Effect of Norepinephrine on Glucose Metabolism in Glioblastoma and Neuroblastoma Cells in Cell Culture

(cyclic AMP/radioactive glucose/glycogenolysis/rat)

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ABSTRACT The addition of norepinephrine to cultured glioblastoma cells results in an inhibition of uptake of radioactivity from D-[2-³H]glucose, D-[1-¹⁴C]glucose, D-[2-¹⁴C]glucose, and D-[6-¹⁴C]glucose. In addition, if the glioblastoma cells are previously labeled with these substrates, norepinephrine causes an increase in the release of radioactivity. These effects were not observed with cultured neuroblastoma cells. It is suggested that the breakdown of glycogen is activated by norepinephrine as a result of an increase in 3':5'-cyclic AMP.

Several years ago Galambos (1) postulated that, in addition to neuron-neuron synapses, neuron-glia synapses occur. A metabolic relationship existing between neurons and glia was suggested originally by Hyden and Lange (2) on the basis of whole animal studies and more recently by Gilman and Nirenberg (3) on the basis of the finding that the neurotransmitter, norepinephrine, causes an increase in 3':5'-cyclic AMP (cAMP) in rat glioblastoma cells in cell culture. These findings were substantiated by Clark and Perkins (4), who also found a rapid increase in the concentration of cAMP of epinephrine and histamine, in addition to norepinephrine, in a tumor astrocyte cell-line derived from a primary culture of a human glioblastoma multiforme. It is known from the early work of Rall and Sutherland (5) that glycogenolysis was increased by cAMP due to activation of a protein kinase that converts phosphorylase *b* to phosphorylase *a*, the active form of the enzyme.

On the basis of these several results, it seemed of interest to examine in cell culture the effect of norepinephrine on glucose incorporation and excretion in glioblastoma and neuroblastoma cells.

MATERIALS AND METHODS

Cell line and culture

Clone C-6, derived from a rat (Wistar strain) astrocytoma induced by *N*-nitrosomethylurea, and clone C-46, derived from a mouse neuroblastoma, were obtained from Dr. G. Sato (6). Clones C-6 and C-46 were grown in Dulbecco's modified Eagle's (7) medium with 10% fetal-calf serum in Falcon flasks or petri dishes at 37° in an atmosphere of 10% CO₂-90% air at 100% humidity.

Radioactivity experiments

For the radioactivity experiments, 60-mm petri dishes were incubated with about 3×10^6 cells for 2 days in the above

medium. By this time the cells were confluent. The medium was then changed to 2 ml of fresh modified Eagle's medium or Earle's (8) balanced salt solutions containing the radioactive compounds. For the studies on the effect of theophylline, the cells were incubated for 1 hr in the culture medium plus 1 mM of theophylline before the addition of the radioactive medium. Norepinephrine (17 µg/ml) was added where indicated. In the experiments in which cells were previously labeled with radioactive compounds, the cells were incubated for 2 hr in a radioactive medium, washed twice with non-radioactive medium, and then incubated for 30 min in a nonradioactive medium.

After incubation in the desired medium the cells were scraped from the petri dish, filtered through a Millipore filter (HAWP 025, HA 0.45 µm, 25 mm) to remove the medium, and washed twice with 0.32 M sucrose. The filter was counted in a Beckman scintillation counter with automatic quench control. It was found that the radioactivity remaining on the filter was constant after the two washings. The radioactivity was then converted to disintegrations per min (dpm) by standard techniques. The operation from the time of scraping to the addition of scintillation fluid was from 5-10 min at 0-5°. In one series of experiments the effect of norepinephrine on ¹⁴CO₂ evolution from [¹⁴C]glucose was determined. Cells were grown in 25-ml Erlenmeyer flasks containing a center well. After addition of the labeled substrate, ¹⁴CO₂ was collected in KOH by standard techniques.

Materials

Theophylline was a product of Nutritional Biochemicals, Inc.; norepinephrine and dibutyl cyclic AMP were purchased from Sigma Chemical Co. The radioactive carbohydrates were purchased from New England Nuclear; the specific activities were: L-[1-¹⁴C]glucose, 1 mCi/0.292 mg; D-[2-³H]glucose, 0.25 mCi/4.45 mg; D-[1-¹⁴C]glucose, 0.1 mCi/2.7 mg; D-[2-¹⁴C]glucose, 0.1 mCi/6 mg; and D-[6-¹⁴C]glucose, 0.1 mCi/9.2 mg.

