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SOME BIOCHEMICAL CHANGES ASSOCIATED WITH NAFENOPIN-INDUCED LIVER GROWTH IN THE RAT*

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Abstract—The response of the liver to a single oral or intraperitoneal dose of the hypolipidemic drug nafenopin has been investigated in rats. Liver weight increased for 3 days and returned to normal in 6 days, although at no time did food consumption differ from that of controls. During the experimental period, liver protein concentrations were unchanged while DNA concentration decreased slightly. Ornithine decarboxylase activity, DNA synthesis and amino acid uptake were markedly stimulated, as has been reported in the regenerating liver. After intraperitoneal nafenopin the onset of these responses was more rapid and the degree of induction greater than that seen after oral nafenopin. Since ornithine decarboxylase and DNA synthesis increased at approximately the same time after orally administered nafenopin, there is some doubt as to whether induction of ornithine decarboxylase is obligatory prior to the synthesis of DNA. The results, however, do support a role for increased amino acid uptake in the pre-replicative phase of liver growth. It is speculated that the oral/intraperitoneal difference in induction rates of these parameters may be attributable to a slow rate of disposition of orally administered nafenopin in the rat or to an unknown alternative mechanism.

Treatment of rats with the hypolipidemic drug nafenopin‡ (NP) (SU-13437) inhibits the hepatic transport of drugs and other foreign chemicals and brings about a profound choloresis [1–6]. It also produces a marked enlargement of the liver, and both hyperplasia and cell hypertrophy are seen [1, 7–11]. In contrast to most other agents that induce liver growth [12], NP has little or no effect on microsomal drug metabolism [1, 5, 9], although it does elicit a proliferation of the endoplasmic reticulum [10] and an increase in peroxisomes and in catalase activity [10, 11].

After treatment with NP, the liver presents a morphologic picture similar to that of the regenerating liver after partial hepatectomy [13]§. Functional changes that occur early during regeneration include induction of ornithine decarboxylase [14, 15], enhanced uptake of amino acids [16] and rapid incorporation of thymidine into DNA [17]. In an effort to determine whether the liver growth stimulated by NP proceeds by mechanisms similar to those seen during regeneration, the above parameters were measured in rats treated with NP.

EXPERIMENTAL

Animals. Male rats (body wt 120–180 g) of the laboratory's Wistar strain were kept under controlled lighting (0900–2300 hr) conditions. Food was available from 1700 to 0900 hr but water was available at all times. For the initial experiments, a weighed amount of food was given to the animals each day at 1700 hr, and the amount consumed was determined the next morning. NP was dissolved in polyethylene glycol-400 (300 mg/ml) and given orally or intraperitoneally in a dose of 200 mg/kg unless otherwise indicated. Control animals received an equal volume of vehicle.

Ornithine decarboxylase. Rat livers were homogenized in 4 vol. of 50 mM phosphate buffer, pH 7.4, containing 1 mM dithiothreitol, 0.1 mM EDTA and 0.1 mM pyridoxal phosphate (added fresh each day) and centrifuged for 1 hr at 100,000 *g*. Supernatant fluid (1.8 ml) was preincubated for 10 min at 37°. The reaction was started by addition to each flask of 0.2 ml (0.5 μCi) DL-[-1-^{14}C]ornithine HCl. The final concentration (0.6 mM) was sufficient to saturate the enzyme [18]. The flasks were capped and shaken at 37° for 30 min. The reaction was stopped by the addition of 0.2 ml of 5 N H_2SO_4 and the shaking continued for 30 min. The $^{14}CO_2$ evolved was trapped by hyamine hydroxide in methanol and counted. Enzyme activity was calculated as pmoles CO_2 evolved/30 min/mg of protein.

DNA synthesis. [^{3}H]thymidine (25 μCi) was injected intraperitoneally. The livers were removed 1 hr later and homogenized with 4 vol. of ice-cold 0.9% NaCl. A sample of the homogenate was mixed with an equal volume of ice-cold 10% trichloroacetic acid and centrifuged. The precipitate was washed twice with 5% trichloroacetic acid, twice with ethanol and twice with hot ethanol–ethyl ether, 3:1 (v/v). The DNA was extracted in hot 5% trichloroacetic acid. A sample of the extract was counted and DNA determined. Thymidine incorporation was calculated as cpm/mg of DNA.

