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Heat-induced changes in intracellular Na^+, pH and bioenergetic status in superfused RIF-1 tumour cells determined by ^{23}Na and ^{31}P magnetic resonance spectroscopy

A. BABSKY†*, S. K. HEKMATYAR†, T. GORSKI†, D. S. NELSON‡ & N. BANSAL†*

†*Department of Radiology, Indiana University School of Medicine, 950 West Walnut Street, R2 E124, Indianapolis, Indiana 46202, USA*
‡*Department of Radiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA*

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Abstract
The acute effects of hyperthermia on intracellular Na^+ (Na_i^+), bioenergetic status and intracellular pH (pH_i) were investigated in superfused Radiation Induced Fibrosarcoma–1 (RIF-1) tumour cells using shift-reagent-aided ^{23}Na and ^{31}P nuclear magnetic resonance (NMR) spectroscopy. Hyperthermia at 45°C for 30 min produced a 50% increase in Na_i^+, a 0.42 unit decrease in pH_i and a 40–45% decrease in NTP/P_i. During post-hyperthermia superfusion at 37°C, pH_i and NTP/P_i recovered to the baseline value, but Na_i^+ initially decreased and then increased to the hyperthermic level 60 min after heating. Hyperthermia at 42°C caused only a 15–20% increase in Na_i^+. In the presence of 3 μM 5-(N-ethyl-N-isopropyl)amiloride (EIPA), an inhibitor of the Na^+/H^+ exchanger, the increase in Na_i^+ during 45°C hyperthermia was attenuated, suggesting that the heat-induced increase in Na_i^+ was mainly due to an increase in Na^+/H^+ anti-porter activity. EIPA did not prevent hyperthermia-induced acidification. This suggests that pH_i is controlled by other ion exchange mechanisms in addition to the Na^+/H^+ exchanger. EIPA increased the thermo-sensitivity of the RIF-1 tumour cells only slightly as measured by cell viability and clonogenic assays. The hyperthermia-induced irreversible increase in Na_i^+ suggests that changes in transmembrane ion gradients play an important role in cell damage induced by hyperthermia.

Keywords: *Hyperthermia, RIF-1, Na^+, pH, NMR*

Abreviations: *$Dy(PPP)_2^{7-}$, dysprosium (III) bis-tripolyphosphate; EIPA, 5-(N-ethyl-N-isopropyl)-amiloride; MEM, minimum essential medium; MPA, methylphosphonic acid; NMR, nuclear magnetic resonance; NTP, nucleoside triphosphate; Na_e^+, extracellular Na^+ level; Na_i^+, intracellular Na^+ level; Na_{ref}^+, ^{23}Na signal intensities of the reference; pH_i, intracellular pH; SR, shift reagent; $TmDOTP^{5-}$, thulium (III) 1,4,7,10-tetraaza-cyclododecane-1,4,7,10-tetrakis (methylene phosphonate)*

*Correspondence: A. Babsky or N. Bansal, Department of Radiology, Indiana University School of Medicine, 950 West Walnut Street, R2 E124, Indianapolis, Indiana 46202, USA. E-mail: ababsky@iupui.edu or nbansal@iupui.edu

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Introduction

With the recent advances in various heating techniques and tissue thermometry, it has become feasible to use hyperthermia (41–46°C) as an investigational agent in the treatment of human neoplastic disease [1, 2]. Many recent clinical trials have demonstrated strong improvements in the results from both ionizing radiation and chemotherapy when hyperthermia is added to the treatment [3]. Hyperthermia can also improve the effectiveness of other anti-tumour therapies, such as gene and photodynamic therapies, anti-tumour vaccines and angiogenesis inhibitors [4–6].

While sensitizing tumours for more effective treatment from radiation and other therapies, hyperthermia also destroys cancer cells directly. Following exposure to heat, virtually every phase of metabolism becomes disrupted. No clear consensus exists, however, as to the primary metabolic events associated with thermal cell kill. A number of reports have proposed the plasma membrane to be a critical target for hyperthermia-induced cell death [7, 8]. Proper functioning of the plasma membrane with respect to permeability and transport processes across the membrane is vitally important to living cells.

Most viable cells maintain a low [Na_i^+] (10–25 mM) against a high [Na_e^+] (~145 mM). The transmembrane Na^+ gradient is achieved by the action of the Na^+/K^+-ATPase, which pumps three Na^+ out of the cell and two K^+ into the cell at the expense of one ATP molecule. This transmembrane Na^+ gradient is very critical for cell survival and is used to drive several vital physiological processes, such as maintenance of normal cell volume, establishment of membrane potential and ion gradients, cellular uptake and release of metabolites, activity of intracellular and membrane-bound enzymes, regulation of cellular bioenergetics and regulation of protein synthesis [9, 10]. In addition, very rapid changes in Na_i^+ are observed upon growth stimulation of quiescent cells with growth factors [11] and during the cell cycle in a number of cell lines [12].

Hyperthermia can alter the [Na_i^+] by several mechanisms. Exposure to elevated temperature may increase the fluidity of the plasma membrane, which in turn can increase the simple diffusion of Na^+ ions into the cells along the concentration gradient and increase the [Na_i^+]. A change in membrane fluidity can also affect the kinetics of membrane embedded proteins such as the Na^+/K^+-ATPase and other ion transporters. Temperature can also alter the activity of ion transport proteins directly and, hence, change the [Na_i^+]. An increase in the ATPase activity would decrease [Na_i^+], while a decrease in the activity would increase [Na_i^+]. Another possibility is that hyperthermia alters [Na_i^+] to maintain the homeostasis of other ions that may be changing in response to heating. For example, hyperthermia can increase glycolytic rates, which will result in increased acid production. This heat-induced intracellular acidification may increase the activity of the Na^+/H^+ anti-porter and other ion exchange mechanisms to drive the protons out of the cells and, in turn, increase the [Na_i^+].

Previous work is contradictory as to whether [Na_i^+] increases or decreases during hyperthermia [13–18]. The ongoing controversy regarding the changes in [Na_i^+] during hyperthermia may stem, in part, from methodological difficulties used in the previous studies. Most of the previous studies used flame photometry, atomic absorption spectroscopy, isotope enrichment techniques or flow cytometry to measure [Na_i^+] [13–17]. Since Na^+ is present in high concentrations only in the extracellular space, exhaustive washing of the extracellular space has to be employed to eliminate all Na_e^+. The cell washing may introduce large errors due to incomplete removal of ions from the extracellular space and resultant perturbation of intracellular ion contents.

In this study, the effects of hyperthermia on [Na_i^+], cellular energy status and intracellular pH (pH_i) in superfused RIF-1 cells were investigated. ^{23}Na nuclear magnetic resonance (NMR) spectroscopy and the paramagnetic shift reagent (SR) thulium (III) 1,4,7,10-tetraaza-cyclododecane-1,4,7,10-tetrakis (methylene phosphonate) ($TmDOTP^{5-}$) were employed for measurement of Na_i^+ and ^{31}P NMR spectroscopy for measurement of nucleoside triphosphate (NTP), P_i and pH_i. The overwhelming advantage of NMR techniques is that they allow noninvasive monitoring of biochemical and physiological processes without disrupting the cell integrity and ionic/metabolic steady state. The effects of EIPA, which is a very potent and specific inhibitor of the Na^+/H^+ exchanger, were also examined to determine the mechanisms of the changes in Na_i^+ and pH_i during hyperthermia. The effect of EIPA on thermo-sensitivity of the tumour cells was also investigated.

