Adrenomedullin reduces intestinal epithelial permeability in vivo and in vitro

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Temmesfeld-Wollbrück B, Brell B, zu Dohna C, Dorenberg M, Hocke AC, Martens H, Klar J, Suttorp N, Hippenstiel S. Adrenomedullin reduces intestinal epithelial permeability in vivo and in vitro. Am J Physiol Gastrointest Liver Physiol 297: G43–G51, 2009. First published May 7, 2009; doi:10.1152/ajpgi.90532.2008.—Leakage of the gut mucosal barrier in the critically ill patient may allow translocation of bacteria and their virulence factors, thereby perpetuating sepsis and inflammation. Present evidence suggests that adrenomedullin (AM) improves endothelial barrier function and stabilizes circulatory function in systemic inflammation. We tested the hypothesis that exogenously applied AM stabilizes gut epithelial barrier function. Infusion of Staphylococcus aureus α-toxin induced septic shock in rats. AM infusion in a therapeutic setting reduced translocation of labeled dextran from the gut into the systemic circulation in animals therapeutically (after onset of septic shock). AM infusion in a therapeutic setting reduced barrier function. Infusion of amylase reduced circulatory function in systemic inflammation. We tested the hypothesis that exogenously applied AM stabilizes gut epithelial barrier function. Infusion of Staphylococcus aureus α-toxin induced septic shock in rats. AM infusion in a therapeutic setting reduced translocation of labeled dextran from the gut into the systemic circulation in animals therapeutically (after onset of septic shock). AM infusion in a therapeutic setting reduced barrier function. Infusion of AM diminished intestinal permeability and transepithelial resistance; cyclic AMP; sepsis (22, 46, 61, 74). As a consequence, gut-derived inflammatory mediators and translocated bacteria and their products may further perpetuate septic and inflammatory events (22, 23, 46, 61). Thus the intestine has to be considered as a particularly important shock organ. Although intestinal epithelial barrier dysfunction is well known as a high risk factor for subsequent translocation of toxic substances and pathogens, there are limited therapeutic options to stabilize gut barrier function in patients suffering from sepsis and septic shock.

Accumulating evidence suggests a pivotal role of the multifunctional regulatory peptide adrenomedullin (AM) in sepsis and septic shock (67, 68). AM is a 52-amino-acid peptide belonging to the calcitonin gene-related peptide family (10, 26, 47). Treatment of virtually all cells presently investigated with AM resulted in a marked increase in cellular cAMP content and subsequent activation of protein kinase A (26, 47). Central body functions like vascular tone regulation or fluid and electrolyte homeostasis are particularly regulated by AM (for review, see Refs. 26 and 50).

Several reports demonstrated high AM plasma levels in vertebrates, including human beings with systemic inflammatory response (28, 36, 66, 67, 69). Under normal conditions, AM is released ubiquitarily by a variety of cells. Interestingly, in sepsis, AM is produced to a great extent in the mucosa of the gastrointestinal tract (49, 75), and splanchic vasculature is highly responsive to AM (15, 16, 42, 52). In previous reports, we demonstrated that AM stabilized endothelial barrier function in vitro, ex vivo, and in vivo (15, 16, 27, 63). Remarkably, AM treatment was shown to abolish ileal mucosal hypoperfusion and to stabilize intramucosal hemoglobin oxygenation in Staphylococcus aureus (S. aureus) α-toxin-exposed ileum (15, 16).

