0026-895X/07/7102-407–415$20.00
MOLECULAR PHARMACOLOGY
Vol. 71, No. 2
25775/3170451
Mol Pharmacol 71:407–415, 2007
Printed in U.S.A.

Acetaminophen Recruits Spinal p42/p44 MAPKs and GH/IGF-1 Receptors to Produce Analgesia via the Serotonergic System[S]

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Received April 17, 2006; accepted November 3, 2006

ABSTRACT

The mechanism of action of acetaminophen is currently widely discussed. Direct inhibition of cyclooxygenase isoforms remains the commonly advanced hypothesis. We combined behavioral studies with molecular techniques to investigate the mechanism of action of acetaminophen in a model of tonic pain in rats. We show that acetaminophen indirectly stimulates spinal 5-hydroxytryptamine $(5\text{-HT})_{1A}$ receptors in the formalin test, thereby increasing transcript and protein levels of low-affinity neurotrophin receptor, insulin-like growth factor-1 (IGF-1) receptor α subunit, and growth hormone receptor and reducing the amount of somatostatin 3 receptor (sst3R) mRNA. Those cellular events seem to be important for the antinociceptive activity of acetaminophen. Indeed, down-regulation of sst3R mRNA depends on acetaminophen-elicited, 5-HT_{1A} receptor-dependent increase in neuronal extracellular signal-regulated kinase 1/2 (ERK1/2) activities that mediate antinociception. In addition, spinal growth hormone (GH) and IGF-1 receptors would also be involved in the antinociceptive activity of the analgesic at different degrees. Our results show the involvement of specific 5-HT_{1A} receptor-dependent cellular events in acetaminophen-produced antinociception and consequently indicate that inhibition of cyclooxygenase activities is not the exclusive mechanism involved. Furthermore, we propose that the mechanisms of 5-HT_{1A} receptor-elicited antinociception and the role of the spinal ERK1/2 pathway in nociception are more intricate than suspected so far and that the GH/IGF-1 axis is an interesting new player in the regulation of spinal nociception.

The antinociceptive action of acetaminophen, a century-old reference compound for the relief of mild pain, is still poorly understood. Discrepancies in its ability to inhibit peripheral cyclooxygenase activity during the inflammatory process have been reported according to the procedure used (Mitchell et al., 1993; Warner et al., 1999) and the redox state of the tissue (Ouellet and Percival, 2001; Boutaud et al., 2002). Some data would also seem to point toward an inhibition of central cyclooxygenase activity (Graham et al., 2001). The existence of a novel isoform of cyclooxygenase, named COX-3, inhibited by acetaminophen with a better affinity than the two other isoforms, was recently proposed (Chandrasekharan et al., 2002). However, the clinical relevance of this result and the mRNA sequence of this COX-3 are disputed (e.g., Dinchuk et al., 2003; Snipes et al., 2005). Inhibition of pros-

This work was supported financially by the Association Nationale de la Recherche Technique and Bristol-Myers-Squibb (grant 170/2001 to J.B.) and the UPSA Pain Institute.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.106.025775.

[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: COX-3, cyclooxygenase 3; 5-HT, serotonin (5-hydroxytryptamine); WAY-100635, *N*-(2-[4-(2-methoxyphenyl)-1-piperazinyl-]ethyl)-*N*-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; GHR, growth hormone receptor; H-1356, CYAAPLKPAKSC disulfide; IGF-1R, insulin-like growth factor-1 receptor; PCR, polymerase chain reaction; TBS, Tris-buffered saline; ERK, extracellular signal-regulated kinase; $p75^{NTR}$, low-affinity neurotrophin receptor; IGF-1Rα, insulin-like growth factor-1 receptor α subunit; sst3R, somatostatin 3 receptor; BDNF, brain-derived neurotrophic factor.

taglandin synthesis by acetaminophen and its importance during antinociception, therefore, remain a matter of debate. It is probably not the only mechanism of action involved because acetaminophen is notably able to exert an analgesic effect in nociceptive tests that are sensitive to central analgesics and in which there is no inflammation (Carlsson et al., 1988; Bustamante et al., 1996).

Over the last decade, a central, serotonin (5-HT)-dependent hypothesis has emerged. Lesions of the serotonergic bulbospinal pathways or depletion of serotonin level significantly reduced the antinociceptive action of acetaminophen in the rat formalin test (e.g., Pini et al., 1996). In addition, its analgesic effect was correlated with a reduction in the maximum number of cortical 5-HT_{2A} receptors (Pini et al., 1996; Srikiatkhachorn et al., 1999) that may lead to an increased activity of the inhibitory serotonergic descending pathways (Barber et al., 1989). In the rat paw pressure test, inhibition of the antinociceptive action of acetaminophen by different intrathecally administered 5-HT receptor antagonists also suggests that the inhibition of nociceptive processing occurs at the spinal level by means of a serotonergic mechanism (Courade et al., 2001b). In agreement with this finding, we recently showed that WAY-100635, injected intrathecally, significantly reversed the action of acetaminophen in the rat formalin test, suggesting that its antinociceptive activity depends on the stimulation of the spinal 5-HT_{1A} receptor (Bonnefont et al., 2003a, 2005).

The molecular mechanisms involved in the modulation of the nociceptive input after acetaminophen administration and indirect stimulation of the 5-HT_{1A} receptors have never been investigated at the spinal level. The aim of our study, therefore, was to examine molecular changes modulated by acetaminophen at the spinal level and to determine whether these changes were associated with the antinociceptive action of acetaminophen and the stimulation of 5-HT_{1A} receptors.

Materials and Methods

Formalin Test. Adult male Sprague-Dawley rats (200–220 g, Charles River Laboratories, L'Arbresle, France) were used. Experiments, including care of the animals, were conducted according to the policy on the use of animals in Neuroscience research approved by the Society for Neuroscience. After acclimatization for 20 min in a Plexiglas box and drug treatments, the rats received 50 μl of formalin (2.5% formaldehyde) s.c. into the dorsal surface of the hind paw. They were then put back in the test chamber, and biting/licking time (in seconds) was monitored during the first 5 min and between 20 and 30 min after formalin administration. Formalin has typically been described to induce two peaks of aversive behavior over 60 min, between 0 and 5 and 20 and 40 min after its injection. The early phase (referred to as phase I) is believed to be an acute pain resulting from a direct activation of the C-type primary afferent fibers by formalin; the late phase (referred to as phase II) is believed to reflect a tonic nociception due to peripheral inflammation and central sensitization leading to the continuous stimulation of nociceptors. Because we observed previously that the peak of the effect of acetaminophen was during phase II (Bonnefont et al., 2003a), we stopped monitoring the nociceptive behavior in the middle of this period to remove the lumbar enlargements of the spinal cord and to investigate in vitro cellular events potentially linked to the antinociceptive activity of the drug. Different animals were used for each experiment (n = 6–10), which were performed blind in a quiet room by a single experimenter, with randomization of the treatments using the method of blocks to avoid any uncontrolled experimental influence.

