Neural control of biosynthesis and secretion of serum transferrin in perfused rat liver

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The effects of sympathetic- and parasympathetic-nerve stimulation on the synthesis of transferrin and other serum proteins from [¹⁴C]leucine and their secretion were studied in rat liver perfused *in situ*. The radioactivities incorporated into perfusate transferrin, albumin and total protein increased with time during 90 min perfusion after an initial lag period of 15–30 min. The increases in the radioactivities of the perfusate proteins were inhibited by electrical stimulation of the hepatic nerve, whereas the increases were enhanced by vagal-nerve stimulation. Measurement of the incorporation of [¹⁴C]leucine into transferrin in the microsomal and cytosol fractions of the liver after 90 min perfusion revealed that the synthesis of this serum protein was suppressed by hepatic-nerve stimulation and increased by vagal-nerve stimulation. The results indicate that the biosynthesis and secretion of transferrin, and possibly other serum proteins, are inhibited by sympathetic-nerve stimulation and enhanced by parasympathetic-nerve stimulation.

INTRODUCTION

The liver has been known to be well innervated by sympathetic- and parasympathetic-nerve fibres, and these autonomic nerves have been implicated in the regulation of certain types of hepatic metabolism (Shimazu, 1983, 1987; Jungermann *et al.*, 1987). Recently, it has also been observed that, in the isolated perfused rat liver, electrical stimulation of the hepatic-nerve plexus around the hepatic artery and the portal vein results in increases of glucose and lactate output and urate formation, and reductions in ketogenesis, urea release and NH₃ uptake (Iwai & Jungermann, 1987; Püschel *et al.*, 1987; Beuers *et al.*, 1986; Ballé & Jungermann, 1986).

However, possible effects of autonomic-nerve stimulation on hepatic protein metabolism have not been studied so far, either *in vivo* or in the isolated perfused organ. The objective of the present work was to clarify this problem by investigating the synthesis and secretion of serum proteins in perfused liver during electrical stimulation of hepatic-nerve and the vagus-nerve bundles. Transferrin was chosen as a rapid-turnover protein of hepatic origin.

MATERIALS AND METHODS

Materials

All chemicals were reagent grade and from commercial sources, except for *Staphylococcus aureus* Cowan-I (SAC-I), which was kindly supplied by Dr. Y. Hitsumoto of the Department of Microbiology of this institution. Rabbit anti-(rat transferrin) (IgG fraction) was purchased from Cooper Biochemical, West Chester, PA, U.S.A. L-[1-¹⁴C]Leucine (50 mCi/mmol) was obtained from ICN Radiochemicals, Irvine, CA, U.S.A. Alanine, BSA (Fraction V) and rat transferrin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Porcine insulin was from Shionogi Pharmaceutical Co., Osaka, Japan.

Liver perfusion and electrical stimulation of the hepatic nerve and the vagus nerve

Male Wistar rats, weighing 280–350 g, were used. They were maintained on a 12 h light/12 h dark cycle (lights on at 06:00 h)

with free access to laboratory chow and water. All experiments were started at about 09:00 h. The animals were anaesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg). Livers were perfused *in situ* at 37 °C by the constant-flow method (see Exton, 1975), 100 ml of recirculating medium being used. The perfusion medium consisted of Krebs–Henseleit bicarbonate buffer, pH 7.4, containing 5.5 mM-glucose, 3 mM-alanine, 2% BSA, 5 units of insulin and sufficient human erythrocytes to give a haematocrit of 20%. Insulin was included to suppress the protein catabolism that is known to occur in perfused livers (Mortimore & Mondon, 1970). The medium was equilibrated with CO₂/O₂ (1:19) and recirculated with a flow rate of approx. 0.8 ml/min per g of liver. After 30 min of pre-perfusion, 10 µCi of [¹⁴C]leucine was added to the perfusion medium and the perfusion was continued for another 90 min. Aliquots (0.5 ml each) of the perfusion medium were removed at intervals for protein analysis. The hepatic nerve or the vagus nerve was stimulated electrically with a bipolar platinum-wire electrode placed around the hepatic artery and portal vein or at the left vagal-nerve trunk just above the divergence of the hepatic branch respectively. Electrical stimuli consisting of 10 s trains of monophasic square pulses (0.5 ms duration, 50 Hz frequency and 10 V amplitude) were repeatedly applied to the nerve at 2 min intervals during 90 min of perfusion.

Determination of [¹⁴C]leucine incorporation into perfusate protein

Incorporation of [¹⁴C]leucine into total secreted protein was determined on perfusate supernatant after removal of erythrocytes by centrifugation. A 0.05 ml portion of the supernatant was precipitated twice with 0.1 ml of 10% trichloroacetic acid, dissolved in 0.1 ml of 1 M-NaOH, neutralized with 1 M-HCl, and the radioactivity was then measured in a liquid-scintillation spectrometer. To determine the radioactivity incorporated into albumin and transferrin, another portion of the perfusate supernatant was subjected to SDS/7.5%-(w/v)-PAGE (Laemmli, 1970). Rat transferrin was used as a standard. After electrophoresis, the gels were stained with 0.125% Coomassie Brilliant Blue R-250. The albumin and transferrin bands on the gels were cut out and solubilized by incubation overnight with 0.5 ml of

Abbreviations used: SAC-I, *Staphylococcus aureus* Cowan-I.

