Desacyl ghrelin prevents doxorubicin-induced myocardial fibrosis and apoptosis via the GHSR-independent pathway

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DOXORUBICIN IS A COMMONLY USED CHEMOTHERAPEUTIC AGENT for treating various malignancies, including breast cancer, leukemia, and sarcomas. The mechanism of the antitumor action of doxorubicin is probably mediated through the inhibition of gene transcription and replication by intercalation into the DNA structure. Although doxorubicin is used extensively in chemotherapy, it is known to induce organ toxicities, including cardiotoxicity, which results in cardiomyopathy and congestive heart failure (50). Cellular mechanisms, including myocardial fibrosis, apoptosis, and altered energy metabolism, have been proposed to account for the cardiomyopathy caused by doxorubicin (4, 36, 43).

Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor-1a (GHSR1a), is synthesized mainly in stomach. Ghrelin exists in two forms in circulation, ghrelin (acylated) and desacyl ghrelin, which lacks octanoyl acid at the serine 3 residue. Desacyl ghrelin is the predominant form of ghrelin in the circulation, with ghrelin in its acylated form contributing only ~5% to the total amount of circulating ghrelin (52). Acylated ghrelin has been shown to be capable of efficient binding and activation of GHSR1a due to octanoylation mediated by ghrelin O-acyltransferase (61), and this mediates most of its endocrinological effects (27, 28). Desacyl ghrelin was considered initially to be the inactive form of ghrelin, devoid of any biological activity because of its inability to bind and activate the classic GHSR1a receptor. However, increasing evidence indicates that desacyl ghrelin is bioactive and exerts cellular effects such as stimulation of adipogenesis and modulation of lipogenic and insulin signaling (11, 19). Besides, the expressions of important genes involved in lipid metabolism, such as fatty acid synthase (FAS), peroxisome proliferator-activated receptor (PPAR)γ coactivator-1α (PGC-1α), and muscle carnitine palmitoyltransferase-1 (mCPT-1), have been shown to be rapidly regulated by desacyl ghrelin in muscle (11). Although the specific receptor responsible for the effects of desacyl ghrelin is still unknown, some findings have illustrated that the effects of desacyl ghrelin could be suppressed by GHSR1a antagonist, suggesting that desacyl ghrelin can be acylated before its action and causes the effects (12). Nevertheless, studies on desacyl ghrelin have been conducted by using GHSR antagonist [e.g., [d-Lys3]-GHRP-6, a widely used compound as a selective GHSR antagonist in the in vitro and in vivo studies for abolishing the effects of acylated ghrelin (3, 39)] and GHSR-ablated mice so as to examine the dependency of GHSR1a signaling pathways (11, 12).

Accumulating evidence suggests that ghrelin and possibly desacyl ghrelin are protective of the cardiovascular system (18). The beneficial cardiovascular effects, such as protection against myocardial ischemia-reperfusion injury, improved prognosis of heart failure, and inhibition of atherosclerosis, have clearly been demonstrated for ghrelin (acylated) (18). The beneficial effects of ghrelin (acylated) on doxorubicin cardiotoxicity in primary cultured cardiomyocytes have also been shown, with effects mediated by antiapoptotic and antioxidative mechanisms (59). Intriguingly, similar to ghrelin (acylated), desacyl ghrelin has been demonstrated to inhibit doxorubicin-induced cardiac apoptosis by activating the extracellular prosurvival signal-regulated kinase (ERK)1/2 and phosphatidylinositol 3-kinase (PI3K)/Akt serine kinase.
signaling pathways through a GHSR-independent pathway in H9c2 cardiomyocytes (2). Of note, desacyl ghrelin has been shown to cause a rapid and strong activation of ERK1/2 and Akt in rat cortical neuronal cells (10). Cardioprotection by ghrelin (acylated) against doxorubicin was later supported by in vivo evidence showing that endogenous ghrelin is increased during the progression of heart failure, and it was suggested to represent a compensatory protective response to maintain the cardiac function during doxorubicin-induced cardiomyopathy (60). To date, the therapeutic role of desacyl ghrelin in doxorubicin cardiotoxicity has not been demonstrated in vivo. Therefore, the present study aimed to examine the cellular effects of desacyl ghrelin on doxorubicin-induced cardiomyopathy by using a mouse doxorubicin cardiotoxicity model. We also tested the hypothesis that desacyl ghrelin would protect against myocardial fibrosis and apoptosis induced by doxorubicin. To further examine whether the effects of desacyl ghrelin are mediated through the GHSR1α pathway, GHSR antagonist [d-Lys3]-GHRP-6 was used to examine the inhibition of GHSR1α signaling in the desacyl ghrelin-treated animals after the administration of doxorubicin. We hypothesized that [d-Lys3]-GHRP-6 would not disrupt the protective effects of desacyl ghrelin so as to confirm that the favorable effects of desacyl ghrelin on doxorubicin-induced cardiotoxicity do not involve the activation of the GHSR1α signaling pathway and thus are not due to the acylation of desacyl ghrelin in causing the effects.

METHODS

**Animals.** Male 10- to 12-wk-old C57BL/6 mice obtained from the Laboratory Animal Services Centre of The Chinese University of Hong Kong were used. Mice were housed in a temperature- and humidity-controlled environment and were exposed to a 12:12-h light-dark cycle in the Centralized Animal Facilities of The Hong Kong Polytechnic University. Mice were allowed to have access to food and water ad libitum. Animal ethics approval was obtained from the Animal Ethics Subcommittee of The Hong Kong Polytechnic University.

