DOCOSAHEXAENOIC ACID AND BUTYRATE SYNERGISTICALLY MODULATE INTRACELLULAR CALCIUM COMPARTMENTALIZATION TO INDUCE COLONOCYTE APOPTOSIS

A Dissertation

by

SATYA SREE N. KOLAR

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2007

Major Subject: Nutrition

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Approved by:

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ABSTRACT

Docosahexaenoic Acid and Butyrate Synergistically Modulate Intracellular Calcium Compartmentalization to Induce Colonocyte Apoptosis. (August 2007)

Satya Sree N. Kolar, B.M.S., Bangalore University, India;

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Chair of Advisory Committee: Dr. Robert S. Chapkin

Docosahexaenoic acid (DHA, 22:6n-3) from fish oil, and butyrate, a short-chain fatty acid fiber-fermentation product, protect against colon tumorigenesis in part by coordinately inducing apoptosis. We have demonstrated that the combination of these two bioactive compounds demonstrates an enhanced ability to induce colonocyte apoptosis by potentiating mitochondrial lipid oxidation. In order to explore the potential involvement of intracellular Ca^{2+} in the pro-apoptotic effect of DHA and butyrate, young adult mouse colonocytes (YAMC) and human colonocytes (HCT-116: p53+/+ and p53-/-) were treated with DHA or linoleic acid (LA) for 72 h ± butyrate for the final 6, 12 or 24 h. Cytosolic and mitochondrial Ca^{2+} levels were measured using Fluo-4 and Rhod-2. In addition, IP_3 pool, store-operated channel (SOC)-mediated changes and apoptosis were measured. DHA did not alter basal Ca^{2+} or apoptosis following 6 h butyrate co-treatment. In contrast, at 12 and 24 h, DHA and butyrate treated cultures exhibited a decrease in cytosolic Ca^{2+} and enhanced apoptosis compared to LA and butyrate. DHA and butyrate also increased the mitochondrial-to-cytosolic Ca^{2+} ratio at 6, 12 and 24 h.

The accumulation of mitochondrial Ca^{2+} preceded the onset of apoptosis which increased only following 12 h of butyrate co-treatment. RU-360, a mitochondrial uniporter inhibitor, abrogated mitochondrial Ca^{2+} accumulation and also partially blocked apoptosis in DHA and butyrate co-treated cells. p53+/+ and p53-/- cells demonstrated similar data with respect to all parameters.

Additionally, mitochondrial Ca^{2+} measurements were also made in rat primary-colonocyte-culture. Rats were fed semipurified diets containing either fish oil (a source of DHA) or corn oil (a source of LA), and colonic crypts were incubated in butyrate ex-vivo and mitochondrial Ca^{2+} was quantified. Crypts from rats fed fish oil incubated in butyrate exhibited an increase in the mitochondrial-to-cytosolic Ca^{2+} ratio compared to fish oil only.

In summary, our results indicate for the first time that the combination of DHA and butyrate, compared to butyrate alone, further enhances apoptosis by additionally recruiting a p53-independent Ca^{2+}-mediated intrinsic mitochondrial pathway. These data explain in part why fermentable fiber when combined with fish oil exhibits an enhanced ability to induce apoptosis and protect against colon tumorigenesis.

DEDICATION

I would like to dedicate this dissertation to my parents, Late Narasimha Murthy, and Mrs. Leela N. Murthy, my sister-in-law Late Naila Manzoor, brother Sathish Cayenne, sister Satyamba K, husband Ashish Sharma and my wonderful daughter Neha Narayan. It is their unconditional love, incessant encouragement and formidable support that has insipired me to stand strong and pursue my goals.

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team without whom this work would not be possible. Her endless patience, effort, hours of work on the microscope, unconditional support and confidence in my skills has gotten me to where I am.

I am very appreciative of the thoughtful advice and moral support extended to me by Dr. Laurie Davidson throughout my tenure not just in the Chapkin lab, but also during my stay at College Station. I'd also like to thank Evelyn Callaway for her willingness to help, technical expertise and timely encouragement to make this project successful. I would like to thank Dr. Yang Yi Fan for her patient instruction at every step of growth of this project. My thanks go out to Wooki Kim who has provided the technical assistance, a sweet smile and kind word when I needed it. It has been a great pleasure and wonderful experience to work with such a nice team. This lab team has not just helped with my work but has helped me understand things better and grow as a person.

This section would be incomplete without a mention of the role of my family in the pursuit of this project. I'd like to thank my parents, brother and sister whose love and guidance is with me in whatever I pursue. My parents have been my ultimate role models. More importantly, I wish to thank my loving husband whose patience, advice and encouragement has taken me a long way through my work. He has provided me with unending inspiration, persistent motivation and has been my forte. Last but never the least, I want to thank my daughter Neha whose level of tolerance has taught me a lot. She has accompanied me to the lab and has stayed awake with me through the wee hours of the morning, during my odd-hour experiments. She has been my greatest supporter

and has been the reason for me to rise every morning and pursue my dream. She has never failed to tell me that I will be “a winner” in whatever I did. She has provided me with the vigor and power to succeed.

I thank all of my friends whose names are not mentioned here due to space constraints. I strongly believe that each of you have made a great impact in my life. I love and thank you all and owe all of you the best part of me and my work.

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LIST OF ABBREVIATIONS

APC, adenomatous polyposis gene

BSA, bovine serum albumin

Ca^{2+}, Calcium

CIF, Ca^{2+} influx factor

CRAC, Ca^{2+} release Ca^{2+} activated channels

DHA, docosahexaenoic acid

DB, docosahexaenoic acid and butyrate combination

EPA, eicosapentaenoic acid

ER, endoplasmic reticulum

FBS, fetal bovine serum

HCT-116, human colon tumor cells

IFN-γ, interferon-γ

IP_3, Inositol-tris-phosphate

IP_3R, Inositol-tris-phosphate receptor

ITS, insulin/transferrin/selenium

KO, p53 knock out cells

LA, linoleic acid

LB, linoleic acid and butyrate combination

LOOH, lipid hydroperoxides

MMP, mitochondrial membrane potential

PTP, permeability transition pore

PUFA, polyunsaturated fatty acid

ROS, reactive oxygen species

SERCA, sacro-endoplasmic reticulum Ca^{2+} ATPase pump

SOC, store-operated channel

VDCC, voltage dependent Ca^{2+} channels

WT, p53 wild type cells

YAMC, young adult mouse colon

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Intestinal physiology and pathogenesis of colon cancer

Statistics of colon cancer. The incidence and mortality rates of colon cancer are similar in both men and women below 50 yrs (1). This is unlike the incidence of breast and prostate cancer which take precedence in women and men, respectively. In the year 2006 it is estimated that about 106,608 new cases of colorectal cancer were diagnosed and approximately 56,000 patients died from the disease. The incidence of colon cancer varies geographically. North America and Northern Europe have a higher incidence compared to Southern Europe. Asia and Africa report the lowest incidence of colon cancer. Nevertheless, the incidence of colon cancer is on the rise in developing countries with an increase in cases among people who migrate from low incidence to high incidence regions (2). The variation in geographical incidence of colon cancer indicates that environmental factors, especially modifiable factors like diet and physical activity play a major role (2). It is estimated that $8.1 billion is spent on treatment of colorectal cancer each year in the USA (3). With health care costs on the rise, dietary intervention to curtail the incidence of colon cancer is the focus of this study.

This dissertation follows the style of Cancer Research.

Anatomy and histology of the colon. The human digestive tract is 28 feet long and the distal 5 feet constitute the colon (about 1.5 -2 m). The colon constitutes ~22% of the length of the alimentary tract. The colon is anatomically divided into 5 parts- the ceacum, the ascending, transverse, descending colon and rectum. The large intestine, unlike the small intestine plays a very minimal role in digestion and absorption.

Its main functions include water and salt reabsorption from the digested material which enters the colon from the small intestine. In addition, the microflora of the colon is very important in the digestion and fermentation of vitamin K and short chain fatty acids like butyrate. The metabolites that are not digested by the intestinal enzymes reach the colon (4). Fiber, which is fermented by the bacterial microflora, yields short chain fatty acids like acetate, butyrate and propionate. Unlike the rest of the organs, butyrate, synthesized in the colon by the bacterial fermentation serves as a major source of the energy for the colonocytes (5-7).

Histologically, the walls of the intestine are made up of four different layers. The outermost serosal layer protects the intestine from its external environment and also lubricates the large intestine so as to aid its movement against the other organs. The muscularis mucosa is the mucosal layer of the large intestine which causes the contraction of the colon. The muscle layer is made up of longitudinal muscle fibers that provide integrity to the colon and contraction, which permits the transit of food through the colon. The submucosal layer consists of loose connective tissue submucosal glands, blood and lymphatic vessels responsible for nourishing the intestinal wall and the removal of metabolic waste. The innermost layer of the intestine is the mucosal layer

which consists of different sub-layers. This sub-layer consists of smooth muscle fibers and connective tissue layer called the lamina propria and a flexible lining of epithelial cells laid out over the flexible sublayers.

The epithelial cells, (i.e. "colonocytes") line the luminal surface of the colon. The luminal surface of the colon is folded into deep cavities lined by finger-like projections known as crypts that are embedded in connective tissue. The crypt openings appear as pits on the luminal surface of the colon. This architectural arrangement of colonocytes increases the surface area of the colon and therefore increases the efficiency of absorption of the salt and water (8, 9). The colonocytes are responsible for secretory, absorptive and barrier function of the colon.

The normal anatomy of the colon as seen in the anterior view of a male abdomen is demonstrated in **Figure 1**.

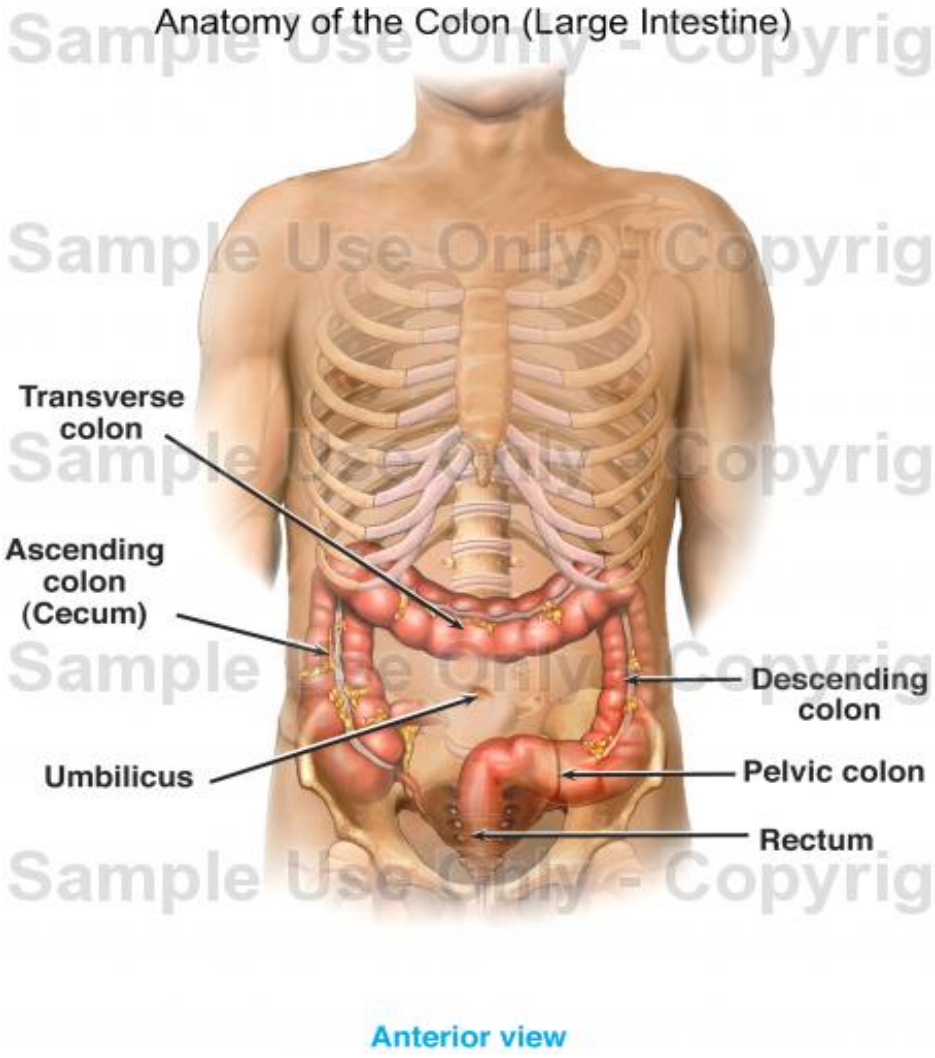

Figure 1. **Anatomy of the Colon.** This image reveals the normal anatomy of the large intestine (colon) as seen in the anterior view of a male abdomen. The distal 5 feet of the digestive tract comprising of the ceacum, ascending, transverse, descending colon and rectum form the 5 parts of the colon. The colon from the ceacum to the mid-transverse colon is known as the right colon while the reminder is known as the left colon. Parts of the colon lie in the abdominal cavity while the other parts are retroperitoneal. Source: Doe Report, 2007 (10).

Turn-over of the colonic epithelium. Colonocytes lining the crypts of the colon are in a continuous process of self-renewal. The cells undergo mitotic division at the base of the crypts and undergo differentiation during migration to the top crypt regions.

The colonocytes undergo apoptosis as they migrate from the base to the top of the crypts and are sloughed off into the lumen of the colon. Colonic cells possess an intrinsic mechanism for their own self-destruction, which is activated following transformation of colonocytes to differentiated cells. Death and sloughing off of the aged cells occurs continously, which is concurrent with replenishment by new colonocytes (8, 9, 11). The colonic epithelium in humans is completely replaced every 4-8 days. An accurate balance between the rate of new cell production and cell death is tightly maintained as is the cell number along the crypt axis. The molecular events responsible for the proliferation, maturation, differentiation, migration, apoptosis and renewal of colonocytes are very tightly regulated by a combination of events. Accumulating evidence suggests that these processes depend on the position of the cell in the crypt and simultaneous expression of genes (12). These findings indicate that the initiation and execution of colonocyte death depends on an apoptosis-permissive environment. A prevailing view is that the apoptotic process is regulated by a complex interplay of local apoptosis-inducing and survival factors which are tightly regulated by complex molecular events (13).

Carcinogenic transformation of colonocytes. Colon cancer is the primary cancer of the digestive system and begins in the form of benign polyps. These growths are usually non-cancerous and have the potential for malignant transformation and metastasis if they are not treated appropriately. Mutations in specific DNA sequences, like APC, K-Ras and tumor suppressor gene- p53, lead to uncontrolled cell proliferation in conjunction to resistance to apoptosis resulting in malignant tumor formation. The

causes for these mutations range from non-modifiable factors like genetic aberrations to modifiable factors like diet. Some diets are known to be pro-carcinogenic while diets rich in n-3 polyunsaturated fatty acids (n-3 PUFAs) and fiber appear to prevent against colon carcinogenesis (14-16). The exact reason why and how diet might prevent colorectal cancer remains uncertain.

Carcinogenesis is a multi-step process that involves genetic mutations resulting in transformation of normal colonocytes to malignant cells. This transformation is due to 6 vital alterations in cell physiology. These include: incessant potential for cell proliferation, ability of the cells to generate their own growth signals, capacity of cells to resist growth-inhibitory signals, evasion of programmed cell death (apoptosis), sustained angiogenesis, and tissue invasion and metastasis (17). The proliferative-apoptotic anticancer defense mechanism is a tightly maintained balance. Any or all of the events that alter cell physiology tip the balance in favor of developing cancer by malignant transformation.

Genetic alterations that predispose tissue for carcinogenesis include mutations of oncogenes and malfunction or loss of function in the tumor suppressor genes (18). Accretion of sequential genetic modifications results in 4 definable stages - the initiation, promotion, progression and malignant transformation stages of colon carcinogenesis. The initiation stage, involves a change in the genetic makeup of a cell. This change may occur randomly or when a carcinogen interacts with DNA causing damage. Initiation stage changes may involve base pair modification which could result in mismatch during gene expression. These mismatches if left unrepaired can lead to genetic mutations. In

addition, alkylation of the single or double strand breaks can lead to an erroneous repair process predisposing the tissue for carcinogenesis. This initial damage rarely results in cancer because the cell has many mechanisms to repair damaged DNA (19). However, if repair does not occur and the damage to DNA is in the location of a gene that regulates cell growth and proliferation, DNA repair, or function of the immune system, then the cell is more prone to be coming cancerous. Genetic alterations seen in the initiation stage predispose the colon to enhanced proliferation and resistance to cellular apoptosis leading to the promotion stage (20).

During promotion, the mutated cell is stimulated to grow and divide faster becoming a population of cells. At this stage there is microscopic evidence of alteration in crypt morphology and formation of aberrant crypts. The aberrant crypts congregate together, the consequences of which are specific regions which form the focus for transformation. These areas are known to be precursors of colon cancer lesions (21). During progression, there is growth and expansion of tumor cells relative to normal cells. The genetic material of tumors is more fragile and prone to additional mutations. These mutations occur in genes that regulate growth and cell function such as oncogenes, tumor suppressor genes, and DNA mismatch-repair genes. These changes contribute to tumor growth until malignant transformation occurs. The tumor then starts invading the submucosal layer of the intestine upon which it is termed as carcinoma. Eventually, the tumor shows signs of malignancy which includes increased angiogenesis, sustained proliferation and resistance to apoptosis. Mutations in at least four or five genes are required to produce a malignant tumor (22-24).

Colorectal tumors occur as a result of activation of oncogenes coupled with inactivation of the tumor suppressor genes. (18, 22). A significant number of studies demonstrates that mutations or truncation of the tumor suppressor gene, adenomatous polyposis gene (APC) results in the development of colon tumors (25-27). A mutation in the APC gene is seen in approximately 60% of patients with an early stage colon tumor (25). Although genetic alterations occur in a specific sequence, the cumulative effect of these changes, rather than their chronology of appearance are responsible for the tumor's properties. In addition to APC gene mutation, Ras oncogene was found to be activated in about 50% of colon carcinomas (22). In addition, cyclin D1, mismatch repair gene like (mismatch repair genes) MSH2, MSH6, MSH3, MLH1, PMS1, PMS2, and p53 are involved in colon tumorigenesis (18, 22, 28-31).

Diet and colon cancer

Epidemiological data related to colon cancer. Despite evidence indicating a strong association between immodifiable genetic factors and colon cancer, clinical and epidemiological studies demonstrate that dietary factors play a significant role. Dietary fat has been long known to have a significant influence in the development of colon cancer (9). Differences in colon cancer incidence and mortality rates between native and migrant populations and the increased risk in populations migrating from low- to high-risk areas suggest that modifiable factors, specifically dietary factors, play a critical role in colon carcinogenesis (2). Epidemiological data in the late 1960's performed in Japan by Wynder et al demonstrated that dietary fat had a major influence in colon cancer

incidence (32). Burkitt et al reported in the early 1970's that the incidence of colon cancer was much lower in African populations who consumed a high fiber and low fat diet compared to their white counter parts (33). Data from these seminal studies resulted in considerable emphasis on the effect of dietary fat and fiber on colon carcinogenesis. Since then substantial progress has been made in acquiring knowledge to study the relationship between dietary components and colon cancer. Currently epidemiological data from population studies indicate that diets high in fat increase the incidence of colon cancer (34-36).

Effect of dietary fat on colon cancer. Dietary fat is one of the key nutritional modulators of colon cancer development (9). Several studies that examined the relationship between dietary saturated, animal fat and colon cancer found a strong positive association between higher intake of fat and an increase in the risk of colorectal cancer development (37-40*).* Data from experimental animals also suggested that both the amount of fat and the composition modulate colon tumor development (41). Studies have consistently shown that the consumption of a diet rich in omega-3 fatty acids (n-3 PUFA's) enriched in fish oil may decrease the risk of colon cancer (42-46). For example, rats fed a fish oil diet produced a smaller number of experimentally induced colon tumors compared to those fed a diet high in corn oil (46, 47). The major difference between fish and corn oil diets was the dietary lipid source. There were significantly higher amounts of eicosapentaenoic acid (EPA, $20{:}5^{\Delta 5,8,11,14,17}$) and docosahexaenoic acid (DHA, $22{:}6^{\Delta 4,7,10,13,16,19}$) in fish oil diet compared to corn oil diet

which had higher amounts of linoleic acid (LA, $18:2^{\Delta 9,12}$), an important source of omega-6 PUFA in the western diet (48).

Colon tumorigenesis, a multistep transformation process ensues due to a disruption in the balance between colonocyte apoptosis and proliferation. Dysregulation in any of the homeostatic processes results in the clonal expansion and carcinogenic transformation. Induction of colonocyte apoptosis is a way by which the cells with damaged DNA are deleted so as to prevent the initiation stage of colon cancer. We have data demonstrating that fish oil enhances the targeted deletion of DNA-damaged cells, which may explain a mechanism by which it protects against colon cancer (45). There is also substantial evidence to demonstrate that colonic epithelial transformation to carcinoma is associated with the progressive inhibition of apoptosis (49-51). Therefore bioactive agents that enhance apoptosis demonstrate a potential for having a chemoprotective effect. Along these lines, our data demonstrate that fish oil fed rats have a higher level of apoptosis with resistance to alkylation and oxidation induced DNA damage (45, 52-54). These findings are consistent with clinical data which demonstrate that increasing intake of n-3 PUFA promoted colonocyte apoptosis in human colon (54). Collectively, these human and rodent data indicate a strong potential for fish oil to be a chemotherapeutic agent against tumor growth.

With respect to the mechanism by which n-3 PUFA acts as a chemoprotective agent, a plethora of studies indicate its ability to incorporate into membrane phospholipids thus altering membrane composition and function. (55-57). Since membranes constituents including phospholipids are an integral part of various signaling

mechanisms, any alteration in phospholipid composition could result in modulation of mechanisms involved in cellular apoptosis, proliferation and differentiation (57, 58). We have demonstrated that n-3 PUFA from fish oil exerts a protective effect against colon tumorigenesis primarily by enhancing colonocyte apoptosis rather than decreasing proliferation (14, 16). More recent investigations have demonstrated that highly unsaturated fatty acids present in fish oil are incorporated into the mitochondrial membrane and have effects on reactive oxygen species (ROS) production (57, 59), membrane lipid oxidation (58, 60), transcriptional or translational regulation (61, 62), mitochondrial Ca^{2+} level (63), eicosanoid biosynthesis (64) and intracellular signal transduction. These cumulative effects are believed to mediate the *n*-3 PUFA-induced pro-apoptotic effect which could in part explain the protective effect of fish oil in colon tumorigenesis.

Effect of dietary fiber and colon cancer. Fiber is one of the most important nutritional factors considered to be protective against colon cancer, with well established biological mechanisms underlying this hypothesis. Experimental and population based studies conducted since the early 1970's have demonstrated that high fiber diet is protective against colon carcinogenesis (33, 65-67). In contrast, large prospective studies from USA, Sweden and Finland showed no protective effects of fiber against colon carcinogenesis (68-70). More recently, the European Prospective Investigation of Cancer and Nutrition Study (EPIC), one of the largest studies performed in 10 European countries, concluded that there was a 40% decrease in risk of colon cancer incidence associated with the increased intake of fiber from food (71). The inconclusive evidence

from epidemiological studies on the chemoprotective effects of fiber challenged the recommendation to increase fiber intake to reduce the risk of colorectal cancer. Some of the inconsistencies in the data could be as a result of the type of fiber investigated or the interaction of fiber with other dietary components like fat (37).

Putative mechanisms by which fiber can be protective are well described (72). Fiber increases stool weight, shortens transit time, dilutes colonic contents, stimulates bacterial anaerobic fermentation, which reduces contact between the intestinal contents and mucosa, and leads to the production of short chain fatty acids (SCFA) like acetate, propionate and butyrate, thus reducing luminal pH and the conversion of primary bile acids to secondary bile acids (72-74). Butyrate, a major source of energy for the coloncoytes is known to have chemoprotective properties (73). Although the chemoprotective effect of butyrate in animal studies is inconclusive (75, 76) its effect in cell lines is well established (77, 78). In cell lines, butyrate reduces cell proliferation and induces apoptosis, factors that are associated with inhibition of the transformation of the colonic epithelium to carcinoma (79-81). With evidence of the chemoprotective effect of dietary fiber and butyrate, recent research has focused on its combined effect with other dietary factors of which fat is one of the most widely investigated.

Fat-fiber interaction. Fat and fiber are two of the most widely investigated dietary components in the prevention of colon carcinogenesis. We have demonstrated that the bioactive compounds generated by dietary fish oil (DHA and EPA) and fermentable fiber (butyrate) work together to protect against colon carcinogenesis primarily by enhancing apoptosis (14, 16, 29, 47, 52). In addition, our in-vivo

experiments have demonstrated that fish oil and fermentable (pectin) fed animals enhanced ROS generation compared to corn oil fed animals (52). Although there is well documented evidence that butyrate alone inhibits histone deacetylases and activates the Fas mediated apoptotic pathway (29, 82, 83), these effects seem to occur as a consequence of its ability to promote cellular oxidation (82, 84). These findings are important because DHA and EPA are highly susceptible to lipid oxidation and this could explain the synergistic effect seen when DHA is combined with butyrate (58, 63). Along the same lines, we have demonstrated that enhanced mitochondrial lipid hydroperioxides which can be blocked by mitochondrion-specific antioxidants play an important role in DHA and butyrate induced apoptosis (58). All of these events culminated in creating an environment conducive for cellular apoptosis. Despite all the scientific evidence indicating the protective effect of fish oil and fermentable fiber, we still lack information regarding the molecular mechanisms by which this combination exerts its protective effect against colon tumorigenesis.

Ion transport across biological membranes

Ion channels. All eukaryotes are made of cell (plasma) membranes which are highly selective barriers separating the contents of the cytosol from the exterior. The plasma membrane consists of lipids and proteins. Membranes permit the development of concentration gradients crucial for the transport of solutes across the membrane. Structurally the plasma membrane is made of a phospholipid bilayer with membrane lipids having a hydrophilic and hydrophobic part. The major kinds of lipids present in

the cell membranes are phospholipids, glycolipids and cholesterol. The phospholipid bilayer is impenetrable to water soluble molecules and ions. However, complex integral protein molecules present within the bilayer permit the flow of selected molecules and ions in and out of the cell. Singer and Nicolson proposed the 'Fluid Mosaic Model' of the structure of cell membranes which is widely accepted today (85). According to this model the integral membrane proteins are embedded in the phospholipid bilayer. The model suggests that three major classes of membrane proteins exist. They are the channels, pumps and receptors. These proteins function to meet the transport needs of the cell. They move ions and other small molecules through the otherwise impermeable phospholipid bilayer. The ion pumps are active and require ATP to move ions against the concentration gradient. In contrast, passive ion channels are transmembrane proteins that form a channel through the cell membrane. Ion channels pass ions down a concentration gradient by facilitated diffusion without the expenditure of energy. The channel can be opened or closed and therefore is referred to as a gated channel. There are three types of gated ion channels: Ligand gated, mechanically gated and voltage gated. Ligand gated channels open or close in response to the binding of signaling-ligand. Voltage gated channels are found predominantly in excitable cells like neurons. They open or close in response to changes in membrane potential across the plasma membrane. Ion channels are highly selective and allow only specific ions through the cell membrane. Ca^{2+} selective channels are extremely important due to the role of the ion in various cellular functions. The focus of this study is based on Ca^{2+} selective

channels present in various cellular membranes, non-excitable colonocyte cells in particular.

Voltage dependent Ca^{2+} channels (VDCC), which are permeable to Ca^{2+} are found to play an important role in membrane potential, release of neurotransmitter, hormones, muscle contraction etc. In excitable cells, VDCC are the preponderant type involved in the influx of Ca^{2+}. VDCC's are classified into N-type (neuron) present in neurons, L-type (long lasting) or T-type (transient type) present in the cardiac muscle, P-type (Purkinje) which are similar to the N-type but present in the heart, and the poorly understood Q-type and R-type which are present in the brain (85, 86). In non-excitable cells, Ca^{2+} influx is essential for regulating distinct cellular processes involving enzyme control, gene regulation, cell growth, proliferation, and apoptosis. In contrast to excitable cells, VDCC are generally absent in non-excitable cells like colonocytes. The store-operated channels (SOC) or Ca^{2+} release–activated Ca^{2+} (CRAC) channels form the major Ca^{2+} entry pathways, in addition to some involvement from second messenger operated channels (87). The SOC entry pathway involves entry of Ca^{2+} through the SOC channels situated on the plasma membrane following emptying of the intracellular Ca^{2+} stores (88). An overview of intracellular Ca^{2+} homeostasis including membrane channels and pumps is demonstrated in **Figure 2**.

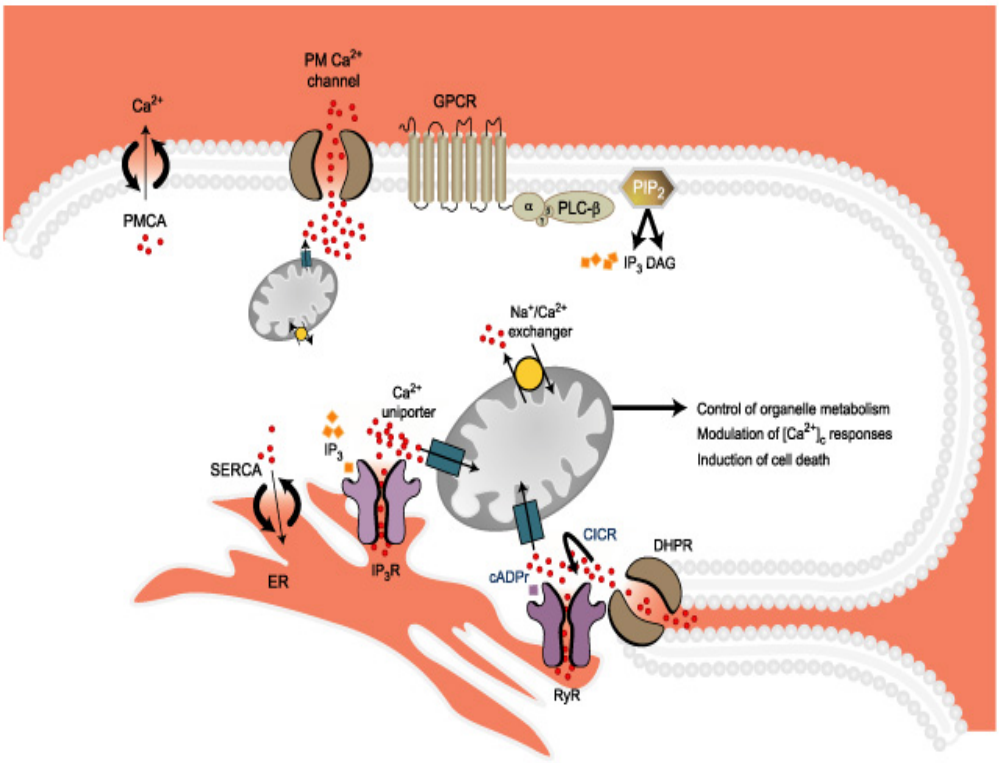

Figure 2. **Overview of intracellular Ca^{2+} homeostasis**. Ca^{2+} enters the cell through the plasma membrane channels and is processed by the ER and mitochondria via the pumps and channels situated on their membranes. Ca^{2+} movement from the ER to mitochondria is a key process in the activation of apoptosis by some stimuli. PM Ca^{2+} channel, plasma membrane calcium channel; PMCA, plasma membrane channel pump; RyR, ryanodine receptor; SERCA, sarcoplasmic-reticulum-Ca^{2+} -ATPase pump. Source: Rizzuto et al, 2004 (90).

Intracellular Ca^{2+} and 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 (89). 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 (89, 90). Extracellular Ca^{2+} concentration is 1 – 2 mM while the intracellular cytosolic concentration of Ca^{2+} is 4 orders of magnitude lower at

approximately 0.1 mM. Free cytosolic Ca^{2+} does not represent the total concentration of 0.1 mM because much of the intracellular Ca^{2+} is sequestered in intracellular organelles such as the endoplasmic reticulum (ER) and mitochondria. In response to a stimulus, the release of Ca^{2+} from these intracellular stores, or its entry into the cells through Ca^{2+} channels in the membrane, rapidly increases the intracellular concentration of Ca^{2+} some 10–100 fold. The transient increase in intracellular Ca^{2+} concentration is a signal for various cellular events like muscle contraction, secretion etc. A consistent increase in Ca^{2+} overload within the cell can induce apoptosis and the activation of Ca^{2+} binding proteins, e.g., calmodulin (91).

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" (92). Eukaryotic cells can increase their cytosolic Ca^{2+} levels by 2 mechanisms: (1) release of Ca^{2+} from intracellular stores or influx via plasma membrane channels. Channels located in the plasma membrane, like 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 the stores into the cytosol. Although the importance of the 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 most importantly, the mitochondria in maintaining intracellular Ca^{2+} homeostasis and cellular physiologic function (88). Mitochondria play a key role in both

apoptosis and necrosis by affecting energy metabolism, participating in Ca^{2+} homeostasis, regulating activation of caspases and releasing ROS (93-95). 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 high affinity and low capacity active pumps situated on the mitochondrial outer membrane, rapidly take up Ca^{2+} that is released from the ER stores and re-circulate it back into the cytosol via exchangers such as the Na^{2+}/ Ca^{2+} exchanger. In summary, mitochondria can sequester cytosolic Ca^{2+} acting as a sink thereby preventing a rapid and sudden change in cytosolic Ca^{2+} level.

