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# Intrathecal adrenomedullin modulates acute inflammatory pain in the rat formalin test

Yuki Sugimoto[a], Seiji Shiraishi[b], Toshimichi Yasuda[a,*], Hiroshi Hamada[a], Masashi Kawamoto[a]

[a] Department of Anesthesiology and Critical Care, Division of Clinical Medical Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan
[b] Division of Cancer Pathophysiology, National Cancer Center Research Institute, Tokyo, Japan

## HIGHLIGHTS

- This study investigated the role of adrenomedullin (AM) in acute inflammatory pain.
- Cerebrospinal fluid (CSF) levels of AM increased 45 min after formalin injection.
- Intrathecal injection of an AM receptor antagonist (AM22-52) reduced the number of phase 2 flinches in the formalin test.
- The effect of AM22-52 lasted for 4 h or more.
- AM in CSF contributes to the modulation of acute inflammatory pain in the formalin test.

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## ABSTRACT

Adrenomedullin (AM), a member of the calcitonin gene-related peptide (CGRP) family, has been demonstrated to be a pronociceptive mediator. This study was undertaken to investigate the role of AM in acute inflammatory pain induced by formalin injection in rats. Interestingly Cerebrospinal fluid (CSF) levels of AM increased 45 min after formalin injection and a selective AM receptor antagonist, AM22–52, administered intrathecally (i.t.) decreased phase 2 flinching in a dose-dependent manner but not phase 1 flinching during the formalin test. This anti-hyperalgesic effect of i.t. AM22–52 lasted for 4 h or more. AM in the CSF contributes to the modulation of acute inflammatory pain in the formalin test, and blocking downstream signaling effects of the AM receptor has the potential to relieve pain associated with acute inflammation.

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## 1. Introduction

Inflammatory pain, which contributes significantly to both acute and chronic pain, is triggered by various pathological conditions, including injuries, infections, burns, arthritis, operations, and vasoconstriction [10]. Therefore, identifying a new pain mediator on which to base the development of improved therapeutic approaches is an effective means to improve pain management.

The formalin test is a widely used model of acute inflammatory pain for screening novel agents to treat pain [2,14]. Despite the prevalence of the formalin test in pain research, relatively little is known about the mechanisms that underlie the nocifensive behaviors produced by formalin.

Adrenomedullin (AM), a 52-amino acid peptide, belongs to the calcitonin gene-related peptide (CGRP) family and is a well-known potent vasodilator released from vascular smooth muscle and endothelial cells during inflammation [11,17,25,30]. AM induces its biological effects by acting on the calcitonin receptor-like receptor/receptor activity-modifying protein 2 or 3 receptor complex [20]. Recently, AM has been found to be a neuromodulator in nociceptive processing [13]. AM is expressed in nociceptive neurons in the dorsal root ganglion (DRG) and superficial layers of the spinal cord [13]. Intrathecal (i.t.) administration of an AM receptor agonist produces long-lasting heat hyperalgesia in a dose-related manner [13]. Intrathecal administration of AM22–52, an AM receptor antagonist, abolishes hyperalgesia in complete Freund's adjuvant (CFA)-induced chronic inflammatory pain [9]. Moreover, AM blockade reverses morphine tolerance [31]. However, It has been recently reported that AM acts as a nociceptive modulator in spinal reflexes whereas it may have analgesic function at higher cognitive levels [4]. The involvement of AM in pain sensitivity is complex.

* Corresponding author at: 734-8553 1-2-3, Kasumi, Minamiku, Hiroshima, Japan. Tel.: +81 82 257 5267; fax: +81 82 257 5269.
*E-mail address:* toyasu@hiroshima-u.ac.jp (T. Yasuda).

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In the present study, to determine the role of AM in acute nociceptive transmission in the spinal cord, we examined the effects of i.t. AM or AM22–52 on pain induced by formalin in the rat.

## 2. Materials and methods

### 2.1. Animals

Experimental protocols were approved by the Institutional Animal Care Committee of Hiroshima University and are consistent with the guidelines of the Committee on the Ethical Issues of the International Association for the Study of Pain [34].

One hundred thirty-seven adult male Sprague-Dawley rats, initially weighing 165–180 g, were used. They were housed individually at a constant room temperature of 23 ± 2 °C under a 12-h light–dark cycle (lights on at 8:00 am) throughout the study.

### 2.2. Agents

Rat AM, vasopressor fragment of human AM (AM1-25) and human AM antagonist (AM22–52) were purchased from Peptide Institute (Osaka, Japan). Ketamine was from Daiichi-Sankyo (Tokyo, Japan), and xylazine was from Bayer (Tokyo, Japan). Formalin was from Wako Pure Chemical Industries (Osaka, Japan), and sevoflurane was from Maruishi Pharmaceutical (Osaka, Japan).

