Advanced glycation end products elicit externalization of phosphatidylserine in a subpopulation of platelets via 5-HT_{2A/2C} receptors

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Advanced glycation end products (AGE) are substantially elevated in individuals with diabetes and/or chronic kidney disease (CKD). These patients are at greatly increased risk of cardiovascular events. The purpose of this study was to investigate the novel hypothesis that AGE elicit externalization of the platelet membrane phospholipid phosphatidylserine (PS). This contributes to hemostasis through propagation of the coagulation cascade leading to thrombus formation. Platelet-rich plasma (PRP) was prepared by differential centrifugation, and PS externalization was quantified by a fluorescence-activated cell sorter using annexin V-FITC. Human serum albumin (HSA)-AGE was generated by incubating HSA with glucose for 2, 4, or 6 wk, and total HSA-AGE was assessed by fluorescence intensity. The 2-wk HSA-AGE preparation (0–2 mg/ml) stimulated a concentration-dependent increase in PS externalization in a subpopulation of platelets that were threefold at 2 mg/ml. In contrast, the 4- and 6-wk preparations were maximal at 0.5 mg/ml and fivefold in magnitude. These effects mirrored the change in total HSA-AGE content of the preparations. The PS response was maximal at 10 min and inhibited by the PKC-δ inhibitor rotterlin and the serotonin [5-hydroxytryptamine (5-HT)]_{2A/2C} receptor antagonist ritanserin in a dose-dependent manner. Moreover, the 5-HT_{2A/2C} receptor agonist 1,2,5-dimethoxy-4-iodophenyl-2-aminopropane mimicked the effect of HSA-AGE on PS externalization. These data demonstrate, for the first time, that HSA-AGE stimulates PS externalization in a subpopulation of platelets via the 5-HT_{2A/2C} receptor. This may have important consequences for platelet involvement in inflammatory responses and the increased cardiovascular risk observed in individuals with diabetes and/or CKD.

Signal transduction; thrombosis; caspase-3

INDIVIDUALS with diabetes and/or chronic kidney disease (CKD) are at increased risk of cardiovascular disease, the major cause of death and disability in these patient groups (5, 10, 11). It is now clear that atherosclerosis, the pathological basis of coronary artery disease and stroke, is an inflammatory disease (27). Moreover, platelet activation and aggregation are crucial pathological events leading to both the induction of inflammatory responses at sites of the injured vessel wall and the development of arterial thrombus (22).

The membrane phospholipid phosphatidylserine (PS) resides in the inner leaflet of the plasma membrane of resting platelets (3), but, following activation of scramblase by PKC-δ, PS translocates to the outer leaflet of the plasma membrane (3, 6). A number of studies have demonstrated that chronically elevated or prolonged exposure of PS on the cell surface both increases vascular damage and results in the formation of a hypercoagulable environment by stimulating adherence of inflammatory cells to the vascular endothelium (e.g., Ref. 16) and providing a catalytic surface for assembly of the prothrombinase complex (22) that accelerates the generation of thrombin (26, 41). Interestingly, a recent study (40) has demonstrated that platelet agonists elicit PS externalization in a subpopulation of these cells and that the size of this population correlates with prothrombinase activity.

Increased platelet PS externalization has also been reported in patients with diabetes (35). Moreover, when platelets isolated from healthy controls were reconstituted with serum prepared from individuals with CKD, a rapid increase in PS externalization in a small, subpopulation was observed (37). However, the factor(s) responsible for this response is unknown.

Advanced glycation end products (AGE) arise by covalent modification of cellular and plasma proteins and form a series of heterogeneous compounds (15). AGE are substantially increased in individuals with either diabetes or CKD compared with the general population (18, 36) and may contribute to the development and progression of cardiovascular disease in these patient groups (30, 32). Although the mechanism(s) by which AGE influence the progression of atherosclerosis is not completely understood, it is thought that, either by acting via specific “receptors for AGE” (RAGE) or through other mechanisms (13, 23), they can promote vascular damage, fibrosis, and inflammation, which are associated with accelerated atherogenesis (32).

