Reduction of Doxorubicin Cytotoxicity by Ouabain: Correlation with Topoisomerase-induced DNA Strand Breakage in Human and Hamster Cells

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ABSTRACT

The cardiac glycoside ouabain, which is a specific inhibitor of the Na+, K+-pump, confers dramatic protection from the cytotoxic effects of doxorubicin (Adriamycin). This effect was documented in cultured A549 cells (human lung adenocarcinoma), CCL210 cells (human fibroblasts), and V79 cells (hamster fibroblasts). Maximum protection from doxorubicin cytotoxicity was achieved using 1 µM ouabain for A549 and CCL210 cells and 300 µM ouabain for V79 cells. These concentrations correlated well with the concentrations of ouabain required to induce Na+, K+-pump blockade, which was assessed using the K+ analogue 86Rb+. This suggests that protection is mediated by pump blockade. Addition of ouabain at the same time as doxorubicin was just as protective as preincubation with ouabain for an hour, demonstrating that the ouabain acts rapidly. Ouabain treatment affected neither influx nor efflux of doxorubicin. Ouabain also had no effect on verapamil-induced inhibition of doxorubicin efflux. However, ouabain partially blocked the verapamil-induced potentiation of the cytotoxic effects of doxorubicin. Therefore, ouabain does not protect by affecting intracellular doxorubicin levels. Fluorescence microscopy showed that the ability of doxorubicin to reach the nucleus was not influenced by ouabain. Alkaline elution studies demonstrated that ouabain greatly decreased doxorubicin-induced DNA strand breakage. Protection from cytotoxicity correlated closely with this decrease in strand breakage. These studies suggest that the stabilization of DNA-topoisomerase II complexes is closely linked to the mechanism of doxorubicin cytotoxicity and that this stabilization is influenced by the intracellular ionic milieu.

INTRODUCTION

Although doxorubicin (Adriamycin) is one of the most active chemotherapeutic agents, its mechanism of action remains unknown (1). Much recent investigation has focused on the effect of doxorubicin on DNA-topoisomerase II complexes. Initially, it was observed that doxorubicin caused DNA-protein cross-links that were revealed as single strand DNA breaks after treatment with proteinase K (2). It now appears that DNA-protein cross-links represent complexes of DNA and topoisomerase II that have been stabilized by doxorubicin (3, 4). Additional support for this model comes from the demonstration that the combination of isolated DNA and purified topoisomerase II exhibits this same behavior when exposed to doxorubicin and other related drugs (5, 6). Although the exact role of DNA-topoisomerase II has not yet been established in eukaryotic cells, it is likely that it plays an important role in DNA replication and transcription (7).

The intracellular ionic environment is known to play a critical role in replication and transcription (8, 9), and it is possible that this role is at least partially mediated by an effect on topoisomerasers. If this were the case, blockade of the Na+, K+-pump, which is an important regulator of the intracellular ionic composition, would affect topoisomerase activity and, potentially, doxorubicin cytotoxicity. Therefore, experiments were conducted to investigate if ouabain, a specific inhibitor of the Na+, K+-pump, affected doxorubicin cytotoxicity. It was discovered that ouabain dramatically protects cells from doxorubicin. Studies were then conducted to characterize the dose dependence and time course of protection. Since many drugs that affect doxorubicin cytotoxicity do so through alteration of intracellular drug levels (10-14), a detailed study of the effect of ouabain on doxorubicin levels was undertaken. When it was determined that ouabain had no measurable effect on intracellular doxorubicin levels, the influence of ouabain on doxorubicin-induced stabilization of DNA-topoisomerase II complexes was assessed.

MATERIALS AND METHODS

Cell Culture. Human adenocarcinoma cells (A549) and normal human lung fibroblasts (CCL210) were obtained from American Type Culture Collection, Rockville, MD. All cell lines were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air. A549 and V79 cells were cultured in RPMI medium and F-12 medium, respectively, each supplemented with 10% fetal bovine serum that was heat inactivated at 56°C for 1 h. CCL210 cells were maintained in F-12 medium with 15% fetal bovine serum that was not heat inactivated. All media were supplemented with penicillin and streptomycin. Under these conditions, the plating efficiencies of V79 cells, A549 cells, and CCL210 cells were 80-90%, 50-75%, and 10-20%, and the doubling times were approximately 9 h, 24 h, and 24 h respectively.

Drug Treatment. Ouabain and verapamil hydrochloride (Sigma) were made fresh prior to each experiment. Doxorubicin hydrochloride (obtained as a freeze-dried powder with lactose; Adria Laboratories) was made as a 500-µg/ml stock in PBS (154 mM NaCl: 1.06 mM KH2PO4: 5.6 mM Na2HPO4), aliquoted, frozen at -70°C, and diluted on the day of the experiment. Stocks were remade every 3 mo. All doxorubicin incubations were for 1 h at 37°C.

