Compartmentation of Glycolytic and Glycogenolytic Metabolism in Vascular Smooth Muscle

Abstract. *Vascular smooth muscle is characterized by a high rate of aerobic lactate production, which may be altered independently of oxidative phosphorylation. This finding suggested a cytoplasmic compartmentation of metabolism. Exogenous glucose was found to be the sole precursor of aerobic glycolysis under unstimulated conditions. Although tissue depolarization with high K^+ resulted in a substantial reduction of endogenous glycogen, exogenous glucose remained the sole precursor of aerobic lactate production. These data showed unequivocally that carbohydrate metabolism is compartmentalized in vascular smooth muscle.*

Traditionally, cellular metabolism has been delineated into distinct, structurally limited compartments, such as nuclear, cytoplasmic, and mitochondrial. During the past two decades, evidence has been accumulating that the cytoplasm itself may be functionally, if not structurally, compartmentalized in relation to intermediary metabolism. Compartmentation of metabolic pathways within the cytosol has been argued on the basis of cellular energetic efficiency (*1*). Experimental evidence obtained from matrix-bound enzyme systems supports this view (*2*) although metabolic compartmentation of the cytoplasm has not yet been definitively demonstrated. Specific localization of enzymes, particularly of membrane-bound complexes, has been shown by the use of histochemical and sedimentation techniques (*3*). Functional compartmentation of metabolism has been postulated on the basis of steady-state isotopic data indicating the existence of multiple pools of metabolic intermediates (*4*). This evidence, however, has been questioned in terms of cellular inhomogeneity and the existence of alternative metabolic pathways (*5*). We present evidence that there are separate pathways for glycolysis and glycogenolysis in porcine carotid artery; that is, "cytosolic" carbohydrate metabolism operates in at least two functionally exclusive compartments.

Under fully oxygenated conditions, the rate of lactate production (J_{lac}) in vascular smooth muscle is often greater than the rate of oxygen consumption (J_{O_2}) on a molar basis (*6*). We showed earlier that J_{O_2} is correlated with isometric force (F_o), whereas J_{lac} is correlated with Na-K transport (*7*). The alteration of aerobic glycolysis independent of oxidative phosphorylation under various experimental conditions (*7*) suggests that there is a compartmentation of metabolism.

This hypothesis was investigated by examining the effect of depolarization with high K^+ on the pattern of carbohydrate utilization in porcine carotid artery. Under unstimulated control conditions, glucose transport is rate-limiting for its catabolism in vascular smooth muscle (*8*). Increasing the potassium concentration of the media (80 m*M*) stimulates J_{O_2}, F_o, and J_{lac} in a manner similar to that found for porcine coronary artery (*7*). Glycogen phosphorylase is also stimulated under this condition (*6*). Within the first 30 minutes of treatment with added K^+, tissue glycogen content decreases by approximately 50 percent (Fig. 1), or an average of 1.41 ± 0.15 μmole/g; this can account for approximately 70 percent of the lactate produced (Table 1). Alternatively, total lactate production can be accounted for by glucose uptake, as indicated by the ratio of J_{lac} to J_{glu} in Table 1. Moreover, the intracellular concentration of glucose remains negligible (*8*), and tissue lactate content is constant after the initial 30 seconds of stimulation (*9*). Therefore it was of interest to determine the relative contribution of glucose uptake and glycogenolysis to aerobic lactate production. This was accomplished by measuring the specific activities of media lactate and glucose after arterial rings were equilibrated with [U-^{14}C]glucose. After a 150-minute incubation under unstimulated conditions, the lactate specific activity ratio (the specific activity of lactate divided by the specific activity of glucose) was 0.50 ± 0.02 (N = 10); this indicates that exogenous glucose was the sole source of aerobic lactate production. In these tissues the specific activity of glycogen was approximately one-tenth that of glucose in equivalent glucosyl units [the ratio of the specific activity of glycogen to the specific activity of glucose was 0.107 ± 0.01 (N = 10)]. Therefore, in the absence of compartmentation, a substantial reduction of the lactate specific activity ratio would be seen after 30 minutes of incubation with added K^+ (80 m*M*) (*10*). This assumption is valid whether or not preferential degradation of labeled glycogen occurs with time of depolarization (*11*).

