Studies on digoxin-14C-acetate incorporation into digoxin and degenerative changes in the brain in rats administered digoxin

A Ravikumar
Department of Neurology, Medical College Hospital, Trivandrum 695011, India
and
Jyothi Augustine & P A Kurup*
Department of Biochemistry, University of Kerala, Kariavattom 695017, India

Received 4 August 2000; revised 1 December 2000

The human hypothalamus produces an endogenous membrane Na+-K+ ATPase inhibitor digoxin. Digoxin is a steroidal glycoside and could be synthesised by the isoprenoid pathway. The other metabolites of the isoprenoid pathway are cholesterol, dolichol and ubiquinone. We have tried to find out the extent of incorporation of 14C acetate into digoxin in rat brain. The effects of digoxin administration on the rat brain was also studied. The results show that the percentage incorporation of 14C acetate into digoxin is low but detectable. The maximum incorporation was observed for cholesterol, followed by dolichol and finally ubiquinone. The histopathological changes observed after digoxin administration were focal degeneration of the ganglion cells in the cerebrum and cerebellum. The carbohydrate components of the glycoproteins were reduced and the concentration of serotonin, dopamine, and epinephrine showed a significant increase. The role of digoxin in mediating neuronal cell death is discussed.

Endogenous digoxin like activity (EDLA), reported to be synthesised by the hypothalamus in mammals has assumed considerable significance in relation to various pathophysiological conditions1,2. The methods used so far to estimate this activity based on immunoreactivity with digoxin antibody were not specific since various intermediates and other substances also crossreact in this reaction. Recent work carried out in this laboratory involving extraction and purification by TLC and HPLC of the purified fraction, could identify the digoxin like activity in brain of rats, positively as digoxin itself3. No information is available at present on the biosynthetic pathway of digoxin in mammalian brain. The steroid nucleus may be synthesized from cholesterol with subsequent oxidation of side chain at position 17, to form the lactone, and followed by glycosylation of OH group in position 3, to form the three digitoxose sugars. As a first step in the elucidation of this pathway we have tried to find out, the extent of incorporation of 14C-acetate into digoxin in rat brain.

This study acquires clinical significance in view of the reports of increased plasma level of digoxin in neurological disorders, namely epilepsy & Parkinson’s disease and psychiatric disorders like schizophrenia and manic depressive psychosis1. Decreased membrane Na+-K+ ATPase activity has also been reported in these neurological and psychiatric disorders consequent to increase in digoxin which is the physiological inhibitor of this enzyme1. In this connection Mallakh et al based on biochemical changes observed, suggested that digoxin administration may provide a useful animal model of mania. Additionally digoxin neurotoxicity in patients frequently presents with symptoms of mania or depression. In human bipolar patients, mania and bipolar depression are both characterised by decreased membrane Na+-K+ ATPase activity.

In view of this, histopathological and the biochemical changes in the rat brain produced on digoxin administration were also studied and the results are discussed in this paper.

Materials and Methods

Experiment 1

Male albino rats (Sprague-Dawley), average body wt 100g, were divided randomly into 2 groups with 5
Experiment 1

14C-acetate incorporation into digoxin of rat brain — The results are given in Table 1. The percentage incorporation of 14C-acetate in digoxin is very low.

Incorporation of 14C-acetate into cholesterol, dolichol and ubiquinone in rat brain

In a separate experiment using 5 rats, (14C-acetate administration as described above) the pooled brain tissue was subjected to Folch extraction. Chloroform was removed from the extract and the residue dissolved in hexane. The hexane solution along with pure standards of dolichol, ubiquinone and cholesterol were subjected to HPLC. The HPLC fractions corresponding to these intermediates were collected and the activity counted in a liquid scintillation counter.

HPLC of pure standards of dolichol, ubiquinone and cholesterol — Results are given in Table 2. The retention time of the cholesterol, dolichol and ubiquinone were 4.6, 3.08 and 2.46 min respectively.

Extent of incorporation 14C-acetate into cholesterol, dolichol and ubiquinone

Results are given in Table 2. The maximum incorporation was observed for cholesterol (1207 cpm), followed by dolichol (91 cpm) and finally ubiquinone (56 cpm).

Experiment 2

Histopathology (Figs 1 & 2) — The histopathological examination of the rat brain indicated definite focal degeneration of the ganglion cells of the cerebrum and cerebellum, suggesting neurodegenerative change in the brain.

2. Concentration of Na+–K+–ATPase activity in brain (Table 3).

There was significant inhibition of this enzyme activity in brain. The percentage inhibition of Na+–K+-ATPase activity was about 74.5%, at the dose of digoxin given.

