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Sulfation of N-Acyl Dopamines in Rat Tissues

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Abstract—Sulfation of N-acyl dopamines has been shown for the first time in cytosolic fractions of rat liver and nervous system. Sulfation of dopamine amides of docosahexaenoic and oleic acids occurred in all tissues studied, N-arachidonoyl dopamine was sulfated in the liver and spinal cord, and N-stearoyl dopamine was sulfated only in the liver. Depending on the substrate and tissue, the sulfation activity varied from 0.5 to 3.5 nmol/min per mg total protein. Kinetic parameters of N-docosahexaenoyl dopamine sulfation in the brain were determined. The findings characterize the sulfation system as the most productive metabolic pathway of N-acyl dopamines, but the role of this system in the body is unclear because of high K_m value.

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Key words: aryl sulfotransferase, neurolipins, metabolism, liver, nervous system

N-Acyl dopamines are signaling lipids belonging to the cannabinoid–vanilloid system. By now, dopamine amides of arachidonic [1], oleic, stearic, and palmitic acids have been found in mammals [2]. Moreover, we detected by mass-spectrometry N-docosahexaenoyl dopamine (DHA-DA) in tissues of freshwater hydras *Hydra magnipapillata* and *H. attenuata* [3]. Signaling effects of N-acyl dopamines manifest themselves mainly in the nervous and immune systems and are realized through interaction with vanilloid (TRPV1) and central cannabinoid (CB1) receptors [1, 2, 4] and also with other intracellular targets [5].

In mammals, acyl dopamines are mainly metabolized through low efficiency hydrolysis with a possible involvement of hydrolase of fatty acid amides [1, 2], methylation of the catechol group by the cytoplasmic catechol-O-methyltransferase [1, 6], and also through oxidation of the dopamine moiety by NADH oxidoreductases from plasma membrane and mitochondria [6]. These data indicated that a fatty acid residue had a poor influence on the recognition of the catechol group in acyl dopamines by different enzymes. Therefore, it was supposed that another enzymatic system responsible for dopamine inactivation could be involved in metabolism of N-acyl dopamines. The system in point is represented by aryl sulfotransferases (AST), which are widely distributed in mammalian tissues [7, 8] and transfer sulfogroups onto hydroxyl groups of an acceptor. Some AST isoforms are known which are substrate-specific to carbohydrates, proteins, phenols, and catecholamines [7], as well as tissue-specific isoforms [9]. Inside the cell, these enzymes are usually localized in the cytosol [10]. The activities of AST specific to phenols and catecholamines increase solubility of phenolic compounds that facilitates their elimination from the body and also inhibits the physiological effect of exogenous dopamine. Although dopamine sulfate has been found in blood plasma and cerebrospinal fluid of mammals, its biological role is still unclear [11].

The purpose of this work was to determine whether sulfation of basic N-acyl dopamines occurs in the nervous system and liver of rats.

Abbreviations: AA-DA, N-arachidonoyl dopamine; AST, aryl sulfotransferase; DHA-DA, N-docosahexaenoyl dopamine; ESI, electrospray ionization; Ol-DA, N-oleoyl dopamine; St-DA, N-stearoyl dopamine.

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MATERIALS AND METHODS

Reagents used were as pure as possible. Adenosine-5′-phosphate-3′-phosphosulfate was from Sigma-Aldrich

(USA), and phosphate-buffered saline was from Helicon (Russia).

Standard N-arachidonoyl dopamine sulfate was synthesized by incubation of N-arachidonoyl dopamine (AA-DA) with a fivefold excess of the pyridine·SO_3 complex (Fluka, USA) in anhydrous pyridine. After the incubation, the reaction mixture was supplemented with 15% aqueous solution of $NaHCO_3$, incubated for 20 min, and then diluted with an equal volume of pyridine. The solution was separated from the precipitated salt and evaporated to dryness. Residual pyridine was removed from the reaction mixture by treatment with 10% solution of CF_3COOH in ethanol. Then the reaction mixture was evaporated to dryness, and the residue was dissolved in a small volume of methanol (20 μl) and used for thin-layer chromatography on Silica gel 60 F_{254} (Merck, Germany) (in the solvent system chloroform–methanol, 7 : 1). The sorbent zone was cut out in the expected product region and successively eluted with 5 ml of methanol and 5 ml of acetonitrile. The eluate was evaporated, and the residue was weighed and dissolved in ethanol (final concentration 10 mg/ml). The structure of the resulting substance was confirmed by mass-spectrometry with electrospray ionization (ESI) in the mode of positive ion generation.

