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AMIODARONE INDUCED PHOSPHOLIPIDOSIS

BIOCHEMICAL, MORPHOLOGICAL AND FUNCTIONAL CHANGES IN THE LUNGS OF RATS CHRONICALLY TREATED WITH AMIODARONE

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Abstract—Amiodarone, an antiarrhythmic drug, causes pulmonary fibrosis in some patients during chronic treatment but the mechanism is unknown. We studied the effects of amiodarone on pulmonary biochemistry, morphology and function at doses of 25 and 50 mg/kg/12 hr given to rats by gavage for four weeks. Plasma and pulmonary phospholipids were significantly augmented, 13% and 88% respectively, in the group given amiodarone 50 mg/kg/12 hr compared to pair-fed controls. Typical phospholipidosis-like light and electron microscopic alterations were seen in the lung, their severity related to the extent of biochemical changes induced by amiodarone. Pulmonary function tests revealed mild but not significant changes in O_2 and CO_2 alveolar exchange efficiency and lung compliance (P–V curve) of treated animals in comparison to pair fed controls. Plasma average concentrations of amiodarone and its main metabolite, desethylamiodarone, after four weeks were 2.46 ± 0.18 and 0.73 ± 0.13 μg/ml, respectively, in the 50 mg/kg/12 hr group. In the same group amiodarone and desethylamiodarone concentrations in lung were 163 ± 26 and 569 ± 153 times higher than those in plasma. A highly significant correlation was found between amiodarone concentrations in plasma and lung and phospholipid content in the lung. A subgroup of animals received amiodarone 50 mg/kg/12 hr for 8 weeks. The pulmonary phospholipidosis-like lesions were similar to those observed after one month of treatment, no fibrosis was evident on light microscopic examination.

Amiodarone is an antiarrhythmic agent, particularly effective against ventricular arrhythmias [1, 2]. The drug is an amphiphilic cationic, iodinated benzofuran derivative. Other amphiphilic compounds are known to cause generalized secondary phospholipidosis in man associated with tissue damage [3–5]. Many tissues including skin, lung, liver, mesenteric lymph nodes, and peripheral nerves can be affected.

A common feature of all these compounds is extensive tissue accumulation, particularly in the lung, adipose tissue and liver. Some clinical reports have described pulmonary toxic effects of amiodarone, such as pneumonitis and interstitial fibrosis leading to restrictive and diffusion defects in lung function in patients taking the drug chronically [6–10], the time to onset ranging from a few months to several years. It has been suggested that amiodarone interferes with lysosomal phospholipase A1 and A2 activities involved in the degradation of phospholipids and their consequent accumulation in the lung is one of the main causes of pulmonary phospholipidosis [4, 11–13], which histologically is characterized by multilamellar lysosomal inclusions chiefly in phagocytes.

It is still not known whether the pulmonary functional and structural lesions found in patients on amiodarone therapy have some pathophysiological relationship with the phospholipidosis-like lesions of the lung. The propensity of amiodarone to cause phospholipidosis-like alterations has been shown experimentally using different doses and dosage schedules, different routes of administration (p.a., i.p., intratracheal insufflation) and different animal models (mouse, rat, hamster, dog) [14–18], but it is not known whether or not lung phospholipidosis causes lung function alteration. Our aim was to reproduce phospholipidosis-like lesions in a rat model in order to investigate the possible cause–effect relationship between these well-known biochemical and histological modifications and the functional lung alterations described in patients [6, 7, 19]. The rat was chosen mainly for two reasons: amiodarone has been shown to induce phospholipidosis-like lesions in the lungs of treated rats [17] and our group had extensive experience in amiodarone kinetics in this animal species [20]. We investigated the lung biochemistry and morphology and assessed lung function tests after repeated oral doses of amiodarone to rats. In view of its peculiar kinetics and tissue distribution [20, 21], we also measured amiodarone and its metabolite, desethylamiodarone, in plasma and tissues by high pressure liquid chromatography, according to the method previously described [20, 21].

