Increase in class 2 aldehyde dehydrogenase expression by arachidonic acid in rat hepatoma cells

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Aldehyde dehydrogenase (ALDH) is a family of several isoenzymes important in cell defence against both exogenous and endogenous aldehydes. Compared with normal hepatocytes, in rat hepatoma cells the following changes in the expression of ALDH occur: cytosolic class 3 ALDH expression appears and mitochondrial class 2 ALDH decreases. In parallel with these changes, a decrease in the polyunsaturated fatty acid content in membrane phospholipids occurs. In the present study we demonstrated that restoring the levels of arachidonic acid in 7777 and JM2 rat hepatoma cell lines to those seen in hepatocytes decreases hepatoma cell growth, and increases class 2 ALDH activity. This latter effect appears to be due to an increased gene transcription of class 2 ALDH. To account for this increase, we examined whether peroxisome-proliferator-activated receptors (PPARs) or

lipid peroxidation were involved. We demonstrated a stimulation of PPAR expression, which is different in the two hepatoma cell lines: in the 7777 cell line, there was an increase in PPAR α expression, whereas PPAR γ expression increased in JM2 cells. We also found increased lipid peroxidation, but this increase became evident at a later stage when class 2 ALDH expression had already increased. In conclusion, arachidonic acid added to the culture medium of hepatoma cell lines is able to partially restore the normal phenotype of class 2 ALDH, in addition to a decrease in cell growth.

Key words: ALDH, hepatocytes, isoenzymes, lipid peroxidation, peroxisome-proliferator-activated receptor (PPAR).

INTRODUCTION

Aldehyde dehydrogenase (ALDH) is a family of several isoenzymes [1,2] important in cell defence against exogenous toxic aldehydes [1,3] and endogenous aldehydes such as those derived from lipid peroxidation [4,5]. The latter appear to influence cell growth and differentiation in some tumour cell lines [6,7]. The ALDH family is widely expressed in tissues and subcellular components, but with some differences regarding the individual isoenzymes. For example, in liver class 2 mitochondrial ALDH is well expressed, whereas class 3 cytosolic ALDH is not present [8–10]. In the cornea and lens, there are high levels of class 3 cytosolic ALDH [11,12], the biological role of which could be important in the oxidation of peroxidic aldehydes, in UV-B photoprotection, and in preventing oxidative damage by free radical species [11].

Compared with normal hepatocytes, cytosolic class 3 ALDH appears in rat hepatoma cells, whereas the levels of class 2 ALDH are significantly decreased [5,8,9,13]. The activity of class 3 ALDH increases with the degree of deviation in hepatoma cell lines, as also occurs in chemically induced hepatocarcinogenesis *in vivo* [5,14–16]. Because class 3 ALDH is important in the metabolism of medium-chain-length aldehydes derived from lipid peroxidation and certain anti-cancer drugs, such as cyclophosphamide [1,5,17], it appears to be important in the induction of selective growth advantage and drug resistance in hepatoma cells.

Class 2 ALDH is expressed in a large number of tissues, with the highest levels occurring in liver, kidney, muscle and heart

[18]. It is synthesized as a high-molecular-mass precursor in the cytosol and transported into the mitochondrial matrix space, where it is processed to the mature enzyme [19]. On the basis of enzymological [20], metabolic [21] and genetic studies [22], it is believed that class 2 ALDH is mainly responsible for the oxidation of acetaldehyde generated during ethanol oxidation *in vivo*. Acetaldehyde has an important role in the effects of ethanol, not only in the liver, but also in the brain [24]. Its levels are governed by the rate of formation and the rate of removal, with the latter being due to the level of ALDH. Approx. 50% of Orientals have significantly decreased class 2 ALDH activity, owing to a single amino acid substitution (Glu⁴⁸⁷ → Lys), which results in non-functional enzyme [25]. This mutant enzyme is encoded by the *ALDH2*2* allele, which might thus contribute to the susceptibility to alcoholic liver disease via acetaldehyde cytotoxicity [26,27].

