Adriamycin-induced changes of creatine kinase activity in vivo and in cardiomyocyte culture

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Abstract

Adriamycin (ADM) is an anthracycline anti-neoplastic agent, whose clinical effectiveness is limited by severe side effects, including cardiotoxicity. The toxic effects of ADM are likely to be the consequence of the generation of free radicals. This study demonstrates that ADM induces significant changes in the activity of the oxidative sensitive enzyme creatine kinase (CK) in the heart in vivo and in a cardiomyocyte culture model. The changes observed are likely to reflect the ability of ADM to damage the plasma membrane of cardiac cells and to induce the direct inactivation of CK. The role for ADM-derived free radicals is one of the possible mechanisms for the CK inactivation observed during the ADM treatment. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Adriamycin (ADM) is an anthracycline derivative and is one of the most frequently used anti-neoplastic agents in the treatment of leukemias and solid tumors (Weiss et al., 1986). Clinical effectiveness is restricted due to dose-limiting toxic side effects, such as myelotoxicity, alopecia, gastrointestinal disturbances and irreversible cardiomyopathy (Lefrak et al., 1973; Arena et al., 1974; Steinherz et al., 1991). ADM-induced cardiotoxicity occurs in a biphasic manner, an acute phase and a delayed phase (Doroshow and Locker, 1982; Villani et al., 1986). EKG abnor-
malities and contractile impairment characterize the acute phase, while the delayed cardiotoxicity resembles congestive heart failure (Doroshow et al., 1985; Monti et al., 1986; Timour et al., 1988; Schuyler and Yarbrough, 1990). Although the pathology has been well described and documented, the mechanism of induced cardiotoxicity by ADM is not fully understood.

One hypothesis for the mechanism for the acute toxicity is the involvement of free radicals (Olson et al., 1981; Doroshow and Locker, 1982; Myers, 1982; Doroshow, 1983). ADM has been demonstrated to be a potent generator of free radicals by either an enzymatic pathway or by forming an ADM–Fe³⁺ complex (Handa and Sato, 1975; Goodman and Hochstein, 1977; Bachur et al., 1978; Sugioka and Nakano, 1982; Gutteridge, 1984; Nakano et al., 1984; Zweier, 1984). In either pathway, molecular oxygen is reduced to form reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radical (Olson and Mushlin, 1990). Conceivably, these ROS could then damage membranes or macromolecules that could lead to myocardial injury. Consistent with this notion, ADM induces oxidative stress in erythrocyte membranes (DeAtley et al., 1998).

Creatine kinases (CKs, EC 2.7.3.2), known to be oxidatively sensitive, are possible macromolecule targets for oxidative modification induced by ADM. The CKs are a family of enzymes catalyzing the reversible transfer of a phosphoryl group between ATP and creatine (Bessman, 1985). In vertebrates, at least four isotypes of CK exist that are products of separate genes: brain CK (BB CK), muscle CK (MM CK), ubiquitous mitochondrial CK (uMtCK), and sarcomeric mitochondrial CK (sMtCK). Cytosolic CK isoenzymes always exist as dimeric molecules composed of two (M and B) types of subunits (MM and BB homodimers or MB heterodimer). The mitochondrial CK isoenzymes self-associate to form either octameric or dimeric molecules and are located on the outer surface of the inner mitochondrial membrane (Schlege et al., 1988). In most tissues that possess CK activity cytosolic and mitochondrial CK isoenzymes are co-expressed.

In the heart, three of the four isotypes of CK are expressed: cytoplasmic BCK (BB form); MCK (MM and MB form); and uMtCK. In the adult rat heart MM CK is the major isof orm expressed and provides 80% of the total CK activity, while MB, BB, and uMtCK only account for 20% of the total CK activity found in the rat heart (Vatner and Ingwall, 1984).

