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Hypothermic activity of acetaminophen; involvement of $GABA_A$ receptor, theoretical and experimental studies

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ABSTRACT

Objective(s): The mechanism of hypothermia action of acetaminophen (APAP) remains unclear even 125 years after its synthesis. Acetaminophen produces hypothermia. The mechanism of this reduction in core body temperature is not clear but evidence shows that it is not dependent on opioid and cannabinoid receptors. Because of strong documents about the roles of GABA and benzodiazepine receptors in hypothemic activity of some drugs such as diazepam, we determined if these receptors also contributes to the hypothermic effect of APAP.

Materials and Methods: Diazepam (5 mg/kg, IP) was used for induction of hypothermia. Flumazenil (10 mg/kg, IP) or picrotoxin (2 mg/kg, IP) used for reversal of this effect. Rats injected with APAP (100, 200 or 300 mg/kg, IP). Baseline temperature measurements were taken with a digital thermometer via rectum. To evaluate the structural correlation between APAP and benzodiazepine receptor ligands, numerous models are selected and studied at HF/6-31G* level of theory. Relative energies, enthalpies and Gibbs free energies were calculated for all selected drugs.

Results: Diazepam induced hypothermia was reversed by flumazenil or picrotoxin. Rats injected with APAP displayed dose- and time-related hypothermia. For combined administration, the hypothermic effect of APAP (200 mg/kg) was strongly reduced by pretreatment with picrotoxin or flumazenil $P<0.0001$and $P<0.01$, respectively. Selective structural data, bond length, dihedral angles, and related distance in pharmacophore of APAP and BZD_R models were the same. Some significant structural analogues were obtained between these drugs.

Conclusion: Results suggest hypothermic action of acetaminophen may be mediate by its effect at $GABA_A$ benzodiazepine receptor.

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Introduction

Acetaminophen (APAP) is widely used as an analgesic and antipyretic drug (1). It also induces hypothermia in non-febrile humans, mice and rats (2-5). APAP is often classified as a nonsteroidal anti-inflammatory drug (NSAID), but differs from other NSAIDs because it does not reduce inflammation (by inhibiting COX-2) or cause ulceration and bleeding of the stomach mucosa (by inhibiting COX-1) (6). In addition, unlike the aspirin-like drugs, APAP does not inhibit platelet aggregation or increase bleeding time (6). The mechanism of APAP hypothermia, especially in regard to the receptors, is poorly understood. APAP induced hypothermia in mice is accompanied by a reduction in brain levels of prostaglandin E2 (PGE 2). COX-3 has been proposed to account for this hypothermic effect (5, 7, 8). However, there is no consistent evidence in the literature that central PGE 2 participates in the control of normal body temperature in any species, and a recent study demonstrated that APAP produces hypothermia in mice by a COX-3-independent mechanism (5).

Benzodiazepine receptors (BZD_R) are an integral part of $GABA_A$ receptor/chloride ionophore complex, which is involved in regulating the coupling between $GABA_A$ receptors and opening of the chloride ion channels (9). Animal studies have shown that diazepam and other benzodiazepines induce hypothermia and reducing fever and pain (just like APAP) and these effects are mediated by benzodiazepine receptors (10, 11). These finding led to the suggestion that the mechanism of induction of hypothermia by acetaminophen is not fully understood until now. It seems another mechanism may also play a role in induction of hypothermia by acetaminophen, perhaps it is unrelated to COX inhibition. The aim of the present study was to find a relationship between GABAergic system and induction of hypothermia by APAP.

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Materials and Methods

Drugs

All chemicals used were of the highest purity. APAP (Sigma-Aldrich, USA) was sonicated and dissolved in 20% 1,2-propanediol (Merck, Germany). Picrotoxin (Sigma-Aldrich, USA) and flumazenil (Sigma-Aldrich, USA) were sonicated and suspended with CMC (1%, Synofarm, Germany) and Tween 80 (5%, Merck, Germany) and were administered 15 and 10 minutes before each acquisition session. Diazepam (Sigma-Aldrich, USA) was sonicated and dissolved in normal saline. Drugs were prepared freshly on test days and administered intraperitoneally in a volume of 5 ml/kg.

