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Free Radical Biology & Medicine 38 (2005) 175–188

www.elsevier.com/locate/freeradbiomed

Serial Review: The Powerhouse Takes Control of the Cell:
The Role of Mitochondria in Signal Transduction
Serial Review Editor: Victor Darley-Usmar

Mitochondrial proteomics in free radical research ☆

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Received 23 August 2004; accepted 6 October 2004
Available online 3 November 2004

Abstract

The importance of mitochondrial dysfunction in numerous diseases has long been appreciated. The impact of oxidative and nitrosative stress on mitochondrial function is complex; however, recent progress in the field using proteomics technologies has begun to shed light on the molecular defects responsible for mitochondrial and cellular dysfunction. This review focuses on the state-of-the-art technologies being used and current research endeavors in the field of mitochondrial proteomics with emphasis on those advancements being made in the field of free radical biology to identify the importance of alterations to the mitochondrial proteome in the development of disease.

Keywords: Mitochondria; Proteomics; Posttranslational modifications; Free radical; Oxidative and nitrosative stress; Blue native gel electrophoresis; Alcohol

Contents

Abbreviations: ALDH, aldehyde dehydrogenase; BIAM, biotinylated iodoacetamide; BN-PAGE, blue native polyacrylamide gel electrophoresis; 1(2)-D, one (two)-dimensional; DNPH, 2,4-dinitrophenylhydrazine; 4-HNE, 4-hydroxynonenal; IBTP, (4, iodobutyl) triphenylphosphonium; IEF, isoelectric focusing; LC-MS/MS, liquid chromatography tandem mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MnSOD, manganese superoxide dismutase; RNS, reactive nitrogen species; ROS, reactive oxygen species; SAM, *S*-adenosylmethionine; STZ, streptozotocin.

☆ This article is part of a series of reviews on "The Powerhouse Takes Control of the Cell: The Role of Mitochondria in Signal Transduction." The full list of papers may be found on the home page of the journal.

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doi:10.1016/j.freeradbiomed.2004.10.011

Introduction

Mitochondria are recognized as having fundamental roles in many cellular processes including energy metabolism via functioning of the oxidative phosphorylation system, the Kreb's cycle, and β-oxidation of free fatty acids. Mitochondria also house steps critical for heme biosynthesis, ketone body formation, and urea degradation. Mammalian mitochondria are unique in that they contain several copies of their own genome, a circular double-stranded DNA molecule (mtDNA), which encodes 13 essential polypeptides that constitute complexes I, III, IV, and V of the oxidative phosphorylation system, 2 large rRNAs, and 22 tRNAs. As such, the majority of oxidative phosphorylation system proteins and other mitochondrial proteins are encoded by the nuclear genome and imported into mitochondria through a highly regulated system of inner and outer transmembrane import complexes and chaperones [1]. Recently though, interest in mitochondrial physiology has been renewed due to growing evidence implicating mitochondrial involvement in cellular signaling pathways through modulation of intracellular calcium stores, production of reactive species, and the interaction of nitric oxide ($^{\bullet}$NO) on mitochondrial functions such as respiration and biogenesis. Given these important roles of the mitochondrion, it is not surprising that alterations in mitochondrial function are considered to play key roles in development of human disease.

As a source for the formation and target of modifications mediated by reactive oxygen and nitrogen species (ROS/RNS), the mitochondrion is recognized as a site critical in cellular responses to oxidative and nitrosative stress. While numerous mechanisms of oxidant-induced injury have been identified, the impact of oxidants on the overall content of mitochondrial proteins, the "mitochondrial proteome," is only now being investigated. It should also be mentioned that the mitochondrial proteome is not only determined simply by protein levels but also by posttranslational modifications to proteins, which might be significant, particularly in cells undergoing an oxidative or nitrosative stress. Fortunately, several significant developments have been made in the field of proteomics to facilitate the determination of alterations to the mitochondrial proteome under conditions of stress. The purpose of this review is to present an overview of the latest research endeavors in the field of mitochondrial proteomics, with emphasis on those advancements being made in the field of free radical biology, to identify the role of posttranslational modification of mitochondrial proteins in development of disease.

Proteomics approaches applied to mitochondria

Mitochondria have frequently been at the forefront of developing technologies and proteomics is not an exception. In this section we review current approaches but discuss in detail the more "routine" approaches accessible to most research laboratories as outlined in Fig. 1. Two-dimensional (2-D) gel electrophoresis is currently the primary tool used for the separation of proteins for proteomic analysis. This technique combines isoelectric focusing (IEF) in the first dimension where proteins are separated according to differences in net charge (isoelectric point), followed by the separation of proteins based on molecular mass in the second dimension using standard SDS–PAGE (Fig. 1A). This technique is capable of resolving hundreds to thousands of proteins in a complex biological sample on a single 2-D gel, which can then be identified by mass spectrometry. Presently, the mass spectrometry method of choice for identification of proteins separated by 2-D gels is matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry due to its high-throughput nature and relatively low cost. Briefly, protein "spots" are excised from gels and digested with trypsin into peptide fragments, which are then spotted onto a target plate for identification by MALDI-TOF. The peptide masses obtained are then entered into a search engine, e.g., "MASCOT," and a database such as NCBI or SwissProt is queried to match the tryptic peptide fingerprint to a parent polypeptide. These search engines calculate a statistical likelihood that the list of submitted peptides matches that predicted for a protein present in the database. Thus, peptide mass fingerprinting can provide very good preliminary data in identifying proteins of interest. Further confirmation of the identity and amino acid sequence of the peptide is accomplished using more sophisticated mass spectrometry techniques like tandem mass spectrometry. For further information regarding the various mass spectrometry techniques used in proteomics research, please refer to several excellent review articles listed here [2–6].