MATERIALS AND METHODS

Male CD-COBS rats (Charles River, Calco, Italy), 250–335 g body weight, were randomly assigned to different treatment schedules. They were given amiodarone (hydrochloride salt), suspended in gum arabic (10% in water) by gavage at loading doses of 50

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(N = 10) or 100 (N = 35) mg/kg/12 hr for the first week, followed by maintenance of 25 or 50 mg/kg/12 hr for three more weeks. Two control groups were used, both receiving the vehicle. Twenty of them had free access to the food and ten were pair-fed, in other words they were given an amount of food equal to that consumed by rats given the highest dose of amiodarone.

The pair-fed group was included because from preliminary experiments we had found that amiodarone could induce reduction of food intake. All rats had water *ad libitum*.

Before and during treatment, 8–10 hr after each dose of amiodarone 3–5 rats from each group underwent a surface ECG using a Battaglia Rangoni Polygraph Mod. ESO 300 UP/S (Casalecchio de Reno, Bologna, Italy). Blood samples were drawn weekly from the same animals for measurement of amiodarone and desethylamiodarone. Each rat and the food consumed by each cage of five rats were weighed 2–3 times a week.

At an average time of 10 hr after the last dose, the animals were anesthetized with chloral hydrate 100 mg/kg/BW i.p. and killed by exsanguination; the investigations made are described below.

Biochemistry. Lipids were extracted from plasma, liver and lung of the treated, pair fed and control animals with chloroform-methanol as described by Carlson [22]. Triglycerides were measured on this extract according to Van Handel *et al.* [23] and cholesterol and phospholipids according to Svanborg and Svennerholm [24] respectively.

Bronchoalveolar lavage was performed on pair fed, control and treated rats. Lungs were washed five times through the trachea, using 5 ml of ice-cold saline [25] for each lavage. An average of 22 ml were recovered, centrifuged and the supernatant precipitated with ice-cold acetone. Total phospholipids and phosphatidylcholine were then measured on the acetone precipitate [26].

Lung function tests. Semi-static compliance (P–V curves) was computed on excised lung. The pressure–volume (P–V) technique was essentially as described by Beckman and Weiss [25]. Lungs were connected to a Harvard infusion pump by a tracheal cannula and cyclically inflated with room air at a flow rate of 27 ml/min up to a maximal pressure of 20 cmH_2O. Pressure was measured by a Gould P23 ID transducer. Five P–V curves were routinely run in sequence, and the third, fourth and fifth were averaged for analysis of compliance, since stability and reproducibility were generally achieved by the third cycle. All measurements were made at room temperature. Leaking lungs, identified by abnormal P–V curves, particularly by failure to return to the initial volume and to maintain a constant pressure, were discarded. Blood samples (0.1 ml) were drawn from the carotid artery during mechanical ventilation with room air (10 min) and pure O_2 (10 min) with tidal volume of 8 ml/kg BW. The arterial pCO_2 and pO_2 were measured by a gas-analyzer Instrumentation Laboratory Mod. 213 to evaluate the gas exchange efficiency.

Tissue distribution study. Amiodarone and desethylamiodarone were assayed in plasma and tissue homogenates of lungs, liver, heart and adipose tissue at the end of treatment by high pressure liquid chromatography according to the method described by Latini *et al.* [21].

Histology. Lung sections of the animals not used for bronchoalveolar lavage were fixed with Zamboni solution, paraffin embedded, and stained with hematoxylin-eosin, PAS, Massou Trichrome. Ultrastructural examination was done by Zeiss EM 109 electron microscope. Since pulmonary fibrosis might depend on time of exposure to amiodarone, a further group of animals was given amiodarone 50 mg/kg/12 hr for 8 weeks, only for lung histological examination.

Statistical analysis. Different treatments were compared by one-way analysis of variance, followed by multiple comparison with Duncan's test. Correlations between concentration of amiodarone and desethylamiodarone in plasma and tissue and biochemical parameters were analyzed by linear regression analysis. The program runs on a HP-85 desk computer [27]. All data are shown as mean ± SE.

