A Newly Identified Role for Superoxide in Inflammatory Pain

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ABSTRACT

Novel classes of pain-relieving molecules are needed to fill the void between nonsteroidal anti-inflammatory agents and narcotics. Our studies have identified superoxide as a novel mediator of hyperalgesia (clinically defined as an augmented sensitivity to painful stimuli) and have exposed potential pathways through which this radical modulates the hyperalgesic response. The role of superoxide in pain was elucidated using a superoxide dismutase mimetic, M40403 [a manganese(II) complex with a bis(cyclo-hexylpyridine-substituted) macrocyclic ligand]. Intraplantar injection of carrageenan in rats led to time-dependent development of peripheral inflammation [measured parameters of inflammation included paw edema, cytokine release in the paw exudates, nitrotyrosine formation (a marker of peroxynitrite formation and oxidative stress), and poly-ADP-ribose-polymerase activation (the nuclear enzyme activated by superoxide/peroxynitrite)] and hyperalgesia. M40403 blocked all measured parameters of inflammation and hyperalgesia. Furthermore, when given therapeutically (2 h after the induction of hyperalgesia) either by intravenous or intrathecal administration, M40403 but not its inactive congener M40404 inhibited hyperalgesia with a rapid onset of action. Our results also show that, at the level of the spinal cord and time of peak hyperalgesia, endogenous manganese superoxide dismutase was nitrated and subsequently deactivated, losing its capacity to remove superoxide. The antihyperalgesic effects of M40403 were not reversed by naloxone excluding the potential involvement of an opiate pathway. Collectively, these studies have unraveled a critical role for superoxide in the nociceptive signaling cascade both peripherally and centrally. The discovery of this pathway opens a new therapeutic strategy for the development of novel nonnarcotic antihyperalgesic agents.

It is now well appreciated that, under normal circumstances, the biological reactivity of superoxide is kept under the control of superoxide dismutase (SOD) enzymes. These enzymes include the Mn enzyme in mitochondria and the Cu/Zn enzyme present in the cytosol or extracellular surfaces (McCord and Fridovich, 1969). In acute and chronic inflammation, superoxide is produced at a rate that overwhelms the capacity of the endogenous SOD enzyme defense system to remove it. Such an imbalance results in superoxide-mediated injury, as shown in numerous animal models of disease (Fridovich, 1999; Muscoli et al., 2003). Important proinflammatory roles for superoxide include endothelial cell damage and increased microvascular permeability (Droy-Lefaix et al., 1991), release of cytokines (Salvemini et al., 1999; Matata and Galinanes, 2002), recruitment of neutrophils at sites of inflammation (Boughton-Smith et al., 1993; Salvemini et al., 1999), single-strand DNA damage (Dix et al., 1996), and poly-ADP-ribose-polymerase (PARP) activation (Inoue and Kawanishi, 1995). Furthermore, superoxide rapidly combines with nitric oxide (NO), removing an important homeostatic signaling molecule and at the same time forming peroxynitrite, a potent cytotoxic and proinflammatory agent (Beckman et al., 1990). One detrimental action of peroxynitrite that results in a loss of function and subsequently elevates the levels of superoxide is the nitration and oxidation of proteins such as manganese superoxide dismutase (MnSOD) (Yamakura et al., 1998; MacMillan-Crow and Cruthirds,

ABBREVIATIONS: SOD, superoxide dismutase; PARP, poly-ADP-ribose-polymerase; NO, nitric oxide; MnSOD, manganese superoxide dismutase; SODm, superoxide dismutase mimetic; TNF, tumor necrosis factor; IL, interleukin; PAR, poly-ADP-ribose; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline/Tween 20; NBT, nitro blue tetrazolium.

2001). The removal of superoxide by the native SOD enzymes (Oyanagui, 1976), synthetic superoxide dismutase mimetics such as Mn(III)tetrakis (4-benzoic acid) porphyrin (Cuzzocrea et al., 1999), or M40403 (Salvemini et al., 1999) has been shown to lessen undesired side effects of inflammation. Inflammation is a key component of pain, as highlighted by the effectiveness of the nonsteroidal anti-inflammatory agents in acute inflammatory pain. Although the role of superoxide in the molecular pathways that induce pain is not known, the participation of this molecule in inflammation led us to hypothesize that superoxide is a critical mediator in nociception. For this purpose, we have tested the effects of a superoxide dismutase mimetic (SODm), M40403, and its inactive SODm congener M40404 in models of inflammatory pain. M40403 is a stable, low molecular weight, manganese-containing, nonpeptidic molecule possessing the function and catalytic rate of native superoxide dismutase enzymes but with the advantage of being a much smaller molecule (MW 483 versus MW 30,000 for the mimetic and native enzyme, respectively) (Salvemini et al., 1999). Furthermore, M40403 is stable in vivo, penetrates cells readily, has wide tissue distribution in rats, is excreted intact with no detectable dissociation, and is recovered in urine and feces intact (Salvemini et al., 1999).

The results presented in this study reveal for the first time that superoxide is a key player in pain. Removal of this mediator is a viable therapeutic target for the development of novel nonnarcotic analgesics.

Materials and Methods

Animals. Male Sprague-Dawley rats (175–200 g) (Harlan, Indianapolis, IN), male Sprague-Dawley rats (250–260 g) purchased with intrathecally implanted cannulas (32 gauge, polyurethane) (Charles River, Raleigh, NC), and male CD-1 mice (28–35 g) (Charles River) were used for these studies. All animals were housed and cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee and the National Institutes of Health guidelines on laboratory animal welfare. The SOD mimetics were synthesized as previously described (Salvemini et al., 1999). Unless specified, all materials were purchased from Sigma-Aldrich (St. Louis, MO). M40403 and M40404 were dissolved in the following vehicle: 26 mM sodium bicarbonate buffered saline, pH 8.1 to 8.3. All other drugs were dissolved in saline. Decomposed superoxide was prepared using a dimethyl sulfoxide solution of potassium superoxide at 2 mM exposed to air for 30 min.

Tail-Flick and Hot Plate Tests. Nociceptive testing was performed by placing the distal third of the tail of a rat in a water bath maintained at 52°C (tail-flick) or by placing rats on a metal surface maintained at 52°C. The latency to withdrawal (tail-flick) or to jumping/licking of a hindpaw (hot plate) was measured once before (control) and at selected time intervals after drug injection. A cutoff latency of 10 s is employed to prevent tissue injury. Groups of at least six rats each received a subcutaneous injection of M40403 (10 mg/kg) or an equivalent volume of vehicle, and testing was performed 15, 30, 45, and 60 min after the injection of the drug or vehicle. Determination of antinociception was assessed between 7:00 and 10:00 AM.

