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Amiodarone Toxicity

II. Desethylamiodarone-Induced Phospholipidosis and Ultrastructural Changes during Repeated Administration in Rats[1]

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Amiodarone Toxicity. II. Desethylamiodarone-Induced Phospholipidosis and Ultrastructural Changes during Repeated Administration in Rats. KANNAN, R., SARMA, J. S. M., GUHA, M., AND VENKATARAMAN, K. (1991). *Fundam Appl. Toxicol.* **16**, 103–109. The contribution of desethylamiodarone (DEA), principal metabolite of the antiarrhythmic drug amiodarone, to the major side effects of amiodarone is unclear. The effects of repeated DEA administration to rats on tissue drug accumulation, ultrastructural changes, and phospholipid concentrations were studied. Two groups (n = 8/group) of male Sprague–Dawley rats (250 g body wt) were administered a 5% aqueous solution of DEA (Dose I, 40 mg/kg/day; Dose II, 60 mg/kg/day) intraperitoneally for 21–23 days, while a third group (control, n = 8) received saline. DEA levels were significantly higher with Dose II compared to Dose I in the lung, liver, kidney, spleen, heart, and serum while the tissue to serum ratios were similar with both doses for all tissues except the heart. DEA administration caused a significant elevation in the lipid phosphorus levels of liver, lung, and alveolar macrophages compared to control levels. A strong positive correlation ($p < 0.01$) was found between tissue DEA levels and lipid phosphorus for the above tissues. Electron microscopy revealed the presence of lipid inclusion bodies in liver, lung, and alveolar macrophages of DEA-treated rats. A dose-dependent increase in the percentage of vacuolar surface area was found in the lung and alveolar macrophages. The tissue ultrastructural changes after repeated DEA dosing were qualitatively similar to our previous findings with amiodarone. Increased lung and liver phospholipid levels with repeated DEA doses may result from a potent inhibitory action of DEA on tissue phospholipase A as has been observed by others in *in vitro* studies.

Amiodarone therapy, although very effective in controlling various types of arrhythmias (Singh, 1983; Mason, 1987), is beset with serious side effects, the major side effects being pulmonary toxicity and hepatotoxicity (Harris *et al.*, 1983; Sobol and Rakita, 1982; Simon *et al.*, 1984). Amiodarone and its principal metabolite, desethylamiodarone (DEA), have been shown to accumulate extensively in the lung and liver in both animal and autopsied human tissue specimens (Holt *et al.*, 1983; Kannan *et al.*, 1985). In addition, DEA's electrophysiological and hemodynamic actions were found to be similar to those of amiodarone (Kato *et al.*, 1988; Talajic *et al.*, 1987). Recently, Abdollah *et al.* (1989) reported that intravenous DEA administration resulted in considerable antiarrhythmic activity in dogs with myocardial infarction and ventricular arrhythmias. The above observations suggest that amiodarone's drug efficacy and toxicity may be mediated, at least in part, by DEA. In isolated hepatocytes, DEA was more toxic than amiodarone (Somani *et al.*, 1990). Cell death as monitored by lactate dehydrogenase

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release and ultrastructural changes (myelinoid inclusion bodies, mitochondrial swelling) occurred sooner with the metabolite than with amiodarone in this culture model.

Our previous work showed that repeated doses of amiodarone to rats produced myelinoid inclusion bodies in the liver tissue and lung alveolar macrophages with a good correlation to tissue amiodarone concentrations (Kannan *et al.*, 1989). Significant phospholipid accumulation accompanied ultrastructural changes in alveolar macrophages in rats treated for 1 week or longer with amiodarone (Reasor *et al.*, 1988). Phospholipid accumulation in the lung and the liver during repeated dosing of amiodarone has also been observed by others (Riva *et al.*, 1987). The accumulation of phospholipids was mainly due to the potent inhibition of phospholipase A_1 and A_2 by amiodarone (Hostetler *et al.*, 1986, 1988; Heath *et al.*, 1985). Recent work by Martin *et al.* (1989) confirmed the above findings in cultured pulmonary endothelial cells in which a concentration-dependent inhibition of lysosomal phospholipase by amiodarone was seen.

