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Rac1 Protein Signaling Is Required for DNA Damage Response Stimulated by Topoisomerase II Poisons*

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Background: Topoisomerase inhibitors are potent anticancer drugs triggering cell death via induction of DNA damage.
Results: DNA damage response stimulated by topoisomerase type I and II inhibitors is affected differently by Rac1 inhibition.
Conclusion: Rac1 signaling is required for the formation of the DNA topoisomerase II cleavable complex.
Significance: Membrane-bound Rac1 GTPase is essential for a full DNA damage response.

To investigate the potency of the topoisomerase II (topo II) poisons doxorubicin and etoposide to stimulate the DNA damage response (DDR), S139 phosphorylation of histone H2AX (γH2AX) was analyzed using rat cardiomyoblast cells (H9c2). Etoposide caused a dose-dependent increase in the γH2AX level as shown by Western blotting. By contrast, the doxorubicin response was bell-shaped with high doses failing to increase H2AX phosphorylation. Identical results were obtained by immunohistochemical analysis of γH2AX focus formation, comet assay-based DNA strand break analysis, and measuring the formation of the topo II-DNA cleavable complex. At low dose, doxorubicin activated ataxia telangiectasia mutated (ATM) but not ATM and Rad3-related (ATR). Both the lipid-lowering drug lovastatin and the Rac1-specific inhibitor NSC23766 attenuated doxorubicin- and etoposide-stimulated H2AX phosphorylation, induction of DNA strand breaks, and topo II-DNA complex formation. Lovastatin and NSC23766 acted in an additive manner. They did not attenuate doxorubicin-induced increase in p-ATM and p-Chk2 levels. DDR stimulated by topo II poisons was partially blocked by inhibition of type I p21-associated kinases. DDR evoked by the topoisomerase I poison topotecan remained unaffected by lovastatin. The data show that the mechanisms involved in DDR stimulated by topo II poisons are agent-specific with anthracyclines lacking DDR-stimulating activity at high doses. Pharmacological inhibition of Rac1 signaling counteracts doxorubicin- and etoposide-stimulated DDR by disabling the formation of the topo II-DNA cleavable complex. Based on the data we suggest that Rac1-regulated mechanisms are required for DNA damage induction and subsequent activation of the DDR following treatment with topo II but not topo I poisons.

Topoisomerase (topo)[3] II poisons such as the anthracycline derivative doxorubicin and the podophyllotoxin etoposide are highly potent antineoplastic drugs. Their clinical use is limited by adverse effects, in particular cardiotoxicity in the case of doxorubicin (1–3), hematologic toxicity in case of etoposide (4) and, for both of them, secondary leukemia (4, 5). Apart from the inhibition of topo II isoforms, anthracyclines have additional biological activities that are believed to contribute to their cytotoxicity. Most frequently discussed are intercalation into the DNA and the generation of reactive oxygen species (6, 7). The redox cycling of doxorubicin leading to oxidative stress and the inhibition of topo II are controversially discussed as critical events responsible for their cardiotoxicity (3, 8, 9). Type II topoisomerases (*i.e.* topo IIα and topo IIβ) are required for DNA replication and transcription because they catalyze the unwinding of the supercoiled DNA double helix (10). During this process both strands of one DNA helix are cut and, following the passage of the second DNA strand, reannealed (11). As an intermediate of this process, covalent binding between DNA and topoisomerase occurs. This DNA-protein complex (*i.e.* cleavable complex) is targeted by topo II poisons. They stabilize the topo II cleavable complex via different mechanisms thereby preventing the religation of the DNA (11). In consequence, DNA double-strand breaks (DSBs) are formed. DSBs are highly cytotoxic lesions and potent inducers of the DNA damage response (DDR), which causes activation of checkpoint control mechanisms and DNA repair (12–14). If DSBs are not properly repaired, they give rise to induction of apoptotic cell death (15). The DDR is regulated by the phosphatidylinositol 3-kinase-like protein kinases ataxia telangiectasia mutated (ATM), ATM and Rad3-related (ATR), and DNA-protein kinase Cs (13). Upon recognition of DSBs by the MRN complex, which consists of the proteins Mre11, Rad50, and NBS, ATM kinase is activated, leading to the phosphorylation of numerous substrates participating in the regulation of cell cycle progression and DNA repair (16, 17). Among others, the histone H2AX is phosphorylated at S139 (γH2AX) in the course of the DDR. Therefore

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[3] The abbreviations used are: topo, topoisomerase; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related; Chk, checkpoint kinase; DDR, DNA damage response; DSBs, DNA double-strand breaks; Gy, gray; γH2AX, S139-phosphorylated histone H2AX; HUVEC, human umbilical vein endothelial cell; PAK, p21-associated protein kinase; Rho, Ras-homologous; TARDIS, trapped in agarose DNA immunostaining.

γH2AX is a frequently used surrogate marker of DNA damage and the DDR (18, 19).

Besides stimulating the DDR, genotoxins also provoke stress signaling by activation of growth factor and cytokine receptors located at the outer cell membrane (20–22). Signaling induced upon activation of these receptors involves small GTP-binding proteins such as Ras and Ras-homologous (Rho) GTPases. Apart from regulating functions related to the actin cytoskeleton (23), Rac1 is essential for activation of stress-activated protein kinases (SAPK/JNK) (24, 25) and transcription factors (26, 27). Moreover, Rac1 seems to have a nuclear function as well because it regulates mitosis (28) and was recently found in the nucleus associated with topoisomerase II enzymes (29). Targeting of Rho signaling, for example by HMG-CoA reductase inhibitors (statins) (30–32), has multiple inhibitory effects on cellular responses following genotoxin treatment. For instance, statins inhibit the activation of the DDR following exposure of human umbilical vein endothelial cells (HUVECs) or smooth muscle cells to ionizing radiation (33, 34). Moreover, statins also attenuate doxorubicin-induced activation of the DDR in HUVECs and rat cardiomyoblasts (H9c2) *in vitro* (35, 36) and have beneficial effects on normal tissue damage provoked by anthracyclines and ionizing radiation (37–39). Yet, the molecular mechanisms involved are still unknown.

In the present study we comparatively analyzed the potency of two different types of topo II inhibitors, namely the anthracycline derivative doxorubicin and the podophyllotoxin etoposide, as well as the topoisomerase type I inhibitor topotecan on DNA damage induction and the activation of the DDR. Moreover, we investigated the effect of lovastatin and the Rac1-specific inhibitor NSC23766 on DNA damage induction and DDR following doxorubicin, etoposide, and topotecan treatment.

