Supra- and sub-baseline phosphocreatine recovery in developing brain after transient hypoxia-ischaemia: relation to baseline energetics, insult severity and outcome

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Following hypoxia-ischaemia (HI), an early biomarker of insult severity is desirable to target neuroprotective therapies to patients most likely to benefit; currently there are no biomarkers within the ‘latent phase’ period before the establishment of secondary energy failure. Brief transient phosphocreatine (PCr) recovery overshoot (measured absolutely or relative to nucleotide triphosphate, NTP) following HI has been observed in cardiac and skeletal muscle; its significance however is unclear. To investigate cerebral PCr recovery levels after HI in relation to (i) baseline metabolism, (ii) insult severity, (iii) energy metabolism at recovery and (iv) subsequent metabolic derangement, cerebral NTP, PCr and inorganic phosphate (relative to the exchangeable high-energy phosphate pool) were measured serially in an in vivo model of perinatal asphyxial encephalopathy using phosphorus-31 magnetic resonance spectroscopy. Measures were compared either in all piglets or between 3 subgroups with no (n = 5, favourable outcome), moderate (n = 8, intermediate outcome) or severe (n = 5, unfavourable outcome) secondary energy failure at 24 h after HI. Maximum NTP, PCr and inorganic phosphate recoveries were observed 2–8 h after HI. Following resuscitation, in subjects with favourable outcome PCr recovered to higher than its baseline level (overshoot); in subjects with unfavourable outcome maximum PCr recovery was lower than baseline and lower than in subjects with favourable and intermediate outcomes. Recovery PCr correlated linearly and negatively with both acute insult severity and baseline PCr/NTP. These results suggest that recovery metabolism 2–8 h after HI may provide an early biomarker of injury severity. PCr recovery overshoot in the developing brain may indicate a protective response to HI leading to cell recovery, survival and protection against subsequent stress. In addition, baseline cerebral metabolism (PCr/NTP) may identify vulnerable infants prior to invasive surgery.

Keywords: hypoxia-ischaemia; MRS; brain protection; energy substrates

Abbreviations: ³¹P = phosphorus-31; MRS = magnetic resonance spectroscopy; HI = hypoxia-ischaemia; PCr = phosphocreatine; NTP = nucleotide triphosphate; SE = standard error; Pi = inorganic phosphate; EPP = exchangeable high-energy phosphate pool


Introduction

Following perinatal hypoxia-ischaemia (HI), a period with apparently normal cerebral energy generation (the ‘latent phase’) is observed before secondary energy failure commences (Lorek et al., 1994; Martin et al., 1996; Taylor et al., 2002; Vannucci et al., 2004); a close
relationship exists between the severity of secondary energy failure and subsequent neurodevelopmental impairment (Azzopardi et al., 1989; Martin et al., 1996; Roth et al., 1997). The importance of the latent phase is increasingly recognized (O’Brien et al., 2006): this is likely to constitute a ‘therapeutic window’ for treatments such as hypothermia (Gunn et al., 1998; Gluckman et al., 2005; Shankaran et al., 2005; Ivata et al., 2005b). Although the neurotoxic cascade is already activated and mitochondrial impairment may exist during the latent phase (Lorek et al., 1994; Nakai et al., 2000; Kristian, 2004; Vannucci et al., 2004), cerebral high-energy phosphates have not been scrutinized in detail during this phase—indeed a robust latent-phase marker of HI severity is desirable to target neuroprotective therapies to patients most likely to benefit.

Stress-induced phosphocreatine (PCr) recovery overshoot (with PCr quantified either as absolute concentration or relative to nucleotide triphosphate (NTP; mainly adenosine triphosphate (ATP)) has been widely recognized in skeletal muscle (Glaister, 2005; Korzeniewski and Zoladz, 2005) and myocardium (Flaherty et al., 1982; Banerjee et al., 1991; Kida et al., 1991; Novel-Chate et al., 1995; Kobara et al., 1996; Straeter-Knowlen et al., 2002; Flogel et al., 2004). This phenomenon has not been described in brain, however it is possible that the recovery level of cerebral PCr during the latent-phase is related to factors such as baseline brain metabolism, acute HI severity and secondary energy failure given the wide variation in cerebral PCr recovery levels observed following HI (O’Brien et al., 2006). To test this hypothesis, a series of experiments were performed using an established model of transient perinatal HI and secondary energy failure.

