Ultrasound-Induced Mild Hyperthermia as a Novel Approach to Increase Drug Uptake in Brain Microvessel Endothelial Cells

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Purpose. Drug delivery to the central nervous system (CNS) is limited by the blood-brain barrier (BBB). Thus, a noninvasive and reversible method to enhance BBB permeation of drugs is highly desirable. In the present work, we studied if ultrasound-induced mild hyperthermia (USHT, 0.4 watts (W)/cm^2 at 41°C) can enhance drug absorption in BBB endothelial cells, and we elucidated the mechanism of USHT on cellular accumulation.

Methods. To accomplish these aims, we studied the effects of hyperthermia (41°C), USHT, P-glycoprotein (P-gp) modulator (PSC 833), and combination of USHT and PSC 833 on accumulation of P-gp substrate (R123) and non-P-gp substrates (sucrose, 2-deoxyglucose, and antipyrine) in monolayers of primary bovine brain microvessel endothelial cells (BBMEC).

Results. USHT, through its thermal effect, produces a significant (relative to controls; no USHT) and comparable increase in R123 accumulation with PSC 833. We also demonstrate that USHT increases permeability of hydrophobic (R123 and [^{14}C]-antipyrine) and not hydrophilic molecules ([^{14}C]-sucrose and 2-[^{3}H]-deoxy-D-glucose). The enhanced permeability is reversible and size dependent, as USHT produces a much larger effect on cellular accumulation of [^{14}C]-antitpyrine (molecular weight of 188 D) than that of R123 (molecular weight of 380.8 D). Although USHT increases membrane permeability, it did not affect P-gp activity or the activity of glucose transporters.

Conclusions. Our results point to the potential use of USHT as a reversible and noninvasive approach to increase BBB permeation of hydrophobic drugs, including P-gp-recognized substrates.

KEY WORDS: hyperthermia; ultrasound; multidrug resistance; P-glycoprotein; *P*-glycoprotein modulating agent; blood-brain barrier.

INTRODUCTION

The transport of solutes between the tissue and the blood within the central nervous system (CNS) differs significantly from that of other organs. In contrast to the systemic circulation, brain microvessels form the so-called blood-brain barrier (BBB), an endothelial barrier that is characterized by its limited permeability to water-soluble or nonhydrophobic solutes (1). Moreover, recent identifications of multidrug (MDR) efflux transporters in the BBB such as P-glycoprotein (P-gp) (2) and MDR resistance-associated protein (MRP) (3) have changed the dynamics of the BBB from a restrictive barrier to hydrophilic molecules to one that limits the transport of both hydrophilic and hydrophobic molecules. There is a critical need to develop novel methods that enhance the delivery of both hydrophilic and hydrophobic centrally active drugs to the CNS.

Hyperthermia has been shown to increase membrane permeability of compounds (4) and has been implicated as a potential approach to enhance CNS drug delivery (5). However, studies indicated that long duration and high temperature (43°C) are needed to induce changes in the BBB permeability (5). Unfortunately, this approach is often associated with substantial BBB disruption, leading to increased accumulation of unwanted molecules in the brain and undesirable disturbances of brain function. The availability of a milder, noninvasive, and reversible method for CNS drug delivery is highly desirable and clinically important to enhance treatment with drugs, the access of which to the CNS is limited by the BBB, as is the case with most anticancer drugs.

Most recently, we showed that we could safely increase cellular drug uptake in cancer cells by induction of mild, short-term hyperthermia (41°C for 20 min) using high-frequency ultrasound (USHT) (6). We hypothesized that this same approach could be used to increase BBB permeation of drugs without severely damaging the BBB. To examine this possibility, we studied how USHT affects the cellular uptake of both the P-gp and non-P-gp substrate in a cell culture model of the BBB, namely primary cultured bovine brain microvessel endothelial cells (BBMEC). We demonstrate that USHT can increase BBMEC accumulation of only hydrophobic drugs by altering membrane permeability in a manner that was selective and reversible. We also show that USHT did not alter the activity of two transmembrane transporter proteins. Together, these data suggest the novel and safe use of USHT to increase BBB permeation of hydrophobic drugs, including P-gp-recognized substrates.

MATERIALS AND METHODS

Materials

Type I rat tail collagen was purchased from Collaborative Biomedical (Bedford, MA). Cell culture medium and fetal calf serum (FCS) were obtained from Gibco (Grand Island, NY). [^{14}C]-Antipyrine (0.056 Ci/mmol) and [^{14}C]-sucrose (401.00 mCi/mmol) were purchased from New England Nuclear (Boston, MA). 2-[^{3}H]-deoxy-D-glucose (11.3 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). PSC 833 was purchased from Norvartis (Basel, Switzerland). A nonradioactive cytotoxicity assay kit was purchased from Promega (Madison, WI). All other reagents, unless specifically stated otherwise, were purchased from Sigma Chemical (St. Louis, MO).

