Journal of Thrombosis and Thrombolysis 12(3), 273–281, 2001.

An Analysis of Mechanisms Underlying the Antifibrinolytic Properties of Radiographic Contrast Agents

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Abstract. Background: **Radiographic contrast agents inhibit fibrinolysis, although by poorly defined pathways. The purpose of this study was to define specific mechanisms by which contrast agents inhibit clot lysis.**

Methods and Results: **Diatrizoate (high osmolar ionic agent), ioxaglate (low osmolar ionic), and ioversol (nonionic) were studied *in vitro*. Diatrizoate inhibited clot lysis by $81.3 \pm 0.6\%$ vs. control ($p < 0.001$). Ioxaglate inhibited clot lysis by $41.7 \pm 11.9\%$, which was of borderline significance ($p = 0.07$). Ioversol did not significantly inhibit clot lysis ($14.9 \pm 11.5\%$ decrease vs. control; $p > 0.3$). Inhibition of fibrinolysis was not explained by the high osmolarities of contrast agents, by their iodine content, or by their effects on the amidolytic activities of t-PA, urokinase, or plasmin. However, plasminogen activation by t-PA, urokinase, or streptokinase was significantly inhibited by contrast agents. Diatrizoate, ioxaglate, and ioversol inhibited plasminogen binding to plasma clots by $51 \pm 4\%$ ($p < 0.001$), $30.1 \pm 4\%$ ($p < 0.01$), and $19.4 \pm 7\%$ ($p = 0.07$), respectively. Plasma clots formed in the presence of contrast agents were resistant to lysis by plasmin. Diatrizoate produced the most potent effect, inhibiting clot lysis by $40 \pm 5.7\%$ ($p < 0.03$). Contrast agents did not inhibit plasminogen binding to fibrin or plasmin-mediated fibrinolysis if they were added after clot formation. Contrast agents altered clot turbidity, an index of fibrin structure, if present during clot formation, but not if added to preformed clots. Contrast agents did not affect plasminogen activator inhibitor-1 or α_2-antiplasmin function.**

Conclusions: **Contrast agents inhibit clot lysis by inhibiting plasminogen activation and by disrupting interactions of plasminogen and plasmin with fibrin by altering fibrin structure. Significant variation in antifibrinolytic properties exists between different contrast agents.**

Abbreviated Abstract. **The purpose of this study was to define specific mechanisms by which contrast agents inhibit clot lysis. In both a purified clot lysis system and a plasma clot lysis system, diatrizoate, an ionic agent, produced the most potent inhibition of fibrinolysis. Contrast agents did not inhibit the active sites of plasminogen activators or plasmin, but did inhibit plasminogen activation. Binding of plasminogen to fibrin and lysis of fibrin by plasmin were inhibited by contrast agents if they were present during clot formation, but not if they were added after clot formation was complete. Contrast agents altered clot turbidity, an index of fibrin structure, if present during clot formation, but not if added to preformed clots. Contrast agents did not affect plasminogen activator inhibitor-1 or α_2-antiplasmin function. The effects of contrast agents on fibrinolytic parameters were not explained by their high osmolarities. These results suggest that contrast agents inhibit clot lysis by inhibiting plasminogen activation and by disrupting interactions of plasminogen and plasmin with fibrin by altering fibrin structure.**

Key Words. **radiographic contrast agents, plasminogen, fibrinolysis**

Radiographic contrast agents are iodinated derivatives of benzoic acid that are used to visualize arteries, veins, and cardiac chambers during angiographic procedures. Radiographic contrast agents inhibit blood coagulation *in vitro*, with ionic-type agents producing a greater anticoagulant effect than nonionic-type agents [1]. The anticoagulant properties of contrast agents appear to be mediated by multiple mechanisms, including effects on thrombin formation, fibrin polymerization, and platelet activation [2–5]. Although controversial, the anticoagulant effect of contrast agents may influence the frequency of thrombotic complications during percutaneous coronary artery interventions [6–9].

Radiographic contrast agents also inhibit fibrinolysis. Dehmer et al. showed that plasma clots formed in the presence of ionic or nonionic contrast agents were more resistant to lysis by tissue-type plasminogen activator (t-PA), urokinase, or streptokinase [10]. Pislaru et al. demonstrated that iohexol (a low-osmolar nonionic agent) and amidotrizoate (a high-osmolar ionic agent) inhibited coronary thrombolysis in

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dogs, while ioxaglate (a low-osmolar ionic agent) did not [11]. Contrast agents could inhibit fibrinolysis by several potential mechanisms, including direct inhibition of plasminogen activators, inhibition of plasminogen activation, direct inhibition of plasmin, and inhibition of plasmin(ogen)-fibrin interactions. Radiographic contrast agents have also been hypothesized to alter the function of serine protease inhibitors [12], such as α_2-antiplasmin and plasminogen activator inhibitor-1 (PAI-1), which are known to play a key role in the regulation of fibrinolysis. However, a systematic analysis of the effects of radiographic contrast agents on component reactions of the fibrinolytic system has not been reported. In this study, we examined *in vitro* the effects of 3 commonly utilized radiographic contrast agents on plasmin formation and activity, plasmin(ogen)-fibrin interactions, and the activities of PAI-1 and α_2-antiplasmin.

