THE ROLE OF DOCOSAHEXAENOIC ACID IN MEDIATING MITOCHONDRIAL MEMBRANE LIPID OXIDATION AND APOPTOSIS IN COLONOCYTES

A Thesis

by

YEE VOON NG

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

MASTER OF SCIENCE

August 2004

Major Subject: Nutrition

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Approved as to style and content by:

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ABSTRACT

The Role of Docosahexaenoic Acid in Mediating Mitochondrial Membrane Lipid Oxidation and Apoptosis in Colonocytes. (August 2004)

Yee Voon Ng, B.S., University of Nebraska, Lincoln

Chair of Advisory Committee: Dr. Robert S. Chapkin

Colon cancer is the second leading cause of cancer death in the United States. Epidemiological data indicate that the consumption of dietary fiber and fish/marine products favorably modulate colon tumorigenesis. Docosahexaenoic acid (DHA, 22:6n-3) from fish oil, and butyrate, a fiber fermentation product generated in colon, protect against colon tumorigenesis in part by inducing apoptosis. We have shown that DHA is incorporated into mitochondrial membrane phospholipids, which enhances oxidative stress and mitochondrial membrane potential (MP) dissipation. To elucidate the subcellular origin of oxidation induced by DHA and butyrate exposure, young adult mouse colonocytes (YAMC) were treated with 0-200 μM DHA, linoleic acid (LA, 18:2n-6) or no fatty acid (control) for 72 h with or without 5 mM butyrate for the final 6-24 h. Real time analysis of cellular membrane lipid oxidation, as indicated by oxidation of a lipophilic vital dye, mitochondrial permeability transition (MPT), as characterized by MP dissipation, and cytosolic ROS production, as depicted by hydrophilic ROS reactive fluorophore accumulation, were measured by living cell fluorescence microscopy. After 24 h of butyrate treatment, DHA primed cells showed a 29% increase in lipid oxidation ($p<0.01$), compared to no butyrate treatment, which could be blocked by a mitochondria targeted antioxidant, MitoQ ($p <0.05$), whereas LA treatment did not show an effect. In the absence of butyrate, DHA treatment, compared to LA, increased resting MP by 14% ($p <0.01$). In addition,

butyrate-induced MP dissipation was greater (20%) in DHA primed cells as compared to LA (10%). This effect was blocked by pre-incubation with MPT inhibitors, cyclosporin A or bongkrekic acid at 1 μM. These data suggest an increase in mitochondrial lipid oxidation and the resultant change in MP may contribute to the induction of apoptosis by DHA with butyrate as shown previously.

This thesis is dedicated ~

to my dearly missed godmother, Mdm. Quay, Siew-Ching,,
whose charisma has touched my soul;

to my incredible parents, Mr. Ng, Ah-Hai and Mdm. Ong, Soo-Jit,
whose unconditional love has nurtured and brought me up to be a decent person;

to my two brothers, Kok-Ming and Kok-Leong,
whose brotherly love has protected and saved me from many hurdles in life;

to my beloved boyfriend, Mr. Wong, Wei-Suei
who has supported me patiently;

to all of my friends, who have taught me many good lessons in life;
and lastly,

to my major professor, Dr Robert S. Chapkin and lab-mates, who have provided me with all kinds of support during my years in graduate school at Texas A&M, College Station.

"Thank you for your love and support!"

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CHAPTER I

INTRODUCTION

Colon cancer is the second most prevalent type of cancer and is a leading cause of cancer death in the United States and in essentially all economically developed countries (1). Over 940,000 cases of colorectal cancer occur annually worldwide, among which North America is one of the continents reporting the highest incidence rate. It is noteworthy that 50% of these cases result in death (1, 2), *i.e.* colorectal cancer is responsible for some 470,000 deaths each year, among which 57,100 are Americans (1). Nevertheless, it is estimated that 70% of colorectal cancers are preventable by moderate changes in diet and lifestyle. For instance, the European Prospective Investigation into Cancer and Nutrition (EPIC) suggests that a daily consumption of 500 grams (1.1. lbs.) of fruits and vegetables can decrease incidence of cancers of the digestive tract by up to 25% (2).

Diet, tumorigenesis and apoptosis—an overview

Epidemiological studies indicate that populations ingesting higher amounts of fish are at a lower risk for colon cancer and a lower mortality rate of colorectal cancer (3-5), compared to those ingesting diets high in saturated fat or other polyunsaturated fatty acids (PUFA). These studies indicate a chemoprotective role for omega-three (n-3) PUFA. Dietary fibers also play a favorable role in the prevention of colon carcinogenesis (5-7). Different combinations of long chain PUFA and fibers in the diet modulate colon tumorigenesis. Most recently, dietary fish oil, and fiber, such as those from fruits and vegetables, in combination has been demonstrated to be beneficial in the prevention of colon cancer (3, 8). In a rat model of colon carcinogenesis, diets containing fermentable fiber and fish oil have been shown to protect against colon tumorigenesis, compared to

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This thesis follows the style and format of Cancer Research.

diets rich in cellulose and corn oil (7, 9).

With regard to dietary intervention, apart from cell proliferation and differentiation, multi-step programmed cell death, *i.e.,* apoptosis, has been extensively investigated as a biological marker regulating colon cancer risk. Cancer studies at the cellular level provide evidence that an abnormality in the molecular expression, configuration, functionality and/or interaction of proapoptotic signaling molecules contribute independently or synergistically to the inhibition of cancer cell growth (see review (10)). For instance, reactive oxygen species (ROS) and their counterparts, intracellular antioxidants, are involved in a multitude of biological effects including mutagenesis, tumor promotion, regulation of proliferation, activation or inactivation of biomolecules, regulation of transcriptional activities as well as the activation and execution of apoptosis.

Effect of dietary long chain fatty acids on cellular and mitochondrial membrane structures

Together with eicosapentaenoic acid (EPA, 20:5n-3), docosahaexenoic acid (DHA, 22: 6n-3) is a member of n-3 PUFA family found in high concentration in fish oil. Dietary fatty acids are incorporated to cell membrane phospholipids and have effects on, but not limited to, cell membrane composition and function (9, 11), membrane lipid oxidation (12, 13), transcriptional or translational regulation (14, 15), eicosanoid biosynthesis (15) and molecular signal transduction (10). These pleiotropic effects are believed to contribute to the observation that dietary fish oil, specifically n-3 PUFA, suppresses the initiation and promotion phases of colon cancer development.

Cumulative evidence shows that n-3 PUFA, in contrast to n-6 PUFA, uniquely affect cell cytokinetics by altering the cellular microenvironment through changes to membrane composition, thereby promoting apoptosis (7, 16-18). In animal studies, supplementing diets with fish oil, a rich source of EPA and DHA, significantly alters tissue levels of these fatty acids. n-3 PUFA from fish oil or purified ethyl esters were incorporated into colonocyte mitochondrial membrane

phospholipids (16, 18), which coincided with the enhancement of apoptosis in the colon (7, 19). A similar trend was observed utilizing colonocyte cell lines, in which the incorporation of DHA into cardiolipin, a mitochondrial membran:e phospholipid, was associated with the induction of apoptotic signaling (11, 17). To date, the significance of fatty acid incorporation into membrane lipids in relation to the induction of apoptosis has not been determined. We hypothesize that the incorporation of n-3 PUFA increases cell susceptibility to oxidative damage, and subsequently perturbs the integrity of mitochondrial membranes.

n-3 PUFA and oxidative stress

Dietary n-3 PUFA may increase apoptosis in colonocytes via a mechanism involving ROS production (20). We have shown that an increase in ROS production following fish oil supplementation in the diet is correlated with the induction of apoptosis in colonic crypts (21, 22). In addition, data from cell culture studies have demonstrated that DHA treatment increases ROS production and induces apoptosis (11, 13, 16). Furthermore, antioxidants, which specifically target membrane lipid oxidation, partially reverse the effect of DHA-induced apoptosis in human colon carcinoma cells (17). This evidence in its entirety suggests that oxidative stress, in particular lipid oxidation may favorably modulate apoptosis in cells enriched with n-3 PUFA. It is noteworthy that even though EPA and DHA are both n-3 PUFA, they may exert protective effects against colon cancer through independent mechanism (23, 24). In the present study, DHA was used to determine the mechanism by which dietary fish oil relative to corn oil, reduce colon cancer risk.

DHA is a long chain n-3 PUFA, and compared to linoleic acid (LA, 18:2n-6), the major PUFA constituent of corn oil, is more susceptible to oxidation (25). The pro-oxidative properties of DHA are attributable to both the position and the number of its double bonds. Specifically, the oxidation of PUFA is linearly dependent on the number of double bonds present (13, 25). Ex vivo data have also shown that colonocytes are capable of incorporating substantial quantities of EPA and DHA, each of which contains five and 6 double bonds, respectively, are highly susceptible to

lipid oxidation (18, 19, 23). Interestingly, the number of fatty acid double bonds, ROS levels, and the proportion of cells undergoing apoptosis in cancer cell lines has been correlated (9, 13). In addition, with respect to fatty acid class, n-3 PUFA, *e.g.,* DHA is more susceptible to oxidation compared to n-6 PUFA, *e.g.,* LA (20). Collectively, these data support our hypothesis that n-3 PUFA, found primarily in fish oil, can induce apoptosis through a mechanism involving the oxidation of membrane phospholipids.

The oxidation of PUFA during metabolic stress gives rise to a series of toxic α,β-unsaturated aldehydes, *i.e.,* enals, including the electrophile, 4-hydroxy-2-nonenal (4-HNE). With respect to additional/alternative mechanisms of action, these class of lipid oxidation products could be cytotoxic and may be pro-apoptotic (26-28). In vivo, animal studies examining the effect of dietary fish oil or DHA supplementation on DNA lesion formation showed that a fish oil/DHA fortified diet significantly increased oxidative damage (20) but decreased the net DNA adduct (8-oxodeoxguanine; O^6-methylguanine) levels in colonocytes (29). These observations suggest that dietary fish oil protects intestinal cells against oxidative DNA damage in part via deletion mechanisms, since the increase of DNA lesion was coupled to an incremental activation of apoptosis (27, 29, 30).

In addition to increasing oxidative stress by giving rise to pro-oxidants, DHA has the ability to modulate gene transcription and the homeostasis between ROS and antioxidants, thereby rendering cells more susceptible to apoptosis. Specifically, DHA has been found to induce lipid oxidation indirectly by influencing gene transcription. PUFA, in general, are capable of enhancing the activation of PPAR-α, which subsequently has been linked to the induction of several genes encoding proteins responsible for lipid transportation and oxidation (14). Collectively, these data suggest that DHA may be pro-oxidative and pro-apoptotic via several overlapping mechanisms.

To further illustrate the importance of oxidative stress and antioxidants balance with regard to apoptosis induction, several studies have examined the activities and expression levels of intracellular antioxidants (31, 32). Endogenous antioxidants may attenuate ROS-induced cell

damage and reduce cell death, initiated by pro-oxidants, such as n-3 PUFA. In vivo, colon epithelial cells expressed a greater endogenous antioxidant capacity in part as a compensatory mechanism towards chronic oxidative stress exposure, compared to the small intestine, sharing similar morphology (33). It is now readily apparent that, besides being pro-oxidative, n-3 PUFA are capable of suppressing the activity and expression of intracellular antioxidant enzymes, thereby enhancing tissue susceptibility towards oxidative stress and subsequent apoptosis induction (20, 32, 34). For example, the activities of endogenous antioxidants, such as glutathione transferase, glutathione reductase, glutathione peroxidase, and catalase appear to be depleted in animals fed a diet enriched with n-3 PUFA compared with diets supplemented with n-9 monounsaturated fatty acid or with n-6 PUFA (35). In a preliminary study, we have also demonstrated that in the colonic crypts of animals fed a fish oil/pectin supplemented diet, the antioxidant activities of catalase and superoxide dismutase were lowered (33) resulting in an increase in ROS and oxidative stress. Thus far, from a disease prevention standpoint, accumulating evidence suggests that n-3 PUFA or DHA promote cancer cell death. These data suggest that the perturbation of redox balance within a cell may be beneficial to the host under certain biological conditions, especially in malignant host cells.

Interaction between dietary fatty acids and butyrate

In conjunction with n-3 PUFA, which have anti-carcinogenic and anti-tumorigenic effects, butyrate in the lumen may synergistically modulate colon carcinogenesis by enhancing or inducing ROS production in vivo (36-38). Our preliminary data show that the feeding of pectin, a fermentable fiber that increases luminal butyrate levels, upregulates ROS production in the proximal colon, primarily in rats fed a fish oil supplemented diet (22, 30, 33). Ex-vivo incubation of rat colonic crypts with physiological concentrations of butyrate also exhibited a similar ROS up-regulation property (9). In contrast, n-6 PUFA incorporation into colonic epithelial cell mitochondrial membranes in corn oil fed rats resulted in a decrease in ROS levels following butyrate treatment (19).

Butyrate, along with other short chain fatty acids (SCFAs), mainly acetate and propionate, is produced in the colonic lumen by microorganisms via the break down of complex carbohydrates and fermentable fibers (*e.g.,* pectin from fruits and vegetables). Among the SCFAs produced, butyrate is the most efficient energy source and the most potent inhibitor of colon cancer invasion (6, 39). It is an essential energy source capable of activating apoptotic pathways in colonocytes. Mice with a homozygous deletion of the gene for short chain acyl dehydrogenase, which encodes an enzyme necessary for the mitochondrial β-oxidation of SCFAs, showed a reduced ability to metabolize butyrate, resulting in a greater than 90% decrease in apoptosis in colonocytes (6).

Localized to the gut, fiber fermentation permits colon epithelial cells to have direct access to an energy source, and creates a channel for butyrate to modulate colon cancer development through epigenetic or genetic means. It is noteworthy that the efficacy of butyrate uptake is dependent on factors such as the co-existing substrate in the lumen (40) and the pathogenesis phase of the mucosa (41). In addition, butyrate could modulate colon tumorigenesis via a gene transcription regulated pathway (42). Hence, the effects of butyrate may take time to appear and could be subtle in nature.

Butyrate can induce cellular oxidative stress and can modulate cellular gene expression leading to changes in cell cycle arrest, maturation, differentiation, as well as apoptosis (36-38, 42). These paradoxical effects of butyrate may be determined by the state of activation of the cells, such as the timing and the amount of butyrate administered, the source of butyrate, and the interaction with dietary fat (43, 44).

With respect to alternative mechanisms to induce apoptosis, early studies showed that butyrate is a potent histone deacetylase (HDAC) inhibitor, which causes nonspecific hyperacetylation of histones and successive alteration in gene expression (42, 45) in favor of colon cancer cell death. In addition, butyrate treatment can increase mitochondrial protein expression, which appears to be related to the enhancement of mitochondrial function and the induction of apoptosis in colonic epithelial cells (46). Our preliminary data, in particular, have shown that

butyrate may activate apoptosis through the activation of Fas/Fas-L and/or a mitochondrial regulated pathway (9, 47). In summary, data from these studies suggest that various mechanisms may account for the protective effects of butyrate against colon cancer. For the purpose of this study, oxidative stress induction and the mitochondrial-targeted apoptosis activation properties of butyrate were investigated.

Due to the complex interaction among dietary components, the ability and the effectiveness of butyrate to upregulate apoptosis and to protect against colon tumorigenesis appears to be dependent on the dietary lipid source, with fish oil being more protective than corn oil (9, 16). In immortalized mouse colon cell lines, the ability of PUFA, including DHA and LA to promote apoptosis was enhanced by a 24 h incubation with ≥ 1mM butyrate (16). Herein, we have hypothesized that butyrate creates a permissive environment for apoptosis in part by enhancing n-3 PUFA-induced oxidative stress.

Reactive oxygen species regulates cellular events

Preexisting oxidative stress levels within a cell differentially modulate cell sensitivity towards successive ROS assault (33, 37, 48). Under basal conditions or when transient oxidative stress is encountered, some cell populations have adapted to the existence of reactive intermediates by evolving defense mechanisms that either scavenge these intermediates or repair the damage they cause. On the other hand, a transient oxidative stress surge may also lead to cell death through apoptosis or necrosis and inhibit proliferation in some cell lines, which most probably does not favor cancer progression (28). It is possible therefore that ROS may represent key elements for selective and efficient apoptosis induction by natural antitumor systems (49).

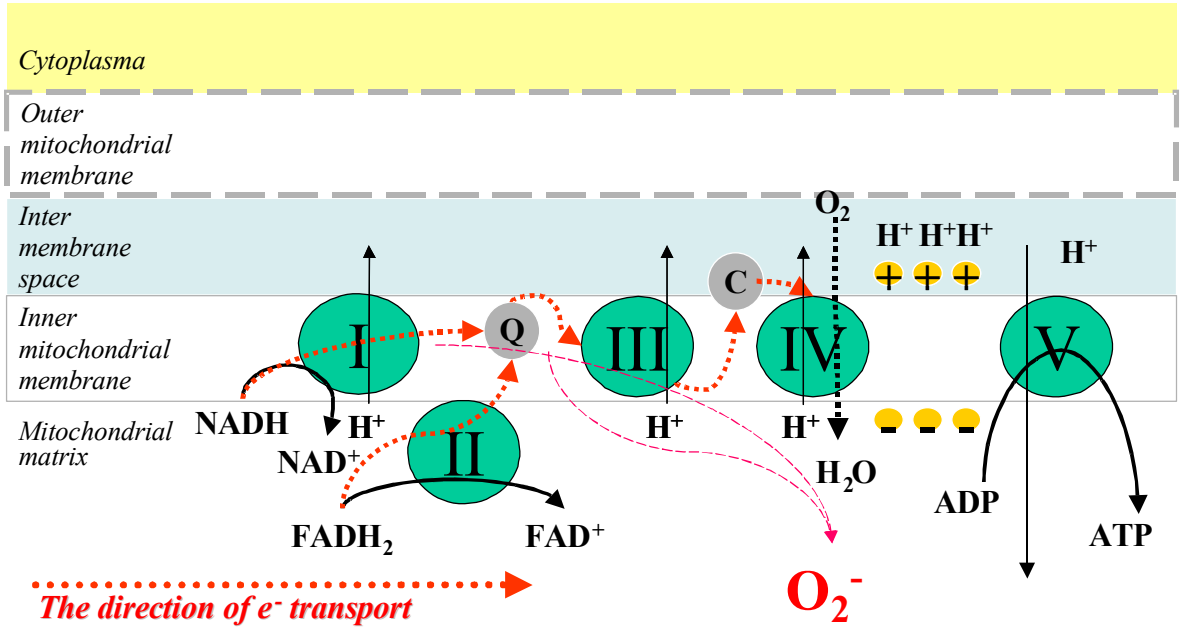
On the other hand, following prolonged exposure to oxidative stress, cells may become hyper-resistant to ROS-induced apoptosis, an event contributing to aging and to carcinogenesis (50). Interestingly, fish oil and butyrate are potent inducers of apoptosis, especially in malignant transformed cells that may be chronically overexposed to oxidative stress. Dietary fish oil or DHA

capable of altering the oxidative balance in colonocytes to rectify an increase in oxidative stress, could promotes apoptosis (3, 5, 17, 29, 34).

Intracellular formation and propagation of ROS

During aerobic metabolism, oxidative damage can accumulate in the mitochondria relative to the rest of the cell because electrons continually leak from the respiratory chain to form damaging ROS (49, 51). This oxidative damage impairs mitochondrial ATP synthesis and Calcium (Ca^{++}) homeostasis and may induce the opening of the mitochondrial permeability transition (MPT) pores, thereby triggering necrotic or apoptotic cell death (52, 53).

Within the inner mitochondrial membrane, NADH is oxidized to NAD^+ by NADH-Ubiquinone reductase, while $FADH_2$ is oxidized to FAD by succinate-Ubiquinone reductase. The electrons from NADH and $FADH_2$ are both transferred to Ubiquinone, generating Ubiquinol. Located near complexes I, II and III is Ubiquinone (Q), which receives 2 electrons (to form Ubiquinol) from complex I and II and further passes electrons to complex III (to form Ubiquinone). In addition, Ubiquinone can donate electrons to oxygen to form superoxide (O_2^-) (and Ubiquinol) thereby transferring ROS into the aqueous mitochondrial matrix. Ubiquinone is recycled to Ubiquinol and continues serving as an antioxidant within the inner mitochondrial membrane (51). Electrons flow from Ubiquinol through a series of cytochromes to oxygen, the ultimate acceptor of electrons, which is reduced to water at complex IV. The transfer of electrons along mitochondrial complexes I, III and IV are coupled to the pumping of protons from the mitochondrial matrix to the inter membranes space (Fig. 1). The extrusion of protons from the mitochondrial matrix generates a pH gradient and an electrical potential difference across the membrane, which together constitute the proton motive force that drives ATP synthesis (54). Protons are translocated down a gradient through complex V to produce ATP.



I: NADH-ubiquinone reductase; **II**: Succinate-ubiquinone reductase; **III**: Ubiquinol-cytochrome C reductase; **IV**: Cytochrome Coxidase; **V**: ATP synthase; **Q**: Ubiquinone/ubiquinol; **C**: Cytochromes

Fig. 1. Electron transportation and mitochondrial complexes. Electrons (e^-) are transported down mitochondrial complexes I, III and IV, a process coupled to the pumping of protons (H^+) from inside of the inner membrane to the inter membrane compartment. This electron transport process results in the leakage of superoxide (O_2^-) and the initiation of ROS propagation.

Incomplete reduction of oxygen during aerobic metabolism generates O_2^-. It is estimated that up to 10% of the reducing equivalents from NADH can leak out of the respiratory chain in the form of O_2^- (54). Mitochondria have the ability to counter oxidative stress hyper-production, *i.e.* electron transport and leakage, and damage by shunting H^+ back to the mitochondrial matrix. Via the effect of a group of Uncoupling Proteins or through allosteric inhibition of the electron transport chain by ATP/ADP ratio through a negative feedback mechanism (54), hyper-production of oxidative stress could be regulated.

In many cases, the initially generated reactive intermediates in the mitochondrial respiratory chain convert cellular constituents into second-generation reactive intermediates capable of inducing further damage. Specifically, the majority of O_2^- generated is dismutated to form H_2O_2, while some serves as a substrate in Fenton reactions or can react with nitric oxide to generate

other reactive nitrogen species and electrophiles. Among all, α,β-unsaturated aldehydes are the most studied membrane phospholipid oxidation products capable of denaturing protein or nucleic acids (20, 28) and causing apoptosis in some models (55).

Mitochondrial structure and membrane potential changes during apoptosis

The permeability transition (MPT) pore is a multiprotein complex formed at the contact site between the mitochondrial inner and outer membranes (Fig. 2). This multiprotein complex is constituted primarily of a voltage dependent anion channel (VDAC), anion nucleotide transferase (ANT) and cyclophylin-D (56-58). The MTP pore is directly involved in the regulation of mitochondrial matrix Ca^{++}, transmembrane potential and cellular activation of apoptosis. The binding of cyclosporin A (CsA) to cyclophylin-D and bongkrekic acid (BKA) to ANT, inhibits MPT activation, subsequently preventing the dissipation of mitochondrial membrane potential (MP) (57, 59, 60).

MPT is characterized by a sustained opening of MPT pores, following a Ca^{++} overload in the mitochondria inner compartment (53, 57, 61), leading to apoptosis. The full-blown activation of MPT pores permits a non-specific release of soluble mitochondrial proteins from the intermembrane space during MPT (53, 57). Some well-studied mitochondrial proteins being released can serve as apoptosis inducing intermediates, *i.e.* cytochrome C and SMAC/DIABLO, by amplifying or transmitting apoptotic signals. On the other hand, transient or full-blown MPT activation has always been coupled by dissipation of the MP as a result of the disruption of the inner mitochondrial membrane and the inability of membranes to maintain a proton gradient. These changes occur in most, if not all, models of cell death and can be taken advantage of to detect apoptosis at an early stage. In addition, changes in the inner mitochondrial membrane and the permeability transition of the mitochondrial membrane are coupled in most cases to a series of catabolic reactions, which result in cell death (62). Interestingly, during the activation of the

energy requiring apoptosis, only a few polarized mitochondria can be detected, because a subpopulation of respiring mitochondria within an individual cell generate ATP needed for the completion of the apoptotic cascade (63).

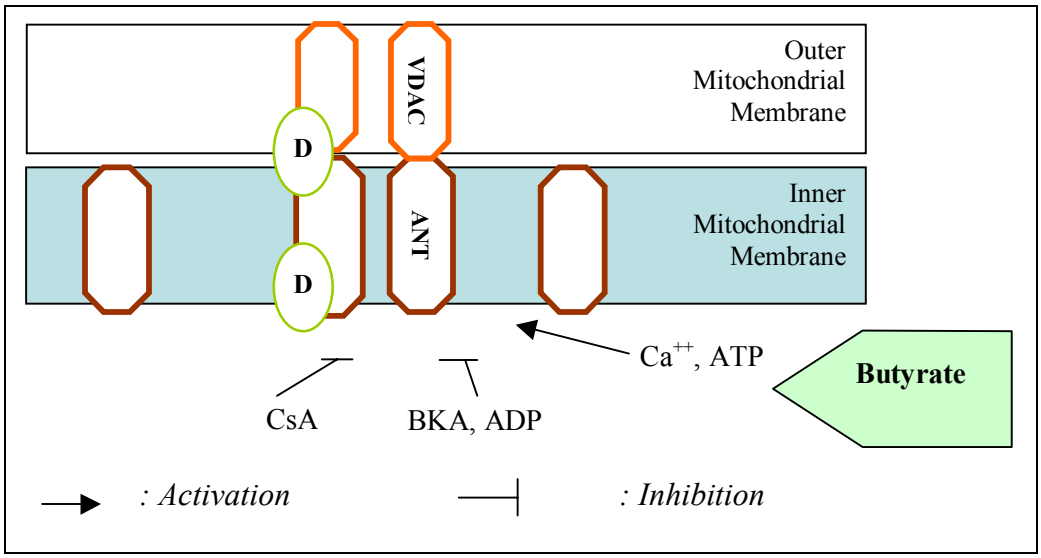


Fig. 2. Mitochondrial permeability transition pores. The mitochondrial permeability transition pore contains the voltage dependent anion channel (VDAC) and the anion nucleotide transferase (ANT). Cyclosporin A (CsA) and Bonkrekic acid (BKA) inhibit membrane permeability transition through binding to Cyclophilin D (D) and ANT, respectively.

Mitochondria related pro- and anti-apoptotic Bcl-2 family proteins

The activation of the MPT and subsequent dissipation of MP appears to be an early apoptotic event regulated by members of the Bcl-2/Bax family, and is irreversible in many cell lines, such as colon epithelial cells (9, 48, 62). Bcl-2 family proteins, which reside within mitochondrial membranes or the inter-membrane space, demonstrate unique binding domains for one another that affect their activation and function (64). These proteins dimerize or oligomerize with one another to induce or inhibit apoptosis, depending on their pro-apoptotic or anti-apoptotic roles (57). For example, the pro-apoptotic Bid protein and its truncated form (tbid) have a BH_3 only domain, which is required for the dimerization or oligomerization with other pro-apoptotic mitochondrial proteins to induce mitochondrial-involved apoptosis (64). In addition, tBid

specifically targets to the mitochondria via cardiolipin binding (65), causing the release of holocytochrome C, which is an effector in a cascade of events leading to apoptosis (65, 66).

We have found that fish oil feeding increases apoptosis while decreasing Bcl-2 expression in colonic crypts of carcinogen injected rats (67). In addition, butyrate via an unknown mechanism, also enhanced cytochrome-C translocation from the mitochondrial to the cytosolic compartment in rats fed a fish oil or n-3 fatty acid ethyl ester rich diet (9). Hence, we have hypothesized that DHA and butyrate induce apoptosis through a mitochondria mediated pathway.

Oxidative stress and mitochondria function

The precise role of mitochondrial ROS and membrane lipid oxidation in colon cell death remains elusive. A plethora of publications have shown that a oxidative assault triggers MPT thereafter activating apoptosis in many cell lines (49, 52, 53, 68). In addition, it is noteworthy that a full-blown MPT and ROS production may amplify each other to warrant an induction of apoptosis (53).

To date, limited evidence links n-3 PUFA and MPT directly to apoptosis in colonic epithelial cells. Herein, we investigated whether DHA and lipid oxidation of mitochondrial membrane phospholipids, followed by oxidative activation of MPT, is capable of triggering colonocyte apoptotic pathways.

Diet modulates mitochondrial membrane permeability transition and apoptosis

Dietary factors may alter the metabolism and redox balance within mitochondria, thereby priming cells for the induction of apoptosis. The acyl groups of mitochondrial phospholipids, *i.e.* cardiolipin, glycerophosphocholine and glycophosphoethanolamine, are altered in rats fed diets differing only with respect to fatty acid content (9, 11). Interestingly, we have shown that the incorporation of n-3 PUFA into glycophosphoethanolamine is associated with the dissipation of MP (19). In addition, butyrate treatment and butyrate/n-3 PUFA co-treatment have been shown to

enhance cytochrome-C relocation (9), MP dissipation and apoptosis in colonocytes (17, 46, 47).

We were able to demonstrate that the pronounced effect of n-3 PUFA or dietary fish oil on apoptosis can be reproduced in a cell culture model. Previously, using young adult mouse colonic (YAMC) epithelial cell lines, we have demonstrated that incubation with DHA increases caspase-3 activity, *i.e.* a marker for apoptosis (16). The conditionally immortalized mouse colon cell line, YAMC, bears a SV-40 heat sensitive large T antigen gene and can escape cell cycle arrest regulated by p53 and retinoblastoma when incubated in a permissive environment (33˚C with 5% CO_2) with γ-interferon (16, 47, 69, 70). p53 signaling regulates cell cycle delay and apoptosis in colon carcinoma cells (71, 72). Studies have suggested that dietary factors may differently affect p53-dependent and p53-independent pathways to colon cancer (72). This is relevant because butyrate is capable of inducing apoptosis in several human colon carcinoma and adenoma cell lines in a p53-independent manner (47, 69, 73). Furthermore, mutations in the p53 tumor suppressor gene have been found in more than 50% of human colon tumor cell types (73-75). These data in its entirety suggests that YAMC cells culture is an appropriate model to further investigate the mechanism by which DHA and butyrate modulate mitochondria function.

CHAPTER II

HYPOTHESIS AND OBJECTIVES OF STUDY

Hypothesis

The incorporation of n-3 PUFA increases cell susceptibility to oxidative damage, and subsequently perturbs the integrity of mitochondrial membranes, a process potentiated by butyrate.

