Exp Brain Res (2004) 156: 20–26
DOI 10.1007/s00221-003-1761-5

RESEARCH ARTICLES

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Redox state of near infrared spectroscopy-measured cytochrome aa_3 correlates with delayed cerebral energy failure following perinatal hypoxia-ischaemia in the newborn pig

Received: 9 June 2003 / Accepted: 10 October 2003 / Published online: 20 December 2003

Abstract Early detection of delayed cerebral energy failure may be important in the prevention of reperfusion injury of the brain after severe perinatal hypoxia-ischaemia (HI). This study investigated whether monitoring of the redox state of cytochrome aa_3 ($Cytaa_3$) with near infrared spectroscopy (NIRS) after severe perinatal asphyxia may allow us to detect early a compromised energy metabolism of the developing brain. We therefore correlated serial $Cytaa_3$ measurements (to estimate mitochondrial oxygenation) simultaneously with the ^{31}phosphorous-magnetic resonance spectroscopy (^{31}P-MRS)-measured phosphocreatin/inorganic phosphate (PCr/Pi) ratio (to estimate cerebral energy reserve) in newborn piglets before and after severe hypoxia-ischaemia. The animals were treated upon reperfusion with either allopurinol, deferoxamine, or 2-iminobiotin or with a vehicle to reduce post-HI reperfusion injury of the brain. Four sham-operated piglets served as controls. Before HI, the individual $Cytaa_3$ values ranged between −0.02 and 0.71 μmol/L (mean value: −0.07) relative to baseline. The pattern over post-HI time of the vehicle-treated animals was remarkably different from the other groups in as far $Cytaa_3$ became more oxidised from 3 h after start of HI onwards (increase of $Cytaa_3$ as compared with baseline), whereas the other groups showed a significant reduction over time (decrease of $Cytaa_3$ as compared with baseline: allopurinol and deferoxamine) or hardly any change (2-iminobiotin and sham-operated piglets). Vehicle-treated piglets showed a significant reduction in PCr/Pi at 24 h after start of HI, but the cerebral energy state was preserved in 2-iminobiotin-, allopurinol- and deferoxamine-treated piglets. With severe reduction in PCr/Pi-ratio, major changes in the redox-state of $Cytaa_3$ also occurred: $Cytaa_3$ was mostly either in a reduced state (down to −6.45 μmol/L) or in an oxidised state (up to 6.84 μmol/L) at these low PCr/Pi ratios. The positive predictive value (PPV) of $Cytaa_3$ to predict severe reduction of the PCr/Pi ratio was 42%; the negative PPV was 87%. A similar relation was found for $Cytaa_3$ with histologically determined loss of neurons.

Keywords Near infrared spectroscopy · Cytochrome aa_3 · Magnetic resonance spectroscopy · Hypoxia-ischaemia · Reperfusion

Abbreviations *$Cytaa_3$*: Cytochrome aa_3 · *HI*: Hypoxia-ischaemia · *NIRS*: Near infrared spectroscopy · *^{31}P-MRS*: ^{31}Phosphorous magnetic resonance spectroscopy · *PCr/Pi*: Phosphocreatin/inorganic phosphate

Introduction

Delayed cerebral energy failure of the developing brain has been demonstrated after severe hypoxia-ischaemia (HI) in the newborn animal and in the human neonate and carries a high risk for adverse outcome. A biphasic impairment in the energy metabolism of the developing brain occurs with an initial decline in high energy phosphates during the actual HI insult followed by a recovery upon reperfusion and reoxygenation. Subsequently, delayed energy failure of brain metabolism develops at 10–24 h after the HI insult (Lorek et al. 1994; Blumberg et al. 1997). Mitochondrial dysfunction seems to play an important role in delayed cerebral energy failure of the brain following severe perinatal HI. An earlier study in a perinatal rat stroke model reported that disruption of cytochrome aa_3 ($Cytaa_3$) activity, the terminal complex of the mitochondrial respiratory chain and generator of ATP via oxidative phosphorylation, was one of the earliest detectable indicators of neuronal cell injury and neurological deficits (Dimlich et al. 1990; Wagner et al. 1990; Nelson and Silverstein 1994).

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Two studies dealing with hypoxic newborn piglets and rats, respectively, found that an augmentation in reduction of near infrared spectroscopy (NIRS)-measured $Cytaa_3$ *during* hypoxia correlated with cerebral energy loss as simultaneously measured with ^{31}phosphorous-magnetic resonance spectroscopy (^{31}P-MRS) (Tsuji et al. 1995; Matsumoto et al. 1996).

