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Inhibition of mitochondrial function: An alternative explanation for the antipyretic and hypothermic actions of acetaminophen

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Abstract

Aims: Acetaminophen is the medication of choice when treating fever because of its limited anti-inflammatory effects. However at overdose it can cause mitochondrial dysfunction and damage, often associated with metabolism to N-acetyl-p-benzoquinone imine (NAPQI). What has never been investigated is whether the inhibition of mitochondrial function, particularly fatty acid uptake and oxidation could be the key to its antipyretic and hypothermic properties.

Methods: Mitochondrial function and fatty acid oxidation (FAO) was determined by measuring oxygen consumption rate (OCR) in isolated mitochondria and in 3T3-L1 adipocytes using the XFp Analyser. Basal fatty acids and adrenergic stimulated OCR of mitochondria and 3T3-L1 adipocytes were assessed with acetaminophen and compared to NAPQI, etomoxir, and various mitochondrial stress compounds.

Key findings: Using the XFp Analyser, acetaminophen (10 mM) decreased FAO by 31% and 29% in basal and palmitate stimulated adipocytes. NAPQI (50 μM) caused a 63% decrease in both basal and palmitate stimulated FAO. Acetaminophen (10 mM) caused a 34% reduction in basal and adrenergic stimulated OCR. In addition acetaminophen also inhibited complex I and II activity at 5 mM. NAPQI was far more potent at reducing mitochondrial respiratory capacity, maximum respiratory rates and ATP production than acetaminophen.

Significance: These studies demonstrate the direct inhibition of mitochondrial function by acetaminophen at concentrations which have been shown to reduce fever and hypothermia in mammals. Understanding how antipyretics directly affect mitochondrial function and heat generation could lead to the development of new antipyretics which are not compromised by the anti-inflammatory and toxicity of the current medications.

Keywords: acetaminophen, NAPQI, antipyresis, lipolysis, mitochondria, electron transport chain

1.0 Introduction

The SARS-CoV-2 pandemic has reminded the world why there is a need for a powerful antipyretic that has limited anti-inflammatory properties. Currently most over-the-counter treatments will also inhibit immune function which is unwanted when the body is fighting infection (Day, 2020). An ideal antipyretic should interact directly with peripheral heat generating systems without inhibiting the cyclooxygenase enzymes, which are involved in other key processes including immune function (Aronoff and Neilson, 2001). Presently the drug of choice is acetaminophen (paracetamol, Tylenol). Despite its effectiveness, every year thousands are hospitalised following acetaminophen overdose (Yoon et al., 2016). Acetaminophen has been shown to reduce fever and cause hypothermia in humans and small mammals in the absence of fever (Foster et al., 2016; Kis et al., 2005). Historically the antipyretic properties of acetaminophen have been attributed to the inhibition of the cyclooxygenase-2 (COX-2) enzyme (Hinz et al., 2008; Chandrasekharan et al., 2002) or a putative cyclooxygenase protein termed COX-3 (Ayoub and Flower, 2019). However, linking the temperature regulatory actions of acetaminophen to the inhibition of cyclooxygenase enzymes has always been problematic, as it is a weak inhibitor of cyclooxygenases which is out of step with its ability to lower body temperature (Chandrasekharan et al., 2002; Graham and Scott, 2005; Censarek et al., 2006; Hanel and Lands, 1982).

In our previous study, we showed that acetaminophen attenuated both lipolysis and mitochondrial function which are both essential for heat generation and thermoregulation (Bashir et al., 2020). It is known small mammals living at ambient temperatures (Ta) below their thermoneutral zone must switch on various thermogenesis processes to maintain core body temperature (Tc), these processes are the same as those required to increase Tc during fever (Gordon, 2012). The process starts with adrenergic stimulation of lipolysis in adipocytes , this leads to β-oxidation of fatty acids and the production of reducing cofactors NADH and

$FADH_2$, and ultimately mitochondrial metabolism and heat generation (De Pauw et al., 2009; Begriche et al., 2011). Compounds such as acetaminophen which uncouple or inhibit mitochondrial electron transport chain (ETC) can also inhibit catecholamine mediated lipolysis (Umbaugh et al., 2021; Ramachandran and Jaeschke, 2020; Bashir et al., 2020; Fassina et al., 1974). This indicates a direct link between the functionality of mitochondrial oxidative phosphorylation, lipolysis and ultimately heat generation in mammals (De Pauw et al., 2009). The acetaminophen metabolite; NAPQI, is likely to mediate the inhibition of mitochondrial function and FAO (Chen et al., 2009; Copple et al., 2008; Jan et al., 2014). Other antipyretic drugs have been shown to directly inhibit one or more mitochondrial FAO enzymes in addition to the inhibition of cyclooxygenases (De Pauw et al., 2009; Fromenty and Pessayre, 1995; Deschamps et al., 1994; Fréneaux et al., 1990; Labbe et al., 2008).

To determine if the antipyretic and hypothermic actions of acetaminophen are due to the impairment of mitochondrial function, OCR was assessed in isolated mitochondria and in 3T3-L1 adipocytes. The impact of acetaminophen on OCR was also assessed in the absence and presence of adrenergic stimulants. In addition, the impact of the metabolite NAPQI on FAO was assessed (Pike et al., 2011).

2.0 Materials and Methods

2.1 Chemicals and Reagents

All chemicals were supplied by Sigma-Aldrich, U.K. unless stated otherwise. Substrate-Limited Medium: DMEM containing 0.5 mM glucose, 1 mM glutamine, 0.5 mM carnitine, and 1% foetal bovine serum (FBS). Carnitine was added to the media on the day of media change. FAO Assay Medium (Krebs Henseleit Buffer): 111 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 2 mM $MgSO_4$, 1.2 mM NaH_2PO_4 dissolved in H_2O and filter sterilized and 2.5 mM glucose, 0.5 mM carnitine, and 5 mM HEPES was added on the day of assay; pH 7.4 at 37°C.

XF Palmitate-BSA FAO substrate and stock respiration reagents were purchased from Agilent Seahorse, U.K.

2.2 Differentiation of 3T3-L1 pre-adipocytes

The 3T3-L1 pre-adipocytes were allowed to become confluent and then allowed to grow for four additional days before being placed in DMEM differentiation medium with 10% FBS, 1 µg/mL insulin, 0.5 mM isobutylmethylxanthine (IBMX), 1 µM dexamethasone, 2 µM rosiglitazone for 48 hours. On day 3, the differentiation medium was switched to DMEM containing 10% FBS and 1 µg/mL insulin and changed every 48 hours from this stage until the 3T3-L1 adipocytes were fully differentiated (Zebisch et al., 2012). The cells were assessed for the impact of acetaminophen and NAPQI on cell viability using MTT assay (Bashir et al., 2020).

