Anesthesiology 2003; 99:976–81

Orally Administered Paracetamol Does Not Act Locally in the Rat Formalin Test

Evidence for a Supraspinal, Serotonin-dependent Antinociceptive Mechanism

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Background: **The mechanism of action of paracetamol (acetaminophen) remains elusive because it is still under discussion as to whether it acts locally and/or centrally. The primary aim of this study was to clarify its site(s) of action (central and/or local) using the rat formalin test.**

Methods: **Spontaneous biting and licking of the injected paw following intraplantar injection of formalin 2.5% was monitored during the two phases of nociceptive behavior (0–5 and 20–40 min after injection), and the authors examined the antinociceptive activity of paracetamol following oral, intravenous, intraplantar, and intrathecal administrations as well as the reversion of this effect by an intrathecal injection of WAY 100,635, a selective 5-HT_{1A} receptor antagonist.**

Results: **The oral administration of paracetamol (300, 400 mg/kg) reduced nociceptive behavior in both phases (400 mg/kg: 36.9 ± 4.6% and 61.5 ± 5.2% of inhibition in phases I and II, respectively, $P < 0.05$), whereas lower doses reduced primarily the score of the second phase of the test. Only high doses of 10 to 20 mg/kg intraplantarly administered paracetamol, which were ineffective when administered subcutaneously, produced a significant but limited reduction in the early phase of the test and had no effect on the second phase or any antiinflammatory activity. Thus, this local effect did not seem to participate in the antinociceptive action of 400 mg/kg orally given paracetamol, which was totally blocked in both phases by an intrathecal injection of 40 μg WAY 100,635 per rat. Such an inhibition was not observed when paracetamol (200 μg per rat) was intrathecally coinjected with WAY 100,635, whereas the antinociceptive action of 5-HT (50 μg per rat, intrathecally) during both phases of pain was inhibited by WAY 100,635 (intrathecally).**

Conclusions: **Orally administered paracetamol does not seem to exert any relevant local action in the formalin model of tonic pain in rats, but it might activate the serotonergic bulbospinal pathways *via* a supraspinal site of action that remains to be elucidated.**

SINCE paracetamol was clinically reintroduced in the 1950s in the United States, many different data have been reported by preclinical studies aiming at understanding the mechanism of its antinociceptive action. It was first suggested that paracetamol had an antihyperalgesic activity similar to that of nonsteroidal antiinflammatory drugs, which was thought to be mainly the result of the peripheral inhibition of the cyclo-oxygenases.[1] *In vitro* inhibition of cyclo-oxygenase activity was reported,[2–5] but it has been suggested that inflammation-induced cyclo-oxygenases were poorly inhibited by paracetamol at the periphery because of a high peroxide level in inflamed tissues.[6–9] Discrepancies about an antiinflammatory effect of paracetamol also exist in the literature.[10–17]

On the other hand, several arguments account for a preferential central action of paracetamol. Pharmacokinetic studies indicated that it readily crosses the blood–brain barrier[18,19] and is distributed uniformly into the brain when administered systemically.[20] In addition, intracerebroventricular and intrathecal administrations of the drug led to antinociception in several nociceptive tests.[21–24] The mechanism of this central action is still widely discussed. Central inhibition of central cyclo-oxygenase activity has also been proposed,[25,26] and a new isoform preferentially expressed in cerebral cortex has been shown to be more sensitive to paracetamol.[27] Another major hypothesis is the involvement of the serotonergic system. A role of 5-HT in the antinociceptive activity of paracetamol has been reported in several studies.[28–33] It has been notably shown that the antinociceptive action of paracetamol, like that of 5-HT, was inhibited by intrathecally injected antagonists of the 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C} receptors[32] and by tropisetron known as a 5-HT_3 receptor[17,24,30] in the rat paw pressure test.

