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Abstract

Statins have been reported to block inflammatory somatic pain and have an anti-cytokine property. Lipopolysaccharide (LPS) or repeated water avoidance stress (WAS) induces visceral hypersensitivity and increases gut permeability in rats, which are mediated through proinflammatory cytokine-dependent pathways. Since visceral hypersensitivity with increased gut permeability plays a crucial role in the pathophysiology of irritable bowel syndrome (IBS), these above animal models are considered to simulate IBS. We hypothesized that lovastatin improves symptoms in the patients with IBS by attenuating these visceral changes. The threshold of visceromotor response (VMR) induced by colonic balloon distention was measured for the assessment of visceral sensation in rats. Colonic permeability was determined in vivo by quantifying the absorbed Evans blue in colonic tissue for 15 min using a spectrophotometer. Subcutaneously (s.c.) injected LPS (1 mg/kg) reduced the threshold of VMR after 3 h. Pretreatment with lovastatin (20 mg/kg s.c. daily for 3 days) abolished this response by LPS. Repeated WAS (1 h daily for 3 days) induced visceral allodynia, which was also blocked by repeated injection of lovastatin before each stress session. The antinociceptive effect of lovastatin on the LPS-induced allodynia was reversed by mevalonolactone, N^G-nitro-L-arginine methyl ester or naloxone. Lovastatin also blocked the LPS- or repeated WAS-induced increased gut permeability. These results indicate the possibility that lovastatin can be useful for treating IBS.

Key words: lovastatin, visceral pain, gut permeability, lipopolysaccharide, water avoidance stress, irritable bowel syndrome

1. Introduction

Disturbed gut motility and altered visceral sensory function are considered to play an important role in the pathophysiology of irritable bowel syndrome (IBS) (Taché et al., 2009). Additionally, the importance of immune system activation has been also indicated (Bercik et al., 2005; Elsenbruch, 2011). There is evidence that increased levels of plasma proinflammatory cytokines and serum lipopolysaccharide (LPS) together with enhanced gut permeability are observed in IBS (Dlugosz et al., 2015; Ortiz-Lucas et al., 2010; Sinagra et al., 2016; Zhou and Verne, 2011). Moreover, LPS-induced stimulation of cytokines release from peripheral blood mononuclear cells is enhanced, and higher symptoms severity such as urgency, diarrhea, etc. are associated with higher cytokines response induced by LPS (Liebregts et al., 2007).

We previously showed that LPS induced visceral allodynia via interleukin (IL)-1 and IL-6 pathways (Nozu et al., 2017b). Furthermore, repeated water avoidance stress (WAS)-induced visceral allodynia, which is considered to be an experimental animal model for IBS (Larauche et al., 2012), was also mediated via IL-1 and IL-6 pathways, similar to LPS (Nozu et al., 2017c). In this context, LPS-cytokine system is considered to be associated with the altered gastrointestinal functions in IBS, and anti-inflammatory therapy by inhibiting LPS-cytokine signaling may be a promising approach for the treatment of this disease.

Statins inhibit the enzyme 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase (Grundy, 1988), and reduce blood cholesterol level, leading to the prevention of

cardiovascular diseases (Kazi et al., 2017). However, the risk reduction of these diseases is observed even in the absence of a significant decrease of cholesterol level (Oesterle et al., 2017), and pleiotropic effects of statins such as inhibition of monocyte activation, the production of inflammatory cytokines, etc. (Inoue et al., 2000; Methe et al., 2005) could be involved with this phenomenon (Oesterle et al., 2017).

Besides, anti-inflammatory and anti-cytokine actions by statins are also showed in the various animal models, such as inflammatory arthritis (Leung et al., 2003), carrageenan-induced paw edema (Goncalves et al., 2011), among others, and the drugs are also known to suppress cytokine production in intestinal intraepithelial lymphocytes (Zhang et al., 2013) and exhibit antinociceptive action in several animal pain models (Garcia et al., 2011; Santodomingo-Garzon et al., 2006).

Therefore, we hypothesized that statins are beneficial for the treatment of IBS by attenuating visceral hypersensitivity through the anti-cytokine action. In this study, in order to examine the hypothesis, we attempted to determine the effects of lovastatin on visceral allodynia and increased gut permeability induced by LPS or repeated WAS in rats.

2. Materials and Methods

2.1. Animals

Adult male Sprague Dawley rats (Charles River Laboratory, Atsugi, Japan) weighing approximately 300 g were used. The animals were housed in groups (3 - 4

rats/cage) under controlled conditions of illumination (12-h light/dark cycle starting at 7 a.m.), and temperature was regulated at 23 - 25 °C with food (Solid rat chow, Oriental Yeast, Tokyo, Japan) and water available ad libitum.

2.2. Chemicals

LPS obtained from Escherichia coli with the serotype 055:B5 (Sigma-Aldrich, St. Louis, MO, USA); naloxone hydrochloride, an opioid receptor antagonist; N^G-nitro-L-arginine methyl ester (L-NAME), a nitric oxide (NO) synthesis inhibitor (Wako Pure Chemical Industries, Osaka, Japan) and mevalonolactone (Tokyo Chemical Industry, Tokyo, Japan) were dissolved in normal saline. Lovastatin (Tokyo Chemical Industry) was dissolved in dimethyl sulfoxide (Sigma-Aldrich). The chemical doses were determined according to previous studies (Mirhadi, 2011; Nozu et al., 2017a; Nozu et al., 2017b).

2.3. Measuring visceral sensation

Visceral sensation was assessed by colonic distention-induced abdominal muscle contractions (visceromotor response; VMR) using electromyogram (EMG) in conscious rats (Ness and Gebhart, 1988; Nozu et al., 2017b, c).

2.3.1. Implanting electrodes and placing colonic distention balloon

Under brief ether anesthesia, the electrodes (Teflon-coated stainless steel, 0.05-mm diameter, MT Giken, Tokyo, Japan) were inserted approximately 2 mm into the left external oblique musculature though a small skin incision. They were fixed to the musculature by cyanoacrylate instant adhesive together with the incised skin, and the electrode leads were directly externalized through this closed incision. A distension balloon (6-Fr disposable silicon balloon urethral catheter, JU-SB0601; Terumo Corporation, Tokyo, Japan) was intra-anally inserted, with the distal end positioned 2 cm proximal to the anus.

2.3.2. Colonic distention and measuring abdominal muscle contractions

After completing electrode implantation and balloon placement, the rats were placed in Bollmann cages and acclimated to experimental conditions for 30 min before measuring. The electrode leads were then connected to an EMG amplifier, and EMG signals were digitized by a PowerLab system (AD Instruments, Colorado Springs, CO, USA) and recorded by a computer software (LabChart 7; AD Instruments). Colonic distension was performed at 30 min after the surgery, as previously described (Nozu et al., 2017b, c). Namely, the ascending method of limits paradigm with phasic distensions was applied by manually inflating the balloon with water using a syringe, and the distention increased progressively in 0.1 ml steps for 5 sec until significant sustained abdominal muscle contractions, i.e., VMR, were detected (Fig. 1A). The VMR threshold was defined as the distended balloon volume (ml) inducing VMR. The threshold was measured twice (2-min interval), and the threshold mean was calculated as the data of the animals. The

136 percentage change threshold, i.e., the threshold value after drug administration divided by
137 the basal threshold value and multiplied by 100, was also calculated.

138

139 *2.3.3. Experimental procedures*

140 First, the basal VMR threshold was measured. The electrodes and distention balloon
141 were then removed, and either the vehicle or LPS at a 1-mg/kg dose was subcutaneously
142 (s.c.) injected. The rats were returned to their home cages, and after 2.5 h, they underwent
143 surgery for electrode implantation and balloon placement again. The second measurement
144 of threshold was performed 3 h after the injection. The vehicle or lovastatin (5, 20 or 50
145 mg/kg) was s.c. injected thrice at 48 h , 24 h and 30 min before injecting LPS or the vehicle
146 (Fig. 1B).

147 The effect of lovastatin on repeated WAS-induced allodynia was also evaluated.
148 First, the basal threshold was measured, and either lovastatin or the vehicle was injected.
149 Ten min later, either WAS or sham stress was applied for 1 h. These treatments such as
150 drug injection and 1-h daily stress session were implemented for 3 consecutive days. The
151 threshold was again measured at 24 h after undergoing the last stress session. Additionally,
152 the drug injection was also performed at 30 min before the second measurement (Fig. 1C).
153 We previously demonstrated that this repeated WAS protocol successfully induced visceral
154 allodynia in rats (Nozu et al., 2017c).

