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Regulation of Glycogen Content in Primary Astrocyte Culture: Effects of Glucose Analogues, Phenobarbital, and Methionine Sulfoximine

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Abstract: Compounds known to affect glycogen metabolism in vivo or in cell-free preparations were used to investigate the regulation of glycogen content in intact astrocytes cultured from newborn rat cortex. Compounds were added with fresh medium to culture dishes, and astrocyte glucose and glycogen content determined 24 h later. Increasing the medium glucose concentration from 7.5 m*M* to 30 m*M* increased cell glycogen content 80%. Addition of 2-deoxyglucose or 3-*O*-methyl glucose (2.5–10 m*M*) also increased cell glycogen content, 50–100%, suggesting a regulatory rather than mass action effect of glucose on astrocyte glycogen content. The phosphorylase *b* inhibitors 2,2',4,4',5,5'-hexabromobiphenyl and riboflavin had no effect on astrocyte glycogen content, consistent with negligible phosphorylase *b* activity in normal astrocytes. Phenobarbital and L-methionine-DL-sulfoximine (MSO) are both known to induce astrocyte glycogen accumulation in vivo. The addition of phenobarbital (2 m*M*) had no effect on the glycogen content of cultured astrocytes, suggesting an indirect mechanism for the in vivo effect. MSO at 1 m*M*, however, induced a 300% increase in glycogen content. The time course of glucose and glycogen content after MSO administration suggests this increase to be the result of slowed glycogenolysis rather than accelerated glycogen synthesis. **Key Words:** Astrocyte culture—Glucose—3-*O*-Methyl glucose—2-Deoxyglucose—Glycogen—Methionine sulfoximine. **Swanson R. A. et al.** Regulation of glycogen content in primary astrocyte culture: Effects of glucose analogues, phenobarbital, and methionine sulfoximine. *J. Neurochem.* **52,** 1359–1365 (1989).

Under normal conditions, brain glycogen has a rapid turnover relative to other tissues (Ibrahim, 1975). However, total brain glycogen content remains relatively constant, independent of wide fluctuations of liver glycogen or plasma glucose (Hutchins and Rogers, 1970; Watanabe and Passonneau, 1973). Although both neurons and glia are capable of metabolizing glycogen (Knull and Khandelwal, 1982), cytochemical studies suggest that in normal brain, glycogen is localized primarily, although not exclusively, to glia (Cataldo and Broadwell, 1986).

Cultured astrocytes exhibit glycogenolysis in response to a number of neuromodulators (Browning et al., 1974; Magistretti et al., 1983; Pearce et al., 1977). This energy mobilization may serve any of the known energy-requiring functions of glia in brain, such as maintenance of extracellular H^+, K^+, Na^+, and water concentrations (Hertz, 1981), and neurotransmitter uptake and metabolism, particularly of glutamate (Schousboe et al., 1977). Glial glycogen might therefore modulate the extent of injury resulting from ischemia, where glucose is limited and extracellular H^+, K^+, osmolality, and glutamate are increasing. Strategies for increasing or decreasing glial glycogen content may prove useful in investigating this possibility.

A number of pharmacological agents have been described in studies of cell-free systems that modulate specific enzymatic activities of glycogen metabolism. However, the effects of these agents in intact cells are largely unknown. Moreover, several agents increase whole brain glycogen in vivo but have an uncertain mode and site of action (Nelson et al., 1968; Hutchins

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Abbreviations used: dBcAMP, dibutyryl cyclic AMP; 2-DG, 2-deoxyglucose; MEM, modified Eagle's minimum essential medium; 3-O-MG, 3-*O*-methyl glucose; MSO, L-methionine-DL-sulfoximine.

relatively stable between 24 and 48 h. Medium glucose appeared to be consumed at a constant rate (0.31 μmol/h/mg of protein) over these intervals. The presence of dBcAMP in the medium (Fig. 1A) resulted in a markedly attenuated initial rise in astrocyte glycogen content as well as lower levels at all subsequent time points. Subsequent experiments, assessing the effects of pharmacologic agents on cell glycogen content, utilized cultures incubated with the test compounds for 24 h in dBcAMP-free medium.

Doubling the initial medium glucose concentration from 7.5 m*M* to 15 m*M* resulted in an approximate doubling of intracellular glucose concentration at 24 h, and a 50% increase in glycogen (Fig. 2). Quadrupling the medium glucose concentration to 30 m*M* again resulted in a proportionate increase in astrocyte glucose content, but a much smaller incremental rise in glycogen content. Mannitol at 25 m*M* was tested to control for osmotic effects of increased medium glucose; there was no significant difference from control values.