Therefore, we now tested the hypothesis that AM stabilizes epithelial barrier function. By using an in vivo rat model of S. aureus α-toxin-induced septic shock, we observed reduced translocation of labeled dextran from the gut into the systemic circulation in animals therapeutically (after onset of α-toxin application) infused with AM. AM diminished α-toxin and hydrogen peroxide (H2O2)-related barrier disruption in Caco-2 cells in vitro and reduced H2O2-related rat colon barrier malfunction in Ussing chamber experiments. AM was shown to protect endothelial barrier function via cAMP elevation, but AM failed to induce cAMP accumulation in Caco-2 cells. cAMP is degraded via phosphodiesterases (PDE), and Caco-2 cells showed high activity of cAMP-degrading PDE3 and 4. However, AM failed to induce cAMP accumulation in Caco-2 cells even in the presence of sufficient PDE3/4 inhibition, whereas adenosyl cyclase activator forskolin induced strong cAMP elevation. Furthermore, PDE3/4 inhibition neither amplified AM-induced epithelial barrier stabilization nor affected AM cAMP-related rat colon short-circuit current, furthermore indicating that AM may act independently of cAMP in Caco-2 cells. Finally, experiments using chemical inhibitors indicated that PKC, phosphatidylinositol 3-kinase, p38, and ERK did not contribute to AM-related stabilization of barrier function in Caco-2 cells. In summary, during severe inflammation, elevated AM levels may substantially contribute to the stabilization of gut barrier function.

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However, the effect of AM on epithelial barrier function in vitro seems not to be dependent on PKC, phosphatidylinositide 3 (PI3)-kinase, p38, or ERK. Overall, in severe inflammation, elevated AM levels may substantially contribute to stabilization of gut barrier function.

**MATERIAL AND METHODS**

Animal model and determination of intestinal permeability. Male Sprague-Dawley rats (Harlan-Winkelmann, Borchern, Germany) weighing 250–300 g were used in all experiments. The study was approved by the local animal care committee (Berlin, Germany; permit No. G0113/04).

Anesthesia and mechanical ventilation of rats as well as volume supply and hemodynamic monitoring were performed as described previously (63). Intestinal permeability was assessed using an in vivo ligated loop model as previously described (63). Briefly, after a median laparotomy was performed, the ileocecal junction was identified. From 3 cm above the junction measured in the oral direction, a 10-cm-long segment of the terminal ileum was exteriorized. The ileum segment was filled with 10 mg of 4-kDa FITC dextran (FD4) (Sigma, St. Louis, MO) in PBS (Sigma) and ligated. The ileal loop ileum segment was filled with 10 mg of 4-kDa FITC dextran (FD4) (Sigma, St. Louis, MO) in PBS (Sigma) and ligated. The ileal loop was replaced, and evaporation via the opened abdomen was prevented by a plastic film.

Rats were allocated to one of four experimental groups, 1) application of isotonic saline (control group, n = 10); 2) application of 4.8 × 10^3 U/kg α-toxin (α-tox group, n = 15); 3) application of 4.8 × 10^3 U/kg α-toxin and 24 μg/kg AM per hour (α-tox + AM group, n = 15); or 4) application of 24 μg/kg AM per hour (AM group, n = 6). Doses of AM and α-toxin were chosen on the basis of previous studies regarding the analysis of endothelial barrier function (15, 16, 27). Purified staphylococcal α-toxin was generously provided by Prof. Dr. S. Bhakdi (University of Mainz, Germany). Activity of α-toxin was 143 U/μg protein. The total duration of the experiments was 6 h. The 45-min α-toxin infusion via central venous catheter was started at t = 0 h. One hour later, a continuous administration of 24 μg/kg AM (Bachem Bioscience, Philadelphia, PA) was initiated via tail vein. Arterial plasma samples were drawn via the carotid artery at hours 0 (before α-toxin), 4, and 6 (after α-toxin). Fluorescence of 0.5 mg/ml arterial plasma was measured at 490 nm wavelength using a Fluoromax 2 Spectrofluorometer (HORIBA Jobin Yvon, Munich, Germany), and FD4 was quantified using standard curves.

Cell culture. Caco-2 cells were purchased from the German Collection for Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cells were maintained under standard cell culture conditions in DMEM with L-glutamine (PAA Laboratories, Pasching, Austria) supplemented with FBS (10% vol/vol), penicillin G (100 U/ml), streptomycin (100 μg/ml), pyruvate (2 mM), and nonessential amino acid supplement (1% vol/vol). For monolayer permeability assays, cells were grown on porous polycarbonate membranes (pore size, 3 μm) in Transwells (12 mm; Costar, Cambridge, MA).

