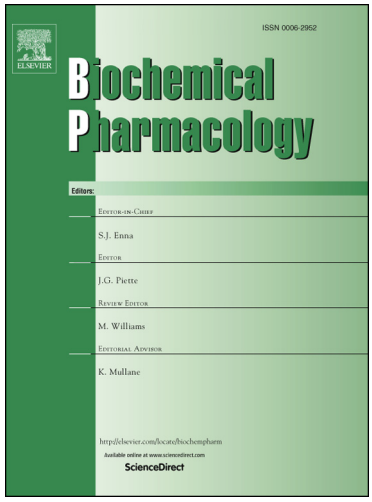

Endocannabinoids and endocannabinoid-like compounds modulate hypoxia-induced permeability in CaCo-2 cells via CB_1, TRPV1, and PPARα

M.A. Karwad, D.G. Couch, K.L. Wright, C. Tufarelli, M. Larvin, J. Lund, S.E. O'Sullivan

PII: S0006-2952(19)30272-2
DOI: https://doi.org/10.1016/j.bcp.2019.07.017
Reference: BCP 13588

To appear in: *Biochemical Pharmacology*

Received Date: 13 March 2019
Accepted Date: 15 July 2019

Please cite this article as: M.A. Karwad, D.G. Couch, K.L. Wright, C. Tufarelli, M. Larvin, J. Lund, S.E. O'Sullivan, Endocannabinoids and endocannabinoid-like compounds modulate hypoxia-induced permeability in CaCo-2 cells via CB_1, TRPV1, and PPARα, *Biochemical Pharmacology* (2019), doi: https://doi.org/10.1016/j.bcp.2019.07.017

Endocannabinoids and endocannabinoid-like compounds modulate hypoxia-induced permeability in CaCo-2 cells via CB_1, TRPV1, and PPARα

MA Karwad[1], DG Couch[1], KL Wright[3], C. Tufarelli[4], M Larvin[2], J Lund[1], and SE O'Sullivan[1]

[1] Division of Medical Sciences & Graduate Entry Medicine, School of Medicine, Royal Derby Hospital, University of Nottingham, Nottingham, UK.

[2]Graduate Entry Medical School and Health Research Institute, University of Limerick, Limerick, Ireland.

[3]Division of Biomedical & Life Sciences, Faculty of Health & Medicine, Lancaster University, Lancaster, UK.

[4]Leicester Cancer Research Centre, Department of Genetics and Genome Biology, University of Leicester, Leicester, UK.

Correspondence

Saoirse E. O'Sullivan, School of Medicine, Royal Derby Hospital, University of Nottingham, Derby, DE22 3DT, UK.

E-mail: Saoirse.osullivan@nottingham.ac.uk

T. 01332724701

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% O_2), 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 ~~H~~ypoxia 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~~A~~rachidonoylglycerol (2-AG) worsened the permeability effect of hypoxia (both via CB_1). Basolateral application of NADA (via TRPV1), OA (via CB_1 and TRPV1), noladin ether (NE, via PPARα), and palmitoylethanolamine (PEA, via PPARα) restored permeability after 4 h hypoxia, whereas OEA increased permeability (via PPARα). 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~~ischemi~~a/reoxygenation~~reperfusion~~, which involves multiple targets, depending on whether the compounds are applied to the basolateral or apical membrane. CB_1 antagonism and TRPV1 or PPARα 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 (CB_1); 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~~A~~rachidonoylglycerol (2-AG); noladin ether (NE); virodhamine (VD).

ABBREVIATIONS:

2-AG	2-a-Arachidonoyl glycerol
AEA	anandamide
AM251	*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carbox-amide
AM630	6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxy-phenyl) methanone
ANOVA	analysis of variance
CB_1	cannabinoid receptor one
CB_2	cannabinoid receptor two
CGRP	Calcitonin gene-related peptide
DMSO	dimethyl sulfoxide
ECS	endocannabinoid system
EVOM	epithelial tissue volt-ohm-meter
FAAH	fatty acid amide hydrolase
GW6471	[(2*S*)-2-[[(1*Z*)-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-*N*-phenylbenzamide
HAEC	Human aortic endothelial cell
MAGL	monoacylglycerol lipase
MEME	Minimal Essential Medium Eagle
JZL184	4-[*Bis*(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester
NADA	N-arachidonoyl-dopamine
NE	noladin ether
OA	oleamide
OEA	oleoylethanolamine
O-1918	1,3-dimethoxy- 5-methyl-2- (1*R*,6*R*)-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 (CB_1 and CB_2) (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-arachidonylglycerol~~ (2-AG) increased intestinal epithelial permeability via the CB_1 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 CB_1 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 37ºC in 5% CO_2 and 95% air. Cells were seeded at $2x10^4$ 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 voltoham^2 meter (EVOM^2) (World Precision Instruments, Sarasota FL, USA).

