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RESEARCH PAPER

Inhibitory effect of bufalin and cinobufagin on steroidogenesis via the activation of ERK in human adrenocortical cells

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BACKGROUND AND PURPOSE
Bufalin and cinobufagin exhibit cardiotonic and natriuretic activities. The aim of this study was to evaluate the effects of bufalin and cinobufagin on aldosterone and cortisol secretion and their mechanisms of action in human adrenocortical cells (NCI-H295).

EXPERIMENTAL APPROACH
H295 cells were incubated with bufalin or cinobufagin in the presence or absence of angiotensin II (Ang II), forskolin, 8-Br-cAMP, corticosterone or deoxycortisol. The role of ERK1/2 was studied by use of the inhibitor of MEK (U0126). The binding of transcription factor steroidogenic factor 1 (SF-1) to steroidogenic acute regulatory (StAR) gene promoter was analysed by EMSA.

KEY RESULTS
Bufalin and cinobufagin markedly inhibited basal, Ang II-, forskolin- or 8-Br-cAMP-stimulated aldosterone and cortisol secretion, and the conversions of corticosterone to aldosterone and deoxycortisol to cortisol. Bufalin and cinobufagin also inhibited StAR protein expression and SF-1 binding to StAR gene promoter. They both increased phosphorylation of ERK1/2, and U0126 fully abolished these effects on ERK1/2 in H295 cells. Furthermore, U0126 reversed the inhibitory effects of bufalin and cinobufagin on StAR protein expression and the binding of SF-1 to StAR gene promoter. However, U0126 did not completely reverse their inhibitory effects on aldosterone and cortisol release.

CONCLUSIONS AND IMPLICATIONS
The inhibitory effects of bufalin and cinobufagin on steroidogenesis of aldosterone and cortisol were associated with inhibition of aldosterone synthase and 11β-hydroxylase, as well as the suppression of StAR protein expression and SF-1 binding to StAR promoter via the phosphorylation of ERK1/2 in H295 cells.

Abbreviations
SF-1, steroidogenic factor 1; StAR protein, steroidogenic acute regulatory protein

Introduction

Cardiotonic steroids (CTS), including cardenolides and bufadienolides, are a class of natural products that are used to increase cardiac contractility in patients with congestive heart failure and cardiac arrhythmia (Chen and Kovarikova, 1967; Hauptman and Kelly, 1999). Digoxin and digitoxin are cardenolides isolated from the plant *Digitalis purpurea* or *Digitalis lanata* (Smith *et al.*, 1984). Bufalin is a bufadienolide purified from the Chinese medicine Chansu, a galenical preparation of the dried white venom of Chinese *Bufo bufo* gargarizans (Chen and Kovarikova, 1967; Hong *et al.*, 1992). Bufalin exhibits several biological activities such as cardiotonic, anaesthetic, blood pressure elevation, natriuresis, respiration and anti-neoplastic effects (Pamnani *et al.*, 1991; Krenn and Kopp, 1998; Schoner and Scheiner-Bobis, 2007). The vasopressor and natriuretic effects of bufalin probably result from inhibition of Na^+/K^+-ATPase and therefore increased contraction of the vascular smooth muscle cells and decreased renal tubular Na^+ reabsorption (Blaustein and Hamlyn, 1984; Pamnani *et al.*, 1991). Several endogenous cardenolides and bufadienolides have been detected in mammalian plasma and tissues (e.g. brain and adrenal gland) (Hamlyn *et al.*, 1982; Ludens *et al.*, 1991; Tymiak *et al.*, 1993; Lichtstein *et al.*, 1998; Lopatin *et al.*, 1999; Fedorova *et al.*, 2000). Increased plasma bufadienolides has been shown in volume-expanded and hypertensive humans and animal models (Lopatin *et al.*, 1999; Fedorova *et al.*, 2000). These findings demonstrates that endogenous CTS may be involved in the regulation of body fluid and the pathogenesis of hypertension via the modulation of Na^+/K^+-ATPase activity in vascular smooth muscle and renal tubules.

In addition to cardiovascular and renal effects, digoxin and bufalin have inhibitory effects on the steroidogenesis of testosterone, progesterone and corticosterone in rats via a mechanism associated with reduced cAMP action (Wang *et al.*, 1997; 2004; Chen *et al.*, 2001). In primates, we (Kau *et al.*, 2005, 2009) have reported inhibitory effects of digoxin on corticosteroids in human adrenocortical cells and *in vivo* in monkeys. However, the effects of the bufadienolides (such as bufalin and cinobufagin) on the secretion of aldosterone and cortisol are still unclear.

A mechanism of action of CTS via the Na^+/K^+-ATPase has been proposed to be involved in the various biological responses of CTS (Dvela *et al.*, 2007; Schoner and Scheiner-Bobis, 2007; Bagrov *et al.*, 2009). The ionic pathway demonstrates that CTS inhibit Na^+/K^+-ATPase and lead to an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) via an effect on the Na^+/Ca^{2+} exchanger (Hougen and Smith, 1978). This ionic pathway may explain the short-term and inotropic action of CTS (Hougen and Smith, 1978, Schoner and Scheiner-Bobis, 2007). The signalling pathway depicts that binding of the CTS to Na^+/K^+-ATPase activates various signalling cascades through intramembrane and cytosolic protein–protein interactions, such as activating Src and the ERK1/2 pathways, phosphoinositide 3-kinase (PI3K) and PKB (Akt), NF-κB and reactive oxygen species (ROS) (Dvela *et al.*, 2007; Schoner and Scheiner-Bobis, 2007; Bagrov *et al.*, 2009). Bagrov *et al.* (2009) also suggested that the ionic and signalling pathways may work both in parallel and synergistically to accomplish physiological responses of CTS binding to the Na^+/K^+-ATPase.