EXPERIMENTAL PROCEDURES

Materials—The following antibodies have been used: γH2AX anti-mouse antibody (pS139) (Millipore); γH2AX anti-rabbit antibody (pS139) (Epitomics, Burlingame, CA); ERK2, β-actin, and talin1 (Santa Cruz Biotechnology); p-ATM (pS1981), p-ATR (pS428), p-Chk1 (pS345), p-Chk2 (pT68), and 53BP1 (Cell Signaling Technology); peroxidase-conjugated secondary antibodies (Rockland, Gilbertsville, PA); topo IIα (Sigma-Aldrich); and Alexa Fluor 488 goat anti-mouse and Alexa Fluor 532 goat anti-rabbit-labeled secondary antibodies from Invitrogen. Lovastatin and Rac1 inhibitor NSC23766 were obtained from Sigma-Aldrich. p21-associated protein kinase type I (PAK1–3) inhibitor IPA3 was from Calbiochem. The topo II poisons doxorubicin, epirubicin, and etoposide as well as the topoisomerase type I-specific inhibitor topotecan were provided by the pharmaceutical department of the University Medical Center Mainz.

Cell Culture and Drug Treatment—Rat cardiomyoblasts H9c2 were grown in DMEM (Invitrogen) containing 20% fetal calf serum (FCS) (PAA Laboratories, Cölbe, Germany) at 37 °C in an atmosphere containing 5% CO_2. If not stated otherwise, treatment of logarithmically growing H9c2 cells with topoisomerase inhibitors was performed for 2 h. Pretreatment with lovastatin was performed overnight, pretreatment with the Rac1-specific inhibitor NSC23766 and PAK inhibitor was performed for 3 h and 2 h, respectively.

Analysis of Cytotoxicity—To measure the cytotoxic effects of topo II targeting drugs, cell viability was monitored by use of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which measures the activity of enzymes that reduce water-soluble MTT to an insoluble formazan. The formazan was then solubilized, and the concentration was determined densitometrically (570 nm). Viability of untreated controls was set to 100%. Experiments were performed in quadruplicate. In addition to cell viability analysis, cell cycle distribution following doxorubicin treatment was monitored by FACS (FACSCalibur; BD Biosciences).

Western Blot Analysis—Total cell extracts were prepared by lysing identical number of cells (approximately $2–5 \times 10^5$) in Roti-load (Roth) sample buffer. Proteins were separated by SDS-PAGE (10–15% gels) and transferred onto nitrocellulose membranes. Membranes were then blocked in 5% non-fat milk in TBS/0.1% Tween 20 for ≥1 h at room temperature. Incubation with the primary antibody (as indicated) was conducted overnight at 4 °C. Incubation with peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (1:2000) (Rockland) was performed for 2 h at room temperature. Bound antibodies were then visualized by chemiluminescence and Hyperfilm ECL (GE Healthcare). Densitometric quantification was performed with the Multi Analyst software (Bio-Rad). Autoradiographies shown are representative of two or three independent experiments. Expression of ERK2 was analyzed as internal protein loading control. Quantitative data shown as mean ± S.D. from $n = 3$.

Immunohistochemical Analysis of H2AX Phosphorylation—Cells were seeded onto cover slides. After treatment, cells were fixed with 4% paraformaldehyde (15 min, room temperature), followed by incubation with ice-cold methanol (−20 °C, 1 h). After blocking (PBS containing 0.3% Triton X-100/5% BSA (w/v), 1 h, room temperature) incubation with an antibody specific for phosphorylated (S139) histone H2AX (1:500) was conducted overnight at 4 °C. Incubation with the secondary fluorescent-labeled antibody (Alexa Fluor 488) was performed for 2 h at room temperature in the dark. For microscopic analysis a Zeiss Axiovert 35 was used. 50 nuclei were evaluated per experimental condition. Values given are the mean ± S.E. from two or three independent experiments with 50 cells being analyzed per treatment.

TARDIS Assay—The TARDIS (trapped in agarose-DNA immunostaining) assay was used to measure the stabilization of the DNA-topoisomerase II cleavable complex, which is provoked by topo II-targeting poisons (40). Cells were embedded in agarose on microscope slides and subjected to a lysis procedure that removed the cell membrane and soluble proteins as described (40). To remove noncovalently bound nuclear proteins, cells were washed with 1 M NaCl. Topoisomerase IIα enzyme, covalently bound to nuclear DNA due to its interaction with topo II-targeting poisons, was detected using anti-topoisomerase IIα antibody (1:200) and FITC-labeled secondary antibodies. The topoisomerase IIβ-specific antibody available to us turned out to be not useful for the TARDIS assay (data not shown). Immunofluorescence images were captured,

