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Involvement of the Proinflammatory Cytokines Tumor Necrosis Factor-α, IL-1β, and IL-6 But Not IL-8 in the Development of Heat Hyperalgesia: Effects on Heat-Evoked Calcitonin Gene-Related Peptide Release from Rat Skin

A. Oprée and M. Kress

Institut für Physiologie und Experimentelle Pathophysiologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, D-91054 Erlangen, Germany

Proinflammatory cytokines contribute to the development of inflammatory and neuropathic pain and hyperalgesia in many *in vivo* models. The rat skin model was used to investigate the effects of proinflammatory cytokines on the basal and heat-evoked release of calcitonin gene-related peptide from nociceptors *in vitro*. In contrast to the excitatory effects of cytokines observed *in vivo*, none of the cytokines tested evoked any calcitonin gene-related peptide (CGRP) release at normal skin temperature of 32°C. However, the cytokines IL-1β, tumor necrosis factor (TNF)-α, and IL-6 but not IL-8 induced a pronounced and transient sensitization of the heat-evoked CGRP release from nociceptors *in vitro*. This heat sensitization was dose dependent, with EC_{50} for IL-1β of 2.7 ng/ml and for TNF-α of 3.1 ng/ml. The maximum IL-1β effect reached almost 600% of the heat-evoked release, and the maximum TNF-α effect induced a rise in CGRP release of 350%. In contrast to IL-1β and TNF-α, IL-6 did not induce heat sensitization when applied alone but was only effective in the presence of soluble IL-6 receptor. This suggests a constitutive expression of signaling receptors for TNF and IL-1β and the signal transduction molecule gp130 but not IL-6 receptor or IL-8 receptor. Furthermore, the acute cytokine signaling observed in the present study was independent of transcriptional pathways because sensitization occurred on short latency *in vitro* and under conditions that excluded chemotactic accumulation of immune cells from blood vessels. Our results demonstrate that interleukins may play an important role in the initiation of heat hyperalgesia in inflammation and neuropathy.

Key words: nociception; heat sensitization; inflammation; neuropeptides; sensory neurons; interleukin

In addition to tissue acidosis, inflammatory mediators and proinflammatory cytokines are found in the vicinity of inflammation and tumor that are produced by leukocytes and thrombocytes that accumulate by chemotactic attraction. This accumulation of various mediators together with tissue acidification acts synergistically to produce pain and hyperalgesia. Although the classic inflammatory mediators and effects of acidosis have been investigated intensively, we are just beginning to understand the importance of cytokines for the development of pain and hyperalgesia as well as their mechanisms of action on nociceptors (for review, see Kress and Reeh, 1996). The most detailed information is available for the proinflammatory cytokines IL-1β, IL-6, and IL-8 and tumor necrosis factor (TNF)-α, which have been traced in inflamed tissue and for which a role in nociception has been established (Alexander et al., 1998; Watkins and Maier, 1999). In tumor patients, cytokines are of dual importance. First, some cytokines, e.g., IL-6 or TNF-α, are produced by malignant tissue (Takeuchi et al., 1996; Antunovic et al., 1998; Matsushima et al., 1999). IL-6 injection produces hyperalgesia, and in IL-6 −/− mutants, heat sensitivity of nociceptors has been shown to be reduced (Zhong et al., 1999). Second, some cytokines such as TNF-α or IL-1β have been used for adjuvant chemotherapy, but approximately one-half of the treated patients developed pain syndromes and complained about local tenderness at the injection site (Kemeny et al., 1990; Del Mastro et al., 1995; Elkordy et al., 1997). In experimental models, TNF-α and IL-1β induced excitation in nociceptors and hyperalgesia (Ferreira et al., 1988; Cunha et al., 1992; Sorkin et al., 1997; Kanaan et al., 1998; Sommer et al., 1998, 1999), and both are suggested to activate receptor-associated kinases, e.g., IL-1 receptor-associated kinase (IRAK) (Cao et al., 1996). Such kinases may phosphorylate ion channels transducing noxious heat in nociceptors, e.g., VR-1. Such phosphorylation has been demonstrated to sensitize native heat-activated currents, and this may contribute to heat sensitization at the nociceptive nerve terminal (Cesare and McNaughton, 1996). The detailed mechanisms leading to hyperalgesia, however, are unknown so far.