Materials and methods

Cell culture

RIF-1 cells were maintained in Waymouth's culture medium (Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum, 10 mM HEPES, 27 mM $NaHCO_3$ and 1% streptozotocin and penicillin under a 5% CO_2 atmosphere at 37°C. The cells in exponential growth phase were cultivated in culture flasks and then dispersed and detached from the flask surface using trypsin (0.05%). Cells were sedimented by centrifugation for 10 min at 1000 rpm and the resulting pellet was suspended in phosphate buffered saline medium to get a final volume of 1–1.5 ml. The number of cells used in each NMR experiment was determined by Coulter counter (Beckman, Miami, FL) for both Control ($316 \pm 52 \times 10^6$) and EIPA groups ($366 \pm 25 \times 10^6$).

Superfusion preparation for NMR experiments

RIF-1 tumour cells were placed in agarose beads to provide a stable environment during NMR experiments [19]. The cell suspension was added to 1.5 ml of gelling agarose (6%) (Sigma type VII: low gelling temperature 70°C) in modified Minimum Essential Medium (MEM, GibcoBRL, Grand Island, NY) containing (in mM) D-glucose (5.55), NaCl (117.2), KCl (5.3), $NaHCO_3$ (26.2), NaH_2PO_4-H_2O (1), $CaCl_2$ (1.8), $MgSO_4$ (0.81), L-glutamine (2), different amino acids and vitamins. In addition, 10 mM HEPES was added to the medium. The cell and agarose suspension was immediately added to 50 ml of mineral oil (37°C) and stirred continuously with a magnetic stirrer for 5 min while decreasing the water bath temperature to 10°C. The interaction of the oil and agarose caused the formation of round beads with diameters of ~800–1500 μm. The oil was removed by suction and the beads were rewashed with 200 ml of modified MEM.

Beads containing the cells were placed in a 10-mm diameter glass NMR tube and maintained in place by a 100 μm pore size filter. A glass capillary filled with 108 mM methylphosphonic acid (MPA) and 10 mM $TmDOTP^{5-}$ was placed vertically on the same area as the beads with cells. The MPA provided a signal intensity reference for ^{31}P spectra and $TmDOTP^{5-}$ shifted the ^{23}Na signal, providing a reference for Na^+ signal intensity. The cells were continuously superfused with MEM at 2.7 ml min^{-1} (37°C). The superfusion medium was oxygenated with 95% O_2 and 5% CO_2. Protein levels in the experimental groups were 21.2 ± 4.3 mg protein (Control group) and 29.1 ± 3.77 mg (EIPA group).

Nuclear magnetic resonance spectroscopy

All NMR experiments were performed on a Varian 9.4 Tesla wide-bore vertical magnet interfaced to an Inova High-Resolution console (Palo Alto, CA). ^{23}Na and ^{31}P spectra of the superfused cells were acquired using a commercial 10 mm NMR probe equipped with variable temperature capabilities. ^{23}Na NMR spectra were collected at 105.8 MHz using a 0.1 s pre-delay and 10.2 μs excitation pulse (90° flip angle) followed by acquisition of 800 data points over a spectral width of 5 kHz. Two hundred and fifty-six free-induction-decays (FIDs) were averaged over 1 min 8 s. The intra- and extracellular ^{23}Na resonances were discriminated with the paramagnetic SR $TmDOTP^{5-}$ (Macrocyclics, Dallas, TX) added to the perfusate buffer. ^{31}P NMR spectra were collected at 161.8 MHz using a 0.7 s pre-delay and 12 μs excitation pulse (60° flip angle) followed by acquisition of 4096 data points over a spectral width of 13 kHz. Three hundred FIDs were averaged over 5 min 4 s.

The temperature in the NMR tube was measured using a non-magnetic fibre-optic thermometer (FISO Technologies Inc., Quebec, Canada), which was calibrated using both a mercury thermometer and a magnetic thermocouple thermometer (Digi-Sense Model, Cole-Parmer, USA). During the baseline superfusion, sample temperature was maintained at 37°C by setting the temperatures on the variable temperature (VT) unit to 55–60°C. In addition, temperature of the water bath used for warming the superfusion medium was set to 39°C. The in-flow and suction lines were covered with a water jacket (39°C) except the part inside the magnet. For hyperthermia at 45°C, the temperature setting on the VT unit was changed to 80–85°C and the water bath and water jacket temperature was raised to 45°C. For hyperthermia at 42°C, the temperature setting on the VT was changed to 68–72°C and the water bath temperature was set to 43°C. The sample temperature inside the NMR tube changed to 42 or 45°C within ~7–8 min after changing the temperature settings. The temperature in the NMR tube was kept at 42 or 45°C for 20–22 min and then returned to 37°C by decreasing the temperature settings on the VT unit to 55–60°C and the water-bath temperature to 39°C. The sample temperature returned to 37°C 8–10 min after decreasing the temperature settings. Plots of temperature in the NMR tube, measured using the fibre-optic probe during the NMR experiments, are presented in Figure 1.

Experimental protocol

Figure 2 shows a schematic representation of the protocol for the NMR experiments. Four groups of RIF-1 cells (A, B, C and D) were studied using ^{31}P and/or ^{23}Na NMR spectroscopy. Groups A ($n=4$) and B ($n=4$) were used to monitor the effects of 45 and 42°C hyperthermia, respectively, on Na_i^+; Group C ($n=4$) was used to monitor the effects of 45°C hyperthermia on cellular energy status and pH; and Group D ($n=5$) was used to examine the effects of 45°C hyperthermia in the presence of EIPA on Na_i^+, cellular energy status and pH.