Methods

Materials

Glu-plasminogen was purified from human plasma [13]. Human fibrinogen, sodium diatrizoate, sodium benzoate, and meglumine were from Sigma. Plasminogen and fibrinogen were fluorescein-labeled as described [14]. ^{125}I-labeled human fibrinogen was from Amersham Life Sciences. Streptokinase, urokinase (u-PA), Spectrozyme UK, Spectrozyme tPA, des-AA-fibrinogen, and α_2-antiplasmin were from American Diagnostica. Plasmin was prepared by activating plasminogen with trace u-PA. S-2251 was from Chromogenix. Human t-PA was from Genentech. Thrombin was from Calbiochem. Recombinant human PAI-1 was from Dr. D. Ginsburg, University of Michigan. Diatrizoate meglumine/diatrizoate sodium (MD-76), ioversol (Optiray 320), and ioxaglate meglumine/ioxaglate sodium (Hexabrix) were from Mallinckrodt Medical.

Clot Lysis Assays

A reconstituted clot lysis assay consisting of Glu-plasminogen (0.2 μM), fluorescein-labeled fibrinogen (1 mg/mL), thrombin (2 U/mL), and t-PA (15 pM) was used. Clots were incubated at 37°C and % clot lysis after 45 min was calculated [14]. Plasma clot lysis assays were performed as described by Bajzar [15]. Human plasma, $CaCl_2$ (10 mM), thrombin (6 nM), and t-PA (0.6 nM) were mixed in 96-well microtiter plates. Plates were incubated at 37°C and absorbance at 650 nm was measured. Lysis time was defined as that required for clot turbidity to decrease by 50%. To study the lysis of fibrin by plasmin, citrated human plasma (500 μL) containing ^{125}I-labeled fibrinogen (1 μCi) was clotted on polypropylene rods by adding $CaCl_2$ (25 mM) and thrombin (1 U/mL). After 45 minutes, clots were washed $\times 5$ with TBS, and clot-bound radioactivity was measured. Plasmin (0.57 μM in 10 mM Tris-HCl, 150 mM NaCl, pH 7.5 [TBS], total volume 500 μL) was added, and clots were rocked gently at 37°C. At timed intervals, 5 μL of the clot supernatant was removed and % clot lysis was calculated from the ratio of supernatant radioactive counts to washed clot counts.

Enzyme Activity Assays

Amidolytic activities of t-PA (3.5 nM), u-PA (50 U/mL), and plasmin (10 nM) were determined by incubating each enzyme in TBS, 0.1% BSA (TBS/BSA) containing synthetic substrate (Spectrozyme tPA, Spectrozyme UK, or S-2251, each at 300 μM) and monitoring absorbance at 405 nm. Enzyme activities were determined from standard curves for each protease. Plasminogen activation in TBS/BSA was studied by incubating plasminogen (1 μM) and S-2251 (300 μM) with u-PA, streptokinase, or t-PA and des-AA-fibrinogen. Plasminogen activation in citrated plasma was studied by adding S-2251 (300 μM), des-AA-fibrinogen (0.1 μM), and t-PA and monitoring absorbance at 405 nm. The effects of contrast agents on the inhibitory activities of α_2-antiplasmin and PAI-1 were studied as described [16].

Binding of Plasminogen to Fibrin

Fibrin-coated microtiter plate wells (96-well) were prepared by incubating fibrinogen (1.5 μM) and thrombin (2 U/mL) in 100 μL of TBS and air drying plates [5]. Wells were blocked with 3% BSA and washed. Fluorescein-labeled plasminogen (0–2.2 μM) and radiographic contrast agent (2.5%) in 100 μL of TBS/BSA were added to wells and incubated at 37°C for 30 minutes. Wells were washed 5 times, and bound plasminogen was eluted by adding 1% SDS (100 μL) and incubating plates at 37°C for 1 hr. Eighty μL were removed and fluorescence was measured [14]. To study plasminogen binding to forming clots, contrast agents and fluorescein-labeled human plasminogen (2.2 μM) were added to citrated human plasma (total volume 500 μL) and clotting was initiated with $CaCl_2$ and thrombin. Retracted clots were washed $\times 5$, blotted dry, weighed, then lysed completely by addition of TBS containing t-PA (15 nm) and unlabeled plasminogen (1.1 μM). Fluorescence was measured and divided by clot weight to determine clot-bound plasminogen.

