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Spinal pharmacology of tactile allodynia in diabetic rats

*,[1]Nigel A. Calcutt & †Sandra R. Chaplan

Departments of *Pathology and †Anesthesiology, University of California San Diego, La Jolla, CA 92093-0612, and *Veterans Administration Medical Center San Diego, CA, U.S.A.

1 Rats develop tactile allodynia to stimulation of the plantar surface of the hindpaw with von Frey filaments within days of the onset of streptozotocin-induced diabetes. This is prevented by insulin and alleviated by systemic lignocaine, but the aetiology is unknown.

2 Using indwelling lumbar intrathecal catheters to deliver pharmacological agents, we have investigated whether tactile allodynia in streptozotocin-diabetic rats is dependent on mechanisms associated with spinal sensitization, by assessing the efficacy of agents that inhibit specific components of spinal nociceptive processing.

3 Dose-dependent inhibition of tactile allodynia in diabetic rats was noted with the N-type calcium channel antagonist SNX 239, the α_2-adrenoceptor agonist dexmedetomidine, the μ-opioid receptor agonist morphine, the N-methyl-D-aspartate (NMDA) receptor antagonist AP5 and the non-NMDA receptor antagonist NBQX.

4 No effect on tactile allodynia was noted after intrathecal administration of the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), the cyclo-oxygenase inhibitor ketorolac, the L-type calcium channel inhibitor diltiazem or any vehicle.

5 These data suggest that the tactile allodynia of diabetic rats involves spinal glutamatergic pathways but is not associated with spinal release of nitric oxide or prostaglandins.

Keywords: Diabetes; diabetic neuropathy; pain; allodynia; glutamate receptor; spinal cord

Introduction

Patients with diabetic neuropathy may experience a variety of aberrant sensations including spontaneous pain, allodynia (pain perception in response to normally non-painful stimuli) and hyperalgesia (exaggerated pain sensations to normally painful stimuli) that are often concurrent with a paradoxical loss of stimulus-evoked sensation (Benbow *et al*., 1994). Treatments such as topical capsaicin (Capsaicin Study Group, 1992) and oral mexiletine (Dejgård *et al*., 1988) are occasionally effective in relieving pain but may be associated with a range of side effects that restrict their use. Unfortunately, the development of mechanistically targeted therapies is impeded by a lack of appreciation of the aetiology of the processes involved and treatment of painful diabetic neuropathy is at present mostly confined to the use of general analgesics (Pfeifer *et al*., 1993).

A number of studies have described abnormal nociceptive behaviour in diabetic rodents. The majority have measured the time to response after application of an increasingly noxious thermal or mechanical stimulus, but their relevance to the pain states associated with diabetic neuropathy, which are often spontaneous or provoked by light touch, remains debatable. Exaggerated responses to noxious chemical stimuli have also been obtained in diabetic rats during the formalin test (Courteix *et al*., 1993; Malmberg *et al*., 1993), in which hyperalgesia is associated with activation of spinal glutamate receptors (Yamamoto & Yaksh, 1992) and release of prostaglandin E_2 (Malmberg & Yaksh, 1992; 1995) and nitric oxide (Malmberg & Yaksh, 1993) in the spinal cord. However, few studies have attempted to describe pain states in diabetic rats that more closely resemble human painful diabetic neuropathy. Allodynia to non-noxiuos thermal stimulation has been observed in diabetic rats (Courteix *et al*., 1993) and we have recently described the presence of tactile allodynia in similar animals, using von Frey filaments to provide a light touch stimulus to the plantar surface of the hind paw (Calcutt *et al*., 1996). Tactile allodynia develops within 7–10 days of the onset of hyperglycaemia, persists for many weeks and is prevented by insulin therapy. Low doses of systemically administered lignocaine alleviated tactile allodynia in diabetic rats and have also been shown to be effective in treating some cases of painful diabetic neuropathy (Kastrup *et al*., 1987).

To investigate the potential aetiology and spinal pharmacology of tactile allodynia in diabetic rats, we have examined the effects of intrathecal delivery of inhibitors of nitric oxide synthase and cyclo-oxygenase, both of which are effective in attenuating spinal sensitization during the formalin test (Malmberg & Yaksh, 1992; 1993; 1995), and also of a variety of agents previously shown to prevent tactile allodynia arising from physical nerve injury (Chaplan *et al*., 1994a).