Objectives

1. To quantify cellular and mitochondrial membrane lipid oxidation in colonocytes following n-3 and n-6 PUFA incubation.

Lipid oxidation was detected using diphenyl-1-pyrenylphosphine (DPPP). DPPP is a novel non-toxic lipophilic fluorochrome that is retained within lipid membrane compartments, wherein it reacts specifically with lipid hydroperoxides to yield fluorescent DPPP-oxide (76). The fluorescence of this oxidized vital dye allows for quantification of membrane oxidant production in living cells.

Our previous data has shown that n-3 PUFA increases cytosolic, *i.e.* water soluble, ROS production in rat colon primary cultures (22, 30). To extend these observations and to assess membrane lipid oxidation, we investigated whether n-3 PUFA incorporation enhances membrane lipid oxidation in colon cell cultures. YAMC cells pretreated with BSA complexed-DHA (n-3 PUFA) or -LA (n-6 PUFA) at 50 μM with or without the addition of 5 mM butyrate were examined with respect to lipid oxidation.

2. To determine the association between membrane lipid oxidation and mitochondrial function.

MPT pore opening is an early and a pivotal event in the mitochondria-dependent apoptotic pathway, resulting in the dissipation of MP ($\Delta\Psi_{mt}$) and the induction of apoptosis (56). The relative levels of MP following butyrate/fatty acid co-incubation and under basal conditions were measured using a cell permeant, cationic lipophilic fluorescent dye, *i.e.*, Rhodamine 123 (Rhd123)

as described previously (9, 77). Briefly, this fluorescence probe is accumulated in mitochondrial matrix by the MP and lipid solubility. In general, a stable real time MP level indicates a dynamic equilibrium of the probes entering and leaving the mitochondrial matrix, *i.e.,* the accumulation of Rhd 123 may fluctuate as the environmental factors (*e.g.* temperature) change, hence a time matched control is used for normalization for each treatment.

To determine if lipid oxidation is localized within mitochondrial membrane phospholipids, we evaluated the effects of MitoQ. MitoQ refers to a mixture of mitoquinone (oxidized) and mitoquinol (reduced) moieties, which are the two redox forms of mitochondria-targeted quinones. The antioxidant activity of MitoQ is associated with mitoquinol's ability to take up electrons from the vicinity of complexes I and II and to donate them to complex III of the respiratory chain or to oxygen to form $O_2^{\cdot-}$ at the aqueous phase (78, 79). This reduced moiety is also recyclable within the respiratory chain through redox reactions. Based on a study by Kelso et al. (78), the covalently attached triphenylphosphine cation and lipophilic alkyl chain of MitoQ contribute to its uptake and accumulation within the matrix face of the inner mitochondrial membrane, where most of the mitochondrial ROS production occurs. The driving force of MitoQ uptake is the membrane potential. While mitochondrial MP is the greatest MP among all organelles, it is assumed the preferential and predominant accumulation of MitoQ is localized to the mitochondrial compartment (61, 79).

3. To determine if butyrate potentiates lipid oxidation and the alteration of mitochondrial function in colonocyte membranes enriched with n-3 and n-6 PUFA.

Select YAMC cultures were incubated with butyrate (0-10 mM) for 6, 12 or 24 h with BSA-complexed DHA or LA (50 μM) or with no fatty acid for 72 h. Butyrate or vehicle (RPMI 1640 complete media) was incubated as indicated in protocol #A-5. Lipid oxidation and MP dissipation were measured using fluorescence microscopy imaging techniques.

CHAPTER III

MATERIALS AND METHODS

Materials

RPMI 1640 and Hanks' balanced salt solution (HBSS) were from Mediatech (Herndon, VA). Fetal bovine serum was from Hyclone (Logan, UT). Insulin, transferrin, selenium without linoleic acid were obtained from Collaborative Biomedical Products (Bedford, MA). *Leibovitz* buffer, Glutamax™ and recombinant mouse interferon-γ (γ-IFN) were from GIBCO BRL (Grand Island, NY). Fatty acids were purchased from Nuchek Prep (Elysian, MN). The fatty acid free bovine serum albumin (BSA) for fatty acid preparation, Cell Death Detection ELISA, Plus and Cytotoxicity-Detection (LDH) kits were from Roche Applied Science (Indianapolis, IN). Fluorescence probes, diphenyl-1-pyrenylphosphine (DPPP), Rhodamine 123 (Rhd 123), 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CMH_2-DCFDA) were from Molecular Probes (Eugene, OR). Cyclosporin A (CsA) was from Calbiochem (San Diego, CA). MitoQ was a gift from Dr. Michael Murphy (Medical Research Council Dunn Human Nutrition Unit, Cambridge, UK). All other reagents were obtained from Sigma (St Louis, MO).

Cell culture

YAMC cells were obtained from R.H. Whitehead, Ludwig Cancer Institute, Melbourne, Australia (70). For all fluorescence assays, prior to fatty acid pretreatment, 4-5x10^3 YAMC cells (passages 17-23) per well were seeded into two wells sterile #1 German borosilicate chambered cover glasses (Nalge Nunc, # 155380) for 24 h to achieve 50-70% confluence. Under a permissive temperature, 33°C, mycoplasma free cultures were grown in RPMI 1640 complete media supplemented with 5000 u/L γ-IFN, 5% fetal calf serum, 1% insulin/transferrin/selenium without LA. For the Cell Death Detection assay, cells were incubated in 35 mm cell culture dishes at 35,000 cells per dish. See Fig. 3 for experimental design and timeline.

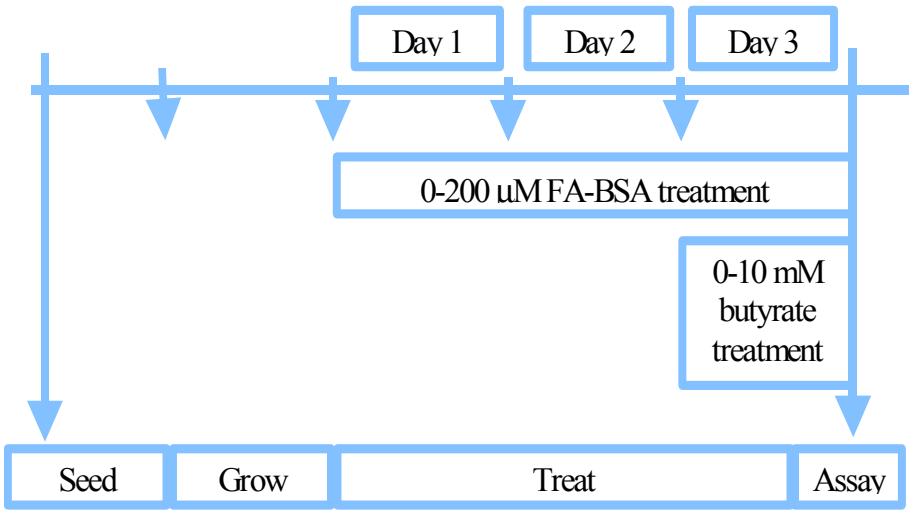


Fig 3. Experimental Design and Timeline

Fatty acid and butyrate preparation and incubation

DHA and LA were delivered to cells complexed to bovine serum albumin (BSA). To make the FA stock solutions, sodium salts of DHA and LA were complexed to fatty acid-free BSA in a 4:1 molar ratio, filter sterilized and stored in smaller aliquots in a freezer at -20° C (47). 2.5 mM BSA complexed fatty acid stock solutions were prepared and used within one-month period. LA is considered chemopromotive and served as the control in all experiments (16, 19).

Upon reaching 50-70% confluence, YAMC cells were pre-treated with or without DHA- or LA-BSA complex for 72 h at 50 µM. Co-incubation with 0, 1 or 5 mM sodium butyrate or isobutyrate (negative control) dissolved in complete RPMI 1640 media was initiated during the final 6, 12, or 24 h of fatty acid pre-treatment. Isobutyrate is a branched-chain isoform of butyrate previously shown to induce neither differentiation nor apoptosis in colonic cell lines (80). The working solutions were filter sterilized before use.

Lipid oxidation and lipid antioxidants

Following fatty acid treatment, cells were washed twice with phosphate buffer saline

solution (PBS) and loaded with 5 μM DPPP (final concentration) for 10 min in the dark at room temperature. The relative levels of DPPP-oxide fluorescence intensities were monitored using 351 nm (excitation) and 380 nm (emission).

Each analysis was performed under basal or oxidative stress conditions, *i.e.* with or without 25-100 μM cumene hydroperoxide (CumOOH) or 10 μM hydrogen peroxide (H_2O_2), to serve as a control. Due to the solubility of these compounds in the lipid bilayers, they differentially react with DPPP. CumOOH initiates lipid oxidation in the membrane, while H_2O_2 generates peroxides primarily and initially in the cytosol without affecting membrane oxidation (76).

To determine the association between PUFA treatment and lipid oxidation in the mitochondrial membrane, select cultures were treated with the mitochondrial targeted antioxidant, 10-(6'-ubiquinoyl) decyltriphenylphosphonium bromide (MitoQ) at 1 - 50 μM (78). 2.5 or 5 μM MitoQ was loaded one or 24 h prior to dye loading, *i.e.* during the last one hour or during the 24 h butyrate loading period. This antioxidant is expected to selectively decrease lipid oxidation within the mitochondrial inner membrane. A lower DPPP-oxide fluorescence intensity following MitoQ treatment would indicate the involvement of mitochondrial membrane lipid oxidation.

In comparison, a general lipid soluble antioxidant, vitamin E (α-tocopherol succinate; VEs), at both 10 μM and 25 μM, was used to estimate the involvement of total cellular lipid oxidation. A 10 mM VEs stock solution prepared in DMSO was filtered sterilized using DMSO safe tuffryn membrane (Acrodisc, #4433) and stored in smaller aliquots under an inert gas, N_2 atmosphere, at –80°C. The stock solution was subsequently diluted in RPMI 1640 media and added to culture.

Mitochondrial membrane potential

Following fatty acid pretreatment, cells were rinsed with PBS, incubated with 656 nM Rhd123 for 15 min at 33˚C in the dark, and washed twice with PBS. Cells were then analyzed for

rhodamine fluorescence by confocal microscopy. The relative levels of Rhd123 fluorescence intensities were monitored at 488 nm (emission) and 530 nm (excitation) using confocal microscopy (Meridian Ultima). A reduction of fluorescence intensity reflects the decrease of MP.

Cytosolic ROS production

To measure the change in cytosolic ROS production, *e.g.* H_2O_2, after n-3 PUFA and n-6 PUFA pretreatment, 5 μM CMH_2-DCFDA (5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate acetyl ester) dissolved in DMSO was loaded into selected cultures for 10 minutes in the dark at 33˚C before imaging (19). Cleavage of acetate moieties by esterases inside the cell causes the 2,7-dichlorofluorescein to be trapped inside the cell, rendering it available for oxidation by peroxides or hydroperoxides (81, 82). ROS production was quantified based on the intensity of the oxidized fluorescent probe trapped within the cell. H_2O_2 (10 μM) served as a positive control to induce cytosolic ROS production. DCFDA was laser excited at 488 nm and monitored with a 530 nm barrier filter.

Fluorescence microscopy and quantification

Laser excitation confocal microscopy (Meridian Ultima, Okemos, MI) was used to capture all images requiring quantification of fluorescence intensity. Cells loaded with fluorophores and washed with PBS were replenished with phenol-free *Leibovitz* media (Gibco, #21083-027) for image analysis. *Leibovitz* media contains pyruvate for cells to be sustained in a CO_2 free environment temporarily. Excitation energy and emission filters were adjusted based on the property of each fluorescent probe. In addition, cells were observed for contamination and other artifacts using phase contrast microscope (Nikon) after each treatment. Groups of cells (>5 cells) from at least fourteen fields for each sample were captured using laser excitation confocal microscopy (Meridian Ultima). Average fluorescence intensities of all the pixels in the image of individual cells were captured to measure the mean brightness of each treatment.

Cell death detection assay

Following fatty acid treatment, floating cells were harvested, washed, lysed and centrifuged at 13,600 x g to sediment nuclei and intact cells. Supernatants containing histone-complexed DNA fragments were collected for the quantification of late stage apoptosis using an ELISA (47). Absorbance and reference wavelengths were set at 405 nm and 490 nm, respectively. Values were normalized by the numbers of adherent cells from each dish. As a control for the mouse cell lines, cultures of cells were incubated for 24 h at a non-permissive (39˚C) temperature, in RPMI 1640 media without γ-IFN supplementation. We have previously demonstrated that both non-permissive (39˚C) temperature and 5 mM butyrate incubation are capable of enhancing YAMC cell apoptosis (16, 69).

Cytotoxicity study

For maximum sensitivity, $1.4x10^4$ YAMC cells per well were seeded into 96-well tissue culture dishes and incubated for 24 h with RPMI 1640 medium supplemented with γ-IFN. Cells were incubated with culture medium containing the compound of interest, *i.e.* MitoQ, at 0-50 μM for 0- 72 h. Supernatants were harvested, and the amount of LDH released was assayed by ELISA. LDH release was compared to untreated wells, which were lysed with 1% Triton-X 100, *i.e.* total releasable LDH present in untreated cells, according to the manufacturer's instructions (Roche, cat#1644793).

Statistical analysis

For all lipid oxidation and cytosolic ROS production studies, the statistical tests were performed using contrast testing under linear mixed models (83). Fixed effects were constructed to indicate the treatments under which the data points were generated. Well level random effects were used to account for the dependency of wells within the same dish. When one set of

experiments were conducted over a prolonged time frame, a factor accounting for the day to day variation was also added into the model.

For all other studies, the effects of independent variables (main treatment effects) were assessed using SuperAnova. A difference between means was tested using the least square means test. A 95% level of probability was accepted as being statistically significant.

CHAPTER IV

RESULTS

DHA and butyrate synergistically enhance lipid oxidation

Dose dependently, butyrate treatment over a 24 h period increased lipid oxidation in DHA (50 μM for 72 h) primed YAMC cultures (Fig. 4A, data are shown at the end of Chapter IV and in Appendix B). DHA pretreated YAMC cells demonstrated an increase ($p<0.05$) in lipid oxidation after co-incubation with physiological levels of butyrate (1-10 mM). However, in LA pretreated cultures, no trend was observed. DHA or LA treatment, in the absence of butyrate, did not increase lipid oxidation, compared to no fatty acid treatment.

In select experiments, in which 5 mM butyrate treatment was carried out for 24 h, DHA primed cells showed a 29% increase in lipid oxidation ($p=0.0013$), when compared to no butyrate treatment (Fig. 4C). The increase in oxidation was detected as early as 12 h following butyrate treatment in DHA primed cell (26%; $p=0.0095$) (Fig. 4B). Specifically, lipid oxidation in response to butyrate (12 h) treatment was not different from the control, *i.e.*, the no fatty acid pretreated cultures. At 6 h, butyrate treatment was insufficient to induce a change in lipid oxidation in any of the fatty acid treatment groups. Figures 4D and E are representative photomicrographs of YAMC cells illuminated following the oxidation of the DPPP fluorescence probe in cell membrane lipid bilayers. Even though the difference in the induction of lipid oxidation with the addition of 24 h butyrate co-treatment was not significantly different between the two fatty acids ($p< ½ \mid t \mid = 0.0579$), DHA pre-treated samples showed a two-fold greater induction of oxidation relative to the LA group (Fig. 4C).

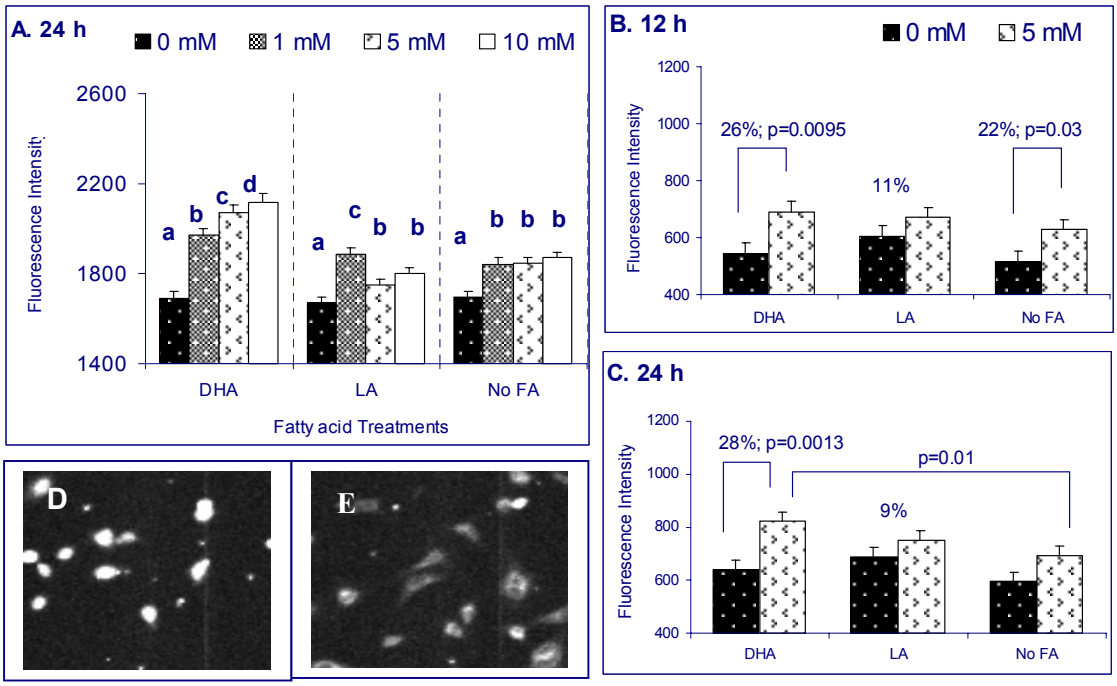


Fig. 4 . Effect of fatty acid and butyrate co-treatment on membrane lipid oxidation. Select YAMC cultures were incubated with butyrate (0-10 mM) for 12 or 24 h with BSA-complexed DHA or LA (50 µM) for 72 h. A) Butyrate dose dependently increased lipid oxidation in DHA primed cells. In comparison, after co-treatment with 5 mM sodium butyrate for the final B) 12 h or C) 24 h of fatty acid treatment, DHA primed cells consistently exhibited a greater increase in lipid oxidation ($p<0.01$), when compared to no butyrate treatment, whereas LA treatment did not show an effect. Lipid oxidation was measured by quantifying the fluorescence intensity of oxidized DPPP incorporated into cellular membranes. Shown are the means (± SE) of changes in average pixel intensities of all cells in each treatment group. The number of cells analyzed (n) ranged from 181 - 280, collected from 5 independent experiments. Values not sharing the same letters are significantly different ($p<0.05$). Panels D and E show representative photomicrographs (200 x) of DPPP loaded YAMC cells treated with DHA and LA, respectively, for 72 h and with 5 mM butyrate for the final 24 h.

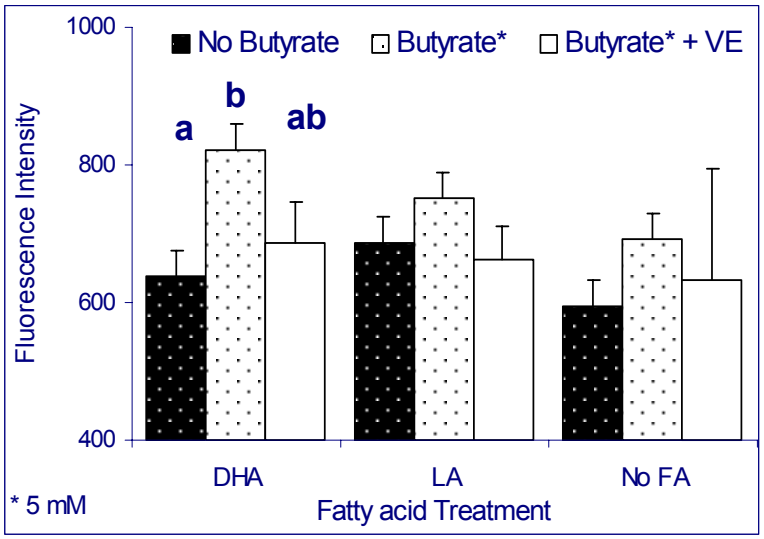


Fig. 5A. Vitamin E succinate reduces butyrate-induced membrane lipid oxidation in YAMC cells. Cultures were treated with 50 µM BSA-complexed DHA, LA or with no fatty acid ± VEs (10 µM) for 72 h with or without butyrate (5 mM) for the final 24 h. Lipid oxidation induced by butyrate in DHA primed YAMC cultures was partially reversed by 10 µM VEs incubation. The number of cells analyzed (n) ranged from 46 -118, collected from 2 independent experiments. Values not sharing the same letters are significantly different (P<0.05).

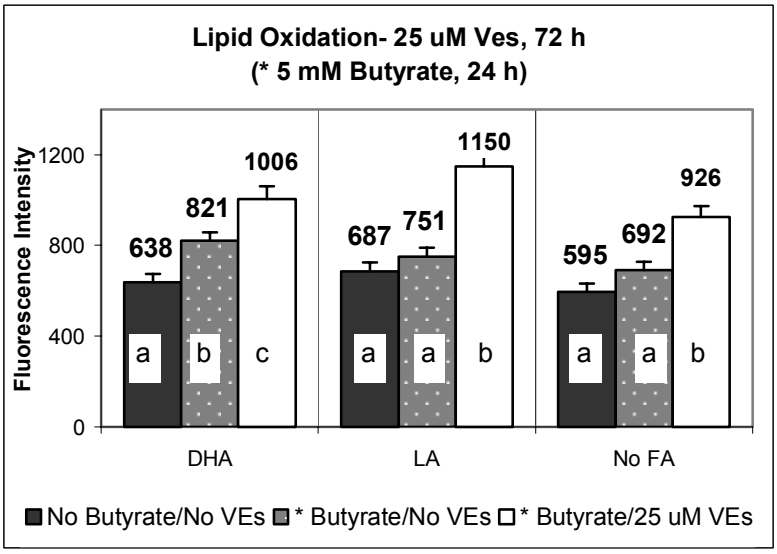


Fig. 5B. VEs (25 µM) appears to promote lipid oxidation in YAMC cells. The number of cells analyzed (n) ranged from 46 -118, collected from 2 independent experiments. Values not sharing the same letters are significantly different (P<0.05).

Effects of vitamin E succinate and MitoQ

The data in Fig. 5A demonstrate that the accumulation of lipid oxidation induced by DHA and butyrate co-treatment for 24 h, were partially reversed by a lipid antioxidant, Vitamin E succinate (VEs) at 10 μM ($p<0.05$). A similar anti-lipid oxidation effect of VEs was also detected in cells co-treated with butyrate for 12 h (Appendix B: Table B1-3). VEs did not affect the basal level of membrane oxidation in both LA-treated and the control groups. The effect of a higher dose of VEs (25 μM) was also evaluated, and appeared to promote lipid oxidation (Fig. 5B). The results depicted in Fig. 6 suggest that the subcellular origin of oxidation induced by DHA and butyrate was localized to the mitochondrial lipid membrane bilayers. Lipid oxidation induced by butyrate in DHA primed cells was blocked by a mitochondria targeted antioxidant, MitoQ (79). MitoQ ($p<0.05$) at as low as 2.5-5.0 μM (Figures 6A-B) reduced DPPP oxidation following a 12 or 24 h co-incubation period with butyrate (5 mM). In comparison, in LA treated cultures, MitoQ did not affect lipid oxidation. MitoQ toxicity was evaluated by determining the percentage of lactate dehydrogenase (LDH) release in YAMC cultures over a 12 - 24 h incubation period using 0 - 50 μM MitoQ. MitoQ concentrations up to 10 μM did not perturb cell viability (Fig. 6C).

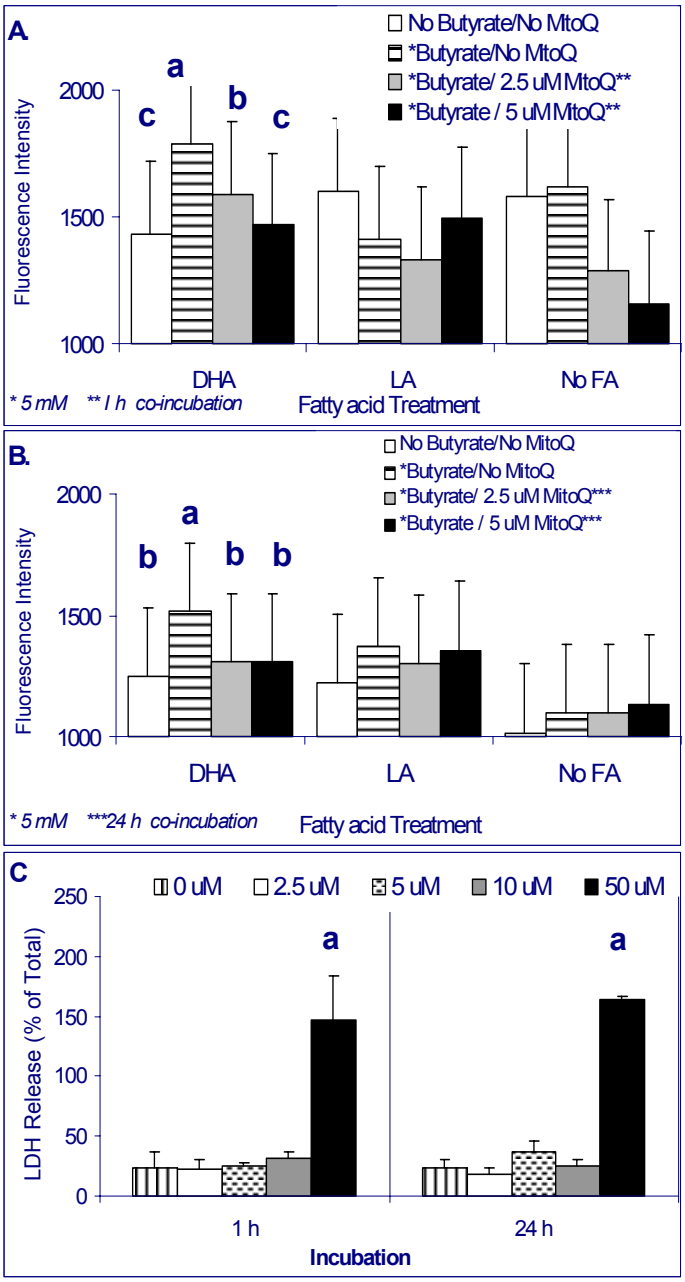


Fig. 6. MitoQ prevents lipid oxidation induced by DHA and butyrate co-treatment. Select YAMC cultures treated with DHA (50 µM) showed an increase in lipid oxidation following 24 h butyrate (5 mM) co-treatment. A) Lipid oxidation was reversed by MitoQ incubation in a dose dependent manner when MitoQ was loaded for the final 1 h of butyrate co-treatment. B) Following 24 h of co-incubation with butyrate, MitoQ (2.5 or 5 µM) reduced oxidation to basal levels. In LA (50 µM) treated cultures, no increase in lipid oxidation was observed initially. C) The level of LDH release induced by MitoQ incubation indicates a lack of cytotoxicity. YAMC cells were incubated with complete medium containing MitoQ at 0-50 µM, for one or 24 h. Supernatants were harvested, and the levels of LDH release were assayed, compared to an untreated well and expressed as percentages LDH present in cultures lysed with 1% Triton-X 100 (total releasable LDH). Data represent n=3 wells. For panels A and B, the number of cells analyzed (n) ranged from 283 - 655, collected from 5 independent experiments. Values not sharing the same letters are significantly different ($P<0.05$).

Effects of fatty acids on mitochondrial MP and MPT

DHA incubation increased the basal level of MP in a dose dependent manner, whereas no effect was observed in LA treated cells (Fig. 7A). Compared to LA, 72 h DHA treatment increased resting MP by 14% ($p<0.01$) (Fig. 7B). This finding is consistent with previous observations (11) showing that DHA is capable of increasing resting MP.

A reduction of fluorescence intensity reflects a decrease in MP (Fig. 8). After 24 h butyrate co-incubation, the dissipation of MP was two fold greater in DHA (20%), compared to the LA (10%) treated cells (Fig. 8A). Fig. 8B shows that CsA at 1 μM, loaded 30 min prior to butyrate incubation and replenished 12 h after the initial dose, blocked the dissipation of MP following 50 μM DHA or LA treatment for 72 h with 5 mM butyrate co-incubation for the final 24 h. Similarly, BkA at 1 μM inhibited MP dissipation induced by the same reagents and treatment (Fig. 8C). Comparable observations were made upon co-incubation with CsA or BkA with butyrate for the final 6 and 12 h. The ability of CsA and BKA to prevent the dissipation of MP, a phenotypic event depicting the opening of MPT pores, indicates the activation of either a transient or a prolonged MPT (59).

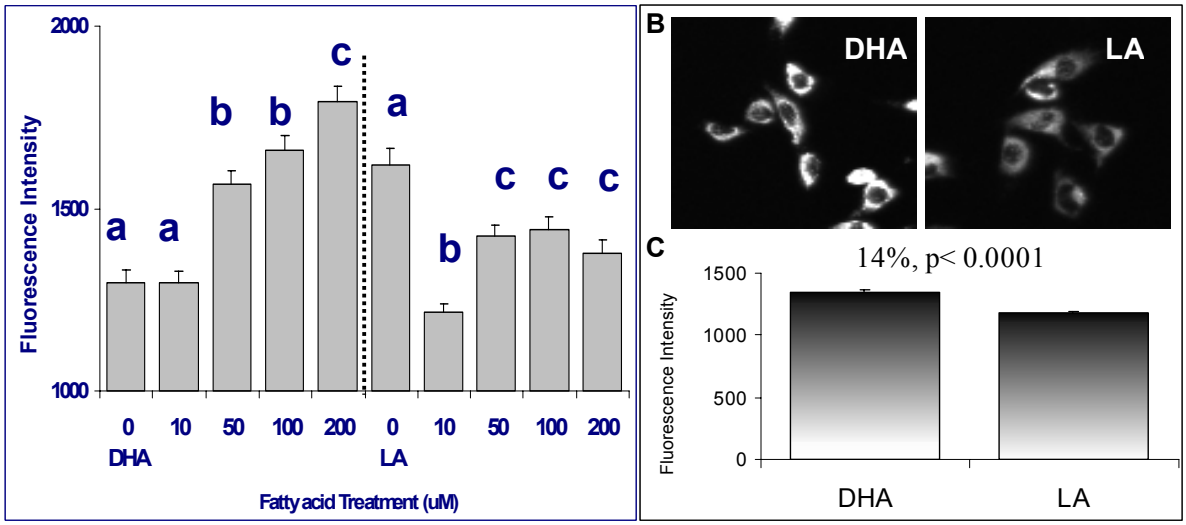


Fig. 7. Mitochondrial membrane potential is enhanced by DHA in a dose dependent fashion in the absence of butyrate. A) YAMC cultures were treated with DHA or LA at 0, 10, 50, 100 and 200 µM for 72 h. To measure the changes in the basal level of mitochondrial membrane potential following fatty acid treatment, cells (post-treatment) were loaded with Rhodamine 123 (656 nM) for 15 min. Each treatment group contained 107 - 221 cells from 2 independent experiments. B) Representative photomicrographs (400X) of cells incubated with 50 µM DHA or LA for 72 h. DHA enriched cells demonstrated a higher fluorescence intensity as compared to LA. C) In another set of experiments (n=4), DHA incubation, compared to LA, at 50 µM increased resting membrane potential by 14% ($P<0.0001$). Mean (± SE) fluorescence values were from 4 wells from 2 independent experiments containing 1058 (DHA group) and 847 (LA group) cells. Values not sharing the same letters are significantly different ($P<0.05$).

