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研究テーマ ①抗癌剤ダウノマイシンによる心筋障害の分子機序。－－
②液性因子による心肥大発生の分子機構。

2．本年度の研究業績

(1) 学会・研究会等においての口頭発表 (有)・無 (学会名・内容)

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別紙（アメリカの Circulation 誌、投稿中）

3．今後の研究計画

別紙

4．研究指導者の意見

朱　偉東さんは

1、研究レポートの提出期限を守ります。

2、自分の意見を進んで発表します。

3、他人の意見をよく聞き、必要なら積極的に取り入れます。

4、共同研究では皆の意見をまとめます。

彼は現在の研究を今後発展させ、将来博士号を取得できると考えています。また、彼は社会性があり、将来中国医学の担い手となることができると思います。

研究指導者氏名 小室一成

5．研究報告

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抗癌剤ダウノマイシン(DM)による心筋細胞アポトーシスの発症機序ーMAPキナーゼ(MAPK) スーパーファミリーの関与

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要旨

抗癌剤ダウノマイシン (DM) による非可逆性心筋障害は致死的であり、その病態解明と予防・治療法の開発が急務となっている。最近、心不全やある種の心筋症において心筋細胞にアポトーシスが起こっていることが報告され、その病態形成における役割が注目されている[1]。また、mitogen-activated protein kinase (MAPK) の新しいファミリーである C-Jun NH_2-terminal kinase (JNK) と p38MAPK が同定され、その働きのひとつとしてアポトーシスとの関連が報告されている[2]。一方、われわれは、DM による心筋障害の一因として知られている活性酸素が p38MAPK を介して培養心筋にアポトーシスによる細胞死を誘導することが報告した[3]。そこで今回、DM による心筋細胞アポトーシスと MAP キナーゼファミリーの関係をさらに詳細に解析した。

KEY WORDS

ダウノマイシン, 心筋細胞, アポトーシス, MAP キナーゼファミリー, 活性酸素.

目的

DMは心筋細胞に傷害を与え、心不全が引き起こされます。この心不全はDM治療における致命的な副作用の一つとして挙げられます。この発症機序の解明を目指し、我々は、DMによる心筋細胞アポトーシスとMAPキナーゼファミリーの関係を解析する。

方法

1）MAPキナーゼ活性測定

ERKとp38MAPKはmyelin basic protein (MBP)を、JNKはc-Jun蛋白を基質にしてその活性を測定した。さらに、p38MAPKについてはリン酸化p38MAPKを特異的に認識する抗体によってそのリン酸化について検討した。

2）心筋細胞のアポトーシスの検出

DM最終濃度10^{-6}Mで24時間の刺激を行い、4%パラフォルムアルデヒドにて固定し、心筋細胞の細胞質をミオシンに対する抗体を用いて、また核をTUNEL法にて二重染色を行った。さらにDNAの電気泳動を行い、ladder形成の有無を調べた。

結果

1）心筋細胞のアポトーシス

10^{-6}M DM刺激により、心筋細胞に多数のTUNEL陽性像 (24%) が確認された。コントロールの培養心筋の染色像ではTUNEL陽性像 (3%) はほとんど確認されなった。アガロースゲル電気泳動法では、アポトーシスに特徴的なladder formationが10^{-6}M DM刺激で見られた。

2）MAPキナーゼの活性化

次にDMに誘導されたアポトーシスの形成機序を解析するために、細胞の増殖・分化・アポトーシスに重要な役割を果たしているMAPキナーゼファミリーの活性について検討した。MAPキナーゼのうち、ERK、JNK、p38MAPKはいずれも活性化されたが、ERKは15分にその活性のピークを認め、JNKとp38MAPKは30分に認めた。また10^{-6}-10^{-3}Mの範囲において濃度依存性に活性化された。

次にERKとp38MAPKの上流について解析を行った。種々の細胞においてRas、
Raf-1は成長因子などの刺激によりERKの上流で活性化されることが知られて

いるが[4]、Ras、Raf-1 の dominant-negative mutant (D.N.) の過剰発現によって、DM 刺激による ERK の活性化は抑制された。一方、DM 刺激による p38MAPK の活性化は Ras、Raf-1 の D.N. では抑制されず、Rho ファミリー（RhoA, Rac1, Cdc42）の D.N. や、Rho を不活性化の状態のままにしてその機能を抑制する Rho GDP dissociation inhibition (RhoGDI) の過剰発現により部分的に抑制された。また逆にRho ファミリーの抑制は ERK の活性化に影響を与えなかった。。このことから ERK の上流に Ras、Raf-1 蛋白が存在し、p38MAPK の上流に Rho 蛋白が存在することが示唆された。

さらに DM による ERK とp38MAPK の活性化のメカニズムを明らかにするために、フリーラジカル消去剤(OH の消去剤 DMSO；H_2O_2 を還元する Catalase；OH, H_2O_2 と O_2^- をともに消去する N-(2-mercaptopropionyl)-glycine）及び細胞内及び細胞外 Ca^{2+} キレート剤 BAPTA-AM と EGTA にて前処置すると、ERK と p38MAPK の活性化が抑制された。一方、PKC、PKA、チロシンキナーゼの阻害薬の前処置では、ERK と p38MAPK の活性化は抑制されなかった。

3）MAP キナーゼの役割

DM 刺激により心筋細胞は一部、細胞質の縮小、核の濃染、縮小といった形態学的にアポトーシスを示し、DNA の ladder 形成も認められた。ERK の特異的な阻害剤である PD98059 の前処置によりアポトーシスを示す細胞数は増加し、DNA の ladder の形成は増加した。逆に、p38MAPK の特異的な阻害剤である SB203580 の前処置によりアポトーシスを示す細胞数は減少し、DNA の ladder の形成も減少した。

考察

近年、細胞内の最も基本的なシグナル伝達経路を構成すると考えられてきた MAP キナーゼのファミリー（ERK, JNK, p38MAPK）がアポトーシスと関連しているとの報告がいくつかなされている[2]。in vitro の研究により、ERK の活性化が心肥大や心肥大時の遺伝子発現に重要であると報告され[5]、JNK/p38MAPK の活性化がアポトーシスの誘導に重要であると報告された[2]。今回の結果により、ラットの培養心筋細胞における抗癌剤 DM 刺激によりアポトーシスが起こっていることが生化学的及び形態学的に証明された。DM により産生されたフリーラジカル（H_2O_2 と OH）が心筋細胞障害を引き起こすことがフリーラジカル消去剤により明らかとなったがその過程において ERK は細胞保護に、p38MAPK は障害に働いていることが示唆された。ERK の上流には

Ras-Raf-1 が、p38MAPK の上流には Rho が存在することも判明した。

Rho 蛋白は最近、細胞の骨格や接着を制御する機能を有することで注目されてきた低分子量 G 蛋白であり、種々の細胞で JNK 及び p38MAPK のカスケードの上流にあることが報告されている[5)]。DM による p38MAPK の活性化を Rho 蛋白の dominant-negative mutant が抑制することより、心筋細胞における DM 刺激のシグナル伝達においても重要な役割を果たしていることが示唆された。どのように Rho 蛋白が活性化されるか、また p38MAPK を活性化するかは今後の課題である。

