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Nitric Oxide Synthase Expressions in ADR-induced Cardiomyopathy in Rats

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In this study, we investigate Nitric oxide synthase (NOS) expressions in adriamycin (ADR)-induced cadiomyopathy in rats. Sixty male Wistar rats were randomly divided into two main groups: control and ADR groups. Myocardial histopathological observation was performed; Expressions of 3 isoforms of NOS genes were examined by RT-PCR analysis; Expressions of 3 isoforms of NOS protein was assessed by Western blot analysis. Myocardium exhibited intensive morphological changes after 8 weeks of ADR treatment. The expression levels of inducible NOS (iNOS) gene and protein were significantly increased in ADR-treated rats after 8 weeks of treatment and then slightly increased at weeks 9 and 10. No significantly difference of neuronal NOS (nNOS) or endothelial NOS (eNOS) gene and protein were observed in the myocardium obtained from the control rats and ADR-injected rats at any time point. iNOS gene expression is selectively induced by ADR in heart. The upregulation of iNOS gene and protein may be somehow correlated with morphological changes seen in heart of rat treated with ADR.

Keywords: Adriamycin, Cardiotoxicity, Nitric oxide synthase

Introduction

The anthracycline antibiotic adriamycin (ADR) is one of the most effective and useful antineoplastic agents for the treatment of various types of cancer (Morabito *et al.,* 2004; Novitzky *et al.,* 2004). Long-term administration of ADR causes a cumulative dose-dependent cardiomyopathy (Nakamura *et al.,* 2000). The mechanism of ADR-induced cardiotoxicity is attributed to the formation of free reactive oxygen radicals, lipid peroxidation, and subsequent changes of membrane fluidity and integrity (Zhou*et al.,* 2001). However, the molecular basis and sequence of the changes in myocardial antioxidant enzyme due to adriamycin remain to be understood.

Nitric oxide (NO) is well known as an important mediator of many physiologic functions, and its role in the pathogenesis of cardiovascular disease is gaining recognition. NO is synthesized by nitric oxide synthase (NOS). In mammals, 3 isoforms of NOS have been identified: inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS). All isoforms have been detected in cardiovascular tissues because neuronal and endothelial cells, as well as monocytes and macrophages, are represented in this organ. NOS expression and its modulatory role on cardiac function have been extensively studied under physiological conditions. However, there was no report about expression of 3 isoforms of NOS genes in ADR-induced cardiomyopathy.

In this study, we evaluated the myocardial effects of ADR by light microscopy and then correlated these data with possible effects of ADR on the expression of 3 isoforms of NOS genes. This study could enable us to illustrate the mechanisms of ADR-induced cardiomyopathy seen in cancer patients undergoing ADR treatment.

Materials and Methods

Animals. Adult male Wistar rats weighing 150-170 g (total $n = 60$) were purchased from the Central Animal Laboratory, the second affiliated hospital of Harbin Medical University, China. Wistar rats were randomly divided into two main groups. One group was injected with physiological saline i.p. once a week and served as control animals. The other group was administered intraperitoneally once per week, with 1 mg/kg of ADR (Wanle Co Ltd Shenzhen) for 6, 8, 9, 10, and 12 weeks. These experimental conditions were similar to those observed in patients undergoing treatment with

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ADR for several months. At 24 hours following the last injection, animals were killed by exsanguination and hearts were removed immediately.

Histological assessment of myocardial damage. The heart was sliced transversally, and a midventricular slice was fixed in 10% formalin for 24 hours, embedded in paraffin, and cut into 3-mm sections for histological assessment. Paraffin sections were stained with hematoxylin and eosin.

