Effect of Heat Preconditioning on the Uptake and Permeability of R123 in Brain Microvessel Endothelial Cells during Mild Heat Treatment

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Received 14 August 2003; revised 13 November 2003; accepted 26 November 2003

ABSTRACT: The purpose of this study was to assess the effect of mild heat and heat preconditioning on the uptake and permeability of a P-glycoprotein (P-gp) substrate, rhodamine 123 (R123), in a cell culture model of the blood–brain barrier (BBB). An immediate goal was to determine whether prior mild heat treatment could render brain microvessel endothelial cells more resistant to future heat stress and affect BBB drug permeation by future ultrasound-induced mild heat (USMH) treatment. To address this issue, the expression level of two proteins, P-gp and heat shock protein 70 (Hsp70), and their effects on uptake of R123 and permeability of R123 and [^{14}C]-sucrose in combination with mild heat and P-gp modulator PSC833 during and after mild heat treatment in heat-preconditioned and heat-unconditioned bovine brain microvessel endothelial cell (BBMEC) monolayers were studied. Mild heat caused a significant increase in BBB permeability of R123 and [^{14}C]-sucrose when compared with control and PSC833. Exposure of BBMECs to heat preconditioning caused a slight but insignificant decrease in cellular uptake of R123 both during and immediately after mild heat treatment. Heat preconditioning also caused a slight but insignificant decrease in permeability of R123 and [^{14}C]-sucrose in BBMEC monolayers during mild heat treatment. Because exposure of BBMEC monolayers to mild heat did not affect P-gp expression but slightly affected Hsp70 expression, a heat preconditioning that results in a reinforcement of the BBB other than increased expression of P-gp is suggested. However, heat preconditioning is not sufficient to override the permeation-enhancing effects of mild heat because mild heat caused a significant increase in R123 uptake and permeability of R123 and [^{14}C]-sucrose in both heat-preconditioned and heat-unconditioned cells. Because Hsp70 is known to play a major role in cellular repair and protective mechanisms, our results would imply a relative benign nature of mild heat treatment. Because heating produced by ultrasonic waves can be controlled and localized to a small volume within the tissue, the present results also suggest that USMH could play a pivotal role in the treatment of brain tumors and other brain-related diseases. © 2004 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 93:896–907, 2004

Keywords: blood–brain barrier; ultrasound; P-glycoprotein; proteins; multidrug resistance; drug delivery; rhodamine 123; cancer; cell culture

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Journal of Pharmaceutical Sciences, Vol. 93, 896–907 (2004)
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INTRODUCTION

Localized heat treatment in which tissue temperatures are elevated to at least 41°C has been used in the clinical management of various cancers,[1] including brain tumors.[2,3] In addition to being used as a direct therapy of cancers, heat treatment has been indicated as an adjuvant to radiotherapy[4] and chemotherapy.[1] The mechanism behind the thermal enhancement of chemotherapy is still controversial,[1] although one explanation for such a phenomenon is that heat treatment increases cellular permeation and uptake of chemotherapy.[5–7] We previously showed that ultrasound-induced mild heat treatment (USMH: 41°C for 20 min) enhances drug uptake in a cell culture model of the blood–brain barrier (BBB) through a thermal mechanism that affects membrane permeability.[8] Our observations imply that USMH could play a pivotal role in brain tumor therapy by enhancing BBB permeation and tumor uptake of chemotherapeutic drugs.

Because the BBB and brain tissue are remarkably sensitive to various physiological and physical insults,[9] one concern of using heat treatment to increase drug distribution in the central nervous system (CNS) is its potential deleterious effects on the brain tissue. Previous studies have indicated that on exposure to high temperatures, cells synthesize a special set of heat shock proteins (Hsps).[10–12] Hsps are highly conserved proteins that are expressed in response to various forms of stress. Hsps are involved in stabilizing cell structure so that cell function may continue. For example, membrane fluid properties change with increased temperature, and these changes can be offset by stabilization of the membrane by Hsps.[13,14] Recent studies suggest that heat shock and viral overproduction of Hsp70 facilitates cell survival during subsequent heat stress.[15–17] In other words, cells can be made more resistant to heat stress than if they have received no prior stress. The conditioning of cells to heat stress following exposure to heat treatment is termed thermotolerance.[12] The attainment of thermotolerance insures membrane and cell functioning in spite of heat- or stress-induced deleterious changes to the cell.[12]

Because it is anticipated that future chemotherapy of brain tumors using USMH would likely require multiple applications, a heat-preconditioned and reinforced BBB would therefore have significant consequence on future applicability of USMH in treatment of brain tumors and other brain-related diseases. To this end, we performed experiments in which we examined the effects of heat preconditioning on bovine brain microvessel endothelial cell (BBMEC) response to drug uptake and permeation on additional mild heat treatment. Because we are interested in using USMH to increase BBB permeation of P-glycoprotein (P-gp) substrate and that highly stress-inducible heat shock protein 70 (Hsp70; also known as Hsp72 or Hsp70i) is the most conserved and best-studied class of Hsps,[10–12] we specifically focused our attention on the expression level of two proteins, P-gp and Hsp70, and their effects on BBB uptake and permeability of a P-gp substrate rhodamine 123 (R123). Here we report that pre-exposure of BBMEC monolayers to mild heat does not affect P-gp expression but slightly affects Hsp70 expression. Heat preconditioning also causes a slight but insignificant decrease in cellular uptake and permeability of R123 in BBMEC monolayers during mild heat treatment, suggesting that pre-exposure of BBMECs to mild heat may protect the cells from future heat stress. However, heat preconditioning is not sufficient to override the permeation-enhancing effects of mild heat. Because heating produced by ultrasonic waves can be controlled and localized to a small volume within the tissue,[18] the present results suggest that USMH could play a pivotal role in treatment of brain tumors and other brain-related diseases.

