Published in final edited form as:

J Alzheimers Dis. 2015 ; 45(1): 305–318. doi:10.3233/JAD-142334.

Mitochondrial Lysates Induce Inflammation and Alzheimer's Disease-Relevant Changes in Microglial and Neuronal Cells

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Abstract

Neuroinflammation occurs in AD. While AD genetic studies implicate inflammation-relevant genes and fibrillar amyloid β protein promotes inflammation, our understanding of AD neuroinflammation nevertheless remains incomplete. In this study we hypothesized damage-associated molecular pattern (DAMP) molecules arising from mitochondria, intracellular organelles that resemble bacteria, could contribute to AD neuroinflammation. To preliminarily test this possibility, we exposed neuronal and microglial cell lines to enriched mitochondrial lysates. BV2 microglial cells treated with mitochondrial lysates showed decreased TREM2 mRNA, increased TNF α mRNA, increased MMP-8 mRNA, increased IL-8 mRNA, redistribution of NF κ B to the nucleus, and increased p38 MAPK phosphorylation. SH-SY5Y neuronal cells treated with mitochondrial lysates showed increased TNF α mRNA, increased NF κ B protein, decreased I κ B α protein, increased A β PP mRNA, and increased A β PP protein. Enriched mitochondrial lysates from SH-SY5Y cells lacking detectable mitochondrial DNA (ρ 0 cells) failed to induce any of these changes, while mtDNA obtained directly from mitochondria (but not PCR-amplified mtDNA) increased BV2 cell TNF α mRNA. These results indicate at least one mitochondrial-derived DAMP molecule, mtDNA, can induce inflammatory changes in microglial and neuronal cell lines. Our data are consistent with the hypothesis that a mitochondrial-derived DAMP molecule or molecules could contribute to AD neuroinflammation.

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Keywords

Alzheimer's disease; amyloid β precursor protein; inflammation; mitochondria; mtDNA; TREM2

INTRODUCTION

Inflammation is observed in brain aging and Alzheimer's disease (AD) [1-4]. Differences in peripheral cytokine levels may distinguish AD and control cohorts [3, 5], and recent genetic studies reveal inflammation-relevant genes influence AD risk [3]. Despite this, the causes and consequences of AD inflammatory changes remain incompletely understood.

Neuroinflammation in AD currently does not appear to reflect the presence of an externally acquired pathogen [6]. This implies an internally produced damage-associated molecular pattern (DAMP) molecule or molecules may contribute to AD neuroinflammation [7, 8]. Amyloid β protein (A β), which aggregates in AD, induces inflammation [9] but this does not exclude the possibility that other intrinsically generated molecules might also contribute.

To this point, mitochondrial dysfunction exists in AD brains [10] and in general mitochondrial dysfunction and inflammation changes co-localize [11, 12]. Mitochondria evolved from a symbiotic relationship with a prokaryote organism, and thus share characteristics with bacteria. Several molecules normally sequestered by mitochondria can induce inflammation, including mitochondrial DNA (mtDNA), mitochondrial transcription factor A (TFAM), cardiolipin, cytochrome c, formyl-peptides, high mobility group B protein 1 (HMGB1), and ATP [8, 13-23].

Mitochondrial-derived DAMPs activate inflammation in non-brain tissues. For example, mtDNA, when not sequestered within mitochondria, induces arthritis and drives inflammatory changes in heart failure models [24, 25]. To date, though, no one has specifically considered the potential contribution of mitochondria, through the release of mitochondrial-derived DAMPs, to chronic brain inflammation. To preliminarily assess the viability of this idea, we exposed microglial and neuronal cell lines to enriched mitochondrial lysates. This approach allowed us to explore ways in which mitochondrial-derived DAMP molecules may affect different central nervous system cell types. Our data suggest an mtDNA-containing DAMP component could indeed contribute to AD neuroinflammation, and also contribute to other AD-relevant molecular changes.

MATERIALS AND METHODS

Cell culture

Human neuronal SH-SY5Y cells and mouse microglial BV2 cells were cultured at 5% CO₂ in high glucose DMEM supplemented with 10% fetal bovine serum (FBS) and 1% of a penicillin-streptomycin stock (catalogue number 30-001-CI, Fisher Scientific). mtDNA-depleted SH-SY5Y cells (p0 cells) were cultured at 5% CO₂ in high glucose DMEM supplemented with 10% FBS, 1% penicillin-streptomycin stock, 100 μ g/mL sodium pyruvate, and 50 μ g/mL uridine. These p0 cells were generated as previously described [26].

p0 cell status was confirmed via PCR, and by demonstrating these cells cannot survive in the absence of uridine and pyruvate supplementation [27].

