HIF-dependent regulation of platelet activating factor receptor as a route for Gram positive bacterial translocation across epithelia

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Running title: PAFr as a route for bacterial translocation
Abstract

Mucosal surfaces, such as the lung and intestine, are lined by a monolayer of epithelia which provides tissue barrier and transport function. It is recently appreciated that a common feature of inflammatory processes within the mucosa is hypoxia (so called inflammatory hypoxia). Given the strong association between bacterial translocation and mucosal inflammatory disease, we hypothesized that intestinal epithelial hypoxia influences bacterial translocation. Initial studies revealed that exposure of cultured intestinal epithelia to hypoxia (pO2 20 torr, 24-48hr) resulted in an increase of up to 40-fold in the translocation of some strains of Gram positive bacteria, independent of epithelial barrier function. A screen of relevant pathway inhibitors identified a prominent role for the platelet-activating factor receptor (PAF receptor) in hypoxia-associated bacterial translocation, wherein pharmacologic antagonists of PAF receptor blocked bacterial translocation by as much as 80±6%. Extensions of these studies revealed that hypoxia prominently induces PAF receptor through an hypoxia-inducible factor (HIF)-dependent mechanism. Indeed, HIF and PAF receptor loss of function studies (shRNA) revealed that apically expressed PAF receptor is central to the induction of translocation for the Gram positive bacteria E. faecalis. Taken together, these findings reveal that some strains of Gram positive bacteria exploit HIF-regulated PAF receptor as a means for translocation through intestinal epithelial cells.
Introduction

The gastrointestinal tract is supported by a rich and complex underlying vasculature. As a consequence, the intestinal epithelial cell layer is particularly susceptible to damage associated with diminished blood flow. The resulting hypoxia is a consequence of both decreased perfusion and increased metabolism within the mucosa (Karhausen et al., 2003). The metabolic shift may result in ‘cytopathic hypoxia’, a form of mitochondrial dysfunction leading to reduced intracellular oxygen and ATP availability (Fink, 1997; Fink, 2001). While this damage poses a risk to the epithelial function of excluding harmful luminal entities (Keely et al., 2005; Keely et al., 2008), recent studies have shown that the intestinal epithelium is equipped with hypoxia-inducible adaptive mechanisms which sustain barrier function under these inflamed conditions (Karhausen et al., 2003; Karhausen et al., 2004a; Karhausen et al., 2005). Indeed, compared to other surfaces, intestinal epithelial cells appear to be uniquely resistant to disruption by hypoxia (Furuta et al., 2001; Synnestvedt et al., 2002; Karhausen et al., 2004a). Such observations may relate to the fact that intestinal epithelial cells are conditioned to a lower pO2 than other tissue sources (Taylor and Colgan, 2007).

Although there is strong correlation between barrier breakdown and bacterial translocation (Fink, 1991; Lenz et al., 2007; Zinkernagel et al., 2007), the molecular mechanisms of bacterial translocation from the lumen of the GI tract to bloodstream are not well understood. While there is an apparent increase in translocated bacteria with hypoxia, most evidence suggests that overall integrity if the intestinal epithelium remains intact even in relatively severe hypoxia (Furuta et al., 2001). This finding may indicate that bacterial translocation during intestinal inflammation is a consequence of increased transcellular bacterial movement, rather than a breakdown of epithelial integrity. As an example, Enterococcus faecalis has become increasingly
important and is now the second most common hospital-acquired infection in the USA (2003; Hageman et al., 2003) with increased prevalence of antibiotic resistant strains (Lam et al., 1995; 2003).

