Theaflavin digallate inactivates plasminogen activator inhibitor: Could tea help in Alzheimer's disease and obesity?

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Abstract. Proteolysis in general and particularly the serine proteases are causally involved in many physiological processes and different diseases. Recently it was reported that plasminogen activator inhibitor type one (PAI-1) inactivation can alleviate the symptoms of Alzheimer’s disease and reduce the body weight of obese individuals. In our broad search for natural compounds and their derivatives that can inhibit PAI-1, we include the polyphenols of teas since teas (green and black) or their components have been reported to alleviate the symptoms of both obesity and Alzheimer’s disease. Inactivation of PAI-1 was measured in human plasma using thromboelastography. We used known PAI-1 inhibitor PAI039 [{1-benzyl-5-[4-(trifluoromethoxy) phenyl]-1H-indol-3-yl}(oxo)acetic acid] as a positive control and (-)-epigallocatechin-3-gallate (EGCG), its prodrug octaacetate EGCG (OcAc EGCG) and theaflavin digallate [TH(2)] as potential PAI-1 inhibitors. We found that inactivation of PAI-1 in plasma by EGCG and OcAc EGCG was low or very low. However, TH(2) inactivated PAI-1 in a concentration-dependent manner with an IC₅₀ of 18 μM which is equal to or better than the IC₅₀ reported for known PAI-1 inhibitor PAI039. Clearly TH(2) inhibits PAI-1 and might play a role in slowing down the progression of Alzheimer’s disease or obesity by a PAI-1-dependent pathway. While the clinical value of TH(2) has not been proven, long-term prospective studies assessing its efficacy are warranted due to the benign nature of the substance.

Introduction

Proteolysis in general and particularly the serine proteases are primary targets in drug discovery and development (1-3). These enzymes are causally involved in many physiological processes and different diseases including cancer, inflammation and blood disorders (4,5). In certain diseases, proteases or their inhibitors are prognostic markers (6,7) as in the case of the plasminogen activation system and breast cancer (4). Also, overexpression or low expression of tissue and urokinase plasminogen activators (tPA and uPA) or their inhibitors (mostly plasminogen activator inhibitor type 1; PAI-1) was reported to be critical in diseases. For example, a high level of urokinase is considered as a marker of unfavorable prognosis in breast cancer, while low levels of the plasminogen activator inhibitor (PAI-1) lead to extended bleeding which in some cases can be life-threatening (4,8). On the other hand low levels of tPA and high levels of PAI-1 are reported to be involved in deep vein thrombosis (9).

Recently it has been reported that PAI-1 and tPA are implicated in Alzheimer’s disease. Evidence suggests that defects in amyloid peptide degradation are at the base of sporadic Alzheimer’s disease (AD). Among the enzymes which can degrade amyloid peptide, plasmin - the serine protease activated by tPA - is particularly relevant. Low levels of plasmin activity have been detected in areas of AD human brains with a high susceptibility to amyloid plaque accumulation (10). Other researchers have demonstrated that plasmin cleaves β-amyloid at certain sites and that exogenously added plasmin blocks β-amyloid neurotoxicity, supporting a physiological role for plasmin in this disease (11,12). Periz and Fortini suggested that β-amyloid levels are normally kept within physiological limits by regulated degradation involving a normal level of plasmin. However, high β-amyloid levels are able to overcome this system by inflammation-related tPA inhibition, consequently suppressing plasmin activity and promoting plaque formation (13). Cacquevel et al found that defective amyloid peptide degradation results, respectively, from a decrease in tPA expression and from an increase in the production of PAI-1 which further diminishes tPA-induced plasmin activity (14).
Thus inhibition of PAI-1 activity can protect residual tPA which results in the degradation of amyloid plaque.

The other promising therapeutic application of PAI-1 inhibitors is obesity. It was reported that in visceral and subcutaneous adipose tissue PAI-1 mRNA expression is positively correlated with body mass index (BMI). Similar results have been found in plasma where PAI-1 activity/antigen are positively and moderately associated with BMI. Furthermore, weight reduction substantially reduces plasma PAI-1 in obese humans (15,16). It has also been shown that inhibitors of PAI-1 attenuate diet-induced obesity in C57BL/6 mice. Specifically, it has been demonstrated in vivo that PAI-1 inhibition has a dose dependent effect on the reduction of body weight, adipocyte volume and circulating active PAI-1 in plasma (15,17). There is no clear mechanism how inhibition of PAI-1 can reduce body weight. However, Crandall et al postulate three possibilities. First, inactivation of PAI-1 may stimulate migration of preadipocytes which would prevent their full differentiation into mature fat cells. Alternatively, PAI-1 inhibition may block angiogenesis that weakens vascularization and consequently the growth of adipose tissue. Finally, PAI-1 may affect fat tissue growth by altering receptor-dependent transport of lipids into lipocytes (17).

