Okadaic acid-induced, naringin-sensitive phosphorylation of glycine N-methyltransferase in isolated rat hepatocytes

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Glycine N-methyltransferase (GNMT) is an abundant cytosolic enzyme that catalyses the methylation of glycine into sarcosine, coupled with conversion of the methyl donor, *S*-adenosylmethionine (AdoMet), into *S*-adenosylhomocysteine (AdoHcy). GNMT is believed to play a role in monitoring the AdoMet/AdoHcy ratio, and hence the cellular methylation capacity, but regulation of the enzyme itself is not well understood. In the present study, treatment of isolated rat hepatocytes with the protein phosphatase inhibitor okadaic acid, was found to induce an overphosphorylation of GNMT, as shown by proteomic analysis. The analysis comprised two-dimensional gel electrophoretic separation of ³²P-labelled phosphoproteins and identification of individual protein spots by matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry. The identity of GNMT was verified by N-terminal Edman sequencing of tryptic peptides. Chromatographic separation of proteolytic peptides and ³²P-labelled amino acids suggested that GNMT was phosphorylated within a limited region, and only at serine residues. GNMT phosphorylation could be suppressed by naringin, an okadaic acid-antagonistic flavonoid. To assess the possible functional role of GNMT phosphorylation, the effect of okadaic

acid on hepatocytic AdoMet and AdoHcy levels was examined, using HPLC separation for metabolite analysis. Surprisingly, okadaic acid was found to have no effect on the basal levels of AdoMet or AdoHcy. An accelerated AdoMet–AdoHcy flux, induced by the addition of methionine (1 mM), was likewise unaffected by okadaic acid. 5-Aminoimidazole-4-carboxamide riboside, an activator of the hepatocytic AMP-activated protein kinase, similarly induced GNMT phosphorylation without affecting AdoMet and AdoHcy levels. Activation of cAMP-dependent protein kinase by dibutyl-cAMP, reported to cause GNMT phosphorylation under cell-free conditions, also had little effect on hepatocytic AdoMet and AdoHcy levels. Phosphorylation of GNMT would thus seem to play no role in regulation of the intracellular AdoMet/AdoHcy ratio, but could be involved in other GNMT functions, such as the binding of folates or aromatic hydrocarbons.

Key words: cytoprotectant, flavonoid, mass spectrometry, okadaic acid, proteomics, *S*-adenosylhomocysteine, *S*-adenosylmethionine.

INTRODUCTION

Glycine N-methyltransferase (GNMT) is a homotetrameric enzyme [1] that is very abundant in rat liver cytosol, making up 0.5–1.0 % of the soluble hepatocellular proteins [2,3]. GNMT catalyses the methylation of glycine by *S*-adenosylmethionine (AdoMet) to form sarcosine and *S*-adenosylhomocysteine (AdoHcy), as first described in guinea pig liver by Blumenstein and Williams [4]. It has been hypothesized that GNMT continuously regulates the AdoMet/AdoHcy ratio and hence the cellular methylation capacity [2], and that it serves to dispose of excess methyl groups [5]. GNMT is also known as a major cytosolic folate-binding protein [6], and it has been suggested (albeit disputed, cf. [7]) that dimeric GNMT is identical to the 4 *S* benzo[*a*]pyrene-binding protein involved in regulation of the cytochrome P450 1A1 gene [8,9].

GNMT can be phosphorylated *in vitro* by cAMP-dependent protein kinase (PKA), which has been reported to enhance its enzymic activity [10]. The benzo[*a*]pyrene-binding ability of GNMT is also stimulated by protein phosphorylation [8], as can be observed even in intact (rat hepatoma) cells after treatment with

the protein phosphatase inhibitor, okadaic acid [11]. However, it is not known whether the phosphorylation of GNMT affects its enzymic activity and the AdoMet/AdoHcy balance in intact cells.

As part of our studies of protein phosphorylation in isolated rat hepatocytes [12–15], we have been using a proteomic approach, i.e. two-dimensional (2D) gel electrophoresis and mass spectrometric protein identification, to search for phosphoproteins that are sensitive to the okadaic acid-antagonistic effect of naringin, a cytoprotective flavonoid [16]. In the present work, we have identified GNMT as an okadaic acid- and naringin-sensitive phosphoprotein, and used this finding to examine the relationship between GNMT phosphorylation and AdoMet/AdoHcy metabolism in intact hepatocytes.

MATERIALS AND METHODS

Biochemicals

Okadaic acid was purchased from Moana Bioproducts (Hawaii, U.S.A.), and [³²P]orthophosphate (800 mCi/ml) from ICN

Abbreviations used: 2D, two-dimensional; 5-MTHF, 5-methyltetrahydropteroylpentaglutamate; AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; AICAR, 5-aminoimidazole-4-carboxamide riboside; AMPK, AMP-activated protein kinase; CaMK-II, Ca²⁺/calmodulin-dependent protein kinase II; DB-cAMP, dibutyl-cAMP; GNMT, glycine *N*-methyltransferase; HCCA, α -cyano-4-hydroxy-*trans*-cinnamic acid; Hcy, homocysteine; MALDI-TOF-MS, matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry; PKA, cAMP-dependent protein kinase; PVDF, polyvinylidene difluoride; TCA, trichloroacetic acid.

