Identification and Quantification of Diadenosine Polyphosphate Concentrations in Human Plasma

Joachim Jankowski, Vera Jankowski, Udo Laufer, Markus van der Giet, Lars Henning, Martin Tepel, Walter Zidek, Hartmut Schlüter

Objective—Diadenosine polyphosphates have been demonstrated to be involved in the control of vascular tone as well as the growth of vascular smooth muscle cells and hence, possibly, in atherogenesis. In this study we investigated the question of whether diadenosine polyphosphates are present in human plasma and whether a potential source can be identified that may release diadenosine polyphosphates into the circulation.

Methods and Results—Plasma diadenosine polyphosphates (Ap$_n$A with n=3 to 6) were purified to homogeneity by affinity-, anion exchange-, and reversed phase-chromatography from deproteinized human plasma. Analysis of the homogeneous fractions with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) revealed molecular masses ([M+H]$^+$) of 757, 837, 917, and 997 d. Comparison of the postsource decay matrix-assisted laser desorption/ionization mass spectrometry mass spectra of these fractions with those of authentic diadenosine polyphosphates revealed that these isolated substances were identical to Ap$_3$A, Ap$_4$A, Ap$_5$A, and Ap$_6$A. Enzymatic analysis showed an interconnection of the phosphate groups with the adenosines in the 5’-positions of the ribose moieties. The mean total plasma diadenosine polyphosphate concentrations (µmol/L; mean ± SEM) in cubital veins of normotensive subjects amounted to 0.89±0.59 for Ap$_3$A, 0.72±0.72 for Ap$_4$A, 0.33±0.24 for Ap$_5$A, and 0.18±0.18 for Ap$_6$A. Cubital venous plasma diadenosine polyphosphate concentrations from normotensives did not differ significantly from those in the hypertensive patients studied. There was no significant difference between arterial and venous diadenosine polyphosphate plasma concentrations in 5 hemodialysis patients, making a significant degradation by capillary endothelial cells unlikely. Free plasma diadenosine polyphosphate concentrations are considerably lower than total plasma concentrations because approximately 95% of the plasma diadenosine polyphosphates were bound to proteins. There were no significant differences in the diadenosine polyphosphate plasma concentrations depending on the method of blood sampling and anticoagulation, suggesting that platelet aggregation does not artificially contribute to plasma diadenosine polyphosphate levels in significant amounts. The Ap$_3$A (with n=3 to 6) total plasma concentrations in adrenal veins were significantly higher than the plasma concentrations in both infrarenal and suprarenal vena cava: adrenal veins: Ap$_3$A, 4.05±1.63; Ap$_4$A, 6.18±2.08; Ap$_5$A, 0.53±0.28; Ap$_6$A, 0.59±0.31; infrarenal vena cava: Ap$_3$A, 1.25±0.66; Ap$_4$A, 0.91±0.54; Ap$_5$A, 0.25±0.12; Ap$_6$A, 0.11±0.06; suprarenal vena cava: Ap$_3$A, 1.40±0.91; Ap$_4$A, 1.84±1.20; Ap$_5$A, 0.33±0.13; Ap$_6$A, 0.11±0.07 (µmol/L; mean ± SEM; each P<0.05 (concentration of adrenal veins versus infrarenal or suprarenal veins, respectively).

Conclusion—The presence of diadenosine polyphosphates in physiologically relevant concentrations in human plasma was demonstrated. Because in adrenal venous plasma significantly higher diadenosine polyphosphate concentrations were measured than in plasma from the infrarenal and suprarenal vena cava, it can be assumed that, beside platelets, the adrenal medulla may be a source of plasma diadenosine polyphosphates in humans. (Arterioscler Thromb Vasc Biol. 2003;23:1231-1238.)

