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Warfarin Untargeted Metabolomics Study Identifies Novel Procoagulant Ethanolamide Plasma Lipids

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Warfarin (Coumadin®) is one of the most widely prescribed anticoagulant drugs used to treat and prevent thrombosis in patients with cardiovascular or other thrombotic disorders (Keeling *et al*, 2011). Warfarin inhibits vitamin K epoxide reductase and decreases vitamin K hydroquinone to attenuate blood coagulation by inhibiting vitamin K-dependent post-translational modifications of plasma coagulation factors in the liver. A recent study showed that R-warfarin interacts with the pregnane X receptor (PXR) which is associated with a variety of metabolic clearance functions in vivo (Keeling *et al*, 2010), suggesting that warfarin could affect a variety of metabolites in addition to vitamin K-related metabolites. There is a paucity of studies about the effects of warfarin on natural plasma metabolites; thus, we sought to identify metabolites that are regulated by warfarin which could affect coagulation.

Mass spectrometry based untargeted metabolomics denotes the profiling of thousands of low molecular weight biochemicals, including lipids, hormones, saccharides, nucleotides, organic acids, and amino acids. The method can identify plasma metabolites “features” and detect statistically significant differences between two subject groups without the specific targeting of individual molecules. (Patti *et al*, 2012a, b). Here we applied this technology to test the effect of warfarin on plasma metabolites using plasma crossover samples drawn from the 17 subjects while on and off warfarin. Among 9,400 plasma features observed, the metabolic features at 32.2 min with 300.2908 m/z and at 38.6 min with 328.3193 m/z were statistically significantly decreased in patients taking warfarin by 4.0 and 7.2 fold ($p=0.018$ and 0.026 by paired t-test, respectively)(figure 1A and supplemental Table S1). The comparison of their fragments and their relative intensities with their model compounds by tandem MS (MS/MS) defined those metabolic features as palmitoylethanolamide (PEA) and stearoylethanolamide (SEA) from the lipid ethanolamide family (Figure 1A and supplemental Table 1S). The change of PEA by warfarin was statistically correlated with the

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Authorship and Disclosures

J.H.G, H.D. and G.S. participated in the conception of the study. G.S., S.T., H.M.Z and E.K. were responsible for untargeted metabolomics analysis. H.D. was responsible for performing experiments with ethanolamine activity and statistical analysis. D.J.E. was responsible for organizing the warfarin crossover study, consenting the patients and obtaining blood specimens. H.D., D.J.E. and J.H.G. were responsible for writing the manuscript.

change of SEA (r^2=0.85, p<0.0001) (Figure 1B). However, the INR which represents the effect of warfarin on vitamin K dependent coagulation factors did not correlate with these lipids (data not shown).

PEA and SEA are naturally occurring saturated N-acylethanolamines (ethanolamides), both of which are structurally related to AEA with a longer unsaturated side chain (20:4) which binds to the cannabinoid receptor. PEA and SEA are devoid of affinity for cannabinoid receptors. The role of PEA in inflammation and nociception via a variety of molecular mechanisms has been documented (Petrosino *et al*, 2010). However, the physiological roles of PEA and SEA remains unclear. Surprisingly, here, we discovered that certain ethanolamide family lipids have procoagulant characteristics. Both the TF/Ca^{2+}-induced and recalcification-induced thrombin generation were increased by PEA in normal plasma (Figure 2B and A). The precursor molecule of PEA, glycerophospho-N-palmitoyl ethanolamine, and analogs of PEA such as SEA and AEA also shortened lag time (p values for linear trend were 0.004, 0.0009, and 0.0006, respectively) (Figure 2C) and enhanced thrombin generation in plasma as seen in values for thrombin peak (p values for linear trend were 0.03, 0.16, and 0.00006, respectively)(Figure 2E), whereas certain other analogs of PEA, palmitoyl N-isopropylamide and N-palmitoyl taurine which are lacking a hydroxyl group in the head group (Figure 1C, 2D and F), showed little influence on thrombin generation. These results suggest that the free hydroxyl group in the head group appears to be a key component for the observed procoagulant activity of certain ethanolamides. Since the plasma concentrations of PEA, SEA and AEA are approximately 5.7, 1.5 and 0.7 nM, respectively (Ozalp *et al*, 2009; Balvers *et al*, 2009), the procoagulant effect of ethanolamides is below or within their plasma concentration ranges. These data suggest that the ethanolamide family possibly stimulates the coagulation pathway to increase thrombin generation in plasma. Interestingly, elevated peripheral AEA levels were found in acute stroke patients (Naccarato *et al*, 2010). The procoagulant activity of AEA and its association with stroke remains to be further evaluated. The physiologic and clinical relevance of the procoagulant properties of various ethanolamides is uncertain at this stage and requires further study.