Two ^{31}P spectra were obtained at the beginning of superfusion in all the experiments to determine the cellular energy status of cells embedded in the agarose beads. In groups A, B and D, 3.6 mM $TmDOTP^{5-}$ was added to the superfusion medium to discriminate between intra- and extracellular Na^+ and 35–45 ^{23}Na spectra were collected over 40–50 min. After ensuring equilibration of the SR, two ^{31}P spectra were collected to determine the influence of the SR on cellular energy status. In Groups A and B, four additional ^{23}Na spectra were collected before hyperthermia and used as baseline data. In Group D, 3 μM EIPA was added to the superfusion medium and allowed to perfuse the sample for

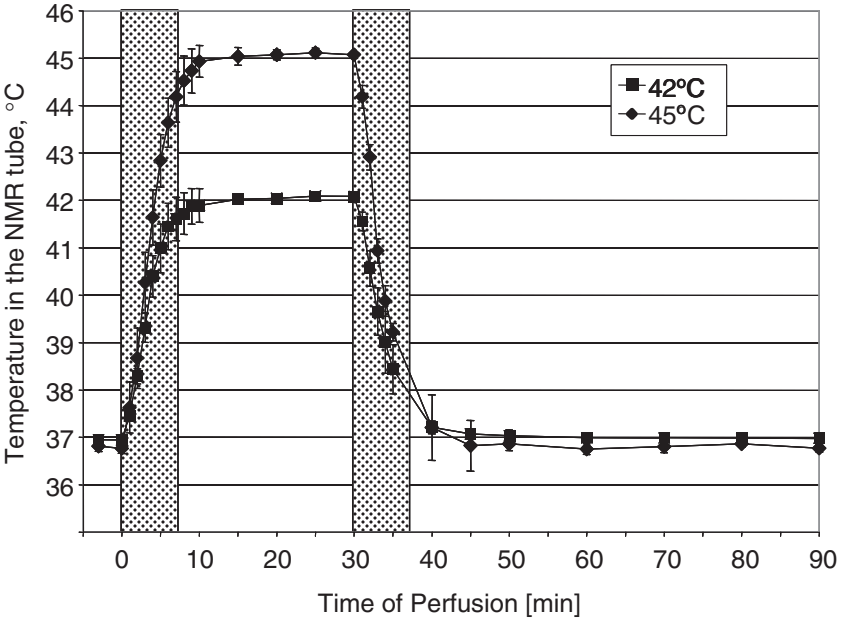

Figure 1. Temperature of superfused RIF-1 tumour cells measured with a fibre-optic probe during the heating experiment. Shading indicates the temperature equilibration periods. Data are presented as mean ± SEM.

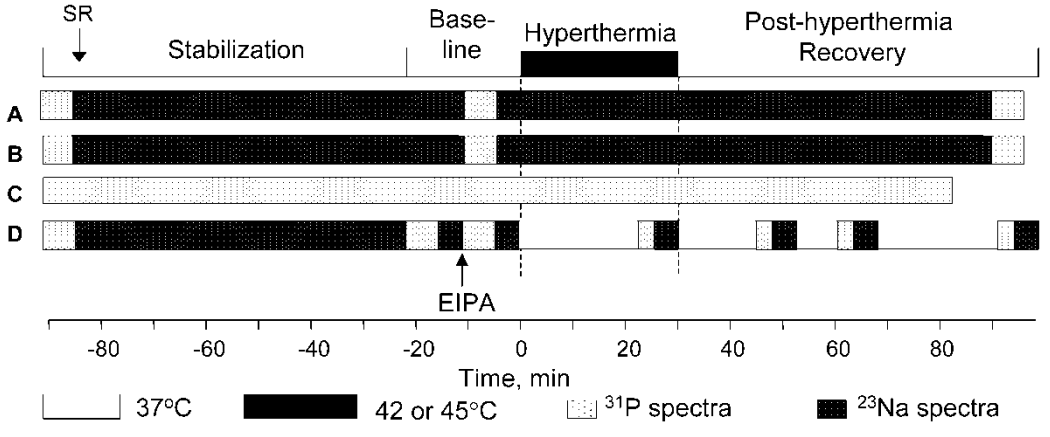

Figure 2. A schematic representation of the experimental protocols. Four groups (A, B, C and D) of RIF-1 cells were studied by ^{23}Na and/or ^{31}P NMR spectroscopy. Groups A and B were used to examine the effects of 45°C and 42°C hyperthermia, respectively, on Na_i^+; Group C was used to monitor the effects of 45°C hyperthermia on cellular energy status and pH; and Group D was used to examine the effects of 45°C hyperthermia in the presence of EIPA on Na_i^+, cellular energy status and pH. See Materials and methods for more details.

the rest of the experiment. Two ^{31}P and four ^{23}Na spectra were then collected before hyperthermia. Hyperthermia was administered by increasing the sample temperature to 45°C (Groups A, C and D) or 42°C (Group B). In Groups A and B, 25–26 ^{23}Na spectra were collected continuously during 30 min of hyperthermia. In Group D, one ^{31}P and

four ^{23}Na spectra were collected during the last 7–10 min of hyperthermia. The sample temperature was then reduced to 37°C, starting post-hyperthermia recovery. During that period, 50–52 ^{23}Na spectra were continuously obtained in Groups A and B during 60 min superfusion at 37°C and two ^{31}P spectra were recorded at the end of experiment. In Group D, one ^{31}P and four ^{23}Na spectra were collected at 15, 30 and 60 min after hyperthermia. In Group C, ^{31}P spectra were continuously collected during baseline, hyperthermia and post-hyperthermia recovery periods.

Cell viability and clonogenic assay

The cell viability and plating efficiency experiments were performed without placing the cells in agarose beads because extraction of cells from the agarose beads could induce large shock and mask the effects of hyperthermia and EIPA. A suspension of RIF-1 cells in exponential growth phase was placed in Hank's buffer (GibcoBRL, Grand Island, NY, USA) and divided into four sets with $6–7 \times 10^6$ cells per ml in each set. Two sets of cells (with and without 3 μM EIPA) were incubated in a water bath at 37°C for 30 min and the other two sets of cells (with and without 3 μM EIPA) were incubated at 45°C for 30 min. After the treatment the cells were washed with Hank's buffer to wash out EIPA. For the cell viability assay, 25 μL cell aliquots from each group were stained with 1% crystal violet in ethanol and the numbers of viable and non-viable cells were counted using a hemocytometer. For the clonogenic assay, a desired number of cells from each of the four groups were cultured in sterilized Petri dishes with 5 ml Waymouth's culture medium in a 95% O_2 and 5% CO_2 incubator at 37°C. After incubation for 7–9 days, the resultant colonies were fixed, stained with 1% crystal violet in ethanol and counted. The plating efficiency was calculated as the ratio of the number of colonies per number of seeded cells. Triplicate cultures were obtained for each experimental group and each experiment was repeated three times.

Data analysis and statistics

All NMR data were transferred to a personal computer and processed with NMR Utility Transform Software (NUTS, Acorn NMR, Fremont, CA) for Windows 95/NT. ^{23}Na FIDs were baseline corrected, zero-filled to 1024 data points, multiplied by a single exponential corresponding to 10 Hz line broadening and Fourier transformed. ^{31}P FIDs were processed in a similar manner with zero-filling to 8192 data points and using a line broadening of 25 Hz. The resonance areas in ^{31}P and ^{23}Na spectra were determined by integration between user-defined points. Cellular energy status was evaluated from the β-NTP to P_i signal intensity ratio (NTP/P_i) because the β-NTP signal does not contain contribution from nucleotide diphosphates. Although ATP is the main constituent of the β-NTP signal, it contains significant contributions from UTP, CTP and GTP, which cannot be resolved in live cells. Intracellular pH was calculated using the chemical shift of P_i signal referenced to α-NTP ($\delta_{P_i-\alpha NTP}$) using the equation

$$pH_i = \frac{1979.5}{T+273} - 5.4409 + 0.018567(T+273) + \log\left(\frac{\delta_{P_i-\alpha ATP} - 10.72 + 0.003579T}{13.18 + 0.001888T - \delta_{P_i-\alpha ATP}}\right),$$

where T is temperature in °C [20].