Key Words: diadenosine polyphosphates ■ plasma ■ human adrenal medulla

In the circulation diadenosine polyphosphates have been shown to be important extracellular mediators affecting vascular tone, growth of vascular cells (for review, see references1,2), and platelet aggregation.3,4 Diadenosine tri- and tetraphosphate (Ap$_3$A, Ap$_4$A) were the first diadenosine polyphosphates to be identified in human platelets.5,6 followed by diadenosine penta- and hexaphosphate (Ap$_5$A, Ap$_6$A)$^7$ and diadenosine heptaphosphate (Ap$_7$A).8 In 1999, diadenosine diphasphate (Ap$_2$A) and Ap$_2$A were shown to be present in human myocardial tissue.9 Dinucleoside diphosphates, Ap$_2$A, Ap$_3$G, and Gp$_2$G are described as a new class of growth-promoting extracellular mediators,
which are released from granules after activation of platelets.10

Dinucleoside polyphosphates can be released into the circulation from activated platelets,5–8,11 from chromaffin cells of the adrenal glands,12–16 or from synaptic vesicles.17 After their release, local concentrations in the range of 10⁻³ mol/L or even higher can be assumed.18 Like ATP, the dinucleoside polyphosphates may be coreleased with catecholamines on sympathetic nerve stimulation and may thus significantly modify the cardiovascular actions of the sympatheticadrenergic system.12

Diadenosine polyphosphates can act as vasoconstrictors or vasodilators in rat mesenteric arteries19 and in the vasculature of the rat kidney.20 Ap₃A is the most potent vasocostrictor diadenosine polyphosphate, followed by Ap₄A and Ap₅A. The ionotropic P2X1 receptor is considered the principal mediator of vasoconstriction.21 The P2X1 receptors cluster on the adventitial surface of vascular smooth muscle cells suggesting that also heteromeric P2X receptors occur.23–25 Not only P2X receptors but also metabotropic P2 years receptors have been reported to mediate vasocostriction.26–29

Besides these purinergic receptors, a specific dinucleotide receptor was described in rat midbrain synaptosomes.30 The dinucleotide receptor is preferentially stimulated by diadenosine polyphosphates and is insensitive to ATP, UTP, adenosine, and their respective analogues.30

Besides the vasoactive actions, growth-stimulating effects of nucleoside polyphosphates have been shown in numerous blood vessels, involving the subsequent activation of protein kinase C, Raf-1, and mitogen-activated protein kinase.31,32 Activation of the P2Y2 receptor increases the expression of c-fos mRNA in cultured aortic smooth muscle cells.33 Dinucleoside polyphosphates also stimulate proliferation in vascular tissue where Ap₃A was shown to be equipotent as ATP.34 The proliferative effect of the Gp₃G and Gp₄G is significantly stronger than ATP in vascular tissues.11

In the present study, an assay for isolation, identification, and quantification of plasma diadenosine polyphosphates (ApₙA with n = 3 to 6) was established. Moreover, the present study was aimed to gain data on the secretion of diadenosine polyphosphates in humans. Therefore, plasma diadenosine polyphosphates were compared with those in vena cava to examine whether human adrenal glands release diadenosine polyphosphates.

Materials and Methods

Chemicals

High-performance liquid chromatography water (gradient grade) and acetonitrile were purchased from Merck (Germany). All other substances were from Sigma-Aldrich (Germany).

Isolation and Identification of Diadenosine Polyphosphates in Human Plasma

Peripheral blood (4 mL) was obtained by catheterization of the cubital vein and was collected in tubes containing K₂-EDTA (7.2 mg). Moreover, adrenal venous blood (4 mL) was obtained by catheterization of adrenal veins, which was performed to further evaluate primary hyperaldosteronism in 6 hypertensive patients with clinical and biochemical evidence of that disease and were collected in tubes containing K₂-EDTA (7.2 mg). Samples were also obtained from inferior vena cava and from cubital veins. All patients had an adrenal vein catheter because of primary hyperaldosteronism with unremarkable adrenal computed tomography scan. The clinical and biochemical characteristics of the patients are given in Table 1. In none of the patients the diagnosis of an adrenal adenoma was made, in all 6 patients bilateral hyperplasia was assumed. For comparison, 6 samples from normotensive age- and sex-matched patients (blood pressure 119±4/81±2 mm Hg) being treated for acute minor illness, such as back pain or dyspepsia, were collected. In 5 hemodialysis patients (Table 1) the arteriovenous concentration gradient was determined using mixed venous and arterial blood, which was taken from the arteriovenous Cimino fistula. The study was approved by the local ethical committee.

To test whether intravascular degradation of diadenosine polyphosphates by capillary endothelial cells affected diadenosine polyphosphate concentrations, the arteriovenous gradient of diadenosine polyphosphates was determined in 5 patients on regular hemodialysis treatment. Arterial blood was taken from the Cimino fistulas and venous blood from the cubital vein.

