Final Progress Report

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Proposal No. IBD-0081R
Applicant Organization: University of Wisconsin-Madison (U.S.A.)
Project Title: Lipoxin metabolism in inflammatory bowel disease
Period of Award: February 1, 2004 - April 30, 2006

i. **Summary of Project Aims**:

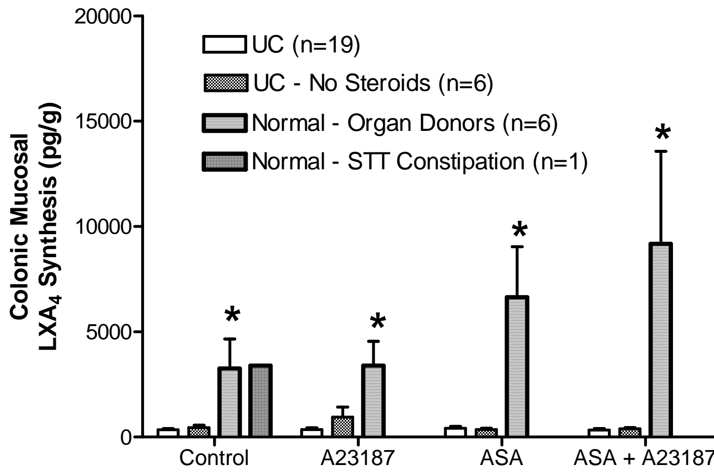
The projects aims were to determine the metabolism of lipoxins in human patient colonic mucosa during IBD compared to normal mucosal lipoxin biosynthesis and metabolism. The activity of the aspirin triggered lipoxin (ATL) pathway was also determined. Following the elucidation of these pathways and ratesof lipoxin biosynthesis, the biological relevancy to IBD would be determined in an animal model of ulcerative colitis in mice which included groups with genetic deltion of key lipoxin producing enzymes. The hypothesis of the study was that chronic inflammation of the colon and bowel in IBD results, at least in part, by a defect in the normal biosynthesis of anti-inflammatory lipoxins in the colonic epithelium. A peripheral aim which was not pursued was to determine lipoxin receptors in human colonocytes and neutrophils.

ii. **Accomplishments towards meeting those aims:** See section iii below.

iii. **Significant results**

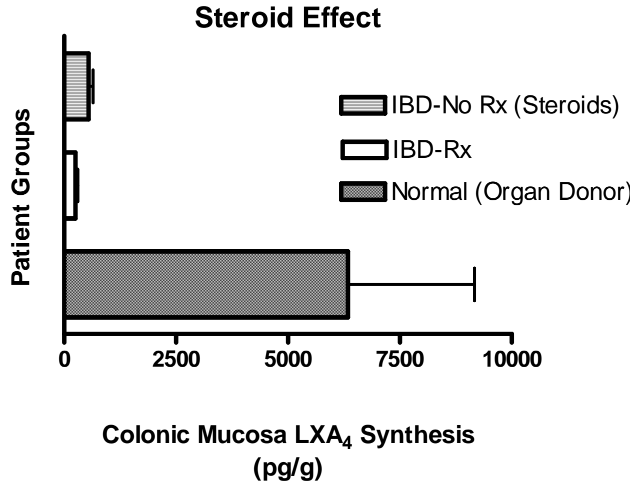
Patient lipoxin biosynthesis: We first began to characterize lipoxin biosynthesis in patients with ulcerative colitis. This was the first test of the hypothesis that chronic inflammation in IBD may be caused, at least in part, by a defect in either local lipoxin biosynthesis or lipoxin biological activity. Patients undergoing surgical resection of colon for treatment of refractory and drug resistant ulcerative colitis were studied along with normal colon obtained from organ donors. Tissue whole wall specimens of the colon were immediately placed in cold saline and processed within 2 hours in the lab. Mucosal scrapings were harvested and placed into tissue culture for 2 hours. After tissue culture, the conditioned media was sampled and assayed for lipoxin A_4 (LXA_4) and aspirin triggered lipoxins (ATL, 15-*epi*-Lipoxin A_4) by competitive binding ELISA assay. The media were also activated with various agonists (aspirin, calcium ionophore) in some conditions. Figure 1 summarizes these results.

