Prostaglandin E₂ produced by *Entamoeba histolytica* binds to EP4 receptors and stimulate IL-8 production in human colonic cells

Running title: Induction of IL-8 by *E. histolytica*

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Abstract

The pathogenesis of *Entamoeba histolytica* in the colon occurs in a step-wise fashion beginning with colonization to the mucin layer followed by stimulation of a pro-inflammatory response that cause non-specific tissue damage that may facilitate parasite invasion to the underlying colonic mucosa. Unfortunately, the parasite and/or host factors that stimulate a pro-inflammatory response in the gut are poorly understood. In this study, we show that live *E. histolytica* or secretory (SP) and soluble ameba components (SAP) can markedly increase interleukin-8 (IL-8) mRNA expression and protein production in colonic epithelial cells. The IL-8 stimulatory molecule produced by live ameba was identified as PGE$_2$ as trophozoites treated with cyclooxygenase inhibitors inhibited the biosynthesis of PGE$_2$ and abrogated IL-8 production induced by live parasites or ameba components. Moreover, using specific prostaglandin EP2 and EP4 receptor agonists and antagonists we have identified that PGE$_2$ binds exclusively through EP4 receptors in colonic epithelial cells to stimulate IL-8. Silencing of EP4 receptors with EP4 siRNA completely abrogated SP- and SAP-induced IL-8 production. These studies have identified bioactive PGE$_2$ as a one of the major virulent factor produced by *E. histolytica* that can stimulate the potent neutrophil chemokine and activator IL-8, which can trigger an acute host inflammatory response. Thus, the induction of IL-8 in response to *E. histolytica*-derived PGE$_2$ may provide a mechanism to explain the initiation and amplification of acute inflammation associated with intestinal amebiasis.
Introduction

*Entamoeba histolytica* is an enteric protozoan parasite and the fourth leading cause of death by a parasitic (1). Humans are the only known host for *E. histolytica* and about 50 million people are affected worldwide each year. The pathogenesis of amebiasis is believed to be a multistep and multifactorial process. Though a large number of studies have attempted to unravel the factors/molecules responsible for the pathogenesis of amebiasis, the processes involved in pathogenesis are not well understood. In most infected individuals, *E. histolytica* trophozoites exist as commensals. However, in a small percentage of infections, ameba can elude luminal and epithelial barrier host defense mechanisms and invade the intestinal mucosa causing ulcers and amebic colitis. Even though host inflammatory responses play an important role in the onset and progression of invasive amebiasis, little is known on the parasite factors that initiate this event. Even less is known on the parasite components that are secreted or released in the gut that can modulate colonic epithelial cell functions.

Some of the important molecules that are involved in the pathogenesis of intestinal amebiasis have been identified. For example, *E. histolytica* trophozoites bind with high affinity to Gal and GalNAc residues on mucus glycoproteins by using their surface adherence N-acetyl-D-galactosamine specific lectin (Gal-lectin, 2) in colonization. The Gal-lectin also mediated binding to target cells where ameba pore-forming proteins (3) can be inserted into lipid bilayers of target cells forming ion-channels and subsequently causing cell death. We have recently showed that cysteine proteases 5 secreted by *E. histolytica* specifically cleaves the C-terminal polymerization domain of mucin polymer and dissolves the protective mucus layer (4). This process allows *E. histolytica* to come in direct contact with epithelial cells. In addition to direct
cytolysis of host cells by ameba, the parasite also activates host epithelial cell immune responses by a contact dependent and independent manner. Lysed epithelial cells release pre-IL-1β, which is processed by ameba cysteine proteinases to its active form (5). Studies in the SCID–human mouse models of intestinal amebiasis have shown stimulation of additional inflammatory mediators including IL-6, growth-related oncogene α (GRO-α), cyclooxygenase 2 (COX-2) and granulocyte–macrophage colony-stimulating factor (GM-CSF) by adjacent intestinal cells through nuclear factor kB dependent signaling pathway (6, 7). Collectively, all these events cumulate into tissue destruction and subsequent tissue invasion by ameba in the colon.