RESULTS

Since the total cells in each dish were collected on Millipore filters for the determination of radioactivity, it was not possible to count the number of cells present nor to do a protein determination to account for any variability due to differences in cell numbers. To account for the latter, it was necessary to use two substrates in each experiment, one labeled with ³H and the other with ¹⁴C. In each experiment, one set of experiments consisted of the addition of L-[1-¹⁴C]-

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TABLE 1. Incorporation of [^{14}C]glucose into glioblastoma cells

Substrate	dpm (total in cells)					
	Prior incubation with theophylline			No prior incubation		
	Control	Norepinephrine	% Inhibition of uptake	Control	Norepinephrine	% Inhibition of uptake
D-[2- ^3H]glucose	23,800 $\pm 1,200^*$	21,800 $\pm 950^*$	5	26,000 $\pm 1,350^*$	16,700 $\pm 1,280^*$	35
D-[1- ^{14}C]glucose						
Exp. 1	3,160	2,340	26	3,400	1,590	52
Exp. 2	—	—	—	780	630	22
Exp. 3	1,100	800	27	1,160	810	30
Exp. 4	—	—	—	2,560	1,350	48
D-[2- ^{14}C]glucose						
Exp. 1	4,600	4,150	10	5,950	2,340	60
Exp. 2	—	—	—	1,520	1,240	20
Exp. 4	—	—	—	5,900	4,350	26
D-[6- ^{14}C]glucose						
Exp. 1	4,300	3,130	28	4,500	2,200	50
Exp. 2	—	—	—	1,480	1,200	20
Exp. 4	—	—	—	3,240	2,120	35

The cells were incubated for 2 days in Dulbecco's modified Eagle's medium plus 10% fetal-calf serum. Each 60-mm petri dish was initially inoculated with 3×10^6 cells in 3 ml of medium. The growth medium was removed and replaced with 2 ml of Earle's balanced salt solution containing the radioactive substrates. The cells were then incubated for an additional 30 min before they were filtered through a Millipore filter. When the cells were previously incubated in the presence of theophylline, growth medium was replaced with new growth medium containing 1 mM of theophylline, and the cells were incubated for 1 hr. This latter medium was then removed, replaced with Earle's balanced salt solution containing the radioactive substrates, and incubated for 30 min. The radioactive medium contained D-[2- ^3H]glucose (1.56×10^6 dpm) plus either D-[1- ^{14}C]glucose (1.63×10^6 dpm), D-[2- ^{14}C]glucose (3.36×10^6 dpm), or D-[6- ^{14}C]glucose (2.24×10^6 dpm). Where indicated, the radioactive medium contained 17 $\mu\text{g}/\text{ml}$ of norepinephrine. The incubations were at 37°, 100% humidity, and 90% air–10% CO_2 .

* Standard error, $n = 3$.

glucose and D-[2- ^3H]glucose to the medium. The assumption in this instance is that the L-glucose would not be metabolized but only incorporated into the cells depending on the number of cells present. That this is reasonable is shown in Fig. 1. The amount of L-glucose incorporated was dependent on the number of cells added. In addition it was found that norepinephrine had no effect on the incorporation of this labeled compound. In the experiments with [^{14}C]glucose, D-[2- ^3H]glucose was also added to account for variability in cell numbers between experiments. As shown in Fig. 1, this also was reasonable since the amount of incorporation was dependent on the amount of cells present. Since several identical experi-

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TABLE 2. Incorporation of [^{14}C]glucose into glioblastoma cells

Substrate	dpm (total in cells)					
	Prior incubation with theophylline			No prior incubation		
	Control	Norepinephrine	% Inhibition of uptake	Control	Norepinephrine	% Inhibition of uptake
D-[2- ^3H]glucose	13,100 $\pm 700^*$	8100 $\pm 490^*$	38	11,250 $\pm 600^*$	9000 $\pm 540^*$	21
D-[1- ^{14}C]glucose						
Exp. 1	1,700	1230	30	1,280	1040	18
Exp. 2	1,350	690	50	—	—	—
Exp. 3	1,060	835	22	—	—	—
D-[2- ^{14}C]glucose						
Exp. 1	1,310	940	28	1,970	1300	34
D-[6- ^{14}C]glucose						
Exp. 1	2,600	1520	40	1,830	1160	35
Exp. 2	670	320	51	—	—	—

The conditions were the same as Table 1, except that the radioactive compounds were in Dulbecco's modified Eagle's medium instead of Earle's balanced salt solution.

