Adrenomedullin gene expression in human placental tissue and leukocytes: a potential marker of severe tissue hypoxia in neonates with birth asphyxia

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Abstract

Objective: The aim of the present study was to investigate the role of adrenomedullin (ADM) as a hypoxia-inducible marker of clinically relevant tissue hypoxia in acute birth asphyxia of term newborn infants.

Methods: For this purpose, ADM mRNA was determined in human placental tissue of 20 term pregnancies complicated by birth asphyxia (pH and base deficit values, clinical score). In addition, ADM mRNA was measured in leukocytes of the asphyxiated newborn infants during the first 12 h of life (n = 12). Controls were available from ten healthy term pregnancies. In vitro, hypoxia-inducible expression of ADM mRNA was evaluated in human choriocarcinoma cells (BeWo) and human leukocytes exposed to hypoxia (1% O2) for 1–24 h. mRNA levels were measured by TaqMan real-time PCR.

Results: In vitro, ADM mRNA related to porphobilinogen deaminase (PBGD) mRNA levels significantly increased in response to hypoxia within a period of 4 h in leukocytes and 12 h in BeWo cells. In human placental tissue, significantly higher levels of ADM/PBGD mRNA were present in asphyxiated newborn infants with severe hypoxic-ischemic encephalopathy (HIE) (n = 5) compared with patients with mild or no HIE (n = 15). Increased levels of ADM/PBGD mRNA levels were found during the first hours of life in leukocytes of neonates with severe HIE compared with controls.

Conclusions: Our results indicate an upregulation of ADM gene expression in human placenta and leukocytes in clinically relevant hypoxic-ischemic birth complications and suggest ADM gene expression as a promising marker for severe complications due to perinatal asphyxia such as HIE.

Introduction

Birth asphyxia in term newborn infants is still a significant cause of neonatal morbidity and mortality. As a result of severe hypoxic-ischemic encephalopathy (HIE) survivors mostly suffer from motor and sensorimotor dysfunctions. Acute birth asphyxia arising from impaired materno-placental or placento-fetal blood flow, as well as chronic (partial) asphyxia, e.g. due to structural and functional placental abnormalities, compromise the fetal oxygen supply and energy production intrapartum and/or during the antenatal period (1). In relation to the degree of tissue hypoxia and endogenous metabolic and hemodynamic adaptive mechanisms, hemodynamic insufficiency, multi-organ failure and global cerebral hypoxic ischemia with impaired vascular autoregulation and neuronal necrosis develop over time. As an ongoing process, secondary neuronal cell death due to excess activation of excitotoxic and apoptotic mechanisms during the phase of reperfusion and reoxygenation extends the hypoxic-ischemic brain damage (2). The complex pathophysiological mechanisms of neonatal asphyxia make it difficult to define early marker systems of severe HIE and worse long-term prognosis during the intrapartum and early postpartum period (3). However, as progress in the development of neuroprotective therapeutic measures arises, the early identification of neonates at risk of severe HIE during the first hours of life is an important goal for appropriate decision making (2, 3).

Adrenomedullin (ADM), a 52 amino acid peptide, widely distributed in human tissues (4) is activated by hypoxia in several cell lines and tissues (5–9) and is assumed to belong to the classic oxygen-related genes up-regulated by the oxygen-sensing transcription factor, hypoxia-inducible factor-1 (6, 9). Apart from its well-known local and systemic vasodilative effects, ADM functions as an autocrine and paracrine growth factor, angiogenic factor and promotes apoptosis (6, 10, 11). ADM and its specific receptors have been
found to be involved in the regulation of the vascular tone and the humoral secretion mechanisms of uteroplacental and fetal tissues in a paracrine and/or autocrine manner (4, 12–14). With highest levels at term (13, 15), immunoreactive ADM has been discovered in maternal and umbilical cord plasma, in amniotic fluid, placental trophoblast cells and fetal membranes (13, 16, 17), whereas expression of ADM mRNA had mainly been detected in maternal decidual and fetal cytotrophoblast cells (15, 16).

