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A transient increase in lipid peroxidation primes preadipocytes for delayed mitochondrial inner membrane permeabilization and ATP depletion during prolonged exposure to fatty acids

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Abstract

Preadipocytes are periodically subjected to fatty acid (FA) concentrations that are potentially cytotoxic. We tested the hypothesis that prolonged exposure of preadipocytes of human origin to a physiologically relevant mix of FAs leads to mitochondrial inner membrane (MIM) permeabilization and ultimately to mitochondrial crisis. We found that exposure of preadipocytes to FAs led to progressive cyclosporin A-sensitive MIM permeabilization, which in turn caused reduction in MIM potential ($\Delta\Psi_M$), oxygen consumption, ATP synthetic capacity, and ultimately death. Additionally, we showed that FAs induce a transient increase in intramitochondrial ROS and lipid peroxide production lasting roughly 30 and 120 minutes for the ROS and lipid peroxides, respectively. MIM permeabilization and its deleterious consequences including mitochondrial crisis and cell death were prevented by treating the cells with the mitochondrial FA uptake inhibitor, Etomoxir; the mitochondrion selective superoxide and lipid peroxide antioxidants, MitoTempo and MitoQ; or the lipid peroxide and reactive carbonyl scavenger, L-carnosine. FAs also promoted a delayed oxidative stress phase. However, since the beneficial effects of Etomoxir, MitoTempo and L-carnosine were lost by delaying the treatment by 2 hours, it suggested that the initial phase was sufficient to prime the cells for the delayed MIM permeabilization and mitochondrial crisis. It also suggested that the second ROS production phase is a consequence of this loss in mitochondrial health. Altogether, our data suggest that approaches designed to diminish intramitochondrial ROS or lipid peroxide accumulation as well as MIM permeabilization, are valid mechanism-based therapeutic avenues to prevent the loss in preadipocyte metabolic fitness associated with prolonged exposure to elevated FA levels.

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Keywords

Preadipocytes; Mitochondria; fatty acids; Bioenergetic; respiration; superoxide; H₂O₂; Lipid peroxidation; Mitochondrial inner membrane permeability; Mitochondrial inner membrane potential; ATP

Introduction

Adipose tissue, once believed to be immune to the cytotoxic effects of fatty acids (FAs), is now recognized to be among the first organs to experience lipotoxicity during the course of high-fat diet-induced loss in metabolic fitness [Strissel, 2007 #159; Alkhoury, 2010 #162; Wueest, 2010 #163]. At the cellular level, metabolic fitness is largely contingent on the mitochondrial ability to match nutrient availability to the energy required to perform cellular processes [Patti, 2010 #383]. Loss of this matching efficiency or bioenergetic fading has been well described in skeletal muscle in states of obesity, insulin resistance and type 2 diabetes [1–3]. With respect to human adipose tissue, studies have shown a decline in oxygen consumption with age and obesity, and documented decreased expression levels of genes implicated in fatty acid oxidation as well as oxidative phosphorylation with obesity, insulin-resistance and type 2 diabetes [4–7]. The cause of mitochondrial dysfunction in adipose tissue, other than possibly a reduced mitochondrial mass, is not known. Although FA-induced mitochondrial dysfunction has been reported in differentiated 3T3-L1, to date, there is no study addressing the mechanisms behind this loss of functionality [8, 9].

Because preadipocytes exhibit a low FA esterification rate it is reasonable to speculate that, like such cells, they would experience some reductive pressure upon nutrient overload [10, 11]. Reductive pressure due to accumulation of NADH and FADH₂ above what is needed for ATP turnover has been proposed to fuel superoxide-derived mitochondrial H₂O₂ emission [12]. However, H₂O₂ emission should be kept minimal via the concerted action of mitochondrial H₂O₂ buffering systems centered on the thioredoxin reductase 2/thioredoxin 2/peroxiredoxin 3, and the glutathione reductase/glutathione/glutathione peroxidase 1 systems [13, 14]. Another of these antioxidant systems is cardiolipin-bound cytochrome c, which uses cardiolipin as a reductant to perform its peroxidase activity [15]. By doing so, it oxidizes cardiolipin, which has been proposed to disturb the proper assembly of the electron transport chain, to lead to the release of proapoptotic factors including cytochrome c itself and to formation/activation of the mitochondrial permeability transition pore (mPTP) [15–17]. In addition, H₂O₂ molecules escaping the buffering systems, after being further oxidized to hydroxyl radicals react with various mitochondrial components, in particular MIM unsaturated FAs initiating a lipid peroxidation cascade that ultimately leads to the generation of reactive aldehydes [18]. Because aldehydes lead to carbonylation of many mitochondrial enzymes, permanently altering their activities, it has been proposed to be the mechanism underlying how high-fat diets alter mitochondrial function in adipocytes [19]. Finally, H₂O₂ itself can alter the activity of redox-sensitive proteins, which include almost all electron transport chain components, the antioxidant systems, and the protein involved in MIM permeabilization and cell death pathways [20, 21].

It has been shown that high-fat diet leads to substantial aldehyde production and protein carbonylation in adipose tissue [22]. It has also been shown that protein carbonylation is elevated in obesity in both mice and humans [23, 24]. Since mPTP formation/activity can be modulated by redox regulation and lipid peroxides, and since cyclophilin D has been demonstrated to be involved in high-fat diet-induced cell death in adipose tissue [25], we sought to test whether prolonged exposure of preadipocytes to FAs can cause a progressive MIM permeabilization and mitochondrial dysfunction. We found that mitochondrial uptake

of FAs, superoxide production and lipid peroxidation causes a delayed MIM permeabilization and that, in turn, MIM permeabilization causes a loss of ATP synthetic capacity and ultimately cell death. This is potentially of clinical importance as losses of preadipocyte adipogenic potential as well as death would exacerbate the adipose tissue dysfunctions that have been repeatedly associated with human obesity.