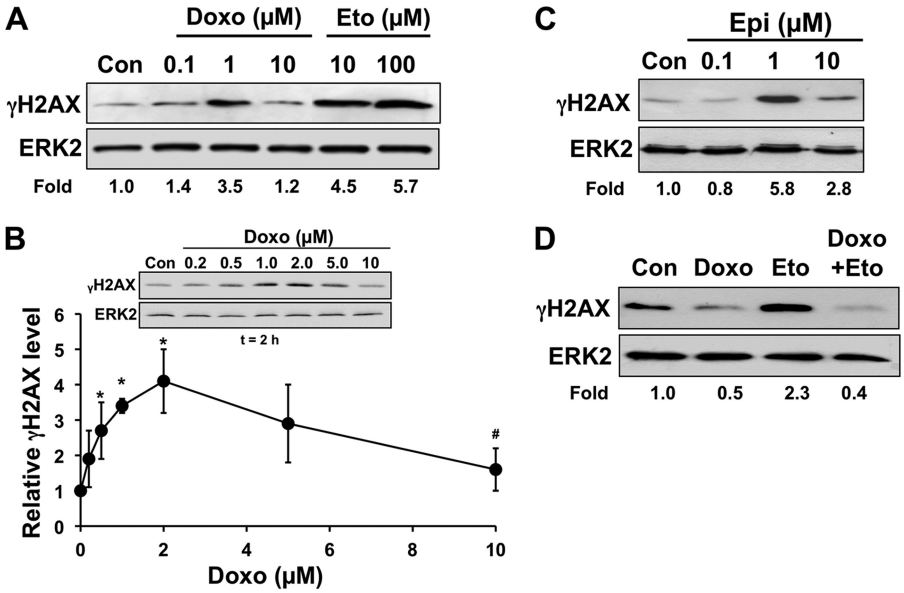

FIGURE 1. **Dose-response analysis of S139 phosphorylation of H2AX by different types of topoisomerase II inhibitors.** *A*, logarithmically growing rat cardiomyoblast cells (H9c2) were treated with increasing concentrations of different types of topoisomerase II poisons. Two h after addition of the anthracycline derivative doxorubicin (*Doxo*) (0.1–10 μM) or the podophyllotoxin etoposide (*Eto*) (1–100 μM), the level of γH2AX was determined by Western blot analysis. For quantitative densitometric analysis, the relative level of γH2AX was set to 1.0 in untreated control. ERK2 protein expression was determined as internal loading control. *B*, logarithmically growing rat cardiomyoblast cells (H9c2) were treated with increasing concentrations of doxorubicin for 2 h before the level of γH2AX was determined by Western blot analysis. Autoradiographies were quantified densitometrically. *, $p \leq 0.05$ compared with untreated control. #, $p \leq 0.05$ compared with treatment with 2 μM doxorubicin. *C*, logarithmically growing rat cardiomyoblast cells (H9c2) were treated with 0.1–10 μM anthracycline derivative epirubicin for 2 h before the level of γH2AX was determined by Western blot analysis. *D*, logarithmically growing rat cardiomyoblast cells (H9c2) were pretreated with a high dose of doxorubicin (10 μM, 1 h) before etoposide (10 μM) was added. After a further incubation period of 2 h, the level of γH2AX was determined by Western blot analysis. *Error bars*, S.D.

and 50 nuclei were counted for the calculation of the percentage of topo IIα-positive cells. Alternatively, fluorescent cells were captured by laser scanning microscopy, and fluorescence intensity of 50 nuclei was determined. If not stated otherwise, quantitative values are given as the mean ± S.E. from two independent experiments with 50 cells being analyzed per experimental condition.

Analysis of DNA Strand Break Induction (Comet Assay)—Formation of DNA strand breaks was assayed by both the alkaline and the neutral comet assay (41). Whereas the alkaline comet assay detects predominantly DNA single-strand breaks, the neutral comet assay favors the detection of DNA DSBs. Comets were visualized by microscopy and quantified by determination of the percentage of DNA in the tail (Komet 4.02; Kinetics Imaging). 50 nuclei were evaluated per treatment. Data shown are the mean ± S.D. from at least two independent experiments.

Statistical Analysis—For statistical analysis the Student's *t* test or the Mann-Whitney *U* test were applied. *p* values of ≤ 0.05 were considered as significant and marked with an *asterisk*.

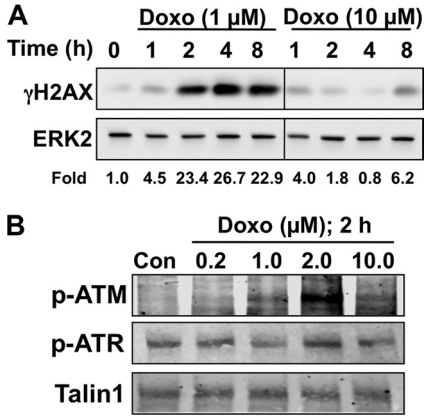

FIGURE 2. **Time kinetic analysis of H2AX phosphorylation by doxorubicin.** *A*, logarithmically growing H9c2 cells were exposed to 1 μM (*A*) or 10 μM (*B*) doxorubicin (*Doxo*). After the indicated periods of time, the level of γH2AX protein was determined by Western blot analysis. Shown is the result of a representative experiment. For densitometric analysis, the amount of γH2AX in untreated control was set to 1.0. ERK2 protein expression was determined as internal protein loading control. *B*, H9c2 cells were treated with the indicated concentrations of doxorubicin for 2 h before the levels of p-ATM and p-ATR were determined by Western blot analysis. For loading control (*Con*), protein expression of talin1 was determined.

RESULTS AND DISCUSSION

Effect of Increasing Doses of Doxorubicin and Etoposide on the Level of γH2AX, DNA Strand Break Induction, and Topo IIα-DNA Complex Formation—Anthracyclines and podophyllotoxins inhibit type II topoisomerases via different mechanisms, thereby inducing cell death (4, 11, 42). Here, we comparatively investigated the outcome of topo II inhibition by either the anthracycline derivative doxorubicin or the podophyllotoxin etoposide on the DDR. To this end, we monitored the effect of increasing concentrations of the aforementioned topo II poisons on S139 phosphorylation of histone H2AX (γH2AX), which is a generally accepted surrogate marker of the DDR triggered by DNA DSBs (43), using H9c2 cardiomyoblast cells. H9c2 cells were used because they are considered an appropriate *in vitro* model when studying anthracycline-induced cardiotoxicity (44, 45). Doxorubicin treatment caused a strong increase in γH2AX protein level at a dose of 1 μM (Fig. 1*A*). At a high concentration (*i.e.* 10 μM) phosphorylation of H2AX was