We hypothesise that monitoring of the redox state of $Cytaa_3$ with NIRS after severe perinatal asphyxia will also allow us to detect *delayed* cerebral energy failure of the developing brain after reperfusion and reoxygenation. Additionally, it will provide the possibility to early assess the efficacy of pharmacological intervention therapy for prevention of reperfusion injury of the developing brain.

We therefore correlated the redox state of serially measured $Cytaa_3$ activity using NIRS with simultaneous estimation of cerebral energy reserve determined using ^{31}P-MRS measured ratios of PCr/Pi in newborn piglets before and in the first 24 h after start of the HI insult. Subgroups of these animals were pharmacologically treated with a free radical scavenger (allopurinol), a free iron chelator (deferoxamine), nitric oxide synthase or a selective inhibitor (2-iminobiotin) in order to reduce post-HI reperfusion/reoxygenation injury of the brain.

Material and methods

Animal preparation and instrumentation

Forty-two newborn Dutch store piglets with gestational age (mean ± S.E.M.) 115±1 days, weight 1.71±0.07 kg, were used at a postnatal age ranging from 1 to 3 days. Anaesthesia was induced with 4% isoflurane in a N_2O/O_2 mixture (79%/21%). After intubation, the piglets were mechanically ventilated (pressure-controlled). Anaesthesia was maintained during the procedure using 1.5% isoflurane in the same N_2O/O_2 mixture. Venous catheters were inserted for continuous infusion of glucose 5%-NaCl 0.45% (5 ml/kg/h) and drug infusion. A catheter was advanced in the right femoral artery for continuous measurement of arterial blood pressure, while the blood was heparinised (5 U/h). Remotely inflatable vascular cuffs (OC2a, In Vivo Metric, Healdsburg, CA, USA) were placed around both common carotid arteries at the level of the thyroid cartilage. Finally, amoxicillin (100 mg/kg/day) and gentamicin (5 mg/kg/day) were given and atropin (0.01 mg/kg) was administered intravenously to prevent bradycardia due to vagal stimuli. During the experiment, rectal temperature was measured and maintained between 38 and 39°C using a heat lamp and/or water blanket. The Animal Research Committee of the Utrecht University, the Netherlands, approved the animal care protocols.

Near infrared spectroscopy

Cerebral oxygenation was assessed with NIRS (Critikon Cerebral RedOx Monitor Model 2020, Johnson and Johnson Medical, Berks, UK). Since the head of the neonate is relatively transparent to near-infrared light, the natural chromophores (i.e. hemoglobin and $Cytaa_3$) can be measured by oxygenation-dependent absorption in this wavelength. The neonatal sensor, consisting of a laser emitter at four wavelengths (776.5, 819.0, 871.4, and 908.7 nm), two light detectors with an interoptode distance of 10 and 35 mm, and a light emitting diode for compensating differences in coupling, was firmly attached with light opaque tape over the parietal brain region in the left parasagittal plane of the piglet skull. The double detector sensor reduces the influence of skull and skin (Wolf et al. 1999) and decreases the variation due to position of or pressure exerted on the sensor (Wolf et al. 1997). The exact position of the NIRS sensor was accurately marked with a waterproof pencil at the skin, so that the NIRS sensor could be reapplied at exactly the same position after returning from the MR scanner. To prevent zeroing of the NIRS during the MR period, the apparatus was kept standby and no baseline reset was applied. By selection of the appropriate wavelength, an algorithm has been developed to convert absorption changes into relative changes in $Cytaa_3$ in the brain (Jobsis 1977). The calculated concentration changes of $Cytaa_3$ were expressed in micrometers. $Cytaa_3$ absorption coefficients were derived from data published by Brunori et al. (1981). The algorithm incorporates wavelength-dependent differential pathlength factor data derived from 'time of flight' studies (Van Der Zee et al. 1992). Changes in $Cytaa_3$ are supposed to indicate changes in the oxidation-reduction level of the intracerebral mitochondrial enzyme cytochrome oxidase and have been used earlier as a relative measure of brain cell oxygenation (Brazy and Lewis 1986; Tsuji et al. 1995): An increase in $Cytaa_3$ relative to baseline indicates an increase in oxidised $Cytaa_3$, whereas a decrease relative to baseline indicates an increase in reduced $Cytaa_3$. Because the enzyme is not maximally oxidised under normal conditions, increased oxygenation or less utilisation of oxygen should further oxidise the enzyme (Jobsis et al. 1977; Thomiley et al. 1993). The calculated concentration of changes in $Cytaa_3$ were recorded in real time and digitised with a sample frequency of 10 Hz using POLY software (Inspektor Research Systems BV, Amsterdam, The Netherlands).

