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CINC-1 mediates the sympathetic component of inflammatory mechanical hypersensitivity in rats



Abstract

The hyperalgesic effect of cytokine-induced neutrophil chemoattractant 1 (CINC-1/CXCL1) was measured in a model of mechanical hyperalgesia in rats. CINC-1 evoked a dose-dependent mechanical hypersensitivity, which was already significant 2 h after the cytokine injection, peaked 4 h after and decreased thereafter. The local pre-treatment of the rats with the β -adrenoceptor antagonist, atenolol (25 mg paw⁻¹), but not with the cyclooxygenase inhibitor indomethacin (100 mg paw⁻¹), inhibited (86%) the

CINC-1-induced hypersensitivity. Conversely, IL-1b-evoked hypersensitivity was inhibited (76%) by local pre-treatment of the animals with indomethacin, but not by atenolol. Carrageenin- and TNF-a-evoked hypersensitivity were attenuated to about the same extent (50%) by antisera neutralising CINC-1 or IL-1b. The association of both antisera abolished the hypersensitivity effect of carrageenin and TNF-a. In addition, carrageenin, LPS and TNF-a were shown to stimulate the production of immunoreactive CINC-1 in the skin of injected paws. These data suggest that CINC-1, released at sites of inflammation, mediates inflammatory hyperalgesia in rats via release of sympathomimetic amines.

Full text

Introduction

Sensitisation of the pain receptor is the common denominator of inflammatory pain. This functional up-regulation of pain receptors is referred to as hyperalgesia [1]. Products of the arachidonic acid/cyclooxygenase pathway, e.g. prostaglandin (PG), and also the sympathomimetic amines are hyperalgesic mediators that directly sensitise the nociceptor [for review see 2]. The capacity of the PGE₂ and PGI₂ to sensitise the primary sensory neurone has been studied extensively in man and in different experimental models, using behavioural and electrophysiological techniques [3-5]. Sympathomimetic amines, such as dopamine and noradrenaline, have been shown to functionally sensitise nociceptors [6].

Although the final hyperalgesic mediators are PG and sympathomimetic amines, the release of these agents is usually preceded by the release of cytokines [7-9]. Therefore, the release of cytokines appears to constitute a link between cellular injury and/or recognition of non-self and the release of the (final) hyperalgesic mediators. In this context, over the past decade, it has been demonstrated that IL-1b and IL-6 stimulate the production of eicosanoids [7, 9] and that IL-8 stimulates the production of sympathomimetic amines [8]. TNF-a also induces mechanical hypersensitivity, which is partially inhibited by antisera neutralising IL-1b or IL-8 and is abolished by the association of both antisera [9], suggesting that IL-1b and IL-8 are involved in the hyperalgesic effect of TNF-a. Confirming this suggestion, inflammatory agents, such as carrageenin and lipopolysaccharide (LPS), evoked mechanical hypersensitivity in rats by

stimulating the release of bradykinin, which stimulated the release of TNF- α , in turn, stimulating the production of IL-1 β /IL-6, and of IL-8 [10].

Chemokines, signifying chemotactic cytokines, are a family of structurally related proteins (8-15 kDa) that play a pivotal role in inflammation, sharing the capacity to induce migration and activation of specific subsets of leukocytes. The chemokines have been subdivided [11] into four subfamilies on the basis of the position of either one or two cysteine residues located near the N-terminus of the protein as in the following list, in which the commonly used name and the newer nomenclature are given for each major chemokine: C (lymphotactin α /XCL-1 and β /XCL2), CC (MCP-1/CCL2, MIP-1 α /CCL3, RANTES/CCL5, MCP3/CCL7, MCP-2/CCL8, eotaxin/CCL11), CXC (IL-8/CXCL-8, CINC-1/CXCL1, MIP-2 α /CXCL2, MIG/CXCL9, IP-10/CXCL10) and CX₃C (fractalkine/CX₃CL1). The CXC chemokines are also subdivided according to the presence of the ELR motif (glutamic acid-leucine-arginine) preceding the CXC in the N-terminus. The ELR-CXC chemokines cause, preferentially, the recruitment of neutrophils, while the non-ELR-CXC chemokines are more specific for lymphocytes. The CC chemokines are attractants for mononuclear cells/macrophages and eosinophils. The C and CX₃C chemokines were recently classified [11], the first class being selective for lymphocytes, and the CX₃C chemokines acting on diverse cell types, including monocytes, T and NK cells.

