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Dual effects of lipophilic extract of *Salvia miltiorrhiza* (Danshen) on catecholamine secretion in cultured bovine adrenal medullary cells

Haoping Mao[a], Han Zhang[a,b], Hong Wang[a], Yuefei Wang[a], Fenrong Zhao[a], Limin Hu[a], Nobuyuki Yanagihara[b], Xiumei Gao[a,*]

[a] *Institute of Traditional Chinese Medicine Research, Tianjin University of Traditional Chinese Medicine, Key Laboratory of Pharmacology of Traditional Chinese Medical Formulae, Ministry of Education; Key Laboratory of Traditional Chinese Medicine Pharmacology, #312 Anshan xi Road, Nankai District, Tianjin 300-193, China*

[b] *Department of Pharmacology, University of Occupational and Environmental Health, School of Medicine, 1-1, Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan*

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ABSTRACT

Ethnopharmacological relevance: Salvia miltiorrhiza (Danshen) is a well known traditional Chinese herb, which has been used widely in China for the treatment of cardiovascular diseases in clinic.

Aim of this study: The aim of the present study is to clarify the effects of lipophilic extract of *Salvia miltiorrhiza* (LESM) on catecholamine (CA) secretion, a traditional Chinese medicine used widely for the treatment of cardiovascular diseases in China.

Materials and methods: LESM was evaluated for its effects on CA secretion using HPLC-ECD method. The effects of LESM on $^{22}Na^+$ influx and intracellular calcium ($[Ca^{2+}]_i$) were also investigated.

Results: Our results showed that LEMS directly stimulated basal CA secretion in an extracellular Ca^{2+}-dependent manner. And the stimulation was not affected by combination of hexamethonium (Hex), an inhibitor of nAChR. LESM also directly elevated $[Ca^{2+}]_i$. In addition, using selective blockers of voltage-dependent Ca^{2+} channels, such as nitrendipine (for L-type), ω-agatoxin-IVA (for P-type) and ω-conotoxin-GVIA (for N-type), it was found that nitrendipine suppressed the elevation of $[Ca^{2+}]_i$ induced by LESM, but not ω-agatoxin-IVA or ω-conotoxin-GVIA. Compared with acetylcholine (ACh) only, however, combination of LESM with ACh inhibited the raise of CA secretion, $^{22}Na^+$ influx and $[Ca^{2+}]_i$ in a concentration-dependent manner. Furthermore, LESM also inhibited CA secretion induced by veratridine (Ver), and 56 mM K^+ at concentrations similar to those for $[Ca^{2+}]_i$ rise. One of the lipophilic active compounds, cryptotanshione (Cryp), also had the same effects on CA secretion with LESM.

Conclusions: All these findings suggest that LESM exerts dual effects on CA secretion in cultured bovine adrenal medullary cells. LESM exerts antagonistic effects on nAChR, voltage-dependent Na^+ and Ca^{2+} channels, whereas it is an agonist of L-type Ca^{2+} channel when it used alone.

1. Introduction

Catecholaminergic system is activated as a part of the central nervous system (CNS) response to stress (Morilak et al., 2005), whereas stress-induced catecholamine (CA) over secretion may be detrimental or directly damage to the cardiovascular system (CVS) when the CVS is already under strain of diseases such as hypertension (both primary and secondary), angina pectoris, cardiac arrhythmia, diabetes, and even congestive heart failure (Thomas et al., 1986; Scardi et al., 1993; Esler and Kaye, 2000; Esler et al., 2006). Therefore, CA played an important role in the regulation of normal functions or inducing dysfunction in cardiovascular systems.

