

## Original Paper

# Mitochondria and Oxidative Stress in the Cardiorenal Metabolic Syndrome

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### Key Words

Cardiorenal syndrome • Overnutrition • Mitochondria • NADPH oxidase • Angiotensin II • MicroRNA • Alcoholic cardiomyopathy

### Abstract

Mitochondria play a fundamental role in the maintenance of normal structure, function, and survival of tissues. There is considerable evidence for mitochondrial dysfunction in association with metabolic diseases including insulin resistance, obesity, diabetes, and the cardiorenal metabolic syndrome. The phenomenon of reactive oxygen species (ROS)-induced ROS release through interactions between cytosolic and mitochondrial oxidative stress contributes to a vicious cycle of enhanced oxidative stress and mitochondrial dysfunction. Activation of the cytosolic and mitochondrial NADPH oxidase system, impairment of the mitochondrial electron transport, activation of p66shc pathway-targeting mitochondria, endoplasmic reticular stress, and activation of the mammalian target of the rapamycin-S6 kinase pathway underlie dysregulation of mitochondrial dynamics and promote mitochondrial oxidative stress. These processes are further modulated by acetyltransferases including sirtuin 1 and sirtuin 3, the former regulating nuclear acetylation and the latter regulating mitochondrial acetylation. The regulation of mitochondrial functions by microRNAs forms an additional layer of molecular control of mitochondrial oxidative stress. Alcohol further exacerbates mitochondrial oxidative stress induced by overnutrition and promotes the development of metabolic diseases.

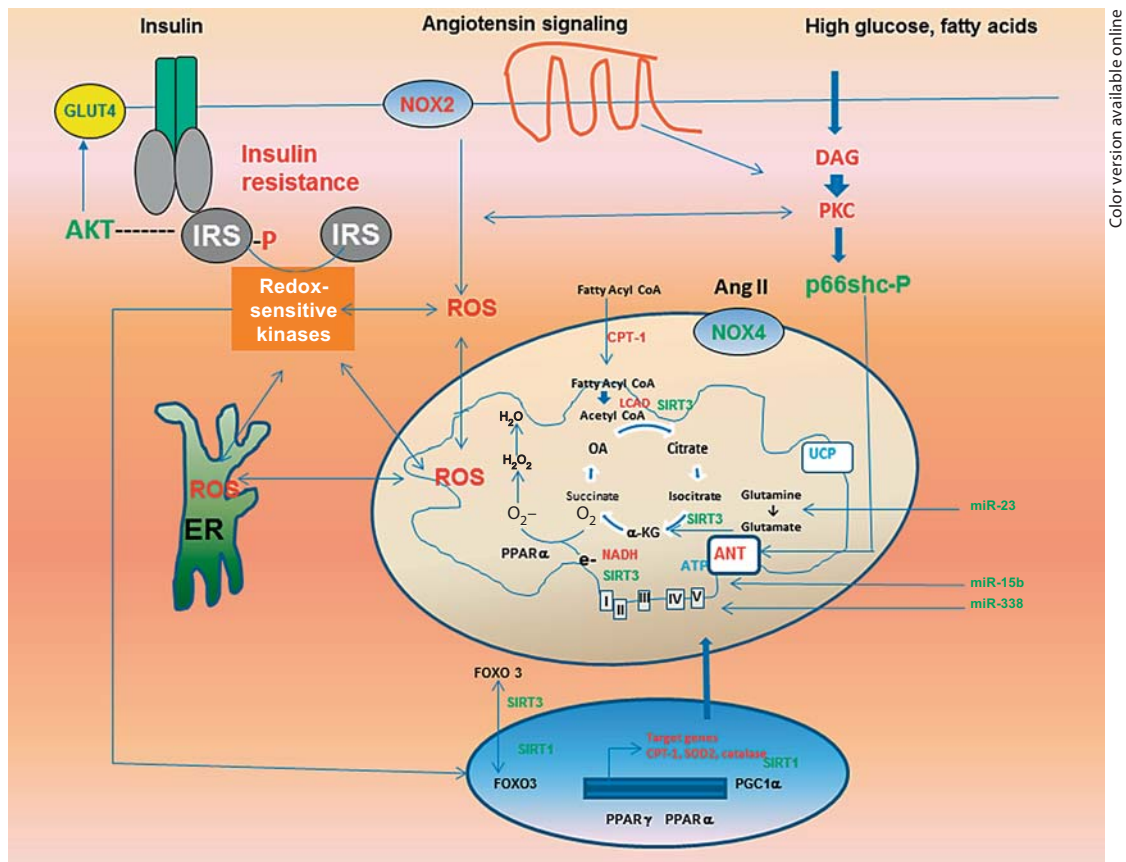
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## Introduction

Mitochondria are critical subcellular components that play a crucial role in energy generation, intermediary metabolism, and cell death. Accordingly, mitochondrial dysfunction is an important mechanism underlying human disease states including cardiac diseases, diabetes, and the cardiorenal metabolic syndrome (CRS) [1–25]. Although genetic disease is a primary cause for mitochondrial dysfunction, oxidative stress is linked to mitochondrial dysfunction either as a cause or as a result in pathological states. Mitochondria are major sources of reactive oxygen species (ROS) such as superoxide within the cell. Yet, they are very susceptible to oxidative damage, thereby contributing to mitochondrial dysfunction in a range of diseases. This review summarizes data that link oxidative stress to mitochondrial dysfunction in the pathogenesis of organ damage and dysfunction in the CRS.

## Maintenance of Normal Mitochondrial Structure and Function

Mitochondria normally play a fundamental role in the maintenance of normal structure, function, and survival of various tissues. Aerobic organisms consume oxygen to produce energy from nutrients, and energy production, mostly in the form of adenosine triphosphate (ATP), is controlled by mitochondria that link oxidative respiration with the metabolism of nutrients (fig. 1) [1–6]. In particular, almost all (>95%) of ATP produced in the heart comes from mitochondrial oxidative phosphorylation (OXPHOS), with the remainder derived from glycolysis. Mitochondria are compartmentalized by outer and inner membranes, and the mitochondrial respiratory chain is located in the inner membrane. The generation of ATP requires two major steps: the oxidation of nicotinamide adenine dinucleotide, its reduced form (NADH), or flavine adenine dinucleotide, its hydroquinone form (FADH<sub>2</sub>), and the phosphorylation of ADP to form ATP (OXPHOS). These two reactions are coupled in mitochondria, and OXPHOS is an efficient and energy-conserving mechanism of producing energy. NADH or FADH<sub>2</sub> are generated during glucose metabolism via glycolysis (tricarboxylic acid cycle) or  $\beta$ -oxidation of fatty acids. NADH or FADH<sub>2</sub> are oxidized to NAD<sup>+</sup> or FAD while protons are pumped to the intermitochondrial membrane space through respiratory complexes I, III, and IV. Electrons from NADH or FADH<sub>2</sub> are then transferred through a series of respiratory chain complexes to O<sub>2</sub>, which finally generates H<sub>2</sub>O. A proton gradient across the membrane is the driving force of F<sub>0</sub>F<sub>1</sub>-ATPase (ATP synthase) to produce ATP from ADP. ATP is transported to the cytosol by exchanging ADP through an adenine nucleotide translocator and is key for various biological events that require energy. On the other hand, mitochondria generate heat by a mechanism called ‘proton leak’. Proton leak from the intermembrane space to the matrix (uncoupling) reduces proton-motive force and generates heat instead of ATP. Uncoupling proteins (UCPs) play a major role in reducing the proton gradient. UCP1 is expressed almost exclusively in brown adipose tissue, UCP2 is ubiquitously expressed, and UCP3 is expressed in skeletal muscle. UCP1, which comprises up to 10% of membrane protein, regulates adaptive thermogenesis, whereas UCP2 and UCP3 do not appear to play a major role in thermogenesis. It was reported that mice with genetic ablation of UCP2 and UCP3 display a normal response to cold, normal basal proton conductance, and normal body weight [6]. In this regard, overexpression of UCP2 or UCP3 lowers ROS production, stimulates the metabolic rate, and protects against weight gain and associated insulin resistance. Collectively, these results suggest that UCPs play an important role in mitochondrial function by regulating both heat and ROS generation.



