b. 代謝調節説

すべての生命現象は，破壊と創造，分解と合成が繰り返されつつ，生から死へ向かっての不可逆的過程をたどる．1950年，シェーンハイマー Schoenheimer は，「生体構成成分は常に流動的に変化している」ことを明らかにした．たとえば，体のすべてのタンパク質には寿命があり，それが個体の寿命と関係があるというのがシュミット Schmidt の代謝調節説である．ネズミのように体が小さく代謝の速い動物ほど寿命は短いという考えである．

c. プログラム説

寿命は遺伝子によって制御されており，老化は遺伝子にプログラムされているという説．これを支持する事実としては，①動物はその種によって最大寿命が異なる，②動物の交配により長命種，短命種，早老種などを作製しうる，③一卵性双生児の寿命の差は2年以内と短い，④ヒトの細胞培養で胎児由来の線維芽細胞は約50回分裂するが，成人由来の細胞では20回，ウェルナー Werner 症候群などの早老症の細胞では2回である．

d. エラー説

DNA-RNA-タンパク質合成系が突然変異，化学修飾により変異し，この集積によって細胞の機能障害，老化をもたらすという説．

e. クロスリンキング説

複数の反応基をもつ物質が架橋となり相異なる複数の高分子と結合して新しい高分子をつくることをクロスリンキング cross-linking というが，こうした物質は分解されにくく細胞傷害を起こす可能性があり，このような物質が老化の原因であるという説．コラーゲンは加齢とともにクロスリンキングが増加し，不溶化する．これらは，皮膚，動脈，関節の硬化をきたす．

3 老年病の特徴

a. 老年病とは

高齢者に認められる疾患を一般に老年病とよぶ．老年病は，①若年期に発症し老年期に及ぶ疾患，②中年期以降に発症するいわゆる生活習慣病，③高齢期に多く発症する疾患，の3者に大別できる．

①の若年期に発症する疾患で，今日の高齢者においてとくに重要なものに肺結核がある．高齢者で認められる活動性結核のほとんどは，若年期に罹患した肺結核の再発である．非活動性であっても，陳旧性(ちんきゅうせい)病巣が大きい場合や胸郭形成術を受けている場合には，高齢期になって慢性呼吸不全を生じることが問題となる．

酸化ストレスの基礎 18

レドックス制御

γ-グルタミルシステインシンテターゼ遺伝子の発現調節と意義

Regulation of γ-glutamylcysteine synthetase gene and its implication

Key point

- 酸化ストレスと消去機構の均衡の破綻は，疾病の発展の原因となる．
- 酸化ストレスに反応するレドックスシステムは，グルタチオンなどのスルフィドリル基によって調節されている．
- 抗酸化ストレス機構の中心をなすグルタチオンの合成は，律速酵素γ-グルタミルシステインシンテターゼ遺伝子の発現調節で行われている．

グルタチオンの役割

グルタチオン（γ-glutamylcyteinylglycine：GSH）は分子量307のトリペプチドで，あらゆる細胞内に高濃度に存在して多彩な生理的機能を有している．GSHは細胞内の非蛋白性硫黄の90％以上を占める．表1に示すように，たとえば，肝や腎には3～6mMのGSHが含まれ，そこでのGSHの代謝回転は，半減期が数10分ときわめて速い．レンズや網膜にも4～8mMのGSHが含まれ，老化とともに減少することからも光線が含む活性酸素から組織を防御するうえでの重要性が指摘されている．細胞内はほとんどが還元型のGSHとして存在し，酸化型グルタチオン（GSSG）は数10μMと低い．すなわちGSH/GSSG比は高い．GSSGの細胞外への輸送は，ATP-binding cassette（ABC）transporterによって行われる．グルタチオンS-トランスフェラーゼの働きで形成された抗癌剤とGSHの抱合体もこのABCで細胞外へ放出される．GSHは癌細胞の耐性獲得とも関連している．

蛋白合成と成熟の過程で，蛋白質の構造や機能を維持するCys残基のsulfhydryl（-SH）基が遊離するかsulfhydryl bridge（S-S結合）を形成するかは重要である．酵素，転写因子や受容体のなかにはその構造と機能活性がSH基の状態で調節されているものもある．GSHによるレドックスシステムは，蛋白質の合成や成熟，

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Yoshishige URATA and Takahito KONDO

表1　正常哺乳動物組織，体液のグルタチオン含量
（文献[22]より引用，一部改変）

標品		GSH* または GSH＋GSSG	GSSG**
肝	ラット	5.5	18
	ヒト	3.5	20
腎	ラット	2.5	38
胃	ラット	7.8	
心臓	ラット	1.1	14
肺	ラット	1.6	
脳	ラット	2.0	14
網膜	ラット	3.6	130
レンズ	ラット	7.5	
脾	ラット	2.0	
精巣	マウス	3.3	
赤血球	ヒト	2.3	3.6
多系核白血球	ヒト	1.5	15
血漿	ラット　大動脈	12	2.7
胆汁	ラット	1.4	380
気道被覆液	ヒト	1.17	90

*：μmol/g湿重量またはml
**：nmol/GSH当量/g湿重量またはml

サイドメモ

GSH/GRXシステム

レドックス調整のTRX/TRX reductaseとともに，GSH/GRXシステムはレドックス制御の中心をなしている．ヒトのGRXにはGRX-1（細胞質型）とGRX-2（ミトコンドリア・核型）の2つのisoformが知られている．TRX superfamilyに属するGRX-1は，分子量約12kDaで1976年にHolmgrenらによって大腸菌から発見された．活性中心にCys-Pro-Tyr-Cysを有している．GSHを基質とし，NADPHを補酵素とする酵素蛋白である．GRXの特徴は，① GSHを基質として直接必要とする，②反応はS-S結合の還元と蛋白-SSGの還元の両作用を示す，③ GRX自体の還元にGSSG還元酵素とTRX reductaseが働く，などがあげられる．これらの作用によってGSH/GRXは，酸化ストレスによるアポトーシスを防ぐうえで，①蛋白質の不可逆的酸化修飾を防ぐ，②アポトーシスシグナルを阻害する，③抗アポトーシスシグナルを活性化する，④ミトコンドリアでは，基質蛋白質のジスルフィド結合の還元や，GSH依存性にリボヌクレオチド還元酵素に水素イオンを提供する．

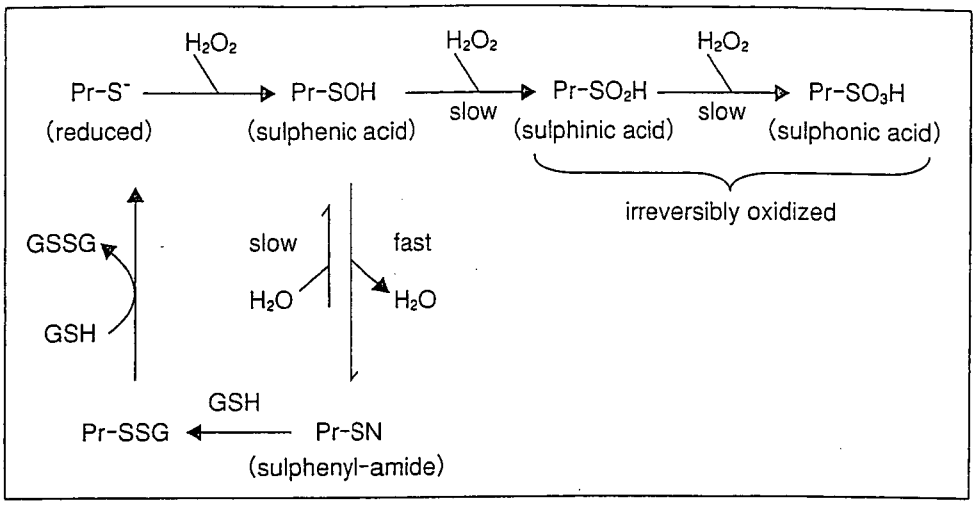

図1　蛋白質システイン残基の酸化修飾（文献[23]より引用，一部改変）

機能の制御のほかに，酸化ストレスを防御する働きもある．シグナル伝達分子においては，リン酸化部位の近傍に存在する特定のCys残基がシグナルのON/OFFを制御することも明らかとなってきた．

酸化ストレスで蛋白質のSH基は容易に酸化され，時として不可逆的な酸化修飾を生じる．S-glutathionylation (Protein-S-SG) はこの不可逆的な酸化修飾から蛋白質の機能と構造を守る役割を担っている．すなわち，過酸化水素は蛋白のCys残基を可逆的に修飾しsulphenic acid (-SOH) をつくる．さらに酸化ストレスが続くと不可逆的なsulphinic acid (-SO_2H) やsulphonic acid (-SO_3H) となる（図1）．GSHはそれを防ぐ働きをもつ．

GSHの合成と機能

GSHの合成は2段階のATP依存性の反応によって行われる．

L-Glu＋L-Cys＋ATP
→L-γ-Glu-L-Cys＋ADP＋Pi　(1)

この反応はγ-グルタミルシステインシンテターゼ（γ-glutamylcysteine synthetase, EC 6.3.2.2, γ-GCS）によって触媒される．

L-γ-Glu-Cys＋Gly＋ATP→GSH＋ADP＋Pi　(2)

この反応はGSH合成酵素による．

γ-GCSは，GSH合成の律速酵素となっている．GSHの濃度は主としてγ-GCS活性で調節されていることから，γ-GCS遺伝子の発現調節は細胞の機能と固く結びついている．GSHがレドックス状態を支配することから，γ-GCSの発現はレドックス制御の面からも重要である．γ-GCSは分子量約100,000で，重鎖（γ-GCSh）と軽鎖（γ-GCSl）とから構成されている．ヒトγ-GCShのcDNA解析からγ-GCShは分子量72,614で，637アミノ酸からなる．一方，ヒトγ-GCSlは分子量30,548で，274アミノ酸からなる．活性中心は重鎖に存在するが，活性発現には軽鎖の存在が不可欠である．γ-GCShにはATP，グルタミンとシステインの結合部位があるが，γ-GCSh単独では酵素活性がきわめて低く，グルタミンとの結合親和性も低い．GSHはグルタミンと競合してγ-GCS活性のfeed back inhibitionを起こす．著者らはγ-GCS欠乏症患者を発見し，その分子異常の解析を試みている[1]．この症例では，γ-GCShのnt 1109のAがTに点変異（アミノ酸配列では370番目のHisがLueに変換）していることが認められた．網状赤血球から分離したcDNAを検索すると，この症例ではheterozygousであった．免疫電気泳動で解析すると，本症例ではγ-GCShの蛋白が欠損しているほかに，γ-GCSl蛋白の低下も認められたことから，変異γ-GCSh蛋白は不安定で存在しえないことと，γ-GCShはγ-GCSl蛋白の安定化に大切であることが推測される．

細胞内小器官のGSH

細胞内でGSHの合成を行っているのは唯一細胞質である．しかしGSHはほとんどすべての細胞内小器官にも含まれ，その器官の種類によって一定の濃度に保たれている．ミトコンドリアにはカタラーゼは局在しないが，多くの活性酸素消去機構，すなわちSODやグルタチオンペルオキシダーゼ，グルタチオン還元酵素などが存在することからもGSHが存在する意義が窺える．ミトコンドリアや核のGSHは細胞質内のGSHを変化させても同様の変動をきたさないことからも，独自のGSH維持機構を所有していると考えられる[2]．アポトーシスと関連した因子Bcl-xを強制発現させるとミトコンドリアの抗酸化能の上昇のみならず，核のGSH濃度が上昇するということからも，酸

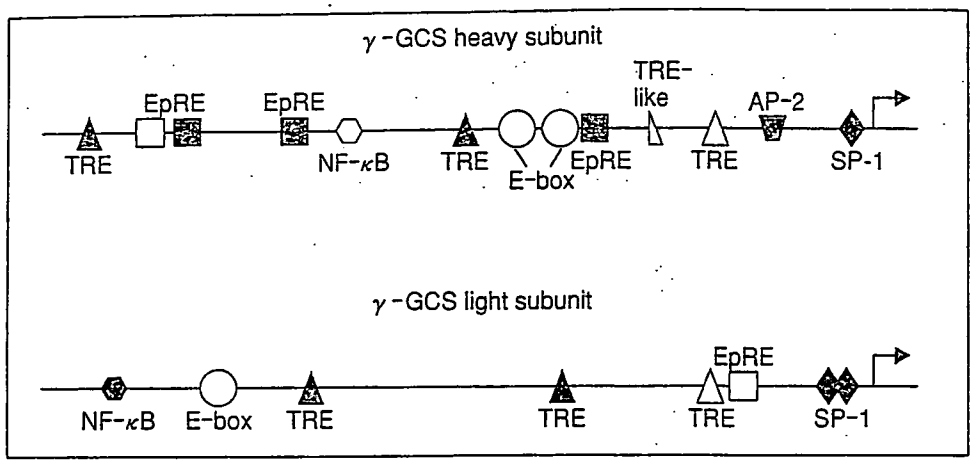

図2　ヒトγ-GCShとγ-GCSl遺伝子プロモーター領域の転写調節因子群[24]

化ストレスに対応する小器官GSHの生理的意義が示唆される[3].

小胞体ではGSH/GSSG比は低く保たれているのが特徴とされている．このことは，小胞体が蛋白質合成と品質管理を行う場所であり，糖鎖による修飾などに重要な分子シャペロンなどの働きが低い還元力の下でのみ可能となることからも生理的必要性が示唆される．細胞外のGSHは細胞内より2～3桁低い．

GSH合成の調節

γ-GCShの遺伝子は染色体1番に，γ-GCSlは6番に位置している．γ-GCSはストレスに対してきわめて反応性に富んでいる．熱ショックや放射線，サイトカインで刺激された細胞は，3時間内にγ-GCSを増加させ6時間でピークを示すことが多い．GSH合成はそれに引き続いて生じる[4].

γ-GCS遺伝子の転写領域にはSp1，AP-1，AP-2，NF-κBなどの結合部位が存在している．γ-GCS重鎖遺伝子の発現は，一般的には酸化ストレス刺激ではAP-1が，TNF-α刺激ではNF-κBが働く．図2にγ-GCShおよびγ-GCSl遺伝子5'上流に存在する転写調節領域の概略を示す．Nrf2はelectrophoretic response element (EpRE) を介した遺伝子発現に働く転写因子であり，γ-GCShの誘導に役割を果たしている．γ-GCShの発現にNrf2が働くことは，β-naphthoflaboneを投与したMulcahyらのグループの研究がはじめての報告であった[5]．その後，Yamamotoらのグループは Nrf2をノックアウトするとacetaminophenに対する肝毒性が上昇することとγ-GCSの発現低下からGSH合成が低下することをみている[6]．またEpREが基本的なγ-GCShの発現に重要であるという報告もなされている[7]．γ-GCShおよびγ-GCSlの発現は細胞種によって異なるほかに原則的に両サブユニットの遺伝子発現は独立しているのでないかと考えられる．

抗癌剤シスプラチン（CDDP）はAP-1依存性に転写活性を増加させる[8]．CDDPによるγ-GCShの転写活性をルシフェラーゼ活性で測定した結果を図3に示す．AP-1, NF-κB, Sp1, AP-2などもそれぞれの結合部位とともに，metal response element, antioxidant response elementやEpREと共同して転写制御に働くと考えられる[9]．γ-GCSh遺伝子の転写において，E-boxの意義も注目されている．酸化ストレスに反応してERKが活性化すると，ERK依存性にc-MycのSer62のリン酸化が起こり，リン酸化c-MycはE-boxに結合して転写活性を上昇させる[10]．ラットの肺上皮細胞では，E-boxがγ-GCSh転写の抑制に働くとの報告がある[11].