In the liver, class 2 ALDH is also important in the metabolism of 4-hydroxynonenal, the most important aldehyde derived from lipid peroxidation [5,16,23], even if it is not the principal metabolizing enzyme. In fact, as we reported elsewhere [5], 4-hydroxynonenal is metabolized preferentially by glutathione S-transferase and alcohol dehydrogenase located in the cytosol. In hepatoma cell lines, there are changes in the pattern of metabolism in correlation with the degree of deviation: class 3 cytosolic ALDH assumes importance in 4-hydroxynonenal metabolism [5,16], whereas class 2 mitochondrial ALDH, glutathione S-transferase and alcohol dehydrogenase decrease their metabolic capability [9]. There is an increase in class 3 ALDH activity, as described above, and a decrease in the activity of other enzymes.

Abbreviations used: ALDH, aldehyde dehydrogenase; PPAR, peroxisome-proliferator-activated receptor; PPARE, peroxisome-proliferator-response element.

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In parallel with the appearance of class 3 ALDH and the decrease of class 2 ALDH isoenzymes, in hepatoma cells there is a decrease in lipid peroxidation owing to the reduction of the percentage content of polyunsaturated fatty acids [5]. In a previous paper we demonstrated that, when lipid peroxidation is restored in hepatoma cell lines, by enriching them with arachidonic acid and treating them with pro-oxidants, there is an inhibitory effect on class 3 ALDH that is due to decreased gene transcription of class 3 ALDH [28,29]; this is accompanied by cell death or a decrease in cell growth, proportionate to the extent of lipid peroxidation. In 7777 hepatoma cells with a low basal level of class 3 ALDH, the products of restored lipid peroxidation almost immediately cause a reduction of cell growth and, when their concentration is such as to result in the complete disappearance of class 3 ALDH, they induce cell death. In JM2 hepatoma cells, which have a high basal level of class 3 ALDH, only when the products of restored lipid peroxidation decrease class 3 ALDH activity by more than 50% is there a considerable decrease in cell growth. The present study examined class 2 ALDH, which is decreased in hepatoma cells, in order to determine if it is possible to restore the normal enzyme activity level by arachidonic acid supplementation. We limited our observations to the effect of enriching hepatoma cell lines with arachidonic acid alone, without adding pro-oxidants, and to the possible mechanisms underlying this effect.

EXPERIMENTAL

Culture conditions

Hepatoma cell lines (7777 and JM2) were seeded (day zero) and maintained for 24 h in medium A [Dulbecco's modified Eagle's medium/F12 supplemented with 2 mM glutamine, 1% (v/v) antibiotic/antimycotic solution] plus 10% (v/v) newborn-calf serum; 24 h later (day 1), medium A was removed and replaced with medium B [medium A supplemented with 0.4% (w/v) albumin, 1% (v/v) insulin/transferrin/sodium selenite ('ITS'), 1% (v/v) non-essential amino acids and 1% (v/v) vitamin solution] with or without arachidonic acid (250, 400 or 600 nmol/10⁶ cells, which corresponds to 40, 60 or 90 μ M respectively). On day 2 (24 h later), medium B supplemented with arachidonic acid was removed and replaced with unsupplemented medium B, and the cells were maintained in culture for a further 2 days (days 3 and 4). Parameters were measured on untreated control cells and on cells treated with arachidonic acid. Cells were harvested at days 2, 3 and 4.

Fatty acid content

The percentage content of fatty acids in phospholipids extracted from cells in total and from mitochondria were measured as described by Canuto et al. [7].

Preparation of the mitochondrial fraction

Cell suspensions obtained from hepatoma cell cultures were sedimented by centrifugation at 600 *g* for 10 min. Homogenates were obtained by disruption of cell pellets with a hand-driven TenBroeck® glass homogenizer; pellets were suspended in a volume of hypotonic medium containing 17.5 mM sucrose, 55 mM mannitol, 5 mM Tris/HCl buffer, pH 7.4, and 0.5 mM EGTA, which corresponded to 2.5 $\times$ the mass of the pellet. The homogenates were then diluted to 20% (w/v) with sucrose and mannitol to generate an isotonic medium, and mild sonication was performed; diluted homogenates were centrifuged at 1500 *g*

for 6.5 min (Beckman centrifuge J-6B). From the collected supernatants, the mitochondrial fractions were isolated by centrifugation at 32000 *g* for 1 min (rotor 50Ti; Beckman ultracentrifuge L8-65). The supernatants were removed and the pellets (mitochondrial fraction) were resuspended in 250 mM sucrose and 20 mM Tris/HCl, pH 7.4, and used for fatty acid and enzyme activity determination.

