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A Newly Identified Role for Superoxide in Inflammatory Pain

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Running Title: Superoxide and hyperalgesia

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Abbreviations*:* NMDA, N-methyl-D-aspartate; PN, peroxynitrite; PBS, phosphate buffered saline; PARP, poly-ADP-ribose-polymerase; SODm, superoxide dismutase mimetic.

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Abstract

Novel classes of pain-relieving molecules are needed to fill the void between the non-steroidal anti-inflammatory agents and the 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, cytokines 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 (two hours 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 at time of peak hyperalgesia, endogenous manganese superoxide dismutase was nitrated and subsequently deactivated, losing its capacity to remove superoxide. The anti-hyperalgesic effects of M40403 were not reversed by naloxone excluding 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

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pathway opens a new therapeutic strategy for the development of novel non-narcotic anti-hyperalgesic agents.

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It is now well appreciated, that under physiological circumstances, the biological reactivity of superoxide is kept under the control of superoxide dismutase (SOD) enzymes. These 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 pro-inflammatory 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 removing an important homeostatic signaling molecule and at the same time forming peroxynitrite (PN), a potent cytotoxic and pro-inflammatory agent (Beckman et al., 1990). One detrimental action of peroxynitrite is the nitration and oxidation of proteins such as MnSOD resulting in loss of function, which subsequently elevates the levels of superoxide (Yamakura et al., 1998; MacMillan-Crow and Cruthirds, 2001). 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 non-

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steroidal 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 M40403 and its inactive SODm congener M40404 in models of inflammatory pain. M40403 is a stable, low molecular weight, manganese-containing, non-peptidic molecule possessing the function and catalytic rate of native superoxide dismutase enzymes, but with the advantage of being a much smaller molecule (MW 483 *vs* 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 non-narcotic analgesics.

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Methods

Animals. Male Sprague-Dawley rats (175-200 g, Harlan, Indianapolis, IN, USA), male Sprague-Dawley rats (250-260 g, Raleigh, NC, USA) purchased with intrathecally-implanted cannulas (32 gauge, polyurethane) and male CD-1 mice (28-35 g, Charles River, USA) 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 in accordance with NIH 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 (St Louis, MO). M40403 and M40404 were dissolved in the following vehicle: 26 mM sodium bicarbonate buffered saline, pH=8.1-8.3. 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 minutes.

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 52ºC (tail flick) or by placing rats on a metal surface maintained at 52^{o}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 cut-off latency of 10 seconds (sec) is employed to prevent tissue injury. Groups of at least 6 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 minutes after injection of the drug or vehicle. Determination of anti-nociception was assessed between 7:00 and 10:00 AM.

Formalin-induced nociception. Mice were allowed to feed *ad libitum* and housed 5-7

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per cage in a temperature-controlled room with a 12-hr light-dark cycle. Determination of anti-nociception was assessed between 7:00 and 10:00 AM. Groups consisted of 8 mice, and each animal was used for one experimental condition. The anti-nociceptive effects of M40403 was tested in the formalin-induced hindpaw licking procedure as described by Hunskaar and Hole (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 the period of 0-5 (early phase) and 10-30 minutes (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 minutes before formalin. Results are expressed as Paw-licking time (sec).

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 (2ml/kg) 15 minutes before injection of superoxide. Hyperalgesic responses to heat were measured at time of maximal hyperalgesic effect. Time course studies revealed that maximal hyperalgesia was reached at 20 minutes 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 hours (h) post-carrageenan injection (therapeutic treatment) and testing performed 0.25, 0.5, 0.75, 1, 2 and 3 h after drug injection. In some experiments, drugs or vehicle were also

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given 30 minutes before carrageenan (prophylactic treatment) and testing performed every hour up to 5 h post carrageenan. Changes in paw-volume were measured as previously described (Salvemini et al., 1996). Briefly, paw-volume was measured with a plethysmometer (Ugo-Basile, 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 (ml) after carrageenan injection relative to the pre-injection value for each animal. Results are expressed as Paw-volume change (ml).

Measurements of thermal hyperalgesia. Hyperalgesic responses to heat were determined as described by Hargreaves (Hargreaves et al., 1988) and a cut off latency of 20 sec was employed to prevent tissue damage in non-responsive animals. Rats were individually confined to plexiglass 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 sec with an electronic clock circuit and thermocouple. If the animal failed to respond by 20 sec the test was terminated. Each point represents the delta change (sec) in withdrawal latency (withdrawal latency of controlateral minus withdrawal latency of injected paw) at each time point. Results are expressed as Paw-withdrawal latency changes (sec).