*Based on these observations, we examined the passage of non-invasive, non pathogenic enteric bacteria* across intestinal epithelial cells subjected to hypoxia in an attempt to identify molecular mechanisms of such translocation. We hypothesized that non-invasive bacteria may exploit existing epithelial cell surface receptors to attach and invade the intestinal epithelium as a mechanism of invading the serosa. Employing established *in vitro* models of infection, we identified PAFr as an entry point into the cell by the Gram positive enteric bacterium *E. faecalis*. We examined the induction and regulation of PAFr by HIF-1 and its regulation in intestinal inflammation *in vivo*. Our finding suggest that *E. faecalis* exploit existing epithelial pathways to overcome the intestinal barrier during inflammation and that competitive antagonism of PAFr may be a therapeutic strategy for patients at risk of sepsis.
Materials and Methods

Cell Culture

Caco-2 intestinal epithelial cells were grown and maintained in T75 cell culture flasks (Costar Corp., Cambridge MA) as described previously (Furuta et al., 2001). For translocation experiments, cells were grown on permeable supports (Costar Corp., Cambridge MA) and maintained over 21 days to achieve fully differentiated, polarized monolayers as previously described (Keely et al., 2009).

Bacterial Cultures

Bacteria were either human isolates or ATCC strains. Primary bacterial cultures of Enterococcus faecalis were streaked on tryptone agar plates and incubated overnight at 37°C. All other strains were grown in LB agar plates. Colonies were removed from the plate and inoculated into 5ml of the appropriate culture broth. This broth was incubated, shaking (250 rpm), overnight at 37°C resulting in a bacterial population of ~1x10^8 CFU/ml.

Infection Studies

Bacterial cultures in broth (1 x 10^8 CFU/ml) was centrifuged at 3000g for 10 minutes. The resulting pellet was resuspended in HBSS (supplemented with magnesium and calcium) buffer to give a concentration of 1 x 10^7 CFU/ml. Caco-2 cells were seeded on 0.33cm^2, 3μm pore transwell® inserts and maintained for 21 days to allow polarization. Monolayers were incubated in either hypoxic (1% O₂) or normoxic conditions at 37°C for 24 hours prior to bacterial challenge. Monolayers were pre-incubated with either vehicle or PAFr antagonist CV-6209 (BioMol International) on the apical side for 30 minutes. Monolayers were then apically
challenged at a multiplicity of infection of 100:1 (3x10^7 bacteria to 3x10^5 epithelial cells) and incubated at 37°C. Samples of 100µl in volume were taken from the basolateral chamber 15, 30, 60 and 120 minutes after challenge. Basolateral samples were diluted 1/100 and 50µl samples were spot plated on tryptone agar plates. Plates were incubated overnight at 37°C and plate counts were carried out to determine bacterial translocation.

For imaging, Caco-2 cells were seeded onto collagen coated glass cover-slips. *E. faecalis* was labeled with BacLight, fluorescent bacterial stain (Molecular Probes) according to manufacturer’s instructions (Berney et al., 2007) before challenge (5 - 45 minutes). Cells were fixed in 4% formaldehyde, permeabilized with 0.01% Triton-X, blocked with 10% BSA and probed with anti-PAFr (Cayman Chemicals). Cells were counterstained with DAPI (Molecular Probes) and imaged by fluorescent microscopy.

**Stable repression of HIF-1α and PAFr by shRNA.**

Lentiviral particles encoding a panel of shRNAs directed against either HIF-1α or HIF-2α (MISSION™ TRC shRNA, Sigma) were used to transduce Caco-2 cells using standard protocols. Non-target shRNA-encoding lentiviral particles were used to generate a control cell line. Stable integration was accomplished by prolonged exposure to puromycin (6µg/ml). For repression of PAFr, hairpin primers with the sequence 5’-ACCTCACCACGGATACGGTCA-CTGAATCAAGAGTTCAGTGACCCTGATCCGTGGTTT-3’ and 5’-CAAAAAACCACGGATACGGTC ACTGAACTGGATTGACCCTGATCCGTGGTTT-3’, corresponding to position 1088-1108 of the PAFR transcript, were annealed and ligated into the BbsI/BbsI digested psiRNA-hH1neo G2 vector (Invivogen). Caco-2 cells were transfected with plasmid using Fugene 6.0 according to manufacturer’s protocols (Roche). A control Caco-2 line was
generated using psiRNA-hH1 neoSCR plasmid, encoding a scrambled shRNA with no known homology to any mouse or human transcript. Stable transfectants were selected with G418 (400ug/ml). Repression was confirmed by real-time PCR and Western blot analysis.

