Original Article

Chronic cardiotoxicity of doxorubicin involves activation of myocardial and circulating matrix metalloproteinases in rats

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Aim: To investigate the role of matrix metalloproteinases (MMPs) in the responses of rats to a prolonged doxorubicin (DOX) treatment. **Methods:** Male Wistar rats were used. DOX was administered by intraperitoneal injections of seven doses (cumulative dose was 15 mg/kg). Control animals were treated with saline. Tissue or plasma samples were collected at four and eight weeks after the application of the last dose. Protein levels were determined by immunoblot assay, and MMP activities were measured by gelatin zymography. Superoxide content was analyzed using a lucigenin chemiluminescence assay and superoxide dismutase (SOD) activities with a SOD assay kit. Qualitative structural alterations of the heart were characterized by transmission electron microscopy.

Results: Systolic blood pressure was higher in DOX-treated rats as compared with the control rats at 8 weeks after treatment. In contrast, there were no differences in the heart rate between the control and DOX-treated rats. DOX treatment caused marked heterogeneous subcellular alterations of cardiomyocytes and structural disorganizations of the cardiac extracellular space. The effects of DOX were linked to a stimulation of plasma MMP-2 and MMP-9 activities that had already increased by 4 weeks after the end of the treatment. In the left ventricle, however, DOX only led to increased MMP-2 activation at 8 weeks after the end of treatment. These changes in tissue MMP-2 were connected with stimulation of Akt kinase activation, inhibition of SOD, an increase in superoxide levels, induction of iNOS protein expression and caspase-3 activation.

Conclusion: Our results show that MMPs are involved in the chronic cardiotoxicity of DOX in rats. The data also suggest that reactive oxygen species (superoxide), NO production (iNOS) and the Akt kinase pathway can modulate MMP-2 activities in rat hearts influenced by DOX.

Keywords: doxorubicin; cardiotoxicity; matrix metalloproteinases; Akt kinase; heart; reactive oxygen species

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Introduction

The anthracycline compound doxorubicin (DOX) is an anti-cancer drug with limited clinical use due to its serious adverse effects, including cumulative dose-dependent cardiac toxicity. The specific mechanisms of doxorubicin cardiotoxicity are complex and, despite decades of research, remain unclear. Several mechanisms may play a role in the effects induced by DOX, including inhibition of nucleic acid and protein synthesis, changes in adrenergic function, mitochondrial abnormalities, lysosomal alterations, altered sarcolemmal Ca²⁺ transport, and an imbalance of myocardial electrolytes^[1]. Oxidative/nitrosative stress plays an important role in the realization of

the effects of DOX. Due to this stress, there is an increased production of free radicals and a modulation of downstream effector signaling pathways^[2]. The effects of DOX are mediated through the induction of reactive oxygen species (ROS) or via a DOX-mediated induction of nitric oxide synthases (NOS), leading to nitric oxide (NO) formation. Identification of the proteins with altered expression and/or activities following DOX treatment and an understanding of their effector cell signaling pathways may provide new insights into the mechanisms responsible for the toxic effects of DOX.

DOX treatment can produce a number of consequences at the subcellular level that may persist for a prolonged time after its application. For example, the delay between anthracycline administration and the occurrence of clinical symptoms is generally 1–2 years in adults but may extend to much longer periods in children^[3]. In experimental animal models,

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some side effects of DOX treatment can take weeks or months to occur^[4]. The cardiotoxic effects of DOX often result in a cardiomyopathy that is similar to dilated cardiomyopathy. This cardiomyopathy manifests as a dilation of all of the chambers of the heart and a severe impairment of left ventricular systolic function that eventually leads to congestive heart failure^[5]. Heart failure develops over a prolonged time period and is associated with myocardial remodeling, which also involves changes in the major structural proteins of the extracellular matrix (ECM). The matrix metalloproteinases (MMPs) play an important role in remodeling of the ECM. These enzymes belong to the zinc-dependent endopeptidases family and are synthesized as zymogens that can be activated by proteolytic cleavage of an amino-terminal domain or through an oxidant-induced conformational change (with no change in molecular weight). The activities of MMPs in tissues are also tightly regulated by the endogenous tissue inhibitors of matrix metalloproteinases (TIMPs)^[6].

MMPs are involved in the degradation and remodeling of the ECM matrix under physiological and pathological conditions. Moreover, there is emerging evidence that MMPs can also degrade other non-matrix substrates, including specific cardiac proteins^[7, 8], to affect a number of biological processes within the myocardium. Circulating MMPs have been found to be increased in patients with heart failure and have been proposed to be a prognostic factor for survival in such patients^[9]. It has also been shown that the intracellular actions of MMP-2 are associated with acute ischemia and reperfusion injury^[7]. Furthermore, using isolated heart preparations, it was demonstrated that myocardial injury induced by peroxynitrite, a product of the reaction of superoxide anion and NO, is mediated through the release and activation of MMP-2 within the coronary circulation^[10]. The activation of MMPs has also been implicated in the realization of acute effects of DOX. In mice, it was found that an acute cardiotoxicity induced by doxorubicin applied in a single dose was connected with the increased activities of MMP-2 and MMP-9^[11]. However, the role of MMPs in processes associated with the chronic effects of DOX remains unclear.

