Involvement of mitogen-activated protein kinases in adriamycin-induced cardiomyopathy

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Lou, H., I. Danelisen, and P. K. Singal. Involvement of mitogen-activated protein kinases in adriamycin-induced cardiomyopathy. Am J Physiol Heart Circ Physiol 288: H1925–H1930, 2005; doi:10.1152/ajpheart.01054.2004.—The current study investigated the phosphorylation of mitogen-activated protein kinases (MAPKs) as well as pro- and anti-apoptotic proteins in adriamycin (ADR)-induced cardiomyopathy (AIC) and heart failure in rats. Modulatory effects of antioxidant probucol on the activation of MAPKs were also examined. Male rats were administered with ADR (15 mg/kg body wt ip, over 2 wk) with and without probucol (120 mg/kg body wt for 4 wk ip). Hearts from these animals were studied at 1- to 24-h as well as at 3-wk posttreatment durations. In the 3-wk group, ADR depressed cardiac function, increased left ventricular end-diastolic pressure (LVEDP), and caused dyspnea and mortality. These changes were prevented by probucol. Phosphorylation of extracellular signal-regulated kinase (ERK)1/2, in the early stage of AIC, showed a biphasic response, with a maximum increase to 513% seen at 4 h, followed by a decrease to 66.8% at 3 wk after the last injection of ADR. Phosphorylation of p38 and c-Jun NH2-terminal kinases (JNKs) showed a steady increase 66.8% at 3 wk after the last injection of ADR. Phosphorylation of ERK1/2 and p38 kinases was decreased, whereas JNK mRNA was undetectable. Probucol completely prevented these MAPK changes. Activation of caspase-3 as well as the increase in the ratio of Bax to Bcl-xl were seen at early time points (1–24 h) as well as in the heart failure stage (3 wk). It is suggested that a transient increase in ERK1/2 at a shorter interval indicate an early adaptive response, and failure of this response corresponded with heart failure. In contrast, a gradual and persistent increase in p38 and JNK MAPKs as well as in caspase-3 and the Bax-to-Bcl-xl ratio may contribute in the initiation of apoptosis and progression of heart failure. Because probucol modulated changes in cellular signaling pathways and cardiac function, it is likely that oxidative stress plays a key role in AIC and heart failure.

Adriamycin (ADR), an anthracycline antibiotic, has been widely used in the treatment of a variety of tumors and carcinomas; however, its clinical application is limited by its irreversible cardiac side effect of cardiomyopathy and congestive heart failure (13, 22). The characteristic features of ADR-induced cardiomyopathy (AIC) include dose dependency, refractoriness to inotropic support, dilation of the membrane tubular system, myofibril drop out, and lack of inflammatory cytokine upregulation (13, 17, 22). Although the mechanism of AIC is multifactorial, increased oxidative stress due to overproduction of free radicals and antioxidant-deficit play an important role (16, 23). In addition, cardiac cell apoptosis has been suggested to be associated with AIC (2, 11). However, the role of oxidative stress in the intracellular signaling pathways and cardiomyocyte apoptosis in AIC remains unknown.

Mitogen-activated protein kinase (MAPK) signaling pathways are the primary intermediate of induction of apoptosis by oxidative stress. There are three major MAPK families, including extracellular signal-regulated kinases (ERKs), p38, and c-Jun NH2-terminal kinases (JNKs). In the cardiovascular system, ERK1/2 are potently and rapidly activated by growth factors and hypertrophic agents thereby mediating cell survival as well as offer cytoprotection (24, 26). 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 (12, 21, 28).

The current study investigated the role of ERK1/2, p38, and JNKs MAPks in the early stage of AIC and heart failure in rats. Because the antioxidant probucol can completely prevent AIC by reducing oxidative stress, modulatory effects of probucol on ADR-induced changes in MAPKs were investigated. Pro- and anti-apoptotic proteins caspase-3, Bax, and Bcl-xl were also examined to define the significance of MAPKs and cardiomyocyte apoptotic signaling in AIC.

MATERIALS AND METHODS

Animal groups and treatment. ADR-induced cardiomyopathy and heart failure were produced according to the established protocol (23). Male Sprague-Dawley rats (250 ± 10 g body wt) were divided into four groups: control, ADR, ADR + probucol, and probucol. ADR was administered in six equal doses (2.5 mg·kg⁻¹·dose⁻¹ ip), on alternate days, over a period of 2 wk for a cumulative dose of 15 mg/kg. Probucol was given in 12 doses for a period of 2 wk before the administration of ADR and 2 wk during the administration of ADR (cumulative dose 120 mg/kg ip). After the final injection, rats in the early stage of cardiomyopathy were anesthetized with an intraperitoneal injection of a ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture and euthanized at 1, 2, 4, and 24 h. Rats for the late-stage studies were observed for body weight, general appearance, behavior, and mortality for 3 wk. At the end of the 3-wk posttreatment period, animals were assessed hemodynamically, and the heart, lung, and liver tissues were collected for the further studies. All animal treatment procedures were according to the guidelines of the Animal Care Committee of the University of Manitoba.

