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FASTING INCREASES THE SUSCEPTIBILITY OF RAT HEPATOCYTES TO THE CYTOTOXIC EFFECTS OF *N*-HYDROXY-ACETYLAMINOFLUORENE

EFFECTS ON MITOCHONDRIAL RESPIRATION AND MEMBRANE POTENTIAL

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Abstract—Isolated rat hepatocytes were incubated with the carcinogen *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF). Cells from fasted rats were much more susceptible to the cytotoxic effects of 1 mM N-OH-AAF than cells from fed rats: after approximately 90 min exposure the former were all dead but the latter still viable. Even after 240 min 25% of the "fed" cells were still viable. The loss of viability was preceded by a decrease in mitochondrial membrane potential (MMP) and inhibition of respiration; the mitochondrial respiration as measured in permeabilized cells appeared uncoupled. Addition of 15 mM fructose prevented cell death and the loss of MMP in cells both from fed and fasted rats to a large extent; however, uncoupling was not prevented. After incubation of hepatocytes from fasted rats with 1 mM [^{3}H]N-OH-AAF for 120 min, 12 nmol [^{3}H]N-OH-AAF became bound per mg cell protein. Addition of fructose decreased this to 7 nmol. In cells from fed animals 4 nmol [^{3}H]N-OH-AAF became bound after 120 min, in this case fructose had no effect. Part of the protective effect of fructose might be explained by a decrease in intracellular ATP, which prevents the formation of reactive intermediates of N-OH-AAF resulting in a decrease of covalent binding, in addition, fructose protects via a yet to be determined mechanism.

Administration of the carcinogen *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF†) to rats induces necrosis of hepatocytes [1]. This necrosis may be relevant for the process of hepatocarcinogenesis because it leads to a regenerative, mitogenic stimulus. The outgrowth of cells initiated by the N-OH-AAF treatment is thought to result from their resistance towards its cytotoxic effect in combination with the mitogenic stimulus [2–7].

We showed previously that sulphation of N-OH-AAF is a major route by which reactive intermediates are formed that may covalently bind to liver macromolecules [8, 9]. Reduced glutathione (GSH) does not seem to play a role in the detoxification of the intermediate; depletion of GSH does not lead to increased binding of N-OH-AAF to liver macromolecules. Moreover, N-OH-AAF binds to liver macromolecules [10] and elicits hepatotoxicity at doses far below those required to deplete hepatic GSH [11]. In a later study we concluded that neither depletion of protein thiols nor lipid peroxidation played a role in the cytotoxicity of N-OH-AAF. The cytotoxicity correlated, however, with a decrease in cellular ATP [12].

Cellular ATP is predominantly generated through mitochondrial oxidative phosphorylation. In this study we have determined the effect of N-OH-AAF on mitochondrial respiration and on the mitochondrial membrane potential (MMP) of isolated hepatocytes, in relation to covalent binding of N-OH-AAF and the onset of cell death. Furthermore, the effect of manipulation of intermediary metabolism by fasting or addition of fructose to the incubation medium was correlated with cytotoxicity, respiration, MMP and covalent binding.

MATERIALS AND METHODS

Chemicals. N-OH-AAF was synthesized as described previously [9]. Collagenase, bovine serum albumin (BSA) type V, rhodamine 123, firefly lantern extract (crude luciferin/luciferase) were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). All other chemicals were of analytical grade.

Isolation and incubation of hepatocytes. Male SPF Wistar rats (230–270 g) of the Sylvius Laboratories, University of Leiden, The Netherlands, were used. They were housed in macrolon cages with hardwood

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† Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulphoxide; GSH, reduced glutathione; HH-buffer, Hanks'-HEPES buffer; LDH, lactate dehydrogenase; MMP, mitochondrial membrane potential; N-OH-AAF, *N*-hydroxy-2-acetylaminofluorene.

bedding and had free access to food (MRH-B, Hope Farms, Woerden, The Netherlands) and tap water. An alternating 12-hr light and dark cycle was maintained in the animal rooms. In a number of experiments rats were fasted for 24 hr prior to the cell isolation.

Rats were anaesthetized with sodium pentobarbital (60 mg/kg; i.p.). Hepatocytes were isolated (between 10 and 12 a.m.) according to a collagenase perfusion method originally described by Seglen [13], with some modifications [14]. Routinely, more than 95% of the freshly isolated hepatocytes excluded trypan blue.