γ-GCS遺伝子発現調節の意義

Kijimaらは，γ-GCSの遺伝子発現を制御する目的で，リボザイムを用いた遺伝子修飾を行った[12]．ハンマーヘッドリボザイムはヌクレオチドを切断する活性を有して特定のRNA配列を切断することが可能である．γ-GCShとγ-GCSlを標的にしたリボザイムを細胞に導入すると，どちらもきわめて効果的にGSH合成を低下させ，酸化ストレスに対する感受性を低下させる．たとえば，γ-GCSh-リボザイムはγ-GCShのみならずγ-GCSlの蛋白発現も減少させている．このことは，合成された各サブユニットは相互に依存して安定であることを示したこの酵素異常症のデータを裏づけるものである.

最近small hairpin RNAを用いたγ-GCSのノックダウン研究では，GSH合成がglutamateや一酸化窒素（NO）による神経細胞死を防ぐうえで重要であることと，γ-GCShとγ-GCSlが独立して制御されていることが報告されている[13].

GSHの上昇は活性酸素の消去に作用するばかりでなく，GSHによるレドックス制御によって細胞の酸化ス

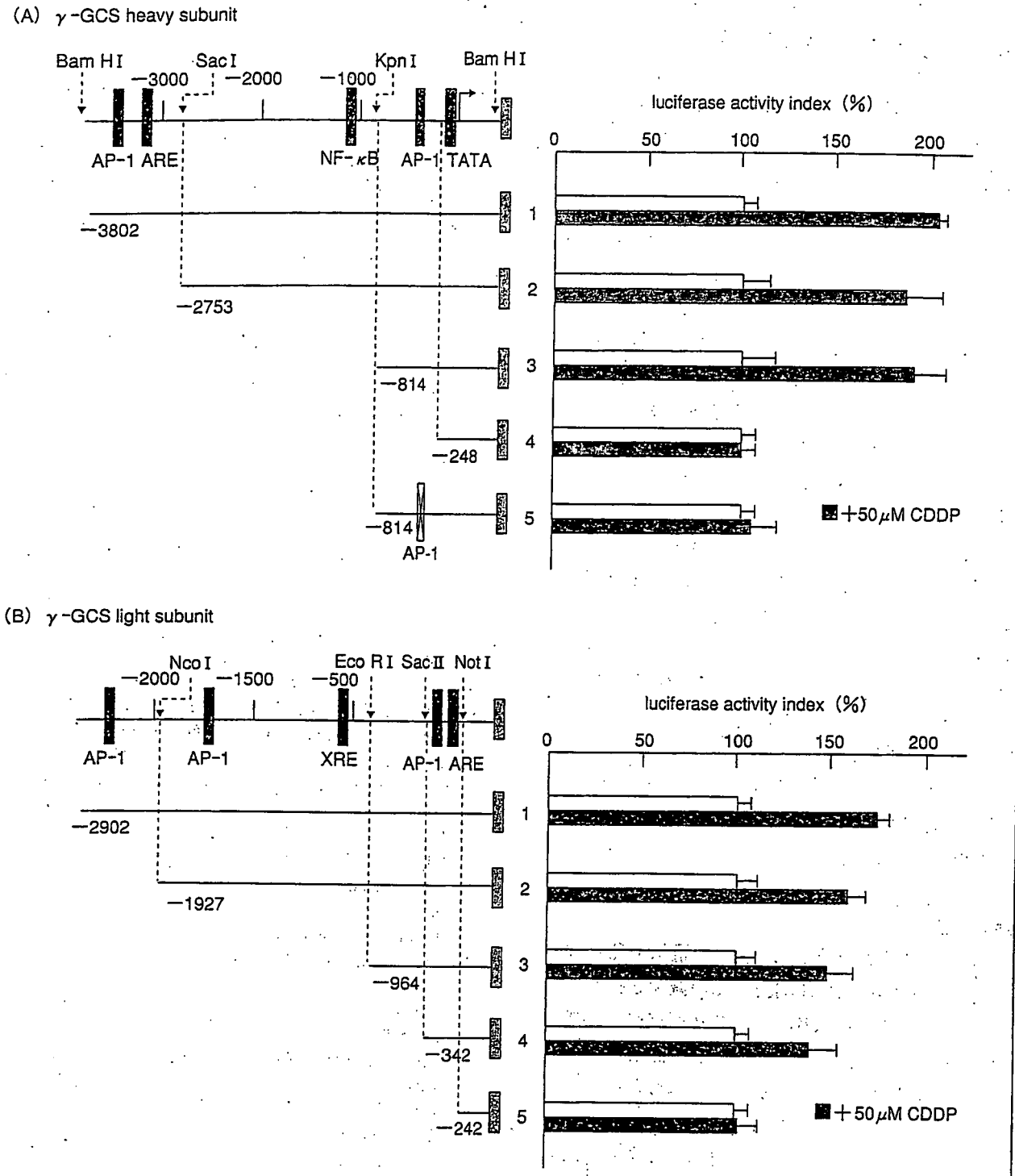

図3　シスプラチン（CDDP）によるγ-GCShとγ-GCSlプロモーター活性の変化

ヒト大腸癌CDDP耐性細胞にγ-GCS遺伝子のプロモーターを含んだルシフェラーゼプラスミドを挿入し，50μM CDDPを1時間添加した後のルシフェラーゼ活性を測定した．

A：γ-GCSh
1：BamHIフラグメント
2：1のAP-1とARE部位を除いたもの
3：2のNF-κB部位を除いたもの
4：3のAP-1部位を除いたもの
5：4のAP-1の変異を起こさせたもの

B：γ-GCSl
1：BamHIフラグメント
2：1のAP-1部位を除いたもの
3：2のAP-1とXRE部位を除いたもの
4：3の−964から−342を除いたもの
5：4のAP-1とARE部位を除いたもの

トレス感受性を制御する因子として重要である．酸化ストレスのある状況では，蛋白質はGSSGの修飾を受けやすい．炎症の際の白血球細胞でこの修飾を受けた蛋白が増加しており[14]，フォスフォフルクトキナーゼ，グリコーゲン合成酵素，guanylate cyclase，グルココルチコイド受容体やクレアチニンキナーゼでS-glutathionylationを受けていることが知られている[15]．c-Junも酸化ストレスでS-glutathionylation修飾を受け，その結果DNA-結合活性が低下する[16]．Glyceraldehyde-3-phosphate dehydrogenaseはGSSGとmixed disulfideを形成する．この変化はグルタレドキシン（GRX）によって還元される[17]．以前から，性ホルモンによる抗酸化作用が知られていたが，その作用機構は不明であった．酸化ストレスによる細胞障害をエストラジオール（E_2）が防御することにγ-GCShの転写調節領域のEpRE様部位にE_2と細胞内受容体が結合してGSH合成を高める機序があることが明らかになった[18]．われわれは，E_2がγ-GCShとともにレドックス制御因子GRXの遺伝子発現をもたらして，細胞内を還元型に維持することで抗細胞死に働くAktシグナルを高めることを報告している[19]．すなわち，Aktのリン酸化部位であるThr308とSer473の近傍にある2つのCys残基が酸化ストレスでS-glutathinylationを起こすと，脱リン酸化酵素によってAktの脱リン酸化が生じてシグナルの低下をきたす．GSHを基質とするGRXは，酸化修飾を受けたCysを還元型に戻すことで脱リン酸化を防ぐ．

疾病とGSH

酸化ストレスが続くと，γ-GCSの遺伝子発現は低下してGSHの減少とGSH/GSSG比の減少を招く．この現象は動脈硬化や糖尿病の血管内皮細胞や，心肥大をきたしている心筋でも著明である．治療によってγ-GCS発現が回復することからも，病態との深い関連性が示唆される．一方，抗癌剤や放射線に耐性を獲得した癌細胞では，γ-GCSの遺伝子発現が著明に上昇しているうえに，抗癌剤投与によってさらに誘導される．GSHと関連したグルタチオンS-トランスフェラーゼなどの解毒酵素やABCも同時に誘導されている．病的老化では酸化ストレス下でのGSH合成を保持することと，癌では低下させることが治療に結びつくと考えられる．

γ-GCSの過剰発現は，TNF-αで生じるミトコンドリア傷害を防御する．ミトコンドリア膜電位，チトクロームCの逸脱，カスパーゼ系の活性化などがおさえられている．前述したようにAP-1を介したシグナルはγ-GCSの発現誘導に働くが，アスベストによる肺線維症で活性化して肺上皮細胞の増殖に働く．このAP-1転写活性がレドックスで制御されていることから，GSHを上昇させるとアスベストによる肺傷害や発癌が軽減する可能性が示されている[20]．

Nagaiらは，気管支被覆液中のGSH/GSSGの変動を喫煙者で検討した．その結果，老年者の喫煙者でGSH/GSSG比の低下が認められた．このことから，慢性肺気腫の進展に肺組織のレドックス低下が関与していることが示唆される[21]．

文献

1) Beutler, E. et al.：*Blood*, 94：2890-2894, 1999.
2) Fernandez-Checa, J. C. et al.：*Semin. Liver Dis.*, 18：389-401, 1998.
3) Voehringer, D. W. et al.：*Proc. Natl. Acad. Sci. USA*, 95：2956-2960, 1998.
4) Urata, Y. et al.：*J. Biol. Chem.*, 271：15146-15152, 1996.
5) Wild, A. C. et al.：*J. Biol. Chem.*, 274：33627-33636, 1999.
6) Enomoto, A. et al.：*Toxicol. Sci.*, 59：169-177, 2001.
7) McMahon, M. et al.：*Cancer Res.*, 61：3299-3307, 2001.
8) Iida, T. et al.：*Int. J. Cancer*, 82：405-411, 1999.
9) Moinova, H. R. and Mulcahy, R. T.：*Biochem. Biophys. Res. Comun.*, 261：661-668, 1999.
10) Benassi, B. et al.：*Mol. Cell*, 21：509-519, 2006.
11) Cheng, L. L. et al.：*Free Radic. Biol. Med.*, 39：1030-1040, 2005.
12) Kijima, H. et al.：*Biochem. Biophys. Res. Commun.*, 247：697-703, 1998.
13) Diaz-Hernandez, J. I. et al.：*J. Biol. Chem.*, 280：38992-39001, 2005.
14) Thomas, J. A. and Sies, H.：Protein S-thiolation and dethiolation. *In*：the Post-Translational Modification of Proteins, Roles in Molecular and Cellular Biology (ed. by Tuboi, S. et al.). CRC Press, Japan, 1992, pp.35-51.
15) Reddy, S. et al.：*Biochem. J.*, 347：821-827, 2000.
16) Klatt, P. et al.：*FASEB J.*, 13：1481-1490, 1999.
17) Lind, C. et al.：*Biochem. Biophys. Res. Commun.*, 247：481-486, 1998.
18) Montano, M. M. et al.：*Oncogene*, 23：2442-2453, 2004.
19) Urata, Y. et al.：*J. Biol. Chem.*, 2006.(in press)
20) Shukla, A. et al.：*Cancer Res.*, 64：7780-7786, 2004.
21) Nagai, K. et al.：*Thorax*, 2006.(in press)
22) 東　胤明：蛋白質・核酸・酵素，33：1365-1369, 1988.
23) Salmeen, A. et al.：*Nature*, 423：769-773, 2003.
24) Dickinson, D. A. et al.：*Free Radic. Biol. Med.*, 37：1152-1159, 2004.

Am J Physiol Cell Physiol 290: C208–C221, 2006.
First published August 31, 2005; doi:10.1152/ajpcell.00075.2005.

Role of calreticulin in the sensitivity of myocardiac H9c2 cells to oxidative stress caused by hydrogen peroxide

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Submitted 22 February 2005; accepted in final form 29 August 2005

Ihara, Yoshito, Yoshishige Urata, Shinji Goto, and Takahito Kondo. Role of calreticulin in the sensitivity of myocardiac H9c2 cells to oxidative stress caused by hydrogen peroxide. *Am J Physiol Cell Physiol* 290: C208–C221, 2006. First published August 31, 2005; doi:10.1152/ajpcell.00075.2005.—Calreticulin (CRT), a Ca^{2+}-binding molecular chaperone in the endoplasmic reticulum, plays a vital role in cardiac physiology and pathology. Oxidative stress is a main cause of myocardiac apoptosis in the ischemic heart, but the function of CRT under oxidative stress is not fully understood. In the present study, the effect of overexpression of CRT on susceptibility to apoptosis under oxidative stress was examined using myocardiac H9c2 cells transfected with the *CRT* gene. Under oxidative stress due to H_2O_2, the CRT-overexpressing cells were highly susceptible to apoptosis compared with controls. In the overexpressing cells, the levels of cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$) were significantly increased by H_2O_2, whereas in controls, only a slight increase was observed. The H_2O_2-induced apoptosis was enhanced by the increase in $[Ca^{2+}]_i$ caused by thapsigargin in control cells but was suppressed by BAPTA-AM, a cell-permeable Ca^{2+} chelator in the CRT-overexpressing cells, indicating the importance of the level of $[Ca^{2+}]_i$ in the sensitivity to H_2O_2-induced apoptosis. Suppression of CRT by the introduction of the antisense cDNA of *CRT* enhanced cytoprotection against oxidative stress compared with controls. Furthermore, we found that the levels of activity of calpain and caspase-12 were elevated through the regulation of $[Ca^{2+}]_i$ in the CRT-overexpressing cells treated with H_2O_2 compared with controls. Thus we conclude that the level of CRT regulates the sensitivity to apoptosis under oxidative stress due to H_2O_2 through a change in Ca^{2+} homeostasis and the regulation of the Ca^{2+}-calpain-caspase-12 pathway in myocardiac cells.

apoptosis; calcium; endoplasmic reticulum

CALRETICULIN (CRT) is a Ca^{2+}-binding molecular chaperone expressed in the endoplasmic reticulum (ER) of a wide variety of eukaryotic cells (35). CRT is involved in many biological processes, including the regulation of Ca^{2+} homeostasis and intracellular signaling, glycoprotein folding, cell adhesion, gene expression, and nuclear transport (17, 23, 35).

CRT is well expressed in the embryonic rat heart, but its expression is suppressed after birth (21). It has been shown that CRT is essential for cardiac development in mice (33, 45). *CRT*-deficient embryonic cells showed an impaired nuclear import of nuclear factor of activated T-cell type 3 (NF-AT3), a transcription factor, indicating that CRT functions in cardiac development as a component of the Ca^{2+}/calcineurin/NF-AT/GATA-4 transcription pathway (33). On the other hand, *CRT*-transgenic mice experience complete heart block and sudden death (42). The CRT-dependent cardiac block involves an impairment of both the L-type Ca^{2+} channel and gap junction connexins 40 and 43 (Cx40 and Cx43, respectively). Also observed was a decrease in phosphorylated Cx43 in the *CRT*-transgenic heart, suggesting that the functions of protein kinases are altered via the regulation of Ca^{2+} homeostasis. CRT is also overexpressed in rat cardiomyocytes under pressure overload cardiac hypertrophy, implying some dysfunction of cardiomyocytes related to the overexpression (51). Furthermore, in cultured myocardiac H9c2 cells, overexpression of CRT after gene transfection promoted apoptosis during cardiac differentiation (24). In that study, the expression of protein phosphatase 2A (PP2A), a Ser/Thr protein phosphatase, was involved in altering the regulation of Akt signaling in H9c2 cells overexpressing CRT via an increase in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Recently, we also reported that Akt signaling is important for cytoprotection against oxidative stress (39) and that a long-term change in $[Ca^{2+}]_i$ regulates *PP2Ac-α* gene transcription via the cAMP response element, resulting in a change in the activation status of Akt and leading to altered susceptibility to apoptosis (56). These studies suggest that CRT plays a vital role in myocardiac development and function, although the mechanism of this phenomenon has not been clarified fully.

