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INSULIN-DEPENDENT SUPPRESSION IN GLUTAMYL HYDROLASE ACTIVITY AND ELEVATED CELLULAR METHOTREXATE POLYGLUTAMATES

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Abstract—Folates and antifolates are converted to polyglutamates, which are better retained in cells and may also bind more tightly to cellular proteins than the parent compounds. The regulation of the process of polyglutamate formation and breakdown is not fully clarified yet and is being studied by a number of approaches. An early observation concerning the potential regulation of polyglutamate formation was that insulin caused a marked increase in the rate and accumulation of polyglutamates of methotrexate (MTX) in rat hepatoma cells. The present study demonstrated that insulin caused a decrease in the activity of γ-glutamyl hydrolase (GH), the enzyme that degrades polyglutamates, that was inversely commensurate with the increase in the synthesis of MTX polyglutamates. The effects of insulin on GH activity with regard to concentration, time of onset, and the effect of N^6, $O^{2'}$ dibutyryl cAMP and theophylline were consistent with the reduction in GH being responsible for the increase in cellular MTX polyglutamate accumulation. Insulin addition also led to an increase in folate polyglutamates. The insulin effects were also seen with H35D cells, a subline with enhanced glutamyl hydrolase activity as a result of having been made resistant to 5, 10-dideazatetrahydrofolic acid. When H35 cells with insulin were compared with H35D cells lacking insulin, there was an 8-fold increase in GH and a 44-fold decrease in the number of γ-glutamate residues added to MTX.

Key words: glutamyl hydrolase; folylpolyglutamates; methotrexate polyglutamate; insulin; hepatic; polyglutamylation

Conversion of classical antifolates to their polyglutamate derivatives is one of the central components of the activity of these drugs due to the selective retention of the polyglutamates by cells [1]. The activity of certain antifolates such as D1694† [2] and DDATHF [3], which inhibit thymidylate synthase (EC 2.1.1.45) and glycinamide ribonucleotide formyl transferase (EC 2.1.2.2), respectively, is increased further because the polyglutamate derivatives bind more tightly to the target enzyme than the parent drugs. The synthesis of the polyglutamate derivatives is catalyzed by the cytosolic enzyme FPGS (EC 6.3.2.17) and their degradation by the lysosomal enzyme GH (EC 3.4.22.12) [4, 5]. The amount of antifolylpolyglutamates in cells is determined by the relative activities of these two enzymes. Numerous studies have been conducted to determine the factors that affect the two enzymes and play a role in defining polyglutamate accumulation. These include studies of transport systems, the role of folate pools, the binding of polyglutamates to cellular constituents, the relative catalytic activities of FPGS and GH and their subcellular location, the substrate affinities for each of these enzymes, and the growth state of the cells.

One observation made concerning the potential regulation of antifolyl- and folylpolyglutamate formation was that insulin causes a marked increase in the rate and accumulation of polyglutamates of MTX [6–11]. These observations were made chiefly in rat hepatoma [8, 9, 11] and human breast cell lines [6, 7, 10]. Detailed studies with H35 hepatoma cells demonstrated that an approximately 2-fold increase in glutamylation occurred in the absence of a change in the activity of FPGS [11]. The present study showed that insulin caused a decreased in GH that was inversely commensurate with the change in cellular MTX polyglutamate synthesis and accumulation. The results were compared with wild-type H35 cells and with a subline (H35D) that has elevated GH and reduced polyglutamate formation [12]. The role of GH in the process of cellular control of polyglutamate accumulation is discussed. A preliminary abstract of some of these data has been presented [13].

MATERIALS AND METHODS

Materials

All culture media and sera were obtained from the Grand Island Biological Co. (Grand Island, NY). Insulin was provided by Eli Lilly & Co. (Indianapolis, IN). DDATHF was provided by Dr. S. S. Kerwar of Lederle Laboratories (Pearl River, NY). [3′,5′,7-^{3}H]MTX and [3′,5′,7,9-^{3}H]folic acid were purchased from Moravek Biochemicals, Inc. (Brea, CA) and purified by means of a DEAE column prior to use [1]. All other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO).

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† Abbreviations: D1694, 2-desamino-2-methyl-5,8-dideazafolic acid; DDATHF, 5,10-dideazatetrahydrofolic acid; FPGS, folylpolyglutamate synthetase; GH, γ-glutamyl hydrolase; MTX, methotrexate; 4-NH_2-10-CH_3PteGlu$_n$, where n is the number of added γ-glutamyl residues; PABAGlu$_n$, *p*-aminobenzoylglutamate, where n is the number of added γ-glutamyl residues; FBS, fetal bovine serum; and dBcAMP, N^6, $O^{2'}$-dibutyryl cAMP.

