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INCREASES IN RAT LIVER CYCLIC AMP AND GLYCOGEN PHOSPHORYLASE ACTIVITY CAUSED BY THE HERBICIDE ATRAZINE

B. MESSNER, J. BERNDT and J. STILL

Gesellschaft für Stralen- und Umweltforschung München, Institut für Toxikologie und Biochemie, Abteil für Zellchemie, Ingolstädter Landstr. 1, 8042 Neuherberg, Germany

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Abstract—The *in vivo* effects of the triazine herbicide atrazine on rat liver glycogen metabolism was investigated. After administration of atrazine, liver c-AMP was elevated up to threefold, the activity of glycogen phosphorylase increased, the content of glycogen in the liver decreased and the level of blood glucose increased. Cyanuric acid, although not a herbicide but a simple triazine, did alter neither liver c-AMP nor phosphorylase activity. *In vitro* adenylate cyclase was not activated or inhibited by triazine. However, phosphodiesterase was inhibited non-competitively by the herbicide (0.1–0.4 mM), resembling theophylline in this respect. When atrazine was added to isolated hepatocytes the effect of glucagon-stimulated c-AMP accumulation was potentiated. Thus, atrazine can inhibit phophodiesterase *in vitro* and in the intact cell. This phophodiesterase inhibition may also occur *in vivo* and this may lead to the observed effects on glycogen metabolism. However, additional hormonal and/or nervous alterations caused *in vivo* by the herbicide can not be excluded.

No changes in the activities of adenylate cyclase and of phosphodiesterase could be detected in response to *in vivo* administration of atrazine. However, these negative results may be the consequence of dissociation of the enzyme–atrazine complex during preparation of the liver fractions.

Herbicides of the triazine type have widespread agronomic use. The mode of action of triazines on plant metabolism has been studied in some detail. For example, atrazine kills plants by a drastic inhibitory effect on the photosynthetic apparatus, especially on the Hill reaction [1–3], and resembles the phenylureas in this respect [3]. The fate of triazines in plants and animals and the mechanism of detoxication was the subject of several investigations [4–7]. Some of these compounds are converted to glutathione conjugates by plants [6, 7]. Some of them are very stable with halflives in the soil extending to many years [8]. Thus, some triazines may be ingested by animals, including man, where they can elicit symptoms of toxic effects [9–12].

In contrast to plants no data are available on the mechanism of action of triazines on the mammalian metabolism. Since cyclic AMP is known to be involved in the regulation of many metabolic events, the effect of atrazine on the level of cyclic AMP in rat liver was examined *in vivo*. Glycogen phosphorylase, the activity of which is very sensitive to the cyclic AMP level, was measured under the same conditions. The contents of liver glycogen and blood glucose and the activities of adenylate cyclase and phosphodiesterase were also determined. Additionally, atrazine was added to liver cell suspensions and the content of c-AMP inside the cells was measured.

MATERIALS AND METHODS

Female rats, Sprague–Dawley (Gassner, Sulzfeld), 120–140 g were kept in a natural day and night cycle and were fed on a standard diet. For feeding experiments, the diet contained 0.05 per cent of atrazine (Riedel-De Haen, Hannover). The herbicide was solved in acetone (0.3 g/20 ml) and throughly mixed (starmix) with 600 g of standard diet. The powder was pasted with water, formed into pellets and dried at 37° for 48 hr. In the preparation of control diet atrazine was omitted.

Before injection, rats were anaesthesized with ether; the period of anaesthesia did not exceed 2 min. Atrazine or cyanuric acid (Fluka, Buchs) was suspended in corn oil. All injections were intraperitoneal. Control animals were injected with corn oil. To avoid any changes in food intake which might have occurred after the injection, food was withdrawn from all animals after injection.

Adenylate cyclase activity was determined after the method of Salomon *et al.* [13] in washed membrane suspensions of rat liver, which were prepared according to the method described by Sweat and Hupka [14]. The activity of phosphodiesterase was determined in the 16.000 *g* supernatant of a crude liver homogenate [15] by the method of Thompson *et al.* [16].

For c-AMP determination the liver was removed under ether anaesthesia, the organ was immediately put into liquid nitrogen, weighed, and the content of c-AMP was determined as described by Michal and Wunderwald [17].

Hepatocytes were prepared from whole rat liver after the method of Berry and Friend [18], modified as described by Baur *et al.* [19]. Cells were used for experiments when they fulfilled the following criteria: trypan blue stainability less than 10 per cent and stimulation of respiration (as measured with an O_2-electrode) by 1 mM succinate less than 20 per cent [19].