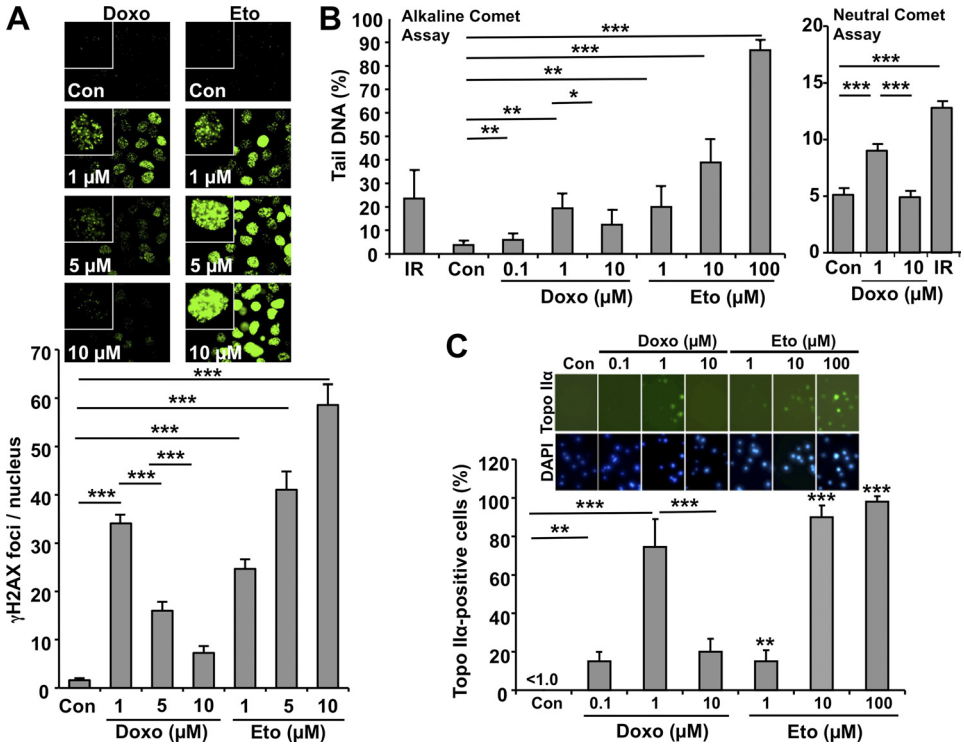

FIGURE 3. **γH2AX foci formation, DNA strand break induction, and formation of DNA-topo II covalent complexes by different types of topoisomerase II inhibitors.** *A*, logarithmically growing rat cardiomyoblast cells (H9c2) were treated with increasing concentrations of the anthracycline derivative doxorubicin (*Doxo*) or the podophyllotoxin etoposide (*Eto*). Two h after drug addition, the number of γH2AX foci was determined by immunohistochemistry. ***, $p \leq 0.001$. *B*, the level of DNA strand break induction was monitored by both the alkaline (*left*) and neutral (*right*) comet assay. For control, cells were irradiated with 5 Gy (*IR*). Data shown are the mean ± S.E. (*error bars*) from two to six independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. *C*, logarithmically growing rat cardiomyoblast cells (H9c2) were treated with increasing concentrations of doxorubicin or etoposide. Two h after drug addition, the formation of DNA-topo IIα covalent complex was analyzed as described under "Experimental Procedures." Nuclei were counterstained with DAPI. The histogram shows the percentage of topo IIα-positive cells. Shown is the result of a single experiment with 50 nuclei being analyzed per treatment. **, $p \leq 0.01$; ***, $p \leq 0.001$.

no longer observed (Fig. 1*A*). Detailed dose-response analyses showed a bell-shaped response of H9c2 cells to doxorubicin, with a maximum level of γH2AX protein found after treatment with 1–2 μM doxorubicin (Fig. 1*B*). Lack of H2AX phosphorylation was also observed following exposure of H9c2 cells with a high dose of the anthracycline derivative epirubicin (Fig. 1*C*), showing that blockage of the DDR at high concentrations is a group property of anthracyclines. By contrast, etoposide treatment of H9c2 cells did not reveal such a bell-shaped response at a high dose (Fig. 1*A*). Notably, pretreatment with 10 μM doxorubicin prevented subsequent H2AX phosphorylation by etoposide (Fig. 1*D*). Time kinetic analysis revealed maximum γH2AX protein levels ≥2 h following treatment with 1 μM doxorubicin (Fig. 2*A*). A high concentration of doxorubicin (*i.e.* 10 μM) did not provoke a comparable response as analyzed up to 8 h after drug addition (Fig. 2*A*). Measuring the activation status of the phosphatidylinositol 3-like kinases ATM and ATR, which catalyze phosphorylation of H2AX at S139 during the DDR, we found that doxorubicin-stimulated S1981 autophosphorylation of ATM was rather poor at high (10 μM) compared with lower (1–2 μM) concentration (Fig. 2*B*). ATR activity, as indicated by the protein level of S428-phosphorylated ATR, was not clearly enhanced following doxorubicin treatment (Fig. 2*B*). At the time of DDR analysis (*i.e.* 2 h), the cells are still fully functional, even if high (10 μM) doses of doxorubicin were used. This is concluded from the fact that up to 8 h after treatment, doxorubicin doses of 1–10 μM did not provoke any signs of cytotoxicity as indicated by assaying cell viability (data not shown). Analyzing viability and cell cycle progression 24 h after treatment with a high dose of doxorubicin (10 μM), ~50% decrease in cell viability and a moderate increase in sub-G_1 phase cells were observed (data not shown).

Bearing in mind that H2AX phosphorylation as monitored by Western blot analysis reflects DNA damage but not necessarily DNA DSBs (46, 47), we investigated the formation of γH2AX foci, which are highly indicative of DSBs (48), by immunohistochemistry. In line with the Western blotting-based data, a low dose (*i.e.* 1 μM) of doxorubicin caused a large increase in the number of nuclear γH2AX foci, which was not observed at a high (*i.e.* 10 μM) doxorubicin dose (Fig. 3*A*). Identical results were obtained when 53BP1 focus formation was analyzed (data not shown). As opposed to doxorubicin, etoposide-induced formation of γH2AX foci increased with dose (Fig. 3*A*). These results were confirmed by measuring the formation of DNA strand breaks by an alternative method, *i.e.* the comet assay (Fig. 3*B*). Both the alkaline comet assay, which detects mainly single-strand breaks, and the neutral comet assay, which reflects predominantly DSBs, showed a reduction in tail moment at high (*i.e.* 10 μM) doxorubicin concentration compared with a low (*i.e.* 1 μM) dose (Fig. 3*B*). The data show that DNA damage induction and subsequent DDR of H9c2 cells treated with topo II poisons depend on agent and dose. At a high dose, anthracyclines do not provoke DNA strand breaks any more, and ATM-catalyzed phosphorylation of the DDR surrogate marker H2AX