BBMEC Isolation and Cell Cultures

BBMECs were isolated from the cerebral gray matter of bovine brain as previously described (7). Purified BBMECs were seeded at a density of 50,000 cells/cm^2 onto collagen-

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coated and fibronectin-treated culture plates (48-well or 10- × 33-mm cell culture plates) in BBMEC plating medium (45% minimum essential medium [MEM], 45% F-12 medium, 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 50 μg/ml polymixin B). The cells were grown in a 37°C incubator with 5% CO_2 and 95% humidity. The plating medium was replaced with BBMEC culture medium (plating medium less polymixin B) on the 3rd day after plating and every day thereafter. BBMECs were allowed to grow to confluence before cellular accumulation studies. Typically, formation of confluent monolayers took about 8 to 10 days.

Ultrasound Apparatus and Exposure

The system used to expose BBMEC monolayers *in vitro* to USHT has been previously described (6). In all studies, ultrasound exposure (0.4 W/cm^2 at 1 MHz) was for a period of 20 min, and the treatment temperature was maintained at 41°C. The accuracy of the power output (watts per squared centimeter) from the ultrasound unit was confirmed by the radiation balance technique using a commercially available radiation balance (UPM DT-10 Ultrasound Powermeter; Ohmic Instruments, Easton, MD).

BBMEC Integrity and Viability as a Function of Ultrasound Intensity

Prior to experiments, BBMEC monolayers in 10- × 33-mm cell culture plates were washed twice and replaced with 12.0 ml of 37°C serum-free MEM. Subsequently, the cells were subjected to USHT treatment at 41°C for 20 min using different intensities of ultrasound (0, 0.4, 0.8, 1.2, 1.6, and 2.0 W/cm^2). Following the ultrasonic exposure, the cells were examined and scored for monolayer integrity (optical microscopy) and cellular viability (measurement of extracellular lactate dehydrogenase [LDH] activity) using the following scale: no effect on monolayer integrity or cellular viability (−), minor effect on monolayer integrity or viability (<10% monolayer detachment and cytotoxicity; +), major effect on monolayer integrity or viability (>20% monolayer detachment and cytotoxicity; ++), and severe effect on monolayer integrity or viability (>50% monolayer detachment and cytotoxicity; +++). The intensity level of ultrasound that produced the least monolayer detachment and cytotoxicity was then chosen for subsequent cellular accumulation studies.

Cellular Accumulation of R123 as a Function of PSC 833, Ultrasound, Hyperthermia, and USHT

Prior to experiments, BBMEC monolayers in 10- × 33-mm or 48-well cell culture plates were washed twice with serum-free MEM. Subsequently, BBMEC monolayers in 10- × 33-mm cell culture plates were replaced with 12.0 ml of 37°C serum-free MEM containing either no R123 or 4 μM R123. The cells then either received no treatment (control, 37°C) or treatment with ultrasound (0.4 W/cm^2 at 37°C or 41°C) or 41°C for 20 min. Conversely, BBMEC monolayers in 48-well cell culture plates were exposed to 0.25 ml of serum-free MEM alone or 0.25 ml of serum-free MEM containing R123 (4 μM) in the presence or absence of 1 μM PSC 833 for 20 min at 37°C. Cellular accumulation studies were then terminated by removing the assay solutions and washing the cells three times with ice-cold phosphate-buffered saline (PBS). The cells were then solubilized by incubation with 0.2 N NaOH (4 ml for 10- × 33-mm, and 1 ml for 48-well cell culture plates) overnight. Aliquots (500 and 50 μl) of the cell lysate solution were then removed for analysis of R123 and protein content (respectively) as previously described (6,7). The amount of protein in each sample was determined by the Pierce BCA method (Pierce Chemical, Rockford, IL). The concentration of R123 in each sample was determined quantitatively by fluorescence spectrophotometry (Shimadzu RF 1501; λ_{ex} = 492 nm, λ_{em} = 535 nm) and was normalized to the protein content of each sample.

Cellular Accumulation of Hydrophilic or Hydrophobic Marker Molecules According to the Elapsed Time after USHT Treatment

BBMECs were seeded and grown in 10- × 33-m cell culture plates to confluence as described above. Prior to experiments, BBMEC monolayers were washed twice and replaced with 12.0 ml of 37°C serum-free MEM. Subsequently, BBMECs were either subjected to no USHT (37°C, control) or USHT (0.4 W/cm^2 at 41°C) treatment for 20 min. At indicated time intervals (0, 20, 40, 60, 120, 180, and 240 min) after USHT treatment, the culture media of both the control and treatment groups were aspirated and the cells were then incubated with 37°C serum-free medium containing either R123 (4 μM), [^{14}C]-antipyrine (0.5 μM), or [^{14}C]-sucrose (1 μM) for 20 min. After the incubation, cellular accumulation studies were terminated by removing the assay buffer solutions and washing the BBMEC monolayers three times with ice-cold PBS. The BBMECs were then solubilized and aliquots of cell lysate solutions were removed for analysis of R123, [^{14}C]-antipyrine, or [^{14}C]-sucrose and protein content, respectively. The level of radioactivity of [^{14}C]-antipyrine or [^{14}C]-sucrose taken into BBMECs was determined using a Beckman LS6000 IC liquid scintillation counter (Beckman Instruments, Fullerton, CA). The concentration of R123 and the amount of protein in each sample were determined as described above.