While conventional 2-D gel electrophoresis is well-suited to identify changes in the levels of the more hydrophilic proteins of the mitochondrion, e.g., matrix proteins, the analysis of membrane proteins is hampered by the fact that many of these proteins precipitate at the basic end of the IEF gels and are thus poorly resolved on conventional 2-D gels [7–9]. To solve this problem alternate protein separation techniques have been used to elucidate the mitochondrial proteome. One approach is the sucrose density gradient fractionation technique developed by Capaldi and colleagues [9–11]. In this technique, mitochondrial extracts are loaded onto a 10–35% step fraction sucrose gradient and centrifuged overnight, and fractions are collected from the

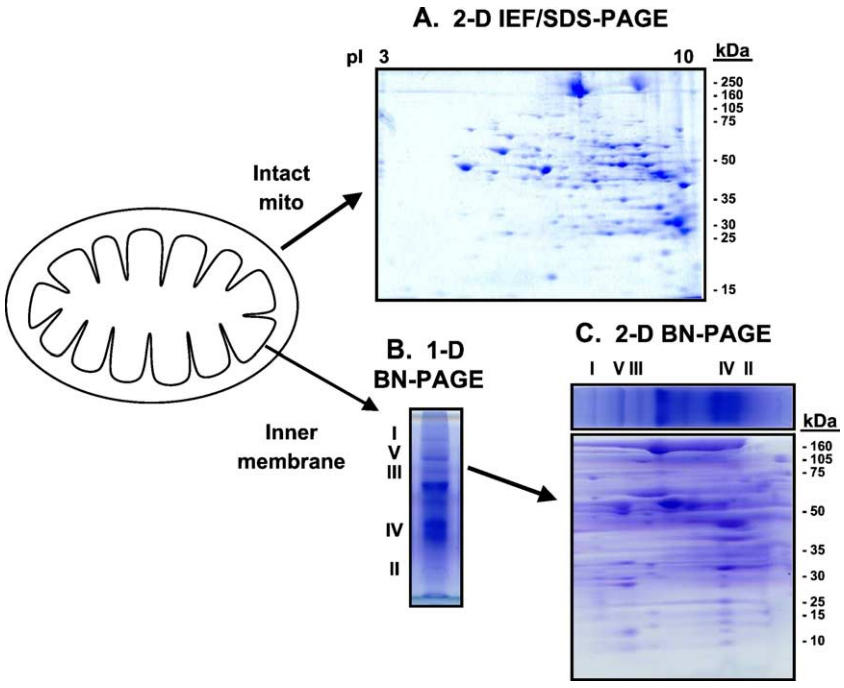

Fig. 1. Scheme illustrating analysis of the mitochondrial proteome. Conventional 2-D IEF/SDS–PAGE is used to generate high-resolution "maps" of mitochondria proteins (A), whereas 1-D (B) and 2-D (C) BN-PAGE is used to visualize changes to proteins present in the inner mitochondrial membrane. (A) 2-D IEF/SDS–PAGE done on whole rat liver mitochondria using a broad-range IEF gel from pH 3 to 10 and 10% homogenous SDS–PAGE resolving gel. Using these conditions typically 200–300 mitochondrial proteins can be visualized. (B, C) BN-PAGE separation of the proteins of the inner mitochondrial membrane. Roman numerals are used to identify the five oxidative phosphorylation complexes. During nondenaturing 1-D BN-PAGE, the five respiratory complexes remain intact (B), whereas under denaturing conditions used for 2-D BN-PAGE, the individual polypeptides of each complex are resolved based on molecular weight (C). Thus, those proteins that constitute a particular respiratory complex are aligned vertically within the gel and can be probed for changes in expression or posttranslational modifications.

bottom of the tubes after the run. The protein composition of the fractions can then be assessed by either 1-D or 2-D gel electrophoresis. By use of this technique, in combination with mass spectrometry and bioinformatics analyses, more than 600 proteins were identified as being part of the human heart mitochondrial proteome [11]. Similar approaches using affinity fractionation by Lopez and colleagues [7] have been used to identify close to 200 proteins of the rat liver mitochondrial proteome.

These novel methodologies have recently been expanded on by Mootha et al. [12] using an integrated proteomic and genome profiling technique to elucidate the mitochondrial proteome of mouse brain, liver, kidney, and heart. In this study, mitochondrial proteins from each tissue were solubilized and size separated using gel filtration chromatography into approximately 15–20 fractions, which were then digested and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). By this approach, 4800 proteins were initially identified when the acquired mass spectra were searched against the NCBI database. However, this list contained a high level of redundancy as identical proteins were found in multiple fractions and different tissues, and corresponded to multiple database entries for the same protein. To correct for these redundancies, the authors used a bioinformatics permissive clustering routine to obtain a final set of 399 proteins, which contained 236 proteins previously identified as mitochondrial and 163 proteins not previously annotated to the mitochondrion. Moreover, DNA microarray analysis demonstrated that mRNA expression levels were for the most part correlated to the detection of these proteins and allowed the authors to generate modules or "neighborhoods" of mitochondrial genes, the expression of which is tightly co-regulated, and thus they share similar functional and regulatory mechanisms. Mitochondrial neighborhoods enriched in genes of oxidative phosphorylation, amino acid metabolism, steriodogenesis, heme biosynthesis, transcriptional regulation, and mtDNA repair were identified. This study is highly significant because it is the first to combine state-of-the-art proteomics, genomics, and bioinformatics technology to elucidate the functional and regulatory organization of mitochondrial genes.

The results of Mootha et al. [12] do, however, highlight one important drawback of many proteomics analyses, that being the underrepresentation of inner mitochondrial membrane proteins. Analysis of inner membrane proteins is hampered due to their physical properties [7–9]. For example, many of the mitochondrial encoded polypeptides do not resolve well using 2-D gel electrophoresis techniques as illustrated by the lack of ^{35}S-labeled mitochondrial translation products in a conventional 2-D gel (Fig. 2). To address this issue, our laboratories have

used the 2-D blue native gel electrophoresis (BN-PAGE) technique originally developed by Schagger and von Jagow [13,14] and recently modified by Brookes and colleagues [15] to facilitate analyses of the mitochondrial proteome under stress. In this technique the five oxidative phosphorylation complexes and other functional complexes of the mitochondrial inner membrane are separated intact in the first dimension under nondenaturing conditions (Fig. 1B). After native electrophoresis, the entire vertical lane containing all the mitochondrial complexes is cut from the gel, rotated 90°, and laid on top of a denaturing SDS–polyacrylamide gel to resolve the individual polypeptides of the complexes based on their molecular mass (Fig. 1C). This procedure in effect produces a two-dimensional proteomic map of the oxidative phosphorylation system. Similarly, the individual oxidative phosphorylation complex band can be excised from the first-dimension BN-polyacrylamide gel and applied to the top of a denaturing gel to resolve the individual proteins in just that complex of interest. By use of this approach, changes in the levels of both mitochondrial and nuclear encoded proteins that constitute the respiratory complexes following stress can be assessed. Moreover, because the first dimension BN-PAGE is done under nondenaturing conditions, information regarding protein:protein interactions and assembly of the oxidative phosphorylation complexes is retained. This is particularly important because it enables probing of the molecular basis by which oxidative and nitrosative stress might decrease the functioning of the oxidative phosphorylation system.