Enzyme activity determination

ALDH activity was determined as described in Canuto et al. [5]. Benzaldehyde (2.5 mM), 4-hydroxynonenal (0.1 mM), propionaldehyde (1 mM) or acetaldehyde (10 mM) were used as substrate.

Cell growth

Cell growth was evaluated as the number of cells present in the monolayer.

Lipid peroxidation

The extent of lipid peroxidation was evaluated by spectrophotometric determination of malondialdehyde as described in Canuto et al. [7].

Western blot analysis

The cells were homogenized in a lysing buffer and used for Western blot analysis, which was performed as described by Canuto et al. [28], except that anti-(class 2 ALDH) antibody was used.

Northern blot analysis

Northern blot analysis was performed as described by Canuto et al. [28], except for the quantity of RNA, which was 30 μ g for peroxisome-proliferator-activated receptor α (PPAR α) and 15 μ g for PPAR γ and class 2 ALDH. Rat class 2 ALDH or PPAR α and PPAR γ cDNA probes were used.

Nuclear run-off assay

Cell suspensions obtained from hepatoma cell cultures were sedimented by centrifugation at 600 *g* for 10 min. Homogenates were obtained by disruption of cell pellets with a hand-driven TenBroeck® glass homogenizer; pellets were suspended in a volume of 0.32 M sucrose and 10 mM MgCl₂, pH 7.4, corresponding to 5 $\times$ the mass of the pellet. The homogenates were centrifuged at 1500 *g* for 6.5 min (Beckman centrifuge J-6B). The supernatants were removed and the pellets (nuclear fraction) were suspended in storage buffer [50 mM Tris/HCl (pH 8.3)/40% (v/v) glycerol/5 mM MgCl₂/0.1 mM EDTA] and stored at -80 °C. Aliquots corresponding to 1.5 $\times$ 10⁷ nuclei for 7777 cells and 3.0 $\times$ 10⁷ nuclei for JM2 cells were sedimented by centrifugation (1500 *g* for 6.5 min), and were then re-suspended in 100 μ l of nuclear run-off buffer [50 mM Hepes/NaOH (pH 7.4)/15% (v/v) glycerol/1.0 mM MgCl₂/50 mM KCl/5 mM dithiothreitol/1.0 mM ATP/1.0 mM CTP/1.0 mM GTP] and 8 μ l of [α -³²P]UTP (3000 Ci/mmol; Amersham International, Milan, Italy). The transcriptional run-off was allowed to proceed at 30 °C for 60 min. The mRNA species were isolated as described by Canuto et al. [28]. The class 2 ALDH and 18 S cDNAs, denatured with 6 $\times$ SSC (where 1 $\times$ SSC = 0.15 M NaCl/0.015 M sodium citrate) for 10 min at 100 °C, were applied to

nitrocellulose membrane (1 µg/slot) in a slot-blot apparatus. Pre-hybridization and hybridization were performed as described by Canuto et al. [28].

Statistical analysis

All data are expressed as means ± S.D. The significance of differences between group means was assessed by variance analysis, followed by the Newman–Keuls test.

RESULTS

Two rat hepatoma cell lines were used: 7777 and JM2, with different degrees of deviation. Similar to chemically induced hepatomas *in vivo*, these hepatoma cells are characterized by a lower percentage content of arachidonic acid than normal hepatocytes [5]. The decrease of this fatty acid was evident in the phospholipids extracted both from whole cells and from mitochondria. The decreases were so large that they did not correlate with the degree of tumour deviation (Table 1). The 7777 and JM2 cell lines were also characterized by differences in ALDH activity (Table 2). The activity determined in the cytosol reflected both class 1 and class 3 ALDH activities, and was higher in the more deviated JM2 cells than in 7777 cells. Cytosolic ALDH activities of both hepatoma cell lines were higher than in hepatocytes,

consistent with earlier studies [1,8,14,15]. On the other hand, the activity of class 2 ALDH determined in mitochondria was similar in both hepatoma cell lines, but was significantly lower than that of hepatocytes, using either acetaldehyde or 4-hydroxynonenal as substrate.

