Ischemic stroke is a leading cause of death and disability in the United States and diabetes mellitus is the fastest growing risk factor for stroke. In addition, hyperglycemia, which is usually associated with diabetes, tends to worsen ischemia/reperfusion injury and to induce more oxidative stress damage. Our laboratory has shown that type II diabetic animals (Goto-Kakizaki rats (GKs) are more susceptible to vascular damage after experimental cerebral ischemia than non-diabetic animals. This made GKs a good model to test vascular protective agents after acute ischemic stroke. Statins possess neurovascular protective properties even when administered after the onset of ischemia. However, the acute vascular effects of statins after ischemic stroke in diabetes have not been studied to date. The goal of these studies was to evaluate the efficacy and magnitude of vascular protection of acute statin therapy in both GKs and their normoglycemic controls after experimental ischemic stroke. I hypothesized that acute atorvastatin treatment after stroke will be vascular protective in both diabetic rats (GKs) and normoglycemic controls (Wistar rats) through the reduction of oxidative stress and restoration of nitric oxide (NO). I investigated the hemorrhagic
transformation, infarct size and neurobehavioral outcome in rats subjected to 3 hours of middle cerebral artery occlusion (MCAo) followed by 21 hours reperfusion. I also investigated the different biomarkers and the plasma concentration involved with atorvastatin neurovascular protection. Results presented in this dissertation show that atorvastatin 30 mg/Kg/day is neurovascular protective after stroke in both normoglycemic and diabetic rats and improves neurobehavioral outcomes. The Phospho-inositol-3-kinase (PI3K)/Akt phosphorylation pathway was found to be involved in the protective effects of atorvastatin. However, atorvastatin did not have an effect on oxidative stress markers or endothelial nitric oxide synthase (eNOS) expression and its phosphorylation in these studies. Neurovascular protection with atorvastatin in rats was achieved at a safe plasma concentration, similar to that seen after 80 mg/day of atorvastatin in humans.

INDEX WORDS: Ischemic stroke, Diabetes, Vascular-protection, Neuro-protection, Statins
VASCULAR PROTECTION WITH ATORVASTATIN AFTER ACUTE ISCHEMIC STROKE IN DIABETES

by

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FORB, Alexandria University, Egypt, 2002

A dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

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VASCULAR PROTECTION WITH ATORVASTATIN AFTER ACUTE ISCHEMIC STROKE IN DIABETES

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CHAPTER 1
INTRODUCTION AND LITTERATURE REVIEW

Statement of the problem and dissertation objectives

Ischemic stroke is a leading cause of death and disability in the United States and diabetes mellitus is one of the major risk factors for stroke. Diabetic stroke patients suffer from an increase in the risk of stroke recurrence, hospital stroke mortality, and worsening in stroke outcomes. Hyperglycemia, which is usually associated with diabetes, tends to worsen ischemia/reperfusion injury and to induce more oxidative stress damage. Data from our laboratory showed that diabetic animals (Goto-Kakizaki rats (GKs) are more susceptible to vascular damage after experimental cerebral ischemia than non-diabetic animals. Vascular and endothelial dysfunction and increased matrix metalloprotease (MMP) -2 and -9 activities are seen in the middle cerebral arteries (MCA) of GKs. Increased cerebral vessel tortuosity has been observed in GKs indicating the presence of processes such as neovascularization and vascular remodeling. All the above findings seem to make GKs vessels more vulnerable to ischemia/reperfusion injury and hemorrhagic transformation than those in normoglycemic rats. Many studies have indicated that statins possess neuroprotective properties even when administered after the onset of ischemia\textsuperscript{1-4}. However, the acute vascular effects of statins after ischemic stroke in diabetes have not been studied to date.
Thus, the objective is to evaluate the efficacy and magnitude of vascular protection of acute statin therapy in both GKS and their normoglycemic controls after experimental ischemic stroke as well as the mechanisms involved.

The central hypothesis of this dissertation is that acute atorvastatin treatment will be vascular protective in both diabetic rats (GKS) and normoglycemic controls (Wistar rats) through the reduction of oxidative stress and restoration of nitric oxide (NO).

![Figure 1.1. Scheme outlining the main hypothesis and objective](image-url)
This hypothesis was tested through the following specific aims:

**Specific Aim#1:** To determine the extent to which atorvastatin can reduce hemorrhagic transformation and improve functional outcome after experimental ischemic stroke in both diabetic rats and normoglycemic controls.

Our working hypothesis is that atorvastatin decreases hemorrhagic transformation, infarct volume and will improve the functional outcome after experimental ischemic stroke.

**Specific Aim#2:** To determine the effect of atorvastatin on oxidative damage and restoration of NO after experimental ischemic stroke in diabetic rats compared to normoglycemic controls.

Our working hypothesis is that atorvastatin upregulates eNOS, increases NO availability and reduces oxidative stress. These effects are more prominent in diabetic animals.

**Specific Aim#3:** To determine the plasma concentration of the atorvastatin dose associated with neurovascular protection after ischemic stroke for future translational studies in humans.

Our working hypothesis is that the plasma concentration of the atorvastatin dose used in rats will be similar to that proven safe in humans.
Vascular Protection: A Novel Target in Stroke

In acute ischemic stroke, both vascular and neuronal tissues are damaged. Most of the previous clinical trials in patients after ischemic stroke focused on neuroprotection; however they failed to demonstrate a real benefit. Vascular protection represents an important and a new target to explore in acute ischemic stroke\(^5\). Vascular damage is an early process that occurs during cerebral ischemia and progresses over time\(^6\). Reperfusion through these injured vessels is likely to cause cerebral edema and hemorrhagic transformation, that further increases brain damage caused by ischemic stroke\(^7\). Up to 30-40% of ischemic strokes undergo spontaneous hemorrhagic conversion, a complication that is more severe and frequent with the use of thrombolytic treatment in stroke. Hemorrhagic conversion or transformation is defined as the transformation of a bland infarct into a hemorrhagic infarct after restoration of circulation, and it accounts for a major cause of early mortality in stroke patients. Prolonged ischemia, aggravated by reperfusion, causes initial dysfunction and later death of capillary endothelial cells. As this process develops, the blood-brain barrier is increasingly compromised, capillaries begin to leak and eventually they lose their physical integrity leading to the formation of hemorrhage\(^7\).

Pathologic processes leading to vascular damage and hemorrhagic transformation can be separated into 3 phases: acute, subacute and chronic. The acute phase, which occurs within minutes to hours from stroke onset, involves excitotoxicity and ionic imbalance. As a consequence, other mediators like endothelin-1 and free radical species are released. Since ischemia leads to impairment in the myogenic tone in the cerebral vasculature, reperfusion is likely to increase brain injury by producing
more free radicals, disrupting the endothelial cell membrane and increasing endothelial and blood-brain barrier (BBB) permeability \(^8, ^9\). In the subacute phase, gene activation plays an important role in the pathophysiology of vascular dysfunction and is likely to start within hours to days from the event. Proinflammatory genes such as interleukin-1\(\beta\) (IL-1\(\beta\)), inducible nitric oxide synthase (iNOS), tumor necrosis factor \(\alpha\) (TNF-\(\alpha\)) and transcription factors including hypoxia inducible factor 1 (HIF1) and nuclear factor \(\kappa B\) (NF\(\kappa B\)) are activated in response to the hypoxia, calcium overload and the free radical release. These proinflammatory products induce the expression of adhesion proteins that are critical for the integrity of the vascular endothelium. These adhesion molecules interact with the neutrophils allowing them to permeate the BBB. In addition, MMP-2 and MMP-9, members of the extracellular protease system, represent another important factor for tissue remodeling in this phase. They are upregulated after ischemic stroke and are able to degrade essentially all components of the neurovascular unit \(^8, ^10\). The chronic phase represents vascular changes on the long term. In this phase, apoptotic-like pathways and cascades are activated. Programmed cell death is triggered by various stimuli that include excessive free radical formation, death-receptor ligation, DNA damage and possibly lysosomal protease activation \(^9\). Several mediators including calpains, cathepsin B, nitric oxide and poly-(ADP-ribose) polymerase (PARP) facilitate cross-communication between cell death pathways.

Vascular protection can be defined as an improvement in the endothelial function to prevent vascular smooth muscle cell proliferation, inflammation, thrombosis and endothelial cells apoptosis. An ideal vascular protective drug would be a therapeutic agent that is safe to be given in humans and able to target mediators involved in the
different vascular damage phases. As mentioned earlier, hemorrhagic conversion due to vascular damage is one of the main limitations of the use of tPA (tissue plasminogen activator) for ischemic stroke and makes it underutilized. A combination of a vascular protective agent with tPA after stroke is very likely to extend the therapeutic window and allow a better use of this thrombolytic therapy ⁴.

Diabetes Mellitus and Stroke: A Vascular Perspective

Type II diabetes is a disease that affects more than 24 million Americans. Stroke is the third leading cause of death in the United States affecting about 750,000 each year ¹¹. In diabetic patients, the risk of stroke is increased by 2-6 fold ¹¹-¹⁴. In stroke patients younger than 55 years, this risk can increase more than 10 folds ¹¹. Additionally, there is an increase in the risk of stroke recurrence, hospital stroke mortality and worsening of stroke outcomes ¹¹, ¹⁵. The high prevalence of stroke in type II diabetes is associated with insulin resistance, impaired glucose tolerance, hyperinsulinemia and poor glycemic control ¹⁵-¹⁷. These factors play a very important role in the worsened stroke outcome mainly due to their pronounced effects on vascular structure and function. Studies from our laboratory showed that type II diabetes induces changes in the structure and integrity of the cerebrovasculature through MMP related mechanisms ¹⁸. Poor glycemic control causes endothelial intracellular hyperglycemia, which represents the basis for many biochemical alterations involved in diabetic complications and vascular injury ¹⁹, and, together with insulin resistance, they cause an increase in oxidative stress and free radical-mediated damage ²⁰.
Reactive oxygen species (ROS), such as superoxide anions, deplete NO by forming toxic peroxynitrite ions and reducing the bioavailability of endothelial NO. This will lead to endothelial dysfunction and increased platelet activation, vascular smooth muscle proliferation and migration \textsuperscript{21-23}. In the presence of hyperglycemia and oxidative stress, advanced glycosylation end product (AGE) formation is enhanced. Once AGE binds to its receptor, intracellular signaling mechanisms are activated and thus, more free radicals are produced \textsuperscript{24}. Hyperglycemia also diverts glucose into the aldose reductase pathway leading to cell damage \textsuperscript{20, 21}. The latter causes an increase in the nicotinamide adenosine dinucleotide phosphate H (NAD(P)H) oxidase activity and ROS production.

Another biochemical pathway affected by hyperglycemia leading to vascular dysfunction is the hexosamine pathway. It produces uridine 5’-diphospho \textit{N}-acetylglucosamine (UDP-GlcNAc) that is involved in the post-translational \textit{O}-acetylglucosamination of proteins. This modification inhibits phosphorylation by either steric hindrance or competition for the same amino acid residues. This pathway is activated in the endothelial cells and leads to the suppression of eNOS phosphorylation and NO production \textsuperscript{14}. Endothelial cells exposed to high glucose levels have less NADP due to the pentose phosphate pathway impairment. NADP is an anti-oxidant and is an important co-factor for a number of enzymes, including glutathione reductase, eNOS and dihydrofolate reductase. Inhibition of this pathway further increases vascular ROS\textsuperscript{14}.

The outcomes of hyperglycemia in permanent occlusion animal models indicate that hyperglycemia, whether it’s acute or chronic as in diabetes, is usually associated
with ischemic damage. In ischemia reperfusion experimental models, hyperglycemia seems to worsen injury even more. This is due to the fact that injury resulting from ischemia reperfusion is due to vascular and neuronal damage rather than just neuronal damage as it is the case in permanent occlusion models. In temporary models with relatively short duration of cerebral ischemia (30 minutes or less) brain injury exacerbation is mediated mainly through metabolic derangement, such as acidosis due to lactate accumulation, with minimal effect on reperfusion impairment. However, a longer duration of brain ischemia (>90 minutes) seems to impair reperfusion and increase the incidence of hemorrhagic transformation. Others have also reported the occurrence of edema, marked blood brain barrier disruption and MMPs activation post ischemia/reperfusion injury probably by altering various signaling pathways leading to the accumulation of free radicals and inflammatory processes.

