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Protein kinase C isozymes regulate matrix metalloproteinase-1 expression and cell invasion in *Helicobacter pylori* infection

DATA SUPPLEMENT

SUPPLEMENTARY MATERIAL AND METHODS

Preparation of cell lysates and immunoblotting

Whole-cell extracts were prepared with a modified RIPA buffer as described.[1] Aliquots of the lysates were boiled with sample buffer comprising 50 mM Tris-HCl, pH 6.8, 2% SDS, 10% Glycerol, 100 mM DTT, and 0.1% bromphenol blue for 5 min. Sub-cellular fractions of AGS cells were prepared with a ProteoExtract kit (Calbiochem/Merk KGaA) according to the manufacturer's instructions. The proteins were separated by SDS-PAGE, electrotransferred onto Immobilon-P transfer polyvinylidene fluoride membranes (Millipore, Schwalbach, Germany) and stained with antibodies overnight at +4°C. Immunoreactivity was detected using the enhanced chemiluminescence detection kit Amersham™ ECL™ (GE Healthcare, Buckinghamshire, UK).

RNA isolation and RT-PCR

Total RNA was extracted with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and further cleaned using RNeasy® Plus Micro kit (Qiagen GmbH, Hilden, Germany). cDNA was synthesized from 1 µg of RNA using a random hexamer primer and RevertAid™ First Strand cDNA Synthesis kit (Fermentas, EU). cDNA was amplified as described[1] using following primers: 5'-CTGAAGGTGATGAAGCAGCC-3' (forward) and 5'-AGTCCAAGAGAATGGCCGAG-3' (reverse) for MMP-1, 5'-TCCAAAATCAAGTGGGGCGATGCT-3' (forward) and 5'-CCACCTGGTGCTCAGTGTGACCC-3' (reverse) for GAPDH, 5'-CTCACCCTAGCAGCAGA ACC-3' (forward) and 5'-GCACGGATGTTGGAGTAGGT-3' (reverse) for mucin 5AC, 5'-

CCAATGACAGTGACCACCAG-3’ (forward) and 5’-CTCAAGTGGGGGAGTTGTGT-3’ (reverse) for mucin 6 (BioTeZ Berlin-Buch GmbH, Berlin, Germany). Serial dilutions of the dipeptidyl peptidase IV gene cloned into a pCR^R2.1-TOPO vector and primers 5’-GATGCTACAGCTGACAGTCGC-3’ (forward), 5’-TGGTGACCATGTGACCCACTG-TGGTGACCATGTGACC’(reverse) served for the generation of a calibration curve.

Transfection

AGS cells (1 x 10^5 cells/35 mm dish) were transfected with 20 nM siRNA (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) using siLentFect™ Lipid Reagent (BioRad) in Opti-MEM™ I culture medium (Invitrogen) supplemented with 5% FCS. A scrambled sequence that does not lead to the specific degradation of any known cellular mRNA was used as a control. For overexpression experiments, the cells were transfected with 0.2 µg of DNA using Effectene® transfection reagent (Qiagen) (DNA/Effectene ratio was 1:10). At 24 h of transfection, cells were starved in RPMI 1640 medium for 16-18 h and then infected with *H. pylori* or stimulated with PMA. Constitutivly active forms of bovine PKCα (A25E) and human PKCθ (A148E) cloned into pEF vector were donated by G. Baier (Innsbruck Medical University, Austria). Alanine to glutamic acid substitution at the pseudosubstrate sequence prevents its binding to the kinase domain and, thus, supports an “open” active conformation of PKC.[2] Constitutively active (T507E) human PKCδ in the pCEFL vector was a gift of S. Shaw (National Cancer Institute, Bethesda, USA). Replacement of the threonine residue with a glutamic acid residue mimics phosphorylation and activates PKCδ.[3]

Reporter gene assay

AGS cells were seeded onto a 24-well plate at a density of 3.5 x 10^4 cells per well in Opti-MEM™ I culture medium supplemented with 5% FCS. Reverse transfection was performed using 0.04:0.001 µg of Firefly AP-1:*Renilla* Luciferase plasmids (Cignal™ AP1 Reporter Assay Kit, SABiosciences,

Frederick, MD, USA) and 0.2 µg of a vector of interest using SureFECT™ transfection reagent (SABiosciences). A construct that encodes firefly luciferase under the control of a TATA box without any additional tandem repeats of the AP-1 transcription response element was used as a negative control. At 48 or 72 h post-transfection, cells were either left untreated or were stimulated with *H. pylori* or PMA and harvested further with Passive Lysis Buffer (Promega, Madison, WI, USA). Luciferase activity was estimated using the Dual-Luciferase Reporter Assay System (Promega) with a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). The firefly luciferase activity was normalised relative to *Renilla*'s activity. In some experiments, cell lysates were boiled with the sample buffer and used for immunoblotting.

