TNFα-induced and berberine-antagonized tight junction barrier impairment via tyrosine kinase, Akt and NFκB signaling

Maren Amasheh[1], Anja Fromm[1,2], Susanne M. Krug[2], Salah Amasheh[2], Susanne Andres[1], Martin Zeitz[1], Michael Fromm[2] and Jörg-Dieter Schulzke[1,3,*]

[1]Department of Gastroenterology, Charité, Campus Benjamin Franklin, Berlin 12200, Germany
[2]Institute of Clinical Physiology, Charité, Campus Benjamin Franklin, Berlin 12200, Germany
[3]Department of General Medicine, Charité, Campus Benjamin Franklin, Berlin 12200, Germany
*Author for correspondence (joerg.schulzke@charite.de)

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Summary

TNFα-mediated tight junction defects contribute to diarrhea in inflammatory bowel diseases (IBDs). In our study, the signaling pathways of the TNFα effect on barrier- or pore-forming claudins were analyzed in HT-29/B6 human colon monolayers. Berberine, a herbal therapeutic agent that has been recently established as a therapy for diabetes and hypercholesterinemia, was able to completely antagonize the TNFα-mediated barrier defects in the cell model and in rat colon. Ussing chamber experiments and two-path impedance spectroscopy revealed a decrease of paracellular resistance after TNFα to 11±4%, whereas transcellular resistance was unchanged. The permeability of the paracellular marker fluorescein was increased fourfold. Berberine alone had no effect while it fully prevented the TNFα-induced barrier defects. This effect on resistance was confirmed in rat colon. TNFα removed claudin-1 from the tight junction and increased claudin-2 expression. Berberine prevented TNFα-induced claudin-1 disassembly and upregulation of claudin-2. The effects of berberine were mimicked by genistein plus BAY11-7082, indicating that they are mediated via tyrosine kinase, pAkt and NFκB pathways. In conclusion, the anti-diarrheal effect of berberine is explained by a novel mechanism, suggesting a therapeutic approach against barrier breakdown in intestinal inflammation.

Key words: Plant alkaloid, TNFα, Barrier function, Tight junction, Claudins, Occludin, Two-path impedance spectroscopy

Introduction

The paracellular barrier of intestinal epithelia prevents luminal antigens or bacteria from coming into contact with the immune system. However, in inflammatory bowel diseases (IBDs), the barrier becomes defective, which might lead to or sustain mucosal inflammation. Paracellular barrier properties are determined by the concerted action of tightening tight junction proteins such as claudin-1, claudin-4, claudin-5 and claudin-8 and pore-forming proteins such as claudin-2 and claudin-10b (Furuse et al., 2002; Amasheh et al., 2005; Amasheh et al., 2009; Yu et al., 2003; Amasheh et al., 2002; van Itallie et al., 2006; Rosenthal et al., 2010).

In vitro studies using the human intestinal epithelial cell line HT-29/B6 (Kreusel et al., 1991) have shown pro-inflammatory effects on epithelial apoptosis, transepithelial resistance and tight junction strands when incubated with the pro-inflammatory cytokine tumor necrosis factor α (TNFα) (Gitter et al., 2000; Goldblum and Sun, 1990; Mullin and Snock, 1990; Mankertz et al., 2000; Schmitz et al., 1999). A number of recent studies have focused on altered barrier function during active IBD (Hollander et al., 1986; Mankertz and Schulzke, 2007) and have identified perturbation of the tight junction (TJ) as the causal pathomechanism. Thus, it is of major importance to know how the TJ is regulated and how this can be influenced therapeutically.

The plant alkaloid berberine has been used in traditional Eastern medicine for a long time in the treatment of gastroenteritis and diarrhea. Recent developments in the therapy of diabetes and hyperlipidemia have brought berberine into the focus of interest (Zhou et al., 2007; Yin et al., 2008a; Kong et al., 2004). Among the plethora of pharmacological actions of berberine that have been described recently, a protective barrier effect in retinal epithelial cells was reported (Cui et al., 2006a; Cui et al., 2006b; Cui et al., 2007). Although its therapeutic benefit in gastrointestinal disease has been attributed in part to antimicrobial and anti-motility actions, berberine has also been shown to prevent ion secretion in vitro in rabbit and rat intestinal models of (Iwasa et al., 1998; Yamamoto et al., 1993; Guandalini et al., 1987; Tai et al., 1981; Taylor and Baird, 1995). In human colonic epithelia, berberine could exert an anti-secretory action directly upon epithelial cells via blockade of K^+ channels (Taylor et al., 1999).

To characterize the influence of TNFα, we analyzed the intracellular localization, protein and mRNA expression, and signaling pathways in intestinal HT-29/B6 cells, which represent an intestinal model epithelium of colonic origin. We used berberine as a tool for mechanistic studies as well as a possible active ingredient against barrier dysfunction. We describe intracellular signaling pathways that contribute to the inflammatory impact of TNFα on expression and distribution of barrier- or pore-forming TJ proteins. In contrast to previous reports in which the contribution of only one signaling compound is often described, here we report the interactive regulation of epithelial barrier function by three pathways: tyrosine kinase Src, NFκB and Akt–GSK.

Results

Effect of berberine and TNFα on transepithelial resistance and fluorescein permeability

As basic parameters of epithelial barrier function, transepithelial resistance (R^t, TER) and the permeability of the paracellular marker fluorescein (P^{fluo}) of HT-29/B6 monolayers was determined 24 hours after addition of berberine (50 μM, mucosal and serosal addition). Berberine alone caused a small but significant, increase in R^t (121±3% of the initial resistance of 107±2 Ω·cm^2, n=12, P<0.001 versus control; Fig. 1A) and no significant change in P^{fluo} (control 0.46±0.07; berberine 0.53±0.06×10^{-6} cm/second, n=7; Fig. 1B). TNFα (500 U/ml) induced a decrease of R^t to 49±2% after 24 hours (n=12, P<0.001) and an increase of P^{fluo} to 1.99±0.28×10^{-6} cm/second (n=7, P<0.001). These effects of TNFα were prevented by the addition of 50 μM berberine. When TNFα was combined with berberine, R^t (105±3% of initial resistance; n=12, Fig. 1A) and P^{fluo} (0.60±0.09, n=5, Fig. 1B) were not significantly different from controls without TNFα.

Effect of berberine and TNFα on paracellular and transcellular resistance

To study the effect of berberine on R^t in more detail, two-path impedance spectroscopy was performed on HT-29/B6 monolayers treated with berberine, with TNFα, or with both, as described above (Fig. 1C, supplementary material Table S1). When TNFα was added to HT-29/B6, a strong decrease in epithelial resistance (R^{epi}) was detectable (control 384±17 Ω·cm^2, n=7; TNFα 132±9 Ω·cm^2, n=8, P<0.001). This was due to a dramatic decrease of paracellular resistance (R^{para}) (control 1909±469 Ω·cm^2, n=7; TNFα 213±26 Ω·cm^2, n=8, P<0.01), which reflects the TJ barrier, whereas transcellular resistance (R^{trans}) was unchanged (control 529±31 Ω·cm^2, n=7; TNFα 411±45 Ω·cm^2, n=8, n.s.). Addition of berberine alone slightly increased R^t to 467±10 Ω·cm^2 (n=7, P<0.01), whereas R^{trans} (755±93 Ω·cm^2, n=7, n.s.) and R^{para} (1849±471 Ω·cm^2, n=7, n.s.) were not significantly altered. When berberine was added with TNFα, an inhibition of the TNFα effect on R^{para} (1842±302 Ω·cm^2, n=5, P<0.001 versus TNFα alone) and on R^{epi} (357±29 Ω·cm^2, n=5, P<0.0001 versus TNFα alone) was observed, whereas R^{trans} (460±50 Ω·cm^2, n=5, n.s.) again remained constant. None of the three resistances after addition of TNFα plus berberine significantly differed from controls.