Times of injection before formalin administration were chosen according to the kinetic parameters previously observed to assess a maximal effect during the whole test. Acetaminophen (400 mg/kg) and diclofenac (40 mg/kg) were given orally 40 min before formalin administration. We chose this dose of acetaminophen because it is the one used most often in experiments in rodents, which are less sensitive to the drug than humans. Diclofenac, a reference nonsteroidal anti-inflammatory drug, has been used to control that our observations were caused by a COX-independent activity of acetaminophen. WAY-100635 (40 μg/rat), a 5-HT_{1A} receptor antagonist, U0126 (2 × 5 μg/rat), a mitogen-activated protein kinase kinase 1/2 inhibitor, pegvisomant (10 μg/rat), a GHR antagonist, and H-1356 (25 μg/rat), an IGF-1R antagonist, were administered intrathecally at times indicated in the text or the figures. Intrathecal injections were performed between L5 and L6 (10 μl) under isoflurane anesthesia (2%).

Drugs. Acetaminophen (Bristol-Myers Squibb, Rueil-Malmaison, France) was suspended in saline (0.9% NaCl; B. Braun, Melsungen, Germany). WAY-100635, diclofenac (Sigma, L'Isle d'Abeau, France), H-1356 (Bachem, Torrance, CA), pegvisomant (Somavert; Pfizer, Paris, France), and formalin (Acros Organics, Noisy Le Grand, France) were dissolved in saline. U0126 (Cell Signaling Technology, Danvers, MA) was dissolved in saline containing 10% dimethyl sulfoxide (Sigma). Saline was used as control treatments except for U0126 (vehicle: saline with 10% DMSO).

Removal of Spinal Cord Lumbar Enlargement. At the end of the behavioral test (i.e., 30 min after the injection of formalin), which corresponds to the peak of the antinociceptive action of acetaminophen, the animals were killed by decapitation. Their spinal cords were rapidly removed and dissected on ice. The lumbar enlargements were frozen in liquid nitrogen and conserved at −80°C until use.

Nylon cDNA Arrays. Spinal cord lumbar enlargement total RNA was extracted using TRI InstaPure reagent (Eurogentech, Liege, Belgium) according to the manufacturer's instructions. The amounts of total RNA were measured spectrophotometrically at 260 nm. The integrity and good quality of the purified RNA were confirmed using formaldehyde-agarose gel containing ethidium bromide. Total RNA reverse transcription and cDNA labeling were performed as recommended by the manufacturer (Clontech, Saint Quentin-en-Yvelines, France). Unincorporated [α-^{32}P]dCTP (MP Biomedicals, Orsay, France) was discarded by filtration on microcon YM-30 tubes (Millipore, Saint Quentin-en-Yvelines, France). Nylon cDNA array membranes (Atlas 1.2 cDNA Expression Arrays; Clontech) were prehybridized at 68°C for 1.5 h with 10 ml of ExpressHyb hybridization buffer (Clontech) and 1 mg of denatured salmon sperm DNA (Invitrogen, Cergy-Pontoise, France). The labeled probe was denatured at 100°C for 5 min and then transferred at 4°C before being added to the prehybridization solution. Hybridization was performed overnight at 68°C. Membranes were then washed twice in 2× standard saline citrate at 68°C for 20 min and twice in 0.5× standard saline citrate, 0.1% SDS at 68°C for 20 min. Membranes were exposed to storage phosphor screen for two days and scanned with a Storm 840 PhosphorImager (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Spot intensities were analyzed using ImageQuant software (GE Healthcare). Background level was subtracted from each spot, and the arrays were normalized by equilibrating the signal intensity of each spot and the sum of their intensities. Two sets (one for formalin and another for formalin + acetaminophen) of three membranes were formed, each membrane for a different animal. For one gene, corrected intensities on each set of membranes were averaged and compared. To be considered significant, an expression variation had to 1) be higher than 2 or lower than 0.5 and 2) to have a limited S.E. (<15%).

Semiquantitative Reverse Transcription-PCR. This technique, based on cDNA amplification linearities, was performed ac-

cording to the manufacturer's instructions (Promega, Charbonnières-les-Bains, France). First, the suitable number of PCR cycles giving linear cDNA amplification was estimated. These amplifications were then normalized to the cDNA amount present in each PCR tube using the gene encoding the rat L32 ribosomal protein as a housekeeping gene. PCR conditions and sense and antisense primer sequences can be found on-line as Supplemental Data (Table S1). The PCR products were analyzed on 2% agarose gels containing ethidium bromide, and gel DNA analyses were made with Kodak Digital Science 1D Image Analysis Software (Paris, France).

Western Blotting Analysis. Spinal cord lumbar enlargements, homogenized in 400 μl of ice-cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM orthovanadate, 100 mM NaF, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, and 100 IU/ml aprotinin; all products from Sigma), were incubated for 20 min at 4°C and then centrifuged at 16,000*g* for 15 min. Proteins from the supernatant were quantified using bicinchoninic acid assay (Pierce, Brebières, France). Two hundred micrograms [for immunoblotting using anti-p75^{NTR} (80 kDa) antibody, 1:100], 25 μg [IGF-1Rα (90 kDa), 1:200], 150 μg [GHR (110 kDa), 1:100], 10 μg [ERK1 (44 kDa) and ERK2 (42 kDa), 1:200], 100 μg (phospho-ERK1/2, 1:2000), or 50 μg [α-actin (42 kDa), 1:400] of protein per lane was diluted in loading buffer and boiled for 5 min before being separated by SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membrane. Membranes were blocked with TBS (10 mM Tris-HCl, pH 7.4, and 140 mM NaCl) containing 5% nonfat dried milk and incubated overnight at 4°C with the antibodies indicated. Membranes were washed three times with TBS buffer containing 0.2% Tween 20 and incubated for 1 h at room temperature with secondary antibody with TBS containing 1% nonfat dried milk and 0.2% Tween 20. Blots were revealed using Super-Signal West Pico Chemiluminescent Substrate (Pierce), and analyses were carried out with Kodak Digital Science 1D Image Analysis Software. All the primary antibodies used were from Santa Cruz Biotechnology (Santa Cruz, CA), except phospho-ERK (Cell Signaling Technology) and α-actin (NeoMarkers, Fremont, CA). Secondary antibodies were from Pierce.

