Allopurinol preserves cerebral energy metabolism during perinatal hypoxia-ischemia: a $^{31}$P NMR study in unanesthetized immature rats

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(Received 26 February 1992; Revised version received 15 June 1992; Accepted 15 June 1992)

Key words: Allopurinol; Nuclear magnetic resonance; Hypoxia-ischemia; Adenosine triphosphate; Immature animal; Rat brain; Neuroprotective; Xanthine oxidase

The effects of high dose allopurinol (ALLOP) pretreatment on the cerebral energy metabolism of unanesthetized 7-day-postnatal rats during exposure to 3 h of cerebral hypoxia-ischemia were serially quantitated using non-invasive $^{31}$P NMR spectroscopy. Adenosine triphosphate, integrated over the last 2 h of hypoxia and expressed as a fraction of baseline, was 0.73±0.16 with ALLOP pretreatment (200 mg/kg s.c.) compared to 0.52±0.05 for saline pretreatment ($P<0.001$). Inorganic phosphate/phosphocreatine (P/PCR), integrated over the same time interval, was 2.63±1.23 relative to baseline with ALLOP versus 5.13±1.45 for saline-treated pups ($P<0.005$). We suggest that the neuroprotection achieved with high dose ALLOP pretreatment may be attributed in part to preservation of energy metabolites.

Perinatal hypoxia-ischemia (asphyxia) is a major cause of mortality and morbidity in the human newborn and can be studied using 7-day-postnatal rats [14, 15, 20]. The rat pups are subjected to right common carotid ligation followed by 3 h of hypoxia in 8% oxygen. This combined hypoxic-ischemic (HI) insult results in brain damage localized to the posterolateral region of the right cerebral hemisphere.

During HI, cellular ATP is degraded sequentially to ADP, AMP, adenosine, inosine and hypoxanthine. Hypoxanthine accumulates as further metabolism requires oxygen. During reperfusion and reoxygenation, hypoxanthine is converted irreversibly by xanthine oxidase into xanthine and uric acid. These steps may limit purine bases for ATP resynthesis. Alternatively, during reoxygenation, hypoxanthine may follow the salvage pathway serving as a substrate for ATP resynthesis. The xanthine oxidase reaction generates superoxide and secondarily derived cytotoxic reactive oxygen species. As such, there has been much interest in the potential role for xanthine oxidase inhibitors like allopurinol (ALLOP) and its active metabolites in the management of ischemia reperfusion injury. Additionally, ALLOP’s antioxidant effects may be enhanced by direct scavenging of hydroxyl radicals [13], and by chelating transition metals [9] in a dose-dependent manner.

We have demonstrated that ALLOP (135 mg/kg s.c.) administered 30 min prior to the onset of cerebral HI markedly reduced brain injury [15]. This neuroprotective effect is dose dependent and amounts in excess of that required to inhibit xanthine oxidase are needed [4, 11, 17]. Other investigators have used ALLOP pretreatment in adult animals to successfully prevent ischemic injury to the heart [21], kidney [3], small intestine [18] and adult rat brain [8, 11, 12]. Pretreatment with ALLOP preserved ATP levels in ischemic heart [10] and kidney [6]. Accordingly, we hypothesized that ALLOP pretreatment would preserve cerebral energy metabolites during an HI insult to the right cerebral hemisphere of 7-day-postnatal rats.

We recently reported that $^{31}$P NMR can be used to measure right cerebral energy metabolism, non-invasively in unanesthetized 7-day-old rat pups [22]. This technique provides a means for integrating serial changes in high energy phosphates during a prolonged period of cerebral hypoxia-ischemia. Therefore we chose to use $^{31}$P NMR to determine if ALLOP pretreatment could preserve energy metabolism during HI insult and early recovery.
Unsexed 7-day-old Wistar (Charles River) rats weighing between 12 and 18 g were subjected to permanent right common carotid artery ligation, via a midline neck incision, using 4-0 surgical silk [14, 15] and were returned to their dams for 2.5 h recovery. During the 5-min surgical procedure each pup was anesthetized with a mixture of halothane (4% induction, 1–1.5% maintenance), 30% oxygen, and balance nitrous oxide. Physiological saline or ALLOP (200 mg/kg s.c.) was injected immediately before placement in the NMR probe, 70 min prior to the onset of hypoxia. This dose was based on pharmacokinetic studies [16], in order to achieve a previously established neuroprotective serum level at the onset of hypoxia [15].

