9-1-2014

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Robert Gilkerson
The University of Texas Rio Grande Valley

Luis Materon
The University of Texas Rio Grande Valley

Recommended Citation

Gilkerson, R., & Materon, L. (2014). Two Roads Converging: Mitochondria and Inflammatory Signaling. Journal of Clinical Immunology & Immunotherapy, 1(1), 1–7. https://doi.org/10.24966/CIIT-8844/100004

Gilkerson R and Materon L, J Clin Immunol Immunother 2014, 1: 004
DOI: 10.24966/CIIT-8844/100004

HSOA Journal of Clinical Immunology and Immunotherapy

Review Article

Two Roads Converging: Mitochondria and Inflammatory Signaling

Robert Gilkerson[1,2] and Luis Materon[1]*

[1]Departments of Biology, University of Texas RGV, 1201 West University Drive, Edinburg, TX 78539, USA

[2]Clinical Laboratory Sciences, University of Texas RGV, 1201 West University Drive, Edinburg, TX 78539, USA

Abstract

As the complexity of cellular signaling in inflammatory response emerges, it is increasingly clear that mitochondria are directly involved in, and in some cases are even required for, activation of inflammatory response. As a bioenergetic organellar network, mitochondria dynamically modulate their organization and function in response to cellular signaling cues and metabolic demand. The NLRP3 inflammasome, a caspase-activating multifactor scaffolding assembly, is directly activated by mitochondrial factors and functional parameters. Mitochondria are also heavily implicated as downstream targets of inflammation in a variety of tissues. Elevated inflammation and cytokine-mediated damage to mitochondria are implicated in the pathogenesis of disparate conditions such as Type 2 diabetes and autism spectrum disorders. Recent findings indicate that mitochondrial factors are released as extracellular mediators of inflammatory response. Here, we discuss the mechanistic interaction of mitochondria in inflammatory signaling, as well as the implications for inflammatory mitochondrial damage as a causative force in highly prevalent human diseases.

Inflammatory Signaling and Innate Immune Response

Inflammation is a crucial mechanism of innate immune response. Macrophages and neutrophils recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), undergoing a complex set of signaling interactions to release pro-inflammatory cytokines (most notably IL-1β and IL-18 [1] that prompt inflammatory response. To mediate innate immune response, pattern-recognition receptors (PRRs) recognize a broad range of markers of infection, stress, and damage. PRRs include membrane-bound receptors such as Toll-like receptors (TLRs), the interleukin receptors (ILRs), and the tumor necrosis factor receptors 1 (TNF-R1) and 2 (TNF-R2). Upon binding of extracellular ligands, these PRRs activate intracellular signaling events, such as activation of NFκB, a transcription factor that upregulates expression of a wide variety of stress-response genes, or through post-translational modification such as activation of c-Jun amino-terminal kinase (JNK) [2] to effect inflammatory response. Within the cytoplasm, inflammatory ligands bind and activate intracellular PRRs that combine with a variety of associated factors to form large cytoplasmic scaffolding assemblies that integrate inflammatory activation and activate secretion of the major cytokines IL-1β and IL-18 by binding and activating caspase-1 [3] (Figure 1). These cytoplasmic PRRs, classed as nucleotide-binding domain leucine-rich repeat-containing receptors (NLRs), recognize a wide variety of intracellular inflammatory stimuli. Four classes of NLRs (NLRP1, NLRP3, NLRC4 and AIM2) share in common a nucleotide-binding oligomerization domain, and have demonstrated an ability to form large oligomeric inflammasome complexes in the cytoplasm [4]. While each of the four sense a variety of inflammatory signals to mediate caspase-dependent activation of inflammation, the NLRP3 inflammasome is the best characterized.