That the heart contains an abundance of CK is consonant with the high-energy requirement for this tissue. Cardiomyocytes utilize the creatine phosphate energy shuttle to sustain a constant level of ATP that is appropriate to maintain cardiac performance even during stressful conditions (Jacobus, 1985). In tissues with high-energy flux or requirements, such as the heart, CK isoenzymes play a key role in energy transfer. Cytosolic CKs are thought to maintain an energy shuttle, whereas creatine phosphate produced in the mitochondria by MtCK regenerates ATP at sites of high-energy consumption (Villani et al., 1986).

Given the importance of CK and its known oxidative susceptibility, together with studies from our laboratory and others that suggest ADM induces oxidative stress, we examined the effect of ADM administration on total CK activity and isoenzyme distribution in rat hearts along with changes in serum CK. Alterations in serum CK levels have been demonstrated to be a marker for cardiac damage. In addition, to gain further insight into the alteration of CK activity by ADM in vivo, we investigated modifications to CK in a cardiomyocyte cell culture system.

2. Materials and methods

2.1. Chemicals

ADM HCI was purchased from the University of Kentucky Hospital pharmacy. Trypan blue, 3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium bromide (MTT) and cell culture materials were purchased from Sigma (St. Louis, MO).

2.2. Animals and drug treatments

All procedures involving rodents were approved by the University of Kentucky Institutional Ani-
mal Care and Use Committee. Rats were housed in a temperature- and humidity-controlled, 12-h light/12-h dark environment. All rodents were fed commercial rat chow and water ad libitum. In vivo studies were performed using adult male Sprague–Dawley rats weighing 350–400 g. Three groups of six animals received ADM (10 mg/kg i.p. for 2 or 3 doses, every third day) or saline according to the same schedule (Minnaugh et al., 1983; Floyd et al., 1986). On days 4 and 7 the rats were anesthetized, ventricular heart tissue was removed, then frozen at −70°C for later analysis. Blood was collected by exsanguination then briefly centrifuged; serum was removed and immediately frozen.

2.3. Cardiomyocytes

Rat heart myoblasts H9c2(2-1) were purchased from American Type Culture Collection (Rockville, MD) and incubated with Dulbecco’s modified Eagle’s medium with 4 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 1.0 mM sodium pyruvate, fetal bovine serum (10%), with 50 units penicillin/ml, 100 μg streptomycin/ml at 37°C in 5% CO₂ and the media changed every 2–3 days. The cultured myocytes were allowed to reach confluence before being used experimentally. Confluent myocytes were defined in accordance by the observations of Kimes and Brandt, who described the dividing myocytes as large, flat, spindle-shaped cells, while confluent myocytes form multinucleated tubular structures (Kimes and Brandt, 1976). In addition the dividing myocyte synthesizes a BB CK isoenzyme, but after myotube formation, the MM isoenzyme is synthesized almost exclusively.

2.4. Adriamycin incubation

Upon reaching confluence, myocytes were incubated with 50 μM ADM for 24 and 48 h at standard culture conditions. Following incubation, myocytes attached on the culture plate were washed with HEPES buffer, then removed and immediately frozen for future analysis. Myocytes that were detached from the culture plate were removed, then centrifuged at 14000 rpm for 6 min. The resulting pellet was resuspended in HEPES buffer and immediately frozen. Prior to analysis, myocyte protein content was determined according to the Pierce method.