Incubation of HT-29/B6 with genistein and BAY11-7082 (Fig. 1D, supplementary material Table S1) caused an increase in R^{trans}, leading to an elevated R^{epi} (R^{para}: 3379±690 Ω·cm^2, n=5, n.s.; R^{trans}: 822±88 Ω·cm^2, n=5, P<0.05; R^{epi}: 651±76 Ω·cm^2, n=5, P<0.05). Adding both blocking components to TNFα-treated cells resulted in a complete inhibition of the TNFα effect (R^{para}: 3087±772 Ω·cm^2, n=8, P<0.01 versus TNFα; R^{trans}: 728±117 Ω·cm^2, n=8, P<0.05 versus TNFα; R^{epi}: 544±71 Ω·cm^2, n=8, P<0.001 versus TNFα). Epithelial capacitance (C^{epi}) in impedance spectroscopy, which reflects the exposed plasma membrane area of the cells (supplementary material Table S1), did not change after incubation with either TNFα or berberine, or when TNFα plus berberine were added. Genistein and BAY11-7082 also had no effect on C^{epi}, either alone or in combination with TNFα (supplementary material Table S1).

Berberine modifies the composition of tight junctions

To determine whether the berberine- and TNFα-induced changes in R^t are due to altered TJ protein expression, quantification of occludin and claudin-1–claudin-5 and claudin-7 was performed by densitometric analysis of western blots with normalization to β-actin as a loading control. Protein expression of claudin-1 was strongly increased by addition of berberine (Fig. 2A; berberine 174±10% of controls, P<0.001, n=8) and TNFα (TNFα 254±11% of controls, P<0.001, n=8). A combination of berberine and TNFα increased protein expression of claudin-1 to 263±16% of controls (P<0.001, n=8).

Another major effect was observed for claudin-2, which was downregulated by berberine (Fig. 2B; berberine 57±9% of controls, n=6, P<0.001). In addition, berberine also downregulated claudin-2 when combined with TNFα (berberine + TNFα, 70±8% of controls, n=6, P<0.001 versus TNFα). Berberine did not change

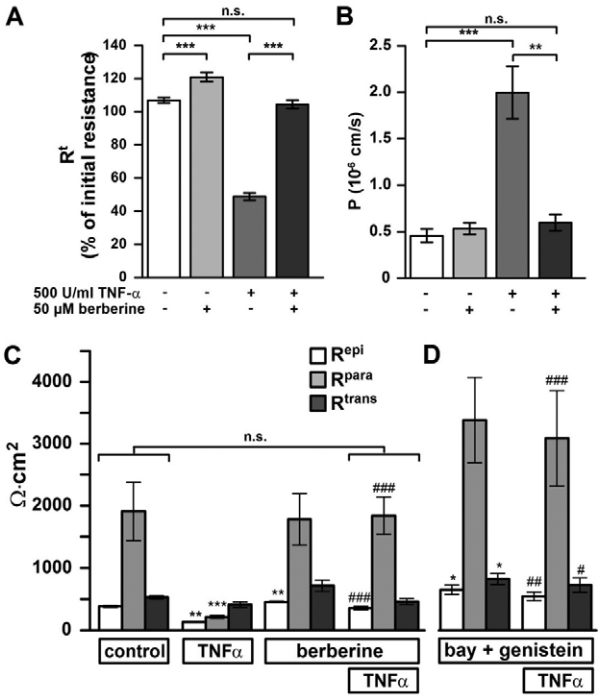

Fig. 1. Effect of berberine on transepithelial resistance. Effects of berberine on transepithelial electrical resistance (R^t) alone and after stimulation with TNFα in HT-29/B6 cells. (**A**) Conventional resistance measurements. R^t of confluent HT-29/B6 cells after stimulation with TNFα (basolateral; 24 hours) and after stimulation with berberine (mucosal + basolateral addition 26 or 2 hours, respectively, before 50 μM TNFα). Data are expressed as mean ± s.e.m. of four independent experiments. ***P<0.001; $^{###}P$<0.001 versus TNFα; n=12. Data are expressed as a % of the initial resistance. (**B**) Permeability to fluorescein (332 Da). Fluorescein permeability after basolateral incubation with TNFα (24 hours) and after stimulation with berberine (26 or 2 hours, respectively, before 50 μM TNFα) on both sides of the monolayer. Data are expressed as means ± s.e.m. ***P<0.001 control versus TNFα; **P<0.01 berberine and TNFα versus TNFα; n=5–7. (**C**) Two-path impedance spectroscopy. Analysis of the two components of epithelial resistance (R^{epi}, white bars), paracellular and transcellular resistance (R^{para}, gray bars, and R^{trans}, black bars) revealed that TNFα lowered R^{epi} by a strong decrease in R^{para}. Treatment with berberine (50 μM) led to an increase in R^{epi} and inhibited the effect of TNFα on R^{para} and R^{epi} when added to TNFα-exposed HT-29/B6 cells. **P<0.01, ***P<0.001 versus control; $^{###}P$<0.001 versus TNFα; n=7–8. (**D**) Incubation of HT-29/B6 cells with genistein (26 or 2 hours, respectively, before 50 μM TNFα) and BAY11-7082 (26 or 2 hours, respectively, before 10 μM TNFα) increased R^{epi} due to an increase of R^{trans}. Incubation of TNFα-exposed HT-29/B6 cells with both blockers inhibited the effect of TNFα on all resistances. *P<0.05 versus control; $^{#}P$<0.05; $^{##}P$<0.01; $^{###}P$<0.001 versus TNFα; n=5–8.

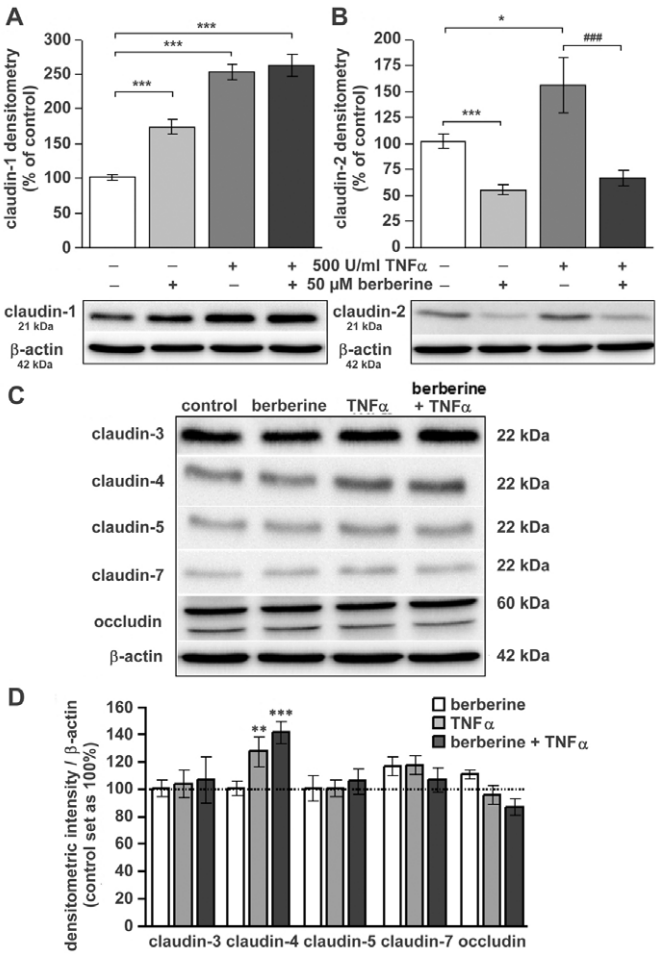

Fig. 2. Effect of berberine on the expression of tight junction proteins. (**A**) Densitometric analysis of claudin-1. Data are expressed as % of controls and normalized with β-actin (***P<0.001 versus control; n=8). Western blot detection of claudin-1 revealed an increase of claudin-1 by berberine and TNFα. Human β-actin served as a loading control. (**B**) Densitometric analysis of claudin-2. Data are expressed as % of controls and normalized with β-actin (***P<0.001 versus control; $^{\#\#\#}P$<0.001 versus TNFα; n=8). Western blot analysis of claudin-2: A decrease of claudin-2 by berberine was detected, whereas TNFα induced an increase of claudin-2. Human β-actin served as a loading control. (**C**) Western blots of the TJ proteins claudin-3, claudin-4, claudin-5, claudin-7 and occludin. An increase of claudin-4 expression by TNFα was detected, whereas claudin-3, claudin-5 and claudin-7 and occludin were not changed. Human β-actin served as a loading control. (**D**) Densitometric analysis. Data are expressed as % of controls and normalized with β-actin (n=7–8; *P<0.05; **P<0.01; ***P<0.001; **P<0.01).

the expression of occludin, claudin-3, claudin-4, claudin-5 or claudin-7 (Fig. 2C,D; n=4–8). Additionally, no effect of berberine or TNFα could be found on claudin-8, claudin-10, claudin-12, claudin-14, claudin-15 or claudin-17 (data not shown).

