Thrombosis Research 136 (2015) 1310–1317

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

N2 extenuates experimental ischemic stroke through platelet aggregation inhibition

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ARTICLE INFO

Article history:
Received 12 March 2015
Received in revised form 13 September 2015
Accepted 28 October 2015
Available online 30 October 2015

Keywords:
N2
Thromboxane A_2
Ischemic stroke
Platelet aggregation

ABSTRACT

Introduction: Thromboxane A_2 (TXA_2) can induce the platelet aggregation and lead to thrombosis. This will cause the low-reflow phenomenon after ischemic stroke and aggravate the damage of brain issues. Therefore, it is potential to develop the drugs inhibiting TXA_2 pathway to treat cerebral ischemia.
Aim: This study aims to prove the protective effect of N2 (4-(2-(1H-imidazol-1-yl) ethoxy)-3-methoxybenzoic acid) on focal cerebral ischemia and reperfusion injury through platelet aggregation inhibition.
Materials and methods: Middle cerebral artery occlusion/reperfusion (MCAO/R) is used as the animal model. Neurological deficit score, Morris water maze, postural reflex test, Limb-use asymmetry test, infarct volume, and water content were performed to evaluate the protective effect of N2 in MCAO/R rats. 9, 11-dieoxy-11α, 9α-methanoepoxyprostaglandin $F_{2\alpha}$ (U46619) or adenosine diphosphate (ADP) was used as the inducer of platelet aggregation.
Results and conclusions: N2 can improve the motor function, learning and memory ability in MCAO/R rats while reducing the infarct volume. N2 can inhibit TXA_2 formation but promote PGI_2, and can inhibit platelet aggregation induced by U46619 and ADP. Further, N2 inhibits thrombosis with a minor adverse effect of bleeding than Clopidogrel. In conclusion, N2 can produce the protective effect on MCAO/R brain injury through inhibiting TXA_2 formation, platelet aggregation and thrombosis.

1. Introduction

As the second most common cause of death, stroke is also the major cause of disability worldwide [1]. On average, every 40 s, there is one person hit by stroke; and approximately every 4 min, a patient with stroke died [2]. Inappropriate activation of platelets, which are under some pathological conditions, leads to thrombosis; and the formation of occlusive thrombi within the circulation will finally lead to stroke [3]. Therefore, it has been proved that the pharmacological suppression of platelet function is effective in the reduction of risk of thrombosis [4]. For the various antiplatelet agents which have been shown to be effective in stroke [5], including Aspirin, Clopidogrel, Ticlopidine, and Dipyridamole, there are always some inevitable side effects with certain severity, such as gastrointestinal disorders and bleeding [6]. Therefore, it is urgent to develop antiplatelet agents with high efficacy but fewer side effects.

The endogenous platelets activator, including collagen, adenosine diphosphate (ADP), thromboxane A_2 (TXA_2) and thrombin, among which TXA_2 is the strongest, plays a significant role in this process. They will activate and adhere to the injury site when vessel wall damage occurs. Afterwards platelets will secrete granule contents and aggregate, initiating thrombus formation at last [7]. The concentration of intracellular Ca^{2+} will be improved once cerebral ischemic stroke and reperfusion occur. The activated Ca^{2+}-dependent phospholipase A_2 (PLA_2) will promote the generation of arachidonic acid (AA) which will be decomposed into TXA_2 by cyclooxygenase and thromboxane synthase [8]. The combination of TXA_2 and G protein coupled receptor on the platelet triggers the original signal [9], which through the cascade reactions of several downstream signaling paths finally actives αIIbβ3-integrin, causes the adhesion and aggregation of platelet as well as vasoconstriction. As a result, the hypoperfusion and secondary

Abbreviations: N2, 4-(2-(1 H-imidazol-1-yl) ethoxy)-3-methoxybenzoic acid; NS, 0.9% sodium chloride; ADP, adenosine diphosphate; PLA_2, phospholipase A_2; MCAO, middle cerebral artery occlusion; MCAO/R, middle cerebral artery occlusion/reperfusion; AA, arachidonic acid; LTs, leukotrienes; TXA_2, Thromboxane A_2; U46619, 9, 11- dieoxy-11α,9α-methanoepoxyprostaglandin $F_{2\alpha}$; PRP, platelet-rich plasma; PPP, platelet-poor plasma; TXB_2, thromboxane B_2; 6-keto-PGF1α, 6-keto-prostaglandin F1α; TTC, 2,3,5-triphenyl-tetrazolium chloride; ELISA, enzyme-linked immunosorbent assay.

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http://dx.doi.org/10.1016/j.thromres.2015.10.039
0049-3848/

injures of brain issues after cerebral stroke will be aggravated. Therefore, TXA_2 pathway could be the potential target for ischemic cerebral stroke.

N2 (4-(2-(1H-imidazol-1-yl) ethoxy)-3-methoxybenzoic acid, MW 284.24), is an analog of Dazoxiben (chemical structure see Fig. 1, a thromboxane synthase inhibitor with antiplatelet aggregative activity, acts by inhibiting the generation of TXA_2). This study established rats Middle cerebral artery occlusion/reperfusion (MCAO/R) model to investigate the protective effect and possible mechanisms of N2 on ischemic stroke. Ozagrel, an antiplatelet agent working as a thromboxane A2 synthase inhibitor, modulating the arachidonic acid cascade thereby reducing TXA_2 and increasing PGI_2, was used to compare the effect of N2 in vitro.

