

Stimulation of Haptoglobin Synthesis by Interleukin-6 and Tumor Necrosis Factor, But Not by Interleukin-1, in Bovine Primary Cultured Hepatocytes

Noriko NAKAGAWA-TOSA, Masami MORIMATSU, Masami KAWASAKI, Hiroki NAKATSUJI¹⁾, Bunei SYUTO, and Masayuki SAITO

Department of Biochemistry, Faculty of Veterinary Medicine, and ¹⁾Department of Animal Science, Faculty of Agriculture, Hokkaido University, Sapporo 060, Japan

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ABSTRACT. The hepatic synthesis of acute phase proteins in ruminants has been suggested to be regulated by some mechanisms different from those in other species such as rodents and human. To explore possible regulatory factors unique to ruminants, we examined effects of interleukin (IL)-6, IL-1 and tumor necrosis factor (TNF), on haptoglobin (Hp) synthesis using a primary culture system of bovine hepatocytes. After bovine primary cultured hepatocytes were incubated in the presence of various concentrations of the cytokines, the synthesis and mRNA level of haptoglobin and albumin were measured by labeling with [³⁵S]-methionine and immunoprecipitation, and by Northern blot analysis, respectively. Hp synthesis was dose-dependently increased by recombinant human (rh) IL-6, and also by rhTNF- α , but to a less extent, while it was not affected by rhIL-1 β . The stimulatory effect is mainly pretranslational, because mRNA level of Hp changed in parallel with protein synthesis. In contrast, albumin synthesis was suppressed by these three cytokines similarly. These results are inconsistent with the previously proposed view that TNF and IL-1 overlap in their pathways leading to the transcriptional activation of many acute phase protein genes. In conclusion, there is a species-specific unique signaling system, especially for TNF, in transcriptional activation of bovine Hp gene.—**KEY WORDS:** acute phase protein, bovine, cytokine, haptoglobin, primary cultured hepatocyte.

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Infection and tissue injury elicit a dramatic change in the plasma level of some specific proteins, so-called acute phase protein (APP). APPs are well preserved through phylogeny and believed to play important protective roles in host defense against tissue damage and infections. It is now accepted that hepatic synthesis and secretion of APP are regulated by diverse inflammatory mediators such as immune cytokines and glucocorticoids. Particularly, the principal roles of interleukin (IL)-6, IL-1 and tumor necrosis factor (TNF) seem well established on the basis of their stimulatory effect on APP synthesis in *in vitro* systems using primary cultured hepatocytes or hepatoma cell lines [4, 6, 11]. According to the difference in the response to these cytokines, APPs are classified into two types: type 1 APP (e.g., α_1 -acid glycoprotein, C-reactive protein, and serum amyloid A) are regulated by IL-1 and TNF, whereas type 2 APP (e.g., α_2 -macroglobulin and fibrinogen) by IL-6 [4, 6]. In both types of APP, glucocorticoids enhance the effect of cytokines synergistically [4, 6, 11].

Cross-species comparisons have indicated that there is a high degree of similarity among species in the qualitative and quantitative patterns of APP responses, and that species differences do not generally exist in the division of two APP types [4]. However, haptoglobin (Hp) is a unique APP so that it is classified as a type 1 APP in the rat while a type 2 APP in human [3, 6, 7]. Moreover, although in both the rat and human serum level of Hp increases only a few folds [11], in the bovine and goat it increases more than 100-folds after inflammatory stimuli [15, 19, 26]. Thus these results suggest that synthesis of ruminant Hp seems to be regulated by some unique

systems different from those in other species. In support of this idea, Higuchi *et al.* [12] recently demonstrated using a primary culture system of bovine hepatocytes that Hp synthesis is increased remarkably by glucocorticoids alone. Their results are apparently different from those in rodent and human hepatocytes, where the major action of glucocorticoids is to enhance the stimulatory effect of cytokines synergistically. However, as far as we know, there has been no report on the regulatory mechanisms of cytokines themselves in ruminant APP synthesis. In this study, we examined effects of IL-6, IL-1 and TNF on Hp synthesis in primary cultured bovine hepatocytes to explore possible regulatory mechanisms unique to this species.

MATERIALS AND METHODS

Animals and chemicals: Four healthy Holstein calves (1–8 weeks old) and one healthy Holstein cow (3 years old) were used in this study.