Many clinical studies investigated the association between hyperglycemia during stroke and outcomes. Both retrospective and prospective trials found an association between admission hyperglycemia and worsened outcomes in stroke. In addition, trials using intravenous thrombolysis within 3 hours of stroke onset (NINID rt-PA Stroke Trial and the case series by Demchuck et al) and the trial using intra-arterial prourokinase within 6 hours after stroke onset (PROACT II) showed increased hemorrhagic conversions with elevated admission glucose levels.
Statins in Stroke: From Prevention to Protection

Currently, statins are the mainstay of lipid lowering treatment. They mediate their effect by inhibiting hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme of the mevalonate pathway of cholesterol biosynthesis. By reducing cholesterol biosynthesis in the liver, this leads to negative-feedback low-density lipoprotein (LDL) receptor upregulation followed by reduction in total serum cholesterol levels. Although cholesterol had established its role in the pathogenesis of coronary artery disease, controversy existed about the relationship between the risk of stroke and serum cholesterol level, until compelling evidence finally showed an association between lowering LDL and a reduced incidence of ischemic stroke. In addition, many studies have shown that statins reduce stroke risk in both primary and secondary prevention trials. Results from these studies in addition to other animal work, have all supported the idea that statins may have benefits over and beyond those generally attributed to the modification of the lipid profile. Statins have been shown to be both neuro- and vasculo-protective when administered prior or acutely after experimental stroke. Most of these beneficial effects are still considered to be due to the inhibition of HMG-CoA reductase. Inhibition of HMG-CoA not only blocks the synthesis of cholesterol but also a number of isoprenoid intermediates. It is the inhibition of Rho isoprenylation by statins which is largely responsible for the upregulation of eNOS expression within the endothelium. However, statins may also directly activate eNOS via additional post-translational mechanisms involving activation of the phosphatidylinositol 3-kinase/protein kinase Akt (PI3K/Akt) pathway. This increase in eNOS augments the production of nitric oxide (NO) which will ameliorate a number of
pathophysiological processes that cause damage within the cerebral vasculature and brain parenchyma during cerebral ischemia and reperfusion. NO improves blood flow to the ischemic brain, increasing angiogenesis and collateral vessel formation through VEGF and by making the brain parenchyma more resistant to the effects of ischemia \textsuperscript{50}. In addition to the statins’ vasodilatory and endothelial protective properties, they also have a potent antioxidant effect. This effect is mediated by the inhibition of Rac and NAD(P)H oxidase. Among the different sources of ROS, NAD(P)H oxidase is considered to be predominant especially in the vascular system \textsuperscript{36}. The antioxidant effect is also very important because it maintains NO bioavailability by preventing its degradation by free radical molecules to peroxynitrite radicals. After acute ischemic stroke and during both spontaneous and therapeutic reperfusion, there is a wide liberation of free radicals. Free radicals induce tissue injury in the ischemic penumbra through lipid peroxidation, protein oxidation and direct damage to nucleic acids. Another important process that contributes to the ultimate injury from cerebral ischemia and reperfusion is inflammation. Mediators and markers of inflammation, such as NFκB and C-reactive protein, have been shown to be reduced by statins. They also reduce monocyte adhesion molecule expression and CNS cytokine production \textsuperscript{50}.

Statins in Diabetes

Recent clinical trials have shown that statins reduce the risk of stroke and other cardiovascular diseases in diabetic patients, even if they were normocholesterolemic \textsuperscript{51, 52}. Hyperglycemia has been shown to increase hemorrhagic transformation in experimental stroke. However, the underlying mechanisms remain unknown. In
addition, clinical data has shown that admission hyperglycemia is associated with hemorrhagic transformation in acute ischemic stroke patients treated with thrombolytic agents. Statins may benefit those patients and allow a better use of these therapeutic options. Statin therapy also improves endothelial function which is one of the main pathophysiological characteristics in diabetes. Lastly, the antioxidant effect of statins may play a positive role as well. A recent study indicates that statin therapy normalized the increased oxidative stress induced by hyperglycemia. A small trial in which consecutive patients with acute ischemic stroke were included showed that better outcome at 3 months (modified Rankin score) were independently associated with prior statin use. Similarly, in an observational study, patients with acute ischemic stroke who were taking statins at the time of admission had better outcome (51%) than patients who were not (38%).

Clinical significance
This dissertation will provide information regarding the vasculoprotective effect of atorvastatin on ischemic stroke in diabetes mellitus where stroke risk is higher and the outcomes are worse. Further, it will provide a better understanding of the mechanisms through which these effects are mediated. A vascular protective agent such as atorvastatin can potentially enhance the use of thrombolytic therapy following acute ischemic stroke by prolonging its therapeutic window. Outcomes may be improved when this combination therapy is used. Since we studied clinically relevant doses of a commercially available therapy, these results can be rapidly translated to human stroke victims.
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CHAPTER 2
EARLY ATORVASTATIN REDUCES HEMORRHAGE AFTER ACUTE CEREBRAL ISCHEMIA IN DIABETIC RATS

Submitted to JPET, 10/10, 2008
Abstract:

Background- Ischemic stroke is a leading cause of death in the United States and diabetes mellitus is a major risk factor for stroke. Our previous work showed that type II diabetic rats (Goto-Kakizaki - GK) have more bleeding after stroke than their normoglycemic controls (Wistar). Our aim was to evaluate the vascular protective properties of acute atorvastatin therapy after experimental ischemic stroke in diabetes and to explore the effect of stroke in GK rats compared to their normoglycemic controls.

Methods- Thirty five male Wistar and 24 GK rats (270-305 g) underwent 3 hours of middle cerebral artery occlusion (MCAO) followed by reperfusion for 21 hours. Animals received either atorvastatin (15 mg/Kg) or vehicle, administered by oral gavage, the first dose 5 minutes after reperfusion and the second dose after 12 hours. At 24 hours, functional outcome was measured and brain tissue was analyzed for infarct volume, hemoglobin content and molecular biomarkers. Plasma was collected for analysis of atorvastatin concentrations.

Results- Atorvastatin-treated groups had significantly lower bleeding rates (p=0.016) and infarct volume (p=0.013) compared to their controls and these benefits were more than 4 times greater in the diabetic animals. Atorvastatin improved neurological outcome in both Wistar and GK rats (p=0.029). Neurovascular protection with atorvastatin was achieved at a peak concentration of (27-77 ng/ml) which is similar to that seen after 80 mg/day of atorvastatin in humans and was associated with an increase in Akt phosphorylation (p=0.0007).

Conclusion- Atorvastatin is a vascular protective agent after experimental ischemic stroke especially in diabetes.
Stroke is the third leading cause of death in the United States and affects about 750,000 individuals each year \(^1\). Type II diabetes mellitus affects more than 17 million Americans. In diabetic patients, the risk of stroke is increased by 2-4 fold\(^1-^3\). Not only is there an increase in the risk of stroke recurrence in diabetics, but these patients also have increased hospital and long-term stroke mortality and a worsening of overall stroke outcomes\(^1,^2\). Hyperglycemia is associated with intracerebral hemorrhage in acute ischemic stroke patients treated with thrombolytic agents and this has been confirmed in animal models\(^4,^5\). Many of the detrimental pathways involved in vascular damage associated with stroke are known to be upregulated in the diabetic vasculature, including oxidative stress, endothelial dysfunction and inflammation \(^6,^7\). A vascular protective agent administered after stroke onset may improve the safety and efficacy of thrombolytic therapy in diabetic patients. We have identified statins as potential vascular protective agent in the acute stroke period\(^8\) and statins are routinely prescribed for lipid lowering in these patients. Statins also have other pleiotropic effects including reduction of oxidative stress, improvement of endothelial function, increase in nitric oxide (NO) bioavailability and inhibition of the inflammatory and cell death mediators \(^9-^{11}\). Many studies have demonstrated that statins reduce stroke risk in both primary and secondary prevention trials \(^12-^{17}\). Statins have also been shown to be neuroprotective when administered acutely after experimental stroke \(^18,^19\), yet, post-stroke effects of statins in diabetes have not been evaluated. Recent data from our group demonstrated that type II diabetic animals (Goto-Kakizaki rats (GK) are more susceptible to vascular damage after experimental cerebral ischemia than non-diabetic animals (Wistar rats) \(^20\) and may serve as a good model to test potential vascular protective agents after stroke.
in diabetes. In this study, we evaluated whether acute atorvastatin therapy is vascular protective in GK rats after experimental ischemic stroke. A set of normoglycemic rats (Wistar) was used to confirm previous reports showing post-ischemic neuroprotective effects of statins 18, 19, 21. We also explored the effect of stroke in the diabetic GK rats compared to their normoglycemic controls.

Materials and Methods

The Institutional Animal care and Use Committee (IACUC) of the Augusta VA Medical Center approved the protocol. Male Wistar rats (n=35), from the Charles River Breeding Company (Wilmington, Massachusetts, USA) and GK rats (n=24), from Taconic farms, Inc. (Germantown, New York, USA) within a narrow range of body weight (270-305) were used.

Experimental cerebral ischemia

Stroke was induced as described previously22. At reperfusion, animals were randomized to receive either atorvastatin (15 mg/Kg) (Pfizer Inc., New York, NY, USA) or vehicle (methyl cellulose - MC - 0.5%) (Sigma Chemical Co., St. Louis, Missouri, USA), administered two doses by oral gavage, the first dose 5 minutes after reperfusion and the second after 12 hours. This dose has been previously shown to be neuroprotective23, 24.

Physiological monitoring

Blood glucose level and body weight were measured in all animals before MCAO and before sacrifice. We also measured hemoglobin A1c at baseline using the A1c+ kit (Metrika, Sunnyvale, California, USA) in a group of 12 rats. In a fourth group (n=5), cerebral perfusion was measured using Periscan PIM 3 System (Stockholm, Sweden).
A skin incision was performed and the skull was exposed. Whole brain scan was performed to measure cerebral perfusion in both hemispheres at baseline, after MCAO and at reperfusion.

**Assessment of infarct volume and hemoglobin content**

At 24 hours after the onset of MCAO, anesthesia was performed with intra-muscular Ketamine 44 mg/Kg and Xylazine 13 mg/Kg. Animals were then perfused with saline, sacrificed and their brains were removed. The brain tissue was sliced and processed as previously described for both infarct volume measurement and hemoglobin quantification using enzyme-linked immunosorbent assay (ELISA) in the brain\textsuperscript{22,25}.

**Neurological assessment**

Neurological function was measured prior to reperfusion and at 24 hours (just before sacrifice) using the Bederson score\textsuperscript{26}. Only animals with a score of 3 prior to reperfusion were included in all analyses. Before sacrifice, animals were tested again using the Bederson score. In addition, spontaneous activity was recorded using telemetry and percentage change in activity after reperfusion was calculated.

**Molecular biomarkers:**

Lipid peroxidation

Lipid peroxide concentration was determined by a method that measures the amount of thiobarbituric acid reactivity (TBARS) by the amount of malondialdehyde formed during acid hydrolysis of the lipid peroxide compound. Thirty μl of sample was incubated with
the reaction mixture containing 10 μl of 8.1% sodium dodecyl sulfate, 150 μl of 20% acetic acid solution (buffered to pH 3.5), and 150 μl of 0.8% thiobarbituric acid at 95°C for 1 hour. After cooling, 1 ml of distilled water and 5 ml of a mixture of n-butanol and pyridine (15:1, v/v) were added and the final mixture was shaken vigorously. After centrifugation at 1500 x g for 10 minutes absorbance of the solvent layer was measured at 532 nm. Tetraethoxypropane was used to establish the standard curve and lipid peroxide level was expressed in terms of μmole/ L malondialdehyde per mg protein.

Detection of nitrotyrosine
Nitrotyrosine immunoreactivity is measured as an indicator of superoxide –dependent peroxynitrite formation by slot blot analysis. Brain homogenates (30 μg) prepared for immunoblotting experiments were immobilized onto a nitrocellulose membrane using a slot blot microfiltration unit. After blocking with 5% nonfat milk, membrane was incubated with an anti-nitrotyrosine antibody from Calbiochem and visualized with Pierce Super Signal Kit. The intensity of bands was analyzed by GelPro Software.

Western blot
Blots were performed for stroke and non-stroke sides of the brain homogenate as described previously. In brief, samples (50 μg protein of the brain homogenate) were separated on a 10% Sodium dodecyl Sulfate-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. The membrane was blocked in 5% milk solution. Endothelial Nitric Oxide Synthase (eNOS), Phosphorylated eNOS (peNOS), serine/threonine protein kinase Akt (Akt), Phosphorylated Akt (pAkt) and actin proteins were determined by probing the membranes with the corresponding primary (cell signaling, Boston, MA) and secondary antibody (Santa Cruz, Santa Cruz, CA).
Blood Pressure:
In a subset of 24 rats (11 GK and 13 Wistar), telemetry transmitters (Data sciences
International, St.Paul, Minnesota, USA) were implanted and data were collected as
previously described 22.

Measurement of plasma atorvastatin levels:
The concentration of atorvastatin in plasma was determined by liquid chromatography
tandem mass spectrometry (LC/MS/MS) as described previously 27. The lower limit of
quantification for atorvastatin was 0.2 ng/ml. The intra- and inter-day coefficients of
variation were below 10% in the concentration range of 0.2 ng/ml to 200 ng/ml for
plasma samples.

Statistical analysis:
The distributions of all continuous variables were checked for normality prior to analysis
and a log transformation was found to be beneficial for infarct volume and excess Hgb.
Baseline blood glucose (BG), weight, activity and mean arterial pressure (MAP) were
compared for Strain and Drug differences using a 2X2 ANOVA without interaction. Log
infarct volume, log excess Hgb values, and Bederson scores were compared using a 2
Strain (Wistar vs. GK) X 2 Drug (vehicle vs. atorvastatin) ANOVA where the interaction
between Strain and Drug was investigated. All molecular biomarkers were analyzed
using a 2 Strain (Wistar vs. GK) X 2 Drug (vehicle vs. atorvastatin) X 2 Sides (stroke vs.
non-stroke) repeated measures ANOVA. Changes from baseline for BG, weight,
activity, and MAP were analyzed using a 2 Strain (Wistar vs. GK) X 2 Drug (vehicle vs. atorvastatin) analysis of covariance (ANCOVA) where baseline values were used as covariates prior to investigating the effects of Strain, Drug and their interaction. For blood pressure (BP) data, the average of all measurements prior to MCAO was the baseline value. Values obtained during the 3 hours of MCAO were averaged for the estimate of BP during stroke, the values for the period of the first 5 hours post-reperfusion were averaged for an estimate of the immediate effects of the drugs, and all remaining values afterwards were averaged for the delayed post-reperfusion value. Means±SD are presented for the various groups. SAS® version 9.1.3 was used for all analyses and p<0.05 was used to determine statistical significance.

