Docosahexaenoic Acid and Butyrate Synergistically Induce Colonocyte Apoptosis by Enhancing Mitochondrial Ca^{2+} Accumulation

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Abstract

We have previously shown that butyrate, a short-chain fatty acid fiber fermentation product, induces colonocyte apoptosis via a nonmitochondrial, Fas-mediated, extrinsic pathway. Interestingly, fermentable fiber when combined with fish oil containing docosahexaenoic acid (DHA, 22:6n-3) exhibits an enhanced ability to induce apoptosis and protect against colon tumorigenesis. To determine the molecular mechanism of action, the effect of DHA and butyrate cotreatment on intracellular Ca^{2+} homeostasis was examined. Mouse colonocytes were treated with 50 μmol/L DHA or linoleic acid (LA) for 72 h ± butyrate (0–10 mmol/L) for the final 24 h. Cytosolic and mitochondrial Ca^{2+} levels were measured using Fluo-4 and Rhod-2. DHA did not alter basal Ca^{2+} or the intracellular inositol trisphosphate (IP$_3$) pool after 6 h butyrate cotreatment. In contrast, at 12 and 24 h, DHA- and butyrate-treated cultures exhibited a 25% and 38% decrease in cytosolic Ca^{2+} compared with LA and butyrate. Chelation of extracellular Ca^{2+} abolished the effect of thapsigargin on the IP$_3$-releasable Ca^{2+} pool. DHA and butyrate cotreatment compared with untreated cells increased the mitochondrial-to-cytosolic Ca^{2+} ratio at 6, 12, and 24 h by 73%, 18%, and 37%, respectively. The accumulation of mitochondrial Ca^{2+} preceded the onset of apoptosis. RU-360, a mitochondrial-uniporter inhibitor, abrogated mitochondrial Ca^{2+} accumulation and also partially blocked apoptosis in DHA and butyrate cotreated cells. Collectively, these data show that the combination of DHA and butyrate, compared with butyrate alone, further enhances apoptosis by additionally recruiting a Ca^{2+}-mediated intrinsic mitochondrial pathway. [Cancer Res 2007;67(11):5561–8]

Introduction

There is substantial experimental, epidemiologic, and clinical evidence indicating that fish oil–containing diets rich in n-3 polyunsaturated fatty acids (PUFA), for example, docosahexaenoic acid (DHA, 22:6$^{\Delta4,7,10,13,16,19}$) and eicosapentaenoic acid (EPA, 20:5$^{\Delta5,8,11,14,17}$), are protective against colon tumorigenesis (1–3). In addition, there is evidence from epidemiologic and observational studies indicating that the consumption of fiber, which increases butyrate levels in the lumen of the colon, is chemo-protective against colorectal cancers (4, 5). In contrast, several systematic reviews have challenged the premise that dietary fiber and fish oil reduce colon cancer risk, fueling a debate regarding the role of dietary fat and fermentable fiber as chemoprotective nutrients (6–9). To address this apparent conundrum, our laboratory has focused on the question as to why a diet containing highly fermentable fiber is only protective when fish oil is the lipid source. We have shown that the bioactive components generated by fermentable fiber (butyrate) and fish oil (DHA) work coordinately to protect against colon tumorigenesis, primarily by increasing apoptosis rather than decreasing cell proliferation (3, 10, 11). With regard to a molecular mechanism of action, we have shown that DHA alters colonocyte mitochondrial membrane composition and function, thereby creating a permissive environment for apoptosis induced by luminal metabolites, such as butyrate (12, 13). More recently, we have shown that mitochondrial lipid oxidation products, membrane phospholipid-derived hydroperoxides (LOOH), play an important role in DHA and butyrate-induced apoptosis (14). Despite the evidence indicating that the combination of dietary fish oil and fermentable fiber enhance apoptosis and suppress colon cancer, we still lack information regarding the precise molecular mechanisms by which the DHA and butyrate combination protect against colon tumorigenesis.

With respect to molecular triggers for apoptosis, Ca^{2+} is one of the most versatile and universal signaling mediators in cells and is required for the activation of many cellular processes. Increasing evidence indicates that alterations in the finely tuned intracellular homeostasis and compartmentalization of Ca^{2+} can lead to cell death either through apoptosis or necrosis. The Ca^{2+} concentration inside the cell is regulated by the simultaneous interplay of various counteracting mechanisms, which can turn cellular signals "on" and/or "off" (15). Eukaryotic cells can increase their cytosolic Ca^{2+} levels via two mechanisms: release of Ca^{2+} from intracellular stores or influx via plasma membrane channels. Channels located in the plasma membrane, for example, store-operated Ca^{2+} channels (SOC), receptor-operated channels, and voltage-operated channels, regulate the influx of Ca^{2+} into the cell. Currently, there is a good understanding of the organelles that function as Ca^{2+} stores and how Ca^{2+} can be released from stores into the cytosol. Although the importance of the endoplasmic reticulum (ER) as the major storage organelle is indisputable, growing evidence indicates that functional compartmentalization of Ca^{2+} exists within the various cellular organelles. More recent studies have identified the contributions of the nuclear envelope, Golgi apparatus, lysosomes, and mitochondria in maintaining intracellular Ca^{2+} homeostasis and cellular physiologic function (16, 17). In fact, it is now recognized that mitochondria play a key role in both apoptosis and necrosis by regulating energy metabolism, intracellular Ca^{2+}

homeostasis, activation of caspases, and the release of reactive oxygen species (18, 19).

Mitochondria are localized in close proximity to inositol 1,4,5-trisphosphate (IP_3)–gated channels situated on the ER. Ca^{2+} uniporters, which are low-affinity and high-capacity active pumps located in the mitochondrial outer membrane, rapidly take up Ca^{2+} that is released from the ER stores and recirculate it back into the cytosol via exchangers such as the Na^{2+}/Ca^{2+} exchanger (20, 21). Therefore, mitochondria can be regarded as critical checkpoints in Ca^{2+} signaling, acting as membrane-bound Ca^{2+} buffers. Under normal conditions, mitochondrial Ca^{2+} uptake serves as a signal to increase cellular metabolism (20). However, in certain situations, mitochondrial Ca^{2+} accumulation is a trigger for cytochrome *c* release and the induction of apoptosis (22).

