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Involvement of interleukin-1β, nerve growth factor and prostaglandin E_2 in endotoxin-induced localized inflammatory hyperalgesia

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1 Intraplantar endotoxin (ET) injection (1.25 μg) into the hind paw of rats resulted in a localized inflammatory hyperalgesia, as assessed by paw pressure (PP), paw immersion (PI), tail flick (TF) and hot plate (HP) tests.

2 ET injection resulted in a significant elevation in the levels of interleukin-1β (IL-1β) and nerve growth factor (NGF) in the injected foot as compared with the non-injected foot. This increase was attenuated by intraperitoneal injections of dexamethasone (200 and 400 μg kg^{-1}) and to a lesser extent by indomethacin (2 and 8 mg kg^{-1}).

3 The tripeptide Lys-D-Pro-Val, which is known to antagonize IL-1β and prostaglandin E_2 (PGE_2) reversed mechanical hyperalgesia, as assessed by the PP test, and reduced significantly thermal hyperalgesia, as assessed by the HP and TF tests.

4 IL-1ra reversed both mechanical (PP) and thermal (PI) nociceptive thresholds tested on the injected leg and significantly reduced thermal hyperalgesia, as assessed by the HP and TF tests.

5 A sheep, anti-mouse NGF antiserum reversed mechanical hyperalgesia (PP test) but had little or no effect on thermal hyperalgesia (PI, HP and TF tests).

6 Our results indicate the importance of IL-1β, NGF and prostaglandin E_2 (PGE_2) in the development of ET induced hyperalgesia and the possible existence of different mechanisms underlying thermal and mechanical as well as central and peripheral hyperalgesia.

Keywords: Inflammation; hyperalgesia; interleukin-1β; nerve growth factor; prostaglandin E_2

Introduction

Inflammatory hyperalgesia is a result of changes in the sensitivity of high threshold nociceptors (Reeh, 1994), in the excitability of spinal neurones (McMahon *et al*., 1993) and in the phenotypic expression of sensory neurones innervating the site of inflammation (Woolf, 1996). Several chemicals, produced by a variety of cells at the inflamed site and within the nervous system, are capable of changing the sensitivity of nociceptors. These mediators, include bradykinin, histamine, neuropeptides, prostaglandin E_2 (PGE_2) and ions like hydrogen and potassium (Dray, 1995). More recently, an important role for inflammatory cytokines has been recognized in sensory hypersensitivity (Ferreira *et al*., 1988; Cunha *et al*., 1992; Watkins *et al*., 1995). The action of cytokines could either be direct upon receptors found on neurones or indirect, by stimulating the release of other substances that can act on neurones, in a cascade-like manner (Safieh-Garabedian *et al*., 1995). Several investigators have demonstrated that the neurotrophin nerve growth factor (NGF) plays a significant role in mediating inflammatory hyperalgesia, an effect which is mediated by NGF binding to the high affinity NGF receptor, trkA (Donnerer *et al*., 1992; Lewin *et al*., 1993; Woolf *et al*., 1994). Also, the pro-inflammatory cytokine interleukin-1β (IL-1β) contributes to the upregulation of NGF, various neuropeptides (Safieh-Garabedian *et al*., 1995) and eicosanoids (Maier *et al*., 1990) during inflammatory hyperalgesia.

We have recently developed a model for localized inflammatory hyperalgesia, an intraplantar injection of endotoxin (ET) administered to the hind foot of both rats and mice (Kanaan *et al*., 1996). The advantages offered by this model include: inflammation and hyperalgesia that remain localized to the injected paw, minimal distress to the animal and almost complete recovery after 24 h (in rats). In the present study, we investigated the changes in IL-1β and NGF levels evoked by injections of ET and the effects of specific antagonists and antisera to the cytokines on ET-induced localized hyperalgesia. A significant increase of IL-1β and NGF levels in ET-injected paw skin was observed which was reversed by treatment with either dexamethasone or indomethacin. Administration of specific antisera or antagonists to IL-1β, NGF and PGE_2 reversed either partially or totally ET-induced hyperalgesia and allowed for discrimination between thermal and mechanical hyperalgesia.

Methods

Animals

Adult male Sprague-Dawley rats (150–200 g) were used in all the experiments. The animals were housed under optimum conditions of light and temperature (12 h light and 12 h dark cycle and $22 \pm 3^{\circ}$C), with food and water provided *ad libitum*. All experiments were carried out with strict adherence to ethical guidelines (Zimmerman, 1983).

