Up-regulation of caveolin expression by cytotoxic agents in drug-sensitive cancer cells

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Caveolin 1 expression is down-regulated in various cancer cell lines. Interestingly, in several drug-resistant cancer cells, a strong induction of caveolin 1 expression has been reported, suggesting a role for caveolin 1 in the acquisition and/or the maintenance of the multidrug-resistance phenotype. Here, we show, in drug-sensitive lung cancer cells (A549, Calu-6 or NCI-H69), that exposure to cytotoxic drugs (taxol, doxorubicin or etoposide) is sufficient to strongly up-regulate caveolin 1 and 2 protein levels. This up-regulation is sustained even 1 week after drug removal. Our results suggest that caveolin up-regulation is an early cellular response to a cytotoxic stress taking place before drug resistance. *Anti-Cancer Drugs* 14:281–287 © 2003 Lippincott Williams & Wilkins.

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Introduction

Up-regulation of caveolin 1 gene expression has been demonstrated in a number of drug-resistant carcinoma cell lines [1,2]. Caveolins, a family of membrane protein, are the main structural protein component of caveolae [3,4]. Caveolins have been suggested to act as scaffolding proteins in order to concentrate and regulate the activity of several signaling molecules [3–6]. Three different genes encode for caveolins. Caveolin 1 and 2 gene products are mainly expressed in adipocytes, endothelial and epithelial cells, as well as in fibroblasts [3]. Caveolin 3 is present exclusively in muscle cells.

Caveolin 1 has been identified as one of 26 candidate tumor suppressor genes that are down-regulated in breast cancer [7]. This observation is supported by the demonstration of caveolin 1 down-regulation in several breast and lung carcinoma cell lines [8,9]. Moreover, caveolin 1 expression levels are dependent upon the level of activity of p53. Caveolin 1 gene expression is shut down in p53-null cells, while it is up-regulated in response to the activation of p53 [9,10].

A somewhat opposite view of the role of caveolin in cancer is suggested from the observation of high levels of caveolin 1 in metastatic tissues from human prostate and breast cancers [11–14]. Caveolin 1 expression is also associated with androgen insensitivity of prostate cancer cells, although the mechanism of action of caveolin in this situation is still unclear [11–13,15,16].

Recently, it was reported in two studies that caveolin 1 levels were dramatically up-regulated in several multidrug-resistant (MDR) cell lines compared to their drug-sensitive counterparts [1,2]. In addition, P-glycoprotein (P-gp) was found to be enriched in caveolin-rich membrane domains after fractionation on a sucrose density gradient [1,17]. This caveolin 1 induction was also reported in drug-resistant cell lines expressing low levels of P-gp, suggesting that caveolin 1 increase was not necessarily correlated with P-gp expression [2].

In this study, we have investigated the effect of various cytotoxic drugs on caveolin 1 and 2 expression in drug-sensitive lung carcinoma cell lines. We now report that up-regulation of caveolin content is an early cellular response to an exposure to a cytotoxic stress.

Material and methods

Materials

Anti-caveolin 1 mouse IgG, anti-caveolin 1 rabbit IgG and anti-caveolin-2 mouse IgG were from Transduction Laboratories (Lexington, KY). Paclitaxel, doxorubicin and etoposide were purchased from Sigma (Oakville, Ontario, Canada).

Cell culture

Cell lines used in this report were obtained from the ATCC (Manassas, VA) and cultured as suggested by the supplier.

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Immunoblotting

Cultured cells were collected using trypsin–EDTA, counted and pelleted by centrifugation. The pellet was then resuspended in Laemmli sample buffer, boiled for 5 min and analyzed by SDS–PAGE using caveolin 1 or 2 antibodies for Western blotting. Volumes of samples loaded on gel were corrected for the number of cells. Quantification of bands by densitometry was made using Scion Image software (Fredericks, MD).

RNA isolation and Northern analysis

RNA was isolated from cultured cells using TRIzol reagent (Life Technologies, Burlington, Ontario, Canada) following the supplier's protocol. Total RNA (5 μg) was separated on a formaldehyde–agarose gel and then transferred to a nylon membrane. A full-length caveolin 1 cDNA was labeled using the BrightStar Psoralen-Biotin kit and the detection was performed using the BrightStar Biodetect kit, both from Ambion (Austin, TX). The membrane was exposed to Kodak BioMax LS film at room temperature for 1–4 h.

