Modulation of Tight Junction Proteins in the Perineurium to Facilitate Peripheral Opioid Analgesia

Heike L. Rittner, M.D.,* Salah Amasheh, Ph.D.,† Rabih Moshourab, M.D.,‡ Dagmar Hackel, Ph.D.,§ Reine-Solange Yamdeu, Ph.D.,§ Shaaban A. Mousa, Ph.D.,|| Michael Fromm, M.D.,# Christoph Stein, M.D.,** Alexander Brack, M.D.*

ABSTRACT

Background: Peripheral application of opioids reduces inflammatory pain but is less effective in noninflamed tissue of rats and human patients. Hypertonic solutions can facilitate the antinociceptive activity of hydrophilic opioids in noninflamed tissue in vivo. However, the underlying mechanisms are not well understood. We hypothesized that the enhanced efficacy of opioids may be because of opening of the perineurial barrier formed by tight junction-proteins like claudin-1.

Methods: Male Wistar rats were treated intraplantarly with 10% NaCl. Pain behavior (n = 6) and electrophysiological recordings (n = 9 or more) from skin-nerve preparations after local application of the opioid [d-Ala2,N-Me-Phe4,Gly5-ol]enkephalin (DAMGO) were explored. Tight junction-proteins as well as permeability of the barrier were examined by immunohistochemistry and Western blot (n = 3 or more).

Results: Local administration of 10% NaCl facilitated increased mechanical nociceptive thresholds in response to DAMGO, penetration of horseradish peroxidase into the


erve, as well as a reduced response of C- but not Aδ-nociceptors to mechanical stimulation after application of DAMGO in the skin-nerve preparation. In noninflamed paw tissue, claudin-1 was expressed in the epidermis, blood vessels, and the perineurium, surrounding neurons immunoreactive for calcitonin gene-related peptide or protein gene product 9.5. Claudin-1 but not claudin-5 or occludin was significantly reduced after pretreatment with 10% NaCl. Intraplantar application of a metalloproteinase inhibitor (GM6001) completely reversed these effects.

Conclusion: Hypertonic saline opens the perineurial barrier via metalloproteinase activation and claudin-1 regulation, thereby allowing access of hydrophilic drugs to peripheral opioid receptors. This principle may be used to specifically target hydrophilic drugs to peripheral neurons.

**What We Already Know about This Topic**
- Opioids have antinociceptive properties in the periphery, but only in inflamed tissue
- Access of opioids to nociceptors might be limited by the perineurium surrounding peripheral nerves

**What This Article Tells Us That Is New**
- Hypertonic saline was used to increase the permeability for opioids to nociceptors via a decrease in tight junction proteins
- Opening the perineurial barrier may be one approach to improve the peripheral analgesic effect of opioids in the clinic

opioids can effectively control inflammatory pain both in the central nervous system and peripherally at the site of injury. In inflamed tissue, opioids bind to opioid receptors on peripheral sensory nerve terminals and induce potent analgesia (antinociception) both in humans and in experimental models. The antinociceptive action of opioids in inflamed tissue is also supported by electrophysiological experiments using skin-nerve preparations. In noninflamed tissue, hydrophilic opioids are particularly ineffective, in behavioral, clinical, and electrophysiological studies. However, intraplantar coinjection of hypertonic solutions with hydrophilic opioids can significantly increase mechanical nociceptive thresholds, but the underlying mechanisms are not entirely clear.

The perineurium forms a barrier composed of a basal membrane and a layer of perineural cells. The membranes