The ERKs belong to the family of MAPKs and are known to be involved in the regulation of many biological functions, including cell proliferation, differentiation, apoptosis and carcinogenesis (Lewis *et al.*, 1998). Several studies have indicated that the ERK1/2 pathway participates in the regulation of steroidogenesis in steroid-producing cells (Cameron *et al.*, 1996; Gyles *et al.*, 2001; Seger *et al.*, 2001; Martinelle *et al.*, 2004). However, the results appear to be contradictory; that is, both stimulating and inhibitory effects have been reported. Seger *et al.* (2001) found that gonadotropin induced ERK activation and progesterone production; however, inhibition of ERK activity enhanced basal and gonadotropin-stimulated progesterone production and the steroidogenic acute regulatory (StAR) protein expression in granulosa-derived cell lines. They also suggested that gonadotropin not only stimulates PKA and induces steroidogenesis but also induces the down-regulation of the ERK cascade. In other words, there is a mechanism of cross-talk between the cAMP/PKA signalling pathway and ERK cascades. ERK activation also contributes to the mechanisms of angiotensin II (Ang II)-mediated gene expression of aldosterone synthase in human adrenocortical cells, inhibition of ERK activation increased basal gene expression of aldosterone synthase (Szekeres *et al.*, 2009). These results demonstrated that ERK cascades play an important role in steroidogenesis; however, it seems the cellular interactions between the ERK cascades and other signalling pathways are complicated. Therefore, the purpose of the present study was to determine the effects of bufalin and cinobufagin on aldosterone and cortisol secretion, and their mechanisms of action, as well as the involvement or role of ERK1/2 cascades in human adrenocortical carcinoma cells (NCI-H295 cells).

Methods

Cell culture

Human adrenocortical carcinoma cells (NCI-H295) were obtained from the Culture Collection and Research Center (Food Industry Research and Development Institute, Taiwan, ROC). Cells were grown in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with hydrocortisone (10^{-8} M), insulin (5 mg·L^{-1}), transferrin (5 mg·L^{-1}), 17β-estradiol (10^{-8} M), sodium selenite (5 μg·L^{-1}) and 2% fetal calf serum (2% FCS, Kibbutz Beit, Haemek, Israel) at 37 °C in a 5% CO_2–95% air-humidified atmosphere. Cells were subcultured onto a 24-well dish or a 100 mm Petri dish and cultured in 2% FCS medium. After 24 h, the medium was removed and replaced with serum-free medium containing ascorbic acid (10^{-7} M), transferrin (5 mg·L^{-1}), BSA (0.01%), bacitracin (0.01%), and cells were cultured for another 24 h.

To examine the effects of bufalin or cinobufagin on aldosterone and cortisol secretion, the cells were incubated with bufalin or cinobufagin (0, 10^{-8}–10^{-6} M) combined with or without Ang II (10^{-7} M), forskolin (10^{-5} M, an adenylyl cyclase activator) or 8-Br-cAMP (10^{-4} M, a cAMP analogue) for 24 h. Our previous study and other laboratory reports (Rainey *et al.*, 2004) found that H295 cells are insensitive to

adrenocorticotropic hormone (ACTH); therefore, we used forskolin as a substitute for ACTH. Bufalin and cinobufagin, a pure compound, were purchased from Sigma Chemical Co. (St. Louis, MO). The activities of aldosterone synthase (P450c11AS, the last steroidogenic enzyme of aldosterone synthesis) or 11β-hydroxylase (P450c11β, the last steroidogenic enzyme of cortisol synthesis) were assessed by the conversion of corticosterone (substrate of P450c11AS, 10^{-6} M) or deoxycortisol (substrate of P450c11β, 10^{-6} M) to aldosterone or cortisol. The levels of aldosterone and cortisol in medium were measured by RIA. It is well established that StAR protein is essential for cholesterol transport to inner mitochondrial membrane and to the site of cytochrome P450 side chain cleavage (P450scc), and that the expression of StAR protein is the key to the regulation of steroid synthesis (Clark *et al.*, 1994; Lin *et al.*, 1995). The effects of bufalin and cinobufagin on protein expression of StAR protein and P450scc in H295 cells were investigated by Western blot analysis.

In order to assess whether bufalin or cinobufagin can affect ERK cascades, we measured the activities of ERK1/2. The protein expression of phospho-ERK1/2 (P-ERK1/2, activated form) and total-ERK1/2 (ERK1/2, for internal control) was determined by Western blot analysis, and the activity of ERK1/2 was indicated by the phosphorylation of ERK1/2 (P-ERK). In order to estimate the role of ERK activation in the effects of bufalin or cinobufagin on steroidogenesis of aldosterone and cortisol, the cells were incubated with bufalin or cinobufagin for 24 h in the presence or absence of U0126 (10^{-5} M, an inhibitor of MEK). The levels of aldosterone and cortisol in medium as well as the protein expressions of P-ERK1/2, ERK1/2, StAR protein, P450scc or β-actin were measured by RIA and Western blot analysis.

