Inhibition of lipolysis: a novel explanation for the antipyretic and hypothermic actions of Acetaminophen

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Abstract

Background: Acetaminophen is both widely used to treat children with fever and is also responsible for thousands being hospitalised annually. Historically the antipyretic actions of acetaminophen was attributed to the inhibition of cyclooxygenase (COX-1/2) enzymes and more recently a novel COX-1 variant (COX-3) located in the brain. However, the evidence for acetaminophen-mediated COX inhibition remains contentious. This study assesses the impact of acetaminophen and other putative COX-3 inhibitors on the release of fatty acids during lipolysis as an alternative mechanism by which antipyretics could reduce body temperature during fever.

Experimental: 3T3-L1 adipocytes, primary brown adipocytes and isolated mitochondria were exposed to COX-3 inhibitors and lipolysis and mitochondrial electron transport chain function assessed.

Results: Acetaminophen, aminopyrine and antipyrine at 1-10 mM caused a significant decrease (up to 70%; $P<0.01$, from control) in lipolysis within 1, 3 and 24 hours without affecting cell viability. The inhibition was observed regardless of where along its signalling pathway lipolysis was stimulated. All three compounds were found to significantly attenuate mitochondrial function by up to 30% for complex I and 40% for complex II ($P<0.01$, from control).

Conclusions: These novel observations combined with the known limited inhibition of the COX enzymes by acetaminophen suggest both the antipyresis and hypothermia induced by acetaminophen and related compounds could be attributed to the direct inhibition of lipolysis and mitochondrial function, rather than cyclooxygenase inhibition centrally. Further these observations could provide new drug targets for reducing fever with the added bonus that fewer individuals being hospitalized by accidental acetaminophen overdose.

Keywords: acetaminophen, hypothermia, cyclooxygenase, lipolysis, mitochondria, electron transport chain.

Introduction

Acetaminophen (Paracetamol) is one of the oldest and most widely used treatments for pain, inflammation and Pyresis (fever) worldwide. With such a broad spectrum of activity many

questions remain about the exact mechanisms of action of acetaminophen. The analgesic properties are thought to be due to actions centrally on opioid, serotonergic and cannabinoid pathways (Sharma & Mehta, 2014) [1] . The anti-inflammatory and antipyretic actions of acetaminophen has traditionally been attributed to inhibition of the cyclooxygenase (COX) enzymes centrally (Hinz *et al*., 2008) [2]. However there is now extensive evidence to show that acetaminophen has only weak anti-inflammatory properties and this has been attributed to its limited inhibition of the COX-1 and COX-2 enzymes (Chandrasekharan *et al*., 2002; Botting & Ayoub 2005[3] [4]). In mammals fever is associated with increased COX-2/PGE_2 induction and activity. With the lack of COX inhibition by acetaminophen another target for the antipyretic actions of this widely used compound needed.

During the last 20 years, many studies have revealed that the administration of acetaminophen (>100 mg/kg) to non-febrile rodents, for which there is no COX-2 induction results in hypothermia. One explanation which initially gained significant support was that acetaminophen is inhibiting a novel COX-1 variant (COX-3). It was proposed that the protein (COX-3) which was suggested to be specifically inhibited by acetaminophen is constitutively expressed in rodents to regulate body temperature (Chandrasekharan *et al*., 2002; Ayoub *et al* 2004) [3] [5]. The COX-3 hypothesis was further supported by the observation that other drugs such as aminopyrine and antipyrine which are also antipyretics, have been suggested to be putative COX-3 inhibitors, also produce hypothermia in mice (Chandrasekharan *et al*, 2002; Ayoub *et al*, 2004) [3] [5]. However, the hypothermic actions of acetaminophen is also observed in non-febrile animals such as the rat and in humans where the COX-3 protein cannot theoretically be expressed (Foster *et al* 2016; Kis *et al*, 2005) [6] [7]. Consequently an alternative hypothesis is needed to explain the acetaminophen-induced hypothermia observed in non-febrile animals and this could provide an explanation for the antipyretic actions of acetaminophen in humans.

In terms of thermoregulation, rodents (homeotherms) are capable of maintaining their core body temperature (Tc) within a fairly constant range (36.0-37.5°C). However, due to their large surface area to mass ratio, small mammals must increase their metabolic heat production to regulate Tc when housed at temperatures below their thermoneutral zone (Gordon, 2012) [8]. To maintain Tc, rodents rely on the stimulation of lipolysis, a catabolic process resulting in the conversion of triglycerides (TG) stored in adipocytes to free fatty acids (FA) and glycerol, the FA are then oxidised in the mitochondria to generate heat (Gordon, 2012; Luo & Liu, 2016) [8] [9]. During periods of cold stress, in the case of

laboratory mice temperatures below 30°C, the process begins with the release of adrenergic neurotransmitters from sympathetic fibres which binds to β_3-receptors located on surface of adipocytes leading to the activation of adenylate cyclase. This initiated an increase in cAMP levels, the activation of Protein Kinase A (PKA) and the phosphorylation of the lipolytic enzymes initiating lipolysis (Luo & Liu, 2016; Ueta *et al*., 2012) [9] [10]. A similar process of brown adipose tissue activation and lipolysis is also observed in humans following prolonged cold exposure (Lesna´ *et al*., 1999) [11].

In most laboratory situations, mice are housed at around 20–24°C (Gordon, 1993, 2004) [12] [13]. With their thermoneutral zone around 30°C they must respond to the mild cold stress by increasing their basal metabolic rate to avoid hypothermia (Gordon, 1985, 1990, 1993) [14] [15] [12]. It is therefore reasonable to hypothesize that animals housed under such conditions may be more susceptible to hypothermia if exposed to compounds which even mildly decrease the availability of FA and or compromise mitochondrial function.

To better understand the mechanisms by which acetaminophen and other related antipyretics could either reduce fever or induce hypothermia in non-febrile animals. Studies were undertaken to assess the impact of acetaminophen, aminopyrine and antipyrine on rate of lipolysis in adipocytes and on the functioning of key complexes in the electron transport chain of isolated mitochondrial.

Methods and Materials

Chemicals

All chemical were supplied by Sigma Chemical, Dorset, UK unless stated otherwise.

Cell culture and differentiation of 3T3 L1 pre-adipocytes.

The mouse 3T3-L1 pre-adipocyte cells (Public Health England, U.K.) were maintained under standard culture conditions (37°C, 5% CO_2, 95% air) with medium containing DMEM supplemented with 10% FBS, 2 mM glutamine, 10,000 units penicillin/ml, 10 mg streptomycin/ml (5 ml/500 ml of medium) and 250 μg/ml amphotericin B (5 ml/500 ml of

medium)**.** Cell were seeded at 10-20,000 cells/cm² and sub-cultured every 3 days at about 70-80% confluent using 0.25% trypsin and 0.03% EDTA.

For differentiation the cells were allowed to become confluent then allowed to grow for four additional days and this was treated as Day 0. At day 1, cells were placed in DMEM differentiation medium with 10% fetal bovine serum, 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 after every 48 hours from this stage until fully differentiated 3T3-L1 adipocytes formation: Public Health England, U.K. (Zebisch *et al*., 2012) [16]. Differentiation was confirmed by Oil Red O staining (Moreno-Aliaga & Matsumura, 1999) [17]. Viability was assessed by the MTT assay (Van Munster, *et al*., 1993) [18].

Isolation of rat primary brown adipocytes

Primary brown fat adipocytes was isolated from Wistar rats (200g) purchased from Envigo UK. The animals were killed by cervical dislocation and brown adipose tissue from the interscapular depots was dissected out and placed on a parafilm in a small volume of Krebs/Ringer phosphate buffer. The cells were isolated as described by (Cannon & Nedergaard, 2008) [19]. Once isolate the cells were incubated in a 24 well place and viability and lipolysis determined.

