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N-3 and n-6 fatty acids stimulate restitution by independent mechanisms in the IEC-6 model of intestinal wound healing

Derek J. Ruthig, Kelly A. Meckling-Gill*

Department of Human Biology and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada, N1G 2W1

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Abstract

We have shown that intestinal epithelial restitution is stimulated by n-3 and n-6 fatty acids. The current studies were undertaken to elucidate the mechanistic pathway(s) involved in this fatty acid modulation of restitution. Inhibition of phospholipase A_2 and eicosanoid synthesis and its effect on fatty acid stimulation of cellular migration in confluent, wounded IEC-6 monolayers was examined. The production of prostaglandin E_2 and transforming growth factor β_1 were also measured in fatty acid supplemented cultures. Inhibition of phospholipase A_2 attenuated the effect of fatty acid stimulation of restitution in both n-3 and n-6 supplemented cultures. The lipoxygenase inhibitor, nordihydorguaretic acid (2 μmol/L), had no effect on stimulation of migration by fatty acids. The cyclooxygenase inhibitor piroxicam (5 μmol/L) and cyclooxygenase-2 specific inhibitors dexamethasone (2 μmol/L) and NS-398 (10 μmol/L) all attenuated the fatty acid stimulation of migration by n-6 fatty acids but had no effect on n-3 stimulated restitution. Prostaglandin E_2 production in n-6 supplemented cultures was significantly greater than in control and n-3 supplemented cultures and was partially inhibited by dexamethasone and NS-398. Latent transforming growth factor β_1 production in n-3 supplemented cultures was significantly higher than baseline and n-6 supplemented cultures. Docosapentaenoic acid supplementation significantly enhanced the restitution process and NS-398 treatment had no effect on this stimulation of cellular migration. The liberation of fatty acid from the *sn*-2 position of phospholipid appears to be necessary for both n-3 and n-6 fatty acid stimulation of restitution. N-6 fatty acid modulation of restitution appears to be mediated through the production of eicosanoid products, however, prostaglandin E_2 does not appear to be the sole prostanoid involved. N-3 supplementation elevates the production of latent transforming growth factor β_1 and may be responsible for n-3 mediated stimulation of restitution. These results further emphasize that n-3 and n-6 fatty acids convey their effects through unique pathways.

Keywords: N-3; N-6; Polyunsaturated fatty acid; Restitution; Intestine; Cell culture

1. Introduction

The intestinal epithelium is susceptible to a number of challenges that can compromise its integrity, including inflammatory bowel disease, ulcers, bacterial infection, chemotherapy and radiation treatment. The lining of the gastrointestinal tract has the capacity to regenerate rapidly and complete turnover can occur within days [1]. Reepithelialization is initiated by rapid migration of cells adjacent to the damaged area in a process termed restitution, followed later by cell division and completed in within 1 to 2 days [2–4].

We recently demonstrated that certain n-3 and n-6 fatty acids have the capacity to stimulate the process of intestinal epithelial cell restitution probably by different mechanisms [5]. A number of growth factors, including a central role for transforming growth factor-beta (TGF-β), and prostaglandins (PG) regulate the restitution process [6–9] so we hypothesized that n-3 and n-6 fatty acids may be exerting their effects on restitution through growth factor and/or prostaglandin signalling pathways.

Here we examined the role of prostaglandins, in fatty acid stimulated restitution, by utilizing eicosanoid synthesis inhibitors or non-steroidal anti-inflammatory drugs (NSAIDS). We also assessed the influence of fatty acid supplementation on the production of TGFβ. We utilized the IEC-6 model which displays many of the *in vivo* characteristics of wound healing [10] and has been used by many researchers to study the process of intestinal epithelial restitution [6–9,11]. The model allows study of the restitu-

* Corresponding author. Tel.: +1-519-824-4120; fax: +1-519-763-5902.

E-mail address: kmecklin@uoguelph.ca.

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tion process without the confounding influence of the heterogeneous cell populations found in intact intestine or primary cell culture.

2. Materials and methods

2.1. Cell culture

IEC-6 (ATCC, Rockville, MD, USA) cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (GibcoBRL, ON, Canada), supplemented with 10% Fetal Bovine Serum (FBS) (GibcoBRL) and 270 U/L insulin (GibcoBRL) at 37°C in a humidified, atmosphere of 10% CO_2. Cell culture stocks were passaged when approximately 80% confluent at 1:15 and media was changed 3 times weekly. Passages 15–25 were used for all experiments.

2.2. Fatty acid supplementation

The fatty acids, docosahexaenoic acid [DHA, 22:6(n-3)], docosapentaenoic acid [DPA, 22:5(n-3)], eicosapentaenoic acid [EPA, 20:5(n-3)], alpha-linolenic acid [ALA, 18:3(n-3)], linoleic acid [LA, 18:2(n-6)] and arachidonic acid, [AA, 20:4(n-6)]. (99% pure, Nu-Chek-Prep, Elysian, MN) were preincubated in FBS for 1 h at 37°C. FBS provides sufficient amounts of bovine serum albumin to bind fatty acids up to at least 1 mmol/L [12].

2.3. Wounding and inhibitor assays

IEC-6 cells were plated in 6 well culture dishes at a density of 2.5×10^5 cells/dish and 24 h later, media supplemented with the various fatty acids (30 μmol/L, [5]) were added. At confluence monolayers were wounded with a single edged razor blade as previously described [5]. Cultures were washed, fresh media supplemented with fatty acid added and 24 h later cells were fixed [5]. Migration was assessed by image analysis using a Nikon DIAPHOT-TMD inverted microscope (Tokyo, Japan) and Northern Exposure software (Empix Imaging Inc., Mississauga, ON, Canada), expressed as the area covered by migrating cells per μm wound in 24 h (μm/24 h).