Invasion assay

AGS cells (1 x 10^5) were loaded into the upper chamber of a 24-well Transwell® plate (Costar, Corning, NY, USA) and cultured on polycarbonate filters (8-µM pore size) coated with collagen I (0.01 mg/insert) in RPMI1640 medium containing 0.5% FCS. After 2 h, cells were left untreated or were stimulated with *H. pylori* or PMA in the presence/absence of BIS I. The cells were allowed to invade toward 5% FCS for 18 h. Invaded cells on the bottom side of the membrane were washed, exposed briefly to trypsin, collected with PBS and counted. Percent invasion was determined by dividing the number of cells that translocated through the collagen I-coated filters by the number of total cells per well. Experimental replicates were performed in triplicate.

Migration assay

Migration of AGS cells was studied using the Oris™ Cell Migration Assay (AMS Biotechnology Ltd., Abingdon, UK) according to the manufacturer instructions. Briefly, 5 x 10^4 cells/well were seeded into a 96-well plate containing cell seeding stoppers. After overnight incubation, a 2-mm diameter cell-free zone at the centre of each well was created by removing of the cell seeding stoppers. Cell growth medium was replaced with fresh RPMI 1640 containing 5% FCS, and cells

were incubated with *H. pylori* or PMA. After 24 h of treatment, cells were rinsed with PBS and stained with Calcein AM reagent. Fluorescent cells in the detection zone (applying the detection mask) were imaged by fluorescence microscope BZ-8100 (Objective CFI Plan Apo 4x; Keyence Corporation, Osaka, Japan). The cell-free area was measured using BZ-Analyzer software (Keyence Corporation).

Immunostaining

HSC (8.4 x 10^4) were seeded onto glass slides coated with 0.04 mg Matrigel^{TM} in a 24-well plate. After 4 days in culture, the cells were fixed with 4% paraformaldehyde (Sigma, Saint Louis, USA) and permeabilized with 0.1% Triton^{R} X-100 (Sigma) in PBS. Unspecific binding was blocked with 10% normal goat serum (Sigma). Mouse anti-pan cytokeratin antibody (recognizes human cytokeratins 1, 4, 5, 6, 8, 10, 13, 18, 19; Sigma) and anti-H^+, K^+-ATPase (recognizes α subunit; Antibodies-online GmbH, Aachen, Germany) were used in dilutions 1:200 and 1:100, respectively. Cy3-conjugated anti-mouse antibody was from Dianova (Jackson ImmunoResearch Laboratories, West Grove, USA). Cells were also stained in the absence of primary antibodies to evaluate non-specific secondary antibody reaction. Hoechst 33342 (Invitrogen, Carlsbad, USA) in dilution 1:1000 was used to stain nuclei. Images were taken using a fluorescence microscope BZ-8100 (Keyence Corporation, Osaka, Japan).

Immunohistochemistry

For retrieval of antigens, deparaffinised sections were heated in citrate buffer (pH 6.0) using a microwave oven for 20 min. Endogenous peroxidase was blocked by 20 min-incubation in 0.3% hydrogen peroxide within absolute methanol. Sections were washed, and non-specific binding was blocked with a pre-immune serum (Merck, Germany). Overnight incubation at +4°C was carried for binding of the primary rabbit polyclonal antibodies (dilution 1:50), including anti-phospho-PKC (pan) (αThr497, δThr505, θThr538) (Abcam, Cambrige, UK) and PKCθ (Abnova, Taipei City,

Taiwan). Then, 30 min-incubation with biotinylated secondary antibody was performed, followed by substrate binding by using streptavidin-biotin-peroxidase method. Counterstaining with haemalaun was carried out additionally in all cases. For all stains, negative and positive controls were performed, and staining was repeated until internal controls showed appropriate results.

PKCθ immunostaining was evaluated semiquantitatively according to the Remmele immunoreactive score (IRS).[4, 5] Briefly, the percentage of positively stained epithelial cells was divided into five grades of 0-4 (0%, <10%, 10-50%, 51-80%, >80%) and multiplied by the intensity the immunohistochemical reaction scaled from 0 to 3. To quantify phospho-PKC (pan) immunostaining, an evaluation of positively stained cells per high power field (hpf) was carried out.

