

RESEARCH ARTICLE

Reactive carbonyl compounds (RCCs) cause aggregation and dysfunction of fibrinogen

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ABSTRACT

Fibrinogen is a key protein involved in coagulation and its deposition on blood vessel walls plays an important role in the pathology of atherosclerosis. Although the causes of fibrinogen (fibrin) deposition have been studied in depth, little is known about the relationship between fibrinogen deposition and reactive carbonyl compounds (RCCs), compounds which are produced and released into the blood and react with plasma protein especially under conditions of oxidative stress and inflammation. Here, we investigated the effect of glycolaldehyde on the activity and deposition of fibrinogen compared with the common RCCs acrolein, methylglyoxal, glyoxal and malondialdehyde. At the same concentration (1 mmol/L), glycolaldehyde and acrolein had a stronger suppressive effect on fibrinogen activation than the other three RCCs. Fibrinogen aggregated when it was respectively incubated with glycolaldehyde and the other RCCs, as demonstrated by SDS-PAGE, electron microscopy and intrinsic fluorescence intensity measurements. Staining with Congo Red showed that glycolaldehyde- and acrolein-fibrinogen distinctly formed amyloid-like aggregations. Furthermore, the five RCCs, particularly glycolaldehyde and acrolein, delayed human plasma coagulation. Only glycolaldehyde showed a markedly suppressive effect on fibrinogenesis, none did the other four RCCs when their physiological blood concentrations were employed, respectively. Taken together, it is glycolaldehyde that suppresses fibrinogenesis and induces protein aggregation most effectively, suggesting a putative

pathological process for fibrinogen (fibrin) deposition in the blood.

KEYWORDS fibrinogen, acrolein, glycolaldehyde, glyoxal, malondialdehyde, methylglyoxal

INTRODUCTION

Fibrinogen is a key protein involved in blood coagulation and hemostasis (Liu et al., 1979; Phillips et al., 1988; Sidelmann et al., 2000). Both the amount (physiological concentrations normally range from 1.5 mg/mL to 4 mg/mL in human blood) and the activity of fibrinogen are important for maintaining the equilibrium of coagulation (Kamath and Lip, 2003). Fibrinogen and its degradation products are known components of stable and unstable atherosclerotic plaques. Furthermore, fibrinogen mediates the atherogenic accumulation of lipoprotein in blood vessel walls (Argraves et al., 2009). *In vivo*, fibrinogen is deposited at atherosclerosis-prone sites before other signs of atherosclerosis are apparent (Orr et al., 2005). *In vitro*, antibody-mediated blocking of tissue factor activity decreases spontaneous fibrinogen deposition, and thus diminishes thrombus formation on atherosclerotic plaques (Stoll and Bendszus, 2006). There are numerous risk factors associated with atherosclerosis, including aging, hyperlipidemia and platelet aggregation (Doruk et al., 2003). However, post-translational modifications such as glycation, nitration and oxidation are also important factors to trigger deposits of fibrinogen and its products as described by Weisel (2005).

The level of interest in reactive carbonyl compounds (RCCs), potential bifunctional compounds, has risen

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dramatically in recent years due to their ability to promote post-translational modifications and cross-linking between proteins and their aggregation. Some familiar and widespread reactive carbonyl compounds such as glycolaldehyde, acrolein, glyoxal, methylglyoxal and malondialdehyde are generated from the peroxidation of membrane lipids (Picklo et al., 2002; Uchida, 1999, 2003; Zarkovic, 2003) and glycation of proteins (Nagai et al., 2000) under oxidative stress *in vivo*. These RCCs all have an active aldehyde group which readily reacts with the side chains of lysinyl and arginyl residues in proteins. These RCCs are present in the blood and their concentrations under physiological conditions have been determined; the order from the highest concentration to the lowest concentration in human plasma is glycolaldehyde (Andrades et al., 2009), malondialdehyde (Nielsen et al., 1997), acrolein (Igarashi et al., 2006), glyoxal and methylglyoxal (Lapolla et al., 2005). Investigations of the effects of RCCs on the conformation and function of fibrinogen are necessary to clarify the mechanisms of hematologic diseases related to RCCs.

Andrades and colleagues have shown that glycolaldehyde causes post-translational modifications of fibrinogen and delays blood clotting (Andrades et al., 2009). As reported, the mechanism for glycolaldehyde-induced delay in the coagulation of human plasma is related to the modification of fibrinogen's lysinyl and arginyl residues. However, whether glycolaldehyde triggers fibrinogen aggregation, which is well known to be associated with protein dysfunction, is also an important risk factor in the glycolaldehyde-induced delay in coagulation. The level of glycolaldehyde is increased during inflammation and hyperglycemia (Kawamura et al., 2000; Anderson and Heinecke, 2003). With the exception of blood

coagulation, however, little is known at present about the effect of glycolaldehyde on fibrinogen aggregation and deposits in blood.

To understand the role of RCCs in protein modification and the role of RCC-induced fibrinogen aggregation in the dysfunction of fibrinogenesis, we have studied how glycolaldehyde triggers aggregation and inactivation of fibrinogen, in comparison with acrolein, methylglyoxal, glyoxal and malondialdehyde, respectively. Our investigation of the endogenous risk factors that affect fibrinogen will help to elucidate the mechanisms underlying fibrinogen aggregation and dysfunction related diseases such as atherosclerosis.

RESULTS

Inactivation of fibrinogen in the presence of RCCs

In order to investigate the effect of reactive carbonyl compounds on the activity of fibrinogen, glycolaldehyde, acrolein, methylglyoxal, glyoxal and malondialdehyde were reacted with fibrinogen (at a final concentration of 3 mg/mL) before thrombin was added to promote fibrinogenesis under the same conditions. In the presence of glycolaldehyde, acrolein, methylglyoxal, glyoxal or malondialdehyde (Fig. 1A and 1B), fibrinogen activity decreased significantly with time. Inactivation of fibrinogen approached 100% within 2 h. Glycolaldehyde and acrolein were markedly effective in suppressing fibrinogenesis compared with malondialdehyde, methylglyoxal and glyoxal (Fig. S1A and S1B). In the absence of RCCs (fibrinogen control) there were no significant changes in the formation of fibrin. These results demonstrate that glycolaldehyde and acrolein suppress fibrinogenesis under the experimental conditions.

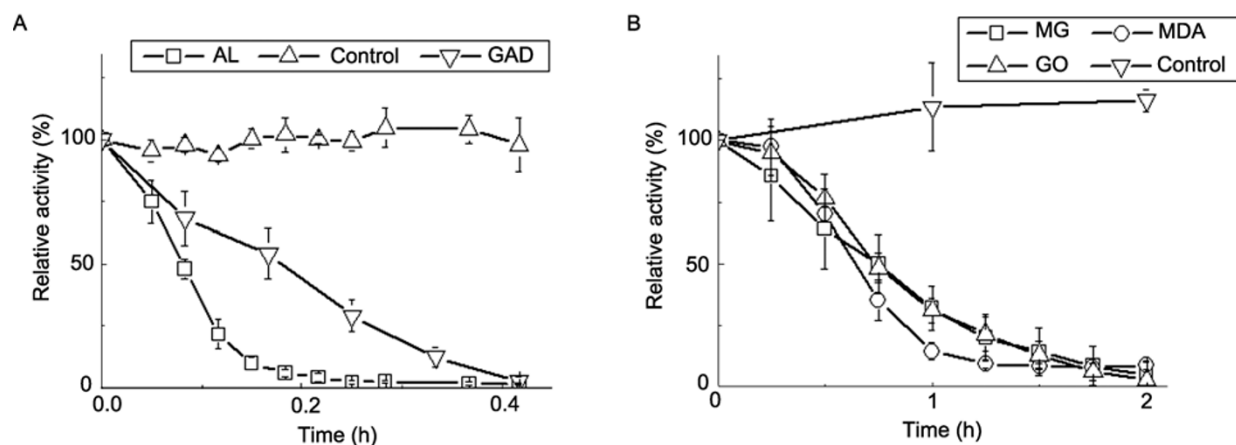


Figure 1. Changes in the activity of fibrinogen in the presence of reactive carbonyl compounds. Fibrinogen (at a final concentration of 3 mg/mL) was incubated with acrolein, glycolaldehyde, malondialdehyde, glyoxal, or methylglyoxal (9 mmol/L each) in PBS buffer (pH 7.4) at 37°C, and aliquots were taken at different time intervals to assay fibrinogen activity by addition of thrombin as described by Zhou et al. (1997). Fibrinogen incubated in the absence of reactive carbonyl compounds (RCCs) served as a control. AL: acrolein, GAD: glycolaldehyde, MDA: malondialdehyde, GO: glyoxal, MG: methylglyoxal.