Under normal conditions, mitochondrial Ca^{2+} uptake may serve as a signal to under certain conditions increase cellular metabolism (96). However, a plethora of studies indicate that mitochondrial Ca^{2+} accumulation is a trigger for cytochrome-c release and therefore apoptosis (97). In addition, studies addressing ER-mitochondria communication indicate that both pro-apoptotic and anti-apoptotic proteins modulate ER Ca^{2+} release by acting on the IP_3Rs (98). Interestingly, pro-apoptotic factors act on both the mitochondria and ER by enhancing mitochondrial Ca^{2+} signal propagation in conjunction with facilitating ER Ca^{2+} mobilization. This results in Ca^{2+}-dependent mitochondrial membrane permeabilization and apoptosis. In addition, ER Ca^{2+} release causes more extensive changes in mitochondrial Ca^{2+} signaling as opposed to cytosolic Ca^{2+} signal (99). Cytochrome *c* released from the mitochondria at the onset of apoptosis is known to bind with high affinity to IP_3R. This blocks the Ca^{2+}-dependent inhibition of IP_3-induced Ca^{2+} efflux which increases mitochondrial Ca^{2+} loading (100). ROS produced by the mitochondria also demonstrates a similar mechanism of increase in IP_3-

induced Ca^{2+} release and mitochondrial Ca^{2+} loading to induce apoptosis (101). Therefore, ER and mitochondria form an intricate network to mediate Ca^{2+}- dependent cellular apoptosis.

Intracellular Ca^{2+} homeostasis

Under normal circumstances there is a constant influx of Ca^{2+} from the extracellular environment into the ER. Ca^{2+} influx from the extracellular medium into the cytosol is mediated by the SOC and second messenger operated channels and other transporters present on the plasma membrane. Once Ca^{2+} enters the cytosol, sarco-endoplasmic reticulum ATPase (SERCA) pumps situated on the ER membrane pump Ca^{2+} into the ER by expending ATP. The mobilization of intracellular Ca^{2+} stores in response to receptor-stimulated formation of IP_3 depends on the IP_3 receptor (IP_3R) present on the ER membrane. According to the capacitative model of Ca^{2+}, mobilization of intracellular Ca^{2+} stores from the ER results in cells refilling their intracellular emptied pool by Ca^{2+} entry through the SOC *(*102). Various theories have been put forth to explain the connection between intracellular emptying and cellular refilling by opening of the SOC. Theories suggest a conformational coupling hypothesis according to which a physical connection between the ER stores and the plasma membrane via protein-protein interaction (103, 104). The release of a diffusible signal – the calcium influx factor (CIF), is discussed below. However, most recently critical new information on the nature of the SOC has been revealed (105, 106). By using interfering RNA technique, Roos et al studied genes encoding for various Ca^{2+} channel proteins and

transient receptor potential genes (105). They demonstrated that knocking out of the gene encoding protein stromal interaction molecule (STIM) substantially decreased Ca^{2+} entry. Two homologs of this protein were found in humans - STIM1 and STIM. STIM1 is found to be present in the plasma and ER membrane. The sequence of STIM1 led to speculation that the protein present on the ER and plasma membrane could interact forming a bridge facilitating SOC entry (105). However, Zhang et al demonstrated very elegantly that STIM1 acts as a Ca^{2+} sensor and upon intracellular Ca^{2+} depletion, migrates from the ER to the plasma membrane to activate the SOC (106). To further strengthen the findings of Roos and Zhang, intracellular Ca^{2+} oscillations were studied. Wedel et al demonstrated that knocking out STIM1 and Orai1 (another component of SOC) abolished intracellular Ca^{2+} oscillations completely (107). These data demonstrate the importance of these proteins in the SOC pathway. Although the precise actions of these proteins are still unclear, their future study will be a critical step towards understanding the mechanism involved in SOC activation, function and signaling. If ER store depletion activates SOC entry, then refilling of the stores should inactivate SOC entry. Recent work has demonstrated that thapsigargin which prevents ER filling, reduces SOC entry (88). There is also evidence that phospholipase-C and diacylglycerol lipase which liberate arachidonic acid from phosphotidyl-inositol (4,5)-bisphosphate, increase cytoplasmic Ca^{2+} due to SOC entry (95). In addition, PLA_2-dependent release of arachidonic acid from membrane phospholipids has been implicated. Arachidonic acid has numerous effects on Ca^{2+} signaling, and there is growing evidence that arachidonic acid inhibits store-operated entry (108). Other SOC inhibitory molecules

include nitric oxide (109) lipoxygenase, calmodulin, sphingomyelin, ceramide (110), protein kinase C (111), and cyclic GMP (112).

The dynamic interaction between the ER and the mitochondria with respect to intracellular signaling and cell death is well established. Recent work has shown that mitochondria are closely juxtaposed with the ER to facilitate efficient mitochondrial Ca^{2+} uptake during IP_3-induced Ca^{2+} release (113, 114). The precise molecular mechanism and regulation of the cross talk between these two storage organelles are still unknown. Nonetheless, there is evidence of clustering of IP_3R's on ER membranes facing the mitochondria (114-116). Besides the close physical association of the ER and mitochondria, IP_3R associated Ca^{2+} signals are found to be associated with an increase in mitochondrial Ca^{2+} levels (117). More recently, PACS2, which is an ER-associated vesicular-sorting protein, was proposed to link the ER to mitochondria. The knockdown of PACS2, led to uncoupling of the organelles and inhibition of Ca^{2+} signal transmission (118). Mitochondria take up Ca^{2+} released from the ER and circulate it back into the cytosol. Therefore, it is obvious that mitochondria modulate cytosolic Ca^{2+} signaling, either by rapidly accumulating the Ca^{2+} released from the ER or by suppressing the positive feed-back of Ca^{2+} via IP_3 receptors, thus acting as a buffer to increase cytosolic Ca^{2+} (119). In addition to sequestering cytosolic Ca^{2+}, mitochondria can trigger cytosolic Ca^{2+} signals via activation of permeability transition pore (120). These findings demonstrate that the mitochondria and ER maintain a local communication pathway for effective transfer of Ca^{2+} between the 2 organelles. During ER release, mitochondria sequester Ca^{2+} without an increase in cytosolic Ca^{2+} levels (120, 121).

Likewise in the case of permeability transition pore (PTP)-mediated mitochondrial Ca^{2+} release, the ER is known to rapidly accumulate Ca^{2+} before it is detected in the cytosol (122).

Mitochondrial Ca^{2+} and apoptosis. There is substantial evidence that intracellular Ca^{2+} fluxes occur during most forms of cell death including apoptosis (123). The role of these fluxes in the induction of mitochondrial apoptosis is still under debate. The transfer of Ca^{2+} from intracellular stores and mitochondria controls cellular respiratory events and activation of respiratory enzymes. Mitochondria are known to function not only in the production of ATP but also in the regulation of cellular apoptotic signaling via maintenance of intracellular Ca^{2+} homeostasis. Cell stimulation leading to increased cytosolic Ca^{2+} level activates the VDAC present on the outer mitochondrial membrane (124, 125). This leads to Ca^{2+} entry into the mitochondrial matrix. Mitochondrial uniporters, proteins that are highly Ca^{2+} sensitive pass the ions across along the electrochemical gradient due the highly negative membrane potential (-180 mV). Additional mitochondrial uniporters like VDAC are also Ca^{2+} sensitive. Therefore, VDAC and the uniporters act in unison to buffer the increase in cytosolic Ca^{2+} levels (125, 126). Ca^{2+} entering the mitochondria stimulate Ca^{2+} sensitive mitochondrial dehydrogenases (CSMDH) which induce the efflux of Ca^{2+} from the mitochondria into the cytosol. This is important for maintaining Ca^{2+} uptake and energy generation (127). Ca^{2+} transfer back to the cytosol is mediated by the Ca^{2+} exchangers Na^{+}/Ca^{2+} and/ or H^{+}/Ca^{2+}. It is long known that mitochondrial sequestration of large amounts of Ca^{2+} contributes to cell death. Sustained elevation of cytosolic Ca^{2+} from a concentration of

100 nM to 1 μM activates both VDAC and the uniporter to enhance mitochondrial Ca^{2+} uptake. Accumulation of large amounts of Ca^{2+} in the mitochondria leads to the interaction of Ca^{2+} with cyclophilin D to induce PTP opening (128). Further elevation of Ca^{2+} in the mitochondrial matrix generates ROS and free fatty acids which also promote formation of PTP (129, 130). Finally, dissipation of the mitochondrial membrane potential, release of pro-apoptotic molecules, and impairment of mitochondrial function leads to apoptosis of the cell.

The accumulation of intra-mitochondrial Ca^{2+} along with the production of mediators such as arachidonate or ceramide can trigger cells to undergo apoptosis. Intra-mitochondrial sequestration of Ca^{2+} alters the architecture of the mitochondria via the induction of swelling and rupture (131). Release of contents from the mitochondrial intermembrane space requires that the outer mitochondrial membrane (OMM) be permeabilized. The mechanism involved is controversial although there is evidence that rupture due to sequestration of Ca^{2+} leading to MPT, activation and translocation of pro-apoptotic proteins like Bax, Bak and tBid to the OMM, and the opening of the PTP could play a significant role in this process (132, 117). A critical event associated with MPT and translocation of pro-apoptotic proteins in mitochondrial apoptotic signaling is the release of normal mitochondrial constituents, i.e., cytochrome-c, mitochondrial activator of caspases (SMAC/ Diablo) and apoptosis inducing factor (AIF) into the cytosol. The released cytochrome-c then binds to apoptotic protease activating factor-1 (APAF-1), which in turn recruits and activates caspase-9 to form an apoptosome. The apoptosome finally activates effector caspase-3 resulting in apoptosis (117, 131).

Ca^{2+}, ROS, mitochondria and apoptosis. Over the past few years, extensive progress has been made in elucidating the role of mitochondrial Ca^{2+} and ROS with respect to PTP and apoptotic signaling. Mitochondria, the respiratory organelle of the cell, are the major source of ROS production. Superoxide ($O_2^{-}\cdot$) is the primary form of ROS generated by mitochondria. $O_2^{-}\cdot$ is converted to H_2O_2 by the enzyme superoxide dismutase (SOD) or by spontaneous dismutation (133). Although the precise mechanism by which mitochondrial Ca^{2+} stimulates ROS generation and apoptosis is unclear, Ca^{2+} can enhance cytochrome *c* dislocation from the mitochondrial inner membrane by inducing the PTP. This results in an effective block of the respiratory chain (complex III) which would enhance ROS generation (134). Along these lines Ng and collogues have demonstrated that the combination of DHA and butyrate potentiate mitochondrial lipid oxidation and the dissipation of mitochondrial membrane potential which could be blocked by the PTP inhibitor, cyclosporine (58). Another pathway by which Ca^{2+} can induce ROS production may involve the perturbation mitochondrial antioxidant status. Consistent with this finding, our laboratory has recently demonstrated that fish oil enhances ROS production, while the combination of fish oil and fermentable fiber suppressed antioxidant enzyme, i.e., superoxide dismutase (SOD) and catalase (CAT) activity and increased the ratio of SOD: CAT (47). Brookes et al have demonstrated that Ca^{2+}-induced PTP opening causes the release of mitochondrial glutathione (GSH) (133). This finding suggests that mitochondria exposed to Ca^{2+} generate more ROS because of diminished GSH levels. Taken together, all these

findings demonstrate that Ca^{2+} may exert direct effects on ROS generation thus contributing to a pro-apoptotic environment.

Apoptosis occurs concurrent to enhanced generation of mitochondrial ROS (94). Studies have indicated a complex interplay between PTP opening and mitochondrial ROS generation in response to Ca^{2+} (93, 135, 136). Along these lines, oxidative stress and Ca^{2+} activate PTP opening while mitochondrial ROS inhibits SERCA pump and activates SOC (137-139). This demonstrates that mitochondrial ROS can modulate extramitochondrial Ca^{2+} pools. Overall, it is clear that mitochondrial ROS and Ca^{2+} accumulation exist as networks which influence each other and eventually modulate cellular apoptosis. In conclusion, experimental evidence associating Ca^{2+} homeostasis and ROS and lipid hydroperoxide (LOOH) with mitochondrial mediated induction of apoptosis forms a strong basis to study intracellular Ca^{2+} homeostasis in n-3 PUFA and butyrate-treated colonocyte cultures.

Tumor suppressor gene- p53

p53 is a tumor suppressor gene found to be mutated in more than 50% of all human cancers, making it the most frequent target for genetic alterations in cancer (140-143). p53-mediated regulation of cell cycle arrest and apoptosis are vital for its tumor suppressor activity (143-145). p53 is latent and not essential for the normal performance of cells within the body as suggested by data that demonstrates p53 knock-out mice also undergo normal development and maturation (146). However, p53 responds to a variety of intrinsic and extrinsic stress signals to trigger various cellular events, like cell-cycle

arrest, apoptosis, inhibition of angiogenesis which indirectly affects metastasis, and DNA repair (147, 148). Furthermore, p53 is also known to contribute, directly or indirectly, to DNA repair processes (149, 150). In response to DNA damage, wild type p53 protein transcriptionally activates downstream target genes that serve as mediators of p53 function (151-152). For example, p21, Bax, and Puma are some of the direct transcriptional targets of p53 (153-155). Activation of down stream signals result in growth control, DNA repair, and apoptosis (56). The deletion of p53 or the presence of a mutated p53 protein enhances proliferation of cells with damaged DNA due to the absence of cell cycle arrest and resistance to apoptosis. Therefore, genetic manipulation used to suppress or knock down the expression of p53 not only facilitates cell survival but also aids metastasis by enhancing angiogenesis.

p53 and apoptosis. The precise molecular pathways of p53-mediated apoptosis are not fully understood. p53 induces several proapoptotic molecules like p53-upregulated modulator of apoptosis (Puma) (153, 156) and Noxa (157) which belong to the BH-3 group of Bcl-2 proteins. Puma, a protein localized to the mitochondria is an essential mediator of DNA-damage induced "intrinsic" form of apoptosis. Puma is known to induce apoptosis by various mechanisms. Yu et al have demonstrated that second mitochondria-derived activator of caspase (SMAC)/Diablo, a mitochondrial apoptogenic protein, mediates Puma induced apoptotic events (157, 158). They demonstrated that SMAC deficient cells do not undergo apoptosis. Puma is also known to bind pro-apoptotic Bax and Bak (159) to induce mitochondrial membrane permeabilization and the release of cytochrome-c (160). In addition, another important

mechanism by which Puma induces apoptosis is by activating caspase-12, a proapoptoic molecule present on the ER. Interestingly, PUFA induces Ca^{2+} release from the ER (161). Activated caspase-12 contributes to mitochondrial membrane permeabilization and the induction of p53-meditated apoptosis.

p53 and Ca^{2+} homeostasis. Shibue et al demonstrated that Puma regulates p53-mediated apoptosis by inducing Ca^{2+} release from the ER (161). The mechanism by which Puma mediates ER Ca^{2+} release is unclear. More recently, studies have also shown direct interaction between antiapoptotic Bcl-2 and IP_3R's (161, 162). Puma decreases the interaction between IP_3R's and antiapoptoic Bcl-2 resulting in a release of Ca^{2+} from the ER (95). Small amounts of cytochrome *c* released during early apoptosis binds to IP_3R to enhance Ca^{2+} release from the ER (163). Therefore, Puma can also act along the same lines releasing a small amount of caspase-12 which binds to the IP_3R's on the ER thus stimulating a release of ER Ca^{2+} (95). Although the precise mechanism by which p53 affects Ca^{2+} homeostasis is unclear, Puma seems to be an important regulator of p53 induced apoptosis.

Summary and purpose

Colon cancer is one of the major causes of mortality in most developed countries. There is substantial epidemiological and experimental evidence to suggest that environmental factors like diet play a very important role in determining risk. Among the various dietary factors, fat and fiber are the most widely investigated components in the chemoprevention of colon carcinogenesis. Evidence emerging from many

experimental and epidemiological studies show that diets rich in n-3 PUFA's found in fish oil, (enriched in EPA and DHA) protect against colon carcinogenesis. However, there is a debate with regard to the effect of dietary fiber on colon cancer. Nonetheless, one of the largest population studies reported that dietary fiber is protective against colon cancer and butyrate a fiber fermentation product has chemoprotective properties (65). The combination of fat (DHA) and fiber (butyrate) is known to be chemoprotecive primarily by enhancing cellular apoptosis (14, 16, 58). Experimental evidence also suggests that DHA primes colonocytes by enhancing butyrate-induced mitochondrial lipid oxidation and apoptosis (58). Although there is evidence for the chemoprotective properties of DHA and butyrate, their precise molecular mechanism of action is still obscure. The role of Ca^{2+} as a key modulator of cellular apoptosis is well established. Local transfer of Ca^{2+} signals between ER and mitochondria and its importance in igniting apoptosis is also well known. Nonetheless, the importance of Ca^{2+} in DHA and butyrate-mediated apoptosis is unclear. Therefore, this study further elucidated the chemoprotective properties of components of fish oil (DHA) and fermentable fiber (butyrate) with respect of alteration in intracellular Ca^{2+} homeostasis both in-vitro and in ex-vivo primary colonocyte cultures. In an attempt to elucidate the molecular mechanisms of action the following hypotheses and specific aims were designed:

Hypotheses

1. DHA and butyrate-dependent induction of lipid hydroperoxides (LOOH) in colonocytes increases mitochondrial Ca^{2+} levels, thereby triggering apoptosis.
2. Changes in intracellular Ca^{2+} homeostasis are consistent in human colonocyte cell lines and primary colonocyte cultures.

Specific aims

1. Determine intracellular Ca^{2+} levels in fatty acid and butyrate treated colonocytes.
2. Determine the source of changes in Ca^{2+} homeostasis using pharmacological agents, e.g., thapsigargin to examine the IP_3 pool (Ca^{2+} released from the ER on stimulation); EGTA and SKF to examine membrane channels.
3. Determine mitochondrial Ca^{2+} levels using pharmacological agents.
4. Determine the role of mitochondrial Ca^{2+} accumulation with respect to fatty acid and butyrate-induced apoptosis by inhibiting the mitochondrial uniporter.
5. Determine the effect of DHA and butyrate on mitochondrial Ca^{2+} accumulation in immortalized mouse (YAMC) human colorectal adenoma cell lines (HCT-116).

6. Determine the role of p53, a tumor suppressor gene in DHA and butyrate induced apoptosis.
7. Determine the role of mitochondrial uniporter in mitochondrial Ca^{2+} accumulation and induction of apoptosis in both mouse and human colonocytes.
8. Measure mitochondrial Ca^{2+} levels in primary cultures from animals fed diets rich in n-3 PUFA and n-6 PUFA following incubation of viable crypts with butyrate ex vivo.

CHAPTER II

DOCOSAHEXAENOIC ACID AND BUTYRATE SYNERGISTICALLY INDUCE COLONOCYTE APOPTOSIS BY ENHANCING MITOCHONDRIAL CA^{2+} ACCUMULATION*

There is substantial experimental, epidemiological and clinical evidence indicating that fish oil-containing diets rich in n-3 polyunsaturated fatty acids (PUFA), e.g. 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 (42-46). In addition, there is evidence from epidemiological and observational studies indicating that the consumption of fiber, which increases butyrate levels in the lumen of the colon, is chemoprotective against colorectal cancers (6, 65). 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 (164-166). In order 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.

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We have demonstrated 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 (14, 16, 52). 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 (56, 57). 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 (58). 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 (103). 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" (92). Eukaryotic cells can increase their cytosolic Ca^{2+} levels via 2 mechanisms: release of Ca^{2+} from intracellular stores or influx via plasma membrane channels. Channels located in the plasma membrane, e.g., 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 (96, 103, 120). 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 (ROS) (96, 131).

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 re-circulate it back into the cytosol via exchangers such as the Na^{2+}/ Ca^{2+} exchanger (88,97). Therefore, mitochondria can be regarded as critical check-points 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 (24). However, in certain situations, mitochondrial Ca^{2+} accumulation is a trigger for cytochrome-C release and the induction of apoptosis (97).

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 or not chemoprotective nutrients modulate intracellular calcium compartmentalization and store-operated channel entry to induce colonocyte apoptosis. The results confirm and extend our previous observations and demonstrate 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 co-treatment.

Experimental procedures

Materials. RPMI 1640 and Hanks' balanced salt solution (HBSS) were purchased from Mediatech (Herndon, VA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Insulin, transferrin, selenium without linoleic acid were purchased from Collaborative Biomedical Products (Bedford, MA). Glutamax, recombinant mouse interferon-γ (IFN-γ) and Leibovitz media were from GIBCO BRL (Grand Island, NY). Fatty acids were obtained from NuChek (Elysian, MN). Fluo-4 AM, Rhod-2 AM and Calcium Calibration buffer kit # 2 were purchased from Molecular Probes (Eugene, OR). RU-360 and SKF-96365 were purchased from Calbiochem (San Deigo, CA). Cell death detection ELISA kit was obtained from Roche Applied Science (Indianapolis, IN). 2-well Lab-Tek Chambered Coverglass slides were purchased from Nunc, Inc. (Naperville, IL). Thapsigargin, staurosporine, EGTA, sodium butyrate and all other

reagents were obtained from Sigma (St. Louis, MO). Stock solutions of 1.0 mM Fluo-4, AM and 4.0 mM Rhod-2 were prepared in DMSO and diluted with medium to 3.0 μM and 2.0 μM, respectively (final concentration of the vehicle DMSO was maintained at 0.1-0.3% in culture). Thapsigargin stock (5.0 mM) was prepared in DMSO and used at a concentration of 5.0 μM (≤ 0.1% DMSO). RU-360 (1 mM) stock was prepared in degassed water and diluted to a final concentration of 10 μM for cell treatment. A (1 mM) stock solution of staurosporine (STS) was prepared in DMSO and diluted to a final concentration of 1 μM 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 interferon-γ) or non-permissive conditions (39°C) as previously described (58, 83). For all fluorescence measurements, cells (passages 12-18) were seeded onto borosilicate 2-chambered cover glass slides at a density of 5-7 x 10^3 to achieve a 50-70% confluence. For apoptosis assays, cells were seeded onto 35 mm cell culture dishes or 6 well plates at a density of 35 x10^3. Bovine serum albumin (BSA) complexed fatty acids were added to cultures 24 h after cell plating as previously described (83). Select cultures were treated with BSA-complexed DHA (22:6, n-3) or LA (18:2, n-6) (0- 200 μM) for 72 h. Cells were co-incubated with sodium butyrate (0-10 mM) in RPMI 1640 media for the final 6, 12, 24 or 48 h of fatty acid pre-treatment.

Quantification of cytosolic Ca^{2+} in mouse colonocytes. Following fatty acid and butyrate treatment, cells were washed with Leibovitz media, free of serum and phenol red. Cells were loaded with 3 μM Fluo-4, AM, at 33°C to determine the levels of

cytosolic Ca^{2+}. Fluo-4 is a visible wavelength non-ratiometric cytosolic Ca^{2+} indicator that exhibits a 40-fold increase in fluorescence intensity with Ca^{2+} binding (167, 168). Following a 1 h incubation with the probe, cells were washed with Leibovitz media and imaged. Images were acquired from groups of 10 – 20 cells, 10-12 areas per well, 4-8 wells per treatment using a Stallion Digital Imaging workstation equipped with 300W xenon fluorescent light source. Fluorescent light was rapidly (<2 msec) switched between excitation wavelengths. Images were captured using a 20X objective 0.75 N.A. and a ROPER CoolSnap HQ camera. For image acquisition, imaging parameters were adjusted for maximum detection of fluorescence with minimal cellular photo bleaching. Cells were excited at 488 nm and fluorescence emission from individual cells was collected at 530 nm. Image acquisition frequency was set to 1 image every 10 sec. Fluorescence intensities were recorded for 300 sec. A minimum of 2 experiments performed on different days were analyzed. Basal intracellular Ca^{2+} was measured prior to the addition of thapsigargin. Thapsigargin, is an irreversible SERCA pump inhibitor which was used to empty the intracellular Ca^{2+} store from the ER (168). Fluorescence intensities following 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 mM EGTA followed by cell stimulation with thapsigargin. Chelation of extracellular Ca^{2+} facilitates quantification of IP_3 Ca^{2+} released from the ER following thapsigargin addition (169, 170). Ca^{2+} was then added back into the media and the contribution of the SOC pool was evaluated (168). In order to further evaluate the association between PUFA and butyrate co-treatment and SOC, cultures were pre-

incubated with 10 μM SKF-96365, a pharmacological inhibitor of SOC, for 5 min prior to image acquisition. Calibration of intracellular Ca^{2+} was performed using the Calcium Calibration Buffer kit #2 from Molecular Probes as previously described (171).

Analysis of mitochondrial Ca^{2+}. Cells treated with fatty acid and butyrate were washed with Leibovitz media and co-loaded with 3 μM Fluo-4 and 2 μM Rhod-2 for 1 h at 33°C. Cells were then washed twice with Leibovitz media 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 nm and 550 nm and fluorescence emissions were collected at 530 nm 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 (172). In other experiments, cells were incubated with RU-360 (10 μM), an inhibitor of the mitochondrial uniporter for 30 min prior to butyrate co-treatment (173). Cells were then washed and co-loaded with fatty acid and butyrate for the final 6, 12 or 24 h following 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 enzyme linked immuno-sorbent (ELISA) (Roche) and caspase-3 activity (Molecular probes) assays as previously described (83,174). To determine the association between mitochondrial Ca^{2+} and apoptosis, select cultures were pre-incubated with RU-360 (10

μM) for 30 min prior to butyrate exposure. Cells were washed and treated with 5 mM butyrate and apoptosis was measured following 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 less than 0.05 were considered to be statistically significant.

Results

DHA and butyrate combination decreases cytosolic Ca^{2+}. Cytosolic Ca^{2+} levels were initially examined following stimulation with thapsigargin, an irreversible SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase) pump inhibitor used to empty ER Ca^{2+} stores. **Figure 3A** shows representative traces of thapsigargin-induced Ca^{2+} response measured over 300 secs. The arrow indicates the time point of thapsigargin addition. Butyrate treatment (5 mM) over a period of 6 - 24 h increased ($p<0.05$) cytosolic Ca^{2+} in cells preincubated with LA (50 μM, a control n-6 fatty acid) by up to 45% compared to untreated control (**Figures 3B, C and D**). In contrast, cells pre-incubated with DHA and butyrate consistently exhibited a lower cytosolic Ca^{2+} level compared to LA and butyrate co-treatment.

Control cultures containing either DHA or LA alone, in the absence of butyrate, exhibited a modest increase in cytosolic Ca^{2+} levels, 8 - 12%, compared to untreated control (**Inset Figures 3C and D**). In comparison, cultures treated with LA and butyrate exhibited a 45% increase compared to untreated control at both time points. Changes in Ca^{2+} levels were detected as early as 6 h following butyrate and fatty acid co-treatment.

Store operated Ca^{2+} channel (SOC) involvement in maintaining intracellular Ca^{2+} homeostasis. To examine the role of store-operated Ca^{2+} 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 utilizes 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+} (30, 31). Consistent with previous experiments (**Figure 3**) following incubation with 5 μM 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 to LA primed cells ($p<0.05$) (**Figure 4**).

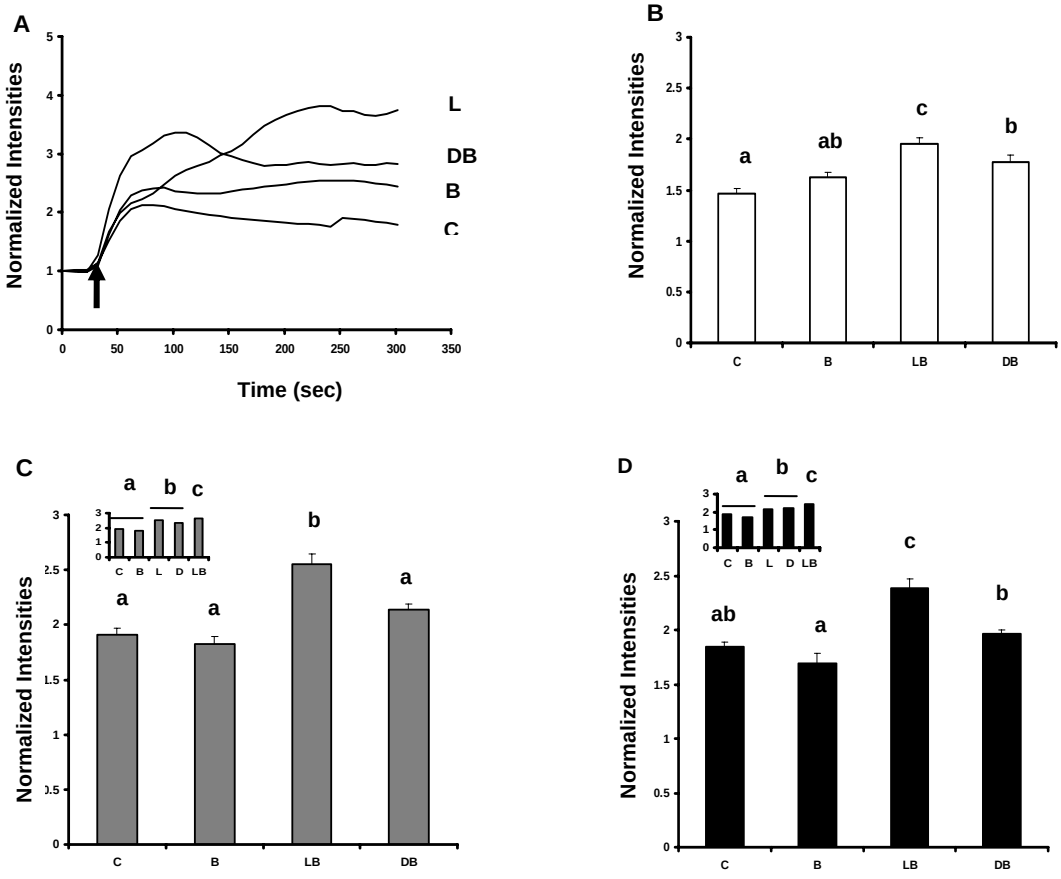

Figure 3. **Effect of fatty acid and butyrate on thapsigargin-induced Ca^{2+} response**. YAMC cells were treated with fatty acid alone (50 μM) for 72 h with butyrate (5 mM) for the final 6 (panel B); 12 (panel C); or 24 h (panel D). Cells were subsequently incubated with Fluo-4 (3 μM), a fluorescent 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 panel A. Arrow indicates point of thapsigargin (5 μM) 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 3-4 fields with 10-12 cells per field. Representative data are from a single experiment, average of 80-120 cells per treatment, n = 4-6 independent experiments. Mean values ± S.E. 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 to LA pretreated cells demonstrated a significant decrease in cytosolic Ca^{2+} levels with 12 and 24 h of butyrate co-treatment. Inset: Cytosolic Ca^{2+} levels measured in untreated, butyrate only, fatty acid only and LB combination cells at 12 and 24 h are shown in the inset of panels C and D, respectively. Representative data are from a single experiment, average of 80-120 cells per treatment, n = 2-4 independent experiments. Mean values ± S.E. not sharing common letters are significantly different, $p < 0.05$. DHA treated cells exhibited no significant difference in cytosolic Ca^{2+} levels when compared to LA treated cells.

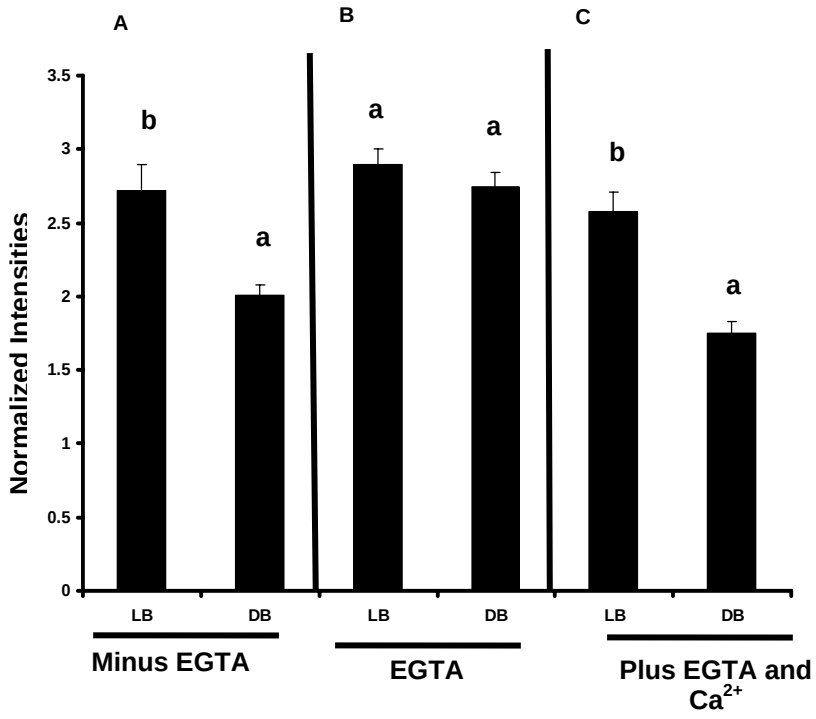

Figure 4. **Effect of fatty acid on thapsigargin-induced Ca^{2+} response in the presence or absence of EGTA**. YAMC cells were treated with fatty acid (50 μM) for 72 h with or without butyrate (5 mM) for the final 24 h. Panel A, Cells were incubated with Fluo-4 (3 μM) for 1 h at 33°C and basal Ca^{2+} was measured. Panel B, Extracellular Ca^{2+} was chelated using 2 mM EGTA and cells were stimulated with thapsigargin (5 μM). Panel C, $CaCl_2$ (10 mM) was added back to the extracellular media and cytosolic Ca^{2+} was quantified. Mean values ± S.E. not sharing common letters are significantly different, $p<0.05$. Representative data are from a single experiment, $n = 2$ independent experiments. LB, LA and butyrate; DB, DHA and butyrate. 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 media with Ca^{2+} reestablished the difference. Taken together, these data demonstrate that plasma membrane channel entry contributed to the difference seen in cytosolic Ca^{2+} level.

