Matrix metalloproteinase-2 and -9 are induced differently by doxorubicin in H9c2 cells: The role of MAP kinases and NAD(P)H oxidase

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Abstract

Objective: Dysregulation of myocardial metalloproteinases (MMPs) is now regarded as an early contributory mechanism for the initiation and progression of heart failure. Doxorubicin is a strongly cardiotoxic anticancer drug. This study investigates the effects of doxorubicin on myocardial MMP-2 and MMP-9 activation.

Methods: After pre-treatment with or without carvedilol or dexrazoxane, we exposed H9c2 cardiomyocytes to doxorubicin to evaluate reactive oxygen species (ROS) formation and MMP-2 and MMP-9 expression and activation. To investigate the signaling pathways leading to doxorubicin-induced MMP activation, we also examined the phosphorylation of three members of the MAPK family (ERK1/2, p38, and JNK), the effects of selective inhibitors of ERK1/2, p38, and JNK on MMP transcription and activity, the transcription of the NAD(P)H oxidase subunit Nox1, and the effects of the NAD(P)H oxidase inhibitor DPI on MMP activation.

Results: Doxorubicin induces a significant increase in ROS formation and a rapid increase of MMP expression and activation. Pre-treatment with carvedilol or dexrazoxane prevented these effects. We also found that p38 is the MAPK that is mainly responsible for MMP-9 activation through an NAD(P)H-independent mechanism. ERK and JNK modulate the transcription of the NAD(P)H oxidase subunit Nox1, while the JNK/ERK NAD(P)H oxidase cascade is an important pathway that mediates doxorubicin signaling to MMP-2. Inhibition of NAD(P)H oxidase attenuates the increase in MMP-2, but augments the doxorubicin-induced increase in MMP-9.

Conclusions: Enhancement of MMP-2 and MMP-9 in cardiac myocytes in response to doxorubicin is mediated by the cooperation of ERK, JNK, and p38 kinase pathways, most of which are redox dependent.

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1. Introduction

Matrix metalloproteinases (MMPs) are an endogenous family of zinc-dependent enzymes that are responsible for matrix remodeling in physiological conditions and in several disease states [1]. MMPs are regulated at multiple levels including transcription, secretion, and activation of inactive zymogens, while their tissue activity is under the strict control of specific inhibitors, i.e., tissue inhibitors of metalloproteinases (TIMPs) [1]. Several studies suggest that Reactive Oxygen Species (ROS) play a major role in the activation of MMPs [2,3], and that NAD(P)H oxidase activation, a major source of ROS, is a key event in this process [4,5]. The cardiac content of MMP-2 and MMP-9 is increased in both experimental and clinical forms of heart failure [6,7]. When MMP activity is increased with insufficient quenching activity by TIMPs, progressive disruption of the collagen network occurs, thus leading to progressive dilation and remodeling of the left ventricle [8,9].
Doxorubicin is one of the most effective antitumor agents, but its clinical use is limited by cumulative dose-related cardiotoxicity, which may ultimately lead to severe and irreversible cardiomyopathy [10]. The cause of doxorubicin cardiotoxicity is multifactorial, even though most doxorubicin-induced cardiac effects can be attributed to the formation of ROS, which ultimately results in myocyte apoptosis (or programmed cell death) [11,12]. The signal transduction pathways that link doxorubicin-induced oxidative stress and cardiac damage is a topic of strong current interest, and emerging evidence indicates that the MAPK family may mediate this apoptotic process [13,14]. It has recently been observed that doxorubicin also induces MMP-2 and MMP-9 expression and activation in the heart, even though the implicated mechanisms have not been elucidated [15].

A great deal of effort has been made to identify which agents might mitigate the cardiotoxic effects of doxorubicin [10]. Among the various molecules, dexrazoxane is the only drug that has been approved for clinical use [16]. Recent experimental evidence suggests that pre-treatment with carvedilol might be useful in reducing the incidence and severity of cardiomyopathy associated with doxorubicin administration [12,17]. Carvedilol is known to work as an antioxidant, and not as a beta blocker, in the prevention of doxorubicin-induced cardiotoxicity, mainly by counteracting the mitochondria-dependent apoptotic pathway [12,17].