**Experimental protocol.** Mice were assigned randomly to one of four groups as follows: control, DOX (doxorubicin), DOX + DAG (doxorubicin treated with desacyl ghrelin), and DOX + DAG + [d-Lys3]-GHRP-6 (doxorubicin treated with desacyl ghrelin in the presence of GHSR1α antagonist [d-Lys3]-GHRP-6). Mice assigned to the DOX, DOX + DAG, and DOX + DAG + [d-Lys3]-GHRP-6 groups were exposed to an intraperitoneal (ip) injection of doxorubicin (Pharmacia & Upjohn, Milan, Italy) at a dose of 15 mg/kg, which has been demonstrated previously to induce cardiomyopathy, cardiac fibrosis, and apoptosis (49). Mice in the control group were injected ip with the same volume of saline instead of doxorubicin. Twelve hours after the injection of doxorubicin, mice were administered with saline (for the control and DOX groups), desacyl ghrelin (for the DOX + DAG group, desacyl ghrelin; Tocris Bioscience), or coadministration of DAG and [d-Lys3]-GHRP-6 (for the DOX + DAG + [d-Lys3]-GHRP-6 group; Tocris Bioscience) by ip injection for 4 consecutive days. In this study, the previously reported dosage of 100 μg/kg body wt of desacyl ghrelin injected twice daily was adopted (33, 38). We adopted a dosage of 3.75 mg/kg [d-Lys3]-GHRP-6 (37.5-fold dose of desacyl ghrelin), which was shown to effectively abolish the ghrelin-GHSR signaling (25). Mice in the DOX + DAG + [d-Lys3]-GHRP-6 group were administrated with [d-Lys3]-GHRP-6 immediately before desacyl ghrelin injection. After the 4-day experimental period, mice were euthanized by an overdose of ketamine and xylazine. Hearts were immediately removed and washed with cold phosphate-buffered saline (PBS). The left ventricle was quickly dissected and frozen in liquid nitrogen and stored at −80°C for subsequent analysis.

**Echocardiographic assessment.** Transthoracic echocardiography was conducted before the doxorubicin injection and after the 4-day experimental period to assess the cardiac structure and function. Echocardiography was performed according to a previously described protocol (45). Mice were anesthetized with an ip injection of ketamine HCl, and the ventral thorax was shaved and covered with ultrasonic transmission gel. Echocardiography was performed in the prone decubitus position with an Esaote MyLab 70 X-Vision Ultrasound System (Esaote, Italy). Two-dimensional grayscale ultrasound scanning was performed to assess the cardiac structures in the parasternal short-axis view at the midpapillary level. The grayscale echocardiographic view was used to position the M-mode echocardiographic line. Left ventricle (LV) internal dimensions and anterior and posterior wall thickness were then measured according to the leading-edge method of the American Society of Echocardiography (29). LV end-diastolic (LVEDD) and end-systolic dimensions (LVESD) were assessed from the M-mode tracing. Fractional shortening (FS), the percent change in LV cavity dimension, was calculated using the equation FS (%) = [(LVEDD − LVESD)/LVEDD] × 100 (13). Ejection fraction (EF) represents stroke volume as a percentage of end-diastolic LV volume and was derived as EF (%) = Y + [(100 − Y) × 0.15], where Y = ([LVESD2 − LVEDS2]/LVEDD2) × 100. All measurements were averaged over three consecutive cardiac cycles.

**TUNEL assay.** Myocardial apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) in ventricular muscle sections by using the in situ cell death detection kit (Roche). Briefly, 5-μm-thick transverse sections were prepared by cutting the frozen tissues at the apex of the LV. Tissue sections were air-dried at room temperature, fixed in 4% paraformaldehyde in PBS, pH 7.4, at room temperature for 20 min, and permeabilized with 0.2% Triton X-100 in sodium citrate at 4°C for 2 min. Slides were incubated with terminal deoxynucleotidyl transferase (TdT) enzyme with buffer and biotin-tagged nucleotides in a humidified chamber at 37°C for 1 h in the dark. Tagged nucleotides were detected by using anti-fluorescein-horseradish peroxidase conjugates. The section incubated with 5 μg/ml DNase for 10 min was used as a positive control. A negative control experiment was performed by omitting the TdT enzyme from the labeling solution. Troponin T was costained in sections for the identification of cardiomyocytes. Sections were treated with 5% horse serum in PBS 30 min and incubated for 1 h with monoclonal mouse Troponin T antibody (Thermo Science) diluted at 1:200 with 2% horse serum in PBS. Sections were incubated with anti-mouse IgG Cy3-conjugated antibody (1:200; Sigma). Sections were then mounted with DAPI Vectashield mounting medium to label all nuclei. Sections were examined under a Nikon 80i microscope. Images were captured with a Nikon DXM 1200C camera, using Nikon ACT-1C software. The number of TUNEL-positive nuclei and total nuclei originating from cardiomyocytes were counted and expressed as TUNEL index.

**Masson’s trichome staining.** Collagen deposition in the LV was determined by Masson’s trichrome staining kit (Sigma). Collagen was stained blue in the frozen tissue transverse sections (5 μm thick) according to the manufacturer’s instructions. Images were obtained in five random fields per section at a total magnification of 200. Analysis was performed with National Institutes of Health Image J analysis software. Area of fibrosis was divided by the total area of microscopic fields.