More recently, expression of ADM in cultured astroglia cells and cerebral vascular endothelial cells and its vasodilator effect on cerebral circulation has been reported (9, 18). Evidence has arisen showing that ADM modifies the regulation of systemic and local blood flow of cardiovascular adaptation during physiological birth conditions (17, 19) as well as during conditions of systemic and/or local hypoxic-ischemic perinatal complications (20, 21).

The aim of the present study was to investigate the expression of ADM mRNA in placental tissue of term gestations complicated by birth asphyxia and in leukocytes of the asphyxiated neonates in order to determine whether the ADM system plays a role as an early indicator of clinically relevant neonatal HIE in vivo.

Materials and methods

In vitro investigations

Term trophoblast choriocarcinoma cells (BeWo) and human leukocytes were incubated at 37°C under either normoxic standard tissue culture conditions (5% CO₂ in 95% air) or hypoxic conditions (1% O₂, 5% CO₂, 94% N₂) for 1, 2, 4, 6, 12 and 24 h. Three experiments per hypoxia period were carried out.

In vivo protocol

Our prospective study included near-term and term neonates of gestational ages above 35 weeks who met the criteria of perinatal asphyxia. Newborn infants with congenital malformations, genetic disorders or inborn errors of metabolism were excluded. Birth asphyxia was defined as an umbilical artery (UA) pH ≤ 7.10, base deficit ≥ 10 mmol/l and/or 5-min APGAR scores ≤ 6. Neurological signs of encephalopathy were recorded according to the criteria of Sarnat & Sarnat (22) who classified three clinical stages of HIE as follows. HIE stage 1: hyperalertness, decreased spontaneous motor activity and activation of sympathetic functions lasting less than 24 h, normal electroencephalogram (EEG). HIE stage 2: generalized muscular hypertonia, strong distal flexion, multifocal seizures and/or pathological EEG. HIE stage 3: stuporous level of consciousness, flaccid muscle tone, suppression of brain stem and autonomic functions, severely pathological EEG with a periodic pattern and isopotential phases. Serial neurological examinations were performed at 24-h intervals for the first 3 days, thereafter at the age of 8–10 days and before hospital discharge (usually at 2–3 weeks of age). EEGs were recorded using the International 10–20 system with modified montages used in routine infant recordings. EEGs are routinely studied first during the first 24 h, thereafter at the age of 48–72 h and at 8–10 days. Serum levels of protein S-100, suggested to be a biochemical marker of glial cell damage (23), were determined 4 and 12 h after delivery (as described previously (23)).

A control group consisted of healthy term deliveries without prenatal or intrapartal complications, normal APGAR index and blood gas analysis (pH > 7.20). The study was approved by the local ethics committee of Friedrich-Alexander University and standardized informed consent was obtained from the parents. Three placental tissue specimens were taken immediately after delivery, rapidly frozen on ice after rinsing with normal saline solution and removal of amniotic membranes and then stored at −80°C until isolation of total RNA. RNA of leukocytes was extracted from mixed umbilical cord blood and venous blood samples (0.5 ml, anticoagulated with EDTA) taken 4 and 12 h after delivery. Placental tissue specimens were available from 20 patients with birth asphyxia (HIE 0–1, n = 15; HIE 2–3, n = 5) and 10 controls, blood samples from 12 asphyxiated neonates (HIE 0–1, n = 8; HIE 2–3, n = 4) and 10 controls.