The hepatocytes (2.5–3.0 mg of protein per ml) were incubated in Krebs–Henseleit buffer, pH 7.2 at 37° under an atmosphere of 95% O_2–5% CO_2. After a 15 min preincubation of the hepatocytes glucagon or other agents were added to the suspension and incubated for 4 min. The incubation was terminated with 0.5 ml of 0.6 $HClO_4$, the precipitate was separated by centrifugation and the supernatant was chromatographed on Dowex 50 (H^+) as described by Pilkis *et al.* [21] and assayed for c-AMP [17].

The activity of glycogen phosphorylase was determined in liver homogenates as described [22], the content of glycogen in the liver was measured according to Bergmeyer and Bernt [23] and that of blood glucose with glucose oxidase [24]. Blood samples (0.1–0.3 ml) were taken during removal of the liver, which was used for glycogen and phosphorylase activity determination.

Theophylline was from Sigma, St. Louis, c-AMP radioisotope dilution test with binding protein (kit), c-AMP, ATP and all other biochemicals were purchased from Boehringer, Mannheim.

RESULTS

When rats were fed a diet containing 0.05 per cent atrazine for 4 to 7 days, the levels of cyclic AMP in the liver rose from 7.7 to 12.4 pmoles/10 mg wet liver tissue. Oral administration, however, was rather time consuming, and it was found that the increase in liver cyclic AMP was much faster and much more pronounced after intraperitoneal injection of the herbicide, being highest 4 hr after the injection of 25 mg of atrazine per 100 g rat (Fig. 1). Other herbicides of the triazine type such as simazine, propacine or prometryne also elevated the level of cyclic AMP in the liver, but atrazine was most effective and was used in further experiments. Cyanuric acid (25 mg/100 g), although not a herbicide but a simple triazine, was tested for comparison and did not significantly alter the content of cyclic AMP in liver.

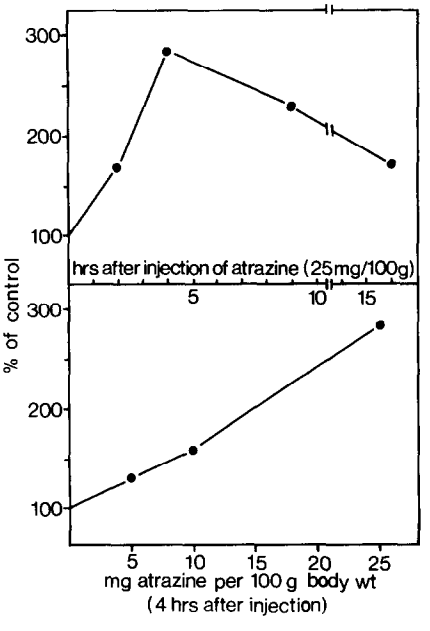

Fig. 1. Effect of atrazine on cyclic AMP content in rat liver. Atrazine was injected i.p. and after the appropriate time cyclic AMP was determined. Values from control rats (injected with corn oil) were 8.2 pmoles per 10 mg wet tissue and were set to 100 per cent. Values in Figs. 1–3 are averages of 8 individual determinations. For the variability of the data see text.

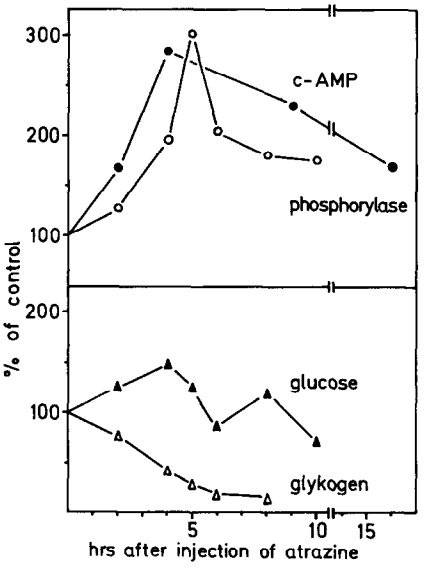

Fig. 2. Effect of atrazine on the activity of glycogen phosphorylase, liver glycogen and on blood glucose. Atrazine (25 mg/100 g) was injected i.p. and, after time intervals as indicated, glycogen phosphorylase, liver glycogen and blood glucose were determined as described in the Methods section. Values from control rats were: glycogen phosphorylase 14.2 ± 1.2 nmol/min/mg of protein; liver glycogen 125 ± 13 μmol glucose units/g wet wt; blood glucose 143 + 7 mg/100 ml and were set to 100 per cent. Values for c-AMP are the same as in Fig. 1. For the variability of the data see text.

