Rapid and Sensitive Differentiating Ischemic and Hemorrhagic Strokes by Dried Blood Spot Based Direct Injection Mass Spectrometry Metabolomics Analysis

Zhansheng Hu,¹ Zhitu Zhu,¹ Yunfeng Cao,^{1,2,3} Lixuan Wang,² Xiaoyu Sun,² Jun Dong,² Zhongze Fang,^{2,4} Yanhua Fang,² Xiaoxue Xu,² Peng Gao,^{1,2,3,5} and Sun Hongzhi^{1,2*}

¹First Affiliated Hospital of Liaoning Medical University, Jinzhou, P.R. China

²Joint Center for Translational Medicine, Dalian Institute of Chemical Physics, Chinese Academy of Sciences and First Affiliated Hospital of Liaoning Medical University, Dalian, P.R. China

³Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, P.R. China

⁴Tianjin Life Science Research Center and Department of Microbiology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin, P.R. China

⁵Clinical Laboratory, Dalian Sixth People's Hospital, Dalian, PR China

Cerebral infarction (CI) and intracerebral hemorrhage are lethal cerebrovascular diseases, sometimes sharing similar clinical manifestations but with distinct therapeutic strategies. Delayed treatment usually resulted in poor prognosis. A timely diagnosis report is highly warranted especially in emergency. One hundred twenty-nine CI patients, 73 intracerebral hemorrhage (ICH) patients, and 98 controls were enrolled in this study. A direct injection mass spectrometry metabolomics approach was adopted using dried blood spot samples. This targeted metabolomics analysis focused on absolute quantitation of 23 amino acids, 26 carnitine/carnitine esters, and 22 calculated ratios parameters.

Key words: stroke; mass spectrometry; metabolomics

Compared to the normal control group, CI and ICH showed distinct metabolite changes, respectively. For stroke differentiation, Tyr, C5-OH/C0, Cit, Asn, Pro, Val, Arg/Orn, Leu, and Val/Phe were elevated in the CI group. On the contrary, C5:1, Phe/Tyr, (C0 + C2 + C3 + C16 + C18:1)/Cit, and Met/Leu were of lower levels in the CI group. Using regression model based on some of the above-mentioned parameters, 79.07% of stroke patients from a new set could be definitely confirmed. This study proved the targeted metabolomics analysis was a promising tool for rapid and timely stroke differentiation. *J. Clin. Lab. Anal.* **30**: 823–830, 2016. © 2016 Wiley Periodicals, Inc.

INTRODUCTION

Stroke has become the second leading cause of death and the third reason of disability in the adult worldwide (1, 2). Basically, stroke can be of ischemic and hemorrhagic origins. The former is usually referred to cerebral infarction (CI), which is caused by the local brain–blood circulation obstacle and results in ischemia, hypoxia, and, most frequently, local necrosis (3). The latter is represented by intracerebral hemorrhage (ICH) and is closely related to cerebral vascular sclerosis lesions and usually brings about higher mortality compared to CI. These two attacks cover more than 95% of the total incidence and, of which, 87% are ischemic strokes (4). Although incidence of stroke decreases in the developed

countries, the annual attack of stroke in the United States, for example, is still about 0.78 million accompanied by one of five to one of four death rate (1). What makes things worse is that mortality of strokes is still increasing in the undeveloped regions (5, 6).

Nearly all the strokes are sudden attacks and neither the occurrence of CI nor the attack of ICH preceded by

Grant sponsor: National Natural Science Foundation of China; Grant number: 81372695.

*Correspondence to: Sun Hongzhi, First Affiliated Hospital of Liaoning Medical University, Jinzhou 121000, P.R. China. E-mail: cmushz@163.com

Received 6 May 2015; Accepted 13 January 2016

DOI 10.1002/jcla.21943

Published online in Wiley Online Library (wileyonlinelibrary.com).