Determinations of cytokine levels in paw exudates. Cytokines (TNFα, IL1β, IL–6) released in the paw exudates were measured by ELISA 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 250 x g for 20 minutes in order to recover a

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sample of the edematous fluid. The volume of fluid recovered from each paw was measured. Cytokines were measured by ELISA (R&D systems, Minneapolis, MN) and results expressed in pg/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 post carrageenan (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. After deparaffinization, endogenous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 30 minutes. The sections were permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS) for 20 minutes. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 minutes with avidin and biotin. Sections were incubated overnight with 1) anti-nitrotyrosine rabbit polyclonal antibody (1:500 in PBS) and with anti-poly (ADP-Ribose) goat policlonal antibody rat (1:500 in PBS). Sections were washed with PBS, and incubated with secondary antibody (FITC-conjugated anti-rabbit and with TRIC-conjugated anti-goat; Jackson, 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 40X oil objective. In order 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

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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 haematoxylin/eosin and studied using light microscopy (Dialux 22 Leitz). All the 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 de-paraffinized and re-hydrated by standard methods. Following re-hydration and immersion in PBS/0.3% Triton X100 (2 x 5 minutes), sections were treated for microwave antigen retrieval (2 x 5 minutes, 1mM EDTA, 4.5 mM Tris, pH 8.0). Sections were blocked for 1 h with 100% normal horse serum (Vector), followed by application of monoclonal anti-nitrotyrosine antibody (1:100 in 100% normal horse serum) at 4^{o} C overnight in a moist chamber. Treatment with secondary antibody, A/B

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complex, and DAB were performed by 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 1:3 w/v ratio. Solubilized extracts were sonicated (5 minutes) using a Sonicator (Fisher Scientific) and after 10 minutes of incubation on ice the lysates were centrifuged (12500 g, 30 minutes 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). 4 mg of the solubilized proteins in 500 µl lysate buffer were incubated with 10 µg of agarose-conjugated anti-nitrotyrosine antibody (Upstate Biotechnology) for 2 h at room temperature. Agarose beads were collected by centrifugation (1 minute at 12000 x g at 4°C) and washed in PBS (pH 7.4) three times. The mixture of the beads-antibody and binding proteins complex were resuspended in 50 µl of sample buffer [2x, 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 minutes). The samples (50 µl) were then loaded in 12% SDS-PAGE mini-gels (Bio-Rad). 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) 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

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50 mM Tris·HCl, (pH 7.4) /150 mM NaCl/0.01% Tween 20 (TBS/T)]. For detection of MnSOD, blots were incubated with rabbit polyclonal anti-MnSOD (2 h, room temperature, 1:1000 dilution; Upstate Biotechnology). After washing with TBS/T, the membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10000 dilution; Amersham) and the specific complex was detected by an enhanced chemiluminescence detection system (Amersham). Quantization of nitration levels was then performed by densitometry using ImageQuant 5.2 software by Molecular Dynamics (Molecular Dynamics, CA). 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 (200V, 1.5 h). Membranes were blocked (1 h, room temperature) with blocking solution, then incubated with mouse monoclonal anti-β actin (2 h, room temperature, 1:3000 dilution; Sigma). After washing with TBS/T, the membranes were incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody (1:20000 dilution; Amersham) 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 and sacrificed by decapitation and exanguined, the vertebral column was opened and spinal cord from L4-L6 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 minute (20 sec, 3 times). The sonicated samples were subsequently centrifuged at 1,100 g for 10 minutes SOD activity was measured in the supernatants as described

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(Beauchamp and Friedovich 1971; Nishida et al., 2002) with some modifications. In brief, a competitive inhibition assay was performed which used xanthine-xanthine oxidase-generated superoxide to reduce nitroblue tetrazolium (NBT) to blue tetrazolium salt. The reaction was performed in sodium carbonate buffer (50 mM, pH 10.1) containing EDTA (0.1 mM), nitroblue tetrazolium (25 μM; Sigma, Milan, Italy), xanthine and xanthine-oxidase (0.1 mM and 2 nM respectively; Boehringer, Germany). The rate of NBT reduction was monitored spectrophotometrically (Perkin Elmer Lambda 5 Spectrophotometer, Milan, Italy) at 560 nm. The amount of protein required to inhibit the rate of NTB 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 pre-incubation for 30 minutes. Enzymatic activity was expressed in units per milligram of protein.

Rotarod Test. Rats were placed on a rotating rod (7 cm diameter) turning at 10 rpm. The animals were exposed to the rotarod for one session of 180 seconds 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 sec at selected time intervals.

Statistical analysis. Results are shown as mean ± s.e.m. for n animals. Unless specified, statistical analysis was done using ANOVA followed by Student-Newman-Keuls test.