Cardiac function and hemodynamic study. After the 3 wk of the last injection, rats were anesthetized by inhalation of isoflurane and oxygen. The right carotid artery was cannulated with a microtip pressure transducer (model SPR-249; Millar Instruments, Houston, TX) to monitor the blood pressure heart function. With the use of the...
software AcqKnowledge for Windows 3.0 (Biopac Systems, Goleta, CA), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), maximal rate of pressure development (+dP/dt\text{max}), the maximum rate of isovolumic pressure development (+dP/dt\text{max}) and the maximum rate of isovolumic pressure decay (−dP/dt\text{max}), aortic systolic pressure (ASP), aortic diastolic pressure (ADP), and aortic diastolic pressure (ADP) were recorded.

**Tissue weights and sample collection.** The livers and lungs were weighed, chopped into small pieces, and dried in an oven at 65°C until a stable weight was recorded. The liver and lung wet-to-dry weight ratios were obtained (23). The hearts were quickly removed, and the ventricles were weighed and cut into two pieces. One-half of the heart was stored in RNA-Later solution (from Ambion) for gene-chip analysis, and the other half was kept in liquid nitrogen for other studies.

**Affymetrix gene-chip probe array analyses.** Total RNA was isolated (TriReagent kits) and purified (QIAGEN kits). Two mRNA samples each from the control, ADR, and ADR + probucol groups were sent to The Centre for Applied Genomics (Hospital for Sick Children) for rat genome array (U34A) analyses.

**Western blot analysis.** Phosphorylated and total ERK1/2, p38, and JNKs MAPKs were examined by Western blot analysis, using PhosphoPlus MAPKs antibody kits (Cell Signaling Technology). For protein isolation, the frozen heart tissues were powdered in liquid nitrogen and then suspended in cell lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, and 1 μg/ml leupeptin]. The samples were homogenized and sonicated. The lysates were centrifuged at 14,000 rpm for 10 min, and the supernatant was transferred into a new tube and stored at 80°C. The quantity of the protein in the samples was measured by a dye-binding assay (Bio-Rad Laboratories) using bovine serum albumin as a standard. The isolated protein was subjected to SDS-PAGE and transferred to 0.45-

**Historical records.** Body weight, g 529.5 ± 19.8
LVSP, mmHg 121.7 ± 6.2
LVEDP, mmHg 3.10 ± 0.54
+dP/dt, mmHg/s 13.2 ± 1.51
−dP/dt, mmHg/s 8.63 ± 1.14
ASP, mmHg 93.2 ± 1.6*
ADP, mmHg 86.2 ± 2.7*
Liver, wet-to-dry wt ratio 3.14
Lang, wet-to-dry wt ratio 4.93 ± 0.02
Heart-to-body wt ratio 2.52 ± 0.07
Mortality, % 0

Data are means ± SE of 5–6 rats in each group. Because of the high mortality in the adriamycin (ADR) group, the starting number of animals was higher. ADR, adriamycin; Pro, Probucol; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt, rate of pressure development; −dP/dt rate of pressure decay; ASP, aortic systolic pressure; ADP, aortic diastolic pressure.

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**Table 1. Effects of probucol on adriamycin-induced changes in the late stage (3 wk) group**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>ADR</th>
<th>ADR + Pro</th>
<th>Pro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>529.5 ± 19.8</td>
<td>335.9 ± 18.0*</td>
<td>406.8 ± 15.9</td>
<td>526.9 ± 16.8</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td>121.7 ± 6.2</td>
<td>96.5 ± 0.7*</td>
<td>108.5 ± 2.8</td>
<td>123.6 ± 4.6</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>3.10 ± 0.54</td>
<td>13.2 ± 1.51*</td>
<td>34.2 ± 0.76</td>
<td>2.9 ± 1.3</td>
</tr>
<tr>
<td>+dP/dt, mmHg/s</td>
<td>10.931 ± 311</td>
<td>8.631 ± 114*</td>
<td>10.469 ± 298</td>
<td>11.235 ± 146</td>
</tr>
<tr>
<td>−dP/dt, mmHg/s</td>
<td>10.245 ± 96</td>
<td>8.797 ± 251*</td>
<td>11.800 ± 198</td>
<td>12.685 ± 306</td>
</tr>
<tr>
<td>ASP, mmHg</td>
<td>120.3 ± 1.3</td>
<td>93.2 ± 1.6*</td>
<td>107.5 ± 2.1</td>
<td>117.6 ± 1.8</td>
</tr>
<tr>
<td>ADP, mmHg</td>
<td>86.7 ± 1.7</td>
<td>62.3 ± 2.7*</td>
<td>84.2 ± 2.9</td>
<td>90.6 ± 1.9</td>
</tr>
<tr>
<td>Liver, wet-to-dry wt ratio</td>
<td>3.14 ± 0.04</td>
<td>3.47 ± 10.7*</td>
<td>3.18 ± 0.08</td>
<td>3.12 ± 0.05</td>
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<tr>
<td>Heart-to-body wt ratio</td>
<td>2.52 ± 0.07</td>
<td>2.13 ± 0.99*</td>
<td>2.54 ± 0.06</td>
<td>2.48 ± 0.04</td>
</tr>
<tr>
<td>Mortality, %</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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**RESULTS**

