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In vivo effects of doxorubicin on kinase C in cultured cells*

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Summary. The ability of doxorubicin to inhibit kinase C in vivo in cultured BALB/c 3T3 mouse fibroblasts was determined by the phosphorylation of the myristoylated alanine-rich C kinase substrate (MARCKS) and the induction of c-fos transcription following treatment with 200 nM phorbol 12-myristate 13-acetate. A concentration of 60 μM doxorubicin did not inhibit MARCKS phosphorylation but completely inhibited c-fos expression. The inhibition of c-fos expression was not specifically mediated via kinase C, since both the total RNA synthesis as measured by [^{3}H]-uridine uptake and the fraction of serum-induced c-fos expression that was not attributable to kinase C were inhibited by doxorubicin. The present data do not support the hypothesis that the cytotoxic effect of doxorubicin is attributable to the inhibition of kinase C in vivo.

Introduction

The anthracycline doxorubicin is one of the most useful drugs in cancer treatment. Anthracyclines are known to intercalate DNA [16] and to inhibit RNA transcription [10]. This interaction with DNA is thought to be important to the cytotoxic effect of this drug. Doxorubicin also causes DNA strand breaks that appear to be mediated by DNA topoisomerase II [25]. However, doxorubicin can be cytotoxic without entering the cell [27] and has been shown to damage the cell membrane [11, 21].

The phospholipid, calcium-dependent protein kinase C appears to be a major signal transducer for growth factors and also seems to be the receptor for the tumor promotor phorbol ester [12]. Activation of kinase C results in rapid induction of c-fos transcription [6] and phosphorylation of the myristoylated alanine-rich C kinase substrate (MARCKS) [9, 22, 30]. Doxorubicin has been found to inhibit kinase C in vitro [8, 15, 19, 31]. In the present investigation, we studied the ability of doxorubicin to inhibit in vivo two kinase C functions in cultured BALB/c 3T3 mouse cells: the induction of c-fos and the phosphorylation of the MARCKS protein following phorbol ester stimulation.

Materials and methods

Cell culture. A31 BALB/c 3T3 mouse fibroblasts [3] were plated at about 2,000 cells/cm^2 in plastic dishes, grown for 5 days in Dulbecco's modified Eagle's media (DMEM) containing 10% calf serum and supplemented with 4 mM glutamine, and then made quiescent and synchronous by 2 days of 0.5% serum arrest prior to experimental manipulations. At the end of low-serum arrest, the cells were washed and placed in media along with different concentrations of doxorubicin for 1 h. The cells were treated with the specified agents and then harvested for measurements of c-fos induction, MARCKS phosphorylation, and total RNA synthesis at the stated times. Doxorubicin was obtained from Cetus Corp. (Emeryville, Calif.).

Two-dimensional gel electrophoresis. In vivo [^{32}P]-orthophosphoric acid-labeled proteins of A31 cells were obtained as previously described [30] following 0.5% low-serum arrest and treatment with 200 nM phorbol 12-myristate 13-acetate (PMA) in minimum essential media supplemented with 20 μM $Fe(NO_3)3^+$. Low-serum-arrested cells were labeled for 1 h, and PMA-treated cells were preequilibrated with ^{32}P for 1 h in low serum prior to treatment. Two-dimensional gel electrophoresis was performed [14] using 1.6% (pH 5–7) and 0.4% (pH 3.5–10) ampholines for isoelectric focusing in the first dimension and a 10% polyacrylamide separating gel together with a 4% stacking gel in the second dimension.

