Meprin A Impairs Epithelial Barrier Function, Enhances Monocyte Migration and Cleaves the Tight-junction Protein Occludin

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ABSTRACT

Meprin metalloproteases are highly expressed at the luminal interface of the intestine and kidney and in certain leukocytes. Meprins cleave a variety of substrates in vitro including extracellular matrix proteins, adherens junction proteins and cytokines, and have been implicated in a number of inflammatory diseases. The linkage between results in vitro and pathogenesis however, has not been elucidated. The current study aimed to determine whether meprins are determinative factors in disrupting the barrier function of the epithelium. Active meprin A or meprin B applied to the Madin-Darby canine kidney (MDCK) cell monolayers increased permeability to fluorescein isothiocyanate (FITC)-dextran and disrupted immunostaining of the tight junction protein occludin but not claudin-4. Meprin A, but not meprin B, cleaved occludin in MDCK monolayers. Studies with recombinant occludin demonstrated meprin A cleaves the protein between Gly100 and Ser101 on the first extracellular loop. In vivo experiments demonstrated that meprin A infused into the mouse bladder increased the epithelium permeability to sodium fluorescein. Furthermore, monocytes from meprin knockout (KO) mice on the C57BL/6 background were less able to migrate through an MDCK monolayer than monocytes from wild-type (WT) counterparts. These results demonstrate the capability of meprin A to disrupt epithelial barriers and implicate occludin as one of the important targets of meprin A that may modulate inflammation.

Keywords: Tight junction proteins; MDCK cells; Knockout mice; Metalloproteinase
INTRODUCTION

Meprins, members of the “astacin” family of metalloproteases, are highly expressed normally in rodent intestine and kidney, and are localized to the apical (brush–border) membrane of polarized epithelial cells lining in these tissues (9). Meprins have also been detected in macrophages of mesenteric lymph nodes, in leukocytes from lamina propria of human inflammatory bowel tissue, and are found at high concentrations in human urine in women with urinary tract infections (13, 53). There are three meprin isoforms composed of α and/or β subunits. Heteromeric meprin A (α2β2 or α3β1) and meprin B (β2) are membrane bound, whereas homomeric meprin A (α2), which forms large complexes, is secreted into the lumen of the intestine and urinary tract.

Meprins are capable of cleaving a wide range of substrates in vitro including extracellular matrix proteins (ECM) such as laminins, collagen and gelatin, adherens junction proteins (E-cadherin), and cytokines (9, 25, 46). Meprins have been implicated in several inflammatory diseases, for example, meprin β has been reported to be a candidate gene for diabetic nephropathy in the Pima Indian population (38). In an experimental model of urinary tract infection, meprin A has been shown to enhance renal damage and bladder inflammation after LPS challenge (53). In addition, polymorphisms of the human MEPIA gene have been correlated with inflammatory bowel diseases (IBD) (5). In an experimental model of ulcerative colitis, meprin αKO mice were more susceptible to injury and inflammation than wild-type counterparts (5). Moreover, meprins are also implicated in cancer invasion and metastasis (30). Previous studies led to the proposal that meprins have an important role in leukocyte transmigration. For instance, Crisman et al. reported that leukocytes lacking meprin β are impaired in
migration through ECM (13). Sun et al. found that meprin αβ deficiency in mice have altered dissemination of monocytes, with decreased egression from bone marrow to peripheral blood (47).

The current study has tested the hypothesis that meprins weaken epithelial barrier function by cleaving tight junction proteins, and thereby facilitate monocyte migration during inflammation. Two meprin isoforms, homomeric meprin A (α2) and meprin B (β2), were examined for their effects on epithelial barrier function and the degradation of tight junction proteins in MDCK cell monolayers and extracts. The epithelial barrier function was determined by the permeability to FITC-dextran and transepithelial electrical resistance. Since tight junction proteins, such as occludin, zona occludens (ZO) and claudins, are essential for optimal epithelial barrier function (15, 42), further experiments were conducted to study the degradation of those proteins after meprin treatment by immunocytochemistry and Western blot assays. To relate the in vitro model to in vivo results, meprin A was infused into the mouse bladder and permeability to sodium fluorescein was measured. Furthermore, monocytes from meprin αKO mice were compared to those from WT to determine whether monocyte migration through a MDCK monolayer was compromised by the lack of meprin A. The work herein is the first to demonstrate interactions between meprins and the tight junction protein occludin, and to show that meprin A allows enhanced migration of inflammatory cells through epithelial barriers.
MATERIALS AND METHODS

Materials. Minimum Essential Medium Eagle (MEM) was purchased from Gibco. All other chemical reagents were obtained from Fisher or Sigma. Monoclonal mouse anti-occludin, anti-claudin-4, anti-ZO-1 and polyclonal rabbit anti-occludin were purchased from Zymed/Invitrogen. Goat anti-mouse Alexafluor 488 was a gift from Dr. W.B Reeves, Penn State University College of Medicine (PSU-COM), while goat anti-rat FITC, and Hoeschst nuclear stain were purchased from Jackson Immuno Research laboratories Inc. EasySep Mouse monocyte enrichment kit was purchased from STEMCELL Technologies Inc.

Animal model. Congenic C57BL/6 meprin αKO and corresponding WT mice were used at 8-9 weeks of age for all experiments. All mice were maintained in the PSU-COM Animal Facility and were allowed free access to water and rodent chow. The derivation of mixed-background (C57BL/6 X 129/Sv) meprin αKO mice was described by Banerjee et al. (5). Congenic meprin αKO mice were generated by crossing mice on the mixed-background with C57BL/6 mice for 10 generations. Mouse tails samples were sent to Charles River to assess the level of genetic homogeneity. The results showed 99.07% homogeneity. All animal protocols were approved by PSU-COM Institutional Animal Care and Use Committee.