Eicosanoid synthesis was inhibited by adding nordihydroguaretic acid (NDGA), 2 μmol/L, piroxicam, 5 μmol/L, dexamethasone (DEX), 2 μmol/L, NS-398, 10 μmol/L, into the culture media 4 h prior to wounding. 4-Bromophenacylbromide (BPB), 10 μmol/L was added similarly to inhibit phospholipase A_2 (PLA_2) activity (all inhibitors were from Sigma Chemical, St. Louis, MO). Following wounding IEC-6 cells were cultured in fresh media supplemented with the appropriate fatty acid and/or drug.

2.4. Assessment of PGE_2 and TGFβ_1 production

Inhibition of PG synthesis in wounded and unwounded IEC-6 cultures was assessed using piroxicam (5 μmol/L) and NS-398 (10 μmol/L). Media was collected 24 h after inhibitor addition and PGE_2 production measured using a PGE_2 immunoassay system (R&D Systems, Minneapolis, MN). TGFβ1 production was measured similarly using a TGFβ_1 E_{max} immunoassay system (Promega, Madison, WI) to determine both latent and bioactive forms of TGFβ_1 in conditioned media. Background PGE_2 and TGFβ1 levels found in 10% FBS were subtracted from values determined in conditioned media.

2.5. Statistics

Data were examined using ANOVA followed by Student Newman-Keuls post-test using GraphPad Instat software (GraphPad Inc., San Diego, CA). All differences were considered significant at the $P < 0.05$ level.

3. Results

Using the PLA_2 inhibitor BPB, we showed that liberation of n-3 or n-6 fatty acids from phospholipids, via PLA_2, was required for the restitution promotion effects of these fatty acids (Fig. 1). Migration rates were unaffected by BPB alone and the degree of attenuation of the migration response was similar for both n-6 and n-3 supplemented conditions (Fig. 1).

None of the lipoxygenase (LOX), NDGA (2 μmol/L) or the cyclooxygenase (COX) inhibitors, piroxicam (5 μmol/L), DEX (2 μmol/L), or NS-398 (10 μmol/L) affected the migration rate in wounded monolayers compared to drug-free controls (Figs. 2–5). Fatty acid stimulated migration was unaffected by NDGA, regardless of which fatty acid was supplemented (Fig. 2). In contrast, the COX inhibitor, piroxicam (5 μmol/L), attenuated the n-6 stimulated migration (LA and AA) but not migration stimulated by the n-3 fatty acids (ALA or EPA, Fig. 3). Similarly the COX2 specific inhibitors DEX (Fig. 4) and NS-398 (Fig. 5) attenuated only the n-6 stimulated restitution process.

DPA, an elongation product of EPA, had previously been shown to be a potent simulator of endothelial cell migration [13]. Because we had observed significant increases in DPA content of EPA and ALA supplemented IEC-6 cells (where we observed enhanced restitution) compared to DHA supplemented cells where there was little DPA produced (and no enhancement of the restitution process) we next examined the effects of DPA alone. DPA supplementation resulted in increased migration of cells across the wound line compared to control cultures (Fig. 6) similar to that observed for EPA and ALA [5]. As with the other n-3 fatty acids this migration was unaffected by COX2 inhibition.

We also measured prostaglandin E_2 (PGE_2) in the absence and presence of piroxicam from conditioned media

