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Differential Role of β-Catenin in VEGF and Histamine-Induced MMP-2 Production in Microvascular Endothelial Cells

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ABSTRACT

Increases in endothelial cell permeability and production of matrix-degrading enzymes are two early steps in the angiogenic process. Factors such as vascular endothelial growth factor (VEGF) and histamine induce the angiogenic process through alterations in both permeability and proteolysis. We hypothesized that β-catenin acts as a positive regulator of MMP-2 and MT1-MMP transcription following VEGF or histamine stimulation. Rat microvascular endothelial cells were exposed to VEGF or histamine overnight and MMP-2 protein production was assessed by gelatin zymography. Latent MMP-2 protein levels were increased following VEGF and histamine treatment as were MMP-2 mRNA transcript levels. Endothelial cells exposed to VEGF and histamine had an increased level of nuclear β-catenin, which was sensitive to inhibition of the PI3-kinase signaling pathway. Promoter assays indicated increased transcriptional activity of both MMP-2 and MT1-MMP in endothelial cells co-transfected with luciferase reporter constructs and β-catenin. Inhibition of β-catenin signaling with inhibitor of catenin and T cell factor (ICAT) revealed that the VEGF-induced increase in MMP-2 mRNA is β-catenin dependent. Interestingly, while MMP-2 mRNA levels increased in response to histamine H1 or H2 receptor activation, significantly larger increases were observed in cells co-treated with ICAT and histamine or the histamine receptor agonists, HTMT and dimaprit. While both VEGF and histamine increase nuclear β-catenin and MMP-2 production, the role of β-catenin in MMP-2 regulation differs between the two stimuli. J. Cell. Biochem. 107: 272–283, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: ANGIOGENESIS; PERMEABILITY; PROTEOLYSIS

Altered vascular permeability is an early hallmark of angiogenesis. Endothelial cell activation follows the increase in permeability and results in cell proliferation and migration towards the angiogenic stimulus. These cells will connect with another pre-existing vessel establishing a new flow channel [Ausprunk and Folkman, 1977]. Although the progression of events making up the angiogenic process has been well established, the mechanism by which each event triggers the next is not well understood.

Permeability factors exert their effects through modulation of adherens junction components. Adherens junctions are sites of contact between adjacent endothelial cells and form through homotypic interactions of vascular endothelial (VE)-cadherin molecules on adjacent cells [Takeichi, 1990, 1995]. Further stabilization of the junction occurs via intracellular association of VE-cadherin with the catenin family of proteins, α, β, γ, and p120 [Stappert and Kemler, 1994; Thoreson et al., 2000; Bazzoni and Dejana, 2004], which link VE-cadherin to the actin cytoskeleton [Ben Ze'ev and Geiger, 1998]. Vascular endothelial growth factor (VEGF) stimulation results in tyrosine phosphorylation of both VE-cadherin and β-catenin [Andriopoulou et al., 1999], and tyrosine phosphorylation of β-catenin is also seen within 5 min of histamine stimulation [Biswas et al., 2006]. Phosphorylation of junctional components has been correlated with a loss or weakening of junction stability and increased permeability [Collares-Buzato et al., 1998; Vestweber, 2000].

Phosphorylation of proteins in the junction can lead to release of β-catenin from the VE-cadherin/α-catenin complex. Under quiescent conditions, cytoplasmic β-catenin is targeted for degradation by a complex composed of glycogen synthase kinase 3β (GSK-3β), axin, casein kinase I, and adenomatosis polyposis coli (APC) [Bienz and Clevers, 2000; Doble and Woodgett, 2003]. Phosphorylation of GSK-3β inactivates this complex, and allows for nuclear translocation of β-catenin [Ding et al., 2000]. Nuclear β-catenin interacts with the T cell factor (TCF) family of transcription factors which together function as transcriptional co-activators [van de

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Wetering et al., 1997]. The β-catenin/TCF complex increases the transcription of genes involved in cell proliferation including cyclin D1 and c-myc [He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999]. Previous studies showed that stimulation of endothelial cells with VEGF led to increased nuclear translocation of β-catenin and histamine stimulation of HeLa cells increased β-catenin/TCF reporter activity, respectively [Diks et al., 2003; Ilan et al., 2003].

The matrix metalloproteinase (MMP) family of zinc- and calcium-dependent endopeptidases is capable of degrading various components of the extracellular matrix [Woessner, 1994] and both MMP-2 and membrane type (MT) 1-MMP have a critical role in the angiogenic process [Sato et al., 1997; Haas et al., 1998; Seiki, 2003]. MT1-MMP is responsible for the activation of secreted MMP-2 and both enzymes cleave a variety of substrates including type I collagen [Seiki, 2003]. Both VEGF and histamine may modulate MMP-2 production. Degranulation of cardiac mast cells, leading to release of chemicals including histamine, increases MMP-2 protein production in cardiac tissues [Chancey et al., 2002] and in skeletal muscle endothelial cells, VEGF stimulation increases levels of MMP-2 [Ispanovic and Haas, 2006].

Takahashi et al. [2002] reported that MT1-MMP expression correlated with nuclear accumulation of β-catenin in SW480 colon cancer cells [Takahashi et al., 2002]. Hlubek et al. [2004] extended this finding by demonstrating β-catenin-dependent activation of MT1-MMP transcription through a TCF-binding element (TBE) located in the MT1-MMP promoter. Recent studies have shown that migrating bovine aortic endothelial cells in a scrape wound assay have increased cytoplasmic and nuclear β-catenin levels [Goodwin et al., 2006]. To date, a relationship between β-catenin and the transcription of MMPs has not been established in microvascular endothelial cells. We hypothesized that stimulation of microvascular endothelial cells with permeability factors, such as histamine and VEGF, would increase nuclear β-catenin levels and enable β-catenin to act as a transcriptional co-activator of both MT1-MMP and MMP-2.

MATERIALS AND METHODS

MATERIALS

All chemicals for cell lysis, electrophoresis, and Western blotting were purchased from Sigma–Aldrich and cell culture components were purchased from Invitrogen. PI3-kinase inhibitor (LY294002) was obtained from Calbiochem, VEGF-165 from Invitrogen, and 6-[2-(4-imidazolyl)ethylamino]-*N*-(4-trifluoromethylphenyl) heptanecarboxamide dimaleate (HTMT) and S-(3-dimethylaminopropyl) isothiourea dihydrochloride (Dimaprit) from Tocris.

CELL CULTURE

Rat microvascular endothelial cells were isolated from the epididymal fat pad and the extensor digitorium longus muscle as previously described [Madri and Williams, 1983; Han et al., 2003] and cultured under conditions of 37°C and 7% CO_2 on 1.5% gelatin-coated flasks with Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 200 mM L-glutamine, 50 U of penicillin, and 0.5 mg/ml streptomycin. For experiments, endothelial cells were plated on type I collagen-coated 60 mm plates at a density of 2 million cells/dish and stimulated with VEGF (10, 20, or 50 ng/ml) or histamine (50, 100, or 400 μM) for 18 h.

GELATIN ZYMOGRAPHY

Cells were lysed for total protein extracts in 120 mM Tris–HCl (pH 8.7), 0.1% Triton X-100, and 5% glycerol supplemented with 0.1% EDTA-free protease inhibitor cocktail (Sigma, P-8340) and 1 μM sodium orthovanadate. Twenty micrograms of protein, as determined by the bicinchonic assay (Pierce), was analyzed by gelatin zymography as described previously [Haas et al., 1998]. Gels were visualized and documented using the Fluorchem gel doc system and analyzed using Alpha ease (Alpha Innotech) software.

REAL-TIME-POLYMERASE CHAIN REACTION (RT-PCR)

Endothelial cells were plated at a density of 250,000 cells/dish on type I collagen, and stimulated with either 400 μM histamine or 20 ng/ml VEGF. Eighteen hours later, cells were lysed using Cells-to-cDNA II (Ambion) according to the manufacturers' instructions. Following DNase treatment, RNA was reverse transcribed using MMLV reverse transcriptase (Ambion). cDNA samples were analyzed by RT-PCR using PCR supermix (Invitrogen), rRNA, GAPDH, HPRT-1, and MT1-MMP or MMP-2 mRNA probe sets containing the probe, forward and reverse sequences (Catalogue # P/N4308329, Rn99999916_S1, Rn01527840_m1, Mm00485954_m1, and Rn01538174_m1, respectively; Applied Biosystems). RT-PCR was performed using an ABI Prism 7700 Sequence Detector (Perkin–Elmer), a Stratagene 3005 MxPro or a BIORAD Chromo 4 and results were analyzed using associated software. The threshold cycle (C_t) was determined and normalization was carried out using the comparative C_t method, as described previously [Milkiewicz and Haas, 2005].

