REPORT

Ouabain inhibits RAW264.7 cells proliferation and induces apoptosis via Bcl-2 and bax expression

Yi Luo[1], Hongxia Qin[2], Lin Zhao[2], Long Jin[1], Anan Jiang[1], Xuewei Li[1], Mingzhou Li[1] and Xun Wang[1]

[1]Institute of Animal Genetics & Breeding, College of Animal Science & Technology, Sichuan Agricultural University, Chengdu, Sichuan, China

[2]College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan, China

Abstract: Ouabain is used in the clinical treatment of the cardiovascular diseases and it can be extensively investigated due to its promising pharmacological, pathological, and physiological properties. The present study is aimed to assess the growth and apoptotic potential of ouabain in RAW 264.7 cells. Varying concentrations of ouabain were applied to cultured RAW 264.7 cells for different time. At 0.01 to 1.00mM doses, ouabain inhibited the proliferation and presented cytotoxicity in both dose- and time-dependent manner. The compound also increased apoptosis after 24 and 48 hours of exposing to 1.00mM dose. Furthermore, the expression of Bcl-2 mRNA significantly decreased following ouabain treatment while Bax mRNA expression increased significantly. In brief, ouabain inhibits cell growth, exerts cytotoxicity and induces apoptosis in RAW 264.7 cells by down-regulating the Bcl-2/Bax ratio at mRNA level.

Keywords: Ouabain, RAW 264.7, apoptosis, Bcl-2, Bax

INTRODUCTION

Ouabain, derived from a plant *Strophanthus gratus*, has been used in the clinical treatment of hypertension, cardiac failure and supraventricular arrhythmias for many years. In recent years, there is widespread confirmation that ouabain not only exists in plants but also presents in mammals (Ferrandi *et al*., 1997), which as a steroid hormone synthesized in the adrenal gland (Laredo *et al*., 1994). The synthesis and secretion of endogenous ouabain will be increased by hypoxemia (Paci *et al*., 2000), low-renin hypertension (Manunta *et al*., 1999) and other stimulus, and participates in many important physiological and pathological processes (Hamlyn and Manunta, 2011; Jacobs *et al*., 2012). Nowadays, the role of ouabain becomes more remarkable, *i.e.* ouabain is the specific and potent inhibitor of the sodium pump, plays an important role in inhibition of sodium-potassium exchange (Morth *et al*., 2011). At the same time, it has been indicated that ouabain was involved in the pathogenesis of the hemolytic uremic syndrome (HUS), hypertension, and other diseases (Burlaka *et al*., 2013; Manunta *et al*., 1999). It has also been suggested that cell proliferation and apoptosis could be regulated by ouabain. *In vitro* culture studies demonstrated that the growth of different types of cells, including rat cardiac myocytes (Bai *et al*., 2013), canine smooth muscle cells (Aarhus *et al*., 1983), and a variety of tumor cells (Da Silva *et al*., 2014; Pezzani *et al*., 2013) were modified by exposing to different concentrations ouabain. Analogous studies have indicated the dual effects of ouabain on proliferation and apoptosis (Ren *et al*., 2014; Winnicka *et al*., 2010). On the one hand, low dose of ouabain could promote cell proliferation and on the other hand the increase in concentration of ouabain could lead to the promotion of apoptosis. The study on a variety of cancer cells indicated that ouabain and ouabain-like cardiac glycosides could promote cancer cells death (Da Silva *et al*., 2014; Joshi *et al*., 2011; Kulikov *et al*., 2007; Pezzani *et al*., 2013), and clinical studies have also revealed that cancer patients on cardiac glycosides treatment had lower mortality rates than those without intake (Prassas and Diamandis, 2008).

