Characterization of Ultrasound-Potentiated Fibrinolysis In Vitro

By Ales Blinc, Charles W. Francis, Janet L. Trudnowski, and Edwin L. Carstensen

We have characterized the effects of ultrasound on fibrinolysis in vitro to investigate the mechanism of ultrasonic potentiation of fibrinolysis and to identify potentially useful ultrasound parameters for therapeutic application. Radio-labeled clots in thin walled tubes were exposed to ultrasound fields in a water bath at 37°C, and lysis was measured by solubilization of radiolabel. Ultrasound accelerated lysis of plasma, whole blood, and purified fibrin clots mediated by recombinant tissue-type plasminogen activator (rt-PA), urokinase, or streptokinase, but ultrasound by itself caused no clot solubilization. The degree of ultrasonic potentiation was dependent on plasminogen activator concentration, increasing from 2.2-fold at a streptokinase concentration of 75 U/mL to 5.5-fold at 250 U/mL in a 1 MHz ultrasound field at 4 W/cm². Ultrasound exposure resulted in heating due to absorption by the plastic tube, but the temperature increase was insufficient to account for the increase in clot lysis rate, indicating that the primary effect was nonthermal. Ultrasound did not accelerate hydrolysis of a peptide substrate by rt-PA and did not alter the rate of plasmic degradation of fibrinogen, indicating that the augmentation of enzymatic fibrinolysis required the presence of a fibrin gel. The acceleration of fibrinolysis by ultrasound was greater at higher intensities and duty cycles and was maximum at frequencies between 1 and 2.2 MHz, but decreased at 3.4 MHz. These findings suggest that ultrasound accelerates enzymatic fibrinolysis by increasing transport of reactants through a cavitation-related mechanism.

© 1993 by The American Society of Hematology.

MATERIALS AND METHODS

Clots and measurements of fibrinolysis Whole blood was collected by venipuncture from healthy, informed volunteers into tubes containing buffered sodium citrate (Becton Dickinson Vacutainer Systems, Rutherford, NJ). Pooled plasma was prepared from five single donor units of citrated fresh frozen plasma obtained through the American Red Cross (Rochester Region, Rochester, NY), aliquotted, and stored at −70°C until use. Fibrinogen (grade I; Helena Laboratories, Beaumont, TX) was diluted to a final concentration of 6 mg/mL in 0.05 mol/L Tris-hydrochloric acid buffer, pH 7.4, containing 0.10 mol/L sodium chloride. Fibrinogen was 125I-radio-labeled using the iodogen technique, and the radioabeled fibrinogen was greater than 90% clottable. Clots were prepared in thin-walled 8-mm diameter nitrocellulose tubes (Beckman, Palo Alto, CA). The platelet phosphodiesterase inhibitor UDCG 212 BS (Boehringer Ingelheim, Ingelheim, Germany) was added to the blood or plasma before clotting in a final concentration of 0.2 mmol/L to prevent clot retraction. Aliquots of 160 μL of blood, plasma, or fibrinogen solution were transferred into the tubes after being mixed with a trace amount of 125I.Fibrinogen. Clotting was induced by adding calcium chloride to a final concentration of 50 mmol/L and bovine thrombin (Calbiochem, La Jolla, CA) to a concentration of 10 U/mL. Clots were overlaid with citrated plasma (840 μL) containing 5 U/mL heparin (Riker Labs, Inc, Northridge, CA) and rt-PA (Activase; Genetech, South San Francisco, CA), urokinase (Abbokinase; Abbott Laboratories, North Chicago, IL), or streptokinase (KabiKinase; Kabi Vitrum, Stockholm, Sweden) was then added to the plasma to induce clot lysis. In some experiments, the activators were incorporated throughout the clot by mixing with the plasma before clotting. Plasmin activity in plasma overriding the clots was inhibited at desired times by adding aprotinin (Trasylo1; Mobay Chemical Co, New York, NY) to a concentration of 500 Kallikrein inhibitor units (KIU)/mL. The samples were mixed and centrifuged at 2,300g for 5 minutes. An aliquot of the supernatant...
was removed and the percent of lysis was calculated from the solubilization of the radio-labeled fibrin.