Transcriptional and protein expression analysis

The transcriptional profile of Caco-2 and repression epithelial cells subjected to control (normoxia, pO2 147 torr) or hypoxia (pO2 20 torr for 6 or 18 hr hypoxia) were assessed from total RNA by quantitative real-time PCR. (PAFr primer set: forward: 5'-AGAAGTTCCGCAAGCACCTC –3', reverse: 5'- GGATCTGGTTGAATGGCACA –3') using iQ SYBR mix (iCycler; Bio-Rad), as described previously (Karhausen et al., 2004b; Louis et al., 2008). Protein expression was ascertained by western blot analysis. Protein isolated from normoxic and hypoxic monolayers was separated by SDS-PAGE, transferred to PVDF membranes and probed with anti-PAFr (Cayman Chemicals). β-Actin was employed as a reference housekeeping protein. Blot analysis was quantified in terms of pixel number using ImageJ (http://rsb.info.nih.gov/ij/).

Chromatin immunoprecipitation (ChIP) of Hif-1α

Chromatin immunoprecipitation was performed as previously described (Kong et al., 2004) using anti-HIF-1α antibody (rabbit polyclonal, Novus Biologicals, Littleton, CO). HIF binding to PAFr promoter DNA was quantified by both standard and real-time PCR using primers (forward: 5’- CTGGCCTCGGGCGCTGCTA -3’ and reverse: 5’-
CCAAGTCACCCCTGGGAGGAA -3’) designed to amplify a 153 bp region of the PAFr
promoter. Chromatin incubated with beads or beads plus rabbit IgG served as controls for nonspecific binding.

**DSS colitis model**

DSS colitis was induced with a modification of the technique of Okayasu et al. (Okayasu et al., 1990). Colitis was induced on day 0 by the addition of 3%, 4.5 or 6% DSS (m.w. = 36,000–50,000; MP Biomedicals) solution in drinking water. Control animals received water alone. On day 6, animals were sacrificed and the GI tract excised. Colon length was recorded and epithelial cells were harvested from the colon by EDTA cell isolation (100mM N-acetyl cysteine, 1mM EDTA, 1.5% v/v HEPES in PBS, vortexed at high speed for 15 minutes).
Results

**Translocation of bacteria through hypoxic monolayers**

As an initial series of experiments, we examined a range of bacteria including non-invasive, non-pathogenic strains, for translocation across intestinal epithelial monolayers and whether this response was influenced by hypoxia. Two Gram positive bacteria, E. faecalis, and S. mitis, showed significantly increased translocation in response to hypoxia (Figure 1a). None of the Gram negative bacteria species tested showed differences between normoxia and hypoxia (Figure 1a). As shown in Figure 1b, these studies revealed that in normoxia, E. faecalis translocated across confluent Caco2 monolayers at very low rates over a two hour period (corresponding to a rate of 4.0±0.6 CFU/min). E. faecalis translocation changed quite significantly when epithelia were subjected to hypoxia (pO$_2$ 20 torr, 48h). Rates of E. faecalis translocation increased by 42±6-fold following hypoxia (corresponding rate of 168.6±7.2 CFU/min, p<0.001). Fractional translocation comparing ratios of normoxia to hypoxia and individual time points (Figure 1c) revealed that the majority of this increase was evident at time points beyond 15 min. Importantly, this translocation occurred without significant changes in transepithelial resistance (TER in the range of 400-500 \( \Omega \) cm$^2$ at all times) or any increases in apparent permeability (Figure 1d, p>0.05), wherein TNF\( \alpha \) (10ng/ml, 48h), which is well established to increase epithelial permeability (Marano et al., 1998; Ma et al., 2005), served as a permeabilizing condition for these studies (p<0.005). Such findings reveal that hypoxia increases bacterial translocation in a manner independent of paracellular permeability.

To further examine whether hypoxia-induced translocation was paracellular or transcellular, we studied the role of epithelial metabolism on bacterial translocation. To do this, we diminished epithelial metabolic rates by lowering incubation temperatures during E. faecalis
translocation across epithelial monolayers, reasoning that transcellular processes, but not paracellular permeability, would not be influenced by decreased metabolic rates (Finlay et al., 1988). As shown in Figure 1e, bacterial translocation decreased with decreasing ambient temperature (p<0.01 by ANOVA), thereby implicating the transcellular and not the paracellular pathway for bacterial translocation.

