Increased Uridine Adenosine Tetraphosphate Concentrations in Plasma of Juvenile Hypertensives


Background—Uridine adenosine tetraphosphate (Up4A) was been recently characterized as a potent vasoconstrictor. Up4A occurs in plasma from healthy subjects at concentrations sufficient to cause strong vasoconstrictive effects. In this study, Up4A concentrations in plasma from juvenile hypertensives and normotensives were determined.

Methods and Results—Up4A was purified to homogeneity by preparative reverse phase high performance liquid-chromatography (HPLC), affinity chromatography HPLC, and analytic reverse phase HPLC from deproteinized plasma of juvenile hypertensives and normotensives. Mean total plasma Up4A concentration was significantly increased in juvenile hypertensives compared with juvenile normotensives (33.0±25.4 versus 3.7±0.9 nmol/L; mean±SEM, n=40 and 38, respectively; P<0.005). Accordingly, Up4A showed a significant association with juvenile hypertension (OR for ln(Up4A): 1.82; 95% CI 1.12, 2.95). Plasma Up4A concentrations correlated with left ventricular mass (Kendall-τ correlation coefficient 0.220, n=40; P<0.05) and intima media wall thickness (Kendall-τ correlation coefficient 0.296, n=40; P<0.05) in the hypertensives. Because the increased intima media thickness may be related to proliferative effects of Up4A, we studied the effects of Up4A on human vascular smooth muscle cell proliferation. The maximum proliferative effect of Up4A was 80.0±24.0% % above control (P<0.01). The proliferative effect of Up4A on smooth muscle cells is cell cycle–dependent, involving stimulation of S phase entry.

Conclusion—Circulating levels of Up4A are strongly associated with juvenile hypertension. The endothelium-derived vasoconstrictor Up4A may contribute to the early development of primary hypertension and is moreover an important risk factor of juvenile hypertension. (Arterioscler Thromb Vasc Biol. 2007;27:1776-1781.)

Key Words: uridine adenosine tetraphosphate ■ hypertension ■ plasma concentration ■ proliferation

Materials and Methods

Chemicals

HPLC water (gradient grade) and acetonitrile were purchased from Merck (Germany); all other substances were from Sigma-Aldrich (Germany).

Study Design

Forty consecutive juvenile patients with primary hypertension were recruited when they presented to an established University Outpatient Department of Pediatric Endocrinology and Cardiology at the University of Rostock (Germany).

Hypertension in children and adolescents was defined as average systolic BP (SBP) or diastolic BP (DBP) ≥95th percentile for gender, age, and height on ≥3 occasions.

The control group consisted of juveniles (n=38) without appreciable cardiovascular risk factors and was selected from patients presenting at the same institution for diagnostic work-up of dizziness and minor orthostatic complaints. Patients with structural or functional cardiovascular abnormalities were excluded from the control group. All patients underwent an identical 2-day screening program.
based on a multiple risk factor approach. Pubertal development based on Tanner stages was assessed by physical examination. The study complied with the Declaration of Helsinki. The local Ethics Committee approved the study plan and written informed consent was obtained from the parents after a detailed interview.

The clinical and biochemical characteristics of the patients and control subjects are given in Table 1.

### Measurement Of Risk Factors

A venous blood sample was collected after overnight fasting. Resting blood pressure (BP) was measured at all extremities by an automatic oscillometric cuff device (Dinamap, Critikon Inc). The 24-hour ambulatory blood pressure (BP) was measured on the right arm (Space Labs Inc). BP data were automatically recorded every 15 minutes from 8:00 AM to 8:00 PM (daytime BP) and every 30 minutes from 8:00 PM to 8:00 AM (nighttime BP). BP studies were not considered meaningful and excluded from analysis if there was an interval of invalid or absent measurements exceeding 2 hours. Hypertension was defined as 24 hour systolic or diastolic BP above the 95th percentile of the reference values according to Soergel et al.3

Echocardiography and vascular measurements were taken with a Devereux-modified American Society of Echocardiography cube (80%(v/v) in water: eluent B) and the following gradient was used for the elution: 0 to 30 minutes: 0% to 40% eluent B; 30 to 33 minutes: 40% to 100% eluent B. The UV-absorption was detected at 254 nm. The columns, dimensions 2100 g, 4°C, 5 minutes).}