**General observations, body weight, tissue weight, and hemodynamics.** At the earlier time points (1–24 h), animals in all of the groups did not show any dyspnea or ascites, and no mortality was seen. During the 3-wk posttreatment period, rats in the ADR group developed a progressively enlarged abdomen, ascites, and dyspnea and showed 30% mortality related to...
heart failure. The ascites was hemorrhagic and ranged 50–140 ml. The livers of ADR-treated rats were enlarged and appeared dark in color. There was a significant increase in the wet-to-dry weight ratio in the liver and lung (Table 1). During the 2-wk treatment period, animals in the ADR and ADR + probucol groups showed a significant decrease in heart and body weights. But there was a gain in body weight in the posttreatment duration. However, this weight gain never reached the weights of animals in the control group.

In the 3-wk group, aortic systolic pressure (ASP), aortic diastolic pressure (ADP), and left ventricular peak systolic pressure (LVSP) were significantly lower in the ADR group. However, LVEDP was elevated more than fourfold in the ADR group. \( \pm dp/dt \) values were depressed in the ADR group. Probucol prevented these changes as well as heart failure (Table 1).

**Myocardial MAPKs mRNA expression in ADR-induced heart failure.** Gene expression of myocardial ERK1/2, p38, and JNKs kinases and their tiered cascade proteins at the heart failure stage was analyzed using DNA microarrays analysis, and the results are shown in Table 2. ERK2 and p38 mRNA expression was decreased in the failing hearts in the ADR group. JNK gene expression was undetectable in both ADR and control groups. Expression of MEKK1, an ERK1/2 kinases upstream stimulator, was also decreased in the ADR group. Probucol modulated these decreases in mRNA levels of ERK2 and p38 and MEKK1 kinases.

**Phosphorylation of different MAPKs in the early stage of AIC.** Phosphorylation of ERK1/2, p38, and JNK kinases in the myocardium at 1, 2, 4, and 24 h after the last injection of ADR was studied, and the data are shown in Fig. 1, A–C. Phosphorylation of ERK1/2, in the early stage of AIC, showed a biphasic response (Fig. 1A). There was a 167% increase at 1 h, 209% at 2 h, and a 513% peak in the 4-h group. At 24 h, it decreased to 197% of control. Phosphorylation of p38 showed a steady increase through 2, 4, and 24 h (119%, 138%, and 148%, respectively), as shown in Fig. 1B. A gradual increase phosphorylation of JNK was also seen through 1, 2, 4, and 24 h (116%, 124%, 127%, and 148%, respectively), as shown in Fig. 1C.

**Phosphorylation of different MAPKs in ADR-induced heart failure.** After 3 wk of posttreatment, phosphorylation of ERK1/2, p38, and JNK kinases was examined in the hearts from the control, ADR, ADR + probucol, and probucol groups, and the data are shown in Fig. 2, A–C. Phosphorylation of ERK1/2 showed a significant reduction by 66.8% of the control, and probucol prevented this decrease (Fig. 2A). In contrast, as shown in Fig. 2, B and C, phosphorylation of both p38 and JNK showed a significant increase to about 148% and 147%, respectively, in the ADR group compared with the control group. Probucol in the ADR + probucol group prevented this ADR-induced activation of p38 and JNKs kinases.

**Caspase-3 and Bax/Bcl-xl in AIC.** Full length as well as cleaved caspase-3 proteins were examined in both early and late stages of AIC by Western blot analysis, and these data are shown in Fig. 3. The activity of caspase-3 was expressed as the ratio of cleaved caspase-3 over full length of caspase-3. When compared with the ratio in the control heart set at 100%, caspase-3 was activated in both the early and late stages of AIC.

![Fig. 1](http://ajpheart.physiology.org/)
AIC, and its activity was increased in a time-dependent manner.

