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Phosphorus-31 Nuclear Magnetic Resonance Analysis of Epidermal Growth Factor Action in A-431 Human Epidermoid Carcinoma Cells and SV-40 Virus Transformed Mouse Fibroblasts†

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ABSTRACT: The effects of epidermal growth factor (EGF) on phosphorus metabolism in perfused A-431 human epidermoid carcinoma cells and SV-40 virus transformed mouse fibroblasts were investigated by using ^{31}P nuclear magnetic resonance. Cells were grown to confluency on microcarrier beads and then placed in a glass sample tube (4.1×10^7 cells/mL) through which medium at 37 °C was perfused. Fourier-transformed ^{31}P NMR spectra were obtained on a Bruker CXP-200/300 spectrometer operating at 80.96 MHz using a solenoidal coil. Prior to treatment of the cells with epidermal growth factor, the spectra were characterized by prominent resonances for ATPβ, ATPα + ADPα, ATPγ + ADPβ, sugar phosphates (including phosphomonoesters), and P_i. Mouse epidermal growth factor (200 ng/mL) was then added to the perfusion medium. In the A-431 cells, no changes were observed up to 80 min; however, after 120 and 160 min relative steady-state levels of ATP were apparently reduced. Concomitantly, a resonance in the region of sugar phosphates was elevated in magnitude. The integrated area assigned to P_i increased significantly at 120 min and returned to the basal value at 160 min. The spectra of SV-40 virus transformed mouse fibroblasts were also altered in response to EGF, although the temporal aspects were different. For example, within 20 min the apparent levels of ATP and ADP were reduced while sugar phosphates were elevated, and these effects persisted through 40 min. These results suggest that epidermal growth factor may lower the cellular levels of ATP and increase the concentration of sugar phosphates in these two cell types in which the factor is not mitogenic. The reduction in ATP levels of A-431 cells following treatment with epidermal growth factor was confirmed by determination of total cellular ATP. Also, immobilization and changes in the relaxation rate (T_1) of cellular ATP after treatment with epidermal growth factor may contribute to the reduced intensity observed in the ^{31}P NMR spectra. This study demonstrates the power and selectivity of ^{31}P NMR in studying acute changes in cellular phosphorus metabolism in response to external stimuli.

Epidermal growth factor is a mitogenic peptide to many cell types both in vivo and in vitro (Carpenter & Cohen, 1978, 1979). A number of investigations into the cellular mechanisms of EGF[1] action have utilized A-431 human epidermoid carcinoma cells as an in vitro model (Carpenter et al., 1979; Chinkers et al., 1979, 1981; Haigler et al., 1979a,b; Ushiro & Cohen, 1980; Cohen et al., 1980; King et al., 1980; Sawyer & Cohen, 1981; Buhrow et al., 1982). The high number of EGF receptors present in these cells [$(2–3) \times 10^6$] has obvious advantages for study over the relatively low numbers [$(4–10) \times 10^4$] present in human fibroblasts (Carpenter et al., 1975). However, in contrast to the mitogenic effects seen in many cells, EGF does not stimulate the growth of A-431 cells, certain breast cancer cells, and other transformed cells (Schonbrunn et al., 1980; Gill & Lazar, 1981; Imai et al., 1981; Barnes, 1982), and moreover the peptide is inhibitory to the growth of these cells. Recent evidence utilizing variant A-431 cell

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[1] Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; P_i, inorganic phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NAD, nicotinamide adenine dinucleotide.

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lines with altered EGF binding and EGF-stimulated tyrosine-specific protein kinase activity suggests that this kinase activity may be involved in mediating the growth-inhibitory effect of EGF (Buss et al., 1982).

^{31}P NMR spectroscopy has been applied as a noninvasive technique to a number of in vivo biological systems (Seeley et al., 1977; Hollis, 1980; Gillies et al., 1981; Chance et al., 1981; Nuccitelli et al., 1981; Ugurbil et al., 1982; Ng et al., 1982; Melner et al., 1982). Recently, this technique has been adapted for use in monitoring anchorage-dependent cells attached to microcarrier beads (Ugurbil et al., 1981) or polymeric membranes (Gonzalez-Mendez et al., 1982) and placed within a perfusion chamber. Under these conditions the cells can be maintained at relatively high densities, and the cellular environment, particularly the state of oxygenation, can be adequately controlled (Smith & Vale, 1980, 1981).

We present herein ^{31}P NMR measurements on both A-431 human epidermoid carcinoma cells and SV-40 virus transformed mouse fibroblasts under basal conditions and following treatment with EGF. These studies were performed with the cells attached to microcarrier beads, perfused with culture media, and situated in a sample tube within the NMR solenoidal coil probe. Our results demonstrate that EGF modulates cellular levels of ATP, ADP, inorganic phosphate, and sugar phosphates.