Confocal laser scanning microscopy

Because berberine induced changes in the expression of the TJ proteins claudin-1 and claudin-2, confocal laser scanning microscopy was applied to localize TJ proteins. In untreated HT-29/B6 cells, berberine did not change the intercellular distribution of claudin-1 in the TJ (Fig. 3A). After incubation with TNFα, claudin-1 showed a re-distribution off the TJ to the subapical cytoplasm (Fig. 3A,B), correlating with a decrease of R^t. Berberine protected claudin-1 from redistribution off the TJ by TNFα and a merged signal of claudin-1 with ZO-1 was detected again in the TJ (Fig. 3A,B). TNFα mediated claudin-1 internalization into intracellular vesicles, which was also inhibited by berberine (Fig. 3B). Claudin-2 was only weakly expressed in TJs of controls (Fig. 3C) and showed a more intense staining in the TJ, as well as intracellularly, after TNFα exposure (Fig. 3C). Addition of berberine inhibited the TNFα-induced increase of claudin-2, and a lower signal intensity of claudin-2 was detected in both the intracellular compartments and in the TJ, when compared with TNFα-treated monolayers. Claudin-3 and claudin-4 were predominantly localized within the tight junction, with no differences between controls and experimental conditions (supplementary material Fig. S1).

Real-time PCR analysis

Real-time quantitative PCR revealed that berberine induced an increased expression of claudin-1 (*CLDN1*) mRNA by upregulation of *CLDN1* gene transcription (3.8±0.5-fold control, n=5, P<0.001; Fig. 4A), whereas TNFα induced a weak increase in *CLDN1* mRNA, which did not reach significance (1.6±0.3-fold control; n=5–6, P=0.08, n.s.). Combined incubation of berberine and TNFα acted synergistically with an increase of *CLDN1* mRNA (berberine+TNFα, 4.5±0.4-fold control, n=5–6, P<0.001). The increased expression of claudin-2 induced by TNFα was – at least in part – the result of increased levels of *CLDN2* mRNA (Fig. 4B; 2.2±0.3-fold control, n=7–8, P<0.05). Berberine had no effect on the mRNA levels of claudin-2 (1.1±0.1-fold control, n=7, n.s.), and berberine in combination with TNFα similarly showed no effect (1.4±0.2-fold control, n=7–8, n.s. vs TNFα).

The mRNA levels of claudin-3 and claudin-4 did not change after TNFα incubation (Fig. 4C; claudin-3: 1.5±0.3-fold control, n=8, n.s.; claudin-4: 1.5±0.3-fold control, n=8, n.s.). Likewise, berberine had no effect on the mRNA level of claudin-3 or claudin-4, either alone (claudin-3: 1.2±0.2-fold control, n=7, n.s.; claudin-4: 1.2±0.3-fold control, n=7, n.s.) or in combination with TNFα (claudin-3: 1.6±0.3-fold control, n=7, n.s.; claudin-4: 1.4±0.3-fold control, n=7, n.s.).

Apoptosis

Apoptotic rates were not altered under different experimental conditions. Measurements of caspase-3 cleavage products were performed by western blotting and TUNEL-staining after 24 hour treatment of HT-29/B6 cells with 500 U/ml TNFα. Data for caspase-3 are given in the supplementary material Fig. S3. TUNEL assay revealed 1.02±0.11% apoptotic cells (n=9) in control versus 0.81±0.07% (n=7, n.s.) after addition of berberine. TNFα, a cytokine with pro-apoptotic activity, did not upregulate apoptosis after 24 hours of exposure (TNFα 1.04±0.17%, n=8, n.s.), at least at the concentration used in our experiments. Co-incubation with berberine and TNFα caused an apoptotic rate that was no different from controls or after TNFα alone (berberine + TNFα, 0.92±0.15%, n=6, n.s.).

Signaling pathways involved in berberine- and TNFα-dependent TJ modulation

Berberine protected intestinal HT-29/B6 cell monolayers from TNFα-induced barrier breakdown. As claudin-1 and claudin-2 are known to have major effects on barrier function, our results indicate a direct effect via these two proteins.

PI3K/Akt

PI3K/Akt signaling is known to be important for intestinal cell proliferation and survival as well as for other cellular functions. It has been recently shown by our group that TNFα-induced claudin-2 expression is mediated by PI3K/Akt signaling (Mankertz et al., 2009). To determine the effect of berberine on this pathway, samples were immunoblotted with an antibody against the active (phosphorylated) form of Akt (Akt-*P*). To ensure that equal amounts of samples were used, β-actin was used as loading control. Phosphorylation of GSK3β, Akt-*P*(Ser473) and Akt-*P*(Thr308) was determined using phospho-specific anti-GSK3β, Akt-*P*(Ser473) and Akt-*P*(Thr308) antibodies, respectively (Fig. 5A). To analyze time-dependent phosphorylation, HT-29/B6 monolayers were incubated with TNFα (500 U/ml) for 0, 5, 30, 60 and 120 minutes. TNFα markedly induced phosphorylation of GSK3β and Akt-*P*(Ser473) (supplementary material Fig. S2A), indicating that TNFα activates Akt and its downstream targets. The TNFα-induced activation reached a maximum at 30 minutes. Berberine alone (40 minute incubation time) inhibited the phosphorylation of Akt-*P*(Thr308) (47±9, n=4, P<0.001; Fig. 5D) and antagonized the TNFα-induced (berberine was added 10 minutes before TNFα, with TNFα incubation for 30 minutes) phosphorylation of GSK3β (TNF, 243±22%, berberine+TNFα, 154±21%, n=5–6, P<0.05; Fig. 5B), Akt-*P*(Ser473) (TNFα, 701±159%, berberine+TNFα 330±47%, n=5–6, P<0.05; Fig. 5C) and Akt-*P*(Thr308) (TNFα, 224±40%, berberine+TNFα, 89±28%, n=4, P<0.05; Fig. 5D).

NFκB

Because TNFα acts as a pro-apoptotic cytokine by activating the NFκB pathway, we examined the effect of berberine on the NFκB pathway. HT-29/B6 monolayers were incubated with TNFα (500 U/ml) for 0, 5, 15, 30, 60 and 120 minutes. TNFα markedly induced phosphorylation of NFκB and IκBα 15 minutes after TNFα exposure (supplementary material Fig. S2B). Berberine (50 μM) pre-incubation (10 minutes before TNFα, with TNFα incubation for 15 minutes) inhibited both (Fig. 5E), NFκB and IκBα phosphorylation by TNFα and antagonized the TNFα effects to a similar extent as the specific blocker BAY11-7082 (10 μM).

Src-*P*(Tyr416)

The activity of tyrosine kinases is essential for many cellular processes, and accumulating evidences suggest a significant contribution to inflammation. Tyrosine kinases have an important role in cytokine function and are implicated in signaling through TNFα. HT-29/B6 monolayers were incubated with TNFα (500 U/ml) for 0, 5, 15, 30, 60 and 120 minutes. TNFα induced maximum phosphorylation of the tyrosine kinase Src-*P*(Tyr416) after 15 minutes (supplementary material Fig. S1C). Berberine (added 10 minutes before TNFα, with TNFα incubation for 15 minutes) partially inhibited the cytokine-induced phosphorylation