P-gp and Glucose Transporters Activity during USHT Treatment

BBMECs were seeded and grown in 10- × 33-mm cell culture plates to confluence as described above. Prior to experiments, BBMEC monolayers were washed twice and replaced with 12.0 ml of 37°C serum-free MEM containing either R123 (4 μM) in the presence or absence of PSC 833 (1.0 μM) or 2-deoxyglucose (50 μM) and 2-[^{3}H]deoxy-D-glucose (0.5 μCi) in the presence or absence of 10 μM cytochalasin B (Sigma Chemical). Subsequently, BBMECs were either subjected to no USHT (control, 37°C) or USHT treatment (0.4 W/cm^2 at 41°C) for 20 min. Afterward, cellular accumulation studies were terminated and cellular R123 contents were determined and normalized by protein as described above. To determine the cellular contents of 2-[^{3}H]deoxy-D-glucose, BBMECs were washed and solubilized as described above. The level of 2-[^{3}H]deoxy-D-glucose radioactivity was determined using a Beckman LS6000 IC liquid scintillation counter and was normalized by protein as described above.

P-gp Activity after USHT Treatment

BBMECs were seeded and grown in 10- × 33-mm cell culture plates to confluence as described above. Prior to ex-

periments, BBMECs monolayers were washed twice and replaced with 12.0 ml of 37°C serum-free MEM. Subsequently, BBMECs were either subjected to no USHT (control, 37°C) or USHT (0.4 W/cm^2 at 41°C) treatment for 20 min. Afterward, the culture media of both the control and treatment groups were aspirated and the cells were then incubated with 37°C serum-free medium containing R123 (4 μM) in the presence or absence of PSC 833 (1 μM). At indicated time intervals (0, 20, 40, and 60 min) after the incubation, cellular accumulation studies were terminated and cellular R123 contents were determined and normalized by protein as described above.

Statistical Analysis

All data are presented as mean ± SD from at least three experiments. Treatment groups were compared with control for significance by unpaired Student *t* tests with $p < 0.05$ being considered significant.

RESULTS

Determination of Optimal Ultrasound Intensity to Induce USHT

Using the ultrasound treatment system, we undertook a study to determine the effects of ultrasound intensity on integrity and cellular viability of BBMEC monolayers. For the study, ultrasound exposure at several intensity levels (0, 0.4, 0.8, 1.2, 1.6, and 2.0 W/cm^2) was continued for a period of 20 min. To maintain the treatment temperature at 41°C, the duty cycle was reduced from 100% to 50% for the low-intensity ultrasound treatment (0.4–0.8 W/cm^2) and from 100% to 10% for the high-intensity treatment (1.2–2.0 W/cm^2). These variations of duty cycle could begin as fast as 1 min into the treatment for high-intensity ultrasound exposure or as slow as several min for low-intensity exposure (5 min for 0.4 W/cm^2 and 2.5 min for 0.8 W/cm^2). Treatment of cells with 20-min exposure of 0.4 W/cm^2 ultrasound did not affect cellular viability as well as the integrity of the BBMEC monolayers (Table I). However, when the treatment time exceeded 20 min (e.g., ≥30 min) while holding the intensity constant at 0.4 W/cm^2, a small percentage of cells was displaced from the cell culture plate. Despite being displaced from the culture plate, the cells retained full viability (data not shown). When the intensity level was increased gradually from 0.4 W/cm^2 while holding the treatment time constant (20 min), more and more cells were displaced from the cell culture plate by the ultrasound. There was no extracellular LDH detected in the culture media, suggesting that both the attached and detached cells survived the ultrasound intensification experiments. Based on these observations, subsequent USHT studies were performed at 0.4 W/cm^2 for 20 min to avoid possible monolayer damage.

Table I. Effect of Different Ultrasound Intensity during Ultrasound-Induced Mild Hyperthermia on Monolayer Integrity and Viability of Bovine Brain Microvessel Endothelial Cells

	Ultrasound intensity (W/cm^2)					
	0	0.4	0.8	1.2	1.6	2.0
Effect on monolayer integrity	–	–	+	++	+++	+++
Effect on viability	–	–	–	–	–	–

Note. The detrimental effects of ultrasound intensity on bovine brain microvessel endothelial cell monolayer integrity and viability were scored as follows: No effect (–), minor effect (+), major effect (++), and severe effect (+++).

Effect of PSC 833, Ultrasound, Hyperthermia, and USHT on Accumulation of R123 in BBMEC

The effects of USHT and putative P-gp modulator PSC 833 in inducing cellular uptake of R123 were compared in BBMEC monolayers. The data indicated that both USHT and 1 μM PSC 833 produced an increase in R123 uptake (74% increase for USHT and 60% increase for PSC 833) when compared with controls (37°C and no P-gp modulating agent; Table II). Because ultrasound can act by either a thermal or nonthermal mechanism (8,9), the effects of ultrasound and hyperthermia on cellular accumulation of R123 in the BBMEC monolayers were examined next. Treatment of BBMEC monolayers with ultrasound alone (i.e., 0.4 W/cm^2 at 37°C for 20 min) resulted in a slight but insignificant increase in cellular accumulation of R123 by the BBMEC monolayers when compared with controls (R123 only at 37°C for 20 min; Table II). In contrast, treatment of BBMEC monolayers with hyperthermia (i.e., 41°C) resulted in a much larger and significant increase (69.7%) in cellular R123 contents. Importantly, when R123 uptake was compared in cells that were either treated with USHT or hyperthermia, similar levels of enhancement were observed (74% vs. 69.7% increase; Table II).