Formalin-Induced Nociception. Mice were allowed to feed ad libitum and housed 5 to 7 per cage in a temperature-controlled room with a 12-h light/dark cycle. Determination of antinociception was assessed between 7:00 and 10:00 AM. Groups consisted of eight mice, and each animal was used for one experimental condition. The antinociceptive effects of M40403 were tested in the formalin-induced hindpaw-licking procedure as described by Hunskaar and Hole (1987). Formalin (20 μl of a 1% stock solution) was injected into the

subplantar region of one hindpaw, and the duration of paw-licking (an index of nociception) was monitored over a period of 0 to 5 (early phase) and 10 to 30 min (late phase) thereafter. M40403 (0.3–10 mg/kg, given subcutaneously, $n = 8$), morphine (10 mg/kg, given subcutaneously, $n = 8$), indomethacin (10 mg/kg, given intraperitoneally, $n = 8$), or an equivalent volume of vehicle was given subcutaneously 40 min before formalin. Results are expressed as paw licking time (s).

Superoxide-Induced Hyperalgesia. Superoxide (final concentration injected, 100 nmol) or an equivalent volume (50 μl) of decomposed superoxide was given by intraplantar injection to lightly anesthetized rats. Drugs or vehicle were given intravenously (2 ml/kg) 15 min before the injection of superoxide. Hyperalgesic responses to heat were measured at the time of maximal hyperalgesic effect. Time course studies revealed that maximal hyperalgesia was reached at 20 min after superoxide injection.

Carrageenan-Induced Edema and Hyperalgesia. Rats received a subplantar injection of carrageenan (0.1 ml of a 1% suspension in 0.85% saline) into the right hindpaw of lightly anesthetized rats [CO_2 (80%), O_2 (20%)]. Drugs or vehicle were administered intravenously (2 ml/kg), subcutaneously (1 ml/kg), or intrathecally (in 10 μl) at 2 h postcarrageenan injection (therapeutic treatment), and testing was performed 0.25, 0.5, 0.75, 1, 2, and 3 h after drug injection. In some experiments, drugs or vehicle were also given 30 min before carrageenan (prophylactic treatment), and testing was performed every hour up to 5 h postcarrageenan. Changes in paw volume were measured as previously described (Salvemini et al., 1996). Briefly, paw volume was measured with a plethysmometer (Ugo Basile, Comerio, Varese, Italy) immediately prior to the injection of carrageenan and thereafter at hourly intervals for 5 h. Edema was expressed as the increase in paw volume (milliliters) after carrageenan injection relative to the preinjection value for each animal. Results are expressed as paw volume change (milliliters).

Measurements of Thermal Hyperalgesia. Hyperalgesic responses to heat were determined as described by Hargreaves et al. (1988), and a cutoff latency of 20 s was employed to prevent tissue damage in nonresponsive animals. Rats were individually confined to Plexiglas chambers. A mobile unit consisting of a high-intensity projector bulb was positioned to deliver a thermal stimulus directly to an individual hindpaw from beneath the chamber. The withdrawal latency period of injected and contralateral paws was determined to the nearest 0.1 s with an electronic clock circuit and thermocouple. If the animal failed to respond by 20 s, the test was terminated. Each point represents the delta change (s) in withdrawal latency (withdrawal latency of contralateral minus withdrawal latency of injected paw) at each time point. Results are expressed as paw withdrawal latency changes (s).

Determinations of Cytokine Levels in Paw Exudates. Cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6] released in the paw exudates were measured by enzyme-linked immunosorbent assay as described previously (Salvemini et al., 1996). Briefly, at 5 h following the intraplantar injection of carrageenan, rats were sacrificed, and each paw was cut at the level of the calcaneus bone. Paws were gently centrifuged at 250g for 20 min to recover a sample of the edematous fluid. The volume of fluid recovered from each paw was measured. Cytokines were measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN), and results were expressed in picograms per paw, normalizing values to the amount of exudates recovered from each paw. All determinations were performed in duplicate. Previous studies indicated that maximal levels of these cytokines were seen at 5 h postcarrageenan (Salvemini et al., 1996). Therefore, animals were sacrificed at these time points.

Immunohistochemical Localization of Nitrotyrosine and Poly-ADP-Ribose (PAR) in the Carrageenan-Inflamed Rat Hindpaw. Indirect immunofluorescence staining was performed on 7-μm thick sections of rat paw tissues embedded in Paraplast (Sherwood Medical, Norfolk, NE). After deparaffinization, endoge-

nous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin. Sections were incubated overnight with 1) anti-nitrotyrosine rabbit polyclonal antibody (1:500 in PBS) and 2) anti-poly (ADP-ribose) goat polyclonal antibody (1:500 in PBS). Sections were washed with PBS and incubated with secondary antibody (fluorescein isothiocyanate-conjugated anti-rabbit and tetramethyl-rhodamine isothiocyanate-conjugated anti-goat) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 2 h at room temperature. Sections were washed as before, mounted with 90% glycerol in PBS, and observed with a Nikon RCM8000 confocal microscope equipped with a $40\times$ oil objective. To confirm that the immunoreaction for the nitrotyrosine was specific, some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity. To verify the binding specificity for PAR, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations, no positive staining was found in the sections, indicating that the immunoreaction was positive in all the experiments carried out.

Histological Examination of the Carrageenan-Inflamed Rat Hindpaw. For histopathological examination, biopsies of paws were taken 2 and 5 h following the intraplantar injection of carrageenan. Tissue from the pads of the rats' hindpaw was removed with a scalpel. Tissue samples were fixed in Dietric solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid) for 1 week at room temperature, dehydrated by graded ethanol, and embedded in Paraplast (Sherwood Medical). Tissue sections (thickness, 7 μm) were deparaffinized with xylene, stained with hematoxylin/eosin, and studied using light microscopy (Dialux 22; Leitz, Wetzlar, Germany). All histological studies were performed in a blinded fashion.

Immunohistochemical Detection of Nitrated Proteins in the Spinal Cord. Central modulation of the nociceptive signal takes place at the lumbar tract of the spinal cord. At that level there is the first synapse where peripheral sensitization transmits the signal to the central nervous system. Paraffin-embedded, formaldehyde-fixed rat spinal cord sections (L4/L6) were deparaffinized and rehydrated by standard methods. Following rehydration and immersion in PBS/0.3% Triton X-100 (2 × 5 min), sections were treated for microwave antigen retrieval (2 × 5 min, 1 mM EDTA, 4.5 mM Tris, pH 8.0). Sections were blocked for 1 h with 100% normal horse serum (Vector Laboratories, Burlingame, CA), followed by application of monoclonal anti-nitrotyrosine antibody (1:100 in 100% normal horse serum) at 4°C overnight in a moist chamber. Treatment with secondary antibody, A/B complex, and diaminobenzidine were performed according to the manufacturer's instructions (Vector ABC Elite Kit; Vector Laboratories).