Cummins et al. (1983*b*), using homogenates of transformed astrocytes, found a decrease in the ratio of phosphorylase *a*/phosphorylase *b* after exposure of the cells to glucose or glucose analogues. Figure 3 depicts the effects of glucose analogues on the content of glycogen and glucose in intact astrocytes. The addition of 3-*O*-methyl glucose (3-O-MG) to the culture medium resulted in a dose-dependent increase in astrocyte glycogen content and small increases in glucose content. 2-Deoxyglucose (2-DG) also caused accumulation of glycogen at 2.5 and 5 m*M*, but less so at 10 m*M*. Glucose content was greatly increased at all concentrations of 2-DG, although the effect was less at 10 m*M* than at 5 m*M*.

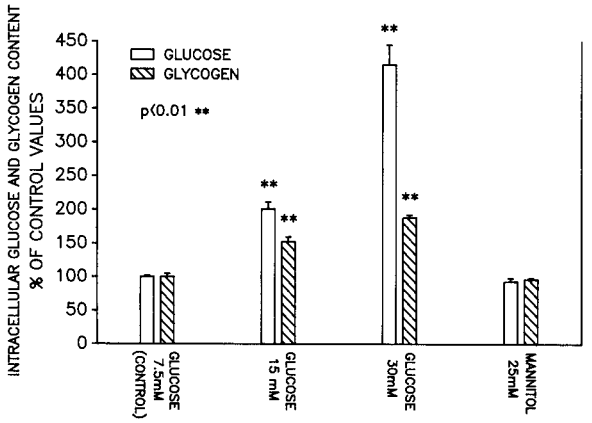

FIG. 2. Effects of the medium glucose concentration on astrocyte glucose and glycogen content. Cells were harvested 24 h after the change of culture media. Values were calculated as nanomoles of glucosyl equivalents per milligram of protein, and expressed as percent of control values + SEM. In each case n = 8, representing pooled results from two experiments. The *p* values indicated are of Student's *t* test comparisons of mean values from cultures incubated in the experimental versus control conditions. ($p < 0.05$ for the difference in glycogen content of cultures incubated in 15 versus 30 m*M* glucose.)

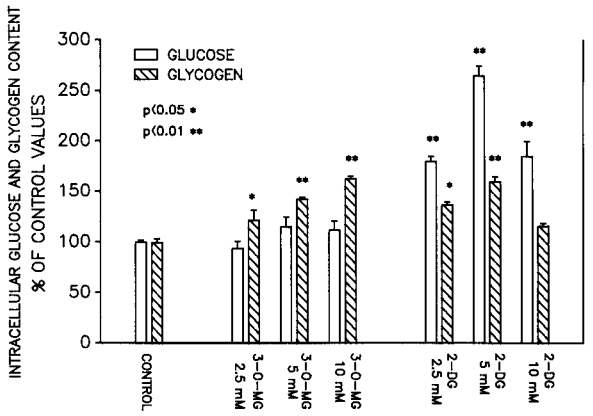

FIG. 3. Effects of the glucose analogues 2-DG and 3-O-MG on astrocyte glucose and glycogen content. Media glucose concentration, 7.5 m*M*; conditions otherwise as in Fig. 2.

Riboflavin inhibits isolated rabbit muscle phosphorylase *b* by competing for the AMP binding site (K_i, 15.6 μ*M*) (Klinov et al., 1986). 2,2',4,4',5,5'-Hexabromobiphenyl is one of several polyhalogenated biphenyls that noncompetitively inhibits isolated rabbit muscle phosphorylase; a 60 μ*M* concentration inhibits the activity of phosphorylase *b* by 90% and phosphorylase *a* by 30% (Mead et al., 1982). Neither of these agents had a significant effect on the glucose or glycogen content of cultured astrocytes (Fig. 4).

Barbiturates at high doses cause a several-fold increase in rat brain glycogen in vivo (Nelson et al., 1968). In the present study phenobarbital at a comparable level (Richards, 1972) caused a small reduction in astrocyte glucose concentration and had no significant effect on glycogen content (Fig. 4).

