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Induction of Antioxidants by Adriamycin in Mouse Heart

*Xianhua Yin, Huiyun Wu, Yan Chen and Y. James Kang**

Departments of Medicine, and Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY 40202, U.S.A.

ABSTRACT. Cardiac oxidative injury is a major limiting factor for clinical application of Adriamycin (ADR) in cancer chemotherapy. ADR depresses some antioxidant systems, thereby further enhancing the cardiotoxicity. Previous studies have shown that ADR inhibits the overall synthesis of DNA, RNA, and protein. It was presumed that the depressed antioxidant activity resulted from the inhibited gene expression. However, there were no experimental data to demonstrate the relationship between the change in antioxidant activities and that in their gene expression. Therefore, the present study was undertaken to examine the effects of ADR on the activities and mRNA abundances of antioxidants in mouse heart. FVB mice (7 weeks old) were treated with ADR (15 mg/kg) by a single i.p. injection. Four days after the treatment, cardiac antioxidant activities and mRNA abundances were measured. The results showed that ADR increased the levels of mRNAs for Cu,Zn-superoxide dismutase (Cu,Zn-SOD), catalase, glutathione peroxidase (GSHpx), and γ-glutamylcysteine synthetase (γ-GCS). On the other hand, ADR increased the activities of catalase and γ-GCS, and slightly decreased total glutathione concentrations in the heart. Cu,Zn-superoxide dismutase, Mn-superoxide dismutase, and glutathione peroxidase activities were not changed significantly. In addition, ADR increased both mRNA and protein levels of metallothionein in the heart. The data demonstrate that up-regulation of antioxidant gene expression occurred in response to ADR in the mouse heart, although the antioxidant activities were not all increased. BIOCHEM PHARMACOL **56**;1:87–93, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. Adriamycin; mRNA; catalase; superoxide dismutase; glutathione; glutathione peroxidase; γ-glutamylcysteine synthetase; metallothionein

The anticancer agent ADR† is one of the most widely used drugs in the treatment of a variety of human neoplasms [1]. However, cardiotoxicity often develops and is a major limiting factor for further use of this drug [2]. Many studies have shown that the cytotoxic effect of ADR is mediated, at least in part, by the production of reactive oxygen species during its intracellular metabolism [3, 4]. Therefore, mechanistic studies have focused on the role of reactive oxygen species in the progress of ADR-induced cardiomyopathy [5–7].

ADR undergoes one-electron reduction through a metabolic activation caused by NADPH-cytochrome-P450 reductase, or other flavin-containing enzymes [8]. This reduction generates an ADR semiquinone free-radical. In the presence of molecular oxygen, the semiquinone rapidly reduces the oxygen to superoxide with regeneration of intact ADR. Superoxide is rapidly converted to hydrogen peroxide spontaneously or by SOD. The ADR semiquinone can then react with the hydrogen peroxide to yield hydroxyl radical [9]. These highly toxic reactive oxygen species react with cellular molecules including nucleic acids, proteins, and lipids, thereby causing cell damage.

Antioxidant systems play an important role in cellular defense from the damage induced by free radicals. It has been well demonstrated that the occurrence of overt oxidative injury is related to the overall balance between the generation of free radicals and the antioxidant defense. Several studies have reported that ADR inhibits the activities of antioxidant systems, such as SOD and GSHpx, and decreases GSH content in myocardial tissue [10–12]. The depression of these antioxidants presumably hastens oxidative injury by ADR to the heart. Previous studies have shown that ADR impairs DNA, RNA, and protein syntheses, both *in vitro* and *in vivo* [13–17]. It is, therefore, postulated that the depression of the antioxidant systems is due to the global inhibitory effect of ADR on DNA, RNA, and protein syntheses [15].

However, this speculation needs to be tested experimentally. The effect of ADR on gene expression of antioxidants in the heart has not been studied. Consequently, the linkage between the effect of ADR on the activities of antioxidant systems and that on the gene expression of

* Corresponding author: Dr. Y. James Kang, Department of Medicine, University of Louisville School of Medicine, Ambulatory Care Building, 3rd Floor, 530 South Jackson St., Louisville, KY 40202. Tel. (502) 852-6788; FAX (502) 852-0846; E-mail: yjkang01@homer.louisville.edu.

