Inflammation-induced up-regulation of ionotrophic glutamate receptor expression in human skin

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Background. N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazolone-4-propionic acid (AMPA), and kainate (KA) receptors are members of the ionotropic glutamate receptor (iGluR) family and are increased in inflamed rat skin. These receptors contribute to inflammatory pain. In this study, we have examined whether there is a similar increase in iGluRs in inflamed human skin in the presence of inflammatory pain.

Methods. Normal and inflamed-skin biopsies were obtained from eight patients undergoing elective wound-debridement surgery. Real-time-polymerase chain reaction (PCR) and western blot analysis were used for quantitation of iGluR mRNA and protein in normal and inflamed human skin.

Results. A significant increase in mRNA and protein for NMDA, AMPA, and KA receptor subunits was detected in inflamed compared with normal skin. The amounts of NMDA (NR1 subunit), AMPA (GluR2 subunit), and KA (GluR6 subunit) mRNA in inflamed skin were mean 6 (SD 1.6-fold), 2.5 (0.6-fold), and 3.8 (0.9-fold) (\(P<0.05\)), respectively, greater than that measured in normal skin. The ratio of NR1, GluR2, and GluR6 protein in inflamed compared with normal skin was 5.7 (1.2), 2.4 (0.5), and 3.6 (0.9) (\(P<0.05\)), respectively.

Conclusions. These results, in human tissue, demonstrate that iGluR mRNA and protein expression are increased during persistent inflammation and that this increased activity may be involved in mediating clinical inflammatory pain in human skin.

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Glutamate is the predominant excitatory neurotransmitter in the central nervous system (CNS), and acts through ligand-gated ion channels (ionotropic glutamate receptors, iGluRs) and G protein-coupled metabotropic glutamate receptors (mGluRs). iGluR subunits have been identified on thin, unmyelinated nociceptive fibres in rat skin. Subcutaneous injection of glutamate and iGluR agonists into the rat hind paw results in a reduction in thermal and mechanical thresholds. Peripherally applied iGluR antagonists attenuate nociceptive scores in the formalin test, used as a rodent model for inflammatory pain. The concentration of glutamate rises in the skin during the formalin test in the rat and in synovial-fluid samples deriving from human arthritic patients. Collectively, these data suggest that glutamate is a peripheral mediator of inflammation released in response to tissue injury and further suggest that peripheral iGluR activation may produce inflammatory pain. Recent psycho-physical behavioural studies in humans have suggested the presence of peripheral glutamate receptors on axons of peripheral primary afferents. In the inflamed state, not only does glutamate content increase in the axons innervating the inflamed region but also significant increases in the numbers of axons labelled for N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazolone-4-propionic acid (AMPA), or kainate (KA) receptors is seen. Quantitation of NMDA, AMPA, and KA iGluRs in the rat has revealed a significant...
increase subsequent to intraplantar injection of complete Freund’s adjuvant. To the best of our knowledge, there are no similar data in inflamed human skin. Real-time quantitative reverse transcription–polymerase chain reaction (RT–PCR) is a useful and reliable method to study mRNA expression. We have used this technique coupled with western blot analysis to quantify both iGluR mRNA and protein expression in inflamed human skin and compare this with normal skin.

**Methods**

The study was performed according to the declaration of Helsinki with approval from the ethics committee of the Kaohsiung Armed Force General Hospital, Taiwan. Sample size was calculated according to previous studies. Eight patients presenting with inflamed wounds and a sensation of hyperalgesia and allodynia on or near the wound region were included in this study for which written informed consent was given. All patients reported pain with an intensity of 3 or more on a numeric rating scale (NRS) ranging from 0 to 10 with 0 meaning ‘no pain’ and 10 ‘worst pain imaginable’ at the time of interview. The following analgesic medications were taken by patients with a painful wound at the time of examination: opioids (n=6) and non-steroidal anti-inflammatory drugs (n=2). Normal and inflamed-skin biopsies were obtained from eight patients undergoing elective wound-debridement surgery. Skin samples of 1 x 0.5 x 0.3 cm, in terms of length, breadth, and thickness, were taken from the surgical site and the other non-inflamed sites with surgical knife, thus two skin-tissue samples taken from each of the eight patients in the study. The dissected-tissue samples were separately placed into individual Eppendorf tubes, and then immediately frozen in liquid nitrogen, and stored at −80°C until use.