The goals of our laboratory were to identify the serum factor(s) responsible for increased platelet PS externalization and investigate the potential involvement of this process in the increased cardiovascular risk observed in those individuals with diabetes/CKD. To address the first of these aims, we examined the ability of human serum albumin (HSA)-AGE to elicit platelet PS externalization and the mechanism involved.

METHODS

Materials and Reagents

Annexin V-FITC was purchased from Immunotech (Marseille, France), and fluorospheres for FACSscan calibration were obtained from DakoCytomation (Glostrup, Denmark). The YSI 2300 STAT...
Plus Analyzer was purchased from Yellow Springs Instruments (Yellow Springs, OH), and the ENDOSAFE LAL Gel Clot Test was obtained from Charles River (Charleston, NC). The Superdex HR75 10/30 column was purchased from Amersham Pharmacia Biotech (Freiburg, Germany). The LS 50B Spectrofluorometer was obtained from Perkin-Elmer (Oberlingen, Germany), and the protein standards for molecular weight calibration were from Sigma (Deisenhofen, Germany), the LS 50B Spectrofluorometer was obtained from Beckman Dickinson (Oxford, UK). Rottlerin, bisindolylmaleimide I, and Ca2+-ionophore A23187 were from Merck Biosciences (Nottingham, UK). HSA, ADP, penicillin, gentamycin, the 5-HT2A2C receptor antagonist ritanerpin, the 5-HT2A2C receptor agonist DOI, and all general chemicals were obtained from Sigma-Aldrich (Dorset, UK). The low-flux Fresenius polysulfone hemodialysis membrane was purchased from Fresenius Medical Care (Bad Homburg, Germany).

Preparation of Platelet-Rich Plasma

Blood was obtained from healthy control subjects who had no history of cardiovascular disease or hypertension and were not using any medication. Ten milliliters of blood were withdrawn using a standard 16-gauge hypodermic needle into acid citrate dextrose and then immediately centrifuged at 100 g for 15 min at 20°C (Beckman J6-MC). The platelet-rich plasma (PRP) fraction was removed and subsequently incubated under various treatment conditions as described below.

Preparation of HSA-AGE

This was prepared by incubating 1 M glucose with 50 mg/ml HSA in 100 mM PBS (pH 7.4) in the presence of 1.5 mM PMSE, 1 mM EDTA, 100 μg/ml penicillin, and 40 μg/ml gentamicin for 2, 4, or 6 wk in the dark at 37°C under sterile conditions as described previously (34). An unmodified protein control was prepared in parallel and, at the end of the incubation, all proteins were extensively dialyzed (3 washes/18 h at 4°C) against PBS (pH 7.4, 4°C) to remove unreacted sugar, separated into aliquots, and stored at −20°C before use.

Analysis of HSA-AGE Proteins

Total HSA-AGE content was determined as arbitrary fluorescence intensity per milligram of HSA. Briefly, HSA concentration was determined by size exclusion chromatography. Protein samples were initially diluted 1:10 in 10 mM PBS containing 300 mM NaCl; 20 μl were then injected onto a Superdex HR75 10/30 HPLC column, and samples were separated using 10 mM PBS containing 300 mM NaCl. The column was calibrated using a set of proteins in the molecular weight range of 6.5–67 kDa (aprotinin, myoglobin, carbonic anhydrase, ovalbumin, and HSA). Proteins were detected by absorbance at 220 nm, and HSA was used for quantitative calibration using peak areas. AGE formation was measured by spectrophotometry as previously described (14). Briefly, protein samples were diluted 1:1,000 in PBS (pH 7.4), and fluorescence emission was detected at 430 nm after excitation at 350 nm using a LS 50B spectrofluorometer. Glucose concentration was determined using a Yellow Springs YSI 2300 STAT Plus Analyzer, and endotoxin levels were assessed using the ENDOSAFE LAL Gel Clot Test.