Although experimental studies have revealed that the electrophysiological, hemodynamic, and antiarrhythmic actions of DEA are similar to those of amiodarone under acute conditions, *in vivo* data on the effects of repeated administration of DEA are not available. Whether DEA contributes to observed tissue morphological changes, as seen with prolonged amiodarone treatment, is also unclear at the present time. This information is particularly relevant since the tissue levels of DEA become equal to or greater than those of amiodarone during repeated dosing of amiodarone (Holt *et al.*, 1983; Kates *et al.*, 1988). In the present study, we report the induction of hepatic and pulmonary phospholipidosis, accompanying ultrastructural changes, and their relationship to tissue DEA levels in rats treated for 3 weeks with DEA.

METHODS AND MATERIALS

Male Sprague–Dawley rats obtained from Charles River Laboratories (Wilmington, MA) were maintained in individual metal cages and were given water and Purina rat chow *ad libitum*. Twenty-four rats with initial body weights of approximately 250 g were used in the study. They were divided into three groups of 8 rats each. The control group received saline ip, while Dose I and Dose II groups were administered DEA in daily doses of 40 and 60 mg/kg, respectively, as a 5% aqueous solution ip, for 21–23 days. The drug was carefully dissolved in deionized distilled water at 60°C. The aqueous solution was stored at 4°C, protected from light. Body weights of rats were monitored weekly. Both DEA doses caused reduced body weight gains compared to controls. In another set of 8 rats given a DEA dose of 80 mg/kg/day ip, all animals became sick and 2 died within 1 week. No pathological evaluations were made, but further treatment with DEA was discontinued in this group which was excluded from the study.

Twenty-four hours after the final dose of DEA, rats were given an overdose of ip Diabutal. An abdominal incision was made and blood was collected from the abdominal artery. Blood was immediately spun and serum stored frozen until analysis. Alveolar macrophages were obtained by lung lavage (Kannan *et al.*, 1989), and known aliquots were used for drug, phospholipid, and electron microscopic measurements. Portions of the lung, liver, kidney, spleen, heart, and brain were dissected for the determination of tissue DEA levels and were kept frozen until analysis. Samples of liver tissue were removed from one lobe from approximately the same location in each rat.

Electron microscopy The ultrastructural changes in the lung, liver, and alveolar macrophages were studied by electron microscopy according to the procedures described in detail earlier (Kannan *et al.*, 1989). Briefly, the lung was fixed *in situ* with 1.5% glutaraldehyde and immersed in the fixative overnight. Freshly dissected liver tissue was immersed in 2% Sorenson's phosphate buffered glutaraldehyde, cut into 1 × 3-mm sections, and fixed overnight. The alveolar macrophages were centrifuged and the washed pellets were fixed in 1.5% glutaraldehyde. The procedure for electron microscopy was the same as that previously described (Kannan *et al.*, 1989).

For each electron microscopic picture used in quantitation, the total area under the same magnification was determined using the automated image analysis system manufactured by Bausch and Lomb (Kannan *et al.*, 1989). The vacuolar area as a percentage of the total surface area covered was determined for lung and alveolar macrophage specimens.

Drug and phospholipid analysis. The concentration of DEA was determined in serum, alveolar macrophages, and the various tissues by an HPLC method standardized in our laboratory (Kannan *et al.*, 1987). Fenethazine was used as an internal standard. Analysis of serum and tissue samples showed a single peak with a retention time similar to that of the DEA standard and there was no evidence for the presence of another metabolite(s) or amiodarone.

Lipid phosphorus (P) was determined in Folch extracts of serum and tissue samples of all rats according to Ames and Dubin (1960). The results were expressed as micro-

TABLE 1

SERUM AND TISSUE CONCENTRATIONS OF DESETHYLAMIODARONE (DEA) IN RATS

Dose/day	Serum (μg/ml)	Lung (μg/g)	Liver (μg/g)	Kidney (μg/g)	Heart (μg/g)	Spleen (μg/g)	Macrophages (μg/10^6 cells)
40 mg/kg	0.077 ± 0.028	11.00 ± 0.76	7.12 ± 0.72	8.55 ± 0.82	3.47 ± 0.60	9.40 ± 2.96	0.057 ± 0.018
60 mg/kg	0.239 ± 0.066	41.55 ± 11.08	17.65 ± 3.85	25.67 ± 4.07	4.69 ± 0.67	43.54 ± 9.34	0.122 ± 0.021
p value	<0.005	<0.005	<0.001	<0.001	<0.005	<0.001	<0.01

Note. All values are means ± SEM; n = 6–8 rats/group except for macrophages (n = 3 or 4 rats/group). DEA concentrations were below the detectable limit of our assay in brain tissue with both doses.

grams lipid P per milliliter serum or milligrams lipid per gram tissue wet weight.

