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Greater stress protein expression enhanced by combined prostaglandin A_1 and lithium in a rat model of focal ischemia[1]

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Key words

prostaglandin A_1; lithium; oxidative stress; heat shock factor 1; cerebral ischemia; Apaf-1; HO-1; HSP90α; HSP90β

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Abstract

Aim: To investigate the effects of lithium (Li) and prostaglandin A_1 (PGA_1) on the expression of heat shock factor 1 (HSF-1), heat shock proteins (HSP), and apoptosis protease activating factor-1 (Apaf-1) induced by permanent focal ischemia in rats. **Methods:** The rats were pretreated with a subcutaneous (sc) injection of Li for 2 d or a single intracerebral ventricle (icv) administration of PGA_1 for 15 min before ischemic insult, or a combination of Li (sc, 1 mEq/kg, 2 d) and PGA_1 (icv, 15 min prior to ischemic insult). Brain ischemia was induced by the permanent middle cerebral artery occlusion (pMCAO). Twenty-four hours after the occlusion, the expression of HSF-1, HSP, and Apaf-1 in the ischemic striatum were examined with Western blot analysis. **Results:** The expression of HSF-1, heme oxygenase-1 (HO-1), HSP90α, and Apaf-1 were significantly increased, but the expression of HSP90β was significantly decreased 24 h after the pMCAO. PGA_1 and Li and their combination significantly enhanced the ischemia-induced elevation in the levels of HSF-1, HO-1, and HSP90α, and recovered HSP90β expression, but decreased Apaf-1 levels in the ischemic striatum. **Conclusion:** The present study demonstrates that PGA_1 and Li have synergistic effects on the enhancement of the expression of HSP, suggesting that the synergistic effects of PGA_1 and Li in the rat model of permanent focal cerebral ischemia may be mediated by the enhancement expression of HSP expression and the downregulation of Apaf-1. Our studies suggest that combined PGA_1 and Li may have potential clinical value for the treatment of stroke.

Introduction

Stroke, most commonly caused by the interruption of blood supply to the brain, is one of the leading causes of disability and mortality in adults. Multiple molecular mechanisms, including energy crisis, oxidative stress, calcium overloading, caspase activation, and inflammation have now been suggested as potential contributors to ischemia-induced neural injury[1,2]. Unfortunately, there has been no satisfactory treatment to prevent neural cell death and the long-term neurological deficits in stroke victims.

In previous studies, we have reported that prostaglandin A_1 (PGA_1) inhibited the excitotoxin-induced apoptosis of striatal neurons *in vivo* and the rotenone-induced apoptosis of cultured SH-SY5Y cells *in vitro*[3,4]. Lithium has been extensively used in the treatment of bipolar mood disorder, and recent study has revealed that it is a neuroprotective drug against a variety of insults, such as glutamate-induced excitotoxicity[5], ischemia-induced neural damage, and other neurodegenerative conditions[6–9]. We have found that PGA_1 protects neurons in rodent models of focal ischemia[10], and the combination of PGA_1 and lithium generates greater neuroprotection under ischemic conditions, possibly through enhancing the expressions of heat shock protein (HSP)70

and glucose response protein 78 (Grp78)[11].

HSP are a group of stress-induced proteins that act as molecular chaperones regulating the formation, folding, and assembly of protein chains and the translocation of newly-formed proteins[12]. Cerebral ischemia of either focal or global type enhances the expression of HSP genes and proteins[13–16], which are speculated to be protective proteins. HSP synthesis is controlled by a family of transcription factors, the heat-shock factors (HSF). Ischemia and heat activate HSF-1, which is present in the cytoplasm as an inactive, monomeric form[17]. Trimerization and phosphorylation, as well as nuclear migration of HSF-1, occur under stress conditions. HSF-1 binds to the heat-shock element, which is present in the promoter of the stress response gene and then initiates HSP transcription and synthesis.

