Enhancement of Fibrinolysis With 40-kHz Ultrasound

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Background—Ultrasound at frequencies of 0.5 to 1 MHz and intensities of ≥0.5 W/cm² accelerates enzymatic fibrinolysis in vitro and in some animal models, but unacceptable tissue heating can occur, and limited penetration would restrict application to superficial vessels. Tissue heating is less and penetration better at lower frequencies, but little information is available regarding the effect of lower-frequency ultrasound on enzymatic fibrinolysis. We therefore examined the effect of 40-kHz ultrasound on fibrinolysis, tissue penetration, and heating.

Methods and Results—¹²⁵I-fibrin–radiolabeled plasma clots in thin-walled tubes were overlaid with plasma containing tissue plasminogen activator (tPA) and exposed to ultrasound. Enzymatic fibrinolysis was measured as solubilization of radiolabel. Tissue attenuation and heating were examined in samples of porcine rib cage. Fibrinolysis was increased significantly in the presence of 40-kHz ultrasound at 0.25 W/cm², reaching 39 ± 7% and 93 ± 11% at 60 minutes and 120 minutes, compared with 13 ± 8% and 37 ± 4% in the absence of ultrasound (P < 0.0001). The acceleration of fibrinolysis increased at higher intensities. Attenuation of the ultrasound field was only 1.7 ± 0.5 dB/cm through the intercostal space and 3.4 ± 0.9 dB/cm through rib. Temperature increments in rib were < 1°C/(W/cm²).

Conclusions—These findings indicate that 40-kHz ultrasound significantly accelerates enzymatic fibrinolysis at intensities of ≥0.25 W/cm² with excellent tissue penetration and minimal heating. Externally applied 40-kHz ultrasound at low intensities is a potentially useful therapeutic adjunct to enzymatic fibrinolysis with sufficient tissue penetration for both peripheral vascular and coronary applications. (Circulation. 1998;98:1030-1035.)

Key Words: fibrinolysis ■ ultrasonics ■ tissue

Fibrinolytic therapy is widely used to accelerate the enzymatic dissolution of thrombotic vascular occlusions and thereby restore blood flow and limit ischemic damage. It has application in the treatment of deep vein thrombosis and pulmonary embolism and also in arterial disease, including peripheral arterial occlusion, stroke, and acute myocardial infarction. Therapeutic success in treatment of arterial occlusion depends on the rapidity of restoring blood flow, because tissue necrosis increases rapidly with the duration of ischemia. Therefore, several approaches have been used to maximize both the rate and completeness of reperfusion, including the use of adjunctive antiplatelet or anticoagulant therapy as well as new fibrinolytic agents and regimens.

Ultrasonic radiation at megahertz frequencies enhances enzymatic fibrinolysis in vitro⁴⁻⁸ and in animal models of arterial⁹⁻¹² and small-vessel¹³ thrombosis with noninvasive transdermal application. The rate of fibrinolytic enhancement is directly related to the temporal average intensity of the field,⁴ which also determines the production of heat in the tissues by absorption of the ultrasound. At frequencies of > 1 MHz, the intensities necessary to achieve significant enhancement of thrombolysis cause tissue heating that approaches levels that could be harmful. Although the elevated temperature can cause minor acceleration of thrombolysis by itself, the predominant mechanism of action of ultrasound is nonthermal.⁴ The present study shows quantitatively that midkilohertz frequencies are more effective in enhancement of thrombolysis than megahertz frequencies. These lower frequencies have important potential advantages for therapy, because the lower absorption of ultrasound in tissues causes less heating and the acoustic field can be broader and more uniform with a greater depth of penetration than occurs at megahertz frequencies.

Methods

Clot Preparation

Fresh-frozen plasma anticoagulated with acid citrate dextrose was obtained through the American Red Cross (Rochester Region), pooled, divided into aliquots, and stored at −70°C until use. Clots were prepared in thin-walled, 8-mm-diameter polyester tubes (Beckman) with an attenuation of < 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 PVDF needle hydrophone (type 80-0.5-4.0 Imotek GmbH). It can be assumed that the attenuation is even less at lower frequencies. To prepare clots, aliquots of 160 μL of plasma were transferred into the tubes after being mixed with a trace amount of ¹²⁵I-radiolabeled fibrinogen.¹ Clotting was induced by addition of calcium chloride to a final concentration of 20 mmol/L and bovine thrombin (Calbiochem) to a concentration of 0.5 NIH U/mL. Clots were incubated for 1 hour at 37°C before use.
Fibrinolysis
Plasma clots were overlayed with 820 µL of plasma containing 2 U/mL heparin (Riker Labs, Inc), and recombinant tPA (Activase, Genentech) was added to the overlying solution. At desired times, tubes were removed from the apparatus, and fibrinolysis was stopped by the addition of 500 kallikrein inhibitory units/mL aprotinin (Trasylol, FBA Pharmaceuticals) to inhibit plasmin. The residual clot was removed, and the remaining fluid was centrifuged at 2300 rpm for 5 minutes. The sediment and supernatant were counted separately, and sedimentable radioactivity was considered to represent clot fragments and nonsedimentable to represent soluble derivatives.

