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Endocannabinoids and endocannabinoid-like compounds modulate hypoxia-induced permeability in CaCo-2 cells via CB₁, TRPV1, and PPAR α

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Abstract

Background and purpose: We have previously reported that endocannabinoids modulate ~~intestinal~~ permeability in Caco-2 cells under inflammatory conditions and hypothesised in the present study that endocannabinoids could also modulate ~~intestinal~~ permeability in ischemia/reperfusion.

Experimental approach: Caco-2 cells were grown on cell culture inserts to confluence. Trans-epithelial electrical resistance (TEER) was used to measure permeability. To generate hypoxia (0% O₂), a GasPak™ EZ anaerobe pouch system was used. Endocannabinoids were applied to the apical or basolateral membrane in the presence or absence of receptor antagonists.

Key results: Complete Hypoxia decreased TEER (increased permeability) by ~35% after 4_h (recoverable) and ~50% after 6_h (non-recoverable). When applied either pre- or post-hypoxia, apical application of N-arachidonoyl-dopamine (NADA, via TRPV1), oleamide (OA, via TRPV1) and oleoylethanolamine (OEA, via TRPV1) inhibited the increase in permeability. Apical administration of anandamide (AEA) and 2-~~a~~Arachidonoylglycerol (2-AG) worsened the permeability effect of hypoxia (both via CB₁). Basolateral application of NADA (via TRPV1), OA (via CB₁ and TRPV1), noladin ether (NE, via PPAR α), and palmitoylethanolamine (PEA, via PPAR α) restored permeability after 4 h hypoxia, whereas OEA increased permeability (via PPAR α). After 6_h hypoxia, where permeability does not recover, only basolateral application PEA sustainably decreased permeability, and NE decreased permeability.

Conclusions and Implications: A variety of endocannabinoids and endocannabinoid-like compounds modulate ~~intestinal~~ Caco-2 permeability in hypoxi~~ischemia~~/reoxy~~genation~~reperfusion, which involves multiple targets, depending on whether the compounds are applied to the basolateral or apical membrane. CB₁ antagonism and TRPV1 or PPAR α agonism may represent novel therapeutic targets against several intestinal disorders associated with increased ~~intestinal~~ permeability.

Key words: intestinal permeability; Caco-2 cells; hypoxia; ischaemia; transepithelial electrical resistance (TEER); endocannabinoids; cannabinoid receptor 1 (CB₁); transient receptor potential vanilloid subtype 1 (TRPV1); peroxisome proliferator-activated receptor alpha (PPAR- α); cannabinoid receptors; N-arachidonoyl-dopamine (NADA); oleamide (OA); anandamide (AEA); 2-~~a~~Arachidonoylglycerol (2-AG); noladin ether (NE); virodhamine (VD).

ABBREVIATIONS:

2-AG	2- <i>a</i> -Arachidonoyl glycerol
AEA	anandamide
AM251	<i>N</i> -(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1 <i>H</i> -pyrazole-3-carbox-amide
AM630	6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1 <i>H</i> -indol-3-yl](4-methoxy-phenyl) methanone
ANOVA	analysis of variance
CB ₁	cannabinoid receptor one
CB ₂	cannabinoid receptor two
<u>CGRP</u>	<u>Calcitonin gene-related peptide</u>
DMSO	dimethyl sulfoxide
ECS	endocannabinoid system
EVOM	epithelial tissue volt-ohm-meter
FAAH	fatty acid amide hydrolase
GW6471	[(2 <i>S</i>)-2-[[[(1 <i>Z</i>)-1-methyl-3-oxo-3-[4-(trifluoromethyl)phenyl]-1-propenyl]amino]-3-[4-[2-(5-ethyl-2-henyl-4-azolyl)ethoxy] phenyl]propyl]-carbamic acid ethyl ester
GW9662	2-chloro-5-nitro- <i>N</i> -phenylbenzamide
<u>HAEC</u>	<u>Human aortic endothelial cell</u>
MAGL	monoacylglycerol lipase
MEME	Minimal Essential Medium Eagle
JZL184	4-[<i>Bis</i> (1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester
NADA	<i>N</i> -arachidonoyl-dopamine
NE	noladin ether
OA	oleamide
OEA	oleoylethanolamine
O-1918	1,3-dimethoxy- 5-methyl-2- (1 <i>R</i> ,6 <i>R</i>)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]benzene
PEA	palmitoylethanolamine
PPAR	peroxisome proliferator-activated receptor
TEER	transepithelial electrical resistance
TRPV1	transient receptor potential vanilloid subtype 1
URB597	3'-(aminocarbonyl)[1,1'-biphenyl]-3-yl)-cyclohexylcarbamate
VD	virodhamine