Mammalian HSP90 is one of the most abundant cytosolic proteins in eukaryotes, amounting to ~1% of soluble proteins, even in the absence of stress[18,19]. Previous studies found that HSP90 could form a cytosolic complex with apoptosis protease activating factor-1 (Apaf-1) and inhibit cytochrome c-mediated oligomerization of Apaf-1 and the activation of procaspase-9 acting as an anti-apoptotic factor[20].

The upregulation of the expression of HSP70 and Grp78 by prostaglandin A1 (PGA_1) and lithium (Li) has been reported[11]. Still, we do not know whether other members of the HSP family are involved in the neuroprotection of combined PGA_1 and lithium. The present study was undertaken to explore whether the expressions of other HSP regulating cell death/survival are affected by PGA_1 and Li. The effect of PGA_1 and Li on Apaf-1, one pro-apoptotic factor regulated by HSP90, was also examined. The results indicated that lithium enhanced the effects of PGA_1 on the expressions of HSF-1, heme oxygenase-1 (HO-1), HSP90α, and HSP90β and downregulated Apaf-1.

Materials and methods

Materials Lithium and PGA_1 were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA).

Rat permanent middle cerebral artery occlusion model Male Sprague-Dawley rats weighing 280–300 g were purchased from the Center for Experimental Animals of Soochow University (Suzhou, China). The National Institutes of Health (NIH) Guidelines for Care and Use of Laboratory Animals were followed in all animal procedures. The rats were anesthetized with an intraperitoneal injection of 4% choral hydrate (350 mg/kg). Through a ventral midline incision, the right common carotid artery (CCA), external carotid artery, and internal carotid artery (ICA) were isolated and ligated. A 30 mm length of monofilament nylon suture (Φ0.22–0.24 mm), with its tip rounded by heating near a flame, was inserted from the right CCA to the ICA through a small incision in the common carotid artery and then advanced to the Circle of Willis to occlude the origin of the right middle cerebral artery. The suture remained there until the rats were killed[21]. The body temperature of the rats was monitored with a rectal probe and maintained in the range of 37.0±0.5 °C with a heating pad (BME-412A ANIMAL REGULATOR, 308005669; Institute of Biomedical Engineering, Hangzhou, Zhejiang, China) during and after surgery until recovery from anesthesia. Sham-operated rats underwent the same procedures, except for the permanent middle cerebral artery occlusion (pMCAO). Rats showing tremor and seizure after surgery were excluded from further experiments. For the study of the time-course of alterations in the expression of HSF-1, HSP, and Apaf-1, 15 rats were killed at 1, 2, 3, 6, 12, and 24 h after ischemic insult (3 rats in each group). For the study of the effects of PGA_1, lithium alone, and a combination of both, 21 rats were divided into 7 groups: sham operated; model; PGA_1 33.3 nmol PGA_1(L); PGA_1 16.5 nmol PGA_1(S); lithium 1 mEq/kg; PGA_1(L)+lithium; PGA_1(S)+lithium.

Lithium and PGA_1 treatment For all the experiments, at the indicated times and doses, lithium was administered subcutaneously to the rats for 2 d (1 mEq/kg, once a day) and PGA_1 (16.5 or 33 nmol, dissolved in 40% ethanol) was injected into the lateral cerebro-ventricle once 15 min prior to the pMCAO. The control animals received the same quantity of vehicle.

Western blot analysis of HO-1, HSP90α, HSP90β, HSF-1, and Apaf-1 Immunoblotting was performed as previously described[3]. The brain tissues from the ischemic striatum of the right middle cerebral artery territory and the corresponding area of the sham-operated rats were rigorously homogenized; the protein concentrations were determined using a BCA kit (Pierce, Rockford, IL, USA). An aliquot of 50 μg proteins from each sample was separated using 10% SDS-PAGE electrophoresis using constant current. Proteins were subsequently transferred to a nitrocellulose membrane, which was then incubated with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBST) at 4 °C overnight. Afterwards, the membranes were incubated with the mAb to HSF-1 (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C for 3 h, then with a horseradish peroxidase-conjugated anti-mouse secondary antibody (1:5000; Sigma, USA) at room temperature for 1 h. Immunoreactivity was detected by enhanced chemiluminescent autoradiography (Amersham, Princeton, NJ, USA) in accor-

dance with the manufacturer's instructions. Between each procedure, the membranes were rinsed 3 times in TBST for 10 min each. The membrane was immediately placed with the protein side up in the film cassette for exposing. The films were used for the final determination. The membranes were reprobed with β-actin (1:5000; Sigma, USA) after striping (TBST with 2% β-mercaptoethanol at 65 °C for 1 h).

