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Induction of heme oxygenase-1 by traditional Chinese medicine formulation ISF-1 and its ingredients as a cytoprotective mechanism against oxidative stress

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Abstract. Traditional medicines are clinically used to treat post-stroke disorders in China. In search of alternative medicines for post-stroke rehabilitation, we recently identified the heme oxygenase-1 (HO-1) pathway as a key mechanism underlying the biological activities of the ischemic stroke formulation ISF-1. This study was designed to further investigate ISF-1 for HO-1 induction in cultured human cells and corresponding cytoprotective effects against oxidative injury. A rat stroke model induced by middle cerebral artery occlusion was employed to verify the activity of ISF-1 *in vivo*. It was found that HO-1 expression was induced by ISF-1 in a dose- and time-dependent manner. Four ingredients from ISF-1 were identified to be responsible for HO-1 induction. The appropriate combinations of these active ingredients or purified compounds resulted in synergistic induction of HO-1 expression. A minimal HO-1-inducing formulation was prepared and showed significant cytoprotection against H_2O_2-induced oxidative stress. Collectively, the herbal formulation ISF-1 was capable of inducing HO-1 expression, *in vitro* and *in vivo*, offering a potential mechanism for post-stroke rehabilitation. This study may shed light on the development of mechanism-defined therapies based on traditional herbal remedies.

Introduction

Oxidative stress has been implicated in the pathogenesis of different diseases including ischemic stroke, organ dysfunctions and chronic progressive neurodegenerative disease (1,2). As an example, ischemia reperfusion injury causes a large number of mortalities and severe disabilities (3,4). Thrombolytic reagents such as recombinant tissue plasminogen activator have been introduced to treat the disease but the pharmacological benefits are restricted to the time window and drug dose (5,6). On the other hand, various antioxidants can protect brain tissues especially the neural cells from oxidative injury. Notably, most of the antioxidants are found to be neuroprotective. The antioxidant activities include neutralizing free radicals, increasing the intracellular level of antioxidant compounds such as glutathione and regulating heme/iron homeostasis. Thus, control of the intracellular redox status may represent an alternative treatment to oxidative injury.

The heme oxygenase (HO) system is an important endogenous mechanism against oxidative stress (1,7). Three isoforms of HOs have been isolated so far and only HO-1 is inducible by various stimuli. HO-1, a key antioxidant enzyme, degrades the intracellular heme into biliverdin, carbon monoxide and the free ferrous ion. Bilverdin is subsequently converted by bilverdin reductase to the more potent antioxidant bilirubin (8). Very much like nitrite oxide, carbon monoxide affects various signaling pathways and suppresses stress-mediated apoptosis (9-11). The free ferrous ion itself may be cytotoxic but is able to induce antiapoptotic proteins such as ferritin (12). Several lines of evidence have demonstrated that HO-1 expression provides an antioxidant defense against oxidative injury (13,14). It is noteworthy that proteolytically truncated HO-1 can be translocated into the nucleus, where HO-1 may modulate the transcriptional responses to oxidative stress (15). Therefore, the HO-1 pathway has been suggested as an important target for pharmacological intervention.

Many Chinese herbal medicines are found to possess antioxidant and anti-inflammatory activities. The active herbal compounds may be flavonoids, saponins and lipids, and are extensively present in plants, fruits and vegetables (16,17). In fact, some herbal medicines may be potential therapeutics for oxidative disorders. As an example, an ischemic stroke formula ISF-1 composed of seven medicinal ingredients has been well-documented for post-stroke rehabilitation (18).

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However, the molecular mechanisms supporting its clinical claims remain elusive. Thus, our laboratory and others have applied DNA microarray technology to determine the biological responses to complex Chinese herbal formulations (18-20). Based on the biological response fingerprints, we suggested that the HO-1 pathway is one of the key mechanisms of action of the Chinese herbal formulation ISF-1.

In this study, we investigated the regulation of HO-1 expression by the formulation ISF-1 and its ingredients in cultured cells. Using the active ingredients/compounds, different combinations were prepared and subsequently tested, *in vitro* and *in vivo*, for the HO-1-inducing activity. We anticipate that this study will shed light on the development of new evidence-based therapeutics based on traditional herbal remedies.

