Reduced spinal microglial activation and neuropathic pain after nerve injury in mice lacking all three nitric oxide synthases

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Abstract

Background: Several studies have investigated the involvement of nitric oxide (NO) in acute and chronic pain using mice lacking a single NO synthase (NOS) gene among the three isoforms: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). However, the precise role of NOS/NO in pain states remains to be determined owing to the substantial compensatory interactions among the NOS isoforms. Therefore, in this study, we used mice lacking all three NOS genes (n/i/eNOS−/− mice) and investigated the behavioral phenotypes in a series of acute and chronic pain assays.

Results: In a model of tissue injury-induced pain, evoked by intraplantar injection of formalin, both iNOS−/− and n/i/eNOS−/− mice exhibited attenuations of pain behaviors in the second phase compared with that in wild-type mice. In a model of neuropathic pain, nerve injury-induced behavioral and cellular responses (tactile allodynia, spinal microglial activation and Src-family kinase phosphorylation) were reduced in n/i/eNOS−/− but not iNOS−/− mice. Tactile allodynia after nerve injury was improved by acute pharmacological inhibition of all NOSs and nNOS. Furthermore, in MG-5 cells (a microglial cell-line), interferon-γ enhanced NOSs and Mac-1 mRNA expression, and the Mac-1 mRNA increase was suppressed by L-NAME co-treatment. Conversely, the NO donor, sodium nitroprusside, markedly increased mRNA expression of Mac-1, interleukin-6, toll-like receptor 4 and P2X4 receptor.

Conclusions: Our results provide evidence that the NOS/NO pathway contributes to behavioral pain responses evoked by tissue injury and nerve injury. In particular, nNOS may be important for spinal microglial activation and tactile allodynia after nerve injury.

Background

Acute pain can act as an early warning device that alerts us to the presence of damaging stimuli, e.g. trauma, chemical irritation and thermal stimuli. This pain usually goes away soon after the noxious stimulus is removed. In contrast, chronic pain persists for a long time, and is categorized into inflammatory pain and neuropathic pain. Neuropathic pain, resulting from peripheral nerve injury, is characterized by spontaneous pain, hyperalgesia and allodynia [1]. Allodynia is nearly always resistant to known treatments such as non-steroidal anti-inflammatory drugs (NSAIDs) or even opioids. After nerve injury, spinal microglia that are the main immunocompetent cells in the central nervous system transform into an activated form. Reactive microglia change their morphology and number, and express several molecules such as interleukin (IL)-1β, IL-6, interferon-γ (IFN-γ), toll-like receptor 4 (TLR4), P2X4 receptor and nitric oxide synthases (NOSs) [2-6]. Therefore, glial cells, especially microglia, have received much attention as a new therapeutic target for the treatment of neuropathic pain. However, the mechanism of neuropathic pain is not fully understood, and there are currently no effective remedies.

Nitric oxide (NO) is a free radical that produces a variety of biological actions under physiological and pathological conditions [7-9] and is synthesized by three isoforms of NOS: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) [10-12]. Previous
studies have shown the involvement of NOSs and NO in acute and chronic pain using pharmacological procedures or mice lacking a single NOS gene among the three isoforms. For instance, intraperitoneal administration of NO precursor or a NO donor potentiates thermal hyperalgesia and tactile allodynia in neuropathic rats [13]. A NOS inhibitor effectively improves tactile allodynia by tight ligation of the fifth and sixth lumbar spinal nerves in rats [14]. Moreover, nNOS-/ mice fail to display nerve injury-induced tactile hypersensitivity [15]. In contrast, iNOS-/ mice do not affect nerve injury-induced thermal allodynia [16]. However, because there is a mechanism to compensate for the quantity of NO in NOSs [17-19], the precise role of endogenous NO still remains to be determined.

In this study, we sought to investigate the role of the NOS/NO pathway in models of acute and chronic pain using mice lacking the iNOS gene (iNOS-/- mice) and mice lacking all three NOS genes (nNOS, iNOS and eNOS triply-NOS-/- mice: n/i/eNOS-/- mice) [20]. Here, we demonstrate that endogenous NO is a critical player for inducing microglial activation in the spinal cord and in pain hypersensitivity after peripheral nerve injury. Furthermore, by using an MG-5 microglial cell-line, we show that NO induces microglial activation. Taken together, inhibition of NOS/NO signaling may be a viable therapeutic strategy for treating neuropathic pain.