**Enzymatic degradation of soluble substrates.** The rate of rt-PA hydrolysis of the chromogenic substrate H-D-isoleucyl-L-prolyl-L-arginine-P-nitroanilide (S-2288; Kabi Vitrum) was measured at 405 nm using a solution of 1 mmol/L S-2288 at 0.2 μg/mL rt-PA. Plasmin degradation of fibrinogen was evaluated using purified fibrinogen (grade I; Kabi Vitrum) containing copurified plasminogen and dissolved in 0.05 mol/L Tris-hydrochloric acid buffer containing 0.10 mol/L sodium chloride to which urokinase was added to a concentration of 250 U/mL. Fibrinolysis was stopped at desired intervals by the addition of aprotinin (500 KIU/mL), and aliquots of the solution were electrophoresed using a 4% to 10% sodium dodecyl sulfate-polyacrylamide gel and a discontinuous buffer as described previously. The rate of fibrinogen degradation was estimated from the appearance of fragment D1 determined by densitometric scanning of Coomasie-stained gels.

**Ultrasound apparatus.** The clots and soluble substrates were exposed to ultrasound in thin-walled nitrocellulose tubes, with an attenuation of less than 0.8 dB at 1 MHz as determined by insertion loss measurements when the water-filled tube was placed between the ultrasound source and a needle hydrophone (Type 80-0.5-4.0, Imotek GmbH, Wurselen, Germany). The tubes were suspended in a circular test tube rack with a diameter of 2 cm and immersed in a tank containing water maintained at 37°C. The axis of the rack was placed 3.5 cm from the ultrasound transducer and rotated with a frequency of 6 to 8 rpm to give equal average exposure to all tubes. A 3-cm thick rubber block was placed behind the rotating rack as an acoustic absorber to minimize standing waves.

The active elements of each source transducer used in this study were piezoelectric crystals with 2.5 cm diameters operating at frequencies of 1, 2.2, and 3.4 MHz. The total acoustic power from each source was measured with a radiation force meter at a distance of 2.2 cm, which corresponded to the closest approach of the sample tubes to the transducer during their rotation. The fields were specified throughout in terms of spatial average acoustic intensity, defined as the total acoustic power divided by the area of the source crystal. However, the intensity profile was spatially complex, as shown in a plot of the intensity as a function of transaxial position at a distance of 2.2 cm from the 1 MHz transducer used in the experiments (Fig 1). Although the spatial average intensity was 1 W/cm², the local intensity experienced by the sample in its transit varied from 0.2 to 2 W/cm².

**Temperature measurements and the temperature dependence of fibrinolysis.** For temperature measurements, clots were formed around 100-μm diameter copper-constantin thermocouples, and the temperature was recorded by a BAT-4 laboratory thermometer (Bailey Instruments, saddle Brook, NJ) at 10-second intervals for the first 3 minutes and at 5-minute intervals for the rest of the 1 hour of exposure to ultrasound. To test for the effects of heating without ultrasound, the temperature dependence of plasma clot lysis with 1 μg/mL rt-PA was measured in the range of 20°C to 54°C using a water bath to control the temperature.

**Statistical analysis.** The statistical significance of the differences between means of multiple groups was tested using two-way analysis of variance with post-hoc discrimination between groups by the Dunnet test. When only two groups were compared, the two-tailed Student's t-test for unpaired data was used. All experiments were performed with the minimum number of 4 samples in a group. Linear regression analysis was used to describe the dependence of clot lysis on temperature and ultrasound duty cycle, and the time course of S-2288 hydrolysis.