Anesthesiology, V 116 • No 6

June 2012

Copyright © 2012, the American Society of Anesthesiologists, Inc. Lippincott Williams & Wilkins. Anesthesiology 2012; 116:1323-34
of these cells express proteins for active transport of, e.g., glucose, and tight junction proteins to limit paracellular permeability. Tight junction proteins include the family of claudins and occludins. Earlier studies demonstrated that hypertonic saline increases permeability of the perineurium as well as the blood-brain barrier. In both barriers several claudins are expressed. Claudin-1 is a major sealing tight junction protein. Claudin-1 knockout mice die during the first day of life because of loss of fluid through the skin. After nerve crush injury, a reduced claudin-1 content in the perineurium parallels increased permeability to the sciatic nerve. Previous studies indicate that metalloproteinases can degrade tight junction proteins like occludin. Indeed, breakdown of the blood-brain barrier under pathophysiological conditions can be ameliorated by treatment with metalloproteinase inhibitors, suggesting a role of these enzymes in maintaining the integrity of the barrier. In this study we examined the effects of hypertonic saline solutions and hydrophilic opioids on the time course of peripheral antinociception; electrophysiological recordings in an in vitro skin nerve preparation; penetration of horseradish peroxidase; and the role and regulation of tight junction molecules in the perineural barrier. We hypothesized that opening of the perineurial barrier formed by tight junction proteins like claudin-1 is responsible for enhanced efficacy of opioids.

Materials and Methods

Animals

Animal protocols were approved by the animal care committee of the Senate of Berlin (Landesamt für Gesundheit und Soziales, Berlin) and are in accordance with the International Association for the Study of Pain. Male Wistar rats weighing 180–220 g were injected intraplantarly under brief isoflurane anesthesia, as described below. Experiments were conducted at indicated time-points.

Measurement of Nociceptive Thresholds

Mechanical thresholds were determined using the paw pressure algometer (modified Randall-Selitto test; Ugo Basile, Comerio, Italy), as described below. The pressure required to elicit paw withdrawal, the paw pressure threshold (PPT), was determined by a blinded investigator. Averages from three measurements per treatment were calculated. Baseline measurements were obtained before the intraplantar injection of 100 μl of 0.9% (control) or 10% NaCl under brief isoflurane anesthesia. In the next step, 3–30 μM DAMGO in 100 μl 0.9% saline were applied either simultaneously or at indicated time intervals. PPT were determined 10 min thereafter. In some experiments, 2 μg D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP; all Sigma-Aldrich Chemical, St. Louis, MO) or 0.02–0.2 mg GM6001 (United States Biological, Swamscott, MA) were injected intraplantarly in 100 μl 0.9% saline together with DAMGO. Doses were chosen based on pilot experiments and literature.

Electrophysiology

In isoflurane-anesthetized rats, 10% NaCl (total volume 300 μl) was injected subcutaneously on the medial aspect of the foot and medial aspect of the rat lower limb, which is innervated by the saphenous nerve. After 30 min, an in vitro skin nerve preparation was dissected and used to record from single primary afferents in microdissected teased filaments of the saphenous nerve, as previously described. The skin nerve preparation was perfused at 15 ml/min with oxygen-saturated synthetic interstitial fluid buffer containing 123 mM NaCl, 5.3 mM KCl, 0.7 mM MgSO₄, 1.7 mM NaH₂PO₄, 2.0 mM CaCl₂, 9.5 mM sodium gluconate, 5.5 mM glucose, 7.5 mM sucrose, and 10 mM HEPES at pH 7.4. The mechanical receptive fields of individual units were identified by manually probing the skin with a glass rod. Aδ- and C-fiber mechanonociceptors were identified by their conduction velocity and threshold to mechanical stimulation by von Frey hairs, as described previously. Stock solutions of DAMGO and CTOP were diluted with synthetic interstitial fluid (pH 7.4). 100 μl of oxygen-saturated synthetic interstitial fluid containing 10 μM DAMGO with or without CTOP was applied directly to the corium through a small metal ring (10 mm ID) placed around the receptive field for separation from the tissue bath. Drugs or vehicle were applied 3 min before mechanical stimulation. A computer-controlled nanomotor (Kleindiek, Reutlingen, Germany) was used to apply controlled displacement stimuli of 10 s duration to the receptive field at regular intervals (interstimulus period, 60 s). The probe of the nanomotor was a stainless steel metal rod and the diameter of the flat circular contact area was 0.8 mm. The signal driving the movement of the linear motor and raw electrophysiological data were collected with a Powerlab 4.0 system (AD Instruments, Spechbach, Germany). Spikes were discriminated off-line with the spike histogram extension of the software. All experiments were carried out at an organ bath temperature of 32°C.