Results
Effect of NOS gene knockout on tissue injury- and chemical-induced pain and acute mechanical and thermal pain behavior responses
First, to examine the role of NOSs in physiological pain, we assessed the acute and chronic pain responses following injection of formalin into the hindpaw and intraperitoneal injection with acetic acid. After formalin injection, wild-type (WT) mice displayed biphasic licking and biting behaviors: the first acute pain phase started immediately after injection and lasted for 5 min, and the second persistent pain phase lasted for 60 min (see Figure 1A). In contrast to WT mice, the magnitude of biphasic behaviors was significantly suppressed in n/i/eNOS-/- mice (Figure 2A). Here, we demonstrate that endogenous NO is a critical player for inducing microglial activation in the spinal cord and in pain hypersensitivity after peripheral nerve injury. Furthermore, by using an MG-5 microglial cell-line, we show that NO induces microglial activation. Taken together, inhibition of NOS/NO signaling may be a viable therapeutic strategy for treating neuropathic pain.

Effect of NOS gene knockout on nerve injury-induced tactile allodynia and spinal microglial activation
In WT mice, withdrawal threshold of the hindpaw ipsilateral to the nerve injury markedly decreased (Figure 2A). While iNOS-/- mice displayed a similar pain response to WT mice after nerve injury, the nerve injury-induced decrease in PWT was significantly suppressed in n/i/eNOS-/- mice when compared with WT mice (day 1, 3, 5 and 10, P < 0.01; day 7 and 14, P < 0.001 vs. WT mice; Figure 2A). In the contralateral hindpaw, both iNOS-/- and n/i/eNOS-/- mice had a similar PWT to that in WT mice (Figure 2B).

Spinal microglia are known to become activated after nerve injury and play an important role in subsequent pain hypersensitivity [2,21,22]. To examine the role of NOSs in microglial activation, we performed immunostaining for ionized calcium binding adaptor molecule 1 (Iba1), a marker of microglia, in the spinal dorsal horn. On day 14 after nerve injury, Iba1 immunoreactivity was markedly enhanced in the ipsilateral dorsal horn in WT mice as compared with that in the contralateral dorsal horn. In the ipsilateral dorsal horn, the changes in morphology of microglial cells after nerve injury (hypertrophy of cell body and retraction of processes) were observed in WT mice and in iNOS-/- mice but were much less in n/i/eNOS-/- mice (Figure 2C). The numbers of ipsilateral Iba1+ microglial cells after nerve injury increased in all three types of mice, but the increase in n/i/eNOS-/- mice was significantly less when compared with those in WT mice (P < 0.01) and in iNOS-/- mice (P < 0.05; Figure 2D). Neither morphology nor number of Iba1+ cells were altered in any three lines of mice in the contralateral dorsal horn (Figure 2C, D).

Suppression in activation of Src-family kinases and expression of IL-6 and TLR4 mRNA after spinal nerve injury in n/i/eNOS-/- mice
Src-family kinases (SFKs) have been reported to be activated in microglia in the spinal dorsal horn after nerve
injury and implicated in neuropathic pain [23]. Increased immunoreactivity of phosphorylated-SFKs (p-SFKs), an active form, in the ipsilateral dorsal horn of WT mice was observed on day 14 after spinal nerve injury. Nerve injury-induced SFK phosphorylation was markedly suppressed in n/i/eNOS−/− but not iNOS−/− mice (Figure 3A, B).

Moreover, expression of inflammatory cytokines and plasma membrane receptors, such as IL-6, IL-1β, TNF-α and TLR4, has been reported to be upregulated in the spinal cord after nerve injury [3-5]. Interestingly, the increase in IL-6 and TLR4 mRNA levels, but not IL-1β and TNF-α (data not shown), were significantly reduced in n/i/eNOS−/− mice (P < 0.05 both vs. WT mice; Figure 3C, D). We have now examined the levels of P2X4R mRNA expression in the spinal cord on day 14 after nerve injury by a real-time PCR analysis. In WT mice, P2X4R mRNA level was increased in 1.61 ± 0.51-fold in the ipsilateral spinal cord as compared with that in the contralateral side. Similarly, 1.61 ± 0.46-fold increase of P2X4R mRNA was observed in iNOS−/− mice. The upregulation of P2X4R mRNA expression was tendency to be suppressed in n/i/eNOS−/− mice (0.77 ± 0.42-fold increase; P = 0.282 vs. WT mice, P = 0.247 vs. iNOS−/− mice, n = 4).

