Regulatory Peptides 178 (2012) 95–101

Contents lists available at SciVerse ScienceDirect

Regulatory Peptides

journal homepage: www.elsevier.com/locate/regpep

GLP-2 enhances barrier formation and attenuates TNFα-induced changes in a Caco-2 cell model of the intestinal barrier☆

G.W. Moran [a], C. O'Neill [a,b], J.T. McLaughlin [a,b,*]

[a] Inflammation Sciences Research Group, University of Manchester, Manchester, M13 9PL, UK
[b] Manchester Academic Health Sciences Centre, University of Manchester, Manchester, M13 9PL, UK

ARTICLE INFO

Article history:
Received 30 October 2011
Received in revised form 30 January 2012
Accepted 5 July 2012
Available online 15 July 2012

Keywords:
Inflammatory bowel disease
Intestinal permeability
Tight junction proteins
GLP-2

ABSTRACT

Introduction: Tight junctions are intercellular permeability seals that regulate paracellular transport across epithelia. Tight junction function, expression and localisation of constituent proteins are significantly altered by cytokines such as TNFα. Glucagon-like peptide-2 (GLP-2) is an intestinotrophic enteroendocrine peptide. It is not known whether GLP-2 regulates the barrier or tight junctions. The aim of this study was to investigate whether GLP-2 has an effect on tight junction function or protein expression, alone or in response to TNFα exposure.

Methods: Caco-2 cells were grown to confluence on filters in the presence or absence of GLP-2. The time course of transepithelial electrical resistance developing across the monolayer was measured; tight junction protein expression was quantified by immunoblotting. At day 20, TNFα in the presence or absence of GLP-2 was added. Changes in TEER and tight junction proteins expression were quantified. Both TNFα and GLP-2 were added on the basolateral side.

Results: GLP-2 exposed Caco-2 cell monolayers showed a significant increase in transepithelial electrical resistance compared to that in untreated control cells. At the same time, expression of the tight junction proteins occludin and zona occludens-1 (ZO-1) was increased at day 17 post-seeding (1.6-fold; $p=0.037$ and 4.7 fold; $p=0.039$ respectively). Subsequent TNFα exposure induced a significant 9.3-fold ($p<0.001$) decrease in transepithelial electrical resistance and a corresponding reduction in the expression of ZO-1 (5.3 fold; $p<0.01$). However, the TNFα-induced reduction in transepithelial electrical resistance in GLP-2-exposed cells was highly attenuated to 1.8-fold ($p<0.01$). No change in tight junction protein expression was noted in GLP-2 exposed cells after cytokine exposure.

Conclusion: GLP-2 enhances formation of the epithelial barrier and its constituent proteins in Caco-2 cells, and diminishes the effects of TNFα. If these effects are replicated in vivo the GLP-2 receptor may present a therapeutic target in intestinal inflammation.

1. Introduction

Tight junctions (TJs) are multi-protein complexes that seal the paracellular space between epithelial cells to regulate movement of molecules and ions by this route. When viewed by electron microscopy, TJs are visualised as areas where the paracellular space is almost obliterated. Many molecular components of TJs have been identified; among these are the (zona occludens-1) ZO-1, occludin and the claudin family of proteins, all key components in the structure and function of TJs [1–3].

Proinflammatory cytokines such as tumour necrosis factor α (TNFα), play an important role in the increased gut permeability present in inflammatory bowel disease (IBD). Apart from activating a complex inflammatory cascade, TNFα plays a direct role in altering tight junction and barrier function as permeability studies on a number of epithelial models have already shown [4–6].

Abbreviations: CD, Crohn's disease; DSS, dextran sodium sulphate; EDTA, ethylenediaminetetraacetic acid; GLP-2, glucagon-like peptide 2; IFNγ, interferon gamma; IL, interleukin; IGF, insulin growth factor; MAGUK, membrane associated guanylate kinase; MLCK, myosin light chain kinase; NfκB, nuclear factor κB; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TEER, transepithelial resistance; TJ, tight junctions; TGF, tumour growth factor; TNBS, 2,4,6 trinitrobenzene sulfonic acid; TNFα, tumour necrosis factor alpha; TNFR, tumour necrosis factor receptor; UC, ulcerative colitis; VIP, vasoactive inhibitory peptide; ZO, zona occludens.