Immunoprecipitation Assay and Western Blot Analysis. Rat lumbar spinal cord enlargements (L4-L6) were homogenized in Lysis buffer (20 mM Tris-base, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1% Chaps, 2 mM EGTA, 1% protease inhibitor cocktail) with a 1:3 w/v ratio. Solubilized extracts were sonicated (5 min) using a sonicator (Fisher Scientific Co., Pittsburgh, PA), and after 10 min of incubation on ice the lysates were centrifuged (12500g, 30 min at 4°C), and supernatants were collected. The supernatants were stored at −80°C immediately. Protein concentration was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL). The solubilized proteins (4 mg) in the 500-μl lysate buffer were incubated with 10 μg of agarose-conjugated anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) for 2 h at room temperature. Agarose beads were collected by centrifugation (12000g, 1 min at 4°C) and washed in PBS (pH 7.4) three times. The mixture of the beads-antibody and binding proteins complex was resuspended in 50 μl of sample buffer [2×, 0.5 M Tris·HCl (pH 6.8), 2.5% glycerol/0.5% SDS/200 mM 2-mercaptoethanol/0.001% bromophenol blue], heated to 95°C (5 min). The samples (50 μl) were then loaded in 12%

SDS-PAGE minigels (Bio-Rad, Hercules, CA). Rat brain treated with peroxynitrite (Upstate Biotechnology) was used as positive control.

After separation by SDS-PAGE, proteins were transferred electrophoretically (200 V, 1.5 h) to nitrocellulose membranes (Bio-Rad). Ponceau Red (Sigma-Aldrich) staining was used to insure successful protein transfer. Membranes were blocked (1 h, room temperature) with blocking solution [1% bovine serum albumin/0.1% thimerosal in 50 mM Tris·HCl (pH 7.4)/150 mM NaCl/0.01% Tween 20 (TBST)]. For detection of MnSOD, blots were incubated with rabbit polyclonal anti-MnSOD (2 h, room temperature, 1:1000 dilution) (Upstate Biotechnology). After washing with TBST, the membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10000 dilution) (Amersham Biosciences Inc., Piscataway, NJ), and the specific complex was detected by an enhanced chemiluminescence detection system (Amersham Biosciences Inc.). Quantization of nitration levels was then performed by densitometry using ImageQuant 5.2 software by Amersham Biosciences Inc. Equal protein loading was determined using β-actin expression as our control. SDS-PAGE was performed using 40 μg of solubilized protein and subsequent transfer to nitrocellulose membrane (Bio-Rad) at 200 V for 1.5 h. Membranes were blocked with blocking solution (1 h, room temperature) and then incubated with mouse monoclonal anti-β-actin (2 h, room temperature, 1:3000 dilution) (Sigma-Aldrich). After washing with TBST, the membranes were incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody (1:20000 dilution) (Amersham Biosciences Inc.), and the specific complex was detected by an enhanced chemiluminescence detection system. No difference for β-actin was detected among the lanes.

Determination of MnSOD Activity in the Spinal Cord. Rats were anesthetized, sacrificed by decapitation, and exsanguinated; the vertebral column was opened, and the spinal cord from L4-L6 was removed, cut in slices, and immediately frozen in N_2 and stored at −80°C for subsequent determination of SOD activity. Tissue samples were homogenized with 10 mM phosphate-buffered saline (pH 7.4) in a Polytron homogenizer and then sonicated on ice for 1 min (20 s, three times). The sonicated samples were subsequently centrifuged at 1100g for 10 min. SOD activity was measured in the supernatants as previously described (Beauchamp and Fridovich, 1971; Nishida et al., 2002), with some modifications. Briefly, a competitive inhibition assay was performed that used xanthine-xanthine oxidase-generated superoxide to reduce nitro blue tetrazolium (NBT) to blue tetrazolium salt. The reaction was performed in sodium carbonate buffer (50 mM, pH 10.1) containing EDTA (0.1 mM), nitro blue tetrazolium (25 μM) (Sigma-Aldrich), xanthine (0.1 mM), and xanthine oxidase (2 nM) (Boehringer Ingelheim GmbH, Ingelheim, Germany). The rate of NBT reduction was monitored spectrophotometrically (PerkinElmer Lambda 5 spectrophotometer; PerkinElmer Life and Analytical Sciences, Milan, Italy) at 560 nm. The amount of protein required to inhibit the rate of NBT reduction by 50% was defined as one unit of enzyme activity. Cu,Zn-SOD activity was inhibited by performing the assay in the presence of 2 mM NaCN after preincubation for 30 min. Enzymatic activity was expressed in units per milligram of protein.

Rotarod Test. Rats were placed on a rotating rod (diameter, 7 cm) turning at 10 rpm. The animals were exposed to the rotarod for one session of 180 s each day for 3 days to adapt the rats to the apparatus. The rats were then injected subcutaneously with SODm (100 mg/kg) and tested again on the rotarod for periods of approximately 180 s at selected time intervals.

Statistical Analysis. Results are shown as mean ± S.E.M. for n animals. Unless specified, statistical analysis was done using analysis of variance followed by Student-Newman-Keuls test.

Results

Inhibition of Carrageenan-Induced Edema and Hyperalgesia by M40403. Using a well established model of inflammatory hyperalgesia (carrageenan-induced inflamma-