2.3 Seeding of 3T3-L1 cells in Seahorse XFp cell culture miniplates

Cells were seeded using two models. In model 1; pre-adipocytes were harvested and resuspended in growth medium to give 5000 cells/well in a XFp cell culture miniplate and allowed to grow until differentiated. In model 2; differentiated 3T3-L1 cells were harvested and resuspended in growth medium to give 5000 cells/well in a XFp cell culture miniplate (Agilent Seahorse, U.K.). The condition of the cells was assessed by light microscopy.

2.4 Hydration of Seahorse XFp sensor cartridge

Two methods were used to hydrate the sensor cartridge (Agilent Seahorse, U.K.). In procedure 1, on the day prior to assay the Seahorse XFp sensor cartridge was filled with calibrant and placed in a non-CO_2 37°C humidified incubator overnight. In procedure 2 the day prior to assay, the calibrant was placed in a non-CO_2 37°C incubator overnight and the cartridge filled with sterile water. The sensor cartridge was then submerged in water and placed in a non-CO_2

37°C humidified incubator overnight. On the assay day the water was replaced with calibrant and replaced in a non-CO_2 37°C incubator for 45 – 60 minutes prior to loading the drug ports of the sensor cartridge (Agilent Seahorse, U.K.).

2.5 Measurement of OCR using Agilent Seahorse XFp Extracellular Flux Analyser

FAO and mitochondrial function were assessed by measuring the OCR of cells and isolated mitochondria using the Agilent Seahorse XFp Analyser and Cell Mito Stress Test (Figure 1). By measuring OCR, the Seahorse equipment allows the determination of key parameters of cellular and mitochondrial function.

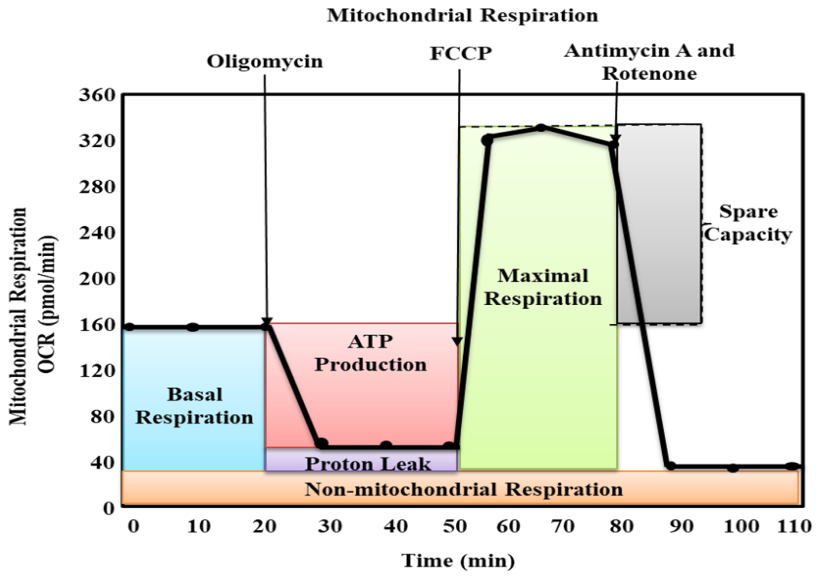


***Figure 1**: Schematic of Agilent Seahorse XF Cell Mito Stress Test Profile (Figure adapted from Agilent Seahorse, U.K.).*

2.6 Effect of Acetaminophen on FAO

2.6.1 Assay preparation

For this assay, model 2 was used for seeding cells and procedure 2 for hydrating the XFp sensor cartridges. The culture medium was removed and substrate-limited medium added 24 hours prior to the assay. The cells were washed twice with FAO assay medium 45 minutes before the assay (t = -45 min) and then placed in a non-CO_2 incubator at 37°C. The assay cartridge was loaded with XFp Cell Mito Stress Test reagents in FAO assay medium; oligomycin (2.5 µg/mL, final); carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP); rotenone/ 40 µM antimycin A (2 µM/4 µM, final, respectively). Etomoxir (40 µM) was added 15 minutes before initiating the assay (t = -15 minutes). For Acetaminophen 10 mM was added and for NAPQI a final concentration of 50 µM was added. The assay was initiated (t = 0 minutes), when the fatty acid XF Palmitate-BSA (1 mM: 0.17 mM) substrate or BSA (0.17 mM) control was added to the appropriate wells. The XFp cell culture miniplate was immediately inserted into the XFp Analyzer and the assay was initiated (Agilent Seahorse, U.K.).

2.6.2 Assessing mitochondrial bioenergetic profile of 3T3-L1 adipocytes using Seahorse XFp Cell Mito Stress Test

For this assay, model no.1 was used for seeding cells and procedure no.1 for hydrating XFp sensor cartridges. 10 mM glucose, 2 mM glutamine and 1 mM pyruvate were added to the medium which was filter sterilised and warmed to 37°C. The contents of each reagent of the Seahorse XFP Cell Mito Stress Test kit were re-suspended with prepared assay medium and loaded as port A, oligomycin (5 µM, final); port B, FCCP (5 µM, final); port C, rotenone/ antimycin A (2.5 µM, final, respectively). The default template of the Seahorse XFp Cell Mito Stress Test was selected on the Seahorse XFp (Agilent Seahorse, U.K.).

2.6.3 Effect of acetaminophen on basal, norepinephrine and isoproterenol stimulated OCR in 3T3-L1 adipocytes

For this assay, model no. 2 was used for seeding cells and procedure no.2 for hydrating XFp sensor cartridges. Using the assay medium, the port injections were: port A, acetaminophen (5 or 10 mM, final). For norepinephrine or isoproterenol stimulated OCR and using assay medium, the port injections were: port A, norepinephrine (1 μM, final) or isoproterenol (1 μM, final). In subsequent assays, using assay medium, the port injections were: port A, norepinephrine (1 μM, final) or isoproterenol (1 μM, final), port B, acetaminophen (1 mM, final); port C, acetaminophen (4 mM, final); port D, acetaminophen (5 mM, final).

