Original Article

Evaluation of the Effects of Novel Nafimidone Derivatives on Thermal Hypoalgesia in Mice with Diabetic Neuropathy

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ABSTRACT

Objective: Diabetic neuropathy (DN) is a common complication in Diabetes Mellitus. The streptozotocin-induced diabetic rodent is the most commonly used animal model of diabetes and increased sodium channel expression and activity were revealed in this model. At this study, we evaluated the effect of three different nafimidone derivatives which have possible anticonvulsant activity on disorders of thermal pain sensation in diabetic mice.

Study Design: Randomized animal experiment.

Material and Methods: Mice were divided randomly into five groups (5 mice per group): Control, Diabetes, Dibetes+C1, Diabetes+C2, Diabetes+C3. We used hot and cold plate, and tail-immersion tests for assessment of thermal nociceptive responses.

Results: Compared with the control group, the hot-plate response time and the number of paw liftings on cold plate as important indicators of loss of sensation increased, but no significant difference (p>0.05) was found in tail-immersion response time test in diabetes group. C3 compound moved it back to control group levels in the all of three tests. C1 and C2 compounds were effective only in cold-plate test.

Conclusion: Nafimidone derivatives may be effective in the cases where epilepsy and diabetes occur together since it has shown efficacy against "loss of sensation" which evolves in diabetic neuropathy over time as well as its antiepileptic effect.

Key Words: Diabet, diabetic neuropathy, loss of sensation, antiepileptic, Na channel blocker, nafimidone derivatives

Introduction

The worldwide diabetes epidemic has shown no signs of abatement. Its prevalence has more than doubled since 1980 (1). Diabetic neuropathy (DN) is the most common diabetic complication, afflicting over 50% of all diabetics. The possible biochemical pathomechanisms include oxidative stress, activation of the polyol pathway, increased advanced glycation end products (AGEs) and their receptors, impaired ionic homeostasis of cells and activation of protein kinases (PKC and PKA), and mitogen-activated protein kinases (MAPK), and inducible nitric oxide synthase (2-6).

Understanding the biochemical mechanisms underlying diabetic neuropathic pain and sensory disorders requires preclinical studies in animal models. The streptozotocin (STZ)-induced diabetic rodent is the most commonly used animal model of diabetes (7, 8). On the other hand, existing animal models of diabetic painful and insensate neuropathy, however, have serious limitations. Diabetic rats and mice have limited life span and rarely show evidence of overt neuropathy, such as demyelination, axonal degeneration, fiber loss,

or axonal regeneration in their peripheral nerves. This makes diabetic rodents unsuitable for studying the contribution of these phenomena of DN to pain or to loss of sensory function (9). Nonetheless, despite these limitations, assessment of behavioral responses to external stimuli in diabetic rats and mice (i.e., thermal and mechanical hyper and hypoalgesia, tactile allodynia, as well as formalin-induced spontaneous nociceptive behavior) has led to identification of a number of mechanisms of loss of sensation and/or pain in diabetes (10).

Decades of research elucidating the pathophysiology of diabetic neuropathy have failed, thus far, to produce a treatment that prevents or reverses its development and progression. Recently, however, numerous competing or parallel pathological pathways have begun to intersect and complement each other, illuminating potential pharmacologic targets. The current foci of diabetic neuropathy research are excessive activation of sodium channels and corrupted molecular mechanisms of cellular pathways of related kinases (11).

Voltage-gated sodium channels (Na_vChs) are a necessary component of normal sensation, emotions, thoughts and movements and are of particular interest as target for neuro-

pathic disorders because they control electrical signals in both the central and peripheral nervous systems (12, 13). Recently, blocking of this channel has been widely accepted as a potential treatment for diabetic pain and insensate neuropathy (14). At this point, the possibility of selectively modulating sodium channels involved in neuropathic disorders has been based on the following three strategies: i) development of non-selective sodium channel blockers that are potent and highly use-dependent; ii) discovery of molecules with peripheral activity, thereby, avoiding CNS side effects and iii) identification of selective sodium channel blockers that specifically target channel subtypes associated with diabetic complications. In this respect, Na_vChs can be considered as 'selective targets' (15).

