Inhibitory effects of omega-3 fatty acids on injury-induced epidermal growth factor receptor transactivation contribute to delayed wound healing

Harmony F. Turk, Jennifer M. Monk, Yang-Yi Fan, Evelyn S. Callaway, Brad Weeks, and Robert S. Chapkin

1Program in Integrative Nutrition and Complex Diseases, Texas A & M University, College Station, Texas; 2Department of Nutrition and Food Science, Texas A & M University, College Station, Texas; 3Department of Veterinary Pathobiology, Texas A & M University, College Station, Texas; and 4Department of Microbial and Molecular Pathogenesis, Texas A & M University System Health Science Center, College Station, Texas

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INTESTINAL WOUND HEALING requires a delicate balance of migration, proliferation, and differentiation of epithelial cells (36). The first step of wound healing is epithelial restitution, in which epithelial cells adjacent to the wound rapidly migrate into the wounded area (11, 12, 48). Restitution begins quickly, within minutes to hours, following injury, and this process has been shown to be independent of cell proliferation (11, 48). The next step of wound healing, which begins hours to days after injury, requires proliferation of the mucosal epithelium to increase the number of enterocytes available to repopulate the injured area (11, 26). The final stage of wound healing requires maturation and differentiation of epithelial cells to restore and maintain intestinal function (48).

Many pathways that are hijacked by cancer cells are integral to the process of wound healing. Importantly, EGFR receptor (EGFR) signaling is involved in all the stages of intestinal wound healing. It has been clearly demonstrated that treatment with EGFR ligands, including EGF, heparin-binding EGF, and transforming growth factor (TGF)-α, promotes intestinal wound healing (15, 17). Additionally, in response to a wounding event, EGFR becomes activated. This activation is regulated in part by G protein-coupled receptors, which signal to activate matrix metalloproteinases (MMPs) (55). Multiple downstream signaling pathways from EGFR are central in regulating distinct stages of wound healing. Restitution requires extensive reorganization of the actin cytoskeleton through activation of Rho family small GTPases, including phosphatidylinositol 3-kinase and Src (12, 16), and EGFR-mediated activation of these mediators is imperative for wound healing. PLCγ1 is also a downstream signaling partner of EGFR that mediates intestinal epithelial cell migration (30, 41), PLCγ1 and Rac1 directly interact to coregulate cytoskeletal remodeling and cell migration in response to EGF (30).

We previously demonstrated that a dietary long-chain n-3 PUFA, docosahexaenoic acid (DHA), suppresses EGFR signaling in mouse colonocytes (50). By altering the plasma membrane localization of EGFR, DHA increased receptor phosphorylation but paradoxically suppressed downstream activation of Ras, ERK1/2, and STAT3. DHA has also been shown to exert similar effects on EGFR-dependent activation of Ras and ERK1/2 activation in several cancer cell lines (42). On the basis of the central role of EGFR-mediated signaling in wound healing, we hypothesized that DHA would be detrimental with respect to intestinal wound healing. This postulate is supported by a recent study conducted by our laboratory demonstrating that mice fed a diet enriched in fish oil containing DHA and its precursor eicosapentaenoic acid (EPA) exhibited reduced overall survival and increased wounding following treatment with dextran sodium sulfate (DSS) (27). An additional study conducted in humans demonstrated that wound healing of the skin is delayed in patients dosed with a combination EPA and DHA (33). Conversely, some studies have demonstrated a beneficial effect of n-3 PUFA in response to injury (18, 25). These conflicting data warrant further evaluation of the role of omega-3 fatty acids on wound healing in various models.
investigation into the mechanisms by which n-3 PUFA modulate wound healing.

MATERIALS AND METHODS

**Cell culture.** Conditionally immortalized young adult mouse colonic (YAMC) cells were obtained from R. H. Whitehead (Ludwig Cancer Institute, Melbourne, Australia). YAMC cells (passages 12–17) were cultured under permissive conditions, 33°C and 5% CO₂, in RPMI 1640 medium (Mediatech, Manassas, VA) supplemented with 5% FBS (Hyclone, Logan, UT), 2 mM GlutaMAX (GIBCO, Grand Island, NY), 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenious acid (Collaborative Biomedical Products, Bedford, MA), and 5 IU/mL murine IFNγ (Roche Applied Science, Mannheim, Germany). Select cultures were treated with 72 h with 50 µM fatty acid [DHA, linoleic acid (LA, 18:2n-6), or EPA; NuChek, Elysian, MN] complexed with bovine fatty acid-free serum albumin (BSA; Roche Applied Science). In select cultures, for the final 16–18 h, complete medium was replaced with low-serum (0.5% FBS) medium. Cells were then stimulated with 0–25 ng/mL recombinant mouse EGF (Sigma, St. Louis, MO) and harvested. For preparation of whole cell extracts of wounded cells, cells were grown to confluence in 150-mm dishes. Monolayers were cross-scraped 25 times in all directions (horizontal, vertical, and diagonals; 100 scrapes total) with a 1-ml pipette tip, as previously described (14). Monolayers were then gently washed three times with ice-cold PBS and harvested.