Reverse transcription-polymerase chain reaction analysis. Total RNA was extracted from fresh-frozen myocardium using the Trizol Reagent (USA Invitrogen). cDNA was synthesized according to Reverse Transcription kit manufacturer's instructions (Promega Corp), and then cDNA was amplified with a Multiplex polymerase chain reaction (PCR) kit (TaKaRa Corp) with the following primers: β-actin: sense: 5' GCCCCTGAGGAGCACCCTGT 3'; antisense: 5' ACGCTCGGTCAGGATCTTCA 3' (300 bp products); iNOS sense: 5' cccttccgaagtttctggcagcagc 3'; antisense: 5' gggtgtcagagtcttgtgcctttgg 3' (497 bp products); eNOS sense: 5' gggctccctccttccggctgcacc 3'; antisense: 5' ggatccctggaaaaggcggtgagg 3' (259 bp products); nNOS: sense: 5' ctgtgacaactctcgatacaacatc 3'; antisense: 5' gagtctatagttgagc atctcctgg 3' (307 bp products). Cycling parameters were as follows: 30 seconds for annealing at 60°C, PCR amplification for 30 cycles. 8 μl of the PCR products was analyzed by electrophoresis on a 1.5% agarose gel. Further semiquantity of PCR product was determined by Kodak 2.0 software.

Western blotting analysis. Proteins were extracted from fresh-frozen left ventricle myocardium. Heart tissues were homogenized and then lysed in a lysis buffer (0.5% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and 5 mM aprotinin) for 1 hour at 4°C. Protein extract (100 ng per lane) was run on a 10% SDS-PAGE gel, and then transferred to a nitrocellulose membrane (Shanghai Huashun Corp). The membrane was incubated with polyclonal rabbit anti-rat iNOS, eNOS or nNOS antibody (Santa Cruz Biotechnology, diluted at 1 : 1000), and visualized by the ProtoBlot®II AP system (Promega Corp).

Statistical analysis. The data were analyzed using the program SPSS 11.5 for Window. Quantitative data were presented as mean ± SD. For comparison between multiple groups, data was analyzed by ANOVA, and with the Student-Newman-Keuls post hoc analysis. Values of $p < 0.05$ were considered significant.

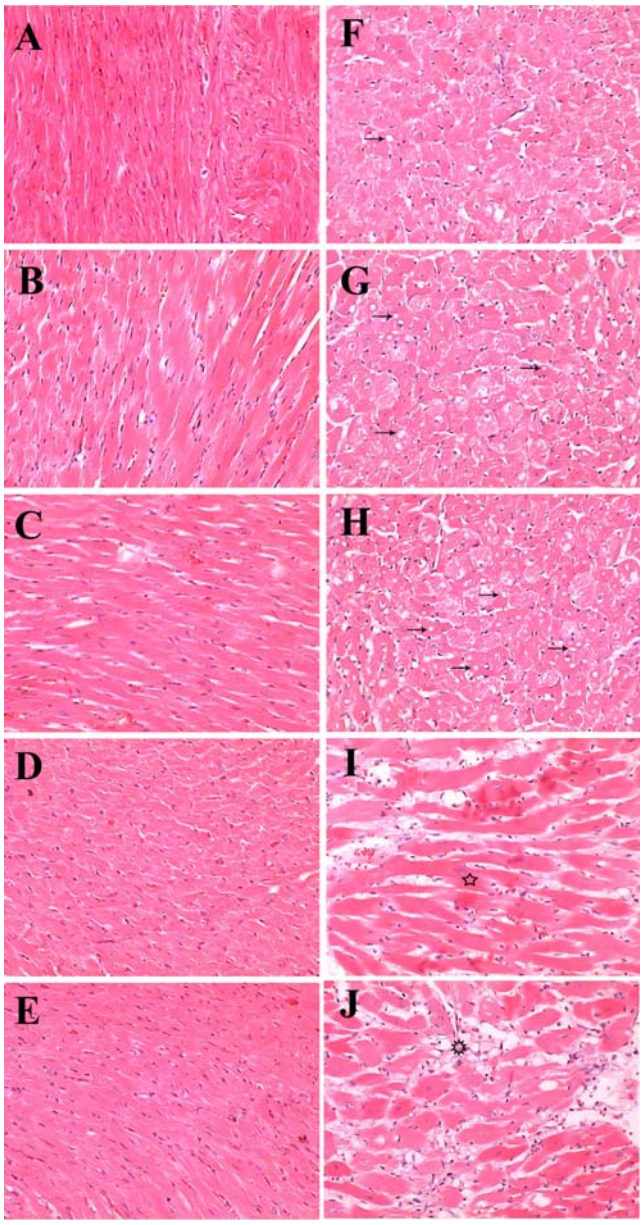


Fig. 1. Microphotographs of myocardium (magnified by 20 times). A~E: Control group (at 6 to 12 w); F~J: ADR group (at 6 to 12 w). Vacuolization (→); myocardial hypertrophy (☆); interstitial fibrosis (○).

