Published in final edited form as:

Curr Neurovasc Res. 2012 August ; 9(3): 207–213.

Short-term High-Dose Effect of Lovastatin on Thrombolysis by rt-PA in a Human Whole-Blood *in vitro* Clot Model

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Abstract

High-dose hydroxymethylglutaryl coenzyme A reductase inhibitor (statin) administration reduces neuronal injury and improves outcomes in experimental models of acute ischemic stroke, and has been shown to be safe in a phase 1 dose-escalation study using lovastatin at doses higher than currently approved for daily use. Statins also affect the hemostatic system by upregulating t-PA expression and decreasing plasminogen activator inhibitor (PAI-1) expression, platelet adhesion and thrombus formation in animal models. Since a thrombolytic agent, recombinant tissue plasminogen activator (rt-PA), is currently the only FDA-approved therapy for use in ischemic stroke patients, it is important to ascertain whether high statin doses impact the efficacy of rt-PA. The main goal of this study was to evaluate the effect of a high dose of lovastatin and its active form, lovastatin hydroxy acid, on rt-PA thrombolysis in an *in vitro* model.

Percentage clot lysis was measured in the presence and absence of rt-PA in three different treatment groups: lovastatin, lovastatin hydroxy acid, and ethanol. The effect of ethanol on clot lysis was studied since ethanol was used to disperse the highly hydrophobic lovastatin. The decrease in clot width over time was measured using microscopic imaging of an *in vitro* human whole blood clot model; an approximately 400 μ m diameter clot was formed on suture silk, suspended in human fresh frozen plasma (hFFP) and exposed to treatment.

In the absence of rt-PA, clot lysis did not show statistically significant differences in the percentage clot lysis between different treatment groups ($p=0.103$). In the presence of rt-PA, clot lysis was greater than in the absence of rt-PA for all groups, but there were no statistically significant differences between treatment groups ($p=0.385$).

In this *in vitro* study, high doses of lovastatin neither impaired nor enhanced the lytic efficacy of rt-PA.

Keywords

Acute Ischemic Stroke; human blood clot model; lovastatin; neuroprotection; rt-PA; thrombolysis

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CONFLICTS OF INTEREST

No conflicts of interest.

INTRODUCTION

Statins are used to lower low-density lipoprotein and total cholesterol in individuals at risk for coronary artery disease and stroke. Statins decrease cholesterol production by blocking the enzyme hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase, which is responsible for conversion of HMG-CoA to mevalonate, the first and rate limiting step in the cholesterol synthesis pathway [1–4]. Lovastatin, like other statins, is a lactone prodrug with high hepatoselectivity attributed to its high lipophilicity. It is absorbed in hepatocytes through passive diffusion and metabolized to its active hydroxy form, lovastatin hydroxy acid [5].

Clinical trials have shown that statins reduce the risk of stroke in patients with a history of coronary artery disease, and improve outcome and reduce mortality after ischemic stroke, independent of their cholesterol lowering effects [6–12]. The AF/TexCAPS primary prevention trial showed a 32% decrease in first cardiac events in patients on lovastatin [13].

The benefits of statins to vascular processes in cardiovascular disease and stroke are widely attributed to both cholesterol-dependent and cholesterol-independent, pleiotropic effects. These pleiotropic effects reflect amelioration of endothelial dysfunction by several mechanisms, reviewed in references [14–21]. Briefly, these include, enhanced NO bioavailability and upregulation of endothelial nitric oxide synthase (eNOS), mainly via inhibition of Rho protein prenylation in the mevalonate pathway [22, 23]. Statins decrease production of superoxide anion via downregulation of angiotensin (AT)-II induced free radical production by Rac-I mediated NAD(P)H oxidase activity [24]. Oxidative stress due to reduction of NO by superoxide anions to form peroxynitrites [ONOO⁻] can cause cell damage and death and is a risk factor for atherosclerosis. Statins also interfere with vascular inflammatory processes, which start early in atherosclerosis and contribute significantly to disease progression. Statins diminish inflammation by lowering serum C-reactive protein (CRP) levels and downregulation of NFκB, mediated by Ras protein prenylation in the mevalonate pathway [18]. Statins also exert anti-inflammatory effects by repressing MHC II (major histocompatibility complex class II) mediated T cell activation, reducing proinflammatory TH1 lymphocytes and enhancing anti-inflammatory TH2 lymphocyte responses [25].