Methods

Animals and surgery

All studies were performed in adult female Sprague-Dawley rats. Rats were made diabetic by a single intraperitoneal injection of streptozotocin (50 mg kg^{-1} body weight, freshly dissolved in 0.9% sterile saline) to ablate pancreatic β cells and induce insulin deficiency. Two days later, diabetes was confirmed in streptozotocin-injected rats by measuring glucose concentration in a blood sample obtained by tail prick, by use of a glucose oxidase-impregnated test strip and reflectance meter (Ames Glucostix and Glucometer II, Myles Inc., Elkhart, IN). Only streptozotocin-injected animals with a blood glucose concentration above 15 mmol l^{-1} were included as diabetic and hyperglycaemia was also confirmed at the time of behavioural testing. Animals were housed in wire-bottomed cages with free access to food and water and maintained in a vivarium approved by the American Association for the Accreditation of Laboratory Animal Care. All experimental procedures were approved by the local animal use sub-committee.

Each diabetic rat was implanted with an intrathecal PE-10 catheter under halothane/oxygen anaesthesia (Yaksh & Rudy, 1976) 6 weeks after the induction of diabetes. The catheters

[1] Author for correspondence.

extended from the cisterna to the rostral edge of the lumbar enlargement and were flushed with 10 μl of preservative-free saline after insertion. Rats that showed neurological deficits after recovering from anaesthesia were immediately killed. Studies were performed in conscious, unrestrained animals, 4–21 days after catheter placement.

Behavioural testing of allodynia

A standardized testing regime was performed to measure tactile allodynia (Chaplan *et al*., 1994a). Briefly, rats were transferred to a testing cage with a wire mesh bottom and allowed to acclimatize for 10–15 min. Von Frey filaments (Stoelting, Wood Dale IL) were used to determine the 50% mechanical threshold for foot withdrawal, by use of a modification of the up-down method of Dixon (Dixon, 1980). A series of filaments, starting with one that possessed a buckling weight of 2.0 g, were applied in sequence to the plantar surface of the right hindpaw with a pressure that caused the filament to buckle. Lifting of the paw was recorded as a positive response and the next lightest filament chosen for the next measurement. Absence of a response after 5 s prompted use of the next filament of increasing weight. This paradigm was continued until four measurements had been made after an initial change in the behaviour or until five consecutive negative (given the score of 15 g) or four positive (given the score of 0.25 g) responses had occurred. The resulting sequence of positive and negative scores was used to interpolate the 50% response threshold. Rats were considered to display tactile allodynia if their thresholds were calculated as <4 g, based on prior comparisons with normal rats (Calcutt *et al*., 1996). Tests were performed immediately before, and at a range of time points from 30 min to 2 h after, delivery of drugs to the spinal cord via the intrathecal catheter. All measurements were performed by an investigator who was unaware of the treatment group of individual animals. Responses were transformed to percentage maximal effect (%MPE) by designating the pre-drug value as 0% effect and thresholds $\geqslant 15$ g as 100% effect (Chaplan *et al*., 1994a) and using the following calculation: %MPE = (post-drug threshold-pre-drug threshold)/(15 pre-drug threshold) $\times$ 100.

Drugs

Ketorolac tromethamine (Syntex, Palo Alto, CA), L-NAME (N^G-nitro-L-arginine methyl ester HCl) (Research Biochemicals International, Natick MA), morphine sulphate (Merck, West Point, PA), dexmedetomidine HCl (Farmos), AP5 ((±)-2-amino-5-phosphonovaleric acid; Research Biochemicals, International, Natick MA), SNX 239 acetate (Neurex Inc., Palo Alto, CA) and diltiazem HCl (Sigma, St. Louis MO) were delivered at a variety of doses in a volume of 10 μl saline followed by 10 μl saline to flush the catheter. NBQX (1,2,3,4, tetrahydro-6-nitro-2,3,-dioxo-benzo[f]quinoxaline-7-sulphonamide) (Novo Nordisk, Copenhagen, Denmark) was also provided in a volume of 10 μl, but was dissolved in a vehicle consisting of a 5.5% dextrose solution made alkaline with NaOH to a pH of 8.5. All drugs were given at doses below those yielding motor dysfunction in control rats and all animals were monitored for motor dysfunction by assessing the righting reflex. Individual rats were used on up to 4 occasions with 2 days allowed between tests. Animals were only employed if their baseline tactile threshold continued to meet criteria for allodynia (<4 g).