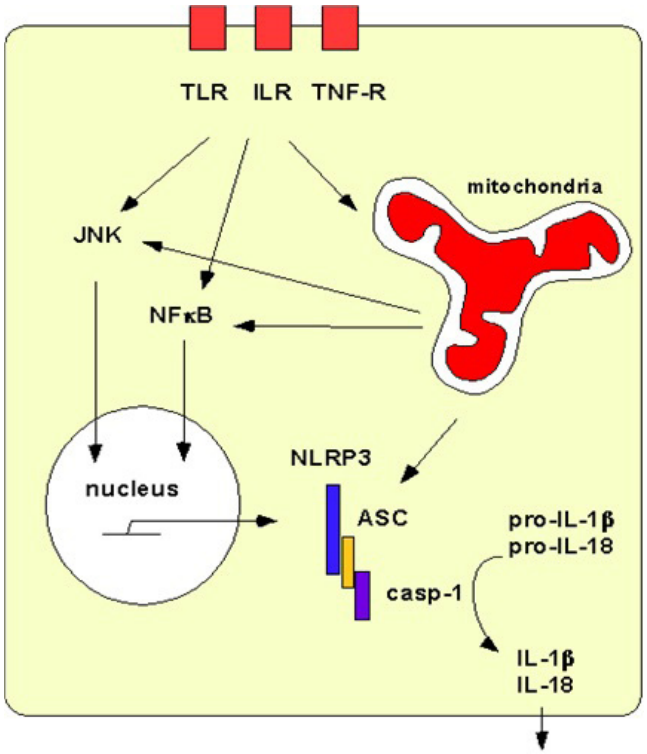

Figure 1: Schematic of mitochondrial interactions with NLRP3 inflammasome signaling.

Plasma membrane PRRs TLR, ILR, and TNF-R (red boxes) bind cytokines and extracellular ligands, activating NFκB and JNK, which activate nuclear transcription of cellular stress factors, particularly NLRP3. NLRP3 (blue), ASC (gold), and caspase-1 (purple) associate in the cytoplasm as the large, macromolecular NLRP3 inflammasome in macrophages. Mitochondria are impacted by membrane-bound PRR signals and aid in activating the NLRP3 inflammasome (via

***Corresponding author:** Luis Materon, Departments of Biology, University of Texas- Pan American, 1201 West University Drive, Edinburg, TX 78539, USA, Tel: 956-665-7140; Fax: 956-665-3657; E-mail: lmateron@utpa.edu

Citation: Gilkerson R, Materon L (2014) Two Roads Converging: Mitochondria and Inflammatory Signaling. J Clin Immunol Immunother 1: 004.

Received: July 11, 2014; **Accepted:** August 18 2014; **Published:** September 01, 2014

ROS or specific interaction with mtDNA, cardiolipin, MFN2, or MAVS). Inflammasome activation results in cleavage of pro-IL-1β and pro-IL-18 to active cytokines IL-1β and IL-18, which are secreted from the cell to spread inflammation.

The NLRP3 Inflammasome

The NACHT, LRR, and PYD domain-containing protein (NLRP3) is a major mediator of caspase-1 activation. To accomplish this, NLRP3 associates with the apoptosis-associated speck-like protein containing a CARD domain (ASC) adaptor protein. ASC contains a caspase recruitment domain (CARD), thus allowing binding of pro-caspase-1 to the inflammasome complex. ASC has a remarkable ability to dimerize and associate with pro-caspase-1, causing formation of a single large (~2 μm diameter) NLRP3 inflammasome in macrophages [5].

Formation of the NLRP3 inflammasome is cued by the membrane-bound PRRs, such as the TLRs, ILRs, and TNF-Rs, which activate NFκB and JNK in the nucleus NFκB is a major stress-response transcription factor, which rapidly increases mRNA levels of pro-inflammatory factors, particular NLRP3 and pro-IL-1β [6,7]. For inflammasome formation, NFκB-mediated increases in transcription of both NLRP3 and pro-IL-1β are required, as these factors are found at low basal levels. Conversely, ASC and pro-caspase-1 (as well as pro-IL-18) are found at sufficiently high levels in the cytoplasm to allow inflammasome assembly [6]. Upon binding of caspase-1 as part of the NLRP3 inflammasome, the inactive pro-caspase-1 is autocatalytically cleaved and forms the active caspase-1 heterodimer [8,9]. Active caspase-1 then cleaves proIL-1β and pro-IL-18 to their active Il-1β and IL-18 forms, which are then secreted as inflammatory cytokines [8-10] (Figure 1). An exciting collection of findings indicates that a variety of factors located in the mitochondria play a crucial role in NLRP3 inflammasome response.