2.5. Creatine kinase activity assay

Ventricular heart samples for enzyme activity measurement were thawed, miniced and suspended in 10 mM HEPES buffer (pH 7.4) containing 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH₂PO₄ and protease inhibitors: leupeptin (0.5 mg/ml), pepstatin (0.7 mg/ml), aprotinin (0.5 mg/ml) and PMSF (40mg/ml). Following centrifugation at 14 000 rpm for 6 min the rat heart homogenate was removed and CK activity was determined colorimetrically by using a commercial kit (Sigma Chemical). One unit (U) of total CK activity was determined as the amount of the enzyme that will convert 1.0 μmol of creatine to creatine phosphate/min at 37°C, pH 9.0. BCK and MCK isoforms were detected by enzyme activity staining following non-denaturing gel electrophoresis (CIBA Corning Diagnostic, Palo Alto, CA). One microliter of rat heart homogenate, serum sample, or cell lysate containing equal amount of protein was applied to each lane of 1.0% agarose Corning multitrac CK Isoenzyme Gel/8 and electrophoresed in CK Isoenzyme Buffer (Corning) at 90 V for 20 min. The enzyme pattern was developed by layering the plates with CK Reagent (Corning). The plates were incubated at 37°C for 20 min and the enzyme bands were visualized with ultraviolet light.

2.6. Cell viability

In order to access myocyte viability of ADM-treated and control cultures, two different procedures were performed. To assess the cellular metabolic integrity, an MTT reduction assay was used, while trypan blue exclusion examined myocytes for compromise of their membrane structure by reverse-phase microscopy. Cardiomyocytes were incubated for 2 h with 30 μl of a 5 mg/ml MTT solution. MTT solution was removed along with detached myocytes then transferred to a microfuge tube and centrifuged
for 6 min at 14 000 rpm. The resulting pellet (detached cells) and the attached myocytes on the culture plate were resuspended in 1 ml of isopropanol with 0.04 N HCl as the color developer and the absorbance values at 570 nm assessed (Hansen et al., 1989). Both readings for the attached and detached cells were combined to estimate the total MTT reading from the plate.

2.7. **Trypan blue uptake**

In addition to MTT reduction myocytes were assessed by trypan blue exclusion. Immediately following the experimental incubation interval, 20 μl of trypan blue was added to control and ADM-treated cultures. After 10 min, the attached and detached myocytes were examined for viability by phase contrast microscopy.

2.8. **Superoxide dismutase activity gel**

Superoxide dismutase (SOD) activity gel was performed according to the method described by Beauchamp and Fridovich with slight modifications (Beauchamp and Fridovich, 1971; Yen et al., 1996). Myocytes were homogenized in 50 mM potassium phosphate buffer (pH 7.8). Two hundred micrograms of protein/lane was electrophoresed through a non-dissociating riboflavin gel consisting of 5% stacking gel (pH 6.8) and a 10% running gel (pH 8.8) at 4°C. To visualize SOD activity, gels were first incubated in 2.43 mM nitro blue tetrazolium (NBT) in deionized water for 15 min and then in 0.028 mM riboflavin/280 mM N,N,N,N-tetramethyl-ethylene-diamine (TEMED) in 50 mM potassium phosphate buffer (pH 7.8) for 15 min in the dark. Gels were then washed in deionized water and illuminated under fluorescent light until clear zones of SOD activity were distinctly evident.

2.9. **Immunoreactive creatine kinase and superoxide dismutase**

Control and ADM-treated myocytes following normalization for protein content was applied to a nitrocellulose membrane. The membrane was then incubated for 1 h at 37°C with either a 1:500 dilution of monoclonal anti-MM CK antibodies (Harlan, Indianapolis, IN) or a 1:300 dilution of monoclonal anti-CuZn SOD antibodies (Sigma). The secondary antibody (anti-rabbit IgG conjugated with alkaline phosphatase; Sigma) was diluted in a blocking solution (1:15000 working dilution) and incubated with the membrane for 1 h at 37°C. Following each incubation the membrane was washed in buffer (phosphate-buffered saline with 0.01% sodium azide and 0.2% Tween 20) for 10 min at room temperature. The washed membrane was developed using a 5-bromo-4-chloro-indolyl phosphate/nitro blue tetrazolium (BCIP-NBT) solution (SigmaFast tablets; Sigma).

2.10. **Image analysis**

CK gels, SOD gels and immunoreactive dot blots were digitized and semi-quantitative analysis was performed by computer assisted imaging using MCID/M4 software supplied by Imaging Research (Ontario, Canada).

