Food Chemistry 132 (2012) 1796–1801

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Antihypertensive effect of mulberry leaf aqueous extract containing γ-aminobutyric acid in spontaneously hypertensive rats

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ARTICLE INFO

Article history:
Received 2 May 2011
Received in revised form 31 October 2011
Accepted 16 November 2011
Available online 16 December 2011

Keywords:
Antihypertension
Morus alba L.
γ-Aminobutyric acid
Angiotensin I-converting enzyme
Spontaneously hypertensive rat

ABSTRACT

We hypothesised that mulberry leaves (ML) exert their antihypertensive effect through γ-aminobutyric acid (GABA) and that the antihypertensive activity of ML extract would be comparable to GABA alone in spontaneously hypertensive rats (SHR). We found that single administration of a water extract from leaves of *Morus alba* L. (WEML) lowered systolic blood pressure (SBP) transiently in a dose-dependent manner. Interestingly, the SBP-lowering effect of WEML was significantly higher than after treatment with GABA alone. We further found that WEML strongly inhibited angiotensin I-converting enzyme (ACE) activity *in vitro* with an IC_{50} value of 29.8 mg/ml. These results suggest that WEML has a transient antihypertensive effect *in vivo* that may involve a mechanism of ACE inhibition in addition to GABA.

1. Introduction

Hypertension is an important worldwide public health challenge, and is a leading cause of cardiovascular, retinal and kidney diseases (DellaCroce & Vitale, 2008; Savica, Bellinghieri, & Kopple, 2010). The first line of treatment for hypertension is lifestyle changes, such as dietary changes, physical exercise and weight loss (Smith et al., 2010). Reduction of blood pressure (by 5 mm of Hg) can decrease the risks of stroke by 34% and ischaemic heart disease by 21%, as well as reduce mortality from cardiovascular disease (Law, Wald, & Morris, 2003). Recently, much effort is being invested in the detection of bioactive components in foods or diets for the treatment and prevention of hypertension (Yoshikawa et al., 2000).

Mulberry (*Morus alba* L.) is cultivated in oriental countries and their leaves have been used for a long time to feed silkworms. In folk medicine, mulberry leaves, root bark and twigs have long been used to reduce fever, protect the liver, improve eyesight, strengthen the joints, facilitate the discharge of urine and lower blood pressure (Chang et al., 2011). To date, different parts of mulberry, from the root bark to the leaves, have been extensively investigated for their health benefits, including antioxidative, hypolipidaemic, antihyperglycaemic, antiatherogenic, antiviral, antimicrobial and neuroprotective effects (Chang et al., 2011; El-Beshbishy, Singab, Sinkkonen, & Pihlaja, 2006; Harauma et al., 2007; Liu et al., 2009; Naowaboot, Pannangpetch, Kukongviriyapan, Kongyingyoes, & Kukongviriyapan, 2009; Yang, Yang, & Zheng, 2010; Zhang et al., 2009). However, there were no reports regarding the antihypertensive effect of mulberry leaves (ML) *in vivo*.

γ-Aminobutyric acid (GABA) is a four-carbon amino acid that acts as one of the major inhibitory neurotransmitters in the central nervous system (Kimura, Hayakawa, & Sansawa, 2002). It has been demonstrated that GABA plays an important role in both central and peripheral nervous system control of blood pressure (Hayakawa, Kimura, & Yamori, 2005; Kimura et al., 2002). GABA is also found in various plants and fermented foods, such as tea leaves (Abe et al., 1995), fermented milk products (Hayakawa et al., 2004), fermented soybean (Aoki, Furuya, Endo, & Fujimoto, 2003), soy sauce (Yamakoshi et al., 2007), rice grains (Akama et al., 2009) and tomato (Yoshimura et al., 2010). Feeding of GABA or GABA-rich foods, such as green tea, fermented milk products and soy sauce, for 4–6 weeks has been found to depress the elevation of systolic blood pressure (SBP) in spontaneously hypertensive rats (SHR) (Abe et al., 1995; Hayakawa et al., 2004; Yamakoshi et al., 2007). In addition, the antihypertensive effect of GABA-rich fermented milk has also been reported in humans (Inoue et al., 2003). Kang et al. (2006) reported that ML contains a considerable amount of GABA. Thus, we hypothesised that ML will have the ability to exert antihypertensive effects *in vivo* through GABA.

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doi:10.1016/j.foodchem.2011.11.143

To test our hypothesis, water extracts from leaves of *M. alba* L. (WEML) were evaluated for their blood pressure-lowering effects in SHR after single and chronic oral administrations by gastric intubation. The antihypertensive effect of GABA alone was used as a positive control for comparison. Theoretically, the WEML-treated group and the GABA-treated group (both containing the same dosage of GABA) should have the same effect on SBP if the antihypertensive effect of ML is solely attributable to GABA. During the chronic administration, we further evaluated liver and renal functions for toxicity assessments of WEML. In addition, the effects of WEML on angiotensin I-converting enzyme (ACE) inhibitory activity, which is known as one mechanism through which antihypertensive agents work, were also evaluated *in vitro*.