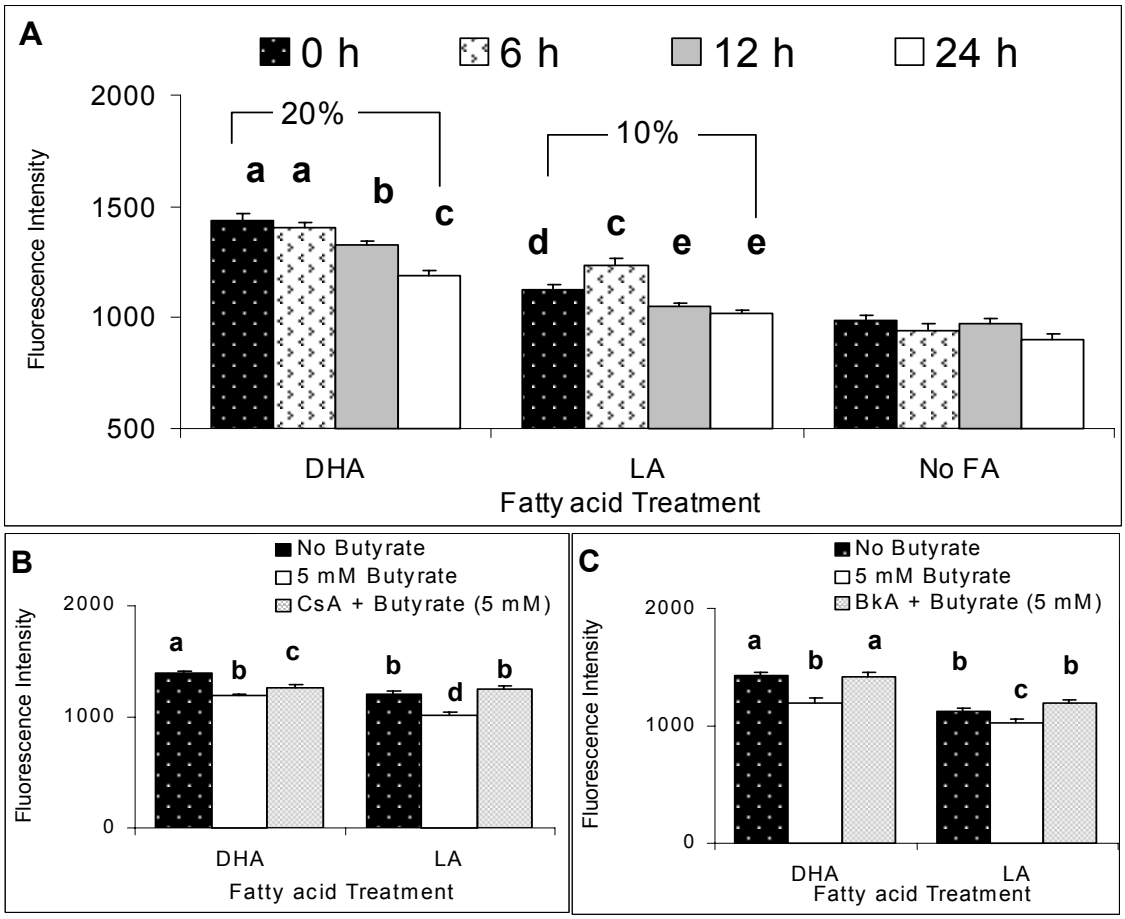


Fig. 8. Effects of fatty acid treatment on mitochondrial membrane potential. A) MP was dissipated by butyrate (5 mM) co-incubation, for the final 6, 12 and 24 h, following 50 µM DHA or LA treatment for 72 h, in a time dependant manner. After 24 h butyrate co-incubation, the dissipation of MP was two-fold greater in the DHA (20%), compared to the LA (10%) treated cells. Mean (± SE) fluorescence values were collected from 4 wells from 2 independent experiments. The number of cells analyzed (n) ranged from 106 - 362. B) Cyclosporin A (CsA) at 1 µM, loaded 30 min prior to butyrate incubation and replenished 12 h after the initial dose, blocked the dissipation of MP following 50 µM DHA or LA treatment (72 h) with butyrate (5 mM) co-incubation for the final 24 h. C) Bongkrekic acid (BkA) at 1 µM inhibited MP dissipation induced by butyrate (5 mM) co-incubation for 24 h. Similar observations were made upon co-incubation with CsA or BkA with butyrate for the final 6 and 12 h (data not shown). Values not sharing the same letters are significantly different ($P<0.05$).

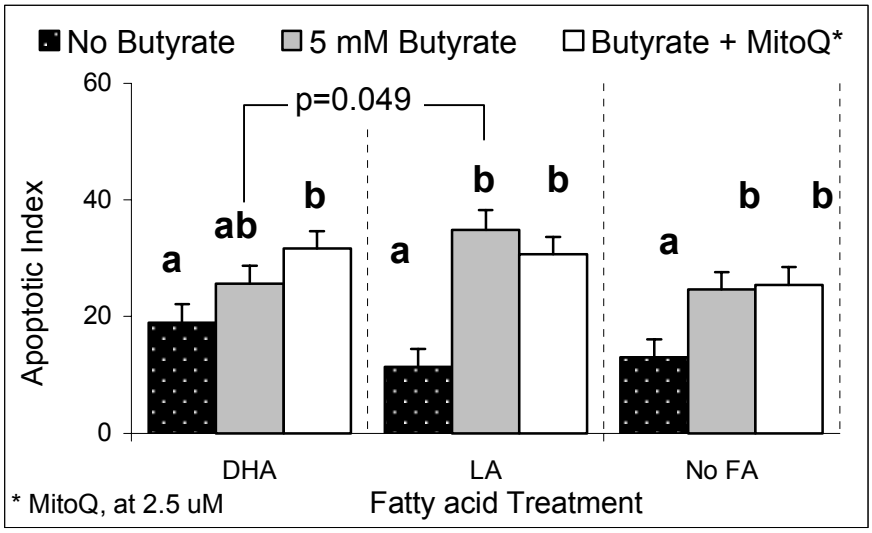


Fig. 9. Selective targeting of a redox active antioxidant (MitoQ) to mitochondria does not block apoptosis induced by fatty acid and butyrate co-treatment as indicated by DNA fragmentation ELISA. Cultures were incubated with 5 mM butyrate and 50 μM DHA or LA or no fatty acid as described in Fig. 3. MitoQ at 2.5 μM was loaded 30 min prior to butyrate incubation. The apoptotic index represents optical density, obtained by ELISA described in Material and Methods, per the number of adherent cells in culture. The number of cultures analyzed (n) ranged from 9-12, collected from 3 independent experiments. Values not sharing the same letters are significantly different ($P<0.05$).

Apoptosis phenotype

DHA and LA treatments (72 h) differentially primed cells for apoptosis induced by butyrate or butyrate plus MitoQ co-treatment. Using a Cell death ELISA (Roche), which measures DNA fragmentation (described in Materials and Methods), the effect of 24 h butyrate incubation to induce apoptosis in LA primed cells was significantly greater than in DHA primed cells ($p=0.049$). The result is in contrast to our other findings, which shows DHA and butyrate synergistically activate pro-apoptotic events. However, the control treatment butyrate at 5 mM incubated for 24 h induced apoptosis [Fig. 9], which is consistent with a previously published study (47). In addition, contrary to our hypothesis, this pro-apoptotic effect of butyrate was not blocked by MitoQ co-incubation. MitoQ co-incubation, which prevented lipid oxidation, did not block apoptosis induced by butyrate, in all treatments.

Cytosolic reactive oxygen species

Using YAMC cell culture model, 5 mM butyrate treatment did not increase cytosolic ROS production, compared to no butyrate treated samples (Fig. 10). This observation is distinct from experiments using animal models (22, 30). The sub-confluent YAMC cell cultures were treated with 50 μM BSA-complexed DHA, LA or with no fatty acid for 72 h with or without 6 - 24 h butyrate (5 mM) co-treatment. The differences in the pair-wise comparison of ROS production between no butyrate and 5 mM butyrate treatments at the same incubation time and with the same fatty acid were obtained by comparing the average fluorescence intensity of YAMC cells loaded with CMH_2-DCFDA (5 μM) for 15 min prior to imaging.

Data collected to generate all of the observations above are summarized in tables 1 to 7 in the following pages.

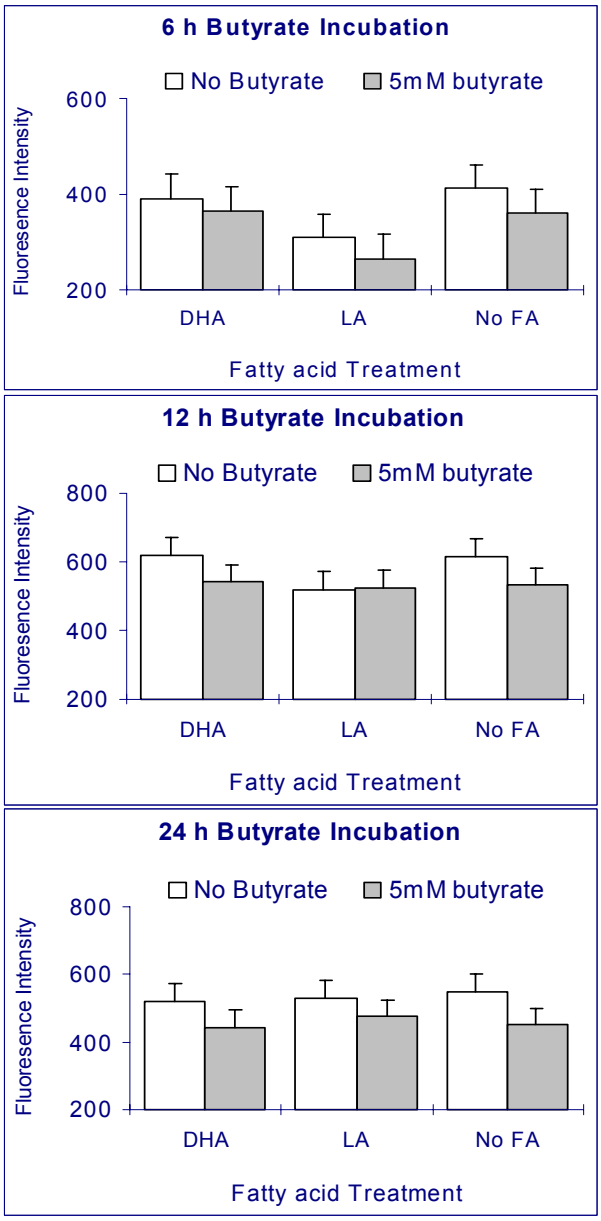


Fig. 10. Pair-wise comparison of reactive oxygen species production between no butyrate and 5 mM butyrate treatment at the same incubation time, treated with the same fatty acid. Cultures were treated with 50 µM BSA-complexed DHA, LA or with no fatty acid for 72 h with or without a) 6, b) 12 or c) 24 h butyrate (5 mM) co-treatment. YAMC cells (post-treatment) were loaded with CMH_2-DCFDA (5 uM) for 15 min prior to imaging. Mean (± SE) fluorescence values were collected from 4 wells from 2 independent experiments. The number of cells analyzed (n) ranged from 434-735. Values not sharing the same letters are significantly different ($P<0.05$).

Table 1 Effect of fatty acid and butyrate co-treatment on membrane lipid oxidation

Fig. 4A	Mean Avg Intensity				SE				Cell analyzed (n)			
	0 mM	1 mM	5 mM	10 mM	0 mM	1 mM	5 mM	10 mM	0 mM	1 mM	5 mM	10 mM
DHA	1690	1972	2071	2113	30.1	29.2	32.7	39.7	306	325	260	176
LA	1669	1887	1749	1802	27.8	27.2	24.4	22.9	360	375	466	531
No FA	1697	1842	1843	1868	21.6	26.0	24.6	28.6	594	409	459	340

Fig. 4B: 12 h	Mean Avg Intensity		SE		Cell analyzed (n)	
	0 mM	5 mM	0 mM	5 mM	0 mM	5 mM
DHA	546	689	38	38	224	213
LA	603	670	37	37	228	230
No FA	516	629	37	37	280	260
Fig. 4C: 24 h						
DHA	638	821	37	37	225	229
LA	687	751	37	37	202	181
No FA	595	692	37	37	230	245

*** Refer to appendix table B-1 to B-5 for more details**

Table 2 Vitamin E succinate (VEs) reduces butyrate-induced membrane lipid oxidation in YAMC cells

Fig. 5	Mean Avg Intensity			SE		
	No Butyrate	Butyrate*	Butyrate* + VEs (10 μM)	No Butyrate	Butyrate*	Butyrate* + VEs (10 μM)
DHA	638.38	821.07	686.99	37.1567	37.1064	86.221
LA	687.16	751.17	662.24	37.1761	37.4652	86.221
No FA	595.17	692.11	632.57	36.6451	36.6513	81.3985

*** 5 mM, 24 h**

Table 3 MitoQ prevents lipid oxidation induced by DHA and butyrate co-treatment

Fig. 6A	Mean Avg Intensity			Standard Error			Cell analyzed (n)		
	DHA	LA	No FA	DHA	LA	No FA	DHA	LA	No FA
No Butyrate/No MitoQ	1433	1601	1579	283.6	283.38	283.9	473	572	227
*Butyrate/No MitoQ	1785	1415	1619	283.65	283.16	283.3	405	548	552
*Butyrate/ 2.5 uM MitoQ	1590	1334	1285	283.43	283.2	283.22	596	628	550
*Butyrate / 5 uM MitoQ	1469	1492	1158	283.3	283.2	283.21	463	458	445
* 5 mM, 24 h									
*Butyrate/No MitoQ	1515	1371	1095	283.19	283.26	283.24	548	552	505
*Butyrate/ 2.5 uM MitoQ	1305	1301	1096	283.22	283.24	283.38	604	598	421
*Butyrate / 5 uM MitoQ	1304	1355	1132	283.25	283.36	283.52	558	482	381

*** 5 mM, 24 h**
Data represent means for a minimum of 4 separate wells and 2 separate experiments.

Fig. 6C: MitoQ-LDH release

	% (LDH Released/Total Releasable LDH)				
Mito Q Concentration (μM)	0	2.5	5	10	50
Duration					
1 hr	23%	22%	26%	31%	147%
24 hr	24%	19%	37%	25%	163%

Number of wells analyzed, n= 3 per treatment.

Table 4 Mitochondrial membrane potential is enhanced by DHA in a dose dependent fashion in the absence of butyrate

Fig. 7A	Mean Avg Intensity					SE					Cell analyzed (n)				
	0 uM	10 uM	50 uM	100 uM	200 uM	0 uM	10 uM	50 uM	100 uM	200 uM	0 uM	10 uM	50 uM	100 uM	200 uM
DHA	1296.4	1296.0	1566.7	1659.7	1792.3	36.0	30.9	36.6	39.3	42.4	107	160	145	115	89
LA	1619.2	1213.5	1423.5	1440.4	1375.3	46.6	24.3	30.1	37.6	38.3	124	221	200	123	139

Fig. 7C	Mean Avg Intensity	SE	Cell analyzed (n)
DHA	1349.786	12.843	1058
LA	1181.188	11.957	847

Table 5 Effect of fatty acid treatment on mitochondrial MP

Fig. 8A	Mean Avg Intensity				SE				Cell analyzed (n)			
	0 h	6 h	12 h	24 h	0 h	6 h	12 h	24 h	0 h	6 h	12 h	24 h
DHA	1432.83	1406.01	1324.69	1188.94	30.07	24.90	20.94	18.57	207	322	362	308
LA	1124.85	1235.15	1052.21	1017.32	28.17	27.31	15.85	17.83	162	199	343	243
No FA	983.73	939.26	973.52	903.57	27.00	28.53	22.40	22.10	124	106	121	117

Fig. 8B (7/30/2002)

	Mean Avg Intensity			SE			Cell analyzed (n)		
	No Butyrate	Butyrate*	CsA + Butyrate*	No Butyrate	Butyrate*	CsA + Butyrate*	No Butyrate	Butyrate*	CsA + Butyrate*
DHA	1392.91	1183.87	1271.15	17.18	26.33	28.10	444	189	166
LA	1212.57	1007.72	1252.61	16.81	35.84	26.98	464	102	108

*** 5 mM, 24 h**

Fig. 8C (9/27/2003)

	Mean Avg Intensity			SE			Cell analyzed (n)		
	No Butyrate	Butyrate*	BKA + Butyrate*	No Butyrate	Butyrate*	BKA + Butyrate*	No Butyrate	Butyrate*	BKA + Butyrate*
DHA	1432.83	1196.98	1420.77	25.95	34.22	36.96	207	118	102
LA	1124.85	1024.31	1189.58	29.33	31.44	32.74	162	141	130

*** 5 mM, 24 h**

Table 6 Pair-wise comparison of ROS production between no butyrate and 5 mM butyrate treatments of the same incubation time, treated with the same fatty acid

Fig. 10	Duration (h)	Mean Avg Intensity		SE		Cell analyzed (n)	
		No Butyrate	5 mM Butyrate	No Butyrate	5 mM Butyrate	No Butyrate	5 mM Butyrate
DHA	6	391.29	365.09	49.93	49.98	627	637
	12	619.87	542.77	50.07	49.99	602	649
	24	521.56	443.12	50.03	50.08	505	534
LA	6	308.57	264.63	49.87	50.01	735	578
	12	520.52	524.64	50.03	50.18	645	553
	24	531.23	474.83	50.06	50.34	666	434
No FA	6	412.28	360.39	50.08	50.06	559	571
	12	615.02	532.53	50.01	50.02	581	509
	24	549.26	451.55	50.04	50.27	486	454

Table 7 Selective targeting of a redox active antioxidant (MitoQ) to mitochondria does not block apoptotic induction by fatty acid and butyrate co-treatment

Fig. 9	Apoptotic Index (OD/ # cell population)			SE			n = cultured plate (35mm dish) per sample		
	No Butyrate	Butyrate*	Butyrate* + MitoQ**	No Butyrate	Butyrate*	Butyrate* + MitoQ**	No Butyrate	Butyrate*	Butyrate* + MitoQ**
DHA	18.99	25.60	31.65	3.11	3.11	2.98	11	11	12
LA	11.33	34.83	30.64	3.11	3.43	2.98	11	9	12
No FA	13.03	24.65	25.42	3.11	2.98	3.11	11	12	11

***Butyrate 5 mM**
****** **MitoQ, 2.5 uM**

CHAPTER V

DISCUSSION AND SUMMARY

The preferential incorporation of n-3 PUFA, including DHA (11, 17), into mitochondrial membrane phospholipids, *i.e.* cardiolipin, increases cell (membrane) susceptibility to damage by oxidative stress (18). Data from our laboratory have suggested that mitochondria and ROS production mediate the apoptotic enhancing effect of butyrate and dietary fish oil in colonic crypts (7, 9, 19). The current experiments support this hypothesis, since DHA, a major fatty acid found in fish oil, enhanced YAMC mitochondrial lipid oxidation induced by co-incubation with physiological concentrations (0 - 10 mM) of butyrate (Fig. 4). It seems likely that the mitochondrial pool of phospholipid is the major site for lipid oxidation induced by butyrate and DHA, since the mitochondrial targeted lipid antioxidant, MitoQ, completely blocked the effect of butyrate (Fig. 6). In comparison, VEs, a lipid soluble antioxidant (Fig. 5) attenuated but did not fully prevent lipid oxidation.

The electrical potential across the inner membrane of mitochondria ranges between 150 and 180 mV, negative inside and is the highest electrical potential difference across any membrane in a typical cell, making up 70 to 80 % of the mitochondrial electrochemical proton gradient (61). In the present study, we show that DHA pretreated YAMC cells exhibit a higher resting MP (Fig. 7C), compared to LA. In addition, DHA up to 200 μM dose dependently increased the resting level of MP (Fig. 7). These findings suggest that incorporation of DHA into mitochondrial membranes induces changes in its bioenergetic properties: electron transport, ATP production, H^+ permeability and MP, congruent with the report by Stillwell et al. (see (84) for review). It appears likely that DHA incorporation (and LA to a lesser extent) increased the function of the proton pump within the respiratory chain. The respiratory chain is coupled to the extrusion of protons from the mitochondrial matrix generates a proton gradient on the both sides of the inner

mitochondrial membrane. This proton gradient makes up part of ΔP (proton motive force), which is the driving force for ATP synthesis through ATP synthase. It is also the driving force for the uptake of various cationic molecules such as Ca^{++} (61). Therefore, the elevated MP associated with increasing DHA concentration is consistent with a fundamental shift in the activity of the respiratory chain.

An increase in mitochondrial bioenergetics is the result of increased ATP production and oxygen consumption (54, 57, 61). Under normal conditions, *i.e.*, mild oxidative stress environments, a high ATP/ADP ratio inhibits the rate-limiting step, cytochrome-C oxidase activity through a feedback loop and causes a regulated mitochondrial respiratory rate. This is an established mechanism within the mitochondria to prevent ROS hyper-production (54). In comparison, the mild uncoupling effect of uncoupling proteins, *e.g.* UCP, to dissipate ΔP by permitting a controlled leakage of protons across the mitochondria membrane, allows electron transport to occur without the constraint of ATP utilization in some cells (54). Studies have shown that free fatty acids are capable of uncoupling energy production in isolated mitochondria. Unsaturated fatty acid incorporation could increase membrane permeability for H^+ while increasing resting state respiration (84, 85). Nevertheless, the potency of this long chain PUFA to collapse MP decreased with increasing carbon chain length (85). DHA incorporation into living cells may increase respiratory chain action through its mild protonophoric action, to sustain a prolonged respiratory chain activation while maintaining an elevated resting level of MP as shown in Fig. 7. Consistent findings have been reported in other studies demonstrating that MP in DHA-enriched cells was higher relative to control groups treated with EPA, LA, arachidonic acid and oleic acid (11).

A population of cells exhibiting an increased MP should produce more cellular oxidants (85). Using the YAMC cell model, our data show that lipid oxidation in cells treated with 50 μM DHA or LA was not different from the control, in the absence of butyrate (Fig. 4A). Moreover, cytosolic ROS was not increased in all treatments with or without butyrate co-incubation (Fig. 9) as

compared to the no fatty acid treatment (control) group. Nevertheless, upon butyrate co-incubation for 24 h, DHA primed cells demonstrated a greater level of lipid oxidation compared to LA and no fatty acid group (Fig. 4C). These data suggest that DHA sensitizes or primes cells to subsequent oxidative stress events.

Since a more negative membrane surface potential, *i.e.,* a greater MP, has been correlated to an increase in the respiration rate (57) and DHA incorporation, which may favor PT pore opening (59), it is likely that DHA treatment primes cells for apoptosis via a mitochondrial PTP dependant mechanism.

Malis et al. (18) showed that the incorporation of PUFA into mitochondrial membrane phospholipids sensitizes cells towards the activation of apoptosis by Ca^{++}. Ca^{++} is a well-known signaling messenger implied in MPT activation. An increase in intracellular free Ca^{++} concentration is in most cases followed by an increase in the mitochondrial free Ca^{++} concentration, of which the Ca^{++} uniporter is believed to be one of the channels for Ca^{++} uptake (52, 61). Ca^{++} overloading has been shown to activate PT pore opening and has been correlated to ROS stress induction (52, 53, 61, 86). Furthermore, Ca^{++} uptake into the mitochondrial matrix may be driven by MP (61). A cytosolic Ca^{++} surge and subsequent mitochondrial Ca^{++} overload would eventually reach a threshold level of Ca^{++} to trigger MPT, resulting in MP dissipation (86). This is consistent with the effect of DHA in the absence of butyrate, where DHA dose-dependently increased resting MP levels, while LA treatment at identical concentrations did not (Fig. 4).

On the other hand, studies have shown that butyrate treatment, which is capable of increasing intracellular ROS stress (36, 37), also triggers the accumulation of endogenous Ca^{++} within the intracellular compartment (87). It is known that supraphysiological concentration of Ca^{++} suffice to induce PT, while lower doses facilitate the induction of PT by other stimuli (57). The increase in the resting level of MP by DHA and release of intracellular Ca^{++} into the intracellular compartment by butyrate may facilitate a more effective uptake of Ca^{++} into mitochondrial matrix. This is noteworthy, because as a ligand, Ca^{++} binds to VDAC-ANT-

Cyclophilin D complex of the mitochondrial PTP, whereby it activates MPT (57).

Ca^{++} accumulation within the mitochondrial compartment has been shown to trigger the release of PUFA enriched at the sn-2 position of phospholipids in mitochondrial membranes (18). Therefore, when cells are treated with butyrate and DHA together, these short and long chain fatty acids may induce Ca^{++} overloading, which has been correlated to the uncoupling effect and the ability to induce MPT of free fatty acid. It is likely that these sequential alterations result in the perturbation of mitochondrial membrane and induce a mitochondrial-dependant apoptosis.

Oxidative damage of mitochondrial membrane proteins and phospholipids (49) can directly trigger MPT (53). We have demonstrated that DHA and butyrate dissipate MP that is preventable by CsA and BKA. It is possible that MPT is involved in the mechanism by which DHA and butyrate protect against colon cancer development. Literature review suggested oxidative stress accumulation within mitochondria activates MPT and is a self-amplifying process, meaning that several consequences of an oxidative reaction may feed forward to amplify MPT activation and ROS hyper-production within cells (57). Focusing on a single mitochondrion level, cardiolipin, a unique mitochondrial phospholipid localized primarily within the inner membrane, is needed for the activity of enzyme complexes involved in electron transport such as cytochrome-C oxidase and NADH reductase (11). The incorporation of DHA into of cardiolipin following dietary fish oil supplementation favors oxidative stress accumulation and apoptosis induction. Specifically, ROS production is correlated to the unsaturation index of cardiolipin acyl chains (9, 11). Moreover, the oxidation of the cardiolipin acyl chains is associated to the release of cytochrome-C from mitochondria triggering caspase activation (49, 68) an event inhibited by α-tocopherol, resveratrol, and Bcl-xl protein expression (49, 88). Furthermore, cardiolipin oxidation and subsequent cytochrome-C release are correlated to MP depolarization (52, 66), ROS generation (49) and MPT-regulated apoptosis (89). On the other hand, redox metabolism, caspases, amphipathic peptides and Bcl-2 family proteins have been shown to act on mitochondria to induce MPT. Further research is required in order to elucidate whether these pathways are involved in the DHA and

butyrate induced MPT activation.

The oxidation of PUFA yields α,β-unsaturated enals, *e.g.*, malodealdehyde (MDA), acrolein (Acr), crotonaldehyde (Cro), t-4-hydroxy-2-nonenal (HNE), and 4-oxo-2-nonenal (26, 28). These enals differentially react with deoxyguanine to form cyclic DNA adducts (an indicator of DNA lesion) or with protein to covalently modify the function within cells (28, 90). Interestingly, oxidized n-3 PUFA as opposed to the native unoxidized n-3 PUFA, are capable of inducing a PPAR-α mediated anti-inflammatory effect (90). With regards to the efficacy of a specific type of PUFA, DHA has been found to be the most efficient precursor for DNA adducts formation in some cell lines, when compared to other PUFA with lower numbers of double bonds (26). Specifically, the formation of DNA adduct is proportional to the number of double bonds of its PUFA precursors (26). It is likely that n-3 PUFA incorporation into mitochondrial membranes provides substrate for lipid oxidation (91) and increases the generation of aldehydes (enals), which are pro-apoptotic.

Concomitant with a role of ROS in the activation of apoptosis, the inhibition of ROS production by Bcl-2 and Bcl-xL is associated with their anti-apoptotic roles (92, 93). Interestingly, butyrate and n-3 PUFA have been found to decrease Bcl-2 expression in colon cancer cell lines (94) and in rat colonic crypts (67), respectively. In comparison, Bax protein (proapoptotic) increases the membrane unsaturation index, especially in cardiolipin, which promotes membrane lipid oxidation (88). Furthermore, this pro-apoptotic effect of Bax is enhanced in cells primed with polyunsaturated fatty acids. This evidence in its entirety suggests that DHA or n-3 PUFA exposure may increase oxidative stress and damage, hence priming cells for apoptosis induction by butyrate. This may explain why DHA and butyrate synergistically enhance apoptosis in the colon (7, 22, 30).

Other than Ca^{++} overload and oxidative stress accumulation, a variety of different pro-apoptotic signal-transduction pathways may be involved in the activation of apoptosis induced by DHA and butyrate. For instance, butyrate incubation may activate apoptosis via a Fas receptor/Fas-L dependant pathway (47), which is an extrinsic pathway that may not involve the

function of mitochondria. This may explain why MitoQ, which blocked lipid oxidation induced by butyrate in DHA primed cells (Fig. 3), did not block apoptosis (Fig. 6), compared to the control treated with fatty acid only. Alleva et al. has shown that coenzymeQ could block biochemical but not the apoptotic pathway that is Fas receptor-mediated (95).