RESULTS

General effects of amiodarone and desethylamiodarone

Rats treated with loading dose of amiodarone

Table 1. Triglycerides (Tg), cholesterol (Ch) and phospholipids (PhL) in plasma and liver of control and treated rats (mean ± SE).

	Plasma (mg/100 ml)			Liver (mg/100 g wet tissue)		
	Tg	Ch	PhL	Tg	Ch	PhL
Control (N = 20)	83 ± 7	77 ± 2	109 ± 3	950 ± 45	258 ± 6	2825 ± 50
Pair fed (N = 8)	57 ± 5§	83 ± 2	117 ± 3	819 ± 47	231 ± 6‡	3009 ± 57
Amiodarone 25 mg/kg/12 hr (N = 5)	67 ± 5	88 ± 3	116 ± 4	864 ± 130	260 ± 23	2875 ± 138
Amiodarone 50 mg/kg/12 hr (N = 17)	56 ± 6§	119 ± 3†§	132 ± 4*§	942 ± 63	263 ± 7*	3228 ± 104§

*P < 0.05 vs pair fed.
†P < 0.01 vs pair fed.
‡P < 0.05 vs control.
§P < 0.01 vs control.

of 100 mg/kg/12 hr presented general weakness. Piloerection, decreased food intake, weight loss and mortality (nearly 50%) were observed (no biochemical or histological tests were performed on the dead animals). The animals reduced their food intake to less than 15 g of pellets per day compared to 20–25 g per day consumed before treatment was started. Gradual recovery was observed when the rats were given the maintenance doses. Body weights, at the end of the chronic treatment, were 406 ± 7 g in controls, 318 ± 12 g in pair fed, 326 ± 10 and 362 ± 12 g in animals given 50 and 25 mg/kg/12 hr, respectively. Due to the significant differences in food intake and body weight between controls and treated animals, pair fed were considered the proper control group. Changes in the ECG were observed after amiodarone 50 mg/kg/12 hr, QT and RR intervals both being lengthened significantly ($P < 0.05$) from 78 ± 4 to 95 ± 5 msec and from 18 ± 1 to 22 ± 1 msec respectively; corrected QT (QTc) according to the Bazett formula [28] was not altered.

Biochemistry

After chronic oral administration of amiodarone 50 mg/kg/12 hr plasma cholesterol and phospholipids were significantly raised ($P < 0.01$ and 0.05) compared to pair fed animals (Table 1). Hepatic cholesterol rose significantly ($P < 0.05$ vs pair fed) after amiodarone 50 mg/kg/12 hr, while hepatic triglycerides and phospholipids were unchanged (Table 1). Table 2 shows the levels of cholesterol, phospholipids and phosphatidylcholine in lung and bronchoalveolar lavages. Rats given the higher dose of amiodarone showed a significant rise in lung phospholipids ($P < 0.05$) and phosphatidylcholine ($P < 0.01$) but not in cholesterol compared to pair fed. The bronchoalveolar lavages of the amiodarone-treated animals presented a marked increase in phospholipids and phosphatidylcholine (3–5 times), but because of the high variability this did not reach statistical significance compared to pair fed. The lower dose of amiodarone, 25 mg/kg/12 hr, did not affect any of the biochemical variables.

Lung function studies

Arterial pCO_2 and pO_2 measured during ventilation with room air were 42 ± 3 and 70 ± 4 mmHg respectively in the group receiving amiodarone 50 mg/kg/12 hr and 39 ± 3 and 73 ± 3 mmHg in controls. During ventilation with pure O_2 the arterial pCO_2 and pO_2 in treated animals were 41 ± 2 and 289 ± 41 mmHg respectively compared to 37 ± 4 and 362 ± 8 mmHg in controls. The P–V curves during air inflation for control and treated animals are plotted in Fig. 1. Pulmonary compliance in the amiodarone 50 mg/kg/12 hr group was not statistically different from pair fed rats. The slightly higher compliance observed in control animals compared to treated and pair fed rats might be due to the higher body weight of the latter group.