The main complication of oral anticoagulant therapy is bleeding, and the bleeding risk is related to the intensity of anticoagulation (Hirsh et al, 2003). Bleeding that occurs at moderate anticoagulation (e.g. INR < 3.0) is frequently associated with trauma or an underlying lesion in the gastrointestinal or urinary tract (Hirsh et al, 2003). However, intracranial hemorrhage frequently occurs without trauma and at moderate or lower levels of anticoagulation (Dowlatshahi *et al*, 2012). Further, mortality rates of intracranial hemorrhage remained high despite the rapid INR correction by the supplementation of coagulation factors (Dowlatshahi *et al*, 2012). This suggests that certain warfarin sensitive factors including plasma lipids which are not reflected by the INR, might have a role in bleeding complications. Curiously there was a subset of subjects in whom the PEA and SEA were decreased by 70% (dotted circle in Figures 1B). The consequent anticoagulant effect by these reductions might be minor compared to the warfarin anticoagulant effect on vitamin K dependent coagulation factors. It would be of interest to investigate the association between ethanolamide decreased levels and warfarin-associated bleeding.

Our study has limitations including small numbers and the lack of a replication study. The cross-over study design lends strength to the small number of patients studied, decreasing intra-individual variability, but it is understood that a study with larger number of participants would be required for the confirmation of the findings reported here. However, it is noteworthy that our untargeted metabolomics identified two molecules from the same family and we newly found their procoagulant activity. This also suggests future study of the

association of ethanolamine family levels with beneficial anticoagulation and unwanted bleeding risk.

