Cardiovascular Research (2008) **80**, 69–77
doi:10.1093/cvr/cvn171

The PGE_2-Stat3 interaction in doxorubicin-induced myocardial apoptosis

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Received 28 November 2007; revised 12 June 2008; accepted 16 June 2008; online publish-ahead-of-print 20 June 2008

Time for primary review: 18 days

KEYWORDS
Prostaglandins;
Apoptosis;
Signal transduction

Aims Both cyclooxygenase-2 (COX-2) and the transcription factor signal transducer and activator of transcription 3 (Stat3) are involved in adaptive growth and survival of cardiomyocytes. In ventricular cardiomyocytes, prostaglandin E_2 (PGE_2), a major COX-2 product, leads to adaptive growth via Stat3 activation, but whether this transcription factor acts as a signalling molecule in PGE_2-induced cell survival is unknown. Therefore, the purpose of this study was to determine whether PGE_2 counteracts cardiac apoptosis induced by doxorubicin (DOX), and if so, whether Stat3 plays a critical role in this cardioprotective effect.

Methods and results Neonatal rat ventricular cardiomyocytes were incubated with DOX (0.5 μM) and/or PGE_2 (1 μM). Apoptosis was assessed by determining caspase3 activation and apoptotic DNA fragmentation. The role of Stat3 was evaluated *in vitro* and *in vivo* by transfecting cardiomyocytes with siRNA targeting rat Stat3 and by using cardiomyocyte-restricted Stat3 knockout (Stat3 KO) mice, respectively. Incubation of ventricular cardiomyocytes with PGE_2 led to a time-dependent decrease in the DOX-induced caspase3 activation, reaching a maximal inhibition of $70 \pm 5\%$ after 4 h. Similarly, PGE_2 inhibited DOX-induced DNA fragmentation by $58 \pm 5\%$ after 24 h. This antiapoptotic action of PGE_2 was strongly reduced by the ERK1/2 inhibitor, U0126, whereas the p38 MAP kinase inhibitor, SB203580, had no effect. Depleting Stat3 expression by 50–60% in isolated ventricular cardiomyocytes markedly reduced the protective effect of PGE_2 on DOX-induced caspase3 activation and DNA fragmentation. Likewise, the stable PGE_2 analogue, 16,16-dimethyl-PGE_2, was unable to counteract cardiac apoptosis induced by DOX in Stat3 KO mice.

Conclusion Our results demonstrate that PGE_2 prevents myocardial apoptosis induced by DOX. This protection requires the activation of the prosurvival pathways of Stat3 and ERK1/2.

1. Introduction

The anthracycline doxorubicin (DOX) is one of the most effective anticancer drugs and frequently used to treat solid tumours and haematological malignances. Unfortunately, its use is limited by cumulative dose-related cardiotoxicity.[1,2] The pathogenesis of DOX has not yet been clearly identified. The presence of a large amount of apoptotic cells in the myocardium of DOX-treated patients could contribute to the dilated cardiomyopathy and heart failure induced by this anthracycline.[3,4] Among the mechanisms involved in DOX-induced cardiotoxicity, there is formation of reactive oxygen radicals,[5,6] leading to apoptosis in cardiomyocytes.[7,8]

Multiple studies indicate that cyclooxygenase-2 (COX-2) and its downstream products such as prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2) play a cardioprotective role, in particular counteracting DOX treatment[9–11] and ischaemia/reperfusion (I/R) injury[12–15] whose deleterious effects are mediated in a similar manner as DOX-induced injury, including the generation of reactive oxygen species.[16] Indeed, COX-2 inhibition aggravates DOX-mediated cardiomyocyte injury including apoptosis *in vitro* and *in vivo*.[9–11] Consistently, the prostacyclin analogue, iloprost, was found to reduce cardiac cell apoptosis and to ameliorate the cardiac function in DOX-treated mice,[11] but the mechanisms of protection are still unclear.

There is increasing evidence that the signal transducer and activator of transcription 3 (Stat3) plays a cardioprotective role in the heart.[17–21] Indeed, conditional knockout (KO) mice harbouring a cardiomyocyte-restricted deletion of Stat3 KO (Stat3 KO) showed increased susceptibility to cardiac injury caused by myocardial ischaemia, inflammation, or

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drug toxicity.[18,22] Stat3 has also been suggested as a critical intermediate to provide cardioprotection from DOX.[22–23]

At the present time, there is little information concerning the effect of PGE_2 on Stat3. In ventricular cardiomyocytes, we have recently demonstrated that PGE_2 leads to an increase in cell size and protein synthesis via a Stat3-dependent pathway involving ERK1/2.[24] However, whether Stat3 acts as a signalling molecule in PGE_2-induced cell survival is unknown. Therefore we determined whether PGE_2 counteracts the apoptotic effect of DOX in neonatal rat ventricular cardiomyocytes and whether Stat3 and/or ERK1/2 are involved. To further evaluate the interaction between PGE_2 and Stat3, we also investigated the role of Stat3 in the antiapoptotic effect of PGE_2 *in vivo* using Stat3 KO and wild-type (WT) mice.

2. Methods

2.1 Cell culture

This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by US National Institues of Health (NIH Publication No. 85–23, revised 1966). Neonatal ventricular cardiomyocytes were isolated from 1–2-day-old Wistar rat ventricles by digestion with trypsin-EDTA and type 2 collagenase as we have previously described.[24] For all experiments described herein, cells were used the third or fourth day of culture, after 16–24 h in DMEM medium depleted in FCS.