Measurement of PS Externalization in PRP

PRP was prepared as described above and then incubated with either 1) 0–2 mg/ml of 2-, 4-, or 6-wk HSA-AGE or HSA control for 10 min or 2) 0.5 mg/ml of 6-wk HSA-AGE or HSA control for increasing times up to 30 min. For comparison purposes, we also examined the effect of ADP (0.5–100 nM), a classic physiological activator of human platelets (7). PS externalization was then determined using cell membrane-impermeant, PS-specific, annexin V-FITC conjugate and CD61-PerCP as a platelet-specific marker with subsequent quantification by a FACScan supporting Lysis II software. Briefly, 7.5 μl Ca2+-binding buffer (10 mM HEPES, 140 mM NaCl, 1% BSA, and 3 mM CaCl2; pH 7.4), 3 μl of 10 mM glycine-proline-arginine-proline (GPRP), 5 μl PerCP-labeled CD61 (glycoprotein-llb/llla) monoclonal antibody, 4 μl FITC-labeled annexin-V, and 3 μl PRP were added to FACScan-specific tubes. Blank samples were prepared as above without FITC-annexin V. Each sample was then left on ice for 15 min in the dark prior to analysis, which counted 10,000 cells from the total platelet population. The FACScan was calibrated monthly with Dako fluorospheres, and a standard curve fit procedure was used to enable data to be expressed as total numbers of binding sites.

Effect of Inhibition of Caspase 3

PRP was preincubated with 20 μM of the caspase-3 inhibitor Z-DEVD-FMK or its negative control Z-FAD-FMK (8) for 30 min and then either 0.5 mg/ml of 6-wk HSA-AGE were added for 10 min or 100 nM of ADP were added for 1 min. The extent of PS externalization was determined as described above.

Effect of Inhibition of PKC-δ

Two inhibitors were used in these experiments. First, PRP was preincubated for 10 min with 10 nM of the PKC inhibitor bisindolylmaleimide I; this inhibits both the classical PKC (PKC-α, -β, and -γ) and novel PKC (PKC-δ and -ε) isoforms (33, 38). Six-week HSA-AGE (0.5 mg/ml) was then added for 10 min, and PS externalization was determined as described above. Second, PRP was preincubated with 10 μM of the PKC-δ inhibitor rottlerin for 5 min. At this concentration, the drug has been reported to block PKC-δ activity by ~80–90% while having no effect on representative examples of either the cPKC isoforms e.g., PKC-α, or atypical PKC isoforms, e.g., PKC-ζ (6). Then, either 0.5 mg/ml of 6-wk HSA-AGE were added for 10 min or 100 nM of ADP were added for 1 min. PS externalization was determined as described above.

Effect of a 5-HT2A2C Receptor Antagonist

PRP was preincubated for 30 min with increasing concentrations (10 nM–1 μM) of the 5-HT2A2C receptor antagonist ritanerpin (19). Then, either 0.5 mg/ml of 6-wk HSA-AGE were added for 10 min or 100 nM of ADP were added for 1 min. The extent of PS externalization was determined as described above.

Effect of a 5-HT2A2C Receptor Antagonist

PRP was incubated with either 1) 1 nM–10 μM of the 5-HT2A2C receptor agonist DOI for 10 min or 2) 1 μM DOI, a concentration...
previously shown to optimally activate extracellular signal-regulated kinases in healthy human platelets (9, 20) for increasing times up to 30 min. We also investigated the ability of the 5-HT2A/2C receptor antagonist ritanserin to block the effect of DOI. This agonist is classified as a category 1 drug and was used as specified in a licence issued by the Home Office. PS externalization was determined as described above.

Collection of Ultrafiltrate From Individuals With CKD or CKD and Type 2 Diabetes

Eight individuals (4 individuals with CKD and 4 individuals with CKD and Type 2 diabetes) gave their informed consent to participate in an initial study and were subject to conventional hemodialysis using a low-flux Fresenius polysulfone membrane. To collect pure ultrafiltrate, the dialysis fluid was disconnected from the dialyser for a short period 20 min after the start of treatment. The dialysis fluid pathway was evacuated, the ultrafiltrate was allowed to flow for 2–3 min, and 3 ml of ultrafiltrate were collected into an endotoxin-free tube. The system was then reconnected, and the treatment was continued.

Response to a 5-HT2A/2C receptor antagonist.