**E. faecalis translocation is inhibited by antagonism of PAFr**

We next surmised that an apical surface receptor could contribute to the translocation of bacteria across intestinal epithelial cells. We approached the task of identifying candidate receptors by means of sterically hindering interaction of bacteria to putative binding sites with antibodies, antagonists or peptides known to interact with the surface protein. Within this screen, an antagonist for the Gram positive bacterial pattern recognition-receptor PAFr (Fillon et al., 2006), CV-6209, significantly reduced the translocation of E. faecalis (Figure 2a, p<0.01), and marginally reduced the translocation of S. mitis (Figure 2b), but not E. coli (Figure 2c) across both hypoxic and normoxic Caco-2 monolayers. Such inhibition of E. faecalis translocation across post-hypoxic monolayers occurred in a time-dependent manner (Figure 2d, p<0.005), thus implying that bacterial translocation of Gram negative and Gram positive species may be differentially regulated in hypoxia, and that PAFr may serve as a pathway for the translocation of Gram positive E. faecalis.

**The Gram positive pattern recognition receptor PAFr is hypoxia-inducible**

Based on our finding that the PAFr antagonist CV-6209 selectively attenuates E. faecalis translocation, we next examined the expression pattern of PAFr in Caco-2 cells. As shown in
Figure 3a, PAFr mRNA transcript was rapidly and prominently induced by hypoxia. Such induction was transient, wherein hypoxia induced PAFr at 2 and 4 hr (p<0.05) and measured levels were near baseline levels by 6 hr. We next examined protein expression of PAFr in hypoxic Caco-2 cells. After 24 hours in hypoxia, PAFr protein expression was increased (Figure 3b) and such induction in protein was shown to be significant by densitometry analysis (Figure 3c, p<0.005). *Analysis of apical and basolateral PAFr expression by differential biotinylation revealed that PAFr expression was predominantly apical in normoxia, but was expressed on both apical and basolateral membranes during hypoxia (Figure 3d). These findings confirmed that the kinetics of bacterial translocation correlates with the increased expression of PAFr in hypoxia.*

**PAFr is induced in epithelial cells during murine DSS colitis**

To place these findings in a physiologic context, we investigated the role of PAFr in an *in vivo* setting. Here, we examined PAFr mRNA expression in epithelial cells isolated from the colon of mice following induction of DSS colitis, conditions known to elicit “inflammatory hypoxia” (Karhausen et al., 2005; Shah et al., 2008). In this setting, PAFr was shown to be induced in DSS colitic mice in a concentration dependent manner (Figure 3e, p<0.01 by ANOVA). Furthermore, PAFr induction correlated with disease severity as measured by correlation of PAFr mRNA and the magnitude of animal weight loss (Figure 3f, p<0.05 by linear regression). These findings suggest that PAFr is induced in severe intestinal inflammation and may implicate that PAFr plays a role in sepsis as a result of inflammatory bowel disease.
Sh-RNA-mediated repression of PAFr attenuates translocation of E. faecalis

To further examine the direct role of PAFr in Gram positive bacterial translocation, we generated a shRNA PAFr repression Caco-2 cell line. Screening of these cells by real-time PCR showed significant repression of PAFr at the transcript level (Figure 4a, p<0.005) compared to non-target control and wildtype cells. Furthermore, hypoxia-inducible protein expression of PAFr was abrogated in PAFr knockdown cell lines exposed to hypoxia (Figure 4b, p<0.01 by densitometry). Upon confirmation of PAFr repression in the cell line, we performed bacterial translocation experiments on hypoxic and normoxic cells. This analysis revealed a significant reduction in the translocation of E. faecalis across hypoxic Caco-2 PAFr-shRNA monolayers (Figure 4c, p<0.01). Furthermore, the PAF antagonist CV-6209, did not further reduce the translocation of E. faecalis, suggesting that the reduction in translocation was PAFr mediated. Notably, the translocation of E. coli was not influenced by the repression of PAFr (ΔCFU/ml = -9.11x10^2 ± 5.44 x10^2 for Caco-2 PAFr-shRNA ), suggesting at least some degree of specificity for Gram positive bacteria.