To compare the effects of different RCCs on the suppression of fibrinogen activity, Tsou's method (Tsou, 1965) was employed to study the kinetics of fibrinogenesis and analyze the first order rate constants of RCC-induced activity suppression. The suppression process followed a biphasic pattern in the presence of all five RCCs (Table 1). The first order rate constant of the fast phase for acrolein was $6.40 \times 10^{-3} \text{ s}^{-1}$ (Table 1), about two folds greater than that of glycolaldehyde, and much higher than that for glyoxal, malondialdehyde and methylglyoxal. Under the experimental conditions used here, the capacity of these RCCs to suppress fibrinogenesis, from strongest to weakest, was: acrolein > glycolaldehyde > methylglyoxal > glyoxal \cong malondialdehyde.

Disruption of fibrinogenesis in the presence of RCCs

For the purpose of determining how RCCs disrupt fibrinogenesis, we observed images of the products (fibrin) of RCC-treated fibrinogen cleaved by thrombin using electron microscopy. Fibrinogen was respectively incubated with glycolaldehyde, acrolein, methylglyoxal, glyoxal and malondialdehyde for 2 h, and thrombin was added to activate the reaction mixture. The samples were allowed to stand at

room temperature for 30 min and aliquots were taken for observations. As shown in Fig. 2, no intact fibrin was observed in RCC-treated fibrinogen in the presence of thrombin but protein granules, aggregations and fibrils were present. RCC-treated fibrinogen cleaved by thrombin appeared as short and indistinct fibrils (though greater quantities of fibrils from thrombin-cleaved glyoxal- and

Table 1 First order rate constants for the suppression of fibrinogenesis in the presence of RCCs*

	Slow phase	Fast phase
Acrolein	12.82 ± 1.84	64.01 ± 8.42
Glycolaldehyde	11.03 ± 1.12	23.74 ± 3.54
Methylglyoxal	2.39 ± 0.24	8.01 ± 1.16
Malondialdehyde	2.46 ± 0.26	5.12 ± 4.36
Glyoxal	2.40 ± 0.21	5.12 ± 4.15
Fib alone as control	-	-

* All rate constants are in the order of $10^4 \cdot \text{s}^{-1}$.

** Suppression of fibrinogenesis in the presence of RCCs is a biphasic process consisting of a slow and a fast phase.

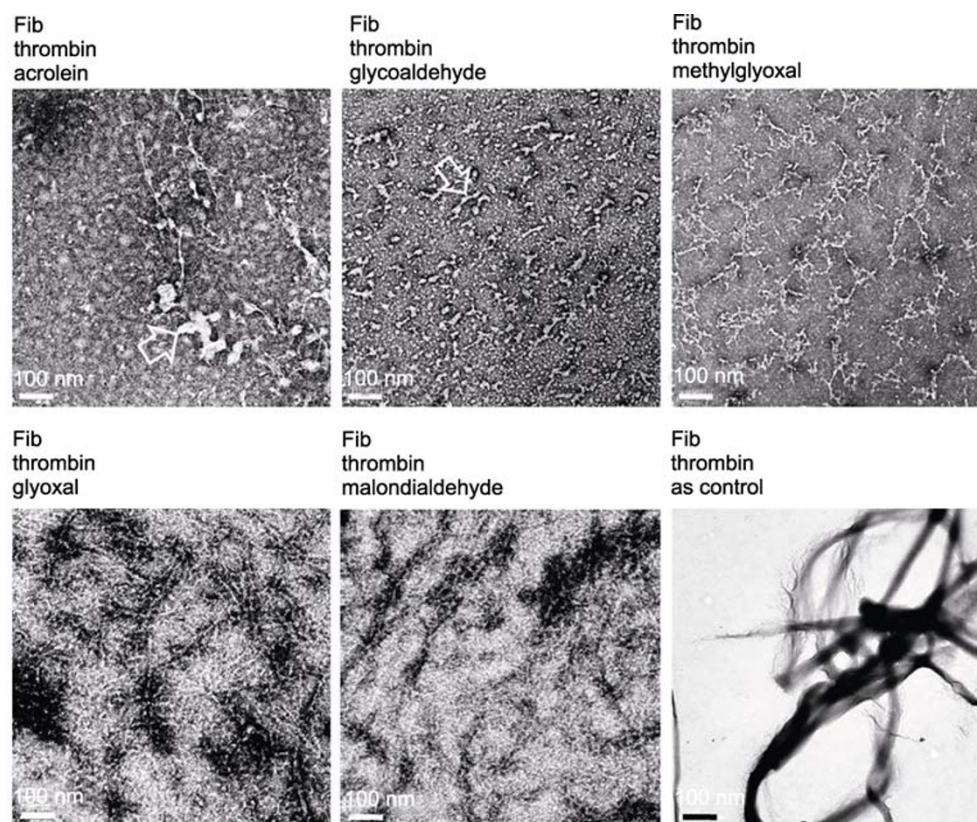


Figure 2. Morphology of fibrils after cleavage of RCC-fibrinogen adducts with thrombin. Reaction conditions were the same as those in Fig. 1, except that fibrinogen was incubated with each RCC for 2 h before thrombin was added. Samples were allowed to stand at room temperature for 30 min before aliquots were taken and observed by electron microscopy. Fibrinogen aggregation was pointed out with arrowheads. The experiment was repeated at least three times. Fib: fibrinogen.

malondialdehyde-treated fibrinogen were present). Furthermore, fibrils of glycolaldehyde and acrolein treated fibrinogen were less than the other three RCCs. Similar results were also observed when the incubation of fibrinogen with the RCCs was prolonged to 8 h (Fig. S2). However, when the fibrinogen positive control in the absence of RCCs was cleaved with thrombin, typical fibrin was formed. Fibrinogen alone as a negative control was observed to maintain its small globular-like profile, which would be shown in the later part. These results suggest that RCCs, glycolaldehyde and acrolein particularly react with fibrinogen and induce protein aggregation, leading to the suppression of fibrinogenesis under the experimental conditions used.