Mice were anesthetized by isoflurane inhalation. For some experiments, ketamine/xylazine (100 mg/kg and 10 mg/kg respectively) were administered i.p. to the mice.

Cell culture. Madine Darby Canine Kidney (MDCK) cells (ATCC accession # CCL-34) were grown in minimum essential medium (MEM) (Gibco) and supplemented with
Earl’s salts, L-glutamine, sodium bicarbonate, and 10% FBS (Atlas Biologicals) at 37°C with 5% CO2 in air.

A confluent monolayer is defined as a group of cells that cover the surface of the culture (e.g., plate) completely but without overlaying each other.

**Meprin purification, activation, and assays.** Recombinant homomeric mouse meprin A and rat meprin B were purified from stably transformed human embryonic kidney (HEK) 293 cells (ATCC accession # CRL 1573) (11). The latent forms of meprins were activated with trypsin at a molar ratio of 1:6 (trypsin:meprin) for 30-60 min at 37°C. Trypsin was removed by filtration through a Sephadex G-25 column. Meprin A and meprin B activities were determined using fluorogenic substrates BK+ (Abz-Arg-Pro-Pro-Gly-Phe/Ser-Pro-Phe-Arg-Lys(Dnp)-Gly-OH) and OCK+ (Abz-Met-Gly-Trp-Met/Asp-Glu-Ile-Asp-Lys(Dnp)-Ser-Gly-OH) respectively (8).

**Recombinant occludin expression and purification.** Recombinant human Occludin (Occludin TM, residue 48 to 290) was cloned into a maltose binding protein (MBP)-fusion plasmid, pMTTH (50), with HindIII and XhoI restriction endonucleases sites. The recombinant plasmid was introduced into *E.coli* BL21 (DE3) RIPL competent cells. Cells were grown in 5 ml LB medium supplemented with Ampicillin (100 μg/mL) at 37 °C overnight, then inoculated in 400 ml M9 media in the presence of antibiotics. Cells were induced with 0.8 mM isopropyl thiogalactoside (IPTG) at an OD600 value of 0.9. After induction, cells were grown at 16 °C overnight. Cells were harvested, resuspended in a buffer of 70mM Tris, 300mM NaCl, and pH8.0, and lysed by sonication. Cell debris was removed by low-speed centrifugation. The supernatant fluid was then subjected to ultracentrifugation at 100,000g at 4 °C for 2 h. Membrane pellet was extracted with 1%
dodecylphosphocholine (DPC) (Anatrace) for 2 h at room temperature. The soluble fraction was then loaded onto a Ni-NTA column (Qiagen) pre-equilibrated with a solution of 20 mM Tris–HCl, pH 8.0, 200 mM NaCl, and 0.2% DPC. The column was washed with 40 ml wash buffer of 20 mM Tris, pH 8.0, 30 mM imidazole, and 0.2% DPC. The column was eluted with an elution buffer containing 20 mM Tris, pH 8.0, 200 mM NaCl, 250 mM imidazole and 0.5% DPC. The eluted protein was dialyzed against a buffer of 70 mM NaCl, 1.35 mM KCl, 5 mM Na$_2$HPO$_4$, 0.9 mM KH$_2$PO$_4$, pH 7.3, and 1.3 mM DPC at 4 °C overnight. The N-terminal fusion MBP was cleaved by thrombin (GE, #27-0846-01; Lot#049k7540-11). After digestion, the solution was reloaded onto a Ni-NTA column and washed with wash buffer. The eluted protein was concentrated and further purified with a Superdex-200 (16 / 60) column.

**Maltose binding protein (MBP) conjugated occludin loop 1 and loop 2.** Cloned DNA sequences expressing human occludin extracellular loop 1 or loop 2 were conjugated respectively into pMTTH (50), using the KpnI and EcoRI endonucleases sites. The ligated vectors were transformed into *Escherichia coli* (E.coli) DH5α strain, and the transformants were selected in the presence of Ampicillin on LB agar plates. The clones thus obtained were sequenced in the PSU-COM Core Sequencing Facility to confirm the DNA sequence of inserted extracellular loops. Loops containing pMTTH vectors were transformed into *E.coli* BL21(DE3) competent cells and the transformants were selected in the presence of Ampicillin. The transformants were inoculated into LB broth with suitable antibiotic and grown to an optical density (O.D.) of 0.6-0.8 at 600 nm, at which point expression of protein was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The culture was collected
after 24 h incubation at 16°C. For a large scale purification of the protein, the culture was centrifuged at 10000 rpm for 25 min at 4°C. Purification of the histidine tagged fusion protein was carried out using Ni-NTA column.

**Preparation of membrane-enriched fractions of MDCK cells.** Membrane-enriched fractions of MDCK cells were prepared according to a modified protocol of Dr. David Antonetti laboratory (PSU-COM). Briefly, MDCK cells were washed with ice-cold PBS, and scraped into 500 ul Buffer A (0.5 M sucrose, 2 M Tris, 0.5 M EDTA, protease inhibitor tablet (41), benzamidine, 10 mM Na₃PO₄, 0.5 M NaF, 0.5 M NaPyrophosphate). EDTA (0.5 M) was added to Buffer A and B for the preparation of membrane-enriched fractions of MDCK cells except for those experiments that included meprin treatments, to avoid inhibition of the protease activity in these experiments. The suspension was homogenized with a Dounce homogenizer (six-strokes with pestle). The preparation was centrifuged at 39,000 rpm for 20 min at 4°C. The sediment was suspended in 2 ml of Buffer B (2 M Tris, 0.5 M EDTA), homogenized again with a pestle, and centrifuged at 39,000 rpm for 20 min at 4°C. The sediment was suspended in 500 μl of Stuart’s lysis buffer (with protease inhibitor tablet) and sonicated for 30 sec, 3 times at 50% pulse (22).

