Role for the Bacterial Signal Indole in Promoting Epithelial Cell Barrier Function

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Abstract: Interkingdom (IK) signaling, in which pathogenic bacteria recognize human hormones in the gastrointestinal tract to initiate infection, has recently emerged as an important contributor to gastrointestinal tract infections. While a majority of studies have primarily focused on the effect of host molecules on pathogens, recent work from our lab has demonstrated that human intestinal epithelial cells can also recognize bacterial signaling molecules. We demonstrated that indole, secreted to ~ 500 μM by commensal *Escherichia coli,* increased expression of tight junction proteins and genes involved in mucin production in HCT-8 intestinal epithelial cells, thus enhancing their barrier properties. These results were consistent with an observed increased in trans-epithelial resistance on exposure to indole over 24 h. Since indole is structurally similar to melatonin, and melatonin is a hormone that regulates the dark-cycle in the eye, we also investigated role of indole on human retinal pigment epithelium (RPE) cells. Our data show that indole increases trans-epithelial resistance of RPE cells, and attenuated their proliferation and migration. Our results suggest a beneficial role for indole in the treatment of eye diseases, and are discussed in detail in this article.

Keywords: Indole, Inter-kingdom signaling.

INTRODUCTION

The human gastrointestinal (GI) tract is home to ~ 10^{14} bacteria belonging to more than 400 known species [1-4], and bacterial cells out-number human cells by a factor of 10 in the GI tract. As a result, the GI tract is rich in a diverse range of eukaryotic and bacterial signaling molecules. The bacterial signaling molecules present include autoinducer-2 [5], autoinducer-3 [6] and indole [7], while eukaryotic molecules include the neuroendocrine hormones such as norepinephrine and dopamine that are locally produced by the enteric nervous system [8]. It is becoming increasingly evident that the close proximity of different kinds of signals and cells belonging to different kingdoms leads to cross-signaling i.e., recognition of bacterial signals by host cells and vice-versa. This cross-recognition of signals has been termed as interkingdom signaling, and is considered an important facet of infection or homeostasis.

Lyte *et al.,* [9] initially demonstrated interkingdom signaling in bacteria when exposure to norepinephrine increased the production of Shiga-like toxins by enterohemorrhagic *E. coli* (EHEC). Sperandio *et al.,* [6] also observed a similar increase in EHEC virulence when grown in the presence of epinephrine and norepinephrine. Previous work from our group [10] showed that both epinephrine and norepinephrine increase EHEC chemotaxis, motility, biofilm formation, and colonization of human epithelial cells. These results were consistent with the increase in EHEC virulence gene expression. These studies provide sufficient evidence for the prevalence of interkingdom signaling from the pathogen side, i.e. recognition of host signals by pathogenic bacteria. However, not many studies have established the occurrence of bacterial signal recognition by the host cells, and its role in the GI tract infection process. Several studies have implicated probiotic culture supernatants in attenuating pathogen infection [11, 12] and modulating infection [13, 14]. However, specific molecules responsible for such protective actions are yet to be identified. In contrast, quorum sensing molecules such as the *N*-(3-oxododecanoyl)-*L*-homoserine lactone produced by *Pseudomonas aeruginosa* have been shown to be deleterious to host cells, in that it helps promote persistent infection by disrupting cell barrier properties, and up-regulating inflammatory pathways [15-17].

Indole is produced in large amounts by commensal *E. coli* [18] in suspension cultures (600 μM) and has been detected in human feces at 250-1100 μM [19, 20], suggesting the presence of copious amounts of indole in the GI tract. We recently demonstrated that indole is an interkingdom

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signaling molecule recognized by intestinal epithelial cells [21]. We studied changes in the intestinal epithelial cell line HCT-8 transcriptome on exposure to 1 mM indole. At 24 h, indole upregulated tight junction, gap junction and adherens junction genes, suggesting an increase in cell barrier properties. Indole also increased polarized HCT-8 cell trans-epithelial resistance (TER), corroborating the microarray data.