Results

Morphological changes in heart. Heart samples removed from rats were examined by light microscopy. Myocardium of saline-injected rats had normal morphology (Fig. 1A~E). There was no histopathological difference between the ADR-treated group and the control group on 6th weeks after the first administration of ADR or saline (Fig. 1F). Myocardium exhibited intensive morphological changes after 8 weeks of ADR treatment. These changes included vacuolization and myofibrillar loss (Fig. 1G). By the 9th to 10th week of ADR treatment, the histological degenerative changes rapidly increased with compensatory myocardial hypertrophy and interstitial fibrosis (Fig. 1H~J).

Expression of RNA of NOS genes in ADR-treated myocardium. To correlate the morphological changes in myocardium in ADR-injected animals with NOS isoforms, we performed RT-PCR analysis with four primers corresponding to three nitric oxide synthase genes (iNOS, eNOS and nNOS) and one housekeeping gene β-actin. The RT-PCR-amplified products were showed in Fig. 2. These data were quantified

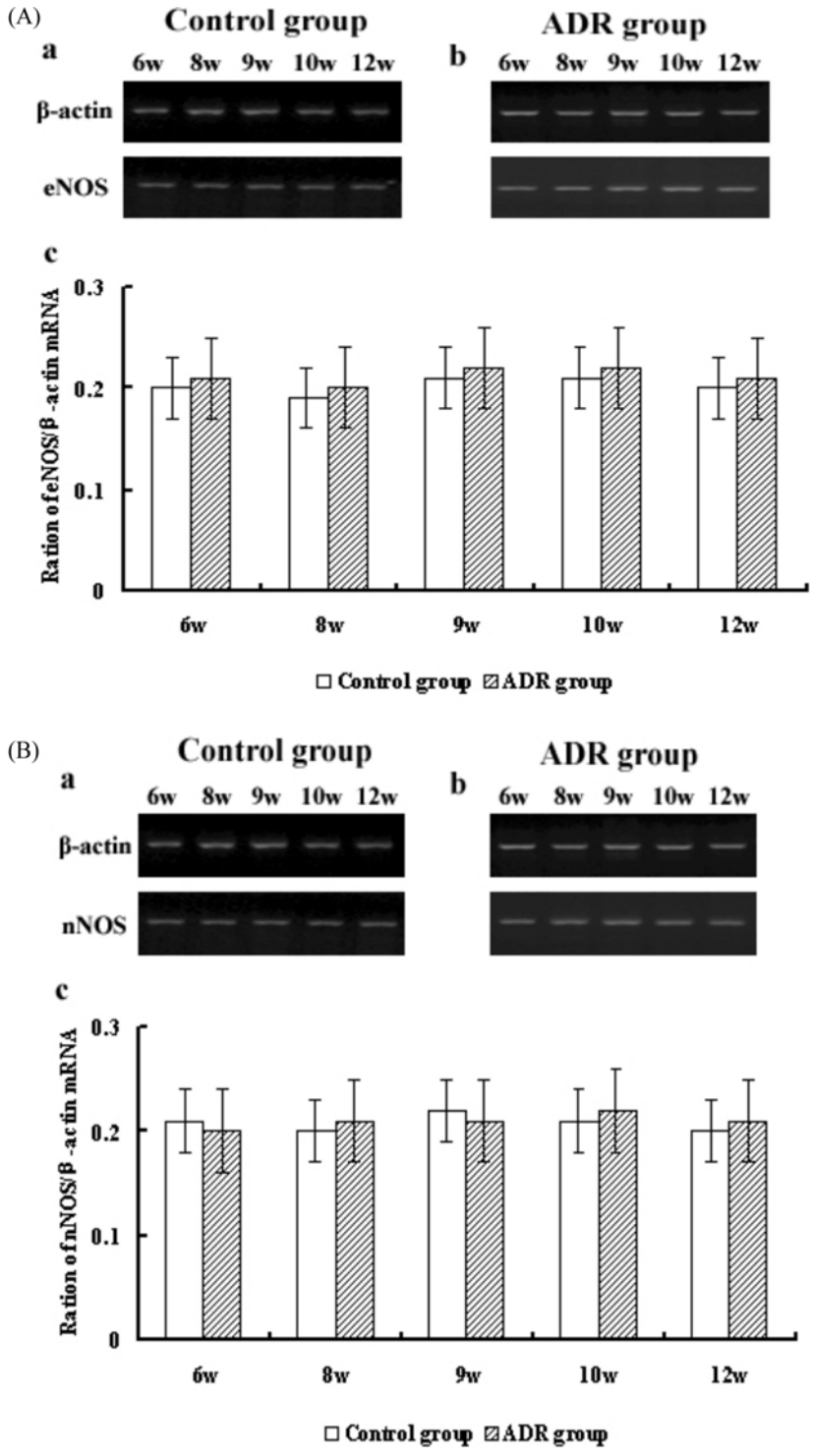