The aim of the present study was to investigate the following: (1) whether doxorubicin increases the transcription and release of MMP-2 and MMP-9 in H9c2 cardiac muscle cells; (2) if MMP-2 and MMP-9 release occurs via NAD(P)H oxidase related redox signaling; (3) the role that members of the MAPK family might play in these signaling pathways; (4) the potential role of the cardioprotective antioxidant agents, dexrazoxane and carvedilol, in the prevention of doxorubicin-induced MMP increases.

2. Methods

2.1. Cell culture

H9c2 rat heart-derived embryonic myocytes (American Type Culture Collection) were cultured as previously described [12]. All studies were conducted under serum free conditions. Cells were always used at less than 70% of confluence.

H9C2 cells were treated with doxorubicin (Sigma) (0.5 μmol/L) [12] for the time indicated for each experiment. The efficacy of pre-treatment with cardioprotective antioxidant agents at preventing doxorubicin-induced effects was also tested. Cells were pre-incubated with carvedilol (10 μmol/L) [12], or atenolol (10 μmol/L) [12], or NAC (10 μmol/L) (Sigma) [18] for 1 h, or with dexrazoxane (20 μmol/L) [19] for 3 h, then the medium was changed and the cells were treated with or without doxorubicin (0.5 μmol/L), for the time indicated for each experiment.

To investigate the role played by MAPK pathways and NAD(P)H oxidase in mediating the doxorubicin signaling to MMP-2 and MMP-9, cells were pre-treated with the ERK1/2 pathway inhibitor PD98059 (50 μmol/L) (Calbiochem) [20], the JNK inhibitor SP600125 (20 μmol/L) [20], or the p38 MAPK inhibitor SB203580 (3 μmol/L) [20] for 1 h, or with diphenyleneiodonium (DPI) (20 μmol/L) [20], an inhibitor of NAD(P)H oxidase, for 90 min, and then were incubated with or without doxorubicin (0.5 μmol/L) [12] for the time indicated for each experiment. Since DPI and the MAPK inhibitors were dissolved in dimethyl sulphoxide (DMSO) 0.1%, an equivalent amount of vehicle was added to control and drug-treated samples when the experiments were performed with these inhibitors.

2.2. Flow-cytometric assay of 2’,7’-dichlorodihydrofluorescein

Determination of intracellular free radical production was based on the oxidation of 2’,7’-dichlorodihydrofluorescein (DCFH) (Sigma) to a fluorescent 2’,7’-dichlorofluorescein (DCF) [21]. DCFH was added at a final concentration of 20 μM and incubated for 30 min at 37 °C [12]. Cellular fluorescence was determined by flow cytometry (Becton-Dickinson). Measurements were taken at 510 to 540 nm after excitation of cells at 488 nm with an argon ion laser.

2.3. RT-PCR

Total RNA from H9c2 cells was isolated using TriPure Isolation Reagent (Roche) and was reverse-transcribed into cDNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) employing random hexamers. The quantity of mRNA was normalized for glyceraldehyde phosphate dehydrogenase (GAPDH).

Amplification of the cDNA by polymerase chain reaction (PCR) was performed using PCR Supermix (Fermentas) with the primers shown in Table 1. The PCR products were resolved on 1.7% agarose gel, and quantified by a gel documentation system with analysis software (Syngene).

2.4. Gelatinolytic zymography

H9c2-conditioned media were concentrated by ultrafiltration with centrifugal filter devices (Millipore Corporation) and then subjected to zymography according to previously described conditions [22,23]. Zymographic results were expressed as MMP proteolytic activity and were measured with the help of an image analyzer system (Syngene). Recombinant MMP-9 and MMP-2 were used as standard (Oncogene).

2.5. Western blotting

Western blotting was performed as previously described [24]. We analyzed the phosphorylated MAPK using anti-
bodies against anti-phospho-JNK, anti-phospho-ERK, anti-phospho-p38 (Santa Cruz Biotechnology).

After immunoblotting analysis, secondary antibodies conjugated with peroxidase were visualized with ECL substrates (Amersham Biosciences) and films were quantified by densitometry. Filters were stripped and re-probed with anti-\( \beta \)-actin (Sigma) to confirm equal protein loading.

2.6. Statistical analysis

Results are reported as mean ± standard error of the mean (SEM) of at least 3 independent experiments. Statistical comparisons were made with unpaired Student’s \( t \) test or ANOVA with Sheffe’s test when appropriate. Statistical significance was set at \( P < 0.05 \).