**Methods**

Experimentation was conducted under United Kingdom (UK) Home Office licence in accordance with UK guidelines.

**Surgical preparation**

Twenty-three Large-White piglets of either sex (body weight 1640 ± 220 g, mean ± standard deviation) were subjected to transient HI aged <24 h. Animals were surgically prepared under general anaesthesia and received continuous physiological monitoring and intensive life support throughout the experimental procedure as described previously (Iwata et al., 2005b). Both common carotid arteries were encircled by remotely controlled vascular occluders (OC2A, In Vivo Metric, Healdsburg, CA, USA). Following surgery, piglets were positioned prone within a plastic pod with the intact head immobilized below a magnetic resonance spectroscopy (MRS) surface coil: the pod was then inserted into the magnetic resonance spectrometer. Warmed air maintained normothermia (rectal temperature 38.5–39.0 °C) throughout the experiment.

**31P MRS acquisition**

Whole-brain phosphorus-31 (31P) spectra were acquired on a 7 Tesla Biospec (Bruker Medizintechnik, Karlsruhe, Germany; 31P frequency 121.6 MHz) with an elliptical surface coil (6.5 × 5.5 cm). A single-pulse acquire sequence was used with 14 286 Hz spectral width and 2048 quadrature data points sampled: repetition time was 10 s therefore spectra were effectively fully-relaxed with peak-area ratios directly proportional to their metabolite concentration ratios. Generally, 192 (baseline), 24 (during HI and ~1 h recovery) and 384 (after recovery) free induction decays were summed to evaluate NTP (quantified using the β resonance), PCr, inorganic phosphate (Pi) and the exchangeable high-energy phosphate pool (EPP = Pi + PCr + 2γ-NTP + β-NTP; incorporation of γ-NTP included the nucleotide diphosphates). The time integral of the NTP/EPP decrement relative to baseline during transient HI and the first 1 h of recovery was used as an index of acute energy depletion (insult severity) as previously described (Iwata et al., 2007).

**Transient hypoxia-ischaemia**

Following baseline MRS, transient global cerebral HI was induced in 20 piglets by reducing the inspired oxygen fraction (FiO2) to 12% and simultaneously inflating both carotid-artery occluders. During HI 31P spectra were acquired every 4 min. After the β-NTP peak amplitude had fallen to 30% of baseline, FiO2 was adjusted to maintain this β-NTP level for 0–24 min to yield a range of insult severities: carotid occluders were then deflated and FiO2 normalized. Further spectra were acquired every 4 min until 1 h after HI and then approximately every 3 h thereafter. In order to correlate endpoint MRS measures with histological cerebral injury, experiments were terminated at various times 24–48 h after HI when NTP/EPP had fallen to less than 60% of baseline thus confirming secondary energy failure. Three sham-operated piglets (controls; no HI) were also studied for at least 24 h.

**Data analysis**

**Sham-operated controls**

Because of the small number of controls their MRS measures are presented without statistical analysis.

**Hypoxia-ischaemia**

One experiment was terminated 14 h after HI because of an extremely short latent phase (in the ‘severe secondary energy failure’ group). The following were excluded from analysis: 1 animal experienced very severe HI and NTP/EPP remained <60% of baseline 4 h after HI, and 1 animal developed lethal arrhythmia which required cardiac massage 6 h after HI.

**Serial metabolite ratios and rates of metabolic change (over all subjects)**

Mixed models with fixed (time and metabolite ratios) and random (subject and time) effects were used to determine the rates of change of NTP/EPP, PCr/EPP and Pi/EPP within the periods (i) first 10 min of HI, (ii) rest of HI (excluding first 10 min), (iii) 0–2 h, (iv) 2–8 h, (v) 8–16 h and (vi) 16–24 h after HI. Although we aimed to acquire at least 2 spectra for each of the time intervals (iii) to (vi), in 3, 2 and 4 animals only a single spectrum was acquired at respectively 2–8, 8–16 and 16–24 h after HI. For the mixed model the missing measurements at 2–8 and 8–16 h (regarded as ‘missing completely at random’), but not at 16–24 h (when the data lack resulted from early termination due to short latent-phase), were surrogated by estimates linearly.
interpolated from the existing measurement in that time interval and the next available measurement: the mixed model during 16–24 h used only the 14 remaining piglets.