Materials and methods

Animals and cell culture. Adult male Sprague-Dawley rats (260-300 g) were obtained from the Laboratory Animal Unit of the University of Hong Kong. Experiments using animals were conducted in compliance with the guidelines of the Use of Live Animals in Teaching and Research for the University of Hong Kong. The human hepatocellular carcinoma cell line HepG2 was obtained from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in MEM medium (Invitrogen, USA) supplemented with 10% v/v fetal calf serum (HyClone, USA) and 1% penicillin/streptomycin (Invitrogen Life Technologies, Inc., CA, USA) at 37˚C in an atmosphere containing 5% CO_2. Plasmid hHO4.9luc DNA was kindly provided by Dr Norbert Leitinger (University of Virginia, Charlottesville, VA, USA) (21). Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of botanical extracts. The dry powders of aqueous herbal extracts were purchased from an established local company Nong's Pharmaceutical Ltd., Hong Kong SAR. The botanical formulations were prepared using seven medicinal ingredients. The chemical and biological fingerprints of ISF-1 were evaluated, and the aqueous extracts of the botanicals were prepared essentially as described previously (18).

Drug treatment and total RNA preparation. Human HepG2 cells ($15x10^6$) were seeded in a 75-cm^2 tissue culture flask and subsequently treated with ISF-1 at the concentration of 5.85 mg/ml, or PBS as the control, at 37˚C for 24 h. Using the RNA-Bee™ RNA Isolation Reagent (Tel-test, Friendswood, TX, USA), total RNAs were isolated from ISF-1-treated and untreated cells, respectively. The RNA samples were cleaned using an RNeasy Kit (Qiagen, Valencia, CA) while DNase I was used to remove genomic DNA contaminants. The quality of total RNA samples was evaluated by A260/A280 ratio and by Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA).

Quantitative RT-PCR analysis. Total RNAs (10 μg) were converted into single-stranded complementary DNAs using random hexamer primers. The mRNAs encoding HMOX1 (heme oxygenase-1, NM_002133) and 18S (ribosome RNA, X03205) were determined using TaqMan Assays-on-Demand reagents (Applied Biosystems, Foster City, CA, USA). The gene expression in HepG2 cells with and without exposure to ISF-1 was calculated using the comparative threshold ($2^{-\Delta\Delta CT}$) method (http://www.appliedbiosystems.com/support/tutorials/pdf/performing_rq_gene_exp_rtpcr.pdf). The threshold cycles (CT) were calculated using ABI PRISM 7900 SDS Software version 2.0 (Applied Biosystems).

Western blot analysis. Total cellular proteins were extracted from human HepG2 cells with and without exposure to various herbal extracts. Briefly, HepG2 cells ($5x10^6$) were seeded in 10 ml of complete growth medium. After overnight incubation at 37˚C, the cells were incubated with and without herbal extracts at 37˚C for 24 h. Total proteins (30-50 μg) extracted from the cultured cells were resolved on 10% gradient SDS-polyacrylamide gels. Proteins were transferred to PVDF membranes using the Trans-Blot Transfer System (Bio-Rad). Goat polyclonal anti-human HO-1 (US Biologicals) and rabbit polyclonal anti-ß-actin (Sigma) antibodies were used to probe the cellular HO-1 and ß-actin, respectively. The bound antibody was detected by horseradish peroxidase conjugated with anti-rabbit or anti-goat IgG. Finally, enhanced chemiluminescence (ECL) detection reagents were employed to visualize the peroxidase reaction products (GE Healthcare). The concentration of the loaded cellular proteins was normalized against the internal control ß-actin.

Luciferase-based assay for HO-1 promoter activity. HepG2 cells were transfected in 100-mm plates with 8.0 μg of pHO-1promoter-Luc plasmid DNA (21) or pEGFP-C1 vector (Clontech, San Diego, CA) using FuGene 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN, USA). Transfected cells were seeded in 96-well tissue culture plates at a density of $1x10^5$ cells/ml and incubated at 37˚C for 24 h. Following treatment with the specific drugs for another 24 h, human HO-1promoter-luciferase reporter activity was determined using Bright-Glo™ Luciferase Assay Reagent (Promega, Boston, MA). Luciferase values were normalized to EGFP expression as a control for transfection efficiency.

