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Effects of Panax ginseng and Ziziphus jujuba on stress-induced apoptosis in rats

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PG has been well studied about effects of stress resistance. Although ZJ has been known that it had stress resistance effect since ancient times, its pharmacological properties and clinical applications have not been studied and reported until recently. Therefore, the purpose of this study is to determine whether effects of stress hormones, mechanism of stress protein could be induced by PG and ZJ of herb extract ingestion during stress exposure. In addition, this study identified expression of apoptosis factors related to stress. 1) Bcl-2 expression of the stressed rats decreased in comparison with the unstressed rats in heart and stomach. Bcl-2 expression of rats administered to PG was higher than the stressed rats in heart and that of rats administered to ZJ was higher than the stressed rats in stomach. 2) Stressed rats were decreased in p53 protein expression than normal rats. Thus, the results suggest stress-induced apoptosis is p53-independent apoptosis. And these results demonstrated that PG or ZJ administration helped to return from stress state to normal. 3) Clusterin expressed markedly in only salivary gland, but that of expression was no difference among four groups in tissues. Clusterin expression has no relation of stress-induced apoptosis.

Key words: Stress, Panax ginseng, Ziziphus jujuba, Apoptosis

Introduction

Traditional Oriental Medicine has been used as a potential source of pharmaceutical remedies for a long time [Liu, 1987; Zheng et al., 1997; Brekhman et al., 1981]. It has been introduced into the Orient that human's body which caught a disease by stress is recovered to function normally metabolism by taking continuously extract of Oriental herbal medicines. As oriental herbs having these effects, there are Panax ginseng, Eleutherococcus senticosus, Astragalus membranaceus, green tea, Ziziphus jujuba etc.

Particularly, Panax ginseng (PG) has been the best known as anti-stress activity, and there is evidence that they increase arousal, stamina and stress resistance [Fulder, 1981; Bhattacharya and Mitra, 1991; Nguyen et al., 1995; Youl Kang et al., 2002]. It has been used for many centuries in the East as an energy booster. Today, it is widely believed that oral administration of ginseng can have beneficial mental and physical effects in humans. In previous studies, Brekhman et al. [Brekhman and Dardymov, 1969] reported that the basic effects of ginseng have been characterized as increased physical stamina and general resistance to a decrease in immune function.

Discovery of PG effects by Rivier and Shen [Rivier and Shen, 1994] that nitric oxide acts as a local hormone with a large array of effects on the body including effects on the hypothalamic-pituitary-adrenal axis (HPA-axis) [Rivier and Shen, 1994] represented a new paradigm, which has been harnessed by Kim et al. [Kim et al.,1998] to explain the previously documented effects of PG on the HPA-axis. It should be noted that there are other reports of adaptogens increasing the stress response. Hiai et al. [Hiai et al., 1979a ; Hiai et al., 1979b] observed a significant and large increase in adrenocorticotrophic hormone (ACTH) and corticosterone within 15 minutes after intraperitoneal injection of PG (7 mg/kg).

PG has been well studied about the effects of stress resistance. Although Ziziphus jujuba (ZJ) has been known that it had stress resistance effect since ancient times, its pharmacological properties and clinical applications have

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not been studied and reported until recently.

In traditional medicine, ZJ has been used for its action on insomia and anxiety [Lee, 1986]. Jujuba fruit contains relatively high amounts of cyclic AMP and cyclic GMP which are enzymes involved in energy production and metabolic processes. It is used traditionally for replenishing Qi (energy) and in conditions marked by lassitude and weakness. Suanzaozentang, a prescription of the Chinese Medicine, possesses the anxiolytic effect in the clinical test. Its action of mechanism was as a result of decreasing the monoaminergic system activity [Hsieh et al., 1986].