Figure 1. ***Colonic mucosal lipoxin synthesis from UC patients and patients without IBD (organ donors and STT constipation).***



The data indicate a large difference in colonic mucosal lipoxin biosynthesis between patients with UC and normal control groups, STT = slow transit time constipation.. This is consistent with our overall hypothesis. The suppression in lipoxin production in the UC patients seems not due to a pharmacological suppression of lipoxin precursors by steroids since stratification of the UC patients into steroid free and steroid groups did not influence lipoxin synthesis rates in that group (figure 1 and 2).

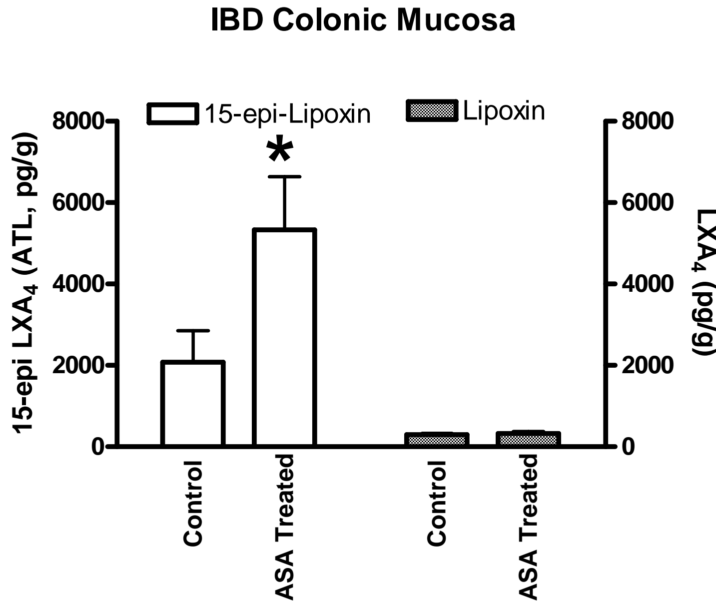
Figure 2. ***Colonic lipoxin synthesis from UC patients and controls (organ donors): Effects of steroid treatment***



Therefore, the differences in lipoxin synthesis between the UC patients and the controls with non-inflamed colon are assumed to be real and not a drug artifact. Although we standardized lipoxin synthesis results to tissue weight, the possibility that the tissue from the patients contained less active mucosal tissue compared to the controls due to atrophy can not be ruled out. For example, patient samples may have had larger fractions of non-lipoxin producing fibrous tissue, relative to the non-UC control patients. This may account for less lipoxin synthesis in the UC group. We will rule this possibility out in our proposed future experiments by determining lipoxin synthesis in purified mucosal tissues rather than gross scrapings. However, even if twice as much patient tissue was derived from non-lipoxin producing scar tissue compared to the controls, this still would not account for the 10 fold difference in lipoxin biosynthesis observed between the two groups (figure 1 and 2). Thus, it seems unlikely that the vast differences in lipoxin biosynthesis in patients compared to normal colon is due to chronic tissue changes alone.

The alternative route for synthesis of lipoxins using aspirin acetylated COX-2 was also studied in UC patient colonic explants. These results are shown in figure 3.

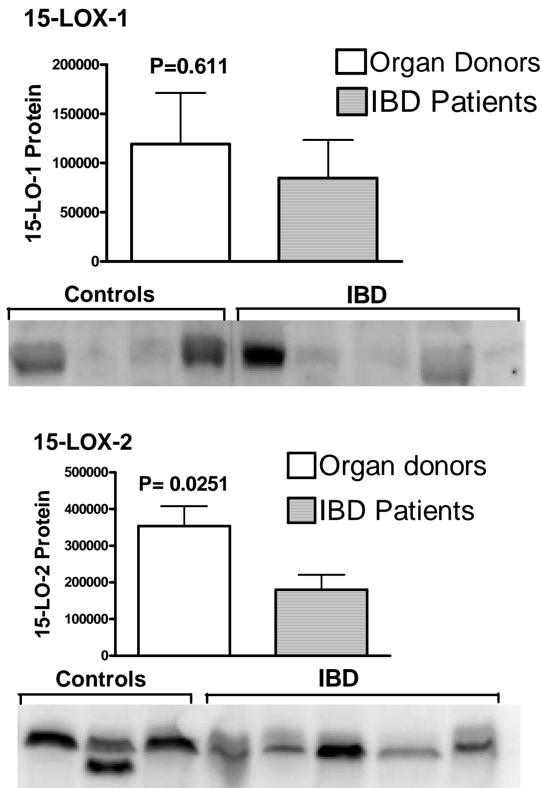
Figure 3. ***Aspirin (ASA) elicited ATL synthesis by colonic mucosa from UC (IBD) patients***



