

Reduction in touch sensitivity and hyperinnervation in vesicant-injured rabbit eyelid by direct injection of corticotropin releasing factor

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Abstract

Skin injury can result in inflammatory responses and increased sensitivity to touch. Corticotropin releasing factor (CRF), when administered locally or systemically, can reduce the inflammatory and hyperalgesic processes after skin injury. However, the mechanisms that control its effects are unclear. Doxorubicin injection produced inflammation, increased sensitivity to touch and more sensitive blink responses in eyelids of adult rabbits, and local injection of CRF reduced these changes. Doxorubicin alone resulted in a significant ingrowth of nerve fibers as determined by morphometric analysis of PGP 9.5 and substance P immunohistochemistry. Treatment with CRF significantly reduced this nerve fiber ingrowth, and a CRF antagonist partially blocked this protective effect. Thus, CRF has a potent tissue protective effect when administered locally after a vesicant-induced injury, and one mechanism of action is the reduction of nerve fiber ingrowth and sensitivity of the eyelid to touch.

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The skin is often exposed to drug treatments and surgical procedures that result in local tissue injury, inflammation and increased sensitivity to touch. Often these negative sequelae of drug application are difficult to treat and cause significant patient discomfort. Intravenous administration of many drugs, including anti-cancer agents like doxorubicin, can result in localized inflammation and tissue injury if extravasation occurs [1,21]. Infiltration of doxorubicin and other vesicant drugs occurs accidentally, and agents that can reduce localized inflammation and tissue injury would be extremely useful.

One agent with anti-inflammatory effects is corticotropin releasing factor (CRF). CRF is a small peptide neurotransmitter released by the hypothalamus in response to stress and has widespread effects on both the autonomic and immune systems. CRF, when administered systemically, reduces edema and vascular leakage after various types of peripheral injuries [8,26]. While CRF is well-known for its systemic effects in the response to stress, local CRF administration can reduce specific aspects

of the inflammatory response [11]. Local CRF administration reduced inflammation and edema after cold-induced injury [2] and after thermal injury [10].

CRF dramatically reduced acute inflammation in eyelids treated with doxorubicin [13,14]. In addition intradermal administration of CRF reduced touch sensitivity of the doxorubicin treated eyelids as assessed by blink response [13]. CRF has a number of potential targets that might reduce inflammation and increased sensitivity at the site of local administration, including a direct effect on inflammatory cell recruitment to the injury site [3] and a direct effect on keratinocyte production of inflammatory cytokines [19]. This study sought to examine another possible mechanism that might explain the effect of CRF on reduction of doxorubicin-induced increased sensitivity to touch.

Rabbit eyelids were injected with doxorubicin only or doxorubicin followed by injection of CRF. Control, doxorubicin-only, and doxorubicin and CRF-treated eyelids were assessed for alteration in the blink response and morphometric quantification of nerve fibers within the treated eyelids as identified with PGP 9.5 and substance-P [7].

Adult New Zealand white rabbits were obtained from Bakkom Rabbitry (Viroqua, WI). All research conformed to the NIH Guide for the Care and Use of Laboratory Animals and was approved by the University of Minnesota Animal Care Commit-

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tee. Doxorubicin injected into human patients caused increased sensitivity to touch, but was not painful [27,28]. All experiments were performed to minimize pain and discomfort.

Prior to injection, to determine baseline blink reflex response both lower eyelids of each rabbit were tested using Von Frey monofilaments (Stoelting, Wood Dale, IL) delivering punctate mechanical stimuli at a range of force intensities (0.373–232.46 mN) [13].