Indole is structurally similar to the indoleamine hormone melatonin (Fig. **1**), which is synthesized in the retina and follows a diurnal rhythm with peak levels occurring at night [22]. Melatonin is involved in the photoreceptor outer segment disc shedding, retinal pigment epithelium (RPE) phagocytosis, photomechanical movements [22], modulation of dopamine release, and circadian changes in intraocular pressure [23]. Melatonin is also a regulator of aging and senescence, controls sexual maturity, sexual cycling, cancer stress, immune response [24], functions as an oncostatic molecule [25], and is an anti-proliferative agent in RPE cells [26]. Hence, we hypothesized that indole regulates RPE cell barrier properties similar to intestinal epithelial cells. We discovered that indole affects RPE trans-epithelial potential (TEP) and increases TER over 24 h. Similar to melatonin, indole also reduced RPE cell proliferation and migration. Our results imply a potential role for indole in modulating cell barrier properties of several different types of epithelial cells.

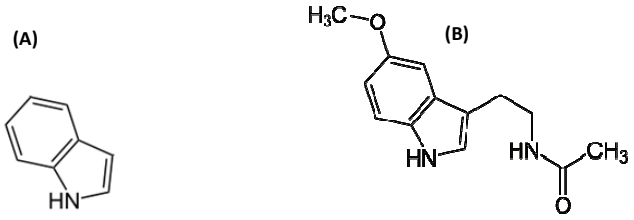

Fig. (1). Chemical structures of indole (**A**) and melatonin (**B**).

RESULTS

Effects of Indole on Intestinal Epithelial Cell Transcriptome

Global changes in gene expression of HCT-8 intestinal epithelial cell line on exposure to 1 mM indole for 24 h were determined [21]. Statistically significant changes in gene expression with indole were calculated at $P < 0.01$ relative to the solvent-only control. Using this approach, a total of 4,102 genes (~ 8% of the HCT-8 transcriptome) was classified as differentially regulated by indole. The 3,083 annotated genes in this dataset were further classified into various functional categories. Several pathways that were populated by this approach belonged to metabolism, DNA repair, protein synthesis, inflammatory and immune response, signaling, and transport (Fig. **2**). The focus of this article is only on genes regulating cell barrier properties (Table **1**). The complete list of differentially regulated genes is available from Gene Expression Omnibus with the accession number GSE14379 in [ref. 21].

Effects of Indole on Intestinal Epithelial Cell Barrier Properties

Genes involved in tight junction regulation, actin cytoskeleton, mucin production, and adherens junction showed increase in expression in presence of indole; thus, suggesting strengthening of overall cell barrier properties (Fig. **3A**). Members of the claudin family of proteins are responsible for tight junction barrier formation, and control paracellular pathway between cells [27]. Claudins are additionally responsible for maintaining polarity of the epithelial cells. Indole upregulated expression of seven claudin genes, implying increased paracellular resistance. Additionally, indole reduced expression of pore-forming claudin-2, which increases paracellular ion permeability [28]. Other major tight junction proteins are zona occludens (ZO), which are present downstream of claudins. Indole increased expression of