Cell culture

H35 rat hepatoma cells were maintained in monolayer culture in Swim's medium containing 20% horse serum and 5% FBS as described previously [1]. H35D cells, the DDATHF-resistant subline with increased GH [12], were maintained in the presence of 21 μM DDATHF and subcultured in the absence of drug for 1 week prior to the experiment.

GH assay

GH activity of cell extracts was measured as described previously [12, 14]. Cell extraction was done in the presence or absence of 0.1% Triton X-100. 4-NH_2-10-CH_3PteGlu$_2$ was used as the substrate at a 50 μM concentration in the presence of 100 mM mercaptoethanol in 0.2 M Tris acetate buffer (pH 6).

Metabolism of 4-NH_2-10-CH_3PteGlu

Cells were grown as above for 72 hr and then were placed in folate-free Swim's medium in the presence or absence of insulin (10 mU/mL or 57 nM) for the next 24 hr. The cells were then incubated with 10 μM [^{3}H]MTX for 4 hr. The metabolites were analyzed by HPLC with acetonitrile gradient as previously described [12].

Cellular folylpolyglutamate chain length

Cells were grown in Swim's medium containing 10% FBS with 4 μM [^{3}H]folic acid for 1 week and then were subcultured in the same medium for 96 hr. Cells were extracted with 0.1 N HCl and treated to form PABAGlu$_n$ according to the method of Shane [15]. PABAGlu$_n$ was analyzed by HPLC using a Partisil SAX column [16].

RESULTS AND DISCUSSION

H35 cells were cultured under conditions that were identical to those used to demonstrate the effect of insulin on the glutamylation of MTX [8, 9]. Although we have shown previously that insulin causes a decrease in GH activity in the cell extract, the previous study did not deal with subcellular compartmentation of the enzyme [14]. It evaluated only the GH extracted from the cytosol. Since GH has been shown to be a lysosomal enzyme in many mammalian cells [4] including H35 cells [13], Triton X-100 treatment was included to release all compartmentalized enzyme [17, 18]. Insulin caused a marked reduction in the measured GH in cell extracts (Table 1). As expected, Triton X-100-treated extracts showed considerably more activity, which was reduced by 50% when insulin was added to the culture medium. The reduction in GH activity was inversely related to the increase in MTX polyglutamates that is observed in the presence of insulin [8, 9, 11]. Following our original report that the activity of FPGS is unaltered by insulin [11], we have verified this repeatedly under different experimental conditions.*

Table 1. Effect of insulin on GH activity in H35 cell extracts

Insulin (mU/mL)	GH activity (nmol/hr/mg)	
	Minus Triton X-100	Plus Triton X-100
None	2.9 ± 0.2	9.1 ± 0.8
10	0.8 ± 0.03	4.5 ± 0.6

Cells were cultured in Swim's medium with serum as described in Materials and Methods for 72 hr. They were then placed in serum-free Swim's with or without 10 mU insulin/mL (57 nM) for 24 hr, extracted with 0.2 M Tris acetate buffer (pH 6.0) in the presence or absence of 0.1% Triton X-100, and assayed for GH. Results are means ± SD (N = 4).

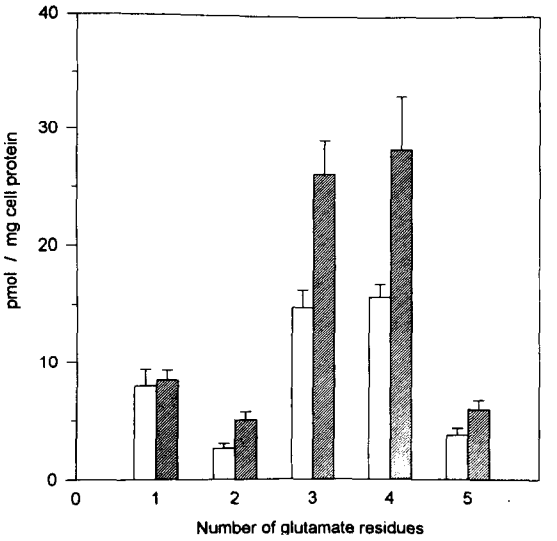

Fig. 1. Effect of insulin on MTX glutamate chain length in H35 cells. H35 cells were grown, and MTX polyglutamates were measured as described in Materials and Methods. The effects of the absence (open bar) and presence (shaded bar) of insulin were measured in triplicate samples (mean ± SD).