EXPERIMENTAL

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]-Sucrose (specific activity, 431 μCi/μmol) was purchased from NEN (Boston, MA). All other reagents, unless specifically stated otherwise, were purchased from Sigma Chemical Company (St. Louis, MO).

BBMEC Isolation and Cell Cultures

BBMECs were isolated from the cerebral gray matter of bovine brain as previously described.[8] Purified BBMECs were seeded at a density of 50,000 cells/cm^2 onto collagen-coated and fibronectin-treated 10×33-mm cell culture plates or

12-mm Transwell (Costar, Cambridge, MA) culture inserts (0.4 μm pore size) in BBMEC culture medium [45% minimum essential medium (MEM), 45% F-12 medium, 10% FCS, 50 U/mL of penicillin, 50 μg/mL of streptomycin, 2.5 μg/mL of amphotericin B]. The cells were grown in a 37°C incubator with 5% CO_2 and 95% humidity. The medium was replaced with fresh BBMEC culture medium on the third day after plating and every day thereafter. BBMECs were allowed to grow to confluence before cellular uptake and permeability studies. Typically, formation of confluent monolayers took ~8–10 days.

Ultrasound Apparatus and Exposure

The system used to expose BBMEC monolayers *in vitro* to USMH has been previously described.[8,19,20] In all studies, ultrasound exposure (0.4 W/cm^2 at 1 MHz) was for a period of 20 min, and treatment temperature was maintained at 41°C. The accuracy of the power output (W/cm^2) 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).

Apical-to-Basolateral Permeability Studies

BBMEC monolayers in Transwell cell culture inserts were preincubated at 37°C in serum-free MEM for 30 min. Prior to the permeability experiments, the media in both the apical (AP) and basolateral (BL) sides were aspirated. Fresh serum-free MEM [1.5 mL at 37°C (control) or 41°C] was then added to the BL side. To start the permeability experiment, serum-free MEM containing 4 μM R123 and 0.4 μM [^{14}C]-sucrose (an impermeable marker) in the presence or absence of 1 μM PSC833 [Norvatis, Basel, Switzerland; 0.5 mL at 37°C (control) or 41°C] was added to the AP side. The cell culture plates holding the inserts were then immediately placed in a 37 or 41°C reciprocal shaking water bath. For permeability studies at 41°C, heat exposure was for a period of 20 min, after which the cell culture plates containing the inserts were immediately transferred to a 37°C reciprocal shaking water bath. In all studies, samples (75 μL) were taken from the BL side at 0, 10, 20, and 30 min and replaced with an equal volume of fresh media. The amount of radiolabeled sucrose in the samples was determined in a Beckman LS6000 IC Liquid scintillation counter (Beckman Instruments, Berkeley, CA). The amount of R123 in the samples was determined quantitatively using fluorescence spectrophotometry (Shimadzu RF 1501; $\lambda_{ex} = 492$ nm, $\lambda_{em} = 535$ nm) as previously described.[8,19] All experiments were performed in triplicate.

Cellular Uptake Studies

One day prior to the uptake experiments, BBMEC monolayers in 10×33-mm culture plates were preincubated at 37°C in serum-free MEM for 30 min. Then the cells were subjected to either no treatment (37°C, heat-unconditioned) or USMH treatment (heat-preconditioned) for 20 min. Afterwards, the media were removed and replaced with 12 mL of 37°C BBMEC culture medium, and the cells were incubated in a 37°C incubator for 24 h. On the next day, the cells received either no treatment (control, 37°C) or USMH treatment for 20 min. At indicated time intervals (0 and 20 min, and 1, 4, and 24 h) after USMH treatment, the culture media of both the control and treatment groups were aspirated, and the cells were incubated with 37°C serum-free MEM containing R123 (4 μM) for 20 min. Cellular uptake studies were terminated by aspiration of media and washing of cells three times with 1.0 mL of ice-cold phosphate buffered saline (PBS). The cells were then solubilized, and aliquots of cell lysate solutions were collected for protein measurements and fluorescence detection of R123 as previously described.[8,19] The amount of R123 was standardized by the protein content of each sample. All experiments were performed in triplicate.

Cellular Uptake and Permeability Studies in Heat-Unconditioned and Heat-Preconditioned Cells

One day prior to the experiments, BBMEC monolayers in 10×33-mm cell culture plates (uptake experiment) or 12-mm Transwell culture inserts (permeability experiment) were preincubated at 37°C in serum-free MEM for 30 min. Subsequently, the cells were either subjected to no treatment (37°C, heat-unconditioned) or mild heat treatment (41°C, heat-preconditioned) induced by ultrasound (uptake experiments) or a 41°C water bath (permeability experiments) for 20 min. Afterwards, the media of both the heat-preconditioned and heat-unconditioned groups were aspirated, and the cells were incubated with 37°C