The hallmark of amebiasis is characterized by infiltration of inflammatory and immune cells in the amebic lesions (8). We hypothesize that the release of IL-8 by colonic epithelial cells is a major factor that can initiate the onset of inflammation. IL-8 is a potent chemoattractant and activator for neutrophils, which can cause nonspecific tissue damage after activation (4). IL-8 is a member of CXC family of chemokines with a molecular weight of 8-10 kDa and is activated after cleavage of 20 amino acid signal sequences. A variety of cells including macrophages, T-lymphocytes, epithelial cells and neutrophils produce IL-8. We have shown (9) that *E. histolytica* synthesizes PGE₂ through a novel COX-like enzyme that is believed to play a major role to maintain cell cycle in ameba. However, the mechanism of IL-8 induction by ameba PGE₂ is not known during invasive amebiasis and it is also not clear if ameba components themselves can directly induce this chemokine in the gut. Herein, we shown that endogenously synthesized PGE₂ by live *E. histolytica* or its presence in soluble ameba proteins (SAP) or in secretory components (SP) can induce IL-8 production by a unique pathway involving EP4 receptors on colonic epithelial cells.
Materials and Methods

Cells, reagents and ameba components. The human adenocarcinoma cell line Caco2 was obtained from the ATCC and grown to confluent monolayers in MEM medium containing 5% fetal bovine serum and 5 mg/ml penicillin-streptomycin. EP receptor specific agonists and antagonists were obtained from Cayman Chemicals unless indicated otherwise. Soluble amebic proteins (SAP) were prepared by three cycles of freeze-thaw lysis of log phase E. histolytica virulent strain HM1:IMSS (sub passaged three times through gerbil livers) and quantified by the BCA protein assay (Pierce). E. histolytica secretory components/proteins (SP) were prepared as described previously (4). For transwell studies, trophozoites were added to Corning transwell inserts with a pore diameter of 0.6 µm, with Caco-2 cells in the bottom well.

Real-time PCR. Total RNA was extracted with TRIzol reagent (Invitrogen) and quantified. One µg of RNA was reverse transcribed by MMLV reverse transcriptase (Invitrogen) and oligo(dT) as per the manufacturer’s instructions. One tenth of cDNA reaction mixture was used for real-time PCR. Amplifications were carried out with Quantitech SYBR green PCR kit (Qiagen) at the following cycling conditions: 94°C hold for 15 min, followed by 45 cycles of denaturation at 94°C for 20s, annealing at 60°C for 30s and extension at 72°C for 60s. Primers used were IL-8 forward 5’CGTGGCTCTCTTGGCAGC3’, IL-8 reverse 5’TCTTTAGCACTCCTTGGCAAC3’, GAPDH forward primer 5’GAAGGTGAAGGTCGGAGT3’ and GAPDH reverse primer 5’GAA GATGGTGATGGGATTTC3’. Specificity of amplification was checked by melt curve analysis. IL-8 mRNA expression was normalized against GAPDH mRNA. Change (n-fold) over control levels was determined according to the comparative cycle threshold method as described (10).
Co-culturing of epithelial cells with ameba. Confluent Caco2 cells (10^6) grown in either regular or transwell plates for 7–10 days were used for all studies. For siRNA experiments, subconfluent (40-50%) Caco2 cells grown for 2-3 days were used. Transwells with 2x10^6 amebae were incubated in culture plates containing 10^6 epithelial cells for 24 h at 37°C, then removed from co-culture and immediately used for subsequent experiments. Epithelial cells were kept under low serum condition (5% fetal bovine serum) during co-culturing and subsequent treatments and without antibiotics for siRNA studies.

IL-8 assays. IL-8 production was measured by Titerzyme kit (Assay designs Inc.) using a monoclonal antibody against human IL-8 as per the manufacturer’s instructions.

Stimulation of cells with agonist, antagonist and inhibitors. Cells were seeded in 6-well plates (10^6 cells/well) and allowed to attach overnight. Prior to the experiments, cells were fasted in serum free medium overnight followed by stimulation with or without PGE_2 along with or without other drugs. Cells were pretreated with AH6809 (EP2 receptor antagonist) or AH23848/L161982 (EP4 receptor antagonist) for 30-60 min prior to PGE_2 or EP receptor specific agonists stimulation. Cells were then processed for RNA extraction by the TRIZol method for real time PCR analysis or used for other studies. All compounds were used at a final concentration of 1 µM except AH6809, which were used at 50 µM. The concentrations of PGE_2 and several EP receptor specific agonist and antagonist used in the experiments were optimal for intestinal epithelial cells, as determined by our previous studies and by others (11-14).
RNA Interference. Wild type Caco2 cells were transfected with EP4 siRNA (Smartpool M-005714-00) or control siRNA (D-001210-02, D-001140-01) from Dharmaco, Inc. (Lafayette, CO) as per manufacturer’s protocol. Briefly, subconfluent (50–60%) cells were transfected using X-Treme Gene siRNA transfection reagent (Roche) for 36h at a siRNA concentration of 40 nM and cells were immediately used for subsequent experiments.