PRP was preincubated with or without the 5-HT2A/2C receptor antagonist ritanserin (1 μM) for 30 min at 37°C. Ultrafiltrate (10 μl) from individuals with either CKD or CKD and Type 2 diabetes was then added, and the incubation was continued for a further 10 min. PS externalization was then determined using annexin V-FITC and CD61-PerCP with subsequent quantification by FACScan. Data are expressed as total binding sites, and statistical analysis was determined as areas under the curve (AUC) (21).

Statistical Analysis

Statistical analysis was undertaken using Minitab 14 (Minitab, State College, PA). Experiments with ADP, caspase-3/PKC-δ inhibitors, the 5-HT2A/2C receptor antagonist, and ultrafiltrate were analyzed using Student’s t-test and are presented as means ± SE, where $P < 0.05$, $P < 0.01$, and $P < 0.001$ with respect to the relevant
control. Serial measurements with HSA-AGE and the 5-HT<sub>2A/2C</sub> receptor agonist were analyzed as summary measures (21), i.e., the areas under the concentration or time curves (AUC). All experiments were performed on at least three occasions.

RESULTS

SDS-PAGE analysis using a 10.5% resolving gel and detection with Coomassie blue showed that all three HSA-AGE preparations had a higher molecular weight compared with their respective HSA controls (Fig. 1). The formation of AGE in the presence of glucose was also confirmed by the initially clear incubation medium becoming brown and by standard fluorescence measurements at 350/430 nm. In all samples, the glucose concentration was below 2 mM and the endotoxin level was ~1 EU/ml, corresponding to <0.2 ng/ml.

When fluorescence intensity was measured as a marker of total AGE formation and expressed relative to HSA content, the controls prepared in the absence of glucose remained constant (1.35–1.65 arbitrary units/mg) during the 6-wk incubation period. In contrast, HSA-AGE increased to 5.57 U/mg at 2 wk, 13.43 U/mg at 4 wk, and 14.9 U/mg at 6 wk.

When PRP from a healthy control was incubated with both annexin V-FITC and CD61-PerCP and then analyzed by flow cytometry, a single large platelet population was observed (Fig. 2, A and B). However, in the presence of 0.5 mg/ml of 6-wk HSA-AGE, a small (~2%) subpopulation, expressing elevated levels of PS externalization, became apparent (Fig. 2, E and F), which was not observed with the corresponding HSA control (Fig. 2, C and D) prepared in the absence of glucose. Moreover, a small platelet subpopulation was also observed in individuals with diabetes and CKD (Fig. 2, G and H). In addition, a similar response was also seen when scramblase was activated directly (3) with a suboptimal concentration (2 μM) of the Ca<sup>2+</sup> ionophore A23187 (Fig. 2, I and J).

![Fig. 4. Effect of ADP on PS externalization in a subpopulation of human platelets. PRP from healthy controls was incubated with (filled bars) or without (open bars) either increasing concentrations of ADP (0.5–100 nM) for 1 min (A) or 100 nM ADP for increasing times up to 10 min at 37°C (B). PS externalization was then determined using annexin V-FITC and CD61-PerCP with subsequent quantification by FACScan. Data are expressed as total binding sites and are shown as means ± SE of 3 independent experiments with statistical analysis by Student’s t-test. *P < 0.05, **P < 0.01, and ***P < 0.001 with respect to the relevant control.](http://ajpcell.physiology.org/)

![Fig. 5. Effect of the Caspase-3 inhibitor Z-DVED-FMK (ZDF) on PS externalization in response to HSA-AGE or ADP in a subpopulation of human platelets. PRP from healthy controls was preincubated with or without 20 μM of the caspase-3 inhibitor ZDF for 30 min at 37°C. Samples were then treated with either 0.5 mg/ml of 6-wk HSA-AGE for a further 10 min or 100 nM of ADP for 1 min. PS externalization was determined using annexin V-FITC and CD61-PerCP with subsequent quantification by FACScan. Data are expressed as total binding sites and are shown as means ± SE of 3 independent experiments with statistical analysis by Student’s t-test. ***P < 0.001 with respect to the relevant control.](http://ajpcell.physiology.org/)