Subsequently, extracellular Ca^{2+} was chelated by addition of 2 mM 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 ($p>0.05$) (**Figure 4**). Following replacement of the medium with $CaCl_2$ (10 mM), 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 following ER Ca^{2+} depletion in colonocytes, cultures were incubated with SKF-96365, an inhibitor of non-voltage gated, SOC channels (175, 176). Typically, butyrate treated cells primed with DHA or LA were pre- incubated with 10 μM SKF for 5 min prior to basal recording. SKF abrogated the fatty acid-induced effect on cytosolic Ca^{2+} levels following 12 h butyrate co-treatment (**Figure 5A)**. Similar effects were observed following prolonged butyrate treatment (24 h) **(Figure 5B**). Thus, the activation of store-operated channels, 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.

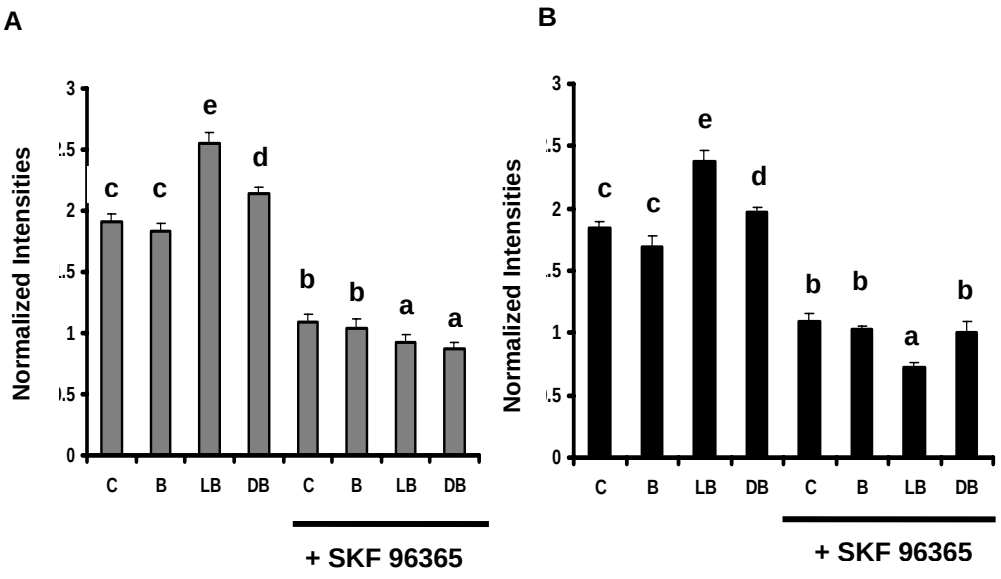

Figure 5. **Thapsigargin-induced Ca^{2+} response in the presence or absence of SOC inhibitor, SKF-96365.** YAMC cells were treated with 50 μM fatty acid for 72 h with or without 5 mM butyrate for the final 12 h (Panel A) and 24 h (Panel B), followed by Fluo-4 (3 μM) incubation for 1 h. Select cultures were pre-incubated with SKF 96365 (10 μM) for 5 min prior to measuring basal Ca^{2+} levels. Cells were stimulated with thapsigargin (5 μM) after 15 sec of basal Ca^{2+} quantification. C, control- no fatty acid or butyrate; B, butyrate only; LB, LA and butyrate; DB, DHA and butyrate. Data are means ± S.E. from a representative experiment, $n = 2$ independent experiments. SKF-96365 significantly blocked SOC entry indicating that SOC are major contributory plasma membrane channels.

Effect of DHA and butyrate co-treatment on mitochondrial Ca^{2+} levels. Since 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 co-treatment on mitochondrial Ca^{2+} uptake. Butyrate co-treatment 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 (**Figure 6A**). In addition, at 12 and 24 h, DHA and butyrate selectively increased ($p<0.0001$) mitochondrial Ca^{2+} by 18 and 37%, respectively (**Figures 6B** 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 to 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 (**Inset, Figures 6B and C**). However, in all cases, cultures pre-treated with DHA and co-incubated with butyrate demonstrated the largest increase in mitochondrial Ca^{2+} levels (**Figure 6**). Representative photomicrographs of untreated cells and cultures co-incubated with butyrate (5 mM for 24 h) and DHA or LA (50 μM) are shown in **Figure 6**, panels D-F.

Effects of a mitochondrial uniporter inhibitor on mitochondrial Ca^{2+} uptake following DHA and butyrate co-treatment-- To investigate the role of the mitochondrial uniporter in Ca^{2+} uptake, cells were treated with RU-360, a mitochondrial Ca^{2+} uniporter inhibitor (131). RU-360 (10 μM) partially inhibited ($p<0.001$) the butyrate-induced increase in mitochondrial Ca^{2+} in DHA primed cells upon addition 30 min prior to butyrate co-treatment at 6 or 12 h (**Figure 7**). In comparison, RU-360 had no effect on mitochondrial Ca^{2+} levels in LA treated cells.

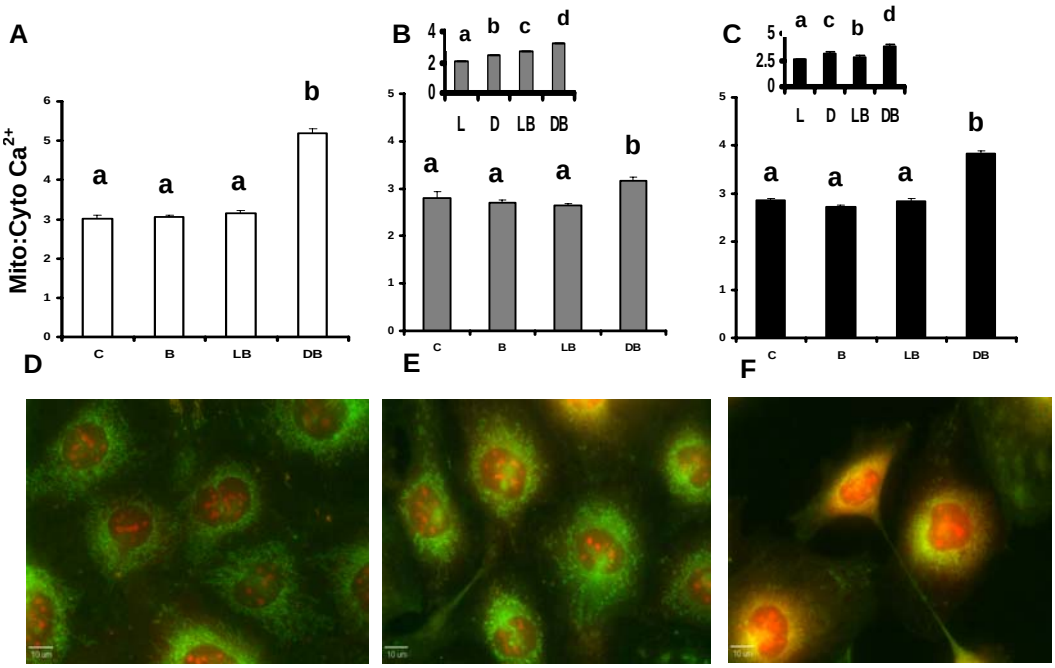

Figure 6. Effect of fatty acid and butyrate co-treatment on mitochondrial Ca^{2+} levels inYAMC cells. YAMC cells were exposed to 50 μM fatty acid for 72 h in the absence or presence of 5 mM butyrate for the final 6 (panel A), 12 (panel B) or 24 h (panel C). Cells were co-loaded with Fluo-4 (3 μM) and Rhod-2 AM (2 μM), and the ratio of mitochondrial-to-cytosolic Ca^{2+} was evaluated as described in the Materials and Methods. Data are means ± S.E. from a representative experiment, $n = 3$ independent experiments. Panels D, E and F are 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. C, control- no fatty acid or butyrate; B, butyrate only; L, LA only; D, DHA only; LB, LA and butyrate; DB, DHA and butyrate. Inset: Represents the effects of fatty acid treatment with or without butyrate on mitochondrial Ca^{2+} levels at 12 (Panel B) and 24 h (Panel C), respectively. Data are means ± S.E. from a representative experiment, $n = 2$-3 independent experiments. Bars not sharing common letters are significantly different, $p < 0.05$. C, control- no fatty acid or butyrate; L, LA only; D, DHA only; LB, LA and butyrate; DB, DHA and butyrate co-treatment.

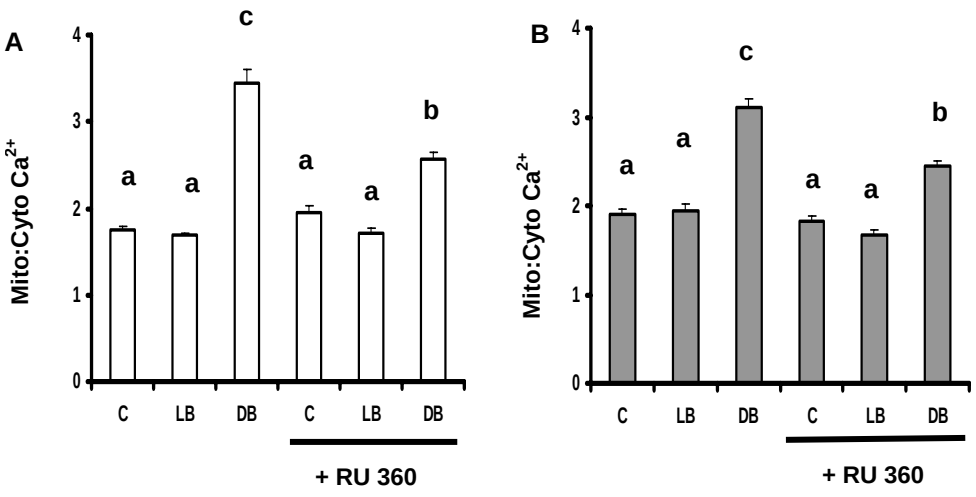

Figure 7. **Effect of RU-360 on mitochondrial Ca^{2+} uptake following fatty acid and butyrate co-treatment**. Cells were pretreated with fatty acid for a total of 72 h and RU-360 (10 μM) for 30 min prior to butyrate co-treatment for the last 6 h (Panel A) and 12 h (Panel B), respectively. Cells were co-incubated with Fluo-4 (3 μM) and Rhod-2 (2 μM) for 1 h and the mitochondrial-to-cytosolic Ca^{2+} ratio was measured. Data are means ± S.E. from a representative experiment, n = 2 independent experiments. C, control- no fatty acid or butyrate; B, butyrate only; L, LA only; D, DHA only; LB, LA and butyrate; DB, DHA and butyrate. Data obtained demonstrate that in DHA and butyrate treated cells, RU-360 partially blocked mitochondrial Ca^{2+} accumulation.

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 to cells treated with LA plus butyrate or butyrate alone (control) at 12 and 24 h **(Figure 8**). In contrast, fatty acid treatment alone had no effect on apoptosis **(Figure 8C**, inset, (**Figure 9A and B**). To further corroborate these observations, complimentary methodology (caspase-3 activity) was also used. Similar results were obtained **(Figure 8D**). For comparative purposes, staurosporine, a broad spectrum protein kinase inhibitor, which induces apoptosis in normal and malignant cells, was used as a positive control (177). As expected, cultures pre-incubated with 1

μM staurosporine for 4 h exhibited an 8-fold increase in apoptotic cells as compared to untreated or fatty acid treated cells ($p<0.0001$) (**Figure 8C**, inset).

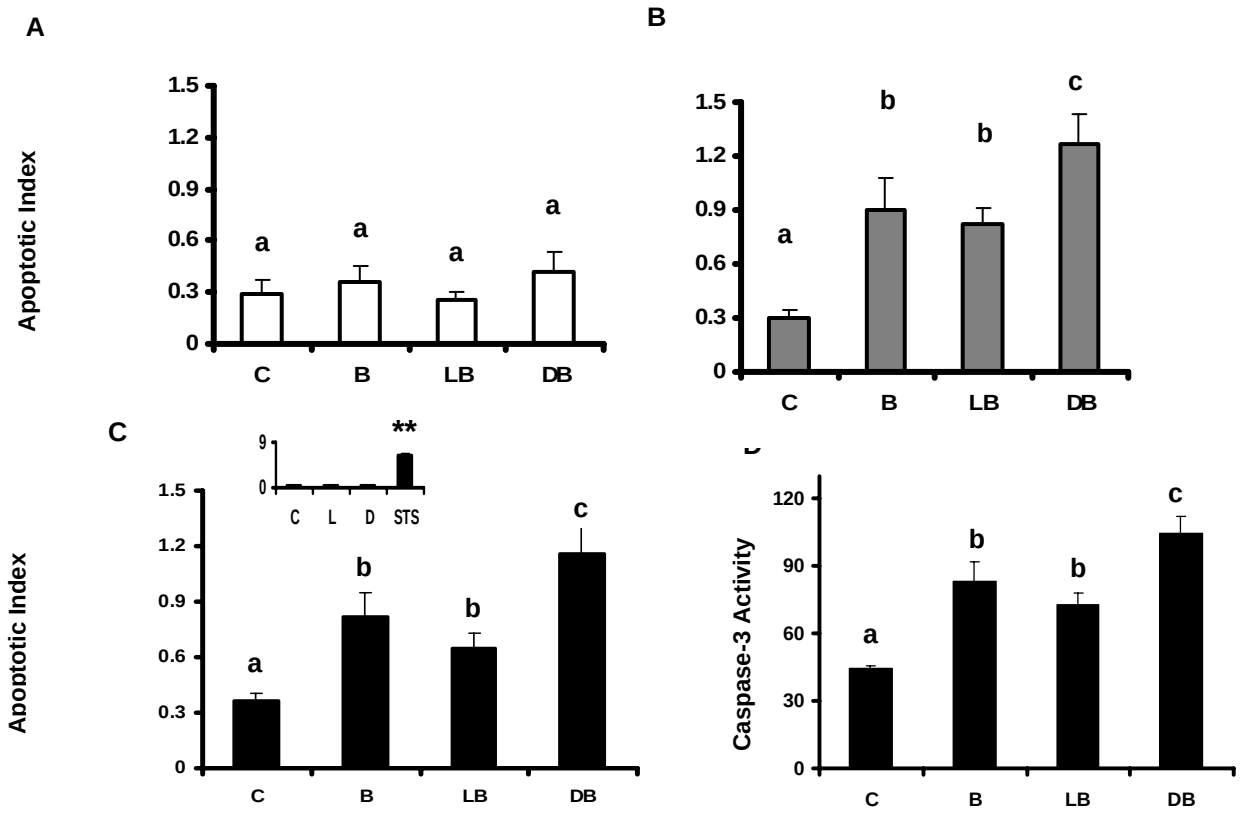

Figure 8. Effect of fatty acid and butyrate treatment on apoptotic index and caspase-3 activity in YAMC cells. YAMC cells were treated with fatty acid (50 μM) for a total of 72 h and 0 or 5 mM butyrate for the final 6 (Panel A), 12 (Panel B) or 24 h (Panels C and D). Nonadherent cells were harvested, and apoptosis was measured by the nucleosomal fragmentation release assay (Panels A, B and C) or both adherent and floating cells were processed and caspase-3 activity was measured as a marker of apoptosis (Panel D). Inset: Control cultures containing no treatment (C), LA only (L), DHA only (D) and 1 μM staurosporine (STS) 4 h prior to the addition of butyrate. Data are means ± S.E. from 2 separate experiments, $n = 6$ wells per treatment. Refer to figure 6 for legend details. Data show that the combination of DHA and butyrate significantly enhanced apoptosis as compared to LA and butyrate or no treatment control.

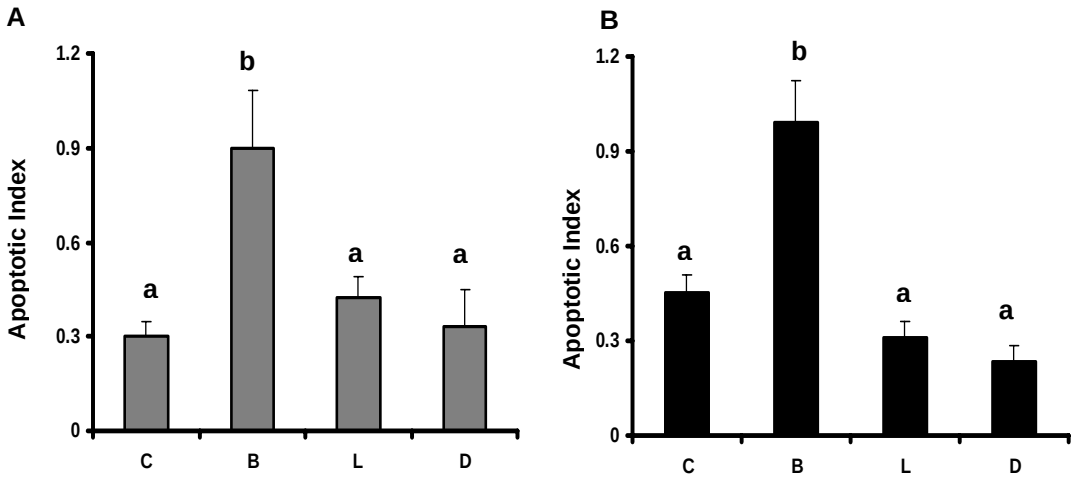

Figure 9. **Effect of fatty acid controls on apoptosis in YAMC cells.** YAMC cells were treated with fatty acid (50 μM) for a total of 72 h or 0 or 5 mM butyrate for 12 (Panel A), or 24 h (Panel B). Nonadherent cells were harvested, and apoptosis was measured by the nucleosomal fragmentation assay. Data are means ± S.E. from 2-4 separate experiments, *n* = 10 wells per treatment. C, control- no fatty acid or butyrate; B, butyrate only; L, LA only; D, DHA only.

The mitochondrial Ca^{2+} uniporter inhibitor, RU-360, suppresses induction of apoptosis following DHA and butyrate co-treatment. 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 following DHA or LA co-treatment are shown in **Figure 10**. RU-360 significantly ($p<0.05$) reduced apoptosis by ~ 45% following 12 h butyrate co-treatment. Similar results were observed in DHA primed cells following 24 h butyrate co-treatment. 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

demonstrate that mitochondrial Ca^{2+} uptake is required for the enhanced apoptosis associated with DHA and butyrate co-treatment.

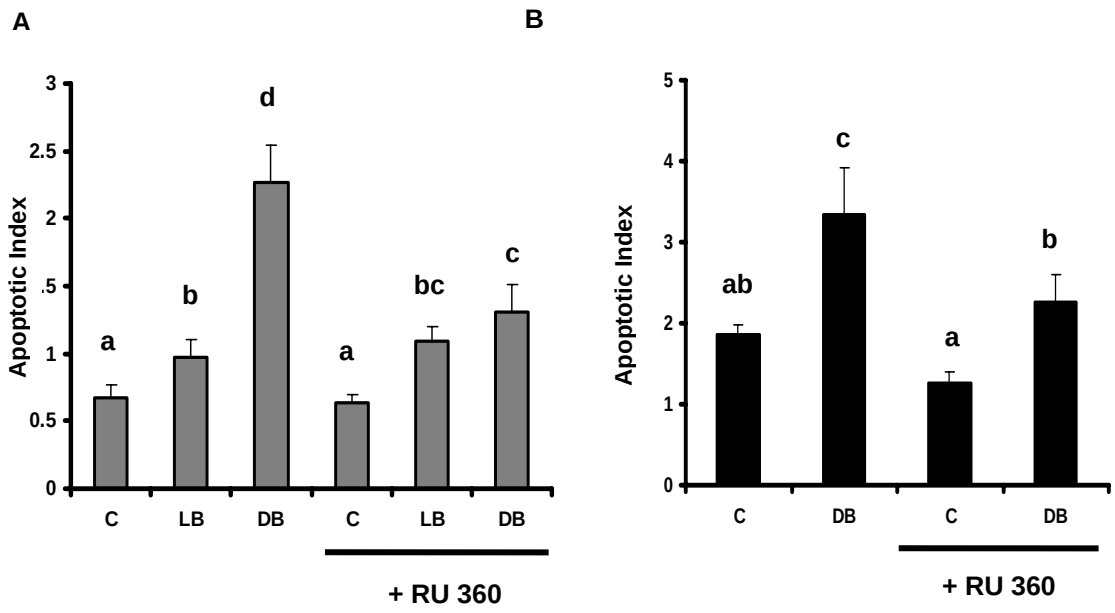

Figure 10. **Effect of RU-360 on apoptotic index in YAMC cells**. YAMC cells were treated with DHA or LA (50 μM) for 48 h and subsequently pre-incubated with 10 μM RU-360 for 30 min. Treated cells were then co-incubated with fatty acid with or without 5 mM butyrate for the final 12 h (Panel A) and 24 h (Panel B). Nonadherent cells were harvested, and nucleosomal fragmentation was used to quantify apoptosis. Data are means ± S.E. from 2 separate experiments, $n = 6$ wells per treatment. C, control- no fatty acid or butyrate; LB, LA and butyrate; DB, DHA and butyrate. Data demonstrate that RU-360 significantly blocked apoptosis with cells pretreated with the combination of DHA and butyrate.

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 **Figure 11**, cells treated with butyrate and DHA exhibited a significantly ($p<0.0001$) higher level of mitochondrial Ca^{2+} compared to 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 demonstrated that DHA, when combined with either a fermentable fiber source or butyrate, enhances ROS and LOOH production, and causes a change in mitochondrial permeability transition (MPT) in colonocytes (56-58, 47). Herein, we report that DHA and butyrate co-treatment also synergistically enhance apoptosis by up to 43% in colonocyte cultures compared to butyrate alone (**Figure 8**). In contrast, colonocytes treated with either DHA or LA alone, demonstrated no significant increase in the level of apoptosis compared to untreated cells (**Figure 9**). From a biological relevance perspective, these data are consonant with animal carcinogen studies demonstrating that the bioactive components of fermentable fiber (butyrate) and fish oil (DHA) coordinately protect against colon tumorigenesis, primarily by increasing apoptosis (14, 16, 45, 52). Further, our studies reveal that the combination of DHA and butyrate, compared to 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 (**Figure 12**).

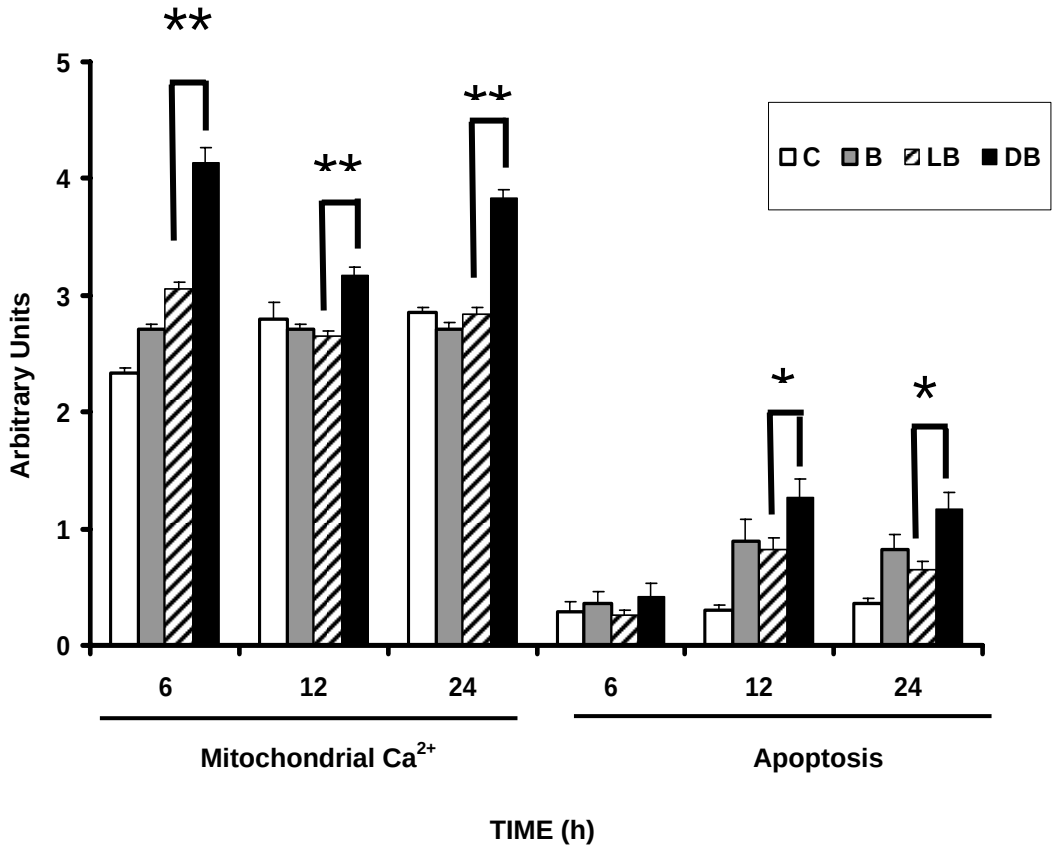

Figure 11. Effect of DHA and butyrate treatment on induction of mitochondrial Ca^{2+} and apoptosis in YAMC cells. The time-dependent effect of fatty acid (72 h) and butyrate co-treatment (6, 12, 24 h) were compared. Induction of mitochondrial Ca^{2+} and apoptosis were measured as described in the Materials and Methods. Data are expressed as a percentage of control (no treatment), mean ± S.E. from $n = 3$ separate experiments; (*, Apoptotic index, control vs treatment at each time point $p < 0.05$; **, Mitochondrial Ca^{2+}, control vs treatment at each time point, $p < 0.0001$).

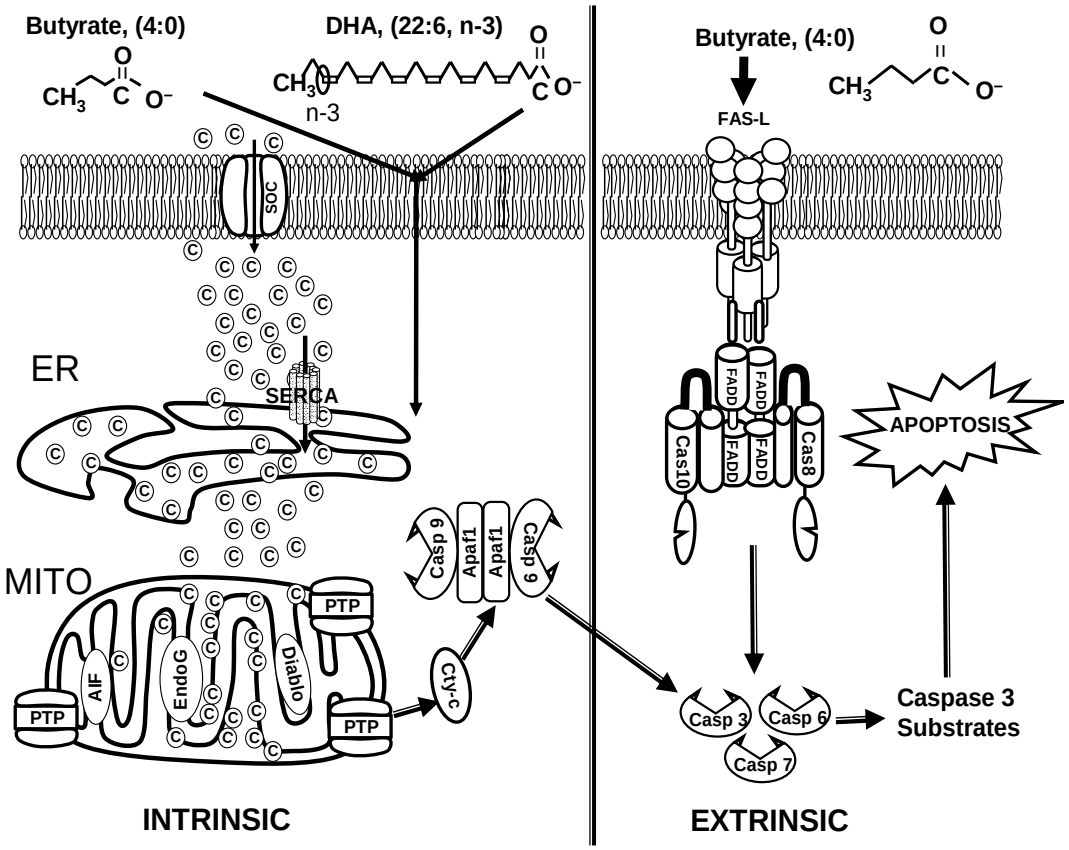

Figure 12. Proposed molecular model of DHA and butyrate-induced apoptosis. Butyrate induces colonocyte apoptosis via a non-mitochondrial, Fas-mediated, extrinsic pathway. DHA and butyrate, in combination, synergistically perturb intracellular Ca^{2+}, stimulating mitochondrial Ca^{2+} uptake. This directly 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 pro-apoptotic 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 pro-caspases and downstream caspases that execute cellular apoptosis.

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+} (66, 103, 177-179). With respect to a molecular mechanism of action, agents which increase mitochondrial ROS/LOOH generation have been linked to a pro-apoptotic cycle involving Ca^{2+} release

from intracellular stores and mitochondrial loading (180). These data suggest that a change in mitochondrial Ca^{2+} homeostasis may mediate the pro-apoptotic 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 (**Figure 6**) while simultaneously decreasing free cytosolic Ca^{2+} levels (**Figures 3C** and **D**). These data are consistent with our previous finding that DHA primes the cell for butyrate induced lipid oxidation (58).

The role of mitochondria in the regulation of intracellular Ca^{2+} homeostasis and apoptotic signaling is well established (87, 96, 120). 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 (116, 181). 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 (97, 117). Consistent with these observations, DHA treated cultures exhibited a significant accumulation of mitochondrial Ca^{2+} within 6 h of butyrate co-treatment (**Figures 6A**), preceding the onset of apoptosis which was not increased until 12 h following butyrate co-treatment (**Figure 8B** and **Figure 11**).

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 following DHA and butyrate co-treatment (**Figure 7**) and partially blocked the induction of apoptosis (**Figure 10**).

These outcomes provide evidence that an increase in mitochondrial Ca^{2+} levels contribute directly to the induction of apoptosis by DHA and butyrate co-treatment 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 demonstrated that multi-factorial 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 (133, 182). There is also evidence that mitochondrial Ca^{2+} sequestration (via the mitochondrial uniporter) results in the opening of the mitochondrial permeability transition (MPT) pore which can induce the release of pro-apoptotic molecules, i.e., Bax, Bak, cytochrome-C, resulting in mitochondrial-mediated (intrinsic) apoptosis (181, 132). This scenario correlates well with our finding of a decrease in colonic mitochondrial membrane potential in fish oil but not corn oil fed rats (56, 57).

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 (**Figure 4**). Following replenishment of extracellular Ca^{2+}, the difference in cytosolic Ca^{2+} between DHA and butyrate versus control (LA and butyrate)

treatment groups was re-established. 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 pre-treatment 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 where DHA was found to regulate intracellular signaling by modulating plasma membrane Ca^{2+} entry (183), 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 MPT pore is activated by both oxidative stress and Ca^{2+}, while mitochondrial ROS inhibit SERCA pumps (138) and activate SOC (137, 139). In accordance with these findings, we have recently demonstrated in HCT-116 cells that a mitochondrion-specific antioxidant blocks the DHA and butyrate-induced induction of apoptosis (manuscript in preparation). Further studies are needed in order to determine if these effects extend to other members of the n-3 PUFA family, since a number of studies have shown that EPA and DHA have similar effects on biological membranes (43, 56, 57).