2.2 Small interfering RNA transfection

Stat3 was silenced by using a siRNA targeting rat Stat3 (Stat3-siRNA) with a mix of the following sequences: 5′-GGCUGAUCAUUUAUAU AAA-3′ and 5′- GAGGGUCUCGGAAAUUUAA-3′. These siRNAs which were provided by Qiagen AG (Hombrechtikon, Switzerland), were annealed according to manufacturer's instructions and stored at $-20^{\circ}C$. Cardiomyocytes were transfected using lipofectamine 2000 (Invitrogen) reagent, following the manufacter's protocol. Briefly, 24 h after cardiomyocytes were plated, transfection was proceeded with a mix containing lipofectamine 2000 reagent and Stat3-siRNA (300 pmol) in OptiMEM+GlutaMAX medium (Invitrogen). Cardiomyocytes were stimulated, with the different drugs 24–36 h after transfection. For control, cells were transfected as described for siRNA experiments with non-silencing siRNA (300 pmol) (5′-UUCU CCGAACGUGUCACGU-3′) which is ineffective in rat cells since it has no mammalian target.

2.3 *In vivo* experiments

WT or Stat3 cardiomyocytes restricted KO (Stat3 KO) mice (13–16 weeks old and weighting 20–40 g) were used in this study. Genotype was confirmed by DNA extraction and PCR analysis as previously described.[25] DOX was administered as a single intraperitoneal (i.p.) injection at a dose of 12 mg/kg. The stable PGE_2 analogue, 16,16-dimethyl-PGE_2 ($dmPGE_2$) was administered (i.p.) at a dose of 10 μg/kg trice daily from 2 days before DOX and continued until 5 days after DOX. The mice were divided into eight groups: (i) WT as control, (ii) Stat3 KO as control, (iii) WT+$dmPGE_2$, (iv) Stat3 KO+$dmPGE_2$, (v) WT+DOX, (vi) Stat3 KO+DOX, (vii) WT+ $dmPGE_2$+DOX, (viii) Stat3 KO+$dmPGE_2$+DOX. When the animals were sacrificed, the hearts were removed and cardiac tissue processed for determination of apoptotic DNA fragmentation. The doses of $dmPGE_2$ and DOX were based on previous work.[11,26]

2.4 Western blotting

Cardiomyocytes were starved of serum overnight and subjected to treatment as indicated in the figure legends. After stimulation in serum-free DMEM, cells were washed, lysed, and analysed by western blotting as previously described.[24] Briefly, total cell proteins were separated by SDS–PAGE and blotted onto nitrocellulose membrane. Afterwards, membranes were probed against cleaved caspase3 (17 kDa fragment) or phosphorylated Stat3 (Cell Signaling Technology, Denvers, MA, USA), and reprobed against glyceraldehyde-3-phosphate dehydrogenase (Chemicon International Inc., Hampshire, UK) or Stat3 total (Upstate Biotechnology, Lake Placid, NY, USA).

2.5 DNA fragmentation

DNA fragmentation was quantified by measuring the content of intracellular nucleosomes. Neonatal rat ventricular cardiomyocytes were cultured in 6-well plates for 2 days (10^6 cells per well). Thereafter, cardiomyocytes were starved of serum for 24 h and subjected to treatment as indicated in the figure legends. After stimulation in serum-free DMEM, cells were washed with ice-cold PBS (phosphate-buffered saline) and lysed with 60 μl of the same buffer used for western blotting.[24] Concerning the *in vivo* studies, minced cardiac tissue was homogenized in the same lysis buffer. The cellular and tissue lysates were used for protein determination and for quantitative evaluation of histone-associated DNA fragments by photometric enzyme immunoassay (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics, Germany) according to the manufacturer's instructions. Results are reported as arbitrary absorbance units normalized to milligram of proteins.

2.6 Determination of cardiomyocyte viability

Cell viability was evaluated by colorimetric MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) which is based on the reduction of MTT into a blue formazan dye by functional mitochondria of viable cells. Briefly, at the end of treatments, cardiomyocytes were washed twice with PBS prior to incubation with 5 mg/mL MTT for 3 h at $37^{\circ}C$ in an atmosphere of 5% CO_2. Thereafter, cells were again washed twice with PBS before lysing cell membranes with dimethyl sulfoxane. The amount of MTT formazan was quantified by determining the absorbance at 540 and 570 nm. Cell viability was also evaluated by staining cardiomyocytes with trypan blue (0.04% in PBS).

2.7 Chemicals

PGE_2 and $dmPGE_2$ were purchased from Sigma-Aldrich GmbH (Buchs, Switzerland), $dmPGE_2$ was prepared as previously described.[27] MTT, SB203580, AG490, and piceatannol were obtained from Calbiochem (Dietikon, Switzerland), while U0126 was from Biomol Research Laboratories (Plymouth Meeting, PA, USA).

2.8 Statistical analysis

All values are expressed as mean ± SEM with n referring to the number of experiments. Linear regression analysis was made using the Origin software (Microcal Software, Inc., Northhampton, MA, USA). Differences between groups were determined using either two-tailed unpaired Student's *t*-tests or ANOVA, followed by Bonferroni's *post hoc* test where applicable. $P < 0.05$ was accepted as statistically significant.

3. Results

3.1 Effect of prostaglandin E_2 on doxorubicin-induced myocardial apoptosis and decrease of cell viability

To investigate whether PGE_2 can counteract DOX-induced apoptosis in ventricular cardiomyocytes, we incubated cells with or without PGE_2 (1 μM) for 30 min before adding

DOX (0.5 μM) for 4–24 h (*Figure 1*). Thereafter, we analysed activation of caspase3 by measuring the expression of the 17 kDa fragment of cleaved caspase3. As is illustrated in *Figure 1A*, PGE_2 caused a significant decrease in the activation of caspase3 induced by DOX after 4 h (70 ± 5%), 8 h (57 ± 6%), 16 h (57 ± 11%), or 24 h (44 ± 3%). DOX also increased apoptotic DNA fragmentation by 109 ± 20 and 319 ± 54% after 16 and 24 h of treatment, respectively, but had no significant effect after 4 and 8 h (*Figure 1B*). The presence of PGE_2 abolished the DNA fragmentation induced after 16 h of incubation with DOX and inhibited that after 24 h of treatment by 49 ± 7%.