The blood samples were centrifuged at 2100g for 10 minutes at 4°C for isolation of plasma (step 1). All blood samples were centrifuged after a standardized interval of 15 minutes after sampling. The plasma was deproteinized with 0.6 mol/L (final concentration) perchloric acid and centrifuged (2100g for 5 minutes at 4°C; step 2). After adjusting pH to 7.0 with 5 mol/L KOH, the precipitated proteins and KClO₄ were removed by centrifugation (2100g for 5 minutes at 4°C).

To test whether the method of blood sampling and anticoagulation might artificially affect plasma diadenosine polyphosphate concentrations as a result of platelet aggregation, the above method of blood sampling was compared with best-practice conditions for limiting platelet activation.35–38 To this purpose, blood (4 mL) of 6 healthy control subjects was collected from the cubital vein using a 19-G winged infusion set and tubes containing citrate 0.11 mol/L at a pH of 8.1. Citrated blood was centrifuged at 120g for 15 minutes to obtain platelet-rich plasma, which was centrifuged at 300g for 20 minutes at room temperature. The supernatant was used for diadenosine polyphosphate determinations.

**TABLE 1. Clinical and Biochemical Characteristics of the Patients and Control Subjects**

<table>
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<tr>
<th>Type of Patients</th>
<th>Patients (N=6)</th>
<th>Hemodialysis Patients (N=5)</th>
<th>Control Subjects (N=6)</th>
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<td>3/2</td>
<td>3/3</td>
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<td>Blood pressure, mm Hg</td>
<td>163±7/98±4</td>
<td>145±6/79±4</td>
<td>119±4/81±2</td>
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<tr>
<td>Red blood cells, pl⁻¹</td>
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<td>3.3±0.5</td>
<td>5.2±0.3</td>
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<tr>
<td>White blood cells, nL</td>
<td>7.7±2.5</td>
<td>8.4±2.7</td>
<td>6.8±2.6</td>
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<tr>
<td>Platelets, nL</td>
<td>253±75</td>
<td>204±72</td>
<td>234±68</td>
</tr>
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</table>

Values are mean±SEM.

Determination of the Amount of Protein-Bound Diadenosine Polyphosphates in Human Plasma

To test the percentage of protein-bound diadenosine polyphosphates, plasma was isolated as described above and divided into 2 parts. One part was ultrafiltrated with a centrifuge filter (size exclusion limit: 10 kD; 3400g for 10 minutes at 25°C), and the other was left untreated. Then, in both portions the diadenosine polyphosphate concentrations were determined as described below.
Extraction of Human Plasma for Quantification of Diadenosine Polyphosphates

After deproteinization diadenosine octaphosphate (5 µg) was added to the sample as internal standard. Triethylammonium acetate (TEAA) in water was added to the deproteinized plasma to a final concentration of 40 mmol/L. The mixture was loaded to a preparative reversed-phase column (step 3, Lichroprep RP-18, 240×10 mm, Merck, Germany; equilibration and sample buffer: 40 mmol/L TEAA in water; flow rate: 2.5 mL/min). Diadenosine polyphosphates were eluted by 30% acetonitrile in water and lyophilized.

Next, the eluate was dissolved in 1 mol/L ammonium acetate at pH 9.5 and concentrated on a phenyl boronic acid resin, prepared according to Barnes et al.38 (step 4). The substances were eluted from the phenyl boronic acid resin by 1 mol/L/L HCl in water (flow rate: 0.2 mL/min). The eluate from the phenyl boronic acid resin at 1 mol/L TEAA to a final concentration of 40 mmol/L added was desalted by a reversed-phase high-performance liquid chromatography (step 5; Supersphere RP-18 endcapped, 250×4 mm, Merck, Germany; eluent A: 40 mmol/L TEAA in water; eluent B: acetonitrile; flow rate: 0.5 mL/min).

The desalted and lyophilized eluate of the phenyl boronic acid resin was chromatographed by anion-exchange chromatography (Uniq-Q-1, 7×35 mm, Bio-Rad, Hercules, Calif; eluent A: 20 mmol/L K2HPO4, pH 8; eluent B: 20 mmol/L K2HPO4, pH 8 with 1 mol/L NaCl; gradient: 0 to 2 minutes, 0% B; 2 to 100 minutes, 0 to 40% B; 100 to 105 minutes, 40 to 100% B, 105 to 116 minute, 100% B; flow rate: 0.5 mL/min). Fractions were collected according to UV absorbance at 254 nm (peak fractionation).