tion and hyperalgesia), the putative role of superoxide in nociception associated with inflammation was investigated. Hyperalgesic responses defined as augmented pain intensity in response to painful stimuli seen upon intraplantar injection of carrageenan involve central and peripheral sensitization (Hargreaves et al., 1988; Urban and Gebhart, 1999). Intraplantar injection of carrageenan leads to a time-dependent development of inflammation and hyperalgesia, which peaks within 2 to 3 h and lasts for another 6 to 8 h (Hargreaves et al., 1988; Salvemini et al., 1999). Administration of M40403 (1–10 mg/kg, $n = 6$) prior to carrageenan injection inhibited the development of edema and thermal hyperalgesia in a dose-dependent manner at all time points. Dose-dependent inhibition curves for inflammation and pain taken at 5 h after carrageenan are shown in Fig. 1a. Dose-dependent inhibition of edema and hyperalgesia were associated with dose-dependent inhibition of the known proinflammatory and pronociceptive cytokines, tumor necrosis factor-α, interleukin-1β, and interleukin-6 ($n = 6$; Fig. 1, b–d). When tested at the same time point and at the highest dose used (10 mg/kg), the inactive SOD catalyst M40404 did not inhibit edema, thermal hyperalgesia ($n = 6$; data not shown), or cytokine release (Fig. 1, b–d). The potential participation of peroxynitrite in superoxide-mediated nociception was evaluated by immunohistochemical detection of nitrated proteins and PARP activation. At the time of maximal inflammation and hyperalgesia (5 h), nitrated proteins are readily detected
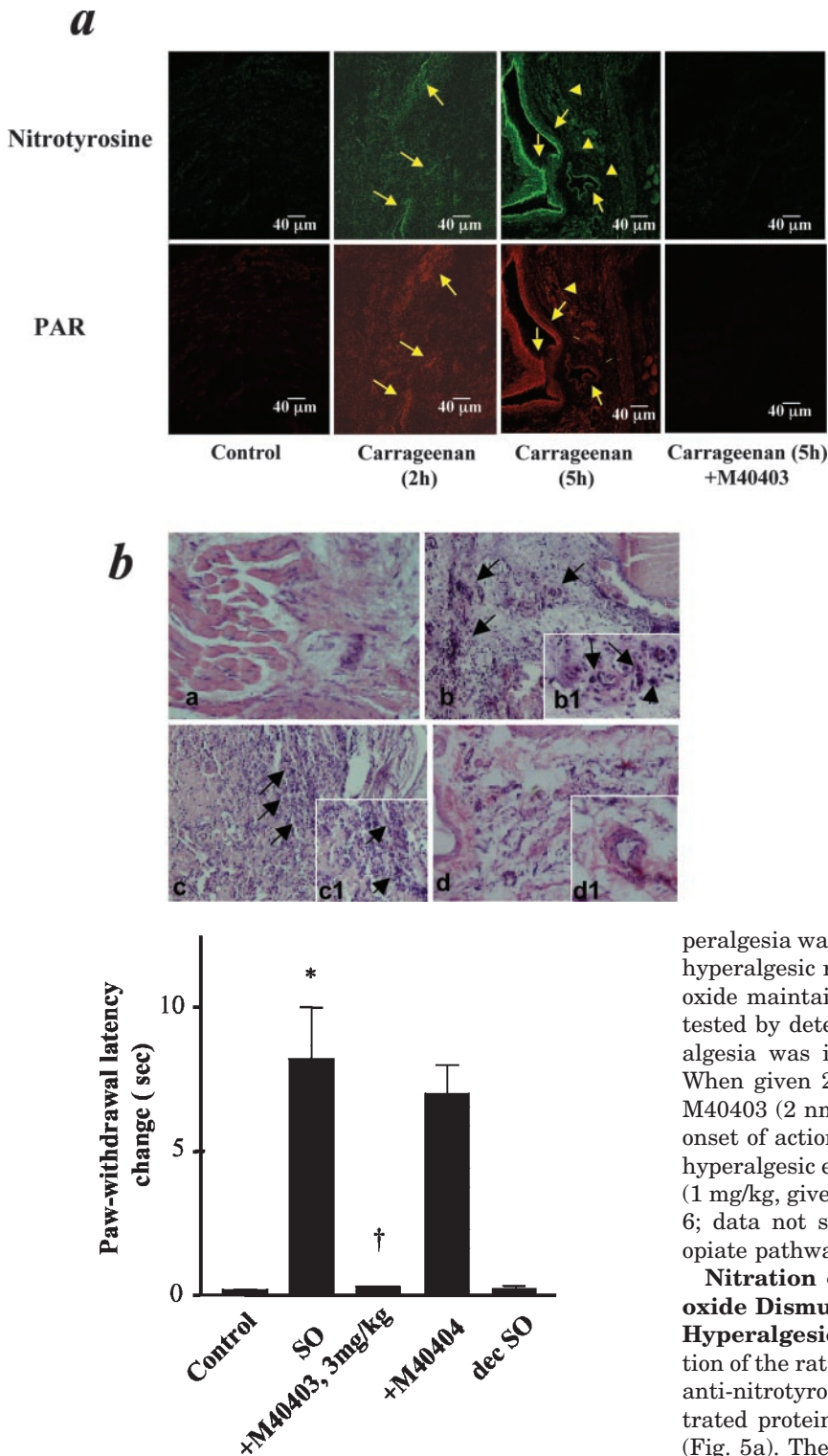
in the inflamed paw and are associated with PARP activation (Fig. 2a). The formation of nitrated proteins and PARP activation was blocked by M40403 (Fig. 2a). Furthermore, the degree of inflammation as determined histologically was reduced by M40403 (Fig. 2b).

The role of superoxide in inducing a hyperalgesic response was further confirmed by showing that intraplantar injection of exogenous superoxide causes pain. Injection of potassium superoxide (100 nmol, $n = 6$) but not decomposed superoxide ($n = 6$) evoked an immediate hyperalgesic response (within 5 min) to noxious heat that peaked at approximately 20 min and then subsided (not shown). The development of thermal hyperalgesia seen after superoxide injection at the time of peak hyperalgesia was inhibited by M40403 (3 mg/kg, $n = 6$; Fig. 3) but not by the catalytically inactive superoxide dismutase mimetic M40404 (3 mg/kg, $n = 6$; Fig. 3).

