Comparative analysis of stress responses of H9c2 rat cardiomyoblasts following treatment with doxorubicin and tBOOH

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ABSTRACT

Cardiotoxicity is the major dose-limiting adverse effect of anthracyclines and is hypothesized to result from damage induced by reactive oxygen species (ROS) or inhibition of topoisomerase II. Here, we comparatively analyzed the effect of doxorubicin and the organic peroxide tertiary-butylhydroperoxide (tBOOH) on stress responses of rat cardiomyoblast cells (H9c2). Moreover, we investigated the impact of serum factors and the novel prototypical protein kinase CK2 inhibitor resorufin on the sensitivity of H9c2 cells exposed to doxorubicin or tBOOH. Measuring cell viability by use of the WST assay as well as cell cycle progression and apoptotic death by FACS-based methods, we found that the sensitivity of H9c2 cells to doxorubicin and tBOOH was differently affected by both serum factors and resorufin. Formation of reactive oxygen species was observed after exposure of H9c2 cells to high doses (i.e. ≥ 5 μM) of doxorubicin only. Moreover, the antioxidant N-acetylcysteine protected H9c2 cells from cytotoxicity provoked by tBOOH but not doxorubicin. Analyzing the phosphorylation level of genotoxic stress responsive protein kinases and histone H2AX, which is indicative of an activated DNA damage response (DDR), we found that resorufin modulates doxorubicin- and tBOOH-induced responses in an agent specific manner. Taken together, the data indicate that (i) oxidative injury is not the most relevant type of damage triggering cell death of H9c2 cells following doxorubicin treatment, (ii) serum factors differently influence the sensitivity of cardiomyoblasts to doxorubicin and tBOOH and (iii) inhibition of CK2 unequally affects doxorubicin- and tBOOH-induced DDR of rat cardiomyoblasts.

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Introduction

Normal tissue damage limits the applicability of anticancer drugs. The clinically most relevant dose-limiting adverse effect of anthracyclines, which are widely used anticancer drugs, is irreversible cardiotoxicity [1-3]. The anti-tumor efficacy of anthracyclines is believed to be mainly due to the poisoning of type II topoisomerases (topo II), which unwind supercoiled DNA during replication and transcription [4-6]. Anthracyclines interrupt the breakage–religation reaction of topo II thereby leading to the accumulation of a
topo II–DNA covalent intermediate, designated as cleavable complex [7]. Apart from inhibition of topo II isoforms (i.e. topo IIα and topo IIβ), exposure to anthracycline results in the formation of reactive oxygen species (ROS) [1]. Formation of ROS, which is triggered by the redox-cycling of anthracyclines, can be blocked by the iron chelator ICRF-187 (dextrazoxane), which is structurally related to EDTA [8]. Since dextrazoxane reduces the risk of anthracycline-induced cardiac damage [9], it was speculated that its cardioprotective potency rests on the prevention of ROS formation in the heart [8]. However, apart from iron chelation, ICRF-187 also acts as a catalytic inhibitor of topoisomerase II [10]. Moreover, it turned out that dextrazoxane triggers the proteosomal degradation of topo IIα, which is a major target of anthracyclines [6]. In addition, topo IIβ mediated generation of DNA double-strand breaks (DSB) has also been suggested to be relevant for the cardiotoxicity of doxorubicin and prevention by dextrazoxane [2]. Thus, apart from lowering ROS formation following doxorubicin exposure, the cardioprotective capacity of dextrazoxane might also be due to its favourable effects on topoisomerase II isoforms. In line with this, the cytoprotective effect of the lipid lowering drug lovastatin, which was observed in human endothelial cells and rat cardiomyoblasts, was related to an attenuated inhibition of topo II and subsequent decrease in DSB rather than to a reduced production of ROS [11,12]. Hence, although ROS are frequently discussed as major mechanism contributing to anthracycline-induced cardiotoxicity [13], the relevance of oxidative damage for anthracycline-induced tumor cell kill and normal tissue damage (i.e. acute and chronic cardiotoxicity) is still under debate [3,14].