2.11. **Statistical analysis**

Statistical comparisons were made using analysis of variance (ANOVA) followed by Student Newman-Keuels’ post hoc test for multiple comparisons. Significance was set at \( P < 0.01 \).

3. Results

3.1. **The effect of adriamycin on creatine kinase activity in vivo**

From the total rat heart CK analysis obtained from rodents that were administered ADM 10mg/kg for either two injections (the day 4 experimental group) or three injections (the day 7 experimental group), a significant decrease in total heart CK activity was observed only in the day 7 group (Fig. 1). A 50% decline in total CK activity was detected for the day 7 group \((n = 6; P < 0.01)\), while the day 4 group yielded a slightly, though not statistically significant, decrease.

The contribution of BB CK and MM CK to the decrease of total heart CK activity was esti-
mated after separation of these CK isoforms by non-denaturing agarose electrophoresis followed by staining for CK activity. BB CK and MM CK were determined by separate experiments due to the dilutions required for the accurate assessment for the two isoenzymes. The results of a typical experiment with two control groups, two day 4 trial groups, and two day 7 trial groups are shown in Fig. 2A,B. MM CK is the predominate isoenzyme present in the rat heart (80%); the BB CK accounts for less than 8% and MB CK less than 12% of total CK activity. Consequently, the observed decrease of total CK activity in the day 7 group was primarily the result of the 40% decrease (n = 6; P < 0.01) in the MM CK isoenzyme (Fig. 3). Although the BB CK isofrom activity declined by 60%, the contribution of BB CK to the total CK activity is very small when compared to MM CK activity. Examination of the heart CK activity of the day 4 group showed no significant changes in either total CK activity or the individual isoenzyme constituent forms.

The analysis of serum CK isoenzyme activity is a clinical marker for the determination of cardiac damage following ischemic/reperfusion injury; upon cardiac membrane damage, CK is able to leak into the serum. The assessment of the activity of serum CK isoenzymes showed that the only significant activity change was observed in the MM CK isofrom from the day 7 experimental group. Fig. 4 illustrates that the rat serum obtained from the day 7 experimental group exhibited a 100% increase of MM CK (n = 6; P < 0.01) activity over that of controls. There were no

Fig. 1. The decrease of total heart creatine kinase activity per mg of protein in control rats injected with saline, day 4 rats injected with adriamycin (ADM) 10 mg/kg × 2 doses, and day 7 rats injected with ADM 10 mg/kg × 3 doses. Error bars represent S.E.M. values, n = 6, * P < 0.01 vs. control.

Fig. 2. Representative fluorescent staining of heart creatine kinase (CK) isoenzymes from control rats injected with saline, day 4 rats injected with adriamycin (ADM) 10 mg/kg × 2 doses, and day 7 rats injected with ADM 10 mg/kg × 3 doses after non-denaturing electrophoresis in agarose gel. Panel (A) represents activity staining for BB isoenzyme. The protein load was adjusted in order to obtain a detectable signal of brain CK activity. Panel (B) represents activity staining for MM isoenzyme. Only the changes of CK homodimers activity were analyzed.

Fig. 3. Semi-quantitative analysis of non-denaturing creatine kinase isoenzyme activity gels of heart tissue from control rats injected with saline, day 4 rats injected with adriamycin (ADM) 10 mg/kg × 2 doses, and day 7 rats injected with ADM 10 mg/kg × 3 doses. Error bars represent S.E.M. values, n = 6, * P < 0.01.
 modifications observed in the isoenzyme activities of the other experimental groups (Fig. 4). This increase in MM CK activity in serum is consistent with the observation of decreased total CK activity in the hearts of the day 7 treatment group, and these data support the notion of cardiac injury resulting from ADM administration.