Ultrasound Treatment
Sample tubes were suspended on a 2.5-cm-diameter circular test-tube rack and immersed in a tank of water at 37°C. The axis of the rack was adjusted so that tubes approached within 1 cm from the face of the transducer, and the rack was rotated at a frequency of 6 to 8 rpm to give equal average exposures to all of the tubes. A 3-cm-thick block of natural rubber mounted on a 1-cm-thick sheet of acrylic was placed behind the sample to minimize reflections. A thin sheet of air-saturated cork-rubber material was attached to the back side of the acrylic to prevent exposure of control samples that were placed behind this barrier.

The source of ultrasound was a 2.5-cm-diameter, 40-kHz piezoelectric transducer (provided by Eastman Kodak Co), driven in continuous mode. The acoustic pressures were measured before and after each experiment with a hydrophone (type 8103, Bruel and Kjær). The face of the hydrophone was placed at the site of closest approach of the samples. Intensities reported here are converted from measured pressures by the relationship $I = \rho^2/2c$, where $I$ is intensity, $\rho$ is the acoustic pressure amplitude, $\rho$ is the density, and $c$ is the speed of sound in water.

Attenuation of Rib Cage
The hydrophone was located on the axis at a distance of 2 cm from the face of the 40-kHz source, and a reference pressure amplitude was measured at this location. A section of porcine rib cage obtained from a meat market was inserted between source and hydrophone, and a series of pressure amplitudes was measured as the rib cage was moved laterally so that several ribs and intercostal spaces passed between the source and hydrophone. Attenuation was calculated from the ratio of the pressure amplitude with the tissue in the field to the reference level measured without the tissue in the field. Beam patterns were measured by recording relative output levels from the hydrophone as it was moved transaxially.

Heating
For direct temperature measurements in porcine rib, a 25-µm copper constantin thermocouple was cemented into a shallow groove cut into the surface of the bone. The entire sample was degassed under vacuum, placed on a plastic film window in a cylindrical container, and embedded by pouring warm agar into the container. These procedures were necessary to eliminate gas bodies near the thermocouple and convection of the coupling medium that might cause errors in the measurement. Absorption of ultrasound in the agar was negligible. The bone sample was placed on the axis of the calibrated field of the 40-kHz source. Temperatures were measured as a function of time and intensity.

Statistics
All data are expressed as mean±SD. Comparison of means was performed with $t$ tests assuming unequal variance between groups, and Bonferroni’s correction was applied for multiple comparisons. The rates of fibrinolysis with tPA in the absence or presence of ultrasound (Figure 1) were fitted by a mixed linear model, and $P$ values are reported as for tests of fixed effects analyzed with the SAS system.

Results
Radiolabeled plasma clots were prepared, overlayed with heparinized plasma containing 1 µg/mL of tPA, and incubated at 37°C in the presence of 40-kHz ultrasound at 0.25 W/cm² or in the absence of ultrasound (Figure 1). Fibrinolysis was increased significantly in the presence of ultrasound and tPA, reaching 39±7% and 93±11% at 60 minutes and 120 minutes, respectively, compared with 13±8% and 37±4% in the absence of ultrasound ($P<0.0001$). Fibrinolysis was <7% at all time points with or without ultrasound exposure in the absence of tPA.

The acceleration of fibrinolysis with 40-kHz ultrasound was dependent on intensity (Figure 2). The extent of fibrino-
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Figure 3. Enhancement of fibrinolysis at various concentrations of tPA. Radiolabeled plasma clots were overlayed with plasma containing no tPA or with tPA at 0.25, 0.5, or 1 μg/mL. They were then incubated at 37°C for 1 hour and exposed to 40-kHz ultrasound (US) at various intensities up to 1.5 W/cm². Solubilization at 1 hour was determined by percentage of radiolabel remaining in solution after centrifugation. Data are mean±SD.

