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Short communication

Effect of isatin on nitric oxide-stimulated soluble guanylate cyclase from human platelets

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Abstract

Isatin, an endogenous indole, has previously been shown to inhibit atrial natriuretic peptide (ANP)-stimulated particulate guanylate cyclase activity. Here, it was shown that it can be transported to human platelets where it inhibited nitric oxide (NO)-stimulated soluble guanylate cyclase activity obtained from human platelets. The effect was most pronounced at 10^{-8} M isatin and is the most potent effect of isatin yet observed. The dose response curve was bell shaped with higher doses becoming less effective. The maximal inhibition observed was of 40%. Isatin had no effect on protoporphyrin IX-stimulated guanylate cyclase. Isatin-dependent regulation of ligand-stimulated guanylate cyclases is suggested to promote a stress-induced switch in metabolism.

Keywords: Isatin; Guanylate cyclase; Nitric oxide; Atrial natriuretic peptide; Platelet; Stress

1. Introduction

Isatin is an endogenous compound, widely distributed in mammalian tissues and body fluids [1]. Its output is increased in conditions of stress [2,3]. The substance was discovered as a component of the endogenous monoamine oxidase (MAO) inhibitory activity, tribulin [4] and is a selective inhibitor of MAO B, with an apparent inhibition constant of 3–20 μM; inhibition of MAO A occurs at higher concentrations (Ki: 60–70 μM) [1]. Isatin also interacts preferentially with MAO B *in vivo* [5]. The most potent known action of isatin, *in vitro*, is the inhibition of ANP binding to its receptor [1,2,6], with an IC_{50} value of 0.4 μM. This is within the physiological range of isatin concentration in the brain [1].

Isatin has been shown to inhibit ANP-stimulated guanylate cyclase of rat brain, heart, and kidney membrane in a dose-dependent manner, reducing formation of (cGMP) [6,7]. Guanylate cyclases are enzymes that produce cyclic GMP from GTP in response to ligand binding. They exist in two forms, a soluble form and a particulate membrane-bound form [8,9], each of which is activated by distinct agonists. Soluble guanylate cyclase is activated by NO, and NO donors such as sodium nitroprusside [10], whereas the particulate form is a plasma membrane receptor for the natriuretic peptides [8,9]. The particulate guanylate cyclases constitute the catalytic domain of natriuretic peptide receptors A and B. These can be discriminated by the differential potency of action of different natriuretic peptides. Guanylate cyclase coupled to the A receptor is more sensitive to stimulation by atrial and brain natriuretic peptides, whereas the B-coupled cyclase is more sensitive to C-type natriuretic peptide [11]. We have demonstrated that isatin interacts with natriuretic peptide receptor A [7]; its effect on the B receptor is not clear. Its effect on soluble guanylate cyclase has not been previously investigated.

In the present study, we investigated the effect of isatin on soluble guanylate cyclase from human platelets, stimulated by the NO donor sodium nitroprusside. Platelets contain only the clearance receptors for natriuretic peptides; these do not exhibit guanylate cyclase activity [12]. We also investigated the possible transport of a physiological concentration of isatin into platelets.

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Abbreviations: NO, nitric oxide.

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2. Materials and methods

[5(*n*)-^{3}H]Isatin (specific activity 26 Ci/mmol) was a custom-made product (Amersham Pharmacia Biotech). All other chemicals were obtained from Sigma–Aldrich unless otherwise specified.

Human platelets were used as the source of soluble guanylate cyclase. Platelets were isolated from the blood of donors as described previously [13]. A suspension of washed platelets in 50 mM Tris–HCl buffer (pH 7.6) containing 0.2 mM dithiothreitol was sonicated in an MSE 5–78 ultrasonic sonicator (UK) for 20 s at 2° and centrifuged at 105,000 *g*. The supernatant was used as the source of human platelet soluble guanylate cyclase.

For determination of isatin transport, platelet-rich plasma was diluted twice with 20 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-ethanesulfonic acid (HEPES) buffer, pH 7.2, containing 160 mM NaCl and glucose (2 g/L). The diluted platelet-rich plasma (200 μL) was incubated with 0.2 μM [^{3}H]isatin for 0, 3, 5, and 10 min at 37 or 0°. The incubation was terminated by 10-fold dilution of the incubation mixture with 20 mM HEPES buffer, pH 7.2, containing 160 mM NaCl and glucose (2 g/L), and platelets were isolated. The amount of transported isatin was calculated as the difference between radioactivity accumulated by the platelet fraction at 37 and 0°. Pilot experiments showed there was no significant binding of [^{3}H]isatin to platelets under these conditions. In the experiments with serotonin reuptake inhibitor, stock aqueous solution of fluoxetine (0.3 mM) was added to the diluted platelet-rich plasma (final fluoxetine concentration: 3 μM) 2 min before adding [^{3}H]isatin; the total incubation time at 37° was 5 min. In control samples water was added instead of fluoxetine.