Cell Survival Assay. After drug treatment, cells were washed twice with PBS, removed from the dishes with PBS containing 0.03% trypsin and 0.27 mM EDTA, and diluted into culture dishes in numbers to yield between 20 and 200 colonies per plate. Dilutions were performed in triplicate. After 6-7 days (for V79 cells) or 12-14 days (for A549 and CCL210 cells), the plates were fixed with methanol-acetic acid, stained with crystal violet, and scored for colonies containing more than 50 cells. Doxorubicin cell survival curves from experiments using preincubations with ouabain and/or verapamil were corrected for the plating efficiency of cells that were treated with the preincubation medium alone.

Rubidium Content. Cells were incubated overnight with 0.01-0.1 µCi/ml of 86Rb+ (1 mCi/ml, 240 µg Rb+/ml; Amersham). The following day, ouabain was added in a small volume of medium into the 86Rb+-containing medium. To assay for 86Rb+ content, cells were washed rapidly 3 times with ice-cold PBS and lysed with distilled water. An aliquot was counted for Cherenkov radiation in a liquid scintillation counter.

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2 The abbreviations used are: PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; AMSA, 4'-[3-acridinylamino]methanesulfonylethylamide; CHO, Chinese hamster ovary.

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RESULTS

Doxorubicin cytotoxicity was assessed in cell lines chosen to allow comparison between human cells (A549 and CCL210) and rodent cells (V79), since the latter are far more resistant to ouabain (20). A 1-h incubation with doxorubicin caused a dosedependent decrease in the survival of clonogenic cells of all 3 lines (Fig. 1). This cytotoxicity was greatly inhibited when the cells were incubated with ouabain for 5 h beginning 1 h prior to doxorubicin exposure. In the cases of A549 and V79 cells exposed to 1.5–2.0 μg/ml of doxorubicin, ouabain caused an approximately 100-fold increase in surviving cells. For CCL210 cells, the presence of ouabain led to a 10-fold increase in cell survival.

This protective effect was dependent on the concentration of ouabain (Fig. 1). For A549 and CCL210 cells, ouabain was effective at concentrations in the submicromolar range, with a maximum effect at 1 μM. For V79 cells, the maximum protective effect of ouabain was seen at a concentration of 300 μM.

The time course of ouabain protection was examined in detail in V79 cells (Fig. 2). In experiments in which cells were preincubated with ouabain for 1 h prior to doxorubicin addition, the protective effect was present immediately after the 1-h doxo-
D to F, A549, CCL210, and V79 cells were exposed to ouabain (1 nM for A549 or concurrent with the doxorubicin exposure). This demonstrates that ouabain's protective effect had a rapid onset. Ouabain incubation was begun either 1 h prior to doxorubicin exposure, (Fig. 3). The same protective effect was seen when ouabain protective effect, the timing of the ouabain exposure was varied incubation was continued for 4 h after doxorubicin exposure. In order to further evaluate the time course of ouabain's protective effect, the ouabain were exposed to the same concentration of doxorubicin (Fig. 7). In both cases, most of the doxorubicin fluorescence was in the nucleus. Therefore, ouabain did not prevent doxorubicin toxicity. In order to rule out the possibility that ouabain exerted its protective effect by decreasing the intracellular doxorubicin level, doxorubicin influx and efflux were assessed in V79 cells under conditions and concentrations identical to those used for cell survival studies (Fig. 5). After a 1-h incubation, intracellular doxorubicin levels were a linear function of the extracellular doxorubicin concentration. These levels were completely unaffected by the presence of 1 mM ouabain, demonstrating that ouabain had no effect on doxorubicin influx. It was particularly important to determine if ouabain affected doxorubicin efflux, since increased efflux has been reported in many instances of doxorubicin resistance (12-14). As was the case for influx, efflux of doxorubicin was unaffected by 1 mM ouabain (Fig. 5). To investigate this further, studies with the Ca2+ blocker verapamil were conducted. Verapamil blocks doxorubicin efflux (Fig. 5) and greatly increased doxorubicin cytotoxicity (Fig. 6). Ouabain had no effect on the verapamil-induced blockade of doxorubicin efflux. However, increased doxorubicin cytotoxicity caused by verapamil was significantly reversed by ouabain. Therefore, the protection by ouabain from doxorubicin toxicity was not caused by a change in intracellular doxorubicin levels. The above data were all measurements of total cellular doxorubicin levels. A possible effect of ouabain could be to block doxorubicin from reaching the nucleus, which is the presumed site of action of this drug. Studies using fluorescence microscopy show that there was no detectable difference in nuclear fluorescence between ouabain-treated and control cells that were exposed to the same concentration of doxorubicin (Fig. 7). In both cases, most of the doxorubicin fluorescence was in the nucleus. Therefore, ouabain did not prevent doxorubicin from reaching its site of action in the cell nucleus. These data indicated that ouabain, by altering the intracellular environment, inhibited doxorubicin that was present inside the cell nucleus from becoming cytotoxic. This suggested that ouabain acted by interfering with the interactions between doxorubicin and DNA. In fact, ouabain greatly inhibited doxorubicin-induced formation of DNA single-strand breaks in V79 cells (Fig. 8). Eighty-three % (±4%, n = 4) of the strand breaks induced by 1.5 μg/ml of doxorubicin was inhibited by preincubation with 1 mM ouabain for an hour. Nearly identical elution
Fig. 5. Ouabain has no effect on intracellular doxorubicin levels. In A, V79 cells were preincubated for 1 h with 1 mM ouabain (●), 10 μM verapamil (▲), both ouabain and verapamil (△), or under control conditions (□) prior to the addition of 1 μg/ml of doxorubicin for 1 h. Cultures were then rinsed twice with PBS and processed immediately for intracellular doxorubicin content (time = 0) or returned to medium containing the preincubation drugs and assessed at varying times after drug exposure. In B, V79 cells were preincubated for 1 h with 1 mM ouabain or under control conditions prior to the addition of varying concentrations of doxorubicin. After 1 h, cultures were assessed for doxorubicin content.