Wistar rats were used in the experiments. The animals were anesthetized with ether and sacrificed by cervical dislocation. Tissue homogenates were prepared at 4°C. The liver, brain, and spinal cord were placed into Petri dishes containing cooled phosphate-buffered saline (pH 7.4); from the brain and spinal cord blood vessels were removed, and the liver was minced to particles not larger than 0.5 cm and washed in the buffer. The tissues under study were minced and homogenized in a tenfold volume of 10 mM potassium-phosphate buffer (pH 7.4) supplemented with 0.5 mM dithiothreitol in a Potter-Elvenheim homogenizer at 800 rpm. The homogenates were successively centrifuged on mLw K24D and Beckman L8 centrifuges (SW-40 rotor) (Beckman, USA) at 1000, 12,500, and 100,000*g* for 10, 30, and 60 min, respectively, and the precipitates were discarded each time. The supernatant fluid resulting at the last centrifugation was taken as cytosolic fraction and was stored at −52°C until use. The protein concentration in the cytosolic fractions was determined by the Lowry method [12]. The protein was precipitated with trichloroacetic acid.

The specific activity of AST relatively to acyl dopamines was measured by the method of Maines [13]. Solutions of N-acyl dopamines in ethanol or DMSO (final substrate concentration in the reaction mixture was 10 μM, injected volume was 4 μl, final solvent concentration in the incubation medium was 1%) were added to the incubation medium (400 μl) containing 50 mM glycine-NaOH buffer (pH 9.0), 7.5 mM 2-mercaptoethanol, and preparation of the cytosolic fraction of the tissue under study with the final protein concentration of 1.25 or 5 mg/ml. The reaction was initiated by addition of adenosine-5′-phosphate-3′-phosphosulfate cofactor solution (final concentration 100 μM). Samples were incubated at 37°C for 30 or 60 min, and the reaction was stopped by addition of an equal volume of methylene blue solution and two volumes of chloroform. Upon careful mixing and separation of the organic and aqueous phases, the chloroform layer was placed into tubes containing small amounts (50-100 mg) of anhydrous Na_2SO_4 to remove residual water, and the tubes were shaken intensively. The absorption at 651 nm was determined 5-10 min later using a LOMO-Fotonika SF-256UVI spectrophotometer (Russia). Incubation mixtures without the cofactor, acyl dopamine, or both these components were used as controls. The amount of product in the extract was determined by a calibration curve constructed with the N-arachidonoyl-O-sulfo dopamine standard synthesized as described above and extracted similarly from the incubation buffer. Generation of the desired product was monitored by mass-spectrometry as described above.

Kinetic parameters of DHA-DA sulfation in the brain were determined by the same method, but because hydrophobic N-acyl dopamines were used in high concentrations, for better dissolution they were added as aqueous solutions supplemented with 5 mg/ml BSA (its final concentration in the incubation medium was 0.5 mg/ml).

RESULTS AND DISCUSSION

AST isoforms have different tissue and substrate specificities and also different pH optimums of activity. The pH optimum for the dopamine-specific isoform was 9.0. Because the reaction products were quantitatively determined by UV absorption, it was necessary to synthesize a standard for the calibration curve. AA-DA was chosen for the standard because among the substances studied it had the intermediate length and number of double bonds in the fatty acid residue. Arachidonic acid is highly polyunsaturated can be easily oxidized and react via its double bonds. Therefore, for sulfation the mildest approach should be chosen. Modification with the pyridine·SO_3 complex in anhydrous pyridine [14, 15] suited these requirements, and we used just this approach for synthesis of AA-DA sulfate as the standard. Generation of the desired product was confirmed by data of ESI mass-spectrometry (Fig. 1).