**Protein fraction preparation.** The protein fraction of cardiac muscles was prepared by adopting a previously described protocol (46, 47). Forty milligrams of tissue samples was minced and homogenized in ice-cold lysis buffer (10 mM NaCl, 1.5 mM MgCl2, 20 mM HEPES, pH 7.4, 20% glycerol, 0.1% Triton X-100, and 1 mM dithiothreitol). The homogenates were centrifuged at 875 g for 5 min at 4°C. The supernatant was collected and further centrifuged at 3,500 g for 4°C. The supernatant was collected and further centrifuged at 3,500 g for 4°C.
5 min at 4°C, and this procedure was repeated three times to collect the final supernatant as the cytoplasmic protein fraction. Protease inhibitor cocktail (P8340; Sigma-Aldrich) was added to a portion of the extracted protein fraction. The protein concentration of the extracted protein fraction was then measured in duplicate by using the Bradford method (Coomassie Protein Assay; Pierce), with bovine serum albumin used as standard. The protein fraction with the addition of protease inhibitor was later used in the apoptotic cell death ELISA and Western blot analysis. Caspase-3 enzymatic activity assay was performed in the protein fraction without the addition of protease inhibitor.

Apoptotic cell death ELISA. Apoptotic DNA fragmentation in ventricle samples was determined by using the Cell Death Detection ELISA Kit (Roche Diagnostics). In brief, 100 μL of protein fraction was added to a streptavidin-coated microplate that had been incubated with mouse monoclonal anti-histone antibody for 1 h at room temperature. After washing, the reaction mixtures were incubated with peroxidase-conjugated anti-DNA-POD mouse monoclonal antibody. The amount of histone-associated DNA fragments, including mono- and oligonucleosomes, was then determined by measuring the absorbance at 405 nm by using ABTS (2,2'-azino-di[3-ethylbenzthiazoline sulfonate-6-diamonium salt]) as a substrate, using a microplate reader (Infinite F200, Tecan). All measurements were performed in duplicate. The absorbance was normalized to the amount of protein content included in the assay and was expressed as OD405 (optical density) normalized to the milligram of protein used.

Fluorometric caspase-3 activity assay. Caspase-3 activity of ventricle muscle tissues was examined by a fluorometric assay, as described previously (46). Fifty microliters of the extracted protein fraction was incubated in 50 μl of reaction buffer (50 mM PIPES, 0.1 mM EDTA, 10% glycerol, and 10 mM DTT, pH 7.2), which contained 7-aminocoumarin (AFC)-conjugated substrate (Ac-DEVD-AFC; Biovision Research Products) at 37°C for 2 h. A negative control experiment was performed by including the caspase inhibitor z-VAD-fmk (BD Pharmingen) in the absence of the protease inhibitor. After washing, the reaction mixtures were incubated with AFC-conjugated substrate. The fluorescence intensity was detected by a microplate reader (Infinite AFC-conjugated substrate. The fluorescence intensity was detected by a microplate reader (Infinite F200, Tecan). All measurements were performed in duplicate. The absorbance was normalized to the amount of protein included in the assay and was expressed as OD405 (optical density) normalized to the milligram of protein used.

RNA extraction and real-time quantitative PCR analysis. Myocardial metabolic regulators [PPARα, mCPT1, uncoupling protein 3 (UCP3), and FAS] and fibrosis signaling factors [transforming growth factor-β1 (TGFβ1), connective tissue growth factor (CTGF), and brain natriuretic peptide (BNP)] were examined in cardiac tissues by quantitative RT-PCR analysis (Table 1). Total RNA was extracted from the ventricle muscles by using TRI Reagent (Molecular Research Center) based on the guanidinium thiocyanate method. Forty micrograms of muscle tissues were minced mechanically and homogenized on ice in ice-cold TRI Reagent. The homogenate was centrifuged following the addition of bromochloropropane to separate the aqueous and organic phases. The RNA in the aqueous phase was precipitated by adding isopropanol and was washed with 75% ethanol. The extracted RNA was then dissolved in DEPC-treated water and quantified in triplicate by measuring the optical density (OD) at 260 nm. The purity of RNA was assessed by examining the OD260/280 ratio. One microgram of RNA was reverse-transcribed with the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan) by using oligo(dT) primers in a total volume of 20 μl according to the manufacturer’s instructions. One microliter of the diluted cDNAs was then used to perform quantitative PCR in FastStart Universal SYBR green master (ROX) (Roche Diagnostics) with forward and reverse primers (Table 1) and RNase/DNase-free water using an ABI 7500 thermal cycler system. PCR was performed using a two-step cycling protocol with pretreatment at 95°C for 2 min, followed by a step of initial denaturing at 95°C for 10 min and then 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min according to the manufacturer’s instructions. β-Tubulin was used as the internal control reference gene. The sequences of PCR products were confirmed by sequencing. All samples were run in duplicate on the same plate. A relative standard curve (concentration vs. threshold cycle) of target and reference genes for quantification of PCR products was generated by dilution of cDNA from the calibrator. Complementary DNAs prepared from all samples were pooled and used as the calibrator to generate the standard curve. Results were expressed as the concentration ratio of the target gene normalized to the internal control β-tubulin gene.

