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Redox Regulation of MAPK Pathways and Cardiac Hypertrophy in Adult Rat Cardiac Myocyte

Koichi Tanaka, MD, PHD, Masaaki Honda, MD, PHD, Toshikazu Takabatake, MD, PHD
Shimane, Japan

OBJECTIVES We analyzed the regulatory function of reactive oxygen species (ROS) on the hypertrophic signaling in adult rat cardiac myocytes.

BACKGROUND The ROS regulate mitogenic signal transduction in various cell types. In neonatal rat cardiac myocyte, antioxidants have been shown to inhibit cardiac hypertrophy, and ROS are suggested to modulate the hypertrophic signaling. However, the conclusion may not reflect the situation of mature heart, because of the different natures between neonatal and adult cardiac myocytes.

METHODS Cultured adult rat cardiac myocytes were stimulated with endothelin-1 (ET-1) or phenylephrine (PE), and intracellular ROS levels, the activities of mitogen-activated protein kinases (MAPKs; ERK, p38, and JNK), and ^{3}H-phenylalanine incorporation were examined. We also examined the effects of antioxidant pretreatment of myocytes on MAPK activities and cardiac hypertrophy to analyze the modulatory function of redox state on MAPK-mediated hypertrophic signaling.

RESULTS The ROS levels in ET-1- or PE-stimulated myocytes were maximally increased at 5 min after stimulation. The origin of ROS appears to be from NADH/NADPH oxidase, because the increase in ROS was suppressed by pretreatment of myocytes with NADH/NADPH oxidase inhibitor diphenyleneiodonium. Extracellular signal-regulated kinase (ERK) activity was increased by the stimulation of ET-1 or PE. In contrast, p38 and *c*-Jun-N-terminal protein kinase (JNK) activities did not change after these stimulations. Antioxidant treatment of myocytes suppressed the increase in ROS and blocked ERK activation and the subsequent cardiac hypertrophy induced by these stimuli.

CONCLUSIONS These data demonstrate that ROS mediate signal transduction of cardiac hypertrophy induced by ET-1 or PE in adult rat cardiac myocytes. (J Am Coll Cardiol 2001;37:676–85)
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Oxygen free radicals or reactive oxygen species (ROS) have been implicated in the pathogenesis of a variety of diseases such as hypoxia-reperfusion injury and drug-induced cardiomyopathy. Recent studies revealed that oxidative stress is increased in patients with congestive heart failure (1) and in cardiac hypertrophy model following decompensation (2). In these situations, ROS have been generally considered to be toxic to the cells. However, recent studies have demonstrated that ROS play a role as second messengers to regulate mitogenic signal transduction in various cell types, such as smooth muscle cells, endothelial cells, and fibroblasts (3).

Cardiac hypertrophy is at least initially an adaptive reaction of the heart against cardiac overloading to maintain cardiac function. As mentioned above, it was reported that ROS are implicated in the transition of compensated hypertrophy to heart failure. In the late stage of cardiac hypertrophy, ROS appear to be toxic for myocardium and induce myocardial dysfunction or injury. Conversely, very few studies have evaluated the role of ROS in the early stage of cardiac hypertrophy. Of interest is a recent report suggesting that antioxidants inhibited neonatal rat cardiac myocyte hypertrophy (4). This suggests the possibility that ROS are involved in the hypertrophic signaling. However, the exact mechanism of how ROS are involved in the signaling pathways of cardiac hypertrophy remains to be elucidated. Furthermore, neonatal cardiac myocytes, which have been widely used for the experimental cardiac hypertrophy models, proved to be different from mature adult cardiac myocytes in several respects (5,6).

In this study we have evaluated the hypothesis that ROS might alter the activities of mitogen-activated protein kinase (MAPK) pathways and might modulate the subsequent development of cardiac hypertrophy in adult rat myocytes. To address this question, in the present study we examined ROS levels and MAPK activities in endothelin-1 (ET-1)- or phenylephrine (PE)-stimulated myocytes, and we analyzed the modulatory function of redox state on MAPK-mediated hypertrophic signaling pathways.

METHODS

Cardiac myocyte isolation and culture. Adult rat cardiac myocytes were isolated from 10-week-old Sprague-Dawley rats as described previously (7). Isolated myocytes were suspended in the M199 culture medium supplemented with 2 mg/ml bovine serum albumin, 2 mmol/liter carnitine, 5 mmol/liter creatine, 5 mmol/liter taurine, 100 IU/ml penicillin, and 100 μg/ml streptomycin, and were plated on

From the 4th Department of Internal Medicine, Shimane Medical University, Shimane, Japan. This study was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

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DCF = dichlorofluorescein
DPI = diphenyleneiodonium chloride
ERK = extracellular signal-regulated kinase
ET = endothelin
JNK = *c*-Jun-N-terminal protein kinase
LDH = lactate dehydrogenase
MAPK = mitogen-activated protein kinase
NAC = *N*-actylcysteine
PE = phenylephrine
ROS = reactive oxygen species

laminin-coated 35-mm dishes. The culture medium was exchanged for fresh medium to remove the damaged myocytes that failed to attach 2 h after plating. After this procedure, 80% to 90% myocytes were viable and showed rod-shape.

Neonatal rat cardiac myocytes were harvested from 2–3-day-old Sprague-Dawley rats by trypsin and collagenase digestion, purified by differential preplating, and cultured in D-MEM/F-12 culture medium supplemented with 2 mg/ml bovine serum albumin, 10 mg/liter insulin, 5.5 mg/liter transferrin, 6.7 mg/liter selenium, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

All experiments in this study were performed on the next day after plating.