Currently, the mechanism of action of paracetamol remains elusive and the localization of the target proteins (central and/or local) involved in its antinociceptive action is still argued. The primary aim of our study was to clarify whether paracetamol was acting peripherally and/or centrally by administering the drug locally, which has been too rarely performed, and by investigating the influence of a centrally injected serotonergic receptor antagonist on the antinociceptive effect of systemically administered paracetamol. The rat formalin test was used because of its clinical relevancy as a model of persistent pain with both peripheral and central nociceptive mediators involved[34] and its advantage of allowing the assessment of spontaneous nocifensive behaviors that limit the influence of the experimenter.[35]

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Received from INSERM E9904, Pharmacologie Fondamentale et Clinique de la Douleur, Faculté de médecine, Clermont-Ferrand, France. Submitted for publication January 15, 2003. Accepted for publication June 12, 2003. The authors received support from the Association Nationale de la Recherche Technique, grant No. 170/2001, Paris, France; Bristol-Myers-Squibb, Rueil-Malmaison, France (to Dr. Bonnefont); a research grant from Institut UPSA de la Douleur, Paris, France; and the Association Claude Bernard, Paris, France. Part of this study was presented as a poster at the 6th Annual Congress of the French Society of Pharmacology, Rennes, France, April 8–10, 2002.

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Methods

Animals

Adult male 200- to 250-g Sprague-Dawley rats (Charles River, France) were housed under standard conditions with food and water *ad libitum* for a week before starting the experiments so they could acclimate. This study, including care of the animals involved, was conducted according to the official edict presented by the French Ministry of Agriculture Paris, France, and the recommendations of the Helsinki Declaration. Thus, these experiments were conducted in an authorized laboratory and under the supervision of an authorized researcher.

Formalin Test

Following acclimatization for 20 min in the test chamber and drug treatment, the rats received 50 μL of 2.5% formalin injected subcutaneously into the dorsal surface of the hind paw. They were then replaced in the Plexiglas box. Biting and licking time, considered as the best nociceptive parameter,[36] was monitored during the two peaks of the typical biphasic nocifensive behavior previously described.[37,38] The spontaneous aversive response corresponding to the early phase was assessed during the first 5 min. The second peak of nocifensive behavior was observed from 20 to 40 min after formalin administration.

Paw Edema Measurement

The rat hind paw was immersed in an electrolyte solution up to the junction between the hairy and nonhairy skin of the ankle, and paw edema was determined electronically as a change in paw volume (in milliliters) using a plethysmometer (Apelex 7150, Massy, France). Volumes were measured before drug treatment, before formalin administration, and at the end of the test. The absolute paw volume was 1.35 ± 0.02 mL.

Treatment Protocols

Antinociceptive Action of Paracetamol after Systemic Administration. To observe the maximal antinociceptive activity of paracetamol during both phases of the test, 100 to 400 mg/kg paracetamol or 5 mL/kg NaCl 0.9% was administered orally or 1 mL/kg intravenously 40 min or 5 min before formalin, respectively, according to the kinetics of action previously described.[30]

Antinociceptive Action of Paracetamol after Intraplantar Injection. According to the protocol described by Park *et al.*,[39] the animals received 0.1 to 20 mg/kg paracetamol or 100 μL vehicle subcutaneously into the dorsal surface of their hind paw 30 min before formalin. Indomethacin at a dosage of 1.8 mg/kg was also administered as a positive control. The effect of both drugs was assessed both on nociceptive parameters and on edema volumes. To examine whether a possible diffusion of paracetamol would not minimize its local effect, the drug was also administered 10 min before formalin. Paracetamol at a dosage of 20 mg/kg and indomethacin were also administered subcutaneously in the rat back 30 min before formalin to verify the lack of any systemic action of the maximal intraplantarly injected dose.

Influence of Intrathecally Injected WAY 100,635 on the Antinociceptive Action of Paracetamol. Intrathecal injections were performed with the rats under isoflurane anesthesia (4% induction, 2% maintenance) between lumbar vertebrae L5 and L6 (10 μL), as previously described.[40] WAY 100,635 at a dosage of 40 μg per rat was administered intrathecally 30 min after 400 mg/kg paracetamol orally (*i.e.*, 10 min before formalin injection). The effect of 200 μg paracetamol per rat and 50 μg 5-HT per rat administered intrathecally 10 min before formalin was also assessed on the two phases of the test, and the influence of WAY 100,635 that was coadministered with either drug was also investigated.