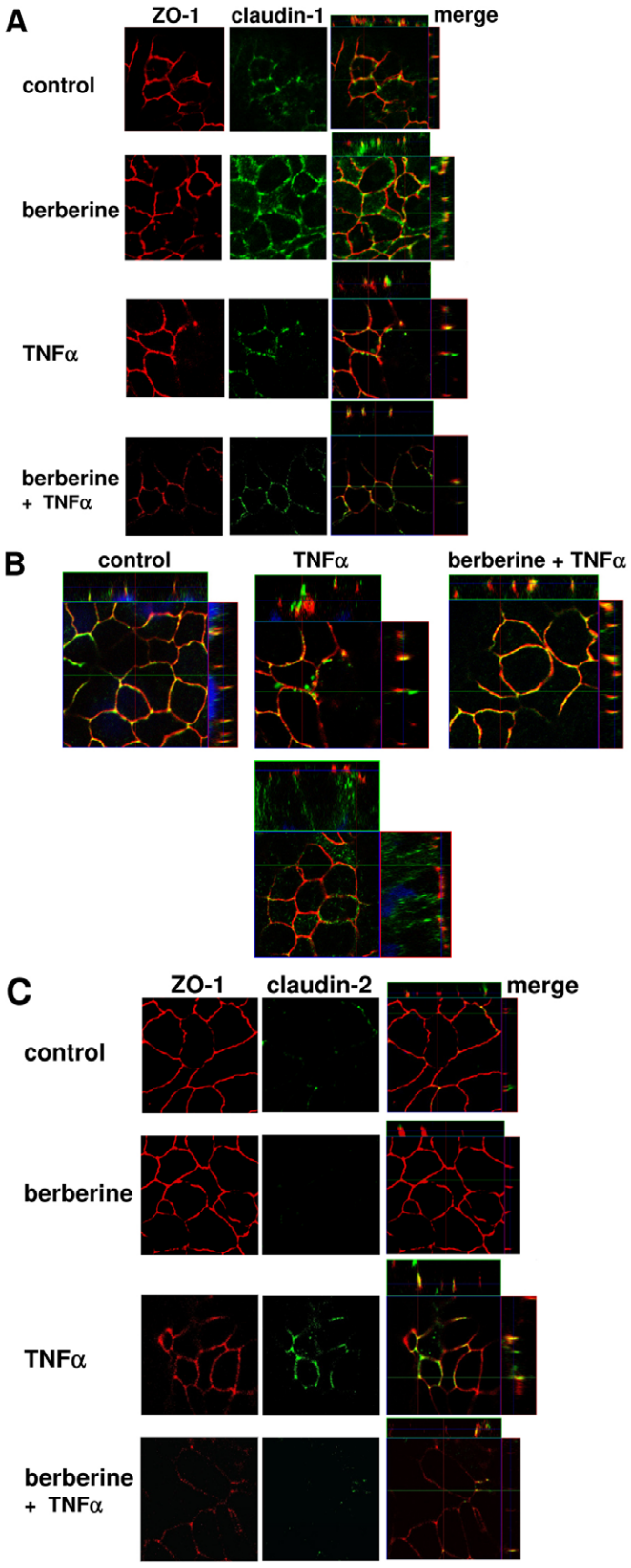

Fig. 3. Cellular distribution of claudin-1 and claudin-2. (**A**) Berberine protects claudin-1 (green) from being redistributed off the TJ by TNFα. Claudin-1 shows a strong colocalization (merge yellow) with ZO-1 (red). Immunofluorescent staining reveals a redistribution of claudin-1 off the TJ into nearby intracellular compartments and vacuoles after TNFα treatment, claudin-1 only sparely remained in the TJ, indicated by a yellow merge with ZO-1, resulting in a weak yellow staining (merge). (**B**) Higher resolution images of claudin-1 and ZO-1. Berberine (50 μM) protects claudin-1 distribution in the epithelial TJ from TNFα-induced disassembly. Here, claudin-1 (green) shows a strong signal in the TJ, merge with ZO-1 (red) results in a yellow color. (**C**) Claudin-2 (green) colocalizes with ZO-1 (red), resulting in a yellow color (merge). After TNFα stimulation (basolateral 24 hours) claudin-2 signal shows a strong staining in the TJ, which is downregulated to control staining by berberin (2 hours before TNFα).

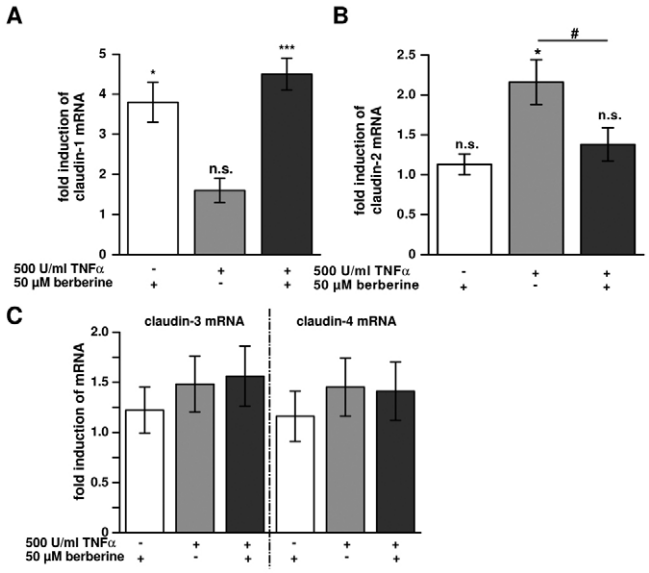

Fig. 4. Effect of berberine on *CLDN1* and *CLDN2* mRNA. (**A**) Quantitative mRNA analysis of claudin-1. TaqMan PCR revealed a nearly fourfold increase of *CLDN1* mRNA after incubation with berberine (26 or 2 hours, respectively, before 50 µM TNFα) and a more than fourfold synergistic effect with TNFα. Data are expressed as % of controls (n=5–6; *P<0.05; ***P<0.001). (**B**) Quantitative mRNA analysis of claudin-2. Berberine showed no regulation of *CLDN2* mRNA, whereas a 24 hour incubation with TNFα (500 U/ml) results in a twofold increase in *CLDN2* mRNA (n=5–6; *P<0.05 versus control; $^{\#}P$<0.05 versus TNFα). (**C**) Quantitative mRNA analysis of claudin-3 and claudin-4. Neither berberine nor TNFα showed any regulation of *CLDN3* or *CLDN4* mRNA (n=7, n.s.).

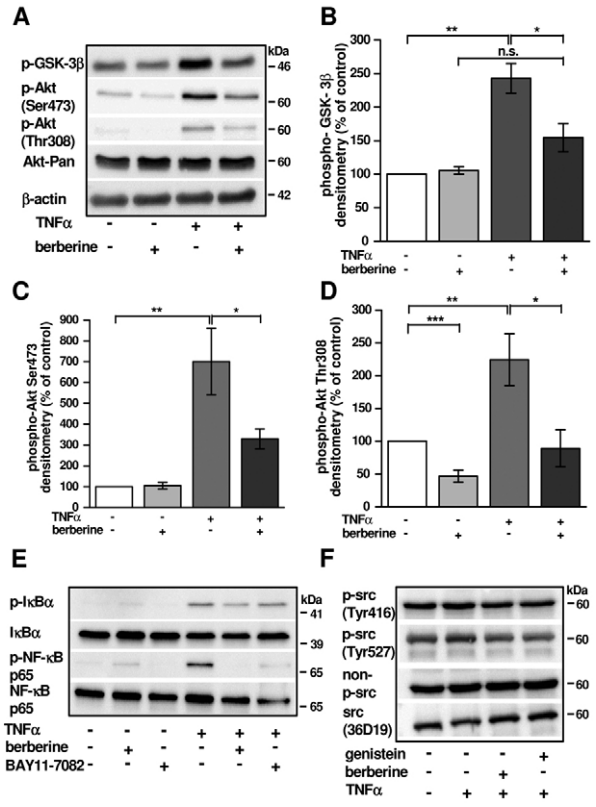

Fig. 5. Activating effects of berberine and TNFα. The effects of berberine and TNFα on activation of phospho-Akt, phospho-NFκB, phospho-IκBα levels and activation of Src-*P*(Tyr416) and Src-*P*(Tyr527) was examined in HT-29/B6 cells. (**A**) For phosphorylation experiments, berberine was added 10 minutes before TNFα, which shows maximum phosphorylation for Akt-*P* [GSK3β-*P*, Akt-*P*(Ser473), Akt-*P*(Thr308)] after 30 minutes, for NFκB-*P* and Src-*P* after 15 minutes. (**B**) TNFα-induced activation of GSK-3β-*P* is inhibited by berberine. (**C**) Berberine also decreases cytokine-dependent phosphorylation of Akt-*P*(Ser 473). (**D**) Even in untreated monolayers of HT-29/B6 cells, berberine reduces phosphorylation of Akt-*P*(Thr308) in native cells, as well as after TNFα stimulation. (**E**) Berberine was added 10 minutes before TNFα. Activation of NFκB-*P* and IκBα-*P* after 15 minutes is inhibited by berberine. (**F**) Berberine was added 10 minutes before TNFα incubation. Activation of Src-*P*(Tyr 416) after 15 minutes is inhibited by berberine.

of Src-*P*(Tyr416) (Fig. 5F). Also, genistein (50 µM) (added 10 minutes before TNFα) partially blocked phosphorylation of Src-*P*(Tyr416) in an experiment that was performed as a positive control (Fig. 5F).

