Suppressed injury-induced rise in spinal prostaglandin E2 production and reduced early thermal hyperalgesia in iNOS-deficient mice

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Suppressed Injury-Induced Rise in Spinal Prostaglandin E_2 Production and Reduced Early Thermal Hyperalgesia in iNOS-Deficient Mice

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It is widely accepted that peripheral injury increases spinal inducible cyclooxygenase (COX-2) expression and prostaglandin E_2 (PGE_2) formation as key mediators of nociceptive sensitization. Here, we used inducible nitric oxide synthase (iNOS) gene-deficient (iNOS−/−) mice to determine the contribution of iNOS-derived nitric oxide (NO) to this process. iNOS−/− mice exhibited reduced thermal hyperalgesia after zymosan injection. Spinal NO and PGE_2 formation both remained at baseline levels, in contrast to wild-type (wt) mice. In wt mice reduced hyperalgesia similar to that seen in iNOS−/− mice was induced by local spinal, but not by systemic treatment with the iNOS inhibitor L-NIL, suggesting that the reduced heat sensitization in iNOS−/− mice was attributable to the lack of spinal rather than peripheral iNOS. Two additional observations indicate that the antinociceptive effects of iNOS inhibition are dependent on a loss of stimulation of PG synthesis. First, intrathecal injection of the COX inhibitor indomethacin, which exerted pronounced antinociceptive effects in wt mice, was completely ineffective in iNOS−/− mice. Second, treatment with the NO donor RE-2047 not only completely restored spinal PG production and thermal sensitization in iNOS−/− mice but also its sensitivity to indomethacin. In both types of mice induction of thermal hyperalgesia was accompanied by similar increases in COX-1 and COX-2 mRNA expression. The stimulation of PG production by NO therefore involves an increase in enzymatic activity, rather than an alteration of COX gene expression. These results indicate that NO derived from spinal iNOS acts as a fast inductor of spinal thermal hyperalgesia.

Key words: nitric oxide; inducible nitric oxide synthase; zymosan; thermal hyperalgesia; paw edema; spinal microdialysis; L-NIL; RE-2047; prostaglandins; cyclooxygenase

Acute tissue damage is often accompanied by the fast development of hyperalgesia and allodynia (Andrew and Greenspan, 1999). Both peripheral mechanisms at the site of injury and central processes particularly in the spinal cord contribute to this phenomenon. Prostaglandins (PGs) (Yaksh and Malmberg, 1993; Brune, 1994) as well as nitric oxide (NO) (Lawand et al., 1997) are produced in response to tissue damage peripherally and centrally. Whereas PGs are generally accepted to play a dominant role in nociceptive sensitization (Bley et al., 1998), the role of NO is less clear. Also some authors claim of an anti-nociceptive action of NO (Goettl and Larson, 1996; Hamalainen and Lovick, 1997), most favor a pronociceptive activity (Malmberg and Yaksh, 1993; Kawabata et al., 1994; Chen and Levine, 1999). Part of this controversy may arise from the existence of three different isoenzymes of NO synthase (NOS) (Gonzalez-Hernandez and Rustioni, 1999), which may have distinct effects on nociception, and from the lack of specific inhibitors for these different isoforms. In the CNS including the spinal cord, NO is thought to be primarily produced by the neuronal isoform of NOS (nNOS) (Downen et al., 1999). However, endothelial NOS is also found in neurons (Wei et al., 1999) and under certain conditions, e.g., after tissue damage (Sinz et al., 1999), inducible NOS (iNOS) can be expressed in the CNS (Meller et al., 1994; Lee and Brosnan, 1996; Barker et al., 1998). Therefore, all three NO-generating isoenzymes appear to be possible sources of NO in the CNS.

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To define the role of NO in spinal processing of nociceptive information more clearly, we investigated nociceptive sensitization in genetically modified mice deficient in the iNOS isoenzyme (iNOS−/− mice). For this purpose, we adapted the Hargreaves model of thermal hyperalgesia (Hargreaves et al., 1988) to mice and developed a technique for spinal microdialysis in mice. We showed that iNOS−/− mice exhibit a delay in thermal sensitization and lack the rise in spinal PG production, which is normally observed in response to peripheral nociceptive stimulation.

MATERIALS AND METHODS

Assessment of thermal hyperalgesia. A modified Hargreaves plantar test (Hargreaves et al., 1988) was used to assess thermal hyperalgesia in mice. A metal grid bottom instead of a glass floor in the observation cage and 10.5 × 13.0 × 4.5 cm boxes to restrict animal movement were used. Zymosan A (Sigma, Deisenhofen, Germany) was injected subcutaneously into the plantar side of right hindpaws, and paw withdrawal latencies (PWL) were determined on exposure of the paws to a defined thermal stimulus were measured using a commercially available apparatus (Hargreaves Test Ugo Basile Biological Research Apparatus, Comerio, Italy).

Mice were kept in the test cages for 1 d to allow accommodation. On day 2, each mouse was tested several times to gain baseline PWL. On day 3 thermal hyperalgesia was assessed for 8 hr starting 15 min after subcutaneous zymosan injection (3.0 mg/ml in 20 μl of PBS, containing NaCl 8 gm/l, Na_2HPO_4 2.9 gm/l, KCl 0.2 gm/l, KH_2PO_4 0.24 gm/l). Experiments were performed in air conditioned rooms (22°C) between 12 A.M. and 8 P.M. In some experiments the assessment of thermal hyperalgesia was continued for 7 d (one measurement per day). Right (injected) and left (noninjected) paws were measured alternately in intervals of 5–10 min. At 1 hr intervals, PWL were averaged.