Cytoprotection against H_2O_2-induced cytotoxicity. Cells were seeded in a 96-well flat bottom plate (BD Biosciences, CA, USA) at a density of $2.0x10^5$ cells/ml. After a 6-h exposure to herbal medicines (six replicates per experiment), cells were challenged with H_2O_2 at 37˚C for 16 h. To verify the role of HO-1, tin-protoporphyrin IX (SnPP, Porphyrin Products, Logan, UT) was used to pretreat the cells at 20 μM SnPP for 20 min. At the end of the incubation, the medium was removed, and 100 μl of 0.5 mg/ml MTT solution was subsequently added to each well. The plates were incubated at 37˚C for 3 h. The colorimetric products were dissolved in 100 μl DMSO with agitation for 30 min. The absorbance was measured at 570 nm on a microplate reader (Bio-Rad, CA, USA).

Rat ischemic stroke model and drug treatment. The rats were divided into the following groups with 4-6 rats in each group: 1) sham control; 2) 1 h ischemia + 14 day reperfusion; 3) 1 h ischemia + 14 day reperfusion + ISF-1 treatment; 4) 1 h

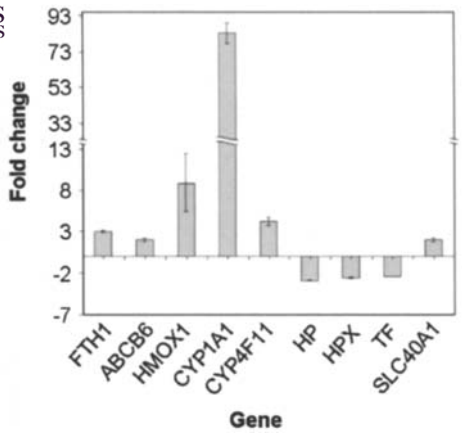

Figure 1. Regulation of heme/iron homeostasis by the formulation ISF-1. The transcriptional profiles of HepG2 cells in response to ISF-1 at its IC50 concentration were determined by Affymetrix HG-U133 Plus2 arrays. The significantly regulated genes were annotated using NetAffx. The genes involved in heme/iron homeostasis were specifically analyzed in terms of their expression values obtained from the microarray analysis. Data represent the mean value of three biological replicates.

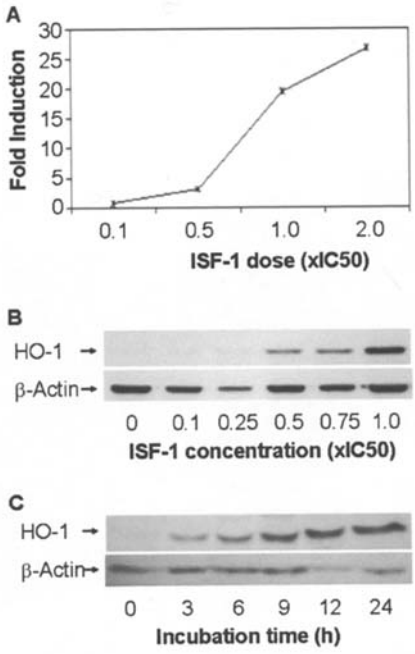

Figure 2. Induction of HO-1 expression by the formulation ISF-1. (A) HO-1 mRNA expression. After a 24-h induction by ISF-1 at indicated doses, HO-1 mRNA expression was determined by the qRT-PCR system (Applied Biosystems Inc.). Data represent the mean value of two biological replicates. (B) HO-1 protein expression. The cellular proteins were analyzed by Western blotting using antibodies against human HO-1 (Stressgen) and ß-actin. (C) Time course. The induction by ISF-1 was performed at its IC50 concentration. The cellular proteins were analyzed by Western blotting using antibodies against human HO-1 (Stressgen) and ß-actin.

ischemia + 28 day reperfusion; and 5) 1 h ischemia + 28 day reperfusion + ISF-1 treatment. Transient middle cerebral artery occlusion (MCAO) was induced using the intraluminal filament technique (22,23). After 1 h of ischemia, the rats in the treatment groups were administered ISF-1 orally twice daily at the dose of 2.5 g dry powder per kg body weight until their sacrifice. At the end of the drug treatment, animals were anesthetized and transcardially perfused with saline. The brains were recovered and lysed with cell lysis buffer (see Western blot analysis in Materials and methods). Sham surgeries were performed in the same way, except that the filament was not advanced to the origin of the middle cerebral artery.

