Quantitative aspects of the effects of insulin, epidermal growth factor and dexamethasone on DNA synthesis in cultured adult rat hepatocytes

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Abstract. Epidermal growth factor (EGF) and insulin in combination have previously been shown to initiate S-phase in primary cultures of adult rat hepatocytes. We here describe the detailed time course and dose-dependency of the effects of EGF and insulin on DNA synthesis in cultured hepatocytes. The DNA synthesis was assessed either biochemically or autoradiographically with a fairly good correlation between the two methods. DNA synthesis started 24–30 h after plating of the cells and peaked at approximately 70 h. Up to 70% of the cells entered DNA synthesis during this period. EGF and insulin acted synergistically on the DNA synthesis. Dexamethasone raised the DNA synthesis slightly, maximal effect occurred at concentrations above 2.5 nM and this agent was routinely used in the experiments with EGF and insulin. In the presence of 0.4 μM insulin from the time of plating, EGF dose-dependently increased the DNA synthesis with maximal effect at 5–15 nM. When added in combination with 1.7 nM EGF, insulin enhanced the DNA synthesis over the concentration range from 0.1 to 3 nM. These studies show that primary cultures of hepatocytes are useful in assessing the quantitative aspects of the interactions between the growth stimulating effects of hormones.

Animal experiments in vivo indicate that humoral factors are involved when quiescent hepatocytes start to replicate their DNA and proliferate during the regenerative response to experimental liver injury (Moolten & Bucher 1967; Lesch & Reutter 1975; Porter & Whelan 1978; Bucher & McGowan 1979). Further evidence for the hormone dependency of liver growth is provided by studies on rat hepatocytes in culture, where DNA synthesis can be induced by a combination of insulin, epidermal growth factor (EGF = urogastrone), glucocorticoids, and either glucagon or isoproterenol (Richman et al. 1976; Brønstad & Christoffersen 1980; McGowan et al. 1981; Friedman et al. 1981; Michalopoulos et al. 1982; Brønstad et al. 1983; Enat et al. 1984). Platelet factors may also be involved in the initiation of hepatocyte DNA synthesis (Russell et al. 1984; Nakamura et al. 1984).

Because of the important implications that these hormone effects may have, both in the general study of hormonal regulation of cell growth and more specifically in the search for liver-stimulatory and liver-protective regimens, it is essential to have detailed quantitative information about the proliferative response elicited in hepatocytes by hormonal treatment. Previous studies demonstrated characteristic biphasic effects of the β-adrenergic agent isoproterenol and of glucagon on hepatocyte DNA synthesis, with stimulation at low concentrations and inhibition at higher levels of the agents (Brønstad & Christoffersen 1980; Brønstad et al. 1983). In the present work we have examined in more detail than has been previously reported the time course of the hormone-induced DNA synthesis and the dose-response relationship for the effects of insulin, EGF and dexamethasone in pri-

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mary cultures of rat hepatocytes. In addition, we have made a comparison of various methods to monitor the effect of these hormones on the hepatocyte DNA synthesis.

Material and Methods

Materials
Collagenase (CLS-II) was from Worthington Biochemicals, Freehold, N.J. Dulbecco’s modified Eagle’s medium, horse serum and foetal calf serum were purchased from Gibco, Grand Island, N.Y. Dexamethasone was obtained from Sigma Chemical Co., St. Louis, Mo. Insulin was obtained from Novo, Copenhagen. Epidermal growth factor (EGF) was purchased from Collaborative Research Inc., Waltham, Mass. [3H]thymidine (22—27 Ci/mmol) was from the Radiochemical Centre, Amersham. RNase was from Boehringer Mannheim GmbH, Mannheim.

Animals and isolation of hepatocytes
Male Wistar rats, 150—200 g fed ad libitum, were used. Hepatocytes were isolated by an in vitro collagenase perfusion (Seglen 1976) with modifications (Christoffersen et al. 1984). Cell viability, measured as the ability to exclude 0.4% trypan blue, was above 95%.

Hepatocyte culture and hormone treatment
The hepatocytes were washed twice by low-speed centrifugation in sterile culture medium without bicarbonate and then seeded into Costar plastic culture flasks using bicarbonate buffered Dulbecco’s modified Eagle’s medium with horse serum (15%), and foetal calf serum (2.5%), penicillin (67 µg/ml) and streptomycin (100 µg/ml). The flasks were gassed with 95% air/5% CO2 and kept at 37°C. Cell density ranged between 12—20 × 103 per cm2 in most experiments. The amount of medium was 0.13 ml per cm2. Medium was not changed during the culture period. Hormones were added at the time of cell plating at concentrations as indicated.

