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# THE EFFECT OF ADRIAMYCIN ON GLYCEROLPHOSPHATE ACYLTRANSFERASE AND LIPID METABOLISM IN RAT HEPATOCYTES IN MONOLAYER CULTURE

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**Abstract**—Total and mitochondrial glycerolphosphate acyltransferase activities were measured after 24 hr exposure of rat hepatocytes to Adriamycin. Both activities decreased with increasing concentrations of Adriamycin. The activity of the microsomal glycerolphosphate acyltransferase, which was determined from the difference between the total and mitochondrial enzyme activity, also decreased with increasing drug concentration. The effect on glycerolphosphate acyltransferase was specific as there was no change in lactate dehydrogenase or cytochrome oxidase activity in this time period. Adriamycin did not inhibit mitochondrial glycerolphosphate acyltransferase activity *in vitro*. After 24 hr exposure of hepatocytes to Adriamycin no change was observed in the biosynthesis of phosphatidylcholine or triacylglycerol. Secretion of lipid into the medium was measured over the subsequent 24 hr. There was a significant reduction in very low density lipoprotein secretion as measured by triacylglycerol secretion from cells incubated with 5 $\mu$M Adriamycin. Cells were damaged by the 48 hr exposure to 1 $\mu$M and higher concentrations of Adriamycin as evidenced by a fall in lactate dehydrogenase activity in these cells. The secretion of lysophosphatidylcholine, as measured by the incorporation of [$^3$H]glycerol into medium lysophosphatidylcholine, was significantly increased when cells were incubated with 5 $\mu$M Adriamycin. The results are discussed in relation to the effect of Adriamycin on hepatic lipid metabolism and the cardiotoxicity of the drug.

The efficacy of Adriamycin* (doxorubicin) as an anti-neoplastic agent is limited because repeated doses lead to cardiotoxicity [1, 2]. The pathogenesis of the cardiomyopathy, that accompanies Adriamycin treatment, is unknown. Adriamycin may also, in some patients, cause liver dysfunction [3].

Adriamycin has a high affinity for DNA [4], which may account for its anti-neoplastic action. The drug inhibits incorporation of thymidine into DNA and uridine into RNA [5], causes breakage of the mitochondrial DNA helix and slows the rate of mitochondrial DNA synthesis [6].

Adriamycin also has a high affinity for cardiolipin and a lower affinity for other acidic phospholipids [4]. The interaction of Adriamycin with cardiolipin may explain the inhibition of oxidative phosphorylation [7], pyruvate uptake by mitochondria [8] and cytochrome oxidase activity [9] by Adriamycin. Heart muscle is rich in mitochondria and hence the interaction of Adriamycin with cardiolipin may contribute to the cardiotoxicity of the drug [10].

Inhibition of succinate oxidation in mitochondria by Adriamycin is enhanced by hexokinase which binds to the outer mitochondrial membrane. As hexokinase is often increased in transformed cells the latter may be more susceptible to the effect of the drug [7]. The action of Adriamycin may be modified by other cell components. For example, *in vitro*, spermine antagonizes the binding of Adriamycin to the inner membrane of heart mitochondria [11]. However, in the intact cell access of the drug to the inner membrane may be limited. Pretreatment of rat liver mitochondria with digitonin or osmotic shock increased their susceptibility to inhibition of respiration by Adriamycin indicating a degree of latency of cariolipin in intact mitochondria [12].

Adriamycin induced cardiotoxicity can be observed in rats [13, 14] so that these can be used as animal models to investigate the development of cardiotoxicity. A dose-dependent increase of lipid peroxides was observed to correspond with that required for the development of cardiomyopathy [14]. There was a concomitant increase in serum triacylglycerol and cholesterol.

This study determines whether the increase of serum triacylglycerol is the result of an increased secretion of very low density liproprotein (VLDL) by the liver by examining the effect of Adriamycin on the secretion of VLDL from rat liver hepatocytes in monolayer culture. The latter system is suitable for studying the regulation of VLDL secretion [15, 16].