**Detection of cleaved product of occludin or claudin-4 by meprin A or meprin B.** MDCK cells were cultured until they reached confluence. Meprin A or B were added to cells or membrane-enriched fractions of cells for various amounts of time. The reactions were stopped by adding sample buffer (60 mM Tris pH 6.8, 25% Glycerol, 2% SDS, 0.1% Bromophenol Blue, 14.4 mM 2-mercaptoethanol), and incubating in boiling water for 5 min. Samples were then subjected to SDS-PAGE electrophoresis. Monoclonal mouse
anti-occludin, or anti-claudin-4 or, polyclonal rabbit anti-occludin antibodies were used to detect potential cleavage products by meprins using Western blot analyses.

**Determination of occludin cleavage site(s).** To determine the cleavage site(s) on occludin cleaved by meprin A, the products of digestion of the extracellular loop 1 or loop 2 were excised from SDS-PAGE gels. Samples were digested with trypsin and subjected to analysis by C18 nanoflow followed by MS/MS analysis. Mass-spectrometric analysis was performed in the Core Facility of PSU-COM, and Proteomics and Mass Spectrometry Core Facility of Cornell University. The amino acid sequence of each fragment was identified by further subjecting it to CID (collision induced dissociation) followed by MS/MS. The masses of ions thus formed are determined and compared with the theoretical masses of ions predicted.

**Immunocytochemistry and confocal microscopy.** MDCK cells were grown in 12-well plates on round glass coverslips until they reached confluence. Cells were treated with 4 μg/ml (47 nM) active or latent recombinant meprin A or meprin B for 5 h. MDCK cells were then fixed in 1% paraformaldehyde, permeabilized with 0.2% Triton-X 100 and blocked in 10% goat serum with 0.1% Triton-X 100 with shaking. Cells were incubated with monoclonal mouse anti-occludin (1:50 dilution), anti-ZO-1 (1:4 dilution) and monoclonal anti-claudin-4 (1:150) antibodies. After 5 washes in 0.1% Triton-X 100, monolayers were incubated with fluorescence-labeled secondary antibodies for 1 h and rinsed as indicated before. Coverslips were mounted on slides and examined using a confocal microscope.

**Permeability assay and Transepithelial electrical resistance (TER).** MDCK cells were cultured on filters with 0.4-μm pores (Millipore) until reaching confluency. MEM
medium was changed to serum-free MEM prior to treatment with meprin. Active or latent meprins (meprin A or B) (47 nM) were added to the apical chamber of insets, and incubated at 37°C for the desired amount of time.

For permeability assays, the apical medium was replaced with fresh medium containing 10 or 15 μg/ml of FITC-dextran (10 kDa). After a 2-h incubation at 37°C, the basal medium was collected and the fluorescence of the permeated FITC-dextran was measured with a fluorescence spectrophotometer (F-2000, Hitachi) at $\lambda_{ex}$492 nm and $\lambda_{em}$ 520 nm (45).

For TER measurements, the degree of resistance of tight junction to ions was assessed by using a voltohmmeter EVOM with a STX2 Electrode (World Precision Instruments) (21).

**Assessment of bladder permeability.** Transurethral catheterization was performed to instill exogenous reagents, such as meprins and sodium fluorescein, into the bladder. Homomeric mouse meprin A was instilled into bladder via transurethral catheterization using a 0.5 mm polyethylene catheter (Intramedic PE 10) attached to the hub of a 50 μl Hamilton #705 syringe with 30 gauge blunt-tipped needle. After 2 h, bladder permeability was determined as previously performed (18, 53). Briefly, 100 μl of 20 mg/ml sodium fluorescein (NaFl) was instilled into bladder via catheterization. After 15 min, blood samples were collected from the interior vena cava, and the plasma fluorescein concentrations were measured with a fluorescence spectrophotometer (F-2000, Hitachi) using a 0.1-100 μg/ml standard curve (excitation 494 nm, emission 516 nm).

**Isolation of monocytes from mouse bone marrow.** Monocytes from wild-type and meprin αKO mice were obtained by negative selection from bone marrow using the
Mouse Monocyte Enrichment Kit (#19761, StemCell Technologies) following the manufacturer’s instructions. Briefly, bone marrow cells were flushed from femurs and tibias with PBS. Mouse Enrichment Cocktail was added to single cell suspension, mixed well and incubated for 15 min. Biotin selection cocktail was added, mixed well and incubated for 15 min. EasySep D Magnetic particles were added and then the tube was placed into the magnet and set aside for 5 min. The magnet and tube were inverted in one continuous motion to collect the desired fraction to a new tube. The magnetically labeled unwanted cells remain bound inside the original tube (52).

Transmigration of monocytes through MDCK monolayers. MDCK cells were cultured on Transwell Permeable Supports (8 μm polyester membrane, Costar) until they reached confluence. Isolated monocytes were added to the top of MDCK monolayer, and monocyte chemotactic protein-1 (MCP-1) (1.5 nM) was added to the bottom chamber. After incubating at 37°C for 3 h, the underside of supports was flushed with trypsin and the cells in the bottom chamber collected by centrifugation at 1,500 rpm for 10 min. The suspended cells were labeled with fluorescence antibodies. The CD11b+/NK1.1-/Ly6g-/Ly6c+ monocyte subsets (inflammatory monocytes) were detected with a flowcytometer (16).

