Correlation of Antioxidative Properties and Vaso-relaxation Effects of Major Active Constituents of Traditional Chinese Medicine

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Abstract

Scutellaria baicalensis Georgi ("huang qin"), *Ligusticum chuanxiong* Hort. ("chuang xiong"), *Panax notoginseng* (Burk.) F.H. Chen ("san qi"), *Uncaria rhynchophylla* (Miq.) Jackson. ("gou teng"), *Rhokiola rosea* L. ("hong jing tian") and *Stephania tetrandra* S. Moore ("fang ji") are commonly used traditional Chinese medicine (TCM) for hypertensive patients. The pharmacologically active compounds found in these TCMs are: baicalin, ligustrazine, notoginsenoside $R_{1,}$ rhynchophylline, salidroside and tetrandrine, respectively, which possess anti-hypertensive properties with diverse cellular mechanisms. In this study, we attempted to evaluate a possible correlation of the anti-oxidative activities (using the cell-free DPPH assay) and the vaso-relaxation effects (using rat isolated thoracic aorta) of these compounds. In the anti-oxidative study, a relative order of free radical scavenging capacity (SR %) of: baicalin $\geq$ tetradrine >> salidroside $\geq$ ligustrazine $\geq$ rhynchophylline $\approx$ notoginsenoside R_1 was demonstrated. In the vaso-relaxing study, a relative order of the maximum relaxation response (at 3 mM) of: tetradrine > baicalin >> ligustrazine > notoginsenoside R_1 $\approx$ rhynchophylline > salidroside was recorded. A positive correlation (R^2 = 0.7741) between the anti-oxidative activity and the vascular relaxation effect of the compounds evaluated was illustrated. In contrast, ascorbic acid only elicited a free radical scavenging activity with no apparent relaxation effect;

whereas nifedipine (a Ca^{2+} channel blocker) caused a marked vascular relaxation with no obvious free radical scavenging activity. Hence, our results suggest, for the first time, that the therapeutic effect (e.g. anti-hypertensive) of these TCM-oriented drugs, unlike the Western medicine, are probably correlated with the unique anti-oxidative potential of these compounds.

Key words: Vaso-relaxation, anti-hypertensive, anti-oxidative activity, traditional Chinese medicine.

Introduction

Hypertension is a complex pathophysiological state that is characterized by high blood pressure. An elevation of the systolic and/or diastolic blood pressure increases the risk of damage to vital organs such as heart, brain, kidneys, adrenal glands and vasculature (Gardon, 2000). Oxidative stress has been implicated in the pathogenesis of hypertension and some of its complications (Redon et al., 2003). It is anticipated that antihypertensive drugs with a prominent anti-oxidative potency would serve as an additional benefit in the treatment of hypertension.

In the market, there are a few anti-hypertensive drugs which possess an additional anti-oxidative function. For instant, carvedilol, a novel β-blocker which has been use clinically for several years, has an anti-oxidative property by scavenging free radicals (Oettl et al., 2001). In addition, thiazide diuretics have been shown to increase the anti-oxidative potency of plasma (in term of better anti-oxidative level measured by the ferric-reducing ability of plasma (FRAP) assay) in animals and hypertensive patients (Uehara et al., 1993; Skalska et al., 2005).

Compounds extracted and isolated from herbs e.g. traditional Chinese medicine (TCM) have been used for more than a thousand years especially in the Asian countries (Japan, Korea and China). It is commonly agreed/believed that the ultimate goals in treating hypertension are to prevent hypertension-related cardiovascular diseases as well as to extend life expectancy with good quality (World Health Organization et al., 2003). Thus, in addition to the mainline research effort in studying

Western medicines, research on finding new drugs on the prevention or treatment of hypertension from nature (Ballew et al., 2001; Li, 2005; Wang et al., 2006; Yan et al., 2006) has gained an enormous momentum recently.

In contrast to most Western medicines with which the therapeutic strategies are mainly based on the cause and effect principle (Li et al., 2007), the philosophy of the uses of TCM are simply based on a holistic and spatial perception of the individual with the environment (i.e. it is a quest for an overall restoration of the "upset equilibrium" of our body). Thus, with a completely different philosophical/therapeutic approach, TCM provides therapeutic effects through a multi-target organs strategy with an array of different mechanisms.

