SUBCELLULAR DISTRIBUTION OF 3H-DIGITOXIN AND ITS METABOLITES IN THE HEARTS OF THE CATS WITH A HYPERSENSITIVITY TO THE DRUG

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Abstract—To clarify the cause of hypersensitivity to digitoxin, an experiment was carried out with cats. The most potent hypersensitivity to digitoxin has been observed 48 hr after the injection of a loading dose. However, 1 hr after this injection, the cats failed to show the hypersensitivity. One, 24 and 48 hr after the injection of 3H-digitoxin, the contents of digitoxin and its metabolites in subcellular fractions of hearts were measured. Digitoxin contents in microsomal fractions 48 hr after the injection only slightly decreased, while those in mitochondrial and nuclear fractions markedly decreased as compared with the check at 1 hr. An increase of sodium ions and a decrease of potassium ions in the hearts were seen 48 hr after the injection. These facts may be related to the cause of hypersensitivity.

The cumulative effect of digitalis is a well-documented phenomenon, and considerable literature (1–6) has been reported, yet the mechanism of the cumulative effect of digitalis is still a matter of speculation. It is known that a marked hypersensitivity to the drug occurs several days after the injection of a loading dose of digitoxin (5, 6). It has been often pointed out that digitoxin tightly binds with purified Na-K ATPase isolated from calf hearts (7) and that this digitoxin-enzyme complex is more stable than the ouabain-enzyme complex (8). It has also been suggested that cardiac glycosides directly act on the sarcoplasmic reticulum (SR) within the cell (9–13).

The present work was designed to clarify the cause of hypersensitivity to the drug, and the results obtained suggest that the cause of the hypersensitivity depends on whether a high concentration exists in the microsomal fraction, which comprises cell membranes and SR, during the 48 hr after administration of digitoxin.

Materials and Methods

Determination of both dysrhythmic and lethal doses: Cats of both sexes, weighing 2.5–3.5 kg, were used in this study. Determinations of both dysrhythmic and lethal doses of digitoxin were made by Hatcher's method 1. 24, 48 and 72 hr after an i.v. injection of a loading dose of digitoxin. As a loading dose, the 20% mean lethal dose, 132 μg/kg, was injected.

3H-digitoxin used: The 3H-digitoxin used in these studies was randomly labeled. The specific activity were 26 mCi/mg. The chromatographic purity of the compound was tested against a digitoxin standard. Ten micrograms of standard digitoxin and tritiated digitoxin were spotted on thin layer silica gel
chromatoplates and developed and visualized according to the method of Katzung and Meyers (14). A single radioactive spot corresponding to digitoxin was present.

**Determination of radioactivity:** One, 24 and 48 hr after the injection of a loading dose of $^3$H-digitoxin (105 μCi/132 μg/kg), glycoside contents (digitoxin and its metabolites) in the blood, heart and each subcellular fraction of the heart were measured by means of the combustion method (Tricarb Sample Oxidizer, Packard Model 305). Radioactivity was determined by a liquid scintillation spectrometer (Beckman LS250). Sufficient net counts were taken to assume an error of less than 5%. All counts were corrected for quenching by means of an external standard. The adequacy of this correction was checked by the use of an internal standard in occasional samples.

**Subcellular fractionation of the heart:** The ventricle muscles were weighed, minced and homogenized in 4 volumes of sucrose solution (0.33 M sucrose, pH 6.8). Homogenization was carried out in an ice bath with a motor-driven homogenizer for a total of 2 min (15 sec of homogenization and 45 sec rest, repeated 8 times). followed by centrifugation at 4°C at 600 x g for 10 min to isolate the nuclear fraction, then at 12,000 x g for 15 min to isolate the mitochondrial fraction. Finally, the microsomal fraction was separated by centrifuging the final supernatant at 100,000 x g for 1 hr. To each particulate fraction, a known amount of distilled water was added, and the pellet was suspended using a glass homogenizer. The total radioactivity of an aliquot of each particulate fraction was counted in a liquid scintillation counter following the combustion procedure. Protein determination of each particulate fraction was done by the Lowry method (15).

**Chloroform extraction:** The radioactivity from biological samples was determined by shaking each sample three times with a five-fold volume of chloroform, combining the extracts, followed by evaporation. The chloroform extracts were counted in a liquid scintillation spectrometer. Recoveries of $^3$H-digitoxin added to the serum and heart averaged 95% in the chloroform extracts.

