ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of
DEVELOPMENT RESEARCH

International Journal of Development Research
Vol. 5, Issue, 02, pp. 3311-3316, February, 2015

Full Length Research Article

INTERPLAY BETWEEN HYPEROSMOTIC STRESS AND PROTEIN KINASE A IN REGULATION OF CILIARY BEAT FREQUENCY IN VITRO

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ARTICLE INFO

Article History:
Received 22nd November, 2014
Received in revised form
24th December, 2014
Accepted 05th January, 2015
Published online 27th February, 2015

Key words:
Ciliary beat frequency,
Protein kinase A,
Hyperosmotic stress.

ABSTRACT

Ciliary beat frequency is an important component of mucociliary clearance. Our aim was to test the hypothesis that hyperosmotic stress reduces ciliary beat frequency and that this reduction can be influenced by protein kinase A manipulation in vitro. Using ciliated nasal epithelial cells ciliary beat frequency was measured at 32°C under different osmotic conditions with or without protein kinase A manipulation. In isoosmotic media at 32°C protein kinase A activation stabilised ciliary beat frequency near baseline and its inhibition significantly reduced ciliary beat frequency. Reversible reduction in ciliary beat frequency on exposure of cells to a hyperosmotic solution of 400mM mannitol at 32°C occurred; and when protein kinase A was activated or inhibited in this hyperosmotic solution ciliary beat frequency was further lowered. In conclusion under isosmotic conditions protein kinase A activation had a positive effect on ciliary beat frequency and its inhibition reduced ciliary beat frequency. Under hyperosmotic stress ciliary beat frequency was reduced and protein kinase A activation or inhibition had a negative impact in that the frequencies were lowered further though this was more pronounced when there was activation.

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INTRODUCTION

Mucus expulsion from the lung results from a complex interaction between the tips of respiratory cilia, the periciliary fluid and the visco-elastic properties of the mucus (Ross and Corrsin, 1974, Duchateau *et al*., 1985, Sleigh *et al*., 1988, Inglis *et al*., 1997). The underlying ciliary beat frequency (CBF) of the airway epithelial cells drives mucociliary clearance (MCC) and small changes in CBF can have large effects on MCC (Salathe *et al*., 1997). A number of regulatory factors including protein phosphorylation and physical factors such as temperature, and osmolyte composition in the fluid bathing the cilia modulate CBF (Luk and Dulfano, 1983, Di Benedetto *et al*., 1991, Mwimbi *et al*., 2003). The administration of mannitol aerosols *in vivo* increases MCC (Daviskas *et al*., 1997, Daviskas *et al*., 1999) by uncertain means. Although β_2 agonists such as terbutaline are commonly used bronchial dilators and their effects on CBF are predicted to be stimulatory, when such drugs are admistered to the osmotically challenged airway, as found in mucus-laden airways in asthma for example, the interaction between hyperosmotic stress and protein kinse A (PKA) manipulation remains unclear. Understanding this interaction is important for conditions such as chronic obstructive airway disease (COAD) where mannitol aerosols in combination with a β agonist may often be co-administered. It was found that mannitol induced an increase in MCC that was transiently inhibited by pre-treatment with terbutaline. Conversely, MCC was transiently enhanced when terbutaline was administered after mannitol (Daviskas *et al*., 2002). This terbutaline effect is mediated through a β_2- adrenoceptor which leads to the activation of the cAMP-PKA signal transduction pathway (Sakuma *et al*., 2000). Thus the complex interaction between MCC and osmotic stress was surprising because mannitol and terbutaline might be expected to give an augmented response. This prompted us to test the hypothesis that such an interaction could involve ciliary function. Here, we model this interaction *in vitro* using human nasal airway epithelium. To determine whether hyperosmotic stress and PKA manipulation affected CBF responses, experiments were carried out using ciliated human nasal epithelial cells treated with PKA modulators in

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iso- and hyperosmolar mannitol solution at 32°C (the normal nasal temperature).