Immunofluorescence and Confocal Microscopy

Immunostaining was performed in paw tissue harvested 1 h after intraplantar injection of 0.9% (control) or 10% NaCl. In certain experiments hypertonic saline was injected together with 0.2 mg GM6001. As a positive control, rats received only 150 μl complete Freund’s adjuvant (CFA, as described previously) intraplantarly 2 h before tissue harvesting.

Tissues were fixed in formalin for 2 h at 4°C, embedded in paraffin, and cut into 8-μm-thick cross-sections. Paraffin was then removed by a xylol-ethanol gradient and sections were boiled in 1 mM EDTA or 10 mM citrate buffer solution. To block nonspecific staining, tissues were bathed in phosphate buffered saline containing 5% (vol/vol) goat serum (blocking solution) and 1% bovine serum albumin for 60 min at room temperature. All subsequent washing procedures were performed with this blocking solution. For immunostaining, we used mouse monocolonal anti-occludin...
and anti-claudin-1; rabbit anti-occludin and anti-claudin-5 antibodies (1:50, Clones OC-3F10, 2H10D10, Z-T22, and Z43JK; Invitrogen, San Francisco, CA);19 guinea pig polyclonal anti-CGRP (1:1000, Peninsula Laboratories, Belmont, CA);20,21 and rabbit polyclonal antiprotein gene product 9.5 antibodies (1:100 Dako, Glostrup Denmark)24 diluted in blocking solution. Tissues were incubated for 60 min and, after two washes, with appropriate secondary fluorescent Alexa Fluor 488 and 594 goat antiantibodies for 45 min (1:500 Molecular Probes, Hamburg, Germany; MoBiTec). Sections were mounted in ProTags MountFluor (BiocyTech, Luckenwalde, Germany). Fluorescence images were obtained with a confocal laser scanning microscope (LSM510Meta, Zeiss, Jena, Germany), using excitation wavelengths of 543 and 488 nm. Concentrations and specific staining in control tissues (gut epithelium19,22) of the antibodies have been shown before by our group.19,23 For quantification of immunofluorescent staining, 12 sections of four animals per group were analyzed, respectively. Black and white images of sections under the specific wave length for claudin-1 staining were obtained with a 40× objective. Perineurial cells in an area of 50 × 50 μm of each section were analyzed densitometrically in a double-blind approach. Images were analyzed densitometrically using AIDA (version 3.53, Raytest Isotopenmessgeräte GmbH; Straubenhardt, Germany). Intensity of specific monochromatic signals was expressed as relative intensity/area.

To assess perineurial permeability rats were treated according to Reference 7. Similar to our previous studies,6 8 mg horseradish peroxidase (HRP) was injected at the indicated time-points after intraplantar injection of 10% saline. After 1 h, rats were perfused with 1.25% glutaraldehyde, 1% paraformaldehyde, 5% sucrose in phosphate buffered saline (all Sigma-Aldrich), and the tissue harvested. Transverse sections (30 μm) of subcutaneous tissue were incubated in the dark with 200 mg of diaminobenzidine tetrahydrochloride in 100 ml of cacodylate buffer (0.1 M, pH 5.1) and 1% H2O2 for 5 min, fractionated on sodium dodecyl sulfate polyacrylamide gels, and subsequently blotted onto polyvinylidene fluoride membranes (PerkinElmer, Boston, MA). Proteins were detected using an antibody against claudin-1 (Invitrogen) and β-actin as protein loading control (Sigma-Aldrich).19 A specific band in Western blot in control tissues (gut epithelium19) has been shown before by our group. Chemiluminescence signal detection and quantification were done by densitometry (FluorChem FC2 Imaging systems, Multimage II; Alpha Innotes, Santa Clara, CA).

Statistical Analysis

Data are presented as mean ± SEM. More than two groups in Western blot and immunohistochemical analyses were compared using ANOVA. The post hoc comparisons were performed by Dunnet’s method compared with control. Repeated measurements in electrophysiological studies were compared by repeated measurements ANOVA with Bonferroni post hoc correction. Multiple measurements with two variables, like in pain behavior studies, were analyzed by repeated measurements two way ANOVA with Bonferroni post hoc correlation. Differences were considered significant if \( P < 0.05 \). Sigma Stat program (Systat Software, Erkrah, Germany) was used for statistical analysis.