Determination of lipolysis assay in differentiated 3T3-L1 adipocytes and brown adipocytes.3

For all experiments the cells were incubated in serum-free medium for 2 hours (Schweiger *et al*., 2014) [20]. The cells were then exposed to different concentrations of the lipolysis stimulants in medium containing 2% BSA (fatty acid free) and glycerol release was measured after 1, 3 and 24 hours, for basal lipolysis no stimulant was added. In tests involving the inhibitors (acetaminophen, aminopyrine and antipyrine) they were added 30 minutes before the stimulants. At the end of the incubation period the 25 µL of the incubation medium would be taken for storage at -20˚C for assay later. To determine the level of glycerol the 25 µL of each sample was transferred to a new 96-well plate and 100 µL of glycerol reagent was added and read at 540 nm (Sigma-Aldrich, U.K.). The concentration of glycerol was determined from a calibration curve (Luo & Liu, 2016) [9].

Isolation of mitochondria homogenate.

Mitochondria was removed and isolated from Wistar rats (200g) as described by Sandoval-Sandoval-Acuna, *et al.*, (2012) [21].

Determination of Complex I activity

The activity of NADH-ubiquinone oxidoreductase (complex 1) was determined by measuring the reduction of 0.05mM of the electron acceptor 2,6-dinitrophenolindophenol (DCPIP) and NADH (0.05mM) in the presence of either acetaminophen, aminopyrine or antipyrine at 600nm. Complex 1 activity was confirmed using complex 1 inhibitor rotenone (0.1mM). The reaction mixture consisted of potassium phosphate buffer (35mM, pH 7.4) in the presence and absence of KCN and antimycin A. The reaction was started by adding the mitochondria homogenate (6.22mg/ml) and incubated for 10 minutes at room temperature (37ºC).

Determination of Complex II activity

The activity of Succinate–Ubiquinone Oxidoreductase activity (Complex II) activity was determined by measuring the reduction of DCPIP (0.05mM) in the presence of succinate (5-100mM) and either acetaminophen, aminopyrine or antipyrine, or a range of antipyretic agents at 600nm. The reaction mixture consisted of potassium phosphate buffer (35mM, pH 7.4) in the presence and absence of KCN (0.5mM) and antimycin A (0.5mM) over 5-100mM of succinate. Complex II activity was confirmed using malonate (0.1mM).The reaction was started by adding the mitochondria homogenate (6.22mg/ml) and incubated for 10 minutes at room temperature (37ºC).

The results were analysed using analysis of variance (ANOVA), followed by Dunnett's Multiple Comparison Test. *$P < 0.05$, ** $P<0.01$, from control was considered statistically significant.

Results:

The effect of Acetaminophen, Aminopyrine and Antipyrine on basal lipolysis in rat primary brown adipocytes.

Prior to the lipolysis studies, the cells were assessed for the impact of acetaminophen (PA), aminopyrine (AP) and antipyrine (AT) at concentrations up to 10 mM on cell viability. The concentrations of the antipyretics used in these studies were within the range normally associated with hypothermia *in vivo*: Fischer *et al*., (1981) [22]; Orbach *et al*., (2017) [23]. The compounds had no effect on the viability of primary adipocytes (24 hours) and up to 48 hours for 3T3-L1 cells (data not shown).

Glycerol release was assayed as an indicator of the extent of lipolysis Schweiger *et al*., (2014) [20]. The primary brown fat adipocytes had a high level of basal glycerol release which did not significantly increase on stimulation. Treatment of primary brown adipocytes with acetaminophen at concentrations (1 mM and 10 mM) reduced basal glycerol release (10% and 19%) at 1 hour and (19% and 26%) at 24 hours (Figure 3.1). Similarly, aminopyrine (1 mM and 10 mM) attenuated glycerol release (22% and 17%) at 1 hour and (34% and 26%) at 24 hours (Figure 3.2). Antipyrine treatment resulted in a smaller but significant decrease in glycerol release (10% and 11%) at 1 hour and (21% and 19%) at 24 hours (Figure 3.3).

Effect of Acetaminophen on basal lipolysis in 3T3-L1 adipocytes

In unstimulated 3T3-L1 adipocytes incubated with acetaminophen at concentrations up to 10mM there was a significant decrease in glycerol release indicating a reduction in basal lipolysis similar to the primary brown adipocytes. This inhibitory effect can be seen from 1 hour with a 47% decrease in glycerol levels, followed by 57% and 52% at 3 and 24 hours respectively suggesting maximum lipolysis occurs within hours of exposure to acetaminophen (Figure 3.4).

Effect of Acetaminophen on Isoproterenol stimulated lipolysis in 3T3-L1 adipocytes

The effect of acetaminophen on lipolysis stimulated by isoproterenol a β-adrenergic agonist was then examined. Addition of isoproterenol elevated glycerol release from adipocytes as compared with that under basal conditions (Figure 3.5A). The glycerol release was both concentration and time dependent over 24 hours although the greater impact was with incubation time (Figure 3.5-3.6).

Addition of acetaminophen attenuated the glycerol release in a concentration dependent manner. Typically at the concentration of 0.001 μM isoproterenol, 1mM of acetaminophen attenuated glycerol release by 41% whereas at 10mM acetaminophen the attenuation was 54%. A similar pattern was seen at 0.01 μM and 0.1 μM isoproterenol (Figure 3.5-6 B-D).

Effect of Acetaminophen on Forskolin stimulated lipolysis in 3T3-L1 adipocytes

In order to assess the post receptor impact of acetaminophen, the adenylate cyclase activator forskolin was used (Bezaire *et al*,. 2009) [24]. Forskolin stimulated lipolysis in the 3T3-L1 cells (Figure 3.7). Prior exposure to acetaminophen (10 mM) attenuated the lipolysis induced by forskolin by 32%, 35% and 46% at 1, 3 and 24 hours in Figure 3.7 B,D,F).

Effect of acetaminophen on 8-Br-cAMP stimulated lipolysis in 3T3-L1 adipocytes

The cAMP analog; 8-Br-cAMP stimulated lipolysis in the 3T3-L1 cells (Figure 3.8). In the cells in the presence of acetaminophen (10 mM) glycerol release was significantly attenuated at 3 hours (45%) and still persisted (40%) at 24 hours (Figure 3.8B) confirming that the acetaminophen effect could also be located beyond the cAMP level.

Effect of Aminopyrine and Antipyrine on basal lipolysis in 3T3-L1 adipocytes

In addition to acetaminophen other putative COX-3 inhibitors have also been shown to induce hypothermia including aminopyrine and antipyrine. In this study both compounds attenuated basal lipolysis at 1 and 24 hours to an extent similar to acetaminophen (Figure 3.9).

The effect of Acetaminophen Aminopyrine and Antipyrine on mitochondrial activity.

Studies were also undertaken to determine the effect of acetaminophen, aminopyrine and antipyrine on the activity of complex I and complex II. At concentrations up to 10mM all the antipyretic compounds were found to inhibit complex I activity by decreasing the rate of rotenone sensitive NADH oxidation between 20-30% (Figure 3. 10). Similarly the

compounds also attenuated complex II activity as demonstrated by the inhibition of malonate sensitive reduction DCPIP between 20-40% (Figure 3.11). The inhibition appears to be more pronounced at higher succinate concentrations.