Statistical Analysis. Microsoft Excel and PRISM GraphPad software were used to plot data and for data analysis. Results are expressed as means ± SE; a P value < 0.05 was considered significantly different.
RESULTS

**Meprin A and B impaired MDCK monolayer’s barrier function.** The permeability of MDCK monolayers to FITC dextran and resistance to ionic flux (TER) was measured to evaluate the epithelial barrier function in response to a meprin A or B challenge. The concentration of 47 nM was chosen on the basis of preliminary experiments, and the fact that previous work had established that the concentration of meprin A monomers in mouse urine is in the range of 20-120 nM [(6); Yura R, Penn State University Thesis, 2008]. After exposure to active meprin A for 5 h, permeability of MDCK monolayers to 40 kDa FITC-dextran showed a tendency to increase but the difference was not significant (Figure 1A). After exposure to active meprin A for 5 h or longer, permeability of MDCK monolayers to 10 kDa FITC-dextran significantly increased as compared to cells incubated with medium only or those treated with latent meprin A (Figure 1B). Exposure to active meprin B for 9 h significantly increased MDCK cell monolayers’ permeability to 10 kDa FITC-dextran, as compared to untreated samples or those treated with latent meprin B (Figure 1C).

Resistance to ionic flux (TER) was also measured to determine the epithelial barrier function in response to meprin A or B. Active meprin A decreased the electrical resistance of MDCK monolayer slightly after 9 h of incubation, as compared to cells incubated with medium only or those treated with latent meprin A (Figure 2A). No statistical significance in resistance to ionic flux was observed between monolayers treated with active meprin B and untreated monolayers or those treated with latent meprin B, even after 9 h of incubation (Figure 2B). These TER data indicate that meprin A
slightly impaired epithelial barrier function of MDCK monolayers while meprin B had no
effect.

**Meprin A and B disrupted TJs on MDCK monolayers.** Tight junctions (TJs) in
epithelium are essential for maintaining barrier function; therefore, MDCK monolayers
were treated with meprin A or B to assess whether immune-staining of tight junctions
was disrupted. In monolayers treated with active meprin A, the immune-stainings of
occludin (Figure 3A) and ZO-1 (Figure 3B) were disrupted. In monolayers treated with
active meprin B, the immune-staining of ZO-1 (Figure 3C) was disrupted. In untreated or
latent meprins treated monolayers, immune-stainings of tight junctions were continuous
at cell borders. The immune-staining of claudin-4 was not disrupted by active meprin A
treatment (Figure 3D). These observations indicated that meprin A and B disrupted TJs
between MDCK cells and affect certain types of TJ proteins.

**Occludin, but not claudin-4, in MDCK membrane fractions was cleaved by meprin
A and B.** To determine whether occludin or claudin-4 was cleaved by meprin A or B, the
membrane-enriched fractions from MDCK cell lysates were incubated with meprin A or
B preparations. Preliminary experiments have shown that ZO-1 protein abundance did
not change as measured by Western blotting after meprin A treatment. The images shown
in Figure 4 were representative images from two to three Western blots of each
experiment. The percentage of hydrolysis from measurements of the optical density of
occludin bands was averaged from these two blots. Western blot analysis showed the
degradation of occludin by both active meprin A and B, but not the latent meprins nor
active meprins with metalloprotease inhibitor EDTA (Figure 4A, Figure 4B). The
percentage of hydrolysis after 1 h active meprin A or B incubation was 83% and 84%
respectively. The antibody used in our experiments has an epitope on the C-terminus of
occludin. Computational prediction revealed multiple cleavage sites for meprin A on the
C-terminal of occludin. Thus, any cleavage products will likely be further degraded by
meprin A and difficult to detect. Western blot analysis did not show degradation of
claudin-4 by either meprin A or B (Figure 4C, Figure 4D).

**Occludin in MDCK monolayer was degraded by meprin A.** To determine whether
occludin in MDCK monolayers was cleaved by meprins, MDCK monolayers were
directly treated with meprin A or B preparations. The images in Figure 5 are
representative of three Western blots of each experiment. The percentage of hydrolysis
from measurements of the optical density of occludin bands was averaged from these
three blots. Western blot analysis showed that the amount of occludin in MDCK cells
treated with active meprin A was decreased (Figure 5A). The percentage of hydrolysis
after treatment with 47 nM active meprin A was 86%. However, meprin B had little
effect on occludin in MDCK monolayers (Figure 5B).

**Claudin-4 in MDCK monolayer was not degraded by meprin A.** To determine
whether claudin-4 is cleaved by meprin A, MDCK monolayers were incubated with
increasing concentrations of meprins (4.7 nM, 23 nM, 47 nM) for 5 h. Western blot
analysis did not show degradation of claudin-4 by meprin A (Figure 5C).

**Recombinant occludin in micelles was cleaved by meprin A.** To identify the site(s)
where meprin A cleaves occludin, a sample of 3 μM recombinant occludin reconstituted
in micelles was incubated with 47 nM of active or latent meprin A. The major
recombinant occludin band is at 28 kDa (Figure 6A). Other observed bands might be
different oligomerization states of occludin or residual *E.coli* proteins. Based on the size
of protein and Western blot analysis, the 28 kDa-band is occludin. Incubation with active meprin A up to 4 h led to 23% and 48% decrease of the occludin band intensity as shown by Coomassie staining and Western blot analysis respectively (Figure 6A, 6B). A potential cleavage product (~22KDa) was observed with Coomassie blue staining, but not with Western blotting. The probable reason is that the cleavage product observed by Coomassie blue staining does not contain the His-tag.

**Occludin extracellular loops were cleaved by meprin A.** To demonstrate cleavage of occludin extracellular loops, 6 μM of maltose binding protein (MBP) conjugated occludin loop 1 and loop 2 were incubated with meprin A (47 nM) for various periods of time up to 4 h (Figure 7A). Cleavage of occludin loop 1 was observed after active meprin A treatment, but not latent meprin A treatment. Similar results were observed in occludin loop 2 for example that the 28 kDa band density decreased after active meprin A treatment, but not after latent meprin A treatment (Figure 7B). To determine whether MBP, a protein of 42 kDa, is cleaved by meprin A, 6 μM MBP was incubated with meprin A for various periods of time up to 4 h (Figure 7C). No cleavage was observed after meprin A treatment. These results confirmed that there were cleavage sites of meprin A on the extracellular loops of occludin.

