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# Mechanical but not painful electrical stimuli trigger TNF alpha release in human skin

T. Eberle [a], B. Doganci [a], H. Krämer [a], M. Fechir [a], I. Wagner [a], C. Sommer [b], F. Birklein [a,*]

[a] Department of Neurology, University Medical Centre Mainz, Langenbeckstrasse 1, 55101 Mainz, Germany
[b] Department of Neurology, University of Würzburg, Germany

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ABSTRACT

Pro-inflammatory cytokines–in particular tumor necrosis factor (TNF)-alpha–play an important role in pain and hyperalgesia. The stimuli inducing TNF-alpha release in humans and the time course of this release are largely unknown. We performed dermal microdialysis in healthy subjects ($n=36$) during three experimental conditions: The first condition (control) was microdialysis without stimulation, the second condition was 30 min of electrical current stimulation (1 Hz, 20 mA, moderately painful), the third condition was 30 min of repetitive mechanical stimulation via an impact stimulator (bullet 0.5 g; velocity 11 m/s, minimally painful). TNF-alpha was quantified in the samples collected at the end of the baseline perfusion (about 1 h of saline perfusion), at the end of stimulation period (exactly 30 min after stimulation commenced) and at the end of the experiment (exactly 90 min after stimulation commenced) using a commercial enzyme-linked immunosorbent assay. The C-fiber-related flare was quantified with a laser-Doppler imager. ANOVA revealed that TNF-alpha levels increased during the eluate sampling period. At 90 min TNF-alpha in the eluate of the mechanical stimulation condition was significantly increased as compared to electrical current or control condition. Flare intensity was highest in the electrical current stimulation condition and only marginally different from control in mechanical stimulation. Our results show that minimal mechanical trauma is sufficient to induce significant TNF-alpha release in the skin. These results may be relevant to the treatment of posttraumatic pain disorders.



## Introduction

Animal and human studies have provided evidence for a pathophysiological role of cytokines in the induction and maintenance of pain, in particular in inflammatory diseases like rheumatoid arthritis. It was only recently that cytokines were also suggested to contribute to neuropathic pain in humans (Heijmans-Antonissen et al., 2006; Uceyler et al., 2007b).

Cytokines can be subdivided into pro-inflammatory cytokines, which also have pro-algesic effects, and into anti-inflammatory cytokines, which mainly exert analgesic effects (Uceyler et al., 2007a; McMahon et al., 2005). Cytokines are produced by immune but also non-immune cells like Schwann cells, fibroblasts, keratinocytes or glial cells, which are in close contact to peripheral or spinal nociceptive neurons (Sommer and Kress, 2004). An early player in the pro-inflammatory cytokine cascade is tumor necrosis factor-alpha (TNF-alpha). TNF-alpha significantly contributes to experimental inflammatory pain and pain after nerve injury (Scholz and Woolf, 2007) (Hao et al., 2006; Milligan et al., 2006), and blocking TNF-alpha, which was mainly increased in keratinocytes, reverses pain, hyperalgesia and spinal Fos expression after fracture-induced pain (Sabsovich et al., 2008b). TNF-alpha acts through direct or indirect mechanisms. Direct mechanisms are peripheral nociceptor sensitization via TNF-alpha receptors and sensitization of ion channels on primary afferent neurons (Junger and Sorkin, 2000; Schafers et al., 2008) or enhanced spinal nociceptive transmission mainly via glutaminergic mechanisms (Cuellar et al., 2004; Kawasaki et al., 2008), indirect mechanisms are via further activation of the immune response, e.g. release of NGF (Schafers et al., 2003), of neuropeptides like CGRP and SP and of inflammatory mediators like bradykinin or prostaglandins (for review see (Uceyler et al., 2009). Accordingly, TNF-alpha antagonists like thalidomide were used to treat experimental pain and hyperalgesia (George et al., 2000), and first successful clinical applications in neuropathic pain have been reported (Bernateck et al., 2007).

In human pain conditions like the posttraumatic complex regional pain syndrome (CRPS), TNF-alpha concentrations in tissue samples and in the blood are increased, in particular when spinal nociceptive sensitization is prevalent, (Maihofner et al., 2005), or if inflammation is obvious like in rheumatoid arthritis (Smith et al., 1997). In peripheral neuropathies, the immunoreactivity for TNF-alpha and other pro-inflammatory cytokines in sural nerve biopsies was correlated with the presence of neuropathic pain (Lindenlaub and Sommer, 2003). Shortly after a mechanical overload like running exercise (Langberg et al., 2002a) or repetitive low-force muscle exercise (Rosendal et al., 2005), the proinflammatory interleukin-6

* Corresponding author. Fax: +49 6131 17 5625.
*E-mail address:* birklein@neurologie.klinik.uni-mainz.de (F. Birklein).

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(IL-6) was increased in the connective tissue. In contrast, heat stimulation of the skin fails to induce significant TNF alpha (Angst et al., 2008b).

These human studies suggest that the kind of the painful stimulus or the tissue might be critical for cytokine release. Physical trauma, but not heat stimuli, might be a natural stimulus for TNF-alpha release into the tissue, and thereby one possible reason for trauma related pain and dysfunction. The posttraumatic course might be aggravated if the pro-inflammatory cytokines are not properly antagonized like in CRPS (Uceyler et al., 2007a). Another possibility is that TNF release from the Schwann cells depends on nociceptor activity, or more indirectly- nociceptors induce cytokine release from keratinocytes by release of neuropeptides like SP, as very recently shown (Sahbaie et al., 2009). In order to address these hypotheses we performed the present study. We used the dermal microdialysis to sample TNF-alpha as a prototypic proinflammatory cytokine *in vivo* from the skin of healthy subjects before and after application of potentially noxious stimuli. Based on the results and hypotheses addressed above, we chose minimally painful mechanical stimuli and moderately painful electrical stimuli, the latter since only electrical stimuli are able to activate all classes of C-fibers including those chemoreceptors which determine neuropeptide release (Schmelz et al., 2000). Since we found a significant TNF-alpha release as early as 1 h after minimally painful mechanical stimulation, our results suggest that the mechanical impact itself may be a potent trigger for dermal cytokine release and posttraumatic inflammation. The significance of this finding for physiological and pathological pain after trauma has to be established in forthcoming experiments.