**Results:**

At stroke, the weights among groups were similar. However, before sacrifice, Wistar rats lost more weight than the diabetic GK rats (-48±12 vs. -35±7 gm, respectively; p=0.002) and change in weight was not affected by atorvastatin treatment. Baseline blood glucose (BG) was significantly higher in the diabetic group than in the normal rats (157±45 vs. 107±19 mg/dl, respectively; p<0.0001) (Table 2.1). Hemoglobin A1c, which is a parameter to monitor chronic glycemic control in people with diabetes, was significantly higher in GK rats (n=9) than in Wistar rats (n=3) (6.7±0.34 vs. 4.2±0.05 % respectively; p=0.0018) indicating that the elevation in BG seen in the GK rats was due to chronic rather than acute hyperglycemia. As expected, BG decreased in all groups when measured again at sacrifice (24 hours from stroke). After adjusting for baseline
values, the decrease in serum glucose levels was found to be similar among the 4 different groups.

In both Wistar and GK rats, there was a decline in cerebral perfusion in the right hemisphere upon MCAO. After reperfusion, blood perfusion in the ischemic hemisphere returned to the baseline level (Figure 2.1).

Infarct volume, hemoglobin content and behavior:

As shown in Figures 2.2 and 2.3, atorvastatin reduced infarct volume and hemorrhage in both Wistar (n=8) and GK (n=5) rats (p=0.013 and p=0.016, respectively) in comparison to their control counterparts. Infarct volume was reduced by 22% with atorvastatin treatment in the normal Wistar rats. In addition, bleeding in the ischemic hemisphere was reduced by 49.6% with atorvastatin. In GK rats, infarct volume was reduced by 79.2% in the atorvastatin treated group and bleeding was reduced similarly by 83.5%. Atorvastatin neurovascular protection was associated with an improvement in the behavioral outcome in both diabetic and non-diabetic animals (Figure 2.4A). Atorvastatin significantly improved the Bederson score in both Wistar and GK rats (2.8±0.4 vs 2.5±0.4 points in Wistar and 3.0±0.1 vs. 2.8±0.3 points in GK rats (p=0.029). Hemorrhage formation in diabetic rats tended to be higher than in normoglycemic rats and they had significantly worse neurobehavioral outcome as shown by Bederson scores (p=0.004), however, their infarct volume was significantly smaller than normal Wistar rats (p=0.001).

Using implanted transmitters, spontaneous movement in the cages were recorded every 10 minutes throughout the experiment. There was a significant increase in the activity
level after stroke in both Wistar and GK rats receiving vehicle compared to the atorvastatin-treated rats (70±23 vs -10±18% change from baseline, respectively, in Wistar and 37±32 vs -6±14% change from baseline, respectively, in GK rats, p=0.049 (Figure 2.4B). This is possibly due to an increase in the discomfort and loss of circadian rhythm in the control groups after stroke.

Molecular biomarkers:
Oxidative stress markers
To examine the differences between strains in oxidative stress levels and whether the beneficial effects of atorvastatin were associated with an anti-oxidant effect, oxidative stress was assessed using lipid peroxidation assay and nitrotyrosine slot blot. Both assays were done on plasma and brain homogenate samples. Diabetes increased oxidative stress systemically as shown by plasma levels of lipid peroxides (p<0.0001) (Figure 2.5A) and nitrotyrosine (p=0.001) (Figure 2.5B) in control GK rats compared to Wistar rats. Interestingly, locally in the brain, diabetic animals had less oxidative stress than their normoglycemic controls as shown by brain lipid peroxides (p=0.001) (Figure 2.5C) and nitrotyrosine levels (p=0.036) (Figure 2.5D) in control GK rats compared to Wistar rats. Atorvastatin did not affect oxidative stress markers. As expected, stroke induced oxidative stress in both strains as shown by the lipid peroxidation (p=0.021) and the nitrotyrosine formation) (p=0.04).
Brain Expression and phosphorylation of eNOS:

To examine the strain differences and whether the beneficial effects of atorvastatin were associated with a change in eNOS expression or activity, eNOS and peNOS protein amounts were quantified by immunoblotting in both the ischemic and the contralateral hemispheres of the brains. There were no differences between the diabetic and non-diabetic animals in eNOS expression or its phosphorylation. Stroke induced eNOS expression in both Wistar (p=0.002) and GK rats (p=0.015) but eNOS phosphorylation was less in the stroke side in Wistar (p=0.007) and GK rats (p=0.011). Atorvastatin did not affect eNOS expression but there was a trend towards an increase in eNOS phosphorylation in the ischemic hemisphere in GK rats treated with atorvastatin compared to control (69±37 % vs. 35±23 %, p=ns). (Figure 2.6A and B)

Brain Akt Phosphorylation:

To further explore the strain differences and the mechanisms related to the neurovascular protection with atorvastatin, expression of pAkt was quantified similar to eNOS and peNOS. There was no difference between diabetic and non-diabetic rats. Atorvastatin significantly increased Akt phosphorylation in the ischemic hemisphere in comparison with controls for both strains (p=0.006 for the interaction) (Figure 2.6C).

Blood pressure:

To explore the effect of diabetes, stroke, atorvastatin treatment and their interactions on blood pressure, we implanted the animals with blood pressure transmitters and MAP was recorded every 10 min on telemetry prior to stroke, during the onset of ischemia, at reperfusion (a and then during the following 21 hrs until sacrifice next day. Baseline
blood pressure was elevated in diabetes as shown in Figure 2.7 (MAP was 112±7 mmHg in GKS vs. 97±5 mmHg in Wistars, p<0.0001). In addition, onset of stroke in diabetes caused significantly higher elevation in blood pressure than in normoglycemic rats (34±5 mmHg in GKS vs. 26±6 mmHg in Wistars, p=0.02). Interestingly, atorvastatin lowered the blood pressure in the diabetic rats for the first 5 post-reperfusion hours (141±9 mmHg in control GKS vs 133±9 mmHg in treated GKS) while there was no treatment effect on the MAP in the normoglycemic Wistar animals (p=0.023 for the interaction).

Atorvastatin maximum concentration (Cmax) after oral gavage:
Administration of atorvastatin (15 mg/Kg) in Wistar rats (n=3) by oral gavage achieved an average maximal concentration (Cmax) (range) of 53.9 ng/ml (27-77 ng/ml) after 30 minutes. We also checked the trough levels (prior to sacrifice) of atorvastatin in Wistar (n=8) and GK (N=5) rats and they were 7.5(1.4) ng/ml and 5.5(0.8) ng/ml respectively

Discussion:
Data from both animal and human studies suggest that hyperglycemia during acute ischemic stroke is associated with an increase in the brain injury due to the impairment in the cerebral reperfusion, subsequently causing an increase in blood brain barrier (BBB) permeability, edema and hemorrhage formation. This subsequently leads to worsening in the functional outcomes and increase in mortality. We have adapted our stroke model to a model of type II diabetes mellitus (GK). We found that GK rats are more vulnerable to reperfusion injury and hemorrhage formation. This is due to the
pathological remodeling characterizing this model which induces blood brain barrier breakdown leading to an increase in hemorrhage and worsening of the functional outcomes after stroke\textsuperscript{20}. In GKS, Laser Doppler scanned images demonstrated a drop in cerebral perfusion at the occlusion time indicating that the small infarct size seen in these rats is not due to inability to occlude the MCA (Figure 2.2). Bederson test showed that diabetic rats were more impaired than Wistar rats regardless of the treatment, which may be due to the increased incidence of bleeding. Increased hemorrhage formation in GK rats confirms that they are a suitable model to test vascular protective agents. As expected, diabetic rats had more oxidative stress than their normoglycemic controls systemically. However, in the brain, oxidative stress markers were lower in GK rats. This might be related to a compensatory response in this mild hyperglycemic stage.

The most important finding in our study is that, in both strains, atorvastatin was not only neuroprotective but it was also vascular protective and improved neurological function. This result differs from that of the Stroke Prevention by Aggressive Reduction in Cholesterol Levels (SPARCL) trial \textsuperscript{17} in which atorvastatin was associated with an increase in hemorrhagic stroke, despite a robust reduction in overall stroke risk. This may be due to the difference between the acute versus the chronic effects of atorvastatin. In this project, we also found that the atorvastatin peak blood level achieved after administering 15 mg/Kg by oral route was similar to that reported after a dose of 80 mg/day of atorvastatin is given to humans\textsuperscript{20}. This gives our results clinical relevance for future translational studies, especially since 80 mg/day of atorvastatin was the dose shown to reduce stroke recurrence in the (SPARCL) trial \textsuperscript{17}. 
Another novel finding is that the vascular protection with atorvastatin in the diabetic rats and their normoglycemic controls was associated with increased levels of phosphorylated Akt kinase in the brain homogenate, an effect that was shown by others to be involved in the acute neuro- and cardio-protection of statins. The phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway is known to be very important in regulating cell growth, proliferation, and survival. The activation of the PI3K/Akt pathway reduces thrombogenicity, vascular permeability, inflammation and apoptosis and thereby protects vascular function. Statins induce Akt translocation to the plasma membrane of endothelial cells. Upon phosphorylation and subsequent activation of Akt at serine 473, it induces phosphorylation of Bad (member of the pro-apoptotic BCL2 protein family), forkhead transcription factor (AFX), caspase 9, eNOS and inhibitor of nuclear factor kappa B (NFκB). Although others have shown the importance of eNOS in statin neuroprotection, our study did not show any treatment effect on eNOS expression or its activation. We think that these differences are due to the prophylactic versus the acute effects of statins in stroke, the route of administration and the dosage administered. In parallel to our results, a previous study showed that atorvastatin in combination with tissue-type plasminogen activator reduced the disruption of vascular integrity in the brain after embolic stroke in rats without affecting eNOS levels, and blocking NOS activity did not inhibit the beneficial effects of the combination treatment on stroke. In addition, low doses of IV rosuvastatin after stroke provided neuroprotection and increased PAkt without altering eNOS expression or its phosphorylation.
Lastly, our study showed that these diabetic rats had slightly elevated blood pressure at baseline compared to their normoglycemic controls. This is in contrast to our earlier work, done in older GK animals, where there was no difference in blood pressure \(^{35}\). It is likely that the endothelial response to the hyperglycemia changes over time and we are investigating this. The animals were also more sensitive to ischemia leading to a higher increase in their MAP than normal rats during MCA occlusion. These results are probably mediated by the endothelial dysfunction and the altered nature of the diabetic vasculature in GK rats \(^{36, 37}\). Interestingly, atorvastatin significantly reduced MAP after reperfusion for 5 hours, an effect that was only seen in GK rats. It is possible that this mild blood pressure lowering contributed to the robust protection seen in the diabetic animals in this study and may have improved their cerebral blood flow. Results from UCSD, a recent large, parallel-design randomized clinical trial, showed that various statins lower blood pressure and this reduction may contribute to the cardiovascular protection of statins \(^{38}\). Although blood pressure lowering is not clinically accepted as a tactic of neurovascular protection, we have already shown that various pharmacologic agents that lower blood pressure are vascular protective after ischemic stroke \(^{22, 39}\).

In conclusion, acute post-ischemic oral administration of high dose atorvastatin has been shown to be vascular- and neuro- protective in both diabetic and normoglycemic rats. Atorvastatin is a novel vascular protective agent, already safely administered to many acute ischemic stroke patients, and may be especially helpful when given at reperfusion in high risk diabetic patients.
Sources of funding;
This work was supported by grants from Pfizer and VA Merit grant (SCF), AHA and JDRF to A.B.R and NIH (DK 074385, NS054688) and AHA Established Investigator Award (0740002N) to AE

Disclosure;
SCF has been a consultant or participated in a Speaker Bureau for Pfizer, Astra-Zeneca and Boehringer Ingelheim.

References


Table 2.1: Table for Wistar and GK rats physiological parameters

Weight, blood glucose (BG) and hemoglobin A1c (HA1c) levels among different treatment groups at baseline and at sacrifice. Wistar rats lost more weight than the diabetic GK rats (p=0.002). Baseline BG was significantly higher in GK than Wistar rats (# p<0.0001). HA1c was significantly higher in GK rats (* p=0.0018)

W: Wistar; C: Control; ATV: Atorvastatin 15 mg/Kg

Figure 2.1: Perfusion Doppler (PIM3) scans and brain infarcts in Wistar and GK rats

Cerebral perfusion measured with PIM3 scanner in both GK and Wistar at baseline, ischemia and reperfusion. Upon ischemia, perfusion dopped in both Wistar and GK rats for 3 hours and then was restored upon reperfusion. The figure also shows that the infarct in the GK brain slices after staining with TTC is smaller than that in Wistar rats, however, more obvious hemorrhage can be seen in GK rats.

Figure 2.2: Effect of atorvastatin on infarct volume in rats 24 hours after Stroke

Atorvastatin significantly reduced infarct volume (mm$^3$) in both Wistars and GK rats compared to their controls (* p=0.0132). Vertical error bars indicate standard error of the mean (SEM).