All data are presented as the mean ± SE. Statistical analyses of the data were performed by Student *t*-test (Statistica/w 5.1 program). A p value ≤ 0.05 was used to denote a statistical significance.

Results

Effect of 42 and 45°C hyperthermia on Na_i^+

Representative examples of ^{23}Na (a) and ^{31}P (b) spectra of the superfused RIF-1 cells after the addition of the Na^+ SR, $TmDOTP^{5-}$ are shown in Figure 3. The ^{23}Na spectrum shows signals at 0, 3.9 and 10.9 ppm from Na_i^+, Na_e^+ and Na^+ in the reference capillary containing 10 mM $TmDOTP^{5-}$, respectively. The intra- and extracellular Na^+ signals are baseline resolved in the spectrum. The ^{31}P spectrum shows three NTP (α, β and γ), a P_i and a phosphomonoester resonance from the tumour cells and an MPA resonance from the reference capillary. The spectrum also shows a relatively weak PCr signal. The PCr signal was detected in most of the experiments, especially when more than 200 million cells were used. Signal intensities and chemical shifts of the ^{31}P resonances did not change significantly after the addition of SR in all the experiments, indicating that cellular energy status and pH_i are unaffected by the SR.

Figure 4 shows the relative changes in average Na_i^+ signal intensity for 45°C (Group A) and 42°C (Group B) hyperthermia experiments during baseline superfusion (37°C), hyperthermia (45 or 42°C for 30 min) and post-hyperthermia recovery (37°C, 60 min). The fluctuations in Na_i^+ signal intensity during baseline superfusion at 37°C were less than 5% in both the groups. The tumour cells in the NMR tube reached the desired temperature 7–8 min after initiating hyperthermia. This temperature equilibration period is indicated by shading in the figure. Heating the cells to 42°C caused a small increase (10–20% compared to baseline) in Na_i^+ for some data points. After decreasing the temperature to 37°C, the Na_i^+ signal intensity returned to the baseline level. When the cells were exposed to 45°C hyperthermia, changes in the Na_i^+ signal intensity were much more pronounced compared to 42°C hyperthermia. The Na_i^+ signal increased to a maximum of 151 ± 9% relative to its baseline value ~20 min after heating was initiated and remained elevated at the same level for the next 10 min during hyperthermia.

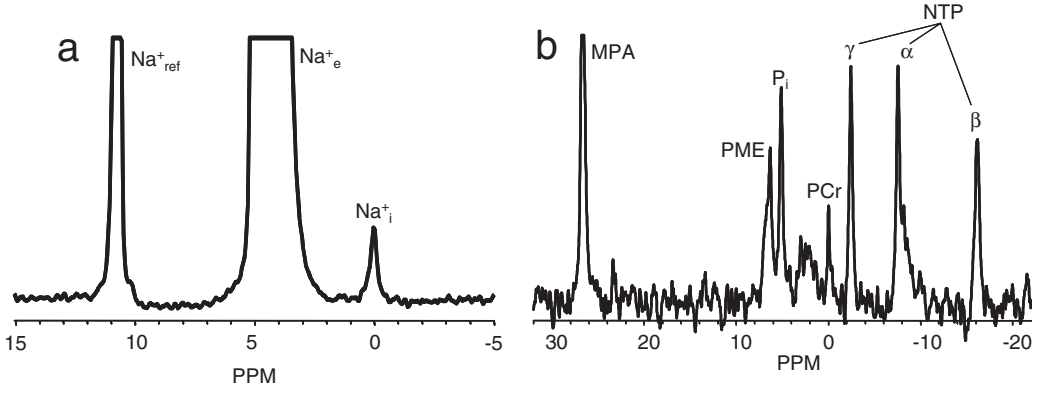

Figure 3. Representative ^{23}Na (a) and ^{31}P (b) spectra of RIF-1 cells at 37°C after equilibration of the SR. The abbreviations used are: Na_{ref}^+, ^{23}Na signal intensities of the reference; PME, phosphomonoester; PCr, phosphocreatine. See text for other abbreviations.

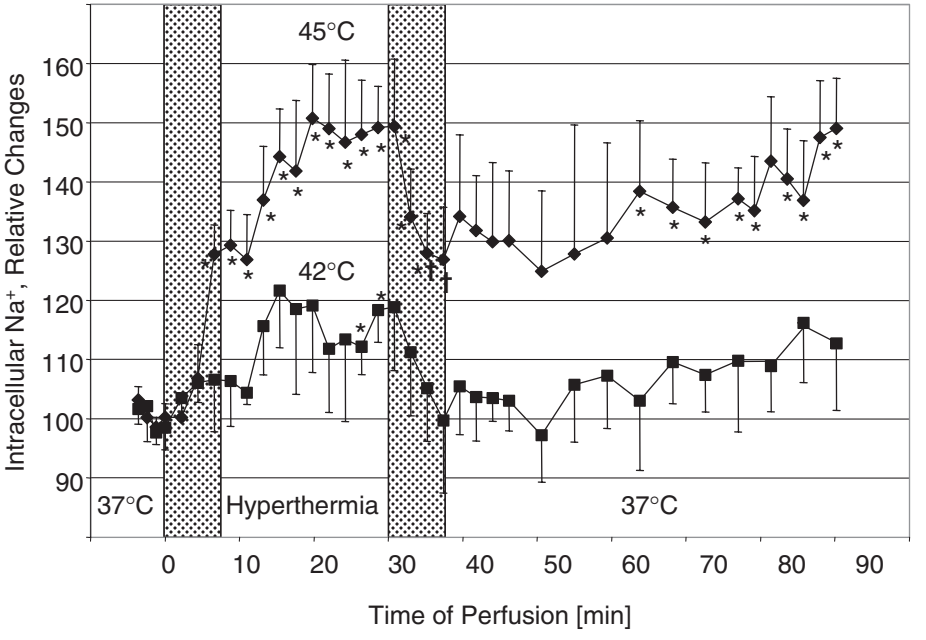

Figure 4. Effects of 42°C (■) and 45°C (◆) hyperthermia on Na_i^+ in superfused RIF-1 tumour cells ($n = 4$). Baseline level of Na_i^+ is normalized to 100. Significance: $^\star p \leq 0.05$ (vs baseline), $\dagger p \leq 0.05$ (vs 45°C hyperthermia level of Na_i^+).

The Na_i^+ signal did not recover to the baseline level when the temperature was returned to 37°C. Initially, the signal decreased during the first 5–10 min after hyperthermia, but it remained ~25% higher compared to the baseline level. This decreased Na_i^+ level was relatively stable for up to ~40 min during 37°C post-hyperthermia superfusion, but then the Na_i^+ signal started to increase and reached the hyperthermia level (150% compared to baseline level) 60 min after hyperthermia. The Na_e^+ signal was constant during the baseline, hyperthermia and post-hyperthermia periods in both 42 and 45°C groups (data not presented).