Cell viability was determined by using the MTT assay. *Figure 2A* illustrates that DOX decreased cell viability in a time- and concentration-dependent manner. As for apoptotic DNA fragmentation, PGE_2 (1 μM) abolished the decrease in cell viability observed after 16 h of incubation with DOX (0.5 μM), and markedly inhibited that after 24 h of treatment with DOX (*Figure 2B*). Similar results were obtained with the trypan blue exclusion method (data not shown).

To compare DOX-induced DNA fragmentation with cell viability, linear regression analysis was used to fit the data described in *Figures 1B* and *2B* (see *Figure 2C*). This analysis shows a high linear correlation between apoptotic DNA fragmentation and cell viability in cardiomyocytes treated or not with DOX (0.5 μM) for 4, 16, and 24 h in the presence and absence of PGE_2 (1 μM).

Our results indicate that in ventricular cardiomyocytes, PGE_2 counteracts DOX-induced apoptosis and the associated decrease in cell viability.

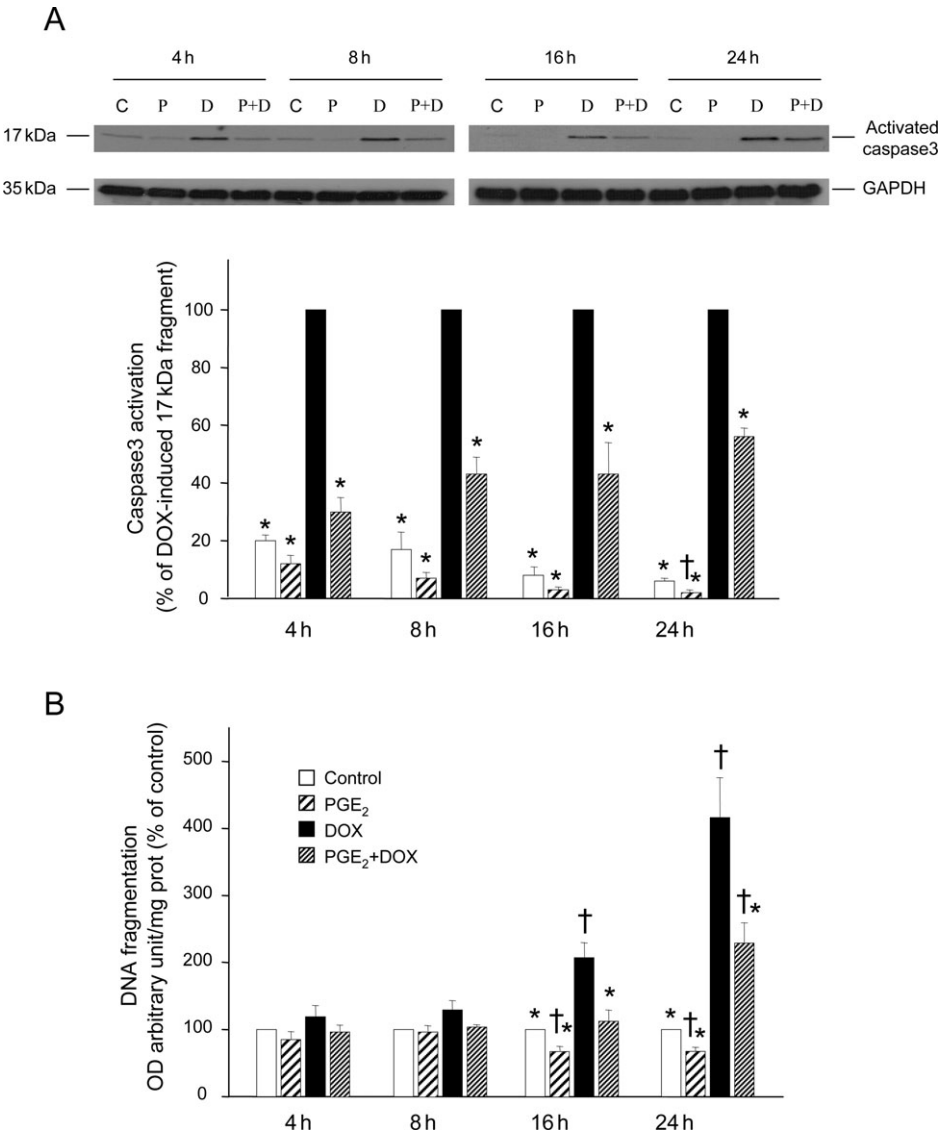

Figure 1 Prostaglandin E_2 inhibits doxorubicin-induced caspase3 activation and apoptotic DNA fragmentation in ventricular cardiomyocytes. Cells were pretreated or not (control, C) with prostaglandin E_2 (P, 1 μM) for 30 min prior to incubation with doxorubicin (D, 0.5 μM) during different time periods (4, 8, 16, and 24 h). (*A*) After these incubation periods, caspase3 activation was analysed in cellular extracts by determining the level of the catalytically active 17 kDa fragment by western blotting. Representative blots are shown at the top. Equal gel loading was assessed using an anti-GAPDH antibody. Specific bands corresponding to activated caspase3 were quantified by densitometry and expressed as percentage of doxorubicin-induced formation of the 17 kDa fragment. (*B*) After the incubation periods with doxorubicin, DNA fragmentation was analysed by measuring histone-associated DNA fragments. Results were calculated as arbitrary absorbance units normalized to mg of proteins and are expressed as percentage of control. $^*P < 0.05$ compared with values from doxorubicin-treated cells, $^\dagger P < 0.05$ compared with control values ($n = 4$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