Results

Local Treatment with Hypertonic Saline Facilitates DAMGO-induced Increase in Nociceptive Thresholds Over Extended Periods

Intraplantar injection of the hydrophilic opioid peptide DAMGO (up to 30 μg) did not change mechanical nociceptive thresholds in paws without inflammation. In contrast, concomitant injection of hypertonic saline permitted a dose-dependent increase in PPT after intraplantar injections of DAMGO (fig. 1A), whereas no difference in baseline PPT was observed (table 1). Contralateral paws were unaffected. The highest dose of DAMGO (30 μg) injected subcutaneously at the animal’s neck did not change mechanical nociceptive thresholds. The injection of hypertonic saline alone did not change these thresholds, either.6 To confirm that the increase in mechanical nociceptive thresholds by DAMGO was dependent on opioid receptors, the selective μ-opioid receptor (MOR) antagonist CTOP was injected concomitantly with DAMGO (30 μg) in rats treated with hypertonic saline intraplantarly (fig. 1B). The effect of DAMGO was fully antagonized by this treatment. To explore the duration of the effect of hypertonic saline, intraplantar DAMGO (30 μg) was injected at different times after 10% saline in separate groups of rats (fig. 1C). PPTs were significantly increased by DAMGO for up to 6 h after a single injection of hypertonic saline. Similar to our previous studies,6 an increase in barrier permeability was observed 10–20 min after
Fig. 1. Long-lasting effects of hypertonic saline solution on DAMGO-induced antinociception. (A) Rats received combined intraplantar injections of different doses of [d-Ala2,N-Me-Phe4,Gly5-ol]enkephalin (DAMGO) and 100 μl of 10% NaCl (green bars) or 0.9% NaCl (red bars). Contralateral paws were shown as blue bars. Mechanical nociceptive thresholds, such as paw pressure threshold (PPT) were determined 10 min after injection in all experiments (n = 6, two-way repeated-measures ANOVA, Bonferroni post hoc correction, *P < 0.05). (B) Baseline nociceptive thresholds were obtained. Subsequently, animals were intraplantarly injected with 30 μg DAMGO and hypertonic saline in the presence (blue circles) or absence (red circles) of a μ-opioid receptor antagonist (2 μg D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂,CTOP) (n = 6, two-way repeated-measures ANOVA, Bonferroni post hoc correction, *P < 0.05). (C) After intraplantar injection of hypertonic saline, five separate groups of rats were reinjected with 30 μg DAMGO intraplantarly at the indicated time-points (n = 6/group, two-way repeated-measures ANOVA, Bonferroni post hoc correction, *P < 0.05). PPT were determined before (baseline, blue circles) and after injection of DAMGO (red squares). The contralateral side is shown for comparison (green triangles). (D) Male Wistar rats (n = 3) were intraplantarly injected with 100 μl 10% NaCl. Control animals did not receive an injection. Staining of horseradish peroxidase of the paw tissue was performed 5–240 min after injection. Intraneural staining was seen at all time points after 10% NaCl (magnification 40×; arrows are pointing at the nerve). A representative example is shown. BL = baseline, TR = treated.
injection of hypertonic saline, as seen by penetration of HRP into the peripheral nerve (fig. 1D).5 No intraneurial staining of HRP was observed under control conditions, in line with our previous findings.6

C- but Not Aδ-Mechanonociceptors Respond to DAMGO after Local Pretreatment with Hypertonic Saline

To support the behavioral data, we studied the electrophysiological effects of hypertonic saline and opioids in in vitro skin nerve preparations (fig. 2).

A total of 81 C-fibers and 32 Aδ-fibers were studied in rats injected with either 0.9% (normal) or 10% (hypertonic) NaCl (saline). The proportion of polymodal C-fibers (heat sensitive), conduction velocities, and mechanical thresholds measured with von Frey hairs were not significantly different among the groups. Using a computer-controlled nanomotor, we applied mechanical stimuli (40–320 μm) to the receptive fields of each single C- and Aδ-fiber nociceptors. The mechanosensitivity of C- and Aδ-fibers from hypertonic saline-injected paws was not significantly different than that from normal saline-injected paws (figs. 2A and 3A). Application of DAMGO did not significantly alter the number of action potentials elicited by incremental mechanical stimuli in C- and Aδ-fiber nociceptors after 0.9% NaCl injections (fig. 2B and 3B). In contrast, DAMGO significantly reduced C-fiber nociceptor discharges in 10% NaCl-injected paws (fig. 2C). This effect was selective for C-fibers, as there was no change in mechanosensitivity in Aδ-fibers under the same conditions (fig. 3C). The effect was fully reversed by coapplication of equimolar concentrations of DAMGO and CTOP (fig. 2D).

