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PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR α (PPAR α) AGONIST, WY-14,643, INCREASED TRANSCRIPTION OF MYOSIN LIGHT CHAIN-2 IN CARDIOMYOCYTES

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ABSTRACT — Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that can be activated by xenobiotics and natural fatty acids. To assess the potential physiological activity of PPAR ligands on cardiac muscular cells, the effects of PPARα agonist, WY-14,643, on both rat hearts and a rat cardiomyocyte cell line (H9c2 cells) were investigated. Male F344 rats were fed a diet containing WY-14,643 at a concentration of 100 ppm for 26 weeks. Cardiac muscular hypertrophy was revealed by morphometric analysis in which the diameter of the muscular fibers in WY-14,643-treated rats was larger than those of control rats. Using H9c2 cells *in vitro*, the protein content per cell was increased in a dose-dependent manner with the treatment of WY-14,643. The transcription of myosin light chain-2 (MLC-2), a parameter of myocardial hypertrophy, was increased in H9c2 cells transfected with the rat MLC-2/luciferase fusion gene by WY-14,643 as well as other peroxisome proliferators, clofibrate and di(2-ethylhexyl) phthalate. In addition, accumulation of myosin light chain protein was confirmed in H9c2 cells treated with WY-14,643 at 10 μg/ml for 7 days or more by immunohistochemistry. These results suggest that PPARα ligands have a potential to regulate MLC-2, which is a contractile protein in cardiomyocytes and may play a part of role in the pathogenesis of cardiac hypertrophy.

KEY WORDS: Peroxisome proliferator-activated receptor α agonist, WY-14,643, Cardiac hypertrophy, Myosin light chain-2

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are a group of PPARα, β, and γ nuclear receptors for peroxisome proliferators, which are a diverse group of chemicals including therapeutic drugs (e.g. hypolipidemic drugs), plasticizers, organic solvents, polyunsaturated fatty acids, and hormones (Gonzales *et al.*, 1998). PPARs act as transcriptional factors after binding to peroxisome proliferator and the retinoid X receptor (Mangelsdorf and Evans, 1995; Wahli *et al.*, 1995). PPAR target genes encode enzymes involved in β-oxidation in peroxisome and mitochondria as well as ketone body synthesis (Wahli *et al.*, 1995). Peroxisomes participate in these processes in liver, retina, heart cardiomyocytes, epithelial cells from kidney proximal tubules, and enterocytes (Hinton and Price, 1993). Cardiomyocytes and epithelial cells of proximal tubules use fatty acids as a main energy source through an efficient peroxisomal β-oxidation pathway for long-chain fatty acid catabolism (Zaar, 1992; Hinton and Price, 1993).

Although the heart is one of the PPARs-rich organs, little is known about how PPAR ligands affect the cardiac function (Bishop-Bailey, 2000). Recently, PPARα appeared to play an important role in mito-

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chondorial fatty acid β-oxidation in cardiac myocytes (Brandt *et al.*, 1998; Yu *et al.*, 1998). Nothing, however, is known with regard to the potential role of PPAR in the pathology of cardiac tissue (Bishop-Bailey, 2000).

Sack *et al.* (1997) reported that the amount of nuclear PPARα decreases in the hypertrophied mouse heart. Barger *et al.* (2000) reported that PPARα is deactivated by several times, leading to diminished capacity for myocardial lipid and energy homeostasis during α-adrenergic agonist-induced cardiac hypertrophic growth in rat neonatal cardiac myocyte. These results indicate that PPARα modulates cardiac hypertrophy.

Cardiac hypertrophy initially acts as an adaptive mechanism to maintain overall pumping performance. Sustained hypertrophy can lead to cardiomyopathy and is a transition to heart failure (Francis *et al.*, 1995; Swynghedauw and Chevalier, 1995). The molecular events in myocardial hypertrophy are characterized by a selective expression of genes for contractile proteins in individual myocardial cells. The amounts of β-myosin heavy chain (Waspe *et al.*,1990), α-actin (Bishopric *et al.*, 1987), and myosin light chain-2 (MLC-2; Kummar *et al.*, 1986) increase 3, 4, and 4 times, respectively. Among these contractile proteins, MLC-2, one of the cardiac myosin molecules (Emerson and Bernstein, 1987), has been used extensively as a marker protein for myocardial hypertrophy (Meidell *et al.*, 1986; Lee *et al.*, 1988; Sen *et al.*, 1988; Henderson *et al.*, 1989; Doud *et al.*, 1995). Phosphorylation of MLC-2 modulates the contractile activity of cardiac cells (Silver *et al.*, 1986; Stull *et al.*, 1985; Morano, 1999).

H9c2 is a cardiomyocyte cell line derived from rat ventricular myocytes. Due to maintaining many molecular markers of cardiomyocytes, H9c2 cells have been used for studying the relationship between oxidants, cardiomyocyte hypertrophy, and cell death (Hescheler *et al.*, 1991).

WY-14,643 is a well-known strong peroxisome proliferator and PPARα agonist (Gray *et al.*, 1983). It increases peroxisomes in hepatocytes and induces liver tumors in rodents (Reddy *et al.*, 1979). In the present study, we first investigated the effect of PPARα ligands on cardiomyocytes using the hearts of rats obtained from a chronic toxicity study of WY-14,643 in rats. For the effect of the PPARα ligand of WY-14,643 on cardiomyocytes in detail, we examined total cellular protein and transcription of MLC-2 in H9c2 cells. To confirm the potential of MLC-2 transcription by PPARα agonists, we applied other two peroxisome proliferators, the hypolipidemic agent of clofibrate and the plasticizer of di(2-ethylhexyl) phthalate (DEHP), as PPARα ligands on the measurement of the transcription of MLC-2 in H9c2 cells.