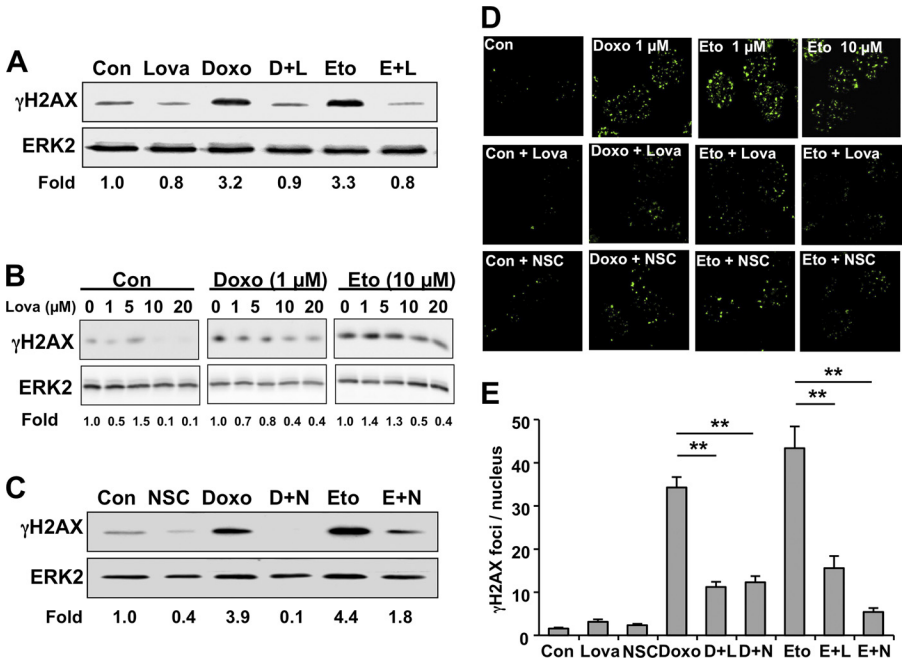

FIGURE 4. **Effect of inhibition of Rho signaling on S139 H2AX phosphorylation following exposure to topoisomerase II inhibitors.** *A*, logarithmically growing rat cardiomyoblast cells (H9c2) were pretreated overnight with lovastatin (*Lova*) (20 μM) before doxorubicin (*Doxo*) (1 μM) or etoposide (*Eto*) (10 μM) was added. After incubation period of 2 h, the level of γH2AX protein was analyzed by Western blotting. The relative amount of γH2AX protein in untreated cells (*Con*) was set to 1.0. Shown is a representative result from at least three independent experiments. *B*, logarithmically growing rat cardiomyoblast cells (H9c2) were pretreated overnight with different concentrations of lovastatin before doxorubicin (1 μM) or etoposide (10 μM) was added. After an incubation period of 2 h, the level of γH2AX protein was analyzed by Western blotting. Relative amount of γH2AX in corresponding non-lovastatin-treated cells was set to 1.0. Shown is a representative result from two or three independent experiments. *C*, logarithmically growing rat cardiomyoblast cells (H9c2) were pretreated for 3 h with the Rac1-specific inhibitor NSC23766 (*NSC*) (100 μM) (64) before doxorubicin (1 μM) or etoposide (10 μM) was added. After an incubation period of 2 h, the level of γH2AX protein was analyzed by Western blotting. Relative amount of γH2AX in untreated cells was set to 1.0. Shown is a representative result from two or three independent experiments. *D*, logarithmically growing rat cardiomyoblast cells (H9c2) were pretreated overnight with lovastatin (20 μM) or for 3 h with the Rac1 inhibitor NSC23766 (100 μM) before doxorubicin (1 μM) or etoposide (1 μM, 10 μM) was added. After an incubation period of 2 h, the formation of γH2AX foci was analyzed by immunohistochemistry as described under "Experimental Procedures." Shown is a representative result. *E*, logarithmically growing rat cardiomyoblast cells (H9c2) were pretreated overnight with lovastatin (*L*) (20 μM) or for 3 h with the Rac1 inhibitor NSC23766 (*N*) (100 μM) before doxorubicin (*D*) (1 μM) or etoposide (*E*) (10 μM) was added. After further incubation period of 2 h, the formation of γH2AX foci was analyzed by immunohistochemistry as described under "Experimental Procedures." Shown are the mean ± S.E. (*error bars*) of two independent experiments with 50 nuclei being analyzed per experiment. **, statistically significant ($p \leq 0.01$).

is disabled. One possible explanation of the striking doxorubicin effects might be related to the DNA intercalating activity of anthracyclines, which is discussed as a predominant toxic effect at high concentrations (6). Upon intercalation of anthracyclines into the DNA, the structural changes that are induced might hamper a proper interaction of topo II enzymes with DNA (49), eventually impairing ATM activation and subsequent γH2AX and 53BP1 focus formation. This view gains support by our aforementioned finding that pretreatment with a high dose of doxorubicin prevents DDR following etoposide treatment (see Fig. 1*D*). To scrutinize this hypothesis further, we investigated whether the formation of covalent DNA-topo II interaction (50) occurs at high doxorubicin concentration. To this end, the TARDIS assay was applied (40). As shown in Fig. 3*C*, complex formation between DNA and topo II was only observed after treatment of the cells with a low (*i.e.* 1 μM) dose of doxorubicin, but not if a high concentration (*i.e.* 10 μM) was used. Etoposide caused cleavable complex formation both at low and high concentrations (Fig. 3*C*). Based on these results, we suggest that the extensive DNA intercalation of doxorubicin, which occurs at high doses, disables covalent DNA-topo II interaction and, in consequence, does not provoke DNA DSB formation and activation of the DDR. The data also led us to speculate that changes in DNA topology provoked by intercalation of chemicals into the DNA are not sufficient to stimulate mechanisms of the DDR as reflected by γH2AX foci formation.

Inhibitory Effect of Lovastatin and the Rac1 Inhibitor NSC23766 on Doxorubicin- and Etoposide-induced H2AX Phosphorylation, DNA Strand Break Induction, and Topo IIα-DNA Complex Formation—In previous reports we have shown that the HMG-CoA reductase inhibitor lovastatin is able to inhibit genotoxin-induced stress responses, including responses evoked by ionizing radiation (33) and doxorubicin (35) in primary HUVECs *in vitro*. Notably, lovastatin also mitigates doxorubicin-induced cardiotoxicity *in vivo* (36, 38, 39). As shown in Fig. 4, lovastatin largely blocked both doxorubicin- and etoposide-induced activation of the DDR, as indicated by a reduced level of S139-phosphorylated H2AX (Fig. 4*A*). Concentrations of ≥5 μM lovastatin are required to attenuate H2AX phosphorylation by topo II poisons (Fig. 4*B*). Notably, the Rac1-specific inhibitor NSC23766 showed inhibitory effects identical to those of lovastatin (Fig. 4*C*), supporting previous reports suggesting Rac1 to be a major target of the pleiotropic statin effects (39, 51). As anticipated from the Western blotting-based results, both lovastatin and NSC23766 largely reduced γH2AX focus formation following treatment of H9c2 cells with topo II inhibitors (Fig. 4, *D* and *E*). The inhibitory effects of lovastatin and NSC23766 on doxorubicin-induced