Cellular Accumulation of Hydrophilic and Hydrophobic Marker Molecules According to the Elapsed Time after USHT Treatment

To determine if USHT mediated its effect on cellular accumulation of R123 by affecting membrane permeability, we compared the cellular accumulation of both a hydrophilic marker molecule ([^{14}C]-sucrose) and a non-P-gp hydrophobic substrate ([^{14}C]-antipyrine) with that associated with R123. [^{14}C]-Sucrose was slightly taken up by the BBMECs, and treatment of BBMEC monolayers with USHT (0.4 W/cm^2 for 20 min) failed to affect cellular accumulation of [^{14}C]-sucrose (Fig. 1a). Furthermore, starting the accumulation studies at

Table II. Accumulation of R123 by Bovine Brain Microvessel Endothelial Cells as a Function of PSC 833, Ultrasound-Induced Mild Hyperthermia Ultrasound, and Hyperthermia

Treatment conditions	Cellular R123 contents (mean ± SD)
Control	100.00 ± 16.24
PSC 833 (1 μM)	160.1 ± 9.9
USHT (0.4 W/cm^2 at 41°C)	174.1 ± 28.8
Ultrasound (0.4 W/cm^2 at 37°C)	123.8 ± 20.1
Hyperthermia (41°C)	169.7 ± 1

Note. All values are the mean ± SD. R123 accumulation for control cells (i.e., no P-gp modulating agent at 37°C) was set as 100. All experiments were carried out in triplicate. *$p < 0.05$ compared with untreated controls. For ultrasound studies, ultrasound exposure was continued for a period of 20 min.

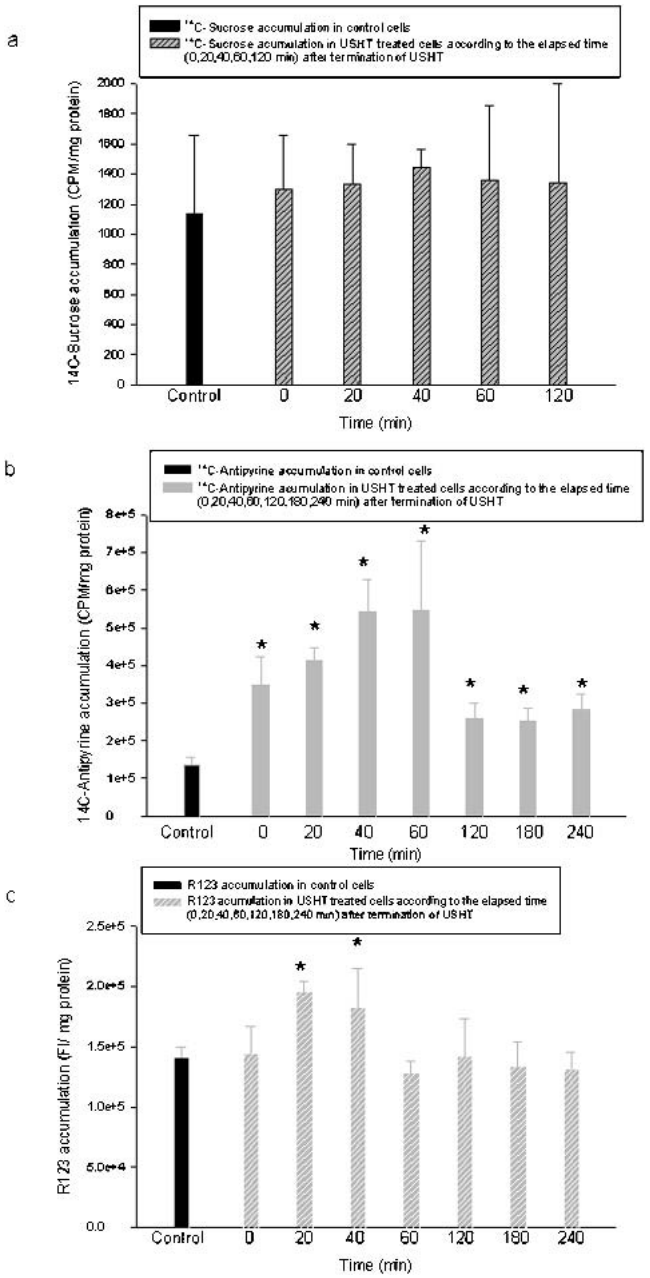

Fig. 1. Cellular accumulation of hydrophilic or hydrophobic marker molecules according to the elapsed time of USHT treatment. BBMECs were either subjected to no USHT (control, 37°C) or USHT treatment (0.4 W/cm^2 at 41°C) for 20 min. At indicated time intervals after USHT treatment, the cells were incubated with 37°C serum-free medium containing either 1 μM [^{14}C]-sucrose (a), 0.5 μM [^{14}C]-antipyrine (b), or 4 μM R123 (c) for 20 min. After the 20-min incubation, the cells were lysed for determination of cellular accumulation of [^{14}C]-sucrose or [^{14}C]-antipyrine or R123, and protein content. All experiments were carried out in triplicate. *$p < 0.05$, compared with control.

various time intervals after the USHT treatment did not seem to change the accumulation levels of [^{14}C]-sucrose in BBMEC monolayers. In contrast, USHT treatment caused a substantial increase in the cellular accumulation of [^{14}C]-antipyrine (Fig. 1b). The accumulation appeared to rise despite the increasing time between USHT treatment and the commencement of accumulation studies. Peak accumulation appeared to occur 60 min after USHT treatment and began to decline thereafter. A similar pattern on cellular accumulation like [^{14}C]-antipyrine was also observed for R123 (Fig. 1c), although the extent of the increase in cellular accumulation appeared to be smaller than that of [^{14}C]-antipyrine. Furthermore, it also took less time after USHT treatment for R123 accumulation in USHT-treated cells to return to that of the untreated cells (60 min for R123 vs. >240 min for antipyrine).