Mitochondrial lysates

Enriched mitochondrial lysates were generated from SH-SY5Y, SH-SY5Y p0, or BV2 cells by first suspending approximately 30 million cells from each line in ice-cold MSHE buffer (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH 7.4). The suspended cells were disrupted via nitrogen cavitation, on ice and over 15 minutes, at a constant 900 PSI chamber pressure. Enriched mitochondrial pellets were subsequently generated by centrifugation (1000 g for 5 min at 4°C, followed by two 10 min spins at 20,000 g and 4°C). Mitochondria were re-suspended in sterile phosphate buffered saline (PBS), ruptured by three rapid freeze/thaw cycles, and protein concentrations were determined using a BCA Protein Assay kit (Bio-Rad). All procedures were completed using aseptic technique.

Isolation and PCR amplification of mtDNA

Mitochondrial-enriched pellets (prepared as described above, only not exposed to the freeze/thaw steps) from BV2 or SH-SY5Y cells were suspended in 600 µl of lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1% SDS) and disrupted using 10 strokes of a Dounce homogenizer (clearance of 0.005-0.0025 inches). The homogenized mitochondria were transferred to microcentrifuge tubes, 60 µl of proteinase K (Qiagen) was added, and the tubes were vortexed and then incubated at 55°C for 2 h. During this 2 h incubation period we continued to vortex the samples every 30 min. Samples were next centrifuged at 8,000 g for 15 min, and the supernatants were transferred to new tubes. 600 µl of phenol:chloroform:isoamyl alcohol (Sigma) were added to the tubes, which were vortexed and centrifuged at 8,000 g for 15 min. Supernatants were transferred to new tubes, 500 µl of chloroform (Sigma) was added, and samples were vortexed and centrifuged at 8,000 g for 15 min. The supernatants were transferred to new tubes, and 40 µl of 3 M sodium acetate and 440 µl of 100% isopropanol were added. Samples were then incubated at -20°C overnight. The following day samples were centrifuged at 8,000 g for 15 min. The supernatants were discarded, and the pellets were washed with 750 µl of 70% ethanol and then centrifuged at 8,000 g for 8 min. The supernatants were discarded and the pellets were washed with 500 µl of 70% ethanol and centrifuged at 8,000 g for 8 min. After discarding the supernatants the pellets were gently air dried and re-suspended in 100 µl of nuclease-free water.

mtDNA was PCR-amplified using a Roche Expand Long Range dNTP pack. Primers used for human mtDNA amplification were previously published [28]. Mouse mtDNA primers were as follows: GCACTGAAAATGCTTAGATGG (forward), AGAGTTTTGGTTCACGGAAC (reverse). PCR amplification was performed using an Applied Biosystems 7300 PCR System. An initial denaturation step at 92°C for 2 min was followed by 10 cycles of 92°C for 15 sec (denaturation) followed by a 12 min 40 sec elongation time at 60°C. For cycles 11-30, for each successive individual cycle 20 sec was added to the elongation time from the previous cycle. An aliquot of the PCR mixture was resolved on a 1% Agarose gel (Bio-Rad) in TBE Buffer (Bio-Rad). PCR products were purified using a Qiagen PCR purification kit (Qiagen).

Treatment of cells with mitochondrial lysates and mtDNA

SH-SY5Y or BV2 cells were plated at approximately 80% confluence in 6-well dishes. The following day cells were treated with 50 µg of post-freeze/thaw mitochondrial lysate, 1 or 2 µg of purified mtDNA, 1 or 2 µg of PCR-amplified mtDNA, or vehicle (PBS) for approximately 24 h.