Permeation of paracellular markers. For paracellular permeability assays, FD4 was dissolved in medium at a concentration of 10 mg/ml. The FITC-dextran solution was added at a final concentration of 0.5 mg/ml FD4 to the apical compartment of the Transwell. In an analogous manner, a solution containing 20% human serum albumin (68 kDa) was enriched with 10 mg/ml Evans blue dye (EBD) and added to the apical chamber at a final concentration of 0.5 μg/ml EBD and 1% albumin. EBD is known to bind to albumin in a very stable way. The completeness of EBD binding to albumin was confirmed by protein precipitation with trichloroethanoic acid in the primary solution and following centrifugation, which resulted in a dark and blue sediment and a clear and uncolored supernatant. To evaluate the permeability of the monolayers, 25-μl media samples were collected from the basal compartment for subsequent FD4 and EBD quantification. EBD possesses a strong fluorescence in the infrared region. Therefore, it could be detected by an infrared fluorescence laser scanner (Odyssey infrared imaging system; LI-COR Biosciences, Bad Homburg, Germany). An EBD-albumin standard in media and all media samples of a permeability assay were transferred into a 96-well plate and scanned at 700 nm. The integrated scanned intensity of each well was stored, and concentration of EBD was calculated via EBD standard curves. FD4 was measured and quantified as described above. Finally a concentration gradient between apical and basal chamber was calculated.

Experimental protocol of Caco-2 permeability assay. Two hours before start of the experiment, the medium in the apical and basal chamber was changed to fresh DMEM, which had been conditioned overnight at 37°C and 5% CO2 gassing. The molecular markers (FD4 and EBD-albumin) were added to the apical chamber (t = −1 h). Thirty minutes later, AM (0.01, 0.05, 0.1, and 0.5 μM; Bachem Bioscience), forskolin (1 μM, Sigma), or solvent (PBS) were added to the basal compartment. Zardaverine (ALTANA Pharma, Konstanz, Germany) was added 10 min before AM. Measurement of transepithelial electrical resistance (TEER) and withdrawal of samples from the basal compartment were performed 1 h after marker application. Amiloride (10 μM) was added 20 min after staphylococcal α-toxin (0.1, 0.5, 1, and 2 μg/ml, H2O2 (1, 2, 5, 10, and 20 mM) or solvent (PBS) was added to the basal compartment (t = 0 h). Thereafter, samples for FD4 and EBD-albumin were collected in hourly intervals, and a final TEER measurement was performed at t = 3 h. Doses of AM and α-toxin were chosen on the basis of previous studies regarding the analysis of endothelial barrier function (27, 29).

Reincubation time and doses of kinase inhibitors were selected from previous studies using these agents [Calphostin C (39, 57), Go 6976 (39, 57), LY29402 (58), U0126 (44, 54), and SB202190 (44, 54)].

Measurement of TEER. The formation of a sealed monolayer of the cells was monitored by serial measurements of the TEER with an endothelial voltmeter (World Precision Instruments, Sarasota, FL). The resistance of the supporting membrane in Caco-2 was subtracted from all TEER measurements. Cells were used for experiments when the TEER had increased above 250 Ω/cm², which usually occurred 9 to 12 days after seeding.

Measurement of cAMP content. Cyclic nucleotide content was measured using a commercially available ELISA (Biotrend, Cologne, Germany) as described previously (55, 59, 60). Preincubation time and doses of PDE inhibitors were chosen from previous studies analyzing cAMP formation or PDE activity (27, 59, 60).

PDE assay. Phosphodiesterase activity was measured as described by Thomson et al. modified by Bauer and Schwabe (9) using [3H]cAMP or [3H]cGMP as substrate as described previously (55, 59, 60). PDE activity was normalized to protein concentrations of the soluble fraction of Caco-2 lysates. Selective inhibitors and activators of PDE isoenzymes were used to determine activities of PDE families as described previously (55, 60).