1.0 Introduction

Ischemia of the gastrointestinal tract, defined as an insufficient delivery of oxygen and other substrates for aerobic metabolism, is a major clinical problem associated with significant morbidity and mortality (1). Mesenteric embolus or arteriosclerosis, strangulated intestinal hernia contents, necrotising enterocolitis, arterial clamping during vascular repair or visceral transplantation, and shock, all causing gastrointestinal ischaemia, are commonly encountered in clinical practice (2-5). Intestinal mucosal enterocytes, particularly at villi tips, are sensitive to periods of ischaemia, and develop characteristic necrotic changes such as increased paracellular permeability and apoptosis after as little as 1 hour (6). These changes can lead to bacterial and lipopolysaccharide translocation from the luminal to submucosal space, leading to further shock, sepsis and possibly death. Currently there are no proven clinical therapies available to prevent or reverse these changes other than reversal of the underlying cause.

Endocannabinoids are lipid signalling molecules synthesised from arachidonic acid on demand from cell membranes (7). Endocannabinoids activate the cannabinoid receptors (CB₁ and CB₂) (8) in addition to other targets such as transient receptor potential ion channels (TRPs) (9), peroxisome proliferator-activated receptors (PPARs) (10) and the orphan G-protein coupled receptors GPR119 (11) and GPR55 (12). These target sites are expressed throughout the gastrointestinal tract. These ligands, the enzymes responsible for their metabolism, and target receptors are collectively referred to as the endocannabinoid system (ECS). The ECS has been shown to govern multiple physiological processes such as regulation of feeding, gastric secretion, intestinal motility and intestinal inflammation. Endocannabinoids and inhibitors of their degradation have been subject of much clinical and pre-clinical work examining their therapeutic potential in gastrointestinal disorders (13).

Previously, we have shown that cannabinoids modulate intestinal permeability *in vitro* during inflammatory conditions. The endocannabinoid anandamide (AEA) and 2-arachidonoylglycerol ~~2-arachidonoylglycerol~~ (2-AG) increased intestinal epithelial permeability via the CB₁ receptor (14, 15). Similarly, within a hypoxia/reperfusion model causing increased permeability of Caco-2 monolayer, inhibition of the metabolising enzymes of AEA and 2-AG, with the fatty acid amide hydrolase (FAAH) inhibitor URB597 and monoacylglycerol lipase (MAGL) inhibitor JZL184 respectively (to enhance local endocannabinoid levels) also prevented recovery (16). We also showed that knock down of the CB₁ receptor had protective effects on epithelial permeability under cytokine-induced inflammatory (16). In contrast, we found that application of the

endocannabinoid-like compounds palmitoylethanolamine (PEA) and oleoylethanolamine (OEA) prevented the cytokine-induced increases in ~~intestinal~~-permeability (17). Other endocannabinoid-like compounds such as N-arachidonoyl dopamine (NADA), noladin ether (NE), oleamide (OA) and virodhamine (VD) have not yet been examined for effects on ~~intestinal~~-permeability in Caco-2 cells.

As the effects of AEA, 2-AG, PEA and OEA on the permeability ~~of the human gut~~ have been established under normal and inflammatory conditions, we sought to examine their effects on hypoxia-induced increases in permeability and the receptor mechanisms of action. Additionally, we investigated the effects of other endocannabinoid-like compounds on ~~intestinal~~-permeability for the first time. Our hypothesis was that some endocannabinoids would reduce the increased ~~intestinal~~-permeability in Caco-2 cells observed after hypoxia.