MATERIALS AND METHODS

Reagents

WY-14,643 was purchased from ChemSyn Laboratories (Lenexa, KA, USA). For administration to rats, WY-14,643 powder was mixed with a diet at a concentration of 100 ppm. Clofibrate was purchased from Sigma Chemical Co. (St. Louis, MO, USA). DEHP was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). For cell culture, WY-14,643 was dissolved in dimethylsulfoxide (DMSO) at 10 mg/ml as stock solutions. Clofibrate and DEHP were dissolved in DMSO at 100 mg/ml as stock solutions. Stock solutions were stored at –80℃ until use. They were thawed just before use and then discarded after one-day use.

Animal experiments

F344 healthy male rats (5 weeks old) weighing approximately 100 g were purchased from Charles River Japan (Atsugi, Japan). The rats were given powder food (MF, Oriental Yeast Co., Ltd. Tokyo, Japan) and tap water *ad libitum*. They were reared in a controlled environment at 22±3℃ with 55±20% humidity on a 12-hr light/dark cycle with humane care in compliance with our institutional guidelines based on the guidelines of animal care and use committees recommendation (American Association for Laboratory Animal Science, 1987). A total number of 24 rats was assigned as controls fed a basal diet or 100 ppm of the WY-14,643-treated groups. After 26 weeks, rats were euthanized under ether anesthesia.

Pathology

Immediately after dissection of rats, fresh hearts were removed, weighed, and fixed in 10% neutral-buffered formalin (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) at room temperature for 24 hr. Fixed tissues were routinely processed for paraffin embedding, sectioned, and stained with hematoxylin and eosin for morphometric evaluation. Using a video image-analyzing system (Mitani-Shoji Co., Chiba, Japan), the diameters of myocardial fibers of the left and right ventricles as well as the septum were measured by the 'least diameter method' (Baandrup and Olsen, 1981).

Cell culture

H9c2 cells, a cardiomyocyte cell line derived from embryonic rat heart (Hescheler *et al.*, 1991; Sipido and Marban, 1991; Mejia-Alvarez *et al.*, 1994), were purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). H9c2 cells were maintained with 4.5 g/L-glucose DMEM (Gibco BRL, Rockville, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37℃ in a humidified atmosphere with 5% CO_2. The cells were differentiated by the method of low serum concentration described in Kimes and Brandt (1976). As practically, 100 μl aliquots of cell suspension at 5×10^5 cells/ml were plated in 24-well plates with 400 μl of differentiation medium (4.5 g/L-glucose DMEM supplemented with 1% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin) or 35 mm dishes with 2 ml of differentiation medium for immunohistochemistry. They were allowed to differentiate for 14 days in the differentiation medium. The media were changed once a week. After confirmation of cell fusion and the presence of multinucleate cells by phase-contrast microscopy as an indication of muscular differentiation, the media were changed to differentiation media containing 1/1000 (vol/vol) DMSO, WY-14,643, clofibrate or DEHP.

Protein content per cell

H9c2 cells were washed 3 times with PBS and dispersed with 0.25% trypsin and 0.01% EDTA for 15 min at 37℃. The cell suspension was collected in microtubes and washed 3 times with PBS. After centrifugation (10,000 rpm×5 min) at 4℃, the cells were solubilized with 100 μl of lysis buffer (50 mM Tris-HCl buffer, pH 6.8; 4% sodium laurylsulfate; 100 μg/ml phenylmethylsulfonyl fluoride; and 1 μg/ml leupeptin). The lysates were centrifuged at 10,000 g for 5 min at 4℃. The supernatants were assayed in triplicate for protein concentration by a BCA Protein Assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin as standard. H9c2 cells in the other wells processed in parallel under the same conditions were washed 3 times with PBS, dispersed with 0.25% trypsin-0.01% EDTA, and the cell number counted with a hemocytometer. Protein content per cell was determined by dividing the total amount of protein by the total number of cells.

MLC immunohistochemistry

H9c2 cells were fixed with 99.5% ethanol for 5 min at room temperature and rinsed 3 times with PBS. They were exposed to 1/1000 (vol/vol) goat anti-human MLC polyclonal antibody that recognizes both rat MLC-1, and MLC-2 (MLC-18, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The avidin-biotin-complex (ABC) method (Hsu *et al.*,1981) was applied to visualize the antigen-antibody complex using a VECTA STAIN ABC kit (Vector Laboratories, Burlingame, CA, USA).