Clot Turbidity Assay

Citrated human plasma (92.5 μL) containing contrast agent (2.5%) or an equal volume of TBS was placed in microtiter wells and thrombin (0.25 U/mL) was added. Turbidity of forming clots was monitored at 608 nm. Maximum turbidity minus turbidity prior to clot formation was used as an index of fibrin fiber density [17–19]. In separate experiments, plasma clots (975 μL) were formed in quartz cuvettes in the absence of contrast agents. After maximum clot turbidity was achieved (approximately 15 minutes), clots were overlaid with 1 mL of 10% diatrizoate and turbidity at 608 nm was monitored for 8 hrs. With each experiment, an identical control clot was overlaid with 10% diatrizoate containing 0.05% methylene blue to allow visual confirmation that the overlaid solution completely perfused the clot.

Statistical Analysis

Data are presented as mean ± 1 standard error of the mean (SEM), unless otherwise indicated. The Student's t test or the Mann-Whitney Rank Sum test was used to compare experimental groups.

Results

Effects of Radiographic Contrast Agents on Clot Lysis

We incubated fluorescein-labeled fibrinogen, plasminogen, t-PA, and thrombin in the presence of contrast agent (2.5%) or TBS, then monitored clot lysis (Fig. 1A). Diatrizoate (MD-76), a high osmolar ionic agent, inhibited clot lysis by 81.3 ± 0.6% ($p < 0.001$ vs. control). Ioxaglate, a low osmolar ionic agent, inhibited clot lysis by 41.7 ± 11.9% compared to control, but this did not achieve statistical significance ($p = 0.07$). Ioversol, a nonionic agent, did not significantly inhibit clot lysis (14.9 ± 11.5% decrease in lysis vs. control; $p > 0.3$). Isosomolar sucrose and/or NaCl solutions did not significantly affect clot lysis (diatrizoate control was 2 M sucrose, 77 mM NaCl; ioxaglate control was 300 mM

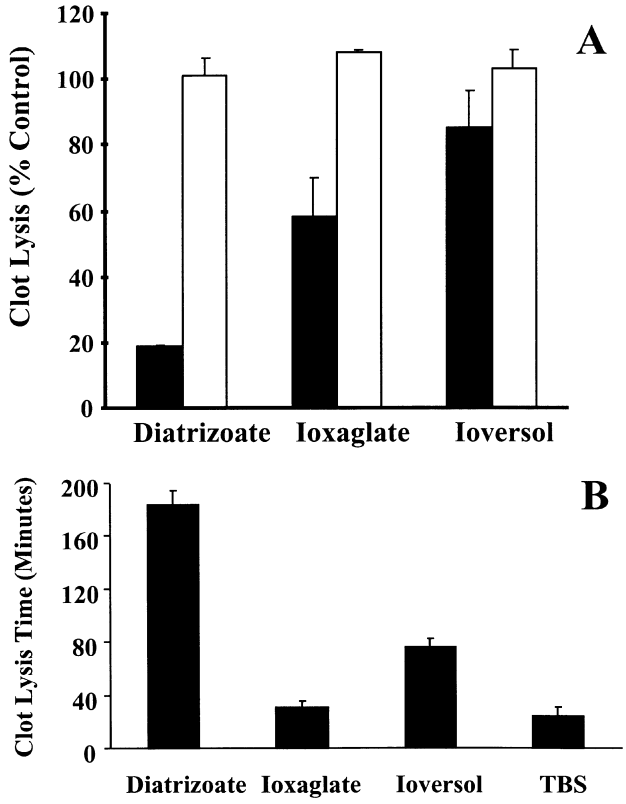

Fig. 1. *Inhibition of clot lysis by radiographic contrast agents. A. Fluorescein-labeled fibrin clots were incubated with t-PA and plasminogen in the presence of radiographic contrast agents (2.5%, black bars) or equal volumes of isosmolar sucrose/NaCl solutions (white bars) or Tris-buffered saline (TBS). Amounts of clot lysis after 45 min are shown. Results are expressed as ratio of % lysis of clots formed in the presence of contrast agent or osmolar control solution to % lysis of clots formed in presence of TBS. Data are mean of triplicate experiments. B. Plasma clots were formed in the presence of radiographic contrast agents (2.5% concentration) or an equal volume of TBS. Clot lysis times were measured. Data represent mean of ≥ 4 experiments.*

