MOLECULAR PHARMACOLOGY, 19:451–455

Effect of Thioacetamide on the Pentose Phosphate Pathway and Other NADP-Linked Enzymes of Rat Liver Cytosol

Chronology of the Perturbations and Metabolic Significance

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Received January 28, 1980; Accepted December 4, 1980

SUMMARY

CERDÁN, S., M. CASCALES, AND A. SANTOS-RUIZ. Effect of thioacetamide on the pentose phosphate pathway and other NADP-linked enzymes of rat liver cytosol: chronology of the perturbations and metabolic significance. *Mol. Pharmacol.* **19:** 451–455 (1981).

Since the NADP oxidation-reduction system plays a primary role in activating and detoxifying chemical carcinogens, the activities of the hepatic enzymes of the pentose phosphate pathway as well as other cytosolic NADP-linked enzymes were studied in the rat *in vivo* during the administration of thioacetamide (TAM). Metabolite couples related to the cytosolic and mitochondrial NADP systems were also investigated. Malic enzyme and transaldolase activities were increased earlier than glucose-6-phosphate and 6-phosphogluconate dehydrogenases. Transketolase and soluble NADP-isocitrate dehydrogenase activities did not change significantly during the 8 weeks of the experiment. This pattern of enzyme activities indicates an increased capacity for generation of NADPH and ribose-5-phosphate and is interpreted to favor detoxifying mechanisms and *de novo* nucleic acid biosynthesis. The state of reduction of the NADP system in TAM-treated livers as calculated by the metabolite indicator method was more oxidized than expected considering the increased capacity for NADPH generation. This finding is interpreted to be the result of a simultaneously increased utilization of NADPH in the synthesis of glutamate as well as in the NADPH-requiring biosynthetic processes.

INTRODUCTION

TAM[2] is a well-known hepatotoxic agent (1). Its administration produces hepatic ultrastructural changes such as increased number and size of nucleoli (2), mitochondrial alterations, and endoplasmic reticulum disruption (3), leading to hepatocellular necrosis in the early phase of poisoning. The chronic phase is characterized by the appearance of nodules (4).

Previous experiments have been carried out in an attempt to elucidate the biochemical mechanisms by which TAM brings about RNA accumulation, nucleolar enlargement (5, 6), and disturbances in RNA transfer from nuclei to the cytosol (7, 8). Andersen *et al.* (9) have also used TAM as a model to study preribosomal RNA biosynthesis in rat liver. However, almost no attention has been paid to changes occurring in the hepatic intermediary metabolism during hyperplastic nodule development. The metabolic imbalance produced by hepatotoxic agents could presumably play some role in the mechanism of action of xenobiotic agents. According to this point of view, significant perturbations of urea cycle enzymes have been reported recently (10).

The pentose phosphate pathway, as well as other cytosolic NADPH-generating systems, may play an important role in the development of hepatotoxicity, since considerable amounts of NADPH and ribose-5-phosphate are needed for activating and detoxifying processes as well as for nucleic acid biosynthesis and repair. The aim of this work has been to study the behavior of the two branches of the hexose monophosphate shunt as well as other cytosolic NADP-linked enzymes [malic enzyme (EC 1.1.1.40) and isocitrate dehydrogenase (EC 1.1.1.42)] during TAM-induced hyperplastic nodulogenesis.

The results obtained show that the pentose phosphate pathway and the other NADPH-generating systems are markedly affected by treatment with thioacetamide. The pattern of change of these enzymes favors NADPH and ribose-5-phosphate generation, and consequently may

This work was supported in part by a grant from the Spanish Comisión Asesora para la Investigación Cientifica y Técnica.

[1] Recipient of a fellowship from the Spanish Ministerio de Educación y Ciencia.

[2] The abbreviation used is: TAM, thioacetamide.

0026-895X/81/030451-05$02.00/0

contribute significantly to detoxifying mechanisms and DNA repair.

MATERIALS AND METHODS

Reagents

Thioacetamide was purchased from Merck and Company, Rahway, N. J. Substrates and coenzymes were obtained from Sigma Chemical Company, St. Louis, Mo. Enzymes were obtained from Boehringer Manheim Corporation, Indianapolis, Ind., with the exception of ribose-5-phosphate isomerase (EC 5.3.1.6) and ribulose-5-phosphate epimerase (EC 5.1.3.1), which were obtained from Sigma Chemical Company. All other biochemicals, obtained from Merck and Company, were of the highest purity available commercially.