Ca^{2+} は細胞内の情報伝達系の中で普遍的なシグナル物質として働いている。細胞内或いは細胞外 Ca^{2+} キレート剤にて前処置すると、ERK と p38MAPK の活性化が同時に抑制されたことから、Ca^{2+} は ERK と p38MAPK の共通の上流に存在することが示唆された。Ca^{2+} が DM による MAPK ファミリーの活性化にどのように関与するのかを今後検討する予定である。また、どのような機序で ERK が細胞保護に、p38MAPK がアポトーシス誘導に働いているかについても、今後の検討課題である。

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研究計画書

（一）研究の背景及び目的

1　抗癌剤ダウノマイシン(DM)による非可逆性心筋障害は致死的であり、その病態解明と予防･治療法の開発が急務となっている。最近、心不全やある種の心筋症において心筋細胞にアポトーシスが起こっていることが報告され、その病態形成における役割が注目されている。また、mitogen-activated protein kinase (MAPK)の新しいファミリーであるC-Jun NH_2-terminal kinase (JNK)とp38MAPKが同定され、その働きのひとつとしてアポトーシスとの関連が報告されている。そこで本研究は、DMによる心筋細胞アポトーシスとMAPキナーゼファミリーの関係を詳細に解析する。

2　高血圧の持続によって標的臓器に障害が起こり、その結果脳卒中、心筋梗塞、高血圧性心不全、突然死、腎不全などが正常血圧者より高率に発症する。そのため本研究は、持続的な高血圧負荷に対する、心筋細胞の適応及び破綻現象について、分子機構の面から病態生理の解明を試みる。

（二）進行状況と今後のスケジュール

1　1997年4月から抗癌剤ダウノマイシンによる心筋障害のメカニズムおよびその分子機序をテーマに研究を行い、この一年間で解明された結果は以下のである。

（1）抗癌剤ダウノマイシンはApoptosisによる心筋障害を引き起こした。

（2）MAPキナーゼスーパーファミリー（ERK, p38MAPK）は抗癌剤ダウノマイシンによるApoptosisにおいて、ERKが細胞保護に、p38MAPKが障害に働く。

（3）ERKの上流にはRas-Raf-1が、p38MAPKの上流にはRhoが存在する。

以上の結果をアメリカのCirculation誌に投稿した。

2　1998年2月から液性因子endothelin 1 (ET-1)による心肥大の分子機序を解析し、現在までに以下の結果を得た。

（1）ET-1は心筋細胞の特異的な胎児遺伝子βMHCを活性化した。

（2）ET-1によるβMHCの活性化にCa^{2+}/calmodulin-dependent kinase IIおよびCa^{2+}/calmodulin-dependent phosphatase calcineurinが関与した。

以上の結果を論文にまとめているところである。また、この研究結果を1998年11月にアメリカ心臓病学会（AHA）で報告（poster）であった。

3　持続的な高血圧負荷に対して、心筋細胞の適応及び破綻現象の病態生理を解明する

ために、細胞肥大を引き起こす心筋細胞特異的な分泌蛋白の clonning と解析を行う。具体的に説明すると、

(1) 培養心筋細胞を ET-1 で刺激して、differential display 法にて新しい遺伝子を探究する。

(2) その新しい遺伝子の配列をもとに、オリゴヌクレオチドを作成し、マウス心筋細胞の遺伝子ライブラリーを用いて分泌蛋白遺伝子を単離、PCR 法で増幅後、塩基配列を決定する。

(3) 心筋特異的な分泌蛋白の遺伝子配列をもとに、この蛋白のアミノ酸一次構造を決定する。次に、特異抗体を作成し、その心筋細胞における局在、受容体の分布、生理作用、圧負荷あるいは AngII、ET-1、カテコラミン負荷による発現の増強を解析する。さらに、プロモーター領域の解析に着手する。

(4) 前述の心筋細胞特異的な分泌蛋白のノックアウトマウスの作成に着手する。さらに、そのノックアウトマウスにおける心肥大反応にどのように関係しているか、ノックアウトマウスにおける蛋白の局在、圧負荷による変動を解析する。

(三) 博士号取得後の計画

以上の研究をさらに発展させ、心肥大・心不全の病態生理の解明を行うとともに新しい機序に基づく治療法の開発を目指す。

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

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Running Title: Daunomycin-induced apoptosis of cardiomyocytes

Abstract

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 μM DM for 24 h, many cells became positive for TUNEL staining with morphological changes characteristic of apoptosis. Fragmentation of DNA into oligonucleosome-sized fragments was recognized by agarose gel electrophoresis in DM-treated myocytes. DM activated three members of mitogen-activated protein kinase (MAPK) dose-dependently, such as extracellular signal-regulated protein kinases (ERKs), c-Jun NH_2-terminal kinases, and p38MAPK in cardiac myocytes. Oxyradical scavengers or Ca^{2+} chelators inhibited DM-induced activation of ERKs and p38MAPK. DM-induced activation of ERKs was also inhibited by overexpression of dominant negative mutants of Ras (D.N.Ras), while the p38MAPK 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 MEK inhibitor PD98059, while pretreatment with a specific inhibitor of p38MAPK SB203580 significantly reduced the number of apoptosis.

Conclusions These results suggest that DM activates MAPKs through reactive oxygen species and Ca^{2+} and that the MAPK family plays important roles in DM-induced apoptosis in cardiac myocytes. ERKs protect cardiomyocytes from apoptosis, whereas p38MAPK is involved in the induction of cardiomyocyte apoptosis.

Key words: daunomycin • ERKs • p38MAPK • apoptosis • cardiomyocyte

Introduction

Anthracyclines such as daunomycin (DM) and adriamycin (AM) are very powerful antineoplastic agents which are widely used in the treatment of cancer. The clinical usefulness of these agents is, however, limited due to serious adverse effects including cardiotoxicity (1, 2). These cardiotoxic effects result in cardiac dysfunction, cardiomegaly, and finally congestive heart failure (3). Although exposure to DM or other anthracycline derivatives has been reported to block cell cycle at G_2 stage (4) and induce apoptosis in tumor cells (5), the mechanism of myocardial impairment by anthracyclines remains uncertain. It has been reported that AM enhances peroxidation of lipids in myocardial membrane (6) and that Sulfhydryl reagents inhibit AM-induced cardiotoxicity (7, 8). These observations suggest that anthracyclines impair myocardium through the production of reactive oxygen species (ROS) (2, 9).

