The Effect of Chronic Doxorubicin Treatment on Mitogen-Activated Protein Kinases and Heat Stress Proteins in Rat Hearts

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Summary

The study has been designed to characterize protein systems involved in the responses of rat hearts to chronic doxorubicin (DOX) treatment. We investigated the influence of DOX on cardiac function, mitogen-activated protein kinases (MAPKs) and heat stress proteins (HSPs). Doxorubicin was administered to rats by intraperitoneal injections over a period of 6 weeks. In control and DOX-treated hearts exposed to 20 min global ischemia and 40 min reperfusion the recovery of contractile function after ischemia/reperfusion (I/R) was determined. The levels and phosphorylation state of proteins in tissue samples were analyzed using specific antibodies. We found an activation of extracellular signal-regulated kinases (ERKs) in rat hearts exposed to DOX treatment and better recovery of contractile function after I/R. Analysis of HSPs showed that DOX induced up-regulation of the levels of HSP60 and down-regulation of HSP70 levels. The levels and/or specific phosphorylation of other studied proteins (p38-MAPK, HSP27, HSP90) were not influenced by DOX. The results point to the possible role of ERKs and some HSPs in mechanisms underlying the response of rat hearts to chronic DOX treatment.

Key words

Doxorubicin • MAPK • Heat stress proteins • Heart

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Introduction

Doxorubicin (DOX) is a type of anti-cancer drug, the clinical use of which is limited by serious adverse effects including cardiotoxicity. These cardiotoxic effects result in cardiac dysfunction and finally congestive heart failure (Mettler *et al.* 1977, Ferrans 1978). Doxorubicin also blocks the cell cycle and induces apoptosis but the mechanisms of myocardial impairment remain uncertain (Wang *et al.* 2004). Several changes may play a role in the pathogenesis of cardiomyopathy induced by DOX. The list includes inhibition of nucleic acid and protein synthesis, release of vasoactive amines, changes in adrenergic function, abnormalities in the mitochondria, lysosomal alterations, altered sarcolemmal Ca^{2+} transport, imbalance of myocardial electrolytes (Singal *et al.* 1987). Moreover, oxidative stress due to overproduction of free radicals and antioxidant-deficit plays an important role in development of DOX cardiomyopathy (Thornalley and Dodd 1985).

Mitogen-activated protein kinase (MAPK) signaling pathways are the primary intermediators of induction of apoptosis by oxidative stress. There are three major MAPK cascades, including extracellular signal-regulated kinases (ERK_S), p38-MAPK and c-Jun NH2-terminal kinases/stress-activated protein kinases ($JNK_S/SAPK_S$). In the cardiovascular system, ERKs are activated by growth factors, cytokines etc. thereby mediating cell survival as well as offer cytoprotection (Sugden and Bogoyevitch 1995, Wang *et al.* 1998). In contrast, JNKs and p38-MAPKs are activated by cellular stresses, including oxidative stress, and are thought to correlate with cardiomyocyte apoptosis and cardiac

pathologies (Kyriakis and Avruch 1996). We have shown previously that MAPKs, especially ERKs play a role in responses of rat hearts to pathological situations (Strnisková *et al.* 2003, Barančík *et al.* 2007). There is also a link between MAPKs and heat stress proteins (HSPs) (Dou *et al.* 2005). HSPs as molecular chaperones play an important role in protein-protein interactions, such as folding and assisting in the establishment of proper protein conformation. These proteins appear to have a significant role in responses and adaptation of myocardium to stress but the role of HSPs in modulation of cardiomyopathy induced by DOX is not yet clear.

The current study investigated the involvement of MAPKs and heat-stress proteins in the responses of rat hearts to chronic doxorubicin treatment.

Methods

Experimental model

Male Wistar rats were divided into two groups. The first group served as a control and animals were treated with saline. In the second group, doxorubicin was administered to rats by intraperitoneal injections over a period of 6 weeks (cumulative dose 15 mg/kg). All animals were housed at a temperature of 22–24 °C in individual cages and fed a regular pellet diet *ad libitum*. After 6 weeks the animals were anaesthetized with sodium pentobarbitone (40 mg/kg, i.p.), heparinized (500 IU) and sacrificed by cervical dislocation. The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 8523, revised 1996).