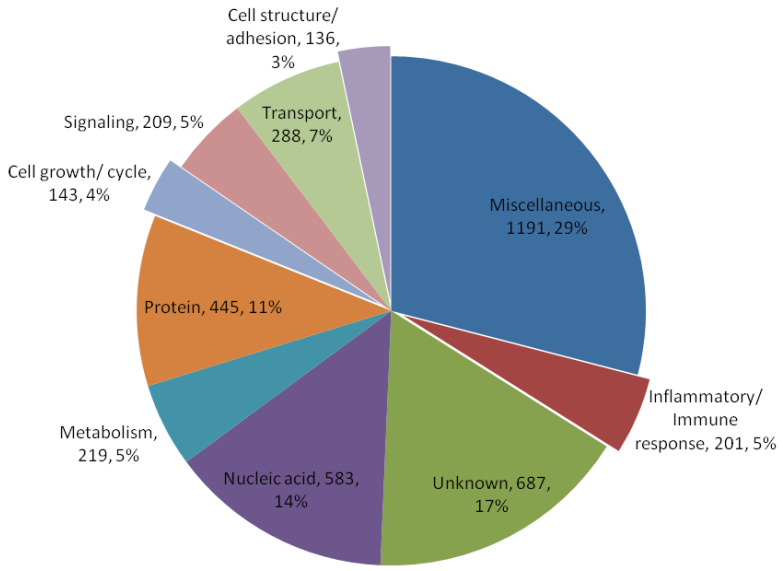

Fig. (2). Differentially regulated HCT-8 genes in presence of 1 mM indole. HCT-8 cells were exposed to 1 mM indole for 24 h, and as a control, solvent-only treated cells were collected. ~ 8% of HCT-8 transcriptome was differentially regulated by indole. The diagram highlights such several important pathways. The three entries in each data label represent, in order, functional category, number of genes differentially expressed, and % of total differentially expressed genes. Reproduced with permission from [21]. ©National Academy of Sciences.

Table 1. Classification of Genes Differentially Regulated on 24 h Exposure to 1 mM Indole

Classification	Pathway	Total Genes in Pathway	Genes Differentially Expressed by Indole
Cell structure/ adhesion	Adherens junction	75	19
	Cell adhesion molecules (CAMs)	133	38
	Gap junction	96	17
	Regulation of actin cytoskeleton	211	50
	Tight junction	135	29
	Cytokine-cytokine receptor interaction	259	45
Cell growth/cycle	Apoptosis	84	23
	Cell cycle	112	25