Experimental part of study

All experiments were carried out on male Sprague–Dawley rats (weight range 200-250 g). The rats housed in a colony room under controlled conditions of temperature (23 ± 1°C) and light (dark cycle of 12:12). Food and water were freely available throughout the experimental protocol. The experiments were performed between 10 a.m. and 2 p.m. On the day of experiments, rats were placed individually in glass boxes (14 cm width, 24 cm length) with ability of free movement (unrestrained), and allowed to rest for 1 hr before drug injection. Seven rats were used in each experiment. Baseline temperature (rectal temperature) was measured with a digital thermometer (Microlife®/M.I Switzerland), lubricated and inserted to a depth of 4 cm. The data are presented as the changes in rectal temperature from the basal values. Basal values are those for the temperature (Time 0) taken immediately before the first drug/solvent administration (1). After each injection, core body temperature were measured every 15 min until 60th min. The animals were preconditioned to the temperature probe by taking temperature measurements 3 days before the experiment and twice on the day of the experiment before drug administration to reduce handling-induced temperature changes associated with stress. Rats were randomly divided into 13 groups consisting of 7 rats each.

Part 1: Picrotoxin (2 mg/kg, IP).

Part 2: Vehicle of acetaminophen; acetaminophen (100 mg/kg, IP); acetaminophen (200 mg/kg, IP); acetaminophen (300 mg/kg, IP).

Part 3: Diazepam (5 mg/kg, IP); vehicle of diazepam (5 ml/kg); diazepam (5 mg/kg) + vehicle of flumazenil (5 ml/kg); diazepam (5 mg/kg) + flumazenil (10 mg/kg).

Based on results obtained from initial experiment (Figure 1), we selected a fixed, submaximal dose of 200 mg/kg of APAP for our combination experiments.

Part 4: Vehicle of acetaminophen (5 ml/kg); acetaminophen (200 mg/kg, IP); acetaminophen (200 mg/kg) + flumazenil (10 mg/kg); acetaminophen (200 mg/kg) + picrotoxin (2 mg/kg).

Single intraperitoneally injection of drugs was given in each group.

Theoretical part of study

Complete geometry optimizations were performed on the APAP, diazepam, lorazepam, temazepam, flurazepam (BZD_R agonists) and flumazenil (BZD_R antagonist) molecules. All structures were optimized at the *HF* level of theory with the *6-31G** basis set. The frequency calculations were done at the same level to determine the nature of the optimized structures to obtain zero-point energies (un-scaled) and thermo-chemical corrections. All calculations were carried out using the *Gaussian 98* program. Structures, thermal energies (*E*), thermal enthalpies (*H*), thermal Gibbs free energies (*G*) for each drug are calculated at *HF* level of theory, using *6-31G** basis sets.

Data analysis

The statistical significance of difference between experimental groups at each point was calculated by student's t-test. Differences with $P<0.05$ were considered statistically significant.

Results

Results of experimental part of study

Effects of APAP on the rat core body temperature

APAP at the doses of 100, 200 and 300 mg/kg, IP, produced a dose- and time dependent hypothermic effect, measured as reduction in the rectal temperature (Figure 1). Acetaminophen caused hypothermia that was maximum (about 3 ˚C below normal) with a dose of 300 mg/kg. Compared with the vehicle control group, acetaminophen after 1 hr produced a -0.5 ± 0.51, -2.77 ± 0.48 and -2.98 ± 0.45 ˚C fall in basal body temperature at doses of 100, 200, and 300 mg/kg, respectively. The mean initial body temperature in the vehicle group was 36.04 ± 0.27 ˚C.

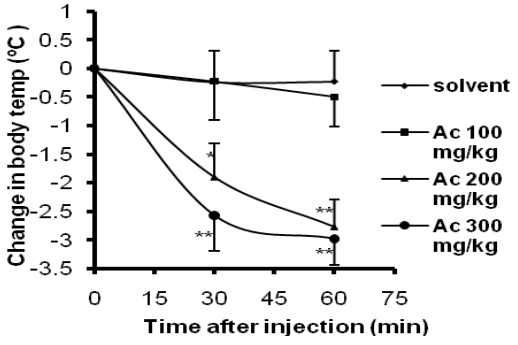

Figure 1. Time-course of the change in core body temperature after intraperitoneally injection of APAP in rats (n=7). Each point represents the mean change in core body temperature. Zero on the abscissa represents body temperature at time of APAP injection. A dose-related fall in temperature was obtained after administration of 100, 200 and 300 mg/kg APAP with a maximum effect at 300 mg/kg. *$P<$ 0.05 and ** $P < 0.01$ in comparison with control group