Colonic mucosa from UC patients maintained the ability to synthesize biologically active lipoxin isomers (ATLs) in response to aspirin (in-vitro). This suggests an active and possibly up regulated COX-2 system, which probably occurs with the inflammatory stimuli. Although patients have diminished lipoxin synthesis ability (figure 1), they are quite capable of producing lipoxins through the alternative route using aspirin acetylated COX-2. It is tempting to speculate that the anti-inflammatory effects of aspirin and perhaps other therapeutic salicylates used to treat UC, may be due to activation of colonic mucosal 15-epi-LXA_4 (ATL) synthesis. In fact, we also demonstrated significant levels of ATL in the non-aspirin treated mucosal tissue from UC patients (control, figure 3). This high background ATL production should only occur via acetylated COX-2 but there was no evidence that the patients were exposed to aspirin before surgery. We therefore speculate that acetylated metabolites of 5-aminosalicylates, which are common drugs used to treat UC (more commonly known as Asacol, Pentasa, and Rowasa) activated local ATL production by acetylating COX-2. This may be a novel mechanism of action for these drugs, which are largely unknown. This would be a further test of our hypothesis. Therefore, we will systematically examine this possibility in future experiments by adding authentic acetylated metabolites of Asacol, which undergoes primary pass hepatic acetylation, to mucosal tissue from UC patients to determine their direct effect on lipoxin and ATL biosynthesis in the colon. We predict that these metabolites may activate ATL synthesis via acetylation of COX-2.

Lipoxin synthesis generally requires conversion of arachidonic acid to 15-HETE, either via a 15-lipoxygenase (lipoxins) or via acetylated COX-2 (to produce ATL). Previous studies from our lab indicate that canine intestinal mucosa produce enantiopure 15 (S)-HETE indicating the presence of a 15-lipoxygenase.. Therefore, we measured 15-lipoxygenase activity in human colonic mucosal scrapings from UC patients and normal control patients (organ donors). Preliminary western blot analysis using antibodies specific for both 15-LOX-1 (leukocyte enzyme) and 15-LOX-2 (epithelial enzyme) are shown I figure 4.

Figure 4. ***15-lipoxygenase enzyme expression in human colonic mucosa***



Significantly less 15-LOX-2 was identified in UC patient (IBD) colonic mucosa, relative to the controls while no statistical difference was observed in the leukocyte enzyme (15-LOX-1). The data are preliminary and the interpretation is unclear since either lipoxygenase subtype should satisfy 15-lipoxygenase activity necessary for the synthesis of lipoxins. The samples used likely contain mucosal epithelium, neutrophils, and platelets, which could all contribute to lipoxin synthesis by the tissue. More detailed analysis will be performed in this proposal by purifying epithelium from mucosal explants from UC patients and non-inflamed controls. Our data suggest that patients with UC may have a deficiency in type-2 15-lipoxygenase. It is plausible that such a defect may alter the normal lipoxin synthesizing capabilities of the tissue. These possibilities will be explored in detail in future studies.

An objective of future proposals is to conduct experiments with purified colonic epithelial and neutrophil populations from patients with UC and normal controls to identify the defect in lipoxin biosynthesis observed in the gross mucosal scraping explants. Agonist and precursor-elicited lipoxin synthesis responses in add back experiments from normal and UC affected patients will help understand these defects. Therefore we have developed methods to purify colonic epithelial cells from human colon for short term tissue culture. These cells (figure 5) demonstrate epithelial cells purified by digestion and centrifugation with add back of neutrophils isolated and purified from peripheral blood by gradient centrifugation.

