The influence of glucocorticoid on the fibrinogen messenger RNA content of rat liver *in vivo* and in hepatocyte suspension culture

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The plasma concentration of fibrinogen, one of the major acute-phase proteins produced by the liver, increases during the acute-phase response as a result of enhanced synthesis in liver. Since adrenal-cortical hormones have been thought to have a key role in the regulation of the fibrinogen synthesis, fibrinogen-polypeptide mRNA sequences were determined in the present study, by using a specific complementary-DNA probe, in RNA fractions obtained from rat hepatocytes exposed to glucocorticoids *in vitro* (hepatocyte suspension cultures) and *in vivo*. Maximal induction of the fibrinogen-polypeptide mRNA (to 400% of the control value) was found *in vitro* at 0.1 μM -dexamethasone after 9 h of incubation. The same magnitude of induction was obtained with 20 μM -cortisol or 60 μM -corticosterone. In contrast with the findings *in vitro*, no induction of the fibrinogen-polypeptide mRNA was observed in the liver at various times after injection of different doses of glucocorticoids into rats. These results suggest that more complex regulatory mechanisms are involved and that glucocorticoids are not the sole regulatory factors *in vivo* in the enhanced synthesis of fibrinogen during the acute-phase response.

In response to physical trauma, infection or inflammation, a co-ordinated sequence of alterations in the concentrations of specific plasma proteins occurs. This response is associated with metabolic changes, which together are referred to as the acute-phase reaction. Fibrinogen or coagulation factor I, one of the major acute-phase proteins produced by the liver, has been investigated intensively in the last few years. It is a glycoprotein and consists of a dimeric molecule of three non-identical polypeptides, namely 2A α , 2B β and 2 γ chains, of M_r 61 000 (A α), 58 000 (B β) and 51 000 (γ) respectively in the rat (van Ruyven-Vermeer & Nieuwenhuizen, 1978; Nickerson & Fuller, 1981). Although it has been documented that the plasma

concentration of fibrinogen increases during the acute-phase response as a result of enhanced synthesis in liver (Weimer & Humelbaugh, 1967; Kampschmidt & Upchurch, 1974; Koj, 1974), little is known about the molecular regulatory mechanism of the fibrinogen biosynthesis in the liver. Previously, using a cDNA probe specific for fibrinogen-polypeptide mRNA, we have demonstrated that the regulation of fibrinogen-polypeptide synthesis *in vivo* after inflammation (Princen *et al.*, 1981b), after partial hepatectomy and surgical trauma (Princen *et al.*, 1982), or in developing organs (Selten *et al.*, 1982), occurs via modulation of the contents of mRNA species coding for fibrinogen polypeptides. Similar findings were also reported after defibrination of rats with Malayan-pit-viper venom (Crabtree & Kant, 1982).

Although the precise molecular mechanisms that bring about the dramatic increase in the production of fibrinogen and the increase in fibrinogen-polypeptide mRNA are still not known, numerous studies showed that a variety of hormones (Crane & Miller, 1977; Grieninger *et al.*, 1978; Hertzberg *et al.*, 1981; Liang & Grieninger,

Abbreviations used: cDNA, complementary DNA; R_0t , the product of initial RNA concentration (mol of nucleotides/litre) and time (s) (assuming that A_{260} of 1.0 corresponds to 40 μg of RNA/ml); R_0t_{50} , the R_0t value at 50% maximal hybridization; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

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1981), fibrinogen degradation products (Barnhardt *et al.*, 1970; Franks *et al.*, 1981; Fuller *et al.*, 1982) and certain leucocytic products (Kampschmidt *et al.*, 1982) may play an important role in the hepatic regulation of fibrinogen synthesis. However, considerable controversy exists as to the result and to the mechanism by which such substances exert their influences on the fibrinogen synthesis.

Investigations on the effect of corticosteroids on the fibrinogen synthesis have been pursued in the intact animal (McKenzie & Fowler, 1968; Cain *et al.*, 1970; Jeejeebhoy *et al.*, 1972), in isolated perfused liver (John & Miller, 1969), in suspensions of hepatocytes (Crane & Miller, 1977) and in monolayer hepatocyte cultures (Griener *et al.*, 1978). In the present study, we report the effects of these hormones on the synthesis in liver of fibrinogen at the level of their specific mRNA species in hepatocyte suspension cultures and in intact animals. The results indicate that there is a differential regulation of the fibrinogen synthesis in isolated hepatocytes and in the liver of intact rats.

