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THE EFFECT OF TRIFLUOPERAZINE ON THE PHOSPHORYLATION OF RIBOSOMAL SMALL SUBUNIT PROTEIN S6 IN D-GALACTOSAMINE-INJURED AND NORMAL RAT LIVER

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Abstract—The effect of the Ca^{2+}–calmodulin antagonist trifluoperazine on the elevation of phosphorylation of rat liver small ribosomal subunit protein S6 induced by the hepatotoxic agent D-galactosamine has been studied. Trifluoperazine applied in various doses (1–100 mg/kg body wt) before injection of D-galactosamine into the rat does not reverse the strong increase in phosphorylation promoted by D-galactosamine. Instead, trifluoperazine has been identified as a potent stimulator of S6 phosphorylation in normal rat liver *in vivo* without causing significant changes in the cyclic AMP content of liver and the overall rate of liver protein synthesis. Both drugs, however, were not effective in stimulating the incorporation of [^{32}P]phosphate into microsomes or crude ribosomes in liver slices *in vitro*. The results suggest that a calmodulin-activated protein kinase probably is not primarily engaged in S6 phosphorylation produced by D-galactosamine. However, further *in vitro* studies are needed to reach a definite conclusion.

Protein S6 in the small ribosomal subunit has been identified as the major phosphorylated protein in eukaryote ribosomes [1]. The degree of phosphorylation of this protein in liver and other tissues is modified by a variety of pharmacologic, pathologic and physiologic factors but the mechanism by which the phosphorylation takes place is only poorly understood (for a recent review see Ref. 2). Although the phosphorylation of the ribosomal protein in the cell can be moderately stimulated by addition of cyclic AMP to the animal or cell culture [3–8] several reports have clearly shown that an increase in the intracellular cyclic AMP concn is not strictly required for enhanced phosphorylation of protein S6 [9–12]. Thus, a cyclic AMP independent, alternative pathway of stimulated S6 phosphorylation may exist under certain conditions [12].

One of the possible mechanisms could involve calmodulin, which has been identified as an ubiquitously distributed, high-affinity intracellular calcium-binding protein (for recent reviews see Refs. 13–16). The calcium–calmodulin complex is implicated in the regulation of important biological processes including the phosphorylation of several tissue and enzyme proteins [16].

The potential involvement of calmodulin in various regulatory mechanisms can be demonstrated by using pharmacologic methods since it is known that certain tricyclic psychotropic drugs including trifluoperazine can bind to and inhibit the calcium-dependent activation by calmodulin of a number of enzymes [17–19]. Thus, trifluoperazine has been used successfully in intact cellular systems to elucidate the involvement of calmodulin in the process under consideration.

In the present communication we describe the effect of trifluoperazine on the stimulated phosphorylation of liver ribosomal protein S6 induced by the hepatotoxic agent D-galactosamine–HCl *in vivo* [20] since a significant rise of the intracellular conc of Ca^{2+} is a very early phenomenon in galactosamine-injured liver tissue [21]. The results show that trifluoperazine does not block galactosamine-stimulated phosphorylation but, in fact, leads to strong phosphorylation of S6 in normal rat liver.

MATERIALS AND METHODS

Treatments of rats. Male Sprague–Dawley rats (270–320 g body wt) which had free access to food and water were i.p. injected between 08.00 and 09.00 a.m. with variable amounts of trifluoperazine–HCl (Röhm Pharma GmbH, F.R.G.), freshly dissolved in 0.154 M saline. The acid solution was neutralized with NaOH before injection. D-Galactosamine–HCl (C. Roth OHG, Karlsruhe, F.R.G.) was dissolved in saline and applied intraperitoneally. Details of the time schedule and the doses of the drugs injected are given in the legends of the tables and figures. Control rats received the same volume of saline alone.

If used, carrier-free [^{32}P]orthophosphoric acid (New England Nuclear, Boston, MA) was administered i.p. to the animals which were killed 20 min after isotope injection.