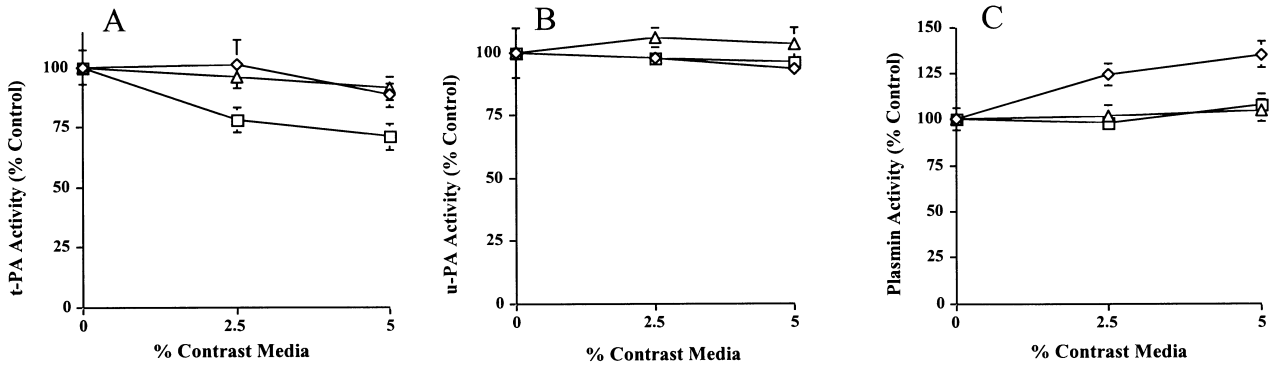

Fig. 2. *Effects of radiographic contrast agents on enzyme amidolytic activity. T-PA (3.5 nm, A), u-PA (50 U/mL, B), or plasmin (10 nm, C) were incubated with radiographic contrast agents and amidolytic activities were measured. Data points represent mean of ≥ 3 experiments ± 1 standard deviation. Diamonds, diatrizoate; triangles, ioversol; squares, ioxaglate.*

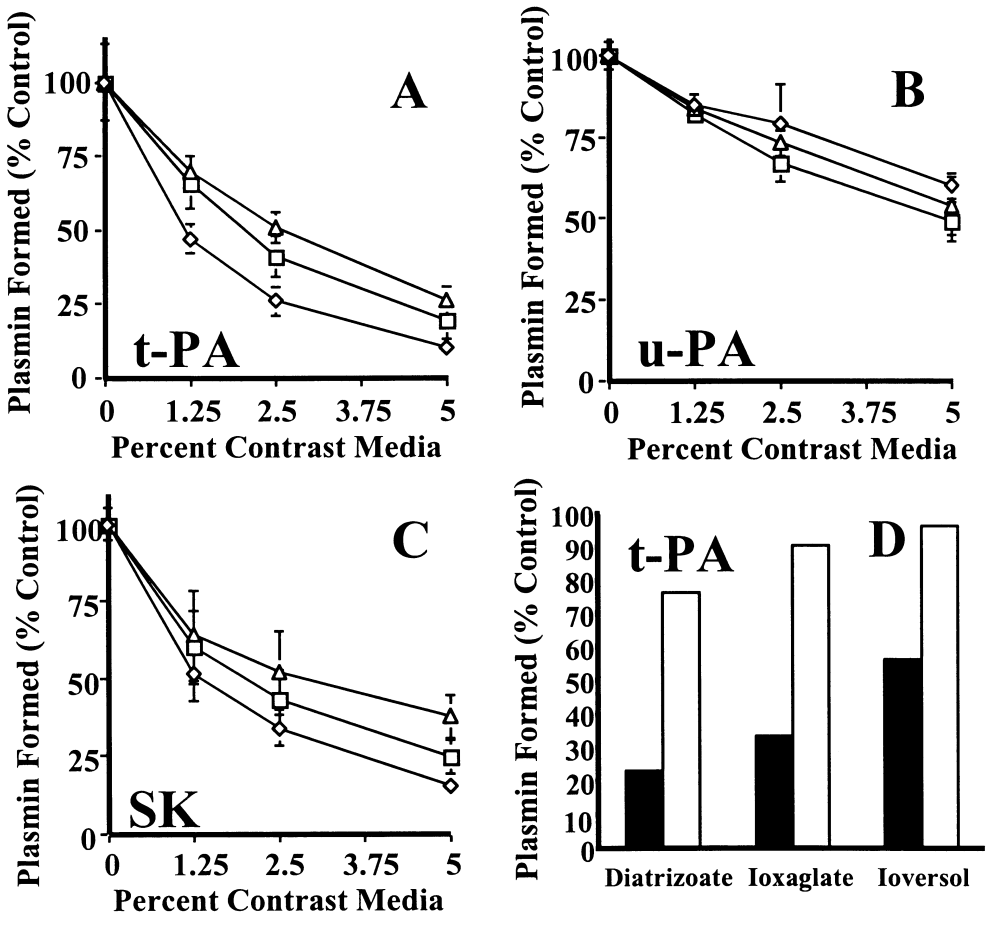

Fig. 3. *Effects of radiographic contrast agents on plasminogen activation. Glu-plasminogen (1 μM), S-2251 (300 μM), and radiographic contrast agents were incubated with t-PA (3 nm, A), u-PA (10 U/mL, B), or streptokinase (0.8 U/mL, C) and plasmin formation was monitored. Data points represent mean of ≥ 3 experiments ± 1 standard deviation. Diamonds, diatrizoate; triangles, ioversol; squares, ioxaglate. D. Osmolar effects of contrast agents on plasminogen activation. T-PA (3 nm), Glu-plasminogen (1 μM), des-AA fibrinogen (100 nm), and S-2251 (300 μM) were incubated with 5% contrast agent (black bars) or equal volumes of isosmolar sucrose/NaCl solutions (white bars, see text for details). Plasmin formation was monitored and expressed as % of control reactions performed in presence of equal volumes of Tris-buffered saline.*

