

Acute Doxorubicin Cardiotoxicity Involves Cardiomyocyte Apoptosis¹

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Abstract

Despite well-documented cardiotoxic effects, doxorubicin remains a major anticancer agent. To study the role of myocardial apoptosis following doxorubicin administration, male Wistar rats were exposed to 1.25, 2.5, and 5 mg/kg of i.p. doxorubicin and terminated on days 1–7 in groups of five. Doxorubicin caused a significant ($P < 0.001$) and dose-dependent induction of cardiomyocyte apoptosis at 24–48 h after the injection. Repeated injections of 2.5 mg/kg given every other day resulted in peaks of apoptosis at 24 h after each injection. However, no additive effect of repeated dosing was noted. In histological samples, alterations in the cytoskeletal apparatus with focal loss of contractile elements were seen after a single injection. Myocyte necrosis was absent. Thus, acute doxorubicin-induced cardiotoxicity involves cardiomyocyte apoptosis, a potentially preventable form of myocardial tissue loss.

Introduction

The anthracycline antibiotic DOX³ is an important antineoplastic agent because of its high antitumor efficacy in hematological as well as in solid malignancies. Its use is limited by the not infrequent induction of dose-dependent chronic cardiomyopathy. The mechanisms of DOX cardiotoxicity include (a) the formation of free reactive oxygen radicals, (b) direct DNA damage and/or interference with DNA repair (1, 2), and (c) induction of immune reactions involving antigen-presenting cells in the heart (3). In addition to nucleic acids and cellular membranes, the cytotoxic action by anthracyclines involves the cytoskeleton of both tumor cells and cardiomyocytes (4). Cytoskeletal changes following DOX administration include reduction in the density of myofibrillar bundles (5), alterations on the Z-disc structure, and disarray and depolymerization of actin filaments (6, 7). Histologically, cytoplasmic vacuolization due to dilation of the sarcotubules and loss of myofibrils characterize DOX cardiomyopathy (8). Recently, myocardial apoptosis has been suggested as a common mechanism of acute and chronic myocyte loss (9–11). DOX induces apoptosis in several cell lines (12) and, in a rat model, in the kidney and the intestine (13). Whether DOX-induced cardiotoxicity involves CA remains unclear. In a rat model, no CA was observed in samples obtained after a 12-week cumulative DOX treatment (13). In human heart samples, however, contraction and ring formation of the nuclei suggestive of apoptosis have been documented within 24 h after DOX injection (14). Furthermore, apoptosis-type cell morphology with shrunken focal cardiomyocytes has been observed in connection with myofibrillar lysis in the classic study by Billingham *et al.* (7). We therefore designed an experimental study to investigate whether acute DOX administration induces CA *in vivo*, the possible dose-response

of such an acute effect, and the relationship of CA to ensuing histological myocardial damage typical of anthracycline cardiomyopathy.

Materials and Methods

Experimental Protocol. Adult male Wistar rats weighing 300–470 g (total $n = 110$) were purchased from the Central Animal Laboratory, Turku University, Finland. The rats had free access to standard rodent chow and water. All procedures for animal care and housing were in compliance with the contemporary guidelines. The Laboratory Animal Committee and the Social and Health Department of the Provincial State Office of Western Finland approved the experimental protocol. DOX was obtained from Pharmacia SPA (Milan, Italy). DOX cardiomyopathy was induced as described by Iliskovic and Singal (15). In brief, after rats received s.c. buprenorphin (0.05 mg/kg; Reckitt & Colman, Hull, England) to provide analgesia, the first 2.5 mg/kg dose of DOX was injected i.p., and the animals were allowed to recover for 48 h before the procedure was repeated as determined by the subprotocols. Control animals ($n = 10$) were treated with only buprenorphin and i.p. saline.

To first study the occurrence of CA after a single *versus* multiple DOX injections, a pilot study with 15 animals was performed. The rats were given 2.5 mg/kg of DOX every other day up to a maximal cumulative dose of 15 mg/kg over 2 weeks. The rats were killed with carbon dioxide on days 1, 3, and 14 after the first injection and on days 1, 7, and 18 after the last DOX injection ($n = 3–5$ in each group).