Rabbits were anesthetized by an intramuscular injection of ketamine and xylazine (1:1; 10 mg/kg:2 mg/kg). The corneas were treated with Proparacaine drops to reduce blinking during injections. All eyelids were injected first medially and then laterally by a midline insertion of the needle to ensure complete eyelid treatment. Needles were left in place for 30 s to minimize leakage. Contralateral eyelids served as either a positive or negative control for the treated eyelid to reduce effects of circulating hormones. Both lower eyelids of rabbits were injected with 2 mg doxorubicin (Pharmacia and Upjohn, Bridgewater, NJ) in 1 ml sterile isotonic saline, followed 20 min later by injection of 75 μ g corticotropin releasing factor (Peninsula Labs., Belmont, CA) in 1 ml saline into one doxorubicin treated eyelid. Four eyelids were injected with either doxorubicin alone or doxorubicin followed by CRF at each post-injection survival interval. A second set of rabbit eyelid pairs received ipsilateral injections of doxorubicin or doxorubicin and CRF and contralateral injections of saline only, CRF only, or doxorubicin followed 20 min later with saline only as a control. Thus, 20 eyelids were injected with doxorubicin only, 20 eyelids injected with doxorubicin followed by CRF, and 4 eyelids each injected with either CRF only, saline only, or doxorubicin followed by saline. A third set of rabbits received injection of CRF antagonist (α -helical CRF 9–41; Peninsula Labs., Belmont, CA) into one lower eyelid. Twenty minutes later, both eyelids received injections of 2 mg doxorubicin, followed by a CRF injection into one eyelid. Antagonist dose was based on previous studies [13,14].

Animals were allowed to survive for 1, 2, or 4 days. Every day, blink reflex was determined the Von Frey mechanical stimuli test. Animals were then deeply anesthetized and perfused through the heart with Zamboni's fixative, which consists of 4% paraformaldehyde and 14% picric acid in phosphate buffered

saline (PBS). Eyelid sections from medial, middle and lateral regions were removed, embedded in tragacanth gum, frozen, and sectioned at 30 μ m on a cryostat. Sections were immunostained for the presence of protein gene product 9.5 (PGP 9.5; Ultraclone, UK), which immunostains all axons, [7] or substance P (Harlan Sera-Lab, Loughborough, England). Sections were post-fixed with 95% ethanol and 5% acetic acid for 10 min, rinsed in PBS, blocked with normal horse serum and incubated overnight with an antibody to PGP 9.5 (1:100). The tissue was incubated using the peroxidase Vectastain Elite ABC kit (Vector Labs., Burlingame, CA), followed by incubation with DAB and heavy metals. For substance P, the sections were quenched in methanol containing 0.1% hydrogen peroxide for 30 min, rinsed with PBS, blocked with rabbit serum and avidin and biotin blocking reagents (Vector Labs.), followed by overnight incubation with an antibody to substance P (1:25). The sections were incubated using the Vectastain Elite ABC kit, and labeling was visualized using DAB and heavy metals. For each antibody, control slides were prepared without primary antibody to verify the absence of non-specific binding.

The total length of PGP 9.5-positive axons and substance P-positive axons in at least three eyelid sections from the three eyelid regions (medial, central and lateral) from each rabbit was quantified morphometrically (Bioquant, Nashville, TN). The length of nerves/0.2 mm eyelid length was determined from mucocutaneous junction to the end of the epithelium on each eyelid sample. The average nerve length/0.2 mm eyelid epithelium length was determined. Nerves were quantified both within the deep layers of the epidermis and within the first 0.5 mm of the dermis directly under the basal layer of the skin. Three-dimensional reconstructions of traced nerve lengths were prepared. All data was analyzed for statistical significance ($p < 0.05$) using analysis of variance (ANOVA) and Dunn's multiple comparison test (Graphpad, San Diego, CA). An *F*-test verified that the variances of control and experimental groups were not significantly different.