Protein kinase CK2 is an ubiquitously expressed serine/threonine kinase, which phosphorylates numerous proteins involved in proliferation and cell survival [15]. CK2 exists as a heterotetrameric holoenzyme and is constitutively active [16]. It is thought to play a key role in tumorigenesis and apoptosis via regulation of tumor suppressor genes, such as p53, and cell cycle regulatory factors [17]. In addition, CK2 is believed to influence the DNA damage response (DDR) and DNA repair since it (i) phosphorylates MDC1 thereby affecting its interaction with apratxin [18], (ii) modulates the activity of the MRN (Mre11/Rad51/NBS) complex [19,20], (iii) regulates XRCC1 [21–23], (iv) accelerates the removal of H2AX foci after ionizing radiation [24] and (v) regulates chromatin changes, which initiate the DDR [25]. Taken together, these data emphasize the physiological relevance of CK2 in cellular response to genotoxic insult, making this kinase an interesting target for pharmaceutical intervention. To this end, CK2 inhibitory compounds, such as 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) or tetrabromobenzodiazole (TBB), have been developed [26,27] and found to induce apoptosis, partially via formation of ROS or DNA double-strand breaks (DSBs) [28]. Recently, it turned out that DMAT and TBB are not highly selective for CK2. Rather, both compounds significantly inhibit PI3-kinases and DNA-PKcs [29]. This is an important concern against their use when analyzing the involvement of CK2 in the DDR, since PI3-kinase related kinases, such as ATM and DNA-PKcs, are key players in the regulation of the DDR and DNA repair, respectively [30]. Previously, resorufin was identified as a novel, highly selective and hence prototypical CK2 inhibitor [31]. The NCi 60 cell one-dose screen for NSC12097 (i.e. resorufin) revealed a large tumor cell-type specificity, with a particular high inhibitory potency observed in melanoma cells. In line with this, the extent of CK2 inhibition mediated by resorufin was also found to be cell line specific [31].

Here, we comparatively analyzed the cytotoxic effects of the anthracycline derivative doxorubicin and the organic peroxide tBOOH using the rat cardiomyoblast cell line H9c2 as a model system of non-malignant and cardiac-like cells. In addition, we examined whether serum factors and the CK2 inhibitor resorufin modulate doxorubicin and/or tBOOH-mediated cytotoxicity. The results obtained indicate that ROS are not the major source of cell death of rat cardiomyoblasts exposed to doxorubicin and, furthermore, show that resorufin influences the viability and stress response of H9c2 cells exposed to doxorubicin and tBOOH.

Materials and methods

Materials

The anthracycline derivative doxorubicin was obtained from the pharmaceutical department of the university hospital Mainz. The organic hydroperoxide tertiary-butylhydroperoxide (tBOOH) and the CK2 inhibitor resorufin were purchased from Sigma-Aldrich (Steinheim, Germany). Antibodies p-JNK, p-p38, p-Chk1 and p-Akt were from Cell Signaling Technology (Beverly, MA, USA). The antibody detecting Ser139 phosphorylated histone H2AX (γH2AX) was obtained from Millipore (Billerica, MA, USA) and the ERK2 specific antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CM-H2DCFDA was from Invitrogen/Molecular Probes (Karlruhe, Germany).

Cell culture and drug treatment

Rat cardiomyoblasts H9c2 cells were grown in DMEM medium (Invitrogen, Paisley, UK) containing 20% (high serum) or 0.1% (serum-deprived) fetal calf serum (FCS) (PAA Laboratories, Colbe, Germany) at 37 °C in an atmosphere containing 5% CO₂. If not stated otherwise, cells were grown in medium with 20% FCS. Treatment of cells cultured in 20% FCS was performed 24 h after seeding, treatment of the serum-deprived cells was performed 72 h after seeding. Resorufin treatment was performed as described before [29]. Briefly, high-dose pre-treatment (i.e. up to 50 μM for 3 h) was followed up by a low-dose post-treatment (i.e. 10 μM) until cells were harvested for analysis.