The cell suspension obtained was diluted to a final concentration of 3×10^6 hepatocytes/mL in pre-oxygenated Hanks'-HEPES buffer (HH-buffer), supplemented with 2.5% (w/v) BSA and 1 g/L glucose, pH 7.4. Cells were incubated in polyethylene vials on a rotary shaker (Adolf Kühner AG, Switzerland) at 200 rpm and 37°. The oxygenation of hepatocytes with 5% CO_2/95% O_2 was performed as described [15].

Hepatocytes were preincubated for 30 min before the addition of the N-OH-AAF solution. Due to its slow rate of dissolution, this compound was dissolved in 0.25 N NaOH and 2 N HCl was added until N-OH-AAF precipitated; 2 N NaOH was then added until N-OH-AAF was just dissolved. The volume was adjusted with water as required. Fructose was added (final concentration 15 mM) to the hepatocyte suspension 30 min prior to exposure to N-OH-AAF.

Analysis. Cell viability was determined by measuring lactate dehydrogenase (LDH) leakage from cells as previously described [14]. Intracellular ATP was determined with the luciferin–luciferase technique as described previously [16]. Cellular protein was determined by the method of Lowry *et al.* [17], using BSA as standard.

Determination of respiration. Oxygen consumption was recorded with a Clark-type oxygen probe (Yellow Springs Instruments Co., Yellow Springs, OH, U.S.A.); the cell was kept at 37°. Cells were incubated as described above; a sample of 1 mL was drawn, 1.5 mL HH-buffer was added and the cells were transferred to the polarographic cell where the oxygen consumption was recorded.

The effect of N-OH-AAF on mitochondrial respiration was studied in more detail by permeabilization of the hepatocyte plasma membrane with digitonin as described before [18]. A 1.6 mL sample of the cell suspension in the incubation vial was transferred to an Eppendorf cup. The cup was centrifuged for 10 sec at 600 *g*. The resulting supernatant was transferred to a second tube and stored. Later the LDH activity in this sample was determined. The resulting pellet was resuspended in 250 μL of buffer (incubation buffer without calcium). Centrifugation and resuspension was repeated once. A 130 μL sample of the cell suspension was transferred to 1200 μL oxygenated test buffer (25 mM Tris–HCl, 10 mM K_2HPO_4, 5 mM $MgCl_2$, 2 mM EDTA and 250 mM sucrose) to which had been added 50 μL 10 mM digitonin in H_2O/dimethyl sulphoxide (DMSO) (1:1, v/v), 50 μL 500 mM succinate, pH 7.4, and 20 μL 50 mM rotenone in DMSO. The mixture was transferred to the polarographic cell (volume 3 mL); subsequently the cell was completely filled with test buffer. The suspension was continuously stirred. Initially state 4 respiration was recorded; subsequently, after addition of 30 μL of 50 mM ADP, state 3 respiration was recorded.

Flow cytometry. Samples of 1 mL were taken from the incubation vial and centrifuged (600 *g*, 10 sec). The supernatants were used for LDH determinations. The cells were incubated at 37° for 7 min in 1 mL HH-buffer containing 2.5% (w/v) BSA and rhodamine 123 (final concentration 1.5 μM), subsequently centrifuged again and resuspended in 2 mL buffer. For each analysis 10,000 cells were monitored in a FACScan flow cytometer (Becton and Dickinson, San Jose, CA, U.S.A.) for rhodamine 123 fluorescence and forward scatter. Figure 1a shows a dot-plot of the analysis of control cells; a cluster R1 of cells with a high MMP (comprising more than 90% of the population) can be defined. Additionally a cluster R2 can be defined which contains cells with a low MMP. Figure 1b shows a typical dot-plot of a population of cells treated with 1 mM N-OH-AAF. Shown in Fig. 3 is the percentage of the cells incubated with N-OH-AAF present in that region vs that of cells incubated without N-OH-AAF; i.e. cells in R1 from Fig. 1b divided by that in R4 from Fig. 1a.

Covalent binding of N-OH-AAF. Cells were incubated with 1 mM [ring-^{3}H]N-OH-AAF (sp. radioact. of 12.1 mCi/mmol) as described above. At the indicated time-points a sample was taken, the cells were washed twice with a 100 mM potassium phosphate buffer (pH 7.4) and stored at −20° for the isolation of macromolecules. The pellet was homogenized in 1 mL of cold methanol (−20°) by sonification of the suspension. The precipitate was separated by centrifugation. The pellet was washed four times with methanol, three times with ethanol and twice with diethyl ether. The final pellet was dried *in vacuo*. The dry powder was dissolved in 1 M NaOH and the amount of radioactivity was determined by liquid scintillation counting after the addition of Plasmasol.

