Regulation of Glucosamine Synthesis in Injury and Partial Hepatectomy[1] (37542)

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Hexosamines play a central role in the structure of extracellular glycoproteins and glycosaminoglycans and in the glycolipid and glycoprotein components of cell membranes. The key enzyme involved in hexosamine synthesis is glucosamine synthetase (L-glutamine:D-fructose-6-phosphate aminotransferase, EC 2.6.1.16), the first enzyme in the pathway and the rate limiting step (1). Kornfeld *et al.* (2) and Kornfeld (3) were the first to show that glucosamine synthetase is subject to strong feedback inhibition by the end product, uridine diphosphate-*N*-acetylglucosamine (UDPAG), *in vitro* and probably *in vivo*. Subsequent work (4–9) has shown that the inhibitory action of UDPAG is markedly influenced by the concentrations of other intracellular metabolites.

Winterburn and Phelps (6, 7) and Hardingham and Phelps (10) have calculated that in liver and skin the normal steady-state concentration of UDPAG is such that glucosamine synthetase must be feedback inhibited by 90%. In spite of this large reserve of latent activity, there is evidence that under conditions of increased hexosamine demand induction of new glucosamine synthetase protein occurs. Shönhöfer and Anspach (11), for example, observed increased glucosamine synthetase activities in liver homogenates of rats injected with carageenin, and Nishii (12) reported similar increases following partial hepatectomy, adrenalectomy and the intraperitoneal transplantation of cancer cells. Chandler, Okubo and Bley (13) and also Akamatsu and Maeda (14) have reported that the increases following stress (laparotomy) and partial hepatectomy can be blocked with actinomycin D and cycloheximide. All of the manipulations mentioned above stimulate the synthesis of hepatically produced plasma glycoproteins (15, 16) and presumably increase the demand on the hexosamine pool.

The present study was carried out to gain a better understanding of the interrelationships between glucosamine synthetase activity and UDPAG pool size by measuring both simultaneously in rat liver extracts as a function of time following laparotomy or partial hepatectomy and after the administration of actinomycin D and cycloheximide.

Materials and Methods. Male, albino rats, 150–200 g, were purchased from Sprague-Dawley, Incorp., Madison, WI and were maintained on standard laboratory chow for at least 1 wk prior to use. Partial hepatectomies were performed according to the procedure of Higgins and Anderson (17) and injuries (laparotomies) were performed in the same manner, but stopped short of ligation and removal of liver lobes. Following surgery, the animals were maintained *ad libitum* on standard chow and water.

At selected time intervals following surgery, the rats were anesthetized with ether and the liver was exposed through an abdominal incision. The liver was perfused via the portal vein with an ice-cold solution of

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0.15 *M* KCl, 1 m*M* EDTA (pH 7.5) (3). The liver was removed, blotted, weighed and placed in 2 vol of ice-cold homogenizing solution [0.154 *M* KCl, 1 m*M* EDTA, 12 m*M* glucose-6-phosphate (pH 7.5)] and minced with scissors. The minced liver was homogenized for 1 min using a loose-fitting Potter-Elvehjem Teflon–glass homogenizer at 1500 rpm. This and all succeeding steps were performed at 0–4°. The crude homogenate was centrifuged for 10 min at 20,000*g*. The postmitochondrial supernatant was centrifuged for 1 hr at 105,000*g* and the clear middle portion of the supernatant (4–7 ml) was withdrawn with a syringe. This material served as the source of the enzyme. Glucosamine synthetase activity was assayed according to the method of Kornfeld (3). Each reaction mixture contained 6 m*M* fructose-6-phosphate, 12 m*M* glutamine, 1 m*M* EDTA, 40 m*M* sodium phosphate buffer (pH 7.5), and 0.1 ml supernatant in a total volume of 0.5 ml. After incubation at 37° for 60 min the reaction was terminated by heating in boiling water bath for 1 min. The protein precipitate was removed by centrifugation and a 0.3 ml portion of the supernatant was assayed for glucosamine-6-phosphate by the method of Levvy and McAllan (18). The optical densities obtained at 585 nm were compared to a standard curve prepared with crystalline glucosamine HCl. No corrections were made for the lower color values of glucosamine-6-phosphate (19).