In this study, six major effective constituents (baicalin, ligustrazine, notoginsenoside R_1, rhynchophylline, salidroside and tetrandrine) were chosen and they are obtained from common TCM that are commonly used/prescribed in treating hypertension in man. These herbs are: *Scutellaria baicalensis* Georgi ("huang qin"), *Ligusticum chuanxiong* Hort. ("chuang xiong"), *Panax notoginseng* (Burk.) F.H. Chen ("san qi"), *Uncaria rhynchophylla* (Miq.) Jacks. ("gou teng"), *Rhokiola rosea* L. ("hong jing tian") and *Stephania tetrandra* S. Moore ("fang ji") (Table 1). Apart from the anti-hypertensive effect, these compounds also possess other pharmacological effects. For example, baicalin (from *Scutellaria baicalensis* Georgi) has been used in treating inflammation, cancer and inhibited bacterial growth (Ciesielska et al., 2002; Woo et al., 2006). Ligustrazine (from *Ligusticum chuanxiong* Hort.) inhibits blood vessel formation and it is used in treating arrhythmia and myocardial

ischemia-reperfusion injury (Shu, 2006). Notoginsenoside R_1 (from Panax notoginseng (Burk.) F.H. Chen) can delay aging, counter shock and it is used to treat cancer (Wang et al., 2007; Yang et al., 2005). Rhynchophylline (from *Uncaria rhynchophylla* (Miq.) Jacks.) caused bradycardia, inhibited cardiac contractility repression effects. In addition, it has been shown to treat ailments in the cardiovascular and central nervous systems (Shi et al., 2003). Salidroside (from *Rhokiola rosea* L) has been used to treat cancer, scald, anoxia and reduced blood glucose (Qu, 2005). Tetrandrine (from *Stephania tetrandra* S. Moore) has been used to treat cardiac arrhythmia, angina and inflammation (Liu et al., 1995; Wang et al., 1995; Xiao et al., 1996).

In addition to all aforementioned effects, compounds isolated from herbs, in general, are favones and flavones with chemical structures/groups that are good anti-oxidants. In view of the important roles of oxidants/free radicals in the development of hypertension and other cardiovascular diseases, in this study we tested the hypothesis that the chief active ingredients isolated from the above mentioned TCM herbs possess both anti-hypertensive effect and anti-oxidative activity. More importantly, there is a correlation between the vascular relaxation effect (using rat isolated aorta) and the free radical scavenging property (using the common cell-free DPPH assay) of the compounds studied.

Materials and Methods

Selection of TCM herbs and Compounds

A total of six TCM herbs were selected for this study and these herbs are commonly used in TCM formulae prescribed to patients with hypertension. The major active constituent, rather than the extracts (water/organic), of each herb, was evaluated in this study.

Animals and Chemicals

Wistar rats (male, 250-300 g) were obtained from Guangdong Experimental Animal Center (Guangzhou, PR of China). Baicalin, ligustrazine, notoginsenoside R_1, rhynchophylline, salidroside and tetrandrine powders (>98% purity) were purchased from Baoji Hongyuan Biotech Ltd. (Baoji, PR of China). DPPH, phenylephrine hydrochloride, ascorbic acid, nifedipine and other reagents (analytical AR grade) for preparing the physiological salt solution were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of compounds and solutions

Baicalin, ligustrazine, notoginsenoside R_1 and rhynchophylline were dissolved in dimethyl sulphoxide (DMSO). Salidroside was dissolved in distilled water and tetrandrine was dissolved in 4 % (vol./vol.) hydrochloric acid (0.1 M) solution.

To measure free radical scavenging property using DPPH assay, individual compound was diluted by distilled water to give a final concentration of 1 mg/mL. Solution of ascorbic acid, which was used as a positive control for comparison, was dissolved in distilled water. In the vaso-relaxation study, stock of individual

compound was firstly diluted in distilled water and further dilutions were made in Tyrode's solution (for composition, see next section).

