Published in final edited form as:

Neurobiol Dis. 2010 October ; 40(1): 277–283. doi:10.1016/j.nbd.2010.05.034.

Niaspan Treatment Induces Neuroprotection After Stroke

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Abstract

Introduction—Niaspan, An Extended-Release Formulation Of Niacin (Vitamin B3), Has Been Widely Used To Increase High Density Lipoprotein (HDL) Cholesterol And To Prevent Cardiovascular Diseases And Stroke. In This Study, We Tested Whether Niaspan Administered Acutely After Stroke Is Neuroprotective.

Methods—Adult Male Rats (N=8/Group) Were Subjected To 2hs Of Middle Cerebral Artery Occlusion (Mcao) And Treated With Or Without Different Doses Of Niaspan (20mg/Kg, 40mg/Kg Or 80mg/Kg) At 2 Hours And 24 Hours After Mcao. A Battery Of Functional Outcome Tests Was Performed, And Serum HDL And Triglycerides Were Measured. Rats Were Sacrificed At 7 Days After Mcao And Lesion Volumes Were Measured. The Optimal Dose Of Niaspan Treatment Of Stroke Was Chosen For Immunostaining: Deoxynucleotidyl Transferase–Mediated Dntp Nick-End Labeling (TUNEL), Cleaved Caspase-3, Tumor Necrosis Factor Alpha (TNF-Alpha), Vascular Endothelial Growth Factor (VEGF), Phosphorylated Phosphatidylinositol 3-Kinase (P-PI3K). Another Set Of Rats (N=4/Group) Were Killed At 7 Days After Mcao For Western Blot Assay.

Results—Niaspan Dose-Dependently Reduced Infarct Volume And Improved Functional Outcome After Stroke. No Significant Difference In HDL And Triglyceride Levels Was Detected Between Niaspan Treatments And Mcao Control Groups. Niaspan Treatment Significantly Decreased The Number Of TUNEL-Positive Cells (105±17) And Cleaved Caspase-3 Expression (381±33) In The Ischemic Brain Compared To Mcao Control (165±18; 650±61, Respectively; P<0.05). Niaspan Treatment Significantly Reduced The Expression Of TNF-Alpha (9.7±1.1% Vs. 16±2.2%; P<0.05) And Negative Correlations Were Observed Between The Functional Tests And The Expression Of TNF-Alpha (R=−0.71, P<0.05). Niaspan Treatment Also Significantly Increased The Expression Of VEGF (5.2±0.9%) And PI3K/Akt (0.381±0.04%) In The Ischemic Brain Compared With Non-Treated Mcao Control (2.6±0.4%; 0.24±0.03, Respectively; P<0.05). The Functional Outcome Was Positively Correlated With P-PI3K (R=0.7, P<0.05).

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Disclosures None.

Conclusions—Treatment Of Stroke With Niaspan At 2 Hours After Mcao Reduces Infarct Volume And Improves Neurological Outcome And Provides Neuroprotection. The Neuroprotective Effects Of Niaspan Were Associated With Reduction Of Apoptosis And Attenuation Of TNF-Alpha Expression. VEGF And The PI3K/Akt Pathway May Contribute To The Niaspan-Induced Neuroprotection After Stroke.

Keywords

Niaspan; Neuroprotection; Apoptosis; Cleaved Caspase-3; TNF-Alpha; VEGF; PI3K/Akt