Immune Complex Assays for ERK1/2 Activation. The spinal cord lumbar enlargement homogenates obtained as described above were immunoprecipitated for 3 h at 4°C with anti-ERK1 or anti-ERK2 antibodies preabsorbed on protein-A Sepharose (Sigma). Precipitated immune complexes were washed twice in lysis buffer and three times in 50 mM HEPES, pH 7.5, containing 150 mM NaCl, 0.1% Triton X-100, 10% glycerol (Eurobio, Courtaboeuf, France), and 200 μM orthovanadate. The phosphorylation reaction was performed for 30 min at room temperature with 0.15 mg/ml myelin basic protein (Sigma) and [γ-^{33}P]ATP (MP Biomedicals). The samples were deposed on P81 Whatman chromatography paper (Whatman, Maidstone, UK), which was then immersed in 1% orthophosphoric acid (Prolabo, Fontenay sous bois, France) to stop the reaction. After three washes in this buffer, radioactivity was counted.

Immunohistochemistry. Animals were anesthetized with pentobarbital (0.1 ml/100 g) and transcardially perfused with 500 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline, pH 7.4. The lumbar enlargements of the spinal cords were dissected and postfixed in the same buffer for 1 h at 4°C. Tissues were then dehydrated in graded ethanol before being included in paraffin. Tissue sections (5 μm) were deparaffinized with xylene before being re-hydrated. Antigen retrieval was performed by bringing slides to boiling in 10 mM sodium citrate, pH 6.0, maintaining them at subboiling temperature for 10 min, and cooling them at room temperature for 30 min. The sections were then washed for 5 min in distilled water, 10 min in wash buffer (0.1% Tween 20, 50 mM NaF in 1× TBS), incubated 10 min in wash buffer containing 3% H_2O_2, and washed again for 10 min. They were then incubated in 1× TBS containing 0.1% Tween 20 and 5% horse serum for 1 h at room temperature to block nonspecific binding and washed 10 min before incubating overnight at 4°C with an anti-phospho-ERK1/2 antibody (Cell Signaling Technology) at a 1:100 dilution in wash buffer. After washing, the slides were treated with anti-rabbit biotinylated secondary antibody (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) at 1:50 in wash buffer for 30 min at room temperature. The sections were then washed in 1× TBS containing 0.1% Tween 20, incubated 30 min at room temperature in ABC reagent, and washed again in 1× TBS containing 0.1% Tween 20. Diaminobenzidine substrate (Dako Denmark A/S, Glostrup, Denmark) was added, and the slides were incubated at room temperature. The reaction was stopped by immersing the sections in distilled water, and the slides were sealed for visualization by light microscopy. The topographic distribution of phospho-ERK changes within the spinal cord was observed and recorded using a charge-coupled device camera (Nikon DS Cooled Camera Head DS-5Mc; Nikon, Tokyo, Japan). Quantification of immunostaining was done using Nikon Lucia software.

Immunofluorescence. Initials steps up to the incubation with the phospho-ERK antibody were performed as described above. After washing 30 min at room temperature, the slides were then incubated with anti-NeuN (1:100; Chemicon, Paris, France) or anti-glial fibrillary acidic protein (1:100; Chemicon) antibody for 2 h at 4°C and washed again for 30 min. They were incubated with both secondary fluorescent (fluorescein isothiocyanate and rhodamine) antibodies (1:200; Interchim, Montlucon, France) for 1 h in the dark at room temperature, washed three times in 1× TBS containing 0.1% Tween 20, and mounted with VECTASHIELD mounting medium. Visualization analysis was performed using a Nikon Labophot microscope equipped for epifluorescence.

Statistical Analysis. Results are displayed as mean ± S.E.M. because the criteria studied are all ratio level measurements (i.e., continuous variables). When assessing acetaminophen versus saline effect in behavioral experiments, gene expression changes and the number of phospho-ERK immunoreactive cells, we performed a Student's *t* test for two group comparisons.

In the whole reminder of the study, when assessing the influence of selective receptor antagonists or inhibitors on the effect of acetaminophen, analyzes were carried out in a completely randomized design because each rat was assessed for each combination of respective acetaminophen factor/selective receptor antagonist factor effects (Mason et al., 2003); i.e., in the overall rat samples, a rat was used for only one of the four possible combinations of two binary factors. These four combinations were designated with an acronym including the factor initial when it was administrated. The analysis emphasized more particularly the effect of the interaction between acetaminophen factor and antagonist/inhibitor factor, because such an interaction is expected to support our biological hypothesis. The analysis was carried out using a linear model assessing the first-order interaction between the 2 factors adjusted on each main effect. In the case of significant first-order interaction, post hoc multiple comparisons procedure was computed to detail differences between each of the four factor combinations controlling for the overall type I error using Tukey honestly significant difference test. For all tests, a P value less than 0.05 was taken as statistically significant.

Results

Antinociceptive Action of Acetaminophen in the Formalin Test—Screening of Transcript Regulation. Acetaminophen administered orally (400 mg/kg, 40 min before formalin) was able to significantly reduce the two peaks of biting and licking of the rat hind paw elicited by the intraplantar injection of formalin [35 ± 7% (P = 0.004) and 61 ± 12% (P = 0.007) of inhibition in phase I and II, respectively; Fig. 1A]. A quantitative assessment of locomotor activity by video tracking indicated that the observed antinociceptive activity of paracetamol was not due to motor impairment (data not shown).

At the acme of the analgesic action of acetaminophen (30 min after the injection of formalin), lumbar enlargements of the spinal cord, where noxious inputs from hind paws transmit nociceptive messages to higher brain centers, were removed for screening the expression of 1176 gene by nylon cDNA arrays. It revealed that acetaminophen increased by more than 2-fold (with a limited dispersion between animals) the mRNA amounts of genes encoding the low-affinity neurotrophin receptor $p75^{NTR}$, the insulin-like growth factor-1 receptor α subunit IGF-1Rα, and the growth hormone receptor GHR, whereas it reduced mRNA level of the *sst3r* gene coding for the somatostatin 3 receptor (Fig. 1B, Table 1). To verify the reproducibility of the hybridizations, different measurements were made on the same batch of total RNA extracts with different probes and different membranes. Similar results were obtained (data not shown).

Semiquantitative reverse transcription-PCR was used to confirm transcript variations. Immunoblotting also showed variations at the protein level because acetaminophen significantly increased protein amounts of $p75^{NTR}$, IGF-1Rα, and GHR (Table 1). No antibody directed against the sst3R was commercially available.

We also observed that those molecular responses elicited by acetaminophen were specific to this model of tonic pain. Indeed, in healthy rats, acetaminophen increased the expression of nine genes that are all different from those screened in the formalin test (see Supplemental Data, Table S2). Furthermore, the injection of formalin did not lead to transcriptional changes compared with healthy rats at this time point (data not shown), which is in agreement with the literature, in that mRNA changes elicited by formalin start to appear at least 1 h after injection of the agent (Bon et al., 2002; Lin et al., 2003).

