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CHANGES IN METHIONINE METABOLISM INDUCED BY D-GALACTOSAMINE IN ISOLATED RAT HEPATOCYTES

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Abstract—We studied several steps of methionine metabolism in isolated rat hepatocytes both with and without the presence of a hepatotoxic agent (D-galactosamine). By use of selective labelling either on methyl or on carboxyl groups, we showed that intracellular methionine is used preferentially for the methylation of phospholipids (42%) and nucleic acids (31%) via *S*-adenosylmethionine. In the presence of D-galactosamine, the incorporation of L-($^{14}CH_3$) methionine into macromolecules is significantly inhibited (>50%). This inhibition is associated with a decrease of *S*-adenosylmethionine and an increase of methionine in the injured cells. These results suggest that hepatotoxicity of galactosamine may be due in part to an inhibition of the methylation of nucleic acids and phospholipids. Consequently, we hypothesize that hypermethioninemia associated with human liver disease could be due, at least partly, to a defect in synthesis and/or utilization of *S*-adenosylmethionine by hepatocytes.

Methionine has several well recognized metabolic functions in mammals. In addition to its role in protein biosynthesis, methionine serves as the precursor of transsulfuration and transmethylation processes and of polyamine biosynthesis. Furthermore, methionine is of great interest in human pathology. Hypermethioninemia can express a specific enzymatic defect in metabolic pathways involving methionine [1] and may also be associated with different hepatocellular injuries [2]. Thus, we attempted to study what effect a hepatotoxic product would have in the several steps of methionine metabolism in isolated rat hepatocytes. The aim of this work is to specify the relations between anomalies of methionine metabolism and hepatocellular injury using an *in vitro* model. Because cell lesions induced in the rat liver *in vivo* [3, 4] and *in vitro* [5, 6] by D-galactosamine (GalN) are in many respects similar to those observed in human viral hepatitis, we chose this model for our studies.

In a recent work [7], we have studied the effect of GalN principally on the first step of methionine metabolism involving the activity of ATP: L-methionine-*S*-adenosyltransferase (EC 2.5.1.6) (MAT), which catalyses the formation of *S*-adenosylmethionine (AdoMet) from ATP and L-methionine. We showed that high concentrations of GalN induced a significant decrease of MAT activity and a drop in ATP levels.

Using methionine labelled either on its methyl or its carboxyl group, we have studied in this present work the fate of these two groups in rat hepatocytes with or without various concentrations of GalN.

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MATERIALS AND METHODS

Isolated rat hepatocytes. Rat hepatocytes were prepared by liver slice method [8] as described in detail previously [7]. Prior to the addition of labelled substrate, 7.5×10^6 cells were preincubated 5 min in 2.5 ml of Eagle's minimal essential medium under an atmosphere of 5% CO_2, 95% O_2 with GalN (2, 10 and 100 mM) or without (control cells). Then, 2.5 μCi of either L-(methyl-^{14}C) methionine (56 mCi/mmol) or L-(carboxyl-^{14}C) methionine (42 mCi/mmol) were added to the cell suspensions. Incubations were carried out for 2 hr at 37° under agitation (60 oscillations/min). The reaction was stopped by cooling in ice and cell suspensions were centrifuged at 150 *g* for 10 min at 4°. The supernatant was examined for extracellular radioactivity and the cells were washed 3 times with 10 ml of MEM. The final cell pellet was extracted with 400 μl of ice-cold 10% TCA, kept at 0° for 30 min and spun at 10,000 *g* for 15 min at +4°. The excess of acid was removed from the supernatant by repeated ether extractions (3 × 1 ml) and TCA soluble material was stored at −20° for the determination of methionine derivatives. TCA precipitate was washed 3 times with 5 ml of 5% TCA and further treated successively for the determination of nucleic acids, phospholipids and proteins.

Nucleic acids. Nucleic acids were first extracted from the TCA precipitate [9]. The pellet was resuspended in 1 ml of 5% TCA and heated for 2 hr at 90°. Radioactivity incorporated into nucleic acids was counted in supernatant obtained after centrifugation (10,000 *g*, 5 min at +4°) using liquid scintillation.