Color version available online

**Fig. 1.** Sources and consequences of mitochondrial oxidative stress and its relationship to insulin resistance in the CRS. ROS-induced ROS release results from several pathways, including hyperglycemia and lipid-induced activation of PKC, Ang II-mediated activation of cytosolic (NOX2) and mitochondrial (NOX) NADPH oxidase, activation of p66shc, ER stress, and dysregulation of nuclear and mitochondrial transcriptional response. The activation of redox-sensitive kinases induces insulin resistance through increased phosphorylation of serine residues in IRS proteins, which in turn suppresses insulin metabolic signaling.

### Mitochondrial Dysfunction in the CRS and Diabetes

There is considerable evidence for mitochondrial dysfunction in association with insulin resistance, obesity, and diabetes [1, 6]. This is especially relevant considering mitochondrial dysfunction compromises glucose-stimulated pancreatic insulin secretion as well as insulin-stimulated skeletal muscle glucose utilization [6]. Mitochondrial dysfunction has been shown to compromise insulin signaling through serine phosphorylation of insulin receptor substrate (IRS)-1 [32]. The contribution of mitochondrial dysfunction to impairments in insulin metabolic signaling is also suggested by gene array analysis showing that reductions in the expression of genes regulating mitochondrial ATP production are associated with insulin resistance [1] and type 2 diabetes mellitus [1–6]. Moreover, reductions in the oxidative capacity of the mitochondrial electron transport chain are manifested in obese, insulin-resistant persons as well as diabetic patients [1, 6]. Genetic and environmental factors, oxidative stress, and alterations in mitochondrial biogenesis can adversely affect mitochondrial function, leading to insulin resistance and various pathological conditions such as the CRS and type 2 diabetes [7–35].

Mitochondrial proteins are encoded by both nuclear and mitochondrial genes. Mitochondrial genes encode 13 protein subunits of the OXPHOS complex as well as mitochondrial-specific ribosomal and transfer (t) RNAs [1, 6, 26]. The oxidative capacity of mitochondria is determined by the expression level of OXPHOS subunits and by the number and size of mitochondria [6]. Because mitochondrial dysfunction and gene expression of mitochondrial OXPHOS genes are related to insulin resistance, mutations in mitochondrial genes caused by metabolic and/or oxidative stress conditions may be one of the mechanisms underlying insulin resistance and other features of the CRS.

The mitochondrial genome appears to be more susceptible to various mutagenic stressors because mitochondrial genes are more proximal to the ROS source and are not protected by histones [26, 27]. In this regard, the mitochondrial genome constitutes only coding sequences, whereas nuclear DNA contains non-coding sequences. Indeed, a naturally occurring thymidine-to-cytidine mutation in the mitochondrial tRNA gene is associated with hypertension, hypercholesterolemia, and other components of the CRS [26]. Another mutation, A3243G, on mitochondrial DNA that encodes tRNA (LeuUUR) causes impaired insulin secretion [27]. In addition, patients with defects in acyl-coenzyme A (CoA) dehydrogenase have phenotypes of cardiomyopathy and liver dysfunction [28]. Polymorphisms in the promoter of UCP2 are associated with a decreased incidence of obesity, reduced insulin secretion, and a high prevalence of diabetes [29, 30]. Alterations of nuclear genes encoding mitochondrial proteins are also associated with insulin resistance [31]. Thus, genetic factors that are inherited through nuclear genes that code for mitochondrial proteins or mitochondrial genes can influence the pathogenesis of the CRS through impairment of the mitochondrial function [6].

There have been reports of alterations in morphology and numbers of mitochondria in skeletal muscle, heart, and the liver in insulin-resistant, obese, and/or diabetic animals and patients [1, 6, 32, 34, 51]. The number and size of mitochondria are related to the mitochondrial oxidative capacity [1, 6]. In this regard, decreased mitochondrial oxidative capacity accompanies the reduction in expression of mitochondrial proteins encoded by both the mitochondrial genome (cytochrome *c* oxidase 1) and nucleus (succinate dehydrogenase and pyruvate dehydrogenase) [6, 32]. The molecular mechanism of mitochondrial biogenesis is driven, in part, through peroxisome proliferator-activated receptor (PPAR) co-activator (PGC)-1 $\alpha$ . PGC-1 $\alpha$  has been known as an inducible integrator of transcriptional circuits controlling mitochondrial biogenesis and function in a variety of tissue and cell types. The expression of PGC-1 $\alpha$  is increased on cellular ATP demand, including exercise, cold exposure, and fasting [35–38]. PGC-1 $\alpha$  is a co-activator of nuclear transcription factors, including nuclear respiratory factor (NRF)-1, PPAR- $\gamma$  and PPAR- $\alpha$  [38, 39]. NRF-1, in turn, regulates the expression of many mitochondrial genes, including OXPHOS genes and mitochondrial transcription factor A, that are crucial for mitochondrial gene expression and replication of the mitochondrial genome [1, 6]. Expression of PGC-1 $\alpha$  is decreased in insulin-resistant and diabetic humans, and NRF-1 expression is reduced in diabetic persons [40]. The reduction of PGC-1 $\alpha$  expression is age dependent [41], and PGC-1 $\alpha$  null mice exhibit serious defects in contractility in both skeletal and cardiac muscles [39–43]. Expression of PGC-1 $\alpha$  is also regulated by the endothelial nitric oxide (NO) synthase (eNOS)/NO/cGMP. The activation of eNOS plays an important role in mitochondrial biogenesis [1, 6, 44–47]. In fact, eNOS-deficient mice are insulin resistant and hypertensive and have defects in fatty acid metabolism and fewer mitochondria [45–47]. Furthermore, exogenous NO or cGMP increases mitochondrial biogenesis [47]. Another important factor that regulates mitochondrial biogenesis is AMP-activated protein kinase (AMPK) [48, 49]. Pharmacological drugs and other factors that activate AMPK promote mitochondrial biogenesis [6]. DNA microarray studies have shown that expression of PGC-1 $\alpha$ , with regard to mitochondrial biogenesis, may be respon-