Preparation of ribosomes and ribosomal protein. The liver was quickly removed and chilled in ice-cold buffer (0.05 M Tris–HCl (pH 7.6), 0.08 M KCl, 0.0125 M $MgCl_2$, 0.25 M sucrose). Ribosomes were

isolated [1], small ribosomal subunits (40S) prepared [1] and the proteins from the 40S subunit were extracted [22]. The specific radioactivity of ribosomal particles (cpm/E_{260} unit) and of the ribosomal protein (cpm/mg protein) was determined as described before [1]. The concn of protein was measured according to Lowry *et al.* [23] using bovine serum albumin as a standard.

Two-dimensional polyacrylamide gel electrophoresis. About 500 μg of extracted 40S ribosomal proteins were separated by two-dimensional electrophoresis as described previously [1]. The radioactivity of protein S6 was estimated after separation of identical amounts of 40S proteins from control and trifluoperazine-treated liver. The area of S6 was excised, the gel dissolved in a mixture of H_2O_2–perchloric acid and counted [24].

Definition and calculation of the elongation angle (e°) and of the area of phosphorylated small subunit protein S6 (ap) in the two-dimensional electropherograms (see Fig. 1) have been described elsewhere [25].

Determination of cyclic AMP content in liver. Immediately after decapitation a portion of the liver (about 500 mg wet wt) was excised and chilled in ice-cold water containing 0.006 M EDTA (0.1 g liver wet wt/ml water–EDTA). The tissue was homogenized in a Potter–Elvehjem homogenizer. A small aliquot was taken for the determination of protein, after which the homogenate was boiled for 3 min. After cooling the samples were centrifuged for 10 min (2000 *g*, 4°) and the supernatants assayed for cyclic AMP content. The cyclic nucleotide was determined with a competitive protein-binding assay (Radiochemical Centre, Amersham, U.K.). The assays were performed in triplicate. The coefficients of variation were about 7%.

Incorporation of [^{14}C]*valine into protein of liver slices.* Slices of 0.5-mm thickness (about 100 mg wet wt) were prepared manually from livers and incu-

