Intrathecal landiolol inhibits nociception and spinal c-Fos expression in the mouse formalin test

[Le landiolol intrathécal inhibe la nociception ainsi que l'expression rachidienne de c-Fos lors du test de formaline chez la souris]

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Purpose: The purpose of this study was to determine if intrathecal landiolol, a β_1 -blocker, can modulate formalin-induced nociception and spinal c-Fos expression in mice, in the absence of anesthesia.

Methods: Thirty-two mice were randomly assigned to one of four groups: the control group ($n = 8$) received intrathecal normal saline 10 μL , while the other three groups ($n = 8$ for each) received intrathecal landiolol at escalating doses of 250 $\mu\text{g}\cdot\text{kg}^{-1}$, 500 $\mu\text{g}\cdot\text{kg}^{-1}$ and 750 $\mu\text{g}\cdot\text{kg}^{-1}$ respectively, immediately after induction of anesthesia with isoflurane. After awakening, inflammatory pain was induced by 10 μL of 5% formalin solution injected into the dorsal surface of the right hind paw. The nociceptive behaviours including licking, biting and lifting of the injected paw were cumulatively recorded as seconds of behaviours/min during phase I (0–10 min) and phase II (10–45 min). The c-Fos protein expressions in the spinal dorsal horn were detected with immunohistochemical techniques in the control and landiolol 750 $\mu\text{g}\cdot\text{kg}^{-1}$ groups.

Results: Compared to the control group, intrathecal injection of landiolol 750 $\mu\text{g}\cdot\text{kg}^{-1}$ significantly decreased pain-related behaviours in phase I, while intrathecal landiolol 250 $\mu\text{g}\cdot\text{kg}^{-1}$, 500 $\mu\text{g}\cdot\text{kg}^{-1}$ and 750 $\mu\text{g}\cdot\text{kg}^{-1}$ significantly decreased pain-related behaviours in phase II during the formalin test. The numbers of c-Fos immunoreactive nuclei in the L5 spinal dorsal horn were significantly lower in the landiolol 750 $\mu\text{g}\cdot\text{kg}^{-1}$ group compared to the control group (landiolol 750 $\mu\text{g}\cdot\text{kg}^{-1}$ 2.4 ± 1.1 vs control 9.2 ± 3.9 ; $P < 0.01$).

Conclusion: The present study indicates that intrathecally administered landiolol produces significant antinociceptive effects in the formalin test. Although further studies exploring the detailed mechanism are needed, these data suggest a potential role of β_1 -adrenoreceptors in spinal nociceptive processing.

Objectif : Le but de cette étude était de déterminer si le landiolol intrathécal, un bloquant β_1 , pouvait moduler la nociception induite par la formaline et l'expression rachidienne de c-Fos chez la souris non anesthésiée.

Méthodes : Après randomisation, trente-deux souris ont été attribuées chacune à l'un des quatre groupes suivants : le groupe témoin ($n = 8$) a reçu 10 μL d'une solution de salin physiologique intrathécal, les trois autres groupes ($n = 8$ chacun) ont reçu du landiolol intrathécal à doses croissantes de 250 $\mu\text{g}\cdot\text{kg}^{-1}$, 500 $\mu\text{g}\cdot\text{kg}^{-1}$ et 750 $\mu\text{g}\cdot\text{kg}^{-1}$ respectivement, immédiatement après l'induction de l'anesthésie avec isoflurane. Après le réveil, une douleur inflammatoire a été provoquée avec 10 μL d'une solution de formaline 5 % injectée dans la surface dorsale de la patte postérieure droite. Les comportements nociceptifs, incluant le léchage, les morsures et l'élévation de la patte injectée ont été enregistrés cumulativement en tant que secondes de comportement / min durant la phase I (0–10 min) et la phase II (10–45 min). Les expressions protéiques de c-Fos dans la corne supérieure dorsale ont été détectées à l'aide de techniques immunohistochimiques dans les groupes témoin et landiolol 750 $\mu\text{g}\cdot\text{kg}^{-1}$.