![Fig. 6. Effect of the PKC-δ inhibitor rottlerin (Rottl) on PS externalization in response to HSA-AGE or ADP in a subpopulation of human platelets. PRP from healthy controls was preincubated with or without 10 μM of the PKC-δ inhibitor rottlerin for 5 min at 37°C. Samples were then treated with either 0.5 mg/ml of 6-wk HSA-AGE for a further 10 min or 100 nM of ADP for 1 min. PS externalization was determined using annexin V-FITC and CD61-PerCP with subsequent quantification by FACScan. Data are expressed as total binding sites and are shown as means ± SE of 3 independent experiments with statistical analysis by Student’s t-test. **P < 0.01 with respect to the relevant control.](http://ajpcell.physiology.org/)
Interestingly, the mean fluorescence intensity of the platelet subpopulation induced by HSA-AGE, A23187 and that present in individuals with diabetes and CKD were comparable. We have therefore subsequently characterized the response of this small population to HSA-AGE in detail.

In a 10-min incubation, the 2-wk HSA-AGE preparation stimulated a significant (AUC, $P < 0.01$ vs. the appropriate control) concentration-dependent ($0–2$ mg/ml) increase in PS externalization in this small platelet population, which was approximately threefold in magnitude at 2 mg/ml (Fig. 3A). The 4- and 6-wk HSA-AGE preparations also elicited significant increases in PS externalization over the same concentration range (AUC, $P < 0.01$ and $P < 0.001$, respectively, vs. the appropriate controls), with their maximal effects being approximately fivefold in magnitude (Fig. 3A). The responses we observed with these two preparations were significantly greater than those seen with 2-wk HSA-AGE (AUC, $P < 0.05$ and $P < 0.01$, respectively), but there were no significant differences between the 4- and 6-wk preparations (AUC, $P = 0.3$). These effects on PS externalization mirrored the changes in total HSA-AGE content of the preparations (arbitrary units/mg HSA) in that there was a large increase between 2 and 4 wk but little further change between 4 and 6 wk (see above). The three HSA control preparations had no significant effect on PS externalization in this subpopulation (Fig. 3A).

When PRP was incubated for increasing times up to 30 min with 0.5 mg/ml of the six-wk HSA-AGE preparation, a significant increase in PS externalization was observed (AUC, $P = 0.018$), which was maximal at 10 min. Beyond this, the response declined but was still elevated above control values after 30 min of incubation (Fig. 3B).

ADP is a classic physiological activator of human platelets (7), but its ability to elicit PS externalization in these cells has not been reported to date. The incubation of PRP with ADP also resulted in the appearance of a small subpopulation of platelets expressing elevated PS externalization. Moreover, the mean fluorescence intensity of this cell fraction was comparable with that observed with HSA-AGE and A23187 and in individuals with diabetes and CKD (data not shown). The effect of ADP was concentration dependent, with a maximal response at 5 nM (Fig. 4A), but, unlike HSA-AGE, it was rapid in onset, being optimal at 1 min and sustained for at least 10 min (Fig. 4B).

In addition to its role in thrombosis, PS externalization has also been implicated in apoptosis via caspase-3-mediated cleavage of PKC-δ (4, 25). When PRP was preincubated for 30 min with the caspase-3 inhibitor Z-DVED-FMK (20 μM) and then subsequently challenged for 10 min with 0.5 mg/ml of 6-wk HSA-AGE, there was no inhibition of the HSA-AGE effect on PS externalization (Fig. 5), suggesting that this response does not involve caspase-3. In contrast, the response to ADP (100 nM) added for 1 min was abolished by Z-DVED-FMK (Fig. 5). The negative control Z-FAD-FMK (20 μM) had no effect (data not shown).

Preincubation of PRP for 10 min with the cPKC/nPKC inhibitor bisindolylmaleimide I (10 nM) inhibited the increase in PS externalization in the small platelet subpopulation elicited by the addition of 0.5 mg/ml of 6-wk HSA-AGE for 10 min. For example, PS externalization increased from 22 ± 1 to 103 ± 4 fluorescent binding sites·10² ($P < 0.001$ vs. control, $n = 3$) in the absence of bisindolylmaleimide I but was not significantly different from control (27 ± 5 fluorescent binding sites·10²) in its presence. Similarly, preincubation of PRP for 5 min with the PKC-δ inhibitor rottlerin (10 μM) completely prevented the increase in PS externalization elicited by the addition of 0.5 mg/ml of 6-wk HSA-AGE for 10 min (Fig. 6), suggesting a role for PKC-δ in the HSA-AGE-mediated response. The effect of 100 nM ADP added for 1 min was also abolished (Fig. 6).