Lipids. Lipids were extracted three times with 1 ml chloroform/methanol (2:1 v/v) from the TCA pellet [10]. Supernatants were pooled and extracted back

by 2 ml methanol/0.1 M potassium chloride (1:1 v/v). The organic phase was evaporated under a nitrogen stream and residues were dissolved in chloroform. An aliquot was removed for the determination of total lipid radioactivity while another aliquot was applied on a silica gel thin layer chromatography (TLC) plate for separation of phospholipids. The mobile phase consisted of chloroform/methanol/water (65:25:3 v/v). Pure phosphatidylethanolamine (PE), monomethyl-phosphatidylethanolamine (PME), dimethyl-phosphatidylethanolamine (PDE), phosphatidylcholine (PC) were added to the residues as carriers. Spots were visualized by iodine, scraped and counted for radioactivity after addition of 0.5 ml of methanol and 10 ml of Picofluor.

Proteins. The TCA pellet was treated with 1 ml of 2 N NaOH for 15 hr and the radioactivity incorporated into solubilized proteins was measured after addition of 10 ml Picofluor to a 100 μl aliquot.

Methionine derivatives. Methionine from its derivatives were separated by TLC of TCA soluble material on cellulose plates (20 × 20 cm) (Eastman Kodak, Rochester, NY). The sheets were developed in 0.1 M sodium phosphate buffer (pH 6.8) saturated with $(NH4)_2SO_4$, 2-propanol (50:1, v/v) [11]. Supernatant was applied on the plates with the following carriers: methionine (Met), methionine sulfoxide (Met(O)), *S*-adenosylmethionine (AdoMet), *S*-adenosylhomocysteine (AdoHCy), 2-keto-4-methylthiobutyrate (2-Keto-4 MTB), sarcosine (Sar). After migration, the plates were dried at 100° and spots were visualized either under u.v. light (AdoMet, AdoHCy) or after ninhydrin revelation. Plates were divided into 0.5 cm strips, scraped and placed with 1 ml of distilled water into scintillation vials. The vials were shaken 15 min and counted with 10 ml Picofluor. R_f of these derivatives were confirmed using another mobile phase: butanol, acetic acid, water (40:10:20, v/v) [7].

Materials. The nutrient medium was Eagle's essential minimal medium (MEM, Eurobio no. 2111, France). Radioactivity was measured by liquid scintillation counting in a Packard Spectrometer. Radioactive compounds were purchased from Amersham (Radio Chemical Center, U.K.). Amino acids and lipid samples were obtained from Sigma Chemical Company (St Louis, MO), Picofluor from Packard Inc. (IL). All other chemical products were purchased from Merck (Darmstadt, F.R.G.).

Statistical analysis. Statistical significance of results obtained for GalN-treated cells was determined using the Student's *t*-test for paired experiments.

RESULTS

Control cells

In order to study certain aspects of methionine metabolism, isolated rat hepatocytes were incubated in the presence of differentially labelled (^{14}C) methionine, and the distribution of radioactivity within hepatocyte suspensions was determined. The distribution of radioactivity after 2 hr of incubation is shown in Table 1. Extracellular radioactivities after incubation with (methyl-^{14}C) methionine and (carboxyl-^{14}C) methionine, accounted for 82% and 87% of the total radioactivity, respectively. After incubation of cells with (methyl-^{14}C) methionine, intracellular radioactivity was 12% of the initial radioactivity and distributed into lipids (5.0%), nucleic acids (3.0%), TCA-soluble material (2.3%) and proteins (0.78%). After incubation in the presence of (carboxyl-^{14}C) methionine, intracellular radioactivity accounted for only 1% of the total radioactivity, approximatively 10 times less than was observed with methionine labelled on the methyl group. This difference was due mainly to the absence of notable incorporation of radioactivity into lipids and nucleic acids (less than 0.1%). There was no significant difference in radioactivities detected in TCA-soluble (0.71%) and protein (0.41%) fractions.

