MAPK Superfamily Plays an Important Role in Daunomycin-Induced Apoptosis of Cardiac Myocytes

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Background—Although anthracyclines, such as daunomycin (DM) and adriamycin, are potent chemotherapeutic agents, they have serious adverse effects, including cardiac toxicity. In the present study, we investigated the molecular mechanisms of DM-induced cardiomyocyte impairment.

Methods and Results—When cultured cardiac myocytes of neonatal rats were exposed to 1 μmol/L DM for 24 hours, many cells became positive for TUNEL staining, with morphological changes characteristic of apoptosis. Fragmentation of DNA into oligonucleosome-size fragments was recognized by agarose gel electrophoresis in DM-treated myocytes. DM activated 3 members of the mitogen-activated protein kinase (MAPK) family dose-dependently, such as extracellular signal-regulated protein kinases (ERKs), c-Jun NH2-terminal kinases, and p38 MAPK in cardiac myocytes. Oxyradical scavengers or Ca2+ chelators inhibited DM-induced activation of ERKs and p38 MAPK. DM-induced activation of ERKs was also inhibited by overexpression of dominant negative mutants of Ras (D.N.Ras), and the p38 MAPK activation was attenuated by D.N.Rho. The number of DM-induced apoptotic cells was markedly increased when the ERK signaling pathway was selectively blocked by a specific MAPK/ERK kinase inhibitor, PD98059, whereas pretreatment with a specific inhibitor of p38 MAPK, SB203580, significantly reduced the amount of apoptosis.

Conclusions—These results suggest that DM activates MAPKs through reactive oxygen species and Ca2+ and that the MAPK family plays important roles in DM-induced apoptosis in cardiac myocytes. ERKs protect cardiomyocytes from apoptosis, whereas p38 MAPK is involved in the induction of cardiomyocyte apoptosis. (Circulation. 1999;100:2100-2107.)

Key Words: daunomycin ● kinase ● apoptosis ● myocytes

Anthracyclines such as daunomycin (DM) and adriamycin (AM) are widely used antineoplastic agents. The clinical usefulness of these agents, however, is limited by serious adverse effects, including cardiotoxicity.1 These cardiotoxic effects result in cardiac dysfunction, cardiomegaly, and finally congestive heart failure.2 Although anthracyclines have been reported to block the cell cycle3 and induce apoptosis in tumor cells,4 the mechanism of myocardial impairment by anthracyclines remains uncertain. It has been reported that AM enhances peroxidation of lipids in myocardial membrane5 and that sulfhydryl reagents inhibit AM-induced cardiotoxicity.6 These observations suggest that anthracyclines impair myocardium through the production of reactive oxygen species (ROS).7

The mitogen-activated protein kinase (MAPK) family is an important mediator of signal transduction and is activated by a variety of stimuli, such as growth factors and cellular stresses.8 Among the MAPK family, 3 members in particular, the extracellular signal-regulated kinases (ERKs),9 c-Jun NH2-terminal kinases (JNKs, also called SAPKs),10 and p38 MAPK,11 have been well characterized. Each MAPK is activated through the specific kinase cascade. ERKs are activated by many growth factors, cytokines, and phorbol esters through a variety of signaling molecules, such as tyrosine kinases (TKs), Ras, protein kinase C (PKC), protein kinase A (PKA), or Ca2+12–14 and through Raf-1 and MAPK/ERK kinase (MEK), and play essential roles in the control of cell growth and differentiation.9 In contrast, JNKs and p38 MAPK are weakly activated by growth factors and phorbol esters but are markedly activated in response to tumor necrosis factor-α, ultraviolet irradiation, and cellular stresses,10,15 and their activation is associated with induction of apoptosis.10 Recently, it was reported that small GTP proteins of the Rho family regulate the JNKs/p38 MAPK pathway.17–20

Despite the many studies on the subject, the molecular mechanism of anthracycline-induced myocardial impair-

Received February 11, 1999; revision received June 21, 1999; accepted June 28, 1999.
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ments is still largely unclear. In the present study, we showed that DM induces cardiomyocyte death, including apoptosis, and that the MAPK family plays a pivotal role in the process.

Methods

CDNA Plasmids
Both HA-tagged ERK2 (HA-ERK2) and Flag-tagged p38 MAPK (Flag-p38 MAPK) were kind gifts from M. Karin, PhD, University of California, San Diego. Expression vectors encoding Rho-GDI, various mutants of RhoA, Rac1, Cdc42, and Ras were provided by J.S. Gutkind, PhD, et al, National Institutes of Health, Bethesda, MD.

