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Combination treatment of experimental stroke with Niaspan and Simvastatin, reduces axonal damage and improves functional outcome

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ABSTRACT

In this study we examined the effect of combination treatment of experimental stroke with Niaspan, a prolonged-release formulation of Niacin (vitamin B3), and Simvastatin, a cholesterol-lowering drug, on functional outcome, axonal damage, axonal density and the of Iba-1 immunoreactive microglia expression in the ischemic brain of rats. Adult male rats were subjected to 2 h middle cerebral artery occlusion (MCAo) and treated with or without Niaspan alone, Simvastatin alone and combination Niaspan and Simvastatin starting 24 h after MCAo and daily for 14 days. Neurological functional tests were performed. Axonal damage and density were evaluated by Amyloid Precursor Protein (APP) and Bielschowsky silver, respectively. Nogo66 Receptor (NgR) expression and immunoreactive microglia (Iba-1) were also measured in the ischemic brain. Niaspan and Simvastatin monotherapy and combination treatment significantly promote functional outcome after stroke ($p<0.05$) compared to MCAo control animals. Combination treatment with Niaspan and Simvastatin induces additive but not synergetic effects when compared to Niaspan or Simvastatin monotherapy groups. Combination treatment significantly decreased APP expression and increased Bielschowsky silver expression. NGR and Iba-1 expression were significantly decreased in the ischemic brain. These data suggest that treatment of experimental stroke with combination of Niaspan and Simvastatin significantly improves functional outcome, reduces axonal damage and increases axonal density. Decreased expression of the NGR and reduced activated microglia may contribute to functional recovery after stroke.

1. Introduction

Niacin is a lipid-altering agent that favorably affects multiple lipid parameters. Niacin is the most effective medication in current clinical use for increasing high density lipoprotein (HDL) cholesterol [1]. Niaspan, a prolonged-release formulation of Niacin (vitamin B3), reduces the Niacin-induced major side effects of flush and hepatotoxicity [2]. Niacin has been evaluated in combination with Simvastatin in several clinical trials and has demonstrated efficacy, with clinical improvement in multiple lipid parameters [3,4]. Simvastatin like other statins is a lipid-altering drug with proven lipid and coronary artery disease (CAD) outcome efficacy [5]. Apart from their lipid-lowering activities, statins have also been shown to mediate pleiotropic effects by enhancement of endothelial function, angiogenesis and reduction of inflammatory responses [6,7]. Several recent clinical trials have demonstrated the efficacy and safety of combined Niaspan and Simvastatin [3,8–10]. The randomized, double-blind ARBITER 2 study [11] demonstrated the benefits of combination of Niaspan and Statin for inhibition of atherosclerosis. Previous studies have shown that Statin or Niaspan monotherapy of stroke animals induces angiogenesis and improves functional outcome [12–15]. Whether combination treatment of stroke with Niaspan and Statin regulates functional outcome has not been investigated.

In the central nervous system (CNS), injured axons not only degenerate but are unable to regenerate and have a limited capacity to sprout and re-establish lost connections [16,17]. Therefore, axonal damage often results in long term disability [16]. Following the immediate primary CNS injury, there is a cascade of down stream events termed "secondary injury" which culminate in progressive axonal degeneration [18–20].

Experimental studies in rodents revealed a rapid, progressive activation of microglia cells in the ischemic brain after stroke [21]. Microglia are thought to orchestrate damage in the penumbra [22,23]. In vitro studies showed that injury from various ischemia-like insults was increased in the presence of microglia [24–26].

In this study, we examined the effect of combination treatment of stroke with Niaspan and Simvastatin on functional outcome, axonal

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damage and axonal density, as well as the expression of Nogo66 Receptor (NgR) and Iba-1 immunoreactive microglia in the ischemic brain in a rat model of middle cerebral artery occlusion (MCAo).

2. Material and methods

2.1. Animal middle cerebral artery occlusion (MCAo) model and experimental groups

Adult male Wistar rats weighing 270–300 g were employed in all experiments. Transient right middle cerebral artery occlusion (MCAo) was induced for 2 h by advancing a 4-0 surgical nylon suture (18.5–19.5 mm), determined by the animal weight, with its tip rounded by heating near a flame, to block the origin of the MCA, using a method of intraluminal vascular occlusion modified in our laboratory [27]. Two hours after MCAo, reperfusion was performed by withdrawal of the suture. Experimental groups consist of rats subjected to 2 h of MCAo. Twenty-four hours after MCAo rats were randomly separated into 4 groups:

1) Control group: rats were gavaged with water daily for 14 days ($N=8$ rats).
2) Niaspan alone treatment group: rats were gavaged with Niaspan (40 mg/kg, Kos Pharmaceuticals, Cranbury, NJ) alone daily for 14 days ($N=6$ rats).
3) Simvastatin alone treatment group: rats were gavaged with Simvastatin (1 mg/kg, Merck, Somerset, NJ) alone daily for 14 days ($N=7$ rats).
4) Combination treatment group: rats were gavaged with Niaspan (40 mg/kg, Kos Pharmaceuticals, Canburry, NJ) in combination with Simvastatin (1 mg/kg, Merck, Somerset, NJ) daily for 14 days ($N=8$ rats).