Hypertonic Saline Decreases Immunoreactivity of Claudin-1

Tight junction protein composition was first studied in paw tissue from untreated animals (fig. 4). Among several tight junction proteins (claudin-1, occludin, claudin-5) that are expressed in the perineurium,9 we observed the most pronounced changes in claudin-1. Claudin-1 immunoreactivity was observed in epidermis, perineurium, and blood vessels (figs. 4A-C). Occludin immunoreactivity was mostly seen in epidermis and blood vessels (figs. 4B, C). Claudin-5 immunoreactivity was only seen in blood vessels (fig. 4D). Claudin-1 distribution in blood vessels and perineurium was differentiated using staining for CGRP, a specific marker of sensory neurons. Peripheral nerves in the subcutaneous paw tissue expressing CGRP were surrounded by a layer of perineurial cells that were positive for claudin-1 immunoreactivity (fig. 4F). Blood vessels were only positive for claudin-1 immunoreactivity, but not for CGRP (fig. 4E). In the blood vessels, the intima of arterioles (fig. 4E), but less in venules (fig. 4C),

### Table 1. Baseline Mechanical Nociceptive Thresholds before Intraplantar Injection of NaCl and DAMGO

<table>
<thead>
<tr>
<th>NaCl (%)</th>
<th>DAMGO (μg)</th>
<th>PPT (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9</td>
<td>—</td>
<td>71.3 ± 1.4</td>
</tr>
<tr>
<td>0.9</td>
<td>30</td>
<td>73.3 ± 0.9</td>
</tr>
<tr>
<td>10</td>
<td>—</td>
<td>72.7 ± 1.9</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>67.7 ± 1.9</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>75.2 ± 0.8</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
<td>73.8 ± 1.1</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. Two-way repeated measures ANOVA (n = 6/group); P > 0.05.

DAMGO = [d-Ala2,N-Me-Phe4,Gly5-ol]enkephalin; PPT = paw pressure threshold.
Facilitation of Peripheral Opioid Effects by Hypertonic Saline Is Dependent on Metalloproteinases

Intraplantar injection of the metalloproteinase inhibitor GM6001 either 10 min or 1 h (data not shown) before behavioral experiments completely prevented the enhancing effect of hypertonic saline on peripheral DAMGO-induced increase in mechanical nociceptive thresholds (fig. 6). GM6001 injection alone did not change mechanical nociceptive thresholds (PPT: baseline 67.71 ± 1.42 g; treated 74.58 ± 4.7 g, \( P > 0.05, n = 4 \), Student paired \( t \) test). Intraplantar treatment with 10% NaCl + GM6001 restored claudin-1 immunoreactivity compared with its reduction by hypertonic saline alone. Peripheral neurons were labeled with protein gene product 9.5 to confirm immunoreactivity of claudin-1 in peripheral neurons (fig. 7A). Semiquantification of the intensity of the claudin-1 staining by densitometric analysis of the sections confirmed a significant reduction after treatment with hypertonic saline, which was restored by pretreatment with GM6001 (fig. 7B). In claudin-1 Western blot, we confirmed that the antibody bound to a protein with the predicted size of claudin-1, 22 kDa. Hypertonic saline reduced claudin-1 content in the membrane fraction of subcutaneous paw tissue, which was restored with GM6001 pretreatment (figs. 7C, D). Western blot analysis by densitometry revealed a significant reduction (61.2%) after 10% NaCl treatment that was inhibited by GM6001 pretreatment.

