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EVIDENCE FOR INDIRECT UTILIZATION OF GLYCINE FOR PRODUCTION OF N-BASES OF GLYCEROPHOSPHOLIPIDS AND SPHINGOLIPIDS IN MOUSE NEUROBLASTOMA CELLS BY USING ^{15}N-LABELED GLYCINE

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SUMMARY: After mouse neuroblastoma cells were cultured with [^{15}N] glycine in Dulbecco's modified Eagle's medium for total four days, they gave significant amounts of ^{15}N-atom% excess in phosphatidylserine, phosphatidylethanolamine, ethanolamine plasmalogen, phosphatidylcholine, sphingomyelin and ceramide. These results were compared with those of [^{15}N]choline, [^{15}N] ethanolamine and [^{15}N]DL-serine used in place of [^{15}N]glycine for the experiments. It was suggested that [^{15}N]glycine is taken into the cells and metabolically converted to [^{15}N]L-serine, whcih is well known to be used for the production of polar head groups of glycerophospholipids and sphingosine.

The authors previously used [^{15}N]choline, [^{15}N]ethanolamine, and [^{15}N]DL-serine for the metabolic study of N-bases of glycerophospholipids and sphingolipids in murine organs and tissues(1,2), although the experiments were restricted due to shortage and high price of these ^{15}N-labeled substances. However, in this study, the experiments have been regained by using cultured murine neuroblastoma cells(3,4) and [^{15}N]glycine that is the simplest of all amino acids and not so expensive. Indeed, Stein et al.(5) reported that [^{15}N]glycine was used for human ^{15}N-tracer studies, and that the urinary ^{15}N-excretion profiles were determined by optical

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; TLC, thin layer chromatography; PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; EtnPla, ethanolamine plasmalogen; PtdCho, phosphatidylcholine.

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emission spectroscopy that we have used. It is in general well-known that glycine is available for the formation of various biochemical substances, such as porphyrin, purine, glycocholic acid, creatine and proteins. However, it has been found for the first time that [^{15}N]glycine is indirectly utilized for the production of N-bases of glycerophospholipids and sphingolipids of the neuroblastoma cells even in DMEM without removing both glycine and serine.

These findings suggested that [^{15}N]glycine is taken into cells and metabolically converted to [^{15}N]L-serine, which is used for the production of N-bases of glycerophospholipids and sphingolipids as already reported by many workers(6-14). For further confirmation, in place of [^{15}N]glycine, other substances, such as [^{15}N]choline, [^{15}N]ethanolamine, [^{15}N]DL-serine were similarly used for the metabolic study in the cultured neuroblastoma cells. The results show that [^{15}N] glycine can be used apparently like [^{15}N]L-serine for the production of N-bases of glycerophospholipids and sphingolipids, but not like [^{15}N]choline and [^{15}N]ethanolamine.

These findings are described and discussed in this paper.

MATERIALS AND METHODS

Materials: [^{15}N]glycine(99 atom%), [^{15}N]choline chloride (95 atom%), [^{15}N]ethanolamine(95 atom%), and [^{15}N]DL-serine (92.3 atom%) were purchased from Shoko Tsusho Co.(Tokyo). Culture medium(DMEM), FCS, penicillin and streptomycine solution (5,000 units/ml and 5,000 µg/ml, respectively) were purchased from Flow Lab.(NR, Australia), trypsin solution (0.25%) from Nacalai Tesque(Kyoto), tissue culture flasks(25 and 80 cm^2) from Nunc(Japan Inter Med., Tokyo), cell scrapers from Greiner (Nurtrigen, Germany), and Silica Gel 60 TLC plates from Merck(Darmstadt, Germany).

Cell cultures: Murine neuroblastoma cell lines, NS-20Y (cholinergic), Neuro 2a(inactive), and N1E-115(adrenergic) were cultured in DMEM supplemented with 10% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycine at 37°C with 5% CO_2 in humidified air. The cells were serially passed with 0.25% trypsin at a split ratio of 1 : 50 in 25 cm^2 flasks.