Besides being able to induce chemotaxis and cell activation, chemokines are also implicated in other 'inflammatory' processes such as angiogenesis and fever [12-14]. In addition, the direct involvement of chemokine receptor activation in nociception and hyperalgesia has been suggested [for review see 15]. CINC-1 (cytokine-induced neutrophil chemoattractant 1) is an 8-kDa polypeptide first isolated and purified [16] from the culture supernatant of the rat kidney epithelial cell line NRK-52E stimulated with IL-1 β or TNF- α or LPS. Rats and mice do not produce IL-8, but they have molecules that are homologues for human GRO (KC and MIP-2 in mice and CINC in rats) [16-18]. CINC-1 has a high degree of homology with IL-8 and shares the same CXCR2 receptor [19, 20].

Although rats do not produce IL-8, they are able to respond to this human chemokine. As mentioned above, human IL-8, when injected into the paws of rats, induced mechanical hypersensitivity and an anti-IL-8 serum inhibited the mechanical hypersensitivity induced by carrageenin and LPS [8-10, 21]. A possible explanation for these findings is that the anti-IL-8 serum cross-reacts

with a rat endogenous hyperalgesic cytokine. The intracerebroventricular (i.c.v.) injection of CINC-1 decreased the nociceptive threshold for mechanical stimuli in a paw-pressure test in rats (a model of mechanical hyperalgesia), suggesting that CINC-1 in the central nervous system facilitates mechanical nociception [22]. In the present study, we investigated the effect of intraplantar injection of CINC-1 on mechanical hypersensitivity in rats, and also the mechanisms underlying the effects of CINC-1 that we describe.

Materials and methods

Animals

Male Wistar rats, weighing 130-180 g, were housed in temperature controlled rooms (22-25°C) and given water and food *ad libitum* until use. All experiments were conducted in accordance with the ethical guidelines of the International Association for the Study of Pain.

Nociceptive test: mechanical nociceptor hypersensitivity

Mechanical nociceptor hypersensitivity was tested in rats [23]. A constant pressure of 20 mm Hg (measured using a sphygmomanometer), was applied (*via* a syringe piston moved by compressed air), to an area of 15 mm² of the dorsal surface of the hind paw, and discontinued when the rat presented a typical "freezing reaction". The freezing reaction was signalled by a brief apnoea, concomitant with a retraction of the head and forepaws and a reduction in the escape movements, frequently made to escape from the position imposed by the experimental situation. Usually, the apnoea was associated with successive waves of muscular tremor. For each animal, the latency to the onset of the freezing reaction (from the time of first application of the pressure) was measured before administration (zero time) and at different times after administration of the hyperalgesic agents. The intensity of mechanical hypersensitivity was quantified as the reduction in the reaction time, calculated by subtracting the value of the second measurement from the first [23]. Reaction times were typically 32-34 s (with standard errors of mean [S.E.M.] of 0.5-1.0 s) before injection of the hyperalgesic agents. Multiple paw treatments with control solutions did not alter basal reaction times. Different individuals prepared solutions to be injected, performed the injections, and measured the reactions times.

Experimental protocol

Mechanical nociceptor hypersensitivity was measured 3 h after intraplantar (i.pl., 100 μ l) injections of CINC-1 (3–300 pg), IL-1b (0.5 pg), TNF- α (2.5 pg) and carrageenin (Cg, 100 μ g) into hind paws of rats. The hypersensitivity induced by CINC-1 (100 pg paw⁻¹) was also evaluated 2, 4, 6 and 24 h after its injection. The larger doses of IL-1b, TNF- α or carrageenin were the smallest that evoked maximum responses and the time interval of 3 h was a time at which responses to the chosen doses of hyperalgesic agents were all at or close to their peak [8–11, 24]. Anti-hyperalgesic agents (indomethacin 100 mg, atenolol 25 mg, or both, in 50 μ l) were injected into hind-paws to be injected 30 min later with CINC-1 (100 pg) or IL-1b (0.5 pg). The anti-CINC-1 serum (50 μ l) anti-IL-1b serum (50 μ l) or control pre-immune serum (50 μ l) also were injected into hind-paws to be injected, 30 min later, with carrageenin (100 μ g), TNF- α (2.5 pg) or CINC-1 (100 pg). Since we previously demonstrated that an anti-hu-IL-8 inhibited carrageenin and TNF- α -induced hypersensitivity in rats, and that this species does not produce IL-8, we tested whether the antibody to human IL-8 cross-reacts with CINC-1. For this, 50 μ l of the anti-hu-IL-8 was injected into hind-paws 30 min before CINC-1 (100 pg) administration and the hypersensitivity was measured 3 h later.