Statistical analysis. Data are represented as means ± standard error (SE) of the means and were analyzed using Student’s t test for unpaired data using Graphpad Prism software. P values ≤ 0.05 are considered significant.
**Results**

*Induction of IL-8 by* E. histolytica. To determine the kinetics whereby *E. histolytica* induces IL-8 production in colonic epithelial cells, we treated confluent Caco2 cells (10^6) with different concentrations of secreted protein (SP) or soluble amebic protein (SAP) or with live ameba. Treatments or co-culture with live ameba was carried out in transwell plates in a contact independent manner.

As shown in Figure 1A/B, there was a dose-dependency for the induction of IL-8 mRNA expression and protein production. Maximum stimulation occurred with 40µg/ml SP which significantly (p>0.001) induced both IL-8 mRNA expression (5 fold) and protein production (9 fold) over untreated controls. With SAP, peak stimulatory responses occurred with 100µg/ml by inducing 7- and 11-fold IL-8 mRNA and protein production over control, respectively. Higher doses of SP and SAP did not induce IL-8 production significantly compared to 40µg/ml of SP and 100µg/ml of SAP respectively. Using these protein concentrations we then determined the time-dependency for IL-8 mRNA and protein production. As shown in Figure 2 A/B, 40µg/ml of SP and 100µg/ml of SAP induced both IL-8 mRNA and protein production significantly (p>0.001) over controls in a time-dependent manner with peak mRNA expression occurring after 4 h and IL-8 protein production after 12 h, respectively. Interestingly, with live *E. histolytica* trophozoites in transwells separated from colonic cells by membranes also significantly stimulated IL-8 mRNA expression and IL-8 production at 6-, 8- and 12-h respectively, as compared to untreated controls. During these co-culture time points, about 90% of ameba were alive as determined by trypan blue exclusion assay (data not shown).
E. histolytica induces IL-8 production through a lipid molecule. To determine the biochemical characteristic of the ameba-derived component(s) that stimulated IL-8, we boiled SP, SAP and PGE₂ for 30 min or delipidized the ameba components with choroform:methanol (2:1 v/v, 11) prior to testing. As shown in Figure 3, SP, SAP, PGE₂ and live E. histolytica (Eh) stimulated robust IL-8 production by 27-, 44-, 57- and 37- fold after 4 h as compared to untreated controls. However, following boiling or delipidization of the ameba components, IL-8 production was completely abrogated (p>0.001). In parallel, we determine that proteinase K treatments on ameba components had no effect in inhibiting IL-8 production (data not shown). These data clearly suggests that lipid or glycolipid molecules maybe responsible for stimulating IL-8 in colonic cells.

Effect of COX1/2 inhibitors on IL-8 production. Based on the results in Figure 3, we suspected that PGE₂ produced by ameba might be a likely candidate molecule that was responsible for stimulating IL-8. We have previously shown (9) that E. histolytica synthesizes PGE₂ by a novel COX-like enzyme that was only inhibited by high doses of the non-specific COX inhibitor (1mM) aspirin but not with other COX-1/2 specific inhibitors. Moreover, live E. histolytica incubated ameba with 100µM arachidonic acid, the precursor of PGE₂, produced high levels of bioactive PGE₂ in a time-dependent manner (9). Thus, to determine the biological function of ameba-derived PGE₂ in stimulating IL-8 in colonic cells, live ameba were treated with COX-1/2 specific inhibitor (Indomethcin), COX-2 specific inhibitor (Nimesulide) and COX1/2 nonspecific inhibitor (Aspirin) for 16 h. As shown in Figure 4, only treatment with aspirin significantly inhibited (8-fold, p>0.001) IL-8 production in Caco2 cells following exposure to live ameba in
transwells as compared to untreated ameba or ameba treated with COX-1/2 specific inhibitors.
Furthermore, SAP and SP derived from aspirin-treated live ameba also significantly inhibited IL-8 production after 12 h (7-fold for both, p>0.001) as compared to untreated controls or cells treated with COX-1/2 specific inhibitors. These data suggest that PGE\(_2\) synthesized by the COX-like enzyme in ameba was responsible for IL-8 induction in colonic epithelial cells.