Résultats : Par rapport au groupe témoin, l'injection intrathécale de landiolol 750 $\mu\text{g}\cdot\text{kg}^{-1}$ a diminué de façon significative les comportements liés à la douleur pendant la phase I, alors que l'administration intrathécale de landiolol 250 $\mu\text{g}\cdot\text{kg}^{-1}$, 500 $\mu\text{g}\cdot\text{kg}^{-1}$ et 750 $\mu\text{g}\cdot\text{kg}^{-1}$ a diminué de façon significative les comportements liés à la douleur dans la phase II durant le test à la formaline. Le nombre de noyaux immuno-réactifs de c-Fos dans la corne supérieure dorsale à L5 était significativement plus bas dans le groupe landiolol 750 $\mu\text{g}\cdot\text{kg}^{-1}$ que dans le groupe témoin (landiolol 750 $\mu\text{g}\cdot\text{kg}^{-1}$ $2,4 \pm 1,1$ vs témoin $9,2 \pm 3,9$; $P < 0,01$).

Conclusion : Cette étude démontre que l'injection intrathécale de landiolol a des effets antinociceptifs significatifs avec le test à la formaline. Bien que d'autres études explorant ce mécanisme soient nécessaires, ces données suggèrent un rôle potentiel des adrénorécepteurs β_1 dans le traitement des stimuli nociceptifs au niveau rachidien.

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SHORT-ACTING β_1 -adrenergic receptor antagonists (β_1 -blockers) have been widely used in clinical anesthesia for their antihypertensive and anti-tachycardia effects. They are effective in blunting adrenergic responses to several perioperative stimuli such as laryngoscopy, intubation and extubation.¹⁻⁴ In addition, the sedative properties of these compounds may decrease anesthetic requirements⁵⁻⁸ and reduce bispectral index, a value reflecting activity of the cerebral cortex and levels of consciousness.³ In recent years, there have been several reports identifying the antinociceptive and analgesic effects of β_1 -blockers in both animal and human studies. For example, Chia *et al.*⁹ reported that intraoperative *iv* infusion of esmolol reduced not only the end-tidal isoflurane concentrations necessary for maintenance of general anesthesia, but also the total morphine consumption in postoperative patient controlled analgesia. Davidson *et al.*¹⁰ also demonstrated that *iv* esmolol reduced pain behaviour in the rat with formalin testing. Several possible mechanisms of β_1 -blockers' nociception have been proposed. However, there has been no direct *in vivo* evidence in animal experimentation showing antinociceptive effects of these drugs.

Landiolol is a newer drug than esmolol which has greater β_1 -adrenoceptor selectivity and inhibition potency.¹¹ As a result of its pharmacological attributes, this study was undertaken to test the hypothesis that intrathecally administered landiolol would reduce pain behaviour and c-Fos expression in the spinal cord of the mouse using the formalin test. The c-Fos protein is a pain-related marker which rapidly expresses neuronal responses to stimulation.^{12,13}

Methods

This protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Yamanashi, Yamanashi, Japan.

Animals

Eight-week-old male Std ddY mice weighing 25–30g were used to eliminate possible sex and age differences in reaction to noxious stimuli. The animals were housed in standard cages (33 × 23 × 12 cm, 2–4 mice/cage) with free access to food and water prior to the experiments. They were maintained under controlled conditions of temperature (23 ± 0.5°C), humidity (50%) and lighting (lights on from 6 a.m. to 6 p.m.).

Drugs

Landiolol hydrochloride (ONO-1101) was obtained from Ono Pharmaceutical Co. (Osaka, Japan) provided

as powder. It was dissolved in 0.9% NaCl and stored at -20°C in a refrigerator before use.

Formalin test and mice behaviour evaluation

The mice formalin test was essentially the same as reported by Hunskaar and Hole.¹⁴ Approximately 30 min before injection, mice were individually placed in another transparent observation cage of the same size for adaptation. Mirrors were placed on sides opposite the observer for easy confirmation of pain-related behaviours. Then, the mice were removed from the chamber to receive anesthesia with 2% isoflurane, followed by intrathecal injection of normal saline 10 μ L or equivalent volumes of landiolol at escalating doses of 250 μ g·kg⁻¹, 500 μ g·kg⁻¹ and 750 μ g·kg⁻¹ respectively. Immediately after intrathecal injection, the mice were awakened and a bolus of 10 μ L of 5% formalin solution was injected into the interplantar dorsal surface of the right hind paw using a micro-syringe (Hamilton co. NV, USA) attached to a 30-G needle. Then, each mouse was returned to the observation cage and the time spent by each animal showing pain-related behaviours was measured by a stopwatch in each five-minute epoch for 45 min after formalin injection. The trained observer was blinded to the intrathecal solutions injected. The nociceptive behaviours consisted of flickering, lifting, biting and licking of the hind paw. The nociceptive responses after formalin injection were documented for two periods: phase I (0–10 min) and phase II (10–45 min).