3.2. Adriamycin-induced toxicity and creatine kinase activity changes in cultured myocytes

In order to study the toxic effect of ADM on CK function in a cell culture model, we co-incubated H9c2(2-1) cultured rat cardiac myocytes with 50 µM ADM for periods of 24 and 48 h. The H9c2 cardiomyocytes are a permanent cell line that has been demonstrated to have identical biochemical and electrophysiological characteristics as in adult cardiac cells (Hescheler et al., 1991).

Confluent H9c2 myocytes incubated with ADM demonstrated morphological changes distinct from non-treated controls. Some of the myocytes shifted from tubular, parallel arrays to independent circular cells upon detachment from the cell plate (Fig. 5). This result is consistent with the results of Demant and Wassermann (1985), who reported cell detachment occurring in a primary rat cardiomyocyte culture during co-incubation with ADM. Unfortunately, those authors did not assess the viability of the detached cardiomyocytes. To determine if the detached cells were alive or dead, cellular viability was assessed by trypan blue exclusion and MTT reduction. The vast majority of detached myocytes were still viable. Consequently, we had to include the detached cell population in the experimental study. As determined by MTT reduction, mitochondrial function of attached cells decreased by 60% (n = 6; P < 0.01) following 24 h of incubation (Fig. 6A). The total level of MTT reduction, calculated as a sum of MTT reduction measured for attached and detached cell populations, was equal to control after 24 h of incubation. After 48 h total level of MTT reduction in ADM-treated cultures decreased significantly, which probably reflects the death of the detached cells (Table 1).

The mature and confluent H9c2(2-1) cell line almost completely expresses CK activity as MM CK (Kimes and Brandt, 1976) and MM CK was
than the findings obtained from the attached myocytes (Fig. 7A,B). CK activity in the detached population declined 67% (n = 6; P < 0.01) at 24 h of ADM incubation which did not significantly change at 48 h. Analysis for CK activity in the media containing the detached myocytes revealed the presence of MM CK at 48 h (data not shown).

3.3. Immunoreactive creatine kinase content assessment and changes of muscle creatine kinase activity normalized to the immunoreactive content in control and adriamycin-treated cultured cardiomyocytes

The content of MM CK present in either attached or detached cardiomyocytes in ADM-treated cultures was determined using an immunochemical detection assay. No significant decrease of immunoreactive CK content was found either in the attached or detached ADM-treated cardiomyocytes. The total level of immunoreactive CK in control and ADM-treated cardiomyocyte cultures was used to normalize the CK activity per the content of immunoreactive protein. The MM CK activity adjusted for the immunoreactive isoenzyme content (the ratio: CK activity staining/anti-MM CK staining) was reduced in the detached cells (Table 2), which suggests the presence of inactive MM CK molecules in this cell subpopulation. After 24 h of ADM treatment the MM CK activity decreased by 45% compared to control value. Following an additional 24 h of treatment, the MM CK activity staining/anti-MM CK immunostaining ratio was decreased by 55%, suggesting further inactivation of the enzyme. No significant decrease of the MM CK activity staining/anti-MM CK immunostaining ratio was observed in the myocytes that remained attached to the plate either after 24 or 48 h of ADM treatment. Moreover, the MM CK activity normalized per the content of the isoenzyme showed the trend to increase following ADM treatment. This increase might reflect a compensatory activation of MM CK in the cells surviving an ADM impact.

To investigate this possibility, we added either a 5 μM ADP solution or vehicle to myocyte lysates.
for 30 min. After the incubation period the samples were stained for enzyme activity following non-denaturing electrophoresis. Myocytes incubated with an ADP solution demonstrated a 255% increase of activity over those treated with a vehicle (Fig. 8).