The peripheral terminals of the nociceptors are complex structures that because of their small size (<0.1 μm) and embedding in the tissue are inaccessible to modern techniques of cellular electrophysiology. In addition to ion channels and receptor proteins, which are integrated in their cellular membrane, they contain neuropeptides, including calcitonin gene-related peptide (CGRP), that are packed in secretory large dense-cored vesicles (Gulbekian et al., 1986; Huang and Neher, 1996). Activation of the terminal results in a uniform reaction of vasodilatation and plasma extravasation that is commonly referred to as neurogenic inflammation. This is maintained by the release of neuropeptides, e.g., CGRP, from the nerve terminals of unmyelinated afferents during activation (Yonehara et al., 1992; Kilo et al., 1997; Kress et al., 1999). Recently, we have demonstrated CGRP release from rat skin *in vitro* induced by noxious heat (Kessler et al., 1999). Such CGRP release from rat skin *in vitro* represents a good model for studying nociceptor function and heat sensitization processes (Kessler et al., 1999).

The aim of the present study was to investigate whether the recombinant proinflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8 could excite nociceptors and thus induce the release of neuropeptides from the peripheral nerve terminals. Furthermore, experiments were performed to assess the sensitizing effects of the cytokines on the heat response.

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Correspondence should be addressed to Dr. Michaela Kress, Institut für Physiologie und Experimentelle Pathophysiologie, Universitätsstrasse 17, D-91054 Erlangen, Germany. E-mail: kress@physiologie1.uni-erlangen.de.

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MATERIALS AND METHODS

Preparation. For the present study, the hairy skin of either hindpaw was obtained from male Wistar rats weighing 40–90 gm that were killed by breathing 100% CO_2. The hairy skin from both hindpaws was subcutaneously excised below knee level without toes and sole; macroscopic vessels and nerves were spared. The weight of the obtained skin flaps ($n = 145$) ranged from 0.15 to 0.35 gm. The skin flaps were wrapped around acrylic glass rods (diameter 6 mm), with the corium side exposed to the surrounding solution, and fixed with surgical silk 3.0. These mounted preparations were rinsed at 32°C for 30 min in synthetic interstitial fluid (SIF) (Bretag, 1969) consisting of (in mM): 107.8 NaCl, 26.2 $NaCO_3$, 9.64 Na-gluconate, 7.6 sucrose, 5.05 glucose, 3.48 KCl, 1.67 NaH_2PO_4, 1.53 $CaCl_2$, 0.69 $MgSO_4$, gassed with 95% oxygen and 5% carbon dioxide at 32°C for 30 min.

Elution procedures and stimulation. A series of six glass test tubes were filled with 1.2 ml SIF each and positioned in a temperature-controlled shaking bath (32°C). The release experiment was started by transferring the mounted skin flap into the first tube. After 5 min incubation, the preparation was forwarded to the second tube for another 5 min and thus moved along the whole series of tubes. Using the first two tubes as control samples, stimulation was performed in the third tube, e.g., adding mTNF-α (0.05–500 ng), mouse IL-1β (0.02–200 ng), human IL-8 (0.1 ng to 1 μg), or mIL-6 (0.02–200 ng) (all from Boehringer Mannheim, Mannheim, Germany) with or without soluble receptor hIL-6 sR (25–250 ng) (R&D Systems, Wiesbaden, Germany). Stock solutions of the proteins were prepared in PBS containing 1% bovine serum albumin (Sigma, St. Louis, MO) and diluted to the final concentration with SIF. For heat stimulation, the third tube was heated to the respective temperature by a thermostatic pump in a second shaking water bath. The mounted preparations were transferred to the hot solution from minute 10 to minute 15, and one extra tube was carried along to monitor the temperature with a calibrated thermometer throughout the experiment. To exclude series effects such as tachyphylaxis or sensitization, each skin flap was used only in a single experiment and exposed only to a single stimulation.