In an attempt to define the association of PAFr expression and bacterial translocation, we examined the association of E. faecalis and Caco-2 monolayers by confocal microscopy. As shown in Figure 5a-c, a marked induction of PAFr expression in Caco-2 monolayers exposed to hypoxia (Figure 5b) over normoxic cells (Figure 5a). However, hypoxic PAFr shRNA knockdown cells (Figure 5c) showed little expression of PAFr and a reduced capacity for bacterial uptake compared to hypoxic scrambled monolayers. X-Z axis imaging of monolayers by confocal showed adhesion and internalization of E. faecalis, confirming transcellular entry of the bacteria (Figure 5d). Together, these findings demonstrate further the central role of PAFr in the translocation of Gram positive bacteria across hypoxic intestinal epithelial cells.
**Sh-RNA mediated repression of HIF-1 reduces PAFr expression and E. faecalis translocation**

We next investigated the molecular mechanisms of PAFr induction by hypoxia. As a global regulator of hypoxia, HIF functions as a central regulator of hypoxia-mediated gene expression. To address the role of HIF-1 in PAFr induction, and consequently E. faecalis translocation, we screened five separate lentiviral shRNAs directed against HIF-1α in Caco-2 epithelial cells. As shown in Figure 6a, Western blot analysis of individual lentivirus-transduced lines revealed significant reduction in HIF-1α in all lines under baseline conditions, but only shRNAs 3, 4 and 5 in hypoxia. There was no significant reduction in HIF-2α levels in any of these cell lines (Figure 6a). Based on these results, we proceeded with our analysis using the shRNA4 cell line.

We next examined PAFr transcript levels in the shRNA HIF-1α knockdown line. As shown in Figure 6b, this analysis revealed the specific loss of hypoxia mediated PAFr induction in response to hypoxia (p<0.005). Further examination of PAFr protein expression revealed a nearly complete loss in cells lacking HIF-1α compared to scrambled controls (Figure 6c). Upon verification of loss of PAFr induction in these cells, we performed bacterial translocation assays and revealed a significant reduction in the translocation of E. faecalis across Caco-2 cells lacking HIF-1α when exposed to hypoxia (Figure 6d).

We extended these findings to determine whether HIF-1α binds to the PAFr gene promoter in response to hypoxia. Sequence analysis of the proximal human PAFr promoter (from chromosome 16, NM_000952) revealed a potential binding site for HIF at positions -133bp relative to the transcription start site). As shown in Figure 5e, ChIP analysis of nuclei derived from Caco-2 cells revealed a prominent band of 153 bp from hypoxic samples. No bands were evident in control IgG immunoprecipitates, and input samples (pre-immunoprecipitation)
revealing the predicted 153 bp band under these conditions. Such results indicate that hypoxia induces HIF-1α binding to the distal 153 bp region of the PAFr promoter. Taken together, these results provide strong evidence for a functional hypoxia-inducible activity, mediated by HIF-1α, in the distal 5′-region the PAFr promoter.
Discussion

In this study, we aimed to elucidate whether conditions found in the inflamed mucosa might influence the translocation of non-invasive bacteria. These studies are founded on the observation that commensal bacteria and/or bacterial products appear to penetrate the mucosa and do so under conditions where the epithelium remains intact. Such translocation contributes to the activation of host immune defense mechanisms, subsequent autointoxication and tissue destruction (Meakins and Marshall, 1986). Here, we propose a mechanism by which non-invasive Gram positive \textit{E. faecalis} override host phosphorylcholine binding receptors (PAFr) during instances of acute inflammation and concomitant tissue hypoxia.

Our initial studies revealed a profound increase in non-invasive bacterial translocation across post-hypoxic intestinal epithelia. As has been demonstrated in the past (Furuta et al., 2001), intestinal epithelia appear to be uniquely tolerant to hypoxia with regard to barrier integrity, and our findings here were no exception. A broader examination revealed that while Gram negative \textit{E. coli} translocation was also increased, the magnitude was far greater for Gram positive \textit{E. faecalis}. This is interesting given the current state of nonsocomial infections. \textit{E. faecalis}, for example, has traditionally been regarded as an opportunistic pathogen, but has become increasingly important as an antibiotic-resistant strain of bacteria associated with hospital-acquired infections in the USA (2003; Hageman et al., 2003).