Figure 5. ***Isolated colonic epithelial cells***

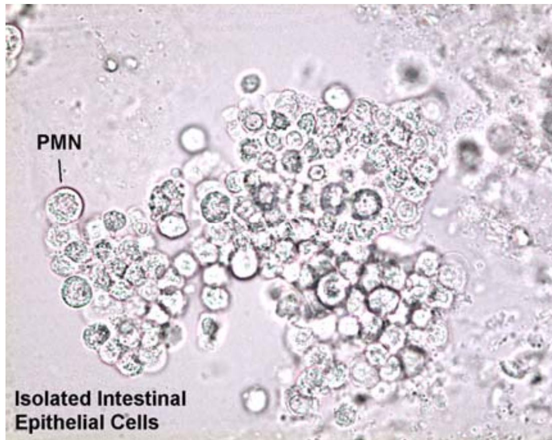
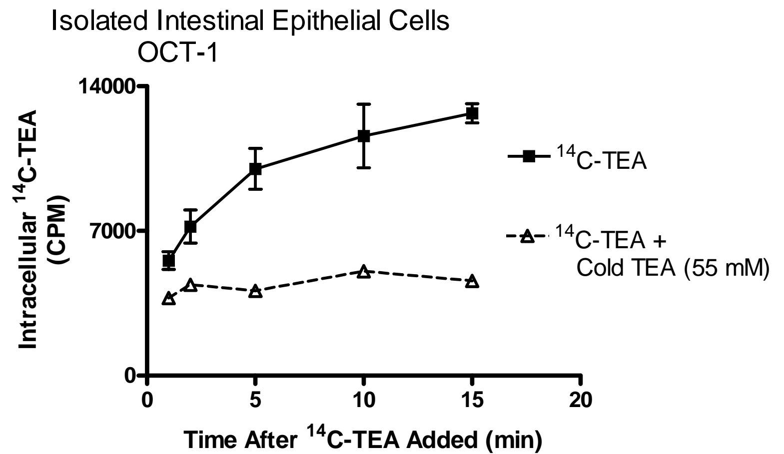


Figure 6. ***Epithelial cell function***

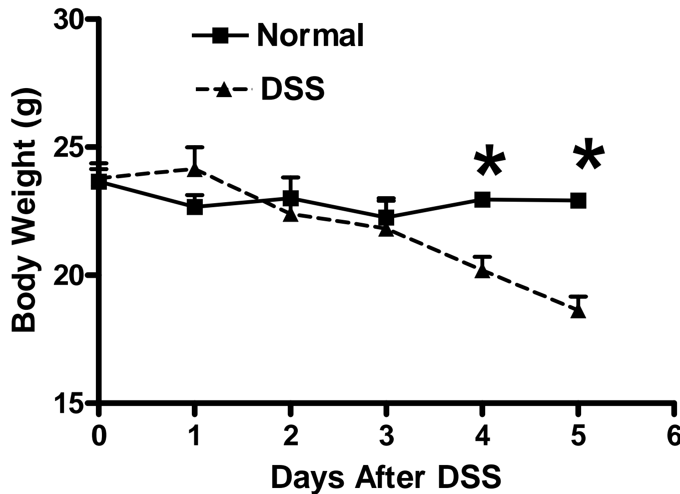


To further validate the purity and function of these cells, we examined the activity of the organic cation transporter (OCT) on these cells. The OCT is common on epithelial cells in the gut mucosa and kidney tubules and represents a functional property of these cells. Purified colonic epithelial cells were incubated with authentic radio-labeled tetraethyl ammonium chloride (TEA, a type 1 small organic cation) and the movement across the cell membrane was monitored at various times after addition of the labeled cation. Figure 6 shows results from these experiment and suggests that the cells isolated are functional epithelia. Displacement of the label with molar excess of cold TEA confirms receptor binding specificity.