Under control conditions, PWL were identical in wt (10.40 ± 0.35 sec; $n = 40$) and iNOS−/−mice (10.25 ± 0.20 sec; $n = 18$). In an initial set of experiments, zymosan (20 μl) was tested in concentrations of 12.0, 6.0, or 3.0 mg/ml. Zymosan injection caused a dose-dependent increase in areas [PWL × observation interval [seconds × hours]; calculated using the linear trapezoidal rule for each mouse] between right and left hindpaw PWL from 0.17 ± 1.75 (PBS) to 10.10 ± 1.81 (3.0 mg/ml) to 17.47 ± 2.55 (6.0 mg/ml) and to 23.30 ± 2.22 (12.0 mg/ml). Injection of vehicle did not affect nociceptive behavior in any of the experiments. For all subsequent

Table 1. Primer sequences

β-actin forward	-TCACCCACACTGTGCCCATCTACGA
β-actin reverse	-GGATGCCACAGGATTCCATACCCA
β-actin probe	-(6FAM)TATGCTC(TAMRA)TCCCTCACGCCATCCTGCGT
COX-2 forward	-TTTGTTGAGTCATTCACCAGACAGAT
COX-2 reverse	-CAGTATTGAGGAGAACAGATGGGATT
COX-2 probe	-(6FAM)CTACCATGGTC(TAMRA)TCCCCAAAGATAGCATCA
iNOS forward	-TCACGCTTGGGTCTTGTTCACT
iNOS reverse	-TTGTCTCTGGGTCCTCTGGTCA

Forward and reverse primer as well as probe sequences for real time RT-PCR of β-actin and COX-2. Forward and reverse primer sequences for RT-PCR of iNOS.

tests an intermediate zymosan concentration of 3.0 mg/ml was used for the detection of pro-nociceptive and anti-nociceptive effects.

Animals. Male iNOS−/− mice weighing 26.7 (22.9–36.8) gm [mean (range)] with the genetic background of C57/Bl6 mice and male C57/Bl6 mice (wt) weighing 21.2 (19.6–26.1) gm were used for all experiments. Breeding pairs of iNOS −/− mice (Laubach et al., 1995) were obtained from The Jackson Laboratory (Bar Harbor, ME). INOS −/− mice show no major abnormalities (Laubach et al., 1995; MacMicking et al., 1995; Wei et al., 1995). Mice were housed under a 12 hr light/dark cycle and cared for according to the guidelines of the Institutional Animal Care and Use Committee. Water and food were given *ad libitum*.

Application of drugs. All drugs were dissolved in isotonic, physiological solvents. Indomethacin was dissolved as described elsewhere (Shen and Winter, 1977). Briefly, for a 10 mM solution, 17.9 mg of indomethacin, 15.3 mg of $Na_2CO_3 \times 10\ H_2O$, and 5 ml of artificial CSF (ACSF) consisting of (in mM): 151.1 Na^+, 2.6 K^+, 0.9 Mg^{2+}, 1.3 Ca^{2+}, 122.7 Cl^-, 21.0 mM $HCO3^-$, 2.5 mM $HPO4^-$, and 3.5 dextrose, pH 7.20, was used. Intraperitoneal drug or vehicle (PBS) injections (50 μl) were given into the lower left abdominal quadrant. Intrathecal injections were performed according to Hylden and Wilcox (1980). In brief, mice were anesthetized with isoflurane, and 5 μl of drug containing solutions or vehicle (ACSF) were injected into the spinal subarachnoid space between L5 and L6 30 min before the administration of zymosan using a 26 gauge needle mated to a 10 μl Hamilton syringe. Mice showing neurological abnormalities were excluded. We added 1% black ink (Pelikan, Hannover, Germany) to all solutions used for intrathecal injections. Proper intrathecal injections were verified by inspection of slices of the spinal cord after lumbar laminectomy.

Tissue samples. After completion of the Hargreaves test, mice were killed under CO_2 anesthesia by intracardial puncture and decapitation. Hindpaws and the thoracolumbar segment of the spinal cord were removed for morphological and biochemical analyses. After intra-articular disconnection at the ankle joint, right and left hindpaws were weighed. Differences in paw weight (Δ PW) were used to measure edema formation.

Spinal microdialysis. The dialysis tube was constructed from a Cuprophan hollow fiber (outer diameter, 216 μm) with a 36 kDa molecular weight cutoff (Hospal, Nuernberg, Germany). This fiber was connected at one side to a polyethylene (PE) tube (inner diameter, 0.4 mm; outer diameter, 0.8 mm) using cyanacrylate glue (number 448 Stabiloplast; Renfert, Chemietechnik, GmbH). A small metal spike was inserted into the other side and fixed with a quick-setting cyanacrylate glue (UHU, Buehl, Germany).

Mice were deeply anesthetized with isoflurane (1.5–2.0% vol; Abbott GmbH, Wiesbaden, Germany) and placed on an electronically controlled heating pad (37°C; CMA/Microdialysis, Stockholm, Sweden). After cutaneous incision of the thoracolumbar region, superficial and deep dorsal lumbar fascia were slit, and muscle tissue was removed from the vertebrae T12–L1. The dialysis tube was introduced through the intervertebral joints between the thoracic and lumbar segments. All accessible parts of the dialysis tube were covered with cyanacrylate glue. After cutting the spike, the free end of the hollow fiber was connected to another PE tube using the same cyanacrylate adhesive. Afterward mice were surgically sewed and permanently anesthetized with urethane (~750 mg/kg, i.p.).

The PE tube was connected to a microdialysis pump (CMA 100; CMA/Microdialysis), and ACSF was perfused at a flow rate of 3 μl/min. ACSF was bubbled with carbogen (5% CO_2 and 95% O_2) and kept at 37°C during the experiments. Samples were collected at 30 min intervals in Eppendorf cups kept on ice and finally stored at −70°C for subsequent analysis of PGE_2, as well as NO_2^- and NO_3^- (NO_x) as the breakdown products of NO. After a washout period of 30 min, baseline samples were collected for 1.5 hr every 30 min. Thereafter, 20 μl of zymosan (3.0 mg/ml) was injected subcutaneously into the right hindpaw, and samples were collected for another 4 hr. After mice were killed, the proper placement of microdialysis tubes was verified by perfusion with black ink (Pelikan), and subsequent microscopic examination.