Western blot analyses

The level of protein expression was determined by western blot analysis using phosphospecific antibodies. Treatment of proliferating cells was performed 24 h after seeding into 6 cm culture dishes (5 × 10⁵ cells/dish). Cells were harvested after different time points. Cell extracts were prepared by lysing the cells in 200 μl SDS sample buffer (37 °C, 5 min) and subsequent sonication (duty cycle 50%, output control 5.5) (Branson Sonifier), followed by heating (95 °C, 5 min). Proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane using the Protean Mini Cell (BioRad, Munich, Germany). After completion of the transfer, membranes were blocked in 5% non-fat milk in TBS/0.1% Tween 20 for ≥ 1 h at room temperature. Incubation with the primary antibody was conducted overnight at 4 °C. Incubation with HRP-coupled anti-rabbit secondary antibody (1:1000) was performed for 2 h at room temperature. Bands
were visualized by chemiluminescence. For quantitative analysis, autoradiographs were densitometrically analyzed using the NIH image J software.

**Determination of protein kinase CK2 activity**

Protein kinase CK2 activity test was performed for 10 min at 30 °C in a total volume of 40 μl containing 25 mM Tris–HCl pH 8.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 125 μM ATP, 0.6 μCi (γ-32P)-ATP (3000 Ci/mmol, Hartmann Analytic, Braunschweig, Germany), 200 μM CK2 peptide (RRRADDSDDDDDD, from KinaseDetect, Odense, Denmark) and 5 μg whole cell extract. Samples were spotted onto P81 phosphocellulose paper and washed extensively in 0.85 mM phosphoric acid. Incorporation of radiolabeled phosphate was measured by counting samples in a liquid scintillation counter (Canberra-Packard, Downers Grove, IL, USA).

**Determination of cell viability**

Cell viability was determined using the WST-1 assay (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s protocol (1.5 x 10⁶ cells were seeded per well (96-well tissue culture plates)). Relative viability in corresponding untreated controls (i.e. cells growing under high or low serum conditions and not treated with doxorubicin or tBOOH) was set to 100%. Data shown are the mean±sd from 2 to 3 independent experiments each performed in triplicate. In case of analyzing the effect of resorufin co-treatment, relative viability in resorufin co-treated cells was set to 100%.

**Measurement of reactive oxygen species (ROS)**

The level of ROS was analyzed by use of the cell-permeable dye 5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA). The oxidized dye can be quantitated fluorometrically or by FACS-based method. H9c2 cells were loaded with 5 μM of CM-H₂DCFDA for 30 min. Afterwards, medium was replaced and cells exposed to different concentrations of doxorubicin or tBOOH. After further incubation period of 2 h, ROS formation was visualized by fluorescence microscopy and quantitated by fluorometry or FACS-based analysis (excitation wave length: 492–495 nm; emission: 517–527 nm). As a positive control, hydrogen peroxide (1 mM) was used. Fluorometry-based data shown are from two experiments performed in quadruplicate, FACS-based ROS data are from a representative single experiment.

**Analysis of cell cycle distribution**

Cell cycle distribution of H9c2 cells was determined by FACS-analysis. To this end, cells were trypsinized, washed twice with PBS and resuspended in 100 μl PBS. For fixation, 2 ml of ice-cold ethanol was added while vortexing and cells were incubated at −20 °C (≥20 min). After centrifugation (1000 ×g, 5 min, 4 °C) the supernatant was discarded. The cells were resuspended in 66 μl PBS and 0.5 μl RNase (1 μg/μl) and incubated for at least 1 h at room temperature. After adding 183.5 μl propidium iodide (50 μg/ml) the cells were immediately measured by FACS (Becton Dickinson, Heidelberg, Germany). Representative results from two independent experiments (performed in duplicate) are shown.

