A new negatively regulated acute-phase phosphoprotein synthesized by rat hepatocytes

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(Received 23 January 1985/10 June 1985; accepted 2 July 1985)

The effect of acute inflammation on the production of the major phosphorylated protein (PP⁶³) excreted by rat hepatocytes was investigated. Both intra- and extra-cellular forms of the protein labelled with [³²P]P_i, [³H]fucose and [³⁵S]methionine were immunoprecipitated with monospecific polyclonal antibodies, and relative rates of PP⁶³ synthesis were measured. The hepatocytes of acutely inflamed rats produced and excreted 85% less ³²P- and ³H-labelled PP⁶³ than did control cells. This decreased amount of PP⁶³ did not result from an impairment in the phosphorylation or glycosylation processes or from a blockade in excretion, but rather was found to be due to extensive shut-off in biosynthesis of the protein as measured by [³⁵S]methionine incorporation. Thus PP⁶³ would appear to represent a new negatively regulated acute-phase protein.

A specific function of liver parenchymal cells is to synthesize and to secrete most plasma proteins (for review, see Crane & Miller, 1983). During acute inflammation, extensive changes in the hepatic synthesis and secretion of a group of proteins called acute-phase reactants have been shown to occur within hours (Koj, 1974; Kushner, 1982). In the rat, α_2 -macroglobulin, α_1 -acid glycoprotein, fibrinogen, haemopexin, transferrin, β -haptoglobin, α_1 -antichymotrypsin, α_1 -antitrypsin and α_1 -acute-phase globulin (i.e. α_1 -cysteine-proteinase inhibitor) represent major acute-phase proteins, all of which are increased to a variable extent during inflammation (Baumann *et al.*, 1983; Andus *et al.*, 1983).

We have recently discovered that rat hepatocytes secrete a major phosphorylated glycoprotein (PP⁶³) that displays strong similarities, with respect to M_r and charge, to several acute-phase proteins of the α -globulin family (e.g. α_1 -antichymotrypsin) (Le Cam *et al.*, 1985). In the present study we show that acute inflammation reduces by about 85% the production of PP⁶³ by hepatocytes, an effect accounted for by inhibition of its biosynthesis.

Abbreviations used: PP⁶³, major excreted 63 000- M_r phosphoprotein; SDS, sodium dodecyl sulphate.

Materials and methods

Materials

Radioactive L-[³⁵S]methionine (sp. radioactivity 1160 Ci/mmol) was purchased from New England Nuclear; [³H]fucose (sp. radioactivity 27 Ci/mmol) and [³²P]P_i (carrier-free) (sp. radioactivity 5000 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Triton X-405 was from Fluka, phenylmethanesulphonyl fluoride from Sigma and protein A-Sepharose CL-4B from Pharmacia.

Methods

Cell preparation. Hepatocytes were isolated from adult male rats (150–200 g) fed *ad libitum* by using the procedure previously described (Le Cam *et al.*, 1976). Inflammation was induced by a single subcutaneous injection of turpentine (0.5 ml/100 g) given 24 h before cell isolation. Cell-suspension viability ranged from 90 to 95% and was similar for control and treated animals.

Labelling of cells and cell-extract preparation. Hepatocytes at $(1.5\text{--}2) \times 10^6/\text{ml}$ were incubated in suspension at 37°C in an amino-acid-rich medium (Salavert & Iynedjian, 1982) devoid of phosphate and of L-methionine. The concentrations of radioactive precursors used were: [³²P]P_i, 400 $\mu\text{Ci/ml}$; [³⁵S]methionine, 250 $\mu\text{Ci/ml}$; and [³H]fucose,

100 μ Ci/ml. Incubations were stopped by centrifuging the suspensions at 800g for 10 s to separate cells from the medium. Cell pellets were washed twice in chilled buffer containing unlabelled phosphate (20 mM) and L-methionine (5 mM). Hepatocytes were then resuspended in 250 μ l of homogenization buffer containing 10 mM-Tris/HCl (pH 7.4), 4 mM-EDTA, 100 mM-Naf (a phosphoprotein phosphatase inhibitor), 0.5 mM-phenylmethanesulphonyl fluoride and 1% Triton X-405, and disrupted by three cycles of freezing and thawing. Cellular homogenates were centrifuged for 15 min at 4°C at 10000g and the supernatants taken up and used as a source of intracellular material.