An increasing body of evidence suggests that apoptosis plays an important role in cardiac development and disease (9, 12). Apoptosis occurs during reperfusion after ischemia in a variety of organs, including the heart (4). Oxidative stress with reactive oxygen species generated during ischemia-reperfusion injury is implied in the mechanism of cardiac damage (4). However, the biological significance of CRT expression levels in cardiomyocytes under oxidative stress has not been revealed to date.

In the present study, we have investigated the biological role of CRT using rat myocardiac H9c2 cells transfected with the *CRT* gene. We show that the level of CRT alters the sensitivity to apoptosis under oxidative stress with H_2O_2 through a change in Ca^{2+} homeostasis and Ca^{2+}-dependent signaling of the calpain-caspase-12 pathway in myocardiac cells.

MATERIALS AND METHODS

Materials. Antibodies against CRT, calnexin (CNX), binding protein (BiP; glucose-regulating protein 78, Grp78), and ER-specific protein 57 (ERp57)/Grp58 were purchased from Stressgen (Victoria,

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BC, Canada). Antibodies against GAPDH, caspase-12, and caspase-3 were obtained from Chemicon International (Temecula, CA), MoBiTec (Gottingen, Germany), and Cell Signaling Technology (Beverly, MA), respectively. Peroxidase-conjugated secondary antibodies against IgG of rabbit and mouse were purchased from Dako (Glostrup, Denmark). The other reagents used in the study were all of high grade and were obtained from Sigma or Wako Pure Chemicals (Osaka, Japan).

Cell lines and culture. H9c2 cells, a clonal cell line derived from embryonic rat heart, were obtained from the American Type Culture Collection (no. CRL-1446). H9c2 cells that had been transfected with the expression vector for mouse *CRT* cDNA were described previously (24). Two cell lines (CRT-S2 and CRT-S8) expressing high levels of CRT protein were used in the study. A 0.6-kb restriction fragment with *Eco*RI containing the translation initiation site was cut from the vector pcDNA3.1/mCRT (24) and inserted in the reverse orientation into pcDNA3.1 (Invitrogen) to obtain antisense CRT (20). The antisense cDNA expression vector was also transfected into H9c2 cells to establish a cell line (CRT-AS) in which the expression of *CRT* was suppressed (20). The established cell lines were used between *passages 12* and *18*. Cells were cultured in DMEM supplemented with 10% FCS in a humidified atmosphere of 95% air-5% CO_2 at 37°C. To induce oxidative stress, cells were cultured with media containing different concentrations of H_2O_2.

Immunoblot analysis. Cultured cells were harvested and lysed in lysis *buffer A* (20 mM Tris·HCl, pH 7.2, 130 mM NaCl, and 1% Nonidet P-40) including protease inhibitors (in μM: 20 4-amidinophenylmethanesulfonyl fluoride, 50 pepstatin, and 50 leupeptin). Protein samples were electrophoresed on 10% SDS-polyacrylamide gels under reducing conditions and then transferred onto a nitrocellulose membrane as described previously (19). The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS; in mM: 10 Tris·HCl, pH 7.5, and 150 NaCl) and then incubated at 4°C overnight with primary antibody in TBS containing 0.05% Tween 20. The blots were coupled with the peroxidase-conjugated secondary antibodies, washed, and then developed using the ECL detection kit (Amersham Biosciences) according to the manufacturer's instructions. The intensity of protein bands was quantified densitometrically, and the value was estimated relative to that for GAPDH.

Fluorescence microscopy. Cells (50,000/ml) were grown on Lab-Tek chamber slides (Nunc) for 24 h. They were fixed with 4% paraformaldehyde in PBS (pH 7.2) and permeabilized for 10 min with PBS containing 1% Triton X-100. The cells were then blocked with 1% BSA in PBS, incubated with the antibody for 1 h, and washed with PBS containing 1% BSA. The immunoreactive primary antibodies were visualized using FITC-conjugated anti-rabbit immunoglobulins (Cappel). After being washed, the stained cells were mounted in VectaShield medium. A Zeiss Axioskop2 (Zeiss, Jena, Germany) with illumination for epifluorescence was used for fluorescence microscopic analysis.

Cell viability assay. The viability of cultured cells was evaluated by performing a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (38). Cells (5,000–10,000) were placed into 100 μl of medium per well in 96-well plates and cultured overnight. After cells were treated with H_2O_2, 10 μl of 0.5% MTT solution was added and the cells were incubated for 4 h. The reaction was stopped by adding 100 μl of lysis *buffer B* (20% SDS and 50% *N,N*-dimethyl formamide, pH 4.7), and then cell viability was evaluated by measuring the absorbance at 570 nm using a microplate reader.

Lactate dehydrogenase release assay. After H_2O_2 treatment, the incubation medium was collected and centrifuged at 10,000 *g* for 20 min, and the supernatant was stored at 4°C for the lactate dehydrogenase (LDH) activity assay. In untreated cells, the medium was removed and the same volume of lysis *buffer A* was added to the cells. The cells were lysed by trituration and centrifuged as described above, and then the supernatant was used to assay the LDH activity of all cells. LDH activity was measured spectrophotometrically using an LDH assay kit (MTX "LDH"; Kyokuto Pharmaceutical, Tokyo, Japan) according to the manufacturer's instructions.

Apoptosis assay. Apoptosis was detected by performing flow cytometry using the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) method (11) with the ApopTag Plus fluorescein in situ apoptosis detection kit (Chemicon International) as described previously (56). Morphological changes of nuclei in apoptotic cells were also characterized using fluorescence microscopy. Cells (50,000/ml) were grown on Lab-Tek chamber slides for 24 h. After undergoing H_2O_2 treatment, cells were fixed with 4% paraformaldehyde in PBS. The cells were stained with 2 μg/ml Hoechst 33342 (Molecular Probes, Eugene, OR) in PBS for 5 min so that we could visualize the nuclei. After being rinsed with PBS, the slides were examined using fluorescence microscopy as described above.

Measurement of cytoplasmic free Ca^{2+}. $[Ca^{2+}]_i$ was measured using a dual-excitation wavelength spectrofluorophotometer (RF-5500; Shimadzu, Kyoto, Japan) with fura-2 essentially as described previously (6, 36), but with a slight modification. Briefly, cultured cells on glass coverslips were loaded with 5 μM fura-2 AM (Dojindo, Kumamoto, Japan) for 20 min in Earle's balanced salt solution (EBSS; in mM: 26 $NaHCO_3$, pH 7.4, 1 NaH_2PO_4, 5.4 KCl, 116 NaCl, 5.5 glucose, and 2 $CaCl_2$) in the presence of 0.01% Pluronic acid F-127. After being washed four times with EBSS, the coverglass was positioned at a 45° angle to both excitation and emission light paths in a quartz cuvette containing 3.5 ml of fresh EBSS. Fura-2 fluorescence was determined at 37°C using the spectrofluorophotometer operating at an emission wavelength of 505 nm and excitation wavelengths of 340 and 380 nm. The maximal signal (R_{max}) was obtained by adding ionomycin at a 4 μM final concentration. Subsequently, the minimal signal (R_{min}) was obtained by adding EGTA at a 10 mM final concentration, followed by Tris-free base to a 30 mM final concentration, to increase the pH to 8.3. R is the ratio (F_1/F_2) of the fluorescence of excitation at 340 nm and emission at 505 nm (F_1) to that of excitation at 380 nm and emission at 505 nm (F_2). The actual $[Ca^{2+}]$ was calculated as $K_d \times (R - R_{min})/(R_{max} - R) \times Sf2/Sb2$, with K_d = 224 nM (16). The Sf2-to-Sb2 ratio is the ratio of fura-2 fluorescence at 380 nm in Ca^{2+}-free medium to that in Ca^{2+}-replete medium.

Assays for uptake and release of Ca^{2+} in the cell. The uptake of Ca^{2+} was measured radiometrically using the Millipore filtration technique as described previously (49), with a slight modification. The cells were cultured with the medium containing H_2O_2 for the periods indicated and then washed with EBSS and cultured for 10 min in EBSS containing $^{45}Ca^{2+}$ (5 μCi/ml). Cells were detached from the culture wells using trypsinization buffer (0.25% trypsin and 0.02% EDTA in EBSS), and the cell suspension was filtered through a 0.45-μm nitrocellulose filter (Bio-Rad Laboratories, Hercules, CA) under vacuum conditions. The filters were rinsed twice with 0.5 ml of washing buffer (in mM: 10 HEPES, pH 7.4, 150 KCl, 2 EGTA, and 2.5 $MgCl_2$). $^{45}Ca^{2+}$ uptake was calculated by measuring radioactivity and standardized according to protein concentrations. For the $^{45}Ca^{2+}$ release assay, cells were cultured for 48 h with the medium containing $^{45}Ca^{2+}$ (1 μCi/ml). After being washed with EBSS, the cells were incubated with EBSS containing H_2O_2. Aliquots were collected at the time points indicated and centrifuged. The radioactivity was measured in the supernatant as the amount of Ca^{2+} released from the cell.

Enzyme assay. Caspase-12 activity was measured with Ala-Thr-Ala-Asp-7-amino-4-trifluoromethylcoumarin (AFC) as a substrate by using a caspase-12 fluorometric assay kit (BioVision, Mountain View, CA) according to the manufacturer's protocol. The assay is based on the detection of cleavage of the substrate, and the activity was quantified using a spectrofluorophotometer to measure fluorescence (excitation, 400 nm; emission, 505 nm) derived from free AFC. Calpain activity was measured using the calpain substrate succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-Leu-Leu-Val-Tyr-AMC) as described by Glading et al. (13), with a slight modification.

Cultured cells were treated with or without H_2O_2 and/or thapsigargin and then harvested and washed with EBSS. Cells were resuspended in EBSS at 2.5 × 10^5 cells/ml and kept on ice for up to 1 h. In each sample, 250 μl were added to a 2.5-ml quartz cuvette with stirring and allowed to warm to 37°C in the spectrofluorophotometer. At time −1 min, ionomycin in DMSO was added to a final 2.5 μM concentration. DMSO alone was added as a control. At *time 0*, Suc-Leu-Leu-Val-Tyr-AMC was added to a final 50 μM concentration, and fluorescence (excitation, 360 nm; emission, 460 nm) was measured immediately for 3 min.

Statistical analysis. Statistical analysis was performed as previously recommended (6a) using Student's *t*-test or ANOVA (StatView software). Significance was set at $P < 0.05$.

RESULTS

Overexpression of CRT enhances cytotoxic sensitivity of H9c2 cells to oxidative stress caused by H_2O_2. Rat myocardiac H9c2 cells were transfected with the expression vector for mouse *CRT* cDNA to obtain cell lines overexpressing CRT (24). Figure 1*A* shows that the expression of CRT increased in the overexpressed cells to ~2.7-fold the level in the parental and mock-transfected (control) H9c2 cells. The transfection had no apparent effect on the expression of other ER proteins such as CNX, BiP, and ERp57. The intracellular localization of CRT was examined using indirect immunofluorescence. As shown in Fig. 1*B*, immunoreactive signals showed a perinuclear reticular pattern in all cases, including the control and CRT-overexpressing cells, although the signal intensity was increased in the transfectants compared with the control cells. To investigate the effect of overexpression of CRT on the cytotoxic sensitivity of H9c2 cells to oxidative stress, control and CRT-overexpressing cells were exposed to different concentrations of H_2O_2 for 1 h and then cell viability was examined by performing an MTT assay as described in MATERIALS AND METHODS. One hour of exposure to H_2O_2 caused cell damage in a dose-dependent manner, and the cytotoxic effect was enhanced more in the gene-transfected cells (i.e., CRT-S2 and CRT-S8) than in the controls (i.e., parental and control vector-transfected cells) (Fig. 2*A*, *left*). As shown in Fig. 2*A*, *right*, in CRT-overexpressing cells, the cell viability was markedly reduced after 1-h exposure to 50 μM H_2O_2, although less reduction was observed in control cells. However, in both control and gene-transfected cells, viability was similarly suppressed after 4 h of exposure to 50 μM H_2O_2. Next, control and CRT-overexpressing cells were exposed to different concentrations of H_2O_2 for 2 h, and then the cytotoxic effect of H_2O_2 was examined by assaying LDH release as described in MATERIALS AND METHODS. Two hours of exposure to H_2O_2 caused cell damage in a dose-dependent manner, and the cytotoxic effect was enhanced more in the gene-transfected cells than in the controls (Fig. 2*B*, *left*). In Fig. 2*B*, *right*, the cells were treated with 50 μM H_2O_2 for the periods indicated, and the LDH released into the medium was quantified and expressed as a ratio to the total intracellular LDH content. The LDH release was enhanced more in the gene-transfected cells than in the controls during the treatment. Figure 2*C* shows that morphological change was observed using phase-contrast microscopy in control and CRT-overexpressing cells treated with 50 μM H_2O_2 for 2 h. In gene-transfected cells treated with H_2O_2, the cell shape was apparently round and had shrunk along with some bleblike structure, although no change was observed in

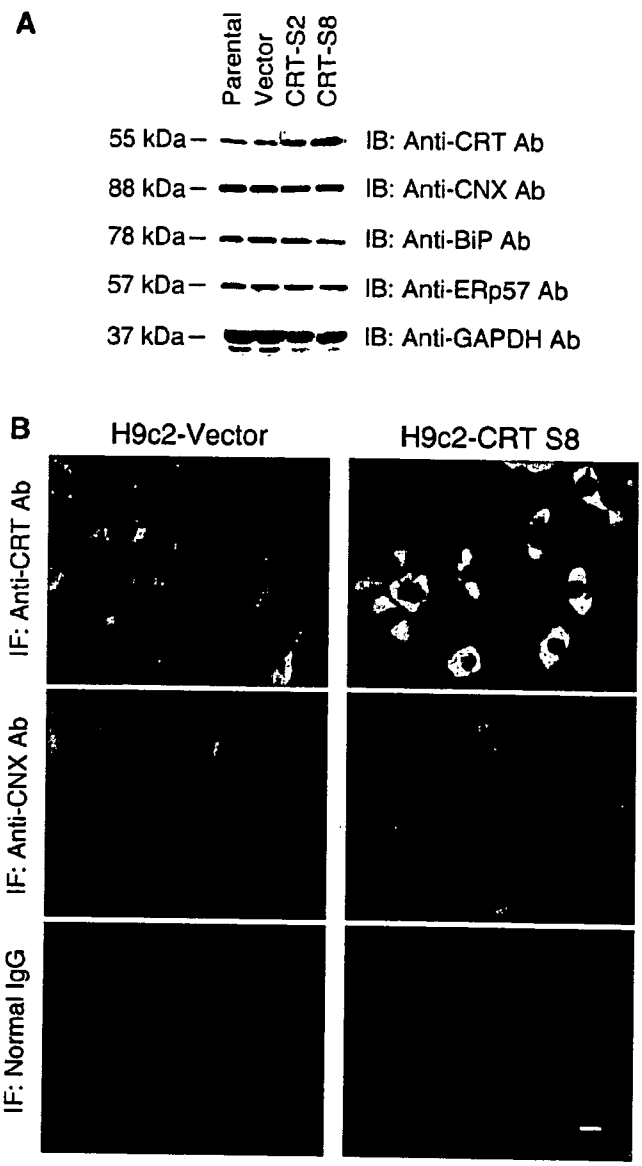

Fig. 1. *A*: expression levels for calreticulin (CRT), calnexin (CNX), binding protein (BiP; glucose-regulating protein 78, Grp78), ERp57/Grp58, and GAPDH were estimated in control (parental and vector) and *CRT* gene-transfected H9c2 (CRT-S2 and CRT-S8) cells using immunoblot analysis with specific antibodies (Ab) as described in MATERIALS AND METHODS. Data represent 3 independent experiments. *B*: intracellular localization of CRT and CNX was evaluated in control and *CRT* gene-transfected H9c2 cells using indirect immunofluorescence (IF) microscopy with specific antibodies. Background signals were obtained in cells stained with normal rabbit IgG. Data represent 3 independent experiments. Bar, 10 μm.

control cells treated with H_2O_2. Collectively, these results indicate that overexpression of CRT enhances the cytotoxic sensitivity to oxidative stress caused by H_2O_2 in myocardiac H9c2 cells.

