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EARLY CHANGES INDUCED BY 2-ACETYLAMINOFLUORENE IN LYSOSOMES IN RAT LIVER PARENCHYMAL CELLS

TROND BERG and THORALF CHRISTOFFERSEN

Institute of Zoophysiology and Institute of Pharmacology, Medical Faculty, University of Oslo, Blindern, Oslo 3, Norway

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Abstract—The effect of the carcinogen 2-acetylaminofluorene (AAF) on the specific activities and the intracellular distribution of rat liver β-glucuronidase and acid deoxyribonuclease was investigated. The enzymes were measured both in whole liver homogenates and in isolated purified parenchymal cells and Kupffer cells. AAF was found to cause a progressive increase in the specific activities of both enzymes during the first 6–8 weeks of carcinogen feeding. The increase was found only in the liver parenchyme: no effect was observed in Kupffer cells. AAF feeding caused a marked redistribution of both enzymes towards heavier cell fractions, indicating that the lysosomes became larger after AAF treatment. An increased size of hepatic lysosomes is consistent with the notion that carcinogen feeding leads to increased cellular autophagy.

CURRENT data indicate that lysosomes are altered during chemical carcinogenesis. Morphological studies have revealed increased numbers of rat liver lysosomes early after treatment with hepatocarcinogens,[1,2] and increased amounts of hepatic lysosomal enzymes have been reported after administration of aflatoxin[3] and dimethylnitrosamine.[4] Many carcinogens have been shown to labilize lysosmes so that increased amounts of enzyme are found in soluble fractions.[5] Lysosomal alterations during carcinogenesis are of interest since the lysosomes are able to degrade, and thereby influence the turnover of many of the cellular constituents[6] (nucleic acids, enzymes, glycogen, organelles) that are known to undergo changes during carcinogenesis.

In the present work we have measured the specific activity and studied the intracellular distribution of two lysosomal enzymes, β-glucuronidase (EC 3.2.1.31) and acid deoxyribonuclease (acid DNase, EC 3.1.4.6), in livers from rats fed 2-acetylaminofluorene (AAF). Since the amount of nonparenchymal tissue in the liver may change during carcinogenesis[2] we have also measured the enzymes in isolated purified Kupffer cells and hepatocytes. The cells have been separated following dispersion of the liver by enzymatic perfusion.

MATERIALS AND METHODS

Animals and diet. Male albino Wistar rats were obtained from Møllegaard Hansens Avlslaboratorier A/S, Copenhagen. The animals weighed about 200 g when the carcinogen feeding was started. The rats were maintained on a 12 hr light/12 hr dark cycle, at constant temperature and humidity. During the experiment they

received basal diet described by Miller *et al.*[7] The diet of the experimental group was supplemented with 0·025% (w/w) AAF. The animals were fed *ad lib.*

Chemicals. AAF was purchased from Fluka, Buchs, Switzerland. Enzyme substrates (calf thymus DNA, type V and phenolphthalein glucuronic acid) were obtained from Sigma Chemical Co., St. Louis. Pronase (type I) was from Calbiochem, Los Angeles and collagenase (type I) from Sigma Chemical Co., St. Louis. All other reagents were analytical grade.

Assay procedures. β-Glucuronidase (EC 3.2.1.31) was determined according to Gianetto and de Duve.[8] Phenolphthalein glucuronic acid served as a substrate. Acid deoxyribonuclease (acid DNase, EC 3.1.4.6) was determined according to de Duve *et al.*[9] Calf thymus DNA was used as a substrate. Both enzymes were measured in the presence of 0·10 M acetate buffer, pH 5. Protein was determined according to Lowry *et al.*[10]

Homogenization and tissue fractionation. Animals were killed by decapitation. The livers were rapidly excised, chilled in ice-cold 0·25 M sucrose solution, cut into small pieces and homogenized in chilled Potter–Elvehjem homogenizers with Teflon pestles. The pestle rotated about 1000 rev/min. The homogenizer was kept in an ice-slurry during the homogenization procedure. Tissue fractionation by differential centrifugation was performed according to de Duve *et al.*[9] in a Sorvall centrifuge equipped with a SM-24 rotor. 0·10% (v/v) Triton X-100 was included in homogenates and fractions to assure that total enzyme activity was measured.