Addition of [^{15}N]glycine, and for comparison, [^{15}N]choline chloride, [^{15}N]ethanolamine, and [^{15}N]DL-serine, respectively to cell cultures: Each cell line(NS-20Y, 1 x 10^4 cells/cm^2; Neuro 2a, 2 x 10^4 cells/cm^2; and N1E-115, 2 x 10^4 cells/cm^2) was seeded in three 80 cm^2 flasks and cultured in 20 ml of 10% FCS-DMEM containing [^{15}N]glycine(200 µg/ml) and for comparison [^{15}N]choline chloride(100 µg/ml), [^{15}N]ethanolamine(100 µg/ml), and [^{15}N]DL-serine(200 µg/ml), respectively for two days. Then, after the media were replaced by the same ^{15}N-substrate containing fresh media, the cells were kept on growing for more two days and observed by phase-contrast microscopy (original magnification x160).

Extraction of lipids: After harvested cells(3 - 7 x 10^7 cells) were washed with phosphate-buffered saline, total lipid of the cells was throughly extracted twice with 100 ml of chloroform-methanol(2:1, v/v) by vigorously shaking. After filtration, the lipid solution was applied to Folch partition with distilled water. The lower phase(chloroform) was removed, washed with the theoretical upper aqueous phase and finally evaporated to dryness under N_2 stream. The lipid residue was weighed and dissolved in chloroform-methanol(2;1, v/v) to a given concentration.

Preparative thin layer chromatography of lipids: Silica Gel 60 plates were activated in microwave oven just before performace of chromatography for favorable resolution of individual lipids(15). After the lipid solution was applied to the plate, it was developed with chloroform-methanol-water(65:25: 4, v/v) for the first dimension. The plate was then exposed to HCl-fume for separation of PtdEtn and EtnPla, dried, and developed with chloroform-acetone-methanol-acetic acid-water (50:20:10:15:5, v/v) for the second dimension. The individual lipid spots were visualized with iodine vapor and scraped from the plate. After the lipid was dissolved with chloroform-methanol(2:1, v/v) and separated from silica gel by centrifuging, the lipid solution was evaporated to dryness under N_2 stream and the residue was obtained.

Analytical procedures of ^{15}N-atom% excess and phosphorus content of individual glycerophospholipids and sphingolipids: After the individual lipid residues were dissolved with at least 0.05 ml of chloroform-methanol(2:1, v/v), a capillary glass tubing(2 mm in external diameter, 0.8 mm in internal diameter and 1 cm long) was dipped into each lipid solution. The solution thus permeated into the tubing was dried at 80°C in oven for about one hour. The lipid-contained tubing was put into a discharge tube(4 mm in e.d., 2.2 mm in i.d. and about 15 cm long) whcih was prepared on a little modification with an instrument for N-15 analysis(Kumazawa Model N-15)(16). While the lipid sample in the sealed tube was decomposed at 580°C for about three hours, water and CO_2 thus released were absorbed with activated CaO and CuO-mixed particles. The remaining N_2 gas in the tube was irradiated by high frequency generator and glowed magenta color light due to $^{14}N^{14}N$ and $^{15}N^{14}N$, which was measured by the optical emission spectroscopic N-15 analyser(JASCO, Model NIA-1)(16). ^{15}N-Atom% excess was determined by subtraction of natural abundance of 0.36 atom% from the ^{15}N-atom% and its mean value was obtained in triplicate. Phosphorus content of individual phospholipid-spots separated by TLC was analyzed by the method of Bartlett (17) for the determination of phospholipid composition.

