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Stress oxydatif, fonction mitochondriale et maladie inflammatoire de l'intestin

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Stress oxydatif, fonction mitochondriale et maladie inflammatoire de l'intestin

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Résumé

CONTEXTE: Bien que la dysfunction mitochondriale et le stress oxydant jouent des rôles prépondérants dans plusieurs conditions pathologiques, ils n'ont pas été étudiés de façon extensive au niveau du tube digestif qui est constamment exposé aux oxydants (provenant de l'alimentation) et à divers agents pathogènes. L'ingestion simultanée de sels ferreux et d'acide ascorbique peut causer le dommage des macromolécules par oxydation. Le ''Nuclear factor erythroid 2 related factor'' (Nrf2) est un important facteur de transcription sensible au potentiel redox et qui protège contre le stress oxydant en induisant des gènes anti-oxydants et de detoxification par sa liaison à l'élément de réponse antioxydante (ARE). Les fonctions anti-oxydantes et anti-inflammatoires de Nrf2 ont été décrites dans une variété de types cellulaires et de tissus. Cependant son rôle est très peu connu au niveau du tube digestif. **OBJECTIF**S: Les objectifs sont d'évaluer comment la peroxydation lipidique médiée par le fer/ascorbate (FE/ASC) affecte les fonctions mitochondriales dans les cellules Caco-2/15, et de déterminer l'ampleur de l'implication de Nrf2. **MÉTHODES:** Le stress oxydant a été induit dans les cellules Caco2/15 en les traitant avec 0.2mm/2mm de FE/ASC. L'augmentation de l'expression de Nrf2 a été obtenue suite au prétraitement des cellules Caco2/15 avec 50 μM d'Olitpraz (OPZ), un puissant activateur. L'invalidation du gène de Nrf2 a été réalisée dans les cellules par transfection avec un vecteur lentiviral contenant un shRNA contre Nrf2. **RÉSULTATS:** Nos résultats montrent que le traitement des cellules Caco-2/15 avec du FE/ASC (0.2 mm/2 mm) augmente les niveaux du malondialdehyde (MDA), réduit la production d'ATP, entraîne une surcharge mitochondriale de calcium, active l'expression protéique du cytochrome C et de l'AIF (apoptotic inducing factor), réduit l'activité des complexes I, II,

III et IV de la chaîne respiratoire mitochondriale, augmente les niveaux de 8-OHdG, un marqueur des dommages à l'ADN mitochondrial, diminue la DNA glycosylase, et altère les expressions génique et protéique des facteurs de transcription mitochondriaux (mtTFA, mtTFB1, mtTFB2).

De plus, nos observations montrent que l'induction et l'activation de Nrf2 dans les cellules Caco-2/15 résultent en: une augmentation des enzymes anti-oxydantes endogènes (catalase, glutathion peroxydase, et superoxyde dismutase), une réduction du facteur nucléaire NFκβ et de TNF-α, une augmentation de la production d' ATP et de l'activité des complexes respiratoires (I, II, III, IV) et de PGC-1α, et une régulation des niveaux de la prohibitine mitochondriale, du Bcl-2 anti-apoptotique et de l'occludine. **CONCLUSION:** Dans l'ensemble, nos résultats montrent que l'exposition aigüe des cellules Caco-2/15 à la peroxydation par le FE/ASC entraîne des effets pathologiques sur les fonctions mitochondriales et l'intégrité de l'ADN, qui sont abolis par l'induction de Nrf2. Il en ressort que Nrf2 joue un rôle majeur dans la protection de l'épithélium intestinal contre le stress oxydant.

Mots-clés : Stress oxydatif, Mitochondrie, Inflammation, Nrf2

Abstract

Background:

Although mitochondrial dysfunction and oxidative stress are key mechanisms in various pathological conditions, they have not been extensively studied in the gastrointestinal tract, which is known to be constantly exposed to luminal oxidants from ingested foods and pathogens. Key among these is the simultaneous ingestion of iron salts and ascorbic acid, which can cause oxidative damage to macromolecules. The protein ‘’Nuclear factor-erythroid 2- related factor’’ (Nrf2) is an important redox-sensitive transcription factor, which protects against oxidative stress by inducing antioxidant and detoxifying genes through binding with antioxidant response element (ARE). Many of Nrf2 antioxidant protective and anti-inflammatory functions have been established in various cells and tissues. However, limited information is available on its role in the gastrointestinal tract.

Objectives:

The objectives are to evaluate how iron-ascorbate (FE/ASC)-mediated lipid peroxidation affects mitochondrion functioning in Caco-2/15 cells, and to mechanistically determine the role of Nrf2.

Methods:

Caco2/15 cells were treated with 0.2mm/2mm of FE/ASC to induce oxidative stress. To increase Nrf2 expression, cultured Caco2/15 cells were pre-treated with 50 μM Olitpraz (OPZ). To down regulate the Nrf2 function, Nrf2 gene was knocked down by transfecting Caco-2/15 cells with a pGFP-RS lentiviral vector containing shRNA against Nrf2.

RESULTS:

Our results show that the treatment of Caco-2/15 cells with FE/ASC (0.2 mm/2 mm): increased the levels of malondialdehyde (MDA), a marker of oxidative stress; reduced ATP production; raised mitochondrial calcium content; regulated the protein expression of cytochrome C and apoptotic inducing factor (AIF); decreased mitochondrial respiratory chain complexes I, II, III and IV activity; prevented mtDNA damage as illustrated by the raised levels of 8-OHdG; lowered DNA Glycosylase, and altered the gene expression and protein mass of mitochondrial transcription factors (mtTFA, mtTFB1, mtTFB2).

Furthermore, our observations indicate that the induction and activation of Nrf2 in Caco2/15 cells resulted in an augmentated endogenous antioxidants enzymes (catalase, glutathione peroxidase, and superoxide dismutase), a reduction of nuclear factor-kappaB (NFκβ) and Tumor Necrosis Factor- Alpha (TNF-α), an increase in the ATP production, mitochondrial respiratory complexes (I, II, III, VI), PGC1α , and **a** regulation of the mitochondrial Prohibitin, anti-apoptotic Bcl-2 protein, and Occludin level.

CONCLUSION:

Findings indicate that acute exposure of Caco-2/15 cells to FE/ASC-catalyzed peroxidation produces pathological effects on mitochondrial functions and DNA integrity, which were diminished by Nrf2 induction. It appears that Nrf2 plays a critical cytoprotective role in intestinal epithelial cells against oxidative stress.

Keywords : Oxidative Stress, Mitochondria, Inflammation, Nrf2

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ATP	Adenosine-5'-Triphosphate
Ca^{2+}	Calcium
CD	Crohn's Disease
DNA	Deoxyribonucleic Acid
IBD	Inflammatory Bowel Disease
IL	Interleukin
GSH;	Glutathione
MDA	Malondialdehyde
Mt	Mitochondrial
NF-κβ	Nuclear factor-kappa B
8-OHdG	8-hydroxy-deoxy-guanosine
OGG1	8-oxo DNA Glycosylate
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
CAT	Catalase
GPx	Glutathione Peroxidase
TNF-α	Tumor Necrosis Factor- Alpha
UC	Ulcerative Colitis
Nrf2	Nuclear factor erythroid 2 related factor
ARE	Antioxidant Response Element (ARE)
$(O_2^{-}\bullet)$	Superoxide Anion
PTP	permeability Transition Pore

TJ;	Tight Junction
PGC-1α	Peroxisome Proliferation Activator Receptor γ-coactivator 1α
OXPHOS	Oxidative Phosphorylation
PHB	Prohibitin
(OH^-)	Hydroxyl Radical
(H_2O_2)	Hydrogen Peroxide
PUFA	Polyunsaturated Fatty Acids
ETC	Electron Transport Chain
Fe^{2+}	Iron
Mt TFA	Mitochondrial Transcription Factor A
Mt TFB1	Mitochondrial Transcription Factor B1
Mt TFB2	Mitochondrial Transcription Factor B2
Mt	Mitochondria
AIF	Apoptosis Inducing Factor
ER	Endoplasmic Reticulum
DSS	Dextran Sulfate Sodium
OS	Oxidative Stress

To my wife Ban and Kids (Abdullah, Hamza, Yusef, Amin)

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1 INTRODUCTION

1.1 Oxidative Stress

Oxidative stress can be defined as an imbalance between Reactive Oxygen Species (ROS) production and anti-oxidant defense as seen in **(Fig.1).**

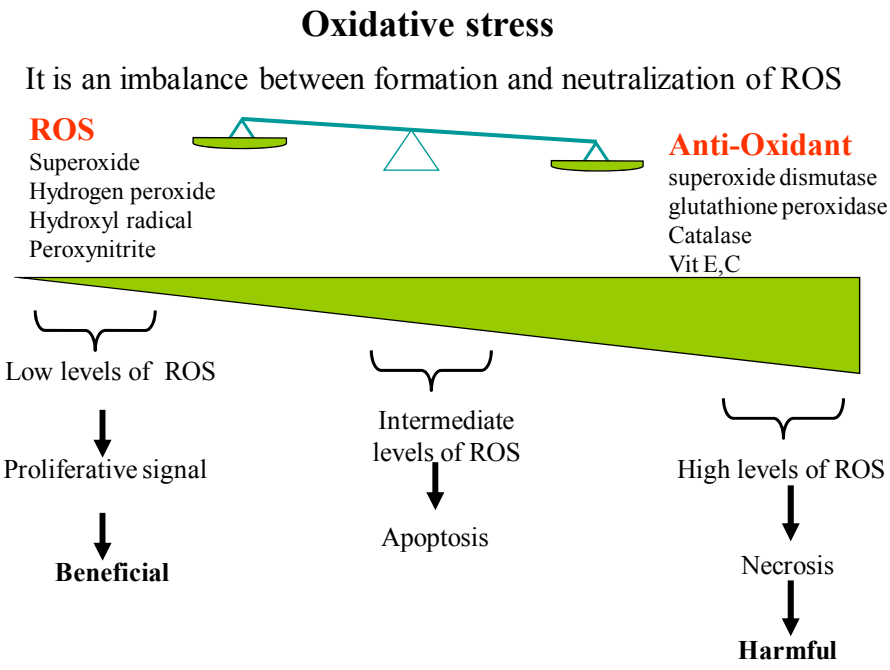

Figure 1: Oxidative stress

The presence of potent cellular detoxification systems minimizes radical generation, terminates radical processes, and repairs damaged macromolecules. However, continued overproduction of ROS and free radicals can overwhelm antioxidant defense and become deleterious to cellular biological processes and tissue functions [1]. Oxidative stress has been implicated in a number of diseases that include atherosclerosis, cancer, and

neurodegenerative diseases, Parkinson's disease, multiple sclerosis, aging and gastro-intestinal. [1-4].

1.1.1 Reactive oxygen species generation and oxidative damage

ROS are free radicals with one unpaired electron derived from molecular oxygen. The most important free radicals are oxygen derivatives, particularly superoxide anion (O_2^{-}•), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2), as well as reactive nitrogen species such as nitric oxide and peroxynitrite. Radical formation in the body occurs through several mechanisms, involving both endogenous and exogenous sources, such as environmental factors that lead to tissue damage as illustrated in **(Fig. 2)** [3, 4]**.**

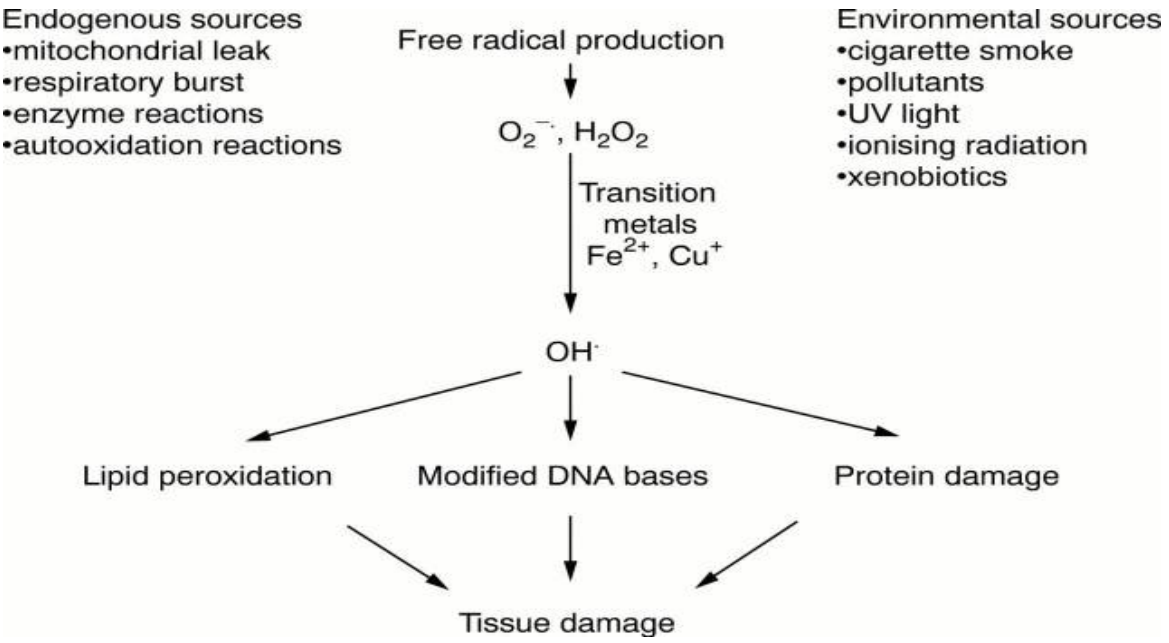

Figure 2: **Major sources of free radicals in the body and the consequences of the free radical damage.** *Young IS J Clin Pathol. 2001*

Under normal physiological conditions, ROS are generated by immune cells as a non-specific defense mechanism against invading organisms, a low level of ROS constitutes inter- and intra-cellular signals, which are vital to maintain proper cellular function [3, 5]. In addition, immune cells, structural cells like epithelial cells, are also involved in the ROS production

However, under pathological conditions, progressive and irreversible generation of ROS increases to a point where the antioxidant system cannot effectively counteract them, and results in impairing physiological functions [4-6]. Thus, depending on their cellular concentrations and duration, ROS can act as either beneficial or harmful agents, as shown in **(Fig.3).**

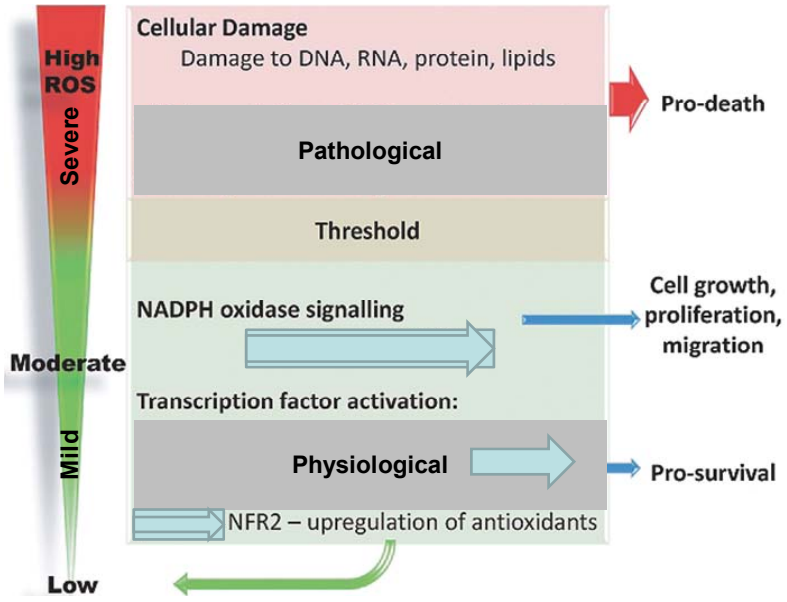

Figure 3: **Mild (physiological) and severe (pathological) oxidative stress**

High ROS concentrations (pathological condition) can affect various subcellular compartments that lead to oxidize mitochondrial enzyme complexes, plasma membrane, nucleic acids (nuclear and mitochondrial DNA), calcium homeostasis, lipid and protein damage as projected in **Fig.4** [1, 3, 6].

Maintaining the protein homeostasis via the regulation of protein unfolding and aggregation is crucial since it shares close relationship with other intracellular pathways, including oxidative stress and mitochondrial dysfunction to impact cell survival and death pathway. Under pathological conditions, the level of abnormal proteins may exceed the ability of the cell to degrade them, allowing aggregation to proceed. Increased oxidative stress results in the accumulation of aggregated proteins that have been suggested to underlie the loss of cellular function.

Oxidative damage modifies a large variety of proteins, including mitochondrial protein and proteins involved in signal transduction, which is often irreversible due to impairment in protein turnover and the need to be degraded by the ubiquitin protein system. However, these damaged proteins are prone to aggregate, poorly degraded and become toxic to the cell [7, 8].

Oxidative damage to mitochondrial and nuclear nucleic acids causes modification of pyrimidine and purine bases and commonly mitochondrial and nuclear DNA damage, measured as increased in 8-hydroxydesoxyguanosine (8-OxO) [9]. Mitochondrial DNA is highly susceptible to ROS induced damage because it is located in close proximity to the production site of ROS, and the mt DNA repair mechanisms are limited. ROS induced mt DNA deletion leads to a decline in the mitochondrial function and enhances ROS production [9, 10].

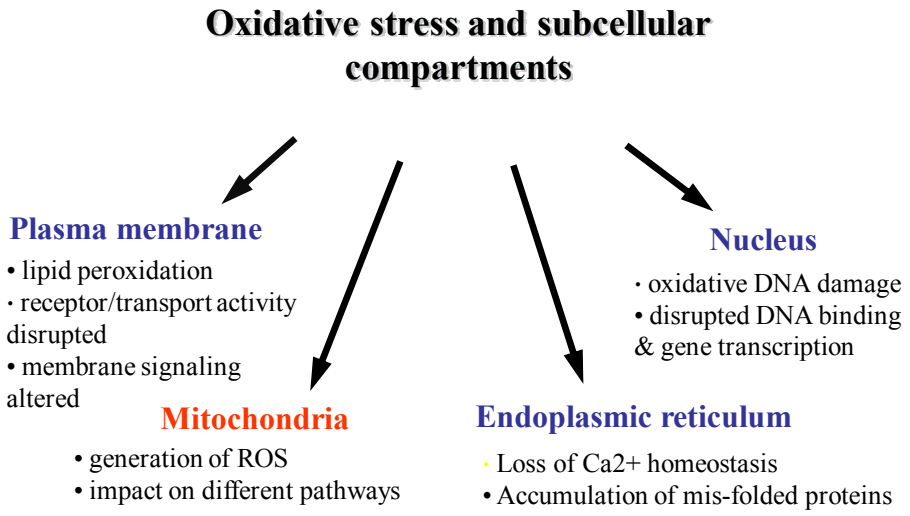

Figure 4: **Oxidative stress affects various compartments with macromolecules damage**

Furthermore, oxidative attacks to polyunsaturated fatty acids (PUFA) in mitochondrial and cellular membranes may trigger lipid peroxidation, resulting in oxidative breakdown of cellular membranes [6, 11].

Mitochondria are a major source of ROS generation. In the mitochondria, O_2^{-}• can be produced by respiratory complexes, enzymes on the outer mitochondrial membrane, inner mitochondrial membrane and in the matrix [7]. Non enzymatic production of O_2^{-}• occurs mainly in the mitochondria, particularly at complex I and III of the mitochondrial electron transport chain (ETC) [12].

Outside of mitochondria, several enzymatic systems are the major sources of O_2^{-}• that are responsible for the production of the intracellular ROS, including NADPH oxidases [13], xanthine oxidase [14] and cytochrome P450-dependent oxygenases [15].

Any condition that results in over-consumption of oxygen can lead to the production of free radicals. It is estimated that up to 1-5% of the oxygen that is passed through the ETC inside the mitochondria results in O_2^{-}• production [16, 17].

$O_2 + e^- \rightarrow O_2^-$ Superoxide radical

$O_2^- + H_2O \rightarrow HO_2 + OH^-$ Hydroperoxyl radical

$HO_2 + e^- + H \rightarrow H_2O_2$ Hydrogen Peroxide

$H_2O_2 + e^- \rightarrow OH + OH^-$ Hydroxyl Radical

Under pathological conditions, O_2^{-}• can react with either luminal or mucosal iron through the Fenton reaction, to produce toxic and highly reactive OH•, leading to a more free radicals production. OH• is also formed from H_2O_2 through the Fenton reaction [18] or from O_2^{-}• through another transition metal-dependent reaction, called the iron-catalyzed Haber–Weiss reaction [19] and is considered to be the most reactive ROS [20].

H_2O_2 is generally considered as a relatively weak ROS that might directly damage proteins and enzymes containing reactive thiol groups. It has the ability to react with partially reduced metal ions, such as Fe^{2+} or Cu^{+}, to form OH• in Fenton reaction [18].

1.1.2 Iron over-consumption induce oxidative stress

Free iron is a potential source of oxidative stress because it catalyses the conversion of H_2O_2 into highly OH•. Iron, like oxygen, is essential for life, but together they can form a highly ROS [21, 22]. The mucosal iron concentration has been observed to be significantly increased in the presence of inflammation, at least in part due to overproduction of free radicals via increased levels of free haemoglobin from mucosal ulceration and bleeding [23]. Subsequent increased in iron may trigger a self perpetuating cycle, resulting in further tissue damage and more inflammation [24].

High concentrations of iron decrease oxidative phosphorylation (OXPHOS) and the total electron transport proteins in the mitochondria. This disturbance of electron movement can lead to increased mitochondrial H_2O_2 production [25]. The combination of increased iron and the physiological concentrations of H_2O_2, generated in the mitochondria during OXPHOS might result in DNA damage [22, 26]. Thus, accumulation of mtDNA damage and subsequent decline in mitochondria function could be a primary cause in various chronic inflammatory diseases.

Free radicals interact with iron and can potentially enhance intestinal inflammation through propagation of lipid peroxidation whereas ascorbic acid can amplify the oxidative potential of iron by promoting metal ion-induced lipid peroxidation [21, 23, 25]. Ascorbic acid, an essential micro-nutrient for the physiological metabolic function, has a significant antioxidant role by scavenging active oxygen species. On the other hand, the interaction of

ascorbic acid with free, catalytic active metal ions could participate in oxidative damage by releasing hydroxyl and alkoxyl radicals. This is further highlighted in patients receiving ferrous as a supplement, as ascorbic ferrous negatively affects the function of intestinal cells.

Iron salts and ascorbic acid, frequently consumed together in multiple-vitamin preparations or ingested foods, form reactive hydroxyl radicals. Clinical observations suggest that oral iron supplementation is not well tolerated and may increase gastrointestinal symptoms and exacerbate disease activity [21, 23, 27]. Thus the cross-talk between iron homeostasis and intestinal inflammation will yield new insights into the pathogenesis of chronic inflammatory diseases and may suggest new therapeutic approaches for these diseases.

Under normal condition, transition metal ions, such as iron and copper, are kept sequestered in cytosol by a number of chelating proteins like ceruloplasmin, ferritin and transferrin given their capacity to aggravate ROS generation. This large amount of chelating iron is released in response to oxidative stress [22]. At the same time, mitochondria are taken up the released chelating iron from cytosol, which may play a critical role in augmenting oxidative stress.

1.1.3 Antioxidant: definition and classification

Antioxidants have been defined as substances that are able, at relatively low concentrations, to significantly reverse the increase of ROS. Antioxidants cannot prevent ROS generation; they rather bring into balance the effects of ROS function.

The physiological role of antioxidants is to prevent cellular damage arising as a consequence of free radicals generation. In many disease states, however, mechanisms that prevent or limit ROS damage may become inadequate. In general, antioxidants prevent free radical-induced tissue damage by preventing the formation of radicals, scavenging them or promoting their decomposition [4, 28].

The most primary antioxidants counteracting free radicals are either enzymatic such as intracellular Superoxide Dismutase (SOD), Catalase (CAT) , or non-enzymatic antioxidants that include vit C, E, and A [3, 7] as seen in Fig. 5

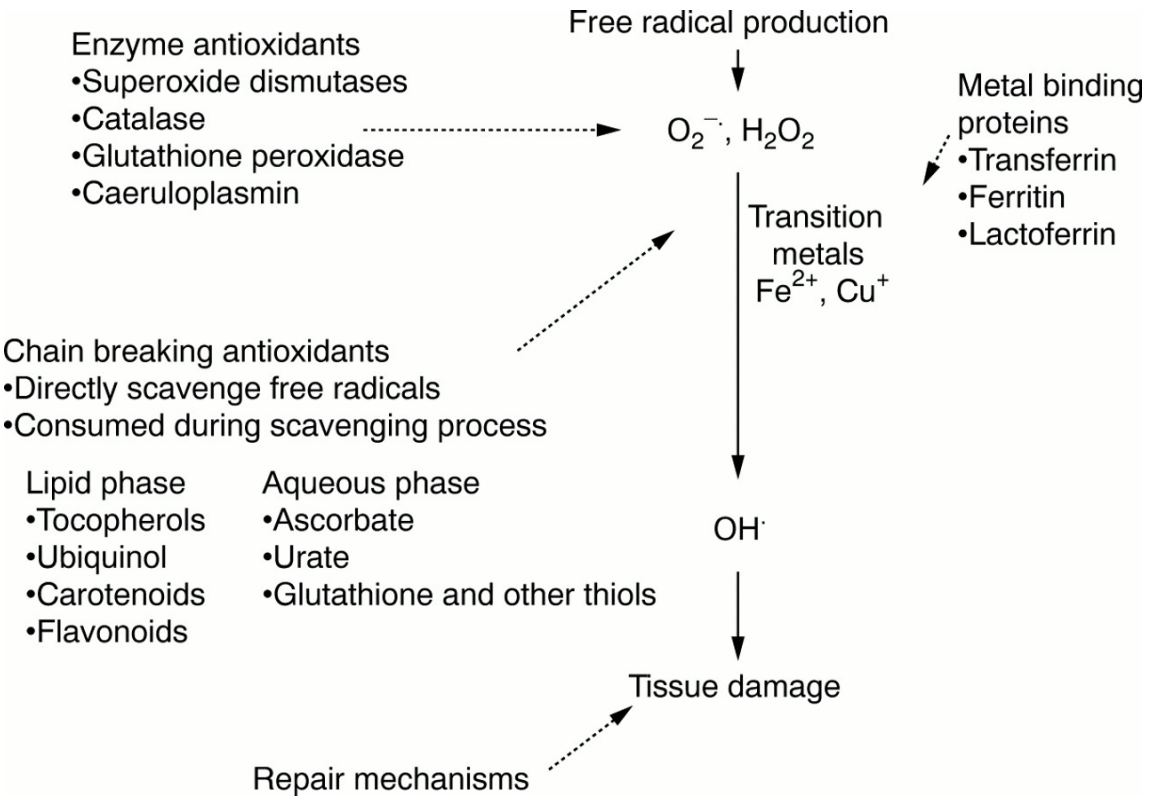

Fig. 5: Antioxidant defenses against free radical attack, **Young IS J Clin Pathol. 2001**

There are three forms of SOD in the mammalian tissues, each with a specific subcellular location and different tissue distributions:

(1) Copper zinc superoxide dismutase, found in the cytoplasm.

(2) Manganese superoxide dismutase, located in the mitochondria.

(3) Extracellular superoxide dismutase, expressed on the cell surface [29, 30].

There are several dietary compounds with antioxidant properties, non-enzymatic antioxidants, normally originating from natural sources, such as fruits, vegetables and plant extracts that play a key role in the host defense and cell survival. In particular, certain minerals (e.g. Zinc), vitamins (C and E) and the flavonoids found in these extracts are considered to be of prime interest [4,31- 35]. Furthermore, the human gut naturally contains a variety of non-enzymatic antioxidant defences. These include water-soluble agents, such as glutathione and ascorbic acid (vitamin C), as well as lipid-soluble defences, such as α-tocopherol (vitamin E) and ubiquinol (reduced co-enzyme Q_{10}) [2, 4, 33-36].

Glutathione (GSH) is a significant intracellular peptide with multiple physiological functions, including antioxidant defensive actions as well as regulatory mechanisms that are able to promote intracellular processes. Additionally, GSH important functions include:

(A) The reduction of various oxidative insults.

(B) The maintenance of the basic structure of protein thiols.

(C) Maintaining of the cysteine reserve.

(D) The modulation of DNA synthesis and immune function.

(E) An anti- apoptotic function [31, 32]

Although evidence shows that antioxidant treatment results in beneficial effects, the clinical benefit from antioxidants is still under wide debate. This remains an important area for future investigation, as researchers attempt to augment intracellular antioxidant, either by dietary supplementation of antioxidants or by overexpressing genes or transcription factors such as Nrf2 encoding antioxidant enzymes, which I will discuss in details in the 2nd part of my thesis.

1.1.4 Oxidative stress and intestinal inflammation

The intestine represents a key defense barrier against luminal toxic agents. Thus, in addition to being exposed to luminal nutrients, the intestinal mucosa is constantly challenged by diet-derived oxidants, mutagens, and carcinogens as well as by endogenously generated free reactive radical [23, 24].

The primary event of the induction of cellular oxidative stress is the inflammatory cascade of neutrophil adherence to vascular endothelial cells, disruption of the endothelial barrier, and subsequent infiltration of inflammatory cells into the intestinal wall, where oxidants and proteases are released to produce mucosal injury [37].

Oxidative stress appears to be a primary causal factor in generating and maintaining a chronic intestinal inflammation, which is characterized by the activation of neutrophils and macrophages, as well as generation of numerous pro-inflammatory mediators, including IL-1β , tumour necrosis factor alpha (TNF-α) , IL-8 and IL-6 [38-40]. It is well known that TNF-α is involved in ROS production; ROS in turn activate nuclear factor- kappa B (NF-

κβ), which then enhances further TNF-α production, making a vicious cycle of excessive oxidative stress production [41-43] as appears in **(Fig.6**).

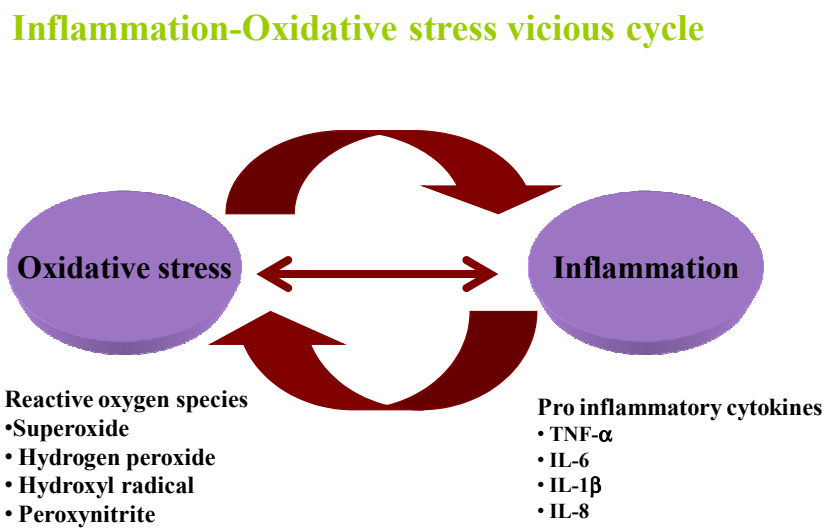

Figure 6: Inflammation and oxidative stress vicious cycle

There is an evidence that ROS and pro-inflammatory cytokines work synergistically as activators of NF-κβ and activator protein-1, thereby modulating their activity [44]. However, activation of NF-κβ has been specifically implicated in maintaining inflammation by the activation of a variety of inflammatory genes [45].

Enhanced NF-κβ signalling in the gut epithelium initiates the primary events of intestinal inflammation and leads to defects in the intestinal epithelial barrier. A recent study by Banan et al suggests that the loss of intestinal epithelial barrier function caused by the exposure to oxidants is lessened by inhibition of NF-κβ activity [27, 40]. There is an

increasing awareness of a role for cellular thiol redox status in NF-κβ activation and gene expression. Staal et al showed that low thiol concentrations promote NF-κβ activation, suggesting that intracellular thiol status has a key role in regulating gene activation [46].

Studies on TNF-α -mediated inflammation have linked TNF-α induced mitochondrial oxidant production with induced NF-κβ activation [29]. Interestingly, Cogswell et al have found that NF-κβ and IκB-α are localized in the mitochondria and can negatively regulate mitochondrial gene expression in response to cellular TNF-α stimulation. Furthermore, mitochondrial exposure to TNF-α has confirmed the loss of expression of cytochrome C oxidase and cytochrome b mRNA, which down-regulates mitochondrial biogenesis [47].

TNF-α–induced oxidative stress and mitochondrial dysfunction in cell lines and tissues are evident in several patho-physiological conditions. In cardiac tissues, Maria pan N et al have shown that TNF-α induction leads to defects in mitochondrial permeability transition pore (PTP) proteins, resulting in pore opening, cytochrome C release and subsequently leading to apoptosis and mitochondrion dysfunction in cardiac tissues [48, 49].

The various mechanisms whereby activation of TNF- α modulates chronic inflammation and mitochondrial damage in gastro-intestinal diseases remain to be elucidated. Such ROS/cytokine-/transcription factor regulatory network loops may contribute to the perpetuation and exacerbation of chronic inflammation and tissue damage, particularly when the local immune response fails to successfully down-regulate the immune reaction.

Increased levels of TNF-α have been found in serum, mucosa and stool of patients with inflammatory bowel diseases (IBD) [50, 51]. Several clinical trials have shown that anti-TNF-α antibody is effective in the treatment of intestinal inflammation, supporting the importance of TNF-α in the ongoing regulation of epithelial barrier functions in association with increased apoptosis [52, 53].