Cell cycle analysis

Cells were collected, counted and pelleted by centrifugation. Cells were then washed twice with phosphate-buffered saline without Ca^{2+} and Mg^{2+}, and fixed in 70% ethanol at $-20^{\circ}C$ for 24 h. Cells were stained in a propidium iodide solution (200 U of DNase-free RNase and 50 mg/ml of propidium iodide in water) for 30 min at room temperature and then put at 4°C for at least 48 h before flow cytometric analysis. Analysis was performed on a Coulter Elite flow cytometer (Becton Dickinson, Fullerton, CA). Ten thousand events were recorded for each sample.

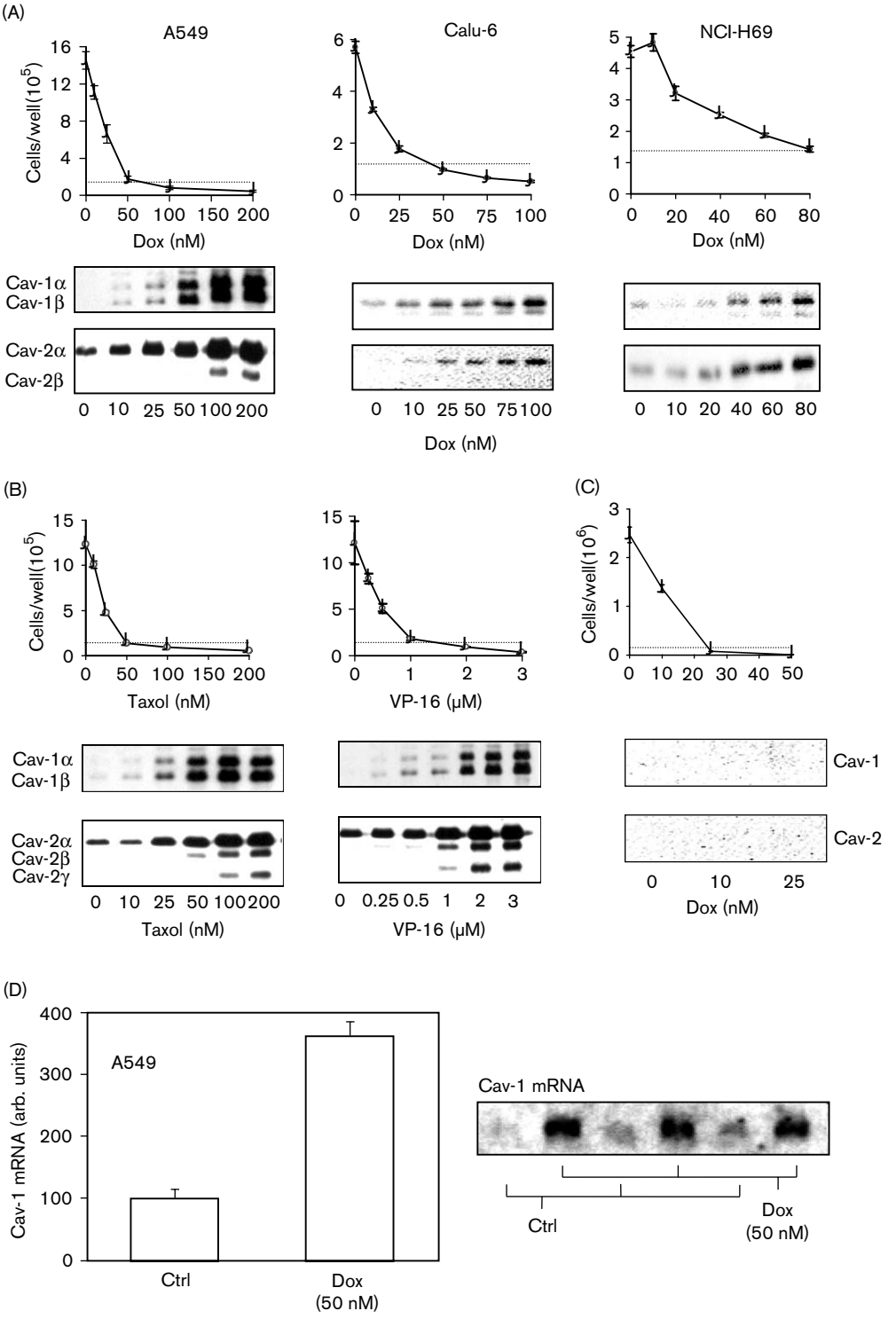
Results and discussion

Exposure to cytotoxic drugs is sufficient to increase caveolin 1 and 2 protein levels in various lung carcinoma cell lines. In this study, A549 and Calu-6 cells (from lung adenocarcinomas) and NIH H69 cells (from a small cell lung cancer) were used. We had previously demonstrated that these cell lines had low levels of caveolin 1 expression compared to normal lung epithelial and alveolar cells [8]. As illustrated in Figure 1(A), exposure for 4 days to increasing amounts of doxorubicin caused a dose-dependent decrease in cellular proliferation. Simultaneously, caveolin 1 and 2 levels were markedly up-regulated. Cytotoxic drugs having other mechanisms of action (VP-16 and taxol) gave similar results, as illustrated in Figure 1(B). A549 cells exposure for 4 days to increasing concentrations of paclitaxel (taxol) or etoposide (VP-16) was accompanied with caveolin 1 and 2 up-regulation of protein levels. A similar trend was observed with Calu-6 and NIH H69 cell lines (not shown). However, cells not expressing endogenous caveolins such as U937 cells (a monocyte-derived cell line) had no detectable caveolin 1 or 2 after doxorubicin treatment (Fig. 1C), suggesting that the cell origin is a factor in the regulation of caveolin levels. Recently, it was reported that taxol at low doses (10 nM) could increase caveolin 1 levels in A549 cells [2]. This effect was observed after 2 days of treatment. Here we show that this effect is not specific to taxol since other types of cytotoxic drugs can similarly