On the basis of the above observations, 7777 and JM2 cells were enriched with arachidonic acid in order to increase the percentage content of this fatty acid to that present in normal rat hepatocytes. Enrichment of culture media with arachidonic acid increased arachidonic acid content in phospholipids extracted from both cell lines (Table 1). Arachidonic acid content returned to normal values in phospholipids extracted from whole cells, and reached about 65% and 53% of the normal value in mitochondrial phospholipids of 7777 and JM2 hepatoma cells, respectively.

Arachidonic acid enrichment slowed the growth of 7777 and JM2 cells (Figures 1 and 2), which remained lower than that of non-enriched cells throughout the experimental period. The decrease in the number of cells in both enriched 7777 and JM2 cells became evident 24 h after the removal of arachidonic acid (day 3 in the Figures).

After 4 days, the numbers of 7777 cells in culture enriched with 250 or 400 nmol of arachidonic acid per 10⁶ cells were 83% or 71% respectively of those of non-enriched cultures. Concomitantly, the numbers of JM2 cells in cultures enriched with 250 or 400 nmol of arachidonic acid per 10⁶ cells were 84% or 80% respectively of those of non-enriched cells. Arachidonic acid at 400 nmol produced a slightly smaller effect on JM2 cells: at this concentration, the difference in percentage survival between enriched 7777 and enriched JM2 cells was significant ($P < 0.05$). It was possible to obtain a further decrease in the numbers of both 7777 and JM2 cells by treating arachidonic-acid-enriched cells with pro-oxidants (results not shown). In this case, increased production of lipid peroxidation aldehydes induced by pro-oxidants was responsible for a more marked decrease in cell numbers, as reported in a previous paper [29].

In hepatoma cells enriched with arachidonic acid, the expression of class 2 ALDH was examined. Both hepatoma cell lines enriched with arachidonic acid possessed more class 2 ALDH mRNA compared with non-enriched cells (Figure 3). Arachidonic acid was thus partially able to restore the expression of the class 2 *ALDH* gene to normal levels, as deduced by comparison with the class 2 ALDH mRNA content in normal hepatocytes (Figure 5; lane H). Interestingly, expression of class 2 ALDH mRNA in both hepatoma cell lines increased with the quantity of arachidonic acid added to the cells (up to 400 nmol per 10⁶ cells). At 600 nmol, the class 2 ALDH mRNA levels were higher than in non-enriched cells, but lower than with 400 nmol. The increased content of class 2 ALDH mRNA in both cell lines, enriched with 250 or 400 nmol of arachidonic acid per 10⁶ cells, is due to increased transcription, as evaluated by using a run-off technique (Figure 4).

Since enrichment with 400 nmol of arachidonic acid caused the highest increase of class 2 ALDH mRNA, we used this concentration to monitor the effect of the enrichment with arachidonic acid after its removal from culture medium (Figure 5). In 7777 hepatoma cells, the increased level of mRNA remained almost constant during the 48 h period. In JM2 cells, class 2 ALDH mRNA levels increased for a further 24 h after arachidonic acid removal, and returned to the non-enriched level after 48 h.

The levels of class 2 ALDH protein and of the enzyme activity were also examined in hepatoma cells 24 h after enrichment with 400 nmol of arachidonic acid, and during the 2 days after removal of arachidonic acid.

Table 1 Percentage of arachidonic acid in phospholipids extracted from hepatocytes and hepatoma cell lines

Data are expressed as percentages of total fatty acids, and are shown as the means ± S.D. from five experiments. The rat hepatoma cells were enriched with arachidonic acid as in the Experimental section. Means with different superscripted letters are statistically different ($P < 0.001$) from one another, as determined by variance analysis followed by the Newman–Keuls test.