Introduction

Niacin Is A Lipid Altering Agent And Has Been Widely Used To Prevent Cardiovascular Diseases, Stroke And Atherosclerosis (Meyers Et Al., 2004). Niacin Is The Most Effective Medication In Current Clinical Use For Increasing High Density Lipoprotein (HDL) Cholesterol (Elam Et Al., 2000). Niaspan, An Extended-Release Formulation Of Niacin (Vitamin B3), Reduces The Niacin-Induced Major Side Effects Of Flush And Hepatotoxicity (Carlson, 2004). We Have Previously Demonstrated That Niaspan Treatment Of Experimental Stroke In Rats Starting 24 Hours After Stroke, Significantly Increases HDL Cholesterol, Enhances Local Cerebral Blood Flow, Promotes Angiogenesis And Arteriogenesis, And Improves Functional Outcome Without Affecting Infarct Volume (Chen Et Al., 2009; Chen Et Al., 2007). Extended-Release Niacin Decreases The Level Of C-Reactive Protein In Patients With Stable Coronary Artery Disease (Kuvin Et Al., 2006) And Inhibits Vascular Inflammation (Ganji Et Al., 2009; Wu Et Al.). The Inflammatory Response Is Characterized By The Local Expression Of Various Inflammatory Cytokines In The Brain (Degraha, 1998). Tumor Necrosis Factor Alpha (TNF-Alpha) Which Is Detected As Early As 1 Hour After The Onset Of Ischemia, Contributes To The Progression Of Neuronal Injury After Stroke (Morales Et Al., 2008; Sehara Et Al., 2007; Suzuki Et Al., 2009). Several Different Strategies To Inhibit TNF-Alpha Effects In Acute Stroke Have Been Reported To Reduce The Degree Of Ischemic Injury In Animal Models (Shohami Et Al., 1999). Niacin Is Metabolized In The Liver To Nicotinamide. Administration Of Nicotinamide Up To 2 Hours After Cerebral Ischemia Protects Against Both Necrosis And Apoptosis, And Reduces Infarct Volume (Mokudai Et Al., 2000; Yang Et Al., 2002a). In This Study We Tested The Hypothesis That Early Treatment Of Stroke With Niaspan Decreases Inflammation After Stroke And Induces Neuroprotective Effects.

The Pathophysiological Events After Cerebral Ischemia, Leading To Neuronal Death And Functional Disabilities Are Complex And Interconnected. The Acute Neuronal Injury Is Followed By A Second Round Of Neuronal Death In The Neighboring Area Of The Ischemic Core (Kirino, 2000). Apoptosis And Inflammation Are Among The Key Factors That Contribute To The Delayed Injury In The Penumbra (Becker, 1998; Graham And Chen, 2001). Caspases, A Superfamily Of Cysteiny-Aspartate Protease Are Essential Players In Cell Death (Schulz Et Al., 1999). Administration Of Broad Spectrum Caspase Inhibitors To Ischemic Rodents Induced Neuroprotection (Rabuffetti Et Al., 2000).

Vascular Endothelial Growth Factor (VEGF) And The Phosphatidylinositol 3-Kinase (PI3K)/Akt Signaling Pathway Promote Neuroprotection Post Stroke. VEGF Is An Angiogenic And Neuroprotective Factor That Can Increase Axonal Outgrowth, Block Neuronal Apoptosis, And Promote Neurogenesis (Jin Et Al., 2000). The PI3-K/Akt Pathway Has Been Implicated In The Ability Of VEGF To Enhance Neuroprotective Effect (Kilic Et Al., 2006). The PI3K/Akt Signaling Pathway Plays A Key Role In Cell Survival And Proliferation (Osaki Et Al., 2004). The Neuroprotective Effect Of PI3-K/Akt Has Also Been

Attributed To Its Anti-Apoptotic Action And Suppression Of Inflammatory Response (Yin Et Al., 2007).

In The Present Study, Using A Rat Model Of Middle Cerebral Artery Occlusion (Mcao), We Tested Whether Niaspan Treatment Would Provide Neuroprotection In Stroke As Measured By Neurological Behavioral Tests And Infarct Volume. We Also Investigated Whether This Neuroprotection Is Associated With Reduced Apoptosis And TNF-Alpha Expression. In Addition, We Examined The Effect Of Niaspan Treatment On VEGF And PI3K/Akt Activity In The Ischemic Brain.