L-Methionine-DL-sulfoximine (MSO) is also reported to cause a several-fold increase in brain glycogen in vivo (Folbergrová, 1973). Comparable doses of MSO added to the culture medium in the present study re-

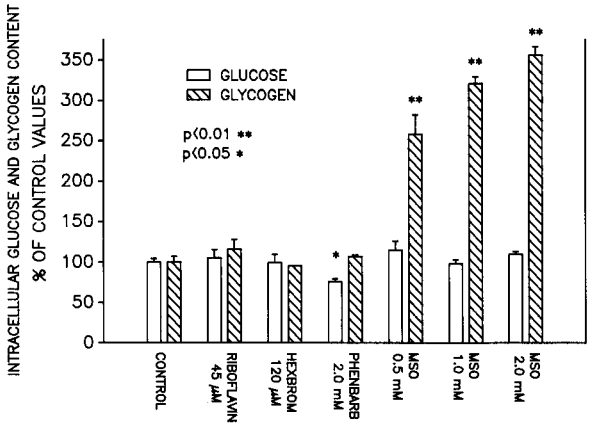

FIG. 4. Effects of phenobarbital, MSO, and phosphorylase *b* inhibitors on cultured astrocyte glucose and glycogen content. HEXBROM, 2,2',4,4',5,5'-hexabromobiphenyl; PHENBARB, phenobarbital.

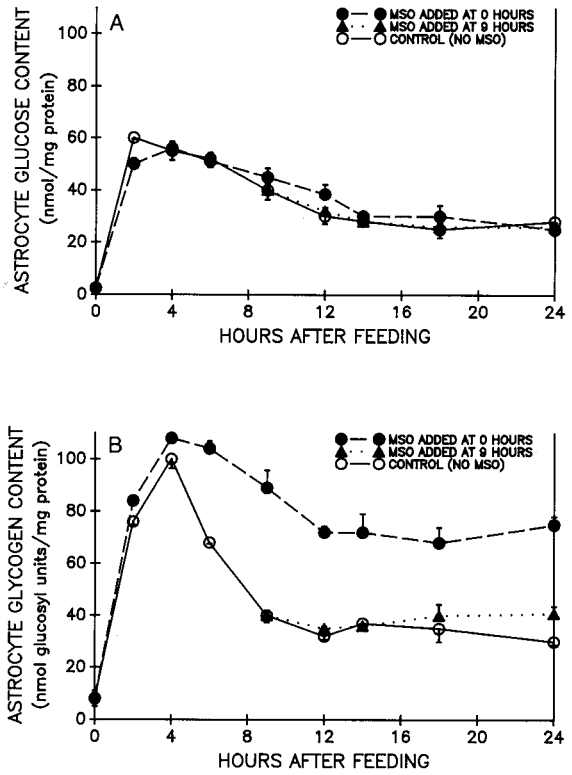

FIG. 5. Effects of MSO on astrocyte glucose (**A**) and glycogen (**B**) content at several times after feeding. Culture medium was replaced at time 0 with fresh medium either with or without 1 m*M* MSO (n = 4). In some dishes (triangles) 1 m*M* MSO was added 9 h after feeding. Data shown are for one of two experiments with similar results.

sulted in 250–350% increases in astrocyte glycogen concentration (Fig. 4). To assess whether MSO was affecting either glycogen synthesis or glycogenolysis primarily, glucose and glycogen were measured at several time points after the addition of fresh medium containing 1 m*M* MSO. The peak glycogen content of the MSO-treated cells was similar to that of control cultures, but the subsequent decline in glycogen content was much slower (Fig. 5B). MSO (1 m*M*) administered 9 h after fresh medium was added, which was after the period of rapid net glycogenolysis, resulted in a minimal deviation from control values (Fig. 5B). Intracellular glucose was unaffected by MSO, whether administered with fresh medium or 9 h later (Fig. 5A).

DISCUSSION

The use of primary astrocyte cultures for pharmacologic studies offers some advantages over other methods. In contrast to cell-free preparations, cell culture allows assessment of pharmacologic effects under conditions existing in the intact cell. Relative to in vivo methods, there is elimination of the confounding effects of systemic metabolism, enabling the delineation of direct effects on astrocytes. Primary astrocytes cultured according to the method employed here have been well characterized (Hertz, 1982; Hertz et al., 1985). The addition of dBcAMP to the media produces morphologic and biochemical differentiation which, in general, more closely approximates the characteristics of astrocytes in vivo (Schousboe et al., 1980; Kimelberg, 1983; Hertz et al., 1985).