W: Wistar; C: Control; ATV: Atorvastatin 15mg/Kg
Figure 2.3: Effect of atorvastatin on hemorrhage in rats 24 hours after Stroke. Hemoglobin excess (μg Hgb/ g protein) decreased in atorvastatin-treated groups compared to their controls (* p=0.0156). Vertical error bars indicate standard error of the mean (SEM). W: Wistar; C: Control; ATV: Atorvastatin 15mg/Kg

Figure 2.4: Effect of atorvastatin on neurobehavioral outcome 24 hours after stroke

A- Effect of atorvastatin 30 mg/Kg/day on Bederson score in the Wistar and GK rats subjected to MCAO. The animals were tested before reperfusion and treatment (white bars), and only animals with a score of 3 were further examined. Before sacrifice, Bederson score was significantly reduced with atorvastatin treatment in both strains (* p=0.0291). Bederson score was also significantly higher in GK compared to Wistar rats (p=0.004). Vertical error bars indicate standard error of the mean (SEM). W: Wistar; C: Control; ATV: Atorvastatin 15mg/Kg

B- Effect of atorvastatin 30 mg/Kg/day on activity level in Wistar and GK rats. Using implanted transmitters, rats’ movement in the cages were recorded every 10 minutes throughout the experiment. There is a significant increase in the activity level in both Wistar and GK rats receiving methylcellulose compared to the atorvastatin-treated animals (* p=0.049). Vertical error bars indicate standard error of the mean (SEM). W: Wistar; C: Control; ATV: Atorvastatin 15 mg/Kg

Figure 2.5: Oxidative stress markers in rats brain and plasma 24 hours after stroke

Effect of strain and treatment on oxidative stress markers in both plasma (A &B) and brain (C & D). In plasma, both lipid peroxidation (A) and nitrotyrosine slot blot (B)
showed significant elevation in the diabetic animals. In the brain, there was less oxidative stress in diabetic rats as shown in the lipid peroxidation assay (C). There were no differences between treated and control animals in plasma or the brain. W: Wistar; C: Control; ATV: Atorvastatin 15 mg/Kg; N: contralateral hemisphere; S: ipsilateral hemisphere.

Figure 2.6: Effect of atorvastatin on eNOS, peNOS and pAkt in rats brain 24 hours after Stroke
(A) Quantitative data of percentage relative density showing increased eNOS expression in the ischemic hemisphere in Wistars (* p=0.002) and GK rats (# p=0.015). (B) eNOS phosphorylation was less in the stroke side in Wistar (* p=0.007) and GK rats (# p=0.011). (C) Quantitative data of percentage relative density showing increased activation of PAkt in atorvastatin-treated groups compared to their controls(* P=0.0007). Vertical error bars indicate standard error of the mean (SEM). W: Wistar; C: Control; ATV: Atorvastatin 15 mg/Kg; N: contralateral hemisphere; S: ipsilateral hemisphere.

Figure 2.7: Effect of stroke and atorvastatin treatment on blood pressure in rats
Mean arterial blood pressure (MAP; mmHg) after acute stroke in the Wistar (triangles) and GK rats (rectangles). MAP was recorded every 10 min (telemetry) prior to stroke (baseline between 6 and 9 AM), during the onset of ischemia (at 10 AM; left arrow), reperfusion (at 1 PM; right arrow) and then during the following 21 hrs until sacrifice next day. At reperfusion, the animals were treated with methylcellulose (dotted) or with atorvastatin (15 mg/Kg) (filled), another dose was given after 12 hours. The black
horizontal bar indicates night-time in the light/dark cycles. Values shown are 1-h averages ± SEM. # p= 0.012, * p=0.023, ** p=0.02

Table 2.1

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<th>GK control (n=7)</th>
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<td>163.8(56.1) # mg/dl</td>
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<td>N/A</td>
<td>6.7(0.34) **</td>
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</tbody>
</table>
Figure 2.1

Baseline | Ischemia | Reperfusion | GK infarct
--- | --- | --- | ---
Baseline | Ischemia | Reperfusion | Wistar infarct

Figure 2.2

Infarct volume (mm³)

- **W C (n=7)**
- **GK C (n=7)**
- **W ATV (n=8)**
- **GK ATV (n=5)**

*
Figure 2.3

![Bar graph showing the Excess hgb (ug/g of protein) for different groups.]

Figure 2.4

A

![Bar graph showing the Bederson score for different groups.]

Baseline, W C after (n=16), GK C after (n=11), W ATV after (n=16), GK ATV after (n=10).
Figure 2.5

A

B

Figure 2.5
Figure 2.6

A

B

C

D
B

PeNOS

eNOS

( % relative to control)

PeNOS/eNOS OD

C

PAkt

Akt

% PAkt/Akt OD relative to control

W C N (n=7) G K C N (n=7) W C S (n=7) G K C S (n=7) W ATV N (n=8) G K ATV N (n=8) W ATV S (n=8) G K ATV S (n=8)

* #

(n=7) (n=7) (n=7) (n=5) (n=5) (n=5)
Figure 2.7

MAP (mmHg) over time (hr) for different groups:
- Wistar control (n=7)
- Wistar Atorvastatin (n=6)
- GK control (n=5)
- GK Atorvastatin (n=6)

Significance:
- * indicates statistical significance
- ** indicates even higher statistical significance
- # indicates a different result

Time (hr) range: 0:00 to 24:00

MAP (mmHg) range: 80 to 160
CHAPTER 3

BLOOD PRESSURE LOWERING AFTER EXPERIMENTAL CEREBRAL ISCHEMIA PROVIDES NEUROVASCULAR PROTECTION

Abstract

There is evidence that acutely elevated blood pressure (BP) after stroke is associated with increased cerebral hemorrhage and edema. Previous experiments in our lab have shown that candesartan 1mg/Kg administered after reperfusion in a model of hypertension after experimental ischemic stroke reduces neurovascular damage and improves outcome. These results could be either mediated by BP lowering or a BP-independent cerebrovascular protective effect.

Purpose: to determine the contribution of BP lowering to the neurovascular protection previously reported with candesartan after stroke.

Methods: Male Wistar rats (280-305 g) underwent 3 hours of middle cerebral artery occlusion (MCAO). At reperfusion, either hydralazine 1 mg/kg (n=8), enalapril 5 mg/kg (n=7) or enalapril 10 mg/kg (n=8) were administered intravenously. BP was measured by telemetry for 2 days before and 24 hours after MCAO. After neurological function was assessed, brain tissue was processed for infarct size and hemoglobin content analyses.

Results: Mean arterial BP (MAP) increased from 92 to 124 mmHg immediately upon MCAO and decreased to 112 mmHg after reperfusion, remaining elevated for 24 hours (p<0.0001) in the saline group. Hydralazine reduced MAP (p=0.048) and infarct size (53% vs. 30%, p=0.0083) and there was a trend toward decreased hemoglobin content. Enalapril 5mg/Kg did not significantly change MAP or other outcomes. Enalapril 10 mg/Kg reduced MAP (p<0.0001) and infarct size (53% vs. 29%, p=0.003). There was an intermediate effect on both hemoglobin content and neurological function, neither one was significant. The time course of BP lowering varied with each treatment.
Conclusion: Acute blood pressure lowering after reperfusion in acute ischemic stroke is an effective strategy to achieve neurovascular protection. The rate, extent and mechanism of blood pressure lowering may determine the magnitude of protection.

Keywords:
Blood pressure, stroke, neurovascular protection
**Introduction**

Elevated blood pressure (BP) after acute ischemic stroke is a very common observation and the management has been a subject of persistent controversy for decades. Most recent comprehensive reviews ¹, ² have called for further study of BP lowering as a strategy to improve functional outcome after ischemic stroke.

Previous experiments in our lab have shown that candesartan 1mg/Kg administered after reperfusion in a model of hypertension after experimental ischemic stroke reduced BP to baseline, reduced neurovascular damage and improved outcome ³. However, it is possible that mechanisms beyond BP lowering are involved. The neuroprotective properties of angiotensin type 1 (AT1) receptor blockade (as in candesartan) have been reported by others ⁴ and may be due to improved cerebral blood flow, restoration of endothelial nitric oxide synthase (eNOS), decreased inflammation or stimulation of the AT2 receptor ⁵-⁷. The aim of the present study was to determine the contribution of BP lowering to the neurovascular protection previously reported with candesartan administered after reperfusion in experimental stroke ³.

**Materials and methods**

The Institutional Animal Care and Use Committee (IACUC) of the Augusta VA Medical Center approved the protocol. Male Wistar rats (n=23), from the Charles River Breeding Company (Wilmington, Massachusetts, USA) within a narrow range of body weight (280-305 g) were used.
**Blood pressure telemetry**

Telemetry transmitters (Data sciences International, St.Paul, Minnesota, USA) were implanted in 23 rats according to the manufacturer’s specifications under sodium pentobarbital anesthesia (65 mg/Kg, i.p.; Abbott Laboratories, Chicago, Illinois, USA). A midline incision was done to expose the abdominal aorta which was shortly occluded to allow insertion of the transmitter catheter into the abdominal aorta. Using tissue glue, the catheter was secured in place. The incision was sutured. The skin was closed using nonabsorbable suture (3-0). Rats returned to their individual cages and were allowed to recover from surgery for 10 days. By placing rats on top of the telemetry receivers arterial pressure waveforms were continuously recorded throughout the study. Data were recorded every 10 minutes for 48 hours before the stroke and until the sacrifice at 24 hours after the onset of stroke. By using inhaled anesthetics during stroke surgery, we were able to collect frequent arterial pressure data in awake animals.

**Experimental cerebral ischemia**

Anesthesia was performed by using 2% isoflurane via inhalation. Cerebral ischemia was induced using the intraluminal suture middle cerebral artery occlusion (MCAO) model. 19-21 mm 3-0 surgical nylon filament was introduced from the external carotid artery lumen into the internal carotid artery to block the origin of the right MCA. The animals were kept under anesthesia for only 10 minutes for the surgical procedure. The suture was removed after 3 hours of occlusion and the animals were returned to their cages. At reperfusion, either hydralazine 119H1650 (Sigma Chemical Co., St. Louis, Missouri,
USA) 1mg/Kg (n=8), enalapril E6888-1G (Sigma Chemical Co., St. Louis, Missouri, USA) 5 mg/Kg (n=7) or enalapril E6888-1G (Sigma Chemical Co., St. Louis, Missouri, USA) 10 mg/Kg (n=8) or saline (n=12) were administered intravenously by tail vein.

**Neurological assessment**

Neurological function was measured prior to reperfusion and at 24 hours (just before sacrifice) using the Bederson score 9. An animal with no apparent deficits obtained a 0; the presence of forelimb flexion = 1; decreased resistance to push = 2; and circling = 3. A score of 3 is consistent with a middle cerebral artery occlusion. Only animals with a score of 3 prior to reperfusion were included in the analysis of infarct size, hemoglobin, and neurological function.

**Assessment of infarct size and hemoglobin content**

At 24 hours after the onset of MCAO, Anesthesia was performed with Ketamine 44 mg/Kg and Xylazine 13 mg/Kg intra-muscular (cocktail), animals were then perfused with saline, sacrificed and their brains were removed. The brain tissue was sliced into seven 2 mm-thick slices in the coronal plane and stained with 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma Chemical Co., St. Louis, Missouri, USA) for 15-20 minutes. Images of the stained sections were taken. Using image analysis software (Zeiss-KS300, Oberkochen, Germany), infarction zones were measured and percentage infarct size was calculated. The ischemic and non-ischemic hemispheres of the slices for the enzyme-linked immunosorbent assay (ELISA) were separated and
processed, using the non-ischemic side as a control. After homogenizing the slices in the core of the infarct and taking the supernatants, ELISA was performed to measure the hemoglobin in the brain tissue\textsuperscript{10}.

**Statistical analysis**

For blood pressure data, the average of all measurements prior to MCAO was the pre-stroke value. Values obtained during the 3 hours of MCAO were averaged for the estimate of BP during stroke, the values for the period 2 hours post-perfusion were averaged for an estimate of the immediate effects of the drugs, and all values 5 hours after the onset of ischemia were averaged for the post-stroke value. A 2-way repeated measures analysis of variance (ANOVA) was used to determine treatment (saline, hydralazine, enalapril 5mg/Kg and enalapril 10 mg/Kg), time (pre-stroke, MCAO, 2hr post-perfusion, post-stroke), and treatment by time differences in mean arterial pressure (MAP). The area under the curve (AUC) for the entire duration of the experiment was also calculated for each rat where baseline was considered to be zero. Maximum (MAX) and minimum (MIN) MAP were also determined. Differences among different treatments and control were determined by one-way ANOVA for AUC, MAX, MIN, average infarct size, hemoglobin content, and post-perfusion values of the Bederson score. A Tukey-Kramer adjustment for multiple comparisons was used for all post-hoc mean comparisons. All analyses were performed using SAS 9.1.3 (SAS Institute Inc., Cary, North Carolina, USA). Statistical significance was measured at an alpha level of 0.05. All values are represented as mean ± standard deviation (SD).
Results

Blood pressure

Figure 3.1 outlines the effect of different treatments on mean arterial BP (MAP) over time pre, during and after stroke. The MAP baseline level was approximately 95 mmHg for all treatment groups with normal circadian variation for the 24 hours prior to stroke. In the saline group, MAP increased from 92 to 124 mmHg immediately upon MCAO and decreased to approximately 112 mmHg after reperfusion, remaining elevated for 24 hours (p<0.0001). In the Hydralazine treated animals MAP dropped from 127 to 94 mmHg but gradually increased again until it reached 108 mmHg before sacrifice (p=0.0448). Enalapril 10 mg/kg reduced MAP from 123 to 96 mmHg and remained lower than saline until sacrifice (p<0.0001). Enalapril 5 mg/Kg didn’t lower MAP when compared to saline. The repeated measures ANOVA showed a significant interaction between treatment and time (p<0.0001). There were no significant differences among the groups for the pre-stroke and the MCAO values. For the two hours post-reperfusion the enalapril 10 mg/Kg and hydralazine lowered BP the most, enalapril 5mg/Kg had intermediate effects, and the saline showed the smallest reduction. For the 19 hours during the post-stroke period the enalapril 10 mg/Kg group and the hydralazine group had MAP that was significantly lower than saline. The post-stroke means for all other groups were significantly higher than the pre-stroke values for the groups (all p<0.01). There was a significant difference among the groups for AUC (p=0.0004), with the enalapril 10 mg/Kg group being significantly lower than the saline group.
Infarct size, hemoglobin content and behavior

As shown in Figures 3.2 and 3.3, there were significant differences among the groups for infarct size (p=0.0004), but not for hemoglobin content (p=0.19) or the post-stroke Bederson score (p=0.42). The enalapril 10 mg/Kg and hydralazine groups had the lowest infarct size that was significantly different from the control group and there was a trend towards reduced hemoglobin content in the hydralazine and the enalapril 10 mg/kg groups (Fig. 3)

Discussion

Our findings show a significant reduction in the infarct area in groups that had significantly lower MAP after reperfusion compared to saline. There was also a trend toward reduction in hemoglobin content, suggesting a vascular protective effect. The reduction in infarct size occurred with both hydralazine and enalapril 10 mg/kg even though the BP lowering was transient with hydralazine and persistent with enalapril, suggesting that elevated BP early after reperfusion is an important therapeutic target. Despite success in reducing infarct size, we were unable to replicate the robust improvement in neurologic outcome at 24 hours we reported in candesartan-treated animals ³. These results support the idea that although elevated BP after reperfusion contributes to ongoing neurovascular damage, the method of BP lowering is also important. Our work and the works of others ³, 4, 6, 11-14, demonstrate that AT1 receptor blockade (candesartan), can provide superior benefit.

