Chiang Mai J. Sci. 2017; 44(x) : 1-16
http://epg.science.cmu.ac.th/ejournal/
Contributed Paper

Proteomics Profiling and Inflammatory Factor Gene Expression in LPS-Stimulated RAW 264.7 Cells Treated with *Crocodylus siamensis* Hemoglobin

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Received: 20 December 2015
Accepted: 5 September 2016

ABSTRACT

Although we previously reported that *Crocodylus siamensis* hemoglobin (cHb) exhibits anti-inflammatory properties by suppressing nitric oxide (NO) production, the actual underlying mechanism has yet remained elusive. Consequently, this report represents the first study aimed to shed light upon the basic mechanistic details of the anti-inflammatory activities of cHb. In the present study, both 100 and 200 μg/ml of cHb were found to reduce the production of NO in LPS-stimulated RAW 264.7 cells. Consistent with the NO testing results, it was observed that co-treatment with cHb also significantly decreased inducible nitric oxide synthase (iNOS), likely due to decreased expression levels of cyclooxygenase-2 (COX-2) and pro-inflammatory cytokines, such as interleukin (IL)-1β and IL-6 mRNA. Therefore, these results indicate that the mechanism of cHb-induced decrease of NO production might be associated with the transcriptional suppression of iNOS. Moreover, the expression of heme oxygenase-1 (HO-1), an anti-inflammatory enzyme, was evaluated in more detail. It could be shown that HO-1 levels were increased in RAW 264.7 cells co-treated with cHb in a concentration-dependent manner. To elucidate the proteomics response of macrophages treated with LPS in the presence or absence of cHb, several proteins with differential expressions were identified via LC-MS/MS analysis. With respect to the individual functions of these proteins, our data indicated involvement in various processes during inflammation, such as cellular metabolism, protein fate, oxidative burst, signal transduction and morphogenesis. Consequently, all results of this study directly indicate that cHb exhibits anti-inflammatory activity on LPS-stimulated RAW 264.7 cells via functioning as an activator or suppressor in the expression of inflammatory factor genes and affects several specific proteins related to important pathways of inflammation.

Keywords: crocodile blood, hemoglobin, inflammatory gene, nitric oxide, proteomics, Siamese crocodile

1. INTRODUCTION

The immune response to tissue injury, which is regulated by cytokine networks and numerous pro-inflammatory genes and proteins, is commonly referred to as inflammation. This process is driven by abundant production of nitric oxide (NO) and pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α. These substances are not only causing cell and tissue damage, but also activate macrophages in immune diseases rheumatoid arthritis, chronic hepatitis, and other related diseases [1]. Correspondingly, these pro-inflammatory mediators have been considered as important targets for the rational development of effective anti-inflammatory agents.

Lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells have been used extensively for inflammatory response studies [2, 3]. During the actual inflammation process, macrophages become activated and play a crucial role in the initiation of many immune responses, involving induction of more than 400 genes responsible for eliminating bacteria, as well as regulation of many other cells through release of cytokines and chemokines [4]. Thus, macrophages represent useful targets for developing new anti-inflammatory agents and exploring molecular anti-inflammatory mechanisms [1]. Recently, numerous biologically active agents of natural origin have been reported to possess anti-inflammatory activity, including crocodile oil [5], anomalin [6], cphycocyanin [3] and ginsenosides [7]. Among natural agents, crocodile blood is considered a rich source of bioactive substances suitable for widespread therapeutic applications. Previous studies have shown that *Crocodylus siamensis* (*C. siamensis*) blood components, such as plasma, white blood cell extract and hemoglobin exhibit anti-inflammatory activity by reducing NO and IL-6 production in a dose-dependent manner [2, 8]. Merchant et al. (2003) reported that crocodile (*Alligator mississippiensis*) serum showed time and concentration-dependent antimicrobial activity against *Escherichia coli*, exceeding that of human serum by 10-fold after 1 h treatment. The antibacterial spectrum of crocodile serum was shown to be much broader than that of human serum [9]. Similar characteristics were observed for *C. siamensis* serum, which displayed activity against a variety of bacterial strains, such as *Salmonella typhi, E. coli, Staphylococcus aureus, S. epidermidis, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Vibrio chorelae* [10]. Another major component of crocodile blood is hemoglobin (Hb), which constitutes the main component of red blood cells in all vertebrates. The Hb molecule exists as a globin tetramer consisting of two identical α-globin chains and two identical β-globin chains. Each individual globin chain further comprises a heme group mediating the binding and transport of oxygen [11]. While the α- and β-globin chains of both human and crocodile (*C. siamensis*) Hb contain 141 and 146 amino acids residues, respectively [11], the amino-acid sequence identity between crocodile (*Crocodylus niloticus*) and human Hb is only 68% for the α-subunit and 51% for the β-subunit. This evidence leads to the idea that a number of entirely new functions enabling species to adapt to a new environment could evolve in a protein by just a relatively small number of amino acid substitutions in key positions [12]. Although

the main function of Hb is clearly the transport of oxygen, it also displays numerous other peculiar biological properties, such as anti-inflammatory, antimicrobial and antioxidant activity [8]. *C. siamensis* hemoglobin (cHb) was shown to reduce NO production during the inflammation process; however, the underlying mechanism of pro-inflammatory factor gene expression and proteomics profiles in LPS-stimulated RAW 264.7 macrophage cells treated with cHb remains unknown. Thus, this study was designed to determine the anti-inflammatory effects of cHb by measuring the mRNA expression of pro-inflammatory factors, such as NO, IL-1β, IL-6, heme oxygenase-1 (HO-1), inducible nitricoxide synthase (iNOS) and cyclooxygenase-2 (COX-2) as well as performing proteomics profiles analysis in LPS-stimulated murine macrophage RAW 264.7 cells.