Incorporation of ^{3}H-phenylalanine. For the evaluation of cardiac hypertrophy, ^{3}H-phenylalanine (Amersham Chemical, Arlington Heights, Illinois) incorporation into myocytes was measured. ^{3}H-phenylalanine (10 μCi) was added to each dish. After 12 h incubation, myocytes were recovered, treated with TCA, and ^{3}H-phenylalanine incorporation into myocytes was measured by liquid scintillation counter. Where indicated, PE (Sigma Chemical, St. Louis, Missouri) or ET-1 (Sigma Chemical) was added simultaneously with or without the treatment of 2 mmol/liter *N*-acetylcysteine (NAC; Sigma Chemical).

Measurements of reactive oxygen species (ROS). After the stimulation of myocytes with ET-1 or PE, intracellular ROS levels were measured using dichlorofluroescein (DCF) as described previously with some modifications (8). Briefly, after these stimulations, culture medium was exchanged for Krebs-Ringer bicarbonate buffer (KRB; composition in mmol/liter: NaCl, 110; KCl, 2.6; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; pyruvate, 5; taurin, 30; HEPES, 20; and glucose, 11, pH 7.4). The DCF-diacetate (5 μg/ml; Molecular Probes, Eugene, Oregon) was loaded to myocytes for 5 min at 37°C. Myocytes were subsequently imaged under fluorescence microscope (Olympus OSP-3; Olympus, Tokyo, Japan), and fluorescence of DCF was quantified. In certain cases, cells were treated with 2 mmol/liter of NAC, 50 U/ml of catalase, 1 μmol/liter of mitochondrial electron transport inhibitor rotenone (Nacalai Tesque, Kyoto, Japan), or 2.5 μmol/liter of NADH/NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI; Calbiochem-Novabiochem, La Jolla, California) for 1 h before assessment with DCF. For each condition, fluorescence intensity was measured from at least 30 individual cells.

Levels of lipid peroxides in myocytes were also measured after these stimulations for 12 h using a lipid peroxidation assay kit (Calbiochem-Novabiochem) according to the manufacturer's recommendation.

The MAPK assay. The activities of MAPKs (extracellular signal-regulated kinase [ERK], *c*-Jun-N-terminal protein kinase [JNK], and p38) were determined by Western blot analysis using polyclonal antibodies that recognize only phosphorylated MAPKs (phospho-ERK, phospho-JNK, and phospho-p38 antibodies; New England Biolabs, Beverly, Massachusetts). Quantitative analysis of MAPK activities was performed using densitometer.

The LDH assay. To examine the cytotoxicity of antioxidant NAC, myocytes were incubated with 2 mmol/liter NAC for 12 h, culture medium was recovered, and the lactate dehydrogenase (LDH) assay was performed using Cytotoxicity Detection Kit (Boehringer Mannheim, Mannheim, Germany).

Statistical analysis. Differences between groups were analyzed by one-way analysis of variance using the Dunnett or Bonferroni procedure. Results are expressed as mean ± SD. A value of $p < 0.05$ was considered statistically significant.

RESULTS

The ROS levels in cardiac hypertrophy. For the demonstration of our hypothesis that hypertrophic singling is mediated by ROS, we measured ROS levels in cardiac myocytes when cells were stimulated by ET-1 or PE. Figure 1 shows the time course of ROS levels after the stimulation of 10^{-7} mol/liter ET-1 (Fig. 1A) or 10 μmol/liter PE (Fig. 1B). Both ET-1 and PE increased ROS levels in cardiac myocytes; ROS levels were maximal at 5 min and declined to basal level by 30 min after stimulation. This increase in ROS levels was also evident by an increase in lipid peroxidation in ligand-stimulated myocytes (Fig. 2).

The origin of ROS. To confirm the antioxidant effect of NAC and catalase, and to determine the localization of ROS origin, myocytes were pretreated with NAC (2 mmol/liter), catalase (50 U/ml), mitochondrial electron transport inhibitor rotenone (1 μmol/liter), or NADH/NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI, 2.5 μmol/liter) for 1 h before the stimulation of ET-1 or PE, and DCF fluorescence was measured. As seen in Figure 3, both NAC and catalase significantly inhibited the increase in DCF fluorescence of myocytes stimulated by ET-1 or PE. The increase in DCF fluorescence induced by ET-1 or PE was inhibited by DPI, however, and not by rotenone. These data are consistent with a NADH/NADPH oxidase as a potential source of the origin of ligand-stimulated ROS.

The MAPK activities. We examined the effects of ET-1 and PE on MAPK activities. The time course of MAPK activities when adult cardiac myocytes were stimulated with ET-1 is shown in Figure 4A. The ERK activity was