**Western blotting.** For Western blotting, cells were homogenized in ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.2, 250 mM sucrose, 2 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 100 mM sodium orthovanadate, 1% Triton X-100, 100 µM activated sodium orthovanadate, 10 mM β-mercaptoethanol, and protease inhibitor cocktail), as previously described (10). After homogenization, lysates were sheared using a 29-gauge needle, incubated on ice for 30 min, and centrifuged at 16,000 g for 20 min. The supernatant was collected, and protein concentration was assessed using Pierce Coomassie Plus Protein assay (Thermo Fisher Scientific, Rockford, IL). Lysates were treated with 1× pyronin sample buffer and subjected to SDS-PAGE in precast 4–20% Tris-glycine mini gels (Invitrogen, Carlsbad, CA). After electrophoresis, proteins were electroblotted onto a polyvinylidene difluoride membrane with the use of a Hoefer Mighty Small Transphor unit at 400 mA for 90 min. After transfer, the membrane was incubated in 5% IgG-free BSA (Roche Applied Science) and 0.1% Tween 20 in Tris-buffered saline (TBST) at room temperature for 1 h with shaking and then with primary antibody diluted in 5% BSA in TBST at 4°C overnight with shaking. Membranes were washed with TBST and incubated with peroxidase-conjugated secondary antibody according to the manufacturer’s instructions. Bands were developed using Pierce SuperSignal West Femto maximum-sensitivity substrate. Blots were scanned using a Fluor-S Max Multimager system (Bio-Rad, Hercules, CA). Bands were quantified using Quantity One software (Bio-Rad). Monoclonal rabbit anti-EGFR (catalog no. 2646), anti-phosphorylated (Tyr1068) EGFR (catalog no. 3777), anti-PLCγ1 (catalog no. 2822), and anti-phosphorylated (Tyr1996) PLCγ1 (catalog no. 2821) were purchased from Cell Signaling. Peroxidase-conjugated goat anti-rabbit IgG was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

**Small Rho GTPase activity assay.** Activation of Cdc42 and Rac1 was assessed using kits from Cytoskeleton (Denver, CO). Samples for these assays were harvested as described above using the lysis buffer provided with the kits and supplemented with protease and phosphatase inhibitors (Sigma). Activation of Cdc42 and Rac1 was analyzed using G-LISA Cdc42 and Rac1 Activation Assay Biochem kits, respectively, in the colorimetric format. The assays were performed using 25 µg of protein according to the manufacturer’s instructions. Absorbance was measured on a microplate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA).

**Bioenergetic profile.** Cellular bioenergetic profiles were measured using an extracellular flux analyzer (model XF24, Seahorse Bioscience, North Billerica, MA). YAMC cells were seeded at a density of 3.0 × 10⁴ cells/well onto 24-well cell culture plates (catalog no. XF; Seahorse Bioscience) and cultured overnight in complete medium at 33°C and 5% CO₂. Cells were then placed into unbuffered assay medium (DMEM supplemented with 11 mM glucose, 2 mM glutamine, and 1 mM pyruvate) and incubated in a CO₂-free environment at 33°C for 1 h. After incubation, the 24-well culture plates were transferred to the extracellular flux analyzer. Hydrated cartridges containing mitochondrial mediators, oligomycin (10 µmol/L), carbonylcyanide p-trifluoromethoxyphenylhydrazone (3 µmol/L), and rotenone (1 µmol/L) were added. The compounds were injected at timed intervals into the wells (final concentration of mitochondrial mediators in each well was 1/10th of the initial concentration), and oxygen consumption rate was monitored continuously.

**Animals.** Male C57BL/6 wild-type mice aged 12–15 wk were purchased from Jackson Laboratories. All procedures followed guidelines approved by the US Public Health Service and were approved by the Institutional Animal Care and Use Committee at Texas A & M University. Mice were maintained under barrier conditions and initially consumed a Teklad commercial mouse nonpurified diet (Harlan, Indianapolis, IN) ad libitum. Subsequently, mice were divided into groups and, for 15–21 days, fed one of three isocaloric semipurified diets that were adequate in all nutrients but differed in their lipid compositions: 3% corn oil (CO) control diet (Dyets, Bethlehem, PA), DHA-enriched (1% DHA + 2% CO) diet, or EPA-enriched (1% EPA + 2% CO) diet. Highly purified lipid sources were utilized, i.e., DHA ethyl ester (>70% pure; Incromega DHA700E SR, Bioriginal Food and Science, Saskatchewan, SK, Canada) and EPA-free fatty acid (>95% pure; SLA Pharma, Watford, UK). Diet composition
(g/kg diet) was as follows: 440 sucrose, 200 casein, 220 cornstarch, 3 dl-methionine, 35 AIN-76 salt mix, 10 AIN-76 mineral mix, 2 choline chloride, and 60 cellulose (Bio-Serv) + 30 CO (CO diet), 10 DHA + 20 CO (DHA diet), or 10 EPA + 20 CO (EPA diet). Diets were replaced daily. Animals were fed the experimental diets for 10 days prior to treatment with 2.5% DSS (36,000–50,000 mol wt; MP Biomedicals, Santa Ana, CA) in the drinking water for 5 days. Some animals suffered adverse symptoms due to the nature of disease progression [inflammatory bowel disease (IBD)] that merited euthanasia. The mice were monitored for lack of fecal pellets, as well as overt appearance of distress. Any animal showing signs of physical distress was removed to an individual cage and monitored. Animals that did not groom, displayed lack of mobility, had multiple days of rectal bleeding, or did not produce enough fecal pellets were terminated. After DSS treatment, mice were allowed to recover for 0, 3, or 6 days. At 2 h before termination, a subset of mice were injected with 2′-deoxy-5-ethyluridine (EdU) for assessment of cell proliferation, and 6 days. At 2 h before termination, a subset of mice were injected with 2′-deoxy-5-ethyluridine (EdU) for assessment of cell proliferation.