Incubation with [3H]thymidine
[3H]thymidine (2 µCi/ml, 0.24 Ci/mmol) was present in the cultures either for extended periods after addition at 24 and at 48 h (also at 72 h in some experiments) to obtain the cumulative labelling index at the end of culturing, or for 60 min (2.5 µCi/ml, 22—27 Ci/mmol) to label the nuclei in S-phase at the time of the pulse and to biochemically determine the rate of incorporation of thymidine into DNA.

Autoradiography
After the incubation with [3H]thymidine the flasks were rinsed twice with saline. The bottoms of the culture flasks were cut out with a circle saw after fixation in methanol for 15 min. They were then dip-coated in Kodak NTB-2 emulsion diluted 1:1 (v/v) with distilled water and were developed after 2 weeks of exposure and stained with haematoxylin. The index of labelled cells was assessed by counting 500 morphologically distinct hepatocytes. The labelled cells were marked intensely with all grains over the nucleus and could easily be distinguished from unlabelled cells.

Biochemical determination of labelled DNA

At the end of the pulse incubation (60 min) with [3H]thymidine the cells were washed twice with saline before the addition of ice-cold 5% trichloroacetic acid. The trichloroacetic acid precipitate (0°C) was collected by scraping and washed 5 times with cold 5% trichloroacetic acid and once with 96% ethanol. The DNA was hydrolyzed by heating in 5% trichloroacetic acid (90°C for 25 min), and an aliquot was counted for radioactivity by liquid scintillation. To ascertain that the radioactivity in the labelled precipitate reflected tritium incorporated into DNA the identity of the TCA-precipitable material was assessed. Hydrolysis by hot acid and DNAase brought 93 and 90% respectively of the radioactive material present in the trichloroacetic acid- and ethanol washed precipitate into solution. We found about 1% of the radioactivity in solution after RNase, trypsin or alkaline hydrolysis. From these experiments we concluded that the radioactivity that could be hydrolyzed by hot acid from the (cold acid) precipitate reflected tritium incorporated into DNA.

The results were expressed either as cpm in DNA, or most often as the incorporation ratio between cpm in DNA by cpm in the acid soluble fraction, to minimize the effect of variation in cellular uptake of precursor for DNA synthesis (Brønstad et al. 1983).

Results

Effect of single and combined hormone administration

Autoradiographs (Fig. 1) showed an increase in the fraction of cells labelled when insulin was added with dexamethasone (Fig. 1B). Labelling with dexamethasone alone was low (Fig. 1A). There was a much more abundant labelling when EGF also was included in the hormone combination (Fig. 1C). Effects of these agents, administered singly or in combination, are summarized in Table 1. Insulin or EGF added at seeding in the presence of dexamethasone both increased the percentage of cells entering S-phase above the level in the cultures that only received dexamethasone. When the two hormones were combined they acted synergistically (Table 1). The synergism between insulin and EGF was present in most experiments, but the degree of synergism varied widely.
Fig. 1.

Autoradiographs of hepatocytes cumulatively labelled from 24 to 72 h of culture. \[^{3}H\]thymidine (7 µCi/flask, 0.24 Ci/mmol) was added at 24 and 48 h. Hormones were all present from the time of plating the cells. (A) Cultures treated with dexamethasone (0.25 µM) show minimal nuclear labelling at 72 h. (B) Culture that received dexamethasone (0.25 µM) and insulin (0.4 µM). (C) Culture treated with combination of dexamethasone (0.25 µM), insulin (0.4 µM) and EGF (1.7 nM).
Table 1.
Dexamethasone (Dxm) 0.25 µM, insulin (Ins) 0.4 µM and epidermal growth factor (EGF) 1.7 nM were added at the time of plating as indicated. The cumulative labelling index was determined by continuously exposing the cells to [3H]thymidine (7 µCi/flask, 0.24 Ci/mmol) with additions at 24 and 48 h and harvesting at 72 h as described in Methods. Values are the mean of duplicate or triplicate cultures in the individual experiments.

<table>
<thead>
<tr>
<th>Hormone treatment</th>
<th>Cumulative labelling index (%)</th>
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<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>Dxm</td>
<td>12</td>
</tr>
<tr>
<td>Dxm + Ins</td>
<td>31</td>
</tr>
<tr>
<td>Dxm + EGF</td>
<td>27</td>
</tr>
<tr>
<td>Dxm + Ins + EGF</td>
<td>51 (46)*</td>
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* Expected if additive only.

Effect of hydroxyurea

Hydroxyurea, a specific inhibitor of replicative DNA synthesis, was found to completely abolish the hormone-induced incorporation of thymidine into DNA. Hydroxyurea at 5 mM was added at 42 h after plating of the cells or immediately before biochemical measurement of the DNA synthesis. DNA synthesis was determined after a 60 min pulse at 72 h of culture. At this concentration hydroxyurea had no effect on cell viability assessed by the ability to exclude trypan blue.