**RNA isolation and real-time RT–PCR**

RNA was isolated and purified from individual skin-biopsy samples using the GeneStrips Reagent (RNAture, Irvine, CA, USA). All cDNA synthesis was performed by reverse transcription of each sample using random hexamer primers and the BcaBEST RNA PCR kit (TaKaRa Biomedicals Company, Shiga, Japan).

Real-time PCR was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with SYBR Green detection by the 7700 Sequence Detection System User Bulletin 2). Results of the RT–PCR analyses were expressed as CT values, which were used to determine the quantity of target-gene mRNA in relation to the quantity of reference-gene mRNA (see ABI Prism 7700 Sequence Detection System User Bulletin 2). ΔCT indicated the difference between the number of cycles necessary to detect the PCR products for NR1, GluR2, GluR6, and the reference gene. ΔΔCT was the difference between the ΔCT values for the inflamed skin groups and the corresponding ΔCT values for the normal skin groups. Data were expressed as 2−ΔΔCT in order to provide an estimate of the quantity of target mRNA present in the inflamed skin tissue relative to the normal skin groups.

**Western blots**

Total skin protein was prepared by the addition of 500 μl of ice-cold solubilization buffer (150 mM NaCl, 50 mM Tris–HCl, pH=8.0, 5 mM EDTA, and 1% Triton X-100) containing protease inhibitors (1 mM phenylmethylsulphonyl fluoride and 5 μg ml−1 each of pepstatin, leupeptin, chymostatin, antipain, and apronitin). The tissue was homogenized with a homogenizer, and subsequent to being placed on ice for 30 min, the homogenate was centrifuged at 10,000 g for 5 min at 4°C. The supernatant was then collected and assayed for protein content using the Bicinchoninic acid (BCA) assay method (Pierce, Rockford, IL, USA), and stored at −70°C until further use. A total of 15 μg of total protein was electrophoresed on an 8% sodium dodecylsulphate–polyacrylamide gel using sample buffer, running buffer, and molecular-weight standards, as suggested by the manufacturer. After electrophoresis, the

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Real-time RT–PCR Primers. Sequences are shown for forward (Fwd) and reverse (Rev) primers</th>
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<tbody>
<tr>
<td>Gene</td>
<td>Direction</td>
</tr>
<tr>
<td>NR1</td>
<td>Fwd</td>
</tr>
<tr>
<td>GluR6</td>
<td>Fwd</td>
</tr>
<tr>
<td>β-actin</td>
<td>Fwd</td>
</tr>
<tr>
<td>GluR2</td>
<td>Rev</td>
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<tr>
<td>GluR6</td>
<td>Rev</td>
</tr>
<tr>
<td>β-actin</td>
<td>Rev</td>
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**PCR (25 μl)** included 10 ng of template, 1X SYBR Green PCR Master Mix, forward and reverse primers (2.0 μM), and water (sterile and UV cross-linked) to the final volume. Primer concentrations for β-actin were 2.5 μM each. To avoid competition, only one mRNA was amplified in each PCR (monoplex). A ‘no-template’ control was used for each primer pair consisting of water (sterile and UV cross-linked). Subsequent to completion, expected sizes of PCR products were confirmed on 3% NuSieve 1:3 agarose (BMA, Rockland, ME, USA) analytical gels.
proteins were transferred to a PVDF membrane and blocked with 5% non-fat dry milk. The primary antibody for anti-NMDA-R1 (a 1:600 dilution of goat polyclonal antibody; Santa Cruz Biotechnology, USA), anti-GluR2 (a 1:400 dilution of goat polyclonal antibody; Santa Cruz Biotechnology), and anti-GluR6 (a 1:400 dilution of goat polyclonal antibody; Santa Cruz Biotechnology) were added for a period of 2 h at room temperature in fresh blocking buffer. The membranes were then washed for 30 min in washing buffer at room temperature, before the secondary antibody (a 1:6000 dilution of horseradish peroxidase-coupled rabbit anti-goat immunoglobulin G; Chemicon, Temecula, CA, USA) was added for a 1 h period at room temperature in blocking buffer. The membranes were washed in washing buffer for another 30 min, and the antibodies were then revealed using Western Blot Chemiluminescence Reagent Plus (NEN, Boston, MA, USA). For densitometric analyses, blots were scanned and quantified using Quantity One analysis software (Bio-Rad, Hercules, CA, USA), and the results were expressed as the percentages of β-tubulin immunoreactivity.