IMMUNOSTAINING

Endothelial cells were plated on 1.5% gelatin-coated cover slips and stimulated with 20 ng/ml of VEGF, 400 μM histamine, or 20 or 50 mM lithium chloride overnight. Cells were fixed in 3.7% paraformaldehyde and permeabilized in 5% goat serum, 0.1% Triton X-100 in PBS. Cells were incubated with primary antibody for β-catenin (Santa Cruz), followed by incubation with AlexaFluor 568 goat anti-rabbit secondary antibody (Molecular Probes, Inc.). Nuclei were visualized using DAPI (Molecular Probes, Inc.). Cells were imaged using an inverted fluorescence microscope (Zeiss, Axiovert 200M), cooled digital CCD camera (Quantix 57; Photometrics) with a computer-controlled filter changer (Lambda 10-2; Sutter Instruments) at a final magnification of 400× and collected using MetaMorph software (Universal Imaging).

CYTOPLASMIC AND NUCLEAR EXTRACT LYSIS

Endothelial cells were lysed for cytoplasmic and nuclear extracts using the NE-PER kit (Pierce). Briefly, cells were trypsinized and centrifuged to pellet the cells. The pellet was resuspended in cytoplasmic extract reagent (CER) I combined with CERII lysis reagent. The lysate was centrifuged and the supernatant (cytoplasmic component) was removed. The pellet was resuspended in

nuclear extract reagent (NER) I. The lysate was centrifuged with the resulting supernatant containing the nuclear component.

WESTERN BLOTTING

Twenty micrograms of protein, as determined by the BCA assay (Pierce), for each condition was prepared under denaturing conditions and separated on a polyacrylamide gel. Proteins were transferred to a PVDF membrane (Millipore) and following transfer were incubated in 5% non-fat milk dissolved in 0.05% Tween 20-TBS. Membranes were probed for β-catenin (Santa Cruz, sc-7199), phospho-GSK-3β (Cell Signaling, 9336), phospho-Akt (Cell Signaling, 4051), total Akt (Cell Signaling, 9272), and β-actin (Cell Signaling, 4967) overnight at 4°C. Membranes were then incubated with donkey anti-rabbit or donkey anti-mouse HRP secondary antibody (GE Biosciences). Bound antibodies were detected using Super West Pico (Pierce) or Imobilon Western (Millipore) ECL as per manufacturer's instructions and imaged on autoradiography film (Hyperfilm; GE Biosciences). Bands were quantified using Fluorchem software.

TRANSIENT TRANSFECTION AND PROMOTER ASSAYS

Endothelial cells were plated on type I collagen-coated 12-well plates at a density of 50,000 cells/well 18 h prior to transfection. Cells were co-transfected, using LipofectAMINE 2000 (Invitrogen), with 0.5 μg of MT1-MMP (full length of −3285, −1398, or −1006 bp) [Haas et al., 1999] or MMP-2 (full length of −1686, −1560, −1375,

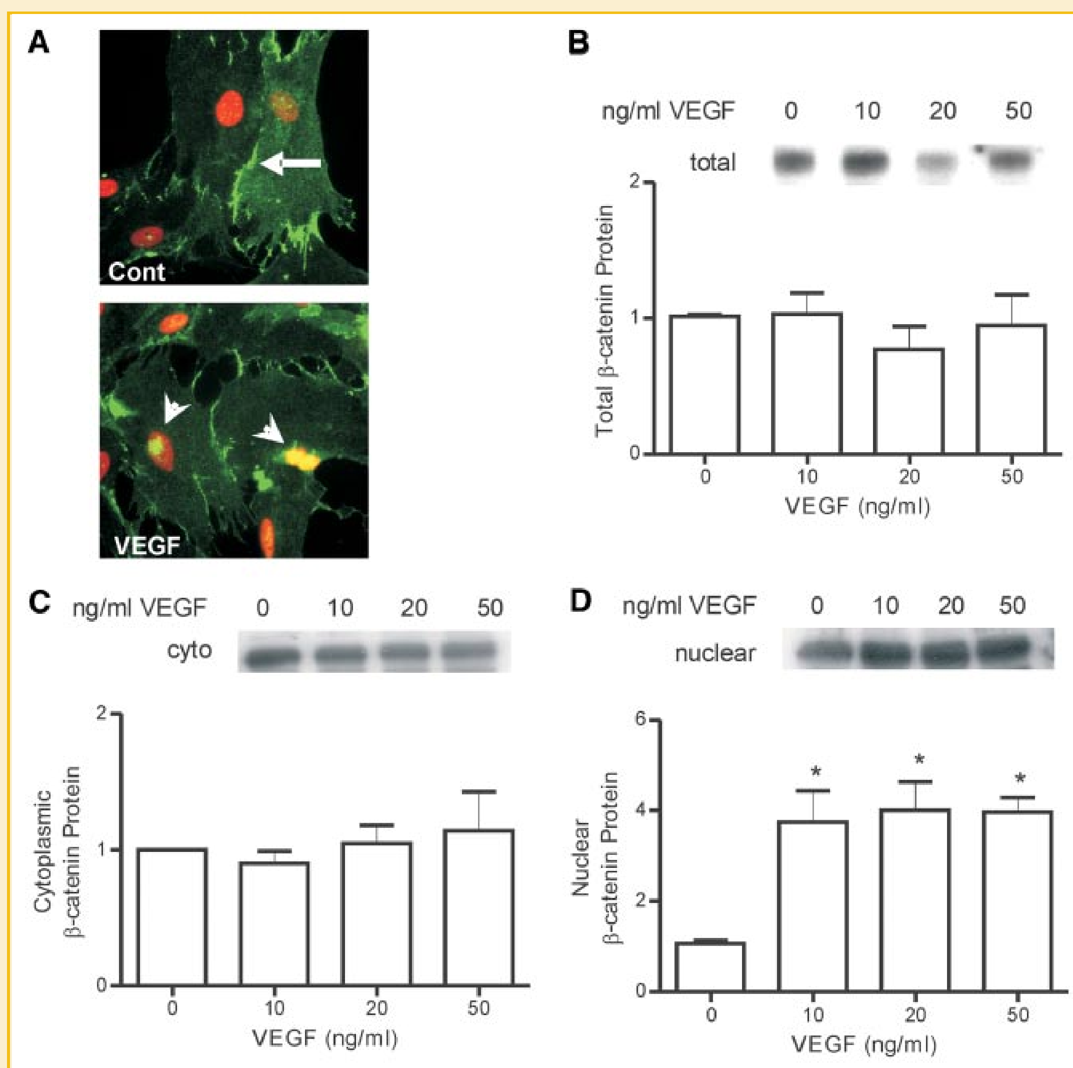

Fig. 1. VEGF increases nuclear levels of β-catenin. A: Endothelial cells were cultured under control conditions (top) or treated overnight with 20 ng/ml of VEGF (bottom). Cells were fixed and stained for β-catenin (green) and DAPI (red nuclei). Arrows indicate β-catenin localized to cell–cell junctions and arrowheads indicate β-catenin localized to the nucleus. Endothelial cells were treated overnight with 0, 10, 20, or 50 ng/ml of VEGF and β-catenin levels were measured. Quantification of total, cytoplasmic, and nuclear β-catenin is shown in panels B, C, and D, respectively. Values are mean ± SEM, $^{*}P < 0.05$ versus control, n = 3. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

or −510 bp) promoter truncations [Han et al., 2003] and 0.5 μg of β-catenin-pcDNA3.1 cDNA (kind gift from O. Tetsu, University of California, San Francisco, CA) or pcDNA3.1 alone. Cells also received 0.05 μg of pRenilla plasmid (Promega) to normalize for transfection efficiency. Cells were lysed 48 h later and analyzed for promoter activity using Dual Reporter Luciferase Assay (Promega) and Wallac Plate Reader (Perkin–Elmer). MMP-2 and MT1-MMP luciferase values from conditions co-transfected with β-catenin were expressed relative to the luciferase values from conditions co-transfected with the empty pcDNA3.1 vector.

ADENOVIRUS TRANSDUCTION

Endothelial cells were plated on type I collagen at a density of 15,000 cells/well 18 h prior to transduction. Cells were transduced with 300 pfu/cell of either Ad-βgal or Ad-ICAT (kind gifts of Sarah George, University of Bristol, Bristol, UK). This was determined to generate >90% of cells expressing the virus based on β-galactosidase staining. 24 h after transduction, media containing the virus were removed and cells were stimulated with 20 ng/ml of VEGF, 50 μM HTMT (H1 receptor agonist), 50 μM dimaprit (H2 receptor agonist), or 100 μM histamine. Twenty-four hours post-stimulation cells were lysed for RT-PCR analysis as previously described. For MMP-2 promoter activity experiments with dimaprit and ICAT (inhibitor of catenin and T cell factor), cells were transfected with 1.6 μg of full-length MMP-2 and 0.16 μg pRenilla, 24 h after plating using LipofectAMINE LTX and Plus Reagent (Invitrogen) according to manufacturer's instructions. Transfection media were removed after 4 h incubation and was replaced with cDMEM containing 300 pfu/cell of either Ad-Bgal or Ad-ICAT. Twenty-four hours later, cells were stimulated with 50 μM dimaprit. Promoter assay was performed as described earlier.