**The cleavage site(s) were determined by Mass-spectrometric analysis.** The cleavage products of extracellular loop 1 and loop 2 were excised from SDS-PAGE gel and samples were trypsin-digested and subjected to analysis by C18 nanoflow followed by MS/MS analysis. These data allowed for identification of consecutive ions and thereby the peptide fragment. The cleavage site identified between Gly100 and Ser101 is on the first extracellular loop of occludin (Figure 8). This cleavage site is consistent with a
computational prediction using PoPS (Prediction of Protease Specificity), a computer program designed by Sarah Boyd of Monash University, based on meprin substrate specificities. The computational program predicted that meprin A has several cleavage sites on occludin extracellular loop 1 and loop 2. For example, there are G-Y (92-93), G-S (100-101), G-Y (103-104) on loop 1, and G-S (100-101) is the most potent predicted site. The predicted fragments size of MBP-loop 1 would be 4 kDa and 43 kDa, which is the correct size-product. On loop 2, potential cleavage sites include: V-N (197-198), G-S (209-210) and V-D (239-240). However, due to the low ion density of the sample, Mass Spec analysis was not able to identify the cleavage site on loop 2.

**Meprin A increased mouse bladder permeability.** The bladder wall serves as a good *in vivo* system to study the alterations in the integrity of the bladder epithelial lining in response to a meprin A challenge. Preliminary studies in our lab showed that the concentration of soluble meprin A in mouse urine is approximately 120 nM (6, 33). In addition, the infiltrating leukocytes that accumulate at inflammation sites also express meprins (13, 47). Thus meprin A concentration in the bladder during inflammation could be in the high nanomolar or micromolar range. To mimic *in vivo* conditions under inflammation, a high concentration of meprin A (4.7 μM) was used to challenge the bladder. The bladder permeability was determined by measurement of sodium fluorescein (NaFl) leakage from the bladder into the serum. Active meprin A increased bladder permeability to sodium fluorescein as compared to Tris buffer, actinonin-inactivated meprin A or actinonin only treatments (Figure 9). The data indicated that active meprin A was able to impair the epithelial barrier *in vivo.*

**Meprin A regulated monocyte transmigration.** A goal of this investigation was to test the hypothesis that epithelium integrity compromised by meprin A allows enhanced
monocyte transmigration. Accordingly, bone marrow derived monocytes from wild-type and meprin αKO mice were cultured on MDCK monolayers. A group of monocytes were added on the apical side of MDCK monolayers grown on inserted filter, without MCP-1 in the bottom chamber as chemo-attractant; the other group of monocytes were added to the apical side of inserted filter without MDCK cell monolayers, but with MCP-1 in the bottom chamber; and the last group of monocytes were added on MDCK monolayers with MCP-1 in the bottom. Transmigration of monocytes was measured by flowcytometry assay (Figure 10). The results showed that the migration of monocyte was driven by chemo-attractant, because when no MCP-1 was present, neither genotype of monocytes transmigrated efficiently through MDCK monolayers. More importantly, the results showed that in the presence of both chemokine and MDCK monolayers, there were significantly fewer meprin αKO monocytes transmigrated through than wild-type monocytes. Since genotypic difference depends on the presence of MDCK monolayers, the results support the hypothesis that meprin A’s effects on epithelial barrier lead to enhanced monocyte migration.
DISCUSSION

In this study, the hypothesis that the meprins are determinative factors in disrupting the barrier function of epithelium has been tested. The results demonstrated that meprin A impairs epithelial barrier function in vivo, with studies of bladder permeability to sodium fluorescein, as well as in vitro, with studies of permeability of an MDCK monolayer to FITC-dextran. A consequence of the activity of meprin A is to enable monocytes to migrate through an epithelial barrier more readily, and in vivo this would allow inflammatory molecules such as cytokines and monocytes to gain access to sites of injury.

Meprin A was more effective than meprin B in impairing the MDCK epithelial barrier function. The difference may be due to the fact that while meprin B is able to cleave occludin in cell fractions, it had little proteolytic activity on occludin in MDCK monolayers. One interpretation of these results is that meprin B is unable to gain access to the vulnerable peptide bonds in the paracellular space of occludin as meprin A does. Meprin A and B have different peptide bond and substrate specificities, and this may explain their differential effects (7, 8). Previous studies showed that meprin B can cleave E-cadherin at the extracellular site and weaken the intercellular contacts. The cleavage site was localized in the extracellular domain adjacent to the plasma membrane (25). Thus a possible explanation for the observed increased permeability of MDCK monolayers to FITC-dextran after meprin B treatment is that meprin B cleaves other junctional proteins, such as E-cadherin.

Meprin A induced permeability change to FITC-dextran was greater than the TER change to ionic flux. There is evidence that specific TJ proteins play different roles in
epithelial barrier functions. Whereas occludin is important for the paracellular transport/diffusion of small molecules, claudins are regulators of cation/anion exchange and charge selectivity through epithelium (23, 40, 44, 48). Thus the observed difference in the extent of increased FITC-dextran permeability and decreased TER after treatment with meprin A can be explained by the fact that meprin A cleaves occludin, a key factor, in permeability to FITC-dextran, but not claudin-4 which is more critical for ionic flux.