2.7. Determination of mitochondrial inhibition using the electron flow assay

2.7.1 Isolation of mitochondria homogenate

Mitochondria were isolated by differential centrifugation from the livers of male Wistar rats (Zavodnik et al., 2011). Briefly, the livers of male Wistar rats (200-250g) was removed, weighed and washed in PBS (4°C) three times, then finely chopped with scissors. The mitochondrial pieces were then resuspended in 7 times (w/v) the volume of buffer containing 70 mM sucrose, 210 mM mannitol, 5 mM HEPES, 1 mM EGTA and 0.5% (w/v) fatty acid-free BSA, pH 7.2 at 4°C. The tissue was then homogenised using a drill-driven Teflon dounce homogenizer for 2-3 strokes. The homogenate was then spun at 800g for 5 minutes at 4°C, the pellet was centrifuged again at 8000g for 10 minutes at 4°C. Finally the pellet was the suspended in the isolation buffer. Total protein (mg/mL) was determined using the Bradford Assay reagent.

2.7.2 Assay preparation

After diluting the mitochondria in mitochondrial assay solution (MAS) to 5 µg/well the substrate was added and then spun at 2000 x *g* for 20 minutes at 4°C (Agilent Seahorse, U.K.). For control group, initial substrate mix of 10 mM and 2 mM of pyruvate and malate + 4 µM FCCP. For known ETC inhibitors controls, 2 µM rotenone, or 10 mM malonate, or 4 µM antimycin A, or 2.5 µg/ml oligomycin was also added in the initial substrate mix. For acetaminophen groups, 1-10 mM acetaminophen was also added in the initial substrate mix. Using procedure 2 for hydrating XFp sensor cartridges, for the control groups, the port injections were made as: port A, rotenone (2 µM, final); port B, succinate (10 mM, final); port C, antimycin A (4 µM, final); port D, ascorbate and N1,N1,N1,N1tetramethyl-1,4-phenylene diamine (TMPD;10 mM and 100 µM final) (Agilent Seahorse, U.K.).

For acetaminophen groups, the port injections were made as: port A, 4 mM, 5 mM, or 10 mM, final; port B, succinate (10 mM, final); port C, acetaminophen (5 mM, final) or acetaminophen (10 mM, final); port D, ascorbate and TMPD (10 mM and 100 µM final) (Agilent Seahorse, U.K.).

The initial substrate mix of pyruvate , malate and FCCP allowed the evaluation of maximal respiration driven by complex I, while the injection of rotenone followed by succinate allowed the assessment of maximal respiration driven by complex II. The injection of antimycin A prevented complex I- and III-mediated respiration due to inhibition of complex III, causing loss of function throughout until ascorbate and TMPD was added, thus allowing the evaluation of respiration driven by Complex IV (Rogers et al., 2011).

2.8 Statistical analysis

The results were analysed using GraphPad Prism 4.02 and presented as mean ± standard deviation (SD). The results were analysed with analysis of variance (ANOVA), followed by Dunnett's Multiple Comparison Test or Bonferroni's Multiple Comparison Test. A *$P < 0.05$, ** $P<0.01$, *** $P<0.001$ from control was considered statistically significant.

3.0 Results

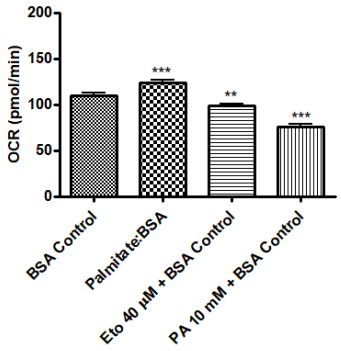
3.1 The Effect of Acetaminophen on FAO

The XF FAO assay measures FAO in cells during basal, stimulated and when energy stressed (Agilent Seahorse, U.K.). Using the XF Cell Mito Stress Test the effect of acetaminophen (10 mM) was investigated under basal, palmitate stimulated and after the addition of oligomycin (2.5 µg/ml), FCCP (4 µM) and rotenone/antimycin A (2 µM/4 µM). The effect of acetaminophen was compared to etomoxir (40 µM), a fatty acid uptake inhibitor. Acetaminophen did not affect cell viability at the concentration used.

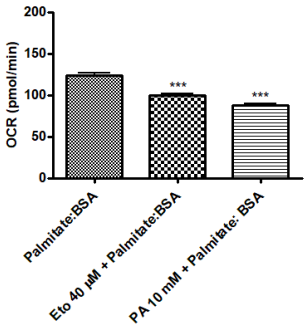
At basal respiration levels there was a significant decrease in OCR by 10% and 31% when cells were treated with either etomoxir or acetaminophen respectively Figure 2(A). A similar reduction in OCR was observed particularly with acetaminophen when respiration was stimulated with palmitate with etomoxir and acetaminophen reducing OCR by 20% and 29% respectively, Figure 2(B). After the addition of oligomycin, both etomoxir and acetaminophen caused a further decrease by 12% and 15% in OCR, Figure 2(C,D). The introduction of FCCP in the presence of palmitate resulted in the expected significant rise in maximum respiration and this was reversed by etomoxir or acetaminophen by 42% and 25% respectively, Figure 3(A,B). By contrast in the presence of rotenone/antimycin (basal respiration), there was a small reduction in OCR of 17% and 16% when etomoxir and acetaminophen were added, Figure 3(C). The addition of palmitate only slightly increased (<10%) in OCR above basal level and again there was also a small reduction in OCR of 23% and 14% when etomoxir and acetaminophen were added respectively, Figure 3(D).

Basal

A.

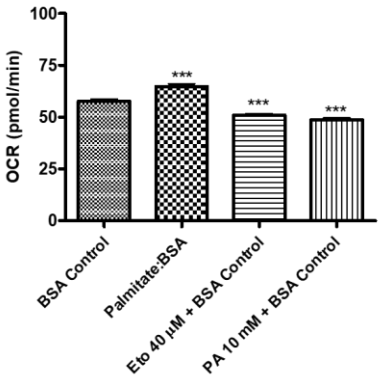


B.



Oligomycin addition

C.



D.