Given the central role of mitochondria in the commitment to apoptosis, we hypothesized that *n*-3 PUFA and butyrate can promote apoptosis by triggering changes in mitochondrial Ca^{2+} levels that contribute to caspase activation and colonocyte cell death. We used an immortalized mouse colonocyte (YAMC) cell line to determine whether chemoprotective nutrients modulate intracellular calcium compartmentalization and SOC entry to induce colonocyte apoptosis. The results confirm and extend our previous observations and show that DHA and butyrate combination synergistically alter intracellular Ca^{2+} compartmentalization by enhancing mitochondrial Ca^{2+} accumulation through an SOC-mediated mechanism. These outcomes provide clear evidence that an increase in mitochondrial Ca^{2+} stores contributes to the induction of apoptosis by DHA and butyrate cotreatment.

Materials and Methods

Materials. RPMI 1640 and HBSS were purchased from Mediatech. Fetal bovine serum was from Hyclone. Insulin, transferrin, and selenium without linoleic acid (LA), were purchased from Collaborative Biomedical Products. Glutamax, recombinant mouse IFN-γ, and Leibovitz medium were from Life Technologies. Fatty acids were obtained from NuChek. Fluo-4 AM, Rhod-2 AM, and Calcium Calibration buffer kit 2 were purchased from Molecular Probes. RU-360 and SKF-96365 were purchased from Calbiochem. Cell death detection ELISA kit was obtained from Roche Applied Science. Two-well Lab-Tek Chambered Coverglass slides were purchased from Nunc, Inc. Thapsigargin, staurosporine, EGTA, sodium butyrate, and all other reagents were obtained from Sigma. Stock solutions of 1.0 mmol/L Fluo-4, AM, and 4.0 mmol/L Rhod-2 were prepared in DMSO and diluted with medium to 3.0 and 2.0 μmol/L, respectively (final concentration of the vehicle DMSO was maintained at 0.1–0.3% in culture). Thapsigargin stock (5.0 mmol/L) was prepared in DMSO and used at a concentration of 5.0 μmol/L (0.1% DMSO). RU-360 (1 mmol/L) stock was prepared in degassed water and diluted to a final concentration of 10 μmol/L for cell treatment. A (1 mmol/L) stock solution of staurosporine was prepared in DMSO and diluted to a final concentration of 1 μmol/L for cell treatment.

Cell culture. Conditionally immortalized YAMC cells were originally obtained from R.H. Whitehead (Ludwig Cancer Institute, Melbourne, Australia). Cells were cultured under permissive (33°C with IFN-γ) or nonpermissive conditions (39°C) as previously described (14, 23). For all fluorescence measurements, cells (passages 12–18) were seeded onto borosilicate two-chambered cover glass slides at a density of 5×10^3 to 7×10^3 to achieve a 50% to 70% confluence. For apoptosis assays, cells were seeded onto 35-mm cell culture dishes or six-well plates at a density of 35×10^3. Bovine serum albumin (BSA)–complexed fatty acids were added to cultures 24 h after cell plating as previously described (23). Select cultures were treated with BSA-complexed DHA (22:6, *n*-3) or LA (18:2, *n*-6; 0–200 μmol/L) for 72 h. Cells were coincubated with sodium butyrate (0–10 mmol/L) in RPMI 1640 for the final 6, 12, 24, or 48 h of fatty acid pretreatment.

Quantification of cytosolic Ca^{2+} in mouse colonocytes. Following fatty acid and butyrate treatment, cells were washed with Leibovitz medium, free of serum and phenol red. Cells were loaded with 3 μmol/L Fluo-4, AM, at 33°C to determine the levels of cytosolic Ca^{2+}. Fluo-4 is a visible wavelength nonratiometric cytosolic Ca^{2+} indicator that exhibits a 40-fold increase in fluorescence intensity with Ca^{2+} binding (24, 25). Following a 1-h incubation with the probe, cells were washed with Leibovitz medium and imaged. Images were acquired from groups of 10 to 20 cells, 10 to 12 areas per well, and 4 to 8 wells per treatment using a Stallion Digital Imaging workstation equipped with 300 W xenon fluorescent light source. Fluorescent light was rapidly (<2 ms) switched between excitation wavelengths. Images were captured using a ×20 objective 0.75 numerical aperture and a ROPER CoolSnap HQ camera. For image acquisition, imaging variables were adjusted for maximum detection of fluorescence with minimal cellular photobleaching. Cells were excited at 488 nm, and fluorescence emission from individual cells was collected at 530 nm. Image acquisition frequency was set to one image every 10 s. Fluorescence intensities were recorded for 300 s. A minimum of two experiments done on different days were analyzed. Basal intracellular Ca^{2+} was measured before the addition of thapsigargin. Thapsigargin is an irreversible sarcoplasmic/ER Ca^{2+} ATPase (SERCA) pump inhibitor that was used to empty the intracellular Ca^{2+} store from the ER (25). Fluorescence intensities after thapsigargin stimulation were normalized to basal Ca^{2+} levels.

To determine the contribution of IP_3 Ca^{2+} pools, extracellular Ca^{2+} was chelated using 2 mmol/L EGTA followed by cell stimulation with thapsigargin. Chelation of extracellular Ca^{2+} facilitates quantification of IP_3 Ca^{2+} released from the ER after thapsigargin addition (26, 27). Ca^{2+} was then added back into the medium and the contribution of the SOC pool was evaluated (25). To further evaluate the association between PUFA and butyrate cotreatment and SOC, cultures were preincubated with 10 μmol/L SKF-96365, a pharmacologic inhibitor of SOC, for 5 min before image acquisition. Calibration of intracellular Ca^{2+} was done using the Calcium Calibration Buffer kit 2 from Molecular Probes as previously described (28).