As can be seen from Fig. 2, the increase in the level of cyclic AMP was followed after a lag period of about one hour by a three-fold increase in the activity of glycogen phosphorylase. As was to be expected from these results, liver glycogen decreased to low values, while the content of glucose in the blood increased by about 50 per cent within 4 hr after the injection and came down thereafter to almost normal values. Cyanuric acid altered only slightly the content of c-AMP, the activity of glycogen phosphorylase in liver and the content of glucose in blood (Fig. 3). Liver glycogen, however, was decreased by about 50-per cent after administration of cyanuric acid. Although the decline of liver glycogen after atrazine injection is probably due to the increased glycogen phosphorylase activity, an unspecific effect of triazines such as cyanuric acid on liver glycogen can not be excluded.

The content of c-AMP, glycogen and blood glucose as well as the activity of phosphorylase varied somewhat from day to day and within groups of animals. A control group was run with each experimental group; these controls were similar and their mean was set to 100 per cent. Values in Figs. 1–3 are given as a percentage of this control. The variability of these

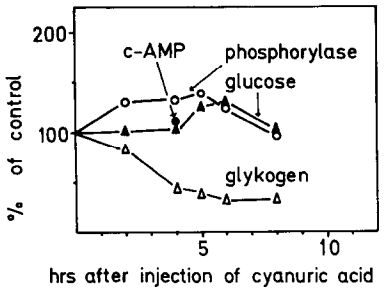

Fig. 3. Effect of cyanuric acid on the activity of glycogen phosphorylase, liver glycogen and blood glucose. Cyanuric acid (25 mg/100 g) was injected i.p. and glycogen phosphorylase, liver glycogen and blood glucose were determined as described in the Materials and Methods section. The c-AMP content was measured 4 hr after administration of cyanuric acid. Values from control rats were: phosphorylase 13.9 ± 1.5 nmol/min/mg of protein; glycogen 154.9 ± 13.7 μmol glucose units/g wet wt; blood glucose 134.9 ± 27.6 mg/100 ml; c-AMP 8.4 ± 1.1 pmol/10 mg wet tissue and were set to 100 per cent. For variability of the data see text.

data therefore can not be indicated in the Figures; S.E.M. was 10–20 per cent within one experimental group.

The increase in liver cyclic AMP could be due to increased rates of synthesis or decreased rates of degradation of cyclic AMP. Therefore we have studied *in vitro* and *in vivo* the effect of atrazine on adenylate cyclase and on phosphodiesterase activity. Whereas adenylate cyclase *in vitro* was neither activated nor inhibited by 0.4 mM atrazine (control 5.0, with atrazine 4.6 pmoles/mg/min), the herbicide *in vitro* inhibited phosphodiesterase. A non-competitive type of inhibition was found (Fig. 4, lower part). In this respect atrazine resembles theophylline (Fig. 4, upper part), a well known inhibitor of phosphodiesterase. These experiments were performed with the enzymes from control rats and the same values were found with the enzymes from atrazine-treated rats (not shown). Cyanuric acid did not influence phosphodiesterase activity when added to the assay mixture (Fig. 4, upper part). This agrees well with the results from *in vivo* experiments, in which cyanuric acid had no or only small effects on the c-AMP level, on blood glucose or on glycogen phosphorylase activity (Fig. 3).

The activities of adenylate cyclase and of phosphodiesterase were also determined in rat liver 4 hr after the injection of atrazine. Adenylate cyclase activity was 5.5 pmoles/mg/min in the control and 6.1 pmoles/mg/min in the atrazine treated rates (25 mg/100 g). For phosphodiesterase the corresponding values were 4.6 and 4.2 nmoles/mg/min, respectively. Thus, no substantial changes in the activity of either enzyme occurred in response to *in vivo* administration of atrazine.

Since it cannot be excluded that the reported effects of atrazine in whole animals are due to hormonal or nervous alterations, some additional experiments were performed with isolated hepatocytes. The liver cells were prepared as described in 'Materials and Methods' and the formation of c-AMP was induced in