Intrathecal injection

Intrathecal injections were performed as described by Hylden and Wilcox.¹⁵ The 30-G needle was inserted into the intervertebral space between the L5 and L6 levels of the spinal cord and intrathecal location of the needle tip was confirmed by a characteristic flick of the tail. In the pilot study, a volume of 5 μ L of 2% lidocaine was injected intrathecally into five mice which elicited immediate hind limb paralysis that lasted for about ten minutes.

Tissue preparation and c-Fos immunohistochemistry

The c-Fos immunocytochemical staining method was similar to that described by Kobelt *et al.*¹⁶ with minor modifications. Briefly, one hour after formalin injection, mice in the landiolol 750 μ g·kg⁻¹ and normal saline groups were anesthetized with isoflurane and oxygen and perfused intracardially with 20 mL normal saline followed by 20 mL of 4% paraformaldehyde in phosphate buffered saline (PBS). After the animal's neck and limbs stiffened, the spinal cords were removed and postfixed overnight with

FIGURE 1 Time-course of formalin-induced pain behaviour in mice treated with intrathecal landiolol 0 $\mu\text{g}\cdot\text{kg}^{-1}$ (control), 250 $\mu\text{g}\cdot\text{kg}^{-1}$, 500 $\mu\text{g}\cdot\text{kg}^{-1}$, and 750 $\mu\text{g}\cdot\text{kg}^{-1}$ after dorsal injection of 5% formalin solution 10 μL into the right hind paw. * $P < 0.05$, † $P < 0.01$, *vs* control group.

the same perfusion solution, and then submerged overnight in 4% paraformaldehyde in PBS containing 30% sucrose, before slicing. The lumbar spinal cord was embedded in optimal cutting compound (O.C.T. compound, Sakura Finetechnical, Tokyo, Japan) and cut into 20- μm -thick frozen serial sections. The tissue sections were mounted on MAS-coated glass slides and incubated in 1% w/v sodium borohydride (Wako Pure Chemical Industries, Tokyo, Japan) in PBS for 15 min. Sodium borohydride was used to minimize aldehyde-induced autofluorescence in the tissue. Then sections were incubated for two hours at room temperature in a blocking solution of 10% bovine serum albumin in PBS with 0.3% TritonX100. Sections were incubated 24 hr at room temperature in a primary antibody to c-Fos (1:10,000 rabbit polyclonal antibody to c-Fos; Oncogene Research Product, San Diego, CA, USA). After incubation, tissue sections were rinsed three times with PBS and incubated in a secondary anti-rabbit antibody (1:400) conjugated with fluorescent marker FITC (sc-2012; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for four hours at room temperature. Then, those sections were washed with PBS three times. Finally, as the counterstaining of cell chromatin, sections were incubated in dilute propidium iodide solutions at 2.5 $\mu\text{g}\cdot\text{mL}^{-1}$ (Sigma-Aldrich, St. Louis, MO, USA) with PBS for 15 min, and washed in PBS three times, then immersed with S-2828 anti-fading reagent in glycerol/PBS (Molecular Probes, Eugene, OR, USA) and cover slipped. Sections were observed using a mirror unit U-MWB2 for FITC and

FIGURE 2 Formalin induced pain behaviours during phase I and phase II. Each block represents the mean of eight animals, and the vertical bars indicate the SEM. * $P < 0.05$, † $P < 0.01$, *vs* control group.

U-MWG2 for propidium iodide with a fluorescence microscope (Olympus BX51-FLA, Tokyo, Japan) and photographed with an Olympus DP70 camera. The above antibodies were controlled with staining the NIH 3T3 (RCB1862, Riken bio resource center, Tokyo, Japan) fibroblast cultures stimulated with 20% fetal calf serum in Dulbecco's modified eagle medium after serum starvation according to the method by Vríz *et al.*¹⁷ Omission of the primary antibody was used as a negative control and resulted in no staining.

Counting of c-Fos protein immunoreactive nuclei

For the quantification of the c-Fos protein expression in the spinal cord, we determined the number of c-Fos-immunoreactive (IR) nuclei in the L5 segment of the spinal cord. The amount of c-Fos-IR nuclei in the dorsal horn lamina I and II of L5 spinal cord was counted visibly. Spinal laminae were defined as described by Weng *et al.*¹⁸

Statistical analysis

The behavioural data are presented as means $\pm$ standard error of the mean of *n* experiments. Statistical analysis was carried out using the non-parametric Kruskal-Wallis test followed by Tukey's test to compare accumulated pain behaviours at each time epoch and total pain behaviours during phase I and phase II among the groups. The Student's *t* test was used to compare c-Fos-IR expression of L5 spinal cord in the landiolol 750 $\mu\text{g}\cdot\text{kg}^{-1}$ group and the normal saline group. A *P* value < 0.05 was considered statistically significant.