It has also been noted that the endoplasmic reticulum (ER) can induce apoptosis independent (96) or dependent of mitochondria (97). Relative to mitochondria, the ER exhibits similar membrane potential maintenance properties and the ability to regulate Ca^{++} homeostasis, and may therefore play a pivotal role in ROS production and subsequent apoptotic events. The involvement of ER in the regulation/activation of apoptosis in colonocytes needs further investigation.

Figure 11 illustrates the putative mechanisms by which the incorporation of DHA into mitochondrial membrane phospholipids results in an increase in MP. Intracellular events related to mitochondrial function are activated subsequent to butyrate treatment.

Significance

Collectively, these data further elucidate the apoptotic cellular targets that are modulated by DHA and butyrate in colonocytes, i.e. by eating a diet high in dietary fish oil and fermentable fiber. We propose that n-3 PUFA alter colonocyte mitochondrial membrane composition and function, thereby creating a permissive environment for apoptosis induced by subsequent pro-apoptotic agents, such as butyrate. Using non-malignant transformed immortalized YAMC, we tested our overall hypothesis that metabolically-induced (butyrate) stress, the result of membrane lipid oxidation and disrupts mitochondria function. We demonstrate for the first time that mitochondrial lipid oxidation was induced by DHA and butyrate co-incubation in living cells. In conclusion, dietary fish oil and pectin may prevent colon cancer tumorigenesis by modulating mitochondrial membranes lipid oxidation and function.

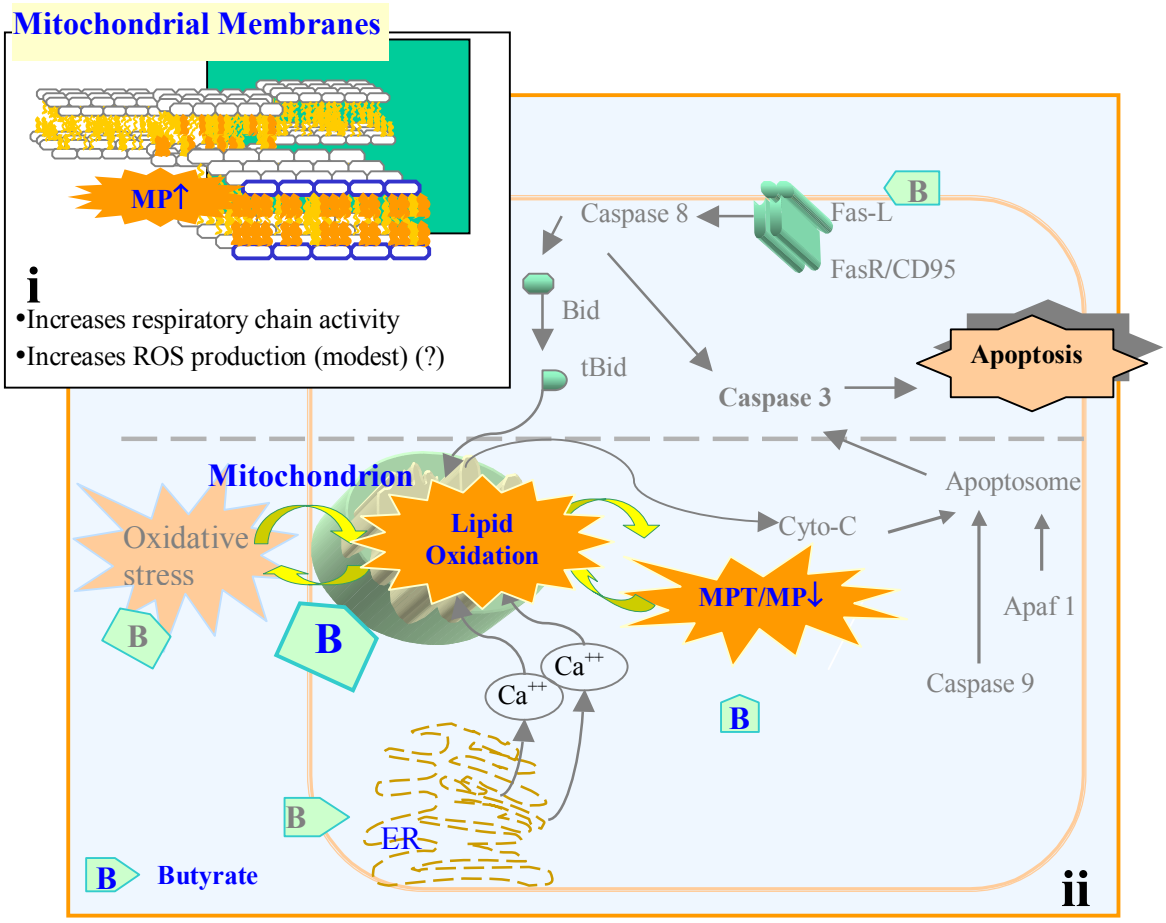


Fig. 11. i) Putative mechanisms by which the incorporation of DHA into mitochondrial membrane phospholipids results in an increase in membrane potential. ii) Intracellular events related to mitochondrial function are activated subsequent to butyrate treatment. The findings from this study show that mitochondrial membrane lipid oxidation and MPT activation are two events likely to be involved in the activation of cell death induced by DHA and butyrate co-treatment.

Abbreviations: B (butyrate), Ca^{++} (calcium), Cyto-C (cytochrome-C), ER (Endoplasmic Reticulum), MP (mitochondrial membrane potential), MPT (mitochondrial permeability transition).

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APPENDICES

A. Experimental Protocols

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Appendix A: EXPERIMENTAL PROTOCOLS

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APPENDIX A

EXPERIMENTAL PROTOCOLS

Protocol # A-1 Preparation of a complete RPMI 1640 media for YAMC culture

Purpose: To prepare a complete RPMI 1640 media for YAMC cell culture.

Reagents (supplier, catalog number), (storage)

- 500 ml (one bottle) RPMI 1640 without Glutamine (Mediatech, #15-040-CV), (walk in freezer)
- 26.6 ml Fetal Bovine Serum (Hyclone, # AK 12434), (chest freezer)
- 5.3 ml *Glutamax*™ (Gibco, 35050-061), (chest freezer)
- 0.532 ml ITS "-" (Collaborative Biomed. Products, #4351 "-"), (P.H. fridge)
  - ___ ITS "-"reconstitution: add 5 ml sterile distilled water into lyophilized powder. 1 ml of reconstituted solution is sufficient to supplement one liter of medium (0.1% dilution).

1. ___ Thaw FBS and *Glutamax*™ in the fridge several hours in advance or overnight.
2. ___ Add FBS, *Glutamax*™ and ITS "-" into 500 ml RPMI 1640 medium.
3. ___ Label bottle as "complete" media and store it at 4˚C.

Final volume: 531.9 ml
Final concentrations: 5% Fetal Bovine Serum, 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, stored in -80˚C freezer) per 1 ml complete RPMI 1640 media.

1. ___ For each 10 ml complete RPMI 1640 medium, supplement 1µl γ-IFN.

Protocol # A-2 Preparation of YAMC cell culture

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

Preparation:

- In warm water bath (37 ˚C), warm tap distilled water in a clean beaker for 30 min.
- Turn on the UV in the hood ~15 min prior to using the hood.

Procedure:

1. ___ Warm prepared complete RPMI 1640 media to RT.
2. ___ Remove a vial of YAMC cells from the liquid nitrogen storage system and thaw it in the pre-warmed water immediately.
3. ___ Add thawed cells into ~10 ml media in a 15 ml conical tube.
4. ___ Spin down YAMC at 200 x g (1096 rpm: tabletop centrifuge in room 307) for 5 min at 6 Acc./Dcc.
5. ___ Aspirate supernatant to remove freezing media.
6. ___ Resuspend pellet in 20 ml complete RPMI 1640 media with 2 µl of γ-IFN.
7. ___ Add cells/media into a T-75 flask.
8. ___ Incubate cell culture at 33˚C under 5% CO_2 atm.

Two-days after:

9. ___ Feed cells every 2 (maximum 3) days by aspiring old media and by replenishing cells with fresh RPMI 1640 media with γ-IFN.
10. ___ When culture reaches 70-90% confluence, passage cell culture through trypsinization (refer to Appendix A: Protocol # A-3 YAMC Cell Culture).

Protocol # A-3 YAMC Cell Culture: Seeding, Passing or Freezing

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

Preparation:

- Warm complete RPMI 1640 and trypsin-EDTA (Gibco, #25300-054) to RT.

Procedure:

For any ~80% confluent cell culture in a T-75 flask:

1. ___ Aspirate old media, rinse monolayer of cells with ~10ml HBSS (Sigma, H-6648) without disturbing the cell monolayer.
2. ___ Aspirate HBSS. Add 5 ml Trypsin-EDTA and incubate cells at 33 ˚C until > 90% cells are lifted.
3. ___ Check cells under microscope. Gently tapping on the side of the flask to loosen adherent cells.
4. ___ Add cells in suspension into a 50 ml conical tube containing ~15 ml complete RPMI 1640 medium.
5. ___ Spin at 200 x g (1096 rpm, countertop centrifuge in room 307) for 5 min to remove trypsin in the media. Aspirate supernatant without disturbing the pellet.
6. ___ Lightly flick the bottom of the tube to loosen the cells from the bottom of the conical tube.

7. A. To seed cells in a culture dish:
   - Resuspend pellet in 4 ml of complete RPMI 1640 medium.

   Count cell (sterility is not critical):
   - Transfer ~ ½ ml of the homogenized cells in suspension into a 1 ml micro-centrifuge tube.
   - Transfer 5 µl of the cell suspension into a 500 µl micro-centrifuge tube.

- Add 5 µl trypan blue, 40 µl PBS into the micro-centrifuge tube. Mix well to yield a homogenized 10% suspension.
- Transfer ~30 µl of the cell suspension onto a hemacytometer.
- Count cell: Cell density (cells count per ml) = $\frac{\text{Living cells count x 10\^4 x 10}}{\text{\# Squares counted}}$
- Seed cell according to desired density.

B. To continue growing more cells in a T-75 flask:

- Divide cell suspension (sterile) at 1:5 ratio. Aliquot each portion into 20 ml media with γ-IFN. Seed cells into a T-75 flask.

C. To freeze (to store) cells in liquid nitrogen.

- Resuspend cells in 4 ml of freezing media (EmbryoMax, # S-002-D).
- Load each cryo vial with 1.0 - 1.5 ml cell suspension. Allow room for expansion. (Yield ~1 million cells per vial).
- Keep vials in Mr. Freezer (Nalgene, #5100-0001) at –80˚C for a day (at most two days over the weekend) before transfering vials into liquid nitrogen for storage.
- Record rack, box and coordinate of storage location into log book.

8. Label vial/flask with name, date, passage number and cell type.

Protocol # A-4: Fatty acid-BSA complex

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

Preparation:

- 2 screw caps and baked items (a spatula, a 100 ml beaker, two 4 ml glass conical vials).

___ Bake glassware and spatula wrapped in foil and taped with autoclave tape at 180˚C for 4 h

Materials:

- FA-free BSA (BM, #100069), MW: 68000
- Na_2CO_3 (JTBaker, #3604-01), MW: 105.99
- RPMI 1640 medium (Mediatech, #150400LV)
- Sterile distilled water (Baxter, 2F7115)
- Others: filters (0.2 µm, tuffryn membrane), syringes, and icebox.
- Fatty acid stocks in ethanol (stored under N_2 at -80˚C):

Recorded density and date of fatty acid stocks:

Example: DHA [10.837 µg/µl in EtOH], (2-01-02)

LA [11.022 µg/µl in EtOH], (8-13-01)

Procedure:

1. ___ Aliquot ~40 ml of RPMI 1640 and 10 ml of sterile distilled water in a sterile hood.
2. ___ Keep stocks on ice before and after vortex stock FA-EtOH vigorously.
3. ___ Add 10 mg of FA (dissolved in EtOH) into 2 ml conical glass vial, respectively. Flush tubes with N_2 before returning stocks to –80˚C for storage.

Example: need 922.76 µl DHA [10.837 µg/µl]

907.28 µl LA [11.022 µg/µl]

4. ___ Clean needle with organic solvent. Dry down the FA with low stream of N_2.

5. ___ Make 0.05 M Na_2CO_3 while the FA-EtOH is drying.
Example: 53 mg Na_2CO_3 in 10 ml sterile H_2O (need ~2 ml per FA-BSA)

5. ___ Make 15% BSA solution.
- Add 20 ml of RPMI 1640 medium into a 100 ml 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 dry down the FA,
- Add 2 ml of 0.05 M Na_2CO_3 to each FA vial.
- Flush the vial with N_2 before vortex extensively.
- Sit the vials at RT for 1 h. Vortex the vials several times to aid FA dissolve completely.

7. ___ Calculate the materials needed to make 2.5 mM FA-BSA complex at FA/BSA: 3/1 molar ratio.
Example: DHA (MW: 328.5): 10 mg in 2 ml 0.05M Na_2CO_3;
BSA (MW: 68000): 15% Solution

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

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

- 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 MW.)/ 2.5 (mM) = 0.012177 (L) = 12.177 ml

- Calculate the volume of basal 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's 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 volume of FA-Na_2CO_3, 15 % BSA, and RPMI to 15 ml conical tubes.

	FA-Na_2CO_3	15 % BSA	RPMI	FA-BSA
DHA	1.9	4.37	5.30	11.57
LA	1.9	5.12	6.53	13.55

9. ___ Flush tubes with N_2. Shake them on a belly dancer for 0.5 ~ 1 h at RT.

10. ___ Use 0.2 μm tuffryn membrane to filter sterilize FA-BSA complex in a cell culture sterile hood. Aliquot the complex.

11. ___ Store aliquots protected from light at –20 °C. Retain for only 1 month.

Protocol # A-5 YAMC Cell Culture: Fatty acids, Butyrate and other Treatments
Ref: Fan et al. Am J Physiol (1999)

Purpose: To treat YAMC cells with butyrate and different polyunsaturated fatty acids.

Pre-preparation:
- ❑ Recover cells from liquid nitrogen (refer to Protocol # A-2)
  Sources: User: _____ Passage: _____ Rack: ____ Box#: ___ R&C: _____
- ❑ Grow cells in a T-75 flask in complete RPMI 1640 medium with γ-IFN.
- ❑ Allow >24 h for cells to adhere and to recover from trypsinization.

Day 1: (for experiment using chambered cover glass)
1. ___ Treat 2 wells chambered cover glasses (Nalge Nunc Intl, # 155380): 1 ml media per well.
   - ❑ Thaw. Filter sterilizes FBS with 0.2 μm tuffryn membrane (Acrodisc, #4192).
   - ❑ Prepare 20 % FBS in RPMI 1640 media (always make 2~3 ml extra).
     Calculation (example): 2 ml FBS + 8 ml Media ---> 10 ml Media/FBS
   - ❑ Load 1 ml media/FBS per well.
   - ❑ Incubate for ≤ 4 h or overnight at 33 ˚C.

Day 2:
2. ___ Warm complete RPMI 1640 to RT.
3. ___ Trypsinize and seed cells (refer to Protocol # A-3)
4. ___ Seed YAMC at 7,000 cells/well in a 2-well chambered cover glass.
   ____ # chambered slides needed.
   ____ml cells/RPMI media + ___μl γ-IFN (at 1μl per 10 ml media)
5. ___ In a conical tube: add together calculated volume of cell/media and fresh media with γ-IFN. Gently invert to mix.
6. ___ Add 1 ml cells/ml (pipette to homogenize) into each pretreated well; incubate at 33 ˚C under 5 % CO_2 atm.

Day 3, 4 and 5: [Note: cells should have recovered from trypsinization.]

7. ___ Treat cells with 50 μM fatty acids.
   - ❑ Check if vitamin E treatment is required. The treatment starts on Day3 and last for 72 h, refer to **Media Preparation** for details.
   - ❑ Defrost FA-BSA stock [2.5 mM].
     *FA stock should be prepared and used within a month and should be stored at –20˚C.
   - ❑ Prepare 50 μM fatty acids with complete RPMI 1640.
     Calculation: (0.02 ml stock + 0.98 ml Media ----> 1 ml FA/media)
     ____ ml stock + ___ ml Media -----> ___ ml FA/media
     *Make fresh FA solution everyday and do not store left over FA stock.
   - ❑ Add γ-IFN (1 μl per 10 ml FA/RPMI 1640 media).

(An additional step for Day 5):

___ Butyrate co-treatment

- Prepare 1 ml 500 mM Butyrate stock in a tube wrapped in foil to protect butyrate stock from light. Refer to **Media Preparation**.
- Start 5 mM butyrate treatment by gently pipetting in 10 µl of 500 mM butyrate during the designated time. Ref: Fan et al. Am J Physiol (1999)
- Gently pipette with a 1000P pipette to mix evenly.

□ Check if any of the following treatments is involved on day 5. Refer to **Media Preparation** for the following treatments.

□ MitoQ □ Cyclosporin A (CsA) □ Bongkrekic acid (BKA)

A sample timetable: estimated butyrate treatment, dye loading and assay time
(** Modify timetable accordingly based on experimental design)

Incubation time (h)/ butyrate treatment		Butyrate loading time	Dye loading time	Assay time	Note
6	+ But				
	- But				
12	+ But				
	- But				
24	+ But				
	- But				

** During planning, expect to spend 7~10 min on imaging per dish.
** Start dye loading for the next dish at ~3 min prior to the finishing of the previous one.

Day 6: Imaging Day (Analysis is done in the Imaging Analysis lab in vet school)
Preparation:

- Warm *Leibovitz* buffer (Gibco, #21083-027) & RPMI 1640 media to RT (<2 h) before start.
- Bring the following to Image Analysis Lab.
  a) In an ice chest with blue ice: fluorescence probes and 500 mM butyrate (and isobutyrate, when applicable).
  b) In a case at RT: Fluorescence probe in media, *Leibovitz* buffer, complete RPMI 1640 with γ-IFN, PBS, gloves, timer, pipettes.
  c) In a warm case: Cell culture covered in foil. In the case, keep a flask of warm water to maintain a warm environment for the cells and a thermometer to gauge the change of temperature during transfer.

8. ___ Dye and inhibitors/control loading**:**

Refer to protocol #5: Lipid oxidation analysis using DPPP.
#6: Mitochondrial membrane potential analysis using Rhodamine 123.
#7: Cytosolic oxidation analysis using CMH_2-DCFDA.

Media preparation [Otherwise stated, all "media" refers to complete RPMI 1640**]**
5 mM (final concentration, i.e. f.c.) Butyrate (Acros, #263190050)
___ Stock preparation: **protect from light and use within 4 h.

- 500 mM butyrate stock: weigh out and dissolve 55 mg sodium butyrate in 1 ml media.
- Filter sterilize stock using a 0.2 µm tuffryn membrane (Acrodisc, #4192).

___ Load 10 µl butyrate [500 mM] stock per 1 ml YAMC cell/media.

5 mM (f.c.) Isobutyrate (Sigma, # I-1754) Ref: Heerdt et al. Cancer Res (1994)

___ Stock preparation:

- 500 mM isobutyrate stock: pipette 44 µl isobutyrate in 1 ml media.
- Filter sterilizes stock using a 0.2 µm tuffryn membrane (Acrodisc, #4192).

___ Load 10 µl isobutyrate [500 mM] stock per 1 ml YAMC cell/media.

10 µM (f.c.) Vitamin E Succinate (MW= 530.8) (Sigma, #95255) (DMSO (v/v%) = 0.1%)

___ Stock preparation:

- 10 mM (VEs): dissolve 53.08 mg in 10 ml DMSO (Sigma, # D8418)
- Filter sterilize 10 ml stock solution (Acrodisc, #4433) and make 1 ml aliquots.
- Flush vials with N_2 and store aliquots in -80˚C.

___ Load 1 µl VEs [10 mM] stock per 1 ml YAMC cell/media.

0, 2.5, 5 µM (f.c.) Mito Q (MW= 665.65) Ref: Kelso et al J Biol Chem (2001)

Stock in storage: 50 mM Mito Q/DMSO stored in inert gas: Argon environment, in dark

___ Working stock solution preparations:

Calculation: a) 50 mM(x) = (0.5 ml) 0.5 mM

x = 0.005 ml

0.5 mM (10 µl) = (1 ml) (5 µM)

b) 0.5 mM (100 µl) = (200 µl) (0.25 mM)

0.25 mM (10 µl) = (1 ml) (2.5 µM)

___ Mito Q [f.c.= 5 µM]:

- Load 5 µl *MitoQ* [50 mM] stock per 495 µl media to yield 0.5 ml Mito Q [500 µM].
- Load 10 µl *MitoQ* [50 mM] stock per 990 µl media to yield 1 ml Mito Q [5 µM].

___ Mito Q [f.c.=2.5µM]:

- Load 100 µl Mito Q [0.5 mM] stock per 100 µl media to yield 200 µl Mito Q [0.25 mM].
- Load 10 µl Mito Q [0.25 mM] stock per 990 µl media to yield 1 ml Mito Q [2.5 µM].

1 µM Cyclosporin A (CsA) (Calbiochem, #239835)

___ Stock preparation:

- 10 mM stock solution: Add 0.8315 ml Ethanol per 100 mg CsA. (aliquot and store stock solution at -80˚C)
- 1 mM CsA working solution: Add 900 µl DMSO per 100 µl CsA [10 mM: stock]
- 100 µM CsA in Media: Add 900 µl Media per 100 µl CsA [1 mM: working stock]
- Filter sterilizes using a 0.2 µm DMSO safe membrane (Acrodisc, #4433).

___ 30 min prior to butyrate loading, load 10 µl (100 µM) CsA per 1 ml cell/media.

___ For CsA txt >12 h, without aspirate old media, add another 10 µl CsA into each well at 12 h. Pipette to mix. Ref: Dr Tjalkens; Bradham et al. Mol.& Cell Biol. (1998)

1 µM Bongkrekic acid (BkA) (MW=486.6) (Sigma, #B6179, in solution)

___ (100x) Stock preparation:

- (For treatment requiring butyrate co-loading, prepare butyrate [500 mM] in advance).
- Load 48.66 µl BKA per 1 ml media (or media with 500 mM butyrate, for co-loading).

___ Load 10 µl of the stock into each well with ~1 ml YAMC cell/media.
___ Pipette to mix evenly.

Protocol # A-6 YAMC Cell Culture: Diphenyl-1-pyrenylphosphine (DPPP) Loading and Control Treatments

Ref: Takahashi et al. Free Radic Biol Med (2001)

Purpose: To study lipid oxidation using DPPP, lipid soluble fluorescents probe.

Cell culture: refer to Protocol # A-4

5 µM (f.c.) DPPP (Molecular Probes #D-7894) (MeOH: 0.0096%; DMSO: 0.096%)
___Stock preparation:

- 4.7 mM DPPP: dissolve 5 mg DPPP in solvents: 250 µl MeOH + 2.5 ml DMSO, aliquot at 100 µl and store in –20˚C for up to a month.
  Or, thaw a 100 µl aliquot.
- Vortex vigorously until all pellets dissolved.
- 5 µM DPPP: add *53* µl DPPP [4.7 mM] in 50 ml PBS.

1. ___ Aspirate old media from each well.
2. ___ Gently, rinse cells once with PBS.
3. ___ Load 1 ml DPPP/PBS [5 µM] into each well.
4. ___ Incubate at RT, protected from light, for 10 min.
5. ___ Wash twice with 1 ml PBS before adding 1 ml fresh *Leibovitz* buffer for imaging.

For control treatments only:

10 µM (f.c.) H_2O_2 (Sigma, #H1009)
___ Prepare DPPP [5 µM] working solution in advance.
___ Stock preparation:

- 50 mM H_2O_2: 5.67 µl H_2O_2 (30%) per 1 ml distilled H_2O.
  Calculation (example): X (50 mM) = (5 ml) (10 µM)
  X= 1 µl
- Add 1 µl H_2O_2 [50 mM] into 5 ml DPPP/PBS [5 µM] for co-loading.

1. ___ Aspirate old media from each well.
2. ___ Wash each well with 1 ml PBS.
3. ___ Load 1 ml solution into each emptied well.
4. ___ Incubate at RT, protected from light, for 10 min.
5. ___ Wash twice with 1 ml PBS before adding 1 ml fresh *Leibovitz* buffer for imaging.

25 µM (f.c.) Cumene hydroperoxide (Sigma, #C-0524) (EtOH: 0.048%)
___ Stock preparation:

- 100 mM CumOOH: 18.6 µl CumOOH per 1 ml solvent: 500 µl 95%EtOH and 500 µl dH_2O.
- Flush the bottle of reagent with Argon gas. Store it in dark at 2-4˚C.
  Calculation (example): X (100 mM) = (5 ml) (25 µM)
  X = 1.25 µl
- Add 1.25 µl CumOOH (100 mM) into 5 ml DPPP/PBS [5 µM] for co-loading.

1. ___ Aspirate old media from each well.

2. ___ Wash each well with 1 ml PBS.
3. ___ Load 1 ml solution into each emptied well.
4. ___ Incubate at RT, protected from light, for 10 min.
5. ___ Wash twice with PBS before adding 1 ml fresh *Leibovitz* buffer for imaging.

Fluorescence Microscopy Imaging
___ Monitor fluorescence intensities at 351 nm (excitation) and 380 nm (emission) using confocal microscopy (Meridian Ultima).

Protocol # A-7 YAMC Cell Culture: Rhodamine 123 Loading and Inhibitor Treatments

Ref: Scaduto Jr, et al. Biophysical J (1999); Fan et al. Am J Physiol (1999)

Purpose: To study mitochondrial membrane potential using Rhodamine 123 (Rhd 123).

656 nM (f.c.) Rhd 123 (Molecular Probes, #R-302)
___ Stock preparation:
- Prepare 13.12 mM Rhd 123: dissolve 5 mg Rhd 123 in 1 ml MeOH), aliquot at 100 µl and store in –20 ˚C for up to a month.
  Or, thaw a 100 µl aliquot.
- Prepare 656 nM working solution:
  Calculation (example): (x) (13.12 mM)= (50 ml) (656 nM)
  X= 2.5 µl
- Add 2.5 µl Rhd 123 [13.12 mM] per 50 ml of media.

1. ___ Aspirate old media from each well.
2. ___ Gently, rinse cells once with PBS.
3. ___ Load 1 ml (656 nM) Rhd 123 per well.
4. ___ Incubate at 33 ˚C protected from light for 15 min.
5. ___ Gently, wash cells with PBS before adding 1 ml fresh *Leibovitz* buffer (Gibco, #21083-027) for imaging.

Inhibitors:
1 µM Cyclosporin A (CsA) (Calbiochem, #239835)
___ Stock preparation:
- 10 mM stock solution: add 0.8315 ml Ethanol per 100 mg CsA (aliquot and store stock solution at –80 ˚C).
- 1 mM CsA working solution: add 900 µL DMSO per 100 µl CsA [10 mM: stock]
- 100 µM CsA in Media: add 900 µL Media per 100 µl CsA [1 mM: working stock]
- Filter sterilize using a 0.2 µm DMSO safe membrane (Acrodisc, # 4433).

___ 30 min prior to butyrate loading, load 10 µl (100 µM) CsA per 1 ml cell/media
___ For CsA txt >12 h: Without aspirate old media, add another 10 µl CsA into each well at 12 h. Pipette to mix. Ref: Dr Tjalkens; Bradham et al. Mol.& Cell Biol. (1998)

1 µM Bongkrekic acid (BKA) (MW=486.6) (Sigma, #B6179, in solution)
___ (100x) Stock preparation:

- ❑ (For treatment requiring butyrate co-loading, prepare butyrate [500 mM] in advance).
- ❑ Load 48.66 µl BKA per 1 ml media (or media with 500 mM butyrate, for co-loading)

___ Load 10 µl stock per well with ~1 ml YAMC cell/media.
___ Pipette to mix evenly.

Fluorescence Microscopy Imaging
___ Monitor fluorescence intensities at 488 nm (emission) and 530 nm (excitation) using confocal microscopy (Meridian Ultima).

Protocol # A-8 YAMC Cell Culture: CMH_2-DCFDA Loading and Control Treatments
Ref: Scaduto Jr, et al. Biophysical J (1999); Barhoumi et al. Fundam Appl Toxicol (1996).

Purpose: To study cytosolic reactive oxygen species production using CMH_2-DCFDA [5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester]

5 µM (f.c.) CMH_2-DCFDA (Molecular probes, #C-6827)
___ Stock preparation:
- ❑ Prepare 5 mM CMH_2-DCFDA: 50 µg into 18 µl DMSO
- ❑ Carefully pipette up and down to mix
  Calculation (example): (x) (5 mM) = (50 ml) (5 µM)
  X= 50 µl
- ❑ Add 50 µl (5 mM) per 50 µl of media.

1. ___ Aspirate old media from each well.
2. ___ Gently, rinse cells once with PBS.
3. ___ Load 1 ml (5 mM) CMH_2-DCFDA per well.
4. ___ Incubate at 33 ˚C protected from light, for 15 min.
5. ___ Gently, wash cells with PBS before adding 1 ml fresh *Leibovitz* buffer for imaging.

Control treatments only:
10 µM (f.c.) H_2O_2 (Sigma, #H1009)
___ Stock preparation:
- ❑ 50 mM H_2O_2: 5.67 µl H_2O_2 (30%) per 1 ml distilled H_2O
  Calculation (example): X (50 mM) = (5 ml) (10 µM)
  X= 1 µl
- ❑ Add 1 µl H_2O_2 [50 mM] per 5 ml PBS.

1. ___ Aspirate old media from each well.
2. ___ Wash each well with 1 ml PBS.
3. ___ Load 1 ml solution into each emptied well.
4. ___ Incubate for 10 min prior to dye loading.

25 µM (f.c.) Cumene hydroperoxide (Sigma, #C-0524) (EtOH: 0.048%)

___ Stock preparation:

- 100 mM CumOOH: 18.6 µl CumOOH per 1 ml solvent: 500 µl 95%EtOH and 500 µl dH_2O.
- Flush the bottle of reagent with Argon gas. Store it in dark at 4 ˚C.
  Calculation (example): X (100 mM) = (5 ml) (25 µM)
  X = 1.25 µl
- Add 1.25 µl CumOOH (100 mM) into 5 ml PBS.

1. ___ Aspirate old media from each well.
2. ___ Wash each well with 1 ml PBS.
3. ___ Load 1 ml solution into each empty well.
4. ___ Incubate for 10 min prior to dye loading.

Fluorescence Microscopy Imaging

___ Monitor fluorescence intensities at 488 nm (emission) and 530 nm (excitation) using confocal microscopy (Meridian Ultima).