3. Results

3.1. Oxidative stress in doxorubicin treated cells

We used DCFH assays to monitor intracellular oxidant production following doxorubicin administration. Fig. 1A shows a typical flow cytometric histogram of DCF fluorescence in cardiac muscle cells. Adding doxorubicin to cardiac muscle cells caused an increase in the DCF fluorescence (Fig. 1B). Pre-treatment with carvedilol or dexrazoxane reduced the doxorubicin-induced increase of DCF fluorescence. Pre-treatment with atenolol had no significant lowering effect on DCF fluorescence.

3.2. Doxorubicin increases MMP-2, MMP-9 and Nox1 transcription levels. Both carvedilol and dexrazoxane attenuate this effect

Treatment with doxorubicin leads to enhanced transcript levels of MMP-2 and MMP-9 (Fig. 2A). The mRNA expression increased at 3 h and was further enhanced until the 18th h. The transcript levels of TIMP-1 and TIMP-2 were not altered by doxorubicin (Fig. 2A). To begin investigating the role played by NAD(P)H oxidase-dependent ROS generation in MMP activation, we examined the expression of different NAD(P)H oxidase subunits after doxorubicin treatment. Expression of the NAD(P)H oxidase subunit, Nox1 mRNA, increased at 1 h, was further enhanced at 3 h, and remained elevated at 24 h. In contrast, p22phox, p47phox, Nox4 expression levels remained unchanged in response to doxorubicin (Fig. 2B). Pre-treatment with NAC, carvedilol or dexrazoxane significantly blunted enhancement of the doxorubicin-induced MMP-2, MMP-9, and Nox1 transcription levels (Fig. 3).

3.3. DPI, the NAD(P)H oxidase inhibitor, exerts different effects on MMP-2 and MMP-9 transcription and activity

In order to examine the role of NAD(P)H in doxorubicin-induced MMP transcription, we assessed the role that NAD(P)H oxidase plays in ROS generation. Pre-treatment with DPI, an inhibitor of NAD(P)H oxidase, almost completely inhibited the doxorubicin-induced increase in DCF fluorescence (Fig. 4A). The effects that DPI had on the transcript levels of MMP-2 were opposite to what was observed on the transcript levels of MMP-9. In fact, while it attenuated the doxorubicin-induced increase of MMP-2, it increased the effects of doxorubicin on MMP-9 expression (Fig. 4B).

Gelatin zymography results were in keeping with mRNA analysis. In fact, gelatin zymography revealed a significant increase in pro-MMP-2 and pro-MMP-9 activity in doxorubicin-treated cells, and showed that such increase diminishes significantly as a result of pre-treatment with NAC (data not shown), carvedilol, or dexrazoxane. Similarly, the doxorubicin-induced increase of pro-MMP-2 and pro-MMP-9 activity was respectively attenuated and enhanced by pre-treatment with DPI (Fig. 5).

3.4. The doxorubicin-induced expression and activity of MMP-2 and MMP-9 is differentially regulated by members of the MAPK family

To investigate the role played by MAPKs on MMP-2 and MMP-9 expression, we used three compounds: SB203580, which inhibits the kinase activity of p38 kinase; PD098059, which inhibits MEK1/2, the upstream activator of ERK1/2; and SP600125, a specific inhibitor of the JNK pathway. Fig. 6A shows the differential effects of these inhibitors on mRNA
transcriptional levels and activity of MMP-2, MMP-9, and Nox1 in doxorubicin treated cells.

p38 kinase inhibition abolished the doxorubicin-induced increase of MMP-9 mRNA transcription levels without affecting either the transcription of MMP-2 or the transcription of Nox1. Pre-treatment with the ERK 1/2 inhibitor did not affect the mRNA transcript level of MMP-9, though it did lead to a significant increase in the mRNA expression of both MMP-2 and Nox1. Pre-treatment with the JNK inhibitor enhanced the doxorubicin-induced increase of MMP-9 transcript levels and completely inhibited the effects of doxorubicin on the expression of MMP-2 and Nox1. Gelatin zymography confirmed the mRNA data (Fig. 6C). Pre-treatment with MAPK inhibitors did not affect MMP expression or activity (Fig. 6B and D).