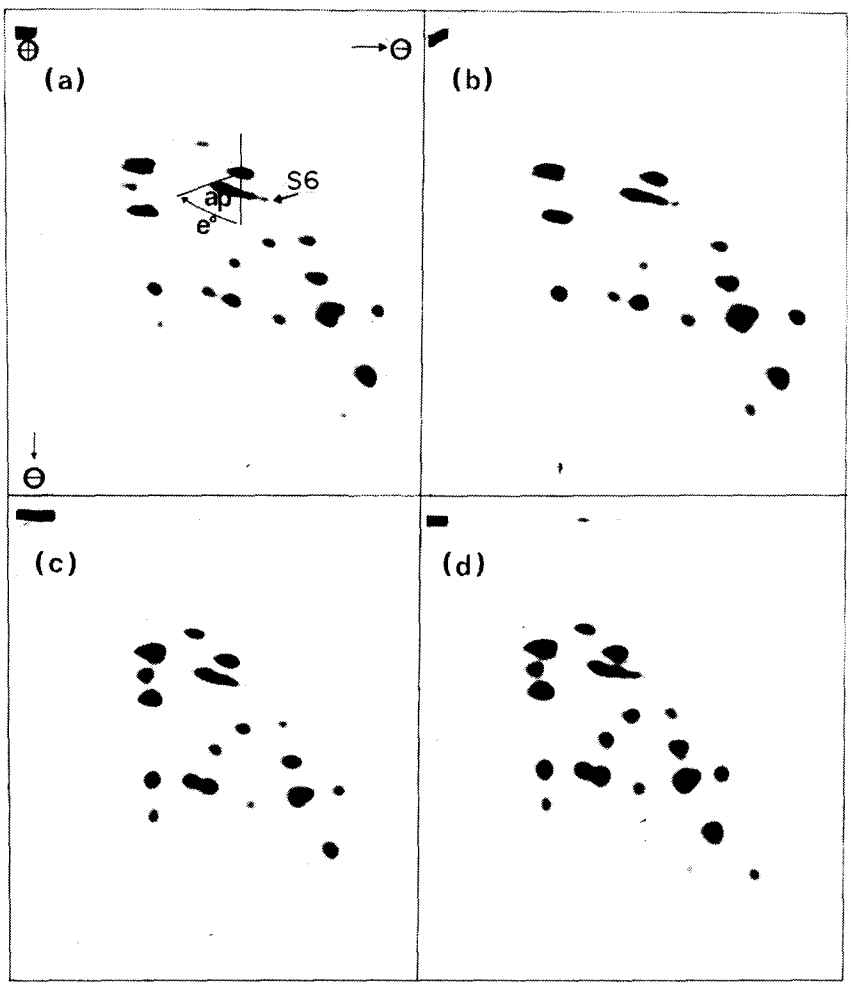

Fig. 1. Two-dimensional electropherograms of 40S ribosomal subunit proteins from the livers of rats treated with D-galactosamine and trifluoperazine. Trifluoperazine was always injected i.p. 30 min prior to the application of D-galactosamine (350 mg/kg body wt). The rats were decapitated 3 hr after application of D-galactosamine and 40S ribosomal subunit protein was isolated from their livers. For electrophoresis 500 μg of extracted protein was used. The position of protein S6, the elongation angle (e°) and the area of phosphorylated S6 (ap) are indicated as defined elsewhere [25]. (a) Only D-galactosamine, (b) D-galactosamine + 100 mg/kg body wt trifluoperazine, (c) D-galactosamine + 5 mg/kg body wt trifluoperazine, (d) D-galactosamine + 1 mg/kg body wt trifluoperazine.