Using chamber experiments of rat colon. In male Sprague-Dawley rats (250–300 g), anesthesia was introduced by a subcutaneous injection of xylcaine (30 mg/kg) and ketamine (100 mg/kg). A catheter was placed in the lateral tail vein. After rats were euthanized by an intravenous overdose of anesthetics, the abdomen was opened widely. A 7-cm segment of proximal colon was removed and placed in ice-cold tyrode buffer solution [(in mM) 141 Na, 85 Cl, 5 K, 25 HCO3, 1 HPO4, 2 H2PO4, 1 Ca, 1 Mg, 13 acetate, 13 propionate, 13 butyrate, and 10 glucose; gassed with 95% O2-5% CO2; pH 7.4]. The colon specimen was opened along the mesenteric side, and full-thickness tissue preparations were mounted in Ussing chambers (area size = 1 cm²). The mounted colon was bathed on both sides with tyrode buffer solution (38°C) and short-circuited with a computer-controlled voltage-clamp device (Mussler, Aachen, Germany).

Rat colon short-circuit current (SCC), which mainly represents chloride secretion (3, 51), was measured as μA/cm². The electrical resistance of colon pieces (Ω/cm²) was calculated by Ohm’s law from...
the displacement in the potential difference caused by bipolar pulses of 100 µA. Therefore, electrical resistance reflected by intestinal barrier function was not influenced by the potential difference related to epithelial ion secretion. For the comparison between experimental groups, the relative changes of SCC and electrical resistance were calculated.

**Cell death detection ELISA and release of lactate dehydrogenase.** A commercially available photometric ELISA was used for the detection of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) in apoptotic epithelial cells (Roche, Mannheim, Germany) as described previously (40, 53). Lactate dehydrogenase (LDH) activity in the supernatants was determined by the colorimetric measurement of the reduction of sodium pyruvate in the presence of NADH (LDH Assay, Roche), and specific LDH release was calculated as described previously (40, 53). In these experiments, Caco-2 cells were exposed to stimuli for 5 h (2 µg/ml α-toxin, 10 mM H₂O₂, respectively) with or without preincubation with AM as indicated.

**Statistics.** All data are presented as means ± SE. Normal distribution of variables was verified by Kolmogorov-Smirnov test. Groups with normally distributed variables were compared with a one-way ANOVA. If normal distribution was not given, a Kruskal-Wallis test was calculated. If normally distributed variables were compared with a one-way ANOVA, *P* < 0.05 was considered significant.

**RESULTS**

**AM reduces α-toxin-induced epithelial permeability in vivo and in vitro.** To test whether AM may stabilize gut epithelial barrier function in vivo, we made use of a rat model of *S. aureus* α-toxin-induced septic shock. Pore-forming α-toxin is the major cytotoxin of *S. aureus*, a pathogen that frequently causes severe sepsis (13, 35). As demonstrated recently, infusion of 4.8 × 10³ U/kg α-toxin in rats resulted in a 24-h mortality of 53%, which was reduced by therapeutic infusion of 24 µg/kg AM per hour starting 1 h after α-toxin exposure to 7% (63). In this model, α-toxin infusion via a central venous catheter caused an over fourfold increase of intestinal permeability for FD4 (Fig. 1), indicated by translocation of FD4 from the gut into systemic circulation. Treatment with 24 µg/kg AM per hour via tail vein infusion started 1 h after α-toxin exposure. AM significantly diminished the α-toxin-related increase of gut-derived FD4 in arterial plasma by 60% (Fig. 1). Application of AM alone induced no significant changes of intestinal permeability. Thus venous infusion of AM seems to stabilize gut epithelial barrier function in α-toxin-exposed rats.

Next, we wondered whether AM may directly stabilize barrier function of gut epithelial cells. In Caco-2 cells, α-toxin dose dependently increased permeation of FD4 through the monolayer. The highest dose of α-toxin (2 µg/ml) induced a significant sixfold increase of FD4 concentration in the basal compartment within 3 h (Fig. 2A) and was used to test AM effects on Caco-2 permeability. AM (0.5 µM) reduced the α-toxin-related increase of monolayer permeability indicated by reduction of FD4 accumulation in the basal compartment 3 h after α-toxin application (Fig. 2B). AM application alone had no effect on FD4 permeation.

