INCREASED EFFECT OF ADRENALINE ON CYCLIC AMP FORMATION AND POSITIVE β-ADRENERGIC MODULATION OF DNA-SYNTHESIS IN REGENERATING HEPATOCYTES

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1. Introduction

Hormonal factors are important regulators of eukaryotic cell proliferation [1–4]. Changes in the way cells respond to humoral agents may, therefore, alter their growth properties. The initiation of DNA-synthesis and proliferation of liver cells occurring after partial hepatectomy [5], seems to require certain growth factors, including insulin and possibly epidermal growth factor (EGF) [6]. Moreover, several observations suggest a stimulatory role of cyclic AMP. Hepatic DNA-synthesis can be induced in vivo by infusion of triiodothyronine, heparin and amino acids in combination with glucagon [7] or dibutyryl cyclic AMP [8]. In primary cultures of adult rat hepatocytes, addition of insulin, glucagon, EGF and dexamethasone stimulates DNA-synthesis [9]. After partial hepatectomy elevation of the cyclic AMP level precedes [10] and may be required for [11] the onset of DNA-replication. It is not clear how this rise in cyclic AMP is produced. We here show that after partial hepatectomy the liver adenylate cyclase activity and the accumulation of cyclic AMP in isolated hepatocytes become more responsive to adrenaline. Furthermore, an indication that adrenergic activation may represent a growth stimulus for hepatocytes, is provided by the demonstration that the β-adrenergic agent isoprenaline in low concentrations (10^{-10}–10^{-8} mol/l), enhanced DNA-synthesis in these cells in culture. It may be noteworthy that increased adrenaline responsiveness has been found also in growing immature [12] and premalignant [13,14] liver.

2. Materials and methods

2.1. *Animals, operations and cell preparation*

Male Wistar rats 150–200 g were used. 70% hepatectomy was performed in ether anesthesia, according to [15]. Sham-operated animals were used as controls. Hepatocytes were isolated as in [16,17].

2.2. *Primary hepatocyte monolayer cultures*

Isolated hepatocytes were washed and seeded in 25 cm^2 Costar culture flasks containing 3.5 ml Dulbecco modified Eagle medium (Gibco, Grand Island, NY) supplemented with 15% horse serum and 2.5% fetal calf serum (Gibco) and with penicillin 100 U/ml, streptomycin 0.1 mg/ml, and nystatin 60 U/ml. The number of cells seeded was 10 000–20 000/cm^2.

2.3. *Assay of adenylate cyclase and cyclic AMP*

Incubations for determination of adenylate cyclase activity were carried out at 30°C in 200 μl mixture containing: [α^{32}P]ATP (The Radiochemical Centre, Amersham) (~10^6 cpm/tube). 1 mM cAMP, 4 mM $MgCl_2$, 30 mM KCl, 50 mM Tris–HCl (pH 7.5), 1 mM methylisobutylxanthine, 1.5 i.u pyruvate kinase, 2.5 mM phosphoenol pyruvate, and homogenate equivalent to 200–300 μg protein. The reaction was terminated by heating (95°C) for 2 min. cyclic [^{3}H]AMP (~2.500 cpm) for recovery determination during the purification step was added to the tubes, and the labeled cyclic AMP was purified by the Dowex-50/alumina double column method in [18] and counted by liquid scintillation.

After stopping the reaction in incubated hepatocyte suspensions with trichloroacetic acid, and subsequently neutralizing the trichloroacetic acid with $CaCO_3$ [19], cyclic AMP was assayed by radioimmunoassay [20].

2.4. *Thymidine incorporation into DNA*

[6-^{3}H]Thymidine (25 Ci/mmol) was added to suspended or cultured hepatocytes as indicated. After

the incubation the cells were washed twice with saline, and ice-cold 5% trichloroacetic acid was added. The trichloroacetic acid-precipitate was washed 5 times with cold 5% trichloroacetic acid and once with 96% ethanol, and an aliquot of the DNA hydrolyzed by hot (90°C) 5% trichloroacetic acid was counted for radioactivity by liquid scintillation.

2.5. *Materials*

EGF was a gift from Dr G. Carpenter, Vanderbilt University, NY. Isoprenaline sulphate was obtained from Norsk Medisinaldepot, Oslo, adrenaline bitartrate from Rhone Poulenc, Paris, insulin and glucagon from Novo, Copenhagen, and dexamethasone from Sigma, St Louis, MO.

3. Results and discussion

3.1. *Increased effect of adrenaline on hepatic adenylate cyclase after partial hepatectomy*

Liver adenylate cyclase from 70% hepatectomized rats showed a marked increase in the responsiveness to adrenaline compared to sham-operated controls (426% vs 66% in homogenates 24 h after the operation, table 1). Full response required homogenate at $\gtrsim$ 1 mg protein/ml, or addition of an equivalent amount of the cytosol if membranes were assayed (not shown), which is consistent with the demonstration that cytosolic factors are necessary for the full expression of the effect of adrenergic agents on the adenylate cyclase [21,22]. Apart from a slight general increase in activity in the hepatectomy group, no other changes in the adenylate cyclase were seen (table 1).