Immunoblotting

After the designated treatment period cells were lysed using MPER (Thermo-Pierce), or else nuclear and cytosolic fractions were generated using NE-PER (Thermo-Pierce) and differential centrifugation. Protein concentrations were determined using a BCA Protein Assay kit. Equal amounts of protein were resolved by SDS-PAGE using 4-20% Criterion TGX Tris-glycine polyacrylamide gels (Bio-Rad) and gel proteins were transferred to nitrocellulose membranes (Invitrogen). Membranes were blocked for 1 h at room temperature while rocking, and incubated in primary antibody at the indicated dilutions (see below) in blocking buffer overnight at 4°C while rocking. Membranes were washed 3 times with PBS-Tween 20 and placed in secondary antibody at 1:2000 (in blocking buffer) for 1 h at room temperature. Membranes were washed 3 times with PBS-Tween 20 and incubated with Supersignal West Femto chemiluminescence reagent (Thermo-Pierce). Images were captured using a Chemidoc imaging station (Bio-Rad). Primary antibodies used were to nuclear factor kappa B (NFκB) (Santa Cruz, 1:200), p38 mitogen activated protein kinase (p38 MAPK) (1:1000, Cell Signaling Technology), phospho-Thr180/Tyr182 p38 MAPK (1:2000, Cell Signaling Technology), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling, 1:2000), actin (Cell Signaling, 1:1000), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα) (Santa Cruz, 1:200), amyloid precursor protein (AβPP) (Cell Signaling, 1:1000), cytochrome c oxidase subunit IV isoform 1 (COX4I1) (Cell Signaling, 1:1000), TATA binding protein (TATA BP) (Abcam, 1:500), Histone deacetylase 1 (HDAC1) (Cell Signaling; 1:1000).

Immunocytochemistry

After receiving a designated treatment, cells were fixed in 4% paraformaldehyde (in PBS, pH 7.4) for 1 h at room temperature. Cells were then incubated in blocking buffer (5% BSA, 0.2% Triton X-100 in PBS, pH 7.4) for 1 h at room temperature. Incubation at 4°C with a primary antibody to NFκB (Cell Signaling Technologies; 1:400) took place overnight in blocking buffer containing 10 µg/mL Hoechst 33258 (Invitrogen). Cells were washed 3 times with PBS followed by incubation with AlexaFluor 488 Donkey anti-Rabbit secondary antibody (Abcam 1:500) for 1 h at room temperature. Cells were washed 3 times in PBS, and placed in anti-fade reagent (Sigma). Images were captured using an Olympus IX71 fluorescent microscope. For each independent sample a minimum of 150 cells were counted.

Aβ enzyme-linked immunoabsorption assay (ELISA)

Conditioned media from cells exposed to different treatments was collected and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (Fisher), a serine protease inhibitor, was added. Media samples were analyzed using an Aβ1-40 ELISA kit (Invitrogen) at a dilution of 1:5 according to the manufacturer's instructions.

Quantitative PCR

RNA was isolated using Tri Reagent (Ambion). Reverse transcription was performed on 1 µg of total RNA using an iScript RT qPCR master mix (BioRad). Amplifications were performed using an Applied Biosystems 7300 PCR System. Quantitative real-time PCR (qPCR) was performed using an iTaq universal probes supermix (Bio-Rad) and TaqMan Gene Expression Assays (Applied Biosystems) to quantify mRNA levels for tumor necrosis factor α (TNF α), matrix metalloproteinase-8 (MMP-8), interleukin-8 (IL-8), triggering receptor expressed on myeloid cells-2 (TREM2), C-C motif chemokine 11 (CCL11), and A β PP. GAPDH mRNA levels were used to normalize the expression levels of these genes.

We used Taqman primers to the nuclear 18S rRNA gene to determine the respective cycle threshold values for nuclear DNA derived from whole cells and from mitochondrial fractions. In qPCR, a 3.3 difference in cycle threshold values approximately reflects a 10-fold change in the amount of amplifiable template. The cycle threshold values obtained from whole cell DNA and enriched mitochondrial fraction DNA were further used to calculate the percentage of nuclear DNA that was eliminated during the preparation of the enriched mitochondrial fractions.

Statistics

Data were summarized by means and standard errors. To compare means between three groups we used one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post-hoc testing. *p*-values less than 0.05 were considered statistically significant.

RESULTS

Mitochondrial lysate characterization

The mitochondrial lysates used for these experiments were assessed for mitochondrial purity and enrichment (Fig. 1). Relative to what was observed in SH-SY5Y and BV2 whole cell lysates, SH-SY5Y and BV2 mitochondrial lysates showed a robust reduction in GAPDH, a primarily non-mitochondrial cytosolic protein, and TATA BP, a nuclear protein. For DNA analyzed from SH-SY5Y whole cells and SH-SY5Y mitochondrial lysates, subtracting the whole cell cycle threshold from the mitochondrial lysate cycle threshold yielded a cycle threshold difference of 2.1. This indicates a 6.4-fold reduction in nuclear DNA occurred during the mitochondrial enrichment process, or that 84.4% of the nuclear DNA was eliminated. For DNA prepared from BV2 whole cells and BV2 mitochondrial lysates, subtracting the whole cell cycle threshold from the mitochondrial lysate cycle threshold yielded a cycle threshold difference of 2.5. This indicates a 7.6-fold reduction in nuclear DNA occurred during the mitochondrial enrichment process, or that 86.8% of the nuclear DNA was eliminated. For DNA prepared from SH-SY5Y ρ 0 whole cells and ρ 0 mitochondrial lysates, subtracting the whole cell cycle threshold from the mitochondrial lysate cycle threshold yielded a cycle threshold difference of 2.7. This indicates an 8.2-fold reduction in nuclear DNA occurred during the mitochondrial enrichment process, or that 87.8% of the nuclear DNA was eliminated.