sible for metabolic disorders, including the CRS and diabetes [40, 50]. These data suggest that decreased mitochondrial function is mainly attributable to the reduced number of mitochondria. Others have reported that subsarcolemmal mitochondrial electron transport activity is lower in obese and diabetic persons [33, 34]. In either case, diminished mitochondrial electron transport activity is partly attributable to the reduced mitochondrial content, but the decrement in mitochondrial function is greater than can be solely explained by mitochondrial content. Alterations in mitochondrial number and/or function that lead to decreased capacity to oxidize fat may be the underlying cause of lipid accumulation in skeletal muscle, heart, and liver which are characteristic of impaired insulin metabolic signaling and other functional abnormalities of the CRS [1, 6, 22–24]. Despite the overwhelming evidence depicting altered expression and activity for the PGC-1 $\alpha$  gene regulatory circuit under pathological conditions such as cardiac hypertrophy and ischemic insult, the jury is still out with regard to whether such changes in PGC-1 $\alpha$  are a cause or a consequence of these pathological changes.

### **Role of Oxidative Stress in Impaired Mitochondrial Respiratory Functions**

#### *ROS-Induced ROS Release*

Cellular oxidative stress involves both cytosolic and mitochondrial oxidative stress. Moreover, cytosolic oxidative stress also contributes to mitochondrial dysfunction, mitochondrial oxidative stress and vice versa. This phenomenon is described as ROS-induced ROS release and contributes to a vicious cycle of enhanced oxidative stress and mitochondrial dysfunction [52]. The magnitude of oxidative stress in cells or tissues is the result of a delicate balance of ROS and antioxidant capacity. ROS are generated either through mitochondrial respiration or through the action of several oxidases including NADPH oxidase, xanthine oxidase, cyclooxygenases, and lipoxygenase [53–55]. Although physiological levels of ROS are required for normal cellular function, overproduction of ROS will result in deleterious effects on cellular function. The major form of ROS is superoxide, and the accumulation of superoxide is caused by activation of NADPH oxidase and/or impaired mitochondrial electron transport. Superoxide is converted into hydrogen peroxide by superoxide dismutase (SOD) either in the cytosol (SOD1) or mitochondria (SOD2). Superoxide is also converted to another ROS peroxynitrite (reactive nitrogen species). Peroxynitrite reacts with proteins, lipids, and nucleic acids to form 3-nitrotyrosine which, in turn, is an indirect index of peroxynitrite formation and oxidative stress in tissues [53–56]. In this context, eNOS may compete with SOD, thereby reducing the catalysis of superoxide and thus superoxide accumulation [57]. The toxicity of hydrogen peroxide is suppressed by its conversion to oxygen and water by catalase or glutathione peroxidase. The effectiveness of glutathione peroxidase is, in turn, dependent on the availability of reduced glutathione, and the overall function of the glutathione system is further linked to vitamin E, vitamin C, and lipoic acid interaction [58, 59]. Therefore, oxidative stress is imposed on tissue when excessive amounts of ROS surpass different antioxidant mechanisms operative in the cell.

#### *Mitochondrial Oxidative Stress*

Mitochondria are a major intracellular source of oxidative stress [60]. Mitochondria-derived ROS are generated during OXPHOS occurring across the respiratory chain in the mitochondria. Although mitochondrial complexes I (NADH dehydrogenase) and III (ubiquinone cytochrome *c* oxidoreductase) are the major sites contributing to the production of ROS, complex II (succinate-ubiquinone oxidoreductase) and complex IV (cytochrome *c* oxidase) may also contribute to increased ROS production [60, 61]. Mitochondrial ROS excess

can be caused either by impaired activity of mitochondrial respiratory chain components or mitochondrial DNA mutations [62, 63]. Mitochondrial oxidative stress is also increased by compromised antioxidant function contributed by manganese (Mn)-SOD, catalase, and the glutathione system [64]. Severe dilated cardiomyopathy is seen in mice with complete deletion of mitochondrial Mn-SOD [65], whereas improved cardiac function and prolonged life span are seen in mice with mitochondrial targeted overexpression of catalase [66]. Moreover, the activity of Mn-SOD is reduced in heart failure [67].

A major contributory factor linked to this increased risk of cardiovascular disease in the metabolic CRS and diabetes is hyperglycemia-induced mitochondrial oxidative stress [68] which can induce cellular injury and dysfunction via several pathways: through the increased flux of glucose and other sugars through the polyol pathway, increased intracellular formation of advanced glycation end products (AGEs), increased expression of the receptor for AGEs and its activating ligands, activation of protein kinase C (PKC) isoforms, and over-activation of the hexosamine pathway. In the diabetic heart, overexpression of Mn-SOD or catalase protects cardiac mitochondria from oxidative damage, improves respiration, and normalizes mass in diabetic mitochondria [69]. Mn-SOD also prevents some of the morphological abnormalities that develop in diabetic hearts and normalizes contractility in diabetic cardiomyocytes [70].

#### *Role of NADPH Oxidase: NOX2 and NOX4*

In the cardiomyocyte, an important source of ROS is the one-electron reduction of O<sub>2</sub> to superoxide by NADPH oxidase, using NADPH as the electron donor [71, 72]. The NADPH oxidase is recognized as an important enzymatic system responsible for ROS production in diseases such as hypertension, atherosclerosis, and both type 1 and type 2 diabetes. Uncoupled eNOS is the predominant source of superoxide following the initial activation of NOX, whereas NOX remains active in the cardiovascular system [73]. Importantly, eNOS uncoupling has been shown to occur in patients with diabetes, contributing to cardiovascular complications [74]. NOX may serve as an upstream signaling molecule in mediating diabetic uncoupling of eNOS in response to angiotensin II (Ang II) [73]. The heterodimeric structure of NADPH oxidase comprises a catalytic subunit, NOX, which has five known isoforms (NOX1–5). These differ in their tissue distribution and kinetics of ROS formation [74]. NOX2-containing NADPH oxidase is mainly localized to plasmalemma and produces superoxide in the extracellular space or within the cytosol. The NOX4-containing oxidase is located in endosomes, focal adhesions, and nuclei and generates superoxide in the intracellular space. Therefore, the subcellular localization of NOX permits the site-specific release of superoxide through distinct regulatory mechanisms, thus making a significant contribution to ROS production, especially in chronic pathologic states [75]. NOX2 is expressed in cardiomyocytes and is often up-regulated in states of oxidative stress. [76]. Ang II is a potent activator of NOX2/NADPH oxidase in the heart and can play an important role in mediating oxidative stress in cardiac pathology [77–79]. Recent studies have shown the localization of the NOX4 subunit of NADPH oxidase in the mitochondria and its up-regulation by Ang II and oxidative stress [80]. Increased expression of NOX4 in the heart also resulted in increased mitochondrial oxidative stress [81]. The cysteine residues in NADH dehydrogenase flavoprotein I, a component of complex I, and in adenine nucleotide transporter 1 (ANT1), a key component of the mitochondrial permeability transition pore (MPTP) complex, together with aconitase-2, an established redox-sensitive protein, are highly oxidized, and their function is inhibited in transgenic mice with cardiac-specific overexpression of NOX4. These data suggest that these factors are directly modulated by O<sub>2</sub><sup>•-</sup> generated by NOX4 [81, 82]. However, using different animal models, a protective role for NOX4 in cardiac hypertrophy has been proposed [83]. Similarly, ROS produced by NOX4 has been identified as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in several studies [84].

