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A Novel Function of Bamboo Extract in Relieving Lipotoxicity

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Abstract

Lipotoxicity is closely related to the etiology and complications of type 2 diabetes mellitus. This study investigated the protective effect of an extract from bamboo Phyllostachys edulis against palmitic acid (PA)- induced lipoapoptosis. The lipo-detoxification function of the bamboo extract (BEX) was evaluated using cell culture models. Cell viability was measured by MTT assay and cell apoptosis was monitored by Annexin V staining. Cellular uptake of fluorescent free fatty acid (FFA) analog was measured by flow cytometry. Protein levels of total protein kinase B (Akt) and phosphorylated Akt (p-Akt) were measured by western blotting. The results show that co-incubating BEX with mouse myoblast C2C12 cells had no effect on the cellular uptake of FFA, but dramatically decreased PA-induced cell apoptosis and protected cell viability. A similar antilipotoxicity effect of BEX was observed in other mammalian cells. BEX significantly decreased the protein levels of both Akt and p-Akt in C2C12 cells under normal cell culture conditions but not under lipotoxic conditions, indicating the regulatory effect of BEX on cell signaling pathways and its response to a high FFA environment. This study demonstrated a novel function of bamboo extract in preventing lipotoxicity in mammalian cells, implicating a promising phytotherapeutic approach for lipo-detoxification.

Keywords

bamboo extract (BEX); palmitic acid; lipotoxicity; lipoapoptosis; free fatty acid; protein kinase B (Akt); phosphorylated protein kinase B (p-Akt)

INTRODUCTION

The prevalence of type 2 diabetes mellitus (T2DM) is high and increasing (Boyle et al., 2001). As one of the diabetic complications, diabetic cardiomyopathy is the leading cause of death in human diabetics (Kannel et al., 1974). A suggested etiology of this heart disease is cardiomyocyte lipoapoptosis (Frustaci et al., 2000). The cardiac apoptosis has been linked to an overload of fatty acid (FA), specifically saturated FA such as palmitic acid (PA), which accounts for 30–40% of total plasma FA (Sparagna et al., 2000). Exposure to high concentrations (70–1000 **μ**M) of long chain saturated FA also induce cell death in other types of cell, such as pancreatic-cells (Shimabukuro et al., 1998), fibroblasts, endothelial cell monolayers (Rosenthal, 1981; Zhang et al., 1992), ventricular cells (de Vries et al., 1997),

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retinal pericytes (Cacicedo et al., 2005) and skeletal muscle cells (Rachek et al., 2007). Lipotoxicity in muscle cells induces insulin resistance, which is a key factor in the etiology of T2DM (Krssak et al., 1999; Perseghin et al., 1999).

Bamboo is a large perennial grass distributed widely from tropical to subarctic zones. In Asian countries, different parts of bamboo have been used for medicinal purposes to treat hypertension, arteriosclerosis, cardiovascular disease and certain forms of cancer. Recent studies demonstrated extracts from bamboo leaves have antioxidant activities and are non-toxic (Xu et al., 2001; Lu et al., 2005; Lu et al., 2006).

The effort of our research team has been directed toward exploring the preventing/therapeutic effects of natural products that convey of high cost-efficiency and ease of widespread dissemination on T2DM. This study investigated the lipo-detoxification effect of an ethanol extract from bamboo Phyllostachys edulis (Carrière) J. Houz. Var. heterocycla J. Houz. [Place of publication: Bambou (Mons) 1:40. 1906]. This is a species known for its fast growth, wide distribution and huge biomass.

MATERIALS AND METHODS

Chemicals and instruments

BODIPY-labeled lauric acid (BLLA) was purchased from Molecular Devices (Sunny Vale, CA); Vybrant Apoptosis Assay Kit #2 was purchased from Invitrogen (Carlsbad, CA); antibodies to total Akt (Cat# 9272) and p-Akt (Cat# 9275S) were purchased from Cell Signaling Technology (Danvers, MA), antibody to alpha tubulin (Cat# DM1A) was purchased from Novus Biologicals (Littleton, CO); Criterion gels were purchased from Biorad (Hercules, CA); ECL plus detection system was purchased from Amersham (Piscataway, NJ); CelLytic MT kit and other chemicals were purchased from Sigma (St Louis, MO).