**AM inhibits H₂O₂-related increase of Caco-2 cell and rat colon permeability.** Next, we tried to find out whether AM may be able to reduce the increase of permeability in the presence of a stimulus with a different mode of action as compared with α-toxin (a pore-forming exotoxin of Gram-positive bacteria, Ref. 13), and we made use of the well-established H₂O₂ as inductor of Caco-2 cell permeability (4, 6, 33, 48, 64). H₂O₂ dose and time dependently increased permeability for FD4 (Fig. 3A) and EBD-albumin (Fig. 3D) and significantly decreased TEER by 60% at 3 h after application (Fig. 3C). The dose of 10 mM H₂O₂, which induced a near maximal effect, was chosen to investigate the effect of various AM doses on epithelial permeability. AM significantly diminished H₂O₂-induced increase of FD4 (Fig. 3D) and EBD-albumin (Fig. 3E) permeation through the Caco-2 monolayer and decrease of TEER (Fig. 3F). The strongest AM effect was achieved at a...
concentration of 0.5 \( \mu M \), causing an over 70% reduction of the permeability increase for the molecular markers. Moreover, \( H_2O_2 \)-induced decrease of relative TEER was strongly inhibited by 0.5 \( \mu M \) AM (\( H_2O_2 \) vs. AM 1000-fold at 5 and 7%).

Furthermore, the effect of AM on \( H_2O_2 \)-related hyperpermeability was expanded to an intact rat colon in Ussing chamber experiments (Fig. 4). \( H_2O_2 \) (0.5 \( \mu M \)) induced within 30 min a significant reduction of colon electrical resistance by 30% (compared to control), which was completely abolished by 1 \( \mu M \) AM (Fig. 4, AM alone data not shown).

Cell viability after exposure of \( \alpha \)-toxin and \( H_2O_2 \). Both the bacterial exotoxin and \( H_2O_2 \) may reduce cell viability, which in turn may result in increased monolayer permeability. However, when exposing our Caco-2 cells to 2 \( \mu g/ml \) \( \alpha \)-toxin or 10 mM \( H_2O_2 \) for 5 h, we did not observe a significant increase in apoptosis (Supplemental Fig. S1A; supplemental material for this article is available online at the American Journal of Physiology Gastrointestinal and Liver Physiology website) or necrosis as evidenced by specific LDH release (Supplemental Fig. S1B). In addition, AM showed no effect on the rate of apoptosis or necrosis.

Effect of AM on gut epithelial permeability seems not to be mediated by intracellular cAMP, PKC, PI3-kinase, p38 MAPK, or ERK. AM-related elevation of the second messenger cAMP is considered as a central mechanism of AM signal transduction in general (26, 47) and in particular for its endothelial barrier-stabilizing activity (27). However, we could not ob-

Fig. 3. AM reduces \( H_2O_2 \)-related barrier dysfunction of Caco-2 monolayers. \( H_2O_2 \) induced time and dose (1–20 mM) dependently an increase in permeability of Caco-2 monolayers for FITC-dextran (A) and Evans blue dye (EBD)-albumin (B) and decreased transepithelial electrical resistance (TEER) determined 3 h after \( H_2O_2 \) addition (C). AM dose dependently (0.01–0.1 \( \mu M \)) reduced permeability for FITC-dextran (D) and EBD-albumin (Fig. 3E) and blocked \( H_2O_2 \)-related TEER decrease (F). All measurements (D–F) were performed 3 h after \( H_2O_2 \) addition. Data presented in A–C are means ± SE of \( n = 8 \) independent experiments. *\( P < 0.05 \) vs. control group. Data presented in D–F are means ± SE of \( n = 8 \) independent experiments. *\( P < 0.05 \) vs. control group. 