### *p66Shc and Mitochondrial Oxidative Stress*

The role of p66Shc the 66-kDa isoform of the mammal ShcA gene, in mitochondrial oxidative stress is an area of active investigation. The significance of p66Shc comes from the finding that genetic deletion of p66Shc in mice confers resistance to oxidative damage and prolongs life span [85]. Although initial studies have implicated its role as a docking adaptor protein for mitogenic signals, recent studies have shown that p66shc acts as a redox enzyme contributing to mitochondrial ROS [86]. When the enzyme becomes phosphorylated on serine 36, it gets translocated into the mitochondrial intermembrane space. In the mitochondria, it catalyzes the production of H<sub>2</sub>O<sub>2</sub>, contributing to mitochondrial permeability transition. One of the consequences of mitochondrial permeability transition is caspase-mediated apoptotic cell death. The expression of p66Shc is increased in diabetic patients, and this increase was correlated to glycemic control and changes in the markers of oxidative stress [87]. p66Shc<sup>-/-</sup> mice are protected from the development of certain diabetes complications, such as nephropathy, endothelial dysfunction, and cardiomyopathy [88–90], thereby favoring the role of p66Shc in mitochondrial stress associated with diabetes mellitus.

### *Role of PPAR-α and Fatty Acid Metabolism*

The normal heart derives most of its energy from fatty acid metabolism, with only about 30% of its energy coming from glucose oxidation. During insulin resistance and diabetes, the rate of glucose uptake is reduced while that of circulating free fatty acids is increased, leading the heart to utilize even more fatty acids for its energy needs [91]. This may result in an increased mitochondrial turnover and increased fatty acid metabolism which are both seen in the early stages of obesity and insulin resistance [92]. In the chronic state, accumulation of free fatty acids and the eventual decrease in fatty acid oxidation create a closed loop of ever-increasing oxidative stress causing deleterious effects on the heart [92]. The lipotoxicity of circulating fatty acids or the intracellular accumulation of lipids contributes to mitochondrial oxidative stress through the activation of PKC, to endoplasmic reticulum (ER) stress, and to increased tissue levels of ceramide [6]. In this regard, the role of PPAR-α should be elucidated, as it is an important regulator of fatty acid metabolism in mitochondria [93–95]. PPARs have three isoforms and are primarily found in metabolic tissues in the body [92]. The expression of PPAR-α is up-regulated under stress conditions to the heart, including during diabetes, and contributes to and correlates with the increased oxidation of fatty acids seen in the early stages of cardiomyopathy [92, 94]. In addition, PPAR-α induces mitochondrial uncoupling and degradation through up-regulation of UCP2 [96] which are thought to be protective mechanisms against increased ROS production from damaged mitochondria. However, PPAR-α also affects the expression of genes involved in cardiac cell fatty acid uptake [92], and thus leads to fatty acid accumulation over time. The level of PPAR-α may be regulated in a negative feedback loop by the fatty acid uptake [similar to glucose transporters (GLUT) regulation being related to glucose uptake], and in the chronic condition leads to the down-regulation of PPAR-α protein expression. As such, PPAR-α has been shown to be down-regulated in human diabetic hearts [97]. This decreased level of PPAR-α leading to reduced fatty acid oxidation and coupled with the detrimental effects of increased fatty acids on the mitochondrial biogenesis results in the toxic accumulation of fatty acids. In compensatory pathologies such as obesity and overnutrition, PPAR-α actually increases β-oxidation of fatty acids, concomitant with limited ROS formation due to the removal of excess or damaged mitochondria through uncoupling mechanisms induced by PPAR-α [97, 98]. In decompensated conditions such as diabetes and eventual heart failure, the β-oxidation of fatty acids is diminished through the reduced expression of PPAR-α, and ROS formation is increased [97, 99]. Thus, one of the antioxidant therapies to improve or ameliorate the metabolic pathology through reduction of ROS formation involves targeting of PPAR-α [98,

100]. Emerging evidence suggests impaired mitochondrial fatty acid oxidation also plays a key role in hepatic steatosis. In this regard, Ang II-induced non-alcoholic fatty liver disease in transgenic TG(mRen2)27(Ren2) rats was characterized by oxidative stress-induced mitochondrial dysfunction and impaired  $\beta$ -oxidation of fatty acids [101]. Mitochondrial dysfunction and oxidative stress also precede insulin resistance and hepatic steatosis in non-alcoholic fatty liver disease in an obese rodent model [102].

#### *70S Ribosomal Kinase, Insulin Resistance, and Oxidative Stress*

The immunosuppressant drug rapamycin has been shown to attenuate ROS formation through the attenuation of mammalian target of rapamycin (mTOR) kinase signaling [103]. This property of rapamycin may be related to its ability to improve insulin signaling, as mTOR/S6K1 has been well studied in mediating insulin resistance by inhibition of IRS-1 through serine phosphorylation of this docking protein. In addition, activation of S6K1 in conditions of overnutrition has been shown to cause oxidative stress and endothelial dysfunction [104]. mTOR is also involved in promoting protein synthesis and translation, and its inhibition could directly reduce S6K1-mediated oxidative stress, and could also be an indirect compensatory effort to prevent the proliferation of cells with dysfunctional mitochondria in chronic stress conditions with elevated ROS formation. As such, mTOR expression is increased in conditions of obesity, insulin resistance, and the CRS, with an even larger increase during type 2 diabetes mellitus and its complications; but it is decreased in some cases during very late-stage decompensated heart failure [104]. In conjunction with this, the endogenous ROS levels increase sequentially, and fall only during very late-stage pathology characterized by extensive necrosis and cell death. On the other hand, ROS have been found to either activate or inhibit mTORC1 and S6K1 signaling. Low doses of ROS and short-term ROS exposure stimulate mTORC1, while high concentrations of ROS or long-term ROS treatment inhibit mTORC1 activity. Importantly, protein phosphatase 2A-mediated dephosphorylation of S6K1 and AMPK-mediated phosphorylation of Raptor (S792) contribute to ROS-induced inhibition of mTORC1 signaling [105]. Therefore, the magnitude and duration of oxidative stress and mitochondrial dysfunction may contribute to cardiac hypertrophy and transition to failure in diabetic cardiomyopathy.