RESULTS AND DISCUSSION

Incorporation of ^{15}N-atom% into N-bases of glycerophospholipids and sphingolipids of neuroblastoma cells with [^{15}N]glycine and for comparison, [^{15}N]choline, [^{15}N]ethanolamine and [^{15}N]DL-serine, respectively in medium during culture : As described in Material and Methods, [^{15}N]glycine and for comparison, [^{15}N]choline, [^{15}N]ethanolamine and [^{15}N]DL-serine, respectively was added to medium, in which NS-20Y,

Neuro 2a and N1E-115 neuroblastoma cells, respectively were cultured for total four days. Under these conditions, normal cell growth was observed in all culture media. The cell number of neuroblastoma cells in each culture medium was counted and listed in Table 1. The cell growth during culture was significantly different between three cell lines independent from ^{15}N-substrates in the media. As shown in Table 1, NS-20Y cells gave the greatest increase in cell number.

Total lipid of harvested NS-20Y, Neuro 2a and N1E-115 cells, respectively was thoroughly extracted as described above. Various glycerophospholipids and sphingolipids were obtained from the total lipid by preparative TLC and subjected to chemical analyses of phosphorus content and ^{15}N-atom% excess. A given amount of each different kind of ^{15}N-substrate had no influence on phospholipid compositions as well as cell growth of neuroblastoma cells(3).

However, as shown in Table 2, when neuroblatoma cells were cultured in [^{15}N]glycine-containing medium for total four days, it was found that they gave significantly different values of ^{15}N-atom% excess in PtdSer, PtdEtn, EtnPla, PtdCho, sphingomyelin and ceramide of each cell line. These findings were compared with those obtained by other ^{15}N-substrates used in place of [^{15}N]glycine. At first, it was noted that [^{15}N]glycine gave results qualitatively similar to [^{15}N]DL-serine rather than [^{15}N]choline and [^{15}N]ethanolamine. It was thus suggested that [^{15}N]glycine is taken to the cells and metabolically converted to [^{15}N]L-serine by 5,10-methyl-

TABLE 1. INCREASE IN CELL NUMBER OF EACH DIFFERENT NEUROBLASTOMA CELL LINE IN EACH DIFFERENT KIND OF ^{15}N-SUBSTRATE CONTAINING MEDIUM FOR A TOTAL OF FOUR DAYS OF CULTURE

Cells	Culture (days)	[^{15}N] Glycine	[^{15}N] Choline	[^{15}N] Ethanolamine	[^{15}N] DL-Serine
NS-20Y	0	2.4×10^6	2.4×10^6	2.4×10^6	2.4×10^6
	4	8.7×10^7	7.0×10^7	8.3×10^7	6.0×10^7
Neuro 2a	0	4.8×10^6	4.8×10^6	4.8×10^6	4.8×10^6
	4	5.7×10^7	6.2×10^7	5.7×10^7	7.1×10^7
N1E-115	0	4.8×10^6	4.8×10^6	4.8×10^6	4.8×10^6
	4	3.7×10^7	3.9×10^7	3.7×10^7	2.5×10^7

TABLE 2. ^{15}N-ATOM% EXCESS IN N-BASES OF GLYCEROPHOSPHOLIPIDS AND SPHINGOLIPIDS OF EACH DIFFERET NEUROBLASTOMA CELL LINE IN EACH DIFFERENT KIND OF ^{15}N-SUBSTRATE CONTAINING MEDIUM FOR A TOTAL OF FOUR DAYS OF CULUTURE

Cells	Lipids	[^{15}N] Glycine	[^{15}N] Choline	[^{15}N] Ethanolamine	[^{15}N] DL-Serine
NS-20Y	PtdEtn	7.50	-	20.75	9.15
	EtnPla	6.25	-	32.56	5.08
	PtdSer	13.99	-	0.94	6.23
	PtdCho	1.29	37.23	1.26	0.66
	SM[a]	1.14	4.47	1.06	1.48
	Ceramide	1.05	-	-	1.19
Neuro 2a	PtdEtn	4.70	-	33.22	11.73
	EtnPla	3.59	-	36.50	6.81
	PtdSer	6.71	-	0.59	8.80
	PtdCho	0.54	36.23	1.85	0.81
	SM	0.79	7.05	1.29	2.50
	Ceramide	0.60	-	-	1.85
N1E-115	PtdEtn	8.90	-	39.84	13.15
	EtnPla	6.54	-	38.61	5.81
	PtdSer	9.90	-	0.59	7.01
	PtdCho	0.54	27.10	2.67	1.71
	SM	2.60	5.85	1.63	2.80
	Ceramide	1.11	-	-	1.98

a, Sphingomyelin.