RNA preparation and analysis. The total RNA of cells was isolated by the guanidium/cesium chloride method [5]. Then, 10 μg RNA/lane was separated by electrophoresis in a 1% agarose-formaldehyde gel [9, 26] and transferred to nitrocellulose for hybridization [28]. Equal RNA loading and transfer were monitored by ethidium bromide staining. Blots were hybridized at 3×10^6 cpm/ml with a ^{32}P-labeled c-fos probe in 40% formamide, 2×SSC (1 × = 150 mmol/l NaCl, 15 mmol/l sodium citrate), 1 × Denhardt's solution, 10% dextran sulfate, 20 μg salmon-

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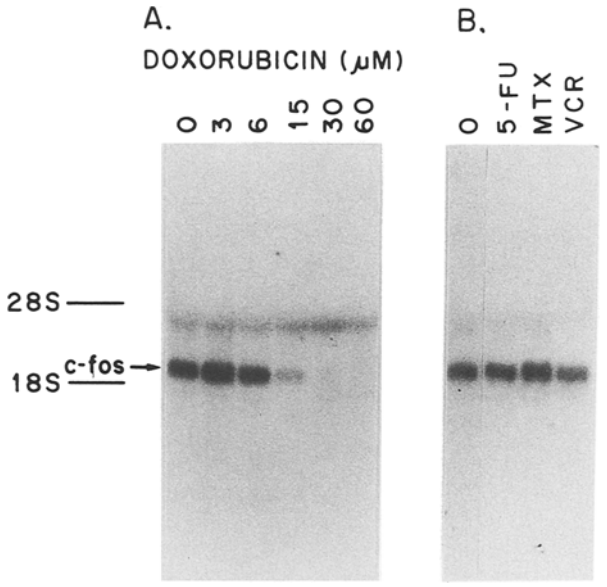

Fig. 1 A, B. Effect of doxorubicin and chemotherapeutic drugs on the expression of c-fos. **A** Different micromolar concentrations of doxorubicin. **B** 4 mM 5-fluorouracil (*5-FU*), 55 μM methotrexate (*MTX*), and 1 μM vincristine (*VCR*). Drugs were added to A31 cells for 1 h at the end of 2 days of 0.5% serum arrest. The cells were then stimulated with 200 nM PMA for 45 min to induce maximal c-fos expression. The positions of the 28S and 18S rRNA are shown on the *left*

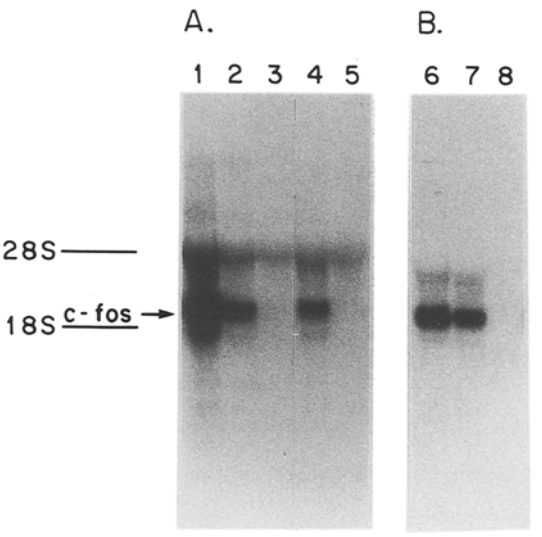

Fig. 2 A, B. Doxorubicin inhibition of c-fos expression that is not kinase-C-dependent. **A** A31 cells were placed in 0.5% serum for 2 days in the presence of nothing else (*lane 1*) and in the presence of 200 nM PMA for both days (*lanes 2, 3*) or for the 2nd day only (*lanes 4, 5*). Either no drug (*lanes 1, 2, 4*) or 60 μM doxorubicin (*lanes 3, 5*) was added for 1 h. The cells were then stimulated with 10% serum for 15 min to induced maximal c-fos expression. **B** A31 cells that had been synchronized by 2 days' incubation in 0.5% serum were treated for 1 h with 0.01% dimethylsulfoxide (DMSO; *lane 6*), 280 nM staurosporine in 0.01% DMSO (*lane 7*), or 280 nM staurosporine plus 60 μM doxorubicin (*lane 8*) and then stimulated with 10% serum for 15 min to induce maximal c-fos expression. The positions of the 28S and 18S rRNA are shown on the *left*

sperm DNA/ml, and 0.02 M TRIS (pH 7.4). Blots were washed to a final stringency of 0.1 × SSC and 0.1% sodium dodecyl sulfate at 52° C and were exposed to Kodak XAR film. Autoradiograms were quantitated by densitometry.