The MAPK family is an important mediator of signal transduction and activated by a variety of stimuli such as many growth factors and cellular stresses (10). Among the MAPK family, especially three members, such as extracellular signal-regulated kinases (ERKs) (11, 12), c-Jun NH_2-terminal kinases (JNKs, also called SAPKs) (13, 14), and p38MAPK (15), have been well characterized. These MAPKs are activated by dual phosphorylation on threonine and tyrosine residues within the motif Thr-X-Tyr in subdomain VIII (10). MAPK kinase kinases phosphorylate and activate MAPK kinases, which in turn phosphorylate and activate MAPKs. Each MAPK is activated through the specific kinase cascade. Many studies on the action of growth factors have elucidated physiological functions of ERKs. ERKs are activated by a variety of signaling molecules such as tyrosine kinases (TKs), Ras, protein kinase C (PKC), protein kinase A (PKA), or Ca^{2+} (16-18), through Raf-1 and MAPK/ERK kinase (MEK), and play essential roles in the control of cell growth and differentiation (19). ERKs are also activated by oxidative stress and play critical roles in survival of NIH 3T3 cells (20), PC12 cells (21), and cardiac myocytes (22). In contrast, JNKs are weakly activated by growth factors and phorbol esters, but markedly activated in response to tumor necrosis factor-α (TNF-α), ultraviolet irradiation, and cellular stresses (13, 14, 23). Recently, cumulative evidence suggests that activation of JNKs has been associated with induction of apoptosis (24, 25). Likewise, p38MAPK, also called RK (26) and CSBP (27), a mammalian homolog of the yeast high-osmolarity glycerol

response-1 kinase (HOG1) (15), is also strongly activated by TNF-α and environmental stresses including osmotic shock and UV irradiation (28). An important physiological substrate of p38MAPK is MAPK-activated protein kinase-2, which phosphorylates heat shock protein (hsp27) as part of the cellular response to stress (26). Recently, it has been reported that small GTP proteins of the Rho family, Rac1 and Cdc42, regulate the JNK/p38MAPK pathway (29-31). More recently, Strutt et al. have also shown that RhoA is also involved in regulation of JNK/SAPK-like kinases, which are required for the generation of tissue polarity (32).

Although many studies have been performed to elucidate the molecular mechanism of anthracycline-induced myocardial impairments (1-3, 6, 8, 9), little is known about the precise intracellular signals which lead to the injury. 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.

Materials and Methods

Materials. [γ-^{32}P]ATP was purchased from Du Point-New England Nuclear Co. (Boston, MA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Tyrphostin (A25) and Genistein was from GIBCO BRL Co. (Gaithersburg, MD). Calphostin C was from Funakoshi Co. (Tokyo, Japan). Anti-hemagglutinin (HA) monoclonal antibody and anti-phospho-specific p38MAPK antibody were from Mitsubishi Biochemical Laboratories (Tokyo, Japan) and New England Biolabs, Inc (Bevely, MA), respectively. M2 Flag monoclonal antibody was from Kodak Co. (New Haven, CT). Horseradish peroxidase-conjugated anti rabbit IgG antibody (HRP-anti-rabbit IgG) and enhanced chemiluminescence reaction system (ECL) were from Amersham Co. (England). Daunomycin and apoptosis ladder detection kit were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and myelin basic protein (MBP) and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

cDNA plasmids. Both HA-tagged ERK2 (HA-ERK2) and Flag-tagged p38MAPK (Flag-p38MAPK) were kind gifts from M. Karin (30). Expression vectors encoding Rho-GDI, various mutants of RhoA, Rac1, and Cdc42 were provided by J.S. Gutkind et al (31), and D.N.Ras was provided by Y. Takai (33). All plasmid DNA was prepared by using QIAGEN plasmid DNA preparation kits (Hilden, Germany).

Cell culture. Primary cultures of cardiac myocytes were prepared from ventricles of one-day old Wistar rats as described previously (34) according to the method of Simpson and Savion (35). In brief, cardiomyocytes were plated at a field density of 1×10^5 cells/cm^2 on 35 mm culture dishes with 2 ml of culture medium (DMEM with 10% FBS). The population of nonmyocytes was <10% of the total cell population. Twenty four hours after seeding, the culture medium was changed to DMEM with 0.1% FBS and cells were cultured for 48 h to 72 h before stimulation.

Transfection. Twenty four hours after plating the cells on culture dishes, DNA was transfected by the calcium phosphate method as described previously (36). For each dish, 2.5 μg of Flag-

p38MAPK or HA-ERK2 plasmid was co-transfected with 7.5 μg of control vector plasmid, Rho-GDI, or various D.N. mutants. After 15 h of transfection, the culture medium was removed, and cells were washed with PBS and maintained in DMEM with 0.1% FBS for 48 h to 72 h before treatment with DM or other reagents.

Assay of ERK activity. The activity of ERKs was measured by "in gel assay" using MBP-containing gel as described previously (37). In brief, cells were lysed with 100 μl of Buffer A (25 mM Tris-HCl pH 7.4, 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 nM okadaic acid, 0.5 mM EGTA, and 1mM phenylmethylsulfonyl fluoride) and 25 μl of cell lysates were applied to an SDS-polyacrylamide gel containing 0.5 mg/ml MBP. ERKs in the gel were denatured in 6 M guanidine HCl and renatured in 50 mM Tris-HCl (pH 8.0) containing 0.04% Triton X-100 and 5 mM 2-mercaptoethanol. The phosphorylative activity of ERKs was assayed by incubating the gel with [γ-^{32}P]ATP. After incubation, the gel was washed, dried, and subjected to autoradiography.

Assay of transfected HA-tagged ERK2. The activity of transfected ERK2 was determined by MBP kinase assay as described previously (37). In brief, cardiomyocytes were lysed with Buffer A and the lysates were incubated with an anti-HA polyclonal antibody for 1 h at 4°C. After incubation, the immunocomplex was precipitated using protein A sepharose, washed, resuspended in 25 μl of the kinase buffer B (25 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 40 μM ATP, 2 μCi [γ-^{32}P]ATP, 2 μM Protein kinase inhibitor, and 0.5 mM EGTA), and incubated with 25 μg MBP as a substrate at 25°C for 10 min. After incubation, the reaction was terminated by addition of Laemmli sample buffer (0.002% bromophenol blue, 10 mM sodium phosphate buffer pH 7.0, 10% glycerol, 0.4% SDS, 1% 2-mercaptoethanol) to the samples, and the samples were boiled for 5 min. The supernatants were subjected to SDS-PAGE, and the gel was dried and subjected to autoradiography.

Assay of JNK activity. Cardiac myocytes were stimulated by 100 μM DM for 20 min at 37°C and lysed on ice with Buffer A. The cell lysates were obtained by centrifugation at 12,000 rpm for 20 min at 4°C and 100 μl of the lysates were incubated at 4°C for 30 min with 1 μg of glutathione S-

transferase (GST) -c-Jun (amino acid number 1-79 of humun c-Jun) fusion protein bound to glutathione sepharose beads in the kinase reaction buffer C containing 25 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM EGTA, 40 μM ATP, and 1 μCi [γ-^{32}P]ATP, as described previously (13, 38). The reaction was terminated by boiling after addition of Laemmli sample buffer. After centrifugation, supernatant was subjected to SDS-PAGE. The gel was dried and subjected to autoradiography.

In addition, 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 (39). In brief, cell lysates were incubated with 2 μg of the anti-JNK antibody at 4°C for 12 h and then incubated with 50 μl of protein A-Sepharose at 4°C for 40 min. After washed twice with ice-cold lysis buffer, each sample was mixed with 1 μg of the GST-c-Jun (1-79) protein in 8 μl of buffer C and incubated at 30°C for 30 minutes. The reaction was terminated by boiling after addition of Laemmli sample buffer. After centrifugation, supernatant was subjected to SDS-PAGE. The gel was dried and subjected to autoradiography.