Measurement of CINC-1 concentration in rat paws

Saline (100 μ l), Cg (100 μ g, 100 μ l⁻¹) and LPS (1 mg, 100 μ l⁻¹) were injected into the hind-paws of rats, and 0.5, 1, 2, 3, 6 or 24 h later the animals were killed and skin paw samples taken and extracted. TNF- α (2.5 pg, 100 μ l⁻¹) was also injected into the hind-paws of rats and skin paw samples taken 3 h later. The samples were processed as described previously [24, 25]. Briefly, the skin samples were placed in 500 μ l of protease inhibitor buffer and homogenised (POLYTRON[®] PT3100). The samples were then centrifuged (1500 $\times$ g for 10 min) and the supernatant stored at -70°C until CINC-1 measurement. The concentrations of CINC-1 were measured by ELISA based upon a previously described protocol [26]. Briefly, microtiter plates (Nunc-Maxisorb) were coated overnight at 4°C with a rabbit anti-rat CINC-1 polyclonal Ab (PeproTech, USA; 2 mg.ml⁻¹). After blocking the plates, rat CINC-1 (PeproTech) standards at various dilutions in RPMI and samples were added in duplicate at room temperature for 2 h. Goat anti-rat CINC-1 biotinylated polyclonal Abs (R&D systems, USA) at a 1:500 dilution were added, followed by incubation at room

temperature for 1 h. Finally, 100 ml of avidin-HRP (1:5000 dilution) were added to each well and, after 30 min, the plates were washed and the colour reagent, OPD (40 mg. well⁻¹), added. After 15 min, the reaction was quenched with H₂SO₄ (1 M) and the O.D. measured at 490 nm. The results were expressed as ng of CINC-1 per paw, obtained by comparing the O.D.s with a standard curve for recDNA CINC-1. This ELISA assay does not recognise CINC-2a or CINC-3 up to the concentration of 10 ng/ml, despite recognising CINC-1 at a concentration of 0.02 ng/ml.

Drugs

Pre-immune serum, anti-rat IL-1b serum, recombinant rat IL-1b and TNF- α were obtained from the National Institute for Biological Standards and Control (NIBSC). Recombinant rat CINC-1 and rabbit anti-rat CINC-1 polyclonal Ab were purchased from PeproTech. Goat anti-rat CINC-1 biotinylated polyclonal Ab was obtained from R & D Systems, carrageenin from FMC Corporation, Philadelphia, USA and indomethacin from Merck, Sharpe & Dohme Ltd, Brazil. Atenolol and other reagents were purchased from Sigma, St Louis, USA.

The LPS content in the cytokines was used in the order of 0.25 iu. mg⁻¹, equivalent to a little over 10⁻¹⁵g of LPS in a hyperalgesic dose of IL-1b (0.5 pg). The threshold hyperalgesic dose of LPS in the above model is approximately 100 ng, i.e. 10⁻⁷ g [10]. Therefore, the doses of the hyperalgesic agents used contained amounts of LPS of up to eight log₁₀'s less than the threshold hyperalgesic dose of LPS.

Data analysis

Results are presented as means $\pm$ S.E.M. for groups of 5 animals for the *in vivo* measurements and for 4 samples for the *in vitro* cytokine measurements. Three independent *in vitro* experiments were performed, each giving similar results. Differences between responses were evaluated by one-way analysis of variance (ANOVA), followed by the Bonferroni t-test. Results with $p < 0.05$ were considered significant.

Results

Mechanical nociceptor hypersensitivity induced by CINC-1

Intraplantar injection of CINC-1 (3-300 pg) into one hind paw of Wistar rats evoked a dose-dependent mechanical nociceptor hypersensitivity, measured 3 h after injections, in both hind paws, except for the smallest doses (3 and 10 pg), which evoked hypersensitivity only in the injected paws (Figure 1A). The hypersensitivity induced by CINC-1 (100 pg) was also pronounced at 2, 4 and 6 h after its administration compared to the response induced by saline injection (Figure 1B). The hypersensitivity response induced by CINC-1 returned to basal level 24 h after its injection (data not shown).

Inhibition by atenolol, but not indomethacin, of CINC-1-induced nociceptor hypersensitivity

Injection (i.pl.) of CINC-1 (100 pg) or IL-1b (0.5 pg) induced mechanical nociceptor hypersensitivity in both hind paws (Figure 2). Local pre-treatment (i.pl.) with indomethacin (100 mg), 30 min before the cytokine injections, inhibited responses to IL-1b (- 76%), but did not affect responses to CINC-1. In contrast, local pre-treatment with atenolol (At, 25 mg) inhibited responses to CINC-1 (- 86%), but not responses to IL-1b (Figure 2).