The following chemicals were purchased; Williams' medium E (WE), methionine-free Dulbecco's modified Eagle's medium (DME) (Gibco, Grand Island, NY, U.S.A.); fetal calf serum (FCS) (Flow Laboratories, McLean, VA, U.S.A.); streptomycin, penicillin (Meiji, Tokyo); fungizone (Bristol-Myers Squibb, Tokyo); phenylmethylsulfonyl fluoride (PMSF) (Nacalai Tesque, Kyoto); L-[³⁵S]-methionine (>37.0 TBq/mmol) (DuPont, Boston, MA, U.S.A.); rabbit anti-bovine albumin anti-serum (Daco Japan, Kyoto); protein A Sepharose FF (Pharmacia-LKB Biotechnology, Uppsala, Sweden); leupeptin, pepstatin (Peptide Institute, Osaka); pre-

stained SDS-PAGE standards (Bio-Rad, Richmond, CA, U.S.A.); nick-translation kit, hybridization transfer membranes (Hybond N) (Amersham International, Buckinghamshire, England); α -[32 P]-dCTP (>111 TBq/mmol) (Hungarian Academy of Sciences, Budapest, Hungary). Recombinant human (rh) IL-1 β (specific activity, 2×10^7 U/mg protein) was kindly provided from Dr. Y. Hirai (Otsuka Pharmaceutical, Tokushima), rhIL-6 (specific activity, 5×10^6 U/mg protein) from Dr. K. Yasukawa (Tosoh, Ayase), and rhTNF- α (specific activity, 2.55×10^6 U/mg protein) from Dr. M. Kitaura (Dainippon Pharmaceutical, Osaka). Collagen was prepared from rat tail tendon according to the method of Elsdale and Bard [9]. Rabbit anti-bovine Hp antiserum was obtained as previously described [16].

Preparation of bovine hepatocyte monolayers: Suspensions of bovine hepatocytes were prepared as previously described [18]. Cell viability was usually $> 90\%$, as assessed by trypan blue exclusion test. Parenchymal cells were easily distinguishable, because they were several fold larger than nonparenchymal cells, and their purity was calculated to be $> 95\%$.

The cells were suspended in a culture medium to give a cell density of 5×10^5 cells/ml. The culture medium was WE supplemented with 5% FCS, 1 μ M insulin, 10 μ M dexamethasone, 50 U/ml penicillin, 50 μ g/ml streptomycin, 0.25 μ g/ml fungizone, 1 mM CaCl_2 , and 1 mM MgCl_2 . Aliquots of 0.3 ml and 10 ml cell suspensions were seeded into 16 mm and 100 mm plastic tissue-culture dishes (Nunc, Roskilde, Denmark) coated with rat collagen and bovine fibronectin for the measurements of protein synthesis and mRNA levels, respectively. The dishes were incubated at 37°C in a humid atmosphere of 5% CO_2 in air for 3 hr, and then the medium was replaced by that lacking CaCl_2 and MgCl_2 . After confluent monolayers were formed by overnight incubation, the monolayers were rinsed once by a medium lacking dexamethasone and cultured for further 24 hr in the same medium, and used for following experiments of stimulation by cytokines.

Stimulation of hepatocytes: rhIL-6, rhIL-1 β and rhTNF- α were diluted in the culture medium immediately before use. Primary cultured hepatocytes were stimulated by various concentrations of rhIL-1 β , rhIL-6 and rhTNF- α for 24 hr, and then used for the measurements of protein synthesis and mRNA level.

Labeling of hepatocytes and immunoprecipitation of Hp and albumin: Isotope labeling of hepatocytes and immunoprecipitation of Hp and albumin were accomplished according to the procedure of Andus *et al.* [1] with some modifications. Briefly, [35 S]-methionine (300 kBq) was added to 0.3 ml methionine-free DME culture medium. After incubation at 37°C for 2 hr, 0.3 ml culture supernatant was separated from the cells by centrifugation at $12,000 \times g$ for 5 min. The proteins in the medium were precipitated with 5% trichloroacetic acid (TCA) containing 30 mg of carrier skim milk (Morinaga, Tokyo). The precipitate was washed three times with 5% TCA, and solubilized in 1 ml of solution A (0.1 M Tris-HCl, pH 7.6,

0.14 M NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS)) containing 1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml pepstatin, and 10 μ g/ml leupeptin, and its radioactivity was counted in a liquid scintillation counter. The solubilized sample containing 2×10^4 cpm in 1 ml was incubated with 5 μ l of a specific antiserum against bovine Hp or albumin at 4°C overnight. The antigen-antibody complexes were bound to 7 mg (dry mass) of protein A Sepharose FF, washed four times with solution A and twice with 50 mM sodium phosphate buffer, pH 7.5, and eluted by incubation with 20 μ l of 0.1 M Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 5% SDS and 10% glycerol at 95°C for 5 min. The eluted protein samples were subjected to 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) [13] and fluorography with Bio-Imaging Analyzer BAS1000 (Fuji Photo Film, Tokyo).