Preparation of purified hepatocytes and reticuloendothelial cells. Heterogenous liver cell suspensions were prepared by perfusing the liver *in vitro* with 0·05% collagenase in Hanks' solution.[11–13] More than 90% of the liver mass was recovered in the cell suspension. Purified hepatocytes were prepared by low-speed centrifugation of the original cell suspension.[12] About 70% of the parenchymal cells were regained and the viability (by the trypan blue exclusion test) of these cells was 90–95%. Kupffer cells were prepared by incubating portions of the original cell suspension (4×10^6 cells/ml) with 0·25% pronase in Hanks' medium buffered with 30 mM HEPES (pH 7·45).[14] All parenchymal cells were destroyed after 1 hr. The suspension was then washed three times by centrifugation (500 *g*, 4 min). About 70% of the Kupffer cells in the original suspension were recovered in the washed fraction, and practically 100% of the cells were viable by the trypan blue test. Purified parenchymal cells and Kupffer cells were resuspended in 0·25 M sucrose to a final cell concentration corresponding to 0·25 mg protein per ml, and homogenized until no intact cells were revealed by light microscopy.

RESULTS

Specific activities of lysosomal enzymes in liver homogenates after AAF treatment. Figure 1 shows the effect of AAF feeding on the specific activities of β-glucuronidase and acid DNase in whole liver homogenates. There is a progressive increase in the specific activities of both enzymes during the first 6 weeks of treatment with AAF, and the values reached after this period were about 2 times higher than in controls for both enzymes. There was a detectable increase as early as 3 days after the initiation of the AAF feeding. Figure 1 also indicates that the time course of the alterations in the specific activities was roughly parallel for the two enzymes. The changes in enzyme activities were reversible during the experimental periods used. Thus, if AAF

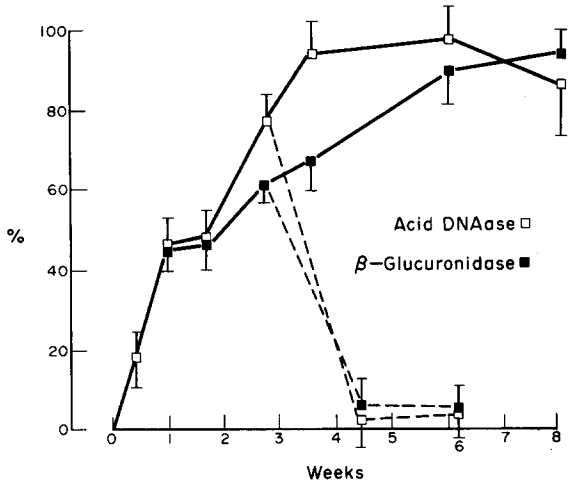

FIG. 1. The time course of changes induced by AAF on the specific acitivities of β-glucuronidase and acid DNase in liver homogenates. Each point represents means ± S.E.M. for seven animals. Enzyme activities are expressed as percentage increase of control values. The dotted curve shows the effect of withdrawal of AAF from the food on the specific activity of the two enzymes. AAF treatment was stopped after 3 weeks of treatment of eight animals. Enzyme activities were determined in liver homogenates prepared from animals that were sacrificed 10 and 20 days (four animals each time) after removal of AAF from the diet.

was withdrawn from the food, both β-glucuronidase and acid DNase returned to normal values in about 7 days (Fig. 1).

Specific activities of lysosomal enzymes in purified Kupffer cells and parenchymal cells. It has been shown that the reticuloendothelial cells of the liver have a high content of acid hydrolases.[14–17] In order to obtain information about the selective contribution from the parenchymal cells and the Kupffer cells to the increase in the specific activities of the two lysosomal enzymes studied here, we purified parenchymal cells and Kupffer cells from control rats and animals that had been treated with AAF for about 4 weeks, at which time the specific activities of the two selected lysosomal enzymes were increased to about twice the control values (Fig. 1). The results of these experiments showed, in agreement with earlier reports,[14,17] that both acid DNase and, to a lesser extent, β-glucuronidase were selectively concentrated in reticuloendothelial cells. Thus, results obtained on control animals showed that the specific activity of acid DNase in Kupffer cells is about 7 times higher than in parenchymal cells (Fig. 2). Figure 2 shows that the specific activities of both enzymes were similar in Kupffer cells prepared from AAF treated animals and control animals, indicating that the AAF feeding did not affect the activities of the lysosomal enzymes in hepatic reticuloendothelial cells. Purified hepatocytes, on the other hand, showed increases in β-glucuronidase and acid DNase after AAF treatment that were at least as large as those found in whole liver homogenates. Figure 2 indicates that purified parenchymal cells contained more than twice the normal amounts of both enzymes after 4 weeks of AAF feeding. The results therefore indicate that the increases in hepatic lysosomal enzyme activities were actually limited to the parenchyme of the liver.