To the eluate of the anion-exchange chromatography, 1 mol/L TEAA was added (final concentration of 40 mmol/L) and were loaded to an analytic reversed-phase column (Supersphere 100 RP-18 endcapped, 250×4 mm, Merck). Nucleoside polyphosphates were eluted by 20% acetonitrile in water. The isolated diadenosine polyphosphates were identified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), postsource decay (PSD)-MALDI-MS, as well as enzymatic cleavage experiments. Moreover, to validate the identification of the diadenosine polyphosphates by their retention time to aliquots of the samples, authentic Ap8A, Ap7A, Ap6A, and Ap5A (each 1 µg) were added. For quantification of diadenosine polyphosphate, peak areas were determined by an integrator (Merck-Integration). The concentrations of diadenosine polyphosphates were calculated by using calibration curves obtained with authentic diadenosine polyphosphates.

Enzymatic Cleavage Experiments

Aliquots of the desalted fractions of anion-exchange chromatography were incubated with enzymes as follows. The samples were dissolved (1) in 20 µL 200 mmol/L Tris buffer (pH 8.9) and incubated with 5'-nucleotide hydrolase (3 mU; from Crotalus durissus, EC 3.1.15.1, from Boehringer Mannheim, Germany, purified according to Sulkowski and Laskowski39 for 9 minutes at 37 °C); (2) in 20 µL 200 mmol/L Tris and 20 mmol/L EDTA buffer (pH 7.4) and incubated with 3'-nucleotide hydrolase (1 µU; from calf spleen, EC 3.1.16.1, from Boehringer Mannheim, Germany) 1 hour at 37°C; and (3) in 20 µL 10 mmol/L Tris, 1 mmol/L ZnCl2, and 1 mmol/L MgCl2 buffer (pH 8) and incubated with alkaline phosphatase (1 µU; EC 3.1.3.1, from calf intestinal mucosa, from Boehringer Mannheim, Germany) for 1 hour at 37°C. The reaction was terminated by ultrafiltration with a centrifuge filter (exclusion limit 10 kd).

After filtration of the enzymatic cleavage products, the filtrate, dissolved in 80 µL of eluent A, was subjected to reversed-phase chromatography (Chromolith SpeedROD, 4.6×50 mm, Merck, Germany; eluent A: 2 mmol/L tetrabutylammonium hydrogensulfate in 10 mmol/L K2HPO4, pH 6.8; eluent B: 80% acetonitrile in water; gradient: 0 to 30 minutes; 0 to 40% B; 30 to 33 minutes; 40 to 100% B; 33 to 36 minutes, 100% B; flow: 1 mL/min).

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS)

Aliquots of the desalted fractions of anion-exchange chromatography were examined by MALDI-MS and PSD-MALDI-MS. A reflector type time-of-flight mass spectrometer (Reflex III, Bruker, Germany) was used according to Hillenkamp and Karas.40 The sample was mounted on an x, y, and z moveable stage allowing irradiation of selected sample areas. In this study, a nitrogen laser (VSL-337 ND, Laser Science) with an emission wavelength of 337 nm and 3 ns pulse duration was used. The laser beam was focused to a diameter of 50 µm at an angle of 45° to the surface of a target. Microscopic sample observation was possible. Single spectra (10 to 20) were accumulated for a better signal-to-noise ratio. In MALDI-MS, large fractions of the desorbed analyte ions undergo PSD during flight in the field free drift path. Using a reflectron type time-of-flight set-up, sequence information from PSD fragment ions of precursors produced by MALDI was obtained.41 Sample preparations for MALDI-MS and PSD-MALDI-MS experiments were identical. The concentrations of the substances analyzed were 1 to 10 µmol/L in bidistilled water. Analyte solution (1 µL) was mixed with 1 µL of matrix solution (50 mg/mL 3-hydroxy-picolinic acid in water). For this mixture cation exchange beads (AG 50 W-X12, 200 to 400 mesh, Bio-Rad) equilibrated with NH4+ as counter ion were added to remove Na+ and K+ ions. The mixture was gently dried on an inert metal surface before introduction into the mass spectrometer. The mass accuracy was in the range of approximately 0.01%.

