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

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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 a minimum of 102% at 24 h. Phosphorylation of p38 and c-Jun NH2-terminal kinases (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 study were observed for body weight, general appearance, behavior, and mortality for 3 wk. At the end of the 3-wk posttreatment period, animals were observed hemodynamically, and the heart, lung, and liver tissues were collected for the further studies.

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 micropipet pressure transducer (model SPR-249; Millar Instruments, Houston, TX) to monitor the blood pressure heart function. With the use of the

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The samples were homogenized and sonicated. The proteins were extracted from the samples, and the protein concentration was measured using a dye-binding assay. The proteins were then separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were probed with antibodies specific to the MAP kinases and their phosphorylated forms. The bands were visualized using chemiluminescence.

**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 the development of heart failure.

**DNA microarray analysis for gene expression of MAPK signaling cascade proteins in the late-stage (3 wk) group.**

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Gene Description</th>
<th>Control</th>
<th>ADR</th>
<th>ADR + Pro</th>
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<td>A00542</td>
<td>BMK1/ERK5 protein</td>
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<td>A</td>
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<td>P</td>
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</table>

A, absent; P, present. Average of two independent assays. ERK, extracellular signal-regulated kinase 1; MAPK, mitogen-activated protein kinase; SAP, stress-activated protein kinase; MKK2, MAPK kinase; MEKK1, MAP kinase; MKP-2, MAPK phosphatase; ADR, adriamycin; Pro, Probucol.
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. \( \pm \frac{dp}{dt} \) 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.

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**Fig. 1.** A–C, phosphorylation of extracellular signal-regulated kinase (ERK1/2) (A), p38 (B), and c-Jun NH2-terminal kinase (JNK, C) mitogen-activated protein (MAP) kinases in adriamycin (ADR)-induced early cardiomyopathy (at 1 to 24 h). UP, myocardial phosphorylated MAP kinase was measured by Western blot analysis with a phospho-specific MAP kinase antibody. Total amounts of MAP kinases were examined from the same stripped membranes for each kinase with the kinase-specific antibodies. LP, densitometric analysis of MAP kinases activities. Values are the ratio of phospho-specific MAP kinases to total MAP kinases. Results were normalized for all experiments by a random setting of the densitometer of the control heart samples as 100%. Data in the LP are means \( \pm \) SE from 4 hearts in independent experiments. *\( P < 0.05 \) vs. control.
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-

![Fig. 2. A–C effects of ADR with and without probucol (Pro) on the activity of MAPKs, ERK1/2 (A), p38 (B), and JNKs (C) in ADR-induced heart failure. UP, myocardial phosphorylated MAP kinases were measured by Western blot analysis with phospho-specific MAPKs antibody. Total amount of MAP kinases were examined from the same stripped membrane for each MAP kinase with the kinase-specific antibodies. LP, densitometric analysis of MAP kinase activation. Values were normalized for all experiments by a random setting of the densitometer of the control heart samples as 100%. Data in the LP are means ± SE from 4 hearts in independent experiments. *P < 0.05 vs. control; #P < 0.05 vs. ADR.](http://ajpheart.physiology.org/)

![Fig. 3. Time course (1 h, 2 h, 4 h, 24 h, and 3 wk) of activation of caspase-3 in AIC. UP, myocardial caspase-3 was measured by Western blot analysis with cleaved caspase-3 antibody, and the same stripped membrane was examined with caspase-3 antibody. LP, densitometry analysis of caspase-3 activation. Values are the ratio of cleaved caspase-3 to full length of caspase-3. Results were normalized by setting the densitometer of the control samples as 100%.](http://ajpheart.physiology.org/)
mic cardiomyopathy and idiopathic dilated cardiomyopathy (8). In another study, only the activities of JNK and p38 were reported to be increased, whereas ERK1/2 had no change in heart failure due to ischemic heart disease (7). In contrast, decreased activity of p38 was reported in the failing myocardium from ischemic and idiopathic dilated cardiomyopathy (14). These are a few examples from many suggesting that MAPKs may be differentially regulated. Thus an unique balance among MAPKs pathways may exist, depending on the stage of heart failure as well as the types of the stimulus.

A study of the time course of changes in MAPKs during the pathogenesis of heart failure, such as is done in this study, is important to sketch a comprehensive picture. Our data show that ERK1/2 was activated early but transiently and downregulated during the heart failure stage. The early upregulation may have been an adaptive and protective response. In contrast, phosphorylation of p38 and JNKs kinases increased early and persisted until the heart failure stage, suggesting that these kinases may play a dominant role in the progression of AIC and heart failure.

MAPKs and ADR-induced oxidative stress. It has been well documented that oxidative stress activates MAPKs. In the perfused rat heart and cultured cardiomyocytes, H2O2 activated all three MAPKs (1, 3, 19). N-(2-mercaptobutyryl)-glycine, DMSO, and catalase, but not SOD, markedly depressed daunomycin-mediated activation of MAPKs, suggesting that –OH and H2O2, but not O2- are mainly involved in the activation of MAPKs (30). All three MAPKs were also reported to be activated in ischemia-reperfusion injury, mediated by oxidative stress (20). It is widely accepted that ADR induces cardiomyopathy by producing reactive oxygen species as well as by decreasing the antioxidant reserve (2). In our previous report, with the use of the same AIC model, lipid peroxidation occurred as early as 1 h and continued to increase up to 24 h, and there was a corresponding decrease in glutathione peroxidase and MnSOD activities (15). In the present study, antioxidant probucol markedly modulated ADR-induced changes in the phosphorylation of all three MAPKs, indicating that the ADR-induced activation of MAPKs may also be modulated through increased oxidative stress.

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, myocardial 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.
REFERENCES


