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Effect of Endotoxin on Tryptophan Pyrrolase and δ -Aminolaevulinate Synthase: EVIDENCE FOR AN ENDOGENOUS REGULATORY HAEM FRACTION IN RAT LIVER

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Endotoxin was administered to rats at a dose shown previously to stimulate hepatic haem oxygenase activity and to block induction of δ -aminolaevulinate synthase, apparently by causing redistribution of haem from cytochrome *P*-450 to a regulatory haem pool in the liver. Within 5 h of the administration of endotoxin (at a time when the effect of the compound on cytochrome *P*-450 is maximal) the relative saturation of tryptophan pyrrolase with intrinsic haem rose, from a basal value of 50% to 90%, indicating that 'free' haem had become available. Concurrently, the activity of δ -aminolaevulinate synthase was decreased to 25% of its basal value. Haem oxygenase reached peak activity 13 h after endotoxin administration. These findings provide new evidence for the existence of an 'unassigned' hepatic haem fraction, which exchanges with cytochrome *P*-450 haem and regulates these three enzyme functions.

Tryptophan pyrrolase (EC 1.13.11.11), which contains haem as prosthetic group, is present in the liver both as holoenzyme and as haem-free inactive apoenzyme. In untreated animals, approx. 50% of the total enzyme protein in the liver is active holoenzyme (Feigelson & Greengard, 1961*a*). The presence of functionally latent apoenzyme is inferred by injection of animals *in vivo* with haem or by addition of haem to the assay mixture *in vitro* (Feigelson & Greengard, 1961*a*; Badawy & Evans, 1975). Either manipulation saturates the apoenzyme, permitting measurement of total tryptophan pyrrolase activity.

The presence of apo-(tryptophan pyrrolase) has been used as a way of examining the presence and regulation of a postulated endogenous 'unassigned' haem pool in the liver. It has been noted, for example, that administration of the porphyrinogenic compound 2-allyl-2-isopropylacetamide (Feigelson & Greengard, 1961*b*; Marver *et al.*, 1966*a*) or of tryptophan (Greengard & Feigelson, 1961) increased the relative haem saturation of tryptophan pyrrolase. Both compounds also stimulated δ -aminolaevulinate synthase (EC 2.3.1.37) (Marver *et al.*, 1966*a*), the rate-determining enzyme of the haem-synthetic pathway in the liver. Thus it was concluded that new synthesis of haem was responsible for activation of tryptophan pyrrolase and, by implication, for an increase in the size of the 'free' haem pool.

In recent studies, we have obtained evidence that compounds such as endotoxin, which stimulates hepatic haem oxygenase activity (EC 1.14.99.3), do so by facilitating dissociation of haem from cyto-

chrome *P*-450 (Bissell & Hammaker, 1976*a*). The dissociated haem appears to mix with the postulated haem pool in the liver; this was deduced from the fact that administration of endotoxin blocks stimulation of δ -aminolaevulinate synthase by 2-allyl-2-isopropylacetamide. Endotoxin blocks the stimulation in proportion to its effect on cytochrome *P*-450 (Bissell & Hammaker, 1976*b*). If this interpretation is valid, then administered endotoxin should also activate tryptophan pyrrolase by making increased amounts of haem available to the apoenzyme. Furthermore, because the increase in haem occurs without new synthesis, endotoxin should simultaneously decrease the basal activity of δ -aminolaevulinate synthase, in contrast with the effect of administered tryptophan or 2-allyl-2-isopropylacetamide. The present studies explore this possibility. Because basal δ -aminolaevulinate synthase activity is at the lower limit of sensitivity of the colorimetric assay for the enzyme (Marver *et al.*, 1966*b*), we have used the more sensitive radiometric method for these experiments.