Effect of mitochondrial lysates on BV2 cell inflammatory gene expression

We measured levels of DAMP response-associated genes in BV2 mouse microglial cells treated with mitochondrial lysates from either BV2 cells (containing mtDNA) or SH-SY5Y human $\rho 0$ cells lacking mtDNA. We used this strategy because a BV2 $\rho 0$ cell line does not currently exist, and because BV2 mitochondrial lysates should overall appear less foreign to BV2 cells than SH-SY5Y mitochondrial lysates. Specific changes observed in BV2 cells treated with BV2-derived mitochondrial lysates but not SH-SY5Y $\rho 0$ mitochondrial lysates, therefore, could indicate mtDNA, an mtDNA-containing complex, mtRNA, or mtDNA-encoded proteins act as DAMP molecules.

TNF α mRNA levels showed a 4.3 fold increase following treatment with mitochondrial lysates containing mtDNA. TNF α mRNA did not significantly increase when mitochondrial lysates from mtDNA-depleted cells were used (Fig. 2A). mRNA levels of TREM2, a gene whose protein product suppresses the production of cytokines and pushes immune cells towards a more phagocytic phenotype, decreased by 48% in cells treated with mitochondrial lysates that contained mtDNA. No change in TREM2 mRNA was observed in cells treated with mitochondrial lysates prepared from mtDNA-depleted cells (Fig 2B).

Prior studies have implicated IL-8 and MMP-8 in mitochondria-induced DAMP responses [13]. We observed an approximate 7.6 fold increase in IL-8 mRNA and an approximate 7.0 fold increase in MMP-8 mRNA in cells treated with mitochondrial lysates from BV2 cells (Fig. 2C,D). IL-8 and MMP-8 mRNA levels were unchanged in cells treated with SH-SY5Y $\rho 0$ mitochondrial lysates (Fig. 2C,D). When BV2 cells were treated with mitochondrial lysates from standard SH-SY5Y cells (which contain mtDNA), the results were similar to those obtained when BV2 cells were treated with mitochondrial lysates from BV2 cells (data not shown). Therefore, mtDNA overall appeared to play either a direct or indirect role in generating these responses.

Effect of mitochondrial lysates on BV2 cell NF κ B expression and compartmentalization

The NF κ B transcription factor helps regulate TNF α production and other inflammation parameters [29]. We therefore examined total cell protein levels of NF κ B and its repressor, I κ B α , and quantified nuclear and cytoplasmic NF κ B protein levels. Cells treated with BV2 cell mitochondrial lysates displayed a 33% increase in total NF κ B protein (Fig. 3A,C). Total NF κ B protein levels did not change in cells treated with mitochondrial lysates prepared from SH-SY5Y $\rho 0$ cells that lacked detectable mtDNA (Fig. 3A,C). I κ B α , which sequesters NF κ B in the cytoplasm and prevents NF κ B-mediated transcription did not change, although a non-significant trend towards a lower total level was perhaps observed in cells exposed to BV2 cell mitochondrial lysates ($p=0.085$) (Fig. 3B,C). Despite this, the NF κ B nucleus:cytoplasm ratio increased by 220% in cells treated with BV2 mitochondrial lysates (Fig 3D-G). The NF κ B nucleus:cytoplasm ratio did not change in cells treated with SH-SY5Y $\rho 0$ mitochondrial lysates.

To validate our Western blot-based protein compartmentalization approach, we additionally performed immunocytochemistry using a primary antibody to NF κ B. With this technique we observed that BV2 cells exposed to mtDNA-containing mitochondrial lysates had a

higher percentage of cells that showed intense nuclear NF κ B staining (Fig. 3H-I). Immunocytochemistry data, therefore, are essentially consistent with Western blot data that indicate mitochondrial lysates from mtDNA-containing cells induce a relative redistribution of NF κ B to cell nuclei.