Indirect immunoprecipitation of PP⁶³ and gel electrophoresis. Details of the preparation and characterization of the antiserum to the major (63000- M_r) excreted phosphoprotein are described elsewhere (Le Cam *et al.*, 1985). This antiserum is monospecific and neutralizes more than 90% of ³²P-labelled PP⁶³ present in incubation media, when used at a 1:50 dilution. Indirect immunoprecipitation of PP⁶³ was carried out by the procedure designed to precipitate the specific liver enzymes phosphoenolpyruvate carboxykinase and tyrosine aminotransferase (Iynedjian *et al.*, 1985), except that the preadsorption step was omitted. Briefly, portions (100 μ l) of incubation media supple-

mented with 0.5 mM-phenylmethanesulphonyl fluoride and 0.6% Triton X-405, or of intracellular cell extract (50 μ l containing 200–300 μ g of proteins), were incubated overnight at 4°C in the presence of 2 μ l of immune serum. Immune complexes were then adsorbed on protein-Sepharose for 3 h at 4°C and the beads were washed four times with phosphate-buffered saline containing 0.6% Triton and three times with the same buffer without Triton. Elution of immune complexes was achieved by heating the beads at 100°C for 10 min in electrophoresis sample buffer containing SDS (3% w/v) and β -mercaptoethanol. Proteins specifically immunoprecipitated and the total proteins present in the cellular extracts and in the media were resolved by one-dimensional polyacrylamide-gel electrophoresis as described by Laemmli (1970). Gels were processed for autoradiography (³²P-labelling) or fluorography (³H and ³⁵S labelling) as reported elsewhere (Iynedjian *et al.*, 1985). Total protein synthesis was quantified by measuring the radioactivity precipitated with 10% (w/v) trichloroacetic acid (Iynedjian *et al.*, 1985). Analysis of the acid-soluble material by SDS/polyacrylamide-gel electrophoresis showed that more than 90% of the ³⁵S-, ³H- and ³²P-labelled proteins were precipitated by the treatment (results not shown). The protein contents of cellular extracts were determined by using the Bio-Rad Assay Kit II.

Fig. 1. Autoradiograms (a) and fluorograms (b) showing the total proteins and the immunoprecipitable PP⁶³ synthesized by hepatocytes from control and 24 h-turpentine injected rats

Cells were labelled with [³²P]P_i (tracks 1–8) and with [³H]fucose (tracks 9–16) for 75 min. To analyse total proteins, 25 μ l of cellular extracts containing 75 μ g of protein (tracks 1, 3, 9, 11) and 50 μ l of incubation media (tracks 5, 7, 13, 15) were diluted in electrophoresis sample buffer and applied on to a 10%-polyacrylamide/SDS gel. Immunoprecipitable intracellular (tracks 2, 4, 10, 12) and extracellular (tracks 6, 8, 14, 16) materials corresponded to twice the volume of those utilized for the direct analysis. The asterisks on the right of track numbers refer to material from inflamed-rat cells. M_r markers were serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000) and lysozyme (14300). Abbreviation used: OR, origin.