Hydralazine and enalapril also have properties apart from a BP lowering effect that could contribute to the neurovascular protection observed. Many researchers have
shown that hydralazine has antioxidant properties\textsuperscript{15-18} and may exert this effect by inhibiting nicotinamide adenine dinucleotide phosphate/ nicotinamide adenine dinucleotide phosphate (reduced form) (NADH/NADPH) oxidase enzyme or by scavenging reactive oxygen species (ROS)\textsuperscript{15, 16}. Hydralazine also inhibits inducible nitric oxide synthase-2 (iNOS-2)/ Cyclooxygenase-2 (COX-2) gene and reduces ROS production by inflammatory macrophages\textsuperscript{18} in addition to being proangiogenic\textsuperscript{17}. The pro-inflammatory actions of Angiotensin II (AngII) in the vascular wall and its role in stimulating ROS, inflammatory cytokines and adhesion molecules production have been established\textsuperscript{19}. As a result, ACE inhibitors would be expected to have protective actions on endothelial and neuronal cells\textsuperscript{20}. In addition, ACE inhibitors improve endothelial dependant vasodilation and improve or maintain cerebral blood flow\textsuperscript{21-23}.

Our study indicates that acute blood pressure lowering after reperfusion in acute ischemic stroke is an effective strategy to achieve neurovascular protection. The most likely mechanisms responsible for the benefits are improvement in post-reperfusion blood flow, reduction in inflammatory damage and the reduction in late oxidant damage. Studies are ongoing to determine the relative contribution of each. The rate, extent and mechanism of blood pressure lowering may determine the magnitude of protection.
References


Legends:

Figure 3.1: Blood pressure in rats after stroke using different blood pressure lowering agents

Mean arterial Blood pressure (MAP) was measured and recorded every 10 minutes using telemetry for the 24 hours after stroke. MAP abruptly increased upon middle cerebral artery occlusion (MCAO) and decreased significantly at reperfusion with different rates in hydralazine (n=8) and enalapril 10 mg/Kg (n=8) compared to the saline-treated group (n=12). In the Enalapril 5 mg/Kg (n=7), MAP remained elevated until sacrifice.

Figure 3.2: Effect of different blood pressure lowering agents on infarct size 24 hours after stroke

Hydralazine (n=8) and enalapril 10 mg/Kg (n=8) reduced infarct size significantly compared to the saline-treated group (n=12). * P<0.05

Figure 3.3: Effect of different blood pressure lowering agents on hemorrhage 24 hours after stroke

Hemoglobin excess in the ischemic hemisphere is expressed as micrograms/gram of tissue. There was no significant difference between treatment groups.

Figure 3.4: Effect of different blood pressure lowering agents on Bederson score 24 hours after stroke

There was no significant difference between treatment groups.
Figure 3.1

- Saline i.v. inj. (n=18)
- Enalapril 5mg/kg i.v. inj. (n=7)
- Enalapril 10mg/kg i.v. inj. (n=8)
- Hydralazine 1mg/kg iv (n=8)
- Control day and night (n=18)
Figure 3.2

% of contralateral hemisphere

Infarct size

Saline
Hydralazine 1mg/Kg
Enalapril 5mg/Kg
Enalapril 10mg/Kg
Figure 3.3

Ug/g tissue

Saline  Hydralazine 1mg/Kg  Enalapril 5mg/Kg  Enalapril 10mg/Kg

Hemoglobin
Figure 3.4

![Bar chart showing Bederson scale (0 - 3) points before and after reperfusion for different treatments: Saline (n=12), Hydralazine 1mg/kg (n=8), Enalapril 5mg/Kg (n=6), Enalapril 10mg/Kg (n=9).]
CHAPTER 4

UNPUBLISHED DATA

A- Atorvastatin Vascular Protection Study

At 24 hours functional outcome was assessed using a battery of neurobehavioral tests. Atorvastatin significantly improved the Bederson score in both Wistar and GK rats (2.7±0.01 vs 2.4±0.1 points in Wistar and 2.96±0.1 vs. 2.83±0.3 points in GK rats, p=0.0291). However, there was no significant difference in any of the other neurobehavioral tests (beam walk, paw grasp, elevated body swing test (EBST)). (Figure4.1).
MMPs are known to be involved in hemorrhagic transformation after ischemia/reperfusion injury specially MMP-2 and MMP-9. To determine if this mechanism is involved in the vascular protection mediated by atorvastatin, we
measured MMP-2 and MMP-9 activity using gelatin zymography. As shown in Figure 4.2, there were no notable differences between groups in both MMP-2 and MMP-9 at 24 hours after MCAO.

![MMP-2 and MMP-9 activity graphs](image)

**Figure 4.2.** Effect of atorvastatin on MMPs activity 24 hours after stroke. There was no difference between groups in MMP-2 and MMP-9 activity. W: Wistar; C: control; ATV: Atorvastatin. Vertical error bars indicate standard error of the mean (SEM).

To evaluate the long term effects of atorvastatin on stroke, 3 hours of MCAO was followed by 72 hours reperfusion to extend the duration of treatment of atorvastatin. Treatment was administered, at 0 hr, 12 hrs, 24 hrs and 48 hrs from reperfusion.

**Neurobehavioral outcome:**

There was no significant difference between groups in all tests performed which is possibly due to the small sample size used (Figure 4.3).
Figure 4.3. Effect of atorvastatin on neurobehavioral outcomes 72 hours after stroke.
There was no difference between groups in the Bederson score (A), beam walk (B), the Paw grasp (C) or the EBS (D) tests. Black bars represent baseline level, Green bars represent control groups before sacrifice and red bars represent treatment groups before sacrifice. Vertical error bars indicate standard error of the mean (SEM).
To assess the effect of atorvastatin on oxidative damage at 72 hours, oxidative stress was measured using a lipid peroxidation assay (TBARS) and nitrotyrosine slot blot assay as shown in Figure 4.4. Atorvastatin treatment did not affect lipid peroxide or nitrotyrosine levels in either Wistar or GK rats’ brains. The TBARS assay showed an increase in oxidative stress in the diabetic animals (40.6±3.4 vs. 28±2.1 μM MDA/mg protein, p=0.006), while nitrotyrosine levels were significantly lower in GK rats (63.7±6 vs 100±10 %, p=0.036). There was a significant increase in the oxidative stress in the stroke side in both strains (100±10 vs 138±10, p=0.031 in W and 63±6 vs 96.5±13, p=0.05)

Figure 4.4. Effect of atorvastatin treatment on oxidative stress at 72 hours in the brain.
There was no treatment effect in both strains. There was an increase in the oxidative stress in the diabetic animals (# p=0.006) as shown in (A). While nitrotyrosine levels showed that GK rats had less oxidative stress than their normoglycemic controls (* p=0.036). Oxidative stress was induced by stroke in both Wistars and GKS (B). Vertical error bars indicate standard error of the mean (SEM).
Statement of the problem and the main hypothesis:

A major challenge in the study of stroke is that most treatments effective in experimental animals have failed in humans. One possible explanation is that targets for protection have been identified in otherwise healthy animals upon which focal cerebral ischemia has been imposed. While it is known that the integrity of cerebral blood vessels is critical in the pathophysiology of stroke, targets to protect the vasculature from ischemic injury in the presence of preexisting vascular disease are yet to be identified. Type 2 diabetes increases the risk of hemorrhagic transformation secondary to ischemic injury as well as poor outcome of stroke. This may be attributable to pathologic remodeling of the cerebrovasculature in diabetics, resulting in an amplified breakdown of the blood brain barrier (BBB) in response to cerebral infarction or primary hemorrhage. We recently demonstrated augmented cerebrovascular remodeling in an experimental model of Type 2 diabetes. These changes were characterized by increased cerebrovascular matrix metalloprotease (MMP)-2 and -9 activities, that are proposed to mediate the BBB breakdown leading to edema and hemorrhage formation in acute ischemic stroke. Diabetes and hyperglycemia are characterized by an increase in oxidative stress and free radical-mediated damage. This damage is exacerbated when accompanied with ischemia/reperfusion injury. Almost 50% of all acute ischemic stroke patients present with elevated blood glucose levels at admission. Preclinical data strongly suggest that regulation of blood glucose improves stroke outcome. However, the relationship of admission glucose levels with plasma levels of acute ischemic injury
markers including MMPs and measures of oxidative stress and the outcome of acute ischemic stroke in patients remains unknown. Furthermore, whether and to what extent glycemic control correlates with these markers and stroke outcome is yet to be determined. Accordingly, the goal of this substudy of the Glucose Regulation in Acute Stroke Patients (GRASP) trial is to test the hypothesis that elevated levels of circulating MMPs and oxidative stress markers (Lipid peroxides and nitrotyrosine) at admission correlate with blood glucose levels and worsened outcome. Tight regulation of blood glucose reduces oxidative stress and improves MMPs and outcome in acute ischemic stroke patients.

Endpoints: The end point is the oxidative stress at discharge, its change from baseline levels and its correlation to the glucose regulation in the different study groups.

Experimental Protocol:

Patients were identified and enrolled by the attending physician or the research nurse as described in the GRASP Trial protocol. They were randomized into 3 groups: tight, loose and blood glucose control with target levels of blood glucose of (70-110, 70-200 and 70-300 mg/dl respectively).

Blood sampling strategy: In addition to the blood assessments described in the GRASP protocol, venous blood (5 mL) was collected into an anticoagulated (citrated) tube at baseline and 5 days (or on the day of discharge if patient discharged prior to hospital day 5). HIPAA regulations were followed and blood samples were logged and labeled as GRASP #1 baseline or discharge as patients enrolled. Blood samples were
collected from all 35 patients enrolled to 3 different study groups (usual care, loose glucose control, and tight glucose control groups) at MCG.

**Oxidative stress markers measurements:**

**Detection of nitrotyrosine:**

Nitrotyrosine immunoreactivity was measured as an indicator of superoxide –dependent peroxynitrite formation by slot blot analysis. Plasma samples (30 μg) were immobilized onto a nitrocellulose membrane using a slot blot microfiltration unit. After blocking with 5% nonfat milk, the membrane was incubated with an anti-nitrotyrosine antibody from Calbiochem and visualized with Pierce Super Signal Kit. The intensity of bands was analyzed by GelPro Software.

**Measurement of TBARS:**

Lipid peroxide concentration will be determined by measuring the amount of thiobarbituric acid reactivity (TBARS) by the amount of malondialdehyde formed during acid hydrolysis of the lipid peroxide compound. Two tenths ml of sample was incubated with the reaction mixture containing 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid solution (buffered to pH 3.5), and 1.5 ml of 0.8% thiobarbituric acid at 95°C for 1 hour. After cooling, 1 ml of distilled water and 5 ml of a mixture of n-butanol and pyridine (15:1, v/v) was added and the final mixture was shaken vigorously. After centrifugation at 1500 x g for 10 minutes absorbance of the solvent layer was measured at 532 nm. Tetraethoxypropane was used to establish the standard curve and the lipid peroxide level was expressed in terms of uMolar malondialdehyde per mg protein.
Results:

A- Nitrotyrosine formation

Nitrotyrosine levels at day 5 or discharge (whichever comes first) were reduced in all different groups compared to their levels at baseline which is possibly due to the huge level of free radicals released after stroke onset. As expected these levels declined by time, however, glucose regulation during the acute and subacute period seems to have a proportional effect on the degree of reduction.

In the usual care group, % reduction from baseline was 8±40% while in the loose control group it was 23±26% and finally, in the tight control group, nitrotyrosine levels reached the lowest values after 5 days and % reduction was 33±45%.