Fig. 2. Expression of eNOS (A), nNOS (B), and iNOS (C) mRNAs in control groups and ADR-induced cardiomyopathy (at 6 to 12 w). a,b, myocardial expressions of NOS and β-actin mRNAs were measured by RT-PCR analysis. c, densitometric analysis of NOS mRNA. Values are the ratio of NOS to β-actin mRNA. Data in the c are means ± SD from 6 hearts in independent experiments. $\Delta p < 0.05$ vs. control.

by densitometry and normalized to β-actin (Fig. 2). No significantly difference of eNOS or nNOS mRNAs were observed in the myocardium obtained from the control rats and ADR-injected rats at any time point (Fig. 2A,B). The expression level of iNOS mRNA was significantly increased in ADR-treated rats after 8 weeks of treatment and then

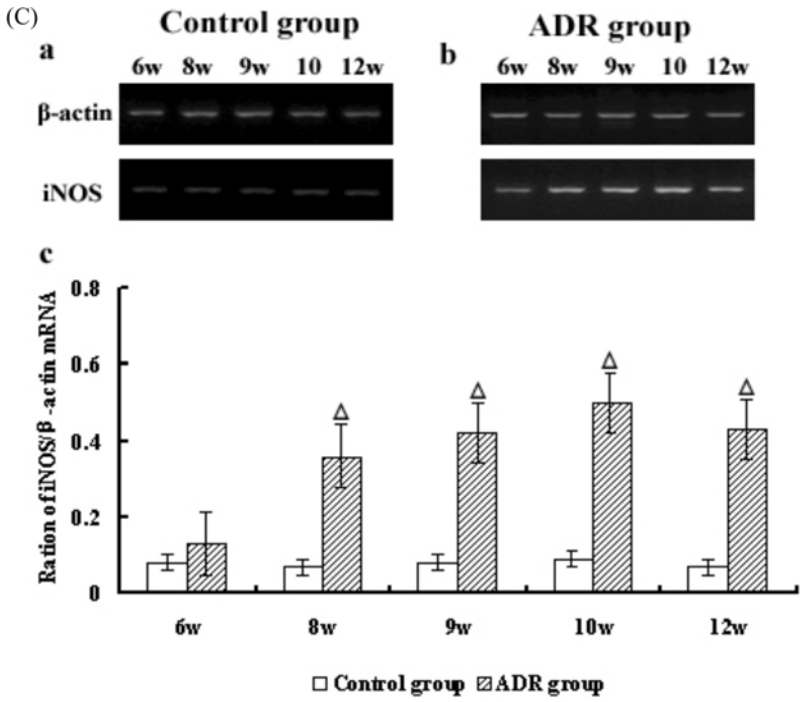


Fig. 2. Continued.

slightly increased at weeks 9 and 10. Although the quantity of iNOS mRNA seemed to be decreased slightly at 12th weeks, but band optical density remained higher than control levels (Fig. 2C). This increase in iNOS mRNA was rather specific, since no significantly difference of nNOS or eNOS mRNAs were observed in the myocardium obtained from the control rats and ADR-injected rats at any time point. This suggests that iNOS gene expression is selectively induced by ADR after 8 weeks of treatment in heart.