#### *ER Stress, Mitochondrial Oxidative Stress, and Insulin Resistance*

The ER is an additional organelle that contributes to mitochondrial oxidative stress and insulin resistance. One of the functions of the ER is protein folding [106]. The accumulation of misfolded proteins results in the initiation of the unfolded protein response, leading to an increased generation of ER-derived ROS [107, 108]. Moreover, the interorganelle communication between mitochondria and the ER may underlie increased mitochondrial ROS generation originating from ER-derived signals [109, 110]. Although the mechanism of ER stress-induced insulin resistance is not yet clearly known, activation of stress-activated MAP kinase c-Jun N-terminal kinase (JNK) has been proposed as one of the signaling pathways linking ER stress and insulin resistance. In conditions of oxidative stress, the redox-sensitive serine kinase JNK also phosphorylates IRS-1 on serine 307 which is inhibitory for insulin metabolic signal transduction [111–115].

#### *Mitochondrial Fission and Oxidative Stress*

Mitochondria are dynamic organelles which are able to interchange their morphology between two distinct arrangements by undergoing the processes of mitochondrial fusion and fission to generate either an elongated interconnected mitochondrial network or a fragmented discrete phenotype, respectively [116, 117]. Changes in mitochondrial morphology are orchestrated by a group of mitochondrial fusion and fission proteins [118, 119]. Recent in vi-



tro data have suggested that hyperglycemia induces mitochondrial fragmentation [120]. In the rat heart myoblast cell line H9c2, sustained hyperglycemia-induced mitochondrial fragmentation and mitochondrial ROS production resulted in cell death by MPTP opening and apoptosis [121]. Importantly, this detrimental process could be prevented by transfecting cells with dynamin-related protein 1 (Drp1)K38A, suggesting that the hyperglycemia-induced mitochondrial fragmentation was a Drp1-dependent process [121]. Coronary endothelial cells isolated from the diabetic murine heart (type I model of diabetes) displayed more mitochondrial fragmentation when compared with those from non-diabetic mice, and this change in mitochondrial morphology was abolished by 4 weeks of pre-treatment with an antioxidant. These findings were associated with reduced levels of optic atrophy 1 (OPA1) and increased levels of Drp1 (levels of mitofusin 1 (Mfn1), mitofusin 2 (Mfn2) and human orthologue of Fis1p (hFis1) were unchanged), although antioxidant therapy did not change the levels of these mitochondrial-shaping proteins [122]. These data have suggested a role for oxidative stress as a mediator of mitochondrial fragmentation, which has been confirmed by the fact that oxidative stress induced further mitochondrial fragmentation in coronary endothelial cells [102]. A recent study has examined the mitochondrial morphology from a streptozotocin-induced diabetic adult mouse heart and has found that the mitochondria with fission were smaller, had lower mitochondrial membrane potential, and were more predisposed to MPTP opening and apoptosis [123].

### **Sirtuins, Oxidative Stress, and Mitochondrial Dysfunction**

Recent proteomic studies have shown that acetylation of mitochondrial proteins is one of the important mechanisms for the fine control of fuel metabolism in mitochondria through acetylation/deacetylation of key pathways including  $\beta$ -oxidation of fatty acids, gluconeogenesis, tricarboxylic acid (TCA) cycle, and urea cycle [124, 125]. Acetylation of mitochondrial proteins is governed by the integrated action of histone acetyl transferases (HATs) and histone deacetylases (HDACs) [126–128]. Classically, HATs and HDACs have been studied in the context of histone modifications and thereby named accordingly. Recent studies have indicated that they can act as lysine acetyl transferases and lysine deacetylases, and expanded their substrate range to transcription factors as well as mitochondrial, cytosolic, and cytoskeletal proteins [126–128]. With regard to cardiac dysfunction in the CRS and diabetes mellitus, they are implicated in cardiac contractile dysfunction, energy metabolism, hypertrophy, and fibrosis [129–134].

The emerging role of the sirtuin (SIR) class of HDACs in mitochondrial biogenesis and mitochondrial oxidative stress, and its regulation by oxidative stress underscore the importance of protein acetylation in mitochondrial oxidative stress in the CRS and diabetes mellitus. SIR proteins (collectively known as sirtuins) belong to a conserved family of nicotinamide adenine dinucleotide (NAD)-dependent deacetylases (class III NAD<sup>+</sup>-dependent deacetylase) implicated in the regulation of life span under calorie restriction. Their key role is in regulating glucose and lipid metabolism [135–138]. In mammals, there are seven homologs of SIR (SIRT1–7). Among these, SIRT1 has been most extensively studied, but the significance of other sirtuins is being increasingly recognized. Recent studies have demonstrated a pivotal role of SIRT1 in the regulation of energy metabolism and cellular survival, in addition to its known role in life span regulation. The differential distribution of sirtuins implicates their distinct roles in cytosolic, mitochondrial, and nuclear events. SIRT1 is predominantly localized to the nuclear compartment and is implicated in regulating the transcriptional and epigenetic regulation of gene expression [135–138]. In contrast, SIRT2 is cytosolic, and SIRT3, SIRT4, and SIRT5 are mainly mitochondrial sirtuins [135–138].

### *SIRT1, Oxidative Stress, and Mitochondrial Dysfunction*

SIRT1 deacetylates a wide variety of substrates implicated in insulin action, including PGC-1 $\alpha$ , UCP2, nuclear factor kappa-B (NF- $\kappa$ B), forkhead Box 01 (FoxO1) proteins, sterol regulatory element-binding protein-1c (SREBP-1c), and PPAR- $\gamma$  [139, 140]. Therefore, it plays a critical role in adipogenesis, and glucose and lipid metabolism in liver and skeletal muscle. Decreased expression or activity of SIRT1 is emerging as one of the major determinants of insulin resistance [141]. SIRT1 has been shown to promote insulin secretion through modulation of UCP expression and deacetylation of FoxO1 [142]. SIRT1 also exerts positive effects on insulin signaling through repression of protein tyrosine phosphatase 1B (PTP1B), a negative regulator of insulin signaling, and stimulation of protein kinase B (Akt) [143]. In adipocytes, SIRT1 promotes glucose uptake and suppression of IRS-1 serine phosphorylation [144]. SIRT1 was shown to modulate the expression of the DNA repair factor growth arrest and DNA damage-inducible protein 45 (GADD45) and the mitochondrial antioxidant enzyme Mn-SOD. This occurs by the SIRT1-mediated regulation of PGC-1 $\alpha$  [145] and FoxO transcription factors [139, 140]. On the other hand, oxidative stress decreases SIRT1 expression, and degradation of SIRT1 occurs by its oxidative modification [146]. SIRT1 also suppresses NADPH oxidase-mediated oxidative stress through PGC-1 $\alpha$  activation, which is sufficient to down-regulate NADPH oxidase expression in endothelial cells [147]. In hamsters treated with resveratrol, nuclear SIRT1 induced mitochondrial Mn-SOD, which reduced oxidative stress and contributed to cardiomyocyte protection. Resveratrol also suppressed myoblast death induced by Ang II through the nuclear activation of SIRT1 and induction of mitochondrial Mn-SOD [148]. Additionally, SIRT1 overexpression or activation by resveratrol leads to down-regulation of Ang II type 1 receptor (AT1R) mRNA in vascular smooth muscle cells and improves insulin resistance [148]. SIRT2 has also been suggested to ameliorate oxidative stress via deacetylation of FoxOs [149].