The increase in ROS and the dysregulation of nitric NO production play very essential roles in promoting the toxic effects of DOX^[2]. The effects of ROS and NO on MMPs can occur in different ways, including direct posttranslational modifications of MMPs, the induction of intracellular factors that can modulate MMP transcription, and as the induction of signaling molecules, which in turn alter the MMP transcription. Several studies have demonstrated that the expression and activation of MMPs is regulated by protein kinases, such as the MAPKs and PI3K/Akt^[12, 13], and emerging evidence indicates that some protein kinases modulate MMPs in response to DOX in cardiac cells^[14].

This study investigated the involvement of MMPs on the chronic effects induced by repeated application of DOX in rats. Our aim was to characterize the alterations in MMP levels, activation and release several weeks after the application of DOX, and to assess the specific mechanisms involved in the

modulation of MMPs as a consequence of DOX treatment.

Materials and methods

Experimental model

Ten-week-old male Wistar rats were purchased from Dobra Voda (Slovakia). The animals were acclimatized to laboratory conditions for one week prior to the start of the study. In the study, 11-weeks old animals weighing 230–260 g were used. All animals were housed at a temperature of 22–24 °C in individual cages and fed a regular pellet diet *ad libitum*. The rats used in the studies were divided into four experimental groups – two control (C – 4 and 8 weeks) groups and two doxorubicin (DOX – 4 and 8 weeks) groups. Eight animals were used for each experimental group. In the DOX groups, the rats received seven intraperitoneal injections of 2.15 mg/kg of DOX over a 3-week period (the total cumulative dose of DOX was 15 mg/kg). The control animals were treated with saline. The animals were anaesthetized with thiopental (50 mg/kg, ip), and samples of tissues or plasma were collected four or eight weeks after the application of the final dose of DOX or saline. All animal experiments were performed in accordance with the rules issued by the State Veterinary Administration of the Slovak Republic, legislation No 289/2003, according to the regulations of the Animal Research and Care Committee of Institute for Heart Research SAS.

Systolic blood pressure and heart rate measurements

Systemic blood pressure (SBP) and heart rate (HR) were measured non-invasively through tail cuff plethysmography (PowerLab 4/30, ADInstruments) in both the control and DOX-treated groups. For the blood pressure measurements, eight animals in each experimental group were used. The measurements were performed prior to the first DOX or saline application and four or eight weeks after the final application.

Sample collection

At the end of the experiment, the animals were anesthetized with injection with thiopental (50 mg/kg, ip) and were euthanized by thoracotomy and a rapid excision of their hearts. The excised hearts were weighed and separated into the left ventricle (LV) and right ventricle (RV). The whole heart weights (HW) and left ventricular weights (LVW) were registered. The LV is the largest part of the rat heart, comprising nearly the entire heart, and its function is critical for physiological function. Moreover, different pathological stimuli significantly affect the physiological and biochemical parameters of the LV and its function. Therefore, our present measurements were focused on changes in the left ventricular tissue. Further processing of the collected left ventricular tissue samples was dependent on the following assay: The tissue ventricular samples for biochemical analysis were immediately frozen in liquid nitrogen and stored at -75 °C until use. For the transmission electron microscopy studies, small blocks of transmural left ventricular tissues were fixed in buffered 2.5% glutaraldehyde immediately after collection. For measurements of superoxide levels, tissues of the left ventricle were collected

and placed into ice-cold, Krebs-Henseleit buffer.

The plasma samples were prepared from whole artery blood drawn from the chest of the rats immediately after excision of the heart. Citrate was immediately added to the collected blood (resulting concentration 0.76%), followed by centrifugation of the blood for 5 min at 1200×g to obtain the plasma. The prepared plasma samples were stored at -20°C until further analysis.

Transmission electron microscopy

Small (1–2 mm³) transmural ventricular heart tissue samples were routinely processed for electron microscopy. The samples were fixed in 2.5% glutaraldehyde in 100 mmol/L cacodylate buffer at 40°C, washed, and then subsequently postfixed in 1% OsO₄ and embedded in Epon812. Ultrathin sections of the tissue were stained with uranyl acetate and lead citrate. The ultrastructure of the myocardial tissue was evaluated using a transmission electron microscope Tesla 500 (Brno, Czech Republic).

Measurement of superoxide levels

The production of superoxide (O₂^{•-}) was evaluated using a Lucigenin Enhanced Chemiluminescence assay^[15]. Left ventricle tissue samples were cut into small pieces of up to 15 mg wet weight and stored in Krebs-Henseleit buffer on ice until measured. Prior to the assay, the tissues were equilibrated for 20 min at 37°C. During the assay, the tissue samples were incubated in a 50 mmol/L solution of lucigenin in Krebs-Henseleit buffer at 37°C and chemiluminescence was measured every 30 s for 5 min using a Turner Designs TD-20/20 luminometer. The data were expressed as relative luminiscent units per mg of tissue (RLU·mg⁻¹ tissue).

