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Protective Effects of Mn(III)Tetrakis (4-Benzoic Acid) Porphyrin (MnTBAP), a Superoxide Dismutase Mimetic, in Paw Oedema Induced by Carrageenan in the Rat

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ABSTRACT. In the present study we investigated the therapeutic efficacy of Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP), a superoxide dismutase mimetic which possesses peroxynitrite scavenging effects, in rats subjected to carrageenan-induced paw oedema. Local administration of MnTBAP (5, 25, and 50 μg/paw) significantly and dose dependently reduced carrageenan-induced paw oedema at all time points. MnTBAP also caused a significant dose-dependent reduction in paw myeloperoxidase activity and lipid peroxidation, as well as preventing histological injury. Immunohistochemical analysis for nitrotyrosine revealed a positive staining in paw from carrageenan-treated rats. No positive nitrotyrosine staining was found in the paws of the carrageenan-treated rats that received MnTBAP. Our study demonstrates that MnTBAP exerts protective effects in carrageenan-induced paw oedema. Part of these anti-inflammatory effects may be related to: 1) reduction of superoxide formation due to the superoxide dismutase-like activity of the compound; and 2) scavenging of peroxynitrite. BIOCHEM PHARMACOL **58**;1:171–176, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. nitric oxide; peroxynitrite; carrageenan; superoxide dismutase; inflammation; paw oedema

Oedema formation in paw is the result of a synergism between various inflammatory mediators that increase vascular permeability and/or mediators that increase blood flow [1]. Several experimental models of paw oedema have been described. Rat paw oedema has been characterised by an early phase caused by the release of histamine, 5-hydroxytryptamine, and bradykinin, followed by a late phase mainly sustained by prostaglandin release [2]. It appears that the delayed onset of the carrageenan oedema has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals, such as hydrogen peroxide, superoxide, and hydroxyl radical, as well as to the release of other neutrophil-derived mediators [3–9]. Recent studies have proposed that the L-arginine-NO pathway plays an important role in the carrageenan-induced inflammatory response. Pharmacological inhibitors of nitric oxide synthase, as well as ablation of the gene for inducible nitric oxide synthase, have been shown to reduce the development of the carrageenan-induced inflammatory response [7, 10–13]. More recent studies have demonstrated the formation of peroxynitrite in a model of the carrageenan-induced paw oedema [7, 8, 12, 13]. In this setting, the early formation of peroxynitrite is related to constitutive NO[‖] production, whereas the delayed production of peroxynitrite is related to the expression of inducible nitric oxide synthase [7, 8, 12, 13].

Peroxynitrite is a strong oxidant that results from the reaction between NO and superoxide [14]. Peroxynitrite plays a role in normal cellular processes, although this activity has not been fully identified [15]; however, it usually causes the oxidation of a variety of types of biomolecules with pathological consequences [16, 17]. Peroxynitrite is cytotoxic via a number of independent mechanisms, with its cytotoxic effects including initiation of lipid peroxidation, inactivation of a variety of enzymes (most notably, mitochondrial respiratory enzymes and membrane pumps) [18, 19], glutathione depletion [20], and DNA damage [21, 22], with subsequent activation of PARS and concomitant cellular energy depletion [23, 24].

Moreover, awareness of the cytotoxic potential of peroxynitrite made it important to seek pharmacological approaches to neutralise peroxynitrite-induced oxidations. These experimental models have demonstrated the anti-

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‖ *Abbreviations:* NO, nitric oxide; PARS, poly (ADP-ribose) synthetase; MPO, myeloperoxidase; MnTBAP, Mn(III)tetrakis (4-benzoic acid) porphyrin; and MDA, malonaldehyde.

inflammatory potential of various therapeutic approaches aimed at inhibition of NO synthesis and peroxynitrite formation [7–10, 12, 13]. In our studies, we utilised MnTBAP, a stable and cell-permeable superoxide dismutase mimetic [25–27]. Recent data have demonstrated that MnTBAP inhibits the oxidation of dihydrorhodamine 123 elicited by authentic peroxynitrite [28]. However, MnTBAP is not a scavenger of nitric oxide [28]. In the current study, we have investigated the biological effects of MnTBAP on the development of inflammation in rats treated with carrageenan.