Inhibitory effects of antibodies against CINC-1 and IL-1b on the mechanical nociceptor hypersensitivity evoked by carrageenin, TNF- α and CINC-1

Carrageenin-evoked mechanical nociceptor hypersensitivity was inhibited by pre-treatment with anti-rat CINC-1 antibody (- 50%), anti-rat IL-1b antibody (- 63%). The inhibitory effects of antibodies to rat CINC-1 and rat IL-1b antibody were additive (- 96%; Figure 3A). TNF- α -evoked mechanical nociceptor hypersensitivity was inhibited by pretreatment with anti-rat CINC-1 antibody (- 54%) and with anti-rat IL-1b antibody (- 55%). Again, the inhibitory effects of the two antibodies were additive (- 94%; Figure 3B). CINC-1-evoked mechanical nociceptor hypersensitivity was abolished by pretreatment with anti-rat CINC-1 antibody (- 94%), but was not affected by pretreatment with anti-rat IL-1b antibody (Figure 3C). The CINC-1-evoked mechanical hypersensitivity was also inhibited by pretreatment with anti-hu-IL-8 antibody (delta reaction time: CINC-1 = 15.52 ± 0.56 ; CINC-1 + anti-IL-8 antibody = 7.1 ± 0.88 sec, $n = 5$; 62% of inhibition). The pretreatment of hind paws with pre-immune serum did not affect the hypersensitivity induced by carrageenin, TNF- α or CINC-1.

Carrageenin, LPS and TNF- α stimulate CINC-1 production in paw skin

The administration (i.pl.) of carrageenin (100 mg. 100 ml⁻¹) and LPS (1 mg. 100 ml⁻¹) stimulated the release of CINC-1 in rat paw skin (Figure 4). The carrageenin-evoked increase in CINC-1 in paw skin was detectable at 2 h, the response peaked at 3 h and returned to baseline at 6 h. In contrast, the LPS-evoked increase in CINC-1 in paw skin peaked at 1 h, was maintained at 3 h and had not quite returned to baseline at 6 h. The administration (i.pl.) of TNF- α (2.5 pg. 100 ml⁻¹) also stimulated the release of CINC-1 in the rat paw after 3 h (saline: 0.07 ± 0.015 ng. paw⁻¹; TNF- α : 0.8 ± 0.06 ng. paw⁻¹; n = 3).

Discussion

CINC-1 belongs to the ELR-CXC sub-family of chemokines which is produced in rats [16, 17, 27]. In common with the other CXC chemokines, CINC-1 induces inflammatory events such as neutrophil migration and activation [28] and has been detected in lung inflammation [29, 30], glomerulonephritis [31], and in inflammatory exudates induced by carrageenin and LPS [27, 32]. Given the presence of CINC-1 in carrageenin-induced and LPS-induced exudates, we utilised a model of inflammatory hypersensitivity (a rat paw pressure test) to investigate the possible hyperalgesic activity of CINC-1. In this test, carrageenin and LPS caused hyperalgesia by stimulating the release of bradykinin, which initiates a cascade of cytokine release [10].

Local injection of CINC-1 into one hind paw (intraplantar, i.pl.) evoked a dose-dependent and time-dependent mechanical nociceptor hypersensitivity in both hind paws although the effects of smaller doses were only observed in the injected paws, suggesting a systemic distribution of the injected cytokine (Figure 1). In support of this suggestion, it was observed that ipsilateral intraplantar administration of atenolol or antibody against CINC-1 inhibited local (i.e. ipsilateral) hypersensitivity but not hypersensitivity in contralateral paws. The lack of effect of indomethacin injected locally on CINC-1-evoked hypersensitivity suggests that local release of cyclo-oxygenase products did not contribute to the IL-8-induced hypersensitivity. The absence of effect of indomethacin was not due to the use of an inappropriate dose of indomethacin since the same dose inhibited IL-1 β -evoked hypersensitivity, which is mediated by prostaglandins [7]. The involvement of sympathomimetic amines in CINC-1-induced hypersensitivity is indicated by the marked attenuation of the response to CINC-1 after treatment with atenolol. The inhibitory effect of atenolol is not due to a possible local analgesic effect because it was not affected by the

hypersensitivity induced by IL-1b [8]. Although rats apparently do not produce IL-8, previous studies showed that i.pl. injection of IL-8 in rats induced mechanical nociceptor hypersensitivity. This response to IL-8 was abolished by atenolol, but not altered by indomethacin, suggesting the involvement of sympathomimetic amines [8]. Since IL-8 and CINC-1 bind to the same CXCR2 chemokine receptor, it is likely that these cytokines activate the sympathetic component of inflammatory hyperalgesia *via* the CXCR2 receptor.

