View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Institutional Repository of the Freie Universität Berlin

Received: 31 March 2019 | Revised: 10 August 2019 | Accepted: 12 August 2019

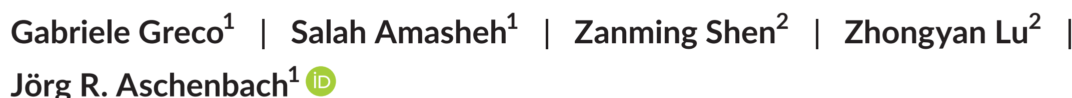
DOI: 10.1111/jpn.13200

Journal of Animal Physiology and Animal Nutrition WILEY

ORIGINAL ARTICLE

Ruminants

Effects of glucagon-like peptides 1 and 2 and epidermal growth factor on the epithelial barrier of the rumen of adult sheep



Gabriele Greco[1] | Salah Amasheh[1] | Zanming Shen[2] | Zhongyan Lu[2] | Jörg R. Aschenbach[1]

[1]Institute of Veterinary Physiology, Freie Universität Berlin, Berlin, Germany

[2]Laboratory of Animal Physiology and Biochemistry, Nanjing Agricultural University, Nanjing, China

Correspondence
Jörg R. Aschenbach, Institute of Veterinary Physiology, Freie Universität Berlin, Oertzenweg 19b, 14163 Berlin, Germany.
Email: joerg.aschenbach@fu-berlin.de

Funding information
Ministry of Agriculture of the People's Republic of China; State of Berlin (Elsa Neumann Stipend); Bundesministerium für Ernährung und Landwirtschaft, Grant/Award Number: 17/12-13-CHN

Abstract

Epidermal growth factor (EGF) and glucagon-like peptides (GLP) modulate the tight junctions (TJ) of the intestinal epithelial barrier (EB) of monogastric animals. This work tried to elucidate whether GLP-1, GLP-2 and EGF can affect the EB of the rumen. Ovine ruminal epithelia were incubated in Ussing chambers for 7 hr with 25 or 250 nM of either GLP-1 or GLP-2 on the serosal side, with 2.5 nM of EGF on the serosal side or with 0.25 or 2.5 nM EGF on the mucosal side. No treatment affected tissue conductance. Short-circuit current (I_{sc}) was affected by time and treatment and their interactions. Only 250 nM of either GLP-1 or GLP-2 decreased I_{sc} in certain periods compared with 25 nM GLP-1 or 0.25 nM mucosally applied EGF; however, not when compared to control epithelia. Fluorescein flux rates (J_{fluor}) of ruminal epithelia were affected by treatment, time and time × treatment interaction. The time × treatment interaction was based on an increase in J_{fluor} between the first and last hour in epithelia incubated with 25 nM GLP-1 or GLP-2 and in epithelia incubated with EGF. After 7 hr incubation, claudin-7 mRNA expression was downregulated in all treatments. Claudin-1 mRNA was upregulated after incubation with 2.5 nM EGF on the serosal side, claudin-4 mRNA was downregulated by 2.5 nM EGF on the mucosal side, and occludin mRNA was increased after incubation with 250 nM GLP-2. The protein abundance of all tested TJ proteins was not influenced by treatment. We conclude that GLP-1, GLP-2, and EGF have no obvious acute effects on the EB of ruminal epithelia under simulated physiological conditions ex vivo. However, by decreasing the mRNA expression of claudin-7 and partly affecting other TJ proteins, they may modulate EB in the longer term or under certain conditions.

KEYWORDS
epidermal growth factor, epithelial barrier, glucagon-like peptide, rumen, tight junction

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2019 The Authors. *Journal of Animal Physiology and Animal Nutrition* published by Blackwell Verlag GmbH

1 | INTRODUCTION

The epithelial barrier (EB) of the rumen provides effective protection against free noxious agents in the ruminal milieu (Aschenbach et al., 2019; Penner, Steele, Aschenbach, & McBride, 2011). However, it can become ineffective under pathological conditions, e.g., when luminal accumulation of short-chain fatty acids and protons challenge key epithelial functions (Aschenbach & Gabel, 2000; Greco et al., 2018; Meissner et al., 2017). Different studies proposed a loss of barrier integrity and selectivity under such circumstances (Liu, Xu, Liu, Zhu, & Mao, 2013; Steele, AlZahal, Hook, Croom, & McBride, 2009). The health consequences of such loss of barrier function appear directly related to the nature and amount of toxins and micro-organisms absorbed into the blood circulation across the leaky epithelium (Aschenbach et al., 2019; Plaizier, Krause, Gozho, & McBride, 2008).

To develop strategies of avoiding or ameliorating a loss of barrier integrity and selectivity, the present study aimed at identifying molecules that may potentially improve the tightness of the EB in ruminal epithelia (RE). Hormones like glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) have been shown to modulate EB of different epithelia (Fukuda et al., 2016; Yu et al., 2014). Such hormones are normally secreted from enteroendocrine cells in the intestine and released into the blood circulation (Gorka et al., 2011; Janssen, Rotondo, Mule, & Tack, 2013). Once reaching the RE, they can directly stimulate their receptors (Taylor-Edwards et al., 2010) or may indirectly affect the EB (Steele, Penner, Chaucheyras-Durand, & Guan, 2016). Likewise, epidermal growth factor (EGF) has also potential to improve the EB of different epithelia (Lamb-Rosteski, Kalischuk, Inglis, & Buret, 2008; Ogawa et al., 2012). It is normally released from a variety of tissues; the release into saliva being of proposed relevance for the RE (Onaga et al., 2006). Hence, EGF may reach the RE from both the luminal and blood side.

To assess the effects of the mentioned GLP hormones and EGF on the ruminal EB, we chose an Ussing chamber approach for the present study. The great advantage of this approach is that functional readouts of barrier integrity like tissue conductance (G_t) and permeability to macromolecules like fluorescein can be monitored continuously over several hours. Secondly, supraphysiological hormone concentrations can be applied without animal welfare concern and compared on epithelia from the same animal. Thirdly, the tissue can be easily harvested at the end of the procedure to study the influence of treatment on the expression of tight junction (TJ) proteins, which physically form the EB (Gonzalez-Mariscal, Betanzos, Nava, & Jaramillo, 2003). Tight junction proteins with a proven role for the ruminal EB are claudin-1, -4, -7, and occludin. Of these, claudin-1 and claudin-4, together with occludin, directly contribute to the formation of the permeation barrier at the level of the *stratum granulosum*; whereas, claudin-7 appears to support the mechanical barrier against abrasion by connecting cells of the *stratum corneum* (Aschenbach et al., 2019; Stumpff et al., 2011).

2 | MATERIALS AND METHODS

2.1 | Animals and tissue sampling

Six healthy adult sheep (*Ovis aries*) with a weight ranging from 65 to 92 kg were purchased from a commercial farm. Animals were fed with hay ad libitum and 200 g concentrate per day for at least 15 days prior to the experiment. The concentrate supply was divided into two equal portions, one in the morning and one in the afternoon. Water was supplied with no restriction. After ≥15 days on the controlled diet, one sheep per day was slaughtered and RE were collected for the experiments. Sheep were stunned with a captive bolt and killed by exsanguination. The abdomen was open by midline incision; the viscera were externalized. A portion of ruminal wall of approximately 30 × 30 cm was cut from the ventral ruminal sac. It was washed first with pre-warmed (38°C) 0.9% NaCl isotonic solution and thereafter in a standard buffered solution containing 10 mM NaCl, 24 mM $NaHCO_3$, 0.6 mM NaH_2PO_4, 2.4 mM $NaHPO_4$, 5.5 mM KCl, 10 mM 2-(N-morpholino) ethanesulfonic acid (MES), 1 mM L-glutamine, 10 mM D-glucose, 100 mM Na-gluconate, 1 mM $CaCl_2$ and 1.25 mM $MgCl_2$ (290 mOsm/L) warmed to 38°C and gassed with carbogen (95% O_2 and 5% CO_2). The washing solution was discarded and the tissue was bathed in fresh standard buffered solution (38°C, carbogen-gassed) in which the *Tunica serosa* and *muscularis* were manually stripped from the *Tunica mucosa* and discarded. The remaining *tunica mucosa* (called epithelium hereafter) was immediately transported to the laboratory in a Dewar container filled with fresh standard buffered solution (38°C, carbogen-gassed).

2.2 | Ussing chamber experiments

Epithelial samples were cut into squares with circa 3.5 cm sides. These epithelial pieces were mounted in Ussing chambers with an inner aperture of 3.14 cm^2 as described by Aschenbach and Gäbel (2000). Ussing chambers were connected to a multichannel voltage-current clamp device (Ing.-Büro Mussler, Aachen, Germany) via platinum wire electrodes for direct current application and Ag/AgCl electrodes for voltage measurement, the latter being connected with the incubation solution via 3 M KCl-agar bridges. The device allowed the continuous recording of short-circuit current (I_{sc}) and G_t (Aschenbach & Gabel, 2000).