Apoptosis is a fundamental form of cell death which plays a major role in the development and homeostasis of multicellular organisms [Arends and Wyllie, 1991]. Disturbances in apoptosis are important in cancer, acquired immunodeficiency syndrome and some neurodegenerative disorders [Thompson, 1995]. Many lines of evidence indicated that apoptotic process requires specialized machinery [Kroemer et al., 1995]. Proteins of the Bcl-2 family together with caspases have among others been identified as essential components of the intracellular apoptotic signaling pathways. Bcl-2 family is composed of anti-apoptotic (bcl-2, bcl-X_L, bag-1, bcl-W, mcl-1 and A1) and pro-apoptotic (bax, bad, bak and bcl-Xs) molecules [Gross et al., 1999], balance between anti- and pro-apoptotic Bcl-2 family proteins may act as a rheostat for the apoptotic program [Gross et al., 1999]. Previous reports demonstrated that some Bcl-2 family members are located on mitochondrial membrane, that can alter the permeability of mitochondrial membrane and trigger the release of cytochrome c [Gross et al., 1999 ; Solange and Martinou, 2000] or caspases [Budihardjo et al., 1999], then activate the post-mitochondrial caspase cascade leading to apoptotic cell death.

A tumor-suppressor gene, p53 encodes a 53 kDa nuclear phosphoprotein (known as the p53 protein) involved in the control of cell cycle, apoptosis and DNA repair [Vogelstein and Kinzler, 1992; Steele et al., 1998]. In a subset of normal tissues, cell death can be induced by activation of the p53 tumor suppressor protein [Gudkov and Komarova, 2003]. Activation of p53 protein induces its function as a target-specific regulator of gene transcription, consequently modulating the expression of downstream proteins, many of which can promote apoptosis, cell cycle arrest, or DNA repair, depending on cell type and context.

Mammalian clusterin is translated with a typical hydrophobic signal peptide, 21 amino acids in length, which is proteolytically removed during translocation of the protein to the ER lumen [Jenne and Tschopp, 1992]. There is extensive evidence of a correlation between clusterin expression and different types of disease (e.g., Alzheimer's disease, gliomas) or pathological stress (e.g., hydrostatic pressure insult or ischemic injury in the kidney) [Jenne and Tschopp, 1992]. This correlation has led in the past to repeated suggestions that clusterin is a stress-response protein. This notion has recently been strengthened by the finding that heat shock factor 1 (a transcriptional activator of heat shock protein genes) binds to a highly conserved region of the clusterin promoter, which is virtually identical to a corresponding element in many heat shock protein promoters, and upregulates clusterin gene expression [Michel et al., 1997].

Therefore, the purpose of this study is to determine effects of PG and ZJ on stress-induced apoptosis in rats. For this purpose, this study identified protein expression of apoptosis factors related to stress following two herb extracts administration.

Materials and Methods

Preparation of herbs

Oriental herbal medicines (Panax ginseng, Ziziphus jujuba) were purched on the Korean market. This study was carried out on animals with extract of commercial Oriental herbal medicines (5 0g/100ml ddH2O) by fumigation with burning.

Animals

Adult male Sprague-Dawley rats weighing 200 ± 2 g were housed five per cage ((W)26× (D)42× (H)18 cm). The room was maintained on a 12:12 reverse light cycle and controlled temperature (25 ± 1°C). Food and water were available *ad libitum* throughout the experiments.

Administration of drugs and restraint stress

The oriental medicine extracts, PG and ZJ were administered (0.5 g/kg) daily during 5 days stress session. In addition, control animals divided into two groups, naive non-stressed and stress control group was administered distilled water for the drug treatments.

Experimental animals were exposed to restraint stress 2 h daily for 5 days, after administration of each drug. Throughout the stress task, animals were restricted to individual in a stress cage made of stainless steel. The stress task took place between 10:00 and 12:00 h in A.M.

Organ collection

The animals were sacrificed by decapitation, and then we dissected the animals and collected hearts, stomachs, and salivary glands tissues within 10 min. Hearts, stomachs, and salivary glands for western blotting were immediately frozen on ice respectively. All organs were stored at −70°C until analyzed.

Western blotting

The isolated tissues were homogenized at 4°C for 30 min in a solubilizing buffer containing complete protease inhibitor cocktail. Insoluble material was removed by centrifugation at 12,000× g for 10 min at 4°C. The supernatant was mixed with sample-buffer× 2, and heat 10 min at 100°C. And the samples were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (12% polyacrylamide) and transferred onto a nitrocellulose membrane (Hybond-

ECL, Amersham Pharmacia Biotech.). Blots were saturated with semi-dry transfer units (Hoefer TE 70 series, Amersham pharmacia biotech.) for 1 h and blots were blocked with 5% non-fat milk in TBST (0.2 M Trizma base, 0.1368 M NaCl, 0.1% Tween^{TM}-20) overnight at 4^{o}C. Blots were incubated for 1 h with bcl-2, p53, and clusterin antibody (Santa Cruz Biotechnology, Inc.) were added with a dilution ratio 1 : 200 for 2 h at room temperature. After three times washes with TBST, blots were revealed with purified goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1 : 1000) (Amersham Pharmacia Biotech.) for 1 h at room temperature followed by incubation with saturate for enhanced chemiluminescence detection (ECL, Amersham Pharmacia Biotech.).