The partial inhibition of carrageenin-evoked and TNF- α -evoked mechanical hypersensitivity by specific antibodies to CINC-1 and to IL-1b suggests that endogenous CINC-1 and IL-1b mediate the hypersensitivity evoked by these substances. The effects of these antibodies upon the hypersensitivity induced by carrageenin and TNF- α are in agreement with a previous study describing a pivotal role for TNF- α in the cascade of hyperalgesic cytokines released after the injection of carrageenin and LPS in rats [9]. In this earlier study, these inflammatory stimuli evoked mechanical hyperalgesia by stimulating the generation of bradykinin, which stimulated TNF- α production that initiated two pathways, each of which involved the release of cytokines and the final nociceptive mediators that sensitize the nociceptor. The two pathways are: (i) carrageenin/LPS $\rightarrow$ bradykinin $\rightarrow$ TNF- α $\rightarrow$ IL-6 $\rightarrow$ IL-1b $\rightarrow$ prostaglandins (which sensitize nociceptors) and (ii) carrageenin/LPS $\rightarrow$ bradykinin $\rightarrow$ TNF- α $\rightarrow$ CINC-1 (present study) $\rightarrow$ sympathomimetic mediators which also sensitize the nociceptors [9, 10].

Consistent with the involvement of the endogenous CINC-1 in carrageenin-evoked and LPS-evoked hypersensitivity, and the knowledge that its production is preceded by TNF- α release, immunoreactive CINC-1 was found to be elevated in rat paw skin subsequent to the i.pl. injection of carrageenin, LPS and TNF- α at doses which evoked mechanical hypersensitivity. The production of CINC-1 after carrageenin and LPS administration has been reported previously [27, 32] and rat macrophages stimulated *in vitro* with LPS release significant amounts of CINC-1 [20]. In a previous study, an anti-human IL-8 serum inhibited carrageenin-evoked and TNF- α -evoked hyperalgesia by approximately 50%, whilst not affecting the hyperalgesia evoked by IL-1b [8, 9]. Since rats do not produce IL-8, the simplest explanation for these results is that the antibody to IL-8 neutralised the endogenously released CINC-1. Confirming this suggestion, we demonstrated that anti-IL-8 serum inhibited CINC-1-induced hypersensitivity.

A recent study showed that intracerebroventricular (i.c.v.) injection of CINC-1 lowers the nociceptive threshold for mechanical stimuli in rats, suggesting that CINC-1 may also have a role within the central nervous system in mediating inflammatory hyperalgesia [22]. In agreement with this, we recently demonstrated that intrathecal administration of CINC-1 induces mechanical hyperalgesia, which is not blocked by atenolol (Cunha *et al.*, manuscript in preparation).

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CONCLUSION

In summary, the data presented above provide evidence that CINC-1, produced at the site of inflammation, in part mediates hyperalgesia induced by inflammatory stimuli *via* activation of the sympathetic component of inflammatory hyperalgesia.