Materials and Methods

Cell culture and transfections

We performed all our experiments using the conditionally immortalized XA15A1 human preadipocytes. These cells were created by Lonza (Walkersville, MD) by stably transfecting primary subcutaneous preadipocytes from an insulin-sensitive donor with a temperature-sensitive large T-antigen. These cells were propagated at 33°C and reverted to their normal phenotype at 37°C for 24 hours. Dulbecco's modified Eagle's medium:F12 (1:1) containing 10% fetal bovine serum (Thermo Scientific HyClone, Logan, UT), 10 mM glucose, 1 mM pyruvate, 33 μ M Biotin, 17 μ M D-Pantothenate, 1 μ M epidermal growth factor, 1 μ M basic fibroblast growth factor, 100 nM insulin, and 10 nM hydrocortisone was used for propagation (growth factors were from Gemini Bio-Products, West Sacramento, CA; chemicals from Sigma-Aldrich, St-Louis, MO). This medium without the added growth factors, insulin and hydrocortisone was used when cells were moved to 37°C. This medium, unless stated otherwise, was replaced on the day of the experiments by DMEM containing 5% of calf serum, 25 mM Hepes, reduced level of pyruvate (0.5 mM), and 600 μ M FA-free BSA (Gemini Bio-Products). For some experiments, XA15A1 cells were transiently transfected with pHyPer Mito or pHyPer Cyto plasmids (Evrogen, Moscow, Russia), which encode mitochondrion-targeted or cytosol-targeted H₂O₂-sensitive dual-excitation ratiometric reporter proteins. These reporters emit at a wavelength of 516 nm, but the excitation maximum switches from 420 to 500 when the protein H₂O₂-sensitive thiols are oxidized and a reversible disulfide bond is formed [26]. 5×10^6 cells were transfected in a 100 μ l final volume of the nucleofection solution V containing 1 μ g of plasmid DNA and the application of the T-030 program from the Amaxa Nucleofactor 2b electroporator (Lonza Walkersville) list. The transfected cells were used 48 hours after transfection.

Preparation of fatty acids

All 50 mM FA stock solutions were prepared in 100 mM NaOH and kept at -20°C. On the day of experiments, these stocks were used to prepare various 2X working solutions in the appropriate medium (DMEM for most and Krebs's ringer bicarbonate buffer for real-time flow cytometry) containing 5% of calf serum, 10 mM glucose, 25 mM Hepes, 0.5 mM pyruvate, and 600 μ M FA-free BSA. The solutions were mixed on an orbital shaker for 60 minutes at 37°C. The FA proportion of these mixes was oleic acid (40%), palmitic acid (25%), linoleic acid (20%) and stearic acid (15%). The FA to BSA ratio was increased from 0.33:1 to 1.67:1 when total FA concentrations were raised from 200 to 1000 μ M. Based on the binding characteristics of the various fatty acids with BSA, the total free FA concentrations of our mixture ranged from 3.6 to 11.2 nM with no individual fatty acid reaching concentrations above 5 nM [27]. As part of a study performed in parallel we found that albumin from this source did not induce the expression of death or proinflammatory mediators at the concentration used.

Determination of the number of dead cells

Live and dead cells were measured by calcein AM and ethidium homodimer-1 (Life technologies, Grand Island, NY). Calcein AM is a live cell dye that is non-fluorescent and accumulates in intact cells, where it acquires a strong green fluorescence upon cleavage by intracellular esterases [28, 29]. Because intracellular esterase activity is quickly lost as cells

die, little to no gain in fluorescence occurs in dead or dying cells. Ethidium homodimer-1 is a dye that enters damaged plasma membranes and interacts with nucleic acids [28]. As this interaction increases the red fluorescence of the dye by 40-Fold, an increase in fluorescence indicates dying or dead cells. At the end of the 6, 12 or 24 hour FA incubation periods, cells were collected and incubated for 15 minutes at room temperature in DPBS containing calcein AM and ethidium homodimer 1 at a final concentration of 0.1 μ M. To detect calcein AM fluorescence, we used an excitation wavelength of 488 and a 530 ± 30 nm emission filter, while for Ethidium Homodimer 1 fluorescence we used an excitation wavelength of 488 and a 675 ± 25 emission filter.

Determination of cellular ATP concentration

For this assay cells were plated in 48 well-plates. Following 24 hours of FA exposure, cells were washed twice with PBS and ATP was extracted by adding 200 μ l boiling water [30]. Following extraction, ATP levels were determined using a bioluminescence ATP determination kit (Life technologies) as described by the supplier. An Infinite 200 Pro Series plate reader was used to quantify the luminescence (Tecan, Morrisville, NC).

Cellular respiration Assay

20,000 preadipocytes in a medium without growth factors were seeded to each well of Seahorse XF24 plates (Seahorse Bioscience, MA) and grown for 24 hours at 37°C before treatment with various inhibitors or FAs. Four to six hours before the experiments, the media was changed to DMEM containing 5% calf serum, 25 mM Hepes, 10 mM glucose, 0.5 mM pyruvate, and 600 μ M FA-free BSA. An hour before FA treatments, inhibitors or their vehicle were added to the cells, and then FAs were added at various concentrations (0 – 1000 μ M) for 24 hours. At the end of the incubation, cells were washed two times with 1 ml of phosphate-buffered saline containing magnesium and calcium. Finally, 500 μ l of the medium described above, with the omission of serum, was added to the cells. Cellular respiration was measured as described previously [31], with the following slight modifications determined to be optimal in preliminary experiments. First, the measurement cycle consisted of 1 min mix, 1 min wait and three min measurement. Second, three basal rate measurements were taken, three after oligomycin injection, three after carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) injection and three after Rotenone injection. Drugs were delivered through the ports at final concentrations of 1 μ M for oligomycin, 500 nM for FCCP and 1 μ M for Rotenone. To calculate coupled respiration, oligomycin-resistant respiration was subtracted to basal respiration. To calculate uncoupled respiration, rotenone-resistant respiration was subtracted to oligomycin-resistant respiration. To calculate the respiratory reserve capacity basal respiration was subtracted to FCCP-elicited respiration. To account for the decrease in cell density we multiplied these oxygen consumption rates by the ratio of live cells (live cells in the control conditions/live cells in the presence of FA). Each of the three independent experiments was performed in triplicate.