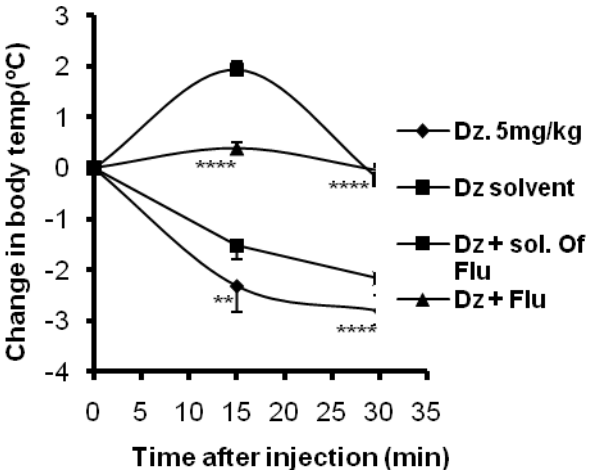

Figure 2. Each point are represents the mean change in core body temperature of 7 rats after intraperitoneally injection of diazepam (5 mg/kg), normal saline (5 ml/kg), vehicle of flumazenil (5 ml/kg) + diazepam (5 mg/kg) and flumazenil (10 mg/kg) + diazepam (5 mg/kg). Zero on the abscissa indicates body temperature at time of saline or flumazenil (BZR-antagonist) administration. ** *P* <0.01 and **** *P* <0.0001 in comparison with control groups

Effect of flumazenil (a BZD_R-antagonist) on diazepam induced hypothermia

As shown in Figure 2, pretreatment of animals with Flumazenil (single dose 10 mg/kg, IP) reduced the hypothermic effect of diazepam (single dose of 5 mg/kg).

Effects of picrotoxin and flumazenil ($GABA_A$ and BZD_R antagonists) on APAP induced hypothermia

Pretreatment of animals with picrotoxin (single dose 2 mg/kg, IP) reduced the hypothermic effect of APAP (single dose 200 mg/kg, IP). Pretreatment of rats with flumazenil (single dose 10 mg/kg, IP) also decreased APAP hypothermia (Figure 3).

Effects of picrotoxin on the rat core body temperature

Unlike the rats injected with picrotoxin (single dose 2 mg/kg, IP) and APAP (single dose 200 mg/kg), single administration of picrotoxin (2 mg/kg) induced convulsion in all treated rats. The results of this administration on basal body temperature are shown in Table 1.

Results of theoretical part of study

Using HF/6-31G*, level of theory, relative energies (E), enthalpies (H), and Gibbs free energies (G) (kcal/mol) are calculated for all selected drugs (Figure 4, Table 2). Some selective structural data, bond length, dihedral angles, and related distance in pharmacophore of APAP and BZD_R models were calculated and some significant structural analogues were obtained between these drugs (Figure 4, Table 3).

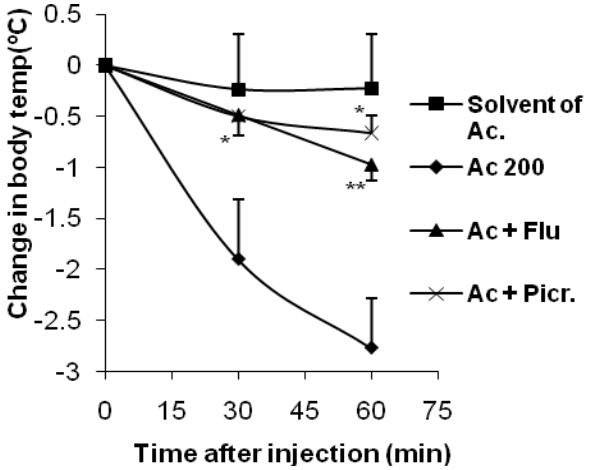

Figure 3. Effect of picrotoxin ($GABA_A$ antagonist) and flumazenil (BZD_R antagonist) on hypothermic effect of APAP. Each tow new point represents the mean change in core body temperature of 7 rats. APAP-treated group; Pretreatment with picrotoxin (2 mg/kg) or flumazenil (10 mg/kg) + APAP (200 mg/kg). * *P*<0.05 and ** *P*<0.01 in comparison with control group

Table 1. Effect of injection of picrotoxin (single dose 2 mg/kg, IP) on core body temperature

T_0	T_{30}	T_{60}	Mean change $_{30}$	Mean change $_{60}$
37.26 ± 0.14	36.7 ± 0.14	36.6 ± 0.32	- 0.23 ± 0.14	- 0.47 ± 0.14