![Fig. 2. Time dependent changes of hepatocyte DNA synthesis in culture.](image)

Time dependent changes of hepatocyte DNA synthesis in culture. The cells were cultured in the presence of 0.25 µM dexamethasone (O-O) or 0.25 µM dexamethasone plus 0.4 µM insulin and 1.7 nM epidermal growth factor (●-●). A: The cumulative labelling index, i.e. the labelling occurring in the continuous presence of [3H]thymidine (7 µCi/flask, 0.24 Ci/mmol) from 24 h (with new additions at 48 h and 72 h) until harvesting of the cultures at the indicated times after plating. B: The labelling index determined after a 60 min pulse of [3H]thymidine (8.25 µCi/flask, 25 Ci/mmol) at the indicated time points. C: The incorporation ratio, i.e. the ratio between radioactivity in DNA and the trichloroacetic acid soluble intracellular radioactivity after a 60 min pulse of [3H]thymidine (8.25 µCi/flask, 25 Ci/mmol) was determined at various times after seeding the cells. The values represent the mean of two flasks from one of several experiments with similar results.
Time course of the hormone-induced S-phase in cultured hepatocytes

We carried out more detailed time studies of the effects of hormones on the onset of S-phase, using different methods for the measurement of DNA synthesis in the hepatocyte cultures. This was done partly in an attempt to describe some of the cell kinetics of the hormone induced G1-S transition in these cells, and partly to evaluate the methods used.

In experiments where the cells were exposed to [3H]thymidine over an extended period (from 24 h after plating to the termination of the culturing) for autoradiographic measurement of cumulative labelling index control cultures contained only a low percentage of labelled nuclei (usually <5% at 70 h), while cultures treated with insulin plus EGF plus dexamethasone showed a 5- to 10-fold increase in nuclear labelling (Fig. 2A). In some of these experiments more than 70% of the cells entered S-phase (at 60–70 h) under the conditions used here. The percentage of cells that had entered DNA synthesis rose rapidly between 40 and 70 h after plating with few cells entering S-phase after this time.

When autoradiography was performed after a brief pulse labelling (60 min), to measure the number of cells engaged in DNA-replication at the time of pulsing, a peak of cells in S-phase was found to coincide in time (70 h) with the most rapid rate of accumulation of labelled nuclei found in the experiments with long-term exposure to [3H]thymidine. The degree of synchrony, defined as the percentage of cells incorporating radioactivity into their nuclei during a 60 min pulse of [3H]thymidine, was 32% at 70 h in the experiment described in Fig. 2B.

In pulse experiments a comparison between labelling index and biochemically determined incorporation of [3H]thymidine into DNA revealed a
Effect of varying concentrations of dexamethasone on hepatocyte DNA synthesis in culture. The cells were either grown with no hormone addition (0—0) or in the presence of 0.4 μM insulin and 1.7 nM epidermal growth factor (●—●) with or without dexamethasone in concentrations as indicated. All hormones were added at seeding. The DNA synthesis was measured as the incorporation ratio during a 60 min pulse of [3H]thymidine after 70 h of culture. The values are the mean ± SEM of triplicate cultures in one experiment.

closely similar time course for the two methods. There was a gradual increase in the DNA synthesis with a peak at 60–70 h (Fig. 2C).

The more detailed time course after addition of dexamethasone, insulin and EGF at plating is shown in Fig. 3A. Incorporation of radioactivity into DNA started between 24 and 30 h after plating the cells and continued to increase until a peak approximately 40 h later. (Results were essentially similar whether expressed as CPM in DNA or as the incorporation ratio (Fig. 3A)). The rate of DNA synthesis approached control levels after 5 days in culture.

Evidence for the linearity between incubation time and incorporation ratio during the pulse period is given in Fig. 3B.

Dose-effect relationships
We also studied the dose-response relationship of dexamethasone, insulin and EGF to obtain more detailed quantitative information about the effect of these hormones in the hepatocyte cultures.

a) Dexamethasone added at the time of plating slightly increased the rate of DNA synthesis above the level in the untreated cultures (Fig. 4). The effect occurred at very low concentrations of the agent and there was no further effect of raising the concentration above 2.5 nM. The shape of the dose-response curve was similar when the effect of dexamethasone on the DNA synthesis was measured in the presence of insulin and EGF.

b) Insulin. Insulin added with dexamethasone and EGF at plating increased DNA synthesis dose-dependently. In the experiment described in Fig. 5
there was a 4-fold increase from 8% labelled cells in the dexamethasone and EGF-treated controls to 36% in the cells that also received 0.4 μM of insulin. When the detailed dose-response relationship was determined (inset Fig. 5) there was a gradual increase in the effect from 30 pM of insulin to near maximal effect at 3 nM of insulin.

c) EGF. (Fig. 6). When the agent was added at plating in the presence of dexamethasone and insulin, maximal effect on rate of DNA synthesis was obtained at an EGF concentration of 5–15 nM. The DNA synthesis started to increase between 0.15 and 1.5 nM of EGF. This increase in incorporation ratio was paralleled by an increase in the percentage of cells entering DNA synthesis as determined by autoradiography.