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30% H_2O_2 at 50 °C in a counting vial with a tight cap. The radioactivity of the solution was then determined by liquid-scintillation spectrometry.

Extraction and determination of transferrin in liver microsomal and cytosol fractions

At the end of 90 min perfusion the left lateral lobe of the liver was excised, immediately cooled on ice and homogenized in 9 vol. of 0.25 M-sucrose. The homogenate was centrifuged at 15000 g for 20 min at 4 °C. A total microsomal fraction was obtained by centrifuging the supernatant at 105000 g for 90 min at 4 °C. The microsomal fraction was extracted with sodium deoxycholate to give a final concentration of 0.9%. Transferrin contained in the extract was quantitatively immunoprecipitated by incubation with an approx. 3-fold excess of anti-(rat transferrin) and protein A from SAC-I. The cytosolic transferrin was also precipitated by the same procedure, except for the deoxycholate extraction. To ensure that precipitation was specific for transferrin, the precipitates obtained from the microsomal and cytosol fractions were further subjected to SDS/PAGE and the transferrin bands were analysed for radioactivity as described above.

For an estimate of the rate of synthesis of total liver protein, a sample (about 0.2 g) of the liver was homogenized with 5 vol. of 5% HClO_4 and the precipitated protein was washed, solubilized in 1 M-NaOH and neutralized with 1 M-HCl for determination of radioactivity. Protein was determined by the method of Lowry *et al.* (1951), with BSA as standard.

Statistical analysis

All values are presented as means $\pm$ S.E.M. The data were evaluated by two-way or one-way analysis of variance, with testing *post hoc* by the *F*-tests for simple effects. Differences where *P* values were less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Effects of autonomic-nerve stimulation on the appearance of ^{14}C -labelled transferrin, albumin and total protein in perfusate

The total and specific serum proteins synthesized by rat liver and released into the perfusion medium were quantitatively analysed in portions of perfusate plasma by measuring the radioactivity incorporated into perfusate protein.

As Fig. 1 shows, perfused liver preparations synthesized and released radioactively labelled protein into the perfusate under the experimental conditions in which the perfusion medium contained ^{14}C leucine and supplemented alanine and insulin. In a control perfusion of rat liver there is a roughly linear appearance of newly synthesized protein in the perfusate during the 90 min perfusion, except for the first 15–30 min. The latter lag period probably represents the time required for synthesis, processing and secretion of radioactive protein. When the hepatic nerve, which comprises mostly post-ganglionic sympathetic fibres, was electrically stimulated intermittently during the 90 min perfusion, the rate of release of total secreted protein was decreased to approx. 45% below the control value. On the other hand, electrical stimulation of the vagal parasympathetic nerve significantly increased the rate of total protein secretion by the perfused liver, especially after 60 min of perfusion.

Fig. 2 depicts the effects of nerve stimulation on the elaboration of serum albumin by the perfused rat liver. The newly synthesized albumin was secreted into perfusate after about 30 min of lag period, and after that period its secretion increased rapidly with the time of perfusion up to 90 min. The time course of the appearance of radioactivity in serum albumin agrees roughly

Fig. 1. Effects of hepatic-nerve and vagus-nerve stimulation on ^{14}C leucine incorporation into total perfusate protein

Each liver was perfused *in situ* in a recirculating system with 100 ml of perfusion medium containing 10 μCi of ^{14}C leucine. Intermittent electrical stimulation of the hepatic nerve and the vagus nerve was started at zero time. At each time point an aliquot of the perfusate was removed and assayed for ^{14}C leucine incorporation into total secreted protein as described in the Materials and methods section. Statistically significant changes from controls are indicated by asterisks.

Fig. 2. Effects of hepatic nerve and vagus-nerve stimulation on ^{14}C leucine incorporation into perfusate albumin

Experimental conditions were as described for Fig. 1. Perfusate albumin was isolated by SDS/PAGE. Statistically significant change from controls is indicated by asterisk.

with previous observations in intact rats (Morgan & Peters, 1971). Electrical stimulation of the vagus nerve led to an increase of about 2-fold in radioactivity found in perfusate albumin compared with controls after 90 min. On the other hand, hepatic-nerve stimulation did not cause a significant change in the secretion of albumin into the perfusate.

The release of radioactively labelled transferrin was also studied (Fig. 3). As in the case of serum albumin, the release of transferrin into the perfusion medium was initiated after about 30 min. Vagal stimulation tended to increase (though not significantly) serum transferrin release, whereas hepatic-nerve stimulation caused a substantial suppression of the release.