In summary, the untargeted metabolomics methods identified endogenous plasma lipids whose plasma levels are regulated by warfarin therapy. Further, this led to the discovery of a new family of plasma procoagulant lipid molecules, ethanolamides. These results show the utility of untargeted metabolomics and have implications for future basic and clinic studies related to warfarin's effects and to the procoagulant activities of ethanolamides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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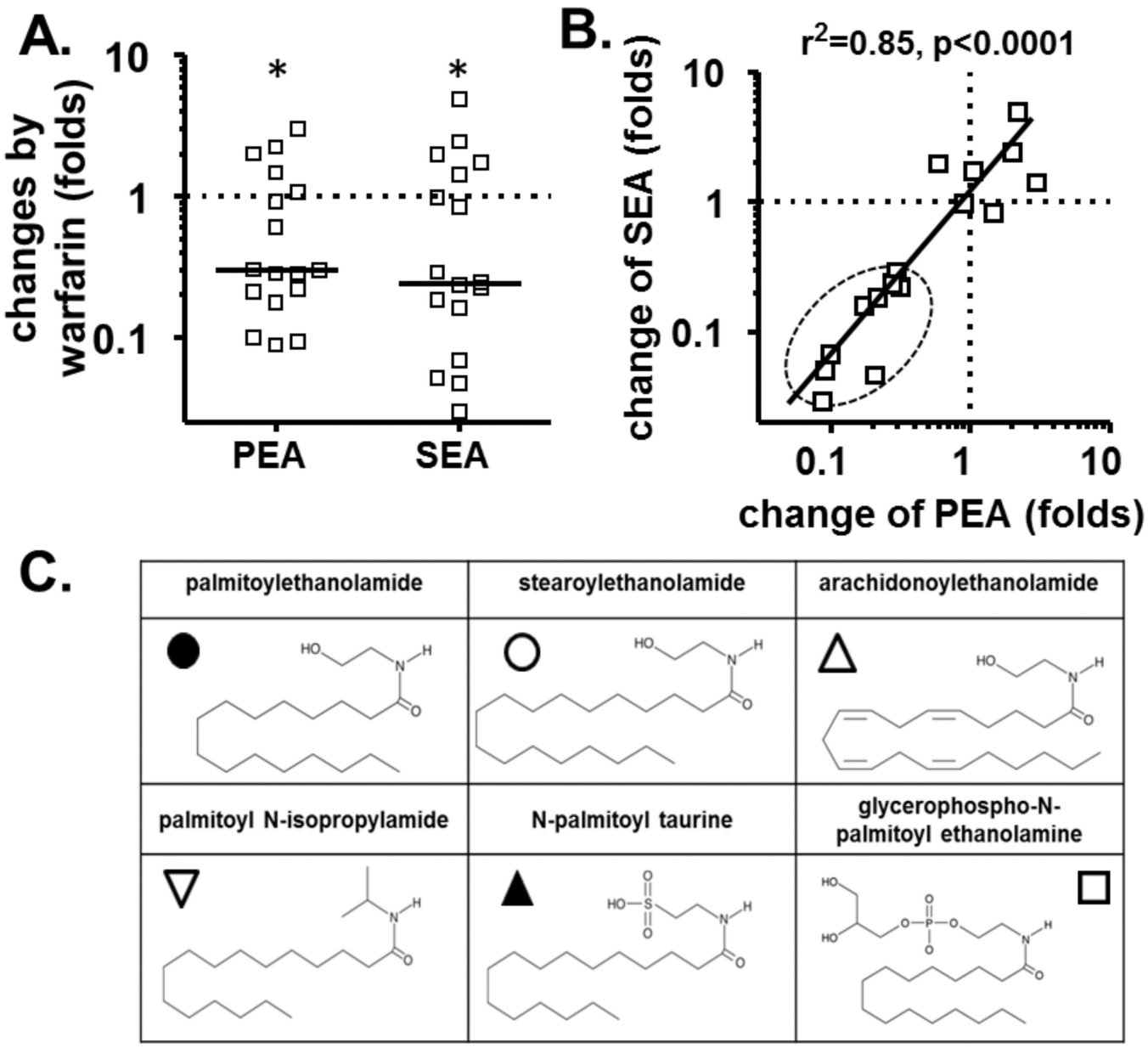

Figure 1. The effect of warfarin on the levels of plasma ethanolamides

The ratios of the integrated ion intensities of plasma metabolites between OFF- and ON-warfarin are shown as fold change caused by warfarin. The ratios were calculated with individual OFF-warfarin values as control. Solid thick lines indicate median values, and * indicates the changes induced by warfarin were statistically significant with $p<0.05$. **[A]** Plasma metabolites identified by untargeted metabolomics as significantly changed in of warfarin crossover subjects; **[B]** The correlation of the PEA change by warfarin with the SEA change by warfarin for each subject; and **[C]** Structure of various kinds of ethanolamides.