Next, the effect of insulin on the MTX polyglutamate profile was evaluated. In this experiment, insulin caused a 75% increase in total MTX polyglutamates. This effect was achieved by an increase in all species of polyglutamates (Fig. 1). To see whether the same effect was extended to cellular folylpolyglutamates, H35 cells were incubated with [^{3}H]folic acid, and the effect of insulin was evaluated. The results were qualitatively similar to those with MTX, with approximately a 40% increase in total folylpolyglutamates (Fig. 2). This was accomplished by large increases in the penta- and hexaglutamates and little significant change in the tetra- and heptaglutamates. The diminished effect on cellular folylpolyglutamates relative to MTX polyglutamates may be due to several factors. There is an extensive opportunity for macromolecular binding and compartmentalization of folates within mammalian cells [19], making them inaccessible to GH. Studies from this laboratory have shown that protein-bound poly-γ-glutamates are not hydrolyzed by GH from H35 cells [20]. It may also be related to the specificity of GH for different substrates. In addition, the effect is measured after the folates have achieved equilibrium within the cells, whereas with MTX it is during the synthetic phase. Further studies evaluating these possibilities are underway.

We have evaluated previously the time and concentration dependence of the effect of insulin on MTX polyglutamate formation [9]. It was found that the rate of glutamylation increases within a few hours after insulin

* Unpublished data.

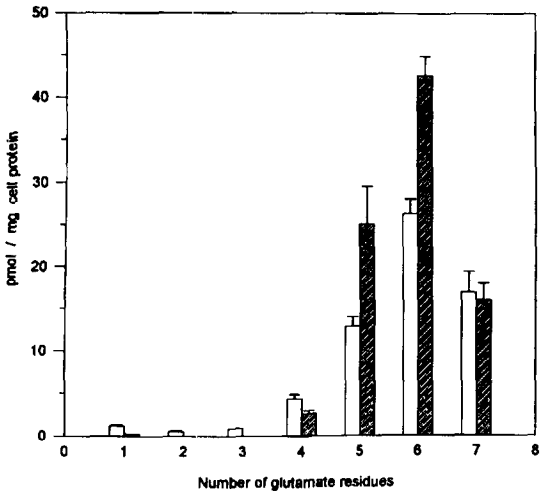

Fig. 2. Effect of insulin on glutamate chain length of folate in H35 cells. Cells were cultured, and folylpolyglutamate chain length was measured as described in Materials and Methods. During the last 24 hr of culture, the serum was removed and insulin was included (shaded bar) or omitted (open bar). Data are means ± SD (N = 4).

addition [9]. A comparison of MTX polyglutamate amounts versus GH activity was measured as a function of the time of incubation with insulin (Fig. 3). The response of the levels of GH activity was measured as a function of time after insulin addition. These data showed that the changes occurring in GH activity bear a temporal correlation to the changes observed earlier with the rate of glutamylation. We had also evaluated the dependence of MTX glutamylation on insulin concentration. Cultures that have been deprived of serum for 24 hr and then incubated with insulin for 16 hr have little response to 0.1 nM insulin, an approximately 50% increase to 10 nM insulin, and a 100% increase to 57 nM [9]. The same study was done with the response of GH to insulin. Baseline values in the absence of insulin were given a value of 0% and the complete decrease seen with 100 nM insulin was given a value of 100%. Insulin at 0.1 nM produced no change, 10 nM caused a 67% decrease, and 57 nM (10 mU/mL) a 100% decrease. Thus, the concentration response of enhanced glutamylation and reduced GH to insulin correlated well.

Earlier studies had also shown that the effects of insulin on glutamylation could be repressed by including dBcAMP and theophylline, which are inhibitors of the signal transduction pathways of the hormone, during the incubation of the H35 cells with insulin. The effect of dBcAMP and theophylline on control cultures was a modest reduction in GH activity, but their effect on insulin-treated cultures was more profound (Table 2). In the latter case, a marked prevention of the insulin-induced reduction of GH activity was observed, which was consistent with the reduction in glutamylation that accompanies dBcAMP and theophylline addition to insulin-treated cultures.