Determination of mitochondrial ROS and H₂O₂, cytosolic H₂O₂ and MIM lipid peroxidation

Mitochondrion-localized steady state levels of ROS were evaluated using MitoSox Red (Invitrogen, Carlsbad, CA), a hydroethidine derived fluorescent probe coupled to the mitochondrion-targeting hexyl triphenylphosphonium cation that detects mitochondrial matrix ROS, essentially superoxide [32, 33]. MIM-localized lipid peroxidation was evaluated using 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-(propionylaminoethyltriphenylphosphonium) bromide (MitoPerOx), a fluorescent probe derived from C11-Bodipy581/591 [34]. However, because the entrance of these probes into mitochondria is dictated by the Nernst equilibrium, it is very likely that their oxidation is underestimated when mitochondria are depolarized. In end-point experiments, cells were

loaded with 4 μM MitoSox Red or 100 nM of MitoPerOx for 30 minutes in the dark at 37°C. After loading cells were washed in DPBS and collected through repeated aspirations and ejections using TrypLE cell dissociation buffer (Invitrogen) and suspended in DPBS. MitoSox red fluorescence was measured using an Accuri C6 flow cytometer (BD Accuri cytometers, Ann Arbor, MI) at an excitation wavelength of 488 and a 585 ± 40 nm emission filter. In some experiments, we performed continuous real-time measurements of MitoSox (Fig 4A and 4B) and MitoPerOx (Fig 4G and 4H) fluorescence in cells pre-loaded with the dye than exposed to the FAs. This method was developed in our laboratory and is an adaptation of a recently published continuous real-time calcium measurement method [35]. For this, we conceived a simple system that consisted of an open 30 ml polypropylene receptacle surrounded by a water coil jacket supplied by a heated-circulating water bath set to maintain the temperature at 37°C. This receptacle sited on a stir plate set at 400 rpm, which allowed a rapid dispersion of added FAs or drugs without interruption as well as maintained a homogeneous cell sampling. For all experiments, cells were counted and suspended in flow buffer which consisted of Krebs's ringer bicarbonate buffer (Sigma-Aldrich) containing 5% calf serum, 25 mM Hepes, 10 mM glucose, 0.5 mM pyruvate and 600 μM BSA to obtain a final concentration of about 400,000 cells in a total volume of 14 mL. The flow rate of the pump was adjusted to analyze fluorescence levels from 2,000 cells per minute after the addition of test compounds. In the experiments depicted in Fig 4C we added the MitoSox in the window of time indicated. With respect to the cells transfected with the pHYPER plasmids, although the pHyPer proteins are dual-excitation ratiometric H_2O_2 sensors, since the Accuri only has one of the two required lasers we only quantified the oxidized form of these proteins using the excitation laser wavelength of 488 coupled to a 535 ± 30 nm emission filter.

Mitochondrial inner membrane potential measurement

Changes in MIM potential ($\Delta\Psi_{\text{M}}$) were measured by use of the lipophilic cationic dye tetramethylrhodamine ethyl (TMRE) ester (Sigma-Aldrich Co) that accumulates in direct proportion to the mitochondrial inner membrane potential in a Nernstian fashion [36]. Samples were incubated with 50 nM TMRE in DPBS for 20 minutes at 37°C. TMRE was measured using an excitation wavelength of 488 and a 585 ± 40 nm emission filter. Mean fluorescence was determined by histogram plots of TMRE fluorescence after the subtraction of auto fluorescence.

Evaluation of mitochondrial inner membrane permeabilization

As mentioned in our description of the use of Calcein AM for the identification of live cells, Calcein AM enters cells and has access to every compartment. Cobalt is known to quench Calcein fluorescence, but since cobalt does not freely enter mitochondria, Calcein AM fluorescence within the mitochondria is not quenched unless there is an increase in membrane permeability [37]. Briefly, cells are incubated with Calcein AM at a final concentration of 0.1 μM in DPBS for 30 minutes followed by 200 μM CoCl_2 for 15 minutes. Calcein AM fluorescence was measured using an excitation wavelength of 488 and a 535 ± 30 nm emission filter. The percent of cells experiencing MIM permeabilization, meaning that the high calcein fluorescence was quenched by cobalt, was determined using the histogram plots of Calcein AM.

Results

The concentration of FAs in the venous drainage of subcutaneous adipose tissue oscillates between postabsorptive/fasting concentrations of more than 1000 μM and postprandial concentrations that can be as low as 200 μM [38]. In certain physiological conditions, for example prolonged fasting, FA levels can be maintained at the upper end of the 200–1000

μM range for more than 24 hours [39]. With these considerations in mind, we incubated human preadipocytes for up to 24 hours in the presence of increasing concentrations of FAs to determine their effects on various parameters of mitochondrial and cellular fitness. No differences were observed when comparing cells incubated with BSA or 200 μM FAs for any of the variables throughout the execution of this work. Therefore, although interchangeable with BSA alone, the 200 μM FA values are presented as the control condition for many of the experiments. In those instances, statistics were also performed using the 200 μM FA values as basal values. All experiments were conducted using a FA composition which portrays the natural FA composition consisting of 40% oleic acid, 25% palmitic acid, 20% linoleic acid and 15% stearic acid [40]. At these proportions the free FA concentration of oleic acid ranged from 0.83 to 4.51 nM, palmitic acid from 0.98 to 2.17 nM, linoleic acid from 2.07 to 4.01 and stearic acid from 0.14 to 0.46 nM.

