Biochem. J. (1982) **202**, 551–553
Printed in Great Britain

Evidence that poly(ADP-ribose) polymerase is involved in the loss of NAD from cultured rat liver cells

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(*Received 26 August 1981/Accepted 13 November 1981*)

Rat hepatocytes cultured for 24h lose 60% of their NAD content. By using the differential response to inhibitors of the two major enzymes that catabolize NAD in mammalian cells, it is shown that poly(ADP-ribose) polymerase is responsible for the loss of NAD. The relevance of this observation to the use of cultured hepatocytes for the study of DNA repair induced by carcinogens is discussed.

The primary culture of rat hepatic parenchymal cells for 24h results in the loss of 60% of their NAD content (Paine *et al.*, 1979). The mechanism underlying this loss has been unknown until now. The present work exploits the differential response to inhibitors of the two major enzymes that catabolize NAD in mammalian cells, poly(ADP-ribose) polymerase and NAD glycohydrolase (EC 3.2.2.5). The results show that the culture of hepatocytes in media containing 3-aminobenzamide, an inhibitor of poly(ADP-ribose) polymerase, prevents the loss of NAD, suggesting that this enzyme activity is responsible for the degradation of NAD in cultured rat liver cells.

Materials and methods

Isolation of hepatocytes

Adult (180–250g) male rats of the Porton-derived Wistar strain, fed *ad libitum* on MRC 41B diet, were anaesthetized by intraperitoneal injection with 60mg of pentobarbitone/kg. Hepatocytes were then isolated by perfusion of the liver with 0.025% (w/v) collagenase (Boehringer Corp., Lewes, Sussex, U.K.) in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (Gibco Biocult, Paisley, Scotland, U.K.) as previously described (Paine *et al.*, 1979). Perfusions were commenced between 10:30 and 11:30h. This process yielded approx. 8×10^8 parenchymal cells per liver, with a viability of $88 \pm 6\%$ (mean $\pm$ S.D. for 18 perfusions) as assessed by Trypan Blue exclusion.

Culture of cells

Hepatocytes were cultured at a density of 20×10^6 cells/20ml of medium in 150mm-diameter Petri dishes (Lux Scientific, supplied by Gateway International, Middlesbrough, Cleveland, U.K.). The medium used was Williams medium E supplemented with 5% (v/v) foetal calf serum and 5mg of gentamycin/100ml (all from Flow Laboratories, Irvine, Scotland, U.K.). Nicotinamide (British Drug Houses, Poole, Dorset, U.K.), 3-aminobenzamide (Tokyo Chemical Co., Tokyo, Japan), nicotinic acid and theophylline (Sigma Chemical Co., Poole, Dorset, U.K.) were dissolved directly into the culture medium.

The cells were cultured at 37C under a humidified atmosphere of air/CO_2 (19:1).

Biochemical determinations

Nicotinamide coenzymes, cytochrome *P*-450 and protein were determined as previously described (Paine *et al.*, 1979).

Poly(ADP-ribose) polymerase and NAD glycohydrolyase were measured in hepatocytes permeabilized by the method of Halldorsson *et al.* (1978), and 10^6 cells were used in each assay. Poly(ADP-ribose) polymerase was measured by the method of Halldorsson *et al.* (1978), and NAD glycohydrolyase by that of Skidmore *et al.* (1979).

Results

Table 1 shows that the total nicotinamide–adenine nucleotide concentration of freshly isolated cells is not significantly different from that found in freeze-clamped liver. In contrast with isolated hepatocytes, cells cultured for 24h without treatment lose 60% of their total nucleotide content, which is due to a loss of NAD^+ plus NADH (referred to as NAD throughout) (Table 1). Supplementation of the

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Table 1. *Effect of nicotinamide, nicotinic acid, theophylline and 3-aminobenzamide on the concentration of cytochrome P-450 and nicotinamide coenzymes in hepatocytes cultured for 24 h*

Samples of freeze-clamped liver, isolated hepatocytes and rat liver cells cultured for 24 h in Williams medium E with or without nicotinamide, nicotinic acid, theophylline or 3-aminobenzamide at the concentrations shown were prepared and their nicotinamide coenzyme and cytochrome *P*-450 concentrations assayed as described in the Materials and methods section. The initial cytochrome *P*-450 concentration of isolated hepatocytes was not significantly different from that found in freeze-clamped liver and was 186 ± 22 ($n = 7$) pmol/mg of protein. The results are means ± S.D. for individual values found in preparations derived from three separate rat livers (i.e. $n = 3$). * denotes not significantly different ($P > 0.05$) from isolated hepatocytes.