Experimental

The experimental approach, including the preparation and injection of endotoxin, has been described in detail elsewhere (Bissell & Hammaker, 1976*a*). δ -Aminolaevulinate synthase was assayed in liver homogenate with α -oxo[5- 14 C]glutaric acid as substrate (Amersham/Searle, Arlington Heights, IL, U.S.A.; 8.9 Ci/mol); the method of Ebert *et al.* (1970) was used, except that the concentration of protein in

Fig. 1. Protein-dependence of δ -aminolaevulinate synthase activity assayed radiometrically in rat liver homogenate. Samples from rats fed *ad libitum* (●) or deprived of food for 24 h (○) were assayed as described in the Experimental section; 0.5% (w/v) homogenate corresponds to a protein concentration in the assay of about 1 mg/ml.

the assay mixture was less than 1 mg/ml. With this modification, enzyme activity was proportional to protein concentration and was higher in unfed than in fed rats (Fig. 1), as described by Bock *et al.* (1971). As a further check of the validity of the assay, individual homogenates from either untreated or 2-allyl-2-isopropylacetamide-treated rats were assayed by both the radioactive method and the colorimetric method of Marver *et al.* (1966b). The results for the two methods were comparable, in agreement with the findings of Bock *et al.* (1971).

Tryptophan pyrrolase was measured during the linear rate of kynurenine formation (Badawy & Evans, 1975). Percentage saturation of the enzyme was calculated from the rate of product formation by the native homogenate, divided by its activity after addition of haem *in vitro* ($\times 100$). Haem oxygenase was determined as described previously (Bissell & Hammaker, 1976a), and protein by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

The effect of administered endotoxin on the saturation *in vivo* of tryptophan pyrrolase with haem is shown in Fig. 2. Saturation was maximal and significantly increased 5 h after endotoxin admin-

Fig. 2. Effect of endotoxin on the relative haem saturation of tryptophan pyrrolase in rat liver homogenate.

Endotoxin was administered at time zero in a single dose of 1.5–2.0 mg/kg body wt. by intraperitoneal injection (●). Control rats received a similar volume of 0.9% NaCl (○). Values represent individual animals, the average of two animals or (points with vertical bars) the mean $\pm$ S.D. for three to five animals. The difference at 5 h between treated and control animals is statistically significant ($P = 0.01$). The continuous line shows the mean value for untreated rats, the dashed lines either side indicating the S.D.

istration, relative to animals injected with comparable amounts of 0.9% NaCl. Total enzyme activity (i.e. that measured after addition of saturating amounts of haem *in vitro*) did not change over this experimental period, in agreement with the studies by Berry *et al.* (1966).

Parallel studies of δ -aminolaevulinate synthase were carried out, and these data are shown in Fig. 3. Activity of the enzyme was diminished after endotoxin administration and, in reciprocal fashion to tryptophan pyrrolase, was maximally decreased 5 h after administration of the compound.

Haem oxygenase activity was determined in post-mitochondrial supernatant from the homogenates used for measurement of tryptophan pyrrolase, and the results are shown in Fig. 4. Although haem oxygenase appeared to be increasing 5 h after endotoxin administration, the difference between the treated and control groups failed to achieve statistical significance ($P > 0.1$; $n = 8$). Peak stimulation of haem oxygenase occurred 13 h after endotoxin administration, in agreement with previous results (Bissell & Hammaker, 1976a).

Fig. 3. Effect of endotoxin on the basal activity of δ -aminolaevulinate synthase in rat liver

Endotoxin (●) or 0.9% NaCl (○) was administered as in Fig. 2 at time zero. At the indicated time points, rats were killed and hepatic δ -aminolaevulinate synthase activity was assayed. The individual points represent the average of duplicate assays from single animals, except at zero time and 5 h when means $\pm$ S.D. for four animals are given. Control and treated animals are statistically significantly different at 5 h ($P < 0.01$).

Fig. 4. Effect of endotoxin (●) or 0.9% NaCl (○) injection on hepatic haem oxygenase activity

Haem oxygenase was assayed in samples of the liver preparation used for Fig. 2, and the representation of the data is the same as in Fig. 2.