Perfusion technique

The hearts were rapidly excised, placed in ice-cold perfusion buffer, cannulated via the aorta and placed into the Langendorff system for perfusion at a constant pressure of 70 mm Hg and at 37 °C. Perfusion solution was a modified Krebs-Henseleit buffer gassed with 95 % O_2 and 5 % CO_2 (pH 7.4) containing (in mmol/l): NaCl 118.0, KCl 3.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.18, $CaCl_2$ 2.5, glucose 7.0. Left ventricular pressure was measured by means of a latex water-filled balloon inserted into the left ventricle via the left atrium (adjusted to obtain end-diastolic pressure of 5-7 mm Hg) and connected to a pressure transducer (P23 Db model, Gould Statham Instruments, USA). Maximal rates of pressure development and fall, +dP/dtmax and

–dP/dtmax, as the indexes of contraction and relaxation were monitored during stabilization and pre-ischemia period and were continuously recorded until the end of reperfusion. Recovery of contractile function after I/R was expressed as percentage of preischemic baseline values.

Protocol of experimental ischemia

The Langendorff-perfused hearts were allowed to stabilize (20 min) before further interventions. The hearts were then exposed to 20 min global ischemia induced by stop of aortal inflow and 40 min reperfusion.

Preparation of tissue protein fractions

The tissue samples were obtained from saline-treated and DOX-treated rats at the end of the application period. The tissues from left ventricle were wiped in liquid nitrogen, resuspended in ice-cold buffer A containing (in mmol/l): 20 Tris-HCl, 250 sucrose, 1.0 EGTA, 1.0 dithiothreitol (DTT), 1.0 phenylmethyl-sulfonylfluoride (PMSF) and 0.5 sodium orthovanadate (pH 7.4) and homogenized with a Teflon glass homogenizer. The homogenates were centrifuged at 700 x g for 5 min at 4 °C, pellets after this centrifugation were discarded and the supernatants were centrifuged again at 13,600 x g for 30 min. The postmitochondrial supernatants after this second centrifugation, termed as soluble fractions, were used for further analysis. The protein concentrations were estimated by the method of Bradford (1976).

Electrophoresis and immunochemical Western blot analysis

Samples of protein fractions containing equivalent amounts of proteins per lane were separated by SDS-PAGE. For Western blot assays, proteins after electrophoretic separation were transferred to nitrocellulose membrane. For primary immunodetection were used antibodies specific against the following proteins: p38-MAPK, ERK, GAPDH (all from Santa Cruz Biotechnology), phospho-p38-MAPK (Thr180/Tyr182), phospho-ERK (Thr202/Tyr204), phospho-HSP27 (Ser82), Hsp90 (all from Cell Signaling Biotechnology), HSP60, HSP70 (both from Sigma). Peroxidase-labeled anti-rabbit or anti-mouse imunoglobulins (Amersham Biosciences) were used as the secondary antibodies. Bound antibodies were detected by the ECL method.
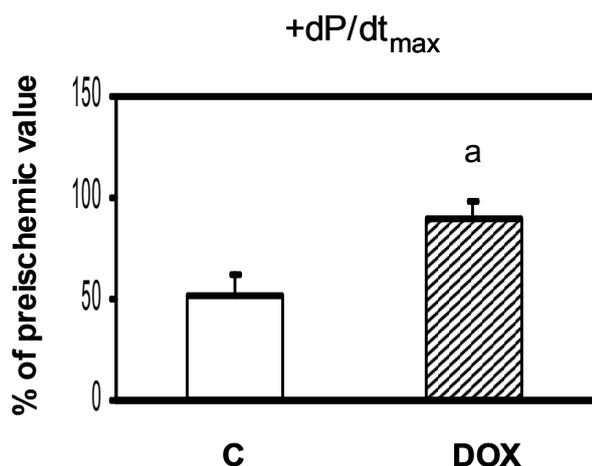
+dP/dt$_{max}$

Fig. 1. Effect of DOX on the recovery of maximal rate of contraction (+dP/dt$_{max}$) after myocardial ischemia and reperfusion. Data are means ± S.E.M expressed in % of baseline values. C – control rat hearts, DOX – doxorubicin-treated hearts, [a] $p < 0.05$ *vs.* control.