P-gp Activity during and after USHT Treatment

We showed that USHT could increase cellular R123 uptake in BBMECs. We hypothesized that USHT could mediate this enhancing effect by transiently reducing P-gp activity. To this end, we investigated the effects of PSC 833 on BBMEC accumulation of R123 during and after USHT treatments. We reasoned that if USHT negatively affected P-gp activity, then the effects of PSC 833 in increasing R123 uptake in USHT-treated cells would be significantly reduced. Figure 2 shows that incubation with PSC 833 (1.0 μM) during USHT treatment caused a 3-fold increase in R123 accumulation when compared with controls. This is significantly more than the 1.7- to 1.8-fold increase in R123 uptake after the cells were treated with either USHT or PSC 833 alone. These results suggest P-gp was not negatively affected during USHT treatment. Similarly, P-gp activity was not significantly affected after USHT treatment (Fig. 3). For instance, incubation with PSC 833 caused a time-dependent and significant increase in R123 accumulation in both the USHT-treated and untreated cells when compared with their respective controls (i.e., no PSC 833). Interestingly, although the maximum R123 accumulation (± PSC 833) was similar in 60 min for both the

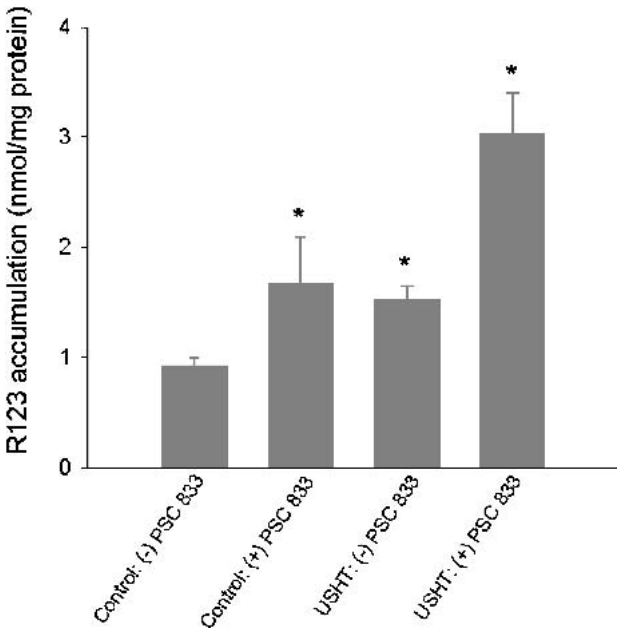

Fig. 2. P-gp activity during USHT treatment. BBMECs were incubated with 37°C serum-free medium containing 4 μM R123 in the presence or absence of 1 μM PSC 833, and were then subjected immediately to no USHT (37°C, control) or USHT treatment (0.4W/cm^2 at 41°C) for 20 min. All experiments were carried out in triplicate. *$p < 0.05$, compared with untreated control (i.e., control less PSC 833).

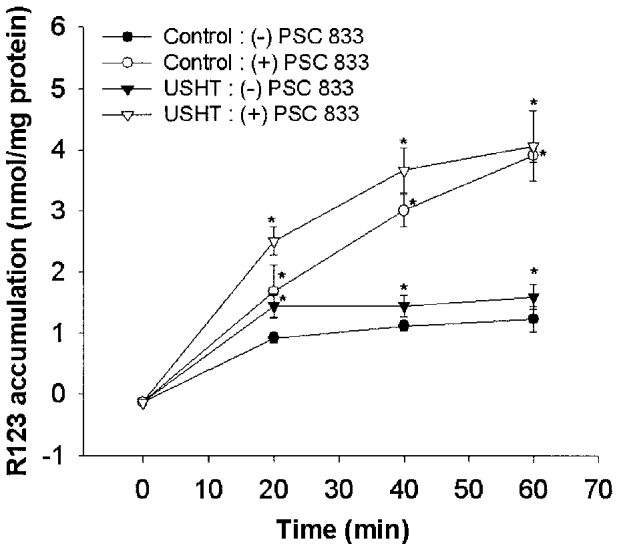

Fig. 3. P-gp activity after USHT treatment. BBMECs were either subjected to no USHT (control, 37°C) or USHT (0.4 W/cm^2 at 41°C) treatment for 20 min. After the treatment, the cells were incubated with 37°C serum-free medium containing R123 (4 μM) in the presence or absence of PSC 833 (1 μM). At indicated time intervals, R123 accumulation was determined. Data represent mean ± SD from three experiments. *$p < 0.05$, compared with untreated control (i.e., control less PSC 833).