Mitochondria: A Dynamic Organellar Network

As organelles of endosymbiontic origin [11], mitochondria occupy a highly unique niche in cellular biology. By combining genetic contributions from both chromosomal and mitochondrial genomes, mitochondria carry out the bulk of cellular ATP production via oxidative phosphorylation. Mitochondrial structure is incredibly dynamic, changing in response to cellular need and organellar bioenergetic function, even in cells with highly constrained architecture such as cardiac [12] and skeletal muscle fibers [13]. This dynamic structure undergoes profound alteration in response to mitochondrial dysfunction, indicating that structural dynamics represent a critical parameter to be explored in mitochondrial-immune interactions.

The dual genetic composition of mitochondria is unique among the organelles of a human cell: both nuclear- and mitochondrially-encoded gene products are required to fully assemble the five complexes of oxidative phosphorylation (OxPhos) in the mitochondrial inner membrane. While hundreds of proteins are present in human mitochondria [14,15], the vast majority of these are encoded by nuclear genes. Mitochondrial DNA (mtDNA) encodes only 2 rRNAs, 22 tRNAs, and 13 polypeptides from a 16,569 bp circular DNA. Despite the small handful of proteins encoded by mtDNA, these polypeptides are essential subunits of the OxPhos complexes in the inner membrane. While Complex II is encoded solely by nuclear DNA, the other four complexes each contain at least one mtDNA-encoded polypeptide. Complexes I-IV transfer electrons supplied by NADH and $FADH_2$, ultimately donated to molecular oxygen to create water, to drive H^+ pumping from the mitochondrial matrix to the intermembrane space. This H^+ pumping activity generates a proton-motive force, which in higher organisms is chiefly comprised of an electrochemical gradient, or transmembrane potential ($\Delta\psi_m$) that is then utilized by the F_1F_0 ATP synthase [16]. By allowing a single H^+ to return to the matrix down the gradient, ADP and P_i are bound at the F_1 portion of the ATP synthase and coalesced to ATP during the rotation-mediated conformational shifting of the holoenzyme [17].

Mitochondrial structure is organized to support bioenergetic function. While traditional models of mitochondrial ultrastructure envisioned a collection of individual organelles dispersed throughout the cytoplasm, advances in cellular imaging and the identification of genetic factors controlling mitochondrial morphology have combined to reveal mitochondrial ultrastructure as a highly dynamic, sensitive process capable of dramatic response to cellular stimuli. Mitochondria were originally named as being 'thread-like granules'. Improved imaging techniques revealed that mitochondria do in fact have the ability to interconnect as a networked reticulum throughout the cell [12,18]. Optic atrophy 1 (OPA1) was identified as a factor that is required for fusion of the mitochondrial inner membrane [19], while mitofusin 1 (MFN1) and mitofusin 2 (MFN2) carry out fusion of the outer mitochondrial membrane (Hoppins et al., 2007). While OPA1, MFN1, and MFN2 carry out mitochondrial fusion, a different set of factors carry out mitochondria fission. FIS1 [20] and MFF [21] are mitochondrial outer membrane proteins that recruit dynamin-related protein 1 (DRP1) from the cytoplasm. Upon docking at the outer membrane, DRP1 will form multimeric rings around a mitochondrion, dividing it in two [23]. Thus, mitochondrial fusion and fission are opposing processes governed by different sets of factors, in which a cell will balance mitochondrial organization between the two (Figure 2).

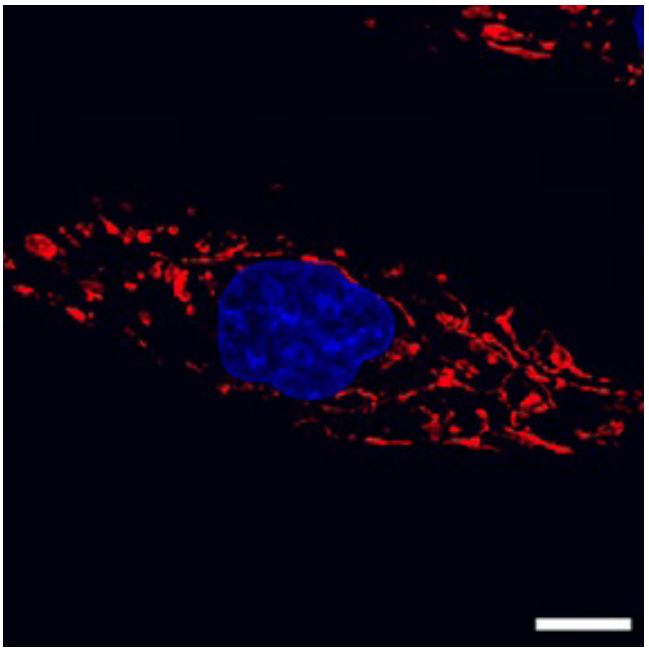

Figure 2: 3T3 mouse embryonic fibroblasts visualized by confocal fluorescence microscopy, labeled for mitochondria (MitoTracker, red) and the nucleus (DAPI, blue).