Overall, these data show that the increase in Na_i^+ in RIF-1 cells is larger and irreversible during 45°C hyperthermia in contrast to 42°C hyperthermia. Therefore, the effects of 45°C hyperthermia were examined further in this study. Although heating at 42°C is more commonly employed in clinical treatment, higher temperatures are often seen near the periphery of lesions that are treated with thermal ablation. Additionally, temperatures in the range of 45°C are sometimes seen in small portions of tumours heated for more traditional hyperthermia treatments.

Effect of 45°C hyperthermia on pH_i and bioenergetic status

The changes in pH_i during baseline, 45°C hyperthermia and post-hyperthermia superfusion are shown in Figure 5. Intracellular pH was 7.14–7.17 during the baseline period. Heating the cells to 45°C led to intracellular acidification. The maximum pH_i decrease ($pH = 6.74 \pm 0.06$) was detected 20 min into hyperthermia. There was no further change in pH_i during the remaining 10 min of hyperthermia. These changes in pH_i parallelled the changes in Na_i^+ during hyperthermia. However, during post-hyperthermia superfusion,

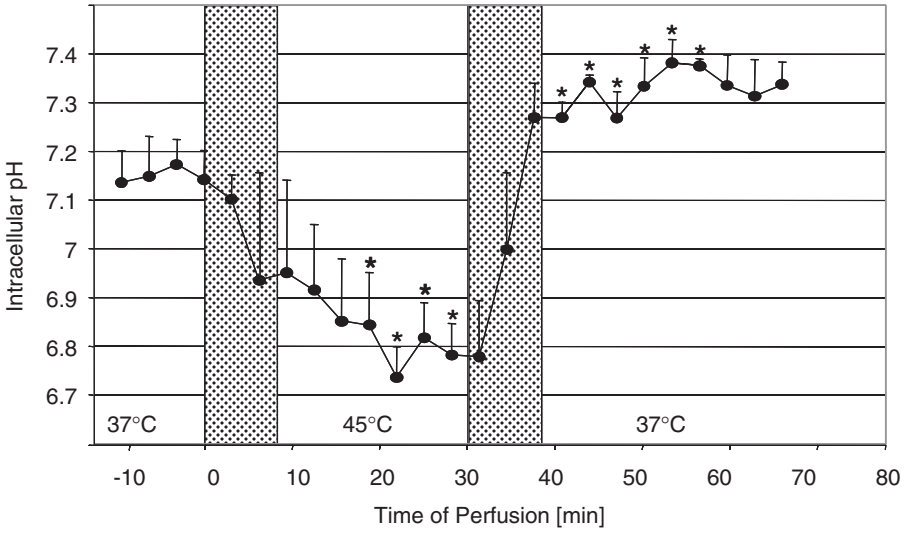

Figure 5. Effects of 45°C hyperthermia on pH_i in superfused RIF-1 tumour cells ($n = 4$). Significance: $^\star p \leq 0.05$ (vs baseline).

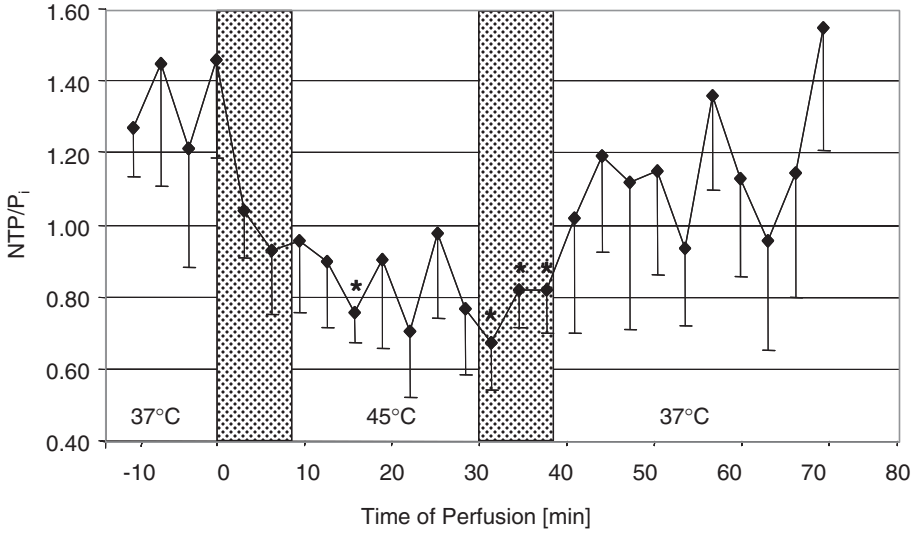

Figure 6. Effects of 45°C hyperthermia on NTP/P_i in superfused RIF-1 tumour cells ($n = 4$). Significance: $^\star p \leq 0.05$ (vs baseline).

pH_i returned to the baseline level and even reached slightly higher values at later time points (7.27–7.33). Thus, unlike the Na_i^+ changes, 45°C hyperthermia produced a reversible decrease in pH_i.

The effect of 45°C hyperthermia on cellular energy status measured from NTP/P_i is shown in Figure 6. The baseline value of NTP/P_i was 1.2–1.4. Heating the cells to 45°C decreased NTP/P_i to 0.7–0.8, largely because of an increase in P_i signal intensity. On returning the temperature to 37°C post-hyperthermia, NTP/P_i increased and was not significantly

different compared to the baseline value. Thus, 45°C hyperthermia for 30 min produced a reversible decrease in the bioenergetic status of the RIF-1 cells.

Effects of Na^+/H^+ exchange inhibition on Na_i^+, pH_i and NTP/P_i

The effects of the Na^+/H^+ exchange inhibitor EIPA on Na_i^+, pH_i and NTP/P_i were investigated to understand the mechanism of the observed changes in Na_i^+ and pH_i during hyperthermia. Figure 7 compares the changes in Na_i^+ (A), pH_i (B) and NTP/P_i (C) for Control and EIPA groups during baseline (before and after the addition of EIPA), at the end (~5 min) of 30 min hyperthermia, and 15, 30 and 60 min after hyperthermia. Addition of 3 μM EIPA did not change the baseline level of Na_i^+. During 45°C hyperthermia in the presence of EIPA, the Na_i^+ signal intensity increased by ~16% compared to the baseline. This increase in Na_i^+ was significantly lower ($p \leq 0.05$) in comparison to the 50% increase without EIPA. After hyperthermia, Na_i^+ initially decreased (15 and 30 min after hyperthermia), but then it increased (60 min after hyperthermia). These changes in Na_i^+ were similar to those observed in the Control group without EIPA. There was no significant difference in Na_i^+ between the EIPA and control group 60 min after hyperthermia. These data show that the increase in Na_i^+ during hyperthermia was mainly caused by activation of the Na^+/H^+ exchanger. The second increase in Na_i^+ after hyperthermia was, however, unaffected by EIPA and may result from changes in plasma membrane structure and/or fluidity, causing a passive influx of Na^+.