TaqMan real-time PCR

Total RNA was extracted from placental tissue using RNeasy Micro Kit (Qiagen, Hilden, Germany). Total RNA from leukocytes was isolated using Qiagen RNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Oligo(dT)15 primer and RNeasy kit were used for the isolation of total RNA from placental tissue. RNA isolation from leukocytes was performed with Qiagen RNA blood kit (Qiagen, Hilden, Germany). One microgram of total RNA was used for each reverse transcription reaction (MMLV reverse transcriptase; Invitrogen, Germany). RT-PCR products were measured by quantitative TaqMan real-time PCR (24). This method makes use of the 5′ exonuclease activity of the DNA polymerase (AmpliTaq Gold, Applied Biosystems, Houston, TX). After the reverse transcription reaction, cDNA was amplified with TaqMan 5′ exonuclease assay probes specific for ADM. The emission of the reporter dye (i.e., 6-carboxyfluorescein; TAMRA) at the 5′-end is quenched by the second fluorescence dye (6-carboxy-tetramethylrhodamine; TAMRA) at the 3′-end. After extension phase or PCR, the polymerase cleaves the 5′-end of the fluorescent dye and thus releases the reporter dye. The increasing amount of reporter dye emission is detected by an automated sequence detector combined with special software (ABI Prism 7700 Sequence Detection System). The algorithm normalizes the reporter signal to
a passive reference. Next, the algorithm multiplies the s.d. of the background reporter signal in the first few cycles (in most PCR systems, cycles 3–15) by a default factor of 10, to determine a threshold. The cycle at which this baseline level is exceeded is defined as threshold cycle (CT). CT depends on the initial template copy number and on the efficiency of both the DNA amplification and the cleavage of the TaqMan probe. The CT values of the samples are interpolated to an external reference curve constructed by plotting the relative or absolute amounts of a serial dilution of a known template vs the corresponding CT values.

Commercial reagents (TaqMan PCR Reagent Kit; Perkin-Elmer Corporation) and conditions were applied according to the manufacturer’s protocol. A total of 300 nmol/l primers and 200 nmol/l TaqMan hybridization probe were analyzed in a 25 l cDNA (RT mixture) and oligonucleotides at a final concentration of 300 nmol/l primers and 200 nmol/l TaqMan hybridization probe were analyzed in a 25 l volume. ADM gene expression was related to the mRNA expression of two housekeeping genes, porphobilinogen deaminase (PBGD) and hypoxanthine-guanine phosphoribosyltransferase (HPRT). The following primers and TaqMan probes were used. PBGD: forward: 5’-TGGTTCATCATCA-3’; reverse: 5’-ACACTGAC-T CCTTCCAG-3’; TaqMan probe: 5’-(FAM)-CGCA- TATCGCTGAAAGGC-(TAMRA)-3’. HPRT: forward: 5’-CCGGCTCCGTATGTCG-3’; reverse: 5’-GGTCTAAACC- TGGTTCTCATCA-3’. ADM: forward: 5’-CCCTGAATGCAAATCGTTCCG-3’; reverse: 5’-GCCCACACTTACCTTTCCG-3’. TaqMan probe: 5’-(FAM)-CGGACATCCACGAG-(TAMRA)-3’.

The thermocycler parameters were 50°C for 2 min (for carry-over prevention with uracil-N-glycosylase), 95°C for 10 min (for hot start PCR), followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

**Statistical analysis**

Data are expressed as means ± S.E.M. Statistical significance was determined by one-way ANOVA and by two-way ANOVA (for repeated measurements). Significance was defined as *P < 0.05*.

**Results**

Clinical data of the asphyxiated newborn infants and controls are summarized in Table 1. Patients were divided into two groups according to the degree of HIE (HIE grade 0–1 vs HIE grade 2–3). There were no significant differences in initial routine laboratory data (UA pH value, base deficit, serum lactate) between the two groups. Serum levels of protein S-100 were significantly higher in neonates with HIE 2–3 compared with controls. In addition, severely asphyxiated newborn infants with clinical and neurophysiological signs of severe encephalopathy (HIE 2–3) developed clinical signs of multi-organ dysfunction during the first days of life, such as acute renal insufficiency (*n* = 2), respiratory distress syndrome (*n* = 3), disseminated coagulation disorder (*n* = 1) and/or adrenal bleeding (*n* = 1). Mean birth weight was slightly lower in patients with severe asphyxia (without statistical significance) including two newborn infants with intrauterine growth retardation.