Preincubation of PRP for 30 min with increasing concentrations (10 nM–1 μM) of the 5-HT₂A/₂C receptor antagonist ritanserin resulted in a dose-dependent inhibition of PS externalization elicited by 10 min of incubation with 0.5 mg/ml of 6-wk HSA-AGE. At 1 μM ritanserin, the response to HSA-AGE was completely abolished (Fig. 7). This suggests that the effects of HSA-AGE on PS externalization in the small subpopulation of platelets are mediated via 5-HT₂A/₂C receptors. In contrast, the response to 100 nM ADP added for 1 min was unaffected by 30 min of preincubation with 1 μM ritanserin (Fig. 7).

Incubation of PRP with the 5-HT₂A/₂C receptor agonist DOI elicited PS externalization in a small platelet subpopulation (Fig. 8, A–D). Moreover, the mean fluorescence intensity of this cell fraction was comparable with that seen with HSA-AGE, ADP, and A23187 and in individuals with diabetes and CKD (Fig. 2). The response to DOI was dose dependent, being

![Fig. 7. Effect of the 5-HT₂A/₂C receptor antagonist ritanserin (Rit) on PS externalization in response to HSA-AGE or ADP in a subpopulation of human platelets. PRP from healthy controls was preincubated with or without the 5-HT₂A/₂C receptor antagonist ritanserin (10 nM–1 μM) for 30 min at 37°C. Samples were then treated with either 0.5 mg/ml of 6-wk HSA-AGE for a further 10 min or 100 nM of ADP for 1 min. PS externalization was determined using annexin V-FITC and CD61-PerCP with subsequent quantification by FACSscan. Data are expressed as total binding sites and are shown as means ± SE of 3 independent experiments with statistical analysis by Student’s t-test. ***$P < 0.001$ with respect to the relevant control.](image-url)
optimal at 1 μM (Fig. 8E), and a significant (AUC, P = 0.011) time-dependent increase in PS externalization was observed when PRP was incubated with 1 μM DOI. This response mimicked the effect seen with HSA-AGE (Fig. 3B) in that it was maximal at 10 min and then returned to basal values (Fig. 8F). In addition, the effect of DOI was inhibited by the 5-HT2A/2C receptor antagonist ritanserin. For example, incubation of PRP with 1 μM DOI for 10 min increased PS exter-

Fig. 8. Effect of the 5-HT2A/2C receptor agonist 1,2,5-dimethoxy-4-iodophenyl-2-aminopropane (DOI) on PS externalization in a subpopulation of human platelets. PRP from a healthy control was incubated with or without the 5-HT2A/2C receptor agonist DOI (1 μM) for 10 min at 37°C. PS externalization was then assessed using annexin V-FITC and CD61-PerCP with subsequent quantification by FACScan. Data are presented as both histograms (a and c) and dot plots (b and d) for control (a and b) and DOI (c and d). PRP from healthy controls was then subsequently incubated either with (filled bars) or without (open bars) increasing concentrations (1 nM–10 μM) of DOI for 10 min (B) or with (filled circles) or without (open circles) DOI (1 μM) for increasing times up to 30 min at 37°C (C). PS externalization was quantified by FACScan. Data are expressed as total binding sites, and statistical analysis was determined either by Student’s t-test (B) or as AUC (C) (21). *P < 0.05 and **P < 0.01 with respect to the relevant control.
nalization from 21 ± 1 to 102 ± 8 fluorescent binding sites·10^2 (P < 0.001 vs. control, n = 3). However, 30 min of preincubation with 1 μM ritanserin reduced PS externalization in the presence of 1 μM DOI to 37 ± 4 fluorescent binding sites·10^2. This was not significantly different from the effect of 1 μM ritanserin alone (26 ± 1 fluorescent binding sites·10^2, n = 3).

