

Palmitoylethanolamide and cannabidiol prevent inflammation-induced hyperpermeability of the human gut in vitro and in vivo – a randomised, placebo controlled, double-blind controlled trial

Short title:

Palmitoylethanolamide and cannabidiol reduce gut permeability

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Abbreviations: Aquaporin-3 (AQP3), aquaporin-4 (AQP4), cannabidiol (CBD), foetal bovine serum (FBS), inflammatory bowel disease (IBD), lactulose:mannitol ratio (LMR), palmitoylethanolamide (PEA), protein kinase A (PKA), phosphate buffered saline (PBS), radioimmunoprecipitation assay (RIPA) , Δ^9 -tetrahydrocannabinol (THC), trans-epithelial electrical resistance (TEER), transient receptor potential vanilloid 1 (TRPV1).

Abstract

Background and aims: We aimed to examine, for the first time, the effect of cannabidiol (CBD) and palmitoylethanolamide (PEA) on the permeability of the human gastrointestinal tract *in vitro*, *ex vivo* and *in vivo*.

Methods: Flux measurements of FD10 and FD4 dextran across Caco-2 cultures treated for 24hr with IFN γ and TNF α (10ng·mL⁻¹) were measured, with or without the presence of CBD and PEA. Mechanisms were investigated using CB₁, CB₂, TRPV1, and PPAR α antagonists, and PKA, NOS, PI3K, MEK/ERK, adenylyl cyclase and PKC inhibitors. Human colonic mucosal samples collected from bowel resections were treated as above. TRPV1, PPAR α , PPAR δ , PPAR γ , CB₁, CB₂, GPR55, GPR119, and claudins -1,-2,-3,-4,-5,-7, and -8 mRNA were measured using multiplex. Aquaporin 3 and 4 were measured using ELISA. A randomised, double blind controlled-trial assessed the effect of PEA or CBD on the absorption of lactulose and mannitol in humans taking 600mg aspirin. Urinary concentrations of these sugars were measured using liquid chromatography mass spectrometry.

Results: *In vitro*, PEA and CBD decreased the inflammation-induced flux of dextrans ($p < 0.0001$), sensitive to PPAR α and CB₁ antagonism respectively. Both PEA and CBD were prevented by PKA, MEK/ERK and adenylyl cyclase inhibition ($p < 0.001$). In human mucosa, inflammation decreased claudin-5 mRNA, which was prevented by CBD ($p < 0.05$). PEA and CBD prevented an inflammation-induced fall in TRPV1 and increase in PPAR α transcription ($p < 0.0001$). *In vivo*, aspirin caused an increase in the absorption of lactulose and mannitol, which was reduced by PEA or CBD ($p < 0.001$).

Conclusion: CBD and PEA reduce permeability in the human colon. These findings have implications in disorders associated with increased gut permeability, such as inflammatory bowel disease.

Keywords: human, permeability, intestinal, cannabidiol, palmitoylethanolamide

Introduction

The gut provides a barrier between the external and internal environment. This selectively permeable barrier allows absorption of nutrients and water from gastrointestinal contents, whilst preventing the transfer of noxious material such as bacteria and lipopolysaccharide. During episodes of inflammation, the barrier becomes compromised, allowing transfer of noxious material into the systemic circulation, leading to disease states such as inflammatory bowel disease (IBD) and septic shock ¹.

The use of *cannabis sativa* for its analgesic and anti-inflammatory effects have been well described ². Interest in the psychoactive properties of *cannabis sativa* lead to the characterisation of its active major compounds Δ^9 -tetrahydrocannabinol (THC) and cannabidiol (CBD) and approximately eighty other compounds ^{3,4}. Discovery of the receptors for these ligands followed, with cloning of the CB₁ and CB₂ receptors ^{5,6}. The endogenous ligands anandamide and 2-arachidonoylglycerol which act at these receptors have been well described ⁷⁻⁹. Endocannabinoid-like compounds acting alongside these such as palmitoylethanolamide (PEA) have emerged, with affinity for receptors such as the GPR55 receptor, and the nuclear peroxisome proliferator activated receptors (PPARs) ¹⁰⁻¹². PEA is widely used as a food additive and is being assessed for clinical use in pain and inflammation ^{13,14}. Similarly, CBD is already in use the treatment of multiple sclerosis (as part of the medicine Sativex), and by itself in childhood epilepsy ^{15,16}.

We previously demonstrated that inflammation causes an increase in the permeability of fully confluent Caco-2 monolayers, measured by trans-epithelial electrical resistance (TEER)¹⁷. We identified that application of CBD or PEA rescued falls in TEER (i.e. reduces inflammation-induced increases in permeability). The effects of CBD were blocked by antagonism of the CB₁ receptor, while PEA was blocked by antagonism of the PPAR α receptor ^{17,18}. Our recent meta-analysis demonstrated that preclinical studies with both CBD and PEA show promise in animal models ¹⁹.

In light of this background, we hypothesised that CBD and PEA reduce inflammation-induced permeability in the human gut, and here examined the effects of PEA and CBD on the human colon using Caco-2 cells and *ex vivo* human colonic tissue, identifying their mechanisms of action. Based on these positive findings, we went on to examine the potential of CBD and PEA to modulate intestinal permeability in humans *in vivo* using a randomised controlled trial.

Materials & Methods

In vitro permeability studies

Caco-2 cells were purchased from European Collection of Cell Culture (Wiltshire, UK; passages 21-42). Cells were cultured in Eagle's minimum essential medium supplemented with L-glutamine, 10% foetal bovine serum (FBS) 1% penicillin/streptomycin and 1% non-essential amino acids mixture (all Sigma-Aldrich). Cells were kept at 37°C in 5% CO₂ and 95% humidity. For permeability experiments cells were seeded at 5x10⁴/ml per well in the apical compartment of 12mm diameter 3.0µm pore polyester membrane inserts within polyester 24-well plates (Corning Incorporated, ME, USA), and cultured for 14-16 days until fully confluent. Randomly assigned wells (n=8) were treated with or IFN γ (basolateral, 10 ng.ml⁻¹, Sigma-Aldrich) for 8 hours, followed by TNF α (basolateral, 10 ng.ml⁻¹, (Sigma-Aldrich)) for 16 hours to induce inflammation. PEA (basolateral, 10µM, Tocris Bioscience, Bristol UK), CBD (apical, 10µM, Tocris Bioscience, Bristol UK) or vehicle (0.01% ethanol) were added with or without inflammation at the start of the 24 hour experimental period. Drug concentrations were based upon previous experiments ¹⁷.

Confluent monolayers cultured on membrane inserts were washed three times with pre-warmed PBS. Clear, non-fluorescent media (FluoroBrite DMEM, ThermoFisher Scientific MA, USA) supplemented with 1% nonessential amino acids and 1% penicillin/streptomycin was added to both the apical (225µl) and basolateral compartment (700µl) as per manufacturer's instructions and left for 30 minutes to equilibrate. FD4 and FD10 fluorescent dextrans (FDs; 25 mg.ml⁻¹) were then added to the apical compartment. At 37°C, baseline (0hr) the fluorescence of 100-µl aliquot basolateral samples were determined (Fluoroskan Ascent FL2.5, ThermoFisher Scientific, Waltham, MA, excitation and emission wavelengths of 485 and 520 nm respectively). Fluorescence was determined at regular intervals for 36 hours.

PEA and CBD we co-applied with antagonists under inflammatory conditions: AM251 100nM (CB₁ antagonist, Tocris Bioscience, Bristol, UK), AM630 100nM (CB₂ antagonist, Tocris Bioscience, Bristol, UK), GW6471 500nm (PPAR α antagonist, Tocris Bioscience, Bristol, UK), and SB366791 500nM (transient receptor potential vanilloid 1 (TRPV1)) antagonist, Tocris Bioscience, Bristol, UK). We co-applied various inhibitors with PEA or CBD under inflammatory conditions: KT5720 (Protein kinase A (PKA) inhibitor, 1 μ M, Tocris Bioscience, Bristol, UK), L-NAME (NOS inhibitor, 10 μ M, Tocris Bioscience, Bristol, UK), PD98059 (MEK/ERK inhibitor, 10 μ M, Tocris Bioscience, Bristol, UK), SQ22536 (adenylyl cyclase inhibitor, 1 μ M, Tocris Bioscience, Bristol, UK) and G06983 (PKC inhibitor, 1 μ M, Tocris Bioscience, Bristol, UK).

mRNA and Protein expression

Caco-2 cells were cultured in 6-well plates (Corning Incorporated, ME, USA) for 14 days. Following 24 h treatments with CBD or PEA, cells were washed twice with ice-cold phosphate buffered saline (PBS), and treated with radioimmunoprecipitation assay (RIPA) buffer containing phosphatase inhibitor and protease inhibitors (Sigma-Aldrich) at 4°C for one h on a rocking platform to cause cell lysis. Cell lysates were then collected and stored at -80 °C until analysis.

Experiments on *ex vivo* human colonic tissue were performed on healthy colonic tissue taken from colon removed at elective resection for bowel cancer at Royal Derby Teaching Hospital NHS Trust, Derbyshire (n=8). After informed consent, samples of macroscopically normal colon at least 10cm proximal or distal to any bowel tumour were obtained immediately after resection within the operating theatre. Ischaemic times following pedicle clamping were not recorded. Sections of tissue 2cm x 2cm were excised and transferred on ice to the laboratory within ten minutes, in pre-chilled Eagle's minimum essential medium supplemented with 1% FBS, 1% penicillin/streptomycin and 1% non-essential amino acids mixture (Sigma-Aldrich). Mucosa with submucosa was dissected free from the underlying muscularis layer. Samples were further dissected into

~2mm x 2mm sections and placed in individual wells of 24-well polystyrene plates (Corning Incorporated, USA) at 37°C in 5% CO₂ and 95% humidity, and treated with PEA and CBD. After 24 h, the tissue was washed with ice cold PBS and stored frozen at -80 °C until homogenisation and analysis. Samples were cryohomogenised as previously described by von Zeigler²⁰. Collected homogenates were then dissolved in 100µl of RIPA buffer containing phosphatase inhibitor and protease inhibitor (Sigma-Aldrich), incubated on an oscillating thermomixer for 30 minutes at 60 °C, and then centrifuged at 10,000G for 15 minutes.

mRNA levels of PPAR γ , PPAR α , PPAR δ , GPR55, GPR119, CB₁, CB₂ and TRPV1 were determined using Quantigene Plex kit QP1013, claudin -1,-2,-3,-4,-5,-7 and-8 were determined using kit QP101 and a Magpix (MAGPX 11326002, Luminex, Texas US). mRNA levels were normalised according to levels of three housekeeping proteins β -actin, peptidyl-prolyl cis-trans isomerase B (PPIB), and hypoxanthine phosphoribosyltransferase 1 (HPRT1), which did not change in expression under the inflammatory protocol.