Materials and methods

Materials

L-[4,5-³H]Leucine (76 Ci/mmol) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Collagenase type I, soya-bean trypsin inhibitor, bovine serum albumin, heparin from pig intestinal mucosa, dithiothreitol and Hepes were purchased from Sigma. Joklik-modified Minimum Essential Medium (MEM) was from Gibco and foetal bovine serum was from Flow Laboratories. All salts and solvents were purchased from E. Merck. Oradexon (dexamethasone disodium phosphate) and dexamethasone were obtained from Organon, Oss, The Netherlands. Cortisol and corticosterone were from Steraloids, Wilton, NH, U.S.A.). All glassware was sterilized, and solutions were freshly prepared and autoclaved, before use. Bovine serum albumin and soya-bean trypsin inhibitor were dialysed against 0.15 M-NaCl and all solutions used in the hepatocyte isolation and culture were filtered through a 0.22 µm Millipore filter and stored frozen.

Animals

Male Wistar rats weighing 160–220 g were used throughout and maintained on standard Purina Chow and water *ad libitum*. Animals were injected with corticosteroids and hepatocytes were always prepared at 09:00 h.

Hepatocyte preparation and incubation

Liver cells were prepared as described by Seglen (1976), with the following modifications. All

buffers were supplemented with 100 units of penicillin and 100 µg of streptomycin/ml and were oxygenated by direct bubbling with oxygen. Collagenase buffer (120 ml, 0.5 mg/ml) was supplemented with 6 mg of soya-bean trypsin inhibitor (Crane & Miller, 1977). After 25 min perfusion the Glisson capsules were removed in 100 ml of (Ca²⁺ and Mg²⁺)-free Hanks' balanced salt solution containing 2% (w/v) bovine serum albumin. Cells were filtered, preincubated and centrifuged (50 g, 5 min) in the same buffer to remove damaged and non-parenchymal cells. After centrifugation the pellet was resuspended in fresh Joklik-modified MEM, containing 25 mM-Hepes (pH 7.5), 0.1 mg of heparin/ml and 10% (v/v) foetal-calf serum. Cells were counted, and viability was determined by Trypan Blue exclusion. In a typical experiment 15 × 10⁶ hepatocytes were incubated in 10 ml of suspension at 37°C in 50 ml polypropylene Erlenmeyer flasks with gentle rotational shaking (90 rev./min) under O₂/CO₂ (19:1). Freshly prepared hormones and labelled precursors were added as concentrated stock solutions. After incubation, cell suspensions were quickly chilled and centrifuged (50 g, 5 min). The cell pellets were frozen in liquid N₂ for subsequent analysis.

Incorporation of labelled precursor into protein

[³H]Leucine (5 µCi/ml) was added to the cell suspension. At the indicated times, duplicate 50 µl samples were withdrawn and spotted on 2.3 cm Whatman 3 MM filter-paper discs, which were immersed in cold 10% trichloroacetic acid containing 5 mM-leucine. Filters were washed to remove free labelled precursor as described (Mans & Novelli, 1960). Protein precipitates were solubilized from the filters by treatment with 2 × 1 ml of 30% H₂O₂/35% NH₃ solution (17:3, v/v) for 10 h at 55°C, and counted for radioactivity in 10 ml of Aqualuma Plus (Lumac), giving a counting efficiency of 41%. Cell protein was determined by the method of Lowry *et al.* (1951).

Immunoprecipitation of ³H-labelled fibrinogen

³H-labelled fibrinogen secreted in the incubation medium was determined as described previously, with minor modifications (Princen *et al.*, 1981a); 10 mM-leucine, 0.1 mM-phenylmethanesulphonyl fluoride and 1 mM-EDTA were added to the immunoprecipitation mixture. ¹⁴C-labelled fibrinogen was prepared chemically from the purified proteins with [¹⁴C]formaldehyde by reductive methylation as described by Crane & Miller (1975).

Preparation of liver and cell RNA

Total post-nuclear liver RNA was prepared as described by Taylor & Schimke (1973). Cell pellets

were quickly thawed and homogenized in a buffer containing 50mM-Tris/HCl, pH 7.4, 25mM-NaCl, 5mM-MgCl₂, 0.25M-sucrose and 0.3mg of heparin/ml. After addition of 0.1 vol. of 10% Triton X-100 and removal of the particulate material by centrifugation for 10min at 16000g, RNA was extracted by addition of 0.5 vol. of 0.3M-NaCl/1.5% SDS/15mM-EDTA/30mM-Tris/HCl, pH 9.0, and an equal volume of phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.). When no detectable interface remained, 2.5 vol. of cold ethanol was added. RNA was precipitated overnight at -20°C and isolated by centrifugation at 16000g for 20min at -5°C.