Statistical analysis
All data are expressed as the mean [standard deviation (SD)]. Data from NR1, GluR2, and GluR6 gene expression were analysed using a paired Student’s t-test in order to determine significant differences between normal and inflamed skin groups. A P-value of <0.05 was considered to represent statistically significant difference between test means.

Results
Patient demographics and details of skin tissue samples are listed in Table 2. On initial presentation, median NRS pain intensity was 6.0 (range 3–10). When expressed normalized β-actin NR1, GluR2, and GluR6 mRNA levels measured by real-time PCR were elevated in inflamed compared with normal skin (Fig. 1A). The amounts of NR1, GluR2, and GluR6 mRNA in inflamed skin were mean 6 (SD 1.6-fold), 2.5 (0.6-fold), and 3.8 (0.9-fold) greater than that for normal skin, respectively (Fig. 1B; P<0.05).

Table 2 Patient demographics and details of the skin samples taken

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Injury interval</th>
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<tbody>
<tr>
<td>Case 1</td>
<td>F</td>
<td>57</td>
<td>Tenosynovitis of hand</td>
<td>29 days</td>
</tr>
<tr>
<td>Case 2</td>
<td>M</td>
<td>46</td>
<td>Ulcer of lower leg</td>
<td>14 days</td>
</tr>
<tr>
<td>Case 3</td>
<td>M</td>
<td>51</td>
<td>2° burn injury of buttock</td>
<td>10 days</td>
</tr>
<tr>
<td>Case 4</td>
<td>M</td>
<td>60</td>
<td>Tenosynovitis of hand</td>
<td>7 days</td>
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<tr>
<td>Case 5</td>
<td>F</td>
<td>65</td>
<td>Cellulitis of foot</td>
<td>11 days</td>
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<tr>
<td>Case 6</td>
<td>F</td>
<td>65</td>
<td>Ulcer of lower leg</td>
<td>12 days</td>
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<tr>
<td>Case 7</td>
<td>M</td>
<td>43</td>
<td>2° burn injury of lower leg</td>
<td>8 days</td>
</tr>
<tr>
<td>Case 8</td>
<td>M</td>
<td>33</td>
<td>Ulcer of lower leg</td>
<td>7 days</td>
</tr>
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</table>

There was a significant increase in NR1, GluR2, and GluR6 protein in inflamed compared with normal skin (Fig. 2; P<0.05). The ratio of NR1, GluR2, and GluR6 protein levels in inflamed compared with normal skin was 5.7 (1.2), 2.4 (0.5), and 3.6 (0.9), respectively (P<0.05). Changes in receptor protein were similar to receptor message.

Discussion
This study is the first to demonstrate the up-regulation of mRNA and protein level for NMDA, AMPA, and KA receptors in clinically inflamed human skin. In addition to iGluRs being present on unmyelinated cutaneous axons in the epidermal layer in rat and human hairy skin, Schwann cells present in both rat and human skin also contain iGluRs. Furthermore, previous reports have demonstrated that approximately 35% of the unmyelinated axons were sympathetic fibres, and 36% and 10% of postganglionic sympathetic axons have also been reported to stain positively for NMDA or AMPA receptors,
respectively. Thus, the total quantity of mRNA and protein for NMDA, AMPA, or KA in the present study should reflect the numbers of receptors on these fibres and cells; however, most of the receptors are likely to be present on unmyelinated cutaneous sensory axons. The findings in this study imply an increased sensitivity of cutaneous axons to the local presence of glutamate, such sensitivity possibly contributing to the mechanical allodynia and heat hyperalgesia that are classic manifestations of inflammatory pain.