A Beckman Coulter Epics XL-MCL flow cytometer (Fullerton, CA) was used to measure the cellular uptake of BLLA and Annexin V labeled apoptosis evens; a Perkin Elmer (Wellesley, MA) Victor 2 plate reader was used for the MTT-based cell viability assay.

Cell culture

Monkey kidney Vero cells were maintained in medium 199 (M199) containing 25 mM of glucose with 5% fetal bovine serum (FBS) supplement; C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, supplemented with 10% FBS; myotubes were differentiated from C2C12 cells by incubating with DMEM containing 2.5% horse serum for 7 days. The cells were maintained in incubators with 5% CO2, 55% relative humidity, at 37 °C. Penicillin/streptomycin (50 **μ**g/mL) was used as antibiotic. All cells were plated in multiwall tissue culture plates 20–24 h before treatment.

Preparation of palmitic acid suspension

PA was dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution of 0.4 M. Before applying to the cells, the required amount of the stock solution was added into culture media and thoroughly dispersed by sonication.

Preparation of bamboo extract

The raw extract used in this study was provided by Golden Basin LLC (Hauula, Hawaii). It was made from fresh leaves and small branches of Phyllostachys edulis in Hunan Province, China. The extraction was in close accordance to a patented procedure (publication number: CN 1287848A). Seventy percent (70%) ethanol was used as an extraction solvent and the ratio of raw materials to the product was 100:7. The raw extract, upon arriving in the laboratory, was further freeze dried and ground into fine powder. The fine powder was further extracted with 100% ethanol (100 mg/mL) at room temperature for 3 h, followed by centrifugation. The supernatant containing ethanol soluble portion of the raw extract was used in this study. This final extract (BEX) was added to cell culture media for up to 0.5% (v/v) to test its lipodetoxification function. An equal amount of ethanol or DMSO was added to media as solvent control.

MTT-based cell viability assay

After desirable treatments, the cells were washed once with warm PBS and incubated with normal medium containing 0.5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) for 3 h, and then a suitable amount of isopropanol containing 0.1 M HCl was added to each well to dissolve the MTTderived formazan crystal. The absorbances at 570 and 690 nm were measured to indicate cell viability and background, respectively.

Flow cytometric assay on apoptosis

The PA-induced apoptosis in C2C12 cells was analysed with a Vybrant apoptosis assay kit #2 following the manufacturer’s instruction.

Flow cytometric assay on cellular FFA uptake

Cell culture media containing the fluorescent FFA analog BLLA was prepared in the same way as PA-containing media. C2C12 cells were incubated with media containing BLLA or the same volume of DMSO, in combination with 0.5% BEX or 0.5% ethanol for 1 and 4 h, then thoroughly washed with warm PBS, trypsinizedresuspended into serum-free DMEM, and kept on ice and protected from light until flow cytometric assay.

Semi-quantitation of protein levels of Akt and p-Akt using western blotting

After suitable treatment, the total protein was harvested from the cells using a CelLytic MT kit supplemented with DTT, proteinase inhibitor and EDTA. Samples were mixed with loading buffer, heated at 100 °C for 5 min and chilled on ice for 5 min. Criterion gels were loaded with the denatured protein samples and run at 200 V for a suitable time. The proteins were transferred to nitrocellulose membrane at 90 mA overnight. Membranes were washed with TBST buffer, blocked with 5% milk in TBST, incubated with primary antibody diluted in blocking solution overnight, washed with TBST and incubated with secondary antibody diluted in blocking solution for 1 h, and then washed with TBST again. ECL plus detection system was used to detect the signal.

Statistical analysis

The **t**-test was used for the statistical analysis. A value of $\mathbf{p} \leq 0.05$ was considered as statistically significant. In the figures, $0.01 < \mathbf{p} \leq 0.05$ is shown by *, $0.001 < \mathbf{p} \leq 0.01$ is shown by **, and $\mathbf{p} \leq 0.001$ is shown by ***.