**RESULTS**

Plasminogen activators were added to plasma overlying plasma clots, and the extent of fibrinolysis was measured by solubilization of radiolabeled fibrin at 1 hour in the presence and absence of 1 MHz ultrasound at an intensity of 4 W/cm² (Fig 2). In the absence of activator, there was mean baseline lysis of less than 5%, and this was not significantly increased by ultrasound exposure. Fibrinolysis increased at higher concentrations of streptokinase, urokinase, and rt-PA in the absence of ultrasound, but was less than 20% at the highest concentrations tested. In the presence of ultrasound, there was greater clot lysis with all activators. The increase was significant at the lowest concentrations tested, ie, 75 U/mL of streptokinase, 62.5 U/mL of urokinase, and 0.125 μg/mL of rt-PA. However, the degree of potentiation by ultrasound was greater at higher activator concentrations. For example, with streptokinase at 75 U/mL, ultrasound increased 1-hour clot lysis 2.2-fold, whereas the potentiation was 5.5-fold at 250 U/mL of streptokinase. For rt-PA, regression analysis defined a relationship between the ratio of clot lysis in the presence compared with the absence of ultrasound (Y) and rt-PA concentration (X) of Y = 1.1X + 2.0 (r = .84) (Fig 2D).

Ultrasound accelerated lysis of clots prepared from whole blood, plasma, or purified fibrinogen, and the degree of augmentation was similar for the three types of clots. For example, exposure to 3.4 MHz at 4 W/cm² increased the 1-hour lysis of purified fibrin clots with 1 μg/mg rt-PA from 23% ± 7% in the absence of ultrasound to 48% ± 9%. The lysis of plasma clots and whole blood clots increased similarly, from 23% ± 5% and 19% ± 5% to 46% ± 6% and 36% ± 8%, respectively (P < .005 in all cases).
Ultrasound could accelerate fibrinolysis by increasing transport of plasminogen activator into the clot from the surrounding plasma. To determine if this mechanism was responsible for the potentiation observed, clots were prepared from plasma mixed with a low concentration of activator before it was clotted by the addition of thrombin. These clots were then overlaid with plasma containing no activator, and lysis was compared in the presence and absence of ultrasound. Ultrasound exposure resulted in a marked increase in 1-hour clot lysis with either rt-PA (50 ng/mL) or streptokinase (10 U/mL).

Fig 2. Effect of plasminogen activator concentration on plasma clot lysis. Plasma clots containing radiolabeled fibrin were overlaid with plasma containing streptokinase (A), urokinase (B), or rt-PA (C) at increasing concentrations. The percent lysis in 1 hour was measured in the absence (□) or presence (○) of 1 MHz ultrasound at an intensity of 4 W/cm². Data are shown as the mean ± SD of four to six measurements for each point. Statistical significance of differences in lysis of insonified and noninsonified clots at each activator concentration was assessed using the two-tailed Student’s t-test (*P < .05; **P < .005). (D) The ratio of lysis in the presence of ultrasound to that in the absence of ultrasound is plotted as a function of rt-PA concentration using data derived from (C). Linear regression analysis derived an equation for the line Y = 1.1X + 2.0 (r = .84).

Fig 3. Degradation of fibrinogen in the presence or absence of ultrasound. Fibrinogen (3 mg/mL) containing copurified plasminogen was incubated with 250 U/mL urokinase at 37°C for 2 hours. At intervals, fibrinogenolysis was stopped by the addition of aprotinin (500 KIU/mL) to inhibit plasmin. The extent of degradation was evaluated by sodium dodecyl sulfate 4% to 10% polyacrylamide gel electrophoresis to identify fragments X, Y, D1, and E. There was no difference in the pattern of plasmic degradation in the presence and absence of ultrasound. The rate of degradation assessed as accumulation of fragment D1 determined by densitometric scanning was not accelerated in the presence of ultrasound.
ULTRASOUND POTENTIATED FIBRINOLYSIS

There was 66% ± 5% lysis of clots containing rt-PA in the presence of ultrasound compared with 18% ± 2% in its absence (P < .01). Similarly, lysis of clots with streptokinase was increased more than 10-fold by ultrasound exposure, from 6% ± 2% to 69% ± 28% (P < .01).