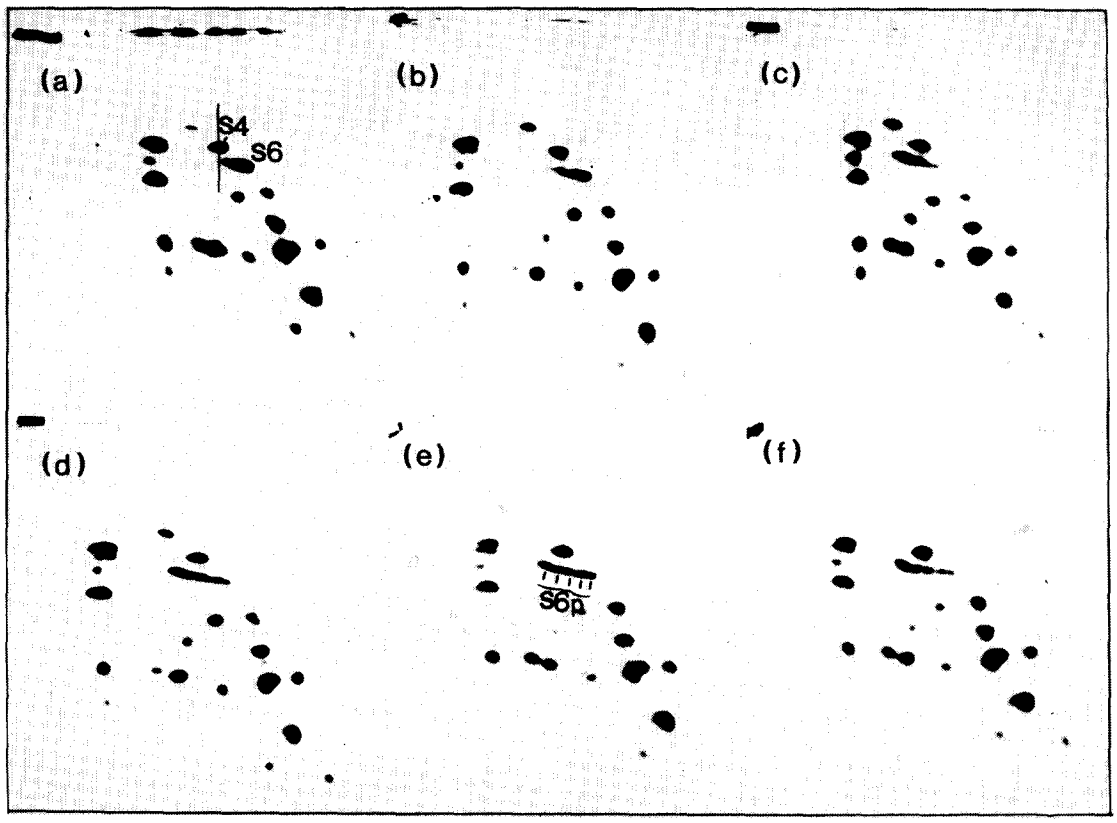

Fig. 2. Two-dimensional electropherograms of 40S ribosomal subunit proteins from the livers of rats treated with variable amounts of trifluoperazine. Trifluoperazine was injected i.p. and the rats were decapitated 3 hr later. Ribosomes and 40S ribosomal proteins were isolated and subjected to two-dimensional gel electrophoresis. (a) No trifluoperazine (control), (b) 10 mg/kg body wt trifluoperazine, (c) 25 mg/kg body wt trifluoperazine, (d) 50 mg/kg body wt trifluoperazine, (e) 100 mg/kg body wt trifluoperazine, (f) 300 mg/kg body wt trifluoperazine. The positions of phosphoprotein S6 and of protein S4 are indicated (a). In (e) the five derivatives of phosphoprotein S6 (S6p) are marked.

bated for 3 hr at 37° and 50 oscillations/min in 3 ml of Dulbecco's modification of Eagle's medium containing 1 μCi of L-[^{14}C]valine [291 mCi/mmole (New England Nuclear)] under 95% O_2–5% CO_2.

At the end of incubation the slices were quickly washed in ice-cold phosphate-buffered saline, boiled for 3 min, dried and homogenized in 2 ml of water from which an aliquot was taken for the determination of protein [22]. Hundred-microlitre portions of the homogenate were spotted on Whatman 3 MM filter paper disks and sequentially extracted according to the technique of Mans and Novelli [26]. The extracted material on the filter was placed in a scintillation vial and digested with 0.5 ml of Soluene 350 (Packard Instrument Co., U.S.A.). After the addition of 10 ml Econofluor (New England Nuclear) the radioactivity was counted.

RESULTS

In accordance with previously reported results [20] the intraperitoneal application of the hepatotoxic agent D-galactosamine causes a strong anodic dislocation of small ribosomal subunit protein S6 visible on the two-dimensional electropherograms (Fig. 1a), which is due to an increased number of phosphorylated serine residues of this protein [1]. The application of various doses of trifluoperazine to the rat prior to the injection of the hepatotoxin does not affect the degree of galactosamine-induced phosphorylation of this protein (Fig. 1b–d). The e° and ap (Fig. 1a) were 70° and 22 mm^2 respectively in galactosamine-treated liver [both are 0 in preparations from control rat liver (Fig. 2a)]. The same parameters for phosphorylated S6 were 72° and 23 mm^2, respectively, in preparations from rat livers treated with 5 mg/kg body wt of trifluoperazine 30 min before injection of 350 mg/kg body wt of D-galactosamine (Fig. 1c). Higher (100 mg/kg) (Fig. 1b) and lower (1 mg/kg) (Fig. 1d) doses of trifluoperazine had essentially the same effects.

In a series of further experiments the effect of trifluoperazine alone on the phosphorylation of ribosomal protein S6 was studied (Fig. 2). It was found that an application of about 10 mg/kg body wt of trifluoperazine caused a significant increase in e° (59°) and ap (9 mm^2) seen on the two-dimensional electropherograms (Fig. 2). Doses higher than 25 mg/kg body wt trifluoperazine caused a maximum shift of S6 (e° = 71°, ap = 27 mm^2) with the occurrence of five phosphorylated derivatives of this small ribosomal subunit protein. Trifluoperazine (1 mg/kg body wt) was without an effect on S6 phosphorylation. The data summarized in Table 1 document

Table 1. The effect of trifluoperazine on the incorporation of radioactivity from *o*-[^{32}P]phosphoric acid into liver microsomes, 80S ribosomes, 40S ribosomal subunits, ribosomal protein of the 40S subunits and protein S6 of the small subunit

