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Aspirin Increases Mitochondrial Fatty Acid Oxidation

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Abstract

The metabolic effects of salicylates are poorly understood. This study investigated the effects of aspirin on fatty acid oxidation. Aspirin increased mitochondrial long-chain fatty acid oxidation, but inhibited peroxisomal fatty acid oxidation, in two different cell lines. Aspirin increased mitochondrial protein acetylation and was found to be a stronger acetylating agent *in vitro* than acetyl-CoA. However, aspirin-induced acetylation did not alter the activity of fatty acid oxidation proteins, and knocking out the mitochondrial deacetylase SIRT3 did not affect the induction of long-chain fatty acid oxidation by aspirin. Aspirin did not change oxidation of medium-chain fatty acids, which can freely traverse the mitochondrial membrane. Together, these data indicate that aspirin does not directly alter mitochondrial matrix fatty acid oxidation enzymes, but most likely exerts its effects at the level of long-chain fatty acid transport into mitochondria. The drive on mitochondrial fatty acid oxidation may be a compensatory response to altered mitochondrial morphology and inhibited electron transport chain function, both of which were observed after 24 hr incubation of cells with aspirin. These studies provide insight into the pathophysiology of Reye Syndrome, which is known to be triggered by aspirin ingestion in patients with fatty acid oxidation disorders.

Keywords

aspirin; fatty acid oxidation; mitochondria; peroxisomes; lysine acetylation; SIRT3

Introduction

Research into the therapeutic benefits of aspirin (acetylsalicylic acid) has been recently reignited by the observation that it may help prevent complex diseases such as diabetes and cancer [1–3]. Mechanisms invoked to explain the benefits of salicylates include increased mitochondrial biogenesis and increased mitochondrial energy metabolism via activation of key metabolic regulators such as AMP-activated protein kinase (AMPK), NF-Kb, and

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SIRT1, among others [4–8]. However, these findings contradict an older literature from the 1970s and 1980s that focused on the link between aspirin and Reye Syndrome. Several published papers from that era demonstrated that aspirin can uncouple mitochondria and inhibit mitochondrial fatty acid oxidation (FAO), among other negative effects [9–12]. This research led to the discontinuation of aspirin as an analgesic in children. Many Reye Syndrome patients were later found to be suffering from undiagnosed genetic disorders, particularly in the pathway of mitochondrial FAO, and aspirin usage was apparently a triggering factor in their metabolic decompensation rather than the root cause [13]. While aspirin use in children has declined sharply in recent decades, there are still instances of severe metabolic decompensation and even death in patients with undiagnosed metabolic disorders after ingestion of aspirin [14–16].

Given the resurging interest in aspirin and its potential for new clinical applications, resolving the contradiction between old and new literature regarding the effects of aspirin on mitochondrial fatty acid metabolism is important. Here, we demonstrate that aspirin increases FAO in two different cell lines. This increase is specific to the mitochondrial pathway, and is most likely the result of a compensatory shift from carbohydrate metabolism to FAO caused by fragmentation of the mitochondrial network and inhibition of the respiratory chain. The ramifications of these data for understanding Reye Syndrome and the use of aspirin to treat complex metabolic disorders such as diabetes and cancer are discussed.

Materials and Methods

Aspirin treatment

HEK293 cells, wildtype mouse embryonic fibroblasts (MEFs), and strain-matched SIRT3 knockout MEFs (a kind gift of Dr. Eric Verdin, Gladstone Institutes) were cultured in DMEM with 10% fetal bovine serum. Aspirin stock was made in ethanol and diluted into DMEM with control cells receiving ethanol only.

Chemical acetylation in vitro

Recombinant human long-chain acyl-CoA dehydrogenase (LCAD) was prepared as described [17] and, 1 μg aliquots were incubated in 25 mM KPO_4 buffer with 5 mM final concentration of either aspirin or acetyl-CoA (Sigma) for 1 hr at 37°C. The reactions were quenched with 100 mM Tris buffer.

Immunoblotting and immunoprecipitation

Rabbit anti-acetyllysine antibody (Cell Signaling Technology, Beverly, MA) was used at 1:2500. Rabbit anti-LCAD, anti-very long-chain acyl-CoA dehydrogenase (VLCAD), and anti-electron transferring flavoprotein (ETF) antisera (kind gifts of Dr. Jerry Vockley, Children's Hospital of Pittsburgh) were used at 1:2500. Note that the anti-ETF antiserum recognizes the α and β subunits of ETF, producing two bands upon immunoblot. For immunoprecipitation, Flag-tagged LCAD and VLCAD proteins were recovered from transiently transfected HEK293 cells using anti-Flag M2 antibody (Sigma). Fractionation of cells into mitochondria and cytosol was conducted exactly as described [18].