To determine if the enhanced enzymatic activity required the presence of a fibrin gel, the activities of plasminogen activators in solution were compared in the presence and absence of ultrasound. The amidolytic activity of rt-PA toward the chromogenic substrate H-D-isoleucyl-L-prolyl-L-arginine-P-nitroanilide dihydrochloride was the same in the presence or absence of ultrasound (data not shown). Urokinase was also incubated with fibrinogen and plasminogen in solution, and the rate and pattern of plasmic degradation were compared electrophoretically (Fig 3). The expected degradation of fibrinogen to fragments X, Y, D, and E was unaltered by ultrasound, and no new bands were identified, indicating that ultrasound did not alter the pattern of plasmic cleavages. The rate of plasmic degradation of fibrinogen was estimated by the accumulation of fragment D1, a terminal derivative. Densitometric scanning of the gels showed no acceleration of the appearance of fragment D1 in the presence of ultrasound, demonstrating that ultrasound did not significantly alter the rate of plasminogen activator-induced degradation of soluble fibrinogen.

Absorption of ultrasound causes heating, and the resulting increase in temperature could thereby accelerate the enzymatic hydrolysis of fibrin. Therefore, the temperature of
was determined by solubilization of radiolabel. Linear regression analysis showed a relation of $Y = 1.1X - 15.6$ ($r = .89$) between 24°C and 50°C.

plasma clots rotating in the ultrasound field was measured to determine the magnitude of this effect (Fig 4). A steady-state temperature was reached by 100 seconds for all frequencies and intensities tested except for 8 W/cm² at 3.4 MHz, at which a steady-state temperature 13°C above baseline was reached at 5 minutes. Temperature increases were dependent on both frequency and intensity of exposure. For example, exposure to 1 MHz at 1, 2, 4, and 8 W/cm² resulted in increases of 1.3°C, 2.1°C, 3.6°C, and 6.5°C, respectively. Heating was greater at higher frequencies, with temperature increases at 3.4 MHz of 6°C and 13°C at 4 and 8 W/cm², respectively. Exposure of the thermocouple to the ultrasound field in the water outside the tubes resulted in no increase in temperature. However, tubes filled with only water had temperature increases equal to those containing clot, indicating that heating was caused by ultrasound absorption by the plastic tube wall. The temperature-dependence of plasma clot lysis with tr-PA was measured independently using a water bath to control the temperature (Fig 5). The percent clot lysis in 1 hour increased from 25°C to 50°C, and then decreased at higher temperatures, probably due to protein denaturation. There was a 1.1% increase in lysis at 1 hour (95% confidence interval, 0.9% to 1.3%) for every 1°C increase in temperature between 24°C and 50°C, as determined by linear regression analysis.

The ultrasonic enhancement of fibrinolysis was dependent on ultrasound intensity and increased progressively up to 8 W/cm² at 1, 2.2, and 3.4 MHz. For example, exposure to 2.2 MHz ultrasound increased 1-hour lysis from a baseline of 23% to 31%, 40%, 48%, 69%, and 88% at 0.5, 1, 2, 4, and 8 W/cm², respectively (Fig 6). The thermal contribution to the ultrasonic enhancement was estimated from data on heating of clots in the ultrasound field (Fig 4) and from the temperature dependence of fibrinolysis (Fig 5). Because heating increased at higher intensities, the thermal component was greatest at 8 W/cm², but remained less than 20% of the increase caused by ultrasound.

The effect of ultrasound frequency on augmentation of fibrinolysis was investigated in the range of 1 to 3.4 MHz (Fig 7). Fibrinolysis was accelerated at all frequencies, with greater effects at higher intensities. The data were adjusted for the expected heating to determine the nonthermal component of augmentation. The greatest adjustments were at the highest frequency and intensity that generated the most heat. After the effect of heating was subtracted, the nonthermal effect of insonification was statistically significantly reduced at 3.4 MHz compared with 2.2 MHz both at 4 and 8 W/cm² ($P < .05$).