up-regulate caveolin 1 expression. Caveolin 2 is not believed to be down-regulated in these cancer cell lines [8]. However, cytotoxic drugs had similar effects on caveolin 2 compared to caveolin 1. This effect of cytotoxic drugs on caveolin 1 and 2 expression became apparent after 24 h of treatment (not shown). Caveolin 1 mRNA is also up-regulated by cytotoxic drugs. As illustrated in Figure 1(D), A549 cells treated with doxorubicin (50 nM) for 48 h exhibit a 3.7-fold increase in caveolin 1 mRNA levels, suggesting a regulation of caveolin cellular content at least in part at the transcription level.

We were then interested to see if these caveolin 1 and 2 levels returned to baseline after drug removal. A549 cells were cultured in the presence of a growth-inhibitory (cytostatic) dose of doxorubicin (50 nM) for 4 days and then put back in normal culture conditions. After 4 days of culture, total cell number was unchanged compared to day 0 (Fig. 2A). Cell counts remained unchanged for at least 3 days after drug removal. After the 4-day interval of drug exposure, caveolin 1 and 2 cell contents were increased by 10- and 15-fold, respectively, compared to those of day 0 (Fig. 2B and C). Three days after drug removal, a slight increase in caveolin 1 levels was observed while caveolin 2 levels doubled as illustrated in Figure 2(B and C). We let cells culture for 6 more days until cell proliferation was readily observable. By microscopic observation, we noted that only a small proportion (less than 5%) of adhering cells resumed their proliferative activity. Even then, caveolin 1 and 2 levels were still clearly elevated compared to those observed at day 0 (7 and 17 times of those of day 0, respectively). We observed such a pattern with Calu-6 cells and with VP-16 in A549 cells (not shown). We are now working to determine which cell population between proliferating and non-proliferating ones are responsible for these lasting elevated caveolin 1 and 2 levels. These results suggested that elevated caveolin content in drug-resistant cells could be induced from the start of the selection, thus well before objective drug-resistance can be measured.

