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Doxorubicin induces ceramide and diacylglycerol accumulation in rat hepatocytes through independent routes

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ABSTRACT

Doxorubicin (DOX) is a potent anticancer drug, whose clinical use is limited due to its toxicity. This toxicity has been associated with free radicals generated during the drug metabolism. We previously found that DOX increased the intracellular diacylglycerol (DAG) levels at 1 h in isolated rat hepatocytes, probably by mobilizing choline-enriched phospholipids. In this work, we studied the effects of DOX on oxidative stress markers, and the possible contribution of ceramide metabolism to DAG accumulation. Other possible routes of DAG production, such as impairment of triacylglycerol (TAG) synthesis, and their connection with oxidative stress were also investigated. Time-course experiments revealed that DOX decreased intracellular GSH at 2 h, but did not affect cell viability, ATP or malondialdehyde (MDA) levels at any time. DOX did not modify the intracellular levels of [^{3}H]-ceramide during the first 90 min of exposure, but increased it significantly at 2 h. [^{3}H]-Sphingomyelin remained unchanged during the whole period. These results indicate that ceramide metabolism is not involved in the early DAG response to DOX. The drug markedly increased the incorporation of [^{3}H]-oleate into intracellular DAG from 60 min. In contrast, DOX reduced the incorporation of [^{3}H]-oleate into intracellular phospholipids and TAG. DOX inhibited TAG synthesis at the DAG acyltransferase step. These results suggest that DOX increases the intracellular levels of the lipid messengers, ceramide and DAG, by independent mechanisms. Activation of the *de novo* synthesis of ceramide is probably involved in the sphingolipid accumulation, while inhibition of TAG synthesis contributes to DAG accumulation, this response being independent of oxidative damage.

1. Introduction

Doxorubicin (DOX) is a potent anticancer drug, whose clinical use is limited due to its toxicity. This toxicity has been associated with reactive oxygen species (ROS) generated during the drug metabolism, leading to the induction of membrane alterations through lipid peroxidation and the impairment of DNA and RNA synthesis (Gewirtz, 1999). Cellular oxidative stress can induce the generation of lipid second messengers, such as phosphatidic acid, diacylglycerol (DAG) or ceramides, and trigger signalling cascades leading to cell survival or apoptosis. Ceramide, a naturally occurring membrane sphingolipid, has long been considered a proapoptotic mediator (Steinbrecher et al., 2004). This lipid mediator is generated by sphingomyelinases-catalyzed sphingomyelin hydrolysis, *de novo* synthesis, or recycling of sphingolipids. Antitumour anthracyclines have been reported to induce apoptosis through sphingomyelinase-mediated ceramide generation (Grazide et al., 2004).

In a previous work, we found that DOX increased the intracellular DAG levels from lipid stores in a short time in isolated rat hepatocytes (Martinez et al., 2002a). This effect was mediated in part by phosphatidylinositol-specific phospholipase C, but was independent of phospholipase D, ruling out DAG production from the sequential actions of phospholipase D, generating phosphatidic acid and phosphatidate phosphohydrolase (Martinez et al., 2002b). DOX also seemed to mobilize lipids from choline-enriched phospholipids (Martinez et al., 2002a). An important source of DAG is sphingomyelin synthesis from ceramide and phosphatidylcholine, a reaction catalyzed by sphingomyelin synthase. In this work, we have studied the effects of DOX on cell oxidative stress markers, and ceramide turnover in response to the drug. Other possible sources of DAG regarding lipid metabolism, such as impairment of triacylglycerol (TAG) synthesis, were also investigated.

2. Materials and methods

2.1. Materials

[^{3}H]-palmitic acid (53 Ci/mmol) and [^{3}H]-oleic acid (7.5 Ci/mmol) were purchased from Amersham Pharmacia Biotech (U.K). Essentially fatty acid-free BSA and HEPES were from Boheringer Mannheim (Germany), collagenase A was from Roche Applied Science (Germany) and fetal calf serum from Biochrom K.G. (Germany). DOX

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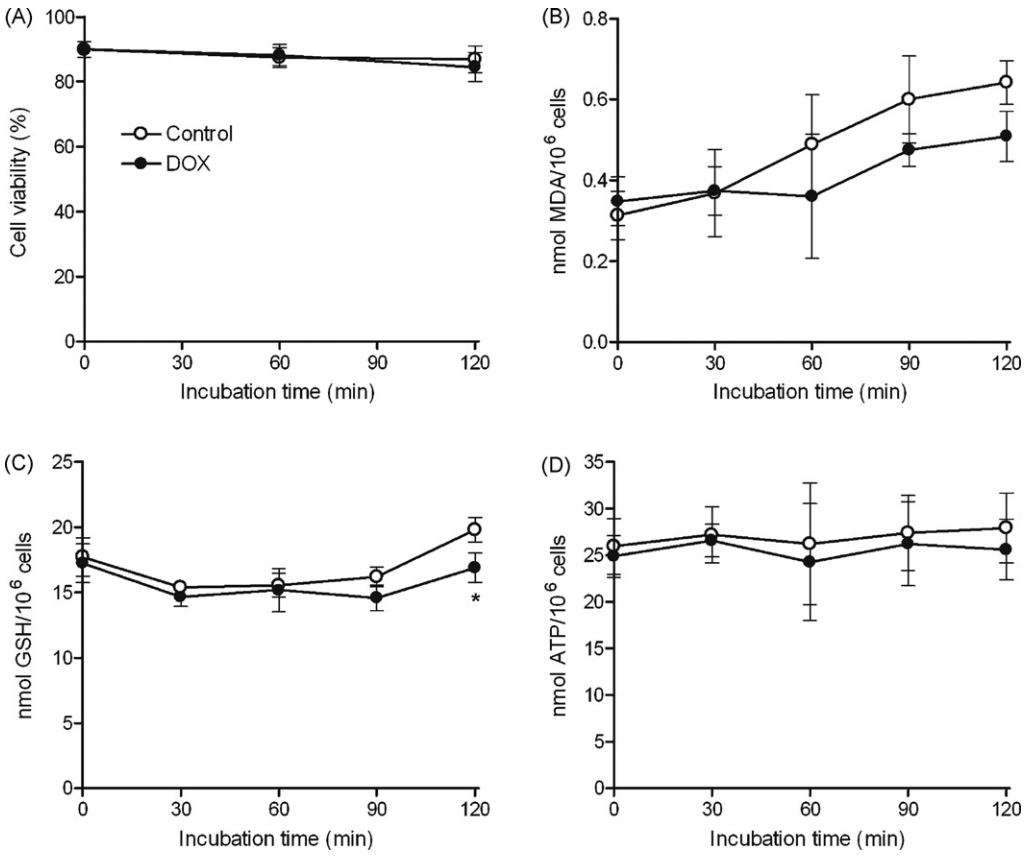