Animals and Experimental Design

Male albino Wistar rats (200–250 g body weight) were used in all experiments. They were maintained on a standard laboratory diet and water ab libitum. The rats received daily i.p. injections of a freshly prepared solution of thioacetamide in 0.15 M NaCl at a dose level of 100 mg/kg body weight, during an 8-week period. The control group received a similar volume of 0.15 M NaCl. All of the animals were weighed daily and their external appearance was checked. The rats, not deprived of food, were killed at approximately the same time of the day.

Groups of at least six animals each were killed after 1, 3, 7, 14, 21, 28, 35, and 57 days of TAM treatment. Each rat was anesthesized with Nembutal (50 mg/kg body weight), its abdomen was opened by a midline abdominal incision, and the portal vein was cannulated. The aorta and inferior vena cava were then severed, and 0.15 M NaCl solution was infused until the liver was essentially free of blood. This procedure prevented contamination of the hepatic soluble fraction with enzymes from red blood cells. The liver was immediately chilled in ice-cold buffer, and a biopsy specimen was taken for homogenization. For metabolite measurements the rats were killed by a blow on the head and liver biopsy specimens were immediately frozen with aluminum tongs precooled in liquid nitrogen (11).

Analytical Procedures

Enzyme determinations. Liver homogenates (1:4), prepared in 0.25 M sucrose, 0.02 M Tris buffer (pH 7.4), and 0.1 mM dithioerythritol, were centrifuged for 20 min at 15,000 × *g*. Postmitochondrial supernatants were centrifuged at 105,000 × *g* for 45 min. The resulting clear supernatant was used for measurements of enzyme activity and protein content.

All enzyme activities were determined spectrophotometrically. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were assayed by the methods of Glock and McLean (12). Transaldolase (EC 2.2.1.2) and transketolase (EC 2.2.1.1) were assayed as described by Novello and McLean (13). Malic enzyme was determined by the method of Rutter and Lardy (14). NADP-dependent soluble isocitrate dehydrogenase was measured according to the method of Bernt and Bergmeyer (15). The soluble protein content was measured according to the method of Lowry *et al.* (16). Results are presented as percentages of control values. Control absolute values of enzyme specific activity are given as milliunits per milligram of soluble protein (nanomoles per minute per milligram of protein).

Metabolite assays. For the assay of intermediate metabolites, perchloric acid extracts of liver biopsies neutralized with potassium carbonate were used. The detailed procedure was as previously described (17). Glutamate, malate, pyruvate, 2-oxoglutarate, and ammonia were determined spectrophotometrically according to previously described methods (18–22). α-Ketoacids were determined immediately after the extraction. Results are expressed as nonomoles per gram of fresh liver.

Ratios of free $NADP^+$ to free NADPH were calculated according to the method of Veech *et al.* (23). The malic enzyme system was used for calculating the cytosolic free $NADP^+$ to free NADPH ratio, and glutamate dehydrogenase was used for calculating the mitochondrial free $NADP^+$ to free NADPH ratio. The equilibrium constants reported by Veech *et al.* (23) were used in the calculations. The liver CO_2 concentration was assumed to be that of the superior vena cava ($1.16\ 10^{-3}$ M) (23).

Statistical significance was evaluated by Student's *t*-test. Values of p greater than 0.05 were considered not significant.

RESULTS

Control animals showed a progressive increase in body weight with time. However, the body weights of the TAM-treated group remained constant. Body weight differences were statistically significant as early as the 3rd day of treatment.

Fig. 1A illustrates the changes in the liver weight to body weight ratio of TAM-treated rats. This ratio, when compared with the control value, showed a progressive increase after the 3rd day of treatment. Differences were statistically significant ($p < 0.001$) from the 1st week. Fig. 1B shows that treatment with TAM decreased the protein concentration in the soluble fraction of rat liver. Statistically significant changes were detected from the 3rd day of treatment. Of the TAM-treated animal population, 10% were dead at the end of the experiments, whereas all animals in the control group were alive. Livers from TAM-treated animals developed nodules starting about the 7th week of treatment.

Table 1 summarizes the effect of TAM on the oxidative and nonoxidative branches of the pentose phosphate pathway as well as on other NADP-linked enzymes (malic enzyme and soluble isocitrate dehydrogenase). Glucose-6-phosphate dehydrogenase did not show apparent changes in activity during the first 3 weeks of treatment, but activity rose drastically from the 21st day (138%; $p < 0.05$) until the 57th day (300%; $p < 0.001$). 6-Phosphogluconate dehydrogenase did not show highly significant changes until the 8th week of treatment (180%; $p < 0.001$). This quantitatively differential increase in the concentration of the two enzymes led to a progressive decrease in the 6-phosphogluconate dehydrogenase to glucose-6-phosphate dehydrogenase ratio. This imbalance has been described (24) for situations in which a strong NADPH supply is required (e.g., lipogenesis).