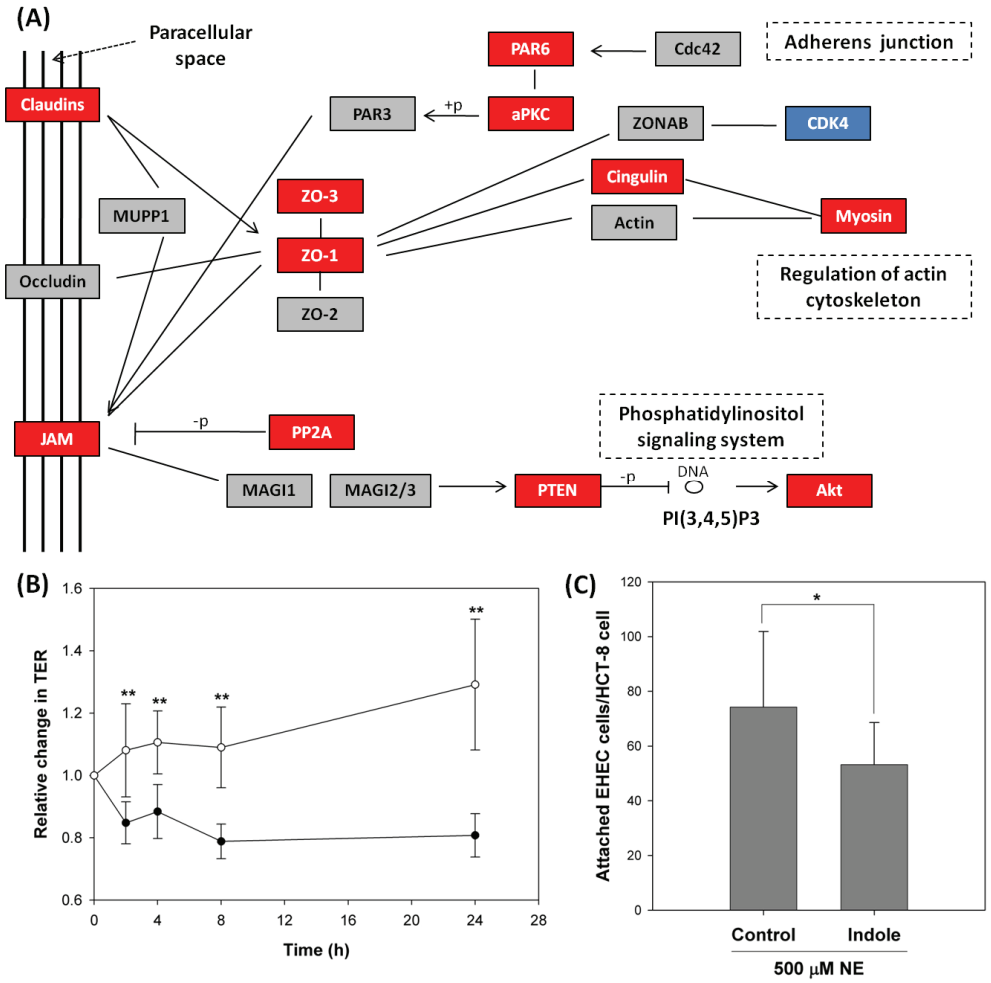

Fig. (3). Changes in HCT-8 tight junction proteins and TER. (**A**) Pathways containing differentially expressed genes involved in tight junction formation were adapted from Pathway Express using KEGG classifications. Red, up-regulation; blue, down-regulation; gray, no change in expression; arrow, molecular interaction leading to activation; blunt line without an arrowhead, molecular interaction leading to inhibition. The pathway scheme shown is based on microarray data and does not include posttranscriptional regulation. (**B**) Changes in the TER of polarized HCT-8 cells exposed to either solvent (filled circles) or indole (open circles) for 24 h. Data represent mean ± SD from seven measurements at each time point and two independent experiments. (**C**) Effect of indole pretreatment on adherence of EHEC to HCT-8 cells in the presence of 500 μM norepinephrine. Data represent mean ± SD from three wells per experiment and three independent experiments. *, statistical significance, using Student's t test, at $P < 0.05$; **, significance at $P < 0.005$. Reproduced with permission from [21]. ©National Academy of Sciences.

ZO-1, -3, and -4, along with gap junction (GJE) proteins GJE-1, -3, -4, and -8. Fifty genes involved in actin cytoskeleton rearrangement and several genes responsible for mucin production were also upregulated. Mucins are high molecular weight glycoproteins important in epithelial cell barrier formation, as Kim *et al.*, [29] have reported that *Lactobacillus acidophilus* reduces the EHEC colonization of HT-29 intestinal epithelial cells by increasing the production of mucin-2. Overall, these gene expression changes imply that indole increases epithelial cell resistance to pathogen colonization.

Phenotypic measurement of trans-epithelial resistance (TER) of polarized HCT-8 cells showed that over a 24 h period, indole increased TER by 1.6-fold (Fig. **3B**). Additionally, pre-treatment of HCT-8 cells with indole reduced norepinephrine-mediated EHEC colonization by 1.4-fold (Fig. **3C**). These results corroborate the microarray data and advocate the importance of indole in promoting epithelial cell barrier properties and defense.

Effects of Indole on Retinal Pigment Epithelium Physiology

As discussed earlier, indole is structurally similar to hormone melatonin present in the eye. Apical perfusion of melatonin has previously been reported to reduce chick retinal pigment epithelium (RPE) trans-epithelial potential (TEP) through depolarization of apical membrane [30]. Hence, we studied the effects of indole on RPE electrophysiology. Native human fetal RPE (hfRPE) cells were extracted and cultured in flasks as described previously [31]. Polarized RPE cells were mounted in a modified Üssing's chamber and intracellular pH (pH_i), TEP, and total tissue resistance (R_t) were monitored according to the established protocol [32]. Application of apical or basolateral 1 mM indole for 2 minutes did not affect the pH_i or the R_t of the RPE (Fig. **4A**). Basolateral perfusion of indole did not affect TEP, but apical perfusion of indole reduced TEP by 1.2 ± 0.2 mV, and similar results (0.95 ± 0.23 mV) were obtained when indole was perfused in both apical and basolateral chambers (Fig. **4B**), as confirmed by data from three independent biological replicates.