Overexpression of CRT enhances apoptosis of H9c2 cells under oxidative stress due to H_2O_2. To examine whether apoptosis contributed to the cell damage observed in the transfectants under oxidative stress, a TUNEL assay was performed using cells treated with H_2O_2. In the study by Turner et

al. (52), maximal fragmentation of DNA was observed in H9c2 cells treated with 250 μM H_2O_2 for 4 h. We compared the extent of DNA strand breaks between control and gene-transfected cells treated with 50 μM H_2O_2 for 1 and 4 h. The TUNEL assay (Fig. 3*A*) showed that an increase in fluorescence intensity derived from DNA strand breaks in the transfectants but not in the control cells after H_2O_2 treatment. After the nucleus was stained with Hoechst 33342

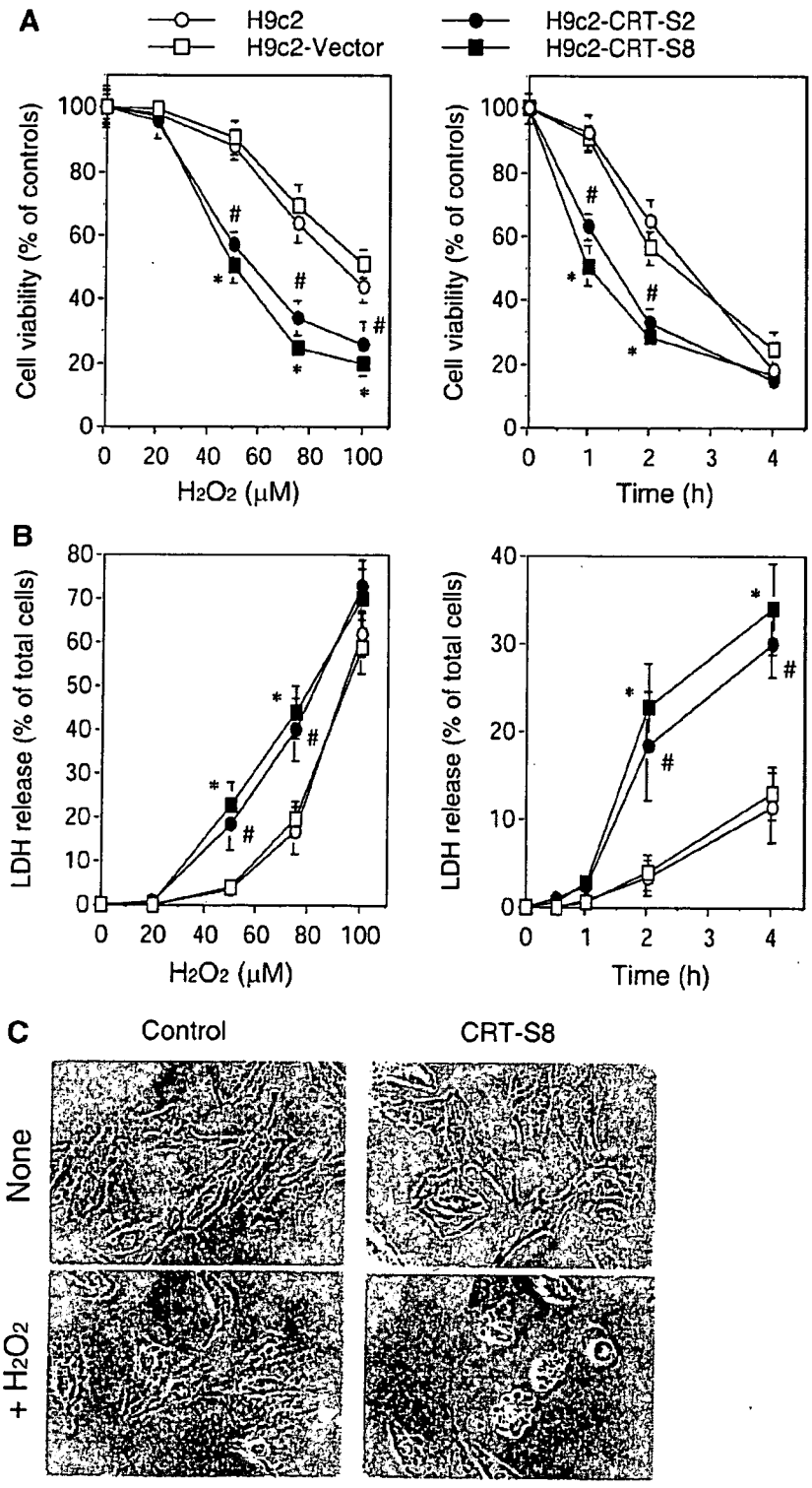

Fig. 2. Overexpression of CRT promotes cell damage in H9c2 cells under oxidative stress due to H_2O_2. *A*: control (H9c2 and H9c2-Vector) and *CRT* gene-transfected (H9c2-CRT-S2 and H9c2-CRT-S8) cells were exposed to different concentrations of H_2O_2 for 1 h, and then cell viability was examined by performing a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described in MATERIALS AND METHODS (*left*). Cells were exposed to 50 μM H_2O_2 for the periods indicated, and then cell viability was examined by performing an MTT assay (*right*). Each value represents the mean ± SD of 4–6 independent experiments. Statistical analysis was performed using a factorial ANOVA test. $*P < 0.05$, $\#P < 0.05$ vs. value at same H_2O_2 concentration (*left*) or time point (*right*) for H9c2 cells treated with H_2O_2. *B*: control and *CRT* gene-transfected cells were exposed to different concentrations of H_2O_2 for 2 h, and then cell damage was examined by performing a lactate dehydrogenase (LDH) release assay as described in MATERIALS AND METHODS (*left*). Cells were treated with 50 μM H_2O_2 for the periods indicated, and the LDH released in the medium was quantified as described in MATERIALS AND METHODS and expressed as a percentage of total intracellular LDH content (*right*). Each value represents the mean ± SD of 4–6 independent experiments. $*P < 0.05$, $\#P < 0.05$ vs. value at same H_2O_2 concentration (*left*) or time point (*right*) as H9c2 cells treated with H_2O_2. *C*: control (H9c2-Vector), *CRT* gene-transfected (H9c2-CRT-S8) cells were exposed to 50 μM H_2O_2 for 2 h, and then cell morphology was examined using phase-contrast microscopy. Bar, 10 μm.

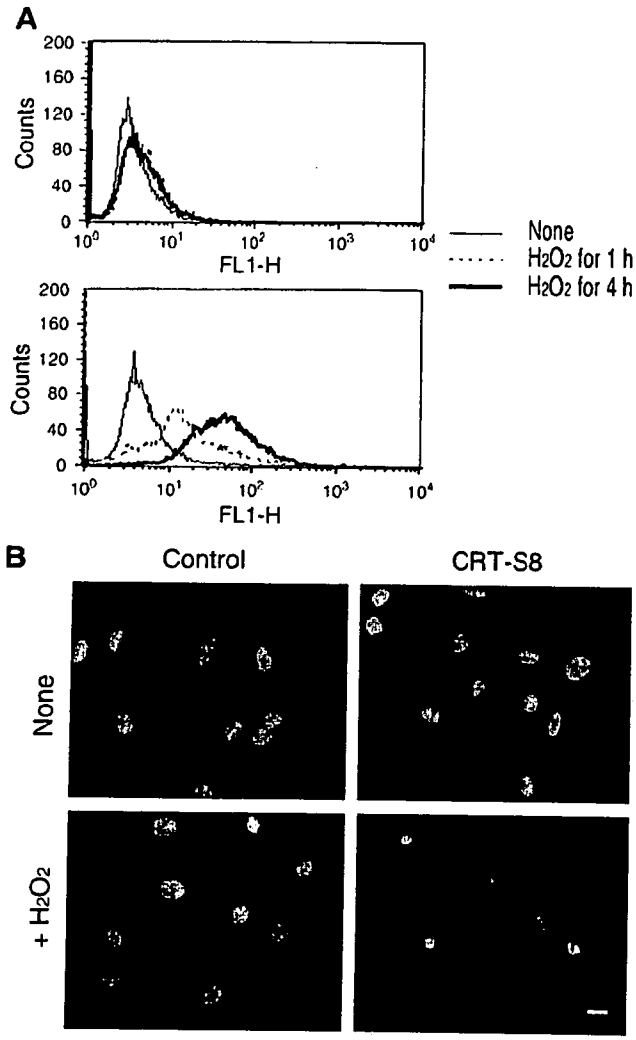

Fig. 3. Overexpression of CRT promotes apoptosis in H9c2 cells under oxidative stress due to H_2O_2. *A*: terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay was performed for control (*top*) and gene-transfected (*bottom*) H9c2 cells under oxidative stress caused by H_2O_2. DNA strand breaks were detected using the TUNEL method as described in MATERIALS AND METHODS. Cells were treated with 50 μM H_2O_2 for the periods indicated. Data represent 3 independent experiments. *B*: morphological changes to nuclei were characterized in Hoechst-stained cells using a fluorescence microscope. Control and gene-transfected cells (50,000/ml) were grown on Lab-Tek chamber slides for 24 h and then treated with 50 μM H_2O_2 for 2 h. After being fixated with 4% paraformaldehyde in PBS, cells were stained with Hoechst 33342 and then visualized using fluorescence microscopy as described in MATERIALS AND METHODS. Bar, 10 μm.

(Fig. 3*B*), chromatin condensation and nuclear fragmentation were observed in the gene-transfected cells treated with H_2O_2, but not in the control cells treated with H_2O_2. Altogether, these results indicate that overexpression of CRT significantly enhances apoptosis in H9c2 cells under oxidative stress caused by H_2O_2.

Overexpression of CRT increases $[Ca^{2+}]_i$ in H9c2 cells under oxidative stress due to H_2O_2. To investigate whether the intracellular Ca^{2+} homeostasis was affected in the cells under oxidative stress, we measured $[Ca^{2+}]_i$ after H_2O_2 treatment (50 or 75 μM). To observe the effect of extracellular Ca^{2+} on $[Ca^{2+}]_i$, we treated the cells with H_2O_2 in the presence of 2 mM Ca^{2+} (+Ca^{2+}) or 10 mM EGTA (+EGTA). The measurement was based on the fluorescence intensity of cells loaded with fura-2 AM as described in MATERIALS AND METHODS. Figure 4 shows that in the presence of extracellular Ca^{2+} (+Ca^{2+}), control cells demonstrated no change in $[Ca^{2+}]_i$ level during treatment with 50 μM H_2O_2, but CRT-overexpressing cells increased to 180 nM after 35 min of treatment. Moreover, with 75 μM H_2O_2 treatment, the difference in the $[Ca^{2+}]_i$ increase was greater. On the other hand, in the absence of extracellular Ca^{2+} (+EGTA), the H_2O_2-induced $[Ca^{2+}]_i$ increase was not observed even in the CRT-overexpressing cells, suggesting that the increase was dependent on the influx of Ca^{2+} from the extracellular space. Altogether, the results indicate that $[Ca^{2+}]_i$ was apparently increased by oxidative stress due to H_2O_2 in CRT-overexpressing cells compared with controls, suggesting some modification of cellular Ca^{2+} homeostasis occurred because of CRT overexpression.

Alteration of Ca^{2+} flux in CRT-overexpressing H9c2 cells under oxidative stress. To investigate the effect of overexpression of CRT on Ca^{2+} influx in cells under oxidative stress, we examined the $^{45}Ca^{2+}$ uptake in control and *CRT* gene-trans-

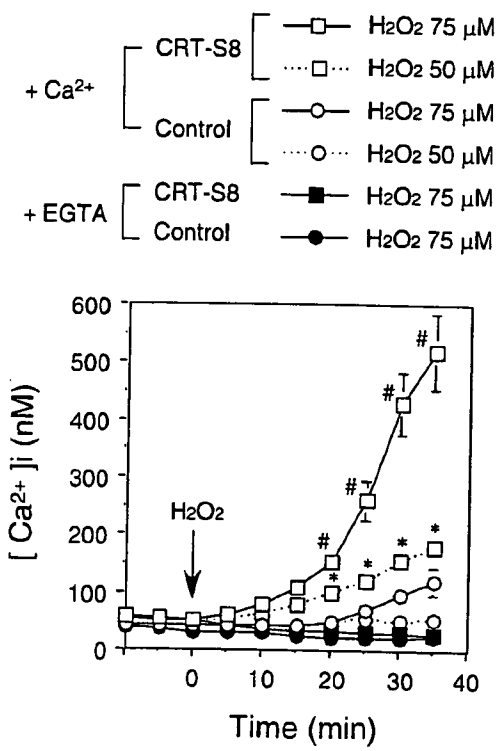

Fig. 4. Cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) increases in CRT-overexpressing H9c2 cells under oxidative stress due to H_2O_2. After being loaded with 5 μM fura-2 AM, control and gene-transfected cells cultured on glass coverslips were treated with H_2O_2 (50 or 75 μM) for the periods indicated. $[Ca^{2+}]_i$ was quantified by measuring fura-2 fluorescence as described in MATERIALS AND METHODS. To observe the effect of extracellular Ca^{2+} on $[Ca^{2+}]_i$, we treated the cells with H_2O_2 in the presence of 2 mM Ca^{2+} (+Ca^{2+}) or 10 mM EGTA (+EGTA). Each value represents the mean ± SD of 4 independent experiments. Statistical analysis was performed using a factorial ANOVA test. *$P < 0.05$ vs. value at same time point for control cells (Ca^{2+}) treated with 50 μM H_2O_2. #$P < 0.05$ vs. value at same time point for control cells (Ca^{2+}) treated with 75 μM H_2O_2.