The addition of EIPA did not change pH_i compared to the baseline value (Figure 7(B)). At the end of hyperthermia, pH_i in the EIPA group decreased to 6.91 ± 0.1 and was not significantly different in comparison to the Control group at the same time point. However, recovery of pH_i with EIPA was slower in comparison to the Control group. Fifteen minutes after hyperthermia, pH_i in the EIPA group was significantly lower than in the Control group (7.36 ± 0.05 for Control and 7.15 ± 0.05 for EIPA, $p \leq 0.05$). During the post-hyperthermia period, this difference decreased. At 60 min after hyperthermia, there was no significant difference in pH_i between groups (7.34 ± 0.05 for Control and 7.24 ± 0.05 for EIPA). These data show that hyperthermia produced a similar decrease in pH_i with and without EIPA. However, the post-hyperthermia recovery of pH_i was faster and more pronounced without EIPA.

Bioenergetic status of the RIF-1 cells did not change significantly at any time point during or after hyperthermia in the presence of EIPA (Figure 7(C)). Although NTP/P_i decreased during hyperthermia in the Control group ($p \leq 0.05$), no significant difference was observed in NTP/P_i between Control and EIPA groups at any time point.

Effects of hyperthermia and EIPA on cell viability and plating efficiency

Effects of 45°C hyperthermia for 30 min with and without EIPA on cell viability and plating efficiency of RIF-1 cells are shown in Figure 8. Hyperthermia did not change viable to total cell ratio in the Control group without EIPA. Hyperthermia in the presence of EIPA, however, slightly decreased the viable to total cell ratio (0.93 ± 0.02 for 37°C set vs 0.76 ± 0.01 for 45°C set, $p \leq 0.05$). The plating efficiency of the cells was decreased in both the Control (0.29 ± 0.01 for 37°C set vs 0.057 ± 0.008 for 45°C set, $p \leq 0.01$) and EIPA (0.44 ± 0.02 for 37°C set vs 0.037 ± 0.003 for 45°C set, $p \leq 0.01$) groups. These plating efficiency results correspond to a decrease in survival fraction for the EIPA group (0.084 ± 0.006) compared to the Control group (0.20 ± 0.03). Thus, 3.0 μM EIPA at 45°C hyperthermia increased the thermo-sensitivity of the RIF-1 tumour cells slightly.

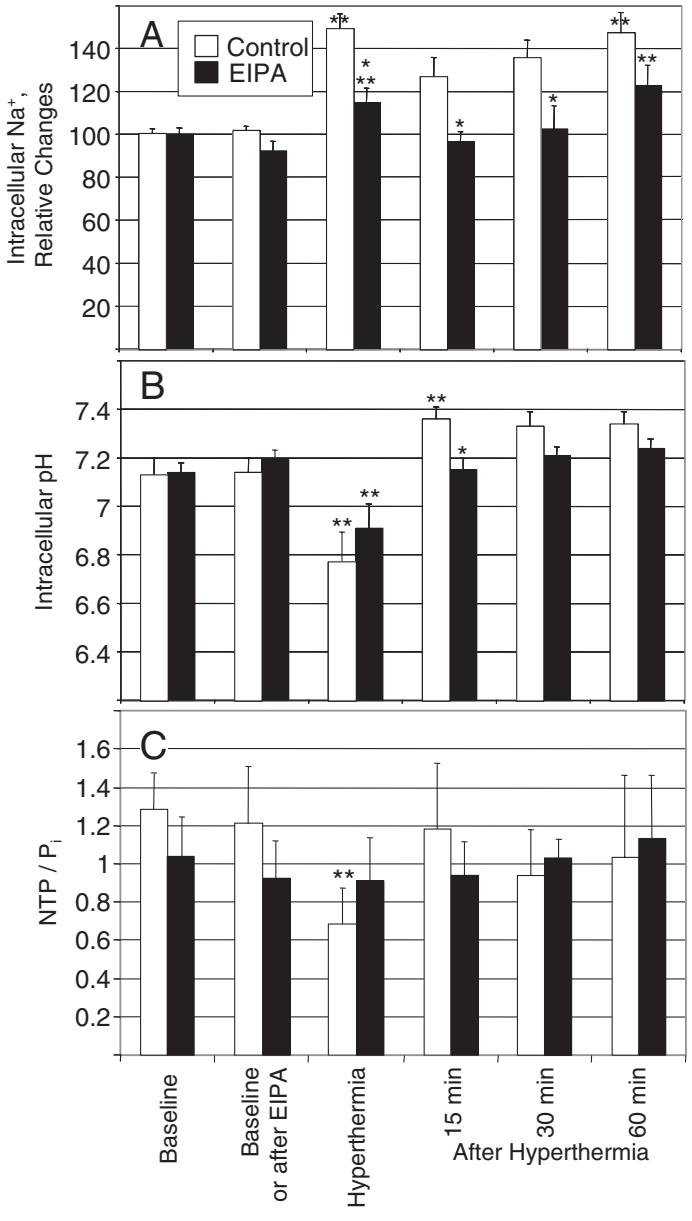

Figure 7. Effects of 45°C (30 min) hyperthermia on Na_i^+ (A), pH_i (B) and NTP/P_i (C) in superfused RIF-1 tumour cells in the absence (Control □, $n = 4$) and presence of 3 μM EIPA (EIPA ■, $n = 5$). EIPA was added to the perfusate before hyperthermia in EIPA group. Significance: ⋆ $p \leq 0.05$ (Control vs EIPA), ⋆⋆ $p \leq 0.05$ (vs baseline).

Discussion

This study mostly examined the effects of 45°C hyperthermia, which belongs to the high range of temperature for clinical therapy. A number of *in vitro* [15–17, 21, 22] and *in vivo* [23, 24] animal studies used 45°C and even higher (46–47°C) temperatures for treatment of cells and solid tumours, including RIF-1, Chinese hamster ovary IS1,

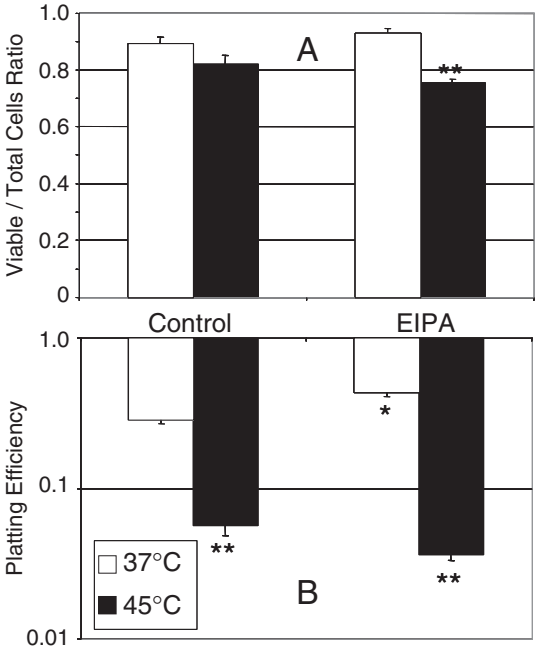

Figure 8. Effects of hyperthermia and EIPA on thermosensitivity of RIF-1 cells measured from viable to total cells ratio (*a*) and plating efficiency (*b*). RIF-1 cells ($15–20 \times 10^6$ per ml) were incubated in water bath for 30 min at 37°C (□) or 45°C (■), without (Control) or with 3 μM EIPA (EIPA). Significance: $*p \leq 0.05$ (EIPA vs Control at 37°C), $**p \leq 0.05$ (37°C vs 45°C).

