

ISOFORMS OF APOLIPOPROTEIN E CAN MODULATE TPA-INDUCED CLOT LYSIS *IN VITRO*

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1. ABSTRACT

Apo E, and its respective isoforms, have been linked to outcome and survival in cerebral vascular and cardiovascular diseases. The effectiveness of intravenous tPA in patients with acute ischemic stroke appears to be enhanced in patients who have an Apo E2 phenotype. The ability of Apo E isoproteins (endogenous Apo E isoproteins or exogenous Apo E isoproteins) to modulate tPA-induced clot lysis *in vitro* was assessed using an *in vitro* clot assay system. Blood samples were obtained from 18-volunteers with three Apo E genotypes: E2, E3 and E4. tPA-induced clot lysis (0-4 µg/ml tPA), was assessed in the presence or absence of supplemental Apo E2, E3 or E4 (9.8 µg/ml). tPA-induced clot lysis was significantly ($P \leq 0.0001$) enhanced by supplementation with Apo E2 ($EC_{50} 0.20 \pm 0.06$ µg/ml) as compared to tPA alone (0.72 ± 0.19). Apo E4 supplementation caused a significant ($P \leq 0.05$) inhibition of clot lysis (0.98 ± 0.23), but there was no significant change caused by Apo E3. The genotype of the volunteer did not significantly affect the ability of the supplemental Apo E from modulating tPA-induced clot

lysis. We conclude that the administration of Apo E isoproteins can modulate clot lysis *in vitro*. Our results suggest that the Apo E isoprotein may have an impact on clot dissolution and the effectiveness of thrombolytic therapy.

2. INTRODUCTION

Apolipoprotein E (Apo E) is a member of a family of lipid-associated proteins whose isoforms have been implicated as an important modifier of several neurologic, vascular and cardiovascular diseases (1, 2, 3, 4). Apo E has three common isoforms - E2, E3, and E4 (1, 3, 4). These monomeric isoforms combine to make six Apo E phenotypes: E2/2, E2/3, E2/4, E3/3, E3/4 and E4/4 (5). The respective isoforms of Apo E have been linked to outcome and survival following acute injury of the central nervous system, incidence or emergence of Alzheimer's disease and a risk factor for cardiovascular disease (1, 5, 6, 7, 8). The presence of an E4 allele has been associated with

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a poor outcome following severe head trauma (9, 10, 11) and with poor survival rates in patients with an intracerebral hemorrhage (ICH) (12).

We have previously reported that the efficacy of intravenous tissue plasminogen activator (tPA) in patients with acute ischemic stroke is enhanced in patients who have an Apo E2 phenotype relative to the Apo E3 and E4 phenotype (5). tPA is an endogenous protein involved in clot degradation, and is found naturally in the blood and in various tissues (13). Although the effectiveness of intravenous tPA in the acute ischemic stroke patient was enhanced in those patients who had an Apo E2 phenotype, the Apo E2 phenotype alone was not associated with a detectable benefit in stroke outcome at 3 months in patients *not* given tPA. Our previous study has suggested a correlative relationship between Apo E and tPA-induced thrombolysis (5), but more work is needed to elucidate whether there is a causal relationship between Apo E genotype, Apo E isoproteins and tPA activity.

Administration of tPA within 3 hours of symptom onset of ischemic stroke is a beneficial therapy for a select population of stroke patients (14, 15, 16, 17). Any treatment capable of increasing tPA's ability to lyse clots (without increasing the risk of hemorrhage (18, 19, 20, 21) should increase its utility and efficacy. Therefore, an enhancement or modulation of tPA therapy could be used as an adjunct/combinational therapy for patients with thromboembolic events. In this paper we have assessed the ability of the Apo E genotype of volunteers to modulate tPA-induced thrombolysis as well as the ability of exogenously added Apo E isoproteins to modulate thrombolysis from the same volunteers. Moreover, we have begun to examine whether Apo E could modulate tPA activity via a direct interaction between the proteins. The data from this study suggests that Apo E supplementation to freshly withdrawn human blood (without anticoagulant) modulates tPA-induced thrombolysis and that this modulation is independent of the genotype of the volunteers' blood. We also provide data that suggests that protein-protein interaction between Apo E2 and E4 can occur and might modulate tPA activity. The results suggest that exogenous Apo E2 will enhance tPA-induced thrombolysis and that exogenous Apo E4 can impede tPA.