155 Next to explore the mechanisms of action of lovastatin on LPS-induced allodynia,
156 the effects of mevalonolactone [20 mg/kg intraperitoneally (i.p.)], L-NAME (10 mg/kg i.p.)

157 or naloxone (1 mg/kg s.c.) was examined. These drugs were administered thrice together
158 with lovastatin or the vehicle.

159

160 *2.4. Stress protocol*

161 Exposure to WAS was performed as previously described (Martínez et al., 1997).
162 Rats were individually placed on a plastic platform (height, 8 cm; length, 6 cm; width, 6
163 cm) positioned in the middle of a plastic cage filled with water up to 7 cm of the platform
164 height. Control animals were also placed in the same plastic cage, but the cage was not
165 filled with water (sham stress).

166

167 *2.5. Measuring colonic permeability*

168 Colonic permeability measurement was performed as previously described with
169 minor modifications (Dai et al., 2012; Nozu et al., 2017a). The permeability was
170 determined 5 h after injecting LPS or 24 h after undergoing the last stress session. The
171 anesthetized rats were placed in a supine position on a heating pad, and laparotomy was
172 performed. The colon was ligated at the junction with the cecum, and the small hole was
173 made at the 1 cm from the ileocecal junction by 18 G needle. Later, an open-tipped catheter
174 (3-Fr, 1 mm internal diameter, Atom, Tokyo, Japan) was inserted into the proximal colon
175 though the hole and secured by a ligature. The colon was gently flushed with phosphate-
176 buffered saline (PBS) using a catheter until all stools were washed out. Next, another

ligation was added on the colon at approximately 4 cm from the junction with the cecum, and 1 ml of 1.5 % Evans blue in PBS was instilled into the colon through a catheter. After 15 min, the rats were killed, and the colons were excised and washed with PBS and 1 ml of 6 mM N-acetyl-cysteine. Then, the colons were opened and placed in 2 ml of N,N-dimethylformamide for 12 h. Permeability was calculated by measuring the Evans blue concentration in the supernatant using a spectrophotometer at 610 nm.

2.6. Statistical analysis

Data are expressed as means ± standard error. Multiple comparisons were performed by one- or two-way analysis of variance followed by Tukey's honestly significant difference test. Comparisons between two groups were performed using Student's t- or paired t-test. The SYSTAT 13 software (Systat Software, Chicago, IL, USA) was used for the study.

2.7. Ethical considerations

Approval by the Research and Development and Animal Care Committees at the Asahikawa Medical University (#15132, approved on April 1, 2015) was obtained for all studies.

3. Results

3.1. Lovastatin abolished LPS-induced visceral allodynia

Lovastatin *per se* did not induce any effect on the basal threshold (ml), i.e., before injection of LPS or the vehicle (0.57 ± 0.022 for lovastatin at 50 mg/kg, n = 10 vs. 0.58 ± 0.015 for vehicle, n = 21, $P > 0.05$).

LPS significantly reduced the threshold of VMR, while the vehicle did not alter it (Fig. 2A). Lovastatin (50 mg/kg) *per se* did not modify the threshold, but it blocked the LPS-induced reduced threshold. Lovastatin at 20 mg/kg also abolished the response, but LPS still evoked the nociceptive effect at 5 mg/kg-dose of lovastatin.

After calculating the percentage change threshold, lovastatin reversed the LPS-induced reduced threshold in a dose-responsive manner ($F = 8.6$, $P < 0.05$, Fig. 2B). Lovastatin at 5 mg/kg did not alter the LPS response significantly, while the drug at 20 or 50 mg/kg completely reversed the LPS-induced response. Since 20 mg-dose of lovastatin was enough to abolish the LPS response, this dose of lovastatin was used for the following experiments.

3.2. Lovastatin blocked repeated WAS-induced visceral allodynia

Repeated WAS reduced the threshold significantly, and lovastatin blocked this response without affecting the threshold change in sham-stressed rats (effect of WAS: F =

34.3, $P < 0.05$; effect of lovastatin: $F = 18.1$, $P < 0.05$; interaction between WAS and lovastatin: $F = 13.6$, $P < 0.05$, Fig. 3).

3.3. Mevalonolactone reversed the antinociceptive effect of lovastatin on LPS-induced visceral allodynia

Repeated intraperitoneal injection of mevalonolactone (20 mg/kg, thrice at 48, 24 h and 30 min prior to injection of LPS or the vehicle) did not alter the basal threshold (ml; 0.57 ± 0.020 for mevalonolactone, $n = 10$ vs. 0.57 ± 0.013 for vehicle, $n = 10$; $P > 0.05$). Moreover, it did not alter the response to LPS (effect of mevalonolactone: $F = 0.004$, $P > 0.05$; effect of LPS: $F = 20.7$, $P < 0.05$; interaction between mevalonolactone and LPS: $F = 0.016$, $P > 0.05$; % change 67.2 ± 5.0 for vehicle + LPS, $n = 5$ vs. 68.5 ± 3.3 for mevalonolactone + LPS, $n = 5$; $P > 0.05$).

Next we determined the effect of mevalonolactone on the antinociceptive effect of lovastatin on LPS-induced visceral allodynia, and the drug blocked it (effect of mevalonolactone: $F = 12.2$, $P < 0.05$; effect of lovastatin: $F = 12.3$, $P < 0.05$; interaction between mevalonolactone and lovastatin: $F = 17.3$, $P < 0.05$, Fig. 4).

3.4. L-NAME reversed the antinociceptive effect of lovastatin

L-NAME (10 mg/kg thrice) neither changed the basal threshold (ml; 0.56 ± 0.020 for L-NAME, $n = 11$ vs. 0.56 ± 0.022 for vehicle, $n = 12$; $P > 0.05$) nor the response to LPS

(effect of L-NAME: $F = 0.03$, $P > 0.05$; effect of LPS: $F = 45.9$, $P < 0.05$; interaction between L-NAME and LPS: $F = 0.09$, $P > 0.05$; % change 68.9 ± 5.1 for vehicle + LPS, n = 6 vs. 68.3 ± 6.1 for L-NAME + LPS, n = 5; $P > 0.05$).

Next, we assessed the effect of L-NAME on the antinociceptive effect of lovastatin, and it blocked the effect (effect of L-NAME: $F = 8.39$, $P < 0.05$; effect of lovastatin: $F = 5.86$, $P < 0.05$; interaction of L-NAME and lovastatin: $F = 5.92$, $P < 0.05$, Fig. 5).

3.5. Naloxone reversed the antinociceptive effect of lovastatin

Naloxone (1 mg/kg thrice) did not alter the basal threshold (ml; 0.55 ± 0.032 for naloxone, n = 11 vs. 0.55 ± 0.018 for vehicle, n = 13; $P > 0.05$). Moreover, it did not modify the response to LPS (effect of naloxone: $F = 0.032$, $P > 0.05$; effect of LPS: $F = 63.3$, $P < 0.05$; interaction between naloxone and LPS: $F = 0.069$, $P > 0.05$; % change 69.7 ± 4.6 for vehicle + LPS, n = 7 vs. 68.0 ± 4.1 for naloxone + LPS, n = 6; $P > 0.05$).

In the following experiment, the impact of naloxone on the antinociceptive effect of lovastatin was explored, and naloxone blocked it (effect of naloxone: $F = 10.5$, $P < 0.05$; effect of lovastatin: $F = 11.4$, $P < 0.05$; interaction of naloxone and lovastatin: $F = 11.1$, $P < 0.05$, Fig. 6).

3.6. Lovastatin abolished LPS- or repeated WAS-induced increased colonic permeability

LPS increased colonic permeability and lovastatin blocked this response to LPS without affecting the basal permeability (effect of LPS: $F = 10.1$, $P < 0.05$; effect of lovastatin: $F = 5.95$, $P < 0.05$; interaction of LPS and lovastatin: $F = 8.48$, $P < 0.05$, Fig. 7A).