Cell Culture and Transfection
Primary cultures of cardiac myocytes were prepared from ventricles of 1-day-old Wistar rats as described previously. Cardiomyocytes were plated at a field density of $1 \times 10^3$ cells/cm$^2$ on 35-mm culture dishes with DMEM with 10% FBS; at 24 hours after seeding, the culture medium was changed to DMEM with 0.1% FBS. DNA was transfected by the calcium phosphate method as described previously.

Assay of MAPK Activity
The activity of ERKs was measured by “in-gel assay” using myelin basic protein (MBP)-containing gel as described previously. The activity of transfected ERK2 was determined by MBP kinase assay as described previously. Kinase activity of JNK was determined by the immune complex kinase assay using an anti-JNK polyclonal antibody (Santa Cruz Biotechnology, Inc) as described previously. Activation of p38 MAPK was examined by Western blot analysis using p38 MAPK–specific antibody (New England Biolabs, Inc) as described previously. Anti-phosphorylated p38 MAPK antibody recognizes only activated p38 MAPK that is phosphorylated on Thr-180 and Tyr-182 and is well correlated with the p38 MAPK activity as reported before. The transfected p38 MAPK was measured with MBP as a substrate after immunoprecipitation as described previously.

Immunofluorescent Cytochemistry
After transfection of HA-ERK2, Flag-p38 MAPK, or myc-tagged dominant negative mutants of Rho (D.N.Rho, the myocytes plated on a cover glass were fixed and incubated for 1 hour at 37°C with the phallolidin-TRITC antibody. Next, the samples were incubated with each anti-tag monoclonal antibody and then incubated with an anti-mouse IgG conjugated to FITC.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling Analysis
Cardiomyocytes plated on a cover glass were incubated with a monoclonal antibody against myosin heavy chain (MF-20) for 1 hour at 37°C and then incubated with an anti-mouse IgG conjugated with rhodamine. Next, 50 μL terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) reaction mixture containing both TdT and FITC-conjugated dUTP was added to each sample.

Agarose Gel Electrophoresis for DNA Fragmentation
To examine the DNA laddering formation, we used the apoptosis ladder detection kit (Wako Pure Chemical Industries, Ltd) as described previously.

Statistics
Statistical comparison of the control group with treated groups was carried out with 1-way ANOVA and Dunnett’s t test. The accepted level of significance was $P<0.05$.

Results

DM Induces Apoptosis in Cardiac Myocytes
We first examined whether DM induces apoptosis of cardiac myocytes. When cultured cardiac myocytes of neonatal rats were exposed to 1 μmol/L DM for 24 hours, a number of myocytes (~24%) showed positive for TUNEL staining compared with the vehicle-treated cells (~3%) (Figure 1, A and B). Many TUNEL-positive cells had condensed nuclei, which are characteristic of apoptosis (Figure 1A). DNA fragmentation was also observed after exposure of cardiomyocytes to 1 μmol/L DM for 24 hours (Figure 1C).

DM Activates MAPKs in Cardiac Myocytes
To elucidate the molecular mechanism of DM-induced apoptosis of cardiac myocytes, we examined whether DM activates MAPKs in cardiac myocytes. When cardiac myocytes were exposed to various concentrations of DM (0.1 μmol/L to 1 mmol/L) for 20 minutes, ERKs were activated in a dose-dependent manner (Figure 2A). After incubation with 1 mmol/L DM for 20 minutes, many cardiac myocytes were dead, and therefore the activity of ERKs per plate was very low (Figure 2A). The increase in ERK activity was first detected 5 minutes after the addition of 100 μmol/L DM, peaked at 15 minutes, and gradually decreased thereafter (Figure 2B). JNKs (Figure 3A) and p38 MAPK (Figure 4A) were also activated by DM. Unlike ERKs, the activity of JNKs and p38 MAPK was high even after treatment with 1 mmol/L DM (Figure 3A and 4A), suggesting that the activity of JNKs and p38 MAPK of each remaining cardiomyocyte should be very high. Activation of JNKs (Figure 3B) and p38 MAPK (Figure 4B) by 100 μmol/L DM was detected from 5 minutes and peaked at 30 minutes.