Results

Streptozotocin treatment and tactile allodynia

All streptozotocin-injected rats were hyperglycaemic at the time of behavioural testing, as indicated by blood glucose concentrations in excess of 22.2 $mmol^{-1}$, the upper limit of the Glucometer. Diabetic rats consistently demonstrated tactile allodynia, as indicated by 50% response thresholds in the range of 0.5–4.0 g. Control animals tested concurrently rarely showed any response to von Frey filaments below the cut-off of 15.0 g (data not shown).

Intrathecal drug action

Intrathecal delivery of SNX 239, dexmedetomidine, morphine, AP5 or NBQX produced a dose-dependent suppression of tactile allodynia (Figures 1 and 2). The rank order was dexmedetomidine = SNX 239 = NBQX > morphine > AP5, although both dexmedetomidine and NBQX exhibited ceiling effects at less than 100% MPE. For all of these compounds, the lowest dose to demonstrate maximal efficacy did so at 1 h post-delivery (Figure 3). Where rats were tested before re-use and thus 48 h after initial drug administration, values had returned to the baseline of tactile allodynia irrespective of the initial drug or dose (data not shown).

Intrathecal administration of saline, NBQX vehicle, diltiazem (up to 500 μg), ketorolac (up to 100 μg) or L-NAME (up to 30 μg) did not alter thresholds from pre-drug levels at 1 h post-delivery (Figure 4) or at time points between 30 min and 2 h post-delivery (data not shown). It was noted that all of the rats treated with 100 μg ketorolac developed a temporary tremor that began some 3 h after treatment and resolved within 12 h, while no effects on general behaviour or motor

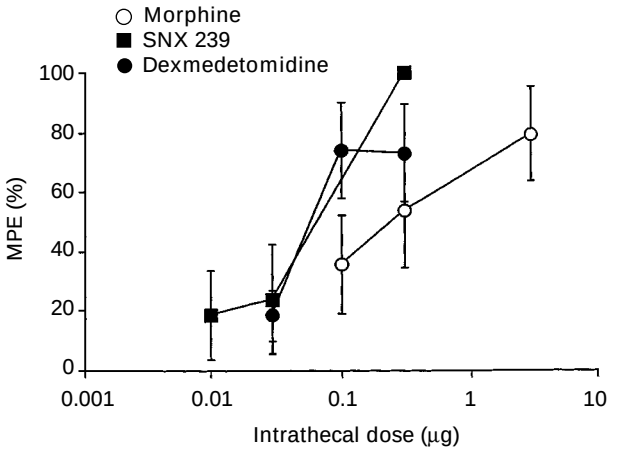

Figure 1 Dose-response curves for suppression of paw withdrawal responses (as % MPE) to von Frey filaments 1 h after intrathecal administration of morphine, SNX 239 and dexmedetomidine. Data are mean and vertical lines show s.e.mean; $n = 4-6$ per point.

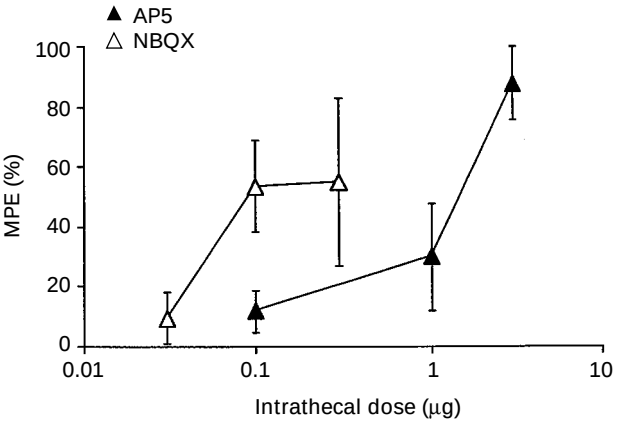

Figure 2 Dose-response curves for suppression of paw withdrawal responses (as % MPE) to von Frey filaments 1 h after intrathecal administration of AP5 or NBQX. Data are mean and vertical lines show s.e.mean; $n = 4-6$ per point.