The incubation solution on the serosal (i.e. blood-directed) side was 16 ml standard buffered solution (see previous section). The 16 ml of incubation solution on the mucosal (i.e. luminally directed) side were of similar composition, except that 40 mM Na-gluconate was replaced by 24 mM Na-acetate, 12 mM Na-propionate, 4 mM Na-butyrate and that pH was titrated to pH 6.1. Before use, all incubation solutions were added 100 mg/L bovine serum albumin to prevent unspecific binding of hormones and EGF to the glassware and with the antibiotics colistin methanesulfonate (25 mg/L) and cefuroxime (100 mg/L). During the whole experiment, mucosal and serosal solutions were thermostated to 38°C and gassed with carbogen.

After an equilibration period of ~45 min, the hormones GLP-1 and GLP-2 (Sigma-Aldrich, St. Louis, MO), and the growth factor EGF (Genscript Biotechnology; Piscataway, NJ) were added in a total of seven treatments plus a control group that received neither hormones nor EGF. Two epithelia per animal were allocated to each group. Treatments with serosal additions of hormones and EGF were 25 and 250 nM GLP-1, 25 and 250 nM GLP-2, and 2.5 nM EGF. Because EGF reaches the RE predominantly from the luminal side in vivo, EGF was also applied at 0.25 and 2.5 nM on the mucosal side. All given concentrations are final concentrations in the incubation solutions. Stem solutions for hormones and EGF were prepared in deionized water containing 100 mg/L bovine serum albumin. The pH values of all incubation solutions were checked after 1 and 6 hr; they did not change. After 7 hr incubation, epithelia were removed from the Ussing chambers. The solution-exposed area was cut out and split into two pieces. One-piece was preserved in 1 ml mRNA-later, stored at 4°C overnight and at −80°C thereafter. The second piece was harvested in a cryovial, shock frozen in liquid nitrogen and stored at −80°C.

2.3 | Fluorescein flux rate monitoring

Measurement of fluorescein flux rates (J_{fluor}) was performed according to Meissner et al. (2017). Immediately after mounting, that is ~45 min before application of hormones and EGF, Na-fluorescein (Carl Roth, Karlsruhe, Germany) was added to a final concentration of 100 µM to the mucosal side of those chambers that received hormones or EGF serosally. Chambers that were treated with EGF mucosally received 100 µM Na-fluorescein on the serosal side.

Immediately after addition of Na-fluorescein and shortly before the end of the experiment, 50 µl of the incubation solution with added Na-fluorescein (hot samples) were collected and stored in a dark vial at 4°C. Starting at the time of hormone addition, samples of the contralateral incubation solution (cold samples) were collected at hourly intervals and two cold sample aliquots for each hour were stored at 4°C in the dark. To continuously refresh the hormone and EGF concentrations in the solutions, a comparatively large sample volume of 2 ml was collected from the cold side at each hourly sampling and replaced with 2 ml fresh solution containing the respective hormone or EGF.

Fluorescence intensity of each sample was measured in 96-well plates for fluorescence-based assays (Thermo-Fischer Scientific, Waltham, MA) in duplicate in three dilution steps (undiluted, 1 in 10, and 1 in 100). Blanks contained incubation solution without Na-fluorescein. Fluorescence measurements were performed at 490 nm (excitation) and 525 nm (emission) using an EnSpire Multimode Plate Reader (Perkin Elmer, Waltham, MA). The total amount of fluorescein in the cold solution was calculated by calibrating the fluorescence intensity of the cold samples to the specific fluorescence intensity of the hot samples from the same chamber. The increase in the total amount of fluorescein between two samplings, corrected for the fluorescein withdrawn by the sampling procedure itself, represented J_{fluor}, either in serosal-to-mucosal (sm; mucosal EGF treatments) or in mucosal-to-serosal (ms; all other treatments) direction, and was normalized per cm^2 of epithelial area.

2.4 | Total RNA extraction, cDNA production and RT-qPCR

Total RNA was isolated with the Nucleo Spin RNA II kit (Macherey & Nagel, Dueren, Germany). and its concentration measured with a nanophotometer (Implen GmbH, Munich, Germany). Quality of RNA was assessed by microchip electrophoresis using RNA 6000 Nano Kit in an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). An amount of 100 ng of total RNA, with RIN values ranging from 8 to 10 for most samples, were converted to cDNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) and adjusted to a concentration of 0.5 ng/µl. A volume of 3.5 µl of such cDNA was pipetted in 384-well plate (Thermo Fisher Scientific) together with the 5.5 µl mastermix which contained iTaq Universal Probes Supermix (Bio-rad Laboratories) and target-specific primers and probes in a final volume of 9 µl. Gene targets were claudin-1, -4, -7, occludin, YWHAZ, RPS 19 and GAPDH, the last three genes were chosen as non-regulated reference genes. Primer sequences and concentrations are listed in Greco et al. (2018). Real-time quantitative PCR was run in triplicates on a thermal cycler ViiA7 (Life Technologies, Carlsbad, CA). After initial denaturation at 95°C for 20 s, a two-step protocol of 40 cycles at 95°C for 1 s and 60°C for 20 s was used for cDNA amplification. Each 384-well plate contained three inter-run calibrators, three blank controls with only deionized water, three negative no-RT controls, and a standard curve to assess amplification efficiencies. Expression values of target genes were normalized with the software qBase (Biogazelle NV, Zwijnaarde, Belgium), which automatically selected RPS 19 as the most stably expressed reference gene. The same software automatically adjusted the C_q values of the amplification curves to the inter-run calibrator.

2.5 | Protein extraction and Western blot analysis

The procedures for protein extraction and Western blot analysis of claudin-1, -4, -7 and occludin were performed as described previously (Greco et al., 2018), except that blotting was performed at 80 V for 60 min and that antibodies for the reference gene (anti-β-actin) were diluted 1 in 2000.

2.6 | Statistical analysis

Time series data (G_t, I_{sc}, J_{fluor}) was analyzed for effects of time and treatment and their two-way interactions using two-way repeated measurement analysis of variance. The results of Western blot and RT-qPCR were analyzed using a one-way analysis of variance. If analysis of variance indicated differences among time points or treatments, post-hoc Holm-Sidak method was used for all-pairwise comparison. Data are reported as standard error of mean (*SEM*).

TABLE 1 Influence of GLP hormones and EGF on short-circuit current (I_{sc}, in µEq·cm^{-2}·h^{-1}; pooled *SEM* = 0.040)

Hour	Control	GLP–1 25 nM	GLP–1 250 nM	GLP–2 25 nM	GLP–2 250 nM	EGF-m 0.25 nM	EGF-m 2.5 nM	EGF-s 2.5 nM
1	1.31	1.43	1.08	1.28	1.07	1.27	1.31	1.23
2	1.20^{ab}	1.31^{a}	0.88^{b}	1.14^{ab}	0.90^{b}	1.16^{ab}	1.18^{ab}	1.14^{ab}
3	1.08^{ab}	1.17^{a}	0.75^{b}	0.94^{ab}	0.76^{ab}	1.06^{ab}	1.09^{ab}	1.03^{ab}
4	0.99^{abc}	1.08^{a}	0.66^{bc}	0.79^{abc}	0.63^{c}	0.94^{abc}	1.02^{b}	0.93^{abc}
5	0.93	1.00	0.66	0.80	0.63	0.96	1.00	0.97
6	0.87	0.92	0.60	0.72	0.59	0.95	0.96	0.89
7	0.74^{ab}	0.77^{ab}	0.52^{b}	0.68^{ab}	0.50^{ab}	0.90^{a}	0.90^{ab}	0.79^{ab}

Note: Data are least square means (LSM; *n* = 9 to 12). The following *P*-vales were calculated: factor treatment, *p* = .004; factor time, *p* < .001; interaction treatment × time, *p* = .018.

[a-c]Differences between multiple treatment means (at *p* < .05) are indicated by letter coding; values within a row differ if they do not share a common letter.

GLP hormones were always applied from the serosal side. EGF was applied either from the mucosal (EGF-m) or the serosal side (EGF-s).

TABLE 2 Influence of GLP hormones and EGF on tissue conductance (G_t, in mS/cm^2; pooled *SEM* = 0.093)

Hour	Control	GLP–1 25 nM	GLP–1 250 nM	GLP–2 25 nM	GLP–2 250 nM	EGF-m 0.25 nM	EGF-m 2.5 nM	EGF-s 2.5 nM
1	2.29	2.30	1.98	2.29	1.95	2.26	2.46	2.35
2	2.56	2.61	2.31	2.67	2.28	2.57	2.83	2.66
3	2.77	2.85	2.54	2.94	2.50	2.78	3.11	2.87
4	2.90	3.00	2.73	3.11	2.68	2.89	3.34	2.85
5	3.02	3.15	2.90	3.27	2.80	3.02	3.54	3.01
6	3.13	3.31	3.07	3.42	2.90	3.12	3.75	3.23
7	3.30	3.58	3.27	3.65	3.02	3.24	4.03	3.35

Note: Data are least square means (LSM; *n* = 12). The following *p*-vales were calculated: factor treatment, *p* = .74; factor time, *p* < .001; interaction treatment × time, *p* = .88.