3. MATERIALS AND METHODS

Recruitment procedure and technique. Following review and approval of the study's protocol and informed consent statement by the University of Cincinnati Institutional Review Board, 50 consented volunteers, over the age of 18 years, were initially screened for specific serum Apo E isoforms by buccal cell swabbing. All volunteers were in good health and were not taking antiplatelet medications. To maintain subject confidentiality, a random study number was assigned to each volunteer by the study nurse coordinator. Only the study nurse coordinator had access to the volunteer identity and their corresponding study number. During the experiments, subjects and experimenters were blinded to Apo E profiles.

Following the determination of each of the 50 subject's specific Apo E genotype, 9 males and 9 females

were contacted by the nurse coordinator to schedule the collection of their blood samples in red top vacutainer tubes using standard sterile technique.

Only after all 18 volunteers were assayed and the data compiled were the genotypes decoded. Decoding was done for genotype only and not for patient identification. Therefore, only the nurse coordinator has the record of patient identity and Apo E genotype.

3.1. Materials

All chemicals and materials were from Fisher Scientific except where indicated. Chemicals were reagent grade.

Apo E genotyping. Buccal samples were used to determine the specific Apo E isoform in the serum of a given volunteer as described previously (5, 22). We screened 50 volunteers and determined their Apo E phenotype. From these we chose 18 people with genotype categories of the following groups: 1) Apo E2/E2, E2/E3, or E2/E4; 2) Apo E3/E3; or 3) Apo E3/E4 or E4/E4. The designations for the Apo E genotype categories were described in our previous paper (5). This genotyping of the groups was necessary because some of the dimer genotypes are relatively rare. For this study, we are assessing the endogenous isoprotein gene product for the ability to modulate thrombolysis.

3.2. Clot lysis

A measure of *in vitro* clot dissolution/disruption was developed and utilized as described below. The weight of liquid liberated from the blood sample was measured to assess tPA (Activase ® Genentech) induced clot lysis. The ability of Apo E isoproteins to modulate tPA-induced clot lysis was assessed using an *in vitro* clot assay system. This system uses the increase in liquid liberated from the clot, in the presence of tPA to approximate the amount of clot lysis. In the absence of exogenous tPA the freshly obtained blood will completely form a clot where no detectable liquid can be obtained.

Two ml of fresh human blood was drawn and added to pre-weighed vials containing known amounts of tPA (0, 0.04, 0.4, 2 and 4 μ g/ml) with or without Apo E2, E3 or E4 (9.8 μ g/ml) (purchased from Pan Vera). The amount of Apo E was chosen as a result of a set of dose response curves (data not shown and (23)). The stock carrier solution (Pan Vera) contains 0.7 M ammonium bicarbonate. After dilution into the blood samples, the final concentration of ammonium bicarbonate in all blood samples was 17 mM. In other control experiments, 17 mM ammonium bicarbonate alone had no detectable effect on clot formation (data not shown). The results are expressed as EC₅₀s, which are the effective concentrations of tPA required to achieve 50% liberation of the clot as liquid, which is a reflection of clot disruption or dissolution. For this reason, we refer to these measurements as an assessment of clot dissolution/disruption.

The blood, tPA and Apo E were mixed and allowed to equilibrate for 40 minutes at 22°C. The vials

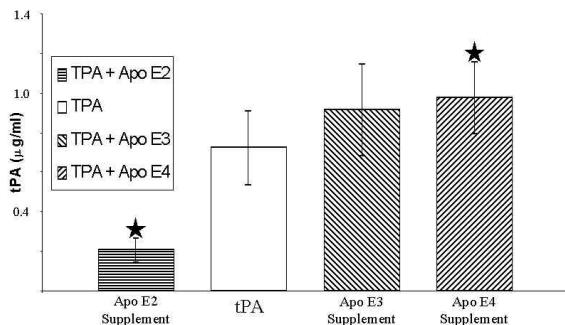


Figure 1. Average EC₅₀ clot dissolution from 18 subjects. Here we present the effective concentration of tPA which can degrade 50% of the clot. These data were obtained using a dose response curve (data not shown). It is evident that the Apo E2 significantly enhances tPA-induced clot lysis. Interestingly, the Apo E4 causes a small but significant decrease in clot lysis and Apo E3 has no significant effect.

