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Oleylethanolamide Activates Ras-Erk Pathway and Improves Myocardial Function in Doxorubicin-Induced Heart Failure

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Oleylethanolamide (OEA) is a natural fatty acid ethanolamide produced in the heart, but its biological actions in myocardium have not yet been defined. This study was carried out to determine whether OEA could be used to prevent the development of heart failure or improve evolving heart failure. We studied *in vivo* and *in vitro* actions of OEA in cardiac muscle. In an animal model of doxorubicin cardiomyopathy, OEA showed robust effects and attenuated the progression of systolic/diastolic dysfunction and ventricular remodeling. During evolving doxorubicin cardiomyopathy, a therapeutic course of OEA treatment partially restored myocardial function. The preventive and therapeutic effects of OEA were associated with significant improvement of survival. To investigate the mechanism of OEA action in cardiac muscle, we have carried out *in vitro* experiments in cultured cardiomyocytes. The results showed that OEA, through activation of Ras-Raf-1-Mek-Erk signaling, inhibited doxorubicin-induced apoptosis. Additional experiments showed that OEA activation of the Erk pathway involved activation of Neu/ErbB2 receptor, which suggests OEA actions in cardiac muscle might require activation of Neu/ErbB2. In summary, OEA improved ventricular remodeling and augmented cardiac function in doxorubicin cardiomyopathy, possibly involving activation of Neu/ErbB2 and Ras-Erk signaling. These findings suggest OEA is a novel cardioprotective compound that may be used to develop new strategies for the management of cardiomyopathy. (*Endocrinology* 147: 827–834, 2006)

H EART FAILURE IS a major cause of death for both men and women in the developed countries. Despite recent medical progress, current therapies for heart failure are limited and the long-term prognosis for heart failure is disappointing (1). The development of heart failure involves abnormal cardiac muscle structure and function. Failing heart manifests unfavorable myocardial remodeling and compromised cardiovascular hemodynamics. The process of myocardial remodeling includes complex anatomical and cellular changes that are not yet fully understood. However, convincing evidence indicate apoptosis of cardiac muscle cells occurs during the development of heart failure and contributes to unfavorable myocardial remodeling and ventricular dysfunction (2–4).

Oleylethanolamide (OEA) is a naturally occurring monounsaturated fatty acid compound in tissues and in circulating blood (5, 6). Although OEA is a natural analog of the cannabinoid anandamide, OEA does not activate cannabinoid receptors. The function of OEA and its mechanisms of actions in cardiovascular system remain largely unknown (5). Pre-

vious studies have shown increased production of *N*-acylethanolamides, including OEA, in the ischemic heart (7, 8). It was speculated two decades ago that increased production of *N*-acylethanolamides might have represented a self-defense mechanisms against ischemia injuries (7), however, whether *N*-acylethanolamides such as OEA can be used to prevent or treat heart failure has never been studied. If OEA can be used to improve cardiac function, its mechanisms of action in the heart should be investigated. In this study, we studied whether OEA can be used to prevent or to treat doxorubicin cardiomyopathy, and discovered robust improvement of cardiovascular function. Furthermore, OEA rapidly activated Neu/ErbB2 receptor and Ras-Erk signaling pathway and suppressed doxorubicin-induced apoptosis in cardiomyocytes. These findings suggest OEA is a new endogenous compound that can be used to modulate myocardial remodeling.

Materials and Methods

Materials

Anti-Mek, anti-Erk, and phosphospecific antibodies were purchased from Cell Signaling (Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies to mouse and rabbit Ig were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Other antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). AG1478 and AG825 were from CalBiochem (La Jolla, CA), and GW2974 was from Sigma Chemical Co. (St. Louis, MO). All other chemicals were purchased from Sigma or Fisher Scientific (Pittsburgh, PA).

Animal model of doxorubicin cardiomyopathy and hemodynamic measurements

Male Sprague-Dawley rats were used for this study. Doxorubicin (3 mg/kg body weight, iv, every other day for three doses) was injected after overnight fasting to induce cardiomyopathy, and the control animals were injected with vehicle. To determine its preventive effect, OEA [5 mg/kg body weight, ip, twice daily (bid) for three doses] was administered before doxorubicin injection when indicated. To define its therapeutic effect, OEA (5 mg/kg, bid every other day for 7 d) was injected during the 4th week after completion of doxorubicin injections. Hemodynamic function was measured at the end of the 5th week after doxorubicin injections. To this end, under anesthesia (ketamine 50 mg/kg plus xylazine 4 mg/kg plus acepromazine 1 mg/kg), the right carotid artery was cannulated with a 1.4 French ultraminiature catheter with dual pressure and volume sensors (Millar Instruments, Houston, TX), and the catheter was advanced into the left ventricle (LV) guided by real-time pulse waveform monitoring. The catheter was interfaced with a transducer control unit and the data acquired with the Aria-1 conductance system and analyzed with PVAN pressure-volume analysis software (Millar Instruments) (9). Changes of LV pressure were measured by millimeters Hg and volume by relative volume unit (RVU).

Transferase-mediated dUTP nick end labeling (TUNEL) assay

For the detection of myocardium apoptosis, myocardial sections were labeled with TUNEL to identify DNA breakage *in situ* with the ApopTag Plus kit (Oncor, Gaithersburg, MD) as we previously reported (10). In brief, after deparaffinization, tissue sections were treated with proteinase K and ribonuclease and then incubated with terminal deoxynucleotidyl transferase and digoxigenin-labeled UTP at 37 C for 1 h. After PBS washing, rhodamine-conjugated antidigoxingenin antibodies were used to identify apoptotic nuclei. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride. The tissue sections were analyzed with a Carl Zeiss Axiophot epifluorescence microscope, and the number of apoptotic and nonapoptotic cardiac muscle nuclei were recorded in 100 high-power fields in each tissue section. The final apoptosis index was calculated as the number of apoptotic nuclei per 10,000 cardiac muscle nuclei.