Western blot analysis. Protein extracts were subjected to Western blot analysis as described previously (22) using p38MAPK-specific antibody. Anti-phosphorylated p38MAPK antibody recognizes only activated p38MAPK that is phospholated on Thr-180 and Tyr-182. The anti-rabbit IgG conjugated with HRP was used as the secondary antibody for p38MAPK and immune complexes were visualized using the ECL detection kit according to the manufacturer's directions. p38MAPK phosphorylation examined by Western blot analysis using phospho-specific p38MAPK antibody is well correlated with the p38MAPK activity as reported before (22).

For the Western blot analysis of Flag-p38MAPK, the Flag-p38MAPK-transfected cells were harvested in Buffer A. Flag-p38MAPK was immunoprecipitated with an anti-Flag M2 monoclonal antibody. The anti-mouse IgG conjugated with HRP was used as the secondary antibody and immune complexes were visualized as mentioned above.

Assay of p38MAPK activity. After transfection of Flag-p38MAPK, the myocytes were incubated with 100 μM DM for 30 min. Cardiomyocytes were lysed with Buffer A and the

transfected p38MAPK was then immunoprecipitated with an anti-Flag M2 monoclonal antibody. The immune complex was resuspended in the kinase buffer B and incubated with MBP as a substrate at 25°C for 10 min. The sample was subjected to SDS-PAGE, and the gel was dried and subjected to autoradiography.

Immunofluorescent cytochemistry. After transfection of HA-ERK2, Flag-p38MAPK or myc-tagged D.N.Rho, the myocytes plated on a cover glass were fixed with 4% paraformaldehyde solution for 30 min at room temperature and treated with 0.3% Triton X-100 for 15 min to improve permeability to the regents. After wash with PBS, the samples were incubated for 1 h at 37°C with the phalloidin-TRITC antibody (red) that identifies filamentous actin. Next, the samples were incubated with anti-HA, anti-Flag M2, or anti-myc monoclonal antibody for 1 h at 37°C and then incubated with an anti-mouse IgG conjugated to FITC (green) for 1 h at room temperature. These samples were analyzed by fluorescence microscopy.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analysis. Cardiomyocytes plated on a cover glass were fixed with 4% paraformaldehyde solution for 30 min at room temperature. After wash with PBS, the samples were incubated with a monoclonal antibody against myosin heavy chain (MF-20) (40) for 1 h at 37°C, and then incubated with an anti-mouse IgG conjugated with rhodamine for 1 h at room temperature. Next, 50 μl terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) reaction mixture containing both terminal deoxynucleotidyl transferase and fluorescein isothiocyanate (FITC)-conjugated dUTP was added on the each sample for 1 h at 37°C. These samples were analyzed by fluorescence microscopy.

Agarose gel electrophoresis for DNA fragmentation. To examine the DNA laddering formation, we used the apoptosis ladder detection kit (Wako Pure Chemical Industries, Ltd. Tokyo, Japan). Briefly, cells ($1X10^5$) were lysed in 180 μl of enzyme reaction solution and 20 μl of enzyme activator solution followed by incubation with 40 μg RNase and 100 μg proteinase K for 30 min at 50°C, and DNA was extracted by 50% isopropanol. The DNA was then ethanol precipitated and

finally resuspended in TE buffer. The DNA was electrophoretically fractionated on 1.5% agarose gel and stained with fluorescent SYBR® Green I as described by the protocol.

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

Results

DM induces apoptosis in cardiac myocytes.

DM has been reported to induce apoptosis in a variety of cells such as Hela S3, Jurkat, ST4, and PF382 cells (5, 41). Thus, we first examined whether DM also induces apoptosis of cardiac myocytes. When cultured cardiac myocytes of neonatal rats were exposed to 1 μM DM for 24 h, a considerable number of myocytes (~24%) showed positive TUNEL staining compared with the vehicle-treated cells (~3%) (Fig. 1A, B). Many TUNEL-positive cells had condensed nuclei, which are characteristic of apoptosis (Fig. 1A). When TdT was not added in the reaction solution, no myocytes were stained positively (data not shown), indicating that the specific labeling targets were the multitude of new 3'-OH DNA ends generated by DNA fragmentation induced by DM. We next examined whether DM induces DNA fragmentation using agarose gel electrophoresis. The characteristic degradation of genomic DNA into oligonucleosomal-length fragments was observed when cardiac myocytes were exposed to 1 μM DM for 24 h (Fig. 1C).

DM activates MAPKs in cardiac myocytes.

Many lines of evidence have suggested that MAPKs are key molecules in intracellular signal transduction pathways and play important roles in cell survival or death (19, 20, 42). To elucidate the molecular mechanism of DM-induced apoptosis of cardiac myocytes, we examined whether DM activates MAPKs including ERKs, JNKs, and p38MAPK in cardiac myocytes. When cardiac myocytes were exposed to various concentrations of DM (0.1 μM—1 mM) for 20 min, ERKs were activated in a dose-dependent manner (Fig. 2A). A significant increase in the activity of ERKs was detected from 1 μM DM, and maximum activation was obtained by 100 μM DM (Fig. 2A). When being incubated with 1 mM DM for 20 min, many cardiac myocytes were dead and therefore the activity of ERKs per plate was very low (Fig. 2A). The increase in the ERK activity was first detected at 5 min after the addition of 100 μM DM, peaked at 15 min, and gradually decreased thereafter (Fig. 2B). One h after the addition of 100 μM DM, the activity of ERKs returned to basal levels. JNKs (Fig. 3A) and p38MAPK (Fig. 4A) were also activated by DM at the dose similar to the case of ERKs. Unlike the ERK activity, the activity of JNKs and p38MAPK was high even after

treatment with 1 mM DM (Fig. 3A and 4A). Since many cardiac myocytes were dead after incubation with 1 mM DM, this result suggests that the activity of JNKs and p38MAPK of each remaining cardiomyocyte might be very high. Activation of JNKs (Fig. 3B) and p38MAPK (Fig. 4B) by 100 μM DM was detected from 5 min and peaked at 30 min. The phosphorylation of JNKs and p38MAPK was observed even at 60 min after addition of 100 μM DM.

ROS is involved in activation of ERKs and p38MAPK.

We next examined how DM activates MAPKs in cardiac myocytes. Since ROS have been reported to be generated from cells which are exposed to DM (2, 9), and to activate MAPKs in many cell types (20, 22), we examined whether ROS are involved in DM-induced activation of MAPKs. Cardiac myocytes were exposed to DM after pretreatment with dimethyl sulfoxide (DMSO) (a diffusible scavenger of ·OH), catalase (a oxidoreductase of H_2O_2), or N-(2-mercaptopropionyl)-glycine (MPG) (a rapidly diffusible scavenger of H_2O_2, $O_2^{-}\cdot$, and ·OH). DMSO and catalase strongly suppressed DM-induced activation of ERKs (Fig. 5A) and p38MAPK (Fig. 5B). In contrast, heat-inactivated catalase did not show any inhibitory effects on the DM-induced activation of these kinases (data not shown). The pretreatment with MPG completely blocked activation of ERKs (Fig. 5A) and p38MAPK (Fig. 5B). However, superoxide dismutase (SOD), a scavenger of $O_2^{-}\cdot$, did not show inhibitory effects on ERKs and p38MAPK (Fig. 5A, B). These results suggest that among ROS, H_2O_2 and ·OH, but not $O_2^{-}\cdot$, may be involved in DM-induced activation of MAPKs in cardiac myocytes.

