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The importance of choice of anaesthetics in studying radiation effects in the 9L rat glioma

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Summary In the present study we demonstrate that the glycolysis of the tumour 9L glioma, *in vivo*, may be manipulated with ketamine/xylazine combinations of anaesthetics. Xylazine alone or in combination with ketamine causes hyperglycaemia which is enhanced by glucose injections. Intracellular tumour pH is acidified when glucose is administered with ketamine/xylazine. However, the combination of inorganic phosphate and insulin with ketamine/xylazine and glucose caused an alkaline shift in the tumour pH as measured by ^{31}P NMR. The anaesthetic combination of ketamine/acepromazine did not produce alterations in blood glucose or in tumour pH status as detected by ^{31}P NMR spectroscopy. These results demonstrate dramatic effects of ketamine/xylazine on the acidification or alkalinisation of the cells of 9L glioma. These altered metabolic states are of potential therapeutic importance. The choice of xylazine alone would be useful for chemotherapy and hyperthermia modalities, both known to be dependent upon glucose metabolism and resultant acidification.

Keywords: ketamine; xylazine; pH glycolysis; glucose; 9L glioma

Glioma is known to be insensitive to chemo- and radiotherapy. Therefore, the use of innocuous agents to alter metabolically glioma tumour pH, would have clinical relevance for enhancing radiation, hyperthermia or chemotherapy effects. Our goal is to improve the radiation response by manipulating the metabolism of a glioma tumour model. We used glucose-mediated inhibition of oxygen consumption in an early attempt to improve the radiation response (Biaglow *et al.*, 1969). Glucose is known to cause a Crabtree effect in many tumours without influencing normal tissue (Aisenberg, 1961). Glucose will acidify tumours (Kallinowski and Vaupel, 1988). The effect is concentration-dependent. Higher concentrations can produce osmotic-related vascular collapse (Vaupel and Okunieff, 1988). A marker for the effect of glucose is tumour pH. Lactate and the associated proton (H^+) production was measured directly by pH electrode (Thistlethwaite *et al.*, 1987) or indirectly by ^{31}P NMR (Vaupel *et al.*, 1989). Recently, increased lactic acid production was reported when inorganic phosphate (P_i), insulin or even respiratory inhibitors were applied together with glucose (Jähde *et al.*, 1992).

Glycolysis is in part controlled by insulin. We have demonstrated previously that insulin was necessary for the enhanced radiation response of Ehrlich tumours when injected with glucose (Biaglow *et al.*, 1970). Glucose-related pH changes *in vivo* to pH 5.6 did not alter the radiation response of Ehrlich ascites cells. On the other hand, insulin was not necessary for the increased radiation responses of irradiated cell suspensions in the presence of glucose (Biaglow *et al.*, 1969). However, insulin did sensitise multicell spheroids *in vitro* when added to the complete growth medium (Biaglow *et al.*, 1979). We have also shown that insulin, when added to spent medium containing glucose, inhibits potentially lethal damage repair (PLDR) *in vitro* (Varnes *et al.*, 1986). In the latter case the effects were in part dependent upon the pH of the medium. Low pH was found to enhance PLDR while immediate adjustment of the spent medium to an alkaline pH inhibited PLDR. Acid or alkaline conditions in tumours may affect the cell cycle or PLDR and influence the overall radiocurability. For that reason, we focused our efforts towards determination of a physiological means for enhancing the radiation response by using glucose in combination with insulin and P_i. For example, tumour acidification may enhance PLDR while alkalinisation would inhibit it (Varnes *et al.*, 1986). Therefore ^{31}P NMR measurements (Vaupel *et al.*, 1989) provide a convenient non-invasive means for determining metabolic alterations caused by the use of a metabolic cocktail.