Neonatal cardiomyocytes

Primary cultures of neonatal cardiomyocytes were prepared from Sprague-Dawley rats according to a protocol we previously described (11). Cardiomyocytes were plated in 100-mm Petri dishes in high-glucose DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin. To study how OEA modulates signal transduction, the cells were serum deprived overnight and then stimulated with OEA at indicated time intervals. To induce apoptosis, doxorubicin (1 μM) was added to serum-free medium as described previously (12).

Western blot

The cells were lysed with lysis buffer [137 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40, 2 mM EDTA (pH 8.0), 3 μg/ml aprotinin, 3 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 10 mM sodium pyrophosphate, and 2 mM Na$_3$VO$_4$]. Equal amounts of proteins were separated by SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA) and incubated with a blocking buffer [5% nonfat milk in 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, and 0.1% Tween 20] for 1 h at room temperature. The membranes were incubated with primary antibodies overnight at 4 C, washed three times [20 mM Tris-HCl (pH 7.5), 137 mM NaCl, and 0. 1% Tween 20), incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000 to 1:10,000 dilution) for 1 h at room temperature, washed three times, and then detected with enhanced chemiluminescence.

Analysis of Ras activation and assay for Raf-1 activities

Ras activation and Raf-1 activities were analyzed, respectively, with Ras activation assay kit and Raf-1 kinase cascade assay kit (Upstate

Biotechnology, Lake Placid, NY). Activated Ras interacts with and binds to Raf-1 (13). For Ras assay, equal protein amounts of cell lysates were immunoprecipitated with glutathione S-transferase fusion protein corresponding to the human Ras binding domain (residues 1–149) of Raf-1. The immunoprecipitates were then resolved with SDS-PAGE and then immunoblotted with anti-Ras antibodies. Raf-1 assay was carried out to measure the phosphotransferase activities in a kinase cascade. The first reaction used recombinant Mek1 as substrate for activated Raf-1. Activated Mek1 in turn activated recombinant Erk2. Finally, the activities of Erk2 were determined with phosphorylation of myelin basic protein in the presence of [γ-P^{32}]ATP.

Measurements of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethybenzimidazolylcarbocyanine iodide (JC-1) staining with epifluorescence microscope

Maintenance of the mitochondrial membrane potential (Δψm) is fundamental for cardiomyocyte survival. JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria (14). JC-1 accumulates in respiring mitochondria and emits red fluorescence. JC-1 emits green fluorescence in the mitochondria that lose cross-membrane electrochemical gradient. The cells were treated with 1.0 μM doxorubicin and OEA as indicated for 1 h, washed twice with 1× PBS buffer, and stained with 10 μg/ml JC-1 in DMEM (without phenol red) containing 0.1% BSA at 37 C (5% CO$_2$) for 30 min. After being briefly rinsed with 1× PBS twice, the cardiomyocytes were incubated in 0.1% BSA DMEM and analyzed under a Zeiss Axiophot epifluorescence microscope at a wavelength of 485 nm (excitation), and the images of green and red fluorescence were recorded with a Sensys digital camera and analyzed with PathVision imaging software.

Cell viability and annexin V assay

To measure cell viability, cardiomyocytes were plated in 48-well plates. Cell viability was performed by staining the cells with calcein AM (Molecular Probes, Eugene, OR) as previously described (10). Calcein AM hydrolyzes to calcein and is retained only in live cells and thus serves as an indicator for cell viability. The plates were analyzed with the Cytofluor 2300 fluorescence measurement system (Millipore) at excitation of 485 nm and emission of 530 nm. Confirmation of apoptosis was determined with fluorescein isothiocyanate-annexin V staining as we previously described (11). The apoptotic cells were stained with annexin V, whereas necrotic cells were simultaneously stained with both annexin V and propidium iodide. Thus, the apoptotic cells were identified and discriminated from necrotic cells. Ten to 15 random microscopic fields from each sample were photographed. The total number of cells on the same microscopic field was calculated by the photograph taken under bright field, and the apoptosis and necrosis indexes were calculated by the ratio of the number of apoptotic (or necrotic) cells to the total number of cells in each field. The final calculation was pooled from the data produced from four to eight experiments in triplicate.

Real-time quantitative PCR

RNA was extracted from cardiomyocytes as we previously reported (15). Two micrograms of total RNA were reverse transcribed with 0.2 μg of oligo(dT)12–18 primer for 50 min at 42 C, and real-time quantitative PCR was carried out with an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA) as previously reported (16). The primer/probe sets were designed with Primer Express software (Applied Biosystems) based on gene sequences from the GenBank database. Primers and fluorogenic probes were synthesized by TIB Molbiol (Berlin, Germany). The primer/probe sequences for the mouse genes were as follows: forward, 5′-CTTCCCAAAGCTCCTTCAAAAA-3′; reverse, 5′-CTGCGCATGCTCCGTG-3′; and probe, 5′-TGGTGGACCTTCG-GCAGCTGG-3′.

Statistical analysis

The data were expressed as mean ± SEM based on data derived from multiple independent experiments. The comparison between multiple groups was performed by a one-way ANOVA with *post hoc* correction by Tukey test. Survival analysis was performed with Kaplan-Meier

model using GraphPad PRISM. A *P* value of <0.05 was considered statistically significant.