The effect of Adriamycin on the activity of glycerolphosphate acyltransferase (EC 2.3.1.15) was also measured. In rat liver glycerolphosphate acyltransferase is associated with both the endoplasmic reticulum and the outer mitochondrial membrane [17, 18]. The mitochondrial enzyme differs in a number of ways from the enzyme in the endoplasmic reticulum [18–23]. One difference, which forms the basis of a method for measuring the two activities in cell homogenates, is the response to *N*-ethylmaleimide: the mitochondrial enzyme is insensitive to this reagent whereas the microsomal enzyme is inhibited [20]. The mitochondrial glycerolphosphate acyltransferase may have a regulatory role in cardiolipin biosynthesis [24].

* Adriamycin is a registered trade mark of Formitalia Carlo Erba.

## MATERIALS AND METHODS

*Materials.* Male Wistar rats (180–200 g) were used. The animals were fed diet 41B; E. Dixon and Sons (Ware, U.K.). This contained (w/w) 46.7% carbohydrate (mainly starch), 15.4% protein, 3.1% fat and 4.4% crude fibre. Food and water were available *ad lib.* [1,3$^3$H]Glycerol (3.8 Ci/mol) and [1-$^{14}$C]oleic acid (54.9 Ci/mol) were purchased from Amersham International (Amersham, U.K.). Cytochrome *c* (type III), Adriamycin and other chemicals were from the Sigma Chemical Co. (Poole, U.K.). Solvents used were of A.R. grade.

*Preparation of hepatocytes.* Hepatocytes were prepared as described by Cascales *et al.* [25]. The last wash of the hepatocytes was in supplemented Liebovitz containing bovine serum albumin (2 g/L). Hepatocytes were put on collagen-coated 60-mm tissue culture dishes as described [25]. After removal of unattached cells the monolayers were incubated overnight in fresh medium.

*Incubation of hepatocytes with Adriamycin and radioisotopes.* On the day following preparation of hepatocytes, the cells were incubated with fresh serum-containing medium in the absence or presence of Adriamycin. The latter was dissolved in the medium at 37°, with stirring and protection from light.

After 24 hr incubation some cells were collected for the determination of enzyme activities: medium was removed and the cells washed with 2 × 2 mL of supplemented Liebovitz (serum-free) containing bovine serum albumin (2 g/L), followed by 2 × 2 mL of supplemented Liebovitz medium. Cells were scraped from the dish in 1 mL of ice-cold 0.25 M sucrose containing 0.5 mM dithiothreitol, 2 mM EDTA and 10 mM Hepes adjusted to pH 7.4 with KOH. The suspension was sonicated for 5 sec at 22 kHz with an amplitude of 8 μm peak-to-peak. The homogenate was stored at −20° before determination of enzyme activities and protein.

Some plates were further incubated in the absence or presence of Adriamycin and 3 mL of serum-free supplemented Liebovitz medium containing 1 mM [1,3-$^3$H]glycerol (1 Ci/mol) and 1 mM (1-$^{14}$C)oleate (0.125 Ci/mol). This medium also contained bovine serum albumin (9.3 g/L) and choline chloride (315 μm). The oleate was added last, to a stirred medium at 37°, from a stock, 80 mM solution, prepared by warming the acid with a 16% excess of KOH. After 2 hr incubation with the radioactive cocktail the cells were collected as described above.

A further group of plates were incubated in the absence or presence of Adriamycin and 3 mL of serum-free supplemented Liebovitz medium containing 1 mM [1,3-$^3$H]glycerol (2 Ci/mol) and 1 mM (1-$^{14}$C]oleate (0.156 Ci/mol). Bovine serum albumin, choline chloride and oleate were added as described above. These plates were incubated for 24 hr with the radioactive cocktail. The medium was collected and centrifuged at 1200 *g* for 10 min at room temperature to remove detached cells and cell debris. Samples (2 mL) of the supernatant were taken for lipid analysis. The monolayer of cells were washed and collected as described above.

*Preparation of mitochondria.* A purified mitochondrial fraction was prepared from whole rat liver as described by Freeman and Mangiapane [26].