FIGURE 3A Typical photomicrograph of a 20- μ m thin section of laminae I and II of the L5 dorsal horn of a “control” mouse. Green: FITC-labelled c-Fos protein. Red: propidium iodide (PI), which is marker of chromatin. Yellow: merged images ($\rightarrow$). c-Fos-immunoreactive (IR) nuclei are shown as yellow spots. Many c-Fos-IR nuclei were expressed by formalin injection. (Scale bar = 100 μ m).

FIGURE 3B Typical photomicrograph of a 20- μ m thin section of laminae I and II of the L5 ipsilateral dorsal horn of a representative intrathecal landiolol 750 μ g $\cdot$ kg $^{-1}$ mouse. Green: FITC-labelled c-Fos protein. Red: propidium iodide (PI), which is marker of chromatin. Yellow: merged images ($\rightarrow$). c-Fos-immunoreactive (IR) nuclei are shown as yellow spots. Few c-Fos-IR nuclei were expressed in the ipsilateral dorsal horn. (Scale bar = 100 μ m).

Results

The intraplantar injection of 10 μ L of 5% formalin solution into the dorsal surface of the right hind paw produced a typical biphasic nociceptive response. The first phase started immediately after formalin injection and lasted for about ten minutes, while the second phase was much longer, starting from 15 min after the injection and lasting for about 30 min. There were no significant differences in the frequencies of the first phase nociceptive behaviours among the groups. However, in the second phase, the frequencies of nociceptive behaviours in the landiolol 250 μ g $\cdot$ kg $^{-1}$, 500 μ g $\cdot$ kg $^{-1}$ and 750 μ g $\cdot$ kg $^{-1}$ groups were less than that in the control group (Figure 1). Figure 2 shows the total time of nociceptive behaviours. In the first phase, the total time of nociceptive behaviours was significantly less in the landiolol 750 μ g $\cdot$ kg $^{-1}$ group compared to the control group (landiolol 750 μ g $\cdot$ kg $^{-1}$

group 5.6 $\pm$ 3.9 sec *vs* control group 13.6 $\pm$ 6.7 sec; $P < 0.05$). In the second phase, the total time of nociceptive behaviours was significantly less in the landiolol 500 μ g $\cdot$ kg $^{-1}$ and 750 μ g $\cdot$ kg $^{-1}$ groups than in the control group (landiolol 750 μ g $\cdot$ kg $^{-1}$ group 80.3 $\pm$ 53 sec, landiolol 500 μ g $\cdot$ kg $^{-1}$ group 113.7 $\pm$ 50.6 sec *vs* control group 300 $\pm$ 78.7 sec; $P < 0.05$). Mice that received injection of normal saline instead of formalin into the right hind paw did not show any pain-related behaviours (data not shown). In addition, the landiolol group mice did not show any differences except for the pain-related behaviours compared to the control mice.

To test whether landiolol would reduce expression of c-Fos, a neural marker of pain, in the mice formalin test, *in situ* fluorescence immunohistochemistry was performed in both landiolol 750 μ g $\cdot$ kg $^{-1}$ and control

FIGURE 4 Changes in total numbers of c-Fos-immunoreactive nuclei in laminae I and II of the L5 dorsal horn one hour after 10 μ L 5% formalin solution was injected into the hind paw. Intrathecal landiolol 750 μ g·kg⁻¹ significantly suppressed c-Fos protein expression in the ipsilateral dorsal horn. * $P < 0.01$ *vs* control group.

groups. Typical photomicrographs of IR Fos neurons in the L5 dorsal horn are shown in Figures 3a and 3b, and the total numbers of c-Fos-IR nuclei in the control and landiolol 750 μ g·kg⁻¹ groups are shown in Figure 4. The c-Fos-IR nuclei expression was rarely observed in the dorsal horn of contralateral sides in both the landiolol 750 μ g·kg⁻¹ and control groups. In contrast, on the ipsilateral sides, many c-Fos-IR nuclei were detected predominantly in the superficial laminae I and II of the spinal dorsal horn in the control group, while there were significantly fewer c-Fos-IR nuclei in the landiolol 750 μ g·kg⁻¹ group (landiolol 750 μ g·kg⁻¹ group 2.4 ± 1.1 *vs* control group 9.2 ± 3.9 ; $P < 0.01$).