NaCl; ioversol control was 0.7 M sucrose). We also studied the antifibrinolytic properties of the individual components of MD-76, which is a mixture of sodium diatrizoate and meglumine diatrizoate. Sodium diatrizoate (15 mg/mL, i.e. concentration yielding same amount of organically bound iodine as 2.5% MD-76) inhibited clot lysis by 67%. Meglumine (4 mg/mL), an organic cation, inhibited clot lysis by only 8%. Sodium benzoate (3.3 mg/mL), the noniodinated parent molecule of sodium diatrizoate, inhibited clot lysis by 72%. These results suggested that the antifibrinolytic effect of MD-76 was attributable to its sodium diatrizoate content, but did not depend on its iodine content or its high osmolarity. To determine the effects of contrast agents on fibrinolysis in a plasma environment, we formed plasma clots in the presence of diatrizoate, ioxaglate, ioversol (each at 2.5%), or TBS, then measured the time until 50% clot lysis (Fig. 1B). Diatrizoate markedly inhibited fibrinolysis (lysis time 184 ± 10.7 min vs. 23.9 ± 6.7 min for TBS control, $p = 0.01$). Ioxaglate did not significantly prolong clot lysis (lysis time 30.8 ± 4.9 min; $p > 0.15$ vs. TBS control). Ioversol prolonged the clot lysis time to 76.0 ± 6.4 min, which was of borderline statistical significance ($p = 0.07$ vs. control). Isosmolar control solutions did not inhibit plasma clot lysis (data not shown).

Effects of Contrast Agents on Fibrinolytic Proteases and Inhibitors

T-PA amidolytic activity was not inhibited by diatrizoate or ioversol, while ioxaglate produced a mild inhibitory effect (Fig. 2). U-PA and plasmin amidolytic activities were not inhibited by contrast agents. However, activation of Glu-plasminogen by t-PA, u-PA, or streptokinase was inhibited by each agent (Fig. 3). The inhibition of Glu-plasminogen activation by contrast agents was not mediated to a significant extent by their osmolar properties (Fig. 3D). Sodium diatrizoate (30 mg/mL) and sodium benzoate (6.6 mg/mL) inhibited t-PA-catalyzed plasminogen activation by 41% and 53%, respectively. The activation of plasminogen in citrated plasma by pharmacologic concentrations of t-PA (1 μg/mL) was inhibited $58.7 \pm 5\%$, $59.6 \pm 0.7\%$, and $63.9 \pm 2.1\%$ by diatrizoate, ioxaglate, and ioversol (each at 2.5%), respectively. Diatrizoate, ioxaglate, and ioversol (each at 2.5%) had no significant effect