Statistical evaluation

Quantification of protein levels was done using Phosphorimager Thyphoon (Amersham Biosciences). Data were expressed as means ± S.E.M. Statistical significance of differences between the groups was analysed by the unpaired Student's t-test. Differences were considered as significant at $p < 0.05$.

Results

Six weeks after the first injection, the body weights were 266±25 g in the DOX-treated group and 278±23 g in the control group. The heart weight/ body weight ratio represented 0.313±0.010 in the DOX-treated group and 0.317±0.020 in the control group.

Effect of chronic doxorubicin treatment on postischemic recovery of contractile function in the rat heart

Figure 1 shows maximal recovery of contraction +dP/dtmax after ischemia/reperfusion in control hearts and hearts of rats after chronic DOX treatment. In DOX-treated hearts significantly improved recovery of +dP/dtmax after ischemia and reperfusion in comparison with control hearts has been observed. The data are expressed as percentage of preischaemic baseline values and represented 89.7±8.8 % in DOX-treated hearts and 51.8±10.9 % in control rat hearts. Recovery of left developed ventricular pressure (LVDP) after ischemia/reperfusion showed similar tendency of changes. In DOX-treated hearts the improved recovery

of LVDP during whole reperfusion has been observed compared to its values in control hearts (data not shown).

Influence of chronic doxorubicin treatment on extracellular signal-regulated kinases (ERKs)

We did not observe significant differences in the levels of total ERKs between the control and DOX-treated rat hearts (Fig. 2A). However, using antibody that reacts specifically with dual phosphorylated ERKs (Thr202/Tyr204) we found that the exposure of rats to DOX was connected with significantly increased specific phosphorylation (activation) of both ERK-1 and ERK-2 (Fig. 2B-D).

Effect of chronic doxorubicin treatment on p38-MAPK

Analysis with antibody specific for p38-MAPK showed that there were not significantly influenced the levels of p38-MAPK in hearts of rats treated with doxorubicin. We also did not observe changes in phosphorylation of p38-MAPK on Thr180/Tyr182 (data not shown). Specific phosphorylation at these sites reflects the activation of p38-MAPK.

Influence of chronic doxorubicin treatment on levels and/or activation of heat stress proteins

Analysis of heat stress proteins showed changes in protein levels of HSP60 and HSP70. Using specific antibody we found that DOX treatment induced up-regulation of the HSP60 levels in comparison with control hearts (Fig. 3). On the other hand, chronic treatment with DOX was associated with a down-regulation of HSP70 expression (Fig. 4). For HSP90, we did not observe significant changes in content of this protein in rat hearts after DOX treatment. Analysis with phospho-specific antibody also did not show significant differences in specific Ser82 phosphorylation of HSP27 after chronic treatment with DOX (data not shown).