Results

Bcl-2 family members have recently been invoked to maintain cell viability by preventing loss of mitochondrial membrane potential, and play a critical role in mitochondrial cytochrome c release. Especially, the bcl-2 protein of Bcl-2 family members protects cells from apoptotic cell death and the functional significance of its association with mitochondria, nuclear envelope and ER.

To explore the possible role of bcl-2 in the stress-induced apoptosis, This study examined the effects of PG and ZJ on the expression of the bcl-2 protein by Western blot analysis. The results identified 28 kDa of bcl-2 protein (Fig. 1) in salivary gland, heart, and stomach. As shown in Fig. 1, the band of bcl-2 protein in stressed rats expressed weakly compared to normal control in heart and stomach tissues. After restraint stress treatment of rats with PG administration, the band of bcl-2 protein expressed strongly in heart tissue compared to only stressed rats. In case of rats with ZJ administration, the band of bcl-2 protein expressed strongly in stomach tissue compared to that of stressed rats.

A tumor-suppressor gene, p53, is located in human chromosome 17 p13.1 and encodes a 53 kDa nuclear phosphoprotein (known as the p53 protein) involved in the control of cell cycle, apoptosis and DNA repair. To identify the relation between the p53 protein and the stress-induced apoptosis and the effects of PG and ZJ on stress, this study examined the expression of the p53 protein using the western blot analysis. The results identified 53 kDa of p53 protein (Fig. 2) in salivary gland, heart, and stomach. As shown in Fig. 2, the band of p53 protein in stressed rats expressed weakly compared to normal control in salivary gland, heart and stomach tissues. After restraint stress treatment of rats with PG administration, the band of p53 protein expressed strongly in salivary gland and stomach tissue compared to that of stressed rats. In case of rats with ZJ administration, the band of p53 protein expressed strongly in salivary gland, heart and stomach tissue compared to that of stressed rats.

One of clusterin roles is the ubiquitous upregulated expression of the gene in e.g. developmental remodeling, apoptotic disease states such as neurodegeneration, and in response to injury and other stresses. To identify the relation between the clusterin protein and the stress-induced apoptosis, and the effects of PG and ZJ on stress, this study examined the expression of the clusterin protein using the western blot

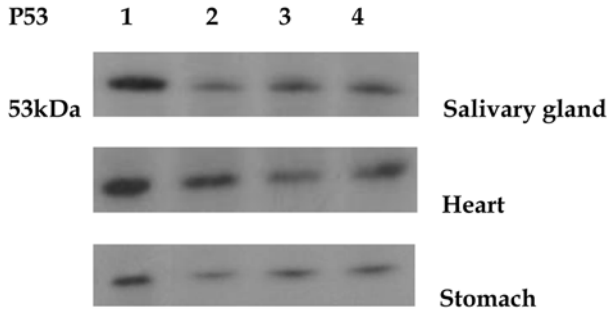

Fig. 2. Western blotting of p53 in rats tissue. Extracts from rats tissue(salivary gland, heart and stomach) were separated by SDS-PAGE and analyzed by western blotting using p53 antibody (Santa Cruz biotech.). Lane 1 : normal, Lane 2 : stress, Lane 3 : stress + PG, Lane 4 : stress + ZJ (n = 5)

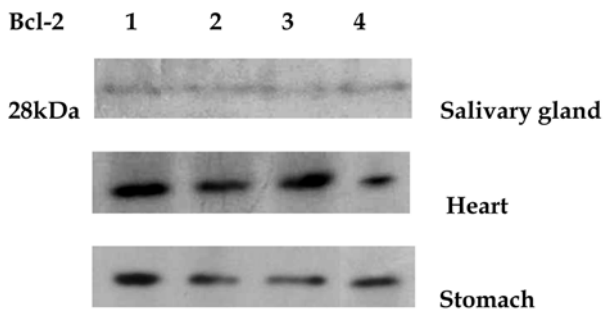

Fig. 1. Western blotting of bcl-2 in rats tissue. Extracts from rats tissue (salivary gland, heart and stomach) were separated by SDS-PAGE and analyzed by western blotting using bcl-2 antibody (Santa Cruz biotech.). Lane 1 : normal, Lane 2 : stress, Lane 3 : stress + PG, Lane 4 : stress + ZJ (n = 5)