Release of Superoxide in the Spinal Cord Contributes to Carrageenan-Induced Hyperalgesic Responses. The central involvement of superoxide in the induction of hyperalgesic responses was defined by administering M40403 after the inflammatory response had developed. Results shown in Fig. 4a show that when given 2 h after carrageenan, intravenous injection of M40403 (0.3–3 mg/kg) produced a time-related and dose-dependent inhibition of hyperalgesia, which was rapid in onset (70–80% inhibition within 15 min), maximal at 1 h ($IC_{50} = 2.6$ mg/kg), and sustained for 3 h (Fig. 4a). The slopes for the time course of
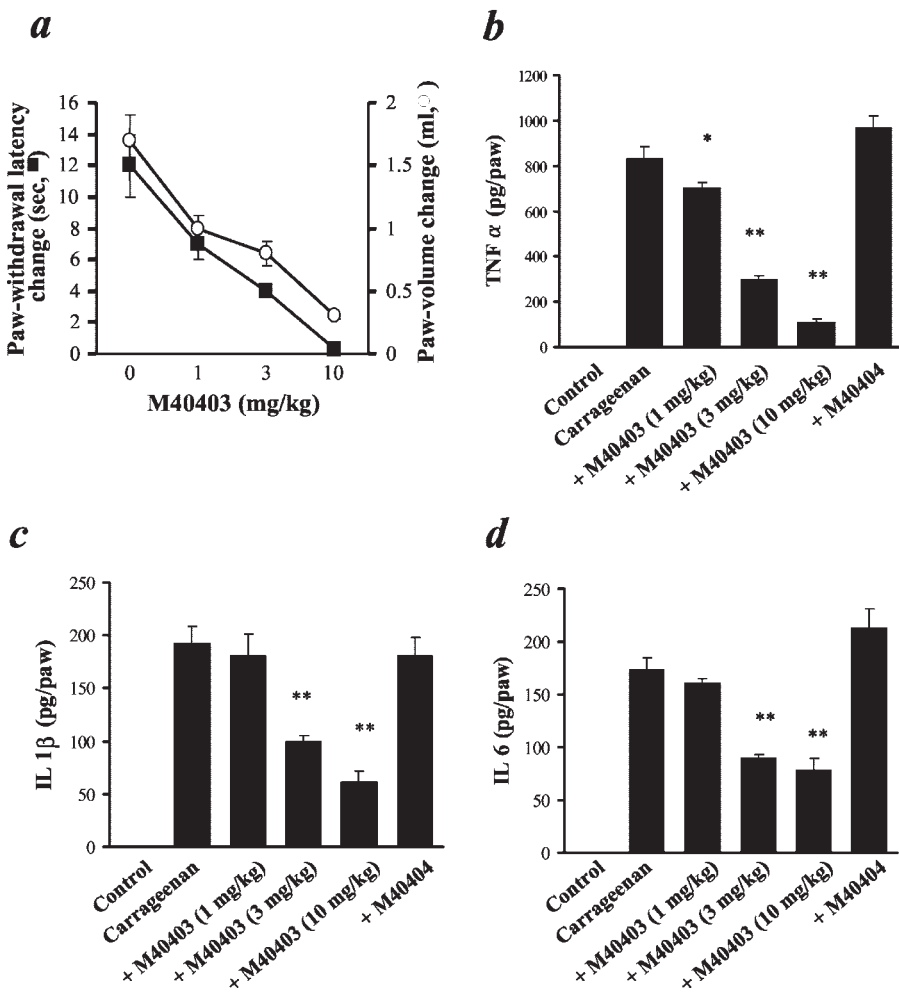
Fig. 1. Antihyperalgesic profile of the superoxide dismutase mimetic M40403. a, M40403, when given intravenously 30 min before carrageenan injection (prophylactically), inhibited edema and hyperalgesia in a dose-dependent manner, as measured at 5 h ($n = 6$). At the same time point, M40403 inhibited the following cytokines in a dose-dependent manner (1–10 mg/kg, $n = 6$): b, TNF-α; c, IL-1β; and d, interleukin-6. The inactive SOD mimetic M40404 did not inhibit cytokine release (b–d). $*$, $P < 0.05$ and $**$, $P < 0.001$ compared with carrageenan alone.
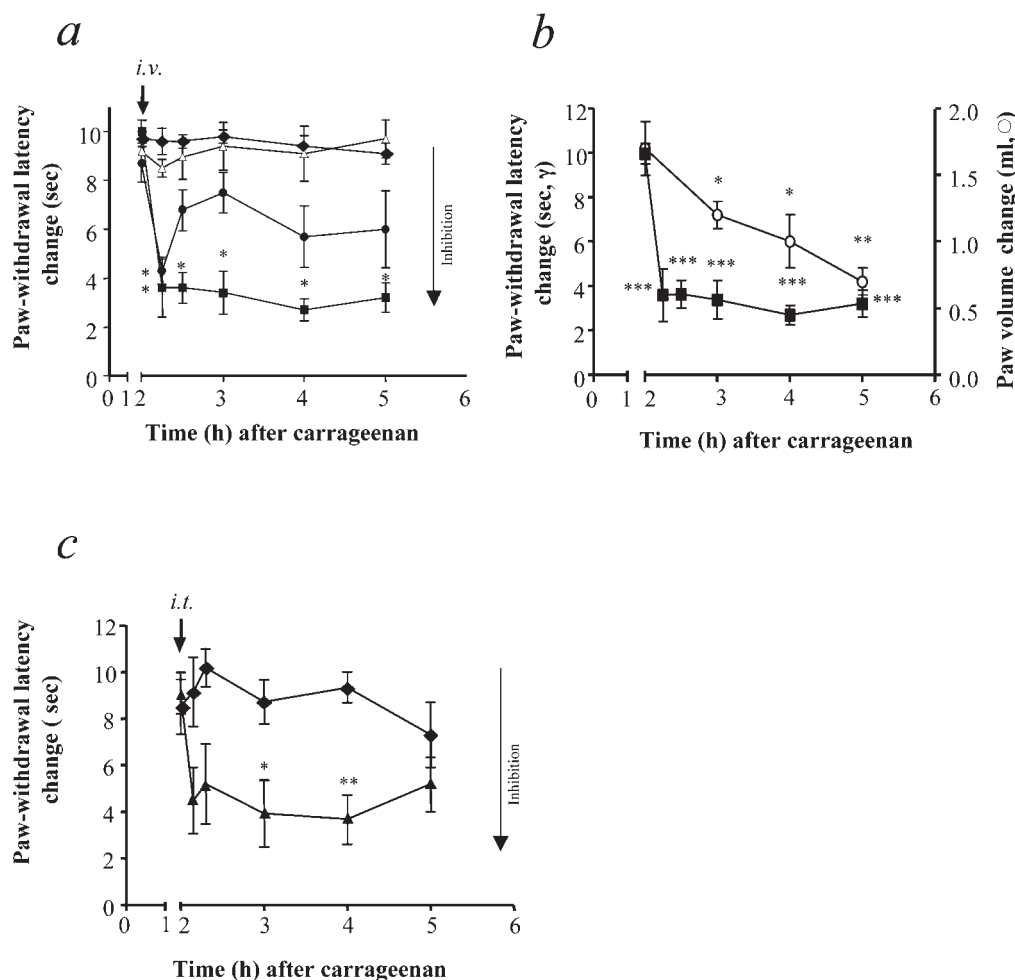
Fig. 2. M40403 inhibits nitrotyrosine formation and PARP activation in the carrageenan-inflamed rat hindpaw. No positive staining for nitrotyrosine and PAR was found in the paw section from control animals (a). At 2 and 5 h after carrageenan, immunohistochemical analysis for nitrotyrosine and PAR shows positive staining mainly localized to the vessels (arrows) and in the infiltrated inflammatory cells (arrowhead) in the tissue section collected at 2 and 5 h after carrageenan administration. The intensity of the positive staining for nitrotyrosine and PAR was significantly reduced by M40403 (given intravenously 30 min before carrageenan injection). Original magnification: 145×. Figure is representative of at least three experiments performed on different experimental days. Overall protective effect of M40403 as determined by histological examination (b); no histological modification was observed in paw tissue from sham-treated rats (a). Representative paw tissue sections collected at 2 (b, b1) and 5 h (c, c1) after carrageenan administration demonstrate marked inflammatory changes, including pronounced cellular infiltration (see arrows). These events were reduced by M40403 (d, d1). Original magnification: 195×. Figure is representative of at least three experiments performed on different experimental days. M40403 was given by intravenous injection 30 min before carrageenan.

Fig. 3. Direct hyperalgesic effects of superoxide. Superoxide (SO), when given by intraplantar injection, causes hyperalgesia, which is blocked by M40403 (3 mg/kg, $n = 6$) but not by the same dose of the inactive SODm M40404 ($n = 6$). ∗, $P < 0.001$ compared with control and †, $P < 0.001$ compared with SO. All drugs were given by intravenous injection 15 min before superoxide.

peralgesia was quick in onset, we hypothesized that once the hyperalgesic response had initiated, spinal release of superoxide maintains the nociceptive input. This hypothesis was tested by determining whether carrageenan-induced hyperalgesia was inhibited by intrathecal injection of M40403. When given 2 h after carrageenan, intrathecal injection of M40403 (2 nmol, $n = 6$) reversed hyperalgesia with a rapid onset of action (Fig. 4c), verifying our hypothesis. The antihyperalgesic effects of M40403 were not affected by naloxone (1 mg/kg, given subcutaneously 30 min prior to M40403, $n = 6$; data not shown) excluding potential involvement of an opiate pathway.