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‡ Nafenopin = 2-methyl-2[*p*-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]-propionic acid.

§ J. Padawar, personal communication.

Amino acid uptake was determined with the use of ^{14}C-labeled 2-amino isobutyric acid, which is rapidly taken up by the liver but not metabolized [19]. Three μCi/100 g was injected intravenously and the livers were removed 5 min later. They were perfused thoroughly with ice-cold 0.9% NaCl and homogenized with 4 vol. of 0.9% NaCl, and the 5% trichloroacetic acid-soluble radioactivity was determined. The uptake was calculated as cpm/g of liver. The amino acid composition of these extracts was determined using a Locarte amino acid analyzer.

Protein was determined by the method of Lowry *et al.* [20], and DNA by the diphenylamine procedure of Munro and Fleck [21].

Chemicals. DL-[1-^{14}C]ornithine HCl (sp. act. 58 μCi/m-mole), [6-^{3}H]thymidine (sp. act. 23.3 Ci/m-mole) and 2-amino[1-^{14}C]isobutyric acid (sp. act. 51 μCi/m-mole) were purchased from The Radiochemical Centre, Amersham, Bucks, U.K.

RESULTS

Groups of three rats were given a single dose of NP orally (500 mg/kg), and body weight, liver weight, protein, DNA and food consumption were determined at various intervals thereafter. The gain in body weight for the treated animals initially lagged behind that of the controls but resumed a normal rate after the second day (Fig. 1A). This was not due simply to anorexia, since food consumption was essentially the same in control and treated groups (Fig. 1F). As expcted, liver weight, as a percentage of body weight, increased by approximately 60 per cent and returned to control levels by day 6 (Fig. 1B). Total liver DNA increased (Fig. 1D) but to a lesser extent than did liver weight, so that the concentration of DNA (Fig. 1E) actually decreased during the experimental period. The concentration of protein within the liver increased slightly during the latter part of the experiment (Fig. 1C).

Ornithine decarboxylase activity was determined at various times after the oral or intraperitoneal administration of NP. Peak activity in treated animals was 19–24 hr after oral administration and 10 hr after the intraperitoneal route (Fig. 2). A second increase after intraperitoneal injection was apparent to 24 hr, but observations were not extended further. Multiple peaks of ornithine decarboxylase during liver regeneration have been observed by others [22–25].

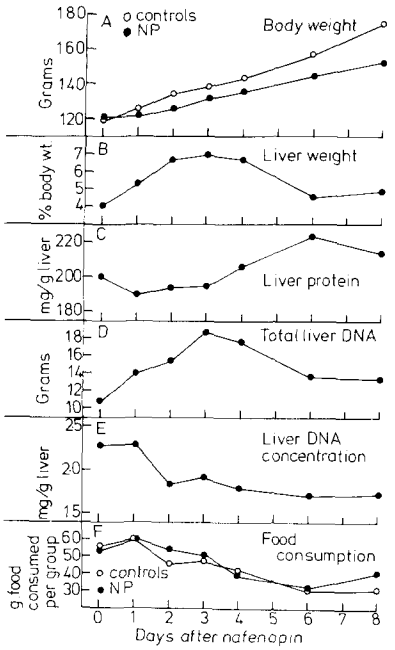

Fig. 1. Effect of a single oral dose of NP (500 mg/kg) on body weight, liver protein, liver DNA and food consumption. Each point is the mean for a group of three rats.

DNA synthesis was also markedly stimulated after NP administration (Fig. 3). Peaks of thymidine incorporation were seen at 17 hr after the oral route and at 21 hr after the intraperitoneal route.

Amino acid uptake by the liver was assessed using 2-amino-isobutyric acid, which undergoes very little metabolism within the body. A fairly broad peak of activity was seen 24 hr after oral NP, and a sharp peak 11 hr after intraperitoneal administration (Fig. 4).

Amino acid analyses revealed that there were no significant differences in the amino acid composition during the maximum periods of uptake after both oral and intraperitoneal NP.