DM-induced ERK activation is dependent on Ras and Raf-1 in cardiac myocytes.

We further examined the DM-induced intracellular signaling pathways leading to activation of ERKs. DM increased the activity of transfected HA-ERK2 in cardiac myocytes (Fig. 6A). Overexpression of D.N.Ras or D.N.Raf-1 strongly suppressed DM-induced ERK2 activation (Fig. 6A). In contrast, overexpression of D.N.Ras or D.N.Raf-1 did not affect activation of p38MAPK induced by DM (Fig. 6B). These data suggest that Ras and Raf-1 play critical roles in DM-induced activation of ERKs and that p38MAPK is activated by DM through pathways different from those of ERKs.

DM-induced p38MAPK activation is dependent on Rho in cardiac myocytes.

It has recently been reported that among Rho family GTP-binding proteins, Rac1 and Cdc42 regulate the activity of JNKs and p38MAPK in a variety of cell types (29-31). More recently, Strutt et al. has also shown that JNK/SAPK-like kinases are regulated by RhoA signaling cascade (32). We thus examined whether small G proteins of Rho family are involved in DM-induced p38MAPK activation in cardiac myocytes. DM activated the transfected p38MAPK in cardiac myocytes (Fig. 7A, top). 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 p38MAPK activation (Fig. 7A, top). Among these dominant negative mutants, RhoGDI most strongly suppressed the activation of p38MAPK by DM. In contrast, DM-induced ERK activation was not affected by overexpression of dominant negative mutants of these Rho family small G proteins (Fig. 7B). To determine whether dominant negative mutants of RhoA, Rac1, and Cdc42 are expressed in cardiac myocytes, we transfected the myc-tagged dominant negative mutants into cardiac myocytes and stained the cultured cells using an anti-myc monoclonal antibody and the phalloidin-TRITC. Expression of the dominant negative mutants of RhoA, Rac1, and Cdc42 was observed in 1-2% of cardiac myocytes (data not shown). To determine whether expression levels of transfected Flag-p38MAPK are equal among various conditions, we performed Western blot analysis using an anti-Flag M2 monoclonal antibody. The transfected each mutant was equally expressed in cultured cardiac myocytes (Fig.7A, bottom). In addition, when constitutively active mutants of RhoA, Rac1, and Cdc42 were cotransfected into cardiomyocytes with p38MAPK, the activity of the transfected p38MAPK was markedly activated (data not shown). These results suggest that Rho family small G proteins play important roles in activation of p38MAPK by DM in cardiac myocytes.

DM-induced activation of ERKs and p38MAPK is dependent on Ca^{2+} but independent of PKC, PKA, and TKs in cardiac myocytes.

We have recently reported that Ca^{2+} plays an important role in activation of ERKs and JNKs in cardiomyocytes (39, 43). To elucidate whether Ca^{2+} is involved in DM-induced activation of MAPKs, we examined the activity of ERKs and p38MAPK after chelation of Ca^{2+} with 5 mM EGTA or 40 μM BAPTA. Chelation of intracellular or extracellular Ca^{2+} by pretreatment with 5 mM EGTA

for 2 min or 40 μM BAPTA for 30 min, respectively, suppressed activation of ERKs and p38MAPK (Fig. 8A, B). Complete depletion of intracellular free Ca^{2+} by BAPTA may cause certain stress to cardiac myocytes and activate ERKs and p38MAPK (Fig. 8A, B). These results suggest that Ca^{2+} is involved in DM-induced activation of ERKs and p38MAPK in cardiac myocytes. On the other hand, downregulation of PKC by pretreatment with 0.1 μM TPA for 24 h (44) or by pretreatment with 1 μM Calphostin C, a specific inhibitor of PKC, for 60 min (45) did not have any effects on DM-induced ERK or p38MAPK activation (Fig. 8C, D). Likewise, inhibition of PKA by pretreatment with 100 μM RpcAMP (18) for 10 min did not affect DM-induced ERK or p38MAPK activation (Fig. 8C, D). After pretreatment for 30 min with 50 μM Tyrphostin (A25) or 20 μM Genistein, two chemically and mechanistically dissimilar tyrosine kinase inhibitors (46, 47), cardiac myocytes were stimulated with 100 μM DM for 20 min. The activation of ERKs or p38MAPK by DM was not affected by the pretreatment with these tyrosine kinase inhibitors (Fig. 8C, D). TPA did not activate ERKs in cardiac myocytes after pretreatment with TPA for 24 h and Calphostin C for 60 min (Fig. 8C). ERKs were not activated by isoproterenol and insulin after pretreatment with RpcAMP for 10 min, or Tyrphostin (A25) and Genistein for 30 min (Fig. 8C), respectively. These results suggest that PKC, PKA, and TKs were fully inhibited by each pretreatment. Taken together, these results suggest that DM induces activation of ERKs and p38MAPK through the pathway independent of PKC, PKA, or TKs in cardiac myocytes.

ERKs and p38MAPK play opposite roles in DM-induced myocardial apoptosis. Finally, to elucidate the roles of MAPKs in the development of cardiomyocyte apoptosis, we examined apoptotic death of cardiac myocytes following the DM treatment in the presence or absence of the MAPK inhibitors, PD98059 for ERKs or SB203580 for p38MAPK. PD98059 has been reported to specifically inhibit MEK1 and MEK2, which are specific activators for ERKs but not for p38MAPK or JNKs (48). When cardiac myocytes were pretreated with 50 μM PD98059 for 60 min, DM-induced ERK activation was completely suppressed, while p38MAPK was not (data not shown). When nuclei of cardiac myocytes were stained by TUNEL method, few cardiac myocytes (less than 3%) were positive in untreated cultures (Fig. 9A). After incubation with 1 μM DM for 24 h, the number of TUNEL-positive cardiac myocytes was increased (~24%)(Fig. 9A). When the ERK