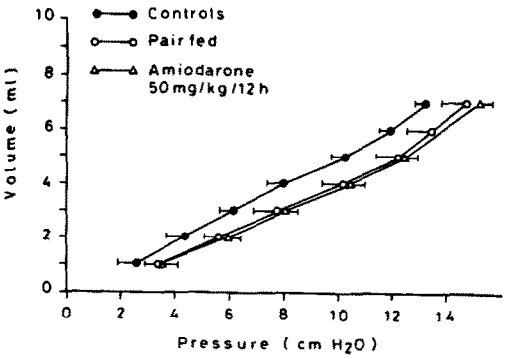

Fig. 1. P–V curves during inflation from control (N = 18) (●—●), pair fed (N = 4) (○—○) and treated rats given amiodarone 50 mg/kg/12 hr (N = 14) (△—△).

Tissue distribution

High concentrations of amiodarone and its major metabolite, desethylamiodarone, were present in rat tissues after long-term treatment of amiodarone (Table 3). The highest concentrations, several hundred times that of plasma, were found in adipose tissue and lungs both after 25 and 50 mg/kg/12 hr of amiodarone. Trough plasma concentrations of amiodarone did not change significantly from the first week to the end of the treatment. After one month of amiodarone 50 mg/kg/12 hr the plasma concentrations of the drug and metabolite range from 1.40 to 4.44 μg/ml and from 0.30 to 2.28 μg/ml respectively. The order of tissue/plasma ratios was adipose

Table 2. Cholesterol (Ch), total phospholipids (PhL) and phosphatidylcholine (PhC) in lungs and bronchoalveolar lavage (BAL) of control and treated rats (mean ± SE)

	Lungs (mg/100 g wet tissue)			BAL (μg)	
	Ch	PhL	PhC	PhL	PhC
Control (N = 20)	357 ± 17	1277 ± 58	687 ± 40	487 ± 32	236 ± 22
Pair fed (N = 8)	329 ± 31	1315 ± 139	671 ± 92	837 ± 218	432 ± 112
Amiodarone 25 mg/kg/12 hr (N = 5)	406 ± 27	1280 ± 170	742 ± 85	411 ± 58	211 ± 53
Amiodarone 50 mg/kg/12 hr (N = 17)	398 ± 26	2103 ± 326*§	1263 ± 214†§	4630 ± 2175‡	1118 ± 508

*$P < 0.05$ vs pair fed.
†$P < 0.01$ vs pair fed.
‡$P < 0.05$ vs control.
§$P < 0.01$ vs control.

Table 3. Amiodarone (A) and desethylamiodarone (DEA) in plasma and tissue of treated animals (mean ± SE)

		Plasma (μg/ml)	Lung (μg/g)	Lung T/P	Liver (μg/g)	Liver T/P	Heart (μg/g)	Heart T/P	Adipose tissue (μg/g)	Adipose tissue T/P
Amiodarone 25 mg/kg/12 hr (N = 5)	A	0.83 ± 0.07	54 ± 7	78 ± 17	34 ± 5	41 ± 2	12 ± 3	17 ± 3	543 ± 31	709 ± 124
	DEA	0.13 ± 0.03	35 ± 5	289 ± 30	12 ± 4	85 ± 8	5.4 ± 2.6	43 ± 6	31 ± 12	227 ± 57
	A/DEA	6.4	1.5		2.8		2.2		17.2	
Amiodarone 50 mg/kg/12 hr (N = 17)	A	2.46 ± 0.18	421 ± 81	163 ± 26	173 ± 32	65 ± 8	70 ± 10	28 ± 3	915 ± 51	383 ± 25
	DEA	0.73 ± 0.13	459 ± 112	569 ± 153	174 ± 55	176 ± 30	57 ± 16	64 ± 9	75 ± 15	104 ± 10
	A/DEA	4.03	0.92		0.99	1.23			11.7	

A = amiodarone, DEA = desethylamiodarone, T/P = tissue/plasma concentration ratio.