Effect of mitochondrial homogenates on BV2 cell p38 protein and phosphorylation levels

NF κ B expression and activation is mediated by the p38 MAPK pathway [30, 31]. No change in total p38 protein was observed in cells treated with mitochondria containing or lacking mtDNA (Fig. 4A,D). Since p38 is activated through distinct phosphorylation events, we assessed p38 Thr180/Tyr182 phosphorylation. In cells treated with mitochondrial lysates that contained mtDNA, phosphorylated p38 levels were significantly increased when normalized to GAPDH (an approximate 4 fold increase) or total p38 protein (an approximate 3 fold increase) (Fig. 4B-D). Cells treated with mitochondrial lysates from ρ 0 cells did not show significant changes in p38 Thr180/Tyr182 phosphorylation when normalized to either GAPDH or total p38 protein.

Effect of mitochondrial lysates on SH-SY5Y cell inflammatory genes

We next assessed cytokine production in SH-SY5Y cells treated with mitochondrial lysates prepared from SH-SY5Y and SH-SY5Y ρ 0 cells. Cells treated with mitochondrial lysates from mtDNA-containing cells showed an approximate 3.3 fold increase in their TNF α mRNA level (Fig. 5A). TNF α mRNA levels did not change in SH-SY5Y cells treated with ρ 0 cell mitochondrial lysates (Fig. 5A). When SH-SY5Y cells were treated with SH-SY5Y cell mitochondrial lysates, CCL11 mRNA levels were unchanged across the groups (ANOVA $p=0.10$), although on post-hoc analysis a trend towards increased CCL11 mRNA was observed (2.3 fold increase, $p=0.04$ on LSD test) (Fig. 5B). Cells treated with SH-SY5Y ρ 0 mitochondrial lysates showed no evidence of CCL11 mRNA upregulation (Fig. 5B). IL-8 and MMP-8 mRNA levels were undetectable in SH-SY5Y cells either preceding or following exposure to mitochondrial lysates (data not shown).

Effect of mitochondrial lysates on SH-SY5Y cell NF κ B protein

SH-SY5Y cells treated with mitochondrial lysates from mtDNA-containing cells displayed a 2.9 fold increase in NF κ B total protein (Fig. 6A,C). No significant change was observed in NF κ B protein levels in SH-SY5Y cells treated with mitochondrial lysates from cells that lacked detectable mtDNA (Fig. 6A,C). The I κ B α protein level fell by 38% in cells treated with mitochondrial lysates that contained mtDNA, but no reduction was observed in cells treated with mitochondrial lysates that lacked mtDNA (Fig. 6B,C). While NF κ B nucleus:cytoplasm ratios were comparable by ANOVA when SH-SY5Y cells were treated with mitochondrial lysates that contained or lacked mtDNA ($p=0.11$), the post hoc analysis showed perhaps a trend towards an increase in cells exposed to the mtDNA-containing mitochondrial lysate ($p=0.05$ on LSD test) (Fig. 6D-G).

Effect of mitochondrial lysates on SH-SY5Y cell A β PP mRNA, A β PP protein, and secreted A β

A β PP transcription is activated by NF κ B [32, 33]. Given the results presented above, as well as reported links between A β PP and AD, we assessed A β PP levels in SH-SY5Y cells treated with SH-SY5Y and SH-SY5Y ρ 0 cell mitochondrial lysates. A β PP mRNA and protein levels increased when cells were exposed to mitochondrial lysates from cells that contained mtDNA (61% and 160% increases, respectively), but did not change following exposure to mitochondrial lysates from cells that lacked mtDNA (Fig. 7A-C). No changes, though, in secreted A β protein were observed. The A β protein concentration in the lysate-untreated control cells was 29.6 ± 0.5 pg/ml, for the cells treated with 50 μ g of ρ 0 cell mitochondrial lysates it was 29.3 ± 0.2 pg/ml, and for cells treated with 50 μ g of SH-SY5Y cell mitochondrial lysates it was 31.5 ± 1.1 pg/ml.

Direct effect of mtDNA on SH-SY5Y and BV2 cell TNF α expression

We next tested the ability of mtDNA to specifically induce TNF α expression. We utilized enriched mtDNA obtained directly from BV2 cells, as well as through a long PCR amplification of BV2 mtDNA (Fig. 8A). When BV2 cells were treated with either 1 μ g of enriched native mtDNA or 1 μ g of PCR mtDNA, TNF α mRNA levels did not change although there was perhaps an upward trend in cells exposed to the enriched native mtDNA ($p=0.085$ on post hoc LSD test) (Fig. 8B). The BV2 TNF α mRNA level did increase when cells were treated with 2 μ g of enriched native mtDNA prepared directly from BV2 cells, but did not change when BV2 cells were treated with an equivalent amount of PCR-generated mtDNA (Fig. 8C). SH-SY5Y cell TNF α mRNA levels did not change following treatment with either 1 μ g or 2 μ g of enriched mitochondrial lysate mtDNA or PCR-amplified mtDNA (data not shown).