		Arachidonic acid (nmol/10 ⁶ cells)	
Treatment...	None	250	400
Whole cells			
Hepatocytes	23.49 ± 1.91 ^a		
7777 hepatoma	5.43 ± 2.01 ^b	18.97 ± 2.88 ^c	24.62 ± 1.98 ^a
JM2 hepatoma	4.82 ± 0.93 ^b	16.50 ± 5.66 ^c	24.50 ± 2.67 ^a
Mitochondria			
Hepatocytes	28.30 ± 3.72 ^a		
7777 hepatoma	1.09 ± 0.15 ^b	14.37 ± 1.65 ^c	18.63 ± 1.98 ^d
JM2 hepatoma	1.23 ± 0.12 ^b	12.53 ± 1.99 ^c	15.08 ± 2.01 ^{c,d}

Table 2 Aldehyde dehydrogenase activity in mitochondria and cytosol isolated from hepatocytes and hepatoma cell lines

Data are expressed as nmol of NADH produced/min per mg of protein and are the means ± S.D. for three experiments. Benzaldehyde (2.5 mM; BA), acetaldehyde (10 mM; ACA), propionaldehyde (1 mM; PA) or 4-hydroxynonenal (0.1 mM; 4-HNE) were used as the substrate. For each substrate, means with different superscripted letters are statistically different ($P < 0.001$) from one another, as determined by variance analysis followed by the Newman–Keuls test.

	Hepatocytes	7777	JM2
Cytosol			
BA	2.27 ± 0.51 ^a	1.23 ± 0.19 ^a	597.63 ± 93.21 ^b
4-HNE	1.64 ± 0.32 ^a	3.32 ± 0.51 ^a	12.25 ± 4.71 ^b
PA	2.33 ± 0.48 ^a	4.35 ± 0.76 ^a	41.73 ± 6.89 ^b
Mitochondria			
ACA	15.20 ± 3.01 ^a	0.51 ± 0.11 ^b	1.67 ± 0.52 ^c
4-HNE	4.31 ± 0.81 ^a	0.69 ± 0.31 ^b	1.51 ± 0.24 ^b

Figure 1 Cell growth in 7777 hepatoma cells enriched with arachidonic acid

Data shown are the means $\pm$ S.D. from eight experiments. Enrichment with arachidonic acid and cell harvesting were as described in Table 3. Means with different letters are statistically different ($P < 0.05$) from one another, as determined by variance analysis followed by the Newman–Keuls test. ●, no. of 7777 hepatoma cells untreated with arachidonic acid; ■, no. of 7777 hepatoma cells treated with 250 nmol of arachidonic acid; ▲, no. of 7777 hepatoma cells treated with 400 nmol of arachidonic acid.

Figure 2 Cell growth in JM2 hepatoma cells enriched with arachidonic acid

Data are means $\pm$ S.D. from eight experiments. Enrichment with arachidonic acid and cell harvesting were as described in Table 3. Means with different letters are statistically different ($P < 0.05$) from one another, as determined by variance analysis followed by the Newman–Keuls test. ●, no. of JM2 hepatoma cells untreated with arachidonic acid; ■, no. of JM2 hepatoma cells treated with 250 nmol of arachidonic acid; ▲, no. of JM2 hepatoma cells treated with 400 nmol of arachidonic acid.

In both 7777 and JM2 cells, changes in class 2 ALDH protein (Figures 6 and 7) were in agreement with changes in class 2 ALDH mRNA levels. Class 2 ALDH protein content was increased by arachidonic acid in both hepatoma cell lines, even if it did not reach the normal level found in hepatocytes. Moreover, reduction in class 2 ALDH protein levels after removal of arachidonic acid was not immediate; at the end of the experiment (day 4), the most marked decrease was in JM2 cells.

Class 2 ALDH activity was measured with 10 mM acetaldehyde as substrate (Table 3) in mitochondria isolated from both cell lines. In agreement with the results obtained for the mRNA and protein content, the activity increased after arachidonic acid enrichment, and remained higher than in the control cells even after removal of the fatty acid.

In an attempt to elucidate the mechanism involved in increasing class 2 ALDH activity, expression of PPARs and the level of

lipid peroxidation were determined. We focused our attention on PPARs and lipid peroxidation because both regulate the expression of several genes. Polyunsaturated fatty acids are known to activate expression of certain genes via direct interaction of PPARs with peroxisome-proliferator-response elements (PPREs) located in the controlling regions of those genes [30]. Polyunsaturated fatty acids are also known to induce lipid peroxidation, being the natural substrate for this process. Some aldehydes derived from lipid peroxidation activate or inhibit gene expression, in correlation with the quantity present in the cells and/or with the type of cells [29,31].