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Pharmaceuticals (Costa Mesa, CA, U.S.A.). 2'-Deoxycoformycin (pentostatin®) was a generous gift from Parke-Davis (Ann Arbor, MI, U.S.A.). Pharmalyte and linear Immobiline dry strips were from Amersham Biosciences (Uppsala, Sweden), TLC plastic sheets (silica gel 60) were from Merck (Darmstadt, Germany), hydrochloric acid (6 M, constant boiling point) from Pierce (Rockford, IL, U.S.A.), polyvinylidene difluoride (PVDF) protein sequencing membrane from Bio-Rad laboratories (Hercules, CA, U.S.A.), and Kodak X-Omat LS film from Kodak (New Haven, CT, U.S.A.). The trypsin used for matrix-assisted laser-desorption ionization-time-of-flight (MALDI-TOF) mass fingerprinting was sequencing grade modified porcine trypsin from Promega (WI, U.S.A.); the acetonitrile was HPLC grade (99.8%) from Fluka Chemie AG (Buchs, Switzerland). All other biochemicals, including naringin, L-homocysteine thiolactone, adenosine, 5-aminoimidazole-4-carboxamide riboside (AICAR), *o*-phospho-L-serine, *o*-phospho-L-threonine, *o*-phospho-L-tyrosine, thermolysin, phenol red, α -cyano-4-hydroxycinnamic acid and trichloroacetic acid (TCA; spectrophotometric grade) were purchased from Sigma (St. Louis, MO, U.S.A.).

Hepatocyte isolation and incubation conditions

Isolated hepatocytes were prepared from 18-h-starved, 250–350 g male Wistar rats by two-step collagenase perfusion [17]. The hepatocytes were incubated, in shaking centrifuge tubes at 37 °C, as 400 μ l aliquots of cell suspension that contained 30–35 mg cellular wet mass. The medium was suspension buffer [17] fortified with 15 mM pyruvate and 2 mM Mg^{2+} .

^{32}P -protein labelling

The hepatocytes were incubated in phosphate-free suspension buffer, to which 500 μ Ci [^{32}P]orthophosphate was added per tube. After incubation for 2 h at 37 °C, four tubes were pooled, the cells were washed twice (5 min at 550 g) in 10% unbuffered, electrolyte-free sucrose, and finally resuspended in 0.5 ml of 10% unbuffered sucrose.

Preparation of cytosol

For preparation of a cytosolic cell fraction, 0.5 ml cell suspension (30–45 mg wet mass in 10% sucrose) was electrodisrupted by a single high-voltage pulse [18,19]. The disrupted cells were mixed with 100 μ l of a homogenization buffer (0.25 M sucrose, 10 mM Hepes, 1 mM EDTA, pH 7.4), containing 100 nM okadaic acid (to prevent dephosphorylation of radiolabelled proteins) and homogenized by 30 strokes in a Dounce homogenizer, with a tight-fitting pestle, at 4 °C. The cell homogenate was centrifuged for 1 h at 50 000 g in a Sorvall SS-34 rotor to obtain a cytosolic fraction.

2D PAGE

A 20 μ l aliquot of cytosol was dissolved in a sample buffer containing ampholytes (2% Pharmalyte, 9 M urea, 65 mM dithiothreitol, 0.5% Triton X-100) and subjected to isoelectric focusing on 18 cm linear Immobiline dry strips, pH 3–10, using the Amersham Bioscience Multiphor II apparatus and procedure. After equilibration in 2% SDS, the strips were placed on top of a vertical SDS/PAGE (10% gel, 18 $\times$ 18 cm) and electrophoresed by use of a BioRad Protean II apparatus. Finally, proteins in the gels were made visually detectable by aldehyde-free silver

staining [20] or by staining with Coomassie Brilliant Blue R-250. Radioactive spots were detected by autoradiography, using Kodak X-Omat LS film and a 24 h exposure. By comparing the staining pattern with an autoradiogram from the same gel, radioactive spots of interest could be unequivocally localized.

MALDI-TOF-MS

A custom-made 2-mm-diameter stainless steel cannula was used to punch out a gel cylinder from the centre of the silver-stained spot corresponding to the okadaic acid- and naringin-sensitive 32 kDa protein. The gel cylinder was dehydrated with 50 μ l acetonitrile for 10 min in an Eppendorf tube. The acetonitrile was then aspirated, and the gel piece was freeze-dried in a SpeedVac AES1010 evaporator (Savant Instruments Inc., Holbrook, NY, U.S.A.). To reduce acrylamidated cysteine sulphhydryl groups, the gel piece was incubated with 30 μ l of 10 mM dithiothreitol in 100 mM NH_4HCO_3 for 1 h at 56 °C. The tube was cooled to 20 °C, the dithiothreitol solution was removed, and the gel piece incubated with 30 μ l of 55 mM iodoacetamide in 100 mM NH_4CO_3 for 45 min at 20 °C in the dark, to carbamidomethylate the cysteine groups. The gel piece was subsequently washed three times for 10 min with 50 μ l of 100 mM NH_4HCO_3 , dehydrated with 50 μ l of acetonitrile, rehydrated with 100 mM NH_4HCO_3 , dehydrated again with acetonitrile, aspirated and Speedvac-dried. The gel piece was then rehydrated at 4 °C for 45 min with just enough trypsin solution (trypsin at 10 μ g/ml in 20 mM NH_4HCO_3 buffer, pH 8.0) to be absorbed by the gel ($\sim 3 \mu$ l/mm²; typically 10 μ l to 3 mm²), and incubated at 37 °C for 16–24 h. Any excess fluid was collected, and the gel piece was extracted once for 20 min at 20 °C with 10 μ l of 20 mM NH_4HCO_3 , and three times with 10 μ l of acetonitrile/formic acid (50%/5%). The pooled supernatants, containing the tryptic peptides, were SpeedVac-dried to a volume of 5–10 μ l.