Plasmid constructs

MLC-2 promoter activity was assayed by measuring luciferase expression of a transfected pMLC LΔ5', a construct in which the MLC-2 5' flanking region is upstream from the luciferase reporter (Henderson *et al.*, 1989). DNA was isolated from rat peripheral white blood corpuscle by the method of Blin and Stafford (1976). The primers for polymerase chain reaction (PCR) were designed using GenBank sequences for the rat MLC-2 promoter (S80994) reported by Doud *et al.* (1995). GENETOX-WIN 4.0 software (Software Development Co., Ltd., Tokyo, Japan) was used to suggest upper and lower primers. The following primer pairs were ordered with Espec Oligo Service Co. (Ibaraki, Japan): MLC-2 upper primer, 5'-CCTTC-CTCAGTGTCCTGGGAT-3' and lower primer 5'-CCACTGACCACTCACCATGGT-3'. The PCR reactions (50 μl) contained 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 25 pmol of each primer, 1.25 units of Taq polymerase (Takara Shuzo Co., Ltd., Shiga, Japan), 20 mM Tris-HCl (pH 8.4), and 50 mM KCl. Amplification cycles were performed in a Takara PCR thermal cycler model MP (Takara Shuzo Co., Ltd.) under the following conditions: 30 sec at 94℃, 30 sec at 58℃, and 60 sec at 72℃ for 35 cycles. Products were separated on 0.8% agarose (Sigma Chemical Co.) gel in 90 mM Tris-borate (pH 8.0) and 2 mM EDTA, stained with ethidium bromide and photographed under UV illumination. To verify authenticity, products were ordered to sequence with Espec Oligo Service Co.. The MLC-2 promoter region was inserted into the firefly luciferase reporter vector, pGL3 (Promega, Madison, WI, USA). MLC-2 was ligated to pRL vector (Novagen, Madison, WI, USA) for 30 min at 16℃ by using a DNA ligation kit (Takara Shuzo Co., Ltd.). The pRL-MLC-2 was cut with *Hind*III and *Nco*I in buffer K (20 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 mM KCl) for 2 hr at 37℃ and a band of about 1,500 bases was collected with a gel extraction kit (Qiagen Co., Tokyo, Japan). The pGL3 vectors were cut with *Hind*III and *Nco*I (Takara Shuzo Co., Ltd.) in buffer K

for 1 hr at 37℃ and incubated with 0.07 units/μl bacterial (*E.coli* K12 SW) alkaline phosphatase (Toyobo Co., Ltd., Osaka, Japan) for 30 min at 65℃. The pGL3 and pRL-MLC-2s vectors were ligated for 30 min at 16℃ by using a DNA ligation kit (Takara Shuzo Co., Ltd.). The pGL3-MLC-2 vectors were cut with *Bam*HI in buffer K for 1 hr at 37℃ and restriction digests were analyzed by agarose gel electrophoresis for authenticity. As a control for transfection efficiency, the *renilla* luciferase vectors, pRL-CMV (Promega), was used for a Dual-Luciferase assay (Promega).

Transfection of MLC-2/luciferase fusion gene (pGL3-MLC-2) into H9c2 cells

The media of differentiated H9c2 cells were switched to a fresh differentiation medium and transiently co-transfected with pRL-CMV (10 μl) and pGL3-MLC-2 (50 μl) vector for 24 hr using the lipid-mediated gene transfer, FuGENE6 (Roche, Indianapolis, IN, USA). Transfected cells were washed twice with PBS and cultured in 4.5 g/L-glucose DMEM containing 1% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin with or without WY-14,643, clofibrate, or DEHP for 48 hr.

Luciferase assay

The luciferase activity was assayed by a Dual-Luciferase Reporter Assay kit (Promega). The H9c2 cells transfected with pGL3-MLC-2 vector were washed twice with PBS and then lysed with 50 μl/well lysis buffer. The plates were incubated at room temperature for 15 min. The lysates were stored at –80℃ until assay. Each 20 μl cell lysate was combined with 100 μl firefly luciferase substrate from the kit. Luciferase activity was measured in triplicate in a microplate luminometer LB96P (Berthold GmbH& Co. KG, Bad Wildbad, Germany). *Renilla* luciferase activity was assayed continuously by adding 100 μl of Stop&Gio solution in the kit. Firefly luciferase activities were normalized to their corresponding *renilla* luciferase activities to correct the variation in transfection efficiency.

Statistical analysis

The data from experiments were analyzed as follows. First, the data were analyzed for homogeneity of variance using Bartlett's test (Bartlett, 1937). If the variance was homogenous, one-way ANOVA was conducted. If the difference was significant, Dunnett's test (parametric type) was used to mean values. If the variance was not homogenous, the Kruskal-Wallis test (Kruskal and Wallis, 1952) was conducted. If the difference was significant, Dunnett's test (non-parametric type) was applied.

RESULTS

Heart weights

To assess the general effect of WY-14,643 on the rat, rats were fed a diet containing WY-14,643 (100 ppm) for 26 weeks. Table 1 shows the effects of WY-14,643 on body weight and heart weight. The absolute heart weight in rats fed WY-14,643 was 0.85±0.03 g, which was 18% lower than that of control rats (1.04± 0.04 g). The absolute body weight of rats fed WY-14,643 was 262±4.2 g, which was 33% lower than that of control rats (393±17.4 g). Therefore, it was inappropriate to compare heart weight of WY-14,643-treated rats with that of controls on an absolute weight basis. For an exact comparison of heart weight, the relative weight of heart per body weight was calculated. The relative heart weight was 23% higher in WY-14,643-administered rats (0.32±0.01%) than in control rats (0.26±0.01%).

Pathology

To assess the effect of WY-14,643 on rat cardiac muscle, morphometry was performed on myocardial fibers of rats fed a diet containing WY-14,643 (100 ppm) for 26 weeks. Microscopically, the diameter of myocardial fibers was 0.015±0.001 nm in rats with

Table 1. Effect of WY-14,643 on body and heart weights of rats administered with WY-14,643 for 26 weeks.