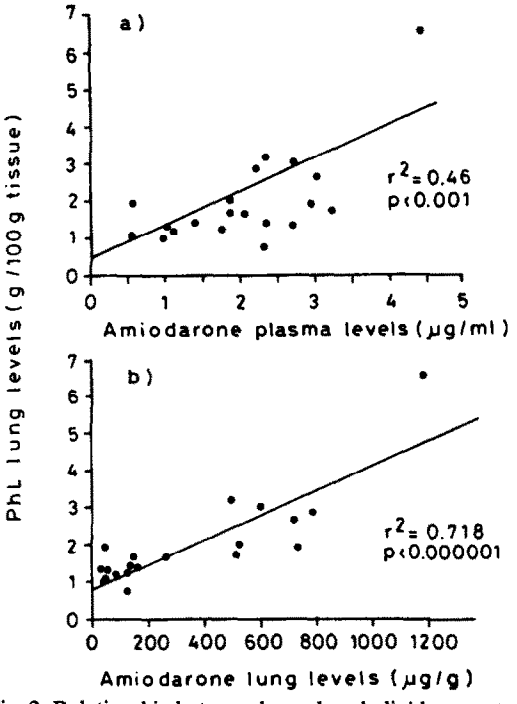

Fig. 2. Relationship between lung phospholipid concentrations and amiodarone in plasma (a) and lung (b) of rats given amiodarone 25 mg/kg/12 hr and 50 mg/kg/12 hr (N = 20).

tissue>lung>liver>heart for amiodarone, and lung>liver>adipose tissue>heart for desethylamiodarone. The amiodarone/desethylamiodarone ratio was close to 1 in lung, liver and heart, about 5 in plasma and 12–17 in the adipose tissue. Lung phospholipid concentrations were correlated with amiodarone concentrations in plasma and lung (Fig. 2). Amiodarone lung concentrations plotted against phospholipid lung concentrations showed a highly positive correlation ($P < 0.000001$), and the intercept of the line on the *y*-axis was comparable to phospholipid lung concentrations in the control group. The correlations were still highly significant when the rat with the highest values (lung phospholipids = 6.2 g/100 g tissue) was discarded.

Histology

Light microscope examinations of the lung sections of animals treated with amiodarone 50 mg/kg/12 hr for 4 weeks, revealed diffuse widening of alveolar septa by edema and dilated blood vessels, focal hyperplasia of type II pneumocytes, marked accumulation of foamy alveolar macrophages which stained positively with PAS reaction; no significant inflammatory cell infiltrates were observed in the interstitial and/or alveolar spaces. Bronchial, bronchiolar and vascular structures were normal, with the exception of some focal "aspecific" peribronchial inflammatory infiltrates by lymphocytes only. Control animals showed only interstitial widening by edema and some hemosiderin-laden macrophage in alveolar lamina. Rats treated for 8 weeks showed an increase of foamy macrophage accumulation in the alveolar spaces.

Electron microscopy showed that all the treated animals (after 4 weeks treatment) presented numerous membrane-bound cytoplasmic inclusions (lamellar bodies) in their lungs. Granular osmiophilic material or concentric layers of membranous material were observed in alveolar macrophages, type II pneumocytes, endothelial cells and occasionally free in alveolar lamina. This inclusion probably represents lysosomal transformation caused by progressive accumulation of indigestible material [29]. Neither light microscopic examination nor electron microscopy showed any microscopic or ultrastructural evidence of fibrosis in the lung section of animals treated chronically with amiodarone.

DISCUSSION

In this investigation we observed pathological, biochemical and morphological changes in the lungs of rats after repeated oral amiodarone. These lesions were obtained by administering the drug orally twice a day in the light of the short half-life of amiodarone in rats [20] compared to humans [30], starting with a one-week loading schedule, then a maintenance schedule for 3 weeks without discontinuing treatment during the week-ends. Trough blood levels were in the same concentration range as those usually found in patients receiving the drug chronically at dosages of 400–800 mg/day [31, 32] indicating that the rat has a higher clearance per kg b. w. than man. The mortality rate, associated with weight loss and decreased food intake, observed with 100 mg/kg/12 hr is in accordance with the report by Mazue *et al.* [17] and Vic *et al.* [18] who described a "malabsorption syndrome" induced in the dog by amiodarone; accumulation of foamy macrophages with lysosomal inclusion bodies were seen in mesenteric lymph nodes [17, 18]. The increase in plasma and hepatic cholesterol and in plasma phospholipids of rats receiving amiodarone 50 mg/kg/12 hr, compared to pair fed rats, appears to be specifically caused by long-term amiodarone.