As shown by our *in vitro* experiments (Fig. 1), hypoxia induced a time-dependent increase in ADM mRNA expression by both human cell types, BeWo and human leukocytes. Whereas ADM/PBGD mRNA levels increased significantly in BeWo cells within 12 h of hypoxia with further increases up to 24 h, a similar increase was observed in human leukocytes within 4 h. Similar results were obtained for normalization to HPRT.

*In vivo*, the placental gene expression of ADM mRNA was significantly higher in severely asphyxiated newborn infants (HIE 2–3) compared with healthy controls and even with patients with mild or no HIE (Fig. 2). To address whether ADM mRNA expression was even related to the further postnatal course of birth asphyxia, ADM mRNA was measured in leukocytes at birth as well as 4 and 12 h postnatally. As shown in

### Table 1 Patients’ clinical data (means ± S.E.M.)

<table>
<thead>
<tr>
<th></th>
<th>HIE 0/1 (n = 15; 8m/7f)</th>
<th>HIE 2/3 (n = 5; 4m/1f)</th>
<th>Controls (n = 10; 6m/4f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age (weeks)</td>
<td>38.4 ± 0.7</td>
<td>37.4 ± 0.8</td>
<td>39.6 ± 0.4</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3099 ± 256</td>
<td>2840 ± 170</td>
<td>3383 ± 123</td>
</tr>
<tr>
<td>Birth weight (SDS)</td>
<td>–0.19 ± 0.31</td>
<td>–1.06 ± 0.14</td>
<td>0.04 ± 0.23</td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>30.4 ± 0.8</td>
<td>31.5 ± 2.9</td>
<td>29.6 ± 4.9</td>
</tr>
<tr>
<td>CS/VD</td>
<td>6/9</td>
<td>4/1</td>
<td>3/7</td>
</tr>
<tr>
<td>UA pH</td>
<td>7.04 ± 0.02</td>
<td>7.01 ± 0.03</td>
<td>7.28 ± 0.02</td>
</tr>
<tr>
<td>UA base deficit</td>
<td>–13.1 ± 1.1</td>
<td>–15.3 ± 1.7</td>
<td>–1.6 ± 1.0</td>
</tr>
<tr>
<td>5-min APGAR (primary ventilation)</td>
<td>7.3 ± 0.3/2</td>
<td>—/5</td>
<td>9.1 ± 0.8/0</td>
</tr>
<tr>
<td>Protein S-100 (ng/ml)</td>
<td>4h postpartum 2.6 ± 0.3</td>
<td>4.0 ± 0.8 *</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>12h postpartum 2.5 ± 0.4</td>
<td>4.4 ± 1.1 *</td>
<td>1.6 ± 0.2</td>
</tr>
</tbody>
</table>

CS, Caesarean section; VD, vaginal delivery; m, male; f, female.

* *P < 0.05 vs controls (one-way ANOVA), venous blood samples were taken 4 and 12 h after delivery.*
Fig. 3. ADM/PBGD mRNA ratios increased significantly in leukocytes of patients with severe HIE with maximum values 4 h postnatally \((n = 4)\) and were significantly above those of healthy controls \((n = 10)\). Results were similar for normalization to HPRT.

**Discussion**

The present study on hypoxia-induced changes of the ADM system in placental tissue and human leukocytes showed a severalfold increase of ADM mRNA under *in vitro* hypoxia and under acute perinatal hypoxic-ischemic conditions *in vivo*.

Our *in vitro* data are in good agreement with previous reports on hypoxia-induced stimulation of ADM mRNA expression in a time-dependent manner that has been found in different cell lines (5, 8). To the best of our knowledge, our observation that human leukocytes respond to hypoxia by the upregulation of ADM mRNA *in vitro* has not yet been reported. The dynamic increase of ADM/PBGD mRNA concentrations during the first hours of life in severely asphyxiated neonates with maximum values after 4 h was similar to the time-dependent ADM/PBGD mRNA activation demonstrated by our *in vitro* data.