Test chemicals	Dose	Final	Heart weight	
	(ppm)	body weight (g)	absolute (g)	relative (% of body weight)
None	–	393 ± 17.4	1.04 ± 0.040	0.26 ± 0.008
WY-14,643	100	262 ± 4.20***	0.85 ± 0.032***	0.32 ± 0.012***

The male F344 rats were fed a diet with or without WY-14,643 for 26 weeks.
Data were expressed as mean±S.D. using 12 rats each.
Significantly different from control: ***p<0.001.

WY-14,643, which was 25% larger than that of control rats of 0.012±0.001 nm (Table 2). These results indicate cardiomyocyte hypertrophy was induced by the feeding with WY-14,643 *in vivo*.

Protein content per cell

To assess the effects of WY-14,643 on protein content per myocardial cell *in vitro*, WY-14,643 was added to the culture medium for H9c2 cells, a rat cardiomyocyte cell line, at final concentrations of 1 μg/ml and 10 μg/ml. The number of cells in each well treated with 0, 1, and 10 μg/ml WY-14,643 for 2 days was 1.2×10^5, 1.5×10^5, and 0.96×10^5 cells/well, respectively. From the amount of cellular protein of H9c2 cells, the protein content per 10^4 cells was calculated as 2.2±0.2, 2.8±0.3, and 4.4±0.1 mg, respectively (Fig. 1). This indicates that cellular protein in each H9c2 cell was increased up to twice with WY-14,643 in a dose-dependent manner.

Table 2. Diameter of myocardial fibers of rats administered withWY-14,643 for 26 weeks.

Test chemicals	Dose (ppm)	Diameter of myocardial fibers (nm)
None	−	0.012 ± 0.001
WY-14,643	100	0.015 ± 0.001***

The male F344 rats were fed a diet with or without WY-14,643 for 26 weeks.
Data were expressed as mean±S.D. using 12 rats each.
Significantly different from control: ***p<0.001.

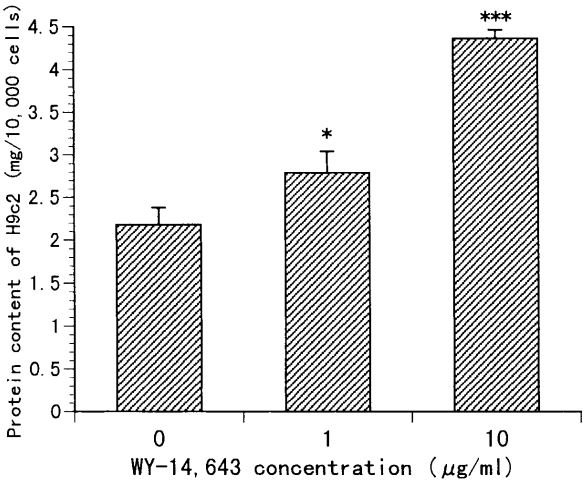

Fig. 1. Protein content per 10^4 cells of H9c2 cells with the treatment of WY-14,643 for 2 days. H9c2 cells were differentiated for 14 days and treated with 0, 1, or 10 μg/ml of WY-14,643. After 2 days, the amount of cellular protein and the number of cells were measured. Bars indicate standard deviation. Mean and standard deviation are obtained from triplicate experiments. *p<0.05, ***p<0.001.

Immunohistochemistry of MLCs in H9c2 cells

To assess the effect of WY-14,643 on the presence of MLCs in H9c2 cells, cellular MLCs were immunohistochemically stained with anti-MLCs antibody. There were a lot of dot-like stains in the cytoplasm of H9c2 cells cultured in the presence of WY-14,643 for 7 and 10 days (Photo 1). The stains were not apparent in H9c2 cells for 2 days (data not shown). These data indicate that WY-14,643 stimulates the expression or interferes with the degradation of MLCs in H9c2 cells.

Augmentation of MLC-2 transcription

To assess the WY-14,643 effects on transcription of the MLC-2 gene, an MLC-2 promoter construct carrying the coding portion of the firefly luciferase reporter gene was transfected into rat myocardial cell line, H9c2 cells. The luciferase activities were increased 1.7-fold and 3.2-fold, respectively, by 1 and 10 μg/ml WY-14,643 for 2 days (Fig. 2). These data indicate that MLC-2 transcription was increased by WY-14,643. To confirm the potential of MLC-2 transcription by PPARα agonists, we examined the clofibrate and DEHP by the same procedure used for WY-14,643. The statistically significant increases of MLC-2 transcription were observed in these two PPARα agonists (Fig. 3). The fold-increases in these chemicals (1.6 in clofibrate, and 1.3 in DEHP at the concentration of 10 μg/ml) were weaker than that in WY-14,643.

DISCUSSION

Peroxisome proliferators are a diverse group of chemicals that include several therapeutic drugs, plasticizers, organic solvents used in the chemical industry, herbicides, and naturally occurring hormones (Ashby *et al.*, 1994). Peroxisome proliferators cause an increase of peroxisomes in liver, kidney, and heart tissues of susceptible species such as rats and mice. One of the potent peroxisome proliferators is WY-14,643, a hypolipidemic drug, that has been extensively characterized as a non-genotoxic rodent hepatocarcinogen (Reddy *et al.*, 1979). WY-14,643 is also known to be a PPARα agonist (Gray *et al.*, 1983).

