Early modifications of gene expression induced in liver by azo-dye diet

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The expression and regulation of the phospho*enol*pyruvate carboxykinase gene were not grossly modified by feeding rats a 3′-methyl-4-(dimethylamino)azobenzene-containing diet despite maximum expression of the L-type pyruvate kinase gene being dramatically reduced as early as the 24th hour of the carcinogenic diet. Inhibition of aldolase B mRNA synthesis occurred more slowly, being maximum at the 3rd day. After stopping administration of the carcinogen, a very rapid, but transient increase of the L-type pyruvate kinase mRNA was observed at the 24th hour, whereas aldolase B mRNA increased only slowly. The amount of aldolase A mRNA fell quickly after termination of carcinogen administration, levels being normal at the 2nd–3rd day. At this time, the histological structure of the liver was indistinguishable from that of animals still receiving the azo-dye diet. It appears, therefore, that in the rat both administration and withdrawal of the azo-dye carcinogen induce rapid modifications of the expression of some genes, before any cellular modification is distinguishable.

Azo-dye diet *mRNA* *Hepatocarcinogenesis* *Phospho*enol*pyruvate carboxykinase* *Aldolase* *Pyruvate kinase*

1. INTRODUCTION

The azo-dye 3′-methyl-4-(dimethylamino)azobenzene (3′-Me-DAB) is a potent carcinogen which, when administered to rats, induces the development of a hepatoma in 18–20 weeks. Schapira et al. [1,2] have shown that this carcinogenesis is associated with a resurgence of fetal proteins and a decrease in the expression of adult specific markers. We have previously demonstrated [3], at the mRNA level, that as early as the first week of carcinogenic treatment expression of some genes is inhibited (e.g. that of L-type pyruvate kinase and aldolase B genes) while other genes encoding 'fetal' enzymes such as aldolase A are induced. These modifications may be related to early cell alterations produced by the carcinogen, or may reflect, at least in part, direct or indirect molecular interactions between the carcinogen or one of its metabolites and the control region of some genes. Such a possibility is of theoretical importance because it could constitute the basis for the carcinogenic action of the dye.

We therefore looked at very rapid modifications in the amount of messenger RNAs for L-type pyruvate kinase (L-PK), aldolase B, phospho*enol*-pyruvate carboxykinase (PEP-CK) and aldolase A in normal rats being fed the azo-dye diet and in rats after termination of feeding the azo-dye diet for 2 or 4 weeks. Results of RNA analyses were compared to those of histological examination of the livers.

2. MATERIALS AND METHODS

3-month-old Wistar rats were fed a solid diet containing 80% sucrose (high carbohydrate diet), with or without the carcinogenic azo-dye (3′-Me-DAB) [3]. Animals were killed by decapitation at different times after introduction or removal of the

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carcinogen from the diet. After killing, livers were immediately removed and homogenized for subsequent RNA purification.

cDNA probes for L-PK, aldolase B [3] and aldolase A [4,5] have been cloned in our laboratory; subclones of L-PK and aldolase A cDNAs were obtained from the M13 single-stranded phage [6]. The PEP-CK cDNA clone was a gift from Dr R. Hanson.

RNA purification, Northern blot and dot blot analysis were as described [3]. The autoradiograms were scanned using a Shimadzu densitometer and the results of mRNA quantification were expressed in arbitrary 'density' units. Histological examination of the livers from animals fed the carcinogenic diet for various times was performed by Dr André Guillouzo (INSERM, U 49, Rennes).

3. RESULTS AND DISCUSSION

3.1. *Histological analysis of the livers of azo-dye-fed rats*

Livers were histologically analyzed after 1 day and 1, 2, 3 and 4 weeks of carcinogenic diet, and 1 and 2 weeks after termination of the 4-week azo-dye treatment.

No histological anomaly could be detected after the 1st day of treatment. After the 1st week, the main anomalies were signs of acute hepatitis with centrolobular cytolytic necrosis. The same pattern was again observed at the 2nd week, here associated with oval cell proliferation and hyperplasia of the Kupffer cells and biliary ducts. After the 3rd and 4th week, during liver regeneration, hyperplastic foci with dysplastic hepatocytes appeared: the hyperplastic foci increasing between the 3rd and 4th week of treatment. When azo-dye administration was stopped after 4 weeks, the histological pattern after 1 week without azo-dye was practically the same as at the 4th week of drug treatment except for a slight decrease in the inflammatory infiltrates.