**Colonic mucosal mRNA expression.** RNA was isolated from colonic mucosal scrapings (n = 4 per dietary group no-DSS controls or n = 6–8 per dietary group at all recovery times post-DSS) using the RNA 4-PCR kit (Life Technologies, Carlsbad, CA). Real-time RT-PCR was used to quantify mRNA expression, and amplification was performed using TaqMan Universal PCR master mix (Life Technologies), as described previously (34). TaqMan gene expression kits (Life Technologies) were used for amplification, namely, IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), IFNγ (Mm01168134_m1), TNFα (Mm00443260_g1), CCL2 [monocyte chemoattractant protein 1 (MCP-1), Mm00441242_m1], IL-10 (Mm00439614_m1), TGFβ1 (Mm01178820_m1), IL-4 (Mm00445259_m1), IL-13 (Mm00434204_m1), and IL-22 (Mm01226722_g1). Amplification of mRNA (fluorescence) was recorded over 40 cycles, and the corresponding cycle numbers (Ct) were used to calculate mRNA expression as follows: 2^−ΔΔCt. Target gene expression was normalized to ribosomal 18S expression (Mm03928990_g1).

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**Fatty acid analysis.** For assessment of dietary lipid incorporation into membrane phospholipids, lipids were extracted from scraped colonic mucosa with 2:1 (vol/vol) chloroform-methanol. Total phospholipids were subsequently separated by thin-layer chromatography with 90:8:10:8 (vol/vol/vol/vol) chloroform-methanol-acetic acid-water. After transesterification, fatty acid methyl esters were quantified by capillary gas chromatography-mass spectrometry (45).

**Statistics.** The effect of independent variables (treatment effects) was assessed using one-way ANOVA, and differences among means were evaluated using Tukey’s post hoc test of contrast or least-squares means. The effect of two independent variables was assessed using two-way ANOVA. Data sets not exhibiting a normal distribution were subjected to the Kruskal–Wallis test (χ² approximation) followed, if justified, by the statistical probability outcome using Wilcoxon’s two-sample test. P < 0.05 was considered statistically significant.

**RESULTS**

**Wounding-induced EGFR transactivation.** We previously showed that EGFR phosphorylation in response to direct stimulation with ligand is increased by DHA (50). However, other long-chain PUFA, including LA and EPA, did not recapitulate this same effect. To expand on these observations, we evaluated the effects of fatty acids on EGFR activation in response to injury. It has been reported that EGFR is transactivated in response to wounding (14). Therefore, we utilized a technique to wound colonic epithelial cells by extensively scratching a confluent monolayer of colonocytes. As expected, wounding strongly induced phosphorylation of EGFR in untreated control and LA-treated cells (Fig. 1A). Treatment of cells with DHA or EPA, however, reduced the injury-induced transactivation of EGFR. This observation is noteworthy, because DHA promotes ligand-induced EGFR phosphorylation, whereas EPA has no effect on phosphorylation of the receptor (50), indicating that n-3 PUFA may modulate EGFR activation in a context-specific manner.