Prolonged exposure to elevated fatty acid concentrations causes cell death and ATP depletion in preadipocytes

We first assessed whether FAs lead to the ultimate cytotoxic outcome, which is death. As shown in figure 1A, incubation of preadipocytes for 6, 12 or 24 hours with increasing concentrations of FAs revealed that exposures of 12 hours or less were not associated with cell death. However, incubation with 800 μM FAs or above, for 24 hours caused an at least a 7-fold increase in cell death as evaluated by flow cytometric measurement of the percent of cells having lost both cellular esterase activity and membrane permeability. To assess if it takes more than 12 hours of continuous exposure to FAs to induce preadipocyte death, or if it takes 24 hours to complete a process already established within the initial 6 or 12 hours, we replaced the medium of cells exposed to FAs for 6 or 12 hours with FA-free medium for the rest of the 24 hour exposure period. As shown in figure 1A, replacing the media prevented cell death (Fig 1A). As an alternative index of cellular vitality, we measured cellular ATP levels in preadipocytes incubated 6, 12 or 24 hours with increasing concentrations of FAs (Fig 1B). ATP levels were not decreased significantly after 6 or 12 hours of exposure to FAs, but were reduced by 48% after a 24 hour exposure to 600 μM FAs and by at least 74% at the concentrations that caused cell death. This disproportional decrease in ATP concentration compared to the loss in cell viability suggested the possibility that prolonged exposure to FAs induced mitochondrial dysfunction.

Prolonged exposure to elevated fatty acid concentrations causes mitochondrial dysfunction in preadipocytes

To test whether FAs induce mitochondrial dysfunction in human preadipocytes, we used cellular respirometry to quantify changes in oxygen consumption rates [41]. As shown in figure 2A, basal respiration using 10 mM glucose and 1 mM pyruvate as substrates was decreased following 24 hours exposure to 1000 μM FAs ($p < 0.01$). Admittedly, this apparent decrease in respiratory rate is an overestimation since cell death occurred during the incubation. However, when accounting for cell death, coupled respiration, which is the portion of respiration coupled to ATP turnover, was reduced by 55% ($p < 0.05$). Maximal respiratory capacity, which was evaluated by the addition of 500 nM of the protonophore FCCP, was decreased after 24 hours exposure to 800 or 1000 μM FAs, respectively (Fig 2A). Taking into consideration cell death, respiratory reserve capacity, which is an approximation of how much respiration can be increased in the context of a given substrate availability, was reduced by 31% ($p < 0.05$) or 34% ($p < 0.01$) after exposure to 800 or 1000 μM FAs, respectively (Fig. 2B). Uncoupled respiration, or the oligomycin-insensitive mitochondrial respiration, was unaffected (Fig. 2A and 2B). To test the possibility that these mitochondrial dysfunctions were the consequence of fatty acid uptake into mitochondria; we pretreated the cells with 10 μM of the carnitine palmitoyltransferase-1 inhibitor etomoxir for 10 minutes prior to the addition of FAs. As shown in figure 2C and 2D, none of the

respiratory rates were affected by FAs in the absence of mitochondrial FA oxidation. Etomoxir completely prevented FA-induced ATP depletion (Fig 2E) and inhibited FA-induced cell death by 83% (Fig 2F).

Prolonged exposure to elevated fatty acid concentrations causes oxidative stress in preadipocytes

Mitochondrial dysfunction can be caused or be the cause of oxidative stress. We first investigated the effects of prolonged exposure to FAs on the propensity of mitochondria to accumulate ROS (Fig. 3A to 3E). In this series of experiments we incubated the cells 3, 12 or 24 hours with FAs and labeled them with MitoSox, a mitochondrial matrix-selective probe that acquires a strong red fluorescence when oxidized [32]. As MitoSox relies on intact mitochondrial membrane potential to accumulate within the matrix, MitoSox red oxidation was likely underestimated at the 24 hour time point. We also measured in real-time the accumulation of MitoSox red fluorescence in the presence of FAs, which will be presented as part of figure 4. As seen in figures 3A to 3D, no significant increase in MitoSox red fluorescence was achieved in cells incubated 12 hours or less with FAs. However, at the 24 hour time point increases in MitoSox fluorescence were significant with FA concentrations of 600 μ M and above. Incubation of the cells with Etomoxir prior to the addition of FAs completely prevented the increases in the mitochondria propensity to accumulate ROS (Fig. 3E). This indicates that FA uptake by the mitochondria is required for this manifestation of the cytotoxic effects of FAs to take place.

FAs induce transient phases of intramitochondrial ROS and MIM lipid peroxidation that are sufficient for FA-induced cell death

To study the more proximal effects of FAs, we adapted a recently described continuous real-time flow cytometry approach [35]. In these experiments superoxide levels were measured in real-time after the addition of FAs. The addition of FAs led to rapid increases in MitoSox fluorescence that were maximal approximately 5 minutes after the addition of FAs and that returned to nadir within 30 minutes (Fig 4A). These increases in mitochondrial matrix ROS were proportional to the concentration of FAs, but the time to nadir was about the same (Fig 4A and 4B). The ability of Etomoxir to decrease the MitoSox fluorescence induced by 1000 μ M FAs by 89% suggests that the increased steady state level of ROS was a consequence of FA uptake by mitochondria (Fig 4B). The 83% prevention of FA-induced ROS accumulation by 10 μ M MitoTempo, a mitochondrion selective agent that prevents superoxide and lipid peroxide accumulation, confirmed that the mitochondrial matrix was the site of ROS accumulation (Fig 4B). Incubation with 10 mM L-carnosine, a dipeptide with lipid peroxide scavenging activity [42], did not prevent the increase in MitoSox fluorescence. As an alternative approach, a series of experiments were performed in which MitoSox was added in 30 minute windows of time. These experiments confirmed that steady state ROS levels were increased in the first 30 minutes of exposure to FAs (Fig 4C). Etomoxir and MitoTempo prevented the increase in ROS accumulation promoted by 1000 μ M FAs using this approach as well. Etomoxir and MitoTempo were used throughout the remainder of the study to assess the role of superoxide or superoxide derived ROS and lipid peroxides on the various FA-induced mitochondrial dysfunctions as well as cell death.