Drugs

Intraplantarly administered paracetamol (UPSA, Bristol-Myers-Squibb Group, Rueil-Malmaison, France) was dissolved in dimethyl sulfoxide (Sigma, Saint Quentin Fallavier, France) and then diluted in saline (0.9% NaCl) with a final concentration of 20% dimethyl sulfoxide. For oral and intrathecal routes of administration, paracetamol was dissolved in saline. Indomethacin (Sigma) was dissolved in saline containing 10% ethanol as previously described.[39] Formalin (Acros Organics, Noisy-Le-Grand, France), 5-hydroxytryptamin hydrochloride, and WAY 100,635 (*N*-(2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl)-*N*-(2-pyridinyl) cyclohexanecarboxamide trihydrochloride) (Sigma) were dissolved in saline.

Drugs and doses were randomized and administered according to the method of blocks to assess the effect of the different treatments at the same lapse of time to avoid any uncontrolled experimental influence. Different animals were used for each experiments (n = 8 per treatment). All the experiments were performed blind in a quiet room by the same experimenter.

Statistical Analysis

The cumulative biting and licking time of each phase of response was generated for every rat. The data (mean ± standard error of mean) expressed in seconds were analyzed by a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls test when appropriate. Paw edema measurements were converted into an increase in paw volume (percentage) compared with the preformalin injection values. Analyses were conducted on the original data using ANOVA followed by the Student-Newman-Keuls test. The level of statistical significance was set at 0.05.

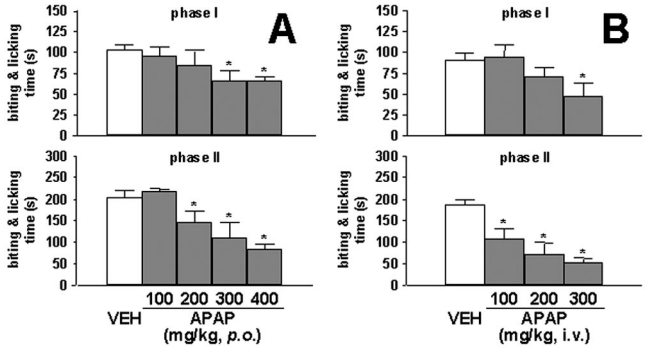

Fig. 1. Antinociceptive activity of paracetamol (APAP) following (*A*) oral or (*B*) intravenous route of administration on hind paw biting and licking time produced by 2.5% formalin. The two phases of nociceptive behavior were monitored 0 to 5 min (phase 1) and 20 to 40 min (phase 2) after intraplantar formalin injection (50 μL). The results are expressed as mean ± standard error of mean of the cumulative of biting and licking time during each phase. *$P < 0.05$ compared with the control group using analysis of variance followed by Student-Newman-Keuls test (n = 8).

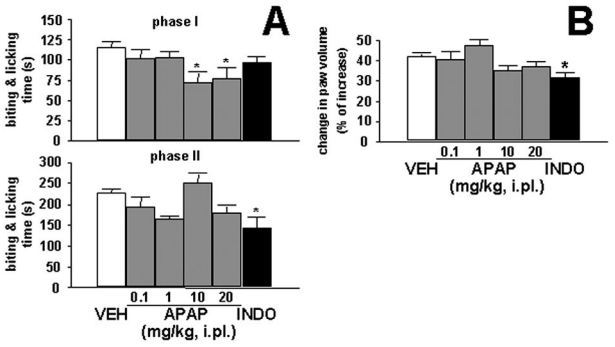

Fig. 2. Antinociceptive (*A*) and antiedematous (*B*) activities of locally administered paracetamol (APAP) or indomethacin (INDO) in the rat formalin test. (*A*) The two phases of nociceptive behavior were monitored 0 to 5 min (phase 1) and 20 to 40 min (phase 2) after intraplantar formalin injection (50 μL). The results are expressed as mean ± standard error of mean of the cumulative of biting and licking time during each phase. *$P < 0.05$ compared with the control group using analysis of variance followed by Student-Newman-Keuls test (n = 8). (*B*) Paw edema measurements determined at the end of the late phase were converted into an increase in paw volume (percentage) compared with the preformalin injection values. *$P < 0.05$ compared with the vehicle group using analysis of variance followed by Student-Newman-Keuls test (n = 8).