Since indole increased TER of intestinal epithelial cells, we investigated its effect on RPE TER. After 24 h, while solvent-only controls showed no statistically significant change in TER, indole treated cells showed a 1.25-fold statistically-significant increase in TER (Fig. **4C**). These re-

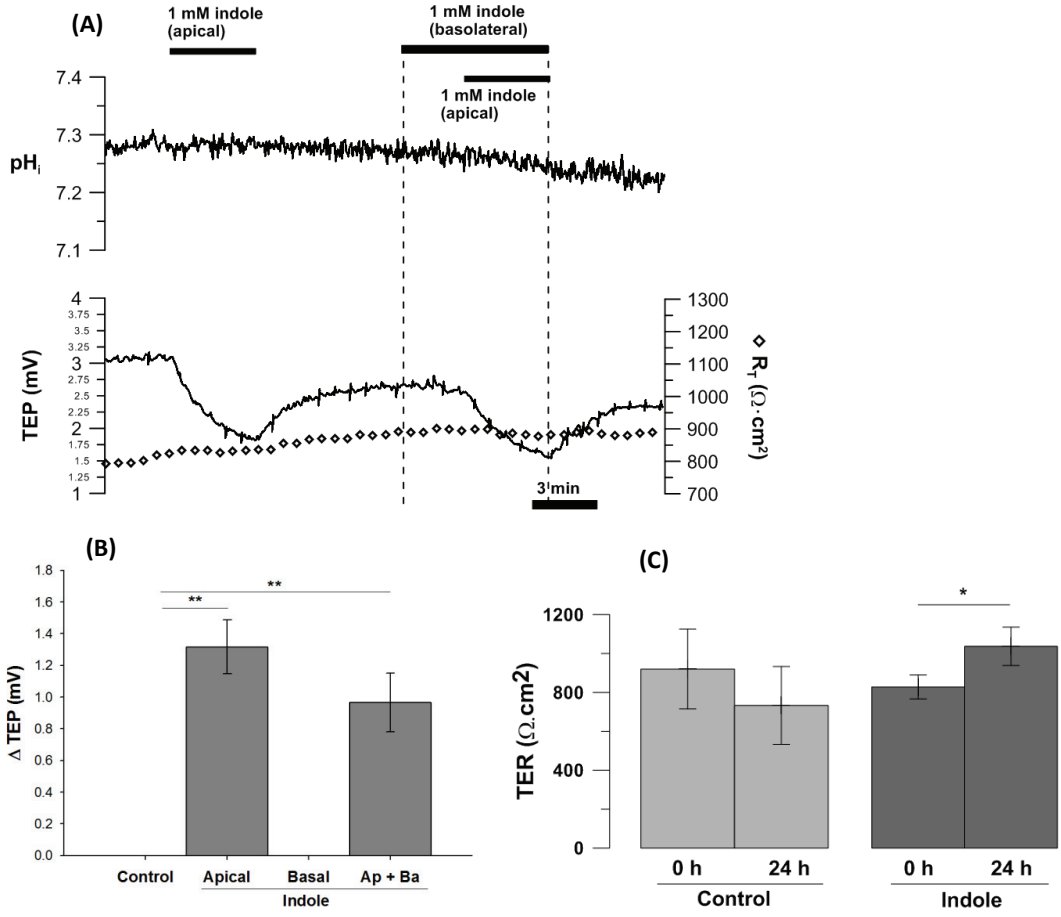

Fig. (4). Changes in RPE TEP and TER on exposure to 1 mM indole. (**A**) Polarized RPE cells were incubated with intracellular pH-indicator dye BCECF. The cells were then mounted on modified Ussing's chamber and were perfused with modified Ringer's solution on apical and basolateral sides. After reaching steady TEP in control solutions, 1 mM indole was introduced at the apical, basolateral or both sides, and the changes in TEP, R_t, and pH_i were recorded. A representative experiment is shown here. (**B**) Average change in TEP with indole. Data represent mean ± SD from three independent experiments. (**C**) Polarized RPE cells were incubated with 1 mM indole or solvent-only control for 24 h and changes in TER were recorded using electrovoltohmmeter. Average change in TER is shown here. Data represent mean ± SD from three independent experiments. *, statistical significance, using Student's t test, at $P < 0.05$; **, significance at $P < 0.005$.

sults, although smaller in magnitude than those obtained with intestinal epithelial cells, suggest an increase in RPE tight junction properties in presence of indole.

Effects of Indole on RPE Migration and Proliferation

Melatonin is reported to suppress proliferation of bovine RPE cells [26]. The authors noted that when human epidermal growth factors (hEGF)–stimulated bovine RPE cells were treated with 10 – 500 pg/ml melatonin, the proliferation of cells reduced in a dose-dependent manner. We studied the impact of indole on migration and proliferation of hfRPE cells. A sterile pipette tip was used to create a 100 – 200 µm scratch on the surface of polarized RPE