Doxorubicin-treated eyelids demonstrated a large increase in amount of PGP 9.5-positive nerve fibers compared to control (Figs. 1 and 2). This correlates with the increased sensitivity to touch reported by patients injected with doxorubicin in this loca-

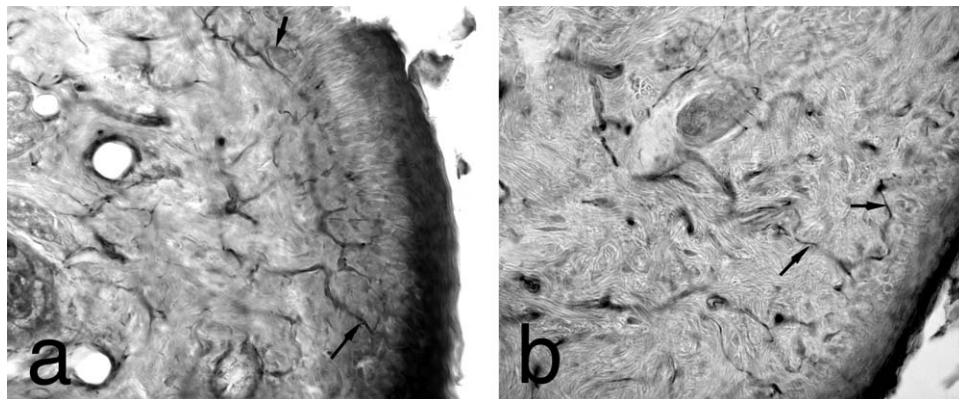


Fig. 1. PGP 9.5 Positive Nerves. PGP 9.5 immunoreactive nerves within treated eyelid dermis and epidermis 1 day after doxorubicin injection (a). PGP 9.5 positive nerves 1 day after a CRF injection 20 min after doxorubicin injection (b). Bar represents 50 μ m.

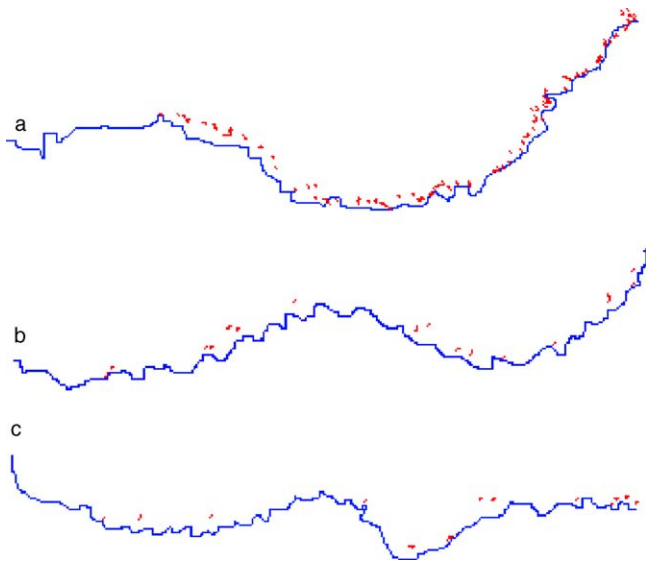


Fig. 2. Three-dimensional reconstruction of all PGP9.5-positive nerves in eyelid sections. Reconstruction of all PGP 9.5-positive nerves along one representative eyelid section after injection with doxorubicin alone (a), doxorubicin and CRF (b), and CRF only (c).

tion [27,28]. This increase in nerve fibers within the epidermis and dermis was substantially reduced when CRF was injected after doxorubicin treatment (Figs. 1 and 2). Eyelids pretreated with a CRF antagonist prior to doxorubicin and CRF injection looked similar to those treated with doxorubicin alone (Fig. 2).

The eyelids were tested for alteration in sensitivity to touch as monitored by alterations in blink reflex due to the various treatments. Doxorubicin alone resulted in a significant increased sensitivity to touch (Fig. 3), which did not develop when CRF was injected after doxorubicin treatment (Fig. 3). The CRF antagonist, α -helical CRF, abolished the effect of CRF on the blink response (Fig. 3).