a specific aura. Owing to the sensitivity of brain to hypoxia and the limited regeneration ability of neurons, therapeutic window for stroke is very narrow (approximately several hours) (7). Delayed stroke treatment contributes to higher mortality and disability, leading to poor prognosis (2). In the undeveloped areas, stroke mortality can even approach to 85%. Therapies for ischemic and hemorrhagic strokes differ greatly and sometimes are opposite. Although their pathological origins are distinct, ischemic and hemorrhagic strokes often share some clinical manifestations in common, such as hemiplegia, aphasia, and other neurological deficits. Currently, image-based strategies are the prevailing clinical manners to diagnose strokes. Computer tomography (CT) scan can achieve relatively accurate diagnosis (~85%) of hemorrhagic stroke, but it cannot detect stroke within 6 hr of onset. CT scan discernable infarct lesions may appear until 12–24 hr after stroke (7). Magnetic resonance imaging (MRI) has some advantages to CT scan, but, in some circumstance, several patient-related factors preclude the application of MRI, such as loss of consciousness, vomiting, and agitation (8). Additionally, MRI is not cost-effective and it is usually not easily accessed in the undeveloped areas (9). Occasionally, lumbar puncture and cerebrospinal fluid (CSF) analysis is helpful (10). Both the patients and the physicians are reluctant to accept the invasive operations. Although angiography is a good method to diagnose and differentiate ischemic and hemorrhagic strokes, this time-consuming method is not an applicable solution in emergency.

In order to make a precise and immediate stroke diagnosis, some serum biomarkers had been explored. Most of them are proteins relevant to vascular injury, metabolic changes, oxidative stress, and inflammation. Owing to their limited specificities, most of them are not well accepted in clinics (7). Besides the macromolecules, there are many fluctuating small molecule metabolites depending upon specific physiological or pathological status. Since the advancement of metabolomics, researchers had tried to find small metabolite markers for stroke diagnosis in a more efficient way. Metabolomics is a newly emerged concept that addresses to quantitative and qualitative analyses of the whole metabolites (metabolome) in a specific system (tissue or biofluid) in a holistic view. Metabolome is thought to be phenotype-specific and metabolomics analysis has been widely employed in disease stratification, biomarker discovery, and drug toxicity evaluation (11–13). Current metabolomics research is primarily based on chromatography–mass spectrometry (MS) and nuclear magnetic resonance (NMR) platform. Through liquid chromatography coupled to MS (LC–MS) analysis, folic acid, cysteine, S-adenosyl homocysteine, and oxidized glutathione were proved to be

TABLE 1. Information of the Patients and the Health Controls

	CI patients	ICH patients	Healthy controls
No. of subjects	129	73	98
Age (median, range)	58.08, 31–80	58.08, 34–84	56.07, 32–80
Male	37 (28.68%)	22 (30.14%)	30 (30.61%)
Female	92 (71.32%)	51 (69.86%)	68 (69.39%)

valuable in CI diagnosis (14). Additionally, a lysophosphatidylcholine (LysoPC(20:4)) was even reported to be utilized to predict stroke reoccurrence (15). For clinical concerns, a rapid stroke diagnosis is highly appreciated. Chromatography separation improved the metabolite discriminating ability, but at the expense of increased analysis time. Venous blood samples are the routine sample type for clinical chemistry analysis, but separating blood cells from plasma or serum is also a time-consuming procedure. Dried blood spot (DBS) samples had been widely used in newborn screening (16, 17), therapeutic drug monitoring (18), and targeted blood metabolite analysis (19, 20). Sampling and preprocessing DBS is very simple. In this regard, a direct injection MS metabolomics analysis was performed using DBS samples collected from CI and ICH patients in this study. The aim of this research was to seek CI and ICH relevant metabolites against normal individuals. Additionally, weather DBS-targeted metabolomics analysis could be used to differentiate CI from ICH would also be discussed.

MATERIALS AND METHODS

Clinical Samples

One hundred twenty-nine CI patients, 73 ICH patients, and 98 age- and gender-matched healthy volunteers from The First Affiliated Hospital of Liaoning Medical University were enrolled in this study. Detailed information is given in Table 1. Informed consent forms were signed by all the participants and the study was carried out in accordance with the Guide of Hospital Ethics Committee of The First Affiliated Hospital of Liaoning Medical University. DBS samples were collected by fingertip puncture less than 12 hr after stroke attacks. Patients with food intake within 2 hr before sampling were excluded. After dried at room temperature, the DBS filter paper was stored at 4°C in a sealed plastic bag individually.