During anticonvulsant drug development studies, the activity of the compounds usually first tested in the conventional acute animal models but recently investigation of the activity of the promising compounds for the other pathophysiological issues such as neuropathic disorders has become rutin (NINDS, ASP). Nafimidone is an example of (arylalkyl) azoles, which possess a profile of activity similar to that of phenytoin or carbamazepine but distinct from barbiturates or valproic acid. Nafimidone alcohol, the major metabolite of nafimidone, also has high anticonvulsant activity. SAR studies of (arylalkyl) azole anticonvulsant compounds have shown that the anticonvulsant properties of this group are associated with the presence of a small oxygen functional group (such as carbonyl, ethylene dioxy, methoxy, acyloxy, hydroxyl and oxime ether substituents) in the alkylene bridge in addition to imidazole ring and lipophilic aryl portion facilitating penetration through blood-brain barrier (16).

In this study, in the light of available literature and as a continuation of our previous studies on anticonvulsant nafimidone derivatives, we aimed to evaluate the effects of some new oxime ester derivatives of nafimidone on disorders of thermal pain sensation in diabetic mice (17-19).

Material and Methods

Animals

A total of 25 female BALB/c albino mice (five months old) were used. The colony was obtained from the Laboratory Animals Research Center of Inonu University, Malatya, Turkey; all handling and maintenance conditions were according to the rules and regulations of the local Institutional Ethic Committee. The animals weighing 28-30 g were housed in a room maintained at 22°C with a 12:12 h light-dark cycle, and had free access to food. Mice were divided randomly into five groups (5 mice per group): Group I served as "control" and mice were untreated. Mice in Group II, III, IV and V were injected intraperitonally each with a single dose of 150 mg/kg body weight of streptozotocin (Sigma Chemicals), dissolved in 0.9% NaCl solution and given in a volume of 0.5 mL (20). Mice in control group received an equivalent volume of 0.9% NaCl solution. The diabetes was confirmed one week after the streptozotocin injection by measuring the blood glucose level with a portable analyzer (Lever Check, Muenster, Germany) using glucose oxidase peroxidase enzyme reagent stripes. Blood was withdrawn from the tail ven of mice with a heparin-ized capillary tube. Animals with a non-fasting serum glucose levels above 275 mg/dL were considered diabetic and used for the all tests after five weeks.

Compounds and administration

New compounds 2-(1H-imidazol-1-yl)-1-(naphthalene-2-yl) ethanone O-pentanoyl oxime hydrochloride (C1), 2-(1H-imidazol-1-yl)-1-(naphthalene-2-yl)ethanone O-3-methylbutanoyl oxime hydrochloride (C2), 2-(1H-imidazol-1-yl)-1-(naphthalene-2-yl)ethanone O-2-propylpentanoyl oxime hydrochloride) (C3) were prepared by esterification of the nafimidone oxime with carboxylic acid and dicyclohexilcarbodiimide/4-dimethylaminopyridine (DCC/DMAP). All compounds were synthesized at Inonu University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry. The structures of the all compounds were confirmed by IR, ^{1}H-NMR, Mass spectral and elementary analysis (19). Thirty minutes before starting the tests, 100 mg/kg b.w. dose - C1 to the first group of the mice; 100 mg/kg b.w. dose-C2 to the second group of the mice; and 100 mg/kg b.w. dose-C3 to the third group of the mice were injected i.p. We tested antiepileptic activity of all new compounds via MES test and found 100 mg/kg b.w. as the most effective dose before main experimental process. All three compounds were suspended in 30% PEG-400 (Polyethylene glycol). To the control group only 30% PEG-400 which is used as solvent was injected i.p.

Assessment of thermal nociceptive responses

Thermal nociceptive responses were assessed by hot and cold plate, and tail-immersion test methods. Animals were always habituated to the testing environment more than 60 min prior to the test. Each mouse was tested only once at 30 min post-injection. The observer did not know which agent was being tested.

Hot plate test

The hot plate test was used to measure latency by the method of Bispo et al. (21) with small modifications. The temperature of the plate (Model MK-350B, Muromachi Kikai Co., Japan) was fixed at 53±0.2°C. A cut off time of 15 sec was maintained at 53°C to prevent tissue damage. The reaction time (jumping, licking one of the hind legs or one of the forelegs, tapping) was measured 30 min after intraperitoneal (i.p.) administration of the drug solution.

Tail-immersion test

The distal part of the tails of the animals was immersed in hot water maintained at 55.0±1.0°C. The time taken to withdraw the tail was noted as reaction time (22). Prior to the experiment the mice were screened for the sensitivity test by immersing the tail of the mice gently in hot water maintained at 55°C (23). A cut off time of 10 sec was maintained at 55°C to prevent tissue damage. This test was carried out at 30 min after i.p. administration of the compounds.