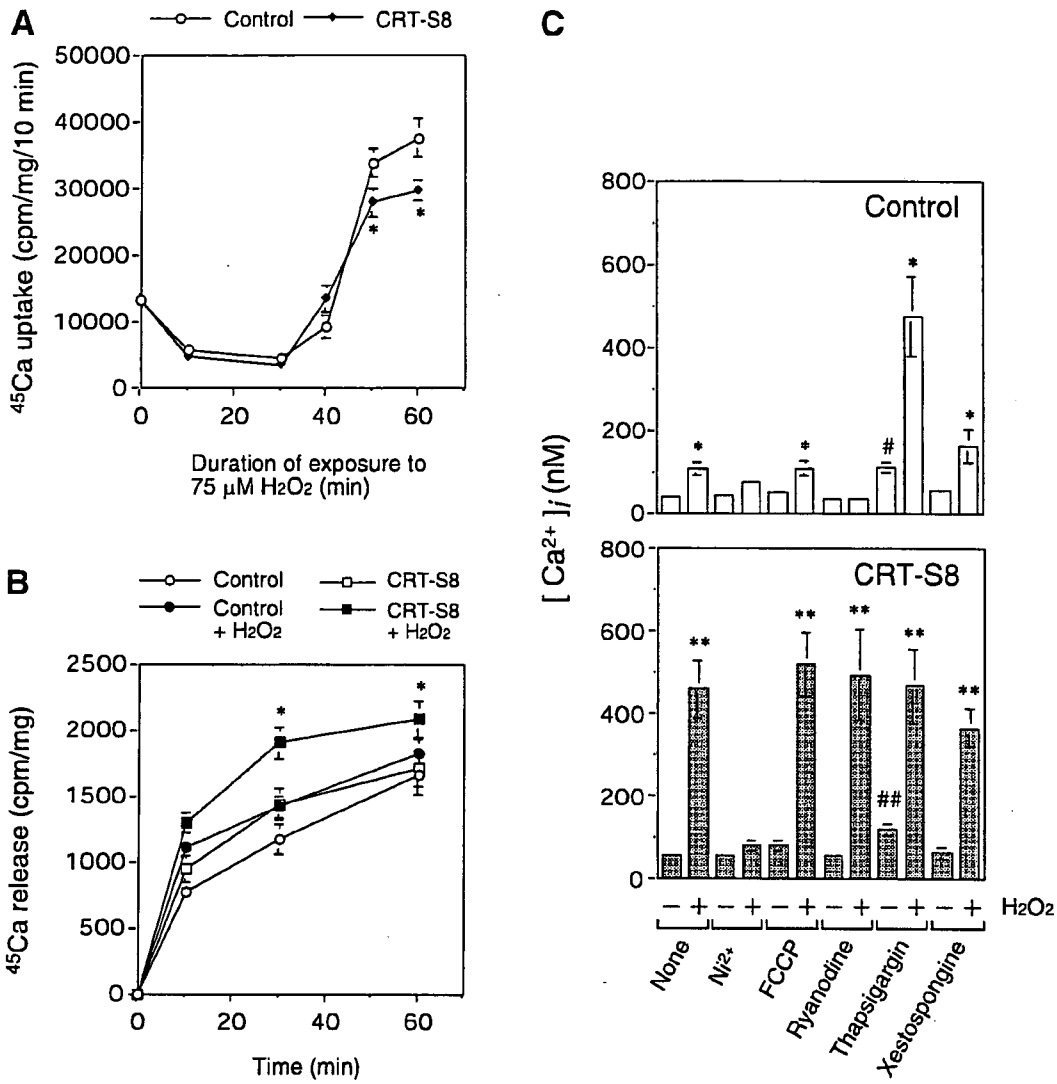

Fig. 5. *A*: uptake of Ca^{2+} in CRT-overexpressing H9c2 cells under oxidative stress due to H_2O_2. The cells were cultured with the medium containing 75 μM H_2O_2 for the indicated periods, washed with Earle's balanced salt solution (EBSS), and then cultured for 10 min in EBSS containing $^{45}Ca^{2+}$ (5 μCi/ml) as described in MATERIALS AND METHODS. After being washed with EBSS, the cells were harvested and $^{45}Ca^{2+}$ uptake was measured as described in MATERIALS AND METHODS. Each value represents mean ± SD of 3 independent experiments. Statistical analysis was performed using a factorial ANOVA test. $^*P < 0.05$ compared with value at same time point for control cells treated with 75 μM H_2O_2. *B*: release of Ca^{2+} from CRT-overexpressing H9c2 cells under oxidative stress due to H_2O_2. Cells were cultured for 48 h with $^{45}Ca^{2+}$ as described in MATERIALS AND METHODS. After being washed with EBSS, the cells were incubated with EBSS containing 75 μM H_2O_2. Aliquots were collected at the time points indicated and then centrifuged. Radioactivity was measured in the supernatant as the amount of Ca^{2+} released from the cell. Each value represents mean ± SD of counts per minute (cpm) recovered in the supernatant normalized to the protein in the total cell pellets. $^*P < 0.05$ vs. same time point for CRT-S8 cells treated without H_2O_2. *C*: effect of Ca^{2+} modulators on $[Ca^{2+}]_i$ in CRT-overexpressing H9c2 cells under oxidative stress with H_2O_2. After being loaded with 5 μM fura-2 AM, control and gene-transfected cells cultured on glass coverslips were pretreated with various modulators containing Ni^{2+} (5 mM), FCCP (1 μM), ryanodine (100 μM), thapsigargin (5 μM), and xestospongin C (1 μM) and then were treated with H_2O_2 (75 μM) for 30 min. $[Ca^{2+}]_i$ was quantified by measuring fura-2 fluorescence as described in MATERIALS AND METHODS. Each value represents mean ± SD of 3 independent experiments. $^*P < 0.05$ vs. corresponding control cells treated without H_2O_2. $^{\#}P < 0.05$ vs. untreated control cells (None). $^{**}P < 0.05$ vs. corresponding CRT-S8 cells treated without H_2O_2. $^{\#\#}P < 0.05$ vs. untreated CRT-S8 cells (None).

fected cells during treatment with 75 μM H_2O_2 as described in MATERIALS AND METHODS. As shown in Fig. 5*A*, in both control and gene-transfected cells, the rate of $^{45}Ca^{2+}$ uptake was suppressed within the first 30 min and then increased during oxidative stress. The uptake rate was higher in the control than in the gene-transfected cells after 40 min. Next, to investigate the effect of overexpression of CRT on Ca^{2+} efflux in the cell under oxidative stress, we examined the $^{45}Ca^{2+}$ release in control and gene-transfected cells during treatment with 75 μM H_2O_2 as described in MATERIALS AND METHODS. After being labeled with $^{45}Ca^{2+}$, the cells were treated with H_2O_2 and the amount of $^{45}Ca^{2+}$ released was measured as described in MATERIALS AND METHODS. As shown in Fig. 5*B*, although the amount of $^{45}Ca^{2+}$ released did not differ between untreated control and gene-transfected cells, the release was increased in gene-transfected cells compared with controls after 30-min treatment with H_2O_2. Collectively, in CRT-overexpressing cells, Ca^{2+} influx seemed to be suppressed and the efflux was

increased in the cells under oxidative stress caused by H_2O_2 compared with control cells treated with H_2O_2. Although these results were not consistent with the finding that $[Ca^{2+}]_i$ was highly elevated in gene-transfected cells treated with H_2O_2 (Fig. 4), they also suggested that an alteration of responses in the intracellular Ca^{2+} stores might lead to increased $[Ca^{2+}]_i$ in CRT-overexpressing cells.

Effect of Ca^{2+} modulators on $[Ca^{2+}]_i$ in CRT gene-transfected H9c2 cells under oxidative stress due to H_2O_2. To investigate whether intracellular Ca^{2+} pools contribute to the altered Ca^{2+} homeostasis, we examined the effect of Ca^{2+} modulators on $[Ca^{2+}]_i$ in cells treated with H_2O_2 (Fig. 5*C*). Ni^{2+} (5 mM) was used to block Ca^{2+} influx from the extracellular space via Ca^{2+} channels and Na^+/K^+ exchangers in the plasma membrane (26, 27). FCCP (1 μM) is a mitochondrial uncoupler that collapses the mitochondrial membrane potential that drives Ca^{2+} uptake into mitochondria (5). A high concentration of ryanodine (100 μM) was used as an antagonist for the ryanodine receptor (55). Thapsigargin (5 μM) was used to inhibit the function of sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) to block the uptake of Ca^{2+} into the ER/sarcoplasmic reticulum (SR) (50). Xestospongin C (1 μM) is a cell-permeable inhibitor of the inositol 1,4,5-trisphosphate (IP_3) receptor (10). The cells were treated with each Ca^{2+} modulator for 10 min after being loaded with fura-2 AM and then were incubated with 75 μM H_2O_2 for 30 min, and $[Ca^{2+}]_i$ was measured as described in MATERIALS AND METHODS. The uptake of Ca^{2+} into mitochondria and the ER was disrupted by treatment with FCCP and thapsigargin, respectively. In control cells, FCCP did not enhance the H_2O_2-induced increase of $[Ca^{2+}]_i$. In CRT-overexpressing cells, $[Ca^{2+}]_i$ was similarly increased by H_2O_2 both cases, with and without FCCP. These results indicate no influence of FCCP on the H_2O_2-induced change of $[Ca^{2+}]_i$ in control and CRT-overexpressing cells. This finding also suggests that the enhancement of H_2O_2-induced increase of $[Ca^{2+}]_i$ observed in CRT-overexpressing cells may not be explained solely by dysfunction of mitochondrial Ca^{2+} uptake. In contrast, the H_2O_2-induced increase in $[Ca^{2+}]_i$ was apparently enhanced in control cells treated with thapsigargin. This indicates that the thapsigargin-sensitive pool (i.e., ER) is involved in the enhancement of H_2O_2-induced increase of $[Ca^{2+}]_i$ and also suggests that dysfunction of SERCA may have a promoting effect on the H_2O_2-induced increase of $[Ca^{2+}]_i$. The ryanodine and IP_3 receptors may be involved in the increase in $[Ca^{2+}]_i$ in response to H_2O_2. However, high concentrations of ryanodine and xestospongin C did not suppress the increase in $[Ca^{2+}]_i$ in CRT-overexpressing cells treated with H_2O_2, suggesting that the ryanodine and IP_3 receptors were not necessarily the main sources of the H_2O_2-induced increase of $[Ca^{2+}]_i$ in CRT-overexpressing cells. Furthermore, it was noteworthy that the H_2O_2-induced increase of $[Ca^{2+}]_i$ was clearly suppressed by Ni^{2+} in gene-transfected cells. This finding is also consistent with the result that the increase was inhibited in gene-transfected cells in the absence of extracellular Ca^{2+} (Fig. 4). These findings indicate that the influx of Ca^{2+} from the extracellular space is important for the H_2O_2-induced increase of $[Ca^{2+}]_i$ in CRT-overexpressing cells, despite the fact that the rate of influx was not increased in the gene-transfected cells treated with H_2O_2 (Fig. 5*A*). Altogether, these results indicate that the ER-stored Ca^{2+} pool plays an important role in the enhancement of the H_2O_2-induced increase of $[Ca^{2+}]_i$ in CRT-overexpressing cells, although Ca^{2+} influx from the extracellular space was also an important contributor to the increase.

$[Ca^{2+}]_i$ level is implicated in the susceptibility of H9c2 cells to apoptosis under oxidative stress due to H_2O_2. To determine whether the increase in $[Ca^{2+}]_i$ is part of the causative mechanism of apoptosis in the gene-transfected cells, H_2O_2-dependent cytotoxicity was examined in CRT-overexpressing cells in the presence or absence of BAPTA-AM, a cell-permeable Ca^{2+} chelator. As shown in Fig. 6*A*, *top*, cell viability was assessed by performing an MTT assay in gene-transfected cells exposed to H_2O_2 treatment (50 μM for 2 h) in the absence or presence of BAPTA-AM (10 μM). The viability was suppressed by H_2O_2 to 28.0 ± 3.4% of that in untreated cells in the absence of BAPTA-AM but returned to 50.5 ± 6.5% in its presence. As shown in Fig. 6*A*, *bottom*, cell damage was also assessed by performing a LDH release assay in gene-transfected cells exposed to H_2O_2 treatment (50 μM for 2 h) in the absence or presence of BAPTA-AM. The release was increased by H_2O_2 to 22.0 ± 5.4% that of untreated cells in the absence of BAPTA-AM but was remitted to 4.7 ± 2.5% in its presence. These results suggest that BAPTA-AM mitigates cell damage due to H_2O_2 by suppressing the increase of $[Ca^{2+}]_i$. The effect of BAPTA-AM on apoptosis was also examined using the TUNEL method in the gene-transfected cells after H_2O_2 treatment (Fig. 6*B*). In the absence of BAPTA-AM, TUNEL-positive cells increased after treatment with 50 μM H_2O_2 for 2 h but were not detected in the presence of BAPTA-AM even 2 h after treatment with H_2O_2. Conversely, the effect of an $[Ca^{2+}]_i$ increase on H_2O_2-induced apoptosis was examined in control H9c2 cells in the absence or presence of thapsigargin (5 μM), an inhibitor for SERCA (50). As shown in Fig. 6*C*, *top*, cell viability was suppressed by H_2O_2 (50 μM for 2 h) to 60.0 ± 4.4% of that in untreated cells in the absence of thapsigargin but was suppressed to 17.1 ± 4.6% in its presence. In Fig. 6*C*, *bottom*, cell damage was assessed by performing a LDH release assay in control cells exposed to H_2O_2 treatment (50 μM for 2 h) in the absence or presence of thapsigargin. The release was slightly increased by H_2O_2 to 7.5 ± 2.2% that of untreated cells in the absence of thapsigargin but was enhanced to 32.7 ± 2.5% in its presence. Furthermore, the effect of thapsigargin on apoptosis was examined using the TUNEL method in control cells with H_2O_2 treatment (Fig. 6*D*). In the presence of thapsigargin, TUNEL-positive cells increased in number after exposure to 50 μM H_2O_2 for 2 h, whereas in its absence, no increase was observed. These results suggest that thapsigargin enhances apoptosis caused by H_2O_2 treatment by increasing the $[Ca^{2+}]_i$ level. This finding was also consistent with our recently reported results (56). Altogether, these results indicate that the increase in $[Ca^{2+}]_i$ plays a causative role in the apoptosis of CRT-overexpressing cells under oxidative stress caused by H_2O_2.