An enhancement of MLC-2 mRNA by WY-14,643 treatment for 2 days, as evaluated with MLC-

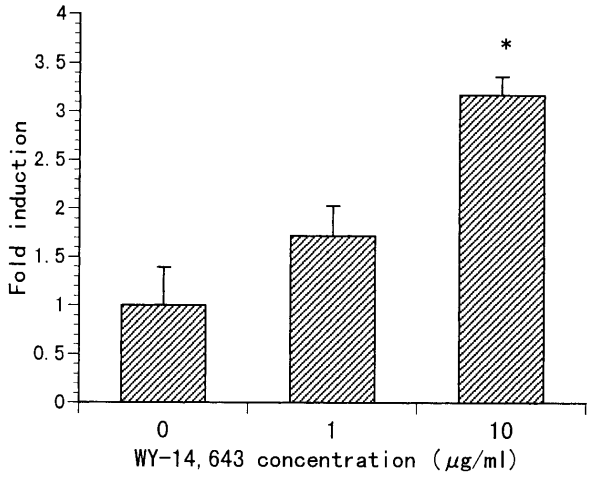

Fig. 2. MLC-2 transcription in H9c2 cells in the presence of 0, 1,or 10 μg/ml of WY-14,643. H9c2 cells were differentiated for 14 days and co-transfected for 24 hr with MLC-2 promoter/firefly luciferase constructs and *renilla* luciferase vector pRL-CMV construct in the presence of 0, 1, or 10 μg/ml of WY-14,643 for 2 days. Cells were harvested and firefly and *renilla* luciferase activities were assayed. Firefly luciferase activities were normalized to the corresponding *renilla* luciferase activities for each sample. Bars indicate standard deviation. Mean and standard deviation are obtained from triplicate experiments. *p<0.05.

2/luciferase fusion gene-transfected H9c2 cells in this study, is especially noteworthy. MLC-2 is one of the contractile proteins of the cardiac myosin and has been reported to be a useful marker for cardiac hypertrophy (Meidell *et al.*, 1986; Lee *et al.*, 1988; Sen *et al.*, 1988; Henderson *et al.*, 1989; Doud *et al.*, 1995). For example, spontaneous cardiac hypertrophy in rats is accompanied by increases in the amount of MLC-2 mRNA (Lee *et al.*, 1988), and α-adrenergic stimulation of neonatal rat myocardial cells produces several features of myocardial hypertrophy, including a several-fold increase in the volume of the MLC-2 gene (Kummar *et al.*, 1986). We also revealed that the transcriptions of MLC-2 in H9c2 cells were increased by other PPARα agonists, clofibrate, and DEHP. These data suggest that PPARα agonists generally have a potential to induce MLC-2 transcription. One cardiac myosin molecule consists of two myosin heavy chains and two pairs of MLC-1 and MLC-2 (Emerson and Bernstein, 1987). The increase of total MLC induced by WY-14,643 was further confirmed immunohistochemically in the present study. Additionally, protein content in H9c2 cells was increased dose-dependently with WY-14,643. This data is supported by a report describing increased expression of uncoupling protein-2 (UCP-2) by WY-14,643 exposure in H9c2 cells (Van Der Lee *et al.*, 2000). Uncoupling protein-2 is known to uncouple

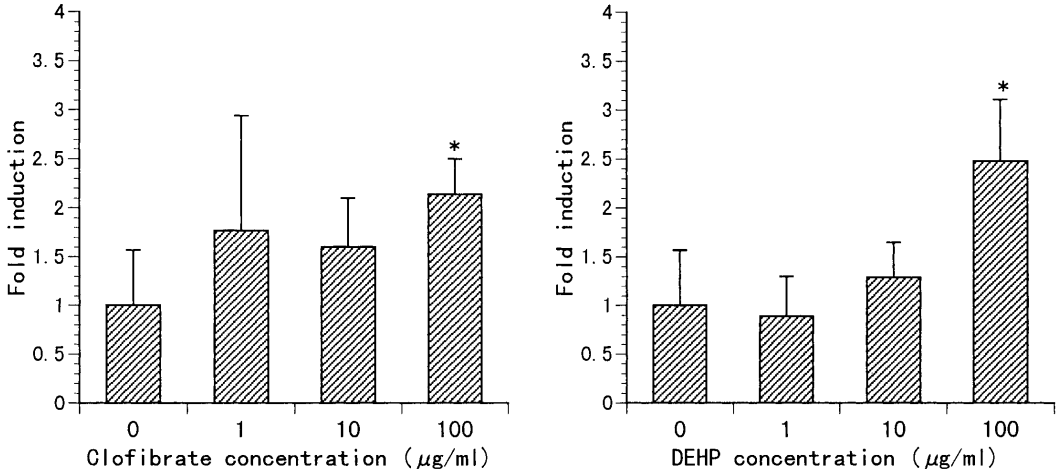

Fig. 3. MLC-2 transcription in H9c2 cells in the presence of clofibrate or DEHP. H9c2 cells were differentiated for 14 days and co-transfected for 24 hr with MLC-2 promoter/firefly luciferase constructs and *renilla* luciferase vector pRL-CMV construct in the presence of clofibrate or DEHP (0, 1, 10, or 100 μg/ml) for 2 days. Cells were harvested and firefly and *renilla* luciferase activities were assayed. Firefly luciferase activities were normalized to the corresponding *renilla* luciferase activities for each sample. Bars indicate standard deviation. Mean and standard deviation are obtained from triplicate experiments. *p<0.05.

oxidative phosphorylation by moving protons across the mitochondrial matrix (Ricquier and Bouillaud, 1997) and mediates the fatty acid-induced uncoupling effects observed in the heart (Fleury *et al.*, 1997).