Discussion

This report confirms the finding that insulin and EGF in combination strongly stimulate the DNA synthesis in primary cultures of rat hepatocytes (McGowan et al. 1981; Friedman et al. 1981; Tomita et al. 1981) and provides new and more detailed quantitative information.

The time course of the DNA synthesis after isolation and plating in primary culture of the hepatocytes was followed either by determining autoradiographically the fraction of the cells entering DNA synthesis or by biochemically measuring the rate of DNA synthesis in the entire culture population. Autoradiography permits an estimation of the percentage of morphologically distinct hepatocytes that enter DNA synthesis and cells morphologically different from the hepatocytes may be excluded. When assessing the DNA synthesis by biochemical measurement of radioactivity in DNA no such distinction is possible and errors due to hormone-induced changes in uptake of precursors for the DNA synthesis may occur. There was a lag phase of approximately 30 h before the DNA synthesis started to increase and it peaked after 70 h of culture. The results with the two methods correlated very well, in accordance with a study on hepatocytes from regenerating liver where various ways of measuring the DNA synthesis were compared (Digernes et al. 1982).

Several studies have indicated that humoral growth-activating signals are involved in liver cell proliferation. These investigations have included inter-animal cross-circulation (Moolten & Bucher 1967), pancreatectomy and evisceration to remove the source of putative necessary factors for liver regeneration (Bucher & Swaffield 1975), hormone injections on intact animals (Short et al. 1975), and studies on cultures (Richman et al. 1976; Brønstad & Christoffersen 1980; McGowan et al. 1981; Friedman et al. 1981; Tomita et al. 1981; Michalopoulos et al. 1982; Brønstad et al. 1983; Enat et al. 1984; Russell et al. 1984; Nakamura et al. 1984). A role for EGF in the induction of hepatocyte proliferation is suggested by studies on primary cultures of hepatocytes (Richman et al. 1976; McGowan et al. 1981; Friedman et al. 1981; Tomita et al. 1981) also on intact animals (Bucher & Wands 1977). Furthermore, it has been shown that insulin has an additive or synergistic effect to EGF in these cultures (McGowan et al. 1981; Friedman et al. 1981).
Dexamethasone improves plating (Laishes & Williams 1976; Christoffersen et al. 1984) of hepatocytes. In several cell types glucocorticoids affect proliferation (Cristofalo & Rosner 1981). At higher concentrations and at somewhat different culture conditions dexamethasone has been reported to inhibit the DNA synthesis of cultured cells from regenerating liver (Richman et al. 1976). However, added at the time of plating in our primary cultures of normal adult hepatocytes dexamethasone slightly stimulated the DNA synthesis.

The combined effects of the glucocorticoid plus insulin and EGF could be of interest in possible future hepato-protective regimens. Insulin or EGF alone in the presence of dexamethasone only moderately increased the DNA synthesis, but the stimulatory effect was synergistic in experiments where either hormone was given to a culture that was also treated with the other. EGF is presumed to have a regulatory function on the DNA synthesis (for review, see St. Hilaire & Jones 1982). The hormone was added at the time of plating and the fairly high concentration of EGF necessary to elicit a strong DNA synthesis response could point to an effect late in the prereplicative phase when presumably some breakdown of this peptide hormone in the medium may have occurred. Later experiments indicate a stronger effect of EGF occurring at lower doses later in the prereplicative phase in culture (Sand & Christoffersen, unpublished).

In an earlier report we demonstrated dose-dependent stimulatory and inhibitory effects of glucagon and cyclic AMP on the DNA synthesis (Brønstad et al. 1983). Present studies in this laboratory focus on the interaction between the effect of cyclic AMP and the hormones described here. Evidence from other cells point to modulatory effects of cyclic AMP on the responsiveness to EGF (Leof et al. 1982), or to insulin plus EGF (Pessin et al. 1983). We believe that adult rat hepatocytes in primary culture represent a good model system to study these interactions. In addition, they may contribute to a more precise understanding of the control of liver regeneration after accidental or experimental injury.

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References


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