Discussion

Although there are reports defining the antinociceptive effects of β_1 -blockers, none has specifically documented direct evidence of action. Our study is the first to provide the evidence that the ultra-short-acting β_1 receptor antagonist, landiolol, decreases both the pain-related behaviour and the expression of c-Fos-IR nuclei in the spinal cord of the mouse in response to the formalin test.

Ultra-short-acting β_1 -blockers have been widely used to blunt excessive hemodynamic changes in clinical anesthesia.¹⁻⁴ The advantages of using landiolol in clinical anesthesia include that it is very short-acting

and highly selective for β_1 receptors,¹¹ thus rendering easy titration and less side effects such as asthma or peripheral vasoconstriction compared to other longer-acting β blockers. As landiolol is metabolized very quickly by serum pseudocholinesterase and carboxyesterase in the liver to an inactive metabolite with a short half-life, it is reasonable to assume that most of the intrathecal landiolol will undergo decomposition and excretion before the drug diffuses into the blood and takes systemic pharmacological effect. In fact, according to our observation, the landiolol group mice did not show any differences except for the pain-related behaviours compared to the control mice.

In a typical formalin test, pain-related behaviours are defined by the mouse's behaviour to shake or hold the paw in the air, or to groom, lick or chew the paw repeatedly. There are two phases of pain-related behaviours after formalin injection which involve a complex series of events. The first phase lasts between five to ten minutes and is attributed to direct chemical stimulation of chemosensitive nociceptors.¹⁹ The second phase starts from 15 to 20 min after injection and is believed to be associated with the release of local inflammatory mediators that lead to spinal dorsal horn neuronal sensitization and subsequent activation of the nociceptors.^{14,20} In the present study, landiolol influenced the second phase since it suppressed the second phase response in a dose-dependent manner, although it reduced the first phase behaviours significantly only at the highest dose (750 μ g·kg⁻¹). In addition, as landiolol is ultra-short acting, it may mainly exert antinociceptive effects for the first 30 min even in the higher dose groups.

There have been numerous reports in the literature relating to expression of the immediate-early-gene product c-Fos as a well-established neuronal marker in response to noxious stimulation. Nociceptive reflexes and other pain-related behaviours similarly modify c-Fos expression in the spinal dorsal horn.^{12,13} The noxious stimulus-evoked c-Fos expression is suppressed by administration of analgesic drugs including morphine,^{21,22} indomethacin,²³ and ketoprofen,²⁴ as well as by other drugs that interfere with nociceptive processing at the spinal level such as noradrenaline,²⁵ and N-methyl-D-aspartate receptor antagonists.^{26,27} We observed that c-Fos-IR nuclei were expressed in the spinal dorsal horn of L5 one hour after formalin injection into the interplantar dorsal surface of the right hind paw, and landiolol markedly inhibited the formalin-evoked expression of c-Fos-IR nuclei in the superficial layers of the ipsilateral dorsal horn of the spinal cord. Suppression of spinal c-Fos-IR expression

clearly indicates that landiolol has antinociceptive effects, since peripheral noxious stimulation is mediated initially to neurons in laminae I and II of the dorsal horn.²⁸

What is the mechanism of β_1 -blocker-induced antinociceptive effects? Berridge *et al.*²⁹ have shown that β receptor antagonists may exert their antinociceptive effects by blocking β receptors within the reticular formation. Hageluen *et al.*³⁰ reported that lipophilic β receptor antagonists might inhibit nociceptive signal transduction through activation of inhibitory G protein in the spinal cord. One report indicates that propranolol, esmolol, landiolol and lidocaine blocked tetrodotoxin-resistant Na^+ channels in the rat spinal dorsal root ganglions dose-dependently, but required very high landiolol concentrations to achieve this effect.³¹ Therefore, blocking tetrodotoxin-resistant Na^+ channels might be one mechanism related to the analgesic effects of landiolol. Nevertheless, there is limited experimental evidence to fully explain the antinociceptive effects of landiolol and other short-acting β_1 blockers and the mechanism of analgesic efficacy remains to be elucidated.

In conclusion, we have shown that the ultra-short-acting β_1 receptor antagonist, landiolol, dose-dependently decreases pain-related behaviour in the mouse formalin test. We also demonstrate that landiolol reduces c-Fos-IR expression in the dorsal horn of the spinal cord after formalin injection. Although further studies exploring the complicated mechanisms of analgesic efficacy are warranted, the results of this investigation indicate that landiolol provides antinociceptive effects at the spinal level.

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