The same method was used to detect HO-1, HSP90α, HSP90β, and Apaf-1. For the detection of HO-1, HSP90α, and HSP90β, the first antibodies used to incubate the membranes were rabbit polyclonal IgG against HO-1 (1:200; Stressgen Bioreagents, Voctoria, BC, Canada), rat monoclonal IgG against HSP90α (1:200; Santa Cruz Biotechnology, USA), rat monoclonal IgG against HSP90β (1:200; Santa Cruz Biotechnology, USA), and rabbit polyclonal IgG against Apaf-1 (1:500; Pharmingen, San Jose, CA, USA), and the second antibody was a horseradish peroxidase-conjugated anti-rabbit IgG (1:5000; Sigma, USA) and anti-mouse IgG (1:5000; Sigma, USA).

Statistical analysis Statistical analysis was performed by one-way ANOVA. $P<0.05$ was considered to be significant.

Results

Enhanced expression of HSF-1 by combined PGA_1 and lithium The Western blot analysis revealed that the increase in the levels of HSF-1 in the ischemic striatum was detected at 12 h and further elevated 24 h after the onset of ischemia. The levels of HSF-1 were further elevated by pretreatment with PGA_1 (33 nmol) and lithium alone. Moreover, the combination of lithium and PGA_1 (16.5 and 33 nmol, respectively) elicited a greater increase in HSF-1 levels compared to PGA_1 alone ($P<0.05$; Figure 1).

Enhanced expression of HO-1 by combined PGA_1 and lithium The elevation of the levels of HO-1 in the ischemic striatum was detected at 12 h and further increased 24 h after the onset of ischemia. The increase in the expression of HO-1 was enhanced by pretreatment with PGA_1 (33 nmol) and lithium alone. The combination of lithium and PGA_1 (16.5 and 33 nmol, respectively), however, induced a greater increase in HO-1 levels compared to PGA_1 alone ($P<0.05$; Figure 2).

Enhanced expression of HSP90α by combined PGA_1 and lithium The upregulation of the expression of HSP90α was detectable at 3 h and was further elevated 24 h after the onset of ischemia. The levels of HSP90α were even further increased by pretreatment with PGA_1 (33 nmol) and lithium alone. The combination of lithium and PGA_1 (16.5 and 33 nmol, respectively) elicited a larger rise in HSP90α levels compared to PGA_1 alone ($P<0.05$; Figure 3).