Chromaffin cells of the adrenal medulla are derived from a population of multipotent neural crest cells in the developing embryo. They share a common sympathoadrenal progenitor with sympathetic neurons. Thus, adrenal medullary chromaffin cells seemed to be sympathetic post-ganglionic neurons and have become specialized in the release of CA to the circulating blood flow. Bovine adrenal chromaffin cells have been widely used as a model system for studying the effects of multiple drugs on CA secretion (Park et al., 2003; Mao et al., 2008). Previous studies have shown that CA secretion in cultured bovine adrenal medullary cells can be stimulated by acetylcholine (ACh) and veratridine (Ver), agonists of nicotinic acetylcholine receptor (nAChR)-ion channels and voltage-dependent Na^+ channels respectively, as well as by high K^+ (56 mM), an activator of voltage-dependent Ca^{2+} channels. In these cells, many studies have reported that both ACh-induced Na^+ influx via nAChR-ion channels and Ver-induced Na^+ influx via voltage-dependent Na^+ channels increase Ca^{2+} influx

* Corresponding author. Tel.: +86 22 23051027; fax: +86 22 23051066.
E-mail address: gaoxiumei@tjutcm.edu.cn (X. Gao).

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via voltage-dependent Ca^{2+} channels, a prerequisite for secretion and synthesis of CAs; in contrast, high K^+ directly gates voltage-dependent Ca^{2+} channels to increase Ca^{2+} influx without increasing Na^+ influx (Wada et al., 1985). Since CA secretion mediated by stimulation of these ion channels in adrenal medullary cells are thought to be similar to those of norepinephrine in the sympathetic neurons, adrenal medullary cells have been a good model for detailed analysis of the actions of cardiovascular drugs (Kajiwara et al., 2002; Toyohira et al., 2005; Shinohara et al., 2007).

Salvia miltiorrhiza (Danshen), which is a well known traditional Chinese herb, has been used widely in China for promoting circulation and improving blood stasis, resolving swelling and tranquilizing the mind. Danshen is frequently used for the treatment of cardiovascular diseases in clinic, including coronary heart disease, hypertension, diabetes, atherosclerosis and chronic heart failure (Zhang et al., 2006; Wang et al., 2006; Yang et al., 2006). Chemical compounds from *Salvia miltiorrhiza* can be classified into two major categories: hydrophilic compounds and lipophilic diterpenoid quinines (LDQ). Both hydrophilic and lipophilic compounds of Danshen have multiple pharmacological activities, such as improving the microcirculatory disturbance, protecting against cardiotoxicity induced by doxorubicin, inhibiting the proliferation of vascular smooth muscle cells, anti-inflammatory, anti-platelet, anti-oxidant and vasorelaxation (Seon-il et al., 2003; Wang et al., 2005; Han et al., 2007; Jiang et al., 2009; Wu et al., 2009).

In our preliminary tests, we examined several extracts from *Salvia miltiorrhiza* including both hydrophilic and lipophilic extracts on CA secretion in bovine adrenal medullary cells. It was found the hydrophilic extracts such as tanshinol, salvianolic acid B had no significant effect on CA secretion; while the major lipophilic extract of Danshen had interesting findings about CA secretion in cultured bovine adrenal medullary cells. The major lipophilic components isolated from the dry roots of Danshen were called tanshinones (Tan) which were diterpenoid compounds with a phenanthrenequinone structure. Many researches have reported that *Salvia miltiorrhiza* Tan may protect against cardiovascular diseases through various mechanisms that may including enhanced vasorelaxation, neuroprotection, anti-inflammation and anti-oxidant properties (Lam et al., 2003, 2006; Jang et al., 2003; Liu et al., 2008). In addition, a recent study reported that Tan IIA directly and specifically activated human cardiac KCNQ1/KCNE1 potassium channels (Iks) independent of PKA activation, PKG activation and channel nitrosylation in HEK 293 cell (Sun et al., 2008). This may be one mechanism underlying the anti-arrhythmia activity of Tan.

However, there is little evidence regarding the effects of LESM on ion-mediated CA secretion. In present study, we examined the effects of LESM on nAChR-ion channels, voltage-dependent Na^+ channels, and voltage-dependent Ca^{2+} channels by investigating the direct effects of LESM on CA secretion, Ca^{2+} influx and/or $^{22}Na^+$ influx, induced by various secretagogues such as ACh, Ver and high K^+ in cultured bovine adrenal medullary cells. We found that LESM inhibited ACh, Ver and high K^+ induced CA secretion via suppression of various ion channels. Meanwhile, one of the lipophilic active compounds cryptotanshinone (Cryp) also had dual effects on CA secretion. Cryp significantly stimulated basal CA secretion while it inhibited various secretagogues-induced CA secretion which is similar with LESM. Whereas LEMS directly stimulated basal CA secretion through L-type Ca^{2+} channel.