Materials and Methods

Cell Culture. A-431 cells and SV-40 virus transformed mouse fibroblasts were grown to confluency in 850-cm^2 roller bottles on Dulbecco's modified Eagle's medium with 20 mM Hepes, 50 μg/mL gentamycin, and 10% calf serum. The cells were harvested by a 5-min incubation at 37 °C in 50 mL of trypsin–EDTA (0.05% trypsin and EDTA disodium salt; GIBCO Laboratories, Grand Island, NY). Following a wash in DMEM/10% calf serum, the cells were seeded on autoclaved microcarrier beads (Superbeads, Flow Laboratories; Bio-Carrier beads, Bio-Rad Laboratories) at densities of (1–3) $\times$ 10^6 cells/mL of hydrated beads. Media changes were made at least every 24 h, and care was taken to avoid acidification of the media.

NMR Measurements. ^{31}P NMR spectra were measured on a Bruker CXP-200/300 spectrometer operating at 80.96 MHz, located at the NMR Core Facility, Comprehensive Cancer Center, University of Alabama in Birmingham. A custom-built probe consisting of a 15-mm i.d. four-turn solenoidal coil was employed. Cells anchored to microcarriers (4 $\times$ 10^7 cells/mL) were located in a glass sample tube (27-mm length, 15-mm outer diameter, 13.2-mm inner diameter) containing center hole rubber end pieces. One center hole was covered with a nylon mesh screen (64 $\times$ 64 μm) held in place by a rubber O-ring. Unless stated otherwise, each spectrum consisted of 2000 free induction decays obtained with 60° pulses and a 1.2-s recycle time. Resolution was enhanced by employing the deconvolution difference method (Campbell et al., 1973). Flow of cell medium at 37 °C through the sample chamber was accomplished by withdrawing with a Buchler peristaltic pump at a flow rate of 1–2 mL/min. The perfusion medium consisted of DMEM with 20 mM Hepes, pH 7.5, 1% calf serum, and 50 μg/mL gentamycin and was immersed in a 37 °C water bath equilibrated with air. Cells were treated with highly purified mouse EGF kindly provided by Dr. Stanley Cohen (Savage & Cohen, 1972) by changing perfusion reservoirs to one with the same medium containing 200 ng/mL EGF. This dose is required to saturate the binding sites for EGF on A-431 cells (Sawyer & Cohen, 1981) and is within the range reported as having inhibitory-like effects on A-431 cell growth (Barnes, 1982). Each experiment reported is representative of at least two independent and reproducible experiments. Following the experiments the microcarrier beads were flushed from the chamber, and cells were counted. This was accomplished with a Coulter cell counter following a 5-min incubation with trypsin–EDTA and another 5-min period to allow the beads to settle out of solution.

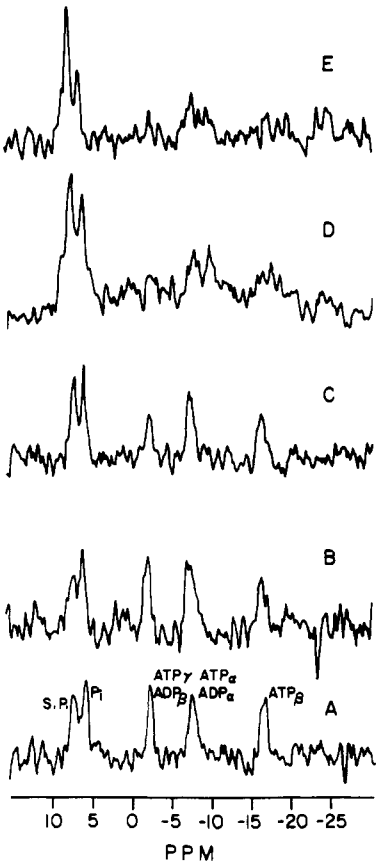

FIGURE 1: ^{31}P NMR spectra of A-431 human epidermoid carcinoma cells attached to microcarrier beads: (A) pretreatment; (B) 40-min post-EGF; (C) 80-min post-EGF; (D) 120-min post-EGF; (E) 160-min post-EGF. Each spectrum consisted of 2000 free induction decays, and the total time per spectrum was 40 min. The spectrum shown at each time point represents the cumulative spectra from the previous time point to the indicated time. S.P. denotes the sugar phosphate region including phosphomonoesters.