We have previously demonstrated that butyrate induces colonocyte apoptosis via a Fas receptor-mediated *extrinsic* pathway (83). In an extension of our findings, we demonstrate for the first time that the combination of DHA and butyrate, compared to 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) appears to reduce tumor formation in the colon by promoting apoptosis (16, 14, 57, and 47). Although previous studies have examined the effects of PUFA to mobilize intracellular Ca^{2+} (183, 184, 185), it is apparent that DHA and butyrate work coordinately in the colon to initiate a pro-apoptotic cycle involving the activation of SOC, leading to rapid entry of Ca^{2+} through the plasma membrane and mitochondrial Ca^{2+} loading (**Figure12**). With regard to putative upstream mediators, we have shown that a DHA - butyrate combination dissipates mitochondrial membrane potential, an effect which was reversed by coincubation with permeability transition pore (PTP) inhibitors (58). This is consistent with the fact that mitochondrial Ca^{2+} interacts with cyclophilin D which can trigger opening of the permeability pore (128). Although the precise chronology of these events have not been elucidated, it is likely that PTP 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 which 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 pro-apoptotic 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.

CHAPTER III

SYNERGY BETWEEN DOCOSAHEXAENOIC ACID AND BUTYRATE ELICITS p53-INDEPENDENT APOPTOSIS VIA MITOCHONDRIAL CA^{2+} ACCUMULATION IN HUMAN COLON CANCER CELLS AND IN RAT PRIMARY COLONOCYTE CULTURE

The incidence of colon cancer is increasing among people who migrate from low incidence to high incidence regions (2). This variation in geographical incidence of colon cancer indicates that environmental factors, especially modifiable factors like diet and physical activity play a major role in disease onset (1, 2). Fat and fiber are two of the most widely investigated dietary components in the prevention of colon carcinogenesis. Currently, there is a plethora of experimental, epidemiological and clinical evidence indicating that diets rich in n-3 polyunsaturated fatty acids (PUFAs), e.g. docosahexaenoic acid (DHA, $22:6^{\Delta 4,7,10,13,16,19}$) and eicosapentaenoic acid (EPA, $20:5^{\Delta 5,8,11,14,17}$) enriched in fish oil are protective against colon tumorigenesis (16, 38, 39, 44, 46, 50, 54, 55, 66, 186, 36 *Fernandez, 1999;Reddy, 1991;*). In contrast, dietary lipids rich in n-6 PUFA (found primarily in vegetable oils), e.g., linoleic acid (LA, $18:2^{\Delta 9,12}$) and arachidonic acid (AA, $20:4^{\Delta 5,8,11,14}$), enhance the development of colon tumors (50, 76, 187, 188, *Reddy, 1991*). Importantly, the typical Western diet contains 10 to 20 times more n-6 than n-3 PUFA (189). There is also evidence that the consumption of fiber, a luminal source of butyrate, is chemoprotective against colorectal cancers (65, 73). Although there is still debate challenging the premise that dietary fiber

reduces colon cancer risk (36, 164, 166), we have demonstrated that the chemoprotective/proapoptotic effect of n-3 PUFA is enhanced when a highly fermentable fiber, pectin (or its fermentation product-butyrate) is added to the diet (29, 47, 58, 50, 181). This is significant because the transformation of colonic epithelium to carcinoma is in part associated with a progressive inhibition of apoptosis (49, 50, 51, 161). Hence, chemotherapeutic agents which restore the normal apoptotic pathways have the potential for effectively treating cancers that depend on aberrations of the apoptotic pathway to stay alive (190). Along these lines, we have demonstrated that measurements of apoptosis have greater prognostic value to detect dietary effects on colon tumor incidence than do measurements of cell proliferation (50, 52).

With regard to mechanisms of action, we have recently demonstrated that dietary DHA is incorporated into mitochondrial membrane phospholipids, thereby enhancing oxidative stress induced by butyrate metabolism (47, 56, 57). We subsequently identified a pathway for the induction of apoptosis in colonic epithelium involving the synergistic enhancement of mitochondrial phospholipid hydroperoxides (PLOOH) by these two bioactive molecules (58). Our results indicate that the effects of individual chemoprotective nutrients (n-3 PUFA and butyrate) may not be as important as the nutritional combination. Indeed, this may explain why a diet containing highly fermentable fiber is only protective when fish oil is the lipid source (29, 50).

Ca^{2+} is one of the most versatile universal signaling mediators of cellular apoptosis. Cumulative evidence indicates a central role for mitochondrial Ca^{2+} accumulation in intrinsically-mediated apoptosis (113, 130, 131, 120). Although the

importance of the endoplasmic reticulum (ER) as the major storage organelle is indisputable, it has been known for some time that mitochondrial sequestration of large amounts of Ca^{2+} contributes to cell death via apoptosis or necrosis (87, 90, 96, 113, 120). Several lines of recent evidence suggest that apoptosis is induced in response to alterations in intracellular Ca^{2+} compartmentalization and enhanced mitochondrial Ca^{2+} accumulation (120, 130, 131, 159). The mitochondrial uniporter, a Ca^{2+} selective ion channel present on the outer mitochondrial membrane, mediates rapid mitochondrial Ca^{2+} uptake of the Ca^{2+} following release from ER stores (125, 126). Furthermore, activation of the uniporter associated with a rise in mitochondrial Ca^{2+} stimulates the generation of reactive oxygen species (ROS) and free fatty acids, which promote the opening of the permeability transition pore (PTP) (129, 120). Opening of the PTP causes dissipation of the mitochondrial membrane potential and eventually the release of Ca^{2+}. However, under certain circumstances, mitochondrial Ca^{2+} accumulation acts as a trigger for release of pro-apoptotic molecules from the mitochondria that leads to execution of the cells (98, 119).

With accumulating evidence favoring a central role for mitochondrial Ca^{2+} accumulation in cellular apoptosis, we recently determined the effect of DHA and butyrate on mitochondrial Ca^{2+} levels. Specifically, we demonstrated using young adult mouse colonocytes (YAMC), that DHA and butyrate combination synergistically alters intracellular Ca^{2+} compartmentalization by enhancing mitochondrial Ca^{2+} accumulation through a store-operated channel (SOC) -mediated mechanism (63*).* In this study, our primary objective was to corroborate these novel findings utilizing human colonocyte

cell lines. In addition, since mitochondria and the p53 tumor suppressor gene play a critical role in damage-induced apoptosis (161, 191), we postulated that the combination of DHA and butyrate significantly enhances mitochondrial Ca^{2+} accumulation and apoptosis in a p53-dependent fashion. To address this hypothesis, we used isogenic p53 wild type and deficient human colon tumor (HCT 116) cell lines to determine the effect of DHA and butyrate on mitochondrial Ca^{2+} levels. Findings from this study demonstrate that an increase in mitochondrial Ca^{2+} accumulation directly contributes to a p53-independent induction of apoptosis by DHA and butyrate. In order to address the in vivo relevance of these observations, parallel experiments were also conducted using intact crypts isolated from rats fed fish oil (contains DHA) or corn oil (contains no DHA, control). Data from these experiments recapitulated observations in the human cell lines, providing conclusive evidence that DHA and butyrate enhance mitochondrial Ca^{2+} accumulation, culminating in the induction of apoptosis.

Experimental procedures

Materials. McCoy's 5A media, GlutaMAX and Leibovitz media were purchased from GIBCO BRL (Grand Island, NY). Hanks' balanced salt solution (HBSS) was from Mediatech (Herndon, VA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Fatty acids were obtained from NuChek Prep (Elysian, MN). Fluo-4 AM, Rhod-2 AM, Mitotracker green FM and LIVE/DEAD Viability/Cytotoxicity Kit were purchased from Molecular Probes (Eugene, OR). RU-360 was purchased from Calbiochem (San Deigo, CA). MitoQ, a mitochondrion targeted antioxidant was a

generous gift from Dr. Michael Murphy, Medical Research Council Dunn Human Nutrition Unit, Cambridge, UK. Cell death detection ELISA kit was obtained from Roche Applied Science (Indianapolis, IN). 2-well Lab-Tek Chambered Coverglass slides were purchased from Nunc, Inc. (Naperville, IL). Pre-cast 4-20% Tris-glycine gels were from Invitrogen (Carlsbad, CA). Electroblotting poly-vinyldiene-difluoride (PVDF) membranes were obtained from Millipore (Burlington, MA). Super Signal West Femto Maximum Sensitivity Substrate was from Pierce (Rockford, IL). Primary rabbit anti-p53 antibody was obtained from Calbiochem (San Deigo, CA) and peroxidase-conjugated goat anti-rabbit IgG secondary antibody was purchased from Kirkegaard & Perry (Gaithersburg, MD). Sodium butyrate, EDTA and all other reagents were obtained from Sigma (St. Louis, MO).

Stock solutions of 1.0 mM Fluo-4, AM and 4.0 mM Rhod-2 were prepared in DMSO and diluted with medium to 3.0 μM and 2.0 μM, respectively (final concentration of the vehicle DMSO was maintained at 0.1-0.3% in culture). 1 mM stock RU-360 was prepared in degassed water and diluted to a final concentration of 10 μM for cell treatment. 50 mM MitoQ stock was prepared in sterile water and a final concentration of 5 μM was used for cell culture.

Cell culture. Isogenic cell lines p53 wild type (p53+/+) and p53 deficient (p53-/-) were obtained from Dr. Bert Vogelstein, Johns Hopkins University, MD. HCT 116 cells were cultured in McCoy's 5A media supplemented with 10% FBS and 1% - 200 mM GlutaMAX. Cells were cultured at 37°C in 5% CO_2. For all fluorescence measurements, p53+/+ (passages 3-8) and p53-/- (passages 15-19) cells were seeded

onto borosilicate 2-chambered cover glass slides at a density of 1.2-1.5 x 10^5 for 72 h to achieve a 50-70% confluence. For apoptosis assays, cells were seeded onto 35 mm cell culture dishes or 6 well plates at a density of 3.5 x10^4. Bovine serum albumin (BSA) complexed fatty acids were added to cultures 24 h after cell plating as previously described (83). Select cultures were treated with BSA-complexed DHA (22:6, n-3) or LA (18:2, n-6) (50 μM) for 72 h. Cells were co-incubated with sodium butyrate (0 - 5 mM) in McCoy's 5A media for the final 6, 12 or 24 h of fatty acid pre-treatment.

Analysis of mitochondrial Ca^{2+}. Cells treated with fatty acid and butyrate were washed with Leibovitz media and co-loaded with 3 μM Fluo-4 and 2 μM Rhod-2 for 1 h at 37°C. For quantification of Fluo-4 and Rhod-2 fluorescence, excitation light was provided at 488 nm and 550 nm and fluorescence emissions were collected at 530 nm and 580 nm, respectively. 10- 15 areas with 20-25 cells per area were imaged and the ratio of mitochondrial-to- cytosolic Ca^{2+} level was calculated. Cells or crypts were analyzed with a Stallion Digital Imaging workstation equipped with 300W xenon fluorescent light source with rapid switching (<2msec) between excitation wavelengths. Images were collected using a 20X objective 0.75 N.A. and a ROPER CoolSnap HQ camera. In select experiments, MitoTracker Green FM was used to confirm the subcellular localization of Rhod-2 fluorescence (172). MitoTracker Green is a green-fluorescent mitochondrial stain which localizes in the mitochondria regardless of mitochondrial membrane potential. This dye has an added advantage in that it is essentially non-fluorescent in aqueous solution, only becoming fluorescent when it accumulates in the lipid environment of the mitochondria. In addition, to reconfirm that

Rhod-2 fluorescence corresponded to mitochondrial Ca^{2+} accumulation, experiments were performed in cultures incubated with RU-360 (10 μM), an inhibitor of the mitochondrial uniporter, for 30 min prior to butyrate co-treatment (173). Cells were subsequently washed and treated with fatty acid and butyrate for the final 6, 12 or 24 h following which the mitochondrial-to-cytosolic Ca^{2+} ratio was determined.

Apoptosis assay. Apoptosis was measured using cellular fragmentation enzyme linked immuno-sorbent (ELISA) (Roche). Floating cells were harvested, washed, lysed and centrifuged to sediment nuclei. Supernatant containing mono and oligonucleosomes were incubated with substrate and subsequently analyzed by ELISA. Absorbance values were measured at 405 nm and subsequently normalized to the number of adherent cells per dish as previously described (83). To determine the association between mitochondrial Ca^{2+} and apoptosis, select cultures were pre-incubated with RU-360 (10 μM) for 30 min prior to butyrate exposure. Cells were washed and treated with 5 mM butyrate and apoptosis was measured following a 6, 12 or 24 h incubation period. To determine the involvement of oxidative stress in fatty acid and butyrate-induced apoptosis, select cultures were pre-incubated with 5 μM MitoQ for 1 h prior to butyrate treatment (58). Cells were then treated with fatty acid and 5 mM butyrate for an additional 12 h and apoptosis quantified.

Western blotting. Cell lysates from p53+/+ or p53 -/- cells were immunoblotted with p53 antibody using the method of Davidson et al. (192). Briefly, samples were treated with SDS sample buffer and subjected to electrophoresis in 4-20% pre-cast Tris-glycine mini-gels. After electrophoresis, proteins were electroblotted onto a PVDF

membrane using a Hoefer Mighty Small Transphor unit (Pharmacia, Piscataway, NJ) at 400 mA for 75 min. Following transfer, the membrane was incubated with rabbit anti-p53 antibody overnight at 4°C, followed by peroxidase labeled goat anti-rabbit IgG incubation for 1 h at room temperature. Bands were developed using Super Signal West Femto Maximum Sensitivity Substrate, and blots were scanned using a Bio-Rad Fluor-S Max MultiImager system (Hercules, CA).

Animals and diets. Animal use was approved by the University Animal Care Committee of Texas A&M University and conformed to NIH guidelines. Male weanling (28-d old) Sprague-Dawley rats (*n* = 60; Harlan Sprague Dawley) were housed individually in raised wire cages. The animals were maintained in a temperature- and humidity-controlled animal facility with a daily 12 h light and 12 h dark photocycle as described by Sanders et al (47). Rats were acclimated for 1 week prior to receiving semi-purified experimental diets. The animals were weighed before the start of the diet and were randomly assigned into 1 of 2 experimental diets (n=15 rats/diet) so that the mean initial body weights did not differ between the two diet groups. Rats were placed on experimental diets for 3 weeks ± 3 days. The experimental diet composition is described in **Tables 1** and **2**. The experimental diets differed only in the type of fat (corn oil or fish oil). The major differences among the fatty acid composition of the dietary lipid sources were significantly higher amounts of EPA (20:5n-3) and DHA (22:6n-3) in the fish oil compared to corn oil diet, and higher amounts of linoleic acid (LA, 18:2n-6) in the corn oil diet (48). Each diet contained 15% dietary fat by weight and 6% dietary fiber by weight. Cellulose, a poorly fermentable fiber, was chosen as the source of fiber.

The percentage of fat and fiber corresponded to 30% energy from fat and 30 g of fiber per day as in a human diet. The fish oil diet contained 3.5 g of corn oil/100 g diet to prevent essential fatty acid deficiency. Food and water were freely available to the animals at all times. To minimize fatty acid oxidation, diets were stored at –80°C and fresh food was provided every 24 h.

Isolation of colonic crypts. Following the feeding period, rats were killed by CO_2 asphyxiation and cervical dislocation. The colon was removed and the colonic crypts were isolated as described previously (193) with slight modification. Briefly, the colon was flushed with phosphate-buffered saline (PBS) to remove feces and incubated at 37° C in calcium and magnesium-free Hank's balanced salt solution (HBSS) containing 25 mM dithiothreitol, 0.1% fatty acid-free BSA, 1 mM glutamine and 30 mM EDTA in order to dislodge crypts from the colonic extracellular matrix. The incubation buffer was developed to minimize the generation of crypt fragments and to reduce mucin levels, thereby improving image background. Following a 15 min incubation period with gentle shaking, the mucosa was gently scraped with a rubber policeman and intact crypts were isolated. Subsequently, the crypt suspension was centrifuged at 100 *g* for 3 min, the supernatant discarded and the pellet gently resuspended in HBSS. The centrifugation step was repeated three times. Crypts were then resuspended in Leibovitz media and immediately analyzed. The time elapsed from animal termination to image capture did not exceed 70 min. The isolation time period was optimized based on preliminary experiments which demonstrated that crypt viability decreased after 70 min.

Table 1. Composition of Experimental Diets

Ingredient	Corn Oil	Fish Oil
Sucrose	32.0	32.0
Casein	20.0	20.0
Corn Starch	22.0	22.0
DL- Methionine	0.3	0.3
AIN-76 Salt Mix	3.5	3.5
AIN-76 Vitamin Mix	1.0	1.0
Choline Chloride	0.2	0.2
Cellulose	6.0	6.0
Corn Oil	15.0	3.5
Fish Oil	0.0	11.5
Total	**100**	**100**

Values are listed as weight percentages.

Table 2. Dietary fatty acid composition[1, 2]

Fatty acid	Corn Oil	Fish Oil
14:0	0.1	6.9
16:0	10.5	13.8
16:1 (n-7)	0.2	9.3
18:0	1.8	2.5
18:1 (n-9)	27.2	11.6
18:2 (n-6)	57.7	15.5
18:3 (n-3)	1.0	1.3
20:5 (n-3)	Trace[3]	8.4
22:5 (n-3)	Trace	1.3
22:6 (n-3)	Trace	8.2

[1] Values are listed as g/100 g fatty acid. Only the major fatty acids (>1 g/100 g) are listed.

[2] CO, diets contain 15% corn oil by weight; FO, 11.5% fish oil + 3.5% corn oil.

[3] Trace, amount (<0.1 g/100 g).

Measurement of mitochondrial to cytosolic Ca^{2+} ratio in primary culture. Isolated colonic crypts were incubated with or without 5 mM butyrate in Leibovitz media for 30 min at 37°C. Crypts were co-loaded with Fluo-4 and Rhod-2 for 30 min. Crypts were subsequently imaged and the ratio of mitochondrial to cytosolic Ca^{2+} was determined as described above. A total of 10-15 areas with intact crypts were imaged for each sample. This technique is well suited for studying significant differences between treatments because it minimizes the variability in dye loading and changes in the size or shape of the crypts.

Crypt cell viability was evaluated in each sample following Ca^{2+} measurement. For this purpose, crypt pellets were co-loaded with Ethidium homodimer-1 and calcein AM (Molecular Probes, OR). Live cells have ubiquitous intracellular esterase activity and convert non-fluorescent calcein AM to a highly fluorescent calcein which is retained inside the live cell to emit a uniform green signal. Ethidium homodimer-1 enters cells with a damaged plasma membrane and is excluded by the intact plasma membrane of viable cells (194). Therefore, Ethidium homodimer enters dead cells with a compromised plasma membrane and undergoes a 40-fold enhancement in fluorescence to produce a bright red fluorescence differentiating it from viable green cells.

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 less than 0.05 were considered to be statistically significant.

In primary culture experiments, to understand how the ratios of the mitochondrial-to-cytosolic Ca^{2+} level vary with diet treatments, linear mixed effect

model (195) were carried out using a procedure "nlme" within the statistical software R (196). A subject-level random effect was modeled to account for similarity of measurements collected from the same rat. The main fixed treatment effects considered in the model include oil (corn vs fish oil), fiber (w/ and w/o butyrate) and fat-fiber interactions. Further, ratios of the mitochondrial-to-cytosolic Ca^{2+} level were considered at the log-scale to reduce potential effects of outlying observations at the right tail of the data distribution. Several inferential statistical analyses within the mixed effects modeling framework were utilized to examine the effect of diet on the mitochondria-cytosol Ca^{2+} ratio.

Results

Effect of fatty acid and butyrate co-treatment on induction of apoptosis in p53 wild type and p53 null colonocytes. The role of fatty acid and butyrate co-treatment on cellular apoptosis was initially investigated. For this purpose, p53+/+ and p53-/- cells (**Figure 13**) were harvested following fatty acid and butyrate co-treatment and analyzed using TUNEL assay. Butyrate co-incubation with DHA in p53+/+ cells induced an 800% and 45% increase in apoptotic index compared to untreated control and to LA and butyrate treated cells, respectively (**Figure 14**). Similarly, isogenic p53-/- cells exhibited a 700% increase in apoptosis compared to untreated control, and a 55% increase relative to LA + butyrate treated cells.

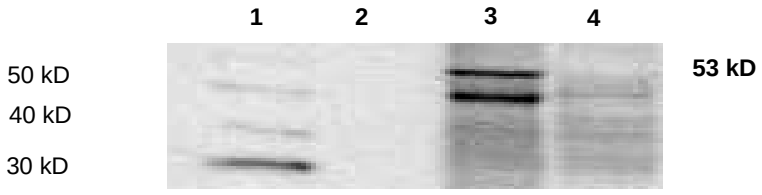

Figure 13. **Representative immunoblot of p53 wild type and knock out cell extracts**. HCT-116 cells (p53 wild type and knock out cells) were separately harvested and total cell lysates containing equivalent amount (10 μg) of protein were probed for p53 expression. Lane 1, molecular marker; lane 2, empty lane; lane 3, lysate from p53+/+ cells; and lane 4, lysate from p53-/- cells.

Effect of DHA and butyrate co-treatment on intracellular Ca^{2+} levels. Since mitochondria are key organelles involved in the regulation of cell death and intracellular Ca^{2+} homeostasis, we measured the ratio of mitochondrial to cytosolic Ca^{2+} ratio in individual cells. Butyrate co-treatment for 6 h increased ($p<0.0001$) the mitochondrial-to-cytosolic Ca^{2+} ratio in cells incubated with DHA by 43% and 57% in comparison with untreated or LA and butyrate co-treated p53+/+ cells, respectively (**Figure 15A, closed bars**). Similarly, p53-/- cells treated with DHA + butyrate exhibited a 44% and 22% increase in mitochondrial Ca^{2+} levels as compared LA + butyrate and untreated cells, respectively (**Figure 15A, open bars**). In addition, following a 12 h incubation with butyrate, DHA enhanced ($p<0.0001$) mitochondrial Ca^{2+} by 31% compared to untreated control in p53+/+ cells (**Figures 15B, closed bars**). In contrast to DHA treated cultures, cells incubated in the presence of LA showed no change (**Figure 15B, closed bars**) in the mitochondrial-to-cytosolic Ca^{2+} ratio compared to untreated cells. p53-/- exhibited a similar pattern of response (**Figures 15B, open bars**). For comparative purposes, 24 h butyrate co-treatment was also examined.

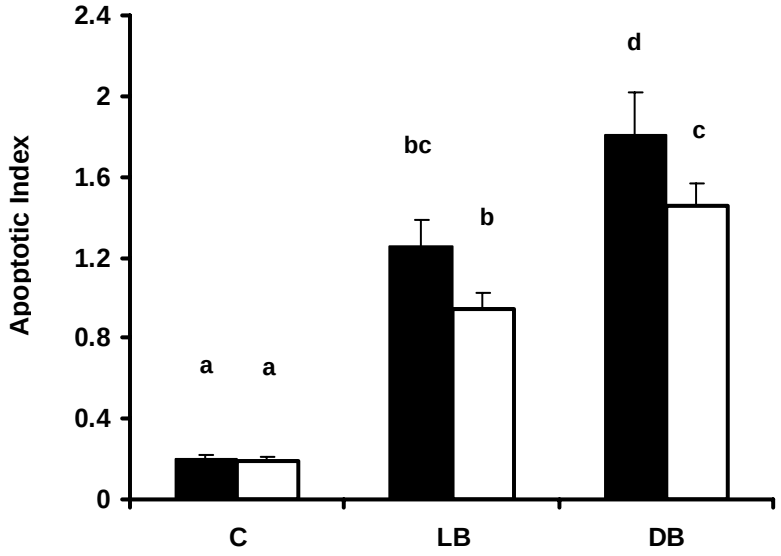

Figure 14. **Effect of fatty acid and butyrate co-treatment on apoptosis in HCT-116 cells.** p53 wild type (p53+/+) and p53 knock out (p53-/-) cells were treated with fatty acid (50 μM) for a total of 72 h and 0 or 5 mM butyrate for the final 12 h. Nonadherent cells were harvested, and apoptosis was measured by the nucleosomal fragmentation release assay. Data are means ± S.E. from 3 separate experiments, $n = 6$ wells per treatment. Solid bars represent data from p53+/+ cells and open bars represent data from p53-/- cells. Control cultures containing no treatment (C), LA and butyrate (LB), DHA and butyrate (DB). Bars not sharing a common letter are significantly different at $p<0.05$.

Consistent with observations made at earlier time points, DHA enhanced the mitochondrial to cytosolic ratio by 30 and 75% compared to LA and butyrate treatment in p53+/+ and p53-/- cells, respectively (**Figure 15C**).

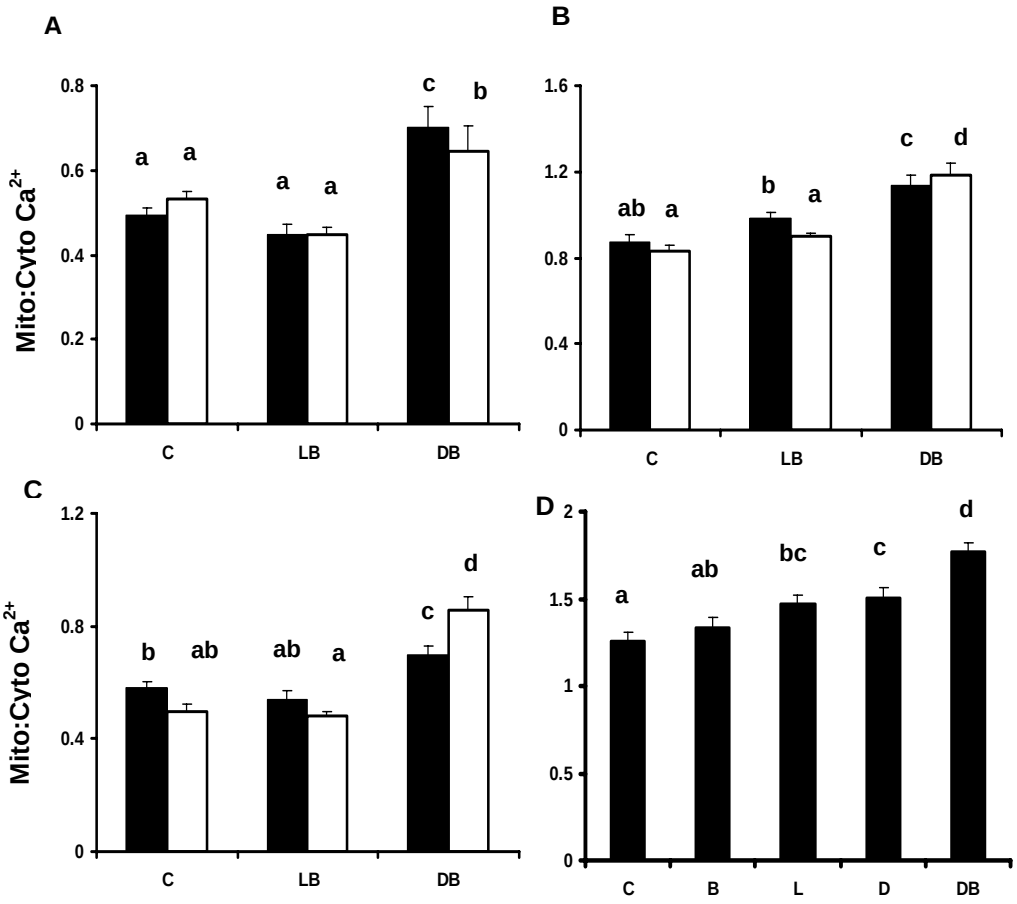

Figure 15. Effect of fatty acid and butyrate co-treatment on mitochondrial Ca^{2+} in HCT-116 cells. p53+/+ and p53-/- cells were exposed to 50 μM fatty acid for 72 h in the absence (panel D) or presence of 5 mM butyrate for the final 6 (panel A), 12 (panel B) or 24 h (panel C). Cells were co-loaded with Fluo-4 (3 μM) and Rhod-2 AM (2 μM) and the ratio of mitochondria-to-cytosolic Ca^{2+} was evaluated as described in the Materials and Methods. Data represent means ± S.E. from a representative experiment, $n = 3$ independent experiments. A significant ($p<0.001$) difference between the combination of LA with butyrate and DHA with butyrate was observed for both cell lines starting from 6 h. In p53+/+ cells, butyrate only treatment did not show any difference compared to untreated cells. DHA or LA in the absence of butyrate did not show any significant difference in the ratio of mitochondrial to cytosolic Ca^{2+} ratio. Control data for p53-/- cells were similar (data not shown). C, control- no fatty acid or butyrate; B, butyrate only; L, LA only; D, DHA only; LB, LA and butyrate; DB, DHA and butyrate.

In addition, control experiments demonstrated that cells treated with butyrate in the absence of fatty acid did not exhibit a significant difference in mitochondrial Ca^{2+} compared to untreated p53+/+ cells. p53+/+ cells treated with DHA in the absence of

butyrate did not demonstrate any significant difference in mitochondrial-to cytosolic-Ca^{2+} ratio compared to LA pre-treated cells (**Figure 15D**). In contrast, butyrate treated cells pre-incubated with DHA demonstrated a significantly higher ($p<0.0001$) level of mitochondrial-to-cytosolic Ca^{2+} ratio.

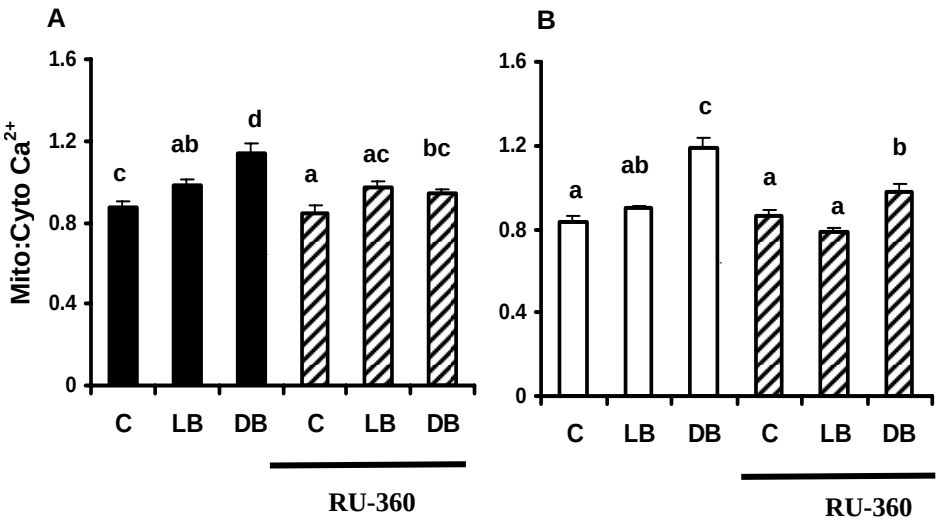

Figure 16. Effect of a mitochondrial uniporter inhibitor (RU-360) on mitochondrial Ca^{2+} levels following fatty acid and butyrate co-treatment in HCT-116 cells. p53+/+ cells (panel A) and p53-/- (panel B) were pretreated with fatty acid for a total of 72 h and RU-360 (10 μM) for 30 min prior to butyrate co-treatment for the last 12 h. Cells were co-incubated with Fluo-4 (3 μM) and Rhod-2 (2 μM) for 1 h and the mitochondria to cytosolic Ca^{2+} ratio was measured. Data represent means ± S.E. from a representative experiment, $n = 2$ independent experiments. Note that RU-360 partially reversed the effects of the combination treatment (DHA and butyrate) on the intracellular ratio in both cell lines. C, control-no fatty acid or butyrate; LB, LA and butyrate; DB, DHA and butyrate.

Effect of a mitochondrial uniporter inhibitor (RU-360) on intracellular Ca^{2+} levels. To investigate the role of the mitochondrial uniporter in Ca^{2+} uptake, cells were treated with RU-360, a mitochondrial Ca^{2+} uniporter inhibitor. As expected, RU-360 (10 μM) partially inhibited ($p<0.001$) the butyrate-induced increase in mitochondrial Ca^{2+} in

DHA primed cells by 20% in p53+/+ cells (**Figure 16A**) and 21% ($p<0.01$) in p53-/- cells at 12 h (**Figure 16B**). In comparison, RU-360 had no effect on mitochondrial Ca^{2+} levels in LA and butyrate and untreated p53+/+ or p53-/- cells.

Inhibition of the Ca^{2+} uniporter partially suppresses the induction of apoptosis following DHA and butyrate co-treatment. The relationship between mitochondrial Ca^{2+} homeostasis and cellular apoptosis was examined by quantifying apoptosis following the blockade of mitochondrial Ca^{2+} uptake. For this purpose, select p53+/+ and p53-/- cultures were treated with RU-360. Results obtained in experiments with butyrate (12 h) following DHA or LA co-treatment are shown in **Figure 17**. RU-360 significantly ($p<0.0001$) reduced apoptosis by 88% following butyrate co-treatment in p53+/+ cells (**Figure 17A**). Similarly, p53-/- cells exhibited a 75% decrease ($p<0.0001$) in apoptosis in the presence of RU-360 (**Figure 17B**). In contrast, inhibition of the uniporter had no effect on cells treated with LA + butyrate or untreated cultures in both cell types. Taken together, these results demonstrate that mitochondrial Ca^{2+} uptake is required for the enhanced apoptosis associated with DHA and butyrate co-treatment.