DNA probe. Plasmid pc-fos-3 containing a 6.8-kbp fragment of mouse c-fos [2] was obtained from the American Type Culture Collection (Rockville, Md.). The Sac I 3.5-kbp fragment was prepared by the hydroxyapatite method [23] and nick-translated [17] with alpha-[^{32}P]-deoxycytidine triphosphate (dCTP) to a specific activity of 1.5×10^8 cpm/μg.

RNA synthesis. Cells were incubated with [5-^{3}H]-uridine (27 Ci/mmol) at 2.5–5 μCi/ml DMEM. After being rinsed with phosphate-buffered saline and incubated with 10% trichloroacetic acid for 15 min at 4° C, the cells were lysed with NaOH. [^{3}H]-Uridine incorporation was quantified by scintillation counting of aliquots.

Results

Effect of doxorubicin on c-fos induction

C-fos is of particular interest because it is the first protooncogene induced (within 15 min) when kinase C is activated and quiescent mouse fibroblasts are stimulated to enter DNA synthesis [6]. Figure 1 A demonstrates that 60 μM doxorubicin completely blocked PMA-induced c-fos expression. Table 1 shows the relative expression of c-fos after doxorubicin treatment as quantified by densitometry. After the data in Table 1 had been plotted, the 50% inhibitory concentration (IC_{50}) was estimated to be approximately 11 μM. To determine whether the inhibition of c-fos expression by doxorubicin was specific to this drug rather than being a general effect of chemotherapeutic agents, the ability of methotrexate, 5-fluorouracil, and vincristine to inhibit c-fos expression was examined. Figure 1 B shows that none of these other drugs inhibited c-fos induction after PMA treatment. A concentration of 60 μM doxorubicin also completely inhibited PMA-induced c-myc expression (data not shown).

Inhibited c-fos expression is not due to specific inhibition of kinase C

Since kinase C is the putative receptor for the phorbol esters, we deemed it important to determine whether the absence of c-fos induction was attributable to the specific inhibition of kinase C by doxorubicin. Previous investigators have shown that a prolonged 24-h preincubation of cells with phorbol ester results in the depletion of kinase C and the loss of phorbol ester-induced c-fos and c-myc

Table 1. Relative densitometry of c-fos expression versus doxorubicin concentration

Doxorubicin (μM)	Relative densitometry value[a]
0	100%
3	132%
6	98%
15	30%
30	6%
60	0%

[a] Arbitrarily defined as 100% in the absence of doxorubicin

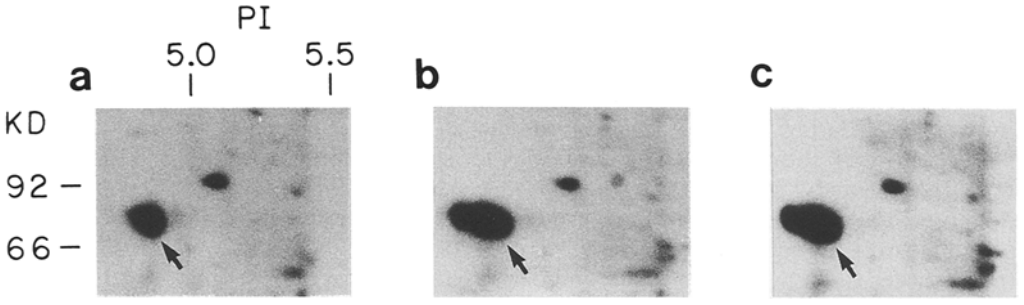

Fig. 3a–c. Effect of doxorubicin on the phosphorylation of the kinase C substrate, the MARCKS protein in A31 cells **a** at the end of 2 days of 0.5% serum arrest and after treatment with 200 nM PMA in the **b** absence or **c** presence of 60 μM doxorubicin. Portions of two-dimensional gels are shown. The *arrows* indicate the position of the MARCKS protein, which migrates anomalously at about 80 kDa