Results

OEA improved heart failure induced by doxorubicin

To define *in vivo* effects of OEA on heart failure, we used a rat model of cardiomyopathy induced by doxorubicin. Doxorubicin is a commonly used antineoplastic agent that causes heart failure (12). In this model, significant dilatation of LV and systolic/diastolic dysfunction occurred 4–5 wk after doxorubicin injections (Table 1). We first investigated whether OEA can be used to prevent doxorubicin-induced heart failure. To this end, OEA (5 mg/kg body weight, bid for 2 d) was administered immediately before each doxorubicin injection. As shown in Table 1, a preventive OEA regimen significantly reversed LV dilatation and preserved systolic and diastolic LV function in nearly all the parameters measured. Doxorubicin-treated rats had lower body weight because of cardiac cachexia, but the beneficial effects of OEA remained similarly robust after LV volumes were adjusted by body weights (Fig. 1). These data indicate that preventive OEA treatment improved LV remodeling in doxorubicin cardiomyopathy.

In this model of heart failure, hemodynamic dysfunction was not evident until 4 wk after doxorubicin injections (Fig. 2). Progression of ventricular dilatation and myocardial dysfunction occurred between the 3rd and 4th weeks; thus, evolving heart failure with progressive ventricular remodeling occurred during this time period. To study whether OEA has any therapeutic effect during evolving heart failure, OEA (5 mg/kg, bid, every other day, for 7 d) was started at the end of wk 3 and continued through wk 4 after doxorubicin injection. Under this therapeutic protocol, OEA was effective and improved hemodynamic function, albeit that the therapeutic effect was somewhat less potent than the aforementioned preventive regimen in all parameters measured (Table 1). Compared with the doxorubicin-only group, significant preventive and therapeutic ef-
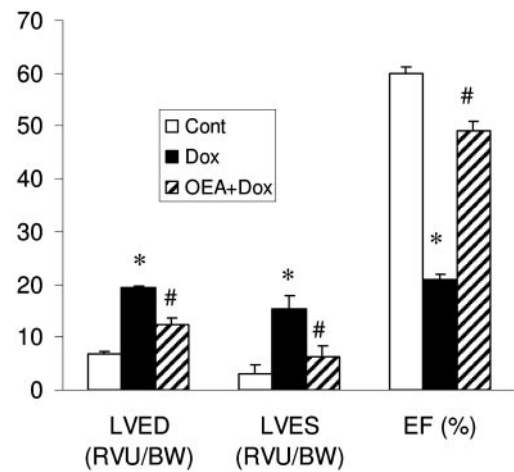

FIG. 1. Body weight-adjusted LV end diastolic (LVED) and end systolic (LVES) volume. LVED and LVES were adjusted by body weight of experimental animals (per 100 g), and the ejection fractions (EF) were accordingly calculated. The data were derived from control, doxorubicin (Dox)-treated, and OEA+Dox-treated rats (preventive regimen) as described in *Materials and Methods*. *, *P* < 0.001 *vs.* control; #, *P* < 0.05 *vs.* Dox.

fects of OEA on cardiovascular function were detected (by ANOVA with *post hoc* analysis).

Apoptosis of cardiac muscle cells contributes to the development of myocardial remodeling and heart failure. To investigate whether the effects of OEA involved modulation of myocardial apoptosis, we performed TUNEL staining on myocardial tissue sections from the experimental animals (Fig. 3). As expected, there was an increased myocardial apoptosis in the doxorubicin-treated animals, and preventive OEA treatment significantly reduced cardiac muscle apoptosis. Survival curve analysis showed that both preventive and therapeutic OEA regimens improved survival in the doxorubicin-treated rats (Fig. 4), suggesting that OEA improved the overall clinical outcome of heart failure.

TABLE 1. Hemodynamic changes

	Control (n = 10)	Dox (n = 10)	OEA immediately before Dox (n = 7)	OEA 4 wk after Dox (n = 8)
LV maximal volume (RVU)	19 ± 1	41 ± 1[a]	27 ± 2[b]	33 ± 3[b]
LV minimal volume (RVU)	6.7 ± 0.4	29 ± 2[a]	12 ± 2[b]	18 ± 5[b]
LV end-systolic volume (RVU)	7.6 ± 0.5	32 ± 2[a]	13 ± 3[b]	20 ± 3[b]
LV end-diastolic volume (RVU)	18 ± 1	40 ± 1[a]	26 ± 3[b]	31 ± 3[b]
LV end-systolic pressure (mm Hg)	126 ± 3	115 ± 4[a]	104 ± 6[b]	100 ± 6[b]
LV end-diastolic pressure (mm Hg)	18 ± 1	25 ± 2[a]	19 ± 1[b]	13 ± 1[b]
LV dP/dtmax (mm Hg/sec)	8877 ± 357	6201 ± 167[a]	6824 ± 473[b]	7245 ± 329[b]
LV dV/dtmax (RVU/sec)	325 ± 35	289 ± 49	365 ± 28	401 ± 39
LV volume at dP/dtmax (RVU)	18 ± 1	41 ± 1[a]	26 ± 2[b]	33 ± 3[b]
LV ejection fraction (%)	65 ± 3	28 ± 4[a]	59 ± 4[b]	47 ± 4[b]
LV stroke volume (RVU)	11 ± 1	11 ± 2	15 ± 1[b]	14 ± 1
Cardiac output (RVU/min)	3085 ± 272	2617 ± 402	3562 ± 301[b]	3729 ± 310[b]
Preload-adjusted maximal power (mW/ml²)	174 ± 14	21 ± 3[a]	80 ± 13[b]	60 ± 12[b]
Arterial elastance (mm Hg/RVU)	12 ± 1	13 ± 3	6.9 ± 0.5[b]	6.9 ± 0.5[b]
Heart rates (beats/min)	260 ± 5	219 ± 8[a]	228 ± 8	249 ± 14
Body weight (g)	371 ± 5	241 ± 13[a]	289 ± 14[b]	263 ± 6[b]

Hemodynamic changes were analyzed with pressure-volume loop. The overall differences among experimental groups were analyzed with ANOVA (F test, *P* < 0.01). *Post hoc* pair comparison between two groups was analyzed with Tukey correction. Dox, Doxorubicin; dP/dtmax, maximal rate of change; dV/dtmax, maximal systolic ejection rate.