Results

In all of the experiments performed, a qualitative assessment of locomotor activity indicated that the observed antinociceptive activity was not the result of motor impairment, because animals displayed a similar behavior regardless of the treatment they received.

Antinociceptive Action of Paracetamol after Systemic Administration

Paracetamol given orally (300, 400 mg/kg) reduced significantly the two phases of spontaneous aversive behavior monitored as biting and licking time of the rat paw injected with formalin (300 mg/kg: 36.7 ± 10.1% and 49.3 ± 14% of inhibition in phases 1 and 2, respectively, $P < 0.05$; 400 mg/kg: 36.9 ± 4.6% and 61.5 ± 5.2% of inhibition in phases 1 and 2, respectively, $P < 0.05$, fig. 1A). The second phase, measured from 20 to 40 min after induction of the noxious stimulus, was more sensitive to paracetamol, as the drug was significantly effective at 200 mg/kg (33.6 ± 9.9% of inhibition, $P < 0.05$), whereas only higher doses reduced both phases. The same profile was observed when the drug was administered intravenously (fig. 1B). Paracetamol reduced biting/licking in the second phase from 100 mg/kg (42.6 ± 13.1% of inhibition, $P < 0.05$). A dose of 200 mg/kg significantly reduced the late phase by 60.9 ± 14.3% ($P < 0.05$), and a dose of 300 mg/kg was necessary to decrease both phases of nociception (47.6 ± 17.6 and 72.5 ± 6.3% of inhibition in phases 1 and 2, respectively, $P < 0.05$).

Antinociceptive Action of Paracetamol after Intraplantar Injection

It was first observed that the 20% dimethyl sulfoxide–containing vehicle used did not affect formalin responses (data not shown). Paracetamol administered intraplantarly 30 min before formalin produced a statistically significant reduction of nocifensive behavior in phase 1 at 10 and 20 mg/kg (38.2 ± 10.0% and 33.3 ± 7.9% of inhibition, respectively, $P < 0.05$, fig. 2A). The dose of 20 mg/kg was the maximum tested because of the poor solubility of the drug and the limited volume injection allowed in the rat paw. However, the second phase (20–40 min) remained unaffected by paracetamol. In contrast, 1.8 mg/kg of indomethacin, a dose similar to that used by Park *et al.*,[39] significantly reduced the late phase only by 37.8 ± 11.9% without attenuating the first one. This difference of profile of action between the two drugs was also seen with the assessment of the antiedematous effect, because only indomethacin decreased significantly the development of paw edema at the end of the second phase (25.2 ± 6.2% of reduction in paw volume, $P < 0.05$, fig. 2B). Paracetamol at a dosage of 20 mg/kg injected intraplantarly 10 min before formalin did not affect the late phase, and its antinociceptive activity in the first phase was comparable to that observed when the injection was performed 30 min before formalin (data not shown).

Influence of Intrathecally Injected WAY 100,635 on the Antinociceptive Action of Paracetamol

Paracetamol at a dosage of 400 mg/kg given orally was significantly effective in inhibiting the two phases of aversive behavior induced by formalin (36.6 ± 9.6% and 39.6 ± 9.9% inhibition in phases 1 and phase 2, respectively, $P < 0.05$; fig. 3). WAY 100,635 at a dosage of 40 μg per rat administered intrathecally 10 min before formalin had no intrinsic action on the duration of the

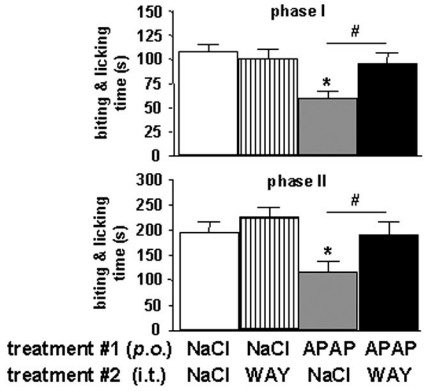

Fig. 3. Reversion of the antinociceptive activity of 400 mg/kg paracetamol (APAP) given orally by 40 μg WAY 100,635 per rat, administered intrathecally, on the biting and licking behavior elicited by 2.5% formalin. Both phases of nociceptive response were assessed 0 to 5 min (phase 1) and 20 to 40 min (phase 2) after intraplantar formalin injection (50 μL). The results are expressed as mean ± standard error of mean *$P < 0.05$ compared with the control group using analysis of variance followed by Student-Newman-Keuls test (n = 8).