Enzyme immunoassays. After incubation, 200 μl aliquots of the eluate were immediately mixed with 50 μl of fivefold concentrated commercial CGRP EIA buffer containing several protease inhibitors and stored on ice until the series was completed. The CGRP content was determined immediately after the end of the experiment using a commercially available enzyme immunoassay EIA for CGRP (SPIbio). All EIA plates were determined photometrically using a microplate reader (Dynatech).

Data analysis. The total concentration of CGRP in the samples was calculated based on 1 gm fresh weight of the cutaneous tissue. Averaged results are given as mean ± SEM. For normalization, the actual raw value was divided by the peptide content of the second baseline sample, which was the last one collected before stimulation. For intra-individual statistical comparisons, the ANOVA with one main effect was calculated for cytokine concentration versus control heat stimulation. In cases in which this test was significant, planned comparisons for *post hoc* tests were used. p values of planned comparisons are given throughout the manuscript, and differences were considered significant at a p value < 0.05.

RESULTS

Rat skin in control solution exhibits a basal release of the neuropeptide CGRP, which varies between 50 and 300 pg/ml with an average ~120 pg/ml. Exposing rat skin at 32°C to IL-1β in a concentration of 20 ng/ml, 200 ng/ml, or 2 μg/ml ($n = 2$ for the lowest and $n = 4$ for the two higher concentrations) yielded no measurable change in basal CGRP release. Also, TNF-α (50 ng/ml, 500 ng/ml, or 5 μg/ml), IL-6 (20 ng/ml, 200 ng/ml, or 2 μg/ml), or IL-8 (100 ng/ml, 1 μg/ml, or 10 μg/ml) at this temperature did not induce any increase in CGRP release from the preparations ($n = 2$ for the lowest, $n = 4$ for the two higher concentrations of each cytokine) (Fig. 1). Between the different series of experiments, the absolute baseline CGRP concentration, however, showed a pronounced interindividual variation. To eliminate such variability, we present the data of all further experiments as normalized values that are obtained by dividing all measured concentrations within one experiment by the CGRP concentration obtained in sample two to assure comparability of the results.

Because the proinflammatory cytokines were not able to excite nociceptors and to directly induce CGRP release but have been suggested to play a role in the development of heat hyperalgesia, their effects on the heat-evoked release of CGRP were investigated. As in a previous study, thermal stimulation with SIF of 47°C ($n = 6$) induced an increase in CGRP of 5.4 ± 0.35-fold during the stimulation period that recovered within 10 min after stimulation. The addition of 20 ng IL-1β during exposure to heat (47°C) significantly increased the amount of released CGRP during heat stimulation from 5.4 ± 0.35 to 28.5 ± 4.6-fold ($n = 6$, $p < 0.001$).