Maintenance of HSP90β expression by combined PGA_1

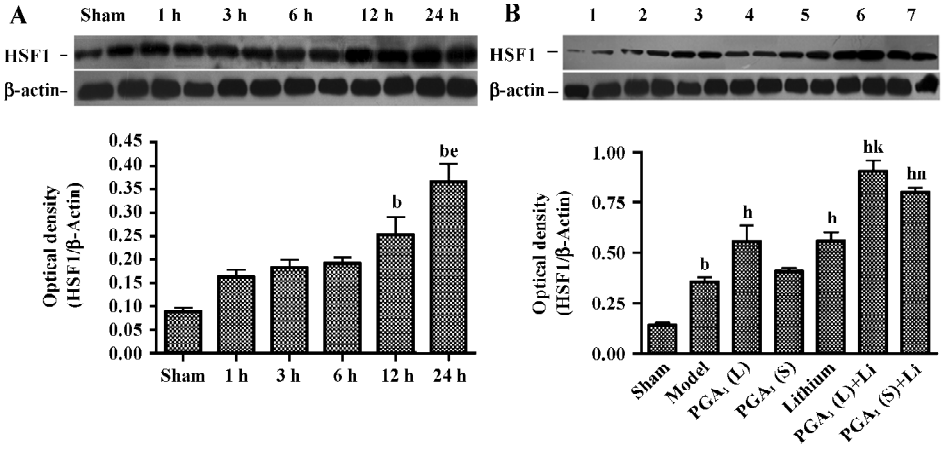

Figure 1. Effects of PGA_1, lithium (Li), and a combination of both on pMCAO-induced HSF-1 expression. Rats were pretreated with a subcutaneous injection of lithium (1 mEq/kg, once a day) for 2 d, a single intracerebral ventricle injection of PGA_1 16.5 nmol PGA_1(S) or 33 nmol PGA_1(S) 15 min prior to pMACO, or a combination of lithium and PGA_1. Extracts from the entire ischemic and sham-operated striata were separated on SDS–PAGE, and protein levels of HSF-1 were detected with immunoblotting. β-Actin was used as a loading control. (A) time-course of pMCAO-induced increases in HSF-1 protein levels. (B) pretreatment of PGA_1 and lithium further enhanced the ischemia-induced expression of HSF-1. The arrangement of lanes are as follows: lane 1: sham; lane 2: model; lane 3: PGA_1(L); lane 4: PGA_1(S); lane 5: lithium; lane 6: PGA_1(L)+lithium; and lane 7: PGA_1(S)+lithium. Bar represents mean±SEM from 3 rats in each group. $^bP<0.05$ *vs* sham-operated group (subjected to surgical procedures, but no MCAO). $^eP<0.05$ *vs* 12 h group. $^hP<0.05$ *vs* model group (subjected to pMCAO and treated with vehicle). $^kP<0.05$ *vs* PGA_1(L) group. $^nP<0.05$ *vs* PGA_1(S) group.