[a] Statistical significance *vs.* control.
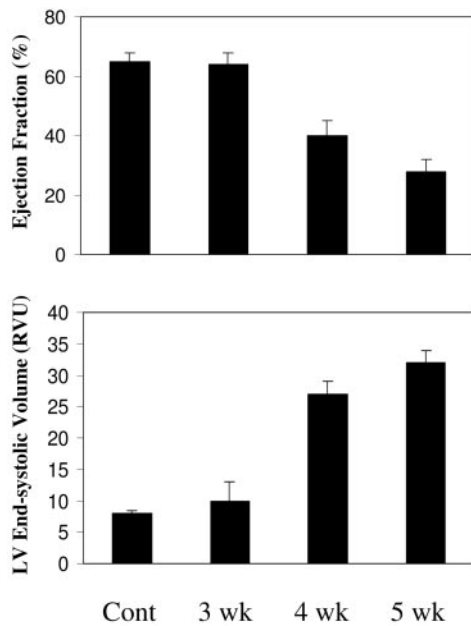[b] Statistical significance *vs.* doxorubicin.

FIG. 2. Evolving heart failure in doxorubicin cardiomyopathy. Cardiomyopathy was induced with doxorubicin injection, and LV ejection fraction and end-systolic volume were measured by a Millar catheter with dual pressure-volume sensors as described in *Materials and Methods* at various time intervals. Data represent the results derived from four to six rats at each time point. Cont, Control.

These *in vivo* experiments indicate two key observations. First, OEA possesses both preventive and therapeutic effects on doxorubicin cardiomyopathy. Second, because the timing of the initial doxorubicin insult on myocardium was far apart from the administration of the therapeutic OEA regimen,
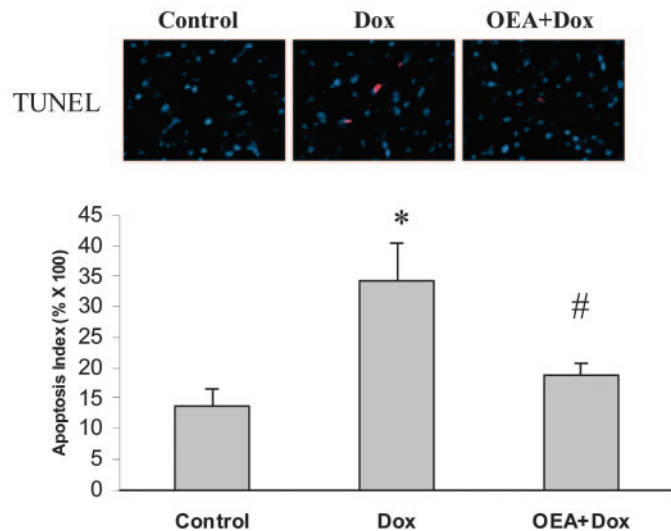

FIG. 3. OEA suppressed myocardial apoptosis *in vivo*. Apoptosis of cardiac muscle cells were analyzed by TUNEL assay in myocardial tissue sections from the control, doxorubicin (Dox)-treated, and OEA+Dox-treated rats (preventive regimen) as described in *Materials and Methods*. *Top*, Representative photographs from each group. The apoptotic nuclei were stained in *red* and the normal nuclei in *blue*. *Bottom*, The number of apoptotic cardiac nuclei is expressed as apoptosis index. These data were derived from 10 control, eight Dox-treated, and seven Dox+OEA-treated rats. *, $P < 0.05$ *vs.* control; #, $P < 0.05$ *vs.* Dox.
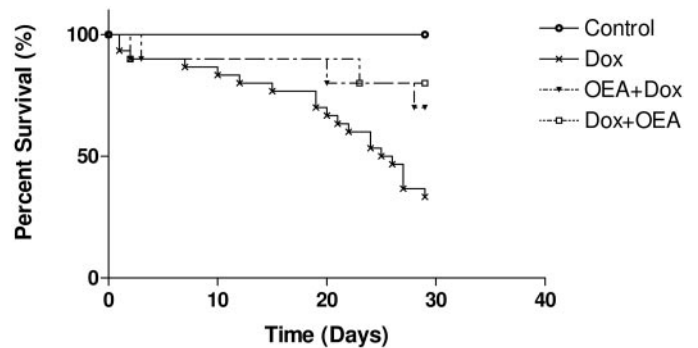
FIG. 4. OEA improved survival. To induce heart failure, Sprague-Dawley rats were injected with doxorubicin (Dox) or vehicle. A preventive OEA regimen (OEA+Dox) was given to a subset of Dox-injected rats before Dox injection as outlined in *Materials and Methods*. In another subset of Dox-injected rats, OEA treatment was administered during the 4th week after Dox injection to determine the therapeutic effect of OEA (Dox+OEA). Survival analysis was performed with Kaplan-Meier model. $P < 0.05$, OEA+Dox *vs.* Dox; $P < 0.05$, Dox+OEA *vs.* Dox.

OEA probably has direct therapeutic action on cardiac muscle and improved myocardial remodeling and function during evolving heart failure.