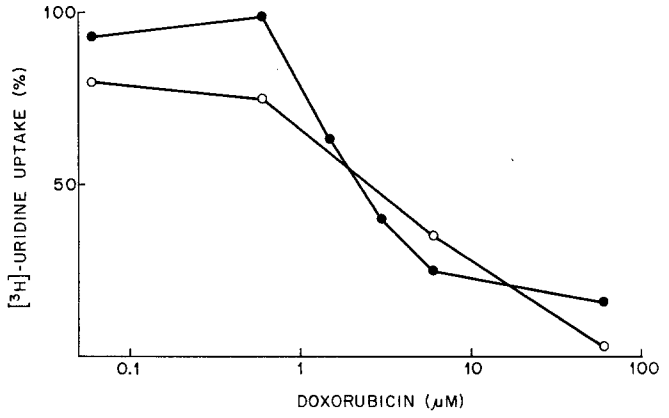

Fig. 4. Effect of doxorubicin on RNA synthesis. Exponentially growing (○) and low-serum-arrested (●) A31 cells were treated with different concentrations of doxorubicin for 1 h. [^{3}H]-Uridine was added for 75 and 45 min, respectively, and aliquots of lyzed cells were taken for scintillation counting. The samples were normalized relative to a sample that had not been treated with doxorubicin and represented 100% [^{3}H]-uridine uptake

Table 2. [^{3}H]-Uridine uptake in A31 cells in the presence of doxorubicin

Doxorubicin (μM)	[^{3}H]-Uridine uptake[a] (cpm/9.6 cm^2 cells)
Low-serum-arrested cells:	
0	3,300
0.06	3,045
0.6	3,275
1.5	2,105
3	1,325
6	830
60	525
Exponential cells:	
0	32,150
0.06	25,540
0.6	24,370
6	11,380
60	970

[a] Low-serum-arrested and exponential cells were labeled with 5 and 2.5 μCi/ml, respectively. The values shown represent triplicate and duplicate counts of a single sample, respectively

expression [1, 18]. Figure 2A demonstrates that after 24–48 h preexposure to 200 nM PMA in low serum, A31 cells expressed a 26%–27% level of serum-induced c-fos but that the induced c-fos that was not kinase-C-dependent was also completely inhibited by doxorubicin. To further test whether doxorubicin inhibited c-fos expression by specific inhibition of kinase C, A31 cells were treated with staurosporine, which is a potent inhibitor of the catalytic fragment of kinase C [18, 20], and then stimulated with serum in the presence of doxorubicin. Figure 2B shows that 280 nM staurosporine inhibited 33% of the serum-induced c-fos expression but doxorubicin inhibited the remaining 67% of the serum-induced c-fos that was not attributable to kinases. The relative values found for c-fos expression in these experiments were quantified by densitometry. Staurosporine inhibited 100% of the PMA-induced c-fos expression (data not shown). The results of these experiments suggest that doxorubicin blocks c-fos induction via a mechanism that is independent of its effect on kinase C or other kinases.

Effect of doxorubicin on phosphorylation of the MARCKS protein

The myristoylated alanine-rich kinase C substrate has been shown to be a specific indicator of kinase C activation in vivo in intact cells [19, 22, 30]. Figure 3 shows that treatment of A31 cells with 60 μM doxorubicin in the presence of 20 μM $Fe(NO_3)^{3+}$ did not block the increase in phosphorylation of the MARCKS protein that followed PMA stimulation.

Effect of doxorubicin on RNA synthesis

The results of the preceding experiments suggest that doxorubicin does not specifically inhibit kinase C and that its inhibition of c-fos expression may be attributable to its known general effect on transcription. Table 2 shows the effect of doxorubicin on RNA synthesis as determined by the uptake of [^{3}H]-uridine by A31 cells. Figure 4 shows the dose-response curve between doxorubicin and [^{3}H]-uridine uptake. The IC_{50} value for doxorubicin was approximately 3 μM for cells that had been low-serum-arrested and

stimulated with PMA for 45 min. Since the proportion of newly synthesized RNA that corresponds to c-fos after PMA stimulation of low-serum-arrested cells is not known, a dose-response curve was also determined for A31 cells in the exponential growth state, during which they do not significantly express c-fos. Figure 4 shows that a similar IC_{50} value of 2 μM was obtained. At 60 μM, doxorubicin inhibited 85% and 95% of RNA synthesis in low-serum-arrested and exponentially growing cells, respectively. Whether the difference between the IC_{50} value obtained for RNA synthesis and that obtained for c-fos expression is significant remains unknown. However, c-fos transcription does not appear to be preferentially sensitive to doxorubicin, and our data are compatible with the hypothesis that the inhibition of c-fos expression by this drug is attributable to its generalized inhibition of transcription.

