

## Antinociceptive Effects of Intrathecal Landiolol Injection in a Rat Formalin Pain Model

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Perioperative beta-blocker administration has recently been recommended for patients undergoing cardiac or other surgery due to the beneficial cardiovascular effects of these agents. In addition, some studies have reported that perioperatively administered beta-blockers also have analgesic effects. In this study, to investigate the antinociceptive effects and the analgesic profile of landiolol, we examined the effects of intrathecal landiolol administration on nociceptive pain behavior and c-fos mRNA expression (a neural marker of pain) in the spinal cord using a rat formalin model. We found that pain-related behavior was inhibited by intrathecal landiolol administration. Moreover, the increase in c-fos mRNA expression on the formalin-injected side was less pronounced in rats administered landiolol than in saline administered controls. Thus, intrathecal administration of landiolol exhibited anti-nociceptive effects. Further investigation of the antinociceptive mechanism of landiolol is required.

**Key words:** beta-blocker, landiolol, formalin, pain behavior, c-fos

The 2006 American College of Cardiology/American Heart Association's guidelines for perioperative cardiovascular evaluation recommend the use of beta-blockers for patients undergoing vascular surgery and for high-risk patients undergoing non-cardiac surgery [1]. In addition to their cardiovascular effects, these beta-blockers also have anesthetic effects, antinociceptive effects, and effects on the central nervous system. The antinociceptive effects of beta-blockers have already been reported in clinical and experimental studies. Clinical studies have reported that the perioperative use of beta-blockers results in analgesic effects [2, 3]. Furthermore,

basic studies using animal formalin models have indicated that beta-blockers inhibit pain-related behaviors [4, 5]. However, there has been no detailed biological examination of the pain-relieving effects of beta blockers, and the mechanism by which they relieve pain remains unclear.

In this study, we investigated the antinociceptive effects of a short-acting beta-blocker, landiolol, using a rat formalin model. We examined the effects of intrathecal landiolol administration on nociceptive behaviors and on c-fos mRNA expression in the spinal cord.

### Materials and Methods

**Animal models.** The Board of Animal Care and Use Committee of Okayama University approved

this study (OKU-2006103). All efforts were made to minimize animal suffering.

Male Sprague-Dawley rats (CLEA Japan, Tokyo, Japan) weighing 300–350 g were used. The rats were housed individually in cages with a 12-h light/dark cycle. Food and water were available ad libitum.

Rats were anesthetized by sodium pentobarbital (40 mg/kg intraperitoneally). Additional inhalation anesthesia with 1.5–2% isoflurane in 100% oxygen was given as needed. An intrathecal catheter (SP-8; Natsume, Tokyo, Japan) was inserted between the L5 and the L6 spinal vertebrae. Landiolol (Ono Pharmaceutical Co., Ltd.) (500 µg, Group L; n = 5) or the same volume of saline (Group C; n = 5) was administered into the subarachnoid space 1 week after intrathecal insertion of the catheter.

Immediately afterwards, 50 µL of 5% formalin was injected into the left plantar. For behavioral assessment, we recorded the behavior of the animals using a digital camera and the flinch counts were measured by playing back. The numbers of flinches were counted at 1 to 2 min and at 6 to 7 min after injection, and every 5 min thereafter up to 50 min after injection. The researcher who analyzed the behavior responses was blinded to the grouping. The flinch counts of the 2 groups during phase I (0–10 min after formalin injection) and phase II (20–50 min after injection) were compared. After observation of pain-related behavior for 50 min, the rats were sacrificed by decapitation under deep anesthesia with a pentobarbital overdose (60 mg/kg). The L4 and L5 spinal cord segments were rapidly dissected and divided into the left (ipsilateral) and right (contralateral) sides, and these sides were collected. Tissues were incubated in RNAlater (Qiagen, Germantown, MD, USA) and total RNA was extracted from each tissue using an RNeasy Lipid Tissue Mini Kit (Qiagen). This RNA was used for quantification of c-fos mRNA expression using the real-time RT-PCR method.

### Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

cDNA was reverse transcribed from 1 µg of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen). cDNA solutions were diluted 10-fold with DNase-free water. Templates were amplified in a 20-µL reaction mixture containing 10 µL SYBR Premix Ex Taq (Takara-Bio, Otsu, Japan), 4.2 µL DNase-free water, 0.2 µM of forward and reverse primers and 5 µL diluted cDNA solution. The primer sequences used are listed in Table 1. Real-time PCR analysis was performed using a Light Cycler (Roche Diagnostics, Mannheim, Germany) and the following amplification conditions: 95°C for 10 sec followed by 45 cycles of 5 sec at 95°C and 20 sec at 60°C.

To construct a plasmid standard, each target was amplified by RT-PCR with the same primers as used for quantitative real-time PCR, and the PCR product was sub-cloned into pCRII-TOPO (Invitrogen). These plasmids were linearized by endonuclease digestion to avoid super-coiled structures. The concentration of the plasmids was measured by spectrophotometry and this concentration was used to calculate absolute copy numbers of the plasmids. Serial dilutions of each plasmid were amplified and quantified by real-time PCR to generate a standard curve. The absolute copy number of c-fos cDNA in the samples was determined based on a corresponding standard curve. Beta-Actin was used as an internal standard. mRNA expression values are expressed relative to the expression on the contralateral side. PCR specificity was confirmed by sequencing, gel electrophoresis, and melting curve analysis.

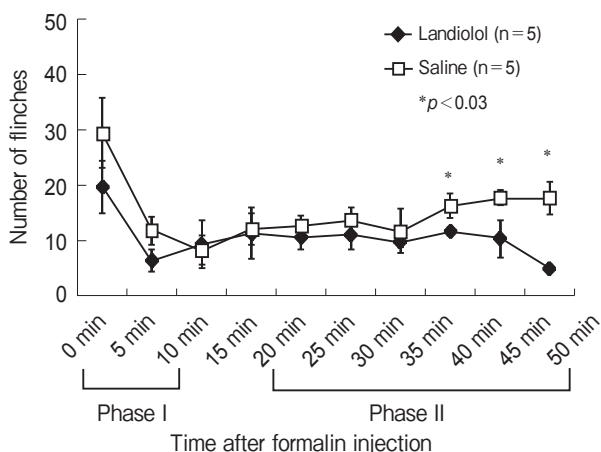
**Statistical analysis.** All the data in this study are presented as means ± standard deviation (SD) and were analyzed using an unpaired *t*-test. *P* < 0.05 was considered significant.

**Table 1** List of primers used for RT-PCR

Gene	Primer Sequence (5'-3')	Amplicon size	Accession No.
c-fos	Forward: ggggacagcctttcctacta Reverse: acggaggagaccagagtggg	185 bp	NM_022197
beta-actin	Forward: ctaaggccaaccgtgaaaag Reverse: accctcatagatgggcacag	170 bp	NM_031144

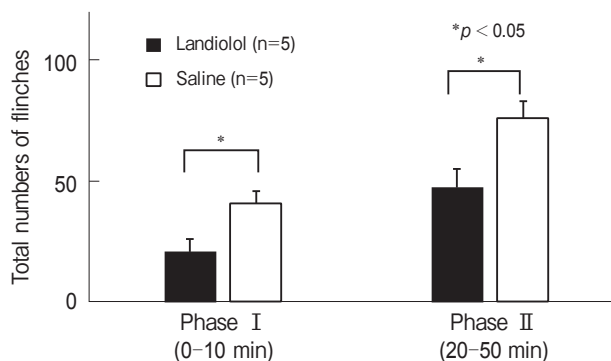
### Results

Injection of 50 $\mu$ L of 5% formalin into the left plantar of rat paws produced a typical biphasic pain behavior, as assessed by the determination of flinch counts, in both the landiolol- and the saline-injected groups of rats. The pain behavior of the 2 groups was then analyzed by comparing their flinch counts. The flinch counts of the 2 groups were compared every 5min and no significant differences were observed for up to 30min. From 35min onwards, there was a significant difference between the 2 groups that persisted to the end of the observation period (Fig. 1). Fig. 2 shows the total number of flinches in phase-I (at 1–2min and 5–6min after formalin injection) and phase-II (at 21–22min and 31–32min, and every 5min up to 50min after formalin injection). In the landiolol group (Group L), both phase-I and phase-II pain-related behavior was inhibited compared with that in the control group (Group C) (total number of flinches, phase-I, Group C vs. Group L:  $38.4 \pm 14.5$  vs.  $26.0 \pm 8.5$ ,  $p < 0.05$ ; phase-II, Group C vs. Group L:  $73.0 \pm 10.5$  vs.  $48.2 \pm 12.2$ ,  $p < 0.05$ ). In both groups, c-fos mRNA expression, as assessed by quantitative RT-PCR, was higher on the formalin-injected side than on the non-formalin-injected side. However, this increase was less marked in Group L