MATERIALS AND METHODS

Our well characterised method has been described in detail elsewhere (Smith *et al*., 1996, Mwimbi *et al*., 2000, Mwimbi *et al*., 2002, Mwimbi *et al*., 2003). Briefly, human nasal epithelial cells from patients (age range, 10-40) undergoing routine operations were collected in medium 199 in theatre In the laboratory the nasal epithelial cells were placed into a perfusion chamber located on an inverted microscope (Nikon TMS Model 301052, Japan) connected to a camera linked visual display unit (VDU). The signal from ciliary motion was digitized, and the frequency of the cilia beating displayed on the VDU. A syringe-operated non-peristaltic infusion pump (Medfusion Model 2010; Medex Medical, Lancashire, UK) delivered 2.5 ml of test solution at 0.125 ml min^{-1}.

The first (time=0 min) reading was defined as the baseline reading (100%) in all cases including the controls. The averaged CBF responses to the modulators were expressed as percentage changes from baseline CBF (± S.E.M.). The experiments were carried out at 32°C, using blinded solutions. CBF measurements were carried out every 5 min except during perfusions when no measurements were undertaken. After 20 min of CBF measurement in M199, test solutions were perfused at a rate of 0.125ml per min over 20 min. The test solutions were M199 (control), the normal mediuml (Sigma, Poole, Dorset BH12 4QH UK), 400mM mannitol (Sigma, Poole, Dorset BH12 4QH UK) in M199, PKA activator 1mM dibutyryl cyclic AMP (dbcAMP) (Sigma, Poole, Dorset BH12 4QH UK) ± 400mM mannitol in M199, 100nM the specific cell permeant PKA inhibitor peptide (MyrPKAI) (Calbiochem-Novabiochem, Nottingham UK) ± 400mM mannitol in M199. The PKA activator is a cAMP analog which activates PKA in the same manner as cAMP (Kultgen *et al*., 2002).

Mannitol is a *cell-impermeant,* nonmetabolized, 6-carbon sugar which induces hyperosmotic stress leading to reduction in cell volume due to efflux of water (Malek *et al*., 1998). The mannitol concentration was chosen because our preliminary studies showed that this concentration was approximately at the $P_{50.}$ Test solution perfusion was followed by one hour of CBF measurements. In order to rule out cell damage and show reversal of mannitol induced hyperosmotic stress on CBF a second perfusion with equimolar urea 400mM (Sigma, Poole, Dorset BH12 4QH UK) in M199 at 0.125ml per min over 20 min was carried out at 100 min. Urea is cell permeant and as a result cannot create an osmotic gradient across cell membranes(Sato *et al*., 1990). In addition our preliminary work showed that equimolar urea perfused at 0.5 ml min^{-1} over 5 min had no significant effects on CBF (data not shown). The pH of the solutions used was between 7.2 and 7.4. Using MINITAB program, differences between mean values at specific time points were determined by a nonparametric Wilcoxon test. Significance of difference was accepted when $p<0.05$. Approval for the study was given by the Tayside Committee on Medical Research Ethics and informed consent was obtained from the patients or from parents of minors in all cases.

RESULTS

Fig 1a shows that there was a small stepped reduction in CBF following perfusion with M199 alone (n=10, baseline CBF 9.7 ± 0.2, squares with crosshairs). CBF was 88.4 ± 2.7 and 65.1 ± 3.6 % of the baseline at 40 and 100 min respectively. Following 400mM urea perfusion at 100 min, CBF increased from 65.1 ± 3.6 to 81.9 ± 5.0 and 71.8 ± 5.1 % of the baseline at 120 and 140 min respectively ($p< 0.05$). In contrast, perfusion of a known PKA stimulant 1mM db-cAMP (Di Benedetto *et al*., 1991, Smith *et al*., 1996) (n=6, baseline CBF 10.1 ± 0.2; small closed circles) at 20 min sustained CBF near baseline (103 ± 2.8 and 97.2 ± 2.3 % of the baseline at 40 and 100min respectively). This stable CBF was significantly higher than the CBF observed following M199 perfusion ($p=0.03$ and 0.0001 at 40 and 100 min respectively). Following perfusion with 400mM urea after db-cAMP, there was a small but significant further elevation of CBF to 104 ± 4.0 and 102 ± 2.0 of baseline at 120 and 140 min respectively ($p< 0.05$ as compared to M199). Thus PKA stimulation stabilised CBF after a flow stimulus that we had previously shown to depress CBF acutely (Mwimbi *et al*., 2000).