Specific blockers in TNFα- and berberine-modulated epithelial barrier function

Specific blockers were used to characterize the signaling of the TNFα- and berberine-induced barrier effects in more detail (Fig. 6) by examining the effect on R^t and on TJ protein expression. Single blocking components LY294002 (10 µM), BAY11-7082 (10 µM) and genistein (50 µM) diminished, but could not completely inhibit the TNFα-induced reduction in R^t. BAY11-7082 and genistein stabilized R^t after 24 hours of TNFα exposure at 82±6% (BAY11-7082) and 69±2% (genistein) of the control values, respectively, whereas LY294002 could not inhibit barrier breakdown at all after TNFα exposure (LY294002 + TNFα, 21±8% of control values). Using BAY11-7082 (10 µM) and genistein (50 µM) together, the TNFα-induced decrease of R^t could completely be blocked (Fig. 6A), similarly to the effects of berberine (BAY11-7082+genistein, 106±8% of control values, n=5).

Densitometric analysis of claudin-1 and claudin-2 protein expression revealed no influence of BAY11-7082 on TNFα-treated HT-29/B6 monolayers (Fig. 6B). Addition of genistein led to an upregulation of claudin-1 protein expression to 151±11% of control values (n=9, P<0.001) and down-regulation of claudin-2 to 62±16% of control values (n=4, P<0.05, Fig. 6B,C).

Confocal laser scanning microscopy showed a strong claudin-1 signal after incubation with genistein or BAY11-7082 in tight junctions of HT-29/B6 monolayers and an increased signal of claudin-1 in the basolateral compartment (Fig. 7A). Results from confocal laser scanning microscopy of genistein-treated cells were in accordance with quantitative results of immunoblotting, showing an increased protein expression of claudin-1. After incubation with BAY11-7082, no increase in claudin-1 protein levels could be detected anymore, whereas strong signals were detected by LSM analysis, indicating a regulation of claudin-1 distribution via NFκB. Both genistein and BAY11-7082 also inhibited TNFα-induced claudin-1 redistribution off the TJ (Fig. 7A).

The TNFα-induced increase in claudin-2 in the tight junction of HT-29/B6 monolayers was reduced by both genistein (50 µM) and BAY11-7082 (Fig. 7B). Whereas genistein reduced claudin-2 protein expression, BAY11-7082 had no influence on tight junction protein expression but regulated TJ distribution into or off the TJ. These substances therefore act synergistically: genistein increases expression of claudin-1 and reduces claudin-2, and BAY11-7082 regulates assembly and disassembly of claudin-1 and claudin-2.

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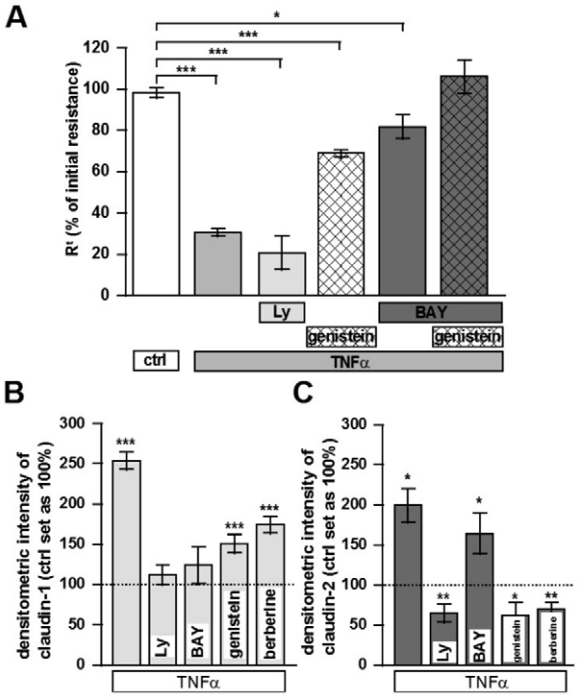

Fig. 6. Specific blockers in TNFα- and berberine-induced TJ regulation. (**A**) PI3K inhibitor LY294002 (10 μM, 2 hours before TNFα added), NFκB inhibitor BAY11-7082 (10 μM, 2 hours before TNFα added) and tyrosine kinase inhibitor genistein (50 μM, 2 hours before TNFα added) alone cannot inhibit the TNFα-induced decrease in R^t after 24 hours of incubation. The combination of genistein and BAY11-7082, however, completely blocks the TNFα-induced R^t decrease (n=5). (**B**,**C**) Densitometric analysis of original blots of claudin-1 and claudin-2 shows an increase of claudin-1 and a decrease of claudin-2 after 26 hours of incubation with genistein (50 μM; n=4; *P<0.05; **P<0.01; ***P<0.001 versus control).

Claudin-4 protein expression did not change after incubation with the inhibitors LY294002, BAY11-7082 or genistein alone, or after combined incubation with genistein and BAY11-7082 (Ly294002, 99±8%; BAY11-7082, 116±11%; genistein, 90±7%; genistein+BAY11-7082, 93±8%). TNFα alone showed an upregulation of claudin-4 protein expression compared with the control (137±15%, n=6, P<0.05; Fig. 2C,D), which was not influenced by the above-mentioned blocking substances LY294002 (Ly294002+TNFα, 109±15%; BAY11-7082+TNFα, 140±13%; genistein+TNFα, 109±13%; n=4–5, n.s.) or by the combination of genistein and BAY11-7082 (genistein+BAY11-7082+TNFα, 107±9%, n=5–7, n.s.).

Specific myosin light chain kinase (MLCK) blockers ML-7 (15 μM) and ipeptide-18 (10 μM), PKC (H-7, 10 μM and calphostin C, 0.1 μM), PKA (H-89, 10 μM), p38 (SB202190, 10 μM) and JUNK (SP600125, 10 μM) did not inhibit the TNFα-induced decrease in R^t. The combination of H-7 (10 μM) and LY294002 (10 μM) showed partial inhibition of cytokine-mediated R^t decrease similarly to genistein. H-7 (10 μM), LY294002 (10 μM) and BAY11-7082 (10 μM), when given together, completely inhibited the TNFα-induced reduction in R^t (data not shown).

Berberine inhibits TNFα-induced barrier breakdown in native rat colon

The effects of berberine were evaluated in native rat colon, focusing on measurement of transepithelial resistance. Protective effects of berberine on the TNFα-induced decrease in R^t in rat colon were examined in Ussing experiments. Viability was maintained for 18 hours, as demonstrated in previous studies (Fromm et al., 1993; Amasheh et al., 2009).

TNFα induced mucosal transformation with crypt surface reduction (control, 1.40±0.05; serosal TNFα, 1.19±0.02; berberine+TNFα, 1.37±0.03 mm^2/mm^2 serosa). The mucosal surface area was reduced in response to cytokine exposure. Without any change in epithelial barrier properties, this decrease in mucosal surface area would lead to an apparent increase in resistance. Thus, resistance values without surface area correction would overestimate epithelial barrier function after cytokine exposure. Therefore, this diminished mucosal surface area was taken into account and resistances were corrected accordingly. As the main result, TNFα reduced resistance from 187±9 Ω·cm^2 (n=5) in the control to 148±6 Ω·cm^2 (n=7, P<0.01). Application of berberine with TNFα prevented a decrease in R^t (221±15 Ω·cm^2, n=7, n.s. versus control; Fig. 8). This effect started 12–14 hours after addition of the cytokines. Results were evaluated in vitro in native rat colon, focusing on measurement of transepithelial resistance.

The LDH levels measured in the mucosal and serosal bathing solution showed no increase in TNFα-treated tissues during the course of experiments (mucosal side: control, 86±4.2, n=5 and TNFα, 90±6.0, n=3, n.s.; serosal side: control, 44±1.3, n=5 and TNFα, 40±0.3, n=3, n.s.), suggesting that the number of apoptotic or necrotic events is not relevant.

Discussion

Relevance of claudins

Among the transmembrane proteins located in TJ strands are occludin, tricellulin, junctional adhesion molecule and the claudin family, the latter has a pivotal role in intestinal barrier function (Amasheh et al., 2002; Furuse et al., 1993; Ikenouchi et al., 2005; Krug et al., 2009a; Bazzoni, 2003; Tsukita et al., 2001; Van Itallie and Anderson et al., 2006; Krause et al., 2008). Although several claudins have been identified to form barriers, a few members of this family also form paracellular channels, for example, claudin-2, claudin-10A and claudin-10B (Amasheh et al., 2002; Günzel et al., 2009).