Given the importance of mitochondrial defects in numerous human diseases, development of proteomics strategies to be used in the clinical setting for screening and diagnosis is important, especially when the size of the tissue sample obtained from a biopsy is limited. Recent studies by Capaldi and colleagues have developed a new microscale procedure, also known as "immunocapture," in which individual oxidative phosphorylation complexes are immunoprecipated intact from small amounts of tissue using monoclonal antibodies against the enzyme [16,17]. With this approach, 42 of the 45 acknowledged complex I polypeptides were identified using MALDI-TOF and LC-MS/MS, with minimal contamination by other inner membrane polypeptides [16]. Similarly, the entire complement of the F_0F_1-ATP synthase complex can be immunoprecipated in fully functional form from detergent-solubilized human tissue or cells [17]. Immunocapture has also been used to purify functional pyruvate dehydrogenase from mitochondria [18]. This new approach is well suited to detect and characterize deficiencies in protein levels of these complexes, as well as in providing detailed proteomic maps of these complexes that can be screened for posttranslational modifications to polypeptides. Thus, these protein separation techniques, along with those described above, are required to define changes in the levels and modification state of membrane proteins, which when used in combination with conventional two-dimensional gel electrophoresis, allow for a more complete study of the entire mitochondrial proteome under stress.

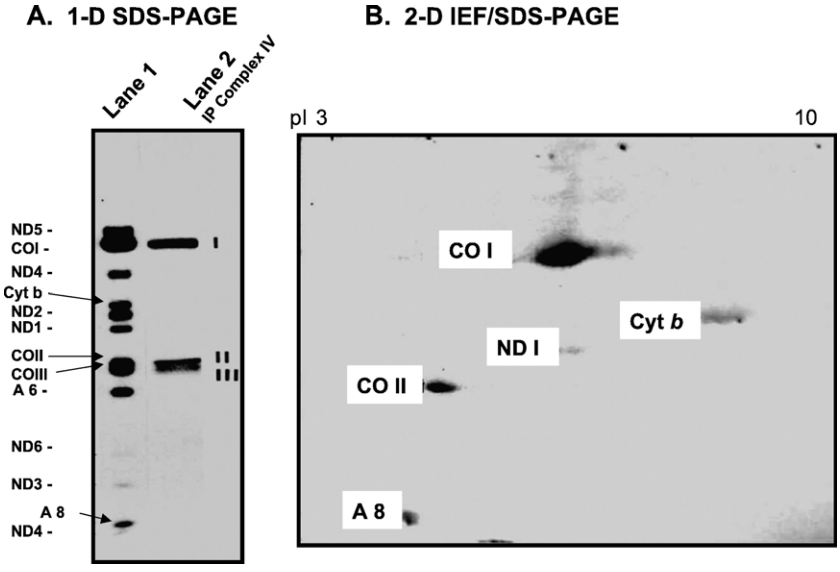

Fig. 2. Visualization of mitochondrial gene products by 1-D SDS–PAGE (A) and 2-D IEF/SDS–PAGE (B). Cells (Raji lymphocytes) were grown to a density of 10^6/ml and mitochondrially encoded proteins labeled with [^{35}S]methionine for 18 h in the presence of emetine (an inhibitor of mitochondrial protein synthesis). Cells were lysed and prepared for SDS–PAGE without heat denaturation and labeled proteins were detected by autoradiography (Lane 1). The mitochondrially encoded proteins were identified by reference to their predicted molecular weights and immunoprecipitation with a cytochrome *c* oxidase specific antibody (Lane 2). The same sample was then subjected to IEF followed by separation on SDS–PAGE. Mitochondrially encoded subunits are labeled. It is clear that very few of the hydrophobic mitochondrially coded proteins enter the second dimension in the 2-D format. CO, cytochrome *c* oxidase subunits; ND, NADH dehydrogenase subunits; A8, ATP synthase subunits; Cyt *b,* cytochrome *b*.

Alteration to the mitochondrial proteome under stress: changes in protein levels

In vitro studies

Recent advances in the field of proteomics have made it possible to begin to determine alterations to the mitochondrial proteome that occur during disease development. Early studies designed to investigate alterations to the mitochondrial proteome in response to oxidative stress were done largely using cultured cells exposed to oxidants such as hydrogen peroxide and *tert*-butyl-hydroperoxide. Using a conventional two-dimensional gel approach, Rabilloud et al. [19] demonstrated that the mitochondrial antioxidants peroxiredoxin III, thioredoxin 2, and manganese superoxide dismutase (MnSOD) were induced in Jurkat cells exposed to both mild and severe oxidative stress. Similarly, Mitsumoto et al. [20] found that sublethal exposure to hydrogen peroxide for 1 h increased the levels of 40 mitochondrial proteins in human endothelial cells, whereas the radical generator AAPH, the nitric oxide ($^{\bullet}$NO) donors PAPA NONOate and GSNO, and $NaAsO_2$ failed to induce these proteins. Of these 40 proteins, 11 were identified by MALDI-TOF and include MnSOD, the molecular chaperones mtHsp70 and Hsp60, three citric acid cycle enzymes, a 51 kDa complex I subunit, complex III core protein 2, and 60S mitochondrial ribosomal L7/L12 protein. Interestingly, their findings indicate that the oxidative stress-induced increase in heat shock proteins and MnSOD was due to the accumulation of their corresponding preprotein forms, which contain the mitochondrial targeting sequence, and is not the result of increased translation of these proteins. This finding suggests that dysfunction of the protein import and processing machinery of the mitochondrion might be impaired by an oxidative insult and contribute to alterations to the mitochondrial proteome.

One reason to explain why Mitsumoto et al. [20] did not observe an alteration to the mitochondrial proteome following $^{\bullet}$NO exposure is that the exposure was too short to induce an effect. Studies by Ramachandran and colleagues [21] demonstrated that chronic exposure of bovine aortic endothelial cells to the $^{\bullet}$NO donor DETA NONOate for 24 h decreased the activities of mitochondrial respiratory complexes I, II, and IV, but had no effect on the specific activity of the mitochondrial matrix enzyme citrate synthase. Proteomic analysis of the mitochondria using one-dimension BN-PAGE demonstrated a significant decrease in the protein content of these three complexes in $^{\bullet}$NO-treated cells as compared with untreated controls. Interestingly, there was no change in the protein levels of the ATP synthase following chronic exposure to $^{\bullet}$NO, suggesting that these decreases are not simply due to $^{\bullet}$NO-mediated inhibition of mitochondrial protein synthesis. It was speculated that the decreases in these protein complexes occurred through targeted disruption of the Fe–S and/or heme molecules in these complexes by $^{\bullet}$NO and other RNS. Chronic treatment with $^{\bullet}$NO did result in a significant increase in the labile iron pool, but whether this iron is derived from the respiratory complexes is not known. However, mitochondrial involvement was indicated by the observation that inhibition of mitochondrial protein synthesis eliminated the increase in the labile iron pool following $^{\bullet}$NO exposure. These results highlight a unique mechanism whereby RNS can alter the functional composition of the mitochondrial proteome, not by modulating synthesis of proteins, but by altering cellular iron metabolism.