Discussion:

Acetaminophen is probably the most widely used over the counter antipyretic worldwide, particularly for infants. Although it is very effective, there is still limited understanding about how it lowers body temperature in humans and in febrile animal models. The situation is further complicated because acetaminophen also causes hypothermia in non-febrile animals. One consequence of our lack of understanding of the exact mechanisms of action of acetaminophen is that every year thousands die or are hospitalised following acetaminophen use. A better understanding would allow the development of safer alternatives

The focus of the present study is to understand how acetaminophen, and other putative COX-3 inhibitors; aminopyrine and antipyrine could disrupt key cellular processes which determine heat generation, with a view to gaining a novel insight into the mechanism of action of antipyretics generally.

For homeotherms such as laboratory rodents housed at temperatures below their thermoneutral zone there is a constant need to increase heat generation to avoid hypothermia. When homeotherms detect the external temperature is below their thermoneutral zone, a number of key responses are observed. One of the most important starts with signals from the preoptic area of anterior hypothalamus (POA) which stimulates peripheral effectors leading to the increase in lipolysis augmenting the amount of free fatty acids available for mitochondrial metabolism and heat generation. Given the lack of agreement about the effectiveness of acetaminophen at inhibiting cyclooxygenases centrally and peripherally, it is reasonable to examine the impact of this key antipyretic at other points along the mammalian heat generation pathway starting with lipolysis.

Lipolysis is a critical metabolic function for adipocytes and is the key process that provides fatty acids for energy generation and to activate uncoupling protein 1, both are important for thermogenesis (Ohlson *et al*., 2004) [25]. Conversion of stored triglycerides into free fatty

acids (lipolysis) is stimulated by β-adrenergic signalling on the cell surface of adipocytes. This leads to the activation of adenylate cyclase, increased levels of cAMP, the activation of PKA and the phosphorylation and activation of lipolytic enzymes including *hormone sensitive lipase*, *adipose triglyceride lipase*, and perilipin (Chrysovergis *et al*., 2014; McKnight *et al*., 1998; MacPherson & Peters, 2015; Schweiger *et al*., 2006; Heeren & Münzberg 2013; Marcelin & Chua, 2010) [26][27] [28] [29] [30] [31]. An essential requirement for all these process, from cell surface receptor signalling pathway, through to lipolysis and oxidation of the fatty acids, is the availability of ATP and functioning mitochondria. Any disruption or inhibition of mitochondrial function could potentially attenuate lipolysis with the consequent impact on heat generation leading to hypothermia.

In this study prior to the lipolysis studies, the 3T3-L1 adipocyte cells were assessed for any direct impact of the antipyretics on viability. The concentrations selected (up to 10 mM) were similar to or lower than the plasma concentrations known to induce hypothermia in animals models and more importantly within the plasma concentrations required for antipyresis or hypothermia in humans (Fischer *et al*., 1981; Orbach *et al*., 2017) [22] [23]. At these concentrations over 24 hours, there was no loss of cell viability indicating that the effects observed could not be attributed to the loss of adipocytes.

To examine whether the acetaminophen, aminopyrine and antipyrine could have a direct impact on lipolysis, the extent of glycerol was assessed. This is the most frequently used *in vitro* model and method to study lipolysis. To ensure detectable levels of lipolysis the adipocytes were incubated in a low glucose medium which forces the cells to metabolise fats for energy. Under basal conditions and without the presence of an adrenergic agonist, acetaminophen at 10 mM was found to significantly reduce glycerol release as early as 1 hour. The fact that acetaminophen inhibited basal lipolysis suggested the effect could also be at a post receptor site or totally independent of adrenergic stimulation.

To further investigate the impact of acetaminophen on the catecholamine receptor pathway, the β-agonist isoproterenol was selected to stimulate lipolysis. Adrenergic stimulated lipolysis is more likely to be observed in animals under cold stress such as rodents housed at 22˚C. In these studies acetaminophen also inhibited isoproterenol induced lipolysis. This observation prompted the question as to where along the adrenergic pathway acetaminophen was acting. The attenuation of the basal lipolysis has already confirmed that at least some of the actions acetaminophen is independent of adrenergic receptor binding. However, to

investigate whether acetaminophen was affecting the binding to the β-receptor, forskolin was used. A direct activator of adenylate cyclase, forskolin was able to stimulate lipolysis and which was also attenuated by acetaminophen (Bezaire *et al*,. 2009) [24]. This observation suggests acetaminophen inhibition is less likely to be at the β-receptor as forskolin work downstream of receptor binding.

The next step was to investigate the effect of acetaminophen beyond the adenylate cyclase stage of the signalling pathway. To determine the impact of acetaminophen post adenylate cyclase, the cAMP analog 8-Br-cAMP was employed. This compound is able to enhance the activity of protein kinase A thereby phosphorylating the lipolytic enzymes without activation of adenylate cyclase. The addition of 8-Br-cAMP induced an increase in lipolysis and this was attenuated by acetaminophen, confirming that the effect of must be distal to the production of cAMP. These observation confirm that the impact of acetaminophen was not on the adrenergic signalling pathway as acetaminophen inhibition was observed regardless of where in the signally pathway lipolysis was stimulated.

In addition to acetaminophen, the effect of aminopyrine and antipyrine were also examined as these compounds are known to cause hypothermia *in vivo* in mice and were reported to be COX-3 inhibitors *in vitro* (Ayoub *et al* 2004; Chandrasekharan *et al*, 2002) [5] [3]. Antipyrine (phenazone) exhibits analgesic, antipyretic and antirheumatic activity. Aminopyrine (aminophenazone) is thought to possess greater antipyretic and analgesic activity, marked anti-inflammatory property but more toxic than antipyrine (Volz & Kellner, 1980) [32]. However like acetaminophen, little is understood about the impact of these compounds on lipolysis and how this relates to thermogenesis. In the present study, both aminopyrine and antipyrine were effective at significantly attenuating glycerol release in a manner similar to acetaminophen. This is a novel observation and like acetaminophen could be linked to antipyresis.

Although the differentiated 3T3-L1 cells are the most widely used *in vitro* model for studying lipolysis and fat metabolism, investigations were also undertaken using freshly isolated rat primary brown adipocytes. As primary cells they should provide a more realistic picture of the effect of the test compound on lipolysis *in vivo*. The primary brown adipocytes cells showed high levels of basal lipolysis which may reflect the fact that they had recently been removed from animals that have acclimatized to living at ambient temperatures which are below their thermoneutral zone and so require constant heat generation. The high level of

basal lipolysis made it difficult to increase the level of lipolysis. However the compounds (acetaminophen, aminopyrine and antipyrine) inhibited lipolysis in the primary cells in a manner similar to the 3T3-L1 cells. Given the surface area to mass ratio of small mammals such as rodents any slight reduction heat generation would affect Tc. Consequently, when such animals are housed at temperatures below their thermoneutral zone, any reduction in lipolysis would compromise their ability to thermo-regulate. This may explain the hypothermia observed when these animals are exposed to acetaminophen, aminopyrine and antipyrine.

Although this is the first time acetaminophen, aminopyrine and antipyrine have been shown to inhibit lipolysis they are not the only antipyretic compounds which have been shown to inhibit lipolysis. Indomethacin has been shown to inhibit enzymes involved in basal and stimulated lipolysis in the kidney (Erman *et al*., 1980) [33]. Other NSAIDs (aspirin, naproxen, nimesulide, and piroxicam) are known to activate the NADPH oxidase (NOX) isoform (NOX4) in adipocytes to produce hydrogen peroxide (H_2O_2), which impairs cAMP-dependent PKA-II activation, thus inhibiting isoproterenol activated lipolysis. H_2O_2 signalling is a novel COX independent effect of NSAIDs in adipocytes and may play a role in antipyresis (Vázquez-Meza *et al*., 2013) [34].