CELL PROLIFERATION ASSAY

Proliferation of skeletal muscle endothelial cells was measured using the CyQUANT Cell Proliferation Assay kit (Molecular Probes, Inc.) as previously described [Uchida et al., 2008] using serial dilutions from 250 to 10,000 cells. Four hours after plating, cells were stimulated with 10, 50, or 100 μM dimaprit and left for 48 h. Proliferation values for treated samples are expressed as a percentage of untreated sample values.

STATISTICAL ANALYSIS

Data are presented as means ± standard error. Comparisons between groups were performed using one-way ANOVA followed by Tukey post hoc or Bonferroni's multiple comparison tests. Statistical significance was set at $P < 0.05$.

RESULTS

VEGF STIMULATION ALTERS β-CATENIN CELLULAR LOCALIZATION IN A PI3K-DEPENDENT MANNER

In microvascular endothelial cells under quiescent conditions, β-catenin is localized to areas of cell–cell contact (arrows) (Fig. 1A, top). However, overnight stimulation with VEGF (20 ng/ml) increased nuclear accumulation of β-catenin (Fig. 1A, bottom; arrowheads). Western blot analysis showed no change in total cellular protein levels of β-catenin in response to VEGF (Fig. 1B). Cytoplasmic extracts also showed no change in β-catenin levels with VEGF (Fig. 1C). However, a significant increase in nuclear β-catenin was observed with VEGF treatment (Fig. 1D).

The nuclear translocation of β-catenin is, in part, dependent on inactivation of the degradation complex through phosphorylation of GSK-3β. This phosphorylation has been shown to occur via

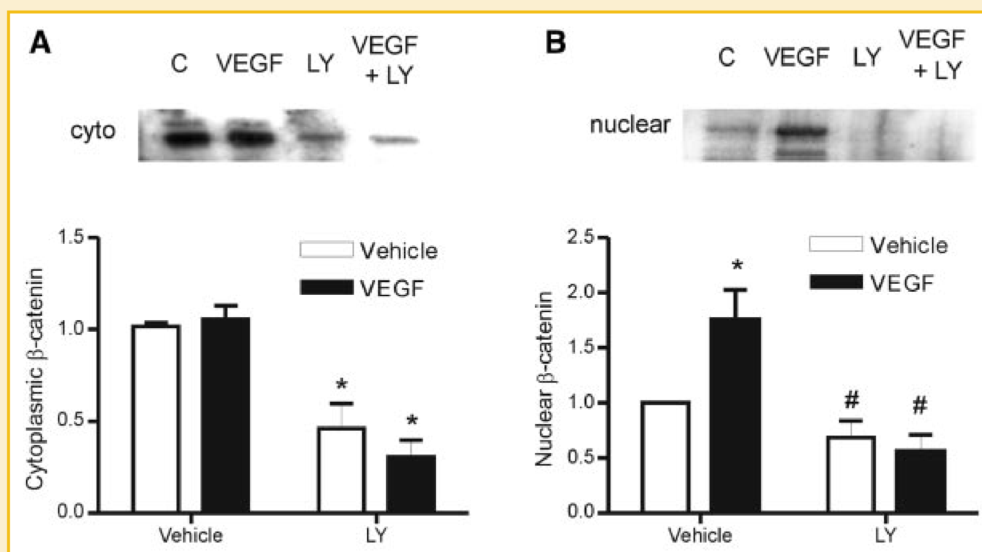

Fig. 2. Inhibition of PI3K pathway blocks VEGF-induced nuclear levels of β-catenin. Endothelial cells were pre-treated for 3 h with the PI3K inhibitor LY294002 (LY) at 50 μM and stimulated with 20 ng/ml of VEGF overnight. Western blotting for β-catenin was performed on cytoplasmic (cyto) (A) and nuclear (B) extracts. Values are mean ± SEM, $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus VEGF, n = 4.

activation of PI3K and Akt and is downstream of VEGF stimulation [Cross et al., 1995; Testa and Bellacosa, 2001; Doble and Woodgett, 2003]. The involvement of the PI3K pathway in increased levels of nuclear β-catenin was assessed by pre-treating microvascular endothelial cells with the PI3K inhibitor LY294002 (50 μM) prior to stimulation with VEGF (20 ng/ml). PI3K inhibition significantly decreased cytoplasmic levels of β-catenin to below control levels, which could not be recovered with VEGF stimulation (Fig. 2A). The VEGF-induced increase in nuclear β-catenin levels was abolished by LY294002 pre-treatment (Fig. 2B).

VEGF ALTERS MMP-2 PROTEIN AND mRNA LEVELS IN MICROVASCULAR ENDOTHELIAL CELLS

VEGF stimulated a significant concentration-dependent increase in latent MMP-2 (72 kDa) levels, but did not alter active MMP-2 levels (62 kDa) (Fig. 3A). RT-PCR confirmed that VEGF stimulation induced a significant increase in MMP-2 mRNA but did not alter MT1-MMP levels (Fig. 3B).

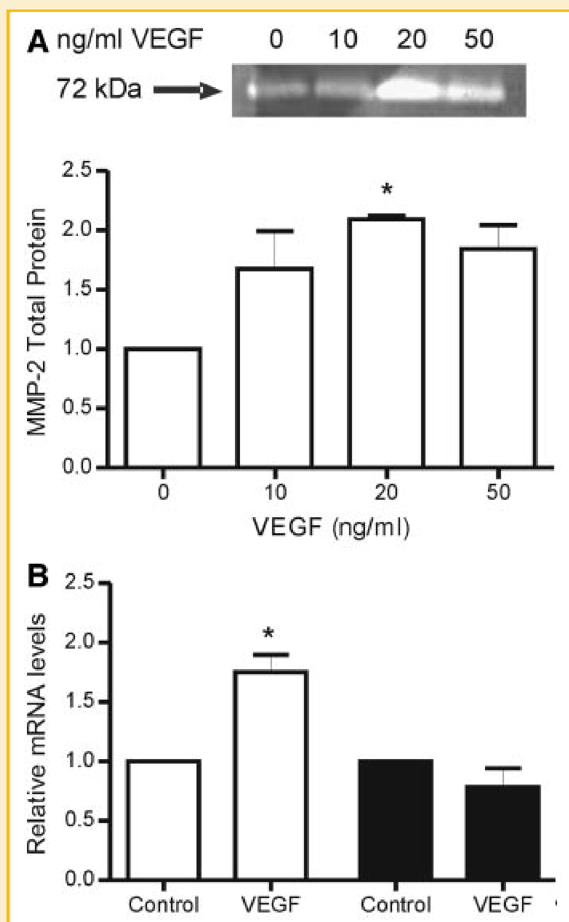

Fig. 3. VEGF increases MMP-2 protein and mRNA levels. Endothelial cells were treated for 24 h with 0, 10, 20, or 50 ng/ml of VEGF. MMP-2 total protein was quantified using gelatin zymography (A). Endothelial cells were stimulated overnight with 20 ng/ml of VEGF and MMP-2 (white bars) and MT1-MMP (black bars) mRNA levels were assessed by RT-PCR (B). Values are mean ± SEM, $^{*}P < 0.05$ versus control, n = 3 for gelatin zymography, n = 4 for MMP-2, and n = 2 for MT1-MMP mRNA.

β-CATENIN UPREGULATES BOTH MMP-2 AND MT1-MMP PROMOTER ACTIVITY

Putative TBEs were identified in both the MMP-2 and MT1-MMP promoters (Fig. 4A). In the murine MT1-MMP promoter, one site, at position −1274 to −1268 relative to the start codon, corresponded to a previously reported TBE defined in the human MT1-MMP promoter. Putative binding elements were also identified at positions −2323 to −2319, −2244 to −2249, and −1794 to −1790. Within the rat MMP-2 promoter, we identified several putative binding elements clustered at bases −710 to −706, −668 to −664, −635 to −631, and −605 to −600 relative to the start codon. Co-transfection of β-catenin cDNA with promoter–reporter constructs resulted in 1.5- and 2.5-fold increases in the activity of the full-length MMP-2 and MT1-MMP promoters, respectively (Fig. 4B,C). MMP-2 promoter truncations containing the putative TCF-binding sites retained increased activity when co-transfected with β-catenin compared to those truncations not containing the putative sites. Truncation of the MT1-MMP promoter at −1398 resulted in decreased β-catenin responsiveness compared to the full-length promoter, despite the continued presence of a TCF-binding site.

INHIBITION OF β-CATENIN BLOCKS VEGF-INDUCED CHANGES IN MMP-2 TRANSCRIPTION

ICAT is an endogenous protein that blocks the interaction of β-catenin with its co-activator TCF, thereby eliminating the ability of β-catenin to bind to and activate the promoter of target genes [Gottardi and Gumbiner, 2004]. Ad-ICAT transduction alone led to a modest, yet significant increase in MMP-2 mRNA levels. Significantly, Ad-ICAT blocked the VEGF-induced increase in MMP-2 mRNA (Fig. 5A).