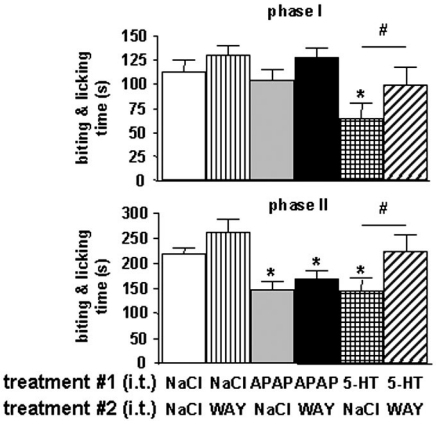

Fig. 4. Examination of the relationship between the antinociceptive activity of 200 μg spinally administered paracetamol (APAP) per rat, administered intrathecally, or 50 μg 5-HT per rat, administered intrathecally, and the 5-HT_{1A} receptor using 40 μg WAY 100,635 per rat, administered intrathecally, on the biting and licking time produced by 2.5% formalin. Nociceptive responses were measured 0 to 5 min (phase 1) and 20 to 40 min (phase 2) after intraplantar formalin injection (50 μL). The results are expressed as mean ± standard error of mean. *$P < 0.05$ compared with the NaCl 0.9%-receiving group using analysis of variance followed by Student-Newman-Keuls test (n = 8).

behavioral response. However, it significantly and totally prevented the antinociception elicited by paracetamol during both phases. Scores of the group treated by paracetamol plus WAY 100,635 were statistically different from those of the paracetamol/saline-treated animals but not from the saline/saline- or saline/WAY 100,635-treated groups.

Reversal by WAY 100,635 of the antinociceptive activity of intrathecally injected paracetamol was then studied. An intrathecal administration of 200 μg per rat of paracetamol did not significantly affect the early phase of aversive behavior, whereas the second phase was significantly inhibited by 32.0 ± 7.1% ($P < 0.05$, fig. 4). This effect was not reversed when paracetamol was coinjected with 40 μg per rat of WAY 100,635 because the score was not significantly different from the paracetamol/saline group (22.7 ± 7.3% of inhibition in biting/licking time, $P < 0.05$). In the same blind experiment, 50 μg 5-HT per rat was administered intrathecally as a positive control. In accordance with previous results,[41] 5-HT inhibited both phases with 43.5 ± 13.7% and 33.5 ± 11% of inhibition in phase 1 and phase 2, respectively. Its efficacy in the second phase was similar to that produced by paracetamol. When coadministered with WAY 100,635, 5-HT lost its antinociceptive property in both phases, the reversal being more marked during the late phase.

Discussion

The primary aim of our study was to investigate the site(s) of action of paracetamol involved in its antinociceptive effect using the formalin model of tonic pain in rats. Several data obtained in this work suggest a limited, if any, local antinociceptive effect of paracetamol in the vicinity of the injury: (1) Intraplantar injection of paracetamol, but not indomethacin, failed to induce any anti-inflammatory effect and did not modify scores of the late phase, whereas high doses were needed to reduce biting/licking behavior in the first phase that could result from a direct activation of C-fiber primary afferents.[34] (2) As seen in another work,[29] paracetamol administered either intravenously or orally primarily reduced behavioral scores in phase 2, which is thought to reflect both ongoing activity of nociceptors and central sensitization.[34] (3) The effect of systemically administered paracetamol in both phases was totally blocked by an intrathecal administration of WAY 100,635, a 5-HT_{1A} receptor antagonist. Few data report any peripheral activity after local administration of paracetamol. The results obtained by Abbott and Hellemans[42] using the same test were not conclusive, whereas Ferreira *et al.*[21] found a locally mediated antinociception in the rat paw pressure test at a dose of 50 μg per rat.