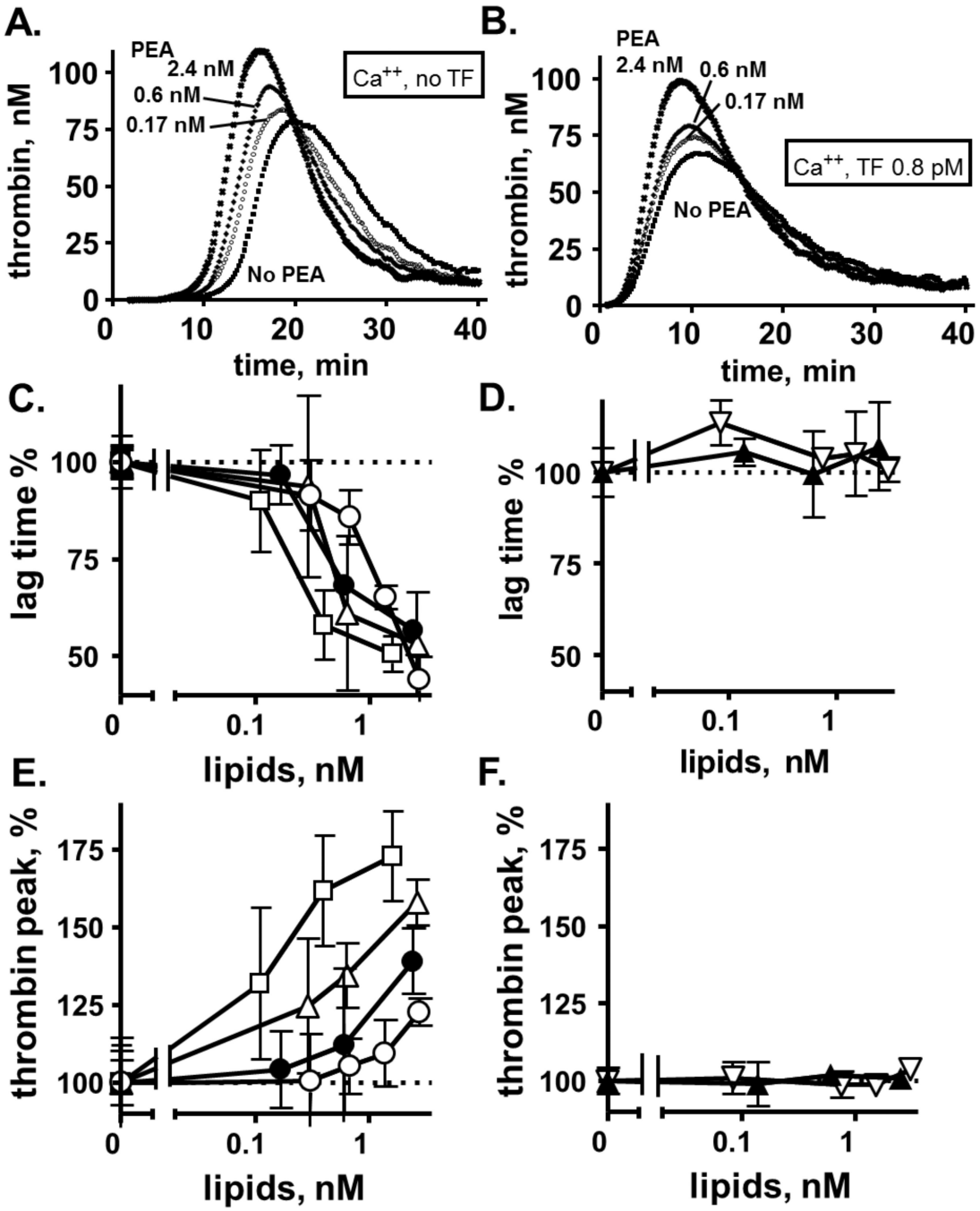

Figure 2. The effect of PEA or ethanolamide analogues on thrombin generation in plasma

Various concentrations of PEA or other ethanolamide analogues were mixed with normal pooled human plasma or factor deficient plasma [George King], and thrombin generation was initiated by adding TF [0.8 pM final] plus $CaCl_2$ or adding only $CaCl_2$ alone. **[A]** $CaCl_2$-induced thrombin generation in normal pooled plasma. Concentrations of PEA were 0, 0.17, 0.6 and 2.4 nM corresponding to the lines from bottom to top; **[B]** TF/$CaCl_2$-induced thrombin generation in normal pooled plasma. Concentrations of PEA were 0, 0.17, 0.6 and 2.4 nM corresponding to the lines from bottom to top; **[C, D]** Effect of various ethanolamides on lag time; and **[E, F]** Effect of various ethanolamides on peak thrombin

generation when 100% was defined as the value for no ethanolamide added. Different symbols in **[C–F]** represent PEA [●], SEA [○], AEA [△], N-palmitoyl taurine [▲], N-isopropylamide [▽] and glycerophospho-N-palmitoylethanolamine [□], respectively. Error bar represents SEM (n=3 or 4).