Ouabain has been extensively investigated due to the promising pharmacological, pathological, and physiological properties (Lucas *et al*., 2012). It is now well established that ouabain regulate apoptosis and proliferation through several signaling pathways. Ouabain has been shown to induce endotheliocyte apoptosis by increased expression of Bax (Qiu *et al*., 2008), meanwhile to protect against neurona excitotoxic apoptosis by increased expression of Bcl-2 (Golden and Martin, 2006). It has also been indicated that ouabain induced cell apoptosis or proliferation may be associated with reactive oxygen species (ROS) production (Pasdois *et al*., 2007; Yan *et al*., 2015), the concentration of intra-cellular calcium ion (Zhao *et al*., 2012), and mitogen-activated protein kinase (MAPK) activation (Barbosa *et al*., 2013; de Souza *et al*., 2014). RAW264.7 cells, the murine peritoneal macrophage cells, were the appropriate *in vitro*-model for studying immunologic function (Maurya *et al*.,

**Corresponding author:* e-mail: wangxun99@163.com

2013), and so far, the study that ouabain regulates apoptosis and proliferation of RAW264.7 cells has not been reported. Therefore, RAW264.7 cells were treated with ouabain to assess the growth and apoptosis potential. And the mRNA expression of Bax and Bcl-2 were analyzed to reveal the mechanism of the action of ouabain.

MATERIALS AND METHODS

Experimental reagents

RAW264.7 cells were obtained from the Center of Cell Culture Collection of Academia Sinica (Shanghai, China). DMEM medium, phosphate buffered saline (PBS), fetal bovine serum (FBS) and trypsin-EDTA were purchased from Hyclone, USA. Ouabain, Hoechst 33342, MTT dye, paraformaldehyde and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich, USA. LDH assay kit was purchased from Beyotime Biotechnology, China. Primers of apoptosis-related gene were synthesized by BGI, China. DNA Reverse Transcription Kit and SYBR-Green Master PCR mix were purchased from TaKaRa, Japan. And trizol RNA extraction reagent was purchased from Invitrogen, USA.

Cell culture

The RAW264.7 cells were routinely cultured in DMEM medium containing 10% FBS and antibiotics at 37°C in a 5% CO_2 humidified atmosphere. When they were 70%-80% confluent in the flask, the cells were lifted by incubation with 0.25% trypsin and 0.02% EDTA. Harvested cells were seeded into 96-well plates at the density of 5×10^3 cells/well and cultured for 12 hours. Then, medium was replaced with medium containing different concentrations of ouabain (0, 0.01, 0.10 and 1.00mM). Cells were cultured for 24h, 48h and 72h respectively.

Cell viability analysis

Cell viability was measured by MTT method (Mosmann, 1983) after 24h, 48h and 72h incubation with medium containing different concentrations of ouabain. The medium was replaced with 200µl of fresh medium and 20µl MTT solution (5mg/mL). After 4 hours incubation (37℃), the medium was removed and 150µl DMSO was added into plate. Then OD_{570nm} was measured by micro plate reader (Thermo Fisher Scientific). The cell viability is calculated by the following formula (Paramasivam *et al*., 2012): Growth Inhibition (%) = (OD_{570nm} of treated cells / OD_{570nm} of control cells) ×100%.

Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase (LDH) leakage ratio was measured using a commercially available kit. According to the manufacturer's instruction, RAW 264.7 cells were seeded into 96-well plates at density of 5×10^3 cells/well and cultured for 12 hours. After treatment, 120µl supernatant was incubated with 60µl LDH substrate solution for 30 minutes. Then OD (490nm) was recorded on a micro plate reader. The LDH release acitivity was calculated using following formula: LDH release (%) = (OD_{490nm} of sample – OD_{490nm} of blank)/($OD_{490nm}$of cell lysate – OD_{490nm} of blank) ×100%.