MATERIALS AND METHODS

Carrageenan-induced Paw Oedema

Male Sprague–Dawley rats (300–350 g; Charles River, Milan) were housed and cared for according to the guidelines of the institutional animal care and use committee. They received a subplantar injection of 0.1 mL saline containing 1% λ-carrageenan in the right hindpaw. The inflammatory agent was given together with vehicle or in combination with MnTBAP (5, 25, and 50 μg/paw). The test agent was solubilised in saline solution and the injection volume was 0.1 mL. Control animals received the same volume of vehicle. The volume of the paw was measured by plethysmometry (model 7140; Ugo Basile) immediately after the injection, as previously described [2]. Subsequent readings of the volume of the same paw were carried out at 60-min intervals and compared to the initial readings.

Myeloperoxidase Activity

MPO activity, an index of polymorphonuclear cell accumulation, was determined as previously described [29]. At the specified time following the intraplantar injection of carrageenan, tissue from the pads of the rat hindpaw was removed with a scalpel and 5-mm pieces were then obtained with a tissue punch. Each piece of tissue was homogenised in a solution containing 0.5% hexadecyltrimethylammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 *g* at 4°. An aliquot of the supernatant was then allowed to react with a solution of tetramethylbenzidine (1.6 mM) and 0.1 mM hydrogen peroxide. The rate of change in absorbance was measured by spectrophotometer at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide min^{-1} at 37° and was expressed in milliunits per gram weight of wet tissue.

Malonaldehyde (MDA) Measurement

Levels of MDA in the paw tissue were determined as an index of lipid peroxidation, as described by Okhawa *et al.* [30]. At the specified time following the intraplantar injection of carrageenan, tissue from the pads of the rat hindpaw was removed with a scalpel and 5-mm pieces were then obtained with a tissue punch. Each piece of tissue was homogenised in 1.15% KCl solution. An aliquot (100 μL) of the homogenate was added to a reaction mixture containing 200 μL of 8.1% SDS, 1500 μL of 20% acetic acid (pH 3.5), 1500 μL of 0.8% thiobarbituric acid, and 700 μL distilled water. The sample was then boiled for 1 hr at 95° and centrifuged at 3,000 *g* for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

Histological Examination

For histopathological examination, biopsies of paws were taken 3 hr following the intraplantar injection of carrageenan, tissue from the pads of the rat hindpaw being removed with a scalpel. The tissue slices 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). Sections (thickness 7 μm) were deparaffinized with xylene, stained with trichromic van Gieson, and observed under the Dialux 22 Leitz microscope.

Immunohistochemical Localisation of Nitrotyrosine

Tyrosine nitration was detected in paw sections by immunohistochemistry as previously described [12]. At the specified time following the carrageenan injection, tissues were fixed in 10% buffered formalin and 8 μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% hydrogen peroxide in 60% methanol for 30 min. The sections were permeabilised with 0.1% Triton X-100 in PBS for 20 min. Non-specific adsorption was minimised by incubating the section in 2% normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (biotin blocking kit, Vector Laboratories). The sections were then incubated overnight with a 1:1000 dilution of primary antinitrotyrosine antibody (Upstate Biotech) or with control solutions. Controls included buffer alone or non-specific purified rat immunoglobulin G (IgG). Specific labeling was detected with a biotin-conjugated goat and anti-rabbit IgG and avidin–biotin peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories).

Materials

Biotin blocking kit, biotin-conjugated goat anti-rabbit IgG, primary antinitrotyrosine antibody, and avidin–biotin peroxidase complex were obtained from DBA. All other reagents and compounds used were obtained from Sigma Chemical Company.