Doxorubicin cardiomyopathy is generally regarded as an irreversible myocardial process with standard therapy. IGF-I is an experimental agent that may partially restore cardiac function in experimental doxorubicin cardiomyopathy. We have compared the preventive efficacy of OEA with IGF-I, and the results are shown in Fig. 5. The preventive effect of OEA was better than IGF-I in preserving LV ejection fraction.

OEA activated the Ras-Raf-1-Mek-Erk pathway

Myocardial remodeling and function can be regulated through the activation of intracellular signaling pathways (17). To explore whether OEA can modulate intracellular signaling pathways in the cardiac muscle, we have studied activation of two major signaling pathways, Mek/Erk and Akt pathways, in primary cardiomyocytes. OEA acutely activated phosphorylation of Mek and Erk within 5 min of incubation (Fig. 6A). OEA activation of Mek/Erk lasted for more than 4 h, which is considerably longer than most peptide growth factors in cardiomyocytes. For example, activation of Erk by IGF-I lasted less

FIG. 5. Comparing the preventive effects of IGF-I and OEA on LV ejection fraction. Cardiomyopathy was induced by doxorubicin. Preventive OEA regimen was administered as described in *Materials and Methods*, and IGF-I (1.5 mg/kg sc) was administered under the same schedule. *, $P < 0.05$ *vs.* control; #, $P < 0.05$ *vs.* Dox+IGF-I.

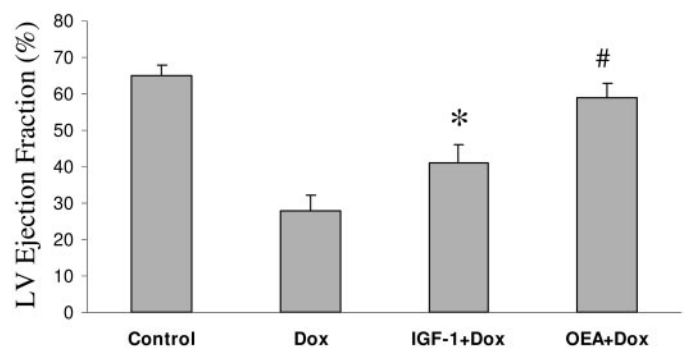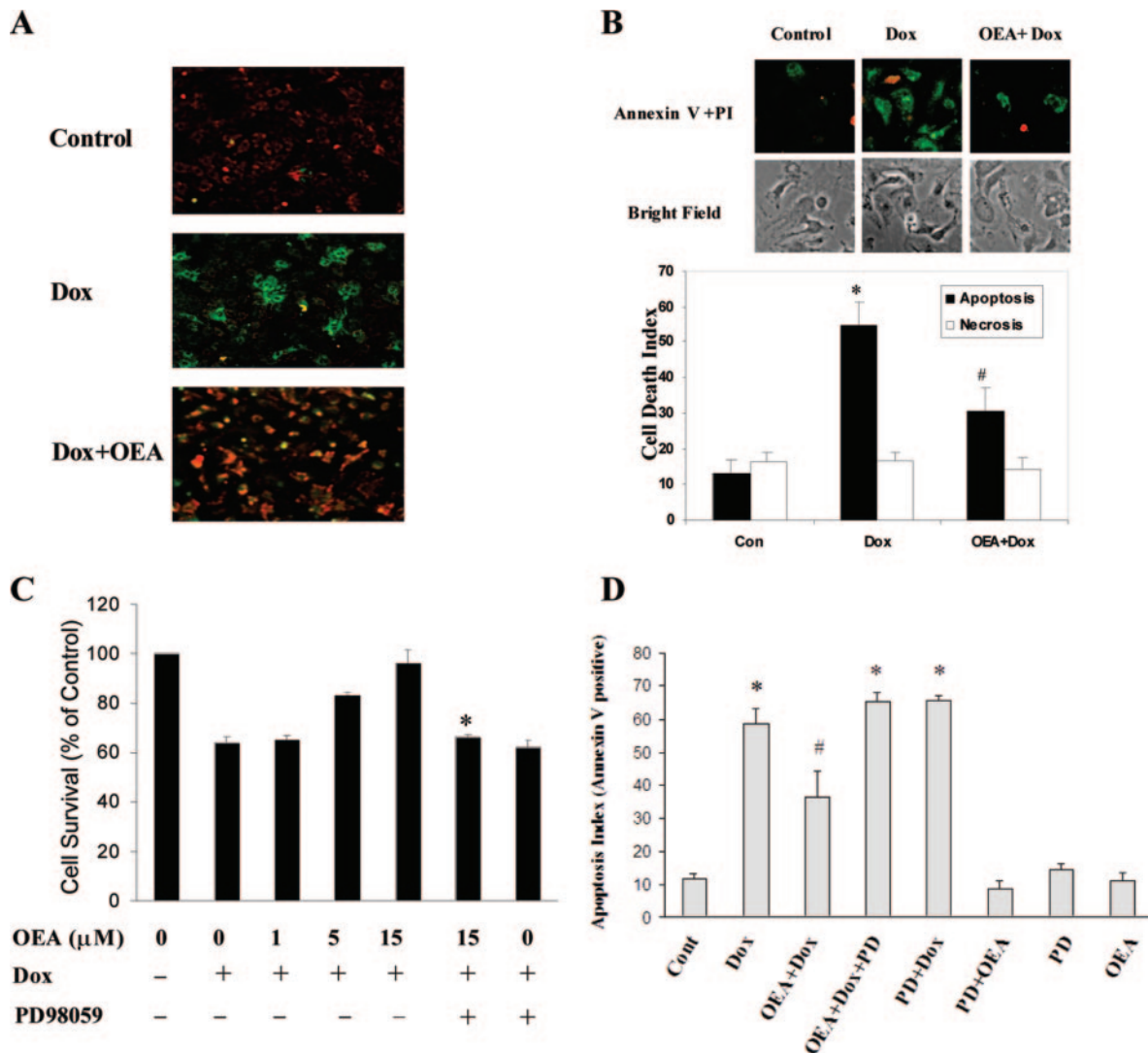FIG. 6. OEA activated Ras-Erk signaling pathway in cardiomyocytes. Cardiomyocytes were serum deprived and then stimulated with OEA (15 μM, except the dose-response study). IGF-I (10^{-8} M) serves as positive control. A, Time-course effect of OEA on activation of Mek, Erk, and Akt. Phosphorylation of Mek, Erk, and Akt were analyzed by Western blots with anti-phospho-Mek, anti-phospho-Erk, and anti-phospho-Akt antibodies. Western blots with anti-Erk and anti-Akt antibodies were used to determine the total amount of Erk and Akt proteins in cell lysates. B, Dose-response effect of OEA on Erk activation in cardiomyocytes; C, activation of Ras by OEA; D, activation of Raf-1 by OEA. Raf-1 activities were assayed with cascades of reactions leading to Erk2 phosphorylation of myelin basic protein.