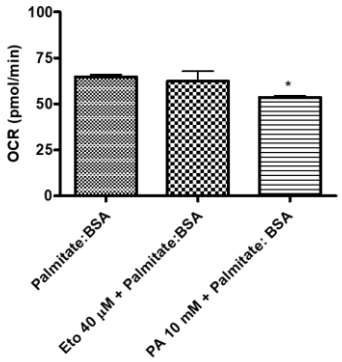
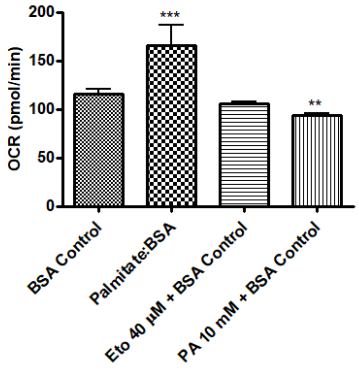


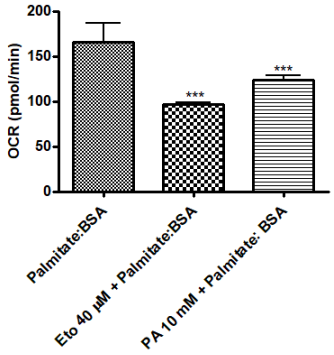
Figure 2: Effect of acetaminophen (PA) and etomoxir (Eto) on FAO during basal and after oligomycin addition in 3T3-L1 adipocytes. *3T3-L1 adipocytes grown in substrate-limited medium overnight and combination of XF Palmitate-BSA FAO Substrate and the XF Cell Mito Stress Test were used. The treatments were basal (A), palmitate (B) and then in the presence of oligomycin basal (C) and palmitate (D). Data are representative of n=3 replicates expressed as means ± Standard deviations (*P < 0.05, **P < 0.01, *** P<0.001 from control).*

FCCP addition

A.

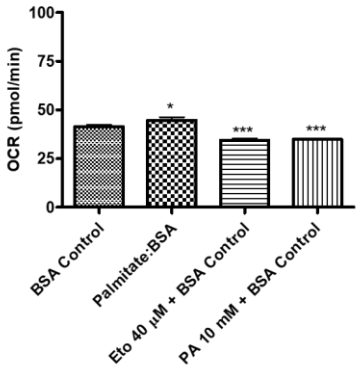


B.



Rotenone/Antimycin A addition

C.



D.

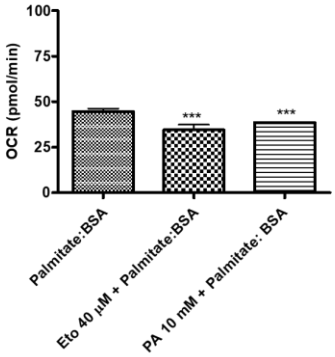


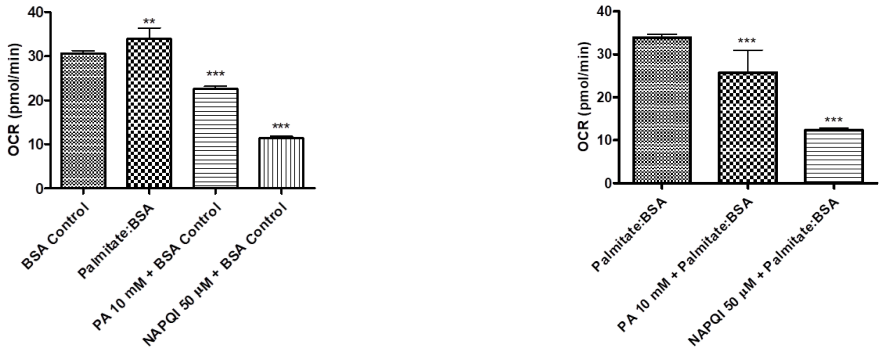
Figure 3: Effect of acetaminophen (PA) and etomoxir (Eto) on FAO after FCCP and rotenone/antimycin A addition in 3T3-L1 adipocytes *3T3-L1 adipocytes grown in substrate-limited medium overnight and combination of XF Palmitate-BSA FAO Substrate and the XF Cell Mito Stress Test was used. The treatments were in the presence of* FCCP *at basal (A), or palmitate (B)stimulated. Then in the presence of* rotenone/antimycin at *basal (C) and palmitate (D). Data are representative of n=3 replicates expressed as means ± Standard deviations (*P < 0.05, **P < 0.01, *** P<0.001 from control).*

3.2 Effect of NAPQI on FAO

In an attempt to determine whether acetaminophen exerted some of its actions through the toxic metabolite NAPQI, the effect of acetaminophen (10 mM) and NAPQI (50 μM) on both endogenous and exogenous FAO was investigated under basal conditions and after oligomycin addition (2.5 μg/ml), FCCP (4 μM) and rotenone/antimycin A (2 μM/4 μM). Acetaminophen and NAPQI did not affect cell viability at the concentrations used. At basal respiration, there was a significant decrease in OCR of 63% by NAPQI, a similar decrease was observed for the palmitate treated cells when treated with NAPQI of 63% Figure 4(A,B). After oligomycin addition, the decrease in respiration caused by NAPQI for endogenous OCR decreased by 46%. A similar decrease was observed with exogenous (palmitate) by NAPQI with OCR decreasing by 44% Figure 4(C,D). The introduction of FCCP resulted in a significant increase in endogenous and exogenous (palmitate) maximal respiration. The addition of NAPQI resulted in a reduction in OCR by 80% and 79% for the exogenous and endogenous respiration respectively, Figure 5(A,B). In the presence of rotenone/antimycin the addition of NAPQI caused a further reduction of 58% and 51% for endogenous and exogenous (palmitate) OCR respectively, Figure 5(C,D).

Basal

A. **B.**



Oligomycin addition

C. **D.**

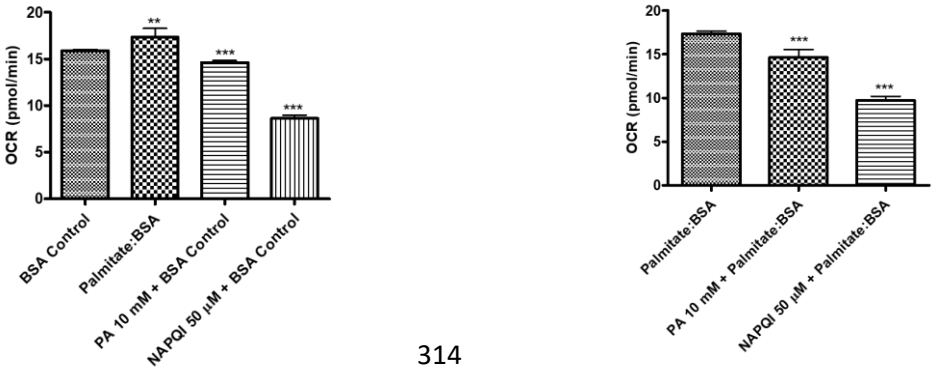
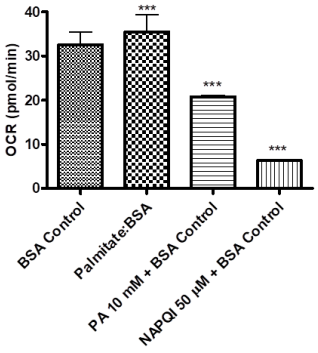