HISTAMINE INCREASES NUCLEAR ACCUMULATION OF β-CATENIN IN ENDOTHELIAL CELLS

Compared to quiescent, unstimulated microvascular endothelial cells, where β-catenin is localized to cell junctions (Fig. 6A, top; arrows), cells stimulated with histamine (400 μM) showed an increase in β-catenin localized to the nucleus (Fig. 6A, bottom; arrowheads). Immunofluoresence results were confirmed using Western blotting. Both total cellular protein levels (Fig. 6B) and cytoplasmic levels of β-catenin (Fig. 6C) were unchanged with histamine stimulation. Histamine induced a significant, concentration-dependent increase in nuclear levels of β-catenin (Fig. 6D).

HISTAMINE STIMULATION ACTIVATES THE PI3K PATHWAY

Histamine stimulation significantly increased phospho-Akt levels within 4 h of stimulation and levels of the phosphorylated protein remained elevated with overnight treatment (Fig. 7A,B). Likewise, endothelial cells stimulated overnight with 400 μM histamine showed a significant increase in phosphorylated levels of GSK-3β (Fig. 7C). To assess if PI3K pathway activation is required for the increase in nuclear levels of β-catenin following histamine stimulation, cells were treated with the PI3K inhibitor LY294002

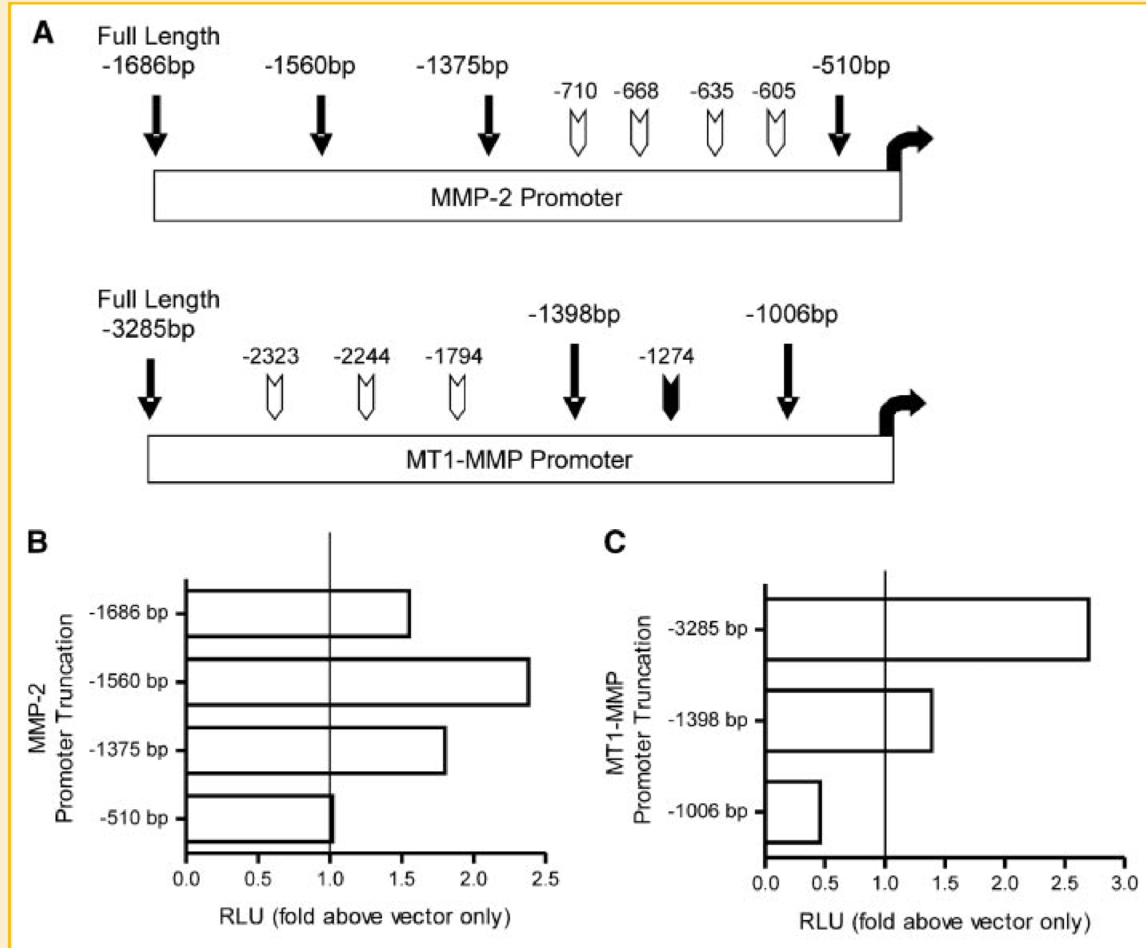

Fig. 4. MMP-2 and MT1-MMP promoter activities are altered by β-catenin transfection. Schematic depicting putative TCF-binding elements in the rat MMP-2 and murine MT1-MMP promoters (A). Arrows indicate location of truncations and chevrons depict putative TCF-binding elements. Endothelial cells were co-transfected with the MMP-2 (B) or MT1-MMP (C) promoter truncations and either β-catenin or pcDNA3. Values are normalized to cells transfected with MMP-2/MT1-MMP promoter truncation co-transfected with empty vector. Results from single representative experiments are shown. Experiments were performed three times with similar results.

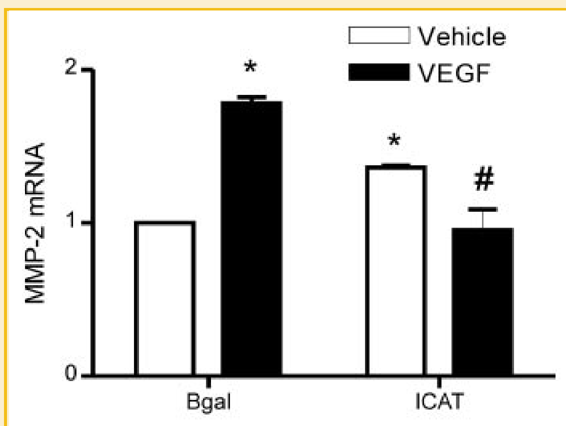

Fig. 5. Inhibition of β-catenin signaling blocks VEGF-induced increase in MMP-2 mRNA. Endothelial cells were transduced with 300 pfu/cell Ad-βgal or Ad-ICAT and 24 h later stimulated with 20 ng/ml of VEGF. MMP-2 mRNA levels were assessed using real-time RT-PCR. $^{*}P < 0.05$ versus βgal, $^{\#}P < 0.05$ versus βgal + VEGF, n = 3.

(50 μM) prior to histamine stimulation. PI3K inhibition significantly reduced cytoplasmic levels of β-catenin below control levels, which could not be increased with histamine stimulation (Fig. 7D). The level of nuclear β-catenin also was reduced significantly following histamine stimulation in cells pre-treated with the PI3K inhibitor (Fig. 7E).

HISTAMINE INCREASES MMP-2 mRNA AND PROTEIN LEVELS

Histamine treatment caused a significant, concentration-dependent increase in latent MMP-2 (72 kDa) levels, but had no effect on active MMP-2 (62 kDa) (Fig. 8A). RT-PCR confirmed that histamine stimulation induced a significant increase in MMP-2 mRNA levels, but did not alter levels of MT1-MMP mRNA (Fig. 8B).