Murine model of ulcerative colitis:

A major objective of future proposals is the demonstration of causation between defects in lipoxin biosynthesis in UC and the disease process. Therefore, we have developed murine models of ulcerative colitis for use in our lab to demonstrate this causation. Our intent is to utilize both genetic and pharmacological blockade of lipoxin synthesis in mice with chemically induced UC to demonstrate an effect on the progression of the inflammatory response and functional changes. We intend to demonstrate potentiation of UC with lipoxin deletion (15-lipoxygenase knockout mice and selective 15-lipoxygenase inhibitors) and therapeutic amelioration of UC with lipoxin active drugs. However, we first developed the model in C57BL/6 mice by oral feeding with 5% dextran sodium sulfate (DSS). Mice were grouped into DSS fed and controls (non-DSS fed). In this model, intestinal function declines as severe colonic inflammation increases with drug exposure. Weight gain or loss is a gross but reliable measure in these animals and is shown in figure 6.

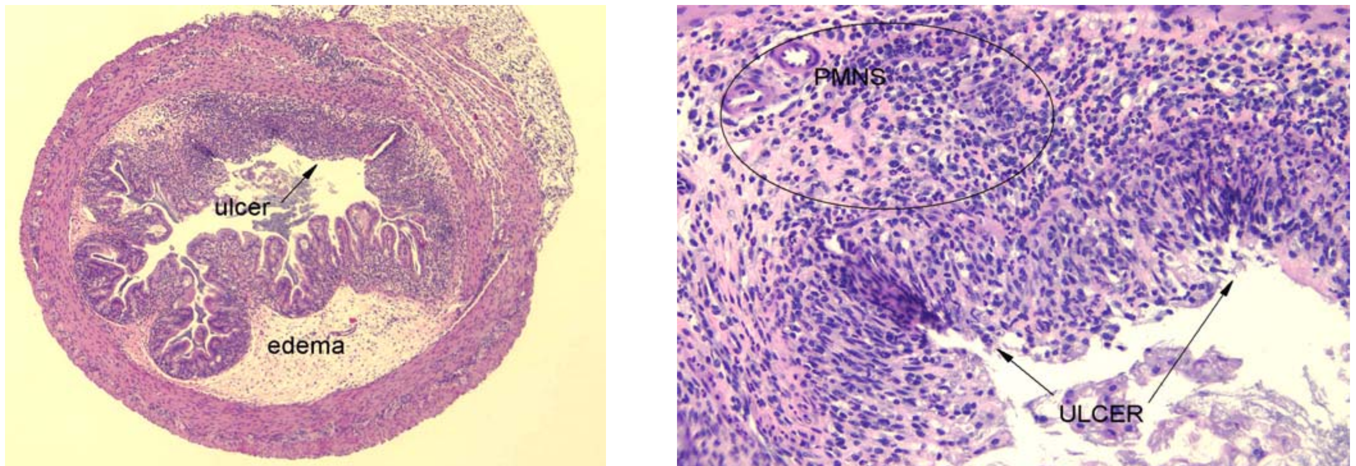
Figure 6. ***Weight loss in DSS treated mice***



The normal control mice maintained their weight over the 5 day observation period while the DSS treated animals steadily lost weight as their intestinal absorptive and digestive function deteriorated. These animals experienced a 25% reduction in body weight after 5 days. Macroscopic findings indicate rectal bleeding, hematochezia, and bloody diarrhea on the cage bedding.

Histological analysis shows characteristics remarkably similar to human UC. A low and higher magnification of a representative colon from a mouse treated with DSS for five days is illustrated in figure 7.

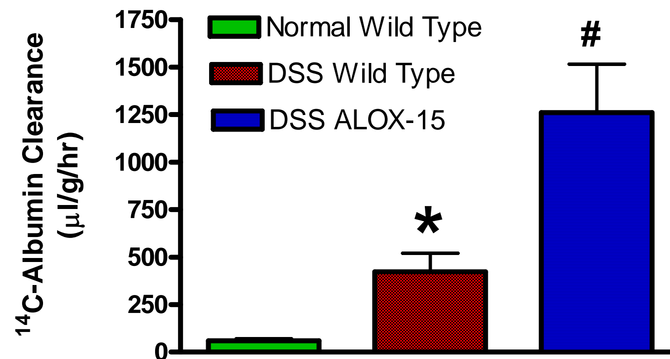
Figure 7. ***Light Microscopy of mouse colon after DSS-induced colitis***



The figure clearly shows characteristic mucosal ulcers formed with loss of villus architecture and edema (left panel) with profound infiltration of neutrophils into the areas around the ulcer (right panel). This highlights the importance of understanding neutrophil inflammatory signaling in a disease with neutrophils as the major effector cell.