Influence of a Selective 5-HT_{1A} Receptor Antagonist. To determine whether these molecular changes were correlated with the antinociceptive action of acetaminophen, the influence of intrathecally injected WAY-100635 (40 μg/rat), a selective 5-HT_{1A} receptor antagonist, was tested on the antinociceptive action of acetaminophen in the formalin model of tonic pain and on transcript levels of the four genes screened. As described previously (Bonnefont et al., 2003a, 2005), we observed a significant interaction between acetaminophen and WAY-100635 ($P < 0.001$), indicating that the 5-HT_{1A} antagonist blocked the antinociceptive activity of acetaminophen during the two phases of nociceptive behavior induced by formalin [36 ± 9% ($P < 0.05$) and 50 ± 9% ($P < 0.05$) inhibition in phases I and II, respectively]. WAY-100635, administered 10 min before formalin had no intrinsic action (data not shown).

Thirty minutes after the injection of formalin, the animals were sacrificed, and lumbar enlargements were removed. Acetaminophen significantly modulated the transcription of the four genes as described above. The antagonist of the 5-HT_{1A} receptor alone had no influence on their transcription but totally blocked the influence of acetaminophen on gene expression regulation (Table 2).

It is noteworthy that we also observed that diclofenac (40 mg/kg, p.o.), a reference nonsteroidal anti-inflammatory drug that inhibits the three COX isoforms (Simmons et al., 1999; Chandrasekharan et al., 2002), exerted an antinociceptive activity only during the second phase of the formalin test, which is in agreement with the literature (Malmberg and Yaksh, 1992), and this action was not blocked by the 5-HT_{1A} receptor antagonist (data not shown).

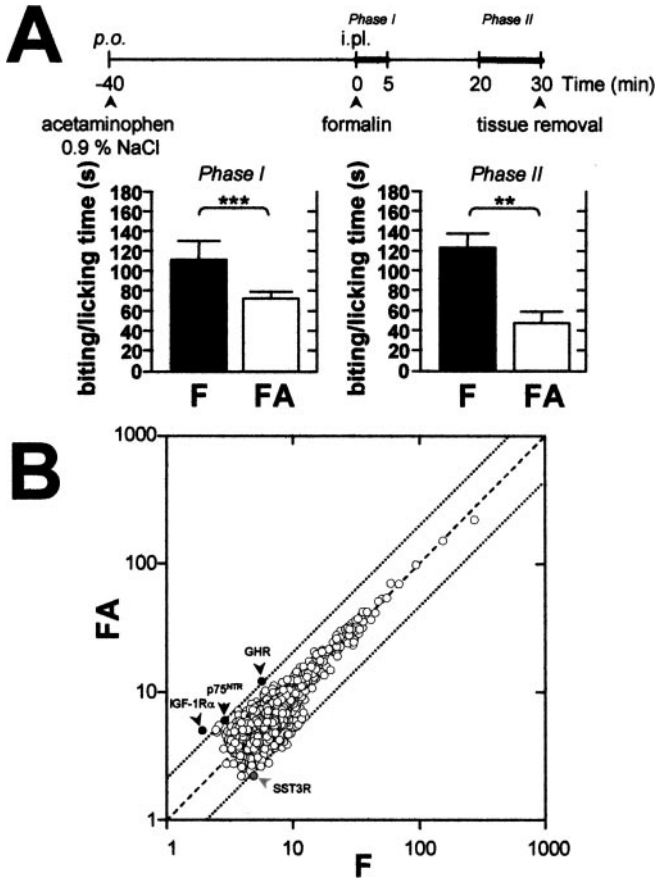

Fig. 1. Antinociceptive action of acetaminophen in the rat formalin test and screening of transcript regulation by nylon cDNA arrays. A, antinociceptive activity of acetaminophen (400 mg/kg, p.o.) in the formalin model of tonic pain in rats. Both phases of nociceptive response (hindpaw biting and licking time) were assessed 0 to 5 min (phase I) and 20 to 30 min (phase II) after intraplantar injection of 2.5% formalin (50 μl). The results are expressed as mean ± S.E.M. of the cumulative biting and licking time during each phase ($n = 6$). **, $P < 0.01$, ***, $P < 0.001$ compared with control group (F) using Student's t test. B, comparison of gene expression changes in the lumbar enlargement of the spinal cord of the two groups of three animals. All rats received an intraplantar administration of 50 μl of 2.5% formalin. Half of them received acetaminophen (400 mg/kg) or saline (p.o.). Normalized expression values in control and treated animals are plotted. Dashed lines indicate 2-fold up or down-regulation. F, saline-treated animals submitted to the formalin test; FA, acetaminophen-treated rats submitted to the formalin test.

TABLE 1

Nylon cDNA array screening of transcript changes promoted by acetaminophen were confirmed by RT-PCR and reflected at a protein level using immunoblotting

Transcript and protein expressions are expressed in -fold changes between acetaminophen-treated and saline-treated rats submitted to the formalin test. End-point RT-PCRs were performed in quadruplicate ($n = 3$), and immunoblots were done in triplicate ($n = 3$).

	cDNA Arrays	RT-PCR	Immunoblots
$p75^{NTR}$	2.12 ± 0.29	2.27 ± 0.39*	1.74 ± 0.25**
IGF1Rα	2.63 ± 0.34	1.91 ± 0.44*	1.17 ± 0.06**
GHR	2.14 ± 0.62	1.99 ± 0.20*	1.15 ± 0.07*
SST3R	0.46 ± 0.09	0.50 ± 0.08*	N.A.

N.A., no antibody raised against sst3R was commercially available.
* $P < 0.05$ vs. control group determined by Student's t test.
** $P < 0.01$ vs. control group determined by Student's t test.