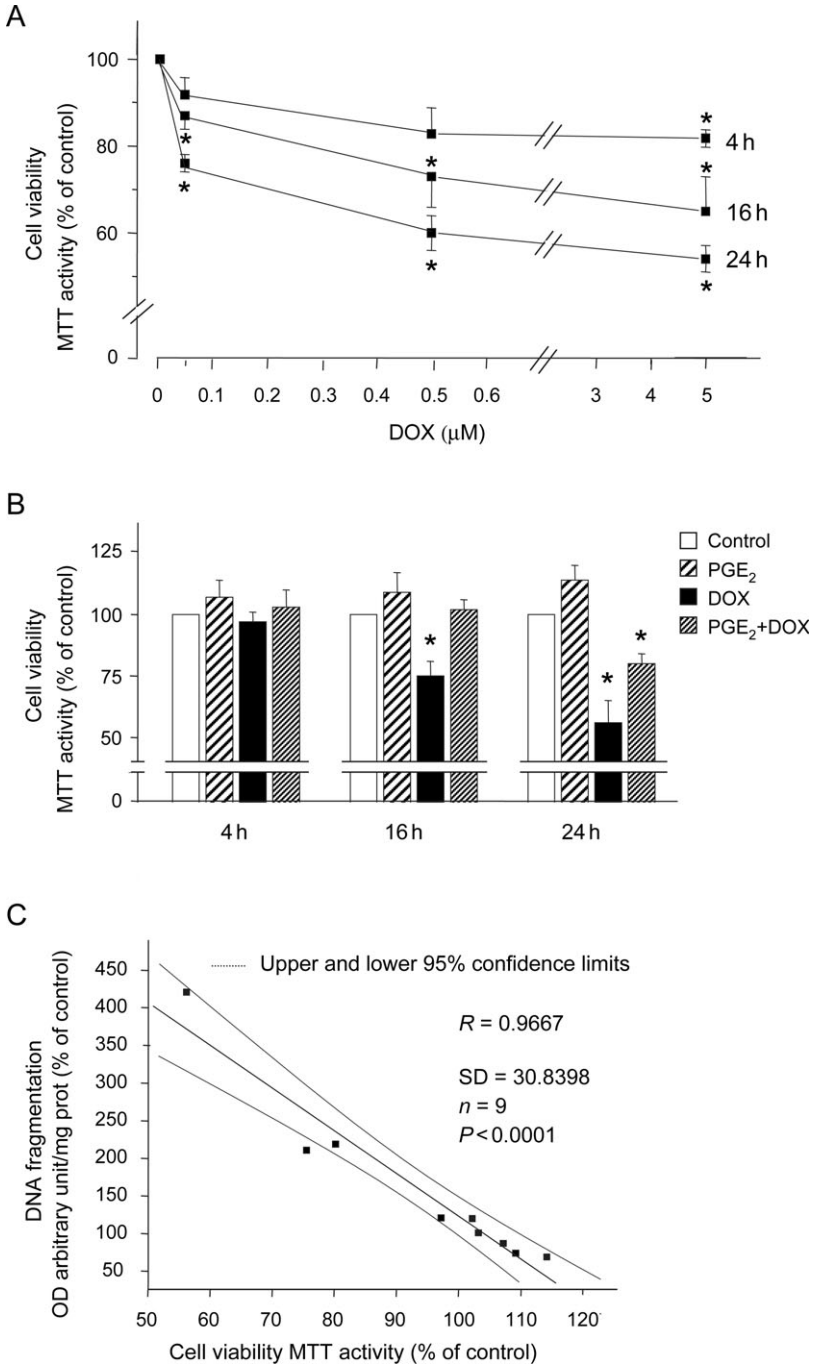

Figure 2 Effect of doxorubicin and prostaglandin E_2 on myocardial cell viability. (*A*) Cardiomyocytes were treated or not with the indicated concentration of doxorubicin (0.05–5 μM) for 4, 16, and 24 h, respectively. Thereafter, cell viability was determined by using the MTT assay. Each line represents mean ± SEM of three separate experiments ($n = 3$ wells in each experiment). Results are expressed as percentage of the corresponding control. (*B*) Cells were pretreated or not with prostaglandin E_2 (1 μM) for 30 min prior to incubation with doxorubicin (0.5 μM) during different time periods (4, 16, and 24 h). After these incubation periods, cell viability was determined by using the MTT assay. Results are expressed as percentage of the control. $^*P < 0.05$ compared with control values ($n = 3$–4). (*C*) The apoptotic DNA fragmentation observed after 4, 16, or 24 h of incubation with 0.5 μM doxorubicin in the presence or absence of 1 μM PGE_2 (see *Figure 1*) was compared with that of cell viability shown in (*A*) by using linear regression analysis to fit the data. *R*, correlation factor, SD, standard deviation.

3.2 Critical role of ERK1/2 in the antiapoptotic effect of prostaglandin E_2

We have previously reported that in ventricular cardiomyocytes, ERK1/2 is critical for PGE_2-induced Stat3 activation as assessed by nuclear tyrosine phosphorylation and DNA-binding activity. Both ERK1/2 and Stat3 have been shown to play an essential role in PGE_2-stimulated cell growth.[24] In this study, we investigated the role of ERK1/2 and p38 MAPK in the antiapoptotic responses induced by PGE_2 in cardiomyocytes exposed to DOX (0.5 μM) for 4 or 24 h. To this purpose we used the specific inhibitors of the ERK1/2 and p38 MAPK signalling pathways, U0126 and SB203580, respectively.[28] U0126 suppresses the activation of MEK1/2 leading to the inhibition of its downstream target ERK1/2.[28] SB203580 specifically inhibits α and β p38 MAP kinases, the main isoforms expressed in cardiomyocytes,[28,29] but it has