GLP hormones were always applied from the serosal side. EGF was applied either from the mucosal (EGF-m) or the serosal side (EGF-s).

The zero hypothesis was considered disproved with *p*-values < .05. The software package used for data assessment and plotting of graphs was SigmaPlot 11 (Systat Software, San José, CA).

3 | RESULTS

3.1 | Electrophysiological data

Values of I_{sc} were affected by the factors time of incubation (*p* < .01) and treatment (*p* < .01) with significant time × treatment interaction (*p* < .05; Table 1). The I_{sc} decreased over time in all treatments. Thus, the interaction was primarily based on lower I_{sc} values of epithelia incubated with 250 nM GLP-1 or GLP-2 compared to those incubated with 25 nM GLP-1 during the second, fourth and partly the third hour of incubation (*p* < .05), and due to lower I_{sc} values of epithelia treated with 250 nM GLP-2 compared to epithelia incubated with 0.25 nM of EGF on the mucosal side in the seventh hour (*p* < .05). However, I_{sc} values of all hormone or EGF-treated groups were not different from Control at any time (Table 1). Values of G_t gradually increased over time (*p* < .01) with no difference among and no interaction with treatments (Table 2).

3.2 | Fluorescein flux rates

Fluorescein flux rates of epithelia were affected by treatment and time of incubation (*p* < .01 each) with significant time × treatment interaction (*p* < .05; Table 3). The main effect of treatment was exclusively attributable to the much lower flux rates in sm direction measured for the mucosal EGF treatments as opposed to the flux rates in ms direction measured for all other treatments (Table 3). The statistical significance of the treatment effect disappeared when ms and sm flux rates were analysed separately (data not shown). The time effect with a time × treatment interaction was mainly caused by an increase in J_{fluor} between the first and last hour of incubation occurring in epithelia incubated with 25 nM of GLP-1 or GLP-2 and in all epithelia incubated with EGF either on the mucosal or on the serosal side (*p* < .05; significances for time within treatment not shown in Table 3 for reasons of clarity). Due to these time-dependent increases, J_{fluor} was greater in epithelia treated serosally with EGF compared to Control in the seventh hour of incubation (*p* < .05). Flux rates of GLP-treated tissues were at no time point different to Control (Table 3).

TABLE 3 Influence of GLP hormones and EGF on fluorescein flux rates (J_{fluor}, in nmol·cm^{-2}·h^{-1}; pooled *SEM* = 0.083)

Hour	Control	GLP–1 25 nM	GLP–1 250 nM	GLP–2 25 nM	GLP–2 250 nM	EGF-m 0.25 nM	EGF-m 2.5 nM	EGF-s 2.5 nM
1	0.52[a]	0.56[a]	0.56[a]	0.53[a]	0.63[a]	0.06[b]	0.07[b]	0.58[a]
2	0.78[a]	0.73[a]	0.84[a]	0.70[a]	0.78[a]	0.12[b]	0.10[b]	0.80[a]
3	0.74[a]	0.82[a]	0.58[a]	0.70[a]	0.61[a]	0.10[b]	0.12[b]	0.83[a]
4	0.51[ab]	0.75[a]	0.51[ab]	0.66[a]	0.70[a]	0.11[b]	0.15[b]	0.81[a]
5	0.52[ab]	0.82[a]	0.61[ab]	0.76[a]	0.60[ab]	0.17[b]	0.19[b]	0.86[a]
6	0.83[a]	0.62[abc]	0.61[abc]	0.59[abc]	0.66[ab]	0.20[c]	0.22[bc]	0.94[a]
7	0.75[bc]	1.08[ab]	0.64[cd]	1.07[ab]	0.63[cd]	0.15[e]	0.29[de]	1.25[a]

Note: Data are least square means (LSM; *n* = 10 to 12). The following *p*-vales were calculated: factor treatment, $p < .001$; factor time, $p = .009$; interaction treatment × time, $p = .048$.

Differences between multiple treatment means (at $p < .05$) are indicated by letter coding; values within one row differ if they do not share a common letter.

GLP hormones were applied from the serosal side, and flux rates were measured in mucosal-to-serosal direction. EGF was applied either from the mucosal (EGF-m) or from the serosal side (EGF-s). Flux rates after mucosal application of EGF were measured in serosal-to-mucosal direction, whereas flux rates after serosal EGF application were measured in mucosal-to-serosal direction.

3.3 | RT-qPCR and mRNA quantification

Results of RT-qPCR are shown in Figure 1 for claudin-1 and claudin-4, and in Figure 2 for claudin-7 and occludin. The mRNA expression of all tested TJ proteins was affected by treatment ($p < .05$). Incubation with 25 nM or 250 nM GLP-1, or 25 nM GLP-2, or 0.25 nM mucosally applied EGF resulted in levels of claudin-1, claudin-4, and occludin mRNA expression not different from control epithelia. Epithelia incubated with 250 nM GLP-2 also showed claudin-1 and claudin-4 mRNA expression similar to control epithelia (Figure 1); however, occludin mRNA expression was upregulated above the level of Control and all other treatments ($p < .05$). Epithelia incubated with 2.5 nM EGF on the mucosal side showed claudin-1 and occludin mRNA expression similar to control epithelia, but claudin-4 mRNA expression was lower than control epithelia and epithelia incubated with GLP-1 or serosally applied EGF ($p < .05$). Finally, epithelia incubated with 2.5 nM EGF on the serosal side showed mRNA expression of claudin-4 and occludin not different from control epithelia; however, claudin-1 mRNA expression was higher than for control and all other treatments, except 0.25 nM mucosally applied EGF ($p < .05$). The mRNA expression of claudin-7 was downregulated in all treatments compared with control epithelia, most prominently by GLP-1, 0.25 nM mucosally applied EGF and 2.5 nM serosally applied EGF ($p < .05$).

3.4 | Western blot analysis

Protein abundance of claudin-1, -4, -7 and occludin were not different among treatments and control epithelia (Figure 1 and Figure 2).

4 | DISCUSSION

In order to understand the factors that modulate the tightness of the ruminal EB, the present study aimed at elucidating the influence of GLP-1, GLP-2 and EGF on ruminal permeability characteristics and TJ protein expression. Our research group had previously studied the effects of pH and SCFA on the EB in the ovine rumen using a comparable experimental setup (Greco et al., 2018; Meissner et al., 2017). Key advantages of the Ussing chamber approach have been mentioned in the Introduction. The present results verified the appropriateness of such approach; data on I_{sc} and G_t confirmed that tissues remained vital throughout the experimental period of 7 hr, which proofed long enough to induce mRNA expression changes of selected TJ proteins.

During the experiments, an unexpected technical peculiarity of fluorescein emerged: The J_{fluor} differed greatly in ms versus sm direction. When planning the study, we had assumed that fluorescein as an established paracellular marker should yield comparable flux rates in either flux direction. Consequently, we had initially interpreted the greatly reduced J_{fluor} after mucosal EGF treatments as barrier-tightening effects of EGF. However, the fact that the discrepancy between ms and sm flux rates was greatest already in the first flux hour and the fact that G_t and TJ protein expression did not mirror this ‘effect’, strongly argued against a true EGF effect of such magnitude on J_{fluor}. The unequal flux rates of fluorescein in ms and sm direction can have two causes. Firstly, there could be active (transcellular) transport of fluorescein across the ruminal epithelium in ms direction. Secondly, lower sm flux rates could be a measuring artefact because fluorescein is a weak acid and changes from the divalent anion (of greater fluorescence) to a monovalent anion (of lower fluorescence) with a pK_a of ~6.4 (Lakowicz, 2006). As a consequence, fluorescein yielded lower fluorescence signals in buffer samples from the mucosal side, which had a pH of 6.1. For the results of the present study, these limitations have the consequence that sm flux rates of fluorescein (mucosal EGF treatments) are not further discussed and results of the ms flux rates of fluorescein will be interpreted cautiously.