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Results

Inhibition of carrageenan-induced edema and hyperalgesia by M40403. Utilizing a well-established model of inflammatory hyperalgesia, (carrageenan-induced inflammation 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 (Haegreaves et al., 1988; Urban and Gebhart, 1999). Intraplantar injection of carrageenan leads to a time dependent development of inflammation and hyperalgesia, which peaks within 2-3 h and lasts for a subsequent 6-8 h (Hagreaves et al., 1988; Salvemini et al., 1999). Administration of M40403 (1-10 mg/kg, n=6) prior to carrageenan injection inhibited in a dose-dependent manner the development of edema and thermal hyperalgesia at all time points. Dose-dependent inhibition curves for inflammation and pain taken at 5 h after carrageenan are shown in Fig. 1*a*. Dose-dependent inhibition of edema and hyperalgesia were associated with dose-dependent inhibition of the known pro-inflammatory and pro-nociceptive 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. 2*a*). The formation of nitrated proteins and PARP activation were

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blocked by M40403 (Fig. 2*a*). Furthermore, the degree of inflammation as determined histologically was reduced by M40403 (Fig. 2*b*).

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 minutes) to noxious heat that peaked at approximately 20 minutes and then subsided (not shown). The development of thermal hyperalgesia seen after superoxide injection at 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. 4*a* 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 minutes), maximal at 1 h (IC_{50}=2.6 mg/kg), and sustained for 3 h (Fig. 4*a*). The slopes for the time-course of reversal of hyperalgesia and inflammation were dissociated. Thus, inhibition of hyperalgesia was rapid in onset, whereas inhibition of inflammation was gradual (Fig. 4*b*). Based on the observation that the onset of reversal of established hyperalgesia 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

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inhibited by intrathecal injection of M40403. When given at 2 h after carrageenan, intrathecal injection of M40403 (2 nmol, n=6) reversed hyperalgesia with a rapid onset of action (Fig. 4*c*) verifying our hypothesis. The anti-hyperalgesic effects of M40403 were not affected by naloxone (given at 1 mg/kg subcutaneously, 30 minutes prior to M40403, *n*=6; 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. 5*a*). The staining for nitrated proteins was nearly undetectable in saline-treated animals and was significantly diminished upon M40403 treatment (Fig. 5*a*). Data in Fig. 5*b* 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. 5*c*). Once nitrated, endogenous MnSOD is deactivated losing its ability to remove superoxide (Fig. 5*d*). Pre-treatment of rats with M40403 (10 mg/kg, given subcutaneously 30 minutes before carrageenan, n=6), prevented MnSOD nitration (Fig. 5*b*) and inhibited hyperalgesia (Fig. 5*c*). At the 5 h time point, the nitrated protein is reduced to near-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. 5*d*). Inactivation of MnSOD is therefore associated with the hyperalgesic response.

Lack of effect of M40403 on basal acute nociception in naïve animals. In rats or mice, M40403 (up to 100 mg/kg, n=6, not shown) has no analgesic activity as measured in the

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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 response (Phase I) and a late response (Phase II, seen between 10-20 minutes post-formalin). The early response, which usually lasts <5 minutes, 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 anti-inflammatory agents (Hunskaar and Hole, 1987). M40403 when given forty minutes prior to formalin, at doses previously shown to inhibit carrageenan induced hyperalgesia, failed to inhibit this phase (not shown). Phase I was as expected blocked by morphine but not by the non-steroidal 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 (sec) 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. M40403 when given subcutaneously at doses as high as 100 mg/kg, 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±6 177±7 sec when measured just before drug injection and subsequently at times 15, 20, 25 and 40 minutes post drug injection (n=6).

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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 the non-steroidal anti-inflammatory agents and the 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 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) are complex in nature and involve peripherally and spinally formed inflammatory mediators including peptides, prostanoids, nitric oxide, cytokines, excitatory amino acids (glutamate) as well as spinal cord glial cells 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 sensitization, by promoting inflammation, and by favouring nitration of endogenous MnSOD in the spinal cord. Thus anti-nociceptive 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. 1*a*, 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

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(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 NF-κB and AP-1 by superoxide which in turn regulates the genes that encode (amongst numerous things) various pro-inflammatory 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 pro-inflammatory 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 which was blocked by M40403. There are at least two well described pathways which can lead to protein nitration. One uses peroxynitrite, (the reaction product of superoxide and nitric oxide, Beckman et al., 1990) 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 which remove superoxide or nitric oxide. Removal of superoxide (with for instance M40403 or

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superoxide dismutase) or of nitric oxide (with for instance a nitric oxide synthase inhibitor) inhibits as shown by numerous investigators the formation of peroxynitrite and indirectly peroxynitrite-mediated protein nitration (Wang and Zweier, 1996; Fries et al., 2003). The finding that in our study nitration was blocked by M40403 support 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. As 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 (Szabo 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 Szabo, 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 time of maximal hyperalgesia and this was blocked by M40403 suggesting that the anti-hyperalgesic effect of M40403 is derived in part by the inhibition of superoxide driven PARP activation.