$\text{31P-MRS}$ measures at baseline and later time intervals were compared using repeated-measure ANOVA: for this analysis, 17 piglets were included during the period 16–24 h due to one early termination.

**Secondary energy failure severity groups**

Secondary energy failure severity was a surrogate for eventual cerebral injury (Iwata et al., 2007) and this was quantified in each piglet as the minimum NTP/EPP 8–24 h after HI determined by fitting a cubic polynomial to the NTP/EPP time-series in order to avoid bias to lower NTP/EPP by random measurement error. Using this secondary-energy-failure severity index piglets were classified into 3 groups: (i) ‘no secondary energy failure’ ($n = 5$, minimum NTP/EPP $\geq 85\%$ of individual baseline), (ii) ‘moderate secondary energy failure’ ($n = 8$, $60\% \leq$ minimum NTP/EPP $< 85\%$) and (iii) ‘severe secondary energy failure’ ($n = 5$, minimum NTP/EPP $< 60\%$). Metabolite ratios were compared with baseline values in each group and between groups using ANOVA.

**Baseline metabolism, insult severity and PCr recovery (over all subjects)**

In our analysis a positive relationship was observed between PCr/EPP 2–8 h after HI and secondary energy failure severity. To identify possible determinants of this relationship, both simple and multiple linear regression models were used with the index of acute energy depletion (insult severity) and/or baseline $\text{31P-MRS}$ measures as independent variables. Due to piglet head size variation we anticipated inconsistent $\text{31P MRS}$ coverage of the brain, if cerebral metabolite ratios varied regionally this might result in dependencies of baseline metabolite ratios on body weight: this possibility was investigated using a simple linear regression model.

**Statistical probability correction**

Statistical results were corrected (Bonferroni) for multiple comparisons between different time intervals and between different groups.

**Table 1** Metabolite-ratio rates of change during and following HI (all piglets)

<table>
<thead>
<tr>
<th></th>
<th>First 10 min HI</th>
<th>Remainder of HI</th>
<th>Time interval after HI (h)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0–2</td>
</tr>
<tr>
<td><strong>NTP/EPP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope ($\times 10^{-3}$ h)</td>
<td>–174.8</td>
<td>–153.5</td>
<td>52.4</td>
</tr>
<tr>
<td>SE ($\times 10^{-3}$ h)</td>
<td>476</td>
<td>31.5</td>
<td>7.7</td>
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<tr>
<td>$P$</td>
<td>0.006</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
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<tr>
<td><strong>PCr/EPP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope ($\times 10^{-3}$ h)</td>
<td>–670.0</td>
<td>–15.3</td>
<td>136.9</td>
</tr>
<tr>
<td>SE ($\times 10^{-3}$ h)</td>
<td>115.3</td>
<td>40.2</td>
<td>170</td>
</tr>
<tr>
<td>$P$</td>
<td>&lt;0.005</td>
<td>NS</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td><strong>Pi/EPP</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Slope ($\times 10^{-3}$ h)</td>
<td>1184.4</td>
<td>520.3</td>
<td>–323.2</td>
</tr>
<tr>
<td>SE ($\times 10^{-3}$ h)</td>
<td>255.1</td>
<td>116.9</td>
<td>36.5</td>
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<tr>
<td>$P$</td>
<td>&lt;0.005</td>
<td>0.006</td>
<td>&lt;0.005</td>
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</table>

**Results**

**Sham-operated animals**

Sham-operated animals showed no apparent temporal change in NTP/EPP, PCr/EPP or Pi/EPP (online supplementary 1).

