Uncoupling protein-2 and non-alcoholic fatty liver disease

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György Baffy

Liver Research Center, Division of Gastroenterology, Rhode Island Hospital and Brown Medical School, Providence, RI 02903

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1. ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) has become the most common form of hepatic disorders in the developed world. NAFLD is part of the metabolic syndrome with insulin resistance as a primary underlying derangement. The natural history of NAFLD may extend from simple steatosis over steatohepatitis into cirrhosis and hepatocellular carcinoma. Among numerous factors shaping these transitions, uncoupling protein-2 (UCP2) may theoretically contribute to every stage of this disease. UCP2 is a recently identified fatty acid-responsive mitochondrial inner membrane carrier protein showing wide tissue distribution with a substantially increased presence in fatty liver. The biological functions of UCP2 are not fully elucidated and the greater part of our current knowledge has been obtained from animal experiments. These data suggest a role for UCP2 in lipid metabolism, mitochondrial bioenergetics, oxidative stress, apoptosis, and even carcinogenesis. Available evidence is reviewed and new concepts are considered to appraise the potential role of UCP2 in the pathogenesis of NAFLD.

2. INTRODUCTION

Obesity has reached epidemic proportions in the economically developed world and reshaped the landscape of medicine (1). Obesity is the gateway to chronic conditions with debilitating consequences such as type 2 diabetes, hypertension, and elevated plasma lipid levels (2). These conditions are termed collectively as the metabolic syndrome with insulin resistance as

common underlying derangement (3). Hepatic manifestations of the metabolic syndrome have become known as non-alcoholic fatty liver disease (NAFLD) (4). Although the 'non-alcoholic' connotation is not particularly fortunate, it indicates that NAFLD is, despite a separate etiology, difficult to distinguish from alcoholic liver disease (5). In Western countries, NAFLD has become by far the most common form of any liver disease with a 14% to 24% estimated prevalence in the general population (6-8).

NAFLD has been classified into three major forms, primarily identified by histology as successive stages that may progress into end-stage liver disease (5, 9). Most patients with NAFLD have pure macrovesicular steatosis with minimal or no clinical symptoms and no perceivable progression (5). Consequently, this initial stage of NAFLD is not always considered a disease. There is evidence, however, that 20% to 30% of these individuals eventually progress into the next stage of NAFLD, termed non-alcoholic steatohepatitis (NASH) and characterized by more frequent clinical symptoms, laboratory abnormalities, distinct histological changes, and a 15% to 20% chance of further progression into cirrhosis and eventually into hepatocellular carcinoma (HCC) (9, 10).

The pathogenesis of NAFLD and the predictors of its progression remain incompletely understood. The 'two hit' hypothesis proposed by Day and James has become a widely accepted framework to guide current research in this area (11). Accordingly, the 'first hit' is a buildup of lipids in the liver along with the development of insulin resistance. These changes may provide the basis for progressive liver disease in response to a 'second hit' in which a variety of mechanisms, some of them self-perpetuating, have been implicated (12, 13). In the late phase of NAFLD, development of cirrhosis and HCC may exhibit distinct sets of pathologic processes.

This review will focus on the potential contribution of uncoupling protein-2 (UCP2), a novel member of the mitochondrial inner membrane carrier protein family (14, 15), to the pathogenesis of NAFLD. Although UCP2 is an appealing molecular target for several reasons discussed below, its biological functions remain poorly defined and we are left with much speculation in this regard. Nonetheless, theoretical considerations and experimental data suggest that UCP2 may have a role in various stages of NAFLD and it is therefore of interest to review current knowledge on UCP2 and outline potential directions for further research.

3. UNCOUPLING PROTEIN-2: GENERAL CONSIDERATIONS

3.1. Historical perspectives

Uncoupling (i.e., disconnection of ATP synthesis from mitochondrial respiration) has long been a tempting target in the treatment of obesity. In fact, dinitrophenol, a potent mitochondrial uncoupling compound, was introduced in the 1930's as a successful anti-obesity agent, just to be withdrawn from the market due to its very narrow therapeutic range and occasionally fatal side effects (16). The subject of uncoupling was later revitalized by two lines of research. In 1961, Peter Mitchell published his groundbreaking chemiosmotic theory, which has defined our current understanding on the mechanism of oxidative phosphorylation (17). The respiratory or electron transport chain (ETC) has four large multi-subunit enzyme complexes embedded in the

inner mitochondrial membrane along with ATP synthase (Figure 1). Electrons are donated to the ETC by NADH (reduced nicotinamide adenine dinucleotide) and succinate and passed along the complexes to ultimately reduce molecular oxygen to water at complex IV (cytochrome c oxidase). According to Mitchell's theory, this series of highly exergonic reactions provides energy for the outward translocation of protons from the mitochondrial matrix into the intermembrane space to create an electrochemical gradient across the inner membrane (17). This mitochondrial membrane potential ($\Delta\Psi m$), also called proton-motive force, will then drive ATP synthesis through the controlled return of protons into the matrix (17).

The chemiosmotic theory has been validated by many experiments (18). It was also noted, however, that oxidative phosphorylation is never 100% efficient and this partial uncoupling is believed to be an inherent characteristic of mitochondrial respiration. In fact, a significant portion of consumed oxygen is 'wasted' and dissipated as heat energy in all examined cell types (19). A backflow of protons independent of ATP synthesis accounts for this portion of oxygen consumption and is termed proton leak (19). The relationship between $\Delta\Psi m$ and uncoupling is non-ohmic, i.e., higher $\Delta\Psi m$ values are associated with disproportionately higher proton leak (20). Proton leak can be estimated by the rate of respiration during resting or 'state 4' conditions, when ATP synthesis is inhibited either directly (e.g., by oligomycin) or by substrate unavailability (lack of ADP) (20). Proton leak in hepatocytes of both endotherm and ectotherm species accounts for 15 to 40% of total oxygen consumption (20). In addition to inherent biophysical properties of the inner membrane (basal proton conductance), proton leak may result from the action of specialized carrier proteins (inducible proton conductance) (20). Uncoupling protein (UCP or thermogenin, later renamed UCP1), a mediator of cold-adapted thermogenesis, performs precisely this function in the mitochondria of brown adipose tissue (BAT) and its identification provided another milestone in bioenergetics (21). UCP1 has been unequivocally proven to be a physiologic mediator of proton leak (21), although this action is restricted to BAT and UCP1 therefore plays a limited role in humans where BAT becomes virtually absent by adulthood.

3.2. Expectations and uncertainties

The search for additional uncoupling proteins was successful in 1997, when UCP2 was identified by cloning strategies in the mouse and human genomes, based on its partial sequence homology with UCP1 (22, 23). UCP3 was described almost simultaneously and 2 further members of the UCP subfamily of mitochondrial anion carrier proteins (UCP4 and UCP5) soon followed suit (24). The *ucp2* gene was mapped to chromosome 7 in mouse and to the syntenic chromosome region 11q13 in man (22, 23). UCP2 mRNA is transcribed from the distal 6 of 8 existing exons of *ucp2* gene (25). UCP2 in humans is a 308 amino acid protein with molecular weight of 33 kDa and a 59% amino acid identity to UCP1 (22, 23). Consequently, the structure of UCP2 protein is very similar to that of UCP1 and is remarkably well conserved between different species. Akin to all members of the mitochondrial carrier family, UCP2 contains six alpha-helical spanners with a total of three mitochondrial energy transfer protein signature regions and is embedded in the mitochondrial inner membrane where it is thought to function as a homodimer (25).