Besides the peripheral role of superoxide, described above, we postulated, investigated, and finally demonstrated 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-

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methyl-D-aspartate (NMDA) 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 NMDA-receptor activation releases superoxide (Lafon-Cazal et al., 1993) and nitric oxide (NO, Kitto et al., 1992), which in turn interacts to form peroxynitrite (Beckman, 1990) which, as discussed, in turn nitrates and deactivates key enzymes including endogenous superoxide dismutase (Yamakura et al, 1998; MacMillan-Crow and Cruthirds, 2001). We noted that 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 once the hyperalgesic response had initiated, spinal release of superoxide maintains the nociceptive input. 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 in 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 over production 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 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 post carrageenan), the levels of nitration of MnSOD were significantly lower than 2 hours post-injection and similar to the saline controls. Despite the decrease in the levels of

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nitration the activity of the protein remains significantly lower than the saline controls (Fig. 5*d*). 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 non-narcotic analgesics.

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Footnotes

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Fig. 1 Anti-hyperalgesic profile of the superoxide dismutase mimetic, M40403. ***a*** M40403 when given intravenously 30 minutes before carrageenan injection (prophylactically) inhibited edema and hyperalgesia in a dose-dependent manner as measured at 5 hours (n=6). At the same time point M40403 inhibited in a dose-dependent manner (1-10 mg/kg, (n=6) the following cytokines: ***b*** tumor necrosis factor-α (TNF-α), ***c*** interleukin-1β (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 to carrageenan alone).

Fig. 2 M40403 inhibits nitrotyrosine formation and poly-ADP-ribose-polymerase (PARP) avctivation in the carrageenan-inflamed rat hindpaw. ***a*** No positive staining for nitrotyrosine and for PAR was found in the paw section from control animals. At 2 and 5 h after carrageenan, immunohistochemical analysis for nitrotyrosine and for PAR shows positive staining mainly localized to the vessels (arrows) and in the infiltrated inflammatory cells (arrowhead) in the tissue section collected at 2 h and 5 h after carrageenan administration. The intensity of the positive staining for nitrotyrosine and for PAR was significantly reduced by M40403 (given intravenously 30 minutes before carrageenan injection). Original magnification: x 145. Figure is representative of at least 3 experiments performed on different experimental days. ***b*** Overall protective effect of M40403 as determined by histological examination. No histological modification was observed in paw tissue from sham-treated rats (a). Representative paw tissues sections collected at 2 h (b,b1) and 5 h (c,c1) after carrageenan administration demonstrate

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marked inflammatory changes including pronounced cellular infiltration (see arrows). These events were reduced by M40403 (d,d1). Original magnification: x 195. Figure is representative of at least 3 experiments performed on different experimental days. M40403 was given by intravenous injection 30 minutes before carrageenan.

Fig. 3 Direct hyperalgesic effects of superoxide. Superoxide (SO) when given by intraplantar injection, causes hyperalgesia, and this 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 to control), † *P* < 0.001 (compared to SO). All drugs were given by intravenous injection 15 minutes before superoxide.

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 (Δ, *n*=4), 1 mg/kg (●, *n*=11) or 3 mg/kg (■, *n*=16) inhibits carrageenan (♦, n=20) induced hyperalgesia with a rapid onset of action. **P* < 0.001 (compared to carrageenan alone). ***b*** When tested at the highest dose (3 mg/kg), M40403 (given at 2 h post carrageenan) inhibited edema, but the time course of inhibition was different to the one seen for anti-hyperalgesic responses **P*<0.05, ***P*<0.01 and ****P*<0.001 (when compared to the value obtained at 2 h post carrageenan before drug injection; statistical analysis was done using unpaired Students t-test). ***c*** M40403 when given intrathecally (2 nmol, ▲) 2 h after carrageenan inhibits carrageenan (♦) induced hyperalgesia with a rapid onset of action (n=6). **P*<0.05 and

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**P<0.01 (compared to carrageenan alone; statistical analysis was done using unpaired Student's t-test).

Fig. 5 Inhibition of carrageenan-induced hyperalgesia by M40403 is associated with nitration and subsequent deactivation of spinal cord (L4/L6) manganese superoxide dismutase (MnSOD). ***a*** Spinal cord sections at the L4-L6 were immunostained with affinity purified monoclonal anti-nitrotyrosine antibodies. Labeling was observes in the motor neurons in the spinal cord layers both at 2 and 5 h post carageenan 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 time of peak nociception (2-5h) is associated with ***b*** nitration of the endogenous MnSOD at the level of the spinal cord. M40403 (10 mg/kg, n=6) attenuates spinal MnSOD nitration ***(b)*** reducing the hyperalgesic response ***(c)***. Immunoprecipation data shown in panel ***b*** is representative of 6 experiments. Rat brain treated with PN was used as positive control (data not shown). βactin has been used for equal loading comparison. * P < 0.001 (compared to control) and † P < 0.001 (compared to 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 minutes before carrageenan.