Persistent pain can cause substantial distress on a patient’s psychological, social, and functional status and quality of life. Although an extensive number of preclinical pain studies have investigated the early stages of inflammatory and neuropathic pain, few appear to have actually addressed aspects of more-persistent and chronic pain combining behavioural and molecular studies. The clinical persistent pain suffered by patients participating in this study did reveal a consistent profile, namely that the characteristics of pain displayed both features of pathological pain described by Woolf, that is, spontaneous pain and hyperalgesia.

Pathological pain, including persistent hyperalgesia and allodynia during inflammation, is an expression of plasticity within the somatosensory system. A significant induction of iGluR mRNA was observed in inflamed skin in this study, and a significant increase in iGluR protein was also detected. The localization of up-regulated iGluR to C- and Aδ-primary afferent fibres is consistent with a role for iGluR in modulating nociception. In general, the predominant effect of iGluR activation is increased excitability and synaptic transmission. Thus, massive induction of iGluR in skin and increased iGluR-mediated activity are thought likely to augment peripheral sensitization and, potentially, behavioural hypersensitivity post-inflammation.

Tissue injury may result in the local release of numerous chemical substance that mediate or facilitate the inflammatory process, including bradykinin, prostaglandins, leukotrienes, serotonin, histamine, substance P, thromboxanes, platelet-activating factor, adenosine and ATP, protons, and free radicals. Cytokines, such as interleukins (ILs) and tumour necrosis factor (TNF), and neurotrophins, especially nerve growth factor (NGF), are also generated during inflammation. Some of these agents can directly activate nociceptors or act indirectly via inflammatory cells, which in turn release algogenic agents. These mediators lead to a sensitization of the nociceptor response to natural stimuli and therefore play a role in hyperalgesia.

Targeting treatment specifically at peripheral iGluR, using selective iGluR antagonists is necessary to determine the functional significance of up-regulated iGluR. Evidence from experimental inflammatory pain models has identified a role for iGluR-mediated activity in the induction of hyperalgesia. Recording from nociceptors in rat glabrous skin indicates that glutamate both excites and sensitizes these units to heat. These studies support the hypothesis that peripheral iGluR activity is crucially involved in the induction and maintenance of altered pain processing. We suggest therefore that targeting iGluR activity both before and during skin injury may be a key step to eliminate a trigger for establishing peripheral sensitization and consequently preventing the development of hypersensitivity. Evidence from human studies has supported this hypothesis. The findings of up-regulation of iGluRs in inflamed tissue in this study further provides evidence for the development of hyperalgesia during inflammatory states and the anti-hyperalgesic effect of iGluR antagonists in humans.

Several sources of glutamate including primary afferents, keratinocytes, macrophages, blood serum, and Schwann cells have been identified. These sources may provide the ligand to activate the increased iGluR numbers and exacerbate pain in inflamed regions.

Up-regulation of NMDA, AMPA, and KA receptors after injection of complete Freund’s adjuvant in rat’s hind

Fig 2 (a) Representative western blot for NR1, GluR2, and GluR6 iGluR in normal (N) and inflamed skin (I). (b) Densitometric quantification of NR1, GluR2, and GluR6 in normal and inflamed skin. Protein is expressed relative to β-tubulin. Each column represents the mean (±SD) for eight skin samples. *P < 0.05 compared with control groups (normal skin).
paw has been reported. This study provides evidence that similar changes in glutamate receptor numbers also occur in inflamed human skin and provides evidence for pain therapy via peripheral approaches. The CNS side-effects that limit the clinical use of glutamate-receptor antagonists could be avoided by local or topical application of glutamate-receptor antagonists.

Funding
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