Cellular respiration

Cellular respiration was measured in an Oroboros Oxygraph-2K. The cells (1 million per chamber) were resuspended in Mir05 respiration media (110 mM sucrose; 0.5 mM EGTA; 3 mM $MgCl_2$; 60 mM K-lactobionate; 20 mM taurine; 10 mM KH_2PO_4; 20 mM HEPES, pH 7.2 and 1 mg/ml fatty acid free BSA) supplemented with freshly made digitonin solution to permeabilize the cell membrane. Then, malate (5 mM), pyruvate (5 mM), and ADP (5 mM) were added in rapid succession to stimulate State 3 respiration through Complex I of the respiratory chain [19]. Upon reaching steady-state, 10 mM succinate was added to assess the combined activity of Complex I + II. Next, 10 μM cytochrome C was added to assess mitochondrial integrity, followed by 0.5 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) to uncouple the mitochondrial membrane and induce maximal respiration.

Substrate oxidation and enzyme activity assays

^{3}H-palmitate and ^{14}C-octanoate were used to measure whole-cell substrate oxidation as previously described [18, 20]. All assays were performed in quadruplicate, normalized to cellular protein content, and expressed as pmoles of substrate oxidized per μg of protein per hr. Acyl-CoA dehydrogenase activity was measured in total HEK293 cell lysates or with recombinant LCAD using the anaerobic ETF fluorescence reduction assay with 25μM palmitoyl-CoA as substrate, as described [21].

Immunofluorescence

Wild-type MEFs were grown on cover slips in 24-well plates to near confluence, treated with aspirin for 24 hr as described above, and then fixed in 4% paraformaldehyde in PBS. Mitochondria were visualized with mouse anti-ATP Synthase (ThermoFisher, Clone 4.3E8.D10), peroxisomes with rabbit anti-PMP70 (Abcam), and nuclei with Hoechst Dye (Sigma), using standard techniques as described [22, 23]. Secondary antibodies used were goat anti-mouse Cy3 (Jackson Immunolaboratories) and goat anti-rabbit Alexa 488 (ThermoFisher). Labeled cells were imaged using a Nikon Structured Illumination Microscope.

Results

Aspirin increases mitochondrial long-chain fatty acid oxidation (FAO) but decreases peroxisomal FAO

Our initial goal was to resolve the conflict in the older literature regarding the effect of aspirin on FAO. We chose a dose of 5 mM aspirin applied to HEK293 cells, which have been previously studied with concentrations up to 30 mM without toxicity [6]. The rate of ^{3}H-palmitate oxidation to $^{3}H_2O$ was significantly increased as early as 3 hr after addition of aspirin (Fig 1A). To determine whether the observed increase in FAO was mitochondrial versus peroxisomal, the 24-hr aspirin experiment was repeated with and without etomoxir, an irreversible inhibitor of carnitine palmitoyltransferase-I (CPT1), which is required for fatty acid transport into mitochondria [18]. The etomoxir-sensitive portion of FAO flux represents the mitochondrial pathway while the etomoxir-insensitive portion reflects the peroxisomal pathway. Aspirin significantly reduced etomoxir-insensitive peroxisomal ^{3}H-

palmitate oxidation while clearly enhancing the etomoxir-sensitive mitochondrial pathway (Fig 1B).

We next investigated potential mechanism(s) by which aspirin drives mitochondrial FAO. The relatively rapid acute effect observed at 3 hr post-aspirin suggested that the mechanism likely does not involve altered expression of FAO proteins. Indeed, 24 hr exposure to aspirin did not significantly change the protein levels of the acyl-CoA dehydrogenase family of FAO enzymes or their redox partner electron-transferring flavoprotein (ETF; Fig 1C). Aspirin is cleaved to salicylic acid, which was recently shown to increase FAO via AMPK [6]. AMPK can indirectly increase CPT1 activity by phosphorylating and deactivating acetyl-CoA carboxylase and thereby reducing cytosolic levels of the natural CPT1 inhibitor malonyl-CoA. To determine whether aspirin and salicylic acid affect FAO by a common mechanism, we first compared them head-to-head. Salicylic acid and aspirin had opposite effects on ^{3}H-palmitate oxidation, as 5 mM salicylic acid had no effect at 3 hr (Fig 1D) and was inhibitory at 24 hr (Fig 1E). We attempted to investigate the role of AMPK by immunoblotting for phospho-AMPK, but the levels of AMPK in HEK293 cells was very low and could not be reliably measured (data not shown). The inability of salicylic acid to increase FAO in our cell model as previously reported [6] may be due to low levels of AMPK in HEK293 cells. We conclude that aspirin increases FAO by a separate mechanism from the AMPK-dependent mechanism previously described for salicylic acid.