The effect of ultrasound duty cycle on fibrinolytic enhancement was also determined, varying both the proportion and absolute duration of on-time. With a 1:1 duty cycle, the 1-hour lysis was not affected by pulse duration, but was 52% ± 12% at 20 milliseconds, 50% ± 8% at 200 milliseconds, and 45% ± 7% at 2 seconds ($P = \text{NS}$). The percentage of on-time was varied between 0% and 100% using cycles of 200 to 500 milliseconds. The temporal average intensity of ultrasound was linearly dependent on the fraction of on-time and was set to correspond to 4 W/cm² at continuous ultrasound. Clot lysis increased linearly with an increasing fraction of on-time from 24% ± 6% in the absence of ultrasound to 62% ± 12% with continuous exposure (Fig 8). Linear regression analysis showed a 0.32% increase in clot lysis for each added
ULTRASOUND POTENTIATED FIBRINOLYSIS

Fig 7. Effect of ultrasound frequency and intensity on plasma clot lysis. Plasma clots were overlaid with plasma containing 1 µg/mL rt-PA and exposed to ultrasound at intensities up to 8 W/cm². The percent lysis at 1 hour increased in the presence of ultrasound at 1, 2.2, and 3.4 MHz. The data are plotted to show the nonthermal effects of ultrasound as a function of frequency and intensity. The expected temperature increase was estimated based on the data in Fig 4; the augmentation of fibrinolysis of the expected temperature increase is estimated from Fig 5. Clot lysis was significantly less at 3.4 than at 2.2 MHz both at 4 and 8 W/cm² (P < .05) (A) 1 W/cm²; (A) 4 W/cm²; (O) 8 W/cm².

Both clinical and in vitro observations show that accessibility of enzyme to fibrin within a clot is an important determinant of lysis rate. The dissolution of both arterial and venous thrombi is more rapid if plasminogen activator can be injected directly into the thrombus than if it is infused systemically or locally into the vessel proximal to the occlusion. Clot lysis in vitro is more rapid and requires a lower concentration of activator if the enzyme is incorporated throughout the clot at the time of formation or if it is directly injected into the clot rather than if the activator diffuses into the clot from its surface. Blinc et al. have shown that the velocity of thrombolysis is primarily determined by the rate of activator penetration into the clot when the plasminogen activator is present initially outside the clot. If activator penetrates into a clot by thermally activated diffusion, its concentration falls exponentially with distance from the plasma-clot boundary, and mathematical modeling has predicted that the velocity of diffusion-limited thrombolysis is a square root function of the activator concentration in plasma. The dependence of lysis rate on activator concentration in the absence of ultrasound (Fig 2) is consistent with this model, demonstrating a plateauing effect at high concentrations. In contrast, lysis increased as approximately a first power function of activator concentration in the presence of ultrasound, suggesting that ultrasound enhanced transport of activator and resulted in greater penetration and a broader zone of lysis.

The ultrasonic potentiation of fibrinolysis in clots already containing a low concentration of activator throughout could also be due to facilitated transport of enzyme. Fibrin strands in clots are inhomogeneously distributed and constitute less

percent on-time (95% confidence interval, 0.27% to 0.38%), with a linear correlation coefficient of .79. To determine whether ultrasound resulted in long-lasting effects of susceptibility to fibrinolysis, clots were exposed to ultrasound before addition of plasminogen activator. Plasma clots exposed to 1 MHz ultrasound at 4 W/cm² for 1 hour before the addition of 1 µg/mL t-PA showed 33% ± 4% 1-hour lysis compared with 30% ± 3% of clots incubated in the absence of ultrasound for 1 hour before the addition of rt-PA (P = .23).

DISCUSSION

The findings presented show that clot lysis mediated in vitro with rt-PA, urokinase, or streptokinase is significantly accelerated by ultrasound. This effect was mediated through plasmin activity, because ultrasound by itself caused no clot dissolution. Although ultrasound caused some heating related to absorption by the plasmic tube, this was insufficient to explain the degree of acceleration, indicating that the primary effect was nonthermal. Ultrasound did not accelerate the hydrolysis of a peptide substrate by rt-PA, and the rate of plasmin degradation of fibrinogen was not increased. The ultrasonic augmentation of enzymatic fibrinolysis required the presence of a fibrin gel, and was seen with clots of whole blood, plasma, and purified fibrin. The need for a gel substrate is consistent with an effect of ultrasound on transport of reactants.