ROS Is Involved in Activation of MAPKs
Because ROS have been reported to be generated from cells that are exposed to DM, we examined whether ROS are involved in DM-induced activation of MAPKs. Pretreatment with DMSO (a diffusible scavenger of ·OH), catalase (an oxidoreductase of H$_2$O$_2$), and N-(2-mercapto propionyl)-glycine (MPG) (a rapidly diffusible scavenger of H$_2$O$_2$, O$_2^-$, and ·OH), but not superoxide dismutase (SOD) (a scavenger of O$_2^-$), strongly suppressed DM-induced activation of ERKs (Figure 5A) and p38 MAPK (Figure 5B). In contrast, heat-inactivated catalase did not show any inhibitory effects on the DM-induced activation of these kinases (data not shown).

DM Activates ERKs and p38 MAPK Through Distinct Pathways
We examined the DM-induced intracellular signaling pathways. DM increased the activity of transfected HA-ERK2 and Flag-p38 MAPK in cardiac myocytes (Figure 6, A and B). Overexpression of D.N.Ras or D.N.Raf-1 strongly suppressed DM-induced ERK2 but not p38 MAPK (Figure 6, A and B). In contrast, inhibition of Rho family functions by overexpression of D.N.RhoA, D.N.Rac1, D.N.Cdc42, or Rho GDP dissociation inhibitor (RhoGDI) suppressed DM-induced activation of p38 MAPK (Figure 7A), but not of ERKs (Figure 7B).
DM-Induced Activation of MAPKs Is Dependent on Ca\textsuperscript{2+} but Independent of PKC, PKA, and TKs

We next examined the role of other signaling molecules in DM-induced activation of ERKs and p38 MAPK. Chelation of extracellular or intracellular Ca\textsuperscript{2+} by pretreatment with 5 mmol/L EGTA for 2 minutes or 40 mmol/L BAPTA for 30 minutes, respectively, suppressed DM-induced ac-

Figure 1. DM induces cardiomyocyte apoptosis. A, After treatment with 1 μmol/L DM for 24 hours, cardiomyocytes were stained with a monoclonal anti–myosin heavy chain antibody (TRITC) (a, c) and TUNEL (FITC) (b, d). a and b, Untreated cardiomyocytes; c and d, cardiomyocytes incubated with 1 μmol/L DM for 24 hours. B, Number of TUNEL-positive cardiomyocytes presented as a percentage of MF-20–positive cardiac myocytes (n=100) from 3 independent experiments (mean±SEM). *P<0.05 vs control.

Figure 2. DM activates ERKs in cardiac myocytes. Cardiac myocytes were incubated for 20 minutes with indicated concentrations of DM (A) or with 100 μmol/L DM for indicated periods of time (B), and activation of ERKs was examined by use of MBP-containing gels. Intensity of each band on autoradiogram was quantified by densitometric scanning, and activity of 42-kDa ERK is shown as percent increase in average from 4 independent experiments compared with unstimulated controls (100%). *P<0.05 vs control.
tivation of ERKs and p38 MAPK (Figure 8, A and B). Downregulation of PKC by pretreatment with 0.1 μmol/L 12-O-tetradecanoylphorbol-13-acetate (TPA) for 24 hours or by pretreatment with 1 μmol/L calphostin C, a specific inhibitor of PKC, had no effects on DM-induced ERK or p38 MAPK activation (Figure 8, C and D). Likewise, inhibition of PKA by 100 μmol/L RpA8C14 or inhibition of TKs by 50 μmol/L tyrphostin (A25) or 20 μmol/L genistein did not affect DM-induced ERK or p38 MAPK activation (Figure 8, C and D).