β-CATENIN INHIBITION ALTERS HISTAMINE-INDUCED EFFECTS ON MMP-2 mRNA

Ad-ICAT was used to inhibit β-catenin-dependent promoter transactivation to examine the role of β-catenin in histamine-induced MMP-2 mRNA expression. Histamine stimulation alone

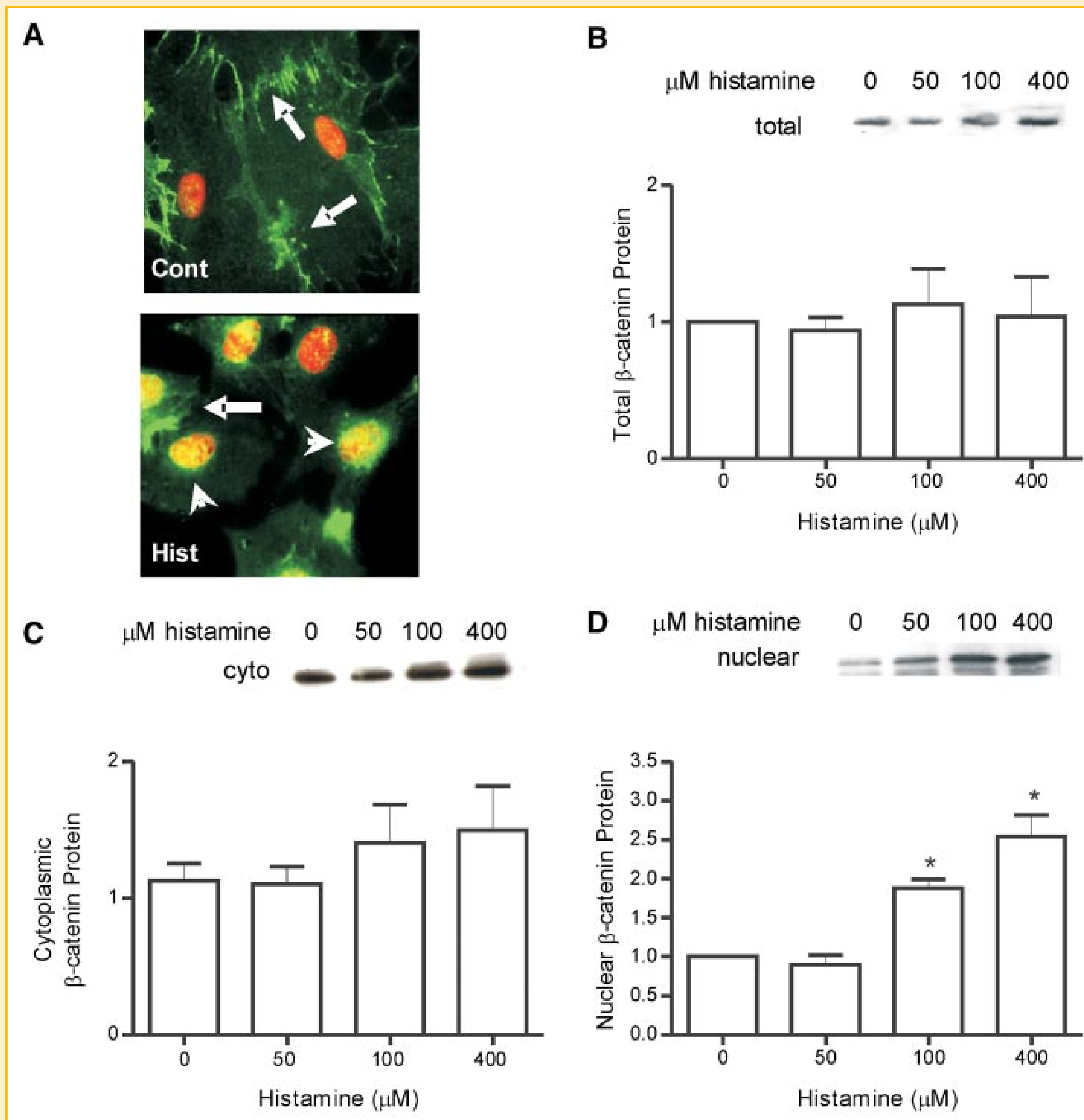

Fig. 6. Histamine stimulation induces nuclear translocation of β-catenin. Endothelial cells were cultured under control (Cont—top) and 24 h histamine-stimulated (400 μM) conditions (Hist—bottom). Cells were fixed and stained for β-catenin (green) and DAPI (red nuclei). Arrows indicate junctional β-catenin and arrowheads indicate nuclear localization. Endothelial cells were treated for 24 h with 0, 50, 100, and 400 μM histamine. Quantification of total, cytoplasmic, and nuclear β-catenin is shown in panels B, C, and D, respectively. Values are mean ± SEM, $^{*}P < 0.05$ versus control, n = 3 for total and cytoplasmic, and n = 5 for nuclear extracts. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

increased MMP-2 mRNA levels. However, Ad-ICAT transduction in combination with histamine stimulation further increased MMP-2 mRNA levels (Fig. 9A). This effect was explored further using histamine receptor-specific agonists HTMT (H1 receptor agonist) and dimaprit (H2 receptor agonist). Both HTMT and dimaprit stimulation overnight induced increases in MMP-2 mRNA levels (Fig. 9 B,C). As was observed with histamine treatment, Ad-ICAT transduction resulted in significantly greater increases in MMP-2 mRNA levels compared to dimaprit-alone (Fig. 9C). Because the response to dimaprit appeared to closely parallel that seen with histamine, the effects of dimaprit on MMP-2 promoter activity and proliferation also were assessed. Dimaprit treatment significantly increased MMP-2 promoter activity. Interestingly, this increase was inhibited partially when dimaprit-stimulated cells were transduced with Ad-ICAT (Fig. 9D). Dimaprit stimulation resulted in a significant, concentration-dependent decrease in endothelial cell proliferation 48 h after stimulation (Fig. 9E).

DISCUSSION

This study revealed that β-catenin functions as a transcriptional co-activator of both MT1-MMP and MMP-2 in microvascular endothelial cells. Increased nuclear translocation of β-catenin and

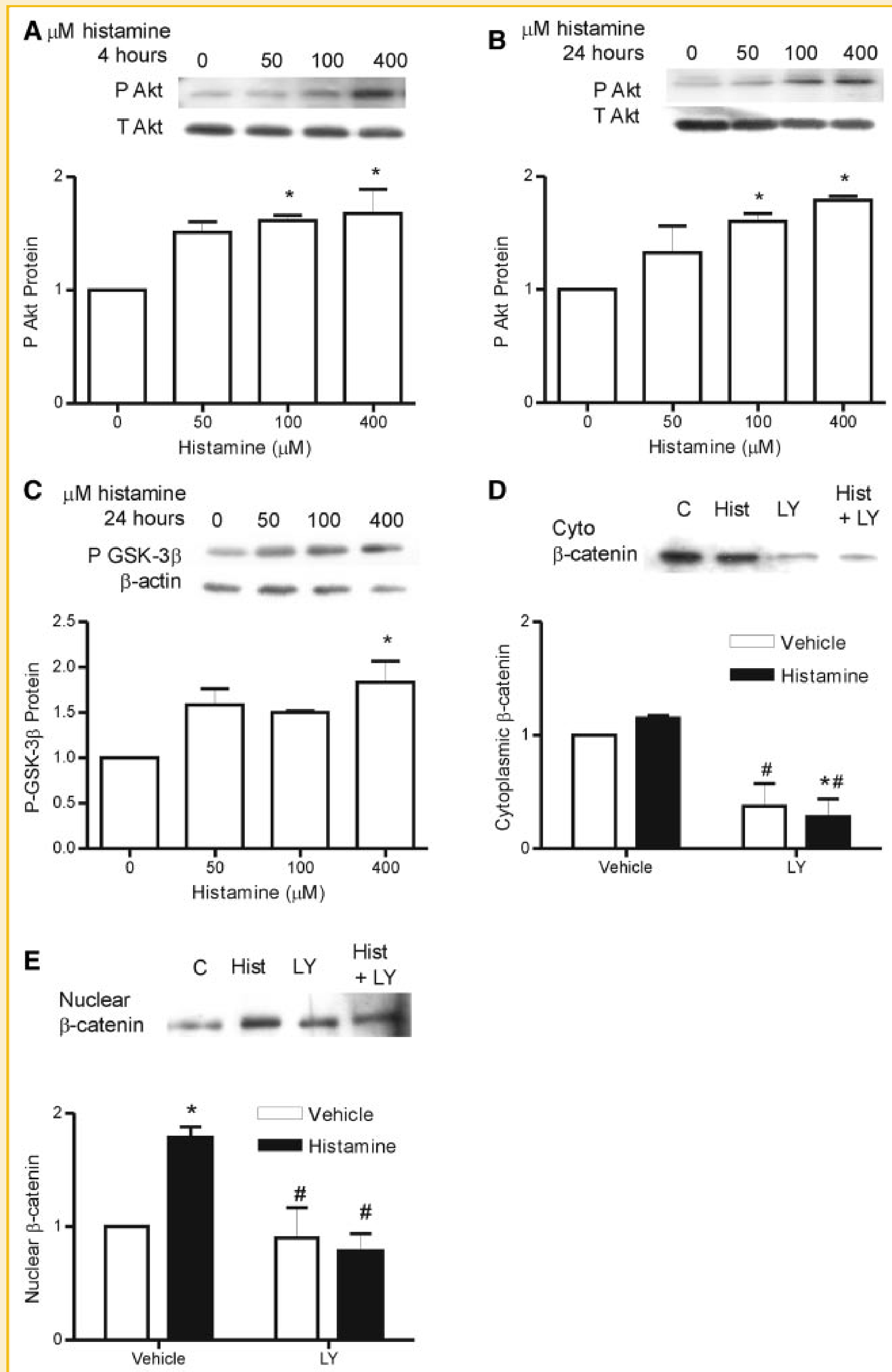

Fig. 7. PI3K pathway is activated by histamine stimulation. Endothelial cells were treated with 0, 50, 100, or 400 µM histamine and Western blotting was used to assess P-Akt following 4 h stimulation (A), or following 24 h stimulation (B). P-GSK-3β levels also were assessed following 24 h histamine stimulation (C). Phospho levels were normalized to total (T) Akt or beta-actin, respectively. Endothelial cells were pre-treated for 3 h with 50 µM PI3K inhibitor LY294002 (LY) and stimulated with 400 µM histamine (HIST) overnight. Western blotting for β-catenin was performed on cytoplasmic (cyto) (D) and nuclear (E) extracts. In all experiments, values are mean ± SEM, $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus histamine, n = 3.