Preparation of purified mRNA and ³H-labelled cDNA

Purification of mRNA species specific for fibrinogen polypeptides and the synthesis of the specific cDNA probes from these purified mRNA species have been reported previously (Strair *et al.*, 1977; Princen *et al.*, 1981b, 1982). Analytical RNA-cDNA hybridization was performed as described (Princen *et al.*, 1982; Selten *et al.*, 1982).

Results

Characteristics of the hepatocyte suspension culture

To study the effects of glucocorticoids on the synthesis of fibrinogen and on the amount of its mRNA in hepatocyte culture, it is necessary to characterize the capabilities of the hepatocytes in this system to synthesize RNA and protein. A high yield of isolated liver parenchymal cells (50×10^6 – 80×10^6 cells/g of liver) was obtained by the isolation technique in our present study. The cells were morphologically intact, showing a viability, as determined by Trypan Blue exclusion, higher than 90%. After 22h of incubation, 75–80% of the hepatocytes was still viable. Isolated liver cells incorporated labelled precursor into hepatic RNA linearly with time (results not shown). Incorporation of [³H]leucine into total hepatic protein continued at an approximately linear rate for over 22h (Fig. 1). Newly synthesized proteins were actively secreted into the medium throughout this period.

Glucocorticoid induction of fibrinogen synthesis and the fibrinogen-polypeptide mRNA content in isolated hepatocytes

When the synthetic glucocorticoid dexamethasone disodium phosphate (0.48 μ M) was added to the culture medium, the synthesis of total protein, as a reflection of the synthesis of secretory proteins, was stimulated (Fig. 1). There was also a marked stimulation of fibrinogen synthesis by glucocorticoid (Fig. 2). After a lag period of 6h,

Fig. 1. Incorporation of [³H]leucine into total and secreted protein of hepatocytes in suspension cultures

Hepatocytes (1.5×10^6 cells/ml) were incubated for 22h in the presence of 5 μ Ci of [³H]leucine/ml. At 3h intervals, total (●) and secreted (▲) protein was measured. The effect of 0.48 μ M-dexamethasone disodium phosphate on protein synthesis by these cells was also determined: total (○) and secreted (△) protein. Each point on the curves is the average value obtained from cell preparations of three different rats. Protein content was 1.20mg/10⁶ hepatocytes.

fibrinogen synthesis increased very rapidly, to 300% of the control value at 22h of incubation. Fibrinogen comprised 4.2% of the secreted proteins during the first 15h of incubation, but decreased to 3.4% after 22h. Addition of 0.48mM-dexamethasone at the beginning of incubation increased fibrinogen synthesis to 8.1% of the secreted proteins at 22h of culture.

In an attempt to gain some insight into the biochemical processes by which glucocorticoids enhance the fibrinogen synthesis, the content of fibrinogen-polypeptide mRNA in these hepato-

Fig. 2. Secretion of fibrinogen by isolated rat hepatocytes. The incubation conditions were as described in Fig. 1. At the indicated times, samples were withdrawn and portions of the medium were frozen until assayed. Fibrinogen was quantitatively determined by duplicate immunoprecipitations from proteins secreted in the culture medium. Fibrinogen secretion in the presence (△) and in the absence (▲) of $0.48 \mu\text{M}$ -dexamethasone disodium phosphate was examined.

cytes was quantified by molecular hybridization with cDNA probes specific for fibrinogen-polypeptide mRNA. Fig. 3 shows some representative results of the hybridization kinetics of various RNA fractions prepared from hepatocyte suspension cultures. When the purified fibrinogen-polypeptide mRNA species were hybridized in RNA excess to their own cDNA probes, a R_{0t_1} value of $4.47 \times 10^{-3} \text{ mol} \cdot \text{l}^{-1} \cdot \text{s}$ was found (Princen *et al.*, 1981b, 1982). By comparing the R_{0t_1} values of various RNA fractions with that of the purified fibrinogen-polypeptide mRNA species, it was calculated that fibrinogen-polypeptide mRNA in control rats comprised 0.056% of the total RNA or 1.6% of the poly(A)-containing RNA. These values were also found with the RNA fraction from freshly prepared hepatocytes ($R_{0t_1} 7.94 \text{ mol} \cdot \text{l}^{-1} \cdot \text{s}$). After a single dose of $0.48 \mu\text{M}$ -dexamethasone disodium phosphate at zero time to a hepatocyte suspension culture, a continuous decrease in the R_{0t_1} value was obtained, with the maximal effect after 9 h of incubation ($R_{0t_1} 2.00$). The time course for the elevation of the fibrinogen-polypeptide mRNA content by dexamethasone disodium phosphate was studied, as shown in Fig. 4. After a lag of