^{31}Phosphorous magnetic resonance spectroscopy

^{31}P-MRS experiments were performed at 81 MHz on a 4.7 Tesla Varian NMR spectrometer (Palo Alto, CA, USA) interfaced to a magnet with a bore size of 40 cm. A ∅ 4-cm inductively coupled surface coil was used for signal excitation and detection and placed onto the intact scalp over the parietal lobes. A 1.0-ms adiabatic half passage pulse was used for excitation. To minimise T1 effects, a repetition time of 10 s was used. Thirty-two acquisitions were averaged at baseline, before HI, and after reperfusion and reoxygenation. Peak amplitudes of PCr and Pi were determined with time-domain fitting procedures using prior knowledge (Magnetic Resonance User Interface 97.2, Barcelona, Spain) and PCr/Pi metabolite ratios were calculated at baseline, continuously during the period of HI until reperfusion and reoxygenation, and at 3 and 24 h post start of HI. To determine whether all groups experienced a comparable amount of HI injury, the surface under the PCr/Pi curve was calculated. Since an earlier study in piglets comparing $Cytaa_3$ with ^{31}P-MRS-measured parameters during severe hypoxia (Tsuji et al. 1995) showed that PCr/Pi-related changes correlated most closely and momentarily with changes in $Cytaa_3$, changes in the metabolite ratio of PCr/Pi were used to assess changes in the cerebral energy level: a reduction of the PCr/Pi ratio indicates a reduction of brain energy level (Wyatt et al. 1989).

Histological quantification of neuronal viability

In the present study the total number of viable neurons in four different parts of the brain (cortex, thalamus, striatum and hippocampus) at 24 h after HI were related to the individual $Cytaa_3$ values at 24 h after HI. In an earlier study we report more in detail on the histological and immunohistochemical findings in the brains of the animals of the various groups (Peeters-Scholte et al. 2002). Briefly, in coronal sections (7 μm) of the brains, stained with cresyl violet, quantification of neuronal viability was performed in the four brain regions using a grid of 100 compartments at 200× magnification. Normal neurons were identified by presence of typical nuclei with clear nucleoplasm and a distinct nucleolus, surrounded by purple-stained cytoplasm. Neurons were defined damaged when no

distinction could be made between nucleus and cytoplasm (pycnotic or necrotic).

Experimental protocol

After completion of the surgical procedure, the piglets were allowed to achieve hemodynamic stability. Arterial blood pressure, heart rate, transcutaneous oxygen saturation using pulse oxymetry (Nellcor, NPB 290, Tyco Healthcare, Pleasanton, CA, USA) and rectal temperature were measured during the study protocol. Subsequently, NIRS-recordings for changes in $Cytaa_3$ relative to baseline (zero) were achieved during 30 min and, after averaging, used as pre-HI values. The piglets were then immediately transferred to the MR unit for pre-HI ^{31}P-MRS spectra. During MR-measurements, anaesthesia was maintained as described earlier (Peeters-Scholte et al. 2002). Animals were paralysed with pancuronium bromide (0.25 mg/kg/i.v.). Normocapnia was controlled using an end-tidal CO_2 probe and ventilatory settings were adjusted when appropriate. Piglets were subjected to 1 h of HI by inflating both occluders and reducing the fraction of inspired oxygen until PCr/Pi metabolite ratios decreased to at least 30% of pre-HI values, as guided by frequent ^{31}P-MRS measurements, for approximately 45 min of the 1 h HI period. Isoflurane administration was discontinued from 10 min after start of HI until 10 min after reperfusion/reoxygenation in order to diminish the effect of anesthetics on cerebral metabolism and on blood pressure during the actual insult. When severe systemic hypotension (mean arterial blood pressure: <30 mm Hg) or bradycardia developed, the O_2 intake was minimally increased to prevent further decrease of blood pressure or to obtain recovery of the heart rate. After 1 h of HI the occluders were deflated and the oxygen intake increased to obtain normoxemia (PaO_2 80–120 mm Hg), carefully avoiding hyperoxemia. The piglets were randomly assigned to receive either vehicle (n=11; 5 ml/kg intravenously 0.9% NaCl), allopurinol (n=8; Apurin, Multipharma, The Netherlands, 20 mg/kg upon reperfusion and a repeated dose of 10 mg/kg at 12 h), deferoxamine (n=9; Ciba Geigy, Switzerland, 10 mg/kg upon reperfusion and a repeated dose of 2.5 mg/kg at 12 h), [injected intravenously by a perfusor pump in 15 min], or 2-iminobiotin (n=10; Sigma, Zwijndrecht, The Netherlands, 0.2 mg/kg intravenously every 4 h). Metabolic acidosis was not corrected following HI. ^{31}P-MRS measurements used for the present study were performed after hemodynamic stabilisation and just before the start of inflation of the carotid occluders and the decrease in FiO_2 (pre-HI value) at 3 and 24 h after start of HI. Between the latter two MRS-measurements the piglets returned to the Piglet Intensive Care Unit and were ventilated until they were stable enough to be extubated. The corresponding monitoring of $Cytaa_3$ at 3 h after start of HI was performed within 15 min after arrival in the Piglet Intensive Care Unit (again an average of a 30-min sampling period). The final measurement, at 24 h after HI, was performed immediately before readmission in the MR unit. Additional $Cytaa_3$ determinations were done at 6 and 12 h (30-min sampling period) after HI. Sampling of arterial blood gases and lactate, arterial pH, hemoglobin and glucose was performed every hour pre-HI, at least every 30 min during HI and every 3 h during the post-HI period. At the end of HI, arterial lactate was measured again. Four piglets served as sham-operated controls and were not subjected to HI. At the end of the experiments, the animals were killed with an overdose of pentobarbital. Brain tissue was obtained for histological analysis (see above).