NO_x and PGE_2 measurements. NO production was assessed indirectly by determining NO degradation products after reduction of NO_3^- to NO_2^- with nitrate reductase (Cytochrome; Sigma) by the Griess reaction-dependent method described elsewhere (Green et al., 1981). We incubated 50 μl of perfusion samples with 50 μl of modified Griess reagent (Sigma), and the absorption was recorded at 540 nm (Flow Titertec, Multiscan Plus MK11; ICN Biochemicals, Frankfurt, Germany).

Tissue samples obtained from the spinal cord and from the hindpaws were weighed, transferred into 99.5% methanol (1 mg of tissue to 10 μl of methanol), and shaken for 2 hr at room temperature. We transferred 100 μl of the supernatants into Eppendorf cups, and methanol was evaporated. The remaining pellet was dissolved in 100 μl of enzyme immunoassay (EIA) buffer. We incubated 20 μl of the microdialysis perfusion samples with 80 μl of enzyme immunoassay (EIA) buffer for PGE_2 measurements.

All further steps were performed as described in the Cayman Chemical Company PGE_2 EIA Kit - Monoclonal, calibration range: 1000–7.8 pg/ml (Cayman Chemicals, Ann Arbor, MI). Measurement was completed by using an ELISA reader (Flow Titertec, Multiscan Plus MK11; ICN Biochemicals) with an absorbency maximum at 405 nm.

RT-PCR. Immediately after preparation, tissue samples of the right hindpaw and spinal cord segment L4 were snap frozen with 800 μl of lysis buffer (Qiagen, Hilden, Germany) in liquid nitrogen, stored at −70°C, and homogenized with a microshredder. RNA was isolated using the RNeasy-kit (Qiagen). Real-time RT-PCR was used to determine expression of mouse-actin, COX-1, and COX-2 mRNA. TaqMan probes were labeled at the 5′ end with the reporter dye molecule FAM (6-carboxy-fluorescein; emission λ, 518 nm), and at the 3′ end with the quencher dye molecule TAMRA (6-carboxy-tetramethyl-rhodamine, emission λ, 582 nm). In addition, the 3′ end was phosphorylated to prevent extension of the probe during PCR.

We used 25 μl of reaction mixture, which contained 2 μl of template, 5 μl of 10 × PCR buffer (100 mM Tris, pH 8.3, 500 mM KCl), 3 μl of $Mn(OAc)_2$, 0.3 μl of dATP, dCTP, dGTP, and dUTP, 0.1 μl of Diagonal DNA polymerase, 0.05 μl of each primer, 0.05 μl of the TaqMan probe, and 14.55 μl of sterile water for the PCR. For RT-PCR and detection of fluorescence signals the ABI Prism 7700 SDS analytical thermal cycler (Perkin-Elmer, Foster City, CA) was used. Thermal cycle conditions were: 2 min at 50°C, 30 min 60°C, 5 min 95°C, and then 45 cycles (15 sec at 94°C, 1 min at 60°C).

The emission of the reporter dye was compared with that of the quenching dye during PCR amplification and the increase of fluorescence signals, ΔRn, was calculated as: $\Delta Rn = (Rn^+) - (Rn^-)$ [Rn^+ = ratio of reporter and quencher dye at any given time during a reaction; Rn^- = ratio of reporter and quencher baseline emission]. Referring to a standard RNA a threshold was defined indicating the exponential phase of the fluorescent signal increase. The C_T value, which correlates to the number of RNA copies present at the start of PCR according to the references of PE Applied Biosystems (User Bulletin 2; ABI PRISM 7700 Sequence Detection System, 1997) was determined as the amplification cycle number when the ΔRn of a sample intersected this threshold value. The quantity of mRNA was given as ΔC_T, which was calculated as $\Delta C_T = C_T^{\text{gene of interest}} - C_T^{\beta\text{-actin mRNA}}$.

iNOS mRNA transcripts were analyzed in spinal cord tissue (thoracolumbar segment) homogenates according to a protocol previously described in detail (Deckert-Schluter et al., 1995). Primer sequences were described previously (Deckert-Schluter et al., 1998; compare Table 1) and were synthesized by TIB MOLBIOL (Berlin, Germany). Briefly, total mRNA was extracted as described above. After reverse transcription of mRNA using the Superscript RT kit (Life Technologies, Frederick, MD) PCR reactions were conducted in a final volume of 25 μl of using the AmpliTaq Gold kit from PE Biosystems (Weiterstadt, Germany). Conditions are: 1 × 2 min 95°C; 45 × 1 min 95°C, 1 min 58°C, 1 min 72°C; 1 × 7 min 72°C. The PCR product was visualized by electrophoresis on 1.5% agarose gels.

Statistical analysis. Results are expressed as the mean ± SEM. The statistical analyses of the behavioral experiments were performed using one-way ANOVA followed by *post hoc* Bonferroni test (the α level was set to 0.05) or by a Student's *t* test ($p < 0.05$ was considered statistically significant).

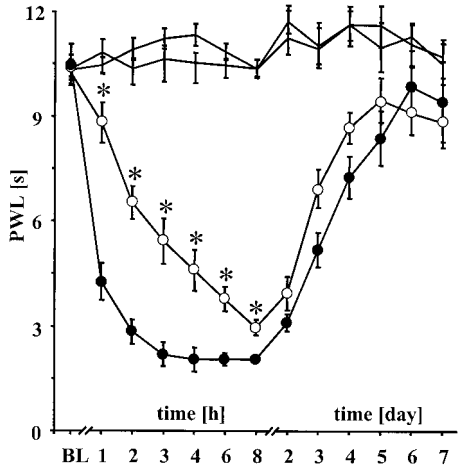

Figure 1. Time course of zymosan-induced thermal hyperalgesia in iNOS−/− mice. Development with time of PWL in iNOS−/− (n = 12; *open circles*) and wt mice (n = 12; *black circles*) after intraperitoneal administration of PBS and after injection of 3.0 mg/ml zymosan subcutaneously into the right hindpaw. Left PWLs (*black lines*) were similar for both lines of mice. Different PWL ($^*p < 0.05$) indicate reduced heat sensitization after zymosan injection in iNOS−/− mice. Data are expressed as means ± SEM.