The increases in phospholipid plasma concentrations and pulmonary phospholipid content in our animals after amiodarone 50 mg/kg/12 hr, but not after amiodarone 25 mg/kg/12 hr, would indicate a dose-related ability of the drug to induce phospholipidosis-like alterations. In fact, this is strongly shown by the highly significant correlation between amiodarone concentrations and increased phospholipid content in lung.

Bronchoalveolar lavage contains both surfactant phospholipids and alveolar cells, mainly alveolar macrophages. The elevated amount of phosphatidylcholine, which is the most represented phospholipid in the surfactant, found in rat bronchoalveolar lavage after amiodarone 50 mg/kg/12 hr, probably reflects the augmented activity of the pneumocyte II cell population induced by amiodarone and also the marked accumulation of enlarged foamy macrophages. Heyneman and Reasor [33], recently showed that alveolar macrophages play a major role in the development of lung phospholipidosis caused by repeated administration of chlorphentermine to rats.

The altered alveolar diffusion capacity in patients with radiological signs of fibrosis after amiodarone therapy was described by Magro *et al.* [34] as the earliest functional alteration of the fibrotic lung. According to them, this event together with the typical radiological picture of interstitial pulmonary fibrosis, precedes clinical symptoms and serves as a marker to detect patients at risk [34, 35]. In our treated animals we did not observe significant changes in the arterial pO_2 and pCO_2 tension compared to controls and even after 8 weeks of amiodarone treatment we detected no morphological indications of lung fibrosis on light microscopic examination.

Pulmonary compliance, which is altered in patients with restrictive pulmonary disease, was not significantly different in treated and control animals. The normal lung function test results of rats given amiodarone are in line with the absence of microscopic fibrotic lesions. We do not know whether longer treatment would induce fibrotic pulmonary lesions, but judging from observations on experimental bleomicin-induced pulmonary fibrosis, two months should be enough [36]. Experimental evidence of amiodarone-induced pulmonary fibrosis was obtained in hamsters given a single intratracheal injection of amiodarone [4]. In patients, the time to onset of pulmonary toxicity varies widely, being usually more than 1 month after starting the therapy. The hypothesis that phospholipidosis-like alterations, well documented by lung biopsy in patients taking long-term amiodarone therapy [19, 37–42], leading to lung interstitial fibrosis in man [43], is not supported by our findings.

Pulmonary fibrosis and liver toxicity induced by amiodarone in patients exposed to long-term therapy are not simply related to phospholipidosis; in fact phospholipidosis-like infiltrations are a common feature of amiodarone chronic treatment, while lung and liver toxicity are rare events. Patients with Fabry's disease, a lysosomal storage disorder, present visceromegaly, cutaneous, ocular and neurologic alterations but neither pulmonary fibrosis nor hepatic cyrrhosis are typical. Costa-Jussa *et al.* [11] suggested, on the basis of their clinical and experimental findings, that lung histiocytosis and interstitial fibrosis represent two separate effects of amiodarone on the lung. The reason why amiodarone should cause one reaction rather than the other is as yet unclear, although pre-existing lung damage in many elderly patients may contribute to amiodarone toxicity [44].

Serious hepatic side-effects have also been recently described [37, 38, 41, 42, 45]. The mechanism of these severe adverse reactions is poorly understood and unstudied. Both metabolic and immunological mechanisms have been proposed.

In conclusion, biochemical lung alterations observed in rats treated with amiodarone are closely related to plasma and tissue concentrations of the drug as well as to the histological picture. The phospholipidosis-like lesions led neither to lung interstitial fibrosis, nor to changes in pulmonary function over the time period studied. We believe that other mechanisms might be responsible for the pulmonary toxicity in humans.

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