Behavioural measurements

Thermal and mechanical pain tests were performed for 3 consecutive days before any injections to establish baseline values. The paw pressure (PP) test was used to assess mechanical hyperalgesia and the hot plate (HP), paw immersion (PI) and tail immersion (TF) tests were performed for the as-

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sessment of thermal hyperalgesia, as described in detail previously (Kanaan *et al*., 1996). Briefly, in the HP test, animals were placed individually on a hot plate (52.8°C – 53.4°C) and the latency of the first sign of paw licking or jumping was taken as an index of the pain threshold. In the TF test, the tail of each animal was immersed into a beaker of distilled water (T = 50.5°C) and the withdrawal latency for tail flicking was recorded; scores were based on 3 trials with a 5 min interval between consecutive tests. For the PI test, injected paw was dipped into a beaker of distilled water (T = 48°C) and the latency to onset of paw removal was recorded. Mechanical hyperalgesia was assessed by the paw pressure (PP) test, by applying a constant pressure of 0.20 kg per cm^2 alternately to the left and right hind paws with a 5 min interval between consecutive applications. The pressure was discontinued when the animals displayed a typical reaction characterized by a vigorous flexion reflex (for more details see Kanaan *et al*., 1996).

Drug administration

Localized inflammation was induced by intraplantar (i.pl.) injection of ET (1.25 μg dissolved in 100 μl sterile physiological saline), prepared from *Salmonella typhosa* (Difco Co., Detroit, Michigan, U.S.A.), into the hind paws of different groups of rats. A separate control group received 100 μl of the sterile saline only, administered in the same manner. One group of rats received intraperitoneal (i.p.) injections of Lys-D-Pro-Val (synthesized at Cambridge Research Biochemicals, Cambridge, U.K. and provided by Dr Stephen Poole, NIBSC) at concentrations of 1 mg, 5 mg or 10 mg kg^{-1}, dissolved in 100 μl saline, 30 min before the ET injection. This tripeptide is known to antagonize both IL-1β and PGE_2-induced hyperalgesia (Poole *et al*., 1992). Another control group received an injection of the tripeptide alone. Recombinant human interleukin-1 receptor antagonist (IL-1ra) (NIBSC preparation 92/672), was administered i.p. as a bolus of 0.625 μg in 100 μl saline 30 min before the ET injection. A sheep anti-mouse NGF antiserum (5 μl g^{-1}; i.p.) was administered to a group of animals, 30 min before the ET injection. The anti-mouse NGF antiserum (a gift from Prof. Clifford J. Woolf, University College London) had been characterized previously for its specificity and titre (Woolf *et al*., 1994). Rats in the control group received normal sheep serum (NSS; 5 μl g^{-1}; i.p.) 30 min before the ET injection. Dexamethasone and indomethacin were administered in doses established as appropriate in previous studies (Benedetti & Butler, 1990; Brochard *et al*., 1992). Dexamethasone phosphate (Laboratory Renaudin, France) dissolved in saline was injected at concentrations of 200 and 400 μg kg^{-1} in different groups of rats, 3 h after the ET injection. Indomethacin was prepared by dissolving indomethacin-lactose (gift from Algorithm, Lebanon) in phosphate buffered saline (PBS; pH = 7.4) and injected at concentrations of 2 and 8 mg kg^{-1}, 3 h after the ET injection (1.25 μg).

Experimental procedures

Each experimental group consisted of 5 or 6 animals. After baseline values had been established for 3 consecutive days, behavioural measurements were carried out at 3 h, 6 h, 9 h and 24 h after ET or saline injection alone or in conjunction with any of the drug treatments detailed above. In the experiments which involved tissue removal, the animals were terminally anaesthetized (sodium pentobarbitone; 50 mg/kg) at 1 h, 2 h, 3 h, 4 h, 6 h and 9 h and the entire hind paw skin (from left and right feet) was removed. The tissue samples were weighed, snap frozen on dry ice and stored at −70°C to be processed for IL-1β and NGF determinations.

IL-1β and NGF assays

Skin tissue was homogenized in phosphate buffered saline (PBS; pH = 7.4) containing 0.4 M NaCl, 0.05% Tween-20, 0.5% bovine serum albumin, 0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 KI ml^{-1} aprotinin. The homogenates were centrifuged at 12,000 *g* for 60 min at 4°C. Both NGF and IL-1β concentrations in the supernatants were measured by two-site enzyme-linked immunosorbent assay (ELISA). The NGF assay was based on the method of Weskamp and Otten (1987), with minor modifications as detailed previously (Safieh-Garabedian *et al*., 1995). A polyclonal rabbit anti-NGF antibody (gift of Peter Frey, Sandoz) was used as the coating antibody. A purified mouse NGF was used as the standard and a rat anti-NGF monoclonal antibody (23c4; Weskamp and Otten) as the recognition antibody. IL-1β was measured by a two-site ELISA in the same supernatant as above and as detailed previously (Safieh-Garabedian *et al*., 1995), using immunoaffinity purified polyclonal sheep anti-rat IL-1β (Taktak *et al*., 1991) to coat high binding microtitre plates. Recombinant rat IL-1β (a gift from Dr Robert Newton, DuPont-Merk, Wilmington, Delaware) was used as the standard and a biotinylated, immunoaffinity purified polyclonal sheep anti-rat. IL-1β was used as the recognition