Statins may also affect clot formation and coagulation. Statins have been shown to decrease tissue factor (TF) mRNA expression, and thrombin generation and platelet activation, through reduced RhoA kinase activation [26, 27]. Undas *et al.* [28] assessed TF initiated coagulation in patients with advanced coronary artery disease and high cholesterol and found reduced blood clotting in the presence of statins related to inhibition of the activation of prothrombin, factor V and factor XIII, and an increased factor Va inactivation.

In animal models, statins have been shown to be both neuroprotective and neurorestorative in a dose dependent manner, related to the aforementioned pleiotropic effects [29–34]. In rodent stroke models, high doses of lovastatin decrease neuronal injury and infarct size and enhance neuronal recovery when administered within 24 hours of acute stroke [35, 36, 37]. Conversely, withdrawal of statins impaired vascular function in mice [38]. Based on these animal studies, the Neuroprotection with Statin Therapy for Acute Recovery Trial (NeuSTART) drug development program is testing the neuroprotective effect of high-dose lovastatin therapy after acute ischemic stroke. In a Phase 1 dose-escalation and safety trial, lovastatin at doses up to 8 times higher than those recommended by the FDA for chronic treatment of hyperlipidemia were safe when administered within 24 hours to patients with acute ischemic stroke [39]. No clinically significant muscle or liver disease was observed and a maximum dose of 8 mg/kg/day was found to be tolerated for 3 days after stroke. In

other clinical trials, withdrawal of statin therapy in patients already taking statins at the time of stroke worsened outcome and increased risk of death and disability, early neurologic deterioration, and infarct volumes [19, 40,41].

Despite the potential emerging role of statins in acute stroke therapy, there is little data on the effect of high doses of statins on the lytic efficacy of recombinant tissue plasminogen activator (rt-PA). As intravenous administration of rt-PA is the only FDA approved therapy for acute ischemic stroke, it is essential to determine the effects of lovastatin on rt-PA-mediated thrombolysis to determine the safety and efficacy of combined therapy with statins and thrombolytics in acute stroke.

There are theoretical reasons to be concerned about the potential effects of lovastatin on thrombolysis and clinical complications of thrombolysis after acute stroke as well. In particular, anti-thrombotic effects of statins might lead to an increased risk of hemorrhagic conversion of cerebral infarcts. There is evidence, for example, that atorvastatin and fluvastatin increase tPA and reduce tPA-inhibitor in endothelial cells [42]. Statins also increase fibrinolysis [43]. Decreased platelet activation and thrombus formation is postulated as one of the mechanisms through which atorvastatin exerts a cerebroprotective effect in experimental stroke [44].

Experiments in rodent models also provide evidence that statins and thrombolytic therapy can be administered together without an increased risk of hemorrhage [37, 45, 46]. However, whether the combination of short-term high-dose statin therapy with rt-PA after acute stroke is safe in humans remains largely unexplored. Findings of an increased risk of hemorrhage among patients randomized to atorvastatin 80 mg daily after stroke in the Stroke Prevention by Aggressive Reduction in Cholesterol Levels (SPARCL) trial do not necessarily provide evidence of an increase in risk of hemorrhagic conversion after acute stroke, especially since treatment in the SPARCL trial was continued for several years [12]. In the NeuSTART phase 1 study, there was no apparent effect of high dose lovastatin on platelet function as assessed by sophisticated platelet aggregation assays [39].

The main goal of this study was to evaluate the effect of lovastatin and lovastatin hydroxyl acid on the lytic efficacy of rt-PA in an *in vitro* human whole-blood clot model. The results of this study could inform research on post-stroke neuroprotection with statins and also provide insight into the potential interaction of rt-PA with chronic statin therapy.