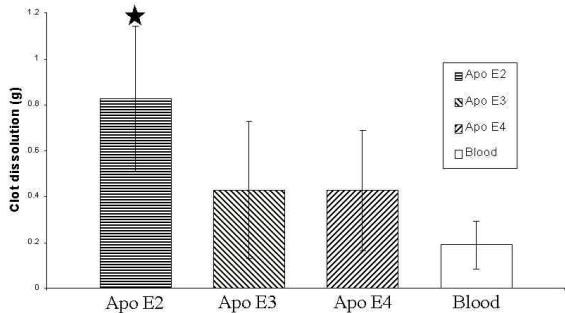


Figure 2. Apo E with blood (no tPA added). When one compares the initial clot lysis or dissolution/disruption caused by Apo E supplementation we see that Apo E2 has a small but significant increase in clot dissolution/disruption compared to blood alone. Apo E3 and E4 have no significant change compared to blood. Note, there is no tPA added to these samples therefore, these results suggest that Apo E2 may enhance endogenous tPA activity to decrease clot formation.

were then centrifuged at 1000g for 15 minutes (4°C) and the supernatant decanted. Clot weight and the decanted solution weight were used to determine clot dissolution/disruption caused by tPA activity. Increasing supernatant weights indicates increased clot dissolution/disruption.

The control blood clot measurements for these experiments are the individual patient's blood with (and without) tPA, and in the presence or absence of Apo E supplementation. Equal volumes of the carrier solutions were used for the control clot lysis measurements. The same amount of Apo E was added to each volunteers' blood.

3.3. Thin layer chromatography (TLC)

Thin layer chromatography is a method for separating molecules, but it can also assess whether

molecules can interact. The methods used here were designed to determine whether Apo E and tPA might interact. Known concentrations of tPA (or tPA carrier solution alone) were applied to the TLC plate and allowed to dry. Following tPA application to the TLC plates, we also applied the respective isoforms of Apo E2, E3 or E4 (or Apo E carrier solution alone). Once all applications were dry, the TLC plates placed in the migration chambers.

The TLC plates were placed into the glass migration chambers containing chloroform:ethanol in a 3:1, 1:1 or 1:3 ratio and allowed to migrate until the solvent front approached 1 cm from the top of the plate. The plates were removed from the chambers and allowed to dry. The TLC plates were then developed and visualized with ninhydrin. The migration distances were marked and rf values (migration distance/solvent front) reported.

3.4. Statistics

Analysis of variance was used to determine statistical differences. A 95% confidence was considered statistically significant.

4. RESULTS

Clot measurements. The ability of Apo E isoproteins to alter tPA-induced clot lysis was assessed using the clot assay system described above. Blood samples were obtained from 18 volunteers and divided into three Apo E genotypes (6 patients in each group). Clot lysis in the presence of varying concentrations of tPA was assessed in the presence or absence of supplemental Apo E2, E3 or E4 for each patient genotype.

Based on our previous work (5), we included two Apo E2/E4 phenotype volunteers in the E2 group. This was supported by the observation that there was no significant difference between the E2/E2 and E2/E3 groups compared to E2/E4 (data not shown). Moreover, this classification is validated because our data, shown below, showed no significant difference in clot dissolution caused by endogenous Apo E genotypes.

In Figure 1 we see that there is a significant increase in clot lysis caused by Apo E2 supplementation and a decrease in clot lysis with Apo E4 supplementation. tPA-induced clot lysis was significantly ($P \leq 0.0001$) enhanced by supplementation with Apo E2 (EC₅₀ of $0.20 \pm 0.06 \mu\text{g/ml}$) as compared to tPA alone ($0.72 \pm 0.19 \mu\text{g/ml}$). When Apo E4 was supplemented to the assay there was a significant ($P \leq 0.05$) inhibition of clot lysis (EC₅₀ of $0.98 \pm 0.23 \mu\text{g/ml}$) as compared to tPA alone ($0.72 \pm 0.19 \mu\text{g/ml}$). There was no significant change in tPA-induced clot lysis caused by Apo E3 supplementation.

Clot dissolution in the absence of exogenous tPA. In Figure 2 the relative clot dissolution produced by Apo E supplementation in the absence of tPA is seen. There is a significant increase in clot dissolution caused by Apo E2 and no significant clot dissolution caused by Apo E3 and E4. The control for this figure is 'blood' which is the blood clot in the absence of tPA and Apo E.

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Table 1. EC₅₀s of the Volunteer's Genotypes

	Apo E2 Genotype ¹	Apo E3 Genotype	Apo E4 Genotype
tPA Alone	0.77±0.42	0.71 ±0.41	0.67±0.36
tPA + Apo E2 Supplementation	0.20±0.088	0.25±0.13	0.15±0.013
tPA + Apo E3 Supplementation	1.08±0.22	1.00±0.60	0.67±0.45
tPA + Apo E4 Supplementation	1.10±0.11	1.00±0.52	0.83±0.35

¹ There were 2 volunteers with the E2/E4 phenotype included in these data. When the results from those two volunteers were discarded the resulting EC₅₀s were as follows: tPA alone; 0.58±0.46, E2 supplementation; 0.21±0.11, E3 supplementation; 1.07±0.28, E4 supplementation; 1.05±0.1 (μg/ml). Errors are SD. There is an N of 6 for each group. The units are μg tPA/ml.