Nuclear extract preparation and EMSA

The human StAR gene contains transcription factor steroidogenic factor 1 (SF-1) binding sites in the promoter. SF-1 plays a major role in controlling the basal and cAMP-stimulated expression of StAR gene (Sugawara *et al.*, 1996). To understand the effects of bufalin or cinobufagin on the binding of SF-1 to StAR gene promoter and the role of the ERK signalling cascade in the regulation of StAR expression, the cells were treated with bufalin or cinobufagin in the absence or presence of U0126 (10^{-5} M). Then the preparation of the nuclear extracts and the EMSA were performed to detect the binding of SF-1 in nuclear extracts to the oligonucleotide probe containing the SF-1 binding site of human StAR gene promoter.

The nuclear extracts were prepared from H295 cells as described previously (Lin *et al.*, 2000). The oligonucleotide probes used for the EMSA were as follows: SF-1 (–42 to –35) 5′-ATGATGCACAGCCTTCAGC-3′ and 5′-CGCTGAAGGCTG TGCATCA-3′ (Sugawara *et al.*, 1997). Double-stranded oligonucleotide probes were 3′-end-labelled with [γ-^{32}P]-ATP by polynucleotide kinase. Five-microgram aliquots of nuclear protein extracts were incubated with 5×10^4 cpm of ^{32}P-labelled oligonucleotide probe at room temperature for 20 min and then electrophoresed on a 6% polyacrylamide gel containing 0.25 × Tris-borate/EDTA at 150 V for 2 h at 4°C. Gels were dried and subjected to autoradiography.

RIAs of steroids

The concentration of aldosterone or cortisol in medium samples was measured by RIA (Kau *et al.*, 2005). The anti-aldosterone serum, no. JJC088, showed the sensitivity of aldosterone RIA was 12 pg per assay tube. The intra- and inter-assay coefficients of variation were 4.0% ($n = 5$) and 4.5% ($n = 5$), respectively. The antiserum, no. YCT74-13, showed the sensitivity of cortisol RIA was 20 pg per assay tube. The intra- and inter-assay coefficients of variation were 5.6% ($n = 4$) and 8.0% ($n = 4$) respectively.

Western blot analysis

After incubation, the proteins of the cell extracts were separated by 10% SDS-PAGE and analysed by Western blotting (Kau *et al.*, 2005). The first antibodies included anti-β-actin antibodies (1:8000, mouse; Sigma Chemical Co., for loading control), anti-P450scc antibodies (1:1000, rabbit), anti-StAR antibodies (1:1000, rabbit; Abcam, Cambridge, UK), phospho-ERK1/2 and ERK1/2 (1:1000, rabbit; Cell Signaling Technology Inc., Danvers, MA). The anti-P450scc antibody was kindly provided by Dr BC Chung (Hu *et al.*, 1991). The secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (1:10 000; Promega, Madison, WI). The specific protein bands were detected by chemiluminescence using the electrogenerated chemiluminescence (ECL) Western blotting detection reagents (Amersham International Plc., Buckinghamshire, UK) and exposed to X-ray film. The density of specific bands was determined by a scanner (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA).

Statistical analysis

The data are expressed as mean ± SEM. The treatment means were tested for homogeneity using ANOVA, and the difference between specific means was tested for significance by using Duncan's multiple-range test or Student's *t*-test (Steel and Torrie, 1980). A difference between two means was considered to be statistically significant when the *P*-value was less than 0.05.

Results

Effects of bufalin or cinobufagin on aldosterone and cortisol release in H295 cells

Figure 1 demonstrates the effects of bufalin and cinobufagin (10^{-8}–10^{-6} M) treatment for 24 h on aldosterone and cortisol secretion by H295 cells. Ang II (10^{-7} M) produced a significant increase in aldosterone secretion (Figure 1A, $P < 0.01$). Bufalin and cinobufagin (10^{-7}–10^{-6} M) significantly inhibited basal and Ang II-stimulated aldosterone secretion in H295 cells ($P < 0.05$ or $P < 0.01$). Corticosterone (10^{-6} M), a substrate of P450c11AS, induced a marked increase in aldosterone secretion ($P < 0.01$). Bufalin and cinobufagin at 10^{-7} to 10^{-6} M inhibited the conversion of corticosterone to aldosterone ($P < 0.01$). Treatment with bufalin or cinobufagin (10^{-7}–10^{-6} M) also reduced cortisol secretion by H295 cells (Figure 1B, $P < 0.01$). Administration of forskolin (10^{-5} M, an adenylyl cyclase activator) or 8-Br-cAMP (10^{-4} M, a cAMP