Thus, management of PAI-1 activity can be therapeutically significant in different diseases. In our broad search for natural compounds and their derivatives that can inhibit PAI-1 we include the polyphenols of tea since tea or its components can alleviate the symptoms of both obesity and Alzheimer's disease (18-21). However, the mechanisms by which tea and epigallocatechin-3-gallate (EGCG) lower body fat and are neuroprotective in Alzheimer's disease are not completely understood. We hypothesize that PAI-1 is inhibited by tea polyphenolic compounds which alleviate the symptoms of these diseases by a PAI-1-dependent pathway.

Materials and methods

Pooled normal human plasma was collected in sodium citrate and was rapidly processed, buffered and freeze-dried in a manner to assure the stability of hemeostasis proteins (product no. 258N; American Diagnostica, Stamford, CT). All hemeostasis proteins were in the normal range. Lyophilized plasma was stored at 4°C, and the aliquoted plasma was reconstituted with 1.0 ml of filtered deionized water. The plasma was used for TEG assays by mixing 1 ml of plasma in 0.4 M HEPES, 0.1 M NaCl; pH 7.4) as a fibrinolytic agent constant amount of tPA was added [10 µl of tPA (2.1 mg/ml in 0.4 M HEPES and 0.1 M NaCl; pH 7.4) was added and incubated for 15 min at 37°C. Immediately after incubation, 3 µl of tPA inhibitor (2.5 mg/ml) in DMSO was added and incubated for 15 min at 37°C. Immediately after incubation, 3 µl of tPA inhibitor (2.5 mg/ml) in DMSO was added and incubated for 15 min at 37°C. Immediately after incubation, 3 µl of tPA inhibitor (2.5 mg/ml) in DMSO was added and incubated for 15 min at 37°C. Immediately after incubation, 3 µl of tPA inhibitor (2.5 mg/ml) in DMSO was added and incubated for 15 min at 37°C. Immediately after incubation, 3 µl of tPA inhibitor (2.5 mg/ml) in DMSO was added and incubated for 15 min at 37°C. Immediately after incubation, 3 µl of tPA inhibitor (2.5 mg/ml) in DMSO was added and incubated for 15 min at 37°C. Immediately after incubation, 3 µl of tPA inhibitor (2.5 mg/ml) in DMSO was added and incubated for 15 min at 37°C. Immediately after incubation, 3 µl of tPA inhibitor (2.5 mg/ml) in DMSO was added and incubated for 15 min at 37°C.

Expression and purification of VLHL PAI-1. The mutation of two amino acids (Gln197-Cys, Gly355-Cys) in human wPAI-1 [SwissPROT P05121 (22)] produces VLHL PAI-1 with half-life >700 h. The composition of this protein and the purification were previously described by our group (23,24). VLHL PAI-1 was chosen to assure stable tPA inhibitory activity over time, which cannot be achieved when wild PAI-1 is used, since its activity changes significantly in a short time.

Tissue plasminogen activator. Fully active human tPA (product no. HTPA-TC) was purchased from Molecular Innovations, Novi, MI.

SDS PAGE analysis. Electrophoresis of proteins was performed at room temperature using 4-12% SDS-polyacrylamide gradient gels under non-reducing conditions. Gels were stained with Colloidal Coomassie Blue (Invitrogen, Grand Island, NY).

Analysis of plasma clot formation with thromboelastography. Thromboelastography, not only allows for the measurement of a global coagulation profile, but also yields data on the kinetics and dynamics of clot formation and clot lysis in whole blood or in plasma (25,26). The critical part of this instrument is a pin hanging on a torsion wire and suspended in a cup holding a sample (360 µl). This pin oscillates at 6 rpm at a 4°-45° angle at 37°C. When plasma changes viscosity during clot formation this pin motion is progressively restrained by the clot and the cup. The strength of the clot determines the degree of force on the pin. The pin movement is converted to a TEG trace (27). Sodium citrated, reconstituted plasma was used for TEG assays by mixing 1 ml of plasma with 20 µl of kaolin (Haemoscope Co., Niles, IL) to which a constant amount of tPA was added [10 µl of tPA (2.1 mg/ml in 0.4 M HEPES, 0.1 M NaCl; pH 7.4) was added and incubated for 15 min at 37°C. Samples were run on a PAGE gel and stained. The optical band intensity of the formed tPA/VLHL PAI-1 complex was measured using densitometry and normalized to the VLHL PAI-1 band.

PAI-1/tPA complex formation assay. VLHL PAI-1 (24 µl of 0.4 mg/ml in PBS) and 5 µl of DMSO or the appropriate inhibitor (2.5 mg/ml) in DMSO was added and incubated for 15 min at 37°C. Immediately after incubation, 3 µl of tPA (2.1 mg/ml in 0.4 M HEPES and 0.1 M NaCl; pH 7.4) was added and incubated for 15 min at 37°C. Samples were run on a PAGE gel and stained. The optical band intensity of the formed tPA/VLHL PAI-1 complex was measured using densitometry and normalized to the VLHL PAI-1 band.