EXPERIMENTAL SECTION

Materials

A 10 mg/ml stock solution of lovastatin (U. S. Pharmacopeia, Rockville, Maryland) in ethanol (ACS Reagent, 99.5%, Acros Organics, New Jersey) was prepared at room temperature. A 10 mg/ml stock solution of lovastatin hydroxy acid sodium salt (Cayman Chemical Company Ann Arbor, Michigan) was prepared by dissolving it in nanopure water. The stock solutions were stored at -20°C when not in use.

Lovastatin and lovastatin hydroxy acid were used at high concentrations of $24\text{ }\mu\text{g/mL}$. This concentration is significantly higher than that recommended by the FDA (10–80 mg/day) [39]. Experimental concentrations of $24\text{ }\mu\text{g/mL}$ were achieved by adding $10\text{ }\mu\text{l}$ of the stock solutions to 4.2 mls of human fresh frozen plasma (hFFP).

The rt-PA was obtained from the manufacturer (rt-PA, Activase[®], Genentech, San Francisco, CA) as a lyophilized powder. Each vial was mixed with sterile water to a concentration of 1 mg/ml as per manufacturer's instructions, aliquoted into 1.0 ml centrifuge tubes (Model 05-408-13, Fisher Scientific Research, Pittsburgh, PA), and stored at -80°C .

The enzymatic activity of rt-PA is stable for years when stored in this fashion [47, 48]. rt-PA was used at a therapeutic concentration of 1 $\mu\text{g/mL}$ [49].

ϵ -Aminocaproic acid (EACA) was purchased from Sigma Aldrich (St. Louis, Missouri, CAS number 60-32-2). A 130 $\mu\text{g/mL}$ solution of EACA was prepared in human fresh frozen plasma with 1 $\mu\text{g/mL}$ of rt-PA. The concentration of EACA was chosen based on literature measurements of steady state plasma concentrations of EACA in human adults [50,51],

HFFP was procured from a blood bank in 250–300 ml units. Each unit was briefly thawed and four were mixed to ensure a representative average sample of FFP for these experiments. These mixed units were then aliquoted into 50 ml polypropylene centrifuge tubes (Model 05-538-68, Fisher Scientific Research, Pittsburgh, PA), and stored at -80°C . Aliquots of rt-PA and plasma were allowed to thaw at room temperature for experiment.

Treatment Groups

All clot lysis experiments were conducted in hFFP. Lovastatin, a hydrophobic drug, was dispersed in ethanol prior to adding it to hFFP. Hence, additional experiments were carried out to determine whether ethanol alone contributes to clot lysis in the presence and absence of rt-PA. EACA added to rt-PA treated plasma was used as positive control. EACA is a lysine analogue, which inhibits rt-PA based thrombolysis by competitive inhibition of lysine binding sites of plasminogen and plasmin [52–55].

The clots were treated with lovastatin, lovastatin hydroxy acid, ethanol alone, or none of these in the presence and absence of rt-PA, and EACA with rt-PA, resulting in a total of 9 different treatment groups (Table 1).

Methods

Human whole blood was obtained from healthy volunteers by sterile venipuncture following local Institutional Review Board approval and written informed consent. The only exclusion criterion was the use of aspirin within 72 hours of the blood draw. Samples of 1–2 ml were placed in sterile glass tubes (3ml, VWR, Batavia, IL) and allowed to form clots in and around a small diameter (~ 1 mm) micropipette (World Precision Instruments Inc., Sarasota, Florida; TW 150-6) through which a segment of 7-0 silk suture (Ashaway Line and Twine Manufacturing Co., Ashaway, Rhode Island) had been threaded. This is similar to clot production methods used in imaging studies by Winter *et al.* [56] and Yu *et al.* [57], and yields clots that are roughly 400 μm in diameter, dimensionally comparable to the brain microvasculature in which stroke-causing clots may form [58, 59]. The clots were incubated for three hours at 37°C , and refrigerated at 5°C for 3 days ensuring maximal clot retraction, lytic resistance and stability [60, 61]. Before each experiment, the micropipette was removed to produce a cylindrical clot adherent to the suture.