Apoptotic nuclear morphology staining

Apoptotic nuclear morphology was observed using Hoechst 33342 staining. Cells were fixed by paraformaldehyde and were washed by PBS (0.01M) twice, followed by 2.5µg/ml Hoechst 33342 staining at 37°C in darkness for 10 min. The morphology was observed by fluorescence microscopy (IX 71, Olympus).

qPCR

RAW264.7 cells were treated with 1.00mM ouabain for 24h and 48h. After treatment, total RNA was extracted from treated RAW264.7 cells by using Trizol (Invitrogen, USA) and synthesized first strand cDNA with Prime Script® RT reagent Kit (TaKaRa, Japan). Primers of genes were designed by using primer3 software (http://primer3.ut.ee/). The sequences of primers were as follows: (i) *β-actin*, forward 5′-AGCCATGTACGTAGC CATCC-3′and reverse 5′-CTCTCAGCTGTGGTGGTGA A-3′; (ii) *Bcl-2*, forward 5′-CTGCAAATGCTGGACTGA AA-3′ and reverse 5′-TCAGGAGGGTTTCC AGATTG-3′; (iii) *Bax*, forward 5′-TGCAGAGGATGATTGCTGAC-3′and reverse 5′-GATCAGCTCGGGCACTTTAG-3′. The expression levels of *Bax* and *Bcl-2* mRNA were quantified using SYBR Green PCR kit on Bio-Rad CFX96 cycler. The $2^{-\Delta\Delta CT}$ method was chose to analysis the data.

STATISTICAL ANALYSIS

The data were expressed as mean ±SE. Statistical analysis was performed with SPSS 14.0 and the data was analyzed using one-way ANOVA followed by Dunnett's test. And $P<0.05$ was considered significant.

RESULTS

Ouabain inhibits cell proliferation

The inhibitory effect of ouabain on cell proliferation was measured by the MTT method. OD value in 1.00mM ouabain treated group significantly decreased when compared with the control group at all selected time points (24,48 and 72h). Thus, 1.00mM ouabain showed strong inhibition effect on cell proliferation (fig. 1). Meanwhile, 0.1mM ouabain treatment group exhibited the inhibition effect to an extent at 72h. Thus, ouabain inhibited proliferation of RAW264.7 cells in a dose-dependent manner.

Cytotoxicity of ouabain

We evaluated the cytotoxicity of ouabain by lactate dehydrogenase (LDH) release assay, which is a reliable