In multicellular organisms epithelia and endothelia delineate the borders between different compartments. This demarcation relies on the establishment of cell-cell contacts, such as tight junctions (TJs) between adjacent cells to withstand mechanical stress and prevent paracellular flux (19). TJs are important for maintaining the epithelium barrier functions, enabling adjacent cell communications as well as influencing migration of mobile cells (17, 39). TJs are maintained through a complex network of interacting proteins, such as occludin, Zona occludens and E-cadherin. Occludin, one of the first TJ proteins to be identified, has a cytoplasmic N-terminus, four transmembrane domains and two extracellular loops (20). It is well conserved in the human, mouse, rat and dog (2). Previous studies by others demonstrated that occludin contributes to barrier function and leukocyte migration. For example, murine epithelial cells with mutant occludin lacking the N-terminus and extracellular domains were unable to form strong tight junctions (4). Elicited expression of occludin in transformed epithelial cells rescues epithelial morphology and promotes reacquisition of the epithelial phenotype (12, 51). The first loop of occludin is required for tight junction resealing resulting from intercellular occludin interactions of adjacent cells (28), while the second loop is required for occludin to localize to cell membrane and assemble the junctional complex with other TJ proteins.
Occludin has also been shown to mediate neutrophil migration across the MDCK cell monolayer (24, 36). In addition, occludin has a critical regulatory function in that it interacts with several other junctional proteins including ZO-1, actin and other cytoskeleton proteins (32).

Several lines of evidence have shown that down-regulation of occludin via either proteolysis or factors such as vascular endothelial growth factor (VEGF) results in elevated permeability (14). For example, proteolysis of occludin by metalloproteases led to increased permeability to FITC-dextran in endothelial cells and disruption of immunostaining, while other junctional proteins (ZO-1, cadherin) were intact (49). Knockdown of occludin by siRNA led to increased permeability to FITC-dextran and decreased TER in epithelial monolayer without affecting ZO-1 or various claudins (1, 36). These findings are consistent with the studies herein with meprin A.

The work herein implicates occludin as one of the important targets of meprin A in the disruption of epithelial barriers. However, the role of occludin in barrier function is controversial, especially in the light of the fact that occludin null mice do not exhibit deficiencies in barrier function (43). It has been suggested that other proteins (such as tricellulin, a TJ protein localized at tricellular junctions) provide functional redundancy that perserves barrier function in occludin null mice (37). In addition, meprin A is capable of cleaving many proteins, and thus the functional effects observed in the present studies may be due to other targets.

Previous studies have indicated that tight junctions regulate leukocyte transmigration (26, 35, 43). The studies herein demonstrated that meprin A enhances leukocytes
transmigration. We suggest that the cleavage of junctional proteins is the molecular basis for increased meprin-mediated leukocytes transmigration.

Meprin metalloproteases are involved in inflammatory processes of both acute and chronic disease conditions (5, 34). The current findings that meprin A impairs epithelial barrier functions can explain previous observations in mouse models of inflammations. In an acute model of bladder inflammation, the host response to intravesicular LPS challenge of meprin αKO mice was less severe than wild-type counterparts, as it was shown that there was less bladder edema, less leukocyte infiltration and less bladder permeability in meprin αKO mice than WT mice (53). Serum cytokine profiles showed that TNFα, IL-1β, and MCP-1 levels were significantly lower in meprin αKO mice than in WT mice after an intraperitonel LPS challenge. Moreover, meprin βKO and WT mice showed similar hypothermia and similar serum nitrate/nitrite levels. The serum cytokine levels in meprin βKO mice were not significantly different from WT counterparts (53). These data indicate that meprin A has a determinative and pro-inflammatory role in the inflammatory response to an acute urinary infection. The current results are consistent with the LPS findings. Both βKO and WT mice produce meprin A in the kidneys and migratory monocytes. The meprin A released by the kidney is predominantly inactive. So the source of active meprin A is likely the monocytes attracted to the site of injury. Thus the breakdown in the bladder barriers and elevated leukocyte infiltration can be attributed to the ability of active meprin A to disrupt cellular tight junctions.

Monocytes and their interactions with endothelial/epithelial cells have been implicated in several kidney disorders, such as diabetic nephropathy and ischemia reperfusion injury
(3, 29). The recruitment of monocytes/macrophages to sites of injury correlates with the progression of kidney damage in diabetic nephropathy. The ability of meprins to affect the movement of monocytes from bone marrow to peripheral sites, as well as to enhance transmigration through epithelial barriers may be factors in the progression of kidney damage. The high concentration of meprin A that is normally present in apical membranes of proximal tubules of rodent kidneys is another factor that may result in kidney damage in response to an acute injury. Studies of kidney ischemia reperfusion (I/R) in mice have shown that redistribution of meprin A to the cytosol and other cellular compartments in response to an insult such as I/R results in renal injury and inflammation (10, 34). Other studies in rats have shown that meprin-deficient mice are less vulnerable to kidney damage in response to acute kidney injury, and that actinonin, an excellent inhibitor of meprin A, prevents renal pathology in acute kidney injury (27). Taken together, these studies indicate that blocking meprin activity could be a promising therapeutic approach for treatment of kidney injuries.
ACKNOWLEDGEMENTS

The authors thank the laboratory of D. Antonetti, currently at the University of Michigan, for assistance with the MDCK cell cultures, preparation of membrane fractions and TER measurement.


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Present address of Pan Shi, Hefei National Laboratory for Physical Sciences at Microscale, School of Life Sciences, University of Science and Technology of China, Hefei, Anhui, 230026, China.