2.1 Effects of hypoxia and reoxygenation on Caco-2 permeability

Complete ~~H~~hypoxic (0% O_2) 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 10µM 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 (CB_1 antagonist), AM630 (CB_2 antagonist), GW9662 (PPARγ antagonist), GW6471 (PPARα antagonist) (all 100nM), capsazepine (TRPV1 antagonist,~~)~~ ~~(~~1µM) and proposed cannabinoid receptor antagonist O-1918 (1µM). 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 µl final volume using 2 µg 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 µl with Zymotaq (ZymoResearch, USA) using 2 µl 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 Biotechne (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 (F~~f~~igure 1). The fall in TEER after hypoxia was recoverable to baseline after up to 4 h of hypoxia (F~~f~~igure 1A). However, the permeability of cells exposed to 6 or 8 h of hypoxia did not recover to baseline by 72 h (F~~f~~igure 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 (F~~f~~igure 2A, ~~figure~~ 3A). Apically applied 2-AG and AEA caused a greater reduction in TEER compared to vehicle (F~~f~~igure 2A). NE, VD and PEA had no significant effect on the fall in TEER compared to vehicle (F~~f~~igure 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 (F~~f~~igure 2B, ~~figure~~ 3B), 2-AG and AEA caused a delayed recovery– of TEER (significantly greater % falls in TEER) compared to vehicle (F~~f~~igure 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 (F~~f~~igure 2C, ~~, figure~~ 3C), OEA caused a greater increase in permeability (F~~f~~igure 2C), and OEA, VD, AEA and 2-AG had no effect (F~~f~~igure 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 (F~~f~~igure 2D, ~~figure~~ 3D), OEA worsened permeability (F~~f~~igure 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 (F~~f~~igure 4B~~A~~), and this effect of NADA was

~~prevented by~~significantly inhibited in the presence of the TRPV1 antagonist capsazepine (but not by antagonism of CB_1, CB_2, the O-1918-sensitive cannabinoid target, PPARα 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 (F~~f~~igure 4C). The effect of AEA and 2-AG at increasing permeability (decreasing TEER) were blocked by the CB_1 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 CB_1 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 PPARα or γ, CB_1, CB_2, 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 CB_1 receptor antagonist AM251 (figure 5B). The positive effects of NE (Figure 5C) and PEA (Figure 5D) on hypoxia-induced permeability were prevented by the PPARα antagonist GW6471. The effect of basolateral application of OEA to further increase CaCo-2 permeability was inhibited by the PPARα 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 PPARα. However, OEA further increases permeability at the basolateral membrane, also via PPARα.

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 PPARα) caused a recovery in TEER throughout the 72 h study period compared to vehicle (figure 6B). NE (which also works through PPARα) 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~~ CB_1 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 PPARα, TRPV1 and CB_1. 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% O_2), all through TRPV1 activation, suggesting this receptor may be expressed at both membrane of differentiated CaCo2 cells. TRPV1 receptors have previosuly been shown~~found~~ to play a major role in the regulation of digestive tract function (22, 23). ~~O~~ur 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).

PPARα 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 PPARα (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 PPARα. The fact that a role for PPARα 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 PPARα agonism reduced intestinal damage and inflammation caused by ischemia and reperfusion (33) (34). Similarly, activation of PPARα 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 PPARα activation, we found that basolateral application of OEA had the opposite effect, also apparently mediated by PPARα. Similarly, we previously found that CB_1 activation by phytocannabinoids decreased Caco-2 permeability while CB_1 activation by endocannabinoids increased permeability (19)–(18). Our more recent unpublished research suggests this is due to agonist bias at the CB_1 receptor, and it is possible that agonist bias also occurs at PPARα.

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 PPARα (17), and suggests that PEA might be effective as a therapeutic after prolonged periods of ischaemia or inflammation, such as during surgery. Since PPARα activation is the common mechanism of action in the present study, it suggests that PPARα 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)~~.