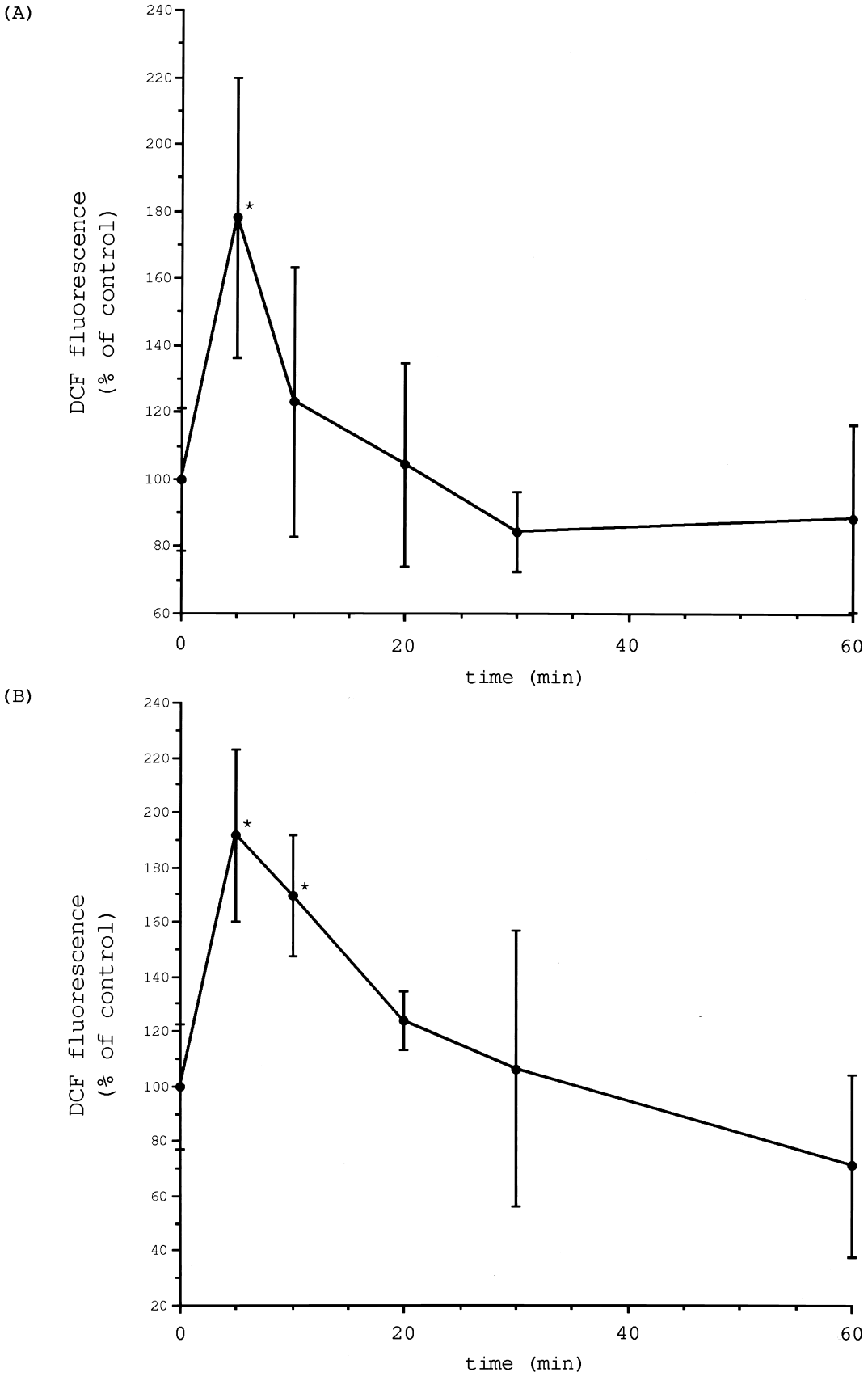

Figure 1. Time course of DCF fluorescence in adult rat cardiac myocytes stimulated with **(A)** endothelin-1 (ET) or **(B)** phenylephrine (PE). Fluorescence intensity was measured from at least 30 individual cells. Data are shown as mean ± SD. *$p < 0.05$ compared to control.

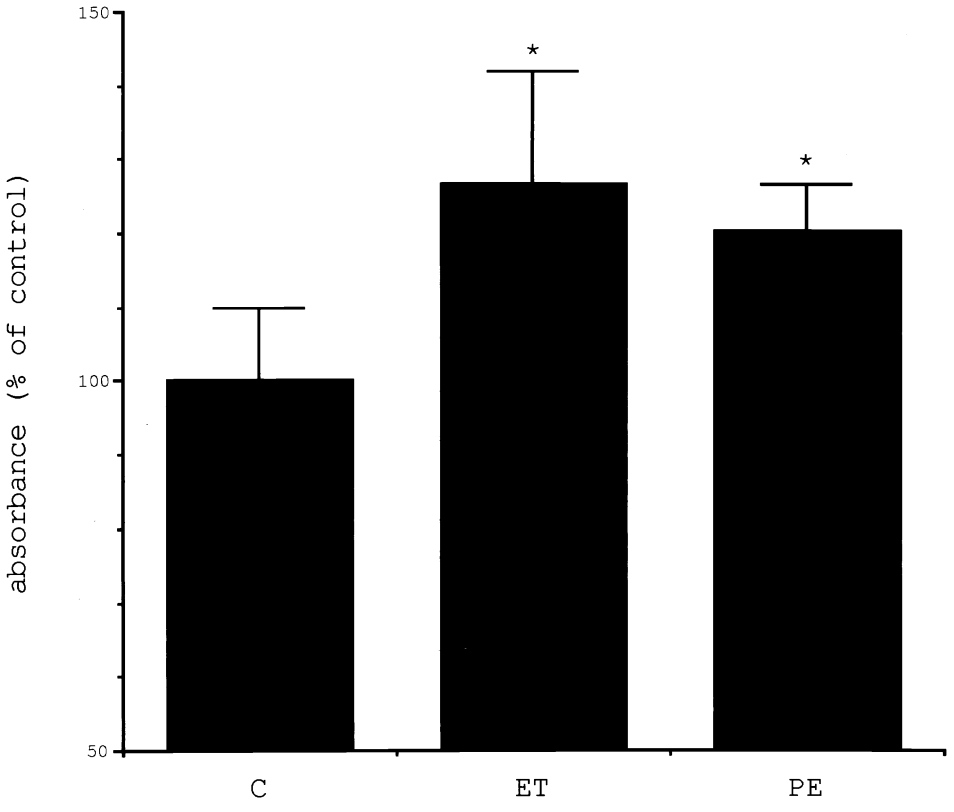

Figure 2. The reactive oxygen species (ROS) levels in cardiac myocytes stimulated with endothelin (ET) or phenylephrine (PE). The ROS levels were measured by lipid peroxidation assay. Data are shown as mean ± SD. n = 5, *$p < 0.05$ compared to control (C).

maximal at 5 min after ET-1 stimulation. However, p38 and JNK activities showed no changes after ET-1 stimulation. The activities of p38 and JNK were also not stimulated by the treatment of myocytes with anisomycin or UV light exposure, which are strong activators of these kinases in other types of cells. In contrast, in neonatal cardiac myocytes (Fig. 4B), p38 and JNK activities were stimulated by ET-1, anisomycin treatment, or UV light exposure. The ERK activity showed similar change to that of adult cardiac myocytes. In neonatal cardiac myocytes, p38 activity remained elevated at least for 60 min, and JNK activity was maximal at 5 min after ET-1 stimulation. Similar results were obtained when myocytes were stimulated with PE (data not shown).