Protocol # A-9 Cell Death Detection ELISA, Plus

Source: Roche, # 1774425

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

Purpose: To quantify apoptosis/histone-complexed-DNA fragments of floating YAMC cells and to normalize values to the number of adherent cells from the same culture dish.

Cell culture:

___ Seed 25,000 cells per 35 mm cell-culture-dish, 24 h prior to fatty acid treatment.
___ Refer to Protocol # A-4 for fatty acid, butyrate and other treatments.
___ Note: load only 1.5 ml total volume per dish on the last day of FA treatment.

Preparation:

__ Label 2 ml epi-tubes (2 sets): floater collection (step 1) & total cell counting.
1.5 ml epi-tubes: supernatant collection (step 11).
__ Reconstitute working solutions:

- Dissolve substances in bottle 1,2 and 3 in 450 µl ddH_2O for 10 min and mix evenly.
  Bottle 1: anti-histone-biotin
  Bottle 2: anti-DNA-peroxidase (store at 2-8°C for upto 2 months)
  Bottle 3: positive control

- ABTS tablets from bottle 7: one tablet/5 ml substrate buffer (bottle 6).
  **Store reconstituted ABTS protected from light for up to a month at 2-8°C.

__ Warm complete RPMI 1640 media and trypsin-EDTA to RT.

Assay Procedures:

Supernatant preparation (35 mm dish): (in room 307)

1. ___ Swirl dish to gather floaters. Collect all (1.5 ml) media with floaters in a 2 ml epi-tube.

(Replenish each dish of adherent cells with 1 ml PBS and keep plates at 33˚C. These cells will be counted within the next 3hs, see Cell Counting).

2. ___ Centrifuge floaters/media at 5,000 rpm (Eppendorf centrifuge) for 5 min at RT.
3. ___ Remove supernatant manually using a pipette *without* disturbing the pellet.
4. ___ Wash: resuspend pellet in 1 ml media (without γ-IFN).
5. ___ Centrifuge at 5,000 rpm (Eppendorf centrifuge) for 5 min at RT.
6. ___ Resuspend cell pellet in 125 µl lysis buffer (bottle 5).
7. ___ Lyse: mix thoroughly and incubate samples for 30 min at 4°C.

At the same time (in lab 321),

8. ___ Prepare immunoreagent, volume: 1/20 anti-DNA-POD, 1/20 anti-histone-biotin, 18/20 incubation buffer. [need 80 µl per sample]
9. ___ Prepare an ice-chest with ice.
10. ___ After 30 min incubation (step 7), centrifuge lysate at max speed (13,600 x g, Eppendorf centrifuge) for 10 min, at 4°C.
11. ___ Prepare a template and a microtiterplate (MP; with enough wells) to include all samples, controls and a blank. Set up the spectrometer to read at 405 nm (reference wavelength at 490 nm) from designated wells.
12. ___ Keep centrifuged lysate on ice, while transferring 100 µl of the supernatant carefully into an epi-tube (1.5 ml) without shaking the pellet.

ELISA procedures:

13. ___ Transfer 20 µl of all samples (include histone-DNA complex, bottle 3) into the middle of the MP well.
14. ___ Add to each well 80 µl of the immunoreagent using multiple channel pipette.
15. ___ Cover the MP with an adhesive cover and a foil. Incubate on a MP shaker under gently shaking (300 rpm) for 2 h at RT.
16. ___ Tape both sides of the modules to secure them. Remove solution thoroughly by inverting and tapping it on a piece of paper towel.
17. ___ Rinse each well (3 x) with 250 µl Incubation buffer (bottle 4). Remove solution carefully by tapping.
18. ___ Lights off. Pipette into each well 100 µl ABTS solution.
19. ___ Incubate on a plate shaker at 250 rpm until the color development is sufficient for a photometric analysis. (~10 min, but check after 5 min of incubation).
20. ___ Measure the optical density at wavelength set (step 11) against ABTS solution as a blank.

Cell Counting:

For each sample from a 35 mm dish:

1. ___ Swirl dish to agitate and to remove non-adherent cells remaining. Aspirate off PBS.
2. ___ Load 0.5 ml trypsin-EDTA into each 35 mm dish containing adherent cells.
3. ___ Incubate at 37˚C for 3 min.
4. ___ Terminate trypsinization by loading 1 ml complete RPMI 1640 media into each dish.
5. ___ Tap and swirl plate lightly. Collect all lifted cells in 1.5 ml epi-tube.
6. ___ Count cells using a hemacytometer.

Protocol # A-10 LDH Activity Assay for Adherent YAMC Cell Culture
Source: Roche, #1644793 Cytotoxicity-Detection Kit (LDH)

Purpose: To measure the cytotoxicity of a reagent on adherent YAMC cultures following a 24 h incubation period.

Cell culture/Assay preparation:
- ❑ All samples and controls should have an n>3. Prepare a 96-well template to include all samples and controls.
- ❑ Wash cells in complete RPMI 1640 medium. Dilute cell density to [~$1.4x10^4$ cells/100µl complete RPMI 1640 media] for optimal sensitivity.
- ❑ Add 100 µl cell-suspension per well in a sterile 96-well tissue culture plate except for wells designated for background control, substance control I and substance control II.
- ❑ Incubate cells overnight (>24 h) at 33˚C for cells to recover from trypsinization.

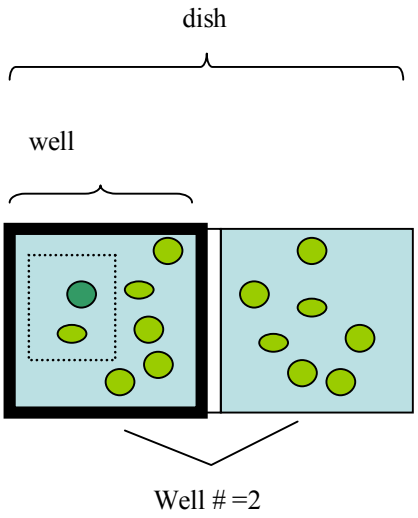
Day 1 Preparation:
- ❑ Prepare complete RPMI medium with γ-IFN.
- ❑ Prepare (2 x) solution of several concentrations of **test substance** (e.g. cytotoxic agents) in medium by serial dilution (will add 100 µl medium to a final vol. of 200 µl/well)

Procedures:
1. ___ **Samples:**
   - ❑ Aspirate old medium and floating cells from wells. Add 100 µl fresh media with γ-IFN into each well.
   - ❑ Transfer 100 µl of the test substance dilutions into corresponding wells containing adherent cells.
2. ___ **Low control**: add 100 µl medium (without test substance) to each of the triplicate wells containing 100 µl/well cells.
3. ___ **High control**: add 100 µl Triton-x-100 (Sigma, x-100) solutions (2% in RPMI 1640, filter sterilized) to each of the triplicate wells containing 100 µl/well cells.
4. ___ **Background control**: fill 200 µl medium into each of the triplicate wells without cells.
5. ___ **Substance control I**: add 100 µl substance (maximum concentration used in the experiment) to triplicate of wells containing 100 µl/well medium without cell.
6. ___ S**ubstance control II**: load 50 µl test substance + 50 µl LDH standard + 100 µl medium to triplicate of wells without cell.
7. ___ Incubate cells in a (33 °C, 90% humidity, 5% CO_2) incubator for 24 h.

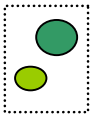
Day 2 Assay:
8. ___ Use a multi-channel pipette, transfer 100 µl/well supernatant carefully from the culture plate into corresponding wells of an optically clear 96 well flat bottom MP.
9. ___ Add 100 µl reaction mixture to each well and incubate for ~30 min at RT, in dark.
10. ___ Set ELISA reader to measure absorbency at 490 nm (reference wavelength: 650 nm).
11. ___ Read.

Protocol # A-11 Image Analysis field selection

Purpose: To show the field of cells analyzed to facilitate statistical analysis and data interpretation.



= one cell (or area)
=> Average intergrated intensity (per pixel)



8-10 fields per well were imaged. Cell number per field is highly dependent on each experiment and/or treatment. ranges 6 ~ 200 cells per well

mean = Average (average integrated intensity) of all cells in each well

Protocol # A-12 Criteria for cell selection

Purpose: To set up the criteria for cell (boundary of a cell) selection for all assays involving image analysis using the Meridian Instrument system.

Include:

a) only a WHOLE CELL. The keys are "whole" (see, **Exclude**, "c") and "cell". Make sure an area selected is a cell and use palette options, e.g., hue-lum to justify cell boundary.

Exclude:

a) Any cell with an odd shape.
It is possible to have contamination of cell type. Select only epithelial cells, which are of research interest.

b) Any cell with an odd size.
Within each screen, select areas of similar sizes. Exclude those that are too big or too small. Refer to Figure 1 (below).

c) Any cell that < 90% of its area is within the screen. Refer to Figure 2.

d) Actively dividing cell.
These cells show different properties compared to the non-dividing cell. They are normally very bright regardless of treatment.

e) Two neighboring cells that do not show clear edges or boundaries. Splitting the two cells subjectively may alter the average integrated values. Refer to Figure 3.

f) When in doubt (exclude the cell)!

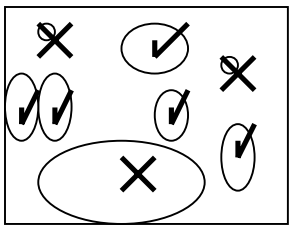


Figure 1

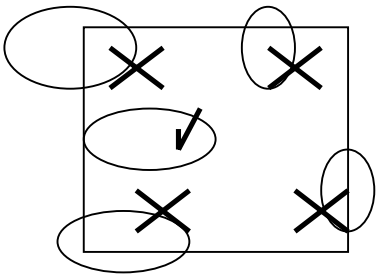


Figure 2

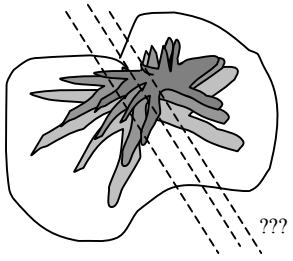


Figure 3

Protocol # A-13 Protein Extraction and Western Blotting

Purpose: To extract cytosolic and mitochondrial protein for protein assay or western blotting.

Protein extraction preparation:

- ❑ Prepare an ice-chest with ice.
- ❑ Turn on and cool down eppendorf microfuge to 4˚C.

Buffer Preparations:

To make 250 ml Stock Mito buffer: (final concentration, i.e. f.c.) 0.3 M sucrose (Sigma, S9378)/ 1 mM EDTA (Sigma, ED4SS)/ 5 mM Hepes (Sigma, H4034), pH 7.4

1. ___ Weigh and add 596 mg Hepes into ~10 ml dH_2O. Adjust pH to 7.4 with 10 M NaOH.
2. ___ Add 51.4 g sucrose, 190 mg Na_4EDTA.
3. ___ Bring up to 250 ml with dH_2O. Store at -20˚C in 10 ml aliquots.

- ❑ Complete mito buffer:

1. ___ Thaw a 10 ml aliquot of mito buffer stock.
2. ___ Add 0.4 ml protease inhibitor cocktail (Sigma, p8340),
3. ___ Add 5 mg DTT (Sigma, D0632) to each 10 ml aliquot to make 3 mM DTT (f.c.).

To make 20 ml Stock RIPA buffer: (f.c.) 50 mM Tris-HCl (Fisher, BP153-500), pH 7.4, 0.5% deoxycholate (Sigma, D6750), 1% NP-40 (Sigma, N6507), 0.1% SDS (Fisher, BP166-100), 50 µM NaF (Sigma, S6521) (Note: use made up 0.4 M NaF stock from Lauire)

1. ___ Dissolve 157.6 mg Tris-HCl in dH_2O, add dH_2O up to 20 ml
2. ___ add 0.1 g deoxycholate
3. ___ add 0.2 ml NP-40
4. ___ add 0.02 g SDS
5. ___ add 2.5 µl (0.4 M) NaF stock from Laurie
6. ___ store at -20˚C in 1.2 ml and 1.5 ml aliquots

- ❑ complete RIPA buffer:

1. ___ Immediately before use, dissolve 1 tablet mini protease inhibitor (Roche, 1836153) in 1.5 ml stock RIPA buffer.
2. ___ Add 171.6 µl (of 1.5 ml RIPA with protease inhibitor) in 1.2 ml stock RIPA buffer.

Procedure:

1. ___ Spin down YAMC cells at 200 x g for 5 min.
2. ___ Wash pellet with PBS (~5 ml), spin at 200 x g for 5 min (Repeat x2).
3. ___ Aspirate supernatant and resuspend pellet with 500 µl complete mito buffer.
   ** Keep everything on ice from this step on.
4. ___ Pass through 27 gauge needle, 3 times.
5. ___ Centrifuge at 600 x g for 10 min at 4˚C in microcentrifuge.
6. ___ Transfer supernatant to 1.5 ml epi-tube and discard nuclei pellet (and unlysed cells)
7. ___ Centrifuge supernatant at 15,000 x g for 10 min at 4 ˚C.
8. ___ Transfer supernatant (**cytosol**) to a new 1.5 ml epi-tube. Save pellet (**mitochondria**).

Cytosolic fraction:

1. ___ Mix, aliquot supernatant into 30 µl each and store at -20 ˚C.

Mitochondrial fraction:

1. ___ Resuspend mitochondria pellet into 100 µl RIPA buffer.
2. ___ Pass it through 29 gauge needle and sit it on ice 20-30 min. Label 500 µl tubes.
3. ___ Centrifuge at 12,000 x g for 10 min to remove unsolubilized material
4. ___ Transfer supernatant (solubilized mitochondrial perparation) to new 1.5 ml epi-tube
5. ___ Mix and make 11 µl aliquots. Store at -20˚C.

6. ___ Save aliquots of complete mitochondrial buffer and RIPA buffer for protein assay.

****

Western blotting

Day 1-Preparation:

- ❑ Thaw samples in a ice chest with ice.
- ❑ Label 0.6 ml epi-tubes.
- ❑ Prepare western template sheet.
- ❑ Transfer membrane (Immobilon-P, Invitrogen, LC2005-2)

1. ___ Pipette samples, H_2O, 5 x pyronin into 0.6 ml epi-tubes (final volume <35 µl/well). Mix by gentle inversion. Quick spin in a table-top centrifuge.
2. ___ Heat at 98˚C, 5 min (or 10 min, if sample >20 µl). Quick spin to collect condensation.
3. ___ Remove tape and comb off the gel cassette. Mark lane with a marker for visibility for sample loading. Align 3rd grid with lower gasket and clamp unit. (For one gel, put a alumine plate to block other side).
4. ___ Add "running buffer" into space between gels and into basin.
5. ___ Load samples (use 10 µl tips, helps to prevent bubble accumulation in the well).
6. ___ Lid on. Bring the unit into 4˚C cold room. Connect electrodes to power supply (red to red, black to black). Run at 45 mAmp (1 gel) or 90 mAmp (2 gels) for 1 h.

* When dye front nears bottom of gel, turn off power supply, set current to 0, and unplug unit.

7. ___ Pour "transfer buffer" into a clean staining tray.
8. ___ Return "running buffer" from unit into original bottle for re-use (*fresh running buffer runs faster).
9. ___ Place gel case on bench, large side down. Crack it open with a spatula along the seam.
10. ___ To mark lane, cut the corner above the 1st lane of gel. Cut gel away from case from the thick bottom lip. Carefully transfer gel to staining tray containing transfer buffer.
11. ___ Set up transfer cassette in large staining tray: black side down, thick sponge on bottom. Immerse cassette and sponge in transfer buffer.
12. ___ Wet 6 mm filter paper with transfer buffer, top with a thick sponge in landscape orientation.
13. ___ Place gel (quickly) onto filter paper, lane 1 on the right for the protein to end up on left side of membrane.
14. ___ Wet transfer membrane with methanol (use dry gloves and tweezers to avoid nonspecific blotching). Layer it onto gel. Prevent bubbles getting between the layers, especially between gel and membrane.
15. ___ Wet another filter paper with transfer buffer. Layer it onto membrane. Use test tube to gently roll out bubbles.
16. ___ Place thin sponge onto filter paper. Roll out bubbles. Close cassette.
17. ___ Insert cassette into transfer unit (black side faces labeled "black", hinges face up).
18. ___ Put stir bar into bottom of transfer unit. Fill unit with transfer buffer. Bang unit to get rid of bubbles. Stir on stir plate in 4˚C cold room.

19. ___ Run unit at constant current 400 mAmp for 75 min (max 90 min, for bigger proteins).

While transferring:

20. ___ Prepare 4% nonfat dry milk (Carnation)/PBS/Tween in a 50 ml tube (1.2 g/30 ml PBS/Tween).
21. ___ After transfer, move cassette to a large tray. Open cassette. Clip off the top right corner of membrane to mark the first lane.
22. ___ Flip membrane with tweezers for the protein side to face up. Rinse and quick shake in PBS/T. Replace PBS/T with 4 % nonfat milk/PBS/T. Shake gently for 1 h at RT.

Optional: Stain gel by immersing gel in Coomassie Staining solution (reusable) on a shaker for 1 h at RT. Rinse and de-stain with de-staining solution by shaking vigorously. Change de-staining solution every 15 min for as many times as needed. Look for any blue band or non-transferred protein.

23. ___ Return "transfer buffer" from unit into original bottle for re-use.
24. ___ Prepare 4% nonfat milk/PBS/T (0.8 g/20 ml PBS/Tween). Immediately before adding transfer membrane, add 1˚Ab. Mix gently. The membrane should always face up.
25. ___ Transfer membrane into 1˚Ab. Shake gently in 4˚C cold room overnight.

Day 2

26. ___ Transfer membrane into tray with PBS/T for brief wash
27. ___ Pour out PBS/T, add fresh PBS/T to tray. Shake vigorously for 10 min. Repeat once.
28. ___ Prepare 4% nonfat milk/PBS/T in a 50 ml tube and pour into small tray. Immediately before immersing membrane, add 2˚Ab. Mix gently.
29. ___ Transfer membrane into 2˚ Ab, shake gently at RT for 1 h.
30. ___ Transfer membrane into a tray with PBS/T for a brief wash.
31. ___ Pour off PBS/T and add fresh PBS/T to wash. Shake vigorously for 15 min. Repeat.
32. ___ Mix Chemiluminescent Super Signal reagents A and B (0.5 ml each) into a 1.5 ml epi-tube. Mix by gentle inversion.
33. ___ Using a pair of tweezers, place membrane in a folded acetate sheet, with protein side facing up.
34. ___ Pipette reagent mixture from step 32 onto membrane. Gently lower acetate sheet to cover membrane with reagent mixture.
35. ___ Incubate for 5 min at RT.
36. ___ Transfer membrane to another new acetate sheet.
37. ___ Immediately, read chemiluminescence on Fluor S-machine in room 435.

In room 435:

38. ___ Open machine doors (top and bottom). Ensure camera is set at 1.4 aperture for maximum light exposure.
39. ___ Software instructions: choose *Quality One* under *file*. Open a "*new file*" and select function "*Fluor-S*". Click on *Select*, *Blotting chemiluminescent,* and *High Resolution.*
40. ___ Focus (at lower aperture, if too bright). Click once on the *focus* button until you hear camera clicks. With a piece of paper with visible text, adjust focus on the camera.
41. ___ To properly position the membrane, make sure the numerical aperture is set at 1.4, close camera door (top). Next, click on *position* until you hear the camera clicks. Position membrane.
42. ___ Acquire for 1 sec to warm up camera. Refocus if necessary. Acquire image for up to 5 min (gradual increase).

43. ___ On a pop up window, press and hold "*Apple" and "B*", and check "*invert display*" and "*autoscale*"
44. ___ Save file and/or export in tif format.

Protocol # A-14 Dual labeling of apoptotic/necrotic YAMC using Annexin-V FITC & Propidium Iodide (PI)
Ref: Fan et al Am J Physiol (1999)

Purpose: To quantify early and late apoptotic events in YAMC cultures; immunofluorescense technique(47).

**Caution: Sodium Azide and PI are poisonous and hazardous substances. Handle with care and dispose of properly.*

Reagents/Materials:
- Annexin-V-FITC/PI Kit (Biosource, # PHN 1018):
  -Annexin V/FITC buffer saline: 1% BSA, 0.1% Sodium Azide, pH7.4
  -PI: 50 µg/ml PBS
  -Annexin Binding Buffer (1x): 10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4
- PBS (sterile) (Invitrogen, 14190-B6)
- RPMI Media (Mediatech # 150400LV) with γ-IFN
- Sterile H_2O
- Sterile100 mm dish for cell culture
- 5 mM Butyrate (Acros, #263190050)
  ___ Dissolve 55 mg sodium butyrate in 1 ml RPMI media ➔ [500 mM] Sodium butyrate stock
  ___ Filter sterilize (0.2µm, tuffryn membrane-REF4192)
  ___ Load 10 µl [500 mM] butyrate per 1 ml YAMC cell/media

Preparation:
- Seed 100k YAMC cells in a 100 mm diameter dish (Falcon, # 353803) to grow for 72 h.
- Treat or induce apoptosis in cells with different treatments (e.g. 5 mM butyrate treatment)

Day of experiment~
- Tube 1: Dilute 10x Annexin-V binding buffer 1:10 in sterile distilled H_2O (10 µl in 90 µl).
- Tube 2: Mix 1x Binding buffer with AnnexinV/FITC and PI buffers (5 µl Annexin-V/FITC and 10 µl PI per 100 µl binding buffer) *****protect from light.**
- Warm binding buffers and RPMI complete media (need ~6 ml per dish) in 37˚C water bath.
- Turn on fluorescence microscope and make sure the system is working before beginning.

Procedure:
A) Floating cells:
1. ___ Collect old media with floating cells in a sterile 15 ml tube.
2. ___ Wash monolayer of cell with 6 ml sterile PBS. Collect sample into the same tube.
   ** Incubate adherent cell (for counting) in RPMI complete media/γ-IFN at 33˚C while working on floating cells

3. ___ Sediment floaters at > 200 x g (*need review*) for 5 min. Carefully pipette to remove supernatant.
4. ___ Wash pellet in PBS (~6 ml). Repeat #3.
5. ___ Remove supernatant (~30 µl left). Gently tap to release cells. Resuspend pellet with 90 µl of Tube 2 (Biosource suggest: 2-3x10^6 cells/ml of tube 2)
6. ___ Remove ~ 40 µl of the mixture to count cell using hemacytometer.
   i) Without further delay,
      ___ Resuspend (~ 50 µl "floaters" left) with 200 µl binding buffer from tube 1.
      ___ Incubate at RT for 15 min in the dark. (Work on 2nd tube while waiting)
   ii) Mix well. Drop ~ 40 µl of sample mixture on a microscope slide and cover with a cover slip, 24 x 60 mm. Invert slide before mounting it on the stage.
   iii) Set magnification power at (10 x 20) X. Use fluorescence microscopy to capture 5 fields per slide, 3 images per field: phase contrast, FITC and TX-Red filters.
   iv) Overlay images. Count positively stained cells and total cell per image.

B) Adherent cells only (-ve control):
1. ___ Aspirate old media.
2. ___ Wash cells with 6 ml sterile HBSS.
3. ___ Trypsinize adherent cells with 6 ml trypsin for 3 minutes.
4. ___ Resuspend cells with 12 ml complete media to stop reaction.
5. ___ Transfer cell to a 50 ml falcon tubes & centrifuge at 200 x g for 5 min.
6. ___ Repeat step A) 5 & A) 6.

Protocol # A-15 Agilant anti-active caspase-3 cell fluorescence assay
Ref: Turner et al Can Lett (2002)

Purpose: To quantify apoptosis by active-caspase-3 immunostaining technique.

Preparation:
- YAMC cell growing and apoptosis induction, e.g.
  Positive control: 39°C w/o γ-IFN, 72 h, **floaters only**
  Negative control: 33°C w/ γ-IFN, **adherent cells only**
  Treatment control: 33°C w/ γ-IFN, 6 h H_2O_2 (150 µM), **adherent and floaters**
  Test sample #1: 33°C, 72 h DHA (50 µM) + 24 h Butyrate (5 mM), **adherent and floaters**
  Test sample #2: 33°C, 72 h LA (50 µM) + 24 h Butyrate (5 mM), **adherent and floaters**

Stock preparations:
A: Staining buffer (s.b.)
B: 1.5 µl SYT016 per 1 ml s.b.. [need: ____ml s.b, + ___ µl SYT016]
C: 5 µg anti-caspase-3 (BD #559565) per 1 ml *BD perm/wash* solution.
[need: ___ml soln + ___ µg Ab]
D: 5 µg Cy5-conj. goat anti-mouse (Jackson IR #111-176-045) per 1 ml s.b.
[need ___ml s.b. + ___ µg Ab]
E: *BD Cyto-fix/Cyto-perm* buffer (diluted in dH_2O) (BD, 554715) [need ____ ml]
F: 1x *BD perm/wash solution* (dilute 10x in dH_2O) (BD, 554715)[(1x) needed ____ ml]

Procedure:

1. ___ Harvest, count and resuspend cells at $5x10^5$/ml. Transfer 1 ml into a 2 ml epi-tube.
2. ___ Adjust cell density to $1x10^6$cells/ml in stock B (0.5 ml stock B for $5x10^5$ cells).
3. ___ Incubate for 10 min, at 33°C.
4. ___ Wash cells in 2 ml s.b. and centrifuge at 200x g for 5 min.
5. ___ Resuspend $5x10^5$ cells in 250 µl stock E.
6. ___ Incubate for 20 min, at 4°C (in the fridge).
7. ___ Wash in 2 ml stock F. Centrifuge.
8. ___ Add 50 µl stock C: 1°Ab (to 5 x 10^5 cells).
9. ___ Incubate on ice, 20-30 min.
10. ___ Wash cells in 2 ml s.b.. Centrifuge.
11. ___ Add 50 µl stock D: 2°Ab.
12. ___ Incubate on ice, 30 min, in **dark.**
13. ___ Wash cells in 2 ml s.b.

Instrument set up:

- Set Bioanalyzer (Agilent 2100) up for cell assay: insert pressure cartridge; set chip selector at **position 2.**

Sample loading:

1. ___ Centrifuge cells (200x g) to remove s.b.
2. ___ Dilute pellet with *cell buffer* (green) to $5x10^5$cells/250 µl.
3. ___ Check visually to make sure no cell clumps or agglomerates left. Pipett up and down to make sure cells are in suspension before loading.
4. ___ Load **10 µl** of the chip *priming solution* (white) into the large *priming well* (PS). Wait for 60 sec for capillary force to fill all channels of the chip.

**Essential practices: To prevent the introduction of air bubbles:
Insert tip of the pipette to the bottom & center of the well when dispensing.
Use inverse pipetting: push slightly over the first resistant when filling; empty the pipette tip only to the first resistance.

** Protect dye solution from light.

5. ___ Load **10 µl** of *focusing dye solution* (**yellow**) into the *focusing well* (FD) to adjust the bioanalyzer optics for each individual chip.
6. ___ Load **30 µl** of *cell buffer* (**green**) into wash of the **2** *cell buffer* wells (CB).
7. ___ Homogenize sample through piptetting. Load **10 µl** per sample well.
   ** Do not leave any well empty or the pressure cartridge may become clogged. Pipette 10 µl of *cell buffer* or a sample replicate into any empty sample well.
8. ___ Loaded chip must be read in bioanalyzer within 5 min.

Appendix B: EXPERIMENTAL RESULTS

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Table #

Table B-1 Lipid oxidation induced by fatty acid treatments and the anti lipid-oxidation effect of Vitamin E at 10 and 25 μM (figure 1&2)

Butyrate incubation (h)	Vit E (μM)	Fatty acids	No butyrate		5 mM butyrate	
			[1] Fluorescence intensity	[2] Standard Error	Fluorescence intensity	Standard Error
12	0	DHA	546	38	689	38
12	0	LA	603	37	670	37
12	0	No FA	516	37	629	37
24	0	DHA	638	37	821	37
24	0	LA	687	37	751	37
24	0	No FA	595	37	692	37
12	10	DHA	597	84	605	86
12	10	LA	610	86	617	86
12	10	No FA	606	86	610	86
24	10	DHA	638	86	687	86
24	10	LA	688	86	662	86
24	10	No FA	664	84	633	81
12	25	DHA	474	55	495	59
12	25	LA	636	55	578	49
12	25	No FA	340	51	464	161
24	25	DHA	869	53	1006	54
24	25	LA	1027	47	1150	45
24	25	No FA	639	45	926	48

[1]Values represent least squares means with (n) found in Table B-2, which is from 5 experiments.

Table B-2 The number of analyzed cells (n) per treatment group for each lipid-oxidation study

Date	well/txt	Treatment	VEs	No FA		LA		DHA	
		Butyrate Incubation		12 h	24 h	12 h	24 h	12 h	24 h
2002.10.18	2	Control		99	91	84	69	77	72
		5 mM butyrate		76	100	73	42	63	67
2002.11.15	4	Control		25	19	23	22	23	20
		5 mM butyrate		21	23	21	21	22	21
2003.01.30	2	Control		16	12	12	12	16	12
		5 mM butyrate		14	12	14	13	12	12
2003.02.10	2	Control	-	14	12	13	14	12	12
(10 μM Vit E)			+	12	13	12	13	13	12
		5 mM butyrate	-	12	12	12	12	13	12
			+	12	14	12	12	12	12
2003.02.21	2	Control	-	126	96	96	85	96	109
(25 μM Vit E)			+	63	117	40	89	43	50
		5 mM butyrate	-	137	98	110	93	103	117
			+	82	77	64	106	34	46

Total	12	Control	-	280	230	228	202	224	225
			+	75	130	52	102	56	62
		5 mM butyrate	-	260	245	230	181	213	229
			+	94	91	76	118	46	58

Table B-3 Fluorescence intensities and number of analyzed cells (n) per treatment group in butyrate dose response study

FA treatment	Fluorescence Intensity				Standard Error				Cell analyzed (n)			
Butyrate (mM)	0	1	5	10	0	1	5	10	0	1	5	10
DHA	1690	1972	2071	2113	30.1	29.2	32.7	39.7	306	325	260	176
LA	1669	1887	1749	1802	27.8	27.2	24.4	22.9	360	375	466	531
No FA	1697	1842	1843	1868	21.6	26.0	24.6	28.6	594	409	459	340

Table B-4 Percentage change in DPPP-oxide intensity following 5 mM butyrate co-incubation

FA Treatment	DHA	LA	No FA
Butyrate incubation			
12 h	*26.2%	11.1%	*21.9%
24 h	*28.7%	9.3%	16.3%

* Indicates a significant change in lipid oxidation following butyrate incubation within a FA group.