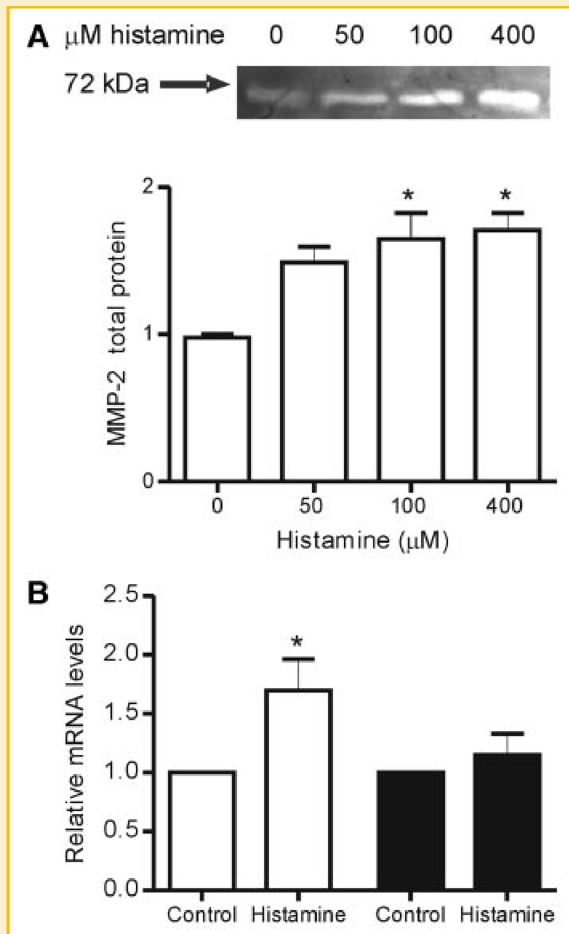

Fig. 8. Histamine stimulation increases MMP-2 protein and mRNA levels. Endothelial cells were treated overnight with 0, 50, 100, or 400 µM histamine. MMP-2 total protein was quantified using gelatin zymography (A). Endothelial cells were stimulated overnight with 400 µM histamine with MMP-2 (white bars) and MT1-MMP (black bars) mRNA levels assessed by qRT-PCR (B). Values are mean ± SEM, $^{*}P < 0.05$ versus control, n = 3 for gelatin zymography, n = 6 for MMP-2, and n = 5 for MT1-MMP.

increased production of MMP-2 mRNA and protein occurred following stimulation with either of two potent permeability factors, VEGF and histamine. MMP-2 promoter activity was increased significantly in endothelial cells co-transfected with β-catenin. Inhibition of β-catenin signaling revealed that the effects of VEGF on MMP-2 transcription are β-catenin dependent as ICAT blocked the VEGF-induced increase in MMP-2 mRNA. In contrast, blockade of β-catenin signaling in histamine, histamine H1, or histamine H2 receptor agonist-stimulated cells augmented the increase in MMP-2 mRNA levels, indicating a more complex involvement of β-catenin in response to histamine receptor activation.

Treatment of endothelial cells with VEGF or histamine increased nuclear β-catenin as detected by immunostaining and protein analysis of nuclear extracts. The increase in nuclear β-catenin in response to VEGF stimulation confirms a previous report in HUVEC [Ilan et al., 2003], which showed increased nuclear β-catenin by immunofluoresence and increased cyclin D1 mRNA levels. We extend these findings by observing an increase in nuclear translocation of β-catenin in endothelial cells following histamine treatment. The increases in nuclear β-catenin in response to histamine and VEGF stimulation can be attributed to changes in protein localization rather than increases in protein synthesis, as no change in total cellular levels of β-catenin was seen with either treatment.

Interestingly, histamine stimulation caused the phosphorylation of GSK-3β on serine 9, which is known to cause GSK-3β inactivation. Both the VEGF and histamine-dependent increases in nuclear β-catenin were attenuated significantly through the use of the PI3K inhibitor LY294002. These findings are consistent with published studies showing that Akt, the downstream target of PI3K, inhibits GSK-3β signaling through phosphorylation [Testa and Bellacosa, 2001]. While our results confirm the previously established finding that VEGFR2 signaling activates Akt, leading to GSK-3β inactivation and reduced degradation of β-catenin [Skurk et al., 2005], the relationship between histamine stimulation and PI3K activation has not been previously reported. Diks et al. [2003] reported that histamine is able to stabilize β-catenin through phosphorylation of GSK-3β, but suggested that this occurs independent of PI3K signaling. In contrast to their observations, which were based on studies in HeLa cells, we observed a significant and prolonged phosphorylation of Akt in endothelial cells treated with histamine. The signal intermediaries linking histamine receptor activation and the PI3K signaling pathway remain to be identified.

Histamine is proangiogenic [Sorbo et al., 1994] and is released from mast cells under angiogenic or inflammatory conditions [Boesiger et al., 1998]; however, its role in the angiogenic process remains largely unexplored. Recent studies using histidine decarboxylase gene knockout mice, which lack histamine production, showed a delayed wound healing angiogenesis response that was improved by exogenous histamine [Numata et al., 2006]. Previous results by Chancey et al. [2002] demonstrated that degranulation of cardiac mast cells increased MMP-2 production. This degranulation however, would also lead to release of VEGF and numerous other factors. Therefore, our study provides further direct evidence of the stimulation of MMP-2 protein production by histamine.

Neither VEGF nor histamine altered active levels of MMP-2 under the experimental conditions we used. Activation of the proMMP-2 enzyme occurs primarily through the formation of a complex with MT1-MMP and tissue inhibitor of MMP-2 (TIMP-2) on the cell surface [Sato et al., 1997; Seiki, 2003]. RT-PCR analysis of MT1-MMP mRNA levels revealed no change in response to VEGF or histamine treatment, consistent with the observed absence of active MMP-2.

Hlubek et al. [2004] showed that binding of the β-catenin/TCF complex to the human MT1-MMP promoter occurs in carcinoma cells. Analysis of the mouse MT1-MMP promoter revealed conservation of this sequence CTTTGTT at position −1274 to −1268. Further analysis of the murine MT1-MMP promoter sequence revealed other putative TBEs containing the core CTTTG sequence in the forward and reverse direction (positions −2323 to −2319, −2249 to −2244, −1823 to −1819, and −1790 to −1794), which could account for the higher MT1-MMP promoter activity of