Redox regulation of MAPK activities. To define the mechanism of how ROS modulate hypertrophic singling, we examined the effects of chemical antioxidant NAC and H_2O_2 scavenger catalase on ERK activity. Myocytes were pretreated with NAC (2 mmol/liter) or catalase (50 U/ml) for 1 h, and ET-1, PE, or phorbol-12-myristate-13-acetate (PMA), an activator of protein kinase C, was added to culture medium. After 5-min stimulation by these agents, cells were recovered and ERK activity was examined by Western blotting. As shown in Figure 5, the increased activity of ERK induced by ET-1 or PE was significantly suppressed by catalase or NAC treatment. However, the enhanced activity of ERK by PMA was not suppressed by these antioxidants. This result demonstrates that redox regulation is capable of altering the activity of ET-1- or PE-dependent MAPK pathway in adult rat cardiac myocytes.

Redox regulation of cardiac hypertrophy. Finally, we examined the effect of antioxidant NAC on cardiac hypertrophy induced by ET-1 or PE. Incorporation of ^{3}H-phenylalanine into adult rat cardiac myocytes was significantly increased by the stimulation of endothelin-1 (ET-1) or phenylephrine (PE) in a dose-dependent manner (Fig. 6). As shown in Figure 7, the increase in ^{3}H-phenylalanine incorporation into myocytes stimulated by 10^{-7} mol/liter ET-1 or 10 μmol/liter PE was significantly inhibited by the treatment of myocytes with *N*-acetylcysteine (NAC). Although NAC can affect the viability of certain cells, at the concentration used we noted no toxicity as assessed by LDH release. The LDH activity released from NAC-treated myocytes showed no significant difference from that released from control myocytes (absorbance; NAC: 0.206 ± 0.019, control: 0.200 ± 0.005).

Taken together, these results demonstrate that in adult rat cardiac myocytes, ROS regulate hypertrophic signaling at least in part by modulating the activities of MAPK cascade.

DISCUSSION

We demonstrated that ET-1 and PE increase ROS levels and induce hypertrophy of adult rat cardiac myocytes, and

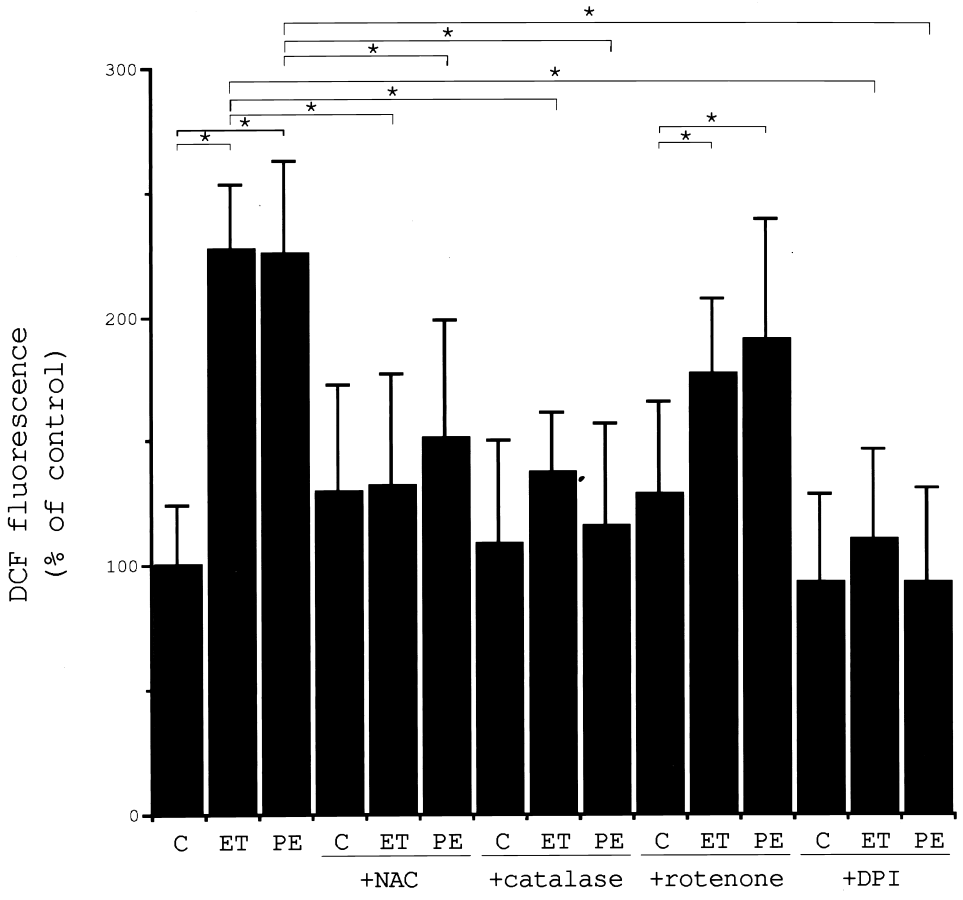

Figure 3. Effects of *N*-actylcysteine (NAC), catalase, rotenone, or diphenyleneiodonium chloride (DPI) on reactive oxygen species (ROS) levels in myocytes stimulated by endothelin (ET) or phenylephrine (PE). The dichlorofluorescein (DCF) fluorescence intensity was measured from at least 30 individual cells. Data are shown as mean ± SD. *$p < 0.05$.

that ET-1- and PE-induced hypertrophy is inhibited by the treatment of cardiac myocytes with chemical antioxidant NAC. These data support the hypothesis that ROS mediate hypertrophic signaling of cardiac myocytes.