Discussion

The current study was undertaken to determine whether the chemotherapeutic agent doxorubicin inhibits kinase C in vivo in cultured cells and, thus, whether such inhibition potentially represents a mechanism of action of this important drug. Our data do not support the intriguing hypothesis that the cytotoxic action of doxorubicin is attributable to its inhibition of kinase C functions in vivo.

Previous studies have documented an inhibitory effect for doxorubicin on purified kinase C in vitro [8, 15, 29, 31]. The reason for the discrepancy between our in vivo data on cultured cells and the in vitro data on purified kinase C is not clear. Previous in vitro studies have used much higher concentrations of doxorubicin, ranging from 150–1,000 μM, to establish IC_{50} values [15, 24]. We used a maximum concentration of 60 μM doxorubicin in our studies, since this dose more closely approximates the clinically achievable 5 μM level [7] and because this concentration completely blocked the expression of c-fos, which is an indicator of kinase C activation. Zhao et al. [31] found that in vitro preincubation of doxorubicin for 1 h with the kinase C activator diacylglycerol reduced the IC_{50} value to about 10 μM, indicating that the sensitivity of kinase C to doxorubicin may be enhanced under specific experimental conditions. Our data show that inhibition of RNA synthesis occurs even at concentrations lower than 10 μM, suggesting that the inhibition of transcription by doxorubicin is physiologically more significant than any inhibition of kinase C.

Hannun et al. [8] have demonstrated that only specific doxorubicin-iron complexes (but not doxorubicin alone or doxorubicin-iron mixtures) are capable of inhibiting purified kinase C and have found an IC_{50} value of about 13 μM for the (doxorubicin)-$3Fe^{+3}$ complex. These investigators report that formation of the (doxorubicin)-$3Fe^{+3}$ complex requires slow titration under acidic conditions and that the complex predominantly acts as a competitive inhibitor with respect to diacylglycerol in the regulatory region of the enzyme.

In the present study, supplementation of the culture media with 20 μM Fe^{+3} to mimic a 3:1 ratio of doxorubicin:Fe^{+3} did not result in decreased phosphorylation of the MARCKS protein, suggesting a lack of kinase C inhibition. The discrepancy between our findings and those of Hannun et al. [8] might be explained by the lack of formation of specific doxorubicin-iron complexes in the tissue-culture media or in the intact cells under our experimental conditions. Alternatively, the regulatory subunit of kinase C could have been cleaved off, leaving the catalytic subunit, which could have continued to phosphorylate the MARCKS protein. The IC_{50} value of 13 μM found for the (doxorubicin)-$3Fe^{+3}$ complex corresponds to 39 μM doxorubicin. This concentration of doxorubicin, which was necessary for the inhibition of purified kinase C in vitro, is substantially higher than the 2 μM IC_{50} value we found for the inhibition of transcription in vivo suggesting that any inhibition of kinase C may not be of physiologic or therapeutic significance.

The observation that there is a correlation between kinase C levels and resistance to doxorubicin in cells suggests a role for kinase C in doxorubicin resistance [4, 13]. However, this observation does not necessarily imply that kinase C is the target for doxorubicin, since enhanced kinase C levels may affect the resistance of cells to this drug via other mechanisms such as a change in the transport of the drug into the cell.

Although our data do not exclude the possibility of an effect of doxorubicin in vivo either in cultured cells or in an intact organism, they indicate that the inhibitory effect of this drug on transcription occurs at lower concentrations and suggest that this inhibition is physiologically more significant than any effect on kinase C.

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