Results

Regulation of heme/iron homeostasis. Heme/iron homeostasis is an important biological pathway, which involves different biosynthesis and degradation enzymes, transporters and transcriptional regulators. Based on the transcriptional profile obtained in human HepG2 cells (18), HO-1 was one of the gene products significantly regulated by the formulation ISF-1, and its expression level was increased up to 9-fold. In addition, a number of other genes involved in heme/iron homeostasis were also significantly regulated (Fig. 1). These genes encode iron or heme transporters including FTH1, ABCB6, HP, TF and SLC40A1; cytochrome P-450 enzymes such as CYP1A1 and CYP4F11; and iron-regulated transcriptional factor HPX. These results suggest that heme/iron homeostasis is one of the important intracellular pathways targeted by ISF-1. On the other hand, some other phase-2 proteins such as NAD(P)H dehydrogenase quinone 1, glutathione *S*-transferase, and glutamate cysteine ligase were also up-regulated to a certain extent. Collectively, the formulation ISF-1 may be able to overcome oxidative stress and promote cell survival.

Induction of heme oxygenase-1 expression. Induced mRNA expression of HO-1 was verified by quantitative RT-PCR whereas HO-1 protein was detected by Western blotting using the specific antibody. In these experiments, the level of HO-1 expression was positively correlated with the drug concentration (Fig. 2A and B). When the drug concentration amounted to 50% of the IC50 value, HO-1 was detectable at both the mRNA and protein levels. HO-1 protein was detectable as early as 3 h after the treatment was initiated, suggesting that HO-1 expression is an early response to ISF-1 (Fig. 2C). Collectively, the activation of the HO-1 pathway may represent a key mechanism that underlies the pharmacological action of ISF-1 in the cells.

Identification of the active ingredients responsible for inducing HO-1. To determine the active ingredients responsible for inducing HO-1 expression, the cells were treated with individual ingredients at their IC50 concentrations. HO-1 expression was detected by Western blotting using the specific antibody. As a result, *Lumbricus*, *Radix Chuanxiong* and *Flos Carthami* were found to be HO-1 inducers (Fig. 3A). In order to measure the activation of HO-1 transcription, a 4.9-kb DNA sequence from the 5'-flanking region of HO-1 was cloned into a luciferase reporter DNA construct and then transiently transfected into HepG2 cells. The transfected cells were treated with individual ingredients and the cellular activity of luciferase was subsequently assayed. Notably, *Radix Astragali* was found to be the strongest activator for

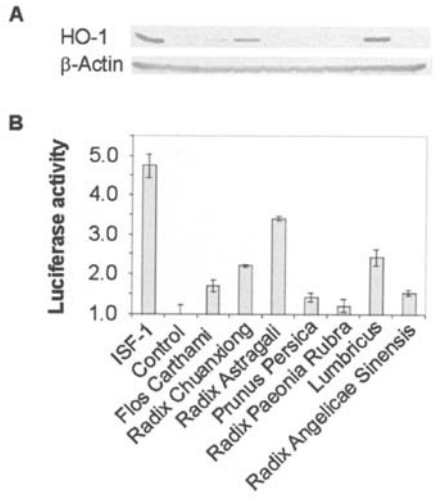

Figure 3. Identification of the active ingredients for inducing HO-1 expression. (A) Screening of medicinal materials for HO-1 induction. Individual ingredients were evaluated at their IC50 concentration. The cellular HO-1 protein was detected by anti-human HO-1 antibody (Stressgen). ß-actin was determined by anti-ß-actin antibodies. (B) Luciferase assay for HO-1 transcription. The drug-induced transcription of the HO-1 gene was correlated to the luciferase activity determined by the Bright-Glo Luciferase Assay Kit.