USHT-treated and untreated cells, their accumulation in the beginning phase (0–20 min) are somewhat different, with the USHT-treated cells staging a slightly higher R123 accumulation than the untreated cells (Fig. 3).

Glucose Uptake during USHT Treatment

Glucose uptake in the BBB occurs by carrier-mediated facilitated diffusion through specific plasma glycoproteins, the glucose transporters (10). To confirm that USHT does not affect the activity of other transmembrane transporter proteins, we studied the effects of cytochalasin B, a high-affinity inhibitor of glucose transport (11), on 2-[^{3}H]deoxy-D-glucose uptake in BBMEC during ultrasound treatment. Figure 4 shows that cytochalasin B significantly reduced 2-[^{3}H]deoxy-D-glucose uptake in cultured BBMECs. The results confirm that glucose uptake in BBMECs is mediated by glucose transporters. USHT did not generate a significant increase in 2-[^{3}H]deoxy-D-glucose uptake by BBMECs (relative to controls, no USHT; Fig. 4). Most importantly, incubation of BBMECs with cytochalasin B significantly reduced 2-[^{3}H]deoxy-D-glucose during USHT treatment (Fig. 4).

DISCUSSION

Effect site pharmacokinetics of centrally acting drugs are determined by the processes governing equilibration between the blood and brain. Previous studies had established hyperthermia as a potential method for enhancing distribution of drugs through the BBB (5). However, it was concluded from those studies that a threshold temperature of at least 43°C was required (5). It was also recognized that these temperatures might have a direct negative effect on brain function, as well as an indirect negative effect by substantially disrupting the BBB and allowing the unwanted accumulation of other molecules, resulting in toxicity (5). The mechanism, namely whether enhanced permeability was mainly due to effects on membranes or efflux transporters, remained unclear. Our study indicated that mild USHT (20 min, 0.4 W/cm^2) is an efficient, noninvasive, and reversible novel method for enhancing drug penetration through the BBB. Our results showed that mild USHT enhances passive diffusion of hydrophobic drugs and allows them to bypass efflux transporters, as demonstrated in our study for P-gp, the activity of which was not significantly affected by mild USHT. Although not assessed in our study, it seems reasonable to expect that short-term mild USHT (41°C) may have less unwanted effects on brain function than hyperthermia of 43°C as used in previous studies (5).

As a first step, we systematically evaluated the optimal intensity of ultrasound to induce hyperthermia (i.e., 41°C) without disturbing the integrity of the BBMEC monolayers. Ultrasound caused an intensity-dependent detachment of BBMECs, with a 20-min exposure of 0.4 W/cm^2 1 MHz Continuous Wave (CW) ultrasound being the highest intensity that did not affect cell monolayer integrity (Table I). As the treatment intensity value increases, the duty cycle is decreased to maintain temperature, but the peak pulse intensity will increase during the on-period of the excitation cycle. Peak intensities on the order of 1 W/cm^2 are known to produce acoustic streaming and bioeffects, even for short pulse durations (12). Streaming results in a steady fluid flow, which in combination with the ultrasonic vibrations acts to dislodge the cells from tissue culture plates. However, this effect is most likely not relevant for the BBB *in vivo* because acoustic streaming and the associated flow will be suppressed in the solid tissue. Based on these observations, an intensity of 0.4 W/cm^2 was chosen for subsequent USHT studies.

Because the MDR transporter P-gp is a major component of the BBB, we studied if USHT could enhance BBB

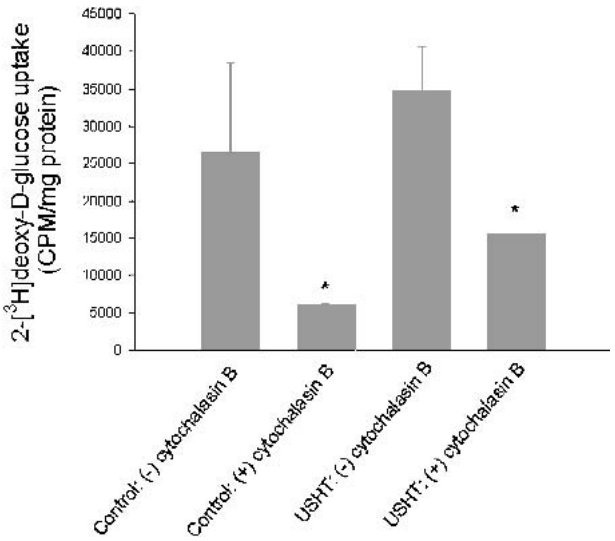

Fig. 4. Glucose uptake during USHT treatment. BBMECs were incubated with 37°C serum-free medium containing 2-deoxyglucose (50 μM) and 2-[^{3}H]deoxy-D-glucose (0.5 μCi) in the presence or absence of 10 μM cytochalasin B, and were then subjected immediately to no USHT (37°C, control) or USHT treatment (0.4W/cm^2 at 41°C) for 20 min. All experiments were carried out in triplicate. *$p < 0.05$, compared with untreated control (i.e., control less cytochalasin B).

endothelial cell uptake of P-gp substrates. USHT (0.4 W/cm^2 at 41°C) increased cellular R123 accumulation to an extent comparable with that induced by putative P-gp modulating agent PSC 833 (Table II). These results are in agreement with our previous findings that USHT treatment of cells increases cellular accumulation of P-gp substrate in P-gp expressing cells (6).