A saturated solution of rinsed α -cyano-4-hydroxy-*trans*-cinnamic acid (HCCA) was made by vortexing 0.25 ml of HCCA powder with 1 ml of acetone for 10 s in an Eppendorf tube and centrifuging for 1 min. The supernatant was discarded, the procedure was repeated, and the second saturated HCCA supernatant was collected. A matrix solution was prepared by mixing 400 μ l saturated HCCA with 200 μ l of nitrocellulose (20 mg/ml in acetone), 200 μ l of isopropanol and 1 μ l of peptide calibrants (calibration mix 1 from Applied Biosystems, Framingham, MA, U.S.A.; containing des-Arg¹-bradykinin at 904.4681 Da, angiotensin 1 at 1296.6853 Da, Glu¹-fibrinopeptide B at 1570.6774 Da and neurotensin at 1672.9175 Da). Equal parts of matrix and peptide solution (usually 2 μ l of each) were mixed in an Eppendorf tube, and 1 μ l of the mixture was spotted onto a MALDI sample plate and allowed to dry in air. The spot was rinsed for 10 s with 5 μ l of 5% formic acid, then with water, and dried in air.

The MS used was a Voyager DE PRO Biospectrometry Workstation from Applied Biosystems. The mass spectral peak list was analysed by the Protein Prospector datamining program (<http://prospector.ucst.edu>), using the Swiss-Prot database (www.expasy.ch) as a source of protein sequence information.

Protein sequencing

Gel pieces containing the 32 kDa protein of interest were pooled from 42 Coomassie-stained 2D gels and subjected to in-gel digestion with trypsin. The peptide mixture (< 10 pmol) was fractionated by reverse-phase HPLC, using a C18 column (1 $\times$ 250 mm) and an Applied Biosystems Model 173 Capillary

HPLC apparatus. The purified peptides were then screened by MALDI-TOF-MS as a guidance for selection of suitable peptides for sequencing. N-terminal sequencing of ~ 2 pmol peptide was performed at the Rockefeller Protein/DNA Technology Center (NY, U.S.A.) by means of an Applied Biosystems/Perkin Elmer 494 pulsed-liquid Procise-HS sequencer equipped with a capillary 140B PTH analyser for detection of amino acid phenylthiohydantoin derivatives [21].

Phosphopeptide mapping and phosphoamino acid analysis

Gel pieces from four 2D gels were washed twice for 2 h with 50 % methanol, once with deionized water and then any remaining methanol was removed by SpeedVac-drying. A 1 ml aliquot of thermolysin (Sigma type X; 1 mg/ml in 0.1 M NH_4HCO_3 , pH 8.0) was then added for digestion overnight at 30 °C. After hydrolysis, the eluted peptides were transferred to a new glass vial and freeze-dried. The peptides were solubilized in a deionized water/formic acid/glacial acetic acid buffer (1800:44:156, v/v/v; pH 1.9) and separated by TLC on a silica plastic sheet using electrophoresis (750 V, buffer pH 1.9) in the first dimension and chromatography with a deionized water/pyridine/glacial acetic acid buffer (189:10:1, v/v/v; pH 3.5) in the second dimension. Radiolabelled phosphopeptides were detected by autoradiography on Kodak X-Omat LS film. For phosphoamino acid analysis, proteins were transferred to a PVDF membrane. The phosphorylated proteins were cut out, and the membrane pieces transferred to a tube with 6 M HCl for a 2 h hydrolysis at 120 °C. The hydrolysate was SpeedVac-dried and the membrane washed in double-distilled water for pooling and drying of the remaining amino acids together with 15 μl of non-radiolabelled phosphoamino acid standards (phosphoserine, phosphothreonine and phosphotyrosine, each at 1 mg/ml). The amino acids were solubilized in the pH 3.5 buffer and separated on a TLC silica plastic sheet using electrophoresis (750 V, buffer pH 3.5) in the first dimension and TLC (pH 1.9 buffer) in the second dimension. Radiolabelled phosphopeptides were detected by autoradiography on Kodak X-Omat LS film, and the position of the amino acid standards was visualized on the sheets with ninhydrin.

HPLC analysis of AdoMet, AdoHcy and sarcosine

Intracellular AdoMet and AdoHcy levels were measured by reverse-phase HPLC essentially as described by She et al. [22], using a SymmetryTM C₁₈ column (5 μm ; 100 Å; 3.9 $\times$ 150 mm) and HPLC instrument system from Waters (Milford, Mass., U.S.A.). The equipment included a number 486 tunable absorbance detector adjusted to 254 nm, a 717plus autosampler and pump, a 600E system controller, a multisolvent delivery system, and a Millennium³² chromatography manager for data analysis.