† *Abbreviations:* ADR, Adriamycin; γ-GCS, γ-glutamylcysteine synthetase; GSHpx, glutathione peroxidase; MT, metallothionein; and SOD, superoxide dismutase.

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these antioxidants has not been delineated. Investigation of this relationship would provide an important insight into the biochemical and molecular mechanisms by which changes in antioxidant systems occur in response to ADR. This information would be of significance in developing experimental and clinical approaches to enhance cardiac protective mechanisms against ADR oxidative injury. For instance, should increased antioxidant capacity be an appropriate approach to decrease ADR cardiotoxicity, it would be important to know the biochemical and molecular events of alterations of the endogenous antioxidant systems by ADR when an exogenous modulation is considered to be applied.

Thus, the present study was undertaken to examine the effects of ADR on the activities and mRNA abundances of antioxidants in the mouse heart. The results demonstrated that ADR increased the levels of mRNAs for Cu,Zn-SOD, catalase, GSHpx, γ-GCS, and MT. However, only the activities of catalase and γ-GCS, and the level of MT were increased. Other antioxidant activities and GSH concentrations in the heart either were not changed or were decreased slightly.

MATERIALS AND METHODS

Chemicals

Doxorubicin hydrochloride (Adriamycin, ADR), GSH, NADH, NADPH, glutathione reductase, lactate dehydrogenase, pyruvate kinase, phosphoenolpyruvate, and ATP were purchased from the Sigma Chemical Co. Sulfosalicylic acid and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from the Aldrich Chemical Co. RNAzol B was obtained from Tel-Tech, Inc., and Gene Screen Plus membrane from DuPont. The random primed DNA labeling kit was the product of Boehringer Mannheim. The SOD-525 assay kit was obtained from BioxyTech S.A. via the Cayman Chemical Co., and BCA protein assay reagents were from Pierce. All reagents were of the highest purity available.

Animals and Drug Treatment

FVB mice were bred and maintained at the University animal facilities. Animals were housed in a plastic cage at 23° on a 12-hr light/dark cycle, and were given lab chow and tap water *ad lib*. Adult male mice (6–7 weeks old, weighing 20–30 g) were randomly assigned to either the drug treatment group or the control group. The drug treatment group received 15 mg/kg of ADR dissolved in physiological saline by a single i.p. injection at a volume of 5 mL/kg; the control group received a sham injection of an equal volume of saline without ADR. On day 4 of postdosing, mice were anesthetized with sodium pentobarbital (65 mg/kg, Vet Labs) and hearts were rapidly excised, sectioned, rinsed briefly in saline to remove blood, frozen immediately in liquid nitrogen, and stored at −80° until processed for enzyme assays and RNA extraction within a week. This time course of tissue harvesting is based on previous studies by us [18] and others [15], which have shown that the level of oxidative injury in the heart, measured by lipid peroxidation and creatine phosphokinase release, and the overt inhibitory effect on DNA, RNA, and protein syntheses peak on day 4. All animal procedures were approved by the American Association of Accreditation of the Laboratory Animal Care Certified Institutional Animal Care Committee.

Northern Blot Analysis

Total RNA was extracted from the myocardium tissue, using the RNAzol B method. RNA was quantified spectrophotometrically and confirmed by ethidium bromide staining of 18S and 28S ribosomal RNA. RNA was denatured in formaldehyde, fractionated by electrophoresis on 1.0% agarose gels, and transferred to nylon membranes. A 1185 bp *Hin*dIII and *Bgl*II fragment of mouse MT-I cDNA (provided by Dr. R. D. Palmiter, University of Washington), a 746 bp *Pst*I fragment of human γ-GCS cDNA (a gift from Dr. R. Timothy Mulcahy, University of Wisconsin Comprehensive Cancer Center), a 900 bp *Hin*dIII and *Bgl*II fragment of rat catalase cDNA (a gift from Dr. Shuichi Furuta, Shinshu University School of Medicine), a 600 bp *Pst*I fragment of human Cu,Zn-SOD cDNA and a 800 bp *Eco*RI fragment of human Mn-SOD cDNA (American Type Culture Collection), and a 687 bp *Eco*RI fragment of rat GSHpx cDNA (provided by Dr. Ambati P. Reddy, Pennsylvania State University) were used as probes for MT-I, γ-GCS, catalase, Cu,Zn-SOD, Mn-SOD, and GSHpx, respectively. The probes were randomly labeled with [α-^{32}P]dCTP (DuPont/NEN Research Products) using the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories), and purified by chromatography (Sephadex G-50, Sigma) before hybridization. Hybridization and wash procedures were conducted using previously published methods [19]. Autoradiographic images were scanned and analyzed using the MCID system from Imaging Research Inc.