Fig. 4. AM reduces \( H_2O_2 \)-related decrease of rat colon resistance in an Ussing chamber model. Electrical resistance of preparations was reduced by addition of 0.5 \( \mu M \) \( H_2O_2 \). Incubation with 1 \( \mu M \) AM completely abolished the \( H_2O_2 \) effect on resistance. Data presented are means ± SE of \( n = 5 \) independent experiments. *\( P < 0.05 \) vs. control group. 

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serve a significant elevation of cAMP in Caco-2 cells exposed to 0.01–1 μM AM, whereas 1 μM forskolin induced significant cAMP accumulation in the same experiment (Fig. 5A). The cyclic nucleotide cAMP is degraded via PDEs, and different cells showed different PDE activity patterns (11, 18), which may furthermore be regulated by inflammatory stimuli (11, 18, 55). Because the PDE activity profile for gut epithelial cells was unknown, we analyzed PDE activity profile in Caco-2 cells (Fig. 5B). Caco-2 cells displayed high activity for cAMP-degrading PDE3/4 and low activity of PDE2, 5, and 7. Furthermore, Caco-2 PDE expression is not modified by exposure of cells to 50 ng/ml TNF-α, a stimulus known to induce PDE2 expression in human endothelial cells (55). Because the PDE activity profile for gut epithelial cells was unknown, we analyzed PDE activity profile in Caco-2 cells (Fig. 5B). Caco-2 cells displayed high activity for cAMP-degrading PDE3/4 and low activity of PDE2, 5, and 7. Furthermore, Caco-2 PDE expression is not modified by exposure of cells to 50 ng/ml TNF-α, a stimulus known to induce PDE2 expression in human endothelial cells (55). Then, we analyzed AM effects on cAMP content in the presence of the well-established dual-selective PDE3/4 inhibitor zardaverine (Fig. 5A). Although the inhibitor potentiated forskolin-related cAMP increase, a significant increase of cAMP in AM-exposed Caco-2 cells was not noted.

Because ELISA measurement of cAMP may not detect low or local AM-related cAMP increases, which in turn could mediate AM effects on epithelial barrier function, we assessed permeability in the presence of zardaverine, thereby inhibiting cellular cAMP degradation (Fig. 6). AM (0.5 μM) alone reduced FD4 translocation, but additional inhibition of PDE3/4 in the presence of 0.1 μM AM was unable to further improve barrier function in H2O2-exposed cells (Fig. 6A). This is in contrast to our results obtained in endothelial barrier regulation in different models including AM-related barrier stabilization (27, 60). Remarkably, although forskolin stimulated cAMP accumulation in Caco-2 cells (Fig. 5A), it failed to reduce FD4 translocation in H2O2-incubated cells (Fig. 6A). In addition, the same response pattern is shown with respect to TEER (Fig. 6B).

Finally, rat colon SCC, which mainly represents cAMP-dependent chloride secretion (3, 51), was measured in the presence of AM and forskolin (Fig. 7). Whereas cAMP-elevating forskolin induced a significant SCC increase, 1 μM AM did not induce changes in SCC. Overall, these data suggest that AM-related stabilization of gut epithelial barrier function occurs via a mechanism that is different from cAMP accumulation.

Several kinases, in particular PKC, PI3-kinase, or the MAPK p38, and ERK, were considered to participate in the regulation of epithelial barrier function and may be part of AM-related signaling. Therefore, we tested whether inhibition of these kinases may impact AM-related stabilization of epithelial barrier function. However, preincubation of Caco-2 cells for 15 min with broad-range PKC inhibitor Calphostin C (10 μM), PKC-α/β inhibitor G6976 (5 μM), PI3-kinase blocker LY29402 (10 μM), ERK inhibitor U0126 (10 μM), as
H2O2-related barrier breakdown in Caco-2 cells in vitro.

In addition, AM diminished H2O2-related translocation of a marker molecule from the gut lumen into the systemic circulation in vivo. AM infusion reduced translocation of a marker molecule within the time frame tested (data not shown).