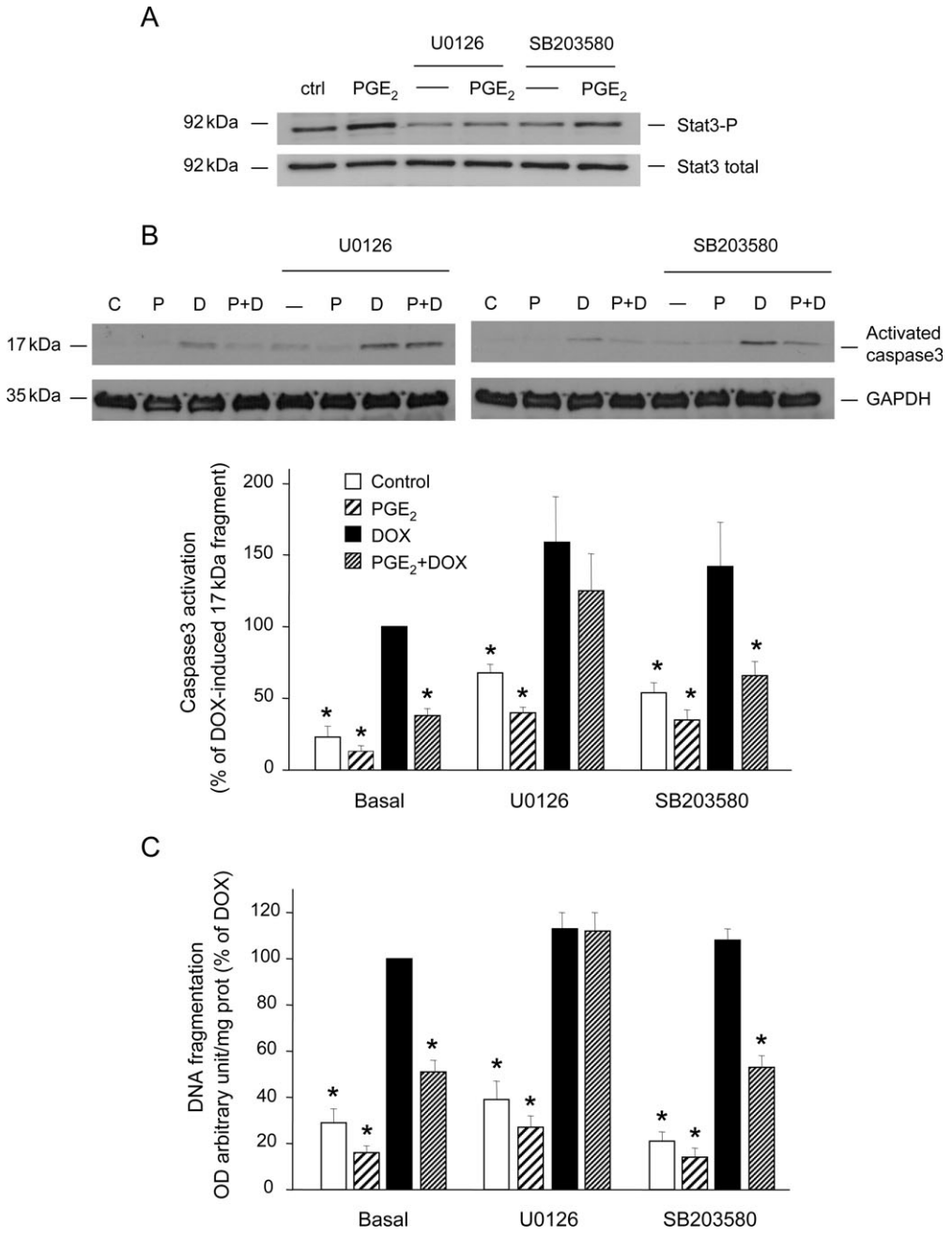

Figure 3 ERK1/2 plays a role in the inhibitory effect of prostaglandin E_2 on doxorubicin-induced activation of caspase3 and DNA fragmentation. Cardiomyocytes were incubated for 30 min with U0126 (10 μM) or SB203580 (SB, 10 μM), prior to stimulation with prostaglandin E_2 (1 μM). (*A*) After 90 min of prostaglandin E_2 stimulation, Stat3 tyrosine phosphorylation (Stat3-P) was analysed in cellular extracts by western blotting. A representative blot is shown: similar results were obtained in four separate experiments. (*B*) After 30 min of prostaglandin E_2 stimulation, cells were incubated with doxorubicin (0.5 μM) during 4 h. At the end of this incubation period, caspase3 activation was analysed in cellular extracts by determining the level of the catalytically active 17 kDa fragment by western blotting. A representative blot is shown at the top of (*B*) (C, control, P, prostaglandin E_2, D, doxorubicin). Equal gel loading was assessed using an anti-GAPDH antibody. Specific bands corresponding to activated caspase3 were quantified by densitometry and expressed as percentage of doxorubicin-induced formation of the 17 kDa fragment. (*C*) After 30 min of prostaglandin E_2 stimulation, cells were incubated with doxorubicin (0.5 μM) during 24 h. At the end of this incubation period, DNA fragmentation was analysed by measuring histone-associated DNA fragments. Results were calculated as arbitrary absorbance units normalized to mg of proteins and are expressed as percentage of doxorubicin-induced DNA fragments. $^*P < 0.05$ compared with values from doxorubicin-treated cells ($n = 4$).

no effect on γ and δ p38 MAP kinases.[30] As shown in *Figure 3A*, U0126, but not SB203580, abolished the PGE_2-induced Stat3 activation which was observed after 90 min of stimulation by determination of cellular Stat3 tyrosine phosphorylation.

To evaluate the role of ERK1/2 and p38 MAPK pathways on DOX-induced apoptosis in the presence and absence of PGE_2, cells were incubated for 30 min with U0126 (10 μM) or SB203580 (10 μM). Thereafter, cardiomyocytes were stimulated for 30 min with PGE_2 (1 μM) before DOX (0.5 μM) was added. Activation of caspase3 was determined after 4 h of DOX treatment, while generation of DNA fragmentation was assessed after 24 h of incubation with DOX. As shown in *Figure 3B*, U0126 strongly reduced the antiapoptotic effect of PGE_2 on DOX-induced caspase3 activation, whereas SB203580 had not significant effect. Similarly, the presence of U0126, but not SB203580, abolished the protective effect

of PGE_2 on DOX-induced DNA fragmentation (*Figure 3*C). It appears that ERK1/2 but not p38 MAPK is involved in the protective influence of PGE_2 counteracting apoptosis in cardiomyocytes exposed to DOX.