Membrane-bound AQP cell membrane fractions were prepared with Mem-PER Plus kit (#89842, ThermoScientific). ELISA for aquaporin-3 (AQP3) and aquaporin-4 (AQP4) were performed as per manufacturer's instructions (#LS-F13079 & #LS-F13078, Lifespan Biosciences, WA USA), and normalised by mean protein content or cell-membrane supernatants using a bicinchoninic acid assay (Sigma-Aldrich).

Randomised, placebo controlled, double-blind controlled trial

In healthy human subjects we induced a state of increased gut permeability with aspirin, measuring the urinary concentrations of sugar probes. Participants were treated with oral PEA and CBD, and the change in gut permeability measured in a randomised double-blind, controlled trial. All experiments and procedures received prior approval of the University of Nottingham Ethics Committee (approval number J16122016), this trial was not registered on a trials registry. Healthy male participants between the ages of 18

and 50 were recruited after informed consent. Participants were screened to exclude any gastrointestinal medical conditions or symptoms, regular medications or recreational drugs, heavy alcohol use, previous abdominal surgery, or personal or family history of IBD. Participants were asked to refrain from the use of any pro or prebiotics the week before the study, and refrain from alcohol or non-steroidal anti-inflammatory use or heavy exercise for three days before the study. Participants separately attended fasted at 08:00. Aspirin 600mg was administered orally with 400mls water with CBD 600mg, PEA 600mg or placebo. At 09:00 1g lactulose and 1g mannitol in 600ml water were further administered, then a baseline urine sample was collected. Urine was collected hourly for 5 hours until 14:00. A further 400ml water was administered at 12:00. Urinary samples were immediately centrifuged at 1,000G for 7 minutes at 3°C and then frozen at -80°C until analysis.

Based on previously published literature a sample population under the same experimental conditions demonstrated a urinary lactulose-mannitol ratio (LMR) of 0.81 when treated with aspirin, versus an LMR of 0.053 (SEM=0.02) ²¹. Based on $\alpha=0.05$ using a paired t-test (effect size = 1.0) it was calculated that to detect a difference in LMR of 0.02 between treatment and placebo arms with a power of 80% and α of 0.05, a sample size of 10 patients was required, should a difference exist between the treatment and placebo groups. Using the block method in a 1:1:1 ratio participants were assigned to receive CBD, PEA or placebo. Both participants and investigators were blinded to the group assignments. Participants were numbered sequentially and were assigned to the treatment groups in the order of recruitment. Code assignment was sealed within an envelope until after the samples were analysed. Participant assignment was only revealed at the end of the study.

CBD was gifted from Artelo Biosciences (CA, USA), extracted from cannabis sativa, and analysed at an independent laboratory to assure >99.65% purity. PEA was obtained from Russel Science (Nicosia, Cyprus). Placebo were manufactured and gifted by Artelo Biosciences using the base cellulose used in the preparation of CBD. Aspirin was

obtained from Aspar Pharmaceuticals (London, UK). Lactulose was obtained from TEVA (Castleford, UK). D-mannitol was obtained from Sigma Aldridge (MO, USA). All products used in the test solution were intended for human use and tested safe for oral consumption. The purity of these sugar probes were reported to be >99.0%.

Concentrations of urinary lactulose and mannitol were determined using a liquid LC-MS method. 20µl aliquots of urine were thawed and diluted to precipitate any excess salt with 980µl 90% acetonitrile to which internal standards xylitol and raffinose were premixed at 0.5µg/ml final concentration. These were vortexed, incubated at -20°C for 4 hours. Previous work demonstrated that it is necessary to monitor sucrose to ensure the assay is specific, correctly identifying lactulose, and therefore included as an analyte²². Calibration standards were made as a dilution series from 2.5 to 250µg/ml of the analytes mannitol and lactulose. The method had been previously validated by creating 6 independently prepared dilutions of 5, 50 and 500µg/ml as above²². The LC method was based on that of Kubica²³. The LC column was a Sequant ZIC-pHILIC pHILIC (150 x 4.6mm, 5µm) kept at 40°C. The mobile phases were A, acetonitrile and B, 5mM ammonium acetate, adjusted drop-wise to pH6.85 with 5mM ammonium hydroxide solution. B Samples were kept in a chilled auto-sampler and 2µl volumes were injected for analysis. The detector was a Sciex 4000 QTrap operating in -ve ion electrospray mode at with the source at 450°C with curtain, nebuliser and auxiliary gases set to 10, 40 and 20 respectively. The ion-spray voltage was -4200V. A minimum of 5 points were used for each analyte. Xylitol was used as the internal standard to normalise the lactulose signal but the raw peak area was used to calculate the mannitol concentrations. Raffinose had been included as an internal standard as it has been used in previous studies^{22,24}. A standard prepared at 50µg/ml was injected every 20 samples was used to monitor precision and accuracy. The CVs were 14.1 and 7.3 for lactulose and mannitol respectively. The precisions were 107 and 89%. Calibration lines are linear over a range greater than 2.5 to 250µg/ml with $R^2 > 0.9995$ for both analytes.

Statistical analysis

For *in vitro* and *ex vivo* experiments data are presented as mean (or mean percentage) change from baseline where indicated \pm standard error of the mean (SEM). Permeability study results were compared using two-way ANOVA. Caco-2 and human tissue group results were compared using one-way ANOVA. The calculated concentration of each urinary sugar was assessed by repeated-measures ANOVA, with probabilities of *post hoc* comparisons subject to the Bonferroni correction. The differences in excretion of lactulose and D-Mannitol over the study period were compared using two-way ANOVA. Normality was assessed using the D'Agostino & Pearson normality test. A p-value of <0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 7.01 for Windows (GraphPad Software, San Diego, USA). All authors had access to the study data and reviewed and approved the final manuscript.

Results

Dextran-transfer studies

Within Caco-2 cultures, inflammation caused an increase in the transfer of labelled FD4 and FD10 dextrans from 2 and 4 h respectively ($p < 0.0001$), until the end of the study period at 36 h (figures 1 A and B). PEA, in the presence of inflammation, reduced FD4 transfer compared to inflammation from 4 hours ($p < 0.01$, fig 2A), increasing with time, with the maximal difference seen at 12 h ($p < 0.0001$). For FD10, PEA in the presence of inflammation caused a reduction in the transfer of dextrans compared to inflammation from 4 h ($p < 0.0001$, figure 1 B) until 36 h. Antagonism of the PPAR α receptor (but not TRPV1, CB $_1$ and CB $_2$) blocked the ability of PEA to reduce transfer of FD10 dextran over the study period (figure 2 A). The effect of PEA in reducing the transfer of dextrans was also blocked by various intracellular signalling inhibitors; KT5720 (PKA inhibitor), PD98059 (MAPK inhibitor) and SQ22563 (adenylyl cyclase inhibitor, $p < 0.0001$, figure 3 C and D), but not by L-NAME (nitric oxide synthase inhibitor), LY294002 (PI3 kinase inhibitor), or G06983 (PKC inhibitor, figure 3 C and D).

CBD in the presence of inflammation caused a reduction in dextran transfer of FD4 compared to inflammation alone from 4 h ($p < 0.01$, figure 1 A). This difference became more pronounced with time, with maximal difference seen at 8 h ($p < 0.0001$). With FD10, CBD in the presence of inflammation caused a reduction in the transfer of dextran from 4 h ($p < 0.0001$, figure 1 B) until 36 hours. Repeating in the presence of various antagonists revealed that only antagonism of the CB $_1$ receptor blocked the effects of CBD (figure 2 B). As with PEA, the effect of CBD on the transfer of dextrans was blocked by the presence of KT5720, PD98059 and SQ22563 but not L-NAME, LY294002 or G06983 ($p < 0.0001$, figure 3 A and B). When applied together PEA and CBD had no added effect on permeability in the presence of inflammation (figure 1 C).

Effects of CBD and PEA on claudin and cannabinoid receptor expression

Compared to vehicle, inflammation, PEA or CBD did not change the mRNA levels of claudins -1, -2, -3, -4, -5, 7, or -8 in Caco-2 cells (figures 4 A, 4 C and supplementary

figure 1 A-E). Inflammation, CBD or PEA treatment did not affect the mRNA of claudins -1, -2, -4, -7, or -8 in human colonic tissue (supplementary figure 1 F-J).

In human colonic tissue, PEA decreased claudin-3 mRNA levels both in the presence of inflammation and alone ($p < 0.01$, figure 4 B). Inflammation decreased claudin-5 mRNA levels compared to control tissue ($p < 0.05$ figure 4 D), and this was prevented by treatment with CBD ($p < 0.05$) but not PEA. PEA treatment alone also decreased claudin-5 mRNA levels ($p < 0.05$ figure 4 D).

In Caco-2 cells, inflammation decreased TRPV1 mRNA levels (figure 5 A), which was prevented by PEA and CBD. In human colonic tissue, inflammation caused a fall in the mRNA of TRPV1, prevented by CBD ($p < 0.05$, figure 5 B). Inflammation increased PPAR α mRNA, but not by treatment with PEA or CBD ($p < 0.05$, figure 5 D).

Inflammation did not affect mRNA levels of PPAR δ , PPAR γ , PPAR α , GPR55, GPR119, CB $_1$ or CB $_2$ in Caco-2 cells or human colonic tissue (figure 5 C and supplementary figure 2 A-L).