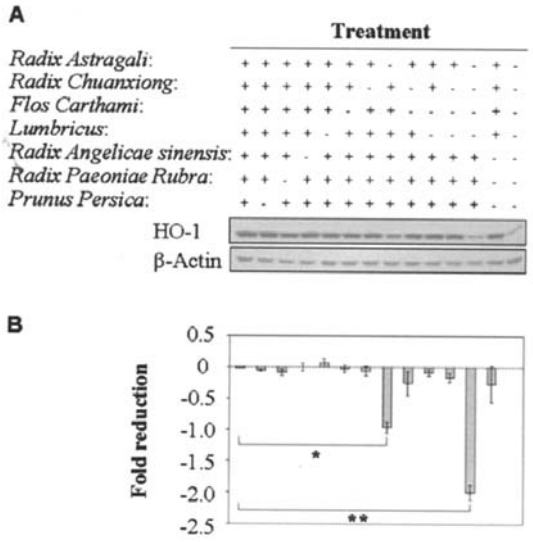

Figure 4. Contribution of individual ingredients to the induction of HO-1 expression. (A) Step-wise deletion of the ingredients from the parent formulation. HepG2 cells were treated with new formulations by mixing ingredients at the ratio specified in the figure. The cellular HO-1 protein was detected by Western blotting using anti-human HO-1 antibody, whereas ß-actin was used as the control. (B) Data analysis. Data represent the mean value of three biological replicates. Significance analysis was performed by the paired Student's t-test, $^{*}p<0.05$; $^{**}p<0.01$.

HO-1 transcription. *Lumbricus*, *Radix Chuanxiong* and *Flos Carthami* also induced HO-1 expression but to a lesser extent, whereas the other ingredients showed no detectable induction of HO-1 expression (Fig. 3B). We noticed that *Radix Astragali* is the major ingredient of the formulation ISF-1. When ISF-1 was used at its IC50 concentration, the concentration of *Lumbricus*, *Radix Chuanxiong* or *Flos Carthami* was much less than their IC50 concentration. Thus, a step-wise deletion strategy was applied to determine the contribution of each individual ingredient to the induction of HO-1. As presented in Fig. 4, deletion of *Radix Astragali* caused a significant decrease in the level of HO-1 protein ($p<0.05$). When the other three HO-1 inducers, *Lumbricus*, *Radix Chuanxiong* and *Flos Carthami*, were further deleted, the capability of the parent formula in inducing HO-1 expression was almost abolished ($p<0.001$). Taken together, these results suggest that *Radix Astragali*, *Lumbricus*, *Radix Chuanxiong* and *Flos Carthami* are the active ingredients for inducing HO-1 expression.

Minimal formulation for inducing HO-1 expression and its cytoprotection against H_2O_2-induced cytotoxicity. The formulation for inducing HO-1 expression was optimized using four ingredients: *Radix Astragali*, *Lumbricus*, *Radix Chuanxiong* and *Flos Carthami*. As presented in Fig. 5A, *Lumbricus* and *Radix Chuanxiong* are two strong HO-1 inducers, whereas *Radix Astragali* significantly enhances the effects of *Lumbricus* and *Radix Chuanxiong*. The maximal induction of HO-1 expression was achieved when all four ingredients were concomitantly presented. Due to the fact that *Flos Carthami* exhibited only trivial activity in the HO-1 induction, only *Radix Astragali*, *Lumbricus* and *Radix Chuanxiong* were used to formulate a new antioxidant formula (AOF) targeting the HO-1 pathway. The new AOF was evaluated for its cytoprotective effects against H_2O_2-induced cytotoxicity. As shown in Fig. 5B, pretreatment with AOF prevented ~50% of the cells from H_2O_2-induced cell death. On the other hand, co-treatment with specific HO-1 inhibitor SnPP significantly attenuated the cytoprotective activity of AOF. Our results indicate that the cytoprotection of AOF is due largely to the induction of HO-1 expression.

Towards the chemically defined formulation targeting the HO-1 pathway. Based on the current knowledge of the chemical compositions of different herbs, multiple active compounds may contribute to the activation of HO-1 expression presumably through different mechanisms. For example, tetramethylpyrazine, a major active compound isolated from *Radix Chuanxiong*, provided protection against ischemic reperfusion injury via inducing HO-1 expression (24). Moreover, astragaloside IV, an important saponin glycoside, is responsible for the broad biological activities of *Radix Astragali* (17,25). In this study, we demonstrated that the induction of HO-1 expression by *Lumbricus* extracts was enhanced by 1.6-fold when astragaloside IV and tetramethylpyrazine were concomitantly present (Fig. 5D). Our results suggest that astragaloside IV and tetramethylpyrazine may coordinate with the active compounds in *Lumbricus* extracts to induce HO-1 expression. When the active compounds from *Lumbricus* are identified, new chemically defined formulations can be developed to achieve the optimal induction of HO-1 expression.