To test this notion of PKA induced stability, we perfused the specific cell-permeant PKA inhibitor peptide, 100nM MyrPKAI (n=6, baseline CBF 10.4 ± 0.2; small open circles) which resulted in a significant augmentation of the decline in post-flow CBF ($p=0.008$ and 0.001 at 40 and 100 min respectively as compared to M199). The decline was no longer stepped but was quasi-linear. Perfusion with 400mM urea at 100 min increased CBF from 52.7 ± 3.4 to 79.8 ± 6.2 and 67.9 ± 7.1 % of the baseline at 120 and 140 min respectively. The CBF after recovery was slightly less that seen with M199 treatment followed by urea wash out. Thus the state of PKA activation regulates the stability of post flow CBF in isoosmotic media. In contrast to the small decline after perfusing with M199 alone, perfusion at 20 min with 400mM mannitol (n=10 baseline CBF 8.6 ± 0.3; closed diamonds) abruptly dropped the CBF to 45.0 ± 3.5 % of the baseline at 40 min and this level was sustained and after an hour it was about 46.4 ± 3.2 % of the baseline ($p<0.05$, as compared to M199) (Fig.2). When PKA was inhibited in the presence of 400mM mannitol (n=10 baseline CBF 9.6 ± 0.3; large open circles) the CBF dropped almost to the same magnitude as in mannitol alone but with a yet significantly lower initial post perfusion frequency ($p=0.02$ at 40 min).

The CBF was now about 10% lower at 35.3 ± 3.9 and 39.2 ± 3.1 % of baseline at 40 and 100 min respectively. Surprisingly, when PKA was activated using db-cAMP in the presence of 400mM mannitol (n=10, baseline CBF 9.6 ± 0.3; large closed circles) the net result was a further lowering of CBF compared to solutions containing mannitol alone or mannitol with PKA inhibited at both 40 and 100min ($p<0.05$ as compared to mannitol). Thus under hyperosmotic stress, PKA activation was unable to prevent the decline in CBF which went down the most to 25.1 ± 3.9 and 29.6 ± 3.7 % of baseline at 40 and 100 min respectively. Thus a stimulus known to activate PKA (Di Benedetto *et al*., 1991, Smith *et al*., 1996) decreased CBF to an extent greater than mannitol alone and co-inhibition of PKA also enhanced the mannitol induced depression of CBF.

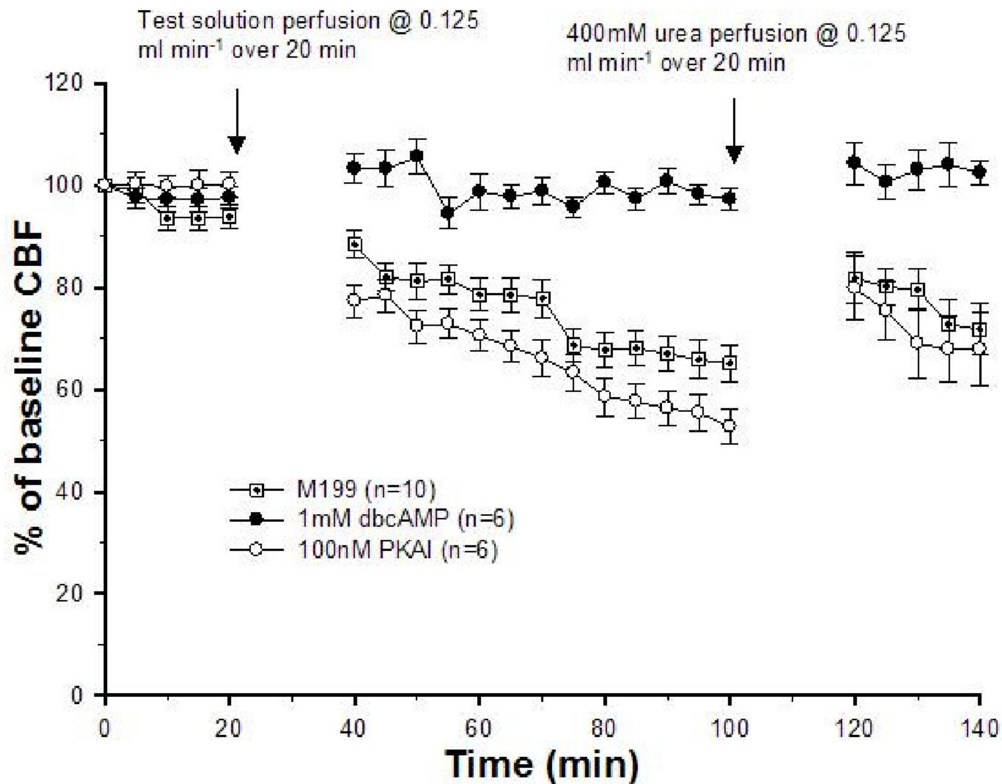

Fig. 1. Effect of PKA manipulation on CBF under isosmotic conditions at 32°C. PKA activation with dbc-AMP in isoosmotic conditions maintained CBF at baseline whereas its inhibition resulted in a significant decline in CBF. There were recoveries in CBF in all experimental series after urea washout