To assess the effects of pharmacologic agents, it was first necessary to establish the relationships between medium glucose concentration, time after feeding, and cell glycogen content. These relationships are shown in Fig. 1. The addition of fresh media (with or without dBcAMP) resulted in a rapid rise of both glucose and glycogen content until 6 h after feeding, followed by a decline until about hour 18. A qualitatively similar rise and fall in glycogen content after feeding has also been observed in C-6 astrocytoma cells (Passonneau and Crites, 1976; Passonneau et al., 1978), and in 6-day-old primary and transformed rat cortical astrocytes (Cummins et al., 1983*a*). The reason for this fall in glycogen content is unclear; the decline in medium glucose over this interval is relatively small in the present study, a result suggesting that a factor other than medium glucose concentration stimulates glycogenolysis. Components of fetal calf or horse serum may well affect glycogen metabolism in this system.

The presence of dBcAMP in the media (Fig. 1) during the experimental period resulted in attenuation of the initial increase after feeding and lower glycogen levels at all subsequent time points. This is not unexpected given the known stimulatory effect of cyclic AMP on astrocytic glycogenolysis (Opler and Makman, 1972; Quach et al., 1978). dBcAMP was therefore deleted from the media when evaluating test compounds in order to avoid masking effects on glycogen content. The initial 24 h after feeding was chosen as the experimental time period because: (1) the glycogen levels are relatively stable at 24 h (Fig. 1); (2) this time interval includes periods with both glycogen synthesis and glycogenolysis predominating, such that agents acting on either pathway might have appreciable effects on net glycogen content; and (3) agents maintaining an effect over several hours may be suitable for use in in vivo studies.

Glucose, in addition to serving as a substrate for glycogen synthesis, may also have a regulatory effect on astrocyte glycogen content. As evident in Fig. 2, doubling and quadrupling the medium glucose concentration at the time of feeding led to proportionate increases in intracellular glucose concentration (measured at 24 h) and increases in cell glycogen content of 50 and 80%, respectively. Similar observations have been reported with astrocytoma cells (Passonneau and Crites, 1976) and transformed astrocytes (Cummins et al., 1983*a*), where increases in medium glucose from beyond 12.5 m*M* did not increase glycogen accumulation 2.5 h after feeding. This apparently saturable effect of glucose on glycogen accumulation suggests either a saturable allosteric regulatory effect of glucose

on glycogen accumulation, a feedback inhibition of glycogen on its synthesis, or both.

Increases in glucose concentration would not be expected to have a mass action effect on glycogen content because glucose and glycogen concentrations are not in equilibrium (McIlwain and Bachelard, 1985). However, other mechanisms by which glucose may affect glycogen metabolism have been described. As reported by Stalmans et al. (1970, 1974), the activity of phosphorylase phosphatase in liver is accelerated in the presence of glucose, resulting in a decrease in the proportion of glycogen phosphorylase in the physiologically more active "a" form as well as an increase in the proportion of glycogen synthetase in the physiologically more active "I" form. Glucose may also have a direct inhibitory effect on phosphorylase *a* (Hue et al., 1975).

The effects of the glucose analogues 3-O-MG and 2-DG provide evidence for a similar regulatory mechanism in astroglia. Cummins et al. (1983*b*), found a decrease in the ratio of phosphorylase *a*/phosphorylase *b* in transformed astrocytes after exposure to glucose or to glucose analogues. 3-O-MG is not phosphorylated in brain and therefore cannot be incorporated into glycogen (Sols and Crane, 1954; Jay et al., 1987). 2-DG can be phosphorylated and eventually incorporated into glycogen, although at a rate much slower than the rate of glucose incorporation (Pentreath et al., 1982). In the present study (Fig. 3), 3-O-MG and 2-DG induced increases in astrocyte glycogen content comparable to the maximal increase achieved with elevated medium glucose concentrations, supporting a regulatory rather than mass action effect of glucose in the intact cell. The effects of 3-O-MG on glycogen accumulation were dose dependent and appeared to be saturable. 2-DG exhibited more complicated effects on glycogen and also increased intracellular glucose, possibly because of inhibition of glycolysis by 2-DG-6-phosphate and inhibition of glucose transport at higher concentrations of 2-DG (Horton et al., 1973). The elevations in intracellular glucose content produced by 2-DG may also have affected glycogen levels.