All 31P NMR studies were performed with a Bruker AM400 wide-bore spectrometer operating at 162.0 MHz. Each unanesthetized rat pup was positioned in a vertical orientation inside a specially constructed probe which included a gas-tight temperature regulated chamber [22]. Ambient temperature was maintained at 32–33°C.

Twenty-three high resolution spectra were acquired in 10- or 20-min time blocks beginning with a 30-min prehypoxic period (baseline). Eight percent O2 was supplied for the 3-h hypoxic period followed by 2.5 h of recovery in air. The animals were then sacrificed by rapid immersion in a liquid nitrogen bath. NMR setup, parameters, and metabolite data analysis have been described [22]. Unless otherwise indicated, the data (mean±S.D.) were compared by a pooled variance 2-sample t-test. The ATP levels were quantitated using the area of the β-phosphate resonance and pH was determined from the chemical shift of inorganic phosphate (P1) [22]. Death during data acquisition was apparent from the very large P1 area, brain pH below 6.8, along with a virtual absence of phosphocreatine (PCr) and β-ATP phosphate resonances.

For all surviving animals, intracellular pH (normal value of 7.16±0.06) declined to a minimum value at a variable time during the first 70 min of hypoxia. This minimum pH was 6.92±0.06 for saline-treated and 7.04±0.04 for ALLOP-treated pups (P<0.0005).

Fig. 1A shows the time course of brain ATP expressed as a fraction of its initial baseline values. The concentration of ATP in normal 7-day-old rat brain is 2.45±0.11 mmol/kg (wet brain wt.) [14]. Saline- (n=8, lower curve) and ALLOP-treated populations (n=13, upper curve) are shown. The curves are equivalent until about 30 min of 8% O2. During the remaining course of hypoxia, the saline group's ATP value decreases to 0.45±0.12 while the ALLOP-treated group declined to 0.64±0.12 (P=0.001). Recovery was not complete in either group after 2.5 h of reoxygenation.

Averaged curves for P1/PCr are shown in Fig. 1B. Since the pH changes were small, this parameter provides a good measure of phosphorylation potential (inversely proportional) [1]. For both populations there was a steady increase in P1/PCr until 2.25 h of hypoxia, followed by a small decline during the remaining 45 min of hypoxia. However, P1/PCr rose to a maximum value of 4.5±1.9 for the saline group but only 2.3±1.2 for the ALLOP-pretreated group (P=0.002). Following reoxygenation, a rapid recovery of phosphorylation potential to the baseline level is observed after 2.5 h for both populations.

Evaluation of the serial results (Fig. 1A,B) from individual points does not properly account for the animal variability with time following the insult. In order to reflect the severity and duration of energy failure we have integrated the NMR data for each animal over the last 2 h of hypoxia as well as initial recovery. These time inter-
vals correspond to expected maximum changes in metabolic levels since ATP drops sharply only after PCr is depleted. Integration of the data also provides an enhanced signal-to-noise ratio.

Fig. 2 summarizes the results of this integrated assessment of high energy phosphates. The ATP level (mean±S.D.) for ALLOP-treated pups (n=13) was 0.74±0.16 (integrated over the last 2 h of hypoxia and expressed as a fraction of baseline) compared to 0.52±0.05 (n=8) for the salines (P=0.001) (Fig. 2A). At the same time, mean P/PCr was 2.63±1.23 relative to baseline with ALLOP compared to 5.13±1.45 with saline (P<0.0005) (Fig. 2B). Both parameters of energy metabolism were also compared using modeled spline curve analysis [7, 22] and by non-parametric statistics. The spline analysis gave increased significance (P<0.0001) during the 2-h range of hypoxia. Non-parametric ranking of the integrated animal metabolite levels gave significance for both ATP and P/PCr (P<0.005, Wilcoxon-Mann-Whitney-test). In all cases, significance was similar during initial recovery.