☆ Moran GW, O'Neill C and McLaughlin JT conceived the study and planned the experimental work. Moran GW carried out the experimental work, analysed the data and drafted the manuscript. O'Neill C and McLaughlin JT participated in analysing the data and critically reviewing the manuscript.

* Corresponding author at: Inflammation Sciences Research Group, School of Translational Medicine, Clinical Sciences Building, University of Manchester, Salford Royal NHS Foundation Trust, Salford, Manchester, M6 8HD, UK. Tel.: +44 161 206 4362/3; fax: +44 161 206 1495.

E-mail addresses: gwmoran@hotmail.com (G.W. Moran), catherine.o'neill@manchester.ac.uk (C. O'Neill), John.Mclaughlin@manchester.ac.uk (J.T. McLaughlin).

0167-0115/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.regpep.2012.07.002

TNFα affects TJ protein expression through activation of NF-kB [7–9]. The increase in TJ permeability is associated with a decrease in transepithelial resistance (TEER), a decrease in ZO-1 and occludin expression and an alteration in their junctional localisation.

It is well established that in IBD, intestinal permeability is increased, at least in part due to defective tight junctions [10–13]. In fact, a defective barrier has been proposed as a possible aetiological factor: increased permeability may allow ingress of larger immunogenic molecules, contributing to the aberrant immune activation to luminal contents. Recent data have shown evidence of morphological changes including increased TJ breaks [14] in both ulcerative colitis (UC) and Crohn's disease (CD) contributing towards a loss of barrier function. In both inflammatory bowel diseases an increase in expression of the 'pore-forming' claudin-2 has been shown. Down-regulation of the 'tightening' TJ protein claudins 1, 4 and occludin has been shown in UC [15,16]. Similarly in CD, down regulation of the 'tightening' claudins 3, 5, 8 and occludin has been suggested as potential contributing factors to the observed increase in intestinal permeability [17,18].

Glucagon-like-peptide 2 (GLP-2), an enteroendocrine peptide secreted from the distal ileal L cells in response to nutrients and mucosal injury has a beneficial effect on gut mucosal integrity. It induces epithelial proliferation [19] and improves intestinal wound healing in a transforming growth factor β (TGF-β) mediated process [20]. GLP-2 decreases mortality in indomethacin-induced murine enteritis [21] and enhances enteric adaptation in rodent short-bowel models [22–24]. Exogenous GLP-2 and its commercially available synthetic analogue Teduglutide, have shown potential beneficial effects on short bowel adaptation in clinical trials [25].

To our knowledge, no data of any effects of GLP-2 on intestinal permeability and TJ protein expression are yet available. In this study we have investigated the effects of GLP-2 on TJ function and protein expression in a cellular model of the intestinal epithelium using Caco-2 cells. The effects of GLP-2 on the barrier development and TJ composition were quantified by measuring the TEER across the cell monolayer. Caco-2 cells were then exposed to TNFα as a reductionist model of intestinal inflammation. Using this model we have shown that GLP-2 has a positive effect on barrier function and TJ protein expression and a protective effect against the response to TNFα.

2. Methods

2.1. Cell culture techniques

Caco-2 cells (passages 45–55) were routinely grown at 37 °C and 5% CO_2 in a constant humidity environment in Minimal Essential Medium (Gibco, Invitrogen), containing non-essential amino acids (1%), 50 U/ml penicillin and 50 μg/ml streptomycin, glutamine (2 mM) and 10% foetal calf serum. Cells were split at confluence at a ratio of 1:10 using 0.05% trypsin/0.02% EDTA (Invitrogen) and then grown on Thincerts™ inserts in 12 well plates (0.4 μm pore size; 1.131 cm^2) (Greiner Bio-one, Gloucestershire, UK).