Fig. 6. Ouabain partially reverses verapamil potentiation of doxorubicin cytotoxicity. V79 cells were preincubated with 1 mM ouabain (●), 10 μM verapamil (▲), both verapamil and ouabain (△), or under control conditions (□). Cells were then exposed to varying concentrations of doxorubicin for 1 h, rinsed twice with PBS, and returned to medium containing preincubation drugs. Cultures were assessed for cell survival 4 h later.

Fig. 7. Ouabain does not affect intranuclear doxorubicin. V79 cells were preincubated for 1 h under control conditions (A and C) or with 1 mM ouabain (B and D). They were then exposed to 0.5 μg/ml (A and B) or 1.5 μg/ml (C and D) of doxorubicin for 1 h and photographed using fluorescence optics as described in “Materials and Methods.”

To better explore this possibility, the data from multiple elutions were combined and normalized as described in “Materials and Methods” to allow comparison with cell survival throughout the range of doxorubicin concentrations used (Fig. 9). Ouabain prevented the formation of strand breaks at both low (0.5 μg/ml) and high (1.5 μg/ml) concentrations of doxorubicin. Cell survival data from Fig. 1 were overlaid on the elution data to facilitate direct comparison. A close association between doxorubicin-induced DNA strand breaks and cell lethality was evident.

DISCUSSION

These results demonstrate that ouabain, a specific inhibitor of the Na+, K+-pump, confers protection from doxorubicin cytotoxicity. This protective effect occurs at the same concentrations of ouabain that result in pump blockade and decrease in intracellular K+ (as assessed by the K+ analogue 86Rb+). This correlation between pump inhibition and protection is clearly demonstrated through comparison of a rodent cell line (V79) and human cell lines (A549 and CCL210). The Na+, K+-pump of rodent cells is approximately 300-fold more resistant to ouabain than other mammalian cells (20), and this difference is quantitatively reflected in the present study by the higher concentrations of ouabain needed to protect V79 cells, compared to A549 or CCL210 cells, from doxorubicin cytotoxicity.

Studies were conducted to elucidate the mechanism by which blockade of the Na+, K+-pump leads to doxorubicin protection.
confirmed these findings and showed that ouabain partially potentiates doxorubicin toxicity through inhibition of Ca2+-dependent doxorubicin efflux (12-14). The present study shows more single-strand breaks after doxorubicin exposure (26). In the present study, direct evidence is presented that doxorubicin cytotoxicity correlates with the induction of single-strand breaks. In the presence of ouabain, both cytotoxicity and strand breakage were decreased dramatically and remained tightly correlated.

The data also demonstrate a critical window beginning at the time of exposure and extending to approximately 1 h after exposure during which doxorubicin damage becomes fixed. Ouabain treatment after this time did not confer any protection from cytotoxicity. It will be of interest to determine if the DNA-topoisomerase II complexes that have been shown to exist many hours after doxorubicin exposure (25) are affected by ouabain at a time when cytotoxicity is not. For instance, if ouabain caused complexes to dissociate under these conditions, it would suggest that the doxorubicin-induced lethal evident occurs within the first hour after exposure and that cell death does not depend on the continued presence of the stabilized complex. Such experiments should better elucidate the link between the formation of DNA-topoisomerase II complexes and cytotoxicity.

