ proteins. Pre-incubation with the Gαi inhibitor, pertussis toxin, resulted in inhibition of EGFR phosphorylation in response to CTF, while the Gαq inhibitor, GP-2A, had no effect [Figure 4A and 4B]. The activation of EGFR by EGF was unaffected by these inhibitors [Figure 4A and 4C]. Pre-incubation with βARK_{CT}, a peptide that functions as a Gβγ protein inhibitor, resulted in a decreased EGFR phosphorylation in response to CTF [Figure 4A and 4B]. Many GPCRs utilize intracellular calcium, src, PKC and oxygen free radical generation to induce HB-EGF release and EGFR phosphorylation. Inhibition of intracellular calcium or src but not PKC blocked EGFR activation in response to CTF [Figure 4D and 4E]. The activation of EGFR by EGF was unaffected by these inhibitors [Figure 4D and 4F]. Inhibition of oxygen free radical production through either the general inhibitor NAC and the more specific inhibitors DPI and apocynin also decreased EGFR activation in response to CTF but not in response to EGF [Figure 4G and 4H]. The proposed pathway defined by these experiments is shown in Figure 5.

DISCUSSION

We have shown that uPA induces smooth muscle cell proliferation through both ERK1/2 and $p38^{MAPK}$-mediated pathways (6) and have demonstrated that there is a distinct difference between uPA and plasmin with regard to the signaling pathways utilized to

induce cell proliferation (13). Plasmin appears only to use ERK1/2, while uPA uses both ERK1/2 and $p38^{MAPK}$ (13, 19, 21). In this report, we use the protease domain of uPA, CTF, to examine the problem more specifically and demonstrate for the first time that CTF-dependent smooth muscle cell proliferation occurs through transactivation of EGFR by a plasmin-mediated, ADAM-induced, HB-EGF-dependent process, which required activation of the intracellular pathways Gαi/Gβγ, src and NAD[P]H oxidase. Rather than signaling through uPAR, the CTF requires a G protein-coupled receptor to activate this cascade; this may occur through its protease domain directly or through generation of plasmin. Our results suggest these actions may activate G protein-coupled protease activated receptors on the cell membrane (15, 16) that signal through ARK1, since the ARK1 inhibitor βARK_{CT} prevents CTF-stimulated phosphorylation of EGFR. Transmembrane molecules such as ADAM are attractive candidate molecules for the transmembrane portion of the signaling pathway. ADAMs are approximately 70 to 90 kDa [mature proteins; the unprocessed precursors are about 20 kDa larger due to their prodomain]. They feature a common modular ectodomain structure, encompassing a variable stalk region; a cysteine-rich domain; a disintegrin domain binding to integrin-class cell adhesion molecules; and a Zn-binding metalloprotease domain. ADAM-9, ADAM-10, ADAM-12 and ADAM-17 have been reported to be involved in the ectodomain shedding of proHB-EGF (22, 23). We have shown that VSMC express ADAM-9, ADAM-10 and ADAM-12. Furthermore, we have recently identified that during migration the predominant pathways that lead to HB-EGF release are ADAM-9 and ADAM-10 dependent in these particular VSMC (20). This study suggests that in proliferation ADAM-10 and ADAM-12 are involved.

The ErbB-1/EGFR consists of a glycosylated ligand-binding domain, a single transmembrane domain and a cytoplasmic tyrosine kinase domain (24, 25) and is expressed in the injured vessel wall (26–28). HB-EGF is a tethered ligand for EGFR whose cleavage is MMP dependent and can be inhibited with heparin. Our data using CRM197 and antibodies against HB-EGF and EGFR support the fact that an extracellular pathway using HB-EGF is required for CTF-mediated EGFR activation. Activation of HB-EGF requires that it be cleaved from the membrane and also requires cell surface heparan sulfate proteoglycans to act as a co-receptors; HB-EGF binding to EGFR can be antagonized in a dose-dependent manner by heparin (29). We also demonstrated that this pathway was protease dependent and in particular dependent on metalloproteinase activity. We further extended these findings to demonstrate that ADAMs were involved. This sequence of events utilizes classical extracellular pathways of receptor transactivation whereby activation of a given receptor activates a heterologous receptor (17). In this model, there is a sequence of three transmembrane signaling events: G protein-coupled receptor activation, followed by ADAM-dependent MMP activation, and then cleavage of and subsequent binding of HB-EGF to the EGFR receptor. ADAM-10, ADAM-12 and ADAM-17 [TNF-α converting enzyme] have been shown to cleave HB-EGF (30). Activation of the ADAM pathway has been shown to involve one or more of the following signaling molecules: src, intracellular calcium, and/or protein kinase C (17). An alternative mechanism may be inactivation of protein tyrosine phosphatases due to the generation of oxygen free radicals by the NAD[P]H oxidase complex.

As with all cell signaling experiments, one must realize that the in vitro analysis does not completely represent the in vivo circumstance due to the fact that the in vivo VSMC is encased in a changing extracellular matrix and is under constant fluctuating hemodynamic and inflammatory forces. When we examine the pathways leading EGFR activation, one must accept that there are redundant pathways (extracellular and intracellular) that may mediate the effects of SMC proliferation and that these could be stimulated by other growth factors/upstream pathways.

Conclusion

In human coronary VSMC, urokinase induces uPAR-independent, domain-dependent smooth muscle cell proliferation through transactivation of EGFR by a plasmin-mediated, ADAM-induced HB-EGF-dependent process, which is mediated by the intracellular pathways involving Gαi, Gβγ, src and NAD[P]H oxidase.