2.0 Methods

Caco-2 cells (European Collection of Cell Culture, Wiltshire, UK; passages 62-86) were used as an *in vitro* model of intestinal epithelial cells and were cultured in Minimal Essential Medium Eagle (MEME) supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin and L-glutamine at 37°C in 5% CO₂ and 95% air. Cells were seeded at 2x10⁴ per ml onto 6.4 mm diameter transparent polystyrene membrane inserts with 0.4 µm pore size in 24 well plates (BD Biosciences, UK) and grown for 14 to 18 days until fully confluent. Transepithelial electrical resistance (TEER) was measured as an indicator of cell confluence and intercellular permeability using a voltohm² meter (EVOM²) (World Precision Instruments, Sarasota FL, USA).

2.1 Effects of hypoxia and reoxygenation on Caco-2 permeability

Complete Hypoxic (0% O₂) conditions, as occurs after embolic blockage leading to total ischaemia of sections of the intestine, were mimicked using a GasPak™ EZ Anaerobe Pouch System (Beckton-Dickinson, Oxon, UK) for up to 8 hours. Using the pouch system, anoxia is achieved within about 20 min. After the hypoxic conditions had ended, the media was changed and inserts were returned to the incubator for a period of reoxygenation -reperfusion- of up to 72 hours. TEER values were measured at baseline (0 h); and then at regular time points.

2.2 Effects of endocannabinoids on increased ~~intestinal~~-permeability caused by hypoxia

AEA, 2-AG, PEA, OEA, NADA, NE, OA and VD (all 10 μ M [based on our previous observations that only micromolar concentrations of cannabinoids are effective in this model](#); (17, 18) or vehicle (0.1% ethanol) were applied to the apical (representing the intestinal lumen) or basolateral (representing the microcirculation) membrane at the onset of hypoxia (0 h) or at the onset of [reoxygenation/reperfusion](#). [Although this is above the reported tissue concentrations of endocannabinoids and endocannabinoid-like substances measured in the gut \(Fu et al., 2007; Igarashi et al., 2015\), it is not known what the local concentrations of these compounds might be when they are produced at the cell membrane.](#) When endocannabinoids were added to fresh media at the beginning of [reoxygenation/reperfusion](#) periods, media was not changed until the end of the 72 h experimental period [\(thus they were present throughout the reoxygenation period\)](#).

To investigate the potential target sites of action, the following receptor antagonists were co-applied with endocannabinoids [as previously published](#) (16-19): AM251 (CB₁ antagonist), AM630 (CB₂ antagonist), GW9662 (PPAR γ antagonist), GW6471 (PPAR α antagonist) (all 100nM), capsazepine (TRPV1 antagonist, 1 μ M) and proposed cannabinoid receptor antagonist O-1918 (1 μ M). Appropriate vehicles (0.1% ethanol or 0.01% dimethyl sulfoxide (DMSO)) were applied to control inserts.

[2.3 RT-PCR](#)

The presence of predicted sites of action was investigated at the mRNA level using reverse transcription followed by polymerase chain reaction (RT-PCR) as previously published (20, 21). Human astrocytes (HAs) were used as a positive control known to express all the target sites of action of interest (21). Total RNA was extracted from HAs and [human aortic endothelial cells \(HAECs\)](#) using Allprep DNA/RNA kit with on column DNaseI treatment (Qiagen, Germany). Reverse transcription with (+) and without reverse transcriptase (-) was performed in 20 μ l final volume using 2 μ g of total RNA and random primers with the High Capacity cDNA Reverse Transcription Kit (Life Technologies, UK). PCR reactions were carried out in a final volume of 25 μ l with Zymotag (ZymoResearch, USA) using 2 μ l of reverse transcription product as the template. After 5 min at 95°C, PCRs were performed for 40 cycles except those for [calcitonin gene-related peptide receptor \(CGRPR\)](#) and CB2R that were carried out for 60 cycles. The cycles included 30 sec at 95°C, 30 sec at the annealing temperature that was optimal for each primer pair (56°C for CB1R and CB2R; 60°C for HPRT; 58°C for TRPV1; 61°C for CGRPR) and a final extension step of 30 sec at 72°C. Amplification products were separated by gel electrophoresis

through ethidium bromide stained 2% agarose (CB1R, CB2R, TRPV1, CGRPR and HPRT) and visualised using a Biorad Chemidoc.