Statistical analysis

Physiologic data of mean arterial blood pressure, blood gases and pH are presented as mean ± S.E.M. Differences in physiological data, $Cytaa_3$ and PCr/P_i at the different points of time within groups were analysed using analysis of variance for repeated measures, whereas differences among groups at a specific time point were analysed using one factorial analysis of variance. When a significant difference was detected, analysis of variance was followed by the

Table 1 Mean values (± SEM) of mean arterial blood pressure (*MABP* in mm Hg), arterial pH (*pH*), $PaCO_2$ (mm Hg), and base excess (mmol/l) in vehicle (n=11), allopurinol (n=8), deferoxamine (n=9) and 2-iminobiotin (2-IB; n=10) pre-HI, and at 3, 6, 12, and 24 h post start hypoxia-ischemia (HI) (=2, 5, 11 and 23 h post infusion of treatment modalities) using near infrared spectroscopy

	Pre-HI	3 h post start HI	6 h post start HI	12 h post start HI	24 h post start HI
MABP					
Vehicle	56±4	53±3	65±5	58±5	58±5
Allopurinol	57±3	63±5	67±8	70±10	46±5
Deferoxamine	52±2	56±4	60±5	65±3	63±4
2-Iminobiotin	53±3	64±6	64±5	60±5	59±5
pH					
Vehicle	7.35±0.02	7.36±0.02	7.37±0.03	7.43±0.01	7.43±0.03
Allopurinol	7.34±0.02	7.35±0.04	7.41±0.05	7.39±0.04	7.47±0.07
Deferoxamine	7.36±0.03	7.33±0.04	7.39±0.03	7.43±0.02	7.43±0.02
2-Iminobiotin	7.31±0.02	7.34±0.04	7.34±0.04	7.41±0.04	7.43±0.06
$PaCO_2$					
Vehicle	45±2	42±3	36±3	34±2	39±3
Allopurinol	50±3	42±4	38±5	44±4	37±6
Deferoxamine	39±2	43±3	37±3	39±2	38±2
2-Iminobiotin	47±2	50±5	47±4	38±3	44±7
BE					
Vehicle	−0.6±1.0	−9.8±0.9*	−3.5±1.7	−1.0±1.3	1.7±2.1
Allopurinol	3.6±1.4	−1.3±1.2	−0.5±1.3	2.5±0.8	4.3±0.9
Deferoxamine	−0.3±1.5	−4.6±1.8	−2.2±1.3	1.4±0.8	0.6±0.1
2-Iminobiotin	−2.9±1.4	−0.5±1.1	−1.5±2.3	−0.4±2.5	2.7±0.5

*$p<0.05$ vs. other groups

Bonferroni test. For estimation of predictive ability, the sensitivity, specificity, and positive and negative predictive values of $Cytaa_3$ at 24 h after HI as an indicator of delayed cerebral energy failure and of neuronal cell damage after severe perinatal HI were calculated. For this aim, we assumed that cerebral energy failure was apparent if the PCr/Pi ratio was two standard deviations (2SD) below the mean value, obtained from all the individual pre-HI measurements (mean: 2.40; −2SD: 1.78); abnormally reduced or oxidised states of $Cytaa_3$were assumed to be 2SD below or above the mean value, respectively, obtained from all the individual pre-HI measurements (−0.02 μmol/L; 2SD: ±0.66 μmol/L). In case of the estimation of predictive ability of $Cytaa_3$ with regard to histologically proven neuronal cell damage, a number of at least 2SD below the mean number of viable neurons in the sham-operated piglets was used (mean: 1,255; −2SD: 650). A p-value <0.05 was considered statistically significant.