The protein content of the 105,000*g* supernatant was determined by the biuret method of Wolfson *et al.* (20) using crystalline bovine albumin as the standard. Enzyme specific activities were expressed as nanomoles of glucosamine-6-phosphate formed per milligram of protein per hour.

One-tenth ml (3–4 mg protein) of the 105,000*g* supernatant catalyzed the linear formation of glucosamine-6-phosphate for at least 120 min. Glucosamine-6-phosphate formation was also linear with increasing enzyme concentration to 0.1 ml extract (4 mg) in 60 min incubations. This relationship was maintained with extracts having the highest activities measured. No extract exceeded a protein concentration of 40 mg/ml. No net glucosamine-6-phosphate formation was observed with extracts heated to boiling prior to incubation nor in reaction mixtures in which glutamine had been omitted.

Hepatic UDP-*N*-acetylhexosamine (UDPAH) concentrations were determined by a modification of the procedure of Bates and Handschumacher (5). One volume of 50% (w/v) trichloroacetic acid was added to 9 vol of 105,000*g* supernatant. After removing the acid-insoluble material by centrifugation, the supernatant was heated for 10 min in a boiling water bath to hydrolyze the UDPAH. The solution was centrifuged again to remove heat-coagulated material and 0.5 ml of the supernatant was assayed for hexosamine content by a modification of the Elson-Morgan reaction as described by Neuhaus and Letzring (21). A standard curve was prepared with glucosamine HCl carried through the same procedure. Pool sizes were expressed as nanomoles of UDPAH per gram of liver. Although this method in reality measures all acid-soluble hexosamine intermediates in the homogenate, it is assumed that it gives a valid indication of UDPAH pool sizes since at least 90–95% of all acid-soluble hexosamine is present as UDPAH (22, 23). The UDPAH pool approximates an equilibrium mixture of 70% UDP-*N*-acetylglucosamine and 30% UDP-*N*-acetylgalactosamine (23).

In experiments where cycloheximide and actinomycin D were used, both were administered intraperitoneally. Cycloheximide (1 mg/ml) was dissolved in sterile isotonic saline and given at a dosage level of 1.0 mg/kg. Actinomycin D (150 μg/ml) was dissolved in a 1:1 mixture of saline–propylene glycol and given at a dosage level of 0.75 mg/kg. Control animals received equivalent volumes of saline or saline–propylene glycol.

Results. Glucosamine synthetase activity as a function of time after surgery. The relative activities of glucosamine synthetase between 1 and 8 days following surgery are shown in Fig. 1. The average specific activity of the synthetase for control animals was 40 nmoles/mg protein/hr. Rats subjected to laparotomy showed a 75% increase in

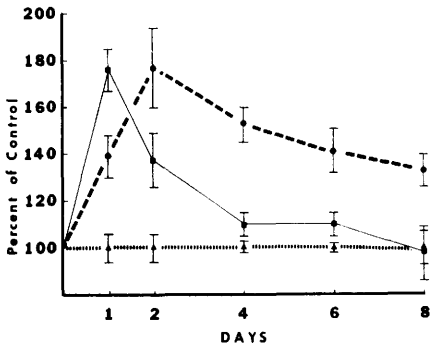

FIG. 1. Relative glucosamine synthetase activity as a function of time following laparotomy and partial hepatectomy. Laparotomy (■—), control (▲···), partial hepatectomy (●—). Values are presented as mean ± SEM. Each point represents the mean of 6 animals.

glucosamine synthetase activity relative to control animals at 24 hr following surgery ($p < 0.001$), which declined but remained significantly above normal at 48 hr ($p < 0.025$), and approached control values by 4 days.

Partial hepatectomy caused a 40% increase in activity above control values at 24 hr after surgery ($p = 0.02$), which continued to rise to a maximum at 48 hr (75% above control levels), then slowly declined over the next 6 days. The activity was still significantly increased over control values at 8 days ($p < 0.001$).