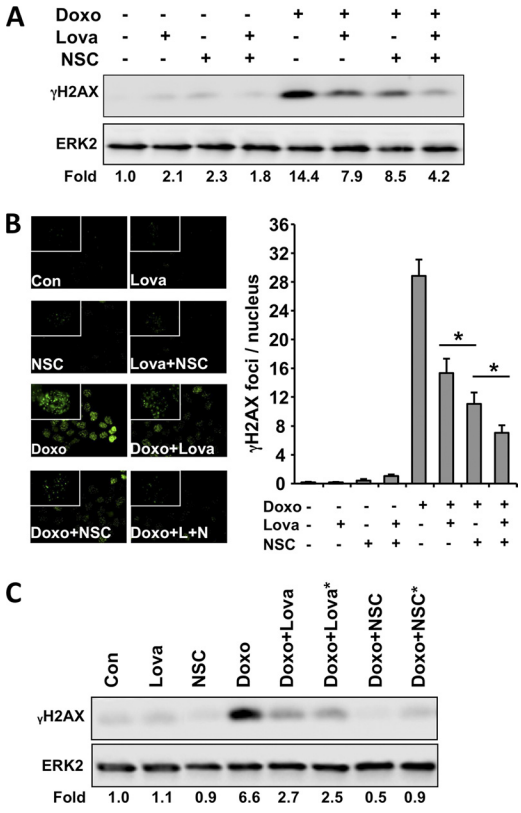

FIGURE 5. **The inhibitory effects of lovastatin and NSC23766 doxorubicin-induced DDR are additive.** *A* and *B*, logarithmically growing H9c2 cells were single pretreated with lovastatin (*Lova*) (overnight) or NSC23766 (*NSC*) (3 h) or were simultaneously pretreated with lovastatin plus NSC23766. Two h after doxorubicin (*Doxo*) treatment, the level of H2AX phosphorylation was analyzed by Western blotting (*A*) or immunohistochemistry (*B*). Relative γH2AX levels detected by Western blot analysis were quantitated densitometrically and set to 1.0 in untreated cells. Data shown under *B* are the mean ± S.E. (*error bars*) derived from one experiment with 50 nuclei being analyzed per condition. *, $p \leq 0.05$. *C*, after pretreatment with lovastatin (overnight, 20 μM) or NSC23766 (3 h, 100 μM), medium was removed and replaced by fresh medium. After a postincubation period of 3 h in the absence of Rac1 inhibitors, doxorubicin (*Doxo*) (1 μM) was added, and H2AX phosphorylation was determined 2 h later by Western blotting. Shown is the result of a representative experiment. Relative γH2AX level in untreated control was set to 1.0.

DDR are additive as shown by Western blot and focus formation analysis (Fig. 5, *A* and *B*). A postincubation period of 3 h was not sufficient to abolish the blocking effect of the lovastatin and NSC23766 pretreatment on doxorubicin-stimulated H2AX phosphorylation (Fig. 5*C*). Moreover, the inhibitory effect of Rac1 inhibitors on doxorubicin-induced increase in γH2AX level was transient. It was only detectable at early time points (*i.e.* ≤2 h) after doxorubicin treatment but not after later time points (*i.e.* ≥4 h) (data not shown).

Assaying the effect of lovastatin and NSC23766 on doxorubicin- and etoposide-induced DNA strand break induction using the comet assay, we found that the increase in DNA strand breaks following doxorubicin and etoposide treatment is significantly reduced by approximately 50–60% in the presence of either lovastatin or NSC23766 (Fig. 6*A*). The observed inhibition of doxorubicin- and etoposide-induced DDR by lovastatin and Rac1-specific inhibitor might be due to their interference with signaling mechanisms either upstream or downstream of topo II. To address this question, the formation of the DNA-topo II cleavable complex, which is stabilized by topo II-targeting poisons, was analyzed by the TARDIS assay as described under "Experimental Procedures" (40). The data show that both lovastatin and NSC23766 attenuate covalent interaction of DNA with topo IIα (Fig. 6*B*), indicating that Rac1 signaling is required for the formation of the DNA-topo II cleavable complex. To figure out whether attenuated doxorubicin-stimulated DDR resulting from pharmacological inhibition of Rac1 signaling is related to ATM/ATR, the activation status of both kinases was analyzed by Western blotting. These analyses showed a clear activation of ATM, as indicated by the increase in the protein level of p-ATM(S1981) (Fig. 7*A*). By contrast, p-ATR(S428) level remained unchanged (Fig. 7*B*), indicating that doxorubicin fails to activate ATR-regulated mechanisms. In line with this, the protein level of activated p-Chk2 (Fig. 7*A*), but not of p-Chk1 (Fig. 7*B*), increased following doxorubicin treatment. Neither lovastatin nor NSC23766 blocked the rise in p-ATM protein induced by doxorubicin (Fig.

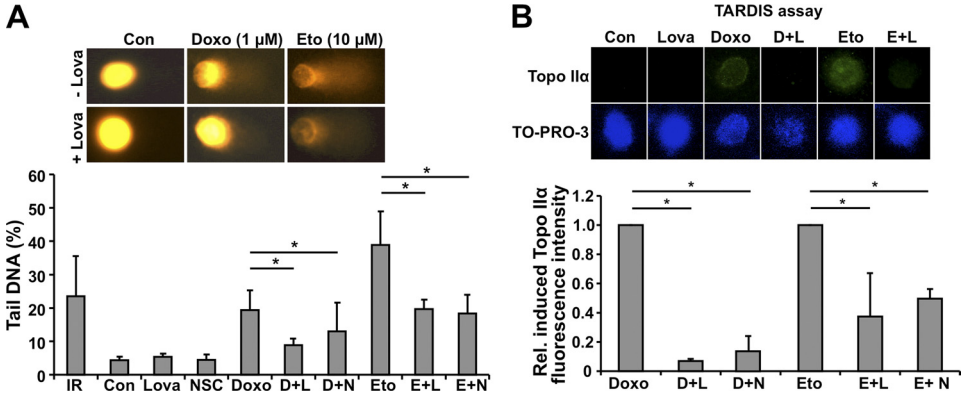

FIGURE 6. **Inhibition of Rac1 signaling reduces the level of DNA strand breaks and attenuates the formation of the DNA-topo II covalent complex following treatment with topo II inhibitors.** *A*, logarithmically growing rat cardiomyoblast cells (H9c2) were pretreated overnight with the lovastatin (20 μM) (*Lova*) or for 3 h with the Rac1-specific inhibitor NSC 23766 (*NSC*) (100 μM) before doxorubicin (*Doxo*) (1 μM) or etoposide (*Eto*) (10 μM) was added. After incubation period of 2 h, formation of DNA strand breaks was analyzed by the comet assay as described under "Experimental Procedures." Shown are the mean ± S.D. (*error bars*) from two to six independent experiments. For reason of control, cells were irradiated with 5 Gy (*IR*), and DNA strand breaks were analyzed 30 min later. *, statistically significant ($p < 0.05$). *B*, logarithmically growing rat cardiomyoblast cells (H9c2) were pretreated overnight with lovastatin (*L*) (20 μM) or for 3 h with the Rac1-specific inhibitor NSC23766 (*N*) (100 μM) before doxorubicin (1 μM) or etoposide (10 μM) was added. After an incubation period of 2 h, the formation of covalent DNA-topo IIα complexes was analyzed by laser scanning microscopy. Relative fluorescence in doxorubicin- or etoposide-treated cells was set to 1.0. Data shown are the mean ± S.E. (*error bars*) from two independent experiments with 50 nuclei being evaluated per experimental condition. *, $p \leq 0.05$.

7, *A* and *C*). Overall, the data indicate that Rac1 interferes mainly with DDR mechanisms downstream of ATM and that ATR-regulated mechanisms are not involved. In this context it should be noted that a low number of DSBs are sufficient to cause a maximum activation of ATM within a few minutes (52). This unique kinetic of ATM activation might explain our observation of a reduced formation of DSBs by Rac1 inhibition without concomitant decrease in p-ATM level.

