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Development of a plasminogen activator inhibitor (PAI-1) assay and comparison of plasma PAI-1 activity in hyperlipidemic/dyslipidemic dogs with either hyperadrenocorticism or diabetes mellitus, and healthy dogs☆

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ABSTRACT

Thrombosis is a serious complication of many canine diseases and may be related to decreased fibrinolytic potential. Plasminogen activator inhibitor-1 (PAI-1) is the key regulator of fibrinolysis with increased levels demonstrated in states of pro-thrombosis and abnormal lipid metabolism. Our objective was to develop and validate a canine PAI-1 activity assay and test whether dogs with hyperadrenocorticism or diabetes mellitus that are hyperlipidemic/dyslipidemic have increased plasma PAI-1 activity. Functionally active PAI-1 in the plasma sample was incubated with recombinant tissue plasminogen activator (tPA), allowing the formation of a 1:1 stoichiometric inactive complex. Residual unbound tPA was then reacted with excess plasminogen in the presence of a colorimetric plasmin substrate. Plasmin production is quantified by computing the area under the curve of time (x) *vs* optical density (y) plot and converted to tPA IU/mL by comparison to a calibration curve of tPA standards. PAI-1 activity was determined by calculating the proportion of exogeneous tPA suppressed by PAI-1 in plasma. Assay verification included assessment of linearity, specificity, precision, sensitivity, and stability. PAI-1 activity was increased in hyperlipidemic compared to healthy dogs, but there was no significant difference between dogs with hyperadrenocorticism and diabetes mellitus. A near significant decrease in activity was detected in thawed plasma stored for 20 h at 4 °C. Our successfully validated assay offers a new tool for investigating fibrinolysis in dogs. Investigation of PAI-1 activity in dogs with other diseases associated with an increased risk of thrombosis would be valuable. Future studies of PAI-1 activity should consider its lability.

1. Introduction

Thrombotic complications occur in dogs with a variety of common diseases including primary endocrinopathies, cancer, inflammatory and auto-immune diseases, protein-losing states, and in association with corticosteroid therapy (de Laforcade, 2012). The relative risk of developing thrombosis is difficult to predict and once present accounts for up to 40% mortality dogs (Respess et al., 2012). Although the hypercoagulable state precipitating thrombosis is complex and multifactorial, a recent study has documented that dogs with diseases that predispose them to thrombosis often have significantly decreased fibrinolytic potential (Spodsberg et al., 2013). Studies investigating the mechanistic links between these disease states and the fibrinolytic pathway in the dog are lacking.

Fibrinolysis depends on the generation of active plasmin, which is regulated largely by the action of two plasminogen activators: tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). The plasminogen-plasmin system of fibrinolysis is tightly controlled by the key negative regulatory enzyme, plasminogen activator inhibitor-1 (PAI-1). Increased PAI-1 activity is considered pro-thrombotic and hypo-fibrinolytic, as a result of decreased generation of active plasmin. Due to its central role in regulating fibrinolysis, PAI-1 has been studied in a number of disease states associated with an increased thrombotic risk in people.

Insulin resistance, hyperglycemia, inflammation, and increases in visceral fat have all been shown to correlate with increased plasma PAI-1 activity in people (Knipe et al., 2010). The profound changes in lipid metabolism seen in these disease states draws attention to a potential link between lipoprotein metabolism and fibrinolytic capacity (Darvall et al., 2007; Binder et al., 2002; Alessi and Juhan-Vague, 2008; Aso, 2007; Lijnen, 2005). This link has been directly investigated in mice. Hyperlipidemic Apo-E −/− mice were found to have increased thrombus weight and attenuated thrombosis resolution, while also

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demonstrating elevated levels of PAI-1 (Diaz et al., 2012). Rabbits on diets formulated to induce hypercholesterolemia or mixed dyslipidemia had significantly increase plasma PAI-1 activity compared to controls (de las Heras et al., 2003). Mechanistically, beta-lipoproteins have been found to increase human endothelial cell secretion of PAI-1 in a concentration and time-dependent manner (Jovin and Müller-Berghaus, 2004). Dogs with both diabetes mellitus (DM) and hyperadrenocorticism (HAC) are commonly hyperlipidemic (Johnson, 2005) and have marked increases in beta-lipoprotein (low density lipoprotein and very low density lipoprotein) fractions with a decrease in high density lipoprotein fraction (Behling-Kelly and Collins-Cronkright, 2014). Despite these associations, to our knowledge, there are no reports investigating the potential relationship between hyperlipidemia or dyslipidemia and PAI-1 in dogs predisposed to thrombosis. The lack of an available means to measure PAI-1 activity in canine plasma is surely a contributory factor to this knowledge gap.

Previous attempts to validate commercially available assays developed to measure human PAI-1 for use in the dog have been largely unsuccessful (Lanevschi et al., 1996). The canine PAI-1 precursor protein sequence shares 88% amino acid identity with mature human PAI-1,[1] limiting the likelihood of cross-reactivity of immunological reagents between these species. Transference of activity assays is complicated by the fact that dogs have a more efficient fibrinolytic capacity than humans (Fletcher et al., 2014); the factors responsible for this difference remain to be elucidated. Alterations in activity or ratios of key proteins involved in fibrinolysis may be divergent enough across species (Dewilde et al., 2010; Declerck and Gils, 2013) to necessitate the development of an assay specifically developed for use in the targeted species. Thus, we sought to develop an assay to measure PAI-1 activity in canine plasma.