Effects of CBD and PEA on membrane-bound AQP channels

In Caco-2 cultures IFN γ and TNF α increased the presence of AQP3 (figure 6 A), and this was prevented by PEA (apical, $p < 0.05$), or CBD (apical, $p < 0.05$). PEA or CBD alone did not affect AQP3 compared to control. In human colonic tissue, inflammation did not change AQP3, though AQP3 levels were increased in the presence of inflammation and PEA, and also inflammation and CBD ($p < 0.01$ and < 0.001 respectively, figure 6 C). PEA or CBD alone did not affect the levels of AQP3 in human mucosal tissue compared to vehicle.

In Caco-2 cells the inflammatory protocol did not affect the presence of AQP4, though treatment with CBD or PEA alone did cause an increase in the presence of AQP4 ($p < 0.05$, figure 6 B). IFN γ and TNF α increased the presence of AQP4 in human colonic mucosa ($p < 0.001$), which was not affected by treatment with PEA or CBD, however

treatment of colonic mucosa with PEA or CBD alone decreased the presence of these proteins compared to vehicle ($p < 0.01$ and < 0.05 respectively, figure 6 D).

Absorption and excretion of urinary sugars *in vivo*

30 male participants aged between 22 and 51 years (median age 28.7) successfully completed the study. No exclusions were made on the grounds of fitness. No participants reported any side effects in experimental sessions. Urinary concentrations of mannitol excreted over the 6-hour study period were normally distributed.

In participants receiving placebo only, aspirin administration caused an increase in the urinary concentration of mannitol and lactulose over the 6-hour study period ($p < 0.0001$ and $P < 0.001$ respectively, table 1). Maximal increases in LMR were found at 2 hours following aspirin and placebo administration ($p < 0.0001$ compared to baseline).

In participants administered both CBD and aspirin, urinary lactulose and mannitol concentrations were also increased across the study period ($p < 0.001$ and $p < 0.01$ respectively, table 1). LMR across the experimental period was also increased ($p < 0.001$ compared to baseline), however compared to the placebo and aspirin group LMR was reduced reaching maximal difference at 2 hours post-administration ($p < 0.0001$, figure 7).

In the PEA and aspirin group, urinary lactulose, but not mannitol concentrations were also increased ($p < 0.01$, table 1). In 6 patients in this group mannitol levels were undetectable at baseline and subsequent timepoints, and hence LMR was unable to be calculated. These patients were excluded from the results. LMR across the study period was increased ($p < 0.01$ compared to baseline). Compared to the placebo and aspirin group LMR was reduced, reaching maximal difference at 3 hours post-administration ($p < 0.001$, figure 7). There was no difference in significance between PEA and CBD in reduction of LMR compared to placebo.

Discussion

The aims of this study were to assess the effect of PEA and CBD on the permeability of the human gastrointestinal tract and identify underlying mechanisms of action. *In vitro* and *ex vivo*, PEA and CBD prevented inflammation-induced permeability, and these effects were mediated by different receptors but similar intracellular pathways, and associated with changes in claudin, AQP and receptor expression. We then measured the excretion of sugar probes *in vivo* using an aspirin-induced pro-inflammatory model, with or without 600mg PEA or 600mg CBD, in healthy volunteers. Aspirin increased the LMR suggesting an increase in intestinal permeability, and this was prevented by both CBD and PEA. Together, these findings suggest that CBD and PEA decrease intestinal permeability, and may have future therapeutic use in IBD.

We chose to examine the effect of CBD and PEA on the transfer of FD4 and FD10 dextrans as these molecules are of a similar size to that of lipopolysaccharide from *Escherichia Coli* and *pseudomonas* species (20-40Å) ^{25,26}. PEA and CBD both reduced FD4 and FD10 transfer across Caco-2 membranes after an inflammatory protocol, blocked by PPAR α and CB₁ antagonism respectively, in line with previous reports ^{18,27}. We hypothesized that there may be additive effects if applied simultaneously, but found this not to be the case. Looking at the intracellular mechanisms of action, we found that inhibition of PKA, ERK/MEK and adenylyl cyclase prevented the actions of both drugs, suggesting that, although the membrane receptors of CBD and PEA are different, they exert their actions through similar intracellular pathways and hence demonstrate no additive effects. This is the first report of the signalling pathways through which PEA acts in human colonic mucosa, and is in line with those explored in murine colitis models where PEA in acts through phosphorylation of ERK ²⁸, and in neuronal tissue where it acts through PKA ²⁹. Similarly, our findings match previously described CBD actions at CB₁ through adenylyl cyclase ⁵.

We hypothesized that permeability changes caused by inflammation may be due to transcriptional changes in tight junction (TJ) proteins such as claudin, as changes in the

presence of these paracellular proteins have been shown in active Crohn's disease ³⁰. TJs are composed of two transmembrane proteins, occludin and claudin, with a third adjacent protein, the junctional adhesion molecule, within the inter-cellular space. These proteins are fused to identical molecules on neighbouring epithelial cells at which point the intercellular space is sealed around a charged pore. The flux of material through this paracellular pore is determined by TJ structure, dependent on claudin type ³¹. Claudin-2 increases the permeability of the TJ, whereas claudin-5 and -8 decrease permeability ³². We found inflammation had no effect on mRNA for claudin proteins in Caco-2 cultures, and this was uninfluenced by PEA or CBD treatment. We compared these findings with experimentally inflamed human colonic tissue, finding a decrease claudin-5 mRNA in response to inflammation and that this change was prevented by treatment with CBD. Claudin-5 is highly expressed in the human colon, and acts to strengthen the mucosal barrier by decreasing permeability through cysteine residues ³³. Inflammation is known to cause a decrease in the presence of claudin-5 and promotes increased permeability across mucosal types, hence this may be a mechanism by which CBD affects permeability ^{30,34}. We also found PEA decreased the transcription of claudin-3, a protein providing a barrier function by decreasing permeability to charged ions in the healthy colon ³⁵. The finding that PEA may decrease this transcription in human colon, yet decreasing hyperpermeability in Caco-2 cultures suggests that either the role of claudin-3 in the gut is incompletely understood, or that PEA does not simply affect permeability in terms of pore formation. In support of our findings, Zeissig and colleagues (2007) found that claudin-3 expression is unaffected by Crohn's disease ³⁰, however in cell culture experiments, expression is reduced by TNF α treatment ³⁶, but not by IL-13 ³⁵. As no other reports have examined the effect of cannabinoids on claudin expression in human tissue, we are unable to determine if the effect of PEA on claudin-3 is a mechanism by which permeability may be affected, and this requires further study. AQP_s have been found to have an increasingly important role in both permeability to water and the immune response, therefore we hypothesized that changes in the

expression of two AQPs, AQP3 and AQP4 may be a mechanism by which cannabinoids affect permeability. These proteins allow transport of water and solute through epithelial barriers via a transcellular route. Similarly to TJs, epithelial AQP populations may change dynamically in response to varying physiological environments³⁷. AQP3 expression in the ileum is reduced in IBD, which was suggested as a mechanism to reduce oxidative stress through limiting water loss³⁸, although it has been shown that knockdown of AQP3 paradoxically impairs gut barrier function and increases permeability³⁹. We found that in Caco-2 cells, inflammation increased levels of membrane-bound AQP3, which was prevented both by PEA and CBD. Conversely, in human tissue, the inflammatory protocol alone had no effect on AQP3 levels, whereas in the presence of the inflammatory protocol and PEA or CBD, AQP3 levels were increased. Previous work demonstrated that PEA and CBD are not anti-inflammatory in Caco-2 cultures, but do prevent the increased secretion of pro-inflammatory cytokines in human colonic tissue⁴⁰. A potential mechanism therefore for PEA and CBD on the inflammatory response may be through upregulation of functional membrane-bound AQP3 and glycerol uptake, although this may not be the direct mechanism through which permeability is affected in Caco-2 cultures⁴¹. Further study is required examining the effect of CBD and PEA on glycerol uptake in conjunction with AQP3 expression in the human colon.

AQP4 is known not to contribute to water transfer in the gut, as knock-down of the channel does not affect permeability⁴², however there is evidence suggesting AQP4 plays a role in the immune response of the colonic mucosa as AQP4 is upregulated in colonic mucosa of IL-10 knock out mice⁴³. In Caco-2 cells we found no change in membrane bound AQP4 expression in response to inflammation, but up regulation in expression caused by PEA and CBD treatment. In comparison with experimentally inflamed human colonic tissue, inflammation increased the presence of AQP4, which was not affected by PEA or CBD, although in the absence of inflammation these levels were reduced compared to vehicle alone. The absence of an immune cell-mediated response to inflammation in Caco-2 cells may explain the difference between the two culture

models' response to TNF α and IFN γ . As AQP4 expression was reduced in human tissue compared to vehicle, it may be possible that prophylactic administration of CBD and PEA may change the response to inflammation. We were unable to examine the effect of PEA and CBD on other AQP subtypes in this study, however this does pose a future avenue of experimentation for cannabinoid and cannabinoid-like compounds.

It has been previously demonstrated that the expression of CB₁ and CB₂ receptors on the gut epithelium, immune cells and enteric nervous system change with inflammation^{44,45}. As PEA has been shown to alter the expression of these receptors in mice, we hypothesized that PEA and CBD might affect their expression (and of other molecular targets of cannabinoids) in experimentally inflamed human colon^{11,28}. Surprisingly we did not find any effect of PEA or CBD on the expression of these two receptors both in Caco-2 and human explant models. However, in both Caco-2 cultures and human colon we found a significant decrease in the expression of TRPV1 in response to inflammation, in line with previous reports¹¹. In both cell culture and explant tissue models CBD prevented these falls, whereas PEA only prevented falls in TRPV1 expression in Caco-2 cultures. We also found that PPAR α expression was increased by inflammation, but this was not affected by PEA or CBD treatment. No other receptors were affected by inflammation. The absence of change in CB₁ or CB₂ transcription is interesting, and contradicts existing evidence that CB₁ and CB₂ both are upregulated in biopsies from IBD patients⁴⁵. One possible explanation for this difference is the role of the enteric nervous system. Peripherally restricted cannabinoid agonists have been shown not to prevent inflammatory changes in murine colitis, suggesting that cannabinoid action at the central nervous system is crucial to their effect on gut inflammation⁴⁶. This was supported by a study from Esposito et al., demonstrating that PEA may act directly on enteric glial cells rather than on mucosal immunocytes²⁸. We may suggest that our normal tissue, which is no longer innervated by the enteric nervous system, did not therefore undergo any nerve-mediated changes in receptor expression. Alternatively, these differences could also be explained by the presence of a secondary immune

response in *ex vivo* tissue, compared to *in vitro* Caco-2 cultures, possessing no specialised immune response, such as macrophages. This presents further evidence that the gut is dependent centrally on endocannabinoid tone for the immune response and barrier function.