It is clear that USHT can mediate enhanced cellular accumulation of P-gp substrates in BBMEC; however, the mechanism is still unknown. Using doxorubicin as a model drug, several investigators have suggested that the site of action of ultrasound may be the cell membrane (13–15), where ultrasound-induced changes may facilitate the penetration of hydrophobic drugs. We followed up on this hypothesis and assessed cellular accumulation of hydrophilic and hydrophobic marker molecules after USHT treatment. Sucrose and antipyrine were chosen as hydrophilic and hydrophobic permeability markers, respectively, because these molecules are not considered substrates of P-gp or of any known transporter proteins in the BBB. USHT failed to affect sucrose accumulation in cells (Fig. 1), suggesting that the integrity of the cell membrane following USHT treatment was still intact and that cells were still viable (Table I). In contrast, cellular accumulation of both R123 (log partition coefficient 0.53) and antipyrine (log partition coefficient 0.4) was increased immediately after USHT treatment (Fig. 1). These observations are consistent with USHT selectively enhancing the permeability of hydrophobic drugs, but not hydrophilic drugs and, therefore, are in agreement with a recent study in which ultrasound increased the uptake of hydrophobic antibiotics into bacterial cells (16).

USHT enhanced the accumulation of the hydrophobic molecules R123 (molecular weight of 380.8 D) and [^{14}C]-antipyrine (molecular weight of 188.0 D). However, the accumulation of antipyrine reached a level that was 4-fold higher than that of controls 60 min after USHT treatment, whereas R123 concentrations increased only 39% over controls 20 min after USHT treatment (Fig. 1). Cellular accumulation of R123 slowly declined to the concentrations in controls 60 min after the USHT treatment. In comparison, cellular concentrations of antipyrine in the USHT-treated cells were still significantly higher than in the controls 240 min after USHT treatment. The uptake of hydrophilic 2-deoxyglucose (molecular weight of 164.2 D, slightly smaller in size than antipyrine) by BBMECs was unaffected by USHT treatment (Fig. 4), indicating that USHT may produce subtle effects on the cellular membrane such that it affects the permeability of hydrophobic molecules rather than hydrophilic molecules. Because R123 is a P-gp substrate, we cannot rule out the possibility that the differences between accumulation of R123 and antipyrine, which is not a P-gp substrate, may relate to the extrusion activity of P-gp. The effect of USHT was reversible. This finding confirmed the benign nature of mild USHT.

Studies have shown that the activity of several membrane-bound proteins is reduced by hyperthermia (17,18), probably through irreversible protein transitions found to occur in membranes (17). Subtle membrane effects produced by USHT may also lead to impairment of P-gp activity. Other reports indicated that hyperthermia may lead to down- (19) or up-regulation of P-gp (20). To assess whether or not changes in P-gp activity are involved in USHT-induced drug accumulation in BBB endothelial cells, we examined the activity of P-gp-modulating agent PSC 833 both during and after USHT treatment on BBMEC accumulation of R123. Our studies focused on functional activity of P-gp rather than on protein expression because P-gp activity correlates better with the importance of this drug efflux system than protein expression (21). P-gp inhibition using PSC 833 was unaffected by USHT regardless of when the procedure was applied (i.e., during or after USHT treatment; Figs. 2 and 3), suggesting that USHT did not alter P-gp activity. Consistently, mild USHT did not significantly affect the activity of glucose transporters (Fig. 4).

We showed that mild USHT enhances cellular uptake of hydrophobic drugs that may also be P-gp substrates by affecting the cell membrane and not by affecting transporter proteins. Because ultrasound can exert its effects by either a thermal or nonthermal mechanism (8,9), studies were undertaken to examine this issue. As shown in Table II, treatment of BBMEC with 0.4 W/cm^2 ultrasound at 37°C (i.e., nonhyperthermic conditions) caused a slight but insignificant increase in R123 uptake when compared with controls (no ultrasound treatment, 37°C). However, when the BBMEC monolayers were treated with 0.4 W/cm^2 ultrasound at 41°C (i.e., USHT treatment), significant increase in cellular uptake of R123 over control (no ultrasound treatment, 37°C) was observed. Most importantly, this increase of cellular uptake of R123 was comparable with that generated using a nonultrasound heat source. Our data implied that at physiological temperature, ultrasound exerts its effect by mostly a nonthermal mechanism, but at hyperthermic conditions (i.e., 41°C), the mechanism of USHT is mostly a thermal effect that increases membrane permeation rather than modulating transport proteins such as P-gp or glucose transporters. Our data suggests that the mild USHT-induced increased diffusion of drugs into BBB endothelial cells transiently overwhelms efflux transporters such as P-gp, yielding increased intracellular drug concentrations.

Because heating produced by ultrasonic waves can be controlled and localized to a small volume within the tissue (22), we envision that mild USHT can be used to selectively increase permeability of the BBB in a specific region such as brain tumor for enhanced drug delivery. In comparison with measures that disrupt the whole BBB, such as P-gp modulators, mild USHT may help to reduce unwanted effects on normal brain tissue.