Figure 4.5. Effect of glucose regulation on nitrotyrosine levels after ischemic stroke in hyperglycemic patients (A) shows the % change from the admission Nitrotyrosine levels among the different groups. The highest reduction was achieved in the tight control group. Vertical error bars indicate standard error of the mean (SEM). (B) Shows the individual values for each patient.
B- Lipid peroxidation

There was an increase in the lipid peroxide levels in the usual care group (97±227%) and that increase was quite similar in the loose control group (162±390%). Interestingly, lipid peroxide levels were not changed from admission levels (18±33%)

Figure 4.6. Effect of glucose regulation on lipid peroxidation after ischemic stroke in hyperglycemic patients (A) shows the % change from the admission lipid peroxides levels among the different groups. The minimum increase in oxidative stress was in the tight control group. There was an increase in oxidative stress in the other two groups. Vertical error bars indicate standard error of the mean (SEM). (B) Shows the individual values for each patient.
CHAPTER 5

DISCUSSION

The aims of the current project were to determine the neurovascular protective properties of atorvastatin in diabetes (GK rats) and the mechanisms through which these properties are mediated. In addition, we determined the concentration associated with the atorvastatin neurovascular protection in rats for future translational studies in humans. Recent data from our group demonstrated that type II diabetic animals (GK rats) are more susceptible to vascular damage after experimental cerebral ischemia than non-diabetic animals (Wistar rats)\(^1\) and may serve as a good model to test potential vascular protective agents after stroke in diabetes. A set of normoglycemic rats (Wistar) was used to confirm previous reports showing post-ischemic neuroprotective effects of statins\(^2-4\).

The most important finding in our study was that atorvastatin 30 mg/Kg/day after ischemic stroke reduced hemorrhage formation (Figure 2.3) and infarct volume (Figure 2.2) in both Wistar and GK rats. Atorvastatin neurovascular protection was associated with improvement in neurobehavioral outcomes (Figure 2.4).

Our second major finding was that, contrary to our hypothesis, acute administration of atorvastatin after reperfusion did not reduce oxidative stress and did not upregulate eNOS expression or its phosphorylation (Figure 2.6). Atorvastatin induced Akt phosphorylation in the brain homogenate (Figure 2.7), an effect that was
shown by others to be involved in the acute neuro- and cardio-protection of statins\textsuperscript{5-7}. The phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway is known to be very important in regulating cell growth, proliferation, and survival. The activation of the PI3K/Akt pathway reduces thrombogenicity, vascular permeability, inflammation and apoptosis and thereby protects vascular function\textsuperscript{8}. Statins induce Akt translocation to the plasma membrane of endothelial cells. Upon phosphorylation and subsequent activation of Akt at serine 473, it induces phosphorylation of Bad (member of the pro-apoptotic BCL2 protein family), forkhead transcription factor (AFX), caspase 9, eNOS and inhibitor of nuclear factor kappa B (NF\textsubscript{k}B)\textsuperscript{9}.

The third finding of this study is that the atorvastatin peak blood level achieved after administering 15 mg/Kg by the oral route was similar to that reported after a dose of 80 mg/day of atorvastatin is given to humans\textsuperscript{10}. This gives our results clinical relevance for future translational studies, especially since 80 mg/day of atorvastatin was the dose shown to reduce stroke recurrence in (SPARCL) trial\textsuperscript{11}. Area under the curve is another important parameter that should be addressed in the future for a more accurate comparison between atorvastatin levels in rats and humans.

Our study shows that diabetic rats are mildly hypertensive compared to the normoglycemic ones. They are also more sensitive to ischemia leading to a higher increase in their MAP than normal rats during MCA occlusion. These results are probably mediated by the endothelial dysfunction, increased tone and altered nature of the diabetic vasculature in GK rats\textsuperscript{12, 13}. Interestingly, atorvastatin significantly reduced MAP after reperfusion for 5 hours, an effect that was only seen in GK rats. It is
possible that this mild blood pressure lowering contributed to the robust protection seen in the diabetic animals in this study and may have improved their cerebral blood flow.

This finding matches our previous study (manuscript #2)\(^1\) where we showed that blood pressure lowering after reperfusion in acute ischemic stroke is an effective strategy to achieve neurovascular protection. In this study, different antihypertensive agents with different doses have been given to the rats at reperfusion. A significant reduction in infarct size was seen only in the treatment groups that lowered MAP after reperfusion (Figure 3.1 and 3.2).

Lastly, our substudy in the GRASP trial shows that glucose control after acute ischemic stroke correlates with reduction in the oxidative stress (Figure 4.5 and 4.6). This result confirms previous animal studies showing the correlation of hyperglycemia in stroke with the generation of ROS\(^\(^1\)\). However, large-scale trials are warranted to correlate glucose control after ischemic stroke with patient functional outcomes and oxidative stress markers.

Our hypothesis stated that atorvastatin is neurovascular protective in both diabetic (GK rats) as well as their normoglycemic controls (Wistar rats) which turned out to be true. We also postulated that atorvastatin would mediate this neurovascular protection through the reduction of oxidative stress and the restoration of nitric oxide bioavailability but our hypothesis was false and that may be due to the following reasons. First of all, atorvastatin are known to reduce oxidative stress through their inhibitory effect on the Rac which inhibits NADPH oxidase and hence decrease ROS production\(^\(^1\)\). However, there are many other sources of oxidative stress and ROS in the brain after ischemia/reperfusion injury that are not inhibited by statins like
cyclooxygenase, myeloperoxidase and mitochondrial electron transport chain. In addition, ROS production seems to occur very early after ischemia which makes it a very hard target to catch with the delayed oral statin treatment (given at 3 hours after the onset of stroke). Second, although statins are known to upregulate eNOS expression, this effect seems to occur after chronic treatment with statins. Dr. Chopp and his group have shown that post-treatment with atorvastatin in combination with delayed tPA in a rat model of embolic stroke was neuroprotective without affecting eNOS levels and inhibition of NOS activity with N-nitro-L-arginine methyl ester did not block the beneficial effects of the combination treatment on stroke. Others have also shown acute neuroprotection with different statins after experimental stroke models without altering eNOS expression. However, we think that the BP lowering effect in atorvastatin-treated GK rats is an indication of cerebral vasodilation that occurred acutely after reperfusion and atorvastatin treatment. That means that atorvastatin may have increased eNOS phosphorylation through PI3K/Akt pathway which has lead to an increase in NO production and improvement in cerebral circulation in both strains. An effect that may have been more prominent in the diabetic rats’ blood vessels because of their increased tone to the extent that it generated a general systemic reduction in their MAP as seen in Figure 2.8. It is very possible also that the eNOS phosphorylation was transient with an early peak after atorvastatin administration followed by a decline to baseline level, a pattern that did not allow us to see any change on eNOS phosphorylation using western blot on samples collected at 21 hours after reperfusion and atorvastatin administration (Figure 2.7).
Figure 5.1. Summary of the project results

Atorvastatin 30 mg/Kg/day given after ischemia/reperfusion injury in both diabetic and normoglycemic rats upregulated Akt phosphorylation which plays an important role in survival, inflammation and vasodilation. These effects mediated neurovascular protection as well improvement in neurobehavioral outcomes in both diabetic and normoglycemic rats.

FUTURE DIRECTIONS

Assessing the vascular protection with the use of only brain extracts without looking at isolated vessels from the cerebral circulation is one of the main limitations of our study. Vascular function is an important component that has not been addressed here. In further studies, contractile and relaxation responses should be examined using arteriograph on isolated MCA.

It is very important also to collect samples at different time points rather than one time point 21 hours after treatment to detect early effects of statin treatment. Western
blots experiments should be ran on isolated vessels from the cerebral circulation, example: MCA or basilar artery, rather than the whole brain extract

We anticipate that prolonging the treatment duration with atorvastatin after stroke would have at least the same impact on the functional outcome if not better. However, we did not see an improvement in the neurobehavioral outcomes at 72 hours. One possible explanation of this result is that the neurobehavioral tests used in our experiments are not sensitive enough to detect an improvement with atorvastatin after such a huge brain injury with the sample size that we had. For that reason, in our 24 hour experiments, we used a large sample size to achieve a power high enough to detect even small differences. The other alternative is to explore other behavioral measures that could be more suitable to our experimental stroke model.

A comprehensive study of atorvastatin dose response on neurovascular protection and the involved mechanisms should be done in the future. It is also very important to determine the optimal duration of treatment with statins after stroke and the best strategy used in treatment discontinuation. That’s because abrupt statin discontinuation is associated with impairment in the vascular function. The underlying mechanism is a negative feedback regulation of different genes leading to an increase in Rho and Rac activity, which eventually will cause suppression of endothelial NO production and increased production of ROS and inflammatory mediators\textsuperscript{16, 20}. 

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REFERENCES


APPENDICES

Included: 2 items

Published Manuscript:

Hazem Elewa, Hend Hilali, Livia S. Machado and Susan C. Fagan
Minocycline for short term neuroprotection.
Pharmacotherapy 2006 Apr;26(4):515-21

Published Manuscript:

Susan C. Fagan, Hazem Elewa and David Rychly
Statin Therapy for Secondary Stroke Prevention: Evidence Catches Up to Practice JPP 2007; 20; 117
APPENDIX A

MINOCYCLINE FOR SHORT TERM NEUROPROTECTION
Minocycline for Short-Term Neuroprotection


Minocycline is a widely used tetracycline antibiotic. For decades, it has been used to treat various gram-positive and gram-negative infections. Minocycline was recently shown to have neuroprotective properties in animal models of acute neurologic injury. As a neuroprotective agent, the drug appears more effective than other treatment options. In addition to its high penetration of the blood-brain barrier, minocycline is a safe compound commonly used to treat chronic infections. Its several mechanisms of action in neuroprotection—antiinflammatory and antiapoptotic effects, and protease inhibition—make it a desirable candidate as therapy for acute neurologic injury, such as ischemic stroke. Minocycline is ready for clinical trials of acute neurologic injury.

Key Words: minocycline, stroke, neuroprotection. (Pharmacotherapy 2006;26(4):515–521)

OUTLINE

Background
Neuroprotective Properties
Mechanisms of Action as a Neuroprotective Agent
  Antinflammatory Effects
  Antiapoptotic Effects
  Inhibition of Matrix Metalloproteinases
Summary
Pharmacokinetic Issues
Adverse Effects
Conclusion

Numerous studies have been conducted to develop an effective neuroprotective agent for acute brain injury, particularly ischemic stroke. To date, no pharmacologic agent has been effective in a context of short-term intervention. Agents such as calcium channel blockers and glutamate antagonists do not provide a benefit in acute brain injury for several reasons, including poor penetration of the blood-brain barrier, dose-limiting toxicity of the agent, and a time window of effectiveness that is too short for clinical use. After carefully reviewing studies of failed agents, we clearly found a need to investigate new approaches to acute brain injury.

Background

Minocycline is a widely used semisynthetic tetracycline antibiotic with known antiinflammatory, antiapoptotic, and glutamate-antagonist properties in several models of brain injury. The drug has been used for decades to treat infections caused by a variety of gram-negative and gram-positive organisms. Minocycline is indicated for the treatment of several diseases including acne vulgaris, central nervous system and urinary tract infections, gonorrhea, meningitis, shigellosis, conjunctivitis, psittacosis, Q fever, relapsing fever, and syphilis. Minocycline is a generic drug, available in oral or intravenous formulations in humans, and subgingival sustained-release microspheres are used in adults with periodontitis.

Like other tetracycline compounds, minocycline interferes with bacterial protein synthesis by binding to the 30S ribosomal subunit, inhibiting messenger RNA–transfer RNA interaction and
In addition to the antibacterial properties, evidence supports anti-inflammatory actions of tetracyclines. Because of its anticollagenase, immunosuppressive, and immunomodulating effects, minocycline hydrochloride has been used to manage rheumatoid arthritis.

**Neuroprotective Properties**

Over the past 5 years, numerous reports have demonstrated the efficacy of minocycline in a variety of animal models of acute neurologic injury (Table 1). The drug had a broad neuroprotective effect unrivaled by those of other agents. Minocycline was effective in animal models of global cerebral ischemia, focal cerebral ischemia, traumatic brain injury, spinal cord injury, and intracerebral hemorrhage. All of these injuries share common pathophysiologic mechanisms and the need for early (probably within 3–6 hrs of onset) interventions and treatment. Minocycline not only reduced tissue injury but also improved functional recovery.

Minocycline is likely to be more successful than other studied neuroprotective compounds in that it avoids the common pitfalls stated above. Minocycline has superior penetration of the blood-brain barrier, and it was protective for longer than 3 hours in the studied animals. In addition, because it is a safe compound, minocycline is particularly well suited for a clinical trial. The drug appears to be an ideal candidate as a therapy that will overcome the issues identified in neuroprotective trials of failed agents.