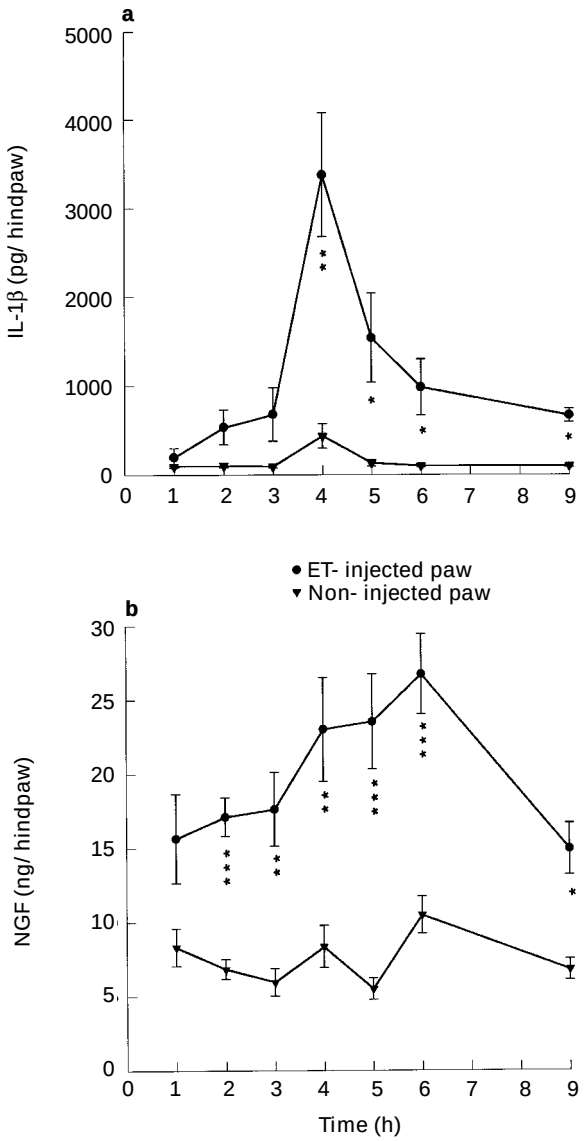

Figure 1 Time course of changes in the levels of interleukin-1β (IL-1β) (a) and nerve growth factor (NGF) (b) in the skin of endotoxin (ET) injected (1.25 μg) and non-injected hindpaws of different groups ($n = 5$ in each) of rats, for each time interval. Each point represents the mean and vertical lines show s.e.mean (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ as compared with non-injected paw).

antibody. For both assays, the colour was developed for 15–20 min by using streptavidin horseradish peroxidase (Dako) and the chromagen, 3,3′,5,5′-tetramethyl-benzidine (Sigma); the optical density (o.d.) was measured at 450 nm. Results for IL-1β and NGF levels in the skin are expressed as pg/hind paw and ng/hind paw, respectively.

Statistical analysis

The degree of significance of differences between experimental groups was performed by the ANOVA test followed by Bonferroni post-test analysis, by use of the Graph Pad Software version 1.13 and Prism version 1. All graphical plots were constructed with Jandel Sigma Plot version 3.0 for Windows '95.

Results

IL-1β and NGF levels in the hind paw

Intraplantar ET injection (1.25 μg) into the hind paw resulted in a significant decrease in mechanical (determined by the PP test) and thermal (determined by PI, HP and TF tests) nociceptive thresholds. The peak of increased sensitivity was obtained at 9 h and by 24 h threshold values were not significantly different from those of saline, or naive controls, data not shown (Kanaan *et al*., 1996).

The time course of elevated IL-1β levels in injected and non-injected paws is given in Figure 1a. At 2 h after ET injection, the level of this cytokine started increasing in the injected paw (537.2 ± 194.8 pg/hind paw) and a peak value