Expression of NOS isoforms Antigen. No significantly difference of eNOS or nNOS protein was observed in the myocardium obtained from the control rats and ADR-injected rats at any time point (Fig. 3A,B). Until 8 weeks after the first administration, no difference was detected by Western blot in the expression of iNOS protein between the control and the ADR-injected groups (Fig. 3C). However, the densitometry of the iNOS-immunoreactive band, with a molecular weight of 130 kDa, increased significantly in ADR-injected rats after 8 weeks of treatment compared with control groups. At weeks 9 and 10, iNOS antigen remained higher in the ADR-injected hearts. At 12th weeks, the iNOS quantity of antigen decreased slightly, but band optical density remained higher than control levels (Fig. 3C).

Disscussion

The present study concerns the expression patterns of NOS mRNAs in ADR-induced cardiomyopathy. From the morphological analysis, we saw gradual myocardial degeneration in rats treated with ADR for 8-12 weeks. Our study provides evidence of upregulation of iNOS expression in ADR-induced cardiomyopathy. The upregulation of iNOS may be somehow correlated with morphological alterations in myocardium in ADR-treated rats.

ADR is one of the most effective and useful antineoplastic agents for the treatment of various types of cancer (Morabito *et al.,* 2004; Novitzky *et al.,* 2004). However, ADR-induced cardiomyopathy has long been a serious side effect in treating human cancers. Despite of various mechanisms ascribed to the DOX-induced cardiotoxicity (Nakamura *et al.,* 2000; Zhou*et al.,* 2001; Koutinos *et al.,* 2002; Dudnakova *et al.,* 2003), the precise mechanism(s) underlying the cardiovascular effects of ADR are not known. In present study, ADR-induced cardiomypathy model in rats showed histological changes similar to those in human ADR-cardiomyopathy: vacuolization and loss of myocytes, compensatory hypertrophy of residual myocytes and interstitial fibrosis. The most pronounced effect of ADR exposure is cumulative dose-dependent morphological alterations. This gradual degenerative change seen in the heart of rat treated with ADR for 8-10 weeks is in agreement with other studies (RVitelli *et al.,* 2002).

To demonstrate whether the morphological changes seen in heart of rat treated with ADR are somehow correlated with the effect of ADR on the biosynthesis of NOS, we detected 3 isoforms of NOS expressions.

Normally, eNOS and nNOS are constitutively expressed in cardiac myocytes. Under baseline conditions, the modulatory effects of eNOS and nNOS on myocardial contractility and