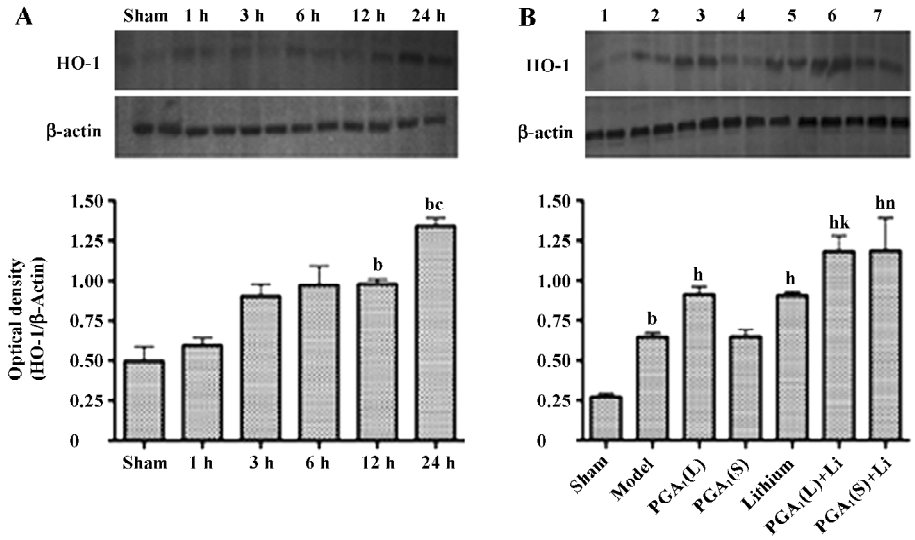

Figure 2. Effects of PGA_1, lithium (Li), and a combination of both on pMCAO-induced HO-1 expression. Rats were pretreated with a subcutaneous injection of lithium (1 mEq/kg, once a day) for 2 d, a single intracerebral ventricle injection of PGA_1 16.5 nmol PGA_1(S) or 33 nmol PGA_1(L) 15 min prior to pMACO, or a combination of lithium and PGA_1. Extracts from the entire ischemic and sham-operated striata were separated on SDS-PAGE, and protein levels of HO-1 were detected with immunoblotting. β-Actin was used as a loading control. Quantitative analysis of HO-1 was performed with Western blot analysis. (A) time-course of pMCAO-induced enhancement of HO-1 expression. (B) pretreatment of PGA_1 and lithium further increased the expression levels of HO-1. Lane 1: sham; lane 2: model; lane 3: PGA_1 (L); lane 4: PGA_1(S); lane 5: lithium; lane 6: PGA_1(L)+lithium; and lane 7: PGA_1(S)+lithium. Bar represents mean±SEM from 3 rats in each group. $^bP<0.05$ *vs* sham-operated group (subjected to surgical procedures, but no MCAO). $^eP<0.05$ *vs* 12 h group. $^hP<0.05$ *vs* model group (subjected to pMCAO and treated with vehicle). $^kP<0.05$ *vs* PGA_1(L) group. $^nP<0.05$ *vs* PGA_1(S) group.

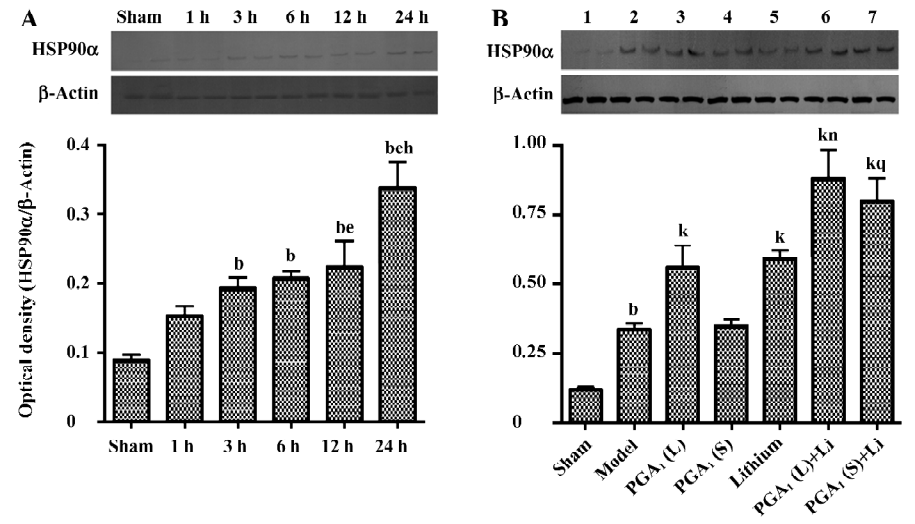

Figure 3. Effects of PGA_1, lithium (Li), and a combination of both on pMCAO-induced HSP90α expression. Rats were pretreated with a subcutaneous injection of lithium (1 mEq/kg, once a day) for 2 d, a single intracerebral ventricle injection of PGA_1 16.5 nmol PGA_1(S) or 33 nmol PGA_1 (S) 15 min prior to pMACO, or a combination of lithium and PGA_1. Extracts from the entire ischemic and sham-operated striata were separated on SDS-PAGE, and protein levels of HSP90α were detected with immunoblotting. β-Actin was used as a loading control. Quantitative analysis of HSP90α was performed with Western blot analysis. (A) time-course of pMCAO-induced enhancement of HSP90α expression. (B) pretreatment of PGA_1 and lithium further increased the expression levels of HSP90α. Lane 1: sham; lane 2: model; lane 3: PGA_1(L); lane 4: PGA_1(S); lane 5: lithium; lane 6: PGA_1(L)+lithium; and lane 7: PGA_1(S)+lithium. Bar represents mean±SEM from 3 rats in each group. $^bP<0.05$ *vs* sham-operated group (subjected to surgical procedures but no MCAO). $^eP<0.05$ *vs* 6 h group. $^hP<0.05$ *vs* 12 h group. $^kP<0.05$ *vs* model group (subjected to pMCAO and treated with vehicle). $^nP<0.05$ *vs* PGA_1(L) group. $^qP<0.05$ *vs* PGA_1(S) group.

and lithium The downregulation of the expression of HSP90β in the ischemic striatum was detected at 12 h and further descended 24 h after the onset of ischemia. However, it was significantly recovered by pretreatment with PGA_1 (33 nmol) and lithium alone. Moreover, the combination of lithium and PGA_1 (16.5 and 33 nmol, respectively) elicited a greater rise in HSP90β levels in the ischemic striatum compared to PGA_1 alone (P<0.05; Figure 4).