The role of ROS in cardiac hypertrophy. Recent clinical and experimental studies demonstrated that oxidative stress plays an important role in the transition of cardiac hypertrophy to heart failure (1,2). Although the exact mechanisms of how ROS are involved in both early and late stages of cardiac hypertrophy are not clear, we propose that low levels of ROS may be essential to regulate the response of cardiac myocytes to hypertrophic stimuli. In contrast, at the late stage of cardiac hypertrophy when the production of ROS significantly exceeds the capacity of an antioxidant defense system such as superoxide dismutase (SOD), glutathione peroxidase and catalase, ROS levels increase in large amounts and may induce myocardial dysfunction and/or injury.

Redox regulation of MAPK pathways and cardiac hypertrophy. Our data demonstrate that the chemical antioxidant NAC and H_2O_2 scavenger catalase suppressed ERK activity stimulated by ET-1 or PE, although these antioxidants did not suppress the activity of ERK enhanced by PMA. The data supports the hypothesis that ROS regulate hypertrophic signaling at least in part by modulating the activities of ET-1- or PE-dependent MAPK pathways in cardiac myocytes. Our experiments using NADH/NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI) and the mitochondrial electron transport inhibitor rotenone showed that the origin of ET-1- or PE-induced ROS might be from NADH/NADPH oxidase in the cell membrane. It is likely that the direct effect of ROS may be on the upstream of the MAPK pathways that exists in the cell membrane or its near site, given that ROS are highly reactive and short-lived.

Previous studies have demonstrated that ROS can modulate multiple signaling pathways upstream of nuclear transcription factors, including Ca^{2+} signaling in vascular smooth muscle cells (VSMCs) (9) protein phosphorylation such as tyrosine kinases in a variety of cell types (10) and MAPK in VSMCs (11). Direct regulatory effects of ROS on transcription factor activities such as AP-1 and NF-kB have also been demonstrated in several cell types (12). In the present study, one of the mechanisms of how ET-1- or PE-induced cardiac hypertrophy is inhibited by NAC may be due to the suppressive effect of NAC on the MAPK cascade. There may be other possibilities that ROS modulate the activity of phosphotidylinositol 3-kinase pathway,

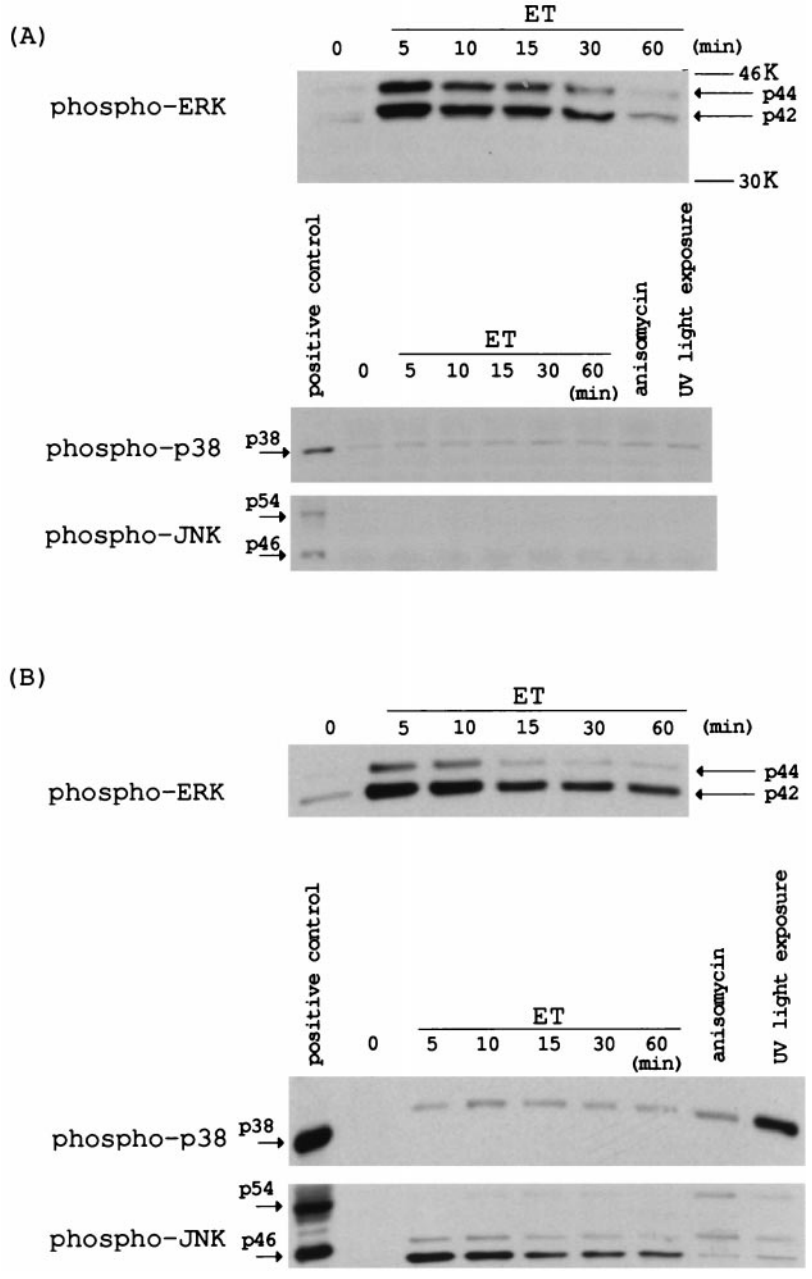

Figure 4. Time course of mitogen-activated protein kinase (MAPK) activities after the stimulation of myocytes with endothelin (ET) in adult **(A)** and neonatal cardiac myocytes **(B)**. Myocytes were also treated with anisomycin (10 μg/ml) for 1 h, or exposed to UV light for 30 min, and phospho-p38 and phospho-JNK expression were also examined. As a positive control for phospho-p38, total cell extracts from anisomycin-treated C-6 glioma cells, which are commercially available (Cell Signaling Technology, Beverly, Massachusetts), were used. As a positive control for phospho-JNK, total cell extracts from 293 cells prepared with UV light treatment (Cell Signaling Technology, Beverly, Massachusetts) were used.