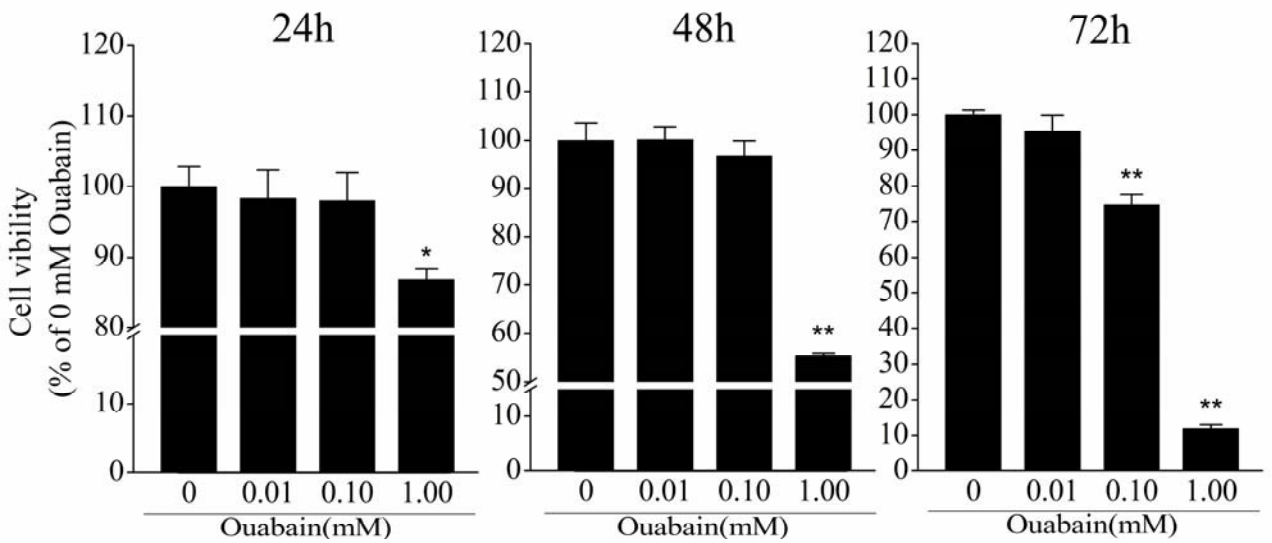

Fig. 1: RAW 264.7 cells were cultured with different doses of ouabain for 24, 48, and 72 hours as indicated in the Materials and Methods. Cell viability was determined based on the MTT assay. Each point represents a mean value and standard error, **P*<0.05, ***P*<0.01 compared to the control.

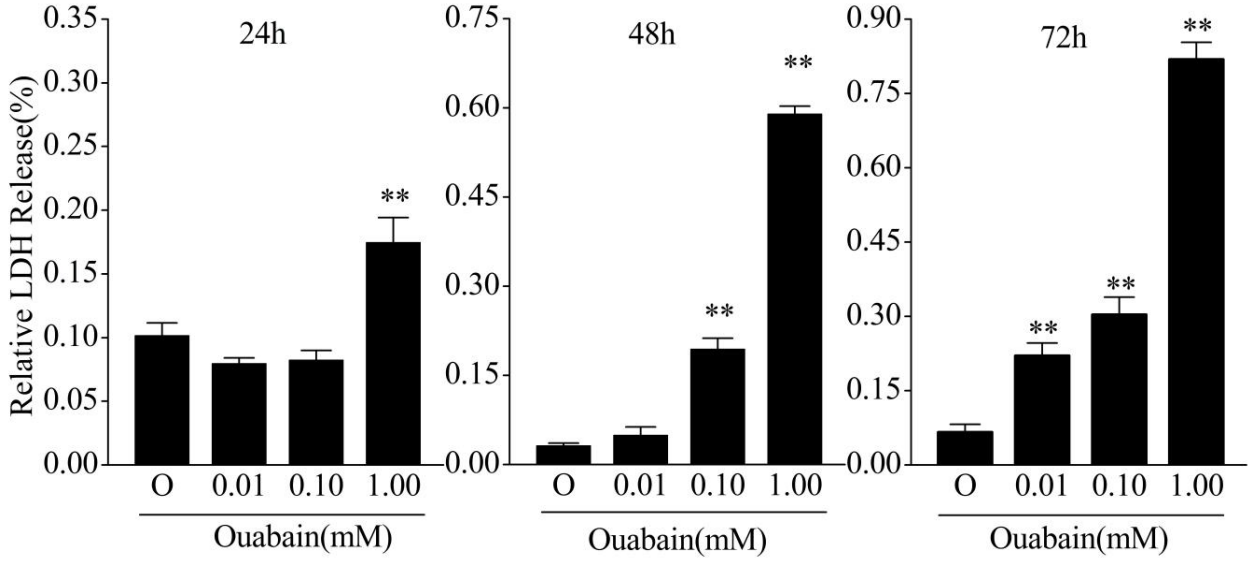

Fig. 2: RAW 264.7 cells were cultured in the presence of varying concentrations of ouabain for 24, 48 and 72 hours as indicated in the Materials and Methods. Cytotoxic was assessed by Lactate dehydrogenase release assay. Each point represents a mean value and standard error; **P*<0.05, ***P*<0.01 compared to control.

method for measuring cellular damage or impairment in cell toxicology studies (Gonzalez-Burgos *et al*., 2013). As shown in fig. 2, LDH release rate was significantly increased after treatment with 0.01mM (72h), 0.10mM (48,72h) and 1.00mM ouabain (24, 48 and 72h). Thus, Ouabain increases RAW264.7 cell LDH release in a time- and does-dependent manner.