Preparation of cDNAs: A bovine liver cDNA library was prepared in pCDM8 vector according to Seed and Aruffo [23]. A bovine Hp cDNA clone (1.3 kbp) was isolated by screening the library with a specific DNA probe prepared by polymerase chain reaction (Morimatsu *et al.*, unpublished result). The cDNA clone was characterized by sequence determination and bacterial expression of their products followed by immunoblotting with specific antibodies and was used for preparing.

Isolation of RNA and Northern blot analysis: Total RNA was extracted from cells harvested from one dish by the method of Chomczynski and Sacchi [8] and glycogen contamination in the RNA pellet was diminished according to the procedure of Puissant and Houdebine [20]. Northern blot analysis was accomplished according to the standard protocols by Sambrook *et al.* [22]. Prior to transfer to nitrocellulose, total RNA, was denatured for 10 min at 65°C in 50% formamide, 2.2 M formaldehyde, and 1 \times running buffer (0.2 M MOPS, 50 mM sodium acetate, and 10 mM EDTA, pH 7.0), and separated on a 1.5% agarose gel containing 2.2 M formaldehyde and 1 \times running buffer. The membranes were prehybridized in 50% formamide, 0.1% SDS, 5 \times Denhardt's solution, 5 \times SSPE (1 \times SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4 , and 1 mM EDTA), and 100 μ g/ml of denatured salmon sperm DNA for 5–6 hr at 42°C. The hybridization was carried out in the same buffer containing the bovine Hp cDNA probe (1.0 kbp *Pvu*II fragment labeled by nick-translation kit) at 42°C overnight. The membranes were washed in 2 \times SSC containing 0.1% SDS at 42°C for 30 min followed by 0.1 \times SSC containing 0.1% SDS at 65°C for 60 min. The membranes were exposed to imaging plate for 2 hr and analyzed with Bio-Imaging Analyzer BAS1000.

Data analysis: Values were expressed as means \pm SE. Statistical analyses were carried out by analysis of variance (ANOVA).

RESULTS

Primary cultured bovine hepatocytes were incubated with various concentrations of cytokines for 24 hr and pulse-labeled with [35 S]-methionine for 2 hr. Figure 1

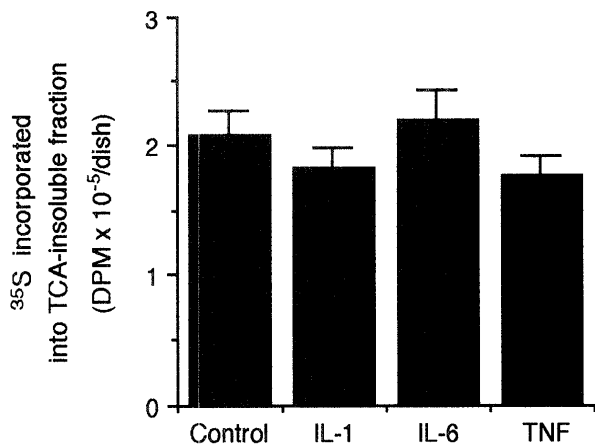


Fig. 1. Effects of rhIL-6, rhIL-1 β , and rhTNF- α on total protein synthesis in bovine primary cultured hepatocytes. Cultured hepatocytes were incubated in the presence of 10 U/ml rhIL-6, 100 U/ml rhIL-1 β , and 100 U/ml rhTNF- α . After incubation at 37°C for 24 hr, hepatocytes were labeled for 2 hr with 300 kBq [35 S]-methionine, and the incorporation of 35 S into TCA-insoluble fractions in the medium was measured. Values are the means \pm SE of four different experiments; *i.e.*, four hepatocyte preparations from three calves and one cow.