### *SIRT3*

The role of SIRT3 in age-induced cardiac hypertrophy and fibrosis was revealed by exaggeration of this process in aged SIRT3 knockout mice [150]. Moreover, SIRT3 knockout mice are more sensitive to cardiac stress induced by pressure overload [151]. The protection of cardiac myocyte death during cardiac failure was induced by overexpression of nicotinamide phosphoribosyltransferase (NAMPT) in a SIRT3-dependent manner [151]. The anti-apoptotic effect of NAMPT through the suppression of translocation of the apoptosis-inducing factor from mitochondria to the nucleus is dependent on SIRT3. SIRT3 has been shown to decrease ROS production in brown adipocytes [152] as well as mitochondrial biogenesis through the transcriptional co-activator PGC-1 $\alpha$  [153, 154]. The significance of SIRT3 in relation to overnutrition has emerged from recent findings of the effects of mitochondrial SIRT3 on hepatic mitochondrial protein acetylation [155]. High-fat diet was characterized by a significant decrease in hepatic SIRT3 activity and concomitant hyperacetylation of proteins involved in gluconeogenesis, mitochondrial oxidative metabolism, and ER stress response, and these changes were further increased in SIRT3 knockout animals. These changes were accompanied by a disruption of OXPHOS complexes II, III, and IV [155]. SIRT3 also stimulates hepatic  $\beta$ -oxidation through deacetylation of long-chain acyl dehydrogenase (LCAD), one of the key enzymes of  $\beta$ -oxidation, and hepatic SIRT3 levels increase during fasting [156]. In primary cultured cardiomyocytes, SIRT3 blocked cardiac hypertrophy by activating the FoxO3a-dependent, antioxidant-encoding genes Mn-SOD and catalase, thereby decreasing cellular levels of ROS [151]. Ang II-induced cardiac hypertrophy was also suppressed in transgenic mice expressing SIRT3 in the heart [157]. In cultured murine tubular epithelial cells, Ang II down-regulates SIRT3 mRNA, and this effect is overcome by AT1R

blockade [158]. These findings suggest that SIRT3 plays a significant role in mitochondrial oxidative stress and dysregulation of mitochondrial biogenesis in metabolic syndrome and diabetes mellitus.

### **MicroRNA-Mediated Regulation of Mitochondrial Dysfunction and Oxidative Stress**

Accumulating evidence implies that the non-coding microRNAs (miRNAs or miRs) impose another layer of regulation of mitochondrial function and oxidative stress. miRNAs are small non-coding RNAs (approx. 22 nucleotides) that modulate mRNA stability and repress post-transcriptional translation by binding to target recognition sequences predominantly located within the 3' untranslated regions of target mRNAs [159–162]. The 'seed sequence' that spans bases 2–8 of the 5' portion of mature miRNAs essentially dictates what target mRNAs would be regulated by a given miRNA. Since one miRNA can regulate hundreds of mRNAs and, conversely, one mRNA can be regulated by multiple miRNAs, it is becoming increasingly clear that a modest repression of the expression of a multitude of mRNAs by a given miRNA plays a major role in modulating related biological functions. Additionally, since many miRNAs may share the same seed sequence and form a miRNA family, they have the regulatory potential to redundantly regulate complex biological functions by targeting multiple genes belonging to a common pathway simultaneously. It is now increasingly recognized that deregulation of miRNAs contributes significantly to the pathophysiological etiology of many chronic diseases such as cancer, diabetes, hypertension, atherosclerosis, and neurological disorders, to name a few. The human genome encodes more than 1,000 miRNA genes; however, details of which miRNAs modulate mitochondrial functions are only beginning to emerge.

#### *miRNA Modulation of Glutamine Metabolism*

Mitochondria are highly abundant and constitute approximately 40% of the total cardiomyocyte volume in the heart [163]. Cheng et al. [164] have shown that the most abundant miRNAs in the heart are miR-1, Let-7, miR-133, miR-126-3p, miR-30c, and miR-26a. Studies on idiopathic end-stage failing human hearts have shown that the expression of miR-24, miR-125b, miR-195, miR-199a, and miR-214 is up-regulated in these tissues [162]. The same study has also shown that miR-23a, miR-23b, miR-24, miR-195, and miR-214 were increased in response to hypertrophy. It is conceivable that the increased expression of miR-23a/b in hypertrophy may actually represent a compensatory mechanism to down-regulate mitochondrial glutaminase (GLS) [165–167]. GLS converts glutamine to glutamate that is further catabolized through the TCA cycle for the production of ATP. Glutamate serves as a substrate for glutathione synthesis as well. Proliferating cells are known to utilize glutamine as a major source for energy, nitrogen for biosynthesis, and a carbon substrate for anabolic processes [165, 166]. Recent reports show that transcriptional regulation of the oncogene Myc coordinates with the expression of genes that promote cells to engage in excessive glutamine catabolism which exceeds the cellular requirement for protein and nucleotide biosynthesis [165]. Such Myc-dependent glutaminolysis results in the reprogramming of mitochondrial metabolism to depend on glutamine catabolism and to maintain cellular viability and TCA cycle anaplerosis. Concomitantly, this stimulation of mitochondrial glutamine metabolism leads to reduced glucose carbon entering the TCA cycle and a decreased contribution of glucose to the mitochondrial-dependent synthesis of phospholipids [163]. Interestingly, miR-23a/b targets mitochondrial GLS [166, 167]. Myc up-regulates glutaminase by down-regulating the expression of miR-23a/b [166, 167]. In age-related macular degeneration patients, it was found that the miR-23 expression was significantly down-regulated. miR-23 was also

down-regulated in oxidant-induced injury [167]. The miR-23 mimic could rescue H<sub>2</sub>O<sub>2</sub>-induced ARPE-19 cell death and apoptosis [167]. Collectively, these observations uncover a role for miR-23 in oxidant-induced injury and mitochondrial metabolism. Moreover, they also suggest a link between Myc regulation of miRNAs, glutamine metabolism, and energy and ROS homeostasis.