The precision of these experiments in detecting alterations of the lactate specific activity ratio was evaluated by incubating tissue rings under anaerobic conditions with added K^+ (80 m*M*). Under these conditions, all glucosyl units derived from glycogen must be released as lactate since oxidative phosphorylation is inhibited by the absence of molecular oxygen. Within 30 minutes of treatment, the lactate specific activity ratio decreased to 0.42 ± 0.02 (N = 4), which shows that an alteration in the lactate specific activity ratio can be measured during a 30- or 60-minute experimental period (Fig. 2B). The lactate specific activity ratio was, however, unchanged after 30 and 60 minutes of depolarization under aerobic conditions (Fig. 2B). The constancy of the lactate specific activity ratio shows that glucosyl units derived from glycogen degradation do not enter the same pool of glycolytic intermediates as that used in the aerobic production of

Table 1. Lactate production rate (J_{lac}) in micromoles per minute per gram for unstimulated control artery rings and alterations with treatment from tissues in Figs. 1 and 2B. Data are presented as the mean ± S.E.M. for all tissues in each treatment group, with the number of arterial segments in parentheses. Treatment-induced alterations are presented as a percentage of the control rate [(experimental − control)/control] × 100. The ratio of lactate produced to glucose uptake (J_{lac}/J_{glu}) was measured on a separate group of tissues that had lactate production rates similar to the rates for control tissues. The media bathing individual tissues were analyzed for glucose and lactate content after 120 minutes of incubation with or without added KCl (80 m*M*). The chemical assays were performed with standard enzyme-linked spectrophotometric procedures (*18*).

Minutes after incubation	Unstimulated	Treatment-induced alterations (percent change)	
		Added KCl	Added KCl in N_2
J_{lac}			
30	0.108 ± 0.01 (22)	+38.0 ± 9.0 (4)	+90.5 ± 9.0 (8)
60	0.102 ± 0.006 (14)	+28.0 ± 3.6 (8)	+90.4 ± 10.7 (4)
J_{lac}/J_{glu}			
120	0.930 ± 0.036* (12)	+0.5 ± 6.1 (4)	

*J_{lac}/J_{glu} is compared in equivalent glucosyl units, so that a ratio of 1 indicates that all lactate can be derived solely from glucose uptake.

lactate. Separate enzymatic pathways for glycogenolysis and glycolysis therefore must be evoked to explain this finding.

It may be argued that the actual compartmentation of glycolytic pathways occurs between different cell types within the artery wall. Histological studies of the intima media have shown that smooth muscle is the predominant cell type in porcine carotid artery (*12*). Thus it is unlikely that the metabolic effects described here could be attributed to the presence of other cell types.

Less than 5 percent of the glucose taken up by unstimulated artery rings is metabolized to CO_2 and water, as calculated from $^{14}CO_2$ production. This is consistent with previous reports on vascular smooth muscle (*13, 14*). Preliminary findings indicate that, upon depolarization, $^{14}CO_2$ production increases by 0.0031 ± 0.0013 μCi/g ($P < 0.05$) after 60 minutes. The additional amount of radioactive label could originate from glycogen since the decrease in the amount of label in glycogen [0.0075 ± 0.0023 μCi/g ($P < 0.05$)] can account for the additional label found in CO_2. If the assumption is made that this additional label does indeed originate from glycogen [specific activity, 6.4 ± 0.2 μCi/mmole ($N = 4$)], then the calculated change in the CO_2 production rate (0.046 ± 0.02 μmole/min-g) is comparable to the increase in the rate of oxygen consumption (0.045 μmole/min-g) measured on an additional segment from the same artery. Similar observations were made on arteries incubated for 30 minutes with added K^+. Thus it is likely that the observed glycogenolysis provides the substrate for the increase in oxidative phosphorylation during a K^+-induced contraction. Vascular smooth muscle—which appears to utilize predominantly lipids for oxidation under resting conditions (*15*)—may, like skeletal muscle, shift to glycogen metabolism under the demand of increased contractile activity. This hypothesis warrants further examination.