A search for potential membrane components which might contribute to increased bacterial translocation identified platelet-activating receptor (PAFr), an apically expressed G-protein coupled cell surface receptor (Claud et al., 2002). Indeed, the finding that the PAFr antagonist CV-6209 reduced the capacity of \textit{E. faecalis} to translocate across hypoxic Caco-2 monolayers was particularly interesting. In addition to it’s role in signaling via PAF, PAFr
functions as an innate immune recognition receptor phosphorylcholine moiety of Gram positive lipoteichoic acid (LTA) (Cundell et al., 1995; Fillon et al., 2006; Barbier et al., 2008), possibly mirroring the role of Toll receptors in Gram negative LPS recognition (Lemjabbar and Basbaum, 2002). Notable were differences in hypoxia-inducible translocation between various Gram positive bacterial strains and species. While we do not know the exact nature of these differences, it is well established LTA structures vary significantly between various Gram positive bacterial species and even within bacterial strains (Draing et al., 2006). It is possible, for example, that these differences in LTA structure could contribute to variability in bacterial uptake across the plasma membrane. Moreover, a number of studies suggest that PAFr may also mediate the recruitment of neutrophils and macrophages to the sites of inflammation and infection (Wallace, 1988; Hirayama et al., 2003; Han et al., 2006). Antagonistic blocking of the PAFr receptor has been shown to reduce the translocation of heat killed bacteria across Caco-2 monolayers and an M-cell co-culture model (Tyrer et al., 2006). Repression of the PAFr gene in Caco-2 monolayers similarly reduced \textit{E. faecalis} translocation across hypoxic monolayers.

Several levels of analysis in Caco-2 epithelial cells revealed a prominent, transcription-dependent induction of PAFr by hypoxia. These observations lead us to define the contribution of HIF to such induction. Studies using HIF-1$\alpha$ loss of function (sh-RNA) revealed a prominent role for HIF-1$\alpha$ in both PAFr induction and in bacterial translocation. Additional studies using ChIP analysis defined a binding site for HIF in the proximal PAFr promoter. PAFr has been shown to be a pathway for nasopharyngeal passage of pneumococci (Cundell et al., 1995) and has been involved the transcytosis of pneumococci through brain microvascular endothelial cells (Ring et al., 1998). More recently PAFr has been implicated in inflammatory response during pneumococcal pneumonia and colitis (Hirayama et al., 2003; Rijneveld et al., 2004). In these
E. faecalis translocation across epithelial barriers. Notable are the observations that epithelial HIF is protective during mucosal inflammation, particularly in murine models of colitis. Studies using conditional loss of intestinal epithelial HIF-1α (Karhausen et al., 2004a) and more recent pharmacologic studies strongly implicate an important adaptive role for HIF in mucosal inflammation. In the current studies, PAFr mRNA was increased in DSS colitic tissue and the degree of PAFr induction positively correlated the degree of disease (weight loss measurements).

It remains to be determined whether HIF-mediated, PAFr-dependent bacterial translocation represents a physiologic clearance mechanism or rather, serves as a pathophysiologic mechanism whereby bacteria exploit PAFr as a route of entry. An example of the former are provided by the observation that Pseudomonas utilizes apically-expressed cystic fibrosis transmembrane regulator (CFTR) in the lung as a route of entry and as a mechanism of bacterial clearance (Pier et al., 1997). Clinically-relevant mutants of CFTR which do not bind Pseudomonas fail to clear bacteria and result in overgrowth pneumonia in some patients with cystic fibrosis. Thus, it is possible that the PAFr functions as a membrane surface sensor, which when bound (e.g. by Gram positive LTA), results in internalization, translocation and clearance. Alternatively, PAFr may represent a previously unappreciated pathway for bacterial uptake and subsequent sepsis. Necrotizing enterocolitis (NEC), for instance, is a mucosal disease of unknown etiology which has been strongly associated with prematurity, enteral feeding and hypoxia (Caplan et al., 2005). Important for the current studies, a primary pro-inflammatory pathway linked to NEC is PAF and a number of studies in murine models have suggested that PAFr antagonism may be beneficial to the development of NEC (Caplan et al., 1997).
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Figure Legends