Additionally, repeated WAS induced increased colonic permeability, and lovastatin abolished this response (effect of WAS: $F = 7.24$, $P < 0.05$; effect of lovastatin: $F = 11.1$, $P < 0.05$; interaction WAS and lovastatin: $F = 11.1$, $P < 0.05$, Fig. 7B).

4. Discussion

Statins exhibit antinociceptive effect on somatic pain animal models (Ghaisas et al., 2010; Santodomingo-Garzon et al., 2006). However, none of the studies has demonstrated this effect on visceral pain. This study clearly showed for the first time that lovastatin abolished visceral allodynia induced by LPS or repeated WAS, which was IL-1 and IL-6-dependent response (Nozu et al., 2017b, c).

Toll-like receptor 4 (TLR4) detects LPS and stimulates nuclear factor-kappa B (NF-κB) pathways resulting in the production of proinflammatory cytokines such as IL-1, IL-6 and tumor necrosis factor-α (Dauphinee and Karsan, 2006). Moreover, WAS elevates the expression of TLR4 in gut (Nebot-Vivinus et al., 2014), and psychological stress activates NF-κB signaling (Topol and Kamyshny, 2013). In this context, LPS- or WAS-induced visceral hypersensitivity is considered to be mediated through TLR4-NF-κB pathways.

Statins inhibit NF-κB activity, thereby reducing the production of proinflammatory cytokines (Ortego et al., 1999). Moreover, the drugs were also demonstrated to decrease TLR4 expression and downstream signaling in human monocytes (Methe et al., 2005). Therefore, lovastatin may inhibit TLR4-NF-κB signaling, leading to blocking visceral hypersensitivity.

We also showed that mevalonolactone reversed the antinociceptive effect, indicating that the action by lovastatin was elicited from specific inhibition of HMG-CoA reductase and affecting the level of mevalonic acid. Previous study showed that the compounds such as isoprenoids arising from mevalonic acid is crucial for the regulation of inflammation-induced production of cytokines (Diomede et al., 2001), which may further support the notion above.

Incidentally, the action of lovastatin was also blocked by L-NAME. It was previously reported that atorvastatin evoked antinociceptive effect on mechanical hypernociception in mouse paws induced by intraplantar injection of LPS, which was blocked by L-NAME but not by selective inhibition of inducible NO synthase (Santodomingo-Garzon et al., 2006). These results suggested that the antinociceptive action by statins was considered to be a NO-dependent response, possibly through activating constitutive NO synthase activity on both visceral and somatic pain. Statins increase endothelial NO production by upregulating endothelial NO synthase (Laufs et al., 1998), through inhibition of isoprenoids production (Laufs, 2003). Besides, it is well known that NO exerts an antinociceptive effect (Chung et al., 2006; Durate et al., 1990), and the

mechanism is thought to be that NO induces cyclic guanosine monophosphate generation to open ATP-sensitive K^+ channels, leading to hyperpolarizing nociceptive neurons (Cury et al., 2011).

This study also showed that the antinociceptive effect of lovastatin was reversed by naloxone, indicating that it was mediated via opioid receptors. Although there is no direct evidence showing that statins activate opioid receptors, several researchers reported that NO stimulated neuronal release of endogenous opioids to stimulate opioid receptors in brain and spinal cord (Cahill et al., 2000; Chung et al., 2006). Therefore, lovastatin may facilitate the production of NO leading to activation of opioid receptors, thereby evoking antinociceptive effect.

There is ample evidence that compromised gut barrier function manifested by increased gut permeability is observed in the patients with IBS (Taché et al., 2009). Repeated WAS or injection of LPS was also demonstrated to increase gut permeability (Bein et al., 2016; Xu et al., 2014). Impaired gut permeability induces bacterial translocation and mucosal inflammation with increased production of proinflammatory cytokines (Moriez et al., 2005). These changes are considered to be an important aspect of pathophysiology of IBS and associated visceral hypersensitivity (Taché et al., 2009).

In the present study, lovastatin inhibited increased colonic permeability induced by LPS or repeated WAS. Sasaki et al. (Sasaki et al., 2003) showed that pravastatin improved gut permeability in dextran-sulfate-induced colitis, which is consistent with our data. Recent studies demonstrated that LPS increased gut permeability through TLR4-dependent

316 pathways (Guo et al., 2015). It is also known that proinflammatory cytokines released by
317 activation of TLR4-NF-κB signaling increase the colonic permeability (Bruewer et al.,
318 2003; Dhawan et al., 2015; Suzuki et al., 2011). Since psychological stress was known to
319 activate TLR4-NF-κB signaling (Nebot-Vivinus et al., 2014; Topol and Kamyshny, 2013),
320 this pathway is considered to contribute to LPS- or WAS-induced increased gut
321 permeability. Therefore, we speculated that lovastatin improved gut permeability by
322 inhibiting TLR4-NF-κB signaling, which may be similar to the mechanism of
323 antinociceptive action.

324 We did not show the direct evidence that lovastatin inhibited the production of
325 cytokines, which was a limitation of the present study. Since the colonic mucosal levels of
326 IL-1β and IL-6 were not significantly elevated in the animal models tested in this study
327 (data were not shown), we could not explore the expected action. In addition, although the
328 antinociceptive effect of lovastatin was blocked by L-NAME, we did not directly show that
329 NO synthesis was increased by the drug. Further studies are needed to determine the
330 precise mechanisms of action in molecular and cellular levels.

331 Despite the above limitations, our results suggest that lovastatin is a promising tool
332 for treating IBS. Since LPS-cytokine system may be involved in the pathophysiology of
333 IBS (Dlugosz et al., 2015; Liebregts et al., 2007; Nozu et al., 2017b, c; Ortiz-Lucas et al.,
334 2010), blocking the system is considered to be novel approach for the treatment. However,
335 biopharmaceutical agents suppressing proinflammatory cytokine cannot be used for the
336 treatment, because they may not have a benefit outweighing their side effects and cost in

the present circumstances. Since statins are some of the most widely prescribed drugs worldwide, their application to IBS treatment seems not to be difficult. Large scale clinical trials to explore the effectiveness of statins in the patients with IBS should be conducted in future.

5. Conclusions

Lovastatin blocked LPS- or repeated WAS-induced visceral hypersensitivity and increased gut permeability in rats. The antinociceptive effect by the drug probably resulted from the inhibition of HMG-CoA reductase, and may be a NO- and opioid receptors-dependent response. Lovastatin may be useful for IBS treatment.

Conflict of interest statement

The authors declare no conflict of interest.

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References

Bein, A., Zilbershtein, A., Golosovsky, M., Davidov, D., Schwartz, B., 2016. LPS induces hyper-permeability of intestinal epithelial cells. J. Cell. Physiol. 232, 381-390.

Bercik, P., Verdu, E.F., Collins, S.M., 2005. Is irritable bowel syndrome a low-grade inflammatory bowel disease? Gastroenterol. Clin. North Am. 34, 235-245.

Bruewer, M., Luegering, A., Kucharzik, T., Parkos, C.A., Madara, J.L., Hopkins, A.M., Nusrat, A., 2003. Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. J. Immunol. 171, 6164-6172.

Cahill, F.J., Ellenberger, E.A., Mueller, J.L., Tseng, L.F., Quock, R.M., 2000. Antagonism of nitrous oxide antinociception in mice by intrathecally administered antisera to endogenous opioid peptides. J. Biomed. Sci. 7, 299-303.

Chung, E., Burke, B., Bieber, A.J., Doss, J.C., Ohgami, Y., Quock, R.M., 2006. Dynorphin-mediated antinociceptive effects of L-arginine and SIN-1 (an NO donor) in mice. Brain Res. Bull. 70, 245-250.

Cury, Y., Picolo, G., Gutierrez, V.P., Ferreira, S.H., 2011. Pain and analgesia: The dual effect of nitric oxide in the nociceptive system. Nitric Oxide 25, 243-254.

Dai, C., Guandalini, S., Zhao, D.H., Jiang, M., 2012. Antinociceptive effect of VSL#3 on visceral hypersensitivity in a rat model of irritable bowel syndrome: a possible action through nitric oxide pathway and enhance barrier function. Mol. Cell. Biochem. 362, 43-53.

Dauphinee, S.M., Karsan, A., 2006. Lipopolysaccharide signaling in endothelial cells. Lab. Invest. 86, 9-22.