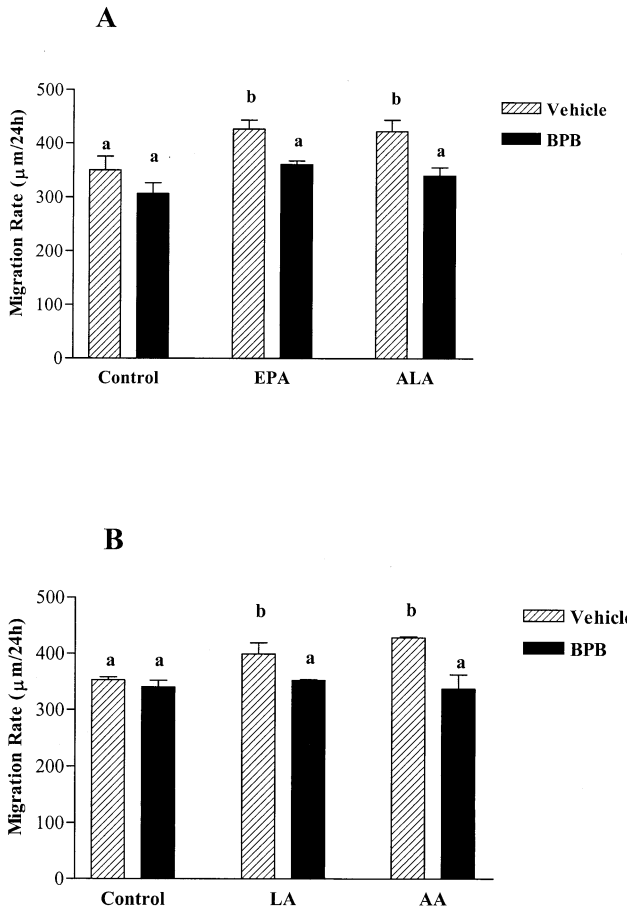

Fig. 1. Effect of BPB on fatty acid stimulation of migration in wounded, IEC-6 monolayers. IEC-6 cells were grown in 6 well culture plates, treated with 30 μmol/L fatty acid and grown to confluence (72 h). Confluent monolayers were treated with BPB (10 μmol/L) 4 h prior to wounding. Cultures were then wounded and maintained for 24 h following replacement with fresh fatty acid supplemented media and BPB. Wounded monolayers were then fixed and analyzed as described in Materials and Methods. (A) Eicosapentaenoic acid, EPA [20:5(n-3)], alpha-linolenic acid, ALA [18:3(n-3)]. (B) Linoleic acid, LA [18:2(n-6)], arachidonic acid, AA [20:4(n-6). Controls represent unsupplemented vehicle and BPB treated cultures. Vehicle treatment represents the addition of ethanol in an equal volume to that required to deliver drug treatment. Data represents mean values ±SEM, n = 4 with eight replicate measurements from various regions of wounds. Values not sharing a letter are significantly different ($P < 0.05$).

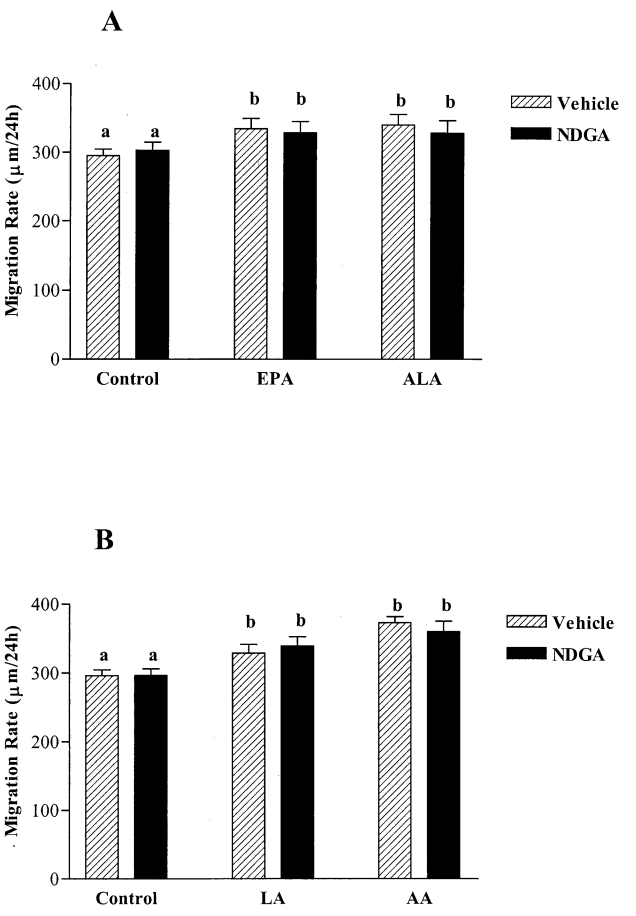

Fig. 2. Effect of LOX inhibitor, NDGA, on fatty acid stimulation of migration in wounded, IEC-6 monolayers. IEC-6 cells were grown in 6 well culture plates, treated with 30 μmol/L fatty acid and grown to confluence (72 h). Confluent monolayers were treated with NDGA (2 μmol/L) 4 h prior to wounding. Cultures were then wounded and maintained for 24 h following replacement with fresh fatty acid supplemented media and NDGA. Wounded monolayers were then fixed and analyzed as described in Materials and Methods. (A) Eicosapentaenoic acid, EPA [20:5(n-3)], alpha-linolenic acid, ALA [18:3(n-3)]. (B) Linoleic acid, LA [18:2(n-6)], arachidonic acid, AA [20:4(n-6). Controls represent unsupplemented vehicle and NDGA treated cultures. Vehicle treatment represents the addition of ethanol in an equal volume to that required to deliver drug treatment. Data represents mean values ±SEM, n = 6 with eight replicate measurements from various regions of wounds. Values not sharing a letter are significantly different ($P < 0.05$).

collected 24 h post-wounding (Table 1). N-3 fatty acid supplementation in unwounded cultures modestly increased the production of PGE_2 compared to unwounded controls and there was little effect of piroxicam. In unwounded cultures, n-6 supplementation increased basal PGE_2 levels but piroxicam had little effect on PGE_2 production. The wounding process, itself, increased PGE_2 levels and this response was inhibited by piroxicam. LA modestly, and AA dramatically, increased the production of PGE_2 above unsupplemented wounded levels. Piroxicam significantly reduced the production of PGE_2 in wounded cultures supplemented with n-3 and n-6 fatty acids, however, levels remained much higher than basal levels in FBS only, piroxicam treated, controls (Table 1). These COX inhibition experiments were repeated in the presence and absence of the specific COX2 inhibitor NS-398 (Table 2). The addition of 10 μmol/L NS-398 significantly reduced the levels of PGE_2 in LA and AA supplemented IEC-6 cells but as seen with piroxicam, levels remained several times greater than control levels in unsupplemented cultures treated with NS-398 alone.

