Oxypurines in Cerebrospinal Fluid as Indices of Disturbed Brain Metabolism

A Clinical Study of Ischemic Brain Diseases

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SUMMARY Using a HPLC method the concentrations of oxypurines were simultaneously measured in CSF of patients with acute cerebrovascular lesions (CVL) and global cerebral ischemia (GCI) in an attempt to study disturbed brain metabolism during cerebral oxygen deprivation. In cerebral infarction both hypoxanthine and xanthine gradually increased from normal levels at admission to pathologically increased on the fourth day from onset of symptoms. There was no correlation between these substances and the clinical score but the maximum CSF-hypoxanthine concentration was significantly correlated to the maximum lesion volume determined by computerized tomography. In GCI the hypoxanthine-xanthine concentrations were considerably increased less than 20 hours from onset of unconsciousness but the initial levels did not predict the final outcome. These findings suggest that the end products of nucleotide degradation accumulate rapidly in acute cerebral hypoxia but more gradually in CVL probably due to growing local edema with subsequent local hypoxia. In controls and patients with CVL the CSF-urate concentrations were positively correlated to those of CSF-albumin. However, in CVL the increase of urate was relatively much more pronounced than the increase of albumin indicating that urate is a sensitive marker of dysfunction of blood-brain barrier.

NUMEROUS ANIMAL EXPERIMENTAL STUDIES have elucidated the changes in metabolism occurring during cerebral oxygen deprivation. As a consequence of decreased oxygen supply the high-energy phosphate utilization exceeds formation, causing a rapid fall of the intracellular adenine and guanine di- and trinucleotides in the brain. In parallel to these events an increase of corresponding monophosphonucleotides occurs in cerebral tissue. Further degradation may result in the end products of the purine metabolism, hypoxanthine and xanthine, while the absence or very low amounts of xanthine oxidase in the brain tissue of various mammalians is not consistent with the formation of urate. The appearance of urate in the cerebrospinal fluid (CSF) has therefore been attributed to a passive transport from the blood over the blood-brain barrier. In this study we have serially measured the oxypurines, i.e. hypoxanthine, xanthine and urate in CSF of patients with acute cerebrovascular lesions and global cerebral ischemia (GCI) in an attempt to elucidate disturbed brain metabolism in these conditions. For this purpose we have developed a HPLC (high pressure liquid chromatography) method which does not require extraction procedures of the CSF samples. The results obtained have been correlated to the magnitude of the lesions and the edema formation as estimated by repeated computerized tomography examinations. In patients with GCI we searched for a relationship between the CSF levels of the oxypurines during the initial period of cerebral deterioration and the final clinical outcome. The obtained data of hypoxanthine, xanthine and urate concentrations in CSF during normal and pathological conditions are discussed with respect to possible purine metabolic pathways in brain, elimination routes of xanthine and hypoxanthine and the possible role of urate as a sensitive marker of CSF-blood barrier function.

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Patients and Methods

The reference group was composed of 11 healthy volunteers and 15 patients admitted due to back pain of short duration. The ages and sexes of the controls are presented in table 1.

The diagnoses and ages of the patients with cerebrovascular diseases are presented in table 2. The clinical diagnosis acute cerebrovascular lesion was based on the rapidly developing signs of focal disturbance of cerebral function with no apparent cause other than vascular. Three cases filled the definition of having experienced a transient ischemic attack (TIA) and one patient filled the definition of reversible ischemic neurological disease (RIND). Six patients had completed strokes attributed to cerebral infarction. The definite diagnoses and ages of the patients with cerebrovascular diseases are presented in table 2. The clinical course and laboratory examination ruled out this possibility. They had a previous history of recurrent paroxysmal headache and the final diagnosis was migraine.

Clinical Methods

The patients with cerebral infarction and TIA-RIND were generally examined on four occasions by the same examiner. The first examination took place as soon as possible after admission and in most cases within 10 hours from the onset of symptoms; the second examination after two days, the third after four days and the fourth after 10–15 days. On each occasion a careful neurological examination was performed followed by CT and lumbar puncture. The neurological findings were transformed to a score ranging from 0 (death) to 100 (normal) according to a modification of the Matthew schedule.\(^5\) CT was performed before and after contrast injection (Isopaque Cerebral\(^6\)) using an EMI 1010 head scanner. The scale of attenuation ranged from -1000 to +1000 Hounsfield units (HU). The volume and the attenuation of the lesion were evaluated as previously described.\(^1\)

In the patients with GCI lumbar puncture was performed within 20 hours from the circulatory arrest. In most patients another lumbar puncture was performed 20–40 hours later and in two patients also after 5 and 10 days.