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 pro-

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inflammatory and pro-nociceptive mediators such as bradykinin, (BK), serotonin (5-HT), histamine, nitric oxide (NO) prostaglandins (PGs) and cytokines. As shown in our study, superoxide (SO) is also formed and plays a major role in the development of pain through direct peripheral sensitization, through the release of various cytokines (for example TNFα, IL1β, IL-6), through the formation of peroxynitrite (ONOO-) and through PARP activation. Furthermore, when released at the level of the spinal cord in response to presumably 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 anti-nociceptive effect of M40403 includes overall inhibition of the effects of superoxide exerted peripherally and centrally.

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Figure 1

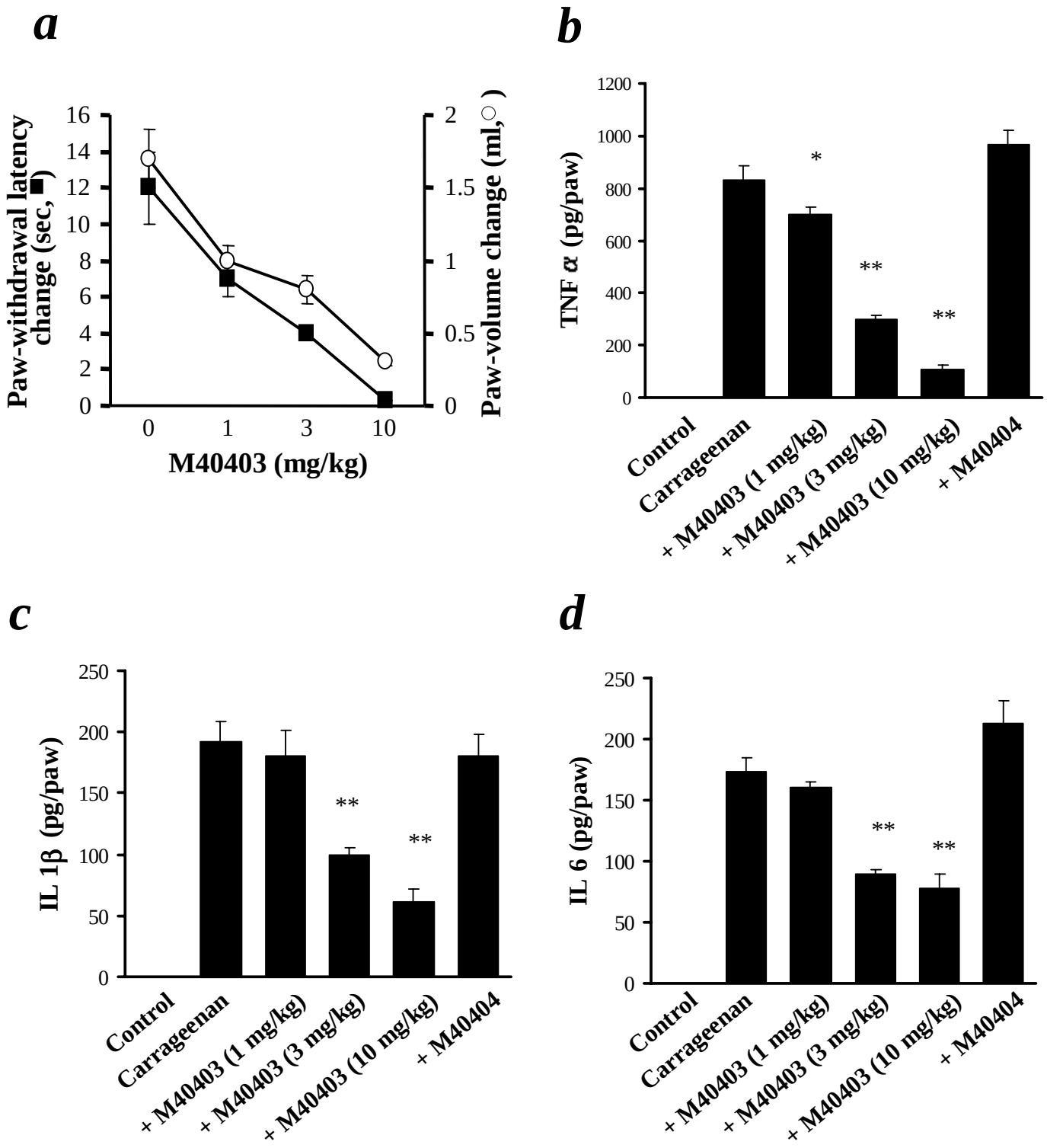

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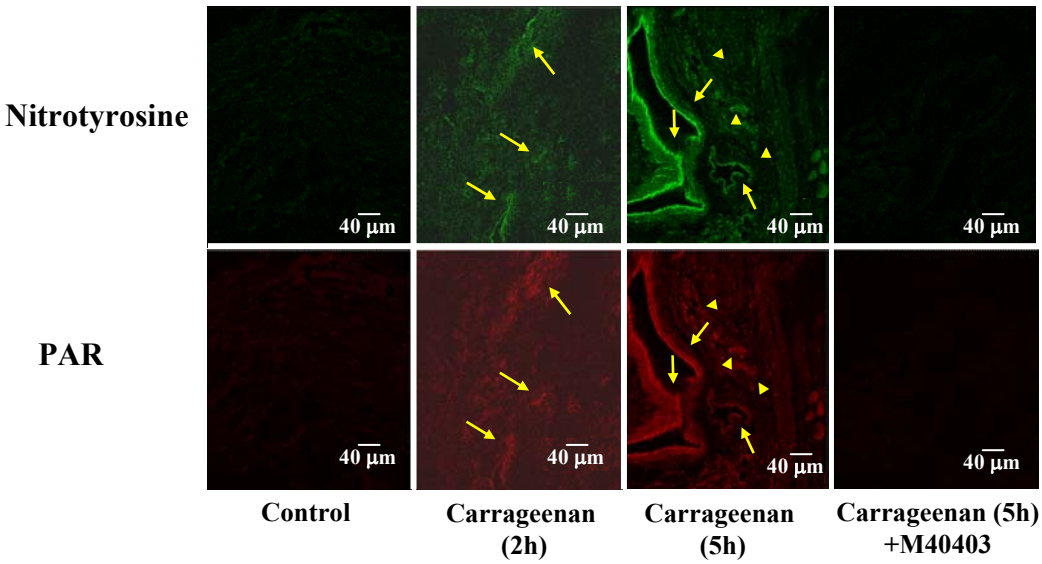
Figure 2

a



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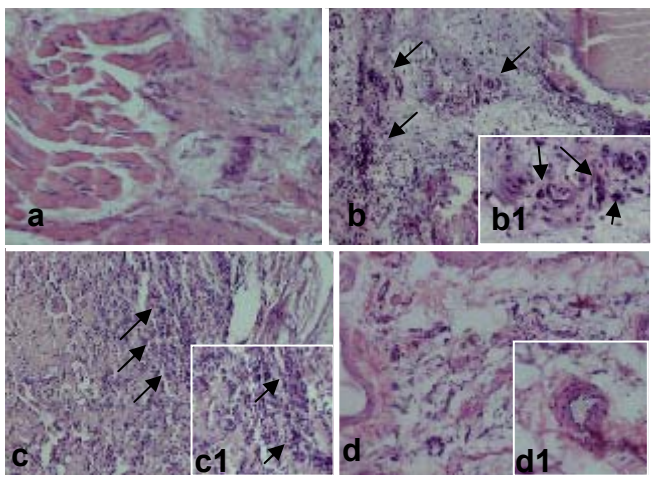