Although the heart contains very high levels of PPAR, little is known about how PPAR ligands affect cardiac function (Bishop-Bailey, 2000). Brandt *et al.* (1998) and Yu *et al.* (1998) have previously described PPARα as having a control myocardial lipid metabolism with the retinoid X receptor α, with a role in mitochondrial fatty acid β-oxidation. In the present study, to assess the potential effect of PPARα agonists in the heart, rats were treated with WY-14,643 for 26 weeks at a dose of 100 ppm that was hepatocarcinogenic in the F344 rats. The heart weights (ratio to the body weight) of WY-14,643-treated rats were greater compared with those of control rats. Additionally, the diameters of myocardial fibers were larger in WY-14,643-treated rats than those in control rats. These results from the 26-week experiment *in vivo* suggest that WY-14,643 directly increases the amount of protein in rat hearts, because it had this effect on H9c2 cells, a cardiomyocyte cell line. Sack *et al.* (1997) reported that the amount of nuclear PPARα is decreased in a hypertrophied heart. The same group also reported that PPARα is deactivated during the α-adrenergic agonist-induced hypertrophic growth in an overexpressed recombinant PPARα system (Barger *et al.*, 2000). Based on our findings and their observations, the modification of PPARα activity is likely to involve the pathogenesis of myocardial hypertrophy. However, the role of PPARα in cardiac hypertrophy remains unknown. Further studies are needed for understanding the pathogenesis of cardiac hypertrophy as well as the safety of PPARα agonist in humans.

Although we could not detect MLCs in H9c2 cells treated with 10 μg/ml WY-14,643 for 2 days by immunohistochemistry, the protein content in H9c2 cells was increased by 2-day WY-14,643 exposure. Knowlton *et al.* (1991) reported the earliest effects of α-adrenergic stimulation on gene expression in neonatal rat myocardial cells is the rapid induction (within 15-30 min) of a program of immediate early gene expression, which includes the c-fos, c-jun, and Egr-1 genes. They also reported that over a later time course (12-24 hr), there is the reactivation or program of embryonic gene expression (atrial natriuretic factor, β-myosin heavy chain, and skeletal α-actin), and then in the following 24-48 hr, constitutively expressed contractile protein genes (MLC-2, cardiac α-actin) are upregulated. Based on their findings, it is considered the proteins induced by WY-14,643 for 2 days in our experiment were not MLCs.

Cardiac hypertrophy is a toxicologically important finding. For example, enlargement of cardiac muscle fibers is reported in dogs and rats after prolonged

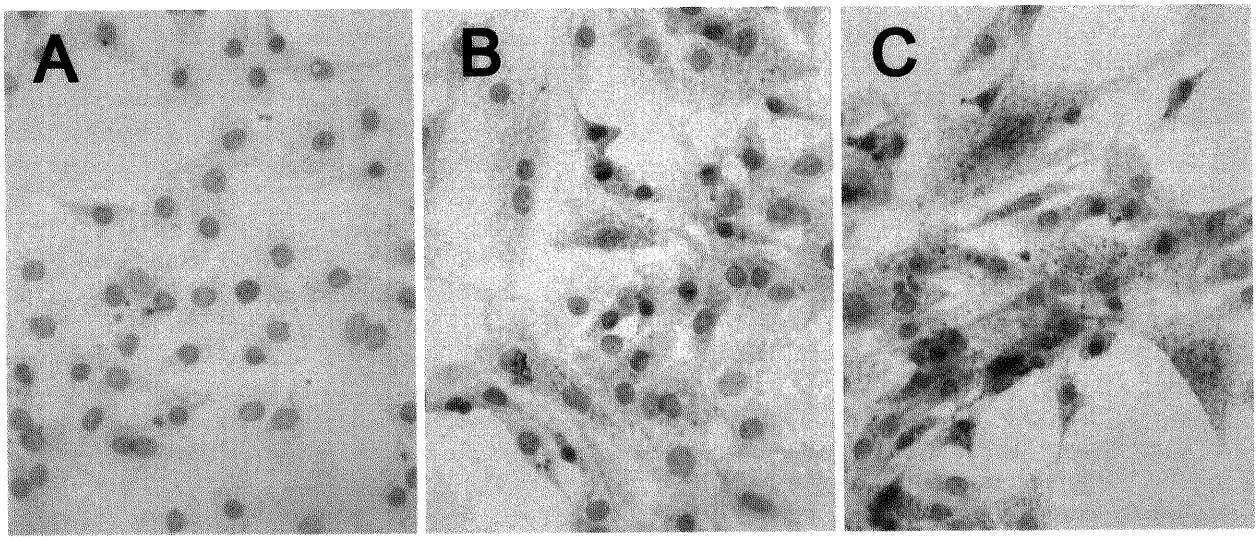

Photo 1. Immunostaining of H9c2 cells with anti- MLCs antibody. H9c2 cells were differentiated for14 days and treated with 0 (A), 1 (B) or 10 μg/ml (C) of WY-14,643. After 10 days, they were stained with goat polyclonal antibody to MLCs. The avidin-biotin-complex (ABC) method was applied to visualize the antigen-antibody complex using a VECTA STAIN ABC kit.

administration of oxfenicine, a modifying agent for muscle metabolism (Greaves *et al.*, 1984). Cardiac hypertrophy also occurs secondarily by an adoptive response to chemical exposure that affects vascular resistance and oxygen delivery. Wagner *et al.* (1999) reported that the rat cardiac MLC-2 gene provides an excellent model system to study the transcriptional and post-transcriptional mechanisms, which contribute to accumulation of contractile proteins during myocardial hypertrophy. The present study employed H9c2 cells, a cardiomyocyte cell line derived from rat ventricles that maintains many of the molecular markers of cardiomyocytes and has been used for studying the relationship between oxidants, cardiomyocyte hypertrophy, and cell death (Hescheler *et al.*, 1991). Thus, our experimental system using MLC-2/lusiferases fusion protein, which can detect the direct hypertrophic effect of the chemicals on cardiomyocytes, is considered useful for assessing cardiac toxicity. It may also provide a good bridge between *in vitro* studies and long-term toxicity studies.