Although lipolysis is a key step in the thermogenesis process, acetaminophen and other hypothermic compounds could also work at sites downstream to the release of fatty acids from triglycerides, for example by directly inhibiting mitochondrial function. Given the lack of inhibition of adrenergic signalling pathway in this study, further studies were conducted using isolated mitochondria. As previously stated, energy generation for thermoregulation require functioning mitochondria. In the present study all three compounds; acetaminophen, aminopyrine and antipyrine were shown to attenuated mitochondrial activity at complex I and complex II. Without fully functioning mitochondria both lipolysis and fatty acid oxidation will be compromised, and this would directly lead to hypothermia in small mammals such as rodents.

It has always been difficult to reconcile the pharmacological actions of acetaminophen particularly the antipyretic effects with the weak inhibition of cyclooxygenase enzymes. The novel observations in this study provide a logical and plausible explanation as to how acetaminophen could cause both hypothermia and antipyresis distinct from any impact on cyclooxygenase activity. Even at the levels of inhibition reported in this study this would

negatively impact on how small mammals thermoregulate at environmental temperatures below their thermoneutral zone. These observations could explain the hypothermia widely reported when some antipyretic compounds are administered to rodents at concentrations normally required for antipyresis. Similarly during fever hyperthermia is driven by increased metabolism including increased lipolysis and mitochondrial function. The attenuating effect on lipolysis and mitochondrial function of these antipyretic agents observed in this study could provide an alternative peripheral mechanism of the action for acetaminophen and many other compounds which is independent of cyclooxygenase inhibition both centrally and peripherally.

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Authors' contributions: WM, and SB participated in the overall planning of experiments, research design, data analysis & interpretation, and manuscript preparation. BE performed the experiments on isolated mitochondria.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, and Immunoblotting and Immunochemistry, and as recommended by funding agencies, publishers, and other organisations engaged with supporting research.

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Figure Legends

Figure 3.1 Effect of acetaminophen on basal lipolysis in rat primary brown adipocytes at 1 and 24 hours: *Brown adipocytes were treated with different concentrations of acetaminophen and glycerol release was measured at 1(A) and 24(B) hours. Data are representative of n=4 replicates expressed as means ± Standard deviations (**P < 0.01 from control).*

Figure 3.2 Effect of aminopyrine on basal lipolysis in rat primary brown adipocytes at 1 and 24 hours: *Brown adipocytes were treated with different concentrations of aminopyrine and glycerol release was measured at 1(A) and 24(B) hours. Data are representative of n=4 replicates expressed as means ± Standard deviations (**P < 0.01 from control).*

Figure 3.3 Effect of antipyrine on basal lipolysis in rat primary brown adipocytes at 1 and 24 hours: *Brown adipocytes were treated with different concentrations of antipyrine and glycerol release was measured at 1(A) and 24(B) hours. Data are representative of n=4 replicates expressed as means ± Standard deviations (**P < 0.01 from control).*

Figure 3.4 Effect of acetaminophen on basal lipolysis in 3T3-L1 adipocytes at 1, 3 and 24 hours: *Differentiated 3T3-L1 adipocytes were treated with serum free DMEM for 2 hours and were then treated with different concentrations of acetaminophen (PA). For basal*

*lipolysis, cells were treated at the same time with an appropriate volume of vehicle. Lipolysis was determined by measuring glycerol released into the culture media at 1(A), 3(B) and 24(C) hours. Data are representative of n=4 replicates expressed as means ± Standard deviations (*P < 0.05, **P < 0.01 from control).*

Figure 3.5 Effect of acetaminophen on isoproterenol stimulated lipolysis in 3T3-L1 adipocytes at 1 hour: *Differentiated 3T3-L1 adipocytes were treated with serum free DMEM for 2 hours. Cells were either treated with different concentrations of isoproterenol (Iso) alone (A) or treated with different concentrations of acetaminophen and isoproterenol added simultaneously (B-D). Lipolysis was determined by measuring glycerol released into the culture media at 1 hour. Data are representative of n=4 replicates expressed as means ± Standard deviations (*P < 0.05, **P < 0.01 from control).*

Figure 3.6 Effect of acetaminophen on isoproterenol stimulated lipolysis in 3T3-L1 adipocytes after 24 hours: *Differentiated 3T3-L1 adipocytes were treated with serum free DMEM for 2 hours. Cells were either treated with different concentrations of isoproterenol alone (A) or treated with different concentrations of acetaminophen and isoproterenol added simultaneously (B-D). Lipolysis was determined by measuring glycerol released into the culture media at 24 hours. Data are representative of n=4 replicates expressed as means ± Standard deviations (*P < 0.05, **P < 0.01 from control).*

Figure 3.7 Effect of acetaminophen on forskolin stimulated lipolysis in 3T3-L1 adipocytes at 1(A), 3(B) and 24(C) hours: *Differentiated 3T3-L1 adipocytes were treated with serum free DMEM for 2 hours. Cells were either treated with forskolin (Fsk) alone (A) or preincubated with different concentrations of acetaminophen for 30 minutes and then forskolin added (B). Lipolysis was determined by measuring glycerol released into the culture media at 1(A,B), 3(C,D) and 24(E,F) hours. Data are representative of n=4 replicates expressed as means ± Standard deviations (*P < 0.05, **P < 0.01 from control).*

Figure 3.8 Effect of acetaminophen on 8-Br-cAMP stimulated lipolysis in 3T3-L1 adipocytes at 3 and 24 hours: *Differentiated 3T3-L1 adipocytes were treated with serum free DMEM for 2 hours. Cells were either treated with 8-Br-cAMP alone (A) or preincubated with different concentrations of acetaminophen for 30 minutes and then 8-Br-cAMP added (C). Lipolysis was determined by measuring glycerol released into the culture media at 3(A,C) and 24(B,D) hours. Data are representative of n=4 replicates expressed as means ± Standard deviations (*P < 0.05, **P < 0.01 from control).*

Figure 3.9 Effect of aminopyrine and antipyrine on basal lipolysis in 3T3-L1 adipocytes at 1, 3 and 24 hours: *Differentiated 3T3-L1 adipocytes were treated with serum free DMEM for 2 hours and were then treated with aminopyrine (AM) or antipyrine (AT). Lipolysis was determined by measuring glycerol released into the culture media at 1(A), 3(B) and 24(C) hours. For basal lipolysis, cells were treated at the same time with an appropriate volume of vehicle. Data are representative of n=4 replicates expressed as means ± Standard deviations (**P < 0.01 from control).*

Fig 3. 10 The effect of antipyretic compounds on mitochondrial complex I activity. *Mitochondria were incubated with 5mM of either acetaminophen (Para) Antipyrine (AT) and Aminopyrine (AM) for 10 mins in the presence of NADH (0.05mM). The reduction of DCPIP at 600nm was monitored for 3 minutes. Values represents the mean ± SD (N= 7). The significance of difference was determined using a one way ANOVA followed by a Dunnett's multiple of comparison post hoc test. **p< 0.001 was statistically significant compared to the control.*

Fig 3.11 The effect of acetaminophen, aminopyrine and antipyrine on complex II activity in rat liver mitochondria. *A concentration of 5mM of the test compounds was added to a reaction mixture containing DCPIP and mitochondria suspension. Varying concentration of succinate (20mM-100mM) was used and absorbance at 600nm was monitored for 3mins. Values are expressed as the mean ± SEM (N= 16).*p<0.05 **p<0.01 and ***p <0.001*

compared to respective control (succinate). AM=Aminopyrine, AT= Antipyrine and PARA = Acetaminophen.