**Acute insult, recovery and secondary energy failure (over all subjects)**

Significant rates of change of NTP/EPP (falling) and Pi/EPP (increasing) were observed during both the first 10 min of HI ($P=0.006$ and $P<0.005$, respectively) and the remainder of HI ($P<0.005$ and $P=0.006$, respectively); falling PCr/EPP was observed only during the first 10 min of HI ($P<0.005$) (Table 1 and online supplementary 2). Compared with baseline, mean PCr/EPP was lower during both the first 10 min and the remainder of HI whereas mean Pi/EPP was higher and mean NTP/EPP lower only during the later HI period (all $P<0.001$, online supplementary 2–3).

After commencing resuscitation, significant rates of change of PCr/EPP and NTP/EPP (both increasing) and Pi/EPP (falling) were observed during the period 0–2 h after HI (all $P<0.005$, Table 1). Metabolite ratios had recovered closest to their baseline values during the period 2–8 h after HI when mean PCr/EPP was similar to baseline but mean NTP/EPP was slightly smaller ($P=0.012$) and mean Pi/EPP greater ($P=0.027$) (online supplements 2 and 3). The earliest time interval displaying overt secondary energy failure was 8–16 h after HI when there were significant rates of change for PCr/EPP (falling, $P=0.006$) and Pi/EPP (rising, $P<0.005$): a significant NTP/EPP rate of change during secondary energy failure (falling, $P=0.042$) was first seen at 16–24 h (Table 1). During 8–16 and 16–24 h, mean PCr/EPP and NTP/EPP were lower and mean Pi/EPP higher compared with baseline (all $P<0.001$, online supplements 2 and 3).
Energy metabolism at 2–8 h and secondary energy failure severity (intra- and intergroup analyses)

Compared with baseline, recovery PCr/EPP 2–8 h after HI was high (overshoot) for ‘no’ and low for ‘severe’ secondary energy failure ($P = 0.043$ and $0.008$, respectively); in the same time interval Pi/EPP was high for both ‘moderate’ and ‘severe’ secondary energy failure ($P = 0.006$ and $0.013$, respectively) but NTP/EPP was similar to baseline in each group (Fig. 1, see online supplementary 4 for representative spectra). The ‘no’ and ‘moderate’ secondary energy failure groups both had higher PCr/EPP ($P < 0.001$ and $0.001$, respectively) and lower Pi/EPP ($P = 0.002$ and $0.019$, respectively) compared with ‘severe’ secondary energy failure; NTP/EPP showed no intergroup difference (Fig. 1, see online supplementary 5 for absolute recovery PCr/EPP ratios).

Fig. 1 NTP/EPP (A), PCr/EPP (B), and Pi/EPP (C) 2–8 h after HI relative to individual baselines for ‘no secondary energy failure (SEF)’, ‘moderate SEF’ and ‘severe SEF’. Symbols: box—first and third quartiles, bold line—median, perpendicular line—range without outliers, open circle—extreme outlier less than 1.5 times the interquartile range from the first quartile.

Baseline metabolism, insult severity and PCr at 2–8 h (over all subjects)

PCr/EPP 2–8 h after HI correlated linearly with both the acute energy-depletion index and baseline PCr/NTP with simple linear regression analysis (Fig. 2; $P = 0.024$ and $0.049$, respectively, see online supplementary 6 for comparisons of recovery PCr/EPP with baseline NTP/EPP and PCr/EPP): correlation significance was higher with multivariate analysis which incorporated the interaction between the acute energy-depletion index and baseline PCr/NTP ($P = 0.008$ and $0.015$, respectively). Baseline PCr/NTP was unassociated with body weight.

Discussion

We demonstrate cerebral PCr recovery above baseline level during the reperfusion phase shortly after transient HI: this
occurred only in subjects in whom secondary energy failure did not arise within 24 h of resuscitation; PCr recovery to below baseline was seen in subjects who eventually developed severe secondary energy failure. This report also describes novel relationships between baseline brain energy metabolism, insult severity and metabolic recovery following transient HI: lower baseline PCr/NTP and milder transient HI were both associated with higher PCr/EPP 2–8 h after HI. Although brief transient PCr recovery overshoot has been described in skeletal and cardiac muscle in response to stress (Flaherty et al., 1982; Kida et al., 1991; Kobara et al., 1996; Flogel et al., 2004; Korzeniewski and Zoladz, 2005), this is the first description of a similar phenomenon in brain after transient HI.