Figure 4. Relative transaxial intensity for 40-kHz transducer. Open symbols show intensities with a porcine rib cage section between source and hydrophone. With 1 beam pattern (○), a rib is centered on axis of sound field. In the other (□), intercostal space is on axis. Solid symbols show intensities in absence of tissue.

field transmitted through a representative sample of porcine rib cage under 2 conditions. In 1, a rib is centered on the axis of the field, and in the other, the intercostal space is centered on the axis. Averaged over 4 samples, the attenuation of the field through the intercostal space was 1.7±0.5 dB/cm, and through the bone, 3.4±0.9 dB/cm. Measurements of heating indicate that temperature increments in rib are <1°C per W/cm².

Discussion

The results demonstrate marked enhancement of fibrinolysis with 40-kHz ultrasound at intensities as low as 0.25 W/cm² and tPA concentrations between 0.25 and 1 μg/mL. At 0.25 W/cm², the ultrasound-induced enhancement of fibrinolysis was greater at higher tPA concentrations, with a 27% enhancement at 0.25 μg/mL, whereas it was 80% at 1 μg/mL. The acceleration of fibrinolysis was also greater at higher ultrasound intensities, which reached a maximum of nearly 4-fold at 1 μg/mL tPA and 1.5 W/cm², increasing from 20% to 77%. Greater fibrinolysis could be achieved with ultrasound at a lower tPA concentration than without ultrasound at higher tPA concentrations.

Increased fibrinolysis with ultrasound was achieved with minimal mechanical disruption of the clot, consistent with prior observations at higher frequencies³,⁴ indicating that enhancement of clot dissolution is due primarily to accelerated enzymatic action. At 1 W/cm², the maximum mechanical disruption observed in any experiment in the absence of tPA was 0.8%. Greater mechanical disruption occurred in experiments with tPA present, reaching a maximum of 3.8% at 1 μg/mL and 1.5 W/cm². This is consistent with the hypothesis that tPA and ultrasound act together, and the mechanical effects of low-intensity ultrasound are observed only after the fibrin network has been proteolytically weakened. Generation of large amounts of clot fragments may be undesirable therapeutically, because they could obstruct distal small arteries.

Low-frequency ultrasound at high intensity has been used in a qualitatively different manner in earlier investigations. Several studies demonstrated the ability of wires vibrating at frequencies of 20 to 25 kHz and high power levels of up to 20
W to disrupt clots in vitro. This approach has been used to fragment thrombi into small particles, resulting in reperfusion in patients with obstructed peripheral arteries. A preliminary study in 20 patients undergoing coronary artery bypass graft surgery for angina demonstrated complete recanalization in 70% of vessels, but arterial perforation occurred in 2 cases. Additional difficulties with this approach for therapy include the unknown effects of distal embolization of fragments, damage to the vessel wall, and heating. Technical problems include limited flexibility of the ultrasonic wire and breakage. In addition, the need for selective catheterization requires specialized facilities and highly trained personnel. Ultrasound at 20 kHz applied transdermally in combination with an echo contrast agent induced reperfusion in thrombosed rabbit femoral arteries without administration of any plasminogen activator, presumably by causing mechanical fragmentation, but excessive heating was a problem. Transdermal application of ultrasound at ≈37 kHz in combination with intravenous stabilized microbubbles was effective in recanalizing thrombosed rabbit iliofemoral arteries in comparison with either ultrasound alone or microbubbles alone.

Our approach is different in concept and uses low-intensity ultrasound to accelerate enzymatic fibrinolysis. A critical issue in developing ultrasound to enhance enzymatic thrombolysis for therapeutic application is identification of the optimum frequency and intensity. Prior reports have demonstrated enhancement of thrombolysis by use of ultrasound at ≈0.5 MHz and intensities of ≈0.5 W/cm². The limited tissue penetration of ultrasound at 1 MHz, however, would restrict potential therapeutic application to vessels in the arms and legs, and the levels required would make tissue heating an additional problem. Miniaturized transducers have also been attached to catheters for endovascular use and this offers the potential to deliver localized ultrasound at the site of thrombosis while limiting exposure of normal tissue. For wide therapeutic application, however, noninvasive external application of ultrasound has greater potential, because it requires neither angiography nor selective catheterization, it eliminates the risk of vessel wall damage by the catheter, and it could be used for vessels too small or inaccessible for catheterization.