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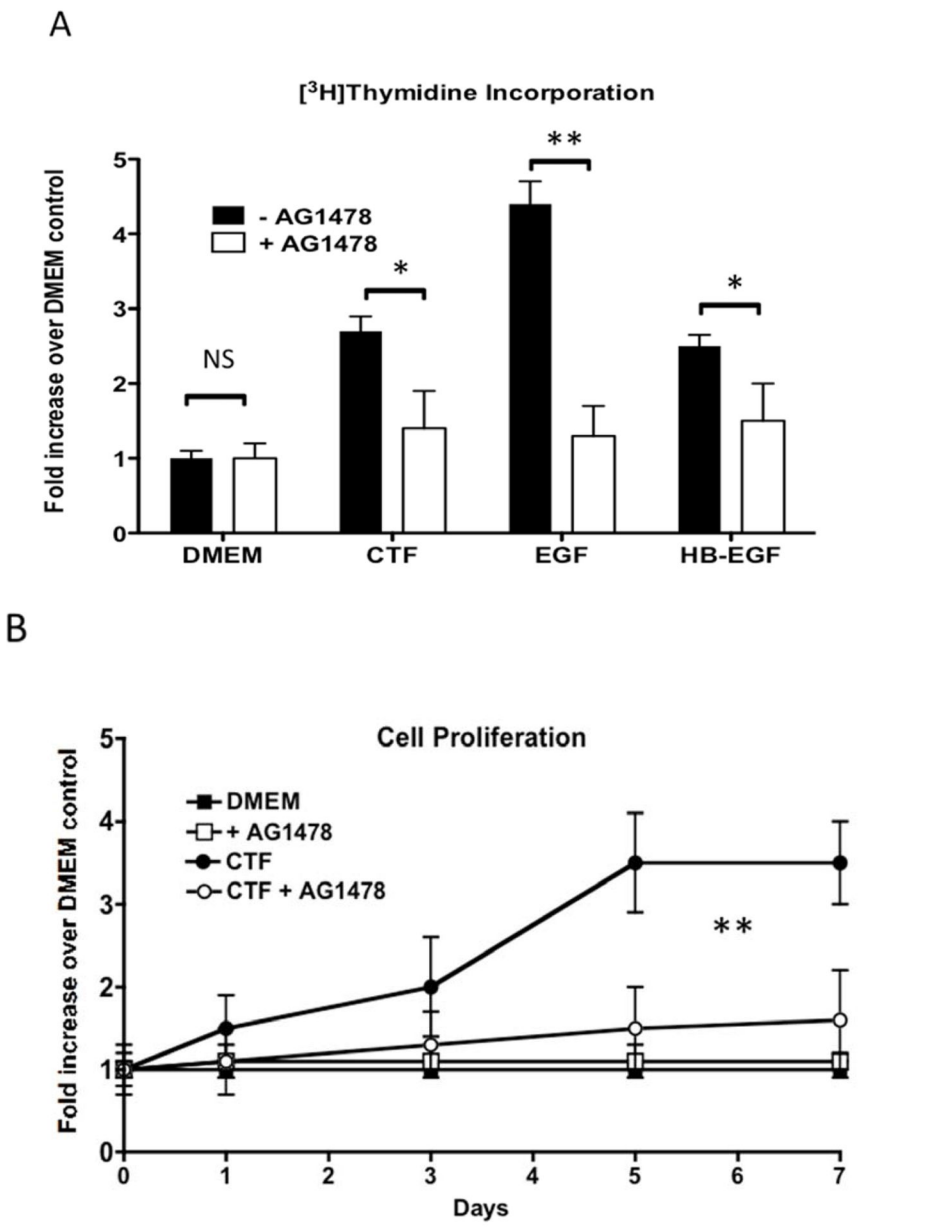
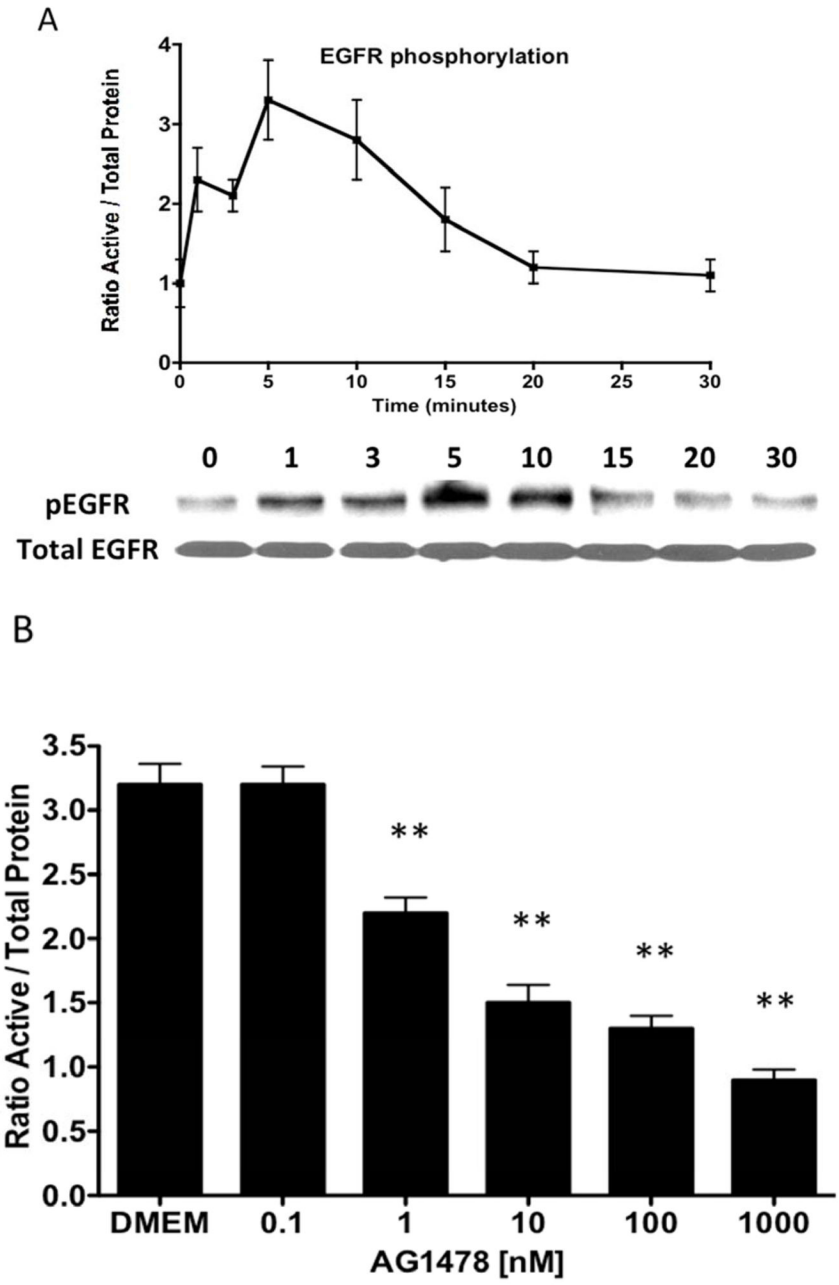
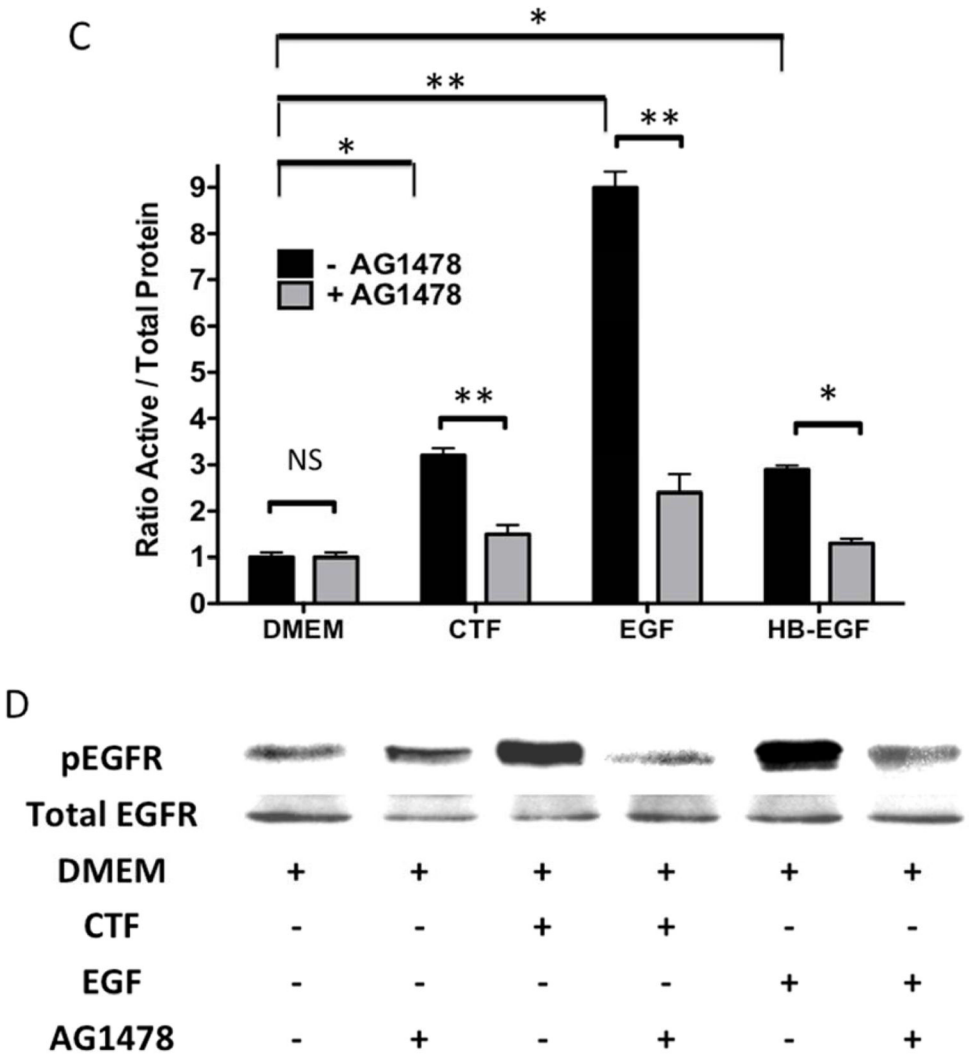