In order to clarify whether thrombin can still recognize the cleavage sites in the N-terminal region of fibrinogen to promote fibrinogenesis (Siebenlist et al., 2005), RCC-treated fibrinogen was incubated with thrombin and aliquots were examined by MALDI-TOF mass spectrometry. Cleavage of fibrinogen releases fibrinopeptide A and B, peptides of ~1900 Da and ~2400 Da in length. While thrombin could still recognize and cleave RCC-treated fibrinogen at the Arg-Gly site of the N-terminal of both the alpha-chain and beta-chain, releasing short peptides with molecular masses of ~1900 Da and ~2400 Da, glycolaldehyde-, acrolein- and glyoxal-treated fibrinogen produced fewer ~2400 Da peptides, suggesting that treatment with these RCCs gives rise to resistance to hydrolysis at the cleavage site of the beta-chain, and delays the release of fibrinopeptide B (Fig. S3B–S3D), in agreement with Riedel et al. (2011). Although glycolaldehyde-, acrolein- and glyoxal-treated fibrinogen hardly released ~2400 Da peptides, the suppression of fibrinogenesis of glycolaldehyde and acrolein is much stronger than that of glyoxal, suggesting the potential to trigger aggregation is most imperative for the RCCs to suppress fibrinogenesis. Cleavage of MDA-treated fibrinogen by thrombin, however, released both fibrinopeptide A and B, as was the case for the fibrinogen control (Fig. S3A and S3E). Neither fibrinopeptide A or B was detected in the MALDI-TOF trace of methylglyoxal-treated fibrinogen cleaved with thrombin (Fig. S3F), just as when fibrinogen alone (background signals) was used as a negative control (Fig. S3G). However, whether aggregation of RCC-treated fibrinogen occurs before or after thrombin cleavage is not clear.

Fibrinogen forms polymers in the presence of reactive carbonyl compounds

In further investigation of whether RCC-treated fibrinogen is prone to aggregation, fibrinogen was incubated respectively with glycolaldehyde, acrolein, methylglyoxal, glyoxal and malondialdehyde. The incubation time was as indicated in Figs. 3 and 4. Aliquots were taken and loaded on a reducing SDS-PAGE gel and subsequently stained with Coomassie blue. During the initial stage of the incubation, α -, β - and

γ -fibrinogen appeared as three distinct protein bands on the SDS-PAGE gel. Of the five RCCs, acrolein induced aggregation of fibrinogen most rapidly (Fig. 4), the protein started to self-polymerize around 30 min. Glycolaldehyde, methylglyoxal, glyoxal and malondialdehyde also produced protein aggregations of their fibrinogen-adducts, however, polymerization occurred more slowly, with a relaxation time of ~4 h. Fibrinogen in the absence of RCCs as control showed no polymerization. These results demonstrate that RCC-fibrinogen adducts self-polymerize, with the speed of polymerization being different for different RCC-treated proteins, That is to say, RCCs first trigger changes in protein conformation and induce aggregation, and this may be the mechanism by which RCCs disrupts fibrinogenesis under our experimental conditions.

The RCC-fibrinogen adducts in the reaction of fibrinogen with glycolaldehyde, acrolein, methylglyoxal, glyoxal and malondialdehyde had some common characteristics listed as follows. (1) RCCs, especially acrolein and glycolaldehyde trigger fibrinogen aggregations. (2) On SDS-PAGE gels, the intensity of the α -fibrinogen band was the first to decrease, suggesting that α -fibrinogen is more vulnerable to reactions with RCCs than β - and γ -fibrinogen, resulting in RCC- α -chain adduct aggregations. (3) Following the disappearance of α -fibrinogen, the intensity of β - and γ -fibrinogen bands also began to decrease. These results suggest that fibrinogen's capacity to react with RCCs to produce aggregations is in the order α -fibrinogen > β -fibrinogen > γ -fibrinogen.

Changes in fibrinogen conformation on reaction with RCCs

Having shown that RCC-fibrinogen adducts are prone to form polymers, we examined their intrinsic fluorescence to probe conformational changes in the protein. As shown in Fig. 5, the intensity of the intrinsic fluorescence of fibrinogen decreased markedly during incubation with RCCs. Glycolaldehyde and acrolein caused a rapid decrease in the intensity of the intrinsic fluorescence of fibrinogen. Although malondialdehyde rapidly quenched the intrinsic fluorescence of fibrinogen, this does not indicate that conformational changes occurred in the protein (see Discussion). Taken together, fibrinogen undergoes rapid conformational changes on reaction with RCCs, in particular with glycolaldehyde and acrolein.

The decrease in the intrinsic fluorescence of RCC-fibrinogen adducts followed a biphasic process, including a fast and a slow phase. Comparing the first order rate constants, acrolein and glycolaldehyde decreased protein intrinsic fluorescence more rapidly than the other RCCs and had greater first order rates for both the fast phase and the slow phase (Fig. 5, Table 2). Methylglyoxal led to a moderate decrease in the intrinsic fluorescence of fibrinogen, while glyoxal gave a slight decrease. The fibrinogen control in the

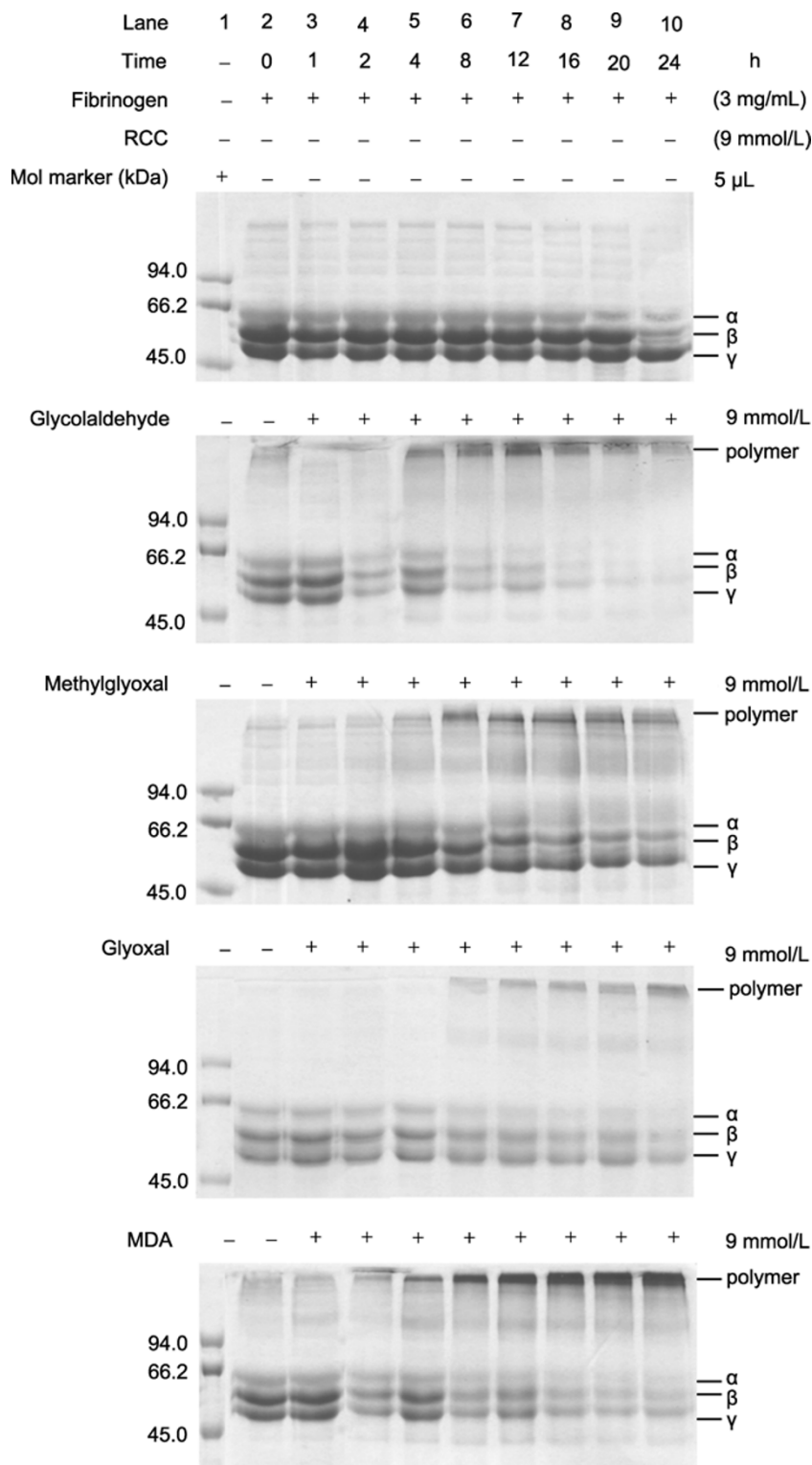


Figure 3. Polymerization of fibrinogen after incubation with different reactive carbonyl compounds for different time intervals. Conditions were the same as those in Fig. 1, except that fibrinogen was incubated with glycolaldehyde, methylglyoxal, malondialdehyde and glyoxal. Aliquots were taken at the time intervals indicated for analysis by 12% SDS-PAGE and gels were stained with Coomassie brilliant blue. Fibrinogen incubated without reactive carbonyl compounds was used as a control (top panel). The experiment was repeated at least three times.

