Intestinal barrier function: regulation of epithelial permeability and mucin expression
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Pivotal role for JNK MAPK in regulation of intestinal epithelial barrier integrity

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Submitted
Abstract
Increased intestinal permeability in Crohn's patients was found to precede relapse to active disease but the signaling pathways regulating epithelial barrier properties are poorly understood. We investigated the importance of the phosphoinositide 3 kinase (PI3K), mitogen activated protein kinase (MAPK), and protein kinase C (PKC) pathways for epithelial barrier integrity. T84 monolayers were incubated with flavonol quercetin or selective inhibitors for PI3K, MAPK (ERK, p38 and JNK), and PKC in the presence or absence of IFN-γ. Both trans-epithelial resistance and apical-to-basolateral fluxes were measured to evaluate barrier integrity and STAT1 phosphorylation was determined. Inhibition of JNK MAPK in particular, dramatically enhanced basal resistance and reduced permeability caused by IFN-γ incubation. Flavonol quercetin, similar to PI3K inhibitor LY294002, ameliorated IFN-γ mediated barrier disruption and additionally inhibited IFN-γ induced STAT1 phosphorylation. In contrast, PKC inhibition had minor effects on basal resistance and aggravated IFN-γ mediated barrier disruption. JNK MAPK plays an essential role in regulation of the epithelial barrier function and is an interesting target in diseases with underlying intestinal permeability, like Crohn's disease. Quercetin ameliorates IFN-γ mediated barrier disruption and is an example of the application of natural food ingredients for the regulation of epithelial barrier function.
**Introduction**

Inflammatory bowel disease, coeliac disease, food allergy, and intestinal infections are associated with enhanced permeability of the intestine. In Crohn’s (CD) patients the barrier function of the gut is impaired during episodes of chronic inflammation and intestinal permeability may precede relapse to active disease. Enhanced permeability of the gut may trigger or sustain inflammation since it results in increased mucosal leakage of antigenic and toxic agents. Defective epithelial tight junctional strands are observed in tissue biopsy specimens from patients with active Crohn’s disease, and in *in vitro* models of immune mediated barrier disruption. IFN-γ has been identified to induce barrier disruption by affecting tight junction proteins and enhancing myosin light chain (MLC) phosphorylation. MLC activation results in contraction of the perijunctional actomysin ring that is connected to the tight junction complex, increasing paracellular permeability. Loss of barrier function in epithelial monolayers has been associated with activation of PKC (protein kinase C), PI3K (phosphoinositide 3-kinase), and the family of small protein GTP-ases (Ras, Rho, Cdc42). Downstream of small protein GTP-ases, MAPKs (mitogen activated protein kinases) (ERK, p38, JNK) are known to be activated and Rho and Rac-1 in particular, were found to regulate MLC phosphorylation and control the perijunctional actomysin ring. At present it is unclear which signaling pathways are involved in IFN-γ mediated barrier disruption. After binding of IFN-γ to its receptor, Janus family tyrosine kinases Jak-1 and -2 are associated with the IFN-γ receptor subunits. Jak-1 and -2 cross phosphorylate STAT1 (signal transducer and activator of transcription-1) and may also activate other signal transducing cascades like PI3K, MAPKs and PKC. Components, including natural occurring flavonoids, that interfere with these signaling pathways may be potential regulators of the intestinal barrier homeostasis and immune mediated barrier disruption. We therefore studied several routes of signal transduction to elucidate their involvement in barrier maintenance and IFN-γ mediated barrier disruption.

**Materials and methods**

**Chemicals**

PD98059, SB203580, Gö-6976 and LY294002 were obtained from Calbiochem (San Diego, CA), SP600125 from Biomol Laboratories (Hamburg, Germany), rotterlin and 4 kDa FITC (fluorescein isothiocyanate)-dextran from Sigma Chemical Company (St. Louis, MO), quercetin from Kaden Biochemicals (Hamburg, Germany) and IFN-γ from HyCult Biotechnology BV. The Bio-Rad DC assay was from Bio-Rad Laboratories (Hercules, CA). Lumi light PLUS substrate solution was obtained from Roche Diagnostics (Mannheim, Germany).
**Chapter 6**