For the HPLC analysis, each incubated cell sample (about 30 mg cells) was sedimented (1500 *g* for 5 min), aspirated and resuspended in 100 μl 10 % TCA, and extracted for 15 min at 4 °C. The sedimentation step did not alter the intracellular AdoHcy/AdoMet levels or ratio, the same results being obtained if TCA was added directly to cells incubating at 37 °C. Usually three to six identically treated samples were pooled and centrifuged for 15 min at 3700 *g*; the supernatant was then transferred to a new tube and stored at -20 °C until the measurement day, when 200 μl was injected for analysis. All samples and solutions used in the HPLC system were filtered through a 0.22 μm Millipore filter prior to injection.

Figure 1 Autoradiographic analysis of cytosolic phosphoproteins from rat hepatocytes, separated on 2D polyacrylamide gels

Hepatocytes were incubated for 2 h at 37 °C in the presence of 500 μCi [^{32}P]orthophosphate, without further additions (A) control (CT), (B) with 100 μM naringin (NA), (C) with 30 nM okadaic acid (OA) or, (D) with both okadaic acid and naringin. Cytosol was prepared from the cells as described in the Materials and methods section and fractionated by 2D gel electrophoresis. Autoradiography of the gels shows that several proteins had become overphosphorylated in okadaic acid-treated cells. The 32 kDa phosphoprotein marked with an arrow in each panel was less phosphorylated if the cells had also been incubated with naringin. The experiment was repeated four times with similar results.

The mobile phase was an aqueous buffer containing 40 mM $\text{NH}_4\text{H}_2\text{PO}_4$, 8 mM 1-heptanesulphonic acid (sodium salt) and 18 % (v/v) methanol, pH 3.0 (adjusted by addition of HCl). Elution proceeded for 40 min at a constant flow rate (1 ml/min) with UV detection at 254 nm. Between samples, the column was washed for 15 min at 1.5 ml/min with water/methanol (1:1, v/v), and equilibrated for 5 min at 1.5 ml/min with the mobile phase buffer. The positions of AdoHcy and AdoMet were identified by co-elution with added standards.

Sarcosine was measured by HPLC according to the Waters AccQ-Tag amino acid derivatization method (Millipore, Milford, MA, U.S.A.) in deproteinized hepatocyte extracts concentrated by freeze-drying. A Waters 486 detector was used for measurement of UV absorbance at 254 nm. Intracellular sarcosine levels (usually < 0.5 $\mu\text{mol/g}$ hepatocellular wet mass) could not be reliably measured due to swamping by a > 15 -fold excess of ammonia, eluting in close proximity to sarcosine.

RESULTS

Effects of okadaic acid and naringin on the phosphorylation of cytosolic proteins

Okadaic acid, an inhibitor of the serine/threonine protein phosphatases, PP1 and PP2A, is known to induce a general overphosphorylation of cellular proteins in isolated rat hepatocytes [23]. As shown in Figure 1(C), cytosolic extracts from hepatocytes treated with okadaic acid (30 nM), fractionated by 2D gel electrophoresis, exhibited overphosphorylation of several ^{32}P -labelled cytosolic proteins relative to control cells (Figure 1A).

The okadaic acid-antagonistic flavonoid, naringin [12,16], had no effect on its own (Figure 1B), but, at 100 μ M, was able to partially antagonize the effect of okadaic acid on a few proteins (Figure 1D). A protein with an apparent molecular mass of 32 kDa and a pI of 6.4–6.5 (Figure 1, arrows) was particularly naringin-sensitive, as indicated by four repeated experiments. By comparing the autoradiograms with the corresponding Coomassie Blue-stained gels, the 32 kDa spot could be shown to coincide with an abundant cytosolic protein (Figure 2A, arrows), suitable for identification by MS and sequencing.

MS identification of the okadaic acid- and naringin-sensitive phosphorylated 32 kDa protein as GNMT

From 2D gels, stained with a gentle silver method compatible with MS [20] (Figure 2B), 2-mm-diameter gel cylinders were punched out of the 32 kDa protein spot and trypsinized overnight. The extracted tryptic peptides were then embedded in an α -hydroxycinnamic acid matrix and subjected to MALDI-TOF mass fingerprinting. A typical mass spectrum is shown in Figure 3(A). A global database search (all species included), based on the 19 most prominent peptide mass peaks, identified rat GNMT (EC 2.1.1.20) on the basis of eight matching masses, with an average mass error of ± 15 p.p.m. The corresponding human enzyme was second on the list with five matching masses; no other protein in the database produced more than four matches. Since both the species and the protein molecular mass (given as 32.5 kDa for GNMT) was right, the identification was concluded to be positive.

A secondary screening at a lower mass threshold retrieved another four GNMT-derived peptides (among a total of 28), thus providing an overall sequence coverage of 62 %. The N-terminal region was particularly well covered (Figure 3B). Unfortunately, the MALDI-TOF method does not detect phosphopeptides with sufficient sensitivity to allow any conclusions regarding phosphorylation sites.

Confirmation of the identity of GNMT by amino acid sequencing

The 32 kDa protein spots excised from a number of 2D gels were pooled and subjected to limited tryptic digestion; selected peptides were then sequenced by N-terminal Edman degradation (Figure 3B). One major peptide consisted of 24 sequenced amino acids whilst another peptide gave a 7-amino acid sequence. The amino acid sequences unequivocally confirmed the identity of the 32 kDa protein as GNMT, previously sequenced from rat liver by Ogawa et al. [24]. The major peptide aligned with amino acid residues 213–236, with three indeterminate positions (213, 231, and 236) whilst the minor peptide aligned with amino acid residues 97–103.