**Activation of downstream signaling in response to EGF or injury.** EGFR signaling is required to activate many important downstream mediators that stimulate cellular events required for wound healing. For example, EGFR-mediated activation of PLCγ1, Rac1, and Cdc42 has been shown to be integral in colonic wound healing (12, 16, 30). Therefore, we assessed activation of each of these signaling mediators in response to direct stimulation with an EGFR-specific ligand, EGF, or in response to injury. Initially, YAMC cells were stimulated with EGF or by wounding, and PLCγ1 activation status was assessed. Phosphorylated (Tyr783) PLCγ1 was quantified, because phosphorylation at Tyr783 is directly mediated by binding of PLCγ1 to EGFR (28). Addition of EGF enhanced phosphorylation of PLCγ1 in all treatment groups. However, PLCγ1 activation in DHA-treated cells was significantly reduced relative to all other treatment groups (Fig. 1B). This is consistent with our previous observation that DHA reduces EGF-stimulated activation of ERK1/2 and STAT3 (50). In contrast, EPA treatment had no effect on EGF-stimulated PLCγ1 compared with control or LA-treated cells. Alternatively, treatment with EPA or DHA inhibited injury-induced activation of PLCγ1 (Fig. 1C), which is consistent with the observation that DHA and EPA reduced injury-induced EGFR phosphorylation (Fig. 1A). Subsequently, we assessed EGFR-induced activation of Rac1 and Cdc42 with an assay that specifically recognizes the activated, GTP-bound forms of these molecules. Incubation with EGF induced activation of Rac1 compared with control or LA-treated cells. Alterna-
Cdc42 to a similar extent in control and LA- and EPA-treated cells (Fig. 2A). Conversely, treatment with DHA significantly reduced EGF-stimulated activation of Cdc42. Furthermore, whereas Rac1 activation was equally induced in control and LA- and EPA-treated cells, DHA significantly reduced activation of Rac1 in response to EGF (Fig. 2B). Additionally, we assessed activation of Cdc42 and Rac1 in response to injury. DHA and EPA significantly reduced activation of Cdc42 relative to control and LA-treated cells (Fig. 2C). Similarly, compared with control cells, DHA and EPA decreased injury-mediated activation of Rac1 (Fig. 2D). Additionally, Rac1 activation was suppressed to a greater extent by DHA than
EPA. These data indicate that DHA reduces ligand-stimulated 
EGFR signaling, whereas DHA and EPA reduce injury-in-
duced EGFR signaling.

**Scratch and migration assays.** On the basis of our initial 
observation that DHA and EPA impede wound-induced EGFR 
signaling, we utilized a functional assay to assess wound 
healing. Cells were grown to confluency, and monolayers were 
subsequently scrape-wounded. Then the cells were treated with 
EGF, and the wound was imaged every 15 min for 24 h. Wound 
healing was measured by counting the number of cells that 
had infiltrated the wounded area at 12 and 24 h after the 
wounding event. Typically, unstimulated (no EGF) cells re-
quired >24 h for complete wound closure (Fig. 3A). In con-
trast, EGF stimulated wound healing, and complete wound 
closure was observed by 24 h in control and LA-treated cells. 
However, EGF-mediated wound healing of DHA- and EPA-
treated cells was significantly delayed. Cell migration was 
additionally assessed using a transwell migration assay. EGF 
was utilized to stimulate cell migration through a membrane 
with 8-μm pores over a 12-h incubation period. A similar 
extent of migration was observed in control and LA-treated 
cells (Fig. 3B). However, treatment with DHA or EPA signif-
icantly reduced cell migration. To ensure that the reduction in 
wound healing was not due to a nonspecific effect on cell 
viability, mitochondrial respiration was assessed (Fig. 4). Fatty 
acid treatment did not influence oxygen consumption rates 
compared with control untreated cells, indicating that DHA 
and EPA did not impact cell viability.

**Survival rates in vivo.** On the basis of the in vitro observa-
tions, we assessed whether the dietary lipid source can alter the 
sequelae associated with acute wound healing in an animal 
model. Mice were fed a diet enriched in CO, purified EPA, or 
DHA for 10 days and then exposed for 5 days to 2.5% DSS in 
the drinking water. DSS is a wounding agent that is often 
utilized to induce a form of colitis that presents clinical and 
histological features similar to human IBDs (29, 54). After 
DSS treatment, mice were allowed no recovery period (0 days) 
or allowed to recover for 3 or 6 days. Mice were maintained on 
the experimental diets throughout the study period, and the 
colonic mucosa phospholipid composition was assessed fol-
lowing the 6-day recovery period (Table 1). Arachidonic acid 
(20:4 n-6) levels were significantly lower in mucosa from 
DHA- and EPA- than CO-fed mice. Additionally, DHA-fed 
mice had significantly enriched levels of DHA and intermedi-

![Fig. 3. n-3 PUFA impede colonocyte wound healing. YAMC cells were untreated (control) or treated with 50 μM fatty acid (LA, DHA, or EPA) for 72 h. For 
the final 16–18 h, cells were serum-starved (0.5% FBS). A: cells were then scratched with a p-200 pipette tip and stimulated with 25 ng/ml EGF. Cell migration 
into the wounded area was imaged every 15 min on a Nikon TiE inverted microscope for 24 h and quantified by counting the number of cells that migrated into 
the wounded area at 12 and 24 h after the initial wound. Four independent experiments were conducted, and representative images are presented. Scale bars, 50 μm. B: after serum 
starvation, cells were trypsinized and counted, and an equal number of cells were seeded into the top chamber of a 2-chamber well in 
serum-free medium. The bottom chamber was filled with medium supplemented with 25 ng/ml EGF. Cells were incubated for 12 h and allowed to migrate 
through the membrane between the chambers. Cells were then stained, and cells remaining in the top chamber were removed. Dye was eluted from the stained 
cells that had migrated through the membrane, and optical density of the dye/elution mixture was measured on a spectrophotometer. Two independent 
experiments were conducted. Values are means ± SE (n = 6). Statistical difference (P < 0.05), as indicated by unique letters (a and b), was measured using 
1-way ANOVA and Tukey’s test of contrast.
ate levels of EPA, whereas EPA-fed mice had the highest levels of EPA and docosapentaenoic acid (22:5 n-3) and intermediate levels of DHA. Supplementation of humans with DHA at 1.6 g/day has been reported to result in a fourfold enrichment of DHA in the serum (9), which is similar to the enrichment observed in the colonic mucosa of the DHA-fed mice. The highest mortality was associated with the EPA-fed mice (Fig. 5A). Additionally, over the 6-day recovery period, EPA significantly reduced survival compared with the CO diet (Fig. 5B). The DHA-enriched diet, however, did not reduce overall survival compared with the CO diet. In addition, the DHA- and EPA-fed mice lost more weight than the CO-fed mice (Fig. 5C). Interestingly, the DHA-fed mice began to regain weight at days 3 and 6 of recovery, whereas mice fed the other diets did not. Assessment of colonocyte proliferation