Nitration of Spinal Endogenous Manganese Superoxide Dismutase: An Important Event in Maintaining Hyperalgesic Responses. Immunohistochemical evaluation of the rat spinal cords using affinity-purified monoclonal anti-nitrotyrosine antibodies revealed the presence of nitrated proteins in motor neurons in the spinal cord layers (Fig. 5a). The staining for nitrated proteins was nearly undetectable in saline-treated animals and significantly diminished upon M40403 treatment (Fig. 5a). Data in Fig. 5b reveal that the endogenous mitochondrial form of SOD, MnSOD, is nitrated (2 h, 2.6-fold increase from control as determined by densitometry) at the time of near-to-maximal hyperalgesia (Fig. 5c). Once nitrated, endogenous MnSOD is deactivated, losing its ability to remove superoxide (Fig. 5d). Pretreatment of rats with M40403 (10 mg/kg, given subcutaneously 30 min before carrageenan, $n = 6$), prevented MnSOD nitration (Fig. 5b) and inhibited hyperalgesia (Fig. 5c).

reversal of hyperalgesia and inflammation were dissociated; thus, inhibition of hyperalgesia was rapid in onset, whereas inhibition of inflammation was gradual (Fig. 4b). Based on the observation that the onset of reversal of established hy-

a

b

c

Fig. 4. M40403, when given at a time of maximal hyperalgesia, blocks nociception. a, M40403, when given intravenously 2 h after carrageenan at 0.3 mg/kg ($\triangle$, $n = 4$), 1 mg/kg ($\bullet$, $n = 11$), or 3 mg/kg ($\blacksquare$, $n = 16$) inhibits carrageenan-induced hyperalgesia ($\blacklozenge$, $n = 20$) with a rapid onset of action. $*$, $P < 0.001$ compared with carrageenan alone. b, when tested at the highest dose (3 mg/kg), M40403 (given at 2 h postcarrageenan) inhibited edema, but the time course of inhibition was different from the one seen for antihyperalgesic responses. $*$, $P < 0.05$; $**$, $P < 0.01$; and $***$, $P < 0.001$ when compared with the value obtained at 2 h postcarrageenan before drug injection. Statistical analysis was done using unpaired Student's t test. c, M40403, when given intrathecally (2 nmol, $\blacktriangle$) 2 h after carrageenan, inhibits carrageenan-induced hyperalgesia ($\blacklozenge$) with a rapid onset of action ($n = 6$). $*$, $P < 0.05$ and $**$, $P < 0.01$ compared with carrageenan alone. Statistical analysis was done using unpaired Student's t test.

At the 5-h time point, the nitrated protein is reduced nearly to control values (1.1-fold increase from control as determined by densitometry). Despite the decrease in the levels of nitration, the activity of the protein remains significantly lower than the saline controls (Fig. 5d). Inactivation of Mn-SOD is therefore associated with the hyperalgesic response.

Lack of Effect of M40403 on Basal Acute Nociception in Naive Animals. In rats or mice, M40403 (up to 100 mg/kg, $n = 6$; not shown) has no analgesic activity as measured in the hot plate/tail-flick assays, suggesting that superoxide does not play a role in normal nociception. This concept was confirmed using the formalin test. Subplantar injection of formalin in mice results in an early (phase I) and late (phase II, seen 10–20 min postformalin) response. The early response, which usually lasts <5 min, occurs a few seconds after the formalin injection and is characterized by intense licking or lifting of the injected paw. This phase is believed to represent a direct irritant effect of formalin on sensory C-fibers and is blocked by opioids but not by antiinflammatory agents (Hunskaar and Hole, 1987). M40403, when given 40 min prior to formalin at doses previously shown to inhibit carrageenan-induced hyperalgesia, failed to inhibit this phase (not shown). Phase I was blocked by morphine as expected but not by the nonsteroidal anti-inflammatory drug indomethacin (both at 10 mg/kg, $n = 8$; not shown). On the other hand, and as expected from the data gathered in this study, the second phase of the formalin response (known to involve central and peripheral sensitization associated with inflammation (Coderre and Melzack, 1992) is blocked in a dose-dependent manner by M40403 (0.3–10 mg/kg, $n = 8$). Thus, paw licking time (s) evoked by formalin in control mice ($n = 8$) was reduced from 63 ± 5 to 45 ± 2, 15 ± 3, 6 ± 2, and 2 ± 0 in the presence of 0.3, 1, 3, and 10 mg/kg M40403, respectively. Phase II was also blocked by morphine or indomethacin (10 mg/kg, $n = 8$; not shown).

Lack of Effect of M40403 in the Rotarod Test. When given subcutaneously at doses as high as 100 mg/kg, M40403 demonstrated no evidence of loss of motor activity as determined by the rotarod test. Thus, the time for the rats to remain on the rotarod was 182 ± 7, 185 ± 4, 176 ± 5, 180 ± 6, and 177 ± 7 s when measured just before drug injection and subsequently at times 15, 20, 25, and 40 min after drug injection ($n = 6$).