Western blot analysis. The protein expression of apoptotic factors (Bcl-2 and Bax), prosurvival ERK-Akt signaling markers (phospho-ERK1/2, ERK1/2, phospho-Akt, and Akt), and metabolic factors [PGC-1α, AMP-activated protein kinase-α (AMPKα), medium-chain fatty acyl-CoA dehydrogenase (MCAD), and long-chain acyl-CoA synthetase isoform 1 (ACS-L1)] were evaluated in cardiac tissues by Western immunoblot. Fifty micrograms of protein was denatured at 95°C for 5 min in Laemmli buffer with 5% β-mercaptoethanol. Protein was fractionated on a 10% SDS-PAGE gel, followed by transfer to polyvinylidene difluoride membrane (Immobilon P, Millipore) by using Bio-Rad Mini Protein II system. The membrane was blocked with 5% skimmed milk powder in PBS-0.1% Tween-20, followed by primary antibody incubation for overnight at 4°C. The primary antibodies used included anti-Bcl-2 (1:500 dilution; Santa Cruz Biotechnology), anti-Bax (1:1,000; Abcam), anti-phospho(Ser473)-Akt, anti-Akt, anti-phospho (Thr422/423)-ERK1/2, anti-ERK1/2, anti-phospho-EGF (1:500; Santa Cruz Biotechnology), anti-phospho (Thr172)-AMPKα, anti-AMPKα, anti-MCAD (1:500; Cayman), and anti-ACSL1 (1:500; Santa Cruz Biotechnology). Membranes were incubated with secondary antibody [anti-mouse IgG or anti-rabbit IgG horseradish peroxidase-conjugated antibodies (1:3,000; Cell Signaling Technology)] after washes. The resulting immunoreactivity was determined using the ECL chemiluminescence reaction kit (Perkin Elmer), and the image was captured by Kodak 4000R Pro

Table 1. Sequence of primers used in real-time PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Full Name</th>
<th>GenBank Accession No.</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>PPARα</td>
<td>Peroxisome proliferator-activated receptor-α</td>
<td>NM_011444</td>
<td>5′GGTACACATCAGGAGTTGCG3′</td>
<td>5′CAGACAGCCACTTGTGAAGAC3′</td>
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<tr>
<td>mCPT-1</td>
<td>Carnitine palmitoyltransferase-1b, muscle</td>
<td>NM_009948</td>
<td>5′AAGCTCACTGCGGCAATTAACT3′</td>
<td>5′TGTAGTGGAGACATCTCTCAT3′</td>
</tr>
<tr>
<td>UCP3</td>
<td>Uncoupling protein 3</td>
<td>NM_009464</td>
<td>5′GGATCTGACCTTTTACTGAAATC3′</td>
<td>5′GGCGACGAGGGCAGTCCCA3′</td>
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<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
<td>NM_007988</td>
<td>5′TTGCTGGCACTACAGAACGTG3′</td>
<td>5′AACACCTTCAAGGGCAAT3′</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Transforming growth factor-β1</td>
<td>NM_011577</td>
<td>5′GCTGACGCTCTGCTGC3′</td>
<td>5′GCTGACGCTCTGCTGC3′</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
<td>NM_001217</td>
<td>5′CAGCTCACTGCGGCAATTAACT3′</td>
<td>5′GAGACAGCCACTTGTGAAGAC3′</td>
</tr>
<tr>
<td>BNP</td>
<td>Natriuretic peptide type B</td>
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<td>5′GGACAGGCTGACCTTTGGAAGAC3′</td>
<td>5′GAGACAGCCACTTGTGAAGAC3′</td>
</tr>
<tr>
<td>β-Tubulin</td>
<td>Tubulin, β</td>
<td>NM_011675</td>
<td>5′GGACAGGCTGACCTTTGGAAGAC3′</td>
<td>5′GAGACAGCCACTTGTGAAGAC3′</td>
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</tbody>
</table>
camera system. Detection of β-tubulin (1:2,000; Sigma) was included as an internal control reference. The arbitrary units of the blot signal were presented as net intensity × band area normalized to β-tubulin signal.

**Statistical analysis.** Data were expressed as means ± SE. Statistical analysis was performed by using Statistics Package for Social Science (SPSS) version 11.0. Differences among groups were evaluated by ANOVA, followed by Tukey’s honestly significantly different post hoc test. Statistical significance was set at $P < 0.05$.

**RESULTS**

**Echocardiographic parameters.** The representative echocardiographic M-mode images obtained in animals of control, doxorubicin, doxorubicin with desacetyl ghrelin treatment, and doxorubicin with desacetyl ghrelin plus [d-Lys3]-GHRP-6 treatment groups are shown in Fig. 1. Doxorubicin-induced cardiac dysfunction was illustrated by a significant decrease from 60.3 to 37.7% in the LV fractional shortening (Table 2). However, this doxorubicin-induced decrease was not found in the animals treated with desacetyl ghrelin (62.4 vs. 64.2%) or desacetyl ghrelin plus [d-Lys3]-GHRP-6 (68.2 vs. 62.7%) (Table 2). Similarly, the EF was decreased significantly from 86.2 to 65.8% after exposure to doxorubicin, but this decrease was not seen in animals treated with desacetyl ghrelin (87.6 vs. 88.1%) and desacetyl ghrelin plus [d-Lys3]-GHRP-6 (90.4 vs. 87.6%) (Table 2). Heart rate, left ventricular anterior wall thickness, posterior wall thickness, and end-diastolic dimension were observed to be significantly decreased following doxorubicin administration, but not all of these changes were found in the animals treated with desacetyl ghrelin (Table 2).

**Myocardial fibrosis and fibrotic regulatory factors.** As shown in Fig. 2, A and B, the fibrotic area in the LVs of the doxorubicin group was markedly increased (15-fold) compared with the control group ($P < 0.05$), and this doxorubicin-induced increase in the BNP expression was reduced by desacetyl ghrelin with or without [d-Lys3]-GHRP-6 ($P < 0.05$). Doxorubicin, desacetyl ghrelin, and [d-Lys3]-GHRP-6 had no effect on gene expression of TGFβ1 ($P > 0.05$; Fig. 2C). However, doxorubicin significantly increased (8-fold) CTGF expression compared with that in the control group (Fig. 2D). This doxorubicin-induced increase was significantly modulated by desacetyl ghrelin, and this modulation was not affected by [d-Lys3]-GHRP-6 (Fig. 2D). The BNP expression was significantly upregulated by doxorubicin, but this change was not affected by desacetyl ghrelin ($P > 0.05$). BNP mRNA level was found to be significantly reduced in the DOX + DAG + [d-Lys3]-GHRP-6 group relative to the DOX group (Fig. 2E).