Guanylate cyclase activity was assayed as described by Garbers and Murad [14]. The reaction mixture (final volume: 150 μL) contained 50 mM Tris–HCl buffer (pH 7.6), 1 mM GTP, 4 mM $MgCl_2$, 4 mM creatine phosphate, 20 μg (120–160 units) creatine phosphokinase, 10 mM theophylline, and 20 μg of human platelet supernatant protein. The effect of isatin was studied in the concentration range from 10^{-9} to 10^{-4} M. The amount of cGMP formed (15 min, 37°) was estimated by enzyme-linked immunosorbent assay method by using Bioimmunogen kits.

Protein was determined by Bradford's method [15].

Statistical differences were evaluated by using the Student's *t*-test.

3. Results

Within the concentration range of 10^{-9} to 10^{-4} M, isatin did not influence the basal activity of soluble guanylate cyclase from human platelets. However, isatin did attenuate sodium nitroprusside-stimulated activity of the enzyme.

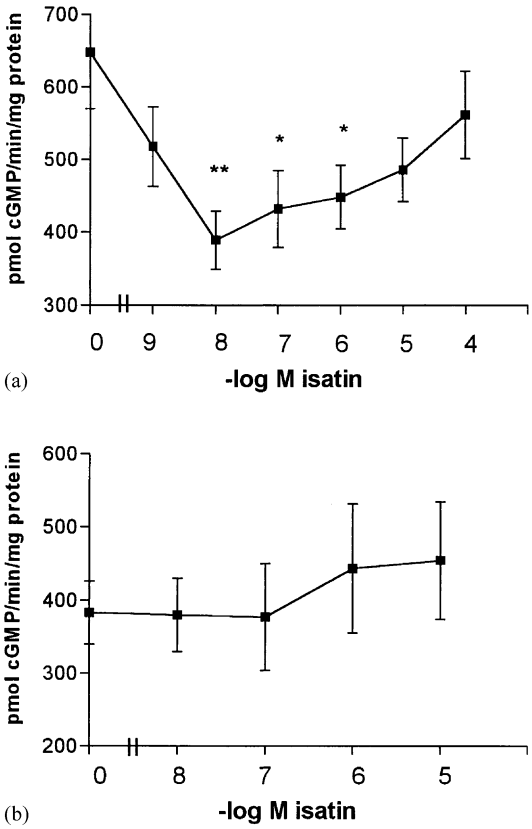

Fig. 1. (a) The effect of isatin on sodium nitroprusside-stimulated soluble guanylate cyclase from human platelets (mean ± SE). (*) $P < 0.05$ and (**) $P < 0.01$: significantly different from with no isatin. (b) The effect of isatin on protoporphyrin IX-stimulated soluble guanylate cyclase from human platelets basal GC activity: 54 ± 8 pmol cGMP/min/mg of protein. Mean of six experiments.

Fig. 1 shows the dose-response curve of the isatin effect on soluble guanylate cyclase. The greatest isatin effect was observed in the range of 10^{-8} to 10^{-6} M. Subsequent increase of isatin concentration, up to 10^{-4} M, reduced inhibition. The maximal inhibition achieved was 40%.

The specificity of the isatin effect on non-stimulated guanylate cyclase was confirmed in a series of independent experiments with enzyme activation induced by protoporphyrin IX. The latter, an immediate heme precursor, is an endogenous stimulator of guanylate cyclase activity [16]. However, in contrast to NO, which requires the guanylate cyclase heme for activation, the latter is not involved in the protoporphyrin IX-induced stimulation of this enzyme [17]. Isatin did not influence guanylate cyclase activation by protoporphyrin IX (5 μM; Fig. 1). Thus, isatin acted specifically on NO-stimulated guanylate cyclase.