Material And Methods

Animal Middle Cerebral Artery Occlusion (Mcao) Model And Experimental Groups

Adult Male Wistar Rats Weighing 270–300g Were Employed In All Experiments. Transient Right Middle Cerebral Artery Occlusion (Mcao) Was Induced For 2 Hours 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 (Chen Et Al., 2006). Rectal Temperature Was Maintained At 37°C Throughout The Surgical Procedure Using A Feedback Regulated Water Heating System. Experimental Groups Consist Of Rats Subjected To Transient Mcao (N=8/Group) And Randomly Treated With Different Doses Of Niaspan (Dissolved In Water; Kos Pharmaceuticals, Canburry, NJ):

1. Control: Water Was Gaviged At 2 Hours And 24 Hours After Mcao.
2. Niaspan 20 Mg/Kg Was Gaviged At 2 Hours And 24 Hours After Mcao.
3. Niaspan 40 Mg/Kg Was Gaviged At 2 Hours And 24 Hours After Mcao.
4. Niaspan 80 Mg/Kg Was Gaviged At 2 Hours And 24 Hours After Mcao.

All Rats Were Sacrificed At 7 Days After Mcao.

Functional Tests

A Modified Neurological Severity Score (Mnss) And Foot-Fault Test Were Performed Before Mcao, And At 1, 3 And 7 Days After Mcao By An Investigator Who Was Blinded To The Experimental Groups.

Modified Neurological Severity Score (Mnss)—Neurological Function Was Graded On A Scale Of 0 To 18 (Normal Score, 0; Maximal Deficit Score, 18). Mnss Is A Composite Of Motor, Sensory, Reflex, And Balance Tests. In The Severity Scores Of Injury, 1 Score Point Was Awarded For The Inability To Perform The Test Or For The Lack Of A Tested Reflex; Thus, The Higher Score, The More Severe Is The Injury (Chen Et Al., 2001b).

Foot-Fault Test—Animals Were Placed On An Elevated Grid Floor (45 Cm × 30 Cm), 2.5 Cm Higher Than A Solid Base Floor, With 2.5 Cm × 2.5 Cm Diameter Openings. Animals Tend To Move On The Grid With Their Paws Placed On The Wire Frame. When Animals Inaccurately Place A Limb, The Limb Falls Through One Of The Openings In The Grid. When The Limb Falls Through And Pulls Back Quickly Without Touching The Solid Base, The Behavior Is Called A Half Fault And Counted As 1 Foot-Fault Score. If The Limb Falls Through And Touches The Base For Support, This Situation Is Called A Full Fault And Counted As 2 Foot-Fault Scores. Both Foot-Fault Scores And The Number Of Total Steps For Both Forelimbs In A Trial With At Least 40 Movements Were Measured. The Asymmetry Score Were Given By Subtracting The Percent Of Ipsilateral Forelimb Faults

From The Percent Of Contralateral Forelimb Faults (Barth Et Al., 1990; Schallert Et Al., 2000).

Histological And Immunohistochemical Assessment

At Seven Days After Mcao, Animals (N=8/Group) 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 (Swanson Et Al., 1990). 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, Malboro, MA) (Swanson Et Al., 1990). Lesion Volume Is Presented As A Volume Percentage Of The Lesion Compared With The Contralateral Hemisphere.

Immunohistochemical Staining

The Optimal Niaspan Dose Which Reduces Infarct Volume And Improves Functional Outcome, Was Selected For Immunostaining. A Standard Paraffin Block Was Obtained From The Center Of The Lesion (Bregma -1 Mm To +1 Mm). A Series Of 6 μ M Thick Sections Were Cut From The Block. Every 10th Coronal Section For A Total 5 Sections Was Used For Immunohistochemical Staining. Antibody Against Cleaved Caspase-3 (Rabbit Polyclonal Igg; Dilution 1:200, Cell Signaling Technology, Danvers, Massachusetts), TNF-Alpha (Rabbit Polyclonal Igg; Dilution 1:200; Abcam PLC, Cambridge, Massachusetts), VEGF (Goat Polyclonal Igg; Dilution 1:200; Cruz Biotech Inc, Santa Cruz, California), And Phosphorylated Phosphoinositide-3 Kinase (P-PI3K; Goat Polyclonal Igg; Dilution 1:200; Cruz Biotech Inc, Santa Cruz, California) Immunostaining Were Performed. Control Experiments Consisted Of Staining Brain Coronal Tissue Sections As Outlined Above, But The Primary Antibodies Were Omitted, As Previously Described (Li Et Al., 1998). Terminal Deoxynucleotidyl Transferase-Mediated Dntp Nick-End Labeling (TUNEL) For Measuring Apoptosis Was Performed Using A Commercial Kit (Apoptag Kit, Chemicon, S7100).