BBMEC culture media for 24 h in a 37°C incubator. Prior to the uptake experiments, BBMEC monolayers were washed twice with 37°C serum-free MEM and replaced with fresh serum-free MEM containing 4 μM R123. Subsequently, the cells either received no treatment (control, 37°C) or USMH treatment for 20 min. Cellular uptake studies were terminated, and the cells were solubilized and collected for protein measurements and fluorescence detection of R123, as already described. Prior to the permeability experiments, the media in both the AP and BL sides of the BBMEC monolayers were aspirated. Fresh serum-free MEM [1.5 mL at 37°C (control) or 41°C] was then added to the BL side. To start the permeability experiment, serum-free MEM containing 4 μM R123 and 0.4 μM [^{14}C]-sucrose [0.5 mL at 37°C (control) or 41°C] was added to the AP side. The cell culture plates holding the inserts were then immediately placed in a 37 or 41°C reciprocal shaking water bath. For permeability studies at 41°C, heat exposure was for a period of 20 min, after which the cell culture plates containing the inserts were immediately transferred to a 37°C reciprocal shaking water bath. In all permeability studies, samples (75 μL) were taken from the BL side at 0, 10, 20, and 30 min and replaced with an equal volume of fresh media. The samples were then assayed for presence of R123 and [^{14}C]-sucrose as already described. As a control uptake study, another group of heat-preconditioned and heat-unconditioned cells were treated with serum-free MEM containing 4 μM R123 plus 1 μM PSC833 for 20 min at 37°C. Afterwards, an R123 uptake study was performed as already described. All experiments were performed in triplicate.

Determination of Apparent BBMEC Permeability Coefficients (P_{app})

The P_{app} for R123 and [^{14}C]-sucrose across BBMEC monolayers were calculated according to the following equation:

$$P_{app} = \mathrm{Flux}_{monolayer}/(A \cdot C) \tag{1}$$

where C is the concentration of R123 and [^{14}C]-sucrose in the AP side (pmol/cm^3) at time zero, A is the surface area of the Transwell (cm^2), and $\mathrm{Flux}_{monolayer}$ is the flux of R123 and [^{14}C]-sucrose across the monolayer calculated by linear regression of amount of R123 and [^{14}C]-sucrose appearing in the BL side (pmol) versus time (s). It should be pointed out that because heat exposure lasted only 20 min for the 41°C permeability study, the fluxes of R123 and [^{14}C]-sucrose at 41°C did not appear to be linear during the time course of the 30-min study. As such, only flux in the first 20 min of the 41°C permeability study was used to calculate P_{app} of R123 and sucrose at 41°C.

Western Blot Analysis

BBMEC monolayers in 10 × 33-mm plates were preincubated at 37°C in 12 mL of serum-free MEM. Subsequently, the cells either received no treatment (control, 37°C) or USMH treatment for 20 min. Following the treatment, the media were removed and the cells were returned for incubation at 37°C in BBMEC culture media. At indicated time intervals (0 and 20 min, and 1, 4, and 24 h) after the USMH treatment, the cells were harvested and stored at −80°C for subsequent Western blot analysis of Hsp70 and P-gp expression. Crude cell lysates were prepared by sonicating the cells in lysis buffer (New England BioLabs, Beverly, MA). Cell debris was pelleted by brief centrifugation, and the supernatant was collected and quantified for protein using the BCA protein assay kit (Pierce, Rockford, IL). For Western blot analysis, total protein samples (20 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4–15% Ready Gels (Bio-Rad, Hercules, CA), and transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA) using a Trans-Blot SD system (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked using 5% nonfat dry milk in Tris-buffered saline (TBS; 150 mM NaCl, 25 mM Tris, 0.1% Tween 20, pH 7.5) and washed three times with TBS. All antibodies were diluted in 1% nonfat dry milk in TBS. The membranes were then either incubated for 2 h at 37°C with 1:1500 diluted primary anti-Hsp70 (Stressgen, San Diego, CA) or overnight at 4°C with 1:100 diluted C219 monoclonal anti P-gp (Signet Laboratories, Inc., Dedham, MA) primary antibodies. The membranes were washed with TBS before incubation for 1 h at room temperature with 1:1500 diluted anti-mouse HRP secondary antibodies (Santa Cruz, Santa Cruz, CA). Membranes were developed using Renaissance WesternBlot Chemiluminescence Reagent Plus (NEN, Boston, MA), and protein bands were visualized on BioMax Film (Eastman Kodak, Rochester, NY). Membranes were reused and incubated with a 1:10,000 diluted anti β-actin antibody (Sigma, St. Louis, MO) for 1 h at room temperature and

processed as already described. Band densities were quantified by densitometry and were normalized to β-actin protein levels. To examine the effect of additional USMH treatment on P-gp expression in heat-preconditioned cells, BBMECs that received prior USMH 24 h earlier were subjected to a second USMH treatment. At indicated time intervals (20 min and 24 h) following the second treatment, the cells were harvested for Western blot analysis of P-gp as already described. As a positive control for Hsp70 expression, BBMEC monolayers in 10×33-mm culture plates were subjected to moderate heat treatment (3 h in a 43°C water bath). Immediately after the treatment, the cells were lysed for Western blot analysis of Hsp70 expression as already described. All experiments were performed in triplicate.

Statistical Analysis

Data, when applicable, are presented as mean ± standard deviation (SD) from at least three experiments. The effects of mild heat, heat preconditioning, and/or PSC833 in permeability studies were compared using analysis of variance (ANOVA) in combination with Scheffe's test as post hoc analyses. Treatment groups in the uptake study were compared with control for significance by unpaired Student *t* tests. All statistical comparisons and distribution statistics were calculated using the SPSS software package (version 10.0, SPSS Inc., Chicago, IL). A significance level of $p < 0.05$ was considered statistically significant.