The cerebrospinal fluid samples were centrifuged and the supernatants stored at -25°C until analysed in sequence for albumin according to the principles outlined by Lizana and Hellsing\(^3\) and for hypoxanthine xanthine and urate by means of a reversed phase high performance liquid chromatography using a U-V absorbance detector (Spectromonitor III, LDC, Florida) operated at 260 mm. 20 \(\mu\)l of untreated CSF was applied on the column (250 \(\times\) 4 mm, Nucleosil, 5 \(\mu\) C8, Macherey-Nagel & Co., Düren, W. Germany), by means of a loop injector. 0.2 mol/l potassium phosphate buffer, pH 7.2 was used as the mobile phase and urate in the chromatogram were identified by comparing the retention times for the pure substances.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>The CSF-concentrations (Means ± SD) of Xanthine, Hypoxanthine and Urate in the Reference Group (Healthy Controls and Patients with Back Pain).</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;n&gt;</td>
<td>&lt;i&gt;</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>11</td>
</tr>
<tr>
<td>+ Back pain</td>
<td>15</td>
</tr>
<tr>
<td>= Reference group</td>
<td>26</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>The CSF-concentrations (Mean Values ± SEM) of Xanthine, Hypoxanthine, Urate and Albumin in Patients with Cerebrovascular Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;n&gt;</td>
<td>&lt;i&gt;</td>
</tr>
<tr>
<td>Cerebral infarction;</td>
<td></td>
</tr>
<tr>
<td>at admission</td>
<td>6</td>
</tr>
<tr>
<td>on day 4</td>
<td>6</td>
</tr>
<tr>
<td>TIA and RIND;</td>
<td></td>
</tr>
<tr>
<td>at admission</td>
<td>4</td>
</tr>
<tr>
<td>on day 4</td>
<td>3</td>
</tr>
<tr>
<td>Global cerebral ischemia;</td>
<td></td>
</tr>
<tr>
<td>at admission</td>
<td>10</td>
</tr>
<tr>
<td>on days 1–2</td>
<td>8</td>
</tr>
<tr>
<td>Migraine</td>
<td>3</td>
</tr>
</tbody>
</table>

Symbols: *p < 0.05, †p < 0.01, ‡p < 0.001, compared to the reference group (t-test).
(xanthine and hypoxanthine was obtained from Sigma Chemicals Co. St. Louis, U.S.A. and urate from E. Merck, Darmstadt, W. Germany) and by addition of the specific enzymes xanthine oxidase and uricase (Boehringwerke, Mannheim, W. Germany) to the CSF samples. Figure 1 illustrates the elution patterns of a known standard and of a CSF specimen. Quantitation of the oxypurines in CSF was performed from standard curves of xanthine, hypoxanthine and urate produced by peak height measurements of standard samples with known amounts. The standard curves were linear for all substances within the concentration ranges of the tested CSF specimens. The coefficient of variation of the measurements of all oxypurines was below 4%. Further details of the technique is presented elsewhere.14

These studies were carried out in conformity with protocols approved by the Human Research Committee of University Hospital, Uppsala.

Results

In table 1 the concentrations of xanthine, hypoxanthine and urate in CSF of healthy individuals and patients with back pain are presented. These two populations having no differences in the CSF concentrations of the measured substances, constitute our reference group. The xanthine, hypoxanthine and urate levels showed no sex-dependency. The urate concentrations were independent of age while the xanthine and hypoxanthine concentrations tended to be positively correlated to the age (r = 0.49 and r = 0.46, respectively p < 0.05). Based on the slopes of the regression lines (xanthine μmol/l = 0.015 age (years) + 1.186 and hypoxanthine μmol/l = 0.017 age (years) + 1.92) the xanthine and hypoxanthine levels should increase with approximately 0.16 μmol/l per decade. The xanthine levels were positively correlated to the hypoxanthine levels (the equation of the regression line was hypoxanthine μmol/l = 0.733 xanthine μmol/l + 1.305, r = 0.57, p < 0.01). No correlations were found between the xanthine or hypoxanthine and urate concentrations.

The reference group had a mean CSF-albumin concentration of 183 ± 68 (SD) mg/l. The albumin levels were related to the urate levels (r = 0.6, p < 0.001) but not to the xanthine and hypoxanthine concentrations.

The CSF Concentrations of Xanthine and Hypoxanthine in Cerebrovascular Disease

Six patients with cerebral infarction were serially followed with xanthine and hypoxanthine measurements in CSF (fig. 2). In the first CSF sample only two patients showed elevations of these substances. During the initial observation period the xanthine and hypoxanthine levels increased and peak levels were observed four days after onset of symptoms (fig. 2, table 2).