### 2.3. I.t. catheter implantation

Rats were anesthetized with intraperitoneal injections of ketamine (75 mg/kg) and xylazine (10 mg/kg) prior to surgical implantation of an i.t. catheter. Anesthetized rats were placed in the prone position on a stereotaxic frame and a small incision was made in the back. A polyethylene catheter (Intramedic PE-10, Becton, Dickinson and Company, Franklin Lakes, NJ) was implanted with the tip positioned at the level of the lumbar enlargement [33]. The end of polyethylene catheter was sealed with a steel wire, and the musculature and skin were closed with surgical sutures [16]. To confirm correct placement of the catheter, 10 μl of 2% lidocaine was injected through the exteriorized end of the catheter and transient paralysis of the both hindlimbs was observed.

Two weeks after catheter placement, the rats were subjected to formalin test. Rats exhibiting neurological deficits or fatigue were excluded from further investigation.

### 2.4. Formalin test

The formalin test was carried out according to the protocol of Malmberg and Yaksh [14]. Rats were placed in a Plexiglas chamber and allowed to habituate for at least 20 min. A mirror was placed on the opposite side of the Plexiglas chamber to allow for easy viewing of behavioral responses. Rats were injected with 50 μl of 5% formalin subcutaneously in the dorsal surface of the right hind paw with a 30 G needle.

Immediately after injection, a rat was placed in the Plexiglas chamber and the number of spontaneous flinches or shaking of the injected paw was counted as pain behavior for 60 min. Rats were observed individually and pain behavior was measured for 1 min at 1 min, 5 min, 10 min (Phase 1), and then at 5 min intervals during 15–61 min after formalin injection (Phase 2).

### 2.5. Measurement of cerebrospinal fluid (CSF)

The procedure for CSF collection was carried out as previously described [5]. All the rats were anesthetized with inhaled sevoflurane. The anesthetized rats were placed in the prone position with a small pillow under the cervix to stretch the posterior neck region. The neck of each rat was shaved and disinfected with ethanol. CSF was directly aspirated from the cisterna magna with a 26 G needle attached to a 1 ml syringe (Terumo, Tokyo, Japan) within 2 min (50–150 μl/rat). There were no neurological deficits observed in the rats upon awakening from anesthesia. CSF samples were immediately placed into siliconized micro-tubes and stored in a −80 °C freezer until radioimmunoassay (RIA) was performed. AM-like immunoreactivity was measured using the commercially available AM125I RIA kit (Phoenix Pharmaceuticals, Burlingame, CA), in which 25 μl of CSF was directly mixed with 75 μl of RIA buffer. We did not pass fluids through a Sep-Pak C18 cartridge. The radioactivity of each pellet was counted with a gamma counter (ARC-380, Hitachi Aloka, Tokyo, Japan). Assays were performed in duplicate and the lower detection limit of the assay was 42.5 pg/ml.

### 2.6. Data analysis and statistics

All values were expressed as means ± SEM. Analysis of variance and post hoc Tukey–Kramer tests were used to analyze the effects of AM and AM22-52. The concentration of AM in the CSF was compared with unpaired *t* tests. A *P* value of less than 0.05 was considered statistically significant.

## 3. Results

### 3.1. CSF concentrations of AM

CSF concentrations of CSF before and after the formalin test ($n = 7$ for each group) were compared. CSF was taken from the cisterna magna 45 min after formalin injection. CSF concentrations of AM before and after the formalin test were 23.0 ± 16.8 fmol/ml and 44.0 ± 16.1 fmol/ml, respectively. CSF concentrations of AM significantly increased after formalin injection.

### 3.2. Effect of i.t. administration of an AM antagonist (AM22–52) on flinching behavior after the formalin test

We investigated the effect of intrathecal administration of AM22–52 on flinching behavior after the formalin test. Fig. 1A shows serial changes, and Fig. 1B represents the total number of flinches in each phase ($n = 9$ for each group). Pretreatment with AM22–52 1 h before formalin injection decreased the number of flinches during phase 2 in a dose-dependent manner. However, it did not decrease the number of flinches during phase 1.

Secondly, we observed the serial effect of pretreatment with AM22–52 1 and 3 h before formalin injection on pain behavior ($n = 8$ for each group, Fig. 2A). Both reduced pain behaviors 30 min after formalin injection, with no difference between the two groups. On the other hand, AM1–25 (20 μg), which is a fragment of AM with vasopressor activity [32], had no effect on phase 1 and phase 2 flinching behavior in the formalin test ($n = 8$ for each group, Fig. 2B).

### 3.3. Effect of i.t. AM on pain behavior after the formalin test

We observed the effect of i.t. AM before intraplantar injection of formalin (Fig. 3). AM was administered i.t. 1 h before formalin injection. I.t. AM before formalin injection increased pain behavior 35 min after formalin injection ($n = 8$). However, i.t. administration of AM before intraplantar injection of saline did not produce spontaneous flinches ($n = 5$).

## 4. Discussion

In the present study, we demonstrated that CSF concentrations of AM increased 45 min after intraplantar formalin injection

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