2. MATERIALS AND METHODS

2.1 Preparation of Crocodile Hemoglobin

Crocodile blood was kindly provided by Sriracha Moda Farm., Ltd., Chon Buri, Thailand and collected using 0.8 × 38 mm needles, a sterile syringe and stored in bottles containing 0.08 g EDTA. The red blood cells (RBC) were collected after allowing the blood settle. Then, cHb was extracted from RBC as previously described [8]. In brief, the RBC was washed three times with ice-cold phosphate buffer saline (PBS), pH 7.0. After centrifugation at 3,000 × *g* for 5 min, the RBC pellet was added to ice-cold distilled water of two-fold volume for breaking the cells. The breaking of RBC cells was performed at room temperature for 10 min. After being centrifuged at 10,000 × *g* for 20 min at 4 °C, the resulting cHb solution (supernatant) was kept at -70 °C until use.

2.2 Cell Culture and Sample Treatment

A murine macrophage cell line (RAW 264.7; ATCC TIB-71) was obtained from the American Type Culture Collection (American Type Culture Collection [ATCC], USA). According to a previously reported method with some modifications [8], RAW 264.7 cells were grown at 37 °C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic:antimycotic (Gibco, USA) in a 5% CO_2 humidified atmosphere. For sample treatment, RAW 264.7 cells were plated at a density of 1×10^5 cells in a 90 × 15 mm dish, and allowed to attach overnight. For stimulation, the medium was replaced with FBS free RPMI 1640 medium, and the cells were further stimulated with 100 ng/ml of lipopolysaccharides (LPS) (Sigma-Aldrich, Germany) in the presence or absence of different concentrations of cHb (100 and 200 μg/ml) for 24 h.

2.3 Nitric Oxide (NO) Determination

NO synthesis in cell cultures was determined by the Griess reaction [8]. Shortly, 100 μl of cell culture medium from each sample treatment was mixed with equal volumes of Griess reagent and incubated at room temperature for 10 min. Subsequently, the absorbance at 540 nm was determined and NO production was calculated as a percentage of control defined by comparison each treated group with the LPS group.

2.4 Total RNA Extraction and First-Strand cDNA Synthesis

Total RNA was extracted from each RAW 264.7 cell treatment using TRIZOL® reagent (Invitrogen, USA). The derived RNA was used as a template for first-strand cDNA synthesis, according to the RevertAid

First-Strand cDNA synthesis kit manual (Fermentas, USA). In detail, 0.5 μg of total RNA was mixed gently with oligo (dT) 18 primer, centrifuged briefly and incubated at 65 °C for 5 min. After that the reaction buffers, RiboLock RNase inhibitor, dNTP mix, and RevertAid M-MuLV reverse transcriptase were added into the mixture with a total volume of 20 μl. After mixing gently and centrifuging briefly, the mixture was further incubated for 60 min at 42 °C. The termination of the reaction was initiated by heating at 70 °C for 5 min and the mixture was kept at -70 °C until use.

2.5 Semiquantitative RT-PCR

To evaluate the inflammatory gene expression (HO-1, iNOS, IL-1β, IL-6, COX-2) in each RAW 264.7 cell sample, 1 μl of each cDNA was used as templates in the PCR reactions and the housekeeping gene, GAPDH, was used as internal control gene. The reactions were carried out in a volume of 20 μl using Taq DNA polymerase (Vivantis, USA). After initial denaturation at 95 °C for 5 min, 35 amplification cycles of 30 s at 95 °C denaturation, 45 s at each annealing temperature, and 45 s at 72 °C extension were performed and repeated at least three times. The PCR primer sequences and annealing temperatures are shown in Table 1. The PCR products were analyzed using ethidium bromide-stained 1-2% agarose gel electrophoresis. The intensity of each DNA band was quantified by Quantity One software (Bio-Rad, USA), and normalized to the level of the housekeeping gene (GAPDH). Relative intensity of each gene was calculated by comparison to that of cells treated with LPS alone. However, the expression of HO-1 was showed by the intensity with normalized to the level of GAPDH.

Table 1. List of primers sequence, PCR product size and annealing temperature used for detection of cytokine gene expression.

Gene		Primer Sequence (5′-3′)	product size (bp)	Annealing temperature (°C)	References
HO-1	sense	GAGCCTGAATCGAGCAGAAC	206	60	[13]
	anti-sense	AGCCTTCTCTGGACACCTGA			
iNOS	sense	TGGGAATGGAGACTGTCCCAG	306	60	[14]
	anti-sense	GGGATCTGAATGTGATGTTTG			
IL-1β	sense	AAGCTCTCCACCTCAATGGACAG	260	57	[14]
	anti-sense	CTCAAACTCCACTTTGCTCTTGA			
IL-6	sense	CCTCTGGTCTTCTGGAGTACCAT	307	60	[14]
	anti-sense	GGCATAACGCACTAGGTTTGCCG			
COX-2	sense	TGTATGCTACCATCTGGCTTCGG	237	58	[15]
	anti-sense	GTTTGGAACAGTCGCTCGTCATC			
GAPDH	sense	GAGAAACCTGCCAAGTATGATGAC	212	55	[16]
	anti-sense	TAGCCGTATTCATTGTCATACCAG			