**Effect of EP receptor specific agonist/antagonist on IL-8 production.** PGE\(_2\) exerts its biological effects by coupling and signaling through EP2/4 receptors to induce intracellular cAMP production. cAMP responsive element (CRE, +828 to +835 bp) regulatory sequences have been identified in the 5'-UTR of IL-8 gene (16). Therefore, to unequivocally demonstrate that PGE\(_2\)-derived from ameba were responsible for the induction of IL-8 production in colonic cells, we treated cells with PGE\(_2\), SP, SAP, live Eh and several EP2 or EP4 receptor specific agonist or antagonist and quantified IL-8 production. As shown in Figure 5, PGE\(_2\), and EP4 receptor specific agonist ONO-AE1-329, SP, SAP and live *E. histolytica* (Eh) induced IL-8 production by 29-, 26-, 9-, 15- and 13- fold over untreated control Caco2 cells. The EP2 receptor specific agonist butaprost and antagonist AH6809 did not induce IL-8 production. Remarkably, pretreatment of cells with EP4 receptor specific antagonists AH23848 or L161982 completely abrogated PGE\(_2\) or SP or SAP-induced IL-8 production (p>0.001). These data clearly indicate that endogenously synthesized PGE\(_2\) by ameba efficiently binds EP4 receptors to induce IL-8 production in colonic epithelial cells.
Silencing of EP4 receptors inhibits IL-8 production. To determine specificity for EP4 receptors in PGE$_2$ induced IL-8 production in epithelial cells we silenced EP4 receptors using EP4-siRNA and determine IL-8 production following stimulation with SP and SAP. As shown in Figure 6A/B, treatment with EP4-siRNA almost completely inhibited EP4 receptor expression (86% over control) in wild type Caco2 cells. More importantly, EP4-siRNA treatment completely inhibited SP- and SAP-induced IL-8 production by 15- and 18- fold as compared to homologous controls (Fig 6C, p>0.001). Interestingly, silencing of EP2 receptor expression by EP2-siRNA did not inhibit PGE$_2$ induced IL-8 production suggesting that EP2 receptor plays a minimal role in this event (data not shown). Using green fluorescent protein as control, the transfection efficiency was routinely found to be between 65–75% (data not shown). During these silencing experiments with EP2-siRNA and EP4-siRNA, more than 90% of transfected Caco2 cells were alive as determined by trypan blue exclusion assay. Taken together, these data clearly demonstrate an important role for EP4 receptors in PGE$_2$ induced IL-8 induction in colonic epithelial cells.
Discussion

*E. histolytica* invades the intestinal mucosa and causes amebic colitis and severe ulcerations. Analysis of the inflammatory response during intestinal amebiasis in human and animal models of the disease has revealed an important regulatory role for chemokines and cytokines. Recruitment and activation of inflammatory cells can also be modulated by secreted amebic factors such as amebapores and monocyte locomotion inhibitory factor. Several Th1/2 cytokines, such as IL-6 and IL-4, and regulatory cytokines, like IL-10 and TGFβ have been shown to be associated with the development of amebiasis (17).

Recent studies have provided evidence that chemokines such as IL-8 are crucial mediators for inflammation and tissue injury in intestinal inflammation. IL-8 is a small, 8–11 kD secreted proteins, may participate in immune and inflammatory responses through the chemoattraction and activation of neutrophils or leucocytes (18). The precise nature of the IL-8 signaling pathway related to epithelial cell signaling is not defined yet. However, the initial signaling events during inflammation ultimately lead to the activation and translocation of various transcription factors that control the transcription of genes encoding the various chemokines and cytokines secreted by epithelial cells (19). It has been found that both the C-X-C and C-C members of the chemokine family of proteins as well as pro-inflammatory cytokines IL-1β, IL-6, TNF-α and cell growth factor GMCSF are released by epithelial cells after bacterial-enterocyte interaction (20). Recently, we have shown that in colonic epithelial cells, MCP-1 is secreted in response to soluble ameba components via PI3 kinase/P65 pathway (21). The release of this cytokines in *vivo* by epithelium would be an effective means of initiating a mucosal inflammatory response. Cytokines, particularly, IL-8, GRO-α and MCP-1 are potent chemoattractants for neutrophils and
monocytes respectively, while GMCSF prolongs the survival of these cells and increases their response to other pro-inflammatory agonists (22).