Table 1. Colonic mucosal fatty acid composition

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>CO</th>
<th>DHA</th>
<th>EPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>1.45 ± 0.20</td>
<td>1.36 ± 0.15</td>
<td>1.41 ± 0.12</td>
</tr>
<tr>
<td>16:0</td>
<td>26.35 ± 0.79</td>
<td>26.13 ± 0.74</td>
<td>24.88 ± 0.40</td>
</tr>
<tr>
<td>16:1 n-7</td>
<td>2.53 ± 0.61</td>
<td>2.45 ± 0.28</td>
<td>2.53 ± 0.18</td>
</tr>
<tr>
<td>18:0</td>
<td>16.35 ± 1.40</td>
<td>19.07 ± 0.99</td>
<td>17.75 ± 0.58</td>
</tr>
<tr>
<td>18:1 n-9</td>
<td>17.87 ± 1.63</td>
<td>16.03 ± 0.49</td>
<td>16.13 ± 0.57</td>
</tr>
<tr>
<td>18:1 n-7</td>
<td>3.49 ± 0.46</td>
<td>3.34 ± 0.28</td>
<td>3.11 ± 0.39</td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>17.74 ± 1.84</td>
<td>15.08 ± 1.77</td>
<td>14.31 ± 1.15</td>
</tr>
<tr>
<td>20:3 n-6</td>
<td>2.91 ± 0.52</td>
<td>2.79 ± 0.43</td>
<td>3.25 ± 0.15</td>
</tr>
<tr>
<td>20:4 n-6</td>
<td>9.24 ± 0.86†</td>
<td>5.60 ± 0.71*</td>
<td>5.58 ± 0.29*</td>
</tr>
<tr>
<td>20:5 n-3</td>
<td>0.43 ± 0.12*</td>
<td>2.91 ± 0.14†</td>
<td>6.44 ± 0.55‡</td>
</tr>
<tr>
<td>22:5 n-3</td>
<td>0.00 ± 0.00*</td>
<td>0.18 ± 0.02*</td>
<td>1.02 ± 0.12†</td>
</tr>
<tr>
<td>22:6 n-3</td>
<td>1.63 ± 0.19*</td>
<td>5.05 ± 0.38‡</td>
<td>3.58 ± 0.19†</td>
</tr>
</tbody>
</table>

Values (means ± SE) are expressed on a weight percent basis (g/100 g fatty acid); n = 5 mice per diet group. CO, corn oil (control); DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid. For each fatty acid within each diet group, values not sharing a symbol (*, †, ‡) differ (P ≤ 0.05).

Fig. 5. Dietary DHA and EPA alter survival of dextran sodium sulfate (DSS)-treated mice. Mice were fed a diet with corn oil (CO) as the sole lipid source or a diet enriched with purified DHA or EPA before and after exposure to 2.5% DSS for 5 days. Mice were then allowed to recover from DSS for 0, 3, or 6 days. A: overall mortality of mice at all time points. Values are means ± SE (n = 65–75 mice per diet). B: Kaplan-Meier curve showing survival of animals that were allowed to recover for 6 days. Values are means ± SE (n = 20 mice per diet). *P ≤ 0.05 (by log-rank test). C: initial (day −10) body weight and body weight at the beginning of DSS treatment (day 0), at the end of DSS treatment (day 5), and following recovery (days 8 and 11). Average body weight was calculated and graphed over time for each diet group (n = 25–75) at each time point. Statistical significance (P ≤ 0.05) between dietary groups, as indicated by different letters (a and b), was determined using a 1-way ANOVA and Tukey’s test of contrast.
DHA-fed mice displayed the highest level of injury at \textit{day 3} of recovery (Fig. 7B). From \textit{day 0} to \textit{day 3} of recovery, injury of EPA-fed mice declined, whereas injury of DHA-fed mice increased (Fig. 7, A and B). Colonic injury was reduced in all diet groups between \textit{days 3} and \textit{6} of recovery (Fig. 7C). No significant differences in mucosal repair were observed between groups at \textit{day 0} or \textit{3} of recovery (Fig. 7, D and E). Significant increase in injury in EPA- and DHA-fed mice at \textit{days 0} and \textit{3} suggests that activation of repair mechanisms is hindered. At \textit{day 6}, however, EPA-fed mice exhibited significantly higher levels of repair than DHA- or CO-fed animals (Fig. 7F). Representative images of colon sections from mice fed each diet are shown in Fig. 7G. Overall, the data indicate that DHA and EPA negatively impact colonic wounding and repair following an acute wounding event.