In the patients with TIA-RIND the xanthine and hypoxanthine levels were significantly raised already at admission and tended to further increase with time reaching maximum levels on the fourth day after admission (fig. 3, table 2). The majority of patients with GCI had considerably elevated levels of both xanthine and hypoxanthine in the CSF samples collected within 20 hours from circulatory arrest. In contrast to the findings in cerebral infarction or TIA-RIND the maximum hypoxanthine levels in GCI were observed in the first CSF sample. Xanthine remained at the same elevated levels during the initial observation period (fig. 4, table 2). One out of three patients with attacks of vascular headache had slightly elevated xanthine-hypoxanthine values.

In the group of patients with cerebrovascular diseases there was a significant positive correlation between the CSF levels of xanthine and hypoxanthine (r = 0.51, p < 0.001).

The CSF Concentrations of Urate in Cerebrovascular Diseases

Already at admission the urate levels were significantly increased in patients with cerebral infarction and GCI and remained elevated throughout the observation period (table 2). In patients with TIA-RIND no increase of the urate concentrations was found (table 2). In patients with cerebrovascular diseases CSF-

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Figure 1. Elution patterns on HPLC of the standard material of hypoxanthine, xanthine, creatinine and urate (left) and of a CSF-specimen. Column: 250 x 4 mm, Nucleosil, 5 μ C8. Mobile phase: 0.2 mol/l KH₂PO₄, pH 7.2. Flow rate: 1.0 ml x min⁻¹. Injected volume: 20 μl.
urate tended to correlate to CSF-albumin ($r = 0.3, p < 0.05$) and CSF-xanthine ($r = 0.33, p < 0.05$) but not to CSF-hypoxanthine.

**The Relation Between the Xanthine, Hypoxanthine and Urate Levels in CSF and the Clinical Picture or CT Findings at Stroke**

The patients with cerebrovascular lesions were scored with respect to symptoms. The lowest score values, i.e. the most pronounced clinical affection were noted at admission and day 2 and were on average $65 \pm 7$ (SEM). None of the patients died. At the end of the observation period the mean score was $75 \pm 8$ (SEM). There was no correlation between the clinical score values and the CSF-oxypurine levels. Only three of the patients with GCI — cases 5, 7, 9 — awoke from their coma. The lack of relationship between the clinical outcome and the CSF-levels of xanthine, hypoxanthine (fig. 4) or urate was evident.

In four patients with cerebral infarction the infarcted area was not visible at the first CT examination but hypo-attenuation areas developed later. All patients developed purely hypo-dense CT-changes, i.e. there was no sign of macroscopically hemorrhagic infarction. There was a rapid increase of the lesion volume reaching maximum size (the mean maximum lesion volume was $35 \pm 12$ (SEM) cm$^3$) on days 2–3. The maximum change of the central hypo-attenuation of the lesion occurred on days 2–4 and was on average $14.7 \pm 1.8$ HU. The individual maximum CSF-hypoxanthine level correlated to the individual maximum lesion volume ($r = 0.69, p < 0.05$).

**Discussion**

Most of the knowledge concerning the purine metabolism has been obtained from bacterial and animal experiments.\textsuperscript{15–17} Purine metabolism differs not only between species but also between organs and cellular systems. The current concept of purine metabolism of the brain is based mainly on animal studies since the reports on humans in various pathological situations are rare and moreover often based on assays with low sensitivity and uncertain accuracy. The development of specific and sensitive HPLC methods for determination of the different purine metabolites at physiological concentrations has provided new possibilities to study purine metabolism in man and the existing co-operation between different organs.\textsuperscript{18–20} In figure 5 purine
metabolism of the brain, as we conceive it today, has been summarized. The main difference from the general scheme is the lack of xanthine oxidase activity and the increased hypoxanthine-guanine phosphoribosyltransferase (HGPRT) activity compared to other tissues. This means that xanthine and to a certain extent hypoxanthine, rather than urate, should be the end products of purine metabolism in brain.

Another factor that may influence purine metabolite concentration in CSF is the function of the blood-brain barrier. However, with the use of the present technique the levels of hypoxanthine and xanthine in CSF from healthy individuals are about two and eight times, respectively, higher than those found in plasma.

During ischemia of the brain the intracellular nucleotide pool is degraded to nucleosides and purines as schematically presented in figure 5. The brain production of adenosine is very rapid during hypoxia and occurs within seconds. Only with prolonged ischemia do the end products of adenosine nucleotide degradation accumulate in the brain of experimental animals. As there is a great concentration difference between the intracellular nucleotides and the oxypurines accessible to analysis in CSF, even minor increases of nucleotide degradation should result in detectable increases of the CSF concentrations of oxypurines.