RESULTS

Effect of Mild Heat on Permeability of R123 and Sucrose in BBMEC Monolayers

We previously demonstrated that USMH could increase cellular uptake of R123 in BBMEC monolayers.[8] As a next step, it will be important to show that this effect will lead to increase in BBB permeability of P-gp substrate. To this end, we studied the effects of USMH on the BBMEC permeability of R123 using a custom diffusion test cell. Unfortunately, the treatment led to significant monolayer damage wherein large sections of viable cell monolayer became detached from the polycarbonate membrane of the cell culture insert by the action of the ultrasonic field (data not shown). At these intensities and consistent with previous findings,[21] we observed significant acoustic micro-streaming in the donor compartment of the cell culture insert. This result was visually evident by watching the surface of the fluid in the compartment during the ultrasonic treatment. In contrast, cell cultures grown on a cell culture plate did not detach when exposed to 0.4 W/cm^2 ultrasound. The detachment of the cells from the polycarbonate membrane of the cell culture insert is believed to be caused by two actions: (a) membrane vibration by the ultrasonic waves and (b) flow induced by acoustic streaming. Unlike the fixed surface of a cell culture plate that we used in our previous study,[8] the membrane in a cell culture insert is free to move with the flow velocity induced by the passage of the ultrasound wave. The ultrasonic waves vibrate the membrane and weaken the attachment of the extracellular matrix to the polycarbonate membrane. After this occurs, the streaming flow acts to further detach the monolayer from the polycarbonate membrane. Based on our previous findings indicating that the mechanism for membrane permeation effect of USMH is primarily thermal,[8] we felt it was logical to conduct BBB permeability study using mild heat produced by non-ultrasound source as a surrogate to USMH.

The time courses of cumulative appearance of R123 and [^{14}C]-sucrose in the BL side under control (37°C) and experimental (37°C + PSC833 or mild heat produced by non-ultrasound source ±PSC833) conditions are shown in Figure 1A,B, respectively. The P_{app} values for R123 and [^{14}C]-sucrose across BBMEC monolayers at either 37 or 41°C in the absence or presence of PSC833 were then calculated from their fluxes as described in the Experimental section and are summarized in Table 1. Mild heat caused a significant increase in BBB permeability of R123 when compared with control (37°C) and PSC833 (Table 1). Consistent with our previous findings, which indicated that the combined use of USMH and P-gp modulating agent produces an additive effect on cellular uptake of P-gp substrate in multidrug resistant (MDR) cells,[20] treatment of BBMEC monolayers with mild heat and PSC833 produced a higher (but insignificant) increase in R123 permeability than treatment with either USMH or PSC833 alone. Mild heat also produced a significant increase in BBB permeability of the impermeable [^{14}C]-sucrose, suggesting that the BBB permeability of R123 at 41°C may contain contributions from both paracellular and transcellular flux. Because the P_{app} for R123 is only slightly higher than that of [^{14}C]-sucrose at 41°C (Table 1) and because

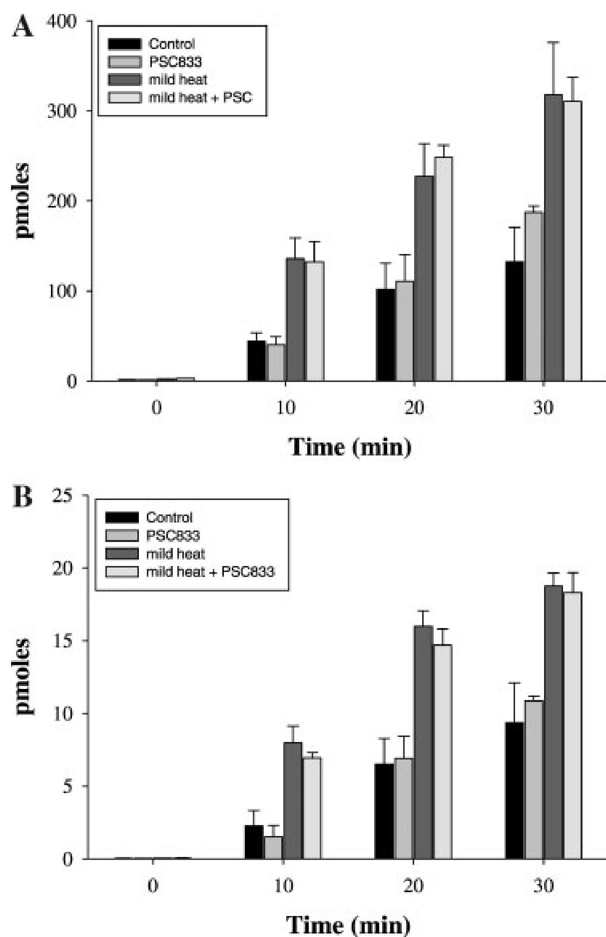

Figure 1. Effect of PSC833 and/or non-ultrasound mild heat treatment on cumulative appearance of R123 and [^{14}C]-sucrose (pmol) in the BL side of BBMEC monolayer versus time. BBMEC monolayers in Transwell cell culture inserts were incubated on the AP side with 37°C serum-free MEM containing 4 μM R123 (graph A) or 0.4 μM [^{14}C]-sucrose (graph B) in the presence or absence of 1 μM PSC833 and then subjected immediately to no mild heat treatment (control, 37°C) or non-ultrasound mild heat (41°C) treatment for 20 min. At indicated time intervals, samples (75 μL) were taken from the BL side and assayed for the presence of R123 and [^{14}C]-sucrose. Data are mean ± SD ($n = 3$).