The addition of ultrafiltrate from either of the two patient groups to platelets from healthy controls increased PS externalization in a small platelet subpopulation. The effect observed with ultrafiltrate obtained from individuals with CKD and Type 2 diabetes was significantly greater than that seen with CKD alone (Fig. 9). Moreover, preincubation of platelets with the 5-HT<sub>2A/2C</sub> receptor antagonist ritanserin (1 μM), under conditions that abolished PS externalization in response to HSA-AGE, also inhibited PS externalization elicited by ultrafiltrate from patients with CKD by ~45% and from individuals with both CKD and Type 2 diabetes by ~35% (Fig. 9).

**DISCUSSION**

The high cardiovascular morbidity and mortality observed in patients with diabetes and/or CKD cannot be fully explained by traditional risk factors, suggesting the involvement of other components (5, 10, 11). Increased accumulation of AGE has been reported in both tissues and plasma of such individuals (18, 36) and is thought to contribute to the development and progression of cardiovascular disease (32), although the precise mechanism(s) involved have yet to be clarified.

We report here, for the first time, that HSA-AGE stimulates PS externalization in a small subpopulation of human platelets. PS externalization via scramblase activation fulfills a dual role. It is critical for the efficient propagation and control of coagulation and thrombosis (41) or can act as an important and necessary signal for apoptotic cell removal by phagocytes (4). In either case, PKC-δ activation is required, and our use of both a cPKC/nPKC inhibitor (bisindolylmaleimide I) and a specific inhibitor of this isoform (rottlerin) supports a role for this signaling molecule in HSA-AGE-mediated PS externalization in human platelets. Moreover, it has previously been shown in other cell types that 5-HT<sub>2A/2C</sub> receptor stimulation (see below) correlates with increased PKC-δ activity (31).

In nucleated cells, the time course of PS externalization relating to thrombin formation is very different compared with that linked to apoptosis, i.e., activation-induced scramblase activity is rapid and transient, whereas it is delayed and sustained during apoptosis (6). The time course we report here for HSA-AGE-mediated PS externalization correlates with cell activation rather than apoptosis in nucleated cells.

PKC-δ is cleaved and activated during apoptosis by caspase-3 (25). In contrast, cell activation-induced scramblase activity, although also dependent on PKC-δ, is independent of caspase-3 (6). Our demonstration that HSA-AGE-mediated PS externalization was not blocked by the caspase-3 inhibitor Z-DEVD-FMK also correlates with cell activation in nucleated cells.

It is however, possible that, in platelets, these two events are interrelated. When activated by a hemostatic agent, platelets undergo a number of changes, including cell shrinkage, PS externalization, and cell disintegration. These changes promote hemostasis by inducing platelet aggregation/adhesion and activation of the coagulation cascade (39). Recent studies (29, 39) have identified the presence of caspase activity (including caspase-3) in human platelets, although, at present, their precise role in these cells remains unclear. Our data imply that the effect of ADP on PS externalization, but not that of HSA-AGE, is mediated via caspase-3. The significance of this requires further investigation.

A heterogeneous response of platelets to PS externalization has been reported previously (2, 40). A recent study (40) demonstrated the effects of thrombin and collagen on PS externalization in a small subpopulation of human platelets. Moreover, these authors reported that the effect of thrombin (5 nM), for example, was mediated on ~4% of the total platelet population (40), a value comparable with that we reported here with HSA-AGE. Wolffs et al. (40) also showed that the mean annexin V fluorescence intensity of the platelet subpopulation was comparable, regardless of the agonist (or combination of agonists) used, with that found upon stimulation with the Ca<sup>2+</sup> ionophore, which elicited a maximal response. We report here a similar observation following incubation of platelets with either HSA-AGE, ADP, and DOI or in platelets from individuals with diabetes/CKD compared with the effect of A23187. A good correlation (r = 0.99) was also seen between the size of the platelet subpopulation and procoagulant activity, and thus these authors concluded that the increased procoagulant activity of a platelet suspension is related to the increase in the fraction of platelets exhibiting maximal PS exposure rather than to the entire platelet suspension (40).