Aspirin induces non-enzymatic protein acetylation in the mitochondria

The structural difference between aspirin and salicylic acid is a single acetyl group. This acetyl group has been shown by others to acetylate cytosolic proteins in cultured cells [24–26], but the effects on mitochondrial proteins have not been investigated. First, we measured aspirin-induced acetylation in whole cell extracts. A 24-hr exposure of HEK293 cells to 5 mM aspirin resulted in a large increase in acetylated proteins as detected with anti-acetyllysine immunoblotting (Fig 2A), while 5 mM salicylic acid had no effect. Next, mitochondria were isolated from HEK293 cells treated with 5 mM aspirin from 0 to 48 hr. Aspirin treatment resulted in acetylation of numerous mitochondrial proteins, with multiple bands appearing on the anti-acetyllysine immunoblot (Fig 2B). Maximal mitochondrial protein acetylation occurred at 24 hrs post-aspirin administration. By 48 hrs the acetylation had largely resolved, possibly due to protein turnover or to removal by mitochondrial deacylases such as SIRT3. Further *in vitro* experiments support the hypothesis that aspirin-induced acetylation occurs via non-enzymatic transfer of the acetyl group from aspirin to lysine residues. Incubation of purified recombinant LCAD, a key FAO enzyme, with 5 mM aspirin for 1 hr resulted in lysine acetylation (Fig 2C). Increasing the pH of the reaction buffer, which would increase the proportion of deprotonated LCAD lysine residues, led to increased acetylation. The reactivity of aspirin with LCAD lysine residues was comparably greater than that between LCAD and the same concentration of acetyl-CoA (Fig 2D) which has been proposed to non-enzymatically acetylate mitochondrial proteins *in vivo* [27].

Reversible acetylation of matrix proteins does not regulate FAO flux in aspirin-treated cells

In general, increased lysine acetylation has been associated with reduced function of mitochondrial pathways, including FAO [28]. There are, however, some notable exceptions,

such as in the heart of obese, diabetic mice where LCAD and other FAO enzymes were shown to be activated by increased lysine acetylation rather than inhibited [29]. We probed the effect of aspirin on acetylation of the FAO proteins LCAD and VLCAD by transiently transfecting HEK293 cells with Flag-tagged-LCAD and VLCAD expression vectors and treating the cells with aspirin for 24 hrs. Anti-Flag immunoprecipitation followed by anti-acetyllysine western blotting revealed aspirin-induced acetylation of both LCAD-Flag and VLCAD-Flag (Fig 3A). We next used a highly-specific ETF fluorescence reduction enzyme activity assay to measure the combined activities of LCAD and VLCAD toward the substrate palmitoyl-CoA in HEK293 cell lysates. Aspirin did not significantly change the activity of these enzymes (Fig 3B). Similarly, *in vitro* treatment of recombinant LCAD with aspirin did not alter its activity (Fig 3C). However, the HEK293 cell experiments were done in wild-type cells which possess endogenous SIRT3 activity. If aspirin-induced acetylation were to activate FAO ala the diabetic heart, then SIRT3 could be deacetylating key sites on the FAO machinery and limiting the observed increase in FAO and LCAD/VLCAD enzyme activity. To test this, we evaluated whether the effect of 24 hr of 5 mM aspirin on long-chain FAO was enhanced in SIRT3 knockout MEFs compared to wild-type cells. Basal palmitate oxidation was not significantly different between SIRT3−/− and wild-type MEFs (black bars, Fig 3D). Wild-type and SIRT3−/− cell lines exhibited a similar magnitude of FAO induction in response to aspirin (gray bars, Fig 3D), indicating that SIRT3 does not play a role in the observed increase in FAO.

Aspirin fragments the mitochondrial network and decreases respiration on pyruvate and succinate

Salicylates alter mitochondrial morphology in plant cells [30] and the state of the mitochondrial network (fused versus fragmented) can change mitochondrial respiration rates as well as substrate selection [31]. In particular, a fragmented mitochondrial network has been associated with reduced mitochondrial carbohydrate oxidation concomitant with increased FAO [32]. To our knowledge, the effect of aspirin on mitochondrial morphology has not been reported. We used immunofluorescence to probe organelle morphology during aspirin treatment, using MEFs rather than HEK293 due to their superior adherence. As shown in Fig 4A, in untreated MEFs the mitochondria (red) form an elongated, largely fused network, while peroxisomes (green) are mostly staining in a punctate pattern. After 24 hr in 1 mM aspirin (Fig 4B), the mitochondrial network showed no overt change, but the peroxisomal signal was increased and the peroxisomes appeared to be forming larger, fused structures. When treated with 5 mM aspirin for 24 hrs, the dose at which the changes in FAO are largest, dramatic changes were seen with regards to both mitochondria and peroxisomes. The mitochondrial network became fragmented and the intensity of peroxisomal staining was diminished (Fig 4C). These imaging studies were replicated in a second set of cells with similar results.