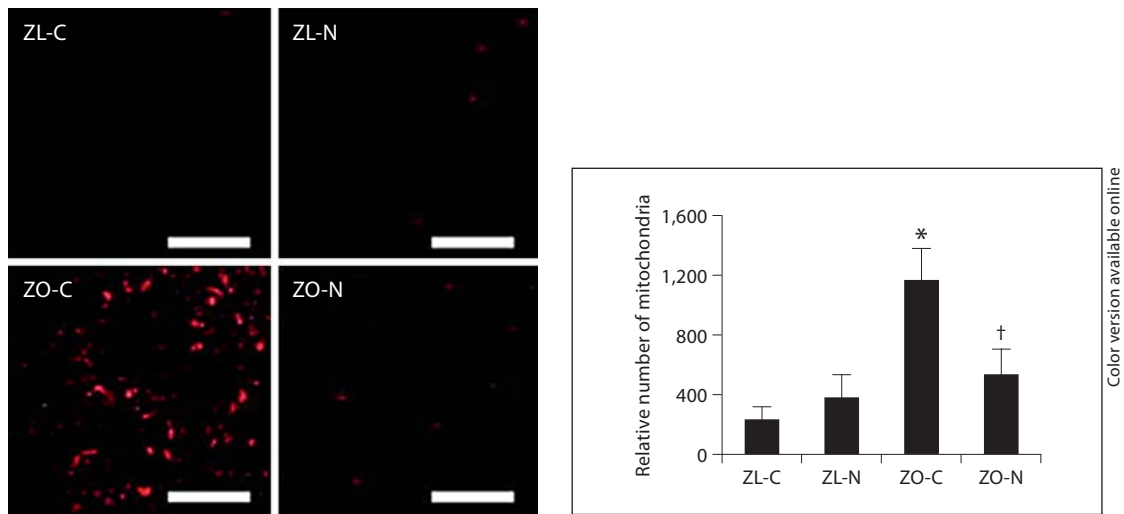
#### *miRNA Regulation of Cardiac Mitochondrial ATP Levels*

In an attempt to determine which miRNAs modulate mitochondria and ATP levels in cardiomyocytes, Nishi et al. [168] evaluated the effects of 23 miRNAs that are known to be expressed in heart tissue by individually over-expressing them in neonatal rat cardiomyocytes using a lentiviral vector. The miRNAs that emerged as regulators of ATP levels from this study were miR-15b, miR-16, miR-195, and miR-424, all sharing the same seed sequence. Over-expressing these miRNAs down-regulated cellular ATP levels and affected mitochondrial integrity. The target of these miRNAs was ADP-ribosylation factor-like 2 (Arl2) mRNA [168]. The knockdown of Arl2 by siRNA also resulted in reduced ATP levels and degeneration of mitochondria [168]. Thus, conditions that induce up-regulation of miR-15b and other miRNAs that share the same seed sequence (such as miR-195, which was shown to be up-regulated in cardiac hypertrophy) reflect mitochondrial degeneration and reduction in ATP levels in cardiomyocytes.

In conditions of overnutrition/obesity, however, an increase in mitochondria is observed in muscles [169–171]. For example, it is reported that maternal diet-induced obesity in mice can cause an increase in mitochondrial potential, mitochondrial DNA content, and biogenesis [169]. Concomitantly, ROS levels were raised, whereas glutathione was depleted and the redox state became more oxidized, suggestive of oxidative stress. Igosheva et al. [169] proposed that the ‘altered mitochondrial properties were associated with significant developmental impairment as shown by the increased number of obese mothers who failed to support blastocyst formation compared to lean dams’. In muscles of mice fed a high-fat diet, it has been reported that the mitochondrial content was actually increased, and it has been proposed that overconsumption, rather than mitochondrial dysfunction, is the underlying reason for the insulin resistance in these animals [170, 171]. We have demonstrated that Zucker obese (ZO) rat heart has increased mitochondria as shown in figure 2 [172]. Compared to Zucker lean (ZL) controls, ZO rats exhibited a prolonged diastolic relaxation time and a reduced initial diastolic filling rate suggestive of cardiac dysfunction. They also showed increases in homeostatic model assessment of insulin resistance, NADPH oxidase activity, 3-nitrotyrosine, and NADPH oxidase-dependent superoxide [172]. Thus, the ZO rat heart tissues exhibit a scenario comparable to that seen in high-fat diet-fed mice. Our miRNA profiling experiments indicated that in fact rat (rno)-miR-16 was slightly down-regulated in ZO rat left ventricular tissue (fig. 3). Other miRNAs that shared the same seed sequence (miR-15b and miR-195) showed comparable expression levels in both ZO and ZL rat left ventricular tissues. Thus in 12-week-old ZO rat left ventricular tissue, down-regulation of Arl2, mediated by up-regulation of miRNAs that share the same seed sequence, does not seem to be occurring.

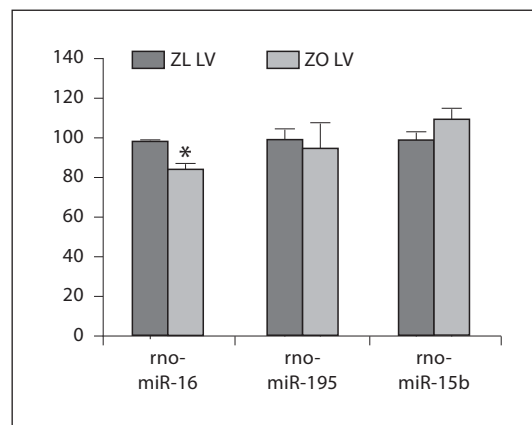
#### *miRNA Regulation of Apoptosis*

We have previously proposed that compensative/adaptive mechanisms are activated in ZO rat heart that may have a role in attenuating the progression to diabetic cardiomyopathy in these animals compared to Zucker diabetic fatty rats [175]. Additionally, we have recently shown that miR-200c, an miRNA only modestly expressed in the heart, is increased in ZO rat heart, and this increase in miR-200c may serve as a compensatory mechanism to down-regulate excessive activation of the nutrient sensor kinase S6K1 [176]. The miR-200 family is



**Fig. 2.** Immunostaining of mitochondrial complex IV-1 in ZO and ZL rats. Representative left ventricular sections immunostained for mitochondrial complex IV-1 of treated and untreated ZL and ZO rats. The increased level of complex IV-1 immunostaining in the ZO-C myocardium indicates an increased mitochondrial number compared with all other groups. The bar graph shows the quantification of converted signal intensities of complex IV-1 protein. ZL-C = Zucker lean-control; ZL-N = Zucker lean-nebivolol; ZO-C = Zucker obese-control; ZO-N = Zucker obese-nebivolol. \*  $p < 0.05$  vs. ZL-C; †  $p < 0.05$  vs. ZO-C. Figure used with permission from Zhou et al. [172].