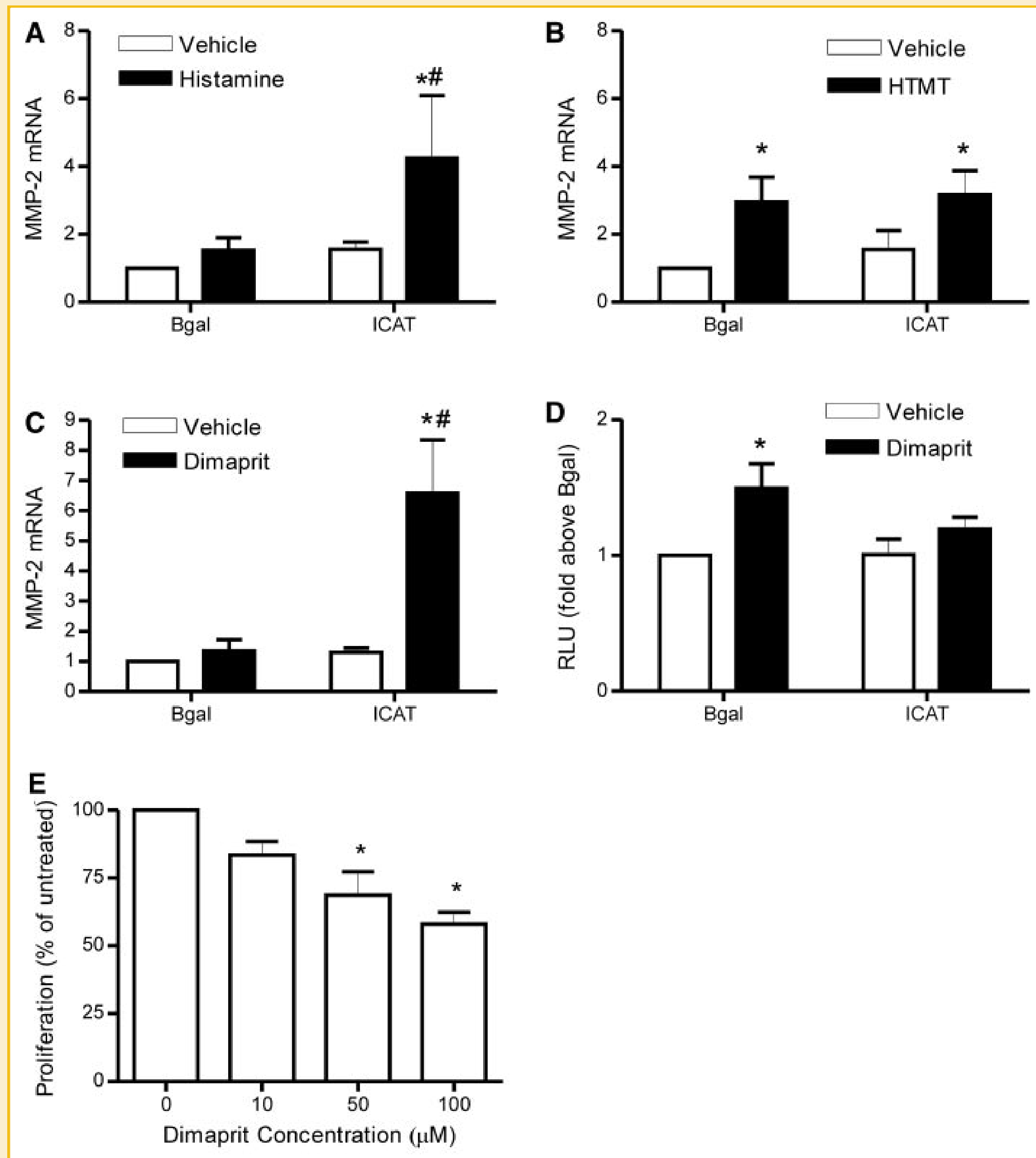

Fig. 9. Inhibition of β-catenin signaling alters the histamine-induced increases in MMP-2 mRNA. Endothelial cells were transduced with 300 pfu/cell Ad-βgal or Ad-ICAT and 24 h later were stimulated with 100 μM histamine (A), 50 μM histamine receptor 1 agonist HTMT (B), and 50 μM histamine receptor 2 agonist dimaprit (C). MMP-2 mRNA levels were assessed by real-time RT-PCR. $^{*}P < 0.05$ versus βgal, $^{\#}P < 0.05$ versus histamine or dimaprit; n = 3 for histamine, n = 6 for HTMT, and n = 7 for dimaprit. (D) Cells were transfected with the full-length MMP-2 promoter, transduced with 300 pfu/cell of Ad-βgal or Ad-ICAT and stimulated with 50 μM dimaprit. Forty-eight hours later, cells were lysed and analyzed for luciferase activity, $^{*}P < 0.05$ versus βgal, n = 6. (E) Skeletal muscle endothelial cells were stimulated for 48 h with 0, 10, 50, and 100 μM dimaprit and proliferation was assessed. Values are presented as a percentage of the proliferation rate of time-matched unstimulated cells, $^{*}P < 0.05$ versus control, n = 4.

the full length versus −1398 bp truncation with β-catenin co-transfection. Currently, it is not clear why endogenous MT1-MMP mRNA levels are not altered following VEGF or histamine treatment. The discrepancy between endogenous gene and promoter–reporter responses suggests silencing of endogenous MT1-MMP through epigenetic mechanisms not affecting the transfected promoter construct.

Recently, MMP-2 transcription by β-catenin via Wnt stimulation was observed in T cells [Wu et al., 2007]. This study revealed interactions between β-catenin and MMP-2 promoter at two TBEs in the human MMP-2 promoter at positions −861 and −804 of the sequence CTTTG [Wu et al., 2007]. We also identified several additional putative TBE in the rat MMP-2 promoter at position −605 to −600 (TTCAAT) [Easwaran et al., 2003] and at positions −706 to

−710, −668 to −664, and −631 to −635 (core CTTTG sequence in reverse and forward directions). Promoter truncations lacking these sites showed a reduced response to β-catenin compared with truncations containing these putative TBE, indicating the functionality of at least one of these sites.

To further define the importance of β-catenin in the response of MMP-2 to VEGF or histamine treatment, we utilized ICAT to disrupt β-catenin co-activation of transcription. ICAT reduced MMP-2 mRNA levels following VEGF stimulation, confirming a positive role of β-catenin-mediated enhancement of MMP-2 transcription in microvascular endothelial cells. We and others previously reported that c-jun activates MMP-2 transcription in response to VEGF [Laderoute et al., 2001; Zhang et al., 2004; Ispanovic and Haas, 2006]. Interaction of c-jun with TCF and β-catenin has been shown to increase c-jun transcriptional activity [Nateri et al., 2005]. It is possible that ICAT suppression of MMP-2 transcription following VEGF treatment occurs as a result of disrupting the formation of both β-catenin–TCF and c-jun–β-catenin–TCF transcriptional complexes.

We did not expect to observe a further increase in MMP-2 mRNA in cells transduced with ICAT and stimulated with histamine. This response was mimicked in cells treated with the histamine H1 receptor agonist HTMT and the histamine H2 receptor agonist dimaprit. β-catenin interacts with numerous transcription factors including c-jun [Nateri et al., 2005], c-fos [Toualbi et al., 2007], CREB/p300 [Hecht et al., 2000], and FOXO [Essers et al., 2005; Hoogeboom et al., 2008] and ICAT likely disrupts these interactions because it binds to the armadillo domain of β-catenin, the region through which β-catenin interacts with almost all other binding partners. Thus, the effects of ICAT treatment may vary depending on the unique set of transcription factors present in the local environment following a given stimulus. We do not think that β-catenin functions as a repressor of MMP-2 transcription, considering that increased levels of mRNA were observed in response to histamine or selective H1 and H2 receptor activation. Rather, our data are consistent with a model in which histamine H1 or H2 receptor activation increases nuclear β-catenin levels and allows for interaction between β-catenin and other factors that are increased/activated following histamine stimulation. This results in low transcriptional activation of MMP-2. Co-treatment with ICAT disrupts the interactions between β-catenin and other nuclear factors, which may allow the formation of other transcriptional complexes that exert greater *trans*-activation of MMP-2. Further work is required to identify specific transcription factor interactions with β-catenin following histamine receptor activation. We used a promoter–reporter assay in order to identify specific promoter regions required for the increased transcriptional activity observed in the presence of ICAT. However, we found that dimaprit-induced MMP-2 promoter activity was inhibited partially with Ad-ICAT transduction, as had been observed in response to VEGF. This suggests that the effect of β-catenin transcriptional complex formation depends on the structure of the native MMP-2 promoter.

β-catenin has a well-defined role as an activator of genes involved in proliferation including cyclin D1 and c-myc [He et al., 1998; Shtutman et al., 1999]. However, our studies show that, despite increases in nuclear β-catenin, the histamine receptor agonist dimaprit inhibits proliferation of microvascular endothelial cells. This response provides further evidence that histamine does not trigger the conventional pattern of β-catenin transcriptional events.

In summary, we have provided evidence that histamine and VEGF, which are potent permeability agents that have documented roles in physiological and pathological conditions including tumor growth and inflammation, are capable of increasing latent MMP-2 production. β-catenin appears to play an integral role in VEGF-induced MMP-2 production. Conversely, while histamine clearly induces nuclear translocation of β-catenin, other transcription factors activated by histamine stimulation appear to play a stronger role in regulating MMP-2 production. These studies help to clarify the mechanisms by which VEGF and histamine promote a cellular environment that promotes capillary growth.

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REFERENCES

Andriopoulou P, Navarro P, Zanetti A, Lampugnani MG, Dejana E. 1999. Histamine induces tyrosine phosphorylation of endothelial cell-to-cell adherens junctions. Arterioscler Thromb Vasc Biol 19:2286–2297.

Ausprunk DH, Folkman J. 1977. Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. Microvasc Res 14:53–65.

Bazzoni G, Dejana E. 2004. Endothelial cell-to-cell junctions: Molecular organization and role in vascular homeostasis. Physiol Rev 84:869–901.

Ben Ze'ev A, Geiger B. 1998. Differential molecular interactions of beta-catenin and plakoglobin in adhesion, signaling and cancer. Curr Opin Cell Biol 10:629–639.

Bienz M, Clevers H. 2000. Linking colorectal cancer to Wnt signaling. Cell 103:311–320.

Biswas P, Canosa S, Schoenfeld D, Schoenfeld J, Li P, Cheas LC, Zhang J, Cordova A, Sumpio B, Madri JA. 2006. PECAM-1 affects GSK-3beta-mediated beta-catenin phosphorylation and degradation. Am J Pathol 169:314–324.

Boesiger J, Tsai M, Maurer M, Yamaguchi M, Brown LF, Claffey KP, Dvorak HF, Galli SJ. 1998. Mast cells can secrete vascular permeability factor/vascular endothelial cell growth factor and exhibit enhanced release after immunoglobulin E-dependent upregulation of fc epsilon receptor I expression. J Exp Med 188:1135–1145.