In vivo studies

As a follow-up to these cell culture studies, recent investigations have focused on the effects of oxidative and nitrosative stress on the mitochondrial proteome using in vivo models of stress. Our work has focused on characterizing the response of the hepatic mitochondria proteome to chronic alcohol-induced stress. Significant changes in liver mitochondria physiology occur in response to chronic alcohol consumption and contribute to the development of pathology, including increased ROS and RNS production [22,23], decreased respiration and ATP synthesis [24,25], and disrupted fatty acid metabolism [26–28]. Moreover, chronic alcohol exposure induces an increased sensitivity of the respiratory chain to inhibition by $^{\bullet}$NO, which we propose contributes to hypoxic liver damage from alcohol [29]. Although previous studies by Coleman and Cunningham [30,31] have demonstrated that chronic alcohol consumption decreases the synthesis of the 13 mitochondrial encoded polypeptides that make up respiratory complexes I, III, and IV and the ATP synthase, little or no information is available regarding the effects of ethanol on the nuclear encoded respiratory complexes or the hundreds of other proteins present within liver mitochondrion. With the advent of new proteomic technologies it is now possible to examine the consequences of ethanol-induced metabolic stress on the mitochondrial proteome. This is important because these studies will provide insights into both the mechanisms of pathology and the adaptation to stress in response to chronic alcohol exposure.

Using both conventional two-dimensional gel electrophoresis and BN-PAGE, we recently reported that levels of expression of 43 mitochondrial proteins are altered in response to chronic alcohol consumption [32]. Of these, levels of 13 were increased and 30 decreased as a consequence of chronic alcohol consumption. Most important was the fact that for 25 of these proteins, levels of expression were not previously known to have been altered following chronic exposure to alcohol. For the first time, both nuclear and mitochondrial encoded gene products of the oxidative phosphorylation system were found to be decreased in response to chronic alcohol exposure. This finding, in combination with the observation that mitochon-

drial chaperone levels were altered, suggests a possible assembly defect in the formation of the respiratory complexes contributing, at least in part, to the ethanol-related decrease in oxidative phosphorylation. Moreover, we observed decreased expression of several key enzymes involved in β-oxidation of fatty acids, which supports the hypothesis that ethanol-mediated inhibition of PPARα-mediated pathways contributes to the development of fatty liver. In addition to this recent work, previous studies by Cahill and Cunningham [33,34] demonstrated an ethanol-elicited alteration in the levels of seven specific proteins of the mitochondrial ribosome. These alterations may be significant in that they may lead to impaired assembly of mitochondrial ribosomes and to a decrease in functional activity [34,35], which will negatively impact energy metabolism via decreased synthesis of the 13 mitochondrial gene products. Taken together, these findings demonstrate the power of proteomics to reveal alterations in biological processes and molecular targets responsible for ethanol-induced mitochondrial dysfunction. Furthermore, it is clear that the response of liver mitochondria to a complex metabolic stress such as chronic alcohol consumption involves changes in the components of multiple biochemical pathways, and not a single modification of one specific enzyme (Fig. 3).

Similar alterations in mitochondrial protein expression were also observed in heart mitochondria from diabetic rats. Using an in vivo model of diabetes, the streptozotocin (STZ)-treated rat, Turko and Murad [36] demonstrated an upregulation of several β-oxidation enzymes, decreases in several of the NADH dehydrogenase complex polypeptides, down-regulation of voltage-dependent anion channel 1 protein, Hsp60, and Grp75, and significant upregulation of catalase 1 week post-STZ injection. Interestingly, many of the alterations in the level of protein expression were preserved 4 weeks after STZ treatment. Like the results in our ethanol studies, these results reveal coordinated up- and downregulation of mitochondrial proteins in response to both the pathological and adaptive responses of the metabolic stress induced in the heart from diabetes.

Recent studies using proteomics approaches in genetic knockout models have also highlighted the important role of mitochondrial dysfunction and oxidative stress in pathology of several diseases. One mouse model that has been used extensively to investigate the role of mitochondrial oxidative stress in numerous diseases is the MnSOD heterogeneous knockout (*sod2*+/−), as the homozygous knockout (*sod2*−/−) typically dies within the first week of life due to numerous mitochondrial, metabolic, and neurological defects. Interestingly, survival and neurological damage in *sod2*−/− mice can be partially rescued by administering the salen–manganese catalytic antioxidant EUK-8 [37]. Thus, to gain further insight into mitochondrial targets of oxidative damage in the brain of *sod2*−/− mice, Hinerfeld et al. [38] recently characterized alterations to the mitochondrial proteome of the cortex of wild-type mice or *sod2*−/− mice supplemented with low and high concentrations of EUK-8. Mitochondrial proteins differentially expressed in the cortex of *sod2*−/− mice treated with EUK-8 as compared with wild-type controls included peroxiredoxin V, 2-oxoglutarate dehydrogenase (E1k), and the succinate dehydrogenase and flavoprotein subunit, which were downregulated, and triosephosphate isomerase and glutathione *S*-transferase

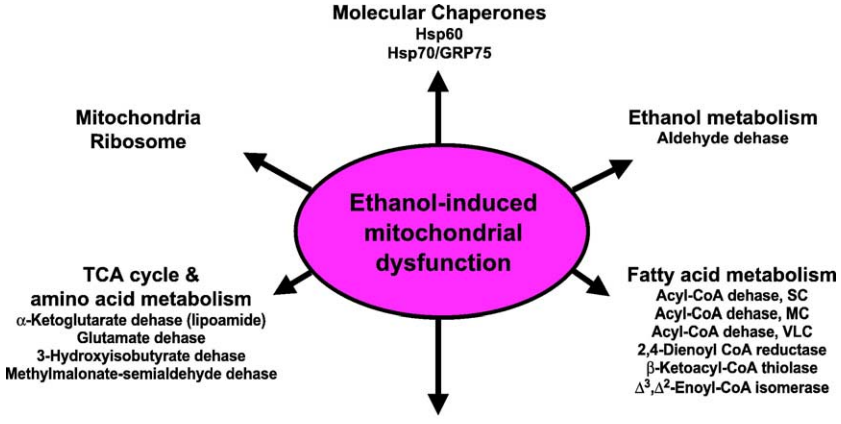

Fig. 3. Summary of alterations to the hepatic mitochondrial proteome in response to chronic alcohol-mediated stress. It is hypothesized that alterations in the levels of these proteins, as well as posttranslational modifications to some of these proteins, contributes to alcohol-induced mitochondrial dysfunction and development of liver disease. This figure summarizes findings from Refs. [30–34,55].