Ouabain-induced RAW264.7 cells apoptosis

The inhibition of proliferation and the increase of LDH release are partly attributed to the induction of apoptosis (Morth *et al*., 2011), to explore the effect of ouabain on apoptosis in RAW264.7 cells, RAW264.7 cell nuclei morphology was examined by Hoechst33342 staining. The results are shown in fig 3, RAW264.7 cells untreated with ouabain had round and homogenously stained nuclei, but cells treated with ouabain showed the characteristic of apoptosis such as chromatin condensation or fragmentation. Especially, following 24 and 48h treatment with 1.00mM ouabain, the large number of apoptotic cells was observed. However, RAW264.7 cells were significantly decreased and hardly observed the stained cells after 72h treatment with 1.00mM ouabain.

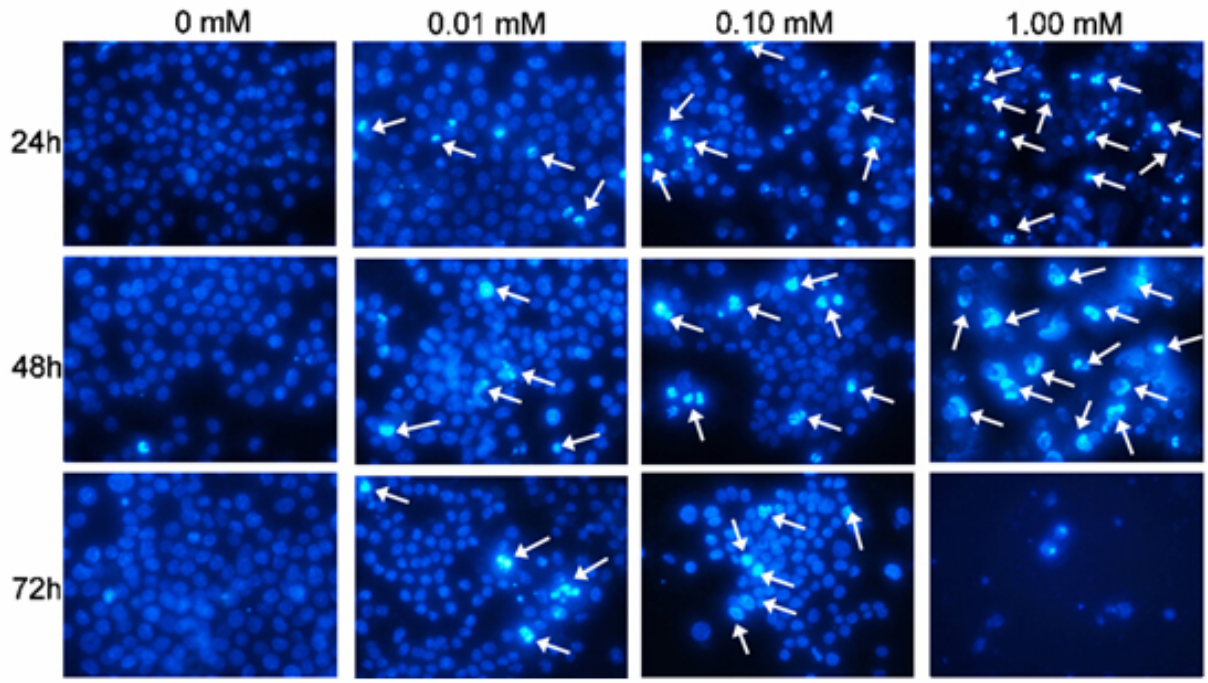

Fig. 3: RAW 264.7 cells were cultured in the presence of varying concentrations of ouabain for 24, 48 and 72 hours as indicated in the Materials and Methods. Fluorescence images of cells stained with Hoechst 33342. Ouabain, especially high dose ouabain treated cells show apoptotic cells with condensed or fragmented nuclei (indicated by arrows).