Measurement of free radical scavenging capacity

The anti-oxidative activity of individual compound was measured by DPPH assay, based on the free radical scavenging capacity of compounds on the stable DPPH free radical. Methanol was used to set the blank at 515 nm. The absorbance ($A_{control}$) of DPPH (0.024 mg/mL) in methanol was measured and recorded as control at 515 nm. Then, each compound (1 mg/mL) was added and mixed with the control and the change in absorbance (A_{sample}) was recorded until the absorbance change was $\leqq$ 0.003 absorbance units per minute, as described previously (Schlesier et al., 2002). The absorbance ($A_{original}$) of individual compound in methanol (without DPPH added) was measured so as to eliminate the "colour interference" of each compound. The percentage free radical scavenging capacity (expressed as SR %) was calculated using the following equation (Li et al., 2007).

$$SR\% = \left(1 - \frac{A_{sample} - A_{original}}{A_{control}}\right) \times 100\%$$

Measurement of the vaso-relaxation effects

The rats were anesthetized and sacrificed by decapitation, and the thoracic aortas were dissected from animals immediately. Fat and connective tissues around the thoracic aortas were carefully removed under the dissecting stereo-microscope (Leica, Germany). Care was taken not to touch the lumen of the thoracic aortas during

dissection to ensure the endothelium intact, unless otherwise stated. Four aortic rings (5 mm in length) were obtained from each aortic preparation and only one ring was used for each drug treatment. The thoracic aortic rings were mounted on an L-shaped metal prongs, which was connected to a force displacement MLT1030/D transducer (AD Instruments, Australia) coupled to the data acquisition programme (PowerLab 8SP, AD Instruments, Australia) for continuous recording of the isometric tension changes.

The mounted thoracic aortic rings were immersed in a 5 mL glass (water-jacketed) tissue baths containing Tyrode's solution of the following composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11 and $CaCl_2$ 1.8 (pH 7.4, 37±1°C), which was aerated continuously with a gas mixture of 95% O_2 and 5% CO_2. An optimal load of 10 ± 1 mN (determined from our preliminary studies) was applied progressively (2 mN per min) to aortic rings. Then, tissues were allowed to equilibrate for about 60 min under the optimal resting tension. During the equilibration period, tissues were washed with drug-free Tyrode's solution every 20 min and the resting tension was readjusted, if necessary, before commencing the experiments. After equilibration, the isolated aortic rings were sensitized with 65 mM KCl until two consecutive contractile responses were reproducible. To exclude the involvement of cyclo-oxygenase cascade, indomethacin (1 μM, a non-selective cyclo-oxygenase inhibitor) was included in the bath solution throughout the experiments. After equilibration, the aortic rings were challenged with phenylephrine (1 μM). After the establishment of a sustained contraction caused by phenylephrine,

individual compound (3 μM - 3 mM) was added cumulatively to the organ baths and the concentration-response curves to each compound were constructed (Cao et al., 2006; Damiani et al., 2003). A 100 % relaxation was considered when the active tension returned to the basal level.

Statistical analysis

Data were expressed as means ± S.E.M., and n denotes the number of replications for each data point. Comparisons of parameters among different groups were made with one-way analysis of variance, followed by Newman-Keul's test for multiple comparisons among means. In all cases, differences between treatment groups were considered significant at a P value < 0.05. Correlation analyses were performed using linear regression and the correlation of determination (R^2). All statistical analysis tests were performed by using GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Measurement of the free radical scavenging capacity

The anti-oxidative activity of all six compounds (1 mg/mL) was measured in term of the percentage free radical scavenging capacity (SR %). The SR % values of six compounds are: baicalin (94.74 ± 1.03 %), ligustrazine (44.45 ± 0.85 %), notoginsenoside R_1 (40.24 ± 2.86 %), rhynchophylline (40.71 ± 1.81 %), salidroside (48.42 ± 1.19 %) and tetrandrine (91.56 ± 0.55 %). Among all six compounds tested,

the anti-oxidative activity (SR %) of baicalin and tetrandrine was comparable to that of ascorbic acid (96.03 ± 0.02 %) ($P > 0.05$) whereas the SR % values of ligustrazine, notoginsenoside R_1, rhynchophylline and salidroside were significantly lower than that of baicalin, tetrandrine and ascorbic acid ($P < 0.001$).