Table B-5 Differences and statistical significance in the induction of lipid oxidation following butyrate co-treatment among different fatty acid treatment groups

Treatment groups:
A = without butyrate and Vitamin E
B = with 5 mM butyrate (no Vitamin E)
C = with 5mM butyrate and 10 μM Vitamin E

To explain notation: DHA vs. LA: A – B 12 h means comparing the difference, A – B, for DHA vs. LA for the 12 h data.

Label	Estimate	Standard Error	DF	t Value	Pr > |t|	
DHA v. LA: A - B 12h	-76.2967	73.3530	33	-1.04	0.3058	
DHA v. no FA: A - B 12h	-29.6799	72.8201	31.7	-0.41	0.6863	
LA v. no FA: A - B 12h	46.6168	72.4843	31	0.64	0.5249	
DHA v. no FA: A - B 24h	-85.7469	72.7508	31.9	-1.18	0.2472	
LA v. no FA: A - B 24h	32.9323	73.0122	33.4	0.45	0.6549	
DHA v. LA: A - C 12h	-46.0405	129.75	220	-0.35	0.7231	
DHA v. no FA: A - C 12h	35.4460	129.66	219	0.27	0.7848	
LA v. no FA: A - C 12h	81.4865	129.49	219	0.63	0.5298	
DHA v. LA: A - C 24h	-73.5267	129.82	221	-0.57	0.5717	
DHA v. no FA: A - C 24h	-11.2017	126.50	199	-0.09	0.9295	
LA v. no FA: A - C 24h	62.3250	126.52	202	0.49	0.6228	
DHA v. LA: B - C 12h	30.2562	129.87	221	0.23	0.8160	

DHA v. no FA: B - C 12h	65.1259	129.66	219	0.50	0.6160	
LA v. no FA: B - C 12h	34.8697	129.64	217	0.27	0.7882	
DHA v. LA: B - C 24h	45.1525	129.89	222	0.35	0.7285	
DHA v. no FA: B - C 24h	74.5452	126.48	197	0.59	0.5563	
LA v. no FA: B - C 24h	29.3927	126.61	201	0.23	0.8167	

Table B-6 Analysis of ROS Data – Pooled Dates (8/11 & 9/1/04)

Compare Time, FA & Butyrate

This analysis was for both dates pooled together. The analysis was completed using a mixed model in SAS to account for the extra variance from subsampling from well. Factors of comparison are FA, time, and butyrate. Date was included as a factor to represent the two separate dates.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F	
Date	**1**	**53.4**	**20.56**	**<.0001**	*****
FA	2	53.3	1.73	0.1862	
butyrate	**1**	**53.3**	**5.77**	**0.0198**	*****
time	**2**	**53.3**	**27.46**	**<.0001**	*****
FA*butyrate	2	53.3	0.31	0.7322	
FA*time	4	53.3	0.81	0.5222	
time*butyrate	2	53.3	0.21	0.8084	
FA*time*butyrate	4	53.3	0.14	0.9674	

Date, butyrate, and time are significant.

The following are the least squares means & standard errors.

Least Squares Means

Effect	FA	time	butyrate	Estimate	Standard Error	DF	t Value	Pr > |t|
FA	DHA			480.62	20.4197	53.2	23.54	<.0001
FA	LA			437.40	20.4477	53.5	21.39	<.0001
FA	No-FA			486.84	20.4460	53.5	23.81	<.0001
butyrate			0mM	496.62	16.6727	53.2	29.79	<.0001
butyrate			5mM	439.95	16.7030	53.6	26.34	<.0001
time		12h		559.22	20.4351	53.3	27.37	<.0001
time		24h		495.26	20.4694	53.7	24.20	<.0001
time		6h		350.37	20.4088	53.1	17.17	<.0001
FA*butyrate	DHA		0mM	510.91	28.8745	53.2	17.69	<.0001
FA*butyrate	DHA		5mM	450.33	28.8797	53.2	15.59	<.0001
FA*butyrate	LA		0mM	453.44	28.8618	53.1	15.71	<.0001
FA*butyrate	LA		5mM	421.36	28.9710	53.8	14.54	<.0001
FA*butyrate	No-FA		0mM	525.52	28.8933	53.3	18.19	<.0001
FA*butyrate	No-FA		5mM	448.16	28.9353	53.6	15.49	<.0001
FA*time	DHA	12h		581.32	35.3811	53.2	16.43	<.0001
FA*time	DHA	24h		482.34	35.3936	53.3	13.63	<.0001
FA*time	DHA	6h		378.19	35.3257	52.9	10.71	<.0001
FA*time	LA	12h		522.58	35.4324	53.5	14.75	<.0001
FA*time	LA	24h		503.03	35.5000	53.9	14.17	<.0001
FA*time	LA	6h		286.60	35.3121	52.8	8.12	<.0001
FA*time	No-FA	12h		573.78	35.3664	53.2	16.22	<.0001
FA*time	No-FA	24h		500.40	35.4646	53.8	14.11	<.0001
FA*time	No-FA	6h		386.33	35.4060	53.4	10.91	<.0001
time*butyrate		12h	0mM	585.14	28.8915	53.3	20.25	<.0001
time*butyrate		12h	5mM	533.31	28.9060	53.4	18.45	<.0001
time*butyrate		24h	0mM	534.02	28.8921	53.3	18.48	<.0001
time*butyrate		24h	5mM	456.50	29.0025	54.1	15.74	<.0001
time*butyrate		6h	0mM	370.71	28.8460	52.9	12.85	<.0001
time*butyrate		6h	5mM	330.04	28.8774	53.2	11.43	<.0001
FA*time*butyrate	DHA	12h	0mM	619.87	50.0748	53.4	12.38	<.0001
FA*time*butyrate	DHA	12h	5mM	542.77	49.9970	53.1	10.86	<.0001
FA*time*butyrate	DHA	24h	0mM	521.56	50.0267	53.2	10.43	<.0001
FA*time*butyrate	DHA	24h	5mM	443.12	50.0807	53.4	8.85	<.0001
FA*time*butyrate	DHA	6h	0mM	391.29	49.9324	52.8	7.84	<.0001
FA*time*butyrate	DHA	6h	5mM	365.09	49.9830	53	7.30	<.0001
FA*time*butyrate	LA	12h	0mM	520.52	50.0324	53.2	10.40	<.0001
FA*time*butyrate	LA	12h	5mM	524.64	50.1842	53.9	10.45	<.0001
FA*time*butyrate	LA	24h	0mM	531.23	50.0631	53.3	10.61	<.0001
FA*time*butyrate	LA	24h	5mM	474.83	50.3441	54.5	9.43	<.0001
FA*time*butyrate	LA	6h	0mM	308.57	49.8718	52.6	6.19	<.0001
FA*time*butyrate	LA	6h	5mM	264.63	50.0050	53.1	5.29	<.0001
FA*time*butyrate	No-FA	12h	0mM	615.02	50.0145	53.2	12.30	<.0001
FA*time*butyrate	No-FA	12h	5mM	532.53	50.0160	53.2	10.65	<.0001
FA*time*butyrate	No-FA	24h	0mM	549.26	50.0359	53.3	10.98	<.0001
FA*time*butyrate	No-FA	24h	5mM	451.55	50.2722	54.2	8.98	<.0001

FA*time*butyrate	No-FA	6h	0mM	412.28	50.0814	53.4	8.23	<.0001
FA*time*butyrate	No-FA	6h	5mM	360.39	50.0609	53.4	7.20	<.0001

Now for pairwise comparisons. Estimates of differences, standard errors are given first, then p-values. I will highlight all <0.05 and for interaction terms, ones that have at least one factor in common:

The Mixed Procedure
Differences of Least Squares Means

Effect	FA	time	butyrate	FA	time	butyrate	Estimate	Standard Error	DF
FA	DHA			LA			43.2168	28.8960	53.3
FA	DHA			No-FA			-6.2198	28.8950	53.3
FA	LA			No-FA			-49.4366	28.9146	53.4
butyrate			0mM			5mM	56.6707	23.5983	53.3
time		12h			24h		63.9670	28.9222	53.5
time		12h			6h		208.85	28.8795	53.2
time		24h			6h		144.88	28.9038	53.4
FA*butyrate	DHA		0mM	DHA		5mM	60.5779	40.8374	53.2
FA*butyrate	DHA		0mM	LA		0mM	57.4690	40.8247	53.1
FA*butyrate	DHA		0mM	LA		5mM	89.5426	40.9018	53.5
FA*butyrate	DHA		0mM	No-FA		0mM	-14.6111	40.8471	53.2
FA*butyrate	DHA		0mM	No-FA		5mM	62.7495	40.8767	53.4
FA*butyrate	DHA		5mM	LA		0mM	-3.1089	40.8283	53.1
FA*butyrate	DHA		5mM	LA		5mM	28.9647	40.9054	53.5
FA*butyrate	DHA		5mM	No-FA		0mM	-75.1890	40.8507	53.2
FA*butyrate	DHA		5mM	No-FA		5mM	2.1716	40.8803	53.4
FA*butyrate	LA		0mM	LA		5mM	32.0736	40.8927	53.4
FA*butyrate	LA		0mM	No-FA		0mM	-72.0801	40.8380	53.2
FA*butyrate	LA		0mM	No-FA		5mM	5.2805	40.8676	53.3
FA*butyrate	LA		5mM	No-FA		0mM	-104.15	40.9151	53.6
FA*butyrate	LA		5mM	No-FA		5mM	-26.7932	40.9446	53.7
FA*butyrate	No-FA		0mM	No-FA		5mM	77.3606	40.8900	53.4
FA*time	DHA	12h		DHA	24h		98.9758	50.0444	53.3
FA*time	DHA	12h		DHA	6h		203.13	49.9964	53.1
FA*time	DHA	12h		LA	12h		58.7414	50.0715	53.4
FA*time	DHA	12h		LA	24h		78.2937	50.1193	53.6
FA*time	DHA	12h		LA	6h		294.72	49.9868	53
FA*time	DHA	12h		No-FA	12h		7.5444	50.0252	53.2
FA*time	DHA	12h		No-FA	24h		80.9173	50.0946	53.5
FA*time	DHA	12h		No-FA	6h		194.99	50.0530	53.3
FA*time	DHA	24h		DHA	6h		104.15	50.0054	53.1
FA*time	DHA	24h		LA	12h		-40.2344	50.0806	53.4
FA*time	DHA	24h		LA	24h		-20.6822	50.1283	53.6
FA*time	DHA	24h		LA	6h		195.75	49.9958	53.1
FA*time	DHA	24h		No-FA	12h		-91.4314	50.0342	53.2
FA*time	DHA	24h		No-FA	24h		-18.0585	50.1036	53.5
FA*time	DHA	24h		No-FA	6h		96.0096	50.0620	53.4
FA*time	DHA	6h		LA	12h		-144.39	50.0326	53.2
FA*time	DHA	6h		LA	24h		-124.84	50.0804	53.4
FA*time	DHA	6h		LA	6h		91.5913	49.9477	52.9
FA*time	DHA	6h		No-FA	12h		-195.59	49.9862	53
FA*time	DHA	6h		No-FA	24h		-122.21	50.0557	53.3
FA*time	DHA	6h		No-FA	6h		-8.1452	50.0140	53.2
FA*time	LA	12h		LA	24h		19.5523	50.1554	53.7
FA*time	LA	12h		LA	6h		235.98	50.0230	53.2
FA*time	LA	12h		No-FA	12h		-51.1970	50.0614	53.4
FA*time	LA	12h		No-FA	24h		22.1759	50.1308	53.6
FA*time	LA	12h		No-FA	6h		136.24	50.0892	53.5
FA*time	LA	24h		LA	6h		216.43	50.0708	53.4
FA*time	LA	24h		No-FA	12h		-70.7492	50.1092	53.5
FA*time	LA	24h		No-FA	24h		2.6237	50.1785	53.8
FA*time	LA	24h		No-FA	6h		116.69	50.1369	53.7
FA*time	LA	6h		No-FA	12h		-287.18	49.9766	53
FA*time	LA	6h		No-FA	24h		-213.80	50.0461	53.3
FA*time	LA	6h		No-FA	6h		-99.7365	50.0045	53.1
FA*time	No-FA	12h		No-FA	24h		73.3729	50.0844	53.5
FA*time	No-FA	12h		No-FA	6h		187.44	50.0428	53.3
FA*time	No-FA	24h		No-FA	6h		114.07	50.1122	53.6
time*butyrate		12h	0mM		12h	5mM	51.8229	40.8678	53.3
time*butyrate		12h	0mM		24h	0mM	51.1205	40.8582	53.3
time*butyrate		12h	0mM		24h	5mM	128.64	40.9359	53.7
time*butyrate		12h	0mM		6h	0mM	214.43	40.8255	53.1
time*butyrate		12h	0mM		6h	5mM	255.10	40.8477	53.2
time*butyrate		12h	5mM		24h	0mM	-0.7024	40.8685	53.3
time*butyrate		12h	5mM		24h	5mM	76.8135	40.9462	53.7
time*butyrate		12h	5mM		6h	0mM	162.60	40.8358	53.2
time*butyrate		12h	5mM		6h	5mM	203.28	40.8580	53.3
time*butyrate		24h	0mM		24h	5mM	77.5160	40.9366	53.7
time*butyrate		24h	0mM		6h	0mM	163.31	40.8261	53.1
time*butyrate		24h	0mM		6h	5mM	203.98	40.8483	53.2
time*butyrate		24h	5mM		6h	0mM	85.7891	40.9040	53.5
time*butyrate		24h	5mM		6h	5mM	126.46	40.9261	53.6
time*butyrate		6h	0mM		6h	5mM	40.6733	40.8156	53

The Mixed Procedure

Differences of Least Squares Means

Effect	FA	time	butyrate	FA	time	butyrate	Estimate	Standard Error	DF
FA*time*butyrate	DHA	12h	0mM	DHA	12h	5mM	77.0993	70.7607	53.2
FA*time*butyrate	DHA	12h	0mM	DHA	24h	0mM	98.3053	70.7819	53.3
FA*time*butyrate	DHA	12h	0mM	DHA	24h	5mM	176.75	70.8199	53.4
FA*time*butyrate	DHA	12h	0mM	DHA	6h	0mM	228.58	70.7152	53.1
FA*time*butyrate	DHA	12h	0mM	DHA	6h	5mM	254.78	70.7508	53.2
FA*time*butyrate	DHA	12h	0mM	LA	12h	0mM	99.3512	70.7857	53.3
FA*time*butyrate	DHA	12h	0mM	LA	12h	5mM	95.2309	70.8929	53.6
FA*time*butyrate	DHA	12h	0mM	LA	24h	0mM	88.6442	70.8073	53.4
FA*time*butyrate	DHA	12h	0mM	LA	24h	5mM	145.04	71.0061	54
FA*time*butyrate	DHA	12h	0mM	LA	6h	0mM	311.30	70.6725	53
FA*time*butyrate	DHA	12h	0mM	LA	6h	5mM	355.24	70.7665	53.3
FA*time*butyrate	DHA	12h	0mM	No-FA	12h	0mM	4.8493	70.7732	53.3
FA*time*butyrate	DHA	12h	0mM	No-FA	12h	5mM	87.3389	70.7744	53.3
FA*time*butyrate	DHA	12h	0mM	No-FA	24h	0mM	70.6124	70.7886	53.3
FA*time*butyrate	DHA	12h	0mM	No-FA	24h	5mM	168.32	70.9553	53.8
FA*time*butyrate	DHA	12h	0mM	No-FA	6h	0mM	207.59	70.8204	53.4
FA*time*butyrate	DHA	12h	0mM	No-FA	6h	5mM	259.48	70.8059	53.4
FA*time*butyrate	DHA	12h	5mM	DHA	24h	0mM	21.2060	70.7270	53.1
FA*time*butyrate	DHA	12h	5mM	DHA	24h	5mM	99.6464	70.7649	53.3
FA*time*butyrate	DHA	12h	5mM	DHA	6h	0mM	151.48	70.6602	52.9
FA*time*butyrate	DHA	12h	5mM	DHA	6h	5mM	177.68	70.6959	53
FA*time*butyrate	DHA	12h	5mM	LA	12h	0mM	22.2519	70.7307	53.1
FA*time*butyrate	DHA	12h	5mM	LA	12h	5mM	18.1316	70.8380	53.5
FA*time*butyrate	DHA	12h	5mM	LA	24h	0mM	11.5449	70.7524	53.2
FA*time*butyrate	DHA	12h	5mM	LA	24h	5mM	67.9432	70.9514	53.8
FA*time*butyrate	DHA	12h	5mM	LA	6h	0mM	234.20	70.6174	52.8
FA*time*butyrate	DHA	12h	5mM	LA	6h	5mM	278.14	70.7115	53.1
FA*time*butyrate	DHA	12h	5mM	No-FA	12h	0mM	-72.2500	70.7182	53.1
FA*time*butyrate	DHA	12h	5mM	No-FA	12h	5mM	10.2396	70.7194	53.1
FA*time*butyrate	DHA	12h	5mM	No-FA	24h	0mM	-6.4869	70.7336	53.2
FA*time*butyrate	DHA	12h	5mM	No-FA	24h	5mM	91.2223	70.9005	53.7
FA*time*butyrate	DHA	12h	5mM	No-FA	6h	0mM	130.49	70.7654	53.3
FA*time*butyrate	DHA	12h	5mM	No-FA	6h	5mM	182.38	70.7509	53.2
FA*time*butyrate	DHA	24h	0mM	DHA	24h	5mM	78.4404	70.7862	53.3
FA*time*butyrate	DHA	24h	0mM	DHA	6h	0mM	130.28	70.6814	53
FA*time*butyrate	DHA	24h	0mM	DHA	6h	5mM	156.47	70.7171	53.1
FA*time*butyrate	DHA	24h	0mM	LA	12h	0mM	1.0459	70.7520	53.2
FA*time*butyrate	DHA	24h	0mM	LA	12h	5mM	-3.0744	70.8593	53.5
FA*time*butyrate	DHA	24h	0mM	LA	24h	0mM	-9.6611	70.7737	53.3
FA*time*butyrate	DHA	24h	0mM	LA	24h	5mM	46.7372	70.9726	53.9
FA*time*butyrate	DHA	24h	0mM	LA	6h	0mM	212.99	70.6387	52.9
FA*time*butyrate	DHA	24h	0mM	LA	6h	5mM	256.94	70.7327	53.2
FA*time*butyrate	DHA	24h	0mM	No-FA	12h	0mM	-93.4560	70.7394	53.2
FA*time*butyrate	DHA	24h	0mM	No-FA	12h	5mM	-10.9664	70.7406	53.2
FA*time*butyrate	DHA	24h	0mM	No-FA	24h	0mM	-27.6929	70.7547	53.2
FA*time*butyrate	DHA	24h	0mM	No-FA	24h	5mM	70.0163	70.9217	53.7
FA*time*butyrate	DHA	24h	0mM	No-FA	6h	0mM	109.29	70.7867	53.3
FA*time*butyrate	DHA	24h	0mM	No-FA	6h	5mM	161.17	70.7722	53.3
FA*time*butyrate	DHA	24h	5mM	DHA	6h	0mM	51.8376	70.7195	53.1
FA*time*butyrate	DHA	24h	5mM	DHA	6h	5mM	78.0316	70.7551	53.2
FA*time*butyrate	DHA	24h	5mM	LA	12h	0mM	-77.3945	70.7899	53.3
FA*time*butyrate	DHA	24h	5mM	LA	12h	5mM	-81.5148	70.8972	53.6
FA*time*butyrate	DHA	24h	5mM	LA	24h	0mM	-88.1015	70.8116	53.4
FA*time*butyrate	DHA	24h	5mM	LA	24h	5mM	-31.7033	71.0104	54
FA*time*butyrate	DHA	24h	5mM	LA	6h	0mM	134.55	70.6767	53
FA*time*butyrate	DHA	24h	5mM	LA	6h	5mM	178.50	70.7707	53.3
FA*time*butyrate	DHA	24h	5mM	No-FA	12h	0mM	-171.90	70.7774	53.3
FA*time*butyrate	DHA	24h	5mM	No-FA	12h	5mM	-89.4068	70.7786	53.3
FA*time*butyrate	DHA	24h	5mM	No-FA	24h	0mM	-106.13	70.7928	53.3
FA*time*butyrate	DHA	24h	5mM	No-FA	24h	5mM	-8.4241	70.9595	53.8
FA*time*butyrate	DHA	24h	5mM	No-FA	6h	0mM	30.8479	70.8246	53.4
FA*time*butyrate	DHA	24h	5mM	No-FA	6h	5mM	82.7308	70.8101	53.4
FA*time*butyrate	DHA	6h	0mM	DHA	6h	5mM	26.1940	70.6504	52.9
FA*time*butyrate	DHA	6h	0mM	LA	12h	0mM	-129.23	70.6853	53
FA*time*butyrate	DHA	6h	0mM	LA	12h	5mM	-133.35	70.7927	53.3
FA*time*butyrate	DHA	6h	0mM	LA	24h	0mM	-139.94	70.7069	53.1
FA*time*butyrate	DHA	6h	0mM	LA	24h	5mM	-83.5409	70.9061	53.7
FA*time*butyrate	DHA	6h	0mM	LA	6h	0mM	82.7169	70.5718	52.7
FA*time*butyrate	DHA	6h	0mM	LA	6h	5mM	126.66	70.6660	53
FA*time*butyrate	DHA	6h	0mM	No-FA	12h	0mM	-223.73	70.6727	53
FA*time*butyrate	DHA	6h	0mM	No-FA	12h	5mM	-141.24	70.6739	53
FA*time*butyrate	DHA	6h	0mM	No-FA	24h	0mM	-157.97	70.6880	53
FA*time*butyrate	DHA	6h	0mM	No-FA	24h	5mM	-60.2618	70.8551	53.5
FA*time*butyrate	DHA	6h	0mM	No-FA	6h	0mM	-20.9897	70.7200	53.1
FA*time*butyrate	DHA	6h	0mM	No-FA	6h	5mM	30.8932	70.7054	53.1
FA*time*butyrate	DHA	6h	5mM	LA	12h	0mM	-155.43	70.7209	53.1
FA*time*butyrate	DHA	6h	5mM	LA	12h	5mM	-159.55	70.8282	53.4
FA*time*butyrate	DHA	6h	5mM	LA	24h	0mM	-166.13	70.7425	53.2
FA*time*butyrate	DHA	6h	5mM	LA	24h	5mM	-109.73	70.9416	53.8
FA*time*butyrate	DHA	6h	5mM	LA	6h	0mM	56.5228	70.6075	52.8
FA*time*butyrate	DHA	6h	5mM	LA	6h	5mM	100.47	70.7016	53.1
FA*time*butyrate	DHA	6h	5mM	No-FA	12h	0mM	-249.93	70.7083	53.1
FA*time*butyrate	DHA	6h	5mM	No-FA	12h	5mM	-167.44	70.7095	53.1
FA*time*butyrate	DHA	6h	5mM	No-FA	24h	0mM	-184.16	70.7237	53.1
FA*time*butyrate	DHA	6h	5mM	No-FA	24h	5mM	-86.4558	70.8906	53.6

FA*time*butyrate	DHA	6h	5mM	No-FA	6h	0mM	-47.1837	70.7556	53.2
FA*time*butyrate	DHA	6h	5mM	No-FA	6h	5mM	4.6992	70.7411	53.2
FA*time*butyrate	LA	12h	0mM	LA	12h	5mM	-4.1203	70.8630	53.5
FA*time*butyrate	LA	12h	0mM	LA	24h	0mM	-10.7070	70.7774	53.3
FA*time*butyrate	LA	12h	0mM	LA	24h	5mM	45.6913	70.9763	53.9
FA*time*butyrate	LA	12h	0mM	LA	6h	0mM	211.95	70.6425	52.9
FA*time*butyrate	LA	12h	0mM	LA	6h	5mM	255.89	70.7365	53.2
FA*time*butyrate	LA	12h	0mM	No-FA	12h	0mM	-94.5019	70.7432	53.2
FA*time*butyrate	LA	12h	0mM	No-FA	12h	5mM	-12.0123	70.7444	53.2
FA*time*butyrate	LA	12h	0mM	No-FA	24h	0mM	-28.7388	70.7586	53.2
FA*time*butyrate	LA	12h	0mM	No-FA	24h	5mM	68.9704	70.9254	53.7
FA*time*butyrate	LA	12h	0mM	No-FA	6h	0mM	108.24	70.7904	53.3
FA*time*butyrate	LA	12h	0mM	No-FA	6h	5mM	160.13	70.7759	53.3
FA*time*butyrate	LA	12h	5mM	LA	24h	0mM	-6.5867	70.8846	53.6
FA*time*butyrate	LA	12h	5mM	LA	24h	5mM	49.8115	71.0832	54.2
FA*time*butyrate	LA	12h	5mM	LA	6h	0mM	216.07	70.7499	53.2
FA*time*butyrate	LA	12h	5mM	LA	6h	5mM	260.01	70.8438	53.5
FA*time*butyrate	LA	12h	5mM	No-FA	12h	0mM	-90.3816	70.8505	53.5
FA*time*butyrate	LA	12h	5mM	No-FA	12h	5mM	-7.8920	70.8518	53.5
FA*time*butyrate	LA	12h	5mM	No-FA	24h	0mM	-24.6185	70.8659	53.6
FA*time*butyrate	LA	12h	5mM	No-FA	24h	5mM	73.0906	71.0324	54
FA*time*butyrate	LA	12h	5mM	No-FA	6h	0mM	112.36	70.8976	53.6
FA*time*butyrate	LA	12h	5mM	No-FA	6h	5mM	164.25	70.8832	53.6
FA*time*butyrate	LA	24h	0mM	LA	24h	5mM	56.3983	70.9978	53.9
FA*time*butyrate	LA	24h	0mM	LA	6h	0mM	222.66	70.6642	53
FA*time*butyrate	LA	24h	0mM	LA	6h	5mM	266.60	70.7582	53.2
FA*time*butyrate	LA	24h	0mM	No-FA	12h	0mM	-83.7949	70.7649	53.2
FA*time*butyrate	LA	24h	0mM	No-FA	12h	5mM	-1.3053	70.7661	53.3
FA*time*butyrate	LA	24h	0mM	No-FA	24h	0mM	-18.0318	70.7803	53.3
FA*time*butyrate	LA	24h	0mM	No-FA	24h	5mM	79.6774	70.9470	53.8
FA*time*butyrate	LA	24h	0mM	No-FA	6h	0mM	118.95	70.8121	53.4
FA*time*butyrate	LA	24h	0mM	No-FA	6h	5mM	170.83	70.7975	53.3
FA*time*butyrate	LA	24h	5mM	LA	6h	0mM	166.26	70.8634	53.5
FA*time*butyrate	LA	24h	5mM	LA	6h	5mM	210.20	70.9572	53.8
FA*time*butyrate	LA	24h	5mM	No-FA	12h	0mM	-140.19	70.9638	53.8
FA*time*butyrate	LA	24h	5mM	No-FA	12h	5mM	-57.7036	70.9651	53.8
FA*time*butyrate	LA	24h	5mM	No-FA	24h	0mM	-74.4301	70.9792	53.9
FA*time*butyrate	LA	24h	5mM	No-FA	24h	5mM	23.2791	71.1454	54.4
FA*time*butyrate	LA	24h	5mM	No-FA	6h	0mM	62.5512	71.0109	54
FA*time*butyrate	LA	24h	5mM	No-FA	6h	5mM	114.43	70.9964	53.9
FA*time*butyrate	LA	6h	0mM	LA	6h	5mM	43.9429	70.6232	52.8
FA*time*butyrate	LA	6h	0mM	No-FA	12h	0mM	-306.45	70.6299	52.9
FA*time*butyrate	LA	6h	0mM	No-FA	12h	5mM	-223.96	70.6311	52.9
FA*time*butyrate	LA	6h	0mM	No-FA	24h	0mM	-240.69	70.6453	52.9
FA*time*butyrate	LA	6h	0mM	No-FA	24h	5mM	-142.98	70.8124	53.4
FA*time*butyrate	LA	6h	0mM	No-FA	6h	0mM	-103.71	70.6772	53
FA*time*butyrate	LA	6h	0mM	No-FA	6h	5mM	-51.8236	70.6627	53
FA*time*butyrate	LA	6h	5mM	No-FA	12h	0mM	-350.39	70.7240	53.1
FA*time*butyrate	LA	6h	5mM	No-FA	12h	5mM	-267.90	70.7252	53.1
FA*time*butyrate	LA	6h	5mM	No-FA	24h	0mM	-284.63	70.7393	53.2
FA*time*butyrate	LA	6h	5mM	No-FA	24h	5mM	-186.92	70.9063	53.7
FA*time*butyrate	LA	6h	5mM	No-FA	6h	0mM	-147.65	70.7712	53.3
FA*time*butyrate	LA	6h	5mM	No-FA	6h	5mM	-95.7665	70.7567	53.2
FA*time*butyrate	No-FA	12h	0mM	No-FA	12h	5mM	82.4896	70.7319	53.2
FA*time*butyrate	No-FA	12h	0mM	No-FA	24h	0mM	65.7631	70.7460	53.2
FA*time*butyrate	No-FA	12h	0mM	No-FA	24h	5mM	163.47	70.9129	53.7
FA*time*butyrate	No-FA	12h	0mM	No-FA	6h	0mM	202.74	70.7779	53.3
FA*time*butyrate	No-FA	12h	0mM	No-FA	6h	5mM	254.63	70.7634	53.3
FA*time*butyrate	No-FA	12h	5mM	No-FA	24h	0mM	-16.7265	70.7472	53.2
FA*time*butyrate	No-FA	12h	5mM	No-FA	24h	5mM	80.9827	70.9142	53.7
FA*time*butyrate	No-FA	12h	5mM	No-FA	6h	0mM	120.25	70.7791	53.3
FA*time*butyrate	No-FA	12h	5mM	No-FA	6h	5mM	172.14	70.7646	53.3
FA*time*butyrate	No-FA	24h	0mM	No-FA	24h	5mM	97.7092	70.9283	53.7
FA*time*butyrate	No-FA	24h	0mM	No-FA	6h	0mM	136.98	70.7933	53.3
FA*time*butyrate	No-FA	24h	0mM	No-FA	6h	5mM	188.86	70.7788	53.3
FA*time*butyrate	No-FA	24h	5mM	No-FA	6h	0mM	39.2721	70.9600	53.8
FA*time*butyrate	No-FA	24h	5mM	No-FA	6h	5mM	91.1550	70.9455	53.8
FA*time*butyrate	No-FA	6h	0mM	No-FA	6h	5mM	51.8829	70.8106	53.4

P-VALUES:

Differences of Least Squares Means

Effect	FA	time	butyrate	FA	time	butyrate	t Value	Pr > |t|
Effect of FA:								
FA	DHA			LA			1.50	0.1407
FA	DHA			No-FA			-0.22	0.8304
FA	LA			No-FA			-1.71	0.0931

None significant at alpha=0.05

Effect of butyrate:

Butyrate	**0mM**	**5mM**	**2.40**	**0.0198 ***

Significant.