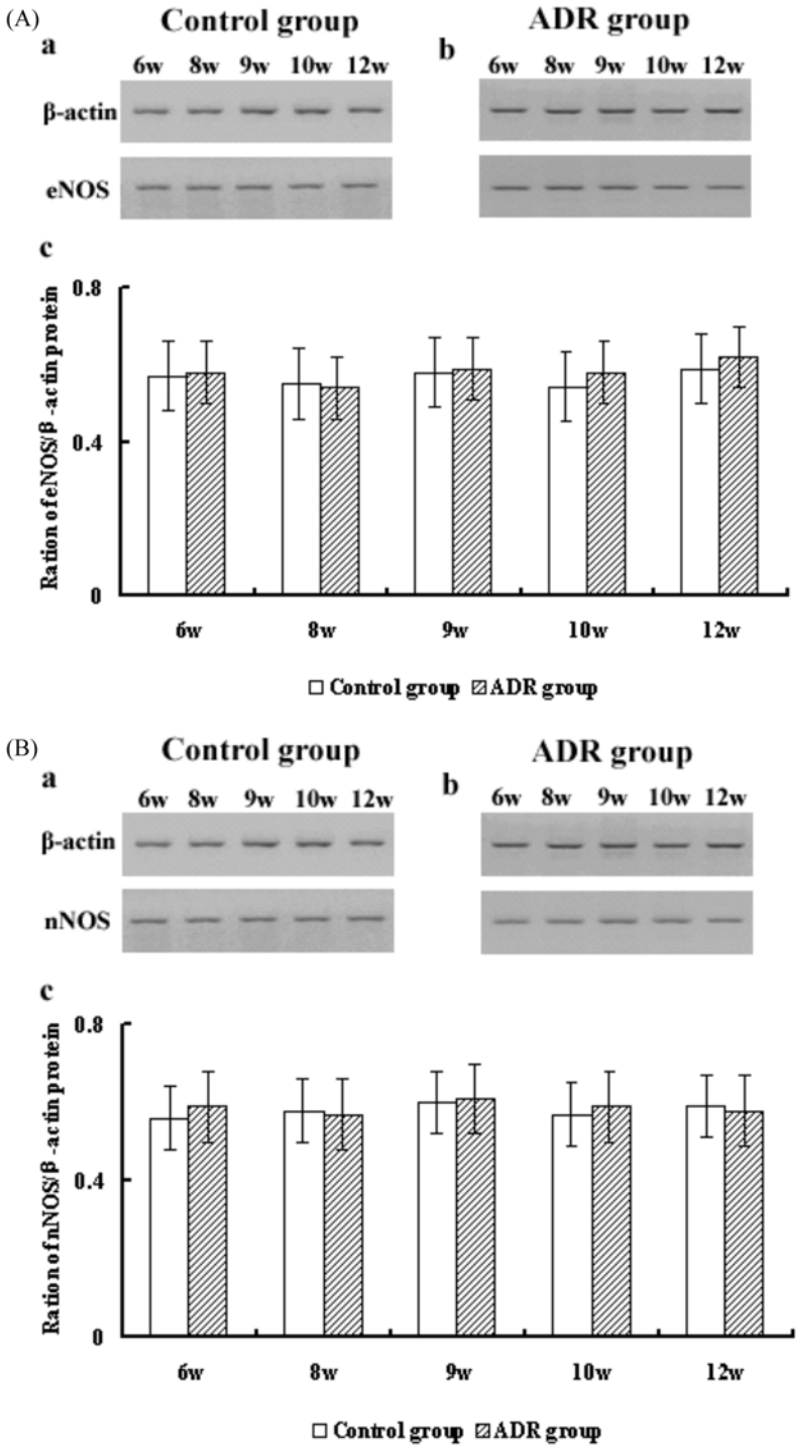
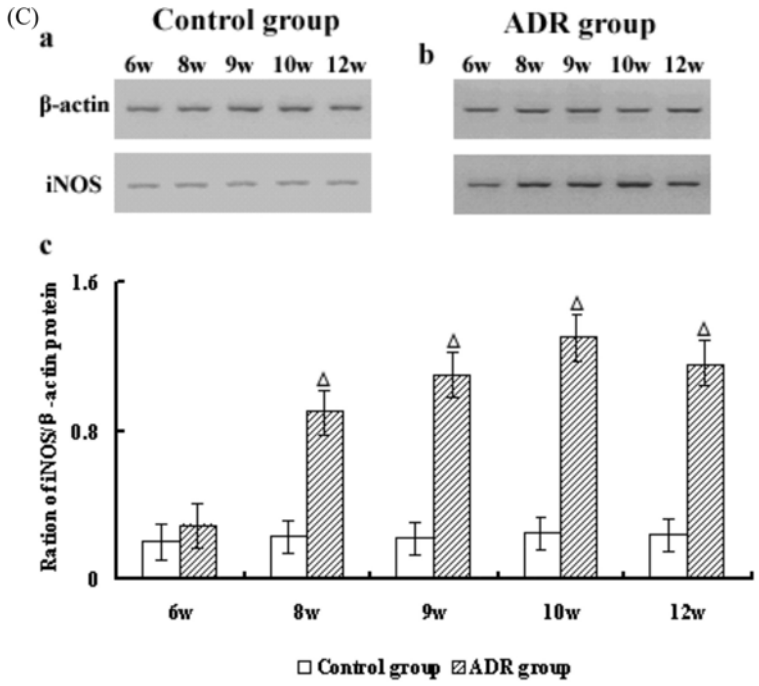