One study [23] failed to show any alteration in adenylate cyclase activity or hormone responsiveness during regeneration after partial hepatectomy. We have no obvious explanation for this discrepancy. These results are supported, however, by further experiments showing an increased number of β-adrenergic receptors on hepatocytes from regenerating liver (Sager, Brønstad, Refsnes, Jacobsen, Christoffersen, unpublished). On the other hand, we have not been able to confirm, either in homogenates or intact cells, the finding [24] that there is a transient increase in adenylate cyclase response to thyroxine and triiodothyronine 4 h after partial hepatectomy.

3.2. *Increased adrenaline responsive cyclic AMP accumulation in intact regenerating hepatocytes*

Consistent with the enhanced adrenergic stimulation of the adenylate cyclase after partial hepatectomy, incubations of intact hepatocytes showed a dramatic increase in cyclic AMP accumulation after exposure to adrenaline (fig.1). The figure also shows that this increase was apparent at 4 h after the operation and progressed up to 24 h. It thus occurred before the onset of the DNA-synthesis (16 h), as measured by incorporation of [^{3}H]thymidine in suspensions of the freshly isolated hepatocytes (fig.1). The adrenaline response was still elevated 1 week after the operation, but had returned to control values after 4 weeks.

In contrast to the adrenaline effect, the responsiveness of hepatocytes to glucagon decreased slightly after partial hepatectomy, with a nadir at ~16 h. This is in accordance with a lowered glucagon binding reported [25], which might be due to a down-regulation of glucagon receptors [26], as a consequence of

Table 1
Adenylate cyclase activity in homogenates from regenerating and control liver

	(pmol cAMP . mg protein^{-1} . min^{-1})	
Addition	Controls (sham-operated)	24 h after partial hepatectomy
None	3.9 ± 0.5	4.5 ± 0.4
Adrenaline (50 μM)	6.5 ± 1.2	19.2 ± 1.1
Prostaglandin E_1 (28 μM)	7.1 ± 0.7	8.5 ± 0.9
Glucagon (14 μM)	31.7 ± 4.2	36.4 ± 6.5
GMP-PNP (100 μM)	19.7 ± 2.9	26.0 ± 1.0
NaF (10 mM)	38.2 ± 5.3	49.0 ± 7.3

Adenylate cyclase activity was assayed in crude homogenates. The results represent mean ± SEM of data from 4 separate expt.

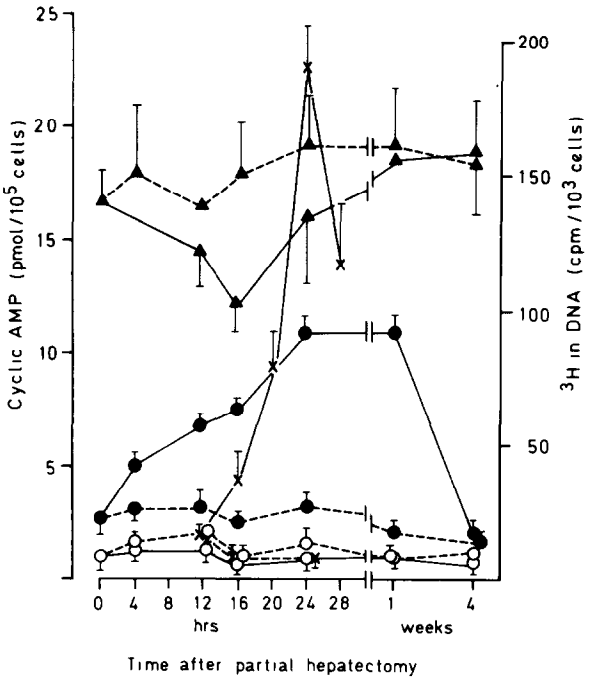

Fig.1. Cyclic AMP response to hormones and DNA-synthesis in suspensions of hepatocytes isolated from partially hepatectomized (——) and sham-operated (– – –) rats. At various times after the operation hepatocytes were isolated and immediately, preincubated (2×10^6 cells in 2 ml in 10 ml Erlenmeyer flasks) for 40 min at 37°C in Krebs-Ringer bicarbonate buffer containing: 118.5 mM NaCl, 3.0 mM KCl, 2.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2.4 mM KH_2PO_4, 24.9 mM $NaHCO_3$ and 10.0 mM glucose (pH 7.4) under continuous aeration with 95% O_2 + 5% CO_2, in a water bath with gyratory shaking (125 rev./min). For the study of cyclic AMP accumulation, incubations were then done for 60 s in the presence of 2 mM theophylline with adrenaline (•, 50 μM), glucagon (▲, 14 μM) or hormone (○, theophylline only). For the measurement of DNA-synthesis (×) 5 μCi [^{3}H]thymidine was added and the incubation was continued for 60 min. The data given represent mean ± SEM of values from 3–4 separate expt.