1.1.5 Intestinal permeability and tight junction proteins

The mucosal barrier is established by the single layer of intestinal epithelial cells. The intestinal epithelium is a polarized monolayer of columnar epithelial cells, which forms a barrier between the luminal contents and the mucosa. Its role is to achieve an efficient absorption of nutrients, water and ions, while preventing the uptake of noxious antigens, microbes and toxins. Epithelial permeability is influenced by the integrity of the epithelial cell layer and the basement membrane, as well as by the surface mucus layer and by autonomic nervous system functions [54, 55].

In intestinal inflammation, lipid peroxidation causes disruption of mucosal barrier function, increased permeability and a reduction of tight junction proteins functions, which ultimately leads to alterations in cellular metabolism [56]. Epithelial barrier dysfunction and inflammation are major contributors to the pathogenesis of gastro-intestinal diseases. However, much remains unknown about how these two processes contribute independently to disease initiation.

The movement of particles in both small and large intestinal parts occurs through two pathways: paracellular and/or transcellular. Numerous stimuli, including microbial components, smoking, anti-inflammatory drug use and proinflammatory cytokines, can regulate paracellular permeability [54]. The integrity of epithelial barrier is dependent on the function of tight junctional proteins (TJs) located near the apical poles of two adjacent epithelial cells. TJs are composed of multiple proteins (claudin family, occludin, zonula occludin-1) that are involved in establishing the epithelial barrier, and they selectively determine which molecules are able to traverse the paracellular space [57-59].

Occludin was the first identified transmembrane protein of this intercellular junction. TJs seem to interact together as networks and it appears that occludin interacts, directly or indirectly, with claudin. Conceivably, occludin has increased electrical resistance by influencing the extracellular conformation of Claudins [57, 58].

The TJs are dynamic structures that are rate limiting for passive absorption of hydrophilic molecules in the intestine, which should be determined by intestinal permeability [59]. Thus, TJs dysfunction may be an important source of the overall intestinal barrier defects, leading to increased permeability very often seen in patients with chronic intestinal inflammation. In vitro experiments examining the effects of inflammatory cytokines on model intestinal epithelial cells suggest that disruption of the epithelial barrier is associated with internalization of transmembrane TJ protein such as occludin [55, 60, 61].

1.2 Mitochondria

1.2.1 Mitochondrion structure and function

Human mitochondrion contains multiple copies of a small double-stranded DNA genome that encodes 13 proteins (subunits of complexes I, III, and IV and the ATP synthase complex), 2 Ribosomal Ribonucleic Acids (RNAs), and 22 transfer RNAs that are needed for mitochondrial DNA translation. Therefore, these organelles represent a critical intracellular target for oxidative damage, which may lead to lethal injury through the loss of electron transport, mitochondrial membrane potential and ATP generation [12, 62, 63].

The mitochondrial genome is transcribed by machinery that includes RNA polymerase, the mitochondrial transcription factor A (mt TFA) and two mitochondrial transcription factors, mt TFB1 or mt TFB2 [64]. Although mitochondria have their own genome, most of the proteins and enzymes that reside in mitochondrial membranes are nuclear gene products. Mitochondria possess both an outer and inner membrane, the latter of which has a larger surface area, is impermeable to all molecules and contains the enzymes responsible for OXPHOS and ATP production as seen in (**Fig. 7)**

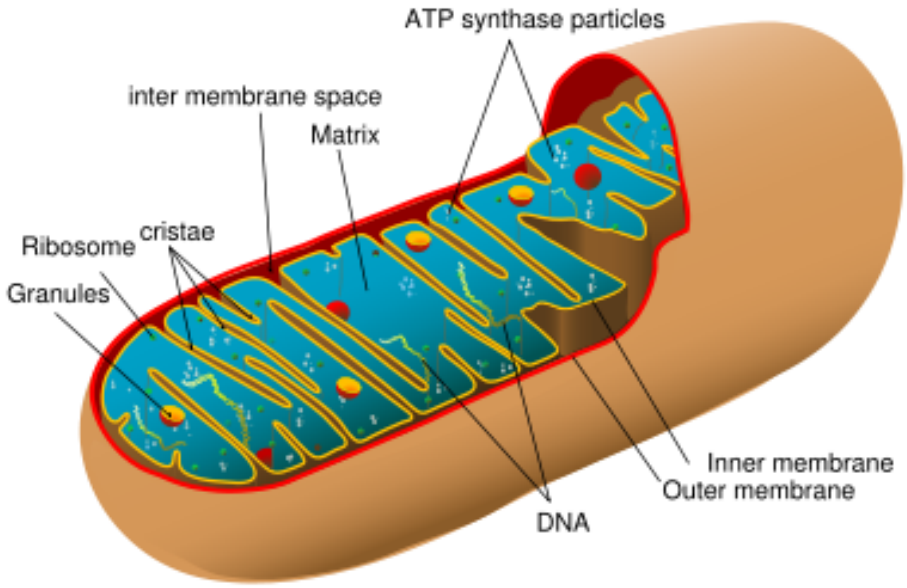

Figure 7: **Mitochondrial structure**

Mitochondria are multifunctional organelles that receive, integrate and transmit signals [65] and are involved in numerous metabolic activities, including ATP production, (OXPHOS), ROS generation and detoxification, calcium (Ca^{2+}) homeostasis and regulation of apoptosis pathways [65-67].

1.2.2 Electron Transport Chain Complexes and ATP production

Mitochondria produce 90% of the required energy necessary for cellular function via adenosine-5'-triphosphate (ATP) production either by anaerobic glycolysis or by mitochondrial OXPHOS. During OXPHOS, electrons from reduced substrates are transferred to O_2 through (ETC), including complex I, III, and IV, which in turn generate a proton gradient across the mitochondrial inner membrane [12, 66, 68]. The electrochemical energy of this gradient is then used by complex V, which couples H^+ reuptake with ADP

phosphorylation in the matrix to generate ATP [69]. It appears that any blockage in the ATP production result in a severe impairment of one or more of these complexes. Excess electrons, however, may react with oxygen to form more ROS production that plays a key role in creating oxidative stress [70] as shown in (**Fig.8)**.

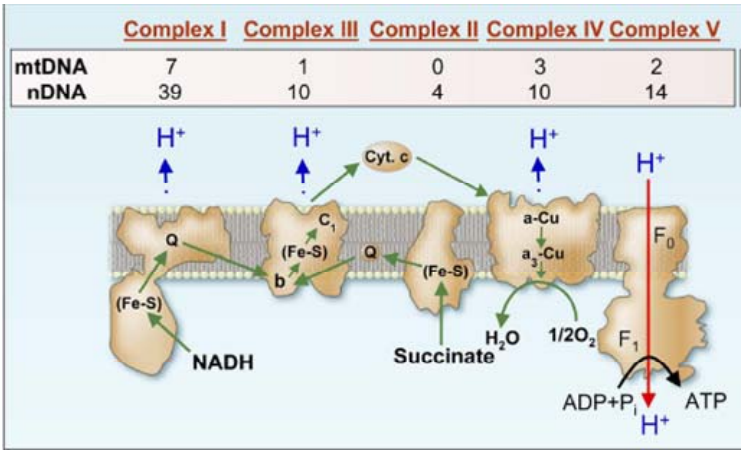

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Figure 8: Summary of protein subunits of the five Respiratory chain complexes encoded by nuclear and mitochondrial genes

1.2.3 Mitochondrial function and oxidative stress

Mitochondria produce the energy needed for normal cellular function and metabolic homeostasis by OXPHOS and serve as biosensors for oxidative stress. Mitochondria constitute a major source of free radicals in cells, resulting in oxidative stress, but are also targets to oxidative stress action. Although the molecular mechanisms responsible for mitochondrion-mediated diseases are not fully elucidated, yet oxidative stress appears to be

a pivotal player. Hence, it is apparent that mitochondrial damage may lead to the impairment of various aspects of tissue functioning.

It is now appreciated that the reduction of mitochondrial oxidative stress may prevent or slow down the progression of many mitochondria dysfunctions related to oxidative stress diseases, including aging, obesity, diabetes and neurodegenerative disorders [10, 70-73]. However, if mitochondria are the major source of intracellular ROS and mitochondria are most vulnerable to oxidative damage, then it would be ideal to deliver the antioxidant therapy to mitochondria [74].

Dietary antioxidants are widely used to ameliorate excessive oxidative stress, but scientific proof of their efficacy is poor. The currently existing antioxidants are not very effective in overcoming oxidative stress-mediated diseases. One of the reasonable answers for the failure of antioxidants to show clear therapeutic effects is their incapacity to reach mitochondria. There are currently growing efforts in developing mechanisms for the targeted delivery of antioxidants to mitochondria [74, 75]. More recently, confocal imaging studies in Caco-2 cells show that intracellular distribution of SS-19 tetrapeptidase antioxidant resembles that of MitoTracker, which localizes in mitochondria [76].

1.2.4 Mitochondrial DNA damage related to oxidative stress

Increased Oxidative Stress may contribute to alterations in the abundance of mitochondria as well as the copy number and integrity of mt DNA in human cells, which is vulnerable to oxidative damage because it is situated much closer to the site of ROS generation, in

particular since mitochondria lack protective histones and have much more reduced base excision repair mechanisms [73].

Oxidative stress has been one of the risk factors that induce gut malignancy. If oxidative stress is irreversible, these damages lead to mutagenesis and carcinogenesis [77]. The accumulation of mt DNA mutations cause the loss of mitochondrion ability to produce sufficient energy to meet cellular needs and serves as a trigger for mitochondrial dysfunction and apoptosis [78]. Mutated mtDNA shows ~10- fold more mutation rates than nuclear DNA in relationship with increased free radical production, thereby leading to a vicious cycle that progressively stimulates the rise of oxidative stress and leads to impaired mitochondrial function.

Oxidative DNA damage was measured by the production of 8-hydroxy-deoxy-guanosine (8-OHdG). This molecule (8-OHdG), which is more specific for mt DNA, is of practical importance because it is easily measurable and has therefore been proposed as a useful marker of oxidative stress [9, 79]. Human cells have developed different repairing enzymes, the most important being 8-oxo DNA Glycosylase (OGG1) that protects against the effects of oxidized DNA bases and preferentially removes 8-OHdG opposite cytosine [79, 80]. In mitochondria, OGG1 is thought to prevent activation of the intrinsic apoptotic pathway in response to oxidative stress by augmenting DNA repairing mechanisms [73].

It has been proposed that mt DNA damage can lead to inhibition of ETC, increased ROS production, loss of mitochondrial membrane potential, and released signals for cell death, such as cytochrome C and AIF [62, 81, 82]. Therefore, mt DNA damage represents an important target for intervention and a biomarker in the course of many human diseases.

1.2.5 Oxidative stress induce mitochondrial calcium overload

Mitochondria may have an impact on Ca^{2+} signals and functions as a buffer to stabilize calcium concentrations within the cell [83, 84]. Thus, mitochondrial Ca^{2+} is considered as a physiological regulator to balance mitochondrial ATP output and cellular ATP demand.

A growing body of evidence suggests that Ca^{2+} channels, which control Ca^{2+} efflux from the endoplasmic reticulum (ER) in response to different biochemical signals, are sensitive to small changes in ROS concentrations, suggesting that these Ca^{2+} channels serve as physiological redox sensors [67, 84]. Under physiological conditions, Ca^{2+} released from the ER during cell activation is taken up by mitochondria to promote OXPHOS.

During oxidative stress, there is a rapid increase in cytosolic Ca^{2+}, which is followed by mitochondrial Ca^{2+} over loading [85]. At its turn, Ca^{2+} overload can lead to induced ROS production and another potential vicious cycle develops, thereby triggering mitochondrial (PTP) opening and cytochrome C release and resulting in apoptosis.

The exact mechanism of mitochondrial Ca^{2+} induced ROS generation is unclear, although it may involve changes in the three-dimensional conformation of the respiratory complexes [72]. Specifically complex results in an increase of H_2O_2 production and inhibits OXPHOS, which may provoke an irreversible reduction in the energy status, thereby initiating patho-physiological processes in certain cells [86, 87].

A recent study suggests that disruptions in intracellular Ca^{2+} mobilization may contribute to the dysmotility of colonic smooth muscles in murine dextran sulfate sodium (DSS)-colitis by enhancing NF-κβ activity [88]. Furthermore, Di Sabatino A et al have provided evidence that the suppression of Ca^{2+} overloading may inhibit pro-inflammatory cytokine release in inflamed gut. Therefore, Ca^{2+} channel inhibitors might reduce mitochondrial Ca^{2+} overload, decrease mitochondrial ROS accumulation, improve mitochondrial energy production and have the potential to ameliorate mitochondrial oxidative stress-mediated diseases [89].

1.2.6 Mitochondrial dysfunction and apoptosis

Apoptosis, known as programmed cell death, plays a vital role in all stages of cell development. Mitochondria play a checkpoint of apoptotic signalling and integrate various types of proapoptotic endogenous signals incoming from other organelles, including nucleus, cytosol and lysosomes as well as exogenous factors including specific viral proteins or xenobiotics [62, 81].

Apoptosis may be triggered by extracellular signals (extrinsic pathway) or by intracellular processes (intrinsic pathway). An increased mitochondrial formation of ROS triggers the intrinsic pathway by increasing permeability of the outer mitochondrial membrane through the opening of PTP, leading to collapse of mitochondrial membrane potential, along with release of apoptogenic mitochondrial proteins into the cytoplasm, which triggers a cascade of events, leading to apoptosis [62, 90] as seen in **(Fig. 8).**

The release of different proteins from the mitochondria is a critical early event in mitochondrial mediated apoptotic cell death. Cytochrome C is such a protein that, upon releasing into the cytosol, forms a complex with procaspase-9, resulting in the activation of the caspase cascade and ultimately in apoptosis [26, 62, 82]. Early release of cytochrome C would be the early sign of apoptosis and suggests the occurrence of mitochondrial dysfunctions along with apoptosis development [76]. The gradual loss of cytochrome C from the intermembrane space during apoptosis favours the mitochondrial formation of O_2^{-}• in two ways: (1) cytochrome C is a scavenger of O_2^{-}• and (2) as cytochrome C is released, the electron flows between Complex III and Complex IV, slows down, and the respiratory chain becomes more reduced [82, 91].

The mechanism of cytochrome C release connects the upstream event of cytochrome C (i.e., dissociation from cardiolipin). Hence, cytochrome C must first dissociate from the inner membrane in order to escape mitochondria. Therefore, oxidation of cardiolipin may be one mechanism by which cytochrome C is solubilized in the intermembrane space [26, 76].

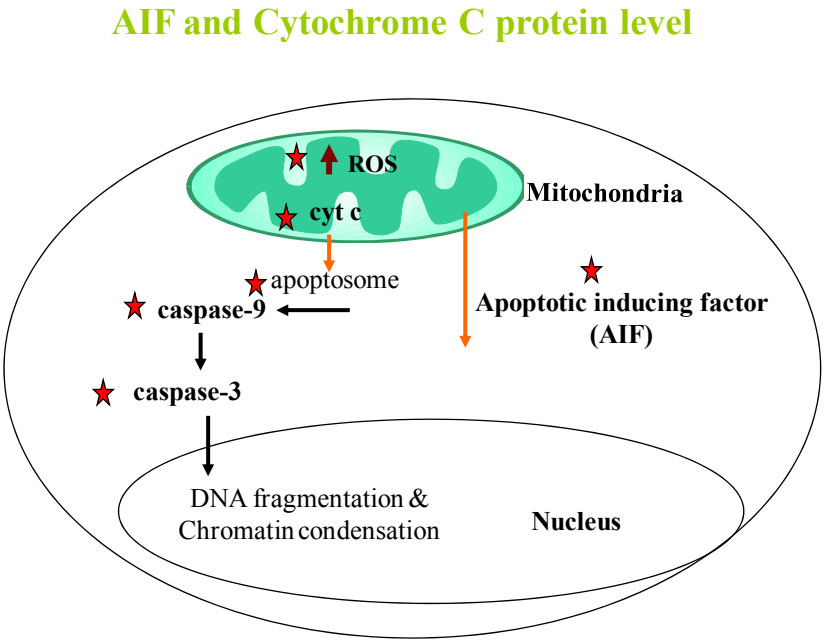

Figure 9: **Caspase-depended (Extrinsic) pathway and caspase- independed (intrinsic) pathway**

Apoptosis is also initiated by mitochondrial intermembrane space protein called AIF, involved in initiating a caspase-independent pathway of apoptosis (positive intrinsic regulator of apoptosis) through DNA fragmentation and chromatin condensation [62]. AIF normally stabilizes mitochondrial membrane permeability and supports OXPHOS. However, if released through the outer membrane into the cytosol, AIF can produce terminal damage to n DNA [92].

Apoptotic pathways contain counter balancing concentrations of anti-apoptotic and pro-apoptotic Bcl-2 family [93]. These proteins are normally found in the cytosol but can be induced to target mitochondria. All of these Bcl-2 family proteins are encoded within the nuclear genome, transported into mitochondria and stored in the space between the inner

and outer membranes [94]. Under physiological conditions, anti-apoptotic Bcl-2 remains bound to the outer membrane and prevent the mitochondrial PTP. In contrast, when pro-apoptotic factor like Bax is induced and translocated to mitochondria, it initiates the apoptosis process [91]. The relative ratio of anti- and pro-apoptotic Bcl-2 family proteins dictate the ultimate sensitivity of cells to various apoptotic stimuli, including oxidants and Ca^{2+} overload [62, 91].

Bcl-2 family proteins regulate the release of cytochrome C, AIF and certain caspases (caspase-2, -3, and -9) from mitochondria. Pro-apoptotic Bcl-2 enhances release of this caspase-activating protein and anti-apoptotic members of the family, which stops cytochrome C release [62, 81]. Furthermore, it has been suggested that the anti-apoptotic functions of Bcl-2 may be (at least in part) associated with depletion of ER Ca^{2+}, thus indirectly reducing mitochondrial Ca^{2+} uptake [67].

1.2.7 Prohibitin protects mitochondrial dysfunction

Prohibitins (PHB) are ubiquitous chaperone proteins forming a ring-like, high-molecular-mass complexes that are mainly localized in the inner mitochondrial membrane, help in the stabilization of mitochondrial respiratory enzymes and mitochondrial membrane proteins, and are implicated in mitochondrial biogenesis, mitochondrial function, mitochondrial morphogenesis and apoptosis [95]. Deficiency of the PHB complex results in increased ROS production or sensitivity to free radicals [96]. Abnormal PHB levels have been reported in mitochondrial dysfunction related to oxidative stress diseases, including obese patients at risk for non-alcoholic steatohepatitis, parkinson disease, schizophrenia [97, 98].

Interestingly, it has been also found that PHB expression is down-regulated in intestinal diseases in experimental colitis *in vivo* and during oxidative stress *in vitro*. Furthermore, Theiss *et al* have shown that TNF-α decreases PHB expression in intestinal epithelial cells, and restoration of PHB expression in these cells can protect against the various effects of TNF-α and NF-κβ on intestinal barrier function [99, 100]. More recently, it has been shown that PHB is a regulator of Nrf2 expression in intestinal epithelial cells during oxidative conditions that prevent inflammation-associated oxidative stress and injury through sustained activation of Nrf2. The above findings emphasize the importance of the PHB complex in maintaining mitochondrial homeostasis that is crucial for human health.

1.2.8 Nucleus-mitochondrial cross talk, role of PGC-1 α

Mitochondrial functions must rely on an orchestrated cross-talk between nuclear and mitochondrial genes and it clearly appears that the nucleus has a dominant role in the regulation of mitochondrial activity. Nuclear transcriptional factors control the activity of mitochondrial genome and coordinate the expression of both nuclear and mitochondrial genes encoding mitochondrial proteins. Nonetheless, nuclear gene expression can be influenced by signals derived from mitochondria, through retrograde communication, so that the regulation of mitochondrial activity is exposed to a bidirectional flow of information [101, 102].

Crosstalk between nucleus and mitochondrial genes contribute to mitochondrial protection against oxidative stress via enhancing antioxidant defense through the Nrf2 pathway that

acts on the genes coding for constituent subunits of the OXPHOS system and mtDNA replication or by stimulating mitochondrial biogenesis through the activation of peroxisome proliferation activator receptor γ-coactivator 1α pathway (PGC-1 α) [68, 103]. PGC-1α may serve as an adaptive set-point regulator, capable of providing an accurate balance between metabolic requirements and cytotoxic protection. Therefore, its dual activities of inducing mitochondrial biogenesis and suppressing ROS make PGC-1α an almost ideal target protein for the control damage associated with mitochondrial dysfunction.

PGC-1α interacts with Nrf-2 to transactivate a number of genes involved in mitochondrial functions such as OXPHOS regulation, protein import and heme biosynthesis. It also mediates mt DNA transcription and replication through two nuclear-encoded genes, the mt TFA and B (mt TFB) [103, 104]. It is suggested that the Nrf-2 pathway could be affected at the levels of transcription, translation or protein levels.

Surprisingly, a more recent study has shown that mice with a targeted disruption of PGC-1α are viable and show no changes in mitochondrial abundance or morphology in liver or brown fat [104]. The retrograde or compensatory pathway has been proposed: free radicals generated from respiratory chain are involved in the signalling pathway from mitochondria to the nucleus in order to enhance the expression of nuclear genes involved in mitochondrial biogenesis such as Nrf2 [101, 102].

1.2.9 Mitochondrial dysfunction related to oxidative stress

Mitochondrial dysfunction has gained more attention in recent years, whereby electrons leaking from the ETC generate ROS that in turn damage ETC components and mitochondrial DNA, which leads to an additional increase in intracellular ROS levels and defects in mitochondrial functions [48, 105, 106]. Because dysfunctional mitochondria will produce more ROS, a feed-forward loop is set up whereby ROS-mediated oxidative damage to mitochondria favours more ROS generation, then resulting in a vicious cycle. This mitochondrial dysfunction causes cell damage and death by compromising ATP production, disruption of calcium homeostasis, damage of mt and nuclear DNA, increases in cytochrome C and AIF release, impairment in various proteins and lipid peroxidation (**Fig 10)**:

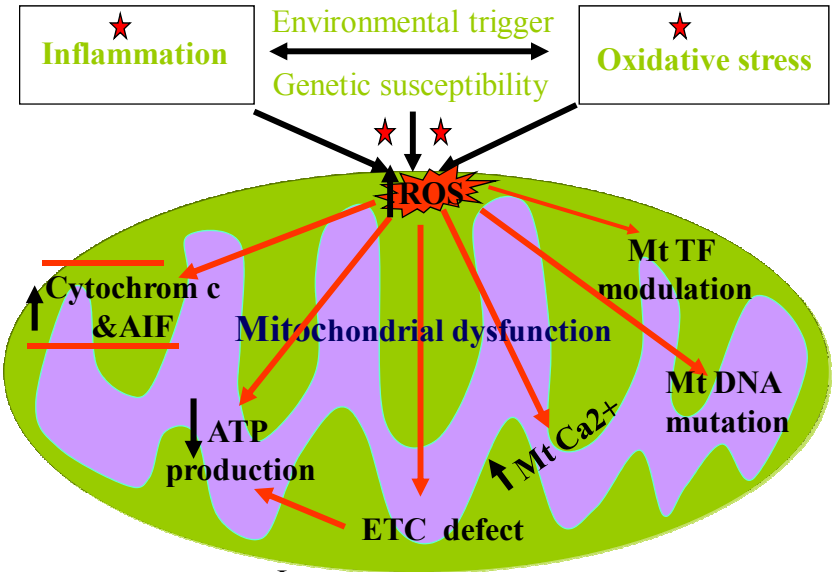

Figure 10: **Mitochondrial dysfunction events related to oxidative stress**

Since mitochondria are attractive targets for drug-delivery strategies, it would be ideal to deliver the antioxidant therapy directly to mitochondria [75]. The mitochondrial outer membrane is permeable to small molecules, and thus the inner membrane represents the major barrier for drug delivery to mitochondria. However, mitochondria-targeted antioxidants research is still in infancy and needs sufficient time to yield more reliable data.

1.2.10 Implication of oxidative stress and mitochondrial dysfunction in inflammatory bowel diseases

Inflammatory Bowel Diseases **(**IBD) are idiopathic, chronic and relapsing inflammatory conditions of the gastrointestinal tract. They are recognized as important causes of gastro-intestinal diseases in children and adults and are common in highly industrialized western countries [107, 108]. Crohns Disease (CD) and Ulcerative Colitis (UC) are the two main clinic-pathological subtypes of IBD [109, 110].

Although the incidence and prevalence of IBD are beginning to stabilize in high-incidence areas such as North America and Northern Europe, they continue to rise in low-incidence regions such as Southern Europe, Asia, and much of the developing world [111, 112]. There are about 201,000 Canadians living with IBD: 112,500 with CD and 88,500 with UC. Canada is among the highest reported prevalence and incidence of IBD in the world [113]. Economic costs in 2008 for IBD are estimated at $1.8 billion per year in Canada [114, 115]. There has been a significant increase in IBD morbidity that exerts enormous economic burden and makes IBD a public health issue [114, 116].

No precise aetiology has been identified for IBD. Epidemiological studies have stressed the involvement of environmental and genetic factors and especially anomalies in the immune system in response to microbial infections [108, 110] as seen in **Fig.11.**

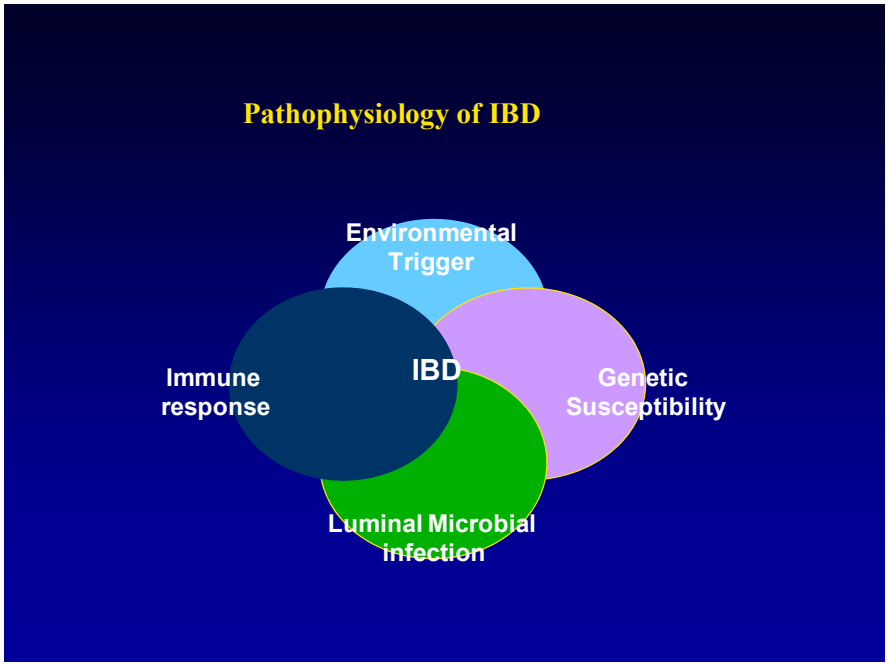

Figure 11: IBD risk factors

The strongest environmental factors being identified are smoking and diet [113, 117, 118]. It is clearly established that smoking is the most consistently environmental risk factor reported to be associated with CD. A meta-analysis suggests that smokers are more than twice likely to develop CD compared to non-smokers [119, 120]. Several other studies have considered the role of dietary factors in the pathogenesis of IBD, while immunological mechanisms have been suggested to link food antigens to the development of intestinal inflammation [121].

It is postulated that the higher incidence of IBD seen in developed countries may be associated with dietary habits. Persson et al suggest that frequent fast food intake and increased simple sugar consumption confer a 3-to-4-fold greater risk for IBD [122]. Whether other suggested factors such as western life-style, social and occupational status, psychological stress, sanitation, appendectomy, drugs and exposure to infections play a role in the expression of IBD remains unclear [123-126] .

Previous findings suggest that IBD incidence appears linked to race. A strong genetic component in IBD aetiology is most likely related. Some epidemiological data support this hypothesis: (1) the heterogeneity in the geographical distribution of IBD, (2) the existence of familial forms of IBD and (3) the high rate of concordance in monozygotic twins [127-130].

Various mechanisms have been discussed concerning immune dysfunction in IBD, including defects in immune response to normal luminal components, and/or defective mucosal barrier to luminal antigens such as diet and enteric bacteria [131, 132] as seen in **Fig. 12**.

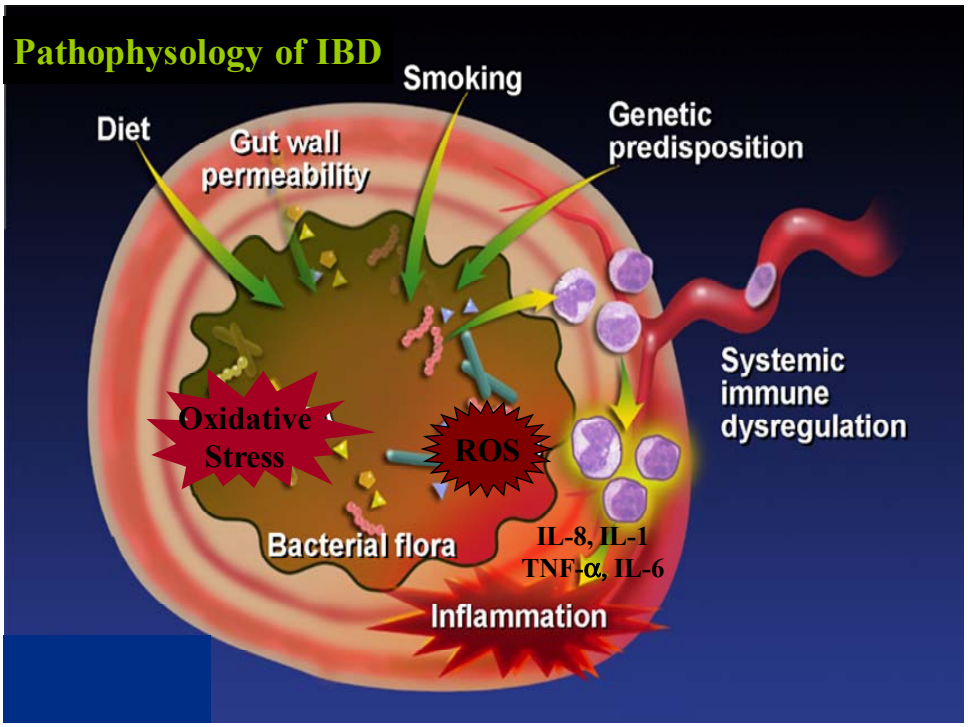

Figure 12: Pathopghysiology of IBD

The intestinal epithelium is the first host defense against invading pathogens and food antigens acting as key mediators of communication between the intestinal lumen and mucosal immune system. The gastrointestinal mucosa is constantly exposed to luminal oxidants from ingested foods that might contain iron salts and ascorbic acid [21, 131].

The ingestion and/or occurrence of peroxides may have significant implications in human health in the long term. In this regards, ROS metabolites have been suspected to provoke injury to intestinal mucosa in various diseases, including intestinal ischemia and subsequent reperfusion, as well as IBD [133, 134].

Oxidative stress is currently considered among the most plausible explanations for IBD in human [3, 35, 134, 135]. Markers for oxidative damage can be measured in plasma, urine,

or post-mortem tissue by means of HPLC and immunohistochemistry [4, 33]. The increased in malonyldialdehyde (MDA), $CH_2(CHO)_2$, levels found in plasma and colonic biopsies from CD patients provide evidence for excess lipid peroxidation reactions [134, 136]. Furthermore, the increased breath ethane and pentane excretion in CD patients, which are non-invasive markers of lipid peroxidation, have been correlated with disease activity [35].

The oxidative stress parameters in CD will be as follow:

1. Breath ethane output ($pmol \bullet kg^{-1} \bullet min^{-1}$)
2. Breath pentane output ($pmol \bullet kg^{-1} \bullet min^{-1}$)
3. F_2-isoprostane (ng/L)
4. MDA

There are many studies supporting the notion that a decline in antioxidant activity occurs with CD. Lih-Brody et al found a decrease in SOD in the mucosa of CD patients [137], which was correlated to the activity of the disease [30]. Furthermore, Buffinton GD et al observed a reduction of total glutathione in inflamed mucosa of patients with CD compared to normal tissue areas [138].

Interestingly, reduced levels of PHB during intestinal inflammation may be one underlying factor that contributes to oxidant-induced mucosal barrier and intestinal permeability dysfunction [99, 100]. Finally, in a more recent report, Beltran B et al have demonstrated that ROS are directly implicated in the oxidative damage that occurs in CD patients. Additionally, mitochondrial membrane potential of CD cells is significantly inhibited compared to control cells and correlates significantly with markers of inflammation [139].

Surprisingly, even if oxidative stress occurs in the intestinal mucosa, limited studies have explored the response of mitochondria to oxidative stress in the intestinal tissue in relation with the key cellular regulatory processes, including (ATP) production, intracellular calcium regulation, cell signaling, ROS generation and apoptosis.

A number of findings have suggested that mitochondrial pathology related to OS may be associated with CD. Mitochondrion morphological changes have been observed in epithelial cells in tissues resected from patients with CD and in animal models of gut diseases [140, 141]. Farhadi et al discussed that mitochondrial damages were noted in intestinal epithelial cells and mucosal protein oxidation in stressed CD patients [100]. Furthermore, Soderholm et al. observed the presence of numerous swollen and irregular mitochondria in the epithelium of colonic segments from stressed rats [39]. O'Morain et al, on the other hand, showed mitochondrial damage through examining rectal biopsies obtained from CD patients [142].

Furthermore, Plasma levels of 8-OHdG was found to be increased in CD patients compared to controls [143, 144]. It has also been shown that 8-OHdG levels were significantly higher in active CD, inactive CD and CD patients in remission compared to controls [139]. A recent report provides evidence on the association between intestinal inflammation, oxidative stress, DNA repair enzyme OGG1 and carcinogenesis. OGG1 (-/-) mice developed a significantly higher number of adenocarcinomas in the DSS colitis model compared to wild type mice [80]. Finally, a recent case report on a young girl with CD disclosed impaired OXPHOS along with abnormalities in Complexes III and IV [103].