Chemicals

HPLC-grade acetonitrile (ACN) and HPLC-grade water were obtained from Thermo Fisher (Waltham, MA). Acetyl chloride and 1-butanol were purchased

from Sigma-Aldrich (St. Louis, MO). Labeled amino acid internal standards (catalog number: NSK-A) containing 12 stable isotope-labeled amino acids and labeled eight carnitine internal standards (catalog number: NSK-B) for absolute quantification were obtained from Cambridge Isotope Laboratories (Andover, MA). Every 1 ml pure methanol was added to NSK-A and NSK-B individually. These dissolved isotope standards were mixed together and stored at 4°C as stock solution. For metabolite extraction, the stock solution was diluted 100-folds as working solution. Two-level quality control (QC) amino acids and carnitines mixed standards were purchased from Chromsystems (Grafelfing, Germany). The QC samples were processed as real samples and randomly inserted in the real sample analysis queue.

Sample Preparation

A paper disc of 3 mm in diameter was punched from the DBS filter paper (equivalent to 3.2 µl whole blood), placed into a well in Millipore MultiScreen HV 96-well plate (Tullagreen, Ireland), and subjected to metabolite extraction. Briefly, every 100 µl of working solution was dispensed into one-well containing a DBS disc. The plate was gently shaken for 20 min at room temperature and centrifuged at $1,500 \times g$ for 2 min to collect the filtrate into a new flat-bottom 96-well plate. For each plate, every two low-level and every two high-level QC control sample solutions were randomly allocated into four blank wells and analyzed as real samples to monitor the stability of the MS analysis. The filtrate and QC solutions were subjected to drying by pure nitrogen gas at 50°C. Each dried sample was derivatized in 60 µl 1-butanol and acetyl chloride mixture (90:10, v/v) at 65°C for 20 min. The derivatized solutions were dried by pure nitrogen gas at 50°C again. Every 100 µl mobile phase solution was added into each well prior to metabolomics analysis.

Metabolomics Analysis

Direct injection MS analysis was performed on AB SCIEX 4000 QTrap system (Framingham, MA) equipped with an electrospray ionization (ESI) source and scanned in positive mode. The injected sample volume was 20 µl. The mobile phase was a mixture of 80% ACN aqueous solution. The elution program was initiated with 0.2 ml/min, decreased to 0.01 ml/min within 0.08 min, and kept constant until to 1.5 min. Then, the flow rate increased to 0.2 ml/min within 0.01 min and held for another 0.5 min. The MS ion spray voltage was set to 4.5 kV. Pressures of ion source gas 1 and gas 2 were all 35 psi. Curtain gas pressure was 20 psi. Auxiliary gas temperature was

kept at 350°C. The detected metabolites, scan modes, and scan parameters were in accordance with the previous report (21). The raw data were collected by Analyst v1.6.0 software (AB Sciex) and quantification of individual metabolites was accomplished by ChemoView 2.0.2 software (AB Sciex) against different isotope standards. The calculated ratios are described elsewhere (21). For each stroke group, screening differential metabolites against the normal controls was conducted by using algorithm of significant analysis of microarrays (SAM) (22). Student's *t*-test was additionally used to ascertain the significance (with $P < 0.05$). For CI and ICH discrimination, 80% of randomly selected samples from each group were compared. Differential parameters were subjected to stepwise regression analysis. Diagnostic ability of the potential markers was evaluated by area under the receiver operating characteristic (ROC) curve (AUC). The left-over 20% case samples in each group were used as testing set to evaluate the diagnosis precision of the regression model. All statistical analysis was conducted by using MINITAB v16.0 software (State College, PA).

RESULTS

Initially, all the stroke samples are mixed together and subjected to separation using principal component analysis. But, the separation failed (data not shown). We thought that mixing the two stroke groups might complicate the data sets and resulted in the inability of group discrimination using limited components.

Next, we separately compared each stroke group with the normal control in order to find the differentially changed metabolites by SAM screening. After through Student's *t*-test confirmation, five parameters were found to be of different blood levels between CI and the control groups (Fig. 1). They were Ala (alanine), Cit (citrulline), C5-OH (3-Hydroxyisovalerylcarnitine), and two C5-OH related ratio parameters. Notably, these parameters were all increased due to the onset of CI. As of the ICH, two carnitine-related parameters were affected (Fig. 2). Similar to the CI situation, these two parameters were also increased in ICH group. What should be mentioned was that the affected metabolites by ICH and CI were totally different, indicating the distinct pathological progress of ischemic and hemorrhagic strokes.