3.4. The activity and immunoreactivity of CuZn superoxide dismutase isoform in myocyte cultures treated with adriamycin

Several studies have demonstrated SOD activity may be decreased as a result of oxidative damage produced by several different free radical generating systems (Fuch and Borders, 1983; Pigeolet et al., 1990; Escobar et al., 1996; Pinteaux et al., 1996). Using the procedure by Beauchamp et al., we determined the CuZn SOD activity in control and ADM-treated cardiomyocyte cultures. The intensity of the CuZn SOD enzyme pattern after the staining of the non-denaturing gel was decreased in ADM-treated cultures, both in attached and detached cell populations. However, when the enzyme activity was normalized to the levels of immunoreactive CuZn SOD protein content (as previously described for MM CK), a decrease of 61% in the detached cell population was found (Table 3).

4. Discussion

CK is considered to be one of the most oxidatively sensitive enzymes, and Thomas et al. demonstrated the ability of ADM-derived free radicals to inactive CK in vitro (Thomas et al., 1994). Together, these results support our observation that, in vivo, ADM causes a significant reduction of total heart CK activity. The loss of CK activity in the heart was accompanied by the detection of increased CK activity in the serum, a clinical marker of cardiac cell damage. However, in our study we could not rule out the possibility that CK from the skeletal musculature could also contribute to the increase of CK observed in the serum. One of the most prevailing hypotheses of cardiac damage resulting from ADM administration is the ability of the drug to produce free radicals (Olson et al., 1981; Doroshow and Locker, 1982; Myers, 1982; Doroshow, 1983). Free radicals are known to damage several macromolecular cellular components (Butterfield, 1997; Butterfield and Stadtman, 1997). For example, we previously demonstrated that ADM induces protein oxidation in erythrocytes (DeAtley et al., 1998). Consistent with this notion, others have shown markers for free radical ROS to be increased in ADM-treated cells (Demant and Wassermann, 1985; Julicher et al., 1985; Thorneley and Dodd, 1985; Ollinger and Brunmark, 1994; Vidal et al., 1996). ADM-induced free radicals may attack the cardiac membrane and cause protein or lipid peroxidation, which would compromise the cellular integrity, potentially accounting for the increase serum CK activity. In addition, the oxidative modification of CK isoenzymes as a result of ADM-induced oxidative stress might also contribute to the decrease of CK activity in cardiomyocytes.

Table 1
Attached and detached cell populations as determined by MTT reduction of cultured H9c2 cardiomyocytes incubated with 50 μM adriamycin (ADM)*

<table>
<thead>
<tr>
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<th>24-h incubation</th>
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<th>48-h incubation</th>
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<tbody>
<tr>
<td></td>
<td>Control (%)</td>
<td>ADM (%)</td>
<td>Control (%)</td>
<td>ADM (%)</td>
</tr>
<tr>
<td>Attached cells</td>
<td>73.7 ± 1.05</td>
<td>24.2 ± 1.17</td>
<td>73.9 ± 0.60</td>
<td>8.0 ± 0.26</td>
</tr>
<tr>
<td>Detached cells</td>
<td>26.3 ± 0.02</td>
<td>73.3 ± 0.84</td>
<td>26.1 ± 68</td>
<td>58.7% ± 0.99</td>
</tr>
<tr>
<td>Totals</td>
<td>100</td>
<td>97.5</td>
<td>100</td>
<td>66.67*</td>
</tr>
</tbody>
</table>

* Values represent mean ± S.E.M. (n = 9) percentage of the total of attached and detached cells in each case.
* P < 0.01.
The intensity of anti-creatine kinase (CK) immunostaining in control and adriamycin (ADM)-treated cardiomyocyte cultures was used to normalize the CK activity per the content of immunoreactive protein. The ratio is expressed as muscle CK activity staining/anti-MM CK immunostaining.