In conclusion, our results suggest that LESM slightly stimulates basal CA secretion partly through L-type calcium channel. On the other hand, LESM also exerts antagonistic effects on nAChR, voltage-dependent Na^+ and Ca^{2+} channels. To our knowledge, this is the first study that investigates the direct action of LESM on CA secretion.

2. Materials and methods

2.1. Materials

Oxygenated Krebs–Ringer phosphate (KRP) buffer was used throughout. Its composition is as follows (in mM): 154 NaCl, 5.6 KCl, 1.1 $MgSO_4$, 2.2 $CaCl_2$, 0.85 NaH_2PO_4, 2.15 Na_2HPO_4 and 10 glucose, adjusted pH to 7.4. Reagents were obtained from the following sources: Eagle's minimum essential medium (MEM) and new born calf serum from Gibco (USA); collagenase from Nitta Zerachin (Japan); cytosine arabinoside, ACh, Ver, hexamethonium, nitrendipine, ω-conotoxin-GVIA and ω-agatoxin-IVA from Sigma (St. Louis, MO, USA); [^{22}Na]Cl from PerkinElmer Life Sciences (Boston, MA, USA); LESM was dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted in a reaction medium before use at a final DMSO concentration not exceeding 0.5%, unless otherwise specified.

2.2. Chemicals

Cryp, Tan I and Tan IIA were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 15,16-Dihyrotanshinone I and methylenetanshinquinone were isolated previously from the dried roots and rhizomes of *Salvia miltiorrhiza* in our laboratory, and their structures were elucidated by comparison of their spectral data. The structures of standards were listed in Fig. 1. Their purities were above 98% using LC analysis. LESM was a gift from Professor Cheng from Zhejiang University, which was extracted from 10 g *Radix Salvia miltiorrhiza* by 100 ml acetoacetate for 3 times. The yield of LESM was 18% (w/w). For total Tan determination, LESM was dissolved in methanol at a concentration of 10 μg/ml. Then it was tested using tanshinone IIA as standard substance by ultraviolet spectrophotometry method with the detection wavelength at 280 nm. The content of total Tan in LESM was 52.6%. Five active compounds, 15,16-dihyrotanshinone I, Cryp, Tan I, methylenetanshinquinone and Tan IIA, were quantified by HPLC–UV (Sun et al., 2007; Zhu, 2007). The HPLC analyses were performed on an Agilent 1100 series HPLC instrument (Agilent, Waldbronn, Germany) composed of a vacuum degasser, a quaternary pump, an autosampler, a column compartment, and a diode array detector (DAD). The chromatographic separation was carried out on a ZORBAX Eclipse plus C18 column (5 μm, φ1.8 mm × 100 mm) by setting the column temperature at 20 °C. The mobile phase consisted of acetonitrile (A) and water containing 0.05% formic acid (B). A gradient program was used as follows: 34–54% A at 0–15 min, 54–68% A at 15–30 min, then held for 20 min. The flow rate was kept at 0.4 ml/min. The detection wavelength was set to monitor at 280 nm. HPLC analysis was performed in triplicate; each with 10 μl of the sample. A typical chromatogram is shown in Fig. 2. The contents of 15,16-dihyrotanshinone I, Cryp, Tan I, methylenetanshinquinone and Tan IIA in LESM were 2.67%, 5.73%, 6.62%, 3.91% and 14.67%, respectively.

2.3. Isolation and primary culture of bovine adrenal medullary cells

Adrenal glands were obtained from the city slaughterhouse. Bovine chromaffin cells were isolated following standard methods (Yanagihara et al., 1979) with some modifications. Briefly, glands were perfused with Ca^{2+}-free KRP buffer to remove erythrocytes out and incubated with 0.5% collagenase, 0.05% trypsin inhibitor and 10% bovine serum albumin for 2 times at 37 °C. The first time took 10 min. The digested cells were thrown away to exclude some other cells and the debris, then the second digestion took 90 min to gain chromaffin cells. To examine the effects of LESM on CA secretion, the cells were cultured at a density of 5×10^5 cells/well in