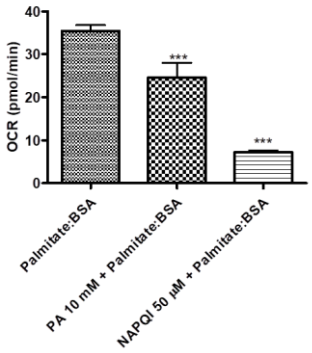
Figure 4: Effect of acetaminophen (PA) and NAPQI on FAO during basal and after oligomycin addition in 3T3-L1 adipocytes. *3T3-L1 adipocytes grown in substrate-limited medium overnight and combination of XF Palmitate-BSA FAO Substrate and the XF Cell Mito Stress Test was used. The treatments were basal (A), palmitate (B) and then in the presence of oligomycin basal (C) and palmitate (D). Data are representative of n=3 replicates expressed as means ± Standard deviations (**P < 0.01, *** P<0.001 from control).*

FCCP addition

A.

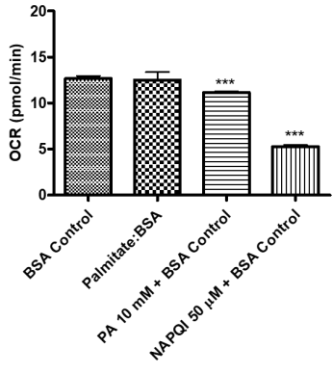


B.



Rotenone/Antimycin A addition

C.



D.

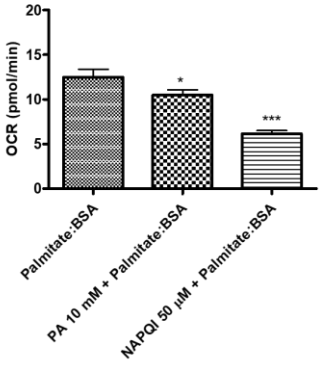


Figure 5: Effect of acetaminophen (PA) and NAPQI on FAO after FCCP and rotenone/antimycin A addition in 3T3-L1 adipocytes. *3T3-L1 adipocytes grown in substrate-limited medium overnight and combination of XF Palmitate-BSA FAO Substrate and the XF Cell Mito Stress Test was used. The treatments were in the presence of* FCCP *at basal (A), or palmitate (B)stimulated. Then in the presence of* rotenone/antimycin at *basal (C) and palmitate (D). Data are representative of n=3 replicates expressed as means ± Standard deviations (*P < 0.05, *** P<0.001 from control).*

3.3 Effect of acetaminophen on basal OCR

In an attempt to examine whether acetaminophen affected basal respiration, 3T3-L1 adipocytes were used. Acetaminophen did not affect cell viability at the concentrations used. Acetaminophen at both 5 and 10 mM attenuated OCR by 32% and 35% when cells were at basal respiration (Figure 6).

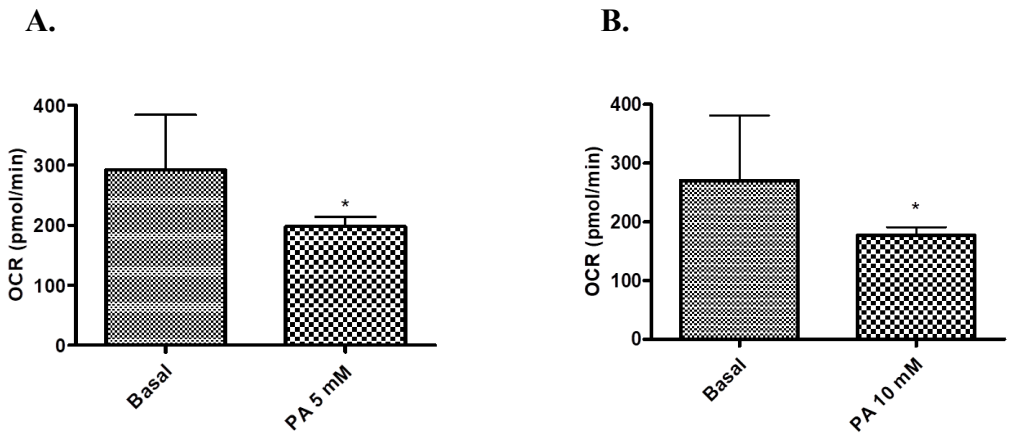


Figure 6: Effect of Acetaminophen (PA) on basal OCR in 3T3-L1 adipocytes. *Measurement of basal OCR in 3T3-L1 adipocytes followed by addition of different concentrations of acetaminophen. The treatments were 5 mM acetaminophen (A) and 10 mM acetaminophen. (B) Data are representative of n=3 replicates expressed as means ± Standard deviations (*P < 0.05 from control).*

3.4 Effect of acetaminophen on norepinephrine and isoproterenol stimulated OCR

3T3-L1 adipocytes were assessed for their ability to respond to an acute exposure of the catecholamines. OCR was significantly increased in response to norepinephrine (53%) or isoproterenol (64%) exposure when compared with untreated cells, Figure 7(A,B). Acetaminophen (10 mM) significantly attenuated catecholamine induced increase in OCR by 27% in case of norepinephrine and 34% for isoproterenol at higher concentrations of the drug, Figure 7(C,D).