Analysis of mitochondrial Ca^{2+}. Cells treated with fatty acid and butyrate were washed with Leibovitz medium and coloaded with 3 μmol/L Fluo-4 and 2 μmol/L Rhod-2 for 1 h at 33°C. Cells were then washed twice with Leibovitz medium, and the mitochondrial-to-cytosolic Ca^{2+} ratio was measured. For quantification of Fluo-4 and Rhod-2 fluorescence, excitation light was provided at 488 and 550 nm and fluorescence emissions were collected at 530 and 580 nm, respectively. The ratio of the cytosolic-to-mitochondrial Ca^{2+} level was subsequently calculated. Although accumulation of Rhod-2 dye in the nucleoli was noticed, MitoTracker was used to confirm that dye loading was predominantly localized within the mitochondria as previously described (29). In other experiments, cells were incubated with RU-360 (10 μmol/L), an inhibitor of the mitochondrial uniporter, for 30 min before butyrate cotreatment (30). Cells were then washed and coloaded with fatty acid and butyrate for the final 6, 12, or 24 h after which the mitochondrial-to-cytosolic Ca^{2+} ratio was determined. Nucleoli were not included in the analysis of mitochondrial Ca^{2+}.

Apoptosis assays. Apoptosis was measured using cellular fragmentation ELISA (Roche) and caspase-3 activity assay (Molecular Probes) as previously described (23). To determine the association between mitochondrial Ca^{2+} and apoptosis, select cultures were preincubated with RU-360 (10 μmol/L) for 30 min before butyrate exposure. Cells were washed and treated with 5 mmol/L butyrate and apoptosis was measured after a 6-, 12-, or 24-h incubation period.

Statistical analysis. The effect of independent variables (treatment effects) was assessed using SuperANOVA. Differences among means were determined using t/F–type tests of contrast. P values <0.05 were considered to be statistically significant.

Results

DHA and butyrate combination decreases cytosolic Ca^{2+}. Cytosolic Ca^{2+} levels were initially examined after stimulation with thapsigargin, an irreversible SERCA pump inhibitor used to empty ER Ca^{2+} stores. Figure 1*A* shows representative traces of

thapsigargin-induced Ca^{2+} response measured over 300 s. The arrow indicates the time point of thapsigargin addition. Butyrate treatment (5 mmol/L) over a period of 6 to 24 h increased ($P < 0.05$) cytosolic Ca^{2+} in cells preincubated with LA (50 μmol/L, a control n-6 fatty acid) by up to 45% compared with untreated control (Fig. 1B–D). In contrast, cells preincubated with DHA consistently exhibited a lower cytosolic Ca^{2+} level compared with LA and butyrate cotreatment. Control cultures containing either DHA or LA alone, in the absence of butyrate, exhibited a modest increase in cytosolic Ca^{2+} levels, 8% to 12%, compared with untreated control (Fig. 1C and D, inset). In comparison, cultures treated with LA and butyrate exhibited a 45% increase compared with untreated control at both time points. Changes in Ca^{2+} levels were detected as early as 6 h after butyrate and fatty acid cotreatment.

SOC involvement in maintaining intracellular Ca^{2+} homeostasis. To examine the role of SOC entry, the increase in plasma membrane influx associated with ER Ca^{2+} emptying was characterized using a Ca^{2+} add-back protocol. This standard procedure uses extracellular Ca^{2+} chelation with EGTA followed by utilization of thapsigargin to induce an initial emptying of the ER Ca^{2+} pool, followed by repletion of the bathing solution with Ca^{2+} (26, 27). Consistent with previous experiments (Fig. 1) after incubation with 5 μmol/L thapsigargin, butyrate (24 h)–treated cells preincubated with DHA in the presence of extracellular Ca^{2+} exhibited a 40% decrease in cytosolic Ca^{2+} compared with LA-primed cells ($P < 0.05$;

Fig. 2). Subsequently, extracellular Ca^{2+} was chelated by addition of 2 mmol/L EGTA, which allows for indirect quantification of the ER Ca^{2+} pool. In these experiments, butyrate-treated cells primed with DHA or LA showed no difference in cytosolic Ca^{2+} levels ($P > 0.05$; Fig. 2). Following replacement of the medium with $CaCl_2$ (10 mmol/L), the phenotype was reestablished, implicating the involvement of plasma membrane channels in the propagation of fatty acid–induced Ca^{2+} signaling.

To identify the plasma membrane channels involved in eliciting a rise in calcium influx after ER Ca^{2+} depletion in colonocytes, cultures were incubated with SKF-96365, an inhibitor of non-voltage-gated, SOC channels (31, 32). Typically, butyrate-treated cells primed with DHA or LA were preincubated with 10 μmol/L SKF for 5 min before basal recording. SKF abrogated the fatty acid–induced effect on cytosolic Ca^{2+} levels after 12 h butyrate cotreatment (Supplementary Fig. S1A). Similar effects were observed after prolonged butyrate treatment (24 h; Supplementary Fig. S1B). Thus, the activation of SOCs, leading to rapid entry of Ca^{2+} through the plasma membrane, is likely to contribute to the observed fatty acid and butyrate-induced perturbation in colonocyte Ca^{2+} homeostasis.