Results

Physiological data

Animal weight, sex, gestational and postnatal ages were equally distributed among groups. No significant differences in the physiological variables were detected, as shown in Table 1, among treatment groups or within groups during the various points of time, except for the base excess. Arterial pO2 values did not significantly differ among groups during HI or reperfusion (data not shown). At the end of the HI period, mean arterial blood pressure and heart rate (not shown) were significantly increased. Heart rate of the 2-iminobiotin-treated piglets remained increased until 24 h post-HI. As expected, a significant decrease in base excess and pH was observed in all groups during the HI insult (data not shown). Arterial lactate values significantly raised from 3.5±0.5 (mean ± SEM) for all groups pre-HI to 10.5±0.9 for vehicle-treated, to 7.6±1.8 for allopurinol-treated, to 13.2±1.1 for deferoxamine-treated, and to 10.9±1.5 for 2-iminobiotin-treated piglets at the end of 1 h of HI. There were no relevant changes in glucose and rectal temperature during the study period for all treatment groups (data not shown). The physiological data of the sham-operated piglets remained perfectly constant over time (not shown).

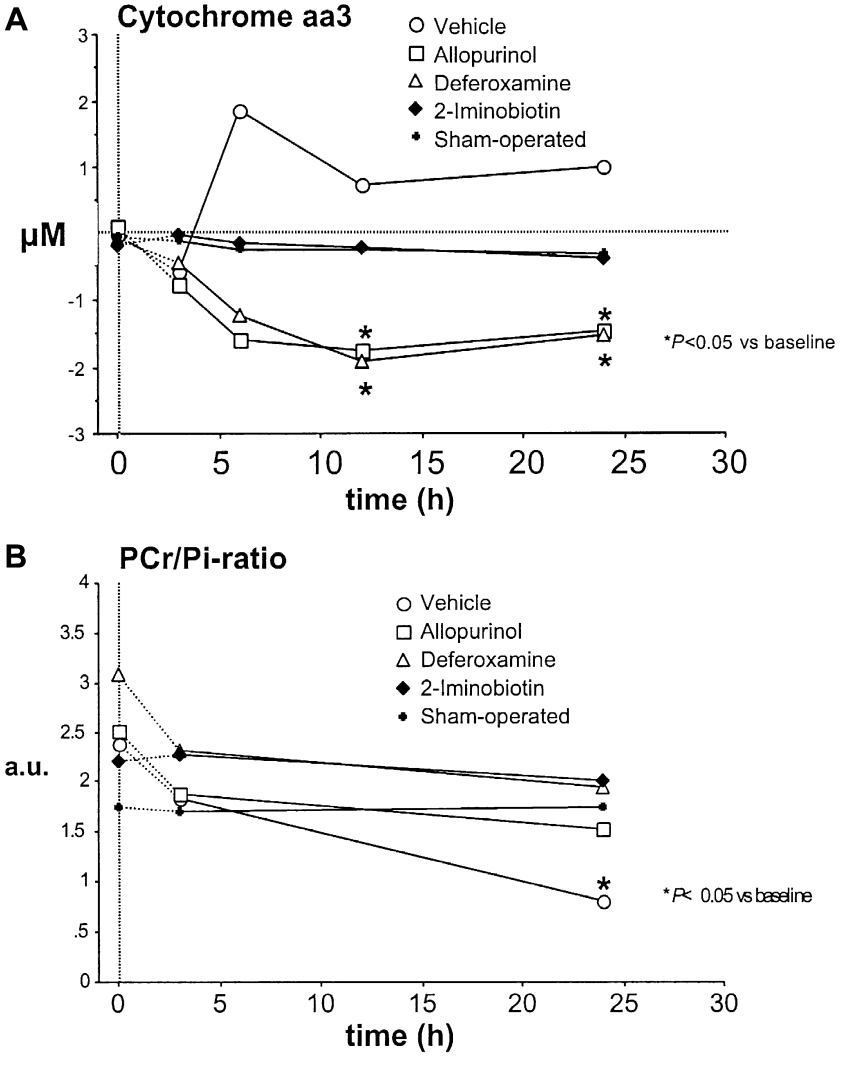

Fig. 1A, B Mean values of cytochrome aa_3 values (**A**) and of phosphocreatine/inorganic phosphate (PCr/Pi)-ratios (**B**) for vehicle (n=11), 2- allopurinol (n=8), deferoxamine (n=9) and 2-iminobiotin (2-IB; n=10)-treated piglets, and of sham-operated piglets (n=4) before hypoxia-ischaemia (HI), and at 3, 6, 12, and 24 h after start of HI using near infrared spectroscopy