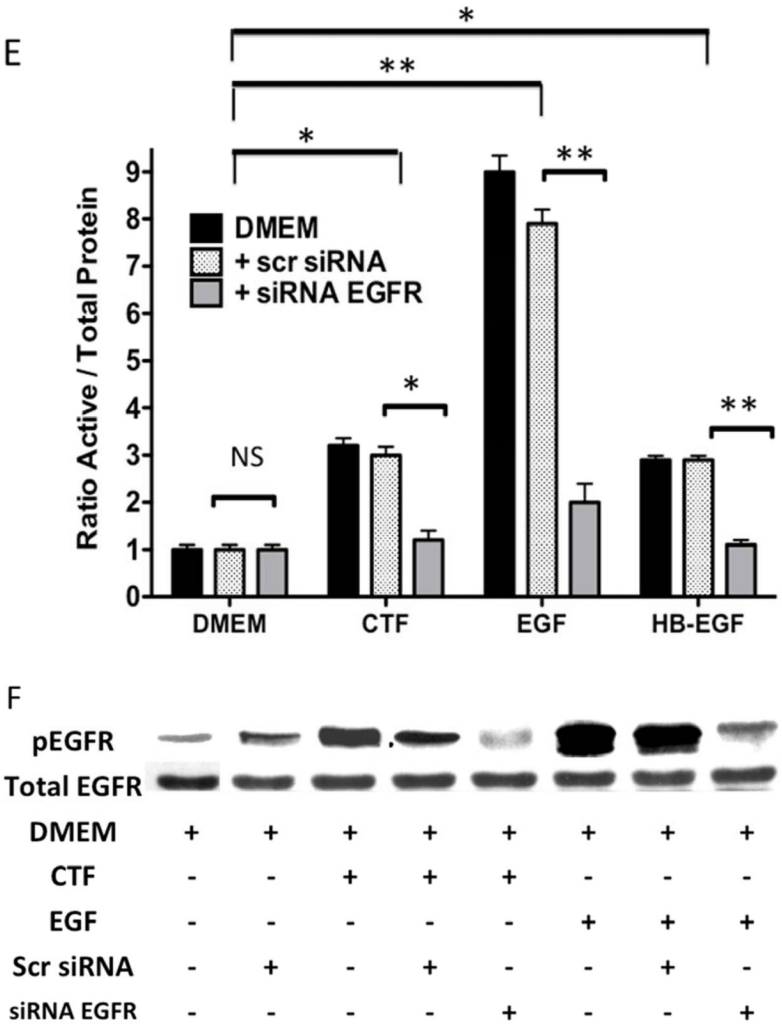
Figure 1.

[A] [^{3}H]-thymidine incorporation of cultured human coronary vascular smooth muscle cells in response to CTF [10 nM], EGF [10 μg/mL] and HB-EGF [10 μg/mL] in the presence and absence of the EGFR inhibitor [AG1478]. Values are the mean±s.e.m. fold increase over control for five experiments [* $p<0.05$, ** $p<0.01$].