<table>
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<tr>
<th></th>
<th>24-h incubation</th>
<th>48-h incubation</th>
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<tbody>
<tr>
<td>Control</td>
<td>2.0 ± 0.30</td>
<td>2.7 ± 0.32</td>
</tr>
<tr>
<td>ADM</td>
<td>Attached cells 2.9 ± 0.28*</td>
<td>0.9 ± 0.17*</td>
</tr>
<tr>
<td></td>
<td>Detached cells 1.1 ± 0.14*</td>
<td>0.5 ± 0.17*</td>
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*Values represent ± S.E.M. (n = 9) for the calculated ratio of MM CK activity staining/anti-MM CK immunostaining ratio in each case.

Several studies have used the primary cultures of cardiac cells or the myocyte cell lines to investigate the toxic effects of ADM (Demant and Wassermann, 1985; Julicher et al., 1985; Thornalley and Dodd, 1985; Ollinger and Brunmark, 1994; Vidal et al., 1996). As it was reported by Demant and Wassermann, ADM treatment induces the significant changes of the cell morphology of cultured cardiomyocytes (Demant and Wassermann, 1985). Our observations are consistent with these findings. ADM induces the appearance of two distinct populations of cells in the treated cultures: those cells that were attached to the culture plate and those which were floating within the media. During our studies we observed the morphological changes in ADM-treated cell cultures precedes cell death. When detached from...
The intensity of anti-CuZn SOD immunostaining in control and adriamycin (ADM)-treated cardiomyocytes was used to normalize the CuZn SOD activity per content of immunoreactive protein.

<table>
<thead>
<tr>
<th>24-h incubation</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.03 ± 0.110</td>
</tr>
<tr>
<td>Attached cells</td>
<td>1.0 ± 0.121</td>
</tr>
<tr>
<td>Detached cells</td>
<td>0.5 ± 0.053</td>
</tr>
</tbody>
</table>

The results obtained from the comparison of CK activity measurement to immunoreactive protein content is suggestive of the notion that CK inactivation occurring during ADM treatment CK is sensitive to oxidative modification (Banerjee et al., 1991; Thomas et al., 1994) and can be a target for oxygen mediated free radicals. Numerous investigations have demonstrated the ability of ADM to induce free radical formation in the presence of oxygen (Doroshow and Locker, 1982; Adachi et al., 1983; Thornalley and Dodd, 1985; Floyd et al., 1986; Thornalley et al., 1986; Rajagopalan et al., 1988; Kalyanaraman et al., 1991; DeAtley et al., 1998). Thus, the formation of ADM-derived free radicals may contribute to the decline in CK activity observed in vitro and in vivo.

In order to acquire evidence for the presence of oxygen free radicals in our experiments, we examined the effect of ADM upon CuZn SOD. Not only was a decrease in SOD activity observed, but also a decline in immunoreactive protein content in ADM-treated myocytes was found. This result is in agreement with other researchers who demonstrated CuZn SOD could be inactivated by free radicals (Fuch and Borders, 1983; Pigeolet et al., 1990; Mao et al., 1993; Escobar et al., 1996; Pinteaux et al., 1996).

During the course of our experiments an increase of CK activity per the immunoreactive content in the attached cell population of cardiomyocytes treated with ADM was noticed. One possible explanation for this increase of activity may be due to an allosteric regulatory mechanism. In myocyte lysates pre-incubated with ADP, a robust increase in their CK activity was observed, suggesting the possibility that not only is ADP a substrate for CK it conceivably could play a role as an allosteric modifier to the enzyme.

In conclusion, the current study shows ADM, a well known generator of oxygen free radicals in biological systems, can induce an identical pattern of CK function damage in heart cells when injected into rats and when added to cultured myocytes. A possible primary event in the development of cardiotoxicity resulting from ADM administration could be the inactivation of CK resulting from generation of oxygen free radi-
cals. Further studies to explore this possibility are in process in our laboratory. Consistent with the findings of this study, ADM-treated cardiomyocytes showed increased production of reactive oxygen species, which were significantly inhibited by pretreatment of the cardiomyocytes with antioxidants (DeAtley et al., 1999).

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