Data are represented as mean change in core body temperature ± SEM of 7 rats in this group. Basal body temperatures (T_0), immediately before administration of picrotoxin (2 mg/kg, IP) and 30 (T_{30}) and 60 (T_{60}) min after injection

Table 2. Energies (E), enthalpies (H), and Gibbs free energies (G) (kcal/mol) for APAP and selected models of BZD_R calculated at HF/6-31G*

Ligand	E (kcal/mol)	H (kcal/mol)	G (kcal/mol)
Diazepam	-788241.16	-788240.04	-788310.85
Lorazepam	-1098716.27	-1098715.15	-1098789.79
Flurazepam	-1007050.83	-1007049.71	-1007144.66
Temazepam	-835201.30	-835200.18	-835273.87
Flumazenil	-845057.24	-845056.12	-845138.08
APAP	-321300.57	-321299.45	-321353.87

Discussion

Acetaminophen (APAP) is a widely used as an antipyretic analgesic and reducing fever caused by bacterial or viral infections and/ or by clinical trauma (1). APAP demonstrated a dose-dependent hypothermic action (1, 5), thus it can improve functional outcome after acute ischemic stroke (2) or other conditions such as status epilepticus, cardiac arrest, convulsion, epilepsy in which controlling the hypothermia is useful and shows therapeutic effectiveness (12, 13). Some studies suggest that this hypothermic action is mediated by inhibition of COX-3 in CNS, in the anterior hypothalamus (7), but more documents will be required to improve this mechanism.

Table 3. Comparison of distances (A°) and angles (°) between the aromatic ring centers and carbonyl group atoms in APAP and selected models of BZD_R calculated at HF/6-31G*

Ligand	Ar- O_4	Ar- C=O	Ar- C_3	Angles (1,2,3)°	Dihedral angles (1,2,3,4) °
Diazepam	4.876	1.195	3.748	122.67	13.47
Lorazepam	4.937	1.192	3.838	129.62	3.37
Flurazepam	4.913	1.197	3.776	123.54	2.6
Temazepam	4.900	1.195	3.770	124.12	8.61
Flumazenil	4.924	1.360	3.787	126.06	3.74
APAP	4.041	1.198	3.843	129.08	0

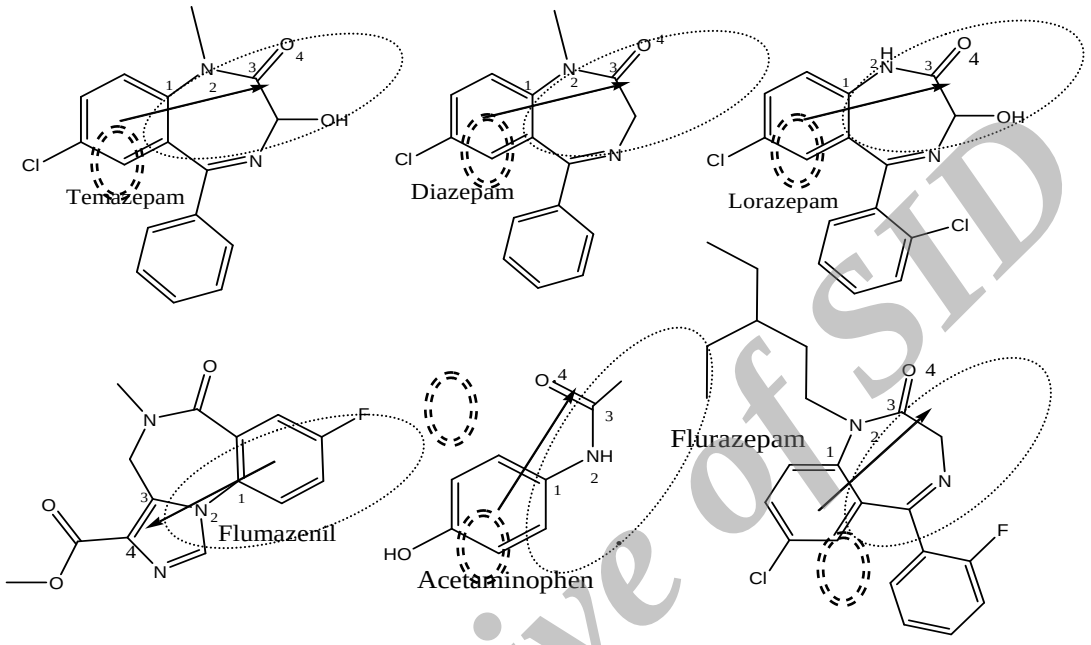

Figure 4. Structural analogues of APAP and selected benzodiazepine receptor ligands