To assess the levels of mitochondrial and cytosolic H_2O_2 , we transiently transfected our cells with plasmids encoding for mitochondrion-targeted or the cytosol-targeted HYPER proteins [26]. In these cells, FAs induced concentration-dependent increases in mitochondrial H_2O_2 levels that were prevented by Etomoxir or MitoTempo, which was to be expected as H_2O_2 is the product of the dismutation of superoxide (Fig 4D). Figure 4E illustrates that the increase in H_2O_2 was transient and lasted about 30 minutes. However, no increase in cytosolic H_2O_2 levels could be documented (Fig 7F). Altogether, with the data

presented in figure 3, these findings suggest that although the delayed ROS production phase reached the cytosol, the transient increase in ROS was confined to the mitochondrial matrix.

We evaluated MIM lipid peroxidation with cells loaded with MitoPerOx, a novel probe that is targeted to the mitochondrial matrix and accumulates in the MIM [34]. Although the initial series of experiments showed that the probe was getting oxidized during the first 30 minutes of FA exposure, it was clear that lipid peroxidation was still actively occurring at the end of the experiments (Fig 4G). To determine the duration of lipid peroxidation phase we incubated the cells longer and found that FA oxidation reached a plateau between 120 and 150 minutes (Fig 4H). Figure 7H also shows that MitoTempo and L-carnitine prevented the increase in lipid peroxidation caused by 1000 μ M FAs by 76% and 85%, respectively.

Finally, because the initial phase in ROS and lipid peroxidation was completed within 2 hours, we compared the efficacy of Etomoxir, MitoTempo, and L-carnosine to prevent cell death when added before or 2 hours after the addition of FAs. Figure 4I illustrates that pretreatment of the cells with Etomoxir, MitoTempo or L-carnosine prevented cell death, but that adding them 2 hours after FAs did not prevent FA-induced cell death. Altogether, these results suggest that although up to 24 hours of continuous exposure to FAs is required for mitochondrial crisis and cell death, the transient increase in lipid peroxidation, or the transient increases in ROS production and lipid peroxidation, participate(s) in an essential priming step(s).

Mitochondrial superoxide production or lipid peroxidation is the trigger of FA-induced mitochondrial dysfunction, ATP depletion and cell death

To assess the role of mitochondrial ROS and/or lipid peroxides caused the mitochondrial dysfunction described in figures 1 to 3, we incubated the cells with Mito Tempo, 1 nM MitoQ (an alternative mitochondrion-targeted superoxide and lipid peroxide scavenger), or L-carnosine. As shown in figure 5, MitoTempo (Fig. 5A), MitoQ and L-carnosine (Fig 5B) all abrogated the increase in MitoSox red fluorescence induced by 1000 μ M FAs. Scavenging superoxide and lipid peroxides prevented a delayed secondary phase of MIM lipid peroxidation (Fig. 5C). Finally, MitoTempo, MitoQ, and L-carnosine prevented ATP depletion (Fig. 5D).

Prolonged exposure to elevated fatty acid concentrations causes depolarization of the mitochondrial inner membrane

Mitochondrial ATP synthesis is dependent on the proton motive force (Δp) of which $\Delta \Psi_M$ is a major component. Since we have established that prolonged exposure to high concentration of FAs induces mitochondrial dysfunction and leads to decreased cellular ATP levels, we wanted to determine the effects of FAs on $\Delta \Psi_M$ (Fig. 6). The results of these experiments show that it took more than 12 hours of exposure to high concentrations of FAs to cause any significant decrease in $\Delta \Psi_M$ (Fig 6A to 6D). This MIM depolarization was prevented by preincubating the preadipocytes with Etomoxir and MitoTempo (Fig 6E) as well as MitoQ and L-carnosine (Fig 6F).

The permeability transition pore is necessary to FA-induced mitochondrial dysfunction, ATP depletion and cell death

Mitochondrial bioenergetics and $\Delta \Psi_M$ rely on an intact inner membrane barrier. One way that FAs could lead to mitochondrial crisis and ATP depletion is through the collapse of this barrier. To test if FA exposure induces activation of the mPTP prior to loss of mitochondrial respiratory function, MIM permeabilization was monitored using a flow-cytometric measurement of calcein AM fluorescence. As shown in figure 7A, 7B, 7C, and 7D, the

percent of cells that experienced MIM permeabilization was already increased by 2.5-fold in the first 12 hours of FA exposure. Also noteworthy is the increase in the percent of cells having MIM permeabilization was, like the decrease in cellular ATP, already significant at 600 μM FAs. Cyclosporin A, a cyclophilin D inhibitor, prevented FA-induced MIM permeabilization by 80% (Fig. 7E). Preincubation with Cyclosporin A also prevented the decrease in $\Delta\Psi_{\text{M}}$ induced by 1000 μM FAs by 84% (Fig. 7F), prevented mitochondrial ROS accumulation (Fig. 7G), totally prevented the decrease in respiratory capacities (Fig. 7H), the decrease in cellular ATP by 79% (Fig. 7I), and cell death by 86% (Fig. 7J). As an alternative approach to inhibit mPTP activation, we preincubated the cells with 10 μM bongkreikic acid, a drug that stabilizes adenine nucleotide translocase in its *m* conformation which does not participate in MIM permeabilization. Bongkreikic acid prevented cell death by 92% as well (Fig. 7J). Altogether the combined evidence suggests that mitochondrial FA uptake and mitochondrial ROS-dependent formation of the mPTP and MIM permeabilization is the underlying cause of the decrease in $\Delta\Psi_{\text{M}}$, loss in ATP synthetic capacity and ultimately cell death.

Discussion

The present study was to characterize the consequences of prolonged exposure to FAs in preadipocytes. Our main conclusion is that transient lipid peroxidation primes for delayed MIM permeabilization, ATP depletion and cell death. This conclusion is based on the combined evidence that the steady state levels of ROS and lipid peroxide were increased in a biphasic fashion, and that prevention of the initial and transient, not the second and delayed phase, abrogated every consequence of prolonged exposure to FAs including cell death. Of all the indices of mitochondrial dysfunction measured, MIM permeabilization was the only one significantly altered in the first 12 hours of incubation. In combination with our results showing that preincubation of the preadipocytes with cyclosporin A prevented every subsequent consequence of exposure to FAs, it suggests that mPTP activation and MIM permeabilization are central to the completion of a lipid peroxide- or aldehyde-primed cell death program.