Effect of DHA and butyrate cotreatment on mitochondrial Ca^{2+} levels. Because the efflux of Ca^{2+} from the ER can lead to coupled increases in mitochondrial Ca^{2+} levels, we assessed the effects of fatty acid and butyrate cotreatment on mitochondrial
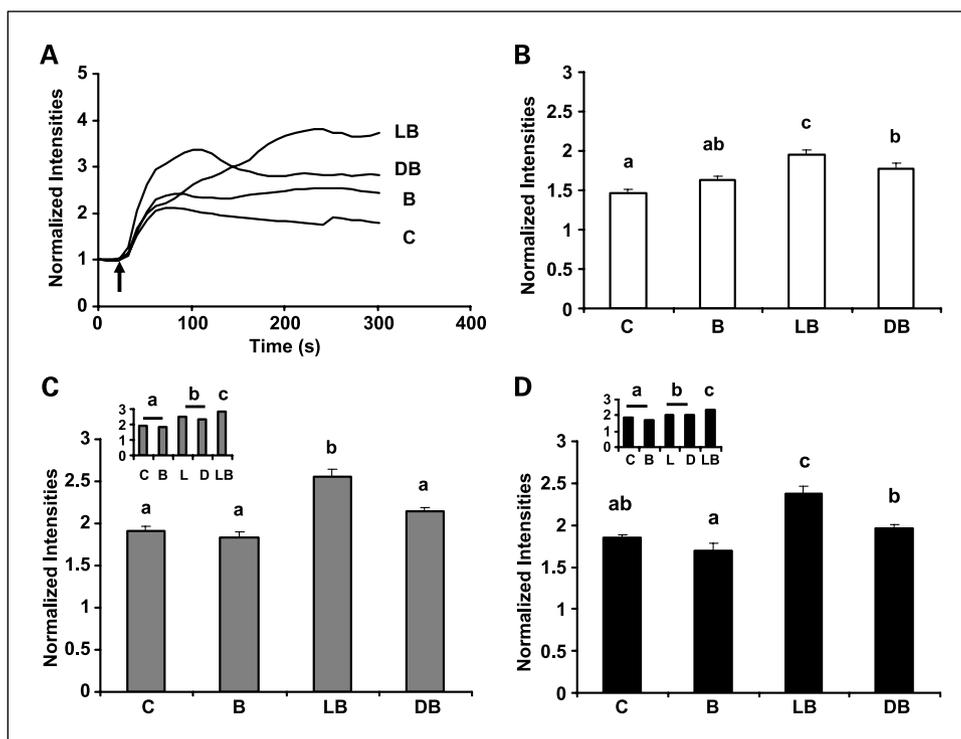

Figure 1. Effects of fatty acid and butyrate on thapsigargin-induced Ca^{2+} response. YAMC cells were treated with fatty acid alone (50 μmol/L) for 72 h with butyrate (5 mmol/L) for the final 6 h (B), 12 h (C), or 24 h (D). Cells were subsequently incubated with Fluo-4 (3 μmol/L), a fluorescent cytosolic Ca^{2+} indicator for 1 h at 33°C, and basal Ca^{2+} was measured. Representative traces (15–20 cells/trace) are shown in (A). Arrow, point of thapsigargin (5 μmol/L) addition. Normalized intensity is the average intensity at any point in time, t divided by intensity at time 0. Cytosolic Ca^{2+} was quantified at the single-cell level by choosing at random three to four fields with 10 to 12 cells per field. Representative data are from a single experiment, average of 80 to 120 cells per treatment, $n = 4$ to 6 independent experiments. Columns, mean; bars, SE. Data not sharing common letters are significantly different, $P < 0.05$. C, control—no fatty acid or butyrate; B, butyrate only; L, LA only; D, DHA only; LB, LA and butyrate; DB, DHA and butyrate. DHA compared with LA-pretreated cells showed a significant decrease in cytosolic Ca^{2+} levels with 12 and 24 h of butyrate cotreatment. Inset, cytosolic Ca^{2+} levels measured in untreated, butyrate only, fatty acid only, and LA and butyrate combination cells at 12 and 24 h (inset in C and D, respectively). Representative data are from a single experiment, average of 80 to 120 cells per treatment, $n = 2$ to 4 independent experiments. Columns, mean; bars, SE. Data not sharing common letters are significantly different, $P < 0.05$. DHA-treated cells exhibited no significant difference in cytosolic Ca^{2+} levels when compared with LA-treated cells.
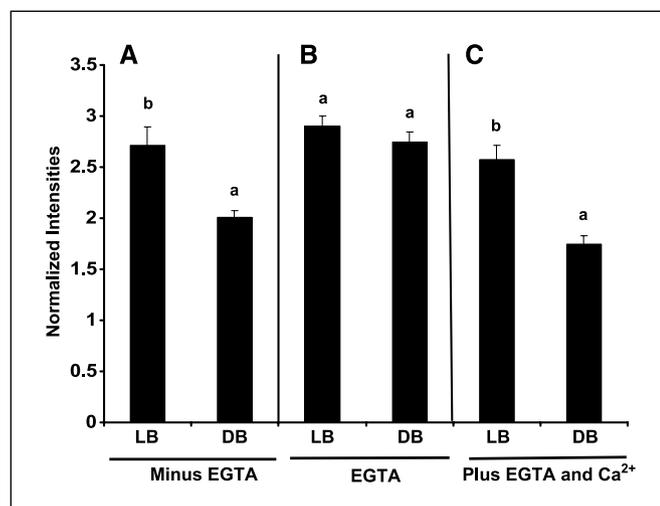
Figure 2. Effect of fatty acid on thapsigargin-induced Ca^{2+} response in the presence or absence of EGTA. YAMC cells were treated with fatty acid (50 μmol/L) for 72 h with or without butyrate (5 mmol/L) for the final 24 h. *A,* cells were incubated with Fluo-4 (3 μmol/L) for 1 h at 33°C and basal Ca^{2+} was measured. *B,* extracellular Ca^{2+} was chelated using 2 mmol/L EGTA and cells were stimulated with thapsigargin (5 μmol/L). *C,* $CaCl_2$ (10 mmol/L) was added back to the extracellular medium and cytosolic Ca^{2+} was quantified. *Columns,* mean; *bars,* SE. Data not sharing common letters are significantly different, $P < 0.05$. Representative data are from a single experiment, $n = 2$ independent experiments. Refer to Fig. 1 for legend details. In colonocytes with the combination of DHA and butyrate, chelation of extracellular Ca^{2+} abolished the difference in cytosolic Ca^{2+} levels and replenishing extracellular medium with Ca^{2+} reestablished the difference. Taken together, these data show that plasma membrane channel entry contributed to the difference seen in cytosolic Ca^{2+} level.