Dhawan, S., Hiemstra, I.H., Verseijden, C., Hilbers, F.W., Te Velde, A.A., Willemsen, L.E., Stap, J., den Haan, J.M., de Jonge, W.J., 2015. Cholinergic receptor activation on epithelia protects against cytokine-induced barrier dysfunction. Acta Physiol. (Oxf.) 213, 846-859.

Diomede, L., Albani, D., Sottocorno, M., Donati, M.B., Bianchi, M., Fruscella, P., Salmona, M., 2001. In vivo anti-inflammatory effect of statins is mediated by nonsterol mevalonate products. Arterioscler. Thromb. Vasc. Biol. 21, 1327-1332.

Dlugosz, A., Nowak, P., D'Amato, M., Mohammadian Kermani, G., Nystrom, J., Abdurahman, S., Lindberg, G., 2015. Increased serum levels of lipopolysaccharide and antiflagellin antibodies in patients with diarrhea-predominant irritable bowel syndrome. Neurogastroenterol. Motil. 27, 1747-1754.

Durate, I.D., Lorenzetti, B.B., Ferreira, S.H., 1990. Peripheral analgesia and activation of the nitric oxide-cyclic GMP pathway. Eur. J. Pharmacol. 186, 289-293.

Elsenbruch, S., 2011. Abdominal pain in irritable bowel syndrome: a review of putative psychological, neural and neuro-immune mechanisms. Brain. Behav. Immun. 25, 386-394.

Garcia, G.G., Miranda, H.F., Noriega, V., Sierralta, F., Olavarria, L., Zepeda, R.J., Prieto, J.C., 2011. Antinociception induced by atorvastatin in different pain models. Pharmacol. Biochem. Behav. 100, 125-129.

399 Ghaisas, M.M., Dandawate, P.R., Zawar, S.A., Ahire, Y.S., Gandhi, S.P., 2010.
400 Antioxidant, antinociceptive and anti-inflammatory activities of atorvastatin and
401 rosuvastatin in various experimental models. Inflammopharmacology 18, 169-177.
402 Goncalves, D.O., Calou, I.B., Siqueira, R.P., Lopes, A.A., Leal, L.K., Brito, G.A., Tome,
403 A.R., Viana, G.S., 2011. In vivo and in vitro anti-inflammatory and anti-nociceptive
404 activities of lovastatin in rodents. Braz. J. Med. Biol. Res. 44, 173-181.
405 Grundy, S.M., 1988. HMG-CoA reductase inhibitors for treatment of hypercholesterolemia.
406 N. Engl. J. Med. 319, 24-33.
407 Guo, S., Nighot, M., Al-Sadi, R., Alhmoud, T., Nighot, P., Ma, T.Y., 2015.
408 Lipopolysaccharide Regulation of Intestinal Tight Junction Permeability Is Mediated
409 by TLR4 Signal Transduction Pathway Activation of FAK and MyD88. J. Immunol.
410 195, 4999-5010.
411 Inoue, I., Goto, S., Mizotani, K., Awata, T., Mastunaga, T., Kawai, S., Nakajima, T.,
412 Hokari, S., Komoda, T., Katayama, S., 2000. Lipophilic HMG-CoA reductase inhibitor
413 has an anti-inflammatory effect: reduction of MRNA levels for interleukin-1beta,
414 interleukin-6, cyclooxygenase-2, and p22phox by regulation of peroxisome
415 proliferator-activated receptor alpha (PPARalpha) in primary endothelial cells. Life
416 Sci. 67, 863-876.
417 Kazi, D.S., Penko, J.M., Bibbins-Domingo, K., 2017. Statins for Primary Prevention of
418 Cardiovascular Disease: Review of Evidence and Recommendations for Clinical
419 Practice. Med. Clin. North Am. 101, 689-699.

420 Larauche, M., Mulak, A., Taché, Y., 2012. Stress and visceral pain: from animal models to
421 clinical therapies. Exp. Neurol. 233, 49-67.
422 Laufs, U., 2003. Beyond lipid-lowering: effects of statins on endothelial nitric oxide. Eur. J.
423 Clin. Pharmacol. 58, 719-731.
424 Laufs, U., La Fata, V., Plutzky, J., Liao, J.K., 1998. Upregulation of endothelial nitric oxide
425 synthase by HMG CoA reductase inhibitors. Circulation 97, 1129-1135.
426 Leung, B.P., Sattar, N., Crilly, A., Prach, M., McCarey, D.W., Payne, H., Madhok, R.,
427 Campbell, C., Gracie, J.A., Liew, F.Y., McInnes, I.B., 2003. A novel anti-
428 inflammatory role for simvastatin in inflammatory arthritis. J. Immunol. 170, 1524-
429 1530.
430 Liebregts, T., Adam, B., Bredack, C., Roth, A., Heinzel, S., Lester, S., Downie-Doyle, S.,
431 Smith, E., Drew, P., Talley, N.J., Holtmann, G., 2007. Immune activation in patients
432 with irritable bowel syndrome. Gastroenterology 132, 913-920.
433 Martínez, V., Rivier, J., Wang, L., Taché, Y., 1997. Central injection of a new
434 corticotropin-releasing factor (CRF) antagonist, astressin, blocks CRF- and stress-
435 related alterations of gastric and colonic motor function. J. Pharmacol. Exp. Ther. 280,
436 754-760.
437 Methe, H., Kim, J.O., Kofler, S., Nabauer, M., Weis, M., 2005. Statins decrease Toll-like
438 receptor 4 expression and downstream signaling in human CD14+ monocytes.
439 Arterioscler. Thromb. Vasc. Biol. 25, 1439-1445.

Mirhadi, K., 2011. Effect of intraperitoneally injection of different doses of lovastatin on pain and inflammatory response induced by formalin in mice. Am J Animal Vet Sci 6, 160-165.

Moriez, R., Salvador-Cartier, C., Theodorou, V., Fioramonti, J., Eutamene, H., Bueno, L., 2005. Myosin light chain kinase is involved in lipopolysaccharide-induced disruption of colonic epithelial barrier and bacterial translocation in rats. Am. J. Pathol. 167, 1071-1079.

Nebot-Vivinus, M., Harkat, C., Bzioueche, H., Cartier, C., Plichon-Dainese, R., Moussa, L., Eutamene, H., Pishvaie, D., Holowacz, S., Seyrig, C., Piche, T., Theodorou, V., 2014. Multispecies probiotic protects gut barrier function in experimental models. World J. Gastroenterol. 20, 6832-6843.

Ness, T.J., Gebhart, G.F., 1988. Colorectal distension as a noxious visceral stimulus: physiologic and pharmacologic characterization of pseudaffective reflexes in the rat. Brain Res. 450, 153-169.

Nozu, T., Miyagishi, S., Kumei, S., Nozu, R., Takakusaki, K., Okumura, T., 2017a. A glucagon-like peptide-1 analog, liraglutide improves visceral sensation and gut permeability in rats. J. Gastroenterol. Hepatol. doi 10.1111/jgh.13808.

Nozu, T., Miyagishi, S., Nozu, R., Takakusaki, K., Okumura, T., 2017b. Lipopolysaccharide induces visceral hypersensitivity: role of interleukin-1, interleukin-6, and peripheral corticotropin-releasing factor in rats. J. Gastroenterol. 52, 72-80.