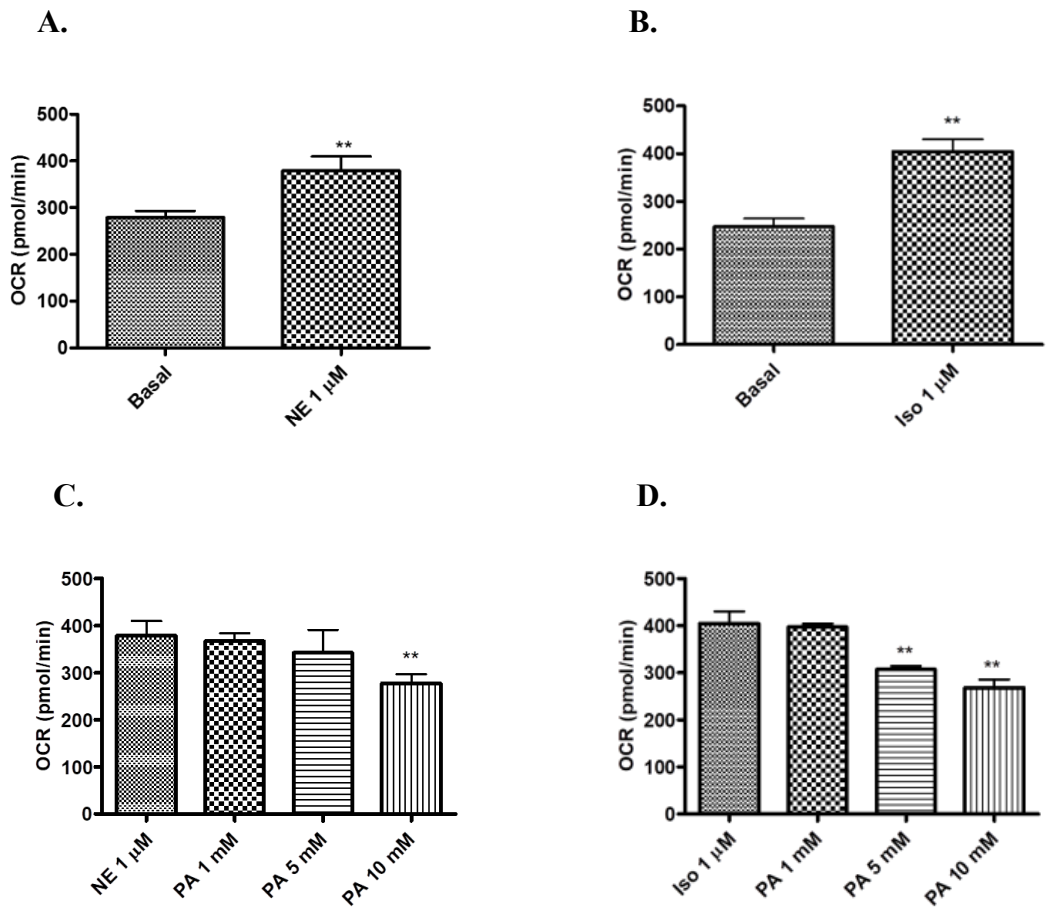


Figure 7: Effect of acetaminophen (PA) on norepinephrine (NE) and isoproterenol (Iso) stimulated OCR in 3T3-L1 adipocytes. *Measurement of basal OCR in 3T3-L1 adipocytes followed by addition of norepinephrine or isoproterenol A, B. Then followed by the addition of acetaminophen with norepinephrine or isoproterenol C, D. Data are representative of n=3 replicates expressed as means ± Standard deviations (** P<0.01 from control).*

3.5 Effect of acetaminophen and NAPQI on individual parameters of mitochondrial function using the FAO assay in 3T3-L1 adipocytes

In an attempt to further investigate the mechanisms by which acetaminophen and NAPQI alter OCR, individual parameters of mitochondrial function were investigated in 3T3-L1 adipocytes (Table 1). Acetaminophen (10 mM) had no effect on proton leak, spare respiratory capacity slightly attenuated basal respiration, non-mitochondrial respiration and coupling efficiency by 14%, 15%, and 19%, but significantly reduced maximal respiration by 43%. The greatest effect was on ATP production in both endogenous and exogenous fatty acid respiration by 52% and 26%. There was no effect on proton leak by NAPQI (50 μM), by contrast basal respiration was significantly reduced by 64% and 65% and spare respiratory capacity was significantly reduced by 81% and 84% for endogenous and exogenous fatty acids respectively. Maximum respiratory rates were greatly attenuated by NAPQI for endogenous and exogenous fatty acids by 93% and 94% respectively. NAPQI reduced non-mitochondrial respiration by 60% and 48%, coupling efficiency by 39% and 34%, and abolished ATP production by 78% and 77% for endogenous and exogenous fatty acids respectively. Finally, spare respiratory capacity was significantly reduced by NAPQI by 81% and 84% for endogenous and exogenous fatty acids respectively.

418 ***Table 1. Effect of acetaminophen and NAPQI on individual parameters of mitochondrial***

419 ***function using the FAO assay in 3T3-L1 adipocytes.***

	Acetaminophen (10 mM)		NAPQI (50 µM)	
	Endogenous	**Exogenous**	**Endogenous**	**Exogenous**
Basal respiration	**-14%****	**0%**	**-64%****	**-65%****
Proton leak	**0%**	**0%**	**0%**	**0%**
Spare respiratory capacity	**0%**	**0%**	**-81%****	**-84%****
Maximal respiration	**-43%****	**0%**	**-93%****	**-94%****
Non-mitochondrial respiration	**-15%***	**0%**	**-60% ****	**-48%****
ATP production	**-52% ****	**-26%****	**-78% ****	**-77%****
Coupling efficiency	**-19%**	**0%**	**-39%**	**-34%**

420 ***Data (% change) are representative of at least 3 replicates expressed as means ± Standard***

421 ***deviations (SD) (*P < 0.05, ** P<0.01 from control).***

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3.6 Elucidation of mechanistic activity of acetaminophen on mitochondrial function

Having established the inhibitory effect of acetaminophen on mitochondrial activity in whole cell systems, studies were undertaken to assess the effect of acetaminophen on the activity of individual electron transport complexes of isolated mitochondria (Table 2). When OCR is driven by the complex I substrates pyruvate/malate there was a 26.7%, 42% and 61.5% inhibition of OCR at 5 mM, 10 mM, and 20 mM acetaminophen respectively. The addition of the complex II substrate succinate, increased OCR by 236%. The addition of acetaminophen to succinate driven OCR resulted an in 28.6%, 48.6% and 60.8% reduction at 5 mM, 10 mM, and 20 mM acetaminophen respectively. By contrast the classical and highly toxic mitochondrial complex inhibitors (rotenone, malonate, antimycin and oligomycin) were far more potent than acetaminophen; inhibiting OCR between 40% and 90% at similar or much lower concentrations (Table 2).

Table 2. Measurement of OCR to determine the mitochondrial complex target of acetaminophen.