The mitochondrion specific antioxidant, MitoQ, inhibits apoptosis induced by DHA and butyrate co-treatment. To probe the association of mitochondrial oxidative stress with the induction of apoptosis, select p53+/+ and p53-/- cultures were treated with 5 μM MitoQ for 12 h in the presence of butyrate and fatty acid. MitoQ significantly decreased apoptosis ($p<0.0001$) in DHA and butyrate co-treated cultures by 40% in p53+/+ cells and 26% in p53-/- cells (**Figure 18A and B**). MitoQ also inhibited apoptosis in LA and butyrate treated cultures by 32% and 39% in p53+/+ and p53-/-

cultures, respectively (**Figure 18A and B**). In contrast, MitoQ had no effect on untreated cell cultures. Collectively, these results demonstrate that mitochondrial ROS/PLOOH production is essential for DHA + butyrate-induced colonocyte apoptosis.

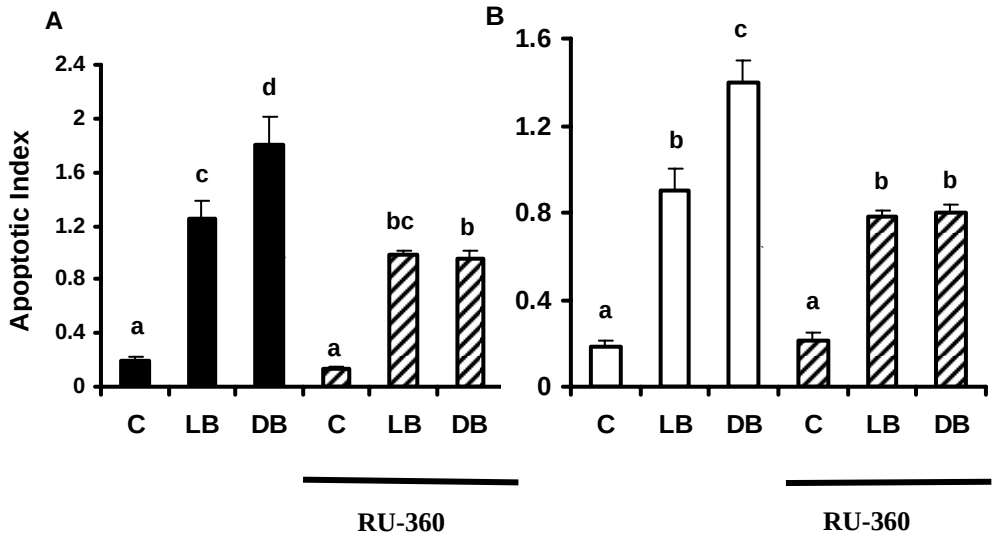

Figure 17. Effect of RU-360 on the induction of apoptosis in HCT-116 cells. p53+/+ cells (panel A), and p53-/- cells (panel B) were treated with DHA or LA (50 μM) for 48 h and subsequently co-incubated with 10 μM RU-360 for 30 min. Cultures were subsequently co-incubated with fatty acid and butyrate following which nucleosomal fragmentation assay was used to quantify apoptosis. Data are means ± S.E., $n = 6$ wells per treatment. C, control- no fatty acid or butyrate; LB, LA and butyrate; DB, DHA and butyrate.

Mitochondrial to cytosolic Ca^{2+} ratio increased in rats fed fish oil followed by butyrate incubation ex-vivo. Intact colonic crypts isolated from rats fed experimental diets containing either LA (corn oil) or DHA (fish oil) were incubated with fluo-4 and Rhod-2 (**Figure 19A**) for the purpose of imaging mitochondrial to cytosolic Ca^{2+} responses following incubation with butyrate (5 mM) *ex vivo***.** Only crypts that exhibited higher than 85% cell viability were included in the analysis (**Figure 19B**).

Dietary fat composition did not significantly (p = 0.527) influence the basal mitochondrial-to-cytosolic Ca^{2+} ratio

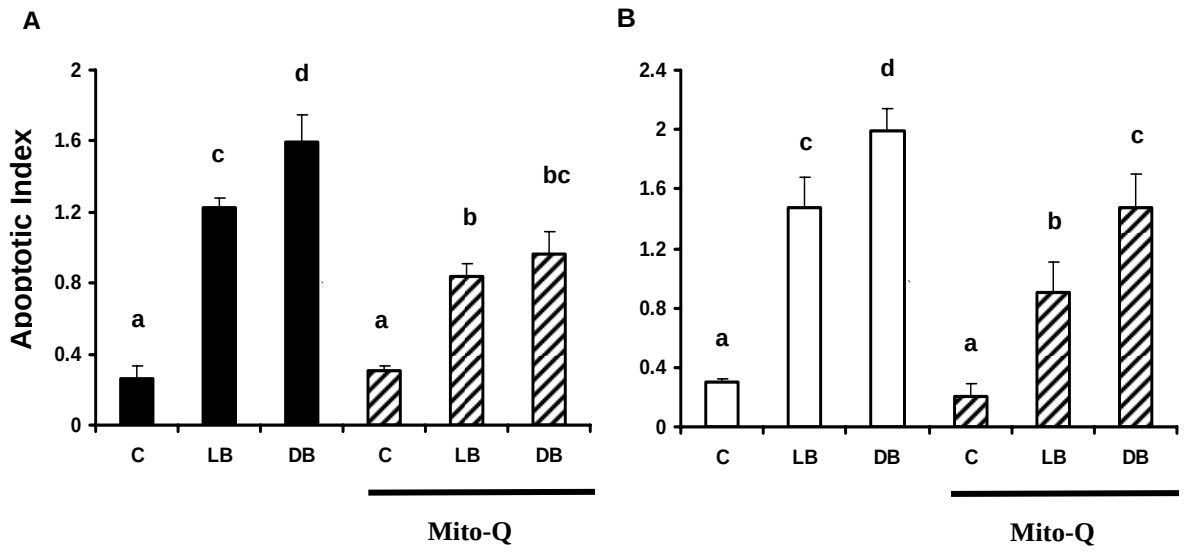

Figure 18. **Effect of a mitochondrial-targeted antioxidant (Mito-Q) on cellular apoptosis following fatty acid and butyrate co-treatment in HCT-116 cells**. p53+/+ and p53-/- cultures were treated with 50 μM DHA or LA for 72 h and butyrate for the final 12 h. Select cultures were pre-treated with 5 μM Mito-Q for 24 h prior to butyrate co-treatment. Nonadherent cells were harvested, and apoptosis was measured by the nucleosomal fragmentation release assay. Data are means ± S.E., *n* = 6 wells per treatment. Panel A represents p53+/+ cells ± Mito-Q incubation. Panel B represents p53-/- cells ± Mito-Q incubation. C, control- no fatty acid or butyrate; LB, LA and butyrate; DB, DHA and butyrate.

(**Figure 19C**). In contrast, butyrate treatment and dietary lipid-butyrate interactions in the main model were significant; both p-values < 0.0001. The colonic crypts from fish oil fed animals incubated in the presence of 5 mM butyrate for 30 min demonstrated a 260% increase ($p<0.001$) in the mitochondrial to cytosolic Ca^{2+} ratio compared to crypts isolated from corn oil fed animals (**Figure 7D**). Collectively, these outcomes demonstrate that DHA + butyrate combination enhance mitochondrial Ca^{2+} stores both in human tumor cell lines and primary rat crypt cultures.

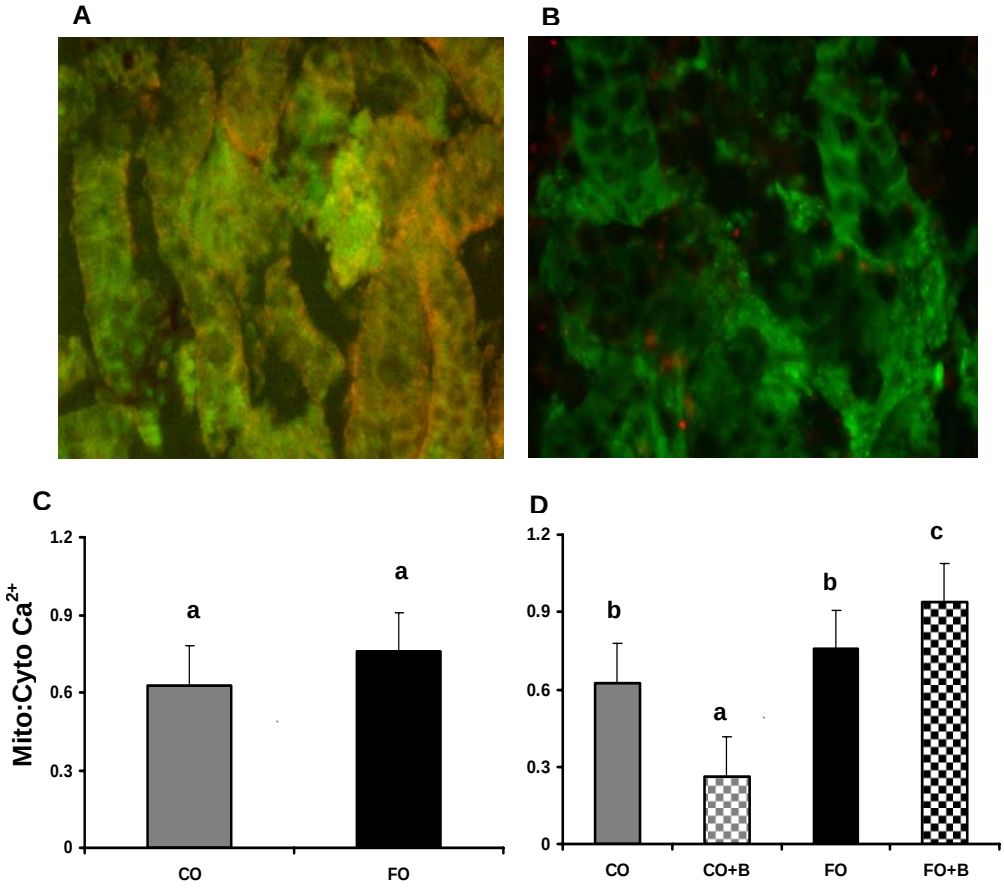

Figure 19. **Measurement of intracellular Ca^{2+} in colonic crypt primary cultures**. Rats fed either fish oil (FO, n-3 PUFA) or corn oil (CO, n-6 PUFA)-enriched diets and colonic crypts were isolated and subsequently incubated with or without 5 mM butyrate (B) for 30 min. Crypts were co-incubated with Fluo-4 (3 μM) and Rhod-2 (2 μM) for 30 min and the mitochondrial to cytosolic Ca^{2+} ratio was determined. Panel A, a representative fluorescence image of crypts co-loaded with Fluo-4 and Rhod-2. Panel B, an example of select cultures incubated with calcein (green) and ethidium homodimer (orange) to assess cell viability. Panel C, relative mitochondria to cytosol Ca^{2+} ratios in isolated crypts under basal conditions, no butyrate added to the culture media. Panel D, effect of exogenous butyrate on mitochondrial Ca^{2+} levels. Data are means ± SE from 15 rats per treatment group with at least 15 images per rat. Bars not sharing a common letter are significantly different, $p<0.001$.

Discussion

The in vivo molecular mechanisms underlying n-3 PUFA-induced apoptosis in the colon are just emerging (43). Furthermore, it is unclear whether observations derived from specialized cell lines accurately reflect endogenous cellular mechanisms. In the current study, we compared the effects of dietary fish vs corn oil, enriched in DHA (22:6n-3) and LA (18:2n-6) respectively, in combination with butyrate, a fiber fermentation product, on mitochondrial Ca^{2+} accumulation in primary colon crypt cultures and in human colonocyte tumor cell lines. Further, our studies examined the relationship between the p53 tumor suppressor gene and mitochondrial Ca^{2+} signals with regard to the induction of colonocyte apoptosis in HCT-116 cells. The present data reveal that: *1)* The combination of DHA and butyrate enhance apoptosis by stimulating mitochondrial Ca^{2+} accumulation in a p53-independent manner; *2)* blockade of the mitochondrial uniporter inhibits mitochondrial Ca^{2+}-induced colonocyte apoptosis; *3)* suppression of mitochondrial oxidative stress with a mitochondrion-targeted antioxidant partially inhibits apoptosis; and *4)* butyrate incubation with primary colonic crypts isolated from fish oil fed animals increases mitochondrial Ca^{2+} stores compared to cultures from corn oil fed animals.

We initially investigated whether p53 plays an essential role in DHA and butyrate-induced apoptosis. This is significant because inactivating mutations in this tumor suppressor gene occur in approximately 50% of all colon tumors (197, 198, 199). Existing data suggest that apoptosis can be mediated by the induction of the p53-regulated modulator PUMA (95, 143, 145), a mitochondrial protein (153, 154). Given

the central role of mitochondria in the commitment to apoptosis, we hypothesized that DHA and butyrate interactively promote p53-dependent apoptosis by triggering changes in mitochondrial Ca^{2+} levels that contribute to caspase activation and colonocyte cell death. We used isogenic p53 wild type and deficient human colon tumor (HCT 116) cell lines to determine whether or not chemoprotective nutrients modulate intracellular Ca^{2+} compartmentalization to induce colonocyte apoptosis. The results confirm and extend our previous observations (58) and demonstrate that DHA and butyrate synergistically enhance mitochondrial Ca^{2+} accumulation which serves as a trigger for apoptosis in a p53-independent manner.

Consistent with previous observations (14, 16, 47, 52), our data demonstrate that pleiotropic bioactive components generated by fermentable fiber (butyrate) and fish oil (DHA) act synergistically to enhance apoptosis. This is noteworthy, because it has now been clearly established that the transformation of colonic epithelium to carcinoma is in part associated with a progressive inhibition of apoptosis (49, 50, 51, 161). Hence, chemotherapeutic agents in combination which restore the normal apoptotic pathways have the potential for effectively reducing cancer risk (195, 200). Although it has been well documented that butyrate is an inhibitor of histone deacetylases and can activate the Fas receptor mediated extrinsic death pathway (201, 83), the role of these mechanisms in the induction of colonocyte apoptosis may be a secondary consequence of its ability to promote cellular oxidation (202, 203). For example, serving as the primary energy source for colonic epithelial cells, butyrate induces cellular ROS generation (201, 204, 205). This is relevant because DHA relative to LA is oxidatively susceptible due

primarily to its high degree of unsaturation (56, 57). Consistent with previous observations (58), both LA/DHA and butyrate-induced apoptosis was partially blocked by coincubation with a mitochondrially targeted antioxidant, Mito-Q (206). These data are further supported by the fact that enhanced mitochondrial lipid peroxidation can directly trigger the release of proapoptotic factors from mitochondria into the cytosol (207, 208, 209).

The overloading of mitochondrial Ca^{2+} can activate the mitochondrial transition (MPT) pore and trigger a series of events culminating in cellular apoptosis (92, 98, 173, 135, 120, 180, 182). Since we have previously observed that butyrate and DHA treatment dissipated the MPT (58), we determined the effect of co-treatment on mitochondrial Ca^{2+} homeostasis. Interestingly, only the combination of DHA and butyrate increased intra-mitochondrial Ca^{2+} levels at 6, 12 and 24 h in p53+/+ and p53-/- cells. Butyrate co-treatment (12 h) in DHA vs LA incubated cells also increased apoptosis by 44 and 35% in p53+/+ and p53-/- cells, respectively. RU360, a mitochondrial Ca^{2+} uniporter inhibitor (173) attenuated ($p<0.05$) DHA and butyrate-induced mitochondrial Ca^{2+} accumulation and apoptosis by ~40% in all cell lines. Based on these data, we propose that the combination of DHA and butyrate, compared to LA and butyrate, enhances apoptosis by additionally recruiting a p53-independent, oxidation sensitive, mitochondrial Ca^{2+}-dependent (*intrinsic*) apoptotic pathway in the colonic epithelium. This could in part explain why the combination of dietary fish oil (containing DHA) and pectin (fermented to butyrate in the colonic lumen) reduces tumor formation in the colon (14, 16 29, 47, 57).

In order to address the physiological relevance of our observations, a series of technically challenging proof-of-principle experiments were conducted. For this purpose, intact viable colonic crypts were isolated from rats fed diets containing either DHA or LA and labeled with with appropriate fluorescent probes to simultaneously measure cytosolic and mitochondrial Ca^{2+} pools. We hypothesized that only animals fed DHA would exhibit an increase in mitochondrial Ca^{2+} following incubation with butyrate. Although no significant difference in the basal mitochondrial to cytosolic Ca^{2+} ratio in colonic crypts from fish oil or corn oil-fed animals was noted, the addition of butyrate to primary cultures induced mitochondrial Ca^{2+} accumulation exclusively in animals fed DHA. These novel data corroborate our cell line observations indicating that DHA and butyrate synergistically trigger colonocyte mitochondrial Ca^{2+} accumulation.

In summary, our results indicate that DHA and butyrate work coordinately in the colon to trigger a previously unrecognized pro-apoptotic cycle involving mitochondrial Ca^{2+} loading. This is noteworthy, in view of the search for toxicologically innocuous cancer chemoprevention approaches which are free of safety problems intrinsic to drugs administered over long periods of time.

CHAPTER IV

SUMMARY AND CONCLUSIONS

n-3 polyunsaturated fatty acids have been promoted for their broad health benefits by virtue of their ability to prevent and treat a wide variety of diseases (183, 187, 210, 211). A growing body of clinical, epidemiological and experimental evidence has emphasized the role of fish oil enriched in n-3 PUFA, like DHA (22:6, n-3) and EPA (20:5, n-3), in a variety of diseases like cancer, heart disease, autoimmune and inflammatory disorders (43, 210, 211). In addition, fiber is one of the most important, although controversial, factors thought to prevent colorectal cancer (71). The rapidly growing incidence of colorectal cancer in the developed countries has intrigued scientists and prompted some to investigate the underlying mechanism involved in the chemoprotective effects of these 2 dietary factors. The favorable effects of the combination of these two dietary components have been demonstrated to be due to enhanced colonocyte apoptosis (14, 16, 52, 53, 58). Nonetheless, a comprehensive understanding of the underlying mechanism has yet to be elucidated. Our previous in-vivo and in-vitro model systems have demonstrated that the combination of DHA and butyrate, the bioactive products of fish oil and fermentable fiber, significantly enhance colonocyte apoptosis, which could in part explain the chemoprotective effect of this dietary combination (47, 58).

Free fatty acids are known to trigger an alteration in intracellular Ca^{2+} homeostasis in various cell types (212, 213). Experimental evidence also indicates that

Ca^{2+} activates calcium-binding non-lysosomal cysteine proteases like calpain (214) and calcium-dependent endonucleases that mediate DNA fragmentation and apoptosis (215, 216). ER-Ca^{2+} depletion in conjunction with enhanced mitochondrial uptake rather than an increase in cytosolic Ca^{2+} have also been identified as a trigger to initiate apoptosis (217). In this investigation we hypothesized that the combination of DHA and butyrate altered intracellular Ca^{2+} homeostasis to induce colonocyte apoptosis. The findings from this investigation may help develop a new model to better understand the fundamental mechanism involved in the pro-apoptotic effect of these bioactive compounds.

The first portion of this study demonstrated that DHA and butyrate increased mitochondrial Ca^{2+} levels while concomitantly decreasing free cytosolic Ca^{2+} levels. This did not come as a surprise considering the well established role of intracellular Ca^{2+} in regulating apoptosis. There is compelling evidence linking the release of Ca^{2+} from intracellular stores and the opening of influx channels on the plasma membrane (87, 102). Therefore, our findings raised an interesting question as to the pool that is involved in the alteration in intracellular Ca^{2+} levels. To determine this we measured intracellular Ca^{2+} release from the ER under basal conditions, followed by chelation of extracellular Ca^{2+} using EGTA, and re-addition of Ca^{2+} back into the extracellular media. To further substantiate the importance of plasma membrane channels we also blocked the SOC using SKF-96365. Data obtained indicated that extracellular Ca^{2+} was primarily responsible for the DHA and butyrate-induced perturbation in intracellular Ca^{2+}. Further studies are needed to examine if SOC are key players in the involvement of DHA and butyrate induced apoptosis.

This study also shed light on the channel involved in mitochondrial Ca^{2+} accumulation. Considering the important role of mitochondrial Ca^{2+} accumulation in cellular apoptosis, RU-360, an inhibitor of the mitochondrial uniporter, was used to determine the association of apoptosis with mitochondrial Ca^{2+}. RU-360 inhibited both mitochondrial Ca^{2+} uptake and apoptosis. These findings demonstrate the role of mitochondrial Ca^{2+} accumulation in the onset of apoptosis induced by DHA and butyrate.

Another objective of this study was to determine if mitochondrial Ca^{2+} accumulation preceded the onset of apoptosis. One potential mechanism by which DHA and butyrate enhances apoptosis could be via enhancing mitochondrial accumulation of Ca^{2+}. To examine this possibility, we measured mitochondrial accumulation of Ca^{2+} and apoptosis at 6, 12 and 24 h of fatty acid and butyrate co-treatment. Indeed, this study demonstrated that mitochondrial Ca^{2+} elevations were seen as early as 6 h of butyrate co-treatment while the onset of apoptosis did not occur until 12 h of butyrate co-treatment. This would not be unexpected as Ca^{2+} accumulation is considered to be important trigger for cytochrome-c release (88, 132) via opening of the permeability transition pore, culminating in apoptosis.

In our previous experiments we demonstrated that butyrate acts via a Fas-mediated pathway (83) and that DHA and butyrate combination enhances mitochondrial lipid hydroperoxide production, and mitochondrial membrane permeability transition (58) to induce colonocyte apoptosis. Results from this investigation have generated additional mechanistic evidence suggesting that DHA and butyrate recruits a Ca^{2+}

dependent mitochondrion-mediated intrinsic pathway to induce colonocyte apoptosis (**Figure 20**).

We next examined if human colonocytes exhibited effects analogous to mouse colonocytes. In addition, we also wanted to elucidate the involvement of p53 in DHA and butyrate-induced apoptosis. p53, a tumor suppressor gene plays a fundamental role in promoting apoptosis in response to cellular stress including DNA damage (145, 218). Previous animal experiments from our lab demonstrate that fish oil-fed animals target DNA damaged cells for deletion by apoptosis (52). In an effort to examine if enhanced apoptosis due to mitochondrial Ca^{2+} accumulation was p53-dependent, experiments were conducted using isogenic (p53 wild type and p53 null human colonic tumor cells) - HCT 116 cell. Results from these experiments demonstrated that DHA and butyrate enhanced both apoptosis and mitochondrial Ca^{2+}, which could be partially inhibited by RU-360 in both p53 wild type and null cells in a comparable manner. These findings indicate that DHA and butyrate induce colonocyte apoptosis and enhance mitochondrial Ca^{2+} uptake via the mitochondrial uniporter in a p53-independent fashion.

In the final phase of this investigation we examined the ability of dietary fish oil to alter mitochondrial Ca^{2+} levels in primary crypt cultures. Rats were fed semi-purified diets containing n-3 PUFA (fish oil) or n-6 PUFA (corn oil) as the lipid source. Colonic crypts were isolated and subsequently incubated with butyrate ex-vivo and the mitochondrial-to-cytosolic Ca^{2+} ratio was measured. Colonic crypts from animals fed fish oil exhibited an enhanced accumulation of Ca^{2+} within the mitochondria compared to cultures not incubated in butyrate. This enhanced accumulation in part explains the

mechanism responsible for the increase in colonoctye apoptosis seen in animals fed a combination of fish oil and fermentable fiber.

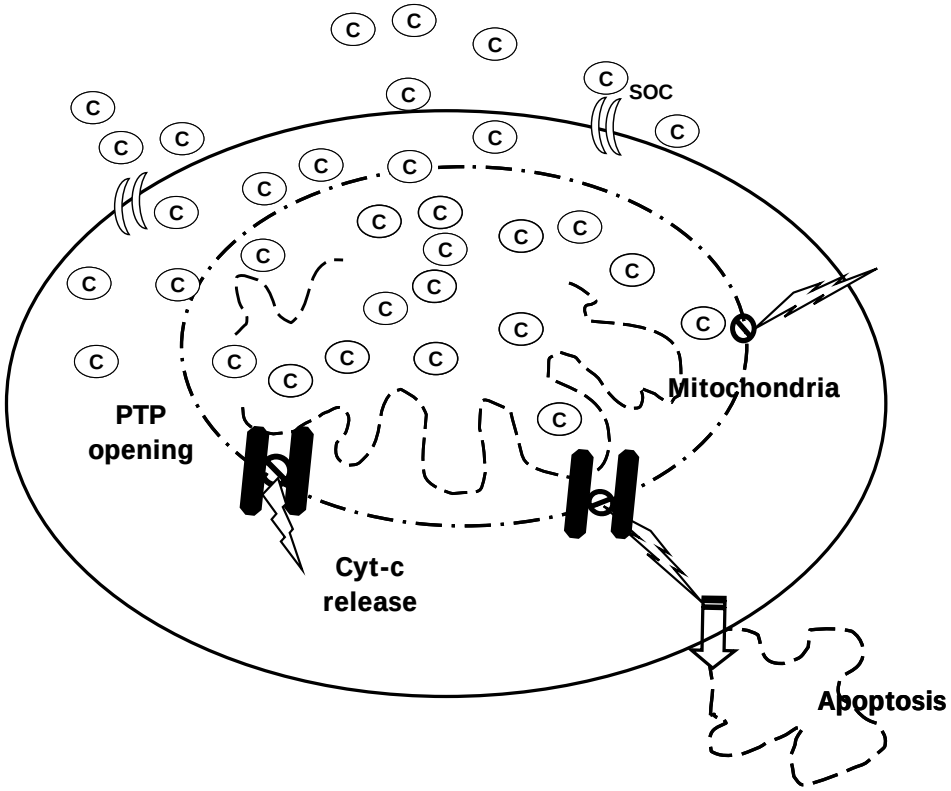

Figure 20. Proposed intrinsic-apoptosis model in DHA and butyrate co-treated cells. DHA and butyrate enhances extracellular Ca^{2+} entry through the SOC. This combination concomitantly decreases the cytosolic Ca^{2+} level and enhances mitochondrial Ca^{2+} accumulation. These events result in opening of the permeability transition pore stimulating the release of pro-apoptoic molecules like cytochrome C resulting in enhanced cellular apoptosis.

Collectively, these data indicate for the first time that the combination of DHA and butyrate, compared to butyrate alone, exhibits a further increase in apoptosis by additionally recruiting a p53-independent, Ca^{2+}-mediated intrinsic mitochondrial pathway. While the recruitment of the intrinsic apoptotic pathway may explain, in part,

the reason for the difference in cancer susceptibility between population consuming diets rich in n-3 PUFA versus n-6 PUFA and fiber, it is most likely not the only determining factor. Nonetheless, investigations like these continue to provide insight into the mechanism/s involved in the chemoprotective effects of fish oil and fermentable fiber. The findings of this study are unique in that they examine the mechanism involved when two important dietary factors interact as seen physiological (in-vivo) environment. However, further studies are required to explain the downstream effects of mitochondrial Ca^{2+} accumulation. Subsequent mechanistic studies may cede compelling evidence regarding the role of intracellular Ca^{2+} compartmentalization in colonocyte apoptosis thus providing a link to the consumption of dietary intake of fish oil and fermentable fiber in the prevention of colon carcinogenesis.

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APPENDIX A

EXPERIMENTAL PROTOCOLS

Protocol # A-1, Preparation of Complete RPMI media for YAMC culture
Ref: Fan et al. Am J Physiol. 1999 Aug;277(2 Pt 1):C310-9; Ng et al. Carcinogenesis. 2005 Nov;26(11):1914-21.

Hood sterility
Purpose: To prepare the hood for all sterile techniques including cell culture

- Turn UV light on in the hood for atleast 15 min prior to use
- Use 95% Ethanol and spray the inside and outside of the hood
- Wipe with a clean paper towel and repeat to ensure sterility
- Spray and clean the outside of media bottles, pipettes etc with ethanol before taking it under the hood

Preparation of Media
Purpose: To prepare complete RPMI media for YAMC cell culture
Reagents (supplier, catalog number)

-500 ml RPMI without glutamine (Mediatech # 15- 040 CV)
-26.6 ml Fetal Bovine Serum (Hyclone AK 12434)
-5.3 ml Glutamax (Gibco, 35050-061)
-0.532 ml ITS "-" minus (Insulin, Transferrin, Selenious acid without Linoleic acid) (Collaborative Biomed products, # 4351)

- Reconstitution of ITS "-": add 5 ml of sterile distilled water into lysophylized powder. 1 ml of ITS is sufficient for 1 L of media (0.1% dilution)

Procedure
-Thaw Glutamax and FBS at 4°C overnight
- Add Glutamax, FBS and ITS "-" into 1 bottle of RPMI 1640 media.
-Gently tilt the bottle to mix
-Label as sterile, complete and store at 4°C

Final volume: 531.9 ml
Final concentration: 5% FBS, 1 % Glutamax, 0.1% ITS

Linoleic acid	0
Insulin	5 μg/ ml
Transferin	5 μg/ ml
Selenious acid	5 ng/ ml

The following reagent is to be added *fresh* into the media before use:

5 units γ-IFN (Gibco BRL, #13284-021) per 1 ml complete RPMI 1640 media.

Add 1μl γ-IFN per 10 ml of complete RPMI 1640 medium just prior to use.

Protocol # A-2, Preparation of Complete McCoy's 5A modified media for HCT116 cells
Ref: Fan et al. Am J Physiol. 1999 Aug; 277(2 Pt 1):C310-9; Ng et al. Carcinogenesis. 2005 Nov;26(11):1914-21.

Preparation of Media
Purpose: To prepare complete McCoy's 5A media for HCT116 cell culture p53+/+ and p53-/- cells
Reagents (supplier, catalog number)
McCoy's 5A (Gibco 16600-082) w/o HEPES
10% FBS (Hyclone AJA9530)
1% Glutamax (Gibco 3505-061)

Procedure
-Thaw 5.5 ml of Glutamax and 56 ml FBS at 4°C overnight.
- Add Glutamax, FBS into 500 ml of McCoy's 5A modified medium without HEPES.
-Gently tilt the bottle to mix.
-Label as sterile, complete, inital and store at 4°C.
- Use separate bottles for p53+/+ and p53-/- cells and have the bottles labeled accordingly.
- Take caution and don't culture p53+/+ and p53-/- under the hood at the same time.

Final concentration: 10% FBS, 1 % Glutamax

Protocol # A-3, Preparation of YAMC Cell Culture
Ref: Fan et al. Am J Physiol. 1999 Aug;277(2 Pt 1):C310-9; Ng et al. Carcinogenesis. 2005 Nov;26(11):1914-21.

Purpose: To start YAMC culture by growing cells in a T-75 flask.

Preparation
-Turn on the UV in the hood for atleast15 min prior to keep it sterile for culture

Procedure
1. Warm complete RPMI 1640 media to room temperature
2. Aliquot 10 ml media in a 15 ml conical tube (to spin down frozen cells) and make up 30 ml complete media with 3 μl γ-IFN (to resuspend and grow cells)
3. Take a vial of YAMC cells from the liquid nitrogen storage system
4. Thaw it in the water-bath immediately
5. Add thawed cells into the media in the conical tube
6. Centrifuge at 200 x g (1096 rpm: tabletop centrifuge, for 5 min) at 6 Acc./Dcc.
7. Using sterile technique Open a T-75 flask and add 12 ml complete media (with γ-IFN)
8. Following completion of spin, aspirate the supernatant to remove the freezing media
9. Resuspend the pellet in 5 ml complete RPMI 1640 (with γ-IFN)
10. Add resuspended cells into the T-75 flask with media, label the flask - specify the cell type, passage number, date and initials
11. Gently rotate the flask to distribute the cells evenly
12. Incubate the flask at 33˚C under 5% CO_2 atmospheric pressure

Feeding cells
Check of cell confluence every day
Feed cells every 48 h (maximum 72 h)

Procedure

1. Aspirate old media from the flask using sterile techniques and replenish with fresh RPMI 1640 media (with γ-IFN)
2. When culture reaches 70-90% confluence, trypsinize the cells and pass them (refer to Appendix A: Protocol # A-5 YAMC Cell Culture)

Protocol # A-4, Preparation of HCT-116 Cell Culture

Ref: Bunz, F et al. Science, Vol.282, Nov, 1998
Purpose: To start HCT 116 (both p53+/+ and p53-/- cells) culture by growing cells in separate T-75 flasks.

Preparation
-Turn on the UV in the hood for atleast15 min prior to keep it sterile for culture.

Procedure

1. Warm complete McCoy's 5A media (both p53+/+ and p53-/- bottles) to room. temperature.
2. Aliquot 10 ml PBS (w/o Ca^{2+} and Mg^{2+}) in a 15 ml conical tube (to spin down frozen cells).
3. Take a vial of p53+/+ cells from the liquid nitrogen storage system thaw rapidly at 37^{o}C with gentle agitation.
4. Add thawed cells into the media in the conical tube.
5. Wash cells in 10 mL PBS (w/o Ca^{2+} and Mg^{2+}).
6. Slowly introduce cells to new environment by adding a few drops of PBS to cryovial before transferring to tube of PBS.
7. Centrifuge at 150 x g for 5 min at room temperature.
8. Resuspend the cell pellet in 1 ml McCoy's 5A growth medium.
9. Add resuspended cells into a T-75 flask with 14 ml McCoy's
10. Gently rotate the flask to distribute the cells evenly
11. label the flask - specify the cell type, passage number, date and initials
12. Incubate at 37^{o}C; under 5% CO_2 atmospheric pressure
13. Check daily until 70-90% confluent, usually 3 days.
14. If cells are not passaged, re-feed cells.
15. Repeat the same procedure for p53-/- cells. Take care not to culture both wild type and knock out cells at the same time.