2.6 Two-dimensional (2D) Polyacrylamide Gel Electrophoresis

Two-dimensional (2D) electrophoresis (2D-PAGE) was carried out following previously documented procedures with some modifications [17]. In brief, RAW 264.7 cells were plated at a density of 2×10^6 cells into a 15 × 60 mm plastic petri dish, and allowed to attach overnight. For stimulation, the medium was replaced with FBS free RPMI 1640 medium, and the cells were further stimulated with 100 ng/ml of LPS (Sigma-Aldrich, Germany) in the presence or absence of 100 μg/ml of cHb for 24 h. Proteins were extracted from each RAW 264.7 cells treatment group using extraction buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (w/v) DTT). Eighty micrograms of each protein sample were mixed with rehydration buffer (7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2 mM DTT, 0.8% (w/v) IPG buffer and 0.2% bromophenol blue) to a final volume of 125 μl. The first dimensional was performed on 7 cm immobilized pH gradient (IPG) strips, linear pH 4-7 (Amersham Bioscience, Sweden) using the following program: rehydration for 12 h followed by focusing of the IPG strips on step 1: 250 Vh, step 2: 500 Vh, and step 3: 8,500 Vh for 9,250 Vh. After the first dimension, SDS-PAGE in the second dimension was performed using a 13.5% polyacrylamide gel. Afterwards, the gel was stained with silver nitrate.

2.7 Protein Profiles Analysis

The stained 2D gels were scanned with a resolution of 300 dpi and 24 bits. The 2D gel images were uploaded into the ImageMaster 2D Platinum 7.0 software (GE Healthcare, Sweden). As described by Khueychai et al. [17], protein spots were detected automatically by applying the same detection parameters (smooth 2, min area 5 and saliency 40) to all 2D gel images. The approximate MW of each spot was calculated according to the standard protein band on the 2D gel (phosphorylase b, 97 kDa; albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.4 kDa). The pI of each protein spot was calculated according to the IPG strip range position. Differentially expressed protein spots were evaluated by spot volumes. After data analysis, the protein spots showing changes in their expressions were excised from preparative 2D gels for protein identification by liquid chromatography mass spectrometry (LC/MS-MS).

2.8 Liquid Chromatography Mass Spectrometry (LC/MS-MS)

The excised protein spot samples were digested with trypsin using sequencing grade reagent (Promega, USA) according to the manufacturer's specifications. Afterwards, the digested peptides were subjected to LC/MS-MS analysis according to previously reported methods [17]. Briefly, the dried digested peptides were resuspended with 10 μl of 0.1% (v/v) trifluoroacetic acid (TFA) and injected into a nanoACQUITY system (Waters Corp., Milford, MA, USA) composed of a Symmetry C18 Trap column and a BEH130 C18 analytical reverse phase column (Waters Corp., Milford, MA, USA). Each tryptic peptide was detected using a SYNAPTTM HDMS mass spectrometer (Waters Corp., UK) in positive nanoelectrospray ion mode. The Quadrupole mass analyzer was adjusted to ensure ions from m/z 200 to 1990 were efficiently transmitted. Bioworks 3.2 software (Thermo Electron, USA) was used to process and convert the data to a Mascot generic file for using Mascot search. Parameters of Mascot search included a specified trypsin

enzymatic cleavage with one possible missed cleavage, +/- 1.2 Da peptide tolerance, +/- 0.6 Da MS/MS tolerances, 1+, 2+, 3+ ions, monoisotopic mass, methionine oxidation variable modification and carbamidomethylation (C) fixed modification. The obtained pI, molecular mass, MASCOT score, number of peptide matches and sequence coverage were used for protein identification.

2.9 Statistical Analysis

All data were derived from at least three independent experiments. The data were calculated by using the analysis of variance. The differences between mean values of all data were compared using the least significant difference test. Statistical significance was set at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Inhibition of NO Production by cHb

In order to evaluate the effect of cHb on pro-inflammatory cytokine genes, 100 ng/ml of LPS was used to stimulate RAW 264.7 macrophage cells. The result showed that NO generation was significantly increased by LPS stimulation, meaning the inflammation occurred within the cell. Significant suppression of NO was found to be effected by both 100 and 200 mg/ml of cHb (Figure 1), which is consistent with previous results reported by Phosri et al. [8]. NO is a small free radical synthesized by nitric oxide synthase (NOS), of which three isoforms have been characterized in mammalian cells: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). Among them, iNOS is expressed in various cell types, such as macrophages, hepatocytes and astrocytes, and is induced in response to various factors, including LPS, IFN-γ, TGF-β, and pro-inflammatory cytokines [18]. Despite, the underlying mechanism for the anti-inflammatory effect of cHb is still not clearly understood in detail. Thus, further experiments were carried out to investigate the effect of cHb on iNOS mRNA expression in each RAW 264.7 cell sample.

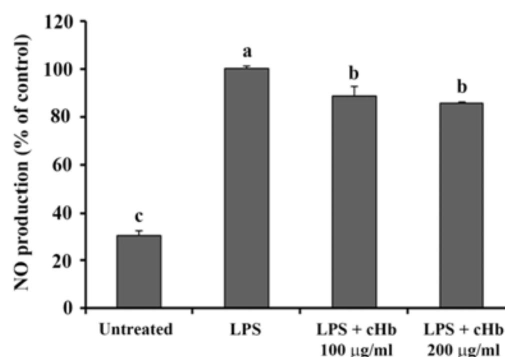

Figure 1. Effect of cHb on NO generation in LPS-stimulated RAW 264.7 cells. The percentage of control was calculated by comparison of each group with the group of LPS-only treated cells. Each value is expressed as the mean ± SD. Different letters (a-c) on top of each bar indicate statistically significant differences ($p < 0.05$) compared to cells treated with LPS alone.

3.2 Inhibition of iNOS mRNA Expression by cHb

To evaluate whether or not the inhibition of NO in RAW 264.7 cells by cHb is correlated with iNOS mRNA expression, the levels of iNOS mRNA were measured by semi-quantitative RT-PCR. In agreement with the results from NO inhibition experiments, LPS induced a remarkable increase of iNOS mRNA levels, while both 100 and 200 μg/ml of cHb suppressed these inductions by more than 20% compared to the expression in LPS-stimulated RAW 264.7 cells (Figure 2A). These results suggest that the inhibitory effect of cHb on LPS-induced NO production is caused by down-regulated iNOS mRNA expression.