To understand the biological function(s) of UCP2, a link of UCP2 to energy metabolism was tested in a hope to find new ways to treat obesity (26). The high homology with UCP1 suggested that UCP2 might also mediate proton leak, only to act on a larger number of targets based on its wide tissue distribution (see below). Moreover, the *ucp2* gene was found in proximity to a cluster of at least 5 additional genes related to energy homeostasis and obesity (22). A common polymorphism in the promoter region of *ucp2* gene (-866A/G) was shown to enhance its transcriptional activity and UCP2 has been linked to increased prevalence of obesity (27), although this association could not be confirmed by others (28).

The physiological role of UCP2-mediated proton leak also remains a matter of debate (20). UCP2 induced proton conductance in yeast only when expressed in amounts a magnitude over the highest known physiological concentrations and still could not account for the degree of basal uncoupling seen in mammalian mitochondria (29). The amount of UCP1, the only uncoupling protein that beyond doubt has been proven to mediate proton leak of physiological relevance, is exceptionally high in BAT and may reach ˜5% of total mitochondrial protein content (30). UCP2 is present to some degree in virtually every mammalian tissue; however, the amount of UCP2 is usually one to two magnitudes smaller when compared to UCP1 abundance in BAT (30). For instance, the presence of UCP2 in spleen, a tissue in which UCP2 is relatively highly expressed, was estimated to be 160 fold less than UCP1 in BAT (31). Consequently, contribution of UCP2 to proton leak is likely to be much more modest, perhaps causing a few mV decrease in $\Delta\Psi m$. This may be, nevertheless, sufficient to result in important consequences as discussed below.

3.3. UCP2 and mitochondrial ROS production

For most electrons that travel down the respiratory chain, cytochrome c oxidase (complex IV) is the final destination where molecular oxygen is converted into water by four-electron reduction (Figure 1). Some 'stray' electrons, however, may convert oxygen into superoxide anion by partial (one-electron) reduction at earlier junctures of mitochondrial respiration (complex I or complex III) (32). There are varying estimates as to what fraction of the totally consumed oxygen would yield superoxide (from 0.15% to a few percents) (33), but this fraction may significantly increase in certain conditions. Any major imbalance between outward proton translocation (gradient buildup) and ATP synthesis (gradient consumption) is reflected in an increased $\Delta\Psi m$. Mitochondrial hyperpolarization prolongs the half-life of mobile electron carriers that will mediate partial reduction of oxygen and generate superoxide (34). This mechanism may be particularly relevant to obesity and other metabolic conditions characterized by fuel surplus that yields excess NADH.

Several years before the identification of novel uncoupling proteins, in vitro experiments using liver mitochondria demonstrated that ROS production correlates with $\Delta\Psi m$ and both can be reduced by artificial uncouplers such as dinitrophenol (35). Subsequently, the concept of mild uncoupling has been introduced, which predicted the existence of a protein-regulated proton leak with the main purpose of controlling mitochondrial oxidative stress (34, 36). It has been demonstrated that small changes of $\Delta\Psi m$ have large effects on rates of superoxide production (37, 38). This indicates at least the

feasibility of such a regulating role for UCP2 often present in insubstantial amounts.

The idea of UCP2 as a negative regulator of ROS production has been successfully tested in a variety of experimental settings. In many studies, absence of UCP2 was associated with increased oxidative stress (39-41). These experiments often implicated the role of monocyte/macrophage cells in which UCP2 expression has been repeatedly found to be high. The first evidence was obtained on nonparenchymal liver cells in which GDP, a natural inhibitor of UCP2, resulted in elevated $\Delta\Psi m$ and hydrogen peroxide production (39). This notion was further supported by in vivo experiments in which peritoneal macrophages of UCP2-/- mice produced 80% more ROS than wild type controls, thus enabling these animals to survive *Toxoplasma* infection (40).

Strategies providing cell protection from oxidative stress by enhanced UCP2 expression have also been successful. Overexpression of UCP2 in cultured neonatal cardiomyocytes made these cells more resistant to oxidative stress and mitigated the detrimental loss of $\Delta\Psi m$ upon exposure to hydrogen peroxide (42). Similarly, cultured cortical neurons overexpressing UCP2 and brain tissue of UCP2-transgenic mice exhibited increased tolerance against glucose and oxygen deprivation, conditions that typically cause neuronal cell death (43).

Of note, uncoupling proteins and mitochondrial ROS appear to have a reciprocal relationship. Exposure of isolated mitochondria to superoxide results in increased proton conductance and correlates with tissue expression of UCP1, UCP2, and UCP3 (44). UCP2 activation can also be elicited by lipid peroxidation end-products (45). According to these observations, UCP2 is part of a negative feedback mechanism that is able to respond to oxidative stress by controlling mitochondrial superoxide production.

3.4. Tissue distribution of UCP2

Initial work detected UCP2 mRNA in a variety of tissues (22, 23). Subsequent reports indicated particularly high UCP2 mRNA expression levels in components of the immune system, such as the spleen, thymus, and macrophages (46-49). Anti-UCP2 antibodies have later become available, but some proved nonspecific when tested on tissues of UCP2-/- mice (47). Further evaluation indicated that UCP2 mRNA and protein levels do not always correlate and translational regulation of UCP2 may be significant (47). These considerations notwithstanding, UCP2 protein expression patterns in various tissues have been fairly reliably mapped in studies using UCP2-/- mouse tissues as negative control (31, 41, 49, 50).

In contrast to the rather ubiquitous presence of UCP2, hepatic UCP2 mRNA expression has been localized to Kupffer cells (46), with very low or undetectable levels in hepatocytes (46). In fetal rodent liver, UCP2 expression reached 30 times higher levels than in adults and UCP2 was exclusively seen in hematopoietic cells by immunohistochemistry (51). It has been argued that high UCP2 expression levels in blood cell lineages, particularly in the monocyte/macrophage system, may confound the detection of UCP2 and could in fact account for its ubiquitous expression pattern (15). However, immunohistochemistry helped to confirm the presence of UCP2 protein in adult mouse hepatocytes, predominantly

in the periportal area (41). UCP2 expression was also detected in healthy and diseased hepatocytes in human percutaneous liver biopsy specimens (52). The latter study also found that UCP2 was relatively abundant in bile duct cells of primary biliary cirrhosis, a finding of unknown significance (52). It is currently unknown whether UCP2 is expressed in other cell types of the liver such as stellate cells or sinusoidal endothelial cells.