This study represents an attempt to establish controlled metabolic states *in vivo* in order to determine the relative importance of anaesthetics and glucose alone and in combination with insulin and P_i in the radiation response. We chose glucose and insulin because of our previous experience with these agents. P_i was chosen because of its well-known role in glucose metabolism. The *in vivo* studies related to glucose metabolism usually require the use of anaesthetics for immobilisation, especially ^{31}P NMR and radiation studies. Anaesthetics are known to influence metabolism. We found it necessary to evaluate the effect of commonly used combinations of anaesthetics: ketamine/xylazine and ketamine/acepromazine on glucose metabolism in our rat glioma model. We use biochemical means for determination of blood glucose changes and ^{31}P NMR estimation of intracellular tumour pH. The effects of P_i and insulin on blood glucose and tumour pH were also determined.

Materials and methods

The 9L glioma tumours were grown as subcutaneous implants on the flank of male, Fischer 344 rats (weight *c.* 200 g) for 2 weeks to 1-1.5 cm^3. Blood glucose level was determined by taking 50 μl samples from the rat tail vein and measured directly through a Hemo Cue Glucophotometer. Unanaesthetised rats were inserted into a tubular plastic holder with the front sealed (with air holes) and tail protruding from a hole in the back. Tails were repeatedly nipped and bled for glucose analysis using either anaesthetised or unanaesthetised rats. Individual rats were used for the time studies on changes in blood glucose following administration of anaesthetics. All experiments were repeated at least 5 times and standard error bars are included. Individual tumour-bearing rats were used for each ^{31}P NMR study. A minimum of three different tumour-bearing rats were scanned following injections. The groups represent ketamine/xylazine, ketamine/acepromazine and xylazine

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anaesthetics alone, with glucose, with glucose–P_i and insulin, and glucose–insulin combinations. Separate tumour-bearing rats were used for blood glucose and pH measurements. All doses of agents were non-lethal and all injections were intraperitoneal. In all cases we were careful to use iso-osmotic concentrations of glucose. Similar considerations were given to the use of P_i.

^{31}P NMR *in vivo* experiments were carried out on a Bruker AMX-300/SWB spectrometer (150 mm bore, 7T vertical magnet) at the phosphorus resonance frequency 121.5 MHz using the custom-built animal probe. The 15 mm double-tuned (^{1}H and ^{31}P) surface coil was placed directly on the tumour. It required approximately 0.5 h to anaesthetise the rat, administer the agents and commence the measurement (Vaupel *et al.*, 1989). The chemical shift of P_i and organic P_i were determined. It was assumed that the chemical shift for phosphocreatine is zero. Rats were kept on a constant temperature board before placement in the constant temperature chamber of the spectrometer.

Results

Figure 1 shows the effect of the combined use of ketamine with xylazine on blood glucose in 9L glioma tumour-bearing rats in comparison with ketamine/acepromazine (K + A) combination. Ketamine alone (not shown) shows no significant increase in blood glucose when compared with control blood glucose (no anaesthetic). The ketamine/xylazine (K + X) mixture produced the same degree of systemic hyperglycaemia in normal and tumour-bearing (data not shown) Fischer 344 rats. Ketamine/acepromazine combination had no effect on blood glucose of normal and tumour bearing rats (cf. bottom curve). The upper curve shows the dramatic increase in blood glucose for injections of glucose [1.5 g kg^{-1} animal body weight (BW) with 2 mg kg^{-1}, (BW) of xylazine]. Reducing the xylazine concentration to 0.2 mg kg^{-1} BW (as in the mixture) decreases the level of hyperglycaemia. The injection of ketamine and xylazine with glucose (K + X + G) also produces hyperglycaemia compared with the rats treated with ketamine (15.0 mg kg^{-1} BW)/acepromazine (0.15 mg kg^{-1} BW) with or without glucose.

Xylazine is known to inhibit insulin secretion (Goldfine and Arieff, 1979). This inhibition would have a dramatic effect on blood glucose because glucose utilisation by peripheral tissue would be blocked. The source of glucose would then come from the breakdown of glycogen stores in the liver. We tested the effects of xylazine alone on blood glucose. Xylazine acts in a dose-dependent manner (cf. insert, Figure 1). The dramatic increase in blood glucose was observed at 1 and 2 h following injection of xylazine. No glucose was administered.