		Basal	Rotenone (complex I inhibitor) 4 µM	Malonate (complex II inhibitor) 10 mM	Antimycin (complex III inhibitor) 8 µM	Oligomycin (complex V inhibitor) 5 µg/ml	PA 5 mM	PA 10 mM	PA 20 mM
Complex I substrate	Mean OCR (pmol/ min) ± SD	60.1 6.45	6.24 2.75	19.3 11.7	6.5 3.45	17.96 6.7	44.1 6.8	34.94 5.4	23.1 7.8
	% of basal (Pyruvate/ Malate)	-	10.3**	32.0**	10.7**	29.8**	73.3*	58.0*	38.5**
Complex II substrate	Mean OCR (pmol/ min) ± SD	142.1 8.7	88.8 8.55	21.2 6.2	15.5 7.0	29.95 4.4	101.5 8.1	73.1 5.4	55.7 4.0
	% of basal (Succinate)	-	62.5*	14.9**	10.2**	21**	71.4*	51.4**	39.2**

Data (from isolated mitochondria) are representative of at least 3 replicates expressed asmeans ± Standard deviations (SD) (*P < 0.05, ** P<0.01 from control), Acetaminophen (PA).

4.0 Discussion

Acetaminophen is widely used to treat fever partly because it only has limited immuno-modulatory effects when compared to NSAIDs such as ibuprofen. The traditional view of acetaminophen is that it alters the central thermoregulatory set points possibly through inhibition of the COX-2 enzyme. However, in a recent study we demonstrated that it is also capable of inhibiting lipolysis and mitochondrial function, thereby linking for the first time, these changes to antipyresis and hypothermia (Bashir et al., 2020; Mitchell et al., 1973a, b). Mammals have evolved various mechanisms to deal with cold stress and to generate heat during fever including adrenergic stimulation of lipolysis in adipocytes (Luo and Liu, 2016). The process also requires the metabolism of substrates in the mitochondria during oxidative phosphorylation. Increased lipolysis and the utilisation of the released fatty acids are the main source of substrates for thermogenesis in small mammals at temperatures below their thermoneutral zone (Lee et al., 2015; Albert et al., 2016; Ueta et al., 2012). The fatty acids derived from lipolysis can also combine with uncoupling protein 1 (UCP1) to increase H^+ permeability leading to the direct production of heat rather than ATP (uncoupling) in a form of non-shivering thermogenesis (Cohen et al., 1997). Attenuation of fatty acid transport into the mitochondria will result in a decrease in the OCR, provided there is no compensatory increase in the utilization of non-fatty acid substrates. The transport of long chain fatty acids inside mitochondria by carnitine palmitoyltransferase 1 (CPT1) on the outer membrane is the main controlling step for FAO (Begriche et al., 2011). The classical fatty acid transport inhibitor etoxomir binds irreversibly to the CPT1 transporter preventing fatty acid uptake into the mitochondria and ultimately preventing the oxidation process (Pike et al., 2011). A similar effect by acetaminophen could lead to antipyresis or hypothermia.

To assess the potential impact of acetaminophen on the capacity of cells to utilize fatty acids liberated following lipolysis, OCR was assessed under different conditions with

505 acetaminophen, etomoxir and different mitochondrial stress compounds. If acetaminophen acts
506 by preventing fatty acid uptake then the impact on OCR should be similar to etomoxir. In
507 adipocytes acetaminophen was effective at inhibiting OCR in the presence and absence of the
508 fatty acid palmitate, suggesting it is capable of attenuating the oxidation of exogenously added
509 fatty acids in addition to inhibiting substrates including fatty acids already inside the
510 mitochondria. This conclusion was confirmed by the lack of inhibition of OCR in cells treated
511 with etomoxir in the absence of exogenously added palmitate. The greater inhibition of OCR
512 in the presence of acetaminophen compared to etomoxir suggest acetaminophen may also be
513 directly inhibiting FAO and possibly delivery of substrates to complex I/II in the ETC. Stress
514 molecules were employed to probe further the impact of acetaminophen on other mitochondrial
515 processes. The use of oligomycin provides evidence that acetaminophen had little effect on
516 proton leak or uncoupling. The reduction of basal ATP production by 50% confirms a
517 significant direct impact on the mitochondrial ETC. The inhibition of maximal respiratory
518 capacity by acetaminophen confirms it is capable of significantly attenuating exogenous FAO
519 although not as effectively as etomoxir.

520 In many cases acetaminophen induced toxicity is enhanced once it is metabolised by the
521 cytochrome P450 systems to the highly reactive metabolite NAPQI. At lower doses of
522 acetaminophen, any NAPQI produced will be efficiently detoxified by glutathione (GSH) and
523 other protective systems, but at higher acetaminophen concentrations NAPQI production
524 would deplete GSH leading to the formation of 3-(cystein-*S*-yl)-acetaminophen adducts and
525 greater toxicity (Mitchell et al., 1973a, b; Jaeschke and Bajt, 2006; Ramsay et al., 1989). In
526 cellular models, NAPQI (400 μM) caused a reduction in GSH levels leading to depletion of
527 mitochondrial ATP content (> 80% depletion after 1 minute exposure). NAPQI is known to be
528 much more toxic than acetaminophen, including inhibiting ADP-stimulated (state 3)
529 respiration of liver mitochondria (Zhang et al., 2015). Given the high levels of acetaminophen

in studies where hypothermia is observed, it is likely that some NAPQI would be generated in the mitochondria of animals given acetaminophen at doses above 100 mg/kg. In this study the concentration of NAPQI (50 μM; Masubuchi et al., 2005; Copple et al., 2008; Jan et al., 2014; Burcham and Harman, 1991) selected appeared to have no impact on cell viability. However, NAPQI had a significant impact on OCR and FAO, a clear indication that once generated NAPQI could attenuate heat generation by causing mitochondrial dysfunction. In adipocytes, NAPQI proved to be a far more potent inhibitor of OCR than acetaminophen at significantly lower concentrations. If replicated *in vivo* this would suggest that even a small amount of NAPQI generated from acetaminophen metabolism could lead to the inhibition of cellular respiration and hypothermia.