2.2. Functional tests

A modified neurological severity score (mNSS) evaluation and foot-fault tests were performed [28,29] before MCAo, and at 1, 7, and 14 days after MCAo by an investigator who was blinded to the experimental groups.

2.3. Histological and immunohistochemical assessment

At fourteen days after MCAo, animals were sacrificed and brains were fixed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde before being embedded in paraffin. Seven coronal sections of tissue were processed and stained with hematoxylin and eosin (H&E) for calculation of volume of cerebral infarction [30]. The indirect lesion area, in which the intact area of the ipsilateral hemisphere was subtracted from the area of the contralateral hemisphere, was calculated using the Global Lab Image analysis system (Data Translation, Marlboro, MA) [30]. Lesion volume is presented as a volume percentage of the lesion compared with the contralateral hemisphere.

2.4. Immunohistochemical staining

A standard paraffin block was obtained from the center of the lesion (bregma −1 mm to +1 mm). A series of 6 μm thick sections were cut from the block. Every 10th coronal section for a total 5 sections was used for immunohistochemical staining. Antibodies against Amyloid Precursor Protein (APP; dilution 1:50, Cell Signaling Technology), Nogo66 Receptor (NgR; dilution 1:50, Santa Cruz Biotech Inc, Santa Cruz, California), and Ionizing calcium-Binding Adaptor molecule 1 (Iba-1; dilution 1:1000, Wako) were employed. Control experiments consisted of staining brain coronal tissue sections as outlined above, but the primary antibodies were omitted, as previously described [31]. Bielschowsky silver immunostaining was used to demonstrate axons. In brief, for Bielschowsky silver staining, slides were placed in 20% $AgNO_3$ in the dark, and then NaOH and sodium thiosulfate were added to the slides in turn. The immunostaining analysis was performed by an investigator blinded to the experimental groups.

2.5. Quantitation

For quantitative measurements of APP, five slides from each brain, with each slide containing 6 fields from the ischemic border area (IBZ) which is adjacent to the ischemic core were digitized under a 20× objective (Olympus BX40) using a 3-CCD color video camera (Sony DXC-970MD) interfaced with an MCID image analysis system (Imaging Research, St. Catharines, Canada) [12,32,33]. For quantitative measurements of Bielschowsky silver staining and NGR, positive area of Bielschowsky silver immunoreactive cells and NGR were measured in the striatal white matter bundles in the ischemic border. For quantitative measurements of Iba-1, positive area of Iba-1 immunoreactive cells was measured in the ipsilateral hemisphere. Data were analyzed in a blinded manner and presented as percentage of positive area for, APP, NGR, Iba-1 and Bielschowsky silver immunoreactive cells, respectively.

2.6. Statistical analysis

Behavior tests (mNSS and foot-fault test) were performed at day 1 after MCAo before treatment (baseline), days 7 and 14 after MCAo. The Global Test using Generalize Estimating Equation (GEE) [34] was implemented to test the group differences on functional recovery measured from the two behavioral tests. The analysis was started by testing the treatment (Niaspan and Simvastatin) interaction, followed by testing the subgroup analysis, if the interaction or main effect was detected at the 0.05 level. The Global Test on multiple outcomes was considered more efficient than a single outcome, when the group effects were consistent on all the outcomes (e.g. positive correlation). The significant treatment interaction may indicate synergy effects of the two treatments and no interaction indicates the additive effects of the two treatments.

Comparisons between treatment groups of data measured by independent *t*-test on day 14 were made using analysis of variance models. Variables included the lesion volume, APP, Bielschowsky silver, NGR and Iba-1. All data are presented as mean ± standard error.

3. Results

3.1. Neurological outcome (Fig. 1)

To test the effect of the combination treatment of stroke rats on functional outcome, GEE statistical analysis was performed to detect the differences in functional recovery among the 4 groups (MCAo control, Niaspan treatment, Simvastatin treatment and combination treatment). The 4 groups were balanced at the baseline and there was no individual functional difference among 4 groups ($p=0.99$ based on the Global Test).

On day 14, a marginal significant interaction was detected based on the Global Test ($p=0.08$), indicating a trend synergestic of the two treatments. A significant effect was observed in the Niaspan treated group ($p=0.0126$), in the Simvastatin treated group ($p=0.0015$) and in the combination treated group ($p=0.0029$), compared to the MCAo control group (Fig. 1A–B). On day 7, no treatment interaction was detected based on the Global Test ($p=0.27$), indicating additivity of the two treatments. Based on the Global Test a significant effect was observed in the Niaspan treatment group ($p=0.038$), in the Simvastatin treatment group ($p=0.027$) and in the combination treatment group ($p=0.0087$), compared to the MCAo control group