Measurement of the vaso-relaxation effect

Individual compound (3 μM - 3 mM) elicited a concentration-dependent relaxation of the phenylephrine pre-contracted aortic preparations. The maximum relaxation (R_{max}) (observed at 3 mM) of six compounds was: baicalin (75.85 ± 6.53 %), ligustrazine (40.16 ± 2.24 %), notoginsenoside R_1 (27.68 ± 3.60 %), rhynchophylline (27.68 ± 4.49 %), salidroside (14.42 ± 1.45 %) and tetrandrine (100.7 ± 2.99 %) (Fig. 1). Nifedipine induced a concentration-dependent relaxation of the phenylephrine pre-contracted aortic rings with R_{max} equal to 96.53 ± 5.45, n = 4.

Relationship between the anti-oxidative activity and the vaso-relaxation effect

In order to investigate whether there was a correlation between anti-oxidative activity and vaso-relaxation effect, a Scatter plot of SR % and the maximum relaxation was performed (Fig. 2). Our results illustrated that there was a positive correlation between the free radical scavenging property (SR %) and the maximum relaxation of the compounds tested ($R^2 = 0.7741$, $P < 0.001$).

Discussion

Our present results illustrated that all six major effective constituents from the TCM herbs, which are commonly used in TCM formulae prescribed to hypertensive patients, possess vaso-relaxation effects. It is consistent with previous data in which baicalin (Huang et al., 2004) and tetrandrine (Wong, 1998) have relaxation effects in rat isolated aortic rings. Interestingly, in the present study, both baicalin and tetradrine are the most potent compounds, among all six herb-originated active constituents examined, in relaxing phenylephrine pre-contracted rat isolated aorta. Despite the fact that all compounds tested possess vaso-relaxation effect (in a differential manner), there is no structural similarity in the chemical structures arguing the involvement of a common receptor type pathway.

In addition to the vaso-relaxation effects, we have also evaluated the anti-oxidative properties by measuring the free radical scavenging activities using the cell-free DPPH assay. Consistent with a previous study (Ng et al., 2000), baicalin demonstrated, in our study, a comparable anti-oxidant effect as ascorbic acid tested in the erythrocyte heamolysis assay. For the first time, our results demonstrated that the free radical scavenging activity of tetrandrine was comparable to baicalin and ascorbic acid. Interestingly, baicalin and tetrandrine are the most potent agents, among all six compounds tested, in scavenging free radical. As stated above, there is no structural similarity which can account for the differential free radical scavenging activities observed.

Oxidative stress plays an important role in the development of atherosclerosis and hypertension. It is now clear not only that diverse reactive oxidative species (ROS)

are produced in the vessel wall, but also that they individually and in combination contribute to many of the abnormalities associated with vascular disease (Blokhina et al., 2003). There is abundant evidence supporting a role of ROS in the pathogenesis of high blood pressure and target organ damage in animal and human hypertension (Rajagopalan et al., 1996; Griendling et al., 2000).

Given the fact that there is a close relationship of ROS generation and cardiovascular diseases, a great deal of effort has been devoted to determine whether antioxidant supplementation can be beneficial in preventing or treating these conditions (Seifried et al., 2007). However, among all antihypertensive drugs used, only thiazides (Uehara et al., 1993; Skalska et al., 2005) and carvedilol (Oettl et al., 2001) have been reported possessing an "additional" anti-oxidative activity.

As stated in Introduction, the "philosophy" of the uses of TCM in treating different diseases is different from the general/common strategy of most Western medicines, we therefore tested whether there is a correlation between the free radical activities and the vaso-relaxation effects of these herbs-based active compounds. Interestingly, our results for the first time illustrated that there is a positive correlation with correlation of determination (R^2) = 0.7741, $P < 0.001$ of the vaso-relaxation effects and the free radical scavenging potentials of these compounds. However, it is important to point out that no such unique correlation was observed with ascorbic acid (a potent anti-oxidant) and nifedipine (a potent Ca^{2+} channel blocker) (both are Western medicines). Taken together, our results demonstrated that compounds isolated from TCM herbs studied in the present study seem to possess unique "dual effects"

which are beneficial in treating hypertension. In addition, our results may help to strengthen the wisdom of the uses of herbal medicines in treating different diseases with an overall/holistic approach with diverse mechanisms.