**Acid hydrolysis of the water soluble phase:** Water soluble metabolites were identified after being extracted with CHCl$_3$ following heating under a concentration of 1/10 N HCl (pH 1.0).

**Thin layer chromatography:** Digitoxin and digitoxigenin in the CHCl$_3$ extracts of various preparations were identified by the thin layer chromatographic procedure. The following solvent system was used: methyl-ethylketone, xylene, chloroform (2:2:1).

**Electron microscopy:** Electron microscopic studies were performed on a microsomal sample. Particles in the microsomal fraction were stained negatively by potassium phosphtungstate (2%).

**Determination of Na-K ATPase activity in the hearts:** Determination of the activity of Na-K ATPase was done by Akera’s procedure (16).

**Determination of sodium and potassium contents in the hearts:** The cellular sodium and potassium contents in the hearts were measured flamephotometrically following routine procedures (17).

**Results**

**Dysrhythmic and lethal doses of digitoxin:** One, 24, 48 and 78 hr after the injection of a loading dose of digitoxin, both dysrhythmic and lethal doses were determined. As shown in Table 1, 48 hr after the injection of digitoxin, the most potent hypersensitivity to the drug was seen in the cats; while 1 hr after the injection, the cats failed to show hypersensitivity to the drug.

**Identification of digitoxin and its metabolites:** One, 24 and 48 hr after the injection of $^3$H-digitoxin, digitoxin and its metabolites
Table 1. Dysrhythmic and lethal doses of digitoxin determined at various times after the administration of the loading dose of digitoxin in cats

<table>
<thead>
<tr>
<th>Time after the administration of a loading dose of digitoxin*</th>
<th>Dysrhythmic dose (Mean±S.E.) mg/kg (%)</th>
<th>Lethal dose (Mean±S.E.) mg/kg (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (without administration) (n=5)</td>
<td>423±45 (100)</td>
<td>660±43 (100)</td>
</tr>
<tr>
<td>1 hour (n=5)</td>
<td>338±32 (80)</td>
<td>541±59 (82)</td>
</tr>
<tr>
<td>24 hours (n=4)</td>
<td>262±31* (62)</td>
<td>386±47* (60)</td>
</tr>
<tr>
<td>48 hours (n=5)</td>
<td>212±19** (50)</td>
<td>330±30** (50)</td>
</tr>
<tr>
<td>72 hours (n=4)</td>
<td>245±46* (58)</td>
<td>382±45* (58)</td>
</tr>
</tbody>
</table>

*: As a loading dose of digitoxin, 20% of the mean lethal dose, 132 μg/kg, was injected. **P<0.05, ***P<0.01, statistically different from the value measured at 1 hr after administration of digitoxin.

were measured. Figure 1 shows a typical radiochromatogram of CHCl₃-extracts of the serum and heart 48 hr after the injection. The radioactivity of each chromatogram was shown as only one spot, and each spot represents digitoxin. Water soluble metabolites in the water phase of the serum were identified after being extracted with CHCl₃ following heating of this water phase under a concentration of 1/10 N HCl (pH 1.0). As shown in Fig. 2, the spot of radioactivity represents digitoxigenin only. The percentage of CHCl₃-extracts from the acid-hydrolysis products of the water soluble phase in the serum and heart represented about 90 and 85 of the total radioactivity, respectively. Water soluble metabolites might represent digitoxin conjugates. Therefore, the radioactivity obtained was calculated as digitoxin.

Figure 3 shows the contents of total digitoxin, digitoxin and its conjugate in heart at 1, 24 and 48 hr after injection. Twenty-four and 48 hr after injection, the contents of total digitoxin and digitoxin decreased significantly compared to those of 1 hr after injection. Figure 4 shows the contents of total...
digitoxin, digitoxin and its conjugate in the pellet and supernatant at various times after injection. Contents of total digitoxin and digitoxin in the pellet were larger than those of the supernatant at various times after injection.

Figure 5 shows a typical electron micrograph of a microsomal fraction isolated from cat hearts. The fragments showed sarcoplasmic (SR) vesicles and fragmented cell membranes. There was no mitochondrial contamination.