2.2. TEER measurement

The electrical resistance of Caco-2 intestinal monolayers was measured by using an epithelial voltohm-meter (World Precision Instruments, Sarasota, FL, USA). For resistance measurements, both apical and basolateral sides of the monolayer were bathed with medium. Electrical resistance was measured until similar values were recorded on three consecutive measurements. The resistances of monolayers were reported after subtraction of the background filter resistance (~100Ω) with the result multiplied with the monolayer area of 1.131 cm^2 leading to TEER as Ω cm^2.

2.3. Treatment of Caco-2 cells with recombinant human (rh) TNFα and GLP-2

Caco-2 cells were plated at confluent density on Thincerts™. At 0 h post plating, GLP-2 (Bachem, Germany) at concentrations of 0, 10 nM, 50 nM or 100 nM, was added to the basolateral side of the monolayer. TEER was measured every 2 days up to a maximum of 21 days. The experiments were performed in duplicate three times. In experiments involving cytokines, cells were grown for 20 days, to ensure a complete barrier had formed, with or without GLP-2 (100 nM). These were then switched to serum free medium for 1 h prior to the basolateral addition of rhTNFα (R&D systems, Abingdon, UK) at 100 ng/ml as previously described.

2.4. Protein extraction

Medium was removed and the cells were washed with ice cold PBS. One hundred microlitres of the extraction buffer (120 mM sodium chloride, 25 mM HEPES, 1% Triton-X-100, 2 mM EDTA, 25 mM sodium fluoride, 1 mM sodium orthovanadate, 0.2% sodium dodecyl sulphate (Fisher Scientific, Louborough, UK) and 1% protease inhibitor enzyme solution (Sigma-Aldrich, St Louis, MO, USA)) was added to each well containing Caco-2 cells. The cells were scraped and the resulting suspension placed into an Eppendorf tube (Eppendorf UK Limited, Cambridge, UK). The well was washed with a second aliquot of extraction buffer and the two suspensions were pooled in the same Epppendorf tube. The suspension was then incubated on ice for 30 min with occasional inversion to re-suspend the cells. The sample was then centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant was removed to a fresh tube for subsequent use in protein quantification studies.

2.5. Immunoblotting

Immunoblotting assays to assess TJ protein expression was performed as previously described [26]. Depending on the size of the protein to be detected, 7.5% (ZO-1), 10% (Occludin) or 12% (β-actin, Claudins 1 and 4) sodium-dodecylsulphate polyacrylamide gels were used. Samples were mixed with Laemmli buffer at 1:4 ratio and then heated to 100 °C for 2 min to denature the proteins and then loaded in equal amounts to each of the wells along with 3 μl of broad range molecular weight marker (BioRad; Hertfordshire, UK).

The proteins were electrophoretically separated for 1 h at 150 V and then transferred (100 V for 1 h) onto polyvinylidene fluoride (PVDF) membranes for western blot analysis. The membranes were blocked using the appropriate blocking solution for 1 h before incubation with the primary antibody at the appropriate concentration overnight at 4 °C. See Table 1.

The membranes were washed three times in 5% fat-free milk in TBS and then incubated for 25 min at RT with the appropriate secondary antibody; goat anti-mouse IgG (H&L) HRP conjugate and goat anti-rabbit IgG (H&L) HRP conjugate (Biorad; Hercules, CA, USA) 1:5000 in 5% milk in TBS. Membranes were then washed three times in 5% milk/TBS, and then twice in TBS.

The membranes were visualised by adding Amersham™ ECL plus Western Blotting Detection System reagent (ECL; GE Healthcare Ltd, UK). Reagents A and B were mixed in a 1:40 ratio, in a volume enough to cover the membrane. The mixture was added to the membrane for 5 min as per the manufacturer's instructions. Amersham Hyperfilm™ ECL (GE Healthcare Ltd) was then exposed to the membranes for the appropriate amount of time and developed using Kodak GBX developer and fixer (Eastman Kodak Company, Rochester, NY).

Films were scanned at 300 dpi in transmission mode using a high-resolution flat bed scanner (Epson Expression 1600, Epson, Hemel Hempstead, UK) and stored in 16-bit grayscale bitmap format using a nondestructive compression algorithm tagged image file