Ca^{2+} uptake. Butyrate cotreatment for 6 h increased ($P < 0.0001$) the mitochondrial-to-cytosolic Ca^{2+} ratio in cells treated with DHA by 73% in comparison with untreated cells (Fig. 3*A*). In addition, at 12 and 24 h, DHA and butyrate selectively increased ($P < 0.0001$)

mitochondrial Ca^{2+} by 18% and 37%, respectively (Fig. 3*B* and *C*). In contrast to DHA-treated cultures, cells incubated in the presence of LA showed a small response or no change in the mitochondrial-to-cytosolic Ca^{2+} ratio compared with untreated cells. With respect to the fatty acid–only controls, LA treatment had no effect on the mitochondrial-to-cytosolic Ca^{2+} ratio. DHA treatment tended to increase the mitochondrial-to-cytosolic Ca^{2+} ratio at all time points (Fig. 3*B* and *C, inset*). However, in all cases, cultures pretreated with DHA and coincubated with butyrate showed the largest increase in mitochondrial Ca^{2+} levels (Fig. 3). Representative photomicrographs of untreated cells and cultures coincubated with butyrate (5 mmol/L for 24 h) and DHA or LA (50 μmol/L) are shown in Fig. 3 (*D–F*).

Effects of a mitochondrial uniporter inhibitor on mitochondrial Ca^{2+} uptake after DHA and butyrate cotreatment. To investigate the role of the mitochondrial uniporter in Ca^{2+} uptake, cells were treated with RU-360, a mitochondrial Ca^{2+} uniporter inhibitor (33). RU-360 (10 μmol/L) partially inhibited ($P < 0.001$) the butyrate-induced increase in mitochondrial Ca^{2+} in DHA-primed cells upon addition 30 min before butyrate cotreatment at 6 or 12 h (Fig. 4). In comparison, RU-360 had no effect on mitochondrial Ca^{2+} levels in LA-treated cells.

Induction of colonocyte apoptosis by fatty acid and butyrate combination. Using the nucleosomal fragmentation assay, DHA significantly ($P < 0.0001$) enhanced butyrate-induced apoptosis compared with cells treated with LA plus butyrate or butyrate alone (control) at 12 and 24 h (Supplementary Fig. S2). In contrast, fatty acid treatment alone had no effect on apoptosis (Supplementary Figs. S2*C, inset*, S3*A* and *B*). To further corroborate these observations, complimentary methodology (caspase-3 activity) was also used. Similar results were obtained (Supplementary Fig. S2*D*). For comparative purposes, staurosporine, a broad-spectrum protein kinase inhibitor, which induces apoptosis in normal and malignant cells, was used as a positive control (34). As expected,
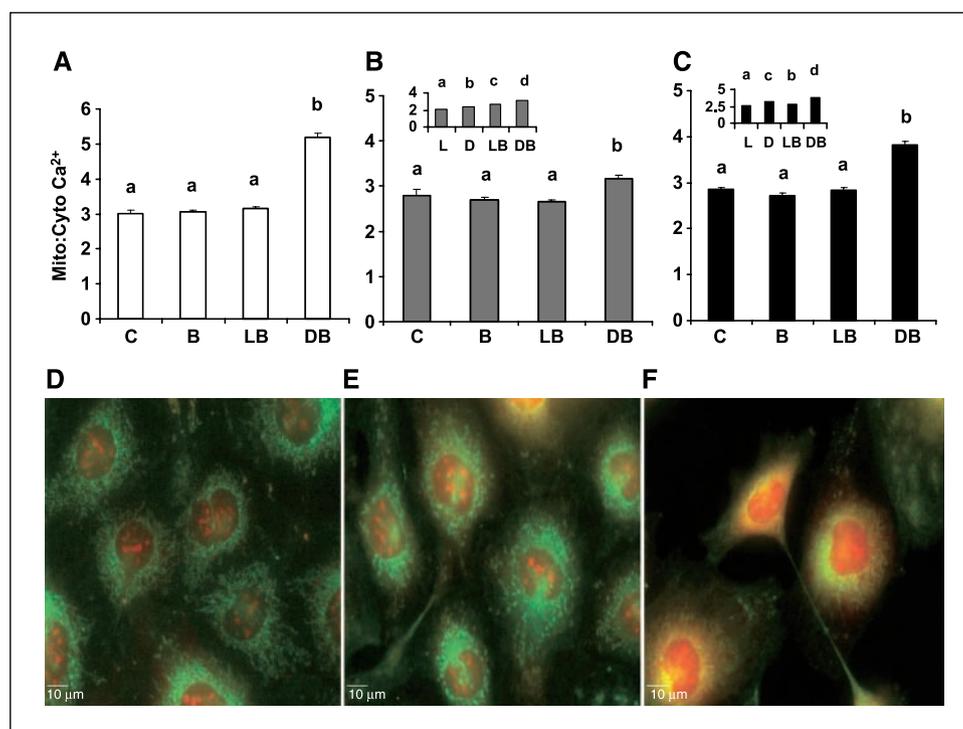

Figure 3. Effect of fatty acid and butyrate cotreatment on mitochondrial Ca^{2+} levels. YAMC cells were exposed to 50 μmol/L fatty acid for 72 h in the absence or presence of 5 mmol/L butyrate for the final 6 (*A*), 12 (*B*), or 24 h (*C*). Cells were coloaded with Fluo-4 (3 μmol/L) and Rhod-2 AM (2 μmol/L), and the ratio of mitochondrial-to-cytosolic Ca^{2+} was evaluated as described in Materials and Methods. *Columns,* mean from a representative experiment, $n = 3$ independent experiments; *bars,* SE. *D* to *F,* representative images from no-treatment (control), LA and butyrate, and DHA and butyrate 24-h cultures. A significant ($P < 0.0001$) difference between the combination of LA with butyrate and DHA with butyrate existed starting from 6 h. Refer to Fig. 1 for legend details. *Inset,* effects of fatty acid treatment with or without butyrate on mitochondrial Ca^{2+} levels at 12 h (*B*) and 24 h (*C*), respectively. *Columns,* means from a representative experiment, $n = 2$ to 3 independent experiments; *bars,* SE. Data not sharing common letters are significantly different, $P < 0.05$.