In order to explore whether the adopted targeted metabolomics analysis could be of any assistance in aiding CI and ICH differentiation, we compared the relevant absolute quantitation and calculated ratio values only using 80% randomly selected samples from each population. This led us to find seven metabolites and six

Fig. 1. SAM analysis results of the CI and control samples. Parameters affected by CI were all increased in the blood.

calculated ratios were significantly different between the two groups. Tyr (tyrosine), C5-OH/C0 (free carnitine), Cit, Asn (asparagine), Pro (proline), Val (valine), Arg/

Orn (arginine/ornithine), Leu (leucine), and Val/Phe (phenylalanine) were elevated in the CI group (Fig. 3). On the contrary, C5:1 (tiglylcarnitine), Phe/Tyr, (C0 +

Fig. 2. SAM analysis results of the ICH and control samples. Only two parameters were increased by ICH attack.

Fig. 3. Statistical analysis of the metabolomics data of CI and ICH. Nine parameters were elevated in CI group compared to ICH group. Most of them were amino acids.

$C2 + C3 + C16 + C18:1$ /Cit (free carnitine + acetylcarnitine + propionylcarnitine + palmitoylcarnitine + petroselinic carnitine)/(citrulline), and Met/Leu (methionine/leucine) were of lower levels in the CI group (Fig. 4).

In view of clinical utilization, these 13 parameters were further processed through a stepwise regression analysis (both alpha to enter and remove were set to 0.15) to construct a model for CI and ICH differentiation. The final model ($P < 0.001$) included five

parameters, Asn, C5:1, Arg/Orn, Val/Phe, and $(C0 + C2 + C3 + C16 + C18:1)/Cit$. ROC curve analysis indicated a diagnosis ability with sensitivity of 77.6% and specificity of 84.8% with an optimal cutoff value of 0.434 (Fig. 5). This curve generated an AUC value of 0.873, which implied the acceptability of the regression model in view of clinical application. Further tested by the remaining 20% stroke patients, diagnosis accuracy of this statistic model approached to 79.07%.

Fig. 4. Statistical analysis of the metabolomics data of CI and ICH. Four parameters were decreased in CI group compared to ICH group. Most of them were calculated ratios.

DISCUSSION

Great effort has been taken to develop stroke diagnosis biomarkers. But the encouraging results are still limited. Heterogeneous pathophysiologies and mechanisms

Fig. 5. ROC analysis of the regression model based on the results of CI and ICH comparison. Only 80% samples of each group were used.

not only complicate stroke clinical manifestations, but also bring about tremendous difficulty in universal biomarker validation (23). Thus, our initial attempt to analyze stroke groups with control group using unsupervised method in a combined manner failed. In fact, even for pure CI and normal population discrimination, a supervised method was necessary (14). Through an alternative supervised method analysis, we found that Ala and Cit increased in CI patients (Fig 1). Ala could increase brain carnosine content (24), which exerted neuroprotective effects by attenuating autophagy taking place in ischemic damaged brain (25). Similarly, Cit supplementation was also demonstrated to be neuroprotective through modulating nitric oxide generation (26). Coincidentally, these two mechanisms were all involved in rescuing mitochondrial functions, indicating that mitochondrial damage might be a very early event in ischemic stroke and an early sensitive response to hypoxia (27). The increased Ala and Cit in CI were thought to be the protective response of the body in acute phase. It would be helpful if the changes of the two metabolites could be monitored dynamically using time serial samples. The other three parameters affected by CI were all related to C5-OH acylcarnitine. C5-OH was thought to be a proapoptotic factor (28). The roles of C5-OH in stroke are elusive currently, but carnitine is an indispensable shutter for fatty acid β -oxidation in mitochondria. Thus, these changed carnitine esters might mirror the impaired mitochondrial oxidation functions. It was very interesting that the affected carnitine esters were confined to short-chain carnitines but not the long-chain ones. Collectively, CI-related metabolite markers were closely related to mitochondrial metabolism.