An Olympus IX-71 microscope (Olympus America Inc., Center Valley, Pennsylvania) equipped with a Charged Coupled Device (CCD) camera was used to take images of the clot, after placing the clot diagonally in a sample holder. A syringe was used to slowly inject 1 ml of medium (hFFP with or without rt-PA and various treatments) into the sample holder. Removing the syringe exposed the ends of the sample holder to atmospheric pressure, and the clot surface to a static fluid column. Clots were exposed to a specific treatment regimen for 33.33 minutes at 37°C ; previous studies have shown that the majority of thrombolysis occurs within a 30 minute time period [62, 63].

Image collection was started immediately after removing the syringe from the sample holder. Light intensity transmitted through the sample clot is reduced with increased clot thickness or clot density. The CCD camera records image light intensity $I(x,z)$ at each pixel

(x,z). By analyzing the light intensity in each pixel, the clot edges can be identified, thus enabling measurement of clot width. The fractional clot loss (FCL, expressed as %) is then defined as

$$\text{FCL} \equiv 1 - CW_{\text{NORM}}(30 \text{ min}) \quad (1)$$

where $CW_{\text{NORM}}(30 \text{ min})$ is the average normalized clot width over the final minute of treatment. Note that as a given sample clot is imaged 6 times per minute, there are 200 measurements for each clot trial. The overall experimental setup is illustrated in Fig. (1), and a full description of the apparatus and techniques can be found in Shaw *et al.* [64]

Statistical Analysis

Analysis of variance (ANOVA) was used to test for the global effect of treatment in the presence of rt-PA, and for the treatment effect in the absence of rt-PA. Analyses used SPSS v 20.0 (IBM Corporation, Armonk, NY).

Results

Table 1 shows the percentage decrease in clot widths with the corresponding standard deviations. Treatments in the absence of rt-PA, show significantly less clot lysis than treatments in the presence of rt-PA, as expected. Fig. (2) shows representative clot images taken over the course of lysis experiments (t=0 seconds (left) and at t=2000 seconds (right) of each panel) for control and lovastatin treated clots in the presence (+) or absence (–) of rt-PA. Debris from clot lysis in rt-PA treated clots can be clearly seen in Fig. (2B, D) at 2000 seconds. Collection of these clot lysis products near the bottom of the raicropipette in which the clot is placed may provide an illusion of clot expansion. However, a careful examination of the images reveals the actual edges of the lysed clots, which have a lower light intensity due to greater clot density compared to the clot lysis products.

The FCL were not found to differ significantly between treatment groups in the absence of rt-PA (p=0.103). The small amount of lysis observed may be attributed to the native rt-PA present in the hFFP for maintaining hemostasis. In the presence of rt-PA, the decrease in clot width did not differ between conditions (Fig. (3); p=0.385). Given the lack of any overall treatment effect, *post hoc* testing comparing conditions was not conducted.

Thrombolysis by rt-PA was suppressed in the presence of EACA. FCL in the presence of EACA and rt-PA combined (mean±SD=1.38±1.41) showed no overall difference from other control treatments in the absence of rt-PA (p=0.266). Compared to treatments where rt-PA was present, the FCL in the presence of EACA and rt-PA combined was significantly lower (p<0.001). Thus, rt-PA activity is effectively inhibited in the presence of EACA, and serves as the positive control for this study.

DISCUSSION

In an *in vitro* clot lysis model, neither high-dose lovastatin nor its acid metabolite were found to have a statistically significant effect on thrombolysis by rt-PA. The experiments showed no evidence of either diminished or enhanced efficacy of rt-PA on thrombolysis in the presence of lovastatin. Based on these results, we expect that coadministration of high-dose lovastatin will neither inhibit nor enhance efficacy of rt-PA in patients receiving intravenous rt-PA for acute ischemic stroke. We also did not find evidence for altered efficacy of rt-PA to patients receiving chronic lovastatin at the time of their stroke. Human trials using clinical endpoints, including intracerebral hemorrhage and hemorrhagic

conversion of infarction will be needed to confirm these findings, however. These studies are planned as part of the NeuSTART drug development program.