Concentration of glycoproteins in rat brain (Table 3) — Significant decrease in total hexose, fucose and sialic acid was observed in the brain in rats

<table>
<thead>
<tr>
<th>Table 1 — 14C-acetate incorporation into digoxin in rat brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Values are mean ± SD of 4 experiments using 5 rats in each experiment</td>
</tr>
<tr>
<td>Total brain wt(g)</td>
</tr>
<tr>
<td>7.079±0.611</td>
</tr>
</tbody>
</table>

Rats in each group as detailed below:

Group I — Control rats

Group 2 — Experimental rats administered 14C-acetate

The rats group II were injected intraperitoneally with 20μ curie of 14C-sodium acetate / 100gm body wt, and fed with normal diet and water ad libitum, while rats of group I were given the same amount of food and water. Three hours after injection, the rats were stunned at the back of the neck and were decapitated. The brain tissue from 5 rats were pooled for extraction and purification of digoxin by TLC as described by Arun et al. The fraction corresponding to digoxin in the TLC was scrapped out, suspended in the scintillant fluid and activity counted in a liquid scintillation counter.

Experiment 2

Male albino rats (Sprague-Dawley strain), average body wt 100 g, were divided randomly into 2 groups with 5 rats in each group as detailed below:

Group I — Control rats

Group II — Experimental rats given digoxin — 0.75 mg/100g body wt orally. (In the previous studies, digoxin was administered at various doses — 0.125 mg to 0.75 mg / 100 g body wt, orally to rats. No mortality was observed even at the highest dose (0.75 mg / 100 g wt) of digoxin. No visible symptoms were observed externally. Therefore the dose of 0.75 mg / 100 g body wt was selected)

Digoxin was administered to rats of group II in aqueous solution orally by tube for 7 days and fed normal diet, and water ad libitum. The control rats were given the same amount of food and water. On the 8th day, the rats deprived of food overnight were killed by decapitation. The brain tissue was removed and used for various estimations — Na+–K+–ATPase activity, neurotransmitter levels (Serotonin, dopamine, epinephrine and norepinephrine), carbohydrate components of glycoproteins and histopathological examination. For the determination of the Na+–K+ ATPase activity, the procedure described by Wallach and Kamat was used. Serotonin was estimated by the method of Curzon et al. and catecholamines by the method of Well-Malherbe. Details of the procedures used for the estimation of carbohydrate components of glycoproteins are described before by Manoj and Kurup. Statistical analysis was carried out by Student's t test.
administered digoxin orally.

Concentration of neurotransmitters in brain (Table 4)—The concentration of serotonin, dopamine and epinephrine showed significant increase in rats administered digoxin, while norepinephrine showed no significant change.

Discussion

The results of $^{14}$C-acetate incorporation into digoxin in rat brain indicate that there is definite incorporation of labelled acetate in digoxin, even though the percentage incorporation is very low. These results indicate that digoxin is synthesized in brain from acetate. To obtain detailed information on the pathway, it is necessary to study the incorporation of labelled intermediates of pathway including labelled cholesterol into rat brain. Detailed work on this aspect is proposed to be carried out, but from the results now available it can only be said that digoxin is synthesized in mammalian brain from acetate.

The results of incorporation studies into cholesterol, dolichol and ubiquinone indicate that relatively small amounts of acetate are used for the synthesis of these substances in rat brain. Of these three intermediates synthesized, the maximum incorporation was in cholesterol, ubiquinone showed only 4.6% and dolichol 7.5% of the incorporation into cholesterol.

The results of this study indicate that digoxin has significant effect on brain metabolism. There does not appear to be any previous report on the effect of oral administration of digoxin on brain metabolism. Digoxin is known to penetrate the CSF in man as reported by Allonen et al$^{12}$. According to Mallakh et al

Table 2—$^{14}$C-acetate incorporation into cholesterol, dolichol and ubiquinone in rat brain

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Cpm</th>
<th>Cpm/g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>1207±77.40</td>
<td>292.96±17.48</td>
</tr>
<tr>
<td>Dolichol</td>
<td>91±6.68</td>
<td>22.00±1.77</td>
</tr>
<tr>
<td>Ubiquinone</td>
<td>56±3.89</td>
<td>13.59±1.15</td>
</tr>
</tbody>
</table>

Fig 1(a)—Normal rat cerebrum (b)—Digoxin administered rat cerebrum The ganglion cells showed focal degeneration. The nucleus has lost its definition. Nucleolus also was not seen.

Fig. 2a—Normal rat cerebellum Fig 2b—Digoxin administered rat cerebellum The ganglion cells showed focal degeneration. The nucleus has lost its definition. Nucleolus also was not seen. x400
ATPase activity and concentrations of serotonin, dopamine and epinephrine in the brain of digoxin administered rats. 