Intracellular distribution of lysosomal enzymes after differential centrifugation. The subcellular distribution patterns of β-glucuronidase and acid DNase in livers from

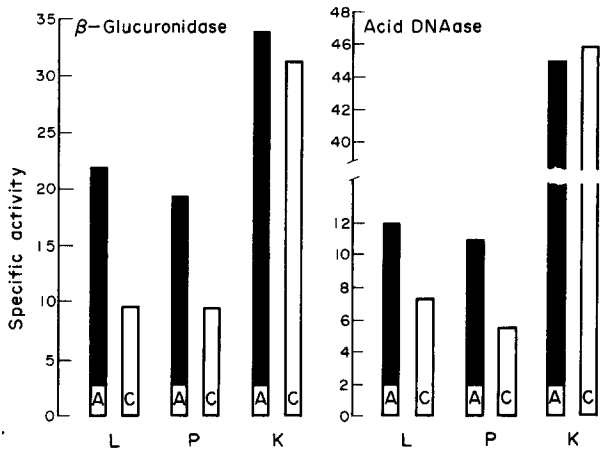

FIG. 2. The effect of AAF treatment for 4 weeks on the specific acitivites of β-glucuronidase and acid DNase in purified Kupffer cells (K) and parenchymal cells (P) and in whole liver homogenates (L) prepared from the livers from which the cells were derived. A: AAF, C: control. Enzyme activities are nmoles/min/mg protein (β-glucuronidase) and nmoles mononucleotide equivalents/min/mg protein (acid DNase).

AAF treated animals and controls are shown in Fig. 3 and Table 1. Results obtained after various times of treatment (1–8 weeks) with AAF were qualitatively similar, and the data in Fig. 3 and Table 1 represent averages for animals that had been treated with AAF for 4–6 weeks. The following features may be noted: (1) The relative specific activities of the enzymes in the nuclear fraction and the heavy mitochondrial fraction were increased after AAF treatment. The increase was particularly evident in the nuclear fraction. After AAF feeding the relative specific activity of both enzymes

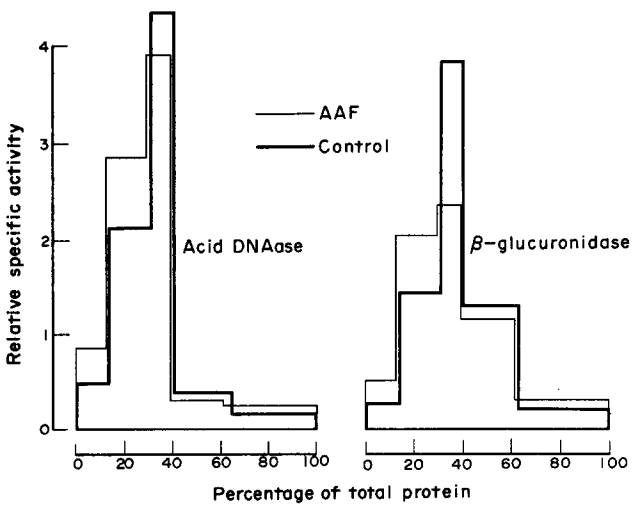

FIG. 3. Distribution patterns of β-glucuronidase and acid DNase after differential centrifugation.[9] Ordinate: mean relative specific activity of fractions (percentage of total recovered activity/percentage of total proteins). Abscissa: relative protein content of fractions (cumulatively from left to right). The data represent means for four experimental and four control animals. Experimental animals were treated with AAF for 4–6 weeks. Fractions are from left to right: nuclear fraction, heavy mitochondrial fraction, light mitochondrial fraction (lysosomal fraction), microsomal fraction and final supernatant.

TABLE 1. ENZYME ACTIVITIES IN HOMOGENATES AND SUBCELLULAR FRACTIONS FROM LIVERS OF CONTROL AND AAF TREATED ANIMALS

	β-Glucuronidase		Acid DNase	
	AAF	Control	AAF	Control
Total activity* in homogenates	19·8 ± 1·2	12·5 ± 0·9	12·9 ± 0·6	9·0 ± 0·9
Distribution† in subcellular fractions				
Nuclear fraction	9·7 ± 1·4	5·3 ± 0·9	10·2 ± 2·0	5·8 ± 1·1
Mitochondrial fraction	32·6 ± 2·7	26·8 ± 2·4	41·3 ± 3·5	35·0 ± 2·2
Lysosomal fraction	24·6 ± 2·2	35·3 ± 1·0	25·9 ± 1·9	41·1 ± 4·2
Microsomal fraction	20·4 ± 1·4	20·6 ± 3·0	8·8 ± 1·0	9·4 ± 0·4
Soluble fraction	12·7 ± 1·5	10·0 ± 1·0	13·7 ± 1·1	8·7 ± 2·2
Total recovery‡ (%)	95·5 ± 2·2	94·3 ± 2·9	99·7 ± 2·1	92·0 ± 2·1

* Enzyme activities in total homogenates are expressed as nmoles/min/mg protein (β-glucuronidase) and nmoles mononucleotide equivalents/min/mg protein (acid DNase).