1.3 Nuclear transcription factor (Nrf2)

1.3.1 Nrf2 structure and functions

Nrf2, a basic leucine-zipper motif transcription factor, plays a key role in the regulation of phase II genes by binding to the ARE element in their promoters in conjuction with small Maf proteins [145, 146]. Nrf2 is one of the redox sensitive transcription factors, which gets activated in response to the increase in ROS. It attempts to restore the redox haemostasis by transcribing antioxidant proteins. Nrf2 is found in most tissues but is mostly abundant in the brain, liver, kidney and systems that are exposed to external environmental stresses like the gastrointestinal tract and skin [146]. Nrf2 has various cytoprotective functions and acts as antioxidant and anti-inflammatory factor capable to regulate mitochondrial biogenesis, mitochondrial functions and apoptosis process [145-148].

1.3.2 Activation of keap1-Nrf2 –ARE pathway, Anti-oxidant role of Nrf2 signalling

In normal conditions, Nrf2 is sequestered by Keap1 which is sensitive to oxidative stimuli owing to the presence of reactive cysteines. Keap1 is a key regulator of the Nrf2 signalling pathway and serves as a molecular switch to turn on and off the Nrf2-mediated antioxidant response [149].

In mild (physiological) oxidative stress, when oxidative modification of one of the Keap1 cysteines occurs, Nrf-2 escapes from this proteolytic pathway, then translocates to the nucleus where it dimerizes with a small Maf protein and binds to DNA sequences of Phase

II antioxidant genes [150, 151]. In severe (pathological) oxidative stress, there is a dissociation of Keap-1 from Nrf-2 in the cytoplasm exposing Nrf2 for ubiquitination. Degradation of Nrf-2 will take place once it is released from Keap-1 to the cytoplasm [150-152] as seen in **Fig.13**.

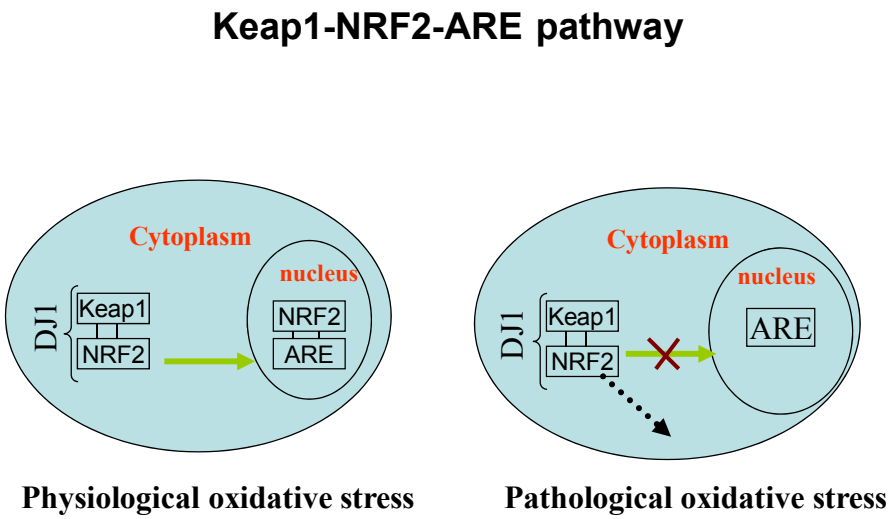

Figure 13: **keap1-Nrf-2-ARE pathway**

Two mechanisms have been proposed for the activation of Nrf2-Keap1 pathway. First, Keap1 contains reactive cysteines that form protein-protein cross links following reaction with electrophiles leading to disruption of the Keap1-Nrf2 interaction and release of Nrf2. The high cysteine content of Keap1 suggested that this would be an excellent candidate

acting as a sensor for oxidative stress [150, 153]. The second mechanism involves the activation of protein kinase signaling pathways resulting in phosphorylation of Nrf2 and enhanced release of Nrf2 from Keap1 [151].

To date, activators of the Nrf2-ARE pathway are widely available and have proven to be well tolerated and have the ability to cross the blood brain barrier. Exposure to a number of these activators leads to dissociation of Nrf2 from Keap1, thereby rescuing Nrf2 from proteasomal degradation and allowing it to enter the nucleus. They include both endogenous activators (ROS and lipid aldehydes) and exogenous agents (heavy metals and electrophilic Xenobiotics) [36]. Various structurally-related plant polyphenolic compound activators have been used to activate Nrf2 like curcumin, caffeic acid phenyl ester, a tocopherol and synthetic antioxidants (ethoxyquin, OPZ, phorbol esters) [36].

OPZ, 4-methyl-5-pyrazinyl-3*H*-1,2-dithiole-3-thione, is a chemo-preventive compound that counteracts the insults of ROS in an Nrf2-dependet manner, induces antioxidant protection (either by a mechanism involving an increase in the production of ROS, which may lead to an increase in antioxidant defense [105]), or disturbs the Keap1-Nrf2 complex by modifying cysteine thiol groups and/or via the phosphorylation of Nrf2-keap1 complex by protein kinases residues of keap1, ultimately leading to Nrf2 release [154, 155]. The liberated Nrf2 then accumulates in the nucleus and, in combination with Maf, transactivates the ARE of many cytoprotective genes, as well as that of Nrf2 itself. The gene families regulated by Nrf2 pathway include phase 2 enzymes (NQO-1, HO-1), antioxidants (SOD, CAT, GPx), NADPH generating enzymes and DNA repair enzymes. Overall, these

antioxidant genes commonly play an important role in cellular membrane protection against oxidative stress, DNA repair and protein damages [151]

Genomic analyses indicated that gene families affected by Nrf2 provide direct antioxidant, increase levels of GHS synthesis, enhance NADPH synthesis, induce the recognition, repair and removal of damaged proteins, regulate expression of other transcription factors, growth factors and molecular chaperones, and inhibit cytokines-mediated inflammation [149, 151-153, 156].

In vitro studies have established the importance of the Nrf2-regulated signalling pathway in the protection against oxidative stress. For example, mouse embryonic fibroblasts from Nrf2 deficient mice had increased sensitivity to superoxide anion relative to mouse embryonic fibroblasts cells from Wild Type (WT) mice [148, 150].

Gong and Cederbaum demonstrated that the knockdown of Nrf2 in a variant line of HepG2 cells, a human liver cell line exposed to increased ROS, resulted in decreased cell viability and failed to induce genes responsible for protection against oxidative stress [157]. Additionally, in a recent study, Khor et al. reported that the aggravation of DSS colitis in *Nrf2*$^{-/-}$ mice was associated with decreased expression of endogenous antioxidant such as HO-1, NQO-1 and GST and increased sensitivity to carcinogenesis [158, 159]**.**

1.3.3 Anti-inflammatory role of Nrf2 signalling

Inflammation-mediated oxidative stress represents a potential stimulus for the activation of Nrf-2-regulated cytoprotective response. The evidence is overwhelming that Nrf2 plays a key role in cellular modulation of the inflammatory response. Nrf2 deficient mice have shown to exhibit increased susceptability to DSS-mediated colitis, cancer, allergen-driven airway inflammation mediated asthma , tobacco smoking and elastase mediated chronic obstractive pulmonary disease compared to WT mice [148, 159, 160]. Additionally, an enhanced expression of Nrf2 and decreased expression of pro-inflammatory mediators, such as IL-1β, IL-6 and TNF-α, were detected in the colons originating from WT but not mice exposed to DSS, a model of colitis [150, 158].

It has been proposed that the protective effect of Nrf2 modulates inflammation by inhibiting the NF-κβ pathway as documented in the case of Nrf2–/– mice. As increased ROS levels activate an NF-κβ signalling pathway (leading to pathogenic states like traumatic brain injury and septic shock), Nrf2 displays ability to limit ROS levels leading to the inactivation of redox sensitive pro-inflammatory NF-κβ pathway, thereby maintaining redox homeostasis [160, 161].

Severe mucosal damages as a consequence of increased infiltration of inflammatory cells, up-regulation of pro-inflammatory cytokine signaling and raised oxidative input, was detected in the colons derived from Nrf2-deficiencient mice but not WT mice during DSS-mediated inflammatory components. These results demonstrated that the Nrf2 signaling pathway is a protective factor against inflammation-associated tumorigenesis and illustrates a potential strategy for chemoprevention of inflammation-associated carcinogenesis. Taken

together, these studies show that the Nrf2 signalling pathway can effectively attenuate pro-inflammatory stimuli leading to decreased inflammation and inflammatory damage [158]. Many studies have provided strong evidence that Nrf2 exerts significant anti-oxidant and anti-inflammatory effects in protecting a variety of tissues (lung, liver, intestine) [160, 162-164]. However; the mechanisms by which Nrf2 contributes to protection of intestinal epithelial cells are only partially understood.

2 RESEARCH PROJECT

2.1 Hypothesis

1. Oxidative stress may affect various mitochondrial functions, including ATP production, Ca^{2+} homeostasis, cellular redox state, apoptosis and mtDNA integrity in intestinal epithelial cells.

2. The Nrf2 pathway maintains cellular redox homeostasis in intestinal epithelial cells, thereby improving mitochondrial functions and cell survival.

2.2 Objectives

We will address the mechanisms whereby increasing the ROS generation leads to mitochondrial dysfunction in intestinal epithelial cells. We will also evaluate the role of Nrf2 as an antioxidant and anti-inflammatory agent with the capacity to maintain cellular redox homeostasis in intestinal epithelial cells, which ultimately results in improving mitochondrial functions and cell survival.

More specifically, we will

1. Characterize the interaction between oxidative stress and mitochondrial dysfunction in the Caco-2/15 cell line using the Iron-Ascorbate (FE/ASC) Oxygen radical-generating system, which participates in lipid peroxidation and represents a powerful tool in our hands

for the initiation of highly reactive hydroxyl radicals and for the down-regulation of endogenous antioxidants

2. Evaluate the anti-oxidant and anti-inflammatory functions of Nrf2 in relationship with oxidative stress, inflammation, apoptosis, intestinal permeability and mitochondrial functions in Caco-2/15 cells.

3 ARTICLE 1

The role of oxidative stress and mitochondrial dysfunction in Crohn's disease

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Running title: oxidative stress in Crohn's disease

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ABSTRACT

Inflammatory bowel diseases (IBD), comprising Crohn's disease (CD) and ulcerative colitis (UC), are common disorders characterized by chronic inflammation of the gastrointestinal tract. CD can affect any region of the gastrointestinal tract and is characterized by a dysregulated mucosal immune response, whereas UC appears as a condition in which the inflammatory response and morphological changes remain confined to the colon . No precise etiology has been identified for CD, and complex interactions between genetic, microbial, immune, and environmental risk factors are thought to underlie the pathogenesis. Very limited evidence is available on the mechanisms that generate the chronicity and relapsing/remitting nature of the disease. A key factor relevant to chronic intestinal inflammation is oxidative stress. Reactive oxygen species, produced in large amount by inflammatory cells, are a major tissue-destructive force in most organ systems, including the gastro-intestinal tract. We have hypothesized that mitochondria are central to the regulation of oxidative stress and play a paramount role in the pathogenesis of IBD. In this review, we highlight the importance of mitochondria and discuss the potential mechanisms linking mitochondrial function to chronic inflammation in CD.

Key words:

Mitochondria, oxidative stress, Crohn's disease , inflammation

Abbreviations:

ATP;	Adenosine-5'-Triphosphate
Ca^{2+};	Calcium
CD;	Crohn Disease
DNA;	Deoxyribonucleic Acid
IBD;	Inflammatory Bowel Disease
IL;	Interleukin
MDA;	malondialdehyde
Mt;	Mitochondrial
NF-κB;	Nuclear factor-kappa B
8-OHdG;	8-hydroxy-deoxy-guanosine
OGG1;	8-oxo DNA glycosylase
ROS;	Reactive Oxygen Species
SOD;	Superoxide dismutase
TNF-α;	Tumor Necrosis Factor- Alpha
UC;	Ulcerative Colitis
PTP	Permeability of transition pores

INTRODUCTION

Crohn's Disease (CD) is a chronic relapsing intestinal inflammation in genetically susceptible individuals and is influenced by environmental factors [1]. In north America, the highest incidences of CD occur in Canada and the Northern United States compared to the South area[2]. The gap in incidence of CD in ethnic or racial groups compared with white has been closing with an increased incidence in African Americans and in second generation South Asians who have migrated to developed countries [3]. The annual incidence of CD in northern climates is 6-15/100.000, with a prevalence of 50-200/100.000 persons. The peak age for CD occur in 20-30 years and is 20%-30% more frequently in women than in men in high incidence areas [2, 4].

Although not associated with increased mortality, CD can cause significant morbidity. There has been a considerable increase in hospitalizations for CD, with stable rates of bowel resection surgery [5]. Recent findings from the United States reveal that the overall hospitalization rate for CD is 18.0 per 100.000 and contributes to disability in ~119.000 patients per year [6]. Another important feature is that over the long-term, up to 75% of CD patients will require surgery [7]. The significant disease-associated morbidity exerts enormous economic burden and makes CD a public health issue [8, 9].

In spite of the tremendous progress in understanding the underlying mechanisms contributing to the chronic intestinal inflammation characteristic of CD, the key processes remain elusive although the implication of oxidative stress has long been promulgated. Oxidative stress in chronic intestinal inflammation is characterized by an imbalance between reactive oxygen

species (ROS) production and anti-oxidant defense [10]. Surprisingly, even if oxidative stress occurs in the intestinal mucosa of CD patients, limited studies have explored the response of mitochondria to oxidative stress in relation with key cellular regulatory processes, including adenosine-5'-triphosphate (ATP) production, intracellular calcium regulation, cell signaling, ROS generation and detoxification, and apoptosis. In this review we highlight the potential role of mitochondria in CD-related oxidative stress and propose several mechanisms linking mitochondrial dysfunction to oxidative stress and the chronic intestinal inflammation.

Pathogenesis of CD

CD is a heterogenus disorder in association with many factors such as environment, microbial flora , dysfunctional immune response, and genetic variables[11]. The role of environmental risk factors in CD is based upon epidemiological, clinical and experimental studies [12-14]. Tobacco smoking, particularly cigarette, is the most consistently environmental risk factor associated with an increased risk for CD and with a poorer prognosis, as defined by higher relapse rates, a more aggressive disease course and increased risk of postoperative recurrence [15]. A meta-analysis suggests that smokers are more than twice likely to develop CD compared to non-smokers [16]. Others suggested factors such as western life-style, social, occupation status, diet, psychological stress, hygiene, drugs, appendectomy and exposure to infections play a role in expression of CD [11, 17-20]. Additionally, changed dietary practices and increased hygiene have been implicated in the immunoregulatory defects in the intestinal mucosa, which appear to be associated with microbial exposure in the pathogenesis of CD[12, 18] . Increased hygiene in developed countries is postulated to have led to the rising incidence of CD and other autoimmune disorders [21]. In particular, evidence linking bacterial flora and intestinal inflammation includes the observation

of relatively high concentrations of bacteria in the normal distal ileum and colon, which is thought partly to explain the predominance of CD in these segments of the gastrointestinal tract [22, 23]. Noteworthy, studies in patients or animal models with CD have demonstrated various changes in the luminal flora with a link to local inflammation and immune function . At present, most efforts are being devoted to better understand which enteric bacteria may have a pathogenic potential, and why their recognition by the mucosal immune system leads to inflammation in CD.

Genetic data have provided strong evidence for the existence of genetic determinants of susceptibility to CD, and it is now accepted that genes contribute to risks, may lead to a clearer understanding of pathogenesis, and ultimately better treatment. Several genetic loci on various chromosomes have been reported to be related to CD. Genome-wide associations (GWAS) studies have show strong association between CD and more than 30 additional genes and variants, such as autophagy-related 16-like 1 gene (*ATG16L1*), immunity-related GTPase family(*IRGM*), interleukin 23 receptor (*IL23R)*, all of which highlight specific pathways of disease pathogenesis involving intestinal flora and immune response (**TABLE 1**). More recently, our group has confirmed the reported GWAS between the *IL23R* gene and CD, where we suggested that *IL23R* is also associated with pediatric-onset CD among Canadian children [24]. Future genetic research should focus on phenotypes, control for environmental variables, geographical localisation and gene-gene interactions.

Inflammation in CD

As mentioned before, inflammation associated with CD is transmural, while inflammatory changes in UC involve only the intestinal mucosal and submucosal layers. In the other hand, inflammation in CD, is discontinuous, segmental with the presence of granulomas in all the gastrointestinal tract [25] . In fact, intestinal chronic inflammation is characterized by infiltration of neutrophils and macrophages into the intestinal mucosa, as well as the disruption of intestinal epithelial cells with augmentation of intestinal epithelial permeability [26]. These activated inflammatory cells generate reactive oxygen species (ROS), which subsequently produce oxidative injury in intestinal mucosa, along with a concomitant imbalance in redox status. The production of ROS is an important factor in the development and perpetuation of inflammation in CD[27]. Importantly, redox imbalance leads to generation of numerous proinflammatory mediators, including interleukin (IL)-1β, tumour necrosis factor alpha (TNF-α), IL-8 and IL-6 [28]. These mediators can, at their turn, generate ROS and reactive nitrogen species, leading to vicious circle of excessive oxidative stress production. There is evidence that ROS and proinflammatory cytokines work synergistically as activators of transcription factors, such as, nuclear factor-kappaB (NF−κβ), activator protein-1, thereby modulating their activity either directly or indirectly by activating other signalling cascades[29, 30]. However, activation of NF-κβ has been specifically implicated in maintaining inflammation by activation of a variety of inflammatory genes. NF−κβ acts as a survival signal for intestinal epithelial cells [26, 31]. Impaired NF-κβ signaling in the gut epithelium initiates the primary events of intestinal inflammation. Recent studies showed that alterations of IkappaB kinase (IkB)/NF−κβ signaling, specifically in intestinal epithelial cells *in vivo,* cause intestinal inflammation in mice [31]. The importance of this transcription factor has been strengthened by the evidence that NOD2, an intracellular sensor of bacteria, is a positive regulator of NF-κβ/IL-1β-mediated innate response

to bacteria challenge [22, 32]. Interestingly, Cogswell *et al*. have found that NF−κβ and IkB-α are localized in the mitochondria and can negatively regulate mitochondrial gene expression in response to cellular TNF-α stimulation [33]. This is particularly important since plays a significant role in CD, as a powerful mediator of intestinal inflammation. Increased levels of TNF-α have been found in serum, plasma, mucosa, and stool of patients with CD [34, 35]. Furthermore, several clincal trials have reported that anti-TNF-α antibody is effective in the treatment of CD, verifying the importance of TNF-α in the ongoing intestinal inflammation [36, 37] .

Oxidative stress in CD

Under physiological conditions, low ROS levels constitute inter- and intra-cellular signals, which are vital to maintain proper cellular function. However, ROS levels are increased in pathophysiological conditions, and their high concentrations can oxidize nucleic acids (nuclear and mitochondrial DNA), lipid peroxidation and protein damage[38, 39]. Thus, depending on their cellular concentrations and duration, ROS can act as either beneficial or harmful biological agents. Free radicals interact with iron and can potentially enhance intestinal inflammation through the production of hydroxyl radicals via its catalytic activity in the fenton reaction[40].

There is growing evidence that oxidative stress leads to many biochemical changes that contribute to numerous chronic disorders, including CD. The intestinal mucosa is vulnerable to oxidative stress from exposure to ROS generated by the lumen contents such as food particles, metals such as iron and copper and bacterial products. Affected CD patients show a misbalance

between production of free radicals and antioxidant defenses[41, 42]. Some studies showed that immune cells of CD patients present a significant ROS increase in both activity and remission phases of the disease, in combination with decreased antioxidant enzymes[42, 43]. Our group has documented that these disturbances are often associated with malnutrition secondary to reduced dietary intake and malabsorption, potentially impairing essential polyunsaturated fatty acid and antioxidant status, as well as the composition of lipoprotein particles[44]. In addition to intestinal inflammation, the generation of ROS may lead to disruption of mucosal barrier function largely characterized by increased permeability[45, 46] .

Lipid peroxidation may be estimated by the determination of malondialdehyde (MDA) concentration. The increased MDA levels found in plasma [44] and colonic biopsies [10] from CD patients provide evidence for excess lipid peroxidation reactions. Furthermore the increased breath ethane and pentane excretion in CD patients, which are non-invasive markers of lipid peroxidation, have been correlated with disease activity [47]. Using biopsies from CD patients, investigators could document an enhanced inflammation and oxidative stress markers along with a decline in antioxidant status in active CD. As the patients improved and became clinically stable, the oxidative parameters decreased, approaching normal values [42]. Other studies support these findings by demonstrating that high ROS production directly caused intestinal epithelial cell injury.

Host defenses include antioxidants enzymes, such as catalase, glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-s-transferase (GsT) and superoxide dismutase (SOD), along with dietary antioxidants (e.g. vitamines C, E, A)[48, 49]. In general,

antioxidants prevent free radical-induced tissue damage by inhibiting the formation of radicals or promoting their decomposition. It has been suggested that the gut may be particularly sensitive to oxidative stress because of the relatively low concentration of endogenous antioxidant enzymes and the presence of high concentrations of xanthine oxidase. It has previously been shown that the levels and the balance between the most important antioxidants are seriously impaired within the intestinal mucosa from CD patients [39, 50, 51]. In active disease, some studies found an increase in SOD and GPx activities, whereas catalase remains constantly inhibited , suggesting that this enzyme is not redox-sensitive but rather a sensor of cellular proceseses[42] .

Oxidative stress and mitochondrial dysfunction in CD

The mechanisms responsible for the increase in oxidative stress in CD patients are not completely understood. Scientific indications stress the importance of investigating the association between oxidative stress and mitochondrion involvement. Mitochondria are multifunctional organelles involved in numerous metabolic activities, including production of ATP, oxidative phosphorylation, calcium (Ca^{2+}) homeostasis and in the regulation of apoptosis pathways [52]. They have emerged as organelles that receive, integrate and transmit signals [53]. Mitochondria constitute a major source of free radicals in cells, resulting in oxidative stress, but are also targets to oxidative stress action [54, 55] . Although the molecular mechanisms responsible for mitochondrion-mediated disease processes are not fully elucidated yet, oxidative stress appears to be a pivotal player. Hence, it is apparent that mitochondrial damage may lead to the impairment of various aspects of tissue functioning.

ROS produced by the mitochondria can modify the mitochondrial (mt)DNA [56]. As a consequence, DNA damage results in mutations which, if not repaired, may lead to an increased risk of cancer [57]. Oxidative stress has been one of the risk factors that induce gut malignancy. The accumulation of mtDNA mutations cause the loss of mitochondrion ability to produce sufficient energy to meet cellular needs and serves as a trigger for mitochondrial dysfunction and apoptosis [58]. Mutated mtDNA may be associated with increased superoxide radical production, thereby leading to a vicious cycle that progressively stimulates the rise of oxidative stress leads to impaired mitochondrial function[59, 60, 61]. Mitochondrial DNA is vulnerable to oxidative damage because it is situated much closer to the site of ROS generation, given that mitochondria lack protective histones and have much more reduced base excision repair mechanisms [62].

Oxidative DNA damage was noted in plasma from CD patient and was measured by production of 8-hydroxy-deoxy-guanosine (8-OHdG). Plasma levels of (8-OHdG) was found to be increased in CD patients compared to the control [50, 51]. It has also been shown that 8-OHdG level was significantly higher in active CD, inactive CD and CD patients in remission compared to control [43]. This molecule (8-OHdG), which is more specific for mtDNA, is of practical importance because it is easily measurable and has therefore been proposed as a useful marker of oxidative stress [63].

Human cells have developed different repair enzymes, the most important being 8-oxo DNA glycosylase (OGG1) that provides protection against the effects of oxidized DNA bases and preferentially removes 8-OHdG opposite cytosine [64]. In mitochondria, OGG1 is thought to

prevent activation of the intrinsic apoptotic pathway in response to oxidative stress by augmenting DNA repair mechanism [56]. Recent data provides accumulating evidence on the association between intestinal inflammation, oxidative stress, DNA repair enzyme OGG1 and carcinogenesis. $OGG1^{-/-}$ mice developed a significantly higher number of adenocarcinomas in the dextran sulfate sodium-induced (DSS) colitis model compared to wild type mice [64, 65]. However, the OGG1 level has not been yet investigated in CD patients. Thus increase in mtDNA damage in CD, might modify mitochondrial function and render the intestinal cell to more environmental influences. This is an area worthy of further exploration.

One of the functions of mitochondria is to store free Ca^{2+}, a key regulator of mitochondrial function, that acts at several levels within the cell to stimulate ATP synthesis [66]. Mitochondrial may have an impact on Ca^{2+} signals, removing Ca^{2+} locally and modulating Ca^{2+} concentrations [67]. Oxidative stress caused a rapid increase in cytosolic Ca^{2+}, which was followed by mitochondrial Ca^{2+} loading [68]. Mitochondrial matrix Ca^{2+} overload, at their turn, can lead to induced production of ROS, triggering the permeability of transition pores (PTP) and cytochrome *c* release, and leading to apoptosis [69, 70]. It has been suggested that PTP opening (triggered by Ca^{2+}) induces a specific conformational change of complex I, which results in an increase of H_2O_2 production when electrons are provided to complex I, and it may also inhibit the electron pathway inside complex I [70]. ROS-mediated mitochondrial damage and dysregulation of Ca^{2+} homeostasis may represent several important mechanisms responsible for amplifying the vicious cycle leading to progressively increasing intracellular ROS concentrations and oxidative stress [71, 72]. Excess Ca^{2+} ions stored in mitochondria can

inhibit oxidative phosphorylation, which may provoke an irreversible reduction in the energy status, thereby initiating pathophysiological processes in certain cells [73].

The exact mechanism of mitochondrial Ca^{2+} induced ROS generation is unclear, although it may involve changes in the three-dimensional conformation of the respiratory complexes [72]. More recent studies suggest that disruptions in intracellular Ca(2+) mobilization lead to increased CaM kinase II activity that may contribute to the dysmotility of colonic smooth muscles in murine DSS-colitis by enhancing NF-kappaB activity [74]. In addition, Di Sabatino A et al, have provided evidence that suppression of Ca^{2+} release may inhibit pro-inflammatory cytokine production and dampen the increased T cell response in inflamed gut. Thus Ca^{2+} channel inhibitor drugs might have a major role in the therapeutic management in CD patients [75]. We believe that increase in mitochondrial Ca^{2+} level in combination with excessive ROS generation in gut epithelium is pivotal pathway in CD pathogenesis. Thus, calcium homoeostasis might be crucial for the maintenance of the mitochondrial function, which is worthy of further investigation and might provide opportunities to develop novel therapeutic interventions to protect cell survival.

In vitro exposure to ROS, or depletion of cellular antioxidants, has been observed to result in apoptosis [76]. Mitochondrial apoptosis-mediated pathways rely on the permeabilization of mitochondrial membranes, due to oxidative stress, leading to collapse of the mitochondrial membrane potential, along with release of apoptogenic mitochondrial proteins (cytochrome C and apoptotic inducing factors) into the cytoplasm, which triggers a cascade of events, leading to activation of caspase 9 and downstream cleavage of caspase 3, 6 or 7 [56, 77, 78]. More

recent data by Catrazi et al suggest neutrophils apoptosis in CD patients and may offer a new target for specific drugs and therapy tools. Elucidation of proapoptotic and antiapoptotic mechanisms in intestinal tissue could delineate potential targets for intervention.[79]

Limited non conclusive results have linked to mitochondrial damage to CD pathogenesis. Several studies suggest that stressors worsen the course of CD. Farhadi *et al*. have demonstrated that mitochondrial damages were noted in intestinal epithelial cells and mucosal protein oxidation in stressed CD patients [80]. Soderholm *et al*. observed the presence of numerous swollen and irregular mitochondria in the epithelium of colonic segments from stressed rats [81]. In addition, O'Morain *et al*. showed mitochondrial damage by examination of rectal biopsies obtained from CD patients [82]. Finally, a recent case report on a young girl with CD disclosed impaired oxidative phosphorylation along with abnormalities in Complexes III and IV [83]. The importance of these observations is that they highlight the potential pathogenetic pathways that might be initiated by genetic or environmental causes of CD, which is worthy of further investigation and might provide opportunities to develop novel therapeutic interventions that improve mitochondrial function and might slow the progression of CD processe.

Recent advances in molecular genetics suggest a role for mitochondrial dysfunction in the development of CD. Previous study, by Theiss *et al*. have demonstrated that prohibitin (PHB), a chaperone protein involved in the stabilization of mitochondrial respiratory enzymes, localizes in the inner mitochondrial membrane and protects against oxidative stress in intestinal epithelial cells. It has been also found that PHB expression is down-regulated in CD patients, in experimental colitis *in vivo* and during oxidative stress *in vitro* [84]. More

recently, Theiss *et al*. have shown that TNF-α decreases PHB expression in intestinal epithelial cells and restoration of PHB expression in these cells can protect against the various effects of TNF-α and NF−κβ on intestinal barrier function [85]. Since overexpression of PHB can protect against oxidative stress and inflammation. Increase in PHB levels in CD patients may represent a potential therapeutic strategy to prevent mitochondrial dysfunction.

Mitochondrial dysfunction in CD is likely to be highly complex processe. It is becoming increasingly important to address the mechanisms by which the interplay between oxidative stress and intestinal inflammation leading to mitochondrial dysfunction, may represent a major role in CD pathogenesis. We propose that chronic intestinal inflammation in genetically susceptible hosts exposed to environmental factors triggers ROS overproduction and disruption of mitochondrial structure and function through mtDNA alterations, Ca^{2+} overload, inhibition of mitochondrial electron transport chain enzymes activity and apoptosis(**Fig 1**). Once we understand the dynamic interplay between these crucial signaling pathways, we will be poised to develop therapies for the mitochondrial dysfunction in this disease.

Therapeutic Intervention: Antioxidants targeted to mitochondria

One of the reasonable answer for the failure of antioxidants to show clear therapeutic effects is their incapacity to reach mitochondria. Currently, there is growing efforts in developing mechanisms for the targeted delivery of antioxidants to mitochondria [86]. More recently, confocal imaging studies in Caco-2 cells show that intracellular distribution of SS-19 tetrapeptidesas antioxidant resembles that of MitoTracker, which localizes in mitochondria [87].

The involvement of mitochondrial dysfunction in a range of clinical diseases and their roles in cellular energy metabolism, apoptosis, Ca^{2+} homeostasis and cell signaling has made the mitochondrion a target for drug delivery [88]. Hence, there is considerable interest in developing strategies to target small and large molecules with therapeutic potential to mitochondria. Several pharmacological agents are currently under investigation, including targeting antioxidants to mitochondria, uncoupling proteins and mitochondrial permeability transition pore inhibitors[89].

There are four various targeting strategies available for the selective delivery of low molecular weight drugs to mitochondria , including (i) targeting based on biophysical properties of mitochondria. (ii) targeting based on binding to mitochondrial membrane components (iii) targeting based on the specific mitochondrial localisation of enzymes that catalyse the release of drugs from prodrugs (iv) and targeting based on transporter-dependent delivery of prodrugs or drugs[86, 88].

The potential therapeutic applications of mitochondrial targeting include: (i) the delivery of antioxidants to mitochondria (ii) the targeting Bcl-2 proteins to mitochondria to trigger apoptosis; (iii) the delivery of drugs to mitochondria to inhibit the mitochondrial permeability transition and (iv) the targeting of drugs to either uncouple the electron transport chain , or activate the uncoupling proteins. More pre-clinical and clinical studies are necessary in order to evaluate the feasibility, effectiveness and toxicity of these molecules.

Developments of non-invasive biomarkers of mitochondrial dysfunction are in need for future clinical studies. Such biomarkers would assess the effectiveness of therapeutic interventions and the progression of the diseases.We hope that work over the next few years will indicate whether these compounds are effective, can decrease mitochondrial oxidative damage in diseases, and improve outcomes for the patients with mitochondrial impariment diseases. If our proposal is proved in large clinical studies, this will clear the way to test those molecules that improve mitochondrial function and might slow the progression of CD.

Perspective and Future Directions

Basic research in mitochondrial diseases related to oxidative stress has made a tremendous progress within the past few years and will undoubtedly provide exciting new insights in the near future. There are clearly more questions than answers in this field related to CD. In this respect, several issues appear to deserve further investigation: It is important to determine whether the mitochondrial dysfunction is one of the early causes rather than a terminal event and whether mitochondrial defects result from inflammatory cytokines that inhibit mitochondrial respiration; (i) can we find mutations in mt DNA and nuclear DNA?; (iii) what is the role of mitochondrial Ca^{2+} overload in the development of CD and what are the pro-apoptotic proteins released by mitochondria, which initiate caspase activation and apoptosis and whether the degree of apoptosis could provide additional prognostic information in CD patients?; (iv) what is the molecular signal pathway capable of orchestrating the cross-talk among mitochondria, cytosol and nucleus?; (v) what are the specific transcription factors implicated?; (vi) does intestinal microbial infection lead to mitochondrial damage?; and (vii)

can we protect mitochondria damage by pharmacological inhibition, targeting antioxidants to mitochondria or nutritional therapy?

Taken together, several exciting mechanisms need to be tested to determine whether the modulation of mitochondrial dysfunction will translate into new treatment approaches for CD. Recognition of the inducing mechanisms could open up ways to improve mitochondrial dysfunction in intestinal tissues and possibly help to define targets for future drug design.

CONCLUSIONS

Few experimental data have been gathered in the past years to support our concept that mitochondrial dysfunctions are more important contributors to CD development than is currently recognized. The detailed mechanisms by which these biochemical events cause CD remain to be established. The molecular genetic approaches to study CD will provide a deeper understanding of the CD-related alterations in the structure and function of mitochondria.

