J Neural Transm [GenSect] (1990) 79: 35–40

Journal of Neural Transmission

Serotonergic effects of isatin: an endogenous MAO inhibitor related to tribulin

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Accepted February 10, 1989

Summary. A study of the acute effects of isatin, an endogenous MAO inhibitor related to tribulin, on rat brain serotonergic function was undertaken. A single dose of isatin significantly increased 5-HT concentrations in the hypothalamus and cortex but did not significantly alter 5-HIAA concentrations. Synaptosomal 5-HT uptake was unaffected but there was a trend for the number of ^{3}H-ketanserin binding sites was to be decreased. The results of the study are discussed in terms of the relationship of isatin to tribulin and their possible causal role in stress.

Keywords: Tribulin, isatin, MAO, serotonin, 5-HT$_2$ receptors, 5-HT uptake.

Introduction

Tribulin, an endogenous monoamine oxidase (MAO) inhibitor and displacer of benzodiazepine binding, has been detected in rat and human urine and rat heart and brain (Glover et al., 1980; Clow et al., 1983; Armando et al., 1986). The chemical nature of tribulin was unknown until recently when Glover and colleagues (1988) reported that the endogenous 2,3-dione, isatin has properties similar to tribulin. Furthermore they succeeded in isolating isatin from rat and human urine and identifying it by mass spectrometry. It remains unclear, however, if all of the MAO inhibiting and benzodiazepine displacing activity previously attributed to tribulin is due to isatin (Glover et al., 1988). "Tribulin" may be several substances of which isatin is one component. Metabolites of isatin or related endogenous compounds may be responsible for some of the biological effects attributed to tribulin.

Tribulin formation in animals is influenced by external stimuli. One of the earliest reports described a significant increase in tribulin output following cold immobilization stress (Glover et al., 1981). Cold immobilization stress has also been shown to be associated with alterations in the serotonergic system, which were attributed to the formation of tribulin (Oxenkrug and McIntyre, 1985).

Cold-immobilization produced a significant increase in pineal melatonin, N-acetylserotonin and serotonin (5-HT) and a decreased turnover of the brain serotonergic system as indicated by a reduction in the 5-hydroxyindole acetic acid (5-HIAA) to 5-HT ratio (McIntyre et al., 1989). Given the relationship between tribulin and serotonin and between tribulin and isatin, we undertook an investigation of the effects of an acute dose of isatin on serotonergic function.

Methods

Animals

Male Sprague-Dawley rats (two months old, weighing 150–200 g) were housed under a constant temperature (22 °C) and diurnal lighting conditions (12 hr light/12 hr dark) with free access to food and water.

Animals were injected (i.p.) with either 80 mg/kg isatin dissolved in dimethyl sulphoxide (DMSO) or DMSO alone. All animals were killed one hour following injection. The dose of isatin for these experiments was selected on the basis of previous reports that doses of 40 mg/kg and higher produce physiological changes (Chocholava and Kolinova, 1981). Isatin was purchased from the Sigma Chemical Co. (St. Louis, U.S.A.).

Animals were killed by decapitation and the brains quickly removed, dissected on ice and stored frozen at — 70 °C until analysis.

Analysis of 5-HT and 5-HIAA

Tissue concentrations of 5-HT and its metabolite 5-HIAA were measured in the hypothalamus and frontal cortex by a high pressure liquid chromatographic (HPLC) procedure. The hypothalamus or frontal cortex (20–40 mg wet weight) was added to 500 µl 0.1 M perchloric acid (on ice), sonicated to provide an homogenous solution and centrifuged for 5 minutes at 10,000 g. Ten microlitre aliquots of the supernatant were then injected directly onto the column. The HPLC conditions were as follows: flow rate of mobile phase 1.2 ml/min, Waters M 460 electrochemical detector set at a potential of 0.70 V and a range of 5 nA. The column was a C_{18} reverse phase HPLC column (Millipore Waters, Milford, MA, U.S.A.).

Mobile phase consisted of 9.8 g KH_2PO_4, 1 g Na_2 EDTA and 5% acetonitrite per litre and pH was 3.0.