PPAR α expression was absent from non-enriched 7777 and JM2 hepatoma cells. It increased after addition of arachidonic acid in 7777, but not in JM2, cells. In 7777 cells, the increase was evident after the addition of either 250 or 400 nmol of arachidonic acid, the concentrations causing an increase in the expression of

Figure 3 Effect of different concentrations of arachidonic acid on mRNA content of class 2 ALDH in hepatoma cells

Cells enriched with arachidonic acid at day 1 were harvested 24 h after fatty acid addition, and examined for mRNA content. The bar graph represents the quantitative analysis. 7777 hepatoma cells were untreated (lane 1) or treated with 250 nmol (lane 2), 400 nmol (lane 3) or 600 nmol (lane 4) of arachidonic acid. Similarly, JM2 hepatoma cells were untreated (lane 5) or treated with 250 nmol (lane 6), 400 nmol (lane 7) or 600 nmol (lane 8) of arachidonic acid.

7777 CELLS

	Class 2 ALDH	18 S
Control		
Arachidonic acid (250 nmoles/10 ⁶ cells)		
Arachidonic acid (400 nmoles/10 ⁶ cells)		

JM2 CELLS

	Class 2 ALDH	18 S
Control		
Arachidonic acid (250 nmoles/10 ⁶ cells)		
Arachidonic acid (400 nmoles/10 ⁶ cells)		

Figure 4 Nuclear transcription in hepatoma cells enriched with different concentrations of arachidonic acid

Nuclear transcripts were isolated from equal numbers of nuclei from hepatoma cells enriched or not with arachidonic acid (250 or 400 nmol per 10^6 cells) for 24 h, as described in the Experimental section. The radiolabelled transcripts were hybridized to slot-blotted class 2 ALDH and 18 S cDNAs.

class 2 ALDH mRNA. PPAR γ expression was absent in 7777 cells before and after enrichment with arachidonic acid, whereas it was marginally detectable in untreated JM2 cells and increased

Figure 5 Effect of arachidonic acid on mRNA content of class 2 ALDH in hepatoma cells over time

Enrichment with arachidonic acid (400 nmol per 10^6 cells) and cell harvesting were as described in the legend to Table 3. The bar graph represents the quantitative analysis. 7777 hepatoma cells were harvested at day 2 (lane 2), day 3 (lane 4) or day 4 (lane 6). Lanes 1, 3 and 5 show control cells for lanes 2, 4 and 6 respectively. JM2 hepatoma cells were harvested at day 2 (lane 8), day 3 (lane 10) or day 4 (lane 12). Lanes 7, 9 and 11 show control cells for lanes 8, 10 and 12 respectively; lane H, hepatocytes.

Figure 6 Protein content of class 2 ALDH in 7777 hepatoma cells enriched with arachidonic acid determined over time

Enrichment with arachidonic acid (400 nmol per 10^6 cells) and cell harvesting were as described in the legend to Table 3. The bar graph represents the quantitative analysis. 7777 hepatoma cells were harvested at day 2 (lane 5), day 3 (lane 6) or day 4 (lane 7). Lanes 2, 3 and 4 show control experiments for lanes 5, 6 and 7 respectively; lane 1, hepatocytes.

very significantly after addition of arachidonic acid. The increase was evident at 250 nmol of arachidonic acid, and was even higher at 400 nmol. The results on PPAR expression are presented in Figure 8, which shows the behaviour of PPAR α in 7777 cells and of PPAR γ in JM2 cells. The behaviour of PPAR α in JM2 cells is not included in Figure 8, since this gene was expressed neither before nor after administration of arachidonic acid. For the same reason, neither is the behaviour of PPAR γ in 7777 cells included in Figure 8.

Lipid peroxidation, determined as the production of malondialdehyde, increased in both cell lines after arachidonic acid treatment, the most marked increase being in 7777 cells (Figure 9).

Figure 7 Protein content of class 2 ALDH in JM2 hepatoma cells enriched with arachidonic acid determined over time

Enrichment with arachidonic acid (400 nmol per 10^6 cells) and cell harvesting were as described in the legend to Table 3. The bar graph represents the quantitative analysis. JM2 hepatoma cells were harvested at day 2 (lane 5), day 3 (lane 6) or day 4 (lane 7). Lanes 2, 3 and 4 show control experiments for lanes 5, 6 and 7 respectively; lane 1, hepatocytes.