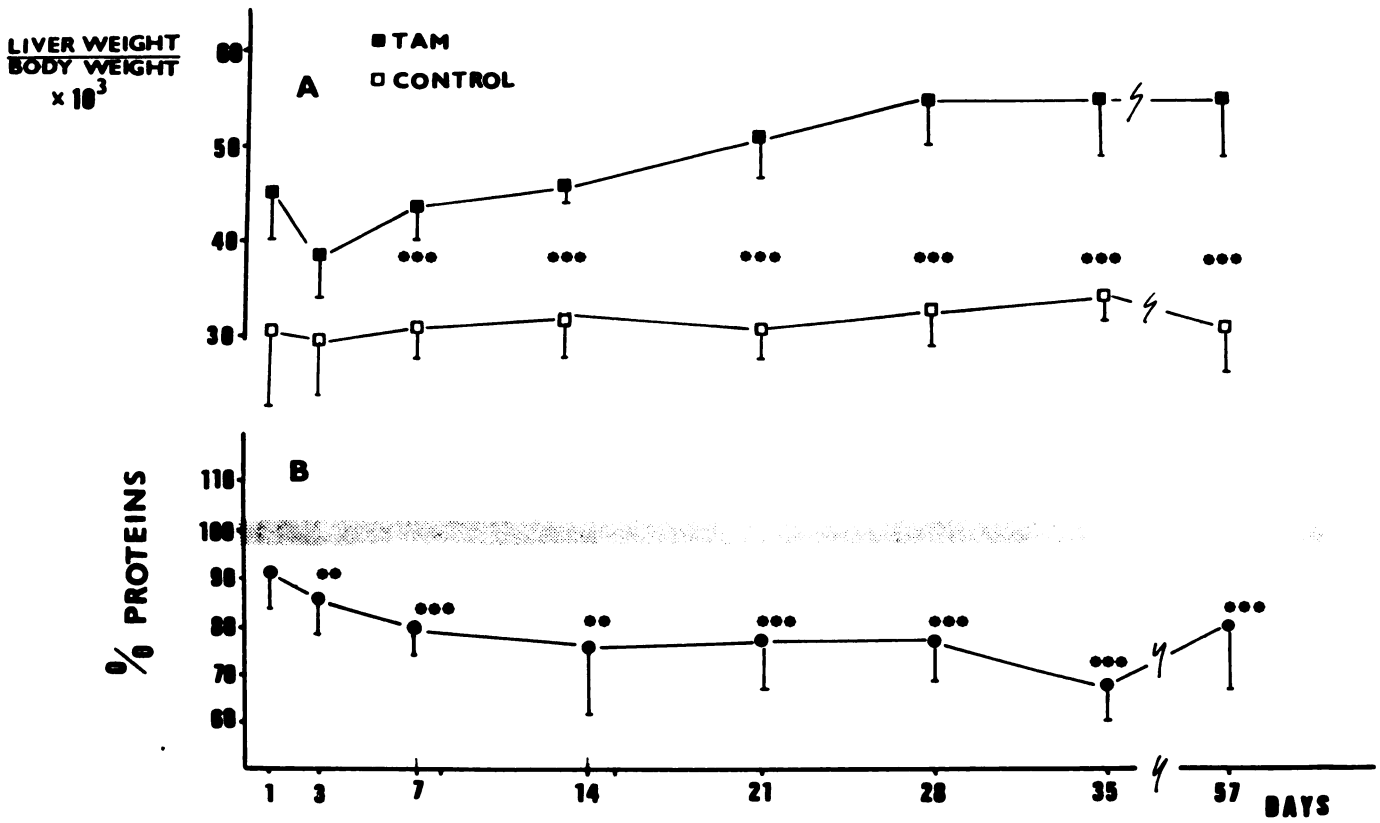

FIG. 1. *Changes in liver weight/body weight ratio (A) and in soluble protein content of liver (B) during TAM treatment*
Thioacetamide treatment was carried out as described under Materials and Methods. A: □—□, control; ■—■, TAM treatment; B: ●—●, soluble protein content of liver. Protein content was determined as described under Materials and Methods. Results are expressed as percentages of control values and are the means of six observations. The mean ± standard deviation of control values was 82 ± 17 mg/g of fresh liver. Statistical significance: $*p < .05$; $**p < 0.01$; $***p < 0.01$.