HeLa and EMT6. Jayasundar et al. [24] showed that only 45°C but not 42°C significantly decreased NTP/P_i and PCr/P_i in solid RIF-1 tumours after 30 min exposure. Liu et al. [25] showed that hyperthermia below ~43°C for 30 and 60 min did not produce any change in surviving fraction and Na^+/H^+ activity in EMT6 cells. However, heating the cells above 44°C decreased these parameters in a dose-dependent manner. Considering these data, 45°C hyperthermia was chosen for 30 min to induce a large acute response.

This study examined the effects of hyperthermia on Na_i^+, pH_i and NTP/P_i in superfused RIF-1 cells. The results demonstrate that among all the NMR parameters measured, the changes in the [Na_i^+] during hyperthermia were most dramatic. Hyperthermia at 42°C produced a 15–20% increase in Na_i^+ in comparison to the baseline, while 45°C hyperthermia produced a 50–55% increase. The 42 and 45°C hyperthermia experiments were designed in such a way that the cells reach the target temperature in approximately the same time. This resulted in a higher heating rate for the 45°C experiments compared to the 42°C experiments. The observed larger increase in Na_i^+ during 45°C hyperthermia may partly be due to the higher heating rate in addition to the higher treatment temperature. No change was observed in Na_e^+ and Na_{ref}^+ signals throughout all the NMR experiments. One would think that a change in Na_i^+ signal should also alter the Na_e^+ signal because the Na^+ has to come from somewhere. The Na_e^+ signal intensity did not change because the volume of the superfused medium (200 ml) was much larger compared to the volume of cells in the beads (~0.5–0.6 ml). A simple calculation shows that a 50% increase in the [Na_i^+] could only produce ~3–5 μM change in the [Na_e^+], which is very small compared to the [Na_e^+] in superfused medium (145 mM). The stable Na_e^+ signal intensity during hyperthermia also suggests that the relative extracellular volume and, hence, the relative

intracellular volume did not change during the experiments. Thus, the increase in Na_i^+ signal intensity observed during heating reflects an increase in $[Na_i^+]$.

The authors are aware of only one other ^{23}Na NMR study where changes in Na_i^+ during hyperthermia were monitored using dysprosium bis-tripolyphosphate ($Dy(PPP)_2^{7-}$) as a SR [18]. In contrast to these results, Skrandies et al. [18] observed a 55% decrease in Na_i^+ in superfused C6 rat glioma cells during 44°C hyperthermia for 15 min. The exact reasons for this discrepancy are not clear, but it may result from differences in the tumour cells investigated or toxic effects of the Na^+ SR used in the previous study. $Dy(PPP)_2^{7-}$ has very strong affinity for divalent cations such as Ca^{2+} and Mg^{2+} and, thus it disrupts the normal ion gradients [26]. In addition, the thermodynamic stability of the complex is very low and it degrades to Dy^{3+} and P_i. Free, unbound Dy^{3+} is acutely toxic even in minute quantities [27]. It has been shown [28] that the SR $TmDOTP^{5-}$ is a vast improvement over the previously available dysprosium-based SRs, such as $Dy(PPP)_2^{7-}$ and $DyTTHA^{3-}$. $DyTTHA^{3-}$ is relatively non-toxic, but it is not very efficient in inducing shifts, thus produces unresolved intra- and extracellular Na^+ resonances. $TmDOTP^{5-}$ has a net negative charge at physiological pH similar to that of $DyTTHA^{3-}$, but has a more favourable geometry for Na^+ binding, which maximizes the induced pseudo-contact shift [28]. Another major advantage of $TmDOTP^{5-}$ over dysprosium(III)-based chelates is that the lower magnetic moment of thulium(III) produces significantly less bulk magnetic susceptibility artifact when introduced into an inhomogeneous sample. The increase in the $[Na_i^+]$ during hyperthermia may result from (1) increased fluidity of the plasma membrane, (2) a decrease in Na^+/K^+-ATPase activity and/or (3) an increase in the activity of Na^+/H^+ exchanger. The Na_i^+ signal did not recover to the baseline level after 45°C hyperthermia. This irreversible increase in Na_i^+ suggests some permanent changes or perhaps damage to the plasma membrane during hyperthermia and/or post-hyperthermia. The plasma membrane damage could lead to passive flux of Na^+ into the cells due to the concentration gradient. Alterations in the plasma membrane may produce changes in PME and PDE resonances in ^{31}P spectra because the metabolites that contribute to these resonances, such as phosphoetanolamine, phosphocholine, glycerophosphoetanolamine and glycerophosphocholine, are involved in membrane lipid synthesis and breakdown [29]. However, in contrast to some previous *in vivo* studies [30], no significant changes were observed in PME and PDE signals from the perfused cells during or after heating.

Changes in the plasma membrane can also alter the activity of Na^+/K^+-ATPase and decrease the efflux of Na^+ from the cells. Na^+/K^+-ATPase activity could also decrease because of a decrease in ATP availability. The results of the ^{31}P NMR experiments show that increasing the tumour cell temperature produced a decrease in NTP/P_i. Temperature could also alter the activity of Na^+/K^+-ATPase directly. Previous studies have demonstrated that cytotoxic temperatures inhibit the ouabain-sensitive Na^+-pump [31, 32]. However, the thermosensitivity of this ion pump seems to depend on cell type because other studies showed no impairment [33] or even an increase in Na^+/K^+-ATPase activity during hyperthermia [8]. The hyperthermia-induced increase in Na_i^+ could also be an active response of the cells to maintain other ion gradients. The increase in Na_i^+ during hyperthermia was significantly attenuated in the presence of 3 μM EIPA compared to the Control group. This observation strongly suggests that the increase in Na_i^+ during hyperthermia was mostly because of increased activity of the Na^+/H^+ exchanger to protect the cells from heat-induced intracellular acidification.

The Na_i^+ signal intensity did not recover to baseline level after 45°C hyperthermia in these experiments. Initially, Na_i^+ signal decreased during the first 5–10 min after

hyperthermia, but it remained ~25% higher compared to the baseline level. This decreased Na_i^+ was relatively stable for up to ~40 min after hyperthermia, but the Na_i^+ signal started to increase, reaching the hyperthermic level (150% compared to baseline level) 60 min after hyperthermia. It is unlikely that the increase in Na_i^+ 40 min after hyperthermia is caused by an increase in Na^+/H^+ exchanger activity because pH_i remained neutral (7.27–7.33 pH units) during this period. The Na^+/H^+ exchanger is relatively inactive at these pH values [34]. In addition, increased Na_i^+ levels were observed in both Control and EIPA groups 60 min after hyperthermia. Another possible reason for the increase in Na_i^+ after hyperthermia is due to a decrease in energy availability for Na^+/K^+-ATPase. However, this is not the case in these experiments because NTP/P_i recovered to the baseline level after the hyperthermia treatment. This increase in Na_i^+ after hyperthermia treatment most likely results from changes in plasma membrane structure [35] and/or activation of membrane lipid oxidation [36], causing membrane leakage and passive influx of Na^+.