Figure 3.1

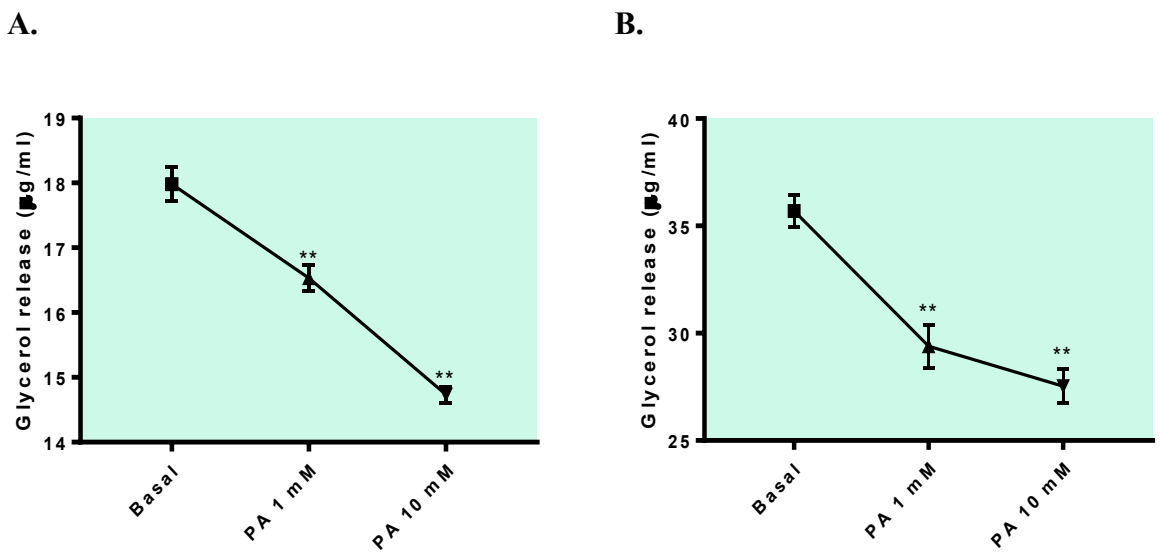
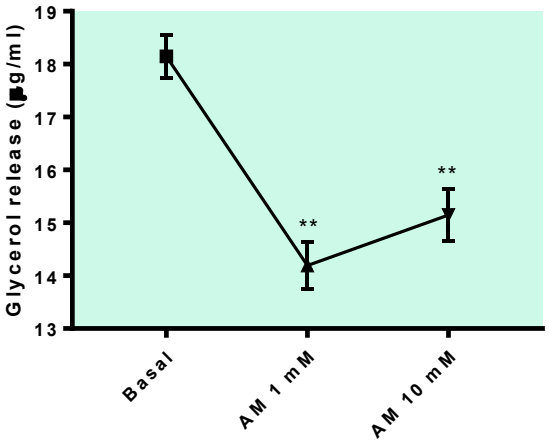

A.
B.
Glycerol release (µg/ml)
19
18
17
16
15
14
**
**
Basal
PA 1 mM
PA 10 mM
Glycerol release (µg/ml)
40
35
30
25
**
**
Basal
PA 1 mM
PA 10 mM

Figure 3.2

A.


Glycerol release (µg/ml)
19
18
17
16
15
14
13
**
**
Basal
AM 1 mM
AM 10 mM

B.

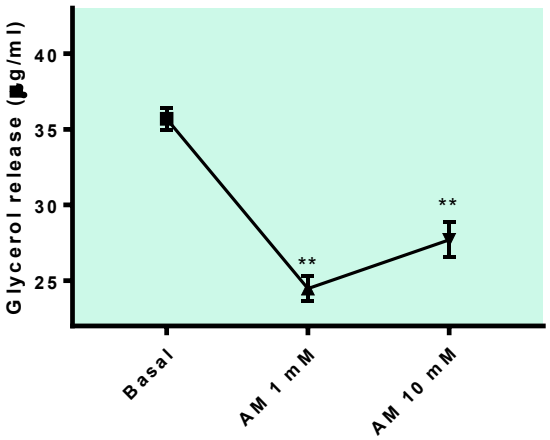

Glycerol release (µg/ml)
40
35
30
25
**
**
Basal
AM 1 mM
AM 10 mM

Figure 3.3

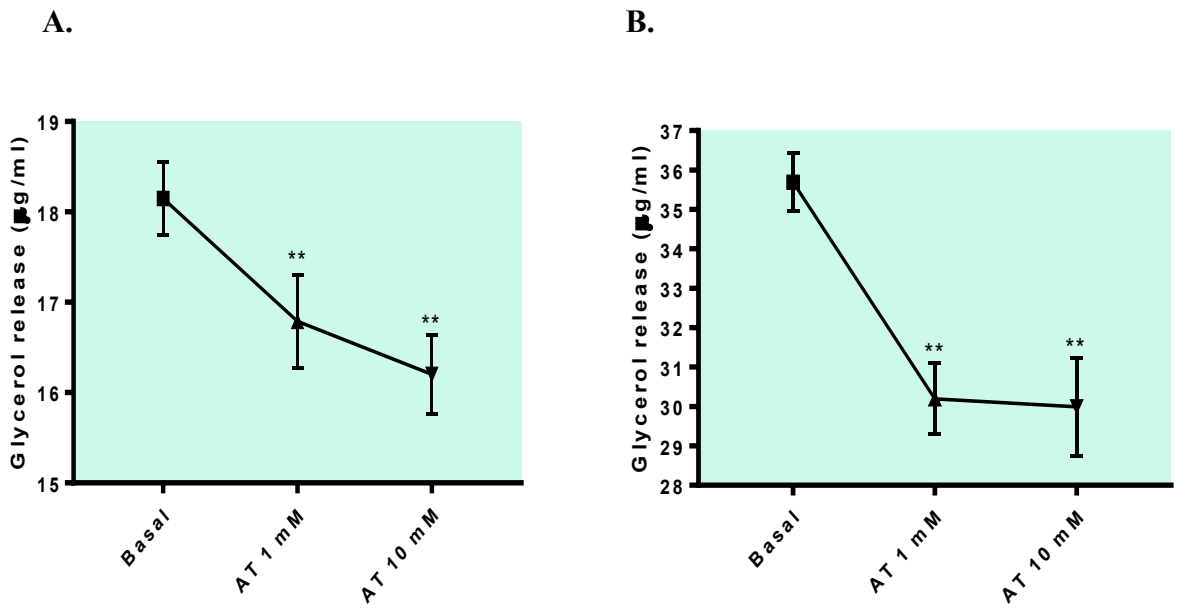

A.
B.
Glycerol release (µg/ml)
19
18
17
16
15
**
**
Basal
AT 1 mM
AT 10 mM
Glycerol release (µg/ml)
37
36
35
34
33
32
31
30
29
28
**
**
Basal
AT 1 mM
AT 10 mM