Discussion

Pain is one of the most prevalent conditions limiting productivity and diminishing quality of life (Anonymous, 1995). Novel classes of pain-relieving molecules are needed, particularly to fill the void between nonsteroidal anti-inflammatory agents and narcotics. Here we show that superoxide is a newly identified mediator of pain, providing an opportunity for novel pain management. The cartoon depicted in Fig. 6
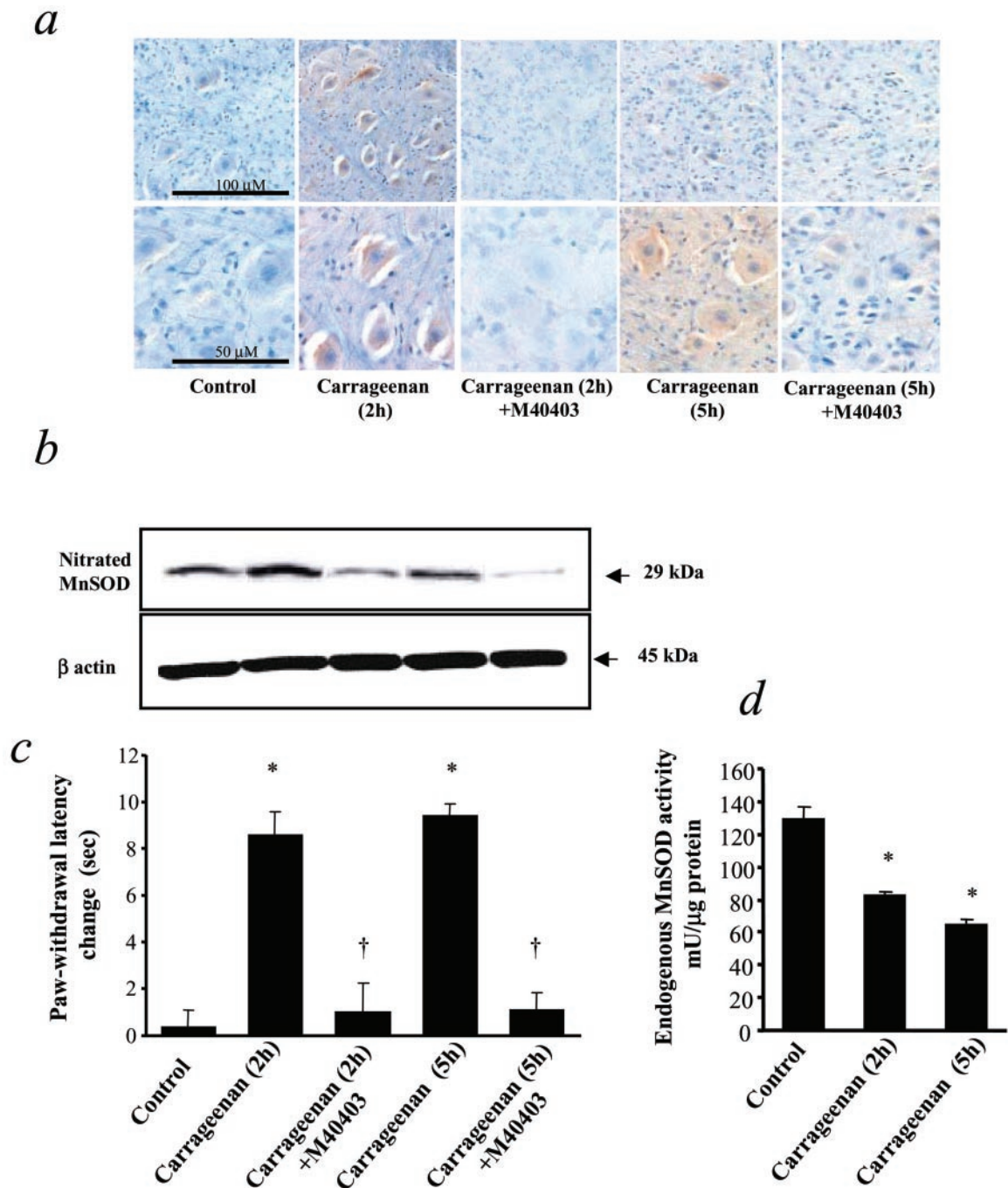
Fig. 5. Inhibition of carrageenan-induced hyperalgesia by M40403 is associated with nitration and subsequent deactivation of spinal cord (L4/L6) MnSOD. Spinal cord sections at the L4-L6 were immunostained with affinity-purified monoclonal anti-nitrotyrosine antibodies (a). Labeling was observed in the motor neurons in the spinal cord layers both at 2 and 5 h postcarrageenan injection. There was no appreciable staining in the saline control sections. The immunostaining of the motor neurons seen after the carrageenan challenge was significantly reduced by M40403. c, carrageenan-induced hyperalgesia seen at the time of peak nociception (2–5 h) is associated with nitration of the endogenous MnSOD at the level of the spinal cord (b). M40403 (10 mg/kg, $n = 6$) attenuates spinal MnSOD nitration (b), reducing the hyperalgesic response (c). Immunoprecipitation data shown in panel b is representative of six experiments. Rat brain treated with peroxynitrite was used as positive control (data not shown). β-actin was used for equal loading comparison. *, $P < 0.001$ compared with control and †, $P < 0.001$ compared with carrageenan alone. d, at 2 and 5 h after carrageenan, nitrated MnSOD is deactivated as shown by the reduced capacity of the enzyme to dismute superoxide. M40403 was given subcutaneously 30 min before carrageenan.

summarizes the key findings of this investigation. It is well appreciated that, during tissue injury and inflammation, hyperalgesia results from a persistent state of peripheral afferent sensitization that subsequently initiates spinal sensitization through the release of the excitatory amino acid glutamate. The mechanism(s) is complex in nature and involves peripherally and spinally formed inflammatory mediators, including peptides, prostanoids, nitric oxide, cytokines, excitatory amino acids (glutamate), and spinal cord glial cell activation (Wall and Melzack, 2000; Watkins et al., 2001). We now show that superoxide is formed and plays a major role in the development of pain through direct peripheral sensitiza-
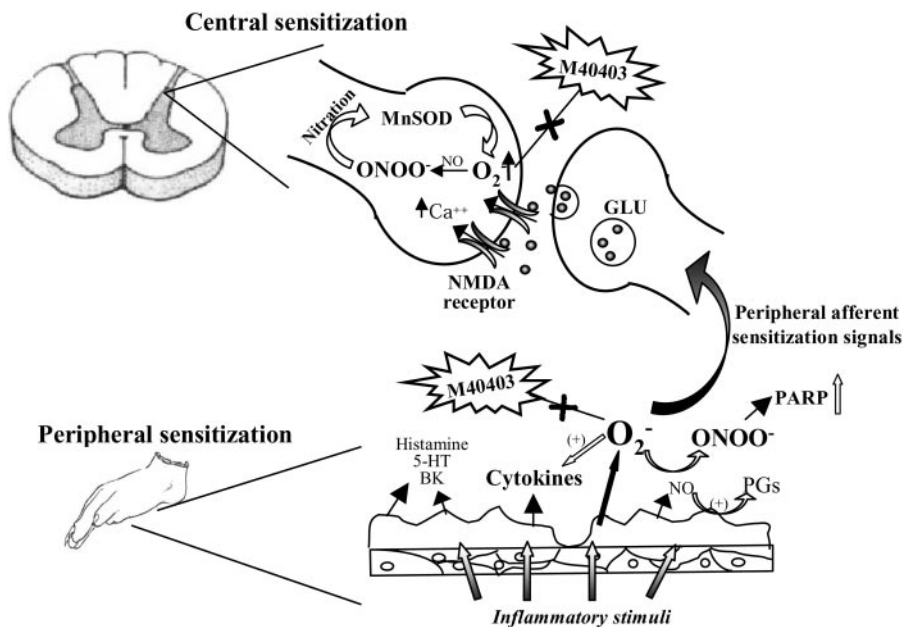
Fig. 6. Proposed role(s) of superoxide in pain. Acute inflammatory hyperalgesia (evoked by an inflammatory stimuli, in this case carrageenan) releases a variety of proinflammatory and pronociceptive mediators, such as bradykinin (BK), serotonin (5-HT), histamine, NO, prostaglandins (PGs), and cytokines. As shown in our study, superoxide is also formed and plays a major role in the development of pain through direct peripheral sensitization, the release of various cytokines (for example, TNF-α, IL-1β, and IL-6), the formation of peroxynitrite (ONOO$^-$), and PARP activation. Furthermore, when released at the level of the spinal cord, presumably in response to glutamate (see Discussion), O$_2^-$ in turn interacts with NO to form ONOO$^-$. The latter in turn nitrates and subsequently deactivates MnSOD in the spinal cord. As a consequence, levels of O$_2^-$ remain elevated, favoring the maintenance of nociceptive signaling. The antinociceptive effect of M40403 includes overall inhibition of the effects of superoxide exerted peripherally and centrally.

tion, by promoting inflammation, and by favoring nitration of endogenous MnSOD in the spinal cord. Thus, the antinociceptive effect of M40403 includes overall inhibition of the effects of superoxide exerted peripherally and centrally.