ERKs and p38 MAPK Play Opposite Roles in DM-Induced Myocardial Apoptosis

Finally, we examined the role of MAPKs in cardiomyocyte apoptosis. After incubation with 1 μmol/L DM for 24 hours, the number of TUNEL-positive cardiac myocytes was increased from ≈3% to ≈24% (Figure 9A). When the ERK signaling pathway was blocked by pretreatment with 50 μmol/L PD98059 for 1 hour, the number of TUNEL-positive myocytes was further increased by >2-fold (≈53%) (Figure 9A). Treatment with PD98059 significantly increased the number of TUNEL-positive cells even
in the absence of DM (~10%) (Figure 9A). DM treatment induced DNA ladder formation (Figure 9B), which became more prominent with pretreatment with PD98059 (Figure 9B). On the contrary, DM-induced apoptosis was significantly suppressed by pretreatment with 10 μmol/L SB203580, the p38 MAPK inhibitor, for 2 hours (~18%) (Figure 9A). DM-induced DNA fragmentation was also reduced by pretreatment with 10 μmol/L SB203580 (Figure 9B). SB203580 (10 μmol/L) is specific to p38 MAPK, 23 and ERKs and JNKs were not inhibited by 10 μmol/L SB203580 (data not shown). The effect of SB203580 on inhibition of apoptosis was well correlated with the activity of p38 MAPK (data not shown).

To further elucidate the role of ERKs and p38 MAPK, we transfected HA-ERK2 and Flag-p38 MAPK into cultured cardiac myocytes. Signals of both HA-ERK2 and Flag-p38 MAPK were observed in the cytoplasm of cardiomyocytes before addition of DM, and both were translocated into the nucleus after DM treatment (Figure 9C). Many p38 MAPK-transfected cells, but not ERK2-transfected cells, were TUNEL-positive after starvation for 48 hours (Figure 9D). When the transfected cells were incubated with 1 μmol/L DM for 4 hours, almost all p38 MAPK-transfected cells were TUNEL-positive (~95%), whereas few ERK2-transfected cells were positive (<3%).

**Discussion**

Apoptosis is an active process induced by a variety of stresses, 16 and apoptosis plays a critical role in a variety of cardiovascular diseases, including myocardial infarction, heart failure, and atherosclerosis. 24 The present study demonstrates that DM induces apoptosis of cardiac myocytes, possibly through the production of ROS. Three members of the MAPK family were activated by DM in cardiac myocytes, and among them, ERKs and p38 MAPK play opposite roles in the induction of apoptotic death. ERKs, which are activated by DM through Ras and Raf-1, protect cardiomyocytes from apoptosis, whereas p38 MAPK, which is activated through the Rho family, is involved in DM-induced apoptosis of cardiomyocytes.
Although 1 μmol/L DM was enough to induce apoptosis of cardiac myocytes, because activation of MAPKs was not prominent with 1 μmol/L DM, we used 100 μmol/L DM to examine the DM-evoked signaling pathway in the present study. It has been reported that the peak plasma concentration of anthracyclines after standard bolus administration in patients is 5 μmol/L, 25 and previous studies on cardiomyocytes demonstrated that treatment with 1 μmol/L AM for 24 to 48 hours produces morphological and ultrastructural changes characteristic of AM cardiotoxicity. 26 Although it is difficult to precisely estimate the amount of necrotic death and apoptotic death of cardiac myocytes, because we can easily detect the ladder formation, 10% of cells may be dead of apoptosis. During the preparation of our manuscript, Sawyer et al 27 reported that DM induced apoptosis in cultured cardiomyocytes of neonatal and adult rats.

Anthracycline-induced cardiotoxicity has been reported to be mediated by ROS. 7 AM stimulates NADPH oxidase–like activity in the cardiac sarcoplasmic reticulum and thereby induces oxidative stress in the myocardium. 7 Cardiac glutathione peroxidase is also reported to be inhibited by AM. 28 We showed here that MPG, DMSO, and catalase, but not SOD, markedly repressed DM-mediated activation of MAPKs. It has been reported that pretreatment with catalase and mannitol (a quencher for -OH), but not with SOD, mitigates the reduction of contractile function and reduces damage due to AM. 29 These results and observations suggest that -OH and H₂O₂, but not O₂⁻, are mainly involved in the DM-induced activation of MAPKs and acute cardiac injury, although it is also possible that SOD, a high-molecular-weight scavenger of O₂⁻, remained in the extracellular space and failed to show effects.

ERKs are activated by serial activation of Raf-1 and MEK in mammalian cells. 9 However, the signal transduction pathways leading to activation of Raf-1 are different according to cell types and stimuli. 12–14 Although PKC is partially involved in ERK activation by H₂O₂ in Jurkat T cells, 30 Ras, but not PKC, is necessary for ERK activation by H₂O₂ in cardiac myocytes. 22 In this study, DM activated ERKs through Ras and Raf-1, but not through PKC, PKA, or TKs, in cardiac myocytes, although we cannot exclude a possibility that tyrphostin (A25)– and genistein-insensitive TKs may mediate DM-induced activation of ERKs.