class mu-1, which were upregulated. Decreased expression of the α-ketoglutarate dehydrogenase subunit and flavoprotein subunit of succinate dehydrogenase correlated with decreased activity of each protein, consistent with previous studies demonstrating the sensitivity of these mitochondrial enzymes to oxidative inactivation [39,40].

Another genetic knockout mouse recently used to investigate alterations in the mitochondrial proteome associated with a neurological disease is the parkin-deficient mouse (*parkin* −/−) [41]. Loss of function mutations in *parkin* are the major cause of familial Parkinson's disease. As the parkin protein has been shown to function as an E3 ubiquitin ligase [42], it is speculated that defective parkin will lead to a loss in nigrostriatal function due to accumulation of proteins normally targeted for proteasome degradation. Moreover, it has been shown that parkin is localized to the mitochondrion and may function to attenuate mitochondria-dependent apoptotic cell death [43]. Proteomic analyses of the ventral midbrain region of *parkin* −/− mice revealed decreases in several mitochondrial proteins including pyruvate dehydrogenase subunit E1α1, NADH dehydrogenase 24 and 30 kDa subunits, and cytochrome *c* oxidase Vb subunit, as compared with wild-type mice [44]. Moreover, these deficiencies in respiratory complex subunits were associated with decreased state 3 respiration with complex I, II, and III/IV oxidizable substrates [44]. Thus, in this first analysis of the mitochondrial proteome using a genetic knockout mouse model of Parkinson's disease, a key role for parkin in the regulation of mitochondrial function in the nigrostriatum was shown.

Finally, recent work by Mato and colleagues [45] demonstrated an interaction of the mitochondrial proteome with the methyl donor and glutathione synthesis intermediate *S*-adenosylmethionine (SAM). Methionine adenosyltransferase is the sole enzyme responsible for the biosynthesis of SAM from methionine and ATP. It has been shown that mice deficient in the methionine adenosyltransferase 1A isoform (*mat1A* −/−) develop nonalcoholic steatohepatitis and hepatocellular carcinoma, which is accompanied by decreased liver SAM levels and oxidative stress [46,47]. While the mechanism responsible for these effects is not known, proteomic analyses have identified altered expression of several mitochondrial proteins in the *mat1A* −/− mouse. Depletion of liver SAM in *mat1A* −/− mice resulted in decreased levels of the mitochondrial encoded cytochrome *c* oxidase subunits COX I and II and the mitochondrial chaperone prohibitin, whereas the ATP synthase β subunit was upregulated [45]. Associated with the altered level of expression of these inner membrane proteins was a decrease in mitochondrial membrane potential. Alterations in the levels of several other mitochondrial enzymes involved in fatty acid metabolism, including the long-chain acyl CoA dehydrogenase and $\Delta^{3,5},\Delta^{2,4}$-dienoyl CoA isomerase, were also noted in *mat1A* −/− mice compared with wild-type controls. Like those changes observed in alcohol-induced fatty liver disease [32] (Fig. 3), these changes in the level of expression of mitochondrial proteins in the *mat1A* −/− mouse define a proteomic profile for the metabolic and pathologic changes to mitochondria in response to SAM deficiency. Furthermore, these studies highlight an important role of SAM and mitochondrial function in the development of fatty liver diseases.

Alteration to the mitochondrial proteome under stress: posttranslational modification to proteins

Cysteine modifications

Exposure of mitochondrial proteins to ROS, RNS, and electrophilic lipids can lead to the posttranslational modification of amino acid residues, which has the potential to alter protein structure and/or induce a permanent loss in function. Recent work also suggests that oxidants can induce reversible modifications, specifically at cysteine residues, which may function to: (1) protect cysteines from irreversible modifications or "overoxidation" and (2) modulate protein function in response to stress. Thus, oxidative, nitrosative, or alkylation reactions can trigger signaling cascades that result in the activation of genes involved in cellular stress responses. Whether posttranslational modification to mitochondrial proteins plays a role in the adaptive response of the cell to stress is not known. Because alterations in the redox status of protein thiols are typically critical in regulating a protein's function, the identification of these proteins and the type of modifications present is of significant interest.

Reversible modifications to thiols that are thought to play key roles in modulating a protein's function include the formation of nitrosothiols (P-SNO), sulfenic acids (P-SOH), and protein mixed disulfides with low-molecular-weight thiols such as glutathione (P-SSG) (Fig. 4). In contrast, cysteine residues can be irreversibly oxidized to higher oxidation states such as sulfinic ($P\text{-}SO_2H$) and sulfonic ($P\text{-}SO_3H$) acids by ROS and RNS (Fig. 4). Each of these thiol modifications has the potential to elicit a biological response that might disrupt mitochondrial function. Indeed, emerging evidence emphasizes a key link between the induction of the mitochondrial permeability transition, alterations in energy metabolism, and protein thiol oxidation in pathways of cell death induced by an oxidative or nitrosative stress [48].

