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INSULIN AND EPIDERMAL GROWTH FACTOR STIMULATE POLY ADP-RIBOSYLATION

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SUMMARY: Treatment of contact-inhibited BALBc/3T3 fibroblasts with insulin or epidermal growth factor elicited a considerable increase in the capacity of poly (ADP-ribose) synthesis. Stimulation of the poly ADP-ribosylation was observed after long-term hormone treatment (18 hours) but not after short-term treatment (1 hour) and it appeared to be correlated with the cell's entry into DNA synthesis. Several cellular components were found to be enhanced in poly ADP-ribosylation. The data suggest a possible role for poly ADP-ribosylation in mitogenic action of polypeptide hormones and growth factors.

INTRODUCTION

The nuclei of eucaryotic cells contain an enzyme that polymerizes the adenosine diphosphate ribose (ADP-ribose) moiety of NAD^+, releasing nicotinamide (1-3). The structure of this polymer and its natural occurrence have been established but the biological function of the poly (ADP-ribose) is not fully understood (2). There is, however, a number of evidences which suggest that poly (ADP-ribose) synthesis may be involved in the determination of chromatin structure which in turn regulates the cell's DNA synthesis, differentiation and transformation (1-3). Also, poly ADP-ribosylation appears to be involved in DNA excision repair mechanisms (4-9) and DNA recombination events (10). Poly ADP-ribosylation of particular nuclear proteins has been identified (11-14).

Since polypeptide hormones and growth factors exert their diverse effects on cellular metabolism through modulation of various proteins (15-17), we investigated the possibility that hormonal stimulation, particularly mitogenic effects, may involve the ADP-ribosylation of cellular components. Here, we report our initial observation that

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indicates the stimulation of poly ADP- ribosylation upon polypeptide hormone treatment in mouse BALBc/3T3 fibroblasts.

MATERIALS AND METHODS

BALBc/3T3 fibroblasts were grown in Dulbecco's modified Eagle medium supplemented with 10% calf serum (heat-inactivated, GIBCO) and penicillin/streptomycin (GIBCO) and otherwise as described before (18).

Insulin (Eli Lilly) or EGF (Collaborative Research) was added to the culture 4 days after its apparent confluency to final concentrations of 0.5 μg/ml and 20 ng/ml, respectively, and incubation was continued for 1 hour or 18 hours. Cells were removed with gentle trypsinization which was terminated by soybean trypsin inhibitor (Sigma). The cells were collected by centrifugation (500 g for 10 min) and permeabilized according to Berger et al. (19). Briefly, the cell pellets were suspended in ice-cold permeabilizing buffer (10mM Tris-HCl, pH 7.8, 1 mM EDTA, 4 mM $MgCl_2$) at 2 x 10^6 cells/ml and placed in an ice-water bath for 15 min. The cells were collected by centrifugation and resuspended at 5 x 10^6 cells/ml in ice-cold permeabilizing buffer containing 0.25M sucrose. Ninety eight percent of the cells were positive for trypan-blue uptake.

Poly (ADP-ribose) synthesis was measured using permeabilized cells by the methods of Berger et al. (19) with slight modification. The reaction mixture contained 1 x 10^6 permeabilized cells, 0.1 M Tris-HCl, pH 7.8, 2.5 mM $MgCl_2$ and 0.1~0.5 μM ^{32}P-NAD^+ (New England Nuclear). Total assay volume was 0.3 ml. Dithiothreitol was eliminated from the original reaction ingredients since it may cause dissociation of insulin receptors into subunits (16). The reaction was terminated by adding ice-cold trichloroacetic acid (TCA) to a final concentration of 10% and the precipitates collected on Whatman GF/C filters. The radioactivity was counted as described before (18).

^{3}H-thymidine incorporation into DNA was measured using intact cells as described before (18).

The samples for polyacrylamide gel analysis were prepared as follows: Thirty three μl of ice-cold 3N perchloric acid (PCA) were added into the reaction mixture to stop poly (ADP-ribose) synthesis. The precipitates were collected by centrifugation (10,000 g for 30 min) and washed twice with 3 ml each of 0.3N PCA. The precipitate was dissolved into the extraction buffer (20), incubated for 30 min at 4°C, and centrifuged at 30,000 g for 120 minutes. The pellet was solubilized with the sample buffer of Laemmli (21).

Polyacrylamide (10%) gel electrophoresis was carried out according to Laemmli (21). Autoradiography was performed using Kodak XRP-5 X-ray film and a HIPLUS-intensifying screen (Dupont). Autoradiograms were traced with a densitometer (EC Apparatus 900).