BBMEC uptake of [^{14}C]-sucrose was not significantly enhanced by mild heat treatment,[8] the results indicate that the observed effects of mild heat on enhanced BBB permeability of R123 are mainly due to changes in paracellular flux. It should be pointed out that because the present cell culture model of the BBB is known to exhibit much higher permeability than its *in vivo* counterpart,[22] the model may be biased towards effects on paracellular flux. Thus, it is possible that distribution of transcellular and paracellular flux for R123 may differ *in vivo*.

Cellular Uptake of R123 in Heat-Preconditioned and Heat-Unconditioned BBMEC According to the Elapsed Time after USMH Treatment

Our previous study indicated that USMH could increase cellular uptake of hydrophobic but not hydrophilic drugs by a thermal mechanism that promotes membrane permeation.[8] Cells are known to synthesize a subset of Hsps in response to thermal and other stresses.[10–12] Although mechanistically not well understood, Hsps have strong cytoprotective effects and serve as molecular chaperones for other cellular proteins.[10–12] Expression of some Hsps has been shown to stabilize cell structure and alter membrane fluid properties.[13,14] It is thought that similar thermotolerance may develop in BBMECs after USHM treatment and render the cells more resistant to future USMH treatment. To this end, we conducted studies in which we compared the uptake of R123 in heat-preconditioned and heat-unconditioned BBMECs according to the elapsed time after USMH treatment. Consistent with our previous findings,[8] USMH caused a slight but reversible increase in cellular uptake of R123 in heat-unconditioned cells compared with control (37°C) 20 min after USMH treatment (Table 2). In contrast, during the first 20 min after USMH treatment, no increase in cellular uptake of R123 compared with control (37°C) was observed in heat-preconditioned cells (Table 2). These results imply that prior USMH treatment rendered BBMEC more resistant to the permeation-enhancing effect of USMH.

Effects of USMH Treatment on R123 Uptake and Permeability in Heat-Preconditioned and Heat-Unconditioned BBMEC

To determine whether heat preconditioning affected the effect of USMH in enhancing cellular uptake and permeability of R123 in BBMEC monolayers, we analyzed cellular uptake of R123 and permeability of R123 and [^{14}C]-sucrose in heat-preconditioned and heat-unconditioned cells during mild heat treatment. The time courses of cumulative appearance of R123 and [^{14}C]-sucrose in the BL side of heat-unconditioned (control) and heat-preconditioned BBMEC monolayers during mild heat treatment are shown in Figures 2A and

Table 1. Effects of PSC833 on the Apparent BBMEC Permeability Coefficients (P_{app}) of R123 and [^{14}C]-Sucrose at 37°C and Mild Heat (41°C)[a]

A	Substrate	Temperature (°C)	$P_{app} \times 10^6$ (cm/s) without PSC833	$P_{app} \times 10^6$ (cm/s) with PSC833
	[^{14}C]-Sucrose	37	13.90 ± 3.86	18.5 ± 0.86
		41 (mild heat)	31.40 ± 2.12	28.9 ± 2.27
	R123	37	16.22 ± 6.26	27.14 ± 7.11
		41 (mild heat)	41.89 ± 6.68	45.72 ± 2.47

B	[^{14}C]-Sucrose	p	R123	p
	37°C versus mild heat (41°C)		37°C versus mild heat (41°C)	
	−PSC833	<0.0002	−PSC833	<0.001
	+PSC833	<0.007	+PSC833	<0.01
	With versus without PSC833		With versus without PSC833	
	37°C	<0.264	37°C	<0.121
	41°C (mild heat)	<0.714	41°C (mild heat)	<0.807

[a]Data are presented as mean ± SD ($n = 3$). Part B shows the statistical comparison of the results presented in A. An analysis of variance in combination with Scheffé's test as *post-hoc* analysis was used. The analyses of variance showed differences with $p < 0.005$. The results of Scheffé's test are presented in part B.

2B, respectively. The P_{app} values for R123 and [^{14}C]-sucrose across BBMEC monolayers at either 37 or 41°C in heat-unconditioned and heat-preconditioned cells were then calculated from their fluxes as already described and are summarized in Table 3. Pre-exposure of BBMEC monolayers to mild heat treatment caused a small but insignificant decrease in BBB permeability of R123 and [^{14}C]-sucrose compared with heat-unconditioned cells during mild heat treatment. Similarly, pre-exposure of BBMEC monolayers to mild heat treatment also caused a small but insignificant decrease in cellular uptake (Fig. 3) of R123 compared with heat-unconditioned cells during mild heat treatment. This attenuation was evident in experiments that were conducted at either 37°C or under mild heat condition (Table 3 and Fig. 3). Consistent with the observations in the uptake studies (Table 2), the data suggest a heat preconditioning that attenuates the permeation enhancing effect of mild heat. However, heat preconditioning is not sufficient to override the effect of mild heat treatment. Mild heat caused a significant increase in uptake of R123 (Fig. 3) and permeability of R123 and [^{14}C]-sucrose (Table 3) in both heat-preconditioned and heat-unconditioned cells compared with control (37°C). Furthermore, because treatment with PSC833 induced a similar but insignificant increase in R123 permeability in heat-preconditioned and heat-unconditioned cells (data not shown) compared with control (37°C, no PSC833), one