Presentation of results. The onset and rate of loss of viability differed slightly between the batches of cells used; especially from fasted rats, as discussed in the results. Therefore, results on oxygen consumption, MMP and ATP concentration of single experiments are shown with the loss of viability in that particular experiment as an internal standard.

RESULTS

Viability

Hepatocytes isolated from livers of fasted or fed rats differed strongly in their susceptibility to the cytotoxic effect of N-OH-AAF. All cells from fasted rats died after 60–120 min (depending on the batch of cells) of exposure to 1 mM N-OH-AAF. In contrast, in all experiments more than 85% of the cells from fed rats were still viable after a 120 min incubation period. Even after a 240 min incubation 25% of the cells obtained from fed rats were still intact (Fig. 2). Fructose inhibited the loss of viability to a great extent; only approximately 30% of the

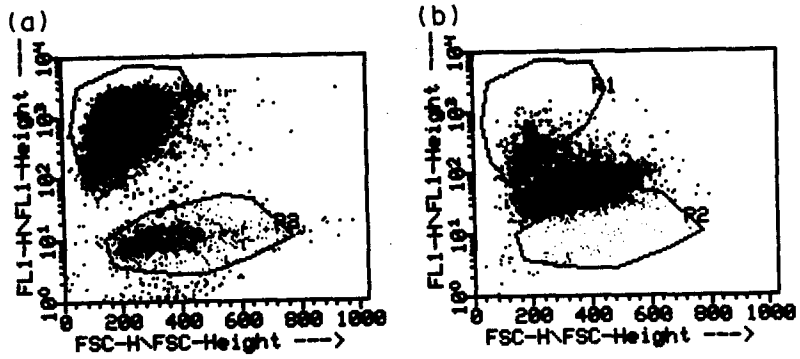

Fig. 1. Flow cytometric dot-plots. Plotted is the forward scatter (*x*-axis) vs rhodamine 123 fluorescence (*y*-axis). Cells were incubated for 50 min in the absence (a) or presence (b) of 1 mM N-OH-AAF.

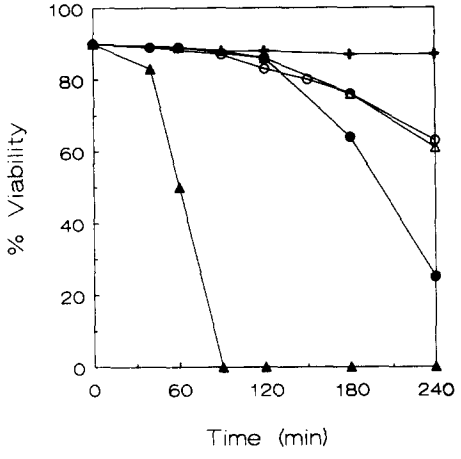

Fig. 2. N-OH-AAF induced loss of viability in hepatocytes. Triangles represent cells from fasted rats, circles cells from fed rats. (+) No addition; closed symbols, 1 mM N-OH-AAF; open symbols 1 mM N-OH-AAF and 15 mM fructose. Viability is expressed as the percentage of LDH that has leaked from the cells vs the total amount present in cells plus medium.

cells, both from fed and from fasted rats, had died after 240 min of exposure.

Respiration

Because previous results suggested involvement of the mitochondria in the development of cytotoxicity, the effect of N-OH-AAF on oxygen consumption of hepatocytes, isolated from fasted rats, was determined (Fig. 3). In a second sample from the same incubation vessel mitochondrial membrane potential (with flow cytometry) and viability of the cells were determined. Figure 3A shows that immediately after the addition of N-OH-AAF the respiration is stimulated, however, after 15 min respiration became progressively inhibited. Directly after the addition of N-OH-AAF cells began to loose their MMP. The loss of viability proceeded in parallel with the decrease in MMP. Fructose (Fig. 3B) prevented the toxic effects of N-OH-AAF, oxygen consumption was increased two-fold compared to the control, while MMP and viability were unaffected and remained constant during the whole incubation period. At longer incubation periods, however, fructose could not totally prevent the cytotoxicity of N-OH-AAF; after 4 hr 30% of the cells had died (Fig. 2).