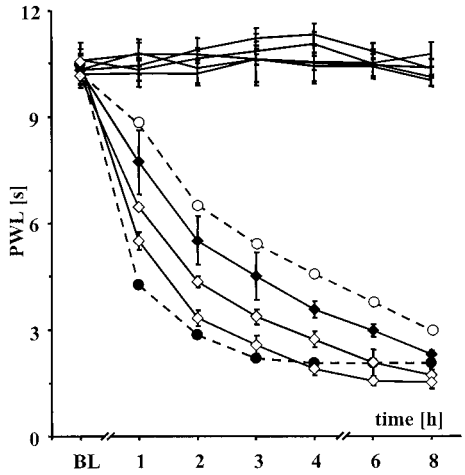

Figure 2. NO donor restores thermal hyperalgesia in iNOS−/− mice. Intraperitoneal injection of RE-2047, a metabolic NO-donor, in doses increasing from 5 (*black diamonds*) to 15 (*gray diamonds*) and 45 mg/kg (*open diamonds*) antagonized the restored heat sensitization of iNOS−/− mice. The difference of heat sensitization between wt (*black circles*) and iNOS−/− mice (*open circles*) after intraperitoneal administration of PBS is drawn as *dotted lines*.

RESULTS

iNOS-derived NO facilitates development of spinal hyperalgesia

A modification of the Hargreaves test was used to assess thermal hyperalgesia in wt and iNOS−/− mice. Both types of mice exhibited a time-dependent sensitization to noxious heat in response to plantar zymosan injection. At the time point of maximum thermal hyperalgesia, PWL were reduced from ~10 sec under control conditions to ~3 sec after zymosan injection. In wt mice, however, maximum sensitization occurred after 3 hr, whereas that of iNOS−/− mice was not reached until 8 hr (Fig. 1). Wild type-like thermal sensitization could be restored in iNOS−/− mice by treatment with the NO-donor RE-2047 (3-methyl-*N*-nitroso-sydnone-5-imine; a generous gift from Prof. Rehse, Department of Pharmaceutical Chemistry, Berlin, Germany) (Rehse and Ciborski, 1995) in a dose-dependent manner. At the highest dose of RE-2047 hyperalgesia in iNOS−/− mice was indistinguishable from that of wt mice, confirming that the reduced thermal sensitization in iNOS−/− mice was caused by the absence of NO generated by iNOS (Fig. 2).

To determine whether the lack of iNOS in the peripheral tissue or in the CNS was responsible for reduced early thermal hyperalgesia, we used the selective iNOS inhibitor L-NIL (L-N6-(I-iminoethyl)-lysine hydrochloride; Alexis Biochemicals, Gruenberg, Germany; Moore et al., 1994) and took advantage of its inability to penetrate the blood–brain barrier in significant amounts (U. Werner, B. Layh, K. Brune, H. and Guehring, unpublished observations). In this series of experiments we compared the effects of L-NIL after systemic peripheral (intraperitoneal) and local spinal (intrathecal) injection in wt mice. After intraperitoneal injection at doses ranging from 15.0 to 135.0 mg/kg L-NIL effectively reduced edema and PGE_2 production in the zymosan-injected paw, but had no effect on thermal hyperalgesia (Fig. 3*A*, Table 2). Increases in the weight of the injected paws were 35.2 ± 3.0% and 48.7 ± 2.1% in L-NIL and vehicle-treated mice, respectively. A similar difference was found for the PGE_2 content in the zymosan-injected paws, which was 25.6 ± 5.0 pg/mg in L-NIL treated and 54.6 ± 2.7 pg/mg in control mice. As expected, L-NIL was completely ineffective in iNOS−/− mice. Both paw edema formation and PGE_2 production in L-NIL-treated mice were not significantly different from those found in iNOS−/− mice (edema, 30.25 ± 4.14%; PGE_2, 23.3 ± 1.9 pg/mg).

By contrast, when L-NIL (0.1 μM; 5 μl) was injected intrathecally the development of zymosan-induced sensitization was significantly retarded compared to vehicle (ACSF; 5 μl)-treated animals and indistinguishable from that of iNOS−/− mice (Fig. 3*B*). The latter finding indicates that the lack of spinal rather than of peripheral iNOS was responsible for the observed delay in thermal hyperalgesia. Interestingly, neither L-NIL nor the nonspecific NOS inhibitor L-NAME (NG-nitro-L-arginine methyl ester; Alexis Biochemicals, Gruenberg, Germany; Amir and English, 1991) affected heat sensitization in iNOS−/− mice (Table 3), indicating that nNOS and eNOS did not significantly contribute to zymosan-induced thermal hyperalgesia.

To investigate whether the facilitating action of iNOS on thermal hyperalgesia was related to the COX system in the spinal cord, we compared the effects of spinal COX inhibition in iNOS−/− and wt mice. In wt mice intrathecal injection of the COX inhibitor indomethacin (Sigma) reduced thermal hyperalgesia in a dose-dependent manner (Fig. 4*A*). This antinociceptive effect was completely absent in iNOS−/− mice (Table 3, Fig. 4*B*) but was restored after substitution of NO with RE-2047, suggesting that the increase in spinal PGE_2 production after zymosan injection required NO, which in wt mice is produced by spinal iNOS.

Spinal NO_x and PGE_2 formation

The difference in heat sensitization between wt and iNOS−/− mice was already present 1 hr after zymosan injection. Because iNOS is generally considered not to be constitutively expressed in the CNS, the fast rise in NO production after zymosan injection would require an at least equally fast induction of iNOS expression. We have therefore analyzed the expression of iNOS mRNA during the early phase of hyperalgesia development by RT-PCR (Fig. 5). Significant amounts of iNOS mRNA were detected 30 and 60 min after zymosan injection and 120 min after zymosan injection in separate experiments (data not shown). As expected, iNOS mRNA was absent under control conditions and in iNOS−/− mice after zymosan injection.