For studies of species differences and extrapolation from these *in vitro* data to human risk assessment in cardiac hypertrophy induced by chemicals, studies that use the other species cells, which include human cells, are expected in future.

REFERENCES

American Association for Laboratory Animal Science (1987): Consensus recommendations on effective institutional animal care and use committees. Lab. Animal. Sci. Special Issue, January, 11-13.

Ashby, J., Brady, A., Elcombe, C.R., Alliott, B.M., Ishmael, J., Odum, J., Tugwood, J. D., Kettle, S. and Purchase, I. F. (1994): Mechanistically-based human hazard assessment of peroxisome proliferator-induced hepatocarcinogenesis. Hum. Exp. Toxicol., **13**, S1-S117.

Baandrup, U. and Olsen, E.G.J. (1981): Critical analysis of endomyocardial biopsies from patients suspected of having cardiomyopathy I: Morphological and morphometric aspects. Br. Heart J., **45**, 475-486.

Barger, P.M., Brandt, J.M., Leone, T.C., Weinheimer, C.J. and Kelly, D.P. (2000): Deactivation of peroxisome proliferator-activated receptor a during cardiac hypertrophic growth. J. Clin. Invest., **105**, 1723-1730.

Bartlett, M.S. (1937): Properties of sufficiency and statistical test. Proc. Roy. Soc., A**160**, 268-282.

Bishop-Bailey, D. (2000): Peroxisome proliferator-activated receptors in the cardiovascular system. Bri. J. Pharm., **129**, 823-834.

Bishopric, N.H., Simpson, P.C. and Ordahl, C.P. (1987): Induction of the skeletal alpha-actin gene in alpha 1-adrenoceptor-mediated hypertrophy of rat cardiac myocytes. J. Clin. Invest., **80**, 1194-1199.

Blin, N. and Stafford, D. W. (1976): A general method for isolation of high molecular weight DNA from eukaryotes. Nucleic Acids Res., **3**, 2303-2308.

Brandt, J.M., Djouadi, F. and Kelly, D.P. (1998): Fatty acids active transcription of the muscle carnitine palmitoyltransferase I gene in cardiac myocytes via peroxisome proliferator-activated receptor α. J. Biol. Chem., **273**, 23786-23792.

Doud, S.K., Pan, L.X., Carleton, S., Marmorstein, S. and Siddiqui, M.A. (1995): Adaptational response in transcription factors during development of myocardial hypertrophy. J. Mol. Cell. Cardiol., **27**, 2359-2372.

Emerson, C.P.Jr. and Bernstein, S.I. (1987): Molecular genetics of myosin. Annu. Rev. Biochem., **56**, 695-726.

Fleury, C., Neverova, M., Collins, S., Raimbault, S., Champigny, O., Levi-Meyrueis, C., Bouillaud, F., Seldin, M.F., Surwit, R.S., Ricquier, D. and Warden, C.H. (1997): Uncoupling protein-2: A novel gene linked to obesity and hyperinsulinemia. Nature Genet., **15**, 269-272.

Francis, G.S., McDonald, K., Chu, C. and Cohn, J.N. (1995): Pathophysiologic aspects of end-stage heart failure. Am. J. Cardiol., **75**, 11A-16A.

Gonzales, F.J., Peters, J.M. and Cattley, R.C. (1998): Mechanism of action of the nongenotoxic peroxisome proliferators: Role of the peroxisome proliferator-activated receptor α. J. Natl. Cancer Inst., **90**, 1702-1709.

Gray, T.J., Lake, B.G., Beamand J.A., Foster, J. R. and Gangolli, S.D. (1983): Peroxisome proliferation in primary cultures of rat hepatocytes. Toxicol. Appl. Pharmacol., **67**, 15-25.

Greaves, P., Martin, J., Michel, M.C. and Mompon, P. (1984): Cardiac hypertrophy in the dog and rat induced by oxfenicine, an agent which modifies muscle metabolism. Arch. Toxicol. Suppl., **7**, 488-493.

Henderson, S.A., Spencer, M., Sen, A., Kumar, C., Siddiqui, M.A. and Chien, K.R. (1989): Structure, organization, and expression of rat cardiac myosin light chain-2 gene. J. Biol. Chem., **264**, 18142-18148.

Hescheler, J., Meyer, R., Plant, S., Krautwurst, D., Rosenthal, W. and Schultz, G. (1991): Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. Circ. Res., **69**, 1476-1486.

Hinton, R.H. and Price, S.C. (1993): Extrahepatic peroxisome proliferation and the extrahepatic effects of peroxisome proliferators. Peroxisomes: Biology and Importance in Toxicology and Medicine. In Inhalated Particles (Gibson, G. and Lake, B., eds.), pp. 487-511, Taylor and Francis, London.

Hsu, S.M., Reine, I. and Fanger, H. (1981): Use of avidin-biotin-complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedure. J. Histochem. Cytochem., **29**, 577-580.