Figure 2 shows the dramatic effect of ketamine/xylazine (2 mg kg^{-1} BW) on blood glucose. The hyperglycaemia peaks at 45 min. There is a slower rise when glucose (1.5 g g^{-1} BW) is injected with insulin (2.70 units kg^{-1} BW) and the peak in blood glucose is at 90 min. The effects are less dramatic when P_i (3.1 mg g^{-1}, BW) is included with insulin and glucose. The combination delays the peak in hyperglycaemia. A maximum increase occurred at 120 min. It is well known that the simultaneous administration of glucose with insulin abolishes insulin's hypoglycaemic effect. Insulin produces hypoglycaemia when given with ketamine/xylazine. Glucose falls to 50% of control in 60 min and begins to rise toward normal at 120 min. These results suggest, in agreement with Goldfine and Arieff (1979), that insulin secretion may have been blocked by xylazine.

Obviously, the xylazine anaesthetic will produce an initially higher blood glucose. The tumour glucose will lag behind the rise in blood sugar. The net steady-state level will depend on the rate of delivery *vs* the rate of utilisation. We chose non- invasive ^{31}P NMR spectroscopy for monitoring the effect of time after injection on metabolic alterations involving pH and presumably increased production of lactate^{-} + proton in the tumour. Although all of the P_i and organic phosphate changes were monitored by this method, the intracellular pH changes appeared (owing to chemical shifts) to be the simplest way of demonstrating the altered metabolism. Thus, Figure 3 shows that glucose with ketamine/xylazine causes significant acidification. The acidification was measured in five different tumour-bearing rats. Ketamine/acepromazine combinations show no changes in tumour pH over a 1.5 h measurement period. However, the upper curve in Figure 3 shows the dramatic effect of using the combination of insulin, glucose and P_i on the alkalinisation of the tumour cells with time (points from five tumour-bearing rats) after ketamine/xylazine injection.

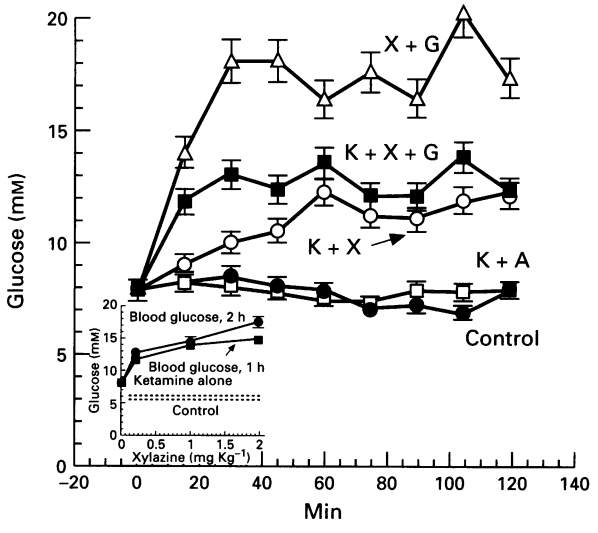

Figure 1 The blood glucose values following various treatments. The curves, from bottom to top are: control glucose values in the unanaesthetised rat, ketamine/acepromazine without glucose, ketamine/xylazine, ketamine/xylazine + glucose and xylazine alone with glucose. All points represent the average of the values obtained from five rats. We measured the effect of xylazine alone and with time on blood glucose. The insert shows the effect of xylazine on blood glucose levels following no treatment (control), ketamine alone and xylazine for 1 h, or xylazine for 2 h after i.p. injection.