Results

Analysis of intracellular and extracellular immunoprecipitable ^{32}P - and ^3H -labelled PP^{63} in hepatocytes from control and inflamed rats

Besides other phosphorylated proteins, the incubation media from control cells contained a major ^{32}P -labelled protein with apparent M_r 63 000 (Fig. 1a, track 5). This phosphoprotein was specifically recognized by PP^{63} antiserum (track 6). In contrast, very little of this phosphoprotein was present in the media of hepatocytes from inflamed rats, as shown by direct analysis (track 7) or after immunoprecipitation (track 8). However, the other ^{32}P -labelled extracellular proteins from control cells (track 5) were also present in comparable amounts in the medium from inflamed rat hepatocytes (track 7). These phosphoproteins most likely corresponded to intracellular material that leaked out of damaged or dead cells during incubation.

In cellular extracts from control cells, small

amounts of ^{32}P -labelled PP^{63} (about 10% of that found outside) were also immunoprecipitated (track 2). Comparatively, the quantity of immunoreactive PP^{63} contained in cellular extracts prepared from inflamed rats was nearly unmeasurable (track 4). This suggests that the dramatic decrease in the amounts of PP^{63} found in the incubation medium did not result from blockade of its excretion. This decrease was also unlikely to be due to non-specific impairment of the phosphotransferase activity, since the groups of ^{32}P -labelled proteins were quantitatively and qualitatively very similar in both types of hepatocytes (tracks 1 and 3).

The same analysis was carried out with cells biosynthetically labelled with [^3H]fucose and gave similar results (Fig. 1b). The amounts of fucosylated protein specifically immunoprecipitated with PP^{63} antibodies present in the incubation medium from inflamed hepatocytes (track 16) was less than 10% of that found in control medium (track 14). The ^3H -labelled PP^{63} was also found in small amounts in the intracellular material from normal cells (track 10) (the same proportion of the amount excreted as for the ^{32}P -labelled PP^{63}), whereas the protein was virtually undetectable in cellular extracts from turpentine-injected rats (track 12). This decrease in production of the fucosylated PP^{63} could not result from an alteration of the cell's overall glycosylation capacity, since [^3H]fucose incorporation, which represents a terminal step in the processing of many glycoproteins (see, for review, Hubbard & Ivatt, 1981), was similar in hepatocytes from acutely inflamed rats (tracks 11 and 15) and in cells from control rats (tracks 9 and 13).

Total and specific PP^{63} protein synthesis in hepatocytes from control and inflamed rats

The capacity of both types of cells to incorporate [^{35}S]methionine into proteins and into material specifically recognized by PP^{63} antiserum was first studied after a short labelling period (20 min) that allowed measurement of synthesis rates. After this 20 min incubation only intracellular material was labelled. The amount of trichloroacetic acid-precipitable radioactivity (total protein synthesis) was 40% higher in inflamed-rat hepatocytes than in cells from control animals (Fig. 2a). In contrast, ^{35}S -labelled polypeptides reacting with the PP^{63} antibodies, which represented 0.8% of total synthesis in control cells, contributed only 0.2% to the overall synthesis in hepatocytes from turpentine-treated rats (Fig. 2b). The same observation was seen after a 75 min labelling period. A difference of approx. 15% between control- and inflamed-rat cells was observed for intracellular and for excreted proteins (Fig. 2a). The excreted proteins represented 30–40% of the newly synthesized proteins in

Fig. 2. Quantitative analysis of total and specific PP^{63} synthesis in hepatocytes from control and inflamed rats. Cells were labelled for 20 min and 75 min with [^{35}S]methionine and measurements of total and PP^{63} synthesis were performed (see the Materials and methods section). Values obtained for immunoprecipitation of PP^{63} were corrected for non-specific adsorption (less than 10%) determined in parallel experiments with non-immune serum. Total intracellular and extracellular protein synthesis (a) were both expressed per mg of protein contained in cellular extracts. Specific PP^{63} synthesis (b) was expressed as a percentage of total protein synthesis in each individual fraction (intracellular and extracellular). The values represent the means of triplicate determinations. □, Control; ■, inflamed.