Figure 4. Effects of RU-360 on mitochondrial Ca^{2+} uptake after fatty acid and butyrate cotreatment. Cells were pretreated with fatty acid for a total of 72 h and RU-360 (10 μmol/L) for 30 min before butyrate cotreatment for the last 6 h (A) and 12 h (B), respectively. Cells were coincubated with Fluo-4 (3 μmol/L) and Rhod-2 (2 μmol/L) for 1 h and the mitochondrial-to-cytosolic Ca^{2+} ratio was measured. *Columns,* mean from a representative experiment, $n = 2$ independent experiments; *bars,* SE. Refer to Fig. 1 for legend details. Data obtained show that in DHA- and butyrate-treated cells, RU-360 partially blocked mitochondrial Ca^{2+} accumulation.
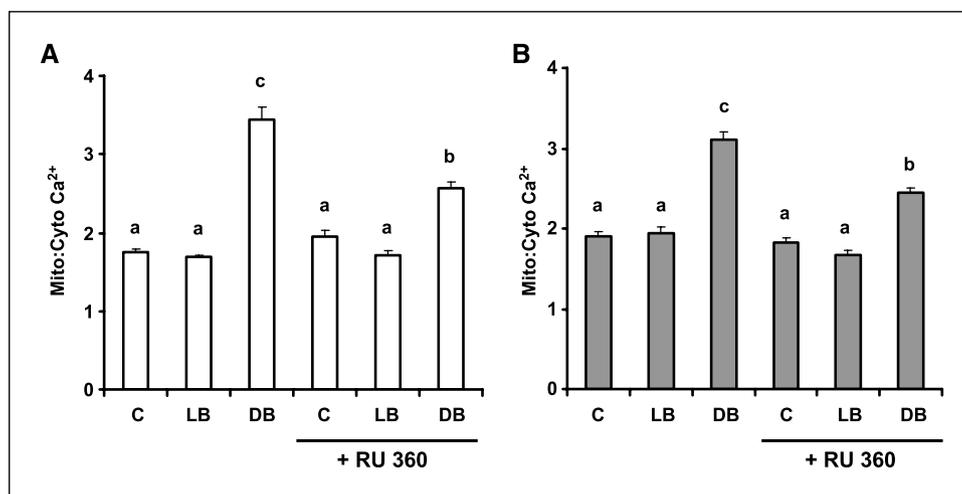
cultures preincubated with 1 μmol/L staurosporine for 4 h exhibited an 8-fold increase in apoptotic cells compared with untreated or fatty acid–treated cells ($P < 0.0001$; Supplementary Fig. S2C, inset).

The mitochondrial Ca^{2+} uniporter inhibitor, RU-360, suppresses induction of apoptosis after DHA and butyrate cotreatment. To investigate the relationship between mitochondrial Ca^{2+} uptake and cellular apoptosis, we compared the levels of apoptosis observed in DHA plus butyrate–treated cells in the absence or presence of the mitochondrial Ca^{2+} uniporter inhibitor RU-360. Results obtained in experiments with butyrate at 12 and 24 h after DHA or LA cotreatment are shown in Fig. 5. RU-360 significantly ($P < 0.05$) reduced apoptosis by ~45% after 12-h butyrate cotreatment. Similar results were observed in DHA primed cells after 24-h butyrate cotreatment. In contrast, inhibition of the uniporter had no effect on cells treated with LA plus butyrate or butyrate alone at 12 and 24 h. Collectively, these results show that mitochondrial Ca^{2+} uptake is required for the enhanced apoptosis associated with DHA and butyrate cotreatment.

Kinetics of DHA and butyrate induction of mitochondrial Ca^{2+} and apoptosis. To probe the functional proximity of mitochondrial Ca^{2+} uptake in relation to the induction of apoptosis, we analyzed the effects of treatment over time (6, 12, and 24 h). As shown in Supplementary Fig. S4, cells treated with

butyrate and DHA exhibited a significantly ($P < 0.0001$) higher level of mitochondrial Ca^{2+} compared with all other treatments as early as 6 h. In contrast, the induction of apoptosis was not observed until 12 h. These results indicate that the increase in mitochondrial Ca^{2+} preceded the onset of apoptosis and therefore plays an important role in triggering programmed cell death in colonic mucosal cells.

Discussion

Dietary DHA ($22:6n$-3) has been extensively studied over the last decade in relation to its role as a bioactive chemopreventive agent. Past results from our laboratory have shown that DHA, when combined with either a fermentable fiber source or butyrate, enhances reactive oxygen species and LOOH production and causes a change in mitochondrial permeability transition in colonocytes (12–14, 35). Here, we report that DHA and butyrate cotreatment also synergistically enhance apoptosis by up to 43% in colonocyte cultures compared with butyrate alone (Supplementary Fig. S2). In contrast, colonocytes treated with either DHA or LA alone showed no significant increase in the level of apoptosis compared with untreated cells (Supplementary Fig. S3). From a biological relevance perspective, these data are consonant with