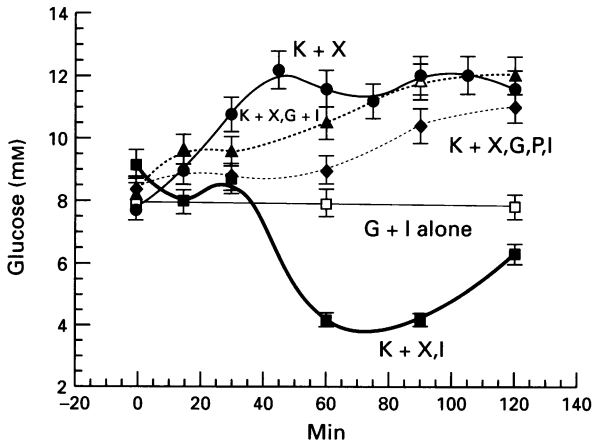

Figure 2 Blood glucose changes following injection with ketamine/xylazine + insulin, glucose + insulin without anaesthetic (G + I), ketamine/xylazine with glucose + insulin + phosphate (K + X, G,P,I), ketamine/xylazine + glucose + insulin (K + X,G + I) and ketamine/xylazine (K + X). All points represent the average of values obtained from five rats.

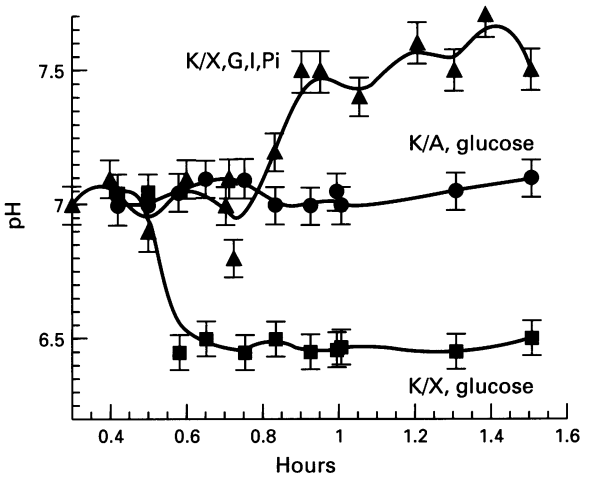

Figure 3 The effect of ketamine/xylazine and ketamine/acepromazine combinations on 9L glioma pH after injection of glucose as measured by ^{31}P NMR. The effect of insulin on 9L glioma pH following i.p. injections of ketamine/xylazine and glucose/P_i. Each variable represents data accumulated from three separate tumour-bearing rats.

Discussion

The results extend and duplicate the report of the effects of xylazine on blood glucose in mammalian systems (Goldfine and Arieff, 1979). The hyperglycaemic effects may prove to be detrimental in studies where tumour glucose needs to be carefully controlled. Nevertheless, xylazine has found widespread use in veterinarian medicine. The most possible mechanism of xylazine action is the agonistic intervention through α_2 post-adrenoceptors in pancreatic β-cells and the consecutive inhibition of insulin secretion (Hsu and Hummel, 1981). The inhibition of insulin secretion causes the release of glucose from the liver glycogen stores; since the liver is the only organ capable of secreting glucose into the blood (Kapit *et al.*, 1987). This feature is based upon the high presence of glucose-6-phosphatase in hepatocytes, the enzyme which is inhibited in the presence and activated in the absence of insulin. Thus, the injection of xylazine creates the mobile pool of endogenous glucose and declining insulin levels decrease peripheral tissue uptake resulting in the net increase in blood glucose. Blood glucose is increased further when it is exogenously injected. The advantage of this glucose metabolism-modifying effect of xylazine is that the undesirable local and systemic hyperosmotic effects of i.p. or i.v. glucose injection can be avoided, by using the glucose solution of lower concentration and osmolarity respectively. Furthermore, it seems reasonable to use the hyperglycaemic effect of xylazine alone and in combination with injected glucose to maximise the tumour glucose. However, xylazine should not be used when tumour glycolysis is blocked by the competitive inhibitor 2-deoxyglucose because it would have to compete with higher tumour glucose. Our data does indicate that small amounts of insulin should be given to rodents receiving xylazine to prevent the hyperglycemia. In addition, blood glucose must be carefully monitored.