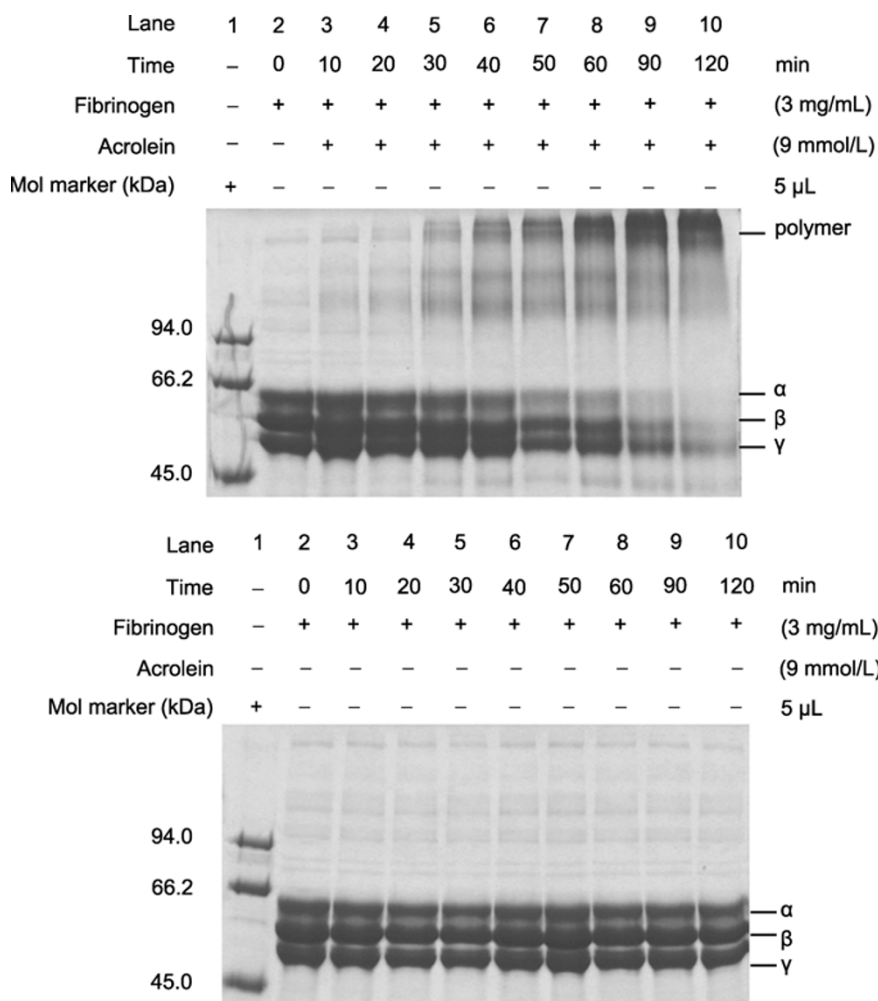


Figure 4. Fibrinogen polymerization on incubation with acrolein for different lengths of time. Conditions were the same as those in Fig. 3, except that acrolein was incubated with fibrinogen. Aliquots were taken at the time intervals indicated for analysis by 12% SDS-PAGE (top panel). Fibrinogen in the absence of acrolein was used as a control (bottom panel). The experiment was repeated at least three times.

absence of RCCs underwent a small but not significant decrease in intrinsic fluorescence. To observe whether the secondary structure of fibrinogen changed in the presence of RCCs, we performed circular dichroism (CD) on the RCC-protein adducts; no significant changes (compared to the control) were detected (data not shown), indicating that the secondary structure of fibrinogen did not change significantly under the experimental conditions used here. These results indicate that the reaction of fibrinogen with RCCs results in marked conformational changes of the protein.

RCC-fibrinogen adducts form molten globules

To further investigate the characteristics of RCC-induced conformational changes in fibrinogen, we observed RCC-treated proteins by electron microscopy (Fig. 6). Acrolein- and glycolaldehyde-treated fibrinogen formed

amorphous aggregations. Methylglyoxal-protein adducts showed short fibril-like aggregations. Reaction with glyoxal or malondialdehyde resulted in tiny fibrils and granules. Fibrinogen alone as a control had the appearance of small granules. Glycolaldehyde and acrolein produced more aggregations than the other three RCCs. That is, the RCC treatment leads to protein aggregation and thus disrupts fibrinogenesis.

In order to clarify the characteristics of RCC-protein adduct aggregations, we stained samples with Congo Red, a dye known to display a characteristic birefringence under polarized light after binding to materials. Congo Red staining serves as a positive indicator of amyloid deposition (Reinke and Gestwicki, 2011). Enhanced staining was observed when fibrinogen was incubated with RCCs (Fig. 7). Acrolein- and glycolaldehyde-fibrinogen adducts showed positive Congo Red staining distinctly.

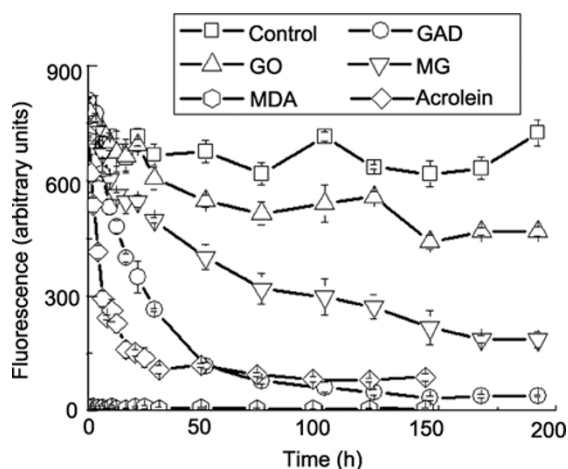


Figure 5. Changes in the intrinsic fluorescence of fibrinogen during incubation with RCCs. Fibrinogen was incubated with each of the RCCs in PBS solution containing 0.05% sodium azide at 37°C. Aliquots were taken to detect intrinsic fluorescence (λ_{ex} 280 nm; λ_{em} 310–500 nm) at different time intervals. Fibrinogen alone was used as a control. Table 2 shows the first order rate constants for changes in intrinsic fluorescence (calculated as described by Tsou (1965)) during the reaction of fibrinogen with different RCCs.

We next investigated the solubility of RCC-fibrinogen adducts by measuring light-scattering of the protein solution

during incubation with RCCs (Fig. 8). The intensity of light-scattering of the solutions of glycolaldehyde- and acrolein-fibrinogen adducts increased significantly with time. However, light-scattering of methylglyoxal-, glyoxal- or malondialdehyde-treated proteins showed little change, as was the case when fibrinogen alone was used as a control. This demonstrates that glycolaldehyde- and acrolein-fibrinogen adducts formed some insoluble deposits and thus exerted stronger suppression of fibrinogenesis than methylglyoxal-, glyoxal- or malondialdehyde-fibrinogen adducts.