SOD

The enzyme activity was assayed as described by Nebot *et al.* [20]. This method is based on the fact that the rate of alkaline autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[*c*]fluorene (BXT-01050) is accelerated by SOD activity. This autoxidation yields a chromophore that is detected at 525 nm. An SOD-525 assay kit based on this method was used. Briefly, tissue samples were homogenized in 9 vol. of 0.25 M of sucrose solution. The homogenate was centrifuged at 1400 *g* for 5 min at 4°. A 250-μL aliquot of the supernatant was dialyzed against distilled water at 4° with 12,000 MW exclusion membrane for 24 hr. Then the sample was mixed with a mercaptan scavenger, 1,4,6-trimethyl-2-vinylpyridinium, for 1 min to eliminate major mercaptan interferences. Total SOD (Cu,Zn-SOD and

Mn-SOD) activity was determined colorimetrically immediately following addition of the chromogenic reagent BXT-01050. One SOD-525 activity unit is defined as the activity that doubles the BXT-01050 autoxidation background [20]. The enzyme activity is expressed as units per gram wet tissue.

Catalase

The heart tissue was homogenized in 1.0% Triton X-100 using a variable speed tissue tearer (Biospec Products, Inc.) at high speed for 10 sec/cycle for 3 cycles on ice. The homogenates were centrifuged at 6000 *g* at 4° for 20 min. The supernatant was diluted with 1.5 vol. of the assay buffer (50 mM of KH_2PO_4/50 mM of NaH_2PO_4, pH 7.0). The enzyme activity was determined by the method described by Aebi [21]. Briefly, in a cuvette 2.0 mL of sample was added. The reaction was initiated by adding 1.0 mL of 30 mM of H_2O_2, and the change in absorbance at 240 nm was monitored at 25° for 1.0 min. A portion of the remaining sample was used for protein determination. Specific activity is expressed as micromoles of H_2O_2 per minute per milligram of protein. Protein was determined by the method of Smith *et al.* [22], using bovine serum albumin as the standard.

GSHpx

Tissue samples were prepared as described above with the exception of the assay buffer (0.1 M of KH_2PO_4/1 mM of EDTA, pH 7.0). The enzyme activity was determined by the method described by Flohé and Gunzler [23]. In a reaction tube, 500 μL buffer containing 2 mM of sodium azide, 100 μL of GSH (10 mM), 100 μL of GSH reductase (2.4 U/mL), and 100-μL sample were incubated for 10 min in a 37° water bath; then 100 μL of NADPH (1.5 mM) was added and the reaction mixture was transferred to a cuvette. Absorbance at 340 nm was monitored at 37° for 3 min before 100 μL of prewarmed H_2O_2 (1.5 mM) was added, followed by an additional 5-min monitoring under the same conditions. Enzyme activity was calculated as described [23]. Specific activity is expressed as nanomoles of NADPH oxidized per minute per milligram of protein.

GSH Reductase (GR)

The enzyme activity was determined by the method described by Carlberg and Mannervik [24]. The tissue preparations were the same as described above for GSHpx. The assay buffer was 0.2 M of KH_2PO_4 (pH 7.0) containing 2 mM of EDTA. In a cuvette, 0.5 mL of buffer, 200 μL of ddH_2O, 50 μL of GSSG (20 mM), and 50 μL of NADPH (2 mM) were combined. Following addition of 200-μL sample, the change in absorbance at 340 nm was monitored at 37° for 2 min. The enzyme activity was calculated as described [24]. Specific activity is expressed as nanomoles NADPH oxidized per minute per milligram of protein.