Even though increased levels of oxidized protein thiols are commonly used as a measure of oxidative damage in mitochondria, the identity of specific mitochondrial proteins containing these modified thiols is largely unknown and identification remains a challenge to researchers. However, with the advent of new thiol labeling reagents used in combination with various proteomics approaches, the identification of specific mitochondrial proteins containing

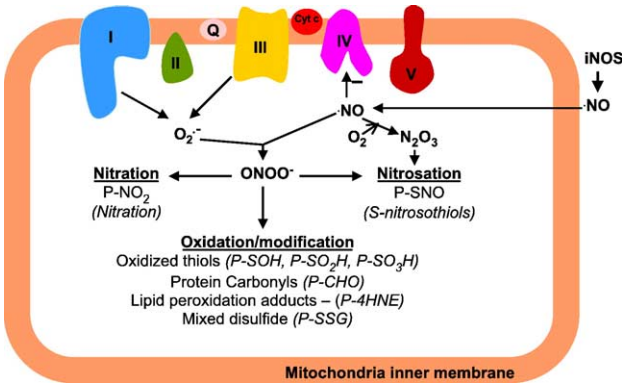

Fig. 4. Modification of mitochondrial proteins by interactions with ROS, RNS, and electrophilic lipids. Superoxide and $^{\bullet}$NO can react to form ONOO$^-$, which can mediate nitration of tyrosine residues (P-NO_2) and nitrosation (P-SNO) of thiols. Peroxynitrite can initiate the sequential oxidation of thiols to sulfenic (P-SOH), sulfinic (P-SO_2H), and sulfonic (P-SO_3H) acid forms. Superoxide and hydrogen peroxide can also mediate the oxidation of thiols. ROS/RNS can also oxidize proteins to generate carbonyl groups (P-CHO). Initiation of lipid peroxidation by ROS/RNS generates electrophilic lipids like 4-HNE that adduct with histidine, cysteine, and lysine residues in proteins. Reaction between protein thiols and low-molecular-weight thiols like GSH can yield mixed disulfides. It is important to note that these reactions of ROS/RNS can occur with membrane, as well as matrix proteins in mitochondria.

thiols susceptible to oxidative modification is becoming feasible as discussed in [49]. In general, these approaches use thiol labeling reagents like biotinylated iodoacetamide (BIAM) [50], which binds to reduced thiols but not oxidized or modified thiols in proteins. These labeled proteins can then be visualized after gel electrophoresis and immunoblotting using streptavidin conjugated to HRP. Thus, proteins containing oxidized thiols that have been oxidized and/or modified by an oxidative or nitrosative stress can be identified by decreased labeling with the thiol reagent. The feasibility of this approach for detecting oxidized mitochondrial proteins following exposure to nitrosative stress is illustrated in recent work by Shiva et al. [39] demonstrating the oxidation of mitochondrial protein thiols after exposure to Angeli's salt, which is a donor of nitroxyl, a very potent RNS.

An alternate approach that can be used to detect *S*-nitrosated proteins, is the three-step "biotin-switch" method developed by Synder and colleagues [51,52], which converts nitrosated cysteines to biotinylated cysteines that can be detected and identified following purification with streptavidin–agarose. Thus, when using the "biotin-switch" method oxidized proteins are detected by increased labeling of proteins. Using this technique, Stamler and colleagues [53] identified several protein targets of *S*-nitrosation in isolated mitochondria including dihydrolipoamide dehydrogenase, hydroxymethylglutaryl-CoA synthase, and sarcosine dehydrogenase. While the identification of these *S*-nitrosated proteins in mitochondria is important, the functional significance of these modifications remains to be determined, as exposure to high concentrations of NO^+ donors failed to inhibit the activity of these enzymes [53].

One disadvantage of the techniques described above is that these thiol labeling strategies can be used only in mitochondrial extracts as these reagents are membrane impermeable. Thus, they provide no information regarding the redox status of mitochondrial protein thiols in fully functional, respiring mitochondria. To circumvent this problem, Murphy and colleagues [54] developed a novel compound, (4-iodobutyl) triphenylphosphonium (IBTP), which selectively labels reduced protein thiol groups in functional mitochondria. IBTP is a lipophilic cation that accumulates several hundred-fold in mitochondria as a function of the large membrane potential across the inner mitochondrial membrane, and has been shown to uniformly label mitochondria and co-localize with mitochondrial enzymes [54]. Once IBTP is accumulated into mitochondria, protein thiolate groups displace the iodo portion of IBTP and form stable phosphonium thioether linkages. These IBTP-labeled proteins can then be visualized after gel electrophoresis and immunblotting with an antibody made against the triphenylphosphonium group. Thus, as with BIAM labeling, proteins containing oxidized or modified thiols can be identified following an oxidative or nitrosative stress by decreased labeling with IBTP. Using this approach Lin et al. [54] demonstrated that the redox thiol status of numerous mitochondrial proteins is modified by exposure to various oxidative/nitrosative stress inducing agents such as peroxynitrite ($ONOO^-$), diamide, and *tert*-butyl hydroperoxide. Thus, this approach has the potential to enable the detection of the thiol redox state of specific mitochondrial proteins in fully functional mitochondria and cells.

Recently, our laboratories have used this approach to identify specific mitochondrial proteins that have an altered thiol status as a consequence of chronic alcohol-induced oxidative stress [55]. In these studies, mitochondria isolated from livers of animals fed control and ethanol-containing liquid diets were incubated with IBTP under State 3 respiration conditions. By labeling respiring mitochondria, we were able to identify those reactive thiols that are exposed in mitochondria during oxidative phosphorylation. Using conventional 2-D gel electrophoresis, 40 individual protein spots immunoreactive for IBTP were observed in immunoblots from mitochondria isolated from livers of control and ethanol-fed animals. Of these, 7 IBTP-labeled proteins were identified by mass spectrometry, with 2 proteins, low-K_m mitochondrial aldehyde dehydrogenase (ALDH) and the molecular chaperone hsp70, possessing a significant and reproducible decrease in IBTP labeling in mitochondria from ethanol-fed animals. Analysis of the low-K_m ALDH showed a significant decrease in the specific activity of enzyme in mitochondria isolated from ethanol-fed animals as compared with controls, which is not restored to control levels following treatment with β-mercaptoethanol. The inability of a strong thiol reductant to restore activity indicates an irreversible modification to the active site cysteine of ALDH

that may have occurred following chronic alcohol consumption. Oxidative modification and subsequent inactivation of the low-K_m ALDH represents a specific molecular mechanism that might contribute to overall mitochondrial dysfunction in response to chronic alcohol exposure due to the inability to detoxify the highly reactive and toxic aldehyde species, acetaldehyde. Taken together, the findings from our work and those of others have demonstrated that experimental protocols have been developed that will enhance efforts to establish a clear link between the posttranslational modification of mitochondrial protein thiols and the development of disease, where oxidative and nitrosative stress is implicated in the etiology.

While modification of cysteine residues has been shown to alter mitochondrial function, other types of posttranslational modification, including tyrosine nitration, protein carbonylation, and adduction via electrophilic lipids, may elicit many of the same detrimental effects on mitochondrial function in response to stress. In general, it is thought that these modifications are responsible for the permanent loss of function and removal of the damaged proteins. Evidence for the presence of each of these specific modifications to mitochondrial proteins following various in vitro and in vivo exposures to oxidative or nitrosative stress has been reported and is discussed in the following sections.