than 20 min in cardiomyocytes. Unlike IGF-I, OEA did not activate Akt. The dose-response effect of OEA is shown in Fig. 6B; Erk was incrementally activated by 2–20 μM OEA. There was no additional activation of Erk when the concentrations of OEA were raised beyond 20 μM. Ras and Raf-1 are signaling steps upstream from Mek; therefore, we next investigated whether OEA can activate Ras and Raf-1. The results show that OEA activated both Ras and Raf-1 (Fig. 6, C and D). These findings suggest that sequential activation of the Ras-Raf-1-Mek-Erk pathway may serve as a biochemical basis of OEA actions in the heart.

OEA directly suppressed apoptosis of cardiomyocytes

Doxorubicin cardiomyopathy involves induction of cardiomyocyte apoptosis (11) and our *in vivo* study showed that OEA treatment inhibited myocardial apoptosis (Fig. 3). To further define the direct effect of OEA on cardiomyocytes apoptosis, we have investigated the effect of OEA in cardiomyocytes. Because mitochondria play a central role in apoptosis signaling, we first studied mitochondria cross-membrane potential, a marker of mitochondria function during induction of apoptosis. Doxorubicin induced depolarization of cardiomyocyte mitochondria cross-membrane potential, whereas OEA restored mitochondria membrane polarization (Fig. 7A). To confirm the direct effect of OEA on cardiomyocytes apoptosis, annexin V staining was performed, and the results show that OEA indeed suppressed doxorubicin-induced apoptosis in cardiomyocytes. In parallel, we also investigated myocyte necrosis, and the results showed that

doxorubicin treatment under this protocol did not induce significant necrosis (Fig. 7B). Analysis of cell survival showed a dose-dependent protective effect of OEA in the cardiomyocytes treated with doxorubicin (Fig. 7C). The dose-response effect of OEA on cardiomyocyte survival parallels the dose-response effect of OEA on Erk activation (Fig. 6B). When activation of Erk was inhibited with a Mek inhibitor PD98059, the prosurvival effect of OEA was inhibited (Fig. 7C). Thus, activation of the Ras-Erk pathway played a key role in the prosurvival action of OEA. Additional analysis with annexin V staining confirmed the involvement of Erk signaling in the antiapoptosis action of OEA (Fig. 7D).

OEA phosphorylated Neu/ErbB2 receptor in cardiomyocytes

It is possible that OEA might have activated the Ras-Erk signaling pathway through interaction with a cell surface receptor. To this end, we have screened various chemical inhibitors of cell surface receptors and found chemical inhibitors of ErbB1/ErbB2 (AG1478) blocked OEA activation of the Mek-Erk pathway (Fig. 8A). To further confirm the involvement of ErbB2, two additional Neu/ErbB2 inhibitors were investigated, GW2974 and AG825. AG825 is a partial/weak inhibitor of Neu/ErbB2 (therefore, higher concentrations were added), whereas GW2974 inhibits ErbB2 and epidermal growth factor (EGF) receptor. Figure 8B shows that AG825 only partially inhibited OEA activation of Erk, and GW2974 inhibited OEA activation of Erk. This experiment rendered additional evidence and support for involvement of ErbB2 in OEA actions in cardiac muscle. Additional ex-

FIG. 7. OEA inhibited apoptosis of cardiomyocytes. Apoptosis of cardiomyocytes was induced with doxorubicin in the presence of OEA (15 μM) or vehicle. A, Depolarization of mitochondrial membrane. Mitochondrial membrane potential is indicated by JC-1 staining (normal, *red*; depolarized, *green*). B, OEA inhibited apoptosis of cardiomyocytes. Apoptosis of cardiomyocytes was determined with annexin V staining, and necrosis of cardiomyocytes was analyzed with concurrent annexin V and propidium iodide (PI) staining. *, $P < 0.05$ *vs.* control; #, $P < 0.05$ *vs.* doxorubicin (Dox). C, Cardiomyocyte survival analysis. OEA rescued cardiomyocyte viability. Mek inhibitor PD 98059 (10 μM) was added 40 min before addition of OEA to inhibit Erk activation. Data represent results from four independent experiments in triplicate. *, $P < 0.05$ *vs.* 15 μM OEA+Dox. D, OEA inhibited apoptosis of cardiomyocytes. Cardiomyocyte apoptosis was determined by annexin V/PI staining, and the graph represents apoptosis index calculated from four independent experiments. PD, PD 98059 (10 μM). *, $P < 0.05$ *vs.* control (Cont); #, $P < 0.05$ *vs.* Dox.