Pharmacological inhibition of nNOS suppresses tactile alldynia after spinal nerve injury

We tested the effects of NOS inhibitors on the development and maintenance of nerve injury-induced tactile
allodynia. Intraperitoneal administration of L-N_{G}^{G}\text{-nitro-arginine methyl ester (L-NAME), a non-specific NOS inhibitor, to rats once daily for 7 days, significantly suppressed the decrease in ipsilateral PWT after nerve injury in a dose-dependent manner without an effect on the contralateral hindpaw (day 1, 3 and 7, P < 0.001; day 5, P < 0.01 100 mg/kg L-NAME vs. vehicle group in ipsilateral; Figure 4A, B). Furthermore, acute intraperitoneal administration of L-NAME and acute intrathecal administration of 7-nitroindazole, a specific nNOS inhibitor, reversed tactile allodynia on day 7 after nerve injury (P < 0.05-0.001 both vs. vehicle group; Figure 4C, D).

IFN-\gamma activated in microglia via NOS/NO
IFN-\gamma levels are known to increase in the spinal cord after spinal nerve injury, and are implicated in microglial activation and tactile allodynia [3,24]. To investigate the
involvement of NOS/NO signaling in IFN-\(\gamma\)-induced microglial activation, we examined the effect of IFN-\(\gamma\) on microglial transcriptional alterations using MG-5 cells, a mouse microglial cell-line [25]. IFN-\(\gamma\) induced a significant increase in the expression of iNOS and nNOS mRNA in a dose-dependent manner in MG-5 cells (39.3 ± 5.0-fold of iNOS and 5.32 ± 0.61-fold of nNOS mRNA in 100 U/mL IFN-\(\gamma\) as compared with non-treatment; \(P < 0.001\) both; Figure 5A, B). Additionally, in IFN-\(\gamma\)-stimulated MG-5 cells, Mac-1 mRNA expression levels, which are known to be upregulated in activated microglia, were significantly increased by 1.50 ± 0.16- and 1.61 ± 0.17-fold as compared with non-treated MG-5 cells (\(P < 0.01\); Figure 5C). This IFN-\(\gamma\)-induced increase was attenuated to control levels by co-treatment with L-NAME (\(P < 0.05\)-0.001; Figure 5C). Conversely, applying sodium nitroprusside (SNP) to MG-5 cells significantly increased

![Figure 3 Reduction in the amount of p-SFKs in inOS\(^{-/-}\)and n/i/eNOS\(^{-/-}\)mice, and changes in IL-6 and TLR4 mRNA expression levels in n/ i/eNOS\(^{-/-}\)mice](image-url)
Mac-1 mRNA expression in a dose-dependent manner (1.46 ± 0.09-fold in the presence of 1.0 mM SNP as compared with the non-treatment group, \( P < 0.01 \); Figure 6A). SNP also increased mRNA expression levels of IL-6, TLR4 and P2X4 receptor in a dose-dependent manner (10.8 ± 0.60-, 1.94 ± 0.14- and 1.67 ± 0.08-fold in the presence of 1.0 mM SNP as compared with the non-treatment group; \( P < 0.001 \) each; Figure 6B-D).

Discussion

The present study provides the first evidence that n/i/eNOS triple gene knockouts attenuate peripheral nerve injury-induced tactile allodynia and spinal microglial activation. Moreover, activating the NOS/NO pathway can promote expressions of microglial IL-6, TLR4 and P2X4 receptor, which are implicated in neuropathic pain [2-6].