Table 1. Distribution of total radioactivity after 2 hr of incubation with L-(methyl-^{14}C)methionine or L-(carboxyl-^{14}C)methionine (2.5 μCi were added to 2.5 ml of medium containing approximately 7.5×10^6 cells)

L-methionine	Extracellular radioactivity	Intracellular radioactivity	
Methyl-^{14}C	82% ± 5 (7)	12% ± 3 (5)	2.3% ± 0.90 (5) TCA soluble material
			5.0% ± 1.10 (5) Lipids
			3.0% ± 1.50 (5) Nucleic acids
			0.78% ± 0.31 (4) Proteins
Carboxyl-^{14}C	87% ± 8 (4)	1.1% ± 0.6 (4)	0.71% ± 0.48 (5) TCA soluble material
			<0.1% (4) Lipids
			<0.1% (4) Nucleic acids
			0.41% ± 0.21 (4) Proteins

Values represent the mean percentage ±SD. The number of experiments is indicated in parentheses.

Table 2. Distribution of radioactivity in TCA soluble material after 2 hr of incubation with L-(methyl-^{14}C)methionine or L-(carboxyl-^{14})methionine

L-Methionine	Met	Met(O)	AdoMet	AdoHCy	Sar
Methyl-^{14}C	45% ± 15 (8)	15% ± 7 (6)	13% ± 4 (8)	—	10% ± 1 (8)
Carboxyl-^{14}C	53% ± 4 (3)	15% ± 5 (3)	17% ± 5 (3)	12% ± 5 (3)	—

Each value (mean ±SD) represents the percentage of the total radioactivity in acidosoluble material. The number of experiments is indicated in parentheses.

Table 3. Distribution of radioactivity into phospholipids (phosphatidylcholine, PC; lysophosphatidylcholine, LPC; dimethyl-phosphatidylethanolamine, PDE; monomethyl-phosphatidylethanolamine, PME) after 2 hr of incubation with L-(methyl-^{14}C)methionine (mean ±SD)

PC + LPC	PDE	PME
95% ± 1.6 (6)	2.0% ± 1.1 (5)	0.8% ± 0.4 (5)

The number of experiments is indicated in parentheses.

The distribution of radioactivity into acid-soluble derivatives is shown in Table 2: using two chromatographic systems, it is possible to identify easily Met, Met(O), AdoMet, AdoHCy and Sar. However, homocysteine, labelled only from (carboxyl-^{14}C) methionine is not well separated from Met(O). Consequently, it is not possible to draw conclusions about this particular metabolite. The proportion of radioactive derivatives (expressed as a percentage of acid soluble material) is nearly identical no matter which of the substrates except for labelled Sar and AdoHCy which are formed exclusively from (methyl-^{14}C) and (carboxyl-^{14}C) methionine respectively. Another methionine derivative, 2-keto-4-methyl-thiobutyrate has not been detected under these experimental conditions.

The chromatographic separation of lipids was achieved only with (methyl-^{14}C) methionine as substrate because no sufficient radioactivity was detected with (carboxyl-^{14}C)methionine. The system used in this study allowed us to resolve the PC (which migrates with lysophosphatidylcholine (LPC)) from PDE and PME. In presence of (methyl-^{14}C) methionine, radioactivity is mostly incorporated into PC, the trimethyl derivative of PE (95%). Only 0.8% and 2% of the radioactivity were recovered in PME and PDE respectively (Table 3).

Galactosamine treated cells

In comparison with the control cells, there are no significant variations of extracellular radioactivity in the presence of GalN (Table 4). However, the intracellular radioactivity decreases significantly with GalN concentration using (methyl-^{14}C) methionine as precursor. These changes are less marked when using (carboxyl-^{14}C) methionine.