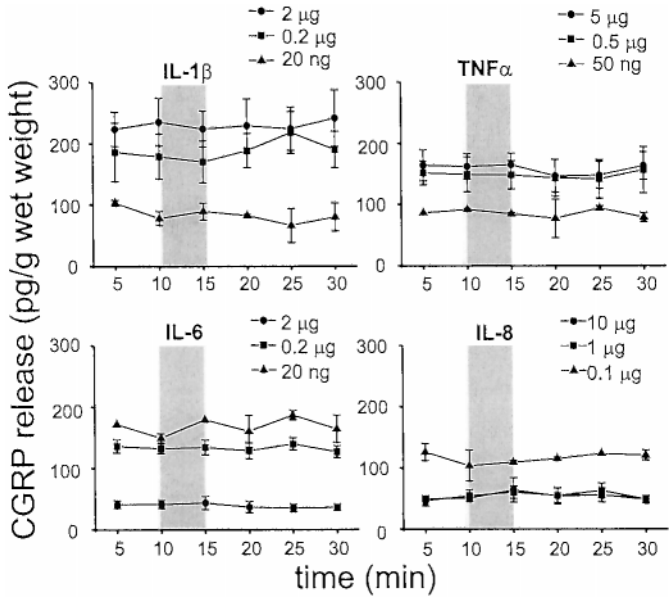

Figure 1. Proinflammatory cytokines do not induce release of CGRP from rat skin *in vitro*. Skin flaps were incubated for 5 min periods, then moved to the next of a series of tubes until the end of the series was reached. The stimulation period is marked by the *gray column*. The CGRP content of the eluate was determined immediately after the experiment using an enzyme immunoassay. Mean baseline values (±SEM) referring to fresh weight of the skin show a pronounced variation from 50 and 250 pg/gm wet weight between the samples. No increase of CGRP release occurs during stimulation with either of the proinflammatory cytokines.

As under control conditions, the CGRP release returned to baseline values within 10 min after stimulation. A similar sensitization was observed when TNF-α (50 ng) was added during heat stimulation. Under this condition, a relative increase of 17.5 ± 2.9 was obtained ($n = 5$; $p < 0.01$).

For both IL-1β and TNF-α, the sensitizing effect on the heat-evoked CGRP release was dose dependent with a threshold of 0.2 ng/ml for IL-1β ($n = 6$; $p < 0.01$), the maximum response of 28.5 ± 4.6-fold increase was reached at 20 ng ($n = 6$; $p < 0.01$) (Fig. 2*A*), and the effect declined again with higher concentrations. For TNF-α we obtained a threshold for sensitization of 0.5 ng/ml ($n = 7$; $p < 0.05$) where the increase was 9.5 ± 1.1-fold. The maximum effect was observed for 50 ng/ml TNF-α, which yielded a 16.5 ± 3.9-fold increase ($n = 5$; $p = 0.05$) (Fig. 2*B*). With the CGRP release evoked by 47°C heat stimulation set as 100%, the maximum IL-1β effect reached almost 600% of the heat-evoked release alone, and the maximum TNF-α effect yielded an increase of ~350%. The EC_{50} for the sensitizing action of the cytokines on the heat-induced CGRP release for IL-1β was 2.7 ng/ml, and for TNF-α it was 3.1 ng/ml (Fig. 2*C*). The coadministration of IL-6 or IL-8 yielded small increases in the heat-evoked CGRP release; neither of them was significant (IL-6, 2–200 ng; IL-8, 0.01–1 μg/ml; data not shown).

Because the administration of IL-6 did not significantly change the heat-induced release, the skin was preincubated with recombinant soluble receptor for IL-6 (sIL-6R) for 10 min starting 5 min before heat stimulation with IL-6. Even in the presence of IL-6, this did not change the baseline CGRP content of the eluates at 32°C ($n = 4$) (Fig. 3). However, the heat-evoked CGRP release at 47°C was significantly increased from 5.4 ± 0.35 with heat stimulation alone to 13.4 ± 2.6 when IL-6 was present together with its soluble receptor ($n = 6$; $p < 0.01$) (Fig. 3).

DISCUSSION

The present study demonstrates for the first time that the proinflammatory cytokines IL-1β, TNF-α, and IL-6 but not IL-8 can transiently sensitize rat cutaneous nociceptors to heat *in vitro* on short latency. None of these cytokines evoked any CGRP release at