signaling pathway was blocked by the pretreatment with 50 μM PD98059 for 1 h, the number of TUNEL-positive myocytes was further increased by over two folds (~53%) (Fig. 9A). Treatment with PD98059 significantly increased the number of TUNEL-positive cells even in the absence of DM (~10%) (Fig. 9A). We further examined DNA fragmentation by agarose gel electrophoresis (49). When cardiac myocytes were exposed to 1 μM DM for 24 h, extracted genomic DNA showed a prominent DNA ladder, characteristic of apoptosis (Fig. 9B). When cardiac myocytes were pretreated with PD98059, DM-induced DNA fragmentation became more prominent (Fig. 9B). Next, we examined apoptotic death of cardiac myocytes following DM exposure in the presence or absence of a p38MAPK inhibitor SB203580, which specifically inhibits p38MAPK, but not ERKs or JNKs even at 100 μM (50). Treatment with less than 5 μM of SB203580 showed no significant inhibition in DM-induced apoptosis. However, DM-induced apoptosis was significantly suppressed by the pretreatment with 10 μM SB203580 for 2 h (~18%)(Fig. 9A). DM-induced DNA fragmentation was also reduced by the pretreatment with 10 μM SB203580 (Fig. 9B). The concentration of 10 μM SB203580 has been widely used to inhibit p38MAPK in many cell types (51, 52). To further elucidate the roles of ERKs and p38MAPK, we transfected HA-ERK2 and Flag-p38MAPK into cultured cardiac myocytes and examined apoptosis. Strong signals of both HA-ERK2 and Flag-p38MAPK were observed in the cytoplasm of cardiomyocytes before addition of DM and they were translocated into the nucleus after DM treatment (Fig. 9C), suggesting that DM activates both ERKs and p38MAPK in cardiac myocytes. When we double-immunostained the cells using TUNEL and either anti-HA or anti-Flag monoclonal antibody, many p38MAPK-transfected cells, but not ERK2-transfected cells showed TUNEL positive after starvation of 48 h (Fig. 9D). Furthermore, when the transfected cells were incubated with 1 μM DM for 4 h, almost all p38MAPK-transfected cells were TUNEL positive (data not shown). In contrast, none of ERK2-transfected cells were TUNEL positive (data not shown). These results also suggest that p38MAPK induces apoptosis of cardiomyocytes while ERKs protect cardiomyocytes from apoptosis. Collectively, ERKs and p38MAPK play opposite roles in DM-induced myocardial apoptosis. ERKs protect cardiac myocytes from DM-induced apoptosis, whereas p38MAPK induces apoptosis.

Discussion

A growing body of evidence suggests that apoptosis is an active process induced by a variety of stresses (21, 25, 42). Even the cells that lose their proliferative ability, like cardiac myocytes, undergo apoptosis by a variety of stimuli (22, 53, 54). 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 in cardiac myocytes by DM, and among them ERKs and p38MAPK 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 p38MAPK, which is activated through the Rho family, is involved in DM-induced apoptosis of cardiomyocytes. Furthermore, activation of ERKs and p38MAPK by DM is dependent on Ca^{2+}, but independent of PKC, PKA, or TKs in cardiac myocytes.

Recently, apoptosis has been suggested to play a critical role in a variety of cardiovascular diseases, including myocardial infarction, heart failure, and atherosclerosis (55). We examined TUNEL staining and DNA ladder formation to determine whether DM induces apoptosis of cardiac myocytes. Although we used 100 μM DM to examine the DM-evoked signaling pathway in the present study, we obtained basically same results even with 1 μM DM. 1 μM DM significantly activated ERKs, JNKs, and p38MAPK in cardiac myocytes through the same signaling pathway as we showed using 100 μM DM. Since activation was not prominant with 1 μM DM, we showed the data obtained with 100 μM DM. To examine the cardiotoxicity of DM, we used 1 μM DM. It has been reported that the plasma kinetics of anthracyclines following standard bolus administration in patients exhibit peak plasma concentration of higher than 5 μM (56) and previous studies on cardiomyocytes demonstrated that treatment for 24-48 h at 1 μM AM produces morphological and ultrastructural changes characteristic of AM cardiotoxicity (57). 1 μM DM has also been used to induce apoptosis in P388 murine leukemia cells in 4-24 h (58). Although it is difficult to precisely estimate the amount of necrotic death and apoptotic death of cardiac myocytes, the present results suggest that at least a part of cultured cardiomyocytes show apoptotic cell death after exposure to DM. Since we can easily detect the ladder formation, at least more than 10% of cells may be dead by apoptosis. Ferrans and colleagues have reported that cells of the kidney and the intestine, but not of

the heart, are dead by apoptosis at 7 days after the administration of AM (59, 60). However, it is possible that apoptosis occurs more rapidly in the heart after administration of AM, leaving no residue at 7 days. In fact, Unverferth et al. have reported that AM markedly alters the morphology of the human myocardial nucleus and nucleolus at 4 hours after treatment, and that these changes diminish with 24 hours (61). Since apoptosis usually occurs in isolated single cell, the remnants of which are engulfed by peripheral phagocytic cells of the macrophage/monocyte lineage, and apoptosis dose not induce persisting changes in tissue such as the inflammation and scarring that characterize necrosis, it should be difficult to detect apoptotic cells in tissue. During preparation of our manuscript, Wang et al. have reported that DM induced apoptosis in the cultured cardiomyocytes of neonatal rats (62).

Anthracycline-induced cardiotoxicity has been reported to be related to the generation of ROS (2, 9). AM stimulates NADPH oxidase-like activity in the cardiac sarcoplasmic reticulum and thereby induces oxidative stress in the myocardium (9). Cardiac glutathione peroxidase is also reported to be inhibited by AM (63). We showed here that MPG (a rapidly diffusible scavenger of H_2O_2 , $O_2^{-}\cdot$, and $\cdot OH$), DMSO (a $\cdot OH$ specific scavenger), and catalase (H_2O_2 oxidoreductase), but not SOD (a scavenger of $O_2^{-}\cdot$), markedly repressed DM-mediated activation of ERKs and p38MAPK. It has been reported that the pretreatment with catalase and mannitol (a quencher for $\cdot OH$) but not with SOD mitigates the reduction in contractile function of the papillary muscles, decreases lipid peroxidase and reduces ultrastructure damage due to AM (2). These results and observations suggest that $\cdot OH$ and H_2O_2, but not $O_2^{-}\cdot$, are mainly involved in the DM-induced activation of MAPKs and acute cardiac injury, although it is also possible that dismutation of $O_2^{-}\cdot$ could result in further H_2O_2 and $\cdot OH$ generation or that SOD, a large molecular weight scavenger of $O_2^{-}\cdot$, remained in the extracellular space.

ERKs are activated by serial activation of Raf-1 and MEK in mammalian cells (19). However, the signal transduction pathways leading to activation of Raf-1 are different according to cell types and stimuli (16-18). It has been reported that angiotensin II (Ang II) activates Src family tyrosine kinases and Ras in smooth muscle cells through G protein-coupled Ang II type I receptor, resulting in activation of ERKs (64, 65). In contrast, we have recently reported that protein kinase C (PKC), but not Src family or Ras, is critical for Ang II-induced ERK activation in cardiac myocytes (45). Although PKC is partially involved in ERK activation by H_2O_2 in Jurkat T cells (66), Ras, but not

PKC, is necessary for ERK activation by H_2O_2 in cardiac myocytes (22). In this study, we demonstrated that DM activates ERKs through Ras and Raf-1, but not through PKC, PKA, or TKs in cardiac myocytes. Although stimulation of TKs often activates ERKs through Ras (16, 17), the DM-induced ERK activation was not inhibited by the pretreatment with Tyrphostin (A25) or Genistein. These results suggest that although we cannot exclude a possibility that Tyrphostin (A25)- and Genistein-insensitive TKs may mediate DM-induced activation of ERKs, TKs may not be involved in activation of DM-induced ERK signaling pathway.