Ca^{2+} signaling or transcription factor activities such as AP-1, and thus regulate hypertrophic signaling in ET-1- or PE-stimulated cardiac myocytes. Further studies are required to define these mechanisms.

Adult versus neonatal cardiac myocyte. In the present study using adult rat cardiac myocytes, ET-1 or PE stimulated the activity of ERK; however, these agents did not stimulate the activities of either p38 or JNK. In contrast, previous studies using neonatal rat cardiac myocytes showed that these stimuli enhance the activities of all these MAPKs (13). Our data using neonatal cardiac myocytes are consistent with the results of these studies. Although the reasons for the different results between adult and neonatal cardiac myocytes are not clear, one obvious explanation may be due to intrinsic differences between neonatal and adult rat cardiac myocytes. Adult cardiac myocytes have no mitogenic properties, and they are considered to be terminally differentiated cells.

In contrast, neonatal cardiac myocytes are still capable of proliferating after birth; therefore, they are not regarded as

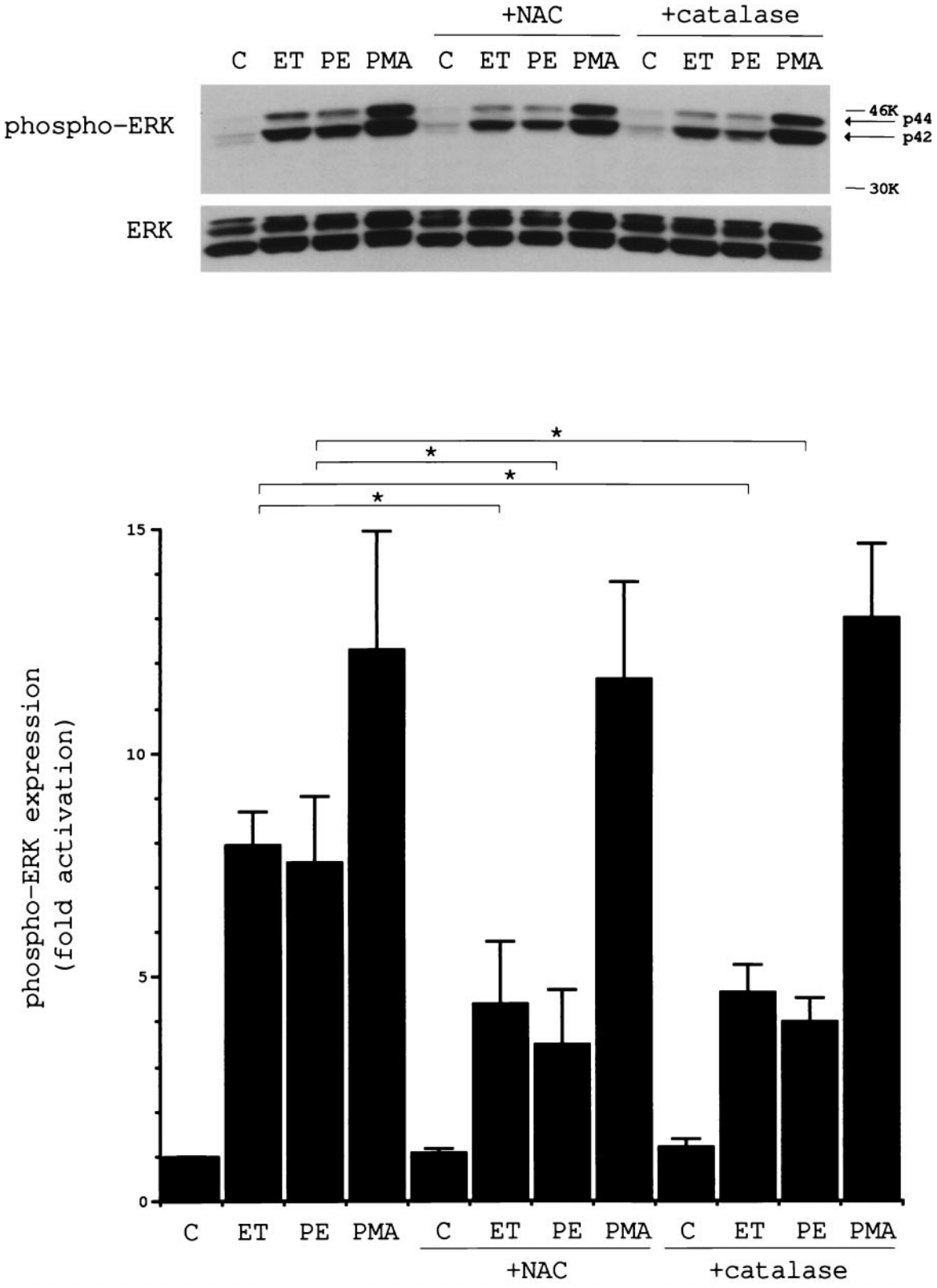

Figure 5. Effects of catalase and *N*-actylcysteine (NAC) on enhanced extracellular signal-regulated kinase (ERK) activity induced by endothelin (ET), phenylephrine (PE), or phorbol-12-myristate-13-acetate (PMA) in adult cardiac myocytes. Activated ERK was detected by Western blot analysis using antibody against phospho-ERK. To confirm that equal amounts of protein were loaded for each lane, membrane was stripped and Western blotting using antibody against whole ERK was performed again. Quantitative analysis was performed using densitometer. Fold activations of phospho-ERK compared to control are expressed as mean ± SD; n = 5. *$p < 0.05$.