PGP 9.5-positive nerve fibers were quantified in eyelid cross-sections from the mucocutaneous junction along the skin towards the bony orbital margin. Total length of nerves was averaged based on 0.2 mm of epithelial length (Fig. 4). All doxorubicin injected eyelids showed a statistically significant increase

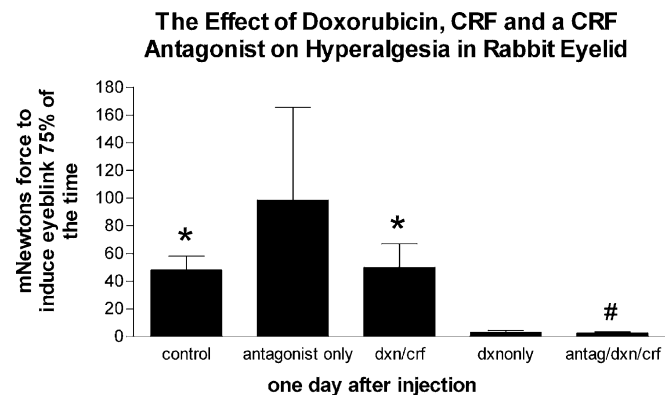
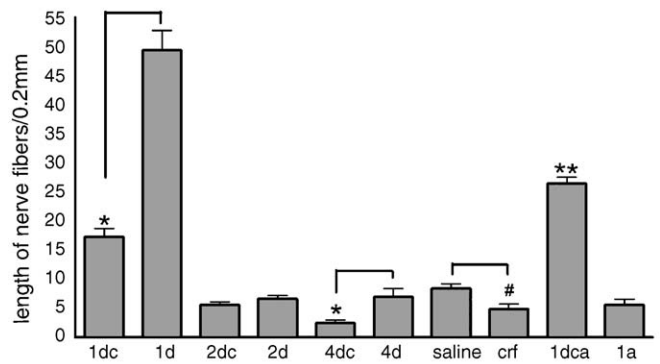


Fig. 3. Blink reflex in eyelids treated with doxorubicin and CRF. Effect of doxorubicin and CRF on blink reflex response. (*) indicates significant difference compared to doxorubicin only. (#) indicates significant difference compared doxorubicin and CRF.

Quantification of PGP 9.5 Positive Nerve Fibers in the Epidermis



Quantification of PGP 9.5 Positive Nerve Fibers in the Dermis

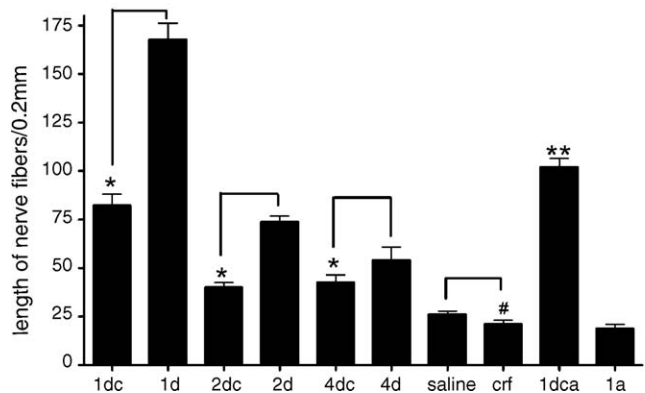


Fig. 4. Quantification of PGP 9.5 positive nerves. Average length of PGP 9.5-positive nerves in eyelids treated with doxorubicin only (d), doxorubicin followed by CRF (dc), saline only (saline), crf only (crf), doxorubicin, CRF and antagonist (dca) and antagonist only (a). (*) indicates significant difference compared to doxorubicin only. (**) indicates significant difference compared to doxorubicin only and doxorubicin and CRF. (#) indicates significant difference compared to saline only control.