DISCUSSION

The hepatic response to the administration of nafenopin is of particular interest since, unlike the vast majority of substances that stimulate liver

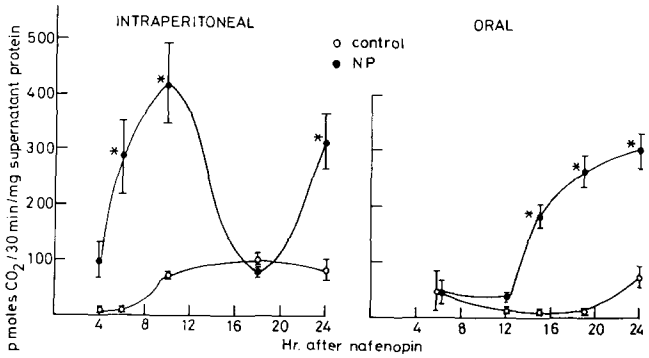

Fig. 2. Ornithine decarboxylase activity in liver supernatant after a single oral or intraperitoneal dose of NP (200 mg/kg). Each point is the mean ± S. E. M. for five or more animals. An asterisk indicates values that differ significantly from those of controls ($P < 0.05$).

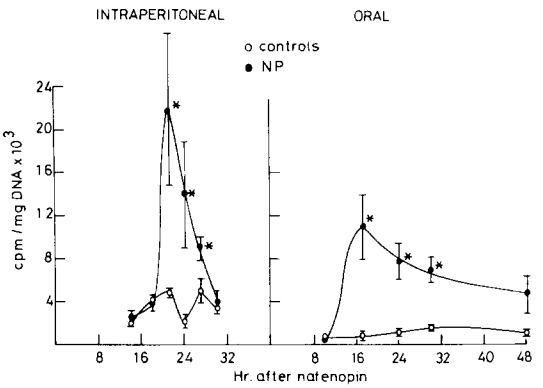

Fig. 3. Hepatic DNA synthesis after a single oral or intraperitoneal dose of NP (200 mg/kg). [^{3}H]thymidine (25 μCi) was injected intraperitoneally and 1 hr later radioactivity and DNA were determined in the hot trichloroacetic extract of whole liver. Values are the mean ± S. E. M. for four or more animals. An asterisk indicates values that differ significantly from those of controls ($P < 0.05$).

growth, it does not induce microsomal drug-metabolizing enzymes [1, 5, 9]. On the other hand, like most inducing agents, it causes a proliferation of the hepatic endoplasmic reticulum [10] and does undergo metabolism in the rat [3] and in man [26]. Clofibrate, a structurally related hypolipidemic agent, similarly stimulates liver growth with little or no effect on drug metabolism [9, 27, 28], although steroid hydroxylation by microsomes is significantly increased [28–30]. Other evidence for the dissociation of liver growth from induction of microsomal drug metabolism is found in experiments using aminotriazole. This inhibitor of haem synthesis prevents the induction of cytochrome P-450 by phenobarbital, while liver growth in response to the barbiturate is unimpaired [31].

In the regenerating liver, dependence of DNA synthesis upon prior induction of ornithine decarboxylase was suggested by Gaza *et al.* [23] and Short *et al.* [25]. These investigators found that after partial hepatectomy, or administration of a mixture of hormones and amino acids, or restoration of a normal diet to protein-deficient rats, hepatic ornithine decarboxylase was markedly induced within a few hours while DNA synthesis was stimulated at 20–24 hr. Certain foreign chemicals which stimulate liver growth also induce ornithine decarboxylase [32–34].

Some doubt as to the causal relationship between induction of ornithine decarboxylase and DNA synthesis is seen in the work of Schrock *et al.* [35], who found that hypertonic glucose given intravenously or Celite given intraperitoneally, procedures that do not stimulate liver growth, induces decarboxylase activity. Hydrocortisone administration at the time of partial hepatectomy delays by several hours the peak of DNA synthesis but has no effect on ornithine decarboxylase induction [36]. Conversely the α-adrenergic blocking agents, phenoxybenzamine and phentolamine, given at the time of partial hepatectomy, partially suppress the rise in ornithine decarboxylase but are without effect on the subsequent increase in DNA synthesis [37, 38]. These drugs do inhibit DNA synthesis if given 18 hr after partial hepatectomy, long after initiation of ornithine decarboxylase induction. Pariza *et al.* [39] found in rats that decarboxylase activity in hepatoma 5123-C was markedly increased while activity in hepatoma 7800 was approximately normal, although the rates of growth, and presumably DNA synthesis, of both tumors were equal. The present work also demonstrates a dissociation between ornithine decarboxylase and DNA synthesis. After intraperitoneal injection of nafenopin, the peaks of ornithine decarboxylase and DNA synthesis were at 10 and 21 hr, respectively, a pattern not unlike that seen after partial hepatectomy. On the other hand, after oral administration of nafenopin, decarboxylase activity was not seen until 19–24 hr, while DNA synthesis showed a peak slightly before this, at 17 hr. Thus, the usual order of these events was actually reversed. This would seem to argue against ornithine decarboxylase playing an essential role prior to the synthesis of DNA associated with liver growth.

