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Biological effects of sarafotoxin in rat liver

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Sarafotoxins (SRTXs*) are a group of 21 amino acid peptides isolated from the venom of the Ein-Geddi burrowing asp *Atractaspis engaddensis* which are highly and rapidly toxic in humans. The most prominent action of the venom is its potent vasoconstrictor–cardiotoxic effect, associated with many local and systemic symptoms including oedema, hypoxia, hypertension, hemorrhage and liver damage [1]. SRTXs show a high degree of sequence homology with the mammalian ETs, a family of three potent vasoconstrictor peptides (ET-1, ET-2, ET-3) and with the vasointestinal contractor peptide (VIC or ET-b) [2], which exert multiple vascular and non-vascular effects [3]. We were interested in the effects of SRTX in the liver.

Materials and Methods

SRTX-b, ET-1, ET-3 and big ET-1 were obtained from Novabiochem (France). [^{125}I]SRTX-b (2000 Ci/mmol) and [^{14}C]glucose-1-phosphate (150 mCi/mmol) were from Amersham (Les Ulis, France). Male, Sprague–Dawley rats (250 g body wt) were from Charles River (Saint Aubin Les Elbeuf, France).

Rat liver plasma membranes were prepared by the method of Prpic *et al.* [4] and hepatocytes were isolated according to the procedure of Seglen [5]. Binding of [^{125}I]-SRTX-b was performed after incubation of liver plasma membranes (30–60 μg/mL) for 90 min at 22° in a 200 μL Krebs–Ringer medium (pH 7.4) containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid, 1% bovine serum albumin, 300 μg/mL bacitracin, with either 50 pM [^{125}I]SRTX-b and varying concentrations of peptides (competition experiments) or varying concentrations of [^{125}I]SRTX-b (saturation experiments), as described previously [6]. Non-specific binding was determined by incubating with 0.1 μM unlabelled SRTX-b. Data from saturation, competition and association experiments were analysed using a non-linear regression program [7]. Activation of rat hepatocytes (7–9 × 10^6 cells/mL) by peptides was performed for 2 min at 37° followed by quick freezing of the tubes in liquid nitrogen as described previously [6]. Glycogen phosphorylase *a* activity was determined as in [6] using 50 mM [^{14}C]glucose-1-phosphate (30,000 cpm/assay). Protein concentrations were determined according to the method of Bradford using bovine serum albumin as standard.

Results and Discussion

Binding of [^{125}I]SRTX-b to rat liver plasma membranes was time-dependent ($T_{1/2}$ = 21 min) and reached an apparent equilibrium at about 90 min (k_{obs} = 0.035 min^{-1}). Dissociation of receptor-bound ligand was minimal, since only 15–20% of dissociation was observed 4 hr after the addition of 1 μM unlabelled SRTX-b (not shown).

As shown in Fig. 1, SRTX-b and ET-1 were equally potent in inhibiting [^{125}I]SRTX-b binding, with IC_{50} values of 0.45 ± 0.01 (n_H = 0.95 ± 0.04) and 0.13 ± 0.06 nM (n_H = 1.07 ± 0.07), respectively. The 39-amino acid intermediate precursor of ET-1, termed big ET-1, was 500-fold less potent (IC_{50} = 224 ± 39; n_H = 1.087 ± 0.15). A second set of competition experiments, performed with [^{125}I]ET-1 indicated that SRTX-b and ET-1 were equally potent in inhibiting [^{125}I]ET-1 binding, with IC_{50} values of 0.15 ± 0.05 and 0.70 ± 0.29 nM, respectively, and with an apparent Hill coefficient close to unity (not shown). Competition experiment data, analysed by a non-linear regression program [7], were better fit by a one site model. Taken together, the data shown in Fig. 1 suggest that SRTX-b and ET-1 may bind with high affinity to the same population of sites in the liver, as indicated by the mutally exclusive binding of the two peptides to these sites.

ET-3 also inhibited [^{125}I]SRTX-b binding and the inhibition curve was better fit by a two site model indicating that ET-3 discriminated between two ET/SRTX binding sites. About 70% of the binding sites had high affinity for ET-3 (IC_{50} = 0.22 ± 0.015 nM, a value similar to that of SRTX-b and ET-1, see Fig. 1) and about 30% were of low affinity for ET-3 (IC_{50} = 40 ± 14 nM).

Incubation of hepatocytes with 10 nM SRTX-b resulted in a rapid and sustained activation of glycogenolysis: a 1.6-fold activation of glycogen phosphorylase *a* was observed after 1 min, and the activity remained elevated for at least 15 min (Fig. 2A). The effect of SRTX-b was dose-dependent, a maximal 1.5-fold stimulation of glycogen phosphorylase *a* being attained at 10 pM, with an EC_{50} of 0.13 ± 0.11 pM (Fig. 2B). SRTX-b was almost as potent as ET-1 in activating glycogenolysis, while ET-3 and big ET-1 were 100- and 300-fold less potent, respectively [6].