The cell's mitochondria display both interconnected, fused mitochondria (to the right of the nucleus) and fragmented, divided mitochondria (to the left of the nucleus). Size bar = 10 μm.

Mitochondrial ultrastructure is directly tied to mitochondrial function, existing in a sensitive balance to maintain energetic homeostasis. Damage or dysfunction to the structure/function balance of mitochondria causes a loss of ability to maintain a fused, interconnected mitochondrial network. Cells with either genetic [23] or pharmacologically-induced mitochondrial dysfunction [24] have mitochondria that are unable to fuse together, instead maintaining an obligately fragmented organization. This loss of organellar fusion under conditions of mitochondrial dysfunction is caused by proteolytic cleavage of the OPA1 fusion protein [25], occuring when the $\Delta\psi_m$ across the mitochondrial inner membrane is low [26]. Subsequently, $\Delta\psi_m$-sensitive cleavage of OPA1 was found to be mediated by OMA1, a metalloprotease located in the inner mitochondrial membrane [27,28]. Thus, mitochondrial function (specifically $\Delta\psi_m$) directly mediates mitochondrial fusion by OPA1, while transgenic ablation of either mitochondrial fission [29] or fusion [30] negatively impacts bioenergetic function, indicating the hand-in-hand relationship between mitochondrial bioenergetic function and structural organization. Moreover, while bioenergetic dysfunction and loss of efficient mitochondrial organization are detrimental in and of themselves, emerging evidence suggests that mitochondrial dysfunction plays a strong role in activation of NLRP3-mediated inflammatory signaling (discussed below). As mitochondrial structural dynamics are directly linked to processes including apoptosis and autophagy [31,32], mitochondrial-inflammatory interactions are likely to be similarly linked to a fascinating set of dynamic alterations, with enormous consequences for the cell and surrounding environment.

Mitochondria in NLRP3 Inflammasome Signaling

Mitochondria are emerging as a major activator of NLRP3 inflammasome signaling, and are in some cases required for NLRP3 inflammasome activation. Recent findings show that a variety of mitochondrial components interacts with and activate the NLRP3 inflammasome as major contributors to innate immune signaling by macrophages. Even more intriguingly, Misawa et al. showed that mitochondria are recruited *en masse* to the inflammasome within the cytoplasm of macrophages [33], suggesting that a host of other mitochondrial factors may have critical roles in inflammasome-mediated signaling.

A role for mitochondria in NLRP3 inflammasome signaling was first suggested when mitochondria were observed to colocalize with the NLRP3/ASC/caspase-1 scaffold assembly upon inflammasome induction. While reactive oxygen species (ROS) had previously been shown to be necessary for NLRP3 inflammasome activity [34], inhibition of the mitochondrial voltage-dependent anion channel (VDAC) abrogated both intracellular ROS levels and inflammasome assembly, indicating that mitochondrial ROS production is directly involved in NLRP3 inflammasome signaling of macrophages [35]. Subsequently, macrophages treated with *E. coli* lipopolysaccharide or ATP (both inflammasome activators) displayed release of mtDNA into the cytoplasm. Moreover, transfection to deliver cytoplasmic mtDNA stimulated secretion of both IL-1β and IL-18, indicating that release of mtDNA from the mitochondrial matrix into the surrounding cytoplasm directly contributes to NLRP3 inflammasome activity. Shimada et al. [36] then found that mitochondrial dysfunction correlates with NLRP3 inflammasome activity, with binding of oxidized mtDNA a required step in NLRP3 inflammasome activation and IL-1β [36]. These results provide an unusual mechanism of inflammasome activation: while mtDNA damage is increasingly appearing as a common form of mitochondrial damage in a variety of cellular settings [37,38], it is unclear how a highly packaged, compacted circle of DNA [39,40] is released from a double membrane-bound organelle into the cytoplasm. Future research will undoubtedly shed new light on how mtDNA escapes the organelle to participate in inflammatory signaling.