In summary, our encouraging findings will provide the mechanistic basis for further *in vivo* studies to demonstrate efficacy of mild USHT and to assess our hypothesis that mild USHT has a favorable safety profile compared with previous approaches using hyperthermia of 43°C (5).

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REFERENCES

1. S. I. Rapoport, K. Ohno, and K. D. Pettigrew. Drug entry into the brain. *Brain Res.* **172**:354–359 (1979).
2. C. Cordon-Cardo, J. P. O'Brien, D. Casals, L. Rittman-Grauer, J. L. Biedler, M. R. Melamed, and J. R. Bertino. Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells

at blood-brain barrier sites. *Proc. Natl. Acad. Sci. USA* **86**:695–698 (1989).
3. S. Seetharaman, L. Maskell, R. J. Scheper, and M. A. Barrand. Changes in multidrug transporter protein expression in endothelial cells cultured from isolated human brain microvessels. *Int. J. Clin. Pharmacol. Ther.* **36**:81–83 (1998).
4. G. M. Hahn and D. P. Strande. Cytotoxic effects of hyperthermia and adriamycin on Chinese hamster cells. *J. Natl. Cancer Inst.* **57**:1063–1067 (1976).
5. R. R. Shivers and J. A. Wijsman. Blood-brain barrier permeability during hyperthermia. *Prog. Brain Res.* **115**:413–424 (1998).
6. Y. Liu, C. W. Cho, X. Yan, T. K. Henthorn, K. O. Lillehei, W. N. Cobb, and K. Y. Ng. Ultrasound-induced hyperthermia increases cellular uptake and cytotoxicity of P-glycoprotein substrates in multi-drug resistant cells. *Pharm. Res.* **18**:1255–1261 (2001).
7. T. K. Henthorn, Y. Liu, M. Mahapatro, and K. Y. Ng. Active transport of fentanyl by the blood-brain barrier. *J. Pharmacol. Exp. Ther.* **289**:1084–1089 (1999).
8. National Council on Radiation Protection and Measurements (NCRP). Report No. 74. Biologic Effects of Ultrasound: Mechanisms and Clinical Implications, 1983.
9. K. Y. Ng and Y. Liu. Therapeutic ultrasound: its application in drug delivery. *Med. Res. Rev.* **22**:204–223 (2002).
10. W. M. Pardridge. Blood-brain barrier transport of nutrients. *Nutr. Rev.* **44(Suppl)**:15–25 (1986).
11. T. T. Rajah, A. L. Olson, and P. Grammas. Differential glucose uptake in retina- and brain-derived endothelial cells. *Microvasc. Res.* **62**:236–242 (2001).
12. F. Duck, A. Baker, and H. Starritt. *Ultrasound in Medicine*, Institute of Physics Publishing, Philadelphia, 1998.
13. P. Loverock, G. ter Haar, M. G. Ormerod, and P. R. Imrie. The effect of ultrasound on the cytotoxicity of adriamycin. *Br. J. Radiol.* **63**:542–546 (1990).
14. G. H. Harrison, E. K. Balcer-Kubiczek, and P. L. Gutierrez. In vitro mechanisms of chemopotentiation by tone-burst ultrasound. *Ultrasound Med. Biol.* **22**:355–362 (1996).
15. G. H. Harrison, E. K. Balcer-Kubiczek, and P. L. Gutierrez. In vitro action of continuous-wave ultrasound combined with adriamycin, X rays or hyperthermia. *Radiat. Res.* **145**:98–101 (1996).
16. N. Rapoport, A. I. Smirnov, A. Timoshin, A. M. Pratt, and W. G. Pitt. Factors affecting the permeability of *Pseudomonas aeruginosa* cell walls toward lipophilic compounds: effects of ultrasound and cell age. *Arch. Biochem. Biophys.* **344**:114–124 (1997).
17. J. R. Lepock, K. H. Cheng, H. Al-Qysi, and J. Kruuv. Thermotropic lipid and protein transitions in chinese hamster lung cell membranes: relationship to hyperthermic cell killing. *Can. J. Biochem. Cell. Biol.* **61**:421–427 (1983).
18. D. A. Bates and W. J. Mackillop. The effect of hyperthermia on the sodium-potassium pump in Chinese hamster ovary cells. *Radiat. Res.* **103**:441–451 (1985).
19. N. Moriyama-Gonda, M. Igawa, H. Shiina, and Y. Wada. Heat-induced membrane damage combined with adriamycin on prostate carcinoma PC-3 cells: correlation of cytotoxicity, permeability and P- glycoprotein or metallothionein expression. *Br. J. Urol.* **82**:552–559 (1998).
20. U. Stein, K. Jurchott, W. Walther, S. Bergmann, P. M. Schlag, and H. D. Royer. Hyperthermia-induced nuclear translocation of transcription factor YB-1 leads to enhanced expression of multidrug resistance-related ABC transporters. *J. Biol. Chem.* **276**: 28562–28569 (2001).
21. M. Fontaine, W. F. Elmquist, and D. W. Miller. Use of rhodamine 123 to examine the functional activity of P-glycoprotein in primary cultured brain microvessel endothelial cell monolayers. *Life Sci.* **59**:1521–1531 (1996).
22. C. J. Diederich and K. Hynynen. Ultrasound technology for hyperthermia. *Ultrasound Med. Biol.* **25**:871–887 (1999).