There are multiple possible explanations for the transient nature of the first ROS and lipid peroxidation phase. First, with respect to superoxide production, preadipocytes have a relatively low esterification rate compared to adipocytes, but the ATP demand associated with the channeling of fatty acids toward triacylglycerol synthesis may still be sufficient to dissipate the reducing pressure [21, 43, 44]. Another possibility is that fatty acids either directly, or via the formation of superoxide or lipid peroxides, increase uncoupling through various mechanisms [45–48]. Elevations in the matrix levels of H_2O_2 also lasted about 30 minutes after the addition of FAs. This is because H_2O_2 accumulation in mitochondria and in cells is normally kept at bay through the concerted action of numerous mitochondrial H_2O_2 buffering systems [13, 14]. *In vitro* studies performed in optimal conditions in isolated mitochondria have established that the maximal rate of H_2O_2 production in the presence of FAs is relatively modest compared to the H_2O_2 dissipating capacity [49, 50]. This makes sense as the H_2O_2 buffering capacity of mitochondria is strongly dependent on mitochondrial NADPH synthesis, which relies on the proton motive force [51]. Therefore, although the H_2O_2 emission capacity of isolated mitochondria has been suggested to be associated with the onset of many metabolic abnormalities [12, 52], it is not too surprising that we could not measure any cytosolic H_2O_2 accumulation in intact preadipocytes. The active phase of lipid peroxidation lasted 2 hours after the addition of FAs. It is important to remember that lipid peroxidation is self propagated and largely self terminated [53]. Altogether, it may explain why adding MitoTempo, MitoQ and L-carnosine after 2 hours had no beneficial effects.

Another finding of our study is that intramitochondrial ROS are the most proximal mediators of FA-induced cytotoxicity and that the delayed ROS production phase appears to be dispensable. Oxidative stress has been described during high-fat diet-induced obesity in mice and in obesity in humans. It has also been described in differentiated 3T3-L1 exposed to FAs for periods ranging from 1 to 7 days though the use of H₂DCF fluorescence reading [8, 9, 22–24, 54–57]. Whether mitochondria are the primary source of ROS, or if there is a hierarchical relationship between mitochondrial ROS production and extramitochondrial sites has not been fully evaluated. Nevertheless, studies in which the investigators have inhibited NADPH oxidases or the toll-like receptor (TLR) 2 or/and 4 have independently suggested extramitochondrial sources of ROS following exposure to FAs [55–57]. This may not be at odds with our data showing that 3 different mitochondrion-targeted drugs prevented oxidative stress and FA-induced cytotoxicity in preadipocytes as there is the possibility that intramitochondrial ROS and lipid peroxides are proximal to TLR2/4- and/or NADPH oxidase-dependent ROS production.

We have established that a lipid peroxidation-dependant priming of the mPTP is central to FA-induced cytotoxicity. With respect to the role of the mPTP and MIM permeabilization, a recent publication of Feng *et al.*, demonstrated that cyclophilin D is required for high-fat diet-induced adipose tissue cell death *in vivo* [25]. The mPTP is a multiprotein complex involved in a myriad of physiological functions, most notably the modulation of mitochondrial matrix calcium levels and adenylate exchanges between the matrix and the intermembrane space, two functions central to mitochondrial bioenergetics [58]. Interestingly, the mPTP has also been shown to be involved in FA-induced uncoupling in adipocytes and as such would have a protective role against FA-induced superoxide and lipid peroxide production [48]. Unfortunately, transition from the physiological transient and low conductance mode to the pathological permanent and high conductance mode leads to the pro-apoptotic and pro-necrotic roles of the mPTP [59–61]. The role of lipid peroxidation and protein carbonylation in the process of mPTP activation and cell death has been described [17, 62]. Our results suggest that in preadipocytes exposed to FAs, there is a two step process, as lipid peroxidation-dependent priming step that occurred in the initial 2 hours, and a delayed execution step identifying MIM permeabilization as essential, but for which we do not have yet a definitive mechanism. The study on how the primed mPTP intersect with various cell death programs is currently underway.

Our results illustrate that FA-induced MIM permeabilization leads to decreased coupled respiration and mitochondrial crisis. Decreased coupled respiration is mostly attributed to decreased ATP demand [63]. However, decreased respiration can also be caused by decreased substrate availability, decreased substrate oxidation, decreased ETC electron transport efficiency, or collapsed proton gradient due to increased MIM permeability [63]. Because the $\Delta\Psi_M$ was decreased at concentrations of FAs associated with decreased respiratory capacity and that cyclosporin A prevented both the decrease in $\Delta\Psi_M$ and reduced respiratory capacities, mPTP activation and MIM permeabilization are clearly a mechanism involved in these processes.

A few studies have addressed some aspects of FA-induced mitochondrial dysfunction in mature adipocytes, none was performed in preadipocytes. First and foremost, an early study established that oxygen consumption is reduced in adipocytes isolated from obese individuals compared to lean individuals [4]. In addition studies addressing the effects of nutrient overload on mitochondrial function performed in differentiated 3T3-L1 have shown that high concentrations of glucose (25 mM) and FAs (the free FA equivalent of 1000 to 2000 μ M total FAs, taking into consideration that they used 2% BSA) for 24 or 48 hours cause mitochondrial dysfunction including decreased mitochondrial content, decreased ATP levels, and increased ROS production [8, 9]. On the other hand the causal involvement of

ROS in inducing mitochondrial dysfunction was suggested by the study of Wang *et al.*, which showed that antioxidants improve respiration in adipocytes [64]. However, our study is the first to address the chronological or hierarchical relationships between FA-overload, increase in steady state ROS and lipid peroxide levels, and mitochondrial dysfunction in preadipocytes. Whether the mechanisms highlighted here are involved in the reduction in preadipocyte number and adipogenic potential that has been reported in human obesity remains to be established [65, 66]. In fact, whether these mechanisms are involved in other dysfunction of adipose tissue like ER stress and inflammation, two downstream consequences of oxidative stress (56, 67), have to be established as well. However, because our study demonstrates that mitochondrion-targeted antioxidants are efficacious at preventing the cytotoxic effects of prolonged FA exposures; *in vivo* experiments testing their efficacy at preventing adipose tissue dysfunctions and/or the onset obesity-associated metabolic abnormality are warranted.