Rac1 signaling is known to be mediated by a variety of effector proteins, notably PAKs (53), which are known to become activated following genotoxin treatment (54). Because PAK2 is considered to be highly specific for Rac1 (55), we made use of the class I PAK inhibitor IPA3, which targets PAK1–3 (56). As shown in Fig. 8 IPA3 reduced doxorubicin and etoposide-induced H2AX phosphorylation (Fig. 8*A*) and γH2AX focus formation (Fig. 8*B*) by approximately 30% only, indicating that Rac1-regulated mechanisms required for DDR are largely independent of PAK1–3. To address the question of the specificity of the observed effects for topo II poisons we included the topoisomerase I poison topotecan in our study. As shown in Fig. 9, treatment of H9c2 cells with topotecan resulted in H2AX phosphorylation (Fig. 9*A*) and caused a strong increase in the number of γH2AX foci (Fig. 9*B*), without any reduction in focus formation at high concentration. Lovastatin did not affect H2AX phosphorylation following topotecan treatment as monitored by Western blot-based analysis (Fig. 9*C*) and immunohistochemistry (Fig. 9*D*). Apparently, the inhibitory effect of lovastatin on the DDR triggered by topoisomerase poisons is specific for topoisomerase type II inhibitors but does not pertain to topoisomerase type I inhibitory drugs. Taken together, the data show that inhibition of Rac1 signaling protects H9c2 cells from DNA DSB formation and subsequent activation of the DDR following administration of topo II but not topo I poisons. The genoprotective statin effect likely rests on the inhibition of the formation of the DNA-topo II cleavable complex. Based on previous reports, both topo IIα and topo IIβ are of relevance for doxorubicin-stimulated DDR (8, 57). Therefore, it is rational to hypothesize that reduced anthracycline-induced DSB formation and attenuated DDR under situations of impaired Rac1 signaling involves both forms of topoisomerase II.

On the basis of the data we suggest the model depicted in Fig. 10. We propose that Rac1-regulated signaling mechanisms are required for low concentration of topo II targeting poisons to provoke the formation of DSBs and to trigger the ATM-regulated DDR. ATR seems to be of minor relevance in this context.

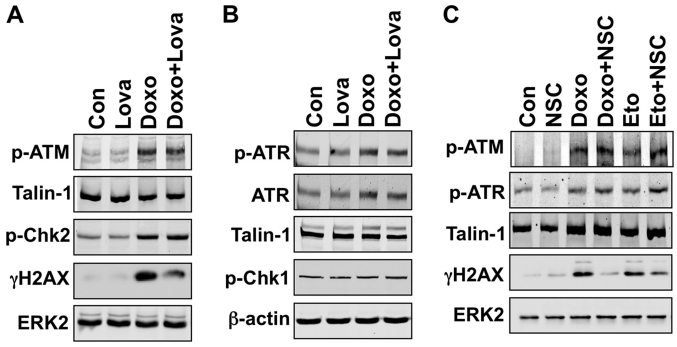

FIGURE 7. **Lovastatin and NSC23766 do not affect doxorubicin-stimulated activation of ATM.** *A* and *B*, logarithmically growing H9c2 cells were pretreated overnight with lovastatin (20 μM) before doxorubicin (1 μM) was added. After a further incubation period of 2 h, phosphorylation status of ATM (p-ATM), checkpoint kinase-1 (p-Chk1), and H2AX (γH2AX) (*A*) as well as of ATR (p-ATR) and checkpoint kinase-2 (p-Chk2) (*B*) was monitored. Protein expression of ERK2, β-actin, and talin1 was analyzed as internal protein-loading controls (*Con*). *C*, H9c2 cells were pretreated for 3 h with the Rac1-specific inhibitor NSC23766 before doxorubicin (1 μM) or etoposide (10 μM) was added. After a further incubation period of 2 h, phosphorylation status of ATM, ATR, and H2AX was analyzed. ERK2 and talin1 protein expression was determined as internal protein loading control. Shown are representative data from two independent experiments.

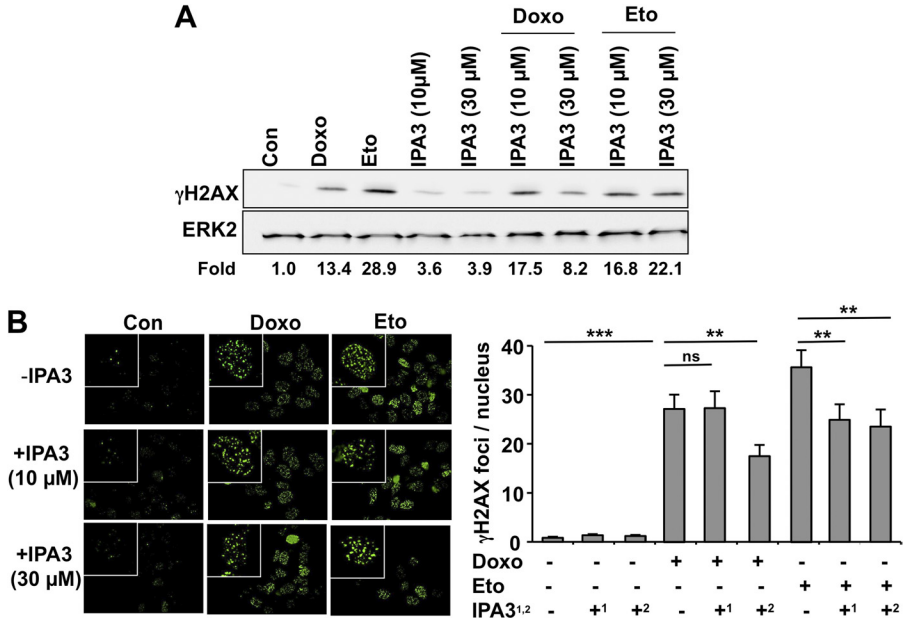

FIGURE 8. **Inhibition of the Rac1 effector PAK partially blocks DDR stimulated by topo II poisons.** Logarithmically growing rat cardiomyoblast cells (H9c2) were pretreated for 2 h with PAK inhibitor IPA3 (10 and 30 μM) before doxorubicin (*Doxo*) (1 μM) or etoposide (*Eto*) (10 μM) was added. After a further incubation period of 2 h, H2AX phosphorylation was analyzed by Western blotting (*A*) or immunohistochemistry (*B*) as described under "Experimental Procedures." *1*, 10 μM IPA3; *2*, 30 μM IPA3. **, $p \leq 0.01$; ***, $p \leq 0.001$; *ns*, not significant. *Error bars*, S.E.