Fig 8. Effect of ultrasound duty cycle on fibrinolytic augmentation. Plasma clots were overlaid with plasma containing 1 µg/mL rt-PA and exposed to 1 MHz ultrasound at an intensity of 4 W/cm². The percentage of on-time was varied between 0% and 100% with 200 to 300 milliseconds on-times. The percent lysis at 1 hour increased progressively as the percentage of on-time increased with a relation of Y = .32X + 26 (r = .79) as determined by linear regression analysis.
than 1% of the total clot mass, which is predominantly composed of serum-filled pores with diameters from 0.3 to 5 μm. The concentration of fibrin in clots prepared from plasma is approximately 10−3 mol/L and that of plasminogen is 2 × 10−6 mol/L. During fibrinolytic therapy, the plasma concentration of rt-PA is less than 5 × 10−8 mol/L. Because solubilization of the polymerized fibrin requires cleavage of several peptide bonds for fibrin unit, each enzyme must cleave multiple bonds during fibrinolysis. Therefore, the overall reaction rate may be limited by enzyme diffusion and could be accelerated by enhancement of transport by ultrasound.

The results are consistent with the action of ultrasound on small gas bubbles that could provide the local agitation needed to transport activators and plasminogen to their target sites on fibrin molecules. Because gas has a much greater compressibility than liquids, particle displacements in response to acoustic pressure changes are much greater in and near gas bodies than in pure liquids. Small amplitude motion of resonance sized bubbles has been termed stable cavitation, and gives rise to local convection currents or microstreaming. Resonance-sized bubbles at frequencies used in diagnostic ultrasound are a few micrometers in diameter, which is approximately the size of pores in plasma and blood clots. Growth of gas bubbles has been observed in agar gels exposed to ultrasound under conditions similar to those used in this study. At a critical value of acoustic pressure, bubble motion increases precipitously with small increases in acoustic pressure, and extremely large pressures and local heating develop when the bubble collapses, a process termed inertial or transient cavitation. Typically, the transition from stable to inertial cavitation is clear and defines a “threshold” for inertial cavitation. The acoustic fields used in this study exceed the threshold for transient cavitation in water, but inhibition of bubble expansion by gel matrix may shift the threshold. Also, the absence of a clear threshold effect on enzymatic fibrinolysis (Fig 7) and the nearly linear dependence on the ultrasound intensity and on-time (Fig 8) are more typical of stable, rather than inertial, cavitation. The frequency dependence of effects is also consistent with this hypothesis because the nonthermal component of the effect decreased at the highest frequency (Fig 8), as expected.

The findings in this report and other in vitro studies suggest that the percutaneous, noninvasive delivery of high-frequency ultrasound has the potential to be a useful adjunct to fibrinolytic therapy for the lysis of arterial or venous thrombi in the extremities. The near-field exposure used in our studies is similar to that routinely and safely applied in diathermy at intensities of up to 1 to 2 W/cm². Although ultrasound at 1 W/cm² produced only an approximately 50% increase in clot lysis in our in vitro study, limited studies on dogs have shown marked shortening of the time to reperfusion with no apparent adverse effects at an ultrasound intensity of less than 1 W/cm². The high-frequency ultrasound used in this study does not cause mechanical fragmentation of the clot or produce particles that could embolize, but rather accelerates plasmin degradation of fibrin into soluble fragments. Advantages of high-frequency ultrasound as an adjunct to fibrinolytic therapy include its noninvasiveness, its potential to reduce time to reperfusion, and its ability to direct application locally, thereby limiting fibrinolytic enhancement to the site of thrombosis and avoiding additional systemic effects.

ACKNOWLEDGMENT

We acknowledge the expert technical assistance of Sally Child and Carol Raeman in the performance of the experiments and the help of Carol Weed in preparation of the manuscript.

REFERENCES

Characterization of ultrasound-potentiated fibrinolysis in vitro

A Blinc, CW Francis, JL Trudnowski and EL Carstensen