The mechanisms underlying the induction of ornithine decarboxylase are not yet understood. Administration of amino acids to starved or protein-deficient animals increases the level of hepatic ornithine decarboxylase [23, 40]. After a single dose of oral nafenopin there was a slight lag in the rate of growth (see Fig. 1A), suggesting the possibility of an induced protein-deficient state. However, at no time after administration of the drug does food consumption decrease or liver protein fall below control values (see Fig. 1C and 1F) [9, 11]. Liver protein concentration is similarly unchanged after clofibrate, which also produces marked hepatomegaly [41]. It is unlikely, therefore, that the effect of nafenopin on ornithine decarboxylase is the result of a temporary protein deficiency.

Enhanced uptake of 2-amino-isobutyric acid is seen after nafenopin (see Fig. 4) as well as after partial hepatectomy [25]. Although significantly increased levels of ornithine decarboxylase are seen slightly prior to increased amino acid uptake, the time courses of these two responses are in the main similar after either route of administration (see Figs. 2 and 4). This parallel is also seen during liver regener-

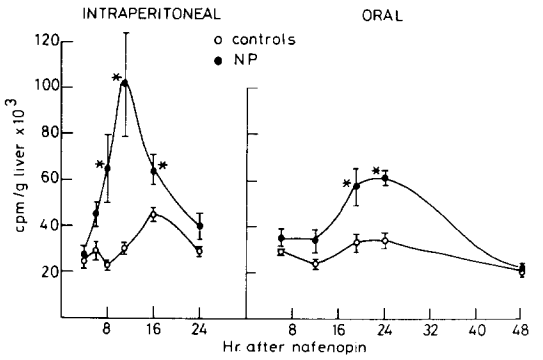

Fig. 4. Amino acid uptake by the liver after a single oral or intraperitoneal dose of NP (200 mg/kg). [^{14}C]-2-amino isobutyric acid was injected intravenously (5 μCi/100 g). Five min later the trichloroacetic acid-soluble radioactivity was determined in the perfused livers. Each value is the mean ± S. E. M. for five or more animals. An asterisk indicates values that differ significantly from those of controls ($P < 0.05$).

ation [25] and suggests the possibility of a functional relationship between these events in the pre-replicative phase of liver growth. However, as in the case of ornithine decarboxylase, peak DNA synthesis actually occurs prior to peak amino acid uptake after the oral route. This again suggests that a re-examination of the temporal relationships of these responses may be in order, or that nafenopin stimulates liver growth through alternative mechanisms.

The difference in the rates of induction of ornithine decarboxylase after oral as compared to intraperitoneal nafenopin is rather large. A significant rise in enzyme activity is apparent 4 hr after intraperitoneal injection, while 12 hr after oral feeding, activity was still within normal range. Thus, the delay between the two routes of administration is greater than 8 hr. In humans, peak plasma levels of nafenopin are reached 1.3 to 3.6 hr after oral administration [26]. A much slower rate of absorption in rats would be required to explain the oral/intraperitoneal differences in induction time. Otherwise an alternative mechanism must be postulated. As yet, the pharmacokinetics of nafenopin in the rat have not been reported.

The possibility was considered that nafenopin causes hepatic necrosis, and therefore the biochemical changes observed would reflect a compensatory growth response. However, after periods of treatment with nafenopin ranging from 2 days to several weeks, morphologic observations have revealed no instance of necrosis [7, 9, 11, 13]*. The stimulation of liver growth, therefore, should be considered to be an adaptive rather than a toxic response.

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