Transaldolase activity increased progressively from the 1st day of treatment thereafter, although statistically significant differences with the control were not obtained until the 1st week of treatment (160%; $p < 0.01$). Transketolase specific activity was not significantly altered during the entire period of treatment.

Malic enzyme displayed a progressive and statistically significant rise in activity after the 3rd day of treatment (133%; $p < 0.01$), whereas isocitrate dehydrogenase activity did not show any significant change with time. This behavior of the soluble NADP-isocitrate dehydrogenase is in accord with previous reports showing a lack of

TABLE 1

Changes in activity of the enzymes of the pentose phosphate pathway and other NADP-linked enzymes (isocitrate dehydrogenase and malic enzyme) during TAM treatment

Thioacetamide was administered as indicated in the text. Enzyme activity determinations were as described under Materials and Methods. Results, expressed as percentage of control specific activities are the means ± standard deviation of six experimental observations.

Time	% Enzyme activity[a]						
	G6PDH	6PGDH	6PGDH/G6PDH	TA	TK	ME	ICDH
days							
1	110 ± 26	118 ± 20	106 ± 12	95 ± 10	87 ± 16	88 ± 20	111 ± 36
3	130 ± 24	113 ± 18	87 ± 10	119 ± 10	82 ± 10	135 ± 34**	65 ± 10
7	112 ± 40	138 ± 15	123 ± 20	155 ± 34**	84 ± 14	155 ± 34**	100 ± 8
14	155 ± 40	126 ± 30	81 ± 22	160 ± 34**	100 ± 21	232 ± 46***	121 ± 30
21	138 ± 40*	106 ± 26	77 ± 22	185 ± 42***	75 ± 24	239 ± 60***	115 ± 30
28	233 ± 46***	155 ± 34**	67 ± 24	189 ± 30***	108 ± 24	270 ± 70***	84 ± 20
35	300 ± 54***	148 ± 36**	48 ± 30*	180 ± 34***	120 ± 26	290 ± 28***	105 ± 20
57	300 ± 36***	180 ± 68***	61 ± 12***	182 ± 30***	107 ± 14	305 ± 46***	100 ± 12

[a] G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; TA, transaldolase; TK, transketolase; ME, malic enzyme; ICDH, NADP isocitrate dehydrogenase. Mean of the control values for G6PDH, 6PGDH, TA, TK, ME, ICDH, and 6PGDH/G6PDH were 28 ± 6, 57 ± 13, 33 ± 5, 30 ± 9, 318 ± 69, 29 ± 9 nmoles/min/mg of protein and 2 ± 0.27, respectively. Statistical significance: $* p < 0.05$; $** p < 0.01$; $*** p < 0.001$.

TABLE 2

Levels of metabolites related to cytosolic and mitochondrial NADP⁺/NADPH oxidation-reduction states of rat liver after 57 days of treatment with TAM

Experimental conditions were the same as described in Table 1. Results are expressed as means ± standard deviation of values from six animals.

Metabolite	Control	TAM	%[a]
	nmoles/g fresh liver		
Malate	238 ± 57	207 ± 35	87
Pyruvate	100 ± 15	82 ± 10	82
[Pyruvate]/[malate]	0.41 ± 0.065	0.385 ± 0.042	94
Glutamate	2318 ± 170	3250 ± 198	140***
2-Oxyglutarate	133 ± 65	109 ± 48	82
Ammonia	300 ± 45	710 ± 37	237**
[2-Oxoglutarate][ammonia]/[glutamate]	15 ± 4	24 ± 3	160*

[a] Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

correlation of this enzymatic activity with other lipogenic activities such as those of glucose-6-phosphate dehydrogenase and malic enzyme (25). No effects were detected when enzyme activities were assayed after adding TAM to the reaction mixture at a concentration similar to that expected *in vivo* (6 mM).

Table 2 shows the alterations of the levels of metabolites related to NADP⁺/NADPH cytosolic and mitochondrial oxidation-reduction states. Ammonia and glutamate were significantly increased to 237% and 140% of control values. Malate, pyruvate, and 2-oxoglutarate concentrations were decreased, although the results were not statistically significant. The [pyruvate]/[malate] ratios were similar in control and TAM-treated livers. On the other hand, the 2-oxoglutarate [ammonia]/[glutamate] ratio was significantly increased in TAM-treated livers.

Table 3 presents the results obtained in the calculation of the state of reduction of the NADP⁺/NADPH cytosolic and mitochondrial oxidation-reduction couples. The cytosolic NADP⁺/NADPH ratios were similar in both situations. In contrast to this observation the mitochondrial NADP⁺/NADPH ratio was significantly increased in TAM-treated livers, indicating a shift to a more oxidized state.