CB_1 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 CB_1 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 CB_1 (45), however these compounds did not have CB_1 mediated effects in our model. This could be due to differences in central versus peripheral/epithelial CB_1 (46), or might reflect agonist bias properties of CB_1 in our cell type as we have already observed for endocannabinoids versus phytocannabinoids. –In other organs, CB_1 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 CB_1 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 PPARα has positive effects on Caco-2 permeability, while CB_1 activation at the apical membrane increases permeability. CB_1 antagonism and TRPV1 or PPARα 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.

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 ± 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 10µM) 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), noladin~~e~~ ether (NE), and oleamide (OA), all 10µM) 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 (NADA, ~~(~~A), oleamide (OA, B) and anandamide, 2-arachidonylglycerol (2-AG) and oleoylethanolamine (OEA, ~~(~~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 CB_1, CB_2, PPARα 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 (NADA, ~~(~~A), oleamide (OA, B) noladin ether (NE, C), ~~and~~ palmitoylethanolamine (PEA) and oleoylethanolamine (OEA, ~~(~~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).

	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	PPARα
PEA	No response	-	↓ permeability	PPARα
NADA	↓ permeability	TRPV1	↓ permeability	TRPV1
NE	No response	-	↓ permeability	PPARα
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.

1. Mallick IH, Yang W, Winslet MC, Seifalian AM. Ischemia-reperfusion injury of the intestine and protective strategies against injury. Dig Dis Sci. 2004;49(9):1359-77.
2. Reiberger T, Ferlitsch A, Payer BA, Mandorfer M, Heinisch BB, Hayden H, et al. Non-selective betablocker therapy decreases intestinal permeability and serum levels of LBP and IL-6 in patients with cirrhosis. J Hepatol. 2013;58(5):911-21.
3. Swank GM, Deitch EA. Role of the gut in multiple organ failure: bacterial translocation and permeability changes. World J Surg. 1996;20(4):411-7.
4. Samel S, Keese M, Kleczka M, Lanig S, Gretz N, Hafner M, et al. Microscopy of bacterial translocation during small bowel obstruction and ischemia in vivo--a new animal model. BMC Surg. 2002;2:6.
5. Russo F, Linsalata M, Clemente C, Chiloiro M, Orlando A, Marconi E, et al. Inulin-enriched pasta improves intestinal permeability and modifies the circulating levels of zonulin and glucagon-like peptide 2 in healthy young volunteers. Nutr Res. 2012;32(12):940-6.
6. Chiu CJ, McArdle AH, Brown R, Scott HJ, Gurd FN. Intestinal mucosal lesion in low-flow states. I. A morphological, hemodynamic, and metabolic reappraisal. Arch Surg. 1970;101(4):478-83.
7. Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC, et al. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature. 1994;372(6507):686-91.
8. Pertwee RG. Pharmacology of cannabinoid CB1 and CB2 receptors. Pharmacology & Therapeutics. 1997;74(2):129-80.
9. Massa F, Sibaev A, Marsicano G, Blaudzun H, Storr M, Lutz B. Vanilloid receptor (TRPV1)-deficient mice show increased susceptibility to dinitrobenzene sulfonic acid induced colitis. Journal of Molecular Medicine-Jmm. 2006;84(2):142-6.
10. O'Sullivan SE. Cannabinoids go nuclear: evidence for activation of peroxisome proliferator-activated receptors. British journal of pharmacology. 2007;152(5):576-82.
11. Fredriksson R, Hoglund PJ, Gloriam DE, Lagerstrom MC, Schioth HB. Seven evolutionarily conserved human rhodopsin G protein-coupled receptors lacking close relatives. Febs Letters. 2003;554(3):381-8.
12. Ryberg E, Larsson N, Sjogren S, Hjorth S, Hermansson NO, Leonova J, et al. The orphan receptor GPR55 is a novel cannabinoid receptor. British Journal of Pharmacology. 2007;152(7):1092-101.
13. Couch DG, Maudslay H, Doleman B, Lund JN, O'Sullivan SE. The Use of Cannabinoids in Colitis: A Systematic Review and Meta-Analysis. Inflammatory bowel diseases. 2018;24(4):680-97.
14. Alhamoruni A, Lee AC, Wright KL, Larvin M, O'Sullivan SE. Pharmacological Effects of Cannabinoids on the Caco-2 Cell Culture Model of Intestinal Permeability. Journal of Pharmacology and Experimental Therapeutics. 2010;335(1):92-102.
15. Alhamoruni A, Wright KL, Larvin M, O'Sullivan SE. Cannabinoids mediate opposing effects on inflammation-induced intestinal permeability. British journal of pharmacology. 2012;165(8):2598-610.
16. Karwad MA, Couch DG, Theophilidou E, Sarmad S, Barrett DA, Larvin M, et al. The role of CB1 in intestinal permeability and inflammation. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2017;31(8):3267-77.
17. Karwad MA, Macpherson T, Wang B, Theophilidou E, Sarmad S, Barrett DA, et al. Oleoylethanolamine and palmitoylethanolamine modulate intestinal permeability in vitro via TRPV1 and PPARalpha. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2017;31(2):469-81.
18. Alhamoruni A, Wright KL, Larvin M, O'Sullivan SE. Cannabinoids mediate opposing effects on inflammation-induced intestinal permeability. Br J Pharmacol. 2012;165(8):2598-610.
19. Alhamoruni A, Lee AC, Wright KL, Larvin M, O'Sullivan SE. Pharmacological effects of cannabinoids on the Caco-2 cell culture model of intestinal permeability. J Pharmacol Exp Ther. 2010;335(1):92-102.