**FACS-based analysis of cell death**

Apoptotic cell death was determined by analyzing the frequency of FITC-Annexin V positive cells by FACS analysis. After trypsinization, cells were washed in PBS, pelleted by centrifugation (1000 ×g, 5 min) and resuspended in 52.5 μl Annexin V-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, 0.1% BSA, pH 7.4) containing 2.5 μl Annexin V-Alexa fluor 488 (Invitrogen). After incubation for 15 min on ice in the dark, 447.5 μl Annexin V-binding buffer containing 20 μl of propidium iodide (50 μg/μl) was added and the sample was immediately subjected to FACS analysis. Representative results from two experiments are shown.

**Results and discussion**

**Serum factors differentially influence the susceptibility of H9c2 cells to doxorubicin and tBOOH**

Assuming that ROS are a major trigger of doxorubicin-mediated toxicity in H9c2 cardiomyoblasts, doxorubicin-induced cytotoxic effects are anticipated to be mimicked by a direct oxidant such as hydrogen peroxide (H₂O₂). To test this hypothesis, cells were treated with doxorubicin or tBOOH in the presence or absence of H₂O₂. As shown in Fig. 1B, the presence of H₂O₂ resulted in a significant increase in cell death, as evidenced by an increase in the percentage of Annexin V-positive cells. These results suggest that ROS are a key factor in the cytotoxicity of doxorubicin and tBOOH in H9c2 cells.

**Fig. 1 – Effect of doxorubicin and tBOOH on cell cycle distribution of H9c2 cells grown in the presence of high and low concentrations of serum factors.** A: Treatment scheme of H9c2 cells. H9c2 cells were seeded in DMEM containing either high (20% FCS) or low (0.1% FCS) concentration of fetal calf serum (FCS). B: Cells growing in serum-rich (i.e. 20% FCS) medium were treated with 0.1 μM doxorubicin or 100 μM tBOOH 24 h after seeding. Serum-deprived cells (i.e. 0.1% FCS) were treated with 0.1 μM doxorubicin or 100 μM tBOOH 72 h after seeding. FACS-analyses were performed 24 h after drug treatment. Graphs shown are representative results from two experiments.
Fig. 2 – Serum factors differently affect the sensitivity of H9c2 cells following doxorubicin and tBOOH treatment. A: H9c2 cells cultured in the presence of high (20% FCS) (upper part) or low (0.1% FCS) (lower part) serum concentration were treated with increasing doses of doxorubicin (0.03 μM–3 μM) for 72 h. Cell viability was measured using the WST-1 assay as described in Materials and methods. Relative cell viability in untreated control cells was set to 100%. Data shown were obtained from 2 to 3 independent experiments each performed in triplicate. B: Cells growing in the presence of high (20% FCS) (upper part) or low (0.1% FCS) (lower part) concentration of serum factors were treated with 20–200 μM tBOOH or 5 μM–50 μM tBOOH, respectively. Cell viability was measured 72 h after addition of tBOOH using the WST-1 assay as described in methods. Relative cell viability in untreated control was set to 100%. Data shown were obtained from 2 to 3 independent experiments performed in triplicate.