#### Vascular Measurement

All subjects were examined in a quiet temperature-controlled room. The procedure was carried out between 7:00AM and 8:00AM after a fasting period of 12 hours. For determination of the intima-media thickness (IMT), a high-frequency (15 MHz) vascular linear transducer was used for imaging the carotid arteries. Patients were examined in the supine position, with the head turned 45° away from the side being scanned. Two segments were identified on each side: the distal 1.0 cm of the common carotid artery, and the bifurcation itself. Five measurements were taken at 2-mm intervals for the near and far wall (distance from the transducer) in each of the 2 segments. Maximum and mean IMT were calculated separately for each side of each segment. Sonography and readings were performed by trained and board-certified sonographers. Intra- and interobserver variability (mean bias) were 0.2% and 1.2%, respectively.

### Extraction Of Human Plasma

Peripheral blood (10 mL) was obtained by catheterization of the cubital vein and was collected in tubes containing K$_2$-EDTA (7.2 mg). The blood samples were centrifuged at 2100g for 10 minutes to isolate plasma after a standardized interval of 10 minutes post-sampling. The resulting plasma was deproteinized with 0.6 mol/L (final concentration) perchloric acid and centrifuged (2100g, 4°C, 5 minutes).

After adjusting the pH to 9.0 with 5 mol/L KOH, the precipitated proteins and KClO$_4$ were removed by centrifugation (2100g, 4°C, 5 minutes).

#### Isolation And Identification Of Uridine-Adenosine Tetraphosphate From Human Plasma

After deproteinisation, P(1)P(2):P(2),P(3)-diadenosine triphosphate (8 μg) was added to the plasma as an internal standard. Thereby we excluded losses during purification as a source of error. Triethylammonium acetate (TEAA) in water was added to the deproteinized plasma to a final concentration of 40 mmol/L. This mixture was loaded onto a preparative reverse phase HPLC column (Chromolith Performance, RP-18 e, 100 to 4.6 mm, Merck; equilibration and sample buffer: 40 mmol/L TEAA in water; flow rate: 1 mL/min).

Up$_A$ was eluted with 30% acetonitrile in water and lyophilized.

Next, the eluate of the preparative reverse phase HPLC was dissolved in 1 mol/L ammonium acetate, pH 9.5 and concentrated on a phenyl-boronic acid resin, prepared according to Barnes et al.6

#### Quantification of Uridine Adenosine Tetraphosphate

Up$_A$ was identified on the basis of its retention time as compared with the synthetic Up$_A$. The lyophilized fractions from the reverse phase HPLC with TEAA as the ion-pair reagent were further separated by analytic reverse phase HPLC using tetrabutylammonium hydrogenosulfate (TBA) as the ion-pair reagent. The fractions, dissolved in 150 μL of 2 mmol/L TBA and 10 mmol/L KHPO$_4$ (pH 6.5), were injected into a reverse phase HPLC column (Chromolith Performance, RP-18 e; 100 to 4.6 mm; Merck). Acetonitrile (80%(v/v) in water: eluent B) and the following gradient was used for the elution: 0 to 30 minutes; 0% to 40% eluent B; 30 to 33 minutes; 40% to 100% eluent B; 33 to 36 minutes: 100% eluent B; flow: 1 mL/min. UV-absorption was detected at 254 nm. The concentrations of Up$_A$ were calculated using calibration curves created with synthetic Up$_A$. The natural logarithm of Up$_A$ concentrations was used for statistical analysis.