Being guided by the results from the pilot study, we then studied the early time course and dose-dependence of apoptosis occurrence. Groups of 15–25 rats received single injections of 1.25, 2.5, or 5 mg/kg of DOX. The rats were killed in groups of five on days 1, 2, and 3 after the injection. For the 2.5 mg/kg injections, rats were also killed at 6 h and on day 7. Finally, to study the effects of consecutive injections, three injections of 2.5 mg/kg each were given every other day. Ten rats were killed on the day after each injection, and five rats were killed on days 2 and 3 after the respective injections.

Immediately post mortem, the entire heart was excised. The heart was sliced transversally, and a midventricular slice was fixed in neutral-buffered 10% formalin for 24 h, embedded in paraffin, and cut into 5- μ m sections for assessment of apoptosis and histological features.

In Situ Assay for Apoptosis. Apoptotic cardiomyocytes were detected with the TUNEL assay and previously described methodology (9, 11, 16). In brief, nuclear DNA strand breaks were end-labeled with digoxigenin-conjugated dideoxy-UTP by terminal transferase and visualized immunohistochemically with digoxigenin antibody conjugated to alkaline phosphatase. The assay was carefully standardized using adjacent tissue sections of each sample, which were pretreated with DNase I (1 unit/ml for 15 min at 37°C; positive control of DNA breaks). The staining of each section was interrupted when intense positivity in the DNase I-treated section appeared. This approach provides optimal sensitivity for double-strand DNA breaks and normalizes the results for differences in tissue permeability of the reagents. The cardiomyocyte origin of TUNEL-positive nuclei was identified by the presence of surrounding myofilaments. The validity of this approach was confirmed by immunofluorescence staining with antimyosin (cat. no. M-8421; Sigma, St. Louis, MO) of randomly selected TUNEL-stained sections. To further confirm the specificity of TUNEL staining in respect to apoptosis, attention was paid to the morphology of positive nuclei and cells.

Quantification of Apoptotic Cells. The number of apoptotic cardiomyocytes in TUNEL-stained sections was counted by use of light microscopy with an ocular grid ($\times 250$ magnification; area of the field, 0.25 mm²). An average of 184 and 210 microscopic fields were analyzed per DOX-treated and control animal, respectively. The average number of cardiomyocytes per field was

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³ The abbreviations used are: DOX, doxorubicin; CA, cardiomyocyte apoptosis; TUNEL, terminal transferase-mediated DNA nick end labeling.