The expression of iNOS is regulated by nuclear factor-kappa B (NF-κB), which is a key regulator of several genes involved in inflammatory responses [19]. NF-κB exists as a heterodimer of p65 and p50, and is located in the cytoplasm as an inactive complex bound to IkB-α. Phosphorylation and subsequent degradation leads to the dissociation of the complex and generates activated NF-κB [19]. Activated NF-κB can then translocate into the nucleus to promote the transcription of target genes and initiate gene expression, including iNOS and cyclooxygenase-2 (COX-2) [19]. In the present study, it was found that cHb-induced down-regulation of NO production was affected by iNOS transcriptional suppression.

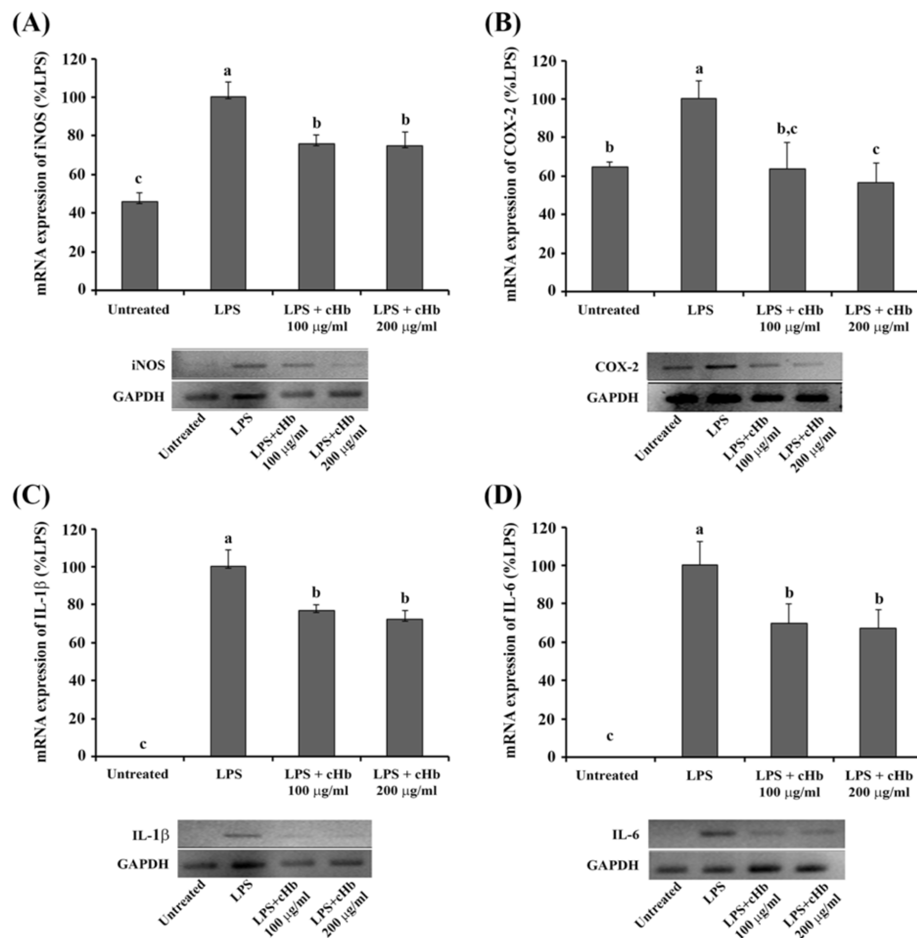

Figure 2. Effect of cHb on LPS-stimulated iNOS (A), COX-2 (B), IL-1β (C) and IL-6 (D) mRNA expression in RAW 264.7 cells using semi-quantitative RT-PCR. The intensity of each DNA band was quantified by Quantity One software (Bio-Rad, USA), and normalized to the level of GAPDH. Relative intensity of each gene was calculated by comparison to that of cells treated with LPS alone. Each value is expressed as the mean ± SD. Different letters (a-c) on top of each bar indicate statistically significant differences ($p < 0.05$) compared to cells treated with LPS alone.

3.3 Inhibition of COX-2 mRNA Expression by cHb

In immune cells, such as macrophages and synoviocytes, COX-2 is induced in response to infection, injury, or other stresses, and produces large amounts of prostaglandins that act to induce inflammatory states [19, 20]. In agreement with these facts, our results revealed that LPS significantly induced COX-2 mRNA expression. This induction was evidently decreased by co-treatment of RAW 264.7 macrophage cells with cHb (Figure 2B). In general, non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used for inflammation treatment worldwide [21]. The class of NSAIDs comprises both traditional nonselective NSAIDs (tNSAIDs), which non-specifically inhibit both COX-1 and COX-2, as well as selective COX-2 inhibitors. Although effective for relieving pain and inflammation, tNSAIDs are also associated with a significant risk of serious gastrointestinal and cardiovascular adverse events in chronic use [21]. Thus, cHb as a natural substance with high efficacy for reducing the COX-2 mRNA level might serve as an excellent naturally derived alternative to tNSAIDs in the treatment of inflammation.