The reason for unusually low UCP2 expression in normal hepatocytes remains puzzling and at this point we may only speculate of several explanations. Supply of ADP may be more abundant in the liver than in other tissues and a state 4-like respiration with unwanted oxidative stress is perhaps less likely to occur. Differences in the mitochondrial inner membrane surface area and its fatty acid composition might allow hepatocytes to adapt their basal proton conductance and avoid mitochondrial hyperpolarization without significant contribution from protein-mediated proton leak (53). Finally, there may be an as yet unknown protein that mediates proton conductance at baseline metabolic activity in hepatocytes. A liver-specific mitochondrial carrier protein has been recently isolated by comparative screening of cDNA libraries from HCC and paired normal liver tissue (54). This novel protein, termed HDMCP (HCC down-regulated mitochondrial carrier protein), exhibits reduced expression in HCC and shows evolutionary conservation between humans, mice, and rats (54). Initial characterization shows that HDMCP, although it only shares an ~25% homology with known uncoupling proteins, lowers $\Delta\Psi m$ and reduces intracellular ATP levels when overexpressed in 293T cells (54).

4. NAFLD AND THE 'FIRST HIT': IS UCP2 LINKED TO STEATOSIS?

4.1. Development of steatosis

Fat is normally stored in the adipose tissue and not in the liver unless there is an imbalance between lipid mobilization and lipid oxidation. When the capacity of hepatic lipid oxidation is exceeded, surplus lipids may be deposited in hepatocytes (Figure 2). Fatty acids in the liver may originate from dietary fat, release from the adipose tissue (lipolysis), or de novo synthesis (55). Fatty acids can be esterified into triglycerides and re-transported into the circulation as very low-density lipoproteins (VLDL) or deposited in the liver. Alternatively, fatty acids can be taken up into mitochondria facilitated by carnitine-palmitoyl-transferase (CPT)-I, undergo beta-oxidation into acetyl-CoA and be further oxidized into carbon dioxide via the tricarboxylic acid (TCA) cycle or serve as substrate for macromolecular synthesis and ketogenesis (56). These pathways are intricately tied to carbohydrate metabolism and form a complex regulation in which insulin has a pivotal function to decide on fuel preference between glucose and fatty acids (57). Although the most perceptible role of insulin is to secure euglycemia, it also regulates lipid metabolism (58). Insulin controls peripheral lipid mobilization by inhibiting hormone-sensitive lipase, prevents secretion of VLDL from hepatocytes by increasing the degradation of apolipoprotein B-100 and blocking VLDL exocytosis, limits mitochondrial fatty acid uptake and beta-oxidation by inhibiting CPT-I, and promotes the synthesis of triglycerides by stimulating the transcription of lipogenic enzymes (58).

Evidence shows that NAFLD is associated with insulin resistance (59, 60). According to the original concept of glucose fatty acid cycle, fatty acids oppose peripheral glucose disposal by inhibiting key enzymes of glycolysis (61). This theory has recently been revisited and it appears that (at least in skeletal muscle and adipose tissue) fatty acids primarily impair insulin signaling and glucose uptake (62). These two concepts agree in that large amounts of fatty acids from various sources (dietary intake, peripheral lipolysis, and de novo synthesis) are destined to generate insulin resistance (62). Importantly, there is a dichotomy among insulin-regulated pathways of carbohydrate and lipid metabolism in which 'glucometabolic' insulin resistance is seen with excessive stimulation of lipogenic pathways that remain responsive to insulin (63). Thus, elevated plasma insulin levels may nurture hepatic fat accumulation and make the steatosis more severe. Interestingly, recent evidence shows that hepatic components of insulin resistance correlate with steatosis and can be abrogated by preventing hepatic fat accumulation (64). A vicious cycle may therefore develop between steatosis and insulin resistance.

4.2. Regulation of UCP2 expression by fatty acids

The association between UCP2 and lipid metabolism was first noted by the ability of serum fatty acids to regulate the expression of *ucp2* gene. The same reports that initially identified UCP2 also demonstrated that white adipose tissue UCP2 transcript levels increased 4 to 6 fold following high-fat feeding (22) as well as in genetic models of obesity including the leptin-deficient ob/ob and leptin receptor-deficient db/db mice (23). A large number of studies on various tissues confirmed that UCP2 expression is generally increased in response to elevated plasma fatty acid levels. Thus, in animal models baseline expression in hepatocytes is often very small or undetectable, but UCP2 shows significant up-regulation in the liver during pathologic conditions associated with elevated plasma fatty acid levels and steatosis (65-68), supporting the view that the *ucp2* gene is regulated by fatty acids and lending credence to the purported involvement of UCP2 in the pathogenesis of fatty liver disease. In situ hybridization and immunohistochemistry performed on livers of ob/ob mice showed that expression of both UCP2 mRNA and protein is increased in hepatocytes compared to lean mice (65). In vivo lipid infusion caused UCP2 up-regulation in rats and in vitro exposure of primary cultured rat hepatocytes to lipids resulted in similar effects, indicating that excess UCP2 may indeed originate in hepatocytes as opposed to nonparenchymal liver cells (69). Finally, UCP2 expression increases considerably in the regenerating liver following partial hepatectomy (70, 71). Post-hepatectomy increase in plasma fatty acid levels (72) has been suggested to contribute to this surge of UCP2 expression (71).

Interestingly, the effect of various fatty acids on hepatic UCP2 expression may be very different. In rat hepatocytes, polyunsaturated fatty acids (PUFA) stimulated liver UCP2 mRNA levels much more than the monounsaturated oleic acid, while the saturated palmitic acid had virtually no effect (73). This differential effect of fatty acids most likely results from the complex interaction of fatty acid-regulated transcription factors and their direct and indirect effects on the *ucp2* gene. Peroxisome proliferator-activated receptors (PPARs), a family of ligand-dependent nuclear transcriptional regulators involved in adipogenesis and lipid metabolism (74), may have a key role in transmitting the effects of

fatty acids on hepatic UCP2 expression (73). The PPAR family has three major subtypes, PPARα, PPARγ, and PPARδ, all activated by fatty acids and their derivatives (74). PPARs form active heterodimers with the retinoid X receptor and bind to PPAR-responsive elements (PPRE) in enhancer sites of regulated genes (74). PPARα promotes hepatic fatty acid oxidation and ketogenesis, PPARγ is important in adipocyte differentiation and lipid storage (preferably in adipose tissue), and PPARδ is involved in cholesterol metabolism and fatty acid oxidation (74). Consequently, all three PPARs have been implicated in the amelioration of insulin resistance (75) and their impact on the course of NAFLD is of tremendous current interest. Direct or indirect stimulation of hepatic UCP2 expression may contribute to the effects of PPARs in fatty liver disease. In vivo activation of PPARα by diet rich in fish oil or by administration of its synthetic ligand, fenofibrate, results in several fold induction of UCP2 in mouse and rat hepatocytes (76). While the presence of PPARγ is insubstantial in healthy liver, its expression increases in rodent models of steatosis (77) and administration of PPARγ ligands may induce UCP2 expression in fatty liver (78). It is worth noting that combination of high-fat diet with fenofibrate was recently reported to induce de novo expression of UCP3 protein in rat hepatocytes (79), indicating that PPAR stimulation may be indeed a powerful tool to manipulate hepatic expression of uncoupling proteins.