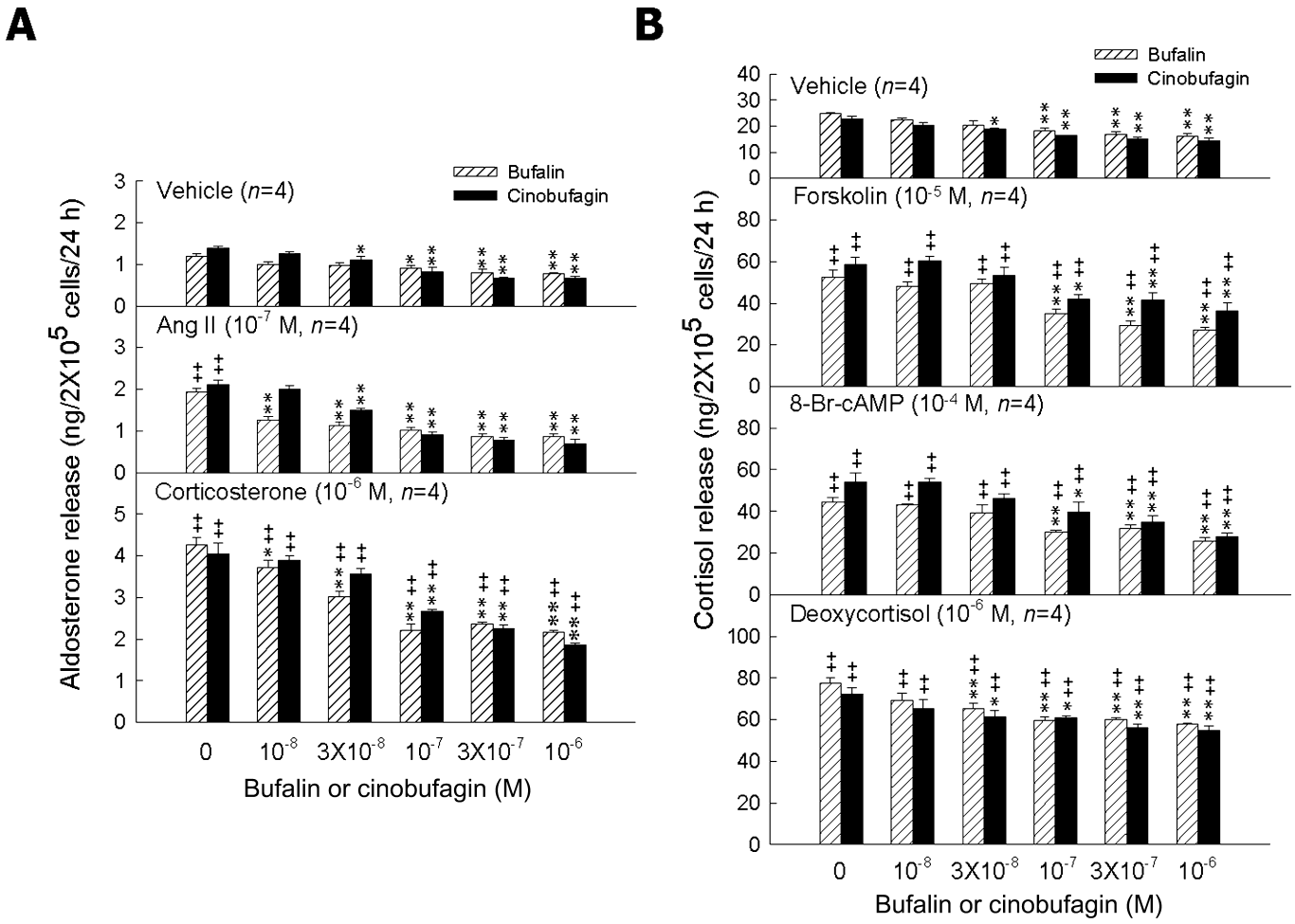

Figure 1

Effects of bufalin and cinobufagin treatment for 24 h on aldosterone and cortisol secretion by H295 cells. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with bufalin or cinobufagin at 0 M, respectively. $^{++}P < 0.01$ as compared with vehicle group. Each value represents the mean ± SEM.

analogue) significantly increased cortisol secretion (Figure 1B, $P < 0.01$). The combination of forskolin or 8-Br-cAMP with bufalin or cinobufagin (10^{-7}–10^{-6} M) decreased the secretion of cortisol as compared with those groups of forskolin or 8-Br-cAMP alone ($P < 0.05$ or $P < 0.01$). Deoxycortisol (10^{-6} M), a substrate of P450c11β, significantly increased cortisol secretion ($P < 0.01$). Both bufalin and cinobufagin (3×10^{-8}–10^{-6} M) reduced the conversion of deoxycortisol to cortisol ($P < 0.05$ or $P < 0.01$).

Effects of bufalin or cinobufagin on the protein expression of StAR and P450scc

The effects of bufalin or cinobufagin (3×10^{-8}, 10^{-7} M) treatment for 10 min, 30 min or 24 h on protein expression of StAR protein and P450scc in H295 cells are shown in Figure 2. Bufalin or cinobufagin treatment for 10 or 30 min did not produce any change in protein expression of StAR protein or P450scc in H295 cells. Incubation of bufalin for 2 h induced a marked decrease in StAR protein expression, but there was no significant effect of cinobufagin treatment for 2 h (data not shown). Bufalin or cinobufagin treatment for 24 h significantly inhibited the protein expression of StAR protein in H295 cells ($P < 0.05$ or $P < 0.01$). However, no significant effects of bufalin or cinobufagin treatment for 24 h on the expression of P450scc protein were observed.

Effects of bufalin or cinobufagin on ERK1/2 phosphorylation

To explore the effects of bufalin or cinobufagin on the activity of ERK1/2 signalling cascade, we examined the protein expression of P-ERK1/2 in response to bufalin or cinobufagin (3×10^{-8}, 10^{-7} M). As shown in Figure 3, ERK1/2 phosphorylation in H295 cells was increased after treatment with bufalin or cinobufagin (3×10^{-8}, 10^{-7} M) for 10 min, 30 min or 24 h ($P < 0.05$ or $P < 0.01$). Neither bufalin nor cinobufagin treatment for 10, 30 min or 24 h altered the protein expression of total-ERK1/2 (ERK1/2) in H295 cells.