3.4 Effect of cHb on Pro-Inflammatory Cytokine mRNA Expression

Although Hb, one of the major components of crocodile blood, was previously shown to exhibit anti-inflammatory activity by reducing NO production, no studies covering effects of cHb on pro-inflammatory cytokines have been reported to date [8]. Pro-inflammatory cytokines represent a special group of cytokines, which are responsible for boosting inflammation pathways. Proteins or other natural bioactive agents that can effectively reduce the biological activity of pro-inflammatory cytokines might also be capable of reducing the brunt of damage arising from diseases that are characterized by cytokine-induced inflammation. Thus, the next investigation was aimed at measuring levels of IL-1β and IL-6, which constitute major pro-inflammatory cytokines produced mainly by activated macrophages [22]. After 24 h incubation with LPS, IL-1β and IL-6, mRNA level was significantly increased, while untreated RAW 264.7 cells did not show mRNA production. As shown in Figure 2C and 2D, the expression levels of IL-1β and IL-6 mRNA were significantly decreased in the presence of both 100 and 200 μg/ml of cHb. IL-1β is typically produced to induce gene expression and synthesis of COX-2, type 2 phospholipase A, whereas iNOS accounts for prostaglandin-E2 (PGE_2), platelet activating factor and NO production [23]. While the IL-6 level is enhanced at the site of inflammation, it plays a key role in the acute inflammatory phase response as defined by a variety of clinical and biological features, such as the production of acute phase proteins [24]. Both IL-1β and IL-6 have thus been recognized as promising therapeutic intervention targets, and the ability to suppress IL-1β and IL-6 expression at the transcriptional level promotes the use of cHb as a valuable natural substance for inflammation treatment.

3.5 Effect of cHb on HO-1 mRNA Expression

Since heme oxygenase-1 (HO-1) is known to contribute to anti-inflammatory processes, the expression level of HO-1 mRNA was evaluated in more detail. The results show that after 24 h incubation with LPS, the HO-1 mRNA level was slightly decreased, albeit not significant when compared to untreated RAW 264.7 cells.

However, cells co-treated with cHb featured significantly increased expression of HO-1 in a concentration-dependent manner (Figure 3). These results are in excellent agreement with previous reports by Abraham and Drummond [25], who found that HO-1 was induced when the cultured macrophages, endothelial cells or tubular cells were exposed to heme or Hb. This induction of HO-1 is widely recognized as an effective cellular strategy to counteract cellular damage and inflammation [25]. It is also well documented that HO-1 mediates the rate-limiting step in heme catabolism. In this transformation, heme is degraded into carbon monoxide (CO), iron and biliverdin, the latter being converted into bilirubin in a subsequent reaction by biliverdin reductase. Both biliverdin and bilirubin are antioxidants, as well as CO, which has anti-apoptotic and anti-inflammatory properties [19, 25].

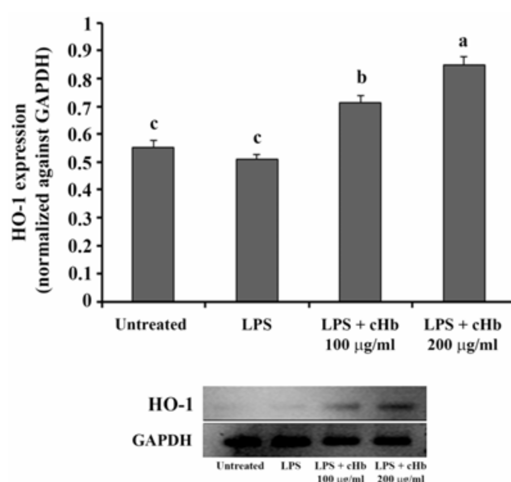

Figure 3. Effect of cHb on HO-1 mRNA expression in LPS-stimulated RAW 264.7 cells. The HO-1 mRNA expression level was determined by semi-quantitative RT-PCR and normalized against GAPDH. Each value is expressed as the mean ± SD. Different letters (a-c) on top of each bar indicate statistically significant differences (p < 0.05).

3.6 Proteomics Change of RAW 264.7 Protein upon Inflammation and cHb Treatment

Because the potential ability to decreased the NO production between 100 and 200 µg/ml of cHb was not significant different, only 100 µg/ml of cHb was selected for this study. As shown in Figure 4, more than 100 individual protein spots were found in each treatment sample. Among these, seventeen proteins were identified by LC/MS-MS and their expression levels were found to be altered considerably (Table 2). Of the seventeen proteins identified, eleven were modulated in response to LPS, including ten up-regulated proteins (i.e. 60-kDa heat shock protein, protein disulfide-isomerase, catalase, vimentin, ATP synthase subunit beta, Protein disulfide-isomerase A6 precursor, β-actin, multiple inositol polyphosphate phosphatase 1, HPr kinase/phosphorylase and betaine reductase) and one down-regulated protein (i.e. ribose ABC transporter ATP-binding protein), whereas three proteins were found only in LPS treatment (i.e. endoplasmin, heat shock cognate 71-kDa protein and 65-kDa macrophage protein). Moreover, out of these LPS-induced changes ten were reversed with decreased levels by cHb co-treatment (i.e. stress-70-kDa protein, protein disulfide-isomerase, catalase, vimentin, ATP synthase subunit beta, protein disulfide-isomerase A6 precursor, β-actin, multiple inositol polyphosphate phosphatase 1, HPr kinase/phosphorylase and betaine reductase), while four displayed increased levels (i.e. coronin-1A, 60-kDa heat shock protein, ribose ABC transport ATP-binding protein, E3 ubiquitin-protein ligase). The above mentioned proteins are associated with multiple functions, including cellular metabolism (e.g. ATP synthase subunit beta, protein disulfide-isomerase A6 precursor and ribose ABC

transporter ATP-binding protein), protein fate (e.g. heat shock cognate 71-kDa protein, 60-kDa heat shock protein and E3 ubiquitin), oxidative burst (catalase and vimentin), signal transduction (HPr kinase/phosphorylase and multiple inositol polyphosphate phosphatase I) and morphogenesis (e.g. coronin-1A and β-actin). Their possible role in response to inflammation will be discussed in the following section.