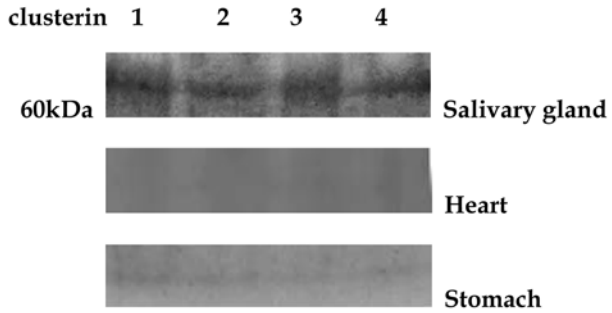

Fig. 3. Western blotting of clusterin in rats tissue. Extracts from rats tissue(salivary gland, heart and stomach) were separated by SDS-PAGE and analyzed by western blotting using clusterin antibody (Santa Cruz biotech.). Lane 1 : normal, Lane 2 : stress, Lane 3 : stress + PG, Lane 4 : stress + ZJ (n = 5)

analysis. The results identified about 60 kDa of clusterin protein (Fig. 3) in salivary gland, heart, and stomach. As shown in Fig. 3, the levels of clusterin protein in stressed rats were similar to normal control in salivary gland, heart, and stomach tissues. After restraint stress treatment of rats with PG or ZJ administration, the levels of clusterin protein had no different expression in salivary gland, heart, and stomach tissue compared to that of stressed rats.

Discussion

Previous studies indicate that oral consumption of PG and ZJ can have beneficial mental and physical effects in human. Thus they are widely marketed and consumed internationally. In addition, studies devoted to clarify its mechanisms of action appear to be of remarkable importance. In the present study, the effect of PG and ZJ on stress-induced apoptosis was examined. The results demonstrated that PG and ZJ inhibited stress-induced apoptosis and helped to return to normal state in the concentration of 0.5 mg/ml. It is intriguing that PG and ZJ reduced stress-induced apoptosis *in vivo*. In recent studies, circulating corticosterone concentrations are elevated during stress, and the present study tested whether these increases in glucocorticoid stimulation *in vivo* were sufficient to be associated with higher frequencies of cell apoptosis.

Apoptosis, a programmed cell death, is characterized by cell shrinkage, chromatin condensation, apoptotic body and DNA fragmentation [Kerr et al., 1972; Wyllie, 1993; Peitsch et al., 1993]. Additionally, the apoptosis is induced *in vitro* by irradiation [Yamada and Ohyama, 1988] or glucocorticoids [Wyllie, 1980; Gavrieli et al., 1992], and *in vivo* by burn [Fukuzuka et al., 1999], hypoglycemia [Morishita et al., 1998], or exercise [Hoffman-Goetz et al., 1999]. Furthermore, immobilization, a well known model of emotional stress [Ueyama et al., 1999], was shown to induce thymus atrophy in the rat [Teshima et al., 1987]. Various stresses trigger adaptative responses in the HPA axis [Selye, 1936]. However, excessive stress induces thymus atrophy, thereby causing immunological depression [Riley, 1975]. The HPA axis activation evokes the overflow of glucocorticoids and catecholamines, either of which can cause thymocyte apoptosis [Cohen and Duke, 1984; Offen et al., 1995]. In support of the involvement of glucocorticoids in the stress-induced thymus atrophy, glucocorticoids treatment, physical stress or hypoglycemia of rats results in thymocyte apoptosis, which is inhibited by a glucocorticoids receptor antagonist RU-486 [Compton and Cidlowski, 1986; Concordet and Ferry, 1993; Morishita et al., 1998].

However, the mechanism and morphology of the apoptosis evoked *in vivo* by stress are largely unknown at present.