Synthesis and Chromatography of Diadenosine Octaphosphate as Internal Standard

Diadenosine octaphosphate was synthesized and chromatographed as described elsewhere.42 Briefly, Ap4A was synthesized by mixing adenosine tetraphosphate (50 mmol/L) with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (2.5 mol/L), HEPES (2 mol/L), and magnesium chloride (125 mmol/L). The substances were dissolved in water, thoroughly mixed with a vortex mixer, and incubated at 37°C at pH 6.5 for 48 hours. The reaction mixture was concentrated on a preparative C18 reversed-phase column (LiChroprep, 310×65 mm, 65 to 40 µm, Merck, Germany) using 40 mmol/L aqueous TEAA in water (eluant A; flow rate: 2 mL/min). After removing substances not binding to the gel with aqueous 40 mmol/L TEAA (flow rate: 2 mL/min), nucleosides containing fraction were eluted with 30% acetonitrile in water (eluant B; flow rate: 2 mL/min). By this procedure, the recovery of Ap4A was about 10% of the diadenosine octaphosphate used.

The concentrate was displaced on 2 reversed-phase columns (columns: Supersphere RP-18 endcapped, 300×8 mm, Merck, Germany; carrier: 40 mmol/L TEAA in water; displacer: 160 mmol/L n-butanol; flow 100 µL/min). As a result of displacement chromatography anion-exchange chromatography yielded baseline separated diadenosine polyphosphates. The identity of the diadenosine polyphosphates was ascertained using with MALDI-MS as described above.

Validity of the Assay

Stock solutions of diadenosine polyphosphates (100 µmol/L) were prepared in water. Stock solutions were stored at −30°C. Solutions with various diadenosine polyphosphate concentrations were obtained by dilution of the stock solutions with water. For validation of the assay, standard diadenosine polyphosphate solutions of different concentrations were added to plasma directly before thawing. The precision of the assay for diadenosine polyphosphates was determined using a plasma sample and authentic diadenosine polyphosphate solutions. The intra- (n=4) and interassay (n=4) variabilities were assessed and expressed as coefficients of variation (C.V.).

Statistics

Results are presented as means±SEM. Two-sided probability values less than 0.05 were considered significant. All values reported are means±SEM.

Results

Figure 1 shows the characteristic anion-exchange chromatogram of an extract from human plasma after precipitation of...
proteins and affinity chromatography. The peaks labeled in Figure 1 represents the diadenosine polyphosphates Ap₃A, Ap₄A, Ap₅A, and Ap₆A.

The identity of the diadenosine polyphosphates was confirmed by MALDI-MS as well as PSD-MALDI-MS (Table 2). A characteristic PSD-MALDI-MS of Ap₅A is given in Figure 2.

To exclude that the diadenosine polyphosphates found are isomers with other than 5’-5’ bonds between the ribose and the phosphate moieties, the isolated substances were incubated with 3’- and 5’-nucleotide hydrolase as well as with alkaline phosphatase. When the fractions containing the diadenosine polyphosphate (Figure 3A) were treated with 5’-nucleotide hydrolase (Crotalus durissus), the UV peak of the intact diadenosine polyphosphate decreased and UV peaks of the hydrolysis products AMP, ADP, ATP, and adenosine tetraphosphate (Ap₄) and adenosine pentaphosphate (Ap₅), respectively, appeared (Figure 3B). Ap₅ was identified by its mass spectrum and by its further sequential degradation to Ap₄, ATP, ADP, and AMP after incubation with alkaline phosphatase (data not shown). Incubation of the fractions containing diadenosine polyphosphates with 3’-nucleotide hydrolase (Figure 3C) or alkaline phosphatase (Figure 3D) yielded no cleavage products. Figure 3 shows representative chromatograms of the enzymatic cleavage experiments with diadenosine pentaphosphate before (Figure 3A) and after incubation with 5’-nucleotidase (Figure 3B), 3’-nucleotidase hydrolyase (Figure 3C), and alkaline phosphatase (Figure 3D).

**TABLE 2.** Molecular Masses of Characteristic Fragments From the PSD-MALDI-MS Spectra of the Desalted Fractions of the Anion-Exchange Chromatography (Figure 1) Labeled Ap₃A, Ap₄A, Ap₅A, and Ap₆A

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M indicates protonated parent ion; A’, adenine; A, adenosine; p, phosphate group, eg, Ap₃ is ATP.