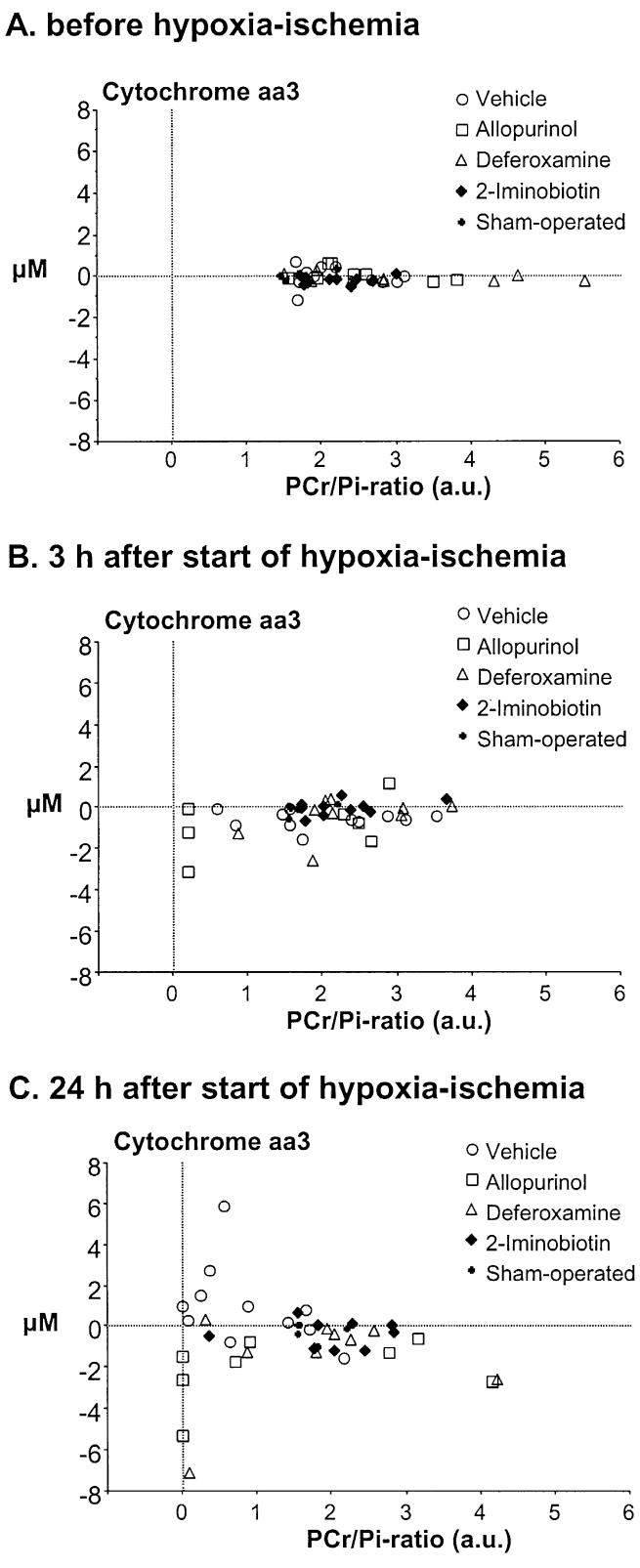

Fig. 2A–C Individual cytochrome aa_3 values as a function of the individual phosphocreatine/inorganic phosphate (PCr/Pi)-ratios of the different treatment groups and sham-operated group before hypoxia-ischaemia (HI) (**A**), and at 3 h (**B**), and 24 h (**C**) after start of HI

Near infrared spectroscopy and ^{31}phosphorous magnetic resonance spectroscopy

Mean values of changes of $Cytaa_3$ of all groups during the different points of time are shown in Fig. 1A. Pre-HI, the individual $Cytaa_3$ values ranged between −0.21 and +0.71 µmol/L (mean value: −0.02). The pattern over time of the vehicle-treated animals was remarkably different from the other groups in as far as $Cytaa_3$ became more oxidised from 6 h after HI onwards, whereas the other groups showed a significant reduction over time (allopurinol and deferoxamine) or hardly any change (2-iminobiotin and sham-operated piglets).

Mean values of ^{31}P-MRS-measured PCr/Pi ratios of all groups pre-HI, at 3 and 24 h after start of HI are shown in Fig. 1B. No significant differences in PCr/Pi metabolite ratios were detected among groups during HI. Vehicle-treated piglets showed a significant reduction in cerebral energy state at 24 h after HI compared with pre-HI, which was not the case for the cerebral energy state of 2-iminobiotin-, allopurinol-, and deferoxamine-treated piglets.