4.3. Impact of hepatic UCP2 expression on steatosis

Does increased hepatic expression of UCP2 have a beneficial or deleterious role in the development of steatosis? One plausible effect of enhanced UCP2 function could be reduction of hepatocellular fat accumulation (Figure 2). First, it should be noted that interaction of UCP2 (and of other uncoupling proteins) with fatty acids seems to be a prerequisite for the 'uncoupling' function (80). In addition to their transcriptional regulatory effects described earlier, fatty acids of varying length and saturation may 'activate' uncoupling proteins and this can be detected as proton leak (80, 81). We may consider two basic mechanisms by which UCP2 action can potentially alleviate hepatic steatosis and the ensuing lipotoxicity. On the one hand, large amounts of fatty acids may enter the mitochondrial matrix and undergo beta-oxidation yielding excess NADH that needs to be re-oxidized by mitochondrial respiration. In case of fuel surplus, mitochondrial respiration may be limited by high proton gradient and become a bottleneck (34, 82). UCP2-mediated proton leak may theoretically resolve this situation and support ongoing fatty acid oxidation as opposed to accumulation. On the other hand, fatty acids enter the matrix in the form of acyl-CoA (83) and insufficient NADH re-oxidation may result in the cleavage of more acyl-CoA into non-esterified fatty acids (NEFA) by matrix acyl-CoA thioesterase (84). NEFA cannot be further metabolized in the matrix or permeate the inner membrane and must be therefore actively transported out to the cytosol for further processing (84). It is worth noting that intracellular accumulation of fatty acids rather than triglycerides is deleterious and triglyceride formation may be in fact protective by converting saturated fatty acids that would otherwise induce apoptosis (85). UCP2 may therefore provide a translocation mechanism and prevent accumulation of NEFA and their harmful effects in the mitochondrial matrix.

Both mechanisms described above involve the interaction of UCP2 with fatty acids. Uncoupling proteins were first characterized as fatty acid transporters once the 'flip-flop' theory was used to explain protein-mediated proton leak (15). According to this hypothesis, uncoupling proteins transport fatty acids anions across the mitochondrial inner membrane. Once in the intermembrane space, fatty acid anions join the protons extruded by respiration and freely re-enter the matrix as non-polar molecules. Although formulated to understand ATP-independent inward proton flux, 'flip-flop' of fatty acids could also be interpreted as a means to free the matrix from accumulation of NEFA (86, 87). It has recently been suggested that a protective effect of novel uncoupling proteins could actually entail the translocation of even more deleterious fatty acid peroxide anions (88). Therefore, if UCP2 mediates transports of fatty acid and fatty acid peroxides in the liver, it may have an important role in protecting the liver from hepatocellular lipotoxicity.

An intriguing and appealing aspect of how the novel uncoupling proteins may affect lipid metabolism is their potential link to AMP-activated protein kinase (AMPK). AMPK phosphorylates a large number of key target proteins and acts as a pivotal metabolic regulator (89). In the liver, AMPK promotes fatty acid oxidation and ketogenesis while inhibits hepatic cholesterol biosynthesis and triglyceride formation (89). In peripheral tissues, AMPK promotes fatty acid uptake and oxidation while inhibits peripheral lipolysis and lipogenesis (89). Combined with its effects on glucose metabolism, AMPK powerfully steers metabolic pathways from energy accumulation to energy expenditure and fuel preference from glucose to fatty acids (89). Consequently, AMPK may improve insulin resistance and prevent hepatic fat accumulation. AMPK activation by AMP involves direct allosteric stimulation, activation of an upstream kinase (AMPKK), and inhibition of an inactivating phosphatase (89). AMPK activation is part of the insulin-sensitizing effect of adiponectin (90), a fat-derived bioactive protein found to have reduced serum levels in NASH and predict extensive necroinflammation in the liver (91). Since interference with efficient oxidative phosphorylation may be reflected by increased intracellular AMP levels, it is reasonable to assume that uncoupling proteins contribute to AMPK activation. Indeed, increased expression of UCP1 in transgenic mice has been shown to augment AMP levels and AMPK activity in skeletal muscle (92) and in white adipose tissue (93). In mice overexpressing human UCP3, skeletal muscle UCP3 protein levels six fold over the control were associated with lower ATP/AMP ratio and increased AMPK activity, presumably accounting for improved glucose tolerance (94). Although a parallel relationship between UCP2 expression and AMPK activity in fatty liver has been suggested (95), the validity of this link remains to be explored.

It came somewhat as a surprise that the degree of steatosis was essentially unchanged in livers of ob/ob mice and high fat-fed mice regardless of whether the *ucp2* gene was intact or disrupted (67). These negative findings cast a significant doubt on any perceivable role UCP2 may have in the development of fatty liver. It must be emphasized, however, that these data pertain to specific animal models and not to the pathophysiology of human NAFLD. Also, mitochondrial fatty acid uptake and a need for enhanced UCP2 action may be mitigated by the effect of high plasma insulin levels on CPT-I activity, although diminished affinity of CPT-I to its physiological

inhibitor, malonyl-CoA, has been reported in experimental diabetes (96). Nevertheless, UCP2 deficiency results in increased hepatic lipid accumulation when fatty acids originate from prolonged fasting and their mitochondrial uptake is not subject to the regulatory effect of insulin (97). To what extent may UCP2 help meet the need for increased beta-oxidation in the 'first hit' of NAFLD characterized by 'glucometabolic' insulin resistance (98) will require further elucidation.

5. NAFLD AND THE SECOND HIT: IS UCP2 LINKED TO STEATOHEPATITIS?

5.1. Oxidative stress and mitochondrial dysfunction in NAFLD

Although steatosis in most patients will not progress into advanced liver disease, severity of steatosis appears to correlate with the progression of NAFLD (99). Lipids accumulated in the liver are not inert and steatosis makes the liver more vulnerable to subsequent injury or a 'second hit' (11). Among the likely mechanisms, oxidative stress appears to have a prominent role in this progression (99). There is increasing evidence in NAFLD for the dysfunction of mitochondria (59, 100, 101), which act not only as powerhouse of the cell and key regulators of apoptosis, but are also considered a major source of intracellular reactive oxygen species (ROS) (32). Consequently, mitochondrial dysfunction may account for a number of pathologic processes that primarily characterize the steatohepatitis stage of NAFLD.

As discussed above (Figure 2), if large amounts of fatty acids enter the mitochondria and undergo beta-oxidation, the respiration machinery slows down due to the stress imposed by increasing $\Delta\Psi m$ and superoxide production may increase significantly (34, 82). Moreover, rate of NADH re-oxidation becomes relatively insufficient and the flux of beta-oxidation also decreases (83). Alternative fatty acid oxidation pathways in peroxisomes and microsomes may gain significance and lead to additional ROS production (102, 103). Potential feed-forward mechanisms that induce further mitochondrial injury and escalate oxidative stress include generation of toxic dicarboxylic acids by microsomal omega-oxidation (104), lipid peroxidation with the formation of reactive aldehydes (105), inhibition of ETC complexes directly by TNF-alpha and ceramide (106, 107) or due to oxidative damage of mitochondrial DNA that encodes several ETC subunits (108). Activity of all ETC complexes including the nuclear-only encoded complex II is decreased in patients with NASH (108), suggesting that ROS may attack the mitochondrial membrane and impair functional assembly of the entire respiratory chain (106). Morphological studies also support the notion that mitochondria are a primary target in NASH and prominent structural changes such as megamitochondria, paracristalline inclusions, and loss of mitochondrial cristae have been reported (59, 101).