The effect of 2, 10 and 100 mM of GalN on the distribution of the radioactivity corresponding to the TCA soluble material is shown in Fig. 1. The results demonstrate that the AdoMet levels strikingly decrease in GalN treated cells. The difference between control cells and GalN treated cells is statistically significant with 2 mM ($P \leqq 0.01$) as well as 10 and 100 mM GalN ($P \leqq 0.001$). This decrease of AdoMet is accompanied with an increase of methionine ($P \leqq 0.05$ with 10 mM GalN). These modifications are in the same order, when (carboxyl-^{14}C)-methionine is used. The variations of Sar (labelled only with the methyl group) and of AdoHCy (labelled only with carboxyl group) are not significant. We observed also an increase in methionine sulfoxide, a derivative formed under mild oxidative conditions from methionine *in vivo* as well as *in vitro* [12]. As its

Table 4. Effect of GalN on the distribution of extra and intracellular radioactivity after 2 hr of incubation with L-(methyl-^{14}C)methionine or L-(carboxyl-^{14}C)methionine (2.5 μCi were added to 2.5 ml of medium containing about 7.5×10^6 cells)

	L-Methionine	GalN 2 mM	GalN 10 mM	GalN 100 mM
Extracellular radioactivity	Methyl-^{14}C	106 ± 3 (7)	109 ± 4 (7)	115 ± 6 (7)
	Carboxyl-^{14}C	98 ± 10 (5)	101 ± 8 (5)	102 ± 7 (5)
Intracellular radioactivity	Methyl-^{14}C	66 ± 13 (6)	49 ± 13 (7)	33 ± 12 (9)
	Carboxyl-^{14}C	95 ± 21 (4)	90 ± 16 (4)	74 ± 22 (3)

The percentage ±SD is calculated with values for untreated cells equal to 100%. The number of experiments is indicated in parentheses.

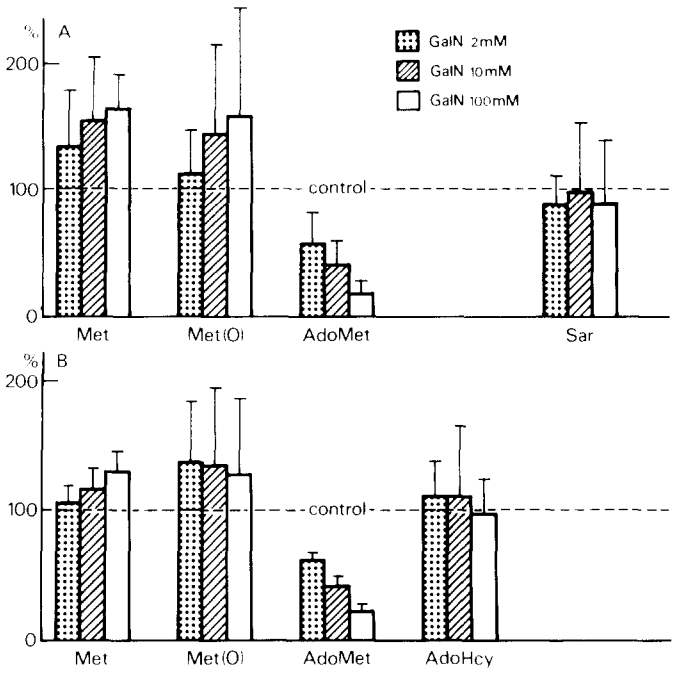

Fig. 1. Distribution of radioactivity in TCA soluble material after 2 hr of incubation with L-(methyl-^{14}C)methionine (A) or L-(carboxyl-^{14}C)methionine (B) in the presence of different concentrations of GalN. Each percentage (control = 100%) represents the mean ±SD of five determinations.

formation could be partially due to the experimental conditions, it is difficult to interpret the meaning of this increase.