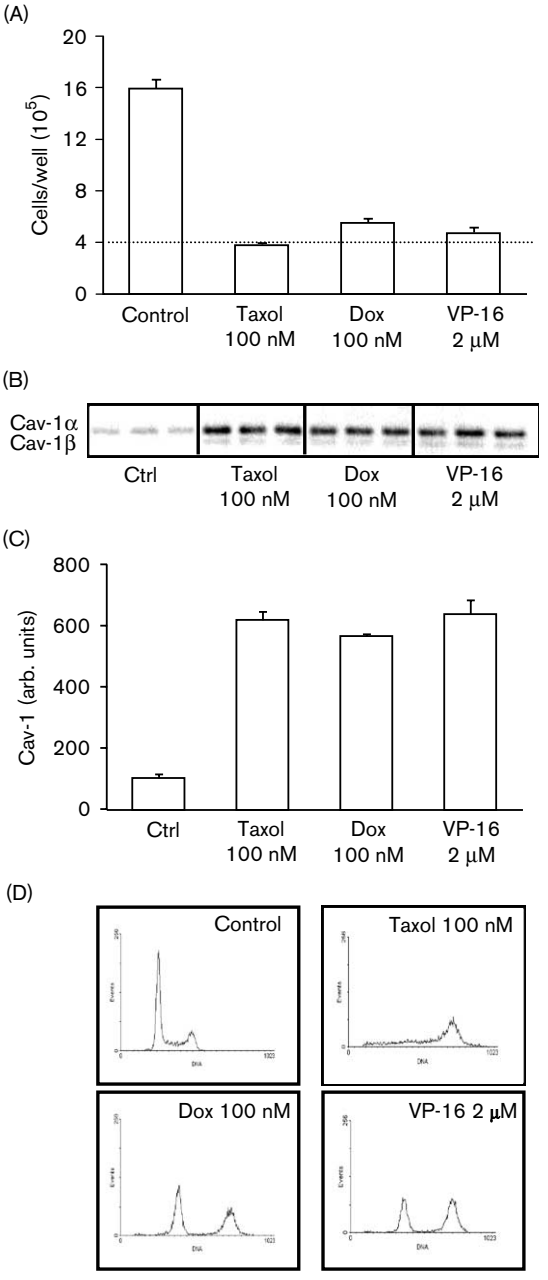
Caveolin 1 protein content is regulated throughout the cell cycle as has been demonstrated by others [9,18]. Since cytotoxic drugs have major effects on the cell cycle, we were interested to correlate this parameter with the caveolin expression up-regulation we observed. In synchronized A549 lung adenocarcinoma cells as well as in

Fig. 1



Cytotoxic drugs regulate caveolin 1 and 2 protein expression in drug-sensitive lung carcinoma cell lines. (A) Doxorubicin inhibits caveolin 1 and 2 expression in A549, Calu-6 and NCI-H69 cells. Cells were treated with increasing doses of doxorubicin (Dox) for 4 days. Cells (125 000/well) were plated on day 0 in triplicate. Cells were trypsinized and counted on a hemocytometer. Volumes of crude cell homogenates corresponding to 30 000 cells were separated by SDS–PAGE and Western blot analysis was performed using caveolin 1 or 2 monoclonal antibodies. (B) Paclitaxel (taxol) and etoposide (VP-16) increase caveolin 1 and 2 levels in A549 cells. Cells were treated with increasing doses of the indicated drug and processed as described above. (C) Doxorubicin does not stimulate caveolin expression in cells having no endogenous caveolin expression. U937 cells were treated as described in (A). Cell counts in (A)–(C) are expressed in cells/well ± SEM ($n=3$). The dotted line indicates the number of cells plated at day 0. (D) Caveolin 1 gene expression is up-regulated by doxorubicin. A549 cells were treated or not (Ctrl) for 2 days with doxorubicin (50 nM) and total RNA extracted for Northern blotting analysis.