periments showed that OEA induced tyrosine phosphorylation of Neu/ErbB2 receptor in cardiomyocytes (Fig. 8, C and D). EGF and heregulin served as positive controls in these experiments. Next, the cell lysates were immunoprecipitated with anti-Neu/ErbB2 antibodies and then immunoblotted with antibodies against phospho-Mek or phospho-Erk. The results show that phosphorylated Mek and Erk were associated with Neu/ErbB2 receptor in the OEA-stimulated cells (Fig. 8C). These results suggested that OEA activation of the Mek-Erk pathway involved activation of Neu/ErbB2. Because GW2974 and AG1478 may inhibit EGF/ErbB1 receptor, we also studied tyrosine phosphorylation of EGF/ErbB1 receptor (Fig. 8E). However, OEA did not phosphorylate EGF receptor in cardiomyocytes. Another potential target of OEA action is peroxisome proliferator-activated receptor-α (PPARα) nuclear receptor because OEA can increase PPARα expression in intestine (16). But in cardiomyocytes, OEA decreased the PPARα mRNA level (Fig. 8F).

Discussion

This is the first report demonstrating that a fatty acid,ethanolamide activated Ras-Erk signaling, at least in part through activation of Neu/ErbB2, and rendered a cardiac protective effect. OEA administration *in vivo* suppressed the cardiotoxic side effect of doxorubicin and improved myocardial remodeling and survival. In most patients with heart failure, it is not possible to correct the underlying causes of heart failure or to
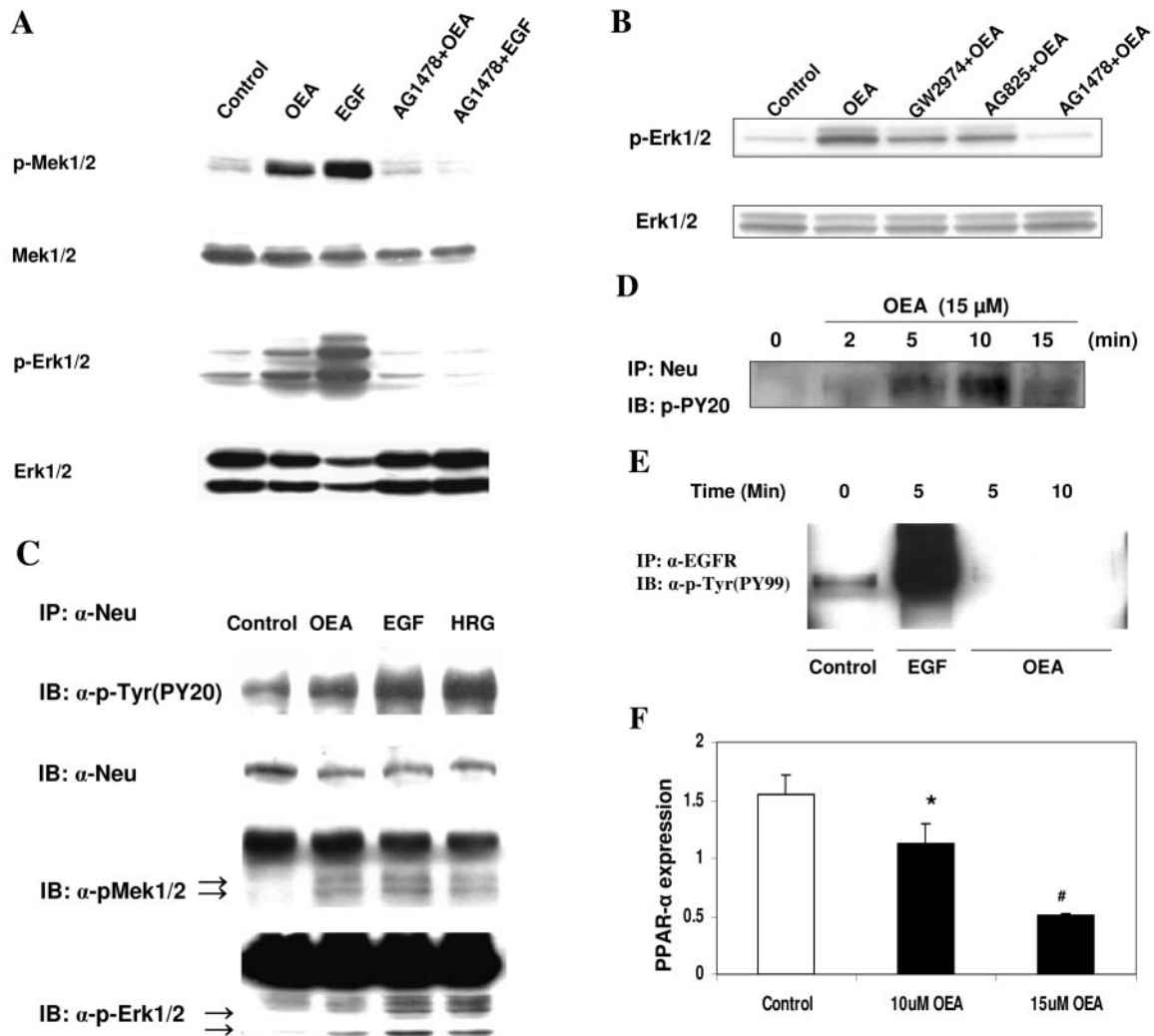
FIG. 8. OEA activation of Erk involved Neu/ErbB2 receptor in cardiomyocytes. A, OEA activation of Erk is suppressed by Neu inhibitor AG1478. AG1478 (5 μM) was added to the culture medium 1 h before addition of OEA (15 μM). EGF (10 nM) provided a positive control. B, Effect of various Neu/ErbB2 inhibitors on Erk activation by OEA. Three inhibitors of Neu/ErbB2 were used in this experiment, GW2974 (5 μM), AG825 (70 μM), and AG1478 (5 μM). GW2974, AG825, or AG1478 was added to the culture medium 1 h before addition of OEA (15 μM). C, Activation of Neu/ErbB2 by OEA. Cardiomyocytes were stimulated with OEA (15 μM), EGF (10 nM), or heregulin (10 nM) for 5 min in the presence of phosphatase inhibitors. The cell lysates were immunoprecipitated with anti-Neu antibodies and then immunoblotted with anti-PY20, anti-Neu, anti-pMek, or anti-pErk antibodies. EGF and heregulin served as positive controls. D, Time course of Neu/ErbB2 activation by OEA. Cardiomyocytes were stimulated with OEA (15 μM) for indicated time intervals. The cell lysate was immunoprecipitated with anti-Neu antibodies and then immunoblotted with anti-PY20. E, OEA did not induce tyrosine phosphorylation of EGF receptor. F, OEA did not increase expression of PPARα. PPARα expression was measured by real-time quantitative PCR. *, $P < 0.05$ *vs.* control; #, $P < 0.05$ *vs.* 10 μM OEA. Data represent the results of three independent experiments.