3.2. *Expression of the PEP-CK, L-PK and aldolase B genes during the first days and weeks of azo-dye administration*

Fig.1 shows that mRNA for PEP-CK was only slightly modified during the whole course of azo-dye hepatocarcinogenesis. In particular, the mRNA concentration observed after 1 week of

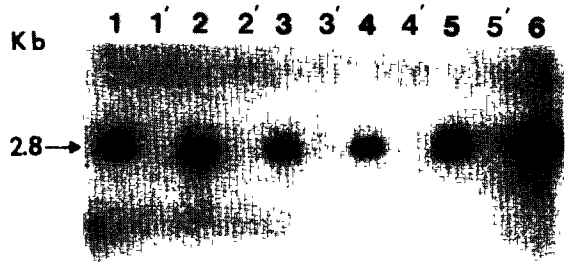

Fig.1. Northern blot analysis was performed in a 1.5% agarose/methylmercury gel. 20 μg total RNA were deposited in each slot. Hybridization was performed using double-strand cDNA probe. The length of PEP-CK mRNA is indicated by an arrow. RNAs were extracted from starved rats – lanes: 1, after 1 week of treatment with 3'-Me-DAB; 2, after 4 weeks treatment; 3, after 9 weeks treatment; 4, after 16 weeks treatment; 5, after 20 weeks treatment. Lane 6 corresponds to RNAs extracted from a normal starved rat. Lanes 1'–5' correspond to rats refed a carbohydrate-rich diet, treated with 3'-Me-DAB for the same respective times.

treatment was the same as in control rats. The PEP-CK gene is regulated by hormones and diet; transcription is induced by fasting, glucagon, cyclic AMP and glucocorticoids and inhibited by carbohydrate diet and insulin [7,9]. Fig.1 demonstrates that the dietary regulation of this gene was not affected by feeding the azo-dye diet; PEP-CK mRNA was undetectable in rats fed the high carbohydrate diet, both with and without 3'-Me-DAB, and was induced in rats fasted for 24 h.

In contrast, mRNA for L-PK fell quickly after the beginning of the carcinogenic diet. After 24 h, it was only 10–20% of the mRNA concentration found in rats fed the same high carbohydrate diet without 3'-Me-DAB (fig.2). Thereafter, as previously shown, L-PK mRNA levels remained low in azo-dye fed rats.

Aldolase B mRNA concentration also decreased in treated rats, but slightly slower than for L-PK mRNA, the concentration at the 3rd day of azo-dye administration being 10–20% of the normal values (fig.3).

3.3. *Rapid modifications of gene expression following cessation of azo-dye administration*

Fig.4 shows the pattern of expression of the aldolase A gene during the first weeks of treatment

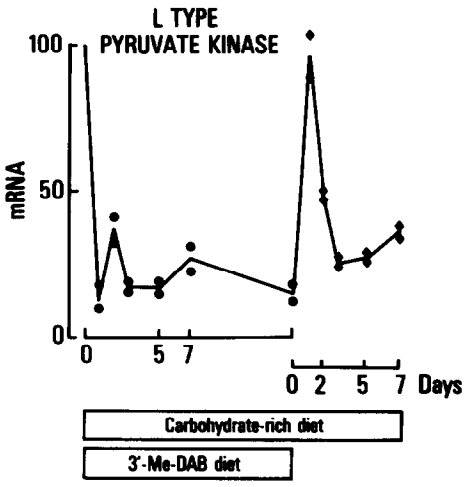

Fig.2. Expression of L-type PK mRNA in rats fed a carbohydrate-rich diet containing 3'-Me-DAB for different times and in rats fed the carbohydrate-rich diet alone after 15 days of azo-dye diet. Each value corresponds to an independent experiment performed on a different rat. The 100% value corresponds to a rat fed a carbohydrate-rich diet for 24 h.

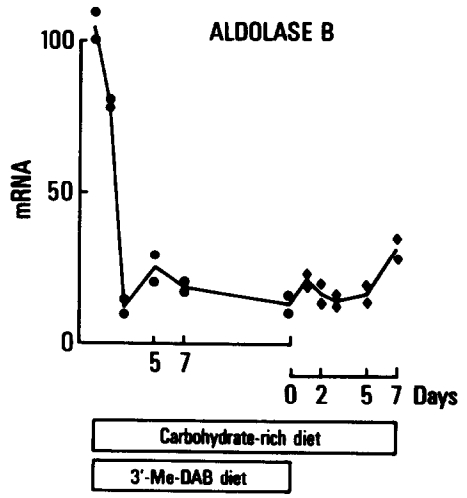

Fig.3. Expression of aldolase B mRNA in rats fed a carbohydrate-rich diet containing 3'-Me-DAB for different times and in rats fed the carbohydrate-rich diet alone after 15 days of azo-dye diet. Each value corresponds to an independent experiment performed on a different rat. The 100% value corresponds to a rat fed a carbohydrate-rich diet for 24 h.