Source of liver	Microsomes (cpm/E_{260} unit)	80S ribosomes (cpm/E_{260} unit)	40S ribosomal subunit (cpm/E_{260} unit)	40S ribosomal protein (cpm/mg protein)	Protein S6 (cpm/protein S6)
Control	835/755	38/22	25/17	720/600	47/29
Trifluoperazine-treated (mg/kg body wt)					
10	3780/3460	158/118	165/115	2660/2400	121/91
20	7245/6395	326/254	300/380	6440/6040	250/190
50	5810/5030	257/203	290/210	5650/5050	220/180

* Rats were injected i.p. with variable amounts of trifluoperazine and received 3 hr later 0.8 mCi of *o*-[^{32}P]phosphoric acid 20 min before death. Controls were treated similarly but trifluoperazine was omitted. 80S ribosomes, 40S ribosomal subunits and 40S ribosomal protein were isolated from the livers and their specific radioactivity was determined. To estimate the radioactivity of protein S6 500 μg of extracted 40S ribosomal proteins were separated by two-dimensional electrophoresis, the area of S6 excised, the gel digested and counted for radioactivity.

The values of duplicate experiments are given.

that trifluoperazine greatly stimulates the incorporation of [^{32}P]phosphoric acid into microsomes and 40S ribosomal subunits of rat liver. A maximum increase in the specific [^{32}P]radioactivity of ribosomal proteins of the 40S subfraction is obtained with 20 mg/kg body wt of the drug. Under this condition the radioactivity associated with S6 is enhanced more than five-fold in comparison to respective control rat livers (Table 1).

The increase in the phosphorylation of S6 obviously is not related to variations in the intracellular cyclic AMP concn (Table 2). Doses of trifluoperazine which induce phosphorylation of protein S6 do not cause a significant elevation of the cyclic AMP content of liver. Only large doses of the drug raise moderately the cyclic nucleotide concn in liver.

Incorporation of [^{14}C]valine into the total protein of slices prepared from livers of rats pretreated with various doses of trifluoperazine *in vivo* was not affected by the drug (Table 3). Since trifluoperazine did not change either the entry of the labeled amino acid into the cells or the specific radioactivity of the intracellular pool of valine (results not shown) it is concluded that the stimulated phosphorylation of protein S6 by trifluoperazine occurs independently from possible changes of overall liver protein synthesis.

It has to be noted that the trifluoperazine-induced phosphorylation of S6 described earlier occurs *in vivo* but, as shown in Table 4, the phenothiazine derivative does not stimulate the incorporation of [^{32}P]orthophosphoric acid into the microsomal fraction and crude (unpurified) 80S ribosomes, respectively, of slices from normal rat liver *in vitro*. Similarly, the addition of D-galactosamine to liver slices incubated *in vitro* did not increase the specific radioactivity of [^{32}P]phosphate of microsomes and crude 80S ribosomes (Table 4). The results strongly suggest that under the conditions applied both drugs, trifluoperazine and D-galactosamine, respectively, do not stimulate the phosphorylation of protein S6 in liver explants *in vitro* although they are potent stimulators of this process *in vivo*.

Table 2. Effect of trifluoperazine on the concn of cyclic AMP in rat liver

Treatment group	Cyclic AMP content (pmoles/mg protein)
Control	3.1 ± 0.1
Trifluoperazine (mg/kg body wt)	
10	3.5 ± 0.2
20	3.9 ± 0.1
50	4.0 ± 0.4
100	4.1 ± 0.5
300	5.1 ± 0.7

Rats were injected i.p. with varying doses of trifluoperazine and decapitated 3 hr later. Control rats were handled similarly but trifluoperazine was omitted. Pieces of liver were extracted and assayed for cyclic AMP using a competitive protein-binding assay as described in Materials and Methods.

The values are the means ± S.D. of triplicate experiments.

Table 3. Effect of trifluoperazine on the incorporation of [^{14}C]valine into protein of liver slices

Treatment group	[^{14}C]Valine (dpm/mg protein)
Control	5610 ± 450
Trifluoperazine (mg/kg body wt)	
10	6230 ± 1050
20	5600 ± 260
50	5650 ± 380
100	4900 ± 860
300	5500 ± 149

Rats were treated for 3 hr with trifluoperazine as described in Table 2. Slices were prepared from the livers and incubated in Dulbecco's medium for 3 hr in the presence of 1 μCi of [^{14}C]valine. Total protein of the slices was extracted by the method of Mans and Novelli [26] and the specific radioactivity was determined.