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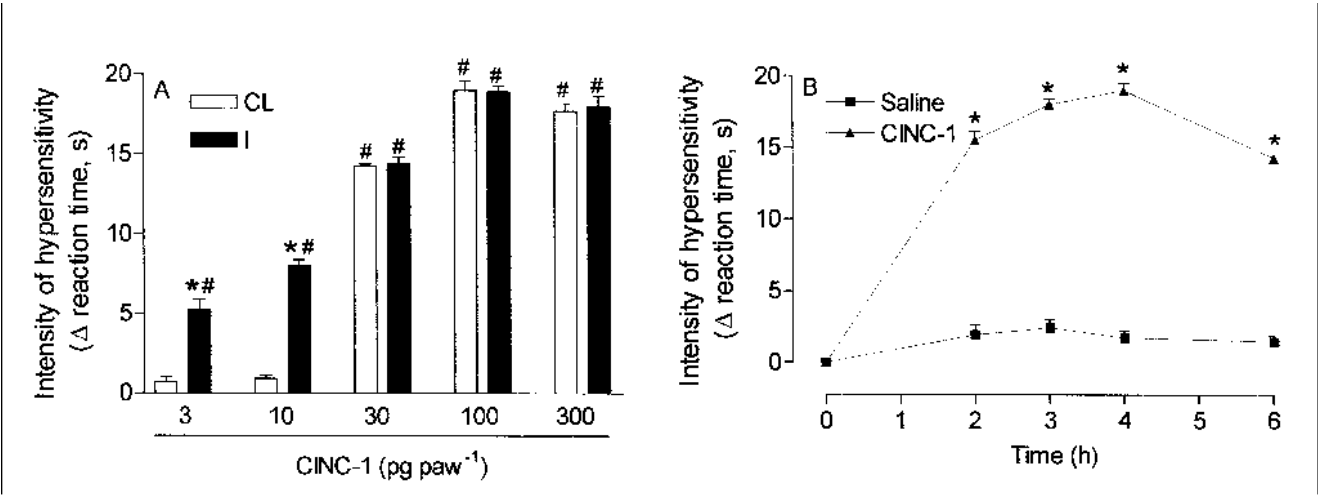
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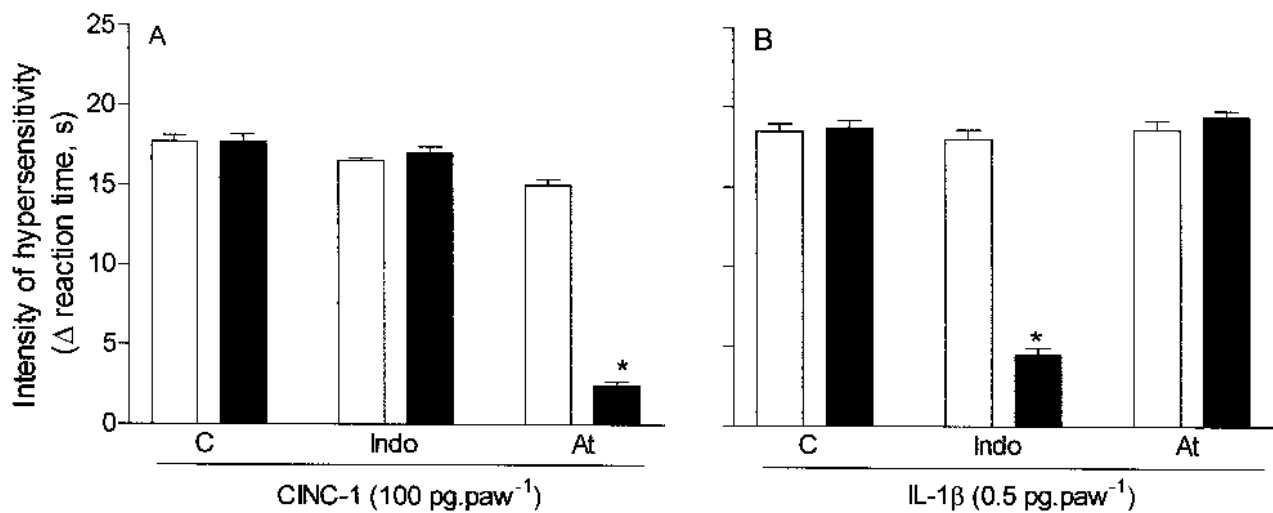
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Figures



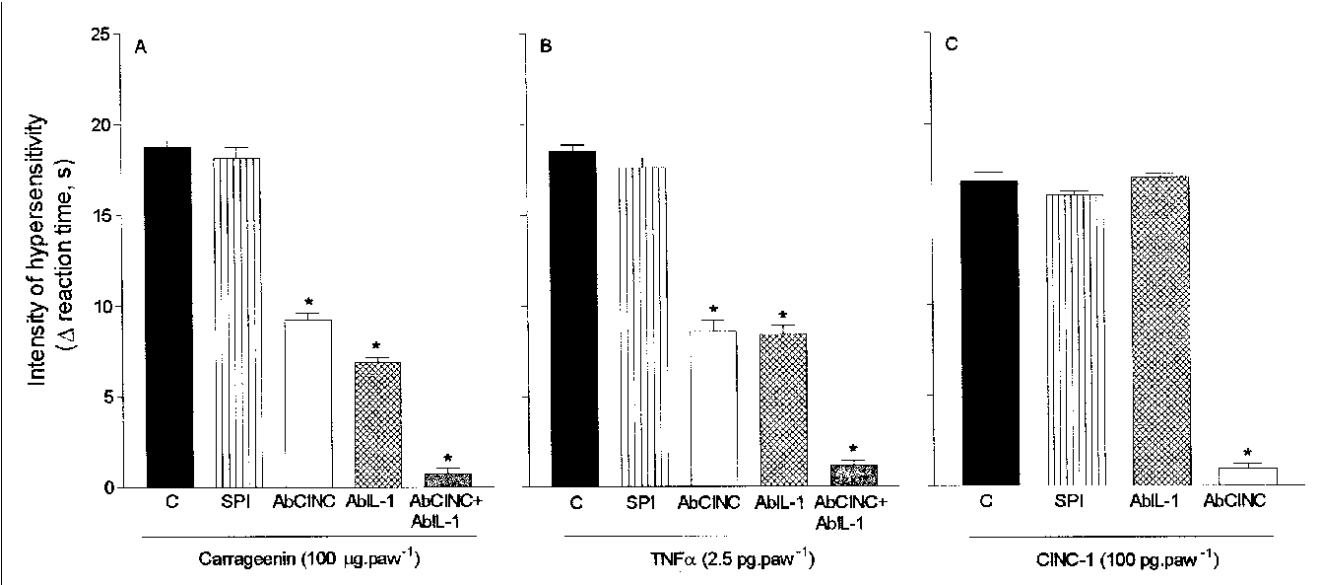
Dose- and time-dependence of mechanical nociceptor hypersensitivity evoked by CINC-1.