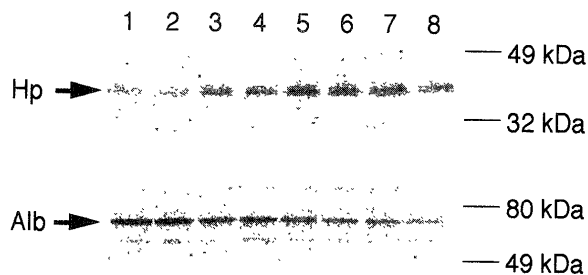


Fig. 2. Fluorograms of Hp and albumin synthesized by bovine primary cultured hepatocytes. Various concentrations of rhIL-6, 0 (lanes 1 and 2), 1 (lanes 3 and 4), 10 (lanes 5 and 6), and 100 (lanes 7 and 8) U/ml, were added to bovine primary cultured hepatocytes for 24 hr, and treated as Fig. 1. Hp and albumin were immunoprecipitated from the medium, subjected to SDS-PAGE and analyzed with Bio-Imaging Analyzer BAS1000 as described in "MATERIALS AND METHODS".

shows effects of rhIL-6, rhIL-1 β , and rhTNF- α on total protein synthesis; *i.e.*, 35 S-incorporation into TCA-insoluble fractions in the culture medium. Neither cytokine showed any significant stimulatory effect on total protein synthesis. To estimate Hp and albumin synthesis, these two proteins in the culture medium were immunoprecipitated and electrophoretically separated. Figure 2 shows a typical example of electrophoretograms. For Hp, as expected, SDS-PAGE gave a band of 35 kDa corresponding to β chain of bovine Hp. Alpha chain (20 kDa) was not detected because it did not contain methionine. For albumin, a band of 67 kDa was detected. It seems obvious that Hp synthesis was increased with increasing

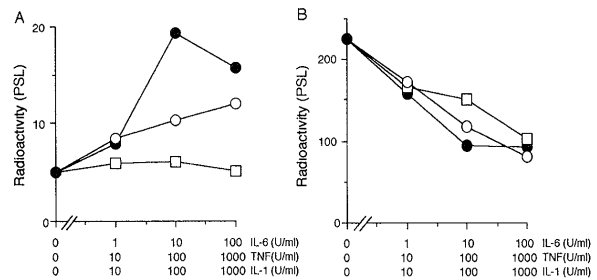


Fig. 3. Dose-response of Hp (A) and albumin (B) synthesis to increasing concentrations of rhIL-6, rhIL-1 β , and rhTNF- α in bovine primary cultured hepatocytes. Hepatocytes were incubated at various concentrations of rhIL-6 (●), rhTNF- α (○), and rhIL-1 β (□), and treated as in Fig. 2. Values are means for four different experiments. SE was less than 30% of the respective means.

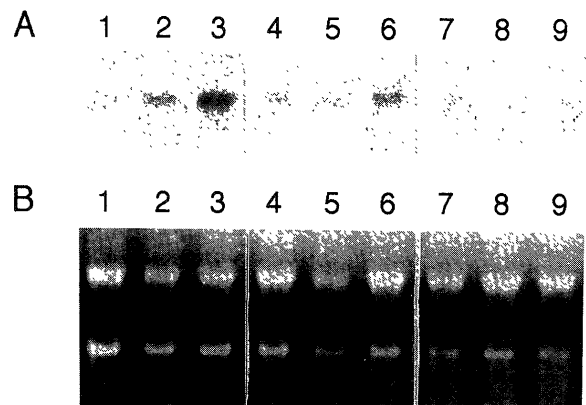


Fig. 4. Northern blot analysis of Hp mRNA in bovine primary cultured hepatocytes. Bovine primary cultured hepatocytes were incubated in the presence of 0 (lane 1), 1 (lane 2), 10 (lane 3) U/ml rhIL-6, 0 (lane 4), 10 (lane 5), 100 (lane 6) U/ml rhTNF- α , and 0 (lane 7), 10 (lane 8), 100 (lane 9) U/ml rhIL-1 β . After 24 hr incubation, total RNA (10 μ g/lane) was isolated from the cells, separated by electrophoresis in 1.5% agarose/formaldehyde gel, transferred to nylon membrane, and hybridized with a 32 P-labeled cDNA probe for Hp (A). B is ethidium bromide stain of the gel for A.

concentrations of rhIL-6, whereas albumin decreased. To analyze the results quantitatively, the radioactivity of each band was determined with a Bio-Imaging Analyzer. ANOVA revealed that rhIL-6 increased Hp synthesis ($P < 0.01$) and decreased albumin synthesis ($P < 0.01$) in dose-dependent manners (Fig. 3). rhTNF- α also stimulated Hp synthesis ($P < 0.01$), although the maximal level at 1,000 U/ml was about two thirds of that by 10 U/ml rhIL-6 (Fig. 3A). In contrast, rhIL-1 β showed no significant effect even at a dose of 1,000 U/ml. On the other hand, rhIL-1 β was as effective as rhIL-6 and rhTNF- α in suppressing albumin synthesis (Fig. 3B).