To investigate in more detail the effect of N-OH-AAF on mitochondrial functioning, oxygen consumption was determined in cells permeabilized with digitonin; first before (state 4) then after addition of ADP (state 3). Figure 4A shows the time course of state 3 and state 4 oxygen consumption with succinate as a substrate. Both were stimulated during the first 90 min of incubation with N-OH-AAF. The respiration rate only slowed down when most cells had died. Table 1 shows the ratio between state 3 and state 4 oxygen consumption. Immediately after addition of N-OH-AAF the ratio was increased but thereafter it decreased which indicated an uncoupling of the mitochondria. Addition of fructose (Fig. 4B) could not prevent the stimulation of the respiration rates by N-OH-AAF. Fructose did prevent the decrease in the ratio state 3/state 4 to a large extent (Table 1).

In cells from fed rats the effects of N-OH-AAF are much less obvious than in cells from fasted rats, although they are similar i.e. eventually a decrease in respiration, in the ratio state 3/state 4, in MMP and in viability. Also, in this case, fructose protected against the effects of N-OH-AAF (not shown).

Cellular ATP

The addition of N-OH-AAF decreased ATP levels in cells from fasted animals (Fig. 5), much faster than we found previously in hepatocytes from fed rats. Fructose decreased ATP in the cells instantaneously, as described before [19]. In control cells from fed rats treated with fructose, ATP levels, after an initial decrease, increased again after 2 hr of incubation (not shown).

Covalent binding

The covalent binding of [ring-^{3}H]N-OH-AAF in hepatocytes is shown in Table 2. The highest binding

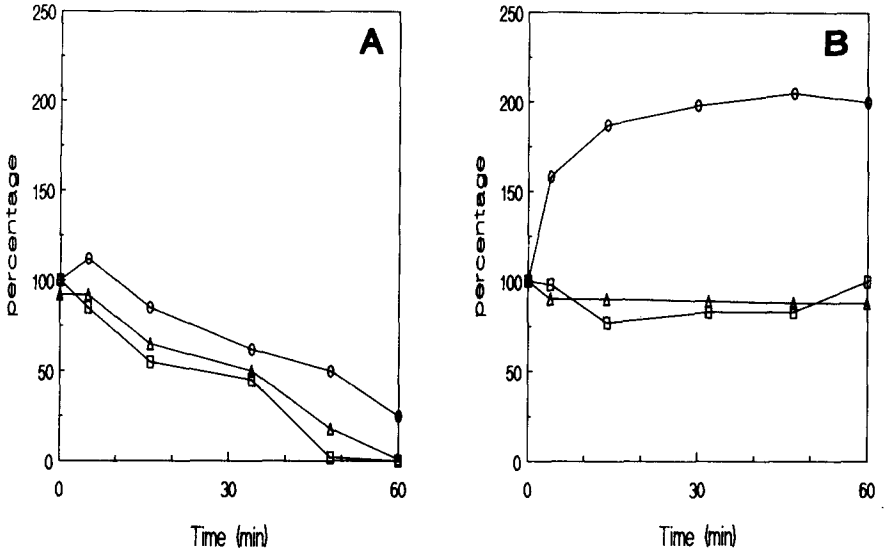

Fig. 3. Effect of N-OH-AAF on respiration, viability and MMP in hepatocytes from fasted rats. (A) Cells incubated with 1 mM N-OH-AAF; (B) cells incubated with 1 mM N-OH-AAF and 15 mM fructose. (○) Rate of respiration; (△) viability; (□) MMP. Respiration and MMP are expressed as percentage of control cells incubated without N-OH-AAF and fructose. Viability is expressed as the percentage of LDH that has leaked from the cells vs the total amount present in cells plus medium.