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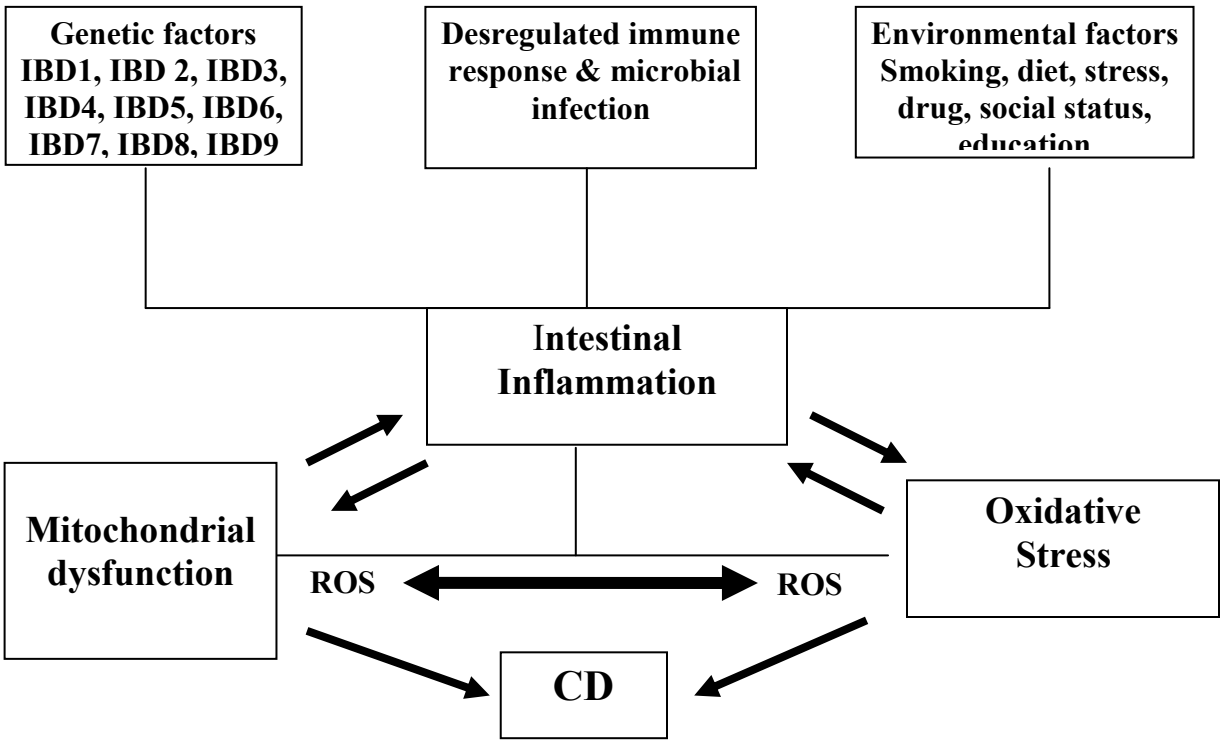
Table 1: Various genes involves in CD and UC pathogenesis

Locus	Chromosomal region	CD	UC	References
IBD1	16	++	-	[90]
IBD2	12	+	+	[91]
IBD3	6	++	++	[92]
IBD4	14	++	-	[91]
IBD5	5	++	-	[93]
IBD6	19	++	++	[94]
IBD7	1	++	++	[95]
IBD8	16	+	NA	[9696]
IBD9	3	++	NA	[97]

CD: Crohn's Disease; UC: Ulcerative Colitis; IBD: Inflammatory Bowel Disease; NA: not available

Figure 1: Proposed model of how inflammation associated with oxidative stress in genetic susiptable promotes the development of mitochondrial dysfunction in CD



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4 ARTICLE 2

Oxidative stress and mitochondrial functions in the intestinal Caco-2/15 cell line

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ABSTRACT

BACKGROUND: Although mitochondrial dysfunction and oxidative stress are central mechanisms in various pathological conditions, they have not been extensively studied in the gastrointestinal tract, which is known to be constantly exposed to luminal oxidants from ingested foods. Key among these is the simultaneous consumption of iron salts and ascorbic acid, which can cause oxidative damage to biomolecules. METHODOLOGY/PRINCIPAL FINDINGS: The objective of the present work was to evaluate how iron-ascorbate (FE/ASC)-mediated lipid peroxidation affects mitochondrion functioning in Caco-2/15 cells. Our results show that treatment of Caco-2/15 cells with FE/ASC (0.2 mM/2 mM) **(1)** increased malondialdehyde levels assessed by HPLC; **(2)** reduced ATP production noted by luminescence assay; **(3)** provoked dysregulation of mitochondrial calcium homeostasis as evidenced by confocal fluorescence microscopy; **(4)** upregulated the protein expression of cytochrome C and apoptotic inducing factor, indicating exaggerated apoptosis; (5) affected mitochondrial respiratory chain complexes I, II, III and IV; (6) elicited mtDNA lesions as illustrated by the raised levels of 8-OHdG; (**7**) lowered DNA glycosylase, one of the first lines of defense against 8-OHdG mutagenicity; and (8) altered the gene expression and protein mass of mitochondrial transcription factors (mtTFA, mtTFB1, mtTFB2) without any effects on RNA Polymerase. The presence of the powerful antioxidant BHT (50 μM) prevented the occurrence of oxidative stress and most of the mitochondrial abnormalities. CONCLUSIONS/SIGNIFICANCE: Collectively, our findings indicate that acute exposure of Caco-2/15 cells to FE/ASC- catalyzed peroxidation produces harmful effects on mitochondrial functions and DNA integrity, which are abrogated by the powerful

exogenous BHT antioxidant. Functional derangements of mitochondria may have implications in oxidative stress-related disorders such as inflammatory bowel diseases.

Keywords

Oxidative stress, Mitochondria, Transcription factors, Inflammation, Calcium

INTRODUCTION

Reactive Oxygen Species (ROS) are by-products of normal aerobic metabolism and are now considered to be important signaling molecules that play a role in gene expression, cell growth and survival as well as oxygen sensing in various cell types [1,2]. The generation of ROS by a cascade of reactions is efficiently blocked by various endogenous antioxidants to overcome their potentially injurious actions [2,3]. However, excessive formation of ROS leads to lasting oxidative stress, characterized by an imbalance between oxidant-producing systems and antioxidant defense mechanisms, which can trigger cell damage by oxidizing macromolecular structures (lipids, proteins and DNA) and modifying their biological functions that ultimately causes cell death [4]. Thus, depending on their cell concentrations, ROS can act as either beneficial or harmful biological agents.

The gastrointestinal tract is frequently exposed to noxious stimuli that may cause oxidative stress and injury. In fact, oxygen free radicals are generated both in the lumen and in the intestinal mucosa. Intraluminal pro-oxidants from ingested nutrients, such as alcohol, cholesterol oxides or iron salts and ascorbic acid, frequently consumed together in multiple-vitamin preparations or ingested foods, can build a pro-oxidant milieu [5–7]. Moreover, local microbes or infections, ischemia/reperfusion, gastric acid production and non-steroidal anti-inflammatory drugs may promote the formation of reactive radicals [8–10]. In addition, the influx of leukocytes, neutrophils and monocytes (associated with inflammation) can produce further ROS via respiratory burst enzymes as well as those involved in prostaglandin and leukotriene metabolism [11]. Clearly, significant oxidative stress has been said to be always associated with mucosal erosions and a causative role in

a variety of gastrointestinal diseases such as Crohn's disease and ulcerative colitis [12–14].

Despite the frequent occurrence of oxidative stress in the gastrointestinal tract and its involvement in the initiation and propagation of the chronic inflammatory response in chronic bowel diseases [15], little is known about mitochondrion response even though this special organelle is both a major source of oxidants and a target for their damaging effects [16]. We have hypothesized that oxidative stress may affect various mitochondrial functions, including ATP production, calcium (Ca^{2+}) homeostasis, cellular redox state regulation, apoptosis, as well as mtDNA integrity [17,18]. Therefore, the specific aim of the present study was to characterize the interplay between oxidative stress and mitochondrial dysfunction in the Caco-2/15 cell line using the iron-ascorbate (FE/ASC) oxygen radical-generating system, which participates in lipid peroxidation in inflammatory bowel diseases (IBD) and represents a powerful tool in our hands for the initiation of highly reactive hydroxyl radicals and for the down-regulation of endogenous antioxidants [19–26].

MATERIALS AND METHODS

Caco-2/15 Cell Cultures

The colon carcinoma cell line, Caco-2/15 (ATCC, Rockville, MD), was cultured at subconfluent stages in MEM (GIBCO-BRL, Grand Island, NY) containing 1% penicillin-streptomycin and 1% MEM non-essential amino acids (GIBCO-BRL) and supplemented with 10% decomplemented fetal bovine serum (FBS) (Flow, McLean, VA) as described previously [27]. Briefly, Caco-2/15 cells (passage 20-30) were maintained in T-75-cm^2 flasks (Corning Glass Works, Corning, NY). Cultures cells were split (1:6) when they reached 90% confluence by use of 0.05% trypsin-0.5 mM EDTA (GIBCO-BRL). For individual experiments, cells were plated at a density of $1x10^6$ cells/well on 24.5 mm polyester Transwell filter inserts with 0.4-μm pores (Coster, Cambridge, MA) in MEM supplemented with 5% FBS. Cells were cultured for 21 days post confluence, at which the Caco-2/15 cells are highly differentiated and appropriate for lipid synthesis and metabolism. The medium was refreshed every second day. To determine the implication of oxidative stress *per se* in alterations in mitochondrial functions, Caco-2/15 cells were incubated with FE/ASC (0.2 mM/2 mM) for 6 h alone and/or with the antioxidant butylated hydoxytoluene (BHT) (2,6-di-*t*-butyl-*p*-cresol, Sigma, St-Louis, MA) (50 μM). Caco-2/15 cells were divided into four groups: control (without any addition), oxidative (FE/ASC), antioxidant (BHT), oxidative and antioxidant (FE/ASC + BHT).

Lipid Peroxidation

Caco-2/15 cells were cultured in the presence or absence of (0.2 mM/2 mM) FE/ASC added to the medium. Incubation periods were terminated with 50 μM BHT to measure malondialdehyde (MDA). The level of MDA formed during the oxidative reaction was

determined by HPLC, as previously described [19]. Briefly, proteins were first precipitated with a 10% sodium tungstate (Na_2WO_4) (Aldrich, Milwaukee, WI) solution. The protein-free supernatants were then reacted with an equivalent volume of 0.5% (wt/vol) thiobarbituric acid solution (TBA; Sigma) at 90ºC for 60 min. After cooling to room temperature, the pink chromogene [(TBA) 2-MDA] was extracted with 1-butanol and dried over a stream of nitrogen at 37ºC. The dry extract was then resuspended in a potassium dehydrogen phosphate (KH_2PO_4)/methanol mobile phase (70; 30, pH 7.0) before MDA determination by HPLC with fluorescence detection.

Assessment of Intracellular ATP

Intracellular ATP was measured by luciferase driven bioluminescence using ATP Bioluminescence Assay Kit from (Calbiochem, EMD Chemicals, Inc. Gibbstown, NJ) as reported previously [28]. Values were then normalized further with regard to the protein content of the respective sample. All Caco-2/15 culture cells were performed in duplicate.

Calcium Measurements by Confocal

For mitochondrial Ca^{2+} monitoring, Caco-2/15 cells were trypsinized, transferred from cell culture flasks to 8-well chamber slides (Lab-Tek™ Nunc, Rochester, NY) at a density of $2{,}5x10^4$ cells in 500 µl of cell culture medium. After a period of three days, cells were serum-starved and incubated with FE/ASC and/or BHT as described above. Cells were rinsed twice in serum-free culture medium and loaded with a mixture of 5 µM Rhod-2/AM (Molecular Probes, Eugene, OR), a fluorescent probe specific for mitochondrial Ca^{2+}, with 0,01% pluronic acid for 30 min at 37ºC as described previously [29–31]. Medium was removed, replaced with dye-free culture medium and incubated for an additional 60 minutes at 37ºC. Thereafter, 1 µl of the fluorescent mitochondria-specific dye MitoTracker™ (green

fluorescence, Molecular Probes) was added to each well at the last 30 min of incubation. Cells were visualized using an inverted laser-scanning confocal microscope equipped with a 40X objective (LSM 510, Zeiss). Excitation wavelength was 488 nm and fluorescence emission was recorded at 543 nm (for Rhod-2) and 516 nm (for MitoTracker™). Six to eight fluorescence images were randomly chosen in selected microscopic fields. Fluorescence intensity was quantified using the Image J software (http://rsb.info.nih.gov/ij).

Mitochondrial Preparations

Mitochondria were isolated using standard differential centrifugation techniques [32]. Briefly, Caco-2/15 cells were treated with (0.2 mM/2 mM) FE/ASC and/or (50 μM) BHT for 6 h at 37°C. Cells were homogenized with a glass pestle Dounce homogenizer in a buffer containing 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 0.5% fatty acid-free bovine serum albumin, and 5 mM HEPES, pH 7.2. The homogenate was centrifuged at 1000 x *g* for 10 min at 4°C. The supernatant was then collected and centrifuged at 10000 x g for 10 min to obtain the pellets containing mitochondria. The pellets were used immediately or stored at –80°C. The protein contents of mitochondrial suspension were determined by the Bradford assay (BioRad, Mississauga, ON) with BSA as a standard.

Evaluation of 8-hydroxy -2-deoxyguanosine

Oxidative DNA damage in whole Caco-2/15 cells, nuclei and mitochondria was evaluated by assessing 8-hydroxy -2-deoxyguanosine (8-OHdG) with high-sensitivity competitive ELISA assays performed with a commercial kit from Genox Corporation (Baltimore, USA). Briefly, 8-OHdG antibody plus sample DNA were added to a 96-well plate percolated with 8-OHdG and incubated overnight at 4°C. After the plate was washed, horseradish peroxidase–conjugated secondary antibody was added for 1 h at

room temperature. After washing, 3,3',5,5'-tetramethylbenzidine was added and incubated for 15 min at room temperature in the dark. The reaction was terminated by the addition of phosphoric acid, and absorbance was measured at 450 nm. All assays were performed in duplicate. Negative controls and 8-OHdG standards (0.125–10 ng/mL) were included in the assay. The average concentration of 8-OHdG was calculated for each sample based on the standard curve.

Mitochondrial Enzyme Assays

The activities of respiratory chain complexes were assayed as previously described in detail [32–34]. Briefly, 20-30 μg of mitochondrial protein were used for each complex every 30 sec for 5 min. The activity of complex I (NADH: ubiquinone oxidoreductase) was measured by monitoring the reduction of decylubiquinone. Complex II (succinate:ubiquinone oxidoreductase) activity was examined by monitoring the reduction of dichloroindophenol when coupled to complex II-catalyzed reduction of decylubiquinone. Complex III (ubiquinol:ferricytochrome C oxidoreductase) activity was assayed using oxidized cytochrome C. The activity of complex IV (cytochrome C oxidase) was determined by oxidation of reduced cytochrome C. Enzyme activities were expressed in nanomoles of substrate used per minute per milligram of protein. Enzyme assays for all complexes were performed in duplicate in mitochondrial fraction of Caco-2/15 cell line. Complex I, II, III, IV chemicals were purchased from Sigma Chemical, St Louis, MO.

Western Blots

To assess the protein mass of mitochondrial transcription factors (mt TF): mtTFA, mtTFB1, mtTFB2 and POLRMT, as well as 8-oxoG-DNA glycosylase (OGG1),

apoptosis-inducing factor (AIF) and cytochrome C, Caco-2/15 cells were homogenized and adequately prepared for Western blotting as described previously [22,23,27,35–39]. The Bradford assay (Bio-Rad) was used to estimate protein concentration. Proteins were denatured in sample buffer containing SDS and β-mercaptoethanol, separated on a 4–20% gradient SDS-PAGE and electroblotted onto nitrocellulose membranes. Nonspecific binding sites of the membranes were blocked with defatted milk proteins followed by the addition of primary antibodies directed against the different proteins. The relative amount of primary antibody was detected with species-specific horseradish peroxidase-conjugated secondary antibody. Even though identical protein amounts of tissue homogenates were applied, the β-actin protein was used to confirm equal loading on SDS-PAGE (results not shown). Blots were developed and the mass of proteins was quantitated using an HP Scanjet scanner equipped with a transparency adapter and software. Rabbit polyclonal mtTFA Ab was obtained from Santa Cruz Biotechnology Santa Cruz, CA; rabbit polyclonal POLRMT from Abcam, Cambridge, MA; and mouse polyclonal mtTFB1 and mtTFB2 Ab, rabbit polyclonal OGG1 Ab, rabbit polyclonal AIF Ab, and mouse monoclonal cytochrome C Ab from Novus Biologicals, Inc.

RT-PCR

Experiments for mRNA quantification as well as for GAPDH (as a housekeeping gene) were performed in Caco2/15 cells using the UNO II thermocycler (Biometra) as reported previously [35,40]. Approximately 30-40 cycles of amplification were used at 95ºC for 30 s, 58ºC for 30 s, and 72ºC for 30 s. Amplicons were visualized on standard ethidium bromide-stained agarose gels. Under these experimental conditions related to RT-PCR, the cycles for mtTFA, mtTFB1, mtTFB2, POLRMT, OGG1 and GAPDH were 31, 31,35,31,31, and 30,

respectively corresponding to the linear portion of the exponential phase. Fold induction and quantification were determined with the software UN-SCAN-IT gel 6.1**.**

Primers Used

GAPDH (**F-** AGAAGGCTGGGGCTCATT/**R**-GGGCCATCCACAGTCTTCT)

H-OGG1 (F-GGGGATTCACAAGGTGAAGA/**R**-GTAAGCTGGCTTGCATCACA)

POLRMT (F-CATCACCTACACCCACAACG/**R**-GTGCACAGAGACGAAGGTCA)

H-mtTFB2 (F-GTCGCTTTTGCATTTTAGGG/**R**-GCTGTCCAAGGAACTGCTTC)

h-mtTFB1 (**F**-CTCCTGGACTTGAGGCTGAC/**R**-TTCTCAGTTTCCCAGGTGCT)

h-mtTFA (F-GGGTTCCAGTTGTGATTGCT/R-TGGACAACTTGCCAAGACAG)

Statistical Analyses

Statistical analyses of data were performed with Prism 4.03 software (GraphPad Software). All values were expressed as the mean ± SEM. The data were evaluated by ANOVA, where appropriate, and the differences between the means were assessed using the Bonferroni's multiple comparison test. A *p*-value of less than 0.05 was considered to be significant

RESULTS

MDA Generation after Iron-Ascorbate Exposure

Before evaluating the role of oxidative stress on mitochondrial function, we evaluated the effectiveness of FE/ASC in initiating lipid peroxidation after incubation with Caco-2/15 cells. At the end of a 6-h culture period, the degree of lipid peroxidation was determined by measuring MDA in cells. As illustrated in Figure 1, FE/ASC induced a significant increase in MDA levels above baseline values compared with control cells. The concentration of MDA was 4-fold higher in cells supplemented with FE/ASC compared with untreated cells. Pre-incubation with the strong antioxidant BHT markedly suppressed the production of MDA, providing direct evidence for the ability of the FE/ASC system to provoke profound lipid peroxidation.

Effect of FE/ASC on Cellular ATP Content

The main function of the mitochondrion is the production of energy in the form of ATP via oxidative phosphorylation and oxygen consumption. We therefore assessed the amount of ATP levels in Caco-2/15 cells exposed to the FE/ASC oxygen radical-generating system. As noted in Figure 2, the administration of FE/ASC led to a four-fold reduction compared with untreated cells. Moreover, pre-incubation with BHT at a concentration of 0.5 mM resulted in a trend of ATP normalization.

Oxidative Phosphorylation Activity

Since mitochondrial oxidative phosphorylation (OXPHOS) is fundamental to all aspects of cell life under aerobic conditions, we evaluated its activity during oxidative stress. The enzymatic activity related to complexes I, II, III, and IV was performed on mitochondrial fraction prepared from Caco-2/15 cells. Our findings documented a significant decrease

in the specific activities of complex I, II, III and IV following FE/ASC treatment (Figure 3). Pre-incubation with BHT abrogated the decline in the OXPHOS enzymatic activities.

Changes in Mitochondrial Calcium Induced by Iron-Ascorbate in Caco-2/15 Cells

We next tested whether FE/ASC caused a change in mitochondrial Ca^{2+} in Caco-2/15 cells using the positively charged and cell permeant Ca^{2+} indicator, Rhod-2/AM, which accumulates predominantly in the negatively charged matrix of the mitochondria. The dye Mitotracker™ was used to confirm the mitochondrial localization of Rhod-2. As presented in Figure 4D, FE/ASC treatment of Caco-2/15 cells induced an increase in Rhod-2 fluorescence that appears predominantly located in the mitochondria as demonstrated by the yellow spots of strong intensity found in the merged image (Figure 4F). In contrast, the distribution pattern of colocalized Rhod-2 and Mitotracker™ observed in control cells revealed spots of less intensity characterized by a more diffuse distribution (Figure 4E). Quantification of Rhod-2 fluorescence intensity is shown in Figure 5. Cells exhibited an increase in Rhod-2 fluorescence after FE/ASC treatment whereas pre-incubation with BHT restored fluorescence intensity to control level.

AIF and Cytochrome C Protein Expression

AIF is normally located in the inter-membrane space of mitochondria and is involved in initiating a caspase-independent pathway of apoptosis by causing DNA fragmentation and chromatin condensation. Furthermore, when cell death is triggered by an apoptotic stimulus, cytochrome C is released into the cytosol, and contributes to the caspase-dependent pathway of apoptosis. Western blot analysis revealed a marked ($P < 0.001$) increase in the level of AIF and cytochrome C protein mass in Caco-2/15 cells following FE/ASC compared with

controls (Figures 6). Pre-incubation with BHT before the addition of FE/ASC prevented the rise in AIF and cytochrome C protein mass.

Quantification of Oxidative DNA Damage in Caco-2/15

ELISA for 8-OHdG, a recognized marker of oxidative DNA damage, was used to quantify oxidative DNA damage in Caco-2/15 cells. Figure 7 shows the average concentration of 8-OHdG detected in the control and experimental groups. Results clearly indicate that the level of oxidative DNA damage in mitochondria was significantly ($P < 0.001$) higher in Caco-2/15 exposed to FE/ASC-mediated lipid peroxidation (Figure 7A). The oxidative DNA damage was attenuated after pre-incubation with BHT. On the other hand, no significant changes were noted in the homogenate or nucleus (Figure 7B and 7C).

OGG1 Repair Enzyme Level

In mitochondria, the base excision repair pathway is primarily responsible for removing 8-OHdG from DNA [41]. In humans, 8-oxodG is repaired by 8-oxoguanine DNA glycosylase (OGG1), an enzyme that recognizes and hydrolyzes the aberrant base from the DNA backbone. We, therefore, examined its gene expression and protein mass in Caco-2/15 cells. As well illustrated in Figure 8, treatment with FE/ASC resulted in a significant ($P < 0.001$) reduction of OGG1 mRNA and protein mass compared with controls. However, pre-incubation of Caco-2/15 cells with BHT prevented the decline in OGG1 expression.

Mitochondrial Transcription Factors

Human mitochondrial transcription requires bacteriophage-related RNA polymerase, POLRMT, mtDNA-binding protein, h-mtTFA/TFAM, and two transcription factors/rRNA methyltransferases, h-mtTFB1 and h-mtTFB2. These crucial proteins define mitochondrial biogenesis and gene expression that together likely fine-tune mitochondrial

functions. Given the deleterious effects of FE/ASC, it was mandatory to explore how oxidative stress modulates the core protein components required for mitochondrial transcription. PCR and Western Blot analyses showed a significant ($P < 0.01$) increase in mtTFA, mtTFB1 and mtTFB2 gene expression (Figure 9A) and protein mass (Figure 9B) without any changes in POLRMT in Caco-2/15 cells treated with FE/ASC compared with controls. Pre-incubation with BHT attenuated the modifications of those transcription factors.

DISCUSSION

The Caco-2/15 cell line has been used to examine a variety of intestinal functions. This intestinal model exhibits many of the features of small intestinal epithelial cells. We employed the FE/ASC oxygen radical-generating system to determine how oxidative stress modulates mitochondrial DNA integrity and function in Caco-2/15 cells [20]. Our results show for the first time that FE/ASC can induce lipid peroxidation accompanied by ATP depletion, mitochondrial transport chain complex inhibition, mitochondrial Ca^{2+} overload, cell apoptosis, mitochondrial DNA lesions and mitochondrial transcription factors alterations.

Iron is the most abundant transition metal in mammalian cells and is essential for the physiological function of multiple proteins [42]. However, excess or non-protein-bound (labile) iron can be detrimental because it can initiate oxygen radical formation and promote ROS [43]. Therefore, iron may cause oxidative damage to biological macromolecules and alter the intracellular redox environment, thereby affecting redox-sensitive cell signaling pathways and transcription factors [44,45]. Although the mechanisms underlying the cytotoxicity of iron in different organs are not fully delineated, many reports have pointed to the participation of iron-mediated peroxidation in numerous pathological states, including atherosclerosis [46,47], cancer [48,49], ischemia-reperfusion injury [50], IBD [51], and conditions of iron overload [52]. Several laboratories [19–21,23–26,52–54] have shown the ability of iron to initiate strong lipid peroxidation, whereas ascorbic acid can amplify the oxidative potential of iron by promoting metal ion-induced lipid peroxidation. The data presented here clearly indicate that the FE/ASC system functioned as a producer of lipid peroxidation and, at the same time, altered the DNA integrity and the function of

mitochondria. It is noteworthy that the iron dose used in the current study is comparable with normal iron concentration in the gut [11]. The deteriorations resulting from the exposure of Caco-2/15 cells to FE/ASC are probably attributable to oxidative stress, because the addition of the BHT antioxidant simultaneously prevented the occurrence of lipid peroxidation and improved the cellular processes of mitochondrial integrity and functions. BHT was selected as an antioxidant because it represents a powerful agent inhibiting iron-mediated oxidative stress and does not have any toxic effects on Caco-2/15 cell culture [21].

Previous reports observed that an accumulation of peroxidation products in mitochondria leads to a decrease in ATP production and compromises the maintenance of cellular homeostasis [55]. In this study, incubation of Caco-2/15 cells with FE/ASC induced a marked decrease in ATP levels. Our data are consistent with previous investigations showing that ATP decreased in the HT-29 intestinal cell-line after oxidative injury by hydrogen peroxide [56]. The fall in ATP synthesis is probably related to the low mitochondrial metabolic activity resulting from the FE/ASC-mediated lipid peroxidation. In fact, electron movement through complexes I, II, III, and IV enables movement of hydrogen ions across the inner membrane into the inter-membrane space creating an electrochemical gradient, which is harnessed into ATP production by ATP synthase in complex V. We reasonably propose that mitochondrial damage from ROS may lead to a degradation in the efficiency of the mitochondrial respiratory chain enzymes and hence a decline in ATP production. The impairment of mitochondrial complex I, II, III and IV activity noted in our experiments may be attributable to ROS-induced cardiolipin damage that has recently been reported in ischemia/reperfusion rat heart, which ultimately led to a decrease in oxidative phosphorylation [57,58]. The phospholipid cardiolipin is found almost exclusively in the

inner mitochondrial membrane where it promotes the optimal function of numerous enzymes involved in mitochondrial energy metabolism. Finally, inactivation of mitochondrial electron transport chain enzymes and/or ATP-synthase may account for the ATP depletion triggered by the administration of FE/ASC to Caco-2/15 cells.

On top of its ATP generation ability, mitochondria also play a part in modulating the amplitude and spatiotemporal organization of Ca^{2+} signals through rapidly accumulating and releasing Ca^{2+} [59,60]. Indeed, intracellular Ca^{2+} plays a key role in cellular metabolism. However, excessive mitochondrial Ca^{2+} overload can trigger ROS overproduction, mitochondrial membrane depolarization and ATP production inhibition, all hallmark events of mitochondrial dysfunction clearly observed in the present work. Additionally, these defective processes may eventually lead to apoptosis [59,60], which was also documented in our studies. In particular, mitochondrial Ca^{2+} overload can favor cardiolipin peroxidation, thereby affecting mitochondrial permeability transition, inducing AIF and cytochrome C release, and culminating in mitochondrial dysfunction and apoptosis [61,62]. Therefore, tools capable of minimizing mitochondrial Ca^{2+} overload would decrease mitochondrial ROS accumulation and improve mitochondrial energy production, which may impact on mitochondrial-oxidative mediated diseases.

The core human mitochondrial transcription machinery comprises a single subunit bacteriophage-related RNA polymerase (POLRMT), mtTFA, and two transcriptional co-activator proteins, h-mtTFB1 and h-mtTFB2. Both factors seem to interact directly with POLRMT forming a heterodimer that, in addition to mtTFA, is required for the accurate initiation on both H_1 and L promoters [63]. The main function of mtTFA is the maintenance of mtDNA replication and transcription during mitochondria biogenesis [64]. In our study,

we observed that mtTFA, mtTFB1, mtTFB2 transcriptional level and protein mass were augmented in the presence of Fe/ASC with no marked difference for POLRMT. Currently, we do not know whether the upregulation of mtTFA, mtTFB1, and mtTFB2 in our experiments represent a compensatory mechanism in response to oxidative stress-related reduction in energy metabolism such as defective electron transport chain, incomplete mitochondrion biogenesis or accelerated apoptosis. Accordingly, mtTFA was found upregulated in response to lipopolysaccharide-induced oxidative damage to mitochondria, presumably to enhance mtDNA levels and OXPHOS activity [65]. Furthermore, over-expression of human TFB2M in HeLa cells induced an increase in TFB1M mRNA levels and protein expression [66], suggesting the existence of a retrograde signaling pathway from mitochondria to the nucleus, which precisely regulates the expression of these related factors. Further investigation is needed to examine these important aspects.

In the present study, FE/ASC raised 8-OHdG that represents one of the most frequently generated oxidative base lesions within DNA, owing to guanine, the lowest redox potential among the nucleic acid bases formed in pathological conditions [67]. Similarly, the double immunofluorescence technique revealed that oxidative DNA damage is induced in colon epithelial cells of the IBD mouse model [68]. Furthermore, nuclei were not affected by FE/ASC-mediated oxidative stress, which confirms that mtDNA is more vulnerable than nuclear DNA to oxidative damage given that it is situated much closer to the site of ROS generation and that mitochondria lack protective histones and far fewer mechanisms that prevent reduced base excision repair activity than DNA from nuclei [69]. Our findings confirm that oxidative DNA damage is one of the most common threats to mitochondrial genome stability.

OGG1 is the DNA repair enzyme that recognizes and excises 8-oxodG [70]. The present study shows that incubation of Caco-2/15 with FE/ASC resulted in a marked decrease in OGG1 transcript level and protein mass. Deficiency in DNA repair enzyme OGG1 has likely important functional consequences, compromising the ability of cells to repair DNA. Therefore, intestinal epithelial cells are as sensitive to lipid peroxidation as other cell types, including kidney cortex cells that accumulate 8-OHdG mainly in the mtDNA and to a lesser extent in nuclear DNA under diabetic conditions [71]. We believe that mtDNA damage is linked to the numerous abnormal processes noted in our study, including ATP generation, Ca^{2+} homeostasis and release of signals for cell death.

In summary, the FE/ASC system in Caco-2/15 appeared to be very effective in promoting lipid peroxidation and, at the same time, altering the mitochondrial function. This mitochondrial dysfunction is probably related to oxidative stress, because the addition of antioxidants prevented the occurrence of lipid peroxidation and improved the mitochondrial function in terms of ATP production, Ca^{2+} homeostasis and apoptotic protein expression. The pattern of our results using the Caco-2/15 cell line may prove useful in elucidating the molecular mechanisms implicated in IBD. Overall, our data suggest that oxidative-mitochondrial dysfunction is not mediated by a single mechanism, but that it may instead be a consequence of multiple vicious circles organized within a complex functional network.

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FIGURE LEGENDS

Figure 1 **Malondialdehyde (MDA) concentrations in Caco-2/15 cells challenged with iron/ascorbate and/or BHT**

At 21 days of differentiation, cells were exposed to (0.2 mM/2 mM) FE/ASC, (50 μM) BHT or both for 6 h at 37°C. Oxidative stress was assessed by measuring MDA as an index of lipid peroxidation. Values are means ± SEM for three independent experiments. $^{*}P < 0.05$

Figure 2 **ATP Levels in Caco-2/15 cells exposed to iron/ascorbate in the presence or absence of BHT**

Caco-2/15 cells were grown on 96-well plates and, after 21 days post confluence, they were treated with (0.2 mM/2 mM) FE/ASC and/or (50 μM) BHT for 6 h at 37°C. ATP levels were measured with a bioluminescence assay and corrected for intracellular protein concentrations. Values are expressed as ng of ATP per μg of cellular protein and represent the means ± SEM for three independent experiments. $^{*}P < 0.001$

Figure 3 **Effect of iron/ascorbate and/or BHT treatment on enzymatic activities of mitochondrial respiratory chain complexes in Caco-2/15 cells**

Enzyme activities of mitochondrial respiratory chain complexes I, II, III, IV were measured by spectrophotometric assays in mitochondrial samples in Caco-2/15 cells treated with (0.2 mM/2 mM) FE/ASC and/or (50 μM) BHT for 6 h at 37°C. Enzyme activities are expressed as nmol/ min/ mg protein. Each value represents the mean ± SEM for 3 separate experiments performed in duplicate. *P < 0.05 vs. controls.