### Table 1. Efficacy of Minocycline in Animal Models of Acute Neurologic Injury

<table>
<thead>
<tr>
<th>Animal Model and Type of Injury</th>
<th>Minocycline Dosage</th>
<th>Therapeutic Window</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat focal ischemia, TMCAo (90 min)&lt;sup&gt;14&lt;/sup&gt;</td>
<td>45 mg/kg i.p. b.i.d. on day 1, then 22 mg/kg i.p. b.i.d. for 2 days</td>
<td>Pretreatment: 76% infarct reduction (72 hrs)</td>
<td>63% reduction</td>
</tr>
<tr>
<td>Rat focal ischemia, embolic clot&lt;sup&gt;15&lt;/sup&gt;</td>
<td>45 mg/kg i.p. b.i.d. on day 1, then 22.5 mg/kg i.p. b.i.d. on day 2</td>
<td>1 hr after insult</td>
<td>42% infarct reduction (48 hrs)</td>
</tr>
<tr>
<td>Mouse focal ischemia, PMCAo&lt;sup&gt;16&lt;/sup&gt;</td>
<td>90 mg/kg i.p.</td>
<td>Pretreat 60 min before or 30 min after insult</td>
<td>Reduced infarction and brain swelling</td>
</tr>
<tr>
<td>Rat focal ischemia, TMCAo (90 min)&lt;sup&gt;17&lt;/sup&gt;</td>
<td>3 and 10 mg/kg i.v.</td>
<td>4 hrs (3 mg/kg), 5 hrs (10 mg/kg)</td>
<td>40–50% infarct reduction (24 hrs)</td>
</tr>
<tr>
<td>Gerbil global ischemia&lt;sup&gt;18&lt;/sup&gt;</td>
<td>45 mg/kg i.p. x 1, then 90 mg/kg i.p. b.i.d. on day 1, then 45 mg/kg after 36 hrs</td>
<td>30 min after insult: Increased survival of CA1 neurons from 10% to 77%</td>
<td></td>
</tr>
<tr>
<td>Rat neonatal hypoxia-ischemia&lt;sup&gt;19, a&lt;/sup&gt;</td>
<td>45 or 22.5 mg/kg i.p.</td>
<td>Immediately before or after insult</td>
<td>Robust protection before and at 30 min, but not at 3 hrs</td>
</tr>
<tr>
<td>Rat spinal cord injury&lt;sup&gt;20&lt;/sup&gt;</td>
<td>50 mg/kg i.p. b.i.d. for 2 days</td>
<td>30 min after insult</td>
<td>Improved function</td>
</tr>
<tr>
<td>Rat spinal cord injury&lt;sup&gt;21&lt;/sup&gt;</td>
<td>90 mg/kg i.p. x 1, then 45 mg/kg i.p. for 5 days</td>
<td>1 hr after insult</td>
<td>Enhanced long-term hind-limb locomotion, coordinated motor function, and hind-limb reflex recovery</td>
</tr>
<tr>
<td>Mouse spinal cord injury&lt;sup&gt;22, b&lt;/sup&gt;</td>
<td>50 mg/kg</td>
<td>1 hr after insult</td>
<td>Improved hind-limb function and strength, axonal sparing, superior to methylprednisolone</td>
</tr>
<tr>
<td>Mouse traumatic brain injury&lt;sup&gt;23&lt;/sup&gt;</td>
<td>90 mg/kg i.p. x 1, then 45 mg/kg i.p. b.i.d. until sacrifice</td>
<td>Pretreatment or 30 min after insult</td>
<td>Improved function (Rotorod test), decreased lesion size</td>
</tr>
<tr>
<td>Rate intracerebral hemorrhage&lt;sup&gt;24, c&lt;/sup&gt;</td>
<td>45 mg/kg i.p. b.i.d. on day 1, then 22.5 mg/kg i.p. x 1</td>
<td>1 hr after insult</td>
<td>Improved function</td>
</tr>
</tbody>
</table>

TMCAo = temporary occlusion of the middle cerebral artery; i.p. = intraperitoneally; PMCAo = permanent occlusion of the middle cerebral artery.

<sup>a</sup>Carotid occlusion plus hypoxia.
<sup>b</sup>Extradural compression with aneurysm clip.
<sup>c</sup>Collagenase.
have been demonstrated in both acute and chronic brain injury. Minocycline has anti-inflammatory effects on neutrophils, monocytes, microglial cells, and neurons. It inhibits neutrophil-mediated tissue injury by inhibiting neutrophilic migration and degranulation and by suppressing the formation of oxygen radicals. In a model of focal cerebral ischemia, minocycline inhibited enzymes that contribute to inflammation, such as the inducible form of nitric oxide synthase and interleukin-1β converting enzyme, it suppressed apoptosis, and it reduced microglial activation. Minocycline inhibited nitric oxide release (likely by suppressing the expression of nitric oxide synthase) from monocytic cells induced by lipopolysaccharide or interferon-γ expression.

In an acute toxin model of Parkinson's disease, minocycline protected neurons (induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in an oxygen radical-based mechanism of injury), where inflammation prominently contributed to neuronal injury. In this model, minocycline prevented microglial activation and expression of interleukin-1 and the inducible form of nitric oxide synthase.

Minocycline was also studied in a rat model of immune-inflammatory encephalitis in which microglia, monocytes, and T-cell activation mediated neuronal injury by means of several inflammatory mediators. The drug delayed and reduced the progression of disease (including demyelination) as well as the infiltration of inflammatory cells.

At nanomolar concentrations, minocycline inhibited glutamate excitotoxic effects in mixed neuron–glial cell cultures in correlation with the inhibition of p38 phosphorylation and interleukin-1 release. The drug protected rat neurons (cereellar granules) from excitotoxic injury induced by reactive oxygen species and nitric oxide. Minocycline neuroprotection in vitro was associated with the inhibition of inflammatory signaling kinases, such as p38. In a model of immune-inflammatory encephalitis, minocycline reduced the release of tumor necrosis factor from activated oligodendrocytes while enhancing the release of interleukin-10, an antiinflammatory cytokine.

The antiinflammatory effects of minocycline have also been demonstrated in humans. At doses commonly used for indications other than neuroprotection, minocycline provided antiinflammatory benefits in rheumatoid arthritis that was not treated with other disease-modifying agents. In a small pilot clinical trial of multiple sclerosis, minocycline 200 mg/day reduced the number of gadolinium-enhancing lesions on magnetic resonance imaging, demonstrating its ability to decrease the inflammatory damage associated with the disease.

In summary, compelling evidence from the last few years suggests that minocycline modulates inflammation and that this drug might be a novel therapeutic approach to diseases characterized by the stimulation of inflammatory cascades, such as acute ischemic brain injury.

Antiapoptotic Effects

Apoptosis, or program cell death, is thought to play a role in both acute and chronic brain injury. Minocycline prevented apoptosis and the release of cytochrome c from mitochondria in both in vitro and in vivo models. Minocycline delayed the progression of amyotrophic lateral sclerosis–like syndrome in superoxide dismutase-1 mutant mice and inhibited mitochondrial release of cytochrome c in vitro and in vivo. The drug inhibited mitochondrial cell death, both caspase dependent (cytochrome c and Smac/Diablo release) and caspase independent (apoptosis inducing factor release), in a Huntington striatal-cell model. Minocycline also protected the renal proximal tubule cells from apoptosis on exposure to azide, hypoxia, staurosporine, and cisplatinum.

Furthermore, minocycline induced the upregulation of the antiapoptotic protein bcl-2 at the messenger-RNA level. In fact, the antiapoptotic effects of minocycline were lost when cells were pretreated with bcl-2 antisense; this finding suggested that the antiapoptotic action of minocycline depended on bcl-2. Moreover, bcl-2 was upregulated in neurons in vitro when they were incubated with equivalent doses of clinically therapeutic concentrations of minocycline. In cardiomyocytes exposed to anoxia and reoxygenation, minocycline inhibited the release of cytochrome c and Smac/Diablo from mitochondria and inhibited both caspase activation and apoptosis.

Inhibition of Matrix Metalloproteinases

Tetracyclines are known to inhibit matrix metalloproteinases. Low-dose doxycycline, the first matrix-metalloproteinase inhibitor the United States Food and Drug Administration approved, is used in periodontal disease.
In a rat model of adjuvant arthritis, doxycycline and tetracycline (two close analogs of minocycline) reduced joint swelling and inflammation and improved radiologic evidence of damage when they were given with a standard nonsteroidal antiinflammatory agent. In this model, the arthritic syndrome was associated with the suppression of matrix metalloproteinase-2 (gelatinase) activation in the inflamed joints.44

Minocycline also reduced levels of matrix metalloproteinase-9 in a model of immune-inflammatory encephalitis.31 In a collagenase-induced model of intracerebral hemorrhage, minocycline reduced MMP-12 and improved functional outcomes.24 In addition, minocycline reduced renal microvascular leakage in a rat model of ischemic renal injury. This action was probably due to diminishing the activity of matrix metalloproteinases.45

Matrix metalloproteinases are increasingly associated with diseases that involve degeneration of extracellular proteins and matrix in the brain.46 For this reason, the inhibition of these proteases with minocycline seems to be an attractive experimental therapy.

Summary

Minocycline has several mechanisms of action, including antiinflammatory, matrix-metalloproteinase inhibitory, and antiapoptotic effects, that make it an attractive neuroprotective agent. It has demonstrated activity in many acute and chronic animal models of neurologic disease, and it is one of few agents that has been shown to be efficacious in animal models of spinal cord injury, traumatic brain injury, intracerebral hemorrhage, or global or focal cerebral ischemia.

These observations point to a key element that distinguishes minocycline from other neuroprotective agents, namely, the diversity of cellular mechanisms affected. Minocycline may likely act by means of vascular mechanisms as well. These mechanisms have been correlated with the intensity of the inflammatory response to injury and with the severity of damage to the brain parenchyma.47 Finally, in humans, minocycline appears to exert antiinflammatory properties with the same dosage regimens as those clinically used for antibacterial treatment. Therefore, minocycline is a likely candidate as a drug for neuroprotection in acute brain injury in its present human formulation and dosage.

Pharmacokinetic Issues

In acute brain injury, the ability to rapidly deliver a potential neuroprotective agent to the systemic circulation is a necessity. In this setting, intravenous administration is most often required.

Because minocycline has been used for decades, its clinical pharmacokinetics are well described in humans. After a 200-mg intravenous dose, the mean peak concentration is 4.0 mg/L,48 and steady-state concentrations after a dose of 100 mg given orally twice/day for 3 days are 1.4–1.8 mg/L.26 Minocycline is the most lipophilic of the commonly used tetracycline antibiotics, and its concentration in the cerebrospinal fluid is 11–56% of plasma concentrations.26 Therefore, concentrations in cerebrospinal fluid after long-term dosing are expected to be approximately 0.5 mg/L. In addition, urinary excretion is lower with minocycline than with other tetracyclines; therefore, minocycline is safer than the other tetracyclines for patients with renal insufficiency.

Since 1999, most reported studies of the neuroprotective effects of minocycline in rodent models of brain injury used large intraperitoneal doses of 10–90 mg/kg.14, 16, 17, 19, 23–25, 39, 49, 50 Even in stroke models, in which timely cerebral neuroprotection is important, intraperitoneal administration was used.14, 19, 24, 39

Because the pharmacokinetics of large intraperitoneal doses of minocycline in rodents were unknown but necessary to extrapolate experimental results to humans, we studied this issue. We found that the intraperitoneal route resulted in widely variable serum concentrations of minocycline and that it delayed absorption in the systemic circulation, with peak concentrations achieved at a mean of 2.5 hours after injection. Compared with intravenous administration, intraperitoneal administration resulted in a bioavailability of 10–80%, which was probably due to the frank deposition of the drug in the peritoneal cavity.51 The intraperitoneal route of administration probably accounts for the wide range of high doses reported in the literature. Intravenous dosing was needed to determine the true therapeutic window and the dose-response relationship in focal cerebral ischemia.51 We determined that peak serum concentrations above 3.5 mg/L and trough concentration above 2 mg/L were neuroprotective in temporary focal cerebral ischemia in rats.17 Low doses are being studied.
When intravenous administration was used to overcome the absorption problems of both oral and intraperitoneal administration, the volume of distribution of minocycline was similar in rats and in humans when adjusted by weight. In other words, the intravenous administration of 3 mg/kg in humans and in rats is expected to achieve peak concentrations of the same magnitude (3–5 mg/L). The main difference in the pharmacokinetic parameters between the species is the half-life, which is approximately 17 hours in humans and only 3 hours in rats.

In summary, intravenous doses of minocycline commonly used in humans should achieve serum and cerebrospinal fluid concentrations that were neuroprotective in animal models.

Adverse Effects

Most available information on the tolerability of minocycline was obtained after long-term oral administration. In studies of ambulatory patients taking minocycline long term, increasing the dosage above 100 mg twice/day was problematic because of the common adverse effect of dizziness (affecting 26–78% of patients). More recently, most adverse effects of oral minocycline therapy in patients with amyotrophic lateral sclerosis were gastrointestinal. The mean tolerated dose was 387 mg/day, and all patients could tolerate at least 300 mg/day. No patient had dizziness, but elevated concentrations of blood urea nitrogen and liver enzymes were reported over the 6-month treatment period.

Doses of up to 400 mg given intravenously have safely been used to treat serious infections in humans. In a case series of 119 patients who received intravenous minocycline 200–400 mg for 2–24 days to treat an infectious disease, 21 (18%) had adverse effects, 50% of which were gastrointestinal. Only one patient discontinued therapy prematurely. This patient developed azotemia, but a chronic urinary tract infection complicated its attribution.

In a search for adverse effects associated with intravenous minocycline that have been reported to the World Health Organization Collaborating Center for International Drug Monitoring (Uppsala, Sweden) since 1975, we found 122 case reports of adverse drug reactions. No assessment of causality was given, and the reports do not represent the opinion of the World Health Organization. The most common event was abnormal hepatic function (19 reports). Thrombocytopenia was reported 11 times, and injection-site reaction was reported once. The data of the World Health Organization were limited because no denominator could be ascertained and because the dosage and duration of intravenous minocycline treatment were unknown.

The dose of minocycline that is neuroprotective and tolerable in humans is still unknown. In addition, the feasibility of rapidly administering intravenous doses of minocycline to patients and the preliminary evidence of the activity of the compound should be determined before minocycline is further developed as a treatment for acute neuroprotection. The question of optimal duration should be addressed by assessing a biomarker of inflammation and by measuring serum levels of minocycline in the patient. In addition, further translational studies in animals will contribute to our understanding of the optimal duration of minocycline treatment for neuroprotection.