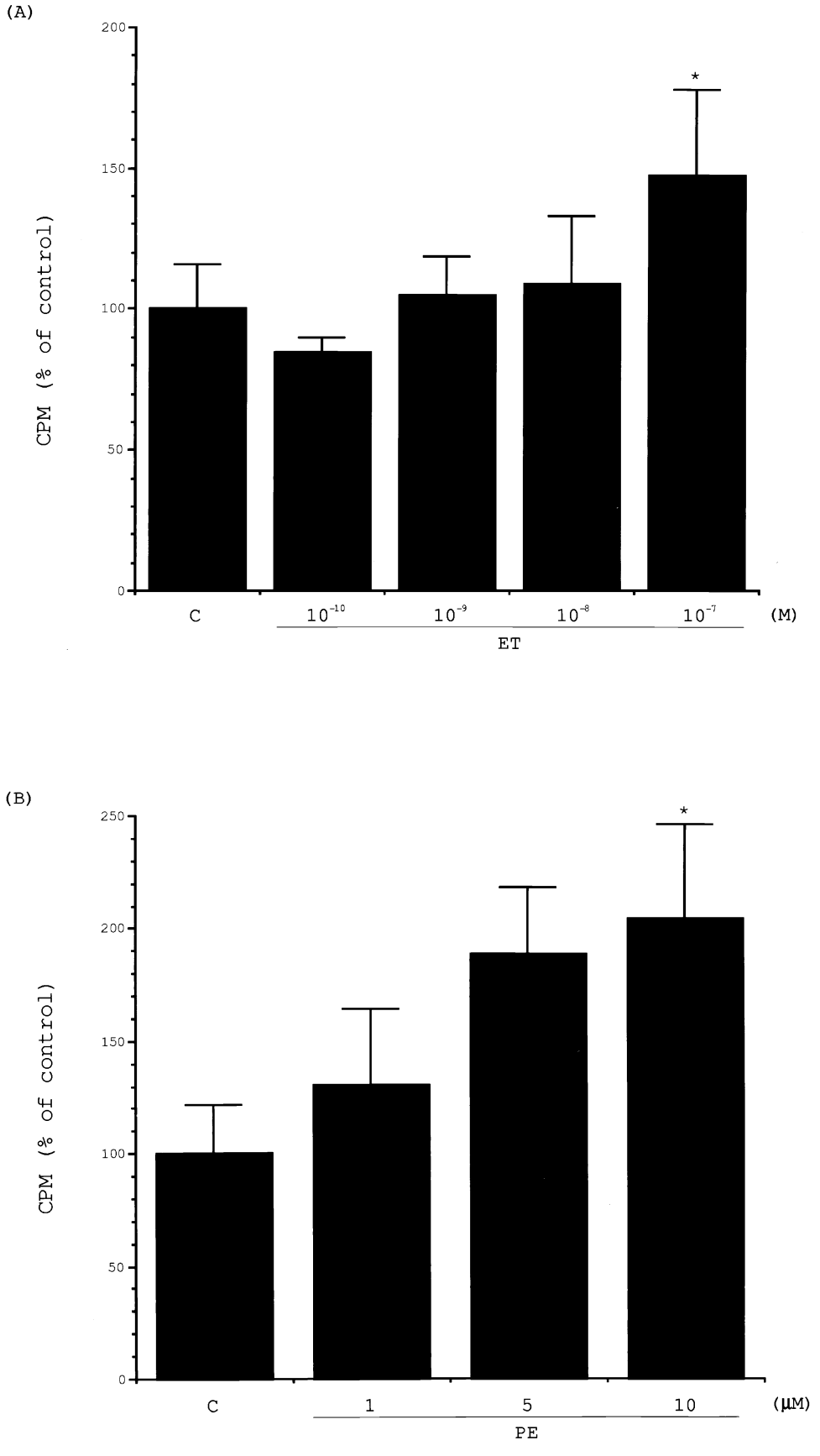

Figure 6. Dose-dependent increase in ^{3}H-phenylalanine incorporation into myocytes stimulated by endothelin (ET) **(A)** or phenylephrine (PE) **(B)**. Percentage of incorporation compared to control was expressed as mean ± SD; n = 5. *$p < 0.05$.