SUPPLEMENTARY TABLES

Supplementary Table 1 Antibodies used for immunoblotting

Antibody	**Source**	**Manufacturer**
Phospho-PKC (pan) Phospho-PKCα (Thr497)	Rabbit	Abcam
Occludin PKCδ PKCθ PLCγ1	Mouse	BD Biosciences Pharmingen, San Jose, CA, USA
MMP-1	Rabbit	Calbiochem/Merk KGaA, Darmstadt, Germany
c-Fos Histone H3 Phospho-ATF-2 (Thr71) Phospho-ERK1/2 (Thr202/Tyr204) Phospho-MARCKS (Ser152/156) Phospho-JNK(Thr183/Tyr185) Phospho-c-Jun (Ser63) Phospho-PKC (pan) (βII Ser660) Phospho-PKCδ (Thr505) Phospho-PKCδ (Ser643) Phospho-PKCμ (Ser744/748) Phospho-PKCθ (Thr538) Phospho-PKCζ/λ (Thr410/403) Phospho-SEK1/MKK4 (Thr261) Phospho-Ser-substrates of PKC PKCμ	Rabbit	Cell Signalling Technology Inc., Danvers, MA, USA
GAPDH	Mouse	Chemicon International, Temecula, CA, USA

MARCKS	Rabbit	Epitomics Inc., Burlingame, CA, USA
HA	Mouse	Roche Diagnostics GmbH, Mannheim, Germany
PEA3 Phospho-PKCε (Ser729) PKCα PLCβ3 Phospho-p38	Rabbit Mouse	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
c-Jun	Rabbit	SIGMA-ALDRICH CHEMIE GmbH, Steinheim, Germany

Supplementary Table 2 Inhibitors and activators used in this work

Compound	Concentration	Manufacturer	Cellular targets
D609	50 µg/mL	Biomol GmbH, Hamburg, Germany	PC-PLC
BIS I LY294002 Rottlerin U73122	5 µM 20 µM 10 µM 5 µM	Calbiochem/Merk KGaA, Darmstadt, Germany	cPKC, nPKC PI3K cPKC, nPKC PI-PLC
PMA	66 nM	Cell Signalling Technology Inc., Danvers, MA, USA	DAG-binding proteins
BAPTA-AM Genistein	10 µM 150 µM	SIGMA-ALDRICH CHEMIE GmbH, Steinheim, Germany	Ca^{2+} Tyrosin kinases

NOTE. Inhibitors were added to the cells 30 min prior to *H. pylori* or PMA treatment.

Supplementary Table 3 PKCθ expression in human gastric mucosa tissue

Gastric mucosa biopsies	Number of specimens	Age	Gender		Mean IRS ±SEM	IRS interpretation, (number of specimens/%)			
			M	F		n	w	m	s
Non-infected	32	19 - 72	20	12	4.72 ± 0.37	1/3.1	10/31.3	20/62.5	1/3.1
Hp-Gastritis	31	32 - 82	20	11	4.74 ± 0.40	0/0	15/48.4	16/51.6	0/0
Adenoma	21	31 - 82	11	10	5.81 ± 0.53	1/4.8	2/9.5	17/80.9	1/4.8
Adenocarcinoma	59	40 - 96	26	33	5.36 ± 0.30	1/1.7	19/32.2	38/64.4	1/1.7

NOTE. No significant variance between the groups was determined by the ANOVA global test. M, male; F, female; n, negative; w, weak; m, moderate; s, strong expression.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1 *H. pylori* activates PKC. AGS cells were infected with *H. pylori* P12 wt (A, C) or were stimulated with PMA (B, C) for the various times indicated, and sub-cellular fractions were prepared. Immunoblotting was performed using antibodies against phosphorylated and unphosphorylated forms of PKC isozymes. GAPDH, occludin and histone H3 were immunodetected to show the appropriate fractionation and equal protein amount in the cell samples. wl, whole lysate of untreated AGS cells. (C) The graphs summarize the densitometric analysis of 3 independent immunoblots (experiments). *$p<0.05$, **$p<0.01$ vs non-stimulated cells (0 hours) within each subcellular fraction. (D) AGS cells were infected with P1wt, P12 wt, its isogenic VacA-deficient mutant or with heat-inactivated bacteria (hi) for 1 or 3 h. Heat-inactivated *H. pylori* were prepared by incubating the bacteria suspension at +70°C for 15 min. In a set of experiments, Transwell® permeable supports (pore size 0.4 µM) were used to separate *H. pylori* P1wt from AGS cells grown in the bottom chamber. Pore-permeable PMA was used as a positive control. The whole cell lysates were prepared and immunoblotting was performed using antibodies as indicated. GAPDH was immunodetected to show equal protein amounts in the cell samples.