In keeping with increased mitochondrial fragmentation, aspirin-treated MEFs showed an overall reduction in oxygen consumption in response to the carbohydrate substrates pyruvate and succinate (Fig 4D). The experiment was replicated in aspirin-treated HEK293 cells with similar results (Fig 4E). Together, these data suggest that aspirin treatment increases FAO not by altering the intra-mitochondrial matrix FAO machinery, but by shifting the

mitochondrial network to a more fragmented state which is associated with reduced utilization of carbohydrate energy sources and a compensatory increase in FAO, likely mediated by increased delivery of long-chain fatty acid substrate through CPT1. In a final experiment, we measured the oxidation of ^{14}C-octanoate, a medium-chain fatty acid that does not require CPT1 for entry into the mitochondria. Aspirin did not stimulate ^{14}C-octanoate oxidation to $^{14}CO_2$ (Fig 4F). These data support the contention that the FAO gain-of-function induced by aspirin is not related to changes in the mitochondrial matrix FAO machinery, but rather, involves stimulated delivery of long-chain fatty acids into the mitochondria via CPT1 and the carnitine shuttle.

Discussion

Nearly 120 years after its introduction, new clinical uses are still being discovered for aspirin and its derivatives [2, 33, 34]. It has shown promising anti-diabetic and anti-cancer properties which have been proposed to be due to its positive effects on cellular energy metabolism and mitochondrial function [2, 3]. However, in past decades aspirin was linked to mitochondrial dysfunction and to Reye Syndrome [13]. While Reye Syndrome mortality has been greatly reduced, aspirin continues to be a trigger for metabolic decompensation and even death among those with undiagnosed inborn errors of metabolism [14]. The current studies confirmed previous findings that aspirin can decrease mitochondrial respiration. The mitochondrial network showed altered morphology after aspirin treatment. Further, a loss of peroxisomes was observed after treatment with 5 mM aspirin. The clumps of peroxisomes visible in MEFs treated with 1 mM aspirin are suggestive of increased pexophagy—the process by which dysfunctional peroxisomes are sequestered by autophagosomes and targeted for degradation [35]—because peroxisomes do not normally fuse. Little is known about the regulation of pexophagy, although it may be triggered by increased reactive oxygen species or possibly by low intracellular ATP [35, 36]. The result of these changes appears to be a drive on mitochondrial FAO. In children with inborn errors of the mitochondrial FAO pathway, this could aggravate the metabolic disease by forcing more substrate through the defective pathway while simultaneously inhibiting peroxisomes as an alternative for eliminating the accumulating toxic long-chain fatty acids. We speculate that such a mechanism contributed to the fatty liver, encephalopathy, and general metabolic decompensation that was commonly referred to as Reye Syndrome. The results presented here would caution against the use of aspirin in any patient with compromised mitochondrial function.

Aspirin and salicylic acid differ chemically by only an acetyl group, but we observed opposite effects on mitochondrial FAO after 24 hr of treatment, with aspirin promoting and salicylic acid inhibiting FAO. The acetyl group on aspirin is thought to be rapidly cleaved by cellular thioesterases, but aspirin has also been reported to acetylate albumin and other blood proteins [37, 38]. When applied to cultured cells, aspirin acetylates many cytosolic proteins [26]. Here, we showed that aspirin acetylates mitochondrial proteins including FAO enzymes, and this is due to direct chemical acylation of lysine residues by aspirin. Therefore, we reasoned that the observed difference with regards to FAO between salicylic and acetylsalicylic acid might be caused by protein acetylation. However, further experiments suggested that this is not the case. First, aspirin treatment did not change acyl-

CoA dehydrogenase activity when applied to HEK293 cells nor when used to chemically acetylate recombinant LCAD. Second, cells lacking the mitochondrial matrix deacetylase SIRT3 displayed the same FAO response to aspirin as wild-type cells. Third, oxidation of labeled octanoate, which bypasses CPT1, was not altered by aspirin, suggesting that aspirin does not exert its effects via changes to the matrix FAO enzymes, including by protein acetylation. However, we cannot rule out the possibility that the effect of aspirin on FAO is secondary to cytosolic protein acetylation. Aspirin has been shown to inhibit glycolysis in human platelets [39], and it is possible that acetylation of the glycolytic machinery is responsible for this effect. Inhibition of glycolysis could result in compensatory changes to the mitochondria. It is likewise possible that acetylation of proteins on the outer mitochondrial membrane, including CPT1 itself, could alter mitochondrial function. Regardless, the opposite effects of salicylic acid and aspirin on mitochondrial FAO indicate that, while nearly the same structurally, these two compounds can have widely varying effects. *In vivo,* this could mean that cell types that abundantly express the thioesterases that hydrolyze acetylsalicylic acid to salicylic acid may experience one metabolic outcome while cell types that poorly express these thioesterases may experience another. In short, the metabolic effects of aspirin may be cell-type specific, an important consideration for understanding its utility in treating diseases with a metabolic component such as diabetes or cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations Used