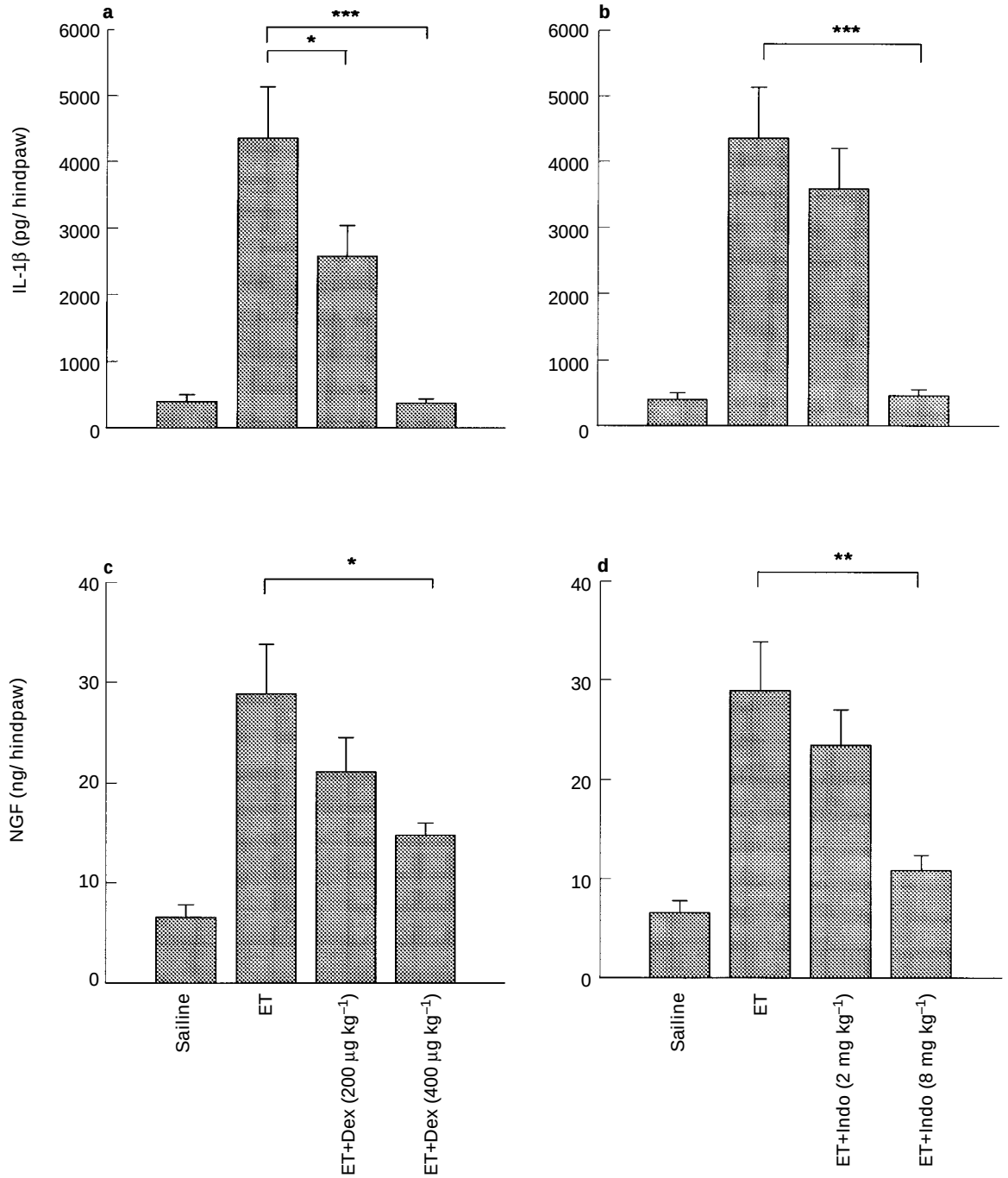

Figure 2 The levels of interleukin-1β (IL-1β) (a and c) and nerve growth factor (NGF) (b and d) measured in the skin of the hindpaws of rats injected with either saline, endotoxin (ET) only (1.25 μg), ET plus dexamethasone (Dex) (200 and 400 μg kg^{-1}), or ET plus indomethacin (Indo) (2 and 8 mg kg^{-1}). Each value represents the mean and vertical lines show s.e.mean (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

(3372.6 ± 701.0 pg/hind paw) was obtained at 4 h which was significantly ($P<0.01$) higher than the non-injected paw (440.0 ± 139.0 pg/hind paw). At 9 h, the level of IL-1β started decreasing (666.9 ± 75.0 pg/hind paw) but was still significantly ($P<0.05$) higher than the control paw (100.0 ± 20.0 pg/hind paw). The NGF level in the injected paw showed an increase starting at 1 h after ET injection, that became statistically significant ($P<0.001$) at 2 h (17.11 ± 1.30 ng/hind paw) as compared with the non-injected paw (6.81 ± 0.66 ng/hind paw), with a plateau obtained between 4 h and 6 h (23.00 ± 3.51 and 26.73 ± 2.70 ng/hind paw, respectively) when compared with the non injected paw (8.34 ± 1.43 and 5.48 ± 0.72 ng/hind paw, respectively). This was followed by a partial recovery at 9 h (14.95 ± 1.75 ng/hind paw) (Figure 1b).

Injection (i.p.) of dexamethasone (200 μg kg^{-1}), 3 h after ET injection, significantly ($P<0.05$) reduced IL-1β levels (from 4353.31 ± 780.71 to 2576.14 ± 462.03 pg/hind paw), but had no significant effect on the level of NGF (28.89 ± 4.90 versus 21.1 ± 3.40 ng/hind paw) at 4 h after ET treatment (Figures 2a and c). The higher dose of dexamethasone (400 μg kg^{-1}) almost completely reduced the level of IL-1β to baseline values (376.93 ± 61.01 pg/hind paw), and significantly ($P<0.05$) reduced NGF levels at 4 h (from 28.89 ± 4.9 to 14.75 ± 1.23 ng/hind paw; Figure 2a and c). Indomethacin treatment (2 mg kg^{-1}; i.p.), 3 h after ET injection had no significant effect on IL-1β (4353.31 ± 780.71 versus 3582.10 ± 622.07 pg/hind paw; Figure 2b), or NGF (28.89 ± 4.90 versus 23.4 ± 3.6 ng/hind paw; Figure 2d) levels. However, indomethacin at 8 mg kg^{-1} (i.p.) significantly reduced IL-1β (459.36 ± 93.70 pg/hind paw; $P<0.001$) and NGF (10.83 ± 1.50 ng/hind paw; $P<0.01$) levels at 4 h (Figure 2b and d).