Cold-plate test

Mice were placed on a cold metal plate maintained at 4±1°C and covered with a transparent plastic box (24×24×21 cm). Brisk lifting of the left hind paw was counted as a noci-

ceptive response, and the number of such responses over a 2 min period was recorded and scored by an observer who was blind to the treatment condition. Walking steps and slow paw lifting related to locomotion were not counted (24).

Statistical analysis

Analysis of Variance, (ANOVA) was performed to all the data and these were followed by a post hoc LSD test for differences between paired groups. It was observed that all the data conformed to normal distribution except blood glucose level. Therefore, nonparametric Kruskal Wallis of variance and Mann-Whitney U tests were performed for the values of glucose. The statistical significance level was set at $p < 0.05$.

Results

Within 3 days of the STZ injection developed hyperglycemia in mice. Diabetic mice showed polyuria, a marked increase in food and water intake, and failed to gain weight (Table 1).

Approximately 20% loss in body weight was determined in STZ injected grups. However, mice showed marked hypoalgesia after 6 weeks of diabetes. According to our findings; compared with the control group, the hot-plate response time and the number of paw liftings on cold plate as important indicators of loss of sensation increased, but no significant difference ($p > 0.05$) was found in tail-immersion response time test in diabetes group. However, used novel therapeutic compounds affected in varying degrees of these responses. Especially, C3 compound was the most effective agent in the all of three tests. C1 and C2 compounds were effective only in cold-plate test.

Discussion

Type I and II diabetic patients are affected by diabetic neuropathy that usually develops as an initial phase of hyperalgesia and allodynia followed by a later phase of hypoalgesia. Associated with DN, assessment of altered responses to both

nociceptive and non-nociceptive stimuli in diabetic rats and mice is performed using a number of behavioural tests, among which paw withdrawal, tail flick, hot-plate and cold-plate are the most widely used. In general, tail-immersion test at spinal level; hot-plate test at both spinal and supraspinal level and cold-plate test through peripheral sensitization give an idea to evaluate the pain perception and transmission (25, 26).

There is a general agreement that diabetic neuropathy in animal models is characterized by a disruption in peripheral nerve function (27, 28). All previous studies describe decreased nerve conduction velocity in diabetic animals, although the magnitude varies (29, 30). Also altered thermal perception has been described and is manifested by thermal hyperalgesia or hypoalgesia (31, 32). We can say that our findings agree with mentioned previous works; we observed thermal hypoalgesia in mice 6 weeks after the STZ injection.

Four main hypotheses about how hyperglycaemia and/or low insulin levels causes diabetic complications are: increased polyol pathway flux, increased advanced glycation end-products (AGEs) formation, activation of protein kinases (PKC and PKA), and increased hexosamine pathway flux (33). Additionally, it has been suggested that, as a result of deteriorated sodium channel activation due to continued hyperglycemia, increased flow of sodium into the cell, in connection with the above mentioned mechanisms, plays an important role in the pathogenesis (34).

Na_vChs in the cell membrane of the nociceptor neurons are integral heteromeric protein complexes that selectively conduct Na^+ ions and amplify membrane depolarisation, thus resulting in the generation and propagation of action potentials that pass into the dorsal horn of the spinal cord (35). It has now been established from electrophysiological and molecular biological techniques that Na_vChs play an important role in the molecular pathophysiology of DN (36). As it is known, high glucose stimulates increased kinases' activity through increased cAMP, which can increase Na_vChs trafficking and thus potentiate the sodium current (37).

The underlying molecular mechanisms of hyper and hypoalgesia observed in different stages of diabetic neuropathy

Table 1. The animals were weighed at the beginning and end of the experiment. Statistically significant difference between control and other three groups were displayed. Also, blood glucose levels (mg/dL) and results of tail-immersion (latency time as seconds), hot-plate (latency time as seconds) and cold-plate (number of brisk lifting of the left hind paw) tests were summarized. Results were expressed as Mean+SE

Groups (n=5)	Blood Glucose (mg/dL)	Tail-immersion response time (sec)	Hot-plate response time (sec)	Cold-plate (the number of paw liftings)	Body Weight (g)	
					Before STZ injection	6 weeks after STZ injection
Control	127.2±4.09[b,c,d,e]	5.4±0.94	7.0±0.36[b,d,e]	6.3±0.79[b]	30.6±2.1	31.3±1.8[b,c,d,e]
Diabetes	293.6±8.68[a]	7.5±1.81	11.5±2.01[a,e]	2.5±0.31[a,c,d]	29.4±1.9	23.7±1.5[a]
Diabetes+C1	277.0±7.76[a]	5.3±0.56	10.9±2.20[e]	6.7±0.86[b]	30.2±2.3	24.9±2.0[a]
Diabetes+C2	286.4±6.82[a]	6.4±1.24	13.7±1.73[a,e]	5.42±0.73[b]	31.1±1.7	25.2±2.2[a]
Diabetes+C3	283.6±9.34[a]	3.9±0.63[b]	4.43±0.25[a,b,c,d]	6.92±1.08[b]	29.7±1.8	24.5±2.1[a]