Determination of superoxide dismutase activity

The superoxide dismutase (SOD) activity in the LV was analyzed using a SOD Assay kit (Fluka), which assays SOD activity by utilizing a highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with superoxide anion. The rate of WST-1 reduction with O₂^{•-} is linearly related to the xanthine oxidase activity and is inhibited by SOD. Therefore, the SOD activity was determined to be inhibition activity and was quantified by measuring the color decrease of WST-1-formazan production at 450 nm. Tissue homogenates from isolated left ventricles were analyzed. The tissue was homogenized in phosphate buffer (pH 7.4). The changes in formazan production were analyzed for 30 min at 37°C using a microplate reader (Thermo Scientific Multiscan FC, Finland). The SOD activities were calculated using a SOD standard curve and expressed as U·mg⁻¹ protein.

Determination of total antioxidant status

The total antioxidant status (TAS) of the samples from the LV was determined using a decolorization assay. This assay makes use of a stable ABTS (2,2'-azinobis-(3-ethylbenzothiazolo-

line-6-sulfonic acid)) radical prepared by the reaction of ABTS and potassium persulfate, as described previously^[16]. It is known that the amount of ABTS radical is reduced in the presence of hydrogen-donating antioxidants, and these changes can be measured spectrophotometrically. We incubated the tissue sample homogenates (used also for assay of the SOD activities) with ABTS radical for 3 min at 37°C. A decrease in absorbance caused by antioxidant addition was registered spectrophotometrically at 720 nm, calculated to the concentration of the antioxidant standard Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and expressed as μmol/L of Trolox equivalent.

Preparation of tissue protein fractions

The tissue samples used for Western blot analysis and zymography were obtained from saline-treated and DOX-treated rats at four or eight weeks after the end of the application period. The tissues from the LV were pulverized in liquid nitrogen, resuspended in ice-cold buffer A (20 mmol/L Tris-HCl, 250 mmol/L sucrose, 1.0 mmol/L EGTA, 1.0 mmol/L dithiothreitol (DTT), 1.0 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 0.5 mmol/L sodium orthovanadate, pH 7.4) and homogenized with a Teflon homogenizer. The homogenates were centrifuged at 800×g for 5 min at 4°C. After centrifugation, the pellets were discarded, and the supernatants were centrifuged again at 16100×g for 30 min. The postmitochondrial supernatants after the second centrifugation, termed as soluble fractions, were used for further analysis. The protein concentrations were estimated using the Bradford assay^[17].

Electrophoresis and immunochemical Western blot analysis

Samples of protein fractions containing equivalent amounts of protein per lane (30 μg per lane) or plasma samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For Western blot assays, after an electrophoretic separation, the proteins were transferred onto a nitrocellulose membrane. The quality of the transfer was verified by Ponceau S staining of the nitrocellulose membranes after transfer, and equal protein loading was verified by blotting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the loading control. Specific anti-Akt, anti-MMP-2, anti-SOD-1, anti-NOS-2 (iNOS), anti-GAPDH (all from Santa Cruz Biotechnology), anti-phospho-Akt, anti-cleaved caspase-3 (from Cell Signaling Technology) antibodies were used for the primary antibody immunodetection. Peroxidase-labeled anti-rabbit immunoglobulins (Cell Signaling Technology) were used as the secondary antibodies. The bound antibodies were detected by the enhanced chemiluminescence (ECL) detection method.

Measurement of MMP activities by gelatin zymography

The gelatinolytic activities of MMPs were analyzed by zymography in polyacrylamide gels containing gelatin as a substrate. Laemmli buffer without 2-mercaptoethanol was added to the protein samples, and non-heated samples were subjected to electrophoresis in SDS-polyacrylamide gels co-polymerized

with gelatin (2 mg/mL). After electrophoresis, the gels were washed twice for 20 min each with 50 mmol/L Tris-HCl (pH 7.4), containing 2.5% Triton X-100, and then incubated overnight at 37 °C in a substrate buffer containing 50 mmol/L Tris-HCl, 10 mmol/L CaCl₂ and 1.25% Triton X-100, pH 7.4. After incubation, the gels were stained with 1% Coomassie Brilliant Blue G-250 and then destained with 40% methanol and 10% acetic acid. The gelatinolytic activities of the MMPs were detected as transparent bands against a dark blue background.

Statistical evaluation

A quantification of the proteins from the Western blot and zymography studies was performed using a Phosphorimager Typhoon (Amersham Biosciences). The data were expressed as the means±SD. The statistical significance of the differences between the groups was analyzed by an unpaired Student's *t*-test. The data from measurements of superoxide dismutase activities, superoxide production and TAS were analyzed by one-way ANOVA. Differences were considered as significant at *P*<0.05 for all tests.