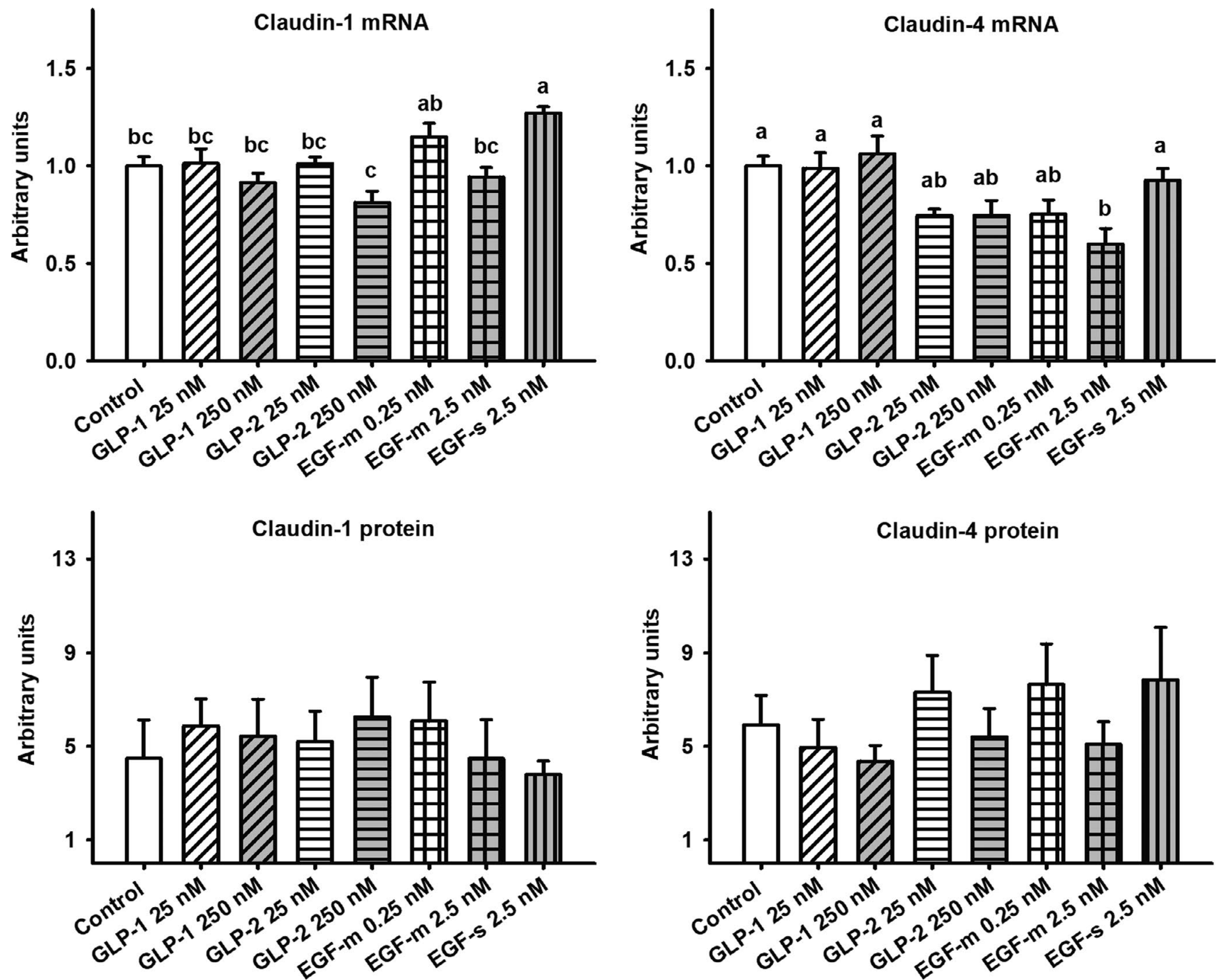


FIGURE 1 Expression of claudin-1 (left panels) and claudin-4 (right panels) at the level of mRNA (upper panels) and protein (lower panels) in ruminal epithelia. Expression was measured after 7-hr incubation with 25 nM and 250 nM GLP-1 on the serosal side, 25 and 250 nM GLP-2 on the serosal side, 0.25 and 2.5 nM EGF on the mucosal side (EGF-m), and 2.5 nM EGF on the serosal side (EGF-s). Data are means ± *SEM* ($n = 12$). Letter code is used to indicate significant differences identified by multiple comparisons post-hoc test. Within each graph, columns differ at $p < .05$ if they do not share a common letter

A great challenge of the experimental design was to choose the right GLP and EGF concentrations. The concentrations of GLP-1 and GLP-2 in blood serum or plasma of ruminants are typically below 100 pM, where they are increased by feed intake (Castro et al., 2016; McCarthy, Faulkner, Martin, & Flint, 1992; Suominen et al., 1998) and further influenced by metabolic and reproductive status (Larsen, Relling, Reynolds, & Kristensen, 2010; Marti, Perez, Aris, Bach, & Devant, 2014; Relling & Reynolds, 2007; Zapata, Salehi, Ambrose, & Chelikani, 2015). Dose-response data for GLP are not available for gastrointestinal epithelia of ruminants; however, GLP-1 and GLP-2 have been tested for dose effects on chloride secretion in the guinea-pig ileum mounted in Ussing chambers with maximum responses at ~100 nM for GLP-1 (Baldassano, Wang, Mulè, & Wood, 2012) and ~30 nM for GLP-2 (Baldassano, Liu, Qu, Mulè, & Wood, 2009). Importantly, those previous experiments observed GLP effects without applying any protease inhibitor to protect against GLP degradation by DDP-4. The latter is a widely distributed GLP-degrading enzyme in the intestine but apparently absent in stomach and oesophagus (Uhlen et al., 2010). Considering that rumen is rather related to stomach and oesophagus than intestine, we assumed that DPP-4 degradation should not be a major issue in our experimental setup and chose 25 and 250 nM as our test concentrations. To yet account for any possibility of GLP degradation, we replenished 12.5% of the incubation solution hourly with fresh solution containing the correct GLP concentration.

Plasma concentrations of EGF are apparently not documented for ruminants in literature. This is due likely to the fact that EGF plasma concentrations are very low and barely measurable in ruminants. In one study on goats, all plasma concentrations were below the detection limit of the assay (<50 pM; Dehnhard, Claus, Munz, & Weiler,