Involvement of the ERK1/2 Pathway. 5-HT_{1A} receptors modulate the transmission of nociceptive messages via opening and closing K^+-/Ca^{2+}-channels, respectively (Millan, 2002). They can also modulate the activity of many intracellular signaling pathways (Raymond et al., 1999), such as the ERK pathway involved in the regulation of gene expression. It is noteworthy that this pathway is known to activate by phosphorylation the Sp family of transcription factors (Strowski et al., 2004), which have consensus sequences on the promoters of the four screened genes. Sp-like transcription factors have even been shown to regulate the transcription of three of them (Poukka et al., 1996; Glos et al., 1998; Yu et al., 1999; Scheidegger et al., 2000). We therefore decided to examine the effect of acetaminophen on ERK1 and ERK2 activities and phosphorylations at the peak of antinociception. Kinase assays revealed that, in the lumbar enlargement of the spinal cord, acetaminophen significantly increased by more than 2-fold the activity of both ERK isoforms, with no modification of their expressions at both protein (Fig. 2) and transcript levels (data not shown). The significant increase in the phosphorylation of ERK1 and ERK2 by 2.17 ± 0.21- and 1.50 ± 0.07-fold, respectively (Fig. 2), would suggest that the augmentation of their activities results from their activation. Such activation of the ERK pathway was mainly located in the superficial laminae of the dorsal horn of the spinal cord (3.7 ± 0.9-fold increase in number of phospho-ERK–positive cells, $P = 0.014$, Fig. 3), where the 5-HT_{1A} receptor is primarily located in the spinal cord (Coggeshall and Carlton, 1997) and where the nociceptive primary afferent fibers connect to projection neurons to transmit nociception toward supraspinal centers (Millan, 1999). This acetaminophen-mediated ERK activation occurred in neurons of the dorsal horn but not in the glial cells (Fig. 4). Analyzing our factorial design involving acetaminophen and WAY-100635 effects on ERK activities showed a significant effect of acetaminophen and a significant interaction between the two factors ($P < 0.001$). WAY-100635 alone did not modify ERK1/2 activities but utterly blocked the increased phosphorylation and activities of both ERKs promoted by acetaminophen (Fig. 2).

Intrathecally injected U0126, a specific inhibitor of mitogen-activated protein kinase kinases 1/2, which are kinases upstream of ERKs, was used to examine the influence of the blockade of the ERK pathway on acetaminophen-modulated transcript levels. We first made sure that U0126 (2 × 5 μg/rat), injected intrathecally 60 and 10 min before formalin, efficiently blocked acetaminophen-elicited increase in ERK1/2 phosphorylation and activities in our conditions. As with WAY-100635, two-way General Linear Model ANOVA indicated a significant acetaminophen-mediated ERK activity increase and a significant interaction between acetaminophen and U0126 on ERK activities ($P < 0.001$). U0126 had no significant intrinsic influence on basal ERK activities but totally prevented the augmentation of phosphorylation and activities of ERK1/2 elicited by acetaminophen. No treatment modified ERK expressions (Fig. 5).

Concerning its influence on acetaminophen-induced transcriptional changes, the inhibitor of the ERK pathway had no influence on the transcription of the four screened genes, and it did not block the increase in $p75^{NTR}$, IGF-1Rα, and GHR mRNAs elicited by acetaminophen. Nonetheless, it significantly prevented the diminution of sst3R transcripts induced by acetaminophen (Table 3).

TABLE 2

Influence of WAY-100635 (40 μg/rat, i.t.) on transcript variations produced by acetaminophen (400 mg/kg, p.o.) in the rat spinal cord

Results are expressed in -fold changes over saline-treated animals submitted to the formalin test (F). Experiments were done in quadruplicate ($n = 3$).

	FA	FW	FAW
$p75^{NTR}$	1.69 ± 0.11*	1.01 ± 0.10	1.07 ± 0.15#
IGF1Rα	1.68 ± 0.09*	1.00 ± 0.07	1.02 ± 0.16#
GHR	1.77 ± 0.20*	1.09 ± 0.11	0.95 ± 0.14#
SST3R	0.71 ± 0.06*	0.92 ± 0.10	0.97 ± 0.22#

FA, acetaminophen-treated rats submitted to the formalin test; FW, WAY-100635-treated rats submitted to the formalin test; FAW, rats treated with acetaminophen and WAY-100635 and submitted to the formalin test.

* $P < 0.05$ vs. F determined by two-way general linear model followed by Tukey Honestly Significant Difference test.

# $P < 0.05$ vs. FA determined by two-way general linear model followed by Tukey Honestly Significant Difference test.

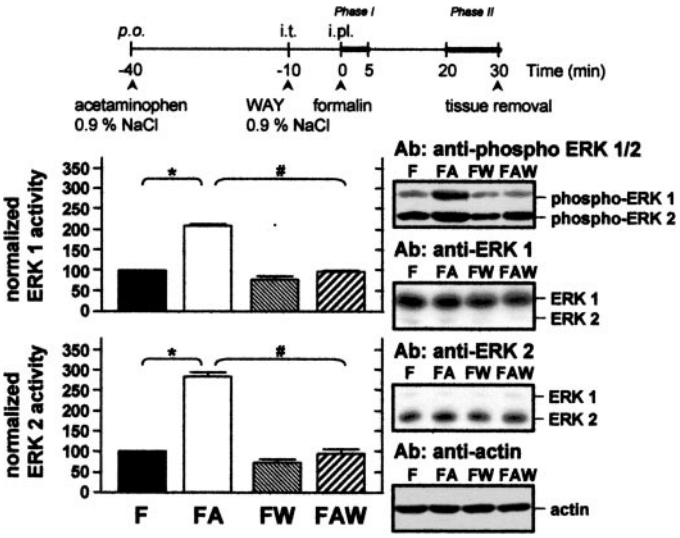

Fig. 2. Acetaminophen activates the ERK1/2 pathway after the stimulation of the 5-HT_{1A} receptor. Kinase assays show activation of ERK1/2 by acetaminophen (400 mg/kg, p.o.) and influence of WAY-100635 (40 μg/rat, i.t.). *, $P < 0.05$ versus F; #, $P < 0.05$ versus FA determined by two-way general linear model followed by Tukey Honestly Significant Difference test. Western blots (right) indicate an acetaminophen-dependent increase in the level of activated phospho-ERKs, blocked by WAY-100635, whereas total ERK1 and ERK2 expressions remained unchanged in all groups of rats. Experiments were done in quadruplicate ($n = 3$). F, saline-treated animals submitted to the formalin test; FA, acetaminophen-treated rats submitted to the formalin test; FW, WAY-100635-treated rats submitted to the formalin test; FAW, rats treated with acetaminophen and WAY-100635 and submitted to the formalin test.

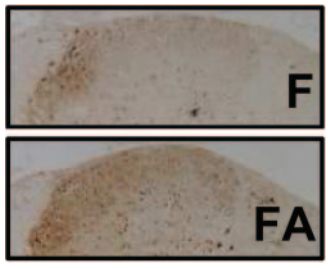

Fig. 3. The activation of the ERK1/2 pathway by acetaminophen occurs in the superficial layers of the spinal cord, ipsilaterally to the injection of formalin. Immunohistochemistry experiments performed on coronal sections of lumbar enlargements of spinal cord show that the level of phosphorylated ERK1/2 in rats receiving a subcutaneous injection of formalin in the right hind paw (F) is increased in the animals treated orally with 400 mg/kg acetaminophen (FA).