Clinically, permeability *in vivo* is not measured, however experimentally, it may be estimated by the ingestion of probe molecules which undergo urinary excretion.

Administering two sugars which are absorbed and excreted at differential rates avoids confounding factors such as delayed gastric emptying, differing volumes of distribution, intestinal transit time and varying renal clearance⁴⁷. D-mannitol is passively absorbed by the small intestine at a steady rate in health. Lactulose, a larger molecule, is not normally absorbed in health, but is passively absorbed during inflammatory intestinal episodes⁴⁸. As both compounds undergo similar degradation in the gut, and regular excretion, the urinary lactulose to mannitol ratio (LMR) gives a measure of intestinal permeability. This ratio has previously been used to calculate intestinal permeability in Crohn's disease, where lactulose absorption proportionally increases to the absorption of D-mannitol⁴⁹. We have shown that aspirin causes an approximate 20-fold increase in LMR, in line with previously published reports⁴⁸. It has been hypothesized that the mechanism by which increased permeability to sugars occurs is through inhibition of cyclooxygenase production, and therefore a decrease in mucosal prostaglandin production⁵⁰. We found that PEA and CBD both decreased the concentrations of these sugars, and therefore may have achieved this through inhibiting cyclooxygenase production or by manipulation of membrane-bound proteins or receptors. Although we have not proven the mechanism in this study, we have shown for the first time in humans, that these compounds can reduce intestinal permeability *in vivo* and may potentially be of clinical use in IBD.

There are several limitations to this study. Although high dose aspirin was used to increase in the absorption and excretion of ingested probes, this may not be a reliable simulation of gut inflammation. Therefore, the effects of PEA and CBD may not translate clinically.

Alternative models have been used to induce a state of hyperpermeability which may resemble clinical disease more closely. One study administered LPS to healthy human subjects, successfully increasing the absorption of orally administered polyethylene glycol⁵¹. However, all 14 participants developed the systemic inflammatory response syndrome, becoming haemodynamically unstable and requiring medical care. In light of these risks, small phase 2 clinical trials examining the effect of CBD and PEA in IBD may now be considered. Secondly, 6 of 10 samples in the PEA cohort were found to contain mannitol levels which were below the level of quantification using our LC-MS method. Therefore, ratios of lactulose to mannitol were not possible to calculate for these 6 participants and were excluded, which may exaggerate group differences. It is not clear if mannitol levels were undetectable because of PEA administration or due to an error in the administration of sugar probes or quantification of sugars at LCMS. If this were due to a PEA effect this would have implications for the clinical use of PEA, as this would mean that it is highly permeability-decreasing. This will be the subject of further study in our research group.

In conclusion, we have demonstrated for the first time in humans that PEA and CBD prevent increases in permeability in the inflamed gut, and may do so through changes in AQP, TJ and receptor expression. These data add the growing body of data demonstrating the anti-inflammatory and permeability-reducing effects of PEA and CBD in the gastrointestinal tract¹⁹. This holds significant promise for the development of future intestinal therapies treating disorders of increased intestinal permeability such as IBD. Their clinical effects should now be assessed in phase 1 and phase 2 clinical trials.

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References

1. Goldenberg NM, Steinberg BE, Slutsky AS, et al. Broken barriers: a new take on sepsis pathogenesis. *Sci Transl Med* 2011;3:88ps25.
2. Izzo a a, Camilleri M. Emerging role of cannabinoids in gastrointestinal and liver diseases: basic and clinical aspects. *Gut* 2008;57:1140–1155.
3. Turner CE, Elsohly MA, Boeren EG. Constituents of *Cannabis sativa* L. XVII. A review of the natural constituents. *J Nat Prod* 43:169–234.
4. Mechoulam R, Hanus L. A historical overview of chemical research on cannabinoids. *Chem Phys Lipids* 2000;108:1–13.
5. Matsuda L a, Lolait SJ, Brownstein MJ, et al. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 1990;346:561–564.
6. Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 1993;365:61–5.
7. Devane WA, Hanus L, Breuer A, et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 1992;258:1946–9.
8. Mechoulam R, Ben-Shabat S, Hanus L, et al. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol* 1995;50:83–90.
9. Sugiura T, Kondo S, Sukagawa A, et al. 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun* 1995;215:89–97.
10. Porter AC, Sauer J-M, Knierman MD, et al. Characterization of a novel endocannabinoid, virodhamine, with antagonist activity at the CB1 receptor. *J Pharmacol Exp Ther* 2002;301:1020–4.

11. Borrelli F, Romano B, Petrosino S, et al. Palmitoylethanolamide, a naturally occurring lipid, is an orally effective intestinal anti-inflammatory agent. *Br J Pharmacol* 2015;172:142–158.
12. Petrosino S, Schiano Moriello A, Cerrato S, et al. The anti-inflammatory mediator palmitoylethanolamide enhances the levels of 2-arachidonoyl-glycerol and potentiates its actions at TRPV1 cation channels. *Br J Pharmacol* 2015.
13. Giammusso B, R DM, Bernardini R. The efficacy of an association of palmitoylethanolamide and alpha-lipoic acid in patients with chronic prostatitis/chronic pelvic pain syndrome: A randomized clinical trial. *Arch Ital Urol* 2017;31:17–21.
14. Marini I, Bartolucci M, Bortolotti F, et al. Palmitoylethanolamide versus a nonsteroidal anti-inflammatory drug in the treatment of temporomandibular joint inflammatory pain. *J Orofac Pain* 2012;26:99–104.
15. Devinsky O, Cross JH, Laux L, et al. Trial of Cannabidiol for Drug-Resistant Seizures in the Dravet Syndrome. *N Engl J Med* 2017;376:2011–2020.
16. Giacoppo S, Bramanti P, Mazzone E. Sativex in the management of multiple sclerosis-related spasticity: An overview of the last decade of clinical evaluation. *Mult Scler Relat Disord* 2017;17:22–31.
17. Alhamoruni A, Wright KL, Larvin M, et al. Cannabinoids mediate opposing effects on inflammation-induced intestinal permeability. *Br J Pharmacol* 2012;165:2598–2610.
18. Karwad MA, Macpherson T, Wang B, et al. Oleoylethanolamine and palmitoylethanolamine modulate intestinal permeability in vitro via TRPV1 and PPAR α . *FASEB J* 2016:fj.201500132.
19. Couch DG, Maudslay H, Doleman B, et al. The Use of Cannabinoids in Colitis: A

- Systematic Review and Meta-Analysis. *Inflamm Bowel Dis* 2018;24.
20. Ziegler LM von, Saab BJ, Mansuy IM. A simple and fast method for tissue cryohomogenization enabling multifarious molecular extraction. *J Neurosci Methods* 2013;216:137–41.
 21. Sequeira IR, Lentle RG, Kruger MC, et al. Standardising the lactulose mannitol test of gut permeability to minimise error and promote comparability. *PLoS One* 2014;9:e99256. Available at:
 22. Gervasoni J, Schiattarella A, Giorgio V, et al. Validation of an LC-MS/MS Method for Urinary Lactulose and Mannitol Quantification: Results in Patients with Irritable Bowel Syndrome. *Dis Markers* 2016;2016:1–6.
 23. Kubica P, Kot-Wasik A, Wasik A, et al. Modern approach for determination of lactulose, mannitol and sucrose in human urine using HPLC–MS/MS for the studies of intestinal and upper digestive tract permeability. *J Chromatogr B* 2012;907:34–40.
 24. D S. 13th Congress of ECCO, Inflammatory Bowel Diseases. In: *MRI T2 relaxometry-based measures of intestinal inflammation in a human intestinal permeability model: a pilot study.*; 2018.
 25. Qiao S, Luo Q, Zhao Y, et al. Structural basis for lipopolysaccharide insertion in the bacterial outer membrane. *Nature* 2014;511:108–111.
 26. Mangoni ML, Epand RF, Rosenfeld Y, et al. Lipopolysaccharide, a key molecule involved in the synergism between temporins in inhibiting bacterial growth and in endotoxin neutralization. *J Biol Chem* 2008;283:22907–17.
 27. Alhamoruni A, Lee C, Wright KL, et al. Pharmacological effects of cannabinoids on the Caco-2 cell culture model of intestinal permeability. *J Pharmacol Exp Ther* 2010;335:92–102.