Figure 4 **Influence of oxidative stress on mitochondrial calcium homeostasis in Caco-2/15 cells**

Representative fluorescence images of control and FE/ASC-treated Caco-2/15 cells loaded with the mitochondrial dye MitoTracker^{TM} (**A, B**) and the mitochondrial Ca^{2+} indicator Rhod-2 (**C, D**). Merged images (**E, F**) indicate colocalization of the two dyes in the mitochondria. Mitochondrial Ca^{2+} accumulation is visible upon oxidative stress (**arrow**). Scale bar-10 μm.

Figure 5 **Quantification of Rhod-2 fluorescence intensity in Caco-2/15 Cells subjected to iron/ascorbate and/or BHT treatment**

Caco-2/15 cells pretreated for 6 h with (0.2 mM/2 mM) Fe/ASC and/or (50 μM) BHT were loaded with 5 μM of Rhod-2 AM for 30 minutes at 37^{o}C. Fluorescence intensity was quantified by image analysis as described in Material and Methods. Six to eight fluorescence images from three independent experiments were randomly chosen. Results were calculated by dividing the pixel intensity by the area of the spot (μm^2). Data illustrated represent the means ± SEM. $^{*}P < 0.001$

Figure 6 **Cytochrome C and AIF expression levels in Caco-2/15 Cells treated with iron/ascorbate and/or BHT**

Caco-2/15 cells were incubated with (0.2 mM/2 mM) FE/ASC and/or (50 μM) BHT for 6 h at 37°C. Gene and protein expression were determined by RT-PCR and Western blotting, respectively. Values are expressed as means ± SEM for three independent experiments. $^{*}P < 0.001$

Figure 7 **Influence of iron/ascorbate treatment in the presence of absence of BHT on 8-hydroxy -2-deoxyguanosine level in Caco-2/15 cells**

The levels of 8-hydroxy -2-deoxyguanosine (8-OHdG) were measured by ELISA kit assay in (A) mitochondrial, (B) homogenate and (C) nucleus samples in Caco-2/15 cells treated with (0.2 mM/2 mM) FE/ASC and/or (50 μM) BHT for 6 h at 37°C. Values are means ± SEM for three independent experiments. $^{*}P < 0.001$

Figure 8 **Effect of iron/ascorbate and/or BHT treatment on 8-oxoG-DNA Glycosylase levels in Caco-2/15 cells**

Caco-2/15 cells were incubated with (0.2 mM/2 mM) FE/ASC and/or (50 μM) BHT for 6 h at 37°C to determine the effects of oxidative stress on 8-oxoG-DNA glycosylase (OGG1) gene expression (A) and protein mass (B). Values are expressed as means ± SEM for three independent experiments carried out in triplicate. $^{*}P < 0.001$

Figure 9 **Effect of iron/ascorbate and/or BHT treatment–on gene and protein expression of mitochondrial transcription factors in Caco-2/15 cells**

Effects of (0.2 mM/2 mM) FE/ASC, (50 μM) BHT or both for 6 h at 37°C on gene expression **(A)** and protein mass **(B)** of mtTFA, mtTB1, mtTB2, POLRMT. A GAPDH cDNA probe was used as a control for RNA loading; β-actin was used as loading control protein. Data originated from three independent experiments. Values are expressed as means ± SEM. $^{*}P < 0.01$

Figure 1.

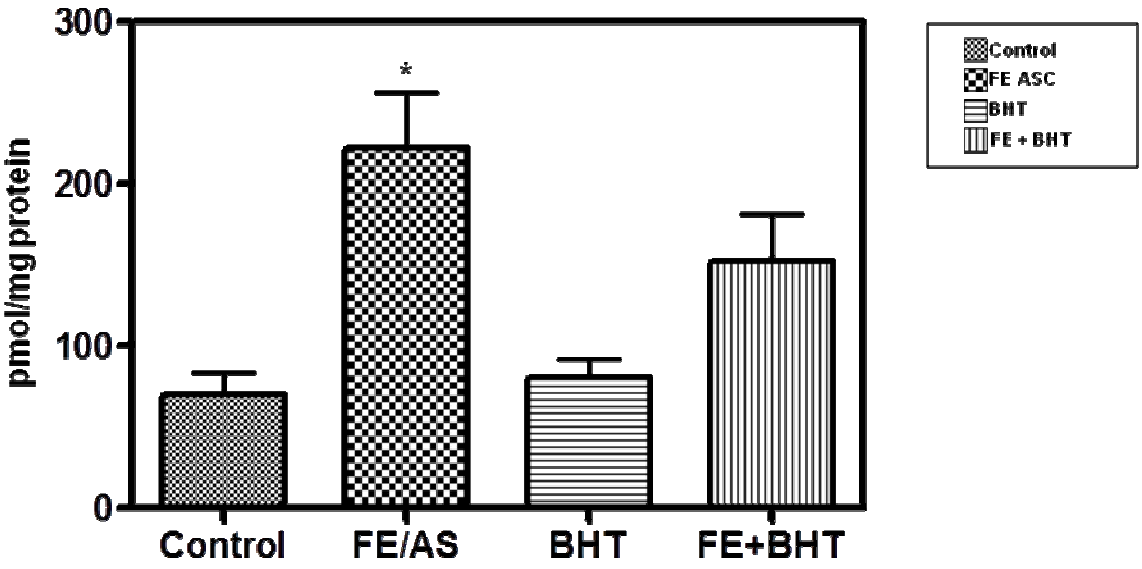


Figure 2.

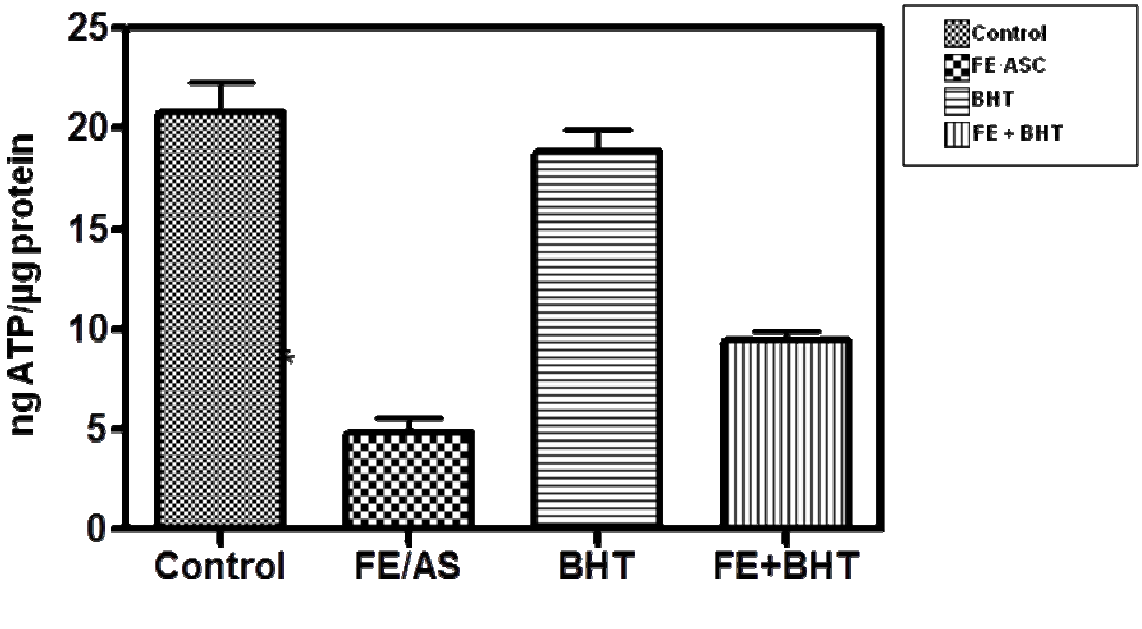


Figure 3.

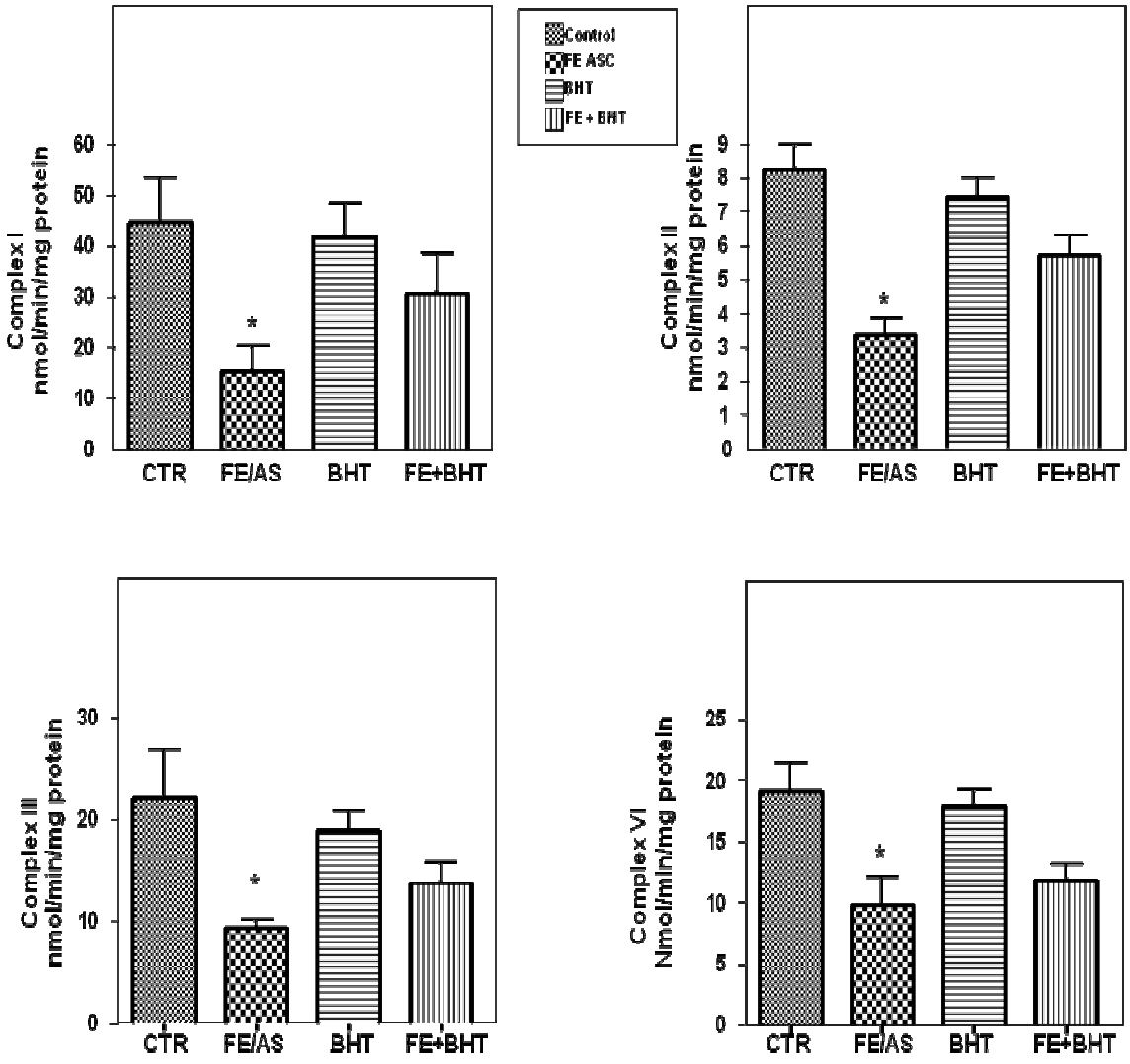

Control
FE ASC
BHT
FE + BHT
Complex I
nmol/min/mg protein
Complex II
nmol/min/mg protein
Complex III
nmol/min/mg protein
Complex VI
Nmol/min/mg protein
CTR
FE/AS
BHT
FE+BHT
*

Figure 4.

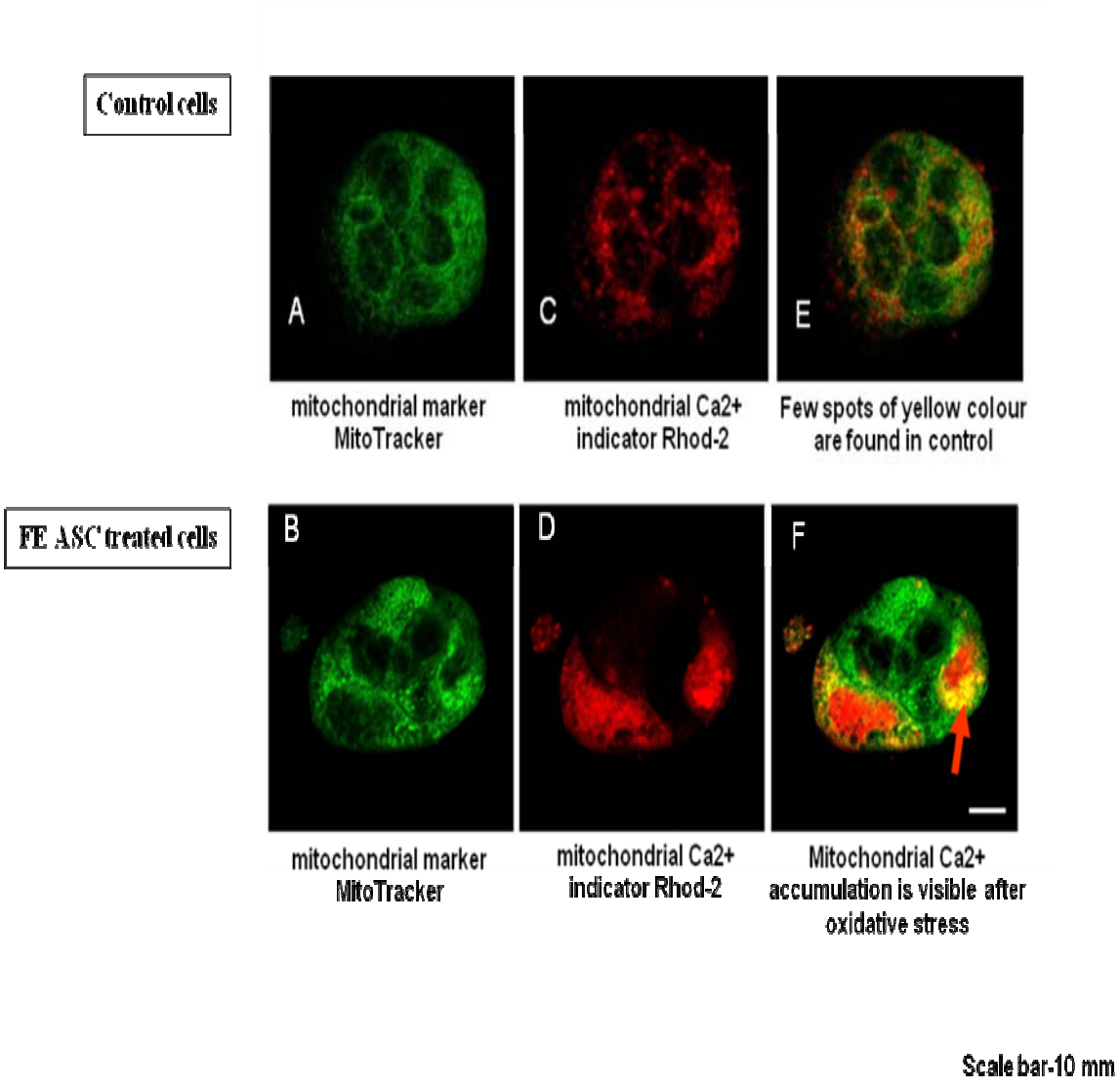

Control cells
A
C
E
mitochondrial marker MitoTracker
mitochondrial Ca2+ indicator Rhod-2
Few spots of yellow colour are found in control
FE ASC treated cells
B
D
F
mitochondrial marker MitoTracker
mitochondrial Ca2+ indicator Rhod-2
Mitochondrial Ca2+ accumulation is visible after oxidative stress
Scale bar-10 mm

Figure 5.

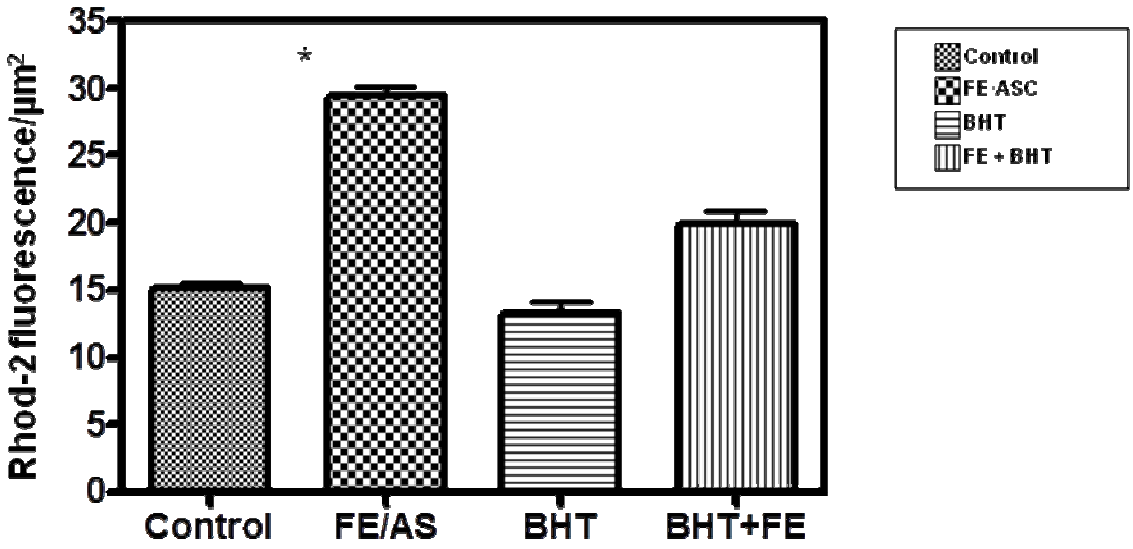


Figure 6.

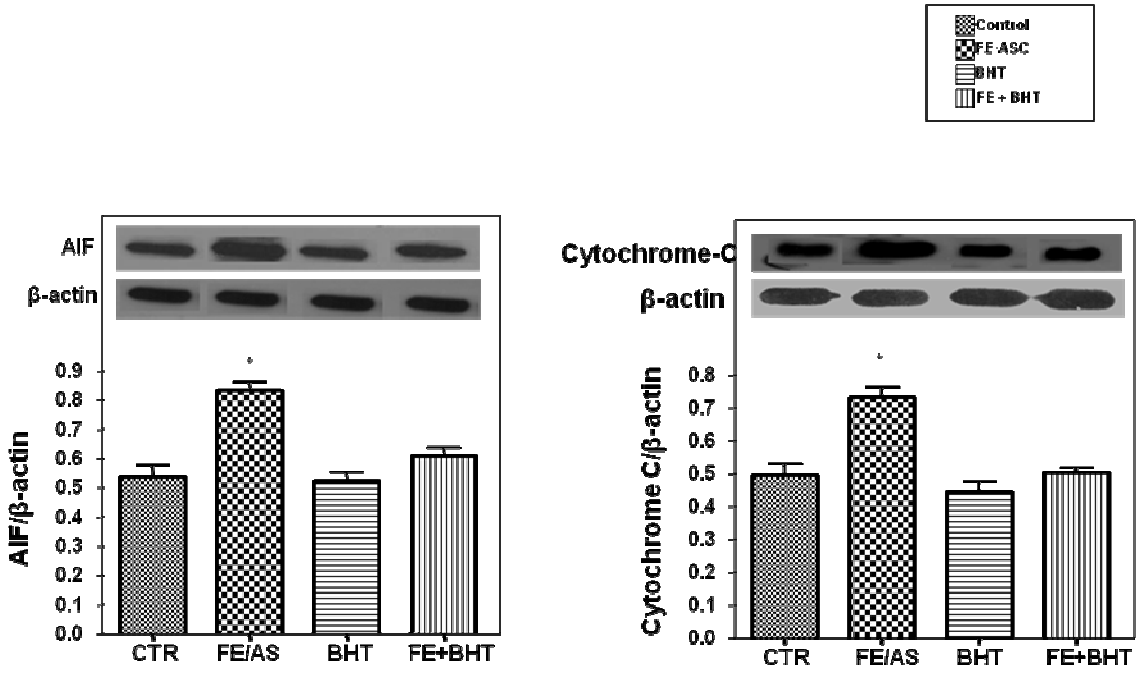


Figure 7.

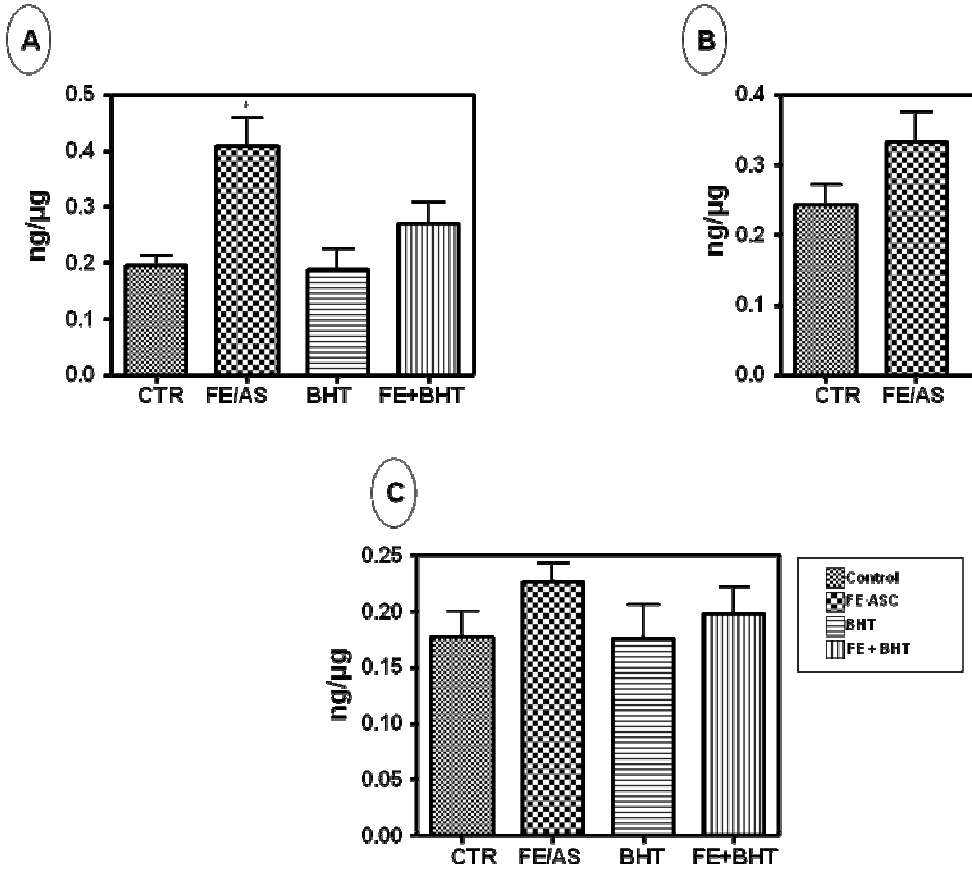


Figure 8.

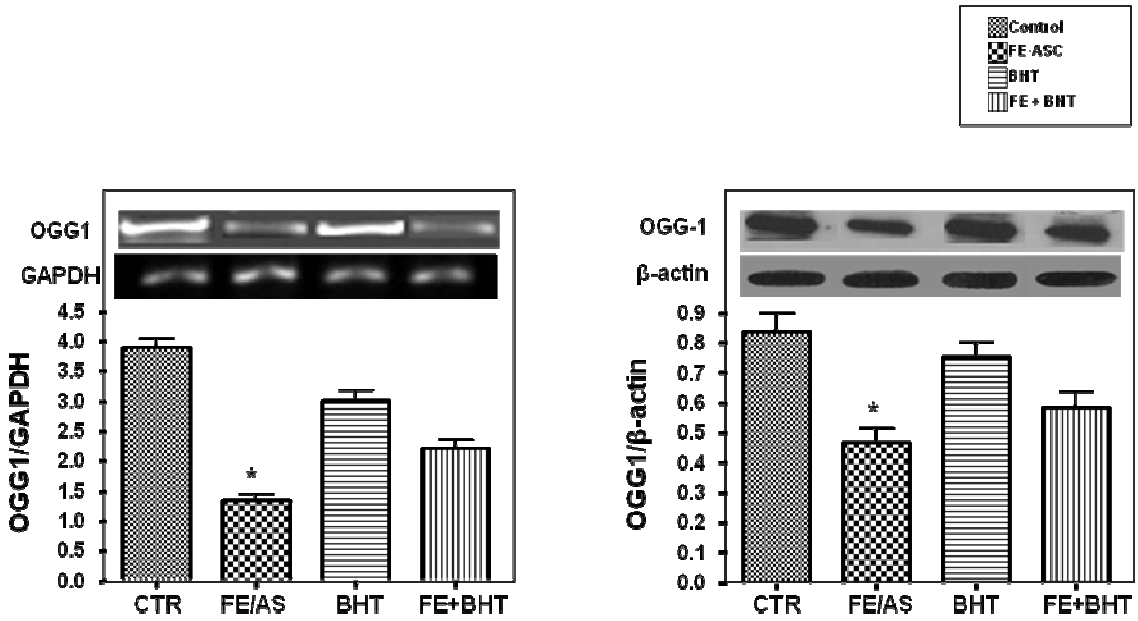
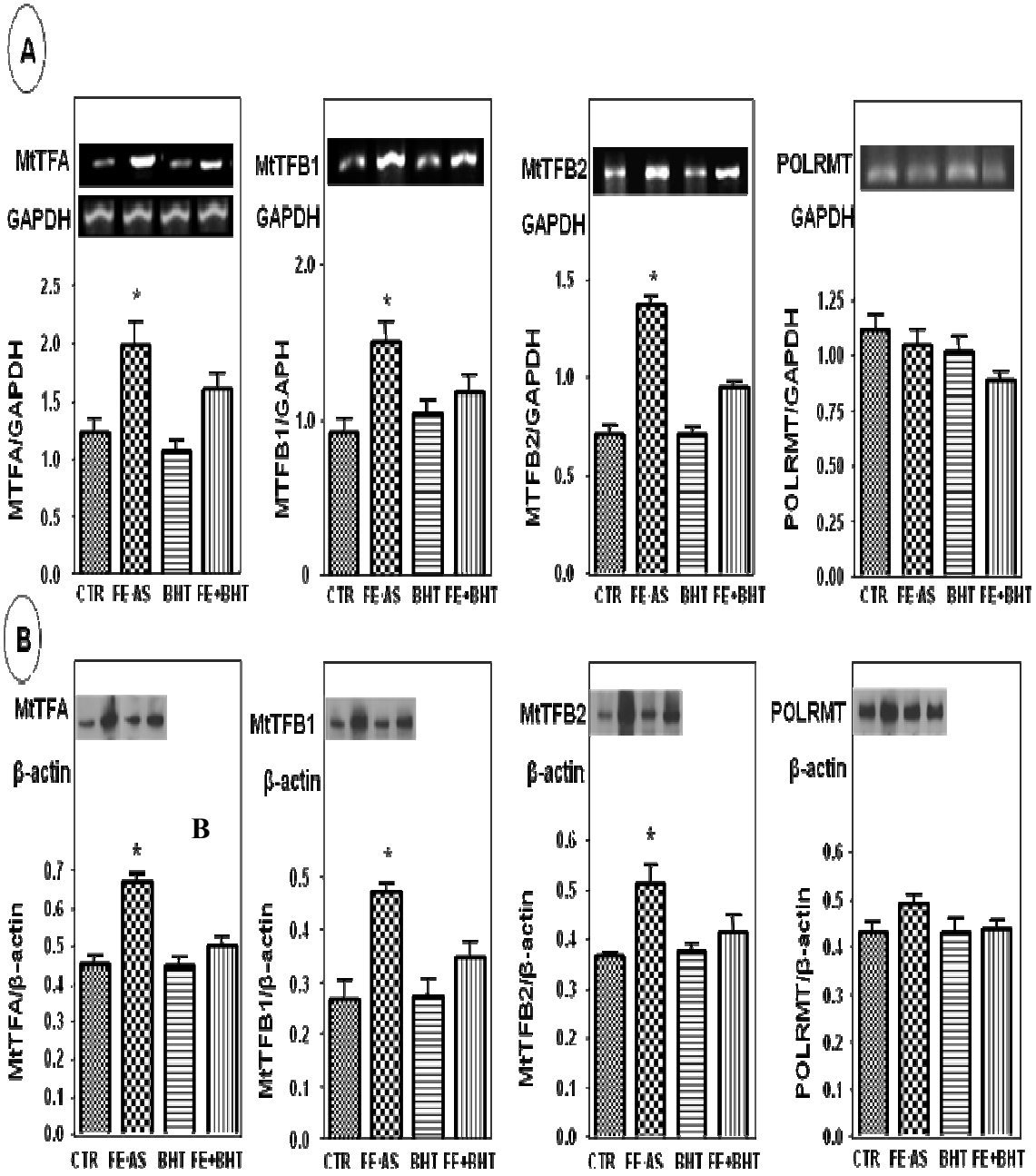


Figure 9.


A
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GAPDH
MtTFB1
GAPDH
MtTFB2
GAPDH
POLRMT
GAPDH
MTFA/GAPDH
MTFB1/GAPH
MTFB2/GAPDH
POLRMT/GAPDH
CTR FE-AS BHT FE+BHT
B
MtTFA
β-actin
MtTFB1
β-actin
MtTFB2
β-actin
POLRMT
β-actin
MtTFA/β-actin
MtTFB1/β-actin
MtTFB2/β-actin
POLRMT/β-actin
CTR FE-AS BHT FE+BHT

5 ARTICLE 3

Role of Nrf2 in Intestinal Cellular Integrity: Impact on Mitochondrial Functions

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Abstract

Oxidative stress can lead to multiple cellular and mitochondrial dysfunctions along with marked inflammation, which in turn can mediate most chronic inflammatory bowel diseases. Although nuclear-factor-erythroid-2-related factor (Nrf2) is a sensitive transcription factor that orchestrates the expression of a battery of antioxidant and detoxification genes, limited information is available on its role in the gastrointestinal tract. The objectives of this study were to evaluate the antioxidant and anti-inflammatory functions of Nrf2 as well as its implication in mitochondrial functions in intestinal epithelial cells. To this end, the *Nrf2* gene was knocked down by transfecting Caco-2/15 cells with a pGFP-RS lentiviral vector containing shRNA against Nrf2 and cells were then treated with various concentrations of iron/ascorbate (Fe/Asc) to induce mild to severe oxidative stress. Inactivation of *Nrf2* resulted in increased lipid peroxidation as demonstrated by raised malondialdehyde levels. Concomitantly, a significant reduction was noted in antioxidant enzymes, including catalase, glutathion peroxidase and superoxide dismutase. This loss of redox homeostasis led to an augmentation in inflammatory mediators such as tumor necrosis factor alpha and nuclear factor-kappa B. Interestingly, Nrf2 silencing or oxidative stress provoked loss of cell junctional sealing as reflected by low occludin expression. Moreover, isolation of mitochondria revealed a decline in ATP production, respiratory complex (I, II, III, IV) activity, Bcl2 anti-apoptotic factor and PGC-1α, a crucial regulator of mitochondrial genesis, energetics and functions. In conclusion, Nrf2 appears to be an active actor in cytoprotection against oxidative stress and inflammation as well as mitochondrial functional integrity.

Keywords: Oxidative stress, inflammation, antioxidant defense, mitochondrial functions, Nrf2

Abbreviations:

CAT	Catalase
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
Fe/Asc	Iron/Ascorbate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-Px	Glutathione peroxidase
GSH	Glutathione
H2O2	Hydrogen peroxide
IBD	Inflammatory bowel disease
IκB	Inhibitor of kappa B
MDA	Malondialdehyde
MEM	Minimal essential medium
NF-κB	Nuclear factor-kappa B
ROS	Reactive oxygen species
shRNAs	short hairpin RNA
SOD	Superoxide dismutase
TBA	Thiobarbituric
TNF-α	Tumor necrosis factor alpha
PHB	Prohibitin
TJ	Tight junction

Introduction

Nrf2 is a transcription factor that regulates the expression of genes containing antioxidant-response element (ARE) in their promoters and represents a crucial factor in the induction of phase II enzyme in response to stressful conditions [1, 2]. It is constitutively controlled by the repressor protein Keap1, which acts as a molecular sensor of disturbances in cellular homeostasis [3-5]. During unstressed states, Keap1 suppresses cellular Nrf2 in cytoplasm, which drives its proteasomal degradation. Upon induction by chemo-preventive agents, Nrf2 is released from Keap-1, translocates to the nucleus, and thus controls the expression of genes involved in oxidative defense [6, 7]. Although knowledge of Nrf2 function and regulation has progressed significantly in the past years, efforts are still needed to uncover the signal-dependent activation of Nrf2 function and the precise mechanisms involved.

Silencing Nrf2 by genetic manipulation renders numerous types of cells and animal models much more sensitive to the damaging effects of oxidants and inflammatory agents [7, 8]. Conversely, activation of the Keap1-Nrf2 pathway allows survival and adaptation under various conditions of stress and has protective effects in many animal models [7, 9]. Furthermore, protective functions of Nrf2 were reported against a number of pathologies that are caused or aggravated by oxidative stress. In many human cancers, missense mutations in *KEAP1* and *Nrf2* genes have been identified [10, 11] and Nrf2 has emerged as a novel target for the prevention of colorectal cancer [12]. Moreover, a growing body of literature suggests that Nrf2-ARE signaling is involved in attenuating inflammation-associated pathogenesis, such as autoimmune diseases, rheumatoid arthritis, asthma, emphysema, gastritis, colitis and atherosclerosis [7].