Conclusion

Minocycline is already in clinical trials for the chronic brain injury of amyotrophic lateral sclerosis and multiple sclerosis and has a strong potential for treating brain diseases that require acute intervention, such as stroke. Minocycline has long been established as a safe drug for clinical use, it has several mechanisms of action, and it had a delayed therapeutic window in experimental models. Minocycline is ready for clinical trials as a short-term neuroprotectant.

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APPENDIX B

STATIN THERAPY FOR SECONDARY STROKE PREVENTION: EVIDENCE CATCHES UP TO PRACTICE
Statin Therapy for Secondary Stroke Prevention: Evidence Catches Up to Practice
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Current approaches for the secondary prevention of ischemic stroke include the aggressive use of antithrombotic therapy, particularly antiplatelet agents, and carotid endarterectomy for eligible patients. Blood pressure lowering with angiotensin converting enzyme inhibitors and diuretics in hypertensives, glucose control to a hemoglobin A1C of less than 7% in diabetics, and statin therapy in patients with hyperlipidemia and/or cardiovascular disease are also important measures for secondary prevention of stroke. Support for the routine use of statin therapy in all patients with ischemic stroke, regardless of lipid profile or a history of cardiovascular disease, has been, until recently, of debate. Clinical evidence found in studies such as the SPARCL trial and the Heart Protection Study now support the routine use of statin therapy in all ischemic stroke patients. This article focuses on the potential and proven benefits of statins in ischemic stroke.

A LARGE PART OF THE MANAGEMENT OF acute stroke patients is focused on identifying the etiology of the event and instituting the appropriate secondary prevention strategies. For the more than 600,000 patients who suffer from an ischemic stroke annually in the United States, antithrombotic treatment with either anticoagulant or antiplatelet therapy has been the cornerstone of secondary prevention for decades. Large, randomized clinical trials published largely in the last 10 years have resulted in a broadening of the guidelines for recurrent stroke prevention to include evidence-based surgical approaches and additional pharmacologic tactics. Although data on the efficacy of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors, “the statins,” in reducing recurrent vascular events in patients with coronary artery disease have been steadily accumulating since the early 1990s, it was not until 2006 that the results of the first true secondary prevention study with statins was published. This article focuses on the evolving status of statin therapy as a secondary stroke prevention strategy.

SERUM CHOLESTEROL AND STROKE

Early studies of serum cholesterol and cardiovascular events pointed to a clear direct relationship between elevated plasma lipids and myocardial infarction and vascular death. This was not the case with stroke. When measurements of plasma cholesterol were similarly studied with relationship to stroke, no clear association could be found. This lack of relationship persisted upon the availability of specific measurements of low density lipoprotein (LDL). Further examination of the data suggested, however, that when hemorrhagic stroke was excluded from the epidemiologic data set, LDL was directly associated with the incidence of ischemic stroke. In fact, low LDL values were associated with hemorrhagic stroke in some data sets. This initiated a debate as to whether the low LDL contributed to the brain hemorrhage or was an epiphenomenon, reflecting a health state or genetic susceptibility. When a case-control study was performed, lipid lowering with statin therapy did not appear to be associated with hemorrhagic stroke. Additionally, a meta-analysis that included...
more recent studies of aggressive LDL lowering in patients being treated for cardiovascular disease conclusively demonstrated a relationship between lowering LDL and a reduced incidence of ischemic stroke. After these important studies, most experts agreed that the controversy was finally put to rest.

STATINS IN STROKE: MECHANISMS OF STROKE PREVENTION AND PROTECTION

Currently, statins are the mainstay of lipid lowering therapy. They mediate their effect by inhibiting the hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of the mevalonate pathway of cholesterol biosynthesis. By reducing cholesterol biosynthesis in the liver, this leads to negative-feedback low-density lipoprotein (LDL) receptor upregulation followed by reduction in total serum cholesterol levels. Despite the controversy regarding the relationship between serum cholesterol and the risk of stroke, both primary and secondary prevention trials have shown that statins reduce stroke incidence. In fact, the reduction in stroke risk is likely to be due to mechanisms beyond lowering of serum cholesterol. Results from these studies, in addition to other preclinical investigations, have all supported the idea that statins may have benefits over and beyond those generally attributed to the modification of the lipid profile. These beneficial effects have been called the “pleiotropic” effects of statins.

By virtue of their pleiotropic effects, statins have been shown to be both neuroprotective and vascular protective when administered acutely after stroke in experimental models. In these acute animal models, no reduction in cholesterol is documented. Most of these beneficial effects are still considered to be due to the inhibition of HMG-CoA reductase enzyme because inhibiting this enzyme reduces the subsequent formation of isoprenoid intermediates. The addition of the fatty isoprenoid moiety to proteins is a major mechanism by which some proteins are anchored or translocated to cell membranes. In the vasculature, for example, the Rho family of GTPases requires isoprenylation to be active. The Rho proteins have been implicated in various mechanisms of cardiovascular pathophysiology, such as smooth muscle cell proliferation and increases in the nuclear factor-kappaB transcription factor which has pro-inflammatory effects. Statin-induced reduction of isoprenoid availability can antagonize these Rho-mediated actions. Furthermore, inhibition of Rho isoprenylation by statins has been shown to upregulate endothelial nitric oxide synthase (eNOS) expression within the endothelium. This leads to an increase in eNOS, causing an augmentation of production of nitric oxide (NO), which can ameliorate a number of pathophysiological processes within the cerebral vasculature and brain parenchyma during cerebral ischemia and reperfusion. Nitric oxide, a potent vasodilator, mediates its beneficial effects by improving blood flow to the ischemic brain and increasing angiogenesis and collateral vessel formation through vascular endothelial growth factor (VEGF), thereby making the brain parenchyma more resistant to the effects of ischemia. Statins may also directly activate eNOS via additional posttranslational mechanisms involving activation of the phosphatidylinositol 3-kinase/proteinkinase Akt (PI3K/Akt) pathway.

In addition to the vasodilation and the endothelial protection effect of statins, they have also demonstrated an antioxidant effect. This effect is largely mediated by the inhibition of the proteins Rac and NAD(P)H oxidase. Among the different enzymatic sources of reactive oxygen species (ROS), NAD(P)H oxidase is considered to be the major one in the vascular system. After acute ischemic stroke and during both spontaneous and therapeutic reperfusion, there is widespread liberation of free radicals and ROS. Free radicals induce tissue injury in the ischemic penumbra through lipid peroxidation, protein oxidation, and direct damage to nucleic acids. Superoxide free radicals also reduce the bioavailability of vasoprotective NO by avidly reacting with it to form peroxynitrite radicals that can subsequently damage proteins via nitration. Other beneficial enzymatic effects exhibited by some statins include the enhancement of the activities of catalase, glutathione peroxidase, paraoxonase, and the superoxide dismutases. Additionally, most of the statins have demonstrated some degree of intrinsic, nonenzymatic antioxidant effects in vitro. The ability to scavenge free radicals may possibly stem from the unsaturated, conjugated bonds in the chemical structures.

Another important process that contributes to the ultimate injury from cerebral ischemia and reperfusion is inflammation. Upregulation of adhesion molecule expression occurs within the infarct and the surrounding penumbra and facilitates postischemic migration of leukocytes through the brain parenchyma. The expression of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α is known to be increased in the brains of experimental animals after stroke, and this appears to contribute to neuronal damage. Other mediators and markers of inflammation, such as nuclear factor-kappaB and C-reactive protein, have been shown to be reduced by statins.
Statins have also demonstrated multifaceted antithrombotic actions in experimental and clinical settings, which may have relevance in the secondary prevention of stroke. Statins appear to exert inhibitory actions on platelets themselves, possibly as a result of increased NO availability, which decreases platelet aggregation.44,45 Other studies have suggested that the antiplatelet effect could be due to reduced thromboxane A2 production,46-48 changes in platelet membrane composition,49 or some other inhibition of platelet reactivity.50 These antiplatelet effects could conceivably reduce the risk of thrombus formation. Statins also have demonstrated inhibition of the extrinsic coagulation pathway, leading to reduced thrombin formation.51 The initiating event in this pathway is the exposure of tissue factor, and statins have been shown to reduce tissue factor expression in a variety of in vitro and experimental animal models.52-54 Other components of the coagulation pathway, such as factors V and VII, may also be inhibited by statins, as suggested by a recent review of the available evidence by Undas et al.51 It has also recently been shown that the endogenous anticoagulant protein C pathway might be stimulated by statins, shifting the balance away from thrombogenesis in the vasculature.55

New and potentially beneficial pleiotropic effects of statins are continually being revealed. Additional benefits with relevance to stroke prevention include increased parasympathetic tone, down-regulation of angiotensin II type-1 receptors, and immunomodulatory effects.35,56-58 These developments further strengthen the theoretical and mechanistic case for statins in secondary prevention.

STATINS AND RECURRENT STROKE PREVENTION

It has been estimated that between 20% and 30% of patients admitted to the hospital for acute ischemic stroke have a history of, or evidence for, coronary artery disease.59 Many believe that this knowledge alone is a sufficiently compelling circumstance to consider statin therapy in all of these patients. In addition, national guidelines published in the 1990s promoted the consideration of ischemic stroke as a “coronary equivalent,” necessitating a target LDL of less than 100 mg/dL.60 It was not until the Heart Protection Study (HPS) was published in 2002, however, that evidence existed in stroke patients to support this claim.16 In this study of more than 20,000 patients with a high risk of vascular events (primary and secondary prevention), simvastatin 40 mg daily reduced the risk of stroke by 25%, which was similar to other studies in primary and secondary prevention of cardiovascular disease. In the more than 3000 patients who had ischemic stroke as a qualifying event in the HPS, recurrent vascular events and vascular death (composite endpoint) were significantly reduced (20% relative risk reduction). This occurred despite the fact that no reduction in recurrent stroke was found in the HPS (10.4% vs 10.5% in statin vs placebo groups, respectively). The HPS led to a measurable change in prescription of statin therapy for secondary prevention in stroke patients,61 and national groups published advisories for placing all ischemic stroke patients on statin therapy.62 Although prescribing practice changed to reflect increased use of statins in ischemic stroke patients with and without coronary artery disease, there was still doubt as to whether statins were effective in the secondary prevention of stroke.1

In the Stroke Prevention by Aggressive Reduction in Cholesterol Levels (SPARCL) trial, high dose statin therapy (atorvastatin 80 mg daily) was studied for the first time for its ability to reduce recurrent stroke.4 In a conservatively powered trial of almost 4800 patients with acute stroke or TIA and no known coronary heart disease, patients were randomized to the statin or placebo and followed for 5 years. The primary endpoint of the study (nonfatal or fatal stroke) was significantly reduced by 16% in the statin group (11.2% vs 13.1% for placebo at 5 years) (see Figure 1). In addition to the primary endpoint, the reduction in coronary events in this population was impressive. There was a 42% reduction in “any coronary event” (5.2% vs 8.6% for placebo) and a 49% reduction in nonfatal myocardial infarction (1.8% vs 3.5% for placebo). As expected, these results were accompanied by a large reduction of mean LDL in the treated group (129

Figure 1. Stroke Prevention by Aggressive Reduction in Cholesterol Levels (SPARCL) study 5-year outcomes. From left to right, all stroke (stroke), any coronary event (ACS), nonfatal myocardial infarction (MI), and intracerebral hemorrhage (ICH). *Difference from placebo statistically significant.
mg/dL vs 73 mg/dL in placebo vs statin, respectively). The aggressive statin therapy was well tolerated in this population with no increase in serious adverse effects, including rhabdomyolysis. There were significantly more patients in the statin group that had persistent elevations in liver enzymes (2.2% vs 0.5%), but there were no cases of liver failure.

One issue of some concern that was raised in the SPARCL results was a significant increase in the incidence of intracerebral hemorrhage (ICH) in the patients treated with atorvastatin. There were 55 (2.3%) patients with at least 1 hemorrhagic stroke in the treated group compared to 33 (1.4%) in the placebo group. This resurrected the concern that reducing the LDL too aggressively may cause intracranial bleeding. However, LDL reduction was much less for HPS subjects taking simvastatin 40 mg daily, subgroup analysis also uncovered an increase (albeit low) in the risk of ICH in the stroke patients (1.3% vs 0.7%). In regards to the SPARCL study, concerns were mitigated by subgroup analysis which revealed that there was no increase in fatal ICH in the atorvastatin-treated patients, suggesting that the ICH seen may have been of a less severe phenotype. Despite the increase in ICH, total stroke was decreased with statin therapy in the SPARCL trial due to the robust reduction in ischemic stroke. An important detail of the SPARCL trial is that the qualifying event could be either ICH or ischemic stroke, and approximately 2% of the patients in each group were ICH patients.

CONCLUSIONS

Statin therapy has been shown to profoundly and significantly reduce the occurrence of stroke in a wide range of patients at risk. First demonstrated in patients with hyperlipidemia and cardiovascular disease, the practice of aggressively introducing statin therapy to ischemic stroke patients slightly preceded the existence of strong secondary stroke prevention data. With the publication of the SPARCL data, there is no doubt that statin therapy is an important secondary prevention strategy for ischemic stroke patients. Although most believe the pharmacologic effect is a class effect of all statin compounds, it is possible that aggressive use of high potency statins (eg, atorvastatin 80 mg daily) may be necessary to achieve the maximum LDL lowering and pleiotropic effects. Future secondary stroke prevention guidelines will likely include a strong recommendation for statin therapy after ischemic stroke.

REFERENCES

STATIN THERAPY FOR SECONDARY STROKE PREVENTION