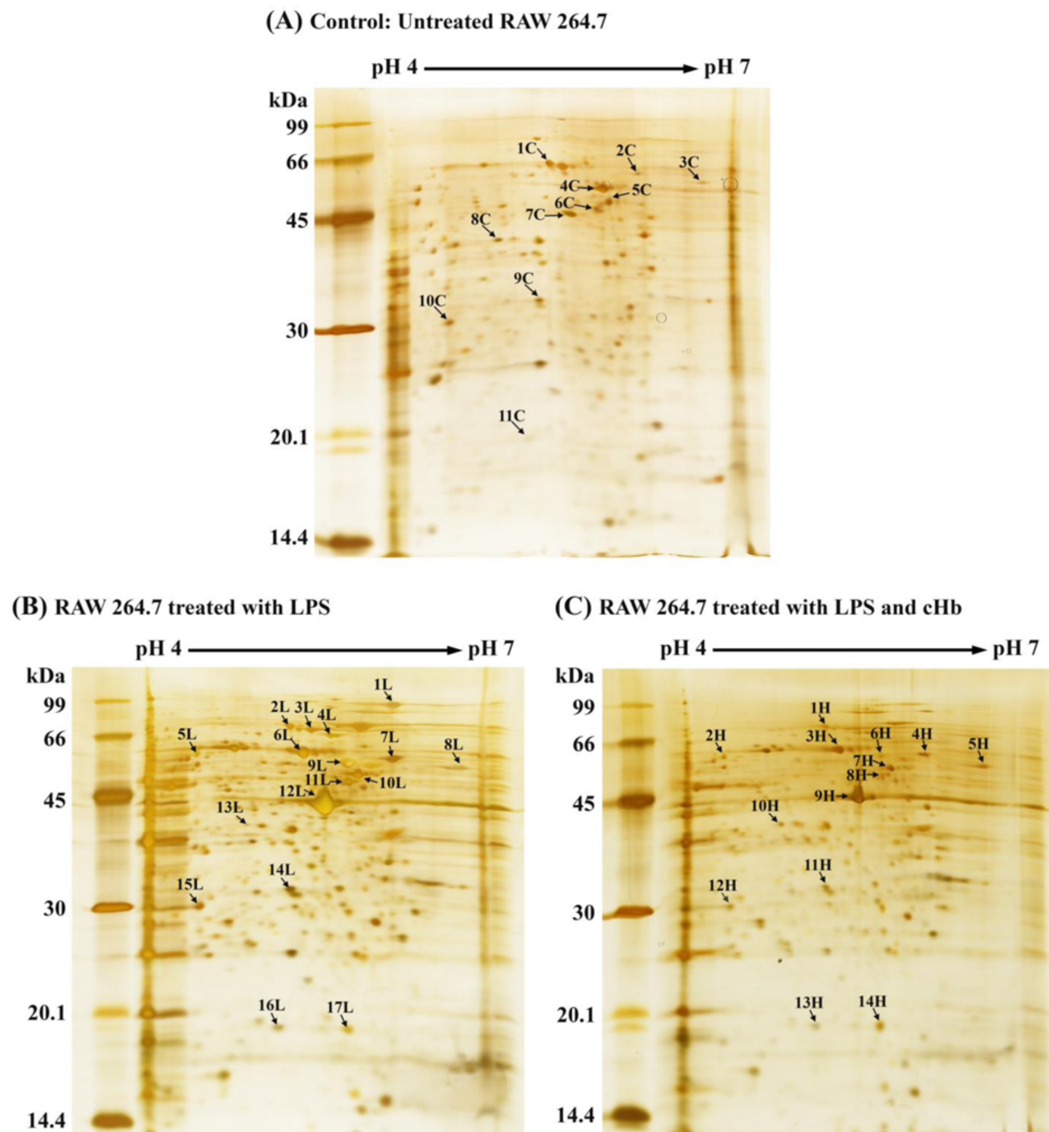

Figure 4. Proteomics profiling of proteins extracted from untreated RAW 264.7 cells (A), RAW 264.7 treated with LPS (B) and RAW 264.7 treated with LPS and co-incubated with cHb (C). In the first dimension (IEF), 80 μg of protein were loaded on a 7 cm IPG strip with a linear gradient of pH 4-7. For resolution of the second dimension (SDS-PAGE), 13.5% polyacrylamide gel was used. Proteins were visualized by silver staining. Arrows indicate protein spots, which were altered in expression in response to LPS with and without cHb.

Table 2. Identification of proteins change in macrophage treated with LPS in the presence or absence of cHb by LC-MS/MS.