Feeding cells
Check of cell confluence every day.
Feed cells every 48 h (maximum 72 h).

Procedure

1. Aspirate old media from the flask using sterile techniques and replenish with fresh complete McCoy's 5A media.
2. When culture reaches 70-90% confluence, trypsinize the cells and pass them (refer to Appendix A: Protocol # A-6 HCT-116 Cell Culture).
3. Repeat the procedure for p53-/- cells.

Protocol # A-5, YAMC Cell Culture: Seeding, Passing or Freezing

Purpose: To pass and to seed or freeze YAMC cell culture.

Preparation

- Use sterile hood conditions for the procedure
-Warm complete RPMI 1640 and trypsin-EDTA (Gibco, #25300-054) to room temperature

Procedure

For any 70% confluent cell culture in a T-75 flask:

1. Aspirate old media, rinse monolayer of cells with ~10ml HBSS (Sigma, H-6648) add and aspirate gently without disturbing the cell monolayer
2. Aspirate HBSS. Add 5 ml Trypsin-EDTA and incubate cells at 33 ˚C for 2-3 minutes or until > 90% cells are lifted
3. Add 15 ml complete media in a 50 ml conical (to spin cells) and add 5 µl γ-IFN to 50 ml media (to grow cells)
4. Check cells under microscope. Gently tap the bottom of the flask to release the adherent cells
5. Add cells in suspension into a 50 ml conical tube containing ~15 ml complete RPMI 1640 medium.
6. Centrifuge cells at 200 x g (1096 rpm, countertop centrifuge for 5 min)
7. Vaccum aspirate supernatant (media with trypsin), taking care not to disturb the pellet
8. Add required volume (5 ml) of complete RPMI 1640 (with γ-IFN) and resuspend the pellet by gently pipetting up and down to make a homogenous supsension.

A. Procedure to count cells and seed

-Once pellet is resuspended in media, cells are counted to seed at a known density
-Transfer 5 µl of the cell suspension into a 500 µl micro-centrifuge tube with 40 µl PBS
-Add 5 µl trypan blue to the tube, and mix well by gently pipetting up and down to get a homogenous suspension (10% suspension)
-Transfer 20 µl of the cell suspension onto a hemacytometer.
-Count cell: Cell density (cells count per ml) = $\frac{\text{Living cells count x 10,000}}{\text{\# Squares counted}}$
-Seed cell according to desired density.

B. Procedure to continue cell growth and expansion in a T-75 flask

-Take 1 ml of the homogenous cell suspension and add to 1 T-75 with ~ 15 ml complete media (with γ-IFN)

- Seed cells into as many flasks as needed

C. Procedure for cell freezing (to store) cells in liquid nitrogen
-At step 8 in the cell passage protocol, resuspend cells in 4 ml of freezing media (EmbryoMax, # S-002-D)
-Add 1 – 1.5 ml of cell suspension (~ 1.5 million per vial)
-Label the vials with the cell type, passage number, date and initials
-Keep vials in Mr. Frosty (Nalgene, #5100-0001) at –80˚C for a day (not more than 48 h) and then transfer vials into liquid nitrogen for storage
-Enter the rack, box # and vial position into cell culture log book

Protocol # A-6, HCT-116 Cell Culture: Seeding, Passing or Freezing

Purpose: To pass and to seed or freeze p53+/+ and p53-/- cell culture.

Preparation
- Use sterile hood conditions for the procedure.
-Warm complete McCoy's 5A media (both p53+/+ and p53-/- bottles) and trypsin-EDTA (Gibco, #25300-054) to room temperature.

Procedure
For any 70% confluent cell culture in a T-75 flask:
1. When cells are 80-90% confluent, remove all medium.
2. Wash cells once with PBS ((w/o Ca^{2+} and Mg^{2+}), aspirate gently without disturbing the cell monolayer.
3. Add Trypsin/EDTA, incubate 3 min at 37^{o}C until > 90% cells detach.
4. Add 5ml medium to Trypsinized cells, pipet cells into tube of medium.
5. Spin cells down at 150 x *g*, 5 min.
6. Vaccum aspirate supernatant (media with trypsin), taking care not to disturb the pellet.
7. Resuspend pellet in complete McCoy's 5A media.
8. Incubate flasks in a humidified, 37^{o}C, 5% CO_2 incubator.
9. Repeat the same for p53-/- cells.

A. Procedure to count cells and seed cells is similar to YAMC cells

-Count cell: Cell density (cells count per ml) = $\frac{\text{Living cells count x 10,000}}{\text{\# Squares counted}}$

-Seed cell according to desired density.

B. Procedure for cell freezing (to store) cells in liquid nitrogen
Freezing Medium: Cell Culture Freezing Medium with DMSO (Specialty Media S-002-D).
1. Label cryovials with cell type, passage number, date and initials.
2. Defrost freezing medium, place both on ice
3. To collect cells, follow Steps 1 - 6 of Procedure: *Passage Cells*
4. Resuspend pellet from T-175 flask in freezing medium to give 5 x 10^6 cells / mL

5. Keep vials in Mr. Frosty (Nalgene, #5100-0001) at –80˚C for a day (not more than 48 h).
6. Transfer vials into liquid nitrogen for storage
7. Enter the rack, box # and vial position into cell culture log book

Protocol # A-7: Fatty acid-BSA complex

Ref: Fan et al. Am J Physiol. 1999 Aug;277(2 Pt 1):C310-9

Purpose: To prepare 2.5 mM fatty acid and bovine serum albumin (BSA) complex.

Materials

a. Spatula (1), 100 ml beaker (1), vials (2). Glassware should be baked at 180°C for 4hr
b. Filter (0.2 μm) and 25 ml syringes (1 for each fatty acid)
c. FA-free BSA (BM, #100069), MW: 68,000
d. Sodium carbonate- Na_2CO_3 (J T Baker, #3604-01), MW: 105.99
e. RPMI medium (Mediatech, #150400LV)
f. Sterile distilled water (Baxter, 2F7115)
g. DHA [21.14 μg/μL in EtOH], MW: 328.5 (2004)
h. LA [22.03 μg/μL in EtOH], MW: 280.48 (20041)

Preparation

Day before preparation: Bake 2 sets (1 stand-by)
On prep day- Ice bucket, vial screw caps, and baked glassware
Turn on the hood U.V light

Procedure

1. Vortex stock tubes of fatty acid (DHA, LA in ethanol) vigorously
2. Add 10 mg (volumes recorded below) of FA-Ethanol into labeled 2 ml glass vials, respectively.

Calculations to make up 10 mg for fatty acid stocks (dated) 10.22.03
LA in EtOH – 11.68 μg/μl
DHA in EtOH – 11.55 μg/μl

$$\text{LA} = \frac{10{,}000\ \mu g}{11.68\ \mu g/\mu L} = \boldsymbol{856.16\ \mu L}$$

$$\text{DHA} = \frac{10{,}000\ \mu g}{11.55\ \mu g/\mu L} = \boldsymbol{865.80\ \mu L}$$

1. Clean Nitrogen tank needles with organic solvent (methanol or ethanol).
2. Dry down the FA with low stream of N_2 (30 psi). Set timer for 1 hour and check every 5- 10 min.
3. Aliquot 10 ml of sterile water and two - 20 ml of RPMI media under the hood
4. Weigh 53 mg (0.053 gm) of sodium carbonate and 3 gm of fatty acid free BSA.
5. Make 0.05M Na_2CO_3

53 mg of Na_2CO_3 in 10 ml sterile H_2O (need 2mL per FA-BSA)

Make 15% BSA solution
Add 20 ml of RPMI medium into 100mL beaker
Gently layer 3 g of BSA onto the medium
Do not stir but let the BSA powder slowly dissolve down to the medium

6. After drying down the fatty acid completely. Close the N_2 tank.
   Add 2 ml of 0.05 M Na_2CO_3 to each FA vial.
   Flush the vial and lid with N_2 and close the vial.
   Set timer for 1 hour and vortex the vials extensively.
   Sit the vials at room temperature for 1 hr (every 10 mins for the first 30 mins and then reduce the frequency to reduce the air bubbles).

7. Calculate the materials needed to make 2.5 mM FA-BSA complex at the FA/BSA 3/1 mole ratio.

Example DHA (MW: 328.5): 10 mg in 2 mL 0.05M Na_2CO_3;
BSA(MW: 68000):15% Solution

(i) Calculate the volume of 15% BSA solution needed for 10 mg DHA to obtain FA/BSA 3/1 mole ratio.

$$\frac{0.01 \text{ g DHA}}{328.5 \text{ (DHA mol wt)}} \times \frac{1}{3} \times 68000 \text{ (BSA MW)} \times \frac{100}{15} = 4.6 \text{ (ml)}$$

(ii)Calculate the total volume of solution needed for 10 mg DHA to make
2.5 mM DHA-BSA complex.

10 (mg of DHA)/ 328.5 (DHA mol. wt)/ 2.5 (mM) = 0.012177 (L) = 12.177 ml

(iii) Calculate the volume of plain RPMI medium needed for making the 2.5 mM DHA-BSA complex.

12.177 (total volume) - 2 (FA in 0.05M Na_2CO_3) - 4.6 (15% BSA) = 5.577

====>>	DHA	15% BSA	RPMI	2.5mM DHA-BSA
	2 ml	4.6 ml	5.577 ml--->	12.177 ml

It is difficult to completely retrieve the 2 ml FA-Na_2CO_3 from the glass vial. Therefore, take only 95% of everything to make the DHA-BSA complex.

1.9 ml DHA + 4.37 ml 15% BSA + 5.298 ml RPMI.

Apply similar calculation to LA.

8. Add the following ml of FA-Na_2CO_3, 15% BSA, and RPMI to 50 ml conical tube for individual FA.

	DHA TUBE	LA TUBE
	10 mg	10 mg
15 % BSA (ml)	4.37 ml	5.12 ml
RPMI (ml)	5.3 ml	6.53 ml
Fatty acid – Na_2 Co_3	1.9 ml	1.9 ml

9. Flush the tubes and lids with N_2. Shake the tubes on belly dancer for 1/2 hr at room temperature.
10. Turn the UV light on for filter sterilization, filter-sterilize the FA- BSA complex using a 10 ml syringe and 0.2 μm Tufryn filter under sterile hood
11. Aliquot 500 μl of each into 1.7 ml eppy's and label the box. Store at -20°C

Protocol #A-8 Cell Culture: Fatty acids, Butyrate and other Treatments
Ref: Fan et al. Am J Physiol (1999)

Purpose: To treat YAMC and p53 cells with polyunsaturated fatty acids (DHA and LA) and butyrate

Preparation

- Grow YAMC cells in a T-75 flask in complete RPMI 1640 medium with γ-IFN and/ or p53 isogenic cell lines in McCoy's A media
- Allow >24 h for cells to adhere and to recover from trypsinization.

Pre-treatment of cover glass slides (Refer to media preparation for calculations)

- Treat 2 wells chambered cover glasses (Nalge Nunc Intl, # 155380) with 25% filter sterilized FBS in serum free media. 1 ml media per well
- Filter sterilize FBS with 0.2 μm tuffryn filter (Acrodisc, #4192)
- Prepare 25 % FBS in serum free RPMI 1640 (for YAMC cells) or McCoys's A (p53 isogenic cell lines) media
- Load 1 ml media/FBS per well and incubate overnight (> 4 h) at 33 ˚C (YAMC) or 37°C (p53 cells) at 5% CO_2

Cell plating- Day 0

Trypsinize cells and seed 2 chambered cover glass slides with 1 ml per well
YAMC required plating density = 5,000 cells/chamber i.e / 1ml
p53 required plating density = 20,000 cells/chamber i.e / 1ml
Dispense 1 ml of the cell suspension into each well/chamber.
Rotate gently and incubate at 33°C (YAMC), 37°C (p53 cells) at 5% CO_2.

Seeding density for Apoptosis experiments

YAMC required plating density in a 100 mm dish = 150,000 cells/ 8 ml/ dish (18750/ ml)
p53 required plating density = 25,000 cells/ 1ml (1.5 ml per well in a 6 well plate)
YAMC required plating density in a 35 mm dish= 30,000 cells / 1.5 ml / dish (20,000 cells/ ml)

Fatty acid treatment- Day's 1 and 2

1. Record cell confluence every day prior to media change or treatment

2. Treat cells with a final concentration of 50 μM fatty acids (DHA or LA) in complete media
3. Defrost FA-BSA stock 2.5 mM, prepared and stored at –20˚C (use before 30 days).
4. Do not to re-use any reconstituted media or fatty acid aliquot
5. Incubate cells in respective incubators at 5%CO_2 O/N

Fatty acid and butyrate co-treament – Day 3

1. Make fresh Butyrate (500 mM) stock for treatments
2. Weigh 0.055 gm butyrate (Acros, #263190050) into a 1.7 ml eppy tube
3. Add 1 ml media and mix well by pipetting up and down
4. Filter sterilize the butyrate solution using a tuffryn filter (Acrodisc, #4192), into a sterile eppy tube and wrap tube in foil to protect from light
5. Final concentration of the butyrate stock is 500 mM
6. Prepare fresh fatty acid-media (DHA or LA) solution for fatty acid only control treatments
7. Add butyrate stock (final concentration of 5 mM) for fatty acid and butyrate combination treatment
8. Use media only, butyrate in media, fatty acid and fatty acid and butyrate combination into the respective treatment slides
9. Add fatty acid and/ or butyrate into the tube with media and mix well by gently pipetting and tilting the tube
10. Start butyrate treatment during the designated time point of the final co-treatment either final 6, 12, 24 or 48 h of fatty acid treatment

Imaging Day (Analysis is done in the Imaging Analysis lab in vet school)
Things to carry to vet school:

Lebovitz media, cell culture media.
Dyes, inhibitors, PBS aliquots, butyrate weighted.
Gloves, timers, pipettes, sterile tubes, syringes, sterile filters.
Cells on slides wrapped in foil.

Preparation:

Warm *Leibovitz* media & cell culture media to room temperature (<2 h) before start.
Check CO_2, water and temperature in the incubator.
Warm the dyes and make up stock solutions.
Make up the inhibitors and necessary solutions as per the protocols below.

Preparation of stock solutions and final concentration for treatment/ loading

[Media refers to complete RPMI 1640 for YAMC cells and complete McCoy's A media for the p53 isogenic cells**]**

1) Preparation of 25% FBS-in serum free media solution for slide pre-coating

Calculation:

C_i = initial stock concentration, C_f = final required concentration, V_f = final required volume, V_i = initial volume of stock to start with

C_i = 100%, C_f = 25%, V_f = 10 ml, V_i = ?

$V_i = \frac{25\% * 10 \text{ ml}}{100\%} = 2.5$ ml *(2.5 ml filtered FBS + 7.5 ml of serum free media, for 10 ml)*

2.5 ml FBS + 7.5 ml Media ---> 10 ml Media/FBS

2) Preparation of 50 µM final concentration of fatty acids in media for treatment

Calculation:

C_i = initial stock concentration, C_f = final required concentration, V_f = final required volume, V_i = initial volume of stock to start with

C_i = 2.5 *1000 µM, C_f = 50 µM, V_f = 10 ml, V_i = ?

V_i = $\frac{50\ \mu M * 1\ ml}{2500\ \mu M}$ = 0.2 ml *(200 µl fatty acid + 9.8 ml of media, 10 ml fatty acid- media)*

0.2 ml stock + 9.8 ml Media ----> 10 ml FA/media

3) Preparation of 5 mM final concentration of butyrate in media for treatment

Calculation:

C_i = initial stock concentration, C_f = final required concentration, V_f = final required volume, V_i = initial volume of stock to start with

C_i = 500 mM, C_f = 5 mM, V_f = 10 ml, V_i = ?

V_i = $\frac{5\ mM * 10\ ml}{500\ mM}$ = 0.1 ml *(100 µl butyrate + 900 µl of media- 10 ml butyrate- media)*

0.1 ml butyrate stock + 9.0 ml Media ----> 10 ml butyrate/media

4) Preparation of 2 mM stock solution and 3 µM final concentration of Fluo-4 (*Molecular Probes # F14201*)

Prepare on the day of experiment

Stock preparation of 2 mM Fluo-4

Molecular weight of Fluo-4 is 1096.95 and the vial contains 50 µg

1096.95 µg in 1ml is 1 mM

50 µg (vial) …. should be dissloved in ? ml to make 1 mM

$\frac{50\ \mu g * 1000\ \mu l}{1096.95\ \mu g}$ = 45.58 µl = ~ 50 µl

Dissolve 50 µg Fluo-4 (vial) in 25 µl DMSO to make up 2 mM Fluo-4 stock solution

Mix well by pipetting up and down

Final concentration of 3 µM Fluo-4

Calculation:

C_i = 1 mM, C_f = 3 µM, V_f = 1 ml (900 µl of Leibovitz) V_i = ?

V_i = $\frac{3\ \mu M * 1 * 1000\ \mu l}{2 * 1000\ \mu M}$ = 1.5 µl per ml of media

1.5 µl of Fluo-4 stock needs to be added to 1 ml Leibovitz to make a final concentration of 3 µM.

5) Preparation of 4 mM stock and 2.5 µM final concentration of Rhod-2 (*Molecular Probes # R1245*)

Stock preparation of 4 mM Rhod-2

Molecular weight of Rhod-2 is 1123.96 and the vial contains 50 µg

1123.96 µg in 1ml is 1 mM

50 µg (vial) …. should be dissloved in ? ml to make 1 mM

$\frac{50\ \mu g * 1000\ \mu l}{1123.96\ \mu g} = 44.49\ \mu l = \sim 45\ \mu l$

45/ 5 = 9

Therefore dissolving 50 µg vial in 10 µl of DMSO will give a stock concentration of 5 mM

Dissolve 50 µg Rhod-2 (vial) in 10 µl DMSO to make up 5 mM Rhod-2

Mix well by pipetting up and down.

Final concentration of 2.5 µM Rhod-2

Calculation:

C_i = 5 mM, C_f = 2.5 µM, V_f = 1 ml (900 µl of Leibovitz) V_i = ?

$V_i = \frac{2.5\ \mu M * 1 * 1000\ \mu l}{5 * 1000\ \mu M} = 0.5\ \mu l$ per ml media

0.5 µl of Rhod-2 stock needs to be added to 1 ml Leibovitz to make a final concentration of 2.5 µM

6) Preparation of 1 mM stock Mitotracker Green FM and 200 nm final concentration (*Molecular Probes # M 7514*)

Stock preparation of 1 mM Mitotracker Green FM

Molecular weight of Mitotracker is 671.88 and the vial contains 50 µg.

671.88 µg in 1ml is 1 mM

50 µg (vial) …. should be dissloved in ? ml to make 1 mM

$\frac{50\ \mu g * 1000\ \mu l}{671.88\ \mu g} = 74.42\ \mu l$

Therefore dissolving 50 µg vial in 74.42 µl of DMSO will give a stock concentration of 1 mM

Dissolve 50 µg Mitotracker (vial) in 74.42 µl DMSO to make up 1 mM Mitotracker

Mix well by pipetting up and down.

Calculation:

C_i = 1 mM, C_f = 200 nM, V_f = 1 ml (900 µl of Leibowitz) V_i = ?

$V_i = \frac{200\ nM * 1 * 1000\ \mu l}{1*1000*1000\ nM} = 0.2\ \mu l$ per ml

Therefore, 0.2 µl of Mitotracker green stock needs to be added to 900 µl Leibowitz for incubation to make a final concentration of 200 nM

7) Preparation of 5 mM stock and 5 µM final concentration of Thapsigargin *(Sigma, # T9033*)
Stock preparation of 5 mM Thapsigargin

Molecular weight is 650.75 and TG is available as a 0.5 m/ vial
3253.75 µg in 1 ml is 5 mM
0.50 mg (vial) …. should be dissolved in ? ml to make 5 Mm

$$\frac{0.5 * 1000\ \mu g * 1000\ \mu l}{3253.75\ \mu g} = \sim 154\ \mu l$$

Dissolve the 0.5 mg Thapsigargin (vial) in 154 µl of DMSO to make 5 mM stock. Mix well by pipetting up and down

5 µM final concentration of Thapsigargin
Calculation:
C_i = 5 mM, C_f = 5 µM, V_i = ?, V_f = 1000 µl (900 µl of Leibovitz+ 100 µl + ? µl of TG - imaging)

$$V_i = \frac{1000\ \mu l * 5\ \mu M}{5 * 1000\ \mu M} = 1\ \mu l$$

1 µl of TG stock needs to be added to 1 ml (900 µl Leibovitz and 100 µl PBS at imaging to make a final concentration of 5 µM

8) Preparation of 1 mM stock and 10 µM final concentration of RU-360
Started experiments using 10 mM stock and then changed to 1 mM stock due to small volumes
Make up RU-360 1 mM stock with degassed nanopure water and use 10 µM final concentration.
- Take ~ 5 ml nanopure water in a 15 ml falcon and blow nitrogen from the nitrogen tank using a clean new glass pipette for 10 min.
- Flush the lid with Nitrogen and tightly close the falcon tube and rush to the hood of cell culture and add the water into the vial of RU-360 and mix well

Calculation for making up 10 mM RU stock
Molecular weight of RU-360 is 550.8
RU vial has 100 ug – should be dissolved in how many ml to make 1 mM stock

$$\frac{100\ ug * 1000\ ul}{550.8\ ug} = 181.6\ ul$$

Ci= 1 mM, Cf= 10 uM, Vf= 10 ml or 12 ml, Vi=?

$$Vi = \frac{10 * 1000\ \mu l * 10\ uM}{1 * 1000\ \mu M} = 100\ ul$$ RU stock into 10 ml media to make 10 µM final conc.

9) Preparation of 10 mM stock and 10 μM final concentration of SKF- 96365 (*Calbiochem # 567310)*

Stock preparation of 10 mM SKF 96365

Molecular weight of SKF is 402.9 and the vial contains 5 mg.

402.9 g in 1000 ml is 1000 mM
402.9 mg in 1000 ml is 1 mM
5000 μg (vial) …. should be dissloved in ? ml to make 1 mM
$\frac{5000\ \mu g}{402.5\ \mu g}$ = 0.01241 ml (12.4 ul of water)

Add 5 mg in 1.241 of sterile water to make the final stock concentration of 10 mM.

Calculation:

C_i = 10 mM, C_f = 10 μM, V_f = 1 ml (900 μl of Leibovitz) V_i = ?

$$V_i = \frac{10\ \mu M * 1 * 1000\ \mu l}{10 * 1000\ \mu M} = 1\ \mu l \text{ per ml}$$

Therefore, 1 μl of SKF stock needs to be added to the 1ml Leibovitz for incubation to make a final concentration of 10 μM

10) Preparation of stock solution of EGTA (10 mM) and 1 M $CaCl_2$ made up with Leibovitz *(Sigma, # E3889)*

Stock preparation of 10 mM EGTA

Molecular weight of EGTA is 380.4
38.04 mg (.038 gm) in 10 ml is 10 mM
-To make 10 mM stock - dissolve the 0.038 gm of EGTA in 10 ml Leibovitz
-Put the tube in warm water bath to ensure complete dissolution of EGTA (difficult to dissolve)
-Use 100 μl of EGTA stock in 1ml media to obtain 1 mM final concentration

Stock of $CaCl_2$ is 1 M made up in water.
Final concentration of calcium chloride used is 5 mM (Vi = 5 μl)

11) Preparation of 50 mM MitoQ stock and 5 μM final concentration (gift from Dr. Micheal Murphy)

Stock preparation of 50 mM MitoQ

Molecular weight of MitoQ is 678.81 and the vial contains 10.7 mg.

678.81 μg in 1ml is 1 mM
67881 μg in 1ml is 100 mM
33940.5 μg in 1ml is 50 mM

10700 μg (vial) …. should be dissloved in ? ml to make 1 mM
$\frac{10.7 * 1000\ \mu g * 1000\ \mu l}{33940.5\ \mu g}$ = 315.26 μl

Assume water density as 1
315-10.7 = 304.3 µl
Dissolve 10.7 mg in 304.3 µl of water
Mix well by pipetting up and down.
Aliquot and store in -20°C until further use.

First make a 500 µM solution and then dilute to 5 µM

Calculation:

C_i = 50 mM, C_f = 500 µM, V_f = 500 µl, V_i = ?

$$V_i = \frac{500\ \mu M * 500\ \mu l}{50 * 1000\ \mu M} = 5\ \mu l \text{ for 0.5 ml media}$$

Take 5 µl of 50 mM stock MitoQ and add 495µl of media and mix well by pipetting up and down. This is 500 µM stock.
Use 10 µl per 1 ml solution to make a final concentration of 5 µM for cell treatment.

12) Stock preparation of 10 mM Decyl TPP bromide and use 5 µM final concetration

Molecular weight of DTPP bromide is 483.46 and the vial contains 9.3 mg (9300 µg).

483.46 µg in 1ml is 1 mM
9300 µg (vial) …. should be dissloved in ? ml to make 1 mM

$$\frac{9300\ \mu g * 1000\ \mu l}{483.46\ \mu g} = 19236.34\ \mu l = 19.24 \text{ ml of ethanol}$$

To make up 10 mM = 19.24/ 10 = 1.92 ml
Therefore dissolving 9.3 mg vial in 1.92 ml of ethanol or methanol will give a stock concentration of 10 mM
Mix well by pipetting up and down.

Calculation:

C_i = 10 mM, C_f = 5 µM, V_f = 20 ml, V_i = ?

$$V_i = \frac{5\ \mu M * 20 * 1000\ \mu l}{10 * 1000\ \mu M} = 10\ \mu l \text{ for 20 ml media}$$

0.5 µl of stock DTTP bromide per ml of media.

Protocol #A-9 Measurement of Basal Cytosolic Ca^{2+} using Fluo-4

Purpose: To study the effect of butyrate and fatty acid combination on basal intracellular Ca^{2+} level using fluo-4, a fluorescent cytosolic Ca^{2+} indicator

Cell culture: YAMC cells, refer to Protocol # A-8 for seeding and treatment

Preparation of 2 mM stock solution and 3 µM final concentration of Fluo-4 (*Molecular Probes # F 14201*)

Stock preparation of 2 mM Fluo-4

Molecular weight of Fluo-4 is 1096.95 and the vial contains 50 µg

1096.95 µg in 1ml is 1 mM

50 µg (vial) …. should be dissloved in ? ml to make 1 mM

$\frac{50\ \mu g * 1000\ \mu l}{1096.95\ \mu g} = 45.58\ \mu l = \sim 50\ \mu l$

Dissolve 50 µg Fluo-4 (vial) in 25 µl DMSO to make up 2 mM Fluo-4 stock solution

Mix well by pipetting up and down

Final concentration of 3 µM Fluo-4

Calculation:

$C_i = 1$ mM, $C_f = 3$ µM, $V_f = 1$ ml (900 µl of Leibovitz) $V_i = ?$

$V_i = \frac{3\ \mu M * 1 * 1000\ \mu l}{2 * 1000\ \mu M} = 1.5\ \mu l$ per ml of media

1.5 µl of Fluo-4 stock needs to be added to 1 ml Leibovitz to make a final concentration of 3 µM.

Procedure

1. Make 50 ml aliquots of Leibovitz media and bring it to 33°C by placing it in the incubator
2. Make up your stock solution of Fluo-4 and bring to room temperature.
3. Aspirate old media from each well of the slide
4. Add 900 µl of warm Leibovitz media into each of the wells gently.
5. Add 3 µl of Flou-4 at the corner of each well and rotate slide gently or use the pipette to mix. Place in 33°C, 5% CO_2 incubator for 1 h.
6. After 1 hour take the slide wash with Leibovitz three times.
7. Add 900 µl of Leibovitz and take it t the microscopy room for imaging.
8. Imaging each slide takes 10- 12 mins, so load the slides at a frequency of 15 min.

Protocol #A-10 Measurement of Thapsigargin induced Ca^{2+} Kinetics

Purpose: To demonstrate a time-dependent effect of butyrate and fatty acid combination on IP-3/ ER calcium pool by Thapsigargin induction using fluo-4, a fluorescent cytosolic Ca^{2+} indicator.

Cell culture: YAMC cells, refer to Protocol # A-8 for seeding and treatment.

Preparation

-Prepare stock solutions of Fluo-4 and Thapsigargin on the day of the experiment (refer to protocol #5 for reagent preparation).

-Keep 100 µl of Thapsigargin aliquots ready for dilution of TG and $Cacl_2$

Procedure

1. Make 50 ml aliquots of Leibovitz media and bring it to 33°C by placing it in the incubator.

2. Make up your stock solution of Fluo-4 and aliquot the required volume of reconstituted TG and bring them to room temperature.
3. Aspirate old media from each well of the slide.
4. Add 900 µl of warm Leibovitz media into each of the wells gently.
5. Add 3 µl of Flou-4 at the corner of each well and rotate slide gently or use the pipette to mix. Place in 33°C, 5% CO_2 incubator for 1 h.
6. After 1 hour take the slide wash with Leibovitz three times.
7. Add 900 µl of Leibovitz and take it t the microscopy room for imaging.
8. Image 3 different fields per well with approximately 8-20 cells per field.
9. Add TG (1 µl into 100 µl PBS) at the 3rd scan.
10. Imaging each slide takes 5- 8 mins, so load the slides at a frequency of 7 min.

Protocol #A-11 Measurement of IP_3 and SOC Ca^{2+} (EGTA and Ca^{2+} add-back)

Purpose: To demonstrate the effect of butyrate (6 and 24 h co-treatment) and fatty acid combination on Ip_3 and SOC Ca^{2+} pools (Zeiss microscope).
-To demonstrate the effect of fatty acid and butyrate combination on SOC by using EGTA to chelate the extracellular $Ca^{2+}$and then to study the reversal of the effect by adding back $CaCl_2$

Cell culture: YAMC cells, refer to Protocol # A-8 for seeding and treatment.

Preparation
-Prepare stock solutions EGTA and $Cacl_2$ ahead of time (previous day). Refer to reagent preparation protocol # A-8.
-Prepare stock solutions of Fluo-4 and Thapsigargin on the day of the experiment.
-Keep 100 µl of Thapsigargin aliquots ready for dilution of TG and $CaCl_2$

Procedure
1. Make 50 ml aliquots of Leibowitz media and bring it to 33°C by placing it in the incubator.
2. Make up your stock solution of Fluo-4 and bring to room temperature.
3. Aspirate old media from each well of the slide.
4. Add 900 µl of warm Leibovitz media into each of the wells gently.
5. Add 3 µl of Flou-4 at the corner of each well and rotate slide gently or use the pipette to mix. Place in 33°C, 5% CO_2 incubator for 1 h.
6. After 1 hour take the slide wash with Leibovitz three times.
7. Add 900 µl of Leibovitz and take it t the microscopy room for imaging.
8. 3 different fields per well with approximately 8-20 cells per field will be imaged (only one well per slide can be recorded, because the cells in the other well were sitting out for too long).
9. Add EGTA (100 µl) and at the 5^{th} scan will add TG (1 µl TG in 100 µl PBS) and then at the 30^{th} scan calcium chloride (5 µl in 100 µl PBS aliquots) will be added and a total of 60 scans will be recorded.
10. Imaging each slide takes 15- 18 mins, so load the slides at a frequency of 18 min

Protocol #A-12 Measurement of IP_3 and SOC Ca^{2+} (using a SOC inhibitor, SKF-96365)

Purpose: To demonstrate the effect of inhibiting store operated channels using SKF-96365 on butyrate and fatty acid combination at 12 and 24 h co-treatment using Fluo-4, a fluorescent Ca^{2+} indicator.

Cell culture: YAMC cells, refer to Protocol # A-8 for seeding and treatment.

Preparation
- Prepare stock solutions of Fluo-4, SKF-96365 and Thapsigargin on the day of the experiment
-Keep 100 µl of Thapsigargin aliquots ready for dilution of TG.

Procedure

1. Make 50 ml aliquots of Leibovitz media and bring it to 33°C by placing it in the incubator.
2. Make up your stock solution of Fluo-4 and bring to room temperature, aliquot 7 µl TG stock into 693 µl of PBS, aliquot SKF (1 µl /well) for 1 set of slides.
3. Aspirate old media from each well of the slide.
4. Add 900 µl of warm Leibovitz media into each of the wells gently.
5. Add 3 µl of Flou-4 at the corner of each well and rotate slide gently or use the pipette to mix. Place in 33°C, 5% CO_2 incubator for 55 m.
6. After 55 min take the slide and add 1µl SKF and incubate for 4 min.
7. Following 5 min remove the Leibovitz with the dye and SKF and wash with 900 µl media.
8. Add 1 µl of SKF and take it for imaging.
9. choose 3 areas and take 5 scans, after the 5^{th} scan add 100 µl TG in PBS and take a total of 90 scans.
10. The process takes 20 minutes per well, so image only one well per treatment slide as the other well would be sitting out at room temp for too long and so cells would be compromised.