Statistical analysis The mean values of tissue and serum drug levels of the two DEA-treated groups were compared by unpaired Student's t test. When three groups were compared for phospholipid levels and vacuolar areas, one-way analysis of variance (ANOVA) was used followed by Scheffe's post hoc test for differences between group pairs. The correlations between the drug and the phospholipid levels in tissues were determined by the least-squares method. ABSTAT Statistical Software (Anderson Bell, Parker, CO) was used. All values were expressed as means ± SEM.

RESULTS

Table 1 gives the serum and tissue concentrations of DEA in rats given 40 (Dose I) and 60 (Dose II) mg/kg DEA ip for 21–23 days. The mean serum DEA levels with Dose I and Dose II were 0.077 and 0.239 μg/ml, respectively. With Dose I, the highest DEA concentrations were found in the lung. Spleen, liver, and kidney had similar but lower concentrations. DEA concentrations were significantly higher with Dose II compared to Dose I for all tissues (Table 1). Concentrations of DEA in alveolar macrophages were 0.057 μg/10^6 cells with Dose I and 0.122 μg/10^6 cells with Dose II. The tissue-to-serum ratios with both dosage regimens were not significantly different from each other except for those of heart (Dose I: 50.9 ± 17.3; Dose II: 20.6 ± 4.4, $p < 0.002$) in which the tissue drug levels might have saturated at the higher dose.

Table 2 lists lipid P levels in control rats and in rats administered 40 or 60 mg/kg DEA for 21–23 days. There was a dose-dependent increase in lipid P in the lung, liver, and alveolar macrophages while serum phospholipids did not change. In the lung, lipid P levels with both doses were higher than those of saline controls, but in the liver, the level with

TABLE 2

SERUM AND TISSUE LIPID PHOSPHORUS CONCENTRATIONS IN CONTROL AND DEA-TREATED RATS

DEA Dose/day	Serum (μg P/ml)	Lung (mg P/g)	Liver (mg P/g)	Macrophages (μg P/10^6 cells)
Control	46.9 ± 3.0	0.91 ± 0.04	1.71 ± 0.13	0.030 ± 0.006
40 mg/kg	48.2 ± 2.8	2.30 ± 0.57	2.15 ± 0.10	0.147 ± 0.013
60 mg/kg	49.0 ± 4.6	4.42 ± 0.72	3.98 ± 0.43	0.184 ± 0.037
p value (ANOVA)	ns	<0.005[a]	<0.001[b]	<0.02[c]

Note. All values are means ± SEM; n = 5 rats/group, except for macrophages (n = 2 rats/group).

[a] All three groups significantly different from each other.

[b] Values for 60 mg/kg DEA group significantly higher than those of control and 40 mg/kg DEA groups.

[c] DEA-treated groups significantly higher than control but the differences between the two DEA groups nonsignificant.

Dose I (2.15 ± 0.10 mg P/g) was not different from that of controls (1.71 ± 0.13 mg P/g) while Dose II showed a significant elevation ($p < 0.001$). Phospholipid levels in alveolar macrophages of treated rats with both doses were higher than those in controls (Table 2).

Figure 1 shows the relationship between tissue DEA concentration and lipid P levels. A significant positive correlation between tissue DEA concentrations and tissue lipid P content was found for liver ($r = 0.92$, $p < 0.001$), lung ($r = 0.96$, $p < 0.001$), and alveolar macrophages ($r = 0.88$, $p < 0.03$).

The ultrastructural changes were examined in the liver, lung, and alveolar macrophages by electron microscopy. Compared to those of saline controls, the above tissues contained an increased number of vacuoles with membrane-bound inclusions filled with material of varying electron density. The electron microscopic pictures of the above tissues of DEA-treated rats (not shown) resembled those from amiodarone treatment (Kannan *et al.*, 1989).