^{3}H-ketanserin binding assay

Tissues were suspended in 40 volumes of buffer (5 mM Tris-HCl, 0.1% Na_2 EDTA, pH = 7.5) at 4 °C and homogenized using an Ultraturrax homogenizer (half-speed, 30 sec). The homogenate was centrifuged at 30,000 g for 10 mins at 4 °C and the resulting pellet resuspended by homogenization in fresh Tris-HCl buffer and respun at 30,000 g for 10 mins at 4 °C. The supernatant was discarded and the pellet resuspended by homogenization in buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.05% ascorbic acid, pH = 7.3) spun at 30,000 g for 10 mins at 4 °C.

The pellet was resuspended in fresh buffer by homogenization to a final volume of 10 mls.

Incubations were performed at 37 °C for 1 h and contained 400 µl membrane preparation, 50 µl ^{3}H-ketanserin (0.2 to 12.8 nM) and 50 µl of incubation buffer with or without methysergide (final concentration 1 µM). The incubations were terminated by the addition of 5 ml of ice-cold Tris incubation buffer and then filtered under reduced pressure through Whatman GF/B filters. The filters were washed twice more with Tris buffer, dried in air

at room temperature and counted in 10 mls of scintillation fluid (Triton, PPO, and POPOP in toluene) in a Liquid Scintillation Counter LKB at an efficiency of approximately 85%. The total bound ^{3}H-ketanserin was less than 5% of radioactivity added. Specific binding was 70% at 2 nM. Protein concentrations were determined by the method of Lowry et al. (1951).

The equilibrium binding characteristics of ^{3}H-ketanserin were calculated from Scatchard analysis of the specific binding using a least squares fitting procedure. The dissociation constant (Kd) and the density of the binding sites (B_{max}) was determined using the computer program EBDA (McPherson, 1983).

Synaptosomal 5-HT uptake

Synaptosomes from prefrontal cortex were prepared by homogenizing the tissue in 0.32 M sucrose at 4 °C. Crude synaptosomes were separated by centrifugation at 1,000 × g at 4 °C. Serotonin uptake was determined by incubating the synaptosomal suspension at 37 °C in the presence or absence of chlomipramine and ^{14}C-5-HT in the concentration range 25 nM to 0.5 μM. Incubations were terminated after 2 mins by vacuum filtration over Whatman GF/B filters. All other conditions of the incubations were as described by Heym and Gladfelter (1982).

Repeated analyses of rat prefrontal cortex have shown acceptable assay precision, with percent coefficient of variation of V_{max} = 11.5% (10.4 ± 1.2 pmol/mg prot/2 min; n = 12); Km (0.12 ± 0.04 μM; n = 12).

Statistical analysis

Student's t-test was used to evaluate the difference between two means for significance by the Statistical Package for the Social Sciences (SPSS-X).

Results

A single dose of isatin increased 5-HT concentrations in the hypothalamus by 60% compared to controls (Table 1; $p < 0.005$, t-test). Concentrations of 5-hydroxyindole acetic acid (5-HIAA) were not significantly different from the controls (Table 1) but the ratio of 5-HIAA/5-HT decreased significantly ($p < 0.0001$). A similar pattern of results was observed in the frontal cortex (Table 2). In general the magnitude of changes in frontal cortex was not as large as that recorded in hypothalamus (e.g. 5-HT was increased 37% cf. 60%).

The number of 5-HT_2 binding sites, as defined with ^{3}H-ketanserin, were

Table 1. Concentration of 5-HT and 5-HIAA in the hypothalamus after 80 mg/kg isatin (i.p.)

Group	N	5-HT (ng/mg tissue)	5-HIAA (ng/mg tissue)	$\frac{\text{5-HIAA}}{\text{5-HT}}$
Control	10	1.00 ± 0.07	1.39 ± 0.17	1.39 ± 0.12
Isatin (1 hr)	5	1.60 ± 0.33*	1.54 ± 0.24	0.97 ± 0.06**

Results expressed as mean ± SD.
* $p < 0.005$ compared with control, t-test
** $p < 0.0001$, compared with control, t-test

Table 2. Concentrations of 5-HT and 5-HIAA in the frontal cortex after 80 mg/kg isatin (i.p.)

Group	N	5-HT (ng/mg tissue)	5-HIAA (ng/mg tissue)	5-HIAA / 5-HT
Control	10	0.35 ± 0.11	0.61 ± 0.23	1.72 ± 0.33
Isatin (1 hr)	5	0.48 ± 0.11*	0.80 ± 0.31	1.62 ± 0.28

Results expressed as mean ± SD.
* $p < 0.05$, compared with control, t-test

Table 3. 5-HT_2 binding by ^{3}H-ketanserin

Group	N	5-HT_2 Binding	
		B_{max} fmoles/mg protein	Kd (nM)
Control	10	148 ± 26	1.1 ± 0.29
Isatin (1 hr)	5	171 ± 12*	1.1 ± 0.29

Results expressed as mean ± SD.
* $p = 0.052$, compared with control, t-test

Table 4. 5-HT uptake following 80 mg/kg isatin (i.p.)