2.4 Chemicals and reagents

All chemicals and reagents used in these experiments were purchased from Sigma Chemical (Poole, UK) unless otherwise mentioned. AEA, 2-AG, PEA, OEA, NADA, VD, NE, OA and the receptor antagonists AM251, AM630, GW9662, GW6471, capsazepine and O-1918 were purchased from Tocris Biotechnie (Bristol, UK) and dissolved in ethanol to a stock concentration of 10mM, with further dilutions in fresh MEME. All antagonists were dissolved in DMSO to 10mM, with further dilution made in MEME.

2.5 Statistical analysis

The effects of endocannabinoids on TERR was statistically analysed by 2-way ANOVA with Dunnett's multiple comparison test comparing data against the vehicle control within that experiment. The effects of endocannabinoids in the presence of antagonists was statistically analysed by 2-way ANOVA with Dunnett's multiple comparison test comparing data against the endocannabinoid alone control within that experiment.

3.0 Results

3.1 Effects of hypoxia on Caco-2 monolayer permeability

Increasing durations of complete hypoxia (1-8 h) caused greater ~~decreases in TEER~~ increases in permeability as suggested by with progressive ~~falls~~ decreases in TEER observed for every additional hour of complete oxygen deprivation (Figure 1). The fall in TEER after hypoxia was recoverable to baseline after up to 4 h of hypoxia (Figure 1A). However, the permeability of cells exposed to 6 or 8 h of hypoxia did not recover to baseline by 72 h (Figure 1B).

3.2 Effects of endocannabinoids on hypoxia-induced falls in TEER

When endocannabinoids were applied apically to Caco-2 cells prior to 4 h hypoxia, NADA, OEA and OA all significantly prevented the fall in TEER compared to vehicle (Figure 2A, ~~figure-3A~~). Apically applied 2-AG and AEA caused a greater reduction in TEER compared to vehicle (Figure 2A). NE, VD and PEA had no significant effect on the fall in TEER compared to vehicle (Figure 2A, ~~figure-3A~~).

When applied apically at the end of the 4 h hypoxia, NADA, OEA and OA caused a faster recovery to baseline compared to vehicle as denoted by significantly lower % falls in TEER (Figure 2B, ~~figure-3B~~), 2-AG and AEA caused a delayed recovery– of TEER (significantly greater % falls in TEER) compared to vehicle (Figure 2B), while NE, VD and PEA had no effect on TEER when applied apically.

When applied basolaterally prior to the onset of hypoxia, OA, NADA, NE and PEA prevented falls in TEER compared to vehicle (Figure 2C, ~~figure-3C~~), OEA caused a greater increase in permeability (Figure 2C), and OEA, VD, AEA and 2-AG had no effect (Figure 2C, ~~figure-3C~~).

When applied basolaterally at the end of the 4 h hypoxia, PEA, NE, NADA and OA improved recovery to baseline compared to vehicle (Figure 2D, ~~figure-3D~~), OEA worsened permeability (Figure 2D), while AEA, VD and 2-AG had no effect.

The effects of all endocannabinoids at each membrane are summarised in table 1.

3.3 Receptor mechanism of action for endocannabinoids

As shown~~found~~ in Figure 3, apical application of NADA (post-hypoxia) significantly limited the fall in TEER caused by hypoxia compared to vehicle (Figure 4BA), and this effect of NADA was

~~prevented by~~ significantly inhibited in the presence of the TRPV1 antagonist capsazepine (but not by antagonism of CB₁, CB₂, the O-1918-sensitive cannabinoid target, PPAR α or γ). The apical application of OA limited the fall in TEER, and this effect was also significantly inhibited by capsazepine (figure 4A,B). Similarly, the effects of OEA at the apical membrane were prevented by the TRPV1 antagonist capsazepine (Figure 4C). The effect of AEA and 2-AG at increasing permeability (decreasing TEER) were blocked by the CB₁ receptor antagonist AM251 (Figure 4C). To summarise, at the apical membrane, NADA, OA and OEA limit the permeability effects of hypoxia, all through TRPV1, while AEA and 2-AG cause further increases in permeability via the CB₁ receptor.

The presence of these target sites at the RNA level in CaCo2 cells was tested by RT-PCR using relevant primers (Figure 4D). This analysis confirmed the presence of PPAR α or γ , CB₁, CB₂, TRPV1 and GPR55 receptors in CaCo-2 cells; expression of these targets in human astrocytes (21) was used as a positive control (Figure 4D).