The morphological changes produced by DEA in lung and alveolar macrophages were quantitatively compared to those from untreated rats. The percentage of total areas covered by the vacuoles in the lung tissue and alveolar macrophages were determined. Figure 2 shows the results. The percentage of vacuole area in the lungs of rats treated with Dose II was significantly higher ($p < 0.001$) than those of controls and Dose I, but there was no difference in the vacuolar area between the latter two groups. The percentage of vacuolar area calculated for isolated alveolar macrophages of rats treated with Dose II (9.73 ± 2.1%) was significantly higher than that of controls (2.37 ± 1.80%) but the mean value for Dose I (5.72 ± 3.13%), although higher, was not statistically different from that of controls.

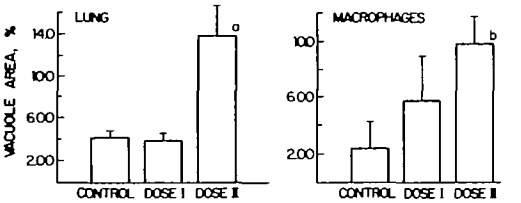

FIG. 2. Vacuolar areas expressed as percentage of total area, from electron micrographs of lung tissue and alveolar macrophages of control and DEA-treated rats. a, significantly higher than control and Dose I groups ($p < 0.001$); b, significantly higher than control group ($p < 0.05$).

DISCUSSION

In the present study, we have shown that prolonged treatment of rats with DEA caused a significant increase of phospholipids in lung and liver tissues and alveolar macrophages compared to those in saline controls. The phospholipid levels in the above tissues showed a positive correlation with DEA concentrations in the same tissues. This study also showed that there is a substantial uptake of DEA by the lung, followed by the spleen, kidney, liver, and heart.

This pattern of tissue DEA uptake is similar to that reported for amiodarone in rats (Plomp *et al.*, 1985) and in rabbits (Kannan *et al.*, 1985) but the absolute serum DEA levels were significantly lower. There was no evidence of

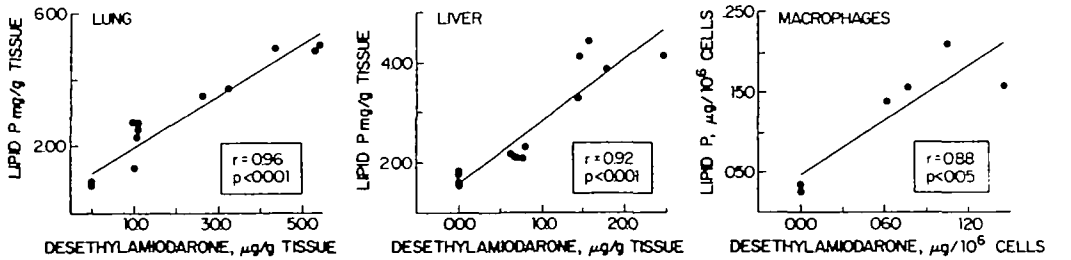

FIG. 1. Correlation between DEA and lipid phosphorus (P) levels in lung, liver, and alveolar macrophages of rats chronically treated with DEA.

further metabolism of DEA since no other peak was seen in HPLC chromatograms. However, the possibility of the formation of metabolic products that were not detected under the present HPLC conditions cannot be ruled out. The urinary excretion pattern of DEA was not determined in the present study. Holt *et al.* (1983) found negligible urinary concentrations of amiodarone and DEA in patients on chronic amiodarone therapy. Very little is known about the metabolism of DEA. Staubli *et al.* (1985) reported that the plasma elimination half-life of DEA was longer than that of amiodarone in amiodarone-treated patients, but they did not characterize or quantify urinary excretions. Further studies in bioavailability of DEA are needed especially since it is known that the bioavailability of the parent drug (amiodarone) was reported to vary over a wide range (Mason, 1987; Kates *et al.*, 1988).