Figure 6 shows a typical radiochromatogram of CHCl₃-extracts of the microsomal fraction or nuclear fraction. The radioactivity of each chromatogram represents digitoxin. Figure 7 shows the subcellular distribution of total digitoxin, digitoxin and its conjugate in the heart at various times after injection. The highest concentration of total digitoxin or digitoxin was seen in the microsomal fraction at various times after injection. There was a large difference in the contents of digitoxin and its conjugate in the nuclear, mitochondrial and microsomal fractions.

Table 2 shows the percent of the contents of digitoxin and its conjugate in the subcellular fractions at various times after injection. Digitoxin contents in the microsomal fraction 24 or 48 hr after the injection only slightly...
Fig. 4. The concentration of digitoxin (CHCl₃-soluble) and its metabolites (water soluble) in the pellet and supernatant at various times after the administration of ³H-digitoxin. *P<0.001, statistically different from the value measured at 1 hr after administration of digitoxin.

Fig. 5. A typical electron micrograph of a microsomal fraction isolated from a cat heart (Negatively stained particles).

decreased as compared with the check at 1 hr.

Table 3 shows changes of both Na-K ATPase activity and electrolyte contents in the hearts at various times after injection of digitoxin. No significant alterations by digitoxin were observed in Na-K ATPase activity or electrolyte contents in the hearts 1 hr after injection. Twenty-four and 48 hr

Fig. 6. Thin layer radiochromatograms of the chloroform extracts of the microsomal fractions and nuclear fractions at 48 hr after the administration of ³H-digitoxin. The radioactivity represents digitoxin. For further explanation, see Fig. 1.
Fig. 7. Subcellular distribution of digitoxin and its metabolites in cat hearts at various times after administration of $^3$H-digitoxin. *P<0.05, **P<0.01, statistically different from the value measured at 1 hr after the administration.

Table 2. Percent of the contents of digitoxin and its conjugate in the subcellular fractions at various times after the administration of $^3$H-digitoxin

<table>
<thead>
<tr>
<th>Time after the administration of a loading dose of digitoxin</th>
<th>Microsom</th>
<th>Mitochondria</th>
<th>Nuclear</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>digitoxin</td>
<td>conjugate</td>
<td>digitoxin</td>
</tr>
<tr>
<td>1 hour</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>24 hours</td>
<td>77</td>
<td>169</td>
<td>66</td>
</tr>
<tr>
<td>48 hours</td>
<td>68</td>
<td>230</td>
<td>47</td>
</tr>
</tbody>
</table>

a: As a loading dose of digitoxin, 20% of the mean lethal dose, 132 μg/kg, was injected.

Table 3. Na-K ATPase activity and electrolyte contents in the cardiac muscles at various times after the administration of digitoxin

<table>
<thead>
<tr>
<th>Time after the administration of a loading dose of digitoxin</th>
<th>Na-K ATPase activity (Mean±S.E.)</th>
<th>Electrolyte (Mean±S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles Pi/mg protein</td>
<td>Na</td>
</tr>
<tr>
<td>Control (no drug) (n=5)</td>
<td>6.42±0.70 (0)</td>
<td>32.0±1.80 (100)</td>
</tr>
<tr>
<td>1 hour (n=5)</td>
<td>6.35±0.58 (1.1)</td>
<td>31.4±2.11 (98)</td>
</tr>
<tr>
<td>24 hours (n=4)</td>
<td>5.32±0.82 (17.1)</td>
<td>36.5±2.72 (116)</td>
</tr>
<tr>
<td>48 hours (n=5)</td>
<td>4.92±0.71 (23.4)</td>
<td>44.1±3.13* (138)</td>
</tr>
</tbody>
</table>

a: As a loading dose of digitoxin, 20% of the mean lethal dose, 132 μg/kg, was injected. *P<0.05, statistically different from the value measured at 1 hr after administration of digitoxin.
after injection, Na-K ATPase activity tended to be inhibited, but the differences from the value measured at 1 hr after injection were not significant. On the other hand, as shown in Table 3, electrolyte contents in the hearts 48 hr after injection were altered significantly compared to those at 1 hr.

Discussion

The most potent hypersensitivity to digitoxin was seen in the cats 48 hr after injection, while 1 hr after injection, the cats failed to show hypersensitivity to the drug (Table 1). On the other hand, contents of total digitoxin and digitoxin in the serum and hearts 48 hr after injection decreased significantly compared to those of 1 hr after injection. Therefore, it is clear that hypersensitivity is independent of the level of digitoxin in the serum or hearts. The most important finding in the present study was that digitoxin contents in the microsomal fraction showed little decrease even though the contents in pellet markedly decreased as compared with the value measured at 1 hr after the injection (Figs. 4 and 7). Total radioactivity in the microsomal fraction 1 hr after the injection was the highest concentration in all the fractions. These results confirm those reported previously using Langendorff’s cat hearts (18). It has also sometimes been reported, using various animals, that glycosides have the highest affinity to the microsomal fraction as compared with other particulate fractions (19–21). From these findings, it is suggested that cardiac glycosides might have a great affinity for the microsomal fraction regardless of the animal species.