RESULTS

BEX prevented PA-induced cytotoxicity

C2C12 cells were exposed to 0.4 mM PA in the presence of either BEX or ethanol at different dosages. After 26 h of treatment without BEX protection, most of the cells died, regardless of the volume of ethanol applied (represented in Fig. 1B). In contrast, BEX protected the cells in a dose-dependent manner (Fig. 1C, D). The same protective effects were observed regardless of the solvents (ethanol or DMSO) used to reconstitute BEX, indicating that the lipodetoxification function of BEX was independent of the solvent used. Similar protective effects of BEX were observed in myotubes and Vero cells under challenge of PA. Cell viabilities of different cells co-incubated with BEX or ethanol during the PA treatment were analysed using MTT assay and are shown in Fig. 2A, B.

BEX prevented PA-induced lipoapoptosis

After incubating C2C12 cells with 0.4 mM PA in combination with BEX or ethanol for 16 h, the apoptotic status of the cells were analysed. Figure 3 shows that 16 h of PA treatment induced about 30% of apoptosis in C2C12 cells, adding 0.2–0.5% of ethanol had no significant effect, but addition of 0.2% BEX decreased the apoptotic rate to 17.3%, and 0.5% BEX brought the rate further down to 5.6%.

BEX did not affect the cellular uptake of FFA

C2C12 cells were incubated with media containing BLLA in the presence of 0.5% BEX or ethanol for 1 or 4 h, and the cellular uptake of BLLA was measured by flow cytometry (Fig. 4A). BEX had no significant effect on the fluorescent intensity caused by the intracellularization of BLLA.

The influence of BEX on total Akt and pAkt

The protein levels of Akt, p-Akt and alpha-tubulin were monitored using western blotting in C2C12 cells incubated with normal medium containing 0.5% BEX or 0.5% ethanol for 4 h, or medium containing 4 mM PA in the presence of 0.5% BEX or 0.5% ethanol for 4 h. The intensities of the bands were normalized against alpha-tubulin that served as a loading control.

After incubating with normal medium containing 0.5% BEX for 4 h, the protein level of total Akt decreased by 22% ($p = 0.034$, Fig. 4B) and that of pAkt decreased by 14% ($p = 0.016$, Fig. 4C). However, when exposed to a high level of FFA, BEX increased the protein level of total Akt by 13% (Fig. 4D) and that of pAkt by 5% (Fig. 4E), although this change was not statistically significant.

DISCUSSION

This study demonstrated a potent lipodetoxification function of BEX in mammalian cell lines. BEX showed no effect on cellular FFA uptake, therefore the antilipoapoptosis effect must be carried out through intracellular pathway(s).

The serine/threonine kinase Akt is a critical signaling factor in higher eukaryotic cells and is one of the most important and versatile protein kinases at the core of human physiology and disease (including type-2 diabetes and cancer). Phosphorylation of Akt leads to its activation and consequently affects various cellular processes such as survival, growth, proliferation, glucose uptake, angiogenesis and metabolism (Manning and Cantley, 2007). Akt signaling regulates lipid metabolism through phosphorylation and inhibition of glycogen synthase kinase 3 (GSK3). Phosphorylation of substrates by GSK3 often targets them for proteasomal degradation, and GSK3 has been shown to promote degradation of the sterol regulatory elementbinding proteins (SREBPs), which are transcription factors that turn on the expression of genes involved in cholesterol and fatty acid biosynthesis (Sundqvist et al., 2005). Therefore, by decreasing total Akt level and subsequently p-Akt level, BEX may decrease SREBPs stability and inhibit lipid production under normal cell culture conditions. However, when exposed to a lipotoxic environment, BEX tends to increase the protein levels of both total Akt and pAkt, which will in turn promote cell survival and inhibit apoptosis (Manning and Cantley, 2007). In summary, BEX has a profound influence on cell signaling pathways through regulating total Akt and subsequently pAkt protein levels, and this regulation response to the FFA content in the environment.

Whether BEX treatment can modify the capacity of fatty acid oxidation in the cells, another metabolic pathway reportedly associated with lipotoxicity (Kelley et al., 1999; Simoneau et al., 1999; Kelley et al., 2002), is to be further investigated.

This is one of the few studies revealing the inhibitory effect of a natural product on lipoapoptosis. The in vitro data obtained indicate BEX as a promising candidate for a phytotherapeutic strategy for T2DMassociated lipotoxicity. But the in vivo function of BEX as a dietary supplement is to be tested in animal models, which will further reveal the bioavailability and metabolism of BEX.