Discussion

These findings provide new information with regard to a regulatory ('free') haem fraction in the liver. In previous work (Bissell & Hammaker, 1976a,b), this postulated haem fraction was shown to exchange with cytochrome *P*-450 haem and to regulate the induction of δ -aminolaevulinate synthase. In the present work, it is shown to affect also the regulation of tryptophan pyrrolase. The evidence that endotoxin causes a relatively specific enlargement of the hepatic 'free' haem pool (rather than a non-specific 'toxic' effect) has been presented previously (Bissell & Hammaker, 1976a). Moreover, endotoxin is probably only one of a large group of compounds that, at pharmacological doses, cause dissociation of haem from cytochrome *P*-450 (Bissell & Hammaker, 1976b).

If the regulatory pool can be augmented by various discrete mechanisms (and if there is functionally only a single pool), specific predictions can be made about the effects of individual experimental agents on haem-sensitive enzymes such as δ -aminolaevulinate synthase and tryptophan pyrrolase. Compounds such as 2-allyl-2-isopropylacetamide may increase the amount of available haem by stimulating δ -aminolaevulinate synthase, in which case activation

of tryptophan pyrrolase may occur as a secondary phenomenon (Marver *et al.*, 1966a). Alternatively, as has been postulated for the effect of administered tryptophan (Marver *et al.*, 1966a), compounds that primarily stimulate synthesis of apo-(tryptophan pyrrolase) may secondarily stimulate δ -aminolaevulinate synthase activity, having triggered a new demand for haem. Regardless of the validity of these hypotheses, the present findings clearly document a third possibility, since endotoxin, while activating tryptophan pyrrolase, concurrently inhibits δ -aminolaevulinate synthase. This combined action resembles that of administered (exogenous) haem (Badawy & Evans, 1975), although there is no evidence that endotoxin causes haemolysis in rats (Bissell & Hammaker, 1976a). Thus the findings are best explained as an endotoxin-mediated redistribution of endogenous hepatic haem and, as such, are fully consistent with the previous postulate about the effect of this compound on cytochrome *P*-450 haem (Bissell & Hammaker, 1976b). To verify that administered tryptophan and endotoxin act by different mechanisms, we evaluated the ability of the amino acid to perturb cytochrome *P*-450, as judged by the previously described approach (Bissell & Hammaker, 1976a). When administered in doses that stimulate

tryptophan pyrrolase (Badawy & Evans, 1975), tryptophan failed to accelerate the degradation of cytochrome *P*-450, as was predicted (D. M. Bissell & L. E. Hammaker, unpublished work).

An additional point of interest is that endotoxin failed to stimulate total enzyme activity, whereas tryptophan and 2-allyl-2-isopropylacetamide increase both haem saturation of the enzyme and its total activity (Feigelson & Greengard, 1961*b*; Marver *et al.*, 1966*a*; Greengard & Feigelson, 1961). The effects of these various agents on the synthesis or degradation of tryptophan pyrrolase may differ (Berry *et al.*, 1966; Rippe & Berry, 1973).

Administered endotoxin exerted essentially reciprocal effects on δ -aminolaevulinate synthase and the haem-saturation of tryptophan pyrrolase, the maximal change in both enzymes occurring at 5 h. This time course corresponds to that for the effect of endotoxin on the dissociation and degradation of haem from cytochrome *P*-450 (Bissell & Hammaker, 1967*b*). By contrast, haem oxygenase reached peak activity only 13 h after endotoxin administration, which appears to be well past the time when 'unassigned' haem was either interacting with other hepatic enzymes (as shown in the present paper) or undergoing degradation to CO (and bile pigment) (Bissell & Hammaker, 1976*a*). Further studies are needed to determine whether changes in haem oxygenase activity under these experimental conditions denote actual rates of haem degradation in the liver or, rather, relate to another process in the turnover of hepatic microsomal haem.

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