The clot lysis model described above has been used to determine the efficacy of co-administration of other medications with rt-PA. Meunier *et al.* have explored combinations of rt-PA with plasminogen, eptifibatide or ultrasound (120kHz and 2MHz) [65–67]. They compared outcomes of several human clinical trials of combination eptifibatide and rt-PA treatment in stroke patients [68]. Interestingly, the predicted concentrations of rt-PA and eptifibatide in trials which showed increased clinical efficacy overlapped with concentrations for maximal lysis *in vitro*, indicating the potential of this model to predict the outcome of clinical trials.

The strengths of our model include the use of *de novo* human whole blood clots, avoiding complications arising from use of citrated human blood or animal blood. The use of hFFP at physiological temperatures to accurately mimics *in vivo* conditions. The concentration of rt-PA used is comparable to concentrations found to be efficient in stroke management. Finally, the clots are modeled to be dimensionally similar to clots in the brain microvasculature where stroke causing clots may form [58, 59].

Despite these strengths, our results should be interpreted with consideration of several limitations. Our system is static in that it is not subjected to blood flow as would be the case *in vivo*. The clots were allowed to retract for 3 days, and as a result are more compact and resistant to lysis than thrombi formed in acute stroke and thus we may be underestimating the lytic efficacy [60, 61]. The only exclusion criteria used in the selection of donors was the use of aspirin; hence, our results may not account for the effects of age, diet, or other blood diseases on thrombolysis. In addition, we only tested lovastatin and not other statins. Lovastatin was chosen because it is being tested in NeuSTART. We cannot say with certainty, however, that our findings are generalizable to other statins.

CONCLUSIONS

Our *in vitro* study did not find any evidence for an effect of lovastatin and lovastatin hydroxy acid on the lytic efficacy of rt-PA. *In vivo* studies are warranted to firmly establish the effects of lovastatin on the hemostatic system, and the corresponding effect on clot lysis by rt-PA.

Acknowledgments

The authors would like to acknowledge funding from NIH/NINDS (P50 NS049060).

FINANCIAL SUPPORT

This work was supported by NIH/NINDS (P50 NS049060).

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Fig. 1.
Experimental set-up for clot-on-a-string model *in vitro* experiments.

Fig. 2. CCD images of control (panels **A**, **B**) and lovastatin (panels **C**, **D**) treated clots in the absence (panels **A**, **C**) or presence (panels **B**, **D**) of rt-PA taken at t=0 seconds (left side of each panel) and t=2000 seconds (right side of each panel) after addition of treatment. Axes represent x and y coordinates in microns.

Fig. 3. Violin plots showing percentage fractional clot lyses of human whole blood *in vitro* clot model suspended in hFFP with different treatment groups in the presence and absence of rt-PA.

Table 1

Experimental Parameters and Fractional Clot Loss.

Treatment	Number of Clots	Number of Donors	Mean $\pm$ SD % Clot Loss
Control (-)	15	4	1.33 $\pm$ 0.99
Control (+)	16	4	10.43 $\pm$ 3.96
Lovastatin (-)	9	3	1.83 $\pm$ 1.15
Lovastatin (+)	11	4	10.42 $\pm$ 4.44
Lovastatin Hydroxy Acid (-)	11	4	0.91 $\pm$ 0.87
Lovastatin Hydroxy Acid (+)	11	4	13.03 $\pm$ 5.03
Ethanol (-)	13	3	0.86 $\pm$ 0.68
Ethanol (+)	8	3	11.29 $\pm$ 2.86
EACA (+)	16	3	1.38 $\pm$ 1.41

(-) Indicates Absence of rt-PA; (+) Indicates Presence of rt-PA. All Experiments Were Conducted in hFFP at 37°C