At the basolateral membrane, the positive effects of NADA on hypoxia-induced permeability were inhibited by the TRPV1 antagonist capsazepine (figure 5A). The positive effects of OA were inhibited by the TRPV1 antagonist capsazepine and the CB₁ receptor antagonist AM251 (figure 5B). The positive effects of NE (Figure 5C) and PEA (Figure 5D) on hypoxia-induced permeability were prevented by the PPAR α antagonist GW6471. The effect of basolateral application of OEA to further increase CaCo-2 permeability was inhibited by the PPAR α antagonist GW6471 but not the TRPV1 antagonist capsazepine. To summarise, at the basolateral membrane, NADA and OA limit the permeability effects of hypoxia through TRPV1 while NE and PEA limit the permeability effects of hypoxia through PPAR α . However, OEA further increases permeability at the basolateral membrane, also via PPAR α .

3.4 Effects of endocannabinoids on increased permeability caused by 6 h hypoxia

As our first experiments had demonstrated NADA, OA and OEA (all through TRPV1) limit the permeability effects of 4 h hypoxia (which is recoverable), we assessed their effects after 6 hours of hypoxia, where permeability does not recover over 72 h. However, in this model, we found that apical application of NADA, OA and OEA had no effect on recovery compared to vehicle (figure 6A).

Similarly, as NADA, NE, PEA and OA all improved permeability when applied basolaterally after 4 hours of hypoxia, we tested their effects after 6 hours of hypoxia. In this model, PEA

(which works through PPAR α) caused a recovery in TEER throughout the 72 h study period compared to vehicle (figure 6B). NE (which also works through PPAR α) increased TEER at 8 hours, though this was transient, with no difference compared to vehicle by 10 h (figure 6B). OA and NADA (which work through TRPV1) had no effect on TEER compared to vehicle in the 6 h hypoxia model.

4.0 Discussion

In this study, we sought to investigate for the first time the effects of a range of endocannabinoids and endocannabinoid-like compounds on hypoxia-induced changes in membrane permeability. This study showed activation of the TRPV1 receptor by endocannabinoids (OEA, NADA and OA) at the apical membrane decreases ~~intestinal~~ permeability, while activation of ~~the~~ CB₁ receptors at the apical membrane (by AEA and 2-AG) increases ~~intestinal~~ permeability. At the basolateral membrane, endocannabinoids (PEA, NADA, NE and OA) decrease ~~intestinal~~ permeability through activation of PPAR α , TRPV1 and CB₁. ~~The~~ expression of these targets in our cell line was confirmed by RT-PCR. VD does not appear to affect ~~intestinal-Caco-2~~ permeability. These findings add to our previous body of working demonstrating the ability of the endocannabinoid system to modulate ~~intestinal~~ permeability in cells and tissues of the intestine.

We have previously demonstrated in a model of inflammation-induced hyperpermeability that OEA decreased permeability at the apical membrane through TRPV1 (17). In the present model of ~~ischaemia-reperfusion~~hypoxia-reoxygenation-induced hyperpermeability, we also found that ~~both~~ apical and basolateral application of OA or NADA, or apical application of OEA, prevented the increased permeability induced by 4 h complete hypoxia (0% O₂), all through TRPV1 activation, suggesting this receptor may be expressed at both membrane of differentiated CaCo2 cells. TRPV1 receptors have previously been shown~~found~~ to play a major role in the regulation of digestive tract function (22, 23). Our findings are in agreement with Du *et al* (2010) who showed that TRPV1 agonists including capsaicin, genistein, and isoflavone have protective role against gastric ischemia and reperfusion when administered prior to reperfusion (24). Capsaicin also protects against damage caused by ischemia and reperfusion in the kidneys (25), lungs (26), brain (27) and heart (28).