28. Esposito G, Capoccia E, Turco F, et al. Palmitoylethanolamide improves colon inflammation through an enteric glia/toll like receptor 4-dependent PPAR- α activation. *Gut* 2014;1300–1312.
29. Lin TY, Lu CW, Wu CC, et al. Palmitoylethanolamide Inhibits Glutamate Release in Rat Cerebrocortical Nerve Terminals. *Int J Mol Sci* 2015;16:5555–5571.
30. Zeissig S, Bürgel N, Günzel D, et al. Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. *Gut* 2007;56:61–72.
31. Weber CR. Dynamic properties of the tight junction barrier. *Ann N Y Acad Sci* 2012;1257:77–84. A
32. Luettig J, Rosenthal R, Barmeyer C, et al. Claudin-2 as a mediator of leaky gut barrier during intestinal inflammation. *Tissue barriers* 2015;3:e977176.
33. Wen H, Watry DD, Marcondes MCG, et al. Selective decrease in paracellular conductance of tight junctions: role of the first extracellular domain of claudin-5. *Mol Cell Biol* 2004;24:8408–17.
34. Kanda T, Numata Y, Mizusawa H. Chronic inflammatory demyelinating polyneuropathy: decreased claudin-5 and relocated ZO-1. *J Neurol Neurosurg Psychiatry* 2004;75:765–769.
35. Prasad S, Mingrino R, Kaukinen K, et al. Inflammatory processes have differential effects on claudins 2, 3 and 4 in colonic epithelial cells. *Lab Invest* 2005;85:1139–62.
36. Mei M, Xiang R-L, Cong X, et al. Claudin-3 is required for modulation of paracellular permeability by TNF- α through ERK1/2/slug signaling axis in submandibular gland. *Cell Signal* 2015;27:1915–1927.
37. Zahn A, Moehle C, Langmann T, et al. Aquaporin-8 expression is reduced in ileum

- and induced in colon of patients with ulcerative colitis. *World J Gastroenterol* 2007;13:1687–95.
38. Ricanek P, Lunde LK, Frye SA, et al. Reduced expression of aquaporins in human intestinal mucosa in early stage inflammatory bowel disease. *Clin Exp Gastroenterol* 2015;8:49–67.
 39. Zhang W, Xu Y, Chen Z, et al. Knockdown of aquaporin 3 is involved in intestinal barrier integrity impairment. *FEBS Lett* 2011;585:3113–3119.
 40. Couch DG, Tasker C, Theophilidou E, et al. Cannabidiol and palmitoylethanolamide are anti-inflammatory in the acutely inflamed human colon. *Clin Sci* 2017;131:2611–2626.
 41. Li Z, Li B, Zhang L, et al. The proliferation impairment induced by AQP3 deficiency is the result of glycerol uptake and metabolism inhibition in gastric cancer cells. *Tumor Biol* 2016;37:9169–9179.
 42. Ma T, Yang B, Gillespie A, et al. Generation and phenotype of a transgenic knockout mouse lacking the mercurial-insensitive water channel aquaporin-4. *J Clin Invest* 1997;100:957–62.
 43. Hansen JJ, Holt L, Sartor BR. Gene expression patterns in experimental colitis in IL-10-deficient mice. *Inflamm Bowel Dis* 2009;15:890–899.
 44. Izzo a a, Fezza F, Capasso R, et al. Cannabinoid CB1-receptor mediated regulation of gastrointestinal motility in mice in a model of intestinal inflammation. *Br J Pharmacol* 2001;134:563–570.
 45. Marquéz L, Suárez J, Iglesias M, et al. Ulcerative colitis induces changes on the expression of the endocannabinoid system in the human colonic tissue. *PLoS One* 2009;4.
 46. Cluny NL, Keenan CM, Duncan M, et al. (SAB378), a Peripherally Restricted

- Cannabinoid CB 1 / CB 2 Receptor Agonist , Inhibits Gastrointestinal Motility but Has No Effect on Experimental Colitis in Mice. 2010;334:973–980.
47. Twiss IM, Burggraaf J, Schoemaker RC, et al. The sugar absorption test in the evaluation of the gastrointestinal intolerance to bisphosphonates: studies with oral pamidronate. *Clin Pharmacol Ther* 2001;69:431–7.
 48. Sequeira IR, Lentle RG, Kruger MC, et al. Differential trafficking of saccharidic probes following aspirin in clinical tests of intestinal permeability in young healthy women. *Clin Exp Pharmacol Physiol* 2014;41:107–17.
 49. Benjamin J, Makharia GK, Ahuja V, et al. Intestinal permeability and its association with the patient and disease characteristics in Crohn’s disease. *World J Gastroenterol* 2008;14:1399–405.
 50. Bjarnason I, Macpherson A, Hollander D. Intestinal permeability: An overview. *Gastroenterology* 1995;108:1566–1581.
 51. Hietbrink F, Besselink MGH, Renooij W, et al. Systemic inflammation increases intestinal permeability during experimental endotoxaemia. *Shock* 2009;32:374–378.

Tables and figures

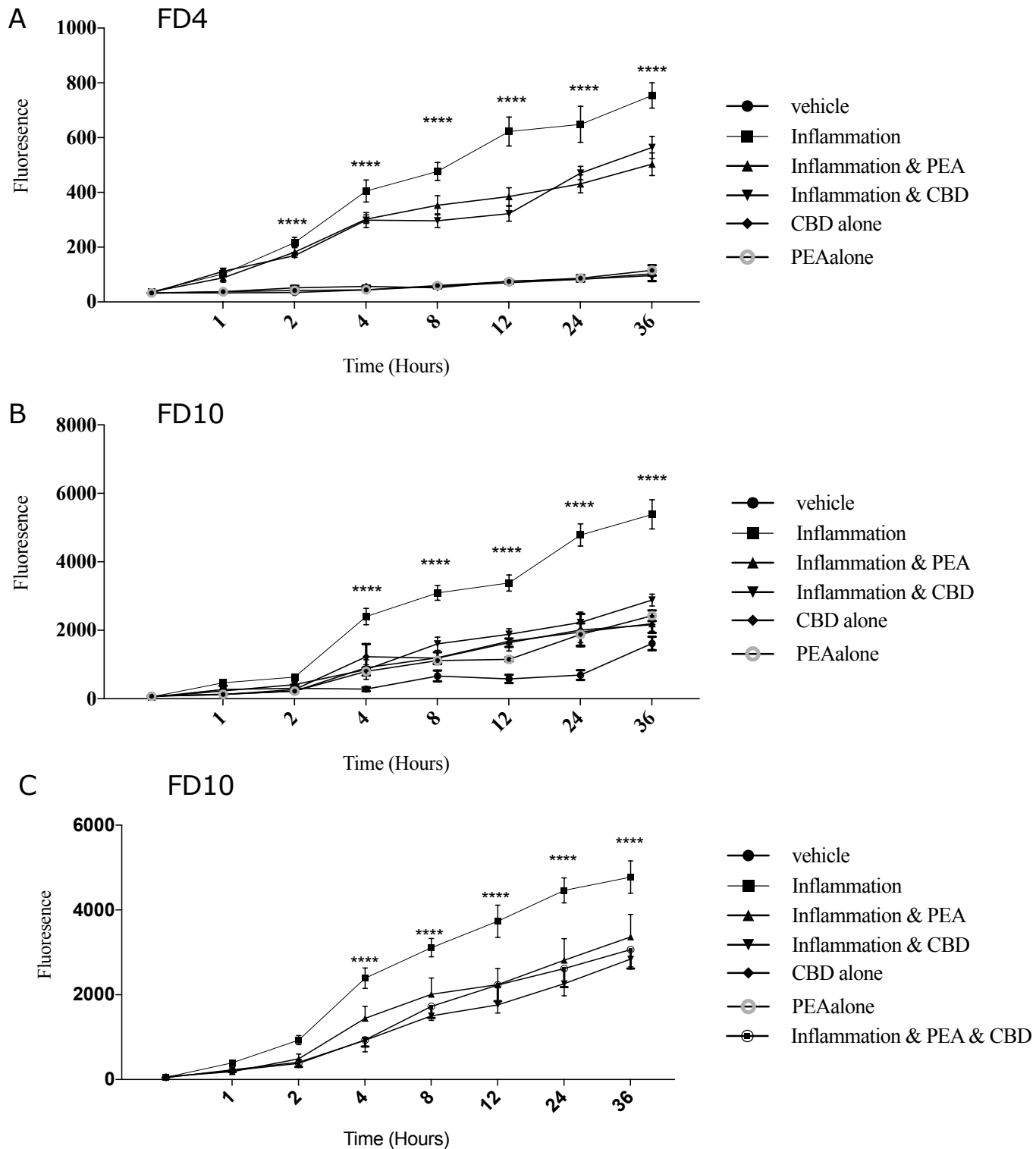


Figure 1: The Effects of PEA, applied basolaterally, and CBD, applied apically, on the permeability of Caco-2 monolayers in response to 24 hr exposure to TNF α and IFN γ measured by transfer of fluorescent dextrans (A:FD4 B:FD10 C:FD10 PEA & CBD). Raw data is expressed as the mean fluorescence per group \pm SEM. N=8 per condition. Data was analyzed by two-way ANOVA using Dunnett's post hoc test comparing against the vehicle control (**** $p < 0.0001$).