[B] Cell proliferation of cultured human coronary vascular smooth muscle cells over a seven-day period in response to CTF [10 nM] in the presence and absence of the EGFR inhibitor [AG1478]. Values are the mean±s.e.m. fold increase over control for five experiments [** $p<0.01$ vs DMEM control].







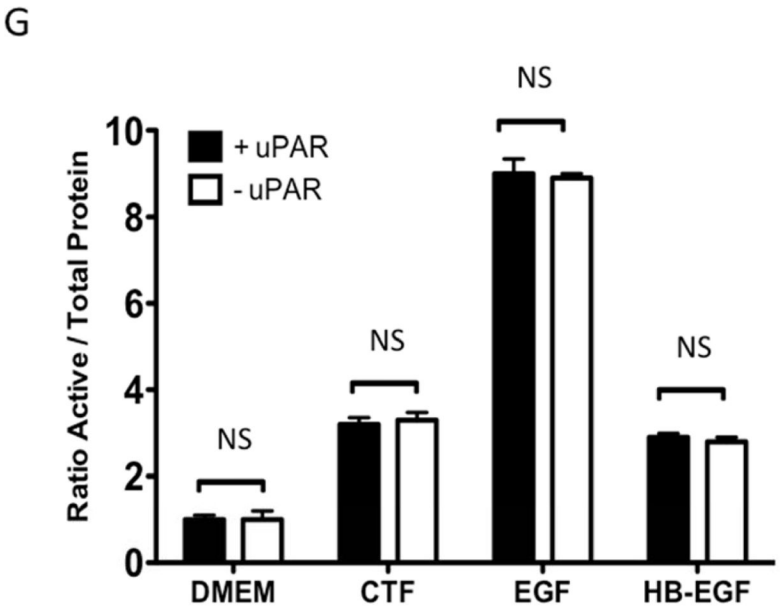


Figure 2. CTF and EGFR activation

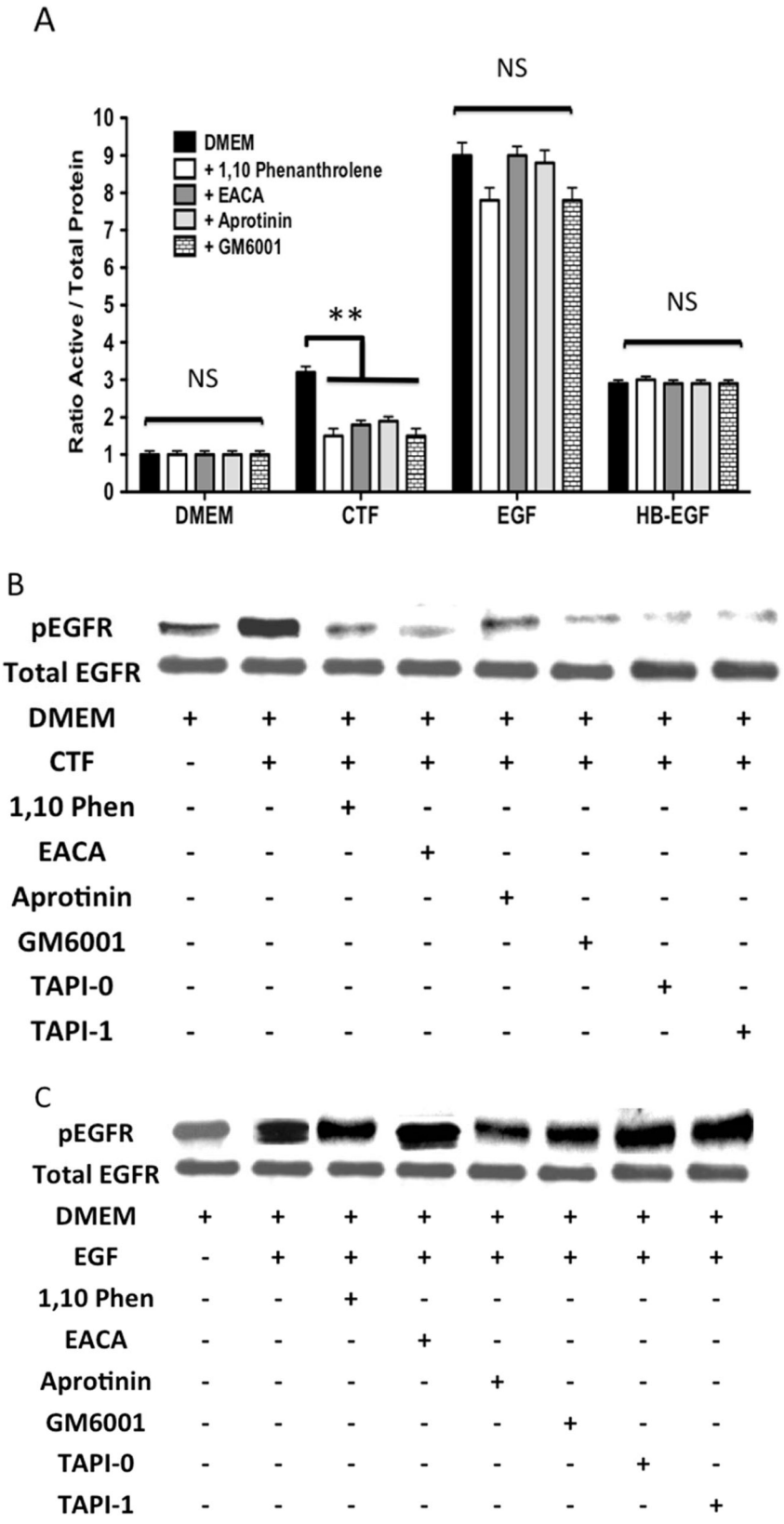
[**A**] Time-dependent EGFR phosphorylation in response to CTF [10 nM] in cultured human coronary vascular smooth muscle cells. Values are the mean±s.e.m. ratio of active /total EGFR protein for five experiments. Representative western blot is shown.

[**B**] Inhibition of EGFR phosphorylation by AG1478 in response to CTF [10 nM] occurs in a concentration-dependent manner. Values are the mean±s.e.m. ratio of active /total EGFR protein for five experiments [**p<0.01].