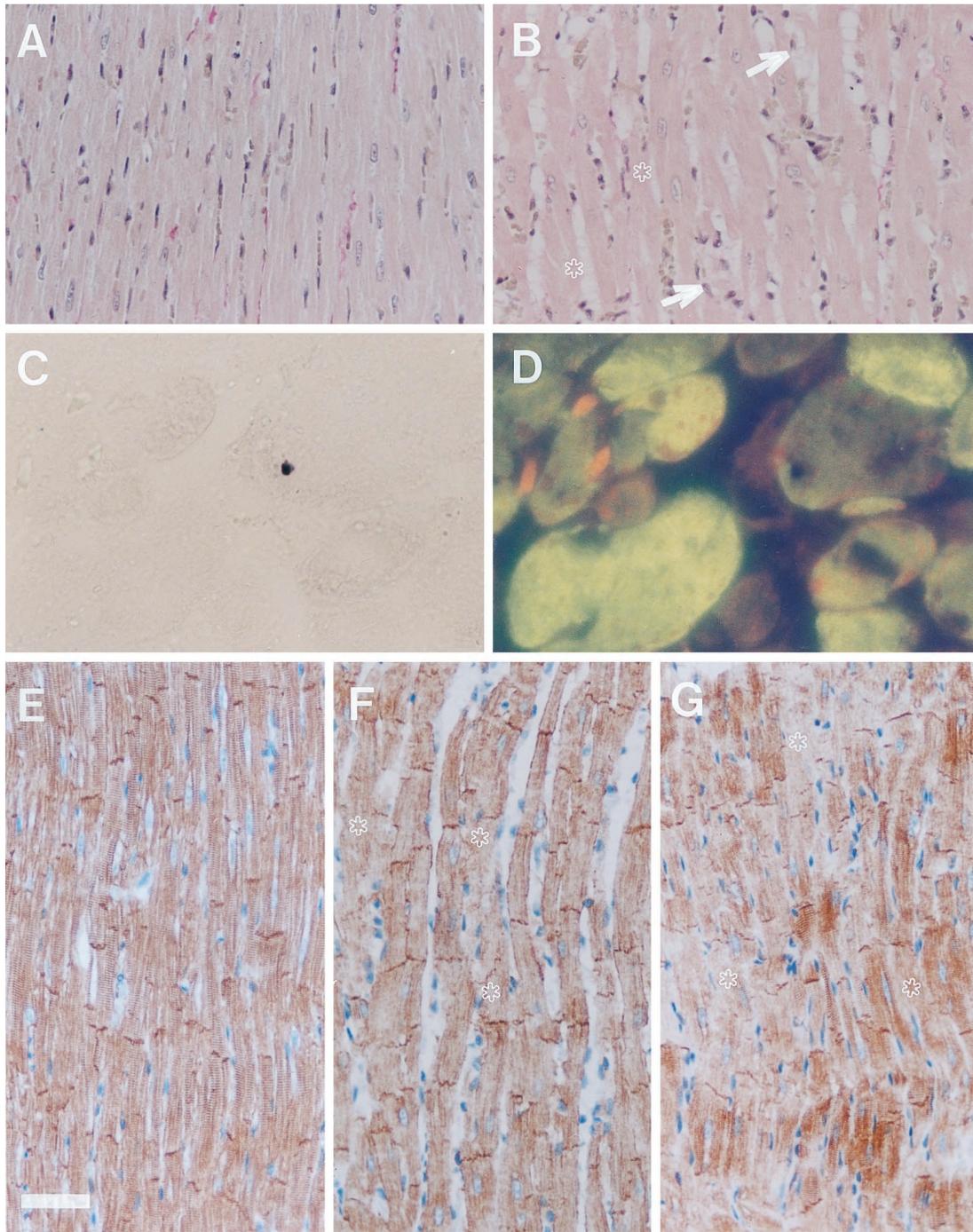


Fig. 1. van Gieson-stained sections of rat myocardium from a control animal (A) and 24 h after a single injection of 2.5 mg/kg DOX (B). Note the vacuolization of cardiomyocytes (arrows) and edema (*) in B. C, dark brown-colored TUNEL-positive nucleus in a sample obtained 24 h after injection of 2.5 mg/kg DOX. Note the condensation of the nucleus and shrunken appearance of the cell. D, double staining with antimyosin shows the same nucleus located within the cardiomyocyte. E–G, immunohistochemical staining for desmin. E, control rat myocardium; F, myocardium after a single injection of 2.5 mg/kg DOX; G, myocardium after the cumulative DOX dose of 15 mg/kg and a follow-up for 7 days. Note the gradual decrease in the intensity of staining and disorganization of cross-striated staining pattern (*). Magnification: A, B, E–G, $\times 200$; bar = 50 μm ; C and D, $\times 1000$; bar = 10 μm .

287 ± 42 (mean \pm SD) in DOX-treated animals and 320 ± 45 in controls. The results are expressed as the percentage of apoptotic nuclei of the total number of labeled cardiomyocyte nuclei, which were counted in the corresponding DNase I-treated section. No apoptotic nuclei in the interstitial space or in the endothelial lining of blood vessels were found. The correlation between the results of repeated procedures with this method is 0.88 ($P < 0.01$; Ref. 11).

Histological Analysis. Formalin-fixed, paraffin-embedded heart tissue sections were stained with van Gieson to demonstrate general histological features. To visualize the cytoskeletal elements, formalin-fixed, paraffin-embedded sections were stained for the intermediate filament protein desmin. Sections were first deparaffinized with xylol, and then boiled twice for 5 min

in a microwave oven in 10 mM citrate buffer (pH 6.0). The primary antibody mouse antidesmin (Zymed Laboratories Inc., San Francisco, CA) was diluted 1:30, and the bound antibody was detected with a biotin-avidin-peroxidase complex (Vectastain ABC kit; Vector Laboratories Inc., Burlingame, CA). The sections were counterstained with hematoxylin.