UDPAH pool size as a function of time after surgery. Figure 2 shows the effects of laparotomy and partial hepatectomy on hepatic UDPAH concentrations relative to controls as a function of time after surgery. The measurements were performed on the same supernatant fractions used to obtain the data in Fig. 1. The UDPAH concentrations in control livers averaged 270 nmoles/g in these experiments. Laparotomy caused no significant alterations in UDPAH concentrations at any time measured as compared to control values. Following partial hepatectomy, UDPAH concentrations fell below normal at 2 days ($p > 0.025 < 0.05$), then rose above control levels at 6 days ($p > 0.025 < 0.05$).

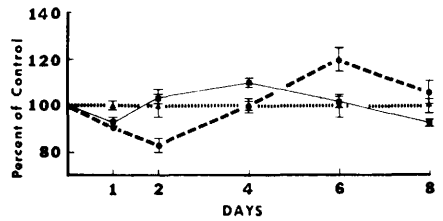

FIG. 2. Relative hepatic UDPAH concentration as a function of time following laparotomy and partial hepatectomy. Assays were carried out in duplicate on the same 105,000*g* supernatants used to assay glucosamine synthetase activities presented in Fig. 1. Values are presented as mean ± SEM. Laparotomy (■—), control (▲···) partial hepatectomy (●—).

Effects of fasting and actinomycin D administration on the response to laparotomy. The effects of fasting and the administration of actinomycin D on the ability of glucosamine synthetase to respond to laparotomy are shown in Table I. Fasting for 24 hr resulted in a 10 to 20% decrease in specific activity of glucosamine synthetase in control animals. Treatment of control animals with actinomycin D also resulted in a 20% decrease in activity. These decreases were statistically significant ($p > 0.01 < 0.025$).

Laparotomy caused a significant increase in both fed and 24-hr fasted rats ($p > 0.001 < 0.005$) when compared to their respective controls. Thus fasting alone for 24 hr had no effect on the increase observed following stress. The administration of actinomycin D, however, completely blocked the increase in glucosamine synthetase activity observed after laparotomy, indicating that the events leading to the increase involved, in part, the prior transcription of one or more species of ribonucleic acid.

Fasted animals were used as controls in these experiments to rule out the possibility that failure to observe an increase in glucosamine synthetase activity after actinomycin D administration was due to a lack of essential nutrients for enzyme synthesis.

Hepatic UDPAH concentrations in these animals are also shown in Table I. No statistically significant differences between the various groups were observed. Again there appeared to be little or no correlation between glucosamine synthetase activities and

TABLE I. Effect of Fasting and Actinomycin D Glucosamine Synthetase Activity and UDPAH Concentration Following Laparotomy and Partial Hepatectomy.[a]

Treatment	Glucosamine synthetase activity (nmoles/mg protein/60 min)	(%) Fed controls
Control–fed (8)	41.6 ± 4.6	100
Control–fasted, 24 hr (4)	35.7 ± 2.5[c]	86
Control–act. D, 24 hr (4)	31.9 ± 5.1[b]	77
Laparotomy–fed (8)	52.2 ± 4.8[b]	125
Laparotomy–fasted, 24 hr (4)	51.4 ± 7.4[b]	124
Laparotomy–act. D, 24 hr (6)	26.6 ± 5.7[b]	64
	Hepatic UDPAH conen (nmoles/g liver)	
Control–fed (8)	252 ± 18	100
Control–fasted, 24 hr (4)	207 ± 18	82
Control–act. D, 24 hr (4)	265 ± 6	105
Laparotomy–fed (8)	270 ± 25	107
Laparotomy–fasted, 24 hr (4)	288 ± 20	114
Laparotomy–act. D, 24 hr (6)	240 ± 20	95

[a] The values are expressed as means ± SD. The number of animals in each group is given in parentheses. Animals were fasted for 24 hr prior to laparotomy and glucosamine synthetase and UDPAH determinations were carried out on the same 105,000*g* supernatant 24 hr following surgery. Animals receiving actinomycin D were injected intraperitoneally and assayed 24 hr later. Animals subjected to laparotomy were injected with actinomycin D at the time of surgery.

[b] $p > 0.001 < 0.005$.

[c] $p > 0.025 < 0.05$.

UDPAH pool sizes.