The constancy of the lactate specific activity ratio not only indicates that glycolysis and glycogenolysis operate via separate enzymatic pathways but also implies that glucose transport must be regulated to match the observed increase in J_{lac} during K^+ depolarization. This follows because glucose transport is rate-limiting for its catabolism in porcine carotid artery (*8*). Thus the regulation of glucose transport is critical to the regulation of this compartmentalized pathway of glucose catabolism. Since aerobic gly-

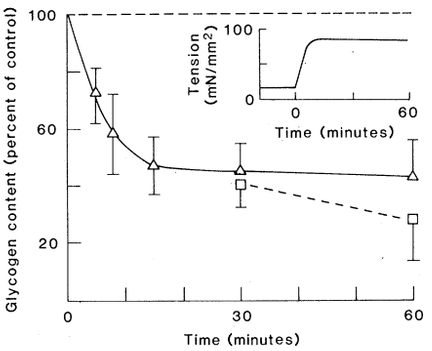

Fig. 1. Glycogen content of K^+-depolarized tissues as a function of the duration of treatment with high K^+ (80 m*M*). The mean value ± standard error (S.E.M.) of glycogen content of all control artery segments ($N = 67$) was 2.82 ± 0.29 μmole/g. The glycogen content of arteries incubated in high K^+ (△) and anaerobic high K^+ (□) media is expressed as a percentage of the glycogen content of their respective experimental control artery segments. Porcine carotid arteries were dissected free from loose connective tissue and adhering fat and cut into segments. The segments were blotted and weighed, then inverted and cannulated isometrically with glass rods. The physiological saline incubation solution (PSS) contained (in millimoles per liter) NaCl, 130; $NaHCO_3$, 14.9; KCl, 4.7; KH_2PO_4, 1.18; $MgSO_4$, 1.17; $CaCl_2$, 1.6; and glucose, 5.40. Solutions were bubbled with a mixture of 95 percent O_2 and 5 percent CO_2 at a *p*H between 7.3 and 7.4 at 37°C. K^+ depolarization was induced by the addition of a concentrated KCl solution to increase the final bath concentration by 80 m*M*. Anaerobic medium was prepared by aeration with 95 percent N_2 and 5 percent CO_2. When incubations were terminated, the glass rods were removed, and the artery segments were frozen in liquid nitrogen. Tissues were then solubilized in NaOH, and the glycogen fraction was precipitated with ethanol as described by Van Handle (*19*). Hydrolysis of the purified glycogen was carried out in the presence of amyloglucosidase, and the final glucosyl content was analyzed with standard enzyme-linked spectrofluorimetric procedures (*18*). Inset: time course of a typical K^+-induced isometric contraction of a segment of porcine carotid artery during the same time period as was used for recording glycogen content.