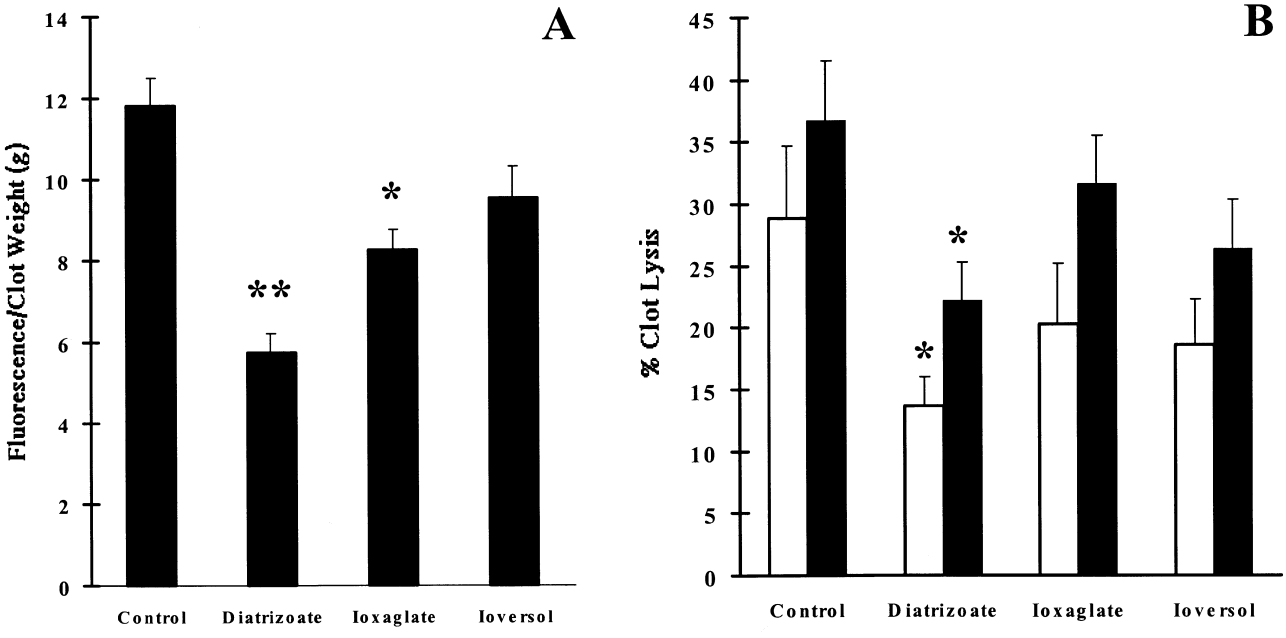

Fig. 4. *Effects of radiographic contrast agents on plasmin(ogen)-fibrin interactions. A. Binding of plasminogen to fibrin. Citrated plasma was clotted in the presence of fluorescein-labeled plasminogen (200 μg/mL) and radiographic contrast agent (5%) or an equal volume of Tris-buffered saline (Control). Clots were washed extensively and bound fluorescence per gram of clot was measured. Data represent mean of 4 experiments. Results statistically different from control are designated by "*" ($p < 0.01$) and "**" ($p < 0.001$). B. Digestion of fibrin by plasmin. Plasma clots were formed in the presence of ^{125}I-labeled fibrinogen and radiographic contrast agent (5%) or an equal volume of Tris-buffered saline (Control). After extensively washing clots, plasmin was added and % clot lysis was measured 30 min (white bars) and 60 min (black bars) later. Data represent mean of 6 experiments. Results statistically different from control are designated by "*" ($p < 0.05$).*

on the capacity of α_2-antiplasmin or PAI-1 to inhibit plasmin or t-PA, respectively (data not shown).

Effect of Contrast Agents on Fibrin-Plasmin(ogen) Interactions

To test the hypothesis that modification of fibrin by contrast agents inhibits plasmin(ogen)-fibrin interactions, we formed plasma clots in the presence of fluorescein-labeled plasminogen and radiographic contrast agent (5%), then measured clot-bound plasminogen (Fig. 4A). Diatrizoate inhibited plasminogen binding by $51 \pm 3.8\%$ compared to control ($p < 0.001$), while ioxaglate inhibited plasminogen binding by $30.1 \pm 4.2\%$ ($p < 0.01$). Ioversol did not significantly inhibit plasminogen binding ($19.4 \pm 6.6\%$ reduction compared to control, $p > 0.06$). Sucrose/NaCl solutions with the same osmolarity as each contrast agent did not inhibit binding of plasminogen to clots (data not shown). Diatrizoate (10%) did not displace plasminogen from lysine-Sepharose (data not shown), suggesting that the inhibition of plasminogen binding to fibrin clots was not mediated by an epsilon-aminocaproic acid (EACA)-like effect. We also examined the effects of contrast agents on plasmin-mediated fibrinolysis (Fig. 4B). Diatrizoate inhibited clot lysis at 60 minutes by $40 \pm 5.7\%$ compared to control ($p < 0.03$), while ioxaglate and ioversol