5.2. NAFLD and cell-specific changes in hepatic UCP2 expression

Fuel surplus due to high fatty acid load and a strained mitochondrial respiration that produces more superoxide may provide a plausible ground for the oxidative stress in hepatocytes typically seen in the steatohepatitis stage of NAFLD. One would expect that fat-laden hepatocytes might be protected from oxidative stress by the enhanced action of UCP2, a protein that

limits mitochondrial superoxide production. However, oxidative stress persists in spite of increased hepatic UCP2 expression, at least in rodent models of fatty liver (109, 110). While higher proton leak in mitochondria isolated from livers of ob/ob mice indicates that UCP2 is operational (65), the rate of ROS production remains significantly increased in these organelles (109). The question why fatty liver in rodents is not protected from ROS production when it has such high levels of UCP2 expression seems difficult to answer. It is important to note that UCP2 expression during NAFLD in human liver is currently unknown. While studies that link ROS to UCP2 activation (44, 45) make it conceivable that UCP2 in fatty liver is not only highly expressed but also functional, there is no good evidence that UCP2 in turn would help to reduce ROS production and protect against steatohepatitis. We may speculate that even increased amounts of UCP2 are not sufficient to control intracellular ROS generation in fatty liver. There is, however, an interesting phenomenon that may help to resolve this apparent paradox (Figure 3).

As noted earlier, UCP2 expression in normal liver dominates in Kupffer cells while it is rather limited in hepatocytes (46). Curiously enough, this pattern appears to revert in certain metabolic conditions. UCP2 expression is decreased and mitochondrial ROS is increased in peritoneal macrophages of ob/ob mice (111). Endotoxin treatment causes similar changes in macrophages of lean mice (111). These observations indicate that the dramatically increased hepatocellular UCP2 expression in fatty liver is associated by a concomitant down-regulation of UCP2 in macrophages, a change that presumably extrapolates to Kupffer cells. It is important to note that UCP2 expression in many studies was assessed in whole liver mitochondria (65, 109) and thus it may reflect the sum of opposing changes in parenchymal and nonparenchymal liver cells. UCP2 mRNA levels diminished in non-parenchymal cells isolated by differential centrifugation from livers of rats fed fish oil or fibrate, although non-parenchymal cells of similarly treated mice showed UCP2 mRNA up-regulation (76). The need to separately analyze UCP2 expression in different liver cell types has been recently addressed with an elegant experimental design (31). Introduction of UCP2$^{+/+}$ macrophages by bone marrow transplantation into UCP2-/- mice and a reverse strategy indicated that UCP2 expression in hepatocytes and liver macrophages is differentially altered in response to various challenges such as endotoxin exposure and atherogenic diet (31). It is easy to recognize that a similar mechanism may pertain to Kupffer cells in NAFLD. Therefore, if UCP2 indeed affects ROS production in steatohepatitis, this role must be related to its diminished expression in liver macrophages rather than to its augmented presence in hepatocytes.

Details of the negative transcriptional regulation of UCP2 in macrophages in response to endotoxin and obesity remain to be elucidated. Interestingly, an open reading frame (ORF) encoding a putative 36 amino acid peptide in exon 2 of the *ucp2* gene has been identified (47). Point mutation of start codons in this upstream ORF in COS cells results in significantly increased UCP2 expression (47). This sequence is conserved across vertebrates and similar ORFs were found in the 5'-untranslated regions of several other mitochondrial carrier proteins (47). Additional evidence for cell-specific negative regulation of the *ucp2* gene was obtained on RAW264 macrophages in which endotoxin-stimulated signals interrupt an enhancer element

localized to intron 2, resulting in increased production of ROS and activation of nitric oxide synthesis (112).

5.3. UCP2 and hepatocellular energy status in NAFLD

While increased mitochondrial ROS production in steatohepatitis is presumably due to down-regulation of UCP2 in liver macrophages, it has been argued that up-regulation of UCP2 expression in fatty hepatocytes could prove deleterious by interfering with ATP synthesis and causing energy depletion (65, 113). Although obesity per se does not appear to alter hepatic ATP stores (114), impaired ATP homeostasis in fatty liver has been observed following various challenges in both human and experimental settings. Graft failure occurs more often if liver transplantation is performed with fatty donor livers (115). Increased body mass index (BMI) correlated with slower recovery of hepatic ATP content following fructose infusion in patients with biopsy-proven NASH (116). In animal experiments, ATP content was diminished in rat liver when steatosis was induced by choline-deficient diet (117). Upon starvation of these animals, further and profound decrease in hepatic ATP stores was found (117). When ob/ob mice were exposed to transient hepatic ischemia and reperfusion, hepatic ATP stores that were already lower at baseline diminished dramatically upon the ischemic challenge and restored only partially by the end of reperfusion (65). It should be again noted that while increased UCP2 expression in experimental fatty liver of various origin has been demonstrated (65, 67, 68), there is currently no data on UCP2 levels in human fatty liver.

In summary, steatosis is likely to provide grounds for additional injury by increasing susceptibility of the liver to one or multiple 'second hits' during the progression of NAFLD. Transcription of the fatty acid-responsive *ucp2* gene undergoes significant changes in fatty liver and it appears that these changes are cell-specific. One the one hand, we may extrapolate from observations on macrophages that down-regulation of UCP2 also occurs in Kupffer cells, a change that may certainly contribute to increased ROS production and oxidative stress in steatohepatitis. One the other hand, up-regulation of UCP2 in hepatocytes may compromise energy homeostasis in these cells by possibly interfering with efficient ATP synthesis. However, these changes may not become manifest unless the liver faces additional challenges (ischemia and reperfusion, infection, etc.) and this may explain the absence of perceivable differences in obesity-related fatty liver of mice with and without UCP2 (67). If alteration of UCP2 expression has a potential to change the outcome of steatohepatitis, any planned intervention may conceivably need a cell-specific design.

6. NAFLD AND LATE EVENTS: IS UCP2 LINKED TO LIVER CANCER?

6.1. Obesity and liver cancer

NAFLD may take an indolent course and progress into cirrhosis and HCC (9, 10). NASH and other features of the metabolic syndrome often precede 'cryptogenic' cirrhosis (10, 101). While history of obesity in cryptogenic cirrhosis is an independent predictor of HCC (118), obesity even without cirrhosis appears to be a risk factor for the development of liver cancer. Perhaps the most striking data on the link of obesity and HCC have been obtained in a prospective US study assessing general cancer prevalence among more than 900,000 subjects wherein the relative risk for HCC of men and women with a BMI of 35 to 40 was 4.52 and 1.68, respectively

(119). Experimental studies support these epidemiological observations. Incidence of HCC is higher in ob/ob mice that have obesity and severe steatosis (120). Since ob/ob mice do not develop cirrhosis, it was suggested that not cirrhosis, but insulin resistance per se might be the cause of HCC in NAFLD (120). Growth effects of elevated plasma insulin and insulin-like growth factor-I levels, characteristic of insulin resistance, may indeed contribute to higher tumor rates in obesity (121).