2. Materials and methods

2.1. Chemicals and reagents

N2 was provided by Hefei Yigong Medicine Co., Ltd. 9, 11-dieoxy-11α, 9α-methanoepoxyprostglandin F2α (U46619) was purchased from Enzo Life Science. Adenosine 5′-diphosphate (ADP) was product of Amresco. Thromboxane B_2 (TXB_2) and 6-keto-prostaglandin $F_{1\alpha}$(6-keto-$PGF_{1\alpha}$) ELISA kits were bought from Shanghai Lengton Bioscience Co., Ltd. All other reagents were of analytical grade and commercially available.

2.2. Animal care

Male and female Sprague–Dawley rats were purchased from Qinglongshan Animal Farm of Nanjing, China. All animals were cared for in compliance with institutional guidelines of China Pharmaceutical University (Nanjing, China). All experiments were approved by Animal Ethics Committee of China Pharmaceutical University.

2.3. Groups and administration

A total of 90 male Sprague–Dawley rats were randomly divided into six groups: 1) Sham group; 2) MCAO/R model; 3) Clopidogrel (15 mg/kg); 4) N2 high dose (30 mg/kg); 5) N2 middle dose (15 mg/kg); 6) N2 low dose (7.5 mg/kg). Sham group and MCAO/R model group received the same volume of 0.9% sodium chloride injection (NS). All drugs were administrated with twice loading dose at 3 h after reperfusion. Loading dose of durgs were administrated for 14 days with a volume of 0.5 ml/100 g/day.

2.4. Establishing model of MCAO.

Rats were fasted for 24 h before operation. MCAO/R injury was induced following the intraluminal suture procedure [10]. Rats were anesthetized by 3% chloral hydrate (1 ml/100 g, i. p) and fixed in the supine position. During the whole experiment, body temperature was maintained at 37 ± 0.5 °C. Briefly, a monofilament nylon suture with silicon coated tip was inserted to occlude the origin of middle carotid artery (MCA). Reperfusion was produced by withdrawal of the intraluminal suture after two-hour occlusion. Sham-operated rats received the same surgical exposure procedures without occlusion of MCA.

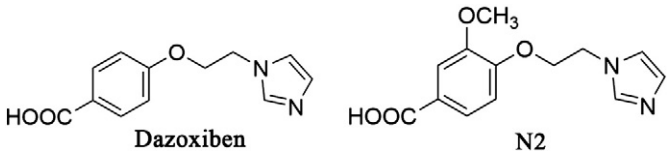

Fig. 1. Chemical structures of Dazoxiben and N2.

2.5. Morris water maze

On the 9th day after reperfusion, rats were tested for learning and memory ability in Morris Water Maze (MWM) as described with minor modifications [11]. Rats received the visible platform trial for the first 2 days, the non-visible platform trial for following 3 days and the probe trial on the last day. The escape latency and swimming path were recorded during the first five days. In the probe trial, the percentage of time spent in the quadrant IV and number of crossing platform was measured.

2.6. Cylinder test

On the 2nd, 5th, 10th and 15th day after reperfusion, the asymmetry of forelimb usage was assessed by the cylinder test. For the limb-use asymmetry test [12], rats would rear up on its hind limbs and explore the vertical surface with its forelimbs. For each rat, 20 times of contacting the wall using forelimbs were observed and the number of wall contacts was measured. The asymmetric score = [(right-left)/(right + left + both)] × 100%.

2.7. Posture reflex test

The postural reflex test was operated on the same day with the cylinder test. In the postural reflex test, the rat was held by the tail and suspended 1 m above the floor and observed its forelimb flexion [13]. Each rat was scored for 20 times in one test according to the following standards [14]: 0 point: both forelimbs extended toward the floor; 1 point: left forelimb affixed to the chest but right forelimb extended toward the floor; 2 point: left forelimb affixed to the chest and the body rotated to left forelimb.

2.8. Neurological defect scoring

At 0.5 h after the final administration, the neurological function was scored according to the method of a 5-point scale [10] by the same experimenter, who was blinded to the different treatments in the experiment. 0 point: normal activities without neurologic deficiency; 1 point: left forepaws with flexion, adduction and affixed to chest; 2 point: circling to the left; 3 point: falling to the left; 4 point: being unable to walk spontaneously and flaccid paralysis.

2.9. Evaluation of infarct and edema

After measuring the above indicates, all rats were sacrificed under deep anesthesia to obtain the brain sample. The samples were weighed and cut coronally into five 2-mm-thick sections, then incubated in 1% TTC at 37 °C [15]. Then the slices were photographed. Infarct rate was calculated as following: infarct rate = (weight of infarction area/weight of brain) × 100%. Edema was determined by measuring the brain water content according to the wet-dry method. Brains were dried in a desiccating oven at 110 °C for 24 h and the cerebral water content was calculated according to the following formula: Cerebral water content (%) = [(1-brain dry weight(g)/brain wet weight (g)) × 100% [16].

2.10. ELISA analysis of TXB_2 and 6-keto-$PGF_{1\alpha}$

Rats were decapitated after evaluation of the neurological deficit scores. Each 0.6 g of cortex was homogenized with 3.6 ml of phosphate buffer (0.1 mM EDTA). The homogenate was centrifuged at 800 *g* for 10 min and then the obtained supernatant was centrifuged at 10,000 *g* for 1 h at 4 °C.The samples were measured using the enzyme immunoassay kit of TXB_2 and 6-keto-$PGF_{1\alpha}$ (stable metabolite of TXA_2 and PGI_2).