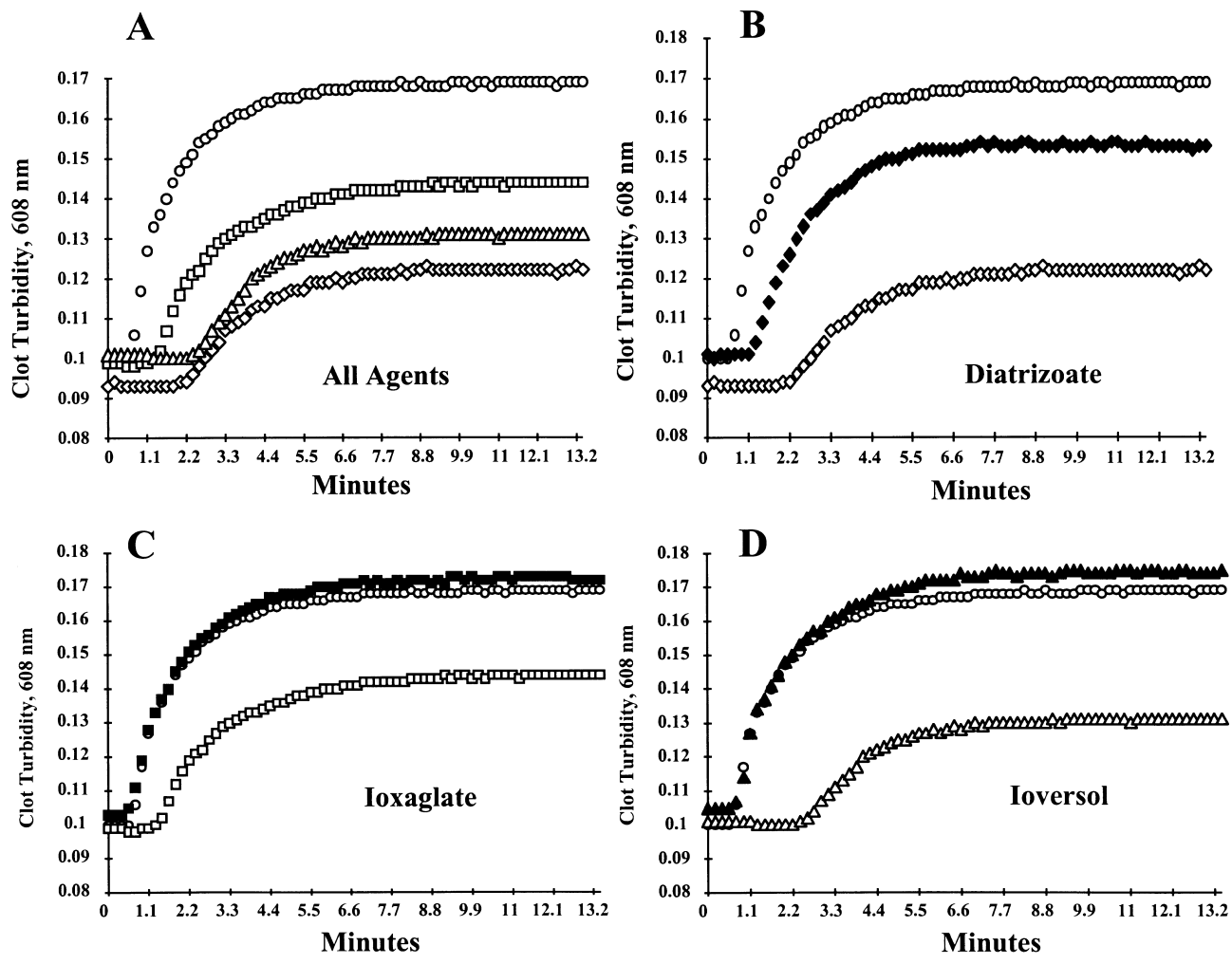

Fig. 5. *Effects of radiographic contrast agents on clot turbidity. A. Thrombin was added to plasma in the presence of 2.5% diatrizoate (diamonds), ioxaglate (squares), ioversol (triangles), or Tris-buffered saline (TBS, circles), and clot turbidity was monitored. B–D. Comparison of effects of diatrizoate (B), ioxaglate (C), and ioversol (D) to those of their respective isosmolar sucrose/NaCl solutions (see text for details). Open diamonds, squares, or triangles represent each contrast agent (2.5% concentration). Filled diamonds, squares, or triangles represent 2.5% concentrations of appropriate isosmolar control solution. Circles represent TBS.*

had no significant effect. However, if fibrin clots were formed in the absence of contrast agents, then incubated with plasmin or fluorescein-labeled plasminogen in the presence of contrast agents (2.5%), fibrinolysis and plasminogen binding were not inhibited (data not shown), suggesting that modification of clot structure during fibrin formation was necessary for contrast agents to inhibit fibrin-plasmin(ogen) interactions.

Effects of Radiographic Contrast Agents on Clot Turbidity

The maximum turbidities of clots formed in the presence of diatrizoate, ioxaglate, or ioversol (each at 2.5%) were 42%, 65%, and 43% that of control clots (Fig. 5A), suggesting that each contrast agent induced formation of thinner fibrin fibers, with ioxaglate producing the least perturbation in fibrin structure. A sucrose/NaCl solution with the same osmolarity as diatrizoate produced only a minor effect on clot turbidity, while the osmolar control solutions for ioxaglate and ioversol exerted no significant effects (Fig. 5B–D). The turbidity of plasma clots formed in the absence of contrast agents, then incubated in the presence of 10% diatrizoate for 8 hrs decreased by $< 5\%$, suggesting that fibrin structure was not altered by diatrizoate if it was added after clot formation was complete.