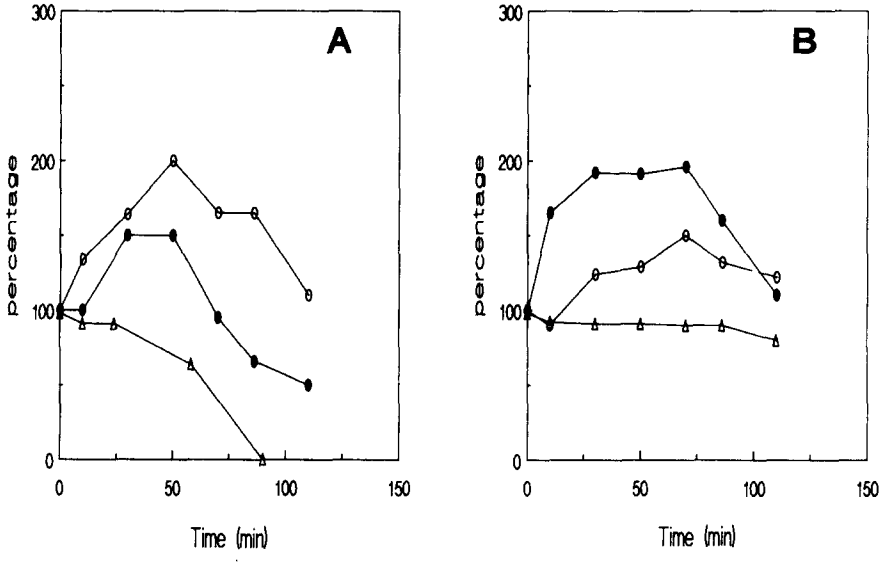

Fig. 4. Effect of N-OH-AAF on respiration and viability in permeabilized hepatocytes from fasted rats. (○) State 4 oxygen consumption; (●) state 3 oxygen consumption; (△) viability. (A) Cells incubated with 1 mM N-OH-AAF; (B) cells incubated with 1 mM N-OH-AAF and 15 mM fructose.

was found in hepatocytes from fasted rats. Incubation in the presence of fructose prevented this binding to a large extent. The binding in cells from fed animals was not affected by addition of fructose.

DISCUSSION

Viability

In a previous paper, we reported that N-OH-AAF lowered intracellular ATP in isolated hepatocytes [12]. We have now studied in more detail the effect of N-OH-AAF on cellular energy status in cells from fed and fasted rats. Also the effect of fructose, a glycolytic intermediate often used [20] to protect cells against cytotoxicity, on N-OH-AAF cytotoxicity was evaluated.

The difference in susceptibility between cells from fed and fasted rats is very striking. The latter were

Table 1. Effect of fructose on the ratio state 3/state 4 respiration in permeabilized hepatocytes incubated with N-OH-AAF with succinate as substrate

Incubation period (min)	Ratio state 3/state 4	
	N-OH-AAF	N-OH-AAF + fructose
0	1.8	2.1
15	2.0	2.7
30	2.0	2.1
60	1.1	2.0
90	0.5	1.4
120	0.5	1.4

Hepatocytes were incubated with 1 mM N-OH-AAF. Respiration was measured with succinate, final concentration 8.3 mM, as a substrate. This is a representative result typical for six separate hepatocyte preparations.

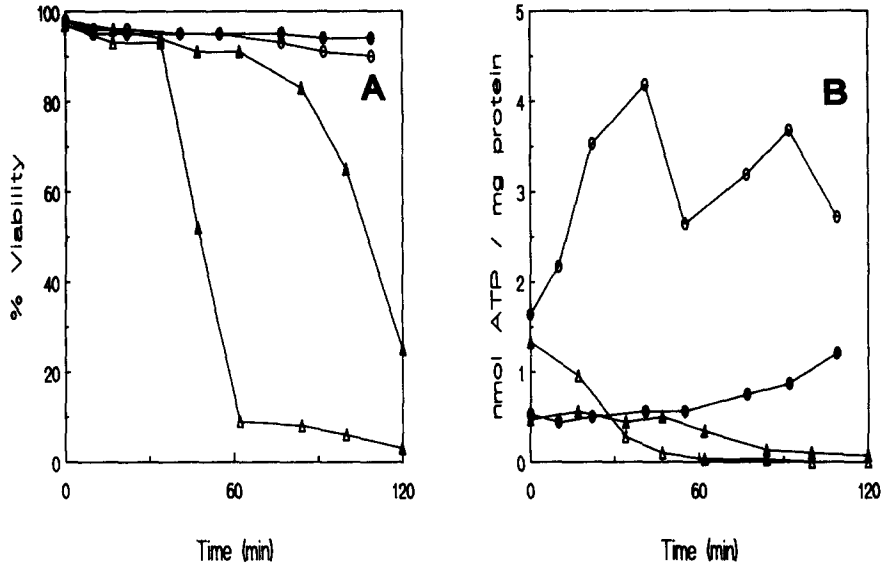

Fig. 5. Effect of N-OH-AAF and fructose on intracellular ATP in hepatocytes from fasted rats. (○) No addition; (●) 15 mM fructose; (△) 1 mM N-OH-AAF and (▲) 1 mM N-OH-AAF and 15 mM fructose. (A) Viability; (B) intracellular ATP.