In this study, we established that endogenously synthesized PGE$_2$ present in soluble and secretory ameba components could induce robust IL-8 production in colonic epithelial cells via activation of the EP4 receptor. During amebic invasion, the epithelium responds by mounting a protective inflammatory response. This may cause release of epithelium cytokines/chemokines such as CXCL1, CXCL8, CCL2, CCL3, CCL5, IL-6, granulocyte/macrophage colony stimulating factor, IFN-$\gamma$ and TNF-$\alpha$. Altogether, histological analyses of human biopsies have shown a mild infiltration of neutrophils, accompanied by hyperplasic lymphoid aggregates with macrophages and dendritic cells in the submucosa at the beginning of amebic ulceration along with neutrophils, macrophages and T cells as the infection progresses (23). A member of the transmembrane kinase family, phagosome-associated TMK96 is required for amebic infection (24). Since neutrophils predominantly recruit to the submucosa during amebic infection, in vivo experiments have shown that neutrophils are not capable of killing the parasite, probably because parasite superoxide dismutases and oxidoreductases are produced, which may inhibit neutrophil respiratory burst (25). Furthermore, neutrophil depletion in murine models of infection resulted in the presence of more severe ulceration of susceptible as compared to resistant strains (26). Moreover, ameba trophozoites interact with $\beta$2 integrins on the surface of neutrophils and induce their apoptosis through PI3-K-mediated pathway (27) and activate intracellular signalling (29). Interestingly, the monocyte locomotion inhibitory factor (MLIF) is an anti-inflammatory oligopeptide produced by *E. histolytica* that inhibits locomotion of human monocytes (29).
In previous studies, it was shown that neutrophils play a major protective role in resolving hepatic *E. histolytica* infection in mice (30). Thus, IL-8 may play an important role in chemotraction and activation of neutrophils during the onset of amebic colitis, which may help to control ameba infection. Our current finding is of considerable interest, as it is not known how *E. histolytica* triggers a host inflammatory response in the gut in the absence of cellular contact. We have previously shown that SP and SAP component are able to induce IL-8 induction in colonic epithelial cells (11); however, the identity of the ameba component responsible for the induction of IL-8 was unknown. We have now identified that ameba-derived PGE$_2$ is responsible for stimulating IL-8 through EP4 receptors in colonic epithelial cells. Thus, it is not surprising that silencing EP4 receptor expression completely abrogated SP- and SAP-induced IL-8 production. All attempts to date to knock down the *in vivo* expression of the COX-like gene using antisense strategy or by silencing the gene using siRNA treatments have failed. As ameba died slowly in culture following these treatments it appears the COX-like enzyme responsible for PGE$_2$ biosynthesis may play a critical role in ameba cell cycle. These findings have unraveled that PGE$_2$ produced in the gut by ameba is a major player in the initiation of inflammation by inducing IL-8 production in the pathogenesis of intestinal amebiasis.

The local induction of chemokines such as IL-8 by epithelial cells could explain the histopathological findings of *E. histolytica* infection and may thus provide a mechanism for initiation and exacerbation of inflammation seen during intestinal amebiasis. Moreover, the modulation of host chemokines like IL-8 could perhaps be used as a virulence marker for *E. histolytica*. In summary, we have shown that PGE$_2$ produced by *E. histolytica* stimulates IL-8 mRNA and protein production in human colonic epithelial cells through an EP4 receptor
signaling mechanism in a contact independent manner. This observation is of great importance to our understanding of ameba pathogenic mechanisms as EP4 antagonists targeted against EP4 receptors can have the potential to become therapeutically important in the treatment of amebiasis. Perhaps, directly targeting the production of IL-8 or blocking EP4 receptors might alter the course of invasive amebiasis but this is only speculation at this time.
Acknowledgement