Suppression of CRT expression by transfection with antisense CRT gene enhances cytoprotection of H9c2 cells against oxidative stress due to H_2O_2. To confirm whether the expression level of CRT influences susceptibility to oxidant-induced apoptosis in H9c2 cells, we transfected the cells with the antisense *CRT* gene expression vector and a cell line (CRT-AS) in which CRT expression was suppressed to a level lower than that in controls as described in MATERIALS AND METHODS (20). The expression level of CRT was compared with that in control

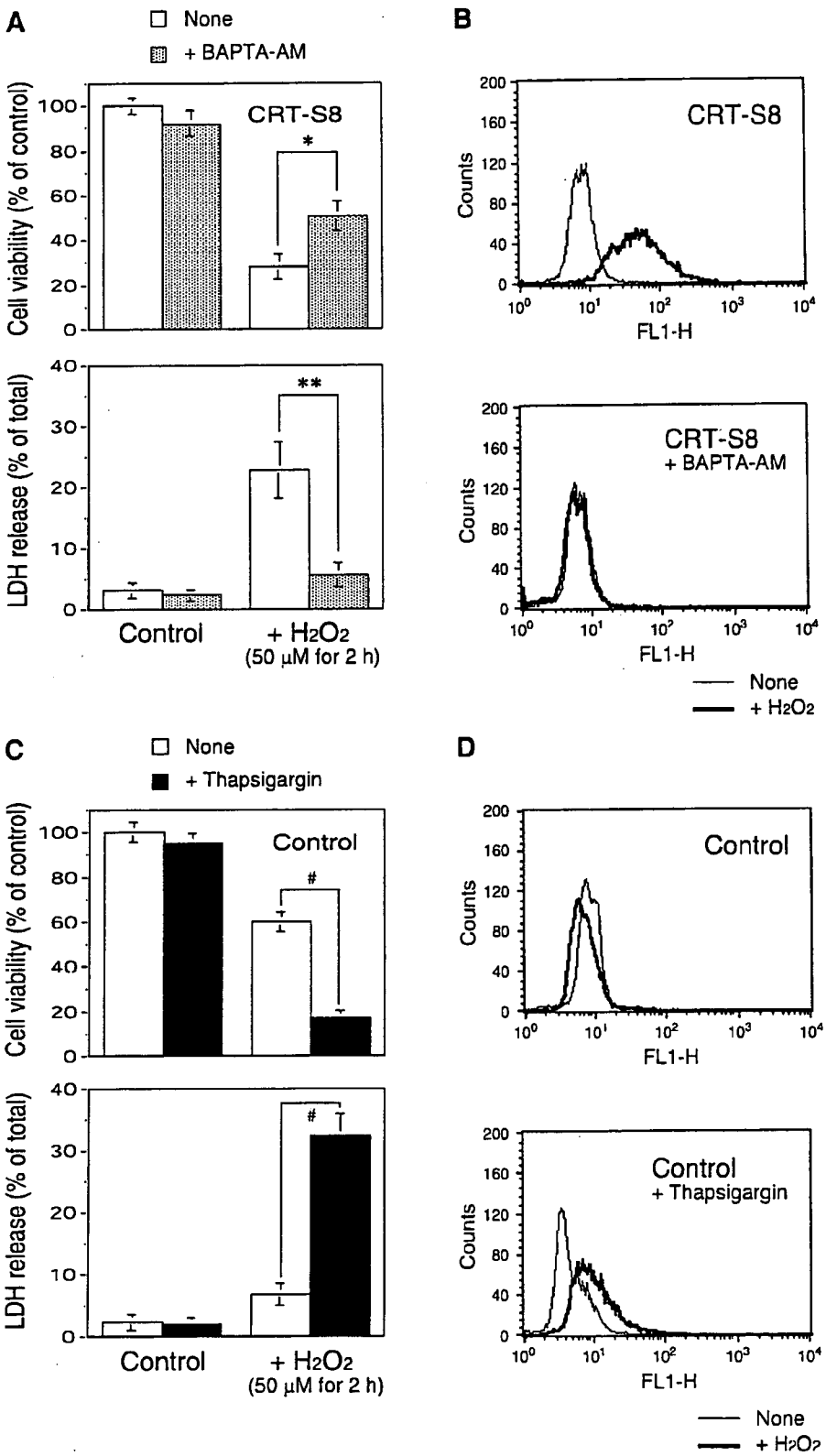

Fig. 6. Effect of Ca^{2+} modulators on apoptosis due to oxidative stress caused by H_2O_2. *A*: CRT-overexpressing (CRT-S8) cells were cultured in the presence or absence of 10 μM BAPTA-AM during the H_2O_2 treatment. The cells were exposed to 50 μM H_2O_2 for 2 h and then cell viability was examined by performing an MTT assay as described in MATERIALS AND METHODS. Each value represents the mean ± SD of 4 independent experiments. Statistical analysis was performed using a paired Student's *t*-test. $^*P < 0.05$, $^{**}P < 0.01$ vs. CRT-S8 cells treated with H_2O_2 without BAPTA-AM. *B*: TUNEL assay for CRT-overexpressing cells treated with or without BAPTA-AM and/or H_2O_2. Cells were cultured in the presence or absence of 10 μM BAPTA-AM during the H_2O_2 treatment. Thin line, no H_2O_2 for 2 h; thick line, 50 μM H_2O_2 for 2 h. DNA strand breaks were detected using the TUNEL method as described in MATERIALS AND METHODS. Data represent 3 independent experiments. *C*: control cells were cultured in the presence or absence of 5 μM thapsigargin during the H_2O_2 treatment. Cells were exposed to 50 μM H_2O_2 for 2 h, and then cell viability was examined by performing an MTT assay as described in MATERIALS AND METHODS. Each value represents the mean ± SD of 4 independent experiments. $^\#P < 0.01$ vs. control cells treated with H_2O_2 without thapsigargin. *D*: TUNEL assay for control cells treated with or without thapsigargin and/or H_2O_2. Cells were cultured in the presence or absence of 5 μM thapsigargin during H_2O_2 treatment. Thin line, no H_2O_2 for 2 h; thick line, 50 μM H_2O_2 for 2 h. DNA double-stranded breaks were detected using the TUNEL method as described in MATERIALS AND METHODS. Data represent 4 independent experiments.

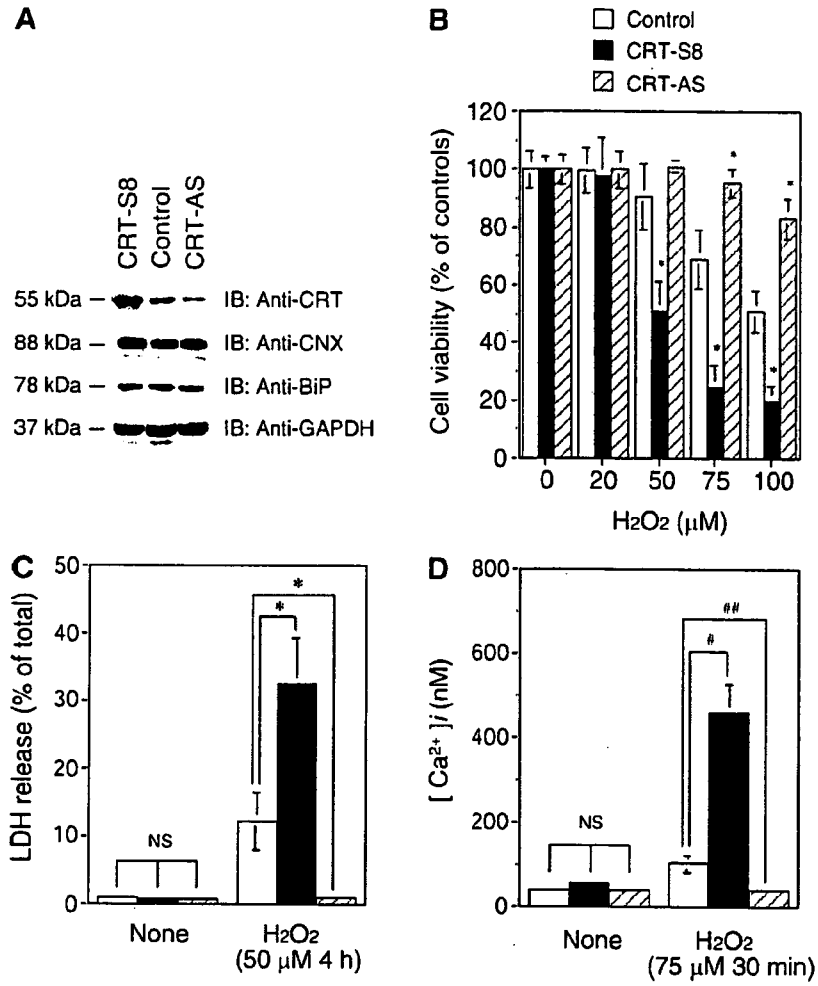

Fig. 7. Suppression of CRT expression showing cytoprotective effects in H9c2 cells under oxidative stress. To observe the effect of the suppressed expression of CRT on cytotoxicity under conditions of oxidative stress, we introduced the antisense gene for CRT into H9c2 cells to obtain cells underexpressing CRT as described in MATERIALS AND METHODS. *A*: expression levels of CRT, CNX, BiP, and GAPDH were examined in control, CRT-overexpressing (CRT-S8), and CRT-underexpressing (CRT-AS) cells using immunoblot analysis with specific antibodies. Data represent 4 independent experiments. *B*: control, CRT-overexpressing, and CRT-underexpressing cells were exposed to different concentrations of H_2O_2 for 1 h, and then cell viability was examined by performing an MTT assay as described in MATERIALS AND METHODS. Each value represents mean ± SD of 4 independent experiments. Statistical analysis was performed using a factorial ANOVA test. $*P < 0.05$ vs. same concentration of H_2O_2 for control cells treated with H_2O_2. *C*: control, CRT-overexpressing, and CRT-underexpressing cells were exposed to 50 μM H_2O_2 for 4 h, and then cell damage was examined by performing an LDH release assay as described in MATERIALS AND METHODS. Each value represents mean ± SD of 4 independent experiments. $*P < 0.01$ vs. control cells treated with H_2O_2. *D*: after being loaded with 5 μM fura-2 AM, control, CRT-overexpressing, and CRT-underexpressing cells cultured on glass coverslips were treated with 75 μM H_2O_2 for 30 min. $[Ca^{2+}]_i$ was quantified by measuring fura-2 fluorescence as described in MATERIALS AND METHODS. $\#P < 0.01$, $\#\#P < 0.05$ vs. control cells treated with H_2O_2.

and CRT-overexpressing cells by immunoblot analysis, and the results showed that the expression level was decreased to ~30% of the control level (Fig. 7*A*). Figure 7*B* shows that control, CRT-overexpressing, and CRT-underexpressing cells were exposed to different concentrations of H_2O_2 for 1 h, and then cell viability was examined by performing an MTT assay as described in MATERIALS AND METHODS. The results showed that cell viability was well maintained in CRT-underexpressing cells treated with H_2O_2 compared with the decreased viability in control and CRT-overexpressing cells. As shown in Fig. 7*C*, control, CRT-overexpressing, and CRT-underexpressing cells were exposed to 50 μM H_2O_2 for 4 h, and then the cytotoxic effect of H_2O_2 was also examined by assaying LDH release as described in MATERIALS AND METHODS. The results showed that the LDH release was suppressed more in CRT-underexpressing cells than in controls during the treatment, although the release was apparently increased in CRT-overexpressing cells. Altogether, the results show that suppression of CRT apparently enhanced cytoprotection against oxidative stress compared with controls, although overexpression of CRT increased the susceptibility to H_2O_2-induced cytotoxicity. This observation indicates that the expression level of CRT is a key factor in determining the susceptibility to H_2O_2-induced apoptosis in H9c2 cells. As shown in Fig. 7*D*, $[Ca^{2+}]_i$ was measured in control, CRT-overexpressing, and CRT-underexpressing cells after H_2O_2 treatment (75 μM) for 30 min. After the treatment with H_2O_2, the $[Ca^{2+}]_i$ increase was apparently suppressed in CRT-underexpressing cells compared with the increase in control and CRT-overexpressing cells. Collectively, the results indicate that the H_2O_2-induced $[Ca^{2+}]_i$ increase is influenced by the expression level of CRT in the cell.

Overexpression of CRT enhances processing and activation of caspase-12 through activation of calpain in H9c2 cells under oxidative stress due to H_2O_2. To further investigate the mechanism of the CRT-dependent enhancement of apoptosis through the alteration of Ca^{2+} homeostasis, we focused on caspase-12, which is activated by calpain, a Ca^{2+}-dependent cysteine protease, in ER stress-induced apoptosis (40). As shown in Fig. 8*A*, the expression levels of caspase-12 and caspase-3 and ER stress-related chaperones were examined using immunoblot analysis in control and CRT-overexpressing cells treated with 75 μM H_2O_2. In control cells, a pro-caspase form of caspase-12 (55 kDa) showed no change after 1 h of treatment with H_2O_2 but was diminished after 2 h of treatment. The level of 35-kDa proteolytic fragment of caspase-12 gradually increased during the 2-h treatment. In CRT-overexpressing cells, the level of the 55-kDa pro-caspase form was low and that of the 35-kDa fragment was rather high even under

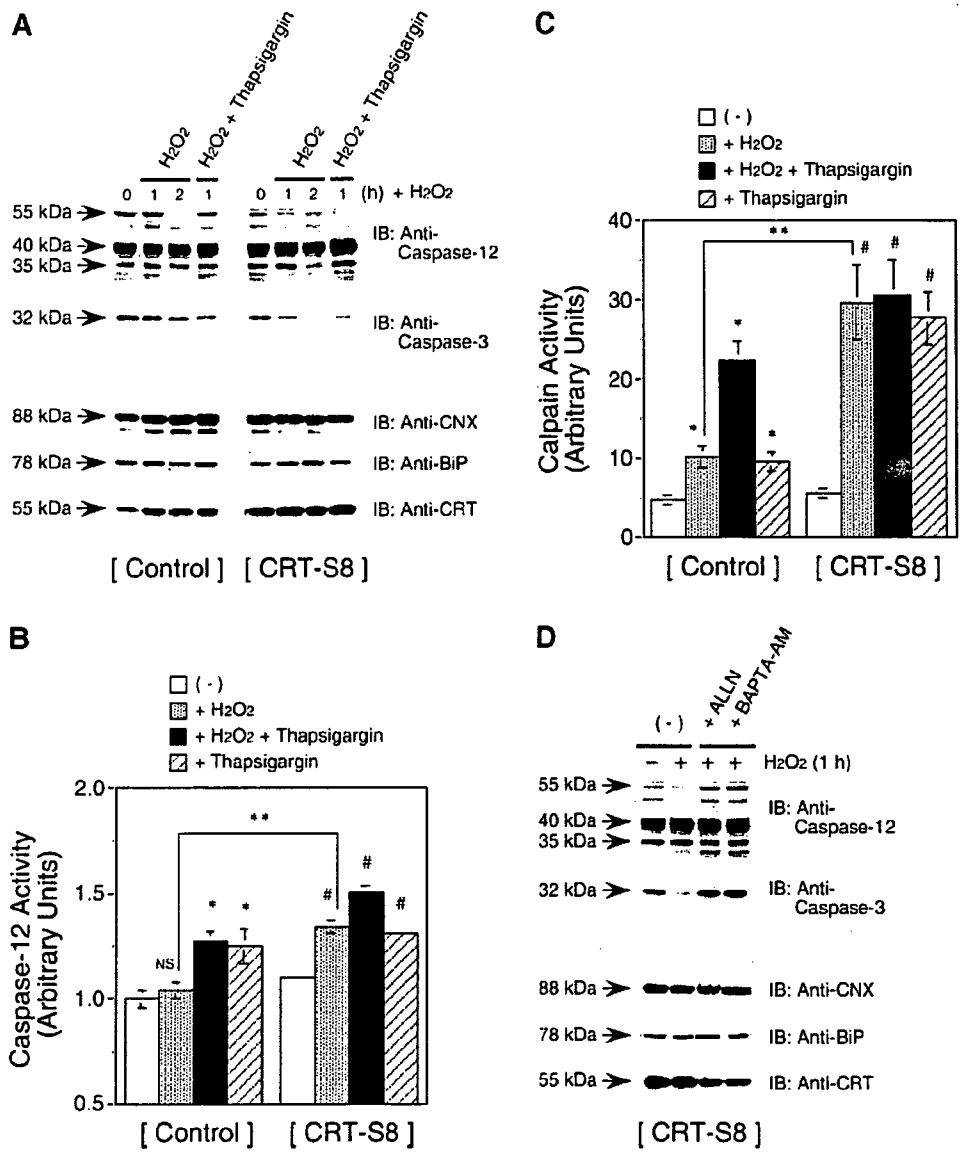

Fig. 8. The calpain-caspase-12 pathway is activated in CRT-overexpressing H9c2 cells under oxidative stress. *A*: control and CRT-overexpressing (CRT-S8) cells were exposed to 75 μM H_2O_2 for 1 and 2 h or to 75 μM H_2O_2 and 5 μM thapsigargin for 1 h, and then the expression levels of caspase-12 and caspase-3, CNX, BiP, and CRT were examined using cell lysates by performing immunoblot analysis with specific antibodies. Data represent 3 independent experiments. *B*: cells were exposed to 75 μM H_2O_2 and/or 5 μM thapsigargin for 1 h. Activity of caspase-12 was assayed with cell lysates using the substrate Ala-Thr-Ala-Asp-7-amino-4-trifluoromethylcoumarin (AFC) as described in MATERIALS AND METHODS. Each value represents the mean ± SD of 3 independent experiments. Statistical analysis was performed using a factorial ANOVA test. $*P < 0.05$ vs. untreated control cells. $**P < 0.05$ vs. control cells treated with H_2O_2. $\#P < 0.05$ vs. untreated CRT-S8 cells. NS, not significant vs. untreated control cells. *C*: cells were exposed to 75 μM H_2O_2 and/or 5 μM thapsigargin for 1 h. Activity of calpain was assayed using the calpain substrate succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-Leu-Leu-Val-Tyr-AMC) as described in MATERIALS AND METHODS. Each value represents mean ± SD of 3 independent experiments. $*P < 0.05$ vs. untreated control cells. $**P < 0.05$ vs. control cells treated with H_2O_2. $\#P < 0.05$ vs. untreated CRT-S8 cells. *D*: CRT-overexpressing (CRT-S8) cells were treated for 1 h with or without 75 μM H_2O_2 in the presence or absence of 50 μM *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) or 10 μM BAPTA-AM, and then the expression levels of caspase-12, caspase-3, CNX, BiP, and CRT were examined using cell lysates by performing immunoblot analysis with specific antibodies. Data represent 3 independent experiments.