Fig. 1. Effects of doxorubicin on (A) cell viability, (B) MDA, (C) GSH, and (D) ATP of rat hepatocytes. Hepatocytes (2×10^6 cells/ml) were incubated in Krebs-Henseleit buffer, pH = 7.4, containing 2% BSA. Reactions were initiated by the addition of 100 μM DOX. At the indicated times aliquots were removed and analyzed as described in Section 2. Results are the means ± SEM of 3–5 experiments. $^{*}p < 0.05$; in comparison with control.

was from TEDEC-MEIJI FARMA, S.A. (Madrid, Spain). Thiobarbituric acid (TBA) was from Sigma Co., St. Louis, USA. All other used reagents were from the highest purity available.

2.2. Preparation of isolated hepatocytes and incubation with DOX

Liver cells were isolated from male Sprague-Dawley rats (180–200 g) by the collagenase perfusion method as previously described (Ruiz-Larrea et al., 1993). The hepatocyte viability was determined by means of the Trypan blue exclusion test and it was typically greater than 90%. Hepatocytes were resuspended (2×10^6 cells/ml) in fresh Krebs-Henseleit buffer plus 2% BSA, pH 7.4. Cells were equilibrated at 37 °C under carbogen (95% O_2, 5% CO_2) for 20 min. DOX (100 μM) was added (zero time) and incubations continued for different periods. At the indicated times, aliquots were removed in order to analyze the different parameters. GSH and ATP were measured in the pellet fractions as previously described (Martinez et al., 2002c). Malondialdehyde (MDA) was measured by an isocratic HPLC method using UV–visible detection (Navarro et al., 2006).

In order to prelabel the cellular lipid stores, hepatocytes (4×10^6 cells/ml) were resuspended in fresh Krebs-Henseleit buffer, plus 2.5 mM $CaCl_2$, fetal calf serum (20%), 20 mM HEPES, pH 7.4, containing [^{3}H]-palmitic acid (0.5 μCi/ml). After 30 min incubation at 37 °C, cells were centrifuged, washed thoroughly 3 times with ice-cold Krebs-Henseleit medium containing 0.5% BSA, resuspended (2×10^6 cells/ml) in the incubation medium and further treated as described above.

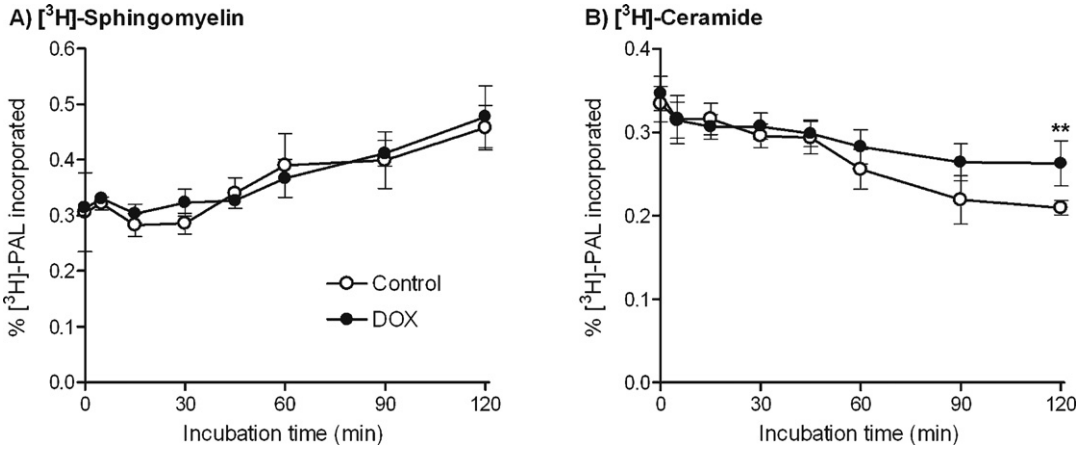

Fig. 2. Time-course of doxorubicin effect on intracellular levels of (A) [^{3}H]-sphyngomyelin and (B) [^{3}H]-ceramide in hepatocytes. [^{3}H]-Palmitic acid prelabelled hepatocytes were exposed to 100 μM DOX for times up to 2 h. At the times indicated in the figure lipids from cellular pellets were extracted, [^{3}H]-sphingomyelin and [^{3}H]-ceramide separated by TLC and counted for radioactivity. Results are expressed as the percentage of [^{3}H]-palmitate incorporated into cells. Values are the means ± SEM of 3–5 experiments. $^{**}p < 0.01$.