monolayer to mimic injury. The cells were then mounted on Zeiss Observer microscope (Carl Zeiss MicroImaging LLC, Thornwood, NY), fitted with an incubation chamber and an automated stage. AxioVision 4.8 software (Carl Zeiss) was used to automatically capture images every hour around the edge of the scratch. After 24-48 h, total distance traveled by cells and speed of cell migration per hour were calculated.

As seen in Fig. (**5A**), 1 mM indole significantly inhibited RPE migration and proliferation over 24 h time period. The inhibitory effect was discernible as early as 6 h into the experiment and reached statistically significant values after 12 h (Fig. **5B**). At the end of 24 h, while solvent-only control RPE cells had migrated and proliferated (39.27 ± 9.84 µm) to cover the entire scratch, indole-treated cells had migrated through a very small distance (14.11 ± 6.72 µm). Thus, these experiments provide a strong evidence of anti-proliferative activity of indole, similar to melatonin.

DISCUSSION

To date, little is known about beneficial bacterial signals recognized by host cells through interkingdom signaling. Several studies report specific pathogenic bacteria signaling molecules that are deleterious to the host cells and help maintain persistent infection. Most of these reports suggest disruption of the epithelial cell barrier properties, through disruption of tight junction proteins and reduction of trans-epithelial resistance, as the mechanism of initiation of infection. *P. aeruginosa* quorum sensing molecule *N*-(3-oxododecanoyl)-*L*-homoserine lactone decreases TER [15] and reduces expression of the JAM-A and ZO-3 tight junction proteins in Caco-2 cells through intracellular Ca^{2+} signaling [33]. Similar mechanism of action of *P. aeruginosa* was reported in bronchial epithelial cells where reduction in TER and disruption of ZO-1 were observed during infection [34]. Strauman *et al.,* [35] showed that enteroaggregative