**Myocardial apoptosis and apoptotic regulatory factors.** The number of myocardial apoptotic nuclei as indicated by the TUNEL index was increased (by 467%, $P < 0.05$) in ventricles of the doxorubicin group compared with those of the control group (Fig. 3A). The TUNEL index was lowered in the desacetyl ghrelin and desacetyl ghrelin plus [d-Lys3]-GHRP-6 groups relative to the doxorubicin group ($P < 0.05$; Fig. 3A). Consistently, the level of apoptotic DNA fragmentation was elevated (by 298%, $P < 0.05$) in the doxorubicin group relative to the control group, and this doxorubicin-induced increase was inhibited significantly by desacetyl ghrelin and desacetyl ghrelin plus [d-Lys3]-GHRP-6 (Fig. 3B). Caspase-3 protease activity was increased (by 32%, $P < 0.05$) in ventricles of the doxorubicin group compared with those of the control group, and this doxorubicin-induced increase was prevented by desacetyl ghrelin and desacetyl ghrelin plus [d-Lys3]-GHRP-6 (Fig. 3C). No significant change was found in the protein content of antiapoptotic Bcl-2 in ventricles of the doxorubicin group compared with the control group (Fig. 3D). However, Bcl-2 protein content was significantly higher (by 138%) in the desacetyl ghrelin and desacetyl ghrelin plus [d-Lys3]-GHRP-6 groups compared with the doxorubicin group (Fig. 3D). The protein content of prosapoptotic Bax was elevated by doxorubicin ($P < 0.05$), but this change was not affected by desacetyl ghrelin ($P > 0.05$) (Fig. 3E). Bax protein level was found to be significantly decreased in the DOX + DAG + [d-Lys3]-GHRP-6 group relative to DOX group (Fig. 3E). The protein expression ratio of Bcl-2 to Bax was further analyzed and found to be lowered (by 46%, $P < 0.05$) in the doxorubicin group compared with control group (Fig. 3F). This doxorubicin-induced decrease in the Bcl-2/Bax ratio was not seen in the desacetyl ghrelin and desacetyl ghrelin plus [d-Lys3]-GHRP-6 groups (Fig. 3F).

**Myocardial ERK/Akt signaling.** The ratio of phospho-ERK1/2-to-total ERK1/2 was significantly decreased (by 52%) in the doxorubicin group relative to the control group (Fig. 4A). This doxorubicin-induced decrease was not seen in the desacetyl ghrelin group (Fig. 4A). However, this restoration of ERK1/2 phosphorylation was inhibited by [d-Lys3]-GHRP-6 (Fig. 4A). The ratio of phospho-Akt to total Akt was reduced (by 28%, $P < 0.05$) in the doxorubicin group relative to the control group, but this decrease was not seen in the animals treated with desacetyl ghrelin or desacetyl ghrelin plus [d-Lys3]-GHRP-6 (Fig. 4B).

**Myocardial metabolic regulators.** The transcript expression of PPARα was increased (by 82%, $P < 0.05$) in the doxorubicin group compared with the control group. This doxorubi-
cin-induced increase was not observed in the desacyl ghrelin and desacyl ghrelin plus [D-Lys3]-GHRP-6 groups (Fig. 5A).

Similar to the pattern of PPARα, the transcript content of mCPT-1 was significantly elevated (by 45%) in the doxorubicin group relative to the control group. This increase was lowered in the desacyl ghrelin and desacyl ghrelin plus [D-Lys3]-GHRP-6 groups compared with the doxorubicin group (P < 0.05) (Fig. 5B). The transcript content of UCP3 was reduced (by 54%, P < 0.05) in the doxorubicin group relative to the control group. This doxorubicin-induced decrease was significantly restored in the desacyl ghrelin and desacyl ghrelin plus [D-Lys3]-GHRP-6 groups (Fig. 5C).

### Table 2. Echocardiographic parameter

<table>
<thead>
<tr>
<th>Echocardiographic parameter</th>
<th>Control (n = 9)</th>
<th>DOX (n = 11)</th>
<th>DOX + DAG (n = 10)</th>
<th>DOX + DAG + [D-Lys3]-GHRP-6 (n = 6)</th>
</tr>
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<tr>
<td>HR, beats/min</td>
<td>474 ± 10</td>
<td>483 ± 11</td>
<td>506 ± 10</td>
<td>509 ± 8</td>
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<td>AWT, cm</td>
<td>0.11 ± 0.00</td>
<td>0.10 ± 0.002</td>
<td>0.10 ± 0.003</td>
<td>0.09 ± 0.002</td>
</tr>
<tr>
<td>PWT, cm</td>
<td>0.10 ± 0.01</td>
<td>0.10 ± 0.004</td>
<td>0.11 ± 0.01</td>
<td>0.10 ± 0.01</td>
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<tr>
<td>LVEDD, cm</td>
<td>0.30 ± 0.01</td>
<td>0.31 ± 0.01</td>
<td>0.32 ± 0.01</td>
<td>0.33 ± 0.01</td>
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<tr>
<td>LVESD, cm</td>
<td>0.12 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>0.12 ± 0.01</td>
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<tr>
<td>FS, %</td>
<td>61.3 ± 1.8</td>
<td>65.4 ± 2.7</td>
<td>60.3 ± 2.1</td>
<td>62.4 ± 2.5</td>
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<td>EF, %</td>
<td>87.0 ± 1.3</td>
<td>89.3 ± 1.6</td>
<td>86.2 ± 1.3</td>
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Results are shown as means ± SE. DOX, doxorubicin; DOX + DAG, doxorubicin with treatment of desacyl ghrelin; DOX + DAG + [D-Lys3]-GHRP-6, doxorubicin with treatment of desacyl ghrelin plus [D-Lys3]-GHRP-6; HR, heart rate; AWT, anterior wall thickness; PWT, posterior wall thickness; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; FS, fractional shortening; EF, ejection fraction. Transthoracic echocardiography was conducted before the doxorubicin injection (Pre) and after the 4-day experimental period (Post) to examine the ventricular parameters. In control rats, saline (administration vehicle of doxorubicin) was administered. *P < 0.05 compared with the corresponding Pre.