Relationship $Cytaa_3$ and PCr/Pi ratio

The relationship between $Cytaa_3$ as an indicator for redox state of the cytochrome oxidase enzyme and PCr/Pi ratios as an indicator of cerebral energy metabolism pre-HI, and at 3 and 24 h after start of HI are shown in Fig. 2A–C. Pre-HI changes in $Cytaa_3$ were minimal, without evidence for a relation with PCr/Pi ratios (Fig. 2A). At 3 h after start of HI there seemed to be a tendency that $Cytaa_3$ was in the reduced state at lower PCr/Pi ratios (Fig. 2B). At 24 h after HI a substantial number of animals, mostly vehicle-treated animals (n=6) and to a lesser degree also allopurinol- and deferoxamine-treated piglets (n=3 and n=1, respectively), showed abnormally low PCr/Pi ratios, indicating severe reduction of brain energy level (Fig. 2C). At these low ratios, a striking subdivision of these vehicle- and

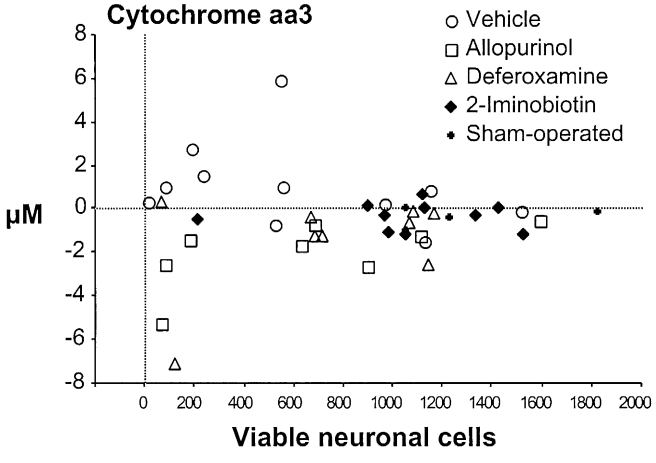

Fig. 3 Individual cytochrome aa_3($Cytaa_3$) values at 24 h after start of HI as a function of the individual total number of viable neurons of the different treatment groups and sham-operated group

allopurinol/deferoxamine-treated piglets took place: $Cytaa_3$ in the six vehicle-treated piglets oxidised further (up to 6.84 μmol/L), whereas $Cytaa_3$ of some allopurinol and deferoxamine-treated piglets was reduced (down to 6.45 μmol/L: 3 allopurinol, 1 deferoxamine-treated: Fig. 2C). The sensitivity, specificity, and positive and negative predictive values of $Cytaa_3$ at 24 h after HI as a marker of cerebral energy failure were 73, 65, 42 and 87%, respectively. The efficiency of $Cytaa_3$, which is the percentage of $Cytaa_3$ measurements that correctly classifies the energy status of the brain, was 67%.

Relationship $Cytaa_3$ and histological score

Figure 3 shows the relationship between changes in $Cytaa_3$ and the histological score of viable neurons (total cell count) at 24 h after HI. There was a remarkable similarity with the $Cytaa_3$-to-PCr/Pi ratio relationship (Fig. 2C): all animals with substantially reduced or oxidised $Cytaa_3$ had the lowest amount of viable neuronal cells. This similarity expressed itself also in the sensitivity, specificity, and positive and negative predictive values of $Cytaa_3$ as marker of neuronal cell loss: 72, 67, 50 and 83%, respectively. The efficiency to indicate gross neuronal cell damage correctly was 68%.

Discussion

As already shown in earlier studies, it was confirmed that a biphasic impairment in the energy metabolism of the developing brain occurs in the early reperfusion and reoxygenation phase after completion of the HI insult. This was especially the case in the vehicle-treated piglets: an initial recovery upon reperfusion and reoxygenation was followed by a delayed (secondary) energy failure of brain metabolism at 24 h after initiation of the insult. As already suggested in an earlier report (Peeters-Scholte et al. 2002), early post-HI treatment with 2-iminobiotin and, to a lesser degree, early post-HI treatment with deferoxamine and allopurinol, showed a (partial) neuroprotective effect as far as this was indicated by a preservation of ^{31}P-MRS-measured brain energy metabolism (all groups) and by the number of viable neurons of the piglet brains (2-iminobiotin).

In the present study it was shown that a substantial change in redox state of $Cytaa_3$ was a reasonable predictor of severe impairment of cerebral energy metabolism at 24 h after the HI insult, as indicated by very low PCr/Pi-ratios. Moreover, the very low number of viable neurons at 24 h after the start of HI in those animals with a marked oxidised or reduced state of $Cytaa_3$ gives room to propose that the pattern of $Cytaa_3$ at 24 h after reperfusion and reoxygenation can be used as a marker of histological brain damage after severe HI. On the other hand, it is reasonable to state that no changes or only moderate changes in redox state of $Cytaa_3$ at 24 h after a HI insult do not always exclude severe histological brain damage.