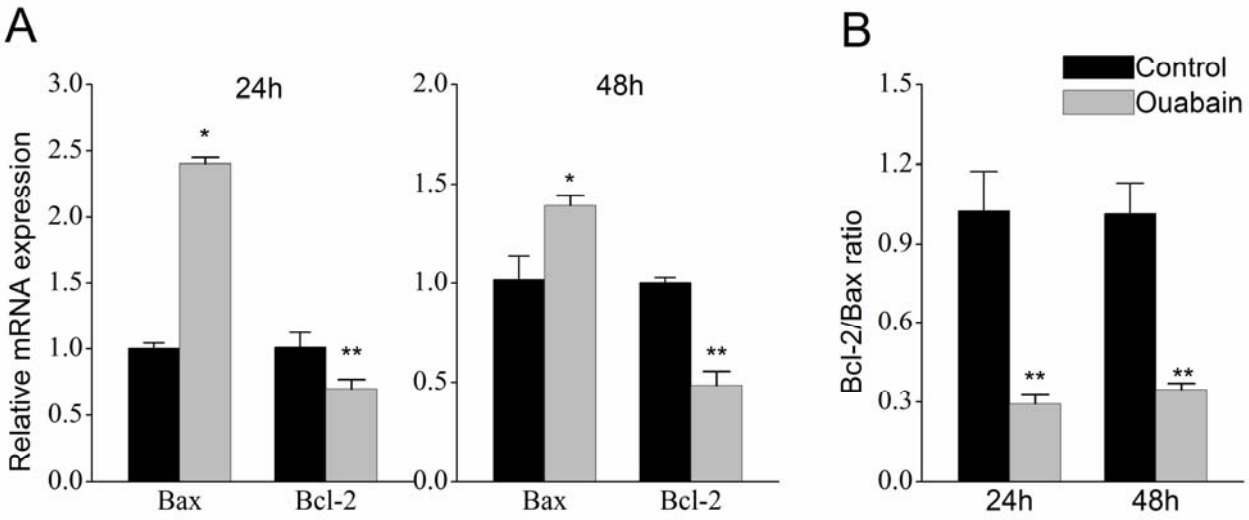

Fig. 4: RAW 264.7 cells were cultured in the absence or presence of 1.00mM ouabain for 24 and 48 hours as indicated in the Materials and Methods. (A)Relative mRNA expression level of Bax and Bcl-2 and (B) the Bcl-2/Bax ratio were observed. Each point represents a mean value and standard error; **P*<0.05, ***P*<0.01 compared to the control.

Relative mRNA expression level

To further clarify the mechanism that ouabain inhibited proliferation and presented cytotoxicity, qPCR was performed to measure mRNA expression of apoptosis-related genes. As shown in fig. 4A, the level of *Bax* mRNA expression was significantly increased after ouabain treatment for 24 and 48 hours. Meanwhile, ouabain down-regulated the *Bcl-2* mRNA expression level after treatment for 48 hours. Fig. 4B shows that the ratio of *Bcl-2/Bax* at mRNA level was significantly down-regulated, and it exhibited 71% and 65% reduction after ouabain treatment for 24 and 48 hours.

DISCUSSION

Previous studies have indicated that the effect of ouabain on proliferation and apoptosis was related to the concentration of ouabain and the type of cells (Aarhus *et al*., 1983; Bai *et al*., 2013;Da Silva *et al*., 2014; Golden and Martin, 2006; Pezzani *et al*., 2013; Ren *et al*., 2014).

Abramowitz *et al*. (2003) found that effective anti-proliferation concentrations (10^{-9}, 10^{-9} and 10^{-6} M) and proliferative concentrations (10^{-8}, 10^{-8}~10^{-7} and 10^{-4}~10^{-3} M) were significantly different after canine, human, and rat vascular smooth muscle cells (VSMCs) were exposed to different concentrations of ouabain. Here, we have evaluated the effect of ouabain (10^{-5}~10^{-3}M) on the proliferation in RAW 264.7 cells. The results showed that concentrations of ouabain ranging from 10^{-5}M to 10^{-3}M showed an obviously inhibitory effect on the proliferation in RAW 264.7 cells, and this finding is consistent with previous studies on the growth-inhibitory activity of ouabain.