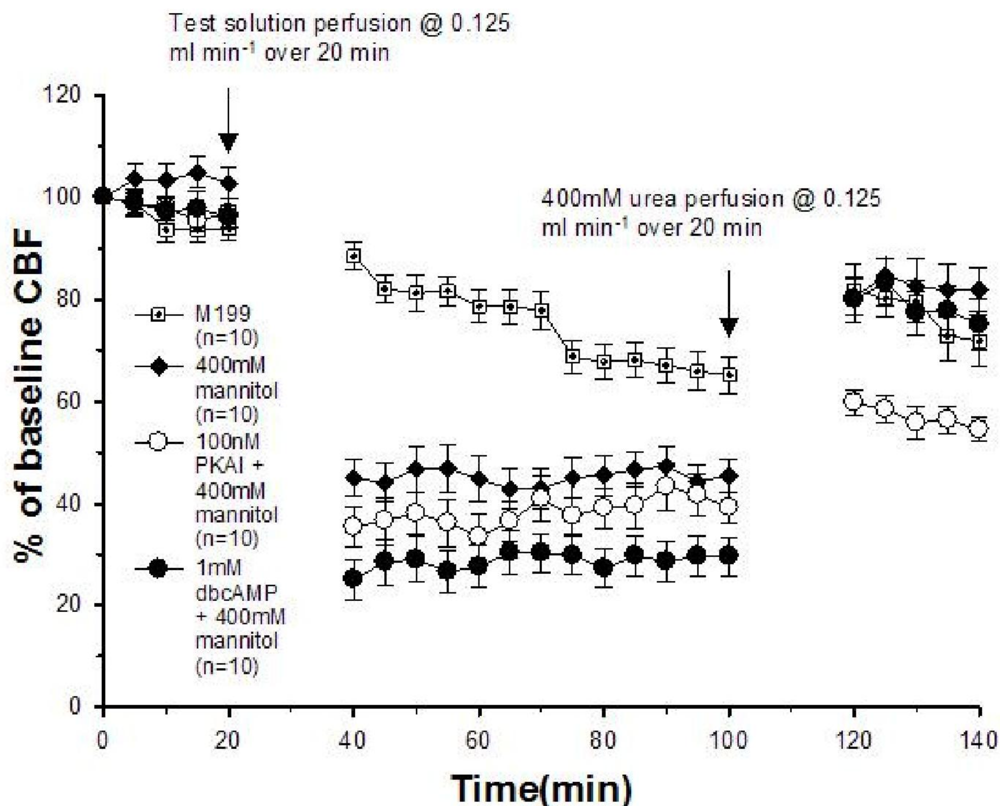

Fig. 2. Effect of hyperosmotic stress and PKA manipulation on CBF at 32°C. Under hyperosmotic stress there was a significant reduction in CBF and PKA activation had an additive effect on mannitol induced reduction in CBF but PKA inhibition had a partial stabilising effect. After urea washout at 120 min there were CBF recoveries in all experimental series. PKA activation in isoosmotic conditions still maintained CBF at or slightly above baseline. Cells that were perfused with a mannitol solution with PKA inhibited had the lowest recoveries

Table1. Raw CBF responses at specific times to different manipulations. Shows CBF responses in Hz (bold) and percentages of the baseline to PKA modulators under iso and hyperosmotic conditions at 32°C