**Cells, antibodies and ELISA**
The human intestinal cell line T84 was purchased from the ATCC (Manassas, VA). Antibodies against STAT1 and phospho( tyr710)-STAT1 were obtained from Cell Signaling Technology (Beverly, MA). HRP-conjugated anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell culture**
T84 (passages 61-64) were seeded in a 5 fold dilution on 12-mm transwell inserts (0.4 μm, Corning BV, Acton, MA) in DMEM/F12 glutamax I with penicillin (100 IU/ml), streptomycin (100 μg/ml) (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 5% heat inactivated fetal bovine serum (Greiner-Bio One, Frickenhausen, Germany). T84 monolayers were used 14 days after confluence.

**Incubation with IFN-γ and chemical inhibitors or flavonoids**
Chemical inhibitors and natural food flavonol quercetin, were applied to the apical compartment and of the transwell. After 2 h human recombinant IFN-γ (100 U/ml) was added basolaterally. Control incubations were performed with DMSO (0.1% v/v), the vehicle for quercetin and the inhibitors. Every 24 h the culture medium was changed and fresh inhibitors and IFN-γ were added.

**Measurement of resistance**
Transepithelial resistance (TER; ohm.cm²) was measured by epithelial voltohmeter (EVOM; World Precision Instruments) prior to medium refreshment at 0, 24, 48 and 72 h of incubation. To normalize for the slight variations between cultures the basal resistances were set to 100%.

**Macromolecular permeability**
The apical-to-basolateral flux of 4kDa FITC-dextran was measured to determine the permeability of the monolayers after 72 h IFN-γ incubation in the presence or absence of inhibitors. One hour prior to dextran fluxes the culture medium was refreshed with culture medium without phenol red (DMEM/F12 including additives). 1 mg/ml 4kDa FITC-dextran was added to the apical compartment. After 30 min 100 μl sample was collected from the basolateral compartment and the fluorescent signal measured at excitation wave length 485 nm and emission 520 nm (FLUOstar Galaxy®, BMG Labtechnologies, Offenburg, Germany). FITC-dextran fluxes were calculated (pmol FITC-dextran/cm²/h). To normalize for the slight fluctuation of the IFN-γ induced fluxes between cultures, these were set to 100%.
Signaling pathways involved in regulation of barrier integrity

Gel electrophoresis and immunoblotting
Cell samples were collected in laemmlini and protein determination was performed using the DC-protein assay according to the manufactures protocol with minor modifications. Samples (20 µg) were run in 10% SDS-page gels and transferred to PVDF membranes (Roche Diagnostics, Indianapolis, IN). Blots were blocked in TBST/5% Protivar (Nutricia, Zoetermeer, The Netherlands) followed by one hour incubation with primary antibodies to STAT1 and PhosphoSTAT1 (1:500). After washing, the blots were incubated with goat anti-rabbit IgG-HRP and Lumi-Light plus as ECL substrate. Signal detection was performed with the Lumi-Imager (Roche Diagnostics).

Data analysis
All data are presented as mean ± SEM. Data were analyzed with the univariate ANOVA using SPSS Version 10 software.

Results

Basal barrier properties and disruption by IFN-γ
T84 monolayers are known to differentiate in culture and develope high transepithelial resistance (TER) which reflexes well developed tight junctional strands. During the course of the experiments basal TER slightly increased as compared to t=0 (p<0.002). The mean basal TER after 72 h of incubation was 1484 ± 86 ohm.cm² and 4 kDa fluxes were consistently low 20 ± 11 pmol/cm²/h. IFN-γ incubation resulted in significant decrease in TER after 48 h of incubation which was more progressive at 72 h. Mean TER after 72 h of IFN-γ incubation was 790 ± 44 ohm.cm², while fluxes were increased to 520 ± 60 pmol/cm²/h.