Figure 3.4

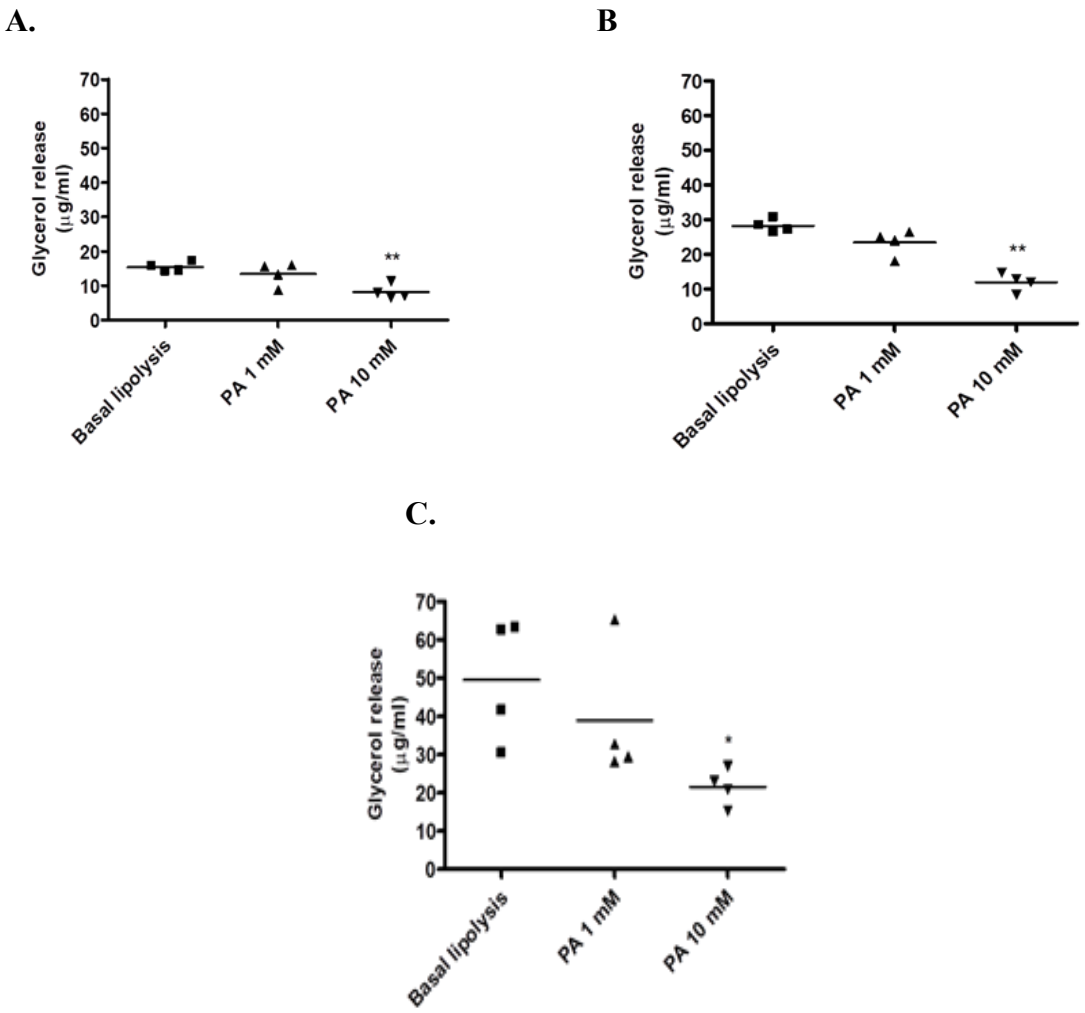

A.
B
C.
Glycerol release (µg/ml)
70
60
50
40
30
20
10
0
**
*
Basal lipolysis
PA 1 mM
PA 10 mM

Figure 3.5

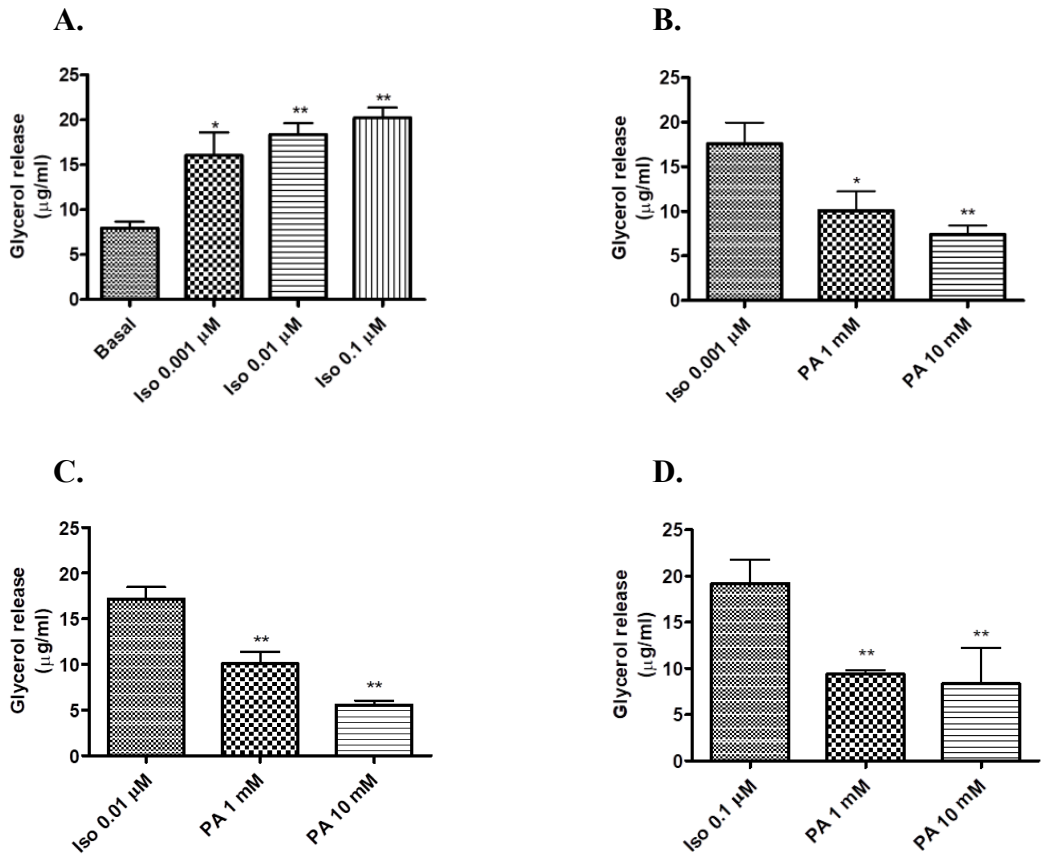

A.
B.
C.
D.
Glycerol release (µg/ml)
Basal
Iso 0.001 µM
Iso 0.01 µM
Iso 0.1 µM
PA 1 mM
PA 10 mM
*
**

Figure 3.6

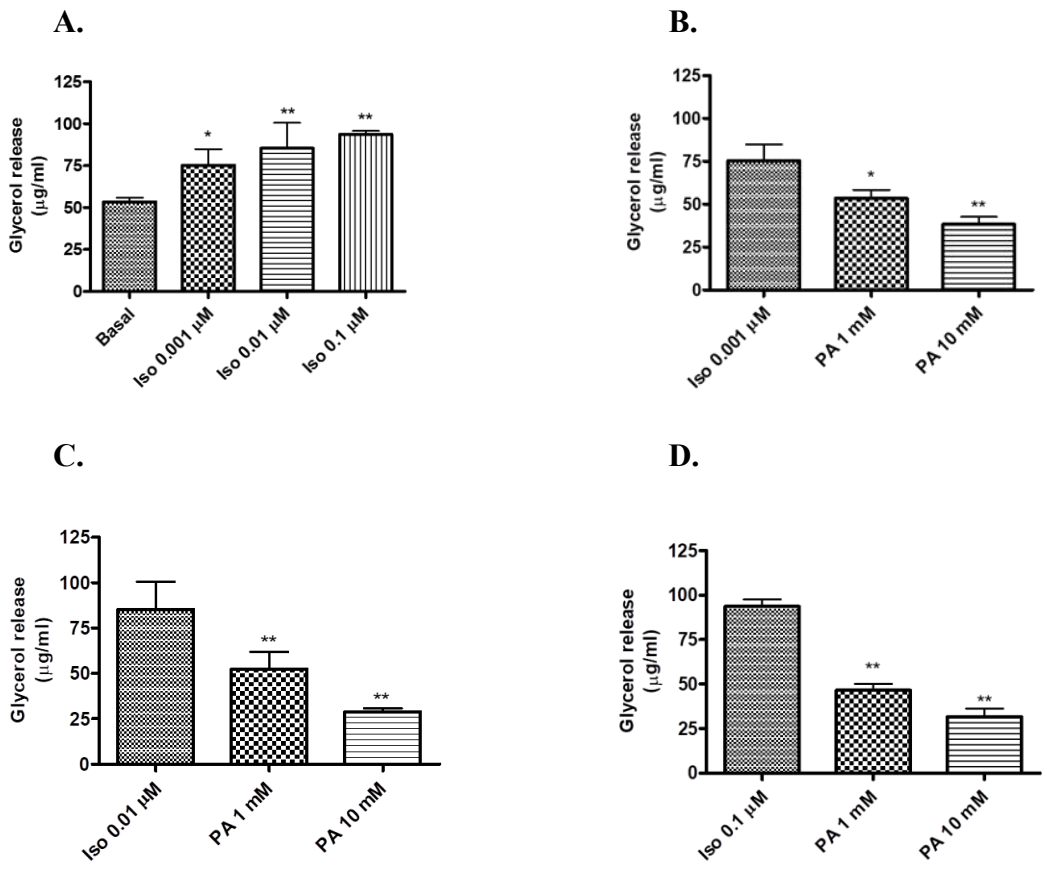

A.
Glycerol release (µg/ml)
125
100
75
50
25
0
*
**
**
Basal
Iso 0.001 µM
Iso 0.01 µM
Iso 0.1 µM
B.
Glycerol release (µg/ml)
125
100
75
50
25
0
*
**
Iso 0.001 µM