Protocol #A-13 Measurement of mitochondrial-to-cytosolic Ca^{2+} ratio

Purpose: To demonstrate the effect of butyrate (6,12 and 24 h co-treatment)and fatty acid combination on mitochondrial Ca^{2+} by co-loading cells with Rhod-2, mitochondrial and Fluo-4, cytosolic Ca^{2+} indicators.

Cell culture: YAMC cells, p53 wild type and knock cell lines refer to Protocol # A-8 for seeding and treatment.

Preparation
- Prepare stock solutions of Fluo-4, and Rhod-2 (refer to protocol #A-8 for reagent preparation)

Procedure

1. Make 50 ml aliquots of Leibovitz media and bring it to 33°C by placing it in the incubator.
2. Make up your stock solution of Fluo-4 and Rhod-2 and bring to room temperature for 1 set of slides at a time.
3. Aspirate old media from each well of the slide.
4. Add 900 µl of warm Leibovitz media into each of the wells gently.
5. Add 3 µl of Flou-4 and 0.5 of Rhod-2 µl at the corner of each well and rotate slide gently or use the pipette to mix. Place in 33°C, 5% CO_2 incubator for 1 h.
6. After 1 h wash three time with 900 µl Leibovitz media.

Image 10 different fields per well with approximately 8-20 cells per field.

Protocol #A-14 Measurement of mitochondrial-to-cytosolic Ca^{2+} ratio using Mito-Tracker FM and Rhod-2

Purpose: To demonstrate that Rhod-2 specifically loads in the mitochondria of the cell by co-loading with Mitotracker Green (Molecular Probes # M 7514).

- Mitotracker is a mitochondrion specific dye and Rhod-2 is a dye which is specific for mitochondrial Ca^{2+}. Therefore co-loading cells with both the dyes specifically indicates that Rhod-2 loads into the mitochondria and not in other peri-nuclear organelles.

Cell culture: YAMC or p53 cells, refer to Protocol # A-8 for seeding and treatment.

Dye information:

1. MitoTracker Green FM is used to confirm the mitochondrial Ca^{2+} accumulation seen with Rhod-2 fluorescence (*Boitier, 1999*).
2. MitoTracker Green is a green-fluorescent mitochondrial stain which localizes in the mitochondria regardless of mitochondrial membrane potential.
3. This dye has an added advantage in that it is essentially non-fluorescent in aqueous solution, only becoming fluorescent once it accumulates in the lipid environment of the mitochondria.

Preparation

- Prepare stock solutions of Fluo-4, Rhod-2 and MitoTracker Green FM (refer to protocol #A-8 for reagent preparation of Fluo-4 and Rhod-2)
-

  Stock preparation of 1 **mM Mitotracker Green FM**

  Molecular weight **of Mitotracker is 671.88** and the vial contains 50 µg.

  671.88 µg in 1ml is 1 mM
  50 µg (vial) …. should be dissloved in ? ml to make 1 mM
  $\frac{50\ \mu g * 1000\ \mu l}{671.88\ \mu g} = 74.42\ \mu l$

  Therefore dissolving 50 µg vial in 74.42 µl of DMSO will give a stock concentration of 1 mM

Dissolve **50 µg** Rhod-2 (vial) in **74.42 µl DMSO to make up 1 mM Mitotracker**
Mix well by pipetting up and down.

Calculation:

C_i = 1 mM, C_f = 200 nM, V_f = 1 ml (900 µl of Leibovitz) V_i = ?

$$V_i = \frac{200\ nM * 1 * 1000\ \mu l}{1*1000*1000\ nM} = 0.2\ \mu l \text{ per ml}$$

Therefore, 0.2 µl of Mitotracker green stock needs to be added to the Leibovitz for incubation to make a final concentration of 200 Nm

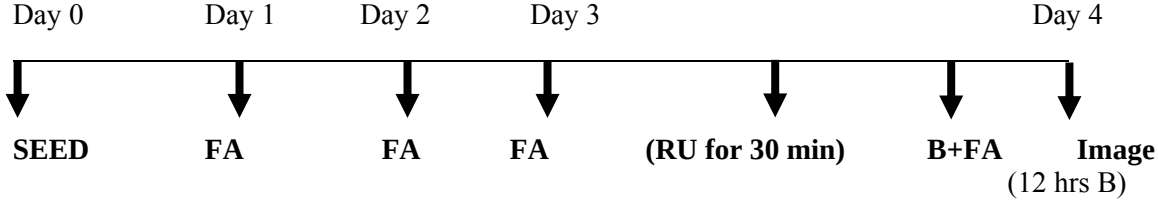
Procedure

1. Make 50 ml aliquots of Leibovitz media and bring it to 33°C by placing it in the incubator
2. Make up your stock solution of Fluo-4, Rhod-2 and Mitotracker after thawing the vials. Prepare dyes for 1 set of slides at a time
3. Aspirate old media from each well of the slide
4. Add 900 µl of warm Leibovitz media into each of the wells gently
5. Add 3 µl of Flou-4 and 0.5 of Rhod-2 µl at the corner of each well and rotate slide gently or use the pipette to mix. Place in 33°C, 5% CO_2 incubator for 1 h
6. After 55 min add 0.2 µl of Mito-tracker green to the corner of each well.
7. Gently rotate the slide and incubate at 33°C, 5% CO_2
8. After 5 min wash the cells (three times with 900 µl Leibovitz media) and take the slide with 900 µl of Leibovitz and start imaging.
9. Dr. Rola will image 10 different fields per well with approximately 8-20 cells per field
10. If magnification is set at 63X then high magnification images (for publication) can be obtained.

Protocol #A-15 Measurement of mitochondrial-to-cytosolic Ca^{2+} ratio by blocking mitochondrial uniporter

Purpose: To demonstrate the effect of butyrate (6, 12 and 24 h co-treatment)and fatty acid combination on mitochondrial Ca^{2+} by blocking the uniporter using RU-360 (blocked for 30 min and 1 h) by co-loading cells with Rhod-2, mitochondrial and Fluo-4, cytosolic Ca^{2+} indicators

Cell culture: YAMC cells, p53 wild type and knock cell lines refer to Protocol # A-8 for seeding and treatment until day 3.



Treatment protocol for day-3

1. Treat cells with 10 μM RU-360 for 30 min or 1 h (2 different sets of slides).
2. After 30 mins remove the media with RU in the 30 min set. Wash gently and add fresh media only, media with DHA, media with LA, media with butyrate and DB and LB media into the respective slides and incubate.
3. After 1 h of RU treatment. Repeat treatment changes for the 1 h + RU group.
4. Use 1 set without RU as the control – RU set.

Preparation

- Prepare stock solutions of Fluo-4, and Rhod-2 (refer to Protocol #A-8 for reagent preparation)

Procedure

1. Make 50 ml aliquots of Leibovitz media and bring it to 33°C by placing it in the incubator.
2. Make up your stock solution of Fluo-4 and Rhod-2 and bring to room temperature for 1 set of slides at a time.
3. Aspirate old media from each well of the slide.
4. Add 900 μl of warm Leibovitz media into each of the wells gently.
5. Add 3 μl of Flou-4 and 0.5 of Rhod-2 μl at the corner of each well and rotate slide gently or use the pipette to mix. Place in 33°C, 5% CO_2 incubator for 1 h.
6. After 1 h wash three time with 900 μl Leibovitz media.
7. Image 10 different fields per well with approximately 8-20 cells per field.

Protocol #A-16 Cell Death Detection ELISA, Plus

Ref: Fan et al. Am J Physiol (1999), Ng et al. (2005)

Purpose: To measure apoptosis by using a nucleosomal assay by quantifying histone-complexed-DNA fragments of floating cell population and to count the cells using the coulter counter so as to normalize the data to the number of adherent cells in the dish.

Cell culture and treatment: YAMC or p53 cells were used.
Seed cells per protocol A-8, 35 mm-cell-culture dish/ 1.5 ml
Refer to Protocol # A-8 for fatty acid, butyrate and other treatments

Preparation

ELISA kit: Cell death detection ELISA Plus from Roche- (BM# 1774425)

1. Reconstitute working solutions (refer to manual, pg 7)
2. Bottle 1: anti-histone-biotin: each bottle, reconstitute in 450 μl double distilled H_2O for 10 min and mix well (store at 2-8°C upto 2 months)
3. Bottle 2: anti-DNA-POD- reconstitute with 450 μl double distilled H_2O for 10 min and mix well (store at 2-8°C upto 2 months)
4. Bottle 3: Positive control - reconstitute with 450 μl double distilled H_2O for 10 min and mix well (store at 2-8°C upto 2 months)

5. Bottle 7: ABTS Tablets- dissolve 1 tablet in 5 ml of substrate buffer (vial 6). (100 µl / sample). This is light sensitive and hence store container in a foil wrap (store for 1 month protected form light)
6. Complete RPMI media (without γ- INF) and McCoy's media
7. Label 3 sets of 2 ml or 1.7 ml eppys (depending on the volume in the plate): floaters collection (step 1), supernatant collection (step 11) and counting.
8. Warm complete RPMI 1640/ McCoy's media and trypsin-EDTA to room temperature
9. Work under sterile hood so turn the hood on and prepare 50 ml tubes of PBS for cell counting, cool the centrifuge to 4°C
10. If using the coulter counter prepare the solutions in the vials the day ahead

Procedure

Preparation of supernatant

1. Under sterile conditions in the hood: swirl the 35 mm dish and collect the media and floating cells into the 1st set of 2 ml eppy-tubes
2. Add 1ml of sterile PBS into the plates and put them in the incubator for counting later
3. Centrifuge at 5,000 rpm (Eppendorf centrifuge) for 5 min at RT. *(pellets all the floaters which are usually apoptotic cells for the assay)*
4. Discard supernatant using 1ml pipette manually without disturbing the pellet *(pellet is imaginary most of the time)*
5. Resuspend pellet in 1ml media without γ-IFN. Finger flick the tubes
6. Wash: centrifuge again at 5,000 rpm (Eppendorf centrifuge) for 5 min at RT
7. Get out as much of the supernatant (without disturbing the pellet) and discard it
8. Resuspend cell pellet with 125 µl lysis buffer (bottle 5 of Cell death detection ELISA kit)
9. Lysis: Mix thoroughly and incubate sample for 30 min at 4°C (cell culture fridge) (*prep immunoreagent) (manual: pg 9)

For 10 tests- 40 µl of reconstituent from bottle 1 + 40 µl of reconstituent from bottle 2 + 720 µl of incubation buffer (bottle 4). Mix well

For 70 tests – 280 µl of reconstituent from bottle 1 + 280 µl of reconstituent from bottle 2 + 5040 µl of incubation buffer (bottle 4). Mix well

10. Centrifuge lysate at max speed (13,600 x g, eppendorf centrifuge) for 10 min, at 4°C. *(Pellets out the intact cells and nuclei etc leaving the cytosolic fraction in the supernatant for the assay)*
11. Transfer 100 µl of the supernatant carefully into another eppy-tube without shaking the pellet. Keep tubes on ice.

Continue in the main lab

ELISA procedure

12. Transfer 20 µl of all samples- cytoplasmic fraction (include Histone-DNA complex, bottle 3 into MP. Pipette into the middle of the MP well.
13. Use 20 µL of

Well (A –1) – Background (Bottle 4 of kit)

Well (B-1) – Blank (ABTS solution - Tablet dissolved in substrate- reconstituted tablet)

Well (C-1) – Positive control (Bottle 3 of kit)

14. Add to each well 80 µl of the immunoreagent using multiple channel pipette/ regular (no immuno-reagent into the blank)
15. Cover the MP with an adhesive cover and foil. Incubate on a MP shaker under gently shaking (300 rpm) for 2 hr at 15-25°C.
16. Remove the solution thoroughly by tapping (tape both sides of the modules to secure them). This step removes the unbound components (antibodies)

17. Rinse each well 3x with 250 µl of incubation buffer (bottle 4). Remove solution carefully by tapping.
18. Turn off lights. Pipette to each well 100 µl ABTS solution.
19. Incubate on a plate shaker at 250 rpm in the cold room until the color development is sufficient for a photometric analysis (approx 2-5min). Check every minute.
20. Measure at 405 nm against ABTS solution as a blank (reference wavelength approx 490nm).

Cell Counting (Hemocytometer)

a. Swirl the plates with the PBS and aspirate the PBS from the plates.
b. Add 0.5 ml Trypsin and put them in the incubator for 3 mints @ 33^0C.
c. Add 1ml media into each of the plates with the trypsin.
d. Swirl the plate and collect all the cell/ trypsin and media into the eppys'.
e. Count the cells using hemocytometer.
f. Calculate the cell density and total number of apoptotic cells.
g. Use this count to calculate the apoptotic index.

Cell counting using Coulter Counter (Ref: Evelyn Callaway)

Prepare the vials with solution for counting the pervious day and check the functioning of the counter

1. Turn power on, open door, lower stage.
2. Remove vial of Coulter Clenz, replace with Isotone/Isoflow, raise stage
3. Readout: Setup #1 (S1). Scroll down to last line and set cell size. This measurement is to be set for the smallest cell of acceptable range. Our lab usually sets 10.00 um.
4. Select: FUNCTIONS on keypad. Scroll to Flush aperture. Hit: START/STOP to flush. Repeat flush, then replace Isotone. Flush twice more.
5. Select: OUTPUT on keypad. Scroll to Count / Concentration and select one. If "concentration" is selected, readout will be in #cells/ml. Scroll to Dilution and enter dilution factor. Leave units on um.
6. Prepare sample cups 24-48 hours ahead: De-gas Isotone by turning red lever to the left. Lift dispensing pump twice. Turn red lever so it is parallel to the arm. Fill Coulter vials with 20 ml per vial (two pumps of Isotone).
7. Add 40 µl sample to Coulter vial (for 1:500 dil). Place vial on stage, raise, and press START/STOP on keypad to count. Count each sample 2-3 times and average.
8. To shut down machine, replace vial of blue Coulter Clenz and flush aperture (under FUNCTIONS) 2-3 times. When flush is complete, turn off power.

Cell Preparation
1. Remove PBS from 35 mm dish or 6 well plate
2. Add 0.75 ml Trypsin and incubate at 37°C for 3.5 min. Swirl dish then incubate 1.5 min more.
3. Add 1 ml media into each plate and collect cells/media into eppendorf tube
4. Set Coulter Counter for "concentration" and count cells.

Calculations
1. Number on readout for Coulter counter set on "concentration" is in #cells/ml of original sample.
2. Multiply #cells/ml by total volume of sample (usually 1.75 ml).
3. Record results as total cells.

Protocol #A-17 Caspase-3 Activity Assay

Purpose: To measure apoptosis in adherent and floating cells and quantify apoptosis using caspase-3 activity kit

Cell Culture and treatment: YAMC cells refer to protocol # A-8 for cell plating and treatment

Procedure: Both floaters and adherent cells were collected and Caspase-3 activity was measured

Preparation: EnzChek Caspase-3 Assay kit #2 (*Molecular Probes, #E-13184*)
**Reagent preparation*
Warm up water bath to ~50°C.
Precool microcentrifuge at 4°C

Bring the vials of Z-DEVD-R110 (component A), DMSO (component B), Ac-DEVD-CHO (component F), R110 reference standard (component G), 20X cell lysis buffer (component C), 5X reaction buffer (component D) to RT.

5 mM stock of Z-DEVD-R110 substrate
Add 264 µl of DMSO directly into the vial of Z-DEVD-R110. (The substrate may need gentle heating (~50°C) to completely dissolve.
(Aliquot the remained substrate, protect from light, store at –20°C, desiccated).

1 M DTT stock solution
Add 650 µl of dd-H_2O directly into the vial of DTT solid (component E).
(Aliquot the remained stock, store at –20°C)

1 mM stock solution of Ac-DEVD-CHO inhibitor
Add 400 µl of DMSO directly into the vial of Ac-DEVD-CHO.
(Aliquot the remained inhibitor, store at –20°C, desiccated).

5 mM stock solution of the R110 reference standard
Add 273 µl of DMSO directly into the vial of R110.

(Aliquot the remained substrate, protect from light, store at –20°C).

1X cell lysis buffer (*Mix right before use*)

Add 100 µl of the 20X cell lysis buffer to 1900 µl of dd-H_2O. (Keep on ice)

2X reaction buffer

Add 800 µl of the 5X reaction buffer and 20 µl of 1 M DTT to 1180 µl of dd-H_2O.
(*The 5X and 2X reaction buffer may contain micelles in suspension, it is normal and won't affect the assay, but, just try to mix the reaction very well immediately before use)

2X substrate working solution*(mix right before use)*

Mix 20 µl of 5 mM Z-DEVD-R110 substrate with 1980 µl of the 2X reaction **buffer**.

Procedure to harvest cells:

1. Scrape adherent cells with robber policeman or plastic cell scraper (in the presence of medium) and tilt the plate and get out as much of the cells (both floaters and adherent cells) into labeled 15-ml falcons.
2. Add 2 ml HBSS with phenol red (w/o Ca^{2+} and Mg^{2+}) and transfer into falcon tube.
3. Centrifuge at 200 x g at RT for 5 min.
4. Aspirate supernatant carefully and resuspend pellets in 1.5 ml PBS, and transfer to 2 mL epi-tube.
5. Centrifuge at 1000 x g at 4°C for 10 min.
6. Aspirate supernatant gently and resuspend cell pellets in 60 µl of the 1X cell lysis buffer.
7. Incubate the lysate on ice for at least 30 min.

Caspase-3 activity assay:

1. After lysate were incubated on ice for 30 min, centrifuge the lysate at 5000 rpm for 5 min at 4°C.
2. Transfer the 65 µL of supernatant to a set of 0.6 µl eppy tubes and mix well. individual wells. (use 50 µl of the supernatant out of the 60 µl into individual microplate wells for caspase assay and save the remaining 15 µl for (10 µl) BCA protein assay).
3. For the samples with inhibitor (one of no-treatment control and one DB combination) add 1 µl of 1 mM Ac-DEVD-CHO inhibitor to 50 µl of sample, as a competitive control.
4. Cover the tube and incubate at RT for 10 min. Then add the sample to wells (keep rest of the samples on ice).
5. Add 50 µl of supernatant into individual wells and save 10 µl for protein estimation.
6. Add 50 µl of 1X cell lysis buffer in one well as background.
7. Add 50 µl of the 2X substrate working solution to each well.
8. Cover the wells and incubate at RT for ~30 min.
9. During the 30 min incubation, prepare the R110 standards
    a. Make 400 µl of 1X reaction buffer by diluting 80 µl of 5X reaction buffer with 320 µl dd-H2O.

b. Make [25 µM] R110 std by diluting 1 µl of 5 M R110 std with 199 µl of 1X reaction buffer.
c. Make [12.5 µM] R110 std by diluting 90 µl of 25 µM R110 with 90 µl of 1X reaction buffer.
d. Make [6.25 µM] R110 std by diluting 60 µl of 12.5 µM R110 with 60 µl of 1X reaction buffer.

10. Add 100 µl of 25, 12.5, 6.25 µM R110 standards to separate wells right before measuring the fluorescence.
11. Measure the fluorescence at Excitation 496/Emission 520 nm continuously (30 min, 1 hr, 2 hr, or overnight)

Protocol #A-18 BCA Protein Micro Assay

Purpose: To estimate protein level in cell lysate using BCA protein assay.

Principle of reaction: Bicinchoninic acid (BCA) is specific for cuprous ion. The BCA assay combines the biuret reaction with properties of the BCA molecule. Protein reacts with Cu^{2+} in an alkaline medium to produce Cu^{1+}. Two molecules of BCA react with one Cu^{1+} to form a purple reaction product that is visible at 562 nm.

Preparation: Pierce BCA Protein Assay- Reagent A, Reagent B
Albumin Std

-Prepare working reagent by mixing 50 parts A with 1 part B
-Standards: 2 mg/ml as is and 1 mg/ml diluted in sample buffer

Assay: Load standards and samples directly into microtitre plate as follows:

Albumin std conc	Std vol (in µl)	Sample Buffer	Working solution
0	0	10	200 µl
0.5	0.5 µl (1 mg/ml)	9.5	200 µl
1	1	9	200 µl
2	2	8	200 µl
4	4	6	200 µl
7	7	3	200 µl
10	5 (2 mg/ml)	5	200 µl
15	7.5 (2 mg/ml)	2.5	200 µl

1. Load 10 µl of sample and the volume of standard as provided in the table above.
2. Mix thoroughly by pipetting up and down.
3. Cover plate and incubate 37°C for 30 min to 1 h.
4. Read at 562 nm.

Protocol #A-19 Coomassie Plus Protein Assay (Pierce)

12*75 mm culture tubes: *VWR International, Cat # 60818-408*
96 well flat bottom microtiter plate
Coomassie Plus Reagent
Homogenization buffer: Triton containing buffer with 6 β-Mercaptoethanol, Protease inhibitor cocktail, 10 mM sodium vanadate.
BSA standards:

1. Perform assay in triplicate – standards and samples
2. Mix samples and standards in 12*75 mm culture tubes.
3. Add the required volumes of water, BSA and homogenization buffer and samples into the respective tubes.
4. Add 500 µl Coomassie plus reagent (repeater pipette) and mix by vortexing.
5. Transfer 300 µl to microtiter plate and read at A595 immediately.

Standard Curve

µg/ml Protein	0.25 µg/µl BSA	1 µg/µl BSA	2 µg/µl BSA	Water µl	Homog. Buffer µl	Reagent µl	Position
0	0	-	-	497.5 µl	2.5 µl	500 µl	A1-3
0.5	2 µl	-	-	495.5	2.5	500	C1-3
1	4 µl	-	-	493.5	2.5	500	A1-3
2	-	2 µl	-	495.5	2.5	500	D1-3
4	-	4 µl	-	493.5	2.5	500	E1-3
10	-	10 µl	-	487.5	2.5	500	F1-3
20	-	-	10 µl	487.5	2.5	500	G1-3

Samples	Amt Sample	Water	Reagent	Position
	2.5 µl	497.5 µl	500 µl	

Change the volume of water based on sample volume to make a total volume 1000 µl.

Protocol #A-20 Total cell lysate preparation for Western Blot

Purpose: To quantify Bcl2 protein expression in n-3 PUFA and butyrate treated YAMC cells versus n-6 PUFA and butyrate treated cells using immunoblotting.

Cell culture and treatment: YAMC cells were grown, expanded in 8- T-175 flasks.

Cells were treated with 50 DHA or LA for 72 h and 5 mM butyrate for the final 24 h and Bcl2 was measured using immunoblotting

1. No treatment (negative control)
2. DHA+B
3. LA+B
4. Butyrate only (positive control)

Following the treatments cells are harvested and a total cell lysate prepared and total protein is quantified by coomasie plus protein assay following which Bcl2 was immunoblotted.

	Reagent	**Volume Needed for 10 ml of Total Cell Buffer**	**Final Conc.**	**Total Volume Needed for 100 mL**
A	500 mM Tris-HCl (pH 7.2) Sigma, T1503, (6.1 g/100 ml)	1	50 mM	10 ml
A	1.0 M Sucrose (17.2g/100ml) Sigma, S9278	2.5 ml	250 nM	25 ml
A	200 mM EDTA (pH 7.6) Sigma, ED4SS (0.76 g/10ml)	100 µL	2 mM	1 ml
A	100 mM EGTA (pH 7.5) Aldrich, 23453-2 (0.38 g/10 ml) add NaOH to get it to dissolve and bring pH up to 7.5	100 µL	1 mMq	1 ml
A	0.4 M NaF (0.168 g/10ml) Sigma S6521	1.25 µL	50 µM	12.5 µL
A	Water	4.79175		4.792 ml
A	10% Triton X-100 Sigma, T6878, 2 ml in 20 ml ddH_2O	1 ml	1 %	10 ml
A	10 Mm activated sodium orthovanadate, Sigma, S6508 (1.839 mg/ml)	100 µl	100 µM	1 ml
B	Sigma Protease Cocktail (P8340)	400 µl		4 ml
B	β-mercaptoethanol, Bio-Rad 161-0710 (14.2 M)	7 µl	10 mM	70 µL

Preparation of Total cell lysate

Prepare 5 ml of membrane Homogenization Buffer (with 10% Triton X to solubilized membranes)

500 mM Tris - 500 µl
1 M sucrose - 1.25 ml
200 mM EDTA - 50 µl
100 mM EGTA - 50 µl
0.4 m NaF - 0.625 µl
10% Triton - 500 µl
10 mM orthovanadate - 50 µl
PI cocktail - 200 µl
β-mercaptoethanol - 3.5 µl

to be added on the day of use

Water – 2395.875 µl

For 4 T-175 flasks – resuspend in 500 µl Homogenization buffer.

1. Trypsinize four (for each treatment and control group) 80-90% confluent T-175 flasks (wash each flask with 20 ml HBSS and 12 ml trypsin) for 7-9 min or till the cells lift off.
2. Stop trypsinization with 25 ml complete media.
3. Spin at 200*g for 5 min at room temperature.
4. Resuspend the pellet in 25 ml PBS.
5. Spin at 200*g for 5 min at room temperature and discard supernatant.
6. Resuspend each pellet in 2 ml PBS and transfer all of the resuspended pellets into one 15 ml falcon.
7. Spin at 200*g for 5 min at room temperature and discard supernatant.
8. Repeat steps 6 and 7 for a total of 3 PBS washes.
9. Following the final wash, carefully remove as much of the supernatant.
10. Resuspend the pellet in 500 µl Homogenization buffer for each treatment and transfer to labeled glass Teflon homogenization tubes.
11. Homogenize with motor and white pestle –***6*** strokes (pestle motion up and down is considered as one stroke).
12. Pass homogenate through a 27-gauge needle into a 1.5 ml eppy tubes.
13. Set the tubes on ice for 20 min.
14. Spin 20 min 14,000 * g, 4^{o}C in eppendroff centrifuge.
15. Aliquot supernatant and save at -80°C for further protein estimation and immunoblotting

Protocol #A-21 Bcl2 quantitation for YAMC cells treated with DHA and butyrate

Refer to appendix A-8 for cell treatment, appendix A-20 for cell lysate preparation and appendix A-18 and A-19 for protein estimation.

Protein concentration and Antibodies for Bcl2 Western blot

1. 30 µg of the protein from each of the treatments are run on a 4-20% polyacriliamide gel and blotted onto a PVDF membrane.
2. Rabbit anti-mouse Bcl2 antibody (dilution 1:3000) from Pharmingen (#554279) is used as a primary antibody
3. Goat anti rabbit IgG from Kirkegaard and Perry Laboratories (# 0741506) is used as a secondary antibody (1:10000 dilution).
4. M1 cell lysate was used as a positive control (Pharmingen # 16296Y, lot #M039093)

Preparation

-Thaw samples on ice
-Label 0.6 ml eppy tubes
-Turn water bath on and set temperature to 98°C
-Prepare the western template sheet
- Cut Immobilon membrane and filter papers ready

Sample Preparation

1. Thaw the samples on ice while you turn on the water bath and set the temperature to 98°C (takes about 15 min).

2. Dye used for the sample dilution is 5X Pyronine. Use 1X of the dye based on the total volume of sample required (usually 25 µl total).
3. With the aid of western template sheet make the necessary dilution (if required) and add the calculated amount of dye and water to the samples and standard.
4. Quick spin.
5. Boil the samples for 5-10 min depending on the volume of the samples (25 µl volume boil for 10 min). *Do not boil the marker*.
6. Quick- spin of the samples on the tabletop.

Gel unit set up

7. Take the pre-made gel (4-20%) and carefully rip off and discard the white tape and the comb. Mark the lanes on the plate.
8. Attach the gel to the gel rack align 3rd with the lower gasket and clamp the unit. (Note that the red clip should have the broad end facing you, broad ends face outside on all 4 clips).
9. Pour running buffer to fill the stand and the trough up to the top mark.
10. Use gel-loading tips (or 10 µl art tips) and load the complete sample volume.
11. Close the unit with the lid and check the leads and make sure black-to-black and red-to-red.
12. In the cold room run the gel at 45-70 amps for 1 hour.
13. After about 40 min check every 10 min to see if the gel is running.
14. Stop the gel just above the lower most mark on the plate.

Gel transfer

15. Take the gel transfer unit in a staining tray and pour transfer buffer into it and the trough.
16. Take the cassette and lay it open.
17. Put a sponge on the black side of the cassette and place a filter paper on top of it.
18. Pour transfer buffer to keep it wet.
19. Take the gel plate out of the running trough and transfer the running buffer into the bottle for reuse.
20. Crack open the plate with a scalpel between the markings on the plate all around by keeping the large side of the gel down.
21. Cut the gel just above the bottom bit by bit with a scalpel and push through the slit in the gel plate to separate the gel.
22. Carefully separate the gel from the plate and cut the gel at lane one to identify the side (left end).
23. Place the gel on the filter paper with lane one on the right (protein side facing the membrane.
24. Take the membrane out of the sandwich (Immobilon—p, Invitrogen, LC 2005-2) and wet it by squirting methanol (better to put it in a dish with methanol).
25. Cut the right hand top corner of the membrane to identify the side.
26. Place the membrane on the gel and place the other filter paper on the membrane. Now, roll a glass tube on the filter paper to eliminate any air bubbles inb/w.
27. Place the sponge and close the white side of the cassette and clip it.
28. Place the cassette in the transfer unit with the hinges facing the top and black side facing back.
29. Put a stir bar into the transfer trough.

30. Fill the trough with transfer buffer just enough to cover the hinges of the cassette. Check the terminals black to black correspond.
31. Place it on the cold room stir plate.
32. Connect black-to-black and red-to-red and set current to 400 m amps and let it transfer for 90 min.

Blocking

33. At the end of 90 min- make 4% nonfat dry milk/ PBS/ Tween in a 50 ml tube (to 30 ml of PBS –Tween and add 1.2 gms of pre-weighted milk powder). Mix gently by inverting.
34. Pour the milk into a dish and keep ready to transfer the membrane into it.
35. After the transfer is complete- open the gel unit and transfer the transfer buffer into the bottle.
36. Use a pair of forceps to take the membrane and place the membrane into the milk dish (with the side facing gel-protein side now facing top)
37. Place it on the shaker for 1 hr at room temperature.

Primary antibody

38. Take a dish with 1.2 gm dry milk powder and 30 ml PBS- Tween. Mix and pour into a new dish.
39. Transfer the membrane from the blocking buffer into the dish with fresh milk.
40. Now, add the appropriate volume of the primary antibody (based on the dilution and add it into the dish).
41. Close the lid of the dish and shake it gently on the cold room shaker overnight.

Washing

42. The next day take the membrane and give a quick wash with PBS –Tween.
43. Then replace the membrane in fresh PBS- Tween in the dish and keep on the shaker at room temp for 10 min. Let it shake vigorously.
44. Repeat the wash 2 times at 10-min interval.

Secondary antibody

45. Make 30 ml milk/ PBS/ Tween and pour into the dish after the second wash.
46. Add the required volume of secondary antibody based on the dilution.
47. Set on shaker for 1 hr at room temperature.
48. Repeat washing with PBS –Tween 3 times.
49. While the 1 wash of 2°dary antibody is going on turn on the imager and set focus.

Developing

50. After the 2nd wash is complete
51. Cut an acetate sheet into 2 halves and remove the black sheet inb/w
52. Mix 0.5 ml of chemiluminescent super signal reagent A with 0.5 ml of reagent B in an ependroff tube.
53. Mix gently by inversion.
54. Now transfer the membrane b/w the layers of the acetate sheet and squirt the developing solution on the top.
55. Slowly close the top layer so that the solution gets evenly distributed on the membrane.

56. Expose for 5 minutes and then transfer the membrane on to the clean acetate sheet
57. Transfer it into the quality one imager for imaging immediately.

Imaging

58. Turn on switch and make sure the lever on the hood is at chemiluminescence.
59. Select Quality on program on desktop.
60. Select scanner – click on chemidoc.xrs.
61. Step 1- option is chemiluminescences.
62. Step 2 – live focus. Focus with a printed sheet and set the iris as you need for brightness. Zoom and focus, as you need for clarification.
63. Freeze. Put the gel in the imager and zoom and freeze again. Close the door.
64. Click on live acquire.
65. Starting exposure time – for Bcl2 imaging - used 30 sec.
    Total exposure time – 150 sec
    Number of exposures – 5
66. Imager takes 5 pictures in 150 sec.
67. Below is the image obtained obtained from one of the representative experiments.