COX	cyclooxygenase
AMPK	AMP-activated protein kinase
SIRT3	sirtuin 3
MEFs	mouse embryonic fibroblasts
LCAD	long-chain acyl-CoA dehydrogenase
VLCAD	very long-chain acyl-CoA dehydrogenase
CPT-1	carnitine palmitoyltransferase-I
ETF	electron transferring flavoprotein
CCCP	carbonyl cyanide 3-chlorophenylhydrazone

References

1. Smith T, et al. Aspirin in the 21st century-common mechanisms of disease and their modulation by aspirin: a report from the 2015 scientific conference of the international aspirin foundation, 28 August, London, UK. Ecancermedicalscience. 2015; 9:581. [PubMed: 26557879]
2. Shirakawa K, et al. Salicylate, diflunisal and their metabolites inhibit CBP/p300 and exhibit anticancer activity. Elife. 2016:5.
3. Mohlig M, et al. Acetylsalicylic acid improves lipid-induced insulin resistance in healthy men. J Clin Endocrinol Metab. 2006; 91(3):964–7. [PubMed: 16384847]
4. Ford RJ, et al. Metformin and salicylate synergistically activate liver AMPK, inhibit lipogenesis and improve insulin sensitivity. Biochem J. 2015; 468(1):125–32. [PubMed: 25742316]
5. He Z, et al. Aspirin regulates hepatocellular lipid metabolism by activating AMPK signaling pathway. J Toxicol Sci. 2015; 40(1):127–36. [PubMed: 25743752]
6. Hawley SA, et al. The ancient drug salicylate directly activates AMP-activated protein kinase. Science. 2012; 336(6083):918–22. [PubMed: 22517326]
7. Yang JM, et al. Acetylsalicylic acid enhances the anti-inflammatory effect of fluoxetine through inhibition of NF-kappaB, p38-MAPK and ERK1/2 activation in lipopolysaccharide-induced BV-2 microglia cells. Neuroscience. 2014; 275:296–304. [PubMed: 24952332]
8. Kamble P, et al. Aspirin may promote mitochondrial biogenesis via the production of hydrogen peroxide and the induction of Sirtuin1/PGC-1alpha genes. Eur J Pharmacol. 2013; 699(1–3):55–61. [PubMed: 23228932]
9. Deschamps D, et al. Inhibition by salicylic acid of the activation and thus oxidation of long chain fatty acids. Possible role in the development of Reye's syndrome. J Pharmacol Exp Ther. 1991; 259(2):894–904. [PubMed: 1941634]
10. Gutknecht J. Aspirin, acetaminophen and proton transport through phospholipid bilayers and mitochondrial membranes. Mol Cell Biochem. 1992; 114(1–2):3–8. [PubMed: 1334228]
11. Glasgow JF, et al. The mechanism of inhibition of beta-oxidation by aspirin metabolites in skin fibroblasts from Reye's syndrome patients and controls. Biochim Biophys Acta. 1999; 1454(1): 115–25. [PubMed: 10354521]
12. Tomoda T, et al. Acetylsalicylate (ASA)-induced mitochondrial dysfunction and its potentiation by Ca2+ Liver. 1994; 14(2):103–8. [PubMed: 8196511]
13. Gosalakkal JA, Kamoji V. Reye syndrome and reye-like syndrome. Pediatr Neurol. 2008; 39(3): 198–200. [PubMed: 18725066]
14. He M, et al. A new genetic disorder in mitochondrial fatty acid beta-oxidation: ACAD9 deficiency. Am J Hum Genet. 2007; 81(1):87–103. [PubMed: 17564966]
15. Whitten R, et al. Liver pathology in Malawian children with fatal encephalopathy. Hum Pathol. 2011; 42(9):1230–9. [PubMed: 21396681]
16. Laster J, Satoskar R. Aspirin-Induced Acute Liver Injury. ACG Case Rep J. 2014; 2(1):48–9. [PubMed: 26157904]
17. Bharathi SS, et al. Sirtuin 3 (SIRT3) protein regulates long-chain acyl-CoA dehydrogenase by deacetylating conserved lysines near the active site. J Biol Chem. 2013; 288(47):33837–47. [PubMed: 24121500]
18. de Moura MB, et al. Overexpression of mitochondrial sirtuins alters glycolysis and mitochondrial function in HEK293 cells. PLoS One. 2014; 9(8):e106028. [PubMed: 25165814]
19. Edmunds LR, et al. Abnormal lipid processing but normal long-term repopulation potential of myc −/− hepatocytes. Oncotarget. 2016
20. Uppala R, et al. Nickel inhibits mitochondrial fatty acid oxidation. Biochem Biophys Res Commun. 2015; 463(4):806–10. [PubMed: 26051273]
21. Goetzman ES. The regulation of acyl-CoA dehydrogenases in adipose tissue by rosiglitazone. Obesity (Silver Spring). 2009; 17(1):196–8. [PubMed: 18948967]
22. Sarin M, et al. Alterations in c-Myc phenotypes resulting from dynamin-related protein 1 (Drp1)-mediated mitochondrial fission. Cell Death Dis. 2013; 4:e670. [PubMed: 23764851]