Identification of serine as the amino acid phosphorylated in GNMT after okadaic acid treatment

Phosphoamino acid analysis by TLC showed that phosphorylation of GNMT in okadaic acid-treated hepatocytes only involved serine residues (Figure 4B). Furthermore, phosphopeptide mapping analysis of GNMT, using limited digestion with thermolysin, indicated that the protein was phosphorylated only in a single peptide (Figure 4C). This peptide was insignificantly phosphorylated in cells treated with both okadaic acid and naringin (results not shown). Treatment of rat hepatocytes with okadaic acid thus seemed to result in naringin-sensitive serine phosphorylation only within a limited region of GNMT.

Figure 2 2D gel electrophoresis of cytosolic proteins from rat hepatocytes

Cytosolic proteins (1.5 mg) from rat hepatocytes were separated by 2D gel electrophoresis and stained (A) with Coomassie Blue BB R-250, or (B) by the gentle silver method used for MALDI-TOF analysis. The arrows point to the okadaic acid/naringin-sensitive 32 kDa phosphoprotein, isolated from several gels and identified by MALDI-TOF-MS peptide analysis or peptide sequencing as GNMT. The protein was isolated from control (untreated) hepatocytes. MW, molecular mass markers.

Activators of cAMP-dependent or AMP-activated protein kinases induce GNMT phosphorylation

To see if GNMT might be phosphorylated through protein kinase activation as well as by protein phosphatase inhibition, hepatocytes were treated with various protein kinase activators. Among these, AICAR, a specific stimulant of AMP-activated

Figure 3 MALDI-TOF-MS of tryptic peptides from the naringin-sensitive 32 kDa cytosolic phosphoprotein

(A) Peptides characteristic of GNMT (G), autolytic trypsin peptides (T) and molecular mass calibration standards (S). The protein was isolated from control (untreated) hepatocytes. (B) Identification of the okadaic acid- and naringin-sensitive 32 kDa phosphoprotein as rat GNMT by MALDI-TOF-MS and amino acid sequencing. Part of the amino acid sequence of rat GNMT is shown. Full underline, tryptic peptides identified by MALDI-TOF-MS; dotted underline, peptides identified by N-terminal Edman amino acid sequencing.

Figure 4 Phosphoamino acid identification and phosphopeptide mapping of GNMT from rat hepatocytes

^{32}P -labelled GNMT from okadaic acid-treated hepatocytes was excised from four gels, hydrolysed with 1 M HCl for 2 h at 120 °C, and subjected to phosphoamino acid separation by TLC. (A) A ninhydrin-stained TLC plate with the positions of phosphothreonine (PT), phosphotyrosine (PY) and phosphoserine (PS) indicated. (B) An autoradiogram of the same plate as in (A), showing ^{32}P incorporation in phosphoserine only. For phosphopeptide mapping, GNMT was excised from four gels and digested with thermolysin. The resulting peptides were separated with electrophoresis in the first dimension and with chromatography in the second dimension, then subjected to autoradiography. Only a single ^{32}P -labelled peptide was detected (C).

protein kinase (AMPK) [25,26], was found to induce GNMT phosphorylation (Figure 5B). Adenosine, which is converted to AMP intracellularly, had a similar effect (Figure 5C). Adenosine

was administered in the presence of the adenosine deaminase inhibitor, 2'-deoxycoformycin, to prevent its intracellular degradation to inosine [15,27]. Both AICAR and adenosine have previously been shown to elevate the hepatocellular levels of their respective ribotides under these conditions [15,27].

Dibutyl-*c*-AMP (DB-*c*-AMP), an activator of PKA, also induced GNMT phosphorylation, albeit to a lesser extent than adenosine or AICAR (results not shown). Both PKA and AMPK, therefore, appear to cause GNMT phosphorylation, directly or indirectly.

Effects of okadaic acid and protein kinase activators on intracellular levels of AdoMet and AdoHcy

To see if GNMT phosphorylation would detectably affect the activity of the enzyme *in situ*, the levels of its substrate, AdoMet, and its product, AdoHcy, were measured by HPLC in hepatocytes treated with the various agents capable of inducing GNMT phosphorylation. Under basal conditions (no agents added), the intracellular concentration of AdoMet was approx. 40 nmol/g cells (wet weight; Table 1), similar to values previously reported from isolated rat livers and hepatocytes [28,29]. The concentration of AdoHcy was approx. 5 nmol/g cells, likewise in agreement with previous studies [22,28,29]. An attempt to stimulate AdoMet utilization by adding the GNMT substrate, glycine (at 1 or 10 mM), did not alter the levels of AdoMet or AdoHcy or their ratio (Table 1; Figures 6A–6C). Serine (at 1 or 10 mM), required for the further metabolism of homocysteine after AdoHcy cleavage, similarly did not cause any detectable change in the

Figure 5 Phosphorylation of GNMT induced by AICAR and adenosine

Hepatocytes were incubated for 2 h at 37 °C in the presence of 500 μ Ci of 32 P-radiolabelled orthophosphate, without further additions (**A**) control, (**B**) with 0.5 mM AICAR, or (**C**) with 0.5 mM adenosine in the presence of 50 μ M deoxycorformycin. Cytosol was prepared from the cells as described in the Materials and methods and fractionated by 2D gel electrophoresis. The arrows points to GNMT. The experiment was repeated four times with similar results.