in PGP 9.5-positive nerve fiber length in the dermis compared to control eyelids. One day after injection of doxorubicin and CRF, there was approximately a two-fold and three-fold decrease in the nerve fibers compared with doxorubicin only in the dermis and epidermis, respectively. These differences were maintained in the dermis both 2 and 4 days after single injections with doxorubicin or doxorubicin and CRF. In the epidermis, there was a significant difference only 1 and 4 days after eyelid treatment. Interestingly, CRF alone reduced the total nerve fibers in the eyelids compared to saline only by 55 and 20% in the epidermis and dermis, respectively. Thus, local injection of CRF resulted in a significant decrease in the vesicant drug-induced increase in PGP 9.5-positive nerves. Pretreatment with the CRF antagonist reduced the potency of the CRF treatment, but did not completely inhibit its effects in both the dermis and epidermis (Fig. 4). Antagonist alone had no significant effect.

Unlike the PGP 9.5 labeling, the increase in substance P-stained nerve fibers induced by doxorubicin was only seen 1 day post-injection (Fig. 5). CRF injection reduced the length of substance P positive nerve fibers compared to doxorubicin by only 19 and 51% in the dermis and epidermis, respectively

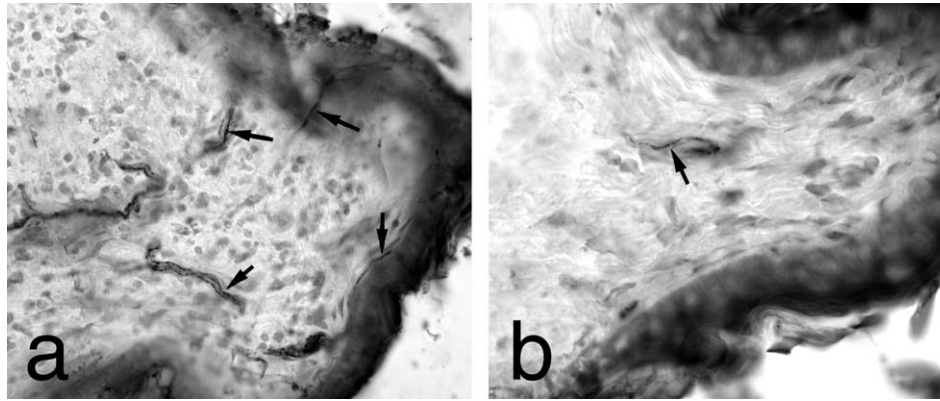


Fig. 5. Substance P positive nerves. Substance P immunoreactive nerves within the injured dermis and epidermis 1 day after doxorubicin injection (a) and 1 day after CRF and doxorubicin injection (b). Bar represents 50 μm .

(Fig. 6). No differences in the length of substance P-positive nerve fibers were seen 2 and 4 days after doxorubicin treatment (data not shown).

Direct subcutaneous injection of doxorubicin results in a pronounced increased sensitivity to touch, which is significantly reduced if CRF is administered locally soon after the injury [13]. Compared to normal eyelid, within 24 h after a localized doxorubicin exposure, nerve fiber length increased significantly within the epithelium and dermis as determined by PGP 9.5 immunostaining. CRF injected 20 min after doxorubicin exposure prevented this increase in nerve ingrowth. Thus, local CRF

administration prevented doxorubicin-induced peripheral nerve sprouting. This may be a primary effect or may be secondary to the dramatic reduction in inflammatory cell infiltration caused by CRF [13,14]. CRF can induce leukocyte recruitment to an injury site, where they locally release opioids and counteract inflammatory increased sensitivity to touch [24].

Systemic administration of CRF results in decreased neurogenic inflammation, specifically by inhibition of sensory neuropeptide release [16]. CRF selectively inhibits trigeminal neuronal responses to noxious heat [20] independent of its effect on systemic levels of beta-endorphin and corticosterone [5]. Systemic administration can affect the central nervous system directly, acting at all levels of the neuraxis [11]. To eliminate possible systemic effects, we examined different treatments using the ipsilateral and contralateral sides of the same animals.