[a] $p < 0.05$ when compared with Control group
[b] $p < 0.05$ when compared with Diabetes group
[c] $p < 0.05$ when compared with Diabetes + C1 group
[d] $p < 0.05$ when compared with Diabetes + C2 group
[e] $p < 0.05$ when compared with Diabetes + C3 group
STZ: Streptozotocin

is very complex. It has been suggested that hyperactivity of small, unmyelinated C-fibers results in hyperalgesia and allodynia (36). Also, Khan et al. (38) reported that A-fiber afferents in diabetic rats developed abnormal spontaneous discharges and increased sensitivity to mechanical stimuli, suggesting a role of large A-fiber neurons in addition to nociceptive C-fibers in the development of diabetic neuropathic pain in early stage of DM.

In our opinion, according to results of previous studies in the literature, a few weeks after induction of hyperglycemia, there is an increased expression and/or activation of Na_vChs that play an important role on developing of hyperexcitability in early stage of DM, but the possible role of Na_vChs on occured hypoexcitability and hyposensation as major symptoms of the later stage of DM 6-8 weeks after induction of hyperglycemia is still unknown. As one of the possible reasons, increase intracellular sodium was attributed to a decrease in the activity of Na^+/H^+ exchange system and may be due to low intracellular pH. Increased acidity may suppress excitability and/or conduction (33). Oskarsson et al. (39) reported that mexiletine, a sodium channel blocker, has proven to be a very safe therapy with negligible side effects in DN. However, all the molecular mechanisms involved in the formation of "loss of sensation" developing over time and hypoalgesia as a result of that loss have not yet been fully clarified.

In the literature, neurodegenerative damage caused by hyperglycemia and oxidative stress, usually due to long term hyperglycemia, is emphasized to characterize by message function disorders occuring along the myelinated and unmyelinated nerves (40). In addition, it is suggested that an increased ion influx to the cell from over-activated sodium and calcium channels due to phosphorylation caused by PKC and PKA activations plays a role in the pathogenesis (33). With a hypothetical approach; a long-term hyper excitability may have caused neural insensitivity in time. Our research findings indicate that increased sodium channel expression and activation in untreated diabetes play a role in formation mechanism of hypoalgesia in time and sodium channel blockage significantly prevents loss of sensation caused by intra cellular excessive sodium presence by obstructing the flow of sodium into the cell.

Valproic acid derivatives among nafimidone oxime esters that we have used in the research; 2-(1*H*-imidazol-1-yl)-1-(naphthalene-2-yl)ethanone *O*-2-propylpentanoyl oxime hydrochloride (C3), were effective in all three thermal response tests and significantly inhibited the loss of sensation which evolves due to diabetes in time. The other two derivative compounds 2-(1*H*-imidazol-1-yl)-1-(naphthalene-2-yl) ethanone *O*-pentanoyl oxime hydrochloride (C1) and 2-(1*H*-imidazol-1-yl)-1-(naphthalene-2-yl) ethanone *O*-3-methylbutanoyl oxime hydrochloride (C2) were only effective at cold-plate test whereas it indicated no activity at tail-immersion and hot-plate tests.

In this context, it can be stated that the C3 derivative molecule is effective on pain transmission ways through both spinal and supraspinal and peripheral sensitization and the other two derivative molecules C1 and C2 were only effective on pain transmission way through peripheral sensitization. Although all these three derivatives are among nafimidone oxime esters, probable reason of why only C3 is more effective may be

the lipophilic character it gained due to valporic acid in comparison with others. On the other hand, Na_vChs are multimeric structures composed of many subunits. Increases in the expression and activity of the different subunits can be observed in different pathologies. Each of the derivative structures that we have synthesized, are likely to cause blockage at different levels by acting on different subunits.

As a result, it can be suggested that valproic acid derivative of nafimidone oxime may be effective in the cases where epilepsy and diabetes occur together since it has shown efficacy against "loss of sensation" which evolves in diabetic neuropathy over time as well as its antiepileptic effect. However, further molecular studies are needed on animals and humans to determine on which subunits of sodium channels those new derivative compounds are effective or what other mechanisms they are active in and the levels of doses and bioavailability.

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