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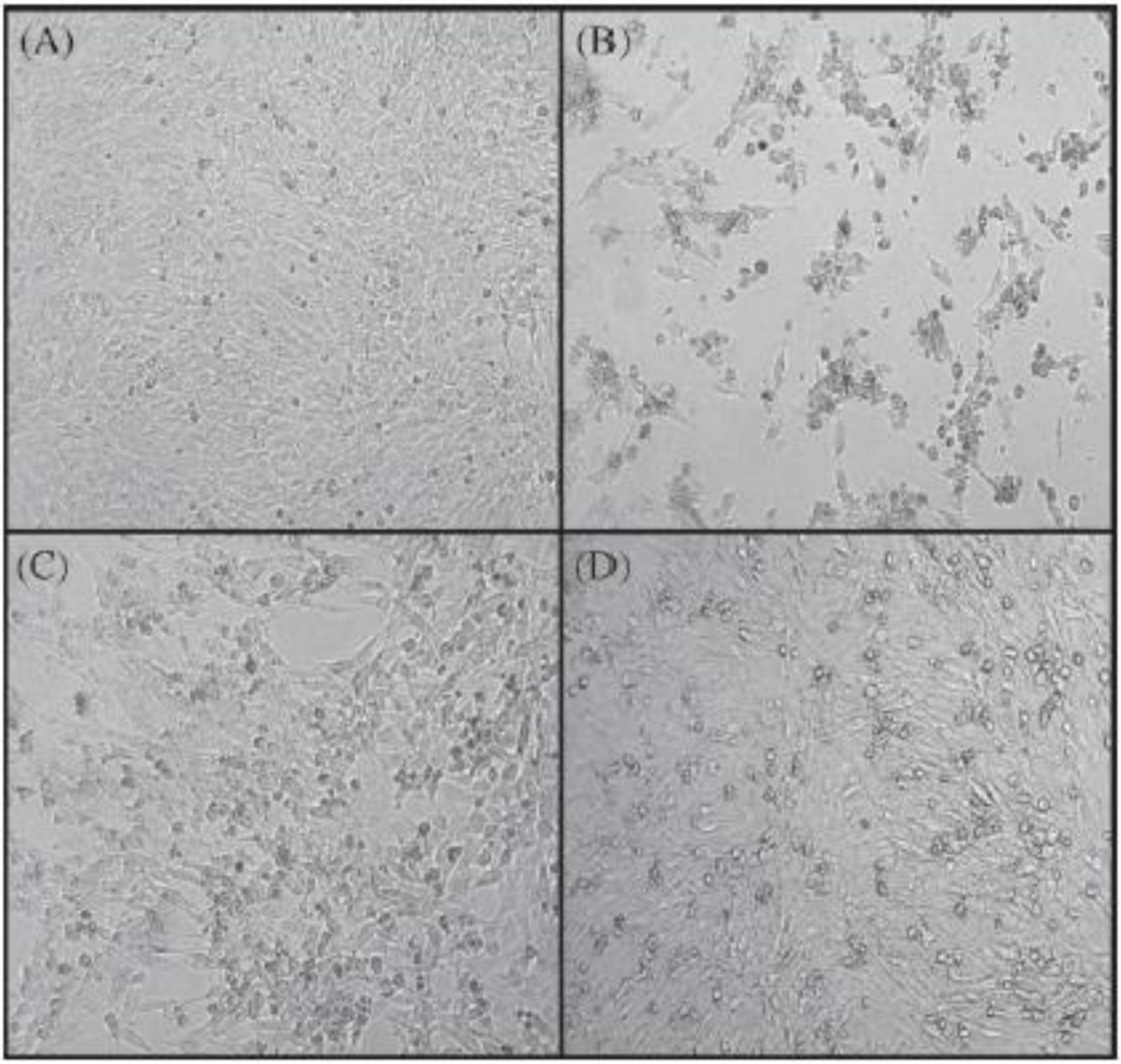

Figure 1.

The morphology of C2C12 cells after exposure to 0.4 mM PA for 26 h. (A) PA free medium + 0.3% (v/v) ethanol as control; (B) 0.4 mM PA + 0.3% ethanol; (C) 0.4 mM PA + 0.3% BEX; (D) 0.4 mM PA + 0.5% BEX.