Table 2. Cellular Uptake of R123 in Heat-Unconditioned and Heat-Preconditioned BBMEC Monolayers According to the Elapsed Time of USMH Treatment[a]

Time after USMH Treatment	R123 Uptake (nmol/μg Protein) in Heat-Unconditioned Cells	R123 Uptake (nmol/μg Protein) in Heat-Preconditioned Cells
20 min	0.47 ± 0.090	0.32 ± 0.037
1 h	0.45 ± 0.056	0.40 ± 0.065
4 h	0.37 ± 0.025	0.39 ± 0.052
24 h	0.32 ± 0.062	0.32 ± 0.014

[a]Heat-unconditioned and heat-preconditioned BBMEC monolayers in 10 × 33-mm culture plates were either subjected to no USMH (37°C, control) or USMH treatment for 20 min. At indicated time intervals after the treatment, the cells were incubated with 37°C serum-free MEM containing 4 μM R123 for 20 min. After the 20-min incubation, the cells were lysed for determination of cellular uptake of R123 and protein content. Data are mean ± SD ($n = 3$). Control R123 uptake in heat-unconditioned and heat-preconditioned cells are 0.33 ± 0.035 and 0.34 ± 0.020 nmol/μg protein, respectively.

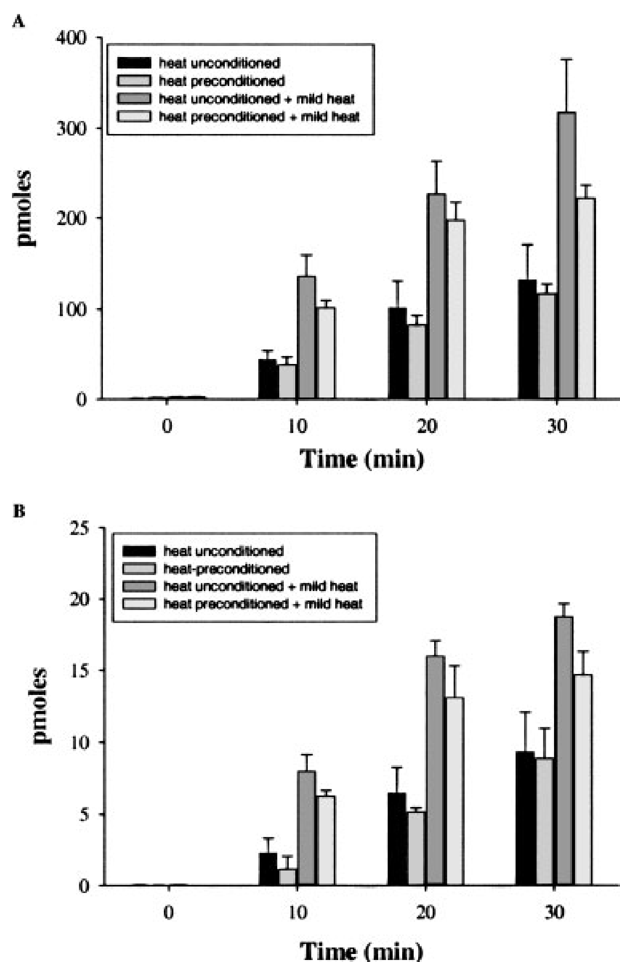

Figure 2. Effect of heat preconditioning on cumulative appearance of R123 and [^{14}C]-sucrose (pmol) in the BL side of BBMEC monolayer versus time at 37 or 41°C. Heat-unconditioned and heat-preconditioned BBMEC monolayers in Transwell cell culture inserts were incubated on the AP side with 37°C serum-free MEM containing 4 μM R123 (graph A) or 0.4 μM [^{14}C]-sucrose (graph B) and then subjected immediately to no mild heat treatment (control, 37°C) or non-ultrasound mild heat (41°C) treatment for 20 min. At indicated time intervals, samples (75 μL) were taken from the BL side and assayed for the presence of R123 and [^{14}C]-sucrose. Data are mean ± SD ($n = 3$).

could argue that heat preconditioning does not alter P-gp expression or activity. This contention is consistent with our previous observations that indicated P-gp activity is not affected by USMH.[8]

Modulation of Hsp70 and P-gp Expression by USMH

Because heat stresses are known to induce Hsps[10–12] and P-gp,[23] we used Western blot analysis to determine whether Hsp70 and P-gp protein levels are altered in BBMEC monolayers exposed to mild heat treatment. In all experiments, USMH induced a very slight but insignificant increase in Hsp70 expression that was time dependent (Fig. 4). This result is in contrast to the large increase seen in Hsp70 expression level after the cells were treated with moderate heating (3 h in a 43°C water bath). Also in contrast, both USMH and moderate heating did not affect P-gp expression in heat-preconditioned and heat-unconditioned BBMECs in all experiments (Fig. 5). The data suggest that both USMH and heat conditioning do not alter P-gp expression.