Effects of cycloheximide administration on the response to laparotomy. Intraperitoneal injections of cycloheximide also blocked the increase in glucosamine synthetase following laparotomy (Table II). Administration of cycloheximide to control animals had no significant effect on glucosamine synthetase activity. No significant changes in hepatic UDPAH concentrations were observed following these treatments. The average hepatic levels of UDPAH in the rats in this experiment were about twice those usually observed. Further investigations showed that the assays were reproducible and not in error. The reasons for the increased levels in this group of rats cannot be ascertained at this time.

Discussion. The patterns of response of glucosamine synthetase after injury and partial hepatectomy (Fig. 1) are quite similar to those previously observed for plasma glycoprotein synthesis (24). Thus it is probable that the changes in enzyme activity are a reflection of the increased demand for hexos-

TABLE II. Effect of Cycloheximide on Glucosamine Synthetase Activity and UDPAH Concentration Following Laparotomy.[a]

Treatment	Glucosamine synthetase activity (nmoles/mg protein/60 min)	(%) Control
Control (9)	43.1 ± 6.8	100
Laparotomy (10)	60.7 ± 7.0[b]	141
Control + cycloheximide (4)	40.9 ± 2.0	95
Laparotomy + cycloheximide (8)	35.9 ± 5.5	83
	Hepatic UDPAH conen (nmoles/g liver)	
Control (9)	589 ± 71	100
Laparotomy (10)	707 ± 250	120
Control + cycloheximide (4)	512 ± 77	87
Laparotomy + cycloheximide (8)	416 ± 52	71

[a] The values are expressed as means ± SD. The number of animals in each group is given in parentheses. Glucosamine synthetase and UDPAH assays were conducted on the same 105,000*g* supernatant and all assays were performed 18 hr after intraperitoneal cycloheximide injection (1 μg/g). Animals subjected to laparotomy were injected with cycloheximide at the time of surgery.

[b] $p > 0.001 < 0.005$.

amines principally for this purpose.

The general pattern of response following partial hepatectomy (Fig. 1) agrees quite well with that reported by Akamatsu and Maeda (14) for the first 72 hr following surgery, with the exception that they observed a much greater rate of increase in enzyme activity and to a much greater extent (250%). In addition, we observed the enzyme levels to peak at 48 hr and then begin to decline, while Akamatsu and Maeda reported a continuing increase, although at a much slower rate. The observations of Akamatsu and Maeda were limited to 72 hr after surgery, therefore, we are unable to compare results beyond that time.

The pattern of response following laparotomy (sham hepatectomy) (Fig. 1) was markedly different from that reported by Akamatsu and Maeda in that the sharp increase we observed at 24 hr was not reported by these investigators. Because of differences in rats, in diets, in assay systems and other factors it is not possible to explain these discrepancies at this time.

The increases in glucosamine synthetase activity following laparotomy and partial hepatectomy were most likely due to the "induction" of new enzyme synthesis since these increases were blocked by the appropriate administration of inhibitors of RNA and protein synthesis. The experiments reported in Tables I and II show that actinomycin D and cycloheximide blocked the increase following laparotomy, and similar experiments by Akamatsu and Maeda (14) showed these agents also blocked the increases after partial hepatectomy. The possibility that the increases were artifactual due to the inhibition of an active glucosamine-6-phosphate deaminase in the extracts was ruled out by experiments performed by us (unpublished data) and by Akamatsu and Maeda (14) which showed that rat liver homogenates contained negligible amounts of the deaminase and that a possible latent activity was not activated by stress or partial hepatectomy.

Despite marked fluctuations in the activity of glucosamine synthetase after laparotomy and partial hepatectomy, the UDPAH pool sizes remained remarkably constant. Fasting and treatment with actinomycin D or cycloheximide also resulted in only minor effects on the UDPAH pool. This finding supports the proposal of Winterburn and Phelps (6) that the control of glucosamine synthetase does not occur by fluctuations in UDPAG concentrations but is due to alterations in the UDPAG binding constant evoked by secondary modifiers.