Increased C4/C2 usually indicated the short-chain acyl-CoA dehydrogenase (SCAD) deficiency. Except SCAD, elevated C5:1 could also be the results of β -ketothiolase deficiency. It had been reported that SCAD deficiency could be observed in pathological cardiomyocytes, but not in physiological cardiomyocytes (29). Additionally, this deficiency was closely related to the deactivation of peroxisome proliferator-activated receptor α (PPAR α) (29). Up to now, we still could not find any hint to link the above-mentioned changes with ICH (Fig. 2). Platelet plays key roles in ICH pathology. Platelet dysfunction means worse outcomes of patients suffering from ICH. Activated platelets augmented PPAR α mRNA expression in human umbilical endothelial cells (30). Except the fact that these esters are linked to mitochondrial oxidation functions, it also implied that ICH might have something to do with SCAD-related pathological progress.

Although the great advance in stroke diagnosis, differentiation of ischemic and hemorrhagic strokes is still a real challenge especially in emergency. Our

metabolomics analysis showed that most of the elevated metabolites in the CI patient blood were amino acids as compared to ICH patients (Fig. 3). Only two calculated amino acid ratios increased in the ICH group (Fig. 4). This finding indicated that amino acid metabolism was differentially perturbed by CI and ICH occurrence.

In order to test if the differentially changed parameters could be used to differentiate stroke types, a regression model was constructed. This model only contained two absolute quantitation metabolites, Asn and C5:1 carnitine ester. The other three parameters were two amino acid ratios and a ratio of combined carnitine esters versus Cit. The model showed an AUC of 0.873 (Fig. 5) and could precisely predict 79.07% of the 40 stroke patients from an independent set. That meant, for differentiation of ischemic and hemorrhagic strokes, quantifying six amino acids and six carnitine metabolites should be helpful.

In this study, we aimed to find a rapid and sensitive method to help differentiate ischemic and hemorrhagic strokes with respect to metabolomics analysis. To circumvent the time consuming chromatography separation, we selected the direct injection MS analysis. For simplifying the sample collection procedure, we paid attention to the DBS sample type. Integrating both advantages, the whole performance could be finished in several minutes, and was a promising alternative in emergency. Limited by the sample and the targeted metabolite amount, the prediction accuracy was not very high enough. The upcoming study will be conducted in larger population and using time serial samples to gain more insight into the substantial difference between CI and ICH.

ACKNOWLEDGMENT

This work was supported by the National Natural Science Foundation of China (81372695).