The mechanism for the direct effects of intradermal CRF is unclear [11]. CRF receptors are present in skin [23]. CRF may down-regulate interleukin-18 production by keratinocytes at the site of local tissue injury [19] or may increase release of enkephalin and dynorphin [4]. Various immune system cells, including neutrophils, macrophages and lymphocytes express the CRF receptor, [16] which upregulates with localized inflammation [6]. In one study the antinociceptive effects were not displaced by opioid or adrenergic compounds [23], yet in another study, antagonists to opioid receptors and naloxone inhibited CRF-induced analgesia [17]. These different responses may reflect whether central or peripheral pathways are involved in modulation of increased sensitivity to touch and/or pain [22]. Doxorubicin results in significant inflammatory cell infiltration at the injection site [13,14], and this is common to a number of models producing localized inflammation and increased sensitivity to touch. The effects of CRF on neurite ingrowth may, at least in part, be secondary to the dramatic reduction in inflammatory cell infiltrate after CRF treatment within the injured eyelid, with the resultant decrease in release of cytokines from these cells [19]. Further studies are needed to differentiate between these possible mechanisms.

Sensory innervation to the skin determines both sensitivity to touch and perception of pain. Many factors modulate the amount of sensory innervation locally in skin and connective tissue. Capsaicin treatment leads to blistering followed by a reduction in

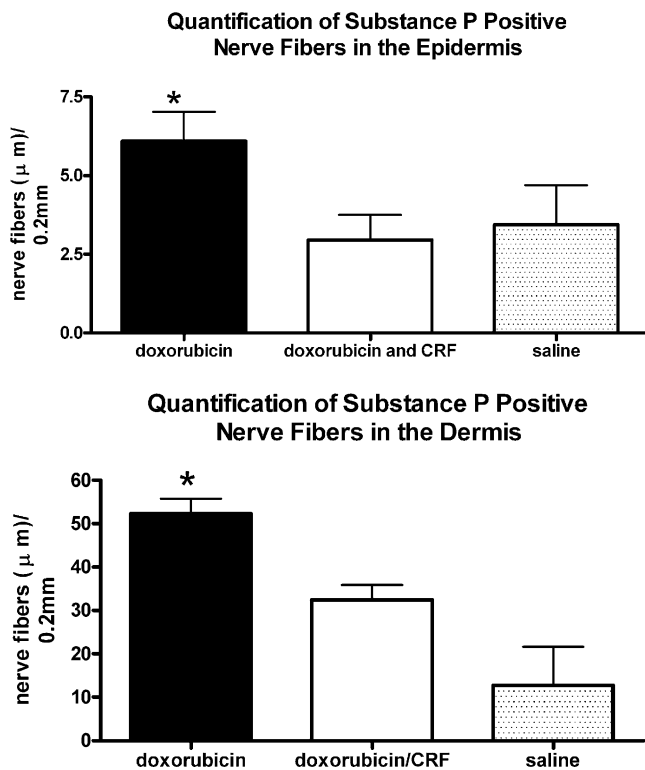


Fig. 6. Quantification of substance-P positive nerves. Average length of substance P-positive nerves in the eyelids treated with doxorubicin only, doxorubicin followed by CRF, and saline only controls. (*) indicates significant difference from the combined injection of doxorubicin and CRF and injection of saline only.

cutaneous nerve fibers [18], while skin innervation increased during the first 6 h following a suction cup blistering injury [9]. Innervation density increases in tumors and is postulated to play a role cancer pain [25]. Growth factors released due to tissue injury can foster sensory nerve ingrowth [12,15], but this was not examined in the present study. The prevention of increased touch sensitivity and the reduction in doxorubicin-induced nerve ingrowth by locally injected CRF suggest a distinct mechanism for its local and immediate anti-hyperalgesic effects after drug-induced tissue injury [13].