Effect of Lys-D-Pro-Val

The tripeptide Lys-D-Pro-Val (10 mg kg^{-1}, i.p.) reversed the ET-induced mechanical hyperalgesia as determined by the PP test (Figure 3a). However, it only reduced thermal hyperalgesia as assessed by the HP test ($P<0.001$) and TF test ($P<0.01$) (Figure 3c and e). Figure 3b, d and f illustrate the effects of various concentrations of this tripeptide as % of baseline value, determined at 9 h after the ET injection.

Effect of IL-1ra

IL-1ra injection (0.625 μg; i.p.) produced an almost complete reversal of the ET induced hyperalgesia (both thermal and mechanical), at the level of the injected foot, as assessed by the PP and PI tests (Figure 4a and b), while hyperalgesia assessed by HP and TF tests was partially reduced, with a minimal effect observed on the TF test (Figure 4c and d). Animals treated with IL-ra alone did not elicit any significant changes in the pain thresholds of the different pain tests (data not shown).

Effect of anti-NGF

Injection of anti-NGF antiserum (5 μl g^{-1}; i.p.) 30 min before the ET injection, produced an almost complete reversal of the mechanical hyperalgesia as assessed by the PP test performed on the injected leg (Figure 5a). Anti-NGF antiserum attenuated the thermal hyperalgesia slightly but significantly ($P<0.05$) as assessed by the PI and TF tests (Figure 5b and c) and more significantly ($P<0.01$) as determined by the HP test (Figure 5d). Animals treated with normal sheep serum 30 min before