A. The intensity of mechanical hypersensitivity was measured 3 h after injection of CINC-1 (3 - 300 pg in 0.1 ml) into one of the hind paws (I, full columns). The contralateral paws were injected with saline (CL, 0.1 ml, open columns). **B.** Time-course of hypersensitivity induced by CINC-1 (100 pg paw⁻¹). The intensity of the mechanical hypersensitivity was determined at the indicated times after CINC-1 or saline injection. Data in both panels are means ± S.E.M. of groups of 5 rats. Results are representative of two different experiments. In panel A, **p* < 0.05 compared with contralateral paws, #*p* < 0.05 compared with saline-injected rats (delta (D) reaction time of saline injected paw: 0.5 ± 0.1 sec, n = 5). In panel B, **p* < 0.05 compared with saline-injected rats. The results were analysed by ANOVA followed by the Bonferroni t-test.



Effect of indomethacin and atenolol on the mechanical nociceptor hypersensitivity evoked by CINC-1 and IL-1b.

Mechanical hypersensitivity was induced by i.pl. injection of CINC-1 (100 pg, panel A) and IL-1b (0.5 pg, panel B) in one of the hind paws (full columns). The contralateral paws were injected with saline (100 ml, open columns). The pre-treatments: saline (C), indomethacin (Indomethacin (In, 100 mg)), atenolol (At, 25 mg) were given i.pl. 30 min before cytokine injection. The intensity of hypersensitivity was measured 3 h after cytokine challenge. Vertical bars are means $\pm$ S.E.M. of groups of 5 rats, and they are representative of two different experiments. * $p < 0.05$ compared with the contralateral paws (ANOVA followed by the Bonferroni t-test).



Effects of pre-treatment with antibodies against rat CINC-1 or IL-1b on mechanical nociceptor hypersensitivity induced by carrageenin, TNF-α and CINC-1.

Mechanical hypersensitivity was induced by i.pl. injection of carrageenin (100 mg; panel A), TNF-α (2.5 pg; panel B) and CINC-1 (100 pg; panel C). Neutralising antibodies, anti-rat CINC-1 (10 mg), anti-rat IL-1b (30 mg), and pre-immune serum (SPI, 50 ml) were given (50 ml, i.pl.) 30 min before stimuli injection. The measurement of hypersensitivity was performed 3 h after injection of nociceptive stimuli. Data are expressed as means ± S.E.M. of groups of 5 rats. **p* < 0.05 compared with the respective control (ANOVA followed by the Bonferroni *t*-test).

In this issue

○ European Cytokine Society president's address

Josef van Damme

○ Losing balance : cytokine signaling and cell death in the context of hepatocyte injury and hepatic failure

Tom Luedde, Christian Liedtke, Michael P Manns, Christian Trautwein
liver failure, apoptosis, tumor necrosis factor, NF-KB, interleukin-6, Fas

○ Presented at the 4th symposium on Cytokines and apoptosis in the cardiovascular system, Halle, Germany, June 6-7, 2002

○ Activated STAT-1 pathway in the myocardium as a novel therapeutic target in ischaemia/reperfusion injury

Anastasis Stephanou
STAT, heart, ischaemia/reperfusion, apoptosis

○ A subset of caspase substrates functions as the Jekyll and Hyde of apoptosis

Jian-Yan Yang, Christian Widmann
RasGAP, MEKK1, caspase substrates, apoptosis, survival

○ Compromised IFN-gamma production in the elderly leads to both acute and latent viral antigen stimulation: contribution to the immune risk phenotype?