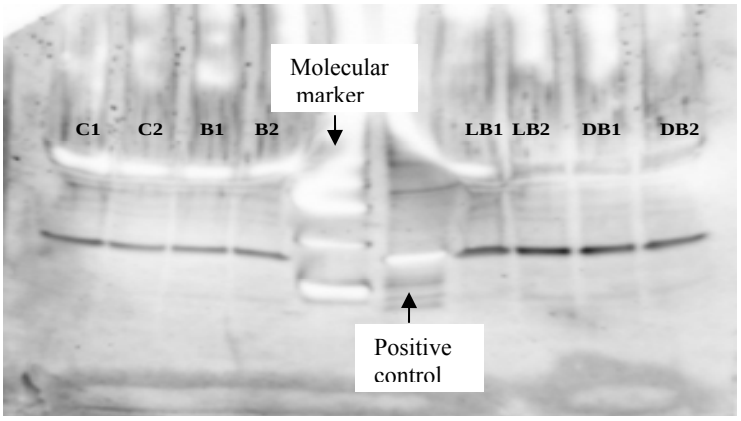


Figure 21. YAMC cell extract immonoblot analysis for Bcl2 protein expression. YAMC cell extracts were harvested for various treatements (in duplicate) and total cell lysates containing 30 μg of protein were probed for Bcl2 expression. Cell lysates - untreated cells (lanes C1, C2), butyrate- (5 mM for 24 h) (B1, B2); LA (50 μM for 72 h) and butyrate (5 mM for 24 h) treated (LB1, LB2); and DHA (50 μM for 72 h) +butyrate (5 mM for 24 h) (DB1, DB2) treated cells were blotted on a 4-30% SDS-PAGE gel to quantitate Bcl2 expression.

Quantitation of Bcl2 protein expression

Treatment	Adjusted Intensity*mm2	Standard Deviation
C1	5709.15	262.55
C2	4702.31	200.67
B1	3962.77	196.59
B2	5275.71	217.02
LB1	9131.85	315.76
LB2	12645.35	421.77
DB1	7948.84	373.85
DB2	5781.76	286.11

Quantitation of Bcl2 protein expression. Table represents the adjusted volume intensity for Bcl2 protein expression (bands quantitated following western blot) in YAMC cell extracts. Refer to appendix A-21 and figure 21 for additional details.

Conclusion: Preliminary data from the representative experiment demonstrates that DHA and butyrate combination downregulated Bcl2 expression in YAMC cell lysates.

Protocol #A-22 Preparation of a Modified AIN-76A Diet (15% fat, 6% fiber)

Ingredient	Bioserve #	Corn Oil	Fish Oil	25 kg CO	25 kg FO
Sucrose	#3900	32.00%	32.00%	8.0	8.0
Casein	#1100	20.00%	20.00%	5	5
Corn Starch	#3200	22.00%	22.00%	5.5	5.5
DL- Methionine	#1340	0.30%	0.30%	0.075	0.075
AIN-76 Salt Mix	#F8505	3.50%	3.50%	0.875	0.875
AIN-76 Vitamin Mix	#F8000	1.00%	1.00%	0.25	0.25
Choline Chloride	#6105	0.20%	0.20%	0.05	0.05
Cellulose	#3425	6.00%	6.00%	1.5	1.5
Corn Oil		15.00%	3.50%	3.75	0.875
Fish Oil		0.00%	11.50%	0.0	2.875
Total		**100%**	**100%**	**25.00 kg**	**25.00 kg**

Materials

1. The following ingredients are stored at room temperature
   Dextrose
   Casein
   Cellulose
   Mineral Mix
   DL-methionine
   Choline Bitartrate (with desiccant)

2. The following ingredients are stored in the cold room:
   Vitamin Mix

3. The following ingredients are stored in the -20°C walk-in cooler in Dr. Chapkin's lab:
   Fish and corn oil (under N_2)

Preparation

- Check all ingredients quantity about 3 weeks prior to mixing diet (ingredients take about 2-3 weeks to arrive).
- Thaw the oils by setting them at 4°C the previous day of mixing.
- Take the 2 mixing bowls and mixers for the Hobart mixer from Dr. Lupton's lab and label them (corn oil- yellow tape and fish oil- blue tape). Transfer the bowls to the mixing room (basement).
- Label color coded (yellow for corn oil and blue for fish oil) cage cars with the name of the diet, date of preparation and initials.
- Make sure that the measuring bowls, spatulas etc are clean before use.

- Bring 3 extra large beakers (5000 ml), 2 medium beakers (1000 ml).
- Don't forget your protocol sheet.

<u>Supplies to be carried to the basement</u>

- Weighing scale, 5000 ml beakers (2) and 2000 ml beakers (2).

- Cart loaded with all the ingredients to be used for the diet preparation.
- 1-2 gallon size Ziploc bags (50) to store the diet.
- 4-5 spatulas and 2 large metal spoons, weight spatula one for each ingredient to be added, few scoops, scrub and soap to wash.
- Pen, paper towels, scissors, gloves and sharpies.
- Weigh the fish and corn oils in the lab into the 5000 ml plastic beakers and take them to the basement and the rest of the ingredients will be weighed downstairs.

Weighing Ingredients

Weigh out the ingredients to prepare each diet (30 kg/diet).

1. Weigh out the amount of oil needed into 5000 ml beakers- 2 for corn oil and 1 for fish oil diet.
2. After weighing, add the small amount of corn oil needed for the fish oil diets to the fish oil.
3. Cover the oil beakers with aluminum foil and set it on the cart to be transferred into the basement.
4. Flush the original stock oil containers with N_2 before closing the lid and storing away in -20°C.

To the Basement

- Take all the equipment and ingredients listed in the preparation paragraph to the basement.
- Don't forget to take the pre-weighted oil beakers.

1. Place one of the large plastic containers on the large scale and tare. Using the plastic scoop, add dextrose until the required weight is reached.
2. Now add the dextrose into the large mixing bowl. Take caution to add the required amounts into either the fish or the corn oil bowls.
3. Using the same procedure as above, weigh out casein and fiber (cellulose) for each diet.
4. Place a small plastic container on the top loading scale and tare. Using a weigh spatula, add mineral mix to the container until the appropriate weight is reached. Remove from the scale, set aside and keep covered.
5. Weigh out vitamin mix, DL-methionine, and choline bitartrate as described in step 4.

Mixing the Ingredients

**IMPORTANT—Prepare corn oil diet first before fish oil to prevent cross contamination of equipment and diets.

1. Now to the large container of casein add the pre-weighted ingredients one by one and stir briefly between the additions of each ingredient.
   Add in order: DL-methionine, Choline bitatarate, Vitamin mix and Mineral mix. Use the metal scoop and mix well as the mineral ix tends to clump.
2. Now add pre-weighted amount of cellulose into the bowl for the Hobart mixer with dextrose. Stir well with a fresh pair of gloves and metal scoop until mixed well.

3. On top of the dextrose/fiber mixture, slowly pour in the container of casein with all the other smaller ingredients (mixed). Mix well with metal scoop until fairly well blended.

4. Place the mixing bowl on the stand of the mixer and latch it down on both sides. Attach the color-coded mixing beater, and raise the mixing stand until it is at its highest point.

5. Cover the bowl with the plastic lid.

6. Set the mixer speed to (1), the slowest speed. Set the timer for 2 minutes and press the start button. The machine will begin mixing.

7. After 2 minutes, use a fresh pair of gloves and check to make sure that the ingredients at the bottom of the bowl are mixed well. Close the lid and turn on the mixer again for 1 minute or until the ingredients are well mixed.

8. Once the mixer stops get the appropriate oil for the diet and pour ½ of it slowly between the beater and the bowl into the dry ingredients. Run it at slow speed (1) for 2 minutes.

9. Once the oil initially poured appears to be well blended, add another ½ of the oil mixture and allow it to mix. Repeat this step until all of the oil mixture is well blended into the rest of the ingredients.

10. Close the bowl, set the timer for 5 minutes and allow the mixer to blend on low speed (1).

11. After five minutes, use clean gloves clean the beater bar and the sides of the bowl.

12. Now, close the bowl and with the mixer speed on 2, blend for another 8-10 minutes to ensure thorough mixing.

Storage of Diets

1. Once the mixing is complete remove the bowl from the mixer and use a clean pair of gloves and scoop and scrap off all the diet sticking on the beater and sides of bowl.

2. Take out a small amount of diet into a clean Ziploc bag and keep in 4°C to be used for diet analysis.

3. Take 2 ziploc bags and put one into the other (double bag). Place the appropriately labeled card between the 2 bags.

4. Use a pair of clean gloves and scoop and take the diet into the inside bag. Fill the bag to 2/3rd and remove all the air from the bag and seal it tightly and store in -20°C in the basement.

Clean-up after mixing the diet

1. Clean the large bowl and beater using soap and the scrub in the large sink. Make sure they are thoroughly clean. Spray with ethanol and dry with paper towels. After the clean bowl and beater are put away on the cart, prepare the next diet.

2. Sweep the floor and leave it clean before you leave.

3. Make sure all the left over ingredients are closed in their respective boxes and sealed. Label the boxes with the date that it was opened and used first. Put the boxes in their respective places.

4. Wash all the small equipment in the lab and dry them thoroughly. Put all the equipment back in their appropriate place.

Protocol # A-23 Crypt Isolation Protocol

Preparation-Several days before

1. Prepare and aliquot stock solutions of BSA (10%), Glutamine (200X), EDTA (50X).

Preparation

BSA: 100 X=10 g BSA / 100 ml HBSS-CaMg
Gln: purchased from GibcoBRL at 200 mM (200 X)
Butyrate: 200 X=2.2 g Butyrate / 100 ml HBSS-CaMg
EDTA: 50 X=56 g EDTA / 100 ml HBSS-CaMg

Prepare 1X PBS
Mix 1 vial of Dulbecco's Phosphate Buffered Saline (w/o CaMg) into 990 ml RNase-free H_2O until dissolved. Store at room temperature.
This will make 10X PBS.
Dissolve 100 ml of 10X PBS into 900 ml of RNase-free H_2O to make 1X PBS.

Store EDTA at 4°C.
Store BSA and Glutamine at -80°C.

Materials

BSA: *Boehringer Manheimer, Cat# 100 018*- Mol. Wt- 68,000
Butyrate: *Acros #263190050*
DTT: *Sigma, Cat # D 0632*

0.75 M, EDTA (tetrasodium)- *Sigma, Cat# ED 45S*
200 mM, Glutamine: *Gibco, Cat# 35050*
HBSS without Calcium and Magnesium- *Sigma, Cat# H-6648*
HBSS with Calcium and Magnesium- *Sigma, Cat# H-8264*
Live-Dead Viability cytotoxicity kit- *Molecular probes, Cat# L3224*

Solutions:
1X PBS : 500 mL warm (37^{o}C), 500 mL cold (4^{o}C)

HBSS (w/Gln+BSA),EDTA,DTT Final Concentration
-1 mL – 200 mM Gln 1 mM
-2 mL - 10% BSA 0.1%
-8 mL - 0.75 M Na_4EDTA 30 mM
-o.77 gm - DTT 25 mM
-Combine Gln, BSA, EDTA, DTT in 150 mL HBSS (Sigma H-6648)
-pH to 7.4 - 7.5 with HCl
-Bring final volume up to 200mL. Warm to 37^{o}C

HBSS (w/Gln+BSA) :
-1mL - 200mM Gln
-2mL - 10% BSA
-Combine Gln, BSA in 200mL final volume HBSS (Sigma H-6648)
-Cool on ice (4^{o}C)

Solutions prepared on day of experiment
Turn on the water bath to 37°C and the Juan centrifuge to cool to 4°C

	Amount to add (in ml)			**Amt to add (in gm)**	
	EDTA 50X	Glutamine 200X	BSA 10% (100X)	DTT (as powder)	Temperature
HBSS+CaMg (300 ml)	----	1.5	3	----	4°C
HBSS-CaMg (300 ml)	12	1.5	3	1.15	37°C (pH-7.4-7.5)
Final Conc.	**30 mM**	**1 mM**	**0.1%**	**25 mM**	

Preparation for the kill
- Surgical equipment (blunt and sharp scissors, forceps)
- Ice bucket with Petri dish and rubber policeman on it to cool
- 50ml centrifuge tubes
- Cups (2) with warm PBS
- Sterile transfer pipettes
- Leibovitz media
- Large and small weigh boats
- Biohazard bags to dispose the rat

Crypt Isolation Procedure
**This procedure describes the isolation of the rat colonocytes.

1. Euthanize rat by CO_2 asphyxiation (3 minutes) and cervical dislocation. Open abdomen with a ventral midline incision and perforate the diaphragm.

2. Cut the large intestine at the rectum. Continue to remove the large intestine up to the cecum. Rinse the colon in the cups with warm PBS by shaking it gently to remove all the feces.

3. Place tissue segment in weigh boats of warm PBS.

4. Cut open the colon to expose lumen and rinse with warm PBS. Place colon in a flask with ~30 ml of HBSS (Gln+BSA) w/ EDTA+DTT buffer (~5ml/cm of tissue).

5. Incubate in the shaking water bath for 15 minutes @ 37°C. Set shaker to level #6.

6. Pour contents of the flask into a Petri dish (on ice). Gently scrape the mucosal side of the tissue with a rubber policeman.

7. Using a sterile pipette, pipette up the scraped cells and transfer the contents of the petri dish into a 50ml centrifuge tube and place in centrifuge.

8. Centrifuge at 100xg (960-1000 rpm) for 3 minutes at 4°C.

9. Remove supernatant and resuspend in 30ml HBSS (w/ Gln +BSA) to wash cells and repeat wash 3 times with centrifugation at 100xg for 3 mins.

10. Discard supernatant and resuspend pellet in 10 ml of Leibovitz buffer.

11. Keep all cell suspensions on ice and take to Image Lab.

On the day of the experiment

Time	Crypt Out	Crypt scrapped	Image lab	Loading time	Imaging time	Total time taken	Parameter measured
Rat #1	8:52 am	9:07 am	9:27 am	9:35 am	10:06 am	1 h, 14 m	Control vs C+RU
Rat #2			10:50 am (given by Ev)	10:54 am	11:14 am		Ratio over time
Rat #3			1:15 pm (given by Ev)	1:20 pm	1:50 pm		Control vs butyrate

At the Image Lab

Don't forget to take

- Warm cell suspensions
- Chamber slides pre-coated with FBS overnight
- 1.7 ml microcentrifuge tubes (labeled)
- 18x18 cover slips
- Pipette tips and pipettes
- Leibovitz media, Fluo-4, Rhod-2, Viability kit
- Centrifuge and microcentrifuge tube racks for incubator
- Protocol and time sheet
- Extra timers if necessary

Testing for Calcium

1. Hold all crypts on ice in cell culture room until needed.

2. Add 25 μL of DMSO into 50 ug vial of Fluo-4 (2 mM stock) and 10 ul DMSO into 50 ug vial of Rhod-2 (5 mM stock) and pipette up and down slowly to mix.

3. Aliquot necessary amount of viability dye, Ethidium Homodimer, (1 μL / tube) into microcentrifuge tube covered with foil.

4. Take 1.7 ml eppy tubes and label them as 0 m, 15 m and + butyrate (2 tubes for each sample). Take 900 μl of Leibovitz in each of the tubes.

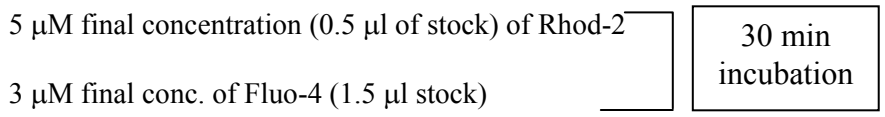
5. Add 25 μl of the appropriate crypt pellet to the '0' labeled microcentrifuge tubes. (We had duplicate tubes for each treatment).

6. Load the 2 tubes with 0.5 μl of Rhod-2 and 3 μl of Fluo-4 for 30 min

7. After 15 minutes take 25 μl of crypt pellet and load the duplicate tubes of 15 minutes time sample with Rhod-2 and Fluo-4.

8. Following 5 minutes take crypt pellet in the + butyrate sample (add 5 μM final concentration). Co-load with Rhod-2 and Fluo-4 for 30 minutes.

9. Rotate the sample with and without butyrate (do 0 and 15 minutes with butyrate followed by no butyrate in one rat and 0 and 15 mintues without butyrate followed by + butyrate sample at 20 minutes in the other). This randomizes the imaging.



10. Invert microcentrifuge tubes to mix cells and incubate the suspensions for the appropriate time at Cells will settle to the bottom of the tube.

11. Load appropriate amount of Rhod-2 and Fluo-4 to each eppy tube of cells for 30 minutes. Invert tube to mix cells and incubate at 37°C. Leave the tubes open to allow CO_2

12. After 30 minutes take the 1 set of tubes and remove the media and add 600 µl of Leibovitz gently along the sides of the tube.

13. Take 6 µl of the washed crypt pellet into the center of the well and hand it over to Dr. Rola.

14. Gently cover with an 18x18 cover slip (try to avoid smashing cells). Give to Dr. Rola for analysis.

15. Image 4 wells per sample. As we are analyzing the last sample. Load 25 µL of crypt pellet with 1 µL of ethidium homodimer and of 1 µL calcein. Incubate at 37°C for 5 minutes.

16. After incubation with the viability dye is completed, transfer 6 µL of the cell pellet to a glass microscope slide. Cover with and 18x18 cover slip. Images for viability will be captured like the rest of the samples. We estimate the percentage that is green (viable) to red cells which are not viable. This gives us an idea of the viability of the crypts.

Protocol #A-24 Crypt Cell Viability Assay

Ref: Sanders, (2004); Hong MY, (2003); Source: Molecular Probes

Purpose: To measure crypt cell (colonocyte) viability by using a The LIVE/DEAD Viability/Cytotoxicity Kit.
The LIVE/DEAD Viability/Cytotoxicity Kit used was from Molecular Probes (L3224).

Information about the kit: The live/ dead kit is a two-color assay to determine viability of cells in a population. The kit identifies live versus dead cells on the basis of membrane integrity (Ethidium homodimer-1) and esterase activity (Calcein AM).

Basis: Live cells have ubiquitous intracellular esterase activity. This is determined by the conversion of cell permeant, non-fluorescent calcein AM to a highly fluorescent calcein which is retained inside the cell to emit a uniform green signal. Ethidium homodimer-1 enters a cell with damaged plasma membrane and is excluded by the intact plasma membrane of live cells. Therefore, Ethidium homodimer enters dead cells with a compromised plasma membrane and undergoes a 40 fold enhancement in fluorescence to produce a bright red fluorescence differentiating it from a viable green cell.

Procedure

1. An aliquot of crypt pellet (at 37°C) is co-loaded with 1 µl of ethidium homodimer-1 and 1 µl of calcein AM.
2. The eppendroff tubes in incubated in the 37°C incubator (keep lid open for free flow of CO_2) for 5 min.
3. Take 6 µl of the viability dye loaded crypt pellet onto 2 wells of the chambered cover-glass.
4. Place a cover slip gently taking care not to crush the crypts.
5. Mount on the Zeiss microscope stage and image the crypts.

APPENDIX B

DATA TABLES

APOPTOSIS- Nucleosomal assay (YAMC)

Apoptosis in YAMC cells following 6, 12, 24 and 48 h butyrate co-treatment

Table B1: 24 h apoptosis data

Treatment	Mean	Std Error	p-value	Count
Control	0.394	0.203	0.05	16
Butyrate	0.904	0.613		12
LA	0.372	0.187		12
DHA	0.242	0.237		12
LB	0.741	0.351		14
DB	1.345	0.189		14

Table B2- 12 h apoptosis data

Treatment	Mean	Std Error	p-value	Count
Control	0.382	0.037	0.05	8
Butyrate	0.5.09	0.089		6
LA	0.423	0.067		6
DHA	0.330	0.121		6
LB	0.758	0.120		7
DB	1.322	0.238		7

Table B3-12 h apoptosis data

Treatment	Mean	Std Error	p-value	Count
Control	0.299	0.047	0.05	14
Butyrate	0.900	0.182		13
LA	0.423	0.067		6
DHA	0.330	0.121		6
LB	0.827	0.089		14
DB	1.263	0.170		11

Table B4- 6 h apoptosis data

Treatment	Mean	Std Error	p-value	Count
Control	0.293	0.084	0.05	6
Butyrate	0.365	0.089		4
LA	0.321	0.086		6
DHA	0.155	0.055		6
LB	0.260	0..044		6
DB	0.414	0.116		6

Table B5- 48 h apoptosis data

Treatment	Mean	Std Error	p-value	Count
Control	0.294	0.025	0.05	6
Butyrate	1.152	0.089		4
LA	0.135	0.055		4
DHA	0.216	0.036		4
LB	1.240	0.202		4
DB	1.011	0.230		4

Caspase-3 assay as an indicator of apoptosis in YAMC cells

Table B6- 24 h apoptosis data

Treatment	Mean	Std Error	p-value	Count
Control	44.716	0.924	0.05	5
Butyrate	83.242	8.440		5
LB	72.765	5.392		6
DB	104.492	7.384		5

Apoptosis in YAMC using RU-360, a blocker of mitochondrial uniporter

Table B7- 24 h Butyrate induced apoptosis in YAMC cells ±RU-360

Treatment	Mean	Std Error	p-value	Count
Control	1.859	0.129	0.0039	3
Control+RU	1.264	0.140		3
Butyrate	1.904	0.094		4
Butyrate+RU	1.781	0.231		4
LB	1.717	0.306		4
LB+RU	2.305	0.224		4
DB	3.335	0.592		4
DB+RU	2.252	0.350		4

Table B8- 12 h Butyrate induced apoptosis in YAMC cells ±RU-360

Treatment	Mean	Std Error	p-value	Count
Control	0.933	0.131	0.0002	5
Control+RU	0.708	0.079		5
Butyrate	0.941	0.112		5
Butyrate+RU	1.019	0.126		5
LB	0.741	0.194		5
LB+RU	0.919	0.095		5
DB	2.153	0.367		5
DB+RU	1.356	0.261		5

Table B9- 12 h Butyrate induced apoptosis in YAMC cells ±RU-360

Treatment	Mean	Std Error	p-value	Count
Control	0.450	0.035	0.0001	6
Control+RU	0.826	0.166		6
Butyrate	1.556	0.170		2
Butyrate+RU	1.657	0.155		3
LB	0.801	0.277		4
LB+RU	1.370	0.084		7
DB	1.940	0.197		4
DB+RU	1.227	0.409		3
STS	6.365	0.320		5
STS+RU	5.508	0.367		3

CYTOSOLIC CALCIUM

Cytosolic Ca^{2+} in YAMC cells with 6, 12, 24, 48 and 72 h Butyrate Co-Treatment

Table B10- 24 h data

Treatment	Mean	Std Error	p-value	Count
Control	1.847	0.043	0.0001	113
Butyrate	1.691	0.094		63
LA	2.381	0.079		103
DHA	2.006	0.107		84
LB	2.228	0.088		53
DB	1.966	0.038		57

Table B11- 24 h data with 10 mM butyrate

Treatment	Mean	Std Error	p-value	Count
Control	0.294	0.240	0.0001	56
Butyrate	1.152	0.226		50
LA	0.135	0.225		81
DHA	0.216	0.161		58
LB	1.240	0.149		65
DB	1.011	0.136		56

Table B12- 12 h data

Treatment	Mean	Std Error	p-value	Count
Control	1.913	0.058	0.0001	91
Butyrate	1.829	0.067		67
LA	2.519	0.094		79
DHA	2.338	0.059		93
LB	2.553	0.094		69
DB	2.139	0.049		76

Table B13- 6 h data

Treatment	Mean	Std Error	p-value	Count
Control	1.466	0.046	0.0001	46
Butyrate	1.629	0.045		58
LB	1.772	0.066		66
DB	1.949	0.068		78

Table B14- 48 h data

Treatment	Mean	Std Error	p-value	Count
Control	2.647	0.240	0.0001	56
Butyrate	3.263	0.361		11
LA	2.580	0.225		58
DHA	3.343	0.161		81
LB	3.246	0.226		12
DB	3.091	0.245		29

Table B15- 72 h data (Dec.'05)

Treatment	Mean	Std Error	p-value	Count
Control	2.647	0.240	0.0001	56
Butyrate	2.907	0.220		18
LA	2.425	0.166		57
DHA	3.343	0.161		81
LB	2.262	0.239		7
DB	2.784	0.140		17

Table B16- EGTA and Ca^{2+} add-back (Feb.18.'05)

Treatment	Mean	Std Error	p-value	Count
Control	1.865	0.053	0.0001	41
Butyrate	1.964	0.120		17
LA	1.751	0.079		47
DHA	1.535	0.897		46
LB	1.790	0.073		29
DB	1.601	0.071		34

Cytosolic calcium following inhibition of SOC with SKF-96365

Table B17- Cytosolic calcium following 24 h butyrate co-treatment with SKF, an inhibitor of SOC

Treatment	Mean	Std Error	p-value	Count
Control	1.847	0.043	0.0001	6
Control+SKF	1.090	0.060		6
Butyrate	1.691	0.094		6
Butyrate+SKF	1.029	0.026		6
LB	2.381	0.088		6
LB+SKF	0.728	0.029		6
DB	1.966	0.038		6
DB+SKF	1.009	0.079		6

Table B18- 12 h butyrate induced cytosolic calcium change with SKF-96365 (Mar.05.'05)

Treatment	Mean	Std Error	p-value	Count
Control	1.913	0.058	0.0001	6
Control+SKF	1.090	0.060		6
Butyrate	1.829	0.067		6
Butyrate+SKF	1.032	0.085		6
LB	2.553	0.094		6
LB+SKF	0.925	0.064		6
DB	2.139	0.049		6
DB+SKF	0.872	0.053		6

MITOCHONDRIAL CALCIUM

Mitochondrial :Cytosolic Ca^{2+} Ratio in YAMC cells with 6, 12 and 24 h butyrate treatment

Table B19 -24 h data

Treatment	Mean	Std Error	p-value	Count
Control	2.855	0.048	0.0001	46
Butyrate	2.716	0.055		50
LA	2.575	0.070		40
DHA	3.152	0.048		47
LB	2.842	0.055		36
DB	3.826	0.072		36

Table B20- 12 h data

Treatment	Mean	Std Error	p-value	Count
Control	2.801	0.137	0.0001	43
Butyrate	2.704	0.047		39
LA	2.001	0.078		44
DHA	2.425	0.058		44
LB	2.650	0.042		44
DB	3.167	0.069		45

Table B21- 6 h data

Treatment	Mean	Std Error	p-value	Count
Control	3.014	0.095	0.0001	44
Butyrate	3.054	0.050		44
LA	2.980	0.053		44
DHA	4.841	0.125		44
LB	3.148	0.065		42
DB	5.189	0.111		40

Mitochondrial :Cytosolic Ca^{2+} Ratio in YAMC cells with RU-360 (6, 12 and24 h) and Butyrate Co-treatment

Table B22- 24 h Mitochondrial calcium with RU-360- 1 h RU-360 data

Treatment	Mean	Std Error	p-value	Count
Control	3.747	0.080	0.0001	18
Control+RU-360	3.379	0.035		21
DB	5.963	0.199		20
DB+RU-360	4.762	0.267		32

Table B23- 12 h Mitochondrial calcium with RU-360- 1 h RU

Treatment	Mean	Std Error	p-value	Count
Control	1.971	0.065	0.0001	24
Control+RU-360	2.010	0.051		23
Butyrate	2.280	0.084		22
Butyrate+RU-360	2.792	0.099		22
LB	2.067	0.040		23
LB+RU-360	2.054	0.061		21
DB	3.468	0.147		22
DB+RU-360	3.039	0.130		23

Table B24- 6 h Mitochondrial calcium with RU-360- 1 h RU

Treatment	Mean	Std Error	p-value	Count
Control	1.743	0.045	0.0001	20
Control+RU-360	1.957	0.069		22
Butyrate	1.667	0.052		24
Butyrate+RU-360	1.965	0.081		22
LB	1.698	0.023		25
LB+RU-360	1.834	0.054		22
DB	3.439	0.159		21
DB+RU-360	2.559	0.090		22

p53 CELL LINE DATA

Apoptosis in p53 wild type (WT) and Knock out (KO) cells

Table B25- 12 h Butyrate induced apoptosis in p53 wild type (WT) cells

Treatment	Mean	Std Error	p-value	Count
WT-Control	0.199	0.022	0.0001	6
WT-LB	1.253	0.132		6
WT-DB	1.807	0.211		5
KO-Control	0.187	0.020		5
KO-LB	0.940	0.086		5
KO-DB	1.458	0.106		5

Apoptosis in p53 cells using RU-360, a blocker of mitochondrial uniporter

Table B26- 12 h Butyrate induced apoptosis in p53 wild type (WT) cells ±RU-360

Treatment	Mean	Std Error	p-value	Count
WT-Control	0.199	0.022	0.0001	6
WT-Control+RU	0.133	0.017		6
WT-LB	1.253	0.132		6
WT-LB+RU	0.976	0.024		6
WT-DB	1.807	0.211		5
WT-DB+RU	0.961	0.059		6

Table B27- 12 h Butyrate induced apoptosis in p53 knock out (KO) cells ± RU-360

Treatment	Mean	Std Error	p-value	Count
KO-Control	0.187	0.020	0.0001	5
KO-Control+RU	0.213	0.038		6
KO-LB	0.903	0.100		4
KO-LB+RU	0.778	0.033		5
KO-DB	1.400	0.103		6
KO-DB+RU	0.801	0.035		6

Apoptosis in p53 cells using Mito-Q, a mitochondrially targeted antioxidant

Table B28- 12 h Butyrate induced apoptosis in p53 wild type (WT) cells ± Mito-Q

Treatment	Mean	Std Error	p-value	Count
WT-Control	0.261	0.068	0.0001	6
WT-Control+MQ	0.310	0.021		6
WT-LB	1.221	0.057		6
WT-LB+MQ	0.834	0.076		6
WT-DB	1.594	0.153		6
WT-DB+MQ	0.963	0.130		5

Table B29- 12 h Butyrate induced apoptosis in p53 knock out (KO) cells ± MitoQ

Treatment	Mean	Std Error	p-value	Count
KO-Control	0.302	0.042	0.0001	6
KO-Control+MQ	0.201	0.091		6
KO-LB	1.475	0.204		6
KO-LB+MQ	0.905	0.207		6
KO-DB	1.996	0.149		6
KO-DB+MQ	1.477	0.226		6

Mitochondrial :Cytosolic Ca^{2+} Ratio in p53 cells with 6, 12 and 24 h Butyrate Co-treatment

Table B30 - 24 h Mitochondrial calcium in p53 wild type (WT) and knock out (KO) cells

Treatment	Mean	Std Error	p-value	Count
WT-Control	0.547	0.030	0.0001	28
WT-LB	0.441	0.021		23
WT-DB	0.689	0.057		17
KO-Control	0.490	0.023		20
KO-LB	0.410	0.027		18
KO-DB	0.797	0.086		9

Table B31- 6 h Mitochondrial calcium in p53 wild type (WT) and knock out (KO) cells

Treatment	Mean	Std Error	p-value	Count
WT-Control	0.492	0.018	0.0001	27
WT-LB	0.448	0.024		24
WT-DB	0.703	0.048		27
KO-Control	0.533	0.017		24
KO-LB	0.446	0.018		22
KO-DB	0.645	0.061		24

Table B32- 24 h Mitochondrial calcium in p53 wild type (WT) and knock out (KO) cells

Treatment	Mean	Std Error	p-value	Count
WT-Control	0.581	0.021	0.0001	38
WT-LB	0.539	0.031		30
WT-DB	0.698	0.032		41
KO-Control	0.497	0.074		7
KO-LB	0.479	0.094		30
KO-DB	0.857	0.230		25

Table B33- 6 h Mitochondrial calcium in p53 wild type (WT) and knock out (KO) cells

Treatment	Mean	Std Error	p-value	Count
WT-Control	0.583	0.028	0.0001	30
WT-LB	0.584	0.035		41
WT-DB	0.877	0.021		38
KO-Control	0.721	0.038		33
KO-LB	0.532	0.011		47
KO-DB	0.960	0.044		32

Table B34- 12 h Mitochondrial calcium in p53 wild type (WT) and knock out (KO) cells

Treatment	Mean	Std Error	p-value	Count
WT-Control	1.921	0.095	0.0001	27
WT-LB	2.101	0.084		30
WT-DB	2.416	0.052		32
KO-Control	2.237	0.044		37
KO-LB	2.132	0.084		29
KO-DB	2.506	0.052		31

Mitochondrial :Cytosolic Ca^{2+} Ratio in p53 cells with RU-360 and 12 h Butyrate Co-treatment

Table B35- 12 h Mitochondrial calcium in p53 wild type (WT) cells ± RU-360

Treatment	Mean	Std Error	p-value	Count
WT-Control	0.872	0.035	0.0001	29
WT-Control+RU	0.843	0.039		35
WT-LB	0.982	0.027		35
WT-LB+RU	0.975	0.028		33
WT-DB	1.136	0.050		34
WT-DB+RU	0.946	0.020		34

Table B36- 12 h Mitochondrial calcium in p53 knock out (KO) cells ± RU-360

Treatment	Mean	Std Error	p-value	Count
KO-Control	0.832	0.028	0.0001	26
KO-Control+RU	0.866	0.023		27
KO-LB	0.897	0.017		35
KO-LB+RU	0.729	0.018		39
KO-DB	1.184	0.056		27
KO-DB+RU	0.977	0.035		32

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Second Prize (Spring, 2007)

Publications (abstracts excluded)

Ng Y, Barhoumi R, Tjalkens RB, Fan YY, **Kolar S**, Wang N, Lupton JR, Chapkin RS. The role of docosahexaenoic acid in mediating mitochondrial membrane lipid oxidation and apoptosis in colonocytes. Carcinogenesis. 2005;26(11):1914-21.

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