The signaling pathway leading to activation of p38MAPK was different from that of ERKs. DM-induced p38MAPK activation was not inhibited by D.N.Ras or D.N.Raf-1. PKC, PKA, and TKs are also not involved in DM-induced p38MAPK activation. It has been reported that small GTP-binding proteins of the Rho family, including Rac1 and Cdc42, regulate the activity of JNKs and p38MAPK (29-31). Recently, Strutt et al. have also shown that RhoA regulates JNK/SAPK-like kinases, which are required for the generation of *Drosophila* tissue polarity (32). During the preparation of our manuscript, Roberts et al. have demonstrated that, in A549 lung carcinoma cells, H_2O_2 induces JNK activation via RhoA-dependent pathway (67). In the present study, we examined whether Rho family small GTP-binding proteins are responsible for DM-induced activation of p38MAPK in cardiac myocytes. Overexpression of Rho-GDI, D.N.RhoA, D.N.Rac1, and D.N.Cdc42 significantly suppressed DM-induced p38MAPK activation, but not DM-induced ERK activation. These results suggest that all three members of Rho family are involved in DM-induced p38MAPK activation in cardiac myocytes. It has been reported that the Rho family proteins regulate p38MAPK through the p21-activated kinase (PAK) (29). It needs further study to elucidate whether PAK is involved in DM-induced activation of p38MAPK.

In the present study, EGTA and BAPTA potently inhibited the DM-induced activation of ERKs and p38MAPK. It is generally regarded that rapidly raised intracellular Ca^{2+}, in response to multiple hormones, neurotransmitters, and immune effecters, regulates myriad cellular processes, including gene expression and cell growth (68, 69). It has been reported that Ca^{2+} activates ERKs by modulating PKC activity (38) or by activating Ca^{2+}-dependent tyrosine kinases (70), and that Ca^{2+}/calmodulin-dependent protein kinases may regulate the JNK cascade (71, 72). It should be determined how Ca^{2+} is involved in DM-induced activation of ERKs and p38MAPK.

Many lines of evidence have suggested that the members of MAPKs, including ERKs, JNKs, and p38MAPK, play important roles in cell survival and death (25, 42). Activation of the ERK signaling pathway may function to protect cells from a variety of cellular stresses (20-22, 42). On the contrary, the JNK and p38MAPK signaling pathways have been suggested as the apoptosis-inducing pathways (24, 42, 73). JNKs and p38MAPK are activated by a variety of stresses such as mechanical stretch (38), ischemia/reperfusion (74), and AngII (39) in cardiomyocytes, but the role of JNK/p38MAPK activation in cardiomyocytes was unknown. DM activated all three members of MAPKs in a different manner. Although the activation of ERKs and JNKs/p38MAPK occurred at almost the same time, the inactivation of JNKs/p38MAPK was preceded by the one of ERKs. Furthermore, unlike the very low ERK activity, the activity of JNKs and p38MAPK remained quite high even after being dead during incubation with DM. From the differences, though uncausal to apoptosis, it at least suggested that ERKs and JNKs/p38MAPK may play distinct role in DM-induced cardiac apoptosis. Pretreatment of PD98059, a specific inhibitor of MEK-1, made the DNA fragmentation prominent and increased the number of apoptotic cells. Moreover, PD98059 treatment itself also induced apoptosis in our system. On the contrary, pretreatment of SB203580, a specific inhibitor of p38MAPK, reduced the number of apoptotic cells. In addition, most of p38MAPK-transfected cells, but not ERK2-transfected cells, became TUNEL positive, suggesting that activation of p38MAPK induces apoptosis in cardiac myocytes. Moreover, when the transfected cells were incubated with 1 μM DM for 4 h, almost all p38MAPK-transfected cells were TUNEL positive. In contrast, none of ERK2-transfected cells were TUNEL positive. These results suggest that ERKs are important to prevent cardiac myocytes from DM-induced apoptosis, whereas p38MAPK is crucial for promoting apoptosis in cardiac myocytes. Like ERKs/p38MAPK, 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, we did not evaluate the role of JNKs in DM-induced cardiac apoptosis in this study.

A major question concerns the mechanism of how MAPKs are involved in DM-induced apoptosis in cardiomyocytes. Many studies have suggested that ERKs play pivotal roles in cell growth and survival (19, 20). ERKs phosphorylate and activate many molecules and some of them may be involved in preventing cells from apoptotic death as well as in promoting cell growth. It

remains to be determined what molecules are targets of ERKs and are involved in inhibition of apoptosis. Some anti-apoptotic factors such as Bcl-2 and Bcl-$_{xl}$ may be such candidates (75). Caspases, such as ICE and CPP32, are critically involved in apoptosis of many cell types (76). JNKs have been reported to activate the caspase-like proteases in U937 cells (77). Caspases are regulated not only by post-translational processing but also by transcriptional control (78). AP-1, a transcription factor downstream of JNKs, may be involved in this transcriptional regulation (79). Another possibility is that DM induces secretion of some factors such as Fas ligand and TNF-α by activating p38MAPK and these factors trigger apoptosis. It has been reported that p38MAPK regulates expression of TNF-α (27), a well known apoptosis-inducing cytokine, and that hemodynamic overload induces TNF-α production in adult feline myocardium (80). These observations suggest that DM may activate p38MAPK which induces cardiomyocyte apoptosis via production of TNF-α.

In summary, the present study demonstrates that DM induces apoptosis of cardiomyocytes through the production of H_2O_2 and ·OH. DM activates ERKs through Ras- and Raf-1-dependent pathways in cardiac myocytes, and activation of ERKs is important for protecting cardiomyocytes from apoptosis. On the other hand, DM activates p38MAPK through the Rho family-dependent pathways in cardiac myocytes, and activation of p38MAPK is critical for induction of cardiomyocyte apoptosis. Although it remains to be determined how each member of MAPK family is involved in cardiomyocyte apoptosis, the present study may pave the way to prevent cardiomyocyte injury from anthracyclines.

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Figure legends

Figure 1. DM induces cardiomyocyte apoptosis.

A. After treatment with 1 μM DM for 24 h, cardiomyocytes were marked by staining with a monoclonal anti-sarcomeric myosin heavy chain antibody (MF-20) followed by incubation with an anti-mouse IgG conjugated with TRITC (a,c). TUNEL staining using FITC-conjugated antibody was performed as described in Materials and Method (b, d). a, b, untreated cardiomyocytes; c, d, cardiomyocytes incubated with 1 μM DM for 24 h. B. The number of TUNEL-positive cardiomyocytes was presented as a percentage of MF-20-positive cardiac myocytes (n=100) from three independent experiments (mean±S.E.). *P<0.05 versus control. C. Genomic DNA was extracted from cultured cardiac myocytes (1×10^5 cells). DNA was fractionated by electrophoresis in 1.5% agarose gel and stained by fluorescent SYBR® Green I . lane 1, molecular weight markers; lane 2, vehicle; lane 3, 1 μM DM for 24 h. Molecular weight is shown at the left.

Figure 2. DM activates ERKs in cardiac myocytes.

Cardiac myocytes were incubated for 20 min with indicated concentrations of DM (A) or with 100 μM DM for indicated periods of time (B) and lysed with ice-cold lysis buffer A. Kinase assays in MBP-containing gels were performed as described in Materials and Methods. The cell lysates were applied to SDS-polyacrylamide gel containing MBP. Phosphorylation of MBP was assayed by incubating the gel with [γ-^{32}P]ATP. After incubation, the gel was washed, dried, and then subjected to autoradiography. The intensity of each band on the autoradiogram was quantified by densitometric scanning, and the activity of ERKs is shown as percent increase in the average from four independent experiments compared with unstimulated controls (100%). *P<0.05 versus control.