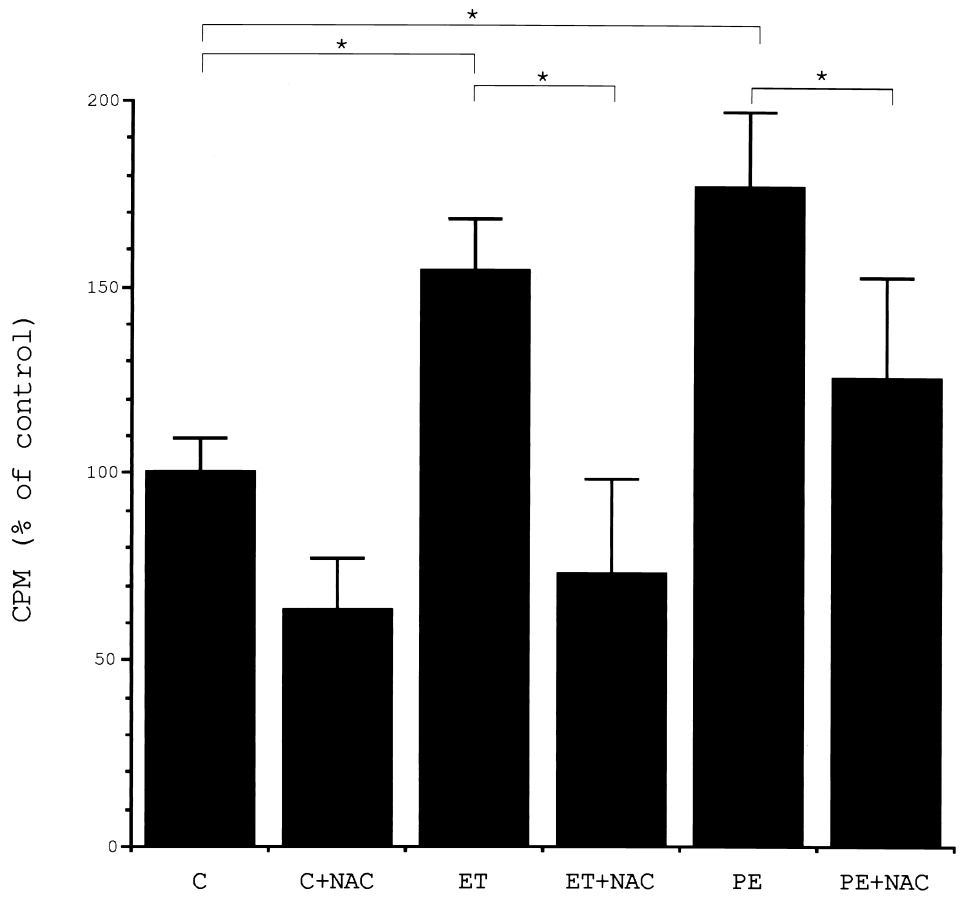

Figure 7. Effects of *N*-actylcysteine (NAC) on ^{3}H-phenylalanine incorporation into myocytes stimulated by endothelin (ET) or phenylephrine (PE). Data are shown as mean ± SD; n = 4. * $p < 0.05$. Differences between groups were analyzed by one-way ANOVA using Dunnett or Bonferroni procedure.

terminally differentiated cells. Furthermore, recent studies revealed different responses to hypertrophic stimuli between neonatal and adult cardiac myocytes in addition to differences in cell size and structure, metabolism, gene expression, and receptor composition (5,6). We believe that adult rat cardiac myocytes are more appropriate for the molecular analysis of hypertrophy of mature heart.

Clinical benefit of antioxidant therapy. Recent clinical and experimental studies have demonstrated that ROS are involved in the pathogenesis of heart failure (14) and that vitamin E supplementation prevents the development of heart failure in an animal model (2). The beneficial effects of a beta-blocker that has antioxidant properties in patients with heart failure have also been reported (15). In the present study, we demonstrated that ROS play an important role in the signaling of hypertrophy in adult rat cardiac myocytes at least in part by modulating the activities of MAPK pathways. These data suggest a potential benefit of antioxidant therapy as inhibitors of cardiac hypertrophy.

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Reprint requests and correspondence: Dr. Koichi Tanaka, The 4th Department of Internal Medicine, Shimane Medical University, 89-1, Enya-cho, Izumo, Shimane 693-8501, Japan. E-mail: tanakak@shimane-med.ac.jp.

REFERENCES

1. Keith M, Geranmayegan A, Sole MJ, et al. Increased oxidative stress in patients wiht congestive heart failure. J Am Coll Cardiol 1998;31:1352–6.
2. Dhalla AK, Hill MF, Signal PK. Role of oxidative stress in transition of hypertrophy to heart failure. J Am Coll Cardiol 1996;28:506–14.
3. Kunsch C, Medford RM. Oxidative stress as a regulator of gene expression in the vasculature. Circ Res 1999;85:753–66.
4. Nakamura K, Fushimi K, Kouchi H, et al. Inhibitory effects of antioxidants on neonatal rat cardiac myocyte hypertrophy induced by tumor necrosis factor-α and angiotension II. Circulation 1998;98:794–9.
5. Decker ML, Janes DM, Barclay MM, Harger L, Decher RS. Regulation of adult cardiocyte growth: effects of active and passive mechanical loading. Am J Physiol 1997;272:H2902–18.
6. Wada H, Zile MR, Ivester CT, Cooper G IV, and McDermott PJ. Comparative effects of contraction and angiotension II on growth of adult feline cardiocytes in primary culture. Am J Physiol 1996;271: H29–27.
7. Kuramochi T, Honda M, Tanaka K, et al. Contrasting effects of an angiotensin-converting enzyme inhibitor and a calcium antagonist on calcium transients in isolated rat cardiac myocytes. Cardiovasc Res 1994;28:1407–13.
8. Tanaka K, Pracyk JB, Takeda K, et al. Expression of Id1 results in apoptosis of cardiac myocytes through a redox-dependent mechanism. J Biol Chem 1998;273:25922–8.

9. Roveri A, Coassin M, Maiorino M, et al. Effect of hydrogen peroxide on calcium homeostasis in smooth muscle cells. Arch Biochem Biophys 1992;297:265–70.
10. Suzuki YJ, Forman HJ, Sevanian A. Oxidants as stimulators of signal transduction. Free Radic Biol Med 1997;22:269–85.
11. Sundaresan M, Yu ZX, Ferrans VJ, Irani K, Finkel T. Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. Science 1995;270:296–9.
12. Winyard PG, Blake DR. Antioxidants, redox-regulated transcription factors, and inflammation. Adv Pharmacol 1997;38:403–21.
13. Nemoto S, Sheng Z, Lin A. Opposing effects of Jun kinase and p38 mitogen-activated protein kinases on cardiomyocyte hypertrophy. Mol Cell Biol 1998;18:3518–26.
14. Signal PK, Khaper N, Palace V, Kumar D. The role of oxidative stress in the genesis of heart disease. Cardiovasc Res 1998;40:426–32.
15. Kukin ML, Kalman J, Charney RH, et al. Prospective, randomized comparison of effect of long-term treatment with metoprolol or carvedilol on symptoms, exercise, ejection fraction, and oxidative stress in heart failure. Circulation 1999;99:2645–51.