Fig. 2. Myocardial fibrosis and fibrotic regulatory factor. Collagen deposition in left ventricle was examined by Masson’s trichome staining. A: collagen was stained blue in the 5-μm-thick frozen tissue transverse sections. B: fibrotic area was presented by %collagen accumulation to total area of the microscopic field. B–E: the mRNA expression of cardiac transforming growth factor-β (TGF-β; C), connective tissue growth factor (CTGF; D), and brain natriuretic peptide (BNP; E) was analyzed by real-time quantitative PCR. Data were expressed as expression ratio normalized to β-tubulin gene. In control rats, saline (administration vehicle of doxorubicin) was administered. Data are expressed as means ± SE (n = 6/group). *P < 0.05 compared with control; #P < 0.05 compared with DOX.
Fig. 3. Myocardial apoptosis and apoptotic regulatory factor. Myocardial apoptosis was examined by terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) in 5-μm-thick transverse sections prepared from frozen tissues at the apex of the left ventricle. The no. of TUNEL-positive cardiomyocyte nuclei relative to the total no. of nuclei were quantified as TUNEL index. A: apoptotic DNA fragmentation was examined in protein fraction extracted from ventricular tissue samples using the Cell Death Detection ELISA Kit. The extent of apoptotic DNA fragmentation was estimated by measuring the cytosolic mono- and oligonucleosomes. B: the optical density at 405 nm (OD405) was normalized to the amount of protein used in the assay. Caspase-3 activity was examined in protein fraction extracted from ventricular tissue samples by a fluorometric assay. C: caspase-3 protease activity was presented as the change of fluorescence intensity normalized to the total mg of protein used (C). D and E: the protein abundances of Bcl-2 (D) and Bax (E) were examined in cytoplasmic protein fraction extracted from cardiac tissues by Western immunoblot. Desitometric quantification was performed, and data were presented as net intensity resulting band area and expressed in arbitrary units. Results were normalized to corresponding β-tubulin signal. F: the ratio of the protein level of Bcl-2 to Bax was shown. In control rats, saline (administration vehicle of doxorubicin) was administered. Data are expressed as means ± SE (n = 6/group). *P < 0.05 compared with control; #P < 0.05 compared with DOX.
of FAS was not significantly affected by doxorubicin and was not significantly different among all groups (Fig. 5D). The protein abundances of PGC-1α and the ratios of phospho-AMPKα to total AMPKα, MCAD, and ACSL1 were not significantly affected by doxorubicin and desacyl ghrelin in all groups (Fig. 6).

**DISCUSSION**

Desacyl ghrelin prevents doxorubicin-induced impairment of cardiac function. Doxorubicin induces cardiomyopathy, which presents a major clinical challenge to its wide therapeutic application in oncology (16). Previous studies demonstrated that the acute cardiotoxicity can be induced by doxorubicin with a single dose of 15–20 mg/kg doxorubicin (5, 42, 66, 68). The experimental period varied from 1 to 7 days after the injection of doxorubicin (5, 62, 66, 68). Additionally, our preliminary study demonstrated that desacyl ghrelin showed the protective effect on cardiac dysfunction induced by doxorubicin when measurements were taken at 4 days after the administration of doxorubicin (5, 62, 66, 68). Taken together, the acute cardiotoxicity was examined at 4 days after the administration of doxorubicin, with a dosage of 15 mg/kg doxorubicin in the present study. Our data indicate that treatment of desacyl ghrelin is effective in preventing the cardiac contractile dysfunction induced by doxorubicin.

Previously, the in vivo data of ghrelin showing cardiac improvement were limited to the acylated form of ghrelin. Ghrelin (acylated) has been shown to improve infarction-induced ventricular dysfunction and ventricular remodeling by inhibiting inflammatory response and expression of matrix metalloproteinases in rats (20). Ghrelin (acylated) has also been demonstrated to protect against ischemia-reperfusion myocardial injury in a rat isolated heart model (6, 17). In a later study, ghrelin (acylated) was demonstrated to protect the isolated rat heart against ischemia-reperfusion injury by attenuating myocardial apoptosis and inhibiting myocardial endoplasmic reticulum stress, as indicated by the decreased glucose-regulated protein-78, C/EBP homologous protein, and caspase-12 (63). With regard to heart failure, ghrelin (acylated) has been shown to improve ventricular dysfunction and ameliorate ventricular remodeling and cardiac cachexia in rats with chronic heart failure induced by experimental ligation of the left coronary artery (38). Specifically for doxorubicin cardiotoxicity, there is in vivo evidence that the endogenous ghrelin (acylated) level is increased during the progression of heart failure induced by doxorubicin (60), leading to the suggestion that ghrelin (acylated) might contribute to a compensatory self-protective mechanism that is linked to antiapoptosis and antioxidative mechanisms and maintains cardiac function (60). Notably, the functional effects of desacyl ghrelin on cardiac disorders are relatively unidentified, although some in vitro data suggest the beneficial effects of desacyl ghrelin on doxorubicin cardiotoxicity by inhibiting cardiomyocyte cell death (2). Here, our study provides novel functional evidence and
molecular insights that substantiate physiological protection by desacyl ghrelin in doxorubicin cardiotoxicity.