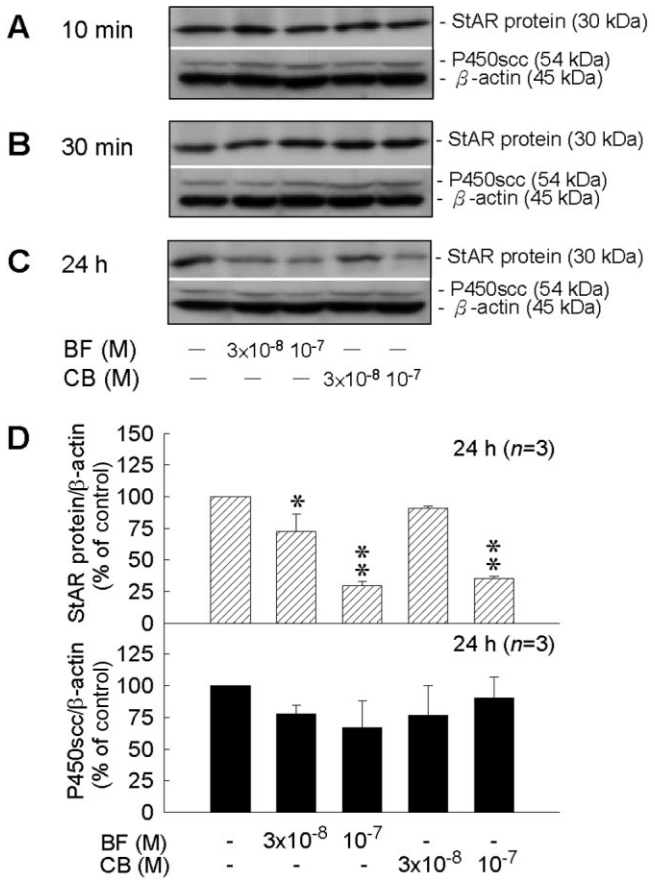

Figure 2

Effects of bufalin (BF) and cinobufagin (CB) treatment for 10 min, 30 min and 24 h on the expression of StAR protein and P450scc in H295 cells. *$P < 0.05$, **$P < 0.01$ as compared with bufalin or cinobufagin at 0 M, respectively. Each value represents the mean ± SEM.

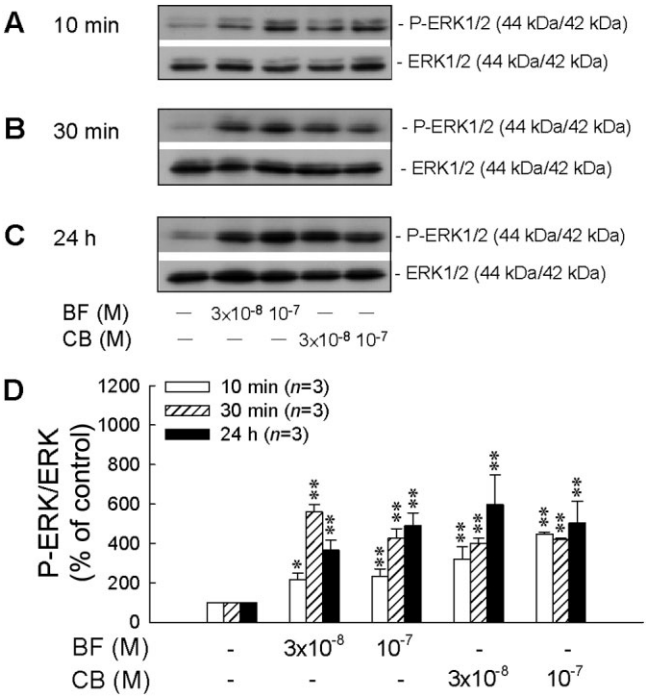

Figure 3

Effects of bufalin (BF) and cinobufagin (CB) treatment for 10 min, 30 min and 24 h on ERK phosphorylation in H295 cells. *$P < 0.05$, **$P < 0.01$ as compared with bufalin or cinobufagin at 0 M, respectively. Each value represents the mean ± SEM.

Role of ERK phosphorylation in the effects of bufalin and cinobufagin on StAR protein expression and steroidogenesis

In order to determine the role of ERK phosphorylation in the effects of bufalin and cinobufagin on StAR protein expression and aldosterone or cortisol secretion, the cells were incubated with bufalin or cinobufagin for 24 h in the presence or absence of U0126 (10^{-5} M). Figure 4 demonstrates that bufalin or cinobufagin (10^{-7} M, 24 h) induced a significant increase in ERK phosphorylation in H295 cells ($P < 0.05$ or $P < 0.01$). U0126 almost completely abolished ERK phosphorylation in the presence of bufalin or cinobufagin in H295 cells. Bufalin or cinobufagin treatment for 24 h markedly reduced the protein expression of StAR protein in H295 cells (Figure 4, $P < 0.01$). U0126 treatment reversed the inhibition induced by bufalin or cinobufagin on StAR protein expression in H295 cells. In addition, in cells treated with U0126 alone the protein expression of StAR protein was higher than that in the control group. There were no significant effects of U0126 on P450scc protein expression in the absence or presence of bufalin or cinobufagin in H295 cells (Figure 4).