3.3 Critical role of signal transducer and activator of transcription 3 in the antiapoptotic effect of prostaglandin E_2 *in vitro*

To evaluate the role of Stat3 in the antiapoptotic effect of PGE_2 *in vitro*, we transfected cardiomyocytes with siRNA targeting rat Stat3. This treatment decreased Stat3 expression by 50–60%, 40 h after transfection of cardiomyocytes when compared with cells transfected with non-silencing RNA (control siRNA) (*Figure 4*, top). This Stat3 knock-down effect remained sustained until 48 h after transfection with anti-Stat3 siRNA (data not shown). After incubation with DOX, caspase3 activation, normalized to Stat3 expression, was increased in both control and anti-Stat3 siRNA transfected cells (*Figure 4*). PGE_2 significantly decreased DOX-induced caspase3 activation in the control siRNA transfected cardiomyocytes, whereas it had no effect in the anti-Stat3 siRNA transfected cells. Exposure of cardiomyocytes to DOX for 24 h also increased apoptotic fragmentation of DNA in both control and anti-Stat3 siRNA transfected cells (*Figure 5A*). As for caspase3 activation, PGE_2 decreased DOX-induced DNA fragmentation in the control siRNA transfected cells. In contrast, this antiapoptotic

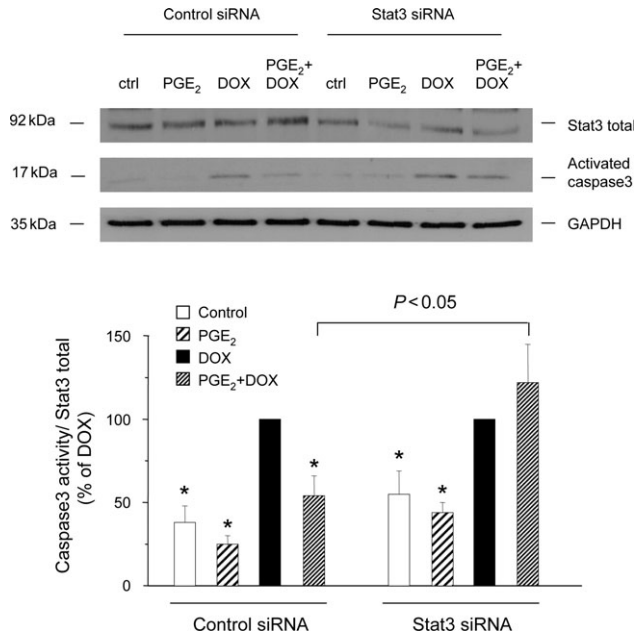

Figure 4 Small interfering RNA targeting Stat3 blocks the inhibitory effect of prostaglandin E_2 on doxorubicin-induced activation of caspase3. Thirty-six hour after transfection of cardiomyocytes with control small interfering RNA or small interfering RNA targeting Stat3 (Stat3 siRNA), cells were pretreated or not (ctrl) with prostaglandin E_2 (1 μM) for 30 min prior to incubation with doxorubicin (0.5 μM) during 4 h. Thereafter, the expression of Stat3 as well as that of the active caspase3 fragment of 17 kDa was analysed in cellular extracts by western blots. Representative blots are shown at the top. Specific bands corresponding to activated caspase3 were quantified by densitometry and normalized to Stat3 expression. Equal gel loading was assessed using an anti-GAPDH antibody. Results are expressed as percentage of doxorubicin-induced formation of the 17 kDa fragment. $^*P < 0.05$ compared with values from doxorubicin-treated cells ($n = 5$).