Effect of time:

time	**12h**	**24h**	**2.21**	**0.0313 ***
time	**12h**	**6h**	**7.23**	**<.0001 ***

time		**24h**		**6h**		**5.01**	**<.0001 ***

All times are significantly different.

Effect of Fa*butyrate:

FA*butyrate	DHA	0mM	DHA	5mM		1.48	0.1439
FA*butyrate	DHA	0mM	LA	0mM		1.41	0.1650
FA*butyrate	DHA	0mM	LA	5mM		2.19	0.0330
FA*butyrate	DHA	0mM	No-FA	0mM		-0.36	0.7220
FA*butyrate	DHA	0mM	No-FA	5mM		1.54	0.1307
FA*butyrate	DHA	5mM	LA	0mM		-0.08	0.9396
FA*butyrate	DHA	5mM	LA	5mM		0.71	0.4820
FA*butyrate	DHA	5mM	No-FA	0mM		-1.84	0.0713
FA*butyrate	DHA	5mM	No-FA	5mM		0.05	0.9578
FA*butyrate	LA	0mM	LA	5mM		0.78	0.4363
FA*butyrate	LA	0mM	No-FA	0mM		-1.77	0.0833 *
FA*butyrate	LA	0mM	No-FA	5mM		0.13	0.8977
FA*butyrate	LA	5mM	No-FA	0mM		-2.55	0.0138
FA*butyrate	LA	5mM	No-FA	5mM		-0.65	0.5157
FA*butyrate	No-FA	0mM	No-FA	5mM		1.89	0.0639 *

Differences of Least Squares Means

Effect	FA	time	butyrate	FA	time	butyrate	t Value	Pr > |t|
Effect of FA*time:								
FA*time	DHA	12h		DHA	24h		1.98	0.0531 *
FA*time	**DHA**	**12h**		**DHA**	**6h**		**4.06**	**0.0002 ***
FA*time	DHA	12h		LA	12h		1.17	0.2459
FA*time	DHA	12h		LA	24h		1.56	0.1241
FA*time	DHA	12h		LA	6h		5.90	<.0001
FA*time	DHA	12h		No-FA	12h		0.15	0.8807
FA*time	DHA	12h		No-FA	24h		1.62	0.1121
FA*time	DHA	12h		No-FA	6h		3.90	0.0003
FA*time	**DHA**	**24h**		**DHA**	**6h**		**2.08**	**0.0421 ***
FA*time	DHA	24h		LA	12h		-0.80	0.4253
FA*time	DHA	24h		LA	24h		-0.41	0.6816
FA*time	DHA	24h		LA	6h		3.92	0.0003
FA*time	DHA	24h		No-FA	12h		-1.83	0.0732
FA*time	DHA	24h		No-FA	24h		-0.36	0.7199
FA*time	DHA	24h		No-FA	6h		1.92	0.0605
FA*time	DHA	6h		LA	12h		-2.89	0.0056
FA*time	DHA	6h		LA	24h		-2.49	0.0158
FA*time	DHA	6h		LA	6h		1.83	0.0723 *
FA*time	DHA	6h		No-FA	12h		-3.91	0.0003
FA*time	DHA	6h		No-FA	24h		-2.44	0.0180
FA*time	DHA	6h		No-FA	6h		-0.16	0.8712
FA*time	LA	12h		LA	24h		0.39	0.6982
FA*time	**LA**	**12h**		**LA**	**6h**		**4.72**	**<.0001 ***
FA*time	LA	12h		No-FA	12h		-1.02	0.3111
FA*time	LA	12h		No-FA	24h		0.44	0.6600
FA*time	LA	12h		No-FA	6h		2.72	0.0088
FA*time	**LA**	**24h**		**LA**	**6h**		**4.32**	**<.0001 ***
FA*time	LA	24h		No-FA	12h		-1.41	0.1638
FA*time	LA	24h		No-FA	24h		0.05	0.9585
FA*time	LA	24h		No-FA	6h		2.33	0.0237
FA*time	LA	6h		No-FA	12h		-5.75	<.0001
FA*time	LA	6h		No-FA	24h		-4.27	<.0001
FA*time	LA	6h		No-FA	6h		-1.99	0.0512 *
FA*time	No-FA	12h		No-FA	24h		1.46	0.1488
FA*time	**No-FA**	**12h**		**No-FA**	**6h**		**3.75**	**0.0004 ***
FA*time	**No-FA**	**24h**		**No-FA**	**6h**		**2.28**	**0.0269 ***
Effect of time*butyrate:								
time*butyrate		12h	0mM		12h	5mM	1.27	0.2103
time*butyrate		12h	0mM		24h	0mM	1.25	0.2163
time*butyrate		12h	0mM		24h	5mM	3.14	0.0027
time*butyrate		**12h**	**0mM**		**6h**	**0mM**	**5.25**	**<.0001 ***
time*butyrate		12h	0mM		6h	5mM	6.25	<.0001
time*butyrate		12h	5mM		24h	0mM	-0.02	0.9864
time*butyrate		12h	5mM		24h	5mM	1.88	0.0661 *
time*butyrate		12h	5mM		6h	0mM	3.98	0.0002
time*butyrate		**12h**	**5mM**		**6h**	**5mM**	**4.98**	**<.0001 ***
time*butyrate		24h	0mM		24h	5mM	1.89	0.0637 *
time*butyrate		**24h**	**0mM**		**6h**	**0mM**	**4.00**	**0.0002 ***
time*butyrate		24h	0mM		6h	5mM	4.99	<.0001
time*butyrate		24h	5mM		6h	0mM	2.10	0.0407
time*butyrate		**24h**	**5mM**		**6h**	**5mM**	**3.09**	**0.0032 ***
time*butyrate		6h	0mM		6h	5mM	1.00	0.3235
Effect of FA*time*butyrate								
FA*time*butyrate	DHA	12h	0mM	DHA	12h	5mM	1.09	0.2808
FA*time*butyrate	DHA	12h	0mM	DHA	24h	0mM	1.39	0.1707
FA*time*butyrate	DHA	12h	0mM	DHA	24h	5mM	2.50	0.0157
FA*time*butyrate	**DHA**	**12h**	**0mM**	**DHA**	**6h**	**0mM**	**3.23**	**0.0021 ***

Differences of Least Squares Means

Effect	FA	time	butyrate	FA	time	butyrate	t Value	Pr > |t|	
FA*time*butyrate	DHA	12h	0mM	DHA	6h	5mM	3.60	0.0007	
FA*time*butyrate	DHA	12h	0mM	LA	12h	0mM	1.40	0.1663	
FA*time*butyrate	DHA	12h	0mM	LA	12h	5mM	1.34	0.1848	
FA*time*butyrate	DHA	12h	0mM	LA	24h	0mM	1.25	0.2161	
FA*time*butyrate	DHA	12h	0mM	LA	24h	5mM	2.04	0.0460	
FA*time*butyrate	DHA	12h	0mM	LA	6h	0mM	4.40	<.0001	
FA*time*butyrate	DHA	12h	0mM	LA	6h	5mM	5.02	<.0001	
FA*time*butyrate	DHA	12h	0mM	No-FA	12h	0mM	0.07	0.9456	
FA*time*butyrate	DHA	12h	0mM	No-FA	12h	5mM	1.23	0.2226	
FA*time*butyrate	DHA	12h	0mM	No-FA	24h	0mM	1.00	0.3230	
FA*time*butyrate	DHA	12h	0mM	No-FA	24h	5mM	2.37	0.0213	
FA*time*butyrate	DHA	12h	0mM	No-FA	6h	0mM	2.93	0.0050	
FA*time*butyrate	DHA	12h	0mM	No-FA	6h	5mM	3.66	0.0006	
FA*time*butyrate	DHA	12h	5mM	DHA	24h	0mM	0.30	0.7655	
FA*time*butyrate	DHA	12h	5mM	DHA	24h	5mM	1.41	0.1649	
FA*time*butyrate	DHA	12h	5mM	DHA	6h	0mM	2.14	0.0367	
FA*time*butyrate	**DHA**	**12h**	**5mM**	**DHA**	**6h**	**5mM**	**2.51**	**0.0150**	*
FA*time*butyrate	DHA	12h	5mM	LA	12h	0mM	0.31	0.7543	
FA*time*butyrate	DHA	12h	5mM	LA	12h	5mM	0.26	0.7990	
FA*time*butyrate	DHA	12h	5mM	LA	24h	0mM	0.16	0.8710	
FA*time*butyrate	DHA	12h	5mM	LA	24h	5mM	0.96	0.3425	
FA*time*butyrate	DHA	12h	5mM	LA	6h	0mM	3.32	0.0017	
FA*time*butyrate	DHA	12h	5mM	LA	6h	5mM	3.93	0.0002	
FA*time*butyrate	DHA	12h	5mM	No-FA	12h	0mM	-1.02	0.3116	
FA*time*butyrate	DHA	12h	5mM	No-FA	12h	5mM	0.14	0.8854	
FA*time*butyrate	DHA	12h	5mM	No-FA	24h	0mM	-0.09	0.9273	
FA*time*butyrate	DHA	12h	5mM	No-FA	24h	5mM	1.29	0.2037	
FA*time*butyrate	DHA	12h	5mM	No-FA	6h	0mM	1.84	0.0707	
FA*time*butyrate	DHA	12h	5mM	No-FA	6h	5mM	2.58	0.0127	
FA*time*butyrate	DHA	24h	0mM	DHA	24h	5mM	1.11	0.2728	
FA*time*butyrate	DHA	24h	0mM	DHA	6h	0mM	1.84	0.0709	*
FA*time*butyrate	DHA	24h	0mM	DHA	6h	5mM	2.21	0.0312	
FA*time*butyrate	DHA	24h	0mM	LA	12h	0mM	0.01	0.9883	
FA*time*butyrate	DHA	24h	0mM	LA	12h	5mM	-0.04	0.9656	
FA*time*butyrate	DHA	24h	0mM	LA	24h	0mM	-0.14	0.8919	
FA*time*butyrate	DHA	24h	0mM	LA	24h	5mM	0.66	0.5130	
FA*time*butyrate	DHA	24h	0mM	LA	6h	0mM	3.02	0.0039	
FA*time*butyrate	DHA	24h	0mM	LA	6h	5mM	3.63	0.0006	
FA*time*butyrate	DHA	24h	0mM	No-FA	12h	0mM	-1.32	0.1921	
FA*time*butyrate	DHA	24h	0mM	No-FA	12h	5mM	-0.16	0.8774	
FA*time*butyrate	DHA	24h	0mM	No-FA	24h	0mM	-0.39	0.6971	
FA*time*butyrate	DHA	24h	0mM	No-FA	24h	5mM	0.99	0.3280	
FA*time*butyrate	DHA	24h	0mM	No-FA	6h	0mM	1.54	0.1285	
FA*time*butyrate	DHA	24h	0mM	No-FA	6h	5mM	2.28	0.0268	
FA*time*butyrate	DHA	24h	5mM	DHA	6h	0mM	0.73	0.4668	
FA*time*butyrate	DHA	24h	5mM	DHA	6h	5mM	1.10	0.2751	
FA*time*butyrate	DHA	24h	5mM	LA	12h	0mM	-1.09	0.2792	
FA*time*butyrate	DHA	24h	5mM	LA	12h	5mM	-1.15	0.2553	
FA*time*butyrate	DHA	24h	5mM	LA	24h	0mM	-1.24	0.2189	
FA*time*butyrate	DHA	24h	5mM	LA	24h	5mM	-0.45	0.6571	
FA*time*butyrate	DHA	24h	5mM	LA	6h	0mM	1.90	0.0624	
FA*time*butyrate	DHA	24h	5mM	LA	6h	5mM	2.52	0.0147	
FA*time*butyrate	DHA	24h	5mM	No-FA	12h	0mM	-2.43	0.0186	
FA*time*butyrate	DHA	24h	5mM	No-FA	12h	5mM	-1.26	0.2120	
FA*time*butyrate	DHA	24h	5mM	No-FA	24h	0mM	-1.50	0.1397	
FA*time*butyrate	DHA	24h	5mM	No-FA	24h	5mM	-0.12	0.9059	
FA*time*butyrate	DHA	24h	5mM	No-FA	6h	0mM	0.44	0.6649	
FA*time*butyrate	DHA	24h	5mM	No-FA	6h	5mM	1.17	0.2479	
FA*time*butyrate	DHA	6h	0mM	DHA	6h	5mM	0.37	0.7123	
FA*time*butyrate	DHA	6h	0mM	LA	12h	0mM	-1.83	0.0731	
FA*time*butyrate	DHA	6h	0mM	LA	12h	5mM	-1.88	0.0651	
FA*time*butyrate	DHA	6h	0mM	LA	24h	0mM	-1.98	0.0530	
FA*time*butyrate	DHA	6h	0mM	LA	24h	5mM	-1.18	0.2439	
FA*time*butyrate	DHA	6h	0mM	LA	6h	0mM	1.17	0.2464	
FA*time*butyrate	DHA	6h	0mM	LA	6h	5mM	1.79	0.0788	
FA*time*butyrate	DHA	6h	0mM	No-FA	12h	0mM	-3.17	0.0026	
FA*time*butyrate	DHA	6h	0mM	No-FA	12h	5mM	-2.00	0.0508	
FA*time*butyrate	DHA	6h	0mM	No-FA	24h	0mM	-2.23	0.0297	
FA*time*butyrate	DHA	6h	0mM	No-FA	24h	5mM	-0.85	0.3988	
FA*time*butyrate	DHA	6h	0mM	No-FA	6h	0mM	-0.30	0.7678	
FA*time*butyrate	DHA	6h	0mM	No-FA	6h	5mM	0.44	0.6639	
FA*time*butyrate	DHA	6h	5mM	LA	12h	0mM	-2.20	0.0323	
FA*time*butyrate	DHA	6h	5mM	LA	12h	5mM	-2.25	0.0284	
FA*time*butyrate	DHA	6h	5mM	LA	24h	0mM	-2.35	0.0226	
FA*time*butyrate	DHA	6h	5mM	LA	24h	5mM	-1.55	0.1278	
FA*time*butyrate	DHA	6h	5mM	LA	6h	0mM	0.80	0.4270	
FA*time*butyrate	DHA	6h	5mM	LA	6h	5mM	1.42	0.1612	
FA*time*butyrate	DHA	6h	5mM	No-FA	12h	0mM	-3.53	0.0009	
FA*time*butyrate	DHA	6h	5mM	No-FA	12h	5mM	-2.37	0.0216	
FA*time*butyrate	DHA	6h	5mM	No-FA	24h	0mM	-2.60	0.0119	
FA*time*butyrate	DHA	6h	5mM	No-FA	24h	5mM	-1.22	0.2280	
FA*time*butyrate	DHA	6h	5mM	No-FA	6h	0mM	-0.67	0.5077	
FA*time*butyrate	DHA	6h	5mM	No-FA	6h	5mM	0.07	0.9473	
FA*time*butyrate	LA	12h	0mM	LA	12h	5mM	-0.06	0.9539	
FA*time*butyrate	LA	12h	0mM	LA	24h	0mM	-0.15	0.8803	
FA*time*butyrate	LA	12h	0mM	LA	24h	5mM	0.64	0.5225	
FA*time*butyrate	**LA**	**12h**	**0mM**	**LA**	**6h**	**0mM**	**3.00**	**0.0041**	*
FA*time*butyrate	LA	12h	0mM	LA	6h	5mM	3.62	0.0007	
FA*time*butyrate	LA	12h	0mM	No-FA	12h	0mM	-1.34	0.1873	

FA*time*butyrate	LA	12h	0mM	No-FA	12h	5mM	-0.17	0.8658	
FA*time*butyrate	LA	12h	0mM	No-FA	24h	0mM	-0.41	0.6863	
FA*time*butyrate	LA	12h	0mM	No-FA	24h	5mM	0.97	0.3352	
FA*time*butyrate	LA	12h	0mM	No-FA	6h	0mM	1.53	0.1322	
FA*time*butyrate	LA	12h	0mM	No-FA	6h	5mM	2.26	0.0278	
FA*time*butyrate	LA	12h	5mM	LA	24h	0mM	-0.09	0.9263	
FA*time*butyrate	LA	12h	5mM	LA	24h	5mM	0.70	0.4865	
FA*time*butyrate	LA	12h	5mM	LA	6h	0mM	3.05	0.0035	
FA*time*butyrate	LA	12h	5mM	LA	6h	5mM	3.67	0.0006	
FA*time*butyrate	LA	12h	5mM	No-FA	12h	0mM	-1.28	0.2076	
FA*time*butyrate	LA	12h	5mM	No-FA	12h	5mM	-0.11	0.9117	
FA*time*butyrate	LA	12h	5mM	No-FA	24h	0mM	-0.35	0.7297	
FA*time*butyrate	LA	12h	5mM	No-FA	24h	5mM	1.03	0.3081	
FA*time*butyrate	LA	12h	5mM	No-FA	6h	0mM	1.58	0.1189	
FA*time*butyrate	LA	12h	5mM	No-FA	6h	5mM	2.32	0.0243	
FA*time*butyrate	LA	24h	0mM	LA	24h	5mM	0.79	0.4305	
FA*time*butyrate	**LA**	**24h**	**0mM**	**LA**	**6h**	**0mM**	**3.15**	**0.0027**	*
FA*time*butyrate	LA	24h	0mM	LA	6h	5mM	3.77	0.0004	
FA*time*butyrate	LA	24h	0mM	No-FA	12h	0mM	-1.18	0.2416	
FA*time*butyrate	LA	24h	0mM	No-FA	12h	5mM	-0.02	0.9854	
FA*time*butyrate	LA	24h	0mM	No-FA	24h	0mM	-0.25	0.7999	
FA*time*butyrate	LA	24h	0mM	No-FA	24h	5mM	1.12	0.2664	
FA*time*butyrate	LA	24h	0mM	No-FA	6h	0mM	1.68	0.0988	
FA*time*butyrate	LA	24h	0mM	No-FA	6h	5mM	2.41	0.0193	
FA*time*butyrate	LA	24h	5mM	LA	6h	0mM	2.35	0.0227	
FA*time*butyrate	**LA**	**24h**	**5mM**	**LA**	**6h**	**5mM**	**2.96**	**0.0045**	*
FA*time*butyrate	LA	24h	5mM	No-FA	12h	0mM	-1.98	0.0533	
FA*time*butyrate	LA	24h	5mM	No-FA	12h	5mM	-0.81	0.4197	
FA*time*butyrate	LA	24h	5mM	No-FA	24h	0mM	-1.05	0.2990	
FA*time*butyrate	LA	24h	5mM	No-FA	24h	5mM	0.33	0.7448	
FA*time*butyrate	LA	24h	5mM	No-FA	6h	0mM	0.88	0.3823	
FA*time*butyrate	LA	24h	5mM	No-FA	6h	5mM	1.61	0.1128	
FA*time*butyrate	LA	6h	0mM	LA	6h	5mM	0.62	0.5365	
FA*time*butyrate	LA	6h	0mM	No-FA	12h	0mM	-4.34	<.0001	
FA*time*butyrate	LA	6h	0mM	No-FA	12h	5mM	-3.17	0.0025	
FA*time*butyrate	LA	6h	0mM	No-FA	24h	0mM	-3.41	0.0013	
FA*time*butyrate	LA	6h	0mM	No-FA	24h	5mM	-2.02	0.0485	
FA*time*butyrate	LA	6h	0mM	No-FA	6h	0mM	-1.47	0.1482	
FA*time*butyrate	LA	6h	0mM	No-FA	6h	5mM	-0.73	0.4666	
FA*time*butyrate	LA	6h	5mM	No-FA	12h	0mM	-4.95	<.0001	
FA*time*butyrate	LA	6h	5mM	No-FA	12h	5mM	-3.79	0.0004	
FA*time*butyrate	LA	6h	5mM	No-FA	24h	0mM	-4.02	0.0002	
FA*time*butyrate	LA	6h	5mM	No-FA	24h	5mM	-2.64	0.0109	
FA*time*butyrate	LA	6h	5mM	No-FA	6h	0mM	-2.09	0.0418	
FA*time*butyrate	LA	6h	5mM	No-FA	6h	5mM	-1.35	0.1816	
FA*time*butyrate	No-FA	12h	0mM	No-FA	12h	5mM	1.17	0.2487	
FA*time*butyrate	No-FA	12h	0mM	No-FA	24h	0mM	0.93	0.3568	
FA*time*butyrate	No-FA	12h	0mM	No-FA	24h	5mM	2.31	0.0250	
FA*time*butyrate	**No-FA**	**12h**	**0mM**	**No-FA**	**6h**	**0mM**	**2.86**	**0.0060**	*
FA*time*butyrate	No-FA	12h	0mM	No-FA	6h	5mM	3.60	0.0007	
FA*time*butyrate	No-FA	12h	5mM	No-FA	24h	0mM	-0.24	0.8140	
FA*time*butyrate	No-FA	12h	5mM	No-FA	24h	5mM	1.14	0.2585	
FA*time*butyrate	No-FA	12h	5mM	No-FA	6h	0mM	1.70	0.0951	
FA*time*butyrate	**No-FA**	**12h**	**5mM**	**No-FA**	**6h**	**5mM**	**2.43**	**0.0184**	*
FA*time*butyrate	No-FA	24h	0mM	No-FA	24h	5mM	1.38	0.1740	
FA*time*butyrate	No-FA	24h	0mM	No-FA	6h	0mM	1.93	0.0583	*
FA*time*butyrate	No-FA	24h	0mM	No-FA	6h	5mM	2.67	0.0101	
FA*time*butyrate	No-FA	24h	5mM	No-FA	6h	0mM	0.55	0.5823	
FA*time*butyrate	No-FA	24h	5mM	No-FA	6h	5mM	1.28	0.2043	
FA*time*butyrate	No-FA	6h	0mM	No-FA	6h	5mM	0.73	0.4669	

Analysis of Controls for Both Days

For 8/11/03, the procedure was run in proc mixed using a subsampling term for well. Some problems with the data – groups are very variable.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
cont_2	2	443	657.44	<.0001

Least Squares Means

Effect	cont_2	Estimate	Standard Error	DF	t Value	Pr > |t|
cont_2	H2O250	667.96	28.0391	443	23.82	<.0001
cont_2	cum100	1677.22	30.0775	443	55.76	<.0001
cont_2	cum25	130.97	31.6323	443	4.14	<.0001

Differences of Least Squares Means

Effect	cont_2	cont_2	Estimate	Standard Error	DF	t Value	Pr > |t|

cont_2	H2O250	cum100	-1009.26	41.1199	443	-24.54	<.0001
cont_2	H2O250	cum25	536.99	42.2705	443	12.70	<.0001
cont_2	cum100	cum25	1546.25	43.6493	443	35.42	<.0001

All significantly different.

9/1/03, since only one well, anova was run. Data set very variable – not confident about the results.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
control	2	712	257.48	<.0001

Least Squares Means

Effect	control	Estimate	Standard Error	DF	t Value	Pr > |t|
control	DH2O2	459.35	18.3452	712	25.04	<.0001
control	H2O2	1123.52	23.7868	712	47.23	<.0001
control	cum	509.58	35.8173	712	14.23	<.0001

Differences of Least Squares Means

Effect	control	control	Estimate	Standard Error	DF	t Value	Pr > |t|
control	DH2O2-50uM	H2O2-5uM	-664.18	30.0393	712	-22.11	<.0001
control	DH2O2-50uM	cum25	-50.239	40.2421	712	-1.25	0.2123
control	H2O2-5uM	cum25	613.94	42.9964	712	14.28	<.0001

Dh2O2 and cum not significantly different from each other.

Table B-7. Summary of Fatty Acid and Butyrate Treatment Effects

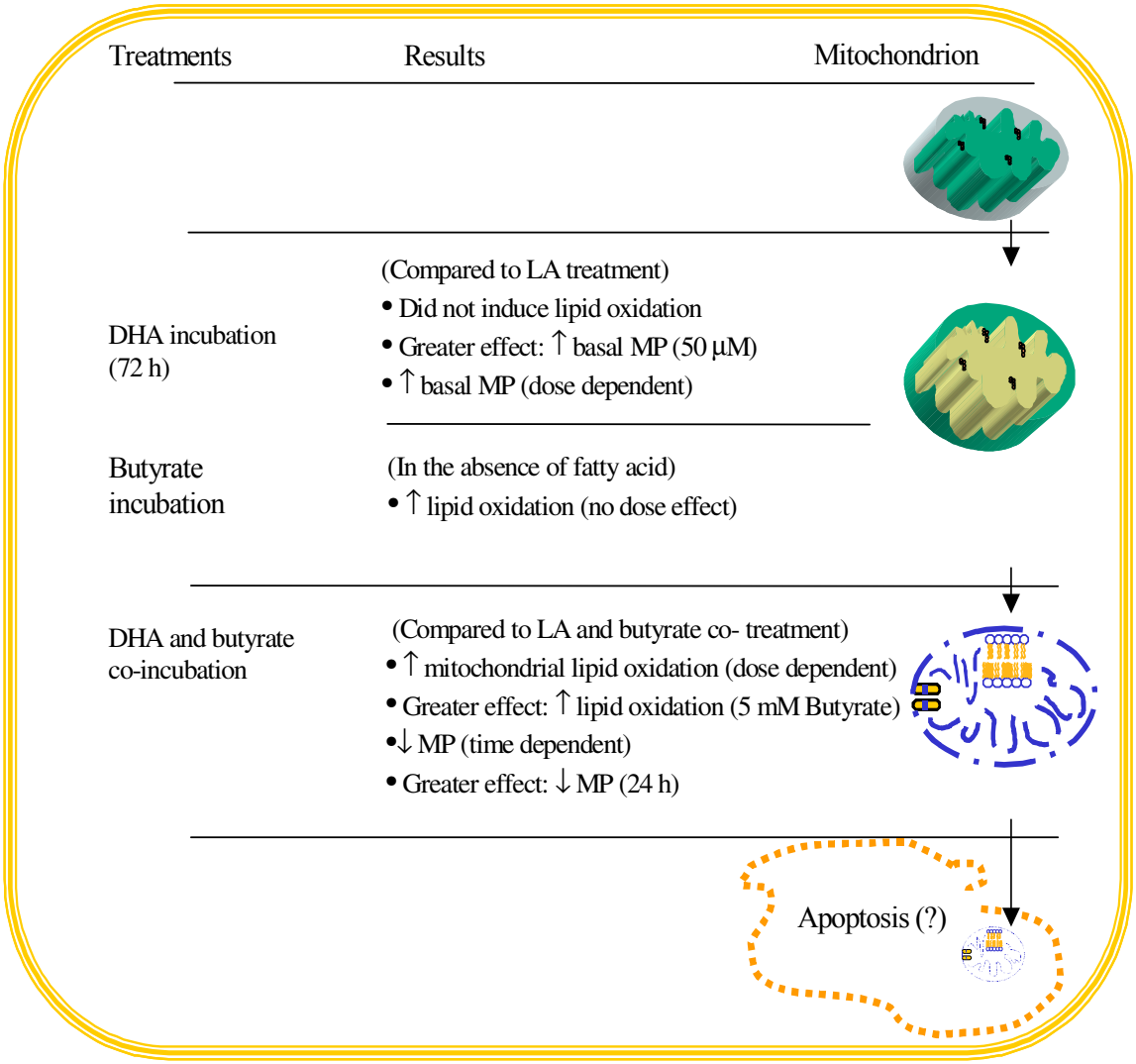


Table B-8. Outline of research proposal

<table>
<tr><td colspan="6">Hypothesis: The incorporation of n-3 PUFA increases cell susceptibility to oxidative damage, and subsequently perturbs the integrity of mitochondrial membranes, a process potentiated by butyrate.</td></tr>
<tr><td colspan="2">Objective 1: To quantify total cellular and mitochondrial membrane lipid oxidation in colonocytes following n-3 and n-6 PUFA incubation.
(FA only)</td><td colspan="2">Objective 2: To determine the association between membrane lipid oxidation and mitochondrial function.

(FA only)</td><td colspan="2">Objective 3: To determine if butyrate potentiates ROS production, mitochondrial functional alteration and lipid oxidation in colonocyte membranes enriched with n-3 and n-6 PUFA.
(FA and Butyrate)</td></tr>
<tr><td>Lipid oxidation: DPPP</td><td>Global membrane effects:
• Does lipid oxidation response to the change in FA dose?
• Does VEs prevent oxidation?

Mitochondrial membrane effects:
• Does MitoQ prevent oxidation?
Does prevention of ROS prevent lipid oxidation?</td><td rowspan="2">Dissipation of MP: Rhd 123</td><td rowspan="2">Mitochondrial membrane effects:
• Does FA or butyrate treatment dissipate MP?
• Is the dissipation of MP inhibitable by Cyclosporin A?
• How and why would MP changes with FA treatment at different doses?
Does the prevention of lipid oxidation by VEs and MitoQ prevent MP dissipation?</td><td>Lipid oxidation:</td><td>• Does butyrate/isobutyrate treatment induce lipid oxidation in global and mitochondrial membranes?
• Does butyrate/FA co-treatment induce lipid oxidation in global and mitochondrial membranes?</td></tr>
<tr><td>Cytosolic ROS: DCFDA</td><td>Global membrane effects:
• Does ROS production response to the change in FA dose?
• Does VEs prevent ROS production?