The mechanism by which ouabain blocks doxorubicin-mediated stabilization of DNA-topoisomerase II complexes is uncertain. The rapid onset of protection correlates best with the rapid decrease in intracellular K+ (as assessed by 86Rb+) that occurs when ouabain inhibits the Na+, K+-pump. This would suggest that doxorubicin-induced stabilization of DNA-topoisomerase II complexes depends directly on the concentrations of intracellular K+ and Na+. Another possibility is that the ouabain-induced increase in intracellular Na+ triggers the Na+, Ca2+ exchange pump that exists in many cells (27). This would cause an increase in intracellular Ca2+ levels. Indeed, ouabain's ability to reverse the acute negative ionotropic effect of doxorubicin on isolated cardiac muscle has been attributed to this mechanism (28). It is conceivable that an elevation of intracellular Ca2+ could also affect doxorubicin-induced DNA-topoisomerase II stabilization. However, it should be noted that the activity of purified topoisomerase II is critically dependent on the concentration of Mg2+ and is independent of Ca2+ (29). In addition, if Ca2+ were the mediator of ouabain's protective effect, one would expect an interaction between the effects of ouabain and the Ca2+ blocker verapamil on doxorubicin cytotoxicity. However, the present study reveals no such interaction.

Ouabain, through its effect on intracellular K+, does have additional intracellular effects, such as inhibition of protein and inhibits the potentialization of cytotoxicity without affecting the verapamil-induced increase in intracellular doxorubicin. Therefore, ouabain does not appear to protect cells from doxorubicin cytotoxicity by affecting doxorubicin influx, efflux, or access to DNA.

It, therefore, appeared likely that ouabain interfered with the ability of doxorubicin to interact with DNA. As described in the “Introduction,” doxorubicin stabilizes DNA-topoisomerase II complexes, which are detected as single- and double-strand DNA breaks. Indirect evidence has suggested that these intercalator-induced strand breaks are related to cell cytotoxicity. For instance, m-AMSA, which causes more single-strand breaks than its analogue o-AMSA, is also more cytotoxic than o-AMSA (25). However, other examples exist in which drugs that cause a similar degree of strand breakage have very different effects on cell survival (4). Stronger support for a direct correlation between cell survival and strand breaks comes from studies of CHO mutants which are more sensitive to doxorubicin cytotoxicity than wild-type CHO cells. These mutants also show more single-strand breaks after doxorubicin exposure (26). The mechanism by which ouabain blocks doxorubicin-mediated stabilization of DNA-topoisomerase II complexes is uncertain. The rapid onset of protection correlates best with the rapid decrease in intracellular K+ (as assessed by 86Rb+) that occurs when ouabain inhibits the Na+, K+-pump. This would suggest that doxorubicin-induced stabilization of DNA-topoisomerase II complexes depends directly on the concentrations of intracellular K+ and Na+. Another possibility is that the ouabain-induced increase in intracellular Na+ triggers the Na+, Ca2+ exchange pump that exists in many cells (27). This would cause an increase in intracellular Ca2+ levels. Indeed, ouabain's ability to reverse the acute negative ionotropic effect of doxorubicin on isolated cardiac muscle has been attributed to this mechanism (28). It is conceivable that an elevation of intracellular Ca2+ could also affect doxorubicin-induced DNA-topoisomerase II stabilization. However, it should be noted that the activity of purified topoisomerase II is critically dependent on the concentration of Mg2+ and is independent of Ca2+ (29). In addition, if Ca2+ were the mediator of ouabain's protective effect, one would expect an interaction between the effects of ouabain and the Ca2+ blocker verapamil on doxorubicin cytotoxicity. However, the present study reveals no such interaction.

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DNA synthesis. However, these effects do not occur until hours after exposure (30–32). This slower time course does not appear to be rapid enough to account for the finding that, when ouabain is added simultaneously with doxorubicin, complete protection occurs.

The current study suggests that it may be possible to influence the efficacy of doxorubicin chemotherapy through manipulation of the Na⁺, K⁺-pump. Pump activity has been shown, in many cases, to be dramatically different in tumor or transformed cells compared to their normal counterparts (33, 34). Although in the current study ouabain protected both tumor (A549) and normal (CCL210 and V79) cells from doxorubicin, it remains possible that differences in Na⁺, K⁺-pump activity between tumor and normal cells could be exploited to improve the therapeutic index of doxorubicin chemotherapy. It will clearly be important to determine if ouabain-induced protection from doxorubicin occurs in other normal and tumor cell lines, and in settings other than cell culture.

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