Group	N	5-HT uptake	
		V_{max} (pmol/mg protein/2 min)	Km (μM)
Control	10	9.3	0.11
Isatin (1 hr)	5	8.9	0.11

Prefrontal cortex of animals in each group was combined

measured in prefrontal cortex. Values of K_d were not changed by isatin (Table 3) but there was a trend for B_{max} to be increased after the dose (Table 3) compared to controls. On the other hand a putative marker of serotonin function, synaptosomal serotonin uptake, was unaltered by isatin injection (Table 4). Both Km and V_{max} parameters of serotonin uptake were not significantly different from controls.

Discussion

The present investigation clearly demonstrates that a single dose of isatin has a rapid effect on the serotonergic system in the hypothalamus. The effects in

the frontal cortex are more varied and not as marked. Given the hypothesis that isatin is identical to, or at least a component of tribulin, this result is not surprising. Previous studies have shown the highest concentrations of tribulin to be present in the suprachiasmatic nucleus (located within the anterior hypothalamus), with substantially smaller concentrations present elsewhere (Armando et al., 1986). Nevertheless the effects on 5-HT concentration in the cortex follow the same pattern and time course as the hypothalamus. The decrease in 5-HIAA/5-HT ratio observed in the hypothalamus has been attributed to an inhibition of monoamine oxidase activity. A similar decrease in 5-HIAA/5-HT has been observed following cold immobilization stress (McIntyre et al., 1989). Furthermore, one of the earliest reported observations on tribulin was a substantial increase in urinary output following this stress (Glover et al., 1981). Taken together, these observations support the claim of Glover et al. (1988) that isatin is at least a component of tribulin. In as much as cold immobilization stress is a model of human conditions such as anxiety, these findings suggest that an investigation of serotonin function is worthwhile in anxiety disorders.

Paradoxically the number of 5-HT_2 receptor sites, as labelled by ^{3}H-ketanserin, is increased in response to increased 5-HT availability. (An uncontrolled analysis of 5-HT_2 receptor number, three hours following the same dose of isatin, found a significant increase in 5-HT_2 number: $B_{max} = 200 \pm 15$ fmol/mg, cf control 148 ± 26 fmol/mg protein.) In vivo manipulation of serotonin does not always lead to predictable changes in 5-HT_2 binding (Conn and Sanders-Bush, 1987). Denervation, for instance, does not produce increased 5-HT_2 density (Blackshear et al., 1981) while chronic treatment with some antagonists may produce down-regulation (Blackshear and Sanders-Bush, 1982). Furthermore, recent studies by Hoyer et al. (1987) have shown that in addition to labelling 5-HT_2 sites, ketanserin also labels α_1-receptors. The effects observed here with isatin may be related to noradrenergic changes, which were not specifically examined in this study. Alternatively, an upregulation of presynaptic autoreceptors could be postulated in order to decrease serotonin synthesis in response to an increased availability of 5-HT. Given that synaptosomal 5-HT uptake is not altered this possibility seems less likely. Further studies using more specific radioligands for serotonin receptor subtypes would be of interest, as would studies of chronic isatin administration. Given the postulate that isatin, tribulin and stress are related an increased number of ^{3}H-ketanserin binding sites would be predicted following cold-immobilization stress. We are currently investigating this possibility in our laboratory.

We previously demonstrated that cold immobilization stress did not alter synaptosomal 5-HT uptake (McIntyre et al., 1989). This lack of effect of isatin on this measure also supports the notion of a relationship between tribulin [the agent(s) responsible for the serotonergic effects of cold-immobilization stress] and isatin. While serotonin turnover and possibly receptor number are altered, a measure of presynaptic serotonin function is not.

In conclusion, these data support the notion that isatin induces similar

serotonergic changes to cold-immobilization stress, which in turn have been linked to an increased production of tribulin.

Acknowledgements

The authors thank Mrs. A. Acevedo for expert technical assistance, and Ms. M. Featherstone who typed the manuscript.

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Received November 14, 1988