In vivo induction of HO-1 expression. In order to verify the activity of ISF-1 *in vivo*, a rat ischemic stroke model was generated by introducing transient MCAO to adult male

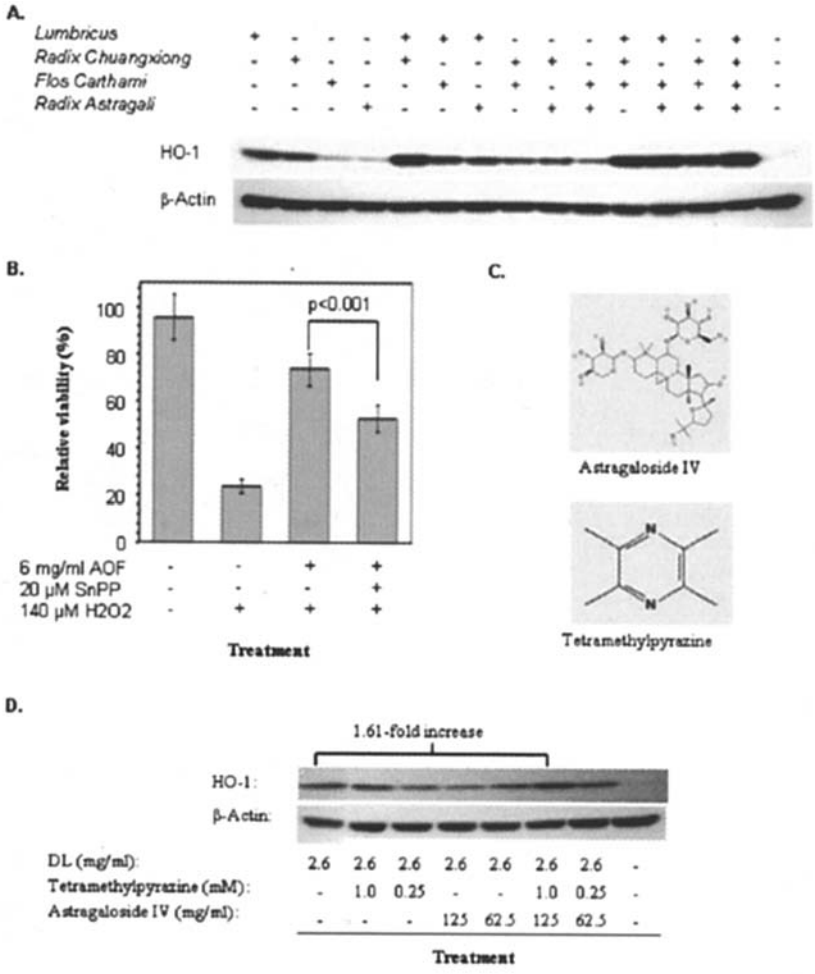

Figure 5. New formulations for HO-1 induction. (A) The individual active ingredients were combined at their IC50 concentration and assayed for their capability of inducing HO-1 expression. (B) The cytoprotective effects of new formulations against H_2O_2-induced oxidative stress. The new AOF formulation was generated by mixing the active ingredients including *Radix Astragali*, *Lumbricus* and *Radix Chuanxiong* at the same ratio as described in ISF-1. Following drug treatment with the new AOF formulation ± Tin-protoporphorin (SnPP), HepG2 cells were challenged with 140 μM H_2O_2. The viable cells were determined by MTT assay. (C) Structures of the active compounds: astragaloside IV and tetramethyl-pyrazine. The chemical structures were downloaded from NCBI PubChem database (http://pubchem. ncbi.nlm.nih.gov/). (D) Combinations of *Lumbricus* extracts with astra-galoside IV and tetramethylpyrazine, active compounds isolated from *Radix Astragali* and *Radix Chuanxiong*, respectively. After HepG2 cells were treated with the new formulation, the total cellular proteins were resolved by 10% SDS-PAGE and probed by anti-human HO-1 antibody (Stressgen) and anti-ß-actin antibodies.

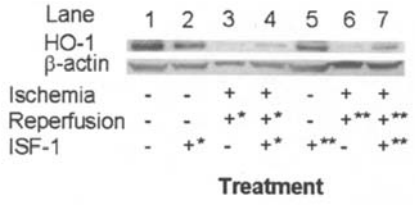

Figure 6. *In vivo* induction of HO-1 expression by the formulation ISF-1. Rats were divided into 7 groups and received corresponding treatment as indicated in the figure. ISF-1 was administered orally at the dose of 2.5 g dry powder twice daily. When the treatment was completed, the animals were anesthetized and transcardially perfused with saline. The brains were recovered and lysed with the cell lysis buffer (see Materials and methods). The total proteins were resolved by 10% polyacrylamide gel electrophoresis and probed by specific antibodies against HO-1 and ß-actin respectively. -, no treatment; +, treatment; *, treated for 14 days; **, treated for 28 days.