Mitochondrial membrane effects:
• TPPB prevents ROS production?</td><td>Cytosolic ROS</td><td>• Does butyrate/isobutyrate treatment induce ROS production in mitochondrial matrix?
• Does butyrate/FA co-treatment induce ROS production in mitochondrial matrix?</td></tr>
<tr><td colspan="4"></td><td>MP</td><td>• Does butyrate/isobutyrate treatment dissipate MP?
• Does butyrate/FA co-treatment dissipate MP?</td></tr>
<tr><td colspan="6">Apoptotic Phenotype</td></tr>
<tr><td colspan="6">Abbreviations used: CMH_2-DCFDA (DCFDA (5-(and-6)-chloromethyl-2',7'- dichlorodihydrofluorescein diacetate, acetyl ester), DPPP (diphenyl-1-pyrenylphosphine), FA (fatty acids), MitoQ (10-(6'-ubiquinoyl) decyltriphenylphosphonium bromide), MP (mitochondrial membrane potential), PUFA (polyunsaturated fatty acids), Rhd 123 (rhodamine 123), ROS (reactive oxygen species), TPPB (2-(2-(triphenylphosphonio)ethyl)-dihydro-H-benzopyran-6-ol bromide), VEs (vitamin E succinate)</td></tr>
</table>

APPENDIX C

RESULTS FROM PRELIMINARY EXPERIMENTS

Result C-1: Mitochondrial Membrane Potential Dissipation in Viable Cells

Purpose: To determine the utility of 2 fluorescence probes, TMRM and calcein AM, to measure mitochondrial membrane potential (MP) in viable YAMC cells.

Rationale:

The tetramethylrhodamine methyl ester perchlorate (TMRM) (Molecular Probes, #T668) is a lipophilic cationic fluorescent probe, which accumulates in the mitochondria in proportion to MP (77). In comparison, calcein AM (Molecular probes, #C3100) is a cell-permeant dye that can be used to determine cell viability in most eukaryotic cells. In viable cells, the nonfluorescent calcein AM is converted to a green-fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases (Molecular probe).

To determine the validity of TMRM and calcein AM to measure the change in MP in viable YAMC cells, following butyrate incubation with or without 50 μM DHA or LA pretreatment, cells were loaded with TMRM and calcein AM as indicated in the protocol.

TMRM and Calcein dye loading protocol:

1. Add 1 ml RPMI complete media with γ-IFN into each well of YAMC cells
2. Add 1.0 μl TMRM (150 nM final conc.)
3. Incubate at 33°C, at least 10 min
4. Add 1.0 μl calcein-AM (1 μM final conc.)
5. Incubate at 33°C, for another 10 min
6. Aspirate off media with dyes
7. Add 1 ml fresh Leibovitz media (without phenol red)
8. Image for 0, 5, 10 and 15 min.

Butyrate, isobutyrate (negative control), DHA and LA were added to the media as described in protocol # A-5. Each experiment was designed to optimize the protocol, hence the order and duration of butyrate incubations varied. Magnification power was set at 200 or 400, images of cells (about 30 cells per field) were captured by a phase-contrast, fluorescent, widefield inverted Nikon microscope. The fluorescence excitation/emission wavelengths were 562/624 nm for TMRM, and 477/536 nm for calcein AM. Ten representative cells from each captured image were randomly selected for fluorescence intensity quantification. Data were plotted using mean (+/- SEM) average pixel intensity of all cells.

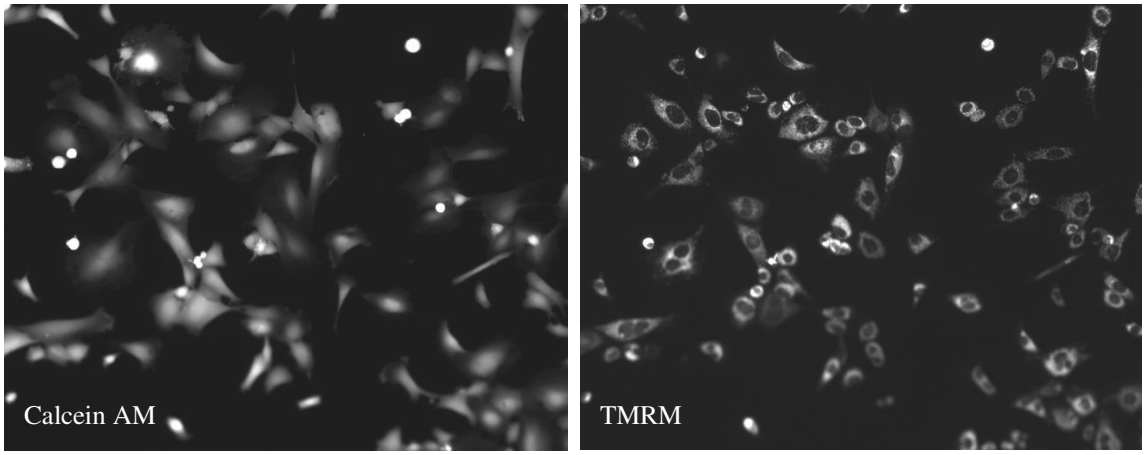


Fig. C-1. Representative photomicrographs of YAMC cells illuminated following loading with TMRM at 150 nM for 10 min and subsequent incubation with calcein AM at 1 μM for 10 min. Two images of each same field were captured using different excitation and emission filters.

Results:

1) A kinetic study (2002.02.15) was also performed to look at the effect of short duration butyrate treatment at 5 mM for up to 45 min, post DHA or LA treatments, on MP and cell viability. Butyrate incubation for up to 45 min did not induce MP dissipation, using TMRM and calcein AM as two fluorescence probes.

2) A representative set of data (2002.03.07) is presented to show that DHA- and LA-BSA pretreatment, at 50 μM, did not alter MP, using TMRM as a fluorescent probe. In this specific set of experiments, 5 mM butyrate treatment was incubated for 2, 6, 12 or 24 h, with or without 50 μM DHA or LA pre-treatment in YAMC cells for 72 h. See table Appendix C-1 for results.

3) To optimize imaging outcome, YAMC cells treated with PUFA and butyrate were analyzed using confocal microscopy and TMRM (2002.05.10). The results from 2003.03.07 (butyrate treatment dissipate MP) were not reproducible.

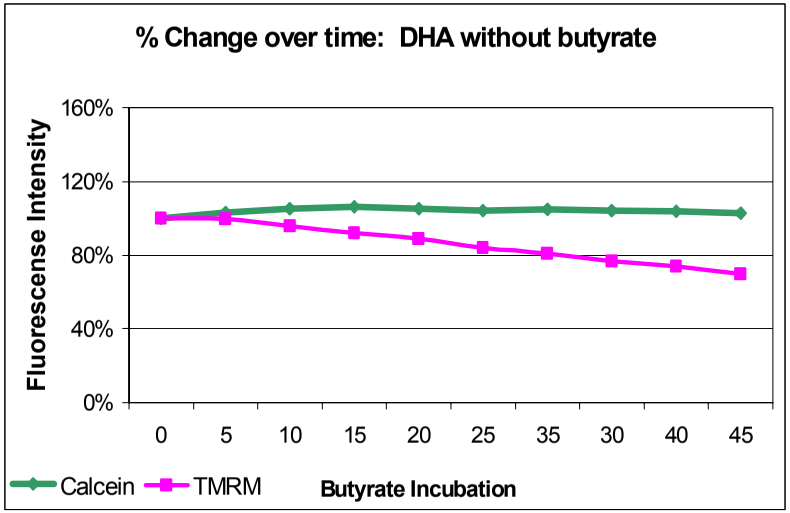
Conclusion:

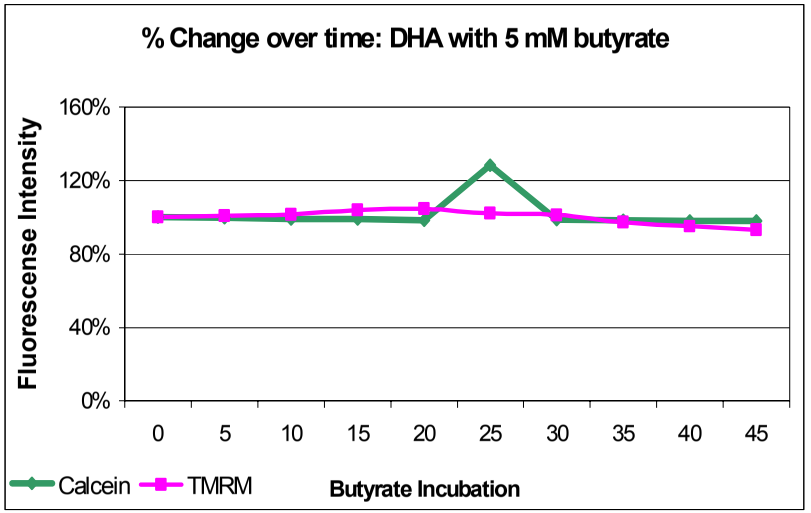
We have previously demonstrated that MP was dissipated by butyrate incubation (5 mM) for 24 h (47). This experiment using TMRM could not consistently reproduce the effect. We conclude that TMRM might not be a suitable probe to measure MP dissipation in YAMC cells.

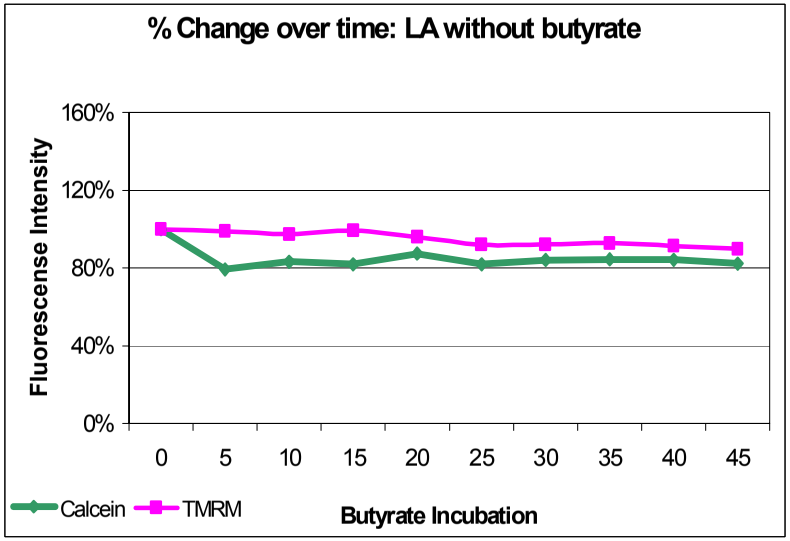
References:

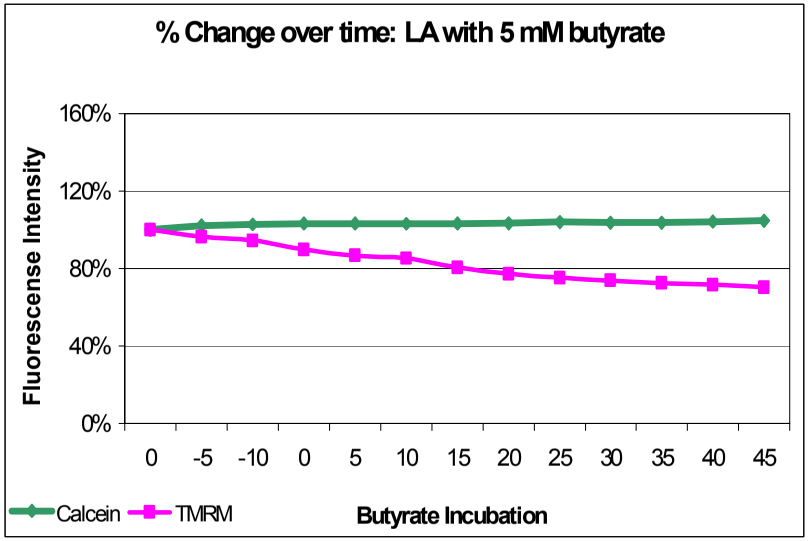
Molecular probes
http://www.probes.com (last accessed: May 20, 2004)

2002.02.15: Kinetic study of 5 mM butyrate incubation (min) following 50 µM (72 h) BSA-DHA or -LA pre-treatment



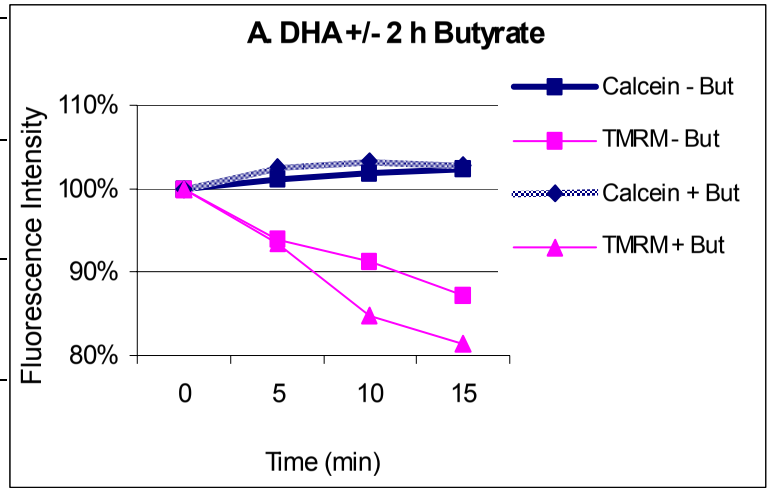


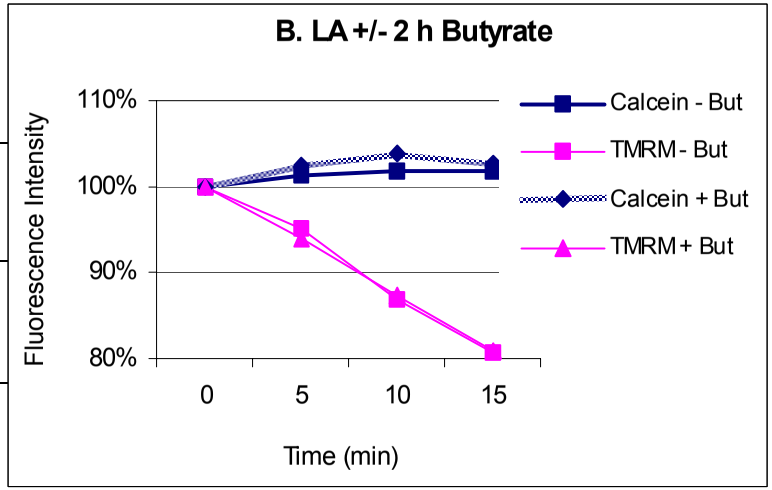




2002.03.07 Preliminary study: YAMC-Fatty acid +/- 2 h butyrate

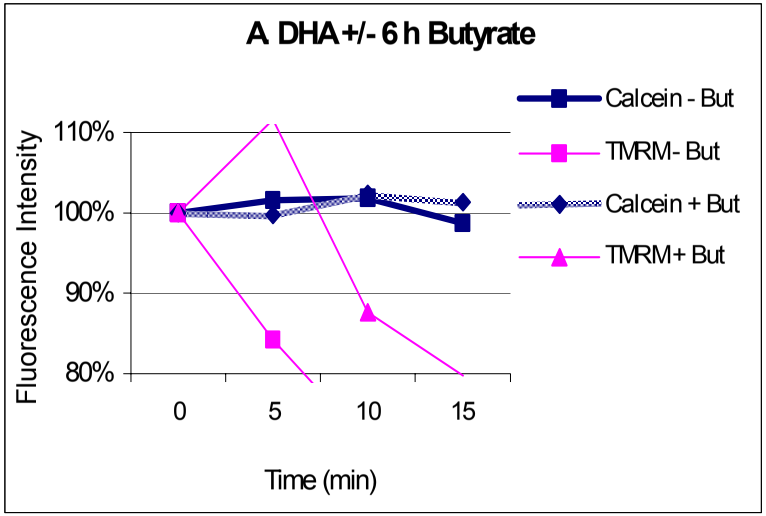
FA	Butyrate	Dye loading	Probe	Average Pixel Intensity	%FI/OI
DHA	+	0 min	Calcein	312.14	100%
DHA	+	5 min	Calcein	320.016	103%
DHA	+	10 min	Calcein	322.486	103%
DHA	+	15 min	Calcein	320.842	103%
DHA	+	0 min	TMRM	272.967	100%
DHA	+	5 min	TMRM	255.065	93%
DHA	+	10 min	TMRM	231.654	85%
DHA	+	15 min	TMRM	222.497	82%
DHA	-	0 min	Calcein	143.146	100%
DHA	-	5 min	Calcein	144.588	101%
DHA	-	10 min	Calcein	145.658	102%
DHA	-	15 min	Calcein	146.554	102%
DHA	-	0 min	TMRM	191.015	100%
DHA	-	5 min	TMRM	179.494	94%
DHA	-	10 min	TMRM	174.476	91%
DHA	-	15 min	TMRM	166.627	87%
LA	+	0 min	Calcein	172.629	100%
LA	+	5 min	Calcein	176.994	103%
LA	+	10 min	Calcein	179.153	104%
LA	+	15 min	Calcein	177.298	103%
LA	+	0 min	TMRM	230.78	100%
LA	+	5 min	TMRM	216.967	94%
LA	+	10 min	TMRM	201.692	87%
LA	+	15 min	TMRM	186.983	81%
LA	-	0 min	Calcein	146.798	100%
LA	-	5 min	Calcein	148.567	101%
LA	-	10 min	Calcein	149.463	102%
LA	-	15 min	Calcein	149.543	102%
LA	-	0 min	TMRM	258.878	100%
LA	-	5 min	TMRM	246.137	95%
LA	-	10 min	TMRM	225.107	87%
LA	-	15 min	TMRM	209.154	81%

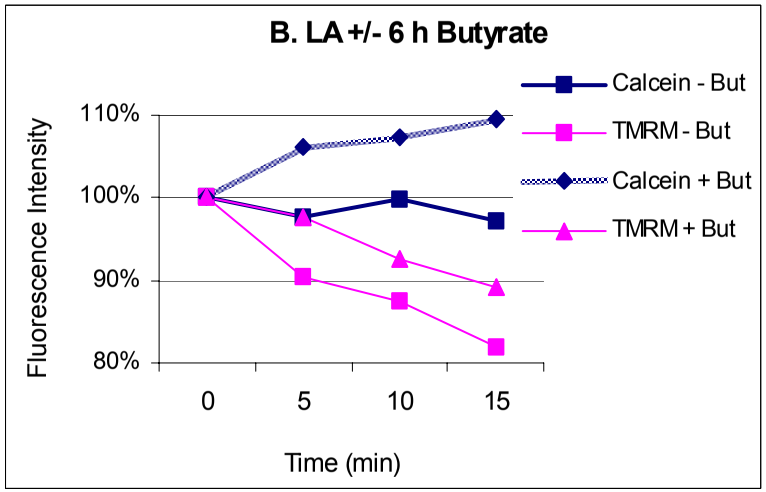




2002.03.07 Preliminary study: YAMC-Fatty acid +/- 6 h butyrate

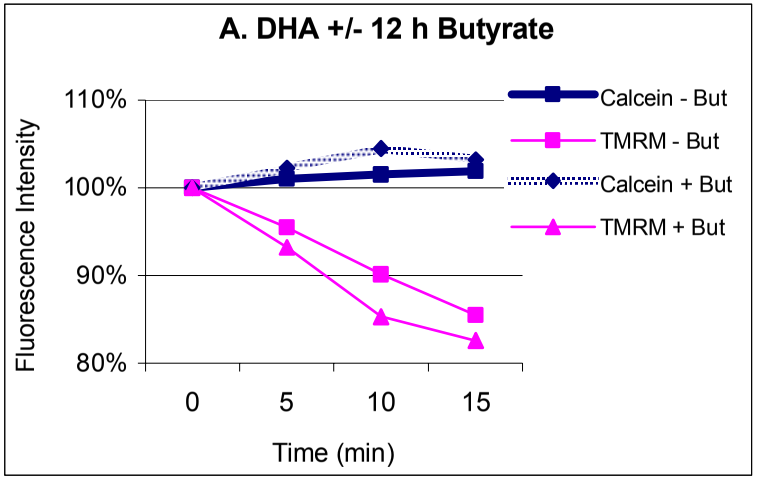
FA	Butyrate	Dye loading	Probe	Average Pixel Intensity	%FI/OI
DHA	+	0 min	Calcein	325.248	100%
DHA	+	5 min	Calcein	324.587	100%
DHA	+	10 min	Calcein	332.729	102%
DHA	+	15 min	Calcein	329.499	101%
DHA	+	0 min	TMRM	242.144	100%
DHA	+	5 min	TMRM	269.925	111%
DHA	+	10 min	TMRM	212.458	88%
DHA	+	15 min	TMRM	192.886	80%
DHA	-	0 min	Calcein	197.385	100%
DHA	-	5 min	Calcein	200.628	102%
DHA	-	10 min	Calcein	201.122	102%
DHA	-	15 min	Calcein	194.639	99%
DHA	-	0 min	TMRM	185.385	100%
DHA	-	5 min	TMRM	156.332	84%
DHA	-	10 min	TMRM	132.222	71%
DHA	-	15 min	TMRM	120.055	65%
LA	+	0 min	Calcein	182.758	100%
LA	+	5 min	Calcein	193.796	106%
LA	+	10 min	Calcein	196.027	107%
LA	+	15 min	Calcein	200.191	110%
LA	+	0 min	TMRM	159.511	100%
LA	+	5 min	TMRM	155.825	98%
LA	+	10 min	TMRM	147.487	92%
LA	+	15 min	TMRM	142.15	89%
LA	-	0 min	Calcein	289.723	100%
LA	-	5 min	Calcein	283.177	98%
LA	-	10 min	Calcein	288.949	100%
LA	-	15 min	Calcein	281.283	97%
LA	-	0 min	TMRM	216.907	100%
LA	-	5 min	TMRM	195.97	90%
LA	-	10 min	TMRM	189.7	87%
LA	-	15 min	TMRM	177.575	82%

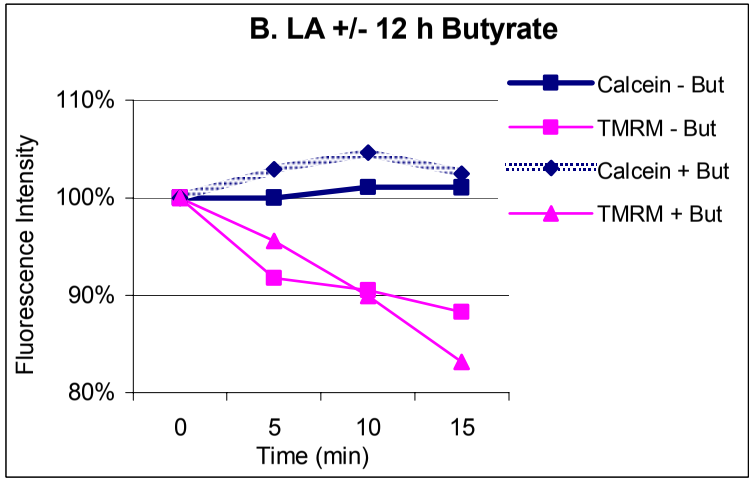




2002.03.07 Preliminary study: YAMC-Fatty acid +/- 12 h butyrate

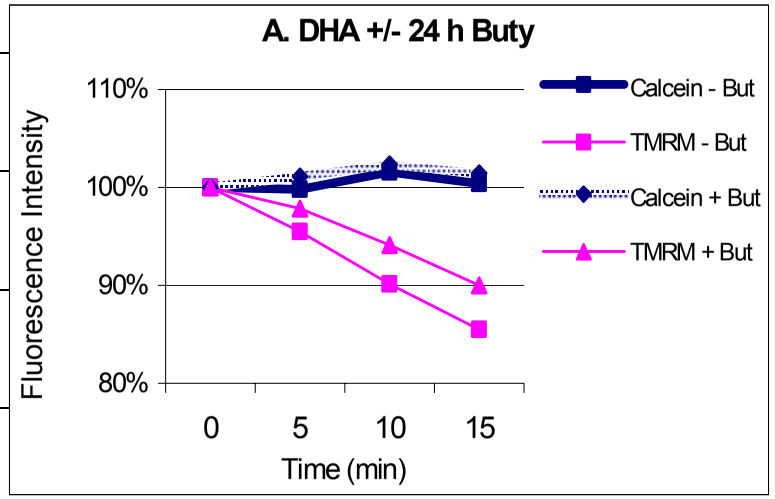
FA	Butyrate	Dye loading	Probe	Average Pixel Intensity	%FI/OI
DHA	+	0 min	Calcein	228.672	100%
DHA	+	5 min	Calcein	233.828	102%
DHA	+	10 min	Calcein	238.943	104%
DHA	+	15 min	Calcein	236.062	103%
DHA	+	0 min	TMRM	319.004	100%
DHA	+	5 min	TMRM	297.336	93%
DHA	+	10 min	TMRM	272.185	85%
DHA	+	15 min	TMRM	263.499	83%
DHA	-	0 min	Calcein	123.337	100%
DHA	-	5 min	Calcein	124.625	101%
DHA	-	10 min	Calcein	125.209	102%
DHA	-	15 min	Calcein	125.717	102%
DHA	-	0 min	TMRM	146.152	100%
DHA	-	5 min	TMRM	137.4	94%
DHA	-	10 min	TMRM	131.274	90%
DHA	-	15 min	TMRM	128.661	88%
LA	+	0 min	Calcein	170.98	100%
LA	+	5 min	Calcein	175.947	103%
LA	+	10 min	Calcein	178.935	105%
LA	+	15 min	Calcein	175.217	102%
LA	+	0 min	TMRM	262.932	100%
LA	+	5 min	TMRM	251.291	96%
LA	+	10 min	TMRM	236.55	90%
LA	+	15 min	TMRM	218.683	83%
LA	-	0 min	Calcein	130.154	100%
LA	-	5 min	Calcein	130.143	100%
LA	-	10 min	Calcein	131.584	101%
LA	-	15 min	Calcein	131.537	101%
LA	-	0 min	TMRM	127.332	100%
LA	-	5 min	TMRM	116.839	92%
LA	-	10 min	TMRM	115.256	91%
LA	-	15 min	TMRM	112.434	88%

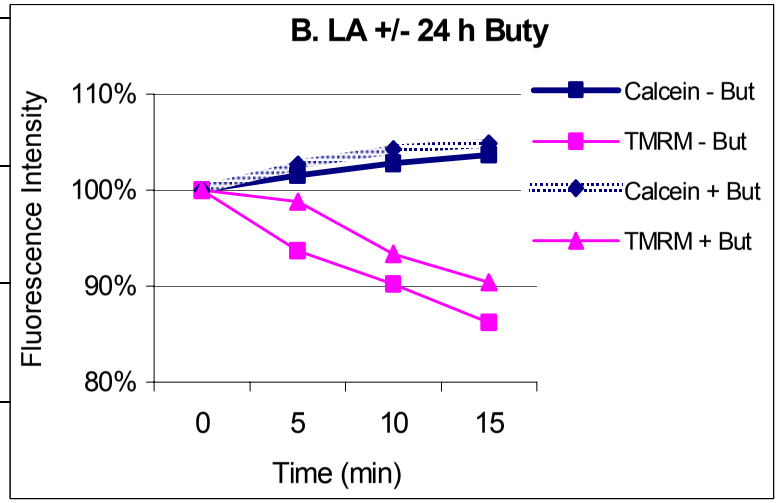




2002.03.07 Preliminary study: YAMC-Fatty acid +/- 24 h butyrate

FA	Butyrate	Dye loading	Probe	Average Pixel Intensity	%FI/OI
DHA	+	0 min	Calcein	157.444	100%
DHA	+	5 min	Calcein	159.194	101%
DHA	+	10 min	Calcein	161.088	102%
DHA	+	15 min	Calcein	159.664	101%
DHA	+	0 min	TMRM	326.143	100%
DHA	+	5 min	TMRM	319.082	98%
DHA	+	10 min	TMRM	306.915	94%
DHA	+	15 min	TMRM	293.546	90%
DHA	-	0 min	Calcein	225.307	100%
DHA	-	5 min	Calcein	224.871	100%
DHA	-	10 min	Calcein	228.884	102%
DHA	-	15 min	Calcein	226.219	100%
DHA	-	0 min	TMRM	432.223	100%
DHA	-	5 min	TMRM	412.704	95%
DHA	-	10 min	TMRM	389.536	90%
DHA	-	15 min	TMRM	369.504	85%
LA	+	0 min	Calcein	164.25	100%
LA	+	5 min	Calcein	168.629	103%
LA	+	10 min	Calcein	171.265	104%
LA	+	15 min	Calcein	172.299	105%
LA	+	0 min	TMRM	226.366	100%
LA	+	5 min	TMRM	223.651	99%
LA	+	10 min	TMRM	211.36	93%
LA	+	15 min	TMRM	204.639	90%
LA	-	0 min	Calcein	208.225	100%
LA	-	5 min	Calcein	211.454	102%
LA	-	10 min	Calcein	214.025	103%
LA	-	15 min	Calcein	215.939	104%
LA	-	0 min	TMRM	326.685	100%
LA	-	5 min	TMRM	306.024	94%
LA	-	10 min	TMRM	294.734	90%
LA	-	15 min	TMRM	281.583	86%





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EDUCATION

Combined Graduate Degree – Dietetic Internship, Texas A&M University, College Station, TX

- General-emphasis Dietetic Internship, July 2004
- Master of Science, Nutrition, August 2004
  - GPR: 3.6/4.0
  - Thesis: To investigate the mechanism by which docosahexaenoic acids and butyrate prevent colon cancer development

Bachelor of Human Resource and Family Sciences-Dietetics

- University of Nebraska, Lincoln, 2001
  - GPR: 4.0/4.0

EXPERIENCES

Volunteer Nutrition Educator, part-time, Scott and White Renal Dialysis Center, Temple, TX, 2004

Teaching Assistant, Texas A&M University, College Station, TX 2002-03

Teaching Assistant, University of Nebraska, Lincoln, NE 2001

Peer Educator, Students Helping Students Nurture Esteem, University of Nebraska, Lincoln, NE 2000-01

Research Assistant, University of Nebraska, Lincoln, NE 2000

Assistant Nutrition Therapist, part-time, Madonna Rehabilitation Hospital, Lincoln, NE, 2000

PROFESSIONAL/HONORS

- Malaysian Student Organization, Treasurer, Texas A&M University, 2003-2004
- American Dietetic Association, member, 2003-present
- American Society for Nutritional Sciences, 2003-present
  - First Prize student poster competition, 2004
- Academic Excellence Award, Kojadi, Malaysia, 2003
- Malaysia Embassy Award, U.S.A., 2001