Claudin-1 and claudin-2 have been detected in intestinal epithelia, and their functional role has been described in detail previously (Furuse et al., 1998; Rahner et al., 2001). Overexpression of claudin-1 in epithelial MDCK cells increases R^t and decreases paracellular permeability to macromolecules (Inai et al., 1999). These alterations are identical to the changes seen here with claudin-1 upregulation after berberine exposure. Overexpression of claudin-2 in MDCK cells induces paracellular channels for cations and water (Amasheh et al., 2002; Rosenthal et al., 2010).

TJ formation and conductance is affected by cytokines via the cytoskeleton and the p75 receptor (Nusrat et al., 2000; Wang et al., 2006). TNFα increases the TJ permeability of Caco-2 cells to mannitol and inulin, requiring upregulation of MLCK protein expression, which is mediated by an increase of *MLCK* mRNA transcription (Ma et al., 2004; Graham et al., 2006). Other stimuli, including bacterial toxins and zonula occludens toxin (ZOT) activate PKC, thereby decreasing R^t (Mullin and O'Brien, 1986; Fasano et al., 1995). In addition, TJ proteins can be affected by transcriptional regulation, because TNFα impaired epithelial barrier function by a reduction of TJ complexity and strand number in HT-29/B6, which has recently been shown to go along with an increase in claudin-2 expression level regulated by PI3K/Akt signaling

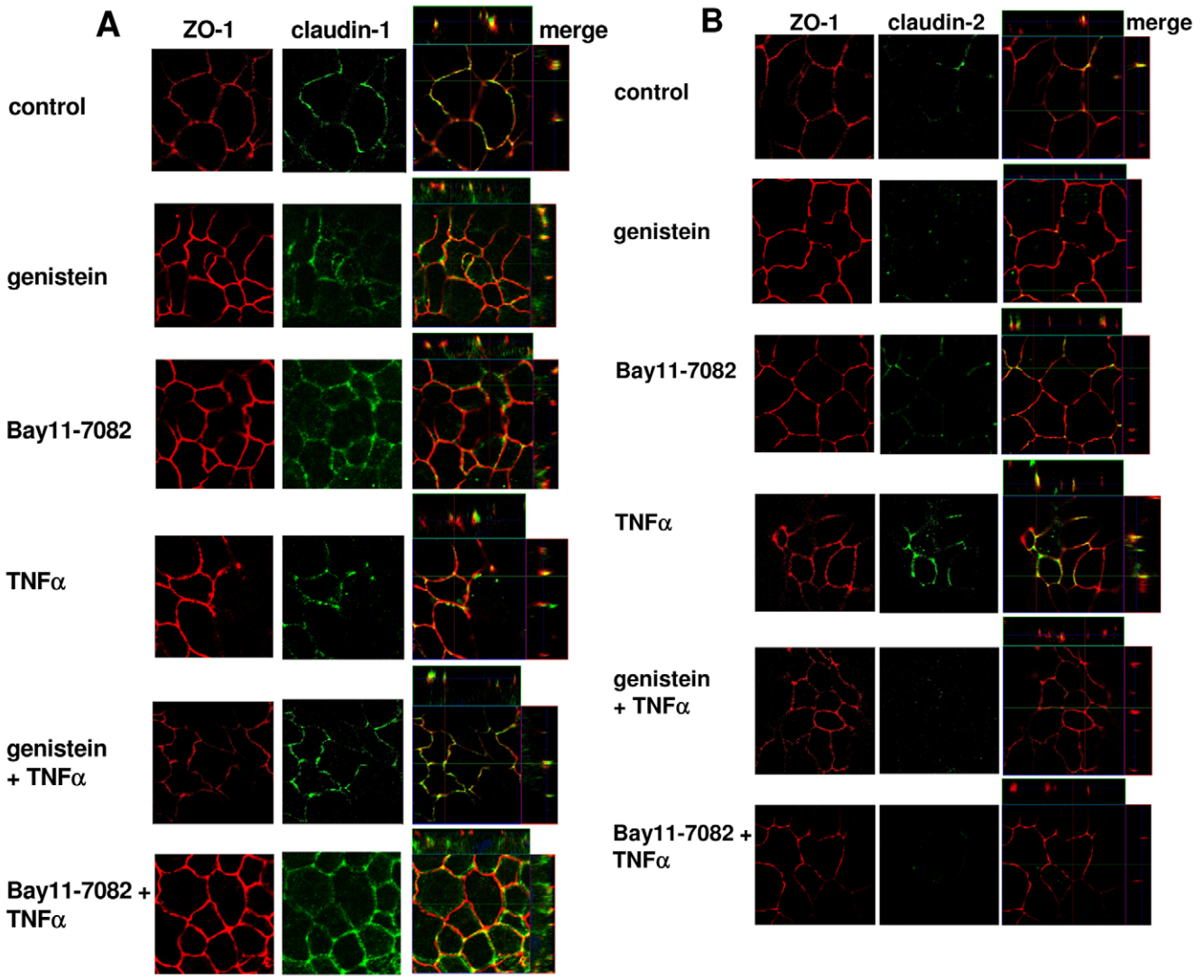

Fig. 7. Specific blockers in TNFα- and berberine-induced TJ regulation. (**A**) Effects of blocking substances on claudin-1 localization (green). NFκB inhibitor BAY11-7082 (10 μM, 2 hours before TNFα added) and tyrosine-kinase inhibitor genistein (50 μM, 2 hours before TNFα added) alone increases the claudin-1 signal but rather basolaterally, whereas the signal colocalization with ZO-1 (red) remains unchanged. Incubation with TNFα induces a disassembly of claudin-1 off the TJ, which is prevented by genistein and BAY11-7082. (**B**) Both substances also regulate claudin-2, which is upregulated by TNFα. Genistein (50 μM) as well as BAY 11-7082 causes claudin-2 to redistribute off the tight junctions so that only residual staining remains.

(Schmitz et al., 1999; Mankertz et al., 2009). In this study, TNFα caused an almost twofold increase in expression of claudin-2 in HT-29/B6 cell monolayers, with an increased amount of claudin-2 located in the TJ, as well as in intracellular compartments. This claudin-2 upregulation leads to increased paracellular permeability for small cations, contributing to a decrease of R^{t} and reflected in a strong decrease of R^{para}. Thus, although the PI3K inhibitor LY294002 did not show a strong effect on the TNFα-induced decrease of R^{t}, two-path impedance spectroscopy revealed that R^{para} is clearly affected.

As second relevant result, the present study demonstrates that berberine is a potent inhibitor of PI3K/Akt signaling. However, PI3K/Akt signaling is not responsible for the regulation of claudin-1 expression, because LY294002 had no effect on claudin-1 protein level and distribution and, in contrast to berberine, LY294002 was not able to inhibit the TNFα-induced decrease of R^{t}. Although berberine, when added to HT-29/B6 cells alone, inhibited TNFα-induced phosphorylation of GSK3β, Akt-*P*(Ser 473) and Akt-*P*(Thr308) and reduced claudin-2 protein expression, no transcriptional downregulation of claudin-2 could be detected. By contrast, elevated mRNA and protein levels for claudin-2 after TNFα stimulation were affected by berberine. This difference in claudin-2 regulation (berberine sensitivity) of the two conditions could be due to a higher claudin-2 gene promoter activity during inflammation, making the promoter sensitive to transcriptional regulation, whereas a second post-transcriptional mechanism might account for the change in claudin-2 protein without concomitant change in its mRNA levels (under non-inflamed conditions).