The induction of phospholipidosis by cationic amphiphilic drugs is a well-established phenomenon (Lullman *et al.*, 1978). Recent reports confirm that repeated amiodarone administration results in an elevation of tissue phospholipid levels both *in vitro* (Martin and Howard, 1985; Yap *et al.*, 1986) and *in vivo* (Reasor *et al.*, 1988; Riva *et al.*, 1987; Heath *et al.*, 1985; Poucell *et al.*, 1984). The increase in phospholipids and lamellar inclusion bodies was dependent on the concentration of amiodarone (Reasor *et al.*, 1988; Kannan *et al.*, 1989). Cellular phospholipidosis, dependent on the cellular amiodarone and DEA concentrations, was also seen in the bronchoalveolar lavage of human subjects (Martin and Standing, 1988). The elevation in phospholipid levels due to amiodarone treatment occurred mainly in phosphatidylcholine in alveolar macrophages. Martin *et al.* (1989) showed that there are significant increases in specific phospholipids including phosphatidylinositol, phosphatidic acid, and bis(monoacylglycerol)phosphate in cultured bovine pulmonary artery endothelial cells treated with amiodarone at equivalent therapeutic concentrations. The phospholipids were not fractionated into individual classes in the present study and therefore, it is not known whether the increase in lipid phosphorus observed with DEA reflects an increase in specific phospholipids.

Work on the mechanism of induction of phospholipidosis by amiodarone has revealed that amiodarone is a potent inhibitor of phospholipase A. The inhibition of phospholipid degradation by amiodarone due to inhibition of phospholipase was shown in the lung by Hostetler *et al.* (1986, 1988) and Heath *et al.* (1985), and more recently in bovine pulmonary artery endothelial cells by Martin *et al.* (1989). Hostetler *et al.* (1988) also showed an inhibition of partially purified lung phospholipase A_1 by amiodarone and DEA. DEA was found to be more potent than the parent drug in this respect. It is likely that inhibition of phospholipase A is the cause for phospholipid accumulation during repeated-dose treatment of DEA *in vivo,* although direct evidence in support of this presumption is not available at the present time.

The relationships among the development of ultrastructural changes, tissue phospholipidosis, and cellular toxicity due to amiodarone (or DEA) are not clearly established. In the present study, the surface vacuolar area increased with increased DEA dosage and tissue DEA concentrations which correlated with increases in phospholipid levels. The ultrastructural changes were more pronounced in alveolar macrophages and in the lung than in the liver. These findings with DEA are similar to those with amiodarone for the liver in a guinea pig model (Pirovino *et al.*, 1988). Foamy inclusion bodies in the cytoplasm of lung cells, suggesting increased storage of phospholipid within cells, were observed in bronchoalveolar lavage in patients with amiodarone toxicity (Marchlinski *et al.*, 1982). Cell lysis as measured by LDH release was more pronounced with DEA than with amiodarone in isolated hepatocytes treated with the two drugs (Somani *et al.*, 1990). However, in the above acute *in vitro* experiments a dissociation of lamellar inclusion bodies from cellular toxicity and cell death was seen. Nevertheless, the possibility that continuous presence of these lamellar inclusion bodies in a chronic situation

may result in cellular toxicity cannot be excluded.

Although phospholipidosis was induced in rat lungs with amiodarone, there was no evidence of pulmonary fibrosis in the rat model (Riva *et al.*, 1987). This suggested that the development of pulmonary fibrosis in patients may be linked to a different mechanism. Other factors such as fibroblast proliferation (Goldstein and Fine, 1986) and production of collagen (Clark and Greenberg, 1986) by alveolar macrophages may play a role in pulmonary fibrosis. In any event, our findings that the biochemical and ultrastructural changes produced by protracted DEA treatment are similar to those of amiodarone will be of use in future investigations designed to evaluate the antiarrhythmic efficacy of DEA. Research aimed at reversal of pulmonary toxicity and fibrosis in animal models will also be of value since amiodarone is increasingly being used as a last resort antiarrhythmic drug. Observations by Kennedy *et al.*, (1988) showing that vitamin E and BHA caused a reversal in pulmonary edema and fibrosis due to amiodarone in a perfused rabbit lung model, and two recent preliminary reports showing that α-tocopherol offered partial protection against amiodarone-mediated injury in rat lung and human pulmonary endothelial cells (Kannan *et al.*, 1990; Martin *et al.*, 1988) offer some promise in this regard.

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