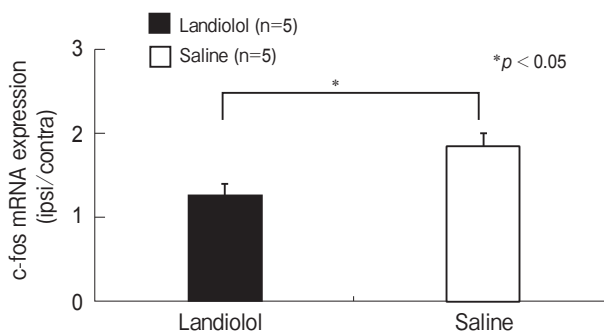


**Fig. 1** Time course of the behavior of landiolol-treated rats in a rat pain model. In the landiolol-treated group of rats, pain-related behavior after formalin injection, as assessed by the number of paw flinches every 5min, was decreased compared with that in the saline-treated group from 35min onwards. \* $p < 0.05$ , landiolol vs. saline group at the indicated time point (n = 5 per group).

than in Group C (c-fos mRNA expression expressed as an ipsilateral: contralateral ratio, Group C vs. Group L:  $1.84 \pm 0.16$  vs.  $1.26 \pm 0.17$ ,  $p < 0.01$ ) (Fig. 3).



**Fig. 2** Phase I and II behaviors -of landiolol-treated rats in a rat pain model. In the landiolol-treated group of rats (Group L), both phase-I and -II pain-related behavior, as assessed by the total number of paw flinches in each phase, was inhibited compared with that of the saline-treated group (Group C). Phase-I : Group C vs. Group L:  $38.4 \pm 14.5$  vs.  $26.0 \pm 8.5$ ,  $p < 0.05$  (n = 5 per group); phase-II: Group C vs. Group L:  $73.0 \pm 10.5$  vs.  $48.2 \pm 12.2$ ,  $p < 0.05$  (n = 5 per group).



**Fig. 3** c-fos mRNA expression in landiolol- and saline-treated rats following formalin injection. In both rat groups, c-fos mRNA expression, as assessed by quantitative RT-PCR at 50 min following formalin injection and expressed as an ipsilateral (ipsi)/contralateral (contra) ratio, was higher on the formalin-injected side than on the non-formalin-injected side. c-fos mRNA expression was decreased in the landiolol-treated group (Group L), compared with that of the saline-treated group (Group C). Group C vs. Group L:  $1.84 \pm 0.16$  vs.  $1.26 \pm 0.17$ ,  $p < 0.01$  (n = 5).

## Discussion

In this study, intrathecal landiolol administration produced antinociceptive effects in the rat formalin model. We also confirmed that *c-fos* mRNA expression in the spinal cord was decreased by administration of landiolol compared to the control. This is the first report in which antinociceptive effects of the beta-blocker landiolol were reported in rats, not only based on behavior assessment but also based on the levels of an objective biological parameter.

The antinociceptive effects of beta-blockers have been reported in previous clinical studies. White *et al.* [2] reported that the postoperative opioid requirement was decreased by the use of esmolol during the intraoperative period. Another report demonstrated that esmolol infusion during hysterectomy decreased patient-controlled intravenous morphine requirements in the postoperative period [3]. In experimental studies, Davidson *et al.* [4] reported that pain-related behaviors decreased with intravenous injection of esmolol in a rat formalin model; however, they did not examine a pain-related biological parameter. Zao *et al.* [5] reported that intrathecal landiolol administration had an antinociceptive effect and decreased the expression of *c-FOS* protein in a mouse formalin model. Although the antinociceptive effects of beta-blockers have been reported in both clinical and experimental studies, the mechanisms that underlie these effects remain unclear.