Despite the high levels of Nrf2 in the small intestine and stomach, little attention has been devoted to its functions in the gastrointestinal tract. Previous work pointed out the protective effect of Nrf2 stimulation against oxidative stress in the gastric mucosa [13]. Recently, Nrf2-deficient mice were found to display increased susceptibility to dextran sulfate sodium (DSS)-induced colitis [12], while an Nrf2 gene promoter polymorphism was associated with ulcerative colitis in humans [14]. This very limited information truly shows little regard for the gastrointestinal mucosa, which is constantly exposed to luminal oxidants originating from ingested nutrients [15-17]. Key among these is the simultaneous consumption of iron salts and ascorbic acid, which can cause oxidative damage to biomolecules [18]. Additionally, the intestinal mucosa is subject to prolonged oxidative stress from reactive oxygen species (ROS) generated during aerobic metabolism [19]. In addition, the influx of neutrophils and monocytes associated with inflammation can generate further ROS via respiratory burst enzymes as well as those involved in prostaglandin and leukotriene metabolism [20].

In order to gain more insight into the physiological and biological significance of Nrf2 in the intestine, molecular strategies were devised in the Caco-2 cell line, a reliable human intestinal model, to thoroughly examine the specific role of Nrf2 in oxidative stress, inflammation, epithelial cell cytoprotection and mitochondrion functions.

Materials and Methods

Caco-2/15 Cell Culture and Treatment Iron/Ascorbate

Caco-2/15 cells (ATCC, Rockville, MD) were cultured at 37^{o}C in Minimal Essential Medium (MEM) (GIBCO-BRL, Grand Island, NY) containing 1% penicillin-streptomycin and 1% MEM non-essential amino acids (GIBCO-BRL) and supplemented with 10% decomplemented fetal bovine serum (FBS) (Flow, McLean, VA). Briefly, Caco-2/15 cells (passage 20-30) were maintained in T-75 cm^2 flasks (Corning Glass Works, Corning, NY) as described previously [21, 22]. Culture cells were split (1:6) when 90% confluence was reached by the use of 0.05% trypsin-0.5 mM EDTA (GIBCO-BRL). Cells were cultured for 21 days post confluence, at which the Caco-2/15 cells are highly differentiated and appropriate for lipid synthesis and metabolism experiments. To determine the implication of oxidative stress *per se* on Nrf2 mRNA and protein mass, Caco-2/15 cells were incubated with different concentrations of iron and ascorbate (Fe/Asc) (0.05/2mm, 0.1/2mm, 0.2 mm/2 mm).

Generation of Stable Short Hairpin RNA Expression in Caco-2/15 cells

Growing 293FT packaging cell line was transiently cotransfected with pLK0.1TRCN000007558 vector (Open Biosystems) harboring short hairpin (sh) RNA expression cassettes against human Nrf2 and the Vira Power Packaging Mix (Invitrogen Corp., Carlsbad, CA 92008) to generate replication-deficient lentivirus. The produced lentivirus stock was five times concentrated with an Amicon Ultra-15 100K centrifugal filter device (Millipore) and 35 μl were used to infect 250,000 Caco-2/15 cells in six-well plates. Forty-eight hours post-infection, cells were transferred to flasks and grown in MEM medium containing 1 μg/ml puromycin (Sigma-Aldrich, USA) for an additional 7

days to establish stable shRNA-expressing Caco-2 cells. Cells were cultivated until 10 days post-confluence and used only following the validation of the suppression of the gene of interest by RT-PCR and Western blot assays. Control cells were obtained by infection with Scramble vector lentivirus harboring the same features as the sh construct.

Iron/Ascorbate Exposure and Treatment of Caco-2/15 with Oltipraz

To determine infected and untreated cell response to oxidative stress, cells were exposed to Fe (0.2 mM)/Asc (2 mM) (1:10) system-mediated lipid peroxidation. At the end of a 6-h culture period, the degree of lipid peroxidation was evaluated by measuring cellular malondialdehyde (MDA). Caco-2/15 cells were then incubated with various concentrations of Oltipraz (4-methyl-5-pyrazinyl-3*H*-1,2-dithiole-3-thione; OPZ) (25, 50 and 100 μM) (Sigma) dissolved in absolute ethanol (ETOH) vehicle and then added to the medium, resulting in a final ETOH concentration of 1.6%. Control cells were treated with the vehicle only. Cells were pre-treated with OPZ for 20 h before the 6-h incubation with Fe/Asc.

Estimation of Lipid Peroxidation

The amount of cellular MDA was determined by HPLC as described previously [23]. Briefly, proteins were first precipitated with a 10% sodium tungstate (Na_2WO_4) (Aldrich Chemical) solution and the protein-free supernatant was then reacted with an isovolume of 0.5% thiobarbituric acid (Sigma) solution at 90°C for 60 min. After cooling to room temperature, the chromogene [$(TBA)_2$-MDA] was extracted with 1-butanol and dried over a stream of nitrogen at 37°C. The dry extract was then re-suspended in KH_2PO_4/methanol (70:30, pH 7.0) mobile phase before MDA detection by HPLC.

Assessment of Intracellular ATP

Intracellular ATP was measured by luciferase driven bioluminescence using an ATP Bioluminescence Assay Kit (Calbiochem, EMD Chemicals, Inc. Gibbstown, NJ) as reported previously [21]. Values were normalized with regard to the protein content of the respective sample.

Mitochondrial Preparations

Mitochondria were isolated using standard differential centrifugation techniques [24]. Briefly, Caco-2/15 cells were treated with Fe/Asc (0.2/2 mM) and/or BHT (50 μM) for 6 h at 37°C. Cells were homogenized with a glass pestle Dounce homogenizer in a buffer containing mannitol (210 mM), sucrose (70 mM), Ethylene-bis tetraacetic acid (EGTA) (1 mM), fatty acid-FBS albumin (0.5%), and HEPES (5 mM, pH 7.2). The homogenate was centrifuged at 1,000 × *g* for 10 min at 4°C. The supernatant was then collected and centrifuged at 10,000 × *g* for 10 min to obtain pellets containing mitochondria. The pellets were used immediately or stored at −80°C. The protein contents of mitochondrial suspension were determined by the Bradford assay (BioRad, Mississauga, ON) with bovine serum albumin as a standard.

Mitochondrial Enzyme Assays

The activities of respiratory chain complexes were assayed as previously described [24-26]. Briefly, 20–30 μg of mitochondrial protein were used for each complex every 30 sec for 5 min. The activity of complex I (NADH: ubiquinone oxidoreductase) was measured by monitoring the reduction of decylubiquinone. Complex II (succinate: ubiquinone oxidoreductase) activity was examined by monitoring the reduction of dichloroindophenol when coupled to complex II-catalyzed reduction of decylubiquinone. Complex III (ubiquinol:ferricytochrome C oxidoreductase) activity was assayed using

oxidized cytochrome C. The activity of complex IV (cytochrome C oxidase) was determined by oxidation of reduced cytochrome C. Enzyme activities were expressed in nmole of substrate used per minute per mg of protein. Enzyme assays for all complexes were performed in duplicate in mitochondrial fraction of Caco-2/15 cell line. Complex I, II, III and IV chemicals were purchased from Sigma Chemical (St Louis, MO).

Enzymatic Antioxidant Activity

The activities of antioxidant enzyme catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured in cell homogenates. For enzymatic activity, a substrate specific for each enzyme was added to the cell homogenates and rates of disappearance of the substrate were measured by spectrophotometry. Caco-2/15 cells were harvested in hypotonic lysis buffer [HEPES (10 mM), $MgCl_2$ (1.5 mM), KCl (10 mM), DTT (0.5 mM), PMSF (0.2 mM)]. Total SOD activity was determined as described by McCord et al. [27]. Briefly, O_2- was generated by the addition of xanthine and xanthine oxidase and the oxidation of the SOD assay cocktail was followed spectrophotometrically (550 nm, 5 min) after adding the sample. The assay for CAT activity was adapted from the protocol reported by Jiang et al. [28]. Briefly, the oxidation of xylenol orange oxidation was measured in the presence of ferrous ions. Absorbance (560 nm, 5 min) was monitored after the addition of H_2O_2 (100 μM). CAT activity was calculated using a standard curve. GPx activity was assessed as described previously [29]. Cell homogenates were added to a PBS buffer containing GSH (10 mM), glutathione reductase (0.1 U) and NADPH (2 mM) with H_2O_2 (1.5%) to initiate the reaction. Absorbance was monitored every 30 sec at 340 nm for 5 min.

Western Blot for Protein Analysis

The Bradford assay (BioRad) was used to estimate protein concentration. Cells were homogenized and proteins (30 μg) were denatured at 95°C for 5 min in sample buffer containing sodium dodecyl sulphate (SDS) and β–mercaptoethanol separated on a 10% SDS-PAGE gel and electroblotted onto nitrocellulose membranes (Amersham). Membranes were blocked in Tris-buffered saline [20 mM Tris-HCl (pH 7.5) plus 137 mM NaCl] dry milk for 60 min at room temperature as described previously [22]. The blots were then incubated overnight at 4°C in blocking solution containing the antibodies for Nrf2 (Abcam, USA), Inhibitor of kappa B (IκB) (Sigma-Alrich), Nuclear factor-kappa B (NF-κB) (Sigma-Aldrich), peroxisome proliferation activator receptor-γ coactivator-1alpha (PGC-1α) (Abcam), Prohibitin (Thermo Scientific, Canada), Tumor Necrosis Factor-alpha (TNF-α) (Sigma), B cells lymphoma-2 (Bcl2) (Abcam), Occludin (Abcam) and β-actin (Sigma-Aldrich). Blots were developed and the protein mass was quantified using an HP Scan jet scanner equipped with a transparency adapter and the UN-SCAN-IT gel 6.1 software.

Semi-Quantitative RT-PCR

Experiments for mRNA quantification as well as for the housekeeping gene GAPDH were performed in Caco-2/15 cells using the UNO II thermocycler (Biometra) as reported previously [30]. Briefly, total RNA was isolated and reverse transcripted into cDNA. PCR amplification was then performed in 50 μl volume using 5 μl PCR Buffer (10 X), 1.5 μl $MgCl_2$ Solution, 2 μl dNTPs (2.5 mM), 0.2 μM of each corresponding primer and 2.5 U of TAQTM Platinum Polymerase (Qiagen). The sequences for mouse Nrf2 primers were 5'-TCTCCTCGCTGGAAAAAGAA-3' and 3'-AATGTGCTGGCTGTGCTTTA-5'. Between 30 and 40 cycles of amplification were used at 95ºC for 30 s, 58ºC for 30 s and

72ºC for 30 s. Amplicons were visualized on standard ethidium bromide-stained agarose gels. Quantification was determined with the software UN-SCAN-IT gel 6.1.

Statistical Analyses

Statistical analyses of data were performed with Prism 4.03 software (GraphPad Software, CA). All values were expressed as the mean ± SEM. The data were evaluated by ANOVA and the differences between the means were assessed using the Bonferroni's multiple comparison test. A *P*-value of less than 0.05 was considered to be significant.

Results

Nrf2 Expression in Response to Oxidative Stress

In order to test the status of Nrf2 expression and its response to oxidative stress, Caco-2/15 cells were incubated with the Fe/Asc oxygen radical-generating system. As illustrated in Fig. 1A, the incubation of differentiated Caco-2/15 cells with increasing amounts of Fe/Asc promoted steady lipid peroxidation, which was assessed by measuring MDA.

The same concentrations of Fe/Asc were employed to determine the modulation of Nrf2. Treatment of Caco-2/15 cells with the low dose of Fe (0.05 mM), combined with Asc (2mM), induced a marked rise in Nrf2 protein expression, whereas high levels of Fe (0.1 and 0.2 mM) lowered Nrf2 protein mass (Fig. 1C). It is worth noting that no significant changes were observed in Nrf2 mRNA with the variable Fe doses combined with 2 mM Asc (Fig. 1B).

Nrf2 Protein Level Modulation by OPZ

OPZ is an Nrf2 activator capable of preventing oxidative stress, which highlights the importance of the Nrf2 antioxidant system. To examine the ability of this chemical agent to regulate Nrf2 in the intestinal system, Caco-2/15 cells were treated with OPZ in the presence and absence of Fe/Asc (0.2/2 mM). The presence of OPZ increased Nrf2 protein expression and prevented Fe/Asc-mediated Nrf2 protein lessening (Fig. 2B). Once again, Nrf2 transcripts were unaltered with OPZ and/or Fe/Asc treatments (Fig. 2A).

Lentiviral Knockdown of Nrf2 in Caco-2/15 Cells

To define the role of Nrf2 in various intestinal processes, including oxidative stress, inflammation and mitochondrial functions, we used stable Caco-2/15 cells together with

shRNA constructs targeting Nrf2-encoding mRNA. Nrf2 expression was measured in several clones and compared with the control and scramble-containing vector (SV) cells. As invariable results were recorded between untreated and SV cells (data not shown), only the latter were employed as control cells for the subsequent studies. Nrf2 was inactivated in a concentration-dependent manner within 10 µl-35 µl as illustrated by decreased gene (Fig. 3A) and protein (Fig. 3B) compared with untreated cells. In order to investigate the effect of OPZ on Nrf2 residual expression, we used the most efficient cellular clone. Under these conditions, OPZ was able to significantly increase Nrf2 in control SV, but without equivalent magnitude in Nrf2 shRNA-infected cells (Fig. 3C).

Nrf2 Knockdown and Endogenous Antioxidant Enzymes

The enzymatic antioxidant pathway protects the cells against free radicals and ROS excess to maintain an oxidative balance. Figure 4 shows that the activity of CAT, GPx and cytoplasmic SOD was significantly reduced in Nrf2 shRNA-infected cells in comparison with control SV cells. Following treatment with OPZ, the activity of the three enzymes was found substantially enhanced in SV, but only moderately elevated in Nrf2 shRNA-infected cells (Fig. 4).

Nrf2 Knockdown and Inflammatory Markers

The NF-κB signaling pathway plays a crucial role in the initiation, amplification and resolution of inflammation by controlling the expression of multiple inflammatory mediators. In the cytosol, NF-κB is linked to its inhibitory molecule IκB. When IκB is degraded in response to various stimuli, NF-κB translocates to the nucleus where it activates the transcription of pro-inflammatory target genes. Administration of Fe/Asc to Caco-2/15 enhanced NF-κB (Fig. 5B) and decreased IκB (Fig. 5C). This effect was

Fe/Asc concentration-dependent. Measurement of TNF-α in Caco-2/15 treated with increasing concentrations of Fe/Asc showed a similar increase pattern (Fig. 5A). In Nrf2 shRNA-infected cells, NF-κB protein expression is increased (Fig. 6B) and IκBα protein expression is reduced (Fig. 6C) compared with SV, suggesting that the inhibitory protein is degraded in the proteasome, leaving NF-κB free to enter the nucleus and activate the transcription of its target genes. Accordingly, the protein expression of TNF-α, a target of NF-κB, was increased in Nrf2 shRNA-infected cells (Fig. 6A). Administration of OPZ prevented the changes of NF-κB, IκB and TNF-α in Nrf2 shRNA-infected cells.

Nrf2 Involvement in Oxidative Phosphorylation Activity

We evaluated the activity of mitochondrial oxidative phosphorylation (OXPHOS) in Caco-2/15 cells treated with Nrf2 shRNA and/or OPZ. Our findings documented a significant reduction in the specific activities of complex I, II, III and IV in Nrf2 shRNA-infected Caco-2/15 cells (Fig. 7) compared with SV. Moreover, OPZ treatment enhanced the activities of complex I, II, III and IV in control Caco-2/15 cells, but only produced a moderate upregulation of these mitochondrial enzymes in the Nrf2 shRNA-infected Caco-2 cells.

Relationship between Nrf2 Silencing and Mitochondrial ATP Levels

The main function of the mitochondrion is the production of energy in the form of ATP via OXPHOS and oxygen consumption. We therefore assessed the amount of ATP levels in Nrf2 shRNA-infected Caco-2 cells in the presence and absence of OPZ. As noted in Figure 8, there was a significant reduction in ATP levels in Nrf2 shRNA cells compared with SV-treated cells. Furthermore, pre-incubation of Caco-2/15 with OPZ resulted in a

significant rise in ATP levels compared with cells without OPZ. These results indicate the regulatory role of Nrf2 in mitochondrial ATP production.

Nrf2 Regulation of Prohibitin

Prohibitin (PHB), a chaperon protein located on the inner mitochondrial membrane, exhibits antioxidant and anti-inflammatory effects [31,32]. Our results show a dramatic reduction in PHB levels after Fe/Asc-induced oxidative stress in Caco-2 cells (Fig. 9A). A similar effect was recorded with Nrf2 inactivation (Fig. 9B). OPZ was able to substantially increase PHB protein expression in SV, but less markedly in Nrf2 shRNA Caco-2/15 cells.

Nrf2 and Intestinal Permeability

Intestinal permeability defects may contribute to intestinal loss of cell junctional sealing by defective tight junctions and may be involved in the pathogenesis of inflammatory bowel diseases [33,34]. Figure 10 shows a significant reduction in occludin protein levels, one of major TJ transmembrane barrier proteins, in Caco-2/15 cells treated with high concentration of Fe/Asc (Fig. 10A) or Nrf2 shRNA (Fig. 10B). OPZ showed little effect on Nrf2 decline in Nrf2 shRNA Caco-2/15 cells.

Nrf2 Anti-Apoptotic Effects via Upregulation of Bcl-2

In the process of programmed cell death, mediators of apoptosis are released from mitochondria through disruptions in the outer mitochondrial membrane and then participate in caspase activation and DNA degradation. In our study, we focused on one specific mediator of the outer mitochondrial membrane, Bcl-2. High concentrations of Fe/Asc lowered Bcl-2 protein expression in control cells (Fig. 11A). Simple ablation of Nrf2 led to similar negative results (Fig. 11B). Treatment with OPZ markedly augmented

Bcl-2 protein expression but was unable to display an equivalent effect in Nrf2 shRNA Caco-2/15.

Nrf2 Contribution to Mitochondrial Biogenesis via Regulation of PGC-1α

PGC-1α is a key player in ROS-induced mitochondrial biogenesis. Nrf2 serves as a cellular sensor for oxidative stress and PGC-1α. We determined the effect of oxidative stress and Nrf2 knock-down on mitochondrial biogenesis by measuring PGC-1α expression in Caco-2/15 cells treated with various doses of Fe/Asc or infected with Nrf2 shRNA**.** We observed a significant reduction in PGC-1α protein expression in Caco-2/15 cells treated with either with Fe/Asc (Fig. 12A) or in Nrf2 shRNA transfection (Fig. 12B) compared with untreated cells. Along with these results, OPZ was capable of inducing PGC-1α protein expression only in SV cells.

Discussion

Multiple studies have shown the importance of oxidative stress and inflammation in the pathogenesis of intestinal diseases following the attenuation of oxidative stress by antioxidant treatment in cell culture and colitis prevention in experimental animal models [35-37]. Despite these promising results, antioxidants have not proven to be effective in human diseases, though the expression of most antioxidant enzymes is tightly controlled upon Nrf2 activation [3,5]. In the present study, we identify several new pathways positively regulated by Nrf2 and we provide evidence for its direct or indirect implication in counteracting oxidative stress, inflammation and mitochondrial dysfunction in intestinal epithelial cells.

In order to examine a variety of intestinal functions, we used the Caco-2/15 cell line that exhibits many of the features of small intestinal epithelial cells and reproduces several of the normal physiological responses to various regulatory agents [21,22]. In addition, we employed the powerful Fe/Asc oxygen radical-generating system to investigate the modulation of Caco-2/15 cell mitochondrial function by oxidative stress [38,39].

In this study, we treat Caco-2/15 cells with increasing concentrations of Fe/Asc (0.05/2mM, 0.1/2mM, 0.2/2mM), which displayed different impacts on lipid peroxidation and on Nrf2 modulation. We found an increase in MDA levels, a marker of lipid peroxidation, in a dose-dependent manner. Interestingly, at low concentrations, Fe/Asc augmented Nrf2 protein levels compared with control cells. This typical response to mild oxidative stress is physiologically relevant, as it could lead to Nrf2 dissociation from its inhibitor Keap1 and its translocation to the nucleus, inducing a protective response. In contrast, increasing the concentration of Fe/Asc (0.2/2mM) led to reduced Nrf2 protein

levels. This may refer to severe oxidative stress observed in pathological states and could possibly be associated with increased Nrf2 degradation, leading to lower translocation to the nucleus.

OPZ is a chemopreventive compound that induces antioxidant protection either by a mechanism involving a cellular response to increased production of ROS [40] or the perturbation of the Keap1-Nrf2 complex secondary to Keap1 cysteine residue modification ultimately leading to Nrf2 dislocation [41]. In our study, optimal concentrations of OPZ (50 μM) induced a significant Nrf2 increase and effectively attenuated cellular oxidative stress. Our findings, showing that the Nrf2 knock-down abrogates the protective effects of OPZ against Fe/Asc provide concrete evidence about the direct contribution of Nrf2 to antioxidant actions.

The increase in SOD, CAT and GPx activities following OPZ treatment is consistent with a previous study by Ramos-Gomez and collaborators, which demonstrated that loss of expression of Nrf2 completely abrogates the protective effect of OPZ. This would be a more direct observation of the relationship between these 2 pathways [42]. Further support for our data is evidenced by a previous report noting that sulforaphane, an OPZ activator, stimulated Nrf2 gene-dependent antioxidant enzyme activity and protected against oxidative stress in gastric mucosa [13]. Finally, Nelson et al. observed that OPZ is effective in protecting retinal pigment epithelial cells from oxidative damage via Nrf2 production [41].

Our results highlighted the anti-inflammatory properties of Nrf2. We observed a reduction in NF-κβ, TNF-α and an upregulation of Iκβ levels upon induction of Nrf2 with OPZ. Conversely, inhibition of Nrf2 shRNA infection enhanced NF-κβ and TNF-α, but down-regulated Iκβ. Similarly, increased concentrations of Fe/Asc stimulated NF-κβ and TNF-α but showed opposite effects on Iκβ. This is consistent with previous studies by Khor et al. who observed raised levels of pro-inflammatory mediators, such as IL-6 and TNF-α, in the colonic tissues of Nrf2-*deficient* mice [12]. It has also been noted that NF-κβ activation could be attenuated by Nrf2 activators, such as phenethyl isothiocyanate and curcumin [43]. Supportive data were noted in cystic fibrosis epithelia where dysfunction of Nrf2 leads to excess intracellular H_2O_2 and pro-inflammatory cytokine production [44]. Nevertheless additional studies are needed to show that Nrf2 can directly inhibit NF-kβ.

PHB is an ubiquitously expressed and highly conserved protein that has multiple functions, including antioxidant defense, anti-inflammatory responses, cellular proliferation, apoptosis regulation and mitochondrial protein folding [45]. PHB is localized on the cell membrane, mitochondrial membrane or nucleus, depending on cell type and physiological conditions [31,46]. PHB has been shown to link two subunits of complex IV in yeast [47] and with subunits of complex I in mammals [48]. A recent study suggests that PHB is a regulator of Nrf2 expression during oxidative states and prevents inflammation-associated oxidative stress through sustained activation of Nrf2 [46]. It has been demonstrated that Caco-2 cells overexpressing PHB are protected from oxidative stress, whereas PHB is decreased in experimental colitis [31]. In the current

investigation, we demonstrated that Nrf2 activation by OPZ enhances mitochondrial PHB expression, while Nrf2 knockdown led to a reduction in mitochondrial PHB. A decrease in PHB was also observed with high Fe/Asc concentrations in the experimental system. Therefore, we suggest that Nrf2 is a master regulator of PHB expression. One possible explanation for these findings is that PHB and Nrf2 regulations are dependent and have synergistic actions within the cells, but such proposal has to be validated. Muraguchi et al. have observed that PHB overexpression inhibits the hypoxia-induced decrease in mitochondrial membrane potential levels and Bcl-2 [49]. Thus, it is reasonable to propose that there is a functional triangle linking Nrf2, PHB and Bcl-2, which share anti-apoptotic and antioxidant functions.

Surprisingly, few studies have focused on the relationship between Nrf2 and apoptosis [4]. Our results provide evidence for the first time that Nrf2 upregulation by OPZ contributes to elevation of Bcl-2 anti-apoptotic protein in Caco-2/15 cells, while Nrf2 knockdown or increasing Fe/Asc concentrations downregulates Bcl-2. Various hypotheses can be proposed to explain Nrf2 antiapoptotic activities. The upregulation of glutathione, SOD and CAT mediated by Nrf2 is likely to be one of the mechanisms by which these antioxidant enzymes prevent inflammation and cell death. Accordingly, recent data have reported that Nrf2 overexpression protects cells from Fas-mediated apoptosis, suggesting an important role for Nrf2 in antiapoptotic pathways [50]. On the other hand, it may be suggested that Nrf2 induces the expression of prosurvival Bcl-2 family members directly or indirectly via upregulation of PHB, since previous work demonstrated repression of proapoptotic BAX/BAK expression via PHB upregulation

[51]. In line with these observations, treatment with resveratrol, an Nrf2 activator, prevented vascular apoptotic cell death in high fat diet Nrf2+/+ mice, without the same success in high fat diet Nrf2-/- mice[52]. In summary, we suggest that Nrf2 acts as an important antiapoptotic mediator and that the underlying mechanism involves activation of Bcl-2.

The disruption of intestinal epithelial barrier function has traditionally been attributed to proinflammatory cytokines. As noted previously, TNF-α generation reduces intestinal epithelial TJ permeability and contributes to intestinal barrier damage [53]. We have explored the possibility of Nrf2 involvement in the regulatory function of intestinal TJs such as occludin. For the first time, we have established an association between Nrf2 and occludin, since Nrf2 induction with OPZ upregulated occludin levels. We have also demonstrated that Caco-2/15 cells treated with Nrf2 shRNA or with increasing concentrations of Fe/Asc display lower occludin levels. Our results are in line with previous data suggesting a reduction in the proteasome-mediated degradation of occludin in the intestinal mucosa of subjects with inflammatory bowel syndrome [54]. Assembly of occludin in the TJ network was also observed with confocal microscopy in Caco-2 cells in response to Nrf2 induction by kaempferol, a natural flavonoid [55]. Overall, these data suggest a central role for Nrf2 in small intestine integrity preservation.

Mitochondrial biogenesis is dependent on external stimuli and an orchestrated crosstalk between the nuclear and mitochondrial genomes and PGC-1α may represent a crucial factor linking external stimuli to mitochondrial biogenesis [56]. For example, PGC-1α

could interact with Nrf2 to transactivate a number of genes involved in mitochondrial functions such as OXPHOS as well as in protein import and heme biosynthesis. PGC-1α also mediates mitochondrial DNA transcription and replication through two nuclear-encoded genes: mitochondrial transcription factor A and B [57]. Our results provide strong evidence that enhancing Nrf2 activity by OPZ in Caco-2/15 cells contributes to mitochondrial biogenesis and function via upregulation of PGC-1α, an increase in mitochondrial enzymatic activity and ultimately a rise in ATP production. Surprisingly, we found a dramatic decrease in PGC-1α in Caco-2/15 infected with Nrf2 shRNA or incubated with high concentrations of Fe/Asc in comparison with SV-treated or control cells. Our results are in agreement with a previous study showing a significant reduction in PGC-1α in retinal epithelial cells treated with oxidative stress [58]. Since mice with a targeted disruption of PGC-1α remained viable and showed no change in mitochondrial abundance or morphology in the liver, we propose that Nrf2 could act in an independent fashion to enhance mitochondrial function and biogenesis [59]. Thus, the absence of a dramatic mitochondrial biogenesis defect in PGC-1α null mice may result from compensatory mechanisms. It remains an open question as to whether Nrf2 can support basal levels of mitochondrial biogenesis independent of PGC-1α and whether Nrf2 overexpression leading to increased PGC-1α levels represents a physiologically meaningful pathway of retrograde regulation in human cells. Accordingly, a recent study showed that Nrf2 could bind to cytochrome oxidase subunit IV promoters prior the activation of PGC-1α [60].

We previously demonstrated that an accumulation of lipid peroxidation products in the mitochondria due to high concentrations of Fe/Asc leads to a decrease in ATP production

and compromises the maintenance of cellular homeostasis [21]. Similarly, in this study, incubation of Caco-2/15 cells with Nrf2 shRNA induced a marked decrease in ATP production. In contrast, ATP production was significantly increased in Caco-2/15 cells following treatment with OPZ. Therefore, our data are consistent with previous investigations documenting a decline in ATP in HT-29 intestinal cells with Nrf2 deletion [61]. In the present investigation, the decrease in ATP synthesis is probably related to the low mitochondrial metabolic activity resulting from Nrf2 knockdown-mediated lipid peroxidation. We reasonably suggest that mitochondrial dysfunction in cells treated with Nrf2 shRNA is due to the low efficiency of mitochondrial respiratory chain enzymes, followed by a decline in ATP production.

Taken together, our data indicate that an increase in Nrf2 in intestinal epithelial cells plays a pivotal role in positively modulating cellular defense and maintaining cellular integrity against toxic insults. For the first time, our study reveals the capacity of Nrf2 to regulate many intestinal genes and enzymes (occludin, PHB, PGC-1α, Bcl-2) related to cell permeability and mitochondrial functions. It is quite possible that some of these expressed proteins could be secondary to or indirectly affected by Nrf2-controlled genes. Therefore, further studies are required to test this assumption.

Figures and Legends

Fig. 1: Nrf2 Expression and MDA Level in Caco-2/15 Cells Treated with Increasing Amount of Fe/Asc

Caco-2/15 cells treated with increasing amount of Fe/Asc. Lipid peroxidation was evaluated by measuring MDA with HPLC (Fig. 1A). RT-PCR and Western blot were performed to assess Nrf2 mRNA (Fig. 1B) and protein levels (Fig. 1C), respectively. Cells that were not treated with Fe/Asc served as basic controls. Results represent the means ± SEM of n = 3 independent experiments. *$P < 0.05$ vs. control cells.

Fig. 2 Nrf2 Modulation in Caco-2/15 Cells Treated with OPZ.

RT-PCR and Western blot were used to evaluate the modulation of Nrf2 gene expression (Fig. 2A) and protein levels (Fig. 2B), respectively, in Caco-2/15 following treatment with 50 μM of OPZ. Results represent the means ± SEM of n = 3 independent experiments. *$P < 0.05$ vs. control cells.

Fig. 3 Nrf2 Expression in Caco-2/15 Cells Infected with Short Hairpin RNAs (shRNAs)

Following lentivirus infection, Caco-2/15 cells were tested for Nrf2 gene (Fig. 3A) and protein (Fig. 3B) expression. RT-PCR was performed for gene expression and Western blot for protein mass. Caco-2/15 cells were infected with various concentrations of Nrf2 shRNA. Cells that were not treated with lentivirus served as basic controls. Western blot

was used to evaluate the level of Nrf2 protein (Fig. 3C) following treatment with 50 μM of OPZ in Caco-2/15 infected with shRNA. Results represent the means ± SEM of n = 3 independent experiments. *$P < 0.05$ vs. control cells or SV cells.

Fig. 4 Endogenous Antioxidant Activities in Caco-2/15 Cells Infected with Nrf2 shRNA

Enzyme activities of CAT, GPx and SOD (respectively, Fig. 4.A, Fig. 4B and Fig. 4C) were measured by spectrophotometric assays in Caco-2/15 cells in response to Nrf2 knockdown with or without OPZ. Enzyme activities are expressed as nmol/min/mg protein. Results represent the means ± SEM of n = 3 independent experiments. *$P < 0.05$ vs. SV cells.

Fig. 5: Inflammatory Mediators in response to Fe/ASC Treatment in Caco-2/15 Cells

Western blots were performed to determine TNFα, NF-κβ and IκB (respectively, Fig. 5A, Fig. 5B and Fig. 5C) protein levels in Caco-2/15 cells treated with increasing concentrations of Fe/Asc. Results represent the means ± SEM of n = 3 independent experiments. *$P < 0.05$ vs. control cells.

Fig. 6: Inflammatory Mediators in Response to Nrf2 Knockdown in Caco-2/15 Cells

Western blots were performed to determine TNFα, NF-κβ and IκB (respectively Fig. 6A, Fig. 6B and Fig. 6C) protein levels in Caco-2/15 cells infected with Nrf2 shRNA. Results represent the means ± SEM of n = 3 independent experiments. *$P < 0.05$ vs. SV cells.

Fig. 7 Regulation of Mitochondrial Respiratory Chain Complexes Activities in Nrf2 shRNA Caco-2/15 Cells

Enzyme activities of mitochondrial respiratory chain complexes I, II, III, IV (respectively Fig. 7A, Fig. 7B, Fig. 7C and Fig. 7D) were measured by spectrophotometric assays in Caco-2/15 cells treated with Nrf2 shRNA and with or without OPZ. Enzyme activities are expressed as nmol/min mg protein. Results represent the means ± SEM of n = 3 independent experiments. **P* < 0.05 vs. control cells or SV cells.

Fig. 8 ATP Levels in Nrf2 shRNA Caco-2/15 Cells

Intracellular ATP was measured by bioluminescence assay. Caco-2/15 cells were treated with Nrf2 shRNA with or without OPZ. Results are expressed as ng of ATP per mg of cellular protein and represent the means ± SEM of n = 3 independent experiments. **P* < 0.05 vs. control cells.

Fig. 9 Modification of PHB Expression in Caco-2/15 Cells Treated with Fe/Asc or Nrf2 shRNA

Western blots were performed to detect the protein level of PHB in Caco-2/15 cells treated with increasing concentration of Fe/Asc (Fig. 9A) or with Nrf2 shRNA (Fig. 9B). Results represent the means ± SEM of n = 3 independent experiments. **P* < 0.05 vs. control cells or SV cells.

Fig. 10 Occludin Regulation in Caco-2/15 Cells Treated with Fe/Asc or Nrf2 shRNA

Occludin protein levels were determined by Western blot in Caco-2/15 cells treated with increasing concentrations of Fe/Asc (Fig. 10A) or with Nrf2 shRNA (Fig. 10B). Results represent the means ± SEM of n = 3 independent experiments. *$P < 0.05$ vs. control cells or SV cells.