Therapeutic hypothermia is the first effective and safe therapy for neonatal encephalopathy (Azzopardi and Edwards, 2007; Jacobs et al., 2007; Schulzke et al., 2007). However, not all infants benefited from this therapy (Gluckman et al., 2005): screening for potential treatment responders at an early stage would be of great clinical advantage. Our results suggest that the cerebral energy profile 2–8 h after perinatal HI might be a potentially useful biomarker.

**Limitations of the study**

The number of subjects (particularly controls) and MRS observations were limited for ethical and practical reasons, however we have seen previously that PCr remains unchanged relative to the total mobile phosphate over 48 h in controls (Lorek et al., 1994). This mirrors human studies in which PCr, Pi, NTP and total mobile phosphate concentrations changed only gradually up to age 20 months (Buchli et al., 1994). The duration of our present study was shorter compared to those in our previous reports (~48 h) (Lorek et al., 1994; O’Brien et al., 2006; Iwata et al., 2007): it is possible that subjects with no or moderate secondary energy failure may have eventually developed severe secondary energy failure with longer observations. Furthermore, the present study did not explore regional variations of cerebral energy metabolism following HI. Further studies are needed using MRS-imaging to identify associations between regional energy metabolism (Iwata et al., 2005a; Iwata et al., 2005b) and long-term MRS and histological outcome.

**High-energy phosphate changes during transient HI and secondary energy failure**

The creatine kinase (CK) reaction can generate ATP from PCr and adenosine diphosphate at sites of high energy demand (Wallimann et al., 1998) constituting a temporal and spatial buffering system for cellular energy stores (Saks et al., 1978; Bessman and Geiger, 1981). In our study there was rapid PCr/EPP reduction only during the first 10 min of HI whereas NTP/EPP declined slowly throughout HI suggesting that NTP was initially maintained at the expense of PCr via the CK reaction (Table 1). We observed a similar sequence of PCr/EPP and NTP/EPP reduction during secondary energy failure suggesting that CK was employed to initially but temporarily maintain the NTP level even during this secondary phase of metabolic derangement. Recent studies have suggested that secondary energy failure occurs concomitantly with irreversible, terminal stage tissue injury (Martin et al., 1996; Cady et al., 1997; Puka-Sundvall et al., 2000b; Vannucci et al., 2004): in addition, our results suggest that secondary energy failure is a consequence of a substantial tissue fraction surviving acute HI only to later undergo further metabolic derangement which eventually leads to more cell death and tissue loss.

**Cerebral stress response at resuscitation and PCr recovery overshoot**

When all piglets were analysed together, resuscitation led to PCr/EPP recovery to baseline 2–8 h following transient HI whereas neither NTP/EPP nor Pi/EPP fully recovered (online supplementary 3). Group analysis revealed that the ‘full recovery’ of PCr/EPP was due to the combined effects of incomplete recovery in the ‘severe’ secondary energy failure group and recovery beyond baseline levels for ‘no’ secondary energy failure: in the latter piglet group PCr/EPP 2–8 h after HI was higher than at baseline and higher than for ‘severe’ secondary energy failure. Even during the latent phase, the neurotoxic cascade (the death programme) and reperfusion-induced recovery processes (the rescue programme) compete to determine the extent of the eventual injury (Kirino et al., 1991; Baiden-Amissah et al., 1998; Vannucci et al., 1998; Kato and Kogure, 1999; Puka-Sundvall et al., 2000a; Johnston et al., 2001; Meng et al., 2005).