[**C**] Inhibition of EGFR phosphorylation in response to CTF [10 nM], EGF [10 μg/mL] and HB-EGF [10 μg/mL] in the presence and absence of the EGFR inhibitor [AG1478]. Values are the mean±s.e.m. ratio of active /total EGFR protein for five experiments [*p<0.05; **p<0.01]. Representative western blots are shown in [**D**].

[**E**] EGFR phosphorylation in response to CTF [10 nM], EGF [10 μg/mL] and HB-EGF [10 μg/mL] in the presence and absence of the siRNA to EGFR. Scr siRNA, scrambled siRNA control. Values are the mean±s.e.m. ratio of active /total EGFR protein for five experiments [*p<0.05; **p<0.01]. Representative western blots for CTF and EGF are shown in [**F**].

[G] EGFR phosphorylation in response to CTF [10 nM], EGF [10 μg/mL] and HB-EGF [10 μg/mL] in the presence and absence of the uPAR on the cell surface. Values are the mean ±s.e.m. ratio of active /total EGFR protein for five experiments.



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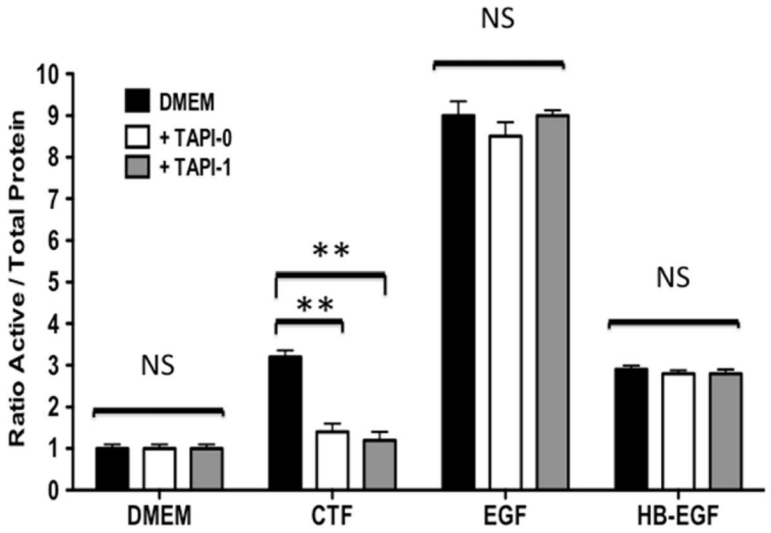

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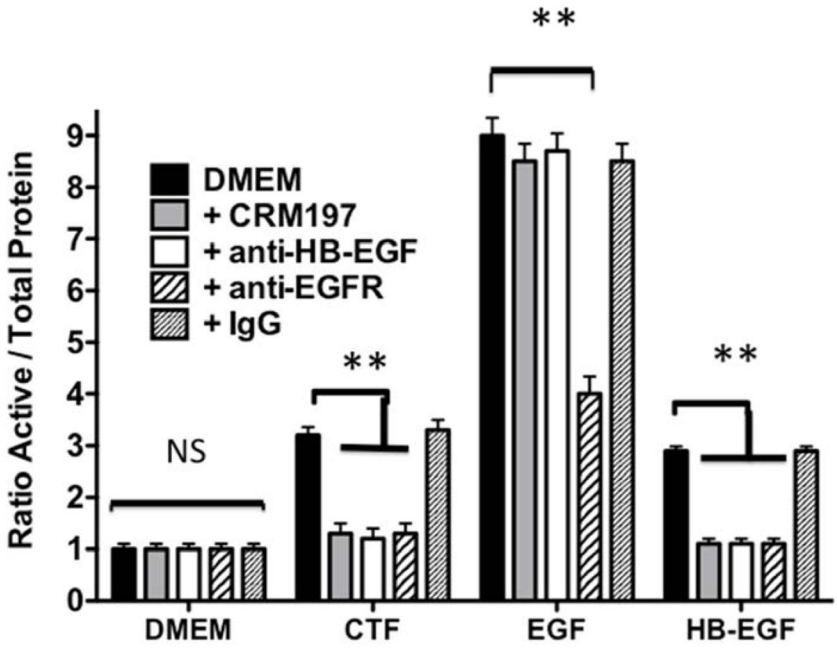