Discussion

In this study we demonstrated that a single intraplantar injection of hypertonic saline facilitates the local antinociceptive effect of the hydrophilic MOR-opioid agonist DAMGO on mechanical nociceptive thresholds in noninflamed paw tissue for up to 6 h. The behavioral data are supported by electrophysiological recordings that showed an inhibition of mechanically evoked discharges in C-fibers by DAMGO following exposure to hypertonic saline. Furthermore, our data suggest that hypertonicity opens the perineurial barrier, facilitating access of hydrophilic opioids. Consistent with this notion, we observed a reduced immunoreactivity and protein content of the tight junction protein claudin-1 in the perineurium of peripheral nerves following injection of hypertonic solutions. This hypertonic effect was fully blocked by a metalloproteinase inhibitor in behavioral, immunohistochemical, and Western blot experiments.

In previous experimental and clinical studies, intraplantar injection of hydrophilic opioid agonists into noninflamed tissue did not induce antinociception. However, coinjection of hypertonic mannitol with opioids increased mechanical nociceptive thresholds. Here we demonstrate that intraplantar DAMGO, a hydrophilic peptide and MOR selective agonist, is only able to increase mechanical nociceptive thresholds if injected with or after another hypertonic solution, 10% saline. CTOP, a selective MOR antagonist, completely blocked the effect of DAMGO, suggesting that the analgesic effects are mediated by opioid receptor activa-

---

**Fig. 3.** Opioid agonist does not affect A\(\delta\)-fiber mechanosensitivity independent of preapplication of hypertonic saline. (A–C) Rat paws were injected with 10% NaCl or 0.9% NaCl, as described in fig. 2. Responses for A\(\delta\)-mechanonociceptors to a suprathreshold mechanical stimulus were recorded in rat skin. (A) A\(\delta\)-fiber stimulus-response plots were unchanged in 10% NaCl- compared with 0.9% injected-rat paws (n = 9 in both groups). (B, C) Mechanically evoked responses were not significantly different in A\(\delta\)-fibers treated with [\(d\)-Ala2,\(N\)-Me-Phe4,Gly5-ol]enkephalin (DAMGO) in synthetic interstitial fluid (SIF) (10 \( \mu \)M, blue circles) compared with controls (SIF; red squares) in 0.9% NaCl-injected paws (n = 9, in both groups) (B), and 10% NaCl-injected paws (C) (DAMGO, n = 5; SIF, n = 9) (\( P > 0.05 \), all above repeated-measures ANOVA, Bonferroni post hoc correction). SIF = synthetic interstitial fluid.

was labeled for claudin-1 as previously described. No staining of perineural cells was seen in negative controls stained with secondary antibody only (fig. 4F). Claudin-1 immunoreactivity was substantially reduced in paws treated with hypertonic saline or in the presence of CFA-induced inflammation (fig. 5A). This pattern was also shown in sections double-stained with protein gene product 9.5 antibody to label peripheral neurons (fig. 5B). No change in the occludin immunoreactivity was observed (fig. 5A).
tion. In the present experiments, the effect of hypertonic saline lasted for more than 6 h, whereas the concentration of saline in the tissue normalizes after 15 min,5 pointing toward structural changes of the perineurium to allow access of hydrophilic compounds. Since we used separate groups of rats to analyze the time course of the DAMGO effect, we excluded the possibility that the reduced response after perineurial DAMGO injection was because of reduced MOR function after repetitive activation. We show that the increase in barrier permeability occurs in small nerve bundles in the periphery. Opioid receptors are expressed along nerve trunks27 as well as in nerve terminals28 in peripheral tissue. Since some nerve terminals lack perineurium at their very tips (discussed in6), our data argue that opioid receptors along the axon may play an important role for the generation of antinociceptive effects in noninflamed subcutaneous tissue. However, this has to be examined in future studies.