APAP penetrates the brain following peripheral administration, and some evidence suggest a centrally acting mechanism for its action (4, 14). But really the mechanism of action of APAP remains unclear even 125 years after its original synthesis (5). The principal inhibitory neurotransmitter in mammalian CNS, γ-amino butyric acid (GABA), exerts its physiological roles by binding to three major classes of receptors, the $GABA_A$, $GABA_B$ and $GABA_C$. The $GABA_A$ receptor belongs to Cysloop super family of ligand-gated ion channels (LGIC) (15). Mammalian $GABA_A$ receptors have been shown to be heteropentameric assemblies of protein subunits, where the most abundant receptor-ion channels have the composition $(\alpha_n)2\ (\beta_n)2\ (\gamma)$ (16). This receptor has received the greatest attention in terms of research because of its importance as a biological target for clinically important drugs such as barbiturates, neurosteroids, anesthetics, ethanol, and benzodiazepines (BZDs) (17, 18). Classically, BZD agonists (anxiolytic, anticonvulsant, muscle relaxant, sedative-hypnotic, reducing pain, fever and basal body temperature) act as positive modulators at $BZD/GABA_A$ receptor (BZD_R) by increasing the frequency of chloride channel openings (19). The allosteric BZD_R has been proposed to reside at the interface between α and γ subunits (16, 20). Exclusive of γ and even β subunits which are respectively necessary for the $GABA_A$ receptor to be able to responds to low concentrations of BZDs and suggested to be responsible for Etomidate-induced hypothermia (21). α subunit ($\alpha_{1,2,3,5}$) is predominant subunit in CNS and is necessary for binding of BZDs. A significant structural analogue exists between APAP and specific ligands of this subunit (TPA-023, TP-003, N-(indol-3-ylglyoxylyl) piperidines (22).

Acetaminophen reduces the core temperatures of febrile and non-febrile in different ways. The hypothermia induced by APAP (Figure 1) has been reported previously in human, mice and rats (1, 2, 5). It has been demonstrated that APAP produces hypothermia independent of opioid or cannabinoid CB1 or NOP receptor activation, in contrary to its antinociceptive effect, which requires opioid and cannabinoid CB1 receptor activation (5). The aim of

the present study was to investigate the mechanism of this hypothermic action in rat. The results suggest that the hypothermic action of acetaminophen (velocity and peak hypothermia respectively in 30 and 60 min after APAP administration) is antagonized by the $GABA_A$ receptor antagonist, picrotoxin and benzodiazepine receptor antagonist, flumazenil (Figure 3). Flumazenil did not alter body temperature (23). Theoretical part of study has neen presented in Table 3. Data suggests that structural similarity exist between APAP and BZD_R ligands (diazepam, lorazepam, flurazepam, temazepam and flumazenil).

The present results are in part consistent with some of previous findings. It has been reported that in the presence of APAP, enoxacin (a quinolone antimicrobial agent) did not decreases GABA responses (24), whilst quinolones such as PNU-101017, a novel, imidazoquinoline amide and benzodiazepine receptor partial agonist that has high affinity for the $GABA_A$ receptor subtypes containing the α_1 and α_3 or α_5 subunits, have shown related to $GABA_A$ receptor (25). Administration of flumazenil antagonized the analgesic effect exerted by acetaminophen (26), and also prevented the lipopolysaccharide anxiety sensitization (27). The latter, can correlates APAP as the most proposed antipyretic drug to $GABA_A$ receptor indirectly. Furthermore, study of analgesic effects of APAP and morphine in a mouse model of bone cancer pain supposed that APAP potentiates opioid inhibition of GABAergic synaptic transmission (28). These results also correlate APAP to GABAergic system too. Body temperature may be a useful test for discriminating between full and partial agonists at the BZD_R (29). It is possible to consider APAP as a partial agonist at benzodiazepine receptor level. This finding will be a new way for understanding the mechanism of action of APAP.

Conclusion

These results support the view that hypothermic activity of acetaminophen is mediated by its effect at $GABA_A$ BZD_R. However, further studies will clarify this hypothesis.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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