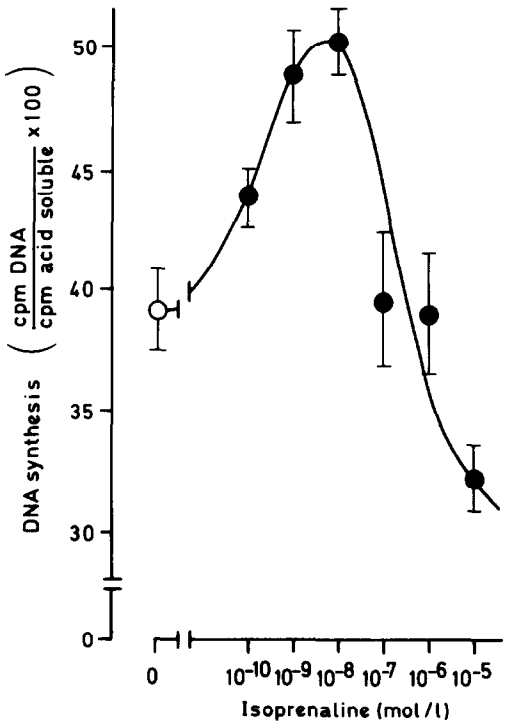

Fig.2. Effect of isoprenaline on DNA synthesis in short time culture of adult rat hepatocytes. Hepatocytes were isolated and seeded 8 h after 70% hepatectomy. Hormones were added to the cultures at the time of seeding in the following concentrations: EGF 10 ng/ml; insulin 400 nM; dexamethasone 100 ng/ml; isoprenaline as indicated. 48 h after hormone addition 8.75 μCi [6-^{3}H]thymidine (27 Ci/mmol) was added for 1 h. The [^{3}H]thymidine incorporated into DNA was determined as described in section 2. The data are expressed as cpm in DNA relative to cpm in the trichloroacetic acid-soluble extract (acid-soluble fraction) to correct for possible errors due to variations in cellular [^{3}H]thymidine uptake. Essentially similar results were obtained, however, if cpm in DNA was plotted. The values given are the mean ± SEM from 5 flasks from 2 expt.

hyperglucagonemia occurring after the partial hepatectomy [27,28].

3.3. *Biphasic effect of isoprenaline on DNA-synthesis in cultured hepatocytes*

The possible direct effect of adrenergic stimulation on growth activation was investigated by measurement of the influence of the β-adrenergic agent isoprenaline on DNA-synthesis in primary cultures of hepatocytes isolated and seeded after partial hepatectomy (fig.2). It was found that if added to cells grown in the presence of EGF, dexamethasone and insulin, isoprenaline in low concentrations (10^{-10}–10^{-8} mol/l) enhanced the incorporation of [^{3}H]-thymidine into DNA, while higher concentrations (>10^{-6} mol/l) were inhibitory. In accordance with [9], glucagon (4×10^{-11}–10^{-7} mol/l in our experiments) also increased the thymidine incorporation (not shown). This effect was of about the same magnitude as that of isoprenaline. Furthermore, under similar conditions, we have found a dose-dependent stimulation of DNA-synthesis by dibutyryl cyclic AMP (10^{-9}–10^{-5} mol/l; Brønstad, Sand,

Christoffersen, unpublished). Stimulatory effects of low concentrations of cyclic AMP have also been found in fetal hepatocytes [29].

3.4. *On the role of cyclic AMP and adrenergic activation in the liver cell proliferation*

DNA replication in regenerating liver is preceded by, and may be dependent on, increased cyclic AMP levels [10,11], and protein kinase activation [30]. The increased adrenergic responsiveness demonstrated in the present report may be a causative factor in these events. This, possibly in combination with an early hyperglucagonemia [27,28] and depressed phosphodiesterase activity [31], which have been reported after partial hepatectomy, may account for the proper timing of the changes in cyclic AMP levels necessary for the initiation of DNA synthesis. A role for catecholamines in liver growth is further suggested by in vivo experiments with adrenergic agonists [32, 33] and antagonists [11] and is supported by the experiments on hepatocyte cultures shown here. Normal hepatocytes (i.e., not isolated from partially hepatectomized rats) gradually aquire increased adrenaline-responsive adenylate cyclase activity when cultured (Christoffersen et al., unpublished), and in such cells isoprenaline also stimulates DNA-synthesis.

Increased adrenaline responsiveness in the liver has also been found in other situations with increased hepatocyte proliferation. During rapid growth in neonatal liver the responsiveness to adrenaline is increased [12]. Elevated response of the adenylate cyclase to adrenergic stimulation is also observed during chemical carcinogenesis [13,14]. While increased adrenaline responsiveness is also seen in states not usually associated with enhanced proliferation [34–36], it may be part of a broad program of regulatory events involved in stimulation of growth in liver cells.

We conclude that regenerating hepatocytes aquire increased ability to form cyclic AMP in response to β-adrenergic activation. This may contribute to the growth initiation. Although inhibitory effects of high concentrations of cyclic AMP on cell growth have been demonstrated in a number of cell culture systems [37], a role for physiological increases of this nucleotide in induction of proliferation in hepatocytes is in accordance with findings in certain other experimental systems [38–40].

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