To further elucidate the role of iNOS in the spinal mechanisms of heat sensitization and its relation to the COX pathway we performed spinal microdialysis to measure NO and PGE_2 production in the spinal cord in response to zymosan injection. In a first series of experiments we tested whether insertion of the microdialysis probe was sufficient to induce spinal PG formation. In these experiments, PBS instead of zymosan was injected subcutaneously into the right hindpaw. Under these conditions only a modest increase in PGE_2 was induced in wt mice (from 18.4 ± 1.9 to 26.3 ± 2.5 pg/ml).

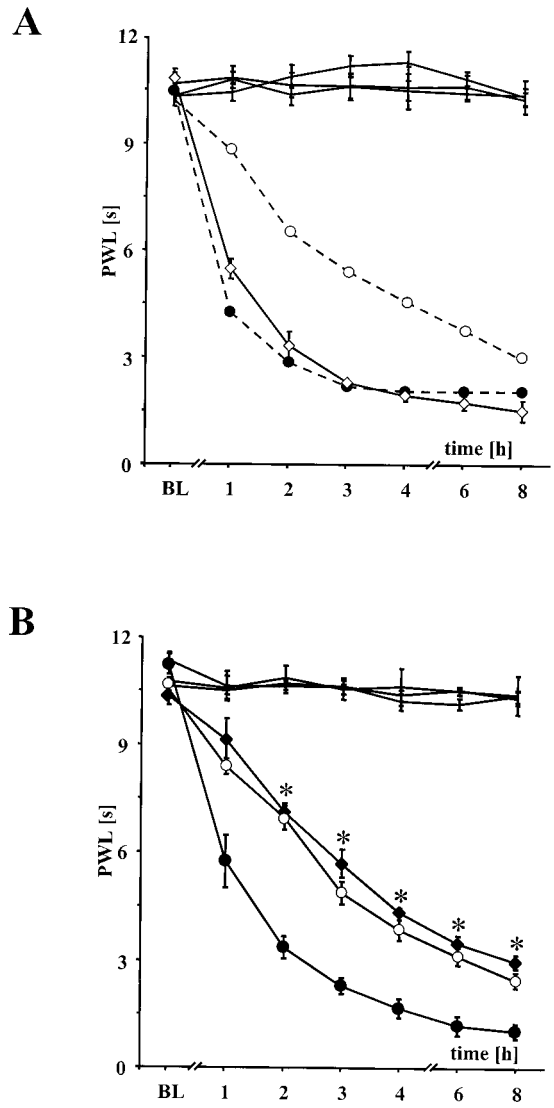

Figure 3. Effects of peripheral versus spinal injection of L-NIL on thermal hyperalgesia in wt mice. *A,* In wt mice (*open diamonds*) the selective iNOS inhibitor L-NIL administered intraperitoneally was without any effect even in doses ranging from 5 to 135 mg/kg. The difference of heat sensitization between wt (*black circles*) and iNOS−/− mice (*open circles*) after intraperitoneal administration of PBS is drawn as *dotted lines*. Data are expressed as mean ± SEM. *B,* In contrast to *A* drugs were administered intrathecally. Intrathecal administration of L-NIL (0.1 μM) to wt mice (*black diamonds*) reduced heat sensitization significantly to the level of iNOS−/− mice. Differences in heat sensitization between wt (*black circles*) and iNOS−/− mice (*open circles*) were also observed after acute intrathecal injection of ACSF (5 μl). Data are expressed as means ± SEM.

Table 2. Thermal hyperalgesia after intraperitoneal administration of drugs

Mouse	Drug mg/kg (i.p.)	Hindpaw stimulus	Area (0–8 hr)	Area (8–168 hr)
wt	PBS	Zymosan	63.0 ± 2.03	593.7 ± 56.20
iNOS −/−	PBS	Zymosan	40.8 ± 2.89	586.1 ± 132.21
wt	L-NIL 15	Zymosan	60.5 ± 2.53	
wt	L-NIL 45	Zymosan	67.6 ± 4.10	
wt	L-NIL 135	Zymosan	65.5 ± 3.01	
wt	Diclofenac 5	Zymosan	34.8 ± 1.32	
iNOS −/−	RE 5	Zymosan	47.3 ± 1.66	
iNOS −/−	RE 15	Zymosan	54.7 ± 0.84	
iNOS −/−	RE 45	Zymosan	60.7 ± 0.83	
wt	PBS	PBS	5.0 ± 1.12	
iNOS −/−	PBS	PBS	3.0 ± 2.50	

Areas [sec*hr] between right and left hindpaws calculated from PWL in two different time intervals from 0 to 8 hr and from 8 to 168 hr in wt (wt) and iNOS-gene deficient (iNOS−/−) mice. Peripheral inflammation was induced with zymosan, and PBS was used as a control. Data are expressed as means ± SEM.

Table 3. Thermal hyperalgesia after intrathecal administration of drugs

Mouse	Drug (i.t.)	Area (0–8 hr)
wt	ACSF	62.0 ± 1.52
iNOS −/−	ACSF	45.1 ± 1.26
wt	L-NIL 0.1 μM	42.1 ± 0.57
iNOS −/−	L-NIL 0.1 μM	43.0 ± 2.96
iNOS −/−	L-NAME 0.1 μM	39.5 ± 1.27
wt	Indo 0.1 mM	60.4 ± 2.22
wt	Indo 1.0 mM	56.4 ± 2.96
wt	Indo 10.0 mM	46.3 ± 3.64
iNOS −/−	Indo 0.1 mM	42.4 ± 0.88
iNOS −/−	Indo 1.0 mM	42.1 ± 1.58
iNOS −/−	Indo 10.0 mM	42.8 ± 1.17
iNOS −/−	RE 45 mg/kg, i.p.	41.5 ± 2.44
	Indo 10.0 mM, i.t.	

Areas [sec*hr] between right and left hindpaws calculated from PWL in the time interval from 0 to 8 hr in wt (wt) and iNOS-gene deficient (iNOS−/−) mice. In every mouse peripheral inflammation was induced with zymosan. Indo, Indomethacin. Data are expressed as means ± SEM.