Implicit in the above discussion is the assumption that in normal astrocytes, phosphorylase *b* activity is suppressed to a low level with essentially all glycogenolysis attributable to phosphorylase *a* activity (Siesjö, 1978). This was tested in the present study by assessing the effects of the phosphorylase *b* inhibitors riboflavin and 2,2',4,4',5,5'-hexabromobiphenyl on glycogen content. Riboflavin acts by competing for the AMP binding site (Klinov et al., 1986) and hexabromobiphenyl inhibits phosphorylase *b* noncompetitively (Mead et al., 1982), using enzyme isolated from rabbit muscle. Neither agent significantly altered astrocyte glycogen content at medium concentrations several-fold higher than the reported K_i values (Fig. 4). Although other possible mechanisms have not been excluded, the lack of effect is consistent with a physiologically low activity of phosphorylase *b*.

Barbiturates and MSO are both known to increase markedly brain glycogen content in vivo, although the mechanisms of these effects are not well understood. Phenobarbital (170 mg/kg i.p.) induces a two- to three-fold rise in brain glycogen content in rats within 8 h (Nelson et al., 1968; Phillips and Coxon, 1973) and the increase can be maintained for days with continued phenobarbital administration. This dose results in a brain extracellular concentration in the range of 1.0–2.0 m*M* (Richards, 1972). The glycogen accumulates almost exclusively in astroglia (Phelps, 1972). In contrast, phenobarbital 2 m*M* added to astrocyte culture medium in the present study had no effect on glycogen content, suggesting the in vivo effect is by a mechanism other than directly upon astrocytes. A lack of effect in vitro has also been noted using C-6 astrocytoma cells (Lust et al., 1975). That the in vivo effect may be a consequence of suppressed neuronal activity, rather than direct action on astrocytes, is supported by similar astrocytic glycogen accumulation in hibernation, hypothermia, after neuronal injury, and in response to a wide range of CNS depressants (Ibrahim, 1975).

Like phenobarbital, MSO also induces a three- to fourfold elevation in brain glycogen, with electron microscopy showing accumulation restricted to astrocytes (Folbergrová, 1973; Phelps, 1975). However, unlike barbiturates, MSO does not depress neural metabolism, and in fact induces seizures at doses that cause glycogen accumulation. The observed effect of MSO on cultured astrocytes was also different; 300–400% increases in glycogen content were seen at doses comparable (on a milligram per volume basis) to doses effective in vivo (Fig. 4).

MSO has a number of effects on brain metabolism (Sellinger et al., 1984), but no direct action on enzymes of glycolysis or glycogenolysis has been identified. Increased synthesis and activity of the gluconeogenic enzyme fructose-1,6-biphosphatase in astrocytes has been reported by Hevor and Gayet (1981), and these authors have proposed that MSO-induced glycogen accumulation is secondary to increased gluconeogenesis. Alternatively, Folbergrová (1973) observed a decrease in phosphorylase *a* activity in mouse brain treated with MSO, and suggested inhibition of glycogenolysis as a cause of glycogen accumulation. These alternatives were evaluated in the present study by measuring astrocyte glucose and glycogen levels at various times after feeding, with or without MSO in the medium (Fig. 5A and B). MSO did not significantly increase glucose content or peak glycogen concentration. However, MSO-treated cultures did exhibit a slower decline in glycogen concentration during the period normally characterized by rapid net glycogenolysis (Figs. 1B and 5B). This effect could be the result of either slowed glycogenolysis, accelerated glycogen synthesis, or both. These possibilities were evaluated by administering MSO to cultures 9 h after feeding. Normally, astrocyte glycogen content remains relatively stable after 9–12 h postfeeding (Figs. 1B and 5B), reflecting either

matched glycogen synthesis and glycogenolysis or, more likely, relatively little activity of either pathway. The relative failure of MSO to elicit an increase in glycogen content when added during this period (Fig. 5B) suggests slowed glycogenolysis, rather than accelerated glycogen synthesis, as the mode of action of MSO.

These observations can be reconciled with the reported failure of MSO to increase glycogen in brain slice preparation (Folbergrová, 1973). In that study, MSO was added with glucose to brain slices depleted of glycogen, and 3 h later the amount of newly synthesized glycogen was determined. Measured levels were not different from control values, as would be expected if MSO acts primarily to inhibit glycogenolysis.

The cell culture system is a useful complement to cell-free and in vivo studies for characterizing pharmacological effects. Data presented here support a regulatory effect of glucose at phosphorylase phosphatase in intact astrocytes. The glucose analogues 3-O-MG and 2-DG were also seen to be effective in increasing cultured astrocyte glycogen content. The in vivo effect of barbiturates on glycogen content appears to be indirect, as no response was seen in pure astroglial culture. In contrast, the effect of MSO on glycogen content was comparable to that seen in vivo, and appeared to be mediated through an inhibition of glycogenolysis.

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