20. Stanley CP, Hind WH, Tufarelli C, O'Sullivan SE. Cannabidiol causes endothelium-dependent vasorelaxation of human mesenteric arteries via CB1 activation. Cardiovasc Res. 2015.
21. Hind WH, Tufarelli C, Neophytou M, Anderson SI, England TJ, O'Sullivan SE. Endocannabinoids modulate human blood-brain barrier permeability in vitro. Br J Pharmacol. 2015.
22. Faussone-Pellegrini MS, Taddei A, Bizzoco E, Lazzeri M, Vannucchi MG, Bechi P. Distribution of the vanilloid (capsaicin) receptor type 1 in the human stomach. Histochem Cell Biol. 2005;124(1):61-8.
23. Peng J, Li YJ. The vanilloid receptor TRPV1: role in cardiovascular and gastrointestinal protection. European journal of pharmacology. 2010;627(1-3):1-7.
24. Du DS, Ma XB, Zhang JF, Zhou XY, Li Y, Zhang YM, et al. The protective effect of capsaicin receptor-mediated genistein postconditioning on gastric ischemia-reperfusion injury in rats. Dig Dis Sci. 2010;55(11):3070-7.
25. Ueda K, Tsuji F, Hirata T, Takaoka M, Matsumura Y. Preventive effect of TRPV1 agonists capsaicin and resiniferatoxin on ischemia/reperfusion-induced renal injury in rats. J Cardiovasc Pharmacol. 2008;51(5):513-20.
26. Wang M, Ji P, Wang R, Zhao L, Xia Z. TRPV1 agonist capsaicin attenuates lung ischemia-reperfusion injury in rabbits. J Surg Res. 2012;173(1):153-60.
27. Pegorini S, Braida D, Verzoni C, Guerini-Rocco C, Consalez GG, Croci L, et al. Capsaicin exhibits neuroprotective effects in a model of transient global cerebral ischemia in Mongolian gerbils. Br J Pharmacol. 2005;144(5):727-35.
28. Vemula P, Gautam B, Abela GS, Wang DH. Myocardial Ischemia/Reperfusion injury: Potential of TRPV1 Agonists as Cardioprotective Agents. Cardiovasc Hematol Disord Drug Targets. 2013.
29. Esposito G, Capoccia E, Turco F, Palumbo I, Lu J, Steardo A, et al. Palmitoylethanolamide improves colon inflammation through an enteric glia/toll like receptor 4-dependent PPAR-alpha activation. Gut. 2013.
30. Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science. 1992;258(5090):1946-9.
31. Mazzon E, Cuzzocrea S. Absence of functional peroxisome proliferator-activated receptor-alpha enhanced ileum permeability during experimental colitis. Shock. 2007;28(2):192-201.
32. Bunger M, van den Bosch HM, van der Meijde J, Kersten S, Hooiveld GJ, Muller M. Genome-wide analysis of PPARalpha activation in murine small intestine. Physiol Genomics. 2007;30(2):192-204.
33. Cuzzocrea S, Di Paola R, Mazzon E, Genovese T, Muia C, Caputi AP. WY 14643, a potent exogenous PPAR-alpha ligand, reduces intestinal injury associated with splanchnic artery occlusion shock. Shock. 2004;22(4):340-6.
34. Di Paola R, Impellizzeri D, Mondello P, Velardi E, Aloisi C, Cappellani A, et al. Palmitoylethanolamide reduces early renal dysfunction and injury caused by experimental ischemia and reperfusion in mice. Shock. 2012;38(4):356-66.
35. Patel NS, di Paola R, Mazzon E, Britti D, Thiemermann C, Cuzzocrea S. Peroxisome proliferator-activated receptor-alpha contributes to the resolution of inflammation after renal ischemia/reperfusion injury. J Pharmacol Exp Ther. 2009;328(2):635-43.
36. Yue TL, Bao W, Jucker BM, Gu JL, Romanic AM, Brown PJ, et al. Activation of peroxisome proliferator-activated receptor-alpha protects the heart from ischemia/reperfusion injury. Circulation. 2003;108(19):2393-9.
37. Ahmad A, Genovese T, Impellizzeri D, Crupi R, Velardi E, Marino A, et al. Reduction of ischemic brain injury by administration of palmitoylethanolamide after transient middle cerebral artery occlusion in rats. Brain Research. 2012;1477(0):45-58.
38. Couch DG, Tasker C, Theophilidou E, Lund JN, O'Sullivan SE. Cannabidiol and palmitoylethanolamide are anti-inflammatory in the acutely inflamed human colon. Clin Sci (Lond). 2017;131(21):2611-26.