Table 2 The first order rate constants of changes in intrinsic fluorescence during fibrinogen reacted with RCCs

RCC	Fast phase	Slow phase
Acrolein	48.02 ± 5.42	12.71 ± 1.68
Glycolaldehyde	10.67 ± 1.12	3.55 ± 0.50
Methylglyoxal	8.53 ± 0.86	1.52 ± 0.29
Glyoxal	4.92 ± 0.54	0.73 ± 0.13
Malondialdehyde*	-	-
Control	-	-

All rate constants are in the order of $10^6 \cdot \text{s}^{-1}$.

* Quenching procedure and calculation of rate constants was according to Tsou (1965).

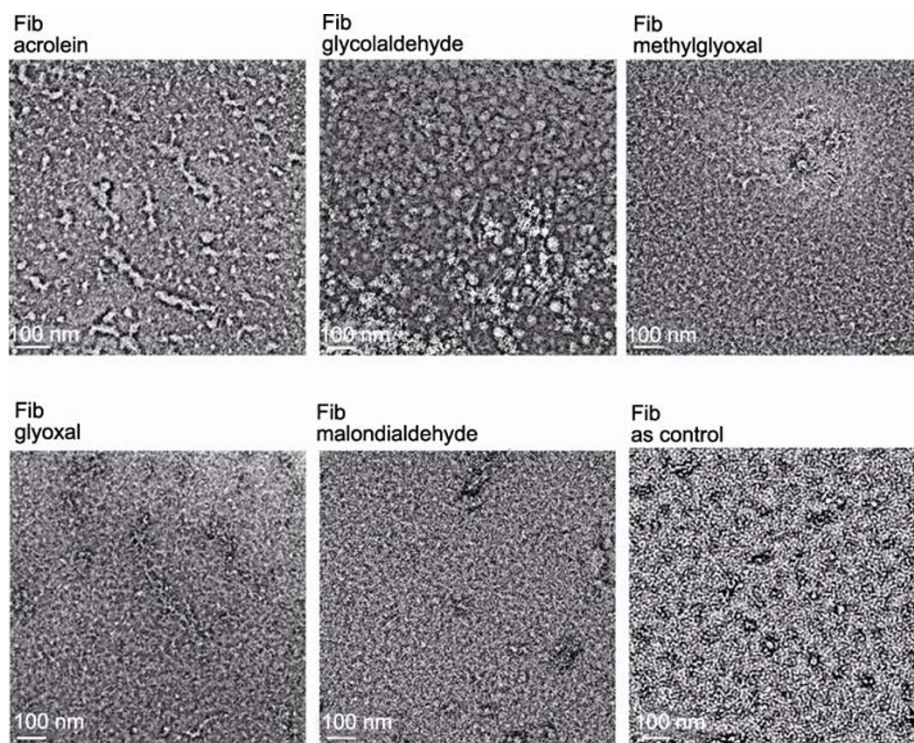


Figure 6. Conditions for the preparation of RCC-fibrinogen derivatives were the same as those described in Fig. 3. Fibrinogen (3 mg/mL) was incubated with 9 mmol/L of each carbonyl compound for 8 h and then observed by electron microscopy after using negative staining. The experiment was repeated at least three times.

As shown in Table 3, kinetic changes in the light-scattering intensity of fibrinogen incubated with glycolaldehyde followed a different pattern from those with acrolein; during the glycolaldehyde treatment, the increase in scattering intensity followed a biphasic process with a slow and a fast phase, while during acrolein treatment a fast phase proceeded a slow phase. This indicates that the formation of protein aggregates induced by glycolaldehyde and acrolein underwent different kinetic processes, even though both processes were biphasic.

Effects of RCCs on coagulation of human plasma

To further investigate the effect of fibrinogen in plasma, we tested whether RCCs are able to interfere with blood coagulation; human plasma was incubated respectively with glycolaldehyde, acrolein, methylglyoxal, glyoxal and malondialdehyde, and calcium (CaCl₂) was added to promote coagulation. Each of the five RCCs suppressed the coagulation of human plasma (Fig. S4A and S4B), but to different extents; the fibrinogenesis (absorbance at 650 nm) was the lowest (22.19% for male, 51.09% for female) in the presence of glycolaldehyde at 40 min of coagulation process (Fig. 9A). The relative coagulating activity of plasma

decreased to ~22% (male) and ~21% (female) when incubated with glycolaldehyde, similar to those with acrolein (Fig. 9B). The level of coagulation of human plasma in HEPES buffer alone was taken as 100% (Fig. 9A and 9B). It's

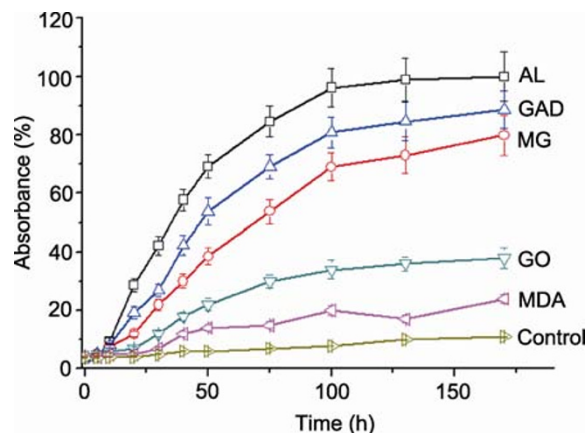


Figure 7. RCC-fibrinogen adducts form amyloid-like aggregations. Congo Red (at a 5 μmol/L final concentration) was added to RCC-fibrinogen adducts at 37°C. The maximal absorbance of sample was measured at different time intervals. The maximal absorbance showed a red shift from 490 nm to 540 nm.