Pro- as well as anti-apoptotic proteins Bax and Bcl-xl were detected in both early and late stages of AIC using Western blot analysis, and the data are shown in Fig. 4. The ratio of Bax over Bcl-xl showed a significant increase starting at 4 h after the last injection of ADR and it continued to rise for 3 wk (Fig. 4).

DISCUSSION

The current study is the first to investigate the MAPK signaling pathways and pro- and anti-apoptotic proteins in both early and late stages of AIC. The major findings are the following:

1. ADR treatment activated all three MAPKs at early time points in the evolution of cardiomyopathy;
2. phosphorylation of ERK1/2 was upregulated at early time points, followed by a decline in the heart failure stage;
3. phosphorylation of P38 and JNK MAPKs was elevated at all time points during the progression of heart failure;
4. antioxidant probucol modulated the phosphorylation of all three MAPKs, indicating the activation of MAPKs through oxidative stress; and
5. myocardial proapoptotic proteins were activated in both early and late stages of AIC, which were shown as the activation of caspase-3 and increase in the ratio of Bax to Bcl-xl.

MAPKs in ADR-induced heart failure. Myocardial MAPKs have been examined extensively in heart failure patients; however, there is a significant variance in the information reported. In this regard, activities of all three MAPKs were reported to be elevated in human patients with heart failure due to ische-
MAP KINASES AND HEART FAILURE

MAPKs and ADR-induced apoptosis. Isolated rat cardiomyocytes exposed to ADR showed the occurrence of apoptosis in terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay, DNA laddering, and electron microscopic evaluation (2, 9, 10). Time-dependent apoptosis was also observed in ADR-treated rats at a cumulative dose of 15 mg/kg. The data revealed a biphasic response of apoptosis, peaking at 4 and 21 days after ADR treatment (11). It is also reported that apoptosis occurred in both cardiomyocytes and endothelial cells in ADR-treated rats at a cumulative dose of 24 mg/kg for over 2 wk (29). Even a single dose of ADR at 15 mg/kg also induced apoptosis in the mice (9). In the current study, activation of caspase-3 as well as the increase in the ratio of Bax to Bcl-xl were shown in both early AIC and heart failure. These changes were preceded as well as accompanied by the activation of p38 and JNK kinases. These MAPKs have been indicated to mediate death signaling pathways in response to ischemia-reperfusion, oxidative stress, hypoxia, and β-adrenergic stimulation (4). ERK1/2 and p38 kinases were reported to play an opposite role in daunomycin-induced apoptosis. Inhibition of ERK1/2 activation was demonstrated to increase daunomycin-induced apoptosis, whereas p38 inhibitor reduced the numbers of apoptotic cells (30). On the basis of the results obtained from the present study as well as existing reports, it is suggested that MAPKs play an important role in the ADR-induced apoptosis.

It was surprising to note that p38 mRNA expression was downregulated while its activity was increased. It is likely that there exists a highly differentiated p38 pathways that is mediated by different isoforms of the p38 MAPKs. For example, the p38α isoform was shown to play an important role in the induction of apoptosis, whereas p38β was capable of inducing a typical hypertrophic response in cardiomyocytes (3, 9, 27). Because cardiomyocyte apoptosis is shown to be induced in the AIC, it is possible that in the heart failure stage of AIC, p38 mRNA expression was decreased due to the downregulation of the p38β isoform, whereas p38α may not change or may even increase. However, when the phosphorylation of p38 was detected, p38α may be the dominant isoform in the determination of the p38 activity. The possibility of posttranslational changes and/or reduced degradation of this protein kinase cannot be excluded.

Apart from inducing apoptosis, the activation of caspase-3 in the heart has also been reported to result in depressed cardiac function, myofibril disruption, and sarcomere disorganization through its ability to cleave cardiac myofibrillar proteins, such as ventricular myosin light chain, α-actin, α-actinin, and troponin T (6, 18). Therefore, the activation of apoptotic signaling proteins may mediate both cardiac dysfunction and apoptosis in the progression of AIC.

In conclusion, the present study suggests that ADR-induced oxidative stress may stimulate MAPKs signaling pathways, leading to activation of proapoptotic proteins, myocardial apoptosis, and progression of heart failure. Exploring the potential for modulating MAPK pathways would likely result in novel approaches to prevent or treat AIC and heart failure.

Fig. 4. Time course of the ratio of Bax to Bcl-xl in the AIC. UP, myocardial Bax and Bcl-xl proteins were measured by Western blot analysis with their specific antibodies. Two proteins were detected from the same stripped membrane. LP, densitometry analysis of Bax and Bcl-xl. Values are the ratio of Bax to Bcl-xl normalized by setting the densitometer of the control samples as 100%.
H1930

MAP KINASES AND HEART FAILURE

GRANTS

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