Quantitation

For Measurement Of Apoptotic Markers, Each Cleaved Caspase-3 And TUNEL Immunostained Coronal Section Was Digitized Using A 20 $\times$ Objective, Via The MCID Computer Imaging Analysis System. Five Sections From The Standard Reference Coronal Section And 8 Brain Field Within Each Section Were Acquired And The Total Number Of Cleaved Caspase-3 And TUNEL Positive Cells In The 8 Fields Of The Ischemic Border Area (IBZ, See Figure 2A) Which Is Adjacent To The Ischemic Core Were Counted Using The MCID Computer Imaging Analysis System (Imaging Research, St. Catharines, Canada). The Total Number Of Positive Cells Per Mm² Area Is Presented.

For Quantitative Measurements Of TNF-Alpha, VEGF And P-PI3K, Five Slides From Each Brain, With Each Slide Containing 8 Fields From The IBZ Were Digitized Under A 40 $\times$ 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) (Calza Et Al., 2001; Chen Et Al., 2003a; Chen Et Al., 2003b). Data Were Analyzed In A Blinded Manner And Presented As Percentage Of Positive Area For, TNF-Alpha, VEGF And P-PI3K, Respectively.

Western Blot

Another Set Of Rats (N=4/Group) Were Killed At 7 Days After Mcao And Brain Tissue Were Extracted From The Ipsilateral Ischemic Area. Equal Amounts Of Cell Lysate Were Subjected To Western Blot Analysis, As Previously Described (Chen Et Al., 2003b). Specific Proteins Were Visualized Using A Supersignal West Pico Chemiluminescence Kit (Pierce). The Following Primary Antibodies Were Used: Anti-B-Actin (Dilution 1:2000; Santa Cruz Biotechnology, Santa Cruz, CA), Anti-Cleaved Caspase-3 (Dilution 1:1000; Cell Signaling Technology), Anti-TNF-Alpha (Dilution 1:500; Abcam PLC.), Anti-Phospho-Akt (Ser473; Dilution 1:1000, Cell Signaling Technology).

HDL Cholesterol And Triglycerides Measurements

Whole Blood HDL And Triglycerides Were Measured Before Treatment And 7 Days After Niaspan Treatment Using Cardiochek P•A Analyzer And HDL And Triglycerides Check Strips (Polymer Technology System, Inc. Indianapolis, IN), According To The Manufacturer's Instructions. 15µl Of Blood Was Collected From The Tail Vein. The Data Are Presented As Mg/Dl Values.

Statistical Analysis

Animals Subjected To Mcao Were Randomized Into One Of Four Groups Including Control Group, Niaspan 20 Mg/Kg, Niaspan 40 Mg/Kg, And Niaspan 80 Mg/Kg. Behavior Tests (Mnss And Foot-Fault Test) Were Performed Before Mcao At 1, 3 And 7 Days After Mcao.

The Global Test Using Generalized Estimating Equations (GEE) (Lu Et Al., 2003) Was Used To Test The Group Difference On Functional Recovery Measured From Multiple Behavior Tests. The Pair-Wise Treatment Comparison Was Conducted If The Overall Treatment Effect Was Detected At 0.05 Level. If The Global Test Was Significant At The 0.05 Level, Tests For Group Differences Were Conducted On Each Functional Test At The 0.05 Level. Otherwise, The Pair-Wise Treatment Comparison Or The Treatment Comparisons On Each Functional Outcome Were Considered As Exploratory. The Global Test On Multiple Outcomes Is More Efficient Than A Single Outcome, When The Group Effects Are Consistent On All The Outcomes (E.G., The Positive Correlation). The Correlation Among Two Outcomes Was Estimated Using GEE. The Most Effective Dose Of Niaspan Treatment Of Stroke Was Identified From The GEE Analysis.