Chronic pain is categorized into inflammatory pain, induced by peripheral tissue inflammation, and neuropathic pain, causing damage and malfunction of the nerve system. The former can have adequate pain control by known analgesics, but the latter shows resistance to existing treatments. We demonstrated that \( iNOS^{-/-} \)-mice showed suppression of formalin-induced acute and persistent pain, but not nerve injury-induced neuropathic pain as compared with WT mice. This result in thermal hyperalgesia is consistent with a previous report showing no attenuation of the nerve injury-induced thermal hyperalgesia in \( iNOS^{-/-} \)-mice [16]. In contrast to the behavioral phenotype of \( iNOS^{-/-} \)-mice in the formalin test, the reduction of nociceptive behaviors of \( n/i/eNOS^{-/-} \)-mice was weak in the late second phase, and in the first phase, \( n/i/eNOS^{-/-} \)-mice facilitated the nociceptive response. However, in a model of neuropathic pain, tactile allodynia induced by nerve injury was markedly inhibited only in \( n/i/eNOS^{-/-} \)-mice. These results suggest that NOS/NO signaling critically contributes to both tissue injurious pain and neuropathic pain, and that each NOS isoform has a different function in pain signaling. In previous studies, N-nitro-L-arginine, a non-selective NOS inhibitor, reduces first phase nociceptive behavior evoked by formalin, whereas the iNOS inhibitor aminoguanidine and the nNOS inhibitor 7-nitroindazole do not have a suppressing effect on the first phase pain response [26]. Furthermore, an
antisense oligodeoxynucleotide targeting the nNOS-2 isoform, one of 10 splicing differentiations, has a significant exacerbation effect against the first phase [27]. While the responses of innocuous mechanical stimuli were indistinguishable between WT mice and the two genotypes of \( \text{NOS}^{-/-} \) mice, the latency of tail flick away from noxious heat stimuli was extended in \( \text{iNOS}^{-/-} \) mice, but not \( \text{n/i/eNOS}^{-/-} \) mice. Acetic acid-induced visceral pain was also enhanced in \( \text{n/i/eNOS}^{-/-} \) mice when compared with WT mice. L-NAME has been reported to have no effect on tail-flick test latencies, but L-NAME and 7-nitroindazole inhibit acetic acid-induced writhing behavior [28]. However, these previous reports illustrate the complexity of NOS in pain perception; our data lead to the hypothesis that nNOS and/or eNOS may have a role in suppressing pain signaling.

Activation of spinal microglia is known to occur after nerve injury [2,21,22]. Because minocycline, which inhibits microglial activation in vivo [29], reduces tactile allodynia on nerve injury [30], the microglial activated restraint leads to pain relief in neuropathic pain states. The activation of spinal microglia as well as tactile allodynia after nerve injury was suppressed in only \( \text{n/i/eNOS}^{-/-} \) mice. The suppression of nerve injury-induced neuropathic pain responses in all NOSs-knockout mice correlated with the reduction in the numerical and morphological changes of microglia after nerve injury. These results suggest that NOS/NO signaling participates in neuropathic pain through activation of microglia.

We have previously shown that SFKs are activated in the spinal cord after nerve injury. The SFKs are the largest family of nonreceptor tyrosine kinases and nine subtypes have been identified: Src, Yes, Fyn, Fgr, Lck, Hck, Blk, Lyn and Frk [31]. We have also showed previously that Lyn is predominantly expressed in microglia. Because transcriptional activity of microglial P2X4 receptor mRNA is reduced in a neuropathic pain model using \( \text{Lyn}^{-/-} \) mice [23], the increase of spinal p-SFKs may result in an enhancement of microglial transcriptional activity in response to nerve injury. After spinal nerve injury,
activated microglia upregulate the expression of P2X4R, IL-6, IL-1β, TNF-α and TLR4 [3-5]. In fact, IL-6 and TLR4 mRNA transcriptional activities increased after nerve injury, and these increases were inhibited in n/i/eNOS-/- mice, which observed a significant decrease of p-SFKs immunoreactivity. P2X4R mRNA expression was also tendency to be reduced in n/i/eNOS-/- mice. It is possible that NOS/NO signaling may be involved in expression of these cytokine/receptor transcripts, presumably through microglial SFK activation after spinal nerve injury. It was reported that the intrathecal administration of the anti-IL-6 antibody attenuates nerve injury-induced tactile allodynia in rats [32]. Additionally, spinal nerve injury-induced tactile allodynia and microglial activation are inhibited in TLR4-/- mice [3]. IL-6 and TLR4 have been implicated in neuropathic pain conditions. Therefore, it is conceivable that suppressing expression of these molecules in microglia may be involved in the attenuation of nerve injury-induced tactile allodynia observed in n/i/eNOS-/- mice.