Inhibition of Apaf-1 expression by combined PGA_1 and lithium The increased expression of Apaf-1 was detected at 6 h and was further elevated 24 h after the onset of ischemia. Pretreatment with PGA_1 (33 nmol) or lithium significantly inhibited Apaf-1 induction. The combination of lithium and PGA_1 (16.5 and 33 nmol, respectively) further reduced Apaf-1 levels in the ischemic striatum compared to PGA_1 alone (P<0.05; Figure 5).

Discussion

HSF-1 has been shown to regulate the expression of HSP in response to ischemia, hypoxia, heat, stress, or injury[41]. During cerebral ischemia, complex and multiple pathological events occur within neural cells, such as the loss of high energy phosphate esters, disturbances in neurotransmitter metabolism, membrane breakdown, mitochondrial failure, an accumulation of intracellular Ca^{2+}, and the subsequent production of oxygen free radicals involving the arachidonic acid metabolic pathway[42]. Although the detailed mechanisms remain to be determined, complex pathological events within ischemic neuronal cells disturb protein metabolism and break down cellular structures, thus possibly producing substrates for the molecular chaperones and activating HSF-1. Conditions that increase the level of oxygen free radicals have also been reported to induce the stress proteins[43]. Hypoxia also activates HSF-1 and the HSF-mediated transcription of HSP70 in myogenic cells[44]. Mosser e*t al* reported that the concentrations of calcium and hydrogen ions, which affect protein conformation, directly activate HSF *in vitro*[45]. Recent studies have reveled that antiproliferative prostaglandins also activate HSF, and extracellular exposure to arachidonic acid activates HSF-1 and heat shock gene transcription[46,47]. It has been reported recently that cerebral ischemia or heat shock could activate HSF-1[48]. Our current findings demonstrate that pMCAO enhanced the expression of HSF-1 in the ischemic striatum, and pre-ischemic PGA_1 and lithium treatments further enhanced the induction of the