Figure 5 illustrates the effects of bufalin and cinobufagin on aldosterone and cortisol secretion in the absence or presence of U0126 (10^{-5} M) in H295 cells. Bufalin and cinobufagin at 10^{-7} M significantly ($P < 0.01$, Figure 5) suppressed aldosterone secretion by 33±7% and 41±4% and cortisol secretion by 32±2% and 36±4%, respectively. Administration of U0126 slightly decreased aldosterone secretion (1.21±0.06 vs. 0.97±0.10 ng per 2×10^5 cells 24 h^{-1}) and cortisol secretion (22.59±1.22 vs. 18.65±0.78 ng per 2×10^5 cells 24 h^{-1}). However, no significant difference was observed between the control group and U0126 group. In the presence of U0126, bufalin and cinobufagin still had a significant ($P < 0.05$ or $P < 0.01$) inhibitory effect on aldosterone secretion, 28±6% and 30±5%, and cortisol secretion, 19±8% and 18±8%, respectively. Although U0126 did not completely reverse the inhibitory effects of bufalin and cinobufagin on aldosterone and cortisol release, it attenuated the inhibitory responses of bufalin and cinobufagin on aldosterone and cortisol release in H295 cells.

Role of ERK phosphorylation in the effects of bufalin or cinobufagin on the binding of SF-1 to the radiolabelled probe of StAR gene promoter

In Figure 6, 'SF-1 binding' indicates the binding signal of SF-1 in nuclear extracts with the radiolabelled probe of StAR gene promoter. 'H_2O' represents the result of nuclear protein replaced by 3DH_2O; therefore, there was no binding signal.

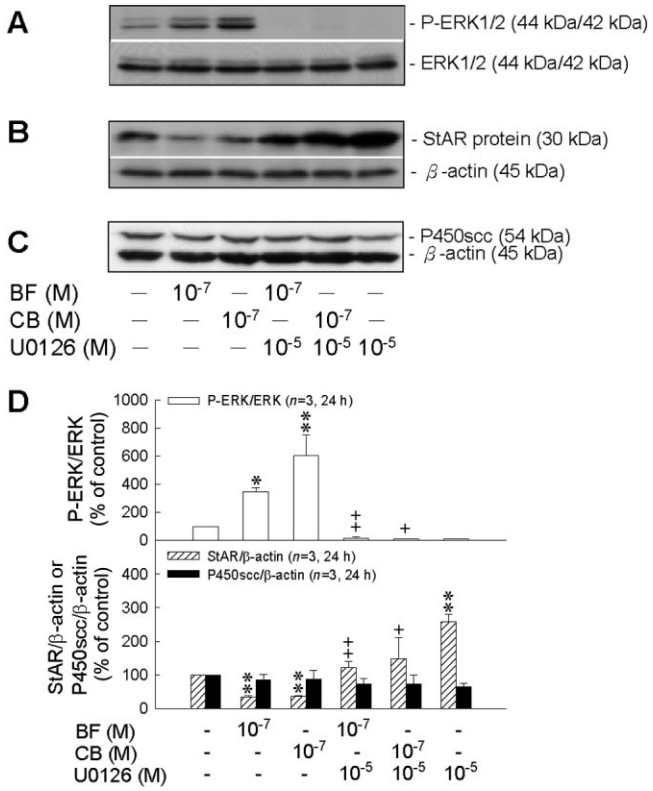

Figure 4

Effects of bufalin (BF) and cinobufagin (CB) treatment for 24 h in the absence or presence of U0126 on the protein expression of phospho-ERK, StAR protein and P450scc in H295 cells. $*P < 0.05$, $**P < 0.01$ as compared with bufalin or cinobufagin at 0 M, respectively. $^{+}P < 0.05$ and $^{++}P < 0.01$ as compared with bufalin or cinobufagin at 10^{-7} M, respectively. Each value represents the mean ± SEM.

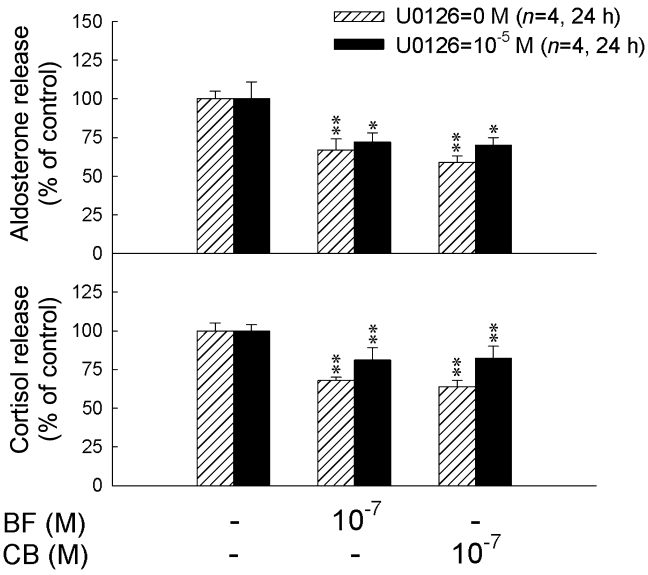

Figure 5

Effects of U0126 (10^{-5} M) on bufalin (BF)- or cinobufagin (CB)-induced inhibition of aldosterone and cortisol release in H295 cells. $*P < 0.05$, $**P < 0.01$ as compared with bufalin or cinobufagin at 0 M, respectively. Each value represents the mean ± SEM.