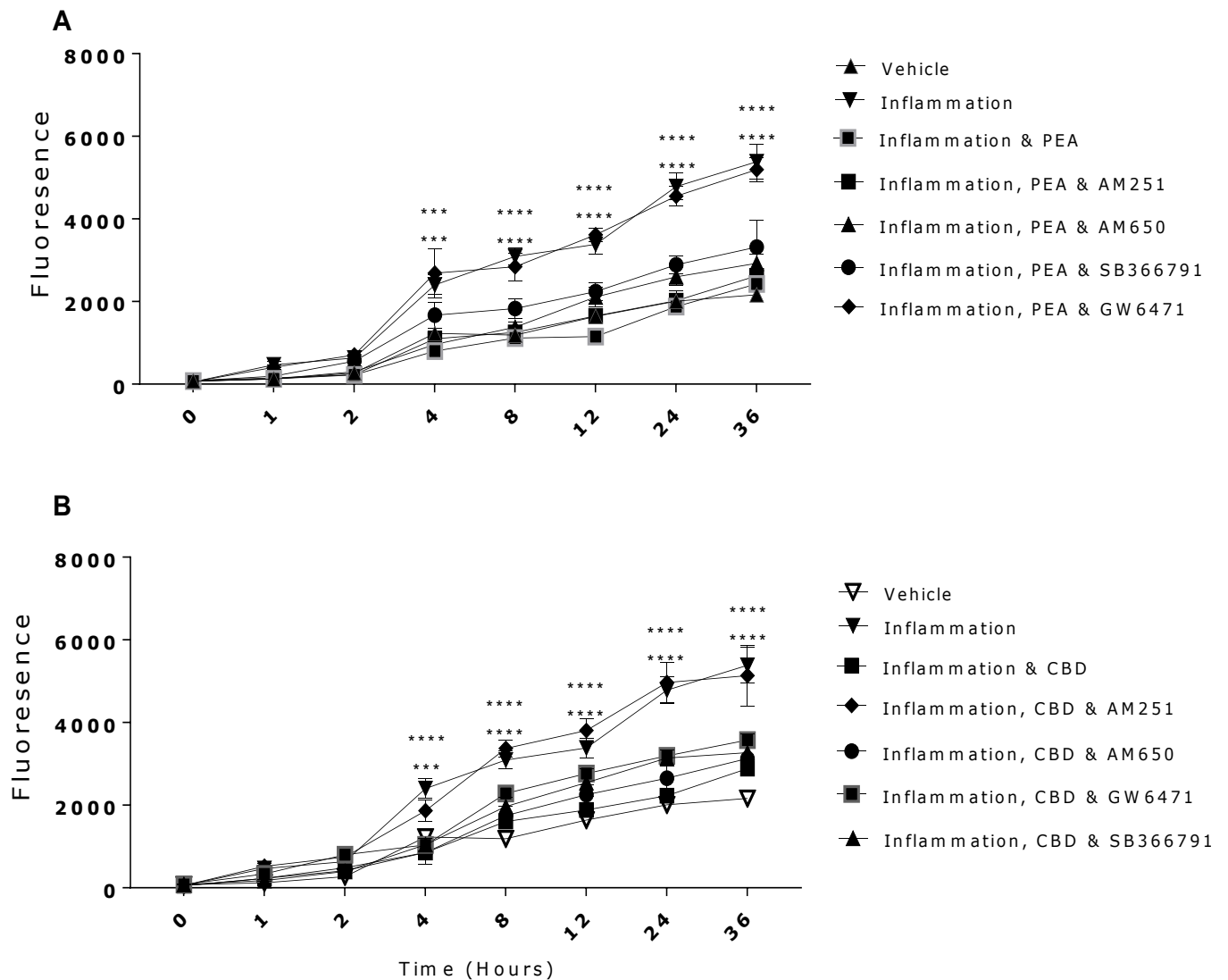


Figure 2: The effects of PEA (basolateral, A) and CBD (apical, B) on the permeability of Caco-2 monolayers in response to 24 hr exposure to TNF α and IFN γ in the presence of various receptor antagonists, measured by transfer of fluorescent dextrans (FD10). Raw data is expressed as the mean fluorescence per group \pm SEM. N=8 per condition. Data was analyzed by two-way ANOVA using Dunnett's post hoc test comparing against the vehicle control (** $p < 0.001$, **** $p < 0.0001$).

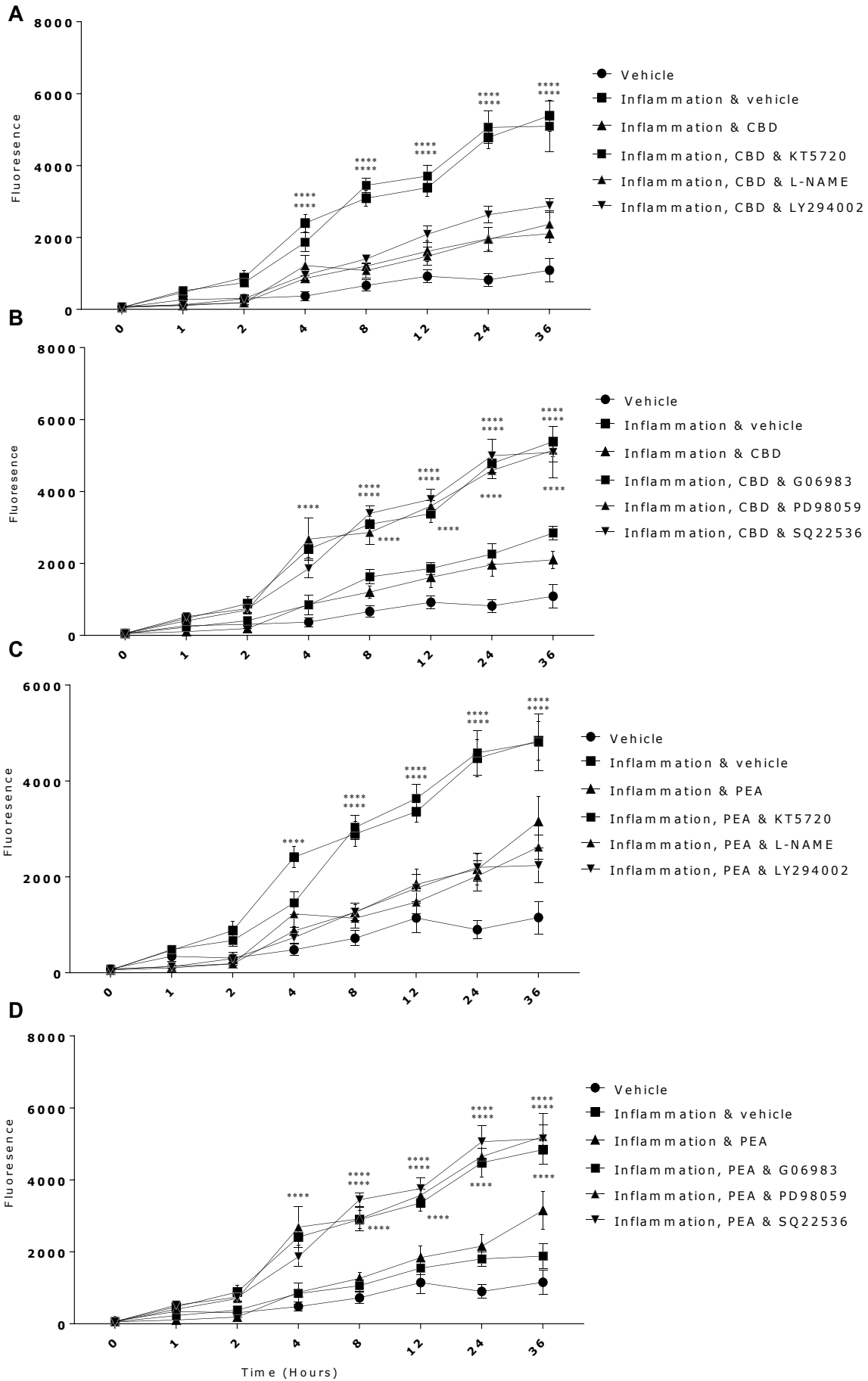


Figure 3: The effects of CBD (apical, A and C) and PEA (basolateral, B and D) on the permeability of Caco-2 monolayers in response to 24 hr exposure to TNF α and IFN γ in the presence of various protein inhibitors (KT5720, L-NAME and LY294002: A and C, and PD88059, SQ22536 and G06983: B and D), measured by transfer of fluorescent dextrans (FD10). Raw data is expressed as the mean fluorescence per group +/- SEM. N=8 per condition. Data was analyzed by two-way ANOVA using Dunnett's post hoc test comparing against the vehicle control (**** p<0.0001).

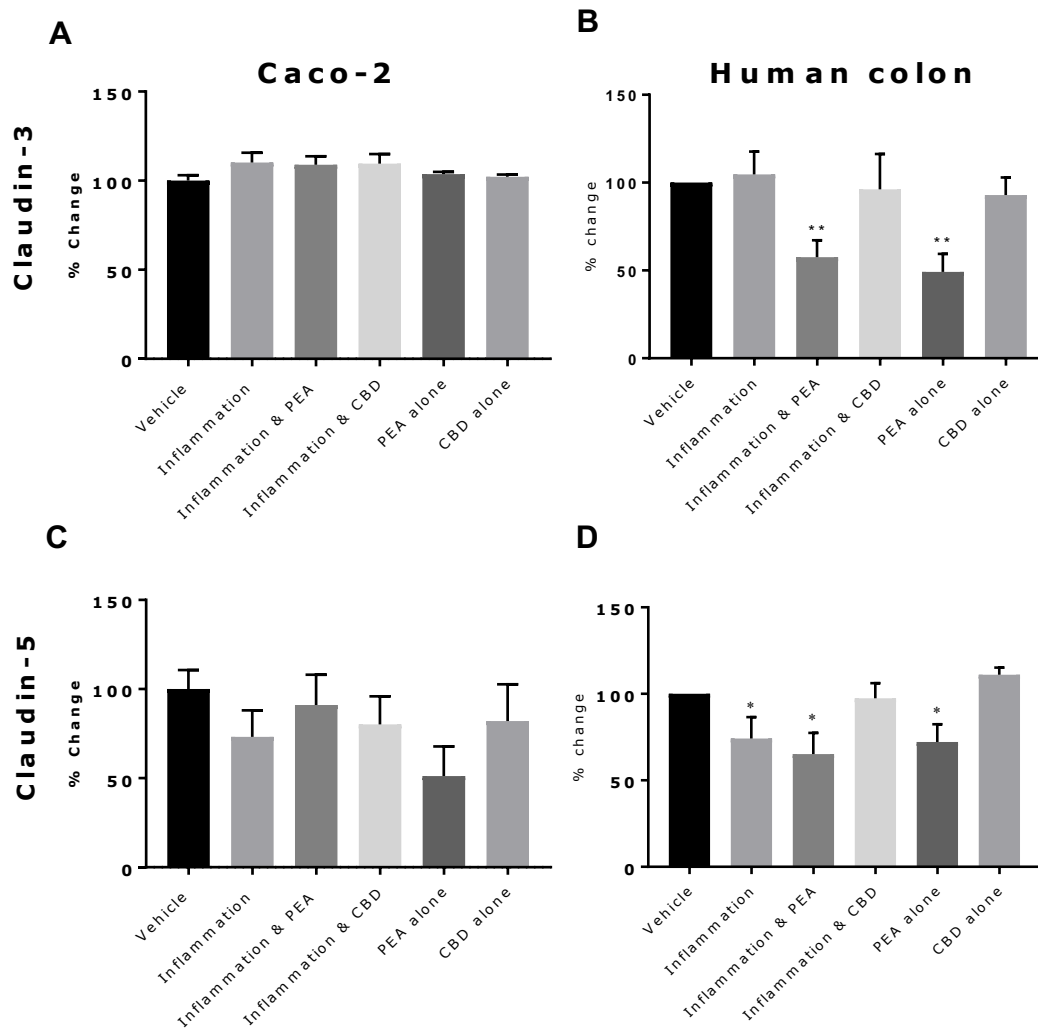


Figure 4. The effects of PEA and CBD on the expression of mRNA levels for tight junction proteins in response to a 24 hr inflammatory protocol in cultured Caco-2 cells (A=claudin 3, C= claudin 5), and human colonic tissue (B=claudin 3, D=claudin 5), measured by multiplex. Data is presented as percentage change from vehicle per plate +/- SEM, n=8 (A&C) and n=7 (B&D) per condition. Data was analysed by one-way ANOVA comparing against vehicle (* p<0.05, **p<0.01).