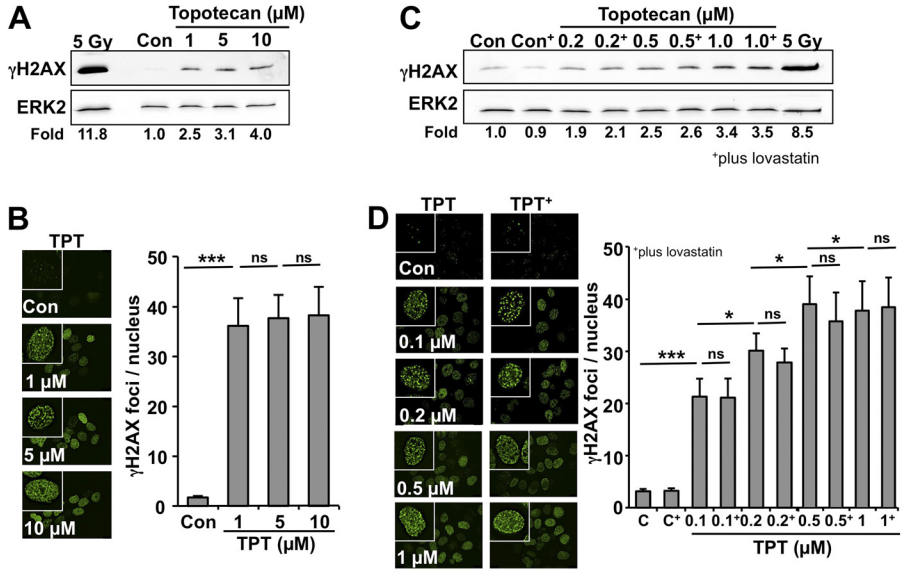

FIGURE 9. **DDR stimulated to the topo I poison topotecan is not inhibited by lovastatin.** *A* and *B*, logarithmically growing rat cardiomyoblast cells (H9c2) were exposed to increasing concentrations of topoisomerase type I inhibitor topotecan (*TPT*). After an incubation period of 2 h, H2AX phosphorylation and the formation of γH2AX foci were analyzed by Western blotting and immunohistochemistry, respectively, as described under "Experimental Procedures." Shown are results from a representative experiment. For control, cells were irradiated with 5 Gy. ***, $p \leq 0.001$; *ns*, not significant. *C* and *D*, logarithmically growing rat cardiomyoblast cells (H9c2) were pretreated overnight with lovastatin (20 μM) before topotecan was added at different concentrations. After further incubation period of 2 h, H2AX phosphorylation was analyzed by Western blotting (*C*) and immunohistochemistry (*D*) as described under "Experimental Procedures." Data shown are from representative experiments. +, pre-treatment with lovastatin; *, $p \leq 0.05$; ***, $p \leq 0.001$; *ns*, not significant. *Error bars*, S.E.

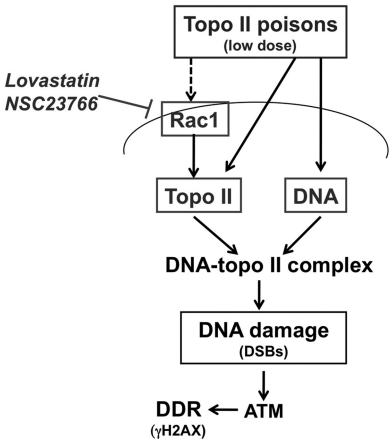

FIGURE 10. **Model of the genoprotective effect of pharmacological inhibition of Rac1 signaling in rat cardiomyoblast cells.** At therapeutically relevant low doses, topo II poisons promote irreversible interaction of topo II proteins with DNA (DNA-topo II complex), eventually leading to the formation of DNA DSBs and activation of the DDR, as reflected by γH2AX. Meanwhile, DSB formation and subsequent DDR do not occur at a high concentration of the anthracycline derivative doxorubicin. Pharmacological targeting of Rac1 signaling by statins (*e.g.* lovastatin) or a Rac1-specific small molecule inhibitor (*e.g.* NSC23766) attenuates DSB formation and subsequent ATM-regulated H2AX phosphorylation by counteracting the formation of the DNA-topo II complex. The data are indicative of a genoprotective function of statins, which is in line with a previous report demonstrating that statins maintain genomic stability (61).

At high doses of doxorubicin, however, there is a preferential intercalation of the anthracycline into the DNA (6), which disables binding of topo II to the DNA. In consequence, cleavable complex formation, subsequent induction of DSBs, and activation of ATM-regulated DDR are hampered. Pharmacological targeting of Rac1 signaling by statins or Rac1-specific small molecule inhibitors mitigates the topo II-DNA complex formation occurring if therapeutically relevant low doses of topo II poisons are applied. Thereby, all subsequent events, *i.e.* DSB formation followed by activation of the DDR, which is reflected by an increase in γH2AX protein level, are blocked. Most important, DNA damage and DDR induced by topo I poisons are not affected by Rac1 signaling, showing the specificity of our findings for topo II-related DNA damage. Although the detailed molecular mechanisms involved in the genoprotective function of statins are unknown, they are likely independent of doxorubicin import and export as well as the expression of topo II proteins (35, 36). Topo II is phosphorylated at numerous amino acids by protein kinase CK2 (58) and further contains several putative consensus sequences for mitogen-activated protein kinases as revealed by database search. Because Rac1 is a key regulator of stress-activated protein kinases (59), it is feasible that phosphorylation of topo II by Rac1-regulated protein kinases is required for its interaction with DNA. Noteworthy, Rac1 has recently been found in the nucleus, where it can interact with various nuclear proteins, including topoisomerase II (29). Bearing this in mind, it is tempting to speculate that it is a so far poorly appreciated nuclear function of Rac1 that is required for stimulation of the DDR following treatment with topo II targeting anticancer drugs. Apart from anticancer drugs, dietary flavanols have also been shown to affect topoisomerase function (62, 63). Whether Rac1 signaling is also involved in this process remains to be elucidated.

Summarizing, the present study provides evidence that inhibition of Rac1 signaling specifically interferes with the genotoxic potency of topo II poisons by preventing DNA-topo II cleavable complex formation, thereby reducing subsequent DSB formation and activation of the DDR. *In vivo* data showed protective effects of statins against doxorubicin-induced cardiotoxicity (36, 38, 39, 60), suggesting that statins might be clinically useful for lowering adverse effects of anthracyclines on heart function. Based on our data we suggest that such putative

beneficial effects of statins might involve inhibition of Rac1 signaling, which promotes DNA damage induction evoked by topo II-targeting poisons.

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