Fig. 11 Disruption of Bcl2 in Caco-2/15 Cells Treated with Fe/Asc or Nrf2 shRNA

Bcl-2 protein levels were determined by Western blot in Caco-2/15 cells treated with increasing concentrations of Fe/Asc (Fig. 11A) or with Nrf2 shRNA (Fig. 11B). Results represent the means ± SEM of n = 3 independent experiments. *$P < 0.05$ vs. control cells or SV cells.

Fig. 12 Decrease of PGC-1α in Caco-2/15 Cells Treated with Fe/Asc or Nrf2 shRNA

PGC-1α protein levels were determined by Western blot in Caco-2/15 cells treated with increasing concentrations of Fe/Asc (Fig. 12A) or with Nrf2 shRNA (Fig. 12B). Results represent the means ± SEM of n = 3 independent experiments. *$P < 0.05$ vs. control cells or SV cells.

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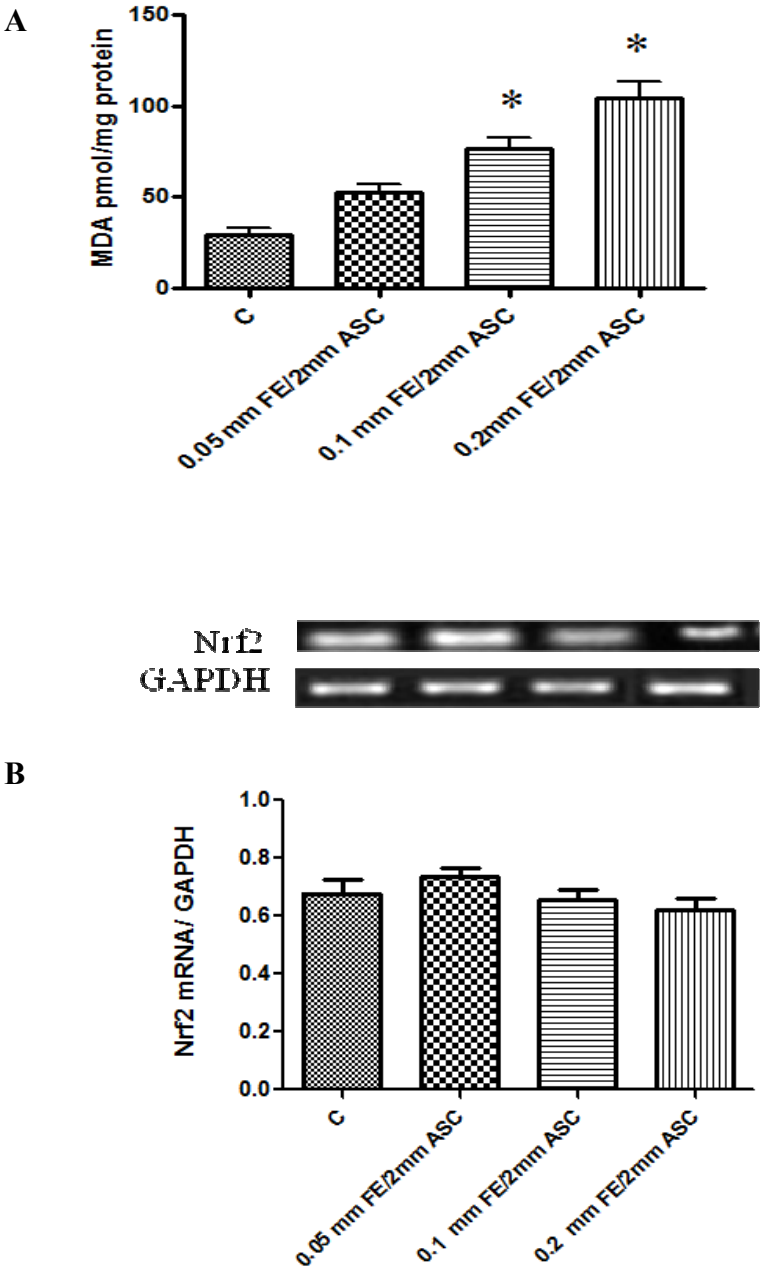
Figure 1.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0.1 mm FE/2mm ASC
0.2 mm FE/2mm ASC

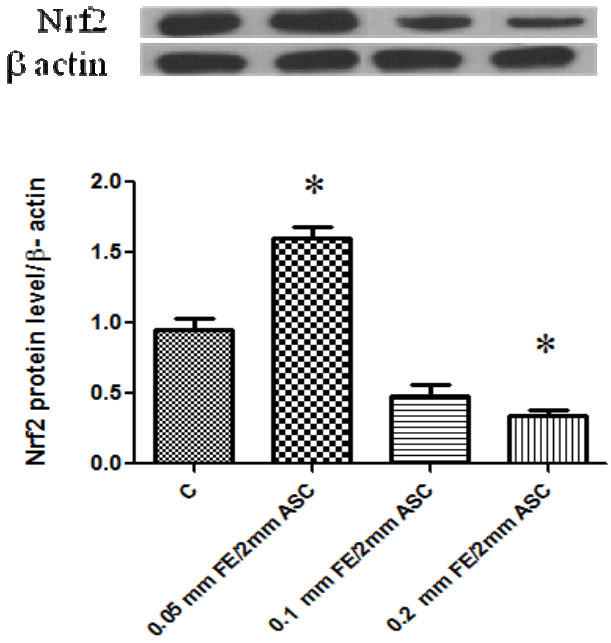
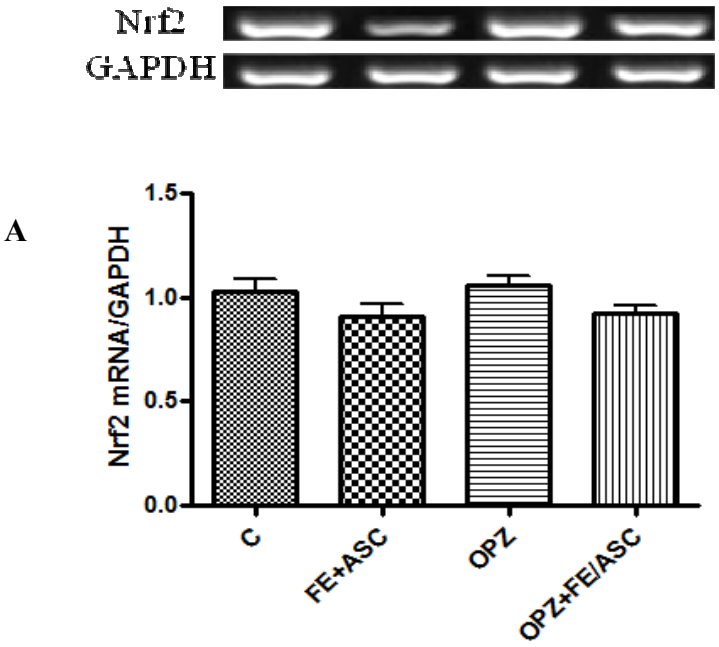

Nrf2
β actin
C
Nrf2 protein level/β- actin
2.0
1.5
1.0
0.5
0.0
*
*
C
0.05 mm FE/2mm ASC
0.1 mm FE/2mm ASC
0.2 mm FE/2mm ASC

Figure 2.


Nrf2
GAPDH
A
Nrf2 mRNA/GAPDH
1.5
1.0
0.5
0.0
C
FE+ASC
OPZ
OPZ+FE/ASC

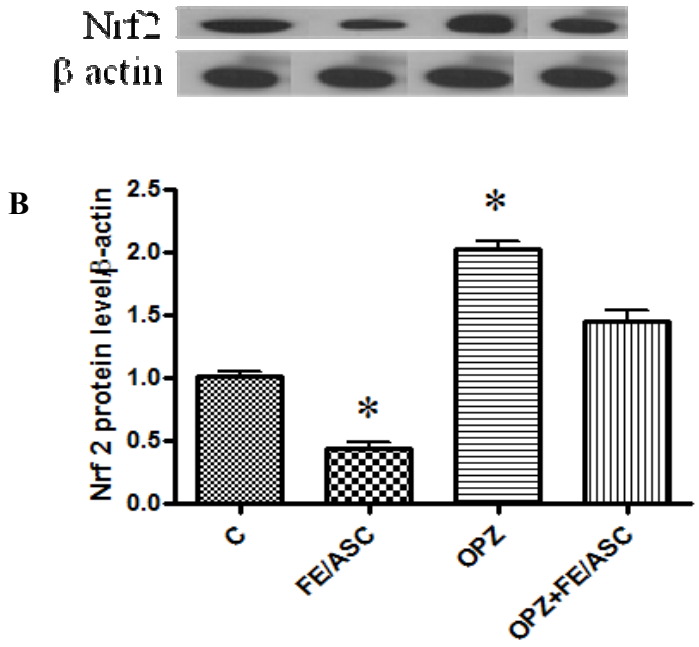
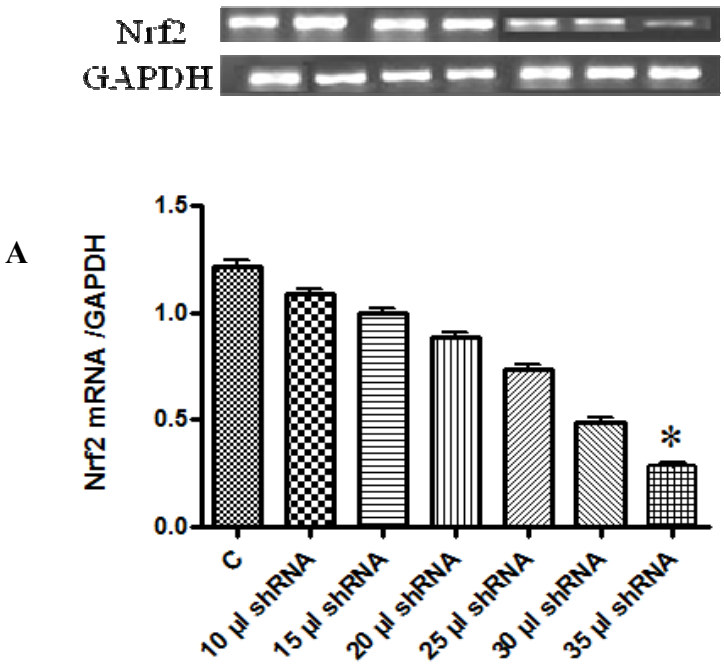
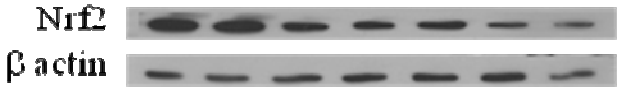
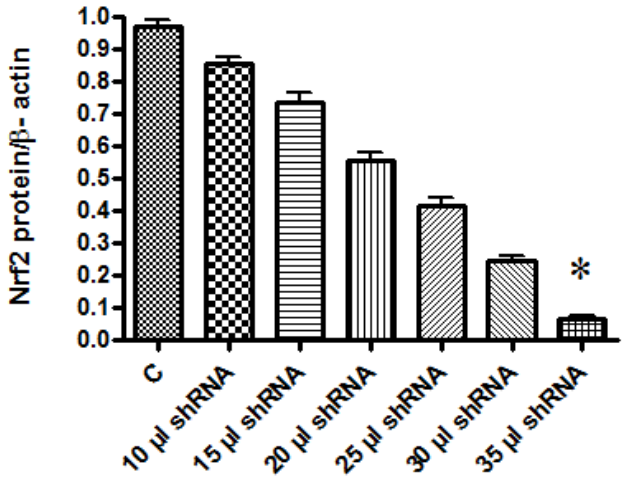

Nrf2
β actin
B
Nrf 2 protein level/β-actin
2.5
2.0
1.5
1.0
0.5
0.0
*
*
C
FE/ASC
OPZ
OPZ+FE/ASC

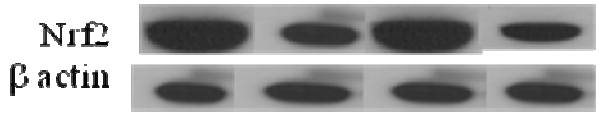
Figure 3.


Nrf2
GAPDH
A
Nrf2 mRNA /GAPDH
1.5
1.0
0.5
0.0
*
C
10 μl shRNA
15 μl shRNA
20 μl shRNA
25 μl shRNA
30 μl shRNA
35 μl shRNA


Nrf2
β actin

B


Nrf2 protein/β- actin
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
C
10 μl shRNA
15 μl shRNA
20 μl shRNA
25 μl shRNA
30 μl shRNA
35 μl shRNA


Nrf2
β actin

C

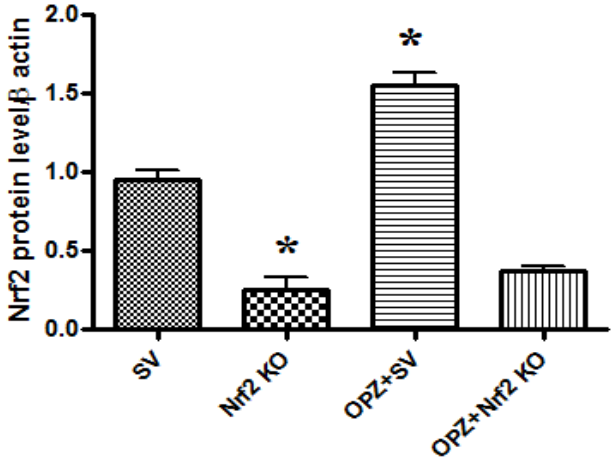

Nrf2 protein level/β actin
2.0
1.5
1.0
0.5
0.0
*
*
SV
Nrf2 KO
OPZ+SV
OPZ+Nrf2 KO

Figure 4.

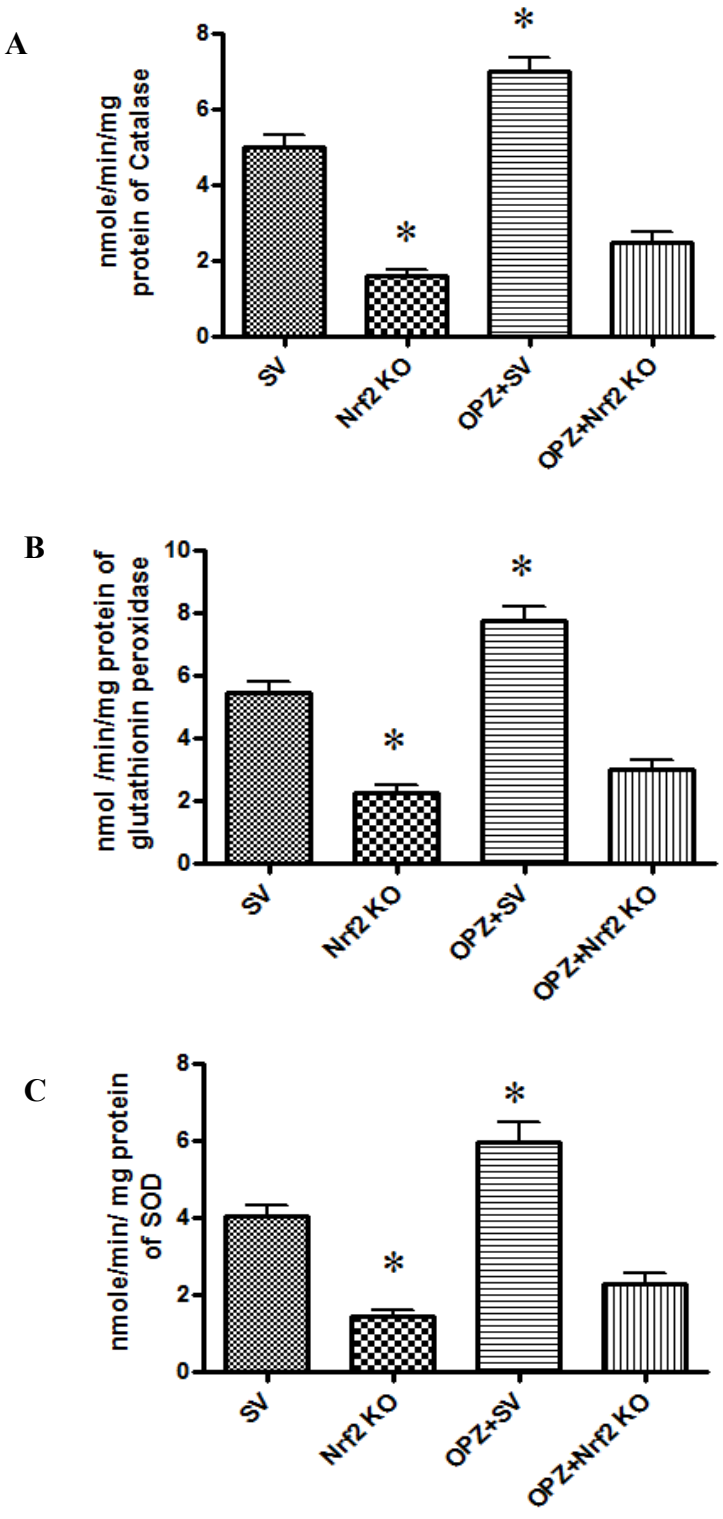
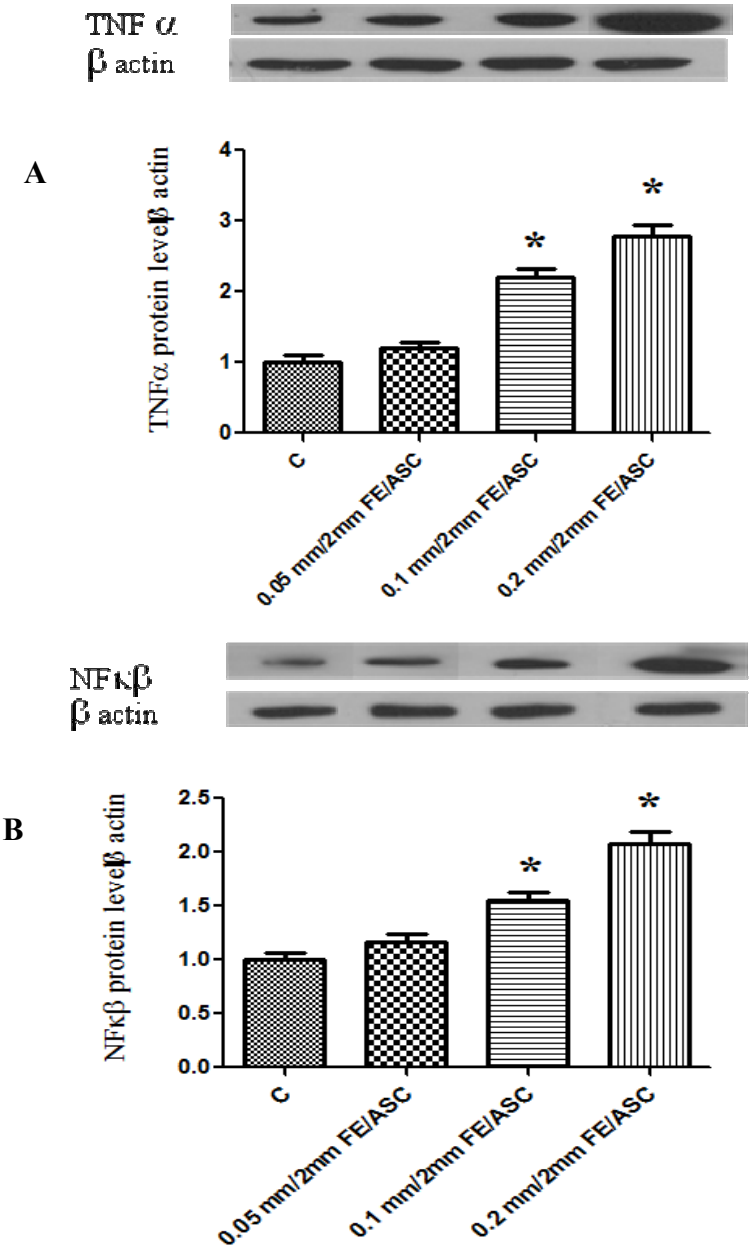

A
nmole/min/mg protein of Catalase
0
2
4
6
8
*
*
SV
Nrf2 KO
OPZ+SV
OPZ+Nrf2 KO
B
nmol /min/mg protein of glutathionin peroxidase
0
2
4
6
8
10
*
*
SV
Nrf2 KO
OPZ+SV
OPZ+Nrf2 KO
C
nmole/min/ mg protein of SOD
0
2
4
6
8
*
*
SV
Nrf2 KO
OPZ+SV
OPZ+Nrf2 KO

Figure 5.


TNF α
β actin
A
TNFα protein level/β actin
4
3
2
1
0
*
*
C
0.05 mm/2mm FE/ASC
0.1 mm/2mm FE/ASC
0.2 mm/2mm FE/ASC
NFκβ
β actin
B
NFκβ protein level/β actin
2.5
2.0
1.5
1.0
0.5
0.0
*
*
C
0.05 mm/2mm FE/ASC
0.1 mm/2mm FE/ASC
0.2 mm/2mm FE/ASC

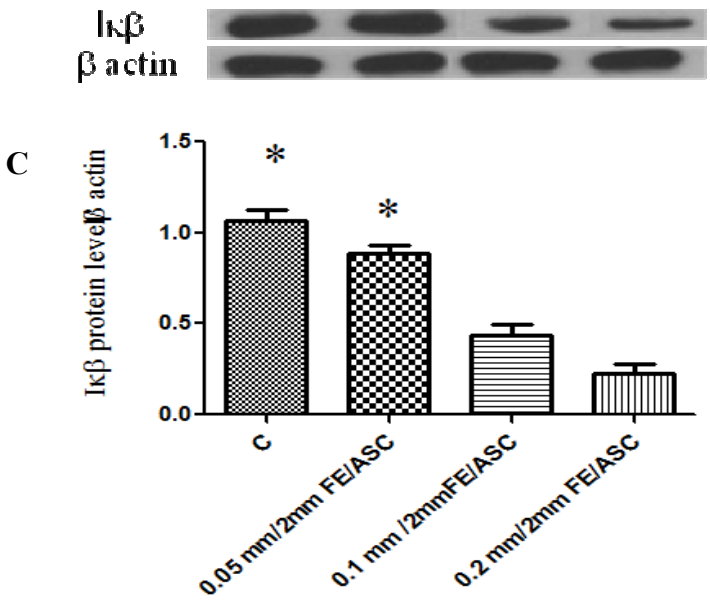
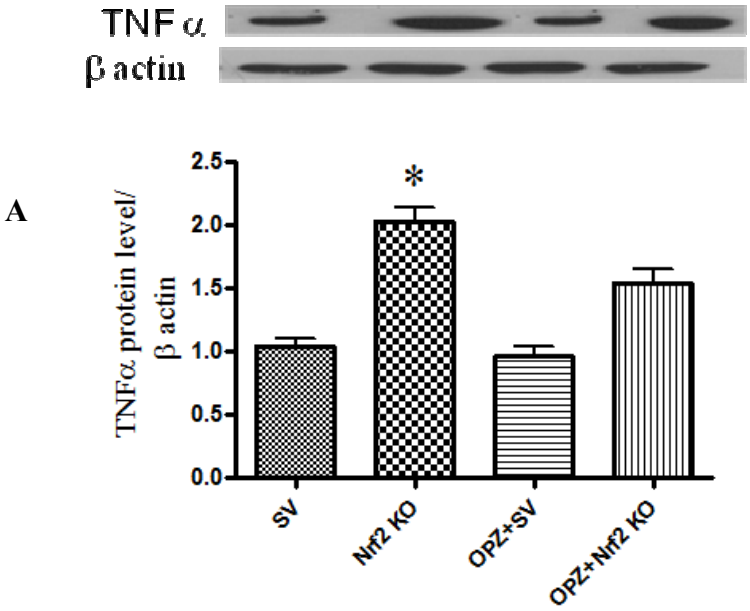
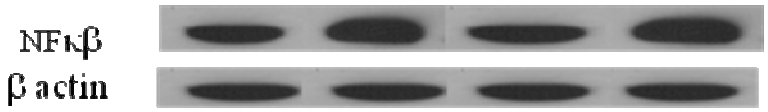
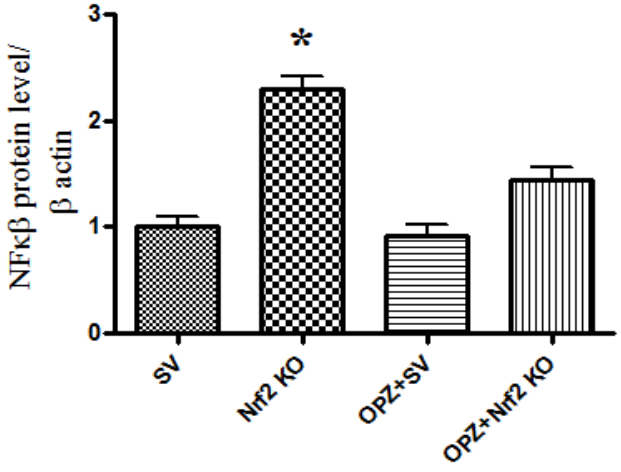

Iκβ
β actin
C
Iκβ protein level/β actin
1.5
1.0
0.5
0.0
*
*
C
0.05 mm/2mm FE/ASC
0.1 mm /2mmFE/ASC
0.2 mm/2mm FE/ASC

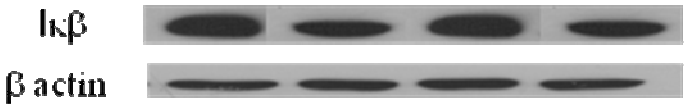
Figure 6.


TNF α
β actin
A
TNFα protein level/
β actin
2.5
2.0
1.5
1.0
0.5
0.0
*
SV
Nrf2 KO
OPZ+SV
OPZ+Nrf2 KO


NFκβ
β actin

B


NFκβ protein level/
β actin
3
2
1
0
*
SV
Nrf2 KO
OPZ+SV
OPZ+Nrf2 KO


Iκβ
β actin

C

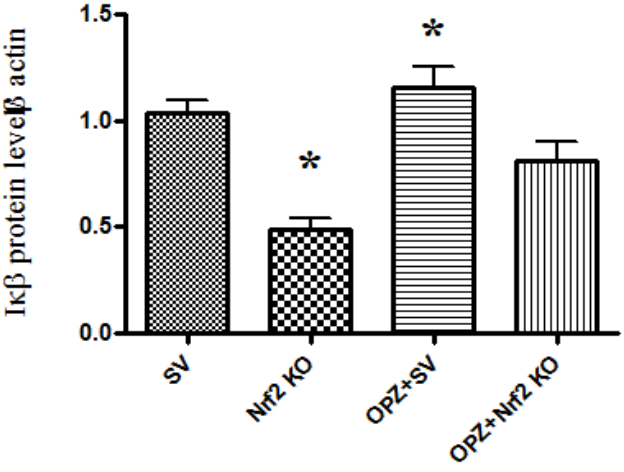
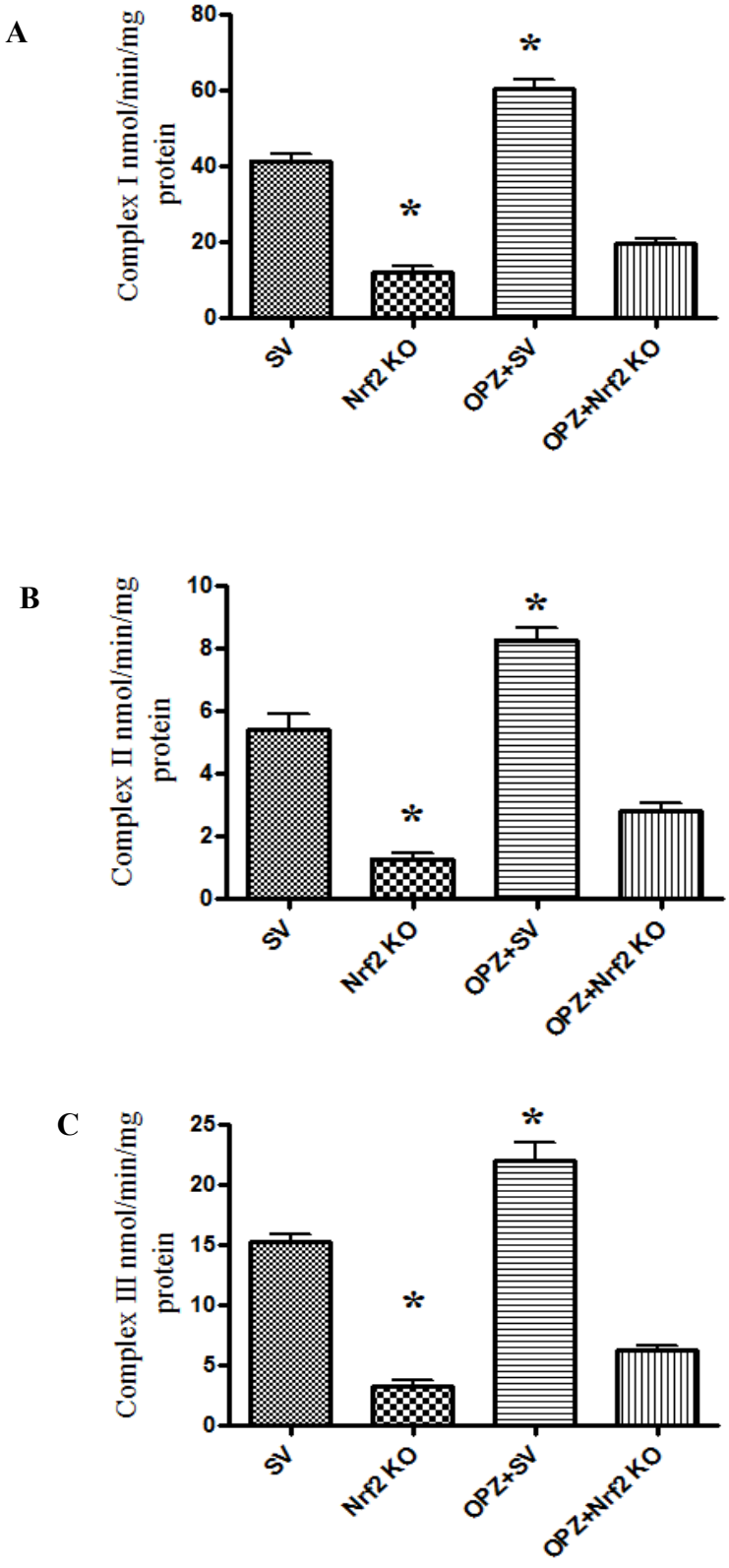

Iκβ protein level/β actin
1.5
1.0
0.5
0.0
*
*
SV
Nrf2 KO
OPZ+SV
OPZ+Nrf2 KO

Figure 7.


A
Complex I nmol/min/mg protein
80
60
40
20
0
*
*
SV
Nrf2 KO
OPZ+SV
OPZ+Nrf2 KO
B
Complex II nmol/min/mg protein
10
8
6
4
2
0
*
*
SV
Nrf2 KO
OPZ+SV
OPZ+Nrf2 KO
C
Complex III nmol/min/mg protein
25
20
15
10
5
0
*
*
SV
Nrf2 KO
OPZ+SV
OPZ+Nrf2 KO

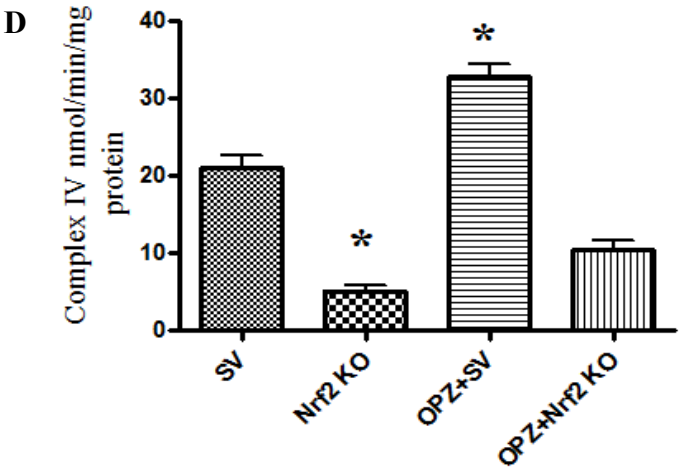

D
Complex IV nmol/min/mg protein
40
30
20
10
0
*
*
SV
Nrf2 KO
OPZ+SV
OPZ+Nrf2 KO

Figure 8.

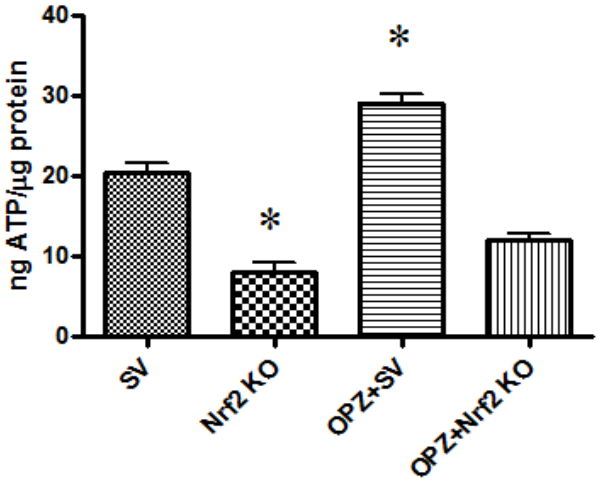

ng ATP/µg protein
40
30
20
10
0
*
*
SV
Nrf2 KO
OPZ+SV
OPZ+Nrf2 KO

Figure 9.