Another method or endpoint for measuring mucosal injury in DSS colitis in this model is the integrity of the capillary/mucosal barrier. Large molecules like proteins do not normally filter across the colonic microcirculation. However, during inflammatory conditions such as ulcerative colitis, the local mediators and cellular inflammation increase capillary permeability to macromolecules. We are able to measure and quantitated this injury by injecting mice with ^{14}C-labelled albumin and tracking the movement of the radiolabel into the lumen of the colon over time. This capillary/mucosal permeability assay is shown in figure 8 for mice with DSS-induced colitis and controls.

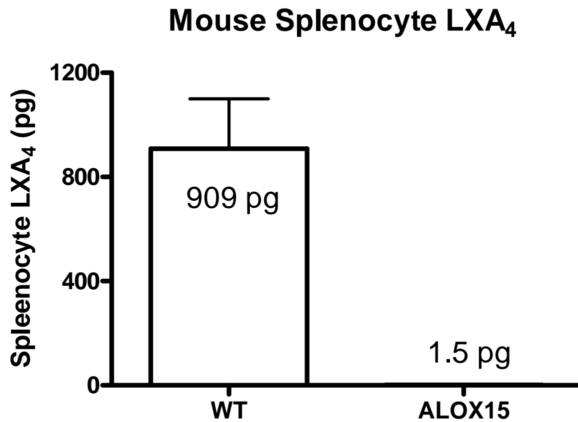
Figure 8. ***Capillary/mucosal permeability in DSS-induced ulcerative colitis: Effects of the 12/15 Lipoxygenase knockout genotype (ALOX-15)***



We routinely observe and quantitated a 6-7 fold increase in colonic capillary / mucosal permeability after DSS treatment in mice. This represents an increase in capillary permeability to macromolecules and a breakdown in the epithelial barrier to the lumen in the colon. This severe barrier defect results in colon malfunction, weight loss, and malnutrition. Maneuvers which mitigate neutrophil trafficking in colitis tend to also reduce permeability of the colon. These outcome parameters will be used to quantitate the disease severity in our future experiments. This design is used in our animal models of UC to establish causation between lipoxin synthesis (biological activity) and the UC pathophysiological phenotype. In fact, this preliminary experiment shows that capillary permeability is significantly **worse** in ALOX-15 knockouts compared to wild type C57BL/6 mice with DSS colitis. This is predicted by our hypothesis and suggests that endogenous lipoxins may serve to limit intestinal inflammation in human inflammatory bowel disease just as they appear to inhibit the inflammatory capillary leak induced by DSS in mice (figure 8). In this experiment, lipoxins (or a 15-lipoxygenase metabolite) appear to limit the functional changes associated with exogenous inflammatory stimuli (DSS) but in human IBD they may in fact prevent the occurrence of persistent gut inflammation by endogenous stimuli (gut bacteria and food antigens). This is our central hypothesis.

To assess the lipoxin synthesis ability of the ALOX15 mice, a preliminary study was conducted in 4 mice. Adult mice were injected (I.P.) with 1 μg LPS in saline to stimulate arachidonic acid metabolism by inflammatory cells. The spleens were harvested 7 hours later. The spleens were macerated in Krebs buffer (2.5 ml) and the suspension was incubated at 37 C under 95% O_2, 5% CO_2 for 60 minutes. The supernatant was harvested, acidified to pH 3.5 with 1.0 N HCl, and applied to open bed C18 extraction columns (Sep-Pak). The lipoxins were eluted from the column with methyl formate after washing with water and petroleum ether, and the residue was concentrated to dryness under N_2. This residue was reconstituted in 200 μl of EIA buffer and 50 μl was assayed for LXA_4 by ELISA (Neogen). The results are summarized below.