The ability of aryl sulfotransferase to recognize substrates acylated by long-chained fatty acids has not been described earlier; therefore, we first tested our hypothesis about a possible sulfation of N-acyl dopamines using cytosolic fraction of liver because many enzymatic systems are the most active in this organ. All N-acyl dopamines studied were sulfated in the liver (Fig. 2), and

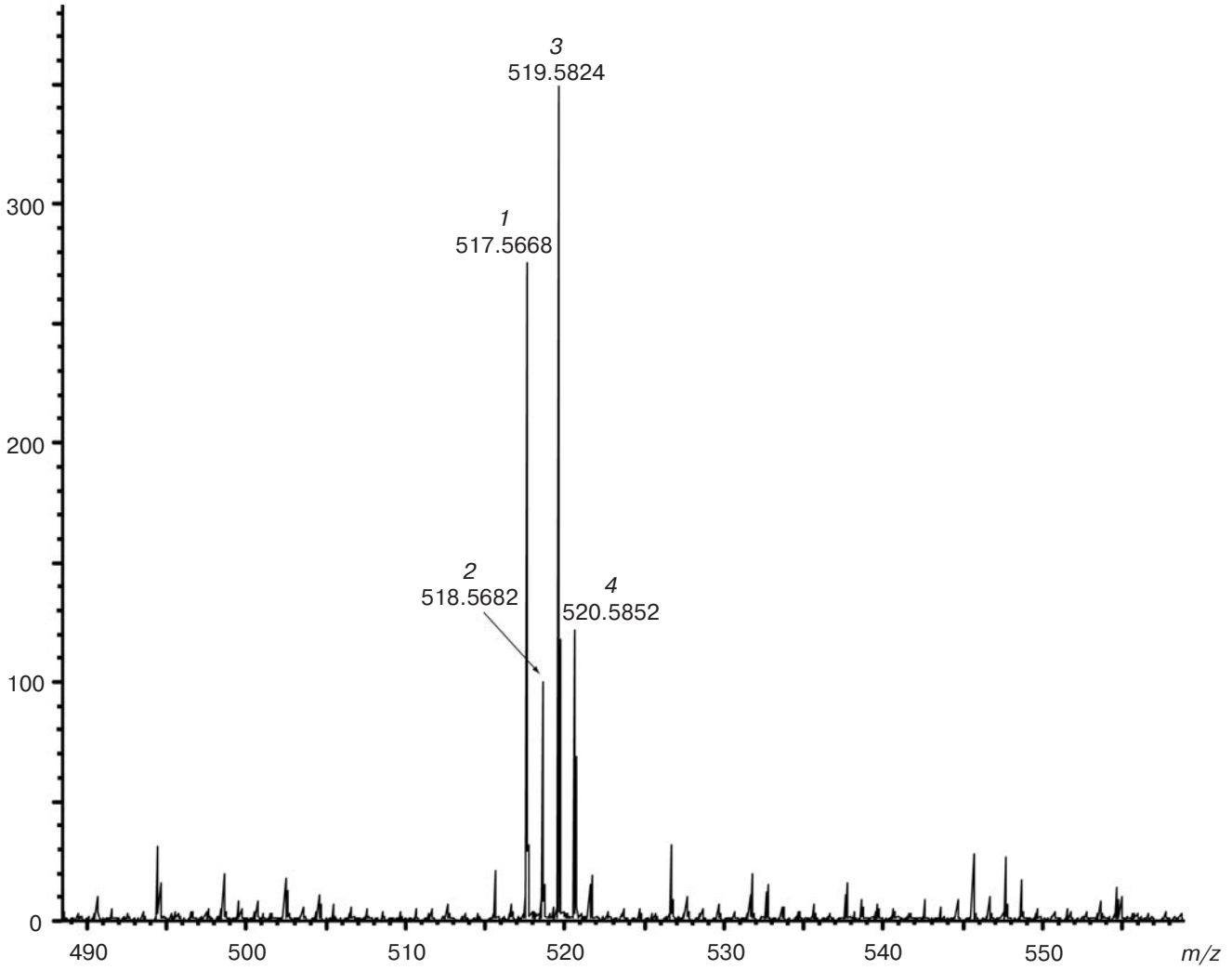


Fig. 1. Fragment of mass-spectrum of the N-arachidonoyl-O-sulfo dopamine standard. The zone of molecular ions: *1*) an ion of unestablished structure with supposed formula $C_{28}H_{39}NO_6S$ ($(m/z)_{calc.}$ = 517.250); *2*) an ion of unestablished structure with supposed formula $C_{28}H_{40}NO_6S$ ($(m/z)_{calc.}$ = 518.258); *3*) [M] ($(m/z)_{calc.}$ = 519.266); *4*) [M+H] ($(m/z)_{calc.}$ = 520.273).

the sulfation rate was maximal in the case of N-oleoyl dopamine (Ol-DA), whereas the enzyme activity relatively to AA-DA was one of the lowest.

A highly hydrophobic substrate, N-stearoyl dopamine (St-DA), could be introduced into the reaction only as solutions in DMSO and not in ethanol, in contrast to the other N-acyl dopamines. AST was shown to be sensitive to solvents [16]. Thus, to compare the sulfation efficiency of different N-acyl dopamines, we studied sulfation of dopamine amides of stearic and docosahexaenoic acids in the liver on introduction of the substrates in the same solvent. To standardize the conditions, DHA-DA solution in ethanol was evaporated, dissolved in DMSO, and the resulting solution was added into the reaction mixture. Under these conditions, the sulfation rates of DHA-DA and St-DA were, respectively, 4.0 ± 0.3 and 0.128 ± 0.007 nmol/min per mg.

In the cytosols of the brain and spinal cord, N-stearoyl dopamine was not sulfated, in contrast to docosahexaenoic and oleic acid dopamine amides. Note that the rates of DHA-DA and Ol-DA accumulation in the brain were twofold higher than in other organs. This suggests importance of this metabolic pathway. Since DHA-DA similarly to AA-DA has a broad activity spectrum and is also a good substrate for AST of the brain, kinetic parameters of DHA-DA sulfation in the brain were determined. The optimal incubation time was found to be 30 min. The enzyme from the cytosolic fraction of the homogenate interacted with DHA-DA with K_m = 18 μM at the maximal activity 3.7 nmol/min per mg.

Production of sulfated neurolipins was confirmed by mass-spectrometry in all experiments. Thus, in the case of Ol-DA sulfation in the brain the initial substance gave