## Methods

### *Subjects*

We investigated 36 healthy subjects (mean age 25.2 ± 2.05 years, 14 women and 22 men), who participated either in the mechanical stimulation experiments ($n = 20$, mean age 23.7 ± 0.47 years, 8 women) or the electrical current stimulation and control experiments ($n = 16$, mean age 26.6 ± 1.5 years, 6 women). All investigations were carried out in a temperature- (23 °C) and humidity- (50% relative humidity) controlled laboratory. All experiments were started not before 1 h of acclimatization period. An informed consent according to the declaration of Helsinki was obtained from each participant and the study was approved by the local ethics committee.

### *Dermal microdialysis*

A rectangular skin area at the right volar forearm was anesthetized by cooling with an ice pack. Thereafter, four single plasmapheresis hollow fibers (0,4 mm in diameter; cut-off 3000 kDa; DermalDialysis, Erlangen, Germany, see below) were inserted intracutaneously with the help of 25-gauge cannulae. The fibers were inserted into the skin over a length of 15 mm. All fibers were transversally orientated to the axis of the forearm. Insertion depth was measured by ultrasound as described in previous studies and found to be 0.65 mm on average with this technique (Schmelz et al., 1997). All plasmapheresis fibers contained 0.1 mm stainless steel wires.

First, hollow fibers were perfused with physiological saline to guarantee function. In order to prevent TNF degradation during sampling the perfusion solution during the experiments contained the peptidase blocker phosphoramidon 0.02% (Sigma-Aldrich, Germany). For constant perfusion of the fibers we used a microdialysis pump (Pump 22/2000, Harvard Apparatus, Holliston, MA, USA), which was set at a constant flow rate of 3 µl/min and connected to the microdialysis fibers via tygon tubes (Novodirect, Germany). The dialysis eluate was collected through glass capillaries in Eppendorf cups.

In order to allow equilibration between the microdialysis perfusate and the interstitial space, a baseline period of at least 60 min with constant saline perfusion is necessary in every microdialysis experiment (Anderson et al., 1994). Some variation of this baseline perfusion could be due to technical factors. After that baseline period, pain stimulation commenced (see below) with either mechanical impact ($n = 20$) or electrical current ($n = 16$). In order to control for the microdialysis-related minimal skin trauma itself, in a third set of experiments microdialysis without stimulation was performed ($n = 16$).

### *Impact stimulation*

The impact-stimulator was described in detail in previous publications (Kohllöffel et al., 1991). In brief, light plastic cylinders (0.5 g; diameter of contact area: 0.2 $cm^2$) were guided and accelerated by compressed air in a barrel to a velocity of 11 m/s, hitting the skin vertically. Impact stimuli were applied to the skin in the middle between the four microdialysis membranes (distance to the nearest membranes: 2 cm). Mechanical impact stimulation started after baseline perfusion of 60 min. Trains of ten impulses (inter-impulse interval 2.5 s) were applied subsequently to three predetermined neighboring skin sites (30 impulses, sites A, B, C, see Fig. 1). This procedure was repeated three times within 5 min (90 impulses). After pauses of 3 min each in order to prevent skin damage, the stimulation was repeated for in total four times. All together repetitive mechanical stimulation lasted 30 min. From previous investigations it is known that the pain threshold for impact stimulation is about 11 m/s depending on the site tested (Sieweke et al., 1999). Accordingly, the last impact stimulation was rated as only 0.9 ± 0.4 on average on the 11-step numeric rating scale (left anchor no pain, right anchor worst pain). None of the subjects reported any aftersensation or tenderness of the surrounding skin.

### *Electrical stimulation*

After baseline perfusion (60 min), electrical stimulation was applied. Electrical impulses (1 Hz, 0.5 ms stimulus duration) were applied intracutaneously via the stainless steel wires inside the microdialysis membranes using a constant current stimulator (DS7, Digitimer, Hertfordshire, UK). We used both pairs of membranes for electrical stimulation. Electrical current was increased by 5 mA steps to 20 mA within the first 5 min and remained unchanged for further 25 min. These electrical stimuli are clearly suprathreshold for pain (Geber et al., 2007). Accordingly at the end of stimulation period, pain was rated as 6.3 ± 1.3 on the 11-step numeric rating scale by the subjects. In accordance with our previous results (Geber et al., 2007) 50% of the participants reported tenderness of the skin surrounding the stimulation site clinically indicating hyperalgesia.

### *TNF-alpha—ELISA*

The microdialysis eluates were collected at the end of the baseline period, after the stimulation period (30 min from baseline sampling), and after further 60 min (90 min from baseline sampling). Sample periods were 30 min each. All samples were immediately stored at −80 °C until analysis. Samples were analyzed for TNF-alpha content using a commercial TNF-alpha enzyme-linked immunosorbent assay (ELISA, Biosource, UK) according to the manufacturer's instructions. The assay system detects human TNF-alpha with a detection limit of <1.7 pg/ml.

### *Laser Doppler imaging*

Superficial blood flow of the skin was quantified using a laser Doppler imager (LDI; Moor, London, UK). The distance between the