Fig. 4. Tight junction protein expression in the paw tissue. (A) Paw tissue from untreated rats was obtained and stained for claudin-1 (red) or claudin-5 (red) and occludin (green) as well as diamidinophenylindole (DAPI) for nuclei (blue) (magnification 10×). Different tissue types, such as epidermis (B), and peripheral nerves and blood vessels (C) known to express claudin-1 were identified by morphology based on DAPI nuclear staining within the structure (magnification 40×). (D) Claudin-5 immunoreactivity was observed in blood vessels, but not in the perineurium of the peripheral nerve (arrows) (magnification 40× and 100×, bar = 50 and 10 μm, respectively). (E, F) Paw tissue from normal rats was double-stained for claudin-1 (green) and calcitonin gene-related peptide (red) as well as DAPI for nuclei (blue) to identify sensory nerves in comparison with blood vessels. One representative example of blood vessels (E) and of peripheral nerves (F) is shown (magnification 100×, bar = 10 μm). (F) Negative control by omission of the primary antibody (anti claudin-1 antibody) is shown on the right (magnification 100×, bar = 10 μm). CGRP = calcitonin gene-related peptide; DAPI = diamidinophenylindole.
Several studies in humans and animals have shown that peripheral applications of low doses of opioids induce significant analgesia under inflammatory conditions. In previous electrophysiological studies, single unit activities of afferent C- and Aδ-fibers in normal rats were increased after local injection of glutamate into the receptive field, which was blocked by morphine and inhibited by naloxone. Peripheral inflammation by CFA sensitizes peripheral cutaneous nociceptors to mechanical and heat stimuli. In studies by Wenk et al., morphine directly inhibited mechanically and thermally activated C-fiber and C/Aδ-fiber nociceptors in inflamed skin 18 h after intraplantar injection of CFA. Here we extend these studies and demonstrate that opioids can inhibit nociceptor excitability in C-mechanoreceptors but not in Aδ-mechanoreceptors also in the absence of inflammation, albeit after hypertonic treatment. This is in contrast to the studies mentioned above, possibly because of different experimental setups. A recent report suggested that MOR are only expressed on C-fibers and Aδ-opioid receptors are restricted to Aδ fibers in normal physiologic condi-

![Figure 5](image-url)
duration of the effect, the anatomical difference between injection in our experiments could be because of the short contrast to others.35

Humans.35,36 The lack of hyperalgesia after hypertonic saline injection (i.e., within 5 min) in rats, or muscle pain in humans.35,36 The lack of hyperalgesia after hypertonic saline injection in our experiments could be because of the short duration of the effect, the anatomical difference between intramuscular and subcutaneous (plantar) tissue, and because we performed all our injections under anesthesia, in contrast to others.35

One possible mechanism underlying the facilitation of opioid-induced antinociception in noninflamed tissue by hypertonic saline might be the alteration of MOR function by hypertonicity. In SH-SY5Y cells the potency of DAMGO to inhibit adenyl cyclase was higher at lower concentrations of NaCl with a maximal level at 50 mM NaCl, but no additional effect was seen at higher sodium concentration.38 In Chinese hamster ovary cells transfected with mouse MOR sodium inhibited spontaneous and agonist-occupied MOR-mediated G-protein activation in a manner inversely proportional to the efficacy of the agonist.39 Neither of those studies, however, examined the influence of hypertonicity versus hypotonicity in depth. In our model, the total paw sodium content increased by up to 250% and returned to normal values within 15 min following intraplantar injection of hypertonic saline in vivo.5 Our present study shows that the effect of hypertonic saline on opioid-induced antinociception can last up to 6 h. Together, these data make a direct influence on MOR function less likely and support the interpretation that hypertonic saline may induce long-lasting structural changes of the perineural barrier.

The perineurium resembles the blood-brain barrier. An epithelial origin is suggested because of the expression of epithelial membrane antigen.6,9,40 The perineurium of mixed peripheral nerves (including motor neurons) seems to develop from central nervous system-derived glia.41 The perineurium surrounding the sciatic nerve expresses claudin-1, claudin-5, occludin, VE-cadherin, and connexin43.6,9,40 We identified neurons in subcutaneous tissue by either CGRP or protein gene product 9.5 and found them surrounded by cells with claudin-1 immunoreactivity. We did not observe claudin-5 in the perineurium as shown before in the humans who express claudin-5 in the endoneurial microvessels.42,43 Nerve damage by crush has been shown to result in a loss of tight junction protein content in the perineurium and opening of the perineurium.9 Here, we prove evidence that either inflammation induced by CFA or hypertonic saline results in a loss of claudin-1 immunoreactivity. No change in claudin was observed. Since claudin-1 is a major sealing tight junction protein,10 it is conceivable that its loss is crucial for the access of hydrophilic opioids to the nociceptive neurons. In parallel to claudin-1 expression we demonstrated increased permeability after hypertonic NaCl treatment, as shown before.6,5 The flux of HRP involves both transcellular and paracellular routes but we did not further examine this question because paracellular markers such as fluorescein-labeled dextran produced high background staining in our tissue. However, our approach might be even superior as an indicator of permeability, as direct functional effects were monitored. In inflammation interferon-γ as well as tumor necrosis factor-α can cause a redistribution and internalization of tight junction proteins like claudin-1.44 Consequently, in CFA inflammation the lack of immunoreactivity for claudin-1 could be because of internalization of claudin-1 and/or its degradation.