Expression of NOS and NO are known to be markedly upregulated in damaged nerves, dorsal root ganglia and glial cells in the spinal dorsal horn after nerve injury [33,34]. Our pharmacological procedure indicated the chronic intraperitoneal administration of L-NAME suppressed the development of tactile allodynia. Furthermore, acute administrations of L-NAME or 7-nitroindazole reversed established tactile allodynia on day 7 after nerve injury. It has been reported that nNOS knockout mice can attenuate neuropathic pain [15]. Because a previous study has shown that intracerebroventricular administration of 7-nitroindazole has no effect on alleviating pain in the chronic constriction injury model [13], nNOS in the brain may have a minor role in nerve injury-induced allodynia. These results suggest that NOS/NO signaling plays a key role in neuropathic pain states, and spinal or peripheral nNOS plays an important role in tactile allodynia during development and maintenance. There is no evidence that eNOS precisely contributes to neuropathic pain, although eNOS expression is upregulated in the injured sciatic nerve [35].

IFN-γ is reported to induce microglial activation and neuropathic pain through Lyn and P2X4 receptor signaling [24]. In our in vitro study, expression of iNOS and nNOS mRNA were increased in IFN-γ-treated MG-5 cells. In IFN-γ- or SNP-treated MG-5 cells, the significant increase in the expression of Mac-1, known as a

| Figure 6 Microglial activation, and increased IL-6, TLR4 and P2X4 receptor mRNA expression levels via NO | Expression levels of Mac-1 (A), interleukin-6 (IL-6; B), toll-like receptor 4 (TLR4; C) and P2X4 receptor (P2X4R; D) mRNA in MG-5 microglial cell line cultures. mRNA expression levels were significant increased by treatment with sodium nitroprusside (SNP), a NO donor (n = 6 samples/group). All data are presented as a mean ± SEM. Statistical analysis was determined by one-way ANOVA with Tukey’s test, *P < 0.05, **P < 0.01, ***P < 0.001. |
marker of activated microglia, were observed. The IFN-γ-induced increase in Mac-1 mRNA expression was prevented by L-NAME co-treatment. From these results, it is possible that IFN-γ upregulates iNOS and nNOS expression, which in turn promotes activation of microglial cells (although it is likely that the role of iNOS in neuropathic pain may be relatively minor because a marked attenuation of allodynia was not observed in iNOS−/− mice compared with n/i/eNOS−/− mice). IL-6 and TLR4 expression, which were attenuated in n/i/eNOS−/− mice with spinal nerve injury, and P2X4 receptor mRNA were increased following SNP treatment. Upregulation of the P2X4 receptor in microglia is an important process in producing neuropathic pain [2]. P2rx4−/− mice do not show nerve injury-induced tactile hypersensitivity [36]. Thus, in addition to IL-6 and TLR4, the P2X4 receptor may also be involved in the suppression of tactile allodynia in n/i/eNOS−/− mice.

**Conclusions**

In summary, we demonstrate that the NOS/NO pathway plays an important role in the development and maintenance of neuropathic pain after nerve injury. Pharmacologic and genetic studies indicate that spinal and/or peripheral NOS, especially nNOS, contributes to upregulation of IL-6, TLR4 and P2X4 receptor expression through microglial SFK activation. These findings are conducive to the understanding of the neuropathic pain mechanism.

**Methods**

**Animals**

All experimental procedures were performed in accordance with institutional guidelines at Kyushu University regarding the care and use of animals. Male iNOS knockout mice (iNOS−/− mice), male n/i/eNOS triple knockout mice (n/i/eNOS−/− mice) and male C57BL/6J WT mice (the genomic status of each mouse was checked) were used in the present study [20]. Male C57BL/6J mice (25-35 g) were bought from Japan SLC (Hamamatsu, Japan). Mice were housed at a constant temperature of 22 ± 1°C with a 12-hour light-dark cycle (lights on between 08:30 and 20:30), and had ad libitum access to food and water.

**Behavioral assays for acute and chronic pain**

In the test of formalin-induced pain, mice were injected intraplantarly with formalin (5% (v/v), 20 μL), and the duration of licking and biting responses to the injected hindpaw were recorded at 5 min intervals for 60 min after injection (formalin pain) [36]. Noxious heat-evoked tail and hindpaw withdrawal responses were detected by the application of radiant heat (Ugo Basile, Italy) to the tail and the plantar surface of the hindpaw, respectively [36]. The intensity of the heat stimulus was adjusted to 30 or 50 V, and the latency of the tail withdrawal response (sec) was measured. The sensitivity to mechanical stimuli was assessed using von Frey filaments (0.02-2.0 g, Stoelting, Wood Dale, IL, USA), and the mechanical stimulus producing a 50% PWT was determined using the up-down method [37,38]. In the chemical visceral pain test, mice were injected intraperitoneally with acetic acid (0.8% (v/v)), and the number of abdominal writhes was counted for 5 min starting from 5 min after the injection [36].