† Enzyme activities in subcellular fractions are presented as per cent of the total recovered activity in all fractions.

‡ Sum of activity in fractions as per cent of activity in the homogenate.

All values represent means ± S.E.M. for four animals. The AAF treatment lasted for 4–6 weeks.

in this fraction was about twice as high as in control fractions. (2) The relative specific activity of the enzymes in the light mitochondrial fraction was decreased after AAF feeding. (3) The relative specific acitivity of the enzymes in the microsomal fraction was unaltered after AAF treatment. Consonant with earlier findings[9] there was a relatively large amount of β-glucuronidase in this fraction. There was, however, no selective increase in microsomal β-glucuronidase after AAF treatment. (4) There was also a slight increase in the amount of soluble enzymes after AAF treatment. (5) The relative amounts of protein in the different fractions are equal in AAF treated and control animals.

DISCUSSION

The results of this study show that AAF treatment leads to increased specific activities of hepatic acid DNase and β-glucuronidase. The data obtained on purified hepatocytes and Kupffer cells further indicate that the increase in enzyme activities is limited to the liver parenchyme which is that part of the liver that gives rise to hepatomas after AAF feeding. Finally, the increased enzyme activities are accompanied by a redistribution of the enzymes towards heavier cell fractions.

We do not know if the increased amount of lysosomal enzymes is due to increased numbers of lysosomes, or elevated enzyme content per lysosomal particle. Data obtained by Flaks[1] indicates that AAF treatment leads to an increased number of lysosomes per hepatocytes during the early stages. An increased number of lysosomes has also been observed after short-time treatment with other hepatocarcinogens.[2]

The intracellular distribution pattern of acid hydrolases following AAF feeding was characterized by relatively large activities of the enzymes in the nuclear fraction and the heavy mitochondrial fraction. Since the main factor governing the distribution of lysosomal enzymes under these experimental conditions is the size of the particle[19] it is likely that the mean size of the lysosomes is increased following AAF treatment. It is tempting to believe that this may be a reflection of increased cellular

autophagy. Consistent with this is the electronmicroscopic observation that treatment with AAF for 4 weeks leads to an increased number of autophagic vacuoles in liver parenchymal cells.[1] Several observations indicate that cyclic AMP participates in the control of cellular autophagy. Many treatments that affect autophagy in the liver (glucagon[20] and insulin[21] administration, fasting,[22] partial hepatectomy[23]) also lead to changes in cyclic AMP metabolism, and it has been demonstrated that dibutyryl cyclic AMP can stimulate cellular autophagy.[24] It may, in connection with the present study, be of particular interest that the response of adenyl cyclase to adrenaline increases after AAF feeding and that the time course of this alteration is very similar to that observed for the activity of the lysosomal enzymes.[25] It is not known if cyclic AMP, in addition to its effect on autophagy, also controls the amount of lysosomal enzymes. It is interesting, however, that the specific activity of hepatic acid hydrolases and the tissue levels of cyclic AMP seem to change in parallel under different conditions. Thus, there is a concomitant increase both in the tissue levels of cyclic AMP and in the specific activities of hepatic acid hydrolases following partial hepatectomy,[18,26] after prolonged fasting[27,28] and during the perinatal period.[29–31]

Increased acid hydrolase activity can be due to increased synthesis and/or decreased degradation of the enzymes. Primary lysosomes, containing newly synthesized enzymes, are formed in the Golgi region. Hypertrophy of this region has been demonstrated early after AAF feeding.[1] It is therefore likely that AAF treatment leads to increased *de novo* synthesis of lysosomal enzymes. Endocytosis of compounds that can be degraded in lysosomes (e.g. proteins) leads to increased synthesis of lysosomes in macrophages *in vitro*.[32] If a similar mechanism is operative in the hepatocytes, it is of interest to find out if AAF treatment is accompanied by changes in the endocytic rate in these cells.

Whether or not increased lysosomal enzyme activity in the premalignant liver persists in neoplasms developed after AAF treatment remains to be seen. Decreased numbers of lysosomes and an atrophied Golgi region have, however, been observed electronmicroscopically in hepatomas developed after AAF treatment.[33] However, a possible decrease in the digestive capacity of the lysosomes in neoplasms does not rule out the possibility that increased activity of lysosomal enzymes or other types of activations of the lysosomes may play an instrumental role in the carcinogenic process. Uptake of acid hydrolases by the nucleus has, for instance, been proposed as an important step in chemical carcinogenesis.[5] Such an event could alter the genotype and eventually allow the cells to grow independently of normal control mechanisms even after the initial stimulus to this alteration had disappeared.

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