Data Analysis

All values in the figures and text are expressed in means ± standard error of the mean of N observations, where N

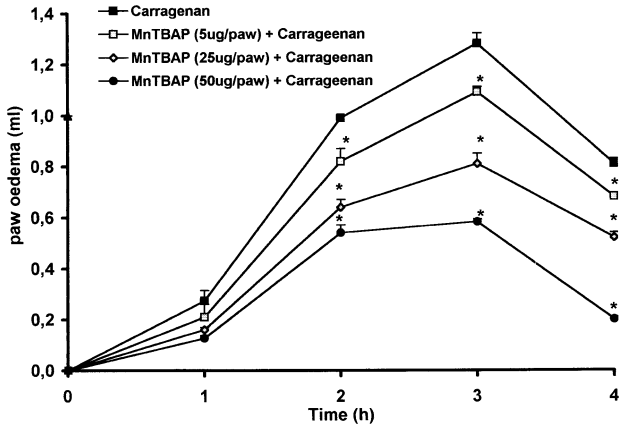

FIG. 1. Effect of MnTBAP (5, 25, and 50 μg/paw) on paw oedema development elicited by carrageenan in the rat. The results are expressed as means ± SEM of $N = 5–6$ rats. MnTBAP treatment significantly inhibited ($*P < 0.01$) oedema formation at the indicated time points.

represents the number of animals studied. Data sets were examined by one- and two-way analysis of variance. Individual group means were then compared with Student's unpaired *t*-test. A *P* value of less than 0.05 was considered significant. In the experiments involving immunohistochemistry, the figures shown are representative of at least 3 experiments performed on different experimental days.

RESULTS

In preliminary experiments, we established that the injection of MnTBAP (5, 25, and 50 μg/paw) into the rat paw did not produce any detectable oedema. The maximal increase in paw volume was observed at three hr after carrageenan administration (maximal in paw volume: 1.16 ± 0.27 mL). However, carrageenan-induced paw oedema was significantly and dose dependently reduced by treatment with MnTBAP (5, 25, and 50 μg/paw) at all time points (Fig. 1). Paw tissue was examined for MPO activity, indicative of neutrophil infiltration, and for MDA, in order to estimate lipid peroxidation. As shown in Fig. 2, A and B, MPO activity and MDA levels were significantly ($P < 0.01$) increased in the paw at 3 hr after carrageenan injection when compared to sham rats. MPO activity and MDA levels were significantly ($P < 0.01$) reduced, in a dose-dependent manner, by MnTBAP (5, 25, and 50 μg/paw) treatment (Fig. 2).

Upon histological examination, the paws revealed pathological changes that correlated closely with the increases in MPO activity and MDA levels. Paw biopsies showed marked inflammatory changes after carrageenan administration, including pronounced cellular infiltration (Fig. 3A). The paw tissues were also examined immunohistochemically for the presence of nitrotyrosine. Immunohistochemical analysis of the paw tissue of control animals showed no nitrotyrosine staining (Fig. 4A). In contrast, 3