Table 1 Intracellular levels of AdoHcy and AdoMet in isolated rat hepatocytes

Isolated rat hepatocytes were incubated for 2 h at 37 °C in the presence of the metabolites and inhibitors indicated. At the end of the incubation, the cells were washed once and extracted with 10 % TCA. The intracellular levels of AdoHcy and AdoMet were measured by HPLC. Values are the means $\pm$ S.E.M., the number of experiments is indicated in parentheses.

Additions during incubation	AdoMet (nmol/g cells)		AdoHcy (nmol/g cells)		AdoMet/AdoHcy ratio	
None	42.8 $\pm$ 5.0	(6)	4.46 $\pm$ 0.41	(6)	9.7 $\pm$ 0.8	(6)
Okadaic acid (30 nM)	44.0 $\pm$ 5.5	(5)	5.63 $\pm$ 0.77	(5)	8.0 $\pm$ 0.4	(5)
Naringin (100 μ M)	39.0 $\pm$ 3.8	(2)	4.77 $\pm$ 0.41	(2)	8.2 $\pm$ 0.1	(2)
Okadaic acid + naringin	32.7 $\pm$ 2.5	(2)	3.69 $\pm$ 0.68	(2)	9.0 $\pm$ 1.0	(2)
AICAR (1 mM)	35.9 $\pm$ 6.2	(2)	5.18 $\pm$ 1.89	(2)	8.5 $\pm$ 4.3	(2)
Dibutyl- γ -cAMP (1 mM)	21.9 $\pm$ 2.2	(3)	3.69 $\pm$ 0.90	(3)	6.4 $\pm$ 1.0	(3)
Adenosine (1 mM)	11.3 $\pm$ 1.3	(2)	68.0 $\pm$ 2.0	(2)	0.17 $\pm$ 0.03	(2)
Adenosine dialdehyde (1 mM)	0	(1)	53.1	(1)	—	
Glycine (1 mM)	42.6 $\pm$ 10.8	(4)	4.28 $\pm$ 1.08	(4)	11.2 $\pm$ 2.4	(4)
Glycine (1 mM) + okadaic acid	46.4 $\pm$ 8.2	(3)	4.86 $\pm$ 0.86	(3)	9.6 $\pm$ 0.3	(3)
Glycine (1 mM) + AICAR	21.8 $\pm$ 12.6	(2)	3.60 $\pm$ 1.62	(2)	9.6 $\pm$ 7.8	(2)
Glycine (1 mM) + dibutyl- γ -cAMP	32.5 $\pm$ 5.7	(2)	3.60 $\pm$ 1.62	(2)	10.4 $\pm$ 3.1	(2)
Glycine (10 mM)	44.4 $\pm$ 13.7	(3)	3.56 $\pm$ 1.26	(3)	12.8 $\pm$ 0.5	(3)
Glycine (10 mM) + okadaic acid	51.9 $\pm$ 20.5	(2)	4.68 $\pm$ 1.89	(2)	11.2 $\pm$ 0.2	(2)
Methionine (1 mM)	184 $\pm$ 50	(5)	35.8 $\pm$ 8.1	(5)	4.9 $\pm$ 0.5	(5)
Met + okadaic acid	192 $\pm$ 57	(4)	36.2 $\pm$ 10.0	(4)	5.1 $\pm$ 0.7	(4)
Met + okadaic acid + naringin	179	(1)	38.9	(1)	4.6	(1)
Met + glycine (1 mM)	81.0 $\pm$ 5.5	(3)	10.4 $\pm$ 1.4	(3)	7.9 $\pm$ 0.6	(3)
Met + glycine (1 mM) + okadaic acid	77.1 $\pm$ 5.7	(2)	9.4 $\pm$ 3.5	(2)	9.3 $\pm$ 2.8	(2)
Met + glycine (10 mM)	76.5 $\pm$ 18.1	(3)	7.7 $\pm$ 2.7	(3)	10.7 $\pm$ 1.3	(3)
Met + glycine (10 mM) + okadaic acid	77.0 $\pm$ 31.5	(2)	10.6 $\pm$ 6.0	(2)	8.3 $\pm$ 1.7	(2)

AdoMet/AdoHcy levels or ratio (Figures 6D and 6E). In contrast, the inclusion of methionine (1 mM), alone or in addition to glycine, stimulated the AdoMet–AdoHcy flux as revealed by an elevation of both metabolites (Table 1 and Figures 6F and 6G).

The intracellular steady-state levels of AdoMet and AdoHcy remained relatively stable during incubation of the cells for 2 h at 37 °C under either low-flux conditions (Figures 6A–6C) or high-flux conditions (in the presence of methionine; Figures 6E and 6F). Hepatocellular levels of sarcosine, the metabolite formed by GNMT-catalysed transfer of methyl from AdoMet to glycine (Scheme 1), were, unfortunately, too low to be reliably measured by HPLC analysis of derivatized amino acids.