Results

Effects of doxorubicin on physiological parameters at four and eight weeks after the end of the treatment

The values of body weight, heart weight, left ventricle weight and heart weight/body weight ratio of saline- and DOX-treated rats obtained at four and eight weeks after the last injection are summarized in Table 1. There were differences in the trends of the body weights of DOX-treated group of rats compared with the control saline-treated animals, and these differences were more pronounced with a prolongation of time after treatment. In animals treated with DOX, the body weights and increases in body weights were lower. However, the weight of the whole heart as well as the weight of LV was not markedly changed compared with control (saline-treated) animals. Eight weeks after the end of treatment, the systolic blood pressure increased in DOX-treated rats compared with control animals (Figure 1A). In contrast, there were no differ-

Figure 1. Effect of doxorubicin treatment on the systolic blood pressure (A) and heart rate (B). These parameters were measured by the non-invasive method of tail cuff plethysmography in both control and DOX-treated groups of rats. The measurements were performed before the first DOX or saline application and 4 or 8 weeks after end of their application. In each experimental group, eight animals were used for blood pressure and heart rate measurements and bars represent mean±SD from these independent measurements. C- control saline-treated rats; DOX- doxorubicin-treated rats; 0- rats before start of treatment; 4 weeks- rats 4 weeks after end of treatment; 8 weeks- rats 8 weeks after end of treatment. ^b*P*< 0.05 vs corresponding control value.

ences in the heart rate between the saline- and DOX-treated groups (Figure 1B).

Electron microscopic analysis

An electron microscopic examination of the hearts of the control rats showed a classic intact ultrastructure of the myocytes, without any abnormalities in the extracellular space (Figure 2A). DOX induced heterogeneous subcellular alterations, characterized by a degeneration and/or loss of myofibrils, and cytoplasmic vacuolization of cardiomyocytes (Figure 2B). These changes are known structural markers of DOX cardiotoxicity. We also observed a structural disorganization of the cardiac extracellular space (Figure 2C), which was manifested by an increased density of extracellular matrix proteins. Simultaneously, ultrastructural analysis also demonstrated the presence of damaged endothelial cells of the capillaries (Figure 2D). The damage was characterized by the occasional disruption of plasmalemmal integrity, injured mitochondria, changes in cytoplasm density, quantity of ribosomes, pinocytic vesicles, and occasional disorganization and clumping of chromatin, indicating endothelial cell activation and dysfunction. Moreover, in DOX-treated rat hearts, an increased accumulation of interstitial collagen fibers, the presence of macrophages, vacuoles forming large membrane-bound spaces, Cajal cell processes, and proliferating fibroblasts were observed. All the

Table 1. The effect of doxorubicin treatment on body weight (BW), BW increase, heart weight (HW), ventricular weight and ratio of heart to body weight. BW increases are values relative to initial body weights prior to drug treatment. C-4: control group, 4 weeks after the end of saline treatment; C-8: control group, 8 weeks after the end of saline treatment; DOX-4: doxorubicin group, 4 weeks after the end of doxorubicin treatment; DOX-8: doxorubicin group, 8 weeks after the end of doxorubicin treatment. Mean±SD. ^b*P*<0.05 vs corresponding control value.

	4 weeks		8 weeks	
	C-4	DOX-4	C-8	DOX-8
Body weight (g)	388±29	359±44	418±39	377±52 ^b
BW increase (g)	132±27	109±33	180±31	152±23 ^b
Heart weight (g)	0.95±0.15	0.94±0.09	1.03±0.10	0.98±0.11
HW/BW (mg/g)	2.47±0.46	2.65±0.49	2.47±0.32	2.64±0.33
LV weight (g)	0.52±0.12	0.53±0.11	0.66±0.09	0.62±0.08

Figure 2. Representative electron microscopic images showing changes in ultrastructure of rat hearts 8 weeks after the application of the last dose of saline or doxorubicin. (A) Electron micrograph of control heart showing normal architecture of cardiomyocytes and without changes in extracellular space. CM – cardiomyocytes; EC – extracellular space. Bar: 1 μ m. (B) Electron micrograph of the myocardium affected with doxorubicin demonstrating injury of cardiomyocytes and dense extracellular space. E – capillary endothelial cells; ECS – extracellular space; CM – cardiomyocytes. Bar: 1 μ m. (C) Extracellular space (ECS) of the myocardium affected with DOX is filled with irregularly distributed dense material, mitochondria (Mi), vacuoles and myelinized structures (arrows) and extravasated red cells (R). E – endothelial cells of capillary; CM – cardiomyocytes. Bar: 1 μ m. (D) Subcellular injury of capillary endothelial cell (E) manifested with disrupted plasmalemma (arrow), reduced cytoplasm density and clumping of chromatin. N – nucleus; ECS – extracellular space; CM – cardiomyocytes, L – lumen. Bar: 1 μ m.

observed changes had occurred at four weeks after the end of treatment and were more pronounced after eight weeks of treatment.

Doxorubicin treatment modulates the activities of matrix metalloproteinases

In our study, we investigated the consequences of DOX treatment on MMPs present in heart tissue as well as on MMPs that were released into the circulation. The MMPs activities were analyzed by zymography using gelatin as a substrate. As a positive control to identify the 63-kDa MMP-2 we used a recombinant active 63-kDa MMP-2 (Calbiochem). Pro-MMP-2 and MMP-9 were identified using fetal bovine serum contain-

ing predominantly activity of the 72-kDa form of MMP-2 and MMP-9.