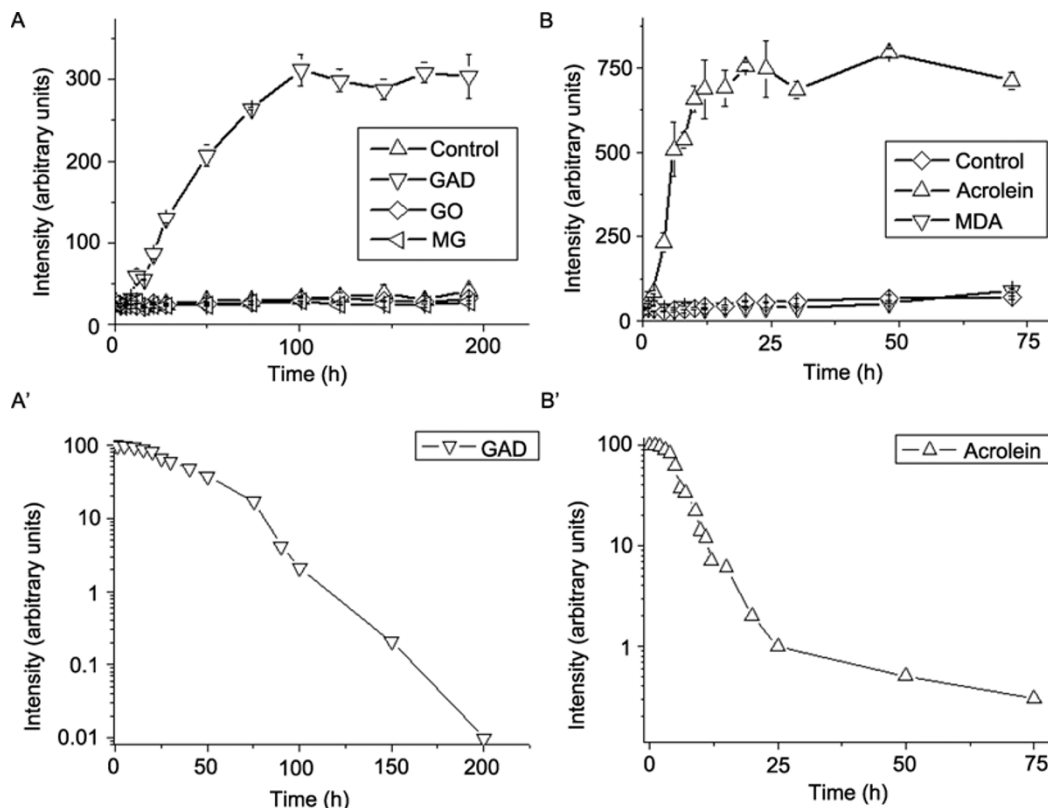


Figure 8. Changes in light-scattering of fibrinogen solutions in the presence of RCCs. After incubation with different RCCs as indicated, fibrinogen was diluted to 1.2 μmol/L and changes in light-scattering at 480 nm were measured (n = 5) at different time intervals in the presence or absence (control) of RCCs (A and B). The same data (A' and B') are plotted on a semi-logarithmic scale (Tsou, 1965). AL: acrolein, GAD: glycolaldehyde, MDA: malondialdehyde, GO: glyoxal, MG: methylglyoxal.

Table 3 The first order rate constants for changes in light-scattering of fibrinogen in the presence of different RCCs

RCCs	Relaxation time (h)	1st phase	2nd phase
Acrolein	1.22 ± 0.14	76.67 ± 8.24	6.10 ± 0.83
Glycolaldehyde	4.24 ± 0.56	7.31 ± 0.86	15.12 ± 1.62

Rate constants are in the order of $10^6 \cdot s^{-1}$.

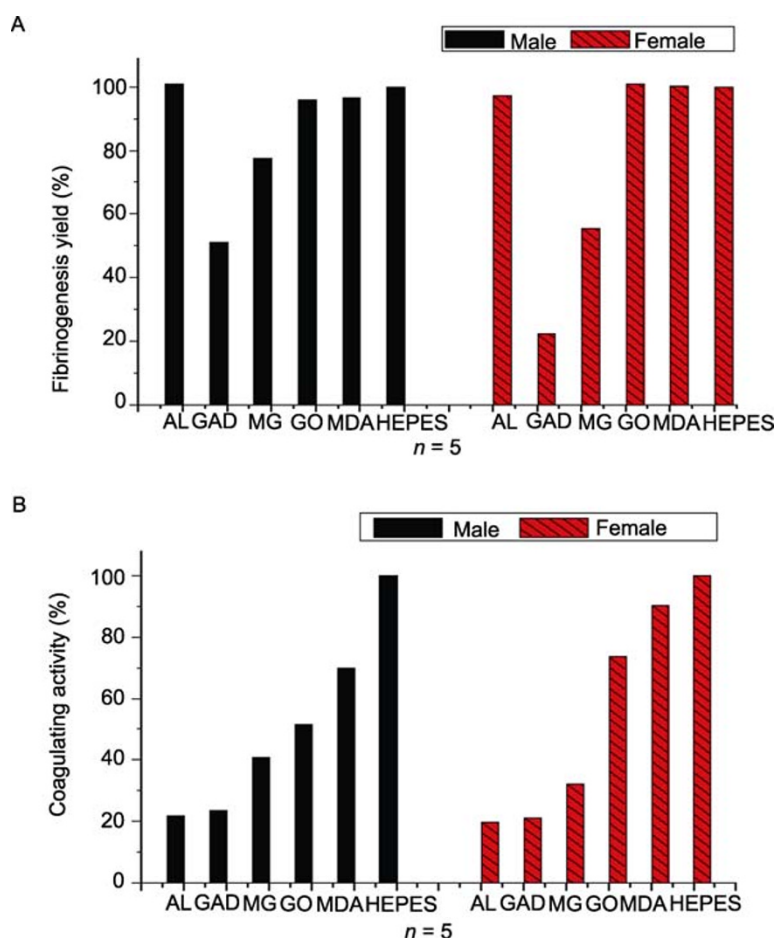


Figure 9. Changes in the coagulation of human plasma in the presence of RCCs. Human plasma (5 mL) from five individuals of the same gender was mixed and aliquots (495 μ L) were incubated respectively with 5 μ L of each of five RCCs (final concentration 1 mmol/L) in aseptic conditions for 8 h at 37°C. 20 mmol/L HEPES (pH 7.4) was used as a control. After incubation, 50 μ L $CaCl_2$ (100 mmol/L) was added to 200 μ L plasma to trigger clotting and then absorbance (650 nm) was measured at 37°C for at least 45 min. The fibrinogenesis yield was calculated by the OD_{650} at 40 min (2400 s) of coagulation process. Coagulating activity was defined as the maximum slope of the absorption curve (Zhou et al., 1997). Bar graphs represent relative fibrinogenesis yield (A) and relative coagulating activity (B). The experiment was repeated at least six times. AL: acrolein, GAD: glycolaldehyde, GO: glyoxal, MDA: malondialdehyde, MG: methylglyoxal.

worth noting that no significant changes were observed when the physiological concentrations of the RCCs were used, except for glycolaldehyde under the same conditions (data not shown).

DISCUSSION

In this study we have compared the effect of glycolaldehyde

with those of acrolein, methylglyoxal, malondialdehyde and glyoxal on the structure and function of fibrinogen and conclude that the RCC-induced protein aggregation is a mechanism by which RCCs disrupt fibrinogenesis and thus blood coagulation. At the physiological concentrations the RCCs in blood (Nielsen et al., 1997; Lapolla et al., 2005; Igarashi et al., 2006; Andrades et al., 2009), only glycolaldehyde shows a significant effect on disturbing fibrinogen-

nesis. However, under the same conditions, glycolaldehyde and acrolein disrupted fibrinogenesis and suppress human blood (plasma) coagulation more strongly than other RCCs. Glyoxal and malondialdehyde, however, did not rapidly suppress fibrinogenesis or disrupt blood coagulation under our experimental conditions. Our results imply, therefore, that an abnormal increase in the concentration of glycolaldehyde or acrolein in the blood could reduce the activity of fibrinogen and affect blood coagulation.

Notably, different RCCs had different effects on fibrinogen but all led to the formation of abnormal protein aggregations. We reasoned that, if RCCs in particular glycolaldehyde react with fibrinogen and induce amorphous aggregations, they may represent an important factor causing reduced blood coagulation. This viewpoint is based on the following observations: (1) RCCs markedly suppress the conversion of fibrinogen to fibrin in the presence of thrombin (Figs. 1 and S1, Table 1), leading to formation of amorphous short fibrils and aggregations which are markedly different from the fibers of intact fibrin (Fig. 2); (2) fibrinogen forms amorphous aggregations and short fibrils in the presence of RCCs (Fig. 6); and (3) RCC-fibrinogen adducts, especially glycolaldehyde- and acrolein-fibrinogen, form high molecular mass polymers which can be observed by SDS-PAGE (Figs. 3 and 4). That is to say, the RCCs not only disrupt fibrinogenesis but also cause protein aggregation.

What are the characteristics of fibrinogen aggregations? Our results suggest that the aggregations are RCC-fibrinogen molten globules (a denatured protein state in which tertiary or higher-grade structures are abnormally changed). This hypothesis is based on the following observations: (1) Protein intrinsic fluorescence decreases markedly on reaction with RCCs, suggesting a change in tertiary structure; (2) no significant changes in secondary structure were observed by analysing CD spectra; (3) the acrolein-, glycolaldehyde- and methylglyoxal-fibrinogen adducts stained positively with Congo Red, showing their amyloid-like characteristics; and (4) RCC-fibrinogen adducts were present as amorphous and fibril-like deposits in electron microscope images. These results indicate that RCCs first inactivate fibrinogen by disrupting protein conformation, leading to the formation of amyloid-like aggregations.