Recent evidence has supported a central role for $TGF\beta_1$ in the process of intestinal epithelial restitution [7] and that the production and efficacy of $TGF\beta_1$ action can be modulated by supplemental fatty acids [14,15]. Therefore, the production of both the latent and bioactive forms of $TGF\beta_1$ were measured to determine any possible involvement of

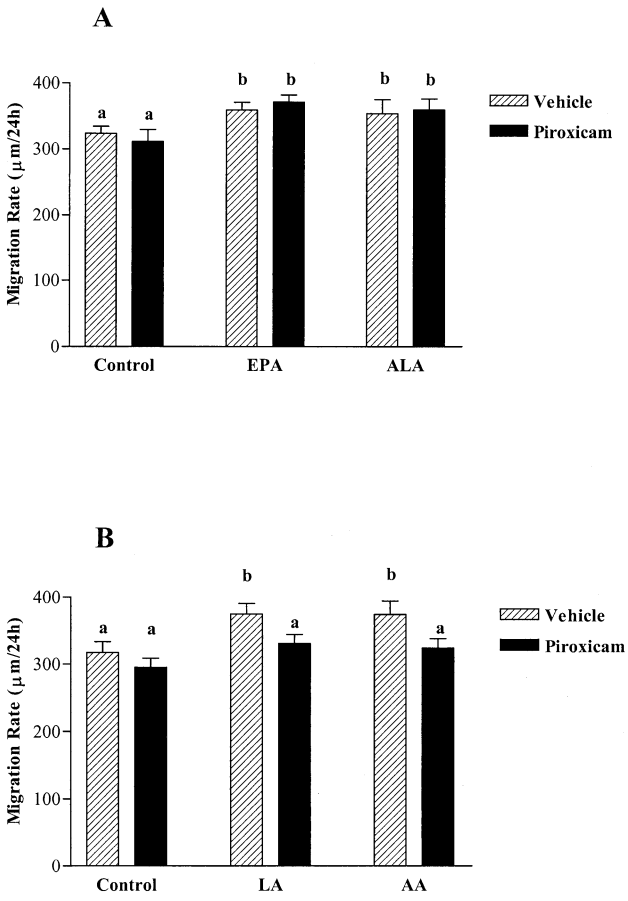

Fig. 3. Effect of COX inhibitor, piroxicam, on fatty acid stimulation of migration in wounded, IEC-6 monolayers. IEC-6 cells were grown in 6 well culture plates, treated with 30 μmol/L fatty acid and grown to confluence (72 h). Confluent monolayers were treated with piroxicam (5 μmol/L) 4 h prior to wounding. Cultures were then wounded and maintained for 24 h following replacement with fresh fatty acid supplemented media and piroxicam. Monolayers were then fixed and analyzed as described in Materials and Methods. (A) Eicosapentaenoic acid, EPA [20:5(n-3)], alpha-linolenic acid, ALA [18:3(n-3)]. (B) Linoleic acid, LA [18:2(n-6)], arachidonic acid, AA [20:4(n-6). Controls represent unsupplemented vehicle and piroxicam treated cultures. Vehicle treatment represents the addition of ethanol in an equal volume to that required to deliver drug treatment. Data represents mean values ±SEM, n = 5 with eight replicate measurements from various regions of wounds. Values not sharing a letter are significantly different ($P < 0.05$).

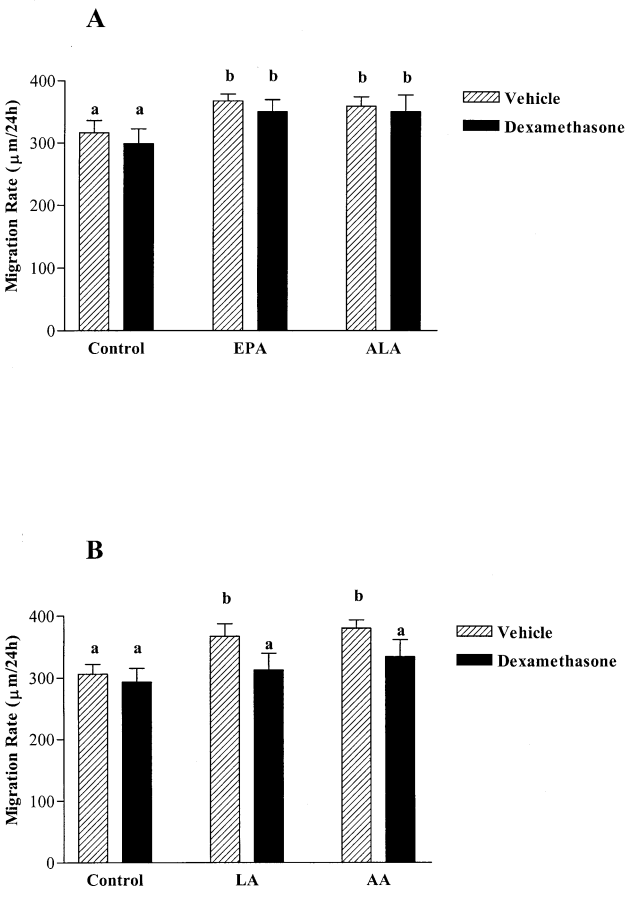

Fig. 4. Effect of the COX2 inhibitor, DEX, on fatty acid stimulation of migration in wounded, IEC-6 monolayers. IEC-6 cells were grown in 6 well culture plates, treated with 30 μmol/L fatty acid and grown to confluence (72 h). Confluent monolayers were treated with DEX (2 μmol/L) 4 h prior to wounding. Cultures were then wounded and maintained for 24 h following replacement with fresh fatty acid supplemented media and DEX. Monolayers were then fixed and analyzed as described in Materials and Methods. (A) Eicosapentaenoic acid, EPA [20:5(n-3)], alpha-linolenic acid, ALA [18:3(n-3)]. (B) Linoleic acid, LA [18:2(n-6)], arachidonic acid, AA [20:4(n-6). Controls represent unsupplemented vehicle and DEX treated cultures. Vehicle treatment represents the addition of ethanol in an equal volume to that required to deliver drug treatment. Data represents mean values ±SEM, n = 6 with eight replicate measurements from various regions of wounds. Values not sharing a letter are significantly different ($P < 0.05$).