It is shown above that cellular polyglutamate content and GH activity have an inverse relationship. In the past, attempts to demonstrate this have been frustrated because of the inability to vary GH activity due to a lack of specific inhibitors of GH and also of mutant cells lacking the enzyme activity. The effects of DDATHF resistance and of insulin in the H35 cell system allow an evaluation of this relationship over a much broader range of GH activity in H35 cells. We have developed antifolate-resistant cells with elevated levels of GH activity and no change in FPGS [12] and also found that insulin modulates GH activity in resistant cells. By evaluating a combination of these variables (Fig. 4), an 8-fold variation of GH in cell extracts caused a 44-fold alteration in the number of glutamate residues added to MTX. As expected, the relationship is the opposite of that observed for FPGS. Human CHO cells lacking FPGS have been transfected with the FPGS gene to evaluate the relationship of FPGS to the glutamylation of MTX. In that case, a 7-fold increase in the enzyme caused approximately a 10-fold increase in MTX polyglutamates following a 24-hr incubation and an 18-hr efflux period [21]. Some limitations exist in making comparisons between differ-

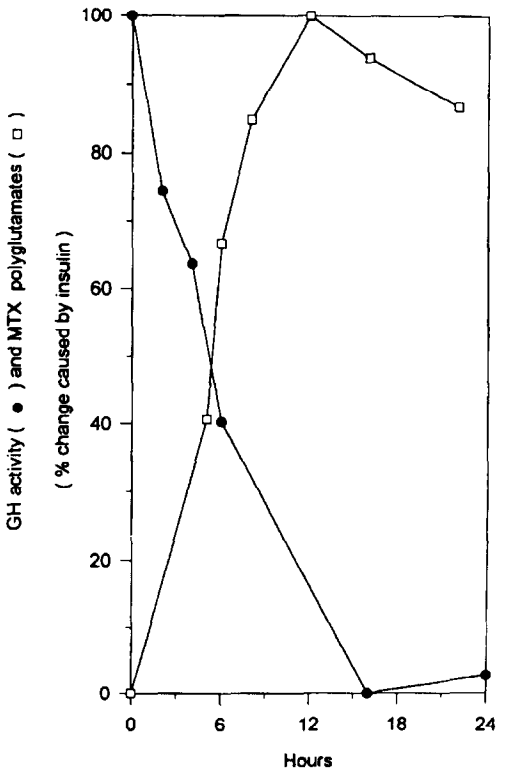

Fig. 3. Effect of the time of incubation with insulin on GH and polyglutamate synthesis. H35 cells were grown in Swim's medium containing 20% horse serum and 5% FBS for 72 hr followed by Swim's medium without serum for the next 24 hr. Cells were then exposed to insulin (10 mU/mL) for varying times, and all cultures were extracted with 0.2 M Tris acetate buffer containing 0.1% Triton X-100 after 120 hr of culture. GH activity was assayed using 50 μM 4-NH_2-10-CH_3PteGlu$_2$ as substrate. GH activity of control samples with no insulin was given a value of 100% (9.6 nmol/mg/hr) and the lowest level of GH, seen at 16 hr was 0% (3.8 nmol/mg/hr). Cellular MTX polyglutamates as a function of time of insulin exposure were measured as described in Materials and Methods. Time includes the preincubation of the cells with insulin plus the 4-hr incubation with insulin and [^{3}H]MTX after which the cells were sampled for polyglutamates. The control for cellular MTX polyglutamates (minus insulin) was given a value of 0% (28.5 nmol/g/4 hr) and the maximal increase observed at 12 hr was given a value of 100% (78.0 nmol/g/4 hr). All values are averages of duplicate observations.

Table 2. Effect of dBcAMP and theophylline on the insulin-dependent reduction in GH

Insulin (mU/mL)	dBcAMP (mM)	Theophylline (mM)	GH activity (nmol/hr/mg)
0	0	0	9.6
0	2	0.1	8.6
10	0	0	5.0
10	2	0.1	8.3

The experiment was conducted as described in Table 1 utilizing Triton X-100 extraction except that the cultures were placed in Swim's medium lacking serum for 24 hr prior to the addition of the 24-hr incubation with insulin (57 nM), $N^6,O^{2'}$-dibutyryl cAMP (dBcAMP 2 mM), and theophylline (0.1 mM). Results are averages of duplicate cultures that were assayed in duplicate.

ent cell systems, and this should be recognized. In the case of CHO and H35 cells, each has nearly the same GH activity [12, 21], but FPGS is several-fold higher in the hepatoma cells [21, 22]. Other factors could also influence these complex processes.