PI3K inhibition ameliorates IFN-γ mediated barrier disruption
The effect of chemical PI3K inhibitor LY294002 (10 µM) was compared to quercetin (25 µg/ml). LY294002 and quercetin did not affect basal barrier integrity (fig 1A/B). LY294002 ameliorated IFN-γ mediated decrease in TER at 48 and 72 h while quercetin showed the same tendency at 48 h and significantly increased TER at 72 h (fig 1A). Typically the maximal effect of LY294002 on TER preservation was observed at 48 h while the quercetin effect appeared to be delayed, thus revealing differential kinetics. The effects of IFN-γ on TER were reflected by enhanced 4 kDa dextran fluxes, whereas combined IFN-γ/ inhibitor incubation tended to decrease IFN-γ induced fluxes (fig 1B).
Figure 1 A) PI3K inhibitor LY294002 (LY) and flavonol quercetin (quer) ameliorate IFN-γ mediated decrease in TER (n=4, #p<0.02, ###p<0.002). The maximal effect of LY294002 and quercetin was observed at respectively 48 h and 72 h. However, TER remained decreased as compared to control incubations (****p<0.002). B) IFN-γ incubation enhanced 4 kDa fluxes (n=4, *p<0.02, ** p<0.002). In line with TER data, a tendency towards decreased 4 kDa dextran fluxes using PI3K inhibitors was observed.
Quercetin reduces STAT1 phosphorylation

STAT1 signaling has been recognized as major route of IFN-γ induced signal transduction. We used western blotting to compare the effects of quercetin with LY294002 on STAT1 phosphorylation. STAT1 phosphorylation was evident at 15 min of incubation and remained during the 72 h incubation period with IFN-γ (data not shown). Quercetin reduced STAT1 phosphorylation after 15 min of IFN-γ incubation while LY294002 was not effective (fig 2).

Inhibitors of JNK and p38 MAPK enhance transepithelial resistance

JNK inhibitor SP600125 (20 μM) dramatically enhanced basal TER at 24 h of incubation and this effect sustained during the 72 h culture period. Similar to effects on basal TER, combined IFN-γ/SP600125 incubations significantly enhanced TER compared with IFN-γ incubations alone (fig 3A). Upto 48 h SP600125 reversed IFN-γ effects on TER, while TER dropped only slightly below control levels at 72 h of combined IFN-γ/SP600125 incubations. Fluxes at 72 h revealed a significant reduced permeability for 4 kDa FITC-dextran (fig 3B). The p38 inhibitor SB203580 (1 μM) enhanced basal TER at t=72 h. Combined incubation of IFN-γ/ SB203580 improved TER at 72 h of incubation as compared to incubations with IFN-γ alone (fig 3A). This was in parallel with the observed effect of SB203580 on basal resistance. Small increase in TER correlated with a tendency towards reduced 4 kDa flux in combined IFN-γ/SB203580 incubations (fig 3B). Incubations with 10 μM SB203580 had similar effects (data not shown). Selective and cell-permeable ERK inhibitor PD98095 (5 μM, nor 50 μM (data not shown)) did not exert any effect on basal barrier integrity (TER and flux) nor IFN-γ induced barrier disruption (fig 3A/B).

PKC inhibitor rottlerin aggravates IFN-γ mediated barrier disruption

We used PKC inhibitors rottlerin and Gö-6976 to study the role for PKC in barrier maintenance and IFN-γ mediated barrier disruption. Incubation with 5 μM rottlerin slightly decreased basal TER in the first 24 h, after this initial effect there appeared to be no further decrease in TER (fig 4A). The kinetics of IFN-γ mediated barrier disruption typically show effects on TER starting at 48 h of incubation. Combined incubation with IFN-γ and rottlerin aggravated barrier disruption compared with incubation of IFN-γ alone. Effects on TER were in agreement with 4kDa FITC dextran fluxes, which were significantly increased during combined incubations of IFN-γ and rottlerin (fig 4B).
Figure 3 A) JNK inhibitor SP600125 but also p38 inhibitor SB203580 enhanced basal TER and improved IFN-\(\gamma\) affected TER with similar amplitude (n=5, */#p<0.02, **/##p<0.002). ERK inhibitor PD98095 did not exert any effect on basal TER nor IFN-\(\gamma\) induced disruption of TER (n=4). B) The 4 kDa FITC-dextran fluxes at 72 h revealed that of all MAPK inhibitors, SP600125 significantly reduced permeability during IFN-\(\gamma\) incubations (n=5, p<0.002). This was in line with pronounced improvement of TER.
Higher concentrations of rottlerin (50 μM) decreased basal TER (<300 ohm.cm²) within 48 h. Gö-6976 (0.05 μM) did not affect basal TER nor flux and did not alter IFN-γ induced changes. Incubations with 0.5 μM Gö-6976 dropped basal resistance with kinetics resembling IFN-γ incubations and did not alter IFN-γ induced changes (data not shown).