Fig. 2



Caveolin 1 and 2 protein level normalization after exposure of A549 cells to doxorubicin. A549 were incubated for 4 days with doxorubicin (50 nM). Treatment was stopped and cells were cultured in normal medium for 9 additional days. (A) Cellular proliferation. Cells were counted from three wells for each time and results are expressed in cells/well ± SEM. The horizontal dotted line indicates the number of cells plated at day 0. The vertical dotted line shows when the treatment has been stopped. (B) Caveolin 1 protein levels during and after treatment with 50 nM doxorubicin. Densitometric measurements of caveolin 1 protein bands on Western analysis are expressed in arbitrary units ± SEM ($n=3$). (C) Caveolin 2 expression during and after treatment with 50 nM doxorubicin. Densitometric measurements of caveolin 2 protein bands on Western analysis are expressed in arbitrary units ± SEM ($n=3$).

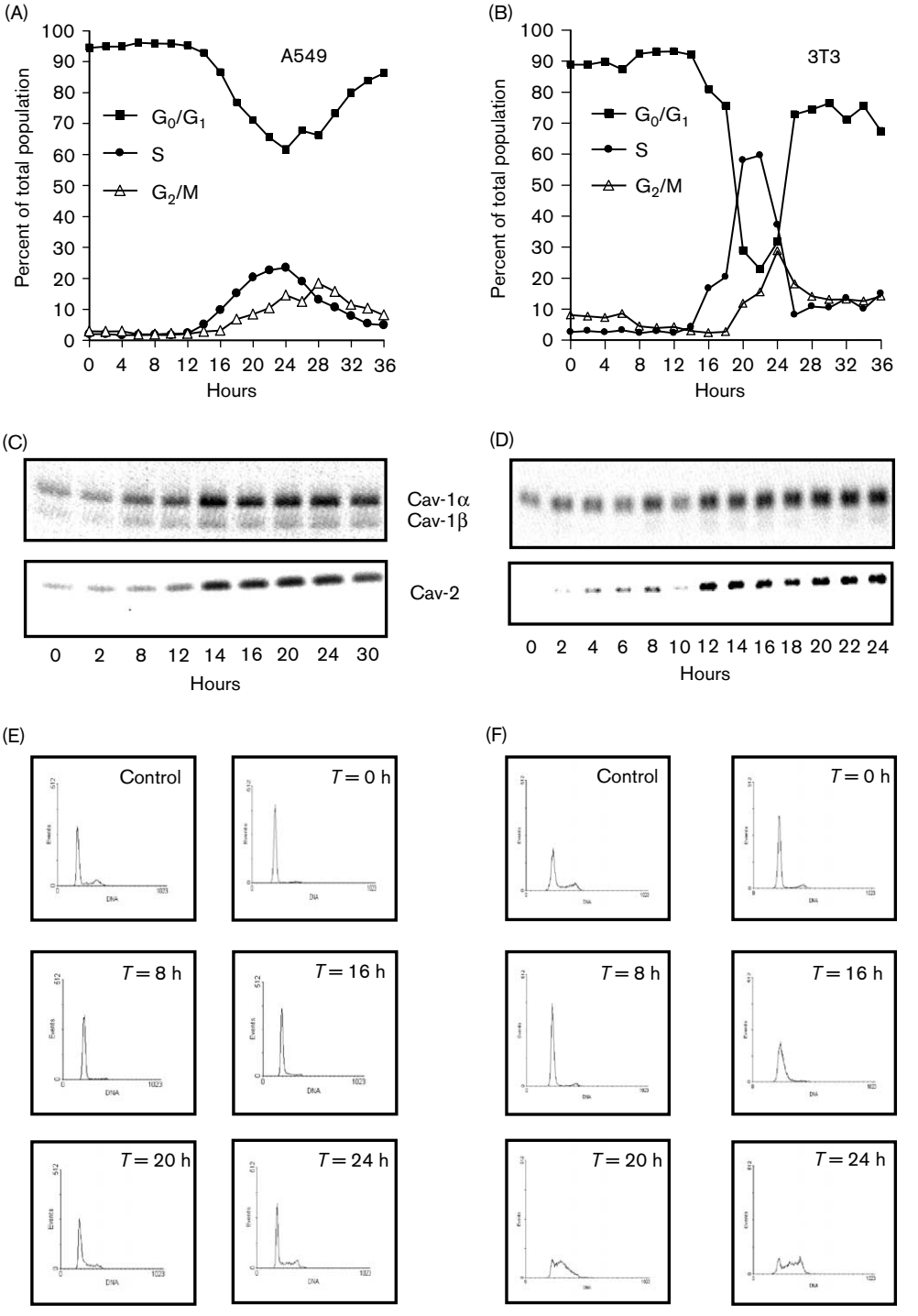
NIH-3T3 fibroblasts, re-entry of cells in the S phase is accompanied by an increase of caveolin 1 and 2 levels (Fig. 3). In fact, this increase probably happens in the late G_1 phase. Caveolin expression is relatively stable thereafter, even after 36 h when cell population distribution in the different cycle phases was similar to normally proliferating cells. Caveolin expression is therefore probably low during the G_0 and G_1 phases. These results seem in contradiction with the observation made by others that caveolin 1 expression is lower in proliferating cells in culture compared to confluent cells [19]. The amplitude of changes in caveolin protein content throughout the cell cycle we have recorded are seemingly less than those observed by Galbiati *et al.* between proliferating and confluent 3T3 fibroblasts, probably reflecting two different mechanisms of caveolin 1 expression control. Although it is clear that caveolin levels are controlled during the cell cycle, we do not know if the number of caveolae at the cell surface follows this trend.