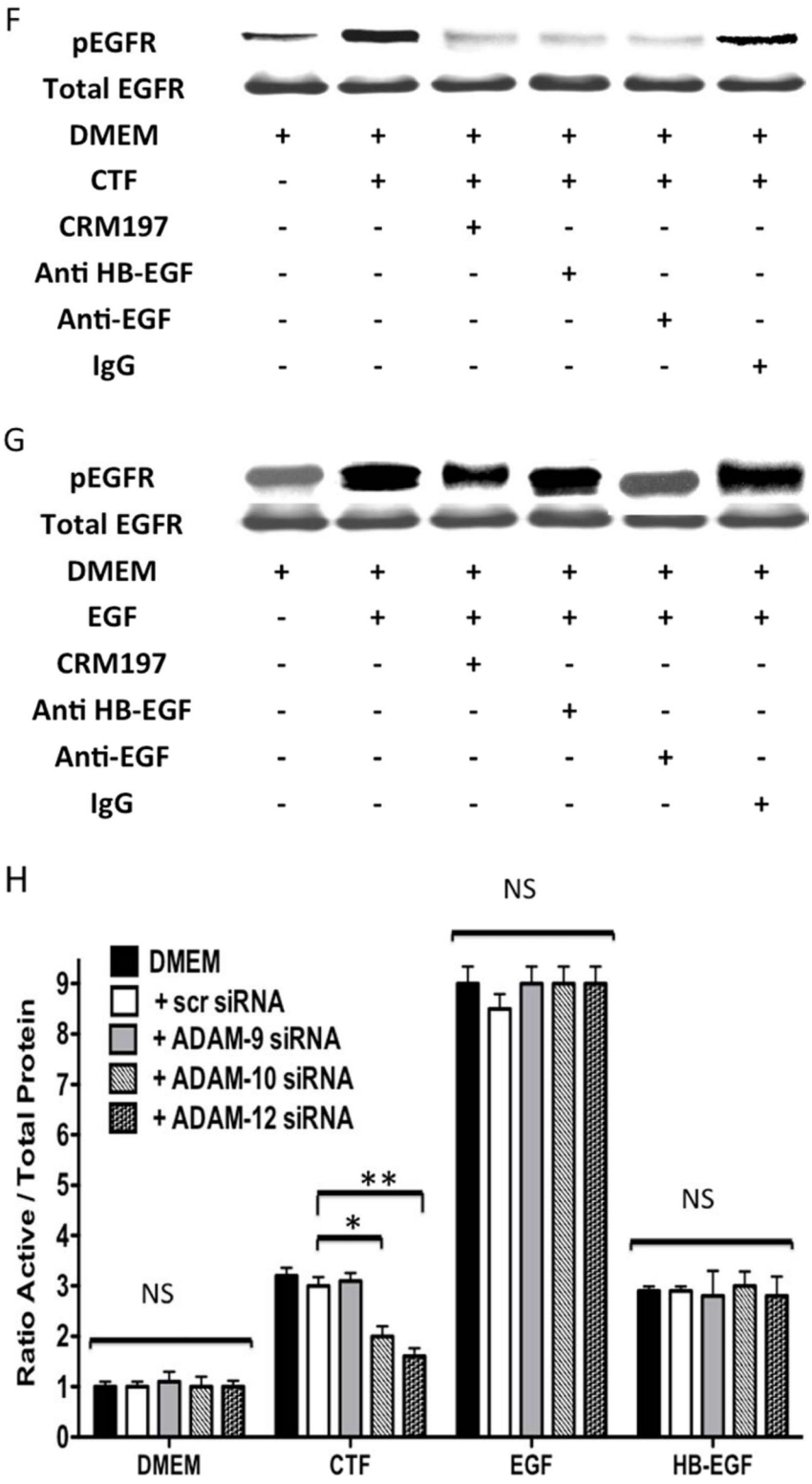


Figure 3. CTF and extracellular pathway

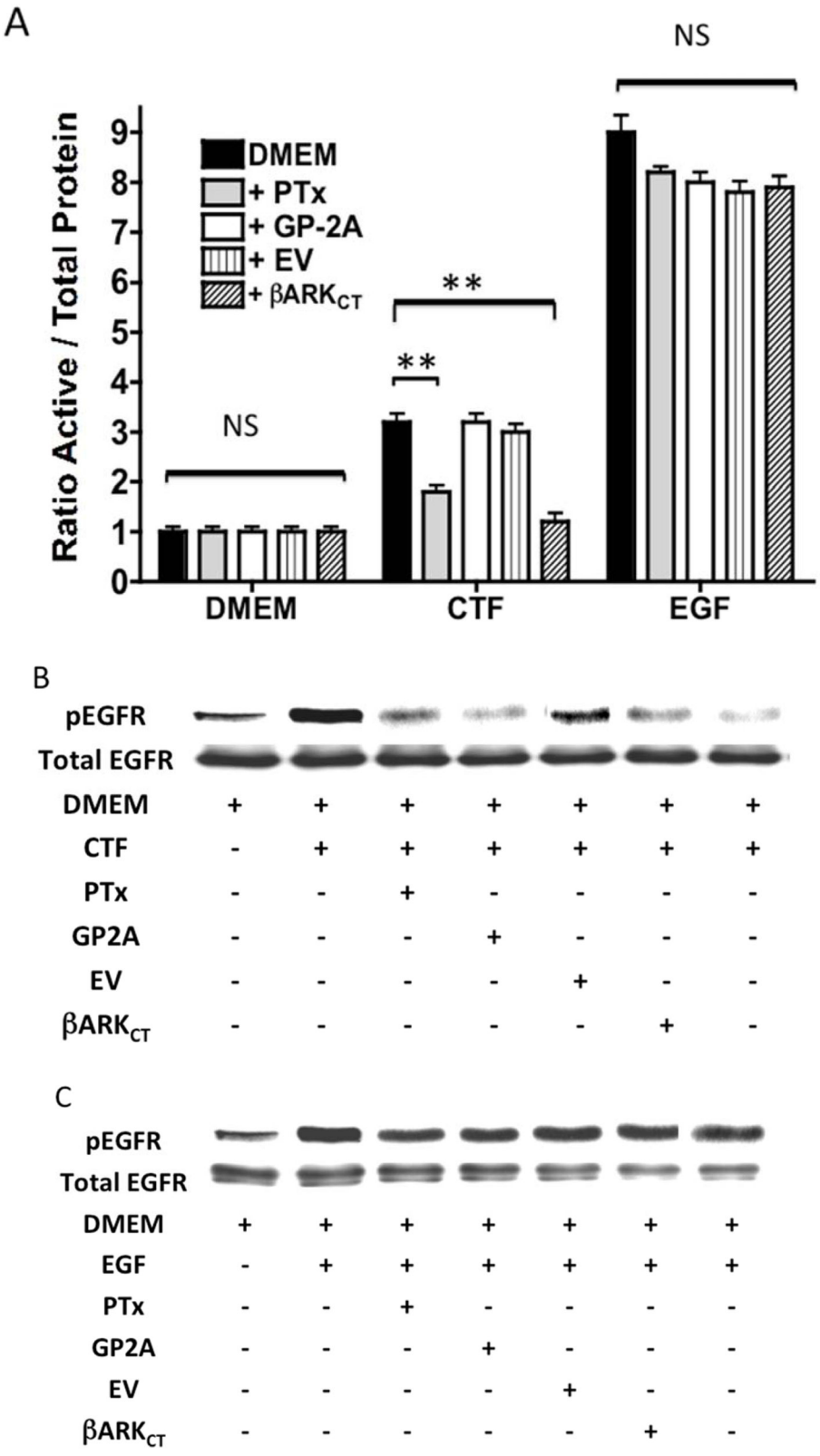
[**A**] EGFR phosphorylation in response to CTF [10 nM], EGF [10 μg/mL] and HB-EGF [10 μg/mL] in the presence and absence of the protease inhibitor 1,10-phenanthrolene, the