Table 2. Hepatocytes were incubated with 1 mM [ring-^{3}H]N-OH-AAF; after 0 and 120 min incubation the amount of covalently bound radioactive material was determined

Source of N-OH-AAF hepatocytes	Time (min)	Addition of fructose	Viability (%)	Covalently bound (nmol/mg protein)*
Fasted rat	0	−	95	1.0 ± 0.3
Fasted rat	120	−	0	12.1 ± 0.5
Fasted rat	120	+	90	7.3 ± 1.7†
Fed rat	0	−	95	1.2 ± 0.3
Fed rat	120	−	85	3.6 ± 0.5
Fed rat	120	+	95	4.1 ± 0.9†

* Data expressed ± SEM (N = 4).

† Significantly different in the presence of fructose compared to the absence by the rank-sum test ($P < 0.05$).

all dead after 60–120 min of incubation while in the former hardly no loss of viability had occurred; even after 4 hr of incubation still 25% of these cells were viable. This suggests a relation between the availability of cellular fuels and the cytotoxicity of N-OH-AAF.

Respiration

In cells from fasted rats exposed to N-OH-AAF the oxygen consumption was impaired. Simultaneously the MMP and the viability were decreased. When cells were permeabilized and succinate was added to the cells, not a decrease as in intact cells, but an increase in oxygen consumption was observed. Due to the permeabilization both state 3 and state 4 oxygen consumption could be determined, both were stimulated during the first 90 min. After 30 min, however, mitochondria became uncoupled. This indicated that N-OH-AAF blocked the influx of substrates into the respiration chain *and* uncoupled the mitochondria in cells from fasted rats. In cells from fed rats the uncoupling of mitochondria is also observed although the onset of cell death was now strongly delayed. Addition of fructose to intact N-OH-AAF-treated cells from fasted rats stimulated respiration and prevented cell death and collapse of the MMP indicating that, although an uncoupling took place, mitochondria still functioned. Determination of intracellular ATP confirmed that N-OH-AAF exhausted cellular ATP; however, initially fructose depleted ATP even more. This is a result of the initial extensive phosphorylation of fructose. A low ATP level by itself can, therefore, not explain the toxicity of N-OH-AAF.

Covalent binding

Covalent binding to cellular macromolecules in "fed" cells was half of that found in "fasted" cells. This can be explained by a higher activity of the glucuronyl transferase in the former cells which competes with the sulphotransferases [21]. The inhibitory effect of fructose on the covalent binding of N-OH-AAF can be explained by the decrease in cellular ATP levels induced by fructose. For the formation of the reactive metabolite via the sulphation pathway ATP is needed indirectly, to form "active sulphate", the cosubstrate of sulphotransferase. The total amount of N-OH-AAF that became bound in 2 hr lies in the same order of magnitude (nmol/mg) as the steady-state concentration of ATP. Therefore, a decrease in ATP could very well explain the decrease in covalent binding which in its turn may explain the decrease in cytotoxicity. The effect of fructose on covalent binding does, however, not explain all its protective effect on the cytotoxicity of N-OH-AAF; in cells from fed rats it also protects against cell death but it has no effect on covalent binding. The possibility mentioned above that fructose provides extra substrate for ATP synthesis cannot explain its protective effect; also in cells from fed rats, which are less susceptible to the toxic effect of N-OH-AAF, fructose provides protection; although it lowers ATP.

Mitochondrial membrane potential

Because we found that prevention of a decrease in MMP also prevented the loss of viability we are now studying the possibility that fructose protects against the development of mitochondrial damage by a different mechanism.

In conclusion, we report that N-OH-AAF is more toxic in hepatocytes from fasted rats than in cells from fed rats. It is found that N-OH-AAF affects the MMP in cells and uncouples mitochondrial respiration. The effects of N-OH-AAF may be explained by covalent binding to mitochondrial macromolecules.

During the revision of this manuscript an abstract was published [22] which describes redox cycling of *N*-hydroxy-aminofluorene (a possible metabolite of N-OH-AAF). At present we cannot exclude the possibility that in addition to a covalent binding redox cycling also plays a role in the cytotoxicity of N-OH-AAF.

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