Treatment and sample origin	Cytochrome *P*-450 (% of initial)	Nucleotide concn. (nmol/mg of protein)		
		NAD^+ + NADH	$NADP^+$ + NADPH	Total
Freeze-clamped liver	100	2.6 ± 0.4	0.8 ± 0.1	3.4 ± 0.3*
Isolated hepatocytes	100 ± 6	3.2 ± 0.2	1.1 ± 0.6	4.3 ± 0.9
Hepatocytes cultured for 24 h				
(a) without treatment	30 ± 3	0.7 ± 0.1	0.7 ± 0.15	1.4 ± 0.3
(b) +2 mM-Nicotinamide	28 ± 1	1.9 ± 0.2	1.3 ± 0.1	3.2 ± 0.4*
(c) +10 mM-Nicotinic acid	31 ± 3	0.8 ± 0.2	0.5 ± 0.2	1.3 ± 0.4
(d) +5 mM-Theophylline	23 ± 6	1.5 ± 0.2	1.5 ± 0.06	3.0 ± 0.2*
(e) +1 mM-3-Aminobenzamide	25 ± 5	1.9 ± 0.1	1.3 ± 0.04	3.2 ± 0.1*

culture medium with a minimum of 2 mM-nicotinamide prevents this loss (Table 1).

Mammalian liver has been shown to form NAD from nicotinamide or nicotinic acid (Ijichi *et al.*, 1966; Chaykin, 1967; Keller *et al.*, 1971), but supplementing media with 10 mM-nicotinic acid, the maximum concentration that does not produce cytotoxic effects (Paine & Hockin, 1980), does not prevent the loss of NAD from cultured hepatocytes (Table 1). The inability of nicotinic acid to prevent the loss of NAD is not due to the failure of cultured hepatocytes to accumulate nicotinic acid (Paine & Hockin, 1980).

An alternative explanation is that nicotinamide, but not nicotinic acid, inhibits the degradation of NAD in cultured rat liver cells. Two enzymes, poly(ADP-ribose) polymerase and NAD glycohydrolase, catabolize NAD in mammalian cells (Skidmore *et al.*, 1979; Durkacz *et al.*, 1980). Nicotinamide, but not nicotinic acid, is known to inhibit both poly(ADP-ribose) polymerase and NAD glycohydrolyase activities (Clark *et al.*, 1971; Durkacz *et al.*, 1980), suggesting that these actions of nicotinamide could be responsible for preventing the loss of NAD in cultured liver cells. This suggestion is supported by the finding that theophylline (1,2-dimethylxanthine), which is not an intermediate in the biosynthesis of NAD, is also able to prevent the loss of NAD (Table 1). Theophylline, like nicotinamide, is known to inhibit both poly(ADP-ribose) polymerase and NAD glycohydrolase activities (Skidmore *et al.*, 1979).

In order to distinguish between the involvement of poly(ADP-ribose) polymerase and NAD glycohydrolase in the loss of NAD from cultured hepatocytes, the differential response of these enzymes to 3-aminobenzamide (Durkacz *et al.*, 1980) was exploited. The activity of poly (ADP-ribose) polymerase in rat hepatocytes was inhibited by 80% by 50 μM-3-aminobenzamide. In contrast, 5 mM-3-aminobenzamide did not inhibit the NAD glycohydrolase activity of rat hepatocytes. Table 1 shows that 3-aminobenzamide prevents the loss of NAD from cultured rat hepatocytes. In view of the differential inhibitory effect of 3-aminobenzamide on poly(ADP-ribose) polymerase and NAD glycohydrolase activity, we conclude that poly(ADP-ribose) polymerase is responsible for the loss of NAD in cultured rat liver cells.

Discussion

The primary culture of rat hepatocytes is receiving increasing attention in studies of the mechanisms of hepatocarcinogenesis, e.g. for review see Sirica & Pitot (1980). However, the usefulness of this model is limited in that the activity of certain liver-specific functions, involved in the response to hepatocarcinogens *in vitro*, alters spontaneously. Of particular significance in this respect is the loss of cytochrome *P*-450 and NAD. The presence of cytochrome *P*-450 is necessary for the activation of pro-carcinogens to carcinogens. The interaction of carcinogens with DNA is followed by a 'repair' DNA synthesis, and it has been shown that NAD is involved in this process (Schacter & Burke, 1978; Jacobson & Narasimham, 1979; Purnell *et al.*, 1980). Paine *et al.* (1979) have shown that the culture of hepatocytes in medium containing 25 mM-nicotinamide not only prevents the loss of cyto-

chrome *P*-450, but also prevents the loss of NAD. Therefore two of the disadvantages of cultured hepatocytes as a model for hepatocarcinogenesis can be removed.

The present results show that the effects of nicotinamide on NAD and cytochrome *P*-450 can be separated, since *in vivo* concentrations of NAD can be maintained in hepatocyte culture by concentrations of nicotinamide (2mM) that are inadequate to prevent the loss of cytochrome *P*-450. In addition, the culture of cells in medium containing 3-aminobenzamide or theophylline prevents the loss of NAD, but not of cytochrome *P*-450 (Table 1).

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