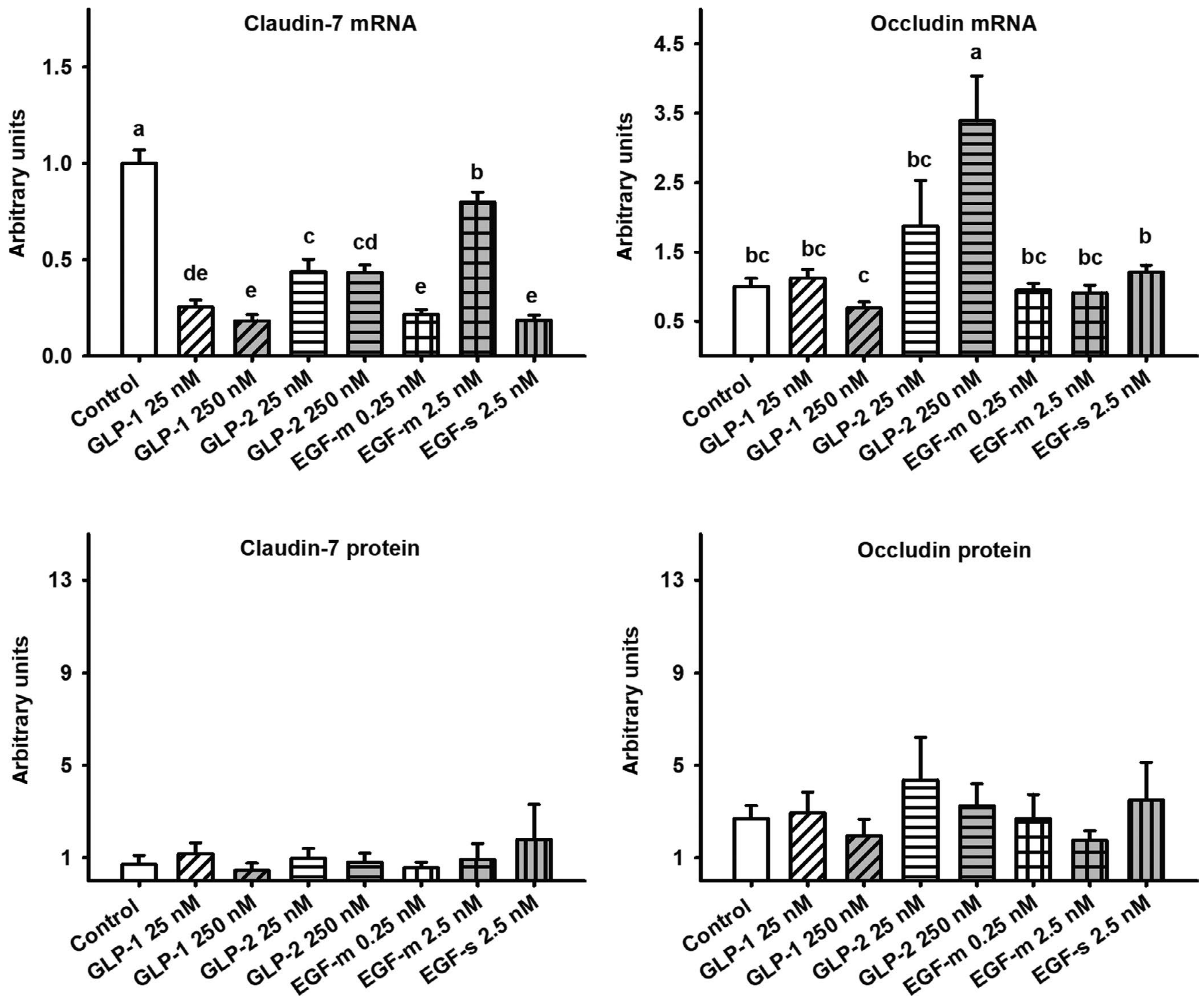


FIGURE 2 Expression of claudin-7 (left panels) and occludin (right panels) at the level of mRNA (upper panel) and protein (lower panel) in ruminal epithelia. Expression was measured after 7-hr incubation with 25 nM and 250 nM GLP-1 on the serosal side, 25 and 250 nM GLP-2 on the serosal side, 0.25 and 2.5 nM EGF on the mucosal side (EGF-m), and 2.5 nM EGF on the serosal side (EGF-s). Data are means ± *SEM* (*n* = 12). Letter code is used to indicate significant differences identified by multiple comparisons post-hoc test. Within each graph, columns differ at $p < .05$ if they do not share a common letter

2000). By contrast, EGF concentrations of ruminal fluid were readily measurable in sheep (73 pM; Onaga et al., 2006), implying that these luminal concentrations are much higher than plasma concentrations. Using the Ussing chamber approach, ion currents in a human colonic adenocarcinoma cell line were influenced by EGF with an EC_{50} of 0.25 nM. Accordingly, we chose concentrations of 0.25 and 2.5 nM for our ruminal permeability measurements. Stability appears less a concern for EGF compared with GLP because EGF is catabolized primarily through internalization together with its receptor. In 3T3 cells, maximal binding between EGF and its receptor occurred after 30–40 min incubation and decreased to 40% after 6 hr (Ahrnonv, Pruss, & Herschman, 1978).

In the present study, GLP-1 and GLP-2 had subtle dose-dependent effects on the active electrogenic current flow (I_{sc}) and the passive permeability to the large anion fluorescein (J_{fluor}) across the RE. The high-dose of either GLP-1 or GLP2 was associated with lower values of both I_{sc} and J_{fluor} compared with the low dose, at least, at certain time points. Decreasing effects of GLP-1 and GLP-2 on I_{sc} had previously been observed in electrical field-stimulated guinea-pig small intestine and were explained by a GLP-mediated depression of acetylcholine release from enteral nerves (Baldassano et al., 2009, 2012). Although the stripped RE preparations used in the present study were devoid of neuronal ganglia, the observed effects on J_{fluor} and I_{sc} yet suggest that both peptides act on ion flow across the epithelium by either direct effects on RE cells or influencing paracrine signals. Given the subtle nature of these responses, interpretations should be seen with care. Nonetheless, the present results appear to extend previous findings, suggesting that receptors

for GLP-1 and GLP-2 are functional in the RE despite comparatively low levels of mRNA expression (Gorka et al., 2011; Pezeshki, Muench, & Chelikani, 2012; Taylor-Edwards et al., 2010).

Alterations in J_{fluor} and G_t are commonly used to assess the permeability of the paracellular space to large (J_{fluor}) and small ions (G_t), assuming that the transcellular route has negligible (J_{fluor}) or rather constant (G_t) contribution to these functional barrier readouts. Nevertheless, we have demonstrated in previous studies that fluorescein is also able to use the transcellular route for permeation across RE, for example, after an acid challenge (Greco et al., 2018; Meissner et al., 2017). Based on the fact that G_t and the protein expression of all tested TJ proteins were unchanged by GLP-1 and GLP-2 in the present study, we thus propose that paracellular barrier function of RE was not affected by these hormones during the 7-hr incubation, despite the fact that J_{fluor} was changed in the last flux period by either of them.

Nonetheless, the mRNA expression of TJ proteins was affected by both GLP-1 and GLP-2. The incubation of RE with GLP-2 and, especially, GLP-1 decreased the mRNA expression of claudin-7 at both tested concentrations. Incubation with GLP-2 simultaneously increased the mRNA expression of occludin mRNA, which was statistically significant at a concentration of 250 nM. Our results align with a previous study where twice-daily injection of GLP-2 (50 µg/kg of BW) selectively increased the mRNA expression of occludin in the jejunum and caecum of calves but had no significant effects on claudin-1 and claudin-4; with claudin-7 being not measured in that previous study (Walker, Evock-Clover, Elsasser, & Connor, 2015).

Earlier studies from our institute have identified claudin-7 as a marker for the terminal differentiation of the RE that is induced in cornifying cells (Greco et al., 2018; Stumpff et al., 2011). Claudin-7 is localized in the *stratum corneum* of the RE and acts as anchor between the cells of the same *stratum corneum* and between the cells of this *stratum* with the cells of the lower *stratum granulosum*. Connection by claudin-7 appears essential to guarantee continuous epithelial integrity under intensive feeding conditions when cornified cells start to balloon. Accordingly, we have suggested previously that decreased expression of claudin-7 may predispose the RE to hyperkeratosis and parakeratosis, especially, when proliferative stimuli are present simultaneously (Greco et al., 2018). Such thesis is reinforced from a study on neoplastic cell models, in which claudin-7 expression was linked to a reduction of cell proliferation and increased cell adhesion (Lu et al., 2015). In contrast to claudin-7, occludin is expressed in all 'living' cell layers of the RE from the *stratum basale* to the *stratum granulosum* (Greco et al., 2018; Meissner et al., 2017; Stumpff et al., 2011). The additional upregulation of occludin mRNA expression in epithelia incubated with higher concentrations of GLP-2 may possibly indicate an increased requirement for junction molecules in the lower strata of the RE due to the proliferative action of GLP-2, as deductible from the proliferative action of GLP-2 in porcine intestine (Sigalet et al., 2014) and the intestine of feed-restricted cows (Kvidera et al., 2017).

Considering that mRNA changes were not followed by changes in TJ protein expression, one may assume that either the time frame of 7 hr GLP treatment was insufficient to initiate changes in TJ protein expression or that additional signals (Yang, Rao, & Wang, 2014) are required to translate the mRNA signals into changes of TJ protein expression. Due to its positive effect on the mRNA expression of occludin, GLP-2 appears to carry a potential to elicit beneficial effects on the EB in RE, albeit the circumstances that translate this into functional protein are currently unknown and despite the risk of parallel induction of parakeratosis via downregulation of claudin-7. Such beneficial effects of GLP-2 have been proposed previously for the development of the EB in the rumen in growing ruminants (Gorka et al., 2011) and could possibly apply to feed adaptation in later life, although studies in vivo clearly indicate that the level of feeding has more prominent effects on the intestinal GLP system (Taylor-Edwards et al., 2010). To further evaluate this, future experiments should test the effect of GLP hormones in premature RE and under challenge conditions. The latter acknowledges that both GLP-1 and GLP-2 display their beneficial effects on intestinal epithelia most prominently under stress conditions like inflammation (Insuela & Carvalho, 2017; Nakame, Kaji, Mukai, Shinyama, & Matsufuji, 2016) and feed restriction (Kvidera et al., 2017; Sigalet et al., 2014).

The effects of GLP-1 and, especially, GLP-2 observed in the present study apparently bear some overlap with the effects of SCFA observed in one of our earlier studies. For example, luminal application of 30 mM butyrate also induced a decrease in I_{sc}, an increase in claudin-1 mRNA expression and a decrease in claudin-7, albeit the latter at the protein level (Greco et al., 2018). It is also accepted that GLP-1 and GLP-2 are released in response to short-chain fatty acids in ruminants (Elsabagh, Inabu, Obitsu, & Sugino, 2017; Fukumori, Mita, Sugino, Obitsu, & Taniguchi, 2012). It can be excluded, however, that the effects of SCFA observed in our previous study were, in part, mediated by GLP hormones. It has been clearly shown that RE has no to negligible mRNA expression of proglucagon (Pezeshki et al., 2012; Taylor-Edwards et al., 2010) and glucagon immune-reactive cells are absent (Bunnett & Harrison, 1986). As such, any GLP-1 or GLP-2 acting on the RE has to be of distant, most likely, intestinal origin (Taylor-Edwards et al., 2010).