γ-GCS

Tissue samples were homogenized in 0.2 M of Tris–HCl buffer (1:5), pH 8.0. The homogenates were centrifuged at 10,000 *g* for 50 min at 4°. The enzyme activity was determined by the assay described by Seelig and Meister [25]. The 1.0-mL reaction mixture contained 0.1 M of Tris–HCl buffer, 150 mM of KCl, 5 mM of Na_2-ATP, 2 mM of phosphoenolpyruvate, 10 mM of L-glutamate, 10 mM of L-α-aminobutyrate, 20 mM of $MgCl_2$, 2 mM of Na_2-EDTA, 0.2 mM of NADH, 17 μg of pyruvate kinase, and 17 μg of lactate dehydrogenase. The reaction was initiated by the addition of 50 μL of tissue homogenate to the reaction mixture, and the decrease in absorbance at 340 nm was followed. A sample blank contained all reagents except the tissue sample for which 50 μL of buffer was substituted. Another control assay was also done by omission of L-α-aminobutyrate in the presence of tissue sample. Specific enzyme activity was expressed as nanomoles of NADH oxidized to NAD per minute per milligram of protein.

Glutathione (GSH and GSSG)

Tissues were homogenized in 10 vol. of 5% (w/v) 5-sulfosalicylic acid at 4°. The homogenate was centrifuged at 10,000 *g* for 15 min, and the supernatant was assayed for GSH by the DTNB-glutathione reductase recycling assay [26]. The 1.0-mL reaction mixture contained 190 μL of stock buffer (143 mM of sodium phosphate and 6.3 mM of Na_4-EDTA, pH 7.5), 700 μL of 0.248 mg NADPH/mL in stock buffer, 100 μL of 6 mM of DTNB and 10-μL sample. The assay was initiated by addition of 10 μL of 266 U glutathione reductase/mL. GSH was used as the standard, and was assayed in parallel under the same conditions as the tissue samples.

MT

Total tissue MT concentrations were determined by the cadmium-hemoglobin affinity assay [27]. Briefly, tissues were homogenized in 4 vol. of 10 mM of Tris–HCl buffer, pH 7.4, at 4°. Following centrifugation of the homogenate at 10,000 *g* for 15 min, 200 μL of supernatant was transferred to microtubes for MT analysis, as described previously [27]. The concentration of MT in the heart is expressed as micrograms per gram of tissue.

Statistical Analysis

Data are expressed as means ± SD, and analyzed by a one-way ANOVA followed by the Scheffe's F-test. The level of significance was set at $P < 0.05$.

RESULTS

The effects of ADR on antioxidant enzyme activities and concentrations of the non-enzymatic antioxidants GSH

TABLE 1. ADR-induced alterations of antioxidants in the heart

	Control	ADR-induced
Cu,Zn-SOD (U/mg protein)	56.03 ± 4.55	53.81 ± 5.58
Mn-SOD (U/mg protein)	10.31 ± 1.52	9.24 ± 1.42
Catalase (μmol H_2O_2/mg protein · min)	27.66 ± 1.63	42.45 ± 2.24*
GSHpx (nmol NADPH/mg protein · min)	24.19 ± 3.66	21.80 ± 2.49
γ-GCS (μmol NADH/mg protein · min)	0.11 ± 0.05	0.21 ± 0.02*
GSH (μmol/g heart)	0.82 ± 0.21	0.58 ± 0.18
MT (μg/g heart)	5.42 ± 0.81	20.14 ± 3.09*

Values are means ± SD for 5–8 mice.
*Significant difference between control and ADR-treated groups ($P < 0.05$).

and MT are presented in Table 1. Among these changes, catalase was increased significantly ($P < 0.05$) in the ADR-treated heart. The activities of GSHpx (Table 1) and GR (data not shown) were not altered in the ADR-treated heart. Interestingly, ADR significantly ($P < 0.05$) increased the activity of γ-GCS, the enzyme that catalyzes the rate-limiting step in *de novo* GSH synthesis. The GSH concentration in the ADR-treated heart, however, was decreased, although this decrease was only marginally statistically significant. As expected, the concentration of MT was increased significantly in the ADR-treated heart ($P < 0.05$).