Air control studies, in which only medical air (21% O₂) was supplied for 6 h, for ALLOP (n=5) and saline (n=3) pretreated pups, confirmed that the experimental conditions did not cause a change in high-energy phosphate levels (Fig. 2).

The HI insult has been shown to produce tissue injury in about 90%, with cystic infarction in over 50% of saline-treated pups [15, 20, 22]. We previously demonstrated an association between 'low' integrated ATP levels (below 0.7 of baseline) and 'high' integrated P/PCr (above 3.5 of baseline) with increased hemispheric water content at 42 h recovery from the HI insult [22]. Applying these threshold criteria to the survivors of the present study, all 8 saline-treated animals and 4/13 ALLOP animals suffered 'low' integrated ATP levels. Similarly, 7/8 saline pups and 3/13 from the ALLOP group displayed 'high' integrated P/PCr levels. As expected, the 3 ALLOP-treated animals with high P/PCr also had low ATP levels. Anderson et al. have shown using 31P NMR that phosphorylation potential (estimated by P/PCr) is a critical index of cellular energy state and a useful predictor of histological outcome [1]. They found a correlation between the duration and degree of change (integrated) in P/PCr with the extent of brain damage.

ALLOP improved energy metabolism significantly from as early as 1 h of hypoxia to initial recovery. Over the last 2 h of hypoxia, integrated ATP levels were 42% higher and integrated phosphorylation potential was 95% higher than without drug treatment. ALLOP could have achieved this effect by either (1) improved cerebral blood flow [2], (2) preservation of purine bases for ATP resynthesis, (3) facilitation of mitochondrial electron transfer [19] or (4) antioxidant activity. Reactive oxygen species can impair mitochondrial function by blocking glycolysis and ATP synthesis [5].

The mortality during this NMR study was 12/25 ALLOP- vs. 2/10 saline-treated (P=0.25, 2-sided Fisher's exact). Fig. 1B shows the P/PCr values obtained about 20 min prior to death for the ALLOP-treated pups that died during hypoxia. Only 2/12 pups that died had levels that appeared significantly higher than the surviving saline population at that time point. Five of the pups had highly preserved P/PCr levels shortly before death. Thus
P/PCr shortly before death was not statistically different from that of surviving saline-treated pups. The saline deaths occurred at 1.5 and 2.5 h into hypoxia, with P/PCr values (20 min prior to death) of 3.8 and above 7.0, respectively (not shown). We suggest that whatever contributed to mortality with ALLOP treatment did not impair cerebral energy metabolism any more than it did with the surviving saline pups. It is conceivable that death was caused by sudden cardiac or respiratory failure rather than from brain injury. Recording of data was incomplete for non-survivors, so a true comparison with survivors cannot be made. However, we observed that neither cerebral ATP nor pH levels were unusually perturbed shortly before death.

In conclusion, this study shows that pretreatment with ALLOP (200 mg/kg) preserves cerebral energy metabolites during a HI insult. This preservation was not accompanied by a reduction in mortality. However, in the ALLOP-treated pups that died, P/PCr shortly before death was not worse than with surviving saline-treated pups. Further attempts to unravel the neuroprotective mechanisms of high dose ALLOP pretreatment should focus on the causes for the preservation of energy metabolites.

We thank Rebecca L. Roberts for technical help with the animals and drugs and Thomas Binja Chang for assistance with the NMR analysis. Allopurinol (Zylorit sodium) was a generous gift from Burroughs Wellcome Co., Research Triangle Park, NC, USA.