**Figure 1.** Typical anion-exchange chromatogram of a plasma extract of adrenal veins (UnoQ-1 7 × 35 mm, Bio-Rad, Hercules, Calif; eluent A: 20 mmol/L K₂HPO₄, pH 8; eluent B: 20 mmol/L K₂HPO₄, pH 8 with 1 mol/L NaCl; gradient: 0 to 2 minutes, 0% B; 2 to 100 minutes, 0 to 40%; B: 100 to 105 minutes, 40 to 100%; B: 105 to 116 minutes, 100%; B; flow rate: 0.5 mL/min).

**Figure 2.** Positive-ion PSD-MALDI-MS of the fraction labeled as Ap₃A in Figure 1. A’, adenine; A, adenosine; M, protonated parent ion; p, phosphate group, eg, Ap₃A is ATP.

**Figure 3.** Chromatograms of the desalted fraction of the anion-exchange chromatography labeled as Ap₃A in Figure 1 after anion-exchange and reversed-phase chromatography before (Figure 3A) and after (Figure 3B) incubation with 5’-nucleotidase, with 3’-nucleotidase (Figure 3C) and with alkaline phosphatase (Figure 3D). Column: Chromolith SpeedROD, 4.6 × 50 mm (Merck, Germany); eluent A: 2 mmol/L tetrabutylammonium hydrogensulfate in 10 mmol/L K₂HPO₄; eluent B: 80% acetonitrile in water; gradient: 0 to 30 minutes, 0 to 40% B; 30 to 33 minutes, 40 to 100% B; 33 to 36 minutes, 100% B; flow rate: 0.5 mL/min.)
The results of the enzymatic cleavage experiments show that the polyphosphate chain interconnects the 2 adenosines via phosphoester bonds to the 5’-positions of the riboses.

The mean diadenosine polyphosphate concentrations (μmol/L; mean±SEM) of cubital veins of normotensive patients amounted to 0.89±0.59 for Ap₄A, 0.72±0.72 for Ap₃A, 0.33±0.24 for Ap₂A, and 0.18±0.18 for Ap₁A. There was no significant difference between cubital venous diadenosine polyphosphate concentrations in the patients with primary hyperaldosteronism and in normotensive subjects (Table 3). Furthermore, Table 3 shows that there were no significant differences between central and peripheral venous diadenosine polyphosphate plasma concentrations. To test whether the capillary endothelium significantly contributes to intravascular degradation of diadenosine polyphosphates, arterial and venous plasma diadenosine polyphosphate concentrations were compared in 5 hemodialysis patients. The measurements revealed no significant arteriovenous difference in any of the diadenosine polyphosphate concentrations (Table 3).

In a further series of experiments, in 6 healthy probands the influence of blood sampling on plasma diadenosine polyphosphate concentrations was examined, comparing the above sampling method with best practice conditions. There were no significant differences in the diadenosine polyphosphate plasma concentrations with either sampling method (0.51±0.09 versus 0.59±0.26 for Ap₄A, 0.38±0.23 versus 0.32±0.12 for Ap₃A, 0.35±0.27 versus 0.42±0.09 for Ap₂A, and 0.29±0.23 versus 0.25±0.09 for Ap₁A (μmol/L); P>0.05; n=6, each EDTA versus citrate).

The concentrations of Ap₁A, Ap₂A, Ap₃A, and Ap₄A in the plasma of infrarenal vena cava, adrenal veins, and the suprarenal vena cava are given in Table 3. All patients had an adrenal vein catheter because of primary hyperaldosteronism with unremarkable adrenal computed tomography scan. The clinical and biochemical characteristics of the patients are given in Table 1. Diagnosis of an adrenal adenoma was made in none of the patients, and in all 6 patients bilateral hyperplasia was assumed.

Filtration experiments of human plasma using a 10-kd cut-off filtration membrane revealed that 5.7±4.5% of the diadenosine polyphosphates isolated from human plasma were not protein bound, with no significant differences depending on the number of phosphate groups.

The plasma concentrations of Ap₁A, Ap₂A, Ap₃A, and Ap₄A in infrarenal vena cava, adrenal veins, and suprarenal vena cava are given in Table 3. The concentrations of left and right adrenal veins were determined separately. In the figure, mean values of left and right adrenal veins are presented.

To test whether the capillary endothelium significantly contributes to intravascular degradation of diadenosine polyphosphates, arterial and venous plasma diadenosine polyphosphate concentrations were compared in 5 hemodialysis patients. The measurements revealed no significant arteriovenous difference in any of the diadenosine polyphosphate concentrations (Table 3).