Statistical Analysis. Quantitative results are expressed as means \pm SD. Percentages of apoptotic cardiomyocytes between DOX-treated and control rats were compared using one-way ANOVA followed by Dunnett's two-sided *t* test. In the case of multiple comparisons (one dose versus multiple doses, different dosages, or consecutive injections) the least significant difference *post hoc* test with Bonferroni correction was used. The software used was

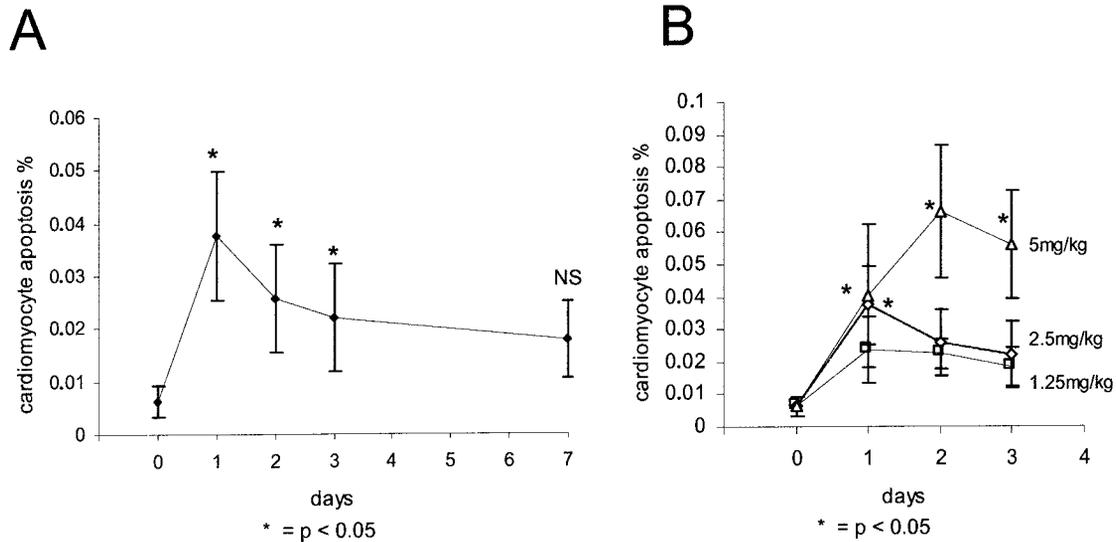


Fig. 2. A, time course of apoptosis occurrence after a single injection of 2.5 mg/kg DOX. B, dose-dependent induction of apoptosis following single injections of 1.25, 2.5, and 5.0 mg/kg DOX.

SPSS 8.0 for Windows (SPSS Inc, Chicago, IL). Differences were considered significant at $P < 0.05$.

Results

General Effects and Histological Analysis. After the total cumulative DOX dose of 15 mg/kg over 2 weeks (pilot study), attenuation in weight gain and signs of clinical DOX-induced cardiomyopathy (ascites, hepatic enlargement, and some degree of general lethargy) were observed as originally described by Iliskovic and Singal (15). The clinical cardiomyopathy syndrome became gradually more evident during the 18-day observation time following the last injection; in particular, greater ascites formation was noted. The pilot study revealed the presence of TUNEL positivity in myocardial samples obtained early during the course of DOX treatment. However, in samples obtained 1–2 weeks after the cumulative treatment, the number of TUNEL-positive cells declined and was not significantly different from controls (data not shown).

In the van Gieson-stained light microscopic sections, alterations consistent with DOX-induced toxicity were seen: cardiomyocyte vacuolization (Fig. 1, A and B) was present after a single injection of 2.5 mg/kg DOX. Notably, necrotic cardiomyocytes were not seen. Neither

polymorphonuclear inflammatory cell infiltration nor lymphocyte infiltration was present.