Table 3 Aldehyde dehydrogenase activity in mitochondria isolated from hepatoma cell lines enriched or not with arachidonic acid

Data are expressed as nmol of NADH produced/min per mg of protein and are the means $\pm$ S.D. for five experiments. Acetaldehyde (10 mM) was used as the substrate. Arachidonic acid (ARA) was added to the cells at day 1, maintained in the culture until day 2, and then removed. Cells enriched or not with ARA were harvested 24 h after fatty acid addition (day 2) and during the days (day 3 and 4) following removal of the fatty acid. For each cell line, means with different letters are statistically different ($P < 0.05$) from one another, as determined by variance analysis followed by the Newman-Keuls test.

Cells	Day ...	ALDH activity		
		2	3	4
7777		0.57 $\pm$ 0.11 ^a	0.93 $\pm$ 0.19 ^b	0.60 $\pm$ 0.21 ^{a,b}
7777 + ARA		1.91 $\pm$ 0.09 ^c	2.82 $\pm$ 0.21 ^d	2.65 $\pm$ 0.41 ^d
JM2		2.18 $\pm$ 0.48 ^a	2.13 $\pm$ 0.26 ^a	1.65 $\pm$ 0.29 ^a
JM2 + ARA		4.60 $\pm$ 0.31 ^b	5.79 $\pm$ 0.31 ^c	4.23 $\pm$ 0.24 ^b

Figure 8 Expression of PPAR α and PPAR γ in hepatoma cells enriched or not with arachidonic acid

The cells enriched with arachidonic acid at day 1 were harvested 24 h after fatty acid addition, and examined for mRNA content. Shown are 7777 or JM2 hepatoma cells untreated (lane 1) or treated with 250 nmol (lane 2) or 400 nmol (lane 3) of arachidonic acid.

DISCUSSION

The decrease in arachidonic acid content in hepatoma cells may be seen as an advantage for tumour cell proliferation. Indeed, increasing the arachidonic acid content decreases hepatoma cell growth in a dose-dependent manner and in correlation with the degree of deviation, as we have shown in previous studies [7,29,32]. These results agree with those of other researchers, who have demonstrated that exogenously supplied ω -6 polyunsaturated fatty acids kill malignant cells selectively [33–35]. 7,12-Dimethylbenz[*a*]anthracene-initiated mouse skin does not develop to the tumour stage if treated with arachidonic acid [36]. HepG2 cells undergo cytotoxicity and apoptosis when treated with arachidonic acid [37], and these effects are probably due to the involvement of increased lipid peroxidation induced by arachidonic acid enrichment [38–39].

This study examined the effect of arachidonic acid on ALDH. We focused our particular attention on the effect of arachidonic acid on class 2 ALDH, which is an isoenzyme located in mitochondria that significantly decreases in hepatoma cells in comparison with normal hepatocytes. In both hepatoma cell lines examined, arachidonic acid content increased in the phospholipids of the cells exposed to this fatty acid, in correlation with the quantity added to the culture medium. In parallel with the increase of arachidonic acid, an increase of class 2 ALDH activity was observed. When arachidonic acid reached the percentage content of normal cells in phospholipids extracted from whole cells, and approx. 50% of the normal level in phospholipids extracted from mitochondria, it caused the highest level of class 2 ALDH expression. The increase of class 2 ALDH mRNA was due to an increase of transcription, as demonstrated by the run-off technique. The increases that were observed in class 2 ALDH mRNA content were in agreement with those of class 2 ALDH protein levels, and both were reflected in increased class 2 ALDH enzyme activity. Thus increasing arachidonic acid levels to those present in normal hepatocytes is able to partially restore the normal phenotype of class 2 ALDH. The two types of hepatoma cells are almost equally responsive to the effect of arachidonic acid on class 2 ALDH, whereas they show a slight difference in growth behaviour in the presence of arachidonic acid, i.e. the 7777 cells are slightly more susceptible than JM2 cells.