DISCUSSION

The prognoses for patients with brain tumors, especially malignant glioblastoma multiforme tumors, have not improved appreciably in the past 20 years.[24] Clinical studies have shown that many brain tumors, including primary malignant glioma, are among the most resistant to chemotherapy.[25] This resistance can be partly attributed to the presence of a tight BBB or the expression of P-gp in brain microvessel endothelial cells.[8] Additionally, there is evidence of P-gp expression as a cause of clinical MDR in certain malignant brain tumors.[26,27] Collectively, these observations are consistent with a role of P-gp, both at the levels of BBB and brain tumors, in the resistance of some brain tumors to chemotherapy agents. Thus, strategies that can promote BBB permeation and brain tumor uptake of chemotherapeutic agents that may also be substrates of P-gp may prove to be beneficial from a therapeutic standpoint.

We recently showed that USMH enhances cellular uptake of hydrophobic (that may also be P-gp substrates) but not hydrophilic drugs in BBMEC, primarily by a nonspecific thermal effect that increases membrane permeation.[8] In the present study, we extended these findings and showed that mild heat treatment could also increase the BBB permeability of a P-gp substrate R123 (Table 1). Because cells are known to develop thermotolerance as a protective mechanism against heat stress,[12] we reasoned that a similar response could have developed in USMH-treated brain microvessel endothelial cells. Development of thermotolerance could significantly undermine USMH therapy by rendering the cells more resistant to subsequent heat treatment and thereby

Table 3. Effect of Heat Preconditioning on the Apparent BBMEC Permeability Coefficients of [^{14}C]-Sucrose and R123 at 37°C and Mild Heat (41°C)[a]

A	Substrate	Temperature (°C)	$P_{app} \times 10^6$ (cm/s) without Heat Preconditioning	$P_{app} \times 10^6$ (cm/s) with Heat Preconditioning
	[^{14}C]-Sucrose	37	13.90 ± 3.86	15.20 ± 5.17
		41 (mild heat)	31.4 ± 2.12	25.7 ± 4.33
	R123	37	16.22 ± 6.26	14.38 ± 1.33
		41 (mild heat)	41.89 ± 6.68	36.43 ± 3.59

B	R123	p	[^{14}C]-Sucrose	p
	37°C versus mild heat (41°C)		37°C versus mild heat (41°C)	
	−Heat preconditioning	<0.002	−Heat preconditioning	<0.005
	+Heat preconditioning	<0.005	+Heat preconditioning	<0.074
	With versus without heat preconditioning		With versus without heat preconditioning	
	37°C	<0.975	37°C	<0.985
	41°C (mild heat)	<0.630	41°C (mild heat)	<0.447

[a]Data are presented as mean ± SD ($n = 3$). Part B shows the statistical comparison of the results presented in A. An analysis of variance in combination with Scheffé's test as *post-hoc* analysis was used. The analyses of variance showed differences with $p < 0.005$. The results of Scheffé's test are presented in part B of the table.

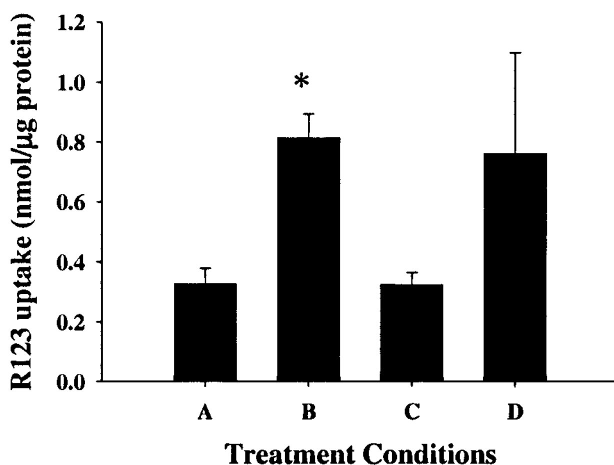

Figure 3. Effect of USMH on the cellular uptake of R123 in heat-preconditioned and heat-unconditioned BBMEC. Heat-preconditioned and heat-unconditioned BBMEC monolayers in 10 × 33-mm culture plates were incubated with 37°C serum-free MEM containing 4 µM R123 and subjected to either no USMH (37°C) or USMH treatment for 20 min. After the treatment, the cells were lysed for determination of cellular uptake of R123 and protein content: A, heat-unconditioned without USMH; B, heat-unconditioned with USMH; C, heat-preconditioned without USMH; and D, heat-preconditioned with USMH. Data are mean ± SD ($n = 3$). Key: (*) significantly different ($p < 0.05$) compared with treatment condition A.

affecting future drug transport. To this end, we performed experiments in which we examined the effects of heat preconditioning on Hsp70 expression in BBMECs and the subsequent response of BBMEC to drug uptake and permeability on additional mild heat treatment. Treatment of BBMEC monolayers with USMH caused a slight increase in Hsp70 expression that was time dependent (Fig. 4). This result is in contrast to the large

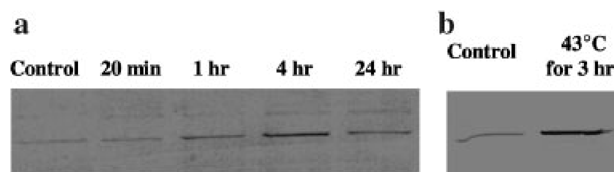

Figure 4. Representative Western blot analysis of Hsp70 in control and BBMECs treated with (a) USMH or (b) 43°C for 3 h. BBMEC monolayers in 10 × 33-mm culture plates were subjected either to no USMH treatment (control) or USMH treatment for 20 min. At indicated time intervals after the treatment, the cells were lysed for Western blot analysis of Hsp70. As positive control of Hsp70 expression, BBMEC monolayers were subjected to no (control) or moderate heat treatment (3 h in a 43°C water bath) and lysed immediately for Western blot analysis of Hsp70. The relative Hsp70 expression levels were obtained by normalizing to β-actin levels.