23. Loughran PA, et al. PEX7 and EBP50 target iNOS to the peroxisome in hepatocytes. Nitric Oxide. 2013; 31:9–19. [PubMed: 23474170]
24. Ai G, et al. Aspirin inhibits glucose6phosphate dehydrogenase activity in HCT 116 cells through acetylation: Identification of aspirin-acetylated sites. Mol Med Rep. 2016
25. Ai G, et al. Aspirin acetylates wild type and mutant p53 in colon cancer cells: identification of aspirin acetylated sites on recombinant p53. Tumour Biol. 2016; 37(5):6007–16. [PubMed: 26596838]
26. Marimuthu S, et al. Aspirin acetylates multiple cellular proteins in HCT-116 colon cancer cells: Identification of novel targets. Int J Oncol. 2011; 39(5):1273–83. [PubMed: 21743961]
27. Wagner GR, Hirschey MD. Nonenzymatic protein acylation as a carbon stress regulated by sirtuin deacylases. Mol Cell. 2014; 54(1):5–16. [PubMed: 24725594]
28. Hirschey MD, et al. SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. Nature. 2010; 464(7285):121-U137. [PubMed: 20203611]
29. Alrob OA, et al. Obesity-induced lysine acetylation increases cardiac fatty acid oxidation and impairs insulin signalling. Cardiovasc Res. 2014; 103(4):485–97. [PubMed: 24966184]
30. Nie S, et al. Mitochondrial-derived reactive oxygen species play a vital role in the salicylic acid signaling pathway in Arabidopsis thaliana. PLoS One. 2015; 10(3):e0119853. [PubMed: 25811367]
31. Liesa M, Shirihai OS. Mitochondrial dynamics in the regulation of nutrient utilization and energy expenditure. Cell Metab. 2013; 17(4):491–506. [PubMed: 23562075]
32. Salabei JK, Hill BG. Mitochondrial fission induced by platelet-derived growth factor regulates vascular smooth muscle cell bioenergetics and cell proliferation. Redox Biol. 2013; 1:542–51. [PubMed: 24273737]
33. O'Brien AJ, et al. Salicylate activates AMPK and synergizes with metformin to reduce the survival of prostate and lung cancer cells ex vivo through inhibition of de novo lipogenesis. Biochem J. 2015; 469(2):177–87. [PubMed: 25940306]
34. Raza H, John A, Benedict S. Acetylsalicylic acid-induced oxidative stress, cell cycle arrest, apoptosis and mitochondrial dysfunction in human hepatoma HepG2 cells. Eur J Pharmacol. 2011; 668(1–2):15–24. [PubMed: 21722632]
35. Nordgren M, et al. Peroxisome degradation in mammals: mechanisms of action, recent advances, and perspectives. Front Physiol. 2013; 4:145. [PubMed: 23785334]
36. Schonenberger MJ, Kovacs WJ. Hypoxia signaling pathways: modulators of oxygen-related organelles. Front Cell Dev Biol. 2015; 3:42. [PubMed: 26258123]
37. Villanueva GB, Allen N. Acetylation of antithrombin III by aspirin. Semin Thromb Hemost. 1986; 12(3):213–5. [PubMed: 3095926]
38. Burch JW, Blazer-Yost B. Acetylation of albumin by low doses of aspirin. Thromb Res. 1981; 23(4–5):447–52. [PubMed: 7324005]
39. Doery JC, Hirsh J, De Grouchy GC. Aspirin: its effect on platelet glycolysis and release of adenosine diphosphate. Science. 1968; 165(3888):65–7. [PubMed: 5729679]

Highlights

- Aspirin increases mitochondrial—but inhibits peroxisomal—fatty acid oxidation.
- Aspirin acetylates mitochondrial proteins including fatty acid oxidation enzymes.
- SIRT3 does not influence the effect of aspirin on fatty acid oxidation.
- Increased fatty acid oxidation is likely due to altered mitochondrial morphology and respiration.