this cytokine in fatty acid modulation of restitution (Table 3). No significant effect was observed on the production of bioactive TGFβ_1 by either wounding or fatty acid treatment. When samples were acid treated to activate the latent form of TGFβ_1, n-3 supplementation, but not n-6 supplementation, increased latent TGFβ_1 production in wounded cultures (Table 3). Wounding alone had no effect on latent TGFβ1 levels in conditioned media.

4. Discussion

The *in vitro* IEC-6 model of intestinal wound healing demonstrates many characteristics similar to *in vivo* intestinal wound repair making it ideal to study the process of restitution without the confounding variables of heterogeneous cell populations inherent to primary cell culture or animal models [10]. We previously demonstrated that the n-3 fatty acids ALA and EPA as well as the n-6 fatty acids LA, GLA and AA stimulate the process of intestinal epithelial restitution in the IEC-6 model [5]. Fatty acid supplementation, *in vivo* and *in vitro*, can modify transport systems, membrane bound enzymes and receptors [16] and are capable of eliciting profound effects on eicosanoid production which can affect many processes in the gastrointestinal tract [17–19]. In the present study we set out to determine which eicosanoid synthesis pathways, LOX or COX, might

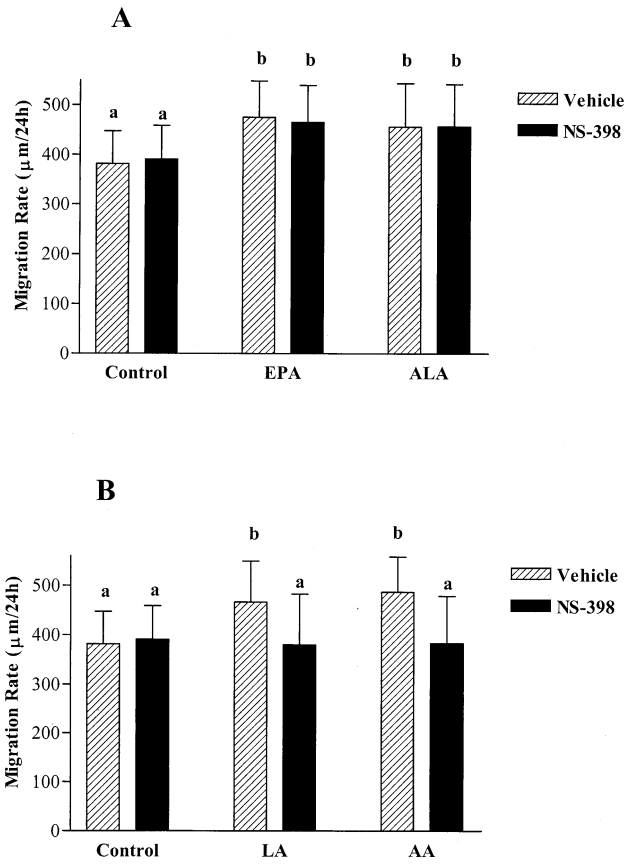

Fig. 5. Effect of COX2 inhibitor, NS-398, on fatty acid stimulation of migration in wounded, IEC-6 monolayers. IEC-6 cells were grown in 6 well culture plates, treated with 30 μmol/L fatty acid and grown to confluence (72 h). Confluent monolayers were treated with NS-398 (10 μmol/L) 4 h prior to wounding. Cultures were then wounded and maintained for 24 h following replacement with fresh fatty acid supplemented media and NS-398. Monolayers were then fixed and analyzed as described in Materials and Methods. (A) Eicosapentaenoic acid, EPA [20:5(n-3)], alpha-linolenic acid, ALA [18:3(n-3)]. (B) Linoleic acid, LA [18:2(n-6)], arachidonic acid, AA [20:4(n-6). Controls represent unsupplemented vehicle and NS-398 treated cultures. Vehicle treatment represents the addition of ethanol in an equal volume to that required to deliver drug treatment. Data represents mean values ±SEM, n = 3 with eight replicate measurements from various regions of wounds. Values not sharing a letter are significantly different ($P < 0.05$).

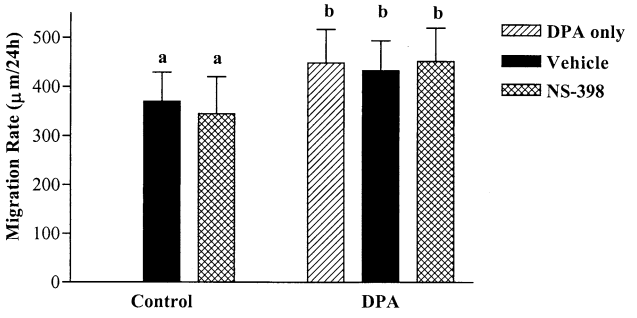

Fig. 6. Stimulation of migration by DPA in wounded, IEC-6 monolayers. IEC-6 cells were grown in 6 well culture plates, treated with 30 μmol/L DPA and grown to confluence (72 h). Confluent monolayers were treated with NS-398 (10 μmol/L) 4 h prior to wounding. Cultures were then wounded and maintained for 24 h following replacement with fresh fatty acid supplemented media and NS-398. Monolayers were then fixed and analyzed as described in Materials and Methods. Docosapentaenoic acid, DPA [22:5(n-3)]. Controls represent unsupplemented vehicle and NS-398 treated cultures. Vehicle treatment represents the addition of ethanol in an equal volume to that required to deliver drug treatment. Data represents mean values ±SEM, n = 3 with eight replicate measurements from various regions of wounds. Values not sharing a letter are significantly different ($P < 0.05$).

be critical for these fatty acids to stimulate intestinal epithelial restitution.