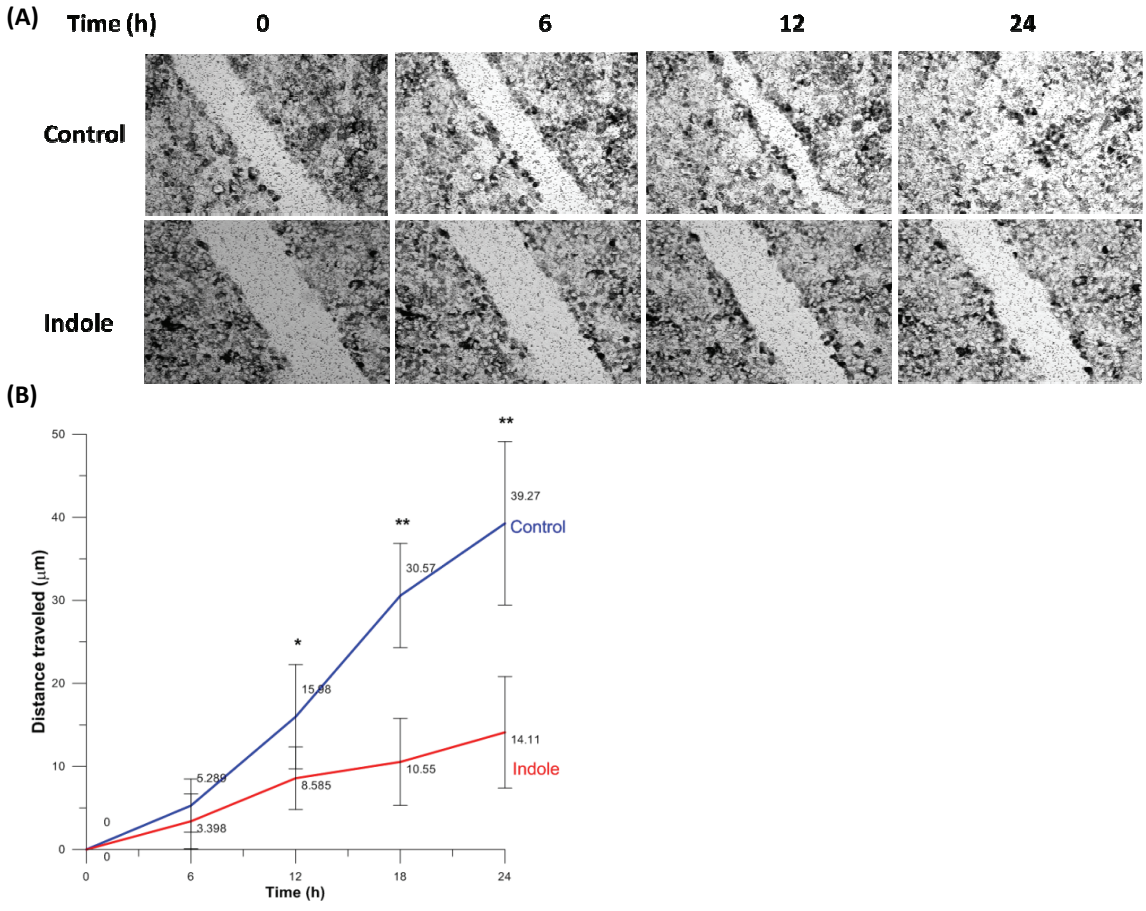

Fig. (5). Changes in RPE cell migration and proliferation with 1 mM indole. A sterile pipette tip was used to create a scratch on the surface of polarized RPE cells. The cells were then incubated in either 1 mM indole or solvent-only control. The migration and proliferation of cells over 24 h was recorded. (**A**) A representative image from one such experiment. (**B**) Average distance traveled by RPE cells over 24 h. Data represent mean ± SD from three independent experiments. *, statistical significance, using Student's t test, at $P < 0.05$; **, significance at $P < 0.005$.

E. coli (EAEC) disrupt TER of polarized T84 intestinal epithelial cells, similar to that seen with *Salmonella*, through the production of AAF/I adhesin, as an *aafA* null mutant of EAEC failed to reduce TER. A concurrent increase in paracellular permeability, and disruption of tight junction proteins ZO-1 and claudin-1 was observed with the EAEC infection of T84 cells *in vitro*.