Fig. 3. Hybridization kinetics of RNA fractions isolated from rat hepatocytes in suspension cultures in the presence and absence of dexamethasone disodium phosphate. $[^3\text{H}]$ cDNA specific for fibrinogen-polypeptide mRNA were hybridized to purified fibrinogen-polypeptides mRNA (▼), to RNA isolated from hepatocytes immediately after preparation (○), to RNA isolated from hepatocytes after 6 h (▲) or 9 h (■) of incubation in the presence of $0.48 \mu\text{M}$ -dexamethasone disodium phosphate. Each hybridization curve was the average result of at least three experiments.

approx. 3 h, an increase in mRNA was observed, to a maximum (400% of the initial value) after 9–10 h of exposure to dexamethasone. A second addition of $0.48 \mu\text{M}$ -dexamethasone led to persistence of the maximal value for a longer period. When glucocorticoid was omitted, the fibrinogen-polypeptide mRNA content was constant for 9–12 h. However, there was a gradual decrease in the mRNA content to 50% of the control value at 22 h. Despite these findings, the hepatocytes cultured for 9 h were still able to synthesize fibrinogen-polypeptide mRNA at a maximal rate in response to a delayed dexamethasone exposure. We can therefore conclude that these hepatocytes were still very viable and metabolically active in the observed experimental period.

The relationship between steroid concentration and degree of stimulation of the fibrinogen-polypeptide mRNA content is demonstrated in Fig. 5. The effect of dexamethasone disodium phosphate was compared with that of the naturally occurring glucocorticoids, cortisol and corticosterone. A significant increase in the fibrinogen-polypeptide mRNA content was detectable at 20 nM and reached a maximum (400% of the initial control value) at $0.1 \mu\text{M}$ -dexamethasone disodium phosphate. Similar findings were obtained with pure dexamethasone (instead of Oradexon, dexamethasone disodium phosphate). In these experiments, however, no induction of fibrinogen-polypeptide mRNA was found with 17-oxo-dexa-

Fig. 4. Time course of the fibrinogen-polypeptide mRNA induction in the presence of dexamethasone disodium phosphate

The relative changes in fibrinogen-polypeptide mRNA contents were determined from the representative hybridization data as partly shown in Fig. 3 by comparison of the R_{0t} value of RNA isolated from hepatocytes immediately after preparation with the R_{0t} values obtained from RNA of cells after various time intervals of incubation. The mRNA content was investigated in the absence (Δ — Δ) or in the presence ($\bullet$ — $\bullet$) of $0.48 \mu\text{M}$ -dexamethasone disodium phosphate at the beginning of the incubation or after 9 h of incubation ($\circ$ — $\circ$); addition of a second dose of $0.48 \mu\text{M}$ -dexamethasone disodium phosphate after the initial addition at zero time ($\bullet$ — $\bullet$) was also examined.

Fig. 5. Dose-response of fibrinogen-polypeptide mRNA induction by corticosteroids

Values were obtained as described in Fig. 4; 1.5×10^6 cells/ml were incubated for 9 h in the presence of various concentrations of dexamethasone disodium phosphate or dexamethasone ($\bullet$), cortisol (Δ), or corticosterone ($\blacksquare$).

methasone, an oxidation product of dexamethasone.

The maximal stimulation of the fibrinogen-polypeptide mRNA content in the experiments using the naturally occurring glucocorticoids was achieved with a concentration of $20 \mu\text{M}$ -cortisol or $60 \mu\text{M}$ -corticosterone, the native corticosteroid of the rat.

Effect of glucocorticoid given *in vivo* on the hepatic content of fibrinogen-polypeptide mRNA

To examine the possible role of glucocorticoid in the regulation of fibrinogen synthesis, it is necessary to investigate the effect of this hormone in intact animals. Knowing that dexamethasone is able to induce the fibrinogen-polypeptide mRNA content in hepatocytes *in vitro* and that there are controversies about the influence of glucocorticoids on the plasma content and the synthesis of fibrinogen *in vivo* (McKenzie & Fowler, 1968; Cain *et al.*, 1970; Jeejeebhoy *et al.*, 1972; Seligsohn *et al.*, 1973; Miller, 1975; Majumdar & Jørgensen, 1977; Gordon & Limaos, 1979; Crabtree & Kant, 1982),

Fig. 6. Hybridization kinetics of RNA fractions isolated from rat liver after injection with corticosteroids