39. Couch DG, Cook H, Ortori C, Barrett D, Lund JN, O'Sullivan SE. Palmitoylethanolamide and Cannabidiol Prevent Inflammation-induced Hyperpermeability of the Human Gut In Vitro and In Vivo-A Randomized, Placebo-controlled, Double-blind Controlled Trial. Inflammatory bowel diseases. 2019;25(6):1006-18.
40. Pazos MR, Tolon RM, Benito C, Rodriguez CF, Gorgojo JJ, Nevado M, et al. Cannabinoid CB1 receptors are expressed by parietal cells of the human gastric mucosa. J Histochem Cytochem. 2008;56(5):511-6.
41. Gomez R, Navarro M, Ferrer B, Trigo JM, Bilbao A, Del Arco I, et al. A peripheral mechanism for CB1 cannabinoid receptor-dependent modulation of feeding. J Neurosci. 2002;22(21):9612-7.
42. Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, et al. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem Pharmacol. 1995;50(1):83-90.
43. Leggett JD, Aspley S, Beckett SR, D'Antona AM, Kendall DA. Oleamide is a selective endogenous agonist of rat and human CB1 cannabinoid receptors. Br J Pharmacol. 2004;141(2):253-62.
44. Bisogno T, Melck D, Bobrov M, Gretskaya NM, Bezuglov VV, De Petrocellis L, et al. N-acyl-dopamines: novel synthetic CB(1) cannabinoid-receptor ligands and inhibitors of anandamide inactivation with cannabimimetic activity in vitro and in vivo. The Biochemical journal. 2000;351 Pt 3:817-24.
45. Porter AC, Sauer JM, Knierman MD, Becker GW, Berna MJ, Bao J, et al. Characterization of a novel endocannabinoid, virodhamine, with antagonist activity at the CB1 receptor. J Pharmacol Exp Ther. 2002;301(3):1020-4.
46. Liu QR, Huang NS, Qu H, O'Connell JF, Gonzalez-Mariscal I, Santa-Cruz-Calvo S, et al. Identification of novel mouse and rat CB1R isoforms and in silico modeling of human CB1R for peripheral cannabinoid therapeutics. Acta pharmacologica Sinica. 2019;40(3):387-97.
47. Muthian S, Rademacher DJ, Roelke CT, Gross GJ, Hillard CJ. Anandamide content is increased and CB1 cannabinoid receptor blockade is protective during transient, focal cerebral ischemia. Neuroscience. 2004;129(3):743-50.
48. Sommer C, Schomacher M, Berger C, Kuhnert K, Muller HD, Schwab S, et al. Neuroprotective cannabinoid receptor antagonist SR141716A prevents downregulation of excitotoxic NMDA receptors in the ischemic penumbra. Acta Neuropathol. 2006;112(3):277-86.
49. Caraceni P, Pertosa AM, Giannone F, Domenicali M, Grattagliano I, Principe A, et al. Antagonism of the cannabinoid CB-1 receptor protects rat liver against ischaemia-reperfusion injury complicated by endotoxaemia. Gut. 2009;58(8):1135-43.