We then used this assay to measure the PAI-1 activity in two cohorts of hyperlipidemic/dyslipidemic dogs: dogs with HAC and dogs with DM. We specifically chose these two groups as HAC dogs have a known propensity to develop thrombosis while DM dogs do not. We hypothesized that PAI-1 activity would be increased in hyperlipidemic/dyslipidemic dogs compared to healthy control dogs and higher in dogs with HAC compared to those with DM.

2. Material and methods

2.1. Reagent preparation

Human plasminogen (Sigma-Aldrich) was reconstituted to a concentration of 0.00001 U/mL in 20 mM lysine buffer at pH 7.4. The plasmin chromogenic substrate S-2251 (D-Val-Leu-Lys p-nitroanilide dihydrochloride, Sigma-Aldrich) was reconstituted to 6.0 mM in 10 mM potassium dihydrogen phosphate, 70 mM sodium dihydrogen phosphate, 100 mM lysine buffer (pH 7.5 at 37 °C). The tPA chromogenic substrate (Sigma-Aldrich) was reconstituted in Milli-Q water to a final concentration of 4 mM. Human recombinant PAI-1 (rPAI-1) (EMD Millipore) was provided as a mixture of the active and latent forms of PAI-1, with >45% in the active form (EMD Millipore, 2016). Single-chain human recombinant tPA (Activase, Genentech Inc) was used and reconstituted in sterile water to a final concentration of 1 mg/mL; its specific activity is reported as 580,000 IU/mg (Genentech Inc., n.d.). Purified chromogen 4-Nitroaniline (pNA) (Sigma-Aldrich) was resuspended in 40% ethanol to generate a 20 mM stock and subsequently diluted in Milli-Q water.

2.2. PAI-1 assay

The PAI-1 assay we developed measures the biological activity of this inhibitor using purified recombinant proteins and a plasmin-specific chromogenic substrate in a two-step procedure (Fig. 1). In the first incubation step, 10 μL of human recombinant tPA (3.125 IU/mL) is added to 10 μL of canine plasma that was pre-diluted 1:4 volumetrically in assay diluent (0.1 M sodium dihydrogen phosphate, 0.01 M EDTA, 0.1 g/L Triton-X pH 7.3) yielding a total volume of 20 μL and a final tPA concentration of 1.5625 IU/mL, and incubated for 30 min at room temperature. We selected tPA as the plasminogen activator because it is primarily involved in fibrinolysis in circulation, whereas uPA is mainly associated with activating plasminogen bound to cell membranes (Rijken and Lijnen, 2009). Studies investigating the interaction of human tPA with PAI-1 have demonstrated a unique mode of inhibition, characterized by the formation of an SDS-stable complex that structurally distorts the catalytic triad (Perron et al., 2003; Huntington et al., 2000; Damare et al., 2014). Similar studies have not been performed using the canine proteins. Given the conservation of their biological activity, the fundamentals of the serpin:proteinase interaction were assumed to be similar in this species for the purpose of developing this assay. Following the initial incubation step, 20 μL of sample or standard was combined with 80 μL of the reaction mixture containing excess human plasminogen (final concentration equal to 0.002 U/μL), colorimetric plasmin substrate S-2251 (final concentration equal to 2.22 mM), and assay diluent in a total reaction volume of 100 μL. The plate was incubated at 37 °C in a microplate reader (SpectraMax 3, Molecular Devices LLC) for 3 h, and optical density (OD) at 405 nm was recorded every minute. Data acquisition and analysis were performed using a commercial microplate reader software package (SoftMax Pro 6, Molecular Devices LLC). All activity assays were performed in clear 96-well microtiter plates (Corning Inc) with samples run in duplicate and the OD of the blank (assay diluent mixture) subtracted. The baseline OD of each plasma sample was determined and substracted from the final result. The concentration of tPA was quantified by fitting area under the curve (AUC) values over the entire 3 h course of the assay to a 4-parameter logistic nonlinear regression model, generating a calibration curve. PAI-1 activity was then calculated by substracting the sample tPA concentration from the 1.5625 IU/mL tPA starting value. Calibration standards were prepared and run with each assay. These standards were a two-fold serial dilution series of human recombinant tPA spanning the range of 3.125 to 0.049 IU/mL, prepared in assay diluent. A correlation coefficient of $R^2 > 0.99$ was considered acceptable for all calibration curves.

2.3. Determination of assay conditions

The amount of tPA and plasmin substrate used in the assay reactions were determined by evaluation of experiments titrating tPA, plasminogen, and plasmin substrate concentrations, as well as the plasma dilution factor. An excess of plasminogen was determined by demonstrating an increase in substrate cleavage with addition of increasing concnetrations of tPA. Substrate depletion was eliminated as a confounding variable by testing to ensure that addition of increasing concentrations of plasminogen generated more substrate cleavage.

2.4. Assay validation

Parameters evaluated were linearity, specificity, precision, sensitivity, and stability.

2.4.1. Linearity

The linearity of the plate reader was evaluated by measuring the OD of serially-diluted pNA (1.25 to 0.019 mM) in Milli-Q water. Analytical linearity was assessed by measuring the PAI-1 in two-fold serial dilutions in canine plasma for 6 dilution points. Human recombinant PAI-1 (rPAI-1) was also used to assess linearity (a validated source of the canine protein was not commercially available). A dose response curve using rPAI-1 was created using a series of dilutions (0.0005 to

[1] BLAST = http://blast.ncbi.nlm.nih.gov/Blast.cgi (standard protein Blast; NCBI Reference Sequence: NP_001184024.1).