With regard to EGF, no changes in electrophysiology were observed during the 7-hr incubation period and, except for the last hour of incubation, no changes were observed for J_{fluor}. Based on the fact that TJ protein expression and G_t were unchanged, we take this as indication that paracellular barrier function was not altered by EGF as extrapolated earlier for GLP-1 and GLP-2. Nevertheless, EGF influenced the mRNA expression of TJ proteins in this study. It appeared that incubation with 0.25 nM of EGF on the mucosal side and 2.5 nM of EGF on the serosal side had comparable effects that were dissimilar to the effects after mucosal application of 2.5 nM EGF. Mucosal application of 0.25 nM EGF and serosal application of 2.5 nM EGF increased or tended to increase claudin-1 mRNA expression and very prominently decreased claudin-7 mRNA expression. At variance, mucosal application of 2.5 nM EGF had no effect on claudin-1 mRNA expression, only moderately decreased claudin-7 mRNA expression and decreased claudin-4 mRNA expression. Previous studies had already demonstrated that EGF may differently affect

the TJs of the same epithelium when changing the epithelial side of growth factor application (Chen, Solomon, Kui, & Soll, 2002; Xiao et al., 2011). One possible explanation for the similar effects of mucosal application of 0.25 nM EGF and serosal application of 2.5 nM EGF in the present study could be that EGF receptors are located primarily on the mucosal (i.e. luminal) side of the RE to bind EGF secreted with saliva (Onaga et al., 2006). In such concept, the similarity of the effect of high-dose serosal EGF with low-dose mucosal EGF could be explained by spill-over of serosally applied, high-dose EGF to the mucosally located receptors. The effect of high-dose (2.5 nM) mucosal EGF is less clear because it ameliorated the decrease in claudin-7 mRNA expression, abolished the stimulation of claudin-1 mRNA expression and, alternatively, caused a decrease in claudin-4 mRNA expression. In support of our findings, however, an increase from 1.6 to 3.3 nM EGF had also reversed the stimulating effect of EGF on transepithelial resistance in previous studies on bronchial epithelium (Xiao et al., 2011). Steele et al. (2015) have concluded from their results that ruminal EGF receptors are downregulated upon sustained exposure to increased EGF concentrations, which could provide one possible explanation for the reversal of EGF effects at very high EGF concentrations.

In the published literature, the role of EGF is mainly seen in a protection of the EB against stressing conditions rather than directly improving the EB (Banan et al., 2003; Basuroy, Seth, Elias, Naren, & Rao, 2006; Guntaka, Samak, Seth, LaRusso, & Rao, 2011; Koepke et al., 2015; Okuyama et al., 2007; Sheth, Seth, Thangavel, Basuroy, & Rao, 2004). It often prevented or decreased the detrimental effect of micro-organisms on the epithelial barrier (Buret, Mitchell, Muench, & Scott, 2002; Kaur, Vaishnavi, Ray, Singh, & Kochhar, 2014; Lamb-Rosteski et al., 2008). EGF does so by stimulation of several intracellular pathways involved in TJ modulation. The MEK/ERK is a frequently cited pathway involved in the TJ modulation after EGF stimulation (Grande et al., 2002; Ikari, Takiguchi, Atomi, & Sugatani, 2011). In other cases, the TJ adaptation was related to PKC, PIK-3, src and STAT signalling (Garcia-Hernandez et al., 2015; Wang et al., 2006; Yoshida et al., 2005). Few studies also reported negative effects of EGF on the EB (Soler, Laughlin, & Mullin, 1993), including the EB of epidermal keratinocyte cultures (Tran et al., 2012). As such, the negative effect of EGF on claudin-7 mRNA and, at very high luminal concentrations, also on claudin-4 mRNA may suggest that EGF may possibly contribute to a weakening the RE barrier under certain conditions in vivo. In this regard, it is interesting to note that the RE down-regulates EGF receptors during the transition to high-energy diets after parturition of dairy cows (Steele et al., 2015).

5 | CONCLUSION

The present study evidenced that a 7-hr exposure to GLP-1, GLP-2 and EGF had no effect on G_t and on the expression of TJ proteins in RE isolated from healthy sheep on a hay-based, low-concentrate diet. This indicates that the paracellular EB of RE was functionally not affected under the experimental conditions, although very high serosal concentration of GLP-1, GLP-2 and EGF increased J_{fluor} in the 7th hour of incubation; the latter may likely be attributable to increased transcellular passage of this permeability marker. Despite no measurable paracellular barrier changes, GLP-2 and EGF increased occludin and claudin-1 mRNA expression respectively, which may be seen as a potential to contribute to barrier stabilization under certain, currently unknown circumstances. However, all tested application modes and concentrations of GLP hormones and EGF decreased the expression of claudin-7 mRNA, which may potentially predispose to disturbed function of the *stratum corneum*, that is, hyperkeratosis and parakeratosis, if translated into similar changes in claudin-7 protein expression. Together with the literature finding that RE downregulate the expression of EGF receptors upon transition to highly fermentable diets, our results suggest that one cannot simply postulate beneficial versus detrimental effects of the tested hormones and EGF on the RE barrier. Further studies are necessary to test the effects under several different conditions and possibly in combination with other endocrine or metabolic signals.

ACKNOWLEDGEMENTS

This study was supported by an Elsa Neumann stipend to G. Greco and coordinated mobility grants from the German and Chinese agricultural ministries to support Sino-German Cooperation in Agricultural Science and Technology (17/12-13-CHN and 2016-2017, No.14). We thank K. Söllig, M. Grunau and U. Tietjen (Freie Universität Berlin) for their reliable support in the laboratories.

ANIMAL WELFARE STATEMENT

All procedures were conducted in compliance with the German legislation on the welfare of experimental animals and communicated with the local authorities, the State Office for Health and Social Affairs Berlin (LAGeSo, Registration No. T 0360/12).

ORCID

Jörg R. Aschenbach https://orcid.org/0000-0001-5080-1007

REFERENCES

Ahrnonv, A., Pruss, R. M., & Herschman, H. R. (1978). Epidermal Growth Factor. Relationship between receptor regulation and mitogenesis in 3T3 cells. *The Journal of Biological Chemistry*, *253* (11): 3970–3977.

Aschenbach, J. R., & Gabel, G. (2000). Effect and absorption of histamine in sheep rumen: Significance of acidotic epithelial damage. *Journal of Animal Science*, *78*(2), 464–470. https://doi.org/10.2527/2000.782464x

Aschenbach, J. R., Zebeli, Q., Patra, A. K., Greco, G., Amasheh, S., & Penner, G. B. (2019). Symposium review: The importance of the ruminal epithelial barrier for a healthy and productive cow. *Journal of Dairy Science*, *102*(2), 1866–1882. https://doi.org/10.3168/jds.2018-15243

Baldassano, S., Liu, S., Qu, M. H., Mulè, F., & Wood, J. D. (2009). Glucagon-like peptide-2 modulates neurally evoked mucosal chloride secretion

in guinea pig small intestine in vitro. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *297*(4), G800–G805. https://doi.org/10.1152/ajpgi.00170.2009

Baldassano, S., Wang, G. D., Mulè, F., & Wood, J. D. (2012). Glucagon-like peptide-1 modulates neurally evoked mucosal chloride secretion in guinea pig small intestine in vitro. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *302*(3), G352–G358. https://doi.org/10.1152/ajpgi.00333.2011

Banan, A., Fields, J. Z., Zhang, L. J., Shaikh, M., Farhadi, A., & Keshavarzian, A. (2003). Zeta isoform of protein kinase C prevents oxidant-induced nuclear factor-kappaB activation and I-kappaBalpha degradation: A fundamental mechanism for epidermal growth factor protection of the microtubule cytoskeleton and intestinal barrier integrity. *Journal of Pharmacology and Experimental Therapeutics*, *307*(1), 53–66. https://doi.org/10.1124/jpet.103.053835

Basuroy, S., Seth, A., Elias, B., Naren, A. P., & Rao, R. (2006). MAPK interacts with occludin and mediates EGF-induced prevention of tight junction disruption by hydrogen peroxide. *Biochemical Journal*, *393*(Pt 1), 69–77. https://doi.org/10.1042/BJ20050959

Bunnett, N. W., & Harrison, F. A. (1986). Immunocytochemical localization of gastric inhibitory peptide and glucagon in the alimentary tract of ruminants. *Quarterly Journal of Experimental Physiology*, *71*(3), 433–441. https://doi.org/10.1113/expphysiol.1986.sp003002

Buret, A. G., Mitchell, K., Muench, D. G., & Scott, K. G. (2002). Giardia lamblia disrupts tight junctional ZO-1 and increases permeability in non-transformed human small intestinal epithelial monolayers: Effects of epidermal growth factor. *Parasitology*, *125*(Pt 1), 11–19.