\textit{EGFR activation and signaling in mice.} For the purpose of assessing EGFR activation status in mice fed each of the experimental diets, EGFR phosphorylation status was measured in lysates derived from scraped colonic mucosa. Specifically, EGFR phosphorylation was measured at \textit{days 0, 3}, and \textit{6} of recovery from DSS exposure. EGFR phosphorylation peaked at \textit{day 3} of recovery in CO-fed mice and decreased by \textit{day 6} (Fig. 8A). Activation of EGFR was significantly higher in CO- than DHA- or EPA-fed mice at \textit{day 3} of recovery. In the DHA- and EPA-fed animals, peak EGFR phosphorylation was observed on \textit{day 6} of recovery, with EGFR phosphorylation progressively increasing from \textit{day 0} to \textit{day 6}. This suggests that regulation of EGFR activation is altered by dietary lipids.

After observing diet-induced differences in EGFR phosphorylation, we compared downstream activation of Rac1 and Cdc42. Consistent with EGFR phosphorylation, Cdc42 activation peaked at \textit{day 3} of recovery in CO-fed mice and then decreased by \textit{day 6} of recovery (Fig. 8B). In contrast, Cdc42 activation continued to increase in DHA- and EPA-fed mice throughout the recovery period. Activation of Cdc42 was significantly higher in CO- than DHA- or EPA-fed mice at \textit{day 3} of recovery but was significantly lower at \textit{day 6}. A similar overall pattern was observed with respect to activation of Rac1 (Fig. 8C). Rac1 activation was significantly higher in CO- than DHA- or EPA-fed mice at \textit{day 3} of recovery, but no significant difference was observed at \textit{day 6}. The data further indicate that EGFR signaling is modified by DHA and EPA.

\textit{Colonic mucosal inflammation.} Since exposure to acute DSS elicits an intestinal inflammatory response, colonic inflammation, e.g., histological changes in immune cell infiltration and colonic mucosal cytokine mRNA expression, was assessed. The degree of colonic inflammation (score 0–3) was graded in a blinded manner by a board-certified pathologist (B. Weeks). At \textit{day 0} of recovery, inflammation scores were significantly lower for DHA- than EPA- or CO-fed mice (Fig. 9A). No significant differences were observed between the dietary groups at \textit{day 3} or \textit{6} of recovery (Fig. 9, B and C). Additionally, no significant difference in colonic inflammation was observed between dietary groups in non-DSS-exposed mice (data not shown). Next, the inflammatory response during the recovery phase following DSS exposure was tracked by analysis of expression of proinflammatory mediators (IFN\textgamma, IL-1\beta, TNF\alpha, IL-6, and MCP-1), anti-inflammatory mediators (IL-10), and cytokines related to wound healing (IL-4, TGF\beta1, IL-13, and IL-22) (Table 2). For all genes assessed, there was no difference between dietary groups in terms of basal mRNA expression in non-DSS-exposed mice (\(P > 0.05\)), with the exception of MCP-1, where basal expression was upregulated in EPA- and DHA- compared with CO-fed mice. After DSS exposure (\textit{day 0} of recovery), mRNA expression of IFN\textgamma and TNF\alpha was reduced in EPA- compared with CO- and DHA-fed mice (\(P < 0.05\)). After \textit{day 3} of recovery, MCP-1 and TNF\alpha mRNA expression was reduced in EPA- and DHA- compared with CO-fed mice (\(P = 0.03\) and \(P = 0.004\), respectively). Gene expression of the inflammatory cytokines IFN\textgamma and IL-6 was reduced only by the EPA-enriched diet (\(P < 0.05\)), whereas only the DHA-enriched diet reduced mRNA expression of IL-1\beta (\(P = 0.03\)) compared with the CO diet. After \textit{day 6} of recovery, the EPA- and DHA-enriched diets reduced mRNA expression of IL-6 and IL-1\beta compared with the CO diet (\(P < 0.05\)), whereas expression levels of the inflammatory cytokines/chemokines IFN\textgamma, TNF\alpha, and MCP-1 did not differ between dietary groups (\(P > 0.05\)). Gene expression of IL-4,
Fig. 7. Dietary DHA and EPA exacerbate colonic injury in mice on exposure to DSS. Mice were fed a diet with CO as the sole lipid source or a diet enriched with purified DHA or EPA for 10 days. Mice were continued on the respective diets and exposed to 2.5% DSS for 5 days and allowed to recover from DSS for 0, 3, or 6 days. Longitudinal halves of the colon were Swiss-rolled, fixed with 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Sections were analyzed, a score of 0–3 for injury (A–C) or repair (D–F) in the distal, middle, and proximal colon was assigned, and the average score for the entire colon was quantified from these 3 scores. Box plots show scores from 11–15 mice per diet. Median score is represented by thick black line on the box plot. Statistical significance between dietary groups, as indicated by different letters (a and b), was assessed by Kruskall-Wallis test followed by, if justified, the statistical probability outcome ($P \leq 0.05$) using Wilcoxon 2-sample testing. G: representative images of hematoxylin-eosin-stained colon sections from mice fed each diet and unexposed (no DSS) or allowed to recover for 0, 3, or 6 days after 5 days of exposure to 2.5% DSS. Scale bars, 500 μm.
TGFB1, and IL-10 in mice from either n-3 PUFA-enriched diet did not differ from that in mice fed the CO diet at any recovery time after DSS ($P > 0.05$). Interestingly, mRNA expression of IL-13, which promotes wounding by inducing apoptosis and delaying epithelial restitution (1, 21, 44), was increased in the DHA-fed mice after days 0 and 3 of recovery from DSS ($P = 0.034$ and $P = 0.015$, respectively), whereas mRNA levels did not differ between the EPA- and CO-fed mice. After day 6 of recovery, mRNA expression of IL-13 was reduced in the EPA- and DHA- compared with the CO-fed mice ($P = 0.018$). Conversely, colonic mRNA expression of IL-22, which promotes epithelial cell restitution and intestinal barrier integrity (5), was increased in the DHA- and EPA- compared with CO-fed mice at all time points after DSS ($P < 0.05$). The data demonstrate that DHA and EPA alter colonic inflammation following injury.