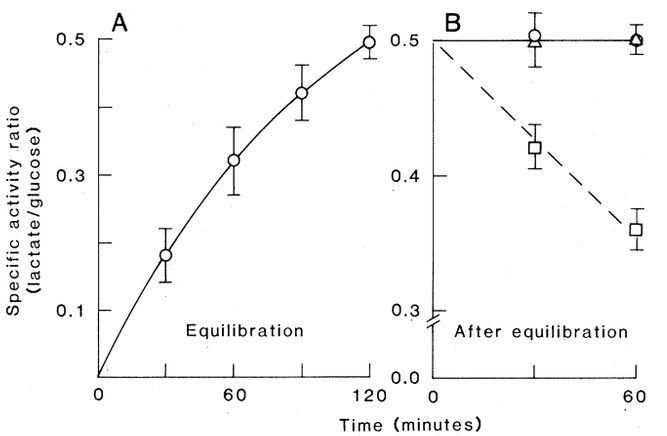

Fig. 2. Equilibration of ^{14}C from media glucose into the lactic acid pool and the effect of K^+ depolarization on the steady-state lactate specific activity ratio under aerobic and anaerobic conditions. (A) Isotope equilibration. Porcine carotid artery rings were incubated individually under isometric conditions for 30-minute time intervals in media containing uniformly labeled [^{14}C]glucose (60 to 100 μCi/mmole). After the initial 30-minute incubation, the rings were removed and placed into another group of 1-ml baths from the same stock PSS solution. This protocol was continued for 3 hours with four tissues. Each individual incubation medium (1 ml) was then run on a Dowex-1-Cl^- anion exchange column (1 by 6 cm; *p*H, 4.75) to separate glucose from lactate. Individual columns were washed with 20 ml of H_2O to collect the glucose fraction. Lactate was removed from the column with 1*M* NH_4Cl (twice, 2 ml each time). The resulting fractions were analyzed for glucose and lactate as well as for radioactivity. The data are presented as a ratio of the specific activity of lactate to the specific activity of glucose. Two moles of lactate are produced from one mole of glucose by glycolysis; therefore, a lactate specific activity ratio of 0.50 indicates that all lactate is derived from glucose, if it is assumed that the specific activities of all other possible sources of lactate are lower than the specific activity of media glucose. The data for the initial four control tissues are shown only through 120 minutes by which time a steady state had been reached (that is, there was no significant difference between the specific activity ratio for 120 minutes and that for either 150 or 180 minutes). Therefore all experimental tissues were incubated in PSS containing [U-^{14}C]glucose for at least 120 minutes before treatment to ensure that the label had reached a steady state. The equilibration of the lactic acid pool with ^{14}C can be fitted to an exponential curve (——) yielding a time constant of 42 minutes. (B) The effect of K^+ depolarization on the lactate specific activity ratio of artery rings incubated under aerobic (△) and anaerobic (□) conditions, and their unstimulated control tissues (○). Control and treated tissues were carried through identical incubations. At the end of the designated experimental periods, individual tissues were frozen in liquid N_2 and extracted as described in Fig. 1; the media were analyzed as described above. The glycogen fraction was also analyzed for radioactivity for determination of specific activity. Approximately 5 percent of the label was recovered as glycogen under unstimulated control conditions, and this percentage decreased with time of depolarization. Approximately 92 percent of the label was incorporated into lactate in both unstimulated and depolarized segments. For control tissues, the glycogen specific activity ratio (ratio of the specific activity of glycogen to the specific activity of glucose) for the 30- and 60-minute experimental periods was 0.107 ± 0.010 ($N = 10$) and 0.105 ± 0.018 ($N = 6$), respectively.

colysis is correlated with Na-K transport, knowledge of the regulation of aerobic glycolysis may be of major significance for understanding the vascular myopathies associated with hypertension, atherosclerosis, and aging, which are known to be correlated with changes in vascular Na-K transport (*16*) and carbohydrate metabolism (*13, 17*).