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CR planned the experiments, collected and analyzed the data; BD provided technical assistance; EJA, PDN and MPM provided access to equipment/materials and revised the manuscript; JR conceived and designed the study, planned the experiments, collected and analyzed the data, and interpreted the data and wrote the manuscript. JR takes the full responsibility for the content of this manuscript.

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MPM holds patents and has financial interests in the development of mitochondria-targeted antioxidants and therapies.

Abbreviations

FA	fatty acid
FAs	fatty acids
MIM	mitochondrial inner membrane

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We studied the mitochondrial consequences of prolonged fatty acids in preadipocytes.

There were two phases of increased steady state levels of ROS and lipid peroxidation.

The delayed phase occurred as a consequence of inner membrane permeabilization.

The initial phase primed the cells to delayed inner membrane permeabilization.

Mitochondrion-targeted antioxidants prevented these effects of fatty acids.

Figure 1. ATP content and cell death in preadipocytes exposed to sustained elevation of FAs. (A) Preadipocytes were incubated 6, 12 or 24 hours with increasing concentrations of FAs (from white to black throughout the figures) and the percent of cells being dead was evaluated by flow cytometry. The cells in the experiments labeled 6 + 18 and 12 + 12 were incubated for a total of 24 hours, but their media were replaced at the indicated time for media without FAs. (B) Preadipocytes were incubated 6, 12 or 24 hours with increasing concentrations of FAs and ATP was measured using bioluminescence. Results are expressed as mean $\pm$ SD of three independent experiments (performed in triplicates). Results of each dose-response were analyzed by one-way ANOVA on repeated measures with Bonferroni's as post hoc test. *** indicates $P < 0.001$ compared to BSA as the (basal) condition.

Figure 2.

Mitochondrial dysfunction, ATP depletion and cell death in preadipocytes exposed to sustained elevation of FAs in the presence or absence of the carnitine palmitoyltransferase-1 inhibitor Etomoxir. (A to D) Preadipocytes were incubated 24 hours with increasing concentrations of FAs and the mitochondrial function was evaluated by respirometry. (A and C) At the end of the 24 hour incubation with FAs, basal, oligomycin-sensitive, FCCP-elicited and rotenone-sensitive respiration were measured. (B and D) Coupled and uncoupled respirations as well as respiratory reserve capacity were calculated from the data presented in A and C taking into consideration the decrease in live cells presented in figure 1A and 2F, respectively. Cells were incubated with FAs following (C and D), or not (A and B), a 10 minute preincubation of with 10 μ M Etomoxir. (E) 10 μ M Etomoxir prevented ATP depletion induced by 24 hour exposure to 1000 μ M FAs. (F) 10 μ M Etomoxir prevented cell death induced by 24 hour exposure to 1000 μ M FAs. Results are expressed as mean $\pm$ SD of three independent experiments. Results presented in A to D were analyzed by two-way ANOVA on repeated measures with Bonferroni's as post hoc test. Results presented in E were analyzed by two-way ANOVA with Bonferroni's as post hoc test. Results presented in F were analyzed by one-way ANOVA on repeated measures. For presentation simplicity, although preincubation with 800 μ M of FAs decreased FCCP-elicited respiration significantly ($P = 0.001$) and that 1000 μ M decreased both basal and FCCP-elicited respiration significantly ($P = 0.01$ and $P = 0.001$ respectively), no symbol was added to figures A and C. **, *** indicates $P = 0.01$ and $P = 0.001$ respectively compared to basal. +++ indicate $P = 0.001$ compared to control respectively.

Figure 3.

Delayed increase in mitochondrial matrix ROS levels in preadipocytes exposed to sustained elevation of FAs in the absence or presence of Etomoxir. (A to E) Preadipocytes were incubated 3, 12 or 24 hours with various concentrations of FAs and mitochondrial superoxide levels were evaluated by flow-cytometric measurement of MitoSox red fluorescence. Representative histograms of MitoSox red fluorescence of cells incubated 3 (A), 12 (B) or 24 (C) hours with (dashed lines) or without (full lines) 1000 μ M FAs. (D) MitoSox red fluorescence of cells incubated 3, 12, or 24 hours with increasing concentrations of FAs. (E) MitoSox red fluorescence of cells incubated with 10 μ M Etomoxir for 10 minutes, then 24 hours with 1000 μ M FAs. Results are expressed as mean $\pm$ SD of three independent experiments. Data presented in D were analyzed by independently by one-way ANOVA on repeated measures. Results in E were analyzed by two-way ANOVA. *, *** indicate $P = 0.05$ and $P = 0.001$ compared to basal, respectively, and +++ indicates $P = 0.001$ compared to control.

Figure 4.