Results

^{31}P NMR spectra from A-431 cells under basal conditions are shown in Figure 1A. Three prominent resonances were observed at the high-field end of the spectrum originating from ATPβ, ATPα + ADPα, and ATPγ + ADPβ. At the low-field region, two major resonances can be assigned to the sugar phosphate region, including phosphomonoesters, and P_i. All chemical shifts are reported relative to ATPα set at −7.5 ppm, which is relatively insensitive to pH fluctuations under the conditions used. This was achieved from independent calibration with creatine phosphate at 0 ppm. Initial studies revealed that the cells and the resulting ^{31}P NMR spectra were unchanged for incubations of 120 min in the perfusion apparatus (unpublished observations). Further studies have also shown no apparent differences between microcarrier beads from two different suppliers, and neither microcarrier exhibits a detectable signal when monitored alone.

When medium containing EGF was introduced, no changes were observed during the first 40 min (Figure 1B). By 80-min posttreatment, slight increases were observed in the P_i and sugar phosphate region resonances (Figure 1C). These

Table I: Integrated Peak Areas[a] for the Phosphorus Signals Observed in A-431 Cells

treatment		ATPβ	ATPα + ADPα + NAD	ATPγ + ADPβ	P_i	S.P.[b]
pretreatment		19 ± 2	25 ± 2	19 ± 2	17 ± 2	20 ± 2
post-EGF:[c]	40 min	17 ± 2	29 ± 2	19 ± 2	20 ± 2	18 ± 2
	80 min	21 ± 3	29 ± 3	19 ± 3	26 ± 3	25 ± 3
	120 min	*d*	*d*	*d*	51 ± 4	80 ± 4
	160 min	*d*	*d*	*d*	17 ± 3	44 ± 3

[a] The areas presented reflect 2000 free induction decays and are given in arbitrary units. Error estimates are shown as (±)one-half of the average integrated noise level. [b] Sugar phosphate region including phosphomonoesters. [c] The time given reflects the cumulative spectra from the previous time point to the indicated time. [d] These signals fell below a sensitivity estimated to be adequate for meaningful measurements.

changes are documented in terms of the integrated peak areas given in Table I. At 120 min after the addition of EGF, reduction was observed in the intensity of the peaks assigned to ATP (Figure 1D), and most of the signals arising from ATP and ADP resonances fell below the sensitivity of the instrument under these conditions. Concomitantly, P_i and a resonance in the sugar phosphate region exhibited significant increases (Table I). Within 160 min, ATP levels remained depressed, P_i levels returned to pretreatment levels, and resonances in the sugar phosphate region decreased from the peak levels seen at 120 min (Figure 1E, Table I). Interestingly, a resonance at about −10 ppm, tentatively assigned to NAD moieties, was pronounced at 120-min post-EGF treatment, but was absent or diminished at the earlier and later time points. The intracellular pH was estimated to be 7.6 from the position of the P_i peak, and this was not altered by addition of EGF.

The EGF-mediated apparent decrease in ATP, as indicated by the ^{31}P NMR results, was confirmed by measuring total cellular ATP (Johnson et al., 1970) in perchloric acid extracts of cells (Chinkers et al., 1981) maintained in static culture at 37 °C. A-431 cells were grown in Dulbecco's medium containing 10% calf serum and then maintained in the medium with 1% calf serum for 24 h. EGF was added, and measurements were made 1, 2, and 4 h later. Relative to control values, the ATP content of the treated cells was reduced by 15–30%. Although the experimental conditions differed (i.e., static cultures vs. perfused cells), there is qualitative agreement between the results of ^{31}P NMR and chemical analysis on cellular extracts.

Similar ^{31}P NMR experiments were performed on SV-40 virus transformed mouse fibroblasts. Pretreatment scans of this cell line revealed prominent resonances for the same metabolites that appeared in A-431 cells (Figure 2A). These cells apparently contain higher levels of ADP as indicated by the ratio of the areas of the ATPγ + ADPβ resonance to the ATPβ peak (Table II). The response of these cells to EGF was much more rapid than to the A-431 cells. Within 20 min after addition of EGF, ATP levels were reduced (Figure 2B), and these effects persisted through the 40-min time point where increases were noted in the sugar phosphate region resonances (Table II, Figure 2C). As in the A-431 cell line, SV-40 virus transformed mouse fibroblasts also showed apparent reduced cellular levels of ADP. The intracellular pH of the transformed fibroblasts was estimated to be 7.6 and was independent of EGF.