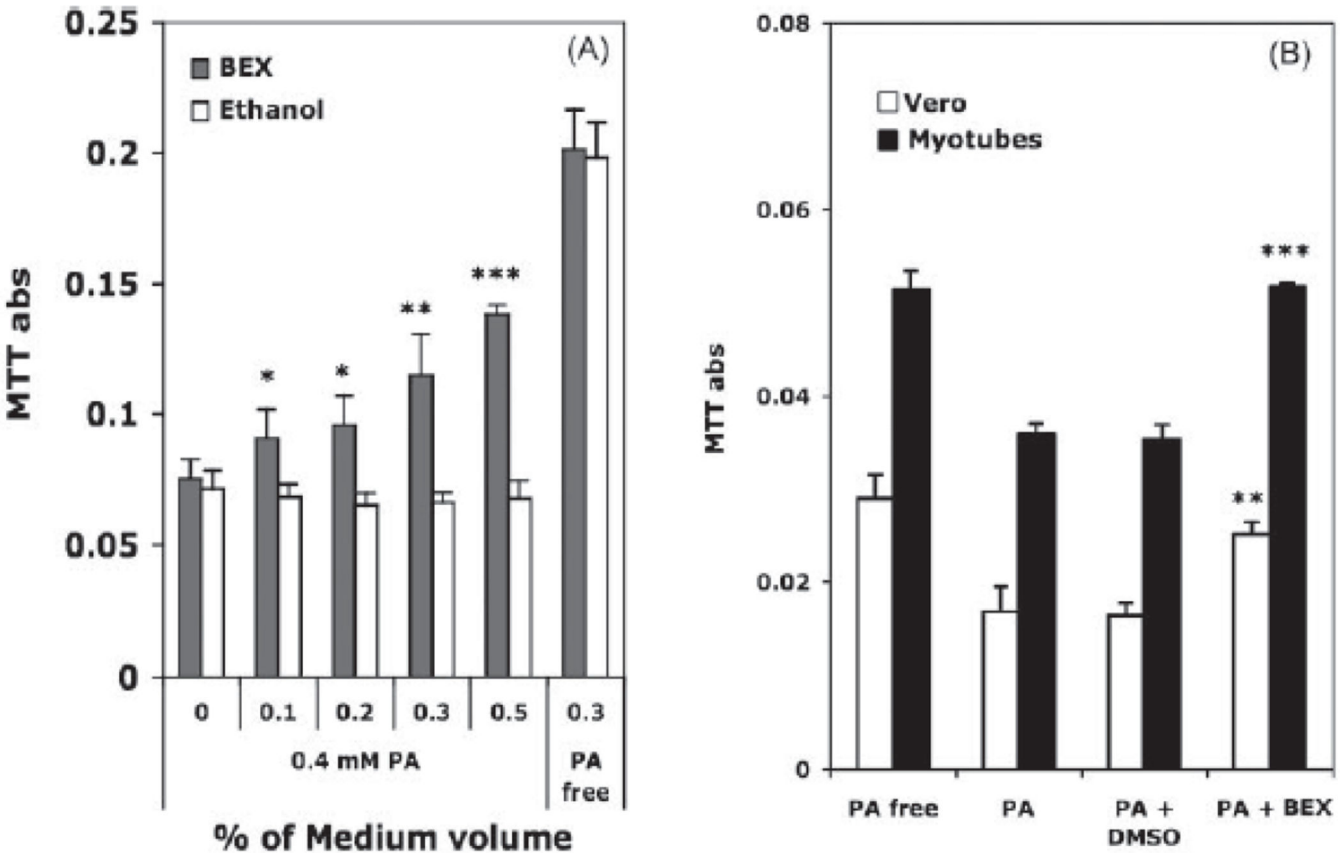

Figure 2.

The MTT-based cell viability of C2C12, myotubes differentiated from C2C12 and Vero cells after PA treatment. (A) C2C12 cells exposed for 26 h to 0.4 mM PA in combination with 0–0.5% (v/v) of BEX or ethanol; PA-free medium + 0.3% BEX or ethanol were used as control. Average ± SD, n = 3. Samples treated with BEX were compared with those treated with ethanol at the same concentration. (B) Myotubes differentiated from C2C12 cells and Vero cells were treated for 24 h with PA-free media, media containing 0.4 mM PA, or 0.4 mM PA in combination with 0.5% BEX or DMSO. Average ± SD, n = 3. Samples treated with PA and BEX are compared with those treated with PA and DMSO.