nonstressed conditions. During treatment with H_2O_2, the 55-kDa band gradually decreased in intensity. On the other hand, the strength of the 35-kDa band was slightly increased after 1 h of treatment with H_2O_2 but was diminished after 2 h of treatment, suggesting enhanced proteolytic processing of the fragment in CRT-overexpressing cells treated with H_2O_2. The level of the 40-kDa band did not show significant change in control and CRT-overexpressing cells during treatment. These results indicate that proteolytic processing or degradation of caspase-12 was more accelerated in CRT-overexpressing cells than in control cells after treatment with H_2O_2. H_2O_2-induced processing also seemed to be enhanced by thapsigargin in both control and CRT-overexpressing cells. In the case of caspase-3, although the proteolytic cleavage was observed in control and CRT-overexpressing cells under stress due to H_2O_2, proteolysis was relatively accelerated in CRT-overexpressing cells

compared with controls. These results indicate that proteolytic processing or degradation of caspase-12 and caspase-3 is apparently accelerated in CRT-overexpressing cells under stress caused by H_2O_2. In contrast, no significant change in the expression levels of ER chaperones such as CNX, BiP, and CRT seemed to be induced by H_2O_2. Next, to investigate the relationship between proteolytic processing and the activity of caspase-12, we examined the enzyme activity of caspase-12 in cells after 1 h with or without 75 μM H_2O_2 and/or 5 μM thapsigargin (Fig. 8*B*). In CRT-overexpressing cells, activity was increased solely by H_2O_2, compared with no significant increase in activity in the control cells. Although activity was increased by thapsigargin in both control and CRT-overexpressing cells, it was elevated more in CRT-overexpressing cells during the combined treatment with H_2O_2. As shown in Fig. 8*C*, the activity of calpain was also examined in the cells after 1 h with or without 75 μM H_2O_2 and/or 5 μM thapsigargin. The activity of calpain was significantly increased by H_2O_2 in CRT-overexpressing cells compared with the small increase observed in control cells. In control cells, treatment with thapsigargin synergistically enhanced the effect of H_2O_2, although the activity was slightly increased solely by thapsigargin. In CRT-overexpressing cells, although calpain activity was markedly increased by thapsigargin, a synergistic effect was not observed. To determine whether the activity of calpain contributes to the processing of caspase-12, we examined the effect of *N*-acetyl-leucyl-leucyl-norleucinal (ALLN), a calpain inhibitor (57), or BAPTA-AM on processing in CRT-overexpressing cells during treatment with H_2O_2 (Fig. 8*D*). CRT-overexpressing cells were treated for 1 h with or without 75 μM H_2O_2 in the presence or absence of 50 μM ALLN or 10 μM BAPTA-AM, and then the expression levels of caspase-12 and caspase-3 and ER chaperones were examined using immunoblot analysis. The H_2O_2-induced processing of caspase-12 was apparently suppressed in the presence of ALLN or BAPTA-AM, resulting in a slight decrease in the level of the 35-kDa fragment and an increase in that of the 55-kDa procaspase form. Proteolytic processing of caspase-3 was also suppressed by ALLN or BAPTA-AM in gene-transfected cells treated with H_2O_2. Altogether, these results suggest that the Ca^{2+}-calpain pathway is involved in the activation of caspase-12 in CRT-overexpressing cells under oxidative stress caused by H_2O_2.

DISCUSSION

In the present study, we used myocardiac H9c2 cells that overexpressed CRT to investigate the effect of overexpression on H_2O_2-induced apoptosis in cardiac myocytes. When exposed to H_2O_2, the CRT-overexpressing cells showed increases in LDH release and DNA strand breaks, indicating that these cells were highly susceptible to apoptosis compared with control cells. Nakamura et al. (41) reported that overexpression of CRT resulted in increased sensitivity of HeLa cells to both thapsigargin- and staurosporine-induced apoptosis. These authors suggested that overexpression of CRT affected communication between the ER and the mitochondria to increase the sensitivity to apoptosis via the altered Ca^{2+} homeostasis, and their hypothesis has been supported by the study of Arnaudeau et al. (1). Pinton et al. (43) reported that the releasable $[Ca^{2+}]$ in the ER is important for ceramide-induced apoptosis and also showed that overexpression of CRT enhanced the ceramide-induced apoptosis in HeLa cells. Recently, it was also reported that CRT controls the susceptibility to apoptosis by regulating p53 functions (34). Furthermore, a necrosis-promoting effect of CRT has been reported to occur in *Caenorhabditis elegans* (53). In contrast, overexpression of CRT provided resistance to oxidant-induced cell death in renal epithelial LLC-PK1 cells treated with iodoacetamide (28), *tert*-butylhydroperoxide (29), or H_2O_2 (18). The function of CRT in the regulation of apoptosis may differ in specific cell types and is still controversial, so further investigation is required.

Ca^{2+} is one of the most versatile biological factors and regulates a variety of cellular events, such as cell development, cell proliferation, and cell death (3). The elevation of $[Ca^{2+}]_i$ has been thought to be an important signal in the mechanism of apoptosis (31). In the present study, we found that the level of $[Ca^{2+}]_i$ increased significantly in CRT-overexpressing cells treated with H_2O_2, although only a slight increase was observed in controls (Fig. 4). As shown in Fig. 6, H_2O_2-induced apoptosis was suppressed by the Ca^{2+} chelator BAPTA-AM in CRT gene-transfected cells but was promoted by thapsigargin in control cells, indicating that the increase in $[Ca^{2+}]_i$ was an important trigger for apoptosis in H9c2 cells under stress due to H_2O_2.

In previous reports, overexpression of CRT led to an increase in the intracellular store of Ca^{2+} (2, 8, 32, 54). CRT also appears to modulate store-operated Ca^{2+} influx (1, 2, 8, 32, 46, 54) and to alter Ca^{2+} transport by SERCA2b (22). In the case of H9c2 cells overexpressing CRT, the total cellular Ca^{2+} content examined on the basis of measuring equilibrium $^{45}Ca^{2+}$ uptake was ~160% of that in control cells and was found mainly within the thapsigargin-sensitive store. However, $[Ca^{2+}]_i$ levels in the resting state were not significantly different between control and CRT-overexpressing cells. On the other hand, store-operated Ca^{2+} influx was examined spectrofluorometrically using cells labeled with fura-2 AM and was suppressed in the CRT-overexpressing cells compared with controls (data not shown). These results indicate that overexpression of CRT influences intracellular Ca^{2+} homeostasis, and the effect in resting H9c2 cells seems to be similar to that in other cell types described in the literature. Recently, Scorrano et al. (48) reported that Ca^{2+} reserved in the ER was an important gateway for apoptosis via the influence on mitochondrial Ca^{2+} homeostasis. Overexpression of CRT also influences the mitochondrial Ca^{2+} homeostasis (1). Altogether, the enhanced susceptibility to H_2O_2-induced apoptosis in CRT-overexpressing H9c2 cells may also be due to the modulation of mitochondrial Ca^{2+} homeostasis by the altered responses in the ER overexpressing CRT.

Under oxidative stress, reactive oxygen and nitrogen can disrupt normal physiological pathways and cause cell death via altered Ca^{2+} homeostasis (7, 30). The $[Ca^{2+}]_i$ level is regulated by Ca^{2+} transport into and out of the ER or SR, in which Ca^{2+} can be stored, as well as by Ca^{2+} transport through the plasma membrane between the cytoplasm and the extracellular space (3). It was reported that oxidative stress causes a $[Ca^{2+}]_i$ increase in a variety of cell types (7). The initial increase in $[Ca^{2+}]_i$ results in part from a rapid release of ER Ca^{2+} through IP_3 receptors after receptor-mediated activation of PLC, and the subsequent generation of IP_3 and the sustained component results from the influx of extracellular Ca^{2+} (54). It is also

known that the uptake of Ca^{2+} from the cytoplasm to the ER/SR by SERCA can be inhibited by O_2^- and H_2O_2 in smooth muscle cells (14, 15). It seems that SERCA can be inhibited both by oxidation of its sulfhydryl residues and by a direct attack of oxidants on the ATP-binding site (7).

In the present study, the influx and efflux of $^{45}Ca^{2+}$ were examined in control and CRT-overexpressing cells with or without H_2O_2 treatment (Fig. 5, *A* and *B*). The results showed that Ca^{2+} influx was suppressed and that efflux was enhanced in the gene-transfected cells during H_2O_2 treatment, suggesting that the H_2O_2-induced increase in $[Ca^{2+}]_i$ might not be caused simply by the change of Ca^{2+} flux between the cytoplasm and extracellular space, but rather by the alteration in intracellular Ca^{2+} pools such as those in the ER and the mitochondria. To investigate the involvement of intracellular Ca^{2+} pools in the H_2O_2-induced increase in $[Ca^{2+}]_i$, we examined the effect of Ca^{2+} modulators on $[Ca^{2+}]_i$ in cells treated with H_2O_2 (Fig. 5*C*). The inhibition of mitochondrial function by FCCP did not enhance the H_2O_2-induced $[Ca^{2+}]_i$ increase in control cells, indicating that suppressed mitochondrial function was not a main cause of the enhancement of $[Ca^{2+}]_i$ observed in CRT-overexpressing cells. On the other hand, thapsigargin, an inhibitor for SERCA, apparently enhanced the H_2O_2-induced $[Ca^{2+}]_i$ increase in control cells, strongly suggesting that the Ca^{2+} store in the ER might be a cause of the $[Ca^{2+}]_i$ elevation observed in CRT-overexpressing cells. This observation also suggested that dysfunction of SERCA2a has a promoting effect on the H_2O_2-induced $[Ca^{2+}]_i$ increase in H9c2 cells. Although ER residents such as the ryanodine and IP_3 receptors may possibly be involved in raising $[Ca^{2+}]_i$ levels in response to H_2O_2, the inhibition of both did not suppress the H_2O_2-induced $[Ca^{2+}]_i$ increase in CRT-overexpressing cells. These results suggest that the H_2O_2-induced $[Ca^{2+}]_i$ increase may be due to a dysfunction of SERCA2a and not to the release of Ca^{2+} from the ER through the ryanodine or IP_3 receptors. However, it is noteworthy that the H_2O_2-induced increase of $[Ca^{2+}]_i$ was clearly suppressed by Ni^{2+}, an inhibitor of Ca^{2+} influx in gene-transfected cells. Collectively, these results indicate that the ER-stored Ca^{2+} pool plays an important role in the enhancement of the H_2O_2-induced $[Ca^{2+}]_i$ increase in CRT-overexpressing cells, although Ca^{2+} influx from the extracellular space was also an important contributor to the increase.

In a recently published report (15), we focused on the function of SERCA2a in CRT-overexpressing H9c2 cells under oxidative stress caused by H_2O_2 because SERCA is an ER/SR resident protein that is highly susceptible to peroxide stress. In that study, we found that in vitro activities of SERCA2a and $^{45}Ca^{2+}$ uptake into the ER were both suppressed by H_2O_2 in CRT-overexpressing H9c2 cells compared with controls (20). This finding indicates that the inactivation of SERCA2a was accelerated by the overexpression of CRT in the microsomes treated with H_2O_2. We also found that CRT transiently interacted with SERCA2a during H_2O_2-induced oxidative stress and that H_2O_2-induced degradation of SERCA2a was apparently enhanced in gene-transfected cells compared with controls. On the other hand, interaction between CRT and the IP_3 or ryanodine receptor was not detected in the cells under the same conditions (data not shown), suggesting that other Ca^{2+}-regulating proteins in the ER had little physical interaction with CRT under oxidative stress. These findings suggest that the increase in $[Ca^{2+}]_i$ may be due partly to the loss of Ca^{2+}-pumping activity of SERCA2a in the ER of CRT-overexpressing cells under oxidative stress.

Sustained elevation of the CRT level in the ER may be a consequence of ER stress. ER stress, also known as the unfolded protein response, is a physiological cellular response against accumulated misfolded proteins in the ER (25). However, prolonged ER stress is known to lead to apoptosis and to be linked to the pathogenesis of several disorders, including genetic diseases (e.g., type 1 diabetes mellitus), neurodegenerative diseases (e.g., Alzheimer disease, Parkinson disease), and metabolic diseases (e.g., hyperhomocysteinemia) (25). Caspase-12, which is associated with the ER, is specifically involved as a cell death effector via ER stress (40, 44). ER stress-induced activation of caspase-12 occurred through proteolytic processing by calpain via $[Ca^{2+}]_i$ elevation in the stressed cell (40). In the present study, we have shown that caspase-12 was highly activated in the CRT-overexpressing cells under oxidative stress through the activation of the Ca^{2+}-calpain pathway (Fig. 8). The results strongly suggest that a Ca^{2+}-calpain-caspase-12 pathway is involved in the mechanism of accelerated susceptibility to H_2O_2-induced apoptosis in CRT-overexpressing cells. Although Morishima et al. (37) did not clarify fully the precise activation mechanism for the caspase-12-related pathway, they reported that ER stress could trigger a specific cascade involving caspase-12, caspase-9, and caspase-3 in a cytochrome *c*-independent manner. This finding may be consistent with our findings that the H_2O_2-induced processing of both caspase-12 and caspase-3 was accelerated in CRT-overexpressing cells under stress and was suppressed in the presence of a calpain inhibitor, ALLN. Furthermore, the processing of caspase-12 and caspase-3 was suppressed by a Ca^{2+} chelator, BAPTA-AM, in the H_2O_2-treated, CRT-overexpressing cells, suggesting an activated linkage of the Ca^{2+}-calpain-caspase-12 signaling cascade in the apoptotic process of CRT-overexpressing cells under oxidative stress.