Incubation of platelet-rich plasma with a physiological concentration of [^{3}H]]isatin (0.2 μM) resulted in its accumulation in the isolated platelets (Fig. 2). This accumulation was found only at 37° and not at 0°. Using the difference between the content of [^{3}H]isatin in platelets incubated at 37 and 0° as the measure of isatin transport, it was found to be linear over 5 min incubation (Fig. 2).

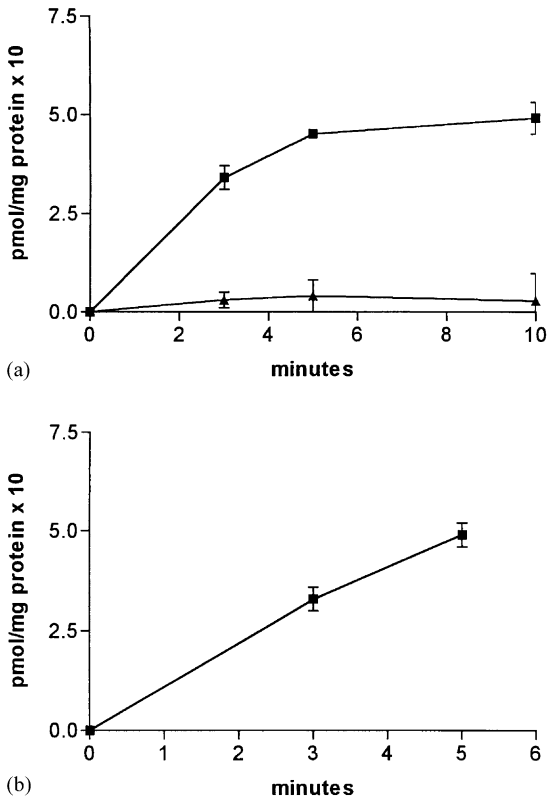

Fig. 2. Isatin transport into human platelets (mean ± SE): (a) at 37° (■) and 0° (▲); (b) at 37–0°. Mean of six experiments.

Increase of the incubation time up to 10 min caused only an insignificant increase of the platelet accumulation of the tritiated isatin. Pre-treatment of the platelets with the selective serotonin uptake inhibitor, fluoxetine, at 3 μM, significantly reduced the isatin accumulation but did not abolished it: control without fluoxetine 0.52 ± 0.008 pmol/5 min/mg of protein; in the presence of fluoxetine 0.23 ± 0.053 pmol/5 min/mg of protein ($P < 0.01$).

4. Discussion

The results of the present study provide the first experimental evidence that low physiological concentrations of isatin, in the 10^{-8} to 10^{-6} M range, may attenuate the NO-dependent stimulation of soluble guanylate cyclase of human platelets.

Isatin concentration in human blood has been reported to vary from 134.2 ± 120.6 ng/mL (assayed in plasma) [18] to 54.7 ± 4.2 ng/mL (assayed in serum) [19]. This approximately corresponds to a range of concentrations 0.3–1.7 μM. The experiments reported here show that [^{3}H]isatin, at the lower end of these values (0.2 μM), can be transported into platelets (Fig. 2) and, therefore, is able to interact with soluble guanylate cyclase.

The mechanism of isatin transport into platelets requires further investigation, with detailed kinetic studies using a wide range of isatin and fluoxetine concentrations. After 5 min incubation, accumulation of isatin became non-linear, suggesting the existence of saturable transport of isatin into platelets. This process was sensitive to the serotonin reuptake inhibitor fluoxetine. However, it remains unclear why a relatively high concentration of this inhibitor (3 μM) only partially inhibited the isatin transport. There may be both active and passive accumulation.

It is of interest that not only particulate guanylate cyclase stimulated by natriuretic peptides [5,7], but also soluble guanylate cyclase, activated by NO, is inhibited by isatin. Moreover, the NO-stimulated guanylate cyclase was even more sensitive to isatin than the particulate enzyme. However, in contrast to the latter, the soluble guanylate cyclase exhibited a bell-shaped dependence on the isatin concentration. Moreover the maximal inhibition achieved was only 40%. Such kinetic behaviour has also been found for the dependence of the auto-oxidation rate of some heme-containing proteins on oxygen concentration [20].

There is increasing evidence that membrane and soluble guanylate cyclases cooperatively regulate cGMP-mediated effects in human and murine vascular tissues [21]. They also may exert concerted effects in other tissues [22]. Thus, it is possible that the increase of isatin concentration under conditions of stress will attenuate ligand-dependent stimulation of both isoenzymes and promote a stress-induced switch in metabolism.

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