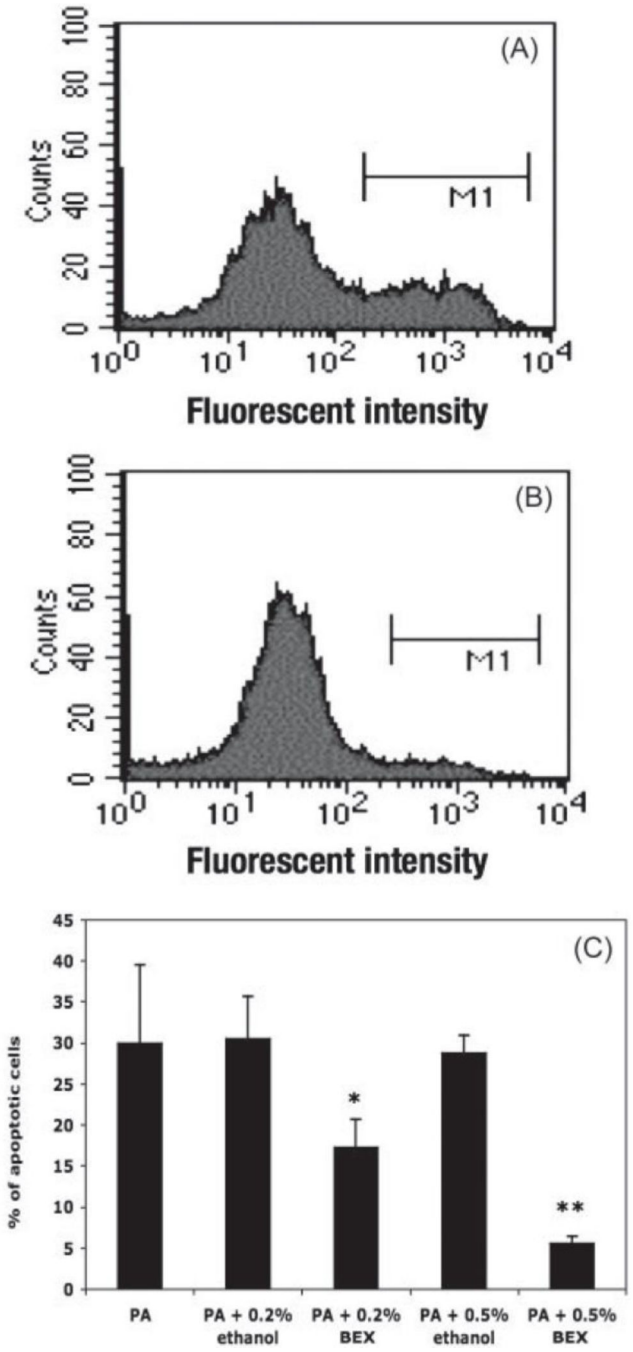

Figure 3.

The apoptosis assay on C2C12 cells after treated by 0.4 mM PA for 16 h in the presence of 0.2% to 0.5% of BEX or ethanol (control). The cells were stained by Annexin V labeled with fluorophore and the fluorescence intensity of the cells was measured by flow cytometer. The histogram in Panel A shows that 30.5% of the control cells treated with PA and 0.5% ethanol became apoptotic as indicated by region M1. Replacing ethanol with 0.5% BEX reduced the apoptotic rate to less than 5.6% (Panel B). The results are summarized in

Panel C, average ± SD, n = 3. Samples treated with BEX were compared with those treated with ethanol at the same concentration.