Immunostaining Measures Including Cleaved Caspase-3, TUNEL, TNF-Alpha, VEGF And P-PI3k, Were Compared Between Rats Treated With Most Effective Dose Of Niaspan And Control Using Two-Sample T-Tests For Normally Distributed Variables And Wilcoxon Rank-Sum Tests For Non-Normal Variables. Spearman Correlation Coefficients Were Calculated For Immunostaining Measurements And The 7 Day Functional Recovery Measures Were Stratified By The Treatment Groups.

Results

Neurological Functional Outcome (Figure 1A And 1B)

To Test Whether Early Niaspan Treatment Of Stroke Rats Regulates Functional Outcome, A Battery Of Functional Tests Was Performed. On Day 3, The Global Test Showed That Rats Treated With Niaspan 40 Mg/Kg Had Significantly Improved Functional Recovery Compared To Mcao Control Group (P=0.029), And Marginally Improved Functional Outcome After Stroke Was Detected In The Niaspan 20 Mg/Kg Treatment Group Compared To The Mcao Control Group (P=0.06). Pair-Wise Comparisons Indicated That Rats In The 40 Mg/Kg And 20 Mg/Kg Treatment Groups Had Significantly Lower Foot-Fault Scores Compared To Control (P=0.012 And P=0.043, Respectively). The Only Significant Pair-

Wise Comparison For Mnss Was Between Rats Treated With 40 Mg/Kg And Mcao Control Rats ($P=0.008$). On Day 7, The Global Test Showed That Niaspan 20mg/Kg ($P<0.05$) And Niaspan 40mg/Kg ($P<0.05$) Treatment Of Stroke Significantly Improved Functional Recovery Compared To Mcao Control, Respectively. Global Test Showed That The Most Effective Dose Is Niaspan 40mg/Kg. Pair-Wise Comparisons Indicated That Rats In The 40 Mg/Kg And 20 Mg/Kg Treatment Groups Had Significantly Lower Foot-Fault Scores ($P=0.009$ And $P=0.005$, Respectively) And Mnss Test ($P<0.0001$ And $P=0.018$) Compared To Controls.

Lesion Volume (Figure 1C)—A One-Way ANOVA Model Was Used To Test The Difference In Ischemic Lesion Volume Between Treatment Groups. Pairwise Comparisons Indicated That The Only Significant Decrease In Infarct Volume Was Detected Between Niaspan 40 Mg/Kg ($18.30 \pm 1.09\%$) And Control ($32.57 \pm 4.37\%$, $P=0.011$) Groups. The Data Suggest That The Most Effective Niaspan Dose In This Experimental Protocol Was 40mg/Kg.

HDL And Triglycerides Levels

To Test Whether The Two Dose Niaspan 40mg/Kg Treatment Regulates HDL And Triglyceride Level, We Measured HDL And Triglycerides Before Treatment And At 7 Days After Niaspan Treatment. The Data Show That No Significant Difference ($P>0.1$) In HDL And Triglyceride Levels Was Detected Before Treatment Between Niaspan 40mg/Kg (33 ± 2.29 And 91.33 ± 9.11 , Respectively; $N=6$) And Mcao Control Group (31.33 ± 2.04 And 98.33 ± 12.6 , Respectively; $N=6$). The Levels Of HDL And Triglycerides Did Not Differ Significantly ($P>0.1$) At 7 Days In The Niaspan Treated Group (30.08 ± 2.28 And 77.4 ± 3.14 , Respectively; $N=6$) Compared With Mcao Control Group (29.83 ± 3.1 And 70.16 ± 3.02 , Respectively; $N=6$).