DISCUSSION

This study tested the ability of mitochondrial lysates to induce inflammation in microglial and neuronal cell lines, as well as the ability of mitochondrial lysates to influence specific AD-associated genes including TREM2 and A β PP. We found that BV2 microglial cells exposed to mitochondrial lysates from cells containing mtDNA, but not mitochondrial lysates prepared from mtDNA-depleted cells, increased mRNA levels of several inflammation-relevant genes (TNF α , IL-8, and MMP-8), decreased TREM2 mRNA, increased total NF κ B protein, shifted NF κ B to the nucleus, and increased p38 MAPK phosphorylation. We also found that in SH-SY5Y neuronal cells mitochondrial lysates from cells containing mtDNA, but not mitochondrial lysates prepared from mtDNA-depleted cells, increased TNF α mRNA, perhaps increased CCL11 mRNA, increased total NF κ B protein, increased A β PP mRNA, and increased A β PP protein. Direct treatment with enriched mtDNA, but not PCR-amplified mtDNA, also increased BV2 cell TNF α expression.

TNF α , an inflammatory cytokine, is activated by inflammatory molecules and can also amplify inflammatory signaling cascades [34, 35]. TREM2 suppresses cytokine production and genetic ablation of TREM2 reduces microglial phagocytosis [36]. IL-8 is a macrophage-generated and secreted inflammatory chemokine that binds Toll-like receptors and plays a

role in innate immune system responses [37, 38]. The matrix metalloproteinase MMP-8, when aberrantly produced by microglial cells, facilitates neuroinflammatory responses through its effects on TNF α [39]. Demonstrating effects on the expression of these genes could prove relevant to AD, a disorder that features neuroinflammation, elevated TNF α levels, reduced TREM2 levels [36, 40], and an increased risk for individuals that carry loss-of-function TREM2 sequence variants [2]. To our knowledge, IL-8 and MMP-8 levels have not been reported in AD brains, although our data are consistent with prior studies that indicate mtDNA induces IL-8 and MMP-8 expression [13].

NF κ B, a transcription factor, plays a role in inflammatory cascades. NF κ B signaling is induced by TNF α , and in turn NF κ B can induce TNF α expression [34]. In our experiments mtDNA-containing mitochondrial lysates increased NF κ B expression, decreased I κ B α levels in SH-SY5Y cells, and caused either absolute or relative (to cytosolic levels) increases in nuclear NF κ B. These findings may prove relevant to AD, a disorder that exhibits NF κ B activation [41].

The canonical inflammatory p38 MAPK pathway also modulates NF κ B activation [42]. Mitochondrial lysates prepared from mtDNA-containing cells, but not mitochondrial lysates prepared from mtDNA-depleted cells, induced p38 phosphorylation. The presence of increased p38 phosphorylation is consistent with a state of increased NF κ B activation. p38 MAPK signaling is increased in human AD subject brains [43].

A β PP expression increases in AD subjects [44, 45]. Therefore, our finding that mitochondrial lysates increase both A β PP mRNA and protein levels could be AD-relevant. Existing data indicate the A β PP gene promoter contains putative NF κ B binding sites [32, 33]. Although we did not mechanistically address this possibility, it seems reasonable to consider that A β PP upregulation in our experiments could reflect increased NF κ B activity. Of further interest to this point, AP1 and SP1, transcription factors that are also linked to inflammatory signaling, may additionally induce A β PP transcription [46].

The observed mitochondrial lysate-induced increase in A β PP protein, though, did not cause a detectable increase in soluble extracellular A β protein. The fate of the A β PP protein in our experiments, therefore, remains unclear. While an increase in A β PP mRNA suggests the increase in A β PP protein reflects increased production, as opposed to decreased degradation, increased A β PP protein production would not necessarily require A β PP degradation rates to also increase. Alternatively, A β PP degradation could be proceeding at an increased rate through the alpha secretase-mediated non-amyloidogenic pathway, or it could be proceeding at an increased rate through an intracellular beta secretase-mediated event. More detailed studies to resolve this question are indicated.