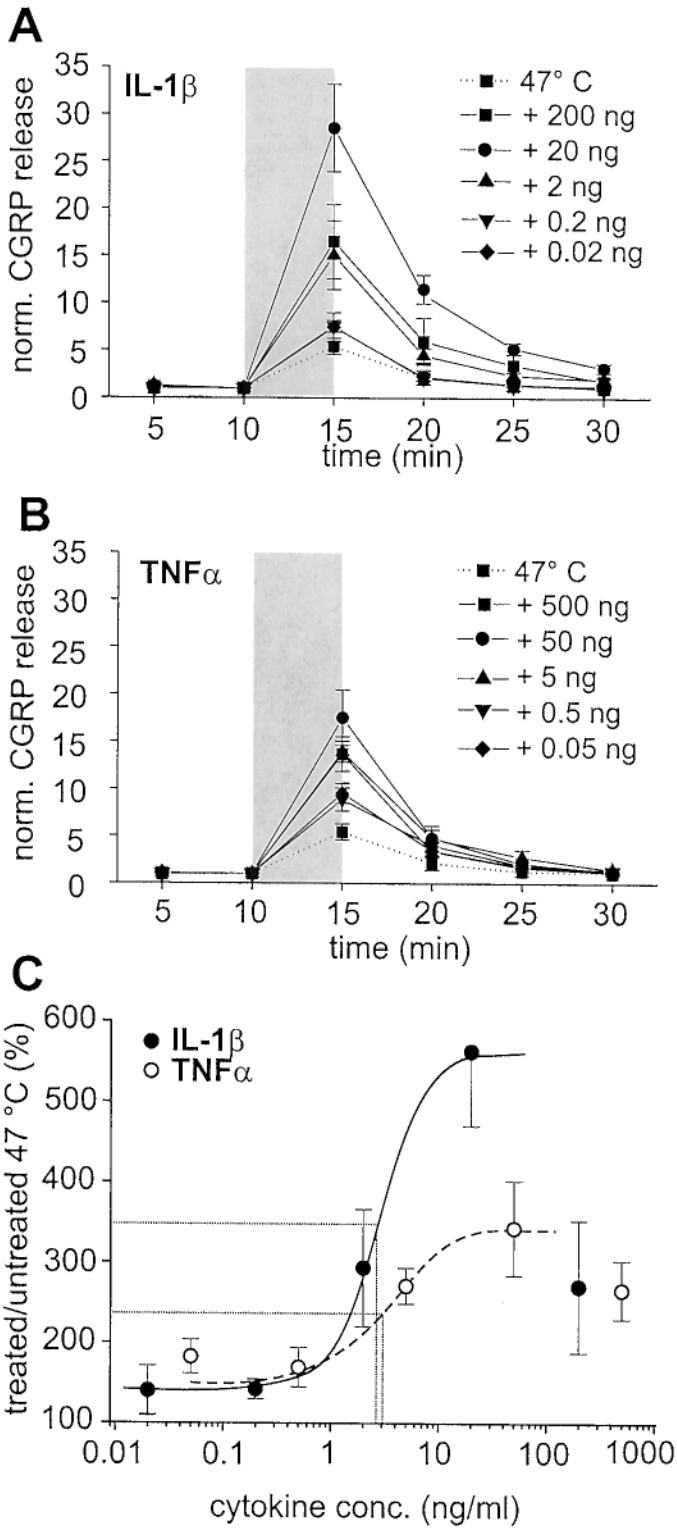

Figure 2. Heat-evoked CGRP release is enhanced during stimulation with IL-1β or TNF-α. After two baseline samples, noxious heat stimulation (47°C, *gray field*) evoked an increase in CGRP release that fully recovered within 5 min. All data points are normalized by division by the value obtained after 10 min of baseline collection of supernatant. The heat-evoked CGRP release was significantly larger in the presence of IL-1β (*A*) or TNF (*B*) than under control conditions and exhibited a slower recovery. *C*, Maximum effects are reached with 20 ng/ml IL-1β and 50 ng/ml TNF-α, respectively, and the EC_{50} was 2.7 ng/ml for IL-1β and 3.1 ng/ml for mTNF-α. At concentrations exceeding 200 ng/ml, the effect decreased again.