Supplementary Figure 2 Histologic examination of phospho-PKC (pan) expression in human antral gastric mucosa. High-power field confocal images are shown. Magnification, 200x. Scale bares = 200 µM.

Supplementary Figure 3 In contrast to rottlerin, BIS I does not affect viability of *H. pylori*. *H. pylori* P12 wt was incubated in RPMI 1640 medium supplemented with 5 µM BIS I or 10 µM rottlerin for 1 h. The bacteria suspension was diluted in PBS and plated onto GC agar supplemented with horse serum, vancomycin, trimethoprim, nystatin and vitamins. The bacterial colonies grew in microaerophilic conditions for 3 days, and then the photos were taken using a VersaDoc™ Imaging System (BioRad, Hercules, CA, USA).

Supplementary Figure 4 *H. pylori* up-regulates MMP-1 in a PKC-dependent manner. (A) BIS I-treated or non-treated HCA-7 cells or HSC were incubated with *H. pylori* P12 wt or PMA for 7 h. MMP-1 expression was analysed by qRT-PCR. *p<0.05, **p<0.01 vs non-stimulated cells; $^{\#}$p<0.05, $^{\#\#}$p<0.01 vs BIS I-free stimulated cells. (B) BIS I-treated or non-treated HCA-7 cells or HSC were incubated with *H. pylori* P12 wt for 4 h. The whole cell lysates were prepared and immunoblotting was performed using antibodies as indicated. GAPDH was immunodetected to show equal protein amounts in the cell samples. (C) Characteristics of cultured human stomach cells (HSC) derived from prenatal stomach tissue. Expression of proteins typical for stomach epithelium (mucin 5AC, mucin 6, pan-cytokeratins and H^+, K^+-ATPase) was analysed in non-treated HSC by RT-PCR and immunostaining. GAPDH expression served as an internal control in RT-PCR. AGS cells served as a positive control. Muc 5AC, mucin 5AC; Muc 6, mucin 6. Cytokeratins and H^+, K^+-ATPase are pseudocolored in *yellow*; Hoechst 33342 nuclear stain is pseudocolored in *margenta*.

Supplementary Figure 5 *H. pylori* activates MAPK. AGS cells were infected with *H. pylori* P1wt or the *cagA* and *virB7* mutants for various times indicated or were stimulated with PMA for 1 h, and the cell lysates were prepared. Immunoblotting was performed using antibodies as indicated.

Supplementary Figure 6 In contrast to PMA, *H. pylori* does not accelerate wound healing. AGS cells grown on an uncoated 96-well plate were infected with different *H. pylori* strains as indicated or stimulated with 4 nM PMA in fresh RPMI 1640 containing 5% FCS in the presence or absence of BIS I for 24 h (A, B). The cells were labelled with Calcein AM and imaged (B). Relative area covered by the cells was calculated (A, C). (C) AGS cells were transfected with siRNAs against PKCα, PKCδ or PKCθ prior to stimulations. *p<0.05, **p<0.01, vs respective control; $^{\#}$p<0.05, vs PMA-stimulated cells.

Supplementary Figure 7 *H. pylori* induces cell scattering in a PKC-independent manner. AGS cells were either pre-treated with BIS I or transfected with PKC-targeting siRNAs and then infected with *H. pylori* P12 wt (A) or PMA (B) for 3 h. Cell morphology was monitored with an inverted Nikon Eclipse TC100 microscope.

SUPPLEMENTARY REFERENCES

1 Sokolova O, Bozko PM, Naumann M. *Helicobacter pylori* suppresses glycogen synthase kinase 3beta to promote beta-catenin activity. *J Biol Chem* 2008;**283**:29367-74.

2 Ghaffari-Tabrizi N, Bauer B, Villunger A, et al. Protein kinase Ctheta, a selective upstream regulator of JNK/SAPK and IL-2 promoter activation in Jurkat T cells. *Eur J Immunol* 1999;**29**:132-42.

3 Liu Y, Graham C, Li A, et al. Phosphorylation of the protein kinase C-theta activation loop and hydrophobic motif regulates its kinase activity, but only activation loop phosphorylation is critical to in vivo nuclear-factor-kappaB induction. *Biochem J* 2002;**361**:255-65.

4 Remmele W, Stegner H. Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. *Pathologe* 1987;**8**:138-40.

5 Allred D, Harvey J, Berardo M, et al. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol* 1998;**11**:155-68.