Spot No.	Treatment effect			Matched protein	Experimental Mw/pI	Theoretical Mw/pI	Score	Sequence coverage (%)	Number of peptides matched	Species
	RAW 264.7 treated with LPS[(A)] compare to Untreated RAW 264.7[(B)]	RAW 264.7 treated with LPS and cHb[(A)] compare to Untreated RAW 264.7[(B)]	RAW 264.7 treated with LPS and cHb[(A)] compare to RAW 264.7 treated with LPS[(B)]							
0 : 1L : 0	P/A	A/A	A/P	Endoplasmin	93.00/6.24122	92.703/4.74	271	8	3	*Mus musculus*
0 : 2L : 1H	P/A	P/A	↓	Stress-70 protein, mitochondrial	76.00/5.40590	73.768/5.91	174	4	2	*Mus musculus*
0 : 3L: 0	P/A	A/A	A/P	Heat shock cognate 71-kDa protein	71.00/5.56674	71.424/5.37	179	7	2	*Bos taurus*
0 : 4L : 0	P/A	A/A	A/P	65-kD a macrophage protein	66.00/5.72834	70.784/5.2	78	2	2	*Mus musculus*
0 : 5L : 2H	P/A	P/A	↑	Coronin-1A	59.50/4.63691	51.641/6.05	47	3	3	*Mus musculus*
1C : 6L : 3H	↑	↑	↑	60-kDa heat shock protein	62.33/5.49055	61.240/5.60	87	5	4	*Bos taurus*
2C : 7L : 4H	↑	↑	↓	Protein disulfide-isomerase	59.33/6.19539	57.629/4.8	130	5	2	*Bos taurus*
3C : 8L : 5H	↑	↑	↓	Catalase	57.00/6.64138	60.106/6.78	30	1	1	*Bos taurus*
4C : 9L : 6H	↑	↓	↓	Vimentin (Fragment)	55.50/5.87718	51.874/4.94	294	17	5	*Cricetulus griseus*
5C :10L : 7H	↑	↑	↓	ATP synthase subunit beta, mitochondrial precursor	52.67/5.94189	56.265/5.19	333	13	2	*Mus musculus*
6C : 11L : 8H	↑	↓	↓	protein disulfide-isomerase A6 precursor	50.33/5.87188	48.659/5.03	60	3	2	*Mus musculus*
7C : 12L : 9H	↑	↑	↓	Put. beta-actin (aa 27-375)	45.00/5.64335	39.446/5.78	131	11	3	*Mus musculus*
8C : 13L : 10H	↓	↓	↑	Ribose ABC transporter ATP-binding protein	42.00/5.03459	52.727/5.82	42	1	1	*Patulibacter medicamentivorans*
9C : 14L : 11H	↑	↓	↓	Multiple inositol polyphosphate phosphatase 1	32.67/5.40686	55.130/7.21	28	3	2	*Mus musculus*
10C : 15L : 12H	↑	↓	↓	HPr kinase/phosphorylase	30.67/4.66733	34.738/5.35	41	4	1	*Granulicatella adiacens*
11C : 16L : 13H	↑	↑	↓	Betaine reductase	19.00/5.32230	17.172/4.41	43	5	1	*Natranaerobius thermophilus JW/NM-WN-LF*
0 : 17L : 14H	P/A	P/A	↑	E3 ubiquitin-protein ligase	19.00/5.85508	19.487/5.65	29	4	1	*Mus musculus*

↑: Proteins was up-regulated.; ↓: Proteins was down-regulated.; A/A: absent in both (A) and (B); P/A: present in (A) but absent in (B); A/P: absent in (A) and present in (B)

Heat shock proteins (HSPs) are originally reported to be induced by heat stress. Nevertheless, other stressful stimuli, such as hypoxia, toxic chemicals, and inflammation also induce HSP expression and it is believed that HSPs represent a mechanism of response to cellular stress that protects intracellular proteins from damaging conditions [26]. HSPs are molecular chaperones that play a critical role in protein folding and conformational changes of proteins [26]. Several studies have shown HSPs to inhibit autoimmune disorders, such as diabetes and arthritis both in animal models and initial clinical trials in patients with chronic inflammatory disease. Furthermore, they have been shown to enhance the production of anti-inflammatory cytokines such as IL-10 [27]. In agreement with these findings, our results demonstrate that LPS induces 2 types of HSPs, including Heat shock cognate 71-kDa protein (HSP71) and 60-kDa heat shock protein (HSP60) in RAW 264.7 cells. The expression of HSP70 was observed only in LPS-treated cells. For HSP60, it was still expressed regardless of cHb treatment. Taken collectively, these findings provide clear support for the assumed function of cHb in anti-inflammatory processes.

ATP-binding cassette (ABC) efflux transporters are transmembrane proteins responsible for the efflux of several endogenous substances. ABC transporters provide an innate defense system against toxins, harmful metabolic compounds and toxic therapeutic agents, indicating that the transporter mediates the export of a wide variety of such substances. Moreover, ABCA1 is expressed in human immune cells (e.g. monocytes and macrophages) in a differentiation-dependent and sterol-sensitive manner, rendering the ABC transporter a likely candidate effector for foam cell formation [28]. ABCA1 imparts a dual role as both a mediator of cellular lipid transport as well as translocator of IL-1β. Therefore, the transporter is an important component of inflammatory responses and lipid transport processes [29]. According to our results, the level of expression of ribose ABC transporter ATP-binding protein was decreased in in response to LPS as implied by an increase of IL-1β. This situation was found to be reversed by cHb treatment, resulting in a high transporter protein expression which enhances the efflux of IL-1β. These results correlate nicely with the gene expression results of IL-1β in response to LPS and co-treatment with cHb.

Coronin 1 (coronin 1A) plays a crucial role in the host defense mechanism against bacterial infection. In mammalians, the protein is predominantly expressed in leukocytes, without detectable expression in any other cell types. Although leukocyte cells normally contain several other coronin isoforms, coronin I has evolved to exert a highly specialized function. The protein is comprised of three domains including a β-propeller (N-terminal) domain (residue 1-355), a linker domain (residue 356-429) and a α-helix-rich domain (residue 430-461) [30]. In swine, however, coronin 1A was shown to suppress the NF-κB activation during *Haemophilus parasuis* infection by inhibiting the degradation of IkB-α and nuclear translocation of p65 [31]. In the same way, NF-κB has been considered as an activator in the pro-inflammatory process. Moreover, the expression of NF-κB is based on the activation by some pro-inflammatory cytokines, especially interleukin 1 (IL-1β). Regarding our work, the expression level of IL-1β in macrophage was found to increase after treatment with LPS and participation of activated NF-κB in the inflammation process appears highly plausible. Therefore, treatment of macrophages with

LPS and co-treatment with cHb would result in the expression of coronin 1A, which, in turn, suppresses the activation of NF-κB and consequently leads to reduced inflammation.