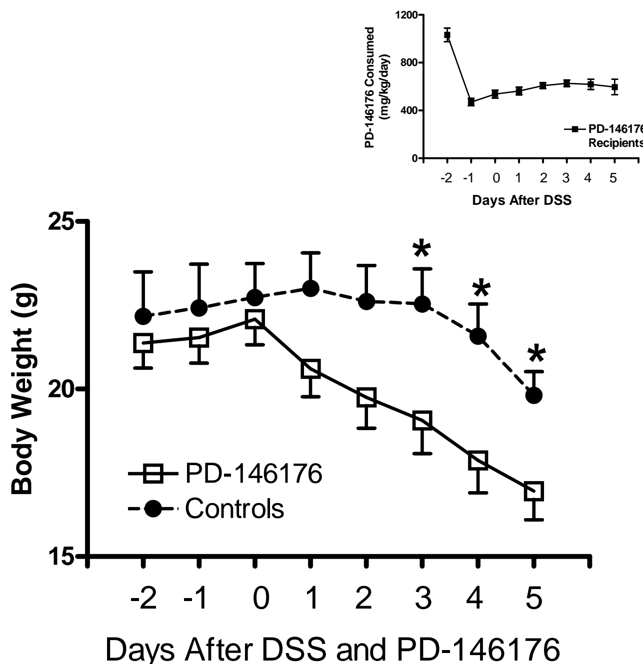
Figure 9. Production of LXA_4 by mouse spleenocytes after LPS injection in wild type (WT) and 15-lipoxygenase knock out mice (ALOX15)



The wild type mice (WT) synthesized immuno-reactive lipoxin A_4 during the cell culture period but no signal above background was generated from spleenocytes derived from the ALOX15 mice.

Another preliminary experiment was designed to test whether lipoxin production plays a causal role in colonic function during the development of ulcerative colitis. We used a putative selective inhibitor of 15-lipoxygenase, PD-146176, to pharmacologically shut down lipoxin synthesis. In this simple experiment we measured gross intestinal function as indexed by weight of the animals over the course of developing DSS-induced colitis. The results are illustrated in figure 10.

Figure 10. Body weight in mice during development of DSS colitis: Effect of the selective 15-lipoxygenase synthesis inhibitor PD-146176.



Rodent food pellets were ground to a powder consistency and fed to both groups (n=8 each). The treated group received PD-146176 mixed into the chow such that their daily intake was over 400 mg/kg/day (upper panel), which has been shown to be effective at inhibiting 15-lipoxygenase in other models.. Both groups were also fed DSS in the drinking water to induce UC. Weight loss was measured daily in both

groups. Clearly, 15-LO inhibitor treated mice had much worse weight loss and intestinal function compared to the untreated UC control animals. Both groups consumed the same amount of food per day. These data suggest that a 15-lipoxygenase metabolite attenuated the disease. We speculate that this 15-lipoxygenase product is a lipoxin. This experiment further supports our hypothesis that lipoxins are involved in regulating inflammatory responses during the course of ulcerative colitis and other forms of inflammatory bowel diseases (IBD).

iv. Lay summary of progress report

Inflammatory bowel disease (IBD) is a chronic autoimmune illness affecting the large intestine, small intestine, or both. The disease causes diarrhea, pain, weight loss, and malnutrition. In severe cases, surgical removal of large amounts of the small and large intestine is required to treat the disease. These patients suffer a poor quality of life and even premature death. Although the exact causes of IBD are unknown, severe and uncontrolled inflammation of the intestine occurs. The body normally uses inflammation reactions to destroy bacteria and remove injured tissue to promote healing. Chemical signals released by white blood cells spark these inflammatory reactions. Other chemicals are released at the end of inflammation that instructs white blood cells to stop the inflammation reaction. Thus, normal inflammatory reactions are self-limiting and rely on these stop signals to end. We believe that the uncontrolled inflammation of the intestine and colon of patients with IBD may be caused by an inability of their tissues to release these chemical stop signals (Lipoxins), which tell the inflammatory response to stop. This could allow inflammation to run uncontrolled. The goal of this project was to identify the metabolic defect in lipoxin metabolism in IBD patients and to determine if this defect in fact contributes to the disease. In our studies, we have in fact identified a significant decrease in lipoxin production from colon obtained from patients with ulcerative colitis, compared to colon tissue obtained from patients without ulcerative colitis. There is further preliminary data in a mouse model of ulcerative colitis that suggests that these molecules are helpful in preventing ulcerative colitis since mice with a genetic defect that prevents them from producing lipoxins seem to suffer severer symptoms compared to normal mice that synthesize lipoxins in the colon.