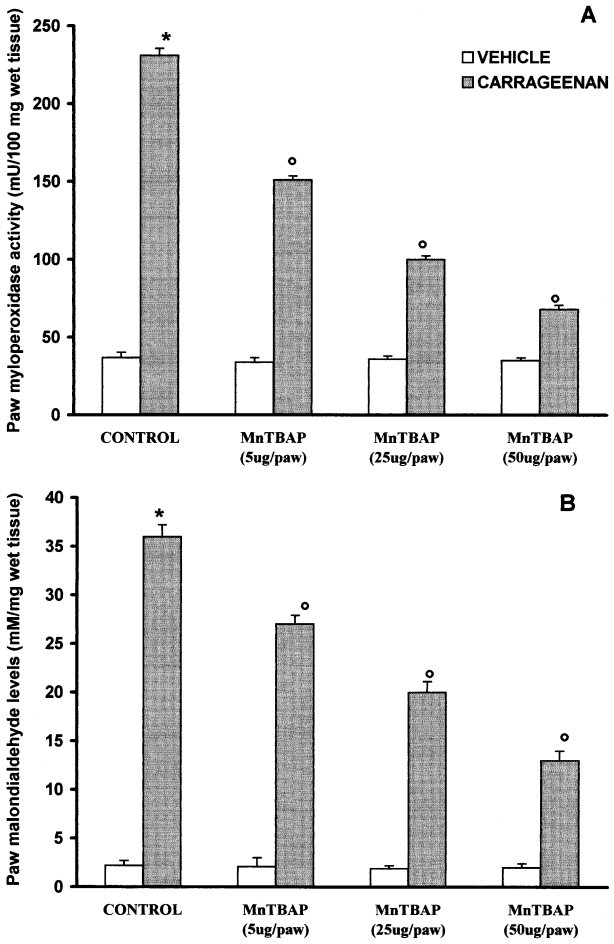

FIG. 2. Myeloperoxidase (MPO) activity (A) and malondialdehyde (MDA) levels (B) in the paw of carrageenan-treated rats killed at 3 hr. MPO activity and MDA levels were significantly increased in the paw of the carrageenan-treated rats in comparison to sham rats. MnTBAP treatment dose dependently reduced the carrageenan-induced increase in MPO activity and MDA levels. Values are means ± SEM of 8 rats for each group. $*P < 0.01$ versus sham; $°P < 0.01$ versus carrageenan.

hr following carrageenan injection, staining for nitrotyrosine was also found to be localised within discrete cells and skeletal muscle fibres in the inflamed paw tissue (Fig. 4, B and D). Treatment with MnTBAP (50 μg/paw) significantly reduced the pathological changes and prevented the appearance of nitrotyrosine in the tissues (Figs. 3B and 4, C and E).

DISCUSSION

Oxygen-derived free radicals and oxidants have been shown to play an important role in various forms of inflammation [1, 8, 9, 12, 13]. Recent data have demonstrated that the expression of the inducible isoform of NO synthase also plays an important proinflammatory role [31]. Moreover, NO and superoxide react to form peroxynitrite, a highly cytotoxic oxidant species [14] which plays important roles in shock and inflammation [8, 9, 12, 13, 32–34].

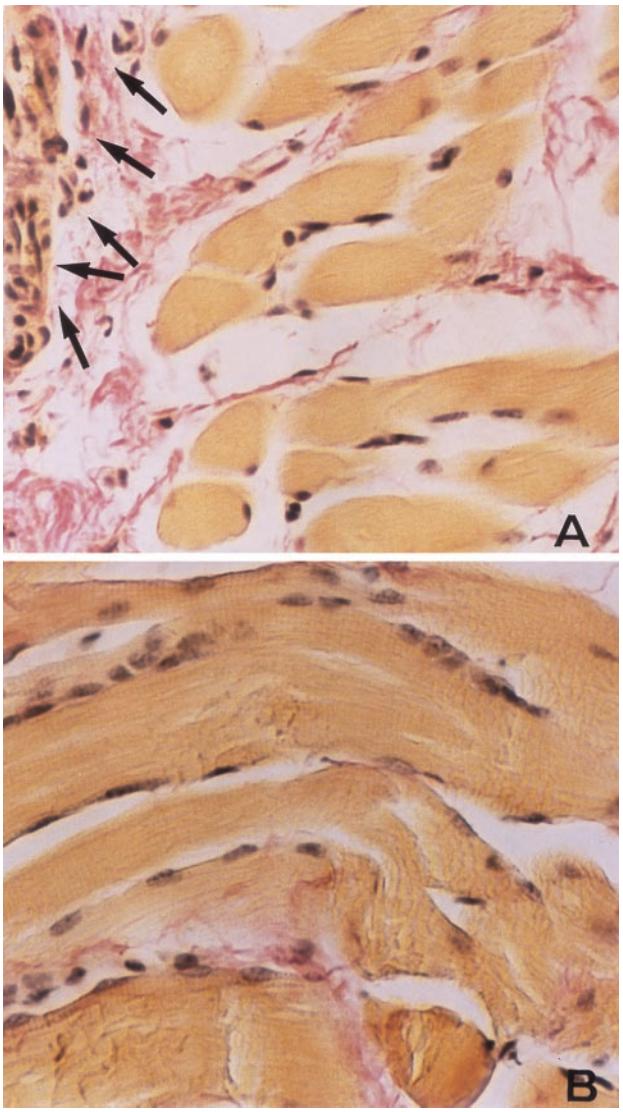

FIG. 3. Representative paw sections from carrageenan-treated rats (A) demonstrate marked inflammatory changes, including pronounced cellular infiltration (see arrows). Paw sections from carrageenan-treated rats that received MnTBAP (50 μg/paw) demonstrate reduced cellular infiltration (B). Original magnification: ×62.5.