Immunohistochemical staining of desmin, which is an intermediate filament protein important in the anchorage of the contractile elements in cardiac myocytes (17), was profoundly altered after a single injection of DOX. The uniformly intense staining for desmin vanished, and the normal cross-striation of the cardiomyocytes (Fig. 1E) disappeared in a focal manner (Fig. 1F). After the cumulative 15 mg/kg of DOX and follow-up for 7 days, the desmin staining showed even more striking changes: the staining of cardiomyocytes appeared patchy; and the sarcomeres and intercalated discs were hardly visible. These heavily damaged cells were, however, often adjacent to cells that appeared normal.

Single DOX Injection: Time Course and Dose-dependent Induction of TUNEL Positivity. Microscopic examination of the TUNEL-stained sections showed the presence of sparse single positive nuclei scattered across the ventricular wall. These were surrounded by cardiomyocyte myofilaments (Fig. 1C) as visualized by double staining with antimyosin (Fig. 1D). In general, TUNEL-positive cardiomyocytes showed condensation of nuclei and, sometimes, shrinking of the cytoplasm consistent with apoptotic morphology (Fig. 1, C and D). Only very few TUNEL-positive inflammatory and other non-myocyte cells were observed equally in all hearts, including the control samples.

TUNEL-positive CA was a very rare event in control animals [$0.0065 \pm 0.0022\%$ (mean \pm SD)]. A significant induction of CA to $0.033 \pm 0.012\%$ ($P < 0.001$) of total cardiomyocytes was seen at 24 h after a single 2.5 mg/kg injection of DOX. The percentage of CA peaked on the first day after the injection and declined thereafter (Fig. 2A). The onset of TUNEL positivity was very rapid: at 6 h, the mean percentage was increased to $0.034 \pm 0.027\%$ ($P < 0.05$). The gradual decline of TUNEL positivity continued at day 7, confirming preliminary observations from the pilot study.

In response to 1.25, 2.5, and 5 mg/kg DOX, a dose-dependent induction of CA was observed. As shown in Fig. 2B, the highest relative percentages of apoptotic cardiomyocytes were observed on day 1 after the 1.25 and 2.5 mg/kg doses and on day 2 after 5.0 mg/kg. Compared with sham-injected controls (day 0; $0.0065 \pm 0.0022\%$), the peak amount of TUNEL-positive nuclei was statistically significantly increased after the 2.5 mg/kg ($0.033 \pm 0.012\%$; $P < 0.001$) and

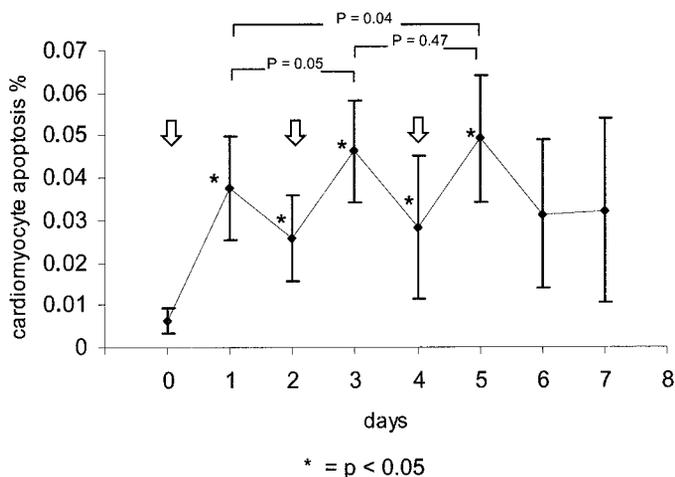


Fig. 3. Three consecutive injections of 2.5 mg/kg DOX administered 1 day apart. Note that the increase of apoptosis is cumulative but not additive. Arrows indicate injections.

5.0 mg/kg ($0.066 \pm 0.020\%$; $P < 0.001$). A gradual decline of TUNEL positivity was observed on day 3, but the percentages still remained above control values.