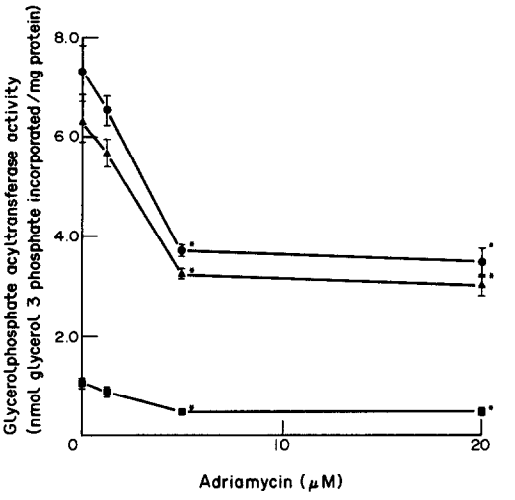


Fig. 1. Effect of Adriamycin on glycerolphosphate acyltransferase activities. Hepatocytes were incubated for 24 hr with various concentrations of Adriamycin. After this incubation cells were harvested as described in Materials and Methods. Total glycerolphosphate acyltransferase activity (●) and *N*-ethylmaleimide insensitive activity (mitochondrial) (■) were measured. The *N*-ethylmaleimide sensitive activity (microsomal) (▲) was calculated from these two values. Results are mean ± SD for triplicate dishes. The significance of difference between Adriamycin-treated and control activities were calculated by an unpaired *t*-test and is indicated by: *$P < 0.001$. Similar results were obtained in two further independent experiments.

*Extraction and analysis of lipids.* Lipids were extracted and analysed as described by Mangiapane and Brindley [15].

*Determination of glycerolphosphate acyltransferase.* Total and mitochondrial (*N*-ethylmaleimide insensitive) glycerolphosphate acyltransferase were determined as described by Lawson *et al.* [27] except that the incubation contained 120 mM KCl and [1,3-$^3$H]glycerolphosphate (6.10 Ci/mol). Butanol (1 mL) saturated with 10 mM glycerol and 10 mM DL-glycerolphosphate was used to stop the reaction and the butanol phase was washed three times with 0.6 mL of 10 mM glycerol, 10 mM DL-glycerolphosphate. Radioactivity of 0.4 mL of the butanol phase was determined.

*Cytochrome oxidase.* Cytochrome oxidase was assayed spectrophotometrically as described by Sottocasa *et al.* [28] except that 0.03% lubrol was added [29].

*Lactate dehydrogenase.* Lactate dehydrogenase was assayed as described by Saggerson and Greenbaum [30].

*Protein.* Protein was determined by the method of Lowry *et al.* [31].

## RESULTS

### *Glycerolphosphate acyltransferase activity*

There was no change in lactate dehydrogenase or cytochrome oxidase activity at the end of the first 24 hr incubation of hepatocytes with Adriamycin (results not shown). However, after this incubation

Table 1. Effect of Adriamycin on the synthesis of glycerolipids in hepatocytes

| Adriamycin (μm) | Phosphatidylcholine | | Triacylglycerol | |
|---|---|---|---|---|
| | Substrate incorporated (nmol/mg protein) | | | |
| | glycerol | oleate | glycerol | oleate |
| 0 | 8.79 ± 0.97 | 16.69 ± 3.21 | 34.86 ± 2.36 | 132.3 ± 9.2 |
| 0.5 | 10.03 ± 0.13 | 23.31 ± 0.6 | 38.55 ± 2.75 | 139.8 ± 12.8 |
| 1.0 | 7.19 ± 0.23 | 15.89 ± 0.77 | 38.71 ± 1.43 | 139.4 ± 6.5 |
| 5.0 | 10.58 ± 0.80 | 21.74 ± 1.67 | 40.81 ± 2.59 | 130.6 ± 5.8 |
| 20.0 | 11.91 ± 1.30 | 25.25 ± 2.59 | 45.87 ± 4.48 | 133.9 ± 19.4 |

Hepatocytes were incubated for 24 hr in the absence or presence of Adriamycin. The medium was replaced with fresh medium containing 1 mM [$^3$H]glycerol and 1 mM [$^{14}$C]oleate and the appropriate concentration of drug. After a further 2 hr incubation the cell lipids were analysed.

Results are expressed in nmol/mg protein and are the mean ± SD of triplicate dishes. Similar results were obtained in another independent experiment.

period, the total, mitochondrial and microsomal glycerolphosphate acyltransferase activities were significantly decreased (Fig. 1). The enzyme activity in the endoplasmic reticulum (microsomal activity) was obtained by subtracting the mitochondrial activity, determined after treatment with *N*-ethylmaleimide, from the total activity, measured in the absence of this reagent. No reduction in any glycerolphosphate acyltransferase activity was observed after 12 hr incubation with 5 μM Adriamycin (results not shown).