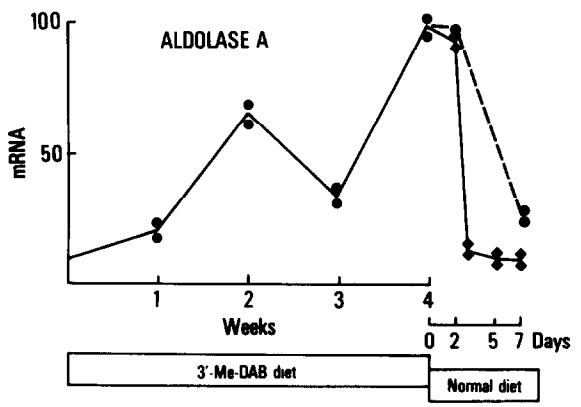

Fig.4. Expression of aldolase A mRNA in rats fed a 3'-Me-DAB diet and in rats fed a normal diet. Each value corresponds to an independent experiment performed on a different rat. The 100% value corresponds to the expression of aldolase B mRNA in rat fetal liver. (●—●) Rats fed a 3'-Me-DAB diet, (◆—◆) rats changed to a normal diet after 4 weeks of the carcinogenic diet.

with 3'-Me-DAB; we again found the biphasic curve reported in [3]. After cessation of drug administration, aldolase mRNA concentration fell very quickly and 5 days after withdrawal of the carcinogen was the same as in nontreated rat livers. However, it is important to recall at this time that the histological pattern of the liver is similar to that of rats receiving carcinogen with in particular the same abundance of oval cells.

Fig.2 shows that removal of the carcinogen from the diet induces a precocious, intense but transient increase in L-PK mRNA concentration. In contrast, the increase in the expression of the aldolase B gene after cessation of 3'-Me-DAB is slow (fig.3).

The very rapid modifications of L-PK gene expression observed after administration and withdrawal of the carcinogenic drug precede any detectable alteration of hepatocytes and seem to be related to a direct or indirect molecular effect of the azo-dye; the L-PK gene is accurately regulated at both transcriptional and post-transcriptional levels in rat liver [10]; it is induced by carbohydrates and insulin and inhibited by glucagon and cyclic AMP [10,11]. It could therefore be hypothesized that the rapid decrease in L-PK mRNA after ingestion of 3'-Me-DAB and its acute

increase after cessation of dye administration are mediated via either changes in intracellular cyclic AMP concentration, or modifications in the systems responsible for carbohydrate and insulin effects. We have, however, previously demonstrated that, though the maximum expression of the L-PK and aldolase B genes in carbohydrate-refed rats was reduced, the capacity of these genes to be regulated by diet was not modified by carcinogen ingestion [3]. Moreover, the PEP-CK gene is also regulated by cyclic AMP and carbohydrates + insulin, but in an opposite direction compared to the L-PK gene [7,9]. We show here that neither the maximum expression of the gene nor the extent of its regulation was modified in azo-dye-treated rats; in particular, mRNA concentrations were very low in carbohydrate-fed rats and abundant in rats fasted for 24 h, which signifies that the response to insulin, carbohydrate and cyclic AMP in the hepatocytes from azo-dye-treated rats was unaffected. The 'molecular' effects of the carcinogen seem therefore to be relatively specific to some genes and not to result from global modifications in cell metabolism. The aldolase B gene exhibits intermediate sensitivity to administration of the azo dye.

The increase in the expression of the aldolase A gene follows proliferation of the oval cells, being maximum at the 2nd and 4th week. In situ hybridization experiments (Lamas et al., unpublished) confirm that these cells are indeed responsible for the increase in aldolase A mRNA. However, the rapid decrease in this mRNA after cessation of the drug administration is striking since, in contrast, histological modifications are very slow, and are undetectable at the time when the aldolase A mRNA concentration returns to a 'normal' value. This result indicates that after removal of 3'-Me-DAB from the diet the oval cells which are still present synthesize only low amounts of aldolase A mRNA, that is to say that the drug (directly or indirectly) is active in stimulating expression of the aldolase A gene in these cells.

In conclusion we demonstrate in this paper that administration of 3'-Me-DAB is able to modify very quickly the expression of some genes, independently of its slower action on the liver cell populations. We can speculate that such molecular effects of the carcinogen constitute the basis for its cellular action and also perhaps for its carcinogenic potency.

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