Tyrosine nitration

The detrimental effects of $^{\bullet}NO$ on mitochondrial function stem largely from the diffusion of $^{\bullet}NO$ into mitochondria and the formation of more reactive secondary intermediates like $ONOO^-$ and nitrogen dioxide ($^{\bullet}NO_2$), which can nitrate tyrosine residues within mitochondrial proteins (Fig. 4). It should be stressed though that not all effects of RNS on proteins are mediated through nitrotyrosine formation, as RNS can affect mitochondrial function through the modification of protein thiols as discussed above. Similarly, $^{\bullet}NO$ and, in some cases, $ONOO^-$ can mediate direct effects on protein function via reaction with iron in heme and Fe/S center-containing proteins. Early studies indicated that $ONOO^-$ binds to and inactivates the Fe/S centers of mitochondrial complex I [56] and that $^{\bullet}NO$ may interact and inhibit complex III [57]. $^{\bullet}NO$ also plays a key role in regulating mitochondrial respiration through reversible binding with cytochrome *c* oxidase. Exposure to low concentrations of $^{\bullet}NO$ results in the reversible inhibition of complex IV activity due to competition of $^{\bullet}NO$ with oxygen at the binuclear center of the enzyme [57–59]. Taken together, these examples illustrate that RNS can modulate the mitochondrial proteome through a variety of mechanisms including interactions with heme iron, Fe/S centers, and cysteine residues, as well as tyrosine residues. A brief overview of the progress made in recent years to identify mitochondrial proteins that constitute the "nitrotyrosine proteome" is presented in the subsequent paragraphs.

Since the influential work of Beckman, Ischiropoulos, and colleagues [60,61] demonstrating the ability of $ONOO^-$ and other RNS to cause the nitration of tyrosine residues, numerous papers have been published illustrating that the mitochondrion is a target of these nitrating species. Before the advent of proteomics technologies, early work in this field focused on identifying nitrated mitochondrial proteins one at a time. With this approach several mitochondrial proteins have been found to be nitrated in vivo and in vitro by exposure to RNS and include MnSOD [62–65], ATP synthase [66,67], and cytochrome *c* [68–71]. Interestingly, Castro et al. [72] have extended their studies on cytochrome *c* nitration and found that cytochrome *c* can serve as a catalyst for both NO_2^-- and H_2O_2-mediated nitration reactions of proteins. These results are significant as they demonstrate for the first time the possibility of a specific protein functioning as a catalyst of nitration reactions not only in the mitochondrial compartment but also in the cytosol following the release of cytochrome *c* during apoptosis. The functional significance of nitrated cytochrome *c* on mitochondrial function and cell signaling pathways remains to be determined.

Since these earlier studies, numerous papers have been published using proteomic methods to identify nitrated mitochondrial proteins under both normal and pathophysiological conditions and from endogenous and exogenous sources of RNS. Typically, these studies have detected nitrated proteins by using antibodies directed against the 3-nitrotyrosine moiety. While many of these studies have observed an increase in nitrated proteins following disease or nitrosative insult, few studies have been successful in identifying the nitrated proteins or, more importantly, the specific modified tyrosine residue within the protein. This lack of information hampers the understanding of the role tyrosine nitration plays in mitochondrial physiology under both healthy and diseased conditions.

Although the field is in a nascent stage, recent studies by Stuehr and colleagues have gone a long way to increase our understanding of the impact of tyrosine nitration in cellular and mitochondrial function. Using an in vivo model of inflammation, Aulak et al. [73] identified more than 40 liver and lung proteins that were nitrated on an exposure to lipopolysaccharide. Proteins identified include voltage-dependent anion channel 1, MnSOD, and aconitase, as well as numerous enzymes involved in glycolysis, the citric acid cycle, and β-oxidation of fatty acids. Similar findings were obtained by Turko et al. [74] in an in vivo model of diabetes when heart mitochondria proteins were probed for nitrotyrosine modifications. Moreover, studies by Brookes et al. [75] demonstrated that several respiratory chain subunits and dehydrogenases involved in intermediary metabolism were endogenously nitrated or nitrated on exposure to $ONOO^-$. More interestingly in this study was the observation that, on induction of the mitochondrial permeability transition, additional mitochondrial targets of nitration were revealed and included glutamate dehydrogenase and two

CoA-containing enzymes [75]. Taken together, these results suggest a role for tyrosine nitration in modulating key mitochondrial functions in energy metabolism, fatty acid metabolism, and apoptosis following oxidative/nitrosative insults.

Following up on their earlier work, Stuehr and colleagues have provided evidence to support the possibility that there is a regulatory cycle of nitration and denitration of proteins within mitochondria that functions like the classic tyrosine phosphorylation signaling model [76,77]. By use of isolated mitochondria, the nitration state of several mitochondrial proteins involved in energy metabolism and antioxidant pathways was found to be rapidly modulated in a model of hypoxia–reoxygenation and to be dependent on the oxygen tension and not protein degradation pathways [76]. Whether these data are sufficient to support the idea of a nitration/denitration cycle functioning within mitochondria and playing a regulatory role in metabolism is not clear at present; however, these data indicate that protein nitration may be more than just a "fingerprint" or "biomarker" of oxidative and nitrosative stress.

Recent studies have also begun to identify the specific tyrosine residues within mitochondrial proteins that are nitrated on exposure to RNS. Using both immunologic and mass spectrometry approaches, Murray et al. [78] demonstrated that in vitro exposure of intact mitochondria to $ONOO^-$ resulted in the selective nitration of several complex I subunits, which was associated with a loss in enzyme activity. Tandem mass spectrometry analysis demonstrated that subunits NDUFB4 and NDUFA6 contained the highest degree of nitration, with Tyr^{122} in NDUFA6 being the most reactive and Tyr^{46}, Tyr^{50}, and Tyr^{51} being the most heavily nitrated in the NDUFB4 subunit. In addition to these findings, subunits of the ATP synthase were found to be nitrated, along with the oxidation of Trp^{61} and Met^{68} residues in the 17.2 kDa subunit of complex I. These findings support those reported earlier for the unique susceptibility of Trp and Met residues to oxidation in several mitochondrial proteins identified in healthy human heart tissue [79]. Identification of these and other reactive tyrosine residues that are susceptible to oxidation and nitration will allow for a more complete understanding of the role of posttranslational modifications in mitochondrial function.