Time (min) / Reagent	M199	Mannitol	dbcAMP	MyrPKAI	MyrPKAI+Mannitol	dbcAMP+Mannitol
0	9.7±0.2 100%	8.6±0.3 100%	10.1±0.2 100%	10.1±0.2 100%	9.6±0.3 100%	9.6±0.3 100%
40	8.4±0.2 88.4 ± 2.7%	3.8±0.3 45.0 ± 3.5%	10.3±0.2 103 ± 2.8%	8.0±0.3 77.3 ± 3.2%	3.2±0.3 35.3 ± 3.9%	2.5±0.4 25.1 ± 3.9%
100	6.1±0.3 65.1 ± 3.6%	3.9±0.3 46.4 ± 3.2%	9.8±0.3 97.2 ± 2.3%	5.4±0.3 52.7 ± 3.4%	3.5±0.2 39.2 ± 3.1%	2.9±0.4 29.6 ± 3.7%
120	7.4±0.4 81.9 ± 5.0%	7.3±0.4 80.3 ± 3.6%	10.5±0.4 104.3 ± 4.0%	8.2±0.6 79.8 ± 6.2%	6.0±0.2 59.8 ± 2.4%	8.0±0.4 79.9 ± 4.5%
140	6.6±0.5 71.8 ± 5.1%	7.4±0.4 81.9 ± 4.3%	10.3±0.3 102.4 ± 2.4%	7.0±0.7 67.9 ± 7.1%	5.5±0.5 54.4 ± 2.3%	7.4±0.5 75.1 ± 5.1%

This suggests that the direction of action of PKA on CBF is osmotically sensitive but the magnitude of the effects of mannitol on CBF predominate over those of PKA manipulation. Cells that were perfused with 400mM mannitol alone recovered their CBF after washout with equimolar urea from 46.4 ± 3.2 to 80.3 ± 3.6 and 81.9 ± 4.3 % of the baseline at 120 and 140 min respectively. In cells which were perfused with 400mM mannitol while PKA was activated CBF rose similarly from 29.6 ± 3.7 to 79.9 ± 4.5 and 75.1 ± 5.1 % of baseline. However, when PKA was inhibited in the presence of mannitol, CBF only rose from 39.2 ± 3.1 to 59.8 ± 2.4 and 54.4 ± 2.3 % of the baseline at 120 and 140 min respectively. Thus after urea washout, cells that had PKA inhibited in 400mM mannitol manifested a significantly lower CBF recovery than cells which had PKA activated in 400mM mannitol ($p<0.05$) suggesting an on-going PKA inhibitory effect.

Overall at the end of the experiment at 140 min and after recovery from hyperosmotic stress, cells which were treated with mannitol alone had the greatest recovery of CBF followed by cells which were treated with mannitol when PKA was activated (81.9 ± 4.3 and 75.1 ± 5.1 % of the baseline respectively). Cells treated with mannitol when PKA was inhibited retained the lowest frequencies at 54.4 ± 2.3 % of the baseline. Table 1 summarises the raw CBF responses at specific times to different manipulations. One observation that was made under the microscope was reduction of cell volume under hyperosmotic stress. Although no measurements were taken this reduction in cell volume seemed to correlate well with reduction in CBF. Upon recovery, after a washout with urea solution, there was evident increase in cell volume which coincided with augmentation of CBF.

DISCUSSION

Our results showed that there was a gradual steady decline in CFB after perfusion with normal medium at 20 min and this decline was briefly reversed after perfusion of a urea solution. This reversal could have been due to replenishment of nutrients for the cells which could have been depleted after being in a perfusion chamber for 100 min. Perfusion of the PKA activator at 20 min however prevented the steady decline in CBF and in fact slightly elevated the CBF above baseline. This was not surprising because other studies before had shown a positive correlation between PKA activation and CBF (Tamaoki *et al*., 1989, Di Benedetto *et al*., 1991, Wyatt *et al*., 1998). Work done so far seems to suggest that PKA activates CBF by phosphorylating a dynein light chain of cilia (Hamasaki *et al*., 1991). Perfusion of a test solution that had a PKA inhibitor at 20 min lowered the observed CBF frequencies below the values in control M199 medium alone. Our result again corroborated other findings which showed that PKA inhibition had a negative effect on CBF(Schmid *et al*., 2006). However when the cells were perfused with a 400mM solution of mannitol (without PKA activation or inhibition) at 20 min there was a very significant drop in CBF which was observed for an hour but after perfusion with 400 mM urea solution at 100 min there was a significant recovery in CBF. This reduction in CBF has also been reported in other studies where hyperosmotic stress with agents such as cell-impermeant mannitol was shown to decreases CBF *in vitro* (Luk and Dulfano, 1983, Shiima-Kinoshita *et al*., 2004).