Steady state levels of mitochondrial ROS, mitochondrial and cytosolic H₂O₂, MIM lipid peroxidation and cell death in preadipocytes exposed to sustained elevation of FAs in the presence or absence of MitoTempo or MitoQ, two antioxidants that prevent superoxide and lipid peroxide accumulation as well as L-carnosine, a lipid peroxide and aldehyde scavenger. (A) Representative traces of preadipocytes loaded with MitoSox Red prior to be incubated with increasing concentrations of FAs. (B) Total area under the curve of MitoSox red fluorescence in cells exposed to increasing concentrations of FAs or pretreated with 10 μ M Etomoxir, 10 μ M MitoTempo, or 10 mM L-carnosine for 10 minutes, then with 1000 μ M FAs. (C) Area under the curve of MitoSox red fluorescence per 30 minute period of cells incubated with 1000 μ M FAs in the presence or absence of 10 μ M Etomoxir or 10 μ M MitoTempo. (D) Total area under the curve of pHyper-Mito fluorescence of cells incubated with increasing concentrations of FAs or pretreated with 10 μ M Etomoxir or 10 μ M MitoTempo, then with 1000 μ M FAs. (E) Area under the curve of pHyper-Mito fluorescence per block of 30 minutes of cells incubated with 1000 μ M FAs in the presence or absence of 10 μ M Etomoxir or 10 μ M MitoTempo. (F) Area under the curve of pHyper-Cyto fluorescence per 30 minute period of cells incubated with 1000 μ M FAs. (G) MitoPerOx fluorescence of cells incubated with increasing concentrations of FAs. (H) MitoPerOx fluorescence at the specified time of cells incubated with 1000 μ M FAs in the presence or absence of 10 μ M MitoTempo or 10 mM L-carnosine. (I) Cell death in cells treated 10 minutes before, or 2 hours after, with 10 μ M Etomoxir, 10 μ M MitoTempo or 10 mM L-carnosine while exposed to 1000 μ M FAs. Results are expressed as mean $\pm$ SD of

three independent experiments. Experiments depicted in A, D, and I were analyzed by one-way and experiments in C, E, F, and H were analyzed by two-way ANOVA. *, *** indicates $P \leq 0.05$ and $P \leq 0.001$ compared to basal, respectively. +, ++, +++ indicates $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ compared to control, respectively.

Figure 5.

Delayed ROS accumulation and lipid peroxidation phase in preadipocytes exposed to sustained elevation of FAs in the absence or presence of MitoTempo, MitoQ, and L-carnosine. (A) MitoSox red fluorescence of cells incubated with or without 10 μM MitoTempo for 10 minutes, then 24 hours with 1000 μM FAs. (B) MitoSox red fluorescence of cells incubated with or without 1 nM MitoQ or 10 mM L-carnosine for 10 minutes, then 24 hours with 1000 μM FAs. (C) Preadipocytes were incubated 3, 12 or 24 hours with 1000 μM FAs in the presence or absence of 10 μM MitoTempo or 10 mM L-carnosine and MitoPerOx fluorescence was measured. (D) ATP levels were determined in cells exposed to 1000 μM FAs in the presence or absence of 10 μM MitoTempo, 1 nM MitoQ or 10 mM L-carnosine. Results are expressed as mean ± SD of three independent experiments. Data presented in A, C and D were analyzed by two-way ANOVA. Data presented in B were analyzed by one way ANOVA. *** indicates $P < 0.001$ compared to basal. ++, +++ indicates $P < 0.01$ and $P < 0.001$ compared to control, respectively.

Figure 6.

MIM potential in preadipocytes exposed to sustained elevation of FAs in the absence or presence of Etomoxir, MitoTempo, MitoQ or L-carnosine. Preadipocytes were incubated 3, 12 or 24 with various FA concentrations and mitochondrial inner membrane potential was evaluated by flow-cytometric measurement of TMRE fluorescence. (A to C) Representative histograms of TMRE fluorescence of cells incubated 3 (A), 12 (B) or 24 (C) hours with (dashed lines) or without (full lines) 1000 μ M FAs. (D) TMRE fluorescence of cells incubated 3, 12, or 24 hours with increasing concentrations of FAs. (E) TMRE fluorescence of cells incubated 24 hours with 1000 μ M FAs in the presence or absence of 10 μ M Etomoxir or 10 μ M MitoTempo. (F) TMRE fluorescence of cells incubated 24 hours with 1000 μ M FAs in the presence or absence of 10 nM MitoQ or 10 mM L-carnosine. Results are expressed as mean $\pm$ SD of four independent experiments. Data presented in D were independently analyzed by one way ANOVA on repeated measures. Results in E were analyzed by two-way ANOVA and data presented in F analyzed by one-way ANOVA. **, *** indicates $P < 0.01$ and $P < 0.001$ respectively compared to basal. ++ indicates $P < 0.01$ compared to control.

Figure 7.

MIM permeabilization, MIM depolarization, mitochondrial matrix ROS accumulation, ATP depletion and cell death in preadipocytes exposed to sustained elevation of FAs in the absence or presence of the two mitochondrial permeability transition pore inhibitors. Preadipocytes were incubated 3, 12 or 24 with various concentrations of FAs and MIM permeability was evaluated by flow-cytometric measurement of calcein AM fluorescence. (A to C) Representative histograms of calcein fluorescence of cells incubated 3 (A), 12 (B) or 24 (C) hours with (dashed lines) or without (full lines) 1000 μ M FAs. (D) Percent of cell having quenched calcein AM fluorescence in cells incubated for 3, 12, or 24 hours with increasing concentrations of FAs. (E) Percent of cell having quenched calcein AM fluorescence in cells incubated 24 hours with 1000 μ M FAs in the presence or absence of 10 μ M Etomoxir, 10 μ M MitoTempo, 110 mM L-carnosine or 10 μ M cyclosporin A. (F) TMRE fluorescence of cells incubated 24 hours with 1000 μ M FAs in the presence or absence of 10 μ M Cyclosporine A. (G) MitoSox red fluorescence of cells incubated 24 hours with 1000 μ M FAs in the presence or absence of 10 μ M Cyclosporine A. (H) Respiratory capacities of cells incubated 24 hours with increasing concentration of FAs in the presence or absence of 10 μ M Cyclosporine A. (I) ATP levels evaluated in cells incubated 24 hours with 1000 μ M FAs in the presence or absence of 10 μ M Cyclosporine A. (J) Percent of dead cells in cells incubated 24 hours with 1000 μ M FAs in the presence or absence of 10 μ M Cyclosporine A or 100 μ M bongkreikic acid. Results are expressed as mean $\pm$ SD of three (A to E and G to J) or four (F) independent experiments. Data presented in D were independently analyzed by one-way ANOVA on repeated measures. Results presented in E, F and J were analyzed by two-way ANOVA. Data presented in H were analyzed by two-way ANOVA on repeated measures. *, **, *** indicates $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to basal, respectively. +, ++, +++ indicates $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to control, respectively.