RESULTS

The contact-inhibited mouse BALBc/3T3 fibroblasts were treated with insulin (0.5 μg/ml) or EGF (20 ng/ml) for 1 hour and 18 hours. Cells were removed from the dish and permeabilized in order to measure the poly

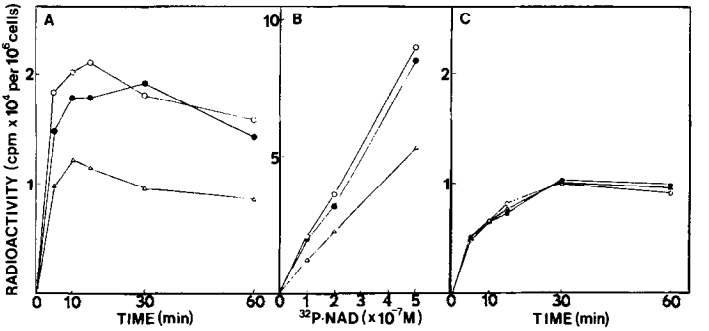

Figure 1-A. Enhanced synthesis of poly (ADP-ribose) after 18 hr-hormone treatment. Permeabilized BALBc/3T3 cells which had been treated with insulin (—O—) or EGF (—●—) were incubated with ^{32}P-NAD$^+$ at 15°C for 10 min. Cells without hormone treatment (—Δ—).

Figure 1-B. NAD$^+$ dependent synthesis of poly (ADP-ribose). Hormone treated, permeabilized BALBc/3T3 cells were incubated with increasing amounts of ^{32}P-NAD$^+$ at 15°C for 10 min. Same symbols were used as Fig. 1-A.

Figure 1-C. No change of poly (ADP-ribose) synthesis after 1 hr-hormone treatment. Permeabilized BALBc/3T3 cells which had been treated with insulin (—O—) or EGF (—●—) for only 1 hr were incubated with ^{32}P-NAD$^+$ at 15° C for 10 min. Control (—Δ—).

(ADP-ribose) synthesis. Figures 1-A and 1-B show that ^{32}P-labeled NAD$^+$ is converted into trichloroacetic acid (TCA)-insoluble materials in a time- and substrate dependent manner. Compared to control cells, maximal levels of the reaction were approximately 2 times higher in the cells that had been treated with insulin or EGF for 18 hours. This stimulation was not seen in 3T3 cells that had been treated with insulin or EGF only for 1 hour (Figure 1-C). Human HeLa cells usually do not respond to insulin or EGF (15,16). No stimulation of poly ADP-ribosylation was observed upon insulin treatment in these cells (data not shown).

The synthesis of poly (ADP-ribose) is evident because the reaction was substantially inhibited by the addition of inhibitors of poly (ADP-ribose) polymerase such as nicotinamide and isobutylmethyl xanthine (Table 1). Also, no TCA-insoluble materials were made when intact cells (not permeabilized) were incubated with ^{32}P-NAD$^+$ (Table 2). We noticed less stimulation at higher temperatures (22°C compared to 15°C) which was

Table 1. Poly (ADP-ribose) synthesis in permeabilized cells

Inhibitors		^{32}P-NAD$^+$ incorporated into TCA-insoluble materials
none		17,652 cpm
Nicotinamide	0.1 mM	1,009
	1 mM	412
	10 mM	421
Isobutylmethyl xanthine	5 mM	2,227
NAD$^+$	1 mM	4,093

The reaction was carried out at 15°C for 10 min using $1x10^6$ EGF-treated and permeabilized 3T3 cells and 0.1 μM ^{32}P-NAD$^+$.

attributed to faster degradation of the reaction products (data not shown).

It is known that the quiescent nondividing BALBc/3T3 cells resume duplication 10 to 18 hours after hormone stimulation (15-17). Data summarized in Table 2 suggest that the increase of ADP-ribosylation coincides with the hormone's mitogenic effects, namely an increase in ^{3}H-thymidine incorporation into DNA.

Figure 2 shows the densitometric tracing of the autoradiograms where the reaction products were analyzed by polyacrylamide gel electrophoresis. ADP-ribosylation was enhanced in the components of molecular weights less than 30,000 and those of molecular weight approximately 100,000.