PA 1 mM
PA 10 mM
C.
Glycerol release (µg/ml)
125
100
75
50
25
0
**
**
Iso 0.01 µM
PA 1 mM
PA 10 mM
D.
Glycerol release (µg/ml)
125
100
75
50
25
0
**
**
Iso 0.1 µM
PA 1 mM
PA 10 mM

Figure 3.7

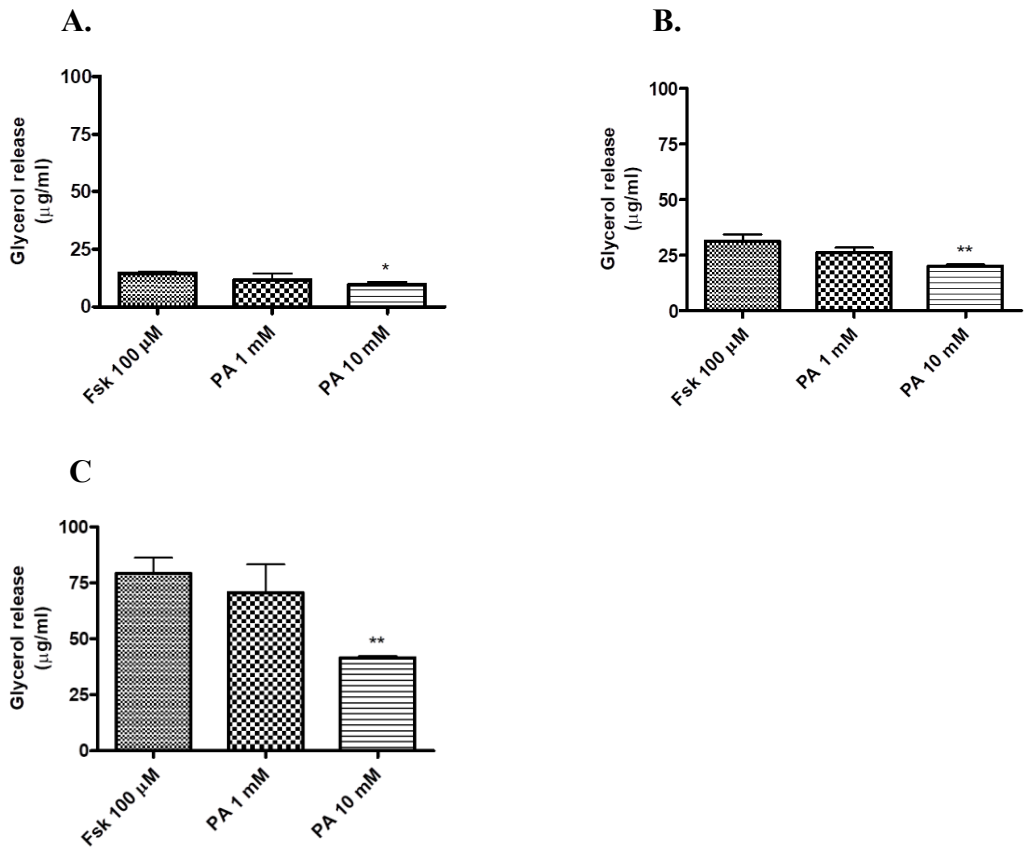

A.
B.
C
Glycerol release (µg/ml)
100
75
50
25
0
*
**
Fsk 100 µM
PA 1 mM
PA 10 mM

Figure 3.8

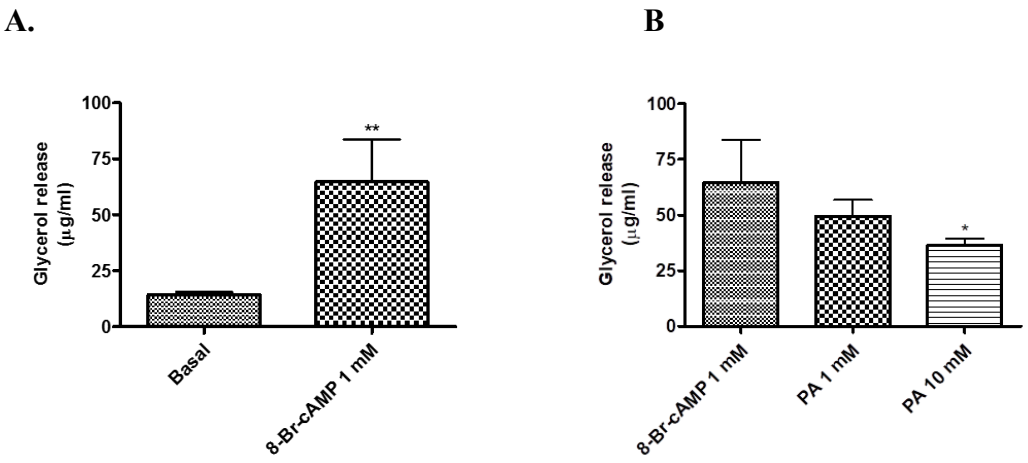

A.
B
Glycerol release (µg/ml)
100
75
50
25
0
**
Basal
8-Br-cAMP 1 mM
Glycerol release (µg/ml)
100
75
50
25
0
*
8-Br-cAMP 1 mM
PA 1 mM
PA 10 mM