A549 cells treated for 2 days with cytostatic doses of taxol, doxorubicin or VP-16 all show a blockade in the G_2/M phase of the cell cycle (Fig. 4). A blockade in the late G_1 phase is also observed after treatment with doxorubicin or VP-16. DNA synthesis was clearly abrogated in doxorubicin- and VP-16-treated cells. In the case of taxol, cells undergoing apoptosis were detected by their DNA content $< 2n$. Caveolin 1 (Fig. 4B and C) and 2 (not shown) protein levels were clearly up-regulated in the presence of the various cytotoxic drugs used. In all cases, the proportion of cells in the G_2 phase was higher than in untreated cells, i.e. in cell cycle phase associated with higher caveolin 1 levels. However, since lower caveolin 1 and 2 levels are detected in normally cycling and synchronized cells after 1 day of serum addition compared to cells treated with cytotoxic drugs, the mechanisms of caveolin up-regulation observed in the presence of cytotoxic drugs remains unclear.

Cytotoxic drugs are well-known activators of p53, which in turn is known to up-regulate caveolin 1 expression when activated. In p53-null keratinocytes, caveolin 1 levels were shown to be strongly down-regulated [9], while p53 activation was shown to increase the expression of a number of genes including caveolin 1 [10]. Although, activation of p53 by cytotoxic drugs could explain the up-regulation of caveolin 1 and 2 levels in cells having a wild-type p53 gene such as the A549 cell line, in cell lines such as Calu-6 [19] and NCI-H69 [20], both having mutated p53 genes, other mechanisms have to be considered.