The CRF antagonist, α -helical CRF 9–41, did not completely block the effect of CRF after the doxorubicin injections. The dose may not have been sufficient, the timing of injections may have resulted in too little antagonist in the eyelid after the sequence of injections, the subsequent injections of doxorubicin and CRF may have diluted its concentration to a less optimal one, or the antagonist may not spread as well as the CRF or doxorubicin within the eyelid. Distinguishing between these alternatives was beyond the scope of this study.

In summary, when given 20 min after doxorubicin injection, locally injected CRF has potent local protective effects that significantly reduce inflammatory cell infiltration [14], hypersensitivity to touch, and doxorubicin-induced increased nerve ingrowth within the dermis and epidermis. Post-injury injection of CRF at a site of accidental local drug or other vesicant exposure has clinical potential to significantly reduce inflammation, tissue injury and increased sensitivity to touch. Further studies are required to extend these results to other injury models.

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References

- [1] D.B. Bowers, J.B. Lynch, Adriamycin extravasation, *Plast. Reconstr. Surg.* 61 (1978) 686–692.
- [2] A. Bozkurt, S. Ghandour, N. Okboy, S. Oner, S. Arbak, T. Coskun, B. Yegen, Inflammatory response to cold injury in remote organs is reduced by corticotropin-releasing factor, *Regul. Pept.* 99 (2001) 131–139.
- [3] A. Brack, H.L. Rittner, H. Machelska, K. Leder, S.A. Mousa, M. Schafer, C. Stein, Control of inflammatory pain by chemokine-mediated recruitment of beta-endorphin and corticosterone, *Regul. Pept.* 118 (2004) 229–238.
- [4] P.J. Cabot, L. Carter, M. Schafer, C. Stein, Methionine-enkephalin- and dynorphin A-release from immune cells and control of inflammatory pain, *Pain* 93 (2001) 207–212.
- [5] M.S. Cepeda, I. Bonney, J. Moyano, D.B. Carr, Corticotropin-releasing hormone produces analgesia in a thermal model independent of its effect on beta-endorphin and corticosterone, *Regul. Pept.* 118 (2004) 39–43.
- [6] S.G. Correa, C.M. Riera, J. Spiess, I.D. Bianco, Modulation of the inflammatory response by corticotropin-releasing factor, *Eur. J. Pharmacol.* 319 (1997) 85–90.
- [7] C.J. Dalsgaard, M. Rydh, A. Haegerstrand, Cutaneous innervation in man visualized with protein gene product 9.5 antibodies, *Histochemistry* 92 (1989) 385–390.
- [8] G.C. Gao, M.R. Dashwood, E.T. Wei, Corticotropin-releasing factor inhibition of substance P-induced vascular leakage in rats: possible sites of action, *Peptides* 12 (1991) 639–644.
- [9] X.H. Gu, G. Terenghi, P.E. Purkis, D.A. Price, I.M. Leigh, J.M. Polak, Morphological changes of neural and vascular peptides in human skin suction blister injury, *J. Pathol.* 172 (1994) 61–72.
- [10] J.G. Kiang, E.T. Wei, Corticotropin-releasing factor inhibits thermal injury, *J. Pharmacol. Exp. Ther.* 243 (1987) 517–520.
- [11] W.R. Lariviere, R. Melzack, The role of corticotropin-releasing factor in pain and analgesia, *Pain* 84 (2000) 1–12.
- [12] L. Li, C.J. Xian, J.H. Zhong, X.F. Zhou, Lumber 5 ventral root transection-induced upregulation of nerve growth factor in sensory neurons and their target tissues: a mechanism of neuropathic pain, *Mol. Cell Neurosci.* 23 (2003) 232–250.