Echocardiographic functional data are physiologically important to confirm the cardioprotective role of desacyl ghrelin. Although echocardiographic examinations were usually performed under anaesthesia with injected anaesthetics in previous studies, ketamine administered at a dose of 100 mg/kg has been suggested to be an acceptable anaesthesia (57). Of note, cardiac ventricular function was measured by echocardiography in anesthetized mice, with ketamine HCl at a dosage of 80 mg/kg administered by intraperitoneal injection in the present study. The cardiac function was determined at a stable status, with the minimal cardiodepressant effects observed according to the structural and contractile results obtained from our echocardiographic examinations.

Antifibrotic effect of desacyl ghrelin on doxorubicin cardiotoxicity. Fibrosis has been suggested to be involved in cardiac stiffness and dysfunction in doxorubicin cardiotoxicity (36). The increased collagen synthesized by the fibroblasts invades and replaces the necrotic or apoptotic myocytes (14). In the present study, we observed that doxorubicin-induced fibrosis was prevented by desacyl ghrelin, as demonstrated by the attenuation of collagen deposition (Fig. 2, A and B). Previously, ghrelin (acylated) treatment has been shown to ameliorate bleomycin-induced acute lung fibrosis (22) and liver fibrogenesis (23, 37). Ghrelin-deficient mice have also been shown to be more susceptible to the development of liver fibrosis and hepatocellular damage after CCL4-induced chronic liver injury (37). Our present finding that desacyl ghrelin protects the heart from doxorubicin by preventing myocardial fibrosis extends the previous demonstration of the effects of ghrelin (acylated) on preventing early left ventricle collagen volume increase in rats with myocardial infarction (48). Additionally, our data exhibit that the inhibitory effect of desacyl ghrelin on the doxorubicin-induced myocardial collagen deposition is mediated through mechanisms that are not associated with the GHSR pathway (Fig. 2, A and B). Consistent with our observations, Li et al. (33) have reported that isoproterenol-induced cardiac fibrosis is relieved by both acylated and desacyl ghrelins in rats. Our present data further demonstrate the antifibrotic role of desacyl ghrelin in doxorubicin myocardial toxicity via GHSR-independent pathway. Of note is that the effect of [D-Lys3]-GHRP-6 itself on fibrosis has not been examined in the present study. Although [D-Lys3]-GHRP-6 has been suggested to exert activities such as stimulation of calcium increase (15), decrease in food intake (1), and reduction of alcohol intake in mice (24), it is unlikely that [D-Lys3]-GHRP-6 would have beneficial effects on the doxorubicin-treated heart. Nonetheless, [D-Lys3]-GHRP-6 has been reported to abolish the antifibrotic effect of hexarelin on interstitial and perivascular fibrosis in the heart of spontaneously hypertensive rats (58), whereas the decrease in cardiac fibrosis induced by desacyl ghrelin in the doxorubicin-treated heart in this study was not found to be abolished by [D-Lys3]-GHRP-6 (Fig. 2, A and B).
CTGF, a profibrotic cytokine, has been shown to be upregulated by TGFβ in the heart with myocardial infarction (7), carotid artery balloon injury (21), and type 1 diabetic cardiomyopathy (41). Wang et al. (53) have found that the interaction between TGFβ and CTGF is important for the development of multiorgan fibrosis, renal fibrosis, and pulmonary fibrosis. In the present study, doxorubicin-induced increase in CTGF expression in the heart is seen in the absence of the increase in TGFβ1 expression (Fig. 2, C and D). This is consistent with the notion that fibrosis in kidney and heart may not be fully regulated by TGFβ but essentially dependent on CTGF (54, 65). Our findings suggest that CTGF might play an important role in doxorubicin-induced fibrotic injury through a TGFβ-independent pathway. BNP serves as an antifibrotic factor by activating guanylyl cyclase-A (51) or interacting directly with CTGF (26). In the present study, stimulation of BNP expression might be a compensatory response to doxorubicin. The balance between CTGF and BNP in cardiomyocytes has been suggested to be a regulatory factor of cardiac fibrosis (26). Our data show that desacyl ghrelin decreases the transcript abundance of CTGF (Fig. 2D), which partly explains the effect of desacyl ghrelin on preventing fibrosis induced by doxorubicin.