ATP synthase is an enzyme encoded by both nuclear and mitochondrial DNA, which is involved in cellular energy production in all living organisms. The beta subunit is an important component of the ATP synthase for binding co-factors involved in ATP synthesis [32]. An alteration of ATP synthase beta subunit expression level in macrophage cells in response to LPS stimulation has been previously reported [32]. Similarly, the present study provided evidence that the mitochondrial precursor for ATP synthase subunit beta was up-regulated in RAW 264.7 cells treated with LPS. The expression level of this protein was reverted to control levels in the presence of cHb. This result implies that the increased abundance of ATP synthase may be necessary to maintain or enhance the energy production capacity of the cell during inflammation.

Actins represent a crucial component of the cytoskeleton participating in numerous cellular processes, e.g. cell migration, cell division, and the regulation of gene expression. Normally, proteins have an inherent ability to form filaments capable of assembling and disassembling based on the needs of the cell. Six different, highly conserved actin isoforms are known in vertebrates [33], of which four are generally expressed in striated (αsk and αca) and smooth (αsm and γsm) muscle cells. However, the two cytoplasmic β-actin and γ-actin isoforms are expressed in separate genes, Actb and Actg1, respectively. In many studies, β-actin serves as a housekeeping gene frequently used as control in gene expression, while showing a different expression behavior in cancer. Moreover, actin fragment reorganization is required for leukocyte activation, adhesion, and transcription of inflammatory factors [34]. In a previous study, actin fragment reorganization was identified as a crucial step in lung inflammation after induction with systemic inflammatory response syndrome. The reorganization is actually critical for two main processes: neutrophil migration into the lung and the secretion of pro-inflammatory cytokines by pulmonary monocytes. This led to the assumption that actin fragments could be a potential target for preventing and treating acute lung injury induced by systemic inflammatory response syndrome [34]. In agreement with these results, the present study revealed that the expression level of actin is increased in response to LPS stimulation, which impedes triggering of inflammatory effects of LPS in macrophages. The fact that co-incubation with cHb led to a reduction in actin levels indicates that cHb exhibits anti-inflammatory activity in LPS-induced macrophage by interacting with the actin fragment.

Several antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH-px), and catalase provide eukaryotic cells with an effective primary defense against reactive oxygen species (ROS). Catalase is one of the most active antioxidant defense enzymes known for being highly cooperative with SOD and other Hydrogen peroxide (H_2O_2) producers at high flux of H_2O_2. It is composed of four identical protein subunits, each of which contains a ferric heme group bound to its active site [35]. Usually, each subunit contains one molecule of NADPH bound to catalase molecule, protecting the enzyme from oxidation by its H_2O_2 substrate. Normally, catalase mediates the dismutation of H_2O_2 to water and oxygen, but it also serves as an efficient scavenger of ROS to prevent cellular damage. Generally, ROS are known to be crucial inflammatory

mediators. During an inflammatory response, the excessive production of ROS can cause major damage to cells, which can lead to DNA damage and mutations [36]. To investigate the effect of cHb on anti-inflammation regulation, the proteomics technique was used to observe the alteration of protein expression in macrophages treated with LPS with or without cHb. In response to treatment with LPS and cHb, the expression level of catalase was down-regulated compared to LPS-treated control. This result revealed that cHb could scavenge ROS, therefore catalase expression is reduced, as it is no longer needed.

Vimentin is an intermediate filament protein that is cleaved in response to caspase-activation by apoptotic and macrophage-mediated processes. The protein is deemed crucial for maintaining cellular integrity and is assumed to provide resistance against stress. Moreover, vimentin has also been reported to be involved in cell migration processes. In cases of tissue and cellular damage, cell migration plays a key role in a number of important defense mechanisms, such as wound healing, inflammation, and immune response [37]. Vimentin is further associated with macrophage differentiation, phagocytosis and ROS production as well as control of the oxidative response [38]. In this study, a reduction of vimentin expression in response to LPS was observed after treatment with LPS and cHb. This implies that the expression of vimentin would be decreased, consequently leading to reduce the inflammation.

4. CONCLUSION

LPS stimulated RAW 264.7 cells have been extensively shown to induce the secretion of numerous pro-inflammatory cytokines, such as IL-1β and IL-6, and inflammatory mediators, such as NO, iNOS and COX-2. The suppression of pro-inflammatory cytokines, iNOS or COX-2 expressions offers new therapeutic strategies for the treatment of inflammation. To the best of our knowledge, this work represents the first report covering the effect of cHb on pro-inflammatory factors mRNA expression. The results of this study revealed that cHb suppresses NO production by decreasing the expression of pro-inflammatory factor genes, including IL-1β, IL-6, iNOS and COX-2, in LPS-stimulated RAW 264.7 cells. It could further be demonstrated that cHb reduces NO production by affecting the transcriptional suppression of iNOS expression. Moreover, cHb significantly increases the expression of HO-1 in a concentration-dependent manner. Proteomics profiling further revealed that LPS exposure affected the expression of several proteins associated with major inflammation pathways, including HSPs, catalase, vimentin and coronin 1A. Treatment with cHb was shown to diminish these alterations and, at least in part, led to restoration of the initial expression levels of several important proteins. Hence, our findings suggest that cHb may be considered as a potential anti-inflammatory drug candidate suitable for topical application.

ACKNOWLEDGMENTS

This work was supported by a grant from the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, through the Food and Functional Food Research Cluster of Khon Kaen University. We would also like to thank the Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Faculty of Science, Khon Kaen University Thailand and, especially, Sriracha Moda co., Ltd., Chon Buri,

Thailand for providing crocodile blood samples.

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