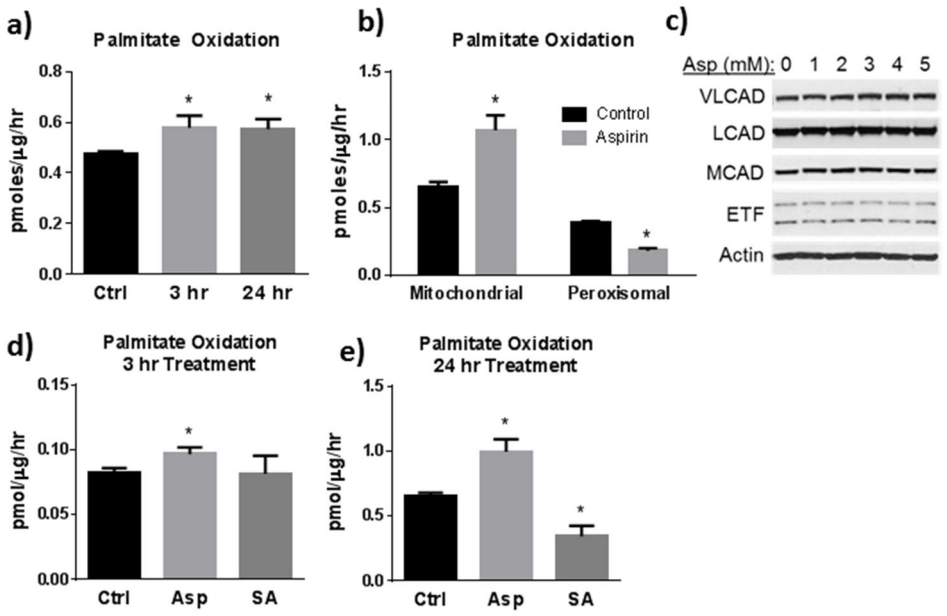

Figure 1. Aspirin increases mitochondrial long-chain FAO

(**a**) HEK293 cells were treated with 5 mM aspirin for either 3 hr or 24 hr and total fatty acid oxidation was measured with ^{3}H-labeled palmitate. (**b**) ^{3}H-palmitate oxidation was measured in HEK293 cells ± 5 mM aspirin for 24 hrs, with or without etomoxir, an irreversible inhibitor of CPT-1. Etomoxir-insensitive fatty acid oxidation represents the peroxisomal pathway. (**c**) Aspirin (0 to 5 mM for 24 hr) did not affect protein levels of the acyl-CoA dehydrogenases or their electron acceptor ETF. Note that ETF is a heterodimer with two different-sized subunits. (**d,e**) The experiment from panel A was repeated with side-by-side comparison to 5 mM salicylic acid (SA) for either 3 hr (d) or 24 hr (e). All palmitate oxidation assays were conducted in quadruplicate wells of cells on a 24-well plate; bars represent means and standard deviations of the four wells. *P < 0.01 versus untreated control cells.

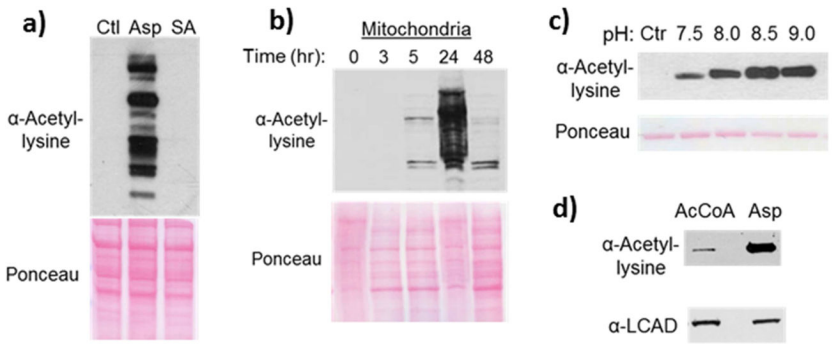

Figure 2. Aspirin chemically acetylates mitochondrial proteins

(**a**) Anti-acetyllysine western-blotting of HEK293 whole-cell lysates after 24 hr of 5 mM aspirin versus 5mM salicylic acid (SA). (**b**) Anti-acetyllysine western-blotting of mitochondria isolated from HEK293 cells treated with aspirin from 0 to 48 hr. (**c**) Anti-acetyllysine western-blotting of recombinant LCAD after incubation with no aspirin (Ctr) or 5 mM aspirin for 1 hr at each indicated pH. (**d**) Anti-acetyllysine western-blotting of recombinant LCAD after 1 hr incubation with either 5 mM acetyl-CoA or 5 mM aspirin.