In addition to PI3K/Akt, two other signaling pathways are relevant for TNFα-mediated barrier disturbance and berberine is a potent inhibitor of all of these signaling cascades. Additionally, we demonstrated that signaling via tyrosine kinase Src-*P*(Tyr416) is

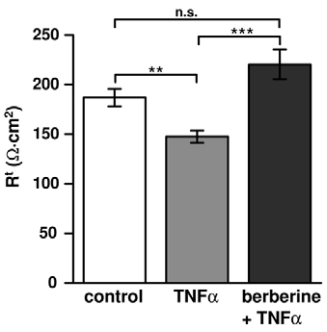

Fig. 8. Berberine inhibits TNFα-induced barrier breakdown in rat colon. TNFα reduces transepithelial resistance (R^t) from 187±9 Ω·cm^2 (n=5) in control to 148±6 Ω·cm^2 (n=7; P<0.01). Application of berberine (50 μM) with TNFα (10,000 U/ml) inhibits R^t decrease by TNFα (n=7; ***P<0.001 versus control, n.s.). This effect starts about 12–14 hours after addition of the cytokines. Ussing experiments were stopped and analyzed after 18 hours.

responsible for claudin-1 protein expression and distribution into the TJ. Berberine inhibits TNFα-induced phosphorylation of Src-*P*(Tyr416) and subsequently of downstream events such as PKC signaling and PI3K/Akt (see Fig. 9). By applying the specific tyrosine kinase inhibitor genistein, claudin-1 protein expression was strongly upregulated and confocal laser scanning microscopy revealed that claudin-1 is distributed into the TJ.

As mentioned above, claudin-2 expression is mediated via PI3K, which is a signaling complex downstream tyrosine kinase Src. Thus, blocking Src activation (phosphorylation) by genistein (or berberine) would lead to a consecutive inhibition of phosphorylation of PI3K (see Fig. 9), resulting in a decrease of claudin-2 expression. Genistein alone could not completely inhibit the decrease in R^t induced by TNFα, although the TNFα-dependent regulation of claudin-1 and claudin-2 was completely inhibited. Only the combined use of genistein and the NFκB inhibitor BAY 11-7082 could block the TNFα-mediated R^t decrease.

The third regulatory pathway involved in barrier modulation of HT-29/B6 cells is NFκB signaling. NFκB is activated by TNFα, and berberine inhibits phosphorylation of NFκB and IκBα. Using BAY11-7082, the TNFα-induced decrease in R^t could be partly inhibited, although not completely. BAY11-7082 itself when given 10 minutes before TNFα showed no effect on claudin-1 or claudin-2 expression regulation. Simultaneous incubation of TNFα-exposed HT-29/B6 monolayers with genistein as specific tyrosine kinase inhibitor and with BAY11-7082 as NFκB blocker, however, was able to disinhibit the R^{para} reduction induced by TNFα, reflecting the maintenance of the TJ barrier. Confocal laser scanning microscopy of immunofluorescent staining showed stronger signals of claudin-1 and reduced signals of claudin-2 in the TJ, although protein expression was not affected. Therefore, we hypothesize that NFκB or IκBα could regulate assembly or disassembly by recruiting intracellular claudin-1 protein and/or by endocytosis of claudin-2 (trafficking). Alternatively, distinct proteasomal influences by NFκB or IκBα and berberine, respectively, were responsible for reduced or increased TJ protein degradation. Ma and co-workers have shown that the TNFα-induced increase in TJ permeability of Caco-2 cells was associated with a downregulation of ZO-1 protein expression, which was accompanied by an altered tight junction localization of ZO-1, and this was prevented by NFκB inhibitors (Ma et al., 2004). In corneal epithelial cells, TNFα disrupted the barrier function of HCE cells, with a disappearance of ZO-1 at TJs resulting in a decrease of R^t, whereas the NFκB inhibitor curcumin blocked these effects of TNFα on R^t and the subcellular localization of ZO-1 (Soler et al., 1999; Kimura et al., 2008). It has been hypothesized that the activation of NFκB can induce transcription and translation of certain target genes, which subsequently lead to the inhibition of TJ protein expression.

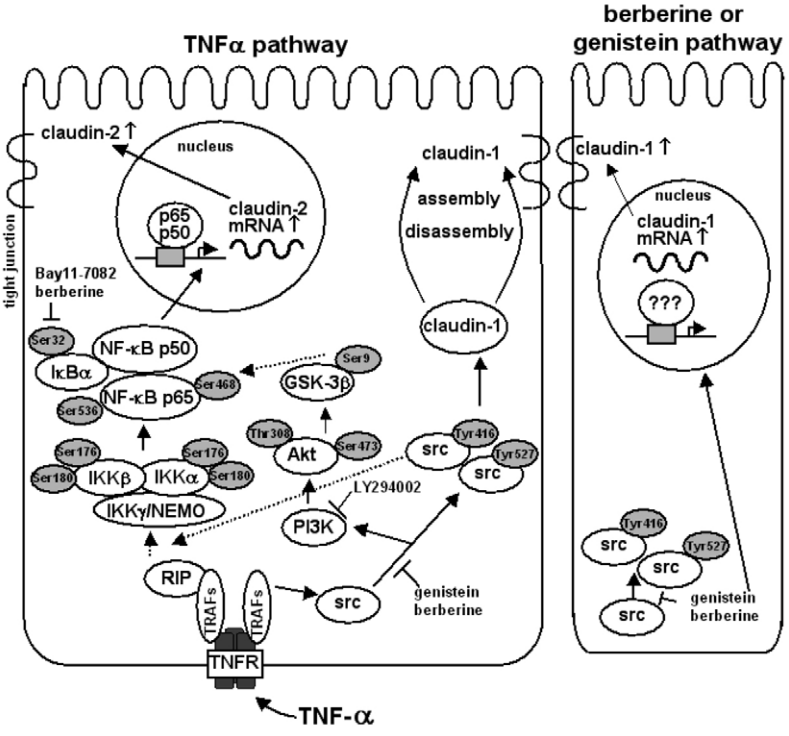

Fig. 9. Scheme of signaling pathways of TNFα and berberine that modulate the epithelial barrier in colonic HT-29/B6 cells. Three pathways were identified to be involved in tight junction protein expression and distribution of colonic epithelial cells, namely tyrosine kinase Src, Akt and NFκB. Tyrosine kinase activity regulates claudin-1 expression and, via downstream PI3K activity, claudin-2 protein expression. However, tyrosine kinase inhibitor genistein alone could not completely block the decrease in R^t induced by TNFα, although the TNFα-dependent regulation of claudin-1 and claudin-2 was completely inhibited. As the third pathway, inhibition of NFκB seems to be important for the arrangement and distribution of tight junction proteins, because inhibition had no influence on TJ protein expression but on distribution of claudin-1 and claudin-2 into or off the TJ. Berberine acts as potent blocker of these three pathways.

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Another possibility is that NFκB activation leads to an accelerated degradation of TJ proteins by proteasome activation (Ma et al., 2005). However, it should be stressed at this point that the present study shows that modulation of TJ proteins and TJ barrier function by TNFα in HT-29/B6 cells is regulated by crosstalk between tyrosine kinase, downstream PI3K and NFκB pathways.

Relevance of epithelial apoptosis

The barrier function of epithelia relies upon the continuity of the monolayer and intact TJs. In general, after incubation with TNFα, however, the number of strands that form the TJ decreases and apoptosis is induced in intestinal epithelial cells (Schmitz et al., 1999; Gitter et al., 2000). These morphological changes lead to a rise of transepithelial ion permeability, because the paracellular ion permeability increases and leaks associated with apoptotic sites increase in number and magnitude. We have analyzed epithelial local conductances associated with apoptoses previously, demonstrating that epithelial apoptoses can account for up to 50% of the TNFα-induced permeability increase, whereas the other half was caused by modulations of TJs in non-apoptotic areas (Gitter et al., 2000). A similar relationship was obtained by blocking apoptosis with Z-VAD for the IL-13 effect on epithelial barrier function (Heller et al., 2005). In the present study, however, TNFα at the dose and time scheme applied did not induce a detectable increase in apoptotic rate in HT-29/B6 cells. The occurrence of very early stages of apoptosis, however, can never be completely ruled out, although neither caspase-3 blots nor TUNEL staining showed an increase in apoptosis. Furthermore, no cleavage fragments of claudin-2 (~10 kDa), which have been interpreted to result from epithelial cell apoptosis (Willemsen et al., 2005), were detectable in our experiments. Additionally, berberine induced no change in cell number or density, indicating no change in linear junctional density. Therefore a change in linear junctional density was not responsible for the observed effects.