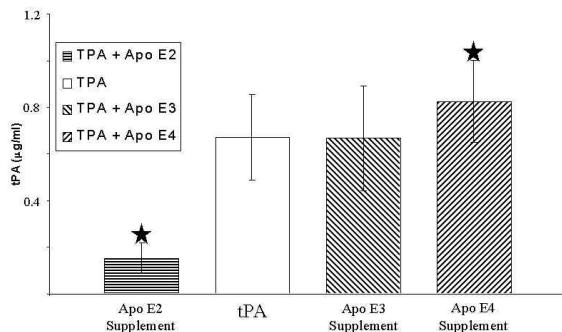


Figure 3. Average EC₅₀ Clot Dissolution Apo E4 Genotype (6 Subjects). In this figure we see that the Apo E4 genotype patients have a significant increase in clot lysis with supplementation of E2. There is no significant difference in the clot degradation in the presence of E3. For this patient population E4 supplementation does not seem to significantly affect the native E4 that is present.

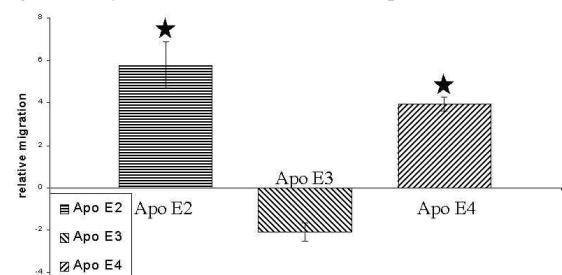


Figure 4. Relative change in migration on TLC of tPA caused by Apo E. In this figure we see that the results of TLC experiments designed to determine if Apo E and tPA could interact. Here we see that Apo E2 and tPA migrated farther when they were together. Importantly so did Apo E4. This migration was enhanced in a hydrophylic environment and suggests that they may both be interacting via hydrophobic interactions. Apo E3 however migrated a shorter distance. This can be interpreted in two ways. One is that there is no interaction and the spotting change was due to random interactions changing migration. The second is that there is specific interaction, but importantly, the interaction is distinctly different than the interaction seen with E2 and E4. N = 3 per isoform.

Volunteers' Apo E genotypes and thrombolysis. When we examined the relationship between patient genotypes (Figure 3 and Table 1) and clot lysis there was a significant increase in clot lysis for all volunteer genotype groups with Apo E2 supplementation (Figure 3). Therefore, supplementation with exogenous Apo E was able to modulate thrombolysis independent of the genotype of the

volunteer's blood. When we compared the volunteers' blood clotting responses according to genotype, there was no significant difference in how the genotype responded to Apo E supplementation (Table 1).

TLC. In Figure 4 there is a relative change in migration (rf value) caused by Apo E supplementation when normalized to the migration of tPA. Apo E2 and E4 caused the tPA spot to migrate significantly farther compared to tPA alone, while Apo E3 impeded the migration of tPA. Apo E2 and E4 increased the tPA migration by similar amounts, suggesting a direct interaction of each isoprotein with tPA.

5. DISCUSSION

In this paper we present experimental evidence that suggests that supplemental Apo E can modulate tPA-induced clot lysis. The mechanism for this effect is not yet clear, but the utility in using the Apo E proteins for modulating thromboembolic events could be very important in treating patients with cerebral vascular, cardiovascular and peripheral vascular diseases. Therefore the potential exists for developing Apo E as a modality for treating some of these patients.

We believe that these data strongly suggest that *in vitro* apolipoprotein E isoproteins impact on tPA-induced clot lysis. This *in vitro* data could be used to enhance the efficacy of tPA therapy or even to decrease detrimental side effects associated with tPA administration.

However, we were not able to demonstrate a significant change in tPA-induced clot lysis by endogenous Apo E (the Apo E genotype of the volunteers). Therefore, we cannot come to a conclusion on the role of the Apo E genotype in altering tPA outcomes (5), but we do believe that exogenous Apo E administration may provide insights into methods for modulating tPA-induced thrombolysis.

When we measure the change in clot weight we believe we are measuring the change in tPA-induced clot lysis. This is because longer incubation times with tPA result in more clot lysis (data not shown). Therefore, when we measure the clot and clot supernatant this is a reflection of competing clot formation and degradation processes, which is the normal physiological situation in the blood stream of patients. tPA administration shifts this process to favor clot lysis and Apo E2 enhances the tPA-induced clot lysis, while Apo E4 inhibits the tPA-induced clot degradation.