'Comp' shows the results of competition of unlabelled probe and radiolabelled probe with SF-1 protein in nuclear extracts. Figure 6A shows the effects of bufalin and cinobufagin (3×10^{-9}–1×10^{-7} M) treatment for 24 h on the binding of SF-1 to the radiolabelled probe of StAR gene promoter in H295 cells. Both bufalin and cinobufagin at 10^{-7} M markedly inhibited the binding of SF-1 to the radiolabelled probe of StAR gene promoter in H295 cells.

As shown in Figure 6B, both bufalin and cinobufagin inhibited the binding of SF-1 with the radiolabelled probe of StAR gene promoter; U0126 reversed these inhibitory effects on binding of SF-1 to StAR gene promoter probe. These results demonstrate that the bufalin- and cinobufagin-induced inhibition of binding to the specific sequences of StAR gene promoter was dependent on the activation of ERK1/2.

Discussion

In the present study, we demonstrated that both bufalin and cinobufagin inhibited basal release of aldosterone and cortisol, the conversions of corticosterone (a substrate of P450c11AS) to aldosterone and deoxycortisol (a substrate of P450c11β) to cortisol in H295 cells. They also suppressed the protein expression of StAR and the binding of SF-1 to the oligonucleotide probe containing SF-1 binding site of StAR gene promoter in H295 cells. In addition, they increased the phosphorylation of ERK1/2 in H295 cells. The MEK inhibitor U0126 significantly inhibited the phosphorylation of ERK and reversed the inhibitory effects of bufalin and cinobufagin on the expression of StAR protein as well as binding of SF-1 to StAR gene promoter probe. These results show that bufalin and cinobufagin inhibit steroidogenesis in H295 cells through a mechanism involving the suppression of StAR protein expression via the phosphorylation of ERK as well as inhibition of P450c11AS and P450c11β.

It is well established that the positive inotropic effect of CTS on the myocardium is due to the inhibition of the cardiac Na^+/K^+-ATPase, causing an increase in intracellular Na^+, which in turn affects the Na^+/Ca^{2+}-exchanger, leading to a rise in $[Ca^{2+}]_i$ and an increase in cardiac contractility (Hougen and Smith, 1978). Accumulated evidence indicates that Na^+/K^+-ATPase acts as a signal transducer; that is, it can respond to extracellular stimuli such as ouabain and relay messengers to MAPKs (Tian *et al.*, 2001; Haas *et al.*, 2002). Ouabain binding to Na^+/K^+-ATPase stimulates Src kinase, which phosphorylates the EGF receptor, leading to activation of the Ras–Raf–MEK–ERK pathway (Dvela *et al.*, 2007; Schoner and Scheiner-Bobis, 2007; Bagrov *et al.*, 2009). Our present results show that bufalin and cinobufagin activate ERK1/2 phosphorylation, U0126 reversed the inhibition of protein expression of StAR induced by bufalin or cinobufagin in H295 cells. In addition, U0126 alone increased the expression of StAR protein above that in the basal group, these observations are in agreement with the findings of several studies (Seger *et al.*, 2001; Ramanjaneya *et al.*, 2008). There-

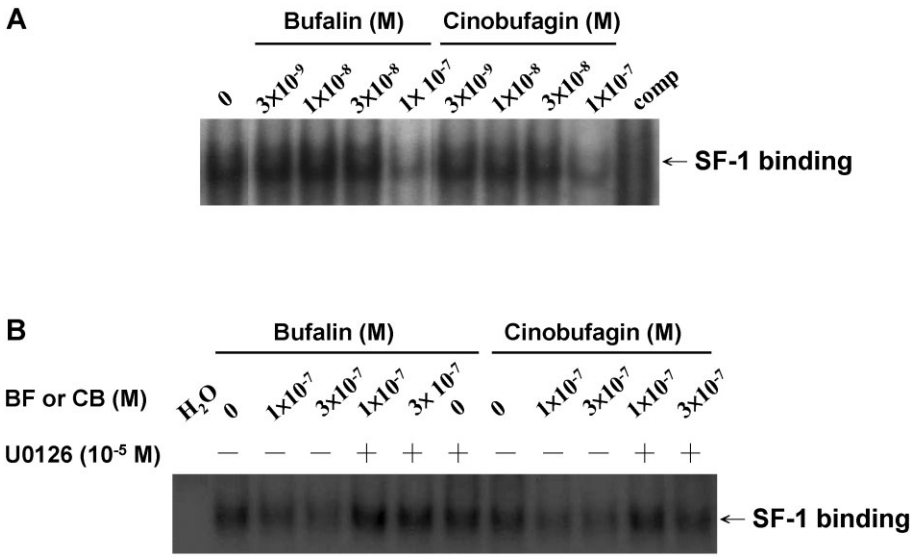

Figure 6

Effects of bufalin (BF) and cinobufagin (CB) treatment for 24 h on the binding of SF-1 to the StAR gene promoter and the role of ERK activation in H295 cells. 'SF-1 binding' indicates the binding signal of SF-1 in nuclear extracts with the radiolabelled probe of StAR gene promoter. 'H_2O' represents the result of nuclear protein replaced by $3DH_2O$; therefore, there was no binding signal. 'Comp' shows the results of competition of unlabelled probe and radiolabelled probe with SF-1 protein in nuclear extracts.