Castro, J. J., Morrison, S. Y., Hosseinni, A., Loor, J. J., Drackley, J. K., & Ipharraguerre, I. R. (2016). Secretion of glucagon-like peptide-2 responds to nutrient intake but not glucose provision in milk-fed calves. *Journal of Dairy Science*, *99*(7), 5793–5807. https://doi.org/10.3168/jds.2015-10519

Chen, M. C., Solomon, T. E., Kui, R., & Soll, A. H. (2002). Apical EGF receptors regulate epithelial barrier to gastric acid: Endogenous TGF-alpha is an essential facilitator. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *283*(5), G1098–G1106. https://doi.org/10.1152/ajpgi.00507.2001

Dehnhard, M., Claus, R., Munz, O., & Weiler, U. (2000). Course of epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) in mammary secretions of the goat during end-pregnancy and early lactation. *Journal of Veterinary Medicine. A, Physiology, Pathology. Clinical Medicine*, *47*(9), 533–540. https://doi.org/10.1046/j.1439-0442.2000.00315.x

Elsabagh, M., Inabu, Y., Obitsu, T., & Sugino, T. (2017). Response of plasma glucagon-like peptide-2 to feeding pattern and intraruminal administration of volatile fatty acids in sheep. *Domestic Animal Endocrinology*, *60*, 31–41. https://doi.org/10.1016/j.domaniend.2017.03.001

Fukuda, S., Nakagawa, S., Tatsumi, R., Morofuji, Y., Takeshita, T., Hayashi, K., … Niwa, M. (2016). Glucagon-like Peptide-1 strengthens the barrier integrity in primary cultures of rat brain endothelial cells under basal and hyperglycemia conditions. *Journal of Molecular Neuroscience*, *59*(2), 211–219. https://doi.org/10.1007/s12031-015-0696-1

Fukumori, R., Mita, T., Sugino, T., Obitsu, T., & Taniguchi, K. (2012). Plasma concentrations and effects of glucagon-like peptide-1 (7–36) amide in calves before and after weaning. *Domestic Animal Endocrinolgy*, *43*(4), 299–306. https://doi.org/10.1016/j.domaniend.2012.05.003

Garcia-Hernandez, V., Flores-Maldonado, C., Rincon-Heredia, R., Verdejo-Torres, O., Bonilla-Delgado, J., Meneses-Morales, I., … Contreras, R. G. (2015). EGF regulates claudin-2 and-4 expression through Src and STAT3 in MDCK cells. *Journal of Cellular Physiology*, *230*(1), 105–115. https://doi.org/10.1002/jcp.24687

Gonzalez-Mariscal, L., Betanzos, A., Nava, P., & Jaramillo, B. E. (2003). Tight junction proteins. *Progress in Biophysics & Molecular Biology*, *81*(1), 1–44. https://doi.org/10.1016/S0079-6107(02)00037-8

Gorka, P., Kowalski, Z. M., Pietrzak, P., Kotunia, A., Jagusiak, W., Holst, J. J., … Zabielski, R. (2011). Effect of method of delivery of sodium butyrate on rumen development in newborn calves. *Journal of Dairy Science*, *94*(11), 5578–5588. https://doi.org/10.3168/jds.2011-4166

Grande, M., Franzen, A., Karlsson, J. O., Ericson, L. E., Heldin, N. E., & Nilsson, M. (2002). Transforming growth factor-beta and epidermal growth factor synergistically stimulate epithelial to mesenchymal transition (EMT) through a MEK-dependent mechanism in primary cultured pig thyrocytes. *Journal of Cell Science*, *115*(22), 4227–4236. https://doi.org/10.1242/jcs.00091

Greco, G., Hagen, F., Meißner, S., Shen, Z., Lu, Z., Amasheh, S., & Aschenbach, J. R. (2018). Effect of individual SCFA on the epithelial barrier of sheep rumen under physiological and acidotic luminal pH conditions. *Journal of Animal Science.*, *96*(1), 126–142. https://doi.org/10.1093/jas/skx017

Guntaka, S. R., Samak, G., Seth, A., LaRusso, N. F., & Rao, R. (2011). Epidermal growth factor protects the apical junctional complexes from hydrogen peroxide in bile duct epithelium. *Laboratory Investigation*, *91*(9), 1396–1409. https://doi.org/10.1038/labinvest.2011.73

Ikari, A., Takiguchi, A., Atomi, K., & Sugatani, J. (2011). Epidermal growth factor increases clathrin-dependent endocytosis and degradation of claudin-2 protein in MDCK II cells. *Journal of Cellular Physiology*, *226*(9), 2448–2456. https://doi.org/10.1002/jcp.22590

Insuela, D. B. R., & Carvalho, V. F. (2017). Glucagon and glucagon-like peptide-1 as novel anti-inflammatory and immunomodulatory compounds. *European Journal of Pharmacology*, *812*, 64–72. https://doi.org/10.1016/j.ejphar.2017.07.015

Janssen, P., Rotondo, A., Mule, F., & Tack, J. (2013). Review article: A comparison of glucagon-like peptides 1 and 2. *Alimentary Pharmacology and Therapeutics*, *37*(1), 18–36. https://doi.org/10.1111/apt.12092

Kaur, S., Vaishnavi, C., Ray, P., Singh, M., & Kochhar, R. (2014). Preliminary investigation on the effect of *Lactobacillus* and epidermal growth factor on tight junction proteins in experimental *Clostridium difficile* infection. *Advances in Microbiology*, *425–435*, https://doi.org/10.4236/aim.2014.48047

Koepke, J., Lim, D., Wales, P., Wizzard, P., Sigalet, D., Li, J. L., … Levesque, C. (2015). Glucagon-like peptide 2 and epidermal growth factor promote intestinal adaptation in two neonatal intestinal failure models. *FASEB Journal*, *29*(1_supplement), abstract 265.1.

Kvidera, S. K., Horst, E. A., Sanz Fernandez, M. V., Abuajamieh, M., Ganesan, S., Gorden, P. J., … Baumgard, L. H. (2017). Characterizing effects of feed restriction and glucagon-like peptide 2 administration on biomarkers of inflammation and intestinal morphology. *Journal of Dairy Science*, *100*(11), 9402–9417. https://doi.org/10.3168/jds.2017-13229

Lakowicz, J. R. (2006). *Principles of Fluorescence Spectroscopy*, 3rd. ed. New York, NY: Springer. https://doi.org/10.1007/978-0-387-46312-4

Lamb-Rosteski, J. M., Kalischuk, L. D., Inglis, G. D., & Buret, A. G. (2008). Epidermal growth factor inhibits *Campylobacter jejuni*-induced claudin-4 disruption, loss of epithelial barrier function, and *Escherichia coli* translocation. *Infection and Immunity*, *76*(8), 3390–3398. https://doi.org/10.1128/IAI.01698-07

Larsen, M., Relling, A. E., Reynolds, C. K., & Kristensen, N. B. (2010). Effect of abomasal glucose infusion on plasma concentrations of gut peptides in periparturient dairy cows. *Journal of Dairy Science*, *93*(12), 5729–5736. https://doi.org/10.3168/jds.2010-3258

Liu, J. H., Xu, T. T., Liu, Y. J., Zhu, W. Y., & Mao, S. Y. (2013). A high-grain diet causes massive disruption of ruminal epithelial tight junctions in goats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *305*(3), R232–R241. https://doi.org/10.1152/ajpregu.00068.2013

Lu, Z., Kim, D. H., Fan, J., Lu, Q., Verbanac, K., Ding, L., … Chen, Y. H. (2015). A non-tight junction function of claudin-7-Interaction with integrin signaling in suppressing lung cancer cell proliferation and detachment. *Molecular Cancer*, *14*, 120. https://doi.org/10.1186/s12943-015-0387-0

Marti, S., Perez, M., Aris, A., Bach, A., & Devant, M. (2014). Effect of dietary energy density and meal size on growth performance, eating pattern, and carcass and meat quality in Holstein steers fed high-concentrate diets. *Journal of Animal Science*, *92*(8), 3515–3525. https://doi.org/10.2527/jas.2014-7832

McCarthy, J. P., Faulkner, A., Martin, P. A., & Flint, D. J. (1992). Changes in the plasma concentration of gastric inhibitory polypeptide and other metabolites in response to feeding in sheep. *Journal of Endocrinology*, *134*(2), 235–240. https://doi.org/10.1677/joe.0.1340235

Meissner, S., Hagen, F., Deiner, C., Gunzel, D., Greco, G., Shen, Z., & Aschenbach, J. R. (2017). Key role of short-chain fatty acids in epithelial barrier failure during ruminal acidosis. *Journal of Dairy Science*, *100*(8), 6662–6675. https://doi.org/10.3168/jds.2016-12262

Nakame, K., Kaji, T., Mukai, M., Shinyama, S., & Matsufuji, H. (2016). The protective and anti-inflammatory effects of glucagon-like peptide-2 in an experimental rat model of necrotizing enterocolitis. *Peptides*, *75*, 1–7. https://doi.org/10.1016/j.peptides.2015.07.025