Having established the restorative effect of arachidonic acid on class 2 ALDH, it was important to identify possible mechanism(s) by which arachidonic acid enrichment increases class 2 ALDH expression. One possible mechanism is via the PPARs, i.e. the increase of class 2 ALDH expression might be mediated by activation of the class 2 ALDH gene by PPARs. PPARs belong to a family of nuclear receptors and are activated by several ligands, such as peroxisome proliferators (phthalates, fibrates, pesticides) and fatty acids [40]. Three types of PPARs, α , β/δ and γ , have been characterized. The α isoform regulates fatty acid metabolism and is the major isoform present in normal rat liver; PPAR β/δ is ubiquitously expressed, but its physiological function is still unclear. PPAR γ is present in liver (PPAR γ 1) and adipose tissue (PPAR γ 2) [41]. PPARs link with the retinoid-X receptor, and therefore activate several genes containing a PPRE. Crabb and colleagues [42] have shown that the human class 2 ALDH gene promoter contains a binding site, designated FP330-3', which can bind members of the nuclear receptor superfamily, including PPARs. The presence of a PPRE site in the class 2 ALDH gene promoter suggests that activation of this gene can be mediated by binding of fatty-acid-liganded PPARs to this PPRE. In the hepatoma cell lines we studied, enrichment with arachidonic acid caused an increase in PPAR expression that differed

Figure 9 Lipid peroxidation in hepatoma cells enriched or not with arachidonic acid

Lipid peroxidation was quantified by determination of malondialdehyde (MDA) production. Data shown are means $\pm$ S.D. from eight experiments. Enrichment with arachidonic acid and cell harvesting were as described in the legend to Table 3. For each cell line, means with different letters are statistically different ($P < 0.05$) from one another, as determined by variance analysis followed by the Newman-Keuls test. The amount (nmol) of MDA produced in hepatoma cells untreated with arachidonic acid is shown by the white bars; also shown are the amounts of MDA produced in hepatoma cells treated with 250 nmol (grey-shaded bars) or 400 nmol (black-shaded bars) of arachidonic acid.

in the two cell lines. In 7777 cells (the less deviated cells), it activated PPAR α , whereas in JM2 cells (the more deviated cells) it increased PPAR γ . A difference between the two cell lines was already present in non-enriched cells: in 7777 cells, both PPAR α and PPAR γ were not expressed, whereas in JM2 cells PPAR γ expression was barely detectable. In conclusion, it may be said that the class 2 *ALDH* gene is activated by arachidonic acid via induction of PPARs. At present, we do not know why arachidonic acid should induce a different PPAR isoform in the two hepatoma cell lines, but it is possible that the difference is due to a different degree of deviation of the two cell lines.

Even if PPARs can transactivate the class 2 *ALDH* gene, we wanted to consider whether a second mechanism might be involved in arachidonic acid up-regulation of the class 2 *ALDH* gene: it appeared possible that malondialdehyde and 4-hydroxynonenal, both derived from lipid peroxidation, also play a role in class 2 *ALDH* gene activation. 4-Hydroxynonenal has been found to be able to induce the expression of glutathione S-transferase A1 via an antioxidant-response element, present in the promoter of this gene [43]. 4-Hydroxynonenal is also able to induce the expression of aldehyde reductase [31], the *c-jun* proto-oncogene [44], and to stimulate transforming growth factor- β ('TGF β ') production [45]. 4-Hydroxynonenal is known to form adducts with proteins in the cytosol [44,46]; in particular, it has been demonstrated to form an adduct with c-Jun N-terminal kinases, and that this event leads to c-Jun N-terminal kinase nuclear translocation [47]. In the present study, the increase in arachidonic acid in cellular phospholipids increased lipid peroxidation in both 7777 and JM2 cells, as measured by malondialdehyde production, but the increase of malondialdehyde production occurred after class 2 *ALDH* mRNA content had already increased. The increase in lipid peroxidation products was better correlated with the decrease in cell growth: this decrease began 24 h after removal of arachidonic acid, i.e. at the same time as the onset of malondialdehyde production. The fact

that polyunsaturated fatty acids might act on tumour cells using different mechanisms has been demonstrated recently in breast cancer cells [48]: γ -linolenic acid has been found to induce changes in the expression of adhesion molecules mediated by PPAR γ activation, and to decrease cell growth mediated by a different mechanism [48].

In conclusion, in hepatoma cells arachidonic acid supplementation partially restores normal hepatocyte levels of class 2 *ALDH* activity, probably via increased PPAR γ gene expression.

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