eliminate comorbidities that precipitate heart failure. Therefore, developing new strategies for the prevention and treatment of cardiomyopathy will have practical clinical implications.

OEA is a fatty acid ethanolamide produced in a variety of tissues and can be found in circulating blood. Despite the fact that OEA appears to be a potential pharmacological agent, previous studies shed little light on its mechanism of action in cardiac muscle. OEA is a natural analog of cannabinoid anandamide, but OEA does not activate putative receptor for anandamide or other cannabinoid receptor subtypes (16). Our data provide novel insight into the potential mechanism of OEA action. The Ras-Erk pathway is known for important biological actions. For example, the antiapoptosis effect of IGF-I in cardiomyocytes involves activation of Erk (18). Ac-

tivation of Erk signaling has also been implicated in the development of myocardial remodeling (17). Suppressing activation of Erk with PD98059 inhibited the protective effect of OEA in cardiomyocytes; thus, this pathway may play a key role in mediating the biological action of OEA. OEA activation of the Ras-Erk pathway is quite unique. Most growth factors that activate Ras also activate Akt, but OEA does not activate Akt. Moreover, OEA activation of Mek and Erk lasted for more than 4 h, a considerably long duration. Distinctive modulation of signaling pathways by OEA suggests possible existence of putative cell-surface receptor for OEA in cardiomyocytes. Our data showed that OEA activation of Erk signaling involved activation of ErbB2. ErbB2 is known to be transactivated along with other cell surface receptors, such

as EGF receptor and leptin receptor (19, 20). At present, we do not know for sure that ErbB2 is the only cell-surface receptor responsible for OEA actions. Whether OEA directly activates ErbB2 or transactivates ErbB2 through another cell surface receptor is beyond the scope of this study and will require additional investigation. However, our data does provide evidence that cardiac OEA signaling involved activation of ErbB2.

The effect of OEA is both preventive and therapeutic and appears quite robust. Doxorubicin cardiomyopathy is notorious for its progressive nature; medical interventions usually do not significantly alter its clinical course. We are pleasantly surprised that the beneficial effects of OEA were observed in most hemodynamic parameters measured. In addition to the aforementioned preventive regimen and therapeutic regimen, we also studied an alternative protocol in an attempt to revive cardiac function after cardiomyopathy has reached its end-stage (5–6 wk after doxorubicin injection). However, our trial on end-stage cardiomyopathy failed to show any benefits of OEA (data not shown). It appears that OEA was effective only during the initial myocardial insult and during evolving heart failure. Together with the *in vitro* data showing the antiapoptosis properties of OEA, these observations suggest that the protective effect of OEA existed only when there was salvageable cardiac muscle left. This study was not designed to test the side effects of OEA. However, in the OEA-treated rats we did not observe any significant change in feeding and defecation pattern or gross anatomical lesion at the end of the study.

Doxorubicin induced unfavorable LV remodeling and myocardial dysfunction; LV dilatation contributed to diastolic dysfunction as evidenced by increased end-diastolic pressure in this model. LV end-systolic pressure was slightly decreased because dP/dt and LV maximal power were decreased in the doxorubicin-treated rats (Table 1). The preload-adjusted LV maximal power represents intrinsic contractility (21). LV maximal power, which is not affected by heart rate, was markedly reduced by doxorubicin and restored by OEA. Because LV contractility was reduced and LV end-diastolic volume was increased, whereas dV/dt remained unchanged, LV ejection fraction significantly worsened in the doxorubicin-treated rats. The modest reduction in heart rate should not have contributed to the hemodynamic changes in the doxorubicin-treated rats in a significant way. The hemodynamic data from OEA-treated rats suggest that OEA minimized LV dilatation and improved systolic as well as diastolic function. One interesting finding is the changes of arterial elastance, arterial elastance derived from LV end-systolic pressure and stroke volume, representing total arterial load and impedance (22). OEA-treated animals showed lower arterial elastance, suggesting lower afterload in these animals. Lower afterload may have contributed to the improvement of hemodynamic function. The contribution of afterload in the context of the therapeutic effects of OEA deserves to be explored in the future.

In summary, our results suggest OEA is a novel cardioprotective compound that suppressed apoptosis of cardiac muscle. OEA actions in the heart might have involved activation of Neu/ErbB2 and Ras-Erk signaling. The robust preventive and therapeutic effect of OEA in this experimental model of cardiomyopathy may provide a new tool to develop new strategies for the management of cardiomyopathy.

Acknowledgments

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