Fig. 3 – Influence of the CK2 inhibitor resorufin on cell viability and cell cycle progression of H9c2 cells. A: Cell viability was measured after pre- and post-treatment of H9c2 cells grown in medium containing either 20% or 0.1% FCS with different concentrations of resorufin. According to our previously used treatment schedule [29], resorufin pre-treatment was performed at a concentration of 10 μM, 25 μM or 50 μM for 3 h, post-treatment was done for 72 h in the presence of 10 μM. Relative cell viability in control cells, which were grown in the absence of resorufin was set to 100%. Data shown were obtained from 2 to 3 independent experiments performed in triplicate. B: Cell cycle distribution was measured after a resorufin treatment period of 24 h. Resorufin pre-treatment (3 h) was performed with 50 μM, post-treatment was performed with 10 μM. Shown is the result of a representative experiment.
as the organic hydroperoxide tBOOH. In a first set of experiments we therefore comparatively analyzed the influence of doxorubicin and tBOOH on cell cycle progression of H9c2 cells cultured in either serum-rich (i.e. 20% FCS) or serum-depleted (i.e. 0.1% FCS) medium. As determined 24 h after exposure, doxorubicin caused a profound G2-arrest in the presence of serum factors, while this response was mitigated in serum-deprived cells (Fig. 1). Following tBOOH treatment, there was only a partial G2-block in high-serum cultured cells, whereas serum-depleted cells showed an accumulation in G1 and subG1 phase (Fig. 1B). Next we assayed the impact of serum factors on doxorubicin- and tBOOH-induced loss of cell viability 72 h after treatment using the WST assay. Regarding doxorubicin, we found that the absence of serum factors has only minor effect on cell viability as concluded from the similar IC50 values of 0.15 μM and 0.25 μM in the presence and absence of serum factors, respectively (Fig. 2A). By contrast, under situation of low serum concentration, the sensitivity of H9c2 cells towards tBOOH increased by about 3-fold as reflected by the IC50 values of 150 μM and 45 μM under serum-rich and serum-deprived conditions, respectively (Fig. 2B). Taken together, cell cycle analyses and viability assays revealed that the response of H9c2 cardiomyoblasts to doxorubicin and tBOOH is differently influenced by serum factors.

**Modulation of doxorubicin- and tBOOH-induced cytotoxicity by the CK2 inhibitory compound resorufin**

In a next step we investigated whether the novel protein kinase CK2 inhibitor resorufin [31] is able to modulate the susceptibility of H9c2 cells to doxorubicin and tBOOH. Under our experimental conditions (i.e. 3 h pre-treatment with 50 μM resorufin followed by 24 h post-incubation with 10 μM), resorufin reduced the activity of protein kinase CK2 by ~20%. This is in line with other report showing large cell-type specific variations in the CK2 inhibitory potency of resorufin, ranging from 40% to 80% inhibition in HCT116, LNCaP, PC3 and DU14 cells, respectively [31]. Resorufin concentration of up to 50 μM did not affect cellular viability of rat cardiomyocytes grown in the presence of high concentration of serum factors (Fig. 3A, upper part), whereas it caused a strong reduction in viability under situation of serum deprivation (Fig. 3A, lower part). Assaying cell cycle progression 24 h after resorufin addition, only moderate cytotoxic effects of the CK2 inhibitor were detectable (Fig. 3B). Co-treatment with resorufin had no major effect on the doxorubicin sensitivity of H9c2 cells in the presence of high serum concentration, whereas serum-deprived cells rendered about 5-fold more resistant to the cytotoxic effects of doxorubicin (Fig. 4A). By contrast, the sensitivity of H9c2 cells towards tBOOH increased by about 2-fold in the presence of resorufin, which was independent of serum factors (Fig. 4B). The data show that resorufin differently influences the viability of H9c2 cardiomyocytes exposed to doxorubicin or tBOOH.

**Influence of resorufin on doxorubicin- and tBOOH-induced apoptosis**

Measuring apoptotic cell death (i.e. early and late apoptosis) by FACS-based quantitation of Annexin V positive cells, we again observed clear modulatory effects of resorufin. In the presence of high concentration of serum factors, resorufin
largely promoted apoptotic death stimulated by either doxorubicin or tBOOH (Fig. 5A). Serum-deprivation caused a clear increase in the basal frequency of apoptosis on its own, in particular if resorufin was administered (Fig. 5B). Doxorubicin treatment further increased apoptotic death. This pro-apoptotic anthracycline effect was largely blocked by resorufin (Fig. 5B). Apparently, resorufin protected H9c2 cells from the cell killing effects of doxorubicin under conditions of serum deprivation. Hence, serum factors influence the outcome of resorufin co-treatment on doxorubicin-induced cell death of rat cardiomyoblasts. By contrast, tBOOH-induced apoptosis was further potentiated by resorufin co-treatment, which was independent of serum concentration (Figs. 5A and B).