When zymosan was injected, both PGE_2 and NO degradation products (NO_x,, i.e., NO_2^- and NO_3^-) increased in wt mice. NO_x concentrations reached their maximum already after 1 hr and thus closely paralleled the development of iNOS-dependent hyperalgesia (Fig. 6*A*). By contrast, PGE_2 showed a more prolonged increase, exhibiting a continuous rise over the whole observation period of 4 hr (Fig. 6*B*).

Next we investigated whether the effects of reduced NO formation on nociceptive sensitization might be secondary to a suppression of PGE_2 formation. iNOS−/− mice lacked not only the zymosan-induced increase in NO, but also showed reduced spinal PGE_2 formation. The rise in PGE_2 production was much smaller compared to that in wt mice. As described above the NO donor RE-2047 reconstituted wt-like hyperalgesia in iNOS−/− mice. As shown in Figure 6*A*, RE-2047 not only increased spinal NO_x concentrations, but also restored spinal PGE_2 formation (Fig. 6*B*).

We analyzed whether the expression of COX-1 or COX-2 differs in iNOS−/− and wt mice. Time course of COX-1 and COX-2 mRNA expression and of the PGE_2 concentration in spinal cord tissue was followed for 7 d. As shown in Figure 7*A,* increases in PGE_2 concentrations reached their maximum 8 hr after zymosan injection, declined, and came back to baseline levels after 7 d. In iNOS −/− mice the rise in PGE_2 was again largely suppressed, and only at 8 hr a modest increase in PGE_2 was seen.

In contrast to the striking difference in PGE_2 production seen between iNOS−/− and wt mice, COX-1 and COX-2 mRNA expression were very similar in both types of mice. This suggests that the NO-induced rise in PGE_2 levels was attributable to an increase in COX-1 and/or COX-2 enzymatic activity, rather than to altered gene expression.

DISCUSSION

Our data indicate that NO production from iNOS in the spinal cord immediately follows the early induction of peripheral tissue damage and inflammation. This process occurs in parallel to the development of thermal hyperalgesia and is required for the increase in spinal PGE_2 production. Our data therefore attribute a decisive role to iNOS in the early phase of development of thermal hyperalgesia.

It appears that NO production immediately after peripheral

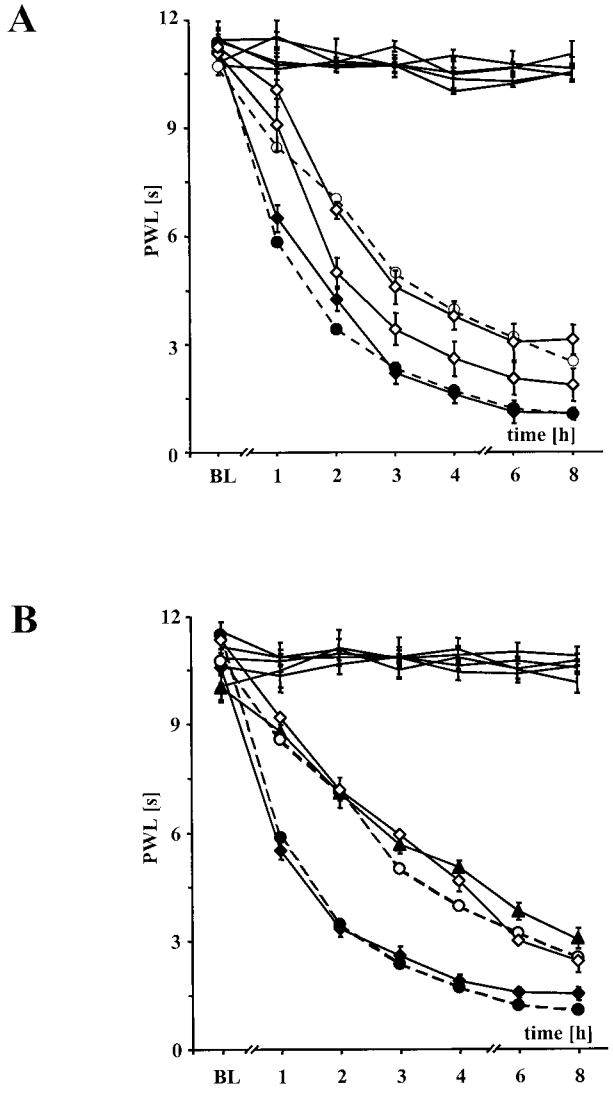

Figure 4. Effects of indomethacin in wt and iNOS−/− mice. *A,* Intrathecal injection of indomethacin, a nonselective COX inhibitor, in ascending doses of 0.1 (*black diamonds*) to 1.0 (*gray diamonds*) and 10.0 mM (*open diamonds*) decelerated the development of thermal hyperalgesia of wt mice. Intrathecal injection was performed to exclude the possibility of a decreased production of prostaglandins in the inflamed hindpaws during systemic application of COX inhibitors. Wt (*black circles*) and iNOS−/− mice (*open circles*) after acute intrathecal injection of ACSF (5 μl). Data are expressed as mean ± SEM. *B,* Intrathecal injection of indomethacin 10.0 mM (*black triangle*) antagonized the development of thermal hyperalgesia after intraperitoneal pretreatment with the NO donor RE-2047 (45 mg/kg). iNOS−/− mice after intraperitoneal pretreatment with 45 mg/kg RE-2047 and after intrathecal injection of ACSF (*black diamonds*) served as controls. Thermal hyperalgesia in iNOS−/− mice was not affected after intraperitoneal pretreatment with PBS and intrathecal pretreatment with indomethacin 10.0 mM (*black triangle*). Wt (*black circles*) and iNOS−/− mice (*open circles*) after acute intrathecal injection of 5 μl of ACSF (*dotted lines*). Data are expressed as means ± SEM.

tissue damage is predominantly iNOS-derived. This is corroborated by our results, which show an induction of iNOS mRNA in the spinal cord as early as 30 min after zymosan injection. Furthermore, administration of L-NAME intrathecally into iNOS−/− mice was without any effect with regard to thermal hyperalgesia. This strongly argues against an extensive contribution of nNOS or eNOS. Observations of Clark et al. (1996), MacNaughton et al. (1998), Haddad et al. (1995), and Salvemini et al. (1995) of an early iNOS expression also support our results. Reconstitution of the fast development of hyperalgesia by the NO donor RE-2047 (Rehse and Ciborski, 1995) demonstrates the importance of early NO production. To relate this to the lack of iNOS genes, we injected the