3.5. Activation of MAPKs

To study the activation of ERK1/2, p38 kinase, and JNKs, H9c2 cells were exposed to doxorubicin after pre-treatment with or without carvedilol or dexrazoxane. Since neither carvedilol nor dexrazoxane alone had any effect on MMP transcription (Fig. 3) or gelatinolytic activity (data not shown), cells were not incubated with either of these drugs alone. Cells were lysed at different time intervals (10, 20, 40, and 60 min). The activation state was determined by immunoblotting using commercially available antibodies that recognize the active phosphorylated form of each protein. MAPKs were maximally phosphorylated at 20 min and remained elevated until the 60th min. Stimulation of cells with doxorubicin caused marked activation of p38 kinase
Fig. 2. Effects of doxorubicin on the mRNA expression levels of MMP-2, MMP-9, TIMP-1 and TIMP-2 (A), and on the mRNA expression levels of NAD(P)H oxidase subunits Nox1, Nox4, p22phox, p47phox (B). Cells were treated with doxorubicin for the indicated time. The upper panels show the results of a single experiment and lower panels are data in mean ± SEM based on three separate experiments. *P < 0.05 vs untreated.

Fig. 3. Pre-treatment with NAC, carvedilol or dexrazoxane, but not with atenolol, blunts the doxorubicin-induced increases of MMP-2, MMP-9, and Nox1 mRNA expression levels. Cells were pre-treated with NAC, or carvedilol, or atenolol for 1 h, or with dexrazoxane for 3 h and then were incubated with or without doxorubicin for 18 h. Ct = control; Dox = doxorubicin; NAC = N-acetyl-L-cysteine; Carv = carvedilol; Dex = dexrazoxane; Aten = atenolol. The upper panel shows the result of a single experiment. Histograms represent normalized band densitometry readings averaged from 3 independent experiments ± SEM. *P < 0.05 vs Ct; †P < 0.05 vs Dox.
which was almost completely abolished by pre-treatment with carvedilol or dexrazoxane. Exposing the cells to doxorubicin also caused a marked activation of JNKs, although this was unaffected by pre-treatment with NAC (data not shown), carvedilol, or dexrazoxane. Doxorubicin failed to activate ERK1/2, but a significant increase in ERK1/2 activity was detectable after pre-treatment with either carvedilol or dexrazoxane (Fig. 7).

4. Discussion

Our results show that low doses of doxorubicin, which had previously been found to induce apoptotic, and not necrotic cell death in cardiomyocytes, also induce early transcription and activation of MMP-2 and MMP-9 in these cells, without changing the transcript levels of their inhibitors, TIMP-1 and TIMP-2. This finding adds a new item to the long list of mechanisms involved in doxorubicin-induced cardiomyopathy [11]. In fact, the disparity between MMP and TIMP levels favors MMP activation, which results in discontinuities within the fibrillar extracellular matrix network, loss of normal structural support, and abnormal stress-and-strain patterns during cardiomyocyte contraction, which, in turn, result in changes in myocardial geometry and function [25]. In addition, detachment from extracellular matrix causes loss of integrin signaling, which leads to myocyte apoptosis [26,27].

Putative signaling pathways involved in MMP-2 and MMP-9 induction by doxorubicin in cardiac myocytes are shown in Fig. 8. Enhancement of MMP-2 and MMP-9 in cardiac myocytes in response to doxorubicin is mediated by the cooperation of ERK, JNK, and p38 kinase pathways, most of which are redox dependent. Our data show that p38, JNK, and ERK play distinct roles in MMP
endothelial and on vascular smooth muscle cells which demonstrated that mechanical stretch or lysophosphatidylcholine — a major constituent of oxidized LDL — enhances mRNA expression and MMP-2 release in an NAD(P)H oxidase-dependent manner [4,5].

Perhaps the most interesting results of the present study are the findings related to NAD(P)H oxidase and MMP-9 regulation. In fact, DPI inhibition of NAD(P)H oxidase not only prevents doxorubicin-induced ROS generation and attenuates the increase of MMP-2, but also augments the doxorubicin-induced increase of MMP-9. van de Loo et al. recently found that NAD(P)H oxidase-deficient mice had more severe joint inflammation, bone erosion and osteolysis, together with enhanced synovial gene expression of MMP-9 that was not counterbalanced by a corresponding up-regulation of its inhibitor [30]. Their experiments suggest that NAD(P)H oxidase-dependent ROS may act as negative anti-inflammatory feedback, and may be important in reducing connective tissue damage. Our data suggest that NAD(P)H oxidase may enhance myocardial remodeling via MMP-2 activation, but at the same time it might self-limit the process by down-regulating MMP-9. A possible explanation for this is that although ROS generally caused an increase in activating transcription factor 1 (AP-1) levels and increased nuclear translocation of NF-kB, at the same time oxidant stress may reduce the transcriptional activity of these molecules by directly oxidizing critical cysteine residues within the DNA binding [31]. Since the promoters of MMP-9 contain at least one AP-1 binding site, it is possible that NAD(P)H oxidase down-regulates the transcription of MMP-9 by an oxidant-dependent mechanism.