* Standard error, $n = 4$.

TABLE 3. Excretion of radioactivity into the medium from glioblastoma cells

Substrate	dpm (total in cells)		% Increased excretion from cells
	Control	Norepinephrine	
D-[2- ³ H]glucose	7420 ±380*	4390 ±370*	40
D-[1- ¹⁴ C]glucose			
Exp. 1	3240	2040	38
Exp. 2	2040	1410	30
Exp. 3	6650	3900	40
D-[2- ¹⁴ C]glucose			
Exp. 1	4270	2480	42
Exp. 3	7300	4800	34
D-[6- ¹⁴ C]glucose			
Exp. 1	2260	1790	22
Exp. 3	9000	7800	16

The cells were grown under the same conditions as Table 1. After 2 days, the growth medium was removed and replaced with Earle's balanced salt solution containing the radioactive substrates as in Table 1. After 2 hr the radioactive medium was removed, the cells were washed quickly, and 2 ml of Earle's balanced salt solution (nonradioactive) were added with or without norepinephrine. The cells were incubated for 30 min and then filtered.

* Standard error, $n = 3$.

ments were done except for cell numbers, it was possible to determine an average value for D-[2-³H]glucose incorporation, and this value was used to correct for variations in cell numbers. It was also found that two washings with 0.32 M sucrose were adequate, in that essentially constant values for incorporation resulted.

For the studies of the effects of norepinephrine on the uptake of D-[¹⁴C]glucose, glioblastoma cells were grown for 2 days in modified Eagle's medium plus 10% fetal-calf serum. The growth medium was removed and replaced with the same medium or medium plus 1 mM of theophylline. After 1 hr the medium was replaced with Earle's balanced salt solution containing different radioactively labeled compounds; the cells were then incubated for 30 min and filtered; (see Table 1). When the cells are not previously incubated in theophylline, there is an inhibition by norepinephrine of from 20–50% in the incorporation of glucose. Less inhibition (10–30%) was found when the cells are previously incubated in theophylline. If the cells are incubated as previously described, except that modified Eagle's medium plus 10% fetal-calf serum is used in place of balanced salt solution, similar results are obtained (Table 2).

In another series of experiments, the cells were incubated for 2 hr in the presence of radioactive substrates and washed; the medium was then changed to balanced salt solution. The cells were incubated for 30 min in the presence of 1 mM of theophylline and norepinephrine. As shown in Table 3, norepinephrine caused an increase in the release of isotope from the cells, as evidenced by the presence of less radioactivity in these cells.

In another series of experiments, the evolution of ¹⁴CO₂ from specifically labeled [¹⁴C]glucose was determined. As shown in Table 4, the addition of norepinephrine caused an

FIG. 1. Incorporation of radioactive glucose into glioblastoma cells. Variable amounts of glial cells were added to petri dishes (1×10^6 cells/ml) containing 2 ml of Dulbecco's modified Eagles medium plus 10% fetal-calf serum. After 48 hr the medium was replaced with 2 ml of medium containing 1 μ l of L-[1-¹⁴C] glucose (1.63×10^6 dpm) and 1 μ l of D-[2-³H] glucose (1.56×10^4 dpm). After 30 min the cells were scraped from the dish and filtered through a Millipore filter. The filter was then counted. Δ — Δ = L-[1-¹⁴C] glucose; $\circ$ — $\circ$ = D-[2-³H] glucose.

increase in ¹⁴CO₂ evolution from [1-¹⁴C]glucose, [2-¹⁴C]glucose, and [6-¹⁴C]glucose.