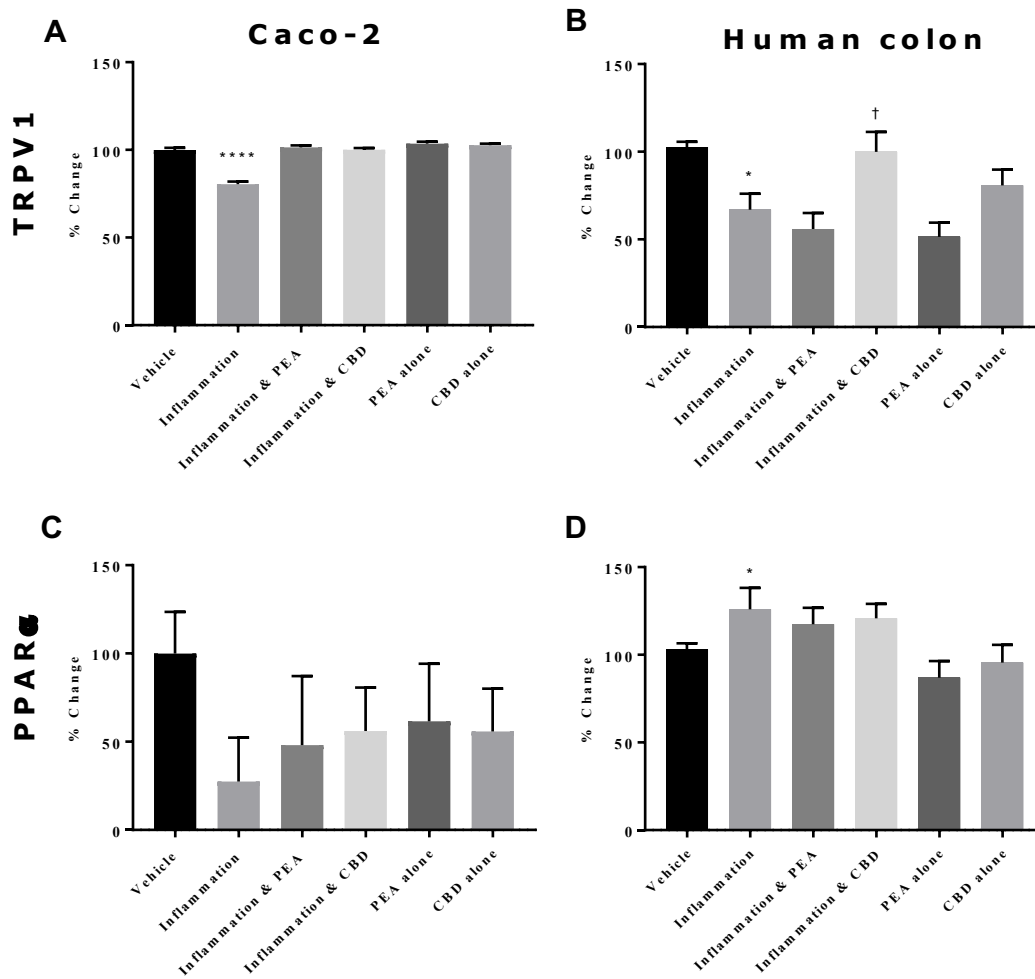


Figure 5. The effects of PEA and CBD on the expression of mRNA levels for receptor proteins in response to a 24 hr inflammatory protocol in cultured Caco-2 cells (A=TRPV1, C=PPAR α), and human colonic tissue (B=TRPV1, D=PPAR α), measured by multiplex. Data is presented as percentage change from vehicle per plate \pm SEM, $n=8$ (A&C) and $n=7$ (B&D) per condition. Data was analysed by one-way ANOVA comparing against vehicle (* $p<0.05$, **** $p<0.0001$), or against inflammation († $p<0.05$).

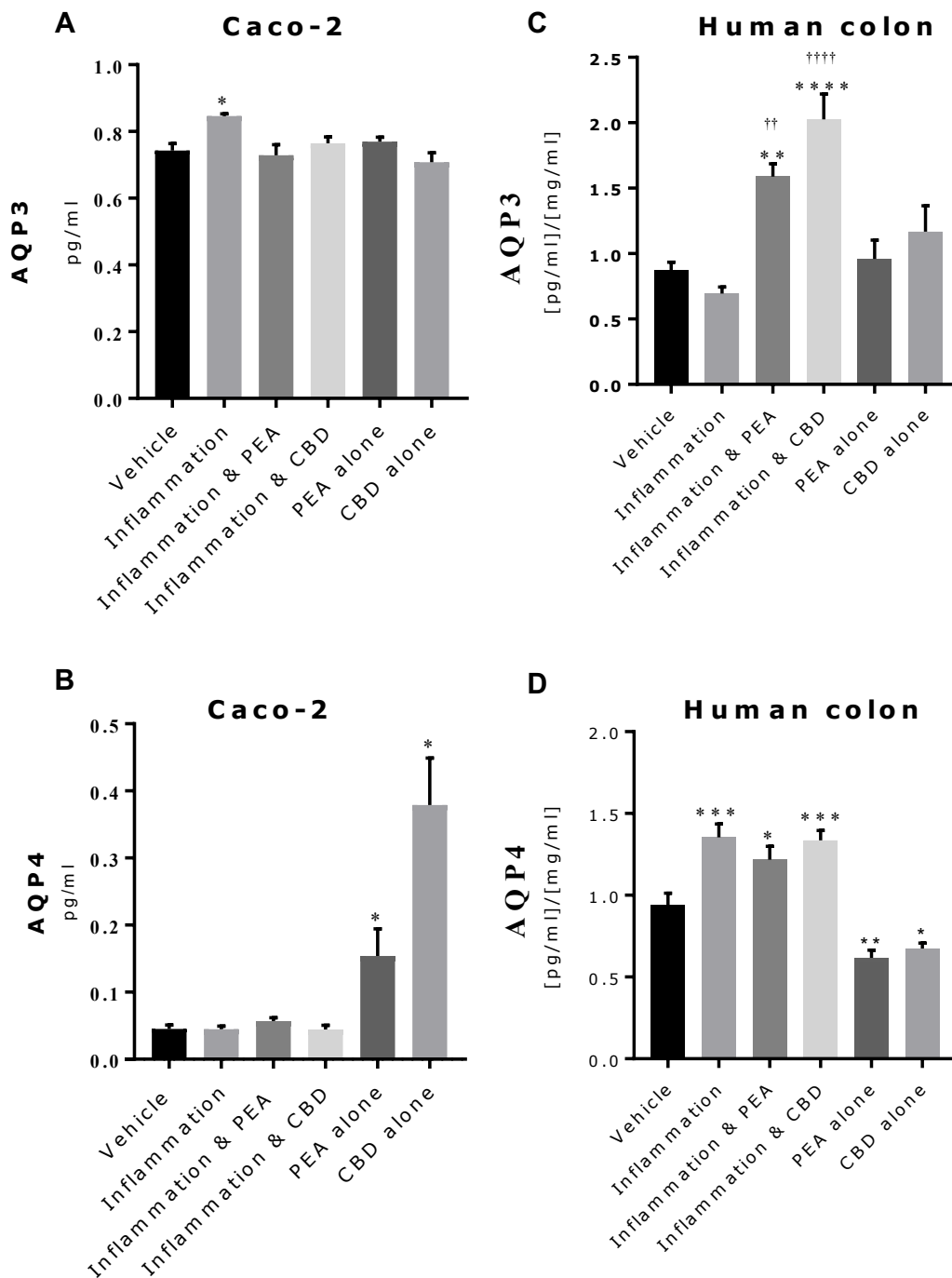


Figure 6. The effects of PEA and CBD on levels of membrane bound AQP3 in Caco-2 cultures (A) and human colonic mucosa (C) and AQP4 in Caco-2 cultures (B) and human colonic mucosa (D) in response to an inflammatory protocol, measured by ELISA. Raw data is expressed as mean protein levels per group +/- SEM. N=6-8 per condition. Data were analysed by one-way ANOVA comparing against vehicle (* p<0.05, ** p<0.01, ***p<0.001, **** p<0.0001) or inflammation (†† p<0.05, ††††p<0.0001).