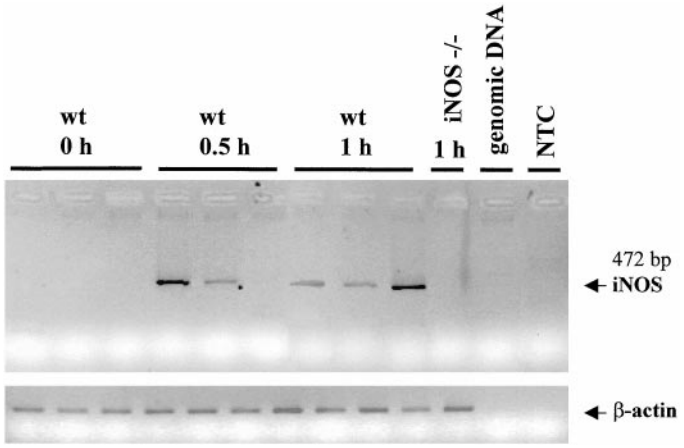

Figure 5. Early iNOS mRNA expression in wt mice. iNOS mRNA expression in spinal cord of wt and iNOS−/− mice after zymosan injection into the right hindpaw. In wt but not in iNOS−/− mice, iNOS mRNA was increased in thoracolumbar segment of spinal cord even 0.5 (3 of 6 mice) and 1 hr (6 of 6 mice) after peripheral inflammation. β-actin RT-PCR product level served as control. This figure represents one of two independent experiments.

selective iNOS inhibitor L-NIL into wt mice; it reduced development of thermal hyperalgesia. The dose of L-NIL chosen (0.1 μM; 5 μl) does not affect the other NOS isoforms (Moore et al., 1994). Furthermore, our results support previous studies showing that selective pharmacological inhibition of iNOS attenuates thermal hyperalgesia in rats (Osborne and Coderre, 1999). Only when given intrathecally did L-NIL inhibit NO production in the spinal cord and result in antinociceptive effects. These data indicate that only spinal iNOS-derived NO production correlates with antinociceptive activity. Interestingly, a substantial part of thermal hyperalgesia was insensitive to inhibition of iNOS and is also considered insensitive to COX inhibition. This insensitivity becomes dominant during the late phase of thermal sensitization ~8 hr after zymosan injection. It is likely that both peripheral mechanisms such as sensitization of capsaicin receptors (Caterina et al., 1997; Kress and Zeilhofer, 1999) and central mechanisms independent from PG and NO contribute to this late phase.

In wt mice, COX inhibition by indomethacin and disruption of the iNOS gene resulted in virtually indistinguishable antinociceptive effects. In iNOS−/− mice, indomethacin failed to display an antinociceptive effect. Antinociceptive activity could be restored in these mice by adding back NO with the NO donor RE-2047. These results demonstrate that the production of spinal PGE_2 requires spinal NO and that spinal iNOS-derived NO appears to mediate thermal hyperalgesia largely if not solely via an increase in spinal PGE_2 formation. In this respect, our data support previous evidence that PGs are key mediators of thermal hyperalgesia (Minami et al., 1994; Ferreira and Lorenzetti, 1996; Yamamoto and Nozaki-Taguchi, 1997). We also confirmed findings that hindpaw inflammation produces enhanced PG levels in the spinal cord (Yang et al., 1996; Hay and de Belleroche, 1997; Ichitani et al., 1997; Yamamoto and Nozaki-Taguchi, 1997; Dirig and Yaksh, 1999; Ebersberger et al., 1999).

In line with several studies, we could show that PG production in the spinal cord is modulated positively by NO release and is diminished by lack of NO production (Salvemini et al., 1994, 1995; Salvemini, 1997; but see also Hamilton and Warner, 1998). Substitution of NO by RE-2047 completely reconstituted PGE_2 production in iNOS−/− mice as well as nociceptive responses in the behavioral tests, indicating once again that iNOS-derived NO amplifies PG production at spinal level. Moreover, the constitutive baseline mRNA expression (Beiche et al., 1996, 1998) and zymosan-induced expression of COX-2 were similar in both types of mice in the dorsal horn of the spinal cord. Consequently, it appears plausible to attribute to iNOS-derived NO a pivotal role as a trigger of spinal enzymatic PG production.