**Figure 4** A) PKC inhibitor rottlerin slightly reduces basal TER (n=5, **p<0.002) and enhances IFN-γ mediated barrier disruption (##p<0.002). B) Combined incubations of IFN-γ and rottlerin enhanced 4kDa dextran flux as compared to flux induced by IFN-γ alone (n=4, ##p<0.002).

**Discussion**

Enhanced intestinal permeability is associated with various diseases and in Crohn’s disease may precede relapse to active disease.²⁻⁶ Recently, mucosal T-cell derived IFN-γ was found to cause increased intestinal permeability, in a mouse stress model.⁴⁶,⁴⁷ Pro-inflammatory cytokines like IFN-γ affect tight junctions structures and MLC phosphorylation hereby enhancing epithelial permeability.¹²,⁴⁷ IFN-γ signaling involves STAT1 regulated gene transcription and several STAT1 independent signaling cascades.³⁸,⁴⁸ Physiological barrier maintenance is a tightly regulated process which is known to be controlled by PKC and small protein GTP-ases (Rac-1, Rho, Cdc42), which also act on MLC phosphorylation.¹⁵,²¹-²³,³²,³⁴,⁴⁹,⁵⁰ However, MAPKs (ERK, p38 and JNK) are downstream effectors of small protein GTP-ases, and therefore may be interesting candidates for the regulation of barrier properties.²⁸-³¹ We evaluated these signaling pathways involvement in maintenance of intestinal barrier integrity as well as immune mediated barrier disruption.

The PI3K-PKB pathway plays an important role in cell growth, differentiation, survival and cytoskeletal modulation.⁵¹,⁵² In our experiments both LY294002 and quercetin did not affect basal barrier integrity but ameliorated IFN-γ mediated barrier disruption. In models of barrier...
disruption with supernatants derived from activated PBMC or IL-4, PI3K also was shown to be involved. PI3K indeed was found to be activated after IFN-γ challenge and has been incriminated to induce serine phosphorylation of STAT1, optimizing gene transcription. Therefore, we investigated the possibility that PI3K inhibition may act through inhibiting STAT1 signal transduction. We observed prolonged STAT1 phosphorylation after IFN-γ incubation (data not shown) in accordance with data obtained by McKay et al. who detected long-term nuclear STAT1 localization after IFN-γ stimulation. LY294002 had no effect on STAT1 phosphorylation, but we found quercetin to decrease STAT1 tyrosine phosphorylation. Besides PI3K, the flavonol quercetin is reported to inhibit PKC, NFκB, PLA2 (phospholipase A2), MAPKs and tyrosine kinases. Because our experiments with LY294002 made a direct involvement of PI3K in STAT1 phosphorylation unlikely, we hypothesize that tyrosine kinase inhibition by quercetin may have resulted in decreased STAT1 phosphorylation. Genistein another tyrosine kinase inhibitor and not affecting PI3K, reduced STAT1 phosphorylation, and was also found to ameliorate immune mediated barrier disruption. Hence, both PI3K and tyrosine kinase inhibitory properties of quercetin may have contributed to the amelioration of barrier disruption caused by IFN-γ.