GRANTS

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DISCLOSURES

The authors declared no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors participated in the design of the studies, interpretation of the results and preparation of the manuscript. Jialing Bao conducted all studies presented in the manuscript and prepared the first draft of the manuscript. Renee E. Yura initially designed the immunohistochemistry, confocal microscopy and bladder permeability experiments. Judith S. Bond served as mentor and coordinated the project and the
preparation of the final manuscript. Specialized contributors included Gail L. Matters for 

studies with permeability assays, S. Gaylen Bradley for bladder permeability 

measurements, Fang Tian and Pan Shi for preparation of recombinant occludin.
REFERENCES


FIGURE LEGENDS

Figure 1. MDCK monolayer permeability to FITC-labeled dextran. MDCK cell monolayers were cultured on filters. Active or latent meprin A or B (47 nM) were added to the apical chamber of filters and incubated at 37°C for various periods of time. The controls were MDCK cell monolayers incubated with medium only. After meprin treatment, apical medium was replaced with FITC-dextran (40 kDa or 10 kDa). After a 2-h incubation at 37°C, the basal medium was collected and the fluorescence was measured by a spectrophotometer at λ_{ex}492 nm and λ_{em} 520 nm. A, there was a tendency for active meprin A to increase permeability to 40 kDa FITC-dextran after 5-h treatment, but the increase was not significant. B, active meprin A significantly increased permeability to 10 kDa FITC-dextran after 5-h treatment, compared to medium controls; active meprin A also significantly increased permeability to FITC-dextran after 7-h and 9-h treatment, compared to medium controls and latent meprin A (*=P<0.05; **=P<0.01). C, active meprin B significantly increased permeability to 10 kDa FITC-dextran after 9-h treatment, compared to medium controls and latent meprin B (*=P<0.05).

Figure 2. The transepithelial electrical resistance (TER) across MDCK cell monolayers. MDCK cell monolayers were cultured on filters. Active or latent meprin A or B (47 nM) were added to the apical chamber of filters and incubated at 37°C for various periods of time. The controls were MDCK cell monolayers incubated with medium only. The transepithelial electrical resistance across the monolayer was measured by a voltohmmeter EVOM with a STX2 Electrode (World Precision Instruments). A, active meprin A significantly decreased the TER value after 9-h treatment. The inset shows the same graph with the expanded Y-axis (*=P<0.05; NS=Not Significant).
Significant). **B**, active meprin B did not significantly decrease TER value after treatment. The inset shows the same graph with expanded Y-axis (NS=Not Significant).

**Figure 3. Immunocytochemistry of tight junctions on MDCK cell monolayers.**

MDCK cells were treated with active or latent meprin A or B (47nM) for 5 h. Cells were fixed and permeabilized, then incubated with anti-occludin (Figure 3A), anti-ZO-1 (Figure 3B and 3C) and anti-claudin-4 antibodies (Figure 3D). Hoescht staining was used (top row of each section) to show nuclei. For each image, three to five random fields of monolayers were observed by microscopy and showed similar disruptions of TJs; one representative field was captured and shown. **A**, occludin immuno-staining was disrupted in monolayers treated with active meprin A (b), while continuous in untreated monolayers and monolayers treated with latent meprin A (a and c). (all images magnification: 20X; scale bar: 50 micron). **B**, ZO-1 immuno-staining was disrupted in monolayers treated with active meprin A (b), while continuous in untreated monolayers and monolayers treated with latent meprin A (a and c). (all images magnification: 20X; scale bar: 50 micron). **C**, ZO-1 immuno-staining was disrupted in monolayers treated with active meprin B (b), while continuous in untreated monolayers and monolayers treated with latent meprin B (a and c). (all images magnification: 40X; scale bar: 50 micron). **D**, claudin-4 immuno-staining was not disrupted in monolayers with active meprin A (b) treatment. (all images magnification: 63X; scale bar: 50 micron).

**Figure 4. Detection of degradation of occludin but not claudin-4 in membrane-enriched fractions of MDCK cells.** Membrane-enriched fractions (80 mg) of MDCK
cells were incubated with active or latent meprins or EDTA inhibited meprins for various periods of time. Occludin and claudin-4 were detected by Western blot analyses. Each experiment was repeated two to three times and a representative one was shown here. The average percentages of band densities over control were calculated and shown in bar graph. The error bars represent range for 4C and 4D, and SEM for 4A and 4B. A, membrane fractions were incubated with 20 nM meprin A. Active meprin A degraded occludin. B, membrane fractions were incubated with 10 nM meprin B. Active meprin B degraded occludin. C, membrane fractions were incubated with 47 nM meprin A. Claudin-4 was not degraded by meprin A. D, membrane fractions were incubated with 47 nM meprin B. Claudin-4 was not degraded by meprin B.

Figure 5. Detection of degradation of occludin but not claudin-4 in MDCK monolayers. MDCK cell monolayers were incubated with exogenous active or latent meprin A or meprin B for 5 h. Occludin and claudin-4 were detected by Western blot analyses. Each experiment was repeated two to three times and representative blots were shown here. The average percentages of band densities over control were calculated and shown in bar graph. The error bars represent range for 5C, and SEM for 5A and 5B. A, Occludin in MDCK monolayers is degraded by active meprin A. B, Occludin in MDCK monolayers is not degraded by active meprin B. C, Claudin-4 in MDCK monolayers is not degraded by active meprin A.

Figure 6. Recombinant occludin is cleaved by homomeric meprin A. Recombinant occludin (3 μM) was incubated with active or latent meprin A (47 nM) for various periods of time.
periods of time. Samples were subjected to SDS-PAGE and occludin was detected by Coomassie staining or Western blot assay. **A** (Coomassie stain), active meprin A treatment for 4 h decreased occludin band intensity (arrow #1). A cleavage product (~22 kDa) was also observed (arrow #2). **B** (Western blot), active meprin A treatment for 2 and 4 h decreased occludin band intensity (arrow).