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References and Notes

1. G. R. Welch, *J. Theor. Biol.* **68**, 267 (1977); R. Goldman and E. Katchalski, *ibid.* **32**, 243 (1971).
2. N. V. Vasil'eva, T. O. Balaevskaya, L. Z. Gogilashvili, K. B. Serebrovskaya, *Biochemistry* **34**, 641 (1969); B. Mattiasson and K. Mosback, *Biochim. Biophys. Acta* **235**, 253 (1971).
3. J. Mowbray and V. Moses, *Eur. J. Biochem.* **66**, 25 (1976); T. K. Shnitka and A. M. Seligman, *Annu. Rev. Biochem.* **40**, 375 (1971); R. N. Margolis, R. R. Cardell, Jr., R. T. Curnow, *J. Cell Biol.* **83**, 348 (1979).
4. N. Kalant and R. Beitner, *J. Biol. Chem.* **246**, 504 (1971); J. Mowbray and J. H. Ottaway, *Eur. J. Biochem.* **36**, 362 (1973); C. C. Dully, R. M. Bocek, C. H. Beatty, *Endocrinology* **84**, 855 (1969).
5. F. Pocchiari, in *Control of Glycogen Metabolism*, W. J. Whelan, Ed. (Academic Press, New York, 1968), pp. 129–137; J. Mowbray and J. H. Ottaway, *Curr. Top. Cell. Regul.* **12**, 108 (1977); R. P. Metzger, D. Piszkiewicz, L. A. Mayer, A. W. Mobley, A. N. Wick, *Biochim. Biophys. Acta* **141**, 231 (1967).
6. R. J. Paul, in *Handbook of Physiology*, section 2, *The Cardiovascular System*, vol. 2, *Vascular Smooth Muscle*, D. F. Bohr, A. P. Somlyo, H. Sparks, Eds. (Williams & Wilkins, Baltimore, 1981), p. 201; *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42**, 63 (1983).
7. ———, M. Bauer, W. Pease, *Science* **206**, 1414 (1979); R. J. Paul, *Am. J. Physiol.* **244**, C399 (1983).
8. R. M. Lynch and R. J. Paul, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 983 (1982).
9. J. M. Krisanda and R. J. Paul, *Am. J. Physiol.* **244**, C385 (1983).
10. The amount of glucose catabolized is directly proportional to the amount of glucose taken up from the bathing media since the intracellular concentration of glucose is zero. As calculated from the data in Table 1, glucose uptake therefore accounts for glucosyl units in the amount of 2.40 μmole/g during a 30-minute incubation with high K^+ (80 m*M*). Approximately 1.41 μmole/g is derived from glycogen during the same time period (Fig. 1), for total glucosyl units of 3.81 μmole/g. If complete mixing of glycolytic intermediates occurs then the predicted lactate specific activity ratio would be [(2.40/3.81) × glucose specific activity ratio (1.0)] + [(1.41/3.81) × glycogen specific activity ratio (0.107)] × 0.5, which is equal to 0.335. The time course of equilibration of the radioactive label (Fig. 2A), which has a time constant of 42 minutes, must also be taken into account. After 30 minutes, equilibration will be 44 percent complete, and a lactate specific activity ratio of 0.43 would be predicted. A lactate specific activity ratio less than 0.46 would be detected as statistically different from control (0.50), given the variance in the measured specific activity ratio in Fig. 2B. Similar derivation of a predicted lactate specific activity ratio for tissues treated with added K^+ after 60 minutes of incubation yields a value of 0.39.
11. Preferential degradation of labeled glycogen would lead to an initial flux of glucosyl units, amounting to 0.30 μmole/g (0.107 × 2.82), from glycogen with a specific activity equal to that of media glucose. Therefore [(2.40 + 0.30)/3.81] × 0.5 yields a lactate specific activity ratio of 0.350. Correction for equilibration of the radioactive label as in (*9*) gives a predicted specific activity ratio of 0.435, again a readily detectable alteration of the lactate specific activity ratio.
12. E. Glück and R. J. Paul, unpublished observations. Smooth muscle comprises at least 25 layers of the porcine carotid artery intima media compared to one layer of endothelium and infrequently dispersed fibroblasts.
13. E. S. Morrison, R. F. Scott, M. Kroms, J. Frick, *Atherosclerosis* **16**, 175 (1972).
14. M. Z. Newmark, C. D. Malfer, C. D. Wiese, *Biochim. Biophys. Acta* **261**, 9 (1972); A. D. Morrison, L. Berwick, L. Orci, A. I. Winegrad, *J. Clin. Invest.* **57**, 650 (1976).
15. K. V. Chace and R. Odessy, *Circ. Res.* **48**, 850 (1981); R. Odessy and K. V. Chace, *Am. J. Physiol.* **243**, H128 (1982).
16. E. T. Garwitz and A. W. Jones, *Am. J. Physiol.* **243**, H927 (1982); A. W. Jones, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **33**, 133 (1974).
17. M. M. Daly, *Am. J. Physiol.* **230**, 30 (1976); S. Yalcin and A. I. Winegrad, *ibid.* **205**, 1253 (1963).
18. O. H. Lowry and J. V. Passonneau, in *A Flexible System of Enzymatic Analysis* (Academic Press, New York, 1972), p. 174; *ibid.*, p. 195.
19. E. Van Handle, *Anal. Biochem.* **11**, 256 (1965); S. R. Nahorsky and K. J. Rogers, *ibid.* **49**, 492 (1972).
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Molecular Basis of Herbicide Resistance in *Amaranthus hybridus*