Many genes participate in the regulation of apoptosis, and activation of caspase cascade is a central effector mechanism promoting apoptosis in response to death-inducing signals from cell surface receptors, from mitochondria or from endoplasmic reticulum [Toshiyuki et al., 2000].

Many evidences now exist to suggest that mitochondrial cytochrome c release is an important control point in caspase activation and apoptosis. Several studies have shown that overexpression of bcl-2 and bcl-XL prevents the mitochondrial release of cytochrome c, thereby inhibiting the activation of caspases cascade and apoptosis [Gross et al., 1999; Budihardjo et al., 1999; Salvesen and Dixit,1999; Solange and Martinou, 2000]. Bax is a proapoptotic member of the Bcl-2 family, bax dimerizes with itself or with bcl-2 or bcl-XL [Gross et al., 1999]. Overexpression of bax has been shown to disrupt the anti-apoptotic properties of bcl-2 and bcl-XL [Gross et al., 1999]. Other studies demonstrated that bcl-2, bcl-XL, bax and bak can act as channel proteins in the mitochondrial membrane [Gross et al., 1999].

This study showed that stress induces apoptosis in rats on the basis of bcl-2 protein expression. The apoptosis of rats tissues induced by immobilization stress, as shown in this study, may lead to involution of the tissues *in vivo* that we observed significant reduction in the body weight for 2h restraint stress (data not shown).

PG or ZJ-administrated rats were undergoing a decrease in apoptosis than stressed rats. We examined the effects of PG and ZJ on the expression of the bcl-2 protein by Western blot analysis. The western blotting results showed that the levels of bcl-2 protein in stressed rats decreased compared to normal control in heart and stomach tissues. After restraint stress treatment of rats with PG administration, the levels of bcl-2 protein increased in heart tissue compared to that of stressed rats. In case of rats with ZJ administration, the levels of bcl-2 protein increased in stomach tissue compared to that of stressed rats. Thus, these results showed that expression of the Bcl-2 family proteins bcl-2 (apoptosis-suppressing molecules) can be differently regulated by stress and PG or ZJ-administration.

Wild-type p53 is considered to participate in apoptosis in response to some chemotherapeutic drugs or irradiation in many tumor cells [Lowe, et al., 1994], and had also been reported as a direct transcriptional activator of the human bax gene [Miyashita and Reed, 1995], suggesting that p53 may be responsible for the activation of certain conserved apoptosis pathways. However, no increase of wild-type p53 protein was detected upon stressed rats than normal rats (Fig. 2). In addition, p53 protein expression of rats with restraint stress treatment and PG administration increased in salivary gland and stomach tissue compared to only stressed rats. In case of rats with ZJ administration, the levels of p53 protein increased in salivary gland and heart tissue compared to only stressed rats. These results suggest that stress-mediated bcl-2 down-regulation and apoptosis occurred independent of p53 protein.

Clusterin is a stress-response protein. Moreover, increased expression of clusterin has been established in diseases where either abnormal cell death or proliferation is occurring.

As shown our results of clusterin expression, the levels of clusterin protein in stressed rats were similar to normal control in salivary gland, heart, and stomach tissues (Fig. 3). After restraint stress treatment of rats with PG or ZJ administration, the levels of clusterin protein had no different expression in salivary gland, heart, and stomach tissue compared to that of stressed rats.

These results indicate that the molecular machinery of stress-induced apoptotic death signaling in rats is suggested as follows.

1) Bcl-2 expression of the stressed rats decreased in comparison with the unstressed rats in heart and stomach. Bcl-2 expression of rats administered to PG was higher than the stressed rats in heart and that of rats administered to ZJ was higher than the stressed rats in stomach.

2) Stressed rats were decreased in p53 protein expression than normal rats. Thus, we suggest stress-induced apoptosis is p53-independent apoptosis. And our results demonstrated that PG or ZJ administration helped to return from stress state to normal.

3) Clusterin expressed markedly in only salivary gland, but that of expression was no difference among four groups in tissues. Clusterin expression has no relation of stress-induced apoptosis.

Apoptosis is a pathological process accompanying histological changes. Therefore histologic investigation should be helpful for the study of apoptosis. And further studies focusing on biological significance and mechanism of stress-induced apoptosis is required to extend our understanding of the mechanisms of chemotherapeutic potency of PG and ZJ in human stress.

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