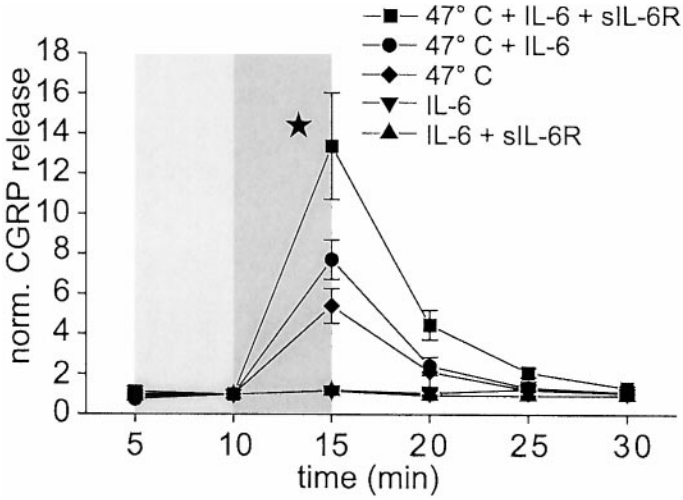

Figure 3. IL-6-induced heat sensitization requires the addition of soluble IL-6 receptor. Addition of IL-6 (20 ng/ml; $n = 5$) produces a marginal rise in CGRP release during heat stimulation. A significant increase is only reached when its soluble receptor (sIL-6R, 25 ng/ml; $n = 6$) is added before and during IL-6 stimulation ($^*p < 0.05$). Preincubation is indicated by the *light gray field*, and heat stimulation with or without IL-6 alone or together with soluble receptor is marked by the *dark gray column*. The *asterisk* marks significant difference.

non-noxious temperature. In contrast to IL-1β and TNF-α, IL-6 was effective only in the presence of soluble receptor. This suggests a constitutive expression of signaling receptors for TNF and IL-1β and the signal transduction molecule gp130 but not of IL-6 or IL-8 receptor in rat nociceptors. These receptors may become activated under pathophysiological conditions to cause acute heat hyperalgesia.

In the present study, the CGRP release from rat skin *in vitro* as a model for nociceptor activation has been used to study the acute effects of IL-1β, TNF-α, IL-6, and IL-8 (Kessler et al., 1999). These mediators are proinflammatory cytokines that share several biological properties. They normally are practically absent from the nervous system but can become upregulated under neuropathic or inflammatory conditions (Jansky et al., 1995). Complex receptor-dependent enzymatic signaling cascades have been reported of which only the IL-8 receptor can activate several types of G-protein, including G_i (Wu et al., 1993), but none of the known receptors is directly linked to an excitatory ionic current. Although in granulocytes IL-8 can trigger a calcium signal via a pertussis toxin-sensitive G-protein-dependent activation of the IP_3 second messenger pathway and calcium release from intracellular stores (Schorr et al., 1996), no comparable changes have been identified in neurons or for the other cytokines tested. A direct excitatory action of the proinflammatory cytokines therefore was not expected, and none of the compounds induced CGRP release directly in the present *in vitro* study. Ectopic excitatory activity that was observed when TNF-α was applied in anesthetized animals thus seem to be attributable to indirect cytokine effects on nociceptors (Sorkin et al., 1997). However, on the basis of several findings, sensitization of nociceptors could be expected to occur during the time course of inflammation, which can be explained by activation of kinases in the signaling pathway of the cytokines investigated in the present study.

IL-1β is one of three endogenous ligands for IL-1RI or IL-1RII. In contrast to its endogenous antagonist IL-1ra, IL-1 (IL-1β and IL-1α) transduces an intracellular signal when bound to the IL-1RI receptor type. IL-1RII, which lacks a transduction molecule, functions as a second inhibitory pathway restricting the effect of IL-1β on its target cell. In the presence of an accessory protein IL-1R AcP, the signaling cascade uses the activation of receptor-coupled kinases and transcription factors. In peripheral nociception, the hyperalgesic action of IL-1β has been well established *in vivo*

(Ferreira et al., 1988; Poole et al., 1992). A first hint to a sensitization of thin afferent nerve fibers came from an enhancement of capsaicin-induced vasodilatation in rat skin *in vitro* because capsaicin as well as noxious heat activate the same ion channel in nociceptive neurons (Herbert and Holzer, 1994; Herbert et al., 1995; Tominaga et al., 1998). The ion channel is modulated by phosphorylation, and the IL-1RI pathway has been suggested to activate several serin kinases, e.g., IRAK (Cao et al., 1996). More evidence for a direct action of IL-1β on primary afferents comes from induced substance P release after long-time exposure of the neurons to the cytokine. The release in this study was connected to the cyclo-oxygenase 2 system (Inoue et al., 1999). Such release has not been observed during short-term exposure in the present study, and therefore it may be related to a translocation or a *de novo* synthesis of proteins.