Fibrinogen monomers are composed of three non-identical polypeptide chains, designated as $\text{A}\alpha$, $\text{B}\beta$, and γ , which are linked together by 29 disulfide bonds (de Maat et al., 1995). Like other blood proteins, fibrinogen is secreted into the plasma and circulates throughout the body. These three chains contain cysteine residues that participate in the cross-linking of the chains. They are also rich in lysinyl residues (40 Lys in $\text{A}\alpha$; 37 Lys in $\text{B}\beta$ and 30 Lys in γ). Cleavage of the N-terminal regions of fibrinogen α (Arg19–Gly20) and β chains (Arg21–Gly22) catalyzed by thrombin releases fibrinopeptide A and B (~1900 Da and ~2400 Da) (Bunce et al., 1992) and is the most important step

in the conversion of fibrinogen into fibrin. Fibrin monomers polymerize end to end to form protofibrils which in turn associate laterally to form fibrin fibers (Hantgan and Hermans, 1979). As mentioned above, RCCs, in particular glycolaldehyde and acrolein, react with the ϵ -amino groups of Lys residues and reduce not only the positive charge of the N-terminal but also of the whole fibrinogen molecule, which results in a marked change in the conformation of fibrinogen and thus affects fibrinogenesis.

We noted that the molecular masses of the peptides released from RCC-fibrinogen in the presence of thrombin here were similar, but somewhat different to other reports. The gamma-chain (NP_001028798.1) was relatively stable under our experimental conditions. The N-terminal region of the alpha-chains (NP_001136389.1) contains an α -amino group and an arginyl residue (Arg19), and the N-terminal region of the beta-chains (NP_776336.1) contains an α -amino group, a lysinyl (Lys15) and two arginyl residues (Arg13 and Arg21). These α - and ϵ -amino groups and δ -guanidine groups have the potential to be modified by RCCs, leading to changes in the molecular mass of the peptides. For this reason, fibrinogen modified with different RCCs may release fibrinopeptide A and B of slightly different molecular masses.

We turn to the mechanism by which the RCCs especially glycolaldehyde suppress fibrinogenesis. There may be a variety of mechanisms involved in the disruption of fibrinogenesis. (1) As has been mentioned above, the reaction of RCCs with fibrinogen blocks ϵ -amino groups of lysinyl residues and the positive charge of the N-terminal region is reduced, possibly disrupting interactions between fibrinogen molecules during fibrinogenesis. (2) RCCs strongly induce fibrinogen aggregation and deposition and aggregated proteins dysfunction in the conversion of fibrinogen to fibrin in the presence of thrombin. (3) Since lysinyl residues in the N-terminal region of fibrinogen molecules modified by RCCs are blocked, it is difficult for thrombin to recognize the cleavage sites (Arg–Gly) (Bunce et al., 1992) and produce fibrin, thus disrupting fibrinogenesis. We believe the second mechanism provides the best explanation for our results. RCC-induced conformational changes result in protein aggregations in which lysinyl residues are partially blocked, since thrombin can still recognize its cleavage sites in RCC-treated fibrinogen (Fig. S3). That is to say, it is protein aggregation that suppresses the conversion of RCC-treated fibrinogen to fibrin in the presence of thrombin.

Of the RCCs employed in this study, glycolaldehyde and acrolein were the most effective at suppressing fibrinogenesis and disrupting human blood coagulation. As is well known, acrolein has an extremely active aldehyde group because of its close proximity to an ethylene double bond. Acrolein- and glycolaldehyde-fibrinogen adducts polymerized and aggregated more rapidly than the other RCC protein adducts in our light scattering assay (Fig. 8). This suggests that both acrolein and

glycolaldehyde-protein aggregations are insoluble in solution. The kinetic process of acrolein-fibrinogen aggregation follows a different pathway from glycolaldehyde-fibrinogen though in both cases their aggregation underwent a biphasic process.

Acrolein-fibrinogen underwent a process with a fast phase followed by a slow phase, while glycolaldehyde-fibrinogen underwent a slow phase followed by a fast phase. These significantly different kinetic processes imply that the formation of insoluble aggregations triggered by acrolein and glycolaldehyde follow different mechanisms.

Although acrolein exerted a strong anti-coagulation effect under the conditions used in this study, the concentration of acrolein (14.4 $\mu\text{mol/L}$, Igarashi et al., 2006) in normal human plasma is much lower than that of glycolaldehyde (1000.0 $\mu\text{mol/L}$, Andrades et al., 2009). Physiological concentrations of methylglyoxal (0.1 $\mu\text{mol/L}$, Lapolla et al., 2005), glyoxal (0.2 $\mu\text{mol/L}$, Lapolla et al., 2005) and malondialdehyde (600.0 $\mu\text{mol/L}$, Nielsen et al., 1997; Lapolla et al., 2005; Igarashi et al., 2006; Andrades et al., 2009) are too low to have a significant effect on fibrinogenesis, unless their blood concentrations were higher than 1 mmol/L . Of note, the concentration of glycolaldehyde is the highest of these RCCs. Each RCC was used at a final concentration of 1 mmol/L in the human plasma coagulation experiment and this concentration is in the same order of magnitude as that of glycolaldehyde in the blood. That is to say, an abnormal increase in glycolaldehyde should be regarded as a non-negligible risk factor for fibrinogen aggregation, deposition, and processes which are involved in blood coagulation disorders and atherosclerosis.

Methylglyoxal-treated fibrinogen was prone to aggregation, and aggregates tended to float in HEPES buffer, thus disrupting MALDI-TOF measurements. We also observed aggregates of methylglyoxal-treated fibrinogen floating in solutions in test tubes. All our attempts to resolve the aggregates before mass spectrometry failed, possibly explaining why the MALDI-TOF trace for methylglyoxal-treated protein resembled background noise. It has been reported that malondialdehyde quenches intrinsic fluorescence when it reacts with a given protein (Traverso et al., 2004). Thus, while in Fig. 5, in the presence of malondialdehyde, the intrinsic fluorescence of fibrinogen was rapidly reduced to the background fluorescence level, which does not represent a rapid conformational change in the protein.

In addition to its well-characterized coagulative activity, it has been proposed that fibrinogen alters the function of cells such as leukocytes, neutrophils, Schwann cells, and monocytes; for example, cell adhesion (Languino et al., 1993; Altieri et al., 1995; Languino et al., 1995; Duperray et al., 1997), migration (Diacovo et al., 1996; Forsyth et al., 2001; Sans et al., 2001), phagocytosis (Rubel et al., 2001, 2002), cytokine and chemokine expression (Fan and Edgington, 1993; RL Perez 1995; Smiley et al., 2001; Szaba and Smiley, 2002), and degranulation (Rubel et al., 2002; Tuluc et al., 2004). Fibrinogen also plays a role in the initiation of angiogenesis

(Chalupowicz et al., 1995; Martinez et al., 2001), and the propagation of infection. Here, while we have only studied changes in the coagulative activity of fibrinogen in the presence of RCCs, we expect that RCCs should affect the function of other blood components, either via the dysfunction of fibrinogen or by direct reactions. Furthermore, human blood coagulation is a complex cascade involving more than ten coagulant factors. We used calcium to promote the coagulation of human plasma, since calcium is able to activate several steps of the coagulation cascade. It is likely that RCCs may also influence other steps in the coagulation process triggered by calcium that have not been investigated here. Further investigation is required to determine the effects of RCCs on the components of the coagulation cascade.