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**TABLE 1. Clinical and Biochemical Characteristics of Juvenile Hypertensives and Control Subjects (Values Are Mean±SEM)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hypertensive Patients (n=40)</th>
<th>Control Subjects (n=38)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>13.8±0.4</td>
<td>13.8±0.4</td>
<td>ns</td>
</tr>
<tr>
<td>Gender, m/f</td>
<td>27/13</td>
<td>18/20</td>
<td>ns</td>
</tr>
<tr>
<td>Blood pressure, mm Hg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>136±2</td>
<td>122±2</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Diastolic</td>
<td>81±1</td>
<td>76±1</td>
<td>ns</td>
</tr>
<tr>
<td>Body mass index, kg/m$^2$</td>
<td>28.3±1.1</td>
<td>22.8±0.9</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>White blood cells, 10$^3$/μL</td>
<td>6.7±2.3</td>
<td>6.6±1.8</td>
<td>ns</td>
</tr>
<tr>
<td>C-reactive protein, mg/L</td>
<td>4.0±8.9</td>
<td>2.3±3.0</td>
<td>ns</td>
</tr>
<tr>
<td>Urea, mmol/L</td>
<td>3.8±1.1</td>
<td>3.5±0.9</td>
<td>ns</td>
</tr>
<tr>
<td>Left ventricular mass, g/m$^2$</td>
<td>99.5±4.8</td>
<td>86.6±3.5</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Intima media wall thickness, mm</td>
<td>0.59±0.09</td>
<td>0.49±0.09</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>Up$_A$ plasma concentration, nmol/L</td>
<td>33.0±25.4</td>
<td>3.7±0.9</td>
<td>P&lt;0.005</td>
</tr>
</tbody>
</table>
Proliferation Of Human Vascular Smooth Muscle Cells

Cell Culture and Cell Counting

Human vascular smooth muscle cells (VSMCs) derived from aorta were purchased from American Type Culture Collection (USA) and cultured in Ham’s F12 (F12 Ham) medium (Invitrogen, Canada) containing 10% fetal bovine serum (FBS). After seeding in the medium with 10% FBS for 24 hours, cells were cultured in medium with 0.5% FBS for 24 hours followed by the presence and absence of 0.1 to 10 μmol/L UpA for 3 days. Cell number was counted using a hemocytometer.

Bromodeoxyuridine Triphosphate (BrdUTP) Incorporation Rate

The BrdUTP staining and cell cycle analysis were performed as described previously. Briefly, cells grown on coverslips were treated with UpA (10 μmol/L) in the presence and absence of suramin (50 μmol/L), PPADS (10 μmol/L), and IpI (100 μmol/L), respectively, as antagonists for 2 days. The antagonists were added 30 minutes before the application of UpA. Cells were labeled with 10 μmol/L BrdUTP for 60 minutes and fixed with ethanol. Cells were then permeabilized with 0.25% Triton-X, and DNA was denatured by 4 mol/L HCl, followed by immunostaining with anti-BrdUTP and Alexa Fluor 488-conjugated secondary antibody. nDNA was counterstained with propidium iodide (PI). Cells were scanned using Laser Scanning Cytometer (LSC) (CompuCyte, Cambridge, UK). BrdU incorporation rate is expressed as the percentage of BrdUTP-positive cells/total scanned cells.

Statistical Methods

All binary and categorical variables were calculated as frequencies. Metric variables were given as mean values with standard error mean (SEM). Because of its large skewness, the UpA variable was transformed using the natural logarithm. The association between hypertension and potential influential variables such as age, gender, ln(conc. UpA), etc was investigated using a logistic regression model. Continuous covariates were centered at the mean. Results are reported as odds ratios and 95% confidence interval. Model selection was based on a stepwise selection approach using the likelihood ratio test and Akaike’s information criterion (AIC). This criterion was computed as -2 (log-likelihood) + B (number of estimated parameters). P<0.05 (2-sided) was considered to indicate statistical significance. All statistical analyses were done using SPSS software (Microsoft SPSS for Windows, version 12.0). The Wilcoxon Mann–Whitney test was used for nonparametric statistical tests, and the Kendall-τ analysis was used for bivariate correlations.

Results

Figure 1 shows a characteristic reverse phase HPLC chromatogram of an extract from human plasma after precipitation of proteins and affinity chromatography. The peak labeled in Figure 1 represents the UV-absorption of UpA.

Figure 2 shows plasma UpA levels in juvenile hypertensives and their healthy control subjects. The mean (±SEM) UpA concentration of cubital veins of juvenile hypertensives was 33.0±25.4 nmol/L and was significantly elevated compared with the control group (3.7±0.9 nmol/L; P<0.005).