Role of berberine

Berberine has been reported to possess antibacterial, anti-inflammatory and anti-tumor properties and effectively inhibits cyclooxygenase-2 transcriptional activity in human colon cancer cells (Kuo et al., 2004; Iizuka et al., 2000; Lin et al., 1999; Fukuda et al., 1999). In further medical settings such as diabetes, berberine seems to have also have pharmacological potential (Zhou et al., 2007; Yin et al., 2008a,b). For centuries, it has been also used as a remedy for gastrointestinal diseases, particularly against diarrhea. In this context, berberine has been shown to inhibit ion transport in human colonic epithelia and to decrease intestinal permeability (Kim et al., 2003). Beneficial effects of berberine on mucosa healing in TNB (trinitrobenzene sulfonic acid)-induced colitis in rats were assumed to be due to inhibition of IL-8 production (Zhou and Mineshita, 2000). However, the molecular background of these effects is unknown. A new signaling pathway by which berberine can elevate low density lipoprotein (LDL) receptors and reduce cholesterol levels in man has been described as a post-transcriptional mechanism that stabilizes the respective mRNA (Kong et al., 2004). In our model of berberine action on TNFα-exposed intestinal epithelial monolayers, *CLDN2* mRNA levels remained unchanged after berberine treatment when compared with the control. Thus, this mechanism does not seem to contribute to the berberine effects here in our study.

Berberine abolished acetaldehyde-induced NFκB activity and cytokine production through the inhibition of IκBα phosphorylation and degradation and could inhibit NFκB p65 nuclear translocation in osteoclasts (Hsiang et al., 2005; Lee et al., 2007; Pandey et al., 2008). In addition, receptor activator of NFκB ligand (RANKL)-induced Akt phosphorylation was inhibited by berberine (Hu et al., 2008). In our HT-29/B6 model, berberine increased claudin-1 via tyrosine kinase inhibition and in addition claudin-2 expression via inhibition of the downstream targets GSK3β or Akt. Inhibition of the NFκB pathway seems to be rather important for the arrangement and distribution of TJ proteins, because inhibition of the NFκB pathway had no influence on TJ protein expression.

In our study, we have analyzed the effect of berberine on epithelial barrier function in both cell monolayers grown on permeable supports and rat colon. Fluorescein fluxes confirmed the protective role of berberine on barrier function. In accordance with a sealing of the paracellular barrier against cations, permeability for macromolecules also decreased after berberine administration. This not only advances our understanding of the regulation of paracellular intestinal barrier function, but might also push forward the future design and refinement of therapeutic study strategies enhancing barrier function during intestinal inflammation e.g. in IBD or in situations with prominent cytokine-induced junctional leaks (e.g. burn injury, multi-organ failure). Berberine has been shown to be safe in the majority of clinical trials. In a study with type II diabetes patients, the incidence of gastrointestinal adverse events was 35% (n=58 berberine treatments, side effects included diarrhea, flatulence and abdominal pain) (Yin et al., 2008b), whereas in a study with patients with congestive heart failure, a tendency for increased gastrointestinal side effects was observed (n=79 berberine treatments) (Zeng et al., 2003). The main limitation of most clinical trials, however, is the relatively small number of patients that are enrolled, so a larger multicenter study needs to be performed.

In conclusion, the present study is the first to present defined barrier-preserving effects of the plant alkaloid berberine, which is used in traditional medicine for gastrointestinal complaints via specific regulation of TJ proteins. Three signaling pathways involved in this regulation were identified, namely tyrosine kinase Src, Akt and NFκB. In addition to improving our understanding of berberine-induced barrier effects, this supplies us with a new laboratory tool for the blockade of a combined set of intracellular signaling events that render berberine suitable for screening studies on signaling pathways in the future.

Materials and Methods

Cells and solutions

Confluent monolayers of the human colon carcinoma cell line HT-29/B6 were grown in 25 cm^2 culture flasks containing RPMI1640 with stable L-glutamine, 10% fetal calf serum and 1% penicillin-streptomycin (Kreusel et al., 1991). Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere. For electrophysiological measurements, HT-29/B6 cells were seeded on Millicell PCF filters (Millipore, Schwalbach, Germany), and experiments were performed after 7 days, giving R^t values of 400–600 Ω·cm^2. The apical compartment was routinely filled with 500 µl culture medium, and the basolateral compartment contained 10 ml. 50 µM berberine was added to the medium mucosal or serosal compartment or on both sides. Application was done with berberine or other inhibitors alone 2 hours before cytokine exposure. 500 U/ml TNFα (TEBU, Offenbach, Germany) was added basolaterally 24 hours before experiments. All cell culture experiments were analyzed after 26 hours. Cell number and cell density were unchanged after berberine treatment. Cell number and cell density were unchanged after berberine treatment (control, 30.95±2.02 per 100 µm; berberine 30.17±1.70 per 100 µm; TNFα, 29.56±1.70 per 100 µm; berberine and TNFα, 31.94±1.94 per 100 µm).

Animals

Colon specimens of male Wistar rats (250–300 g) were obtained from distal colon. As described earlier, they were placed with the mucosal side down on a plastic plate, and the muscularis propria was removed using fine forceps (Fromm et al., 1993).

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Then, the tissue was turned with the mucosal side facing up, and the epithelial sheet was stripped off manually. This step was mostly performed under microscopic control and took about 5 minutes for each specimen. Thus, after stripping only epithelium, lamina propria and the outer layer of the muscularis mucosae remained and was mounted in the Ussing chamber. Low scatter of the results turned out to depend on the consistency of the stripping procedures. Two segments of the colon termed late distal colon (LDC) and early distal colon (EDC) were used in this study. Specimens of LDC were obtained from the very last part of the colon, located between the lymph node at the pelvic brim and the anus. To prepare this extraperitoneal segment of the colon, it was necessary to cut open the pelvic bones. EDC was obtained 6–7 cm proximal to the anus.

Electrophysiological measurements

Measurements on rat distal colon were performed in Ussing chambers equipped with gas lifts. The circulating bathing solution was composed of: 140 mM Na^+; 123.8 mM Cl^-; 5.4 mM K^+; 1.2 mM Ca^{2+}; 1.2 mM Mg^{2+}; 2.4 mM HPO_4^{2-}; 0.6 mM $H_2PO_4^-$; 21 mM HCO_3^-; 10 mM D(+)-glucose; 2.5 mM glutamine; 10 mM D(+)-mannose; 0.5 mM β-OH-butyrate and antibiotics piperacillin (50 mg/l) and imipenem (4 mg/l). The solution was gassed with 95% O_2 and 5% CO_2 at pH 7.4 and kept at 37°C. The area exposed to the 10 ml bathing solution on each side was 0.54 cm^2 for rat colon. Monitoring of tissue resistance was carried out using programmable voltage clamp devices (CVC6 and CVC8, Fiebig, Berlin, Germany). After an equilibration period of 30 minutes, heat-inactivated FCS (10%) was added to avoid adhesion of the cytokines to the Ussing chamber's glass surface. After incubation with or without berberine (50 μM, both sides addition, 1 hour), TNF-α (10,000 U/ml) was added to the serosal compartment. After 18 hours, tissue was fixed with 4% formalin. Measurements on cell cultures were performed using permeable filter inserts for conventional R^t measurements and for two-path impedance spectroscopy. Both techniques were described previously (Kreusel et al., 1991; Krug et al., 2009b).

RNA isolation and qRT-PCR

Total RNA extraction was performed using RNAzol B (WakChemie, Steinbach, Germany) according to the manufacturer's instructions and RNA was quantified by NanoDrop® ND-1000 UV-Vis Spectrophotometer (PEQLAB Biotechnologie GmbH, Erlangen, Germany). 2 μg total RNA per reaction were reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Mannheim, Germany) as described by the manufacturer. Quantitative RT-PCR was performed on a 7500 FAST Real-time PCR System using the TaqMan® Gene Expression Assay No. Hs00221623_m1 (claudin-1), Hs00252666_s1 (claudin-2), Hs00265816_s1 (claudin-3) and Hs00533616_s1 (claudin-4) and human GAPDH according to the manufacturer's instructions (Applied Biosystems).

Immunochemistry

Western blot experiments, and immunofluorescence staining and analysis by confocal laser scanning microscopy were performed as described previously (Amasheh et al., 2002; Weiske and Huber, 2006; Florian et al., 2003; Zeissig et al., 2007).

Apoptosis

Apoptosis was tested by means of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay (Roche, Mannheim, Germany), as reported previously (Bürgel et al., 2002).

Statistical analysis

Data are expressed as means ± s.e.m. Statistical analysis was performed using the unpaired *t*-test. For multiple testing, Bonferroni-Holm correction was applied. $P<0.05$ was considered significant.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/23/4145/DC1

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