Soluble TNF-α, the second cytokine tested, belongs to a family of cytokines that forms homotrimers to bind to the highly conserved cystein-rich TNF receptor (TNFR)1 (p55) and TNFR2 (p75), leading to receptor homotrimer formation. The intracellular domains of the two receptors do not show homology, and they exert different actions on target cells that probably involve the activation of NFκB or a death domain. Of the two TNF receptors, mainly TNFR1 seems to convey intracellular signaling, whereas TNFR2 is responsible for ligand passing. Although TNFR1 is constitutively expressed at low levels, TNFR2 is induced in inflammation (Durum and Muegge, 1998). The development of thermal hyperalgesia in inflamed tissue seems to depend on the elevation in the levels of TNF-α that can be detected from ~3 hr after complete Freund's adjuvant injection, with a peak after 24 hr and elevated levels up to 5 d. Part of this hyperalgesic action is attributed to its capacity to induce nerve growth factor (NGF) (Safieh-Garabedian et al., 1995). TNF-α in the present study, however, sensitizes nociceptors to heat *in vitro* and with short latency, and therefore the effect is unlikely caused by NGF induction. Possible signaling pathways of either receptor subtype that may be responsible for such short-term sensitization, however, are so far unknown. Because IL-1β and TNFα share a number of biological properties, common signaling may be assumed that may involve receptor-associated kinases. This would be in line with a pronounced sensitization of heat-activated ion channels by phosphorylation (Cesare and McNaughton, 1996; März et al., 1999).

In contrast to IL-1β and TNF-α, no transient heat sensitization was seen after acute application of IL-6. This could suggest that in normal tissue the IL-6 receptor and/or the signaling protein pg130 is not expressed. Because the coadministration of IL-6 together with its recombinant soluble receptor readily induced heat sensitization, it is concluded that the gp130 is present in normal tissue but the IL-6 receptor protein is not. This is in partial contrast to, e.g., human vascular smooth muscle cells that express only a scant amount of IL-6R and no gp130 (Klouche et al., 1999). In the same model, an autocrine activation loop for gp130 by IL-6/IL-6R has been found (Klouche et al., 1999). In contrast, nociceptors seem to constitutively express appreciable amounts of gp130 that probably dimerize and become functional in the presence of IL-6/IL-6R to activate several members of a family of cytoplasmic tyrosine kinases as known from other cell types (Durum and Muegge, 1998). The cytokine IL-6 thus represents an exception in the group of proinflammatory cytokines because its signaling molecule gp130 seems to be constitutively expressed, whereas IL-6 receptor is not. The additional presence of soluble IL-6 receptor is required, and this may represent dual regulation of IL-6 signaling by the cytokine and its receptor. From other models it is known that neural activities of IL-6-like cytokines often depend on soluble receptor (März et al., 1999). A potential role for IL-6 in the inflammatory heat hyperalgesia is further supported by a recent report of a reduced heat sensitivity in IL-6 null mutant mice in the hot plate test (Zhong et al., 1999). Apart from its known signaling through the IL-6R/gp130 system, IL-6 has been found to cooperate with high affinity neurotrophin receptor Trk (tyrosine kinase) signaling mechanisms that are known to regulate the heat-responsive nociceptor phenotype (Sterneck et al., 1996; Bergmann et al., 1998; Koltzenburg et al., 1999). These phenotype changes seem to involve receptor (e.g., VR-1) upregulation and thus seem to be distinct from the acute heat sensitization observed in the present *in vitro* study. Interestingly, similar long-term effects also are known for IL-1β and TNF-α (Sommer et al., 1998, 1999; Sweitzer et al., 1999). The acute effects presented here, however, reveal a dual role for proinflammatory cytokines: on one hand they induce sensitization in the acute phase of inflammation possibly via receptor associated kinases and phosphorylation of ion channels, whereas on the other hand hyperalgesia is maintained in chronic inflammation by upregulation of receptors and secondary signaling mechanisms.

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