Besides PI3K, the MAPK signaling pathways are involved in many cellular processes and regulate inflammatory responses. The MAPK family consists of JNK (c-Jun N-terminal kinase), p38 and ERK (extracellular signal regulated kinase). JNK and p38 MAPK are known as stress activated protein kinases regulating cellular growth, differentiation and death and are activated cell type specifically downstream of the Rho family of small GTP-binding proteins Rho, Rac-1 or Cdc42. Inhibition of JNK, and to a lesser extent p38, was found to importantly increase basal TER. Since inhibitors exert their effect on basal TER this suggests constitutive activation of JNK and p38. Indeed JNK is slightly phosphorylated during normal culture conditions and also constitutive Rho activation and MLC phosphorylation is also observed in epithelial cells. Growth factors are known to activate JNK and VEGF is known to enhance vascular tight junction permeability via JNK activation resulting in actin reorganization and redistribution of tight junction proteins. Activation or inhibition of Rho and/or Rac-1 may enhance MLC phosphorylation via the ROCK- or PAK-1-MLCK (myosin light chain kinase) pathway and/or affect tight junction molecules resulting in barrier disruption. Since Rho and Rac-1 activation may also result in JNK activation we propose JNK to regulate epithelial barrier function via similar mechanisms. Alternatively, JNK activation has been incriminated to induce apoptosis, which would decrease TER. However, basal apoptosis in the in vitro model used in our experiments is low and was not enhanced by IFN-γ, making an effect on apoptosis an unlikely explanation for the effects observed after JNK inhibition.
Signaling pathways involved in regulation of barrier integrity

JNK inhibition not only increased basal TER, but also prevented IFN-γ-related decreases of TER and increase in flux. At this point it is unclear whether IFN-γ induced barrier disruption involves JNK activation, or that the protective effect of JNK inhibition is accomplished solely by the increase in basal TER. Improved barrier function of the gut results in decreased antigenic load into the mucosa. This might have implications for Crohn’s patients in whom enhanced permeability is known to precede active disease. Crohn’s patients treated with a JNK/p38 inhibitor indeed were found to have improved mucosal healing, however inhibition of JNK activation in lamina propria immune cells may have contributed to the effect. Like JNK inhibition but much less effective, p38 inhibition increased basal TER and also combined IFN-γ/SB203580 incubations resulted in slight improvement of TER. SB203580 was ineffective in ameliorating barrier disruption caused by pro-inflammatory cytokines and EPEC infection, however in these studies incubations lasted maximal 24 hours. IFN-γ receptor activation results in signaling via the Ras-Raf-MKK1/2-ERK1/2 route in several cell types, but in endothelial cells this route was found not to be activated by IFN-γ. Oncogenic activation of Raf or Ras has been shown to disrupt barrier integrity via alteration of tight junction structures. However in our experiments even high doses of ERK inhibitor PD98095 did not exert any effect on basal barrier function nor IFN-γ mediated barrier disruption. This is in line with reports by Czerucka et al. and Savkovic et al. who did not find ERK to be involved in EPEC and salmonella induced decrease in resistance, while ERK activation did result in IL-8 secretion.

Like we have suggested for JNK also PKC controls epithelial barrier integrity via regulation of the peri-junctional actomyosin ring. Activation of PKC was found to decrease MLC phosphorylation, enhancing TER by relaxation of the peri-junctional actomyosin complex. Furthermore, inhibition of PKC was found to decrease TER via disruption of this complex. The PKC inhibitor rottlerin slightly affected basal barrier properties, but enhanced IFN-γ mediated barrier disruption. This might imply that IFN-γ and PKC-mediated stimuli share downstream pathways that converge on the peri-junctional actomyosin complex, leading to barrier disruption Indeed, IFN-γ has been shown to cause barrier disruption by enhancing MLC phosphorylation.

In conclusion, JNK MAPK inhibition dramatically improved basal barrier integrity suggesting that JNK is involved in constitutive regulation of epithelial barrier function. Of all inhibitors used, JNK inhibition most profoundly ameliorated IFN-γ mediated barrier disruption. Although this effect may have been independent from IFN-γ signaling pathways organizing barrier disruption, therapeutically targeting JNK seems to hold most promise for diseases with underlying permeability defects. Quercetin ameliorates IFN-γ mediated barrier disruption and is an example of application of a natural food component to reduce immune mediated barrier disruption.
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References
Signaling pathways involved in regulation of barrier integrity

Chapter 6


Signaling pathways involved in regulation of barrier integrity