The present study demonstrates that, in the liver, SRTX-b binds to high affinity binding sites and activates glycogenolysis. The low reversibility of SRTX-b binding may explain the long-lasting stimulation of glycogenolysis (Fig. 2A), as suggested previously for the action of ET-1 [6] and ET-3 [8] in the liver and in other tissues (for a review, see Ref. 3). We have characterized previously high affinity ET-1 binding sites in liver plasma membranes [6]. We show here that SRTX-b and ET-1 bind to the same population of sites in the liver as indicated by the mutually exclusive binding of the two peptides to these sites and as reported in uterus, heart and brain [9–11]. Competition experiments (see Fig. 1) indicate that two populations of SRTX/ET binding sites coexist in the liver, which bind ET-3 with different (high and low) affinities, SRTX-b and ET-1 binding to the two sites with similar high affinities. Recently, molecular cloning of two distinct ET receptors

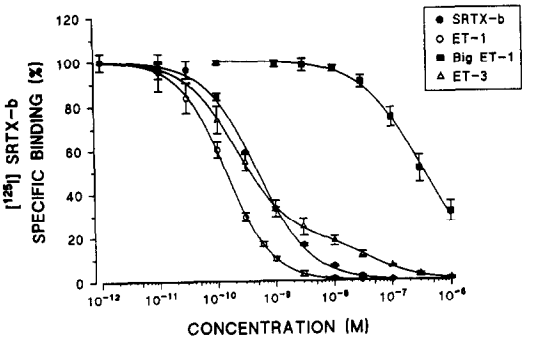

Fig. 1. Competitive inhibition of the binding of [^{125}I]SRTX-b to rat liver plasma membranes by unlabelled peptides. Each point represents the mean ± SD from three independent experiments.

* Abbreviations: SRTX, sarafatoxin; ET, endothelin.

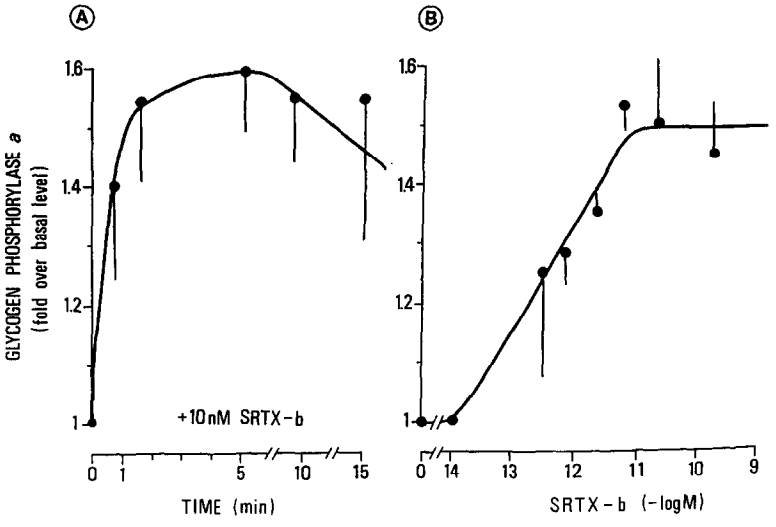

Fig. 2. Time-course (A) and dose-dependent activation (B) of glycogenolysis by SRTX-b. Data are expressed as fold increase over basal glycogen phosphorylase *a* activity, which was 35 ± 5 nmol glucose-1-phosphate transformed/mg protein/min. Results are the means ± SEM of three experiments.

has been reported; one (ET_A) with high affinity for ET-1 and SRTX-b and low affinity for ET-3 [12], the other non-selective (ET_B), binding the three peptides with similar high affinities [13]. This raises the possibility that the two liver binding sites correspond to the two ET subtypes, ET_A and ET_B.

The biological effects of SRTX-b in the liver are similar to that induced by ET-1, since both cause activation of glycogenolysis with similar potencies and affinities (see Fig. 2B and Ref. 6). The rank order of potencies of related peptides to activate glycogenolysis is ET-1 ≥ SRTX-b ≫ ET-3 > big ET-1, suggesting that, of the two ET binding sites existing in the liver, only the one with high affinity for ET-1 and SRTX-b and low affinity for ET-3 (ET_A subtype?) mediates activation of glycogenolysis. It should be noted that, as observed previously for ET-1 [6], the apparent affinity of SRTX-b for its binding sites is 1000-fold lower than that for activation of glycogen phosphorylase *a*. This may be attributed both to the difficulty in estimating binding parameters due to the poor reversibility of SRTX-b binding and to the possible existence of "spare" receptors, implying that only a small fraction of ET receptors occupied would be needed to obtain maximal stimulation of glycogenolysis.

We report here that SRTX-b binds to the same population of sites as ET-1 in the liver and exerts similar biological effects. Inhibition curve of [^{125}I]SRTX-b binding by ET-3 was better fit by a two site model indicating the presence of two binding sites for SRTX/ET in liver plasma membranes: one with high affinity for ET-1 and SRTX-b and low affinity for ET-3, and a non selective subtype which binds the three peptides with similar high affinities. Based on the order of potencies of related peptides to activate glycogen phosphorylase *a*, only the ET-1/SRTX selective subtype would be coupled to glycogenolysis, the function of the non-selective ET subtype being under investigation. It should be stressed that to date, SRTX-b and ET-1 are the most potent activators of hepatic glycogenolysis. Moreover, this study points out the role of liver as one target organ for the action of SRTX which may account for some of the toxic effects of this peptide.