$[^3\text{H}]$ cDNA species specific for fibrinogen-polypeptide mRNA were hybridized to RNA isolated from livers of control rats ($\circ$ — $\circ$), or to RNA isolated after various time intervals from livers of rats injected with different amounts of corticosteroids ($5 \mu\text{g}$ – 10mg) ($\bullet$ — $\bullet$). For comparison, the hybridization kinetics of fibrinogen-polypeptide $[^3\text{H}]$ -cDNA with the total cellular RNA from hepatocytes isolated 9 h after incubation in the presence of $0.48 \mu\text{M}$ -dexamethasone disodium phosphate ($\bullet$ — $\bullet$) and with the total liver RNA isolated from rats 24 h after turpentine injection ($\blacktriangle$ — $\blacktriangle$) (Princen *et al.*, 1981b) are shown.

we decided to examine the hepatic contents of fibrinogen-polypeptide mRNA in intact rats after dexamethasone administration. After injection of this synthetic hormone either intraperitoneally or intravenously into the animals with a dose ranging from 5 μ g to 10 mg per rat, there was no detectable change in fibrinogen-polypeptide mRNA content up to 24 h, as compared with findings in control animals (Fig. 6). In addition, no induction of fibrinogen mRNA was observed after repeated injections with 10 mg of dexamethasone at 6, 9–16 and 21 h after the initial injection (10 mg). These findings show that glucocorticoids given *in vivo*, in sharp contrast with the findings *in vitro*, are not able to increase the content of fibrinogen-polypeptide mRNA in the liver.

Discussion

An elevated plasma concentration and an enhanced hepatic synthesis of fibrinogen have been observed after exposure to a variety of noxious stimuli or tissue injuries (Weimer & Humelbaugh, 1967; Kampschmidt & Upchurch, 1974; Koj, 1974). Since the plasma concentrations of the adrenal-cortical hormones are increased in all these 'stress' conditions, these hormones have been thought to have a key role in the regulation of the fibrinogen synthesis. To investigate the possible stimulatory effect of glucocorticoids in the genesis of the acute-phase fibrinogen response, two experimental approaches have been used by different groups of investigators. In the first, glucocorticoids have been administered *in vivo* and *in vitro* with the aim of stimulating fibrinogen synthesis. In the other approach, it was determined whether adrenalectomy prevents an increase in fibrinogen synthesis caused by inflammatory stimuli known to be effective in control experiments. By using these approaches in the isolated perfused rat liver (John & Miller, 1969), in suspensions of hepatocytes (Crane & Miller, 1977) or in monolayer cultures (Grieninger *et al.*, 1978), the stimulatory effect of glucocorticoids on the fibrinogen synthesis has been reported. In the present study, we have demonstrated that the enhanced synthesis of fibrinogen in a hepatocyte suspension culture after exposure to glucocorticoids is regulated at the mRNA level as found in intact animals during the acute-phase response (Princen *et al.*, 1981, 1982). Whether this increase in mRNA is caused by increased transcription or mRNA stabilization remains to be determined.

There is a substantial degree of controversy in the literature about the effect of glucocorticoids on fibrinogen synthesis in intact animals, with or without adrenalectomy. Several investigators presented evidence that glucocorticoids are able to

stimulate (Cain *et al.*, 1970; Jeejeebhoy, 1972) or have only a limited 'permissive' role in the stimulation of the fibrinogen synthesis (Miller, 1975; Crabtree & Kant, 1982).

In the present experiments, glucocorticoids may influence the synthesis of fibrinogen by more than one mechanism. As *in vitro*, these hormones may exert their effects directly on the liver cells by affecting one or more of the many subcellular processes involved in protein synthesis. Alternatively, the changes in fibrinogen synthesis could be induced indirectly, occurring secondarily to the effects of these hormones on the metabolic pathway of other organs or tissues. In our present study we demonstrated that there was no detectable increase in the fibrinogen-polypeptide mRNA content after dexamethasone exposure *in vivo*. These contradictory findings from studies *in vivo* and *in vitro* suggest that there is a differential effect of glucocorticoids on fibrinogen synthesis in hepatocyte suspension cultures and in intact animals, although the details of the molecular mechanism remain unclear. Since glucocorticoids are not able to stimulate the fibrinogen mRNA content in the liver and to increase the plasma fibrinogen concentration, we believe that glucocorticoids are not the sole regulatory factor in the acute-phase fibrinogen response *in vivo*. Further study on the molecular mechanisms for the dramatic increase in the production of fibrinogen and the enhanced content of fibrinogen-polypeptide mRNA during the acute-phase reaction is required.

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