Next, we studied the association between juvenile hypertension and potential risk factors, based on a logistic regression model. Body mass index (BMI) and the natural logarithm of UpA were found to be important predictors of hypertension. The odds ratio for ln(UpA) was equal to 1.82 (95% CI 1.12; 2.95). The odds ratio for BMI was equal to 1.24 with 95% CI (1.10, 1.40; Table 2). A correlation of BMI and UpA concentration was not detected.

Plasma UpA concentration levels were scattered over a significantly wider range in hypertensives compared with normotensives. Plasma UpA was not uniformly elevated in all juvenile hypertensive patients. Therefore, correlations of plasma UpA levels with phenotypic data other than blood pressure were studied.

There was a significant difference in both left ventricular mass (99.5±4.8 versus 86.6±3.5 g/m²; P<0.05) and the intima media wall thickness in juvenile hypertensives versus their healthy control subjects (0.59 mm±0.09 versus 0.49 mm±0.09; P<0.005). Although the number of patients analyzed in this study was comparatively low, the left ventricular mass and intima media wall thickness was correlated with UpA plasma concentration in hypertensives (Kendall-τ correlation coefficient of left ventricular mass: 0.220, n=40 P<0.05; Kendall-τ correlation coefficient of intima media wall thickness: 0.296, n=40 P<0.05). Patients with plasma UpA levels exceeding the upper quartile were more likely to show an increased left ventricular mass and an increased intima media wall thickness. Furthermore, the left ventricular mass correlates with the systolic RR (Kendall-τ correlation coefficient of RRsys: 0.216, n=78 P<0.01) as well as with the diastolic RR (Kendall-τ correlation coefficient of RRDia: 0.187, n=78 P<0.05). The intima-media thickness correlates with the systolic RR (Kendall-τ correlation coefficient of RRsys: 0.247, n=78 P<0.01) as well as
with the diastolic RR (Kendall-τ correlation coefficient of RR\text{dia}: 0.208, n=78 \ P<0.05).

To investigate whether Up4A plasma concentration not only correlates with intima wall thickness but also stimulates the growth of human VSMCs, the effect of Up4A on the proliferation rate was tested in vitro. Up4A induced a strong dose-dependent stimulation of the proliferation of human VSMCs at physiologically relevant concentrations (Figure 3A). The maximum effect of Up4A was obtained at a concentration of 10 \ \mu\text{mol/L}, which induced an increase of human VSMC proliferation of 80.0±24.0% above the control (means±SEM from 4 independent experiments). The logEC\text{50} value (log mol/L) was in the range of −5.9±0.1.

To determine whether the growth stimulating effect of Up4A on proliferation rate of VSMCs is cell cycle–dependent and whether P2Y receptors are involved in the Up4A-mediated effect, we measured the BrdUTP incorporation rates in the presence and absence of P2 receptor antagonists. We found that the addition of Up4A significantly stimulated an increase in the BrdUTP incorporation rate, which was abolished in the presence of suramin and PPADS, respectively (Figure 3B). However, the presence of diinosine pentaphosphate (Ip5I), a potent and selective P2X\text{1} and P2X\text{3} antagonist, had no significant effect on the stimulatory effect of Up4A on BrdUTP incorporation rate (Figure 3B).

**Discussion**

The novel endothelium-derived circulating vasoconstrictor Up4A is elevated in the plasma of juvenile primary hypertensives. To test the hypothesis that Up4A plays a role in the development of primary hypertension we decided to study juvenile hypertensives for several reasons:

First, in adult hypertensives several secondary processes of hypertension may be superimposed on pathologies directly related to the pathogenesis of hypertension; eg, structural changes of the arterial wall and the subsequent development of isolated systolic hypertension may induce secondary hormonal adaptive responses. Hence, humoral changes may be dependent on the duration of hypertension.

Second and most importantly, endothelial dysfunction represents a critical step in vascular pathology associated with primary hypertension. However, it is unknown whether endothelial dysfunction is an initial step in the development of primary hypertension or whether it is the consequence of secondary functional and structural vascular changes.

Because the mean duration of hypertension was less than 1 year in our patients, it is unlikely that increased plasma Up4A levels were a result of secondary, adaptive structural, and functional vascular processes. Therefore, it can be concluded that increased Up4A secretion may be an early step in the development of hypertension. On the other hand, experimental studies have shown that mechanical stress stimulates endothelial cells to secrete Up4A. Therefore, increased shear stress attributable to hypertension could contribute to elevated plasma Up4A levels. Moreover, there may be a mutual causal relationship between hypertension and increased endothelial Up4A secretion.