The effects of ADR on the abundances of mRNAs for the antioxidants were determined as shown in Fig. 1. The RNA samples were isolated from the whole heart. ADR significantly ($P < 0.05$) elevated the levels of mRNAs for all of the antioxidants, with the exception of Mn-SOD. Analysis of autoradiographic images indicated that ADR increased the levels of mRNAs for catalase about eight-fold, Cu,Zn-SOD about four-fold, GSHpx more than two-fold, γ-GCS about three-fold, and MT-I about 25-fold, as shown in Fig. 2.

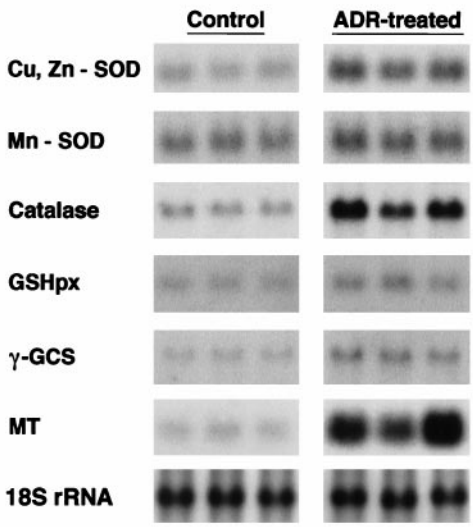

FIG. 1. Northern blot analysis of mRNA from the hearts of mice treated with or without 15 mg/kg of ADR for 4 days. Total heart RNA was isolated as described in Materials and Methods, and 10 μg of total RNA from triplicate samples was fractionated by 1% agarose gel, and transferred to nylon membrane. Blots were hybridized with Cu,Zn-SOD, Mn-SOD, catalase, GSHpx, γ-GCS, and MT-I probes as described in Materials and Methods. Ethidium bromide staining of 18S rRNA was examined to ensure equal loading. A representative ethidium bromide staining of the 18S rRNA was shown on the lower panel; others were the same and are not shown.

DISCUSSION

The data presented here demonstrate that ADR increased the mRNA levels for all the antioxidants examined, with the exception of Mn-SOD. Cellular concentrations of RNA are determined by transcription activity and RNA stability. Since ADR does not seem to alter the turnover rate of RNA [28], the increase in mRNA levels of antioxidants most likely results from a transcriptional activation. In contrast to the increased gene expression of antioxidant systems, ADR inhibits overall RNA synthesis [15, 29, 30]. Particularly, cardiac muscle gene and nonmuscle gene expression were depressed by ADR, both *in vivo* and *in vitro* [31–33]. It seems that the myocardium tissue offsets the oxygen free

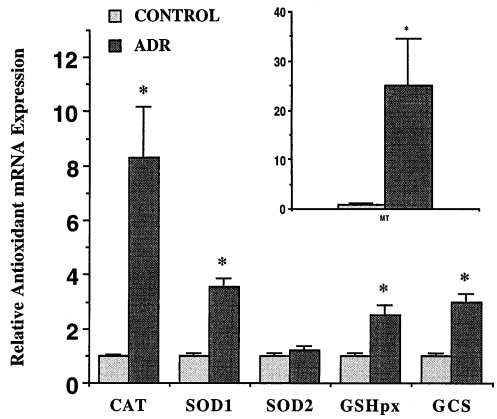

FIG. 2. Effect of ADR on the abundance of mRNAs for antioxidants, as shown in Fig. 1. Results of the northern blots were quantitated by an autoradiographic image analysis system. The elevated levels of mRNAs for the antioxidants by ADR were expressed relative to the levels detected in the controls. Values are means ± SD of triplicate measurements. Control values are arbitrarily designated as 1. SOD1 = Cu,Zn-SOD, and SOD2 = Mn-SOD. The insert shows the MT result. *Significantly different from the controls ($P < 0.05$).

radical generation by selectively increasing mRNA transcripts encoding antioxidants, although a nonspecific inhibitory effect on gene expression is imposed by ADR.