tetrahydrofolate:glycine hydroxy-methyltransferase localized in the mitochondria(18,19), and that [^{15}N]L-serine thus produced is transported to the endoplasmic reticulum and used to synthesize [^{15}N]PtdSer(8), which is then transported to the mitochondria and easily decarboxylated to [^{15}N]PtdEtn(8,9). The ^{15}N atom% excess in EtnPla was thought to be due to [^{15}N] ethanolamine released from the [^{15}N]PtdEtn thus produced, although [^{15}N] ethanolamine used for the experiment gave ^{15}N-atom% excess in EtnPla as high as in PtdEtn, as shown in Table 2. The putative metabolic pathway of [^{15}N]glycine described above seemed to explain the significantly different values of ^{15}N-atom% excess between PtdSer and PtdEtn given by [^{15}N]glycine or [^{15}N]DL-serine used for the experiment.

On the other hand, even low values of ^{15}N-atom% excess in PtdCho, sphingomyelin and ceramide which were resulted from the metabolism of [^{15}N]glycine or [^{15}N]L-serine in the cell cultures were unable to be neglected, indicating that [^{15}N] PtdCho was produced by direct and successive methylation of [^{15}N]PtdEtn previously produced(20,21), and that [^{15}N]sphingo-myelin was produced by transfer of [^{15}N]phosphorylcholine from

[^{15}N]PtdCho thus produced to ceramide (22,23), whose [^{15}N]sphingosine was synthesized from [^{15}N]L-serine (11-14). Although the low value of ^{15}N-atom% excess in PtdCho appeared to be due to the isotope dilution with a large amount of PtdCho which accounts for about 50% of total phospholipids in the cells(3), [^{15}N]choline gave high value of ^{15}N-atom% excess in PtdCho (Table 2), indicating a very active de novo synthesis via the CDP-choline pathway, whereas it was also suggested that de novo syntheses of sphingomyelin and ceramide are much slower than those of glycerophospholipids, but that they are faster than the synthesis of PtdCho by the methylation of PtdEtn, because [^{15}N] DL-serine indicated that values of ^{15}N-atom% excess in sphingomyelin and ceramide were a little higher than that in PtdCho(Table 2). It remains to be clarified how [^{15}N]ethanolamine gave very low ^{15}N-atom% excess in PtdSer(Table 2). However, [^{15}N]ethanolamine gave no ^{15}N-atom% excess in ceramide, thus indicating that it is not converted to [^{15}N]L-serine which is used to produce sphingosine. Also, [^{15}N]choline gave no ^{15}N-atom% excess in glycerophospholipids and sphingolipids except for choline-containing lipids, thus indicating that it is not converted to [^{15}N]ethanolamine or [^{15}N]L-serine. In the present paper a fairly amount of [^{15}N]glycine or [^{15}N]DL-serine was required to get the sufficient ^{15}N atom% excess in glycerophospholipids and sphingolipids of cultured cells in DMEM, but if glycine and serine are in advance removed from the media to avoid isotope dilution, it is likely that a relatively smaller amount of [^{15}N]glycine or [^{15}N]DL-serine is enough to get sufficient ^{15}N atom% excess in them. Various effects of each different kind of ^{15}N-substrate on the metabolism of glycero-phospholipids and sphingolipids were rather similar between and NS-20Y, Neuro 2a, and N1E-115 cell lines which have different neurotransmitters. It has been reported that glycine, together with gamma-aminobutyric acid, is the predominant inhibitory neurotransmitter and potentiates the action of glutamate, the main excitatory neurotransmitter on postsynaptic N-methyl-D-aspartate receptor, and that plasma membrane transporters for glycine are widely distributed in the central nervous system(24). However, the physiological functions of glycine on the cultured cells and cell membrane transporters for glycine are unclear in this study.

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