This work was supported by grants from the Canadian Institute for Health Research and the Canadian Association of Gastroenterology-Axcan Pharma-CIHR Research and Fellowship Award. We thank Dr. Mark Giembycz from the University of Calgary for providing the EP4 agonist ONO-AE1-329 and EP4 antagonist ONO-AE3-208 (Ono Pharmaceutical Co. Ltd, Japan).
References


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Figure legends

Fig. 1. *E. histolytica* components induce IL-8 mRNA and protein production from colonic epithelial cells. (A) Confluent Caco2 cells (10^6) were treated with different concentrations of SP or SAP or live *E. histolytica* (2x10^6) for 4 h. Live *E. histolytica* trophozoites were incubated in transwells with colonic cell monolayers. Total cellular RNA was extracted by the TRIzol method and real-time PCR was performed as described in Materials and Methods. Data represent changes (n-fold) of mRNA expression over controls. (B) IL-8 production was quantified using ELISA kits following treatment of Caco2 cells with SP or SAP or live Eh for 12 h. Data represent the mean ± SE of the mean from three different experiments. * P<0.1; ** P<0.01; *** P< 0.001 over controls.

Fig. 2. Induction of IL-8 mRNA and protein production. Confluent Caco2 cells (10^6) were treated with 40µg/ml of SP or 100µg/ml of SAP or live *E. histolytica* (2x10^6) for different time points as described in Fig. 1. (A) Fold increase of IL-8 mRNA were determined by real time PCR over controls as previously described. (B) IL-8 production was quantified using ELISA kit. Data represent the mean ± SE of the mean from three different experiments. * P<0.1; ** P<0.01; *** P< 0.001 over controls.

Fig. 3. Involvement of lipid mediators (PGE_2) in IL-8 production. Confluent Caco2 cells (10^6) were treated with 40µg/ml of SP or 100µg/ml of SAP or 1µM PGE_2 or live *E. histolytica* (Eh, 2x10^6) for 12 h and IL-8 production measured. All components were boiled for 30 min or delipidized before treatment and IL-8 production was assayed. Control (Ctl) represents no
treatment. Data represent the mean ± SE of the mean from three different experiments. *** p<0.001.

Fig. 4. Role of COX inhibitors in inhibiting *E. histolytica* induced IL-8 production. Confluent Caco2 cells were treated with 40µg/ml of SP or 100µg/ml of SAP or live *E. histolytica* (Eh, 2x10^6) for 12 h and IL-8 production quantified. For inhibition studies, live ameba was pretreated with 50µM COX-1/2 inhibitor (Indomethcin), 40µM COX-2 specific inhibitor (Nimesulide) and 1mM COX1/2 nonspecific inhibitor (Aspirin) for 16 hr and SP/SAP were prepared from drug-treated ameba as previously described. Caco2 cells were treated with SP and SAP components and IL-8 production quantified by ELISA. Control (Ctl) represents no treatment. Data represent the mean ± SE of the mean from three different experiments. *** p<0.001.

Fig. 5. Effect of EP2 and EP4 receptor agonist and antagonist on IL-8 production. Confluent Caco2 cells were treated with 1µM of PGE2, ONO-AE1-329 (EP4 receptor agonist) or butaprost (EP2 receptor agonist) for 12 h. Cells were pretreated with either 1µM of AH23848/L161982 (EP4 receptor antagonist) or 50µM of AH6809 (EP2 receptor antagonist) for 60 min prior to treatment with PGE2 or SP or SAP for 12 h and IL-8 production measured by ELISA. Data represent the mean ± SE of the mean from three different experiments. *** p<0.001.

Fig. 6. Treatment with EP4 siRNA inhibits IL-8 production. Caco2 cells were transfected with or without EP4 receptor siRNA and control siRNAs as described in Material and Methods.
A. Expression of EP4-receptor was determined by immunoblot analysis. B. Densitometric analysis of EP4 receptor expression was normalized against β-actin. C. Colonic cells were treated with SP and SAP for 16 h and IL-8 production measured by ELISA. Data represent the mean ± SE of the mean from three different experiments. *** p<0.001 relative to respective untransfected control cells.
Fig. 1

A.

Fold increase in IL-8 mRNA/GAPDH

- SP
- SAP
- Live Eh

μg/ml

B.

IL-8 production (pg/ml)

μg/ml
Fig. 3

IL-8 Production (pg/ml)

- Ctl
- SP
- SAP
- PGE₂
- Eh

Boiled
Delipidized

***