Fatty acid supplementation of culture media results in rapid accumulation of eicosanoid precursors into membrane phospholipid [5,20,21]. The use of NSAIDs, which inhibit key enzymes necessary for eicosanoid synthesis, have been shown to cause gastric ulcers and impair wound healing in the gastrointestinal tract [22]. Here we exploited a number of inhibitors of eicosanoid metabolism to elucidate which pathways were responsible for inducing restitution. NDGA, an inhibitor of the LOX pathway, had no effect on restitution in either vehicle treated controls or fatty acid stimulated cultures. The use of the COX pathway inhibitor, piroxicam, reduced migration rates of n-6 fatty acid enhanced restitution back to control levels but had no effect on n-3 stimulated migration rates. Although conditions at wounding were somewhat disparate, Zushi et al. [9] also demonstrated similar results with NDGA and piroxicam during stimulated resealing. Fetal calf serum contains both eicosanoids and various cytokines that may be responsible, in whole or in part, for the effect of fetal calf serum on resealing speed seen in Zushi's work [9]. DEX and NS-398 have been described as selective COX2 inhibitors [23] although some controversy exists with respect to the specificity of DEX. DEX was previously described as an inhibitor eicosanoid synthesis by modulating PLA_2 activity, but has more recently been described as a selective inhibitor of COX2 *in vitro* and *in vivo* [24–29]. Nonetheless, in our experiments both DEX and NS-398 reduced the n-6 fatty acid stimulated migration rate back to control values with no significant effect on n-3 stimulated restitution. Therefore, the results from eicosanoid inhibitor experiments would suggest that activity of LOX pathway is either not required or not sufficient for fatty acid stimulated restitution. On the other hand, the use of indomethacin, [5] piroxicam, DEX and NS-398 indicate that the n-6 fatty acid stimulation of restitution requires intact COX activity. Inhibition of eicosanoid synthesis in our previous work [5] as well as in the present study did not affect n-3 stimulated restitution suggesting that n-3 and n-6 fatty acids are exerting their effects through independent mechanisms. If one accepts the specificity of DEX and NS-398, then this data indicates that COX2 is central to the n-6 fatty acid stimulation of cellular migration.

Prostaglandins have been described as having potent effects on the process of wound healing [9,22]. COX2 production of PGs is known to be induced by a number of inflammatory mediators, cytokines and other factors asso-

Table 1
Prostaglandin E_2 (pg/ml) production in conditioned media from wounded and unwounded, confluent IEC-6 cultures treated with fatty acid and COX inhibitor, piroxicam (5 μmol/L)[a]

Treatment	Vehicle[b]	Piroxicam	Vehicle	Piroxicam	Vehicle	Piroxicam
	Control	Control	DHA	DHA	EPA	EPA
Wounded	366 (±123)	34 (±8)	144 (±19)	81 (±1)	305 (±52)	111 (±26)
Unwounded	42 (±6)	36 (±9)	76 (±14)	103 (±11)	116 (±17)	143 (±43)
	Control	Control	ALA	ALA		
Wounded	329 (±81)	46 (±4)	362 (±86)	73 (±11)		
Unwounded	56 (±1)	44 (±2)	73 (±18)	56 (±10)		
	Control	Control	LA	LA	AA	AA
Wounded	374 (±134)	42 (±3)	534 (±101)	163 (±27)	3344 (±586)	1643 (±71)
Unwounded	57 (±8)	50 (±7)	169 (±37)	183 (±20)	1682 (±357)	2506 (±151)

IEC-6 cells were grown in 6 well plates and maintained in media supplemented with 30 μmol/L fatty acids to confluence. Cultures were treated with COX inhibitor, piroxicam (5 μmol/L) 4 h prior to wounding. IEC-6 monolayers were wounded as described in Materials and Methods. Fatty acid supplemented media and drug treatment were replaced following wounding in both wounded and unwounded cultures. Cultures were maintained for 24 h at which time conditioned media was collected and processed as described in Materials and Methods. Docosahexaenoic acid, DHA [22:6(n-3)], eicosapentaenoic acid, EPA [20:5(n-3)], alpha-linolenic acid, ALA [18:3(n-3)], linoleic acid, LA [18:2(n-6)], arachidonic acid, AA [20:4(n-6)]. Control represents cultures not supplemented with fatty acid.

[a] Values represent means (±SEM) from three independent experiments performed in duplicate.