The absolute recovery of diadenosine-5’-5’-octaphosphate, 5 μg of which were added as internal standard, was found to be 43.3±18.6%. The calibration graphs showed good linearity for concentrations of the diadenosine polyphosphates (Ap₁A, Ap₂A, Ap₃A, Ap₄A) ranging from 0.05 to 15 μmol/L (r=0.999). The peak area ratios of the diadenosine polyphosphates were linear in the concentration ranges investigated. Based on a signal-to-noise ratio of 3, the detection limit for the diadenosine polyphosphates was 16 nmol/L. The precision of the assay for diadenosine polyphosphates was determined using a plasma sample and authentic diadenosine polyphosphates.

To evaluate the quantification of diadenosine polyphosphates in human plasma, the intra- (n=4) and interassay (n=4) variabilities were assessed. The intra-assay variability for a human plasma sample and standard solutions, assessed on 4 consecutive working days, was 8.2%. The interassay coefficient of variation for a plasma sample and a standard solution, assessed on 4 consecutive working days, was 11.1%.

**Discussion**

Quantification of diadenosine polyphosphates from human plasma requires several sample preparation steps. First, the large amounts of proteins and peptides have to be removed. These substances were denatured by perchloric acid and removed by centrifugation. Second, the large number of small hydrophobic and hydrophilic substances has to be separated. Third, carbohydrates have to be removed from the sample before affinity chromatography because of the ability of
carbohydrates to bind to the affinity gel. These aims were achieved by reversed-phase chromatography.

Next, a highly selective concentration step with a boronate derivative of a cation exchange gel was used, which selectively retains nucleoside polyphosphates containing 2 or more sets of 1,2 cis-diol groups. Whereas mononucleoside polyphosphates like ATP with less than 2 cis-diol groups do not bind in ammonium acetate at buffer concentrations of 1 mol/L to the boronate gel because of charge repulsions between the negative phosphate groups and the carboxyl groups of the cation-exchange gel, the boryl ester formation of the 2 cis-diol groups of diadenosine polyphosphates is sufficient to overcome charge repulsion.37 The eluate from the boronate gel contained salts that prevented diadenosine polyphosphates from binding to the anion exchanger. Therefore, the eluate was desalted by concentration on a reversed-phase gel. For quantification of the diadenosine polyphosphates the plasma extract from the reversed-phase chromatography was subjected to anion-exchange chromatography.

The diadenosine polyphosphate concentrations in human plasma were found to be in the μmol range. From the concentration–response curves published, it appears that circulating diadenosine polyphosphates affect vascular tone. Moreover, in concentrations $>10^{-6}$ mol/L diadenosine polyphosphates have a growth-stimulating effect on vascular smooth muscle cells.10,42,43 Given the EC$_{50}$ value for Ap$_4$A in the 10$^{-7}$ mol/L range, the plasma levels reported here seem surprisingly high. Because it is highly unlikely that the plasma diadenosine phosphate concentrations exceed the EC$_{50}$ by 2 orders of magnitude, the question arises whether or not a part of the circulating diadenosine polyphosphates exists in a bound form. Indeed, the results show that a considerable portion of plasma diadenosine polyphosphates is protein bound, as they are retained by a 10-kd filter. Therefore, it may be assumed that only a small portion of total plasma diadenosine polyphosphates directly affects vascular tone.

The findings show that diadenosine polyphosphate concentrations vary within the venous system and depend on the number of phosphate groups contained in the molecule. The adrenal venous diadenosine polyphosphate concentrations higher than those in other veins leave 2 alternative explanations: either diadenosine polyphosphates are produced by the adrenal medulla or adrenal vascular endothelium is less effective in degrading diadenosine polyphosphates by its ectonucleases than other endothelial cells. Between both hypotheses, a decision can be made on the basis of arterial diadenosine polyphosphate concentrations. The measurements revealed that there is no significant gradient between nonadrenal venous and arterial diadenosine polyphosphate concentrations. This finding implies that the ectonucleases located on vascular endothelial cells do not degrade sufficient amounts of diadenosine polyphosphates to lower venous diadenosine polyphosphate concentrations significantly. Therefore, the increased adrenal venous plasma diadenosine polyphosphate concentrations compared with nonadrenal venous plasma cannot be due to a decreased endothelial degradation restricted to adrenal vascular endothelium.