The drop in CBF observed coincided with the observed reduction in cell volume noted under the microscope. The reduction in cell volume was due to the efflux of water from the cells due to increased extracellular solute concentration. It is not clear how hypertonicity could result in reduction of CBF. One plausible explanation is that with reduction in cell volume the immediate response of the cells was to try and reverse the decline in cell volume through reversed volume increase (RVI) to avoid irreversible damage leading to apoptosis. RVI involves activation of ion channels so that water can follow passively as ions enter cells and this requires energy. As a result of this urgent extra demand under hyperoosmotic stress there was energy redistributed within cells to accommodate the changing environment and this is critical for coping with stressful conditions (Olz *et al*., 1993). It has actually been observed that energy pool management is the cells top priority under stress conditions (Varela *et al*., 2004). What we can speculate is that under hyperosmotic stress energy is shunted away from powering ciliary molecular motors to activation of ion channels.

This early process of RVI is mediated by preexisting ion transport systems, mainly the Na^+ –K^+–$2Cl^-$ co-transporter (NKCC), the Na^+/H^+ exchanger and the Cl^-/HCO_3^-exchanger (McManus *et al*., 1995, Lang *et al*., 1998). For instance the NKCC transporter requires electrochemical sodium gradient to move Na^+, K^+ and $2Cl^-$ into the cell and the movement of these ions is accompanied by an influx of water. And this gradient is

established by the Na-K ATPase which requires energy from ATP. So in a way the activity of NKCC is indirectly dependent upon ATP by way of secondary active transport. Therefore it is possible that under hyperosmotic stress there was diversion of ATP from ciliary motion to maintenance of ion channels in order to reverse the cellular volume decrease. Exactly what triggers onset of immediate adaptive responses in RVI is not known but what has been deduced from other studies is that the decrease in cell volume may be the trigger to the signalling pathway (Petronini *et al*., 1990, Klein *et al*., 1999). When perfusion of a mannitol solution was carried out with PKA activated the surprising result was CBF dropped below the level seen when the test solution was mannitol alone without PKA manipulation.

We had expected an augumentation of CBF since PKA activation in normal control M199 stabilized CBF at baseline. This can again be explained in terms of energy redistribution. Activating PKA under hyperosmotic stress could have lead to phosphorylation of other proteins involved in adaptive responses and this could have diverted more ATP away from sustaining CBF. Perhaps this can be the reason why inhibiting PKA stabilized CBF in a hyperosmotic solution better than in a hyperosmotic solution with PKA activated. The effect of PKA manipulation in a hypertonic solution is the reverse of what is observed in isoosmotic solution. On recovery from hyperosmotic stress after urea washout, there was a variable reversal of the effects of PKA manipulations on CBF. Cells that were in mannitol with PKA activated had higher CBF on recovery than cells that were exposed to mannitol with PKA inhibited. This suggests firstly, ongoing action of intracellular PKA manipulators despite washout of the external solution. In conclusion our data suggests that at the normal nasal temperature, PKA activation in isoosmotic media maintains CBF at or slightly above baseline whereas PKA inhibition with a selective inhibitor of PKA reduces CBF significantly below the M199 control whilst simultaneously eliminating the stepped reduction in CBF observed in medium alone.

In contrast, a hyperosmotic solution of 400mM mannitol reduces CBF significantly. When PKA is inhibited in the presence of mannitol, the abrupt decline in CBF is slightly greater than after perfusion with mannitol alone. In contrast, activation of PKA in the presence of mannitol results in an equally abrupt and yet significantly lower CBF than in mannitol alone or with the presence of PKA inhibition. Thus it appears that the stimulatory effect of PKA activation on CBF is reversed under hyperosmotic stress. Further work will have to establish the cause but the need to divert energy supply to more immediate needs under cellular stress is a promising line of inquiry. This study had some limitations as the in vitro conditions under which work was carried out did not reflect the in vivo situation. We didn’t have the equipment to monitor changes in cell volume under different osmotic conditions. It would have been better if volume estimations were done as that was going to help understand the relationship between cell shrinkage due to hypertonicity and CBF.

Acknowledgements

The authors would like to thank the ENT surgeons, the anaesthetists and patients at Ninewells Hospital and Medical School, Dundee, and patients for taking part in the study.

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