Discussion

In the present study we showed that rat hearts exposed to chronic doxorubicin (DOX) treatment show better recovery of contractile function after I/R. The investigation of changes associated with action of DOX revealed that prolonged exposure of rat hearts to DOX led to an activation of extracellular signal-regulated kinases and modulation of protein levels of HSP60 and HSP70.
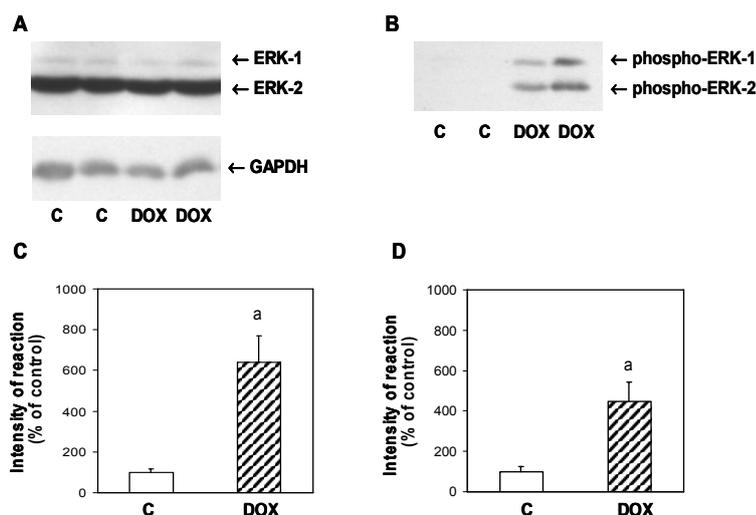
Fig. 2. Effect of chronic DOX treatment on protein levels and activation of extracellular signal-regulated protein kinases (ERKs). **A.** Upper blot record shows ERKs levels in fractions isolated from the left ventricular tissue of control and DOX-treated rat hearts. The ERKs levels were determined using specific antibody. Blot in lower part shows the protein levels of GAPDH. **B.** The changes in specific phosphorylation of ERKs in control and DOX-treated rat hearts. The activation of ERKs was determined using phospho-specific antibody (Thr202/Tyr204). **C.** Quantification of phospho-ERK-1 content in the left ventricular tissue after DOX treatment. **D.** Quantification of phospho-ERK-2 content in the left ventricular tissue after DOX treatment. Data are expressed as a percentage of values for control tissue. Each bar represents mean ± S.E.M. of 5 tissue samples *per* group. ^{a} $p<0.05$ *vs.* control group. C – control hearts; DOX – doxorubicin-treated rat hearts.
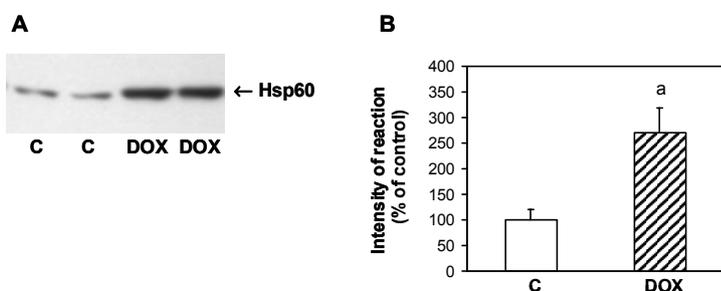

Fig. 3. Effect of chronic DOX treatment on protein levels of HSP60. **A.** Record showing HSP60 protein levels in fractions isolated from the left ventricular tissue of control and DOX-treated rat hearts. The HSP60 levels were determined using specific antibody. **B.** Quantification of HSP60 content. Data are expressed as a percentage of values for control tissue. Each bar represents mean ± S.E.M. of 5 tissue samples *per* group. ^{a} $p<0.05$ *vs.* control group. C – control rat hearts; DOX – doxorubicin-treated hearts.
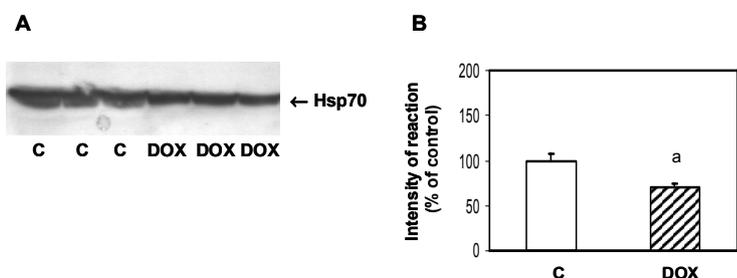

Fig. 4. Effect of chronic DOX treatment on protein levels of HSP70. **A.** Record showing HSP70 levels in protein fractions isolated from the left ventricular tissue of control and DOX-treated rat hearts. The HSP70 levels were determined using specific antibody. **B.** Quantification of Hsp70 content. Data are expressed as a percentage of values for control tissue. Each bar represents mean ± S.E.M. of 5 tissue samples *per* group. ^{a} $p<0.05$ *vs.* control group. C – control rat hearts; DOX– doxorubicin-treated hearts.