As shown in this study, M40403 exerted a profound anti-inflammatory effect, which included inhibition of edema, cytokine release, formation of peroxynitrite, and PARP activation. Furthermore, as shown in Fig. 1a, inhibition of the inflammatory response closely correlated with inhibition of hyperalgesia. The observation that M40403 inhibits cytokine release is consistent with data obtained in other models of acute (Salvemini et al., 1999) and chronic inflammation (Salvemini et al., 2001). A mechanism by which M40403 blocks cytokine release is by preventing the activation of redox-sensitive transcription factors, including nuclear factor-κB and activator protein-1, by superoxide, which in turn regulates the genes that encode (among numerous things) various proinflammatory and pronociceptive cytokines (Gius et al., 1999; Matata and Galinanes, 2002). Other than the well recognized role of these cytokines in inflammation, the same inflammatory molecules play a role in pain, as shown by their ability to directly induce hyperalgesia (Watkins et al., 2001). Furthermore, when released from activated spinal cord glial cells and other inflammatory cells, these cytokines participate in the induction of pain through several pathways (Watkins et al., 2001).

Another important mechanism by which superoxide dismutase mimetics attenuate inflammation and hyperalgesia is by reducing peroxynitrite formation by removing superoxide before it can react with nitric oxide. This is important since the proinflammatory and cytotoxic effects of peroxynitrite are numerous (Squadrito and Pryor, 1995; Salvemini et al., 1998). Results of our studies suggest that a mechanism by which superoxide modulates hyperalgesia is through the formation of peroxynitrite. Carrageenan injection leads to nitration of proteins, as detected in the periphery and in the spinal cord that was blocked by M40403. There are at least two well described pathways that can lead to protein nitration; one uses peroxynitrite (the reaction product of superox-

ide and nitric oxide) (Beckman et al., 1990), and the other uses hydrogen peroxide and myeloperoxidase (Eiserich et al., 1998). The involvement of these pathways in a particular setting can be dissected pharmacologically by the use of agents that remove superoxide or nitric oxide. Removal of superoxide (for instance, with M40403 or superoxide dismutase) or nitric oxide (for instance, with a nitric-oxide synthase inhibitor) inhibits the formation of peroxynitrite and, indirectly, peroxynitrite-mediated protein nitration, as shown by numerous investigators (Wang and Zweier, 1996; Fries et al., 2003). The finding in our study that nitration was blocked by M40403 supports the role of peroxynitrite and not hydrogen peroxide/myeloperoxidase in this process.

Superoxide and peroxynitrite have been shown to induce DNA single-strand damage, which has been associated with PARP activation (Inoue and Kawanishi, 1995) resulting in the depletion of its substrate NAD$^+$ in vitro and a reduction in the rate of glycolysis. Because NAD$^+$ functions as a cofactor in glycolysis and the tricarboxylic acid cycle, NAD$^+$ depletion leads to a rapid fall in intracellular ATP and, ultimately, cell injury (Szabó and Dawson, 1999). PARP activation has been implicated, for instance, in the development of hyperalgesia seen upon chronic use of opioids (Mayer et al., 1999). Furthermore, substantial evidence provided by the utility of PARP inhibitors exists to support that PARP activation is important in inflammation (Virag and Szabó, 2002). SOD mimetics of the M40403 class reduce PARP activation in models of acute and chronic inflammation (Salvemini et al., 2001), supporting the role of superoxide in this pathway. Here we have found that PARP was in fact activated in the paw at the time of maximal hyperalgesia, and this was blocked by M40403, suggesting that the anti-hyperalgesic effect of M40403 is derived partly by the inhibition of superoxide-driven PARP activation.

In addition to the peripheral role of superoxide described above, we postulated and finally demonstrated in our investigation that superoxide released centrally in response to carrageenan is a key event in the maintenance of nociception. It is well established that glutamate released at the level of

the spinal cord and subsequent activation of the *N*-methyl-D-aspartate receptor, a subclass of excitatory amino acid receptor, is fundamental in the development of hyperalgesic responses associated with pain of various etiologies (Bennett, 2000). It is also well known that *N*-methyl-D-aspartate-receptor activation releases superoxide (Lafon-Cazal et al., 1993) and NO (Kitto et al., 1992), which in turn interacts to form peroxynitrite (Beckman et al., 1990). We noted that the removal of superoxide by M40403 once the hyperalgesic response has been initiated leads to a rapid reversal of hyperalgesia. Taken together, this allowed us to hypothesize that spinal release of superoxide maintains the nociceptive input once the hyperalgesic response has been initiated. Indeed, intrathecal injection of M40403 led to a rapid reversal of hyperalgesia, supporting our hypothesis. As shown in several studies, an important mechanism in maintaining high levels of superoxide and sustaining superoxide-driven pathological effects is nitration and deactivation of endogenous superoxide dismutase, the enzyme that normally lowers the levels of superoxide (Yamakura et al., 1998; MacMillan-Crow and Cruthirds, 2001). Nitration of the enzyme is in fact closely linked to those disease states driven by overproduction of superoxide, for example, ischemia and reperfusion, organ transplantation, shock, and inflammation (MacMillan-Crow and Cruthirds, 2001). In the present study, we have found that at the time of near-to-maximal hyperalgesia, MnSOD was found to be nitrated in the lumbar spinal cord. M40403 blocked MnSOD nitration and hyperalgesia. At the later time point (5h postcarrageenan), the levels of nitration of MnSOD were significantly lower than 2 h postinjection and similar to the saline controls. Despite the decrease in the levels of nitration, the activity of the protein remains significantly lower than the saline controls (Fig. 5d). This data suggest that the activity of the MnSOD maybe inhibited by other posttranslational modifications in addition to nitration, as has been reported previously (MacMillan-Crow and Cruthirds, 2001). Alternatively, and consistent with previous observations (Souza et al., 2000, Aulak et al., 2004; Elfering et al., 2004), the nitrated MnSOD may have been degraded by the proteasome or other proteolytic pathways. Taken together, these results support the concept that inactivation of the MnSOD enzyme is a critical event in the hyperalgesic response, possibly by allowing the levels of superoxide to remain elevated and in turn maintain nociceptive signaling.

Collectively, our studies provide evidence that superoxide is a newly identified mediator of pain (Fig. 6) and that its removal by low molecular weight synthetic enzymes of superoxide dismutase represents a viable therapeutic target for the development of novel nonnarcotic analgesics.

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