Fig. 3. Effects of hepatic-nerve and vagus-nerve stimulation on [^{14}C]leucine incorporation into perfusate transferrin

Experimental conditions were as described for Fig. 1. Perfusate transferrin was isolated by SDS/PAGE. Statistically significant change from controls is indicated by an asterisk.

Effects of autonomic-nerve stimulation on the synthesis of transferrin in liver-cell fractions

In preliminary experiments, samples of the perfused livers were homogenized with HClO_4 , and the precipitated protein was analysed for its radioactivity (due to [^{14}C]leucine incorporation) during a 90 min perfusion. It was found that the mean radioactivity recovered from total liver protein was considerably higher in livers after vagus-nerve stimulation (810.8×10^3 d.p.m./g of liver) and lower in livers after hepatic-nerve stimulation (306.8×10^3 d.p.m./g of liver) than in controls (428.8×10^3 d.p.m./g of liver).

An attempt was then made to measure the incorporation of [^{14}C]leucine into transferrin extracted from the microsomal and cytosol fractions of the livers perfused for 90 min. Transferrin in

Fig. 4. Synthesis of transferrin in the perfused liver after autonomic nerve stimulation

Experimental conditions for liver perfusion and nerve (V, vagal; H, hepatic) stimulation were as for Figs. 1–3. At the end of 90 min perfusion the livers were excised and the microsomal (M) and cytosol (Cyt) fractions of the liver homogenate were obtained by centrifugation. Transferrin contained in each cell fraction was isolated by immunoprecipitation and SDS/PAGE and assayed for its radioactivity. Statistically significant changes from controls (C) are indicated by asterisks.

each cell fraction was isolated by immunoprecipitation with anti-(rat transferrin) and by SDS/PAGE. The results in Fig. 4 show that the incorporation of [^{14}C]leucine into transferrin in both microsomal and cytosol fractions was increased by vagal-nerve stimulation and suppressed by hepatic-nerve stimulation compared with controls, although the increase in the microsomal fraction after vagal-nerve stimulation was not statistically significant ($n = 4$).

These results suggest that vagal-parasympathetic-nerve stimulation increases, and hepatic-sympathetic-nerve stimulation reduces, not only the secretion, but also the synthesis, of transferrin and probably other serum proteins in liver. These effects of autonomic-nerve stimulation on hepatic protein metabolism might be secondary, owing to haemodynamic changes. It is generally accepted that electrical stimulation of the hepatic sympathetic fibres produces an increase in inflow resistance with a reduction of total liver blood flow, whereas stimulation of the vagal parasympathetic fibres has little effect on overall blood flow to the liver. In the present experiments the livers were perfused by the constant-flow method to minimize changes in portal blood supply. We further confirmed that the overall flow out of the liver did not change significantly during hepatic-nerve stimulation or vagal-nerve stimulation in separate experiments. In the case of hepatic-nerve stimulation, however, a redistribution of blood flow within the liver parenchyma may occur, and this leads to a partial O_2 deficiency for some parenchymal areas (Ji *et al.*, 1984). The possibility cannot be excluded, therefore, that the decreases in synthesis and release of proteins into the perfusate after hepatic-nerve stimulation might be due to a change in the intrahepatic microcirculation. Since an apparent change of haemodynamics and an increase in lactate output could not be observed after vagal-nerve stimulation (results not shown), it seems unlikely that the increases in synthesis and secretion of proteins in response to vagal-parasympathetic-nerve stimulation are simply due to change in blood supply.

Work on the neural regulation of liver metabolism has been advanced by utilizing liver preparations perfused *in situ* (Shimazu, 1983; Jungermann *et al.*, 1987), which makes it possible to study a completely isolated organ whose efferent-nerve supply to the liver has been kept intact and where any possible effects of hormonal factors of extra-hepatic origin can be excluded; some of the metabolic changes caused by nerve stimulation have been shown to be due to direct intrinsic innervation of hepatocytes, whereas others are affected indirectly through haemodynamic changes (Jungermann *et al.*, 1987). By exploiting the advantages of this liver preparation perfused with heterospecies blood, the present study represents a distinct advance with regard to the reciprocal role of the sympathetic and parasympathetic nerves in regulating the net synthesis of specific serum protein. In addition, the present study confirms and extends the previous notion that the parasympathetic nervous system originating from the lateral hypothalamus can stimulate a series of anabolic processes, whereas the ventromedial hypothalamus–sympathetic nervous system leads to an activation of catabolic processes in body metabolism (Shimazu, 1987).

In the present study, however, nerve action was examined in the presence of insulin, which is indispensable in ensuring protein synthesis in the isolated liver. Hence, the observed effects of the nerve stimulation on serum protein synthesis might have been due to neural modification of insulin action. Moreover, it is not known from the present experiments whether the changes in hepatic synthesis of serum protein induced by autonomic-nerve stimulation are derived from alterations in the transcription of a specific gene or translational activity. With the use of inhibitors of transcription and translation, molecular mechanisms of this kind could be studied.

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