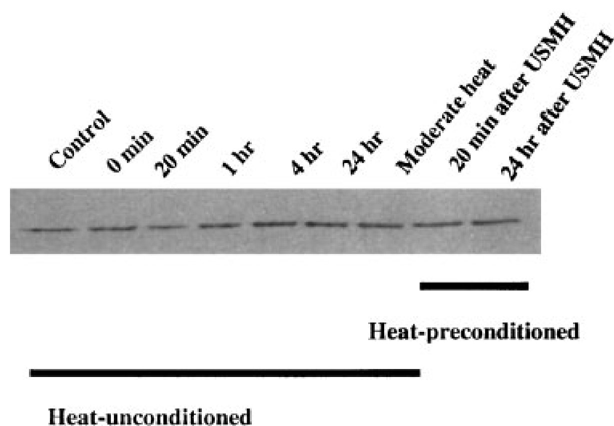

Figure 5. Representative Western blot analysis of P-gp in control and BBMECs treated with either (a) USMH or (b) 43°C for 3 h. BBMEC monolayers in 10×33-mm culture plates were subjected either to no (control) or USMH treatment for 20 min. At indicated time intervals after the treatment, the cells were lysed for Western blot analysis of P-gp. To examine the effect of additional USMH treatment on P-gp expression in heat-preconditioned cells, heat-preconditioned cells were subjected to a second USMH treatment and harvested at indicated time intervals (20 min and 24 h) after the second treatment for Western blot analysis of P-gp. As another control, BBMEC monolayers were subjected to moderate heat treatment (3 h in a 43°C water bath) and lysed immediately for Western blot analysis of P-gp. The relative P-gp expression levels were obtained by normalizing to β-actin levels.

increase in Hsp70 expression level seen in BBMEC after exposure to moderate heating (43°C for 3 h; Fig. 4). Taken together, the data imply that USMH is a mild process that can be safely used to enhance BBB drug permeation. This contention is supported by our recent two-dimensional gel study in which pair-wise comparisons between untreated and USMH-treated BBMEC revealed no apparent differences in protein expression patterns (data not shown).

USMH causes a slight increase in Hsp70 expression level in BBMEC that may protect BBMEC against future heat stress. Indeed, R123 accumulation in BBMEC at 20 min and 1 h after the USMH treatment in heat-preconditioned cells was substantially reduced compared with control cells that had not received prior heat treatment (Table 2). This contention is further supported by the findings that pre-exposing BBMECs to mild heat reduced R123 uptake (Fig. 3) and permeability of R123 and [^{14}C]-sucrose (Table 3) during mild heat treatment. Because R123 is a putative substrate of P-gp and that P-gp expression level in BBMECs was not changed after USMH treatment (Fig. 5), the observation indicates that heat preconditioning results in a reinforcement of the BBB other than increased expression of P-gp. Indeed, heat preconditioning has been shown to prevent disruption of the BBB induced by a hypoxic-ischemic insult.[28] A reinforced BBB may lead to reduction in membrane perturbation and hence drug permeation. Because heat preconditioning occurred without inducing a large and significant increase in endogenous Hsp70 (Fig. 4), the data imply that one or more Hsp70-independent mechanisms, potentially involving other Hsps or non-Hsp-related processes,[11,29] may also be involved in the protection process. Consistent with this suggestion are findings that indicated heat-induced expression of Hsp27 and Hsp32[30] and other Hsps[12] are responsible for preservation of cellular functions of various cell types.

Our data indicate that heat preconditioning affords slight protection to brain microvessel endothelial cells against future heat stress (Fig. 3, Tables 2 and 3). However, this protection was not enough to override the enhancing effect of mild heat on BBB permeation (Fig. 3 and Table 3). Mild heat caused a substantial increase in R123 uptake (Fig. 3) and significant increase in permeability of R123 and [^{14}C]-sucrose (Table 3) in BBMEC monolayers that had received prior mild heat treatment. The results suggest that pre-exposure of BBMEC to mild heat treatment (whether induced by ultrasound or non-ultrasound heat source) did not render the cells enough protection against future mild heat treatment, and support the contention that USMH has potential for enhanced BBB transport of hydrophilic and, in particular, hydrophobic (including P-gp substrates) drugs as either a stand-alone or combined therapy with P-gp modulating agent (Table 1).

Because the BBB and brain tissue are remarkably sensitive to various physiological and physical insults,[9] one concern of using heat to increase drug distribution in the CNS is its potential deleterious effects on the brain tissue. Interestingly, not all hyperthermic temperatures could cause these injuries. The threshold for thermal damage in normal brain tissue is ~40–60 min at 42–42.5°C or 10–30 min at 43°C.[31] These observations imply that a selective heating of the brain, using carefully controlled technique or a heating below the threshold for thermal damage, should suffice to increase BBB transport of hydrophobic drugs with acceptable toxicity. Based on the

present findings and the ability of ultrasound to target a small volume within the tissue,[18] we believe that an ultrasound-guided heating system will one day be developed for enhanced therapy of brain tumors and other brain-related diseases.

ACKNOWLEDGMENTS

This work was supported by Grant £CA79708 from the NIH awarded to K. Ng.

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