fore, we hypothesize that ERK phosphorylation plays a down-regulating role in StAR protein expression in H295 cells. Although U0126 abolished the inhibition of the expression of StAR protein induced by bufalin or cinobufagin (Figure 4), it did not completely reverse bufalin- or cinobufagin-induced inhibition of aldosterone and cortisol release in H295 cells (Figure 5). Moreover, both bufalin and cinobufagin suppressed forskolin- and 8-Br-cAMP-stimulated cortisol release as well as the conversion of deoxycortisol to cortisol in H295 cells (Figure 1B). Based on these results, we suggest that in addition to activation of ERK cascades, bufalin and cinobufagin suppress cortisol release via other pathways, e.g. inhibition of adenylyl cyclase and cAMP action as well as P450c11β in H295 cells. Hence, their precise mechanisms of action remain to be elucidated.

Several transcription factors (e.g. NURR-1, NGFI-B, SF-1) are involved in the modulation of gene expression of steroidogenic enzymes and control of adrenocortical steroidogenesis via multiple signalling pathways, such as PKC, MAPKs and calmodulin kinases (Bassett *et al.*, 2004; Spat and Hunyady, 2004; Nogueira *et al.*, 2009). StAR has a transcription factor-rich promoter region that is under the regulation of many transcription factors (Manna *et al.*, 2003). The transcription factor SF-1 not only regulates StAR protein but may also modulate many other steroidogenic genes, including P450 steroid hydroxylases (Parker *et al.*, 2002; Cartier *et al.*, 2005). Although we cannot rule out the involvement of other transcription factors, U0126 treatment reversed both the bufalin- and cinobufagin-induced inhibition of StAR protein expression and the binding of SF-1 to the StAR promoter probe (Figure 6). These data show that the inhibition of the expression of StAR protein induced by bufalin or cinobufagin is dependent on the activation of ERK1/2 and the involvement of SF-1 transcription factor in H295 cells.

In summary, we conclude that both bufalin and cinobufagin decrease aldosterone and cortisol secretion, StAR protein expression and SF-1 binding to StAR promoter via ERK phosphorylation in H295 cells. Our data also show that bufalin and cinobufagin suppress cortisol secretion by inhibiting the post-cAMP pathway and P450c11β activity in H295 cells. These mechanisms of action of bufalin and cinobufagin on aldosterone and cortisol release in H295 cells involving ERK and post-cAMP pathways are shown in Figure 7. Aldosterone and cortisol, the principal adrenocorticosteroids, are secreted by the adrenal cortex. Aldosterone, a mineralocorticoid, stimulates the kidneys to retain Na^+ and water and increases the blood volume and blood pressure. Cortisol, a glucocorticoid, stimulates gluconeogenesis and inhibits glucose utilization, which help to raise the blood glucose level. Cortisol also has modest mineralocorticoid activity, which becomes significant during the hypercortisolaemia of Cushing's syndrome. Aldosterone has been shown to have potent detrimental effects on left ventricular remodelling, including stimulation of myocardial fibrosis (Weber, 2001; Cohn, 2007). Clinical and experimental studies have shown that modulation of the renin–angiotensin–aldosterone system during heart failure by angiotensin converting enzyme inhibitors, Ang II type 1 receptor blockers and mineralocorticoid receptor antagonists provides substantial cardiovascular protection (Pitt *et al.*, 1999; Tokmakova and Solomon, 2006). Taken together, the results of the present study indicate the potential of bufalin and cinobufagin as a therapeutic strategy for congestive heart failure and Cushing's syndrome.

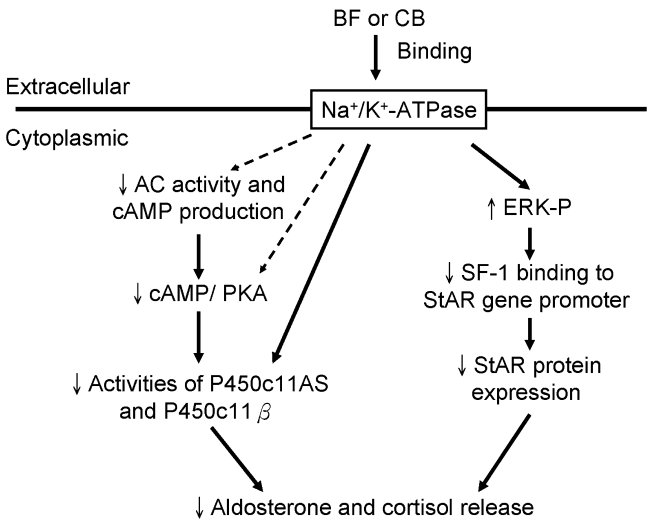

Figure 7

Schematic diagram explaining the mechanisms involved in the inhibitory effects of bufalin (BF) and cinobufagin (CB) on aldosterone and cortisol release in H295 cells. The continuous arrows indicate reactions that are supported by the results of our present study. The discontinuous arrows represent reactions that are rather speculative. AC, adenylyl cyclase; P450c11AS, aldosterone synthase; P450c11β, 11β-hydroxylase.

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Conflict of interest

The authors declare no conflict of interest.

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