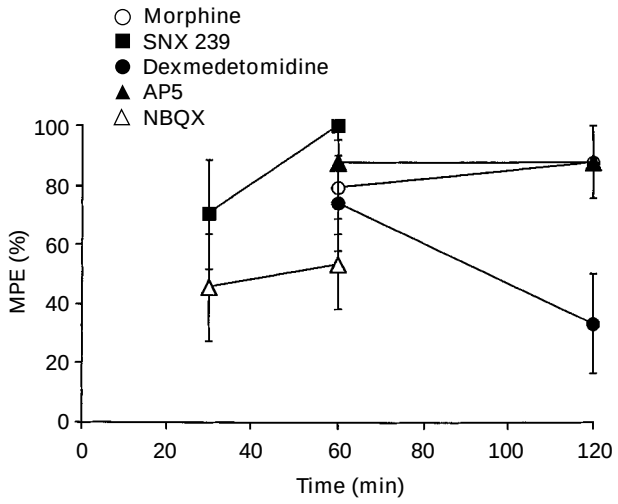

Figure 3 Suppression of paw withdrawal responses (as % MPE) to von Frey filaments at various time points after intrathecal administration of morphine (3 μg), SNX 239 (0.3 μg), dexmedetomidine (0.1 μg), AP5 (3 μg) and NBQX (0.1 μg). Data are mean and vertical lines show s.e.mean; $n=4-6$ per point.

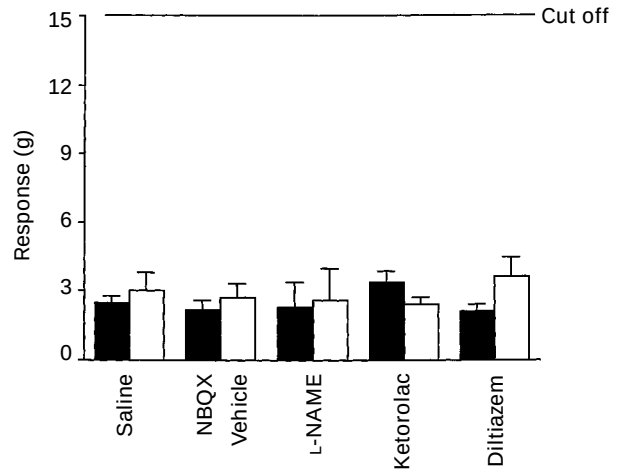

Figure 4 Response thresholds to von Frey filament stimulation of the hindpaw of diabetic rats before (solid columns) and 1 h after (open columns) intrathecal administration of 0.9% saline, NBQX vehicle, L-NAME (30 μg), ketorolac (100 μg) or diltiazem (500 μg). Data are mean$\pm$s.e.mean; $n=4-7$ per group. No statistically significant differences in responses before or after delivery were noted by use of paired t test.

function were observed after administration of any other agent.

Discussion

We have recently described tactile allodynia in streptozotocin-diabetic rats (Calcutt *et al*., 1996). This is evident within 7–10 days of the onset of diabetes and persists for at least 8 weeks without any indication of remission. Our studies used female rats in random oestrous cycle yet the magnitude of tactile allodynia was similar in all animals and reproducible over many weeks, indicating that levels of female sex hormones are unlikely to be involved in this phenomenon. Because insulin therapy at doses that maintained normoglycaemia prevented the development of tactile allodynia, the disorder is also unlikely to arise from streptozotocin neurotoxicity *per se* and appears to be secondary to insulin deficiency or its consequences. Acute normalization of blood glucose with insulin did not reverse the allodynia. Moreover, the disorder was also present in diabetic rats treated with enough insulin to prevent muscle wasting and weight loss in the foot whilst remaining hyperglycaemic. These observations suggest that allodynia is unrelated to acute hyperglycaemia or decreases in muscle mass. Tactile allodynia in diabetic rats was temporarily alleviated by low doses of systemic lignocaine, as has been demonstrated for a similar phenomenon induced by nerve injury (Chaplan *et al*., 1995) and for painful diabetic neuropathy in man (Kastrup *et al*., 1986; 1987; Bach *et al*., 1990). However, the mechanisms by which low doses of lignocaine or its metabolites influence nociception is unknown and may be unrelated to the underlying aetiology of diabetic tactile allodynia.