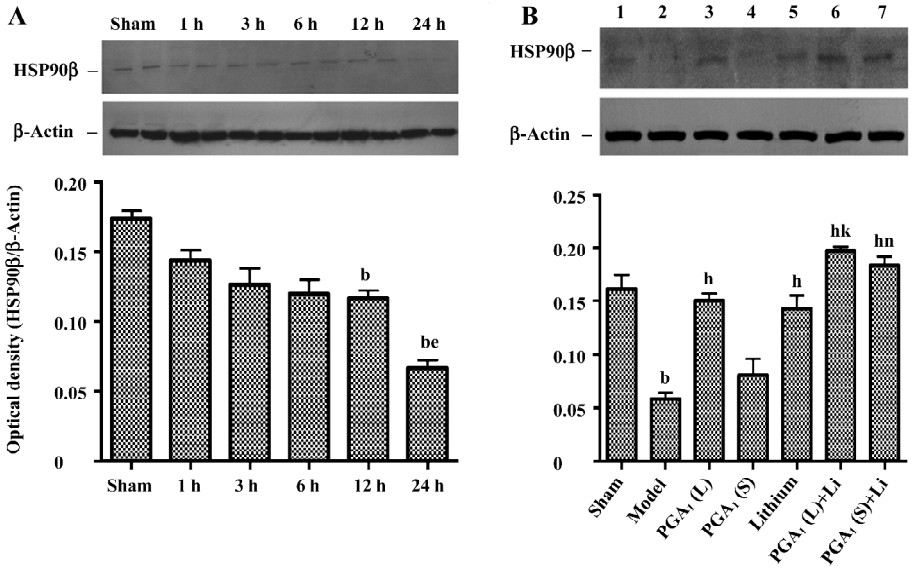

Figure 4. Effects of PGA_1, lithium (Li), and a combination of both on pMCAO-induced alterations in HSP90β protein levels. Rats were pretreated with a subcutaneous injection of lithium (1 mEq/kg, once a day) for 2 d, a single intracerebral ventricle injection of PGA_1 16.5 nmol PGA_1(S) or 33 nmol PGA_1(L) 15 min prior to pMACO, or a combination of lithium and PGA_1. Extracts from the entire ischemic and sham-operated striata were separated on SDS–PAGE, and protein levels of HSP90β were detected with immunoblotting. β-Actin was used as a loading control. Quantitative analysis of HSP90β was performed with Western blot analysis. (A) time-course of pMCAO-induced decrease in HSP90β expression. (B) pretreatment of PGA_1 and lithium maintained the expression levels of HSP90β. Lanes in (B) are as follows: lane 1: sham; lane 2: model; lane 3: PGA_1(L); lane 4: PGA_1(S); lane 5: lithium; lane 6: PGA_1(L)+lithium; and lane 7: PGA_1(S)+lithium. Bar represents mean±SEM from 3 rats in each group. ^bP<0.05 *vs* sham-operated group (subjected to surgical procedures, but no MCAO). ^eP<0.05 *vs* 12 h group. ^hP<0.05 *vs* model group (subjected to pMCAO and treated with vehicle). ^kP<0.05 *vs* PGA_1(L) group. ^nP<0.05 *vs* PGA_1(S) group.

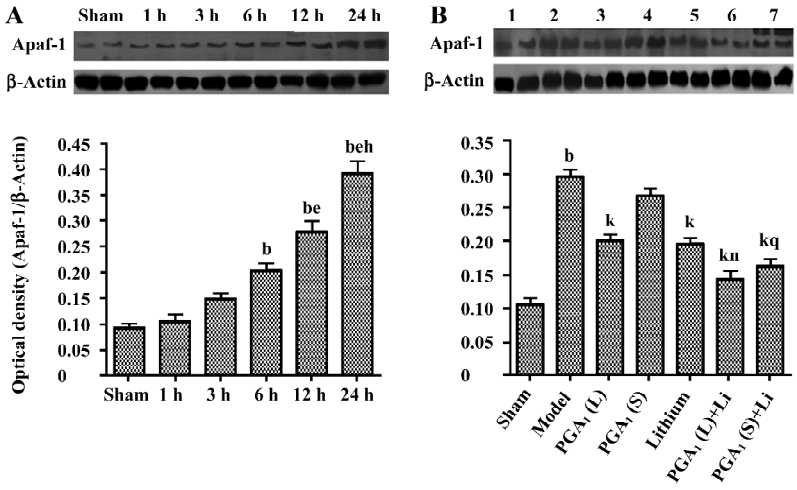

Figure 5. Effects of PGA_1, lithium, and a combination of both on pMCAO-induced Apaf-1 expression. Rats were pretreated with a subcutaneous injection of lithium (1 mEq/kg, once a day) for 2 d, a single intracerebral ventricle injection of PGA_1 16.5 nmol PGA_1(S) or 33 nmol PGA_1(L) 15 min prior to pMACO, or a combination of lithium and PGA_1. Extracts from the entire ischemic and sham-operated striata were separated on SDS-PAGE, and protein levels of Apaf-1 were detected with immunoblotting. β-Actin was used as a loading control. Quantitative analysis of Apaf-1 was performed with Western blot analysis. (A) time-course of pMCAO-induced enhancement of Apaf-1 expression. (B) pretreatment of PGA_1 and lithium decreased the levels of Apaf-1. Lanes in (B) are as follows: lane 1: sham; lane 2: model; 3: lane PGA_1(L); lane 4: PGA_1(S); lane 5: lithium; lane 6: PGA_1(L)+lithium; and lane 7: PGA_1(S)+lithium. Bar represents mean±SEM from 3 rats in each group. $^bP<0.05$ *vs* sham-operated group (subjected to surgical procedures, but no MCAO). $^eP<0.05$ *vs* 6 h group. $^hP<0.05$ *vs* 12 h group. $^kP<0.05$ *vs* model group (subjected to pMCAO and treated with vehicle). $^nP<0.05$ *vs* PGA_1(L) group. $^qP<0.05$ *vs* PGA_1(S) group.