Because the activation of the ERK pathway was not involved in the modulation of all the four genes screened, we examined changes in the activation of other mitogen-activated protein kinases (p46 and p54 c-Jun NH_2-terminal kinase, $p38^{MAPK}$) by immunoblotting with antibodies directed against the active phosphorylated forms of those kinases. No modification of their activities was detected in our conditions after a treatment with acetaminophen (data not shown).

The Cellular Events Modulated by Acetaminophen Were Necessary for Its Antinociceptive Activity. Little is known about sst3R, which has been recently cloned, and no selective antagonist is yet available. We therefore looked at the involvement of the ERK pathway in the antinociceptive action of acetaminophen to examine whether the intracellular signaling events leading to the down-regulation of sst3R mRNA levels were participating in the effect of the analgesic in the rat formalin test. This experiment revealed that the main effects of acetaminophen and U0126 were both significant ($P < 0.001$ and $P = 0.029$, respectively). The interaction between acetaminophen and U0126 was bordering our significance level ($P = 0.0547$), suggesting that the influence of U0126 on the antinociceptive activity of acetaminophen was only partial. Indeed, if acetaminophen significantly reduced the biting and licking time in both phases of the test by 35 ± 8% ($P < 0.05$) and 55 ± 10% ($P < 0.05$), its effect was not modified by U0126 in the early phase of the test but significantly reduced the one elicited during the second phase ($P < 0.05$). This reversal was only partial as biting/licking time in the late phase was still significantly shorter by 28 ± 13% in animals treated with both acetaminophen and U0126 compared with the control group (Fig. 6).

We also looked at the implication of the GH/IGF-1 axis in the antinociceptive activity of acetaminophen in the formalin test using pegvisomant and H-1356, selective antagonists of GHR and IGF-1R, respectively. As previously observed, acetaminophen significantly reduced the biting and licking time in both phases of pain [38 ± 9% ($P < 0.05$) and 54 ± 10% ($P < 0.05$) inhibition in phase I and II, respectively]. Interaction between acetaminophen and pegvisomant did not reach significancy suggesting a limited implication of the GH receptor during the antinociceptive activity of acetaminophen. Nonetheless, it is noteworthy that if pegvisomant, used at the highest ineffective dose (10 μg/rat, i.t.), did not modify the activity of acetaminophen during the early phase but significantly altered the effect of the analgesic drug in the late phase ($P < 0.05$). This remained partial in that the reduction of biting/licking time still reached a significant level in animals treated with both acetaminophen and pegvisomant (29 ± 7% of inhibition compared with the control group, $P < 0.05$; Fig. 7A). In the second experiment using H-1356, a significant interaction with acetaminophen was observed ($P < 0.001$). The antinociceptive activity of acetaminophen [47 ± 9% ($P < 0.05$) and 52 ± 10% ($P < 0.05$) inhibition in phases I and II, respectively] was totally blocked by the IGF-1R antagonist (25 μg/rat, i.t.) in both phases (Fig. 7B).

Discussion

Acetaminophen-elicited stimulation of spinal 5-HT_{1A} receptors, thought to be due to the reinforcement of 5-HT descending pathways, modulates pain transmission in a complex manner. Among the cellular events involved, we particularly described that acetaminophen was up-regulating the expression of GH and IGF-1 receptors, which would participate in its antinociceptive activity more or less robustly. We also depicted the partial involvement of the ERK pathway in both regulation of gene expression and mediation of antinociception.

Two main hypotheses are advanced to explain the mechanism of the antinociceptive activity of acetaminophen. Acetaminophen has been linked to the family of nonsteroidal anti-inflammatory drugs inhibiting cyclooxygenases and therefore prostaglandin production. More recently, an interaction with the 5-HT system has been evidenced (for review, Bonnefont et al., 2003b). In agreement with this latter hypothesis, we show that blocking spinal 5-HT_{1A} receptors inhibits the antinociceptive action of acetaminophen in the formalin test, confirming previous results (Bonnefont et et al., 2005). We suspect that the stimulation of those receptors is due to the reinforcement of bulbospinal 5-HT pathways at the supraspinal level (Bonnefont et al., 2003a). Some data suggest that interleukins, responsible for prostaglandin synthesis, may increase cerebral 5-HT catabolism (e.g., Barkhudaryan and Dunn, 1999). One could suggest that reinforcing the activity of the 5-HT descending pathways by acetaminophen would result from inhibition of COX activi-

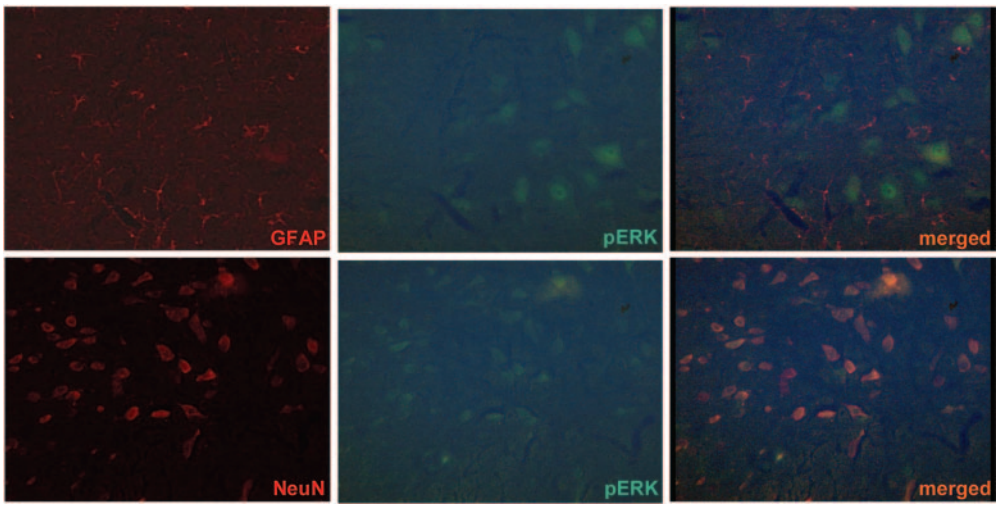

Fig. 4. The ERK1/2 pathway is stimulated in neurons of the dorsal horn of the spinal cord in rats treated by acetaminophen (400 mg/kg, p.o.) and submitted to the formalin test. Immunofluorescence studies indicate that phosphorylated ERK1/2 are colocalized with the neuronal marker NeuN (lower lane) but not glial fibrillary acidic protein (GFAP) (upper lane), a marker of the glial cells.

ties. We therefore assessed the involvement of spinal 5-HT_{1A} receptors in the antinoceptive effect of diclofenac, a nonspecific COX inhibitor, which has been reported to be the most efficient inhibitor of the putative COX-3 (Chandrasekharan et al., 2002). The antinociceptive activity of diclofenac, which has a profile different from that of acetaminophen, was not inhibited by WAY-100635, suggesting that the involvement of the 5-HT system in the effect of acetaminophen is an intrinsic mechanism of action, but not the result of COX inhibition. We do not exclude, however, the idea that some cellular events we observed downstream to the spinal 5-HT_{1A} receptors may depend on the impact of acetaminophen upon both COX activities and 5-HT system.