**Figure 7. Occludin extracellular loops are cleaved by meprin A.** MBP conjugated loop-1 and loop-2 (6 μM) were incubated respectively with active or latent meprin A (47 nM) for the times shown. After incubation, samples were subjected to 10% SDS-PAGE electrophoresis and stained with Coomassie blue. Each experiment was repeated two times and a representative gel is shown here. **A**, Cleavage of occludin loop 1 was observed after active meprin A treatment (arrow), but not after latent meprin A treatment. **B**, Cleavage of occludin loop 2 was observed after active meprin A treatment (arrow), but not after latent meprin A treatment. **C**, When MBP itself was treated with meprin, no cleavage was observed after active meprin A treatment up to 4 h (lane4).

**Figure 8. Mass spectrometry analysis of occludin cleavage products.** The cleavage products of recombinant extracellular loop 1 and loop 2 were excised from SDS-PAGE gel and samples were subjected to Mass spectrometry analysis. **A**, Peptides highlighted in gray were identified from loop 1 with more than 95% confidence. The peptide closest to C-terminus is HHHHHR. The rest of C-terminus has an excess of Glycine residues thus it is difficult to obtain more accurate identification. **B**, Based on the predicted cleavage site, the precursor ions of the possible C-terminal peptides (GYGTSLLGG, GYGTSLLGGS,
GYGTSLLGGSV, GYGTSLLGGSVG, and GYGTSLLGGSVGY etc) were analyzed. Based on the XIC profile, the measured mass (m/z 824.41217) is very close to theoretical mass m/z 824.4148 of GYGTSLLGG. The mass difference is 3.2 ppm, which indicates the cleavage site is very likely to be Gly 385 (pointed out by arrow in Figure 8A.) It corresponds to Gly 100 on first extracellular loop of full length occludin.

**Figure 9. Bladder permeability to sodium fluorescein was increased after exposure to active meprin A.** C57BL/6 mice were anesthetized, then active, or actinonin inhibited meprin A (4.7 μM), or actinonin alone was injected into the bladder via transurethral catheterization. Mice in the control group were instilled with Tris buffer. After 2 h, 100 ml of 20 mg/ml sodium fluorescein was injected into bladder. After 15 min, blood samples were collected from the interior vena cava, and the plasma fluorescein concentrations were measured at λ<sub>ex</sub>494 nm and λ<sub>em</sub>516nm. The results are an average of two independent experiments, and are expressed as means ±SE, with n=3 per group (* =P<0.05).

**Figure 10. Meprin A regulates monocyte transmigration.** The same number of monocyte cells were isolated from wild-type and meprin αKO mice bone marrow. Monocytes were added to the apical side of MDCK monolayers grown on inserted filters; the lower chamber contained MCP-1 (1.5 nM) as chemoattractant. Control groups included monocytes added to the apical side of inserted filter without MDCK cell monolayers, but with MCP-1 (1.5 nM) in the bottom chamber; and a group of monocytes added on MDCK monolayers without MCP-1 in the bottom. After incubating at 37°C for
3 h, monocytes in the bottom chamber were collected and measured by flow cytometry for the CD11b\(^+\)/NK1.1\(^-\)/Ly6g\(^-\)/Ly6c\(^+\) subsets, which represent the infiltrating inflammatory monocytes. As shown above, significantly fewer meprin \(\alpha\)KO monocytes transmigrated through MDCK monolayers than wild-type monocytes. The results are expressed as means \(\pm\) SE, with \(n=3\) per genotype, per group \((*=P<0.05)\) for two independent experiments.
Figure 1.

A. 40kDa-FITC-dextran concentration (ng/μl)

B. 10kDa-FITC-dextran concentration (ng/μl)

C. 10kDa-FITC dextran concentration (ng/μl)
Figure 2.

A. Graph showing the Ohms $\times$ cm$^2$ over time (h) for Control, Latent meprin A, and Active meprin A.

B. Graph showing the Ohms $\times$ cm$^2$ over time (h) for Control, Latent meprin B, and Active meprin B.
Figure 3.

A. (a) Control (b) Active Meprin A (c) Latent Meprin A

Nuclei

Occludin

B. (a) Control (b) Active Meprin A (c) Latent Meprin A

Nuclei

ZO-1
### Figure 4.

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22 kDa -

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22 kDa -

Densitometry:

- 5 h: 102
- 12 h: 97
- Latent: 105
- w/EDTA: 95

% control:

- 5 h: 125%
- 12 h: 107%
- Latent: 115%
- w/EDTA: 124%
Figure 5.

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65 kDa –

Densitometry

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65 kDa –

Densitometry

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22 kDa –

![Densitometry Graph]

![Image of gel with bands at 22 kDa]
Figure 6.

A. Recombinant occludin incubated with meprin A

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B. Recombinant occludin incubated with meprin A

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![Image of gel electrophoresis](image-url)

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Figure 8.

A.

1 MKIEEGALVI WINGGKGYNG LAEVGKIFK DTVYKTVVEH PKLEENKFQ VAATGDPDE IFWAHIDFGG YASSGLLAEI TPDIAFQQDL YPFTQDAVRY
101 NGGLIAVPIA VEALSLVYNK DLNPVPPKTW EEIPEALKEL RAKGKSALMF NLQEPYFTWPL LAIAOGNYAF KNYKGYDKV DVGDNAGAK AGLTFYVGLI
201 KNNPNMDTID YSIAEAEFKN GETAMING WAKSNIDTSK VnyaGYTVLPT FKQGKSKPFEV QVLSAGINAA SPIKELAEK LENYLLTDEG LEAVNKDKPL
301 GAVALSVEE ELAKOPRIAA TNENAOQGETI MPNIPQMSAF WYAVRTAVIN AASGRQTVDE ALKDAQTGTG HHHHRQGYT STLGGSVGYP YGSGFOSYG
401 SQIGYUYGYG VGGVQYIDPR A

B.
Figure 9.
Figure 10.