Consecutive Injections. Fig. 3 shows the amount of CA after three consecutive injections of 2.5 mg/kg DOX administered 1 day apart. After each injection, a new increase in the mean amount of CA was observed (0.033, 0.042, and 0.049% on days 1, 3, and 5, respectively; Fig. 3). Notably, the effect of each new DOX dose was attenuated so that the relative increase was 7-fold after the first injection, 33% after the second injection, but only 16% after the third injection.

Discussion

Our data demonstrate a significant induction of CA in the early phase following DOX administration. The percentage of TUNEL-positive cells peaked within 24–48 h after a single injection, followed by a gradual decline to baseline levels by day 7 after the moderate 2.5 mg/kg dose. The number of TUNEL-positive cardiomyocytes also declined rapidly during the follow-up after a cumulative high-dose DOX treatment. Thus, our findings are not inconsistent with those obtained by Zhang *et al.* (13), who could not demonstrate CA after prolonged (42–84 days) DOX administration and follow-up.

The fact that DOX is toxic to and interferes with DNA raises concern regarding the specificity of the TUNEL assay in assessing apoptosis as a mechanism of DOX cardiotoxicity. The validity of our method has been discussed previously in detail (9, 11, 16). In brief, the standardization procedure using DNase I-treated control sections results in optimization of the TUNEL assay for apoptosis-specific double-strand DNA breaks. During microscopic examination, typical nuclear and cellular morphological features of apoptosis were found in association with TUNEL positivity. Recently, induction of significant CA by DOX in a rat ventricular cell line was reported by Delpy *et al.* (18). A protective effect by thermal preconditioning in this *in vitro* model was also described by Ito *et al.* (19). In addition to the TUNEL assay, these researchers used also agarose DNA gel electrophoresis (DNA ladder assay). In our experience, DNA ladders are only demonstrable in the TUNEL-positive areas when the amount of positive cells exceeds $\sim 0.04\%$ (9).

A theoretical basis for reduced toxicity by fractional DOX dosing is provided by the observation that the single high dose of 5 mg/kg increased TUNEL positivity more ($0.066 \pm 0.020\%$) than did two 2.5 mg/kg injections ($0.051 \pm 0.018\%$). The induction of TUNEL positivity was cumulative after repeated injections, but the relative increase was blunted. This further supports dose fractionation and suggests induction of protective mechanisms. Because the number of apoptotic cardiomyocytes was reduced to nonsignificant levels 3 days after the cumulative dose was achieved, induction of apoptosis by DOX seems to be an acute effect in DOX cardiotoxicity. Cardiomyocyte necrosis is characterized by swelling of the cell, which eventually leads to leakage of cellular components, causing an inflammatory response in adjacent areas. In principle, cells may proceed to or transform to a necrotic type of cell death. However, immediate or delayed histological signs of necrosis did not develop in our model even after a dose considered to be pharmacological or after three repeated injections.

Myocardial apoptosis is not, however, the only mechanism of deteriorating contractile force because the remaining myocardium is also dysfunctional. In addition to the apparent loss of cellular elements, our data show that the very first injection of DOX induces changes in the cytoskeleton and in the contractile element of the cardiomyocyte. The rapid damage observed in the cytoskeleton is likely to cause myocardial dysfunction and contribute to clinical heart

failure syndrome (20). Whether this effect is due to direct DOX toxicity or a failure in protein synthesis reflecting DNA damage remains unclear. These cytoskeletal interferences are likely to favor cell shrinkage and affect tension development (20). This effect can modify or even speed up apoptosis.

Attenuation of apoptotic cell death by a caspase inhibitor has been demonstrated in a myocardial reperfusion injury model. The caspase inhibitor pretreatment effect was achieved in cells receiving a signal that usually promotes apoptosis (21). In cultured cardiomyocytes, exposition to thermal preconditioning before DOX administration attenuates apoptosis occurrence (19). Thus, myocardial apoptosis is a potentially modifiable and preventable form of myocardial tissue loss.

The acute cardiotoxicity of DOX is multifactorial. The very first injection of DOX alters the organization of the cardiomyocyte; in addition, an early induction of apoptosis is observed. This potentially novel mechanism is transient, but it may be of key importance to the ensuing heart failure. Inhibition and modification of this mechanism warrants further study.

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