**DISCUSSION**

To our knowledge, this is the first examination of the differential effects of DHA and EPA on EGFR signaling within the context of wound healing. There is an abundance of interest in this field, especially since many Americans take fish oil supplements because of their well-established health benefits (52). Interestingly, our data indicate that n-3 PUFA may not always promote optimal health, which may explain some of the inconsistencies in the literature.

DHA and EPA reduced wound-induced transactivation of EGFR (Fig. 1A). Multiple signaling pathways are known to modulate transactivation of EGFR. For example, PGE$_2$ mediates activation of MMPs, which cleave EGFR ligands from the plasma membrane (2, 38, 39). It is well established that DHA and EPA reduce production of PGE$_2$ (6, 49), which could dampen transactivation of EGFR. Similarly, Src has been
shown to mediate activation of MMPs to induce activation of EGFR (39, 53). Interestingly, Src signaling has been shown to be facilitated by lipid rafts (22), and n-3 PUFA incorporation into raft mesodomains perturbs downstream signaling events (7, 8, 46). It is likely, therefore, that the effect of DHA and EPA on the activation of EGFR could be caused by inhibition of one of these mechanisms. Clearly, further work is required to pinpoint the mechanism by which n-3 PUFA inhibit transactivation of EGFR in response to injury.

Next, we observed that, in response to ligand (i.e., EGF), DHA, but not EPA, inhibited EGFR signaling through PLCγ1, Rac1, and Cdc42 (Figs. 1 and 2). This is consistent with our previous observations that DHA suppresses EGFR signal transduction (50). In contrast, in response to wounding, EPA and DHA reduced activation of PLCγ1, Rac1, and Cdc42 (Figs. 1 and 2), which is consistent with the observation that EPA and DHA inhibit wound-induced transactivation of EGFR. In addition to these mediators, we previously showed that n-3 PUFA reduce activation of ERK1/2 and STAT3 (35, 50). All these factors are integral in mediating wound healing, which further suggests that n-3 PUFA could be unfavorable for recovery from injury.

With respect to the analysis of wound healing and cell migration in vitro, EGF enhanced wound healing in control and LA-treated cells (Fig. 3A). However, EGF-mediated wound healing was strongly impeded by treatment with DHA or EPA. Similar results were observed for cell migration (Fig. 3B). It is intriguing that although EPA did not impair ligand-induced EGFR signaling, it did impair EGF-induced biological responses. This suggests that the effects of EPA on wound healing and migration may be partially independent of EGFR. Additional studies are needed to clarify the role of EPA in EGF-induced wound healing. Interestingly, EGF-induced cytoskeletal remodeling has been shown to be mediated by products of 5-lipoxygenase and cyclooxygenase pathways (40). DHA and EPA compete with arachidonic acid as substrates for these enzymes, which could affect cytoskeletal remodeling and cell migration in a manner independent of activation of EGFR signaling mediators.