We also have shown that overexpression of CRT promotes apoptosis during cardiac differentiation in H9c2 cells (24). In that study, we showed that Akt signaling was suppressed in H9c2 cells overexpressing CRT via $[Ca^{2+}]_i$ increase. In addition, we recently reported (56) that cAMP response element-dependent transcriptional upregulation of the *PP2Ac-α* gene is involved in the inactivation of Akt, leading to the enhancement of oxidant-induced apoptosis in H9c2 cells under conditions in which $[Ca^{2+}]_i$ elevation is prolonged. With regard to the differentiation of cardiomyocytes, the importance of the intracellular generation of reactive oxygen species is implicated (47). In this respect, the altered Ca^{2+} homeostasis leading to accelerated apoptosis in CRT-overexpressing cells during differentiation may be related to a similar mechanism in cells to which reactive oxygen species are exposed. Moreover, in addition to the mechanism related to Akt signaling, the results of the present study also suggest that the Ca^{2+}-calpain-caspase-12 pathway is part of another mechanism of the differentiation-induced apoptosis of CRT-overexpressing H9c2 cells (24).

In conclusion, the results of the present study indicate that the level of CRT regulates susceptibility to oxidative stress through a change in Ca^{2+} homeostasis and a Ca^{2+}-dependent calpain-caspase-12 pathway in myocardiac H9c2 cells, suggesting a pathophysiological significance of CRT in myocardiac disorders under conditions of oxidative stress.

ACKNOWLEDGMENTS

We are grateful to Midori Ikezaki and Akiko Emura for technical assistance.

GRANTS

This work was supported in part by Grants-in-Aid for the 21st Century Centers of Excellence program from the Ministry of Education, Science, Sports, Culture, and Technology of Japan, and by grants from the Ministry of Health, Labor, and Welfare, Japan.

REFERENCES

1. **Arnaudeau S, Frieden M, Nakamura K, Castelbou C, Michalak M, and Demaurex N.** Calreticulin differentially modulates calcium uptake and release in the endoplasmic reticulum and mitochondria. *J Biol Chem* 277: 46696–46705, 2002.
2. **Bastianutto C, Clementi E, Codazzi F, Podini P, De Giorgi F, Rizzuto R, Meldolesi J, and Pozzan T.** Overexpression of calreticulin increases the Ca^{2+} capacity of rapidly exchanging Ca^{2+} stores and reveals aspects of their lumenal microenvironment and function. *J Cell Biol* 130: 847–855, 1995.
3. **Berridge MJ, Bootman MD, and Lipp P.** Calcium: a life and death signal. *Nature* 395: 645–648, 1998.
4. **Bishopric NH, Andreka P, Slepak T, and Webster KA.** Molecular mechanisms of apoptosis in the cardiac myocyte. *Curr Opin Pharmacol* 1: 141–150, 2001.
5. **Buckler KJ and Vaughan-Jones RD.** Effects of mitochondrial uncouplers on intracellular calcium, pH and membrane potential in rat carotid body type I cell. *J Physiol* 513: 819–833, 1998.
6. **Chen Q, Jones TW, and Stevens JL.** Early cellular events couple covalent binding of reactive metabolites to cell killing by nephrotoxic cysteine conjugates. *J Cell Physiol* 161: 293–302, 1994.

6a. **Curran-Everett D and Benos DJ.** Guidelines for reporting statistics in journals published by the American Physiological Society. *Am J Physiol Cell Physiol* 287: 243–245, 2004.

7. **Ermak G and Davies KJA.** Calcium and oxidative stress: from cell signaling to cell death. *Mol Immunol* 38: 713–721, 2001.
8. **Fasolato C, Pizzo P, and Pozzan T.** Delayed activation of the store-operated calcium current induced by calreticulin overexpression in RBL-1 cells. *Mol Biol Cell* 9: 1513–1522, 1998.
9. **Fisher SA, Langille BL, and Srivastava D.** Apoptosis during cardiovascular development. *Circ Res* 87: 856–864, 2000.
10. **Gafni J, Munsch JA, Lam TH, Catlin MC, Costa LG, Molinski TF, and Pessah IN.** Xestospongins: potent membrane permeable blockers of the inositol 1,4,5-trisphosphate receptor. *Neuron* 19: 723–733, 1997.
11. **Gavrieli Y, Sherman Y, and Ben-Sasson SA.** Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 119: 493–501, 1992.
12. **Gill C, Mestril R, and Samali A.** Losing heart: the role of apoptosis in heart disease—a novel therapeutic target? *FASEB J* 16: 135–146, 2002.
13. **Glading A, Chang P, Lauffenburger DA, and Wells A.** Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway. *J Biol Chem* 275: 2390–2398, 2000.
14. **Grover AK and Samson SE.** Effect of superoxide radical on Ca^{2+} pumps of coronary artery. *Am J Physiol Cell Physiol* 255: C297–C303, 1988.
15. **Grover AK, Samson SE, and Fomin VP.** Peroxide inactivates calcium pumps in pig coronary artery. *Am J Physiol Heart Circ Physiol* 263: H537–H543, 1992.
16. **Grynkiewicz G, Poenie M, and Tsien RY.** A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260: 3440–3450, 1985.
17. **Holaska JM, Black BE, Love DC, Hanover JA, Leszyk J, and Paschal BM.** Calreticulin is a receptor for nuclear export. *J Cell Biol* 152: 127–140, 2001.
18. **Hung CC, Ichimura T, Stevens JL, and Bonventre JV.** Protection of renal epithelial cells against oxidative injury by endoplasmic reticulum stress preconditioning is mediated by ERK1/2 activation. *J Biol Chem* 278: 29317–29326, 2003.
19. **Ihara Y, Sakamoto Y, Mihara M, Shimizu K, and Taniguchi N.** Overexpression of *N*-acetylglucosaminyltransferase III disrupts the tyrosine phosphorylation of Trk with resultant signaling dysfunction in PC12 cells treated with nerve growth factor. *J Biol Chem* 272: 9629–9634, 1997.
20. **Ihara Y, Kageyama K, and Kondo T.** Overexpression of calreticulin sensitizes SERCA2a to oxidative stress. *Biochem Biophys Res Commun* 329: 1343–1349, 2005.
21. **Imanaka-Yoshida K, Amitani A, Ioshii SO, Koyabu S, Yamakado T, and Yoshida T.** Alteration of expression and distribution of the Ca^{2+}-sorting proteins in endo/sarcoplasmic reticulum during differentiation of rat cardiomyocytes. *J Mol Cell Cardiol* 28: 553–562, 1996.
22. **John LM, Lechleiter JD, and Camacho P.** Differential modulation of SERCA2 isoforms by calreticulin. *J Cell Biol* 142: 963–973, 1998.
23. **Johnson S, Michalak M, Opas M, and Eggleton P.** The ins and outs of calreticulin: from the ER lumen to the extracellular space. *Trends Cell Biol* 11: 122–129, 2001.
24. **Kageyama K, Ihara Y, Goto S, Urata Y, Toda G, Yano K, and Kondo T.** Overexpression of calreticulin modulates protein kinase B/Akt signaling to promote apoptosis during cardiac differentiation of cardiomyoblast H9c2 cells. *J Biol Chem* 277: 19255–19264, 2002.
25. **Kaufman RJ.** Orchestrating the unfolded protein response in health and disease. *J Clin Invest* 110: 1389–1398, 2002.
26. **Kimura J, Miyamae S, and Noma A.** Identification of sodium-calcium exchange current in single ventricular cells of guinea-pig. *J Physiol* 384: 199–222, 1987.
27. **Kurebayashi N and Ogawa Y.** Depletion of Ca^{2+} in the sarcoplasmic reticulum stimulates Ca^{2+} entry into mouse skeletal muscle fibres. *J Physiol* 533: 185–199, 2001.
28. **Liu H, Bowes RC III, van de Water B, Sillence C, Nagelkerke JF, and Stevens JL.** Endoplasmic reticulum chaperones GRP78 and calreticulin prevent oxidative stress, Ca^{2+} disturbances, and cell death in renal epithelial cells. *J Biol Chem* 272: 21751–21759, 1997.
29. **Liu H, Miller E, van de Water B, and Stevens JL.** Endoplasmic reticulum stress proteins block oxidant-induced Ca^{2+} increases and cell death. *J Biol Chem* 273: 12858–12862, 1998.
30. **Lounsbury KM, Hu Q, and Ziegelstein RC.** Calcium signaling and oxidant stress in the vasculature. *Free Radic Biol Med* 28: 1362–1369, 2000.
31. **McConkey DJ and Orrenius S.** The role of calcium in the regulation of apoptosis. *Biochem Biophys Res Commun* 239: 357–366, 1997.
32. **Mery L, Mesaeli N, Michalak M, Opas M, Lew DP, and Krause KH.** Overexpression of calreticulin increases intracellular Ca^{2+} storage and decreases store-operated Ca^{2+} influx. *J Biol Chem* 271: 9332–9339, 1996.
33. **Mesaeli N, Nakamura K, Zvaritch E, Dickie P, Dziak E, Krause KH, Opas M, MacLennan DH, and Michalak M.** Calreticulin is essential for cardiac development. *J Cell Biol* 144: 857–868, 1999.
34. **Mesaeli N and Phillipson C.** Impaired p53 expression, function, and nuclear localization in calreticulin-deficient cells. *Mol Biol Cell* 15: 1862–1870, 2004.
35. **Michalak M, Corbett EF, Mesaeli N, Nakamura K, and Opas M.** Calreticulin: one protein, one gene, many functions. *Biochem J* 344: 281–292, 1999.
36. **Morgan-Boyd R, Stewart JM, Vavrek RJ, and Hassid A.** Effects of bradykinin and angiotensin II on intracellular Ca^{2+} dynamics in endothelial cells. *Am J Physiol Cell Physiol* 253: C588–C598, 1987.
37. **Morishima N, Nakanishi K, Takenouchi H, Shibata T, and Yasuhiko Y.** An endoplasmic reticulum stress-specific caspase cascade in apoptosis: cytochrome *c*-independent activation of caspase-9 by caspase-12. *J Biol Chem* 277: 34287–34294, 2002.
38. **Mosmann T.** Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55–63, 1983.
39. **Murata H, Ihara Y, Nakamura H, Yodoi J, Sumikawa K, and Kondo T.** Glutaredoxin exerts antiapoptotic effect by regulating redox state of Akt. *J Biol Chem* 278: 50226–50233, 2003.
40. **Nakagawa T and Yuan J.** Cross-talk between two cysteine proteases: activation of caspase-12 by calpain in apoptosis. *J Cell Biol* 150: 887–894, 2000.
41. **Nakamura K, Bossy-Wetzel E, Burns K, Fadel MP, Lozyk M, Goping IS, Opas M, Bleackley RC, Green DR, and Michalak M.** Changes in endoplasmic reticulum luminal environment affect cell sensitivity to apoptosis. *J Cell Biol* 150: 731–740, 2000.
42. **Nakamura K, Robertson M, Liu G, Dickie P, Nakamura K, Guo JQ, Duff HJ, Opas M, Kavanagh K, and Michalak M.** Complete heart block and sudden death in mice overexpressing calreticulin. *J Clin Invest* 107: 1245–1253, 2001.
43. **Pinton P, Ferrari D, Rapizzi E, Di Virgilio F, Pozzan T, and Rizzuto R.** The Ca^{2+} concentration of the endoplasmic reticulum is a key deter-

minant of ceramide-induced apoptosis: significance for the molecular mechanism of Bcl-2 action. *EMBO J* 20: 2690–2701, 2001.

44. **Rao RV, Ellerby HM, and Bredesen DE.** Coupling endoplasmic reticulum stress to the cell death program. *Cell Death Differ* 11: 372–380, 2004.
45. **Rauch F, Prud'homme J, Arabian A, Dedhar S, and St-Arnaud R.** Heart, brain, and body wall defects in mice lacking calreticulin. *Exp Cell Res* 256: 105–111, 2000.
46. **Roderick HL, Llewellyn DH, Campbell AK, and Kendall JM.** Role of calreticulin in regulating intracellular Ca^{2+} storage and capacitative Ca^{2+} entry in HeLa cells. *Cell Calcium* 24: 253–262, 1998.
47. **Sauer H, Rahimi G, Hescheler J, and Wartenberg M.** Role of reactive oxygen species and phosphatidylinositol 3-kinase in cardiomyocyte differentiation of embryonic stem cells. *FEBS Lett* 476: 218–223, 2000.
48. **Scorrano L, Oakes SA, Opferman JT, Cheng EH, Sorcinelli MD, Pozzan T, and Korsmeyer SJ.** BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis. *Science* 300: 135–139, 2003.
49. **Sumida M and Tonomura Y.** Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from the skeletal muscle: X. Direct evidence for Ca^{2+} translocation coupled with formation of a phosphorylated intermediate. *J Biochem (Tokyo)* 75: 283–297, 1974.
50. **Thastrup O, Cullen PJ, Drøbak BK, Hanley MR, and Dawson AP.** Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc Natl Acad Sci USA* 87: 2466–2470, 1990.
51. **Tsutsui H, Ishibashi Y, Imanaka-Yoshida K, Yamamoto S, Yoshida T, Sugimachi M, Urabe Y, and Takeshita A.** Alteration in sarcoplasmic reticulum calcium-storing proteins in pressure-overload cardiac hypertrophy. *Am J Physiol Heart Circ Physiol* 272: H168–H175, 1997.
52. **Turner NA, Xia F, Azhar G, Zhang X, Liu L, and Wei JY.** Oxidative stress induces DNA fragmentation and caspase activation via the c-Jun NH_2-terminal kinase pathway in H9c2 cardiac muscle cells. *J Mol Cell Cardiol* 30: 1789–1801, 1998.
53. **Xu K, Tavernarakis N, and Driscoll M.** Necrotic cell death in *C. elegans* requires the function of calreticulin and regulators of Ca^{2+} release from the endoplasmic reticulum. *Neuron* 31: 957–971, 2001.
54. **Xu W, Longo FJ, Wintermantel MR, Jiang X, Clark RA, and DeLisle S.** Calreticulin modulates capacitative Ca^{2+} influx by controlling the extent of inositol 1,4,5-triphosphate-induced Ca^{2+} store depletion. *J Biol Chem* 275: 36676–36682, 2000.
55. **Yang F, He XP, Russell J, and Lu B.** Ca^{2+} influx-independent synaptic potentiation mediated by mitochondrial Na^{+}-Ca^{2+} exchanger and protein kinase C. *J Cell Biol* 163: 511–523, 2003.
56. **Yasuoka C, Ihara Y, Ikeda S, Miyahara Y, Kondo T, and Kohno S.** Antiapoptotic activity of Akt is down-regulated by Ca^{2+} in myocardiac H9c2 cells: evidence of Ca^{2+}-dependent regulation of protein phosphatase 2Ac. *J Biol Chem* 279: 51182–51192, 2004.
57. **Zhang L, Song L, and Parker EM.** Calpain inhibitor I increases β-amyloid peptide production by inhibiting the degradation of the substrate of γ-secretase: evidence that substrate availability limits β-amyloid peptide production. *J Biol Chem* 274: 8966–8972, 1999.