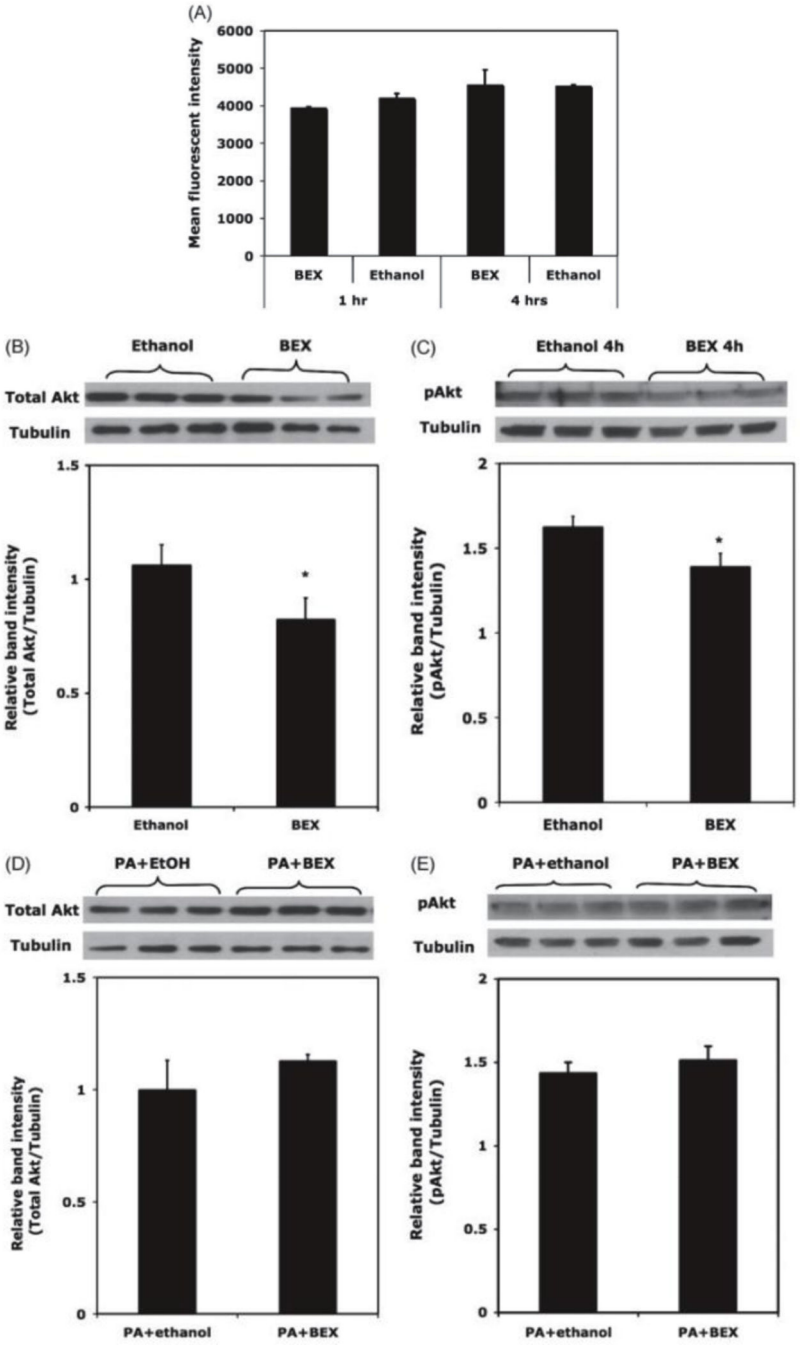

Figure 4.

Influence of BEX on cellular properties of C2C12 cells. (A) BEX did not alter the cellular uptake of FFA. C2C12 cells were incubated with BLLA in the presence of 0.5% BEX or ethanol for 1 and 4 h. The intracellular level of BLLA is indicated by the mean fluorescent intensity. The background was adjusted with DMSO treated cells. Average ± SD, n = 3. (B, C) BEX significantly decreased the protein level of total Akt and phosphorylated Akt in C2C12 cells under normal cell culture condition. C2C12 cells were incubated with normal medium containing 0.5% BEX or 0.5% ethanol for 4 h. The protein levels of total Akt and

p-Akt were normalized against alpha-tubulin. Average ± SD, n = 3. (D, E) BEX tended to increase the protein level of total Akt and phosphorylated Akt in C2C12 cells under lipotoxic condition. C2C12 cells were incubated with medium containing 0.4 mM PA in the presence of 0.5% BEX or 0.5% ethanol for 4 h. The protein levels of total Akt and p-Akt were normalized against alpha-tubulin. Average ± SD, n = 3.