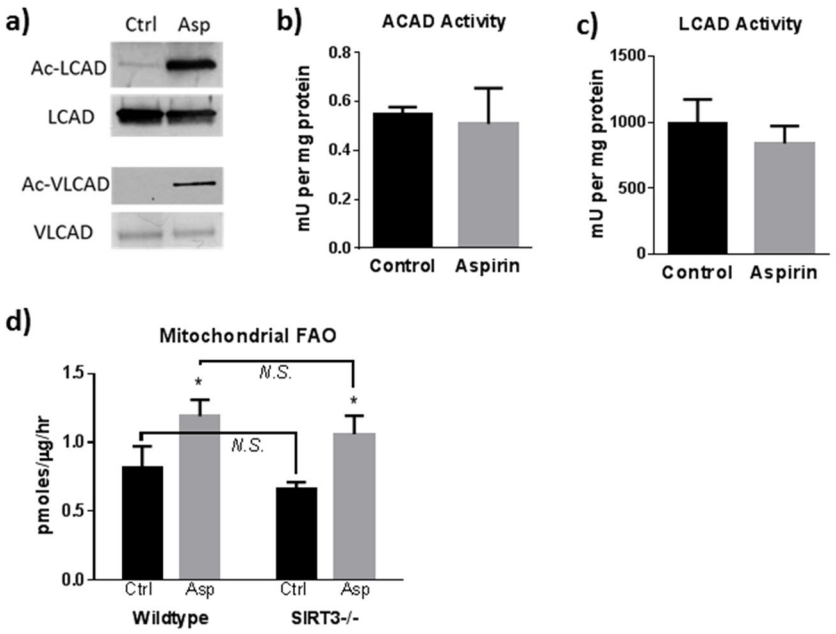

Figure 3. Reversible acetylation of matrix proteins does not regulate FAO flux in aspirin-treated cells

(**a**) Anti-acetyllysine western-blotting of LCAD-Flag and VLCAD-Flag recovered from HEK293 cell lysates by immunoprecipitation ± 24 hr of 5 mM aspirin. (**b**) Acyl-CoA dehydrogenase activity against palmitoyl-CoA in HEK293 cell lysates ± 24 hr of 5 mM aspirin. This reflects combined activities of LCAD and VLCAD. (**c**) LCAD activity against palmitoyl-CoA ± 1 hr incubation with 5 mM aspirin. (**d**) Etomoxir-sensitive (mitochondrial) 3H-palmitate oxidation in wild-type versus SIRT3−/− MEFs ± 24 hr of 5 mM aspirin. All enzyme activity assays and palmitate oxidation assays were conducted on quadruplicate samples of aspirin-treated cells or LCAD protein. Bars represent means and standard deviations. *P < 0.01 of aspirin versus untreated control cells; *NS*= difference not statistically significant.

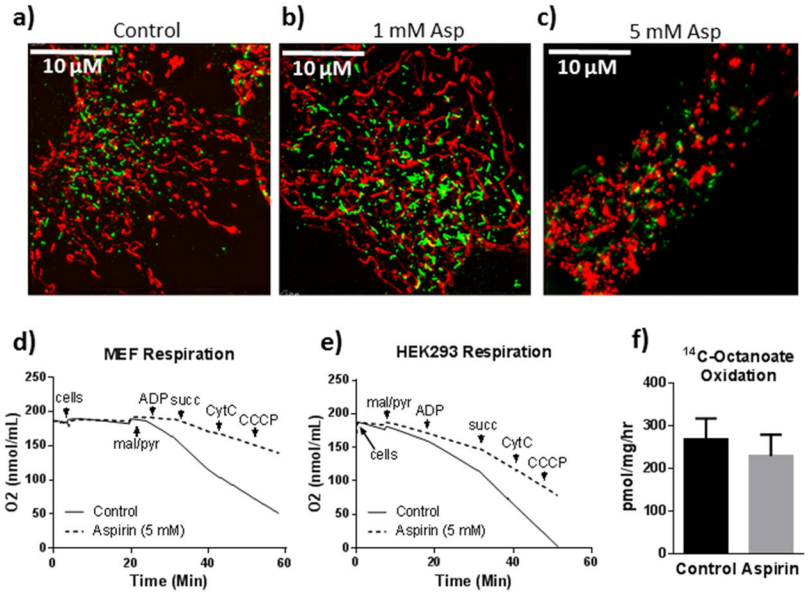

Figure 4. Aspirin fragments the mitochondrial network and decreases respiration on pyruvate and succinate

(**a–c**) Wild-type MEFs treated with vehicle (a), 1 mM aspirin for 24 hr (b), or 5 mM aspirin for 24 hr (c). The mitochondrial network (red) was visualized with anti-ATP synthase antibody and peroxisomes (green) with anti-PMP70 antibody. MEFs were used rather than HEK293 cells because of better adherence. The experiment was repeated with a separate plate of cells yielding similar images.(**d,e**) Oxygen consumption traces for digitonin-permeabilized wild-type MEFs (d) and HEK293 cells (e) ± 24 hr of 5 mM aspirin. Both cell lines show inhibited malate/pyruvate/succinate-driven mitochondrial respiration. These experiments were repeated three times witch each cell line, yielding similar oxygen traces. (**f**) Oxidation of ^{14}C-octanoate, a medium-chain fatty acid which can enter mitochondria independently of CPT-1, in HEK293 cells ± 24 hr of 5 mM aspirin treatment. Cells were assayed in suspension with released $^{14}CO_2$ captured in hanging baskets. Bars represent means and standard deviations of four separate preparations of cells.