Figure 5. Effect of RU-360 on apoptotic index. YAMC cells were treated with DHA or LA (50 μmol/L) for 48 h and subsequently preincubated with 10 μmol/L RU-360 for 30 min. Treated cells were then coincubated with fatty acid with or without 5 mmol/L butyrate for the final 12 h (A) and 24 h (B). Nonadherent cells were harvested, and nucleosomal fragmentation was used to quantify apoptosis. *Columns,* mean from two separate experiments, $n = 6$ wells per treatment; *bars,* SE. Refer to Fig. 1 for legend details. Data show that RU-360 significantly blocked apoptosis with cells pretreated with the combination of DHA and butyrate.
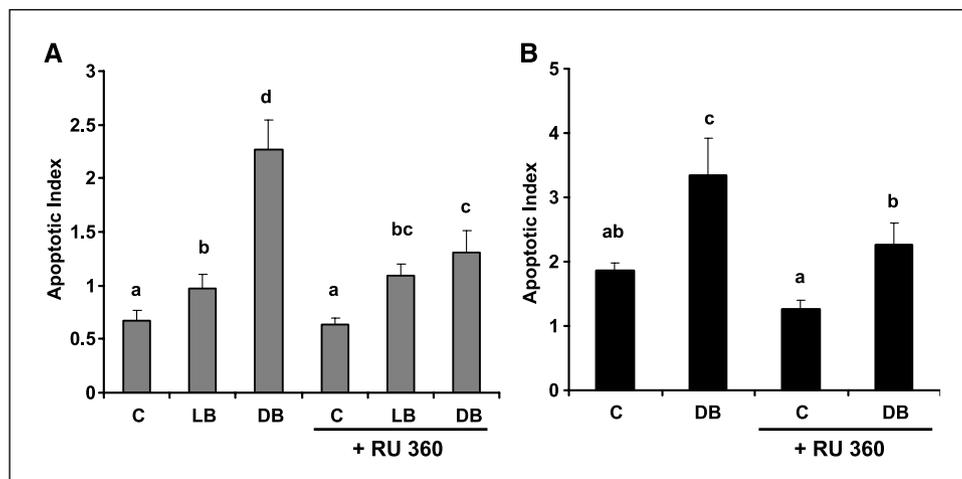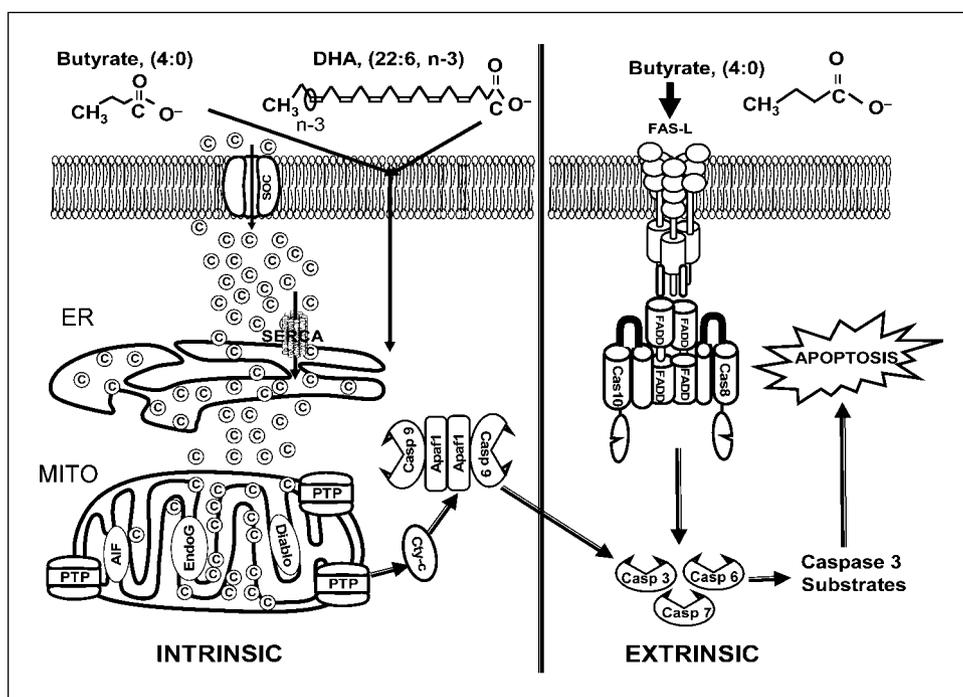
Figure 6. Proposed molecular model of DHA and butyrate-induced apoptosis. Butyrate induces colonocyte apoptosis via a nonmitochondrial, Fas-mediated, extrinsic pathway. DHA and butyrate, in combination, synergistically perturb intracellular Ca^{2+}, stimulating mitochondrial Ca^{2+} uptake. This directly or indirectly decreases cytosolic Ca^{2+} and promotes SOC-mediated entry via plasma membrane channels. Mitochondrial Ca^{2+} accumulation subsequently triggers the opening of the permeability transition pore (*PTP*) and release of proapoptotic molecules like cytochrome *c* and other factors such as apoptosis-inducing factor (*AIF*), and second mitochondrial activator of caspases (*smac/DIABLO*). Together, these effects culminate in the induction of procaspases and downstream caspases that execute cellular apoptosis.

animal carcinogen studies showing that the bioactive components of fermentable fiber (butyrate) and fish oil (DHA) coordinately protect against colon tumorigenesis, primarily by increasing apoptosis (3, 10, 11, 36). Further, our studies reveal that the combination of DHA and butyrate, compared with butyrate alone, further enhances apoptosis by additionally recruiting a Ca^{2+}-mediated intrinsic mitochondrial pathway. Based on our findings, we propose a pathway for the induction of apoptosis in colonic epithelium that involves the synergistic action of DHA and butyrate on enhanced mitochondrial Ca^{2+} accumulation (Fig. 6).

Over the last decade, extensive progress has been made in establishing the effects of long-chain PUFA on various ions and ion channels, including Ca^{2+} (37–39). With respect to a molecular mechanism of action, agents that increase mitochondrial reactive oxygen species/LOOH generation have been linked to a proapoptotic cycle involving Ca^{2+} release from intracellular stores and mitochondrial loading (40). These data suggest that a change in mitochondrial Ca^{2+} homeostasis may mediate the proapoptotic effect of butyrate and DHA in colonocytes. The current experiments support this hypothesis because the combination of DHA and butyrate produced the highest mitochondrial Ca^{2+} accumulation (Fig. 3) while simultaneously decreasing free cytosolic Ca^{2+} levels (Fig. 1*C* and *D*). These data are consistent with our previous finding that DHA primes the cell for butyrate-induced lipid oxidation (14).

The role of mitochondria in the regulation of intracellular Ca^{2+} homeostasis and apoptotic signaling is well established (16–18). Recent work has shown that mitochondria are closely juxtaposed with the ER to effectively take up Ca^{2+} that is released from the ER. There is also evidence of clustering of IP_3 receptors on the ER membrane facing the mitochondria (41, 42). Besides close physical association of the ER and mitochondria, IP_3 receptor–dependent Ca^{2+} signals are associated with an increase in mitochondrial Ca^{2+}, which is capable of triggering apoptosis (22, 43). Consistent with these observations, DHA-treated cultures exhibited a significant

accumulation of mitochondrial Ca^{2+} within 6 h of butyrate cotreatment (Fig. 3*A*), preceding the onset of apoptosis that was not increased until 12 h after butyrate cotreatment (Supplementary Figs. S2*B* and 4).