Figure 3.9

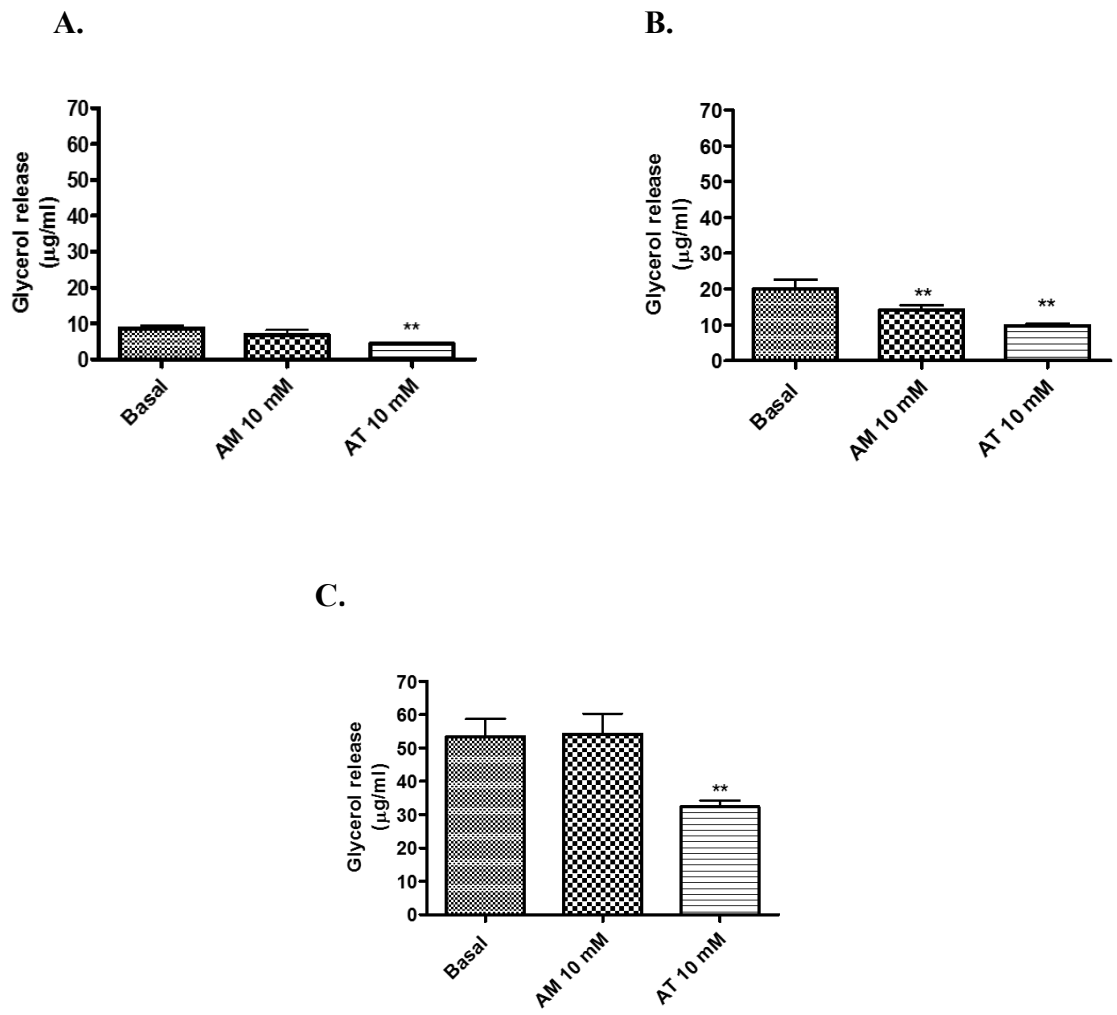

A.
B.
C.
Glycerol release (µg/ml)
70
60
50
40
30
20
10
0
**
Basal
AM 10 mM
AT 10 mM

Figure 3.10

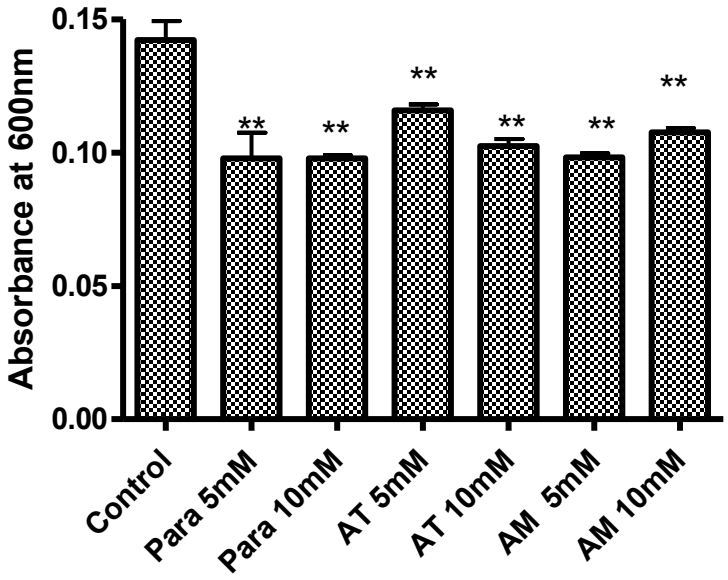
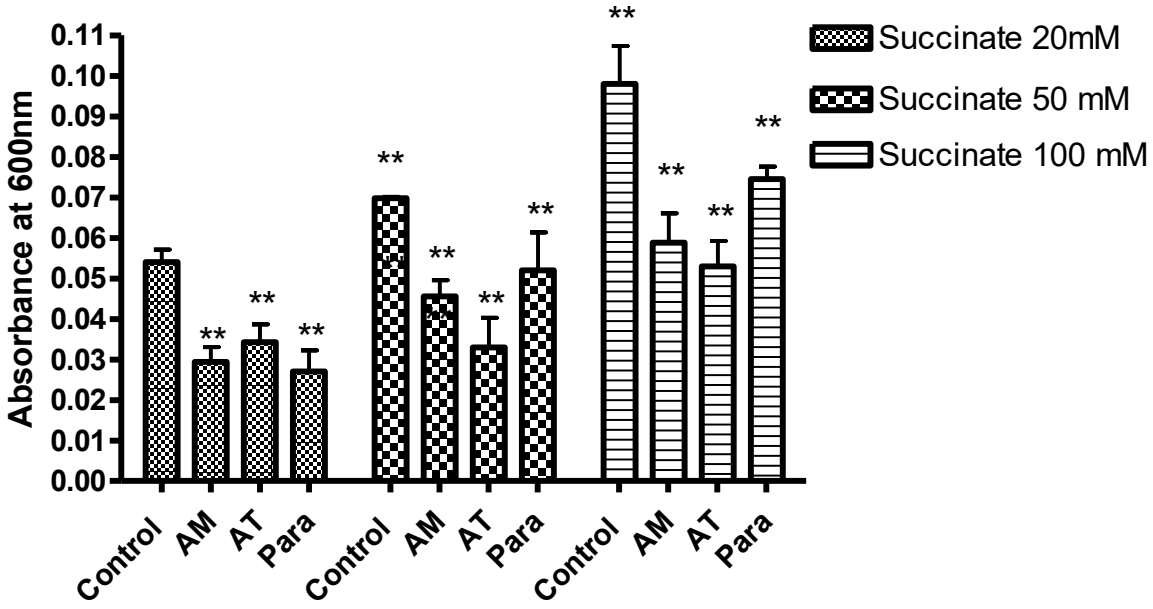

Absorbance at 600nm
0.15
0.10
0.05
0.00
**
**
**
**
**
**
Control
Para 5mM
Para 10mM
AT 5mM
AT 10mM
AM 5mM
AM 10mM

Figure 3.11


Absorbance at 600nm
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Control
AM
AT
Para
Control
AM
AT
Para
Control
AM
AT
Para
Succinate 20mM
Succinate 50 mM
Succinate 100 mM
**

Highlights

- In terms of antipyresis and hypothermia, the peripheral actions of acetaminophen and related compounds are more important than inhibition of cyclooxygenases centrally.
- The antipyresis and hypothermic effects of acetaminophen may be due to the inhibition of lipolysis.
- The inhibition of mitochondrial electron transport chain by acetaminophen may explain both the antipyresis and hypothermic effects.