Although GNMT is but one among many methyltransferases that convert AdoMet to AdoHcy, on the basis of its high intracellular concentration, it would be expected to make a measurable

impact on the overall AdoMet/AdoHcy flux and ratio [27]. However, the GNMT phosphorylation stimulant, okadaic acid (30 nM), had no detectable effect on the hepatocellular levels of AdoMet or AdoHcy (Figures 7A and 7B). The hepatocellular levels of AdoMet or AdoHcy were also unaffected by the okadaic acid antagonist, naringin, either in the presence or absence of okadaic acid (Table 1). Higher concentrations of okadaic acid (100 μ M or 1 mM) were, likewise, without effect (results not shown). The AdoMet/AdoHcy ratio was not altered by either okadaic acid or naringin (Table 1), indicating that the cellular methylation capacity remained unaffected.

Since okadaic acid strongly suppresses autophagic protein degradation [12], and hence the release of glycine from degraded protein, one could imagine that the intracellular supply of glycine might become rate-limiting for the GNMT reaction in the presence

Figure 6 Hepatocellular levels of AdoMet and AdoHcy during incubation with amino acids involved in their metabolism

Freshly prepared rat hepatocytes were analysed without incubation (dotted lines), or after incubation for 1 h (dashed lines) or 2 h (unbroken line) at 37 °C with (A) no additions (CONTROL), (B) 1 mM glycine (GLY 1 mM), (C) 10 mM glycine (GLY 10 mM), (D) 1 mM serine (SER 1 mM), (E) 10 mM serine (SER 10 mM), (F) 1 mM methionine plus 1 mM glycine (MET 1 mM + GLY 1 mM), (G) 1 mM methionine plus 10 mM glycine (MET 1 mM + GLY 10 mM). After incubation, the cells were homogenized in 10% TCA, and the acid extracts fractionated by HPLC.

Figure 7 Effects of glycine and methionine on AdoMet and AdoHcy levels and okadaic acid responsiveness

The effect of okadaic acid on hepatocellular levels of AdoHcy and AdoMet was examined after 2 h of incubation at 37 °C in the absence of added amino acids (A) without OA (CONTROL), (B) with OA (OA 30 nM), (C) without OA and with 1 mM glycine (GLY 1 mM), (D) with 30 mM OA and 1 mM glycine (GLY 1 mM + OA), (E) without OA and with 10 mM glycine (GLY 10 mM), (F) with 30 mM OA and 10 mM glycine (GLY 10 mM + OA), (G) without OA and with 1 mM methionine (MET 1 mM), (H) with 30 mM OA and 1 mM methionine (MET 1 mM + OA), (I) without OA, with 1 mM methionine and 1 mM glycine (MET + GLY 1 mM), (J) with 30 mM OA, 1 mM methionine and 1 mM glycine (MET + OA + GLY 1 mM), (K) without OA, with 1 mM methionine and 10 mM glycine (MET + GLY 10 mM), (L) with 30 mM OA, 1 mM methionine and 10 mM glycine (MET + OA + GLY 10 mM). After incubation, the cells were homogenized in 10% TCA, and the acid extracts fractionated by HPLC.

Scheme 1 Metabolic flow through GNMT

Ado, adenosine; AdoDi, adenosine dialdehyde; AdoHcy (SAH), S-adenosylhomocysteine; AdoMet (SAM), S-adenosylmethionine; AHC, adenosylhomocysteinase; MeX, methylated X; MTs, methyltransferases; Nar, naringin; Sarc, sarcosine; X, any methyltransferase substrate.

of the drug. However, the effect of okadaic acid on AdoMet or AdoHcy levels in the presence of 1 or 10 mM glycine was undetectable (Figures 7C–7F and Table 1). The levels or ratio

of these metabolites under high-flux conditions (induced by the addition of methionine) were also unaffected by okadaic acid, either in the presence or absence of a glycine supplement (Figures 7G–7L and Table 1). Thus, the detectability of a phosphorylation-induced change in GNMT activity did not seem to be limited by the availability of the enzyme substrates AdoMet and glycine.

AICAR, which, like okadaic acid, stimulates GNMT phosphorylation, had little or no effect on the levels of AdoMet and AdoHcy (Figures 8A and 8B and Table 1). The levels of the two metabolites were, likewise, only slightly affected by DB-cAMP (Figure 8C), despite its ability to stimulate GNMT phosphorylation in intact cells (present study) as well as under cell-free conditions [10]. Phosphorylation of GNMT by these agents would thus seem to have no significant influence on intracellular AdoMet and AdoHcy levels. Although adenosine, another inducer of GNMT phosphorylation, raised the level of AdoHcy but not the level of AdoMet (Table 1), this accumulation is more likely to reflect the role of adenosine as a direct AdoHcy precursor and an inhibitor of further AdoHcy metabolism [30], than an effect on the flux through GNMT. In fact, adenosine

Figure 8 Effects of AICAR and DB-cAMP on hepatocellular AdoMet and AdoHcy levels under various metabolic flux conditions