Abstract. *Resistance of different species of weeds to* s-*triazines, a commonly used class of herbicides, has been shown to involve a change in the binding affinity of the herbicide to a chloroplast polypeptide of 32,000 daltons. A single amino acid difference in this 32,000-dalton protein appears to be responsible for resistance to the herbicide in* Amaranthus hybridus.

Many commercially important herbicides inhibit photosynthesis. One such class of herbicides, the *s*-triazines, inhibit electron transfer on the reducing side of photosystem II (PS II) (*1, 2*). The mechanism of action of the triazines involves the initial binding of the herbicide to chloroplast thylakoid membranes and blocking of electron transport at the second stable electron acceptor of PS II (*3*). The high-affinity binding site for the herbicide is a thylakoid-membrane polypeptide of the PS II complex (*4*) and has been identified as the "rapidly turned over" 32,000-dalton chloroplast-encoded membrane polypeptide (*5*).

In agricultural areas where atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine) has been used extensively, atrazine-resistant biotypes of many species of weeds have appeared. The first evidence of intraspecific resistance to the *s*-triazines was reported for *Senecio vulgaris* in 1970 (*6*). Since this initial report, herbicide-resistant biotypes have been identified among 28 different species of weeds in North America and Europe (*7*). In all cases, the appearance of resistant weeds followed a continuous use of triazines (usually atrazine or simazine) over extended periods of time. In the examples of triazine resistance thus far examined, the biochemical basis of resistance has been identified as a change in the herbicide-target site in the chloroplast (*2, 8*). When chloroplasts were isolated from the resistant biotype of *Amaranthus hybridus*, it was demonstrated that a 32,000-dalton polypeptide of PS II is present in the thylakoids but does not bind the triazine (*8*).

Triazine resistance is maternally inherited (*9*), suggesting that this trait is coded for by the chloroplast genome. A chloroplast-encoded protein with a molecular size of 32,000 daltons has been characterized in many higher plants (*10*). This protein is tightly bound to thylakoid membranes and rapidly metabolized, and its synthesis is modulated by light (*11*). Translation of the messenger RNA (mRNA) for this protein occurs on chloroplast ribosomes (*12*) to produce a precursor polypeptide of 33,500 daltons in *Spirodela oligorhiza* and 34,500 daltons in maize and spinach (*13*). In order to investigate the genetic basis of triazine resistance, we have studied the gene which encodes this polypeptide, now designated psbA, in herbicide-resistant and herbicide-susceptible biotypes of the weed *Amaranthus hybridus*. Seeds used to propagate atrazine-resistant and atrazine-susceptible plants were from plant material originally collected in the state of Washington and previously characterized by chloroplast electron transport studies (*14*).

Chloroplast DNA was isolated (*15*) from both atrazine-susceptible and atrazine-resistant biotypes of *A. hybridus*. There is no apparent difference in the pattern of DNA fragments generated by Eco RI and Bam HI restriction endonucleases when chloroplast DNA's from the two biotypes are compared (*8*). Furthermore, with the use of the maize psbA gene cloned in the recombinant plasmid pZmc427 (*10*) as a probe, it was discovered that psbA in *A. hybridus* is present, in both susceptible and resistant plants, within a single Bam HI fragment [6 kilobase pairs (kb)] (*8*) and a single 3.68-kb Eco RI fragment (*16*).