The incorporation of (methyl-^{14}C)methionine into proteins, nucleic acids and lipids is inhibited in a dose-dependent fashion by GalN. Figure 2 shows this inhibition. The inhibition of the methylation of macromolecules is significant even with only 2 mM GalN, for proteins ($P \leqq 0.01$), nucleic acids ($P \leqq 0.001$) as well as for phospholipids ($P \leqq 0.05$). At higher concentrations of GalN, the inhibition of the incorporation of (methyl-^{14}C)methionine into macromolecules becomes more pronounced ($P \leqq 0.001$). The effect of GalN on the distribution of radioactivity into the derivatives of phosphoethanolamine is shown in Fig. 3. These results indicate that the decrease of incorporation of the methyl group is especially marked in phosphatidylcholine even with only 2 mM of GalN ($P \leqq 0.05$). The decrease of incorporation into PDE is not statistically significant.

Using (carboxyl-^{14}C)methionine, we have only studied the effect of GalN on protein synthesis (Fig. 2), as no sufficient incorporation of radioactivity was detected into nucleic acids and lipids with the controls. The protein synthesis inhibition is noteworthy: 60% with 2 mM of GalN as we have previously described using leucine ^{14}C [7].

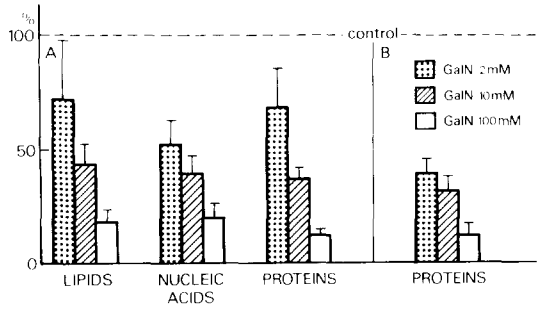

Fig. 2. Effect of GalN (2, 10 and 100 mM) on the incorporation of L-(methyl-^{14}C)methionine (A) into lipids, nucleic acids and proteins and of L-(carboxyl-^{14}C)methionine (B) into proteins. Each percentage (control = 100%) represents the mean ±SD of five determinations.

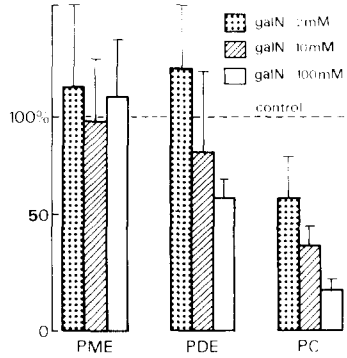

Fig. 3. Effect of GalN (2, 10 and 100 mM) on the incorporation of L-(methyl-^{14}C)methionine in different fractions of phospholipids. Each percentage (control = 100%) represents the mean ±SD of five determinations.

DISCUSSION

The use of methionine labelled either on its methyl or carboxyl group allowed us to characterize certain aspects of methionine metabolism in isolated rat hepatocytes. Figure 4 summarizes the metabolic pathways undergone by methionine in the cell. The methyl-group is mainly incorporated into macromolecules via AdoMet, while carboxyl group is mainly eliminated as CO_2 through transsulfuration and transmethylation pathways. As expected, a significant difference in the distribution of intracellular radioactivity occurs depending on whether (methyl-^{14}C) or (carboxyl-^{14}C) was used. This difference is mainly due to the rapid incorporation of (methyl-^{14}C) into lipids and nucleic acids (Tables 1 and 3). In contrast, radioactivities incorporated into these fractions from (carboxyl-^{14}C) were barely detectable (less than 0.1% of the total radioactivity). The findings concerning the incorporation of the methyl group of methionine into macromolecules are in agreement with the data of Phi and Söling [13] and Hoffman *et al.* [14].

The study of the TCA insoluble material reflect the use of methionine in macromolecule biosynthesis. When (carboxyl-^{14}C) methionine was used as substrate, sufficient amount of radioactivity was only recovered in the protein fraction, although a very low fraction is used for the formation of methionyl tRNA. No incorporation into the lipid pool can be observed. On the other hand, methyl groups are mainly incorporated into lipids and especially in PC, as previously described by Van Phi and Söling [13]. In addition, a significant fraction of (methyl-^{14}C) methionine was utilized for methylation of nucleic acids, via the transmethylation pathway. These results reflect the low rate of protein methylation as compared to phospholipids and nucleic acids.