5-HT_{1A} receptors are known to induce neuronal hyperpolarization to modulate spinal nociceptive processing (Millan, 2002) but also to activate different signaling pathways (Raymond et al., 1999). Activation of these pathways is often related to regulation of mRNA transcription and/or stabilization (Hoffmann et al., 2002). Despite the fact that a direct impact of transcriptional changes on physiological response is difficult to appreciate, regulation of gene expression in nociceptive neurons contributes to neuronal plasticity and development of hyperalgesia and allodynia (Woolf and Costigan, 1999). Thus, gene regulation could exert an important role in promoting antinociception as well.

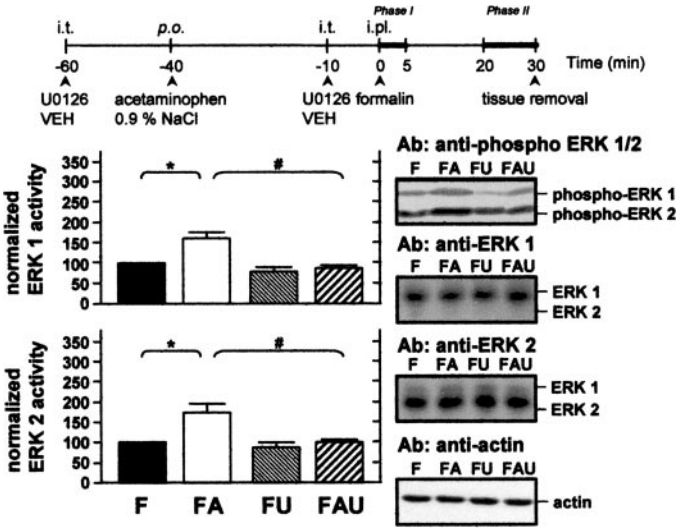

Fig. 5. Intrathecally injected U0126 (2 × 5 μg/rat) blocks the activation of the ERK1/2 pathway elicited by acetaminophen (400 mg/kg, p.o.). *, $P < 0.05$ versus F; #, $P < 0.05$ versus FA determined by two-way general linear model followed by Tukey Honestly Significant Difference test. Western blots (right) indicate an acetaminophen-dependent increase in the level of activated phospho-ERKs, blocked by U-0126, whereas both ERK1 and ERK2 expressions remained unchanged in all groups of rats. Experiments were done in quadruplicate ($n = 3$). F, vehicle-treated animals submitted to the formalin test; FA, acetaminophen-treated rats submitted to the formalin test; FU, U0126-treated rats submitted to the formalin test; FAU, rats treated with acetaminophen and U0126 and submitted to the formalin test.

TABLE 3

Influence of U0126 (2 × 5 μg/rat, i.t.) on transcript variations promoted by acetaminophen (400 mg/kg, p.o.) in the rat spinal cord

Results are expressed in -fold changes over saline-treated animals submitted to the formalin test (F). Experiments were performed in quadruplicate ($n = 3$).

	FA	FU	FAU
$p75^{NTR}$	1.84 ± 0.34*	1.03 ± 0.15	1.74 ± 0.30*
IGF1Rα	1.84 ± 0.34*	1.25 ± 0.25	2.00 ± 0.40*
GHR	1.97 ± 0.44*	1.11 ± 0.22	2.39 ± 0.45*
SST3R	0.50 ± 0.09*	0.99 ± 0.23	0.98 ± 0.14#

FA, acetaminophen-treated rats submitted to the formalin test; FU, U0126-treated rats submitted to the formalin test; FAU, rats treated with acetaminophen and U0126 and submitted to the formalin test.

* $P < 0.05$ vs. F determined by two-way general linear model followed by Tukey Honestly Significant Difference test.

# $P < 0.05$ vs. FA determined by two-way general linear model followed by Tukey Honestly Significant Difference test.

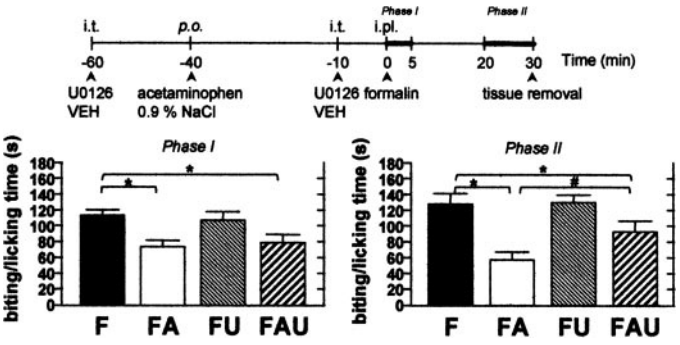

Fig. 6. Intrathecally injected U0126 (2 × 5 μg/rat) partially blocks the effect acetaminophen (400 mg/kg, p.o.) in the rat formalin test. Both phases of nociceptive response (hindpaw biting and licking time) were assessed 0 to 5 min (phase I) and 20 to 30 min (phase II) after intraplantar injection of 2.5% formalin (50 μl). The results are expressed as mean ± S.E.M. ($n = 10$). Vehicle for U0126 was saline containing 10% dimethyl sulfoxide. *, $P < 0.05$ versus F; #, $P < 0.05$ versus FA using two-way general linear model followed by Tukey Honestly Significant Difference test. F, vehicle-treated animals submitted to the formalin test; FA, acetaminophen-treated rats submitted to the formalin test; FU, U0126-treated rats submitted to the formalin test; FAU: rats treated with acetaminophen and U0126 and submitted to the formalin test.