The description of tactile allodynia in diabetic rats adds another facet to the variety of nociceptive disorders present in this animal model, which include thermal allodynia (Courteix *et al*., 1993) and hyperalgesia in the formalin test (Courteix *et al*., 1993; Malmberg *et al*., 1993; Calcutt *et al*., 1994). In control animals, injection of formalin into the hindpaw causes a biphasic behavioural response characterized by periods of lifting and flinching of the afflicted limb separated by a period of inactivity. While the first phase of flinching is driven by primary afferent input to the spinal cord from the injury site, the second phase exhibits exaggerated activity of wide dynamic range second order neurones compared to the minimal primary afferent input (Dickenson & Sullivan, 1987). This has led to the suggestion that the second phase represents a period of spinal sensitization which may be substantially mediated by release of prostaglandins and nitric oxide in the spinal cord (Malmberg & Yaksh, 1992; 1993; 1995). Because prostaglandin production is enhanced in some diabetic tissues (Chang *et al*., 1991) and there is also exaggerated hyperalgesia during phase 2 of the formalin test in diabetic rats (Calcutt *et al*., 1995), we speculated that tactile allodynia may also result from a diabetes-induced spinal sensitization by prostaglandins. However, tactile allodynia was not alleviated by maximum usable doses of either the cyclo-oxygenase inhibitor ketorolac or the nitric oxide synthase inhibitor L-NAME, both previously shown to suppress formalin hyperalgesia (Malmberg & Yaksh, 1992; 1993; 1995). This suggests that spinal production of prostaglandins or nitric oxide is unlikely to be involved in the tactile allodynia of diabetic rats unless effects occur outside the scope of our time course. A peripheral action of NSAIDs on diabetic tactile allodynia also cannot be discounted by the present study, although our findings are consistent with clinical experience, where NSAIDs rarely benefit patients with painful diabetic neuropathy.

Other than in diabetic rats, tactile allodynia also occurs in rats following nerve injuries, such as ligation of the L_5 and L_6 spinal nerves (Kim & Chung, 1992) and is of similar magnitude in both models (Calcutt *et al*., 1996). In recent studies, we have shown suppression of tactile allodynia in this nerve injury model by intrathecal delivery of a variety of agents, including α_2-adrenoceptor agonists (Yaksh *et al*., 1995), adenosine agonists (Lee & Yaksh, 1996), and N-type calcium channel antagonists such as SNX 111 and SNX 239, of which the latter produces less marked motor dysfunction (Chaplan *et al*., 1994b). Agents that were ineffective included L-type calcium channel antagonists (Chaplan *et al*., 1994b), α_1-adrenoceptor agonists (Yaksh *et al*., 1995), or opiates (Lee *et al*., 1995). Because diabetic rats show morphological evidence of nerve fibre disruption in their spinal roots (Tamura & Parry, 1994), we investigated the efficacy of agents previously shown to be effective against nerve injury-induced tactile allodynia.

Consistent with studies of the spinal pharmacology of tactile allodynia induced by nerve injury, both the N-type calcium channel antagonist SNX 239 and the α_2-adrenoceptor agonist dexmedetomidine were effective against tactile allodynia in diabetic rats, while the L-type calcium channel antagonist diltiazem was ineffective. It is notable that SNX 239 produced a complete effect (100% MPE) at a dose of 0.3 μg in diabetic rats, while intrathecal doses of up to 3 μg produced only 56% of the MPE in rats with tactile allodynia resulting from spinal

nerve ligation and 100% MPE was not achieved (Chaplan *et al*., 1994b). Similarly, the efficacy of dexmedetomidine against diabetic tactile allodynia, with 50% effect lying between 0.03 and 0.1 μg, appears to be greater than nerve injury-induced tactile allodynia, where the ED_{50} was 0.9 μg (Yaksh *et al*., 1995), although in this case 100% inhibition of tactile allodynia was not achieved in either model. Other than these differences in potency, diabetic tactile allodynia also differed from the nerve injury-induced disorder in that intrathecal morphine was effective in diabetic rats, whereas it was effective only when delivered systemically or supraspinally, but not intrathecally in the nerve injury model (Lee *et al*., 1995). It appears, therefore, that the tactile allodynia of diabetic rats may not share the same aetiology as that of rats after spinal nerve injury, despite the similarity of behavioural responses (Calcutt *et al*., 1996). However, it should be acknowledged that the spinal root injury of diabetic rats is mild and largely restricted to myelin disruption (Tamura & Parry, 1994) without the axonal degeneration that accompanies injury caused by nerve ligation. It remains plausible that these two models reflect extreme ends of a spectrum of injury-induced tactile allodynia, with diabetic tactile allodynia more amenable to therapy due to the limited axonal injury.