PPAR α is highly expressed in human intestine and plays a key role in [the](#) reduction of intestinal inflammation (29, 30), decreasing intestinal permeability (31) and cell viability (32). We previously found in a model of inflammation-induced hyperpermeability that PEA decreased permeability through PPAR α (17) and similar findings were observed in the present study with hypoxia as the stimulus for increasing permeability. NE application at the basolateral also prevented increased permeability through PPAR α . [The fact that a role for PPAR \$\alpha\$ activation was only observed when endocannabinoids were applied to the basolateral membrane may reflect a more basolateral location of the nucleus in these cells, however there could be other explanations for these findings such as differential expression of the hydrolysing enzymes or transport processes of the endocannabinoid system across polarised cells such as intestinal epithelium.](#) These data are in agreement with *in vivo* data showing PPAR α agonism reduced intestinal damage and inflammation caused by ischemia and reperfusion (33) (34). Similarly, activation of PPAR α improves ischemia and reperfusion injury in other organs and systems including the kidneys (34, 35), heart (36) and brain (37). Interestingly, we found that PEA does not have an anti-inflammatory effect (reduce cytokine secretion) in Caco-2 cells (38), suggesting that the positive effects of ~~OEA and~~ PEA (and possibly NE) on [cellular](#) permeability are separate from any anti-inflammatory effects.

[Although basolateral application of PEA and NE improved the Caco-2 permeability response to oxygen deprivation through PPAR \$\alpha\$ activation, we found that basolateral application of OEA had the opposite effect, also apparently mediated by PPAR \$\alpha\$. Similarly, we previously found that CB₁ activation by phytocannabinoids decreased Caco-2 permeability while CB₁ activation by endocannabinoids increased permeability \(19\)–\(18\). Our more recent unpublished research suggests this is due to agonist bias at the CB₁ receptor, and it is possible that agonist bias also occurs at PPAR \$\alpha\$.](#)

Although PEA, NADA, OA and NE all had protective effects on ~~intestinal~~ permeability in the 4 hour, recoverable model of hypoxia, after 6 hour hypoxia, only basolateral application of PEA or NE were able to partially improve permeability. This is consistent with our previous finding that basolateral application of PEA was able to decrease permeability 72 h after the induction of inflammation via PPAR α (17), and suggests that PEA might be effective as a therapeutic after prolonged periods of ischaemia or inflammation, such as during surgery. Since PPAR α activation is the common mechanism of action in the present study, it suggests that PPAR α may be a more

robust therapeutic target (than TRPV1, [whose activation was only beneficial in the 4 h model](#)) for treating increased intestinal permeability. This may be because we recently showed that TRPV1 is downregulated in Caco-2 cells or in human colonic tissue after inflammation(39) ([Couch et al., 2019, in press](#)).

CB₁ receptors are expressed in the gastrointestinal tract epithelium (40, 41) and in our Caco-2 cells, and are activated by many endocannabinoids including AEA, 2-AG (30, 42), OA (43) and NADA (44). In this study, we found that activation of CB₁ at the apical membrane with AEA and 2-AG worsened hypoxia-induced hyper-permeability, consistent with our previous observations examining their effect on inflammation-induced hyper-permeability (18). Of interest, NADA, NE and VD have been shown previously to be able to activate CB₁ (45), however these compounds did not have CB₁ mediated effects in our model. [This could be due to differences in central versus peripheral/epithelial CB₁\(46\), or might reflect agonist bias properties of CB₁ in our cell type as we have already observed for endocannabinoids versus phytocannabinoids.](#) –In other organs, CB₁ receptor antagonism decreases infarct size in cerebral ischemia (47, 48) and preserves hepatocyte function in ischemic liver injury (49). Our data from this and previous studies (18)–(16) suggests that CB₁ antagonism may prevent the hyperpermeability caused by ischaemia and hypoxia. This may be a useful clinical tool for prophylaxis against the effects of clinical gastrointestinal ischaemia.

In conclusion, this is the first report of endocannabinoids modulating [Caco-2 intestinal permeability](#) through numerous targets in a recoverable model of intestinal epithelial [hypoxia ischemia](#) and [reperfusion reoxygenation](#). Endocannabinoid activation of TRPV1 and PPAR α has positive effects on Caco-2 permeability, while CB₁ activation at the apical membrane increases permeability. CB₁ antagonism and TRPV1 or PPAR α agonism may represent novel therapeutic targets against several intestinal disorders associated with increased intestinal permeability. Endocannabinoid effects on permeability should now be investigated in *ex vivo* models of inflammation and ischaemia and also in animal models to evaluate potential roles in disease modification and prophylaxis for maintenance of intestinal mucosal integrity.

Conflicts of interest

[None. SOS is a scientific advisor for the Centre for Medicinal Cannabis, Artelo Bioscience and Dragonfly Bioscience. The research in this manuscript was not funded by these parties.](#)

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Figure legends

Figure 1. The effect of short-term and recoverable (1-4 h, A) and non-recoverable (6 and 8 h, B) hypoxia and reoxygenation/reperfusion on Caco-2 permeability. Data are given as means \pm S.E.M. (n=3).