Recently, we have shown the formation of peroxynitrite in carrageenan-induced inflammation [8, 9, 12, 13]. Recent data challenge the prevailing view that NO is independently toxic and propose that much of the NO-related cytotoxicity and oxidant reactions are, in fact, due to peroxynitrite formulation [14]. In fact, potent novel, cell-permeable superoxide dismutase mimetics prevent peroxynitrite formation and protect against cellular injury in various models of inflammation [14, 27].

In the present study, we found that: 1) MnTBAP reduces the development of carrageenan-induced paw oedema; 2) MnTBAP reduces morphological injury, neutrophil infiltration, and lipid peroxidation in carrageenan-induced models of local inflammation; and 3) MnTBAP reduces nitrotyrosine immunostaining, an indicator of peroxynitrite formation in inflammation. Recent reports have shown that nitrotyrosine formation may also result from the reaction between nitrite and MPO [35]. Thus, it is possible that the cytotoxic effects observed in response to carrageenan represent the sum of a complex interaction between various oxygen- and nitrogen-derived radicals and oxidants. It is logical to assume that the pharmacological effects of MnTBAP arise from the combination of the following actions: 1) inactivation of superoxide, thus preventing the formation of peroxynitrite, with consequent protection against the development of peroxynitrite-induced cellular energetic failure; 2) scavenging of peroxynitrite, the direct inhibition of the peroxynitrite-induced oxidative process; and 3) a reduced neutrophil recruitment into the inflammatory site, perhaps representing an important additional mechanism for the anti-inflammatory action. At present we cannot envisage a suitable pharmacological approach to separate the above factors. The mechanism of reduced neutrophil infiltration into the inflamed tissue is not clear at present. It may be related to prevention by MnTBAP of endothelial oxidant injury and to a preservation of endothelial barrier function. Clearly, the delineation of the relative contribution of MnTBAP's multiple modes of action to the anti-inflammatory effects observed in the current study requires further investigation. A novel pathway of inflammation, governed by PARS, has been proposed in relation to hydroxyl radical- and peroxynitrite-induced DNA single strand breakage [24, 34, 36]. This pathway plays an important role in various forms of shock and reperfusion injury [32–34]. It is clear that MnTBAP has potent protective effects in an experimental system where the injury is likely to be mediated by superoxide alone [26]. However, recent studies have demonstrated that peroxynitrite potently triggers DNA single strand breakage in a number of cell types studied, including thymocytes, macrophages, and vascular smooth muscle cell PARS, with consequent reduction of mitochondrial respiration [23, 32, 36].

Zingarelli *et al.* [27] have recently demonstrated that this mesoporphyrin compound protects against the suppression of mitochondrial respiration induced by authentic peroxynitrite in vascular smooth muscle cells and against the cellular energetic failure in a rodent model of endotoxin shock.

Therefore, protection by MnTBAP against the development of inflammation may be related to either superoxide or peroxynitrite and thus prevent the activation of PARS in inflammation.

In conclusion, in the present study we have demonstrated that MnTBAP exerts beneficial anti-inflammatory effects in carrageenan-induced paw oedema. The mechanism of action of MnTBAP may be related to: 1) reduction of superoxide formation; and 2) scavenging of peroxynitrite. The relative contribution of the two effects needs to be determined in further studies.