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Figure 3

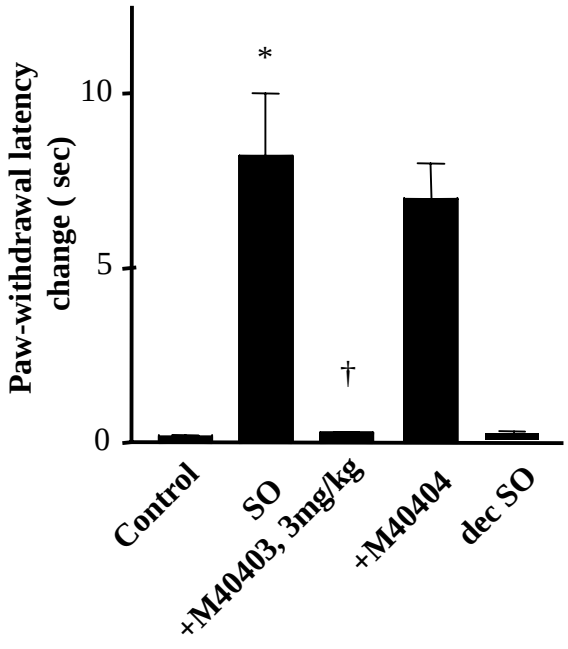

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Figure 4

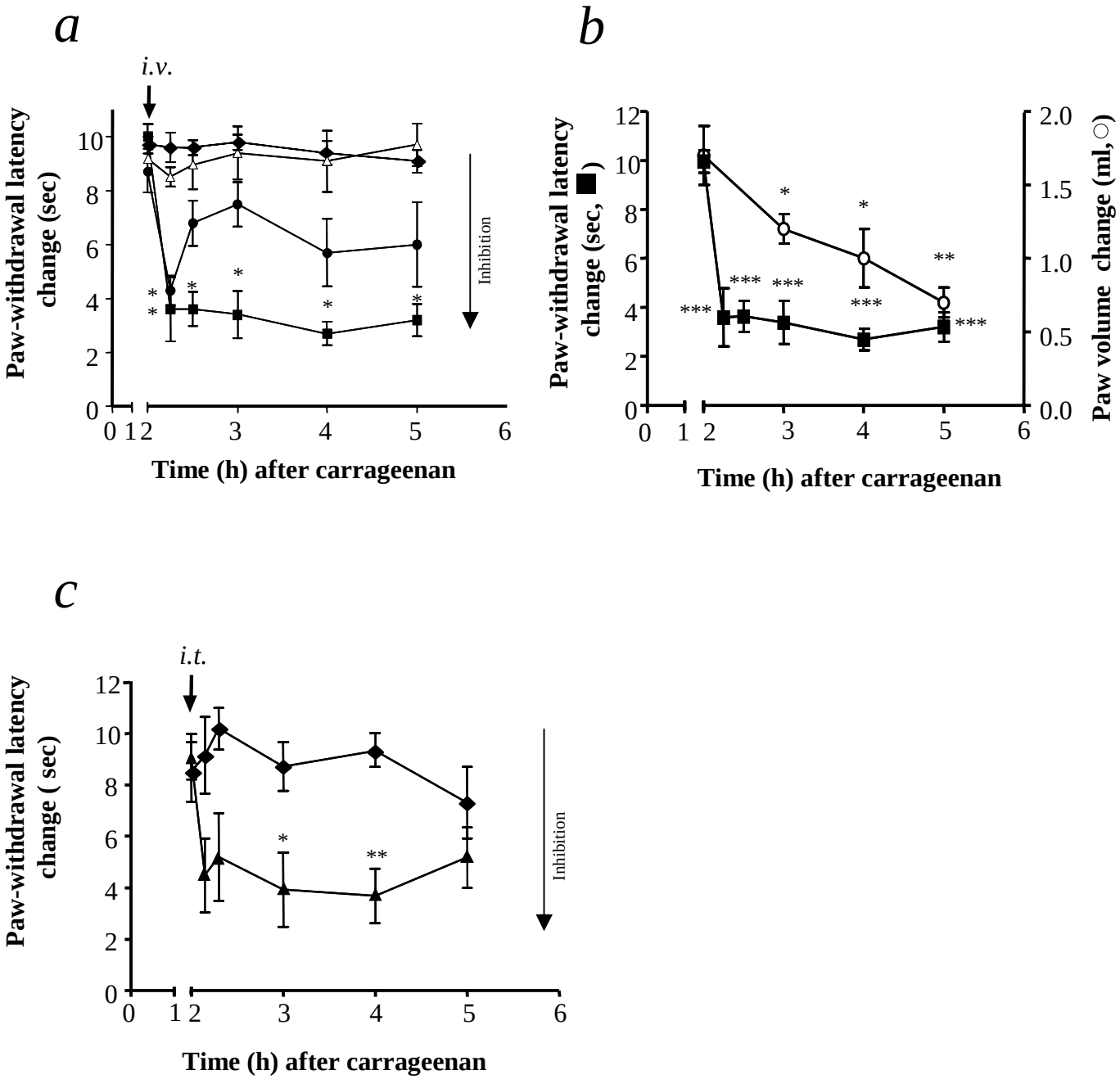

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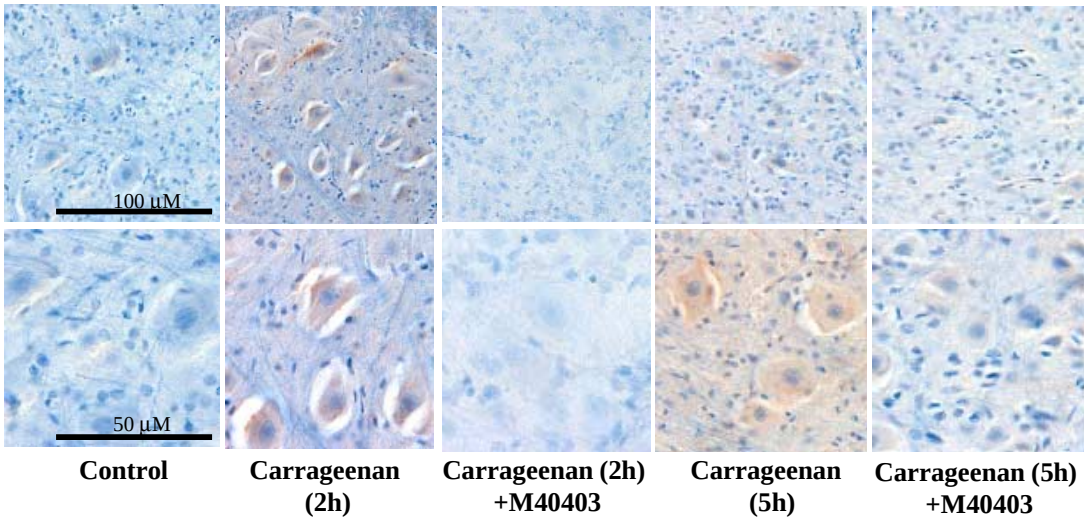