Analysis of ROS levels following treatment of H9c2 cells with doxorubicin and tBOOH

The aforementioned findings indicate that cytotoxicity induced by anthracyclines might not be mainly driven by the formation of ROS, at least not in H9c2 cells in vitro. To scrutinize this hypothesis, we analyzed ROS formation and investigated the effect of the antioxidant N-acetylcysteine on doxorubicin-induced cytotoxicity. Measuring ROS level after treatment of H9c2 cells with different concentrations of doxorubicin or tBOOH for 6 h, we found that high doses of doxorubicin (i.e. $\geq 5 \mu M$) are required for a clear increase in ROS (Figs. 6A and B, left panel). At low dose of $\leq 0.5 \mu M$, which is most relevant for inducing cytotoxicity (see Fig. 2A), no clear increase in ROS levels could be observed (Figs. 6A and B). In contrast, tBOOH treatment considerably increased ROS levels already at a concentration of $100 \mu M$ (Figs. 6A and B), which is a relevant dose for reducing viability (see Fig. 2B) and, notably, is equitoxic to $0.1 \mu M$ doxorubicin (see Fig. 2). Measuring ROS levels at earlier time points (i.e. 2 h after exposure) identical results were obtained (data not shown). Taken together, doxorubicin-derived ROS are likely not of major relevance for doxorubicin-induced loss of viability of H9c2 cells.

To further address the question as to the contribution of doxorubicin-derived ROS for damaging H9c2 cells, we made use of the antioxidant N-acetylcysteine (NAC). This compound is anticipated to protect H9c2 cells from doxorubicin-induced cytotoxicity in case doxorubicin-formed ROS majorly contribute to cytotoxicity. As shown in Fig. 7A, NAC did not protect H9c2 cells from doxorubicin-induced loss of viability. Yet, as expected, NAC protected H9c2 cells from loss of viability observed after treatment with high concentration of tBOOH (Fig. 7B). Together with the aforementioned lack of ROS production at relevant (i.e. low) dose of doxorubicin, these data argue against a major relevance of doxorubicin-induced ROS formation...
formation as an initial trigger of cytotoxicity in cultured rat cardiomyoblasts. Of course, we can’t exclude that the formation of ROS at late times following doxorubicin treatment might amplify cytotoxicity. Yet, in this case, ROS have to be considered as a secondary (promoting) effect of doxorubicin-induced damage rather than as a primary trigger of toxicity.

Fig. 6 – Comparative analysis of doxorubicin- and tBOOH-induced formation of reactive oxygen species in H9c2 cells. A: Logarithmically growing H9c2 cells were treated with increasing concentrations of doxorubicin (0.2–1.0 μM) or the organic hydroperoxide tBOOH (20–500 μM). After incubation period of 6 h, the level of intracellular ROS was measured using the CM-H2DCFDA assay as described in Materials and methods. For reason of control, cells were also treated with hydrogen peroxide (H2O2) (1 mM, 1 h). Shown is the increase (Δ) in fluorescence (relative light units (RLU)) in drug-treated cells as compared to untreated control. Identical results were obtained when cells were treated for a time period of 2 h. Data shown are the mean ± SEM of quadruplicate determinations from two experiments. * Increase in fluorescence (RLU) ≤ 100. B: Cells were treated with doxorubicin and tBOOH as described under A. ROS formation was analyzed by FACS. Data shown are from a representative experiment.

Fig. 7 – Impact of antioxidant N-acetylcysteine on doxorubicin and tBOOH-induced loss of cell viability. A, B: Logarithmically growing H9c2 cells were pre-treated or not for 1 h with the antioxidant N-acetylcysteine (NAc) (10 mM) before increasing concentrations of doxorubicin (0.1–3.0 μM) (A) or the organic hydroperoxide tBOOH (50–300 μM) (B) were added. After further incubation period of 8 h in the presence of NAc, medium was replaced by drug-free medium and viability was assayed 72 h later. Data shown are the mean ± SEM of quadruplicate determinations from one experiment.
Stress responses of H9c2 cells following exposure to doxorubicin and tBOOH