The mode of action of SNX 239, dexmedetomidine and morphine in blocking tactile allodynia is likely to be via inhibition of spinal neurotransmitter release. SNX 239 blocks a substantial fraction of glutamate release (Graham & Burgoyne, 1995; Takizawa *et al*., 1995), while the α_2 receptor has prominent presynaptic inhibitory effects on release of both glutamate and substance P (Go & Yaksh, 1987; Anwyl, 1991). Morphine has both presynaptic effects on neurotransmitter release and depresses excitability of postsynaptic neurones (Yaksh, 1987). At present there is little evidence to support the idea of increased sensitivity of peripheral sensory neurones in diabetic rats. Indeed, electrophysiological studies have shown that the properties of Aβ fibres, which normally respond to tactile stimuli, are not altered in diabetic rats (Ahlgren *et al*., 1992). There are also no spontaneous discharges or change in the sensitivity of C fibres to von Frey filaments (Ahlgren *et al*., 1992; 1997). It has recently been shown that inward calcium currents are prolonged in small sensory neuronal cell bodies from the dorsal root ganglia of diabetic rats (Kostyuk *et al*., 1995), so that it is possible that synaptic release of neurotransmitters may also be enhanced for any given stimulus. However, this is balanced by findings that the mRNA, synthesis and axonal transport of neuropeptide neurotransmitters, such as substance P and calcitonin gene-related peptide (CGRP), are reduced in diabetic rats (Robinson *et al*., 1987; Calcutt *et al*., 1990; Diemel *et al*., 1992). Studies examining the release of sensory neurotransmitters in the spinal cord of diabetic rats in response to tactile or noxious stimuli have yet to be performed.

Both NMDA and non-NMDA receptor antagonists were effective in inhibiting the tactile allodynia of diabetic rats, indicating the involvement of excitatory amino acid neurotransmitter pathways. Postsynaptic non-NMDA receptors are widely held to be involved in nociceptive pathways responding to acute painful stimuli evoked by C and Aδ fibre activity, rather than to activity of the large Aβ fibres stimulated by the light touch stimuli of von Frey filaments. In contrast, NMDA receptors participate in states of persistent pain and spinal sensitization. NMDA antagonists have been shown to reduce or abolish tactile allodynia in other models, including that following spinal nerve ligation, and to reduce flinching in the formalin test (Chaplan *et al*., 1997), while numerous studies have shown reduction of thermal hyperalgesia with these agents (Yamamoto & Yaksh, 1992; Mao *et al*., 1993). In the present study, there were differences in the dose-inhibition response of tactile allodynia between the two types of antagonist, with the non-NMDA receptor antagonist being more potent but ultimately less efficacious. It is possible that, while divergent pathways may operate in various types of hyperalgesia, the NMDA receptor constitutes a final common pathway for these abnormal sensory states. Interestingly, NMDA receptors are also present on the presynaptic terminal of primary afferent fibres, where they may serve as mediators of a positive feedback system (Liu *et al*., 1994). Whether such a system is involved in the tactile allodynia of diabetic rats requires further investigation.

Our present studies have indicated that tactile allodynia in diabetic rats does not arise from spinal sensitization mechanisms involving prostaglandins or nitric oxide. Spinal glutamatergic pathways appear to be involved, despite the probable absence of exaggerated primary afferent input, and the therapeutic potential of agents that inhibit presynaptic transmitter release or glutamate receptors has been illustrated. The aetiology of tactile allodynia development in diabetic rats remains uncertain and the possibility that diabetes enhances excitatory neurotransmitter release or causes a reduction in pre- and/or postsynaptic spinal inhibitory mechanisms awaits investigation.

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