DISCUSSION

The main role of the pentose phosphate pathway is to provide reducing power (NADPH) needed for biosynthetic processes and ribose-5-phosphate used in *de novo* nucleic acid biosynthesis. This pathway is particularly efficient in tissues requiring a large supply of NADPH, ribose-5-phosphate, or both, as in the case of adipose tissue, lactating mammary gland, and intestinal mucosa. A particular arrangement allows the cycle to act as an NADPH-generating system via its oxidative branch or as a ribose-synthesizing system using the oxidative and/or nonoxidative branches, which can act as two parallel mechanisms for the conversion of hexose monophosphates into pentose phosphates (26).

The sequential analysis of several enzymes of the hexose monophosphate shunt was carried out to study the time dependence of the hepatotoxic agent treatment on the activity of these soluble proteins. As illustrated in Table 1, different enzyme activities were increased or depressed at different times during the treatment, which seems to indicate a chronologically ordered response to administration of the hepatotoxin. Of the enzymes analyzed, malic enzyme and transaldolase activities increased earlier than those of glucose-6-phosphate and 6-phosphogluconate dehydrogenases. The activity of the latter two rose only after 4 weeks of treatment. Transketolase and soluble NADP-isocitrate dehydrogenase activities were significantly decreased from the beginning of the experiments, but their specific activities did not change because of the lower protein content of TAM-treated livers (see Fig. 1B).

These changes in soluble enzyme activities could be related to one or more of the following known thioacetamide effects: qualitative alterations in the level of nonhistone proteins (27); multistep regulation of RNA biosynthesis (9, 28) and processing; selective RNA transfer from TAM-treated nuclei to the cytosol (7, 8), or a direct influence on the ribosomal protein biosynthesis machinery (29). Although it has not been described, the possibility exists that TAM also acts by varying the rates of enzyme degradation.

By whatever means the mechanism is mediated, it does not perturb to the same extent the activities of all of the soluble enzymes assayed. Moreover, the effects of different proteins are chronologically separated. This pattern of change in enzyme activities indicates an increased capacity for production of NADPH and ribose-5-phosphate which could be used for detoxifying mechanisms (e.g., ammonia withdrawal through glutamate biosynthesis) and/or *de novo* nucleic acid biosynthesis and repair during TAM-induced nodulogenesis.

On the other hand, the more oxidized state of the mitochondrial NADP system could be explained as a result of an increased glutamate biosynthesis. This point

TABLE 3

Cytosolic and mitochondrial NADP⁺/NADPH oxidation-reduction states of rat liver after 57 days of treatment with TAM

Experimental conditions were the same as in Table 1. Calculations were as described under Materials and Methods, using the equilibrium constants previously reported (23).

Compartment	Related metabolite ratio	Condition	Oxidation-reduction state	%[a]
Cytosol NADP⁺/NADPH	[Pyruvate][CO_2]/[malate]	Control	0.0138 ± 0.002	
		TAM	0.0129 ± 0.001	93
Mitochondria NADP⁺/NADPH	[2-Oxoglutarate][NH_4^+]/[glutamate]	Control	0.006 ± 0.002	
		TAM	0.009 ± 0.001	138*

[a] Statistical significance: * $p < 0.05$.

of view is supported by the increase in glutamate concentration in TAM-treated livers (Table 2). This result indicates that TAM-treated livers apparently use more NADPH to synthesize glutamate (30) than do control livers, and is in agreement with the expected *in vivo* increased activity of NADPH-generating systems. However, the increased glutamate biosynthesis was still unable to remove the excess of NH_4^+ secondary to the urea cycle inhibition produced by TAM (10), since its concentration is still higher than control values.

The lack of parallelism in the variations of the free $NADP^+$/NADPH cytosolic and mitochondrial ratios was striking. Sies *et al.* (31) have described a similar situation in isolated rat liver cells treated with high amounts of ammonium chloride. In this condition, as in TAM-treated rats, the ammonium supply far exceeded the capacity of the urea cycle. The increased supply of NH_4^+ apparently does not allow transport of reducing power from the cytosol sufficient to equilibrate the NADP system in both compartments.

The metabolic events reported herein and their particular chronology preceding nodule formation during TAM treatment suggest that the alterations produced in the pentose phosphate pathway as well as in other NADPH-generating and -consuming systems localized in the cytosol and/or mitochondria may be important in the mechanism of adaptation of the liver to prolonged administration of xenobiotic agents.

ACKNOWLEDGMENTS

We wish to thank Dr. R. Parrilla for helpful criticism and Miss Dolores Velasco for her valuable technical assistance.

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