In ventricular tissue samples, we predominantly detected enzymatic gelatinolytic activities of the 72-kDa MMP-2. DOX treatment markedly increased the activity of this form of the enzyme, as assayed at eight weeks after end of treatment. However, activity of the 72-kDa MMP-2 in the LV did not change at four weeks after the end of DOX treatment (Figure 3A, 3B). For the 63-kDa form of MMP-2 and MMP-9, we did not observe intensive gelatinolytic bands in the samples isolated from the LV, and the application of DOX also did not induce additional activation of these forms of MMPs. As shown in Figure 3C, the observed effects of DOX on activity of the 72-kDa form of MMP-2 were not connected with a modulation of the protein levels of this enzyme. The protein levels of MMP-2 were determined using a specific antibody that reacts with both the 72-kDa and 63-kDa forms of MMP-2, and our results did not show any differences in the protein levels of tissue MMP-2 between saline- and DOX-treated hearts at four or eight weeks after the end of the treatment. The observed increase of 72-kDa MMP-2 activity at eight weeks after the end of the DOX application was also not related to changes in levels of TIMP-2 (tissue inhibitor of MMPs) (Figure 3C).

A zymographic analysis of blood plasma samples revealed several bands corresponding to the gelatinolytic activities of proteinases. Using positive controls, we identified the activities of 72-kDa MMP-2 and 92-kDa MMP-9. We found that the activities of both MMPs increased in the plasma of DOX-treated rats at both four and eight weeks after the end of DOX treatment (Figure 4A, 4C, 4D). Using a specific antibody, we predominantly detected the presence of the 72-kDa form of MMP-2 in the plasma, but we did not observe differences in protein levels of this enzyme between the plasma samples of saline- and DOX-treated rats (Figure 4B).

Doxorubicin treatment increases superoxide production, iNOS levels and cleaved caspase-3 levels in the left ventricle of rat hearts

An activation of the non-cleaved oxidatively activated 72-kDa form of tissue ventricular MMP-2 suggested a potential alteration of its enzyme activity due to the action of radicals. To determine a possible role for superoxide radical formation, we measured the levels of superoxide in the LV after application of DOX in conditions where the 72-kDa MMP-2 had been activated. As shown in Figure 5A, DOX treatment induced an increase in superoxide levels at eight weeks after the end of the DOX application. This increase in superoxide levels was connected with a decreased enzymatic activity of SOD (Figure 5B) and a significant lowering of total antioxidant status (TAS). The TAS was determined using ABTS radical for antioxidant determination, and the value obtained in the DOX group at eight weeks after the end of the DOX application (8.90 ± 1.40 μ mol/L Trolox equivalent) represented 73% of the corresponding control value (12.23 ± 0.76 μ mol/L Trolox equivalent). However, superoxide levels, SOD activities and TAS had not changed at four weeks after DOX treatment. To

Figure 3. Effect of doxorubicin treatment on tissue matrix metalloproteinases and TIMP-2. The MMPs and TIMP-2 were analyzed in protein fractions isolated from the left ventricular tissue of control (C) and doxorubicin-treated (DOX) rat hearts obtained 4 weeks (C-4, DOX-4) or 8 weeks (C-8, DOX-8) at the end of the treatment. Tissue samples obtained from six animals in each experimental group were used for the analysis. (A) Records showing the influence of DOX-treatment on gelatinolytic activity of tissue 72 kDa MMP-2. The MMP activities were analyzed by zymography in polyacrylamide gels containing gelatin as a substrate and enzymatic gelatinolytic activities of 72 kDa MMP-2 were detected predominantly in samples from left ventricle. (B) Quantitative analysis of MMP-2 activities. Data are expressed as a percentage of value for corresponding control. Each bar represents mean $\pm$ SD of 6 independent tissue samples per group. ^b $P < 0.05$ vs corresponding control value. (C) Effect of DOX treatment on protein levels of MMP-2, TIMP-2, and GAPDH. The levels of proteins were analyzed by Western blot analysis using specific antibodies. An antibody specific for MMP-2 reacted with both 72 kDa and 63 kDa forms of MMP-2. Protein loading was confirmed by using GAPDH.

investigate whether the changes in superoxide levels and SOD activities are associated with a modulation of SOD expression, we measured the protein levels of SOD-1. We did not observe any changes in SOD-1 protein levels as a consequence of DOX application (Figure 5C, 5D).