From the present results, the following may be considered as a possible mechanism responsible for the hypersensitivity: digitoxin occupying the Na-K ATPase of cell membranes which are contained in the microsomal fraction produces a hypersensitivity to the drug as a result of the cumulative effect of the drug. Matsui and Schwarz (7) have shown that Na-K ATPase, highly purified from the membrane fraction (microsomal fraction) of calf hearts, has an ATP-dependent and ion dependent ability to bind digitalis glycosides. Indeed, 24 and 48 hr after injection, Na-K ATPase activity tended to be inhibited; although the differences from the value measured at 1 hr after injection were not statistically significant (Table 3). It follows then that long lasting inhibition, though weak, Na-K ATPase activity by the drug occupying the cell membrane proceeds. Thus, it may be considered that the inhibition elicits a significant increase of sodium ions and a decrease of potassium ions in the heart 48 hr after injection (Table 3). A decrease of the membrane potential of cardiac muscles is elicited as a result of the electrolyte changes produced by the drug. A firing of action potential in the heart muscles may occur more easily when membrane potential decreased. Therefore, digitalis-induced dysrhythmia may occur more easily when inhibition of Na-K ATPase activity occurs in the cardiac muscles. Indeed, it was demonstrated that a decrease of the lethal dose of digitoxin in cats with a hypersensitivity to the drug was paralel with a decrease in the dysrhythmic dose of digitoxin (Table 1).

The microsomal fraction comprises SR vesicles in addition to cell membranes. Several investigators have reported that cardiac glycosides may produce inotropic action as a result of the drug action on the SR vesicles (9–12, 19). These recent results suggest the possibility that digitoxin occupied in the SR may act on the SR in cardiac muscles at the toxic stage: A digitalis-induced contracture, namely, insufficient myocardial relaxation might occur due to an incomplete Ca-uptake by the digitoxin occupying SR.

Several studies on the nature of the water soluble metabolites of digitoxin have been published (14, 22, 23). It has been reported
that water soluble metabolites were observed in the human heart a considerable time after the administration of digitoxin (14). Furthermore, Yoshida (23) reported that the water soluble metabolites in cat bile yielded digitoxin only after enzymatic separation. In this experiment, acid hydrolysis of the water soluble metabolites of digitoxin yielded only \(^3\text{H}\)-digitoxigenin as determined radiochromatographically. Therefore, the majority of the water soluble metabolites in the heart may represent digitoxin conjugates. As we have already reported (5), cats with interrupted enterohepatic circulation of digitoxin, i.e., biliary fistula cats, failed to show both the accumulation of digitoxin in the heart and hypersensitivity to digitoxin. Contents of the water soluble metabolites in the microsomal fraction 48 hr after injection of digitoxin were greater than those at 1 hr. Therefore, this increase in the contents of water soluble metabolites in the microsomal fraction may depend on the drug concentration in the liver after the administration of digitoxin (4, 23), the liver supplying the drug to other organs, such as the heart for a long period of time.

In conclusion, it may be suggested that the marked hypersensitivity to the drug in the cat is produced by the high concentration of digitoxin in the microsomal fraction and that the hypersensitivity may be produced as a result of the cumulative effect of the drug occupying the Na-K ATPase of cell membranes contained in this fraction.

References

1) Gold, H. and Degraff, A.G.: Studies on digitalis in ambulatory cardiac patients. JAMA 92, 1421-1424 (1929)
7) Matsui, H. and Schwarz, A.: Mechanism of cardiac glycosides inhibition of the \(\text{Na}^+\text{-K}^+\)-dependent ATPase from cardiac tissue. Biochim. Biophys. Acta 151, 655-663 (1968)
12) Fujino, S., Igarashi, T. and Hoshi, K.: Ouabain potentiation of Ca release from fragmented cardiac sarcoplasmic reticulum from isolated cat heart. Experientia 35, 1220–1221 (1979)