<table>
<thead>
<tr>
<th>Group</th>
<th>Hexose (mg/g)</th>
<th>Fucose (mg/g)</th>
<th>Sialic Acid (mg/g)</th>
<th>Na^+K^-ATPase activity (µ mole Pi/hr/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>81.4±</td>
<td>35.4±</td>
<td>38.5±</td>
<td>0.242±</td>
</tr>
<tr>
<td>Experimental</td>
<td>60.9±</td>
<td>37.32±</td>
<td>30.24±</td>
<td>0.062±</td>
</tr>
</tbody>
</table>

‘t’ values: 7.18 (8.83) 4.18 (17.65)  

Group 2 has been compared with group 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serotonin (mg/g)</th>
<th>Dopamine (mg/g)</th>
<th>Epinephrine (mg/g)</th>
<th>Norepinephrine (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.872±</td>
<td>2.19±</td>
<td>1.39±</td>
<td>4.34±</td>
</tr>
<tr>
<td>Experimental</td>
<td>1.570±</td>
<td>3.41±</td>
<td>1.77±</td>
<td>4.53±</td>
</tr>
</tbody>
</table>

‘t’ values: 6.43 (7.32) 5.59 (12.8)  

Group 2 has been compared with group 1.

*P<0.01

al, administration of ouabain intraventricularly in rats at sublethal doses, increased the motor activity of rats significantly, which agrees with our results of increased neurotransmitter levels. Another report of Kaji et al is on the effect of digitalis on clinical symptoms in patients with multiple sclerosis.

Histopathological examination of rat brain indicated a definite focal degeneration of ganglion cells of cerebrum and cerebellum on oral administration of digoxin, suggesting neurodegenerative changes in the brain. The levels of neurotransmitters in the brain, viz. serotonin, dopamine and epinephrine all showed statistically significant increase in our studies on administration of digoxin. Since the whole brain tissue was used for the study, it is not possible to say whether the increase is in intracellular and extracellular levels. Digoxin administration has been found to result in elevated serotonin, dopamine and epinephrine concentration in rat brain in this study. In this concentration digoxin has been reported to promote dopamine release by exocytic and carrier mediated process in rat brain. It has also been shown to inhibit serotonin uptake by mouse brain synaptosomes. Digoxin like glycoside have also been reported to affect adrenergic and noradrenergic transmission in the sympathetic ganglia.

Digoxin has been reported to inhibit glutamate transport via the dicarboxylic acid carrier system into a glial cell resulting in synaptic accumulation of glutamate and increased NMDA transmission. The Na^+K^-ATPase inhibitory action of digoxin may deplete the cell of magnesium and remove the Mg block on the NMDA receptor. Digoxin by decreasing Na^-Ca^2+ exchange could also increase the intracellular calcium which activates the signal transduction mechanism involved in NMDA transmission. Digoxin thus leads to glutamate excitotoxicity and neuronal degeneration.

Increased binding of digoxin to Na^+K^-ATPase could lead to altered sodium-calcium exchange and intracellular calcium accumulation leading to cell destruction. Digoxin also has an inhibitory effect on nucleoside transport into a cell. Increased levels of digoxin could thus regulate the nucleoside reserve of the cell and DNA repair. DNA repair defects have also been reported in neuronal degeneration.

Digoxin administration to rats has been found to significantly decrease the hexose, fucose and sialic acid content of brain glycoproteins. Digoxin has been reported to have an inhibitory effect on Na^+K^-ATPase related sugar transport into a cell. This leads to decreased availability of sugar residues for N-linked and O-linked glycosylation of proteins and glycoprotein synthesis. This may result in altered glycoproteins resistant to lysosomal catabolism and produce a lysosomal dysfunction with consequent accumulation of defectively processed proteins in the neuronal cell. Protein processing defects could lead on to neuronal degeneration. Defective processed protein like amyloid accumulates in Alzheimer’s disease as a results of the golgi body-lysosomal dysfunction.

Thus digoxin administration results in degenerative changes in the brain as a result of its effects on neurotransmitter levels, NMDA excitotoxicity and protein glycosylation.

References
2. Haupert G T, Sodium pump regulation by endogenous
4 Christo DJ & El Mallakh RS, Possible role of endogenous ouabain like compounds in the pathophysiology of bipolar illness, Med Hypotheses, 41 (4) (1993) 378.
10 Bennet C A, Franklin N L, Statistical analysis in chemistry and chemical industry, 1 (1967) 105.
16 Reih M E & O'Reilly C A, Inhibition of serotonin uptake in to mouse brain synaptosome by ionophores and ion channel agents, Brain Res, 521 (1990) 347.
21 Lees G J, Inhibition of Na⁺-K⁺ ATPase a potentially ubiquitous mechanism contributing to CNS neuropathology, Brain Res Rev, 16 (1991) 283.
22 Plagemann P G & Aran J M, Sodium dependent active nucleoside transport in mouse spleen lymphocyte, leukemia cells, fibroblasts and macrophages but not in equivalent human and pig cells–dipyridamole enhances nucleoside salvage by cells with both active and facilitated transport, Biochim Biophys Acta, 1025 (1990) 32.