Effects of AICAR (1 mM) or DB-cAMP (1 mM) on hepatocellular AdoHcy and AdoMet levels were examined after 2 h of incubation at 37 °C in the absence of added amino acids and with (A) nothing (CONTROL), (B) 1 mM AICAR (AICAR 1 mM), (C) 1 mM DB-cAMP (DB-cAMP 1 mM); or with 1 mM glycine plus (D) nothing (GLY 1 mM), (E) 1 mM AICAR (AICAR 1 mM + GLY 1 mM), (F) 1 mM DB-cAMP (DB-cAMP 1 mM + GLY 1 mM); or with 10 mM glycine plus (G) nothing (GLY 10 mM), (H) 1 mM AICAR (AICAR 1 mM + GLY 10 mM), (I) 1 mM DB-cAMP (DB-cAMP 1 mM + GLY 10 mM); or 1 mM methionine plus (J) 1 mM glycine (MET 1 mM + GLY 1 mM), (K) 1 mM AICAR plus 1 mM glycine (AICAR 1 mM + MET 1 mM + GLY 1 mM), (L) 1 mM DB-cAMP plus 1 mM glycine (DB-cAMP 1 mM + MET 1 mM + GLY 1 mM). After incubation, the cells were homogenized in 10% TCA, and the acid extracts fractionated by HPLC.

dialdehyde, a specific inhibitor of adenosylhomocysteinase [31], similarly elevated the level of AdoHcy but not that of AdoMet (Table 1).

Since okadaic acid, AICAR and DB-cAMP are all inhibitors of hepatocytic autophagy [12–15], the possibility was considered that they could inhibit the flux through GNMT by reducing the endogenous supply of glycine from autophagic protein degradation, thereby masking any phosphorylation-dependent activation of the enzyme. However, the addition of glycine at 1 mM (Figures 8D–8F) or 10 mM (Figures 8G–8I) did not affect the basal levels of AdoMet or AdoHcy, nor significantly alter the lack of response to AICAR or DB-cAMP (cf. also Table 1). The inclusion of methionine (1 mM) to raise AdoMet and AdoHcy levels similarly failed to induce any responsiveness towards AICAR or DB-cAMP (Figures 8J–8L). Thus, neither glycine nor AdoMet would seem to be rate-limiting for the detection of a GNMT-activity change following AICAR- or DB-cAMP-induced phosphorylation of the enzyme.

DISCUSSION

The present study shows that GNMT becomes phosphorylated in isolated hepatocytes after treatment with a protein phosphatase

inhibitor, okadaic acid, as well as after treatment with activators of protein kinases like AMPK and PKA. Since serine was the only amino acid phosphorylated after okadaic acid treatment, the phosphorylation site(s) would have to be found among the 18 serine residues present in GNMT [24]. The amino acid sequence surrounding Ser-10 (Val-Tyr-Arg-Thr-Arg-Ser-Leu-Gly-Val-Ala-Ala) could possibly qualify as a recognition site for AMPK as well as for PKA [32], the latter enzyme being capable of phosphorylating GNMT in intact hepatocytes (present study) as well as under cell-free conditions [10]. The GNMT phosphorylation observed after okadaic acid is, however, unlikely to be mediated by PKA, since it was so effectively antagonized by naringin. This flavonoid, believed to be a protein kinase inhibitor [12,16], is only moderately antagonistic against the biological effects of PKA-activating cAMP analogues [13]. AMPK, on the other hand, was recently shown to be a naringin-sensitive enzyme [33], and thus remains a plausible GNMT kinase candidate.

GNMT catalyses the conversion of glycine to sarcosine, using AdoMet as a methyl donor. Since sarcosine has no known biological role, it was thought that the real function of GNMT is to regulate biological methylations by controlling the cellular AdoMet/AdoHcy ratio [2]. If this were the case, GNMT phosphorylation, which has been shown to enhance the catalytic activity of GNMT under cell-free conditions [10], would be expected to alter the AdoMet/AdoHcy ratio in intact cells. GNMT is known to be partially phosphorylated in intact cells [10], and its phosphorylation can be enhanced, e.g. by treatment with the protein phosphatase inhibitor, okadaic acid [11]. However, the present study has demonstrated that neither okadaic acid nor other agents capable of inducing GNMT phosphorylation in intact rat hepatocytes (AICAR, DB-cAMP) were able to alter the intracellular concentrations of, or the ratio between, AdoMet and AdoHcy. These results would suggest either that the metabolic flux through GNMT is unaffected by the phosphorylation state of the enzyme, or that the contribution by GNMT to the overall AdoMet flux is quantitatively insignificant. The fact that hepatocellular sarcosine levels were too low to be detectable by standard HPLC analysis would tend to indicate that the enzymic activity of GNMT *in situ* is low. GNMT phosphorylation would thus seem to play no part in the regulation of cellular methylation by AdoMet and AdoHcy.

It is possible, however, that GNMT may influence cellular methyl metabolism by virtue of its function as a major cytosolic folate binding protein [6]. GNMT binds tetrahydrofolate in the form of 5-methyltetrahydropteroylpentaglutamate (5-MTHF), which can serve as a methyl donor in various biological methylations [34]. 5-MTHF is also an effective inhibitor of the enzymic activity of GNMT, regardless of the phosphorylation state of the enzyme [10,35]. This inhibition may conceivably account for the apparent inactivity of GNMT in intact hepatocytes. Furthermore, the phosphorylation-independent inhibitory effect of 5-MTHF suggests that phosphorylation does not substantially impair the ability of GNMT to bind this folate. If anything, phosphorylation should increase the ability of GNMT to bind 5-MTHF, as is the case with its ability to bind aromatic hydrocarbons [8,11]. However, in the absence of any correlative evidence, the functional significance of GNMT phosphorylation will have to remain an open question.

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