460 Nozu, T., Miyagishi, S., Nozu, R., Takakusaki, K., Okumura, T., 2017c. Repeated water
461 avoidance stress induces visceral hypersensitivity; role of IL-1, IL-6 and peripheral
462 corticotropin-releasing factor. J. Gastroenterol. Hepatol. doi 10.1111/jgh.13787.
463 Oesterle, A., Laufs, U., Liao, J.K., 2017. Pleiotropic Effects of Statins on the
464 Cardiovascular System. Circ. Res. 120, 229-243.
465 Ortego, M., Bustos, C., Hernandez-Presa, M.A., Tunon, J., Diaz, C., Hernandez, G., Egido,
466 J., 1999. Atorvastatin reduces NF-kappaB activation and chemokine expression in
467 vascular smooth muscle cells and mononuclear cells. Atherosclerosis 147, 253-261.
468 Ortiz-Lucas, M., Saz-Peiro, P., Sebastian-Domingo, J.J., 2010. Irritable bowel syndrome
469 immune hypothesis. Part two: the role of cytokines. Rev. Esp. Enferm. Dig. 102, 711-
470 717.
471 Santodomingo-Garzon, T., Cunha, T.M., Verri, W.A., Jr., Valerio, D.A., Parada, C.A.,
472 Poole, S., Ferreira, S.H., Cunha, F.Q., 2006. Atorvastatin inhibits inflammatory
473 hypernociception. Br. J. Pharmacol. 149, 14-22.
474 Sasaki, M., Bharwani, S., Jordan, P., Joh, T., Manas, K., Warren, A., Harada, H., Carter, P.,
475 Elrod, J.W., Wolcott, M., Grisham, M.B., Alexander, J.S., 2003. The 3-hydroxy-3-
476 methylglutaryl-CoA reductase inhibitor pravastatin reduces disease activity and
477 inflammation in dextran-sulfate induced colitis. J. Pharmacol. Exp. Ther. 305, 78-85.
478 Sinagra, E., Pompei, G., Tomasello, G., Cappello, F., Morreale, G.C., Amvrosiadis, G.,
479 Rossi, F., Lo Monte, A.I., Rizzo, A.G., Raimondo, D., 2016. Inflammation in irritable
480 bowel syndrome: Myth or new treatment target? World J. Gastroenterol. 22, 2242-
481 2255.

Suzuki, T., Yoshinaga, N., Tanabe, S., 2011. Interleukin-6 (IL-6) regulates claudin-2 expression and tight junction permeability in intestinal epithelium. J. Biol. Chem. 286, 31263-31271.

Taché, Y., Kiank, C., Stengel, A., 2009. A role for corticotropin-releasing factor in functional gastrointestinal disorders. Curr Gastroenterol Rep 11, 270-277.

Topol, I., Kamyshny, A., 2013. Study of expression of TLR2, TLR4 and transckription factor NF-kB structures of galt of rats in the conditions of the chronic social stress and modulation of structure of intestinal microflora. Georgian Med. News, 115-122.

Xu, D., Gao, J., Gillilland, M.r., Wu, X., Song, I., Kao, J.Y., Owyang, C., 2014. Rifaximin alters intestinal bacteria and prevents stress-induced gut inflammation and visceral hyperalgesia in rats. Gastroenterology 146, 484-496 e484.

Zhang, J., Osawa, S., Takayanagi, Y., Ikuma, M., Yamada, T., Sugimoto, M., Furuta, T., Miyajima, H., Sugimoto, K., 2013. Statins directly suppress cytokine production in murine intraepithelial lymphocytes. Cytokine 61, 540-545.

Zhou, Q., Verne, G.N., 2011. New insights into visceral hypersensitivity--clinical implications in IBS. Nat. Rev. Gastroenterol. Hepatol. 8, 349-355.

Figure legends

Figure 1

A The threshold of visceromotor response (VMR) was determined by the distended balloon volume (ml) inducing apparent sustained abdominal muscle contractions. Demonstrable EMG recording is depicted. The threshold was 0.4 ml in this animal. **B** Schematic representation of the experimental protocol. The basal VMR threshold was measured at 30 min after the surgery for implanting EMG electrodes and placing the balloon, and LPS (1 mg/kg) or the vehicle was administered. Later, the surgery and balloon placement were performed again, and the threshold was measured at 3 h after the injection. Lovastatin (5, 20 or 50 mg/kg) or the vehicle was injected thrice at 48 h, 24 h and 30 min before injection of LPS. **C** The basal threshold was measured, and the rats were subjected to either water avoidance or sham stress for 1 h daily for 3 consecutive days. The second threshold measurement was performed at 24 h after the last stress session. Lovastatin or the vehicle was injected 4 times, i.e., at 10 min before each stress session and 30 min before the second measurement.

Figure 2

A Effect of lovastatin (Lova) on LPS-induced visceral allodynia. LPS significantly reduced the threshold of visceromotor response (VMR), and lovastatin at 20 and 50 mg/kg abolished this response. Lovastatin *per se* did not alter the threshold. * $P < 0.05$ vs. basal threshold by paired t-test. **B** Percentage change threshold of VMR was significantly

522 reduced in the vehicle + LPS, and lovastatin dose-dependently reversed this response by
523 LPS. * $P < 0.05$ vs. vehicle + vehicle, # $P < 0.05$ vs. vehicle + LPS by one-way analysis of
524 variance followed by Tukey's honestly significant difference test. Each column represents
525 the mean ± standard error. The number of rats examined is shown in parentheses.

526

527 Figure 3

528 Effect of lovastatin (Lova) on repeated water avoidance stress (WAS)-induced visceral
529 allodynia. Repeated WAS significantly reduced the threshold, and lovastatin abolished this
530 response. Sham; sham stress. * $P < 0.05$ vs. vehicle + sham, # $P < 0.05$ vs. vehicle + WAS
531 by two-way analysis of variance followed by Tukey's honestly significant difference test.
532 Each column represents the mean ± standard error. The number of rats examined is shown
533 in parentheses.

534

535 Figure 4

536 Mevalonolactone reversed the antinociceptive effect of lovastatin (Lova) on LPS-induced
537 visceral allodynia. * $P < 0.05$ vs. vehicle + vehicle + LPS, # $P < 0.05$ vs. vehicle + Lova +
538 LPS by two-way analysis of variance followed by Tukey's honestly significant difference
539 test. Each column represents the mean ± standard error. The number of rats examined is
540 shown in parentheses.

541

Figure 5

L-NAME abolished the antinociceptive effect of lovastatin (Lova) on LPS-induced visceral allodynia. * $P < 0.05$ vs. vehicle + vehicle + LPS, # $P < 0.05$ vs. vehicle + Lova + LPS by two-way analysis of variance followed by Tukey's honestly significant difference test. Each column represents the mean ± standard error. The number of rats examined is shown in parentheses.

Figure 6

Naloxone blocked the antinociceptive effect by lovastatin (Lova) on LPS-induced visceral allodynia. * $P < 0.05$ vs. vehicle + vehicle + LPS, # $P < 0.05$ vs. vehicle + Lova + LPS by two-way analysis of variance followed by Tukey's honestly significant difference test. Each column represents the mean ± standard error. The number of rats examined is shown in parentheses.