DOX and its derivatives are used as chemotherapeutic drugs and it is known that long-term treatment with DOX can result in the development of cardiomyopathy and congestive heart failure. This involves multiple factors including the generation of free radicals, alterations in intracellular Ca^{2+} homeostasis, myocardial cell apoptosis (Kang *et al.* 2000, Singal *et al.* 2000). On the other hand, several cardioprotective substances are produced by the heart in response to the exposure of DOX which may attenuate cardiac injury. The exact mechanisms and biochemical pathways (events) that influence the cytotoxicity of DOX have not been well-characterized. Some studies have shown that

cell growth and survival/death signaling pathways, PI3K/Akt and MAPKs pathways, play important roles in regulating DNA damage agents-induced cell death (Sawyer *et al.* 2002, Wang *et al.* 2002). We found that prolonged exposure of rats to DOX was connected with an increased activation of ERKs and this suggests their possible role in adaptive responses of myocardium to DOX treatment. The important role of ERK pathway in regulation of responses to DOX has been demonstrated in cultured rat neonatal cardiomyocytes (Arai *et al.* 2000). The authors found that DOX activated ERKs, p38-MAPKs and SAPK/JNKs but only inhibition of ERK pathway suppressed the effects of DOX on SERCA2

expression. The ERKs activation usually confers a survival advantage to cells and this indicates that the observed ERKs activation may play a role in adaptive responses that modulate doxorubicin-induced injury in the ischemic myocardium. Also in adult rat ventricular myocytes it has been shown that concerted activation of HER2, ERK1/2, and Akt serves as a salvage pathway against the damaging effects of DOX (Sawyer *et al.* 2002). Moreover, in a recent study it was found that DOX induced the activation of both caspase-3 and Akt in the left ventricle, with the latter effect likely reflecting a protective response to counteract the induction of cardiomyocyte apoptosis (Ichihara *et al.* 2007). Doxorubicin-induced cytoxicity on cardiac cells was found to be prevented also by survival factors (IGF-1) acting through PI3K/Akt kinase pathway (Wu *et al.* 2000). In contrast to ERKs, JNKs and p38-MAPK are thought to correlate with cardiomyocyte apoptosis and cardiac pathologies. DOX was also found to induce apoptosis by activating p38-MAPK and inhibitors of this kinase prevented DOX-induced apoptosis (Kang *et al.* 2000). We did not observe the effects of DOX on p38-MAPK pathway. This was confirmed by the determination of p38-MAPK activation and also phosphorylation of HSP27. This small heat stress protein is a physiological substrate for MAPKAP kinase-2 which is activated by p38-MAPK. However, we did not observe changes in specific phosphorylation of HSP27 in response to DOX treatment. HSPs are essential for maintenance of normal cellular function and recovery after insult in essence acting as a chaperone, thereby preventing protein aggregation and participating in the refolding of damaged proteins following stress (Mayer and Bukau 1998). It was shown that these proteins could

also play a role in modulation of apoptosis (Kim *et al.* 2006) and cell responses to DOX (Ohtsubo *et al.* 2000). We found different efects of DOX on expression of HSPs. The levels of HSP60 were up-regulated and the levels of HSP70 down-regulated in DOX-treated rat hearts. On the other hand, the HSP90 was not significantly influenced by the effect of DOX. The observed data suggest a link between HSP60 and HSP70 and cardiac function in hearts influenced by DOX. The connection between DOX and HSP60 was observed also in cardiomyocytes where it was found that overexpression of HSP60 significantly reduced DOX-mediated induction of pro-apoptotic Bad protein (Shan *et al.* 2003). The influence of HSPs on activation of some protein kinases is also possible, and a recent study reported that the HSP90 inhibitor can reduce the phosphorylation of ERK without changing its total protein level. Furthermore, treating cells with HSP90 inhibitors decreased the protein level of Raf (Dou *et al.* 2005).

In conclusion, the data demonstrate the changes in signaling pathway of extracellular signal-regulated protein kinases and heat-stress proteins as a consequence of chronic treatment with doxorubicin. The latter might be potentially involved in the mechanisms underlying the modulation of cardiac functions.

Conflict of Interest
There is no conflict of interest.

Acknowledgements

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