Evaluation of the role of GH in regulating polyglutamate formation may be more complicated than that of FPGS. It is a lysosomal enzyme in many systems [4], so that its compartmentation plays a role in the availability of substrates. Centrifugal isolation of lysosomes shows that GH is clearly lysosomal in H35 cells [13]. Recently, we found that its subcellular distribution is unaltered in H35 and H35D cells in response to insulin when measured by differential centrifugation.* Further studies are underway to understand the dynamics of lysosomal–polyglutamate interactions in the H35 system. Sirotnak and coworkers have already studied this in sarcoma cells and found a carrier-mediated transport system for entry of polyglutamates with lysosomes [23]. In that system, both the lysosomal transport system and the state of activity of GH appear to be rate-limiting [17]. In the H35 system, the mechanisms of altered GH activity as a result of insulin and acquired DDATHF resistance are not yet resolved. Adding to these features is the fact that large amounts of the enzyme are secreted in cell culture systems, including the H35 cells [14]. These problems are under active scrutiny and may yield further clues to the control of polyglutamate accumulation and to understanding GH.

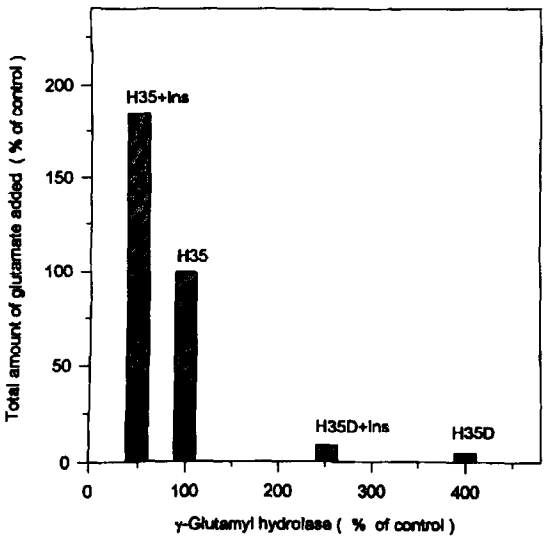

Fig. 4. Effect of insulin on γ-glutamyl hydrolase activity and total amount of glutamate residues added to MTX in H35 and H35D cells. Normalized data are from the same experiment utilizing H35 and H35D cells, each in the presence and absence of insulin. The experiment was constructed with GH measured as described in Table 1 following Triton X-100 extraction and the glutamylation of MTX measured as described in Fig. 1. The control for H35 cells (no insulin) was given a value of 100% and had absolute values of 9.1 nmol/hr/mg cell extract for GH and 73.3 pmol Glu added to MTX/mg cell protein/4 hr. Results are the averages of triplicate measurements of one experiment. The standard deviation for GH activity was between 7.5 and 10.8% of the mean and for glutamate added was between 6.2 and 13.3% of the mean.

One observation that requires further evaluation is the effect of insulin on MTX polyglutamate turnover. An earlier study showed that turnover, which occurs at approximately 50% the rate of synthesis of MTX polyglutamates, is increased by the presence of insulin [8]. Since insulin reduces GH activity, it would be expected to decrease turnover, and that does not appear to be the case. However, insulin alters numerous cellular events, and one that we do not currently understand may be impinging on this process. In the turnover experiment, the cells had been equilibrated with MTX for 24 hr which may affect FPGS or GH activities or other processes that alter turnover. Additionally, the amount of GH in the cell exceeds the activity of FPGS by approximately 10-fold. Thus, the sequestering of GH by lysosomes [13] and secretion [14] must play an important role in altering its effects on MTX polyglutamates. Any subtle changes in substrate availability caused by prolonged exposure to MTX could alter the balance of the effect of GH. An additional factor to be considered is the fact that GH acts only on free and not bound polyglutamates [20]. In the turnover experiment, the amount of MTX polyglutamates in the insulin-treated cells exceeded the amount in control cells. If large amounts of the MTX polyglutamates are bound, then the amount of free material may be proportionately much greater in insulin-treated cells. Under these conditions, even with reduced hydrolase, greater turnover may occur due to the greater availability of substrates. This could be a major contributor if the polyglutamate concentration is in the range of the K_m for the substrates. As we continue the study to understand the mechanism of the effect of insulin on GH, further studies will be needed to understand the results of the turnover study.

In summary, the present results have shown that a decrease in the activity of GH appears to be the reason for enhanced polyglutamylation of MTX in response to insulin in the H35 cell system. The same conditions, insulin addition with a resulting decrease in GH, also lead to an increase in folylpolyglutamates. The effect of GH decrease on the glutamylations of MTX correlates with regard to the time of addition and concentration of this hormone and also the effects of compounds that block the effects of insulin. By the utilization of two parameters that alter GH activity in the H35 cell system,

* Unpublished data.

insulin and acquired drug resistance, an 8-fold change in GH results in a 44-fold inverse change in the number of glutamate residues added to MTX.

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