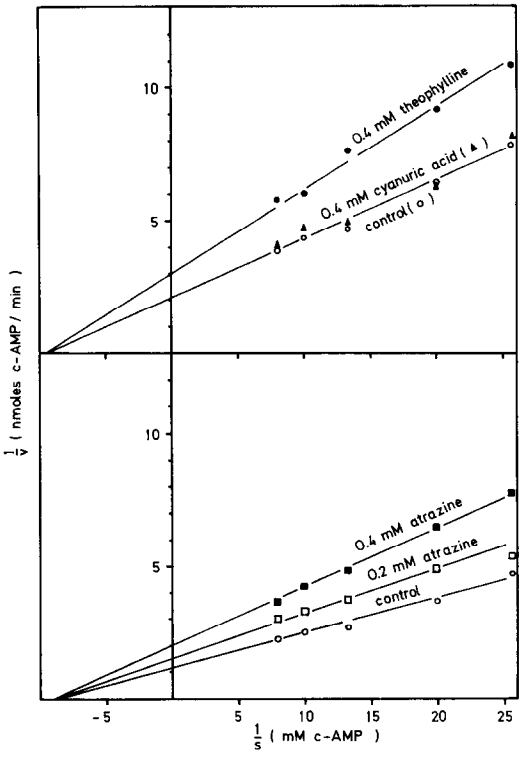

Fig. 4. *In vitro* effect of atrazine, theophylline and cyanuric acid on phosphodiesterase activity (Lineweaver–Burk plot). Lower part: 0.164 mg of protein of the 16.000 *g* supernatant from rat liver (spec. act. 4.8 nmol/min/mg) was used. Upper part. phosphodiesterase was purified 3-fold (two steps, [15]) from the 16.000 *g* supernatant, 0.042 mg of protein (spec. act. 12.5 nmol/min/mg) was used. Assay of the enzyme was as described in the Methods section. The compounds were added to the incubation mixtures without preincubation.

the cells by glucagon in the presence of atrazine. The results are summarized in Table 1. Atrazine alone had only a small effect on basal c-AMP levels in isolated hepatocytes. However, the herbicide caused a significant increase in the glucagon stimulated c-AMP levels, producing at least three times the response

Table 1. Effect of atrazine on the c-AMP content in isolated rat hepatocytes

Additions	c-AMP (pmoles/mg of protein) Expt 1	Expt 2
None (control)	1.28 ± 0.13	2.70 ± 0.09
Glucagon (5×10^{-9} M)	6.84 ± 0.55	11.90 ± 0.68
Atrazine (4×10^{-4} M)	2.12 ± 0.11	4.59 ± 0.32
Glucagon + atrazine	36.90 ± 2.19	38.70 ± 1.75

Hepatocytes were prepared and incubated as described in the Methods section. Atrazine was solved in acetone (0.08 M) and 5 μl of this solution or of acetone (control) were added to the liver cell suspension. After incubation was terminated, the content of c-AMP in the hepatocytes was measured as described in the Methods section.

to the hormone. The identity of the product formed in the glucagon plus atrazine-treated hepatocytes to be c-AMP was proved by the following experiment: after incubation the cell suspension was acidified, centrifuged and chromatographed as for c-AMP extraction (see 'Materials and Methods'). The column eluate was divided into two parts: part 1 was used for c-AMP determination giving 38.70 pmoles/mg of protein (Expt 2 in Table 1); part 2 was incubated with (commercial) purified phosphodiesterase and c-AMP was assayed. No c-AMP could be detected in the phosphodiesterase-treated sample. Thus, the product in fact is c-AMP.

DISCUSSION

From these results it is evident, that atrazine (triazines) after *in vivo* administration can elevate the content of cyclic AMP in rat liver. The activities of adenylate cyclase and of phosphodiesterase were found to be unchanged when measured in liver homogenates of atrazine-treated rats. This indicates that the herbicide does not affect the synthetic rates of these enzymes. Other pesticides, such as *p,p'*-DDT were found to produce a significant rise in the amount of adenylate cyclase after *in vivo* administration [25].

It has to be considered that, during the homogenization of the liver, atrazine may dissociate from adenylate cyclase and/or phosphodiesterase, resulting also in unchanged activities. The *in vitro* inhibition of phosphodiesterase by atrazine (Fig. 4) was observed with concentrations between 0.1–0.4 mM, which was in the same range as the concentration of the substrate, cyclic AMP. When atrazine was added in similar concentrations to isolated hepatocytes, the herbicide potentiated the effect of glucagon on c-AMP accumulation in the cells while having alone no substantial effect on the c-AMP level (Table 1). The herbicide resembles theophylline and other potent inhibitors of phosphodiesterase [20] in this respect. Thus, atrazine inhibits phosphodiesterase not only *in vitro* but also in the intact cell.

The intracellular concentration of these herbicides during *in vivo* experiments is not known and caution must always be applied in drawing conclusions about the *in vivo* situation from *in vitro* results. However, one can assume that the inhibitory effect of atrazine may also occur *in vivo* and may induce an increase in the cyclic AMP level. This, in turn, would be expected to lead to an increase in phosphorylase activity and hence to glycogen degradation.

On the other hand, it cannot be excluded that in whole animals, in addition to a phosphodiesterase inhibition, the herbicide causes hormonal and/or nervous alterations (increased blood levels of catecholamines and glucagon) leading to increased c-AMP levels.

The experiments with atrazine described here were performed to study the effects of a triazine on mammalian metabolism. The concentrations used were much higher than those expected to occur in the environment, but due to their chemical nature and stability some of these herbicides may accumulate in the lipid part of mammals and can alter the intermediary metabolism. i.e. liver cyclic AMP. A simple triazine, cyanuric acid, was much less effective, indicating that substituents other than OH-groups in the triazine ring moiety are necessary.

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