Kimes, B.W. and Brandt, B.L. (1976): Properties of a clonal muscle cell line from rat heart. Exp. Cell Res., **98**, 367-381.

Knowlton, K.U., Baracchini, E., Ross, R.S., Harris, A. N., Henderson, S.A., Evans, S.M., Glembotski, C.C. and Chien, K.R. (1991): Co-regulation of the atrial natriuretic factor and cardiac myosin light chain-2 genes during α-adrenergic stimulation of neonatal rat ventricular cells. J. Biol. Chem., **266**, 7759-7768.

Kummar, C.C., Cribbs, L., Dalaney, P., Chien, K.R. and Siddiqui, M.A.Q. (1986): Heart myosin light chain 2 gene. Nucleotide sequence of full length cDNA and expression in normal and hypertensis rat. J. Biol. Chem., **261**, 2866-2872.

Kruskal, W.H. and Wallis, W.A. (1952): Use of ranks in one-criterion variance analysis. J. Am. Stat. Assoc., **47**, 583-621.

Lee, H.R., Henderson, S.A., Reynolds, R., Dunnmon, P., Yuan, D. and Chien, K.R.J. (1988): Alpha-1 adrenergic stimulation of cardiac gene transcription in neonatal rat myocardial cells. Effects on myosin light chain-1 gene expression. J. Biol. Chem., **263**, 7352-7358.

Mangelsdorf, D.J. and Evans, R.M. (1995): The RXR heterodimers and orphan receptors. Cell, **83**, 841-850.

Morano, I. (1999): Tuning the human heart molecular motors by myosin light chains. J. Mol. Med., **77**, 544-555.

Meidell, R.S., Henderson, S.A., Slahetka, M.F. and Chien, K.R. (1986): Alpha 1-adrenergic stimulation of rat myocardial cells increases protein synthesis. Am. J. Physiol., **251**, H1076-H1084.

Mejia-Alvarez, R., Tomaselli, G.F. and Marban, E. (1994): Simultaneous expression cardiac and skeletal muscle isoforms of the L-type Ca^{2+} channel in a rat heart muscle cell line. J. Physiol., **478**, 315-329.

Reddy, J.K., Rao, M.S., Azarnoff, D.L. and Sell, S. (1979): Mitogenic and carcinogenic effects of a hypolipidemic peroxisome proliferator, [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid (WY-14,643), in rat and mouse liver. Cancer Res., **39**, 152-161.

Ricquier, D. and Bouillaud, F. (1997): The mitochondrial uncoupling protein: Structural and genetic studies. Prog. Nucleic Acid Res. Mol. Biol., **56**, 83-108.

Sack, M.N., Disch, D.L., Rockman, H.A. and Kelly, D.P. (1997): A role for Sp and unclear receptor transcription factors in a cardiac hypertrophic growth program Proc. Natl. Acad. Sci. USA., **94**, 6438-6443.

Sen, A., Dunnmon, P., Henderson, S.A., Gerard, R.D. and Chien, K.R. (1988): Terminally differentiated neonatal rat myocardial cells proliferate and maintain specific differentiated functions following expression of SV40 large T antigen. J. Biol. Chem., **263,** 19132-19136.

Silver, P.J., Buja, L.M. and Stull, J.T. (1986): Frequency-dependent myosin light chain phospholylation in isolated myocardium. J. Mol. Cell. Cardiol., **18**, 31-37.

Sipido, K.R. and Marban, E. (1991): L-type Ca^{2+} channel, potassium channels, and novel nonspecific cation channels in a clonal muscle cell line derived from embryonic rat ventricle. Circ. Res., **69**, 1487-1499.

Stull, J.T., Nunnally, M.H., Moore, R.L. and Blumenthal, D.K. (1985): Myosin light chain kinases and phosphorylation in skeletal muscle. Adv. Enzyme Regul., **23**, 123-140.

Swynghedauw, B. and Chevalier, B. (1995): Biological characteristics of myocardium during the regression of cardiac hypertrophy due to mechanical overload. J. Heart. Valve. Dis., **4**, S154-S159.

Van Der Lee, K.A., Willemsen, P.H., Van Der Vusse, J.J. and Van Bilsen, M. (2000): Effects of fatty acids on uncoupling protein-2 expression in the rat heart. FASEB. J., **14**, 495-502.

Wagner, M., Mascareno, E. and Siddiqui, M.A. (1999): Cardiac hypertrophy: Signal transduction, transcriptional adaptation, and altered growth control. Ann. N. Y. Acad. Sci., **874**, 1-10.

Wahli, W., Braissant, O. and Desvergne, B. (1995): Peroxisome proliferator-activated receptors: Transcriptional regulators of adipogenesis, lipid metabolism and more. Chem. Biol., **2**, 261-266.

Waspe, L.E., Ordahl, C.P. and Simpson, P.C. (1990): The cardiac beta-myosin heavy chain isogene is induced selectively in alpha 1-adrenegic receptor-stimulated hypertrophy of cultures rat heart myocytes. J. Clin. Invest., **85**, 1206-1214.

Yu, G.S., Lu, Y.C. and Gulick, T. (1998): Co-regulation of tissue specific alternative human carnitine palmitoyltransferase I β gene promoters by fatty acid enzyme substrate. J. Biol. Chem., **273**, 32901-32909.

Zaar, K. (1992): Structure and function of peroxisomes in the mammalian kidney. Eur. J. Cell Biol., **59**, 233-254.