Discussion

These results describe a heretofore unreported effect of EGF in vitro, namely, an apparent decrease in cellular ATP. It is

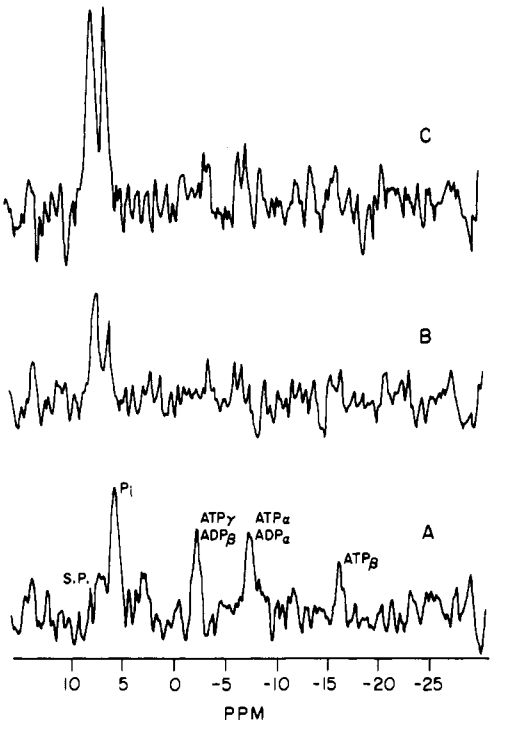

FIGURE 2: ^{31}P NMR spectra of SV-40 virus transformed mouse fibroblasts anchored to microcarrier beads: (A) pretreatment; (B) 20-min post-EGF; (C) 40-min post-EGF. The pretreatment spectrum was based on 3000 free induction decays, and the spectrum at each time point was derived from 1000 free induction decays. The spectrum at 20 min denotes the cumulative spectra from 0 to 20 min and that at 40 min represents the cumulative spectra from 20 to 40 min. S.P. denotes the sugar phosphate region including phosphomonoesters.

Table II: Integrated Peak Areas[a] for the Phosphorus Signals Observed in SV-40 Virus Transformed Mouse Fibroblasts

treatment	ATPβ	ATPα + ADPα + NAD	ATPγ + ADPβ	P_i	S.P.[b]
pretreatment	17 ± 2	48 ± 2	37 ± 2	62 ± 2	23 ± 2
post-EGF: 40 min	*c*	*c*	*c*	55 ± 2	67 ± 2

[a] The areas are in arbitrary units, and error estimates are shown as (±)one-half of the average integrated noise level. The results for the control or pretreatment spectrum are based on 2000 free induction decays, while those for EGF-treated cells reflect 2000 free induction decays and refer to cumulative data over the time interval 0–40 min. [b] Sugar phosphate region including phosphomonoesters. [c] Sensitivity, coupled with a relatively low signal-to-noise ratio in these regions, was not sufficient to permit a meaningful estimate of peak area.

possible, of course, that immobilization and changes in T_1[2] of cellular ATP may contribute to the reduction in peak intensity in the high-field region of the spectrum. However, with the A-431 cells we know from chemical measurements that ATP levels do decrease following treatment with EGF. Concomitant with the decrease in cellular ATP there is an increase in the sugar phosphate region in both cell types, and P_i was found to increase in the A-431 cells 120 min after the addition of EGF. Although EGF is not mitogenic to A-431 cells, it elicits a number of biochemical and morphological effects. For example, following EGF binding, the cells exhibit extensive ruffling, extension of filopodia, formation of multilayered

[2] The possible contribution of T_1 changes is under investigation, but it seems unlikely that with our conditions of a 60° radio-frequency pulse and a 1.2-s recycle time a T_1 change would be sufficient to account for the alteration in intensity that we measured.

colonies, and rounding in calcium-free medium (Chinkers et al., 1979, 1981). It is not known if the spectral changes that we measured are related to these morphological changes. The effect of EGF on SV-40 virus transformed mouse fibroblasts has not been extensively investigated, although Barnes & Colowick (1976) have reported that EGF stimulates deoxyglucose uptake in cells maintained in serum-free medium.

Our results demonstrate the responses of both A-431 human epidermoid carcinoma cells and SV-40 virus transformed mouse fibroblasts to EGF in vitro by monitoring cellular phosphorus metabolism in a noninvasive manner. This technique allows relatively high concentrations of cells to be placed in a small volume and yet maintain cellular functions. Further studies into the actions of mitogenic factors on cellular energetics may prove useful in understanding the responses elicited by these agents. Similar studies on cell types for which EGF is mitogenic are under current investigation.

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Registry No. ATP, 56-65-5; ADP, 58-64-0; P, 7723-14-0; epidermal growth factor, 62229-50-9.

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