The pH measurements from the chemical shift of P_i signal in ^{31}P spectra showed a 0.42 unit decrease in pH_i during 45°C hyperthermia. The acidification of cells during hyperthermia has been reported in several previous studies; however, the exact mechanism of the pH change is not clear. Previous reports have suggested that the decrease in pH_i during hyperthermia results from a decrease in Na^+/H^+ exchanger activity [18]. In this study, this does not appear to be the case. In the presence of EIPA, the increase in Na_i^+ during hyperthermia was significantly attenuated, indicating that Na^+/H^+ activity in the superfused RIF-1 cells is in fact increased during hyperthermia. If Na^+/H^+ exchanger were solely responsible for maintaining pH_i, then one would expect a more pronounced acidification during hyperthermia in the presence of EIPA. However, the changes in pH_i with and without EIPA were similar during hyperthermia. Thus, other ion-exchange mechanisms, such as HCO_3^-/Cl^- exchangers [37] and monocarboxylic acid symporters [38], may also be involved in pH_i regulation during hyperthermia.

The observed decrease in pH_i during hyperthermia may result from a temperature-induced change in intracellular buffer systems [39, 40]. According to Rahn et al. [40], the average change in pH_i in tissues of vertebrates as a result of a change in temperature is -0.016 pH/°C. Thus, in these experiments, a temperature increase of 8°C should decrease the pH_i by ~0.13 units as a result of change in buffering processes. This mechanism accounts for only ~30% of the observed 0.42 unit drop in pH (Figure 5) and suggests that other metabolic processes may also be involved in the heat-induced acidosis.

A shift of tumour cell metabolism from glucose oxidation to anaerobic glycolysis and, hence, lactic acid production, can also contribute to the observed acidification of the RIF-1 cells during hyperthermia. Previous reports have shown that hyperthermia increases the production of acidic metabolites including lactic acid in a number of human and animal tumours [39, 41]. Owen [42] suggested that glycolysis generates five to seven times more acid within the tumour cell compared to complete oxidation of glucose if the two are operating at the same level in terms of ATP generation per unit time. In addition, intensified hydrolysis may also result in acidification during hyperthermia.

The decrease in pH_i during hyperthermia can be useful for delivery of some chemotherapeutic agents to tumour cells. Raghunand et al. [43] showed that gavage and/or ip administration of $NaHCO_3$ improved the therapeutic effect of a weak-base drug, mitoxantrone. The authors proposed that the primary mechanism of the increased therapeutic effect resulted from a decreased transmembrane pH gradient, which increased mitoxantrone uptake by the tumour cells. Because pH_e in most solid tumours is acidic compared to pH_i, the transmembrane pH gradient could also be decreased by decreasing pH_i.

The intracellular acidification during hyperthermia can, thus, improve the delivery of weak-base drugs into tumour cells.

The ^{31}P NMR data show that cellular energy status, measured from NTP/P_i, decreased during hyperthermia but then returned to the baseline level immediately after hyperthermia. The authors are not aware of any other study in which NTP/P_i was monitored during hyperthermia in superfused cells or solid tumours. The exact mechanism for the observed decrease in NTP/P_i during hyperthermia is not clear, but it may result from decreased oxidative metabolism or increased ATP hydrolysis. A number of *in vivo* studies show that NTP/P_i is dramatically decreased immediately after hyperthermia and remains depressed for several hours [29, 44]. In contrast to the *in vivo* data, NTP/P_i in the cell experiments returned to the baseline level within 15–20 min after hyperthermia and remained at this level for the remainder of the experiment. The decrease in cellular energetics in the *in vivo* tumours has been suggested to result from vascular modifications and a decrease in tumour blood flow [45]. Despite the complete recovery of NTP/P_i after hyperthermia, a second wave of increase in Na_i^+ signal intensity was observed. This increase in Na_i^+ after hyperthermia appears to be a direct cellular effect of the treatment, independent of perfusion, and may play an important role in cellular damage caused by heat.

A relatively weak PCr signal was observed from the superfused RIF-1 cells compared to the previously published ^{31}P spectra from RIF-1 tumours implanted in mice [46]. *In vivo* ^{31}P spectra of sc-implanted tumours may show a relatively large PCr signal because of contamination from adjacent muscle tissue, which has high concentration of PCr. Bhujwalla et al. [47] have applied one-dimensional chemical-shift imaging to obtain localized ^{31}P spectra from sc-implanted RIF-1 tumours. The spectra from the slices that were entirely within the tumour did not show a PCr signal, while the spectra from the slices that contained both tumour and normal tissue showed a large PCr signal. Thus, consistent with superfused cell data, RIF-1 tumour cells do not appear to contain a significant amount of PCr.

The results of the clonogenic assay experiments show a small enhancement in the thermosensitivity of RIF-1 cells with EIPA. This may be because inhibiting the Na^+/H^+ anti-porter with EIPA did not sufficiently alter the pH_i profile of the superfused tumour cells during hyperthermia. Furthermore, the recovery of pH_i in both Control and EIPA groups 60 min after the hyperthermia treatment was similar. Intracellular pH has been shown to be an important factor for cell killing by hyperthermia in both *in vitro* and *in vivo* studies [37, 48]. Although a large difference was observed in the Na_i^+ changes during hyperthermia with and without EIPA, thermo-sensitivity of the cells was changed only slightly. These results suggest that the increase in Na_i^+ during hyperthermia (in the absence of EIPA) is not a direct cause of cellular damage by hyperthermia. Rather, it appears to be triggered as an active response of the cells to compensate for other metabolic/ionic changes caused by heating. On the other hand, the second increase in Na_i^+ after hyperthermia, observed both in the absence and presence of EIPA, appears to be caused by passive influx of Na^+ and suggests that heat induced plasma membrane damage.

In conclusion, the data presented here show that hyperthermia produced an irreversible increase in Na_i^+ and a reversible decrease in pH_i and NTP/P_i in the superfused RIF-1 cells, as measured by ^{23}Na and ^{31}P MR spectroscopy. Experiments in the presence of EIPA showed that the increase in Na_i^+ was mediated mostly by activation of the Na^+/H^+ exchanger; however, multiple ion transport processes may be involved in the maintenance of pH_i. The second increase in Na_i^+ after hyperthermia suggests

heat-induced plasma membrane changes and cellular damage. EIPA increased the thermo-sensitivity of RIF-1 cells only slightly, perhaps because the inhibitor was ineffective in acidifying the tumour cells.

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