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In recent studies on the relationship between outcome following head trauma and Apo E4 genotype, the Glasgow Coma Scale (GCS) score at presentation to the hospital, which is the most important predictor of outcome in patients with head trauma, were substantially lower in patients with an Apo E4 allele (9, 10, 11). The relationship between Apo E4 genotype and severe disability or death was only of borderline significance. Further, the authors found no difference in the likelihood of an unfavorable outcome (severe disability or death) in persons with and without an E4 allele. The authors hypothesize that the presence of the Apo E4 allele resulted in impaired neuronal sprouting and poorer reorganization following brain injury (9, 10, 11).

A relationship between other apolipoproteins and fibrinolysis has been reported (24, 25, 26). Apolipoprotein A isoforms are known to modify fibrinolysis *in vitro* and *in vivo* by binding to fibrin and competing with plasminogen for fibrin binding sites. In an *in vivo* model of clot lysis, Biemond and colleagues (24) found that high concentrations of Apo A incorporated into clots significantly reduced tPA-induced thrombolysis. Sangrar and colleagues have also demonstrated, *in vitro*, that Apo A attenuates tPA mediated plasminogen activation (26). We hypothesize that the Apo E2 isoform, like Apo A isoforms, may directly or indirectly modify the thrombolytic activity of tPA because of its binding properties (Figure 4).

If the Apo E2 isoform directly or indirectly modifies the effect of tPA in acute ischemic stroke or the brain's response to reperfusion, this might occur because of interaction with tPA (Figure 4), and is consistent with Apo E's unique binding properties (27, 28). The TLC data reported in this study support the possibility of Apo E2 and E4 interacting directly with tPA. This interaction may be similar for E2 and E4, but in the case of Apo E3, the interaction with tPA was distinctly different than E2 and E4. This similar interaction between Apo E2 and E4 with tPA does not, however obviate distinctly different activity changes. We suggest that Apo E2 and E4 could possibly bind to the same or close allosteric binding sites, but cause distinctly different conformational and or activity changes because of the structural difference between Apo E2 and E4. There is, however, a possibility that Apo E could be interacting with other components of the blood clotting system (29), though the mechanisms of such interactions do not diminish the importance of our results because the blood clot was being dissolved as observed by the increase in clot supernatant.

The effectiveness of intravenous tPA in patients with acute ischemic stroke was enhanced in those patients in the NINDS tPA Stroke Trial who had an Apo E2 phenotype. No such benefit for an Apo E2 phenotype was demonstrated in placebo patients, as measured either by clinical outcome or by CT-lesion volume at three months post stroke (5). We have proposed that the three most likely explanations for these observations are: 1) An Apo E2 phenotype (or another factor associated with an Apo E2 genotype) enhances thrombolysis in the presence of tPA, 2) An Apo E2 phenotype is associated with decreased

reperfusion injury, 3) these findings occurred by chance or represent an artifact due to imbalances in other variables (known or unknown) that may affect outcome following ischemic stroke (5). From the data reported in this paper (Figures 1 and 2), it is not yet possible to speculate on the role of endogenous Apo E in the effectiveness of tPA. But the exogenous application of Apo E2 or E4 might be useful in increasing or decreasing tPA-induced clot lysis for treating thromboembolic events.

Clot lysis by administration of tPA to patients is a beneficial therapy when administered within 3 hours following the onset of ischemic stroke, but tPA is not without risks (14, 18, 19, 20, 21, 30, 31, 32). A treatment that increases tPA's therapeutic effect (Apo E2's enhancement of thrombolysis) should increase tPA's utility and potency as a thrombolytic, assuming that there is no increased risk with such an adjunct therapy. Therefore an enhancement of tPA therapy may be exploited as an adjunct/combinational therapy. This therapeutic potential is true for vascular thrombosis dysfunction in any organ system. Moreover, it may be possible to develop a series of compounds based on Apo E2 fragments that will increase tPA's clot lysis inducing activity (and possibly enable one to give lower doses of tPA) and decrease activity with Apo E4. We suggest therefore, that Apo E2 and E4 (or rationally designed fragments thereof) may someday be used to modulate tPA's activity.

6. CONCLUSION

Our data suggest that tPA-induced thrombolysis can be enhanced with Apo E2 and impeded with Apo E4. It is possible that the interaction of tPA and E2 or E4 may be useful in modulating tPA-induced thrombolysis. More studies are required to assess the therapeutic potential of supplementation with exogenous Apo E2 and E4.

7. ACKNOWLEDGEMENTS

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Key Words: Apolipoprotein E, Thrombolysis, Clot, Blood vessel, Perfusion

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