Another radical implicated in the toxic effects of DOX is nitric oxide produced by NO synthases (NOS). We assayed for changes in iNOS expression and found that DOX treatment induced an upregulation of iNOS protein levels (Figure 6A, 6B). These changes had already occurred by four weeks after the end of DOX treatment and were more pronounced at eight

Figure 4. Effect of doxorubicin treatment on plasma matrix metalloproteinases. The MMPs released into circulation were analyzed in plasma samples prepared from whole artery blood of control (C) and doxorubicin-treated (DOX) rats obtained 4 weeks (C-4, DOX-4) or 8 weeks (C-8, DOX-8) after the end of the treatment. Blood plasma samples obtained from six animals in each experimental group were used for the analysis. (A) Records showing the influence of DOX-treatment on gelatinolytic activities of MMPs. Zymographic analysis of blood plasma samples revealed several bands corresponding to the gelatinolytic activities of several proteinases and using positive controls the activities of 72 kDa MMP-2 and 92 kDa MMP-9 were identified. (B) The records showing protein levels of MMP-2 determined in plasma samples by Western blot analysis using a specific antibody which detected predominantly the presence of 72 kDa form of MMP-2 in plasma. (C) Quantitative analysis of MMP-2 activities. Data are expressed as a percentage of value for corresponding control. Each bar represents mean $\pm$ SD of 6 independent plasma samples per group. ^b $P < 0.05$ vs corresponding control value. (D) Quantitative analysis of MMP-9 activities. The data are expressed as a percentage of value for corresponding control. Each bar represents mean $\pm$ SD of 6 independent plasma samples per group. ^b $P < 0.05$ vs corresponding control value.

weeks after the treatment. We also observed an increased amount of cleaved (activated) caspase-3 in the hearts of DOX-treated rats, and these changes were more evident at eight weeks after the end of the treatment (Figure 6C, 6D).

Chronic effects of doxorubicin treatment are connected with stimulation of Akt kinase

To characterize the influence of DOX treatment on the protein

Figure 5. Effect of doxorubicin treatment on levels of superoxide anion (O_2^-), activities of SOD and protein levels of SOD-1. Left ventricular tissue of control (C) and doxorubicin-treated (DOX) rat hearts obtained 4 weeks (C-4, DOX-4) or 8 weeks (C-8, DOX-8) at the end of the treatment was used for the analysis. Tissue of left ventricle obtained from six animals in each experimental group was used for the analysis. (A) Doxorubicin treatment induced increase in levels of superoxide (O_2^-) 8 weeks after the end of the DOX application. On the other hand, superoxide levels were not changed 4 weeks after DOX treatment. The superoxide levels were evaluated using Lucigenin Enhanced Chemiluminescence assay and the obtained data were expressed as relative luminiscent unit per mg of tissue ($RLU \cdot mg^{-1}$ tissue). Each bar represents mean $\pm$ SD of six independent measurements. $^bP < 0.05$ vs corresponding control value. (B) Doxorubicin treatment induced inhibition of enzymatic activities of superoxide dismutase (SOD) 8 weeks after the end of the drug application. The SOD activities in left ventricle were analyzed using the SOD Assay kit (Fluka) and are expressed in $U \cdot mg^{-1}$ of protein. Each bar represents mean $\pm$ SD of six independent measurements. $^bP < 0.05$ vs corresponding control value. (C) The record showing the effects of DOX treatment on protein levels of SOD-1. The protein levels of SOD-1 were analyzed by Western blot analysis. (D) Quantitative analysis of SOD-1 protein levels. The data are expressed as a percentage of value for corresponding control. Each bar represents mean $\pm$ SD of 6 independent tissue samples per group. $^bP < 0.05$ vs corresponding control value.

blot analysis of heart left ventricular tissue samples obtained at four or eight weeks after treatment with saline (controls) levels and activation of Akt kinase, we performed a Western

Figure 6. Effect of chronic DOX treatment on protein levels iNOS and cleaved caspase-3. For the analysis protein fractions isolated from the left ventricular tissue of control (C) and doxorubicin-treated (DOX) rat hearts obtained 4 weeks (C-4, DOX-4) or 8 weeks (C-8, DOX-8) at the end of the treatment were used. Tissue samples obtained from six animals in each experimental group were used. (A) Western blot record showing the influence of saline and DOX on iNOS protein levels induction. The protein levels of iNOS were analyzed by Western blot analysis. (B) Quantitative analysis of iNOS protein levels. The data are expressed as a percentage of value for corresponding control. Each bar represents mean $\pm$ SD of 6 independent tissue samples per group. $^bP < 0.05$ vs corresponding control value. (C) Western blot record showing the influence of saline and DOX on caspase-3 activation. Levels of fragment of activated caspase-3 resulting from cleavage adjacent to Asp175 were determined using a specific antibody. (D) Quantification of cleaved caspase-3 content in the left ventricular tissue after DOX treatment. The data were obtained from Western blot records and are expressed as a percentage of value for corresponding control. Each bar represents mean $\pm$ SD of 6 independent tissue samples per group. $^bP < 0.05$ vs corresponding control value.

or DOX (DOX-treated rats). This analysis used an antibody specific for Akt kinase and revealed no differences in the levels of Akt kinase between the control and DOX-treated hearts at either four or eight weeks after the treatment (Figure 7A). However, detection with a phosphospecific antibody (Ser473) showed an increased phosphorylation of Akt kinase specifically on Ser473 in the LV of DOX-treated rat hearts at eight weeks after the end of the treatment (Figure 7A). The observed levels of active Akt kinase at eight weeks after the end of DOX treatment were also increased in relation to total