- [13] L.K. McLoon, A.M. Sandnas, K. Nockleby, J.D. Wirtschafter, Reduction in vesicant-induced cellular inflammation and hyperalgesia by local injection of corticotropin releasing factor in rabbit eyelid, *Inflamm. Res.* 51 (2002) 16–23.
- [14] L.K. McLoon, J.D. Wirtschafter, Local injections of corticotropin releasing factor reduce doxorubicin-induced acute inflammation in the eyelid, *Invest. Ophthalmol. Vis. Sci.* 38 (1997) 834–841.
- [15] D.C. Molliver, J. Lindsay, K.M. Albers, B.M. Davis, Overexpression of NGF or GDNF alters transcriptional plasticity evoked by inflammation, *Pain* 113 (2005) 277–284.
- [16] S.A. Mousa, C.P. Bopaiah, C. Stein, M. Schafer, Involvement of corticotropin-releasing hormone receptor subtypes 1 and 2 in peripheral opioid-mediated inhibition of inflammatory pain, *Pain* 106 (2003) 297–307.
- [17] S.A. Mousa, M. Schafer, W.M. Mitchell, A. Hassan, C. Stein, Local upregulation of corticotropin-releasing hormone and interleukin-1 receptors in rats with painful hindlimb inflammation, *Eur. J. Pharmacol.* 311 (1996) 221–231.
- [18] M. Nolano, D.A. Simone, G. Wendelschafer-Crabb, T. Johnson, E. Hazen, W.R. Kennedy, Topical capsaicin in humans: parallel loss of epidermal nerve fibers and pain sensation, *Pain* 81 (1999) 135–145.
- [19] H.J. Park, H.J. Kim, J.H. Lee, J.Y. Lee, B.K. Cho, J.S. Kang, H. Kang, Y. Yang, D.H. Cho, Corticotropin-releasing hormone downregulates interleukin-18 expression in human HaCaT keratinocytes by activation of p38 mitogen-activated protein kinase pathway, *J. Invest. Dermatol.* 124 (2005) 751–755.
- [20] L.R. Poree, A.H. Dickenson, E.T. Wei, Corticotropin-releasing factor inhibits the response of trigeminal neurons to noxious heat, *Brain Res.* 502 (1989) 349–355.
- [21] R. Rudolph, R.S. Stein, R.A. Pattillo, Skin ulcers due to adriamycin, *Cancer* 37 (1976) 1087–1094.
- [22] M. Schafer, L. Carter, C. Stein, Interleukin 1 and corticotropin releasing factor inhibit pain by releasing opioids from immune cells in inflamed tissue, *Proc. Natl. Acad. Sci. U.S.A.* 91 (1994) 4219–4223.
- [23] A. Slominski, G. Ermak, J. Hwang, A. Chakraborty, J.E. Mazurkiewicz, M. Mihm, Proopiomelanocortin, corticotropin releasing hormone and corticotropin releasing hormone receptor genes are expressed in human skin, *FEBS Lett.* 374 (1995) 113–116.
- [24] C. Stein, M. Schafer, H. Machelska, Attacking pain at its source: new perspectives on opioids, *Nat. Med.* 9 (2003) 1003–1008.
- [25] P.W. Wacnik, C.M. Baker, M.J. Herron, T. Kren, B.R. Blazar, G.L. Wilcox, M.K. Hordinsky, A.J. Beitz, M.E. Ericson, Tumor-induced mechanical hyperalgesia involves CGRP receptors and altered innervation and vascularization of DsRed2 fluorescent hindpaw tumors, *Pain* 115 (2005) 95–106.
- [26] E.T. Wei, G.C. Gao, Corticotropin-releasing factor: an inhibitor of vascular leakage in rat skeletal muscle and brain cortex after injury, *Regul. Pept.* 333 (1991) 93–104.
- [27] J.D. Wirtschafter, Clinical doxorubicin chemomyectomy: an experimental treatment for muscle spasms, *Ophthalmology* 98 (1991) 357–366.
- [28] J.D. Wirtschafter, L.K. McLoon, Long term efficacy of local doxorubicin injections in eyelid spasm patients, *Ophthalmology* 105 (1998) 342–346.