**Figure 1: Increased translocation of E. faecalis across hypoxic Caco-2 monolayers.** A) Influence of hypoxia on the translocation of a selection of Gram negative and Gram positive over 60 minutes. B) Influence of hypoxia on the translocation of E. faecalis over time. C) The rate of increased E. faecalis translocation with hypoxia. D) Apparent permeability of Caco-2 monolayers after infection, TNF-α (10ng/ml) is a positive control. E) Translocation of E. faecalis across Caco-2 monolayers is temperature dependent. Results are derived from 6 or more experiments. Hx: Hypoxia, Nx: Normoxia, *P<0.05, **P< 0.01, *** P< 0.005; Students t-test.

**Figure 2: PAFr antagonist CV-6209 (60 μM) prevents E. faecalis but not S. mitis or E. coli translocation across hypoxic Caco-2 monolayers.** A) Influence of CV-6209, a PAFr antagonist and C-PAF a PAFr agonist on the translocation of E. faecalis across Caco-2 monolayers . B) Influence of PAFr antagonism on the translocation of S. mitis across Caco-2 monolayers. C) Influence of PAFr antagonism on the translocation of E. coli across Caco-2 monolayers. D) Inhibition E. faecalis translocation by CV-6209 over time. Bacterial translocation over 60 minutes. Results are derived from 6 or more experiments. Hx: Hypoxia, Nx: Normoxia, **P< 0.01, *** P< 0.005; Students t-test.

**Figure 3: Induction of PAFr in hypoxic Caco-2 monolayers and in DSS colitis.** A) Induction of PAFr mRNA transcript over time in Caco-2 monolayers incubated in hypoxia . B) Expression of PAFr protein in Caco-2 monolayers after 24 hours in hypoxia 48kDa represents apical cell surface PAFr (verified by biotinylation, densiometry analysis 3191±248 pixels for apical PAFr
vs 319±254 pixels for basolateral PAFr) while the 69kDA band represents cytoplasmic PAFr. C) Densiometric analysis of PAFr protein expression by ImageJ pixel count. Results are derived from 3 experiments. D) Apical (ap) and basolateral (bas) expression of PAFr in hypoxic and normoxic Caco-2 monolayers. E) Induction of PAFr in DSS colitis. mRNA was isolated from epithelial cells from mice 6 days after induction of DSS colitis F) PAFr correlation with disease severity (weight loss) Results are derived from 3 experiments. For A)*P<0.05; One Way ANOVA, For B) P<0.05 Linear Regression Analysis. Hx: Hypoxia, Nx: Normoxia. *P<0.05, *** P< 0.005; Students t-test.

**Figure 4: ShRNA-mediated repression of PAFr in Caco-2 monolayers reduces translocation of E. faecalis.** A) PAFr is not induced by hypoxia in PAFr knockdown Caco-2 monolayers incubated in hypoxia . B) Loss of protein induction by PAFr knockdown Caco-2 monolayers after 24 hours in hypoxia . C) Translocation of E. faecalis across hypoxic and normoxic PAFr knockdown Caco-2 monolayers. CV-6209 (60 μM) does not reduced translocation across  PAFr knockdown Caco-2 monolayers. Bacterial translocation was over 120 minutes. Results are derived from 6 experiments Hx: Hypoxia, Nx: Normoxia. **P< 0.01, *** P< 0.005; Students t-test.