Table 2. DNA synthesis and poly ADP-ribosylation

Hormone treatment	Time [a] (hrs)	^{3}H-thymidine [b] incorporation (cpm/10^6 cells)	ADP-ribosylation (cpm/10^6 cells)	
			intact cells	permeabilized cells
None	1	2,291	N.T.[c]	9,978
	18	1,826	196	11,368
Insulin	1	3,100	N.T.	9,982
	18	8,555	248	21,020
EGF	1	2,165	N.T.	10,343
	18	163,000	565	19,125

a) Confluent BALBc/3T3 cells were incubated with hormone for the indicated time.

b) Intact 3T3 cells were used.

c) not tested.

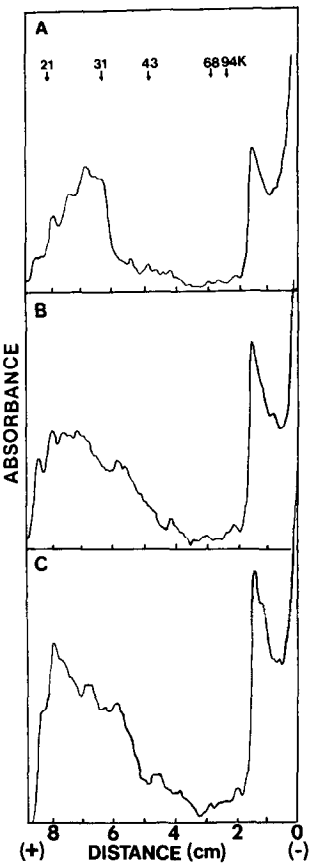

Figure 2. Analysis of the poly ADP-ribosylated cellular components on 10% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. The electrophoresis conditions were 100 volts, 15 mA per gel for 4 hrs. Densitometric trace of the autoradiograms are shown. The markers used were: 94K (phosphorylase b), 68K (bovine serum albumin), 43K (ovalbumin), 31K (carbonic anhydrase) and 21K (soybean trypsin inhibitor). 0 is the top of the slab gel. A. Control, B. Cells had been treated with insulin for 18 hrs., C. Cells had been treated with EGF for 18 hrs.

DISCUSSION

Insulin and EGF are known to act through specific cell surface receptors and appear to modulate various intracellular proteins by phosphorylation/dephosphorylation mechanisms (15-17). The data presented here provide an additional biochemical parameter, namely poly ADP-ribosylation, in which a change is recognized upon hormonal treatment. Our data also support the suggestion that the events of hormonal action

leading to DNA synthesis are correlated at least in part with the synthesis of poly (ADP-ribose).

It has been shown that poly ADP-ribosylation occurs on nuclear proteins including histones and nonhistone proteins (11-14). It may therefore control the structure and function of chromatin resulting in cellular growth and differentiation (1). Recent reports suggest that single-strand breaks in DNA cause an increase in poly (ADP-ribose) synthesis (4-9) and that inhibiting poly ADP-ribosylation by nicotinamide increases sister chromatid exchange (10). These argue that poly ADP-ribosylation is involved in DNA excision repair mechanisms and recombination events.

Our data on stimulation of poly ADP-ribosylation in hormone-treated, permeabilized cells implies three major possibilities: (i) an increase in endogenous sites for ADP-ribosylation, (ii) an increase in poly (ADP-ribose) polymerase activity, and (iii) a decrease in poly (ADP-ribose) degrading enzymes. With regard to possibility (i), we suggest that hormonal signals of EGF and insulin may cause single strand breaks in DNA, possibly by activating specific endonucleases, resulting in the initiation of DNA synthesis. This view is supported by the observation that the increase in ADP-ribosylation is 2-fold regardless of which hormone is added and that patterns of the ADP-ribosylated cellular components by insulin and EGF are reasonably similar to those of histones and nonhistone chromosomal proteins (11-14). There is presently no evidence indicating a covalent attachment of the polymer to the DNA but this is still a possibility (8, 9, and Figure 2). The degree of ^{3}H-thymidine incorporation is quite different (Table 2), and this may reflect a difference in the precise mechanism of DNA chain elongation. Possibility (ii) can be ruled out because, if it is the case, saturation levels of the reaction would remain unchanged and the initial velocity should be faster. This, however, is opposite to the data in Figure 1.

Possibility (iii) is also unlikely because the similar rate of degradation in Figure 1-A indicates no appreciable change in poly (ADP-ribose) degrading enzymes in control and hormone treated cells. Therefore, we favor possibility (i) and hope that further investigation will clarify and lead us to a better understanding of the molecular mechanisms of mitogenic action of polypeptide hormones.

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