REFERENCES

- Petrea RE, Beiser AS, Seshadri S, Kelly-Hayes M, Kase CS, Wolf PA. Gender differences in stroke incidence and poststroke disability in the Framingham heart study. *Stroke* 2009;40:1032–1037.
- O'Donnell MJ, Xavier D, Liu L, et al. Risk factors for ischaemic and intracerebral haemorrhagic stroke in 22 countries (the INTERSTROKE study): A case-control study. *Lancet* 2010;376:112–123.
- Leppala JM, Virtamo J, Fogelholm R, Albanes D, Heinonen OP. Different risk factors for different stroke subtypes: Association of blood pressure, cholesterol, and antioxidants. *Stroke* 1999;30:253–2540.
- Lloyd-Jones D, Adams RJ, Brown TM, et al. Heart disease and stroke statistics–2010 update: A report from the American Heart Association. *Circulation* 2010;121:e46–e215.
- Krishnamurthi RV, Feigin VL, Forouzanfar MH, et al. Global and regional burden of first-ever ischaemic and haemorrhagic stroke during 1990–2010: Findings from the Global Burden of Disease Study 2010. *Lancet Glob Health* 2013;1:e259–e281.
- Feigin VL, Forouzanfar MH, Krishnamurthi R, et al. Global and regional burden of stroke during 1990–2010: Findings from the Global Burden of Disease Study 2010. *Lancet* 2014;383:245–254.
- Miao Y, Liao JK. Potential serum biomarkers in the pathophysiological processes of stroke. *Expert Rev Neurother* 2014;14:173–185.
- Singer OC, Sitzer M, du Mesnil de RR, Neumann-Haefelin T. Practical limitations of acute stroke MRI due to patient-related problems. *Neurology* 2004;62:1848–1849.
- Kim BJ, Kang HG, Kim HJ, et al. Magnetic resonance imaging in acute ischemic stroke treatment. *J Stroke* 2014;16:131–145.
- Fiebach JB, Schellinger PD, Jansen O, et al. CT and diffusion-weighted MR imaging in randomized order: Diffusion-weighted imaging results in higher accuracy and lower interrater variability in the diagnosis of hyperacute ischemic stroke. *Stroke* 2002;33:2206–2210.
- Nicholson JK, Lindon JC, Holmes E. “Metabonomics”: Understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* 1999;29:1181–1189.
- Nicholson JK, Connolly J, Lindon JC, Holmes E. Metabonomics: A platform for studying drug toxicity and gene function. *Nat Rev Drug Discov* 2002;1:153–161.
- Shyr LF, Yang NS. Metabolomics for phytomedicine research and drug development. *Curr Opin Chem Biol* 2008;12:66–71.
- Jiang Z, Sun J, Liang Q, et al. A metabonomic approach applied to predict patients with cerebral infarction. *Talanta* 2011;84:298–304.
- Jove M, Mauri-Capdevila G, Suarez I, et al. Metabolomics predicts stroke recurrence after transient ischemic attack. *Neurology* 2015;84:36–45.
- Guthrie R, Susi A. A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. *Pediatrics* 1963;32:338–343.
- Therrell BL, Adams J. Newborn screening in North America. *J Inher Metab Dis* 2007;30:447–465.
- Berm EJ, Brummel-Mulder E, Paardekoooper J, Hak E, Wilffert B, Maring JG. Determination of venlafaxine and O-desmethylvenlafaxine in dried blood spots for TDM purposes, using LC-MS/MS. *Anal Bioanal Chem* 2014;406:2349–2353.
- Duthaler U, Keiser J, Huwyler J. LC-MS/MS method for the determination of two metabolites of tribendimidine, deacetylated amidantel and its acetylated metabolite in plasma, blood and dried blood spots. *J Pharm Biomed Anal* 2015;105:163–173.
- Shah NM, Hawwa AF, Millership JS, Collier PS, McElnay JC. A simple bioanalytical method for the quantification of antiepileptic drugs in dried blood spots. *J Chromatogr B Analyt Technol Biomed Life Sci* 2013;923–924:65–73.
- Wang Q, Sun T, Cao Y, Gao P, Dong J, Fang Y, Fang Z, Sun X, Zhu Z. A dried blood spot mass spectrometry metabolomic approach for rapid breast cancer detection. *Oncotargets Ther* 2006;9:1389–1398.
- Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* 2001;98:5116–5121.
- Kim SJ, Moon GJ, Bang OY. Biomarkers for stroke. *J Stroke* 2013;15:27–37.
- Hoffman JR, Ostfeld I, Stout JR, Harris RC, Kaplan Z, Cohen H. Beta-alanine supplemented diets enhance behavioral resilience to stress exposure in an animal model of PTSD. *Amino Acids* 2015.

25. Baek SH, Noh AR, Kim KA, et al. Modulation of mitochondrial function and autophagy mediates carnosine neuroprotection against ischemic brain damage. *Stroke* 2014;45:2438–2443.
26. El-Hattab AW, Emrick LT, Williamson KC, Craigen WJ, Scaglia F. The effect of citrulline and arginine supplementation on lactic acidemia in MELAS syndrome. *Meta Gene* 2013;1:8–14.
27. Sayre NL, Chen Y, Sifuentes M, Stoveken B, Lechleiter JD. Purinergic receptor stimulation decreases ischemic brain damage by energizing astrocyte mitochondria. *Adv Neurobiol* 2014;11:121–150.
28. Ferrara F, Bertelli A, Falchi M. Evaluation of carnitine, acetylcarnitine and isovalerylcarnitine on immune function and apoptosis. *Drugs Exp Clin Res* 2005;31:109–114.
29. Huang J, Xu L, Huang Q, et al. Changes in short-chain acyl-coA dehydrogenase during rat cardiac development and stress. *J Cell Mol Med* 2015.
30. Wu YQ, Chen XS, Chai JB. The involvement of the CD40-CD40L pathway in activated platelet-induced changes in HUVEC COX-2 and PPARalpha expression. *Inflammation* 2012;35:1184–1190.