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Results

VLHL PAI-1 and tPA form a complex observed on SDS PAGE gel as a single band of molecular weight equal to the sum of the MW for both proteins. Addition of inhibitors reduces the intensity of this band. We measured it by densitometry and normalized it to the intensity of the VLHL PAI-1 band. As noted in Fig. 2, PAI039 inhibited the complex formation almost completely, while the other agents were less efficient but progressively more effective in the following order: OcAc EGCG < EGCG << TH(2). However, Gorlatova et al. reported that PAI039 exhibited reversible inactivation of PAI-1, and the site of interaction of the PAI039 drug with the PAI-1 molecule is inaccessible when PAI-1 is bound to vitronectin and may overlap with the PAI-1 vitronectin binding domain (31). Human vitronectin is a major plasma glycoprotein and thus could interfere with the inhibition of PAI-1 by inhibitors identified in vitro.

Consequently, all experiments involving PAI-1 inhibition were carried out with normalized human plasma, and kinetics of clot formation and its lysis were measured by thromboelastography. Normalized human plasma clots after addition of Ca²⁺ and kaolin, tPA activates plasminogen to lyse the clot, while VLHL PAI-1 inhibits tPA thus protecting the clot from tPA-induced lysis. Inhibitors of PAI-1 reduce VLHL PAI-1 protection and allow complete or partial tPA-induced lysis. Expected thromboelastograms are documented in Table I.

Table I. Changes in the thromboelastogram as a function of proteolysis status.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>tPA treated</th>
<th>tPA + VLHL PAI-1</th>
<th>tPA + VLHL PAI-1 + Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>tPA</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VLHL PAI-1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inhibitor</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Expected thromboelastogram</td>
<td>-</td>
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electrophoretic mobility) (32). Hence we investigated whether addition of DMSO alters the clotting characteristics of plasma. Differences in all of the determined parameters between DMSO-treated and control samples were not statistically significant (Fig. 3). Thus, we concluded that DMSO does not change the clotting parameters of plasma. In the same experiment we assessed how tPA would perform alone and when opposed by VLHL PAI-1 in the presence of DMSO. Most clotting parameters for tPA or tPA+VLHL PAI-1 were within experimental errors. However, we observed
statistically significant lower MA (represents clot strength) for tPA-treated samples (P<0.05) (Fig. 3). Furthermore, LY30 for tPA was 90.7±0.7% while addition of VLHL PAI-1 decreased LY30 to 0.3±0.9%. LY30 for all other samples (control, DMSO) was 0 indicating lack of inhibitory activity toward PAI-1.

PAI039 inhibited PAI-1 in human plasma as determined by thromboelastography. Even more potent was theaflavin digallate while EGCG showed much less PAI-1 inhibitory activity. No activity was observed for OcAc EGCG under the same conditions.

Inhibition of PAI-1 by TH(2) was concentration-dependent as illustrated in Fig. 4. The calculated IC50 in plasma was 18 μM. All other clotting parameters (Fig. 5) were within experimental errors with the exception of An (α) which was slightly elevated for two lower concentrations of TH(2). The α angle is an indication of the rate of clot formation (33), but explanation of the possible mechanism is highly speculative and was outside the scope of this study.

Discussion

Green tea polyphenol EGCG and black tea polyphenols are traditionally perceived as simple radical scavengers, although a growing body of evidence reveals their previously unsuspected biochemical activity (4,34,35). The PAI-1 inhibitory activity of theaflavin digallate falls into this category. Inhibition of PAI-1 by tea polyphenols has not been reported in the scientific literature to date. Yet, inhibition of PAI-1 by EGCG among the other compounds was quoted in a 2009 patent application (36) independently confirming our findings.

Of the 4 tested compounds TH(2) showed the highest PAI-1 inactivation. PAI039 with a IC50 reported by different authors between 2.7 (37) and 10-20 μM (36) was used as a positive control. IC50 in plasma for TH(2) in our experiments was 18 μM. Inactivation of PAI-1 by PAI039, measured by thromboelastometry, was slightly less effective than TH(2) but clearly within the reported range (36,37). The PAI-1 inhibitory activity of EGCG was much less effective that the two previously mentioned compounds. EGCG interacts with many targets including MAP kinases, phosphatases, DNA, RNA, DNA methyltransferase and topoisomerases (38). It has also been reported that the majority of such interactions result in inhibitory effects (38). Thus it is possible that the high PAI-1 inhibitory activity reported in isolated systems will somehow be lower when measured in plasma, where the abundance of different proteins can compete for EGCG binding. OcAc EGCG did not inhibit PAI-1 under the same conditions as TH(2). OcAc EGCG is considered a prodrug of EGCG to which OcAc EGCG hydrolyzes (39,40). However, it must be stated that when the sensitivity of the assay was greatly increased, we observed some PAI-1 inhibitory activity for both compounds (data not shown).

In conclusion, TH(2) markedly inhibits PAI-1 and might play a role in slowing down the progression of Alzheimer’s disease or obesity by a PAI-1-dependent pathway. While the clinical value of TH(2) has not been confirmed, long-term prospective studies evaluating its efficacy are warranted due to the benign nature of the substance. TH(2) can also be a leading compound for future development of more potent compounds.

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References