Niaspan Treatment Decreases Expression Of TUNEL And Cleaved Caspase-3

To Test Whether Niaspan 40mg/Kg Treatment Regulates Apoptosis, Two Apoptotic Markers, TUNEL And Cleaved Caspase-3, Were Employed. Figure 2B–C Show That Early Niaspan Treatment Significantly Decreased The Number Of TUNEL-Positive Cells In The Ischemic Brain, As Well As The Number Of Cleaved Caspase-3 Positive Cells Compared To Mcao Control Rats ($P<0.05$). Cleaved Caspase-3 Expression In Niaspan Treatment Group Significantly ($P<0.05$) Decreased In The Ischemic Brain Compared To Mcao Control As Measured By Western Blot (Figure 3). These Data Indicate That Niaspan Has An Antiapoptotic Role In The Treatment Of Stroke.

Niaspan Treatment Attenuates TNF-Alpha Expression In The Ischemic Brain

To Further Investigate The Effect Of Niaspan 40mg/Kg Treatment On The Inflammatory Reaction In The Ischemic Brain, We Measured The Expression Of TNF-Alpha In The Ipsilateral Ischemic Border Area. Figure 2D Shows That Niaspan Treatment Significantly Reduced The Expression Of TNF-Alpha In The Ischemic Border Compared With Mcao Control Rats ($P<0.05$). Figure 3 Shows That TNF-Alpha Expression Decreased ($P=0.06$) In The Niaspan Treatment Group Compared To Mcao Control. Significant Negative Correlations Were Observed Between The Functional Tests At Day 7 And The Expression Of TNF-Alpha ($R = -0.71$, $P<0.05$).

Niaspan Treatment Increases VEGF, PI3K/Akt Activity In The Ischemic Brain

In Order To Investigate Mechanisms Underlying The Niaspan-Induced Neuroprotection After Stroke, VEGF And P-PI3K Activity Was Measured In The Ischemic Border. Figure 2E–F Show That Niaspan 40mg/Kg Treatment Significantly Increased The Expression Of

VEGF (E) And P-PI3K (F) In The Ischemic Border Compared With Non-Treatment Mcao Control Rats ($P<0.05$). The Western Blot Data (Figure 3) Show That P-Akt Activity Significantly ($P<0.05$) Increased In The Treatment Group Compared To Mcao Control Group. The Functional Outcome At Day 7 Was Positively Correlated With P-PI3K ($R=0.7$, $P<0.05$).

Discussion

The Results Of The Current Study Indicate That Niaspan Treatment Of Stroke Starting At 2 Hours After Mcao Is Neuroprotective. The Neuroprotective Effect, Observed In The 40mg/Kg Niaspan Treatment Group Rather Than In 20mg/Kg And 80mg/Kg, Was Demonstrated By Significant Infarct Volume Reduction And Functional Improvement After Mcao In Rats. The Neuroprotective Effects Of Niaspan 40mg/Kg Were Associated With Reduction Of Apoptosis And Attenuation Of TNF-Alpha Expression. Niaspan Treatment Also Significantly Increased VEGF And PI3K/Akt Activity In The Ischemic Brain.

Niaspan Treatment Of Stroke Decreases Expression Of TUNEL And Cleaved Caspase-3

Apoptotic Cell Death Is A Highly Regulated Process That In Many Cases Requires Activation Of Caspases (Manabat Et Al., 2003). Caspase-3 Is The Most Abundant Caspase Family Member In The Adult And Neonatal Nervous System (Le Et Al., 2002). During Ischemia, Caspase-3 Is Cleaved And Activated Where Upon It Degrades Multiple Substrates In Cytoplasm And Nucleus Leading To Cell Death (Le Et Al., 2002; Namura Et Al., 1998). Genetic Deletion Of Caspase-3 Rendered Neurons Resistant To Ischemic Injury Both In Vivo And In Vitro (Le Et Al., 2002). Moreover, Inhibition Of Caspase-3 Like Activity In The Brain Prevented DNA Fragmentation And Other Apoptotic Nuclear Changes Resulting From Ischemic Injury (Luo Et Al., 2002). Niacin And Nicotinamide Are Water-Soluble B Group Vitamins. Nicotinamide, The Amide Form Of Niacin, Is The Precursor Of The Coenzyme Beta-Nicotinamide Adenine Dinucleotide (NAD^+) And Plays A Significant Role In Cell Survival And Metabolism. Nicotinamide Is Formed Through The Conversion Of Niacin In The Liver Or Through Hydrolysis Of NAD^+ (Li Et Al., 2006). Nicotinamide Prevents Neuronal Degeneration Against Trauma, Axonal Degeneration, Oxidative Stress, Cerebral Ischemia, And Spinal Cord Injury (Li Et Al., 2006). Nicotinamide May Foster Cellular Protection Through Modulation Of Caspase Activity (Maiese And Chong, 2003) During Cerebral Vascular Endothelial Injury (Chong Et Al., 2002) And Prevent Nitric Oxide-Induced Neuronal Injury (Lin Et Al., 2000). Our Data Demonstrate That Niaspan 40mg/Kg Treatment Inhibits Cleaved Caspase-3 Expression And Decreases DNA Fragmentation In The Brain After Transient Focal Cerebral Ischemia. Therefore, Niaspan Has An Anti-Apoptotic Role In The Treatment Of Stroke After Focal Ischemia In Rats.