Fig. 3. Expression of eNOS (A), nNOS (B), and iNOS (C) proteins in control groups and ADR-induced cardiomyopathy (at 6 to 12 w). a,b, myocardial NOS proteins were measured by Western blot analysis with NOS antibody. c, densitometric analysis of NOS proteins. Values are the ratio of NOS to β-actin. Data in the c are means ± SD from 6 hearts in independent experiments. $\Delta p < 0.05$ vs. control.

relaxation or heart rate have been recently confirmed by several studies (Lefkothea *et al.,* 1999; Massion *et al.,* 2003). Some researches suggested that nNOS is up-regulated and translocated from the sarcoplasmic reticulum to the sarcolemma in post-infarction heart failure (Damy *et al.,* 2003; Damy *et al.,* 2004). Under various pathophysiological conditions, eNOS expression has different alteration. In the acute phase of myocardial infarction, cardiac eNOS expression remains



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unchanged (Prabhu *et al.,* 2000) or is transiently increased (Horinaka *et al.,* 2004); however, in the chronic phase after myocardial infarction (more than 4 weeks) cardiac eNOS are consistently decreased (Frutos *et al.,* 2001). We detected no significantly different expression of eNOS and nNOS gene and protein between control and ADR groups at any time point. Although we have no clue as to the reason for this discrepancy, we surmise that it may be due to the different animals and the nature of stimuli.

Studies have shown that iNOS in cardiomyocytes is only induced in stimulated or pathophysiological conditions (Takimoto *et al.,* 2002). iNOS produces high amounts of cytosolic NO, responsible for the pro-inflammatory and defensive effects of NO as part of the innate immunity. In present study, we observed significantly high levels of iNOS gene and protein expression in ADR-treated rats after 8 weeks of treatment before morphological degeneration had begun. Meanwhile, a prolonged up-regulation of iNOS gene and protein expression took place at 9th, 10th and 12th weeks. High concentrations of NO produced by iNOS might result in damage to myocardium in ADR-injected rats. However, other research showed that iNOS might be beneficial in failing hearts, potentially through a reduction of myocardial oxygen consumption, or an increase of angiogenesis (Heger *et al.,* 2002).

In a word, our study provides evidence of upregulation of iNOS gene and protein expressions in ADR-induced cardiomyopathy. The upregulation of iNOS gene and protein may be somehow correlated with morphological changes seen in heart of rat treated with ADR.

References

Damy, T., Ratajczak, P., Shah, A. M., Camors, E., Marty, I., Hasenfuss, G. (2004) Increased neuronal nitric oxide synthase-derived NO production in the failing human heart. *Lancet.* **363**, 1365-1367.

Damy, T., Ratajczak, P. and Robidel, E. (2003) Up-regulation of cardiac nitric oxide synthase 1 derived nitric oxide after myocardial infarction in senescent rats. *FASEB J.* **17**, 1934-1936.

Dudnakova, T. V., Lakomkin, V. L., Tsyplenkova, V. G., Shekhonin, B. V., Shirinsky, V. P. and Kapelko, V. I. (2003) Alterations in myocardial cytoskeletal and regulatory protein expression following a single doxorubicin injection. *J. Cardiovasc. Pharmacol.* **41**, 788-794.

Frutos, D. E., Sanchez, D. M., Farre, J., Gomez, J., Romero, J. and Marcos-Alberca, P. (2001) Expression of an endothelial-type nitric oxide synthase isoform in human neutrophils: modification by tumor necrosis factor-alpha and during acute myocardial infarction. *J. Am. Coll. Cardiol.* **37**, 800-807.