DISCUSSION

The presented study demonstrates that exogenously applied AM contributes to the stabilization of gut epithelial barrier function in vitro and in vivo. In a therapeutic setting, intravenous AM infusion reduced translocation of a marker molecule from the gut lumen into the systemic circulation in α-toxin-exposed rats. In addition, AM diminished H2O2-related reduction of resistance in rat colon model as well as α-toxin- and H2O2-related barrier breakdown in Caco-2 cells in vitro.

Reduction of gut mucosal barrier function allowing the translocation of bacteria and their products from the gut lumen into surrounding tissues and the systemic circulation is considered a detrimental event in critically ill patients (22, 23, 46, 61). However, little is known about therapeutic interventions to stabilize gut barrier, particularly in sepsis and septic shock.

AM is increased in sepsis and septic shock, and the endogenous peptide AM may be considered as a counterregulatory peptide in these situations. Enhancing endogenous AM levels by infusing exogenous AM has shown benefit in a couple of experimental settings (67, 68, 73). AM reduced mortality in models of polymicrobial sepsis (24, 72, 73), endotoxin- (24) and exotoxin-related (63) shock, and hemorrhage (19). Norepinephrine, AM seems to be of particular importance for the regulation of endothelial barrier function under different inflammatory conditions (15, 16, 27, 29, 63). Therefore, we tested the hypothesis that AM stabilizes gut epithelial barrier function, thereby reducing the risk of translocation of gut content, which in turn has the potential to reduce the risk of the extent of a systemic inflammation.

We noted that therapeutic infusion of AM reduced gut barrier dysfunction in an infection model, which starts with the systemic application of staphylococcal α-toxin (63), the major staphylococcal virulence factor (13). In addition, AM reduced the loss of barrier function in cultured α-toxin- or H2O2-exposed Caco-2 epithelial cells. Finally, AM normalized resistance in H2O2-exposed rat colon in Ussing chamber experiments. Hence, AM stabilized gut epithelial barrier function in different models.

The massive production of AM in the mucosa of the gastrointestinal tract observed in sepsis (75) may thus be considered a barrier-protective response of the gut. Furthermore, we and others found that the splanchnic vasculature is highly responsive to AM (14–16, 42, 52). In particular, AM treatment was shown to abolish ileal mucosal hyperperfusion and to stabilize intramucosal hemoglobin oxygenation in S. aureus α-toxin-exposed ileum (15, 16). From these studies, it appears that AM provides gut barrier protection at different levels, 1) direct stabilization of barrier function of epithelial cells, 2) promotion of intramucosal perfusion and hemoglobin oxygenation in the microcirculation, and 3) optimization of splanchnic blood flow.

However, it should be noted that the exact mechanism how α-toxin impairs barrier function in vivo (and in vitro) is unknown. In vivo, for example, α-toxin may act directly by affecting the mucosal layer or indirectly by perturbation of the microvascular environment in the gut. In the in vivo model used in this study, α-toxin was infused intravenously, and AM has been shown to stabilize endothelial barrier function under certain circumstances (16, 27, 29, 63), including the application of α-toxin (16, 29, 63). Thus it could not be ruled out that the beneficial effect of AM is, at least in part, dependent on the tightening of the endothelial barrier, thus limiting direct access of the toxin to the epithelial compartment.

Besides its barrier-stabilizing activity, the potent direct antimicrobial activity of AM (1, 2) produced by the gastrointestinal mucosa may help to kill invading bacteria at sides of disturbed barrier function. Interestingly, most recently, Gonza-
lez-Rey et al. (25) showed a therapeutic effect of AM in a murine model of Crohn’s disease, indicating that AM may be a counterregulatory peptide in both acute and chronic gut diseases.