NIRS-determined relative changes in $Cytaa_3$ are supposed to indicate changes in the oxidation-reduction level of the intracerebral enzyme cytochrome oxidase, the terminal member of the mitochondrial respiratory chain and can therefore be used as a relative measure of cellular oxygenation (Jobsis 1977). Earlier studies showed that disruption of the enzyme cytochrome oxidase represents one of the earliest detectable indicators of neuronal injury in the perinatal stroke model (Nelson and Silverstein 1994). A substantial decrease in oxygen supply and/or dysfunction of the mitochondrial electron transport chain in neurons will result in an increase of the reduced form of the $Cytaa_3$ enzyme. Since $Cytaa_3$ is normally almost (but not entirely) oxidised (Jobsis et al. 1977; Thomiley et al. 1988), the NIRS-determined $Cytaa_3$ will show the reduction of this enzyme as a decrease from baseline. On the other hand, during decreased utilization of oxygen because of disruption of the mitochondrial chain but with a sufficient supply of oxygen, further oxidation of the enzyme occurs, since $Cytaa_3$ is not yet maximally oxidised under baseline conditions (Reynolds et al. 1988). The NIRS-determined $Cytaa_3$ will show this oxidation as an increase from baseline. In pathological conditions such as during delayed energy failure of the perinatal brain after reperfusion/reoxygenation, failure of electron transport due to severe disruption of the mitochondrial chain leading to less utilization of oxygen may indeed cause a highly and maximally oxidised $Cytaa_3$. This may explain that in the vehicle-treated piglets, the treatment group with the most extensive brain damage (Peeters-Scholte et al. 2002), several piglets showed a substantial increase of $Cytaa_3$ as compared with baseline, as indicated by Figs. 1A and 2C.

However, it must be stated here that NIRS-measured changes in $Cytaa_3$, as a relative measure of changes in neuronal cell oxygenation, have been widely disputed. The normally low energy requirement of especially the newborn brain may mask fluctuations in the oxidation-reduction level of the enzyme and effect the reliability of $Cytaa_3$ as a marker of actual changes of oxidation of this enzyme (Astrup 1982). Moreover, there is concern that NIRS-measured $Cytaa_3$ does not properly reflect changes in cell oxygenation because of the use of wrong algorithms for calculation of $Cytaa_3$ signal changes and the fact that concentrations of deoxygenated and oxygenated cerebral hemoglobin are at least ten times higher than those of $Cytaa_3$, thereby obscuring measurements of relative changes in $Cytaa_3$ (Pryds et al. 1990; Wickramasinghe et al. 1995). Despite the above-mentioned considerations, a study of Cooper et al. in rats (1998) showed that in spite of substantial changes in oxygenated and deoxygenated cerebral hemoglobin signals, no interference occurred with the much smaller $Cytaa_3$ signal. Furthermore, a study of Matsumoto et al. (1996) in adult rats during hypoxia showed that an increase in reduced $Cytaa_3$ reflected brain energy depletion as measured by ^{31}P-MRS-determined PCr. Tsuji et al. (1995) showed that this was also true in severely hypoxic piglets. Finally, our study confirmed that there was indeed a relation between ^{31}P-MRS-determined delayed brain energy depletion and $Cytaa_3$ 24 h after a

severe perinatal HI insult. These $Cytaa_3$ changes appeared to be also indicative for the amount of neuronal cell death at that point of time. We therefore suggest that substantial reduction or extreme oxidation of $Cytaa_3$ in the reperfusion/reoxygenation phase after a severe perinatal HI insult, can be used as an additional bedside tool to assess substantial delayed cerebral energy failure and brain damage due to severe perinatal asphyxia.

In summary, substantial reduction or extreme oxidation of $Cytaa_3$, at 24 h after an HI insult in the newborn piglet was related to simultaneously determined abnormally low ^{31}P-MRS-measured PCr/Pi-ratios, indicating a delayed energy failure of the piglet brain. These rather extreme $Cytaa_3$ changes were also related to substantial neuronal cell death. On the other hand, normal or only moderately reduced or oxidised $Cytaa_3$ values at 24 h after an HI insult did not always exclude substantial neuronal cell death. Further studies are warranted to further elucidate and confirm the use of NIRS-measured $Cytaa_3$ as a reliable additional means to assess disturbances in energy failure of the developing brain before this particular application of NIRS can be considered in clinical practice.

Acknowledgements We thank Tyco Healthcare, Pleasanton, CA, USA, for putting a Nellcor NPB 290 oxymeter at our disposal. We also thank the biotechnicians of the Central Laboratory Animal Institute of the Utrecht University for their enthusiastic help during the experiments. Finally, we thank Kees Braun, Wouter Veldhuis and Prof. K. Nicolay for their help during the MRI/MRS experiments and Prof. P.R. Bär for critically reviewing the manuscript.

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