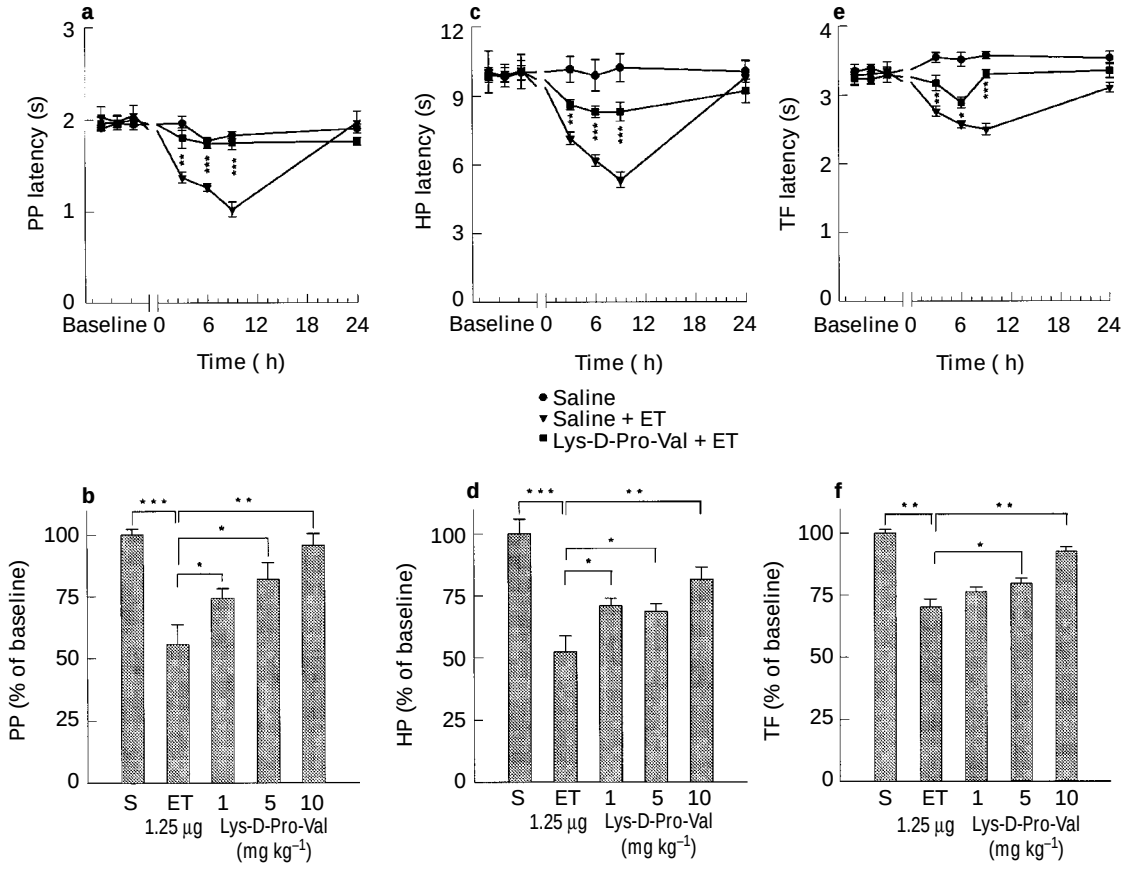

Figure 3 The effects of Lys-D-Pro-Val on endotoxin (ET)-induced (1.25 μg) hyperalgesia as assessed by the paw pressure (PP; a and b), hotplate (HP; c and d) and tail flick (TF; e and f) tests. (a), (c) and (e) show the time course of the effect of 10 mg kg^{-1} dose. Each point represents mean and vertical lines show s.e.mean for each experimental group ($n=5$). The significance of differences is based on comparison between values of ET and ET plus Lys-D-Pro-Val. (b), (d) and (f) show the effect of different doses of Lys-D-Pro-Val, measured at 9 h in separate groups of rats ($n=5$ for each dose) on the responses to ET (1.25 μg); S, saline. (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

the ET injection were not significantly different from animals treated with the ET only.

Discussion

The results of this study demonstrate that in the ET model of localized inflammatory hyperalgesia, that we have described previously (Kanaan *et al*., 1996), there is a significant elevation in the levels of both IL-1β and NGF, the latter showing a more sustained increase, but both declining before the peak of hyperalgesia, observed at 9 h. Similar elevations in IL-1β level and NGF have been demonstrated in rats, with i.pl. injections of complete Freund's adjuvant (CFA) (Woolf *et al*., 1994; Safieh-Garabedian *et al*., 1995). Furthermore, intraplantar injections of either IL-1β (Ferreira *et al*., 1988) or NGF (Woolf *et al*., 1994; Andreev *et al*., 1995) were also shown to produce hyperalgesia. Therefore, we assume that both IL-1β and NGF may contribute to the ET-induced hyperalgesia. This assumption is further supported by the significant reduction of ET-induced increase in IL-1β and NGF levels by dexamethasone (a steroidal anti-inflammatory drug, which is known to inhibit the early phenomenon of inflammation and the synthesis of various algogenic or pro-algogenic substances such as eicosanoids and interleukins) (Masferrer *et al*., 1992; Victorov & Hoek, 1995) and to a lesser extent indomethacin (a non-steroidal anti-inflammatory drug known to inhibit prostaglandin-forming cyclo-oxygenase cascade and more specifically cyclo-oxygenase-1) (Mitchell *et al*., 1993). We have already shown that these drugs significantly reduce both thermal and mechanical hyperalgesia as a result of the ET injection (Jabbur *et al*., 1996). Similar observations were obtained for the CFA model of inflammation, with the exception that dexamethasone did not significantly affect thermal hyperalgesia (Safieh-Garabedian *et al*., 1995). Even though the inhibitory effect of glucocorticoids on IL-1β is well characterized (Lee *et al*., 1988; Dawson *et al*., 1993), the effect of indomethacin on the production of this cytokine is less clear (Dawson *et al*., 1993; Utsunomiya *et al*., 1994). However, our study shows that indomethacin, at high doses only, prevented the increase in both IL-1β and NGF levels induced by i.pl. injection of ET.

Whether the possible mediation of the ET-induced hyperalgesia through IL-1β is via PGE_2-dependent (Ferreira *et al*., 1988; Maier *et al*., 1990) or independent (Follenfant *et al*.,