Figure 7. Effect of chronic DOX treatment on protein levels and activation of Akt kinase. For the analysis protein fractions isolated from the left ventricular tissue of control (C) and doxorubicin-treated (DOX) rat hearts obtained 4 weeks (C-4, DOX-4) or 8 weeks (C-8, DOX-8) at the end of the treatment were used. Tissue samples obtained from six animals in each experimental group were used for the analysis. (A) Western blot record showing the influence of saline and DOX on protein levels and specific phosphorylation of Akt kinase. Levels of Akt kinase were determined by Western blot analysis using a specific antibody. The changes in the specific phosphorylation (activation) of Akt kinase were determined by Western blot analysis using an antibody which reacts with Akt kinase phosphorylated specifically on Ser473. (B) The quantification of Akt kinase phosphorylation and activation. The data were obtained from Western blot records and are expressed as a ratio of content of phosphorylated Akt kinase to total Akt kinase. Each bar represents mean $\pm$ SD of 6 independent tissue samples per group. ^b $P < 0.05$ vs corresponding control value.

Akt kinase levels (Figure 7B).

Discussion

The results of this study provide new insights into the chronic effects of prolonged DOX administration on MMPs several weeks after DOX treatment. We have shown that the activation of myocardial and circulating MMPs, especially MMP-2, is closely associated with the progression of the toxic effects of DOX. Our results also add new evidence that specific cellular signaling mechanisms, such as the Akt kinase pathway, are involved in the regulation of myocardial MMP-2 in relation to the chronic effects of DOX.

The consequences of DOX administration in humans can be acute (occurring during or immediately after treatment), early (occurring within one year of exposure), and late (occurring one or more years after initial exposure)^[3, 4]. This is important from a clinical point of view because in most patients, the cardiotoxicity of DOX develops long after the patient's initial exposure to the drug. The severity of the toxic effects induced by DOX is also dependent on the specific protocol of the DOX treatment (dosage, time, *etc.*). Some strategies to prevent the cardiac toxicity of DOX involve reducing dosages and pro-

longing the DOX infusion time to limit its serum concentrations. In our animal model, we used repeated administration of lower doses of DOX during a prolonged period of three weeks, and we investigated the effects of a pre-treatment with DOX by assessing the outcomes several weeks after the end of the treatment. The time periods of four weeks and eight weeks after the last dose of DOX for functional and other (morphological, biochemical) assessments in the present study were chosen because during these time periods, chronic changes initiated by DOX can already be observed. DOX treatment is often accompanied by cardiotoxic effects that manifest as cardiomyopathy associated with severe systolic dysfunction of the left ventricle^[5]. Our previous study also showed that the application of DOX significantly affected the physiological and several biochemical parameters of the LV^[18]. Therefore, the measurements performed in the present study focused on changes in tissues from the LV. Using electron microscopy, tissue and cardiomyocyte damage following DOX treatment was demonstrated, and no such changes were observed in the control rat hearts. Structural disorganizations of the cardiac extracellular space were also observed, and these alterations could be related to the observed changes in activities of MMPs, enzymes that are known to play an important role in processes of extracellular matrix remodeling.

It has been shown that an activation of myocardial MMPs may be an early marker of acute cardiotoxicity induced by a single ip injection of 25 mg/kg DOX in mice^[11]. However, the role of MMPs in the processes associated with the chronic effects of DOX remains unknown. In a recent study it was documented that chronic anthracycline cardiotoxicity in rabbits is associated with profound changes in left ventricular morphology and function as well as with alterations in the collagen network^[19]. However, despite the alterations of collagen proteins, no changes in MMP activities were found. In the present study, an induction of gelatinolytic activity of the 72-kDa MMP-2 in the LV of DOX-treated rats was found. This increase in MMP activity occurred eight weeks after the end of DOX treatment and was not connected with changes in protein content of this enzyme. The latter finding suggests that the regulation of MMP-2 activity observed in rats exposed to DOX treatment does not occur at the transcriptional level. Moreover, the unchanged protein levels of TIMP-2 suggest that this specific tissue inhibitor also does not play a role in the observed changes of MMP-2. However, the results of some studies indicate that a transcriptional regulation of MMPs, in consequence to DOX action at some time points, cannot be completely ruled out. The increased expression of the genes for myocardial (ventricular) MMP-2 and MMP-9 was documented after an acute treatment with DOX in the mouse model^[2, 20], and the transcriptional activation of several MMPs has also been found in pig myocardium^[21]. One explanation for the differences obtained in specific experimental animal models could be that the changes in MMPs expression are time dependent. This hypothesis is also supported by the results

of a study where MMP-2 mRNA expression increased significantly in DOX-treated mice on days one and two; however, on day four, the MMP-2 mRNA levels declined^[20].