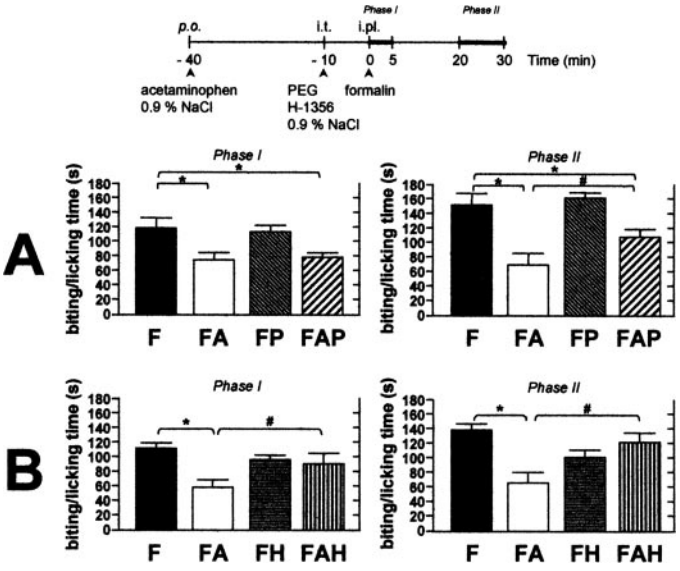

Fig. 7. Acetaminophen-elicited reinforcement of the GH/IGF-1 axis is involved in the antinociceptive activity of the analgesic. Intrathecally injected pegvisomant (10 μg/rat; A) and H-1356 (25 μg/rat; B), selective antagonists of the GH and IGF-1 receptors, respectively, block the effect acetaminophen (400 mg/kg, p.o.) in the rat formalin test. Both phases of nociceptive response (hindpaw biting and licking time) were assessed 0 to 5 min (phase I) and 20 to 30 min (phase II) after intraplantar injection of 2.5% formalin (50 μl). The results are expressed as mean ± S.E.M. ($n = 10$). *, $P < 0.05$ versus F; #, $P < 0.05$ versus FA using two-way general linear model followed by Tukey Honestly Significant Difference test. F, vehicle-treated animals submitted to the formalin test; FA, acetaminophen-treated rats submitted to the formalin test; FP, pegvisomant-treated rats submitted to the formalin test; FAP, rats treated with acetaminophen and pegvisomant and submitted to the formalin test; FH, H-1356-treated rats submitted to the formalin test; FAH, rats treated with acetaminophen and H-1356 and submitted to the formalin test.

Screening 1176 genes using nylon cDNA arrays indicated that acetaminophen modulates the expression of four genes in the lumbar enlargement of the rat spinal cord. We observed a 2-fold increase with a limited dispersion between animals in mRNA levels of $p75^{NTR}$, IGF-1Rα, and GHR and a decrease in sst3R transcript amount. These modulations were dependent on spinal 5-HT_{1A} receptor stimulation, which was in line with our hypothesis that gene expression changes could be important for acetaminophen-produced antinociception. Nonetheless, one could suggest that the screened receptors could be regulated in response to nonspecific effects of acetaminophen, because they are known to be primarily involved in cellular events such as regulation of the cellular cycle, cellular differentiation, apoptosis, or survival (Dechant and Barde, 2002; Jeay et al., 2002; Dupont et al., 2003). The fact that their expressions were not modified in healthy animals treated with the same dose of the analgesic allows us to reasonably exclude this hypothesis. In addition, the completely different set of genes screened in healthy rat lets us suggest that acetaminophen-modulated gene expressions are specific to the pathophysiological state. This is not surprising because the 5-HT_{1A} receptor does not participate in the antinociceptive activity of acetaminophen in every nociceptive test (Bonnefont et al., 2005).

Increases in $p75^{NTR}$, IGF-1Rα, and GHR transcript amounts had repercussions on protein levels further suspecting their potential antinociceptive influence. If gene regulations were not after a simple "on/off" pattern (i.e., that formalin was modulating gene expression toward a pronociceptive phenotype and that acetaminophen was reversing it to the basal state), some evidence suggests that the activity of those up-regulated receptors could be involved in producing antinoception. GHR stimulation provokes IGF-1 synthesis (Schwartzbauer and Menon, 1998) and spinal IGF-1R stimulation produces a dose-dependent antinociception in the rat tail-flick reflex (Bitar et al., 1996). It is noteworthy that reduced GH and IGF-1 secretions are observed in fibromyalgic patients (Leal-Cerro et al., 1999). In line with these data, we observed that reinforcing the GH/IGF-1 axis would participate, at least partially, in spinal nociceptive processing, because both GHR and IGF-1R antagonists seemed to significantly alter the effect of acetaminophen with different intensities.

No selective antagonist of $p75^{NTR}$ is currently available to investigate the involvement of this receptor. However, some studies reported that the stimulation of spinal $p75^{NTR}$ can promote inhibitory neurotransmission and reduce hypersensitivity (Paqueron et al., 2001; Yang et al., 2002).

The meaning of the decrease in sst3R expression is also difficult to appreciate. The physiology of this recently cloned receptor is elusive, partly because of the lack of selective ligands. Therefore, no involvement of sst3R in pain modulation has been described so far, but somatostatin, its endogenous ligand, is known to antagonize the synthesis and release of pituitary GH (Tsuzaki and Moses, 1990). Having observed that the activation of the ERK pathway was responsible for the reduction in sst3R mRNA, we examined the influence of this signaling pathway on the activity of acetaminophen. Our results suggest that ERKs participate in antinociception, and further studies will be needed to indicate whether this is due to regulation of gene expression or direct modulation of protein activity. Studies mainly reported that ERKs were contributing to pain hypersensitivity (Karim et al., 2001; Ji et al., 2002; Obata et al., 2003), including in the formalin test (Ji et al., 1999). In our experiments, we observed a slight but significant increase in ERK1 activity of 24 $\pm$ 9% ($P < 0.05$), but not ERK2 activity, in animals submitted to the formalin test compared with healthy animals (data not shown). However, U0126 exerted no antinociceptive effect, which may be due to an experimental design different from that of Ji et al. (1999). Whatever the case, we propose that the influence of the ERK pathway on nociception is not as simple as observed so far. It has been recently shown that spinal ERK activation by BDNF could produce either pronociception (endogenous BDNF) or antinociception (exogenous BDNF). It has been suggested that the outcome would depend on the nature and/or localization of the cells stimulated (Pezet et al., 2002; Malcangio and Lessmann, 2003). Finally, despite the fact that the role of ERKs is important for the antinociceptive activity of acetaminophen, the fact that their influence remains partial for both gene expression changes and antinociception, suggests that other important pathways are modulated by the analgesic via the 5-HT_{1A} receptor.

In conclusion, the antinociceptive activity of acetaminophen in the rat formalin test is a complex, multistep process that relies on a spinal serotonergic mechanism through 5-HT_{1A} receptor stimulation, subsequent activation of ERK1/2 pathway (among others that remain to be identified), and regulation of protein expressions such as GH and IGF-1 receptors. These cellular events may be specific to acetaminophen because the antinociceptive and pharmacodynamic profiles of diclofenac, the most potent COX-3 inhibitor, are different in this test. Our results indicate that acetaminophen does not exclusively inhibit cyclooxygenase activities and offer new insights into its mechanism of action and the pharmacology of pain.

Acknowledgments

We dedicate this article to the memory of Eliane Duroux and thank her for technical assistance with this work. We also would like to thank Dr. E. Bourinet and Pr. A. I. Basbaum and his group for helpful discussions.

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