6.2. UCP2 as a modulator of apoptosis

Most cancers result from an imbalance between cell proliferation and cell death (122). Mitochondria occupy a central role in the regulation of apoptosis (123). While the intrinsic apoptosis pathway primarily responds to metabolic perturbations and intracellular oxidative stress, the extrinsic pathway is activated through cell surface death receptors (123). There is evidence for crosstalk between these two pathways and for mitochondrial involvement in both processes (123). Major apoptosis events include oligomerization of pro-apoptotic Bcl-2 proteins and their translocation to the mitochondria, permeabilization of the mitochondrial outer membrane, disruption of $\Delta\Psi m$, release of intermembrane constituents (e.g., cytochrome c and apoptosis-inducing factor) into the cytosol, activation of initiator and executioner caspases, concluding into disassembly of the cell (123).

Based on its presumed biological functions, UCP2 may modulate apoptosis at several checkpoints. One such checkpoint may be mitochondrial hyperpolarization (MHP), a brief increase in $\Delta\Psi m$ that appears very early in apoptosis and is often overlooked (Figure 4). MHP was initially observed during the apoptosis of T lymphocytes (124) and then demonstrated in various cell types including hepatocytes (125-127). MHP seems to initiate ROS production and thus may have an important role in triggering apoptosis (126). Notably, apoptosis is delayed or may not occur if MHP (and subsequent ROS production) is prevented by mild uncoupling or by other measures (126, 128). However, it remains to be seen whether this mechanism accounts for the cytoprotective effect of in vitro and in vivo UCP2 overexpression.

6.3. UCP2 and cancer cell adaptation to oxidative stress

It has been known for many years that ROS production is increased in cancer cells (129, 130), but the significance of this finding is still poorly understood. ROS may elicit a wide range of cellular responses from proliferation to growth arrest to cell death (131). Under 'optimal' conditions, ROS may confer a growth advantage to tumor cells (130). Adverse environmental conditions such as nutritional deprivation, hypoxia, and reduced substrate adhesion, however, may be detrimental to cancer cells by further increasing oxidative stress that is already at a higher level (130). Stimulation of ROS production in cancer may be exploited for therapeutic purposes and in fact many anticancer treatment modalities have been developed based on this concept (132). Recent anti-cancer treatment efforts specifically target cellular mechanisms that lead to increased ROS levels. Thus, enhancement of mitochondrial ROS production by the respiratory complex I inhibitor rotenone has been shown to increase apoptosis in human leukemia cells (133) and in fibrosarcoma cells (134). However, cancer cells may adapt to harsh conditions, become more tolerant to oxidative stress, and avoid apoptosis (135, 136).

Is there a link between increased expression of UCP2 and the propensity for HCC in NAFLD patients? In fact, there is very little known about the effects of UCP2 on carcinogenesis. In principle, even small changes in $\Delta\Psi m$ and the rate of mitochondrial ROS production affected by UCP2 may cause significant shifts in the level of oxidative stress and in subsequent decisions on the fate of cancer cells. Drug-resistant subclones of various cancer cell lines that exhibit lower $\Delta\Psi m$ are less susceptible to oxidative stress and express more UCP2 (137). This suggests that cancer cells may eventually use enhanced UCP2 function to gain survival advantage. Expression of UCP2 in human colonic epithelium correlates with neoplastic changes along the adenoma-adenocarcinoma sequence, although UCP2 may serve here only as a marker of oxidative stress (41). More support for the role of UCP2 in cancer cell adaptation has been provided by studies in which high UCP2-expressing human cholangiocarcinoma cell lines were more resistant to apoptotic stimuli than low UCP2-expressing cell lines (138). Furthermore, UCP2 expression was found continuously higher and $\Delta\Psi m$ lower in primary cultured hepatocytes when followed up to 5 days (139). Increased UCP2 expression was also found in two out of four examined HCC cell lines and this coincided with hypomethylation of the *ucp2* gene (140). These studies suggest that increasing UCP2 expression may accompany hepatocyte dedifferentiation and coincide with reversion to a fetal phenotype of energy metabolism. Whether chronically high UCP2 expression in NAFLD contributes to the increased risk of HCC formation, however, is presently unknown.

In summary, it is an intriguing possibility to consider UCP2, a negative regulator of ROS production, to act as a modulator of apoptosis and a factor in the adaptation of cancer cells to oxidative stress. Further studies are needed to establish the role of UCP2 in cancer in general, and in NAFLD-related HCC formation in particular.

7. CONCLUSIONS AND PERSPECTIVES

The relationship of UCP2 and NAFLD appears to be more than serendipitous. For the time being, however, it is difficult to avoid speculations and there is much to be elucidated. Most importantly, the biological functions and transcriptional regulatory mechanisms of UCP2 are still debated. Also, most of our knowledge about the effects of UCP2 in liver disease is obtained from animal models not fully representative of human NAFLD. In spite of these limitations, we may identify several areas in which UCP2 can contribute to the pathogenesis of NAFLD linked to the metabolic syndrome. In addition to promoting hepatic fat accumulation and insulin resistance, increased levels of plasma fatty acids and certain fat-derived bioactive factors increase UCP2 expression and activation in fatty liver. UCP2 may alter hepatic fat distribution and metabolism, but there is currently no convincing evidence that up-regulation of UCP2 has an impact on the development of steatosis. In line with the 'two-hit' theory of NAFLD progression, it seems more feasible that UCP2 contributes to transition of steatosis into steatohepatitis. UCP2 has the ability to affect hepatocellular bioenergetics upon additional challenges. Oxidative stress is a major pathogenic factor in steatohepatitis and UCP2 has been shown to control ROS production at their primary source in the mitochondrial respiratory chain. Recognition of liver cell-specific regulation of UCP2 expression allows us to differentiate these effects in parenchymal and non-

parenchymal cells and supports customized strategies to modify UCP2 transcription and function. Finally, UCP2 may mitigate ROS-triggered apoptosis and be a factor in the development of hepatocellular carcinoma related to obesity and insulin resistance.

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FIGURE LEGENDS

Figure 1—Major determinants of mitochondrial membrane potential (ΔΨm)

ΔΨm is the result of proton translocation from mitochondrial matrix to intermembrane space by the electron transport chain (ETC) that includes complexes I to IV and mobile electron carrier proteins ubiquinone (U) and cytochrome c (C). ETC is supplied by reducing equivalents primarily formed during combustion of glucose and fatty acids. ΔΨm is consumed through oxidative phosphorylation mediated by ATP synthase (FoF1) and through proton leak mediated by carrier proteins (UCP2 shown here) and by basal proton conductance (BPC). Production rate of superoxide ($O_2^{-\cdot}$) at various ETC sites correlates with ΔΨm explaining why processes that consume the proton gradient may alleviate mitochondrial oxidative stress.