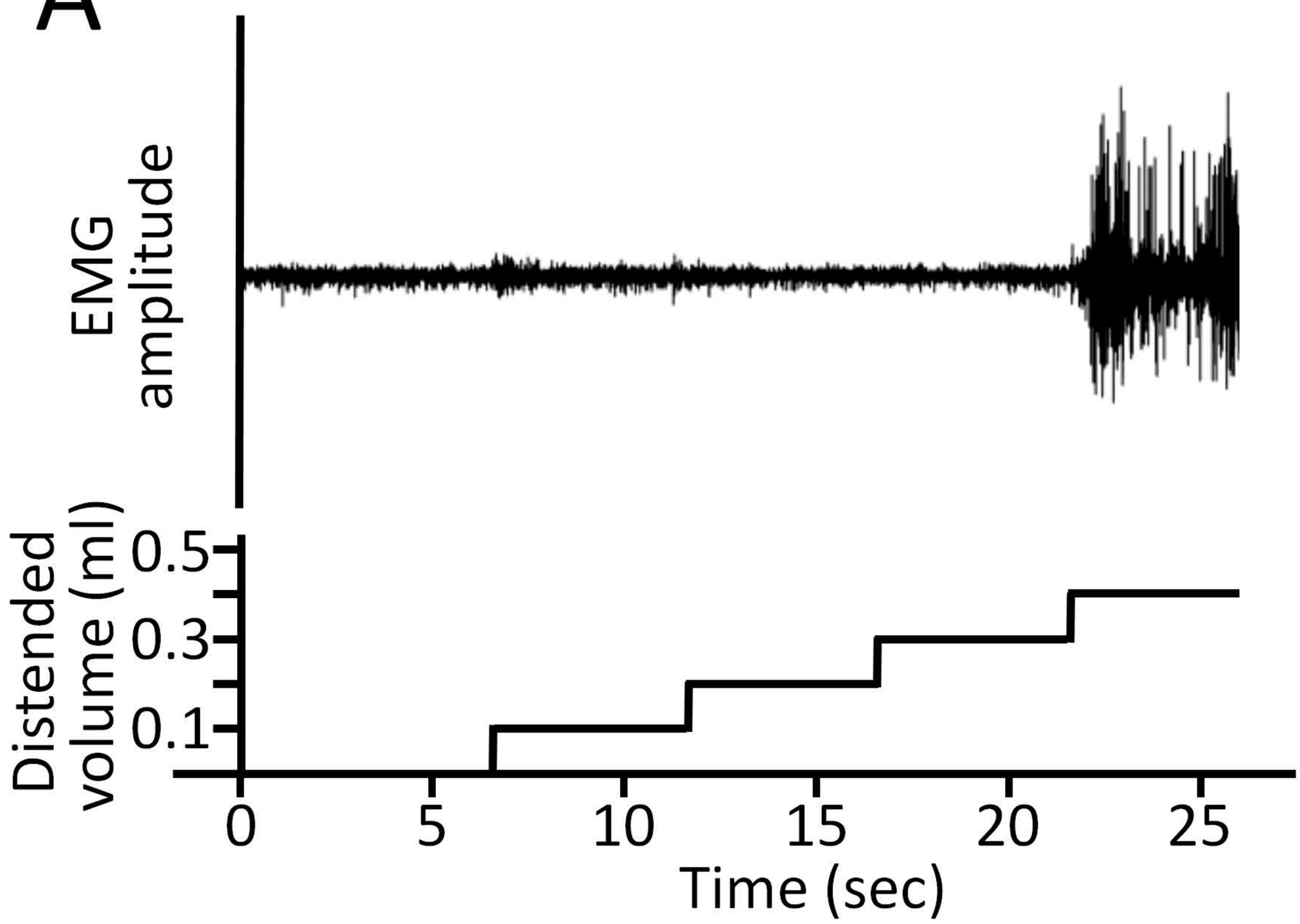
Figure 7

Effect of lovastatin (Lova) on colonic permeability. **A** LPS increased the permeability, which was blocked by lovastatin. **B** Repeated water avoidance stress (WAS) increased the permeability, and lovastatin abolished this response. Sham; sham stress. * $P < 0.05$ vs. vehicle + vehicle or vehicle + sham, # $P < 0.05$ vs. vehicle + LPS or vehicle + WAS by two-way analysis of variance followed by Tukey's honestly significant difference test. Each

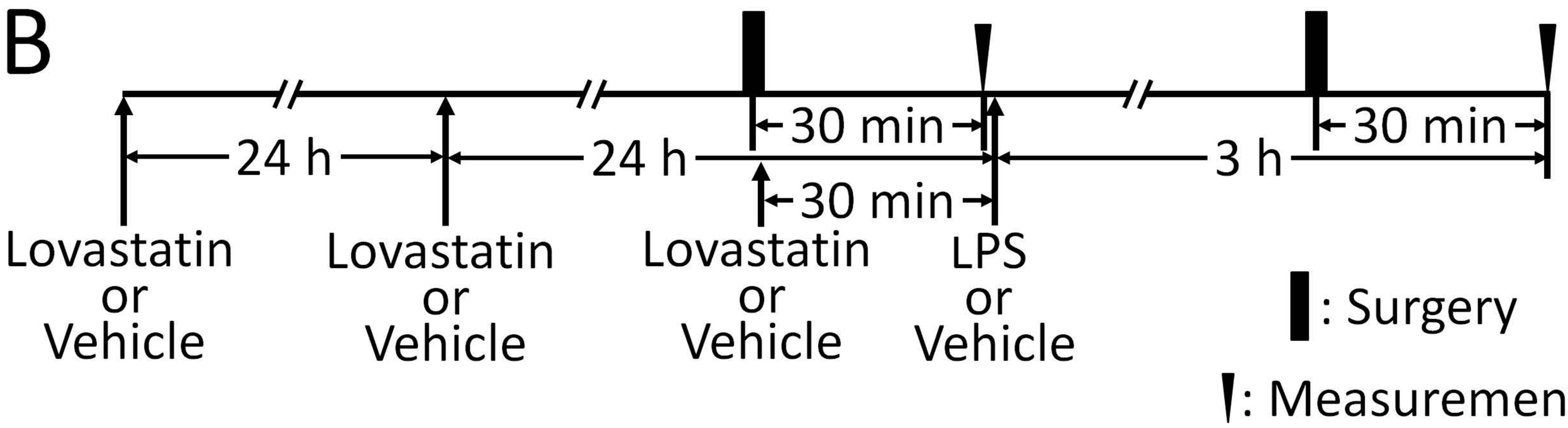
562 column represents the mean ± standard error. The number of rats examined is shown in

563 parentheses.

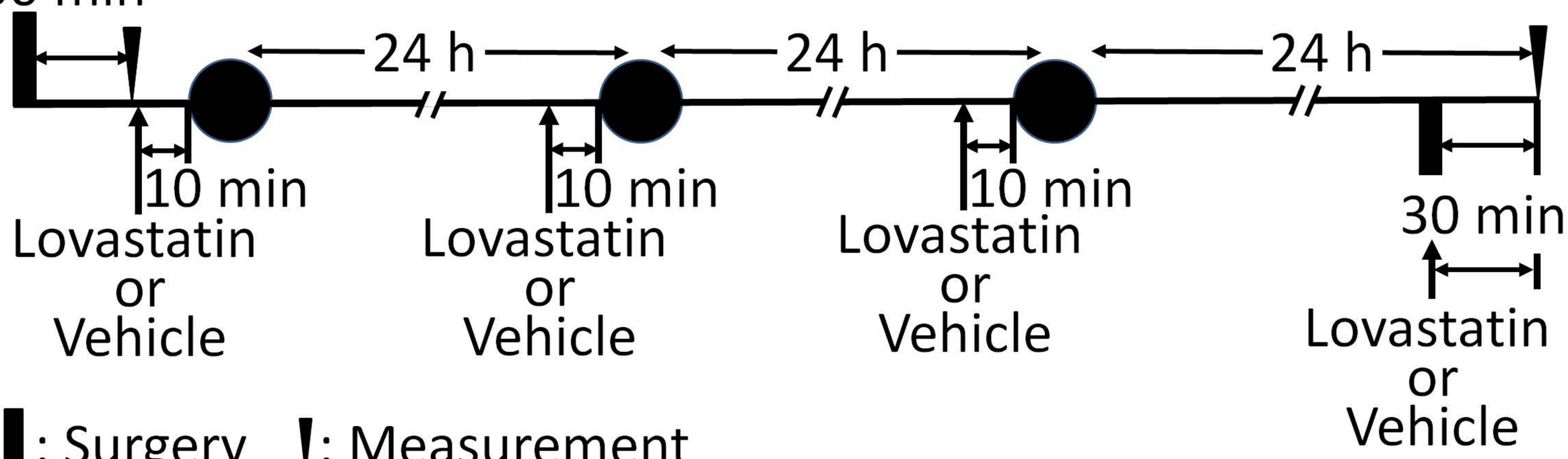
A


EMG amplitude
Distended volume (ml)
0.5
0.3
0.1
0
5
10
15
20
25
Time (sec)

B


24 h
24 h
30 min
3 h
30 min
30 min
Lovastatin or Vehicle
Lovastatin or Vehicle
Lovastatin or Vehicle
LPS or Vehicle
: Surgery
: Measurement

C


30 min
24 h
24 h
24 h
10 min
10 min
10 min
30 min
Lovastatin or Vehicle
Lovastatin or Vehicle
Lovastatin or Vehicle
Lovastatin or Vehicle
: Surgery
: Measurement
: Water avoidance or sham stress

A

Basal

After vehicle injection

After LPS injection

VMR threshold (ml)

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0

(5) (5) (16) (6) (6) (5)

* *

Vehicle Lova 50 Vehicle Lova 5 Lova 20 Lova 50

B

% change threshold

140
120
100
80
60
40
20
0

(5) (5) (16) (6) (6) (5)