The effect of norepinephrine on glucose metabolism in neuroblastoma cells was also studied. The cells were grown for 2 days in modified Eagle's medium plus 10% fetal-calf

TABLE 4. The effect of norepinephrine on the evolution of ¹⁴CO₂ from [¹⁴C]glucose in glioblastoma cells

Substrate	dpm/mg protein in ¹⁴ CO ₂	
	Control	Norepinephrine
D-[1- ¹⁴ C]glucose		
Exp. 1	950	1400
Exp. 2	3400	5600
D-[2- ¹⁴ C]glucose		
Exp. 2	1560	2750
D-[6- ¹⁴ C]glucose		
Exp. 1	860	970
Exp. 2	260	300

The cells were incubated for 2 days in Dulbecco's modified Eagle's medium in 25-ml Erlenmeyer flasks containing a center well. The growth medium was then removed and replaced with Earle's balanced salt solution containing the radioactive substrates. To the center well was added 0.2 ml of 2 N KOH and a fluted filter paper. The flasks were then sealed with a rubber serum-bottle stopper. After incubation at 37° for 30 min, 1 ml of 1 N HCl was injected into the flask through the rubber stopper. The cells were incubated another 2 hr, and the filters and KOH were then removed and placed in a scintillation vial. The cells were scraped from the flasks for the protein determinations.

FIG. 2. A glioblastoma cell is illustrated showing how its glucose metabolism is affected by norepinephrine by activation of glycogenolysis and subsequent induction of CO_2 production by glycolysis.

serum. The growth medium was removed and replaced by balanced salt solution containing different labeled compounds, and the cells were incubated for 30 min and filtered. As shown in Table 5, norepinephrine had no effect on the incorporation of label from radioactive glucose in these cells.

DISCUSSION

In these experiments we examined the question of whether norepinephrine affects the uptake of radioactive glucose, the release of radioactive compounds derived from radioactively labeled glucose, or the metabolism of radioactive glucose in glioblastoma and neuroblastoma cells in culture. From the results presented it appears that the addition of norepinephrine affects these processes in glioblastoma cells and not in neuroblastoma cells in cell culture.

We suggest that the process occurring in glioblastoma cells can be explained as shown in Fig. 2. The breakdown of glycogen is activated by norepinephrine as a result of an increase in cAMP (2, 3), which activates the protein kinase for conversion of phosphorylase *b* to phosphorylase *a*. This process results in an increase of glucose within the cell. If radioactive glucose is added to the medium less will be taken into the cell because of an increase in an intracellular pool of glucose or compounds derived from glucose. This is the result that was obtained (Tables 1 and 2). If the cells are previously labeled with glucose and the medium containing the radioactive compounds is removed, then it might be expected that radioactivity would be lost from the cell in the presence of norepinephrine. This occurred (Table 3). In addition, it might be expected that if norepinephrine caused an increased conversion of glycogen to glucose, increased oxidation of the glucose would also occur. The results shown in Table 4 are consistent with this postulate.

Although we may speculate that the effect of norepinephrine is on the breakdown of glycogen, the nature of the

TABLE 5. Incorporation of [^{14}C]glucose into neuroblastoma cells

Substrate	dpm (total in cells)	
	Control	Norepinephrine
D-[2- ^3H]glucose	7150 $\pm 550^*$	7400 $\pm 600^*$
D-[1- ^{14}C]glucose	1140	1270
D-[2- ^{14}C]glucose	1400	1320
D-[6- ^{14}C]glucose	1440	1510

The conditions were the same as for Table 1, except that neuroblastoma cells were used in place of glioblastoma cells.

* Standard error, $n = 3$.

experiments do not permit an unequivocal conclusion in this regard. This would require determination of the products derived from the radioactive glucose and the nature of the compounds lost from the cell in the presence of norepinephrine.

Nevertheless, we propose that *in vivo* these products from glia cells are made available to neurons and used for metabolic processes in the neurons, assuming the present experiments might have relevance to the intact brain. Thus, one can envision the relation of neurons and glia cells to be one whereby a neurotransmitter from neurons triggers a reaction in glia cells that results in the latter providing metabolites to the neuron for various synthetic reactions or generating energy for cellular processes.

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