The regulation of glucosamine synthetase is, apparently, an extremely complex process. The recent work on the regulation of this enzyme by Kornfeld *et al.* (2) and Kornfeld (3), Hardingham and Phelps (10), Winterburn and Phelps (6–8), Bates and Handschumacher (4, 5), and Miyagi and Tsuiki (9), although still unsettled in some areas, may be summarized as follows: (a) UDPAG is the major feedback inhibitor of the enzyme; (b) the degree of feedback inhibition by UDPAG is not dependent on changes in its concentration, but upon changes in the concentration of secondary effectors which alter the binding constant of UDPAG to the enzyme; (c) secondary effectors identified to date include uridine triphosphate (UTP) which increases the apparent K_i of UDPAG, leading to activation of the enzyme, and glucose-6-phosphate (G6P) and adenosine monophosphate (AMP) which decrease the apparent K_i of UDPAG, enhancing its inhibition; (d) these secondary effectors exert their effects at physiological concentrations; (e) the secondary effectors are not active in the absence of UDPAG; and (f) the normal steady-state level of UDPAG is such that the enzyme should be 90% inhibited at all times, with the 10% activity remaining still in excess of the normal demand for new hexosamines.

This latter observation prompted Winterburn and Phelps (7) to the reasonable conclusion that "the availability of this latent potential will permit the rapid synthesis of hexosamine when triggered by the appropriate metabolic control without biosynthesis of enzyme *de novo*." Nevertheless, the evidence presented in this paper, and the work of Akamatsu and Maeda (14) and others (11, 12) indicates that *de novo* synthesis of enzyme does occur after appropriate stimuli in spite of the large latent activity avail-

TABLE III.

	ATP	ADP	AMP	G6P
Control	2.95 ± 0.06[a]	0.86 ± 0.05	0.18 ± 0.02	0.051 ± 0.004
Sham hepat.	2.52 ± 0.16	0.92 ± 0.13	0.25 ± 0.06	0.063 ± 0.003
Part. hepat.	1.60 ± 0.18	1.07 ± 0.04	0.39 ± 0.05	0.062 ± 0.023

[a] Values (mmoles/kg ± SEM).

able.

How does one explain this apparent paradox? We suggest that laparotomy and partial hepatectomy cause changes in the concentrations of the secondary effectors in directions that enhance the binding of UDPAG to the enzyme, leading to markedly greater inhibition. This occurs even though the demand for new hexosamine is greater than normal. In order to meet this demand in the face of increased feedback inhibition the cell must respond by synthesizing more enzyme.

Support for this explanation can be found in the work of Mandel *et al.* (25) who showed that hepatic UTP concentrations decreased about 50% 17 hr after partial hepatectomy and from Hornbrook[3] (Table III) who has shown that the levels of AMP and G6P change +38.9, and +23.5%, respectively, 24 hr after laparotomy, and +116.7 and +21.6%, respectively, 24 hr after partial hepatectomy. The direction of the changes of UTP, AMP and G6P are such that as a result of their collective action a marked inhibition of glucosamine synthetase should be expected *in vivo*.

We suggest that all future studies on the regulation of glucosamine synthetase *in vivo* should involve, as a minimum, the measurements of the intracellular concentrations of UTP, AMP and G6P, as well as UDPAG. This will add considerably to the effort involved but it is difficult to see how meaningful interpretations of results can be made without this data.

Summary. The activity of glucosamine synthetase and the concentration of UDPAH were measured concomitantly in rat livers as a function of time following laparotomy and partial hepatectomy. Glucosamine synthetase activity increased significantly 24 to 48 hr after these treatments. The activity returned to normal levels 4 days after laparotomy but remained significantly elevated at 8 days following partial hepatectomy. UDPAH concentrations were not affected significantly at any time after laparotomy and were only slightly affected after partial hepatectomy. Fasting for 24 hr caused a significant decrease in glucosamine synthetase activity but did not affect the ability of the enzyme to respond to laparotomy or partial hepatectomy. Actinomycin D or cycloheximide administration completely blocked the increase in glucosamine synthetase activity after laparotomy. UDPAH pool sizes were not affected significantly by these treatments.

[3] Data kindly supplied by Dr. Roger Hornbrook, Department of Pharmacology, University of Oklahoma Health Sciences Center. The percentage changes were calculated from the data in Table III.

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