Mitochondria, which resemble bacteria in many ways, contain a pool of known DAMP molecules. mtDNA, TFAM, cardiolipin, cytochrome c, formyl-peptides, HMGB1, and ATP have previously been shown to induce a DAMP response in various models [8, 13-23]. Our data argue mtDNA, at least in its native form, may play a particularly important role. We cannot, however, completely rule out the possibility that other lysate components acted as DAMP molecules and contributed to DAMP responses in our experiments. Specifically, due

to their lack of mtDNA p0 cells cannot synthesize mitochondrial RNA or mtDNA-encoded proteins and this, in turn, should eliminate formyl-peptide synthesis. It therefore remains possible that mitochondrial RNA or formyl-peptides may have partly driven changes in our inflammation-associated endpoints. TFAM protein levels may also change in p0 cells.

The mitochondrial fractions used throughout this study were robustly enriched for mitochondrial content but were not completely pure. We thus need to consider the possibility that potential non-mitochondrial contaminants, such as nuclear DNA, may have contributed to our observed DAMP responses. While the enrichment procedure we used eliminated approximately 85% of the nuclear DNA initially present in our cell cultures, substantial amounts of nuclear DNA were no doubt present in our mitochondrial lysate and mtDNA preparations. However, equivalently enriched mitochondrial lysates obtained from p0 cells, which also contained nuclear DNA contamination but lacked mtDNA, consistently failed to elicit an inflammatory response. We therefore conclude, as have others, that even if nuclear DNA can act as a DAMP molecule in this respect it is less functionally potent than mtDNA [25]. We further conclude that in our experiments, either mtDNA or an mtDNA-containing complex functioned as a DAMP molecule. We again wish to emphasize, though, that this does not rule out the possibility that other mitochondrial components contributed to the DAMP responses observed in these experiments.

Several factors could account for the finding that PCR-amplified mtDNA did not increase TNF α mRNA levels despite the fact that native enriched mtDNA did. In general, certain DNA features are not perpetuated during PCR amplification. These include DNA tertiary structure, epigenetic modifications, oxidative changes, and bound proteins (although one would predict proteinase K treatment should remove most or all mtDNA-bound proteins).

A recent study reported that retinal ganglion cells release mitochondria from axons, and that these mitochondria are then engulfed and degraded by surrounding glial cells [47]. Therefore, neurons have the ability to shed mitochondria, and if this process becomes compromised inflammation could ensue. Given this context, our findings are consistent with the possibility that bioenergetic dysfunction and failure of axonal mitochondrial degradation could give rise to chronic brain inflammation through the release of mitochondrial components. Further studies are warranted to determine if mitochondrial components induce chronic brain inflammation *in vivo*.

ACKNOWLEDGEMENTS

This work was supported by the University of Kansas Alzheimer's Disease Center (P30AG035982), the Frank and Evangeline Thompson Alzheimer's Treatment Program Fund, the Hugh and Betty Libby Foundation, the Greater Kansas City Automobile Dealers Association, the Gene and Marge Sweeney Chair, the University of Kansas Alzheimer's Disease Center Pilot Program, the University of Kansas Landon Center on Aging, the University of Kansas Frontiers Heartland Institute for Clinical and Translational Research, and the University of Kansas Medical Center's Biomedical Research Training Program.

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Fig. 1.

Characterization of mitochondrial lysates. (A) 50 μ g of whole cell lysate (WCL) or mitochondrial lysate were resolved by electrophoresis and transferred to a nitrocellulose membrane. Representative immunochemistry data for COX-4I1 (mitochondrial marker), TATA BP (nuclear marker), and GAPDH (cytosolic marker) protein are shown. (B) The cycle threshold difference between DNA from whole cells and DNA from enriched mitochondria was used to calculate the amount of nuclear DNA that was eliminated during the mitochondrial enrichment process.

Fig. 2.

Effect of mitochondrial lysates on microglial cell inflammatory gene expression. BV2 cells were treated overnight with either PBS vehicle, 50 μ g of mitochondrial lysate from BV2 cells, or 50 μ g of mitochondrial lysate from SH-SY5Y ρ 0 cells. Nine independent samples were analysed for each condition. (A) TNF α mRNA level. (B) TREM2 mRNA level. (C) IL-8 mRNA level. (D) MMP-8 mRNA level. Values shown are relative group means $\pm$ SEM, with the control group set to 1. *indicates $p < 0.05$; ** indicates $p < 0.005$.

Fig. 3.