AM-mediated elevation of the second messenger cAMP is considered as a central event in AM signaling (10, 26, 47) and was shown to contribute to AM-related stabilization of endothelial barrier function in several models (15, 16, 27, 63). However, we did not observe any effect on cAMP content in AM-exposed Caco-2 cells although the cells respond with strong cAMP elevation in the presence of the adenyl cyclase activator forskolin. PDE metabolizes cAMP (and cGMP) to its biologically inactive nucleotide 5’-monophosphates (11, 18). On the basis of sequence similarity, mode of regulation, and preference for cAMP or cGMP as substrate, PDEs are categorized into 11 gene families (11, 18). Because the PDE isoenzyme pattern for gut epithelial cells has not been shown so far, we first analyzed PDE activity in Caco-2 cells. These cells expressed high activity of PDE3/4, which both are able to hydrolyze cAMP efficiently. There was very low PDE2, PDE5, and PDE7 activity. In contrast to human endothelial cells, PDE enzyme activity is not altered by TNF-α exposure of cells (55). Thus we investigated the effect of AM on cAMP content in the presence of the dual selective PDE3/4 inhibitor zardaverine.

AM failed to increase cAMP content in this situation although zardaverine was shown to potentiate forskolin-induced cAMP levels in Caco-2 cells. Because we cannot rule out that low or a local cAMP increases below the detection limit of the ELISA, we tested the effect of AM on Caco-2 barrier function in the presence of the PDE3/4 inhibitor. Again, though, there was no effect of PDE inhibition on the AM response with respect to barrier function. This is in contrast to the data shown for other cells such as endothelial cells (27, 55, 60), in which inhibition of cAMP-metabolizing PDEs potentiated AM-related cAMP elevation and resulted in synergistic stabilization of barrier function. Furthermore, forskolin, shown to strongly elevate cAMP levels in Caco-2 cells, did not stabilize Caco-2 barrier function (but protects endothelial barrier function, Ref. 60). Finally, we analyzed SCC, mainly representing chloride secretion (3, 51), which is known to be stimulated by cAMP-elevating agents in rat colon pieces. In contrast to forskolin, AM did not increase SCC. Overall, several lines of evidence suggest that the effect of AM on Caco-2 cell barrier function is unrelated to cAMP-dependent mechanisms. This is in line with the positive inotropic function of AM in rat hearts, which appeared to be cAMP independent (62), although cAMP elevation is considered a major signaling molecule in AM-related cardiovascular effects (14, 17, 26–28). However, it should be mentioned that, by using the kinase inhibitor H-89, Fernandez et al. (21) provided evidence that AM effects on sugar transport in rat intestine were possibly regulated by cAMP elevation, but cAMP levels were not measured in this study.

A variety of complex dynamic structures like tight junctions and the actin cytoskeleton impact on gut epithelium to regulate the tightness of the barrier (12, 30, 56). AM activates a variety of pathways including protein kinase C (20), PI3-kinase (41, 43), and ERK kinases (45). All these pathways are known to impact gut epithelial barrier function (4, 5, 7, 32, 37, 71). However, it turned out that inhibition of these kinases (PKC, PI-3K, ERK, and p38) by specific chemical inhibitors did not reduce the beneficial effect of AM on epithelial barrier function with respect to TEER and FITC-dextran permeability in vitro in our experimental setting. The regulation of epithelial junctions and cell contraction is of outstanding complexity (23, 30) and may be regulated by a concerted action of different kinases together, which may explain the negative result obtained. Furthermore, other kinases like RhoA-associated coiled-coil forming kinase (38, 70) or members of the Src-family (8) not addressed in our study may account for the observed effect. Therefore, to finally decipher the cAMP-independent AM-effect on gut permeability, a more explorative effort (e.g., large-scale siRNA screen for kinases) may be used in a further study.

In conclusion, this study demonstrates a protective effect of AM on gut epithelial barrier function in vitro and in vivo. Analysis of PDE activity identified PDE3/4 as major cAMP-degrading enzymes in Caco-2 cells. AM failed to induce cAMP elevation in Caco-2 cells even in the presence PDE3/4 inhibition, and PDE3/4 inhibition did not enhance AM effects on barrier function. Moreover, cAMP elevation by forskolin did not stabilize Caco-2 barrier function, suggesting that cAMP-independent signals lead to barrier stabilization by AM. Further studies are needed to define the AM-related signaling pathways stabilizing gut barrier function.

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