We are aware that incubating the H9C2 cell line with doxorubicin is a rough simulation of doxorubicin-induced cardiotoxicity, and thus our results should be viewed as hypothesis-generating rather than definitive. Indeed H9c2 cells are phenotypically distinct from cardiac myocytes, even though they share several properties. The cardiomyocyte culture-based system is considered a more relevant cellular model for this type of investigation, even though this approach itself has some limitations. Neonatal cardiomyocytes often show differences in the MAPK signaling as compared to adult cardiomyocytes, and most importantly, discrepancies do exist between in vitro (cultured myocytes) and in vivo (animal heart) model systems [32]. On the other hand, H9c2 cells provide technical advantages for studying cellular mechanisms, and they have often been used to investigate the pathways implicated in cardiac cell death secondary to oxidative stress, energy deprivation, iron exposure, or doxorubicin treatment [12,33–35]. As far as the role of ROS in doxorubicin-induced cardiotoxicity and the protective role of carvedilol are concerned, it must also be kept in mind that the results obtained with H9c2 cells have been confirmed in animal studies [12,17,36].

Carvedilol has recently become the backbone for the treatment of patients with congestive heart failure. By means of a comprehensive adrenergic blockade (β1, β2, and α),

**Fig. 5.** Cells were pre-treated with carvedilol for 1 h, or with dextrazoxane for 3 h or with DPI for 90 min, and then incubated with or without doxorubicin for 24 h. Supernatants were tested for MMP-2 and MMP-9 by gelatin zymography. Ct=control; Dox=doxorubicin; Carv=carvedilol; Dex=dextrazoxane; DPI=diphenyleneiodonium. In the upper panel data are the results of a single experiment. In the lower panel results are mean±SEM of 4 separate experiments. *P<0.05 vs Ct; **P<0.05 vs Dox.
Carvedilol provides greater protection of the heart against the deleterious consequences of sympathetic activation compared to the selective, conventional adrenergic blockade [37,38]. Carvedilol also acts as a potent antioxidant and prevents cardiomyocyte apoptosis in experimental models of both anthracycline cardiomyopathy and ischemia/reperfusion [12,39]. Lastly, treatment of heart failure patients with carvedilol attenuates left ventricular remodeling and reduces the increased circulating levels of MMPs, which are remarkably increased in congestive heart failure [40,41,23]. Briest et al. demonstrated that carvedilol blunts the norepinephrine induced increase in cardiac MMP-2 transcription and activity elicited by norepinephrine infusion [40]. Since nisoldipine had no effect on MMPs, even though it was able to abolish the norepinephrine induced afterload increase, the authors attributed carvedilol efficacy solely to the adrenergic blockade. However, their study did not compare carvedilol to traditional beta blockers. Our present and previous findings demonstrate that carvedilol is a potent antioxidant and works as an antioxidant,
and not as a beta blocker in the prevention of doxorubicin-induced cardiotoxicity [12]. Since ROS generation, myocardial apoptosis, and MMP over-expression do not only occur in the context of doxorubicin cardiotoxicity, but also in failing heart of all etiologies, our investigation can also serve as a useful experimental model of cardiomyopathy. In keeping with the previous literature, our findings also suggest that the antioxidant activity exerted by carvedilol may also have an ancillary, and perhaps not negligible role in preventing cardiac damage in the setting of congestive heart failure [42].

References

Fig. 7. The effects of 20 min of exposure to doxorubicin on the activation of MAPK pathways in H9c2 cells pre-incubated in the presence or absence of carvedilol (1 h) or dexrazoxane (3 h). Total cell lysates were analyzed by Western blotting using phospho-specific antibodies. Ct=control; Dox=doxorubicin; Carv=carvedilol; Dex=dexrazoxane. The left panels show the results of a single experiment and right panels are data in mean±SEM based on 3 separate experiments *P<0.05 vs Ct; †P<0.05 vs Dox.

Fig. 8. Proposed signaling pathways involved in MMP-2 and MMP-9 induction by doxorubicin in cardiac myocytes.