Our in vivo results further demonstrate that DHA and EPA alter wound healing, in part by modifying EGFR-mediated signaling events. Consistent with the in vitro effect of EPA on wound healing, EPA-fed animals displayed the lowest survival levels (Fig. 5, A and B). Additionally, body weight loss in response to DSS treatment tended to be greater in DHA- and EPA- than CO-fed mice (Fig. 5C). Furthermore, DHA-fed mice displayed significant reductions in cell proliferation during the early stages (days 0 and 3) of recovery (Fig. 6A), whereas no effect of diet was observed on apoptosis (Fig. 6B). Collectively, these data suggest that early wound-healing events are delayed by DHA and EPA. Upon analysis of colonic injury, we observed that the levels of injury were highest at day 0 of recovery in EPA-fed mice (Fig. 7A) and subsequently declined progressively to day 6 (Fig. 7, B and C). However, in DHA-fed mice, injury largely increased from day 0 to day 3, suggesting that colonic repair was impeded. Upon assessment of downstream signaling, peak activation of EGFR and downstream signaling mediators was delayed in animals fed EPA or DHA (Fig. 8). This suggests that the CO-fed mice recover more quickly than the n-3 PUFA-fed mice. Although it is premature to conclude that the perturbations in downstream signaling are only due to changes in EGFR regulation, the data clearly demonstrate differential regulation of important wound-healing signaling events in n-3 PUFA- compared with CO-fed mice.
Because of the well-documented effects of DHA and EPA on inflammation, we additionally assessed the degree of colonic inflammation and gene expression of inflammatory mediators to further understand how the colonic mucosal microenvironment is impacted by dietary lipids. DHA significantly reduced the inflammation histological score at day 0 of recovery compared with CO- and EPA-fed mice (Fig. 9A), which is indicative of reduced immune cell infiltration into the colonic mucosa. In contrast, on day 0, EPA-fed mice exhibited increased colonic injury scores (Fig. 7A). At day 0 of recovery, colonic mRNA expression of IFNγ and TNFα was reduced in EPA- compared with CO- and DHA-fed mice (Table 2). The anti-inflammatory effects of EPA and DHA were apparent at later stages of DSS recovery, as both n-3 PUFA reduced mRNA expression of MCP-1, TNFα, IL-6, and IL-1β (Table 2). In the early stages of recovery, mRNA expression of IL-13, a cytokine that antagonizes epithelial restitution (21, 44), was upregulated in DHA-fed mice and may additionally contribute to the increased injury scores observed at day 3 of recovery in these mice (Fig. 7B). Collectively, the response to an intestinal wounding event is a complicated process representing the sum of multiple inputs that promote or inhibit the process of epithelial restitution. Further studies are required to determine the mechanisms through which n-3 PUFA affect these pathways.

Although it remains unclear how DHA and EPA alter EGFR activation in colonic wound healing, we present cogent data indicating that these bioactive lipids present a barrier to wound healing due to altered mucosal cell signaling. Several decades of research indicate that DHA and EPA suppress inflammatory mediators in experimental models and clinical trials (6). Although early immune-mediated events are required for wound healing, prolonged inflammation can be detrimental to this process. DHA and EPA can be metabolized into a number of lipid mediators, including resolvins, protectins, and maresins, which facilitate resolution of inflammation (3). These lipid mediators have varying biological activities, which may mediate the effects of EPA and DHA. In this study, we observed that the DHA-fed animals started to gain weight between days 3 and 6 of recovery, whereas the EPA- and CO-fed animals did not. This suggests that DHA may facilitate events that expedite the later stages of wound healing, perhaps by resolving inflammation. We have presented a putative model depicting the complicated role that n-3 PUFA play in regulating EGFR signaling and inflammation and the effects they may have on wound healing and colon carcinogenesis (Fig. 10).

Findings from our study are particularly relevant to individuals who repeatedly experience colonic wounding, e.g., IBD. These patients undergo multiple cycles of active disease and recovery. They have low expression of EGFR ligands (37), and current work suggests that supplementation with EGF elicits a positive response in these patients (47). Conflicting data suggest a beneficial role (4, 18, 25), no effect, or even a detrimental role (20, 24, 31) for n-3 PUFA in IBD. A systematic review and meta-analysis of human studies indicated no clear beneficial role for n-3 PUFA in IBD patients (51). The data presented here suggest that the inhibition of EGFR signaling by n-3 PUFA could delay wound healing in these patients. However, the anti-inflammatory effects of n-3 PUFA may be beneficial during some disease stages, e.g., resolution of chronic inflammation. Future work should focus on determining optimal timing and dosing of n-3 PUFA to elicit the beneficial effects of these dietary lipids while avoiding the detrimental effects.

In summary, we propose that the inhibitory effects of DHA and EPA on injury-induced transactivation of EGFR likely contribute to delayed wound healing. This provides a potential mechanism to explain why n-3 PUFA do not always facilitate recovery and remission in IBD patients.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

H.F.T., J.M.M., and R.S.C. are responsible for conception and design of the research; H.F.T., J.M.M., Y.-Y.F., and E.S.C. performed the experiments; H.F.T., J.M.M., and B.W. analyzed the data; H.F.T. and J.M.M. interpreted the
results of the experiments; H.F.T. prepared the figures; H.F.T. drafted the manuscript; H.F.T., J.M.M., and R.S.C. edited and revised the manuscript; H.F.T., J.M.M., Y.-Y.F., E.S.C., B.W., and R.S.C. approved the final version of the manuscript.

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C916 n-3 PUFA ALTER COLONIC INJURY-INDUCED EGFR SIGNALING

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