Several lines of evidence support the notion that hypertonic saline may affect tight junctions. In isolated frog sciatic nerve, the permeability of 14C was enhanced after exposure to hypertonic solution.7 At the blood-brain barrier, osmotic changes change the distribution of occludin, ZO-1, and claudin-5.8 Hyperglycemia reduces ZO-1 and occludin content in rat cerebrovascular cells. The latter finding was accompanied by an increase in plasma MMP activity.

Metalloproteinases form a large group of enzymes secreted as proenzymes.45 Substrates of MMP9 and MMP2 include most of the extracellular matrix components, which are recognized by their secondary structure. Several studies demonstrated that MMPs effectively degrade tight junction molecules such as occludin.11–13 In intestinal epithelial cells,
interferon-\(\gamma\) induces MMP-mediated degradation of claudin-2.\(^{46}\) However, claudin-1 proteolysis has not been examined. \textit{In vivo} changes in the blood-brain barrier that function under pathologic conditions are in part mediated by MMPs.\(^{47}\) For example, in focal ischemia in the rat, MMP activity is increased, and treatment with an MMP inhibitor reversed the degradation of claudin-5 and occludin.\(^{47}\) In addition, it has been shown that hypertonic solutions can increase the activity of MMPs in corneal epithelial cells.\(^{48,49}\) Consistently, we show here that a broad-spectrum MMP inhibitor reversed the effect of hypertonic saline on the accessibility of MOR and on the immunoreactivity of claudin-1 in the perineurium. The particular MMPs involved, as well the exact mechanism of action, needs to be explored in future studies.

In summary, we have shown that hypertonicity facilitates exogenous peripheral opioid actions in noninflamed tissue both in behavioral and electrophysiological experiments. Hypertonic saline also decreases the immunoreactivity of claudin-1 in the perineural barrier. These effects can be fully blocked by an MMP inhibitor. Specifically and reversibly modulating tight junction proteins in the perineurium might also allow controlled access of other substances for treatment.

Fig. 7. Metalloproteinase inhibition blocks hypertonicity-induced decrease of claudin-1 expression. Rat paws were treated with 100 \(\mu\)l hypertonic saline, or with hypertonic saline together with a metalloproteinase inhibitor (0.2 mg GM6001). Tissue was stained for claudin-1 (\textit{red}) and protein gene product 9.5 (\textit{green}) (A). Nuclei were counterstained with diamidinophenylindole (DAPI, \textit{blue}). Representative sections are shown (magnification 40\(\times\); \textit{bar} = 10 \(\mu\)m). (B) The relative intensity of the staining was semiquantified by densitometric analysis of the stained sections (\(n = 12, \*P < 0.05\), ANOVA, \textit{post hoc} Dunnet’s method vs. control). (C) Tissue was analyzed by Western blot. Representative blots are shown. (D) Western blot analysis by densitometry (integrated density value, relative to \(\beta\)-actin) revealed a significant reduction after 10\% NaCl treatment that was inhibited by GM6001 pretreatment (\(n = 4\) or 5/group, \(\*P < 0.05\), ANOVA, \textit{post hoc} Dunnet’s method vs. control). IDV = integrated density value; PGP = protein gene product.
of pain or other diseases. However, studies on long-term safety and toxicity will be needed before clinical application.

References

37. Sánchez EM, Bagüés A, Martín MI: Contributions of peripheral and central opioid receptors to antinociception in rat muscle pain models. Pharmacol Biochem Behav 2010; 96: 488–95