Effect of mitochondrial lysates on microglial cell NFκB expression and protein compartmentalization. BV2 cells were treated overnight with PBS vehicle, 50 μg of mitochondrial lysate from BV2 cells, or 50 μg of mitochondrial lysate from SH-SY5Y p0 cells. In (A) through (G), seven to ten independent samples were analysed for each condition. (A) Total cell NFκB protein. (B) Total cell IκBα protein. (C) Representative whole cell immunocytochemistry data. (D) Cytosolic NFκB protein. (E) Nuclear NFκB protein. (F) Nucleus:cytosol NFκB protein ratio. (G) Representative nucleus and cytosol immunocytochemistry data. (H) Immunocytochemistry with a primary antibody to NFκB and Hoechst staining was performed on BV2 cells treated with PBS vehicle or 50 μg of BV2 cell mitochondrial lysate. Cells were visualized using confocal microscopy. The NFκB staining is green and the nuclear Hoechst staining is blue. Arrowheads indicate cells with intense nuclear NFκB staining. (I) The percentage of BV2 cells in each group with intense NFκB nuclear staining is indicated. Six independent samples were prepared and analysed for each condition. Values shown in (A) through (F) are relative group means ± SEM, with the control group set to 1. * indicates $p < 0.05$. In (H), ** indicates $p < 0.005$ by Student's T-test.

Fig. 4.

Effect of mitochondrial lysates on microglial cell p38 protein and phosphorylation levels. BV2 cells were treated overnight with PBS vehicle, 50 μ g of mitochondrial lysate from BV2 cells, or 50 μ g of mitochondrial lysate from SH-SY5Y p0 cells. Cells were lysed using MPER, equal protein concentrations were resolved by electrophoresis, gel proteins were transferred to nitrocellulose membranes, and the membranes were immunoblotted for p38, GAPDH, and phosphorylated p38 protein. (A) Total p38 normalized to GAPDH. (B) Phosphorylated p38 normalized to GAPDH. (C) Phosphorylated p38 normalized to total p38. (D) Representative immunoblot data. Values shown are relative group means $\pm$ SEM, with the control group set to 1. ** indicates $p < 0.005$.

Fig. 5.

Effect of mitochondrial lysates on neuronal cell line inflammatory gene expression. SH-SY5Y cells were treated overnight with PBS vehicle, 50 µg of mitochondrial lysate from SH-SY5Y cells, or 50 µg of mitochondrial lysate from SH-SY5Y p0 cells. Twelve independent samples were prepared and analysed for each condition. (A) TNFα mRNA level. (B) CCL11 mRNA level. Values shown are relative group means ± SEM, with the control group set to 1. ** indicates $p < 0.005$; # indicates a non-significant ANOVA, but with $p < 0.05$ between the control group and the indicated group on LSD post hoc testing.

Fig. 6.

Effect of mitochondrial lysates on neuronal cell NFκB protein levels and compartmentalization. SH-SY5Y cells were treated overnight with PBS vehicle, 50 μg of mitochondrial lysate from SH-SY5Y cells, or 50 μg of mitochondrial lysate from SH-SY5Y p0 cells. Ten to twelve independent samples were prepared and analysed for each condition. (A) Total cell NFκB protein. (B) Total cell IκBα protein. (C) Representative whole cell immunoblot data. (D) Cytosolic NFκB protein. (E) Nuclear NFκB protein. (F) Nucleus:cytosol NFκB protein ratio. (G) Representative nucleus and cytosol immunoblot data. Values shown are relative group means ± SEM, with the control group set to 1. * indicates $p < 0.05$; # indicates a non-significant ANOVA, but with $p < 0.05$ between the control group and the indicated group on LSD post hoc testing.

Fig. 7.

Effect of mitochondrial lysates on neuronal cell AβPP mRNA and protein levels. SH-SY5Y cells were treated overnight with PBS vehicle, 50 μg of mitochondrial lysate from SH-SY5Y cells, or 50 μg of mitochondrial lysate from SH-SY5Y ρ0 cells. Ten to twelve independent samples were prepared and analysed for each condition. (A) AβPP mRNA levels. (B) AβPP protein levels. (C) Representative immunoblot data. Values shown are relative group means ± SEM, with the control group set to 1. * indicates $p < 0.05$; ** indicates $p < 0.005$.

Fig. 8.

Effect of mtDNA on microglial cell TNF α expression. (A) Long PCR of mtDNA. The band shown is approximately 15kb in size. Neg=negative control PCR. (B) BV2 cells were treated overnight with either 1 μ g of native enriched mtDNA from BV2 cells or 1 μ g of PCR-amplified mtDNA. (C) BV2 cells were treated overnight with either 2 μ g of native enriched mtDNA from BV2 cells or 2 μ g of PCR-amplified mtDNA. In (B) and (C), twelve independent samples were prepared and analysed for each condition. Values shown are relative group means $\pm$ SEM, with the control group set to 1. * indicates $p < 0.05$.