Physiologically, ADM is suggested to play a functional role from early pregnancy as has been shown by immunohistochemical and gene expression studies (17, 24). ADM is regarded as a placental growth factor (14) and might be involved in the regulation of trophoblast invasion during implantation and/or in embryonic development including growth and tissue differentiation (25). At term, placental ADM was shown to modulate utero-placental and fetal circulation in a paracrine manner or by interactions with other vasoactive substances such as nitric oxide and endothelin-1 (24, 26). In addition to systemic and local vasodilative effects, ADM is involved in the regulation of endocrine systems such as adrenocorticotropin, thyroid hormone, progesterone and insulin.
secretion (18). Tissue hypoxxygenation was demonstrated to be an important stimulus of ADM mRNA activation in several organs (liver, kidney, brain, lung) (8, 27, 28). Hobauer et al. (27) and others (8, 10) reported a concomitant increase in ADM protein synthesis due to tissue hypoxia. We found increased levels of placental ADM/PBGD mRNA in term pregnancies complicated by clinically significant birth asphyxia resulting in severe neonatal HIE, indicating that even acute and subacute severe tissue hypoxxygenation stimulates placental ADM gene expression similar to observations in pregnancies associated with chronic placental insufficiency (12, 15, 20). When considering the underlying mechanisms of birth asphyxia at term, the activation of the placental ADM system might indicate vasoactive adaptation mechanisms to impaired placentation and fetal blood flow and oxygen delivery in the clinically most affected newborn infants. In general, acute interruption of the fetal oxygen supply, e.g., by obstetric complications, as well as chronic (partial) asphyxia, e.g., arising from relevant placental abnormalities, compromise the fetal oxygen supply intrapartum (1). We assume from our data that in neonates who developed severe HIE the presence of acute/subacute prenatal distress could be one explanation for the increased ADM mRNA concentrations found in the clinically most affected cases. In this context, the finding of lower mean birth weight in the group with HIE grade 2—3 (including two patients with intrauterine growth retardation: not significant) might indicate prenatal partially impaired fetoplacental function. Well-established, initial routine biochemical markers such as single pH values or lactate levels do not significantly allow differentiation between an acute intrapartum distress of short duration and prolonged intrauterine hypoxia/ischememia. Whether ADM mRNA in placental tissue or in leukocytes of asphyxiated newborn infants may serve as a prognostic factor for severe HIE remains to be shown in a large prospective trial.

Our preliminary data show that ADM/PBGD mRNA levels in leukocytes were higher in newborn infants with severe HIE than in patients with mild/no HIE and controls. We cannot exclude the possibility that our observation of postnatally increasing ADM/PBGD mRNA ratios in leukocytes of the severely asphyxiated newborn infants with maximum levels after 4 h might derive from a systemic activation of the ADM system rather than from central nervous system-related ADM mRNA upregulation. However, from recently published in vitro data it is obvious that the hypoxia-inducible activation of ADM mRNA expression plays a functional role in cerebral hypoxia and ischemia (9, 20). While ADM mRNA expression was found to be activated in cells of the blood–brain barrier and in astrocytes, upregulation of the specific receptor mRNA (RDC-1) was present in endothelial cells of the cerebral microvessels, indicating cell-specific hypoxia-induced regulation mechanisms (9).

We cannot fully exclude additional factors other than hypoxia/ischemia known to induce ADM expression such as bacterial endotoxins and cytokines (29), but none of our patients showed clinical signs of prenatatal or neonatal infection/sepsis.

In summary, our preliminary results indicate placental activation of ADM mRNA expression in near-term and term deliveries complicated by severe birth asphyxia and confirm the hypothesis that the hypoxia-induced upregulation of the ADM system might modulate local adaptive hemodynamic mechanisms during acute tissue hypoxia in vivo. Clinically, our observations of ADM mRNA activation in placental tissue and leukocytes of the neonates with severe HIE could suggest ADM mRNA as a promising marker of clinically relevant hypoxic-ischemic conditions of the feto-placental unit during the perinatal period. Further long-term evaluation of newborn infants is necessary to prove the prognostic value of the ADM system in severely asphyxiated newborn infants.

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References