To determine whether the effects of cytokines are at the step of transcription, we measured mRNA level of Hp by

Northern blot analysis after stimulation of hepatocytes with the cytokines for 24 hr. As shown in Fig. 4, Hp mRNA level was increased by rhIL-6 and rhTNF- α , but not by rhIL-1 β .

DISCUSSION

In this study, we demonstrated that, in bovine primary cultured hepatocytes, Hp synthesis was increased by rhIL-6 and rhTNF- α , but not by rhIL-1 β . These effects are specific to Hp synthesis, since total protein synthesis was not changed by these cytokines. The stimulatory effects of cytokines were mainly pretranslational, because mRNA level of Hp changed in parallel with protein synthesis. These results are consistent with recent studies demonstrating that almost all APPs are transcriptionally regulated by cytokines through the activation of transcription factors such as NF-IL6, acute-phase response factor (APRF), and NF κ B [6, 14, 17, 24, 27].

It has been proposed that APPs can be classified into two major types: type 1 APP is regulated by IL-1 and TNF, whereas type 2 APP by IL-6 [4, 6]. For example, rat Hp is a type 1 APP while human Hp type 2 [3, 6, 7]. This classification is based on the difference in the responses to these cytokines of rodent and human APPs. In fact, it is known that IL-1 and TNF shared considerable overlap in their signal transduction mechanism of APP genes expression [2, 5, 6, 21]. In the present study, however, we demonstrated that bovine Hp belonged to neither type: that is, Hp synthesis in bovine hepatocyte primary cultures was increased by rhIL-6 and rhTNF- α , but not by rhIL-1 β . It is possible that rhIL-1 β is incapable of acting on bovine hepatocytes because of species specificity. However, the IL-1-specific signal pathways of our bovine hepatocytes were obviously activated during incubation with rhIL-1 β , because albumin synthesis was effectively suppressed by this cytokine. Thus, unlike in rodent or human hepatocytes, TNF and IL-1 certainly do not overlap in their signal transduction in bovine hepatocytes, at least in Hp gene expression. A similar dissociation between the responses to IL-1 and TNF was also suggested in complement component 3 and α_1 -acid glycoprotein expression in a rat hepatoma cell line [5]. Taken together, the classification of type 1 APP requires further careful examinations. Although the synthesis of bovine Hp was strongly stimulated by rhIL-6 as shown in this study, it is not an ordinal type 2 APP. TNF and IL-1 do not stimulate any type 2 APP gene in other species [10]. On the contrary, rhTNF- α , effectively stimulated the synthesis of bovine Hp. All these results collectively suggest that there is a species specific unique signaling system in TNF-induced transcriptional activation of bovine Hp gene.

In bovine, serum Hp level increases more than 100-fold by inflammatory stimuli or injection of rhIL-6 [15, 19, 26]. A maximal level appears in 24 hr after injection of turpentine oil, a typical inducer of inflammation [15]. A single injection of high doses of rhIL-6 induces a smaller response, whereas continuous infusion of low doses of

rhIL-6 produces a great response comparable to that after turpentine oil injection [19]. In consistent with these previous *in vivo* findings, the present study confirmed that rhIL-6 actually stimulated Hp synthesis in *in vitro* primary cultured bovine hepatocytes. However, the extent of increase in Hp synthesis by rhIL-6 *in vitro* was only several fold, being much less than that by *in vivo* inflammatory stimuli. These findings suggest that rhIL-6 alone is not enough to induce maximal Hp response and that the IL-6 action is mediated in part by some other factor(s). Although we found unexpectedly the unique stimulatory effect of rhTNF- α on *in vitro* Hp synthesis, the elevation was still small by rhTNF- α alone and even in combination with rhIL-6. Recent reports indicate that some growth factors such as transforming growth factor- β and hepatocyte growth factor modulate hepatic APP synthesis [5, 25], leading a suggestion that these and other unknown factors may influence Hp synthesis in bovine liver. Alternatively, it is also possible that the difference of the activity of *in vivo* and *in vitro* hepatocytes may be due to some changes of properties of the cultured cells. In fact, while Hp is undetectable in sera of normal healthy cows, considerable amount of Hp was secreted even in unstimulated control hepatocyte cultures. These problems make it difficult to compare directly *in vivo* and *in vitro* data. Further studies are required to identify factors contributing to *in vivo* Hp responses.

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