**Fig. 3.** miRNA profile in the heart of ZL and ZO rats. The miRNA was isolated with an mirVana miRNA Isolation Kit (Ambion Inc.) from fresh-frozen tissues ( $n = 3$  for each group), and was labeled with a FlashTag™ Biotin HSR RNA Labeling Kit. An Affymetrix miRNA GeneChip was used for this study (46,228 probes comprising 7,815 probe sets, including controls). The probes on this chip are derived from the Sanger miRBase miRNA database v11 (April 15, 2008, <http://microrna.sanger.ac.uk>) [173]. Data analysis was performed by miRNA QC tool and Significance Analysis of Microarrays (SAM) software [174]. LV = Left ventricle. \*  $p < 0.05$  for ZL versus ZO.



implicated in the epithelial-to-mesenchymal transition (EMT) that is accompanied by mitochondrial biogenesis and involved in organ fibrosis and carcinoma progression [177–184]. It was reported that miR-200 is down-regulated in EMT, and that overexpression of miR-200b can attenuate EMT [179]. miR-200 regulates stem cell factors and functions as a cell growth inhibitor by targeting apoptosis inhibitor FAS-associated phosphatase-1 (FAP-1) [182]. It also regulates zinc finger E-box binding homeobox 1 (ZEB1), the EMT activator that promotes tumorigenicity [183, 184]. Expression of the miR-200c/141 cluster is down-regulated by DNA methylation in cancer, suggesting that an epigenetic silencing mechanism regulates these miRNAs. These observations have led to the idea that the members of the miR-200 family (miR-200a, b, c, miR-141, miR-429) serve as guardians of differentiation [185]. An analysis

of rat (rno)-miR-200c targets predicted by miRecords [186] revealed that mitochondrial enzyme holo-cytochrome-c synthetase (HCCS) is also a target for miR-200c. HCCS functions as heme lyase and during apoptotic stimuli, it translocates to outside the mitochondria and suppresses the X-linked inhibitor of apoptosis protein, thus causing the activation of caspase-3 [187]. Up-regulation of miR-200c can lead to the suppression of HCCS expression that can attenuate cardiomyocyte apoptosis [188]. In this context, the increase in miR-200c observed in the ZO rat heart further supports the notion that in 12-week-old ZO rat heart compensatory/adaptive mechanisms to attenuate the progression of obesity-induced heart disease are activated via up-regulation of miR-200c.

#### *Control of Cytochrome C Oxidase IV Expression by miR-338*

miR-338 is another miRNA that modulates OXPHOS and mitochondrial functions, since it targets cytochrome *c* oxidase IV (COX IV) mRNA [189]. In neurons, miR-338 overexpression by transfection resulted in decreased COX IV mRNA and protein levels and mitochondrial activity, due to reduced ATP levels. In contrast, expression of anti-miRNA oligonucleotides increased COX IV levels and improved OXPHOS. Thus, it was concluded that miR-338 overexpression is detrimental to mitochondrial functions. In this context, it is noteworthy that miR-338 is one of the most abundant miRNAs seen in hepatocellular carcinoma [190].

In brief, the above examples show that we are beginning to comprehend the fundamental roles of miRNAs in the modulation of oxidative stress and mitochondrial dysfunction. While a wealth of data is available regarding the miRNA-mediated regulation of mitochondrial functions in cancer and neuronal diseases, the miRNA modulation of overnutrition-related mitochondrial dysfunction is yet to be elucidated.

#### **Alcohol, Oxidative Stress, and Mitochondrial Dysfunction**

Heavy and prolonged consumption of alcohol results in both myocardial and hepatic injuries [191, 192]. A number of theories have been put forward for the onset and development of alcoholic complications, including oxidative damage, deposition of triglycerides, altered fatty acid extraction, decreased myofilament Ca<sup>2+</sup> sensitivity, impaired protein metabolism, and mitochondrial anomalies [193–199]. Not surprisingly, oxidative stress and mitochondrial damage have been shown to contribute to the toxic effects of alcoholic liver and myocardial injuries [200–202]. The significance of a link between alcohol and obesity/diabetes stems from the increased risk of progressive alcoholic liver and myocardial injury in obesity/diabetes [203–205]. These interrelationships may be caused by several common mechanisms of mitochondrial dysfunction and oxidative stress induced via alcohol and obesity/diabetes. The factors contributing to mitochondrial dysfunction include decreased expression of SIRT1 [206], decreased phosphorylation of AMPK [207, 208], altered mitochondrial permeability transition contributed by dysregulation of SIRT3 [209], and impaired transport of glutathione across mitochondria with decreased mitochondrial glutathione levels [210]. Long-standing alcohol consumption also leads to decreased SOD2 and catalase, thereby suppressing mitochondrial antioxidant capacity [193, 200, 204, 205, 211, 212]. In addition, chronic heavy alcohol consumption also results in insulin resistance in cardiomyocytes [213–216]. The effects of alcohol are, in part, caused by ethanol metabolite acetaldehyde and increased metabolism of alcohol caused by induction of cytochrome P450 2E1 [213, 214]. The role of acetaldehyde in alcoholic-induced mitochondrial dysfunction, insulin resistance, ER stress, and activation of redox-sensitive kinases and AMPK signaling has been demonstrated in a novel transgenic mouse model with cardiac-specific overexpression of alcohol dehydroge-

nase, which mimics the acetaldehyde-overloaded model of alcoholic cardiomyopathy. Moreover, ethanol effects are attenuated in mitochondrial aldehyde dehydrogenase-overexpressing mice. These observations underscore the significance of oxidative metabolism of ethanol in ethanol-mediated mitochondrial toxicity. In this regard, ethanol potentiation of Ang II activation of extracellular signal-regulated kinase (ERK1/2) is noteworthy [217] since phosphorylation of NADPH oxidase by ERK1/2 contributes to increased oxidative stress [218], and oxidative stress affects SIRT1 levels [146].

## Conclusion

In summary, mitochondrial dysfunction is an important player in the development of heart disease and other components of the CRS. The interplay between cytosolic and mitochondrial oxidative stress is associated with impaired mitochondrial electron transport and mitochondrial dynamics. Dysregulation of insulin and angiotensin signaling including activation of NADPH oxidase, ER stress, and activation of ROS-sensitive kinases contribute to a vicious cycle of enhanced oxidative stress and mitochondrial dysfunction. Altered acetylation of proteins in both the nuclear and mitochondrial compartment and modulation of mitochondrial function by miRNAs are also emerging as regulators of mitochondrial oxidative stress. Alcohol further augments mitochondrial oxidative stress in the CRS. Thus, in addition to being a major source of ROS generation, mitochondria are very susceptible to oxidative damage.

## Acknowledgements

This research was supported by the NIH (R01 HL73101-01A1 and R01 HL107910-01 to J.R.S.), the Veterans Affairs Merit System 0018 (to J.R.S.) and the University of Missouri Mission Enhancement Fund and Forest Laboratories (to L.P.). The authors would like to thank Brenda Hunter for her assistance in editing the manuscript.

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