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Tables and Figures captions

Figure 1. Percentage relaxation of the six compounds. Data are expressed as mean±SEM, n=4.

Figure 2. Relations of percentage free radical scavenging capacity SR% and maximum relaxation percentage of the six compounds

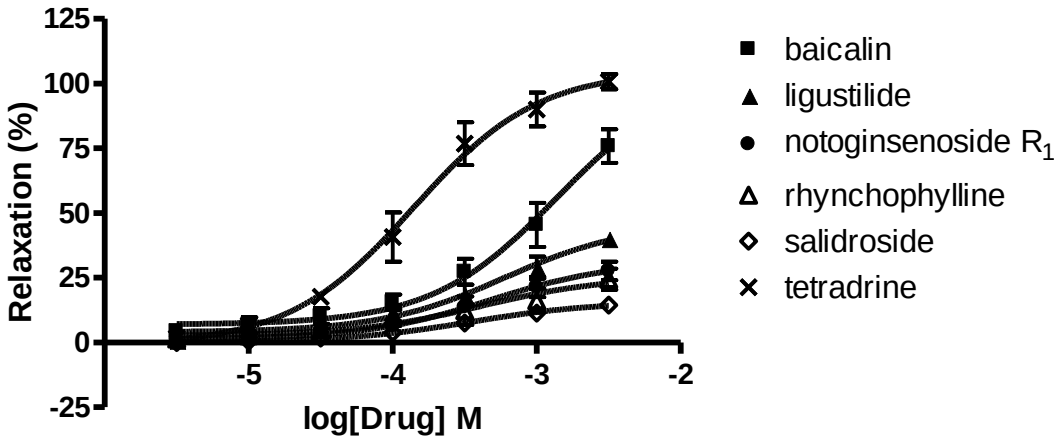
125
100
75
50
25
0
-25
Relaxation (%)
-5
-4
-3
-2
log[Drug] M
baicalin
ligustilide
notoginsenoside R1
rhynchophylline
salidroside
tetradrine

Figure 1.

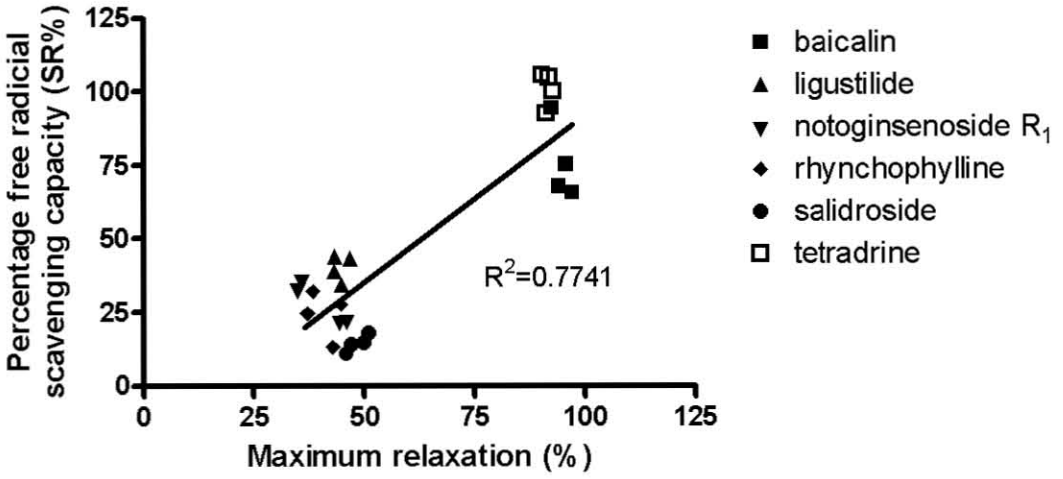
Percentage free radicial scavenging capacity (SR%)
125
100
75
50
25
0
0
25
50
75
100
125
Maximum relaxation (%)
R2=0.7741
baicalin
ligustilide
notoginsenoside R1
rhynchophylline
salidroside
tetradrine

Figure 2.