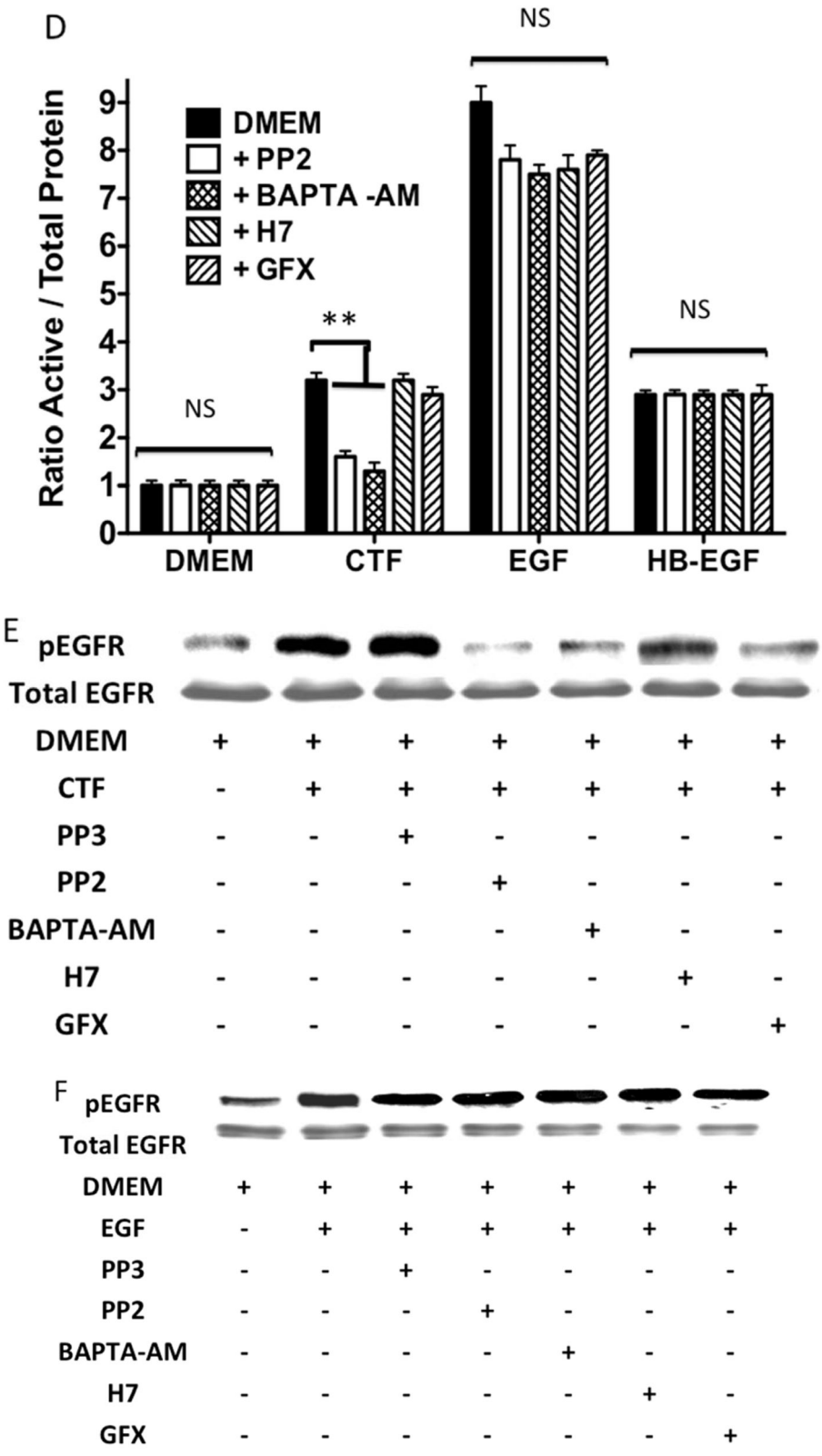
plasmin inhibitors [aprotinin and ε-aminocaproic acid, (EACA)] and the MMP inhibitor [GM6001]. Values are the mean±s.e.m. ratio of active /total EGFR protein for five experiments [**p<0.01]. Representative western blots are shown in [**B**] and [**C**].

[**D**] EGFR phosphorylation in response to CTF [10 nM], EGF [10 μg/mL] and HB-EGF [10 μg/mL] in the presence and absence of the ADAM inhibitors TAPI-0 and TAPI-1. Values are the mean±s.e.m. ratio of active /total EGFR protein for five experiments [*p<0.05; **p<0.01].

[**E**] EGFR phosphorylation in response to CTF [10 nM], EGF [10 μg/mL] and HB-EGF [10 μg/mL] in the presence and absence of the HB-EGF inhibitor CRM197, HB-EGF blocking antibody and EGFR blocking antibody. Values are the mean±s.e.m. ratio of active /total EGFR protein for five experiments [**p<0.01]. Representative western blots are shown in [**F**] and [**G**].

[**H**] Incubation with siRNA to ADAM-10 and -12 significantly reduced EGFR activation in response to CTF. Responses to EGF and HB-EGF were unaffected. Scrambled siRNA and siRNA against ADAM-9 had no effect. We had previously shown that siRNA to ADAM-9, -10 and -12 resulted in a ~50% decrease in their respective A Disintegrin And Metalloproteinase Domains (ADAM) protein expression compared to DMEM control or scrambled siRNA. (20). Values are the mean±s.e.m. ratio of active /total EGFR protein for five experiments [*p<0.05; **p<0.01].