HSF-1 level in the ischemic striatum. This suggests that the enhanced expression of HSP by PGA_1 and lithium is mediated at the transcriptional level through HSF-1.

HO-1, a HO family member, is a 32 kDa HSP (HSP-32), which is inducible by a variety of stress factors, including oxidative stress, exposure to heavy metals, heat, focal cerebral ischemia, and traumatic brain injury[22–24]. In the human central nervous system, HO-1 expression was detected in brain tumors and in neurodegenerative diseases including Pick's disease, Alzheimer's disease, Parkinson's disease, and cerebral ischemia[25–27]. It should be noted that the functional role of HO-1 is still unclear. There are several indications of the protective function of HO-1. Oxidative stress has been proposed to be a contributing factor in the pathophysiology of ischemia/reperfusion[28]. The products of heme degradation, bilirubin, and biliverdin were shown to be protective because of their antioxidant activity[29,30]. HO-1 was shown to protect vessels against heme and hemoglobin-mediated injury[31], to protect kidney against ischemia/perfusion-induced injury[32] and to reduce hyperoxia-induced lung injury in rats[33]. The increased expression of the HO-1 protein following transient global and permanent focal ischemia may reflect an elevation of antioxidant defense mechanisms as a response to ischemia-induced oxidative stress. In our ischemic model, the HO-1 level was maximally increased at 24 h post-insult in the ischemic striatum, when the brain area suffered severe damage after pMACO. Moreover, pre-ischemic PGA_1 and lithium treatments robustly enhanced the induction of the HO-1 level in the ischemic striatum.

HSP90 is a highly conserved and essential stress protein for the viability of eukaryotic cells. It is not only abundantly expressed in cells, but also significantly induced after stress[34]. In eukaryotes, HSP90 has dual chaperone functions. They are involved in the conformational maturation of signal transduction molecules (for example, nuclear hormone receptors and kinases) and in the cellular stress response[18]. The finding that HSP90 is induced in response to diverse apoptotic stimuli, such as UV, sodium arsenite and cerebral ischemia, has supported its involvement in cell survival[35–37]. Recently, it was reported that HSP90 could form a cytosolic complex with Apaf-1 and inhibit cytochrome c-mediated oligomerization of Apaf-1 and the activation of procaspase-9[20]. It also could stabilize the receptor-interacting protein, a major anti-apoptotic adaptor, to activate anti-apoptotic pathways through NF-κB and mitogen-activated protein kinases (MAPK)[38]. In addition, Akt is a substrate of HSP90 and forms a complex with HSP90. The inhibition of Akt–HSP90 binding results in Akt dephosphorylation, a decrease in Akt

kinase activity, and the induction of apoptosis[39]. In the present study, although HSP90α expression was enhanced significantly by pMCAO, HSP90β expression was significantly decreased in the ischemic models. Pre-ischemic PGA_1 and lithium treatment robustly enhanced the induction of HSP90α levels and maintained the expression of HSP90β in the ischemic striatum. Our findings in changes in HSP90β expression is consistent with those reported by Sun *et al*, in which HSP90β descended significantly in the ischemic/reperfusion model, but was maintained by the treatment of acupuncture[40]. This may contribute to PGA_1 and lithium's protection against cerebral ischemia in our study.

Taken together, pretreatment with PGA_1, lithium, and a combination of both just before cerebral ischemia, significantly enhanced the expressions of HO-1, HSP90α, and HSF-1, and maintained HSP90β, but decreased the expression of Apaf-1 in the ischemic striatum. This is the first *in vivo* study showing that pretreatment with PGA_1, lithium, and a combination of both could activate heat shock gene transcription, suggesting that the combined treatment of PGA_1 and lithium may be considered for the clinical testing of PGA_1 and lithium against ischemic neuronal damage.

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