There is evidence from this study (cf. Figure 2) on the role of P_i in maintaining blood glucose and also affecting 9L glioma tumour alkalinisation, judged by ^{31}P NMR data. P_i is known to be a very sensitive modifier of glycolysis by increasing hexokinase and phosphofructokinase activities. These enzymes catalyse the first two steps of cellular glucose metabolism. Our findings are similar to those obtained by others (Osinski and Bubnovskaja 1984; Jähde *et al.*, 1992) who found the significant pH drop in rodent tumour models other than glioma, after continuous intravenous infusion of glucose and P_i solution. However, our results indicate that insulin injected with glucose and phosphate causes an opposite effect, namely, the alkalinisation of 9L glioma tumour cells, following the injection of ketamine/xylazine and glucose/P_i. Insulin stimulates glucose phosphorylation (hexokinase) and the turnover of ADP requires P_i. Glycolysis will cause acid production if pyruvate is not consumed via respiration. Pyruvate is reduced to lactate and is pumped out of the cell with protons. The classical studies of Warburg showed that almost all tumour cells produce lactate under aerobic conditions. $Lactate^- + H^+$ production is increased under hypoxia (cf., Aisenberg, 1961). The esterification of P_i will alkalinise cells ($P_i + H^+ + ADP \rightarrow ATP + H_2O$) because of the use of hydrogen ion. Pyruvate will also consume protons if metabolised via the mitochondria ($pyruvate^- + H^+ + O_2 \rightarrow CO_2 + H_2O$). However, if the rate of tumour lactate production is greater than the circulation's ability (blood buffers are bicarbonate and haemoglobin) to carry away the proton, the tumour will become acid. Insulin is known to alkalinise cells internally when acting as a mitogen (Lammers *et al.*, 1989). Therefore, it is not surprising that alkalinisation is occurring *in vivo*. Further studies are needed to determine the net effect of alkalinisation on tumour metabolism.

We tested the effect of radiation on regrowth delay in tumour bearing rats receiving the cocktail of ketamine/xylazine followed by i.p. injection of glucose/P_i with insulin. Our preliminary data indicated the time to reach 3 × initial size after 10 Gy is significantly delayed by 3–5 days compared with radiation alone. The results presented herein motivated us to plan detailed studies on the mechanism for insulin–glucose–P_i alkalinisation of tumours and the effects on PLDR and cell survival following irradiation with rodents anaesthetised with different combinations of ketamine, xylazine and acepromazine. Our study shows that the glycolysis of tumours may be manipulated with ketamine/xylazine or xylazine alone in conjunction with glucose, P_i and insulin.

Xylazine produces hyperglycemia in normal and 9L glioma-bearing Fischer rats, in a dose-dependent manner. For that reason, xylazine could be considered as a candidate for enhancing the glucose metabolism-modifying effect in tumour-bearing rats when the lowering of pH is desired as in the case of hyperthermia or chemotherapy.

Ketamine/acepromazine has no effect on blood glucose. No effect on tumour cell pH is produced when glucose is injected following ketamine/acepromazine anaesthetic. Ketamine/acepromazine should be used in the studies where it may be necessary to maintain constant intracellular pH following glucose administration.

Glucose, P_i and insulin alkalinise the 9L glioma judged by *in vivo* ^{31}P NMR data. Further studies are necessary to determine if the time for maximal pH change is related to an increased radiation response. Estimation of lactate production by proton NMR will be useful in establishing treatment regimens.

Glioma is known to be resistant to chemo- and radiotherapy. The use of innocuous and well-established clinical agents to alter tumour cell pH metabolically, would have clinical relevance for enhancing radiation, hyperthermia or chemotherapy effects.

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