Unlike the parent compound NAPQI appeared to partly uncouple mitochondria; however this conclusion is complicated by the fact that NAPQI was such a potent inhibitor of the mitochondrial processes generally. As induction of mitochondrial permeability transition (MPT) is more relevant to NAPQI associated damage (Masubuchi et al., 2005; Umbaugh et al., 2021) this could also explain the observation of partial uncoupling induced by NAPQI in the FAO assay results. However, these studies are not about toxicity but functionality. As with acetaminophen the impact of NAPQI on other key mitochondrial parameters was also investigated using stress compounds. Because NAPQI had such a devastating effect on OCR, it is difficult to evaluate the impact on any of the parameters except to say even small amounts of NAPQI could be devastating for mitochondrial energy generation. It is worth mentioning that nonmitochondrial OCR contributes to a small proportion of total OCR in most of the cells including this study and involve processes that are not directly relevant to thermogenesis and therefore was not a prime focus of our current study. In addition, the reduction of basal OCR with no further impact on nonmitochondrial OCR as well as proton leak confirms that NAPQI had a significant direct impact on the processes linked to mitochondrial OCR.

555 The inhibition of mitochondrial fatty acid uptake and oxidation by either acetaminophen or
556 NAPQI may explain the accumulation of long chain acylcarnitines and fatty acids in the serum
557 of acetaminophen treated WT mice (Chen et al., 2009). This study is the first to report the
558 direct inhibition of fatty acid uptake and oxidation by acetaminophen and the metabolite
559 NAPQI in adipocytes. These novel observations suggest the probable targets of acetaminophen
560 and other compounds which cause a reduction in fever or induce hypothermia may not be
561 related to cyclooxygenase enzyme inhibition alone as previously assumed. Instead these studies
562 provide an alternative explanation as to why despite their weak inhibition of the
563 cyclooxygenase enzymes these compounds are such potent antipyretics.

564 To determine exactly where in the mitochondria acetaminophen may be targeting, an electron
565 flow assay was used along with specific mitochondrial complex inhibitors to assess electron
566 flow through different ETC complexes. Acetaminophen caused a significant reduction in OCR
567 when either complex I or complex II substrates were used suggesting acetaminophen may be
568 disrupting electron transport generally in the mitochondria. Previous i*n vitro* and *in vivo* studies
569 have shown that this drug has a number of targets including mitochondrial membrane potential,
570 the movement of electrons from complex I to complex III and ATP production (Ohba et al.,
571 2016; Nazareth et al., 1991; Masubuchi et al., 2005; Kon et al., 2004; Porter and Dawson, 1979;
572 Martin and McLean, 1995; Mingatto et al., 1996). Other studies show inhibition of state 3
573 respiration by acetaminophen which reflects some interference with the ETC but not
574 uncoupling of oxidative phosphorylation (Somasundaram et al., 1997, 2000). Reduction in
575 mitochondrial function has also been reported for other antipyretics including aspirin,
576 diclofenac sodium, mefenamic acid, and piroxicam, all with antipyretic properties. However in
577 some areas the pattern of inhibition of these compounds was different to acetaminophen.
578 Indomethacin, aspirin, naproxen, and piroxicam were shown to uncouple oxidative

phosphorylation of isolated rat liver mitochondria in micromolar concentrations whereas acetaminophen did not.

In terms of how the results in this study can be linked to hypothermia and antipyresis, there are widespread in vivo studies particularly in mice which have been used to explain the effect of acetaminophen in humans. The studies generally show that at doses of 100-500 mg/kg, antipyresis or hypothermia and toxicity is observed, and this translates to about (1-10 mM) in vitro according to many studies (Flower and Vane, 1972; Ayoub and Flower, 2019; Ayoub et al., 2004; Fukushima et al., 2017, Gentry et al., 2015; Massey et al., 1982; Walker et al., 1981; Orbach et al., 2017;Rivera et al., 2017; Ahmed et al., 2011; Messner et al., 2013; Agarwal et al., 2011). By contrast at lower doses; <100 mg/kg there is no evidence of antipyresis or hypothermia in mice, we see a similar effect for humans with a low equivalent dose of 480-560 mg/day not being antipyretic (Nair and Jacob, 2016; Reagan-Shaw et al., 2008; Mirrasekhian et al., 2018; Li et al., 2008). Regardless of the extent of inhibition *in vivo*, during fever or under cold stress conditions only a minor attenuation of energy production would be required to reduce fever or cause hypothermia in small mammals in temperatures below their thermoneutral zone. We are arguing that you need some mitochondrial disruption (without cell death) to see antipyresis or hypothermia. More importantly many studies show that these antipyretic and hypothermic doses in mice also translate to the therapeutic dose equivalents of acetaminophen in humans, making these in vitro studies (and the related concentrations) relevant to our understanding of the mechanism of antipyresis and hypothermia (Jetten et al., 2012; Reagan-Shaw et al., 2008; Nair and Jacob, 2016; Heard et al., 2011; Foster et al., 2016). The observation of a negative impact of acetaminophen on various aspects of mitochondrial function is not new, however this is the first time that these observations have been suggested as an explanation for hypothermia or antipyresis. These studies should provide the momentum for the development of new safer antipyretics which do not compromise the immune system

and can be used to treat fever in patients with infections such as COVID-19 where continued immune function is essential.

Conclusion

This is the first study to suggest that the antipyresis and hypothermia observed in vivo are the direct effects of the impact on mitochondrial function rather than any putative inhibition of the cyclooxygenase enzymes which has remained an unproven hypothesis. Adipocytes play a critical role in thermogenesis, particularly in small mammals at temperatures below their thermoneutral zone. The current study explores for the first time using acetaminophen and the metabolite NAPQI, other potential targets in the thermogenesis pathway including fatty acid uptake, β-oxidation of fatty acids liberated from lipolysis, and most of the complexes of the ETC in both 3T3-L1 adipocytes and isolated mitochondria and using the Agilent Seahorse XF technology. Ultimately this study reveals both new knowledge and new understanding about how acetaminophen could work in relation to antipyresis and hypothermia.

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Abbreviations

NAPQI	N-acetyl-p-benzoquinone imine
FAO	Fatty acid oxidation
OCR	Oxygen consumption rate
COX-2	Cyclooxygenase-2
Ta	Ambient temperature
Tc	Core body temperature

ETC	Electron transport chain
FBS	Foetal bovine serum
IBMX	Isobutylmethylxanthine
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
MAS	Mitochondrial assay solution
MPT	Mitochondrial permeability transition
TMPD	N1,N1,N1,N1tetramethyl-1,4-phenylene diamine
SD	Standard deviation
ANOVA	Analysis of variance
UCP1	Uncoupling protein 1
CPT1	Carnitine palmitoyltransferase 1
FFA	Free fatty acids

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