Figure 3. DM activates JNKs in cardiac myocytes.

Cardiac myocytes were incubated for 20 min with indicated concentrations of DM (A) or with 100 μM DM for indicated periods of time (B) and lysed with ice-cold lysis buffer A. A. The cell lysates were incubated at 4°C for 30 min with 1 μg of GST-c-Jun (1-79) fusion protein in the kinase reaction buffer C containing [γ-^{32}P]ATP. B. Cell lysates were immunoprecipitated with an anti-JNK

antibody and protein A-Sepharose and incubated with [γ-^{32}P]ATP and GST-c-Jun (1-79) protein as a substrate. The samples were applied to 12% SDS-polyacrylamide gels. The gel was washed, dried, and then subjected to autoradiography. The intensity of each band on the autoradiogram was quantified by densitometric scanning, and the activity of JNKs is shown as percent increase in the average from four independent experiments compared with unstimulated controls (100%). *P<0.05 versus control.

Figure 4 . DM activates p38MAPK in cardiac myocytes.

Cardiac myocytes were treated for 20 min with indicated concentrations of DM (A) or with 100 μM DM for indicated periods of time (B) and lysed with ice-cold lysis buffer A. Cell lysates were subjected to SDS-PAGE, and Western blot analysis was performed using an anti-phosphorylated p38MAPK specific antibody. The blot was developed by ECL as described in Materials and Methods. The intensity of each band on the autoradiogram was quantified by densitometric scanning. The activity of p38MAPK is shown as percent increase in the average from four independent experiments compared with unstimulated controls (100%). *P<0.05 versus control.

Figure 5. DM activates ERKs and p38MAPK through ·OH and H_2O_2.

Cardiac myocytes were preincubated with SOD (50 mM) for 90 min and DMSO (0.5%), catalase (500 U/mg), or MPG (20mM) for 30 min. After incubation for 20 min with 100 μM DM, the activity of ERKs (A) and p38MAPK (B) was assayed as described under the Fig. 2 and 4 legend, respectively. Representative autoradiograms from three independent experiments are shown.

Figure 6. Ras and Raf-1 are critical for DM-induced ERK activation.

HA-ERK2 or Flag-p38MAPK was transfected into Cardiac myocytes, and the myocytes were treated with 100 μM DM for 15 min for ERKs or 30 min for p38MAPK. The transfected ERK2 and p38MAPK were immunoprecipitated with an anti-HA polyclonal antibody and an anti-Flag monoclonal antibody, respectively. The immunecomplex was incubated with [γ-^{32}P]ATP and MBP as a substrate. Aliquots of the reaction mixture were subjected to SDS-PAGE, and the gel was

washed, dried, and subjected to autoradiography. A representative autoradiogram of ERK2 (A) and p38MAPK (B) from three independent experiments is shown.

Figure 7. DM-induced p38MAPK activation is dependent on Rho.

After transfection of expression plasmids encoding Flag-p38MAPK or HA-ERK2 with D.N.RhoA, D.N.Rac1, D.N.Cdc42, or RhoGDI, cardiac myocytes were maintained in DMEM with 0.1% FBS for 48 h and then treated with 100 μM DM for 30 min for p38MAPK or 15 min for ERK2. The activity of the transfected p38MAPK or ERK2 was assayed as described under Fig. 6 legend. Representative autoradiograms of p38MAPK (A, top) and ERK2 (B) and from three independent experiments are shown. Western blot analysis using anti-Flag M2 monoclonal antibody was performed to show equal expression of Flag-p38MAPK in each case (A, bottom).

Figure 8. DM-induced activation of ERKs and p38MAPK is dependent of Ca^{2+} but independent of PKC, PKA, and TKs in cardiac myocytes.

A, B. Extracellular and intracellular Ca^{2+} was chelated by incubation for 2 min with 5 mM EGTA and for 30 min with 40 μM BAPTA, respectively. Cardiac myocytes were treated with 100 μM DM for 20 min, and activities of ERKs (A) and p38MAPK (B) were assayed as described in the Fig. 2 and 4 legend, respectively. Representative autoradiograms from three independent experiments are shown.

C, D. PKC was downregulated by incubation of cardiac myocytes with 0.1 μM TPA for 24 h or inhibited by pretreatment with 1 μM Calphostin C for 60 min. PKA was inhibited by pretreatment with 100 μM RpcAMP for 10 min. Receptor or cytoplasmic TKs were inhibited by pretreatment with 50 μM Tyrphostin (A25) or 20 μM Genistein for 30 min. Cardiac myocytes were treated by 100 μM DM for 20 min or by TPA (0.1 μM), isoproterenol (1 μM), or insulin (1 μM) for 8 min and activities of ERKs (C) and p38MAPK (D) were measured as described under Fig. 2 and 4 legend, respectively. Representative autoradiograms from three independent experiments are shown.

Figure 9. ERKs and p38MAPK play opposite roles in DM-induced cardiac apoptosis.

After preincubation with 50 μM PD98059 for 60 min or 10 μM SB203580 for 2 h, cardiomyocytes were incubated with 1 μM DM for 24 h. A. TUNEL staining was performed as described under Fig. 1 legend. One hundred of MF-20–positive cardiac myocytes were counted, and the number of TUNEL-positive cells was presented as a percentage from three independent experiments (mean±S.E.). $*P<0.05$ versus control; $**P<0.05$ versus DM treatment. B. Genomic DNA was extracted and fractionated in a 1.5% agarose gel as described in Fig. 1 legend. lane 1, DNA size markers; lane 2, vehicle; lane 3, 1 μM DM for 24 h; lane 4, pretreatment with 50 μM PD98059 for 60 min+1 μM DM for 24 h; and lane 5, pretreatment with 10 μM SB203580 for 2 h+1 μM DM for 24 h. C. After transfected with HA-ERK2 or Flag-p38MAPK, cardiomyocytes were stimulated by 100 μM DM for 20 min and stained using phalloilin-TRITC (g-l) and either anti-HA or anti-Flag M2 antibodies, followed by incubation with an anti-mousé IgG conjugated with FITC (a-f). a and g, untreated cardiomyocytes; b and h, cardiomyocytes with DM treatment; c and i, Flag-p38MAPK-transfected cardiomyocytes without DM treatment; d and j, Flag-p38MAPK-transfected cardiomyocytes with DM treatment; e and k, HA-ERK2-transfected cardiomyocytes without DM treatment; f and l, HA-ERK2-transfected cardiomyocytes with DM treatment. D. After transfected with HA-ERK2 or Flag-p38MAPK, cardiomyocytes were stained with TUNEL method (d-f) and either anti-HA (c) or anti-Flag M2 (a) antibodies and anti-HA plus anti-Flag M2 antibodies (b), followed by incubation with an anti-mouse IgG conjugated with TRITC. b and e, untreated cardiomyocytes; c and f, HA-ERK2-transfected cardiomyocytes; a and d, Flag-p38MAPK-transfected cardiomyocytes.