Adriamycin, at a final concentration of 1, 5, 20, 100, 250 and 500 μM, was preincubated with a purified mitochondrial fraction, (that had been prepared from whole rat liver, frozen and thawed), for 30 min at 37° prior to addition of palmitoyl CoA. The drug had no effect on glycerolphosphate acyltransferase activity at any of these concentrations of drug except at 500 μM where a small inhibition (<20%) was observed.

*Biosynthesis of cell lipids*

No change in the rate of biosynthesis of phosphatidylcholine or triacylglycerol was observed after 24 hr incubation of hepatocytes with Adriamycin (Table 1).

*Secretion of lipid by hepatocytes*

At the end of the second 24 hr period of incubation with Adriamycin there was a significant reduction of lactate dehydrogenase activity at Adriamycin concentrations of 1 μM and above. Lactate dehydrogenase activity at 1 and 5 μM Adriamycin was approximately 50% that of cells incubated in the absence of the drug. There was also visible (by light microscopy) damage to the cells showing that Adriamycin toxicity had occurred.

Despite the loss of lactate dehydrogenase activity the incorporation of both glycerol and oleate into secreted triacylglycerol was unaffected at 1 μM Adriamycin (Table 2). Similar results were obtained at 0.5 μM Adriamycin, at which concentration lactate dehydrogenase activity was also unaffected (results not shown). However, at 5 μM Adriamycin there was a significant decrease in secretion of triacylglycerol, as determined by glycerol and oleate incorporation into medium triacylglycerol (Table 2). No change was observed in the incorporation of oleate into secreted lysophosphatidylcholine. However, there was a significant elevation of incorporation of glycerol into lysophosphatidylcholine at 5 μM Adriamycin (Table 2).

## DISCUSSION

In the present work, although lactate dehydrogenase and cytochrome oxidase activities were unaffected, the activities of both mitochondrial and microsomal glycerolphosphate acyltransferase were significantly decreased after 24 hr incubation of hepatocytes with Adriamycin at 5 and 20 μM (Fig. 1). At the latter concentration the mitochondrial enzyme activity was inhibited by 55% and the microsomal activity by 52%.

The contribution to total glycerolphosphate acyltransferase activity of the mitochondrial form is rather low compared with other reports [20, 27]. This may be due to the assay employed in which the palmitoyl-CoA concentration was optimal for microsomal rather than mitochondrial activity [20]. High concentrations of palmitoyl-CoA can cause substrate inhibition of the mitochondrial glycerolphosphate acyltransferase [20]. Enhanced mitochondrial activity can be achieved at higher glycerolphosphate concentrations [20] than used in the present work. Under the assay conditions employed enzyme activity was proportional to sample protein.

Direct inhibition of glycerolphosphate acyltransferase by Adriamycin or indirect inhibition, by alteration of the lipid micro-environment either by binding the drug to acidic phospholipids [4] or by lipid peroxidation [32], appears to be unlikely as Adriamycin had little effect on mitochondrial glycerolphosphate acyltransferase *in vitro*. The extent to which peroxidation occurs *in vivo* is uncertain but lipid peroxides appear to be increased in kidney, heart and to a lesser extent in liver in rats treated chronically with Adriamycin [33].

Adriamycin is unstable in the medium (50% loss in 24 hr) but very little is degraded intracellularly, in rat hepatocytes in culture (6% in 24 hr) [34]. Adriamycin uptake from the medium may be gradual

Table 2. Effect of Adriamycin on secreted lipid

| Adriamycin (μm) | Lysophosphatidylcholine | | Triacylglycerol | |
|---|---|---|---|---|
| | Substrate incorporated (nmol/mg protein) | | | |
| | glycerol | oleate | glycerol | oleate |
| 0 | 2.57 ± 0.30 | 6.91 ± 0.83 | 2.40 ± 0.31 | 13.04 ± 0.99 |
| 1.0 | 3.06 ± 0.39 | 5.54 ± 0.51 | 2.79 ± 0.41 | 13.28 ± 1.36 |
| 5.0 | 5.56 ± 0.30* | 8.33 ± 1.35 | 0.46 ± 0.04† | 4.06 ± 1.38† |