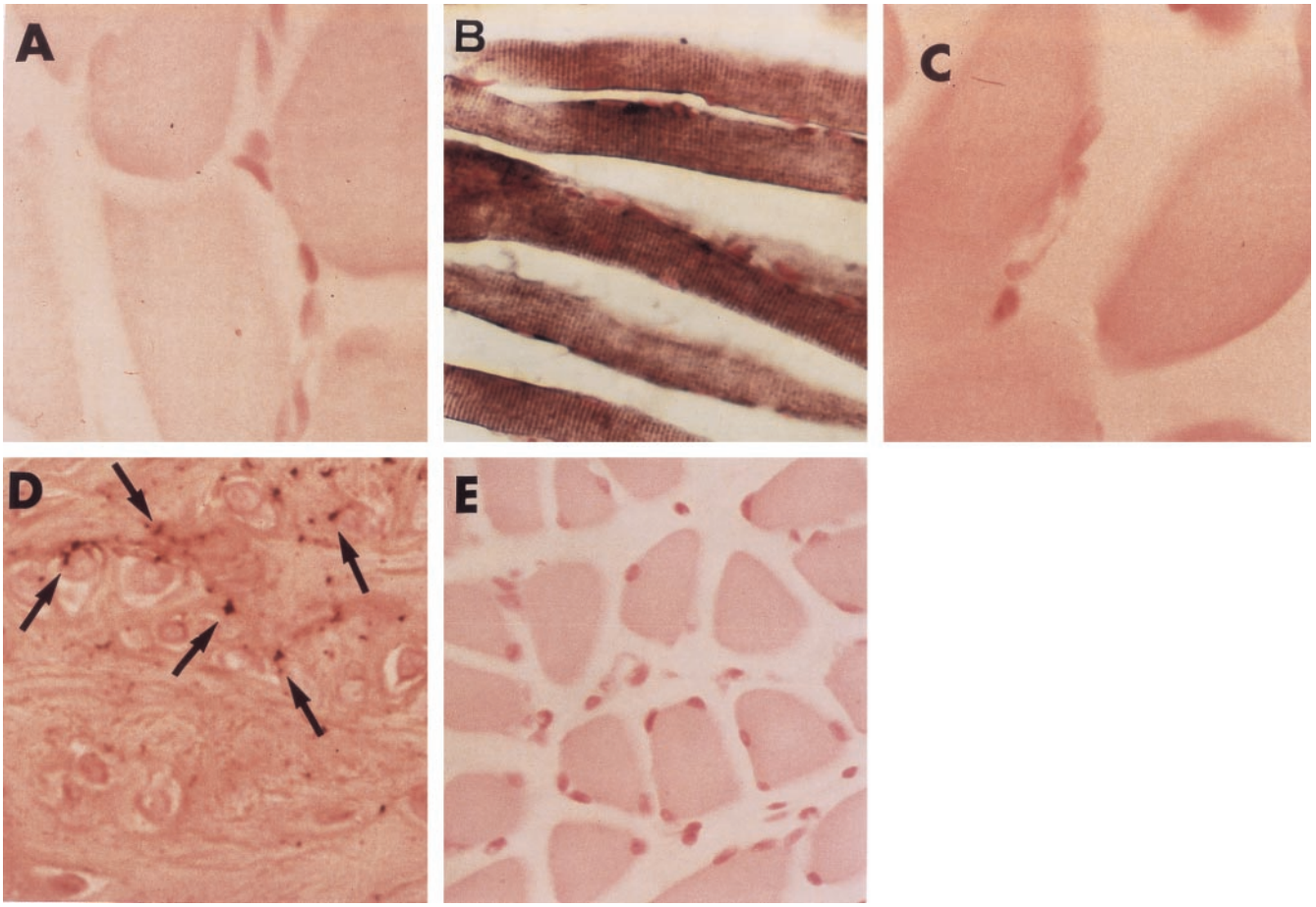

FIG. 4. Immunohistochemical localisation of nitrotyrosine in the rat paw. Staining was absent in control tissue (A). Three hours following carrageenan injection, nitrotyrosine immunoreactivity was localised in skeletal muscle (B) and within discrete cells in the inflamed paw tissue (see arrows) (D). Treatment with MnTBAP (50 μg/paw) reduced the appearance of nitrotyrosine (C and E). Original magnification: ×62.5.

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