Qin Ouyang, Wolfgang M Wagner, Anders Wikby, Ed Remarque, Graham Pawelec
Aging, T-cell, IFN-g, CMV, EBV, immune risk phenotype, longitudinal study

○ Cytokines in chronic heart failure: possible involvement in the neurohormonal and the cytokine systems at the cAMP level?

Claudia Werner, Harald Loppnow, Mathias Rauchhaus, Armin Wessel, Karl Werdan, Reiner Buchhorn
Adult, b-blocker, children, chronic heart failure, cyclic AMP, cytokine, infant, interleukin, soluble TNF receptor, tumor necrosis factor

○ Effects of tumor necrosis factor-alpha and interleukin-6 in elderly populations

Helle Bruunsgaard

TNF- α , IL-6, aging, mortality, inflammation

○ Energetic depression caused by mitochondrial dysfunction

Frank Norbert Gellerich, Sonata Trumbeckaite, Ying Chen, Marcus Deschauer, Tobias Müller, Stephan Zierz

Mitochondria, outer membrane, inner membrane, mitochondrial disease, sepsis, phosphorylation potential

○ Senescence: the cost of being young and healthy?

Graham Pawelec

○ The regulation of death-associated protein (DAP) kinase in apoptosis

Galit Shohat, Taly Spivak-Kroizman, Miriam Eisenstein, Adi Kimchi

apoptosis, DAPk, autophosphorylation, negative regulation

○ Neisseria meningitidis can induce pro-inflammatory cytokine production via pathways independent from CD14 and toll-like receptor 4

Tom Sprong, Peter van der Ley, Liana Steeghs, Will J Taw, Trees JW Verver-Janssen, Mihai G Netea, Jos WM van der Meer, Marcel van Deuren

Neisseria meningitidis, lipopolysaccharide, endotoxin, cytokines, CD14, TLR4

○ Evaluation of tumor necrosis factor alpha production in ex vivo short term culture whole blood from women with polycystic ovary syndrome

A Veronica Araya, Adam Aguirre, Carmen Romero, Cristian Miranda, Maria Carmen Molina, Arturo Ferreira

LPS, polycystic ovary syndrome, TNF- α , whole blood cell culture

○ Plasma levels of IL-12, IL-18 and TGF-beta in Plasmodium falciparum malaria

Lucia Malaguarnera, Salvatore Pignatelli, Jacques Simporè, Mariano Malaguarnera and Salvatore Musumeco

IL-12, IL-18, TGF- β , Plasmodium falciparum

○ In vitro analysis of IGN-gamma and IL-12 production and their effects in ileal Crohn's disease

Stefaan Colpaert, Kathleen Vastraelen, Zhanju Liu, Philippe Maerten, Chong Shen, Freddy Penninckx, Karel Geboes, Paul Rutgeerts, Jan L Ceuppens
IL-12, IFN-g, anti-IL-12/ anti-IFN-g, in vitro, Crohn's disease

○ Differential modulation of IL-2- and IL-4-mediated early activation of normal human B lymphocytes by the caspase inhibitor zVAD-fmk

Shahul Mouhamad, Damien Arnoult, Marie-Thérèse Auffredou, Jérôme Estaquier, Aimé Vazquez
IL-2, IL-4, B lymphocyte, caspase, proliferation, cell cycle entry

○ Prolactin-cytokine network in the defence against *Acanthamoeba castellani* in murine microglia

Nunzia Benedetto, Claude Auriault
Prolactin, cytokine, *A. castellani*, MGC.

○ CINC-1 mediates the sympathetic component of inflammatory mechanical hypersensitivity in rats

Berenice B Lorenzetti, Fabiane H Veiga, Claudio A Canetti, Stephen Poole, Fernando Q Cunha, Sergio H Ferreira
Inflammatory hyperalgesia, CINC-1, tumour necrosis factor alpha, interleukin-1b, interleukin-8, sympathetic mediators, prostaglandin E2

○ A CXC chemokine sequence isolated from the rainbow trout *Oncorhynchus mykiss* resembles the closely related interferon-gamma-inducible chemokines CXCL9, CXCL10 and CXCL11

Kerry J Laing, Niels Bols, Christopher J Secombes
Chemokine, rainbow trout, IP10, I-TAC, Mig

○ Meeting report of the “Club Cytokine” (French Society of Immunology) meeting, in Le Croisic, France

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