Additional studies suggest that entire mitochondrial organelles are active players in NLRP3 inflammasome activity. Cardiolipin, a diphosphatidylglycerol lipid, is found nearly exclusively in the mitochondrial inner membrane. However, upon both ROS-dependent and –independent induction of the NLRP3 inflammasome, cardiolipin translocates to the outer membrane of the mitochondria, where it interacts with the leucine-rich repeat (LRR) domain of NLRP3, concurrent with ASC and caspase-1 recruitment to the inflammasome leading to IL-1β secretion [41]. Cardiolipin translocation to the outer membrane appears to be mediated by phospholipid scramblase-3, contributing to mitochondrial autophagy in rat cortical neurons [42], suggesting that binding of cardiolipin by cytoplasmic signaling molecules is a general stress-response mechanism in cells, with profound disease implications. As a large-scale cytoplasmic macromolecular scaffolding assembly, the NLRP3 inflammasome requires the tubulin cytoskeleton to transport mitochondria to inflammasome sites, where they bind to ASC [33]. The mitochondrial anti-viral signaling (MAVS) factor is a likely adaptor protein mediating NLRP3-mitochondrial interaction, required for IL-1β maturation and secretion in THC-1 monocytes, as well as macrophages [43,44]. MFN2 is required for NLRP3 inflammasome activation following infection with RNA virus: Ichinohe et al. found that this association requires an intact $\Delta\psi_m$ for MFN2-NLRP3 interaction [45]. These studies indicate that a diverse set of mitochondrial factors mediate activation of the NLRP3 inflammasome (Figure 1). MFN2 and MAVS, as proteins located in the outer mitochondrial membrane, are easier to envision as 'docking partners' with the NLRP3 protein, while translocation of cardiolipin to the mitochondrial outer membrane and the release of mtDNA from the organelle into the cytoplasm represent highly dynamic events in inflammasome activation. These studies clearly demonstrate that much remains to be understood regarding the roles these factors play in inflammatory response, and further indicate that additional mitochondrial factors localized to any part of the organelle may be similarly involved in dynamic recruitment to the NLRP3 inflammasome. Further, the studies above (except where indicated) have characterized NLRP3 signaling interactions in macrophages, as major mediators of innate immune response. It is highly likely that NLRP3-mediated signaling will show a range of specific responses in different cell types throughout the body.

Inflammatory Damage to Mitochondria

In addition to their emerging role as integral contributors to inflammatory response in the innate immune system, mitochondria are increasingly implicated as cellular targets of cytokine-mediated inflammation in a host of tissues. While macrophages and similar immune cells involve mitochondria in inflammatory signaling, many of the same cytokines have been shown to damage mitochondria in the pathogenesis of prevalent human diseases, particularly Type 2 diabetes mellitus.

Mitochondria have long been implicated in the pathogenesis of Type 2 diabetes mellitus and associated metabolic disorders. Decreased mitochondrial function [46,47] and gene expression [48,49] have been strongly correlated with Type 2 diabetes in diverse tissues such as skeletal muscle and peripheral blood. As such, an abundance of clinical and experimental data indicates that decreased mitochondrial function and bioenergetics capacity plays a contributing role in Type 2 diabetes. However, it has been unclear what genetic or environmental factors this can be attributed to. Inherited mutations of mtDNA cause insulin resistance and diabetes mellitus in patients [50,51], indicating that mitochondrial dysfunction can be a causative determinant of insulin resistance. Despite this, inherited pathogenic mtDNA mutations do not occur frequently enough (1 in 5,000-10,000 individuals [52,53] to explain the rapidly-expanding prevalence of Type 2 diabetes worldwide. However, the emergence of cytokine-mediated inflammation as a causative mechanism of Type 2 diabetes suggests that cytokine-mediated damage to mitochondria plays a major role in the pathogenesis of diabetes and co-morbid conditions.

Cytokine-mediated inflammation has gained recognition as a major causative force in the development of insulin resistance and diabetes [54-56]. While the initial studies demonstrating this causative mechanism explored the ability of TNF-α to mediate insulin resistance [55], subsequent studies built upon these findings to include IL-1β, IL-6, IL-18, resistin, leptin, adiponectin, and others. Many of these are expressed both by macrophages and adipocytes, providing a mechanistic link for the co-morbidity of Type 2 diabetes and obesity [57]. Upon binding of these cytokines to PRRs at the plasma membrane, the NFκB and JNK pathways are activated, mediating crucial stress-mediated transcription of inflammatory factors (such as IL-1β and NLRP3, above). The NLRP3 inflammasome is activated in cytokine-mediated insulin resistance [58,59] and mediates impaired wound healing through sustained inflammation in diabetic patients [60].