Figure 2. The effects of endocannabinoids and endocannabinoid-like compounds (anandamide (AEA), 2-arachidonylglycerol (2-AG), oleoylethanolamine (OEA) and palmitoylethanolamine (PEA), all 10 μ M) applied before (A, C) or after 4 h hypoxia (B, D) to the apical (A,B) or basolateral (C,D) membrane of Caco-2 cells. Arrows denote the time of application of endocannabinoids and the solid line denotes the period of hypoxia. Data are given as means and standard error bars S.E.M. (n=3) and was analysed by 2-way ANOVA with Dunnett's multiple comparison test comparing data against the vehicle control (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Figure 3. The effects of endocannabinoids (N-arachidonyldopamine (NADA), virodhamine (VD), noladine ether (NE), and oleamide (OA), all 10 μ M) applied before 4 h hypoxia (A, C) or after 4 h hypoxia (B, D) to the apical (A,B) or basolateral (C,D) membrane of Caco-2 cells. Arrows denote the time of application of endocannabinoids and the solid line denotes the period of hypoxia. Data are given as means and standard error bars S.E.M. (n=3) and was analysed by 2-way ANOVA with Dunnett's multiple comparison test comparing data against the vehicle control (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Figure 4. Mechanisms of action for N-arachidonyldopamine (NADA₂-A), oleamide (OA, B) and anandamide, 2-arachidonylglycerol (2-AG) and oleoylethanolamine (OEA₂-C) applied to the apical membrane after 4 h hypoxia in the presence of various receptor antagonists. Arrows denote the time of application of endocannabinoids and the solid line denotes the period of hypoxia. Data are given as means and standard error bars S.E.M. (n=3) and was analysed by 2-way ANOVA with Dunnett's multiple comparison test comparing data against the endocannabinoid alone control (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). D. RT-PCR showing the presence of CB₁, CB₂, PPAR α and γ , TRPV1 and CGRP receptors in CaCo-2 cells. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a house-keeping gene. The columns shown with + are with reverse transcriptase and those with – are without reverse transcriptase. Human astrocytes (HA) are shown as a positive control for cannabinoid target expression.

Figure 5. Mechanisms of action for N-arachidonyldopamine (NADA₂-A), oleamide (OA, B) noladin ether (NE, C), and palmitoylethanolamine (PEA) and oleoylethanolamine (OEA₂-D) applied to the basolateral membrane after 4 h hypoxia in the presence of various receptor antagonists. Arrows denote the time of application of endocannabinoids and the solid line denotes the period of hypoxia. Data are given as means and standard error bars S.E.M. (n=3) and was analysed by 2-way ANOVA with Dunnett's multiple comparison test comparing data against the endocannabinoid alone control (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Figure 6. A. The effects of apical application of N-arachidonyldopamine (NADA), oleoylethanolamine (OEA) and oleamide (OA) on Caco-2 permeability after 6 h hypoxia. B. The effects of basolateral application of NADA, noladin ether (NE), palmitoylethanolamine (PEA) and OA on Caco-2 permeability after 6 h hypoxia (A). Data are given as means and standard error bars S.E.M. (n=3) and was analysed by 2-way ANOVA with Dunnett's multiple comparison test comparing data against the vehicle control (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

ACCEPTED MANUSCRIPT

	Apical		Basolateral	
	Effect on IP	Target	Effect on IP	Target
AEA	↑ permeability	CB ₁	No response	-
2-AG	↑ permeability	CB ₁	No response	-
OEA	↓ permeability	TRPV1	↑ permeability	PPAR α
PEA	No response	-	↓ permeability	PPAR α
NADA	↓ permeability	TRPV1	↓ permeability	TRPV1
NE	No response	-	↓ permeability	PPAR α
OA	↓ permeability	TRPV1	↓ permeability	CB ₁ & TRPV1
VD	No response	-	No response	-

Table 1 Summary of the target sites of action of endocannabinoids on intestinal permeability (IP) in ischemia/hypoxia/reoxygenation/reperfusion at the apical and basolateral membranes of Caco-2 cells.

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