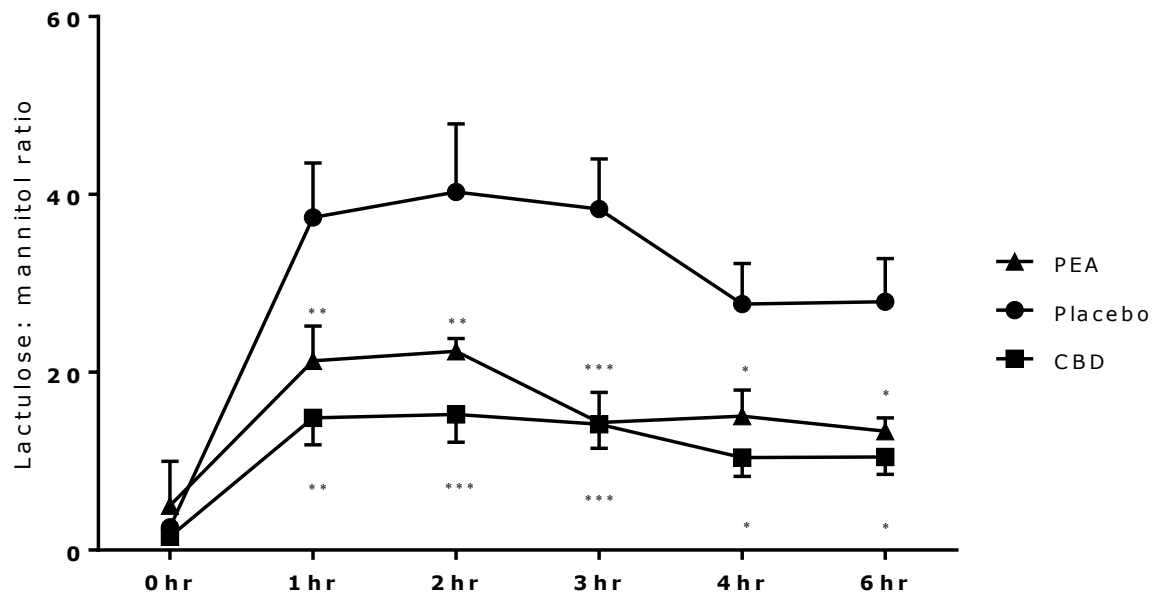
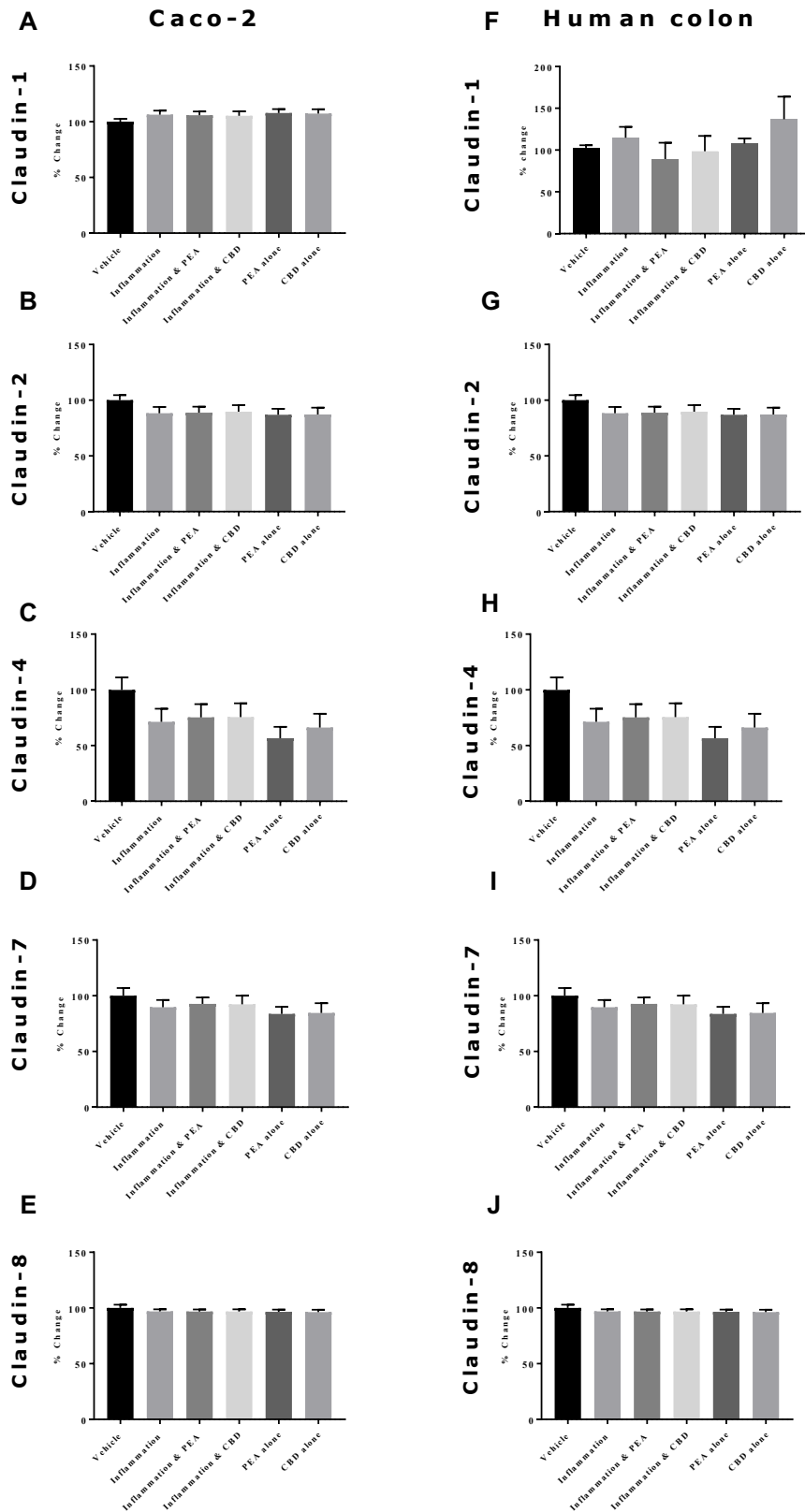


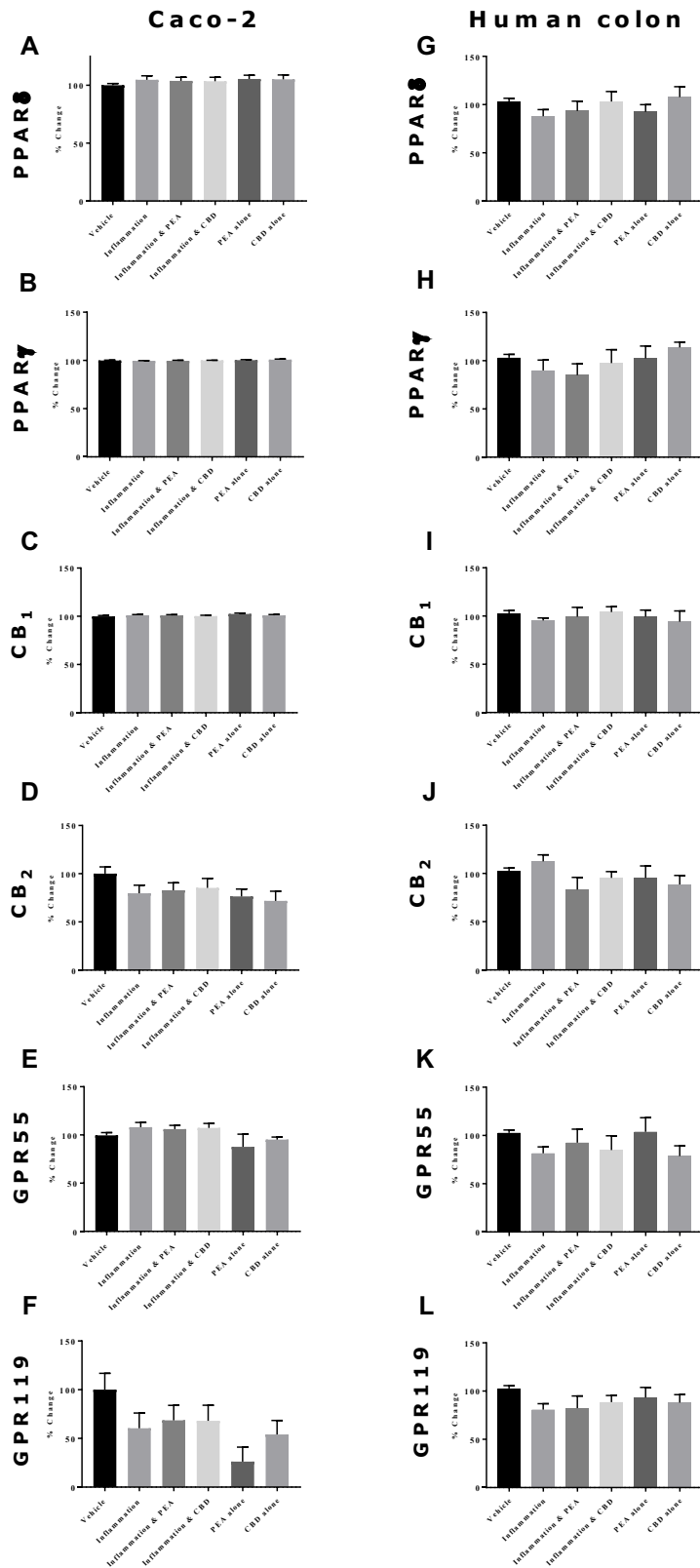
Figure 7. The concentration ratios of urinary lactulose and mannitol over time in healthy participants treated with aspirin and either placebo, CBD or PEA, measured by LC MS. Results are expressed as mean ratios \pm SEM. Time points between groups were compared using two-way ANOVA using Dunnett's multiple comparisons test comparing to placebo at the same time point (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Table 1. Urinary concentrations of lactulose and mannitol in healthy volunteers receiving aspirin and placebo (n=10), CBD (n=10) and PEA (n=4) therapy. Results are expressed as mean concentrations +/- standard deviation. Concentrations were compared to baseline using repeated measures ANOVA and Dunnett's multiple comparison test (*<0.05, **<0.01, ***<0.001 ****<0.0001). LMR – Lactulose: mannitol ratio.



Supplementary figure 1. The effects of PEA and CBD on the expression of mRNA levels for tight junction proteins in response to a 24 hr inflammatory protocol in cultured Caco-2 cells (A=claudin 1, B=claudin 2, C=claudin 4, D=claudin 7, E=claudin 9), and human colonic tissue (F=claudin 1, G=claudin 2, H=claudin 4, I=claudin 7, J=claudin 8),

measured by multiplex. Data is presented as percentage change from vehicle per plate \pm SEM, $n=8$ (A-E) and $n=7$ (F-J) per condition. Data was analysed by one-way ANOVA comparing against vehicle.



Supplementary figure 2. The effects of PEA and CBD on the expression of mRNA levels for receptor in response to a 24 hr inflammatory protocol in cultured Caco-2 cells (A=PPAR δ , B=PPAR δ , C=CB₁, D=CB₂, E=GPR55, F=GPR119), and human colonic tissue (G= PPAR δ , H= PPAR δ , I= CB₁, J= CB₂, K=GPR55, L=GPR119), measured by multiplex. Data is presented as percentage change from vehicle per plate +/- SEM, n=8 (A-F) and n=7 (G-L) per condition. Data was analysed by one-way ANOVA comparing against vehicle.