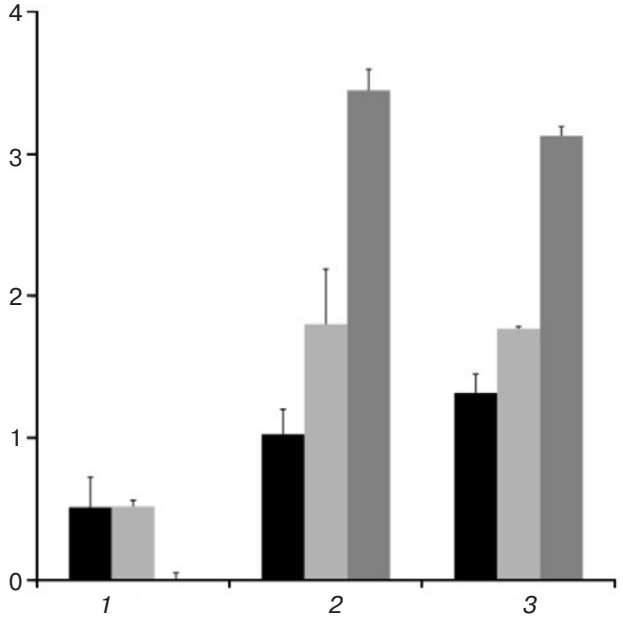
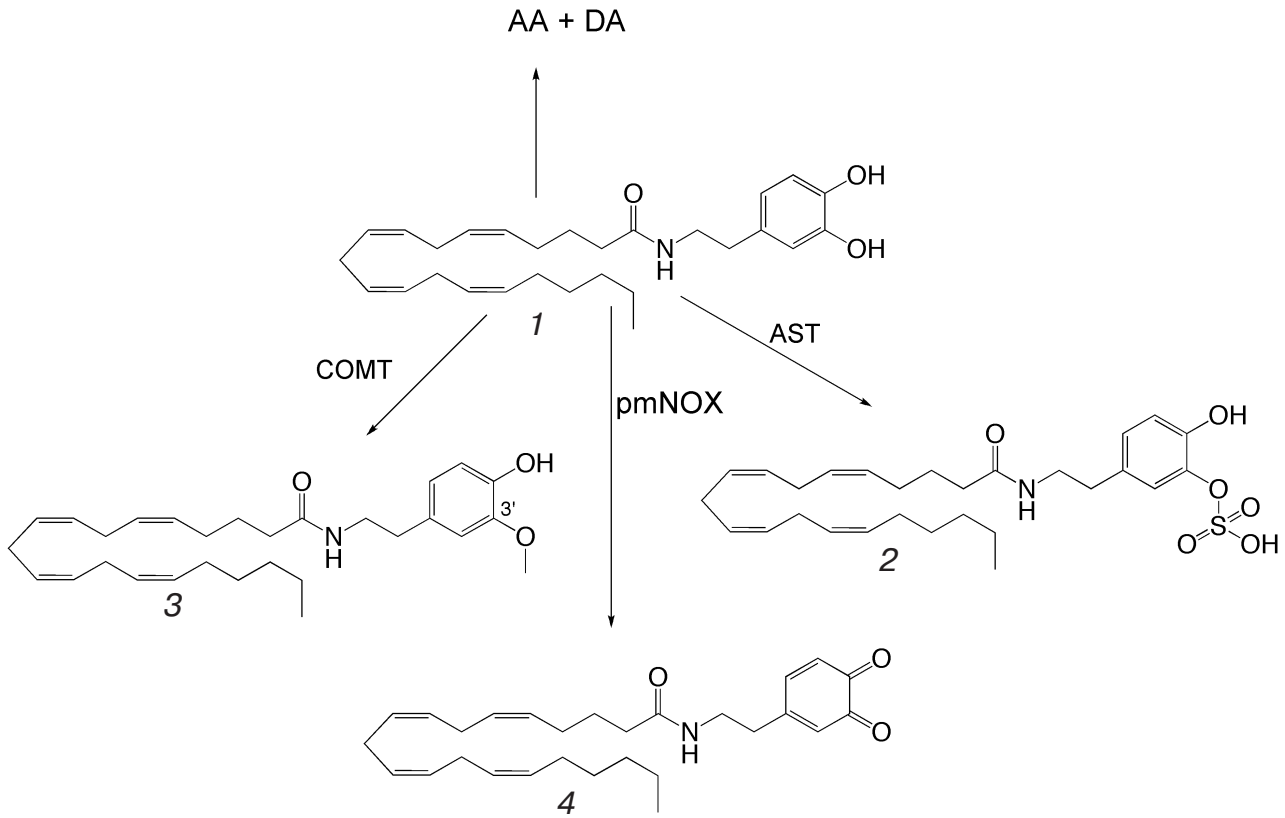


Fig. 2. Specific activities of AST (nmol/min per mg total protein) from cytosolic fractions of rat organs relative to different neurolipins: *1*) AA-DA; *2*) DHA-DA; *3*) Ol-DA. Black, light-gray, and dark-gray columns represent liver, spinal cord, and brain, respectively.

three molecular ions as follows: [M+H] ($(m/z)_{calc.}$ = 418.328, $(m/z)_{exp.}$ = 418.478), [M+Na] ($(m/z)_{calc.}$ = 440.310, $(m/z)_{exp.}$ = 440.469), and [M+K] ($(m/z)_{calc.}$ = 456.284, $(m/z)_{exp.}$ = 456.458), whereas the sulfation product gave ions as follows: [M] ($(m/z)_{calc.}$ = 497.281, $(m/z)_{exp.}$ = 497.494), [M+H] ($(m/z)_{calc.}$ = 498.289, $(m/z)_{exp.}$ = 498.480), and an ion of unestablished composition with supposed formula $C_{26}H_{42}NO_6S$ ($(m/z)_{calc.}$ = 496.273, $(m/z)_{exp.}$ = 496.474).