The idea that profound and prolonged cerebral ischemia may increase the CSF concentrations of hypoxanthine was confirmed by our observations in patients with global cerebral ischemia (GCI). Less than 20 hours from cardiac arrest and onset of unconsciousness most of the patients had considerable elevations of the hypoxanthine as well as the xanthine concentrations.
Based on the hypothesis that longstanding hypoxia is a prerequisite for hypoxanthine increase the initial hypoxanthine levels might have a prognostic value but in this study the hypoxanthine levels did not predict the outcome. During the next two days the hypoxanthine levels in the majority of patients with GCI decreased considerably while xanthine remained at the same elevated levels. This difference in the CSF-pattern of these substances may reflect differences in the production but also in the elimination rate. While both xanthine and hypoxanthine are probably transported through the blood-CSF barrier into the peripheral circulation, hypoxanthine but probably not xanthine — due to the low affinity of HGPRT for xanthine (fig. 5) — can be reutilized and incorporated into brain nucleotides when brain circulation returns.  

In cerebral infarction there was a gradual increase of both hypoxanthine and xanthine from normal levels at admission until pathologically raised levels on the fourth day. This pattern may indicate that elevated hypoxanthine and xanthine levels originate from a local ischemia developing as a consequence of the growing edema around the infarcted area having its maximum 3–4 days after the infarction. Degradation of DNA and other cellular substances containing purine components may occur in necrotic brain tissue and could also contribute to increased concentrations of oxypurines in CSF. In support of these ideas we observed a positive correlation between the maximum CSF-hypoxanthine level and the maximum brain lesion volume. In patients with TIA and RIND a similar hypoxanthine-xanthine pattern as in cerebral infarction was noted indicating that an established lesion is not a prerequisite for a disturbance of the production of energy-rich phosphate compounds. One patient with an acute attack of migraine also had raised levels of both substances. The observations in TIA-RIND and in vascular headache may illustrate a functional aspect of the oxidative metabolism in brain arteries.  

Hypoxanthine and xanthine measurements in suspected cerebral hypoxia seem to have clinical usefulness and are made easier by the present HPLC technique which enables quantitation of the oxypurines in a single chromatogram without the need of extraction procedures of the samples.  

Acknowledgment

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References

Rapid, Transient Drop in Brain Glucose After Intravenous Phloretin or 3-0-Methyl-D-Glucose

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SUMMARY Rats were injected intravenously with either phloretin (100 mg/kg) or 3-0-methyl glucose (2 g/kg) to reduce the carrier-mediated flux of glucose into brain. Plasma glucose and brain free glucose (BFG), lactate, and glycogen were measured over a 16 min time course. Injection of these substances caused a rapid drop in BFG to 60% of control at one minute and a minimum (50% of control values) at 4 min., followed by a gradual rise to control levels at 16 min. While plasma glucose fell, and then increased after injection, brain lactate and glycogen content was unaffected. Repeated injections of phloretin eventually caused a drop in brain glycogen; but with either competitor, BFG never fell below 50% of normal values. The i.v. injection of the glucose analog, 3-0-methyl glucose (the less toxic of the two drugs) is proposed as a possible means of cutting off the potentially hazardous supply of blood glucose to the postischemic brain.

Methods

Radioisotopes were purchased from New England Nuclear Corp., Boston, MA. The specific activities were [3H] H2O, 5 mCi/g, and D-[2-14C]-glucose, 57 uCi/μmol. The isotopes were greater than 98% pure as determined by radioscanning after thin layer chromatography, using solvent systems recommended by the manufacturer. Phloretin and 3-OMG were obtained from Sigma Chemical Co., St. Louis, MO. Soluene-100 tissue solubilizer and Instagel liquid scintillation fluid were purchased from Packard Instrument Co., Downers Grove, IL.

Intravenous Injection

Male Wistar rats (Mission Laboratory Supply, Inc., Rosemead, CA), 200-300 g, were given free access to Purina rat chow and water and maintained on a 12 hour light-dark cycle (lights on, 7 a.m.—7 p.m.). The rats were prepared surgically on the afternoon before the experiment. The animals were anesthetized with diethyl ether and a polyethylene catheter (PE-10) was inserted into a tail vein. The catheter was filled with 10% (v/v) heparin (1000 U/ml) in Ringers solution, heat sealed, and the tail covered with an aluminum sheath to prevent catheter dislocation. The next morning the animals were weighed and the sheaths removed immediately prior to injection.

THE LARGE FLUX OF GLUCOSE into brain is possible by virtue of the hexose carrier system in brain capillary endothelial cells, the blood-brain barrier (BBB).1,2 The purpose of this study was to determine the content of BFG after intravenous injection of substances known to interfere reversibly with transport of glucose into brain via the BBB hexose carrier. The inhibitory substances used were phloretin, which has approximately 150 times the affinity for the hexose carrier as does glucose,3 and 3-0-methyl-D-glucose (3-OMG) which has an affinity similar to that of glucose.4,5 These substances, in appropriate intravenous doses, cause an abrupt drop of BFG content to about 50% of normal values which persists for several minutes. The drop in BFG is dose-dependent; but even large doses of the inhibitors are ineffective in driving BFG below the 50% threshold.

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