Ouabain not only has the anti-proliferative activity but also shows the promotion effect on apoptosis and cell death. When a variety of cancer cells (Da Silva *et al*., 2014; Kulikov *et al*., 2007; McConkey *et al*., 2000; Pezzani *et al*., 2013; Ramirez-Ortega *et al*., 2006), smooth muscle cells (Abramowitz *et al*., 2003), fibroblasts (Winnicka *et al*., 2010) or renal cells (Akimova *et al*., 2005) were exposed to ouabain, cell death and apoptosis were observed. In order to confirm whether ouabain promoted cell apoptosis, we stained cells with Hoechst 33342. The present results revealed that ouabain was able to inhibit proliferation and induce apoptosis of RAW264.7 cells in concentration-dependent manner. These results were similar to those mentioned above.

The Bcl-2 family, a critical regulatory factor in response to apoptosis, which is composed of anti-apoptotic and pro-apoptotic genes (García-Sáez, 2012). Both of these are known to be linked to the apoptotic cascade (Czabotar *et al*., 2014; Kumari and Kakkar, 2012; Liang *et al*., 2012). Bcl-2 could inhibit cytochrome c release, disturb the cellular damage effect of the free radical, regulate cellular redox status, and exert an inhibiting role in the apoptosis signaling pathway (Low *et al*., 2011; Sarafian *et al*., 1994; Voehringer, 1999; Yang *et al*., 1997). On the contrary,bax can promote apoptosis by activating Caspase-3 and triggering cytochrome c release (Alamri *et al*., 2014; Choudhuri *et al*., 2002; Sun *et al*., 2012).

Trenti *et al*. found that JNK activation by ouabain lead to a decrease of Bcl-2 levels and promoted non-small cell lung cancer cells death (Trenti *et al*., 2014). And Kulikov *et al.* (2007) also indicated that ouabain causes a down regulation of Bcl-2 at the time it causes the stimulation of p53 phosphorylation. Previous evidence demonstrated that over expression of Bcl-2 conferred protection against TRAIL induced apoptosis in various carcinoma cells (Fulda *et al*., 2002). In addition, a study of human neuroblastoma indicated that the increase in cytosolic cytochrome c protein is clearly ouabain concentration-dependent and indirectly proportional to the disappearance of Bcl-2 (Kulikov *et al*., 2007).

Activation of Bax results in mitochondrial outer membrane permeabilization (MOMP) and release of pro-apoptotic proteins, including cytochrome c, from the inter-membrane space into the cytosol (Fulda *et al*., 2002). Bax could increase cytochrome c release, cytochrome c binds Apaf-1 and promotes the formation of an oligomeric Apaf-1 apoptosome to activate the caspase family and damaging mitochondria to induce apoptosis (Pinkoski *et al*., 2001; Sun *et al*., 2012). Previous studies have also indicated that Bcl-2 could form dimers with Bax and is involed in the apoptosis process (Czabotar *et al*., 2014; Korsmeyer, 1999). Thus, the decrease of Bcl-2/Bax ratio will promote apoptosis to occur.

According to the present study, the mRNA expression of *Bcl-2* in RAW264.7 cells was found to be down regulated, while the Bax mRNA expression was unregulated after ouabain treatment. Thus, the Bcl-2/Bax ratio was decreased and is consistent with the earlier studies. An apparently decreased ratio of Bcl-2/Bax would lead to cell apoptosis; and evidence further demonstrate that ouabain inhibits RAW264.7 cell proliferation and induces apoptosis via Bcl-2 and Bax expression.

CONCLUTIONS

Taken together, the present data suggest that ouabain significantly inhibited the proliferation and promoted apoptosis of RAW 264.7 cells. Furthermore, ouabain induces apoptosis of RAW 264.7 cells by regulating mRNA expression of the Bcl-2 and Bax.

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