The formalin test is a widely used pain research model, since the tissue injury and inflammation that result from formalin injection mimic human clinical pain conditions [6]. The formalin test results in 2 phases of rat pain behavior. Subcutaneous injection of diluted formalin into the rat hindpaw produces a biphasic pain behavioral response that consists of an early, acute phase (phase I) and a late, tonic phase (phase II). The early phase starts immediately after formalin injection and lasts for about 10 min. The second phase starts 20 min after formalin injection and lasts for 60 min or longer. During these 2 phases, pain behaviors such as flinching (a brisk raising and shaking), licking or biting of the affected paw are observed, and these behaviors are readily quantifiable.

In our study, the frequency of flinches that were measured over each 5-min interval after formalin injection was not significantly different between the

landiolol- and saline-injected rats until 30 min after formalin injection. However, from 35 min onwards there was a significant difference between the 2 groups, which persisted to the end of the experiment. The total number of flinches of rats that were administered landiolol was inhibited in both phase-I and phase-II compared to the controls. Regarding the pain mechanisms of the formalin test, it is generally accepted that the pain in the first phase is caused by direct peripheral nociceptive stimulation. In contrast, the mechanism that induces pain in the second phase seems to be dependent on the combination of the intensity of the nociceptive stimulus and on an inflammatory reaction in the peripheral tissue [7]. The second phase is thought to reflect ongoing peripheral inflammatory input and the development of central sensitization. In previous studies, it was reported that only high (150 mg/kg/h) doses of esmolol infusion in rats decreased pain-related behaviors in phase-II, whereas low (40 mg/kg/h) or high infusion doses did not affect these behaviors in phase-I [4]. It has also been demonstrated that, during the formalin test, only high-dose (750  $\mu$ g/kg) intrathecal administration of landiolol in mice decreases pain behaviors in both phase-I and phase-II, while intrathecal administration of 250 and 500  $\mu$ g/kg landiolol displays antinociceptive effects only in phase-II [5]. These results, including the results of our study, indicate that landiolol is more effective in phase-II than in phase-I. This finding implies that landiolol provides analgesic efficacy for inflammatory pain rather than for a noxious stimulus.

We measured *c-fos* mRNA as a neural marker in addition to the pain behavior. *C-fos* mRNA and its protein product Fos have been widely used as neural markers in pain research since their use by Hunt *et al.* [8]. *C-fos* is known as one of the immediate early genes (IEGs) which become activated within a short period of time after various stimuli, including formalin injection, and its expression does not require the synthesis of other proteins. Fos, produced by translating *c-fos*, is a transcription factor which promotes the expression of a target gene by binding to an activator protein-1 site along with Jun. Although IEG *c-fos* encodes for the nuclear protein Fos, the decrease of *c-fos* mRNA by intrathecal landiolol injection in this study does not directly indicate pain relief. We demonstrated the change of neural activation by measuring

c-fos mRNA when the landiolol was injected intrathecally in a rat formalin model.

Regarding potential mechanisms of the antinociceptive effects of beta-blockers, it has been considered that beta-blockers might block tetrodotoxin-resistant Na channels [9] or contribute to antinociception via a central site [10]. Furthermore, it has been reported that beta-blockers act on peripheral anti-inflammatory sites [11]. Further studies are required to elucidate the antinociceptive mechanisms of landiolol.

The dose of landiolol used in this study was determined based on the report of Zhao. However, in the end our dose was much higher because we could not observe sufficient analgesic effects with the dose used by Zhao et al. Their research was performed on mice, and we considered that the difference in species was probably responsible for the difference in efficacy. We cannot conjecture as to how these doses translate to clinical usage because, to our knowledge, there have been no reports on intrathecal landiolol administration in human.

In conclusion, we have demonstrated that intrathecal landiolol administration produced analgesic effects in a rat formalin model. We also showed that landiolol inhibits c-fos mRNA expression in the spinal cord. The analgesic effects of landiolol might be exerted via an effect on anti-inflammatory sites.

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