Many of these same cytokines have been shown to directly damage mitochondria. TNF-α cause rapid damage to mtDNA and increased ROS production [61], and inhibits mitochondrial bioenergetics [62]. This TNF-α-induced damage is dependent on TNF-R1 binding, and appears to involve stress-response translocation of p53 to mitochondria [61,63]. Similarly, heat-inactivated *E. coli* activates TLR-4, causing mtDNA depletion [64]. These findings are concordant with experimental and clinical data showing loss of mtDNA content and bioenergetic function [65-67]. This mitochondrial damage has wide-ranging effects on the cell at large. Mitochondrial involvement in the development of insulin resistance appears to occur via elevated mitochondrial ROS production, rather than decreased OxPhos activity *per se* [68]. Mitochondrial dysfunction has been suggested to cause insulin resistance by decreasing insulin receptor substrate-1 (IRS1) expression [69,70]. Mechanistically, loss of mtDNA has been shown to effect broad changes in nuclear transcription via 'retrograde' mitochondria-to-nucleus signaling, in which mitochondrial dysfunction affects pathways including NFkB and JNK to add to cellular stress response [71,72]. The specific impacts of mitochondrial dysfunction on gene expression of various inflammatory factors are likely to provide insight into a critical cell-wide consequence of mitochondrial dysfunction.

While the connections between inflammatory signaling and mitochondria have been best characterized in diabetes and metabolic disorders, these interactions are increasingly found across a range of other prevalent diseases. Gene expression profiling [73,74] and biomarker studies find increases in cytokines in autistic subjects [75], particularly IL-6 and TNF-α [76-]. These studies are consistent with findings of mitochondrial dysfunction and increased organellar fission in brain samples of autistic individuals [79]. Similar involvement of NLRP3-associated inflammation is found in Alzheimer's disease [80] and cardiomyopathy [81]. These associations strongly suggest that inflammation and mitochondria comprise a pathogenic axis that is likely to play a role in a wide range of prominent human diseases.

Mitochondrial Factors as Inflammatory Messengers

As discussed above, the release of mtDNA from the organelle in response to NLRP3-associated inflammatory stimuli is a highly novel, dynamic response, suggesting that mtDNA is a key mediator of intracellular inflammation. Recent evidence suggests that mtDNA and associated factors may have further roles as *extracellular* inflammatory mediators. Mathew et al. observed that extracellular, partially degraded mtDNA causes induction of cytokine secretion, most notably IL-1β, suggesting that mtDNA is itself a category of damage-associated molecular pattern (DAMP) for recognition by PRRs [82]. Similarly, Chaung et al. [83] found that transcription factor A, mitochondrial (TFAM), the major mtDNA-packaging protein, mediates inflammation in hemorrhagic shock [83]. These findings strongly indicate that mtDNA and the other protein factors directly associated with it [39] are released from mitochondria and act as pro-inflammatory signaling factors, appearing as clinically-relevant indicators of inflammation in experimental systems and patients [84,85]. Just as the adaptive nature of the mitochondrial network has become evident as a key element of cellular homeostasis, these findings indicate the importance of mitochondrial factors as mediators of inflammatory signaling. The mechanisms of mitochondrial factor release will reveal exciting, highly novel molecular dynamics of factors previously thought to reside exclusively within mitochondria.

Conclusion

The specific interactions of mitochondrial factors with innate inflammatory factors, particularly NLRP3, bring together two fields of cell biology that previously had little apparent connection. These interactions are of enormous importance medically, as increased inflammation has emerged as a causative or contributory factor in a wide range of many of the most rapidly-expanding diseases today. As the dynamics and causes of both bioenergetic stress and inflammation in human disease are uncovered, understanding the intrinsic mechanistic connections of inflammatory signaling with mitochondrial biology is increasingly vital to cellular homeostasis and human health.

Acknowledgements

This work is supported by a Research Grant from the Diabetes Action Research and Education Foundation (to RG).

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