For time kinetic comparative analyses of stress responses of H9c2 cells two different concentrations of doxorubicin and tBOOH were used. As shown in Fig. 8A, 1 μM of doxorubicin resulted in a time-dependent increase in the level of S139 phosphorylated H2AX (γH2AX), which is indicative of the induction of DNA damage. Interestingly, high dose of doxorubicin (i.e. 10 μM) did not trigger this response (Fig. 8A). Also activation of checkpoint kinase 1 (Chk1), which occurred in the presence of a low (i.e. 1 μM) concentration of doxorubicin, was no longer observed following high dose doxorubicin exposure (Fig. 8A). This finding indicates that the molecular mechanisms underlying doxorubicin-induced geno- and cytotoxicity might vary with the dose. Moderate activation of protein kinases JNK and Akt by doxorubicin was observed with high (i.e. 10 μM) doses of doxorubicin only. Regarding tBOOH, phosphorylation of H2AX was found following treatment with both low (i.e. 200 μM) and high (i.e. 500 μM) concentration of the organic peroxide (Fig. 8B). The same holds true for the activation of Akt, whereas a weak JNK stimulation was observed with high tBOOH concentrations (i.e. 500 μM) (Fig. 8B).

Modulation of doxorubicin- and tBOOH-stimulated stress responses by resorufin

Next we examined the influence of the CK2 inhibitor resorufin on doxorubicin- and tBOOH-induced stress responses. The effects observed were rather complex and depended on dose and time of analysis. For example, densitometrical analysis revealed that the phosphorylation level of H2AX stimulated by low doxorubicin dose (i.e. 1 μM) was reduced by ~40% in the presence of resorufin (Fig. 9A). Also phosphorylation of checkpoint kinase 1 (pChk1) was attenuated by ~60% if resorufin was present, whereas phosphorylation of Akt was increased by ~2.3-fold (Fig. 9A). The latter is likely due to the fact that resorufin is able to slightly stimulate Akt phosphorylation on its own in some cell lines [31], including H9c2 (data not shown). The phosphorylation status of stress activated protein kinases (SAPK/JNK), which was highest following treatment with high dose (i.e. 10 μM) of doxorubicin was not affected by resorufin (Fig. 9A). Regarding tBOOH, resorufin promoted H2AX phosphorylation following treatment with 200 μM for 4 h and 8 h, but not with 500 μM (Fig. 9B). Resorufin at a dose of 50 μM did not cause DNA strand breaks on its own, as analyzed by the alkaline comet assay and measuring the level of γH2AX by western blot analysis (data not shown).

Summarizing, the data show that the susceptibility of rat cardiomyoblast cells towards the anthracycline derivative doxorubicin and the oxidant tBOOH is differently affected by serum factors. Furthermore, the protein kinase CK2 inhibitor resorufin modulates cell viability, apoptosis and stress responses of H9c2 cells in an agent specific manner, indicating...
CK2 to be involved in the regulation of the DDR following anthracycline and tBOOH exposure. Based on the data we suggest that the mechanisms influencing the cytotoxicity of doxorubicin and tBOOH in H9c2 cells are largely different. The results obtained from ROS analysis and the use of the antioxidant NAC let us to conclude that early ROS formation is of minor relevance for doxorubicin-induced cytotoxicity in cultured H9c2 rat cardiomyoblasts. Recently we found a cytoprotective effect of the lipid lowering drug lovastatin on doxorubicin-induced cytotoxicity in both HUVEC [11] and H9c2 cells [12], which was reflected by cardioprotection in mice [12]. This finding indicates that the analysis of endothelial cells and cardiomyocytes in vitro might be a suitable model for an initial assessment of cardiotoxicity caused by anthracyclines in vivo. Discussing the contribution of anthracycline-derived ROS for cardiac damage in vivo, it remains possible that different oxygen tension in cultured cells versus cardiac cells in the heart might influence the informative value of in vitro experiments. To clarify this concern, future in vitro experiments under condition of different oxygen tension are suggested.

**Conflict of interest statement**

There is no conflict of interest of the authors regarding this manuscript.

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