If a limited local antinociceptive effect of paracetamol can be observed only after high doses administered intraplantarly, a central serotonin-mediated action is demonstrated after oral administration. This observation is in agreement with previous results from our group[30,32] or from other authors.[28,29,31] However, the present data give information on the central site(s) of action of paracetamol and the nature of the 5-HT receptors involved. Concerning the central site(s) of action, the results obtained on the early phase suggest a supraspinal action of paracetamol. Orally, but not intrathecally, administered paracetamol reduced scores in phase 1. This effect was inhibited by WAY 100,635 (intrathecal), a 5-HT_{1A} recep-

tor antagonist, which also inhibited the antinociception induced by intrathecally injected 5-HT in the early phase. Thus, paracetamol injected intrathecally does not seem to be able to activate the 5-HT system. The spinal serotonergic effect observed after oral administration of paracetamol could be the result of an activation of a supraspinal component able to activate this system. This is in agreement with previous results showing that the action of paracetamol was reduced in the early phase of the formalin test after lesion of the bulbospinal 5-HT neurons using 5,6-DHT[28] and with the lack of binding of paracetamol on the 5-HT reuptake sites.[30] Activation of the 5-HT descending pathways could be correlated with the diminution of cortical high-affinity 5-HT_{2A} receptors evoked by paracetamol,[29] because a close relationship between this decrease and the analgesic activity of paracetamol has been reported.[31] Moreover, some studies proposed that the stimulation of supraspinal 5-HT_2 receptors could produce a decrease in the activity of bulbospinal pathways.[43,44]

The data observed in phase 2 tend to confirm a supraspinally mediated activation of the serotonergic bulbospinal pathways. The antinociceptive effect of orally administered paracetamol was inhibited by an intrathecal injection of WAY 100,635, similar to the effect of intrathecally injected 5-HT. On the other hand, the antinociceptive effect of high doses of intrathecally injected paracetamol produced an antinociception comparable to that of 5-HT in the second phase only as previously described.[45] Besides, this action was not reversed by the 5-HT_{1A} receptor antagonist, suggesting a WAY 100,635-insensitive spinal activity. Thus, the serotonergic effect observed after an oral administration of paracetamol might not involve a spinal but instead primarily a supraspinal site of action, which could promote the activation of bulbospinal pathways. However, a direct spinal effect of paracetamol, WAY 100,635-insensitive, cannot be ruled out for high doses, at least in the late phase. Indeed, it has been observed that paracetamol still exerts a part of its antinociceptive effect in the late phase following the inactivation of the 5-HT system.[28,29]

Finally, this work also informs on the nature of the 5-HT receptors involved in the effect of paracetamol and shows, in the rat formalin test, the involvement of the spinal 5-HT_{1A} receptors, which are also involved in the antinociceptive effect of 5-HT.[46] However, we must keep in mind that we do not control rostral spreading of WAY 100,625 into the brain. These results disagree with those obtained with paracetamol and 5-HT in the rat paw pressure test by Courade *et al.*[32] and Bardin *et al.*[47] Indeed, their antinociceptive actions in this mechanical pain test were inhibited by several 5-HT receptor antagonists, but not by WAY 100,635. This indicates that various 5-HT receptors are involved according to the nature of the noxious stimulus and, consequently, to the nature of the afferent fibers involved. This is in line with the comments recently reported by Millan[48] about the pharmacologic complexity of the serotonergic system in controlling nociceptive pathways.

In conclusion, our data seem to indicate that paracetamol, long thought to act by a mechanism similar to the nonsteroidal antiinflammatory drugs, exerts an antinociceptive action in both phases of the rat formalin test that is not mediated by a local antinociceptive action contrary to indomethacin. Its antinociceptive effect observed during the early phase might result from a modulation of the activity of supraspinal neuronal areas leading to the activation of the serotonergic inhibitory descending pathways and the involvement of spinal 5-HT_{1A} serotonergic receptors. Reduction of the nocifensive behavior in phase 2 might also largely involve the bulbospinal serotonergic system and spinal 5-HT_{1A} receptors when the drug is given systemically, but a direct modulation of other spinal pro- or antinociceptive systems might also be implicated in this phase. Finally, a potential interaction between supraspinal and spinal sites of action could also occur as proposed by Raffa *et al.*[49]

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