* # #

Vehicle + Vehicle

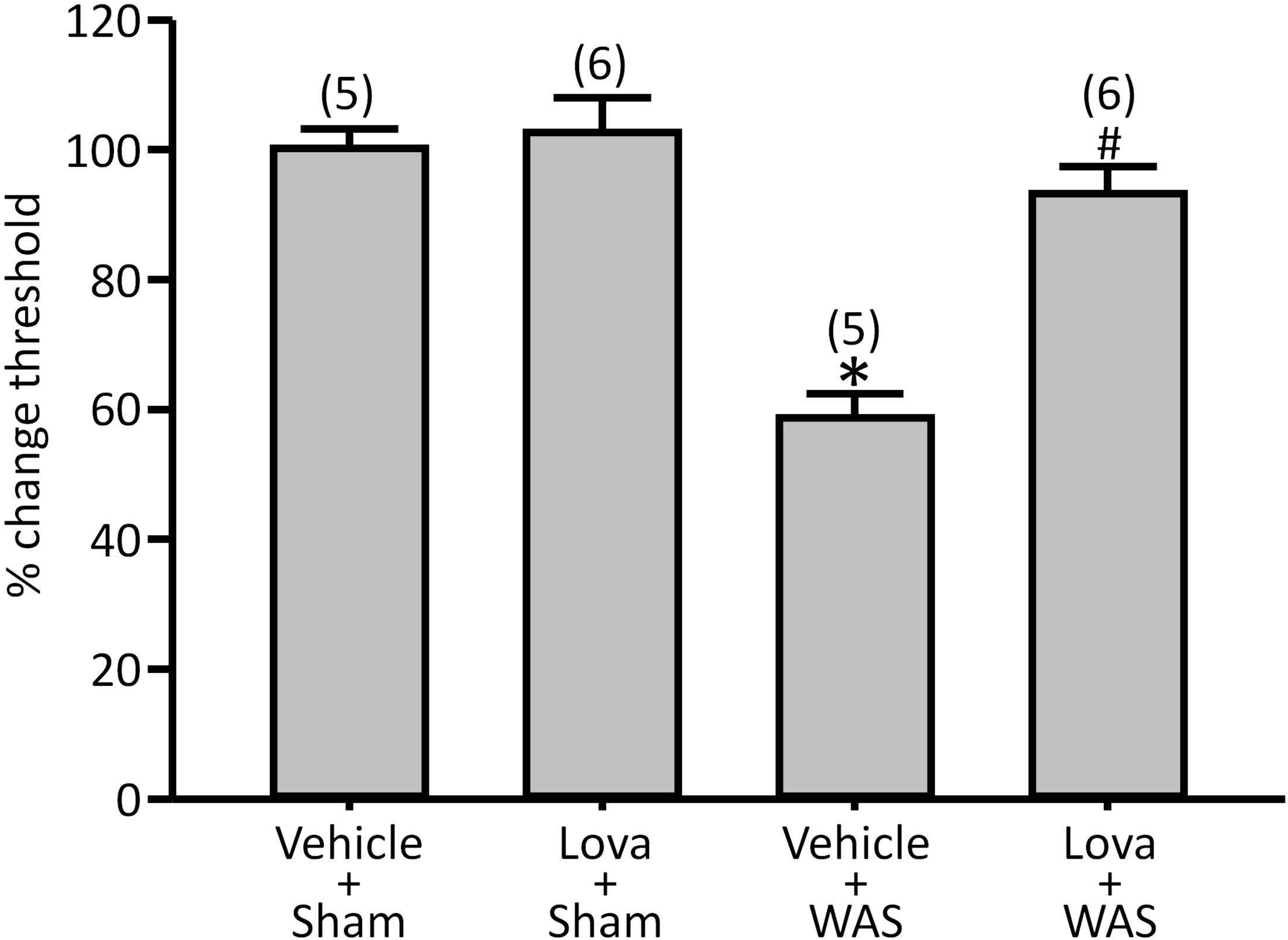
Lova 50 + Vehicle

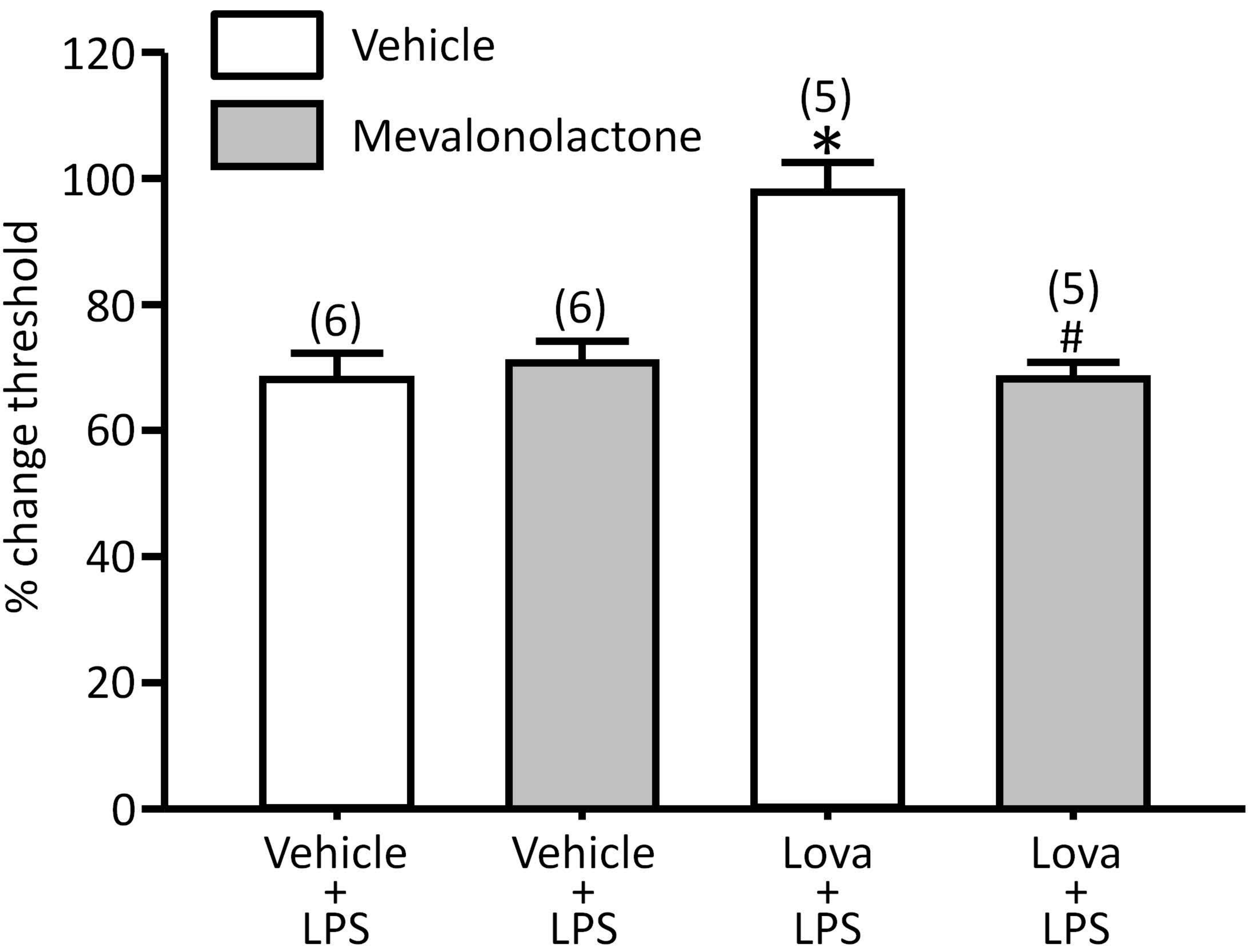
Vehicle + LPS

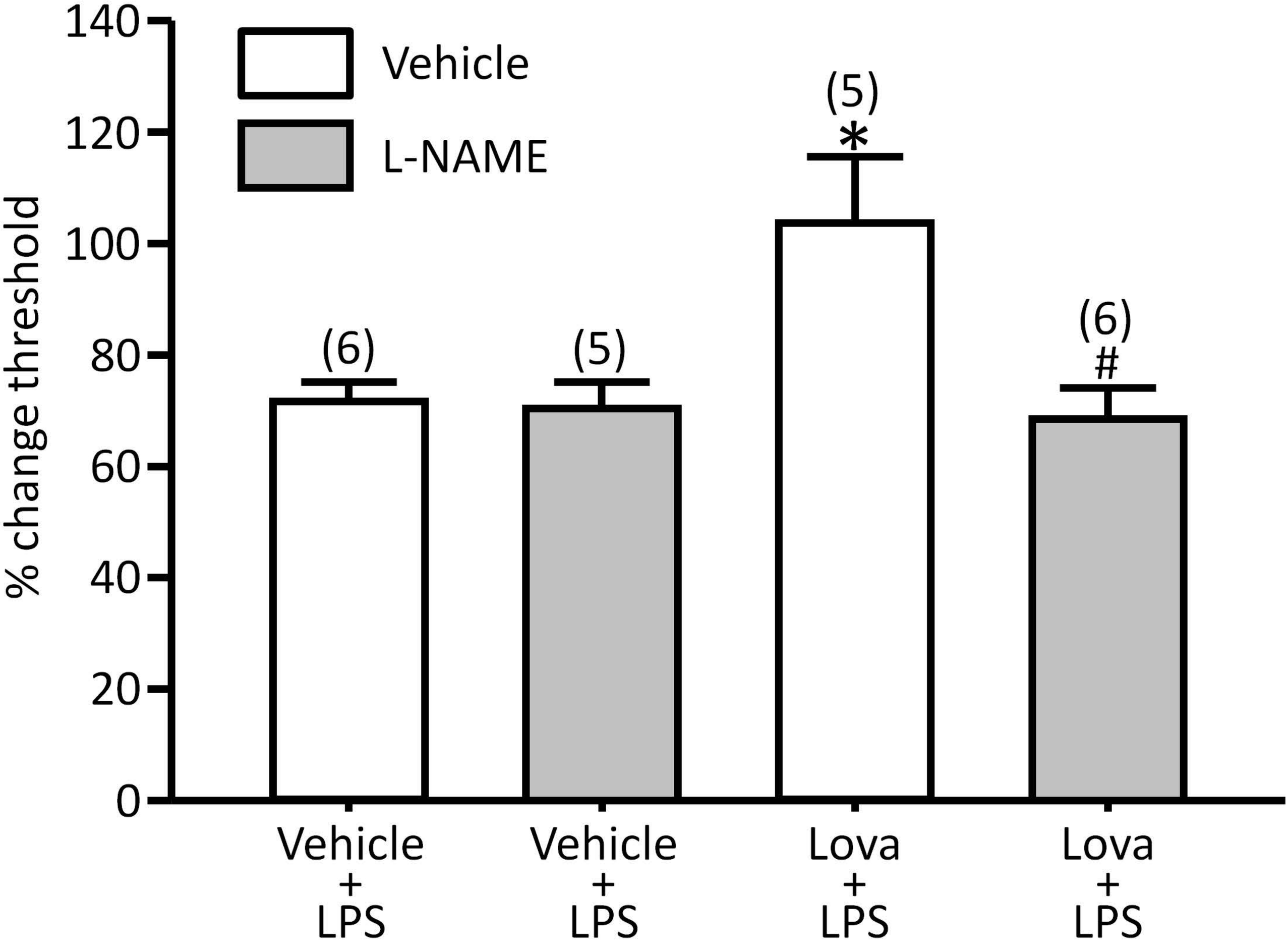
Lova 5 + LPS

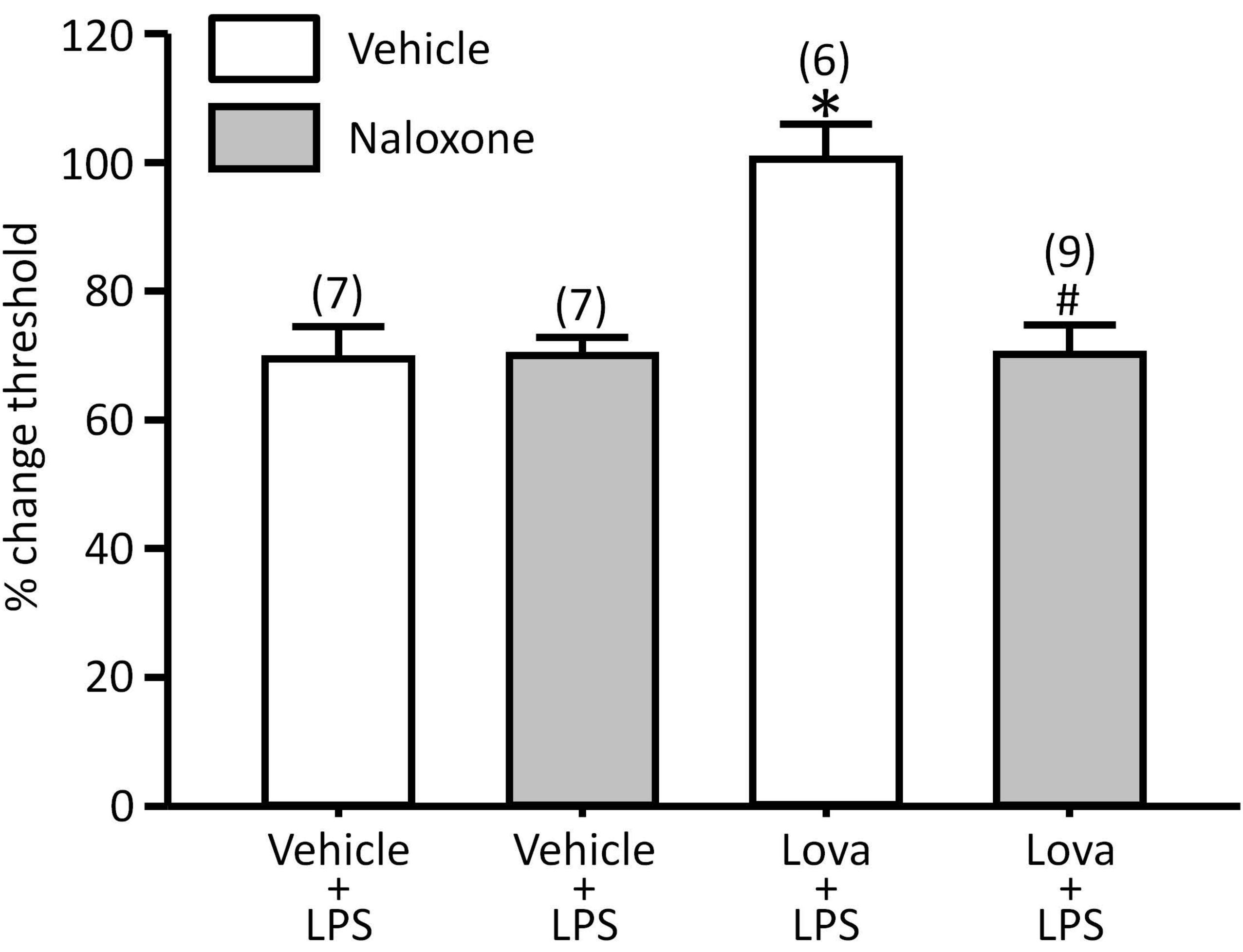
Lova 20 + LPS

Lova 50 + LPS


120
100
80
60
40
20
0
% change threshold
(5)
(6)
(5)
*
(6)
#
Vehicle + Sham
Lova + Sham
Vehicle + WAS
Lova + WAS


Vehicle
Mevalonolactone
% change threshold
120
100
80
60
40
20
0
(6)
(6)
(5)
*
(5)
#
Vehicle + LPS
Vehicle + LPS
Lova + LPS
Lova + LPS


Vehicle
L-NAME
% change threshold
140
120
100
80
60
40
20
0
(6)
(5)
(5)
*
(6)
#
Vehicle + LPS
Vehicle + LPS
Lova + LPS
Lova + LPS


Vehicle
Naloxone
% change threshold
120
100
80
60
40
20
0
(7)
(7)
(6)
*
(9)
#
Vehicle + LPS
Vehicle + LPS
Lova + LPS
Lova + LPS

A

Colonic permeability (µg/g tissue)

60
50
40
30
20
10
0

(5)
(5)
(6)
*
(6)
#

Vehicle + Vehicle
Lova + Vehicle
Vehicle + LPS
Lova + LPS

B

Colonic permeability (µg/g tissue)

80
60
40
20
0

(6)
(6)
(5)
*
(6)
#

Vehicle + Sham
Lova + Sham
Vehicle + WAS
Lova + WAS