The concentrations of the various diadenosine polyphosphates differ from each other. Obviously, those diadenosine polyphosphates with a higher number of phosphate groups show lower concentrations than those with a lower number of phosphates. This pattern may either be the result of an increased degradation of diadenosine polyphosphates by circulating and endothelial enzymes or by a decreased rate of synthesis with increasing number of phosphate groups. Although this question cannot be solved on the basis of the present data, findings reported in the literature may give an answer: concerning platelets, several studies revealed that the diadenosine polyphosphate contents decreased with an increasing number of phosphate groups.42,44 Because the intraplatelet diadenosine polyphosphates are not accessible by the extracellular degrading enzymes, it appears more likely that the synthetic pathway is less effective with increasing number of phosphate moieties to be incorporated.

In earlier studies, platelet diadenosine polyphosphates had been quantified referring the platelets content to the pertinent whole blood volume. In the platelets contained in 1 L of whole blood, the following amounts of diadenosine polyphosphates were found44: Ap$_3$A, 192.5±14.7 nmol; Ap$_4$A, 223.8±16.8 nmol; Ap$_5$A, 100.2±7.9 nmol; Ap$_6$A, 32.0±1.9 nmol (mean ± SEM). Conceivably, even if 100% of the platelet diadenosine polyphosphates are assumed to be released and hence to be distributed within the pertinent volume, the resulting plasma concentrations would be far less than those reported here. Therefore, a significant artificial contribution to plasma diadenosine polyphosphates by platelet aggregation appears to be unlikely. This conclusion is further supported by the fact that the method of blood sampling and anticoagulation does not significantly affect plasma diadenosine polyphosphate concentrations when best-practice conditions are compared with those initially applied.

Therefore, human plasma diadenosine polyphosphates cannot solely stem from platelets. Thus, we tested the hypothesis that diadenosine polyphosphates in human plasma are, at least partially, derived from the adrenal glands. Adrenal venous plasma diadenosine polyphosphate concentrations are significantly higher than those in the vena cava, both infrarenally and suprarenally (Table 3). This result suggests that human adrenal glands release Ap$_3$A, Ap$_4$A, Ap$_5$A, and Ap$_6$A.

These results are in accordance with results from animal experiments, which showed that the adrenal medulla contains diadenosine polyphosphates.16 They are released from perfused bovine adrenal glands and also from isolated chromaffin cells activated with carbachol. The ratio of the released diadenosine polyphosphates to released ATP and catecholamines is in the same order as that found in isolated chromaffin granules.14 With regard to these results, it can be inferred that diadenosine polyphosphates are also released by the chromaffin granules of human adrenal glands.

To what extent may these findings be relevant for human physiology and pathophysiology? If the adrenal medulla secretes not only adrenaline and noradrenaline into the circulation but also diadenosine polyphosphates, this seems to be of minor clinical significance because substitution of adrenal steroids is generally sufficient to restore well-being and normal hemodynamics after bilateral adrenalectomy.
Severe adrenal insufficiency, eg, Addison’s disease, which is caused by several diseases, may be another example that the systemic actions of diadenosine polyphosphate are limited. The clinical picture after destruction of the medulla and cortex, which occurs after adrenal tuberculosis, does not show significant differences to the clinical picture after destruction of the cortex alone, which occurs after autoimmune adrenalitis. However, the role of adrenal medulla in vascular and metabolic regulation has not been ultimately defined, and a potential role of either catecholamines or other secretory products, such as diadenosine polyphosphates, has not yet been examined in detail.

Furthermore, over the last decades it has been repeatedly documented that plasma catecholamines are elevated in essential hypertension.45 Therefore, the present report showing increased platelet diadenosine polyphosphate contents in essential hypertension.45 Indeed, there is a report showing increased platelet diadenosine polyphosphate secretion by the adrenal medulla may be a potential mechanism underlying increased platelet diadenosine polyphosphates in essential hypertension.45

Plasma diadenosine polyphosphate levels in essential hypertension have not been determined in this study because the patients studied had all exhibited a primary hyperaldosteronism. In these patients, peripheral venous plasma diadenosine polyphosphate concentrations were similar to those seen in normotensive controls.

In summary, plasma concentrations of diadenosine polyphosphates in the range of 10^-6 mol/L are compatible with systemic effects of these agents. By this finding, our view on diadenosine polyphosphates as hormones may be modified and extended.

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References


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