To explore the connection between the accumulation of mitochondrial Ca^{2+} and apoptosis, we examined the effect of a mitochondrial Ca^{2+} uniporter inhibitor (RU-360). RU-360 significantly blocked mitochondrial Ca^{2+} uptake after DHA and butyrate cotreatment (Fig. 4) and partially blocked the induction of apoptosis (Fig. 5). These outcomes provide evidence that an increase in mitochondrial Ca^{2+} levels contribute directly to the induction of apoptosis by DHA and butyrate cotreatment and the change in intracellular Ca^{2+} homeostasis is not an epiphenomenon.

Despite the difficulties inherent to measuring mitochondrial Ca^{2+} levels, both imaging and uniporter inhibitor data indicate that Rhod-2 loads primarily into the mitochondria. With respect to how mitochondria translate/interpret Ca^{2+} signals that ultimately trigger apoptosis, it has been shown that multifactorial cross-talk among Ca^{2+}, ATP, and oxidative stress enhance cytochrome *c* dislocation from the inner mitochondrial membrane and activate Ca^{2+}-dependent endonucleases, which are responsible for the induction of the DNA fragmentation and apoptosis (44). There is also evidence that mitochondrial Ca^{2+} sequestration (via the mitochondrial uniporter) results in the opening of the mitochondrial permeability transition pore, which can induce the release of proapoptotic molecules (i.e., Bax, Bak, cytochrome *c*), resulting in mitochondrial-mediated (intrinsic) apoptosis (42, 45). This scenario correlates well with our finding of a decrease in colonic mitochondrial membrane potential in fish oil– but not corn oil–fed rats (12, 13).

To elucidate the relative contribution of ER Ca^{2+} store emptying versus SOC Ca^{2+} entry in maintaining intracellular Ca^{2+} homeostasis, thapsigargin-evoked Ca^{2+} responses were measured in a Ca^{2+}-free environment using EGTA. The removal of extracellular Ca^{2+} effectively abolished the DHA and butyrate-induced decrease

in cytosolic Ca^{2+} levels (Fig. 2). Following replenishment of extracellular Ca^{2+}, the difference in cytosolic Ca^{2+} between DHA and butyrate versus control (LA and butyrate) treatment groups was reestablished. These data suggest that Ca^{2+} influx through SOC partly mediates the DHA and butyrate perturbation of intracellular Ca^{2+}. To corroborate the involvement of plasma membrane non–voltage-gated Ca^{2+} channels, SOC-dependent influx was also antagonized using SKF-96365. SKF pretreatment eliminated the difference in thapsigargin-evoked cytosolic Ca^{2+} levels between LA plus butyrate and DHA plus butyrate treatment groups. These data are consistent with previous observations in which DHA was found to regulate intracellular signaling by modulating plasma membrane Ca^{2+} entry (46), suggesting that the combination of DHA and butyrate alters colonocyte-free cytosolic Ca^{2+} levels in part by modulating SOC entry. Along these lines, growing evidence suggests that the mitochondrial permeability transition pore is activated by both oxidative stress and Ca^{2+}, whereas mitochondrial reactive oxygen species inhibit SERCA pumps (47) and activate SOC (48). In accordance with these findings, we have recently shown in HCT-116 cells that a mitochondrion-specific antioxidant blocks the DHA- and butyrate-induced induction of apoptosis.[4] Further studies are needed to determine if these effects extend to other members of the *n*-3 PUFA family, because a number of studies have shown that EPA and DHA have similar effects on biological membranes (1, 12, 13).

We have previously shown that butyrate induces colonocyte apoptosis via a Fas receptor–mediated extrinsic pathway (23). In an extension of our findings, we show for the first time that the combination of DHA and butyrate, compared with butyrate alone, further enhances apoptosis by additionally recruiting a Ca^{2+}-dependent mitochondrial-intrinsic pathway. Notably, DHA-enriched mitochondria were sensitized to rapidly sequester Ca^{2+},

which served to trigger apoptosis in the presence of butyrate. This could, in part, explain why the combination of dietary fish oil (containing DHA) and pectin (which generates butyrate in the lumen of the colon) seems to reduce tumor formation in the colon by promoting apoptosis (3, 10, 13, and 35). Although previous studies have examined the effects of PUFA to mobilize intracellular Ca^{2+} (46, 49), it is apparent that DHA and butyrate work coordinately in the colon to initiate a proapoptotic cycle involving the activation of SOC, leading to rapid entry of Ca^{2+} through the plasma membrane and mitochondrial Ca^{2+} loading (Fig. 6). With regard to putative upstream mediators, we have shown that a DHA-butyrate combination dissipates mitochondrial membrane potential, an effect that was reversed by coincubation with permeability transition pore inhibitors (14). This is consistent with the fact that mitochondrial Ca^{2+} interacts with cyclophilin D, which can trigger opening of the permeability pore (50). Although the precise chronology of these events have not been elucidated, it is likely that permeability transition pore opening, mitochondrial lipid oxidation, mitochondrial Ca^{2+} accumulation, and the induction of apoptosis are all intimately linked.

In summary, recent data indicate that chemotherapeutic agents that restore normal apoptotic pathways have the potential for effectively treating cancers that depend on aberrations of the apoptotic pathway to develop. Hence, it is of interest that DHA and butyrate work coordinately in the colon to trigger a previously unrecognized proapoptotic cycle involving mitochondrial Ca^{2+} loading. This may explain why a diet containing highly fermentable fiber is only protective when fish oil is the lipid source.

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[4] In preparation.

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Docosahexaenoic Acid and Butyrate Synergistically Induce Colonocyte Apoptosis by Enhancing Mitochondrial Ca $^{2+}$ Accumulation

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