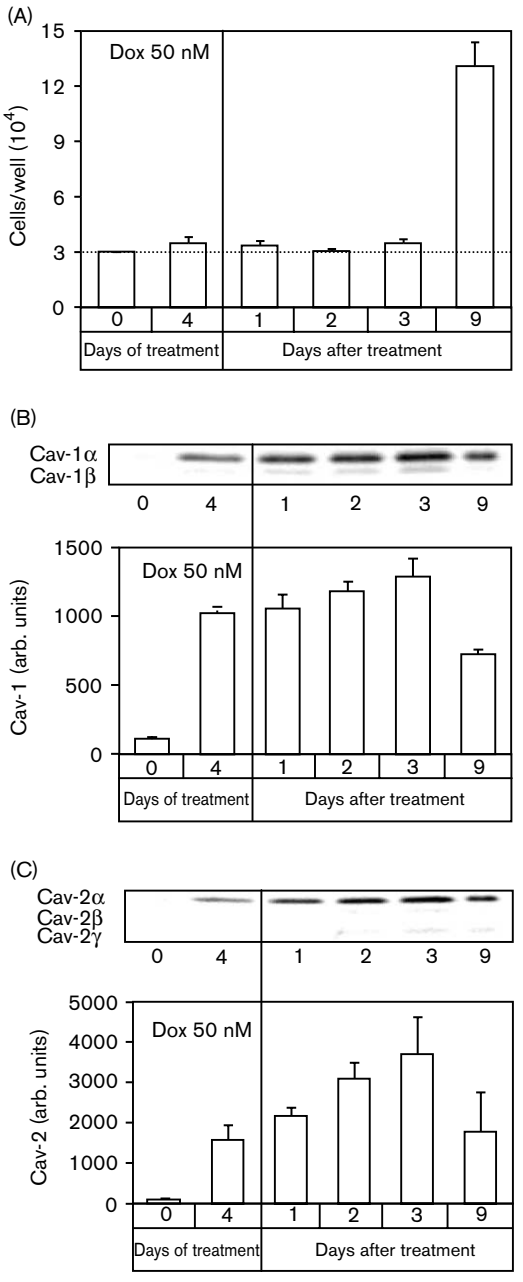
The state of activation of the *ras*–mitogen-activated protein kinase (MAPK) signaling pathways is well-documented for its control of caveolin 1 levels [21–23]. Activation of the p42/44 MAPK pathway has been shown

Fig. 3



Caveolin 1 and 2 modulation throughout the cell cycle in synchronized A549 and NIH-3T3 cells. (A, C and E) A549 cells were cultured in serum-deprived medium for 48 h in six-well culture dishes. Cells were then cultured in complete medium ($T=0$). Cells were collected (one culture well) every 2 h for 36 h for analysis of caveolin 1 and 2 protein content by Western blotting (C) and cell cycle analysis (A and E) as described in legend of Figures 1 and 2, and in Materials and methods. (B, D and F) NIH 3T3 fibroblasts were incubated in serum-deprived medium for 24 h and re-exposed to fetal bovine serum afterwards for the time indicated. Analysis was as for A549 cells.

Fig. 4



Cytotoxic drug stimulation of caveolin 1 and 2 expression is associated with an accumulation of cells in the G_2/M phase of the cell cycle. (A) A549 cells were treated with growth inhibitory doses of taxol, doxorubicin and VP-16 for 48 h ($n=3$). Dotted line indicates the number of cells plated at day 0 in six-well culture dishes. Error bars represent the SEM. (B) Caveolin 1 levels are increased after 48 h by cytotoxic drugs as demonstrated by Western blot analysis. (C) Densitometric quantification of bands illustrated in (B). Results are expressed in arbitrary units. (D) Cell cycle analysis of A549 after exposure for 48 h to cytotoxic drugs. Note that the calibration gates were different for the control cell analysis.

to down-regulate caveolin 1 gene expression [21,22]. On the other hand, activation of the p38 MAPK pathway during two differentiation processes, i.e. conversion of 3T3-L1 formation in adipocytes [24] and myotube formation in C2C12 cells [25], has been associated with caveolin 1 and 3 induction of gene expression, respectively. Interestingly, activation of the p38 signaling pathway has been reported in cells treated with various cytotoxic drugs [26,27]. Finally, activation by many chemotherapeutic drugs of the c-jun N-terminal protein kinase has been reported [28] but, in this case, it is not known if this MAPK family member is implicated in the regulation of caveolin expression. We are now investigating if these signaling pathways are implicated or not in this caveolin 1 and 2 induction by cytotoxic drugs in lung cancer cells.

In summary, cell exposure to cytotoxic drugs strongly increases caveolin 1 and 2 protein levels in lung cancer cell lines. This effect is regulated, at least in part, at the expression level. Although, caveolin 1 and 2 levels are modulated during the cell cycle, the magnitude of the up-regulation in caveolin levels observed after cell exposure to cytotoxic drugs is much larger, suggesting a different mechanism of regulation.

In conclusion, increases in caveolin 1 and 2 expression reported in several drug-resistant cell lines could possibly reflect exposure to cytotoxic drugs instead of being a step in the development of drug resistance. It remains to be determined if high levels of caveolin have a specific role *per se* in the MDR phenotype.

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