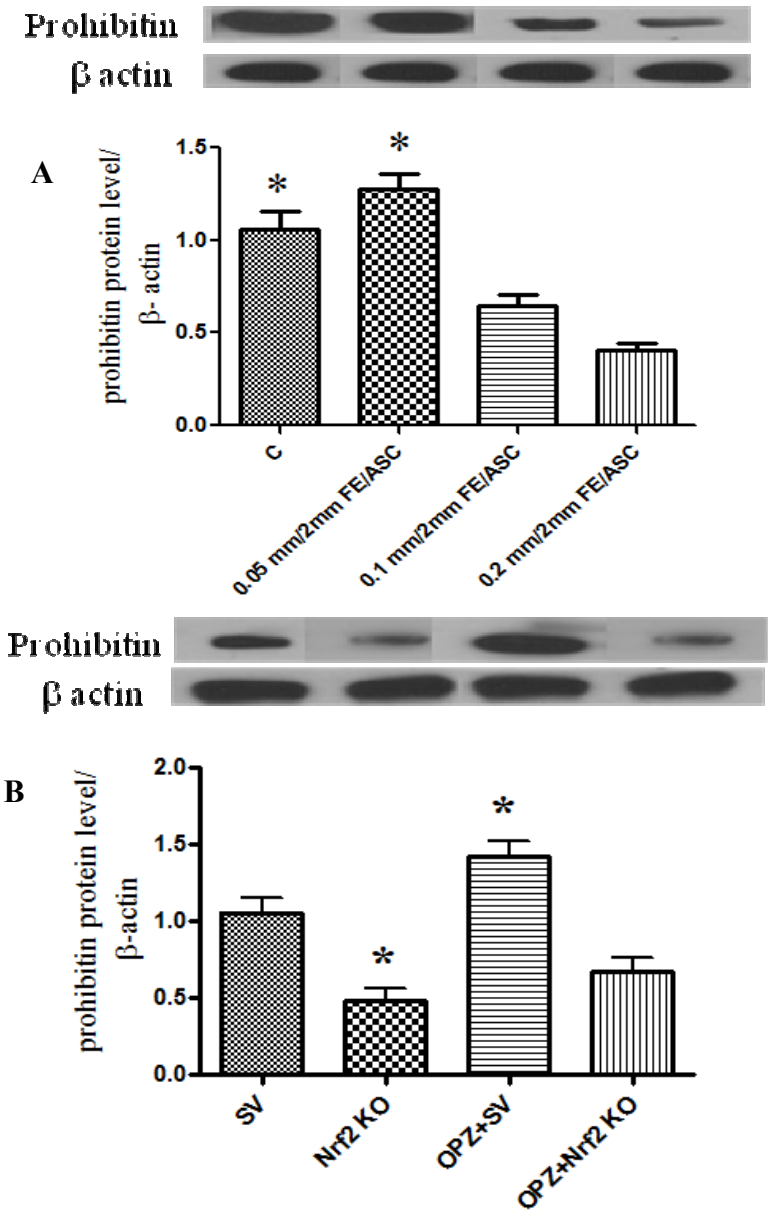

Prohibitin
β actin
A
prohibitin protein level/ β- actin
1.5
1.0
0.5
0.0
*
*
C
0.05 mm/2mm FE/ASC
0.1 mm/2mm FE/ASC
0.2 mm/2mm FE/ASC
Prohibitin
β actin
B
prohibitin protein level/ β-actin
2.0
1.5
1.0
0.5
0.0
*
*
SV
Nrf2 KO
OPZ+SV
OPZ+Nrf2 KO

Figure 10.

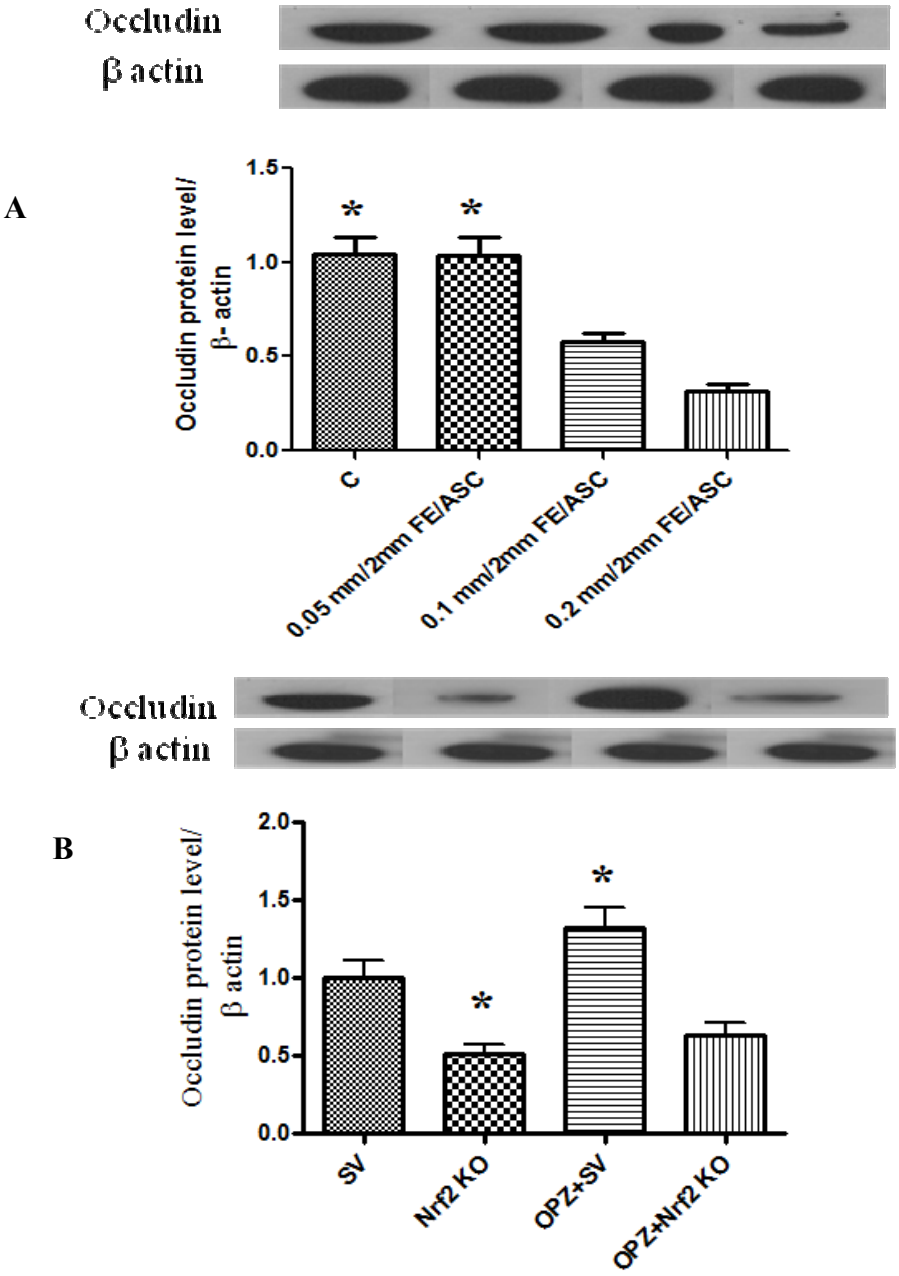

Occludin
β actin
A
Occludin protein level/ β- actin
1.5
1.0
0.5
0.0
*
*
C
0.05 mm/2mm FE/ASC
0.1 mm/2mm FE/ASC
0.2 mm/2mm FE/ASC
Occludin
β actin
B
Occludin protein level/ β actin
2.0
1.5
1.0
0.5
0.0
*
*
SV
Nrf2 KO
OPZ+SV
OPZ+Nrf2 KO

Figure 11.

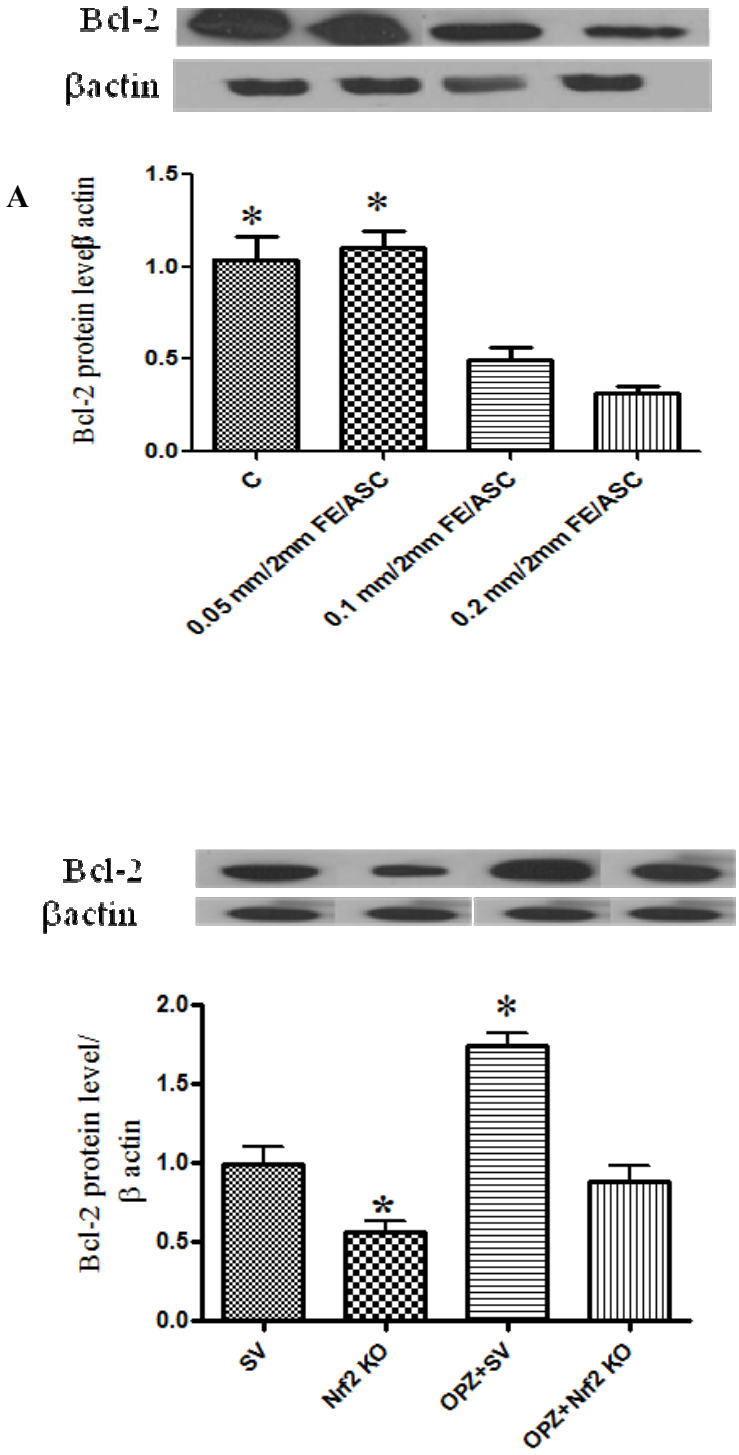
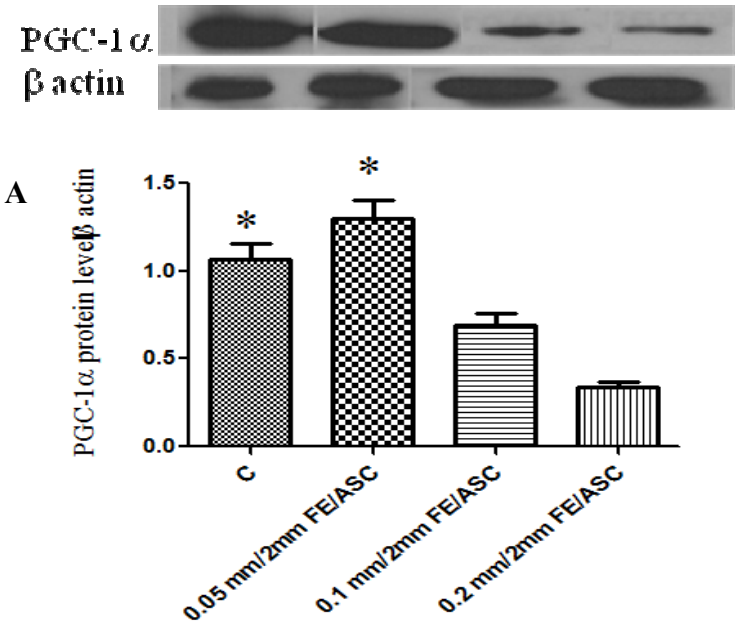
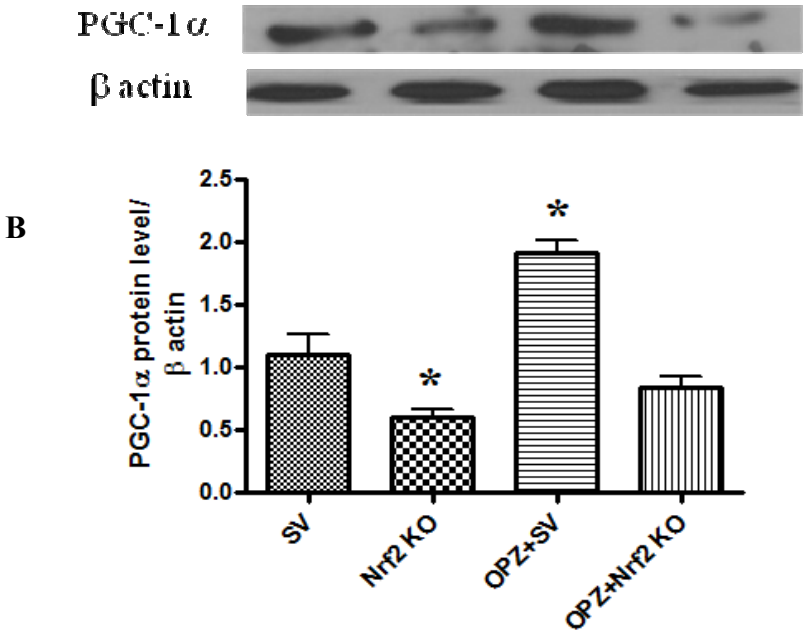

Bcl-2
βactin
A
Bcl-2 protein level/β actin
1.5
1.0
0.5
0.0
*
*
C
0.05 mm/2mm FE/ASC
0.1 mm/2mm FE/ASC
0.2 mm/2mm FE/ASC
Bcl-2
βactin
B
Bcl-2 protein level/
β actin
2.0
1.5
1.0
0.5
0.0
*
*
SV
Nrf2 KO
OPZ+SV
OPZ+Nrf2 KO

Figure 12.


PGC-1α
β actin
A
PGC-1α protein level/β actin
1.5
1.0
0.5
0.0
*
*
C
0.05 mm/2mm FE/ASC
0.1 mm/2mm FE/ASC
0.2 mm/2mm FE/ASC


PGC-1α
β actin
B
PGC-1α protein level/
β actin
2.5
2.0
1.5
1.0
0.5
0.0
*
*
SV
Nrf2 KO
OPZ+SV
OPZ+Nrf2 KO

6 DISCUSSION

The Caco-2/15 cell line has been used to examine a variety of intestinal functions [136, 165, 166]. Because Caco-2/15 cells exhibit many of the features of intestinal epithelial cells and reproduce several of the normal physiological responses to various regulatory agents, we have employed this intestinal model to determine whether the FE/ASC oxygen radical-generating system modulates mitochondrial functions.

Iron is the most abundant transition metal in mammalian cells and is essential for the physiological functions of multiple proteins. However, excess iron can be detrimental because it can initiate oxygen radical formation and promote ROS [21, 33].

It has been found that iron increases disease activity in colitis and this is associated with oxidative stress, neutrophilic infiltration and increased pro inflammatory cytokines [27]. Thus, iron may cause oxidative damage to biological macromolecules and alter the intracellular redox environment, thereby affecting redox-sensitive cell signaling pathways and transcription factors [18, 19]. Although the mechanisms underlying the cytotoxicity of iron in different tissues are not fully investigated, data have indicated the contribution of iron-mediated peroxidation in numerous pathological states, including atherosclerosis, cancer, ischemia-reperfusion injury, IBD and conditions of iron overload [22, 25, 33, 167].

Several studies have shown the ability of iron to initiate strong lipid peroxidation, whereas ascorbic acid can amplify the oxidative potential of iron by promoting metal ion-induced

lipid peroxidation [21, 168]. Our data indicate that the FE/ASC system functioned as a producer of lipid peroxidation and, at the same time, altered the function of mitochondria.

In this study, the incubation of Caco-2/15 cells with FE/ASC induced a marked lowering in ATP production level that is probably related to the low mitochondrial metabolic activity resulting from the FE/ASC-mediated lipid peroxidation. In fact, electron movement through complexes I, II, III and IV enables the movement of hydrogen ions across the inner membrane into the inter-membrane space, thus creating an electrochemical gradient, which is harnessed into ATP production by ATP synthase in complex V. We suggest that mitochondrial damage from ROS may lead to a degradation in the mitochondrial respiratory chain enzymes (complexes I, II, III, IV), and hence a decline in the ATP production. Finally, blockage of mitochondrial electron transport chain enzymes and/or ATP-synthase may account for the ATP inhibition triggered by the administration of FE/ASC to Caco-2/15 cells.

The increase in ROS production might interrupt the electron transport chain and affect ATP production. The possible mechanism may be attributable to ROS-induced cardiolipin damage. Under normal physiological conditions, cardiolipin anchors cytochrome C protein to the inner mitochondrial membrane where it participates in the electron transport between complexes III and IV of the respiratory chain. During oxidative stress, cardiolipin peroxidation could be crucial for enabling cytochrome C dissociation from the mitochondrial inner membrane along with anomalies of electron transport (between complexes III and IV of the respiratory chain) and ATP production.

Mitochondria also play a part in modulating the amplitude of Ca^{2+} signals through rapidly accumulating and releasing Ca^{2+} [67, 83]. However, excessive mitochondrial Ca^{2+} overload can trigger ROS overproduction, mitochondrial membrane depolarization and ATP inhibition [72, 83, 85]. Additionally, these defective processes may eventually lead to apoptosis [72, 87]. Therefore, molecules capable of minimizing mitochondrial Ca^{2+} overload would decrease mitochondrial ROS accumulation and improve mitochondrial energy production, which may impact on mitochondrial-oxidative mediated diseases.

The core human mitochondrial transcription machinery comprises a single subunit bacteriophage-related RNA polymerase (POLRMT), mt TFA and two transcriptional co-activator proteins, h-mtTFB1 and h-mtTFB2 [103]. Both factors appear to interact directly with POLRMT forming a heterodimer that, in addition to mt TFA, is required for the accurate initiation of both H_1 and L promoters. We demonstrated that mt TFA, mt TFB1, mtTFB2 transcriptional level and protein mass were augmented in the presence of FE/ASC. This increase in the experiments represents a compensatory mechanism in response to the oxidative stress-related condition.

In our experiments, we found that FE/ASC increase in 8-OHdG. It has been observed previously by other researchers that 8-OHdG represents one of the most frequently generated oxidative base lesions within DNA, and one of the most common threats to mitochondrial genome stability [73, 77, 86, 143]. Furthermore, nuclei were not affected by FE/ASC-mediated OS, which confirms that mt DNA is more vulnerable than nuclear DNA to oxidative damage given that it is situated close to the site of ROS generation and,

concomitantly, mitochondria lack protective histones and have far fewer mechanisms that prevent reduced base excision repair activity compared to nucleus DNA [143].

OGG1 is the DNA repair enzyme that recognizes and excises 8-oxodG [79, 80]. The present study shows that the incubation of Caco-2/15 cells with FE/ASC has resulted in a marked decrease in OGG1 transcript level and protein mass. Deficiency in DNA repair enzyme OGG1 has likely important functional consequences that compromise the ability of cells to repair DNA. A previous study suggests that increased 8-OHdG levels in colorectal carcinoma are attributed to increased formation and are maintained elevated by diminished 8-OHdG repair activity at appropriate high levels [79]. Finally, these results indicate that mt DNA damage following oxidative stress can lead to a vicious cycle of ROS propagation and mt DNA oxidation.

The cascade initiated by oxidative mt DNA damage leads to the deficiency of key electron transport enzymes, and subsequent ROS generation may ultimately dramatically release death signals, such as cytochrome C and AIF. Mt DNA damage represents a key target for intervention and a biomarker in the course of many human diseases. We believe that mt DNA damage is linked to the numerous abnormal processes noted in our research, including ATP generation, Ca^{2+} homeostasis and release of signals of cell death.

In summary, the FE/ASC system in Caco-2/15 appeared to be very effective in promoting lipid peroxidation and, at the same time, altered the mitochondrial function. This mitochondrial dysfunction is probably related to oxidative stress because the addition of antioxidants (BHT) prevented the occurrence of lipid peroxidation and improved

mitochondrial functions in terms of ATP production, Ca^{2+} homeostasis and apoptotic protein expression.

Our results show for the first time that FE/ASC can induce lipid peroxidation accompanied by a defect in ATP production, mitochondrial transport chain complex inhibition, mitochondrial Ca^{2+} overload, cell apoptosis, mitochondrial DNA damage and mitochondrial transcription factors alterations in intestinal epithelial cells. All hallmark events of mitochondrial dysfunction are clearly found in our study.

Our findings support the clinical observation that iron supplementation enhances disease activity in patients with chronic intestinal inflammation. The iron dose used in the current study is comparable to normal iron concentration in the gut [5].

Overall, our data suggest that oxidative-mitochondrial dysfunctions are not mediated by a single gene or mechanism, but it instead may be a consequence of multiple factors organized within a complex functional network. The pattern of our results may prove to be useful in elucidating the molecular mechanisms implicated in IBD.

Multiple studies have shown that the significance of oxidative stress and inflammation in the pathogenesis of intestinal diseases. In fact, antioxidant treatments attenuate the oxidative stress in cell culture and improve colitis in experimental models [134, 169, 170]. Despite these studies, antioxidants have yielded disappointing results and have not proven to be effective in human diseases. High amounts of antioxidants are needed to achieve protective effects in the intestine, as most exogenous antioxidants do not cross the blood

brain barrier owing to their hydrophilic nature. Furthermore, administration of antioxidants is limited owing to their toxicity at high dose, resulting in small therapeutic window of these molecules.

Thus, it is important to emphasize the need for alternative strategies to therapeutically counteract the oxidative stress-mediated diseases. In recent years, scientific interest has focused increasingly on other mediators that have shown important regulatory roles in various body functions. A promising molecule to inhibit ROS-mediated damage is the activation of endogenous antioxidant enzymes present in the intestine via activation of nuclear transcription factors such as Nrf-2.

Nrf2 is an important redox-sensitive nuclear transcription factor which protects against oxidative stress by inducing the transcription of antioxidant and detoxifying genes through binding with ARE [151, 171]. It has the potential to be a target to intervene in a variety of diseases, such as neurodegenerative, cardiovascular, pulmonary and chronic inflammatory diseases, where oxidative stress occurs and Nrf2 is degraded [146, 160, 164, 172]. Several approaches can be utilized to activate Nrf2 or reduce its degradation through modulating its post-translational modifications (e.g. phosphorylation and acetylation) or Keap1 stability [151, 153]. Therefore, the development of a specific Nrf2 activator to switch on its responsive genes would bring novel avenues for the treatment of oxidative stress related disease.

To evaluate the anti-oxidants function of Nrf2 in oxidative stress, inflammation, apoptosis, intestinal permeability and mitochondrial functions in Caco-2/15 cells, we employed the

FE/ASC oxygen radical-generating system to determine how Nrf2 modulates mitochondrial dysfunction related to oxidative stress in Caco-2/15 cells.

Our results revealed that the increase in Nrf2 activity plays a pivotal role in modulating the classical ARE-driven gene and in regulating many genes and enzymes, including PHB, PGC-1α, occludin, TNFα and Bcl-2 that have not been verified before as being under direct control of Nrf2. The up-regulation of these enzymes and proteins may represent an adaptive response occuring in face of exposure to oxidative insults. This adaptive response is regulated by an interaction between Keap1 and Nrf2 in which the exposure to ROS results in the activation of Nrf2 transcriptional expression.

In our study, we incubate Caco-2/15 cells with increasing concentrations of FE/ASC (0.05/2mM, 0.1/2mM, 0.2/2mM), which displayed different impacts on lipid peroxidation and on Nrf2 modulation. We observed an increase in MDA levels, a marker of lipid peroxidation, in a dose-dependent manner. Interestingly, at low concentrations, FE/ASC increased Nrf2 protein levels compared to control cells. This typical response to mild oxidative stress is physiologically relevant, as it could lead to Nrf2 dissociation from its inhibitor Keap1 and its translocation to the nucleus, thereby inducing a protective response. In contrast, increasing the concentration of FE/ASC (0.2/2mM) led to reduced Nrf2 protein levels. This may refer to severe or pathological oxidative stress observed in disease states and could possibly be associated with an increased Nrf2 degradation, leading to a lower translocation to the nucleus and an increase in oxidative stress.

OPZ is a chemo preventive compound that induces antioxidant protection either by increasing the production of ROS or by modification of Keap1 cysteine residue, ultimately leading to Nrf2 translocation to nucleus. In our study, optimal concentrations of OPZ (50 μM) induced a significant increase in Nrf2 protein mass. Our findings, indicating that the Nrf2 knock-down blocks the protective effects of OPZ against FE/ASC, provide concrete evidence about the direct contribution of Nrf2 to antioxidant actions.

Furthermore, the enzymatic activity of SOD, CAT and GPx was increased following OPZ treatment. It has been found that the level of this antioxidant was decreased in Nrf2-depleted Caco-2/15 cells. Our findings were in agreement with a previous study showing that OPZ significantly reduced colitis in Nrf2-deficient mice, thereby suggesting a dependence of the Nrf2-ARE pathway by OPZ [159]. Further support for our data is evidenced by a previous report noting that sulforaphane, an OPZ activator, stimulated Nrf2 gene–dependent antioxidant enzyme activity and protected against oxidative stress in gastric mucosa [172].

Our results expressed the anti-inflammatory function of Nrf2. We found a significant decrease in NFκβ and TNFα, as well as an increase in Iκβ level upon induction of Nrf2. Conversely, blockage of Nrf2 induces NFκβ and TNFα up-regulation along with Iκβ down- regulation. Our results are consistent with a previous study by Khor et al who noted that the levels of pro-inflammatory mediators, such IL-6 and TNF-α, were significantly increased in the colonic tissues of $Nrf2^{-/-}$ mice as compared to their wild-type mice [158].

Since inflammation has damaging effects on intestinal permeability, we suggest that Nrf2 activation may play a protective role in intestinal permeability defects via the up-regulation of occludin (as it has been observed in our results) and down-regulation of pro-inflammatory cytokines.

Since the overexpression of PHB can protect against oxidative stress and inflammation [99], we suggest that OPZ enhances Nrf2 activation which in turn enhances the activation of PHB. In the current study, we demonstrated that Nrf2 activation by OPZ enhances mitochondrial PHB expression, while Nrf2 knockdown led to reduction of mitochondrial PHB. A decrease in PHB was also observed with high FE/ASC concentrations in the experimental system. Thus, we propose that Nrf2 is a master regulator of PHB expression.

Previous data indicated that there is a regulatory mechanism between PHB and Bcl-2 [173]. It has been found that the activation of Nrf2 up-regulated Bcl2 expression. Bcl-2 is a well-known molecule that has many protective functions such as antioxidant and anti-apoptotic ability. Taken together, our previous observations will support the Nrf2 anti-apoptotic function. Therefore, we suggest that there is a functional triangle linking Nrf2, PHB and Bcl-2, which share anti-apoptotic and antioxidant functions.

We provide for the first time evidence that Nrf2 acts as an important anti-apoptotic mediator and that the underlying mechanism involves activation of Bcl-2. Nrf2 induction by OPZ contributes to the elevation of Bcl-2 anti-apoptotic protein in Caco-2/15 cells, while Nrf2 knockdown or increasing FE/ASC concentrations down-regulates Bcl-2. In agreement with our observations, treatment with resveratrol, an OPZ activator, inhibited

vascular apoptotic cell death in Nrf2+/+ mice, without the same success in Nrf2-/- mice [174].

A primary defect of the tight junctions leading to increased intestinal epithelial permeability has been proposed as a basic pathogenic event in chronic intestinal inflammatory diseases [60]. We have demonstrated the possibility that Nrf2 contributes to the regulatory function of intestinal TJs such as occludin. For the first time, we have shown an association between Nrf2 and occludin in view of the induction of Nrf2 with OPZ and the simultaneous up-regulation of occludin. We have also observed that Caco-2/15 cells treated with Nrf2 shRNA or with increasing concentrations of FE/ASC display reduction in occludin levels. Assembly of occludin at the TJ network was also observed with confocal microscopy in Caco-2 cells as a response of Nrf2 induction by kaempferol, a natural flavonoid [175]. Overall these data suggest a key role of Nrf2 in the protection of the small intestine integrity.

Much progress has been made in uncovering the transcriptional mechanisms that govern the function and biogenesis of mitochondria. Mounting evidence suggests that Nrf2 acting on both nuclear and mitochondrial genes serves to coordinate the expression of gene products required for maintaining mitochondrial functions. A recent discovery of the PGC-1α family provides important information regarding the crosstalk between nucleus and mitochondria [103]. PGC-1α has the potential to integrate and utilize Nrf2 activities that have been implicated in the expression and function of the mitochondrial oxidative machinery.

Our results provide strong evidence that inducing Nrf2 activity by OPZ in Caco-2/15 cells contributes to mitochondrial biogenesis and function via up-regulation of PGC-1α, increased mitochondrial enzymatic activity and raised ATP production. Surprisingly, we noted a dramatic decrease in PGC-1α in Caco-2/15 cells infected with Nrf2 shRNA or incubated with high concentrations of FE/ASC. Our results are in agreement with a previous study showing a significant reduction of PGC-1α in retinal epithelial cells treated with oxidative stress agents [162]. Therefore, we suggest that Nrf2 acts as an independent transcription factor in regulating mitochondrial biogenesis.

Mitochondria constitutes a primary targets for oxidant-induced intestinal epithelial injury and may have a central role in intestinal epithelial cell survival. As we noted previously, we have demonstrated that an accumulation of peroxidation products in mitochondria leads to a decrease in ATP production and compromises the maintenance of cellular homeostasis [106]. Incubation of Caco2/15 cells with Nrf2 shRNA induced a marked decrease in mitochondrial complex activities (Complexes I, II, III, IV) and ATP levels. In contrast, ATP production was significantly increased in Caco-2/15 cell following treatment with OPZ. Accordingly, mitochondrial functions monitored by the levels of ATP production were reduced in cells treated with Nrf2 shRNA in comparison with control cells. We reasonably suggest that mitochondrial dysfunctions in cells treated with Nrf2 shRNA are due to degradation in mitochondrial respiratory chain enzymes, resulting in the decline in ATP production. The impairment of mitochondrial complexes activity noted in our experiments may partly be attributable to decreased antioxidant enzymes and increased oxidative stress, which ultimately led to a decrease in oxidative phosphorylation. These

results provide evidence to support the proposal that Nrf2 is an effective mitochondrial cell survival and biogenesis.

Taken together, our data indicate that increased Nrf2 in intestinal epithelial cells plays a pivotal role in positively modulating cellular defense and maintaining cellular integrity against toxic insults. For the first time, our study reveals the capacity of Nrf2 to regulate many intestinal genes and enzymes (occludin, PHB, PGC-1α, Bcl-2) related to cell permeability and mitochondrial functions. It is quite possible that some of these expressed proteins could be secondary or indirectly attributed to Nrf2 effects. Therefore, additional studies are required to test this assumption.

Finally, the Nrf2 multifunction protection phenomenon raises an interesting question about how a single protein can protect many different organs from toxic insults. A possible molecular mechanism explaining this phenomenon is that Nrf2 coordinately up-regulates classic ARE-driven genes as well as cellular type-specific genes that are required for the defense system.

7 CONCLUSION

Considerable evidence provided in this thesis supports the importance of mitochondria as a critical target and event responsible for toxic oxidative stress. This is in part supported by the dramatic protection of cells against toxic oxidative stress following the enrichment of intestinal epithelial cells with Nrf2. The specific oxidative damage to mitochondrial targets that are directly responsible for cell injury and disease, are more difficult to define. However, this thesis provides compelling evidence to support the importance of different mitochondrial targets in oxidative stress.

Evidence evidence was also provided as to the importance of mt DNA integrity in the maintenance of cell viability and calcium homeostasis since calcium overload was a critical event in mitochondrial cytochrome C release and apoptosis initiation. Furthermore, oxidative stress-mediated inhibition of mitochondrial complexes and limited ATP production appear to play a critical role in chronic intestinal inflammation.

Moreover, our results have shown that Nrf2 has multiple functions beyond the classic acute stress responses to oxidative insults. These functions include involvement of Nrf2 in anti-apoptotic process, mitochondrial functions, independent PGC-1α biogenesis and modulation of tight junction proteins such as occludin that contributes to intestinal permeability.

The failure of antioxidant therapy strategies in clinical trials has encouraged efforts to find novel approaches for strengthening the endogenous antioxidant defense system, which may

induce mitochondrial biogenesis and anti-inflammatory responses. In this regards, Nrf2, a master regulator of the induction of a battery of genes able to positively influence several cytoprotective systems, may be a promising target to counteract mitochondrial dysfunctions and its consequences in IBD. Thus, exogenous Nrf2 inducers should be tested for therapeutic potential in IBD.

Undoubtedly, future research should address several exciting issues:

1. Do Nrf2-activating drugs (such as OPZ) display comparable effects like nutritional agents (such as resveratrol)?
2. Are Nrf2 target genes or their expression level quite different depending on the Nrf2-activating signal?
3. Can Nrf2 activity be pharmacologically or nutritionally manipulated in a safe manner to help ameliorate IBD or other intestinal inflammatory diseases?

We hope that further work in the next years will uncover Nrf2 effectiveness in terms of the reduction of mitochondrial oxidative damage in IBD and the beneficial outcome of patients with mitochondrial diseases. If our proposal is proved true in large clinical studies, this strategy might slow the progression of IBD.

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