Discussion

Our studies demonstrate that contrast agents inhibit fibrinolysis by multiple mechanisms, and that the antifibrinolytic properties of different classes of contrast agents vary significantly. The antifibrinolytic properties of contrast agents have been attributed to their high osmolarities [11]. However, our results suggest that the effects of contrast agents on clot lysis are not mediated by their high osmolarities, but rather by specific, iodine-independent, chemical properties. Inhibition of clot lysis by contrast agents did not appear to result from interactions with the active-sites of plasminogen activators or plasmin. However, contrast agents inhibited plasminogen activation in a concentration-dependent manner. Our results are consistent with those of Schilvold et al. [20]. However, these studies involved significantly higher concentrations of contrast agents than our experiments, did not examine plasminogen activation in plasma, and did not investigate the osmolar effects of contrast agents on plasminogen activation. Previous studies demonstrated that contrast agents inhibit the capacity of plasminogen activators to lyse fibrin clots [10,21]. However, in these experiments it was not possible to differentiate effects of contrast agents on plasminogen activation from "downstream" effects on the lysis of fibrin by plasmin. Our experiments show that clots formed in the presence of contrast agents, particularly diatrizoate, are resistant to lysis by plasmin. Since plasmin activity was not inhibited by contrast agents, their capacity to inhibit clot lysis is most likely explained by a direct effect on fibrin. Our studies of clot turbidity suggested that the contrast agents used in our experiments modified fibrin structure, with diatrizoate and ioversol exerting greater effects than ioxaglate. These changes are consistent with earlier studies demonstrating that contrast agents induce formation of fibrin fibers with lower mass-length ratios [22,23]. However, a functional consequence (i.e. reduced sensitivity to lysis by plasmin) of contrast-induced changes in fibrin structure has not been demonstrated previously. In addition, we showed that clots formed in the presence of contrast agents, particularly diatrizoate, demonstrate reduced plasminogen binding capacity. This may represent an important mechanism by which contrast agents downregulate fibrinolysis, since plasminogen recruitment to fibrin is a critical determinant of clot lysis [24,25]. However, our results suggested that clot structure, capacity to bind plasminogen, and sensitivity to lysis by plasmin were altered by contrast agents only if they were present during clot formation. These data support the hypothesis that radiographic contrast agents modify fibrin by affecting its polymerization, but do not significantly affect the structure or sensitivity to lysis of preformed clots.

It therefore appears that radiographic contrast agents can inhibit both blood coagulation and fibrinolysis, with ionic agents generally producing a more potent effect on both processes than nonionic agents do. It is reasonable to hypothesize that the ratio of the anticoagulant effect to antifibrinolytic effect of a contrast agent determines its net antithrombotic effect. It is of interest that ioxaglate, a low osmolar ionic agent, produced the least inhibition of plasma clot lysis and the least perturbation of fibrin fiber density, since in previous studies we found that ioxaglate (in comparison to diatrizoate and ioversol) produced the most potent inhibition of thrombin generation. Thus, ioxaglate exhibits the highest ratio of anticoagulant/antifibrinolytic effects—i.e. the least thrombogenic profile. This observation is consistent with clinical studies suggesting that ioxaglate is associated with a lower risk of thrombosis during coronary angioplasty than nonionic contrast agents are [6–8,26,27]. Although the relative antithrombotic effects of ioxaglate observed in these trials may be explained by its more potent inhibition of blood coagulation, the relative lack of inhibition of clot

lysis by ioxaglate compared to other contrast agents could also contribute to its apparent clinical benefits.

Although our studies identify potential mechanisms by which contrast agents may modulate fibrinolysis, several factors must be taken into account when considering their clinical significance. Our experiments did not examine the potential effects of contrast agents on coronary blood flow, platelets, and vascular endothelial cells, all of which can modulate thrombolysis. In addition, our experiments used steady-state concentrations of contrast agents. During angiography the concentration of contrast agents in the coronary circulation varies substantially. Plasma levels are very high for several seconds during contrast injection, but decay rapidly in the absence of coronary occlusion. Although adult patients may receive as much as 200 mL of contrast agent during cardiac catheterization, the circulating concentration of contrast agents between injections is probably less than 2% [28]. Nevertheless, Dehmer et al. demonstrated that plasma clots prepared from peripheral blood samples obtained at the completion of catheterization procedures were resistant to lysis *in vitro*, suggesting that even low concentrations of contrast agents can affect clot lysis [10]. In addition, Pislaru et al. demonstrated that contrast agents inhibit of t-PA-mediated coronary thrombolysis in dogs [11]. Even minor effects of contrast agents on fibrinolysis could potentially influence the results of clinical trials in which thrombolytic drug efficacy is determined by serial injections of contrast agents into an occluded coronary artery in order to determine the time required to restore patency. Therefore, it may be important for these types of angiographic studies to rigorously standardize the type and amount of contrast agent that is administered after infusion of the thrombolytic drug.

Acknowledgments

This work was supported by National Institutes of Health grants HL-02728 (WPF) and HL-07853-01 (PMF) and by Research Fellowship Award 26F967 (PMF) and Grant-in-Aid Award 15GS9789 (WPF) from the American Heart Association, Michigan Affiliate.

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