The mean values ± S.D. of triplicate measurements are listed.

Table 4. The effect of trifluoperazine and D-galactosamine on the incorporation of [^{32}P]phosphoric acid into microsomes and crude 80S ribosomes of liver slices

	Microsomes (cpm/E_{260} unit)	80S ribosomes
Control	1320 ± 95	166 ± 15
Trifluoperazine	1520 ± 102	170 ± 9
D-Galactosamine	1550 ± 88	110 ± 14

Thirty slices were prepared from normal rat liver and incubated in 50 ml of Dulbecco's modification of Eagle's medium at 37° in 95% O_2–5% CO_2 in the absence of any additions (control) or in the presence of 50 μmoles/l of trifluoperazine and 500 μmoles/l of D-galactosamine, respectively. After 1 hr of incubation 0.5 mCi of *o*-[^{32}P]phosphoric acid was added to each incubation. Twenty minutes later the slices were washed in ice-cold buffer, microsomes and crude 80S ribosomes were isolated and their specific radioactivities were determined.

The values are the means ± S.D. of triplicate experiments.

DISCUSSION

The present study shows that the treatment of rats with trifluoperazine prior to the application of D-galactosamine does not block the increase in phosphorylation of liver ribosomal small subunit protein S6 induced by the hepatotoxic agent. Considering the antagonistic effect of this phenothiazine derivative on Ca^{2+}–calmodulin [17–19] the failure of total or partial reversibility of S6 phosphorylation by trifluoperazine suggests that a Ca^{2+}–calmodulin-dependent protein kinase is probably not implicated in the mechanism of S6 phosphorylation promoted by D-galactosamine and maybe also by other stimuli. However, before a definite conclusion on the non-participation of a Ca^{2+}–calmodulin-activated protein kinase in the process of S6 phosphorylation can be reached the effect of this drug and also of other inhibitors of calmodulin [27] on S6 phosphorylation in cultured cells promoted by defined agents [12] should be examined.

Instead, we identified the phenothiazine as a potent stimulator of S6 phosphorylation in normal rat liver. The mechanism by which the drug acts on the small ribosomal subunit protein is not clear. Obviously it does not involve elevation of total intracellular cyclic AMP. This observation supports the view of the existence of a cyclic AMP independent pathway of S6 phosphorylation suggested by others [12] which seems to be a frequently effective alternative to the cyclic AMP sensitive phosphorylation of this protein [9–12]. Depending on the nature of the stimulatory agents one of these mechanisms is operating which leads to the phosphorylation of different sites within the S6 polypeptide [28]. Clearly, if the action of trifluoperazine on intact cells is to be assessed other effects than inhibition of calmodulin, e.g. impairment of insulin secretion [29] and selective receptor blockade [30–32], have to be kept in mind.

The action of trifluoperazine on S6 is independent from the rate of overall protein synthesis in liver (Table 3). Under the conditions applied the incorporation of [^{14}C]valine was not modified by the phenothiazine derivative. This confirms for trifluoperazine that the correlation between phosphorylation of S6 and protein synthesis is not stringent, which was also found under cell culture conditions [9].

For further studies on the mechanism of trifluoperazine- and D-galactosamine-induced phosphorylation of S6 it should be emphasized that both drugs were ineffective in stimulating *in vitro* the incorporation of [^{32}P]phosphate into microsomes and unpurified 80S ribosomes of liver slices.

It seems unlikely that the time of exposure of the slices to the drugs was too short, since other cells respond to stimulators of S6 phosphorylation within this time period [12]. Rather, the results suggest that the action of the compounds on S6 requires certain humoral, e.g. hormonal, factors which have not been identified yet. The direct or indirect involvement of serum factors in S6 phosphorylation is well documented [11, 12, 33] and it was reported that the omission of serum from medium decreases the ribosomal protein kinase activity of cultured cells [34]. Thus, the effect of serum from trifluoperazine-treated rats on S6 phosphorylation in isolated hepatocytes needs to be examined.

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