Probiotic cultures and supernatants are reported to enhance epithelial cell barrier function or attenuate pathogen virulence in several studies. *Bifidobacteria infantis* cell-free spent medium increased T84 cell TER and reduced the paracellular permeability [36]. Enhanced protein production of claudin-4, ZO-1, and occludin, and reduction of pore-forming claudin-2 was also observed under these conditions. *In vivo*, oral administration of *B. infantis* spent medium attenuated colonic permeability in mice [36]. *L. plantarum* attenuated enteroinvasive *E. coli* infection by restoring the TER of epithelial cells and preventing redistribution of tight junction proteins [37]. Probiotics *L. rhamnosus* GG [38], *L. acidophilus* and *Streptococcus thermophilus* [39] have been reported to antagonize infections by increasing TER and repressing pro-inflammatory signaling. However, specific signals responsible for such protective actions of probiotics are not known. The data presented in our previous study [21] imply that indole-mediated increase in TER and tight junction gene expression, and decrease in pathogen colonization is similar to the one seen with probiotic bacteria. Indole is produced by several GI tract commensal bacteria species like *E. coli, Bacteroides* and *Clostridia* [40], and it is possible that the beneficial effects of probiotics are mediated, in part, through indole.

Not much data exist in literature on the effects of either indole or the indoleamine melatonin on the RPE cell electrophysiology or barrier function. In the human RPE cells, as seen with intestinal epithelia, exposure to indole increased TER, suggesting that this signaling molecule is not tissue specific and causes an improvement in cell barrier properties in various epithelia. Apical melatonin is reported to depolarize chick RPE apical membrane; thus, reducing overall TEP [30], and it is speculated that apical K^+ conductance is decreased in the presence of melatonin. Basolateral perfusion of melatonin hyperpolarizes both membranes and it hyperpolarizes basolateral membrane more, resulting in overall TEP decrease [30]. In our study, apical perfusion of indole caused a similar reduction in TEP, suggesting that indole might act through a similar mechanism, while basolateral indole caused no TEP change. Overall, more research needs to be done to determine the specific ion channels, transporters and exchangers affected by indole.

Multiple studies have reported anti-proliferative properties of melatonin in several different cell types. In bovine RPE cells, nuclear density was doubled in presence of hEGFs. Treatment of hEGF-induced cells with 500 pg/ml melatonin reduced the nuclear density to control levels [26]. Additionally, proliferation of mitotically active SV40 transformed human fetal RPE cells was significantly reduced in presence of melatonin. The authors reported that melatonin, unlike 5-fluorouracil and daunomycin, was anti-proliferative in mitotically active RPE cells, but not on normal confluent cells; thus, highlighting a possible use for melatonin in cancer therapy. Indeed, melatonin-mediated inhibition in mitotically active breast cancer MCF-7 cells has been observed [41], and that melatonin exerts its antitumor effect by delaying mitosis in MCF-7 cells [42]. It has been observed that melatonin levels are reduced in aged individuals that suffer from prostate cancer, and melatonin reduces proliferation of prostate cancer cells, suggesting a connection between the two events [43]. Similar results have been reported in human choriocarcinoma JAr cells, where melatonin inhibits proliferation and delays G1/S cell cycle transition [44]. Our data are consistent with above mentioned studies and imply that indole, similar to melatonin, is an anti-proliferative agent in the RPE cells. This observation is clinically important since the functionally differentiated RPE cells in adults are non-proliferative [45]. However, in retinal diseases such as proliferative vitreoretinopathy (PVR) and exudative age-related macular degeneration, RPE cells begin to migrate and proliferate [46]. Thus, arresting RPE migration and proliferation becomes an important therapeutic target, and it is intriguing to speculate that indole can be a promising candidate.

CONCLUSION

In conclusion, current data strongly suggest the role of indole as a beneficial interkingdom signaling molecule in the GI tract. Currently, no drug-based treatment exists for chronic intestinal inflammatory diseases, and our data provide promise for new indole-based therapy to promote epithelial cell barrier function. Additionally, preliminary data with indole on RPE cells suggest the potential application of indole as an anti-proliferative agent in the treatment of the diseases of the eye. Together, these results are indicative of the promise for indole and indole-like molecules as novel therapeutic modalities designed to promote epithelial cell barrier properties and function.

CONFLICT OF INTEREST

None declared.

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