Some published information indicates that enzymatic thrombolysis may be enhanced at lower ultrasound frequencies. Tachibana found acceleration of urokinase-induced lysis of whole blood clots in a Chandler loop model with 48-kHz ultrasound, and Olsson et al demonstrated increased fibrinolysis in vitro with streptokinase using 170-kHz pulsed ultrasound at 0.5 W/cm² and 1% duty cycle. Luo et al reported that 28-kHz ultrasound at 18 W/cm² applied transcutaneously significantly accelerated streptokinase-induced thrombolysis in rabbit femoral arteries in comparison with ultrasound alone or streptokinase alone. Thermal injury to the dermis also occurred. A more recent report showed a small acceleration of whole-blood clot lysis with urokinase in combination with 170-kHz ultrasound at 0.5 W/cm². A catheter-mounted transducer at 225 kHz accelerated thrombolysis with urokinase in vitro as did a 20-kHz catheter-mounted transducer. The latter demonstrated enhancement of fibrinolysis with 20-kHz ultrasound at intensities of 1 and 1.5 W/cm² and reported up to 40% "fibrinolysis" that may have represented mechanical clot disruption. The intensity of the acoustic field was not fully characterized in the latter reports because of the difficulty in calibration of the very small transducer, which approximated a point source. None of these earlier studies, however, make possible a direct comparison of the relative effectiveness of midkilohertz and megahertz frequencies. This study, however, used the same sample preparations and exposure conditions as were used at megahertz frequencies in earlier experiments in this laboratory. Figure 5 is a quantitative comparison of the data at 1 MHz (Reference 3) with the results at 40 kHz reported here. The advantages of 40-kHz exposures are striking.

The capacity to enhance fibrinolysis at a frequency as low as 40 kHz is important for several reasons. At 1 MHz, the attenuation of soft tissues, such as liver and muscle, is ≈0.5 dB/cm, and this reduces the intensity by 50% in propagation paths on the order of 5 cm, which should be adequate for the treatment of clots in peripheral blood vessels. The attenuation of bone, however, is at least an order of magnitude greater, and use of high-frequency ultrasound for noninvasive treatment of heart or brain is essentially precluded. In contrast, the depth of penetration of 40-kHz ultrasound in soft tissues is for practical purposes infinite, and the rib cage transmits ≈50% of the incident intensity into the heart. Wavelengths of several centimeters mean that beam patterns are broad and relatively uniform even after passing through the chest wall. Also, there is a rather large intensity window between the minimum values that are effective in enhancing thrombolysis (≈0.25 W/cm² in situ) and the levels that would be thermally hazardous (1 to 2 W/cm² at the bone).

Taken together, these factors suggest that an effective treatment need not require critical placement of the transducer relative to the clot or precision calibration of the acoustic fields. In fact, a treatment procedure similar to that used for ultrasonic diathermy in a physical therapy setting may be appropriate for cardiac application. The transducers would be comparable in size and shape, coupling could be via gel on the skin, and stroking of the...
transducer over the target area all might be the same. Only the frequency of the transducer would be different.

For clinical application, it will be necessary to balance the risk of adverse effects of ultrasonic heating against the benefit of an increased rate of thrombolysis. As an illustration, contrast 40 kHz with 1 MHz in a hypothetical application in which the site of interest is 5 cm below the skin surface, the path of the site is soft tissue, and the goal of the treatment is to achieve 50% thrombolysis in 60 minutes (Figure 5). At 1 MHz, 6 W/cm² is required at the site or ≈12 W/cm² at the surface of the body. This is an order of magnitude greater than ordinarily would be used in physical therapy and would produce unacceptably large heating rates. At 40 kHz, the required intensity at the skin (and at the skin surface) is only 0.5 W/cm². Thus, the required surface intensity is lower by more than an order of magnitude, and because the absorption coefficient of the tissue is also much smaller at the lower frequency, concern for heating is essentially eliminated. Two possible exceptions may be mentioned. If a coupling gel is used to make contact between the source transducer and the skin, highly localized heating can occur near air bubbles that become entrapped in the gel, but this is a relatively trivial problem. However, a more serious problem might be encountered if the clot under treatment is located in a vessel near a bone. At 1 MHz, the excess absorption of sound in bone at levels great enough to achieve the target rate of thrombolysis would produce unacceptable heating. In contrast, the 40-kHz measurements of bone heating reported above show that bone heating should not be ignored, but the problem would be minimized by at least an order of magnitude by the lower intensities required and the lower absorption coefficient of bone at 40 kHz.

At midkilohertz frequencies, even the brain may be accessible for noninvasive treatment with ultrasound. Figure 6 is a summary of the published data on the attenuation of skull bone.28-32 Even at 300 kHz, the intensity transmitted through the skull should be >33% of the level incident on the scalp. Attenuation above this frequency is roughly proportional to the frequency. This suggests that at 40 kHz, even with the loss of intensity in passing through the skull, the intensity reaching the brain with thermally acceptable incident levels would be great enough to significantly enhance enzymatic thrombolysis.

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