2. Materials and methods

2.1. Materials

All chemicals used were of analytical grade. Methyl red, gallic acid, bromocresol green, triethylamine (TEA), γ-aminobutyric acid (GABA), potassium sulphate, aluminium nitrate, ammonium acetate, angiotensin I-converting enzyme (ACE), *N*-hippuryl-His-Leu tetrahydrate (HHL), phenyl isothiocyanate (PITC), Folin–Ciocalteu's reagent, sodium dihydrogenphosphate dehydrate, sodium phosphate, dibasic and hippuric acid were obtained from Sigma (St. Louis, MO, USA). Ethanol, acetic acid, acetonitrile, *n*-hexane, methanol, hydrochloric acid, sodium hydroxide, sodium carbonate, ethyl ether, sulphuric acid and all standards of trace elements were purchased from Merck (Darmstadt, Germany). Potassium acetate was obtained from J.T. Baker (Phillipsburg, NJ, USA). Laboratory animal diets, MF-18, were purchased from Oriental yeast Co., Ltd. (Tokyo, Japan).

2.2. Plant materials

Leaves from *M. alba* L. were obtained form Tainan District Agricultural Research and Extension Station, Council of Agriculture, Executive Yuan, Taiwan. The code name of the investigated mulberry leaves is Tai-Sang 203.

2.3. Preparation of WEML

After cleaning and cutting, ML were dried, crushed and put through a 40 mesh sieve. ML powder (1500 g) was extracted with 6000 ml of distiled water at 100 °C for 1 h. After filtration, the solutions were dehydrated in a vacuum (VirTis 12ES, the VirTis Co., Inc., Gardiner, NY, USA) and the dried extract was used as WEML. The extraction rate of WEML was 4.6%.

2.4. Determination of compositions and GABA of WEML

General compositions of WEML, including moisture content and crude protein, fat, ash and fibre contents, were analysed by the methods referred to as AOAC 15.950.02, AOAC 15.976.05, AOAC 15.920.39, AOAC 15.955.03 and AOAC 15.962.09, respectively. GABA and other amino acid contents were determined by the acid hydrolysis method (Krause, Bockhardt, Neckermann, Henle, & Klostermeyer, 1995). Briefly, dried samples (100 mg) were degraded in 6 N HCl solution at 110 °C for 24 h. After filtration, GABA and other amino acids were analysed using an automatic amino acid analyzer (Hitachi L8500, Hitachi Co., Tokyo, Japan). Trace elements, including Na, K, Ca and Mg, were analysed by a flame atomic absorption analyzer (Varian AA-1275, Varian Twchtron, Springvale, Australia).

2.5. Animals

Male spontaneously hypertensive rats (SHR) were purchased from BioLasco Taiwan Co., Ltd. (Taipei, Taiwan). The SHR strain was obtained by selective breeding of Wistar–Kyoto (WKY) rats with high blood pressure during the 1960s by Okamoto and colleagues (Kundu & Rao, 2008). The rats were caged individually in a room under the following conditions: temperature 25 ± 1 °C; relative humidity 40–60%; 12 h light/dark cycle. All rats were fed a standard diet (laboratory animal diets MF-18, Oriental yeast Co., Ltd., Tokyo, Japan) and tap water with *ad libitum* access to both. The animals were used for the experiments after a 1 week quarantine period. All animal procedures were performed according to a Guideline for the Care and Use of Laboratory Animals from the Chinese-Taipei Society of Laboratory Animal Sciences.

2.6. Blood pressure and heart rate measurements

Systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) were measured using the tail-cuff method with a blood pressure monitor (Model MK-1030, Muromachi Co., Tokyo, Japan). Before the measurements, the rats were kept in a heated box at 37 °C for 10 min to raise their body temperature. SBP, DBP and HR were measured in triplicate with results reported as the average.

2.7. Single administration study

In total, 30 male SHR rats (13 weeks old, with body weights (BW) between 265 and 309 g) were used for the single administration study. The rats were randomly divided into five groups of six rats: control (normal saline 10 ml), low dose (0.53 g/kg BW of WEML; equivalent to 2 mg/kg BW of GABA), medium dose (1.05 g/kg BW of WEML; equivalent to 4 mg/kg BW of GABA), high dose (5.26 g/kg BW of WEML; equivalent to 20 mg/kg BW of GABA), and 20 mg/kg BW of GABA. WEML and GABA were dissolved in normal saline (10 ml) and given orally by gastric intubation. SBP, DBP and HR were measured before the administration (baseline), and at 4, 8 and 24 h after administration.

2.8. Chronic administration study

In total, 50 male SHR rats (13 weeks old, with BW ranging from 276 to 318 g) were randomly divided into five groups of 10 rats. The groups were dosed equivalently as in the single administration study, once each week, throughout the study. All samples were given orally to the rats by gastric intubation. The animals were allowed free accesses to the diet and drinking water during the 8 weeks of study. Body weight, blood pressure and heart rate were measured once a week at a consistent time of a day. At the end of the 8-week treatment period, the rats were fasted for 12 h and then sacrificed. Rat serum was obtained from the heart for biochemical analysis and organs including heart, liver, kidney and lung were weighed and expressed as relative organ weight (organ weight/body weight $\times$ 100%). Biochemical analyses of serum, including creatinine (Cr), blood urea nitrogen (BUN), uric acid (UA), cholinesterase (ChE), aspartate transaminase (AST) and alanine transaminase (ALT), were determined by an automatic biochemical analyzer (Chiron/Diagnostics Express Plus, East Walple, USA) with commercial kits (Randox Laboratories Ltd., Crumlin Co., Antrim, UK).

2.9. Determination of ACE-inhibitory activity in vitro

Angiotensin I-converting enzyme (ACE) inhibitory activity was measured by the spectrophotometric assay with some modifications