MMP activity can also be regulated through post-translational modifications that include either proteolytic removal of the propeptide domain or zymogen modification by oxidant stress. In the latter mechanism, a powerful endogenous oxidant, such as peroxynitrite, can activate MMPs by reacting with a critical cysteine residue without a loss of the propeptide domain^[22]. The observed activity of the 72-kDa MMP-2 corresponds to the form of MMP-2 that is activated through conformational changes induced by oxidative stress. Thus, the observed increase in the activities of the 72-kDa form of MMP-2 in DOX-treated left ventricular tissue can also be explained through the observed reduction of SOD activities and increased levels of superoxide anion. Furthermore, the observed increase in iNOS protein levels in the left ventricle of DOX-treated rats suggests that the induction of NO production from increased iNOS expression is also a consequence of DOX treatment. Recent reports also indicated that elevated iNOS activity and nitrosative stress are involved in DOX-induced cyto- and cardiotoxicity^[2, 23]. The findings that at eight weeks after DOX treatment, there are higher levels of superoxide anion as well as increased levels of iNOS in the left ventricular tissue point to an increased formation of peroxynitrite, a product of the reaction between NO and superoxide anion. This powerful oxidant could be involved in the observed MMP-2 activation. Moreover, peroxynitrite was also found to stimulate the release of MMP-2 into the circulation from rat hearts during reperfusion^[24].

Our results showed that, in parallel to the observed activation of tissue MMP-2, the levels of the phosphorylated (activated) form of the Akt kinase also increased. The signal transduction pathways linked to DOX-induced ROS formation and oxidative stress have been investigated, and several lines of evidence point to a role for MAPK and PI3K/Akt kinase signaling^[14, 25]. The Akt kinase activation observed in the present study can have several possible roles. First, it may represent an attempt to overcome apoptosis induced as a consequence of DOX action. It has been shown that DOX treatment *in vivo* causes cardiomyocyte apoptosis^[26], and we also observed increased levels of cleaved (activated) caspase-3 in the hearts of DOX-treated rats. It is also known that oxidative stress induced by ROS is accompanied by an activation of Akt kinase, and this enzyme has been proposed to be a reperfusion injury salvage kinase (RISK), counteracting myocardial cell damage through a compensatory protective pathway^[27]. The role of Akt kinase in a salvage pathway to protect against the damaging effects of DOX was documented in rats^[28], and other studies have documented that a single injection of DOX can induce the activation of both caspase-3 and Akt in the left ventricles of mice^[25]. The activations of these factors in mice were observed five days after the onset of treatment, suggesting

that the observed Akt kinase activation likely reflects a protective response to counteract the induction of cardiomyocyte apoptosis. A second possible role for Akt kinase activation may be related to the observed activation of MMP-2. It was demonstrated that the enhancement of MMP-2 and MMP-9 in cardiac myocytes in acute responses to DOX could be mediated through redox-dependent MAPK and PI3K/Akt kinase pathways^[14]. The positive influence of PI3K/Akt kinase pathway on the induction of MMP-2 expression and activation was also demonstrated in studies showing that the activation of Akt kinase resulted in upregulation of MMP-2^[29, 30]. Similarly, our present results show that Akt kinase activation in cardiac tissue (eight weeks after DOX treatment) is connected with a stimulation of tissue MMP-2 activity. However, in contrast to the above studies, we did not observe changes in MMP-2 protein expression. Moreover, our recent observation that activation of Akt kinase induced by a short cycle of ischemia and reperfusion (realized within several minutes) is associated with inhibition of MMP-2 activation^[13] suggests that the roles of Akt kinase in MMP-2 regulation can be different, and this may be attributed to substantial differences in time (acute, chronic effects) and experimental models.

In the present study, we found increased activities of MMP-2 and MMP-9 in the plasma of DOX-treated rats at both four and eight weeks after the end of the treatment. MMPs are synthesized within a wide variety of tissue types. Therefore, the observed changes in plasma MMP activities may not necessarily reflect changes found in the heart. Circulating MMPs have been proposed to be a prognostic factor for survival in patients with heart failure^[9], and increased circulating and tissue MMP-2 and MMP-9 levels have also been reported to play a role in the development of hypertension^[31]. Thus, the observed increases in plasma MMPs activities can also explain the observed elevation of systolic blood pressure in rats eight weeks after DOX treatment. This finding is also supported by the results of a recent study demonstrating that MMPs inhibition may attenuate increases in systolic blood pressure^[32].

In conclusion, our data demonstrate that alterations in MMP activation and release occur several weeks after the application of DOX. These findings suggest that MMPs, especially MMP-2, are closely related to the progression of the toxic effects of DOX and are responsible for the pathogenesis of DOX-induced cardiomyopathy. Moreover, our results show that changes in the activation and release of MMPs can undergo dynamic changes during the time course of developing the toxic effects of DOX. A study of the cellular mechanisms that are involved at the onset of the chronic effects of DOX demonstrated an important role for Akt kinase signaling and oxidative/nitrosative stress. These results also suggest that increased oxidative/nitrosative stress and activation of downstream effector of Akt kinase pathway may play a role in the modulation of MMP-2 activities that occurs in rat hearts as a chronic consequence of DOX treatment.

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Author contribution

Monika IVANOVÁ, Petra ŠIMONČÍKOVÁ, Jana VLKOVIČOVÁ, Monika BARTEKOVÁ and Miroslav BARANČÍK performed the physiological and biochemical experiments; Ľudmila OKRUHLICOVÁ and Narcisa TRIBULOVÁ contributed to the electron microscopic examinations; Ima DOVINOVÁ contributed to the examination of parameters related to oxidative stress; Miroslav BARANČÍK wrote the paper.

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