Niaspan Treatment Attenuates TNF-Alpha Expression In The Ischemic Brain

TNF-Alpha Is One Of The Key Immunomodulatory And Pro-Inflammatory Cytokines Up-Regulated After Focal Stroke And Plays A Detrimental Role In Neuronal Survival (Hallenbeck, 2002; Wang Et Al., 2004). Administration Of TNF-Alpha During An Ischemic Brain Insult Has Been Shown To Augment The Injury, As Evidenced By Increased Tissue Damage And Neurological Deficits (Barone Et Al., 1997). TNF-Alpha Is Neurotoxic And Capable To Induce Apoptosis Through The Activation Of Caspase Cascade (Hsu Et Al., 1996; Rabuffetti Et Al., 2000). Inhibition Of The Effect Of TNF-Alpha In Acute Stroke, Such As Using An Anti-TNF Neutralizing Antibody (Hosomi Et Al., 2005) And Inhibition Of Soluble TNF-Alpha Receptor Type1 (Nawashiro Et Al., 1997) Reduce Ischemic Damage And Improves Functional Outcome After Stroke. Nicotinamide Is A Potent Inhibitor Of Proinflammatory Cytokines Such As TNF-Alpha In Vitro And In Vivo (Chen Et Al., 2001a; Fukuzawa Et Al., 1997; Ungerstedt Et Al., 2003). In Addition, Niacin Also Has Anti-

Inflammatory Properties And Inhibits Vascular Inflammation By Processes Independent Of Changes In Plasma Lipid And HDL Levels (Ganji Et Al., 2009; Wu Et Al.). Our Results Indicate That Niaspan 40mg/Kg Treatment Significantly Reduced The Expression Of TNF-Alpha In The Ischemic Border Compared With Mcao Control Rats ($P<0.05$), And TNF-Alpha Expression Significantly And Negatively Correlated With Functional Outcome After Stroke. This Anti-Inflammatory Effect Of Niaspan Was Not Associated With Changes In HDL Or Triglycerides. Therefore, The Reduction Of TNF-Alpha Expression May Contribute To Niaspan Treatment Induced Neuroprotection Effects Which Appear Independent Of Increasing HDL.