**Antipapoptotic effect of desacyl ghrelin on doxorubicin cardiotoxicity.** Doxorubicin exposure causes activation of mitochondrial apoptosis in cardiomyocytes (9). Doxorubicin-induced cardiac dysfunction has been shown to be improved by interventions that inhibit apoptosis in cardiomyocytes, such as administration of erythropoietin and transgenic overexpression of the Nd1 Kelch family protein in the heart (8, 35). Previously, the antipapoptotic effect of ghrelin has been reported in an in vitro study showing that both acylated and desacyl ghrelinins inhibit doxorubicin-induced apoptosis in H9c2 cardiomyocytes (2). Consistent with these in vitro findings, the presently observed improvement of the doxorubicin-induced cardiac dysfunction with desacyl ghrelin was accompanied by the suppression of the TUNEL index, apoptotic DNA fragmentation, and protease activity of caspase-3 (Fig. 3, A–C). This is in line with the observed Bcl-2/Bax ratio, which is significantly decreased by doxorubicin, a change that is prevented by desacyl ghrelin (Fig. 3F). It is noted that our findings also agree with the previous in vitro findings demonstrating that desacyl ghrelin is protective against apoptosis in isolated cardiomyocytes (30). Of note is that the antipapoptotic effect of desacyl ghrelin on doxorubicin-treated heart was not found to be abolished by [D-Lys3]-GHRP-6 in the present study. According to the findings suggesting that [D-Lys3]-GHRP-6 might promote apoptosis in dorsal root ganglia cells (15) and that [D-Lys3]-GHRP-6 was shown to abolish the antipapoptotic effect induced by acylated ghrelin in human umbilical vein endothelial cells (67), it is unlikely that our observed responses of apoptosis and apoptotic regulatory factors in the hearts of the DOX + DAG + [D-Lys3]-GHRP-6 group (relative to DOX group) were attributed to [D-Lys3]-GHRP-6. Nonetheless, the direct effects of GHSR1a antagonist [D-Lys3]-GHRP-6 on doxorubicin-induced cardiac apoptosis were not examined in this study.

Experimental expression of constitutively active PI3K has been demonstrated to inhibit the activation of caspase-3 and apoptosis in cardiomyocytes (55). There are in vitro data showing that acylated and desacyl ghrelinins inhibit doxorubicin-induced apoptosis by activating the intracellular prosurvival signaling pathways conveyed by ERK1/2 and PI3K/Akt in cultured cardiomyocytes (2). Also, desacyl ghrelin has been shown to activate Akt activities in skeletal muscle of mice (40). Consistent with these findings, our data show that the phosphorylation statuses of ERK1/2 and Akt are suppressed in the heart tissues in response to doxorubicin exposure, and these suppressions are opposed by desacyl ghrelin (Fig. 4). Of note is that inconsistent responses of ERK1/2 signaling to doxorubicin exposure have been reported previously. Liu et al. (34) have shown that doxorubicin induces time-dependent activation of ERK1/2 from 1 to 48 h of incubation in H9c2 cardiomyocytes. However, the level of phosphorylated ERK1/2 was observed to be reduced in neonatal rat cardiomyocytes at 24 h after doxorubicin treatment (64). Xiang et al. (56) also reported that the level of ERK activation was decreased at week 11 in rat heart with chronic doxorubicin administration. Our present ERK data are in accord with the previous results showing that ERK1/2 phosphorylation in heart was inhibited by a single administration of doxorubicin with a dose of 15 mg/kg in mice (32). The observation that the reverse of Akt activation induced by the treatment of desacyl ghrelin is not affected by the GHSR antagonist [D-Lys3]-GHRP-6 (Fig. 4B) suggests that desacyl ghrelin activates Akt signaling through a GHSR-independent pathway. However, according to our analysis, the desacyl ghrelin treatment-induced increase in ERK1/2 phosphorylation was surprisingly found to be inhibited by GHSR antagonist [D-Lys3]-GHRP-6 (Fig. 4A). The treatment of [D-Lys3]-GHRP-6 has been shown to inhibit ERK1/2 activation induced by acylated ghrelin in primary oligodendrocytes (31). Nonetheless, the expression of ERK1/2 was demonstrated to be increased in porcine ovarian granulosa cell after being treated with [D-Lys3]-GHRP-6 (44). In the present study, the observed inhibition of ERK1/2 phosphorylation by [D-Lys3]-GHRP-6 suggests that the desacyl ghrelin-induced restoration of ERK1/2 phosphorylation in doxorubicin-treated cardiomyocytes might be associated with GHSR-dependent mechanisms. Although the exact mechanisms remain to be elucidated, these prosurvival signaling results are generally in support of the changes in Bcl-2/Bax ratio, suppression of caspase-3 activity, and inhibition of myocardial apoptosis in the heart treated with desacyl ghrelin following doxorubicin exposure (Fig. 3). Collectively, our data suggest that desacyl ghrelin prevents the activation of myocardial apoptosis induced by doxorubicin, and the antipapoptotic effect of desacyl ghrelin is probably mediated through cellular signaling of the ERK1/2 and PI3K/Akt pathways.

In conclusion, the present investigation demonstrated that desacyl ghrelin significantly modulated several cardiotoxic
effects of doxorubicin, including contractile dysfunction, myocardial fibrosis and apoptosis, suppression of cellular pro-survival signaling, and disruption of some myocardial metabolic regulators, and this was probably mediated through a GHSR-independent pathway. These results are consistent with the hypothesis that desacyl ghrelin protects the heart against doxorubicin-induced cardiomyopathy. It is worth noting that our data were collected in a mouse model of acute doxorubicin cardiotoxicity (i.e., 4 days after a single administration of doxorubicin). Further research is warranted to further investigate the cardioprotective effects of desacyl ghrelin in response to chronic prolonged exposure to doxorubicin. Furthermore, the potential clinical application of desacyl ghrelin in resolving or preventing the cardiotoxic effect of doxorubicin during chemotherapy is worthy of further exploration.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

X.P., B.Y.Y., S.-p.Y., and P.M.S. contributed to the conception and design of the research; X.P. performed the experiments; X.P. analyzed the data; X.P., B.Y.Y., S.-p.Y., M.Y., I.F.B., and P.M.S. interpreted the results of the experiments; X.P. and P.M.S. prepared the figures; X.P. and P.M.S. drafted the manuscript; X.P., B.Y.Y., S.-p.Y., M.Y., I.F.B., and P.M.S. edited and revised the manuscript; X.P., B.Y.Y., S.-p.Y., M.Y., I.F.B., and P.M.S. approved the final version of the manuscript.

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