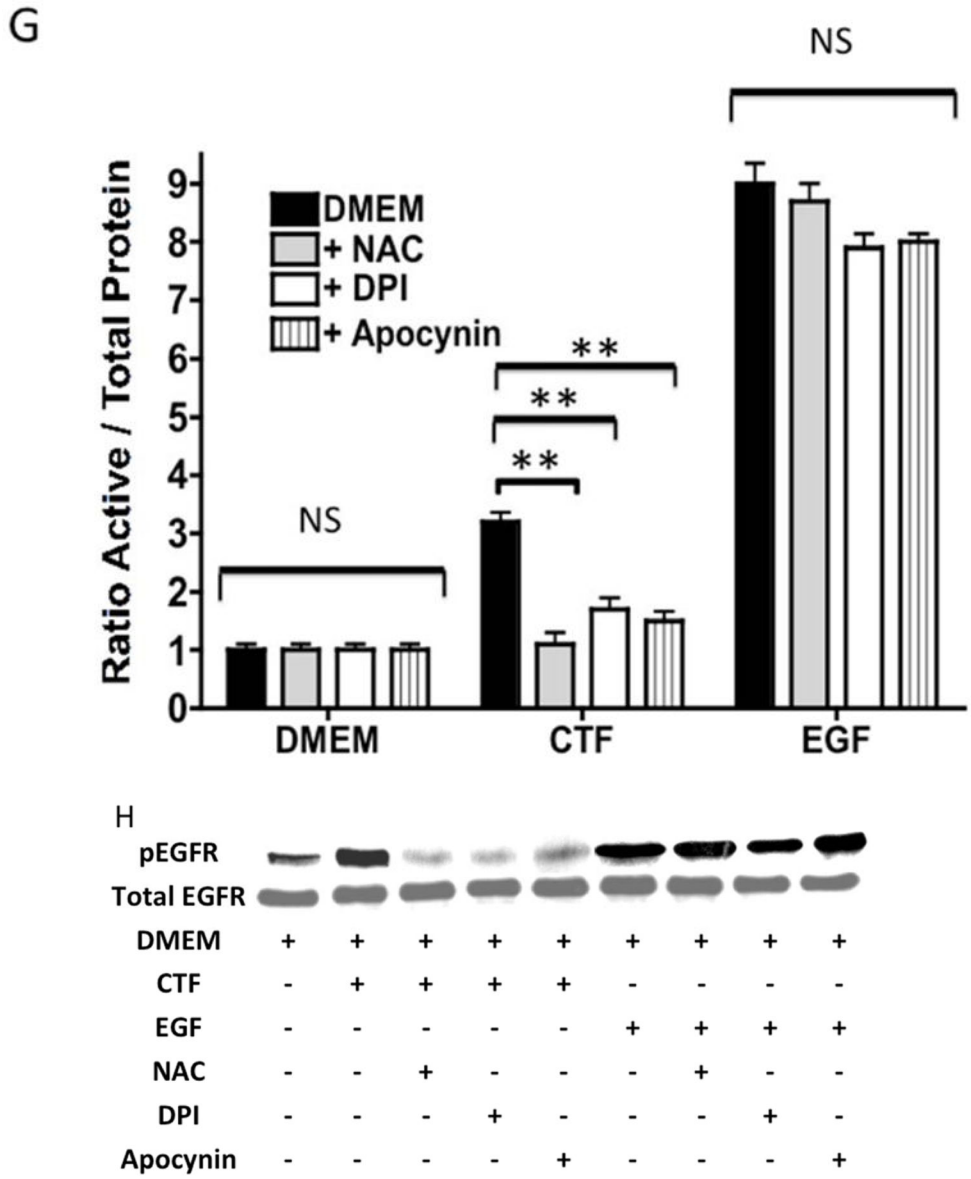


Figure 4. CTF and intracellular pathway

[**A**] EGFR phosphorylation in response to CTF [10 nM] and EGF [10μg/dL] in the presence and absence of the pertussis toxin [PTx] or Gαq inhibitor [GP-2A]. Preincubation with $bARK_{CT}$ resulted in a decreased EGFR phosphorylation in response to CTF. EV, empty vector. Values are the mean±s.e.m. ratio of active /total EGFR protein for five experiments [**p<0.01]. Representative western blots are shown [**B**] and [**C**].

[**D**] Inhibition of intracellular calcium [BAPTA-AM], src [PP2] and PKC [H7 and GFX] blocked EGFR activation in response to CTF [10 nM] and EGF [10 μg/dL]. Values are the mean±s.e.m. ratio of active /total EGFR protein for five experiments **p<0.01]. Representative western blots are shown [**E**] and [**F**].

[**G**] Inhibition of oxygen free radical production through either the general inhibitor NAC and the more specific inhibitors DPI and apocynin also decreased EGFR activation in response to CTF [10 nM] and EGF [10 μg/dL]. Values are the mean±s.e.m. ratio of active /

total EGFR protein for five experiments [**p<0.01]. Representative western blots are shown in [**H**].

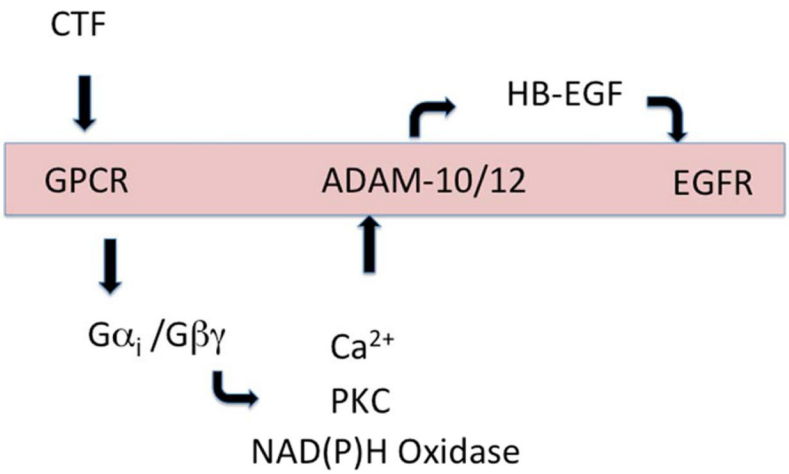


Figure 5. Representative pathway for CTF transmembrane signaling

CTF interacts with a G protein-coupled receptor which activates activates both Gαi and Gβγ. In turn, a series of intracellular molecules are involved which lead to activation of A Disintegrin And Metalloproteinase Domains (ADAM) proteases (ADAM-10 and ADAM-12). Activation of ADAM-associated MMP activity leads to release of the tethered ligand, HB-EGF, which in turn activates the EGFR. Activation of EGFR is necessary for CTF-mediated proliferation.

Table 1

Action	Inhibitor
Plasmin Activation inhibitors	ε-aminocaproic acid and aprotinin,
Matrix Metalloproteinase [MMP] inhibitor	GM6001
ADAM inhibitors	TAPI-0
ADAM inhibitors	TAPI-1
HB-EGF inhibitor	CRM197
HB-EGF inhibitor	HB-EGF antibodies
EGFR inhibitor	AG1478
Gαi inhibitor	pertussis toxin [PTx]
Gαq inhibitor	GP-2A
Gβγ inhibitor	βARK_{CT}
src inhibitor	PP2;
src inhibitor control	PP3
PKC inhibitor	H7
PKC inhibitor	GF109203X for PKC;
Calcium singalling inhibitor	BAPTA-AM
Free radical scavenger	N-acetylcysteine [NAC]
NAD[P]H inhibitor	diphenyleneiodium [DPI]
NAD[P]H inhibitor	apocynin