Sprague-Dawley rats. The rats were divided into seven groups and subjected to corresponding treatment. Transient cerebral ischemia induced HO-1 expression in glial and neuronal cells while no effects on HO-2 were observed (26). Probably due to its sensitivity to stress, HO-1 was also detected in the sham control. As shown in Fig. 6, ISF-1 did not alter HO-1 expression in animals without introduction of MCAO. In contrast, HO-1 expression was barely detectable in rats with MCAO. Such a phenomenon may be associated with severe neurodegeneration induced by ischemia (27). Intriguingly, HO-1 expression was recovered by ISF-1 administered concomitantly with reperfusion. Thus, induction of HO-1 may represent a key mechanism underlying the protective effects of ISF-1 in ischemia reperfusion injury.

Discussion

The chemical compositions of botanical drug products are complex and have not been fully characterized. Different herbal compounds act on specific protein targets and subsequently modulate various biological processes such as transcriptional control, RNA processing control, translation control and protein activity control. It is conceivable that

multiple components from botanical drugs could coordinate with each other to achieve the therapeutic effects. However, it has been a difficult task to elucidate the mechanisms of action of complex herbal drugs until genome-targeting DNA microarray technology became available. High density DNA microarray technology allows parallel determination of the expression level of thousands of genes, offering a global view of drug-regulated genes and intracellular pathways.

We recently fingerprinted the cellular responses of human HepG2 cells to the botanical formulation ISF-1 (18). According to the genome-wide transcriptional profiles, the biological effects of ISF-1 on HepG2 cells are rather diverse but mostly afffect metabolism, transcription and signal transduction. Notably, a number of phase-1 and -2 drug metabolizing enzymes such as cytochrome P-450 1A1 and 4F11, glutathione *S*-transferase, glutamate cysteine ligase and HO-1, and phase-3 drug transporters including ATP-binding cassette B6, C2 and G2 were dramatically induced. Phase-1, -2 and -3 proteins play critical roles in overcoming the cellular stress due to general xenobiotic exposure (28). As a key member of phase-2 proteins, HO-1 breaks down intracellular heme into equimolar amounts of biliverdin, carbon monoxide and the free ferrous ion, conferring protection against oxidative injury (29). In addition, heme is essential for the activity of hemeoproteins such as cytochrome P-450 enzymes. Thus, a better understanding of the HO-1 pathway will facilitate further investigation of its role in regulating the intracellular redox status.

The 5'-flanking region of the human HO-1 gene contains a 500-bp promoter and multiple enhancer binding sequences. Previous genomic mapping has identified several cis-regulatory elements such as the heme-responsive element, antioxidant responsive element, and xenobiotic-responsive element (30-32). Moreover, several specific DNA sequences have been annotated for binding to corresponding transcriptional factors including AP-1, NF-κB and HIF-1. Coordinated activation of these regulatory elements may enhance HO-1 expression. Notably, the heme and nitric oxide donor were found to synergistically activate the HO-1 pathway (33). However, little is known about how the induction of HO-1 expression could be optimized by coordinating multiple regulatory elements. By investigating the ingredients of the formulation ISF-1, we successfully identified *Radix Astragali*, *Lumbricus*, *Radix Chuanxiong* and *Flos Carthami* as HO-1 inducers. Surprisingly, the appropriate combinations of the active ingredients or the active compounds led to synergistic induction of HO-1 expression. In the rat stroke model, the formulation ISF-1 restored HO-1 expression that was down-regulated by middle cerebral artery occlusion. Thus, our results may lead to the development of new antioxidant therapies for post-stroke disorders.

In conclusion, this study used genome-wide biological response fingerprints of human cells to define the intracellular pathways as the targets for complex herbal medicines. We have thus proposed a general strategy for developing new chemically defined formulations targeting specific pathways. Our strategy involves two major procedures: step-wise deletion of one or more individual ingredients from complex herbal formulations and formulation of new combinations using the active ingredients. While the development of conventional drugs becomes increasingly difficult, this study may elucidate the development of mechanism-based therapies based on well-documented traditional remedies.

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