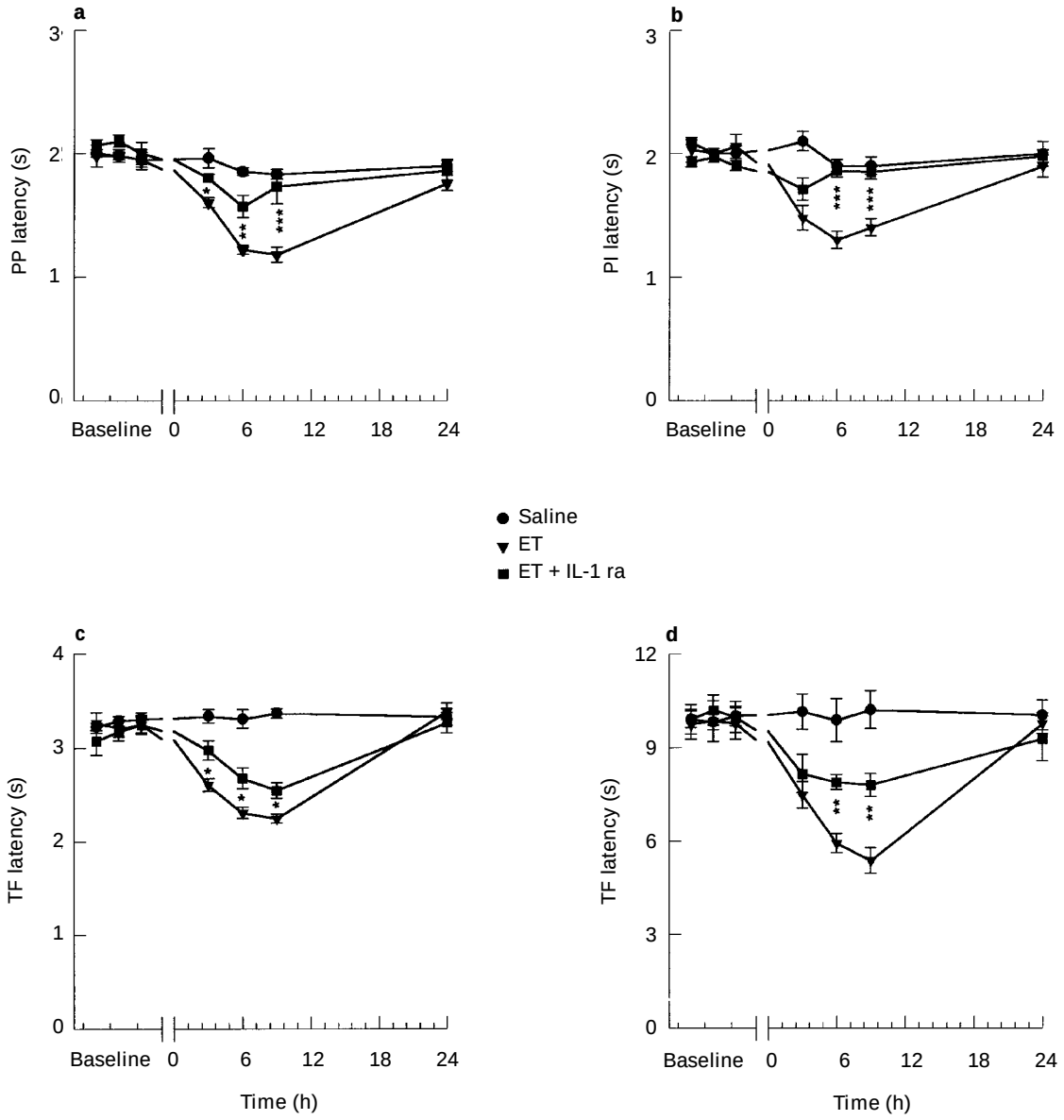

Figure 4 Time course of the effects of an interleukin-1 receptor antagonist (IL-1ra; 0.625 μg) on endotoxin (ET)-induced (1.25 μg) hyperalgesia. Each graph shows the effect of either saline, ET or ET plus the IL-1ra on a specific pain test. Each point on the curve represents the mean and vertical lines show s.e.mean, for each experimental group ($n=5$ in each). The significance of differences is based on comparison between the values of ET and ET plus the IL-1ra. (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