Brief PCr recovery overshoot (quantified either as PCr absolute concentration or measured relative to NTP) is one of such stress responses widely recognized in skeletal muscle (Glaister, 2005; Korzeniewski and Zoladz, 2005) and myocardium (Flaherty et al., 1982; Banerjee et al., 1991; Kida et al., 1991; Novel-Chate et al., 1995; Kobara et al., 1996; Straeter-Knowlen et al., 2002; Flogel et al., 2004), however it remains unclear if this phenomenon is beneficial, detrimental, or inconsequential. In skeletal-muscle, endurance training up-regulates PCr recovery and improves exercise performance (Glaister, 2005), whereas in cardiac muscle, PCr recovery overshoot following reperfusion has been related both to adversities including depressed contractile function (‘myocardial stunning’), poor ATP restoration and worse histological injury (Flaherty et al., 1982; Flogel et al., 2004), and to favourable remote effects such as more efficient energy consumption and tolerance to forthcoming potentially lethal HI (‘ischaemic preconditioning’) (Kida et al., 1991; Novel-Chate et al., 1995; Kobara et al., 1996). Although cerebral PCr recovery overshoot following HI has not been described previously, several stimuli such as hypothermia and creatine supplementation, both of which...
are potent neuroprotective treatments, induce an elevation in cerebral PCr (Wilken et al., 1998; Adcock et al., 2002; Erecinska et al., 2003). In our current study PCr recovery above baseline occurred only in the ‘no’ secondary energy failure group, and the PCr level correlated linearly and negatively with insult severity (Fig. 2), suggesting that PCr recovery overshoot in brain is a welcome response to HI potentially heralding cell recovery and survival. Given that induced high cerebral PCr (e.g. incurred by hypothermia or creatine supplementation) is associated with increased resistance to HI, cerebral PCr recovery overshoot following HI might also protect against subsequent potentially lethal stressors.

Our study was not designed to investigate the mechanism of PCr recovery above baseline in developing brain. However in skeletal muscle this stress response is attributed to delayed downgrading of oxidative phosphorylation from the activated high-demand status (Korzeniewski and Zoladz, 2005). In myocardium a potential explanation is mitochondrial PCr synthesis proceeds unperturbed but cytoplasm-specific CK deficiency due to oxygen radical toxicity reduces PCr utilization (Banerjee et al., 1991; Flogel et al., 2004). Another possibility is that cardiac function is down-regulated either passively due to severe damage or actively as a ‘hibernation period’ for stressed tissue (Kloner and Jennings, 2001). A transient reduction of intracellular adenine nucleotides by loss into the interstitial and vascular space observed in myocardium following HI could also explain why neither NTP/EPP nor Pi/EPP regained baseline levels after HI (Reimer et al., 1981; Hoffmeister et al., 1987). Indeed, in the developing brain, a transient, reversible reduction in total cerebral adenine nucleotides following HI has been observed although the relationship with PCr recovery level was not investigated (Vannucci et al., 1998; Vannucci et al., 2004). However, given the prolonged PCr recovery above baseline observed in the current study, it is possible that the underlying mechanism may be entirely different from that in skeletal and cardiac muscle.

We also observed that lower baseline PCr/NTP was associated with higher recovery PCr/EPP. Although we used term-born piglets of similar size and maturation, high brain PCr/NTP under normal physiological conditions may imply differences in maturity, metabolic activity and subsequent vulnerability to HI. Interestingly, pre-existing low PCr (relative to NTP or creatine) leads to greater PCr following exercise (skeletal muscle) and creatine supplementation (skeletal muscle and brain) (Rawson et al., 2002; Korzeniewski and Zoladz, 2005; Pan and Takahashi, 2007). Further studies are required to elucidate the relationship between cerebral PCr recovery overshoot and background metabolism.

**Clinical implications**

Early prediction of the extent of cerebral injury following transient HI is desirable for the refining and appropriate targeting of neuroprotective therapies in the newborn. Our current results suggest that (i) elevated PCr/EPP and Pi/EPP 2–8 h after birth might predict minimal or severe cerebral injury respectively following HI and (ii) a high baseline PCr/NTP might identify infants with low tolerance to HI prior to cardiac surgery.

**Conclusions**

PCr recovery overshoot has been reported elsewhere in skeletal muscle after exercise and in myocardium after reperfusion. We report cerebral PCr recovery above baseline in a newborn porcine model of transient HI studied with serial 31P MRS: piglets in whom secondary energy failure did not develop displayed this phenomenon; PCr/EPP recovery levels correlated negatively with baseline PCr/NTP and insult severity. Our findings give novel insight into the cerebral stress response to HI and suggest that, as well as Pi/EPP, cerebral PCr/EPP might be an early favourable biomarker in neonatal encephalopathy.

**Supplementary material**

Supplementary material is available at *Brain* online.

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