Electrophilic lipid adduction

In addition to the oxidation of proteins via classic ROS and RNS, several lines of evidence suggest that electrophilic lipids generated during enzymatic or nonenzymatic metabolism of polyunsaturated fatty acids can also act to posttranslationally modify proteins. Examples of reactive aldehyde intermediates that have been shown to adduct to proteins include malondialdehyde, acetaldehyde, and 4-hydroxynonenal [80–83], as well as the more recently recognized reactions with the cyclopentenone prostaglandins [84,85]. Adduction occurs largely at nucleophilic centers within proteins, particularly at cysteine, lysine, and histidine residues [86] (Fig. 4). While it is generally thought that adduction of lipid peroxidation products to proteins is considered an important mediator of cellular damage due to disruption of protein function, recent studies highlight these oxidizing lipids as being important activators of signal transduction pathways involved in the adaptation to stress [87].

Since the initial observations of Szweda and colleagues [88] that modification of mitochondrial proteins by the lipid peroxidation product 4-hydroxynonenal (4-HNE) contributes to mitochondrial dysfunction, several studies have been conducted to identify 4-HNE-modified proteins in mitochondria with limited success. Most studies have focused on the inactivation of α-ketoglutarate dehydrogenase by 4-HNE through its interaction with sulfhydryl groups present within the lipoic acid moiety in the enzyme [40]. The susceptibility of this mitochondrial enzyme to inactivation has also been verified in in vivo models of oxidative stress [89] and via alterations in mitochondrial glutathione status [90]. In contrast, recent work by Hagen and colleagues demonstrated that while 4-HNE adduction to rat heart α-ketoglutarate dehydrogenase increases with age, this is not associated with a decrease, but rather an increase, in enzymatic activity due to a lowered K_m for α-ketoglutarate in older animals [91]. These conflicting results demonstrate the critical need to determine whether these modifications truly have an impact on protein functionality and cellular pathways of metabolism or are simply bystander effects from oxidative insult.

Studies conducted by Chen et al. [92,93] have demonstrated the formation of malondialdehyde and 4-HNE protein adducts to subunits of cytochrome *c* oxidase in liver from animals exposed to ethanol. More important is the observation that formation of these adducts resulted in a decrease in oxidase activity, thus demonstrating a functional role of these adducts in ethanol-induced mitochondrial dysfunction. Additional studies by this group have demonstrated that 4-HNE adduct formation with cytochrome *c* oxidase subunits also plays a role in enzyme inactivation from cardiac reperfusion injury [94]. Similarly, recent work by Papaconstantinou and co-workers has shown the presence of endogenous levels of 4-HNE modified subunits of complexes I, III, IV, and V, as well as the adenine nucleotide transporter and the voltage-dependent anion channel in bovine heart submitochondrial particles analyzed by BN-PAGE [95]. Inactivation of the citric acid cycle enzyme isocitrate dehydrogenase by 4-HNE has also been shown in an animal model of hypertension and cardiac hypertrophy [96]. Like those findings presented for nitration reactions, it appears that many of the same proteins and enzymes involved in mitochondrial energy metabolism are also susceptible to modification by reactive aldehyde species. Whether this is a consequence of special characteristics of these proteins that make them susceptible to

oxidation or simply because they are present in high abundance and thus represent a large "target" that is hard for oxidants to miss is not clear. Either way the evidence is strong to suggest that these modifications have the potential to play critical roles in altering mitochondrial function under both normal and pathological conditions.

Carbonyl formation

Measurement of protein carbonyls has been used for many years as a general marker of protein oxidation and damage from oxidative stress since the seminal work of Levine, Berlett, and Stadtman illustrating its utility [87,97–99]. Carbonyl groups can be introduced into proteins via the direct oxidation of the protein backbone, oxidation of lysine, arginine, and histidine residues, and adduction by carbonyl-containing lipids (i.e., 4-HNE) or sugars (Fig. 4). In general the detection of protein carbonyls is done after their derivatization with 2,4-dinitrophenylhydrazine (DNPH) to generate hydrazones that can be detected spectrophotometrically. While this assay has been adapted to an electrophoresis/immunblotting approach as a anti-DNPH antibody is available to detected the DNPH-modified proteins [100] few studies have been successful in identifying specific proteins that have an altered carbonyl status following stress [101,102], with no mitochondrial proteins identified. However, with the development of newly improved strategies and reagents to label carbonylated proteins, the number of proteins identified as containing this posttranslational modification is likely to grow rapidly [103–105]. Indeed, recent work by Choksi et al. [95] identified several carbonylated proteins within respiratory complexes I, II, III, and V of heart mitochondria. While these are important new findings for the field, the relevance of these modifications to normal functioning of mitochondria has not been established.

Summary

Recent advancements in proteomics technologies have now made it possible to critically examine the molecular targets within the mitochondrion that contribute to cellular dysfunction. While a tremendous amount of progress has been made in this area in recent years, it should be pointed out that there are still deficiencies in these techniques and approaches that greatly impede these analyses. For example, progress in the field is hampered by the fact that only a small number of modifications can be detected in proteins due to the limited number of antibodies that are available to detect these modifications. Similarly, the specificity of these antibodies is an issue, as it is likely that differences in epitope specificity pose a far bigger problem than generally acknowledged. This is especially the case with antibodies directed against 3-nitrotyrosine residues and 4-HNE protein adducts. And, even though significant advancements have been made in instrumentation, mass spectrometry is still not sufficiently sensitive to detect, quantify, and localize these posttranslational modifications, particularly when these alterations may encompass only a few amino acids within the protein. Thus, quantification of the degree of modification within a protein remains a challenge. However, even with these limitations, cutting-edge research in the field has now begun to identify specific molecular modifications to selective amino acids within mitochondrial proteins that occur from exposure to ROS, RNS, and electrophilic lipids under both normal and pathophysiological conditions.

Previously, oxidative modification of proteins was thought to represent a detrimental process in which the modified proteins were irreversibly inactivated, leading to cellular dysfunction. While this is still the case in many situations, it is now clear that posttranslational modifications of mitochondrial proteins can be specific and reversible and, thus, may play a key role in normal mitochondrial physiology. Thus, the challenge for future studies will be to incorporate this knowledge into a framework whereby these complex functional and regulatory alterations in the mitochondrial proteome can be used to increase our understanding and treatment of human disease.

Acknowledgments

This work was funded by NIH Grants AA13682 and AA15172 (S.M.B.) and AA13395 and HLO70610 (V.D.U.) and by a grant from the Alcoholic Beverage Medical Research Foundation (S.M.B.).

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