[b] Vehicle treatment is an equal volume of ethanol as that used to deliver drug.

ciated with wounding in the gastrointestinal tract [30,31]. In the current study, piroxicam and NS-398 had no measurable effect on basal production of PG but did indeed inhibit production in cases where PG synthesis was stimulated by either wounding or fatty acid supplementation. One exception to this was observed in unwounded, AA supplemented cultures treated with piroxicam. Unexpectedly, here we saw an increase in PG synthesis. Reddy and Herschmann have proposed two alternative and independent pathways for PG synthesis, one of which focuses on COX1 utilization of exogenous AA and secretory PLA_2 and the other that shows preference for COX2 and endogenous AA liberated by cytosolic PLA_2 [32]. Chulada and co-workers have actually demonstrated this preference for AA source in rodent fibroblasts [33]. Using this model one might suspect that the source of higher than basal PG levels in unwounded, AA-supplemented cultures was via COX1. Further increases in PG synthesis would then be stimulated by wounding in a COX2 specific fashion as has been observed in a number of stress and mitogen activated pathways that utilize cytosolic PLA_2 [32]. AA itself can modulate COX1 activity and the inhibitory action of COX inhibitors through allosteric regulation [34]. Though piroxicam has been described as inhibiting both isoforms of COX, at the concentrations used in these experiments piroxicam is suggested to be relatively more selective for COX1. In the wounded, AA-supplemented situation, COX2 conversion of AA to PGE_2 would be less affected by piroxicam acting primarily on COX1,

Table 2
Prostaglandin E_2 (pg/ml) production in conditioned media from wounded and unwounded, confluent IEC-6 cultures treated with fatty acid and COX2 inhibitor, NS-398 (10 μmol/L).[a]

Treatment	Vehicle[b]	NS-398	Vehicle	NS-398	Vehicle	NS-398
	Control	Control	EPA	EPA	ALA	ALA
Wounded	40 (±21)	20 (±8)	81 (±30)	66 (±8)	152 (±6)	45 (±3)
Unwounded	16 (±1)	13 (±7)	72 (±5)	68 (±20)	77 (±4)	78 (±27)
	Control	Control	DHA	DHA		
Wounded	40 (±25)	12 (±9)	105 (±19)	87 (±6)		
Unwounded	15 (±1)	14 (±9)	106 (±10)	96 (±4)		
	Control	Control	LA	LA	AA	AA
Wounded	40 (±17)	21 (±4)	313 (±67)	155 (±36)	1301 (±25)	555 (±25)
Unwounded	23 (±4)	20 (±3)	310 (±81)	214 (±36)	1423 (±148)	899 (±132)

IEC-6 cells were grown in 6 well plates and maintained in media supplemented with 30 μmol/L fatty acids to confluence. Cultures were treated with COX2 inhibitor, NS-398 (10 μmol/L) 4 h prior to wounding. IEC-6 monolayers were wounded as described in Materials and Methods. Fatty acid supplemented media and drug treatment were replaced following wounding in both wounded and unwounded cultures. Cultures were maintained for 24 h at which time conditioned media was collected and processed as described in Materials and Methods. Docosahexaenoic acid, DHA [22:6(n-3)], eicosapentaenoic acid, EPA [20:5(n-3)], alpha-linolenic acid, ALA [18:3(n-3)], linoleic acid, LA [18:2(n-6)], arachidonic acid, AA [20:4(n-6)]. Control represents cultures not supplemented with fatty acid.

[a] Values represent means (±SEM) from three independent experiments performed in duplicate.

[b] Vehicle treatment is ethanol in an equal volume to that used to deliver drug.

Table 3
Bioactive and Latent TGFβ_1 production in conditioned media.[a]

Treatment	Control	DHA	EPA	ALA	LA	AA
Bioactive TGFβ_1 (pg/ml)						
Wounded	290 (±66)	352 (±147)	316 (±112)	251 (±80)	309 (±112)	194 (±31)
Unwounded	249 (±65)	267 (±63)	206 (±59)	200 (±51)	135 (±68)	199 (±12)
Latent TGFβ_1 (pg/ml)						
Wounded	975 (±79)[a]	1166 (±137)[a]	2796 (±170)[b]	3035 (±430)[b]	852 (±94)[a]	933 (±69)[a]
Unwounded	1143 (±89)[a]	1257 (±54)[a]	3095 (±427)[b]	2809 (±279)[b]	972 (±173)[a]	974 (±33)[a]

IEC-6 cells were grown in 6 well plates and maintained in media supplemented with 30 μmol/L fatty acids to confluence. IEC-6 monolayers were wounded as described in Materials and Methods. Fatty acid supplemented media was replaced following wounding in both wounded and unwounded cultures. Cultures were maintained for 24 h at which time conditioned media was collected and processed as described in Materials and Methods. Latent TGFβ (pg/ml) was determined by acid activation of samples with 1N HCl. Bioactive TGFβ (pg/ml) was determined in samples not acid activated. Docosahexaenoic acid, DHA [22:6(n-3)], eicosapentaenoic acid, EPA [20:5(n-3)], alpha-linolenic acid, ALA [18:3(n-3)], linoleic acid, LA [18:2(n-6)], arachidonic acid, AA [20:4(n-6)]. Control represents cultures not supplemented with fatty acid. Values not sharing a superscript are significantly different ($P < 0.05$).