**Figure 5: E. faecalis adherence-to and invasion-of scr-shRNA and PAFr-shRNA Caco-2 monolayers.** A) Fluorescent staining of PAFr and E. faecalis on Caco-2 scr-shRNA monolayers B) Fluorescent staining of PAFr and E. faecalis on hypoxic Caco-2 scr-shRNA monolayers C) Fluorescent staining of PAFr and E. faecalis on hypoxic Caco-2 PAFr-shRNA monolayers. D) Internalization of E. faecalis by hypoxic hypoxic Caco-2 PAFr-shRNA monolayers over time.
Magnification at 40X for top-down fluorescent imaging and 63x for confocal X-Z imaging. Blue stain = DAPI nuclear stain (Molecular Probes), Red = anti-PAFr antibody (Cayman Chemicals), Green = BacLight™ labeled E. faecalis (Molecular Probes). Results are derived from 3 experiments and images are representative.

**Figure 6: Knockdown of HIF-1 in Caco-2 monolayers prevents PAFr induction in hypoxia and the translocation of E. faecalis.** A) Expression of both HIF-1α and HIF-2α isoforms in intestinal epithelial cells. Western blot analysis of HIF knockdown Caco-2 cells cultured for 16 hours under normoxic conditions (Nx) or in hypoxia (Hx) with the PHD inhibitor DMOG (0.5mg/ml). Nuclear lysates were immunopробed with antibodies against HIF-1α and HIF-2α. TATA-binding protein levels were monitored as a reference loading control. Highest level of repression was observed in the stable cell line harboring shRNA 4 (70% repression); this cell line was used in all further experiments. sh; short hairpin RNA. B) Loss of PAFr is not induced by hypoxia in HIF-1 knockdown Caco-2 monolayers incubated in hypoxia. C) PAFr protein induction is reduced in HIF-1 knockdown Caco-2 monolayers after 24 hours in hypoxia. D) Translocation of E. faecalis across hypoxic and normoxic HIF-1 knockdown Caco-2 monolayers. E) ChIP analysis to examine HIF-1 binding to the PAFr promoter in hypoxic Caco-2 cells. Reaction controls included immunoprecipitations using nonspecific IgG and beads only, as well as PCR performed using total Caco-2 DNA (Input). Results are derived from 3-6 experiments. Hx: Hypoxia, Nx: Normoxia. *P<0.05, **P<0.01, ***P<0.005; Students t-test.
Figure 2

**S. mitis**

**E. faecalis**

**E. coli**

**N. mitis**

**S. mitis**

**E. faecalis**

**E. coli**

**S. mitis**

**E. faecalis**

**E. coli**
Figure 3

A. Graph showing fold change in PAFr transcript over time in hypoxia (hours).

B. Western blot images of PAFr and β-actin under normoxic (Nx) and hypoxic (Hx) conditions.

C. Bar graph comparing ratio of β-actin:PAFr under normoxic and hypoxic conditions.

D. Western blot images of PAFr under apical (ap) and basolateral (bas) conditions under normoxic and hypoxic conditions.

E. Graph showing fold change in PAFr transcript with different percentages of DSS.

F. Scatter plot showing the relationship between peak % weight loss (%) and fold change in PAFr transcript, with a significant linear correlation (P<0.05).
Figure 4

A

Fold Change in PAFr Transcript

Wildtype Scr-shRNA PAFr-shRNA

B

PAFr -

β-actin -

Wildtype Scr-shRNA PAFr-shRNA

C

CFU/ml

Scr-shRNA PAFr-shRNA
A

<table>
<thead>
<tr>
<th>Nx</th>
<th>Hx + DMOG</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>![Image]</td>
</tr>
<tr>
<td>HIF-2α</td>
<td>![Image]</td>
</tr>
<tr>
<td>TATA BP</td>
<td>![Image]</td>
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</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Fold Change in PAFr Transcript</th>
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</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>![Graph]</td>
</tr>
<tr>
<td>Scr-shRNA</td>
<td>![Graph]</td>
</tr>
<tr>
<td>HIF-1-shRNA</td>
<td>![Graph]</td>
</tr>
</tbody>
</table>

C

- **PAFr**
  - Scr-shRNA: ![Image]
  - HIF-1-shRNA: ![Image]
- **β-actin**
  - Nx: ![Image]
  - Hx: ![Image]

D

<table>
<thead>
<tr>
<th>Translocated Bacteria (CFU/mL)</th>
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</thead>
<tbody>
<tr>
<td>![Graph]</td>
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</tbody>
</table>

E

- ![Image]