**Spinal nerve injury-induced neuropathic pain model**

The spinal nerve injury model in mice was produced as described previously [23,39]. For the experiments using mice, the left fifth lumbar spinal nerve of mice was transacted under 2% (v/v) isoflurane anesthesia.

**Immunohistochemistry**

The mice were anesthetized by pentobarbital (100 mg/kg, intraperitoneally) and perfused transcardially with 4% (w/v) paraformaldehyde. The fifth lumbar segment of the lumbar spinal cord was quickly removed, post-fixed in the same fixative, and placed in 30% (w/v) sucrose solution for 24 hrs at 4°C. Immunohistochemistry and analysis of transverse spinal cord sections (30 μm) were performed in accordance with methods described previously [40]. The rabbit anti-ionized calcium binding adaptor molecule 1 (anti-Iba1) antibody (1:2,000, Wako, Osaka, Japan) was used as the primary antibody, and the anti-rabbit immunoglobulin G (IgG)-conjugated Alexa Fluor 488 (1:1,000, Molecular Probes, Eugene, OR, USA) was used as the secondary antibody. The intensity of p-SFKs immunoreactivity was analyzed using a software Photoshop CS3 (Adobe, San Jose, CA).

**Drug administration**

Phosphate buffered saline (PBS; as a vehicle control) or L-Nω-nitro-arginine methyl ester (L-NAME) (Sigma, St. Louis, MO, USA; 30 or 100 mg/kg) was intraperitoneally administered to rats once a day for 7 days with a 26 G needle. Measurement of PWT was performed on 0, 1, 3, 5 and 7 days after administration. For intrathecal drug administration, under 2% (v/v) isoflurane anesthesia, mice were injected with a 32 G needle. After peripheral nerve injury, rats were administered intrathecally with PBS (5 μL, as a vehicle control) or L-NAME (100 mg/kg) at day 7 after nerve injury. Measurement of PWT was performed at 0.5, 1, 1.5, 2, 3, 6 and 12 hrs after administration. Similarly, 7-nitroindazole (Calbiochem, Darmstadt, Germany; 3 or 10 μg in 5 μL) was intratheca administered and measurement of PWT was performed at 0.5, 1,
1.5, 2 and 3 hrs after administration. Intrathecal drug administration was performed just after pre-measurement, and PWT was measured at each time point.

MG-5 Culture
The MG-5 mouse microglial cell line was cultured as described previously by Ohsawa et al. [25]. Conditioned medium from the supernatant of A1 cells cultured overnight in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) containing 10% (v/v) fetal bovine serum, penicillin and streptomycin (20 U/mL) was used as the culture medium for MG-5 cells. MG-5 cells were exposed to IFN-γ and SNP for 1 hr under serum-free conditions.

Real-time reverse transcription-PCR
The fifth lumbar spinal cord was quickly removed. Total RNA preparation and real-time RT-PCR amplification/detection were performed in accordance with methods described previously [40]. All expression values were normalized by the expression values of 18S ribosomal RNA. The TaqMan probe and the forward and reverse primers used in this study were designed according to Table 1. Ribosomal RNA was measured using TaqMan Ribosomal RNA Control Reagents (P/N 4308329, Applied Biosystems, Carlsbad, CA, USA). The TaqMan probe and primers for Mac-1 were obtained from Applied Biosystems.

Statistical analysis
Data are expressed as the means ± SEM. Statistical analyses of the results were conducted with one-way ANOVA, one-way repeated ANOVA, two-way ANOVA or two-way repeated ANOVA with a post hoc test (Tukey’s multiple comparison test). Statistical significance was set at a P value < 0.05.

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Authors’ contributions
KK performed the experiments, analyzed the data and wrote the manuscript; MTsuda designed, performed and supervised the experiments, and wrote the manuscript; MTsutsui, YT, HS and NY provided i/cNOs-/− and i/eNOS-/− mice, and edited the manuscript; HT-S supervised the experiments and edited the manuscript; MTsuda designed, performed and supervised the experiments, and wrote the manuscript; YT, HS and NY provided i/cNOs-/− and i/eNOS-/− mice, and edited the manuscript; MTsuda designed, performed and supervised the experiments, and wrote the manuscript; HT-S supervised the experiments and edited the manuscript; All authors have read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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