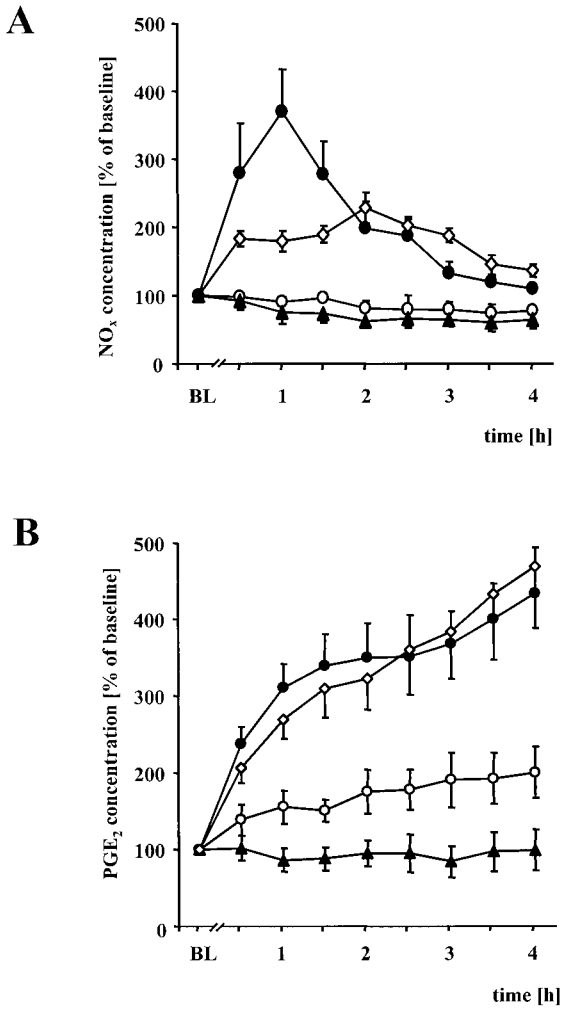

Figure 6. Spinal microdialysis in wt and iNOS−/− mice. *A,* Time course of percentile NO_x level changes in the spinal cord of wt mice (*black circles*) and iNOS−/− mice (*open circles*) after subcutaneous injection of 3.0 mg/ml zymosan into the right hindpaw. Samples were collected via microdialysis of the thoracolumbar segment. In wt mice zymosan injection led to a rapid increase of breakdown products of NO, whereas only a slight increase of NO_x was observed in iNOS−/− mice. After treatment with RE-2047, an NO donor, NO_x levels in the spinal cord of iNOS−/− mice increased (*open diamonds*). *Black triangles* represent NOx levels in wt mice after injection of PBS instead of zymosan into the right hindpaw. Data are expressed as means ± SEM. *B,* Time course of the percentile PGE_2 changes in the spinal cord of wt (*black circles*) and iNOS−/− mice (*open circles*). The additional curve (*black diamonds*) shows the changes of PGE_2 levels after injection of RE-2047 intraperitoneally into iNOS−/− mice. *Black triangles* represent wt mice after PBS administration into the right hindpaw instead of zymosan. Data are expressed as means ± SEM.

PGE_2 and NO production did not run in parallel. NO exhibited a fast peak within ~1 hr, whereas increases in PGE_2 occurred at a markedly prolonged time scale. Our results suggest that PGE_2 production is not attributable to increased COX-1 or COX-2 expression. It may be speculated that the enhanced early PG production is caused by a free radical driven modulation of phospholipase A_2 activity and therefore of the concentration of arachidonic acid, which is the rate-limiting substrate of PG production (but see also Zingarelli et al., 1997; Sahnoun et al., 1998). However, a direct or indirect interaction of NO or one of its metabolites with the COX-1 or COX-2 proteins by enhancing their enzymatic activity is also possible (Salvemini, 1997). In either case one would assume that the NO-induced change in COX enzyme activity outlasts the rise in spinal NO, e.g., via sustained changes in enzyme activity of COX-1, COX-2, or phospholipase A_2.

An intriguing question raised by our study is whether inhibition of iNOS provides a novel target for analgesic therapy. Our experiments have shown that inhibition of iNOS via gene disruption or by selective drugs almost completely abolishes the PG-mediated part of thermal hyperalgesia. With the possible exception of inhibition of resistance to certain microbial agents, iNOS−/− mice are not only viable but also show no major abnormalities indicating that pharmacological inhibition of iNOS may be considered as largely safe. The use of iNOS inhibitors as analgesics would be largely limited by the fact that they would have to be administered very early, before spinal PG production has been triggered.

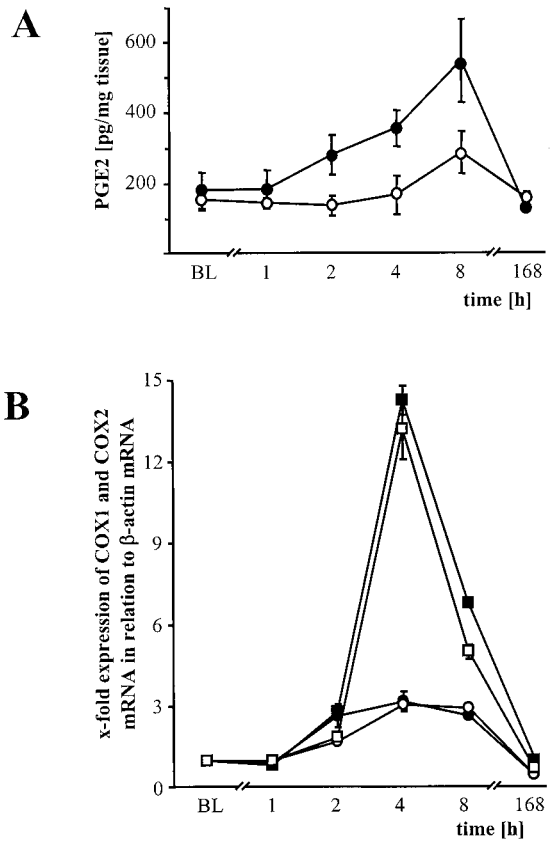

Figure 7. PGE_2 concentration and COX-1 and COX-2 mRNA expression in spinal cord tissue. *A*, Time course of absolute PGE_2 levels in the spinal cord tissue samples of wt (*black circles*) and in iNOS−/− (*open circles*) mice after zymosan injection into the right hindpaw. PGE_2 measurement was performed from tissue samples after perfusion with 100 ml of ice-cold PBS. Data are expressed as means ± SEM. *B*, Time course of real time RT-PCR measurement of spinal COX-1 (*circles*) and COX-2 (*squares*) mRNA expression in wt (*black*) and iNOS−/− (*open*) mice after peripheral administered zymosan. There are no differences between COX mRNA expression in both lines of mice. Data are expressed as means ± SEM.

Our data also indicate that NO donors reaching the CNS may be enhancers of pain development and in this respect be problematic. This observation is in line with findings of Urban et al. (1999), Inoue et al. (1997), and Aley et al. (1998) (but see also Lauretti et al., 1999) and should be subjected to further research.

Our data offer no clues indicating whether the decrease of NO production seen in our animals before hyperalgesia reaches its peak is of importance for the later normalization of hyperalgesia that goes along with healing processes in the damaged tissue (Reichner et al., 1999).

In summary, we have shown that NO generated by iNOS not only plays an important role as a mediator of tissue inflammation acting in peripheral tissue, but that it also serves an important role in the spinal cord, where it triggers PGE_2 formation, enhances COX-2 activity, and facilitates the development of thermal and possibly other forms of hyperalgesia.

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