Sulfation rates of two of the studied substances are especially noteworthy: those of arachidonoyl dopamine and N-stearoyl dopamine. The low efficiency of St-DA sulfation seems to be caused by its strong hydrophobicity and the resulting limited availability for the cytoplasmic enzyme. Because solubility of AA-DA is comparable with the solubilities of oleic and docosahexaenoic acid dopamine amides, this factor is unlikely to determine the low rate of AA-DA metabolism in the brain. But it must not be ruled out that the AA-DA sulfation observed in the liver and spinal cord can be manifestations of activity of a specific isoenzyme that is absent in the brain. An idea that the AA-DA metabolism, which is apparently independent of the aryl sulfotransferase system, plays a special role seems more reasonable. The arachidonic acid residue is likely to inhibit either the interaction of AA-DA with the



Metabolism of N-acyl dopamines exemplified by AA-DA: *1*) AA-DA; *2*) N-arachidonoyl-3-O-sulfo dopamine; *3*) N-arachidonoyl-3-O-methyl dopamine; *4*) N-arachidonoyl-*p*-ethylamino-*o*-benzoquinone. COMT, catechol-O-methyltransferase; pmNOX, different oxidoreductases

enzyme or the enzyme activity, but details of this influence need special study.

The investigations indicate that sulfation is one of the most productive catabolic pathways of acyl dopamines (Scheme). In fact, these compounds are hydrolyzed very slowly. Dopamine amides of arachidonic, oleic, and stearic acids are virtually not inhibitors of hydrolysis of anandamide by hydrolase of fatty acid amides, which inactivates endocannabinoids [2], and, thus are unlikely to be its substrates. Hydrolysis of AA-DA by other enzymes is possible, but its rate is extremely low: 20 and 12.2 pmol/min per mg in the brain and liver, respectively [1]. There are no data on hydrolysis of docosahexaenoic acid dopamine amide. Methylation, which is another possible pathway of inactivation, is also not very productive: the specific activity of cytosolic catechol-O-methyltransferase is 0.1 nmol/min per mg in the liver, and its productivity is an order of magnitude lower in the nervous system [6]. Thus, sulfation is the most efficient metabolic pathway for Ol-DA and DHA-DA. Only the rate of oxidation is close to the sulfation rate [6], but, because dopamine amide quinones are highly reactive and seem to be dangerous for cellular proteins [17], this pathway seems to play a special, possibly a signaling role. The AA-DA case in the brain is ambiguous: the sulfation system does not recognize it and no other productive pathways of its inactivation are known. It was suggested that either mechanisms of inactivation of this neurolipin should be activated in response to a special signal, or include transformation pathways that had not been considered from this viewpoint (e.g. transport into the excretory system). Metabolism of N-stearoyl dopamine is still unclear: it is not methylated in the liver and central nervous system and seems not to be oxidized in the liver mitochondrial fraction [6], but it is sulfated only in the liver and not very efficiently. Studies on neurolipin glucuronidation seem promising for elucidating this problem because this pathway of preparation for elimination of xenobiotics and some endogenous compounds has been also shown for dopamine [18]. We have already obtained some preliminary data on glucuronidation of acyl dopamines in the liver, and results of more detailed studies will be published elsewhere.

Note also that at the relatively high rate the K_m value for DHA-DA is rather high, at least as compared with the acyl dopamine content (thus, the AA-DA content in brain is not higher than 6 pmol per g wet tissue [1]). However, the intracellular concentration of neurolipins can be markedly higher, and, thus, the enzymatic system under study can also contribute to termination of neurolipin signaling. Sulfation of N-acyl dopamines can be biologically significant not only for termination of endogenous acyl dopamine action (under normal and pathological conditions) but also for protection against exogenous acyl dopamines.

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