Niaspan Treatment Increases VEGF And PI3K/Akt Activity

VEGF Was First Found In Vascular Endothelial Cells And Was Characterized As An Angiogenic And Vessel Permeability Factor (Keck Et Al., 1989; Leung Et Al., 1989). VEGF Also Has Neuroprotective Effects In Vitro (Jin Et Al., 2000), And Protects Degenerating Motoneurons In Rodent Models Of Amyotrophic Lateral Sclerosis (Storkebaum Et Al., 2005). VEGF Antisense Treatment Increased Ischemia-Induced Neuronal Damage, Including Increased Numbers Of TUNEL-Positive Cells, Larger Infarct Volume (Yang Et Al., 2002b). Furthermore, Exogenous Administration (Sun Et Al., 2003) Or Overexpression Of VEGF Reduces Hypoxic Ischemic Brain Infarct And Decreases Hypoxic Neuronal Death (Sun And Guo, 2005) And Reduces Neurological Deficit (Zhang Et Al., 2000). Two PI3K Inhibitors, Wortmanin And LY294002, Reversed The Neuroprotective Effect Of VEGF In Immortalized Hippocampal Neuronal Cell Line (HN33). Inhibition Of PI3K/Akt Pathway, Completely Prevented VEGF Neuroprotection In In Vivo And In Vitro Models Of ALS (Lunn Et Al., 2009). PI3K/Akt Promotes Niaspan-Induced Neurogenesis (Chen Et Al., 2009; Chen Et Al., 2007). Activation Of Protein Kinase B (Akt1) Is A Necessary Requirement For Nicotinamide Cytoprotection And The Inhibition Of PI3K Phosphorylation Of Akt1 Eliminated The Cytoprotective Effect Of Nicotinamide Of Hippocampal Neurons (Chong Et Al., 2004). Our Data Show That Niaspan 40mg/Kg Treatment Significantly Increased The Expression Of VEGF, P-PI3K, And P-Akt In The Ischemic Brain Compared With Non-Treatment Mcao Control Rats And The Increased P-PI3K Expression Correlated With Functional Outcome After Stroke ($R=0.7$, $P<0.05$). Therefore, The PI3K/Akt Pathway May Be Involved In Niaspan-Induced Neuroprotective Effect.

In Summary, We Demonstrated That Niaspan Dose-Dependently Provides Neuroprotection In A Rat Stroke Model. Treatment Of Stroke With Niaspan 40mg/Kg At 2hs After Mcao Reduces Infarct Volume And Improves Neurological Outcome After Stroke. The Neuroprotective Effects Of Niaspan (40mg/Kg) Were Associated With Reduction Of Apoptosis And Attenuation Of TNF-Alpha Expression. VEGF And PI3K/Akt Pathway May Contribute To The Niaspan-Induced Neuroprotection After Stroke.

Acknowledgments

The Authors Wish To Thank Qinge Lu And Supata Santra For Technical Assistance.

Sources Of Funding This Work Was Supported By National Institute On Aging RO1 AG031811 (JC), National Institute Of Neurological Disorders And Stroke PO1 NS23393 (MC) And 1R41NS064708 (JC), And American Heart Association Grant 09GRNT2300151 (JC).

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Fig. 1. Neurological Outcome And Lesion Volume After Stroke

Panels **A–B** Show Mnss (A) And Foot-Fault (B) Tests After Stroke In The 4 Experimental Groups (Mcao Control, Niaspan 20mg/Kg, Niaspan 40mg/Kg And Niaspan 80mg/Kg Treatment Groups). Panel **C** Shows The Lesion Volume In The 4 Experimental Groups. SE = Standard Error. *P< 0.05 Vs Mcao (N=8/Group).

Fig. 2. Niaspan Treatment Of Stroke Reduces Apoptosis, Attenuates TNF-Alpha Expression And Increases VEGF And PI3K Activity

Panel A Shows Quantification Field Of View In The Ischemic Border Zone. Panels **B–D** Show That Niaspan 40mg/Kg Treatment Significantly Decreased The Number Of TUNEL-Positive (**B**), Cleaved Caspase-3 (**C**), And TNF-Alpha (**D**) Expression In The Ischemic Brain Compared To Mcao Control Rats ($P<0.05$). Panels **E–F** Show That Niaspan 40mg/Kg Treatment Significantly Increased The Expression Of VEGF (**E**) And P-PI3K (**F**) In The Ischemic Border Compared With Non-Treatment Mcao Control Rats ($P<0.05$). SE = Standard Error. * $P<0.05$ Vs Mcao ($N=8$ /Group).

Fig. 3. Western Blot Assay: Niaspan Treatment Of Stroke Reduces TNF-Alpha And Cleaved Caspase-3 Expression And Increases P-Akt Activity In The Ischemic Brain

Panel **A** Shows Western Blot Assay. Panel **B** Shows Western Blot Quantification Data (N=4/Group).