The significant correlation of plasma Up4A levels with left ventricular mass and with the intima media wall thickness may be explained by the proliferative effect of this nucleotide. The proliferative effect of Up4A on smooth muscle cells is cell cycle–dependent, involving stimulation of S phase entry. Because suramin inhibiting P2Y and P2X purinoceptors, and PPADS preferentially inhibiting P2Y purinocep-

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>Standard Error</th>
<th>Odds Ratio</th>
<th>95.0% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>ln(Up4A)</td>
<td>0.597</td>
<td>0.248</td>
<td>1.818</td>
<td>1.118 - 2.954</td>
</tr>
<tr>
<td>BMI</td>
<td>0.216</td>
<td>0.063</td>
<td>1.241</td>
<td>1.096 - 1.404</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.329</td>
<td>0.304</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
involved are most likely either the P2Y2 or P2Y4 subtype. The receptor subtypes involved are most likely either the P2Y2 or P2Y4 subtype. The mitogenic effects via P2Y receptors are linked to stimulation of phospholipase C and Ca\(^{2+}\) release from inositol-phosphate-sensitive intracellular stores, and they are synergistic with those induced by conventional polypeptide growth factors.

The signal transduction includes nucleotide-stimulated inositol trisphosphate and 1,2-diacylglycerol formation and the subsequent Ca\(^{2+}\) mobilization, protein kinase C activation, and prostaglandin E\(_2\) synthesis. The stimulation of phospholipase D and the activation of the P42/P44 mitogen-activated protein kinase (MAPK) pathway and subsequent cell proliferation. Furthermore, ERKs can be activated via P2Y receptors and the upstream activator MEKs such as MEK1–3; the intracellular signaling cascades vary between receptor subtypes.

Plasma Up\(_4\)A levels in juvenile hypertensives are in a range for effective stimulation of purinergic receptors. Hence it is conceivable that by increased plasma Up\(_4\)A levels proliferation of both vascular smooth muscle cells and cardiomyocytes is stimulated.

In this context it is noteworthy that not only arterial hypertrophy has been found in primary hypertension, but also venous hypertrophy, which should not be mechanically but also neurohumorally mediated. Although there are numerous neurohumoral factors potentially stimulating VSMCs and cardiomyocyte growth, such as angiotensin II or catecholamines, in the group of juvenile hypertensives examined the correlation of plasma Up\(_4\)A levels with arterial wall thickness suggests that this circulating factor may significantly contribute to the morphological changes in these patients.

As there is a large overlap between plasma Up\(_4\)A levels in normotensives and hypertensives, there is currently no diagnostic threshold for plasma Up\(_4\)A measurements currently. However, plasma Up\(_4\)A levels may yield other information relevant for the assessment of hypertensive patients. Because Up\(_4\)A has been isolated from human endothelial cells, it may be assumed that elevated plasma Up\(_4\)A levels indicate changes in endothelial function. Thus, endothelial dysfunction may not only be characterized by diminished NO release, but also by an increased secretion of endothelial vasoconstrictors. The data suggest that, at least in a subset of juvenile hypertensives, altered endothelial function is an early event in the course of hypertensive vascular disease. Endothelial dysfunction defined as a decreased capacity to secrete NO has also been found to develop late in the course of hypertension, suggesting that endothelial dysfunction may also occur as a phenomenon secondary to hypertensive vascular damage. Recent insights by Stasch et al demonstrate that endothelial oxidative stress and related vascular disease states leads to a soluble guanylyl cyclase, which is indistinguishable from the oxidized/heme-free enzyme. This soluble guanylyl cyclase variant may be a novel cyclic GMP signaling entity that is unresponsive to NO and prone to degradation.

In summary, the findings revealed increased plasma Up\(_4\)A levels in juvenile hypertension. Increased left ventricular mass and increased wall thickness of muscular arteries may be phenotypic of juvenile hypertension associated with elevated plasma Up\(_4\)A levels. Additionally, endothelium-derived vasoconstrictors may contribute to the early development of primary hypertension.

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**Disclosures**

None.

**References**


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