Hepatocytes were incubated for 24 hr in the absence or presence of Adriamycin. The medium was replaced with fresh medium containing 1 mM [$^3$H]glycerol and 1 mM [$^{14}$C]oleate and the appropriate concentration of drug. After a further 24 hr incubation the medium lipids were analysed. Results are expressed as nmol/ml cell protein and are the mean ± SD of triplicate dishes. The significance of difference between Adriamycin-treated and control values were calculated by an unpaired *t*-test and is indicated by *$P < 0.001$ where drug-treated value is higher than the control and †$P < 0.001$ where the drug-treated value is lower than the control. Similar results were obtained in three further independent experiments.

but high concentrations can accumulate in membranes. Thus when K562 cells were incubated in a medium containing 1 μm Adriamycin the concentration in nuclei, determined by microspectrofluorimetry, reached 100 μm in 4 hr [35]. Subcellular fractionation of L1210 cells, which had been incubated with Adriamycin, showed that most of the drug was found in the nucleus but a significant amount was associated with mitochondria and some was recovered in plasma membrane and endoplasmic reticulum [36]. The rate of uptake of Adriamycin by rat hepatocytes and its subsequent subcellular distribution are not known. The observation that glycerolphosphate acyltransferase activity was inhibited by 5 μM Adriamycin after 24 hr but not after 12 hr suggests that the effect of the drug on glycerolphosphate acyltransferase activity does depend on the accumulation of the drug within the cells.

Despite the large inhibition of glycerolphosphate acyltransferase at 5 and 20 μM Adriamycin no effect of the drug was observed on the rate of lipid biosynthesis confirming the earlier conclusion that the glycerolphosphate acyltransferase does not have a regulatory role in the pathway [37].

It was surprising to find no effect of Adriamycin on lipid biosynthesis as the major regulatory enzyme, phosphatidate phosphohydrolase [38], acts on phosphatidate, an acidic phospholipid to which Adriamycin can bind [4]. Perhaps this lipid is not the initial site of interaction of the drug with the cell and consequently phosphatidate phosphohydrolase is not immediately affected.

Prolonged incubation of hepatocytes with Adriamycin led to damage to the cells. The impairment of triacylglycerol secretion by 5 μM Adriamycin (Table 2) may be a consequence of the toxic effect of Adriamycin on hepatocytes. This toxic effect was not seen in the first 24 hr incubation with the drug. The reduction of triacylglycerol secretion seen in the present work may explain the moderate steatosis observed in livers of patients treated with Adriamycin [3].

No evidence has been obtained in the present work for an increase of lipid secretion from hepatocytes incubated with Adriamycin. Thus the increase in serum lipids of rats given a chronic treatment with Adriamycin may reflect impaired removal of lipid from the serum. This could result from the decreased activity of heart lipoprotein lipase that occurs in the presence of Adriamycin treatment [39].

An increased incorporation of glycerol into secreted lysophosphatidylcholine was observed in hepatocytes incubated with 5 μM Adriamycin (Table 2). This lysophosphatidylcholine may be oleate-depleted as there was no corresponding increase in incorporation of oleate into the secreted lysophosphatidylcholine. This lipid is not secreted as part of VLDL [15] but its secretion is stimulated by albumin [40] which was included in the incubation medium. It has been suggested that the transport of lysophosphatidylcholine from the liver may be of physiological importance in supplying extrahepatic tissues with essential fatty acid and choline [41]. The increased lysophosphatidylcholine secretion in the presence of Adriamycin may be caused by increased phospholipase activity, either in the plasma membrane [42] or as the result of disruption of lysosomes.

Various effects of Adriamycin, observed *in vitro*, require high concentrations of the drug. Half maximal inhibition was observed at: 100 μm (of protein kinase C [43]), 125 μm (of pyruvate transport by rat heart mitochondria [8]) and 175 μm (of complexes III and IV of the mitochondrial respiratory chain [9]). The inhibition of import of mitochondrial precursor protein into mitochondria was 80% at 360 μm [44]. However, as described above, high membrane concentrations of Adriamycin can occur as cells accumulate the drug. Thus, a number of cell activities are affected by Adriamycin. One or more of the effects of Adriamycin ultimately lead to cell death.

Hepatocytes in culture offer a simple system in which to study Adriamycin toxicity. The effects of the drug on myocytes in culture may be a better model for studying cardiotoxicity. The latter is the most serious problem of the use of Adriamycin in the chemotherapy of cancer.

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