Figure 2—Potential effects of UCP2 on fatty acid metabolism in NAFLD

Large amounts of fatty acids (FA) are available in NAFLD that enter mitochondria of hepatocytes as FA-CoA, undergo beta-oxidation, and yield ketones or oxidize fully within the tricarboxylic acid cycle (TCA). This fuel surplus feeds reducing equivalents into ETC and drives up ΔΨm, promoting ROS production and shifting excess FA into alternative pathways. These include increased synthesis of triglycerides leading to ectopic fat accumulation as well as oxidation of fatty acids in non-mitochondrial compartments of the cell. FA may be freed of CoA by hydrolysis and trapped in the matrix as anions (FA-). Interaction of ROS with accumulated FA may produce lipid peroxide anions (HOO-FA-), similarly trapped in the matrix. In principle, UCP2 may alleviate these consequences by reducing ΔΨm and the rate of ROS production and allowing efficient mitochondrial beta-oxidation. UCP2-mediated proton leak may in fact occur through FA 'flip-flop' moving either FA- or HOO-FA- anions across the inner membrane. FA 'flip-flop' may also result in removal of trapped FA- and HOO-FA- anions from the matrix.

Figure 3—Consequences of cell-specific hepatic UCP2 expression in NAFLD

Opposing regulation of UCP2 expression may occur in non-parenchymal and parenchymal liver cells during NAFLD. UCP2 in normal liver tissue is primarily detected in Kupffer cells and is scarcely present in hepatocytes. This cell-specificity appears to swap in fatty liver disease with large amounts of UCP2 found in hepatocytes and diminishing expression in Kupffer cells and other macrophages. Based on the presumed functions of UCP2, Kupffer cells appear to be a key source of oxidative stress in NAFLD, while increasing competition of UCP2-mediated proton leak may contribute to impaired hepatocellular bioenergetics. Details of the transcriptional regulation of UCP2 remain to be seen.

Figure 4—Hypothetical model for the link between UCP2 and carcinogenesis

A wide spectrum of cell fate outcomes is associated with ROS production and includes such extremes as autonomous proliferation and destruction of the cell (inset). Cancer formation is normally offset by apoptosis in which ROS-mediated mitochondrial outer membrane

permeabilization (MOMP) is often a point of no return, leading to cytochrome c release into the cytosol and activation of the caspases cascade. Mitochondria provide much of intracellular ROS and this process is very sensitive to changes in $\Delta\Psi m$. Apoptosis is often initiated by a surge in ROS resulting from an early increase in $\Delta\Psi m$ and UCP2 may in principle mitigate this brief mitochondrial hyperpolarization. Therefore, even subtle shifts in $\Delta\Psi m$ could modulate apoptosis and tilt the balance of ROS effects toward cancer formation. This model may be valid in NAFLD where prolonged elevation of UCP2 expression in hepatocytes would contribute to the development of liver cancer.

Figure 1

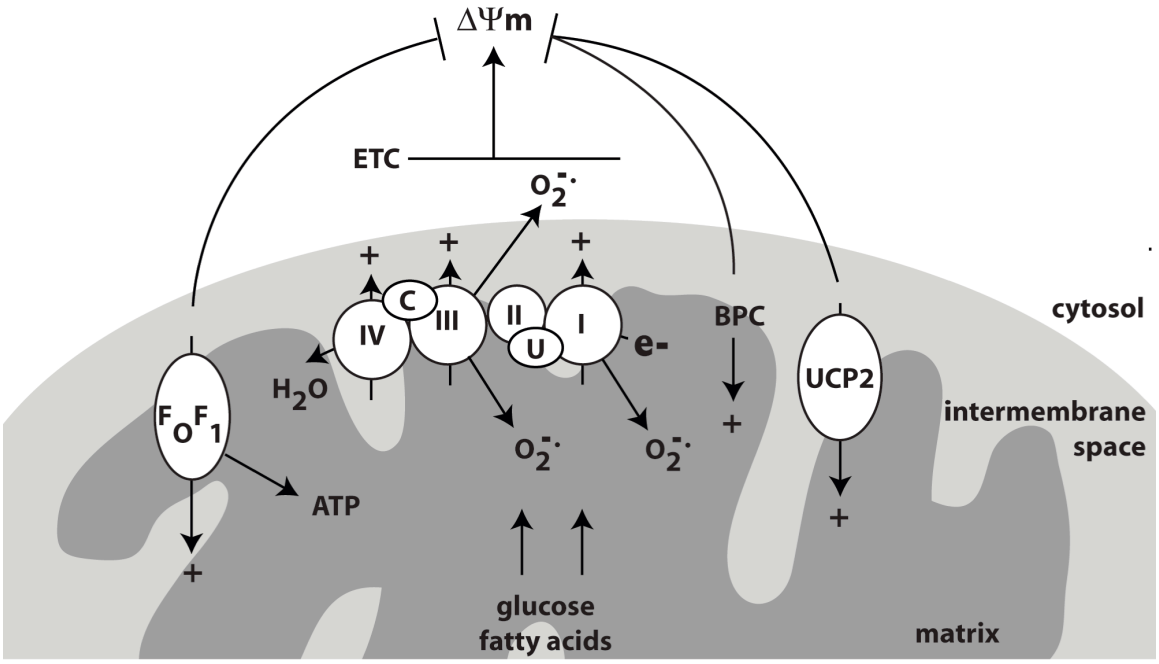

ΔΨm
ETC
$O_2^{-\cdot}$
+
+
+
C
IV
III
II
U
I
e-
H_2O
BPC
UCP2
cytosol
intermembrane space
F_OF_1
ATP
$O_2^{-\cdot}$
$O_2^{-\cdot}$
+
+
+
glucose
fatty acids
matrix