Fig. 3. Fluorograms showing immunoprecipitable [^{35}S]methionine-labelled intracellular and excreted proteins synthesized by hepatocytes from control and inflamed rats

Incubation conditions and immunoprecipitation of PP^{63} were performed as described in Fig. 2, and gel analysis was as in Fig. 1. Fluorograms were all obtained after a 72 h exposure of the film at -80°C . Thus the differences in intensity of the different bands can be directly compared, and the difference in the production of PP^{63} by hepatocytes from control (tracks 1, 3 and 5) and inflamed (tracks 2, 4 and 6, marked with an asterisk) cells is clearly seen.

both cell types (Fig. 2a). The relative amount of intracellular immunoreactive material found in hepatocytes from inflamed rats was decreased in the same proportion after a 75 min (0.25% of total) as after a 20 min labelling period, as compared with the 75 min control (0.9% of total) (Fig. 2b). This difference was even more pronounced for the extracellular immunoreactive material, which represented about 1.9% of total excreted proteins in control cells, but only 0.3% in hepatocytes from turpentine-treated rats (Fig. 2b). Thus the specific PP^{63} synthesis was reduced by about 85% in inflammation (1.25% versus 0.20% of total synthesis). To rule out the possibility that this decrease in the production of ^{35}S -labelled PP^{63} resulted from the synthesis of an abnormal protein that would be weakly or not at all recognized by PP^{63} antibodies, we analysed the immunoprecipitable material by one-dimensional gel electrophoresis. The autoradiograms shown in Fig. 3 demonstrate that the molecular species present in both types of cells were similar, the differences being only quantitative. In intracellular cell extracts (tracks 1–4) the antiserum precipitated two major polypeptides: a 55000- M_r species (the more abundant) and a 52000- M_r species. After a 75 min labelling period, a small quantity of mature 63000- M_r protein was also detected in control cells (track 3). The antibodies reacted with two proteins present in the incubation media: a 63000- M_r species that corresponded to the mature PP^{63} protein and a 55000- M_r species that might correspond to the major intracellular precursor (tracks 5 and 6).

Discussion

The nature of the stimuli responsible for changes in rates of liver protein synthesis in acute inflammation remains poorly understood (Baumann *et al.*, 1984; Gross *et al.*, 1984). A striking feature of the

inflammatory response resides in the fact that plasma levels of most acute-phase proteins synthesized by liver are increased. The converse situation (i.e. decreased concentration in blood) appears much more uncommon, since only albumin (Koj, 1980) and $\alpha_2\text{-}\mu$ -globulin (Baumann *et al.*, 1983) have been identified as negatively regulated acute-phase proteins. The present study revealed the existence of an as-yet-unrecognized negatively regulated rat acute-phase protein, namely the major 63000- M_r phosphorylated glycoprotein produced by hepatocytes. As demonstrated for many acute-phase reactants (Schreiber *et al.*, 1982; Jamieson *et al.*, 1972; Koj, 1974), the mechanism underlying the decrease observed in production of this phosphoprotein in hepatocytes from inflamed rats involved mainly an extensive biosynthesis shut-off. Indeed, not only did cells from turpentine-injected animals produce considerably less mature PP^{63} , but they also synthesized less of the precursor forms. This was observed after a long labelling period, but, more importantly, after a short labelling period during which protein degradation is minimal and therefore cannot significantly account for lower rates of precursor-polypeptide synthesis. It has been demonstrated that alterations in the rates of hepatic synthesis of several acute-phase proteins (e.g. α_1 -acid glycoprotein, α_2 -acid glycoprotein, α_2 -macroglobulin, transferrin, α_1 -proteinase inhibitor, β -hepatoglobulin and haemopexin) induced by inflammation is accounted for by changes in levels of translatable mRNA (Chandra *et al.*, 1981; Northemann *et al.*, 1983; Baumann *et al.*, 1983). Whether this will hold for the negatively regulated PP^{63} rat protein remains to be investigated.

We thank Mr. G. Visciano for art work, Miss A. Rainaud for typing the manuscript and Dr. M. Fehlmann and Dr. D. Vesely for criticisms and advice.

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