Fibrinogen (fibrin) aggregates in the presence of RCCs leading to the suppression of fibrinogenesis and plasma coagulation suggesting that the aggregated fibrinogen may be related to fibrinogen deposition in blood vessels (Fig. S5). This viewpoint is supported by these observations: (1) We have observed that RCC (malondialdehyde)-treated bovine serum albumin (BSA) forms deposits in SH-SY5Y cells *in vitro* (unpublished data); (2) Hyung and colleagues have shown that β -amyloid induces fibrinogen oligomerization and co-deposits in blood vessels (Ahn et al., 2010); and (3) proteins, especially amyloid-like aggregations, are prone to become deposited after they have aggregated (Kourie and Henry, 2001). RCC-treated fibrinogen observed here had features similar to amyloid.

MATERIALS AND METHODS

Materials

Fibrinogen and the reactive carbonyl compounds glycolaldehyde, glyoxal and methylglyoxal were from Sigma Co. (USA). Acrolein was from Beijing Ouhe Technology Limited Company (China). Human citrated plasma was kindly provided by the Physical Examination Center of Zhongguancun Hospital (Beijing, China). Malondialdehyde was prepared as described previously (Li et al., 2010). A malondialdehyde stock solution (200 mmol/L , 4.05% *w/w*) was freshly prepared by hydrolyzing 1,1,3,3-tetramethoxypropane (TMP) (purity $\geq 98\%$, Fluka Chemie AG, Buchs, Switzerland) (Li et al., 2010).

Modification of fibrinogen with reactive carbonyl compounds

RCC-fibrinogen adducts were prepared according to the method described by Kuhla et al. (2007). Bovine fibrinogen (final concentration of 3 mg/mL) was dissolved in 10 mmol/L PBS buffer (pH 7.4) and incubated respectively with acrolein, glycolaldehyde, glyoxal, malondialdehyde and methylglyoxal at 37°C for the time as indicated. As fibrinogen contains 214 lysinyl residues and 160 argininy residues, a 9 mmol/L final concentration of each of the RCCs was used since this gave a 3-fold excess of reactive aldehyde groups to lysinyl and argininy residues thus allowing maximal modification of the fibrinogen protein. Fibrinogen alone was used as a control.

Fibrinogen activity assays

The light scattering assay described by Zhou et al. (1997) was used to determine the blood-coagulant activity of fibrinogen. Both incidence and scattering wavelengths were set at 480 nm. Fibrinogen (final concentration 0.2 mg/mL) was dissolved in 750 μ L of reaction buffer (0.05 mol/L Tris-HCl, 0.05 mol/L NaCl, pH 7.2), and the resulting solution was incubated for 5 min at 25°C. Scattering intensity was measured for 10 min at 25°C immediately after addition of thrombin (final concentration 43 U/L). Fibrinogen activity was calculated as the change in the scattering intensity during the time interval indicated as described by Tsou (Tsou, 1965).

SDS-PAGE of RCC-fibrinogen adducts

RCC-fibrinogen adducts were boiled for 5 min in Laemmli SDS sample loading buffer and separated by 12% SDS-PAGE. Gels were stained with Coomassie brilliant blue. Native fibrinogen was used as a control.

Electron microscopy of RCC-fibrinogen adducts

Preparation of electron microscopy sample was described as Liu et al. (2011). Briefly, RCC-fibrinogen adducts (2.3 μ mol/L) were placed on a formvar-coated brass grid incubated at room temperature for 1 min. Samples were washed twice with ddH₂O and stained with 3% uranyl acetate for 1 min. Grids were examined on a Philip Tecnai 20 Electron Microscope (FEI Co., Eindhoven, Netherlands) equipped with a digital camera.

Fluorescence measurements

Fluorescence measurements were performed on a Hitachi F-4500 spectrofluorometer (Japan). Samples (fibrinogen protein diluted to 0.5 mg/mL) were dissolved in 10 mmol/L PBS buffer (pH 7.4) unless stated otherwise. All the fluorescence measurements were repeated at least three times.

Protein intrinsic fluorescence. To detect intrinsic fluorescence of fibrinogen, the excitation wavelength was set at 280 nm, and the emission spectrum was scanned from 310 nm to 500 nm.

Fluorescence of RCC-fibrinogen adducts. The fluorescence of RCC-fibrinogen adducts modified by malondialdehyde ($\lambda_{\text{ex}} = 400$ nm), acrolein ($\lambda_{\text{ex}} = 420$ nm), methylglyoxal ($\lambda_{\text{ex}} = 320$ nm), glyoxal ($\lambda_{\text{ex}} = 340$ nm) and glycolaldehyde ($\lambda_{\text{ex}} = 350$ nm) was measured at 37°C.

Congo Red binding assay

This assay (Rojas Quijano et al., 2006) was performed by adding a freshly-prepared stock solution of Congo Red in 100 mmol/L potassium phosphate (pH 7.2) to RCC-fibrinogen adducts (3 mg/mL) to give a final Congo Red concentration of 5 μ mol/L as described previously (Nie et al., 2007).

Light scattering analysis of RCC-fibrinogen adduct aggregations

Fibrinogen was incubated with each of the RCCs in PBS solution containing 0.05% sodium azide at 37°C. After

fibrinogen was suspended in solutions of RCCs, aliquots were taken from each RCC solution at different time intervals, and the intensity of light scattering was measured using the same spectrofluorometer with wavelengths of incident light of 480 nm and scattering light of 480 nm.

CD measurements

CD spectra were recorded using a Jasco J-720 CD spectrometer. The spectra were measured in 1-mm pathlength quartz cuvettes, and data were collected from 195 nm to 250 nm at 0.5-nm intervals. The sample buffer used was 100 mmol/L sodium phosphate buffer (pH 7.2) and bandwidth was set at 1.5 nm (37°C). Spectrum baselines were corrected using a spectrum for the buffer alone measured under identical conditions.

Anticoagulation tests

5 mL of plasma from five individuals of the same gender was mixed and incubated for 10 min at 37°C before use. Mixed plasma (495 μ L) was incubated respectively with 5 μ L of (100 mmol/L) acrolein, glycolaldehyde, methylglyoxal, glyoxal and malondialdehyde under aseptic conditions for 8 h in a 37°C water bath. 20 mmol/L HEPES (pH 7.4) was used as a control.

After incubation, clotting was triggered by adding 50 μ L CaCl₂ (100 mmol/L) to 200 μ L plasma. The mixed liquid was quickly added to a cuvette and monitored spectrophotometrically (650 nm) at 37°C for at least 45 min. The fibrinogenesis yield was defined as the OD₆₅₀ value at 40 min of the coagulation process. Coagulating activity of each RCC was defined as the maximum slope of its RCC-induced plasma coagulation curve.

MALDI-TOF mass spectrometry measurements

Fibrinogen (at a final concentration of 4.5 mg/mL) was incubated respectively with 10 mmol/L of each of the five RCCs at 37°C for 8 h. 200 μ L of thrombin (final concentration of 78 U/L) was then added to 800 μ L of the reaction sample. After incubating with thrombin for 15 min, the proteolytic RCC-fibrinogen adducts were examined using MALDI-TOF mass spectrometry. Fibrinogen incubated with 20 mmol/L HEPES in the presence or absence of thrombin was used for positive and negative controls.

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YJ Xu and M Qiang carried out the fluorescence measurements, CD, FM, SDS-PAGE, human plasma coagulation detection and mass spectrometry experiments and drafted the manuscript. JL Zhang participated in fluorescence experiments. RQ He and Y Liu conceived the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript; there is no conflict of interests.

ABBREVIATIONS

AL, acrolein; GAD, glycolaldehyde; GO, glyoxal; MDA, malondialdehyde; MG, methylglyoxal; RCCs, reactive carbonyl compounds

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