It is intriguing to speculate on the criteria that sensitize a subpopulation of cells (or desensitize the majority of platelets) to these agonists. Two possibilities are differences in Ca<sup>2+</sup> homeostasis that may then be reflected in scramblase activity and/or in specific agonist receptor density. The relationship between platelet age and susceptibility to PS externalization is also unknown. The development of novel treatments for vari-

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**Fig. 9.** Effect of the 5-HT<sub>2A/2C</sub> receptor antagonist ritanserin on PS externalization in response to ultrafiltrate from individuals with either CKD or CKD and Type 2 diabetes in a subpopulation of human platelets. PRP from healthy controls was preincubated with or without 1 μM of the 5-HT<sub>2A/2C</sub> receptor antagonist ritanserin for 30 min at 37°C. Ultrafiltrate (UF20) from individuals with either CKD or CKD and Type 2 diabetes who were undergoing low-flux hemodialysis was then added, and the incubation was continued for a further 10 min. PS externalization was determined using annexin V-FITC and CD61-PerCP with subsequent quantification by FACSScan. Data are expressed as total binding sites and are shown as means ± SE of 4 independent experiments with statistical analysis by Student’s t-test. *P < 0.05 and ***P < 0.001 with respect to the relevant control.
ous pathophysiological states may be facilitated by a more detailed understanding of these phenomena.

It is clear that many effects of AGE on a number of cell types are mediated via RAGE (23). However, as far as we are aware, RAGE has not been identified on human platelets, and, in preliminary experiments with anti-RAGE antibody, using methodology that confirmed the presence of RAGE on mononuclear phagocytes (28), we were unable to detect this receptor on human platelets (unpublished observations). A previous study in human platelets demonstrated that ADP-induced aggregation could be enhanced by the addition of AGE but that this effect was diminished by the addition of sarpogrelate (13). This suggests that the effects of AGE on human platelets are mediated via the 5-HT2A receptor. As sarpogrelate is not available commercially, we employed ritanserin, a 5-HT2A/2C receptor antagonist (9), to establish that HSA-AGE-mediated PS externalization is mediated via this receptor in these cells. The presence of 5-HT2A/2C receptors on human platelets has long been established (e.g., Ref. 1). The inability of ritanserin to inhibit the response to ADP established that it does not act in a nonspecific manner on other components of the pathway such as PKC-δ or scramblase activity. To then confirm that activation of this receptor is indeed linked to PS externalization, we employed the 5-HT2A/2C agonist DOI (9) and demonstrated that this agent mimics the effect of HSA-AGE in eliciting a transient, dose-dependent stimulation of PS externalization in a subpopulation of human platelets that is maximal at 10 min. Thus, our data support a signaling pathway in which HSA-AGE mediates PS externalization via a mechanism involving PKC-δ but that is independent of caspase-3.

Although we did not assess HSA-AGE in terms of units of AGE per milligram of protein, data from previous publications indicate that the concentration range we used is similar to that detected in disease states in vivo (13, 17). Similarly, we did not attempt to establish the active component(s) among those likely to be present in our HSA-AGE preparation, which was responsible for the effects we have described. However, a previous study (12) examining the ability of BSA-AGE to potentiate ADP-mediated platelet macroaggregation demonstrated that neither pentosidine nor Nε-(carboxymethyl)lysine was responsible (12). Thus, the epitope(s) of HSA-AGE responsible for mediating platelet PS externalization remains to be established.

Evidence that the pathway we have described is of physiological and clinical significance is provided by the demonstration that the 5-HT2A/2C receptor antagonist ritanserin, under conditions that abolished PS externalization in response to HSA-AGE, also caused substantial inhibition of the effect of HSA-AGE, also caused substantial inhibition of the effect. This suggests that a significant fraction of the elevated platelet PS externalization observed in these individuals is mediated via the same receptor as the HSA-AGE generated in vitro.

Our study represents an important first step toward identifying the serum factor(s)/mechanism(s) responsible for increased platelet PS externalization and potentially facilitates the development of novel treatments aimed at manipulating platelet responses in pathophysiological states such as diabetes, CKD, myocardial infarction, and stroke.

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REFERENCES