The same experiments were carried out in the presence of GalN and the effect of this hepatotoxin on different steps of methionine metabolism was studied. Several years ago, Stramentinoli *et al.* [15] showed that GalN induces *in vivo* a decrease in MAT activity accompanied by a decline in liver AdoMet. In the same way, we demonstrated recently, in isolated rat hepatocytes, that GalN induces a decrease of MAT activity and a large drop of ATP levels [7]. In the present work, using methionine labelled on its methyl or carboxyl group, we showed a significant decrease of AdoMet levels in relation to GalN concentration. This decrease of synthesis of AdoMet probably explains the great inhibition of methylation of macromolecules such as nucleic acids and phospholipids. It is likely that the decrease in AdoMet could be due to the large drop of ATP levels induced by GalN [7], since the AdoMet synthesis requires the presence of ATP as adenosyl donor. It is possible that, under these conditions, there is not sufficient available ATP to assure AdoMet biosynthesis, thus leading to an accumulation of methionine. An excessive utilization of AdoMet in polyamine synthesis, for example, could also cause this AdoMet decrease.

These results demonstrated a rapid inhibition of methylation (in less than 2 hr) of macromolecules in isolated hepatocytes by GalN with concentrations as low as 2 mM. These modifications may be secondary to other known effects of GalN in hepatocytes (e.g. uridine trapping, aminoglycogen formation, cell membrane injury, etc.) [3]. It may, also, be related to the decrease of cellular ATP levels that is seen as early as 15 min after the addition of the GalN to the

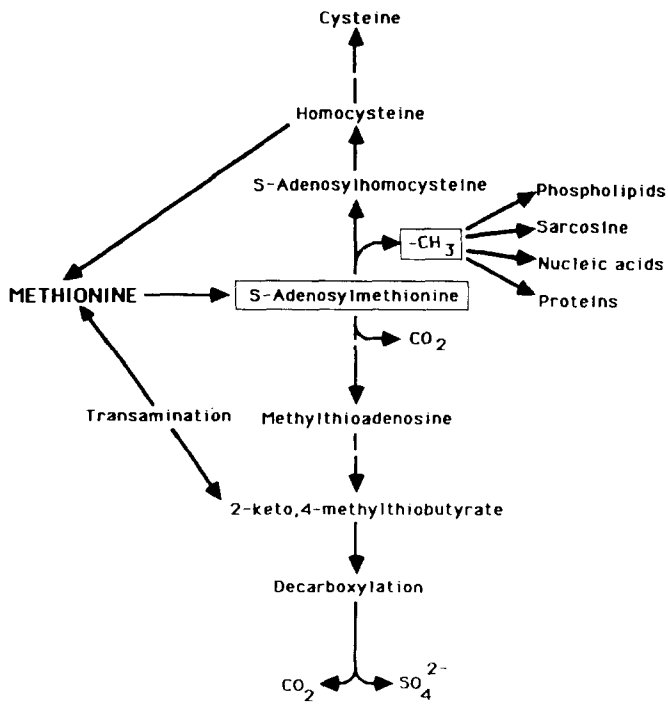

Fig. 4. Metabolites and products referred to in this study.

cell incubation medium [7]. It is difficult to draw conclusions about a major effect by the inhibition of macromolecule methylation on cell injury induced by GalN. For this, further studies are necessary particularly using an *in vivo* model of GalN hepatotoxicity.

On the other hand, our results point out that a hepatotoxic product induces *in vitro* a decrease of AdoMet associated with an accumulation of methionine in hepatocytes. Based upon these results, we hypothesize that hypermethioninemia in human hepatocellular injury could be due, at least partly, to a defect in the use and/or synthesis of AdoMet. If this hypothesis is true, an abnormality in AdoMet synthesis could have numerous consequences in cell metabolism, considering the fundamental role of this metabolite in transmethylation and transsulfuration processes.

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