Chancey AL, Brower GL, Janicki JS. 2002. Cardiac mast cell-mediated activation of gelatinase and alteration of ventricular diastolic function. Am J Physiol Heart Circ Physiol 282:H2152–H2158.

Collares-Buzato CB, Jepson MA, Simmons NL, Hirst BH. 1998. Increased tyrosine phosphorylation causes redistribution of adherens junction and tight junction proteins and perturbs paracellular barrier function in MDCK epithelia. Eur J Cell Biol 76:85–92.

Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378:785–789.

Diks SH, Hardwick JC, Diab RM, van Santen MM, Versteeg HH, van Deventer SJ, Richel DJ, Peppelenbosch MP. 2003. Activation of the canonical beta-catenin pathway by histamine. J Biol Chem 278:52491–52496.

Ding VW, Chen RH, McCormick F. 2000. Differential regulation of glycogen synthase kinase 3beta by insulin and Wnt signaling. J Biol Chem 275:32475–32481.

Doble BW, Woodgett JR. 2003. GSK-3: Tricks of the trade for a multi-tasking kinase. J Cell Sci 116:1175–1186.

Easwaran V, Lee SH, Inge L, Guo L, Goldbeck C, Garrett E, Wiesmann M, Garcia PD, Fuller JH, Chan V, Randazzo F, Gundel R, Warren RS, Escobedo J, Aukerman SL, Taylor RN, Fantl WJ. 2003. Beta-catenin regulates vascular endothelial growth factor expression in colon cancer. Cancer Res 63:3145–3153.

Essers MA, Vries-Smits LM, Barker N, Polderman PE, Burgering BM, Korswagen HC. 2005. Functional interaction between beta-catenin and FOXO in oxidative stress signaling. Science 308:1181–1184.

Goodwin AM, Sullivan KM, D'Amore PA. 2006. Cultured endothelial cells display endogenous activation of the canonical Wnt signaling pathway and express multiple ligands, receptors, and secreted modulators of Wnt signaling. Dev Dyn 235:3110–3120.

Gottardi CJ, Gumbiner BM. 2004. Role for ICAT in beta-catenin-dependent nuclear signaling and cadherin functions. Am J Physiol Cell Physiol 286: C747–C756.

Haas TL, Davis SJ, Madri JA. 1998. Three-dimensional type I collagen lattices induce coordinate expression of matrix metalloproteinases MT1-MMP and MMP-2 in microvascular endothelial cells. J Biol Chem 273:3604–3610.

Haas TL, Stitelman D, Davis SJ, Apte SS, Madri JA. 1999. Egr-1 mediates extracellular matrix-driven transcription of membrane type 1 matrix metalloproteinase in endothelium. J Biol Chem 274:22679–22685.

Han X, Boyd PJ, Colgan S, Madri JA, Haas TL. 2003. Transcriptional up-regulation of endothelial cell matrix metalloproteinase-2 in response to extracellular cues involves GATA-2. J Biol Chem 278:47785–47791.

He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. 1998. Identification of c-MYC as a target of the APC pathway. Science 281:1509–1512.

Hecht A, Vleminckx K, Stemmler MP, van Roy F, Kemler R. 2000. The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. EMBO J 19:1839–1850.

Hlubek F, Spaderna S, Jung A, Kirchner T, Brabletz T. 2004. Beta-catenin activates a coordinated expression of the proinvasive factors laminin-5 gamma2 chain and MT1-MMP in colorectal carcinomas. Int J Cancer 108:321–326.

Hoogeboom D, Essers MA, Polderman PE, Voets E, Smits LM, Burgering BM. 2008. Interaction of FOXO with beta-catenin inhibits beta-catenin/T cell factor activity. J Biol Chem 283:9224–9230.

Ilan N, Tucker A, Madri JA. 2003. Vascular endothelial growth factor expression, beta-catenin tyrosine phosphorylation, and endothelial proliferative behavior: A pathway for transformation? Lab Invest 83:1105–1115.

Ispanovic E, Haas TL. 2006. JNK and PI3K differentially regulate MMP-2 and MT1-MMP mRNA and protein in response to actin cytoskeleton reorganization in endothelial cells. Am J Physiol Cell Physiol 291:C579–C588.

Laderoute KR, Calaoagan JM, Knapp AM, Mendonca HL, Johnson RS. 2001. c-jun cooperates with SV40 T-antigen to sustain MMP-2 expression in immortalized cells. Biochem Biophys Res Commun 284:1134–1139.

Madri JA, Williams SK. 1983. Capillary endothelial cell cultures: Phenotypic modulation by matrix components. J Cell Biol 97:153–165.

Milkiewicz M, Haas TL. 2005. Effect of mechanical stretch on HIF-1{alpha} and MMP-2 expression in capillaries isolated from overloaded skeletal muscles: Laser capture microdissection study. Am J Physiol Heart Circ Physiol 289:H1315–H1320.

Nateri AS, Spencer-Dene B, Behrens A. 2005. Interaction of phosphorylated c-Jun with TCF4 regulates intestinal cancer development. Nature 437:281–285.

Numata Y, Terui T, Okuyama R, Hirasawa N, Sugiura Y, Miyoshi I, Watanabe T, Kuramasu A, Tagami H, Ohtsu H. 2006. The accelerating effect of histamine on the cutaneous wound-healing process through the action of basic fibroblast growth factor. J Invest Dermatol 126:1403–1409.

Sato H, Okada Y, Seiki M. 1997. Membrane-type matrix metalloproteinases (MT-MMPs) in cell invasion. Thromb Haemost 78:497–500.

Seiki M. 2003. Membrane-type 1 matrix metalloproteinase: A key enzyme for tumor invasion. Cancer Lett 194:1–11.

Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben Ze'ev A. 1999. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci USA 96:5522–5527.

Skurk C, Maatz H, Rocnik E, Bialik A, Force T, Walsh K. 2005. Glycogen-synthase kinase3beta/beta-catenin axis promotes angiogenesis through activation of vascular endothelial growth factor signaling in endothelial cells. Circ Res 96:308–318.

Sorbo J, Jakobsson A, Norrby K. 1994. Mast-cell histamine is angiogenic through receptors for histamine1 and histamine2. Int J Exp Pathol 75: 43–50.

Stappert J, Kemler R. 1994. A short core region of E-cadherin is essential for catenin binding and is highly phosphorylated. Cell Adhes Commun 2:319–327.

Takahashi M, Tsunoda T, Seiki M, Nakamura Y, Furukawa Y. 2002. Identification of membrane-type matrix metalloproteinase-1 as a target of the beta-catenin/Tcf4 complex in human colorectal cancers. Oncogene 21:5861–5867.

Takeichi M. 1990. Cadherins: A molecular family important in selective cell-cell adhesion. Annu Rev Biochem 59:237–252.

Takeichi M. 1995. Morphogenetic roles of classic cadherins. Curr Opin Cell Biol 7:619–627.

Testa JR, Bellacosa A. 2001. AKT plays a central role in tumorigenesis. Proc Natl Acad Sci USA 98:10983–10985.

Tetsu O, McCormick F. 1999. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 398:422–426.

Thoreson MA, Anastasiadis PZ, Daniel JM, Ireton RC, Wheelock MJ, Johnson KR, Hummingbird DK, Reynolds AB. 2000. Selective uncoupling of p120(ctn) from E-cadherin disrupts strong adhesion. J Cell Biol 148:189–202.

Toualbi K, Guller MC, Mauriz JL, Labalette C, Buendia MA, Mauviel A, Bernuau D. 2007. Physical and functional cooperation between AP-1 and beta-catenin for the regulation of TCF-dependent genes. Oncogene 26:3492–3502.

Uchida C, Gee E, Ispanovic E, Haas TL. 2008. JNK as a positive regulator of angiogenic potential in endothelial cells. Cell Biol Int 32:769–776.

van de Wetering M, Cavallo R, Dooijes D, van Beest M, van Es J, Loureiro J, Ypma A, Hursh D, Jones T, Bejsovec A, Peifer M, Mortin M, Clevers H. 1997. Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. Cell 88:789–799.

Vestweber D. 2000. Molecular mechanisms that control endothelial cell contacts. J Pathol 190:281–291.

Woessner JF, Jr. 1994. The family of matrix metalloproteinases. Ann N Y Acad Sci 732:11–21.

Wu B, Crampton SP, Hughes CC. 2007. Wnt signaling induces matrix metalloproteinase expression and regulates T cell transmigration. Immunity 26:227–239.

Zhang G, Dass CR, Sumithran E, Di Girolamo N, Sun LQ, Khachigian LM. 2004. Effect of deoxyribozymes targeting c-Jun on solid tumor growth and angiogenesis in rodents. J Natl Cancer Inst 96:683–696.