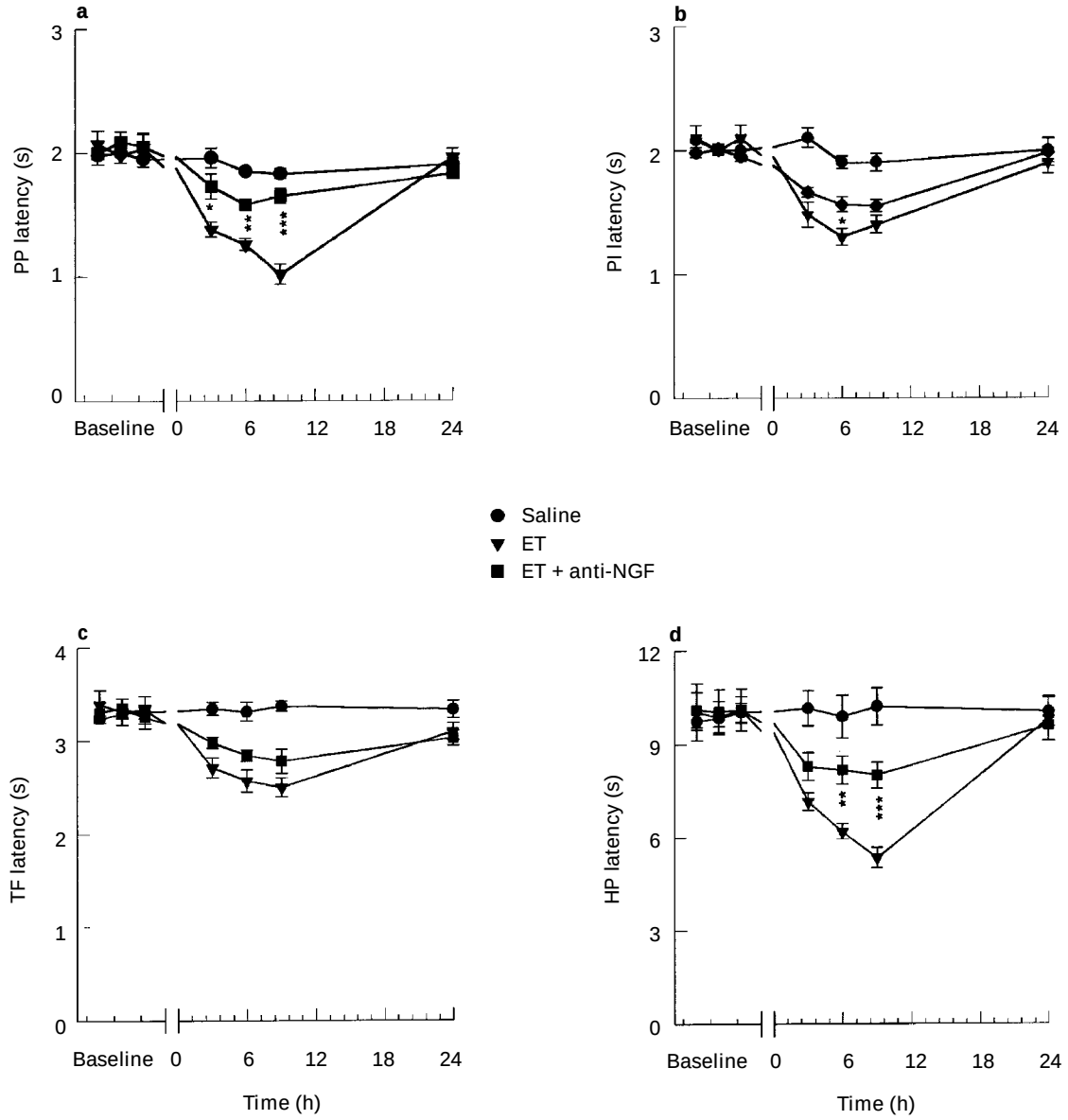

Figure 5 Time course of the effects of anti-nerve growth factor (anti-NGF; 5 μl g^{-1}) on endotoxin (ET)-induced (1.25 μg) hyperalgesia. Each graph shows the effect of either saline, ET or ET plus anti-NGF on a specific pain test. Each point in the curve represents the mean and vertical lines show s.e.mean for each experimental group ($n=5$ in each). The significance of differences is based on comparison between the values of ET and ET plus anti-NGF. (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

1989; Watkins *et al*., 1994) mechanisms is still debatable. The fact that Lys-D-Pro-Val, which is known to antagonize PGE_2 and IL-1β induced hyperalgesia, reversed completely the mechanical hyperalgesia (tested at the site of the injected leg) but did not completely reverse the thermal hyperalgesia (assessed by the HP and TF tests), strongly suggests the involvement of PGE_2. This involvement is further supported by several studies showing that ET induces cyclo-oxygenase-2 activity in macrophages, monocytes (Lee *et al*., 1992) and kupffer cells (Victorov & Hoek, 1995).

Our results also indicate that both mechanical and thermal hyperalgesia can exist independent of each other and that there is dissociation between peripheral and central mechanisms of the ET-induced inflammatory hyperalgesia (Kanaan *et al*., 1996). The use of specific antagonists for different cytokines lends further support to this hypothesis. IL-1ra was more effective in reversing the mechanical and thermal hyperalgesia in the injected leg (PP and PI tests) than the thermal hyperalgesia assessed by the HP and TF tests, the latter being the least affected. This difference could be explained by data from our previous study (Kanaan *et al*., 1996), showing that the tail flick hyperalgesia reflects mainly a central hyperexcitable state induced by the ET injection, while HP hyperalgesia could be attributed to both central and peripheral components since the injected paw is in contact with the heating pad that is used to perform the experimental test. Furthermore, this finding clearly demonstrates that IL-1β release by ET plays a key role in triggering hyperalgesia, but this does not exclude the possibility that other factors could also be involved. In fact, this assumption is in agreement with a previous study (Ferreira *et al*., 1993) showing that each of the antisera neutralizing IL-1β, IL-6 and IL-8, inhibited partially the ET-induced mechanical hyperalgesia. There are several ways in which IL-1β can contribute to hyperalgesia; for example, it can directly activate nociceptors (Fukuoka *et al*., 1994), upregulate NGF level (Lindholm *et al*., 1987; Safieh-Garabedian *et al*., 1995) and subsequently, as demonstrated previously, it can regulate both substance P and calcitonin gene-related peptide in adult dorsal root ganglion neurones (Lindsay *et al*., 1989). The fact that anti-NGF antiserum is capable of reducing hyperalgesia, further substantiates the importance of NGF in inflammatory hyperalgesia. This antiserum reversed the mechanical hyperalgesia, as assessed by pressure applied to the injected leg, while the thermal tests (performed on the injected paw either com-

pletely, (PI), partially (HP) or independently from it (TF)) were less affected by these antisera. These results lend further support to the assumption we made earlier that different pathways may exist for both thermal and mechanical hyperalgesia.

In conclusion, ET induced (i.pl.) localized inflammation and hyperalgesia are mediated via IL-1β, NGF and PGE_2, with the possible involvement of other factors. This increase in the levels of proinflammatory mediators was attenuated by dexamethasone and to a lesser extent by indomethacin. Hyperalgesia was partially or totally reversed by specific antisera and antagonists which allowed the dissociation between thermal and mechanical hyperalgesia and between the peripheral and central mechanisms of the ET-induced hyperalgesia.

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