[a] Values represent means (±SEM) from three independent experiments performed in duplicate.

and AA may interfere with piroxicam inhibition of COX1 through an allosteric mechanism, resulting in an apparent increase in PGE_2. PGE_2 synthesis in the presence of n-3 fatty acids also appears slightly elevated above basal levels in vehicle treated conditions. This may result from a slight cross reactivity, inherent to the PGE_2 assay, to PGE_3 that is a product of n-3 metabolism by COX. When prostaglandin precursors (as fatty acids) are supplemented into culture media we saw a dramatic increase in PGE_2 released into conditioned media. Although the use of COX inhibitors, piroxicam and NS-398, reduced PGE_2 production in n-6 fatty acid supplemented cultures, it did not reduce PGE_2 production back to control levels. PGE_2 levels remained several fold greater than controls in these cultures and yet the fatty acid stimulated restitution was completely attenuated. This suggests that PGE_2 is not likely to be the only important metabolite responsible for enhanced restitution or that a critical threshold of production is necessary before increased restitution is observed. Zushi et al. [9] identified a role for PGI_2 in serum enhanced restitution. Erickson et al. [35–37] showed that although PGE_2 was capable of reducing injury, it did not influence the restitution process in an *in vivo* model of wound healing. *In vivo*, fatty acid supplementation and the subsequent increase in PGs might prove beneficial in maintaining the integrity of this important mucosal barrier in the face of intestinal challenge.

A variety of growth factors enhance intestinal restitution and a central role for TGF-β has been defined [6–9]. In the present work supplementation of culture media with EPA and ALA but not DHA, LA or AA, resulted in increased secretion of the latent form of TGF-β. Modulation of cytokine metabolism by n-3 fatty acids is corroborated by several studies. Enhanced migration of bovine endothelial cells by fibroblast growth factor (FGF) and tumour necrosis factor-α was potentiated by EPA but not DHA or AA [38]. Fernandes et al. [14] observed that a fish oil diet, but not a corn oil diet (rich in n-6 fatty acids), increased TGF-β mRNA and protein in mouse spleen. In our work, DHA had no effect on the production of TGF-β, therefore the effects observed by Fernandes et al. [14] could be specifically due to the EPA, rather than the DHA in the fish oil diet. From our results we propose that EPA and ALA are exerting their effects on the restitution process through increased production of TGF-β.

We have also identified enhanced migration of wounded IEC-6 monolayers by DPA, consistent with studies demonstrating an enhanced migratory effect of DPA on bovine endothelial cells [13]. Kanayasu et al. [38] observed a stimulation of cellular migration by EPA but not DHA supplementation and furthermore Kanayasu-Toyoda et al. showed that inhibition of EPA elongation to DPA, prevented the enhanced migration [39]. We and others have shown that supplementation with DHA, DPA, EPA and ALA are all capable of increasing membrane phospholipid composition of EPA but only DPA, EPA, and ALA increase incorporation of DPA into membrane phospholipid [5,38]. However, only supplementation with DPA, EPA and ALA both elevate levels of DPA and enhanced restitution [5] (this study), suggesting that DPA is the critical metabolite.

What is the *in vivo* relevance of this level of fatty acid incorporation? Can similar changes be induced through dietary supplementation? The *in vivo* situation may be different in that retroconversion of DHA through dietary supplementation could be achieved by other tissues and DPA retransported back for incorporation into the intestinal mucosa. Thus, while DHA had little/no effect in cultured IEC-6 intestinal epithelial cells, this does not preclude an ability of DHA to effect such a change in the intact organism. In fact we have shown, in rat models [40,41] that DHA supplementation results in a significant increases in DPA content in multiple tissues. In tissues such as bone marrow and liver DPA was undetectable in control animals and rose to 2% of

fatty acids in DHA fed animals. This is similar to the 1–3% increase in DPA that we observed in IEC-6 cells under the ALA and EPA supplemented conditions, respectively. Hansen Petrik et al. [42], recently fed ethyl ester supplemented diets to tumor susceptible mice and showed similar levels of DPA in the mucosae isolated from ALA and EPA fed mice. Linseed fed as whole seed to finishing pigs results in an up to 3% increase in DPA with a concomitant decrease in AA in liver and other tissues [43]. While many authors do not report complete fatty acid compositional data, once can infer that if EPA and DHA are increased (*in vivo*) that DPA is likely also increased. Gee et al. reported substantial increases in colonic mucosal content of EPA and DHA prior to surgery in patients consuming fish oil, approximating the *in vitro* changes we have observed [44]. Therefore it is clearly possible to achieve these levels of fatty acid change *in vivo* that could have clinical benefit.

PLA_2 hydrolyses fatty acids, including AA, from the *sn*-2 position of phospholipid and this action is attenuated by BPB. Minami et al. [11] showed that in the IEC-6 model of intestinal wound healing, BPB treatment blocked the migration-promoting effect of PLA_2 from *Naja naja* venom by a mechanism independent of eicosanoid synthesis. In the present experiments the inhibition of PLA_2 by BPB abolished both n-6 and n-3 enhanced migration. Inhibition of the release of eicosanoid precursors from the *sn*-2 position of phospholipid could account for the loss of stimulation of restitution in LA and AA enriched cultures. Because eicosanoid metabolism is not required, but PLA_2 activity is necessary for the EPA and ALA effect, it may be that n-3 fatty acids need to be released from the plasma membrane by PLA_2 to be elongated and desaturated to DPA prior to their stimulation of migration.

We had previously proposed that n-3 and n-6 fatty acids were exerting their stimulatory effects on intestinal epithelial cell restitution by differing mechanisms [5]. The present studies demonstrate that only n-3 fatty acids, probably through DPA, are modulating the production of TGF-β and that only n-6 fatty acids are exerting their effects through a COX dependent pathway. Future work should address the molecular mechanisms for these fatty acid specific effects so that these nutrients might be used effectively in a clinical setting in the treatment and recovery from intestinal challenges.

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