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Hormonal regulation of 3α-hydroxysteroid/dihydrodiol dehydrogenase in rat liver cytosol

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Abstract—In rat liver, dihydrodiol dehydrogenase and 3α-hydroxysteroid dehydrogenase activities are catalyzed by the same protein. This study shows that estrogen and growth hormone can induce the enzyme in hypophysectomized rats. This implies that estrogen can exert an effect on hepatic steroid and carcinogen metabolism in the absence of the anterior pituitary.

Dihydrodiol dehydrogenase (DD*; EC 1.3.1.20) prevents the formation of *anti*-diol epoxides of polycyclic aromatic hydrocarbons (ultimate carcinogens) by converting their *trans*-dihydrodiol precursors to reactive *ortho*-quinones [1–3] which can be detoxified as glutathionyl and mercapturic acid conjugates [4, 5]. In rat liver, DD is identical to 3α-hydroxysteroid dehydrogenase (3α-HSD; EC 1.1.1.50) [6]. Understanding the mechanisms which induce 3α-HSD/DD activity may have important implications for the regulation of chemical carcinogenesis and steroid hormone metabolism. Previous studies have shown that this enzyme is not regulated by classical inducers of polycyclic aromatic hydrocarbon metabolism such as 3-methylcholanthrene [7, 8]. The present communication shows that estrogens and growth hormone have a direct effect on the induction of rat hepatic 3α-HSD/DD.

Materials and Methods

Androsterone, testosterone and progesterone were products of Steraloids (Wilton, NH). Benzenedihydrodiol (*trans*-1,2-dihydroxy-3,5-cyclohexadiene) was synthesized as described [9]. β-NAD^+ and $NADP^+$ (monosodium salts) were obtained from Pharmacia PL Biochemicals (Piscataway, NJ). Enzyme grade ammonium sulfate and sucrose were products of Schwarz/Mann, Inc. (Spring Valley, NJ). 17β-Estradiol-3-sulfate and growth hormone (bovine somatotrophin) were purchased from Sigma (St. Louis, MO).

All animals were obtained from Charles River (Wilmington, MA). Female rats were ovariectomized on day 28 and treated 2 weeks later. Immature male and female rats were hypophysectomized on day 21, and animals that demonstrated no weight gain over 10 days were then treated. Animals received growth hormone (1.5 units bovine somatotrophin s.c./day for 6 days) or a single dose of one of the following: 17β-estradiol-3-sulfate, testosterone or progesterone (1.0 mg of steroid in saline + 2% EtOH, s.c.). Animals were decapitated 48 hr after the last treatment. Control animals received vehicle alone which had no effect on enzyme activity.

Livers (1 g) from single animals were homogenized in 50 mM Tris–HCl pH 8.6, containing 250 mM sucrose, 1 mM EDTA and 1 mM 2-mercaptoethanol. Cytosol was prepared by differential centrifugation and a 40–75% ammonium sulfate fraction was prepared which precipitated all the soluble 3α-HSD/DD activity [6].

3α-HSD (androsterone oxidation) and DD (benzenedihydrodiol oxidation) activities were measured spectrophotometrically with the 40–75% ammonium sulfate fraction as described previously [9]. Reactions were initiated by the addition of the enzyme preparation. Activities were expressed as nanomoles substrate oxidized per minute per milligram protein. Protein determinations were made by the method of Lowry *et al.* [10].

Results and Discussion

We have shown that livers from adult female rats contain twice as much 3α-HSD/DD activity as adult males and that this difference is reflected by a comparable increase in enzyme protein [9]. Enzyme activity in adult female rats can be reduced to the level observed in males by ovariectomy [9], confirming previous reports that soluble 3α-HSD activity is elevated in female rat liver cytosol [11]. Furthermore, treatment of ovariectomized female rats with 17β-estradiol-3-sulfate restores enzyme activity to the level observed in normal adult female rats [9]. Administration of 17β-estradiol-3-sulfate to intact males also produced a significant increase in enzyme activity, suggesting that feminization of the enzyme phenotype has a dependence on estrogen (see Fig. 1). Similar findings have been reported for the regulation of soluble 3α-HSD in female rat kidney [12].

* Abbreviations: DD, dihydrodiol dehydrogenase, *trans*-1,2-dihydrobenzene-1,2-diol:dehydrogenase (EC 1.3.1.20); 3α-HSD, 3α-hydroxysteroid dehydrogenase, 3α-hydroxysteroid:$NAD(P)^+$ oxidoreductase (EC 1.1.1.50); benzenedihydrodiol, *trans*-1,2-dihydroxy-3,5-cyclohexadiene; androsterone, 5α-androstan-3α-ol-17-one; 17β-estradiol-3-sulfate, 1,3,5(10)-estratriene-3,17β-diol-3-sodium sulfate; GH, growth hormone; IGF-1, insulin-like growth factor I; and HYP, hypophysectomized.