Figure 2

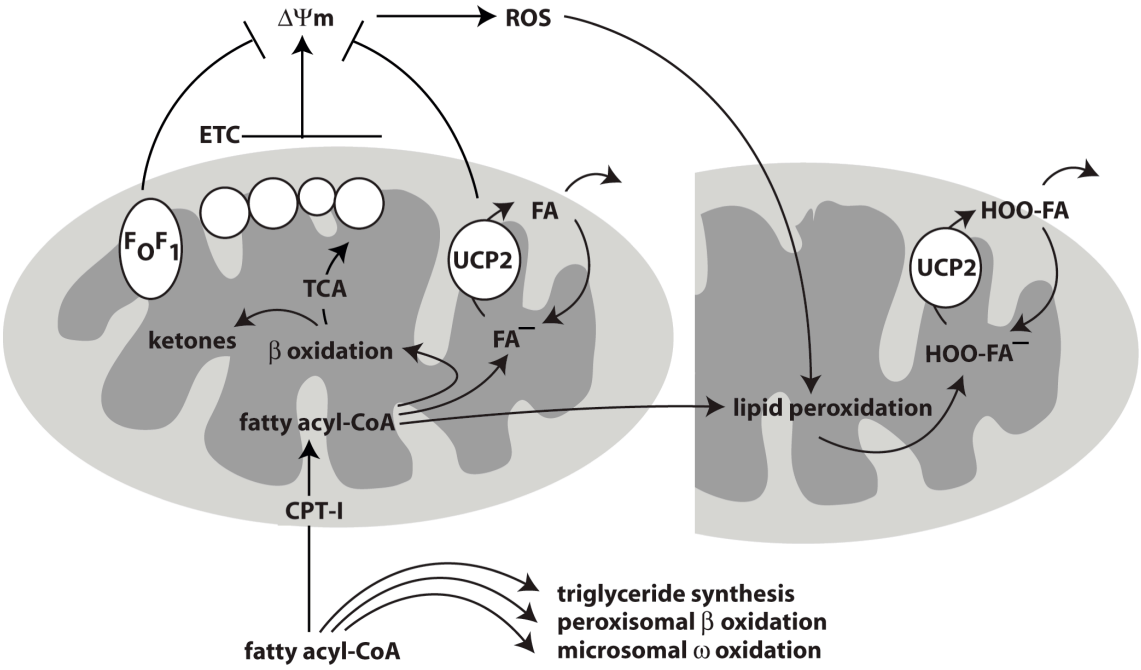

ΔΨm
ROS
ETC
FA
HOO-FA
F_OF_1
UCP2
UCP2
TCA
ketones
β oxidation
FA^-
$HOO\text{-}FA^-$
fatty acyl-CoA
lipid peroxidation
CPT-I
triglyceride synthesis
peroxisomal β oxidation
microsomal ω oxidation
fatty acyl-CoA

Figure 3

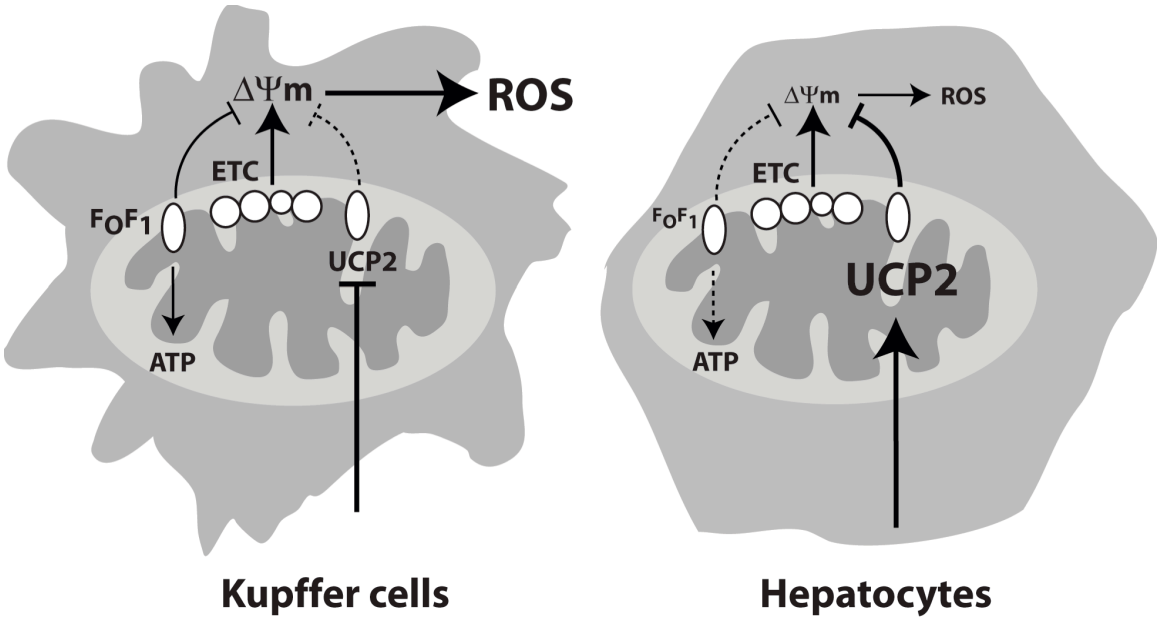
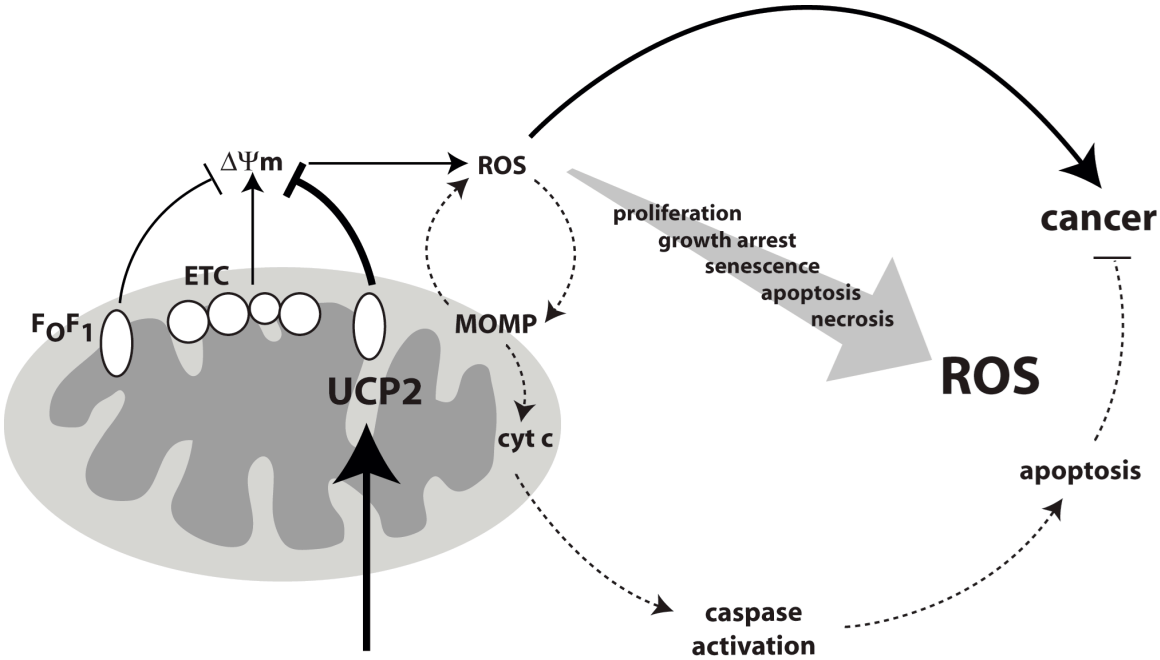

ΔΨm
ROS
ETC
FOF1
UCP2
ATP
Kupffer cells
ΔΨm
ROS
ETC
FOF1
UCP2
ATP
Hepatocytes

Figure 4


ΔΨm
ROS
cancer
proliferation
growth arrest
senescence
apoptosis
necrosis
ETC
FOF1
MOMP
ROS
UCP2
cyt c
apoptosis
caspase
activation

Send correspondence to: György Baffy, M.D., Ph.D., Liver Research Center, 55 Claverick Street, Room 413, Providence, RI 02903. Tel: (401) 444-2536, Fax: (401) 444-2939, E-mail: gbaffy@brown.edu

Running title: UCP2 and NAFLD

Key words: uncoupling protein-2, mitochondria, electron transport chain, oxidative phosphorylation, proton leak, mitochondrial membrane potential, reactive oxygen species, fatty acids, AMP-activated kinase, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, cryptogenic cirrhosis, hepatocellular cancer