Ogawa, M., Kojima, T., Someya, M., Nomura, K., Takasawa, A., Murata, M., … Sawada, N. (2012). Epidermal growth factor modulates claudins and tight junctional functions in ovarian cancer cell lines. *Histochemistry and Cell Biology*, *138*(2), 323–338. https://doi.org/10.1007/s00418-012-0956-x

Okuyama, M., Fujiwara, Y., Tanigawa, T., Watababe, K., Shiba, M., Tominaga, K., … Arakawa, T. (2007). Roles of ZO-1 and epidermal growth factor in esophageal epithelial defense against acid. *Digestion*, *75*(2–3), 135–141. https://doi.org/10.1159/000106454

Onaga, T., Shimizu, Y., Hayashi, H., Tsuji, M., Endoh, D., & Okada, H. (2006). Localization and secretion of epidermal growth factor in the parotid gland and its intragastric kinetics in sheep. *Life Sciences*, *79*(17), 1616–1629. https://doi.org/10.1016/j.lfs.2006.05.016

Penner, G. B., Steele, M. A., Aschenbach, J. R., & McBride, B. W. (2011). Ruminant Nutrition Symposium: Molecular adaptation of ruminal epithelia to highly fermentable diets. *Journal of Animal Science*, *89*(4), 1108–1119. https://doi.org/10.2527/jas.2010-3378

Pezeshki, A., Muench, G. P., & Chelikani, P. K. (2012). Short communication: Expression of peptide YY, proglucagon, neuropeptide Y receptor Y2, and glucagon-like peptide-1 receptor in bovine peripheral tissues. *Journal of Dairy Science*, *95*(9), 5089–5094. https://doi.org/10.3168/jds.2011-5311

Plaizier, J. C., Krause, D. O., Gozho, G. N., & McBride, B. W. (2008). Subacute ruminal acidosis in dairy cows: The physiological causes, incidence and consequences. *The Veterinary Journal*, *176*(1), 21–31. https://doi.org/10.1016/j.tvjl.2007.12.016

Relling, A. E., & Reynolds, C. K. (2007). Plasma concentrations of gut peptides in dairy cattle increase after calving. *Journal of Dairy Science*, *90*(1), 325–330. https://doi.org/10.3168/jds.S0022-0302(07)72633-4

Sheth, P., Seth, A., Thangavel, M., Basuroy, S., & Rao, R. K. (2004). Epidermal growth factor prevents acetaldehyde-induced paracellular permeability in Caco-2 cell monolayer. *Alcoholism: Clinical and Experimental Research*, *28*(5), 797–804. https://doi.org/10.1097/01.ALC.0000125358.92335.90

Sigalet, D. L., de Heuvel, E., Wallace, L., Bulloch, E., Turner, J., Wales, P. W., … Holst, J. J. (2014). Effects of chronic glucagon-like peptide-2 therapy during weaning in neonatal pigs. *Regulatory Peptides*, *188*, 70–80. https://doi.org/10.1016/j.regpep.2013.12.006

Soler, A. P., Laughlin, K. V., & Mullin, J. M. (1993). Effects of epidermal growth factor versus phorbol ester on kidney epithelial (LLC-PK1) tight junction permeability and cell division. *Experimental Cell Research*, *207*(2), 398–406. https://doi.org/10.1006/excr.1993.1207

Steele, M. A., AlZahal, O., Hook, S. E., Croom, J., & McBride, B. W. (2009). Ruminal acidosis and the rapid onset of ruminal parakeratosis in a mature dairy cow: A case report. *Acta Veterinaria Scandinavica*, *51*, 39. https://doi.org/10.1186/1751-0147-51-39

Steele, M. A., Penner, G. B., Chaucheyras-Durand, F., & Guan, L. L. (2016). Development and physiology of the rumen and the lower gut: Targets for improving gut health. *Journal of Dairy Science*, *99*(6), 4955–4966. https://doi.org/10.3168/jds.2015-10351

Steele, M. A., Schiestel, C., AlZahal, O., Dionissopoulos, L., Laarman, A. H., Matthews, J. C., & McBride, B. W. (2015). The periparturient period is associated with structural and transcriptomic adaptations of rumen papillae in dairy cattle. *Journal of Dairy Science*, *98*(4), 2583–2595. https://doi.org/10.3168/jds.2014-8640

Stumpff, F., Georgi, M. I., Mundhenk, L., Rabbani, I., Fromm, M., Martens, H., & Günzel, D. (2011). Sheep rumen and omasum primary cultures and source epithelia: Barrier function aligns with expression of tight junction proteins. *Journal of Experimental Biology*, *214*(Pt 17), 2871–2882. https://doi.org/10.1242/jeb.055582

Suominen, A. H., Glimm, D. R., Tedesco, D., Okine, E. K., McBurney, M. I., & Kennelly, J. J. (1998). Intestinal nutrient-gene interaction: The effect of feed deprivation and refeeding on cholecystokinin and proglucagon gene expression. *Journal of Animal Science*, *76*(12), 3104–3113. https://doi.org/10.2527/1998.76123104x

Taylor-Edwards, C. C., Burrin, D. G., Matthews, J. C., McLeod, K. R., Holst, J. J., & Harmon, D. L. (2010). Expression of mRNA for proglucagon and glucagon-like peptide-2 (GLP-2) receptor in the ruminant gastrointestinal tract and the influence of energy intake. *Domestic Animal Endocrinology*, *39*(3), 181–193. https://doi.org/10.1016/j.domaniend.2010.05.002

Tran, Q. T., Kennedy, L. H., Leon Carrion, S., Bodreddigari, S., Goodwin, S. B., Sutter, C. H., & Sutter, T. R. (2012). EGFR regulation of epidermal barrier function. *Physiological Genomics*, *44*(8), 455–469. https://doi.org/10.1152/physiolgenomics.00176.2011

Uhlen, M., Oksvold, P., Fagerberg, L., Lundberg, E., Jonasson, K., Forsberg, M., … Ponten, F. (2010). Towards a knowledge-based Human Protein Atlas. *Nature Biotechnology*, *28*(12), 1248–1250. https://doi.org/10.1038/nbt1210-1248

Walker, M. P., Evock-Clover, C. M., Elsasser, T. H., & Connor, E. E. (2015). Short communication: Glucagon-like peptide-2 and coccidiosis alter tight junction gene expression in the gastrointestinal tract of dairy calves. *Journal of Dairy Science*, *98*(5), 3432–3437. https://doi.org/10.3168/jds.2014-8919

Wang, Y., Du, D., Fang, L., Yang, G., Zhang, C., Zeng, R., … Chen, Z. (2006). Tyrosine phosphorylated Par3 regulates epithelial tight junction assembly promoted by EGFR signaling. *The EMBO Journal*, *25*(21), 5058–5070. https://doi.org/10.1038/sj.emboj.7601384

Xiao, C., Puddicombe, S. M., Field, S., Haywood, J., Broughton-Head, V., Puxeddu, I., … Davies, D. E. (2011). Defective epithelial barrier function in asthma. *The Journal of Allergy and Clinical Immunology*, *128*(3), 549–556 e541–512. https://doi.org/10.1016/j.jaci.2011.05.038

Yang, H., Rao, J. N., & Wang, J. Y. (2014). Posttranscriptional Regulation of Intestinal Epithelial Tight Junction Barrier by RNA-binding Proteins and microRNAs. *Tissue Barriers*, *2*(1), e28320. https://doi.org/10.4161/tisb.28320

Yoshida, K., Kanaoka, S., Takai, T., Uezato, T., Miura, N., Kajimura, M., & Hishida, A. (2005). EGF rapidly translocates tight junction proteins from the cytoplasm to the cell-cell contact via protein kinase C activation in TMK-1 gastric cancer cells. *Experimantal Cell Research*, *309*(2), 397–409. https://doi.org/10.1016/j.yexcr.2005.06.019

Yu, C., Jia, G., Jiang, Y., Deng, Q., Chen, Z., Xu, Z., … Wang, K. (2014). Effect of glucagon-like peptide 2 on tight junction in jejunal epithelium of weaned pigs though MAPK signaling pathway. *Asian-Australas*

Journal of Animal Science, *27*(5), 733–742. https://doi.org/10.5713/ajas.2013.13540

Zapata, R. C., Salehi, R., Ambrose, D. J., & Chelikani, P. K. (2015). Effects of prepartum fat supplementation on plasma concentrations of glucagon-like peptide-1, peptide YY, adropin, insulin, and leptin in periparturient dairy cows. *Journal of Dairy Science*, *98*(10), 6876–6885. https://doi.org/10.3168/jds.2014-9283

How to cite this article: Greco G, Amasheh S, Shen Z, Lu Z, Aschenbach JR. Effects of glucagon-like peptides 1 and 2 and epidermal growth factor on the epithelial barrier of the rumen of adult sheep. *J Anim Physiol Anim Nutr.* 2019;00:1–12. https://doi.org/10.1111/jpn.13200