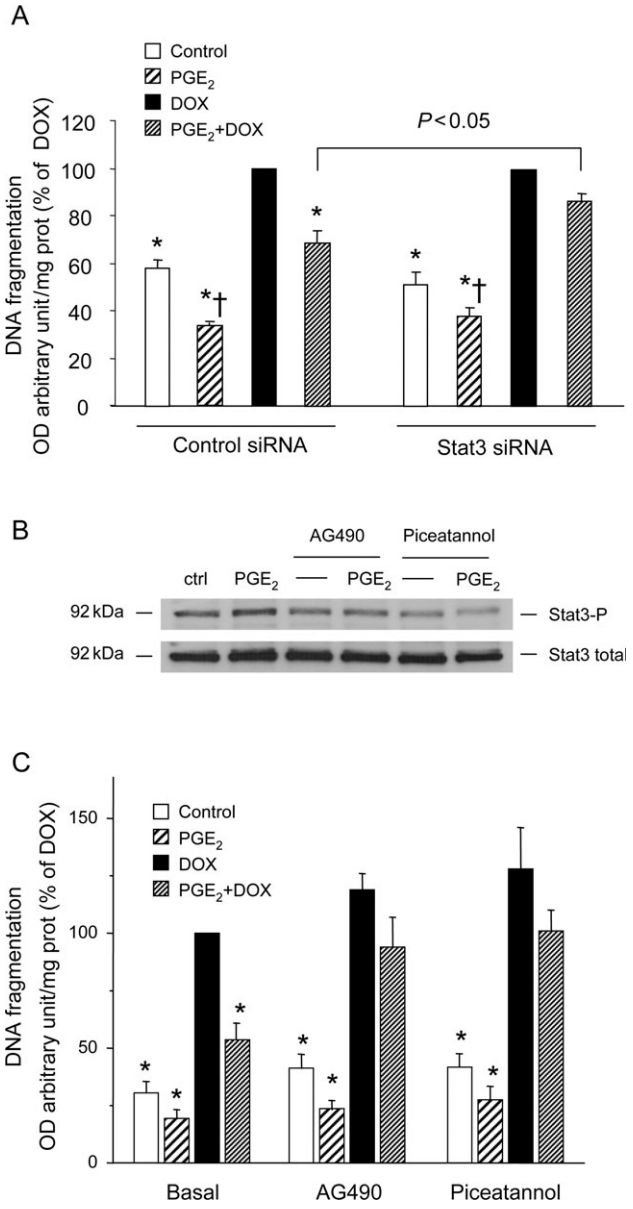

Figure 5 Small interfering RNA targeting Stat3 and pharmacological inhibition of Stat3 reduce the inhibitory effect of prostaglandin E_2 on doxorubicin-induced apoptotic DNA fragmentation. (*A*) 24 h after transfection of cardiomyocytes with control siRNA or siRNA targeting Stat3 (Stat3 siRNA), cells were pretreated or not (control, ctrl) with prostaglandin E_2 (1 μM) for 30 min prior to the incubation with doxorubicin (0.5 μM) during 24 h. Thereafter, DNA fragmentation was analysed. (*B* and *C*) Cells were incubated for 1 h with the Stat3 inhibitors piceatannol (10 μM) or AG490 (10 μM) prior to stimulation with prostaglandin E_2 (1 μM). (*B*) After 90 min of prostaglandin E_2 stimulation, Stat3 tyrosine phosphorylation (Stat3-P) was analysed in cellular extracts by western blotting. A representative blot is shown: similar results were obtained in three separate experiments. (*C*) After 30 min of prostaglandin E_2 stimulation, cells were incubated with doxorubicin (0.5 μM) during 24 h. At the end of this incubation period, DNA fragmentation was analysed. $^*P < 0.05$ compared with values from doxorubicin-treated cells; $^{\dagger *}P < 0.05$ compared with values from control cells ($n = 4$).

effect of PGE_2 was virtually abolished in the anti-Stat3 siRNA transfected cardiomyocytes. Since the transfection technique appears to increase basal DNA fragmentation (compare *Figure 1*), resulting in apparently smaller effects of DOX and PGE_2, we also investigated the role of Stat3 in the antiapoptotic effect of PGE_2 on DOX-induced DNA fragmentation by using the Stat3 pathway inhibitors AG490[31]

and piceatannol.[32] Both these inhibitors strongly inhibited PGE_2-induced Stat3 phosphorylation (*Figure 5B*) as well as the antiapoptotic effect of PGE_2 on the DNA fragmentation induced by DOX (*Figure 5C*).

3.4 Critical role of signal transducer and activator of transcription 3 in the antiapoptotic effect of prostaglandin E_2 *in vivo*

In order to evaluate the role of Stat3 in the antiapoptotic effect of PGE_2 *in vivo*, we used Stat3 KO mice[25] and their littermate controls (WT). Stat3 KO and WT mice were each divided in four groups: control, +dmPGE_2, +DOX, and + dmPGE_2+DOX. The survival rate of animals was 90–100% in all groups. DOX treatment did not impair survival rate, neither in WT animals nor Stat3 KO mice. Likewise, the heart weight/body weight ratio was similar in all animal groups. After treatment, cardiac tissue ($n = 5$–7 animals per group) was assessed for apoptosis by measuring DNA fragmentation. As shown in *Figure 6*, no difference was observed in myocardial DNA fragmentation between WT and Stat3 KO mice at baseline. Treatment with DOX induced an increase in cardiac DNA fragmentation which reached a higher significance level in Stat3 KO mice than in WT animals. Our results confirm previous data from the literature reporting that Stat3 KO mice are more sensitive to DOX treatment than WT animals.[22] As expected, the increase in DOX-induced cardiac apoptosis was markedly attenuated by dmPGE_2 injections in WT mice. In contrast, the same treatment remained without effect in Stat3 KO mice. Of note, in dmPGE_2-treated animals, cardiac apoptosis was significantly different between WT and Stat3 KO mice, whether or not animals had been injected with DOX.

Our investigations, both *in vitro* and *in vivo*, demonstrate that the protective effect of PGE_2 against DOX-induced cardiac apoptosis is Stat3-dependent.

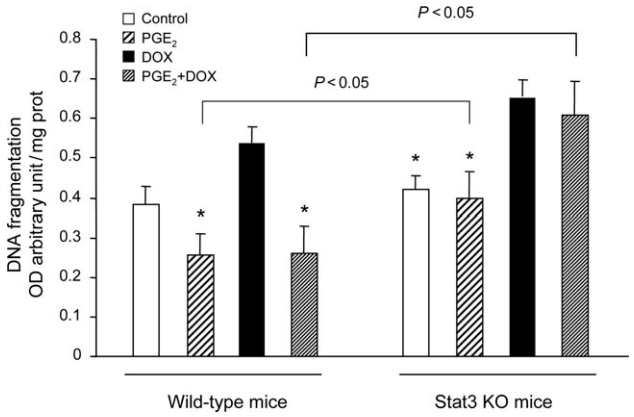

Figure 6 Stat3 plays a role in the inhibitory effect of 16,16-dimethyl prostaglandin E_2 on doxorubicin-induced myocardial DNA fragmentation *in vivo*. Wild-type and Stat3 knockout mice were treated or not (control) with the stable prostaglandin E_2 analogue 16,16-dimethyl prostaglandin E_2 (PGE_2) for 2 days before and 5 days after a single injection of doxorubicin (DOX) (12 mg/kg, i.p.). Thereafter, mice were killed, hearts were removed and cardiac tissue processed for DNA fragmentation analysis. Apoptotic DNA fragmentation in cardiac tissue was assessed by measuring histone-associated DNA fragments. Results are expressed as arbitrary absorbance units normalized to mg of proteins. *$P < 0.05$ compared with values from doxorubicin-treated mice ($n = 5$–7 animals per group).

4. Discussion

Our study revealed that in ventricular cardiomyocytes, PGE_2 exerts antiapoptotic activities which are mediated by Stat3 involving ERK1/2. Indeed, PGE_2 was found to counteract DOX-induced caspase3 activation and apoptotic DNA fragmentation which was associated with impaired survival in ventricular cardiomyocytes. By transfecting cardiomyocytes with siRNA targeting specifically rat Stat3, we obtained 50–60% inhibition of Stat3 expression. In these Stat3 silenced cells, the antiapoptotic effect of PGE_2 on DOX-induced caspase3 activation and DNA fragmentation was significantly reduced. Likewise, the Stat3 pathway inhibitors, piceatannol and AG490, abolished the antiapoptotic effect of PGE_2 on DNA fragmentation generated by DOX. The role of Stat3 in PGE_2-induced antiapoptotic activity was further confirmed *in vivo* using WT and Stat3 KO mice. While treatment with the stable analogue dmPGE_2 significantly inhibited the DOX-induced increase in myocardial apoptotic DNA fragmentation in WT mice, it had no effect in Stat3 KO mice.