The present results also clearly demonstrated that the previously observed depression of antioxidant activities should not result from the global inhibitory effect of ADR on gene expression. For instance, previous studies have shown that cardiac GSHpx was depressed constantly by ADR (15 mg/kg) between day 1 and day 5 after a single i.p. injection in mice [15]. Other studies have shown that the same dose of ADR depressed the cardiac GSHpx activity during the first 2 days after the treatment and the enzyme activity returned to control level by day 4 in mice [5]. However, on day 4, severe inhibitory effects of ADR on DNA, RNA, and protein syntheses in mouse heart have been observed [15]. In the present study, the cardiac GSHpx activity on day 4 after ADR treatment was not changed significantly, although it was slightly decreased consistently (repeated by 3 experiments). The concentration of cardiac GSHpx mRNA, however, was elevated significantly. Therefore, the depressed GSHpx activity unlikely results from the inhibitory effect of ADR on gene expression, but the restoration is likely to be the result of the activation of GSHpx gene transcription. The same explanation would apply to the Cu,Zn-SOD.

How were the genes of these antioxidant systems selectively activated? Studies on gene regulation of MT have demonstrated that the proximal upstream region of the MT gene contains an antioxidative responsive element and a metal responsive element, both of which confer the response of MT to reactive oxygen species [34], and its induction occurs mainly at the transcriptional level [35, 36]. However, such effects of reactive oxygen species on gene expression of other antioxidant systems have not been demonstrated in mammalian systems.

ADR produces reactive oxygen species, leading to oxidative injury to the heart [37]. Among the most important antioxidant defense mechanisms is GSH. Several studies have demonstrated the role of GSH in cardiac protection against ADR toxicity [38–40]. For example, supplement of exogenous glutathione by i.p. injection at 500 mg/kg to mice provided significant protection against ADR cardiotoxicity [38]. Depletion of cardiac GSH by diethyl maleate sensitizes the heart to ADR toxicity [39]. Decreased concentrations of cardiac GSH by ADR were observed previously [38–40] and in the present study, although the depression of cardiac GSH by ADR in this study was only marginally statistically significant. In contrast, the mRNA abundance and the activity of γ-GCS were increased significantly. This phenomenon may be explained by increased consumption of GSH in the enzymatic and nonenzymatic detoxification of reactive oxygen species generated by ADR, which would, in turn, require enhanced gene expression of γ-GCS to increase the synthesis of GSH for maintaining a stable cellular level under the stress of ADR. Other studies have shown that GSH levels in red blood cells and myocardium were transiently reduced in mice following ADR treatment (15 mg/kg) and returned to control values in 24 hr [40]. The transient decrease of GSH could be a result of its consumption and the enhanced γ-GCS gene expression, and, thereby, the increased enzyme activity would restore the cellular GSH level.

Consistent with the increased levels of mRNAs for catalase, the enzyme activity was increased significantly in the hearts of ADR-treated mice. Previous studies have also shown that the increase in catalase activity occurs from 4 to 6 days after ADR treatment (10 mg/kg, i.v.) in mice [41, 42], which is in agreement with the result of the present study.

MT is a ubiquitous, low-molecular-weight metal-binding protein [43]. Recently, it has been received much attention for its diverse functions including detoxification of free radicals [43, 44]. Chemicals including ADR that induce oxidative stress increase MT production in most of the organs of mice [43, 45]. Our findings showed that ADR at a dose of 15 mg/kg markedly increased both MT mRNA levels and protein content in the heart, suggesting that MT may be a critical determinant of ADR cardiotoxicity. Several studies [46, 47] have shown that pre-induction of MT has a protective effect against ADR cardiotoxicity. A recent study using cardiac specific MT overexpressing transgenic mice has demonstrated convincingly the role of MT in cardiac protection against ADR toxicity [48].

In summary, our findings showed that ADR selectively induced cardiac mRNA transcripts for Cu,Zn-SOD, catalase, GSHpx, and γ-GCS, likely through up-regulation of gene transcription. However, only catalase and γ-GCS activities were increased. Activities of other antioxidants either did not change or were depressed, suggesting that enhanced production of the transcripts may reflect a compensatory response to the consumption of the antioxidants, such as GSH and GSHpx, in detoxification of ADR. The fact that both MT mRNA and protein were elevated markedly suggests that MT may play an important role in cardiac protective responses to ADR.

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