The signaling pathway leading to activation of p38 MAPK was different from that of ERKs. It has been reported that small GTP-binding proteins of the Rho family, including RhoA, Rac1, and Cdc42, regulate the activity of JNKs and p38 MAPK. 17–20 Roberts et al 31 reported that in A549 lung carcinoma cells, H₂O₂ induces JNK activation via a RhoA-dependent pathway. In the present study, overexpression of Rho-GDI, D.N.RhoA, D.N.Rac1, and D.N.Cdc42 significantly suppressed DM-induced activation of p38 MAPK, but
Figure 9. ERKs and p38 MAPK play opposite roles in DM-induced cardiomyocyte apoptosis. After preincubation with 50 µmol/L PD98059 for 60 minutes or 10 µmol/L SB203580 for 2 hours, cardiomyocytes were incubated with 1 µmol/L DM for 24 hours. A, One hundred MF-20–positive cardiac myocytes were counted, and number of TUNEL-positive cells was presented as percentage from 3 independent experiments (mean ± SEM). *P<0.05 vs control; **P<0.05 vs DM treatment. B, Genomic DNA was extracted and fractionated in a 1.5% agarose gel. Lane 1, DNA size markers; lane 2, vehicle; lane 3, DM; lane 4, pretreatment with 50 µmol/L PD98059 + DM; and lane 5, pretreatment with 10 µmol/L SB203580 + DM. C, After transfection with HA-ERK2 or Flag-p38 MAPK, cardiomyocytes were stimulated by 100 µmol/L DM for 20 minutes and stained with phalloidyne-TRITC (g through l) and either anti-HA or anti-Flag M2 antibodies, followed by incubation with an anti-mouse IgG conjugated with FITC (a through f). a and g, untreated cardiomyocytes; b and h, cardiomyocytes with DM treatment; c and i, Flag-p38 MAPK-transfected cardiomyocytes without DM treatment; d and j, Flag-p38 MAPK-transfected cardiomyocytes with DM treatment; e and k, HA-ERK2–transfected cardiomyocytes without DM treatment; and f and l, HA-ERK2–transfected cardiomyocytes with DM treatment. D, After transfection with HA-ERK2 or Flag-p38 MAPK, cardiomyocytes were stained with TUNEL method (FITC) (d through f), anti-HA antibody (c), anti–Flag M2 antibody (a), and anti-HA plus anti-Flag M2 antibodies (b) (TRITC). b and e, untreated cardiomyocytes; a and d, Flag-p38 MAPK-transfected cardiomyocytes; and c and f, HA-ERK2–transfected cardiomyocytes.
not that of ERKs. These results suggest that all 3 members of the Rho family are involved in DM-induced p38 MAPK activation in cardiac myocytes.

Many lines of evidence have suggested that members of MAPKs, including ERKs, JNKs, and p38 MAPK, play important roles in cell survival and death.16 Activation of the ERK signaling pathway functions to protect cells from a variety of cellular stresses.16,22 On the contrary, the JNK and p38 MAPK signaling pathways have been suggested to be apoptosis-inducing pathways.16 DM activated all 3 members of MAPKs in a different manner. Pretreatment with SB203580 reduced the number of apoptotic cells. Moreover, almost none of ERK2-transfected cells were TUNEL-positive even after exposure to DM. On the contrary, pretreatment with SB203580 reduced the number of apoptotic cells. In addition, most p38 MAPK-transfected cells became TUNEL-positive.

These results suggest that ERKs prevent cardiac myocytes from DM-induced apoptosis, whereas p38 MAPK promotes apoptosis in cardiac myocytes. Like ERKs/p38 MAPK, DM-induced activation of JNKs was inhibited by various inhibitors, such as catalase, EGTA, BAPTA, and DMSO (data not shown). However, pharmacological specific inhibitors of JNKs are not available at present, and the role of JNKs in DM-induced cardiac apoptosis was not evaluated in this study. It remains to be determined how the MAPK family modulates DM-induced apoptosis of cardiac myocytes.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research, Developmental Scientific Research, and Scientific Research on Priority Areas from the Ministry of Education, Science, Sports, and Culture of Japan and by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Drug ADR Relief, R&D Promotion, and Product Review of Japan.

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*Circulation.* 1999;100:2100-2107
doi: 10.1161/01.CIR.100.20.2100
*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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