According to a recently published, fundamental hypothesis,[33] the myocardium adaptation to stress depends on the nature of the signalling stimulus. The cardiomyocyte can either survive leading to beneficial or adaptive hypertrophy[34] or undergo apoptosis, which induces left ventricular failure and dilation (maladaptive hypertrophy).[35] Including observations from a previous study,[24] our results suggest that PGE_2 is beneficial for the heart, favouring an adaptive pattern of hypertrophy by its dual action on cell survival and hypertrophic growth, via the activation of Stat3.

COX-2 has been shown to mediate PGE_2 production in both neonatal and adult cardiac myocytes.[36,37] Moreover, several *in vitro* and *in vivo* studies reported that COX-2 and its prostanoid products as well as Stat3 similarly counteract DOX- and oxidative stress-induced myocardial damage in both neonatal and young adult ventricular cardiomyocytes.[9–11,19,21–23] However, there is little information concerning the effect of PGE_2 or other COX-2-derived products on myocardial Stat3 activation and its cardioprotective role. Very recently, Qian *et al.*[38] using cardiac EP4-deficient mice, reported that this prostaglandin receptor plays a role in hypertrophy via activation of Stat3 which seems cardioprotective in mice with myocardial infarction.

There is growing evidence that Stat3 plays an important role in cardiac remodelling, particularly by promoting cardiomyocyte survival and hypertrophy.[19,20,23] Moreover, it has been demonstrated that Stat3 activation confers cardioprotection at the time of reperfusion in response to ischaemic preconditioning and TNFα.[15,17,25] Interestingly, a recent study reported a decrease in myocardial Stat3 levels in the aged mouse heart associated with a loss of cardioprotection induced by ischaemic post-conditioning.[39]

Consistent with our results, the prostacyclin analogue iloprost was shown to prevent the release of lactate dehydrogenase induced by DOX in ventricular cardiomyocytes, while COX-2 inhibition increased this deleterious response.[9] *In vivo*, it has been shown that inhibition of COX-2 aggravates DOX-induced cardiac injury as detected by cardiomyocyte apoptosis and the release of lactate dehydrogenase and cardiac troponin C.[10] Moreover, Neilan *et al.*[11] observed that DOX-induced cardiac apoptosis and dysfunction was significantly higher in COX-2 KO than in WT mice, and that this DOX-induced myocardial toxicity was attenuated

by the stable prostacyclin analogue iloprost which was administered 2 days before and 5 days after DOX injection. The latter finding is in agreement with the myocardial antiapoptotic effect of dmPGE$_2$ we observed *in vivo*, using a similar experimental protocol.

Interestingly, Delgado *et al.*[40] showed in a murine model, that when COX-2 inhibition treatment was initiated after heart failure had been established by prolonged DOX administration, it attenuated further progression of this cardiomyopathy. The authors reasoned that in their experimental setting, the effects of COX-2 inhibition cannot be related to an interaction with the effects of DOX. They further speculate that the role of COX-2 is initially adaptive, attenuating the deleterious effects during DOX treatment but later, after removal of this stress, it becomes maladaptive, leading to the progression of heart failure.

Confirming the cardioprotective role of COX-2, several studies have demonstrated that this enzyme and its products reduce I/R injury[12–15] and mediate the late phase of preconditioning.[15,41] Indeed, both prostaglandin receptor EP3 and EP4 signalling have been reported to protect the heart from I/R injury.[13,14] Moreover, Xuan *et al.*[15] have shown that activation of Stat3 leading to up-regulation of COX-2 underlies the protective effect of late preconditioning against myocardial I/R injury. From the point of view of a positive feed back phenomenon, the stimulatory role of PGE$_2$ in Stat3 activation which we demonstrated in our work, could contribute to this cardioprotective process in cardiac myocytes.

In this study, we also found a role for ERK1/2 in the Stat3-dependent, cardioprotective effect of PGE$_2$, counteracting DOX-induced apoptosis in cardiomyocytes. Indeed, inhibition of the ERK1/2, but not of the p38 MAPK pathway, strongly reduced the antiapoptotic effect of PGE$_2$ on DOX-induced caspase3 activation and DNA fragmentation in ventricular cardiomyocytes. These observations are in agreement with our previous results showing that in ventricular cardiomyocytes, PGE$_2$-induced Stat3 activation and hypertrophic growth involves ERK1/2 but not p38 MAPK.[24] Consistent with our present results, it has been shown that ERK1/2 is implicated in myocardial survival signalling, while p38 MAPK appears to have pro- or antiapoptotic effects depending on the experimental model.[35] In neonatal ventricular cardiomyocytes, ERK1/2 was found to prevent apoptosis in cells exposed to the anthracycline daunomycin, whereas p38 MAPK appeared to be involved in the induction of apoptosis.[42]

Taken together, our data demonstrate that PGE$_2$ prevents ventricular cardiomyocytes from apoptosis induced by DOX, and that Stat3 activation involving ERK1/2 plays a key role in this protective effect. Our *in vivo* studies confirm the importance of Stat3 in the protective role of PGE$_2$ against DOX-induced cardiac apoptosis. Thus, our investigations underline the potential significance of the PGE$_2$–Stat3 interaction for the development of novel therapeutic strategies offering increased cardioprotection.

Conflict of interest: none declared.

Funding

This work was supported by the Swiss National Science Foundation (grant 310000-108342/1), the Swiss University Conference Foundation, the South African National Research Foundation and the South African Medical Research Council.

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