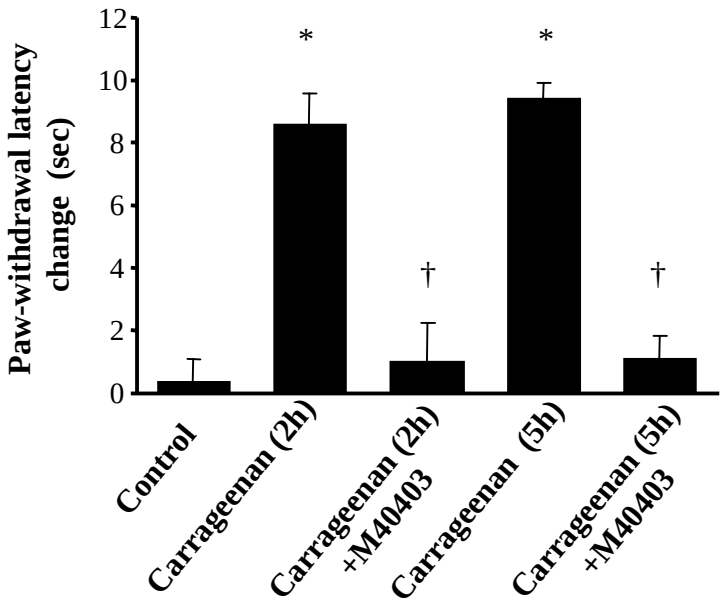
Figure 5

a



b

c



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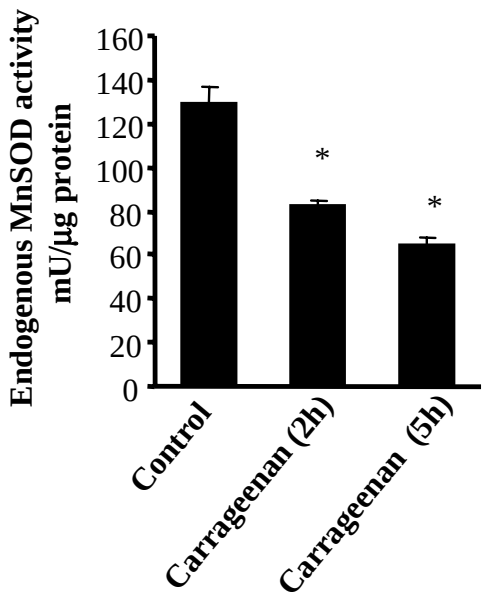

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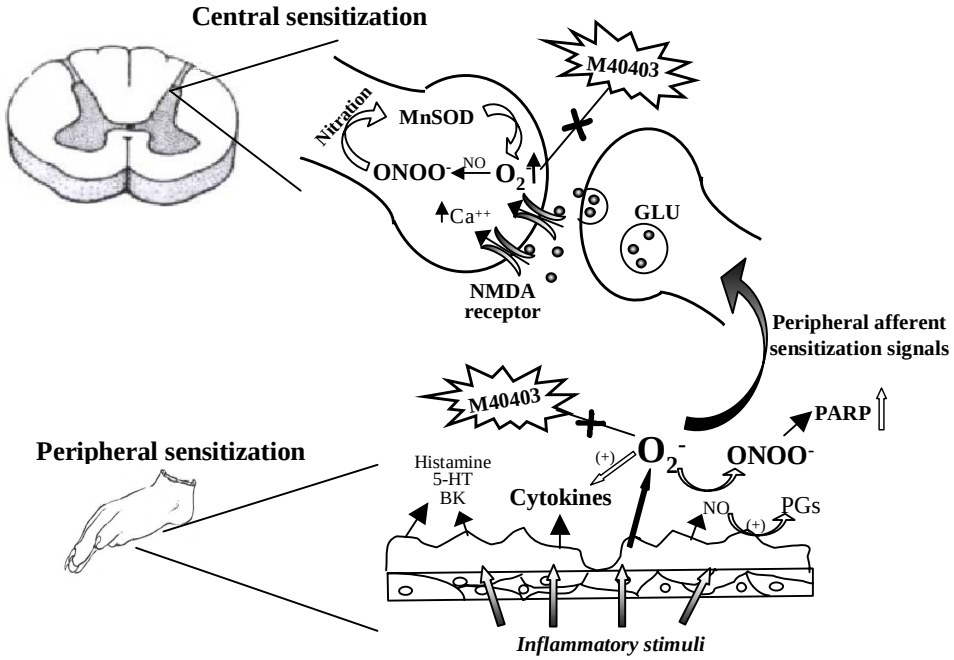
Figure 6



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