Heger, J., Godecke, A. and Flogel, U. (2002) Cardiac-specific overexpression of inducible nitric oxide synthase dose not result in severe cardiac dysfunction. *Circulation Res.* **90**, 93-99

Horinaka, S., Kobayashi, N., Yabe, A., Asakawa, H., Yagi, H. and Mori, Y. (2004) Nicorandil protects against lethal ischemic ventricular arrhythmias and up-regulates endothelial nitric oxide synthase expression and sulfonylurea receptor 2 mRNA in conscious rats with acute myocardial infarction. *Cardiovasc. Drugs Ther.* **18** 13- 22.

Koutinos, G., Stathopoulos, G. P., Dontas, I., Perrea-Kotsarelis, D., Couris, E., Karayannacos, P. E. and Deliconstantinos, G. (2002) The effect of doxorubicin and its analogue mitoxantrone

on cardiac muscle and on serum lipids: an experimental study. *Anticancer Res.* **22**, 815-820.

Lefkothea, C., Theophilidis, P. G., Thomopoulos, G. N. and Tsiftsoglou, A. S. (1999) Structural and Functional Impairment of Mitochondria in Adriamycin-induced cardiomyopathy in mice: Suppression of cytochrome *c* oxidase II gene expression. *Biochem. Pharmacol.* **57**, 481-489.

Massion, P. B. and Balligand, J. L. (2003) Modulation of cardiac contraction, relaxation and rate by the endothelial nitric oxide synthase (eNOS): lessons from genetically modified mice. *J. Physiol.* **546**, 63-75.

Massion, P. B., Dessy, C., Desjardins, F., Pelat, M., Havaux, X. and Belge, C. (2004) Cardiomyocyte-restricted overexpression of endothelial nitric oxide synthase (NOS3) attenuates beta-adrenergic stimulation and reinforces vagal inhibition of cardiac contraction. *Circulation* **110**, 2666-2672.

Morabito, A., Gattuso, D., Stani, S. C., Fanelli, M., Ferrau, F., De Sio, L., Castellana, M. A., Lorusso, V., Priolo, D., Vitale, S., Sarmiento, R., Lo Vullo, S., Mariani, L. and Gasparini, G. (2004) Safety and activity of the combination of pegylated liposomal Doxorubicin and weekly docetaxel in advanced breast cancer. *Breast Cancer Res. Treat.* **86**, 249-257.

Nakamura T., Ueda, Y., Juan, Y., Katsuda, S., Takahashi, H. and Koh, E. (2000) Fas-mediated apoptosis in adriamycin-induced cardiomyopathy in rats in vivo study. *Circulation* **102**, 572-578.

Novitzky, N., Thomas, V., Abrahams, L., Toit, C. and Donald, M. (2004) Increasing dose intensity of anthracycline antibiotics improves outcome in patients with acute myelogenous leukemia. *Am. J. Hematol.* **76**, 319-329.

Prabhu, S. D., Chandrasekar, B, Murray, D. R. and Freeman, G. L. (2000) beta-adrenergic blockade in developing heart failure: effects on myocardial inflammatory cytokines, nitric oxide, and remodeling. *Circulation* **101**, 2103- 2109.

RVitelli, M., Filippelli, A., Rinaldi, B., Rossi, S., Palazzo, E., Rossi, F. and Berrino, L. (2002) Effects of docosahexaenoic acid on [Ca(2+)](i) increase induced by doxorubicin in ventricular rat cardiomyocytes. *Life Sci.* **71**, 1905-1916.

Takimoto, Y., Aoyama, T., Tanaka, K., Keyamura, R. and Yui, Y. (2002) Augmented expression of neuronal nitric oxide synthase in the atria parasympathetically decreases heart rate during acute myocardial infarction in rats. *Circulation* **105**, 490-496.

Zhou, S., Palmeira, C. M. and Wallace, K. B. (2001) Doxorubicin-induced persistent oxidative stress to cardiac myocytes. *Toxicol. Lett.* **121**, 151-157.