Compartmentation of Glucose and Fructose 1,6-Bisphosphate Metabolism in Vascular Smooth Muscle†

Christopher D. Hardin* and Tina M. Roberts

Department of Physiology, University of Missouri, Columbia, Missouri 65212

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ABSTRACT: We examined the metabolism of exogenously added ^{13}C-labeled fructose 1,6-bisphosphate (either labeled at the first and sixth carbons or labeled at the first carbon only) and of [2-^{13}C]glucose in well-oxygenated and well-superfused hog carotid artery segments. Exogenously added fructose 1,6-bisphosphate was utilized by hog carotid artery and primarily participated in gluconeogenesis while the production of [3-^{13}C]lactate was not significantly different from zero. When [1,6-^{13}C]fructose 1,6-bisphosphate or [1-^{13}C]fructose 1,6-bisphosphate was utilized individually, gluconeogenic flux occurred without metabolism through aldolase and triosephosphate isomerase resulting in formation of [1,6-^{13}C]-glucose and [1-^{13}C]glucose respectively. When [2-^{13}C]glucose was the sole exogenous substrate, it was utilized and exclusively participated in glycolytic flux with production of [3-^{13}C]lactate and no gluconeogenic flux from the trioses to [5-^{13}C]glucose. When both glucose and fructose 1,6-bisphosphate were provided together as exogenous substrates, glucose still participated exclusively in glycolytic flux with no trioses participating in gluconeogenesis while fructose 1,6-bisphosphate participated in glycolytic flux with [3-^{13}C]lactate production approximately being approximately half of the [1,6-^{13}C]glucose production from [1,6-^{13}C]fructose 1,6-bisphosphate. In the presence of glucose, [1-^{13}C]fructose 1,6-bisphosphate also participated in glycolytic flux and gluconeogenic flux simultaneously. However in the presence of [2-^{13}C]glucose, [1-^{13}C]fructose 1,6-bisphosphate underwent isomerization through the trioses prior to gluconeogenesis since [6-^{13}C]glucose was produced. Therefore, the intermediates of glucose utilization and catabolism to lactate do not appear to mix with the intermediates of exogenous fructose 1,6-bisphosphate metabolism. Thus we observed a simultaneous yet separable flux of glycolysis and gluconeogenesis indicating a structural organization of carbohydrate metabolism in vascular smooth muscle.

Glycolysis has been extensively studied in vascular smooth muscle since this tissue is characterized by a high rate of lactate production under well-oxygenated conditions (Paul, 1980; Hardin & Paul, 1995). Hog carotid artery, a tonic vascular smooth muscle, has been considered by many to be a model system for the study of compartmented metabolism. Paul and his colleagues have shown that oxygen consumption and lactate production can vary, often in opposite directions (Paul, 1983), and have suggested that oxidative metabolism may provide ATP specifically for contraction while a membrane-associated glycolytic pathway may provide ATP to membrane-associated ATPases such as the sodium pump (Campbell & Paul, 1992) or the calcium pump (Hardin et al., 1992). In addition, there has been considerable evidence that the pathways for glucose breakdown and for glycogen breakdown may be spatially and functionally separate. Using uniformly labeled [^{14}C]glucose, Lynch and Paul (1983) demonstrated that the specific activity of the lactate produced was equal to that of the glucose exogenously applied despite breakdown of unlabeled glycogen. In addition, at least two separate pools of glucose 6-phosphate were found in hog carotid artery (Lynch & Paul, 1986). Using ^{13}C NMR, we have shown that when glucose and glycogen were labeled at different positions within the glucose and glucosyl units, the intermediates of the two pathways did not completely mix despite simultaneous breakdown of glucose and glycogen (Hardin & Kushmerick, 1994). Therefore, it is becoming established that carbohydrate metabolism in hog carotid artery may be structured with spatially distinct sets of glycolytic enzymes carrying out glucose breakdown separately from glycogen breakdown. Indeed, substantial evidence exists for a spatial localization of glycolytic enzymes in smooth muscle. Glycolytic enzymes have been localized to the plasma membrane (Paul et al., 1989), to the contractile apparatus (Hardin & Paul, 1992), and to the endoplasmic reticulum (C.D.H., unpublished observations) of smooth muscle. Therefore, there exist multiple cytoplasmic locations for binding of the sets of glycolytic enzymes.

The study of the compartmentation of carbohydrate metabolism in smooth muscle has focused on the metabolic fate of either glucose or glycogen. However, we have recently shown that exogenously applied fructose 1,6-bisphosphate, a glycolytic intermediate, is metabolized by hypoxic hog carotid artery with significant lactate production resulting from fructose 1,6-bisphosphate metabolism (Hardin & Roberts, 1994). In the current study, we examined the metabolism of [1,6-^{13}C]fructose 1,6-bisphosphate and [1-^{13}C]-fructose 1,6-bisphosphate under well-oxygenated and well-superfused conditions. We found that fructose 1,6-bisphosphate primarily participates in gluconeogenic flux with production of appropriately labeled glucose while exog-

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* Address correspondence to this author at Department of Physiology, MA-415 Medical Sciences Building, University of Missouri, Columbia, MO 65212. Phone: 314-884-6209. Fax: 314-884-4276. E-mail: physcdh@muccmail.missouri.edu.

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enously applied [2-^{13}C]glucose participates exclusively in glycolytic flux with production only of appropriately [2-^{13}C]-lactate. Since none of the glycolytic intermediates (trioses) derived from exogenous glucose metabolism participated in gluconeogenic flux, we conclude that the metabolism of glucose to lactate occurs without the intermediates mixing with other sets of glycolytic enzymes. Therefore, the metabolism of exogenously applied fructose 1,6-bisphosphate and of exogenously applied glucose appear to be compartmentalized in hog carotid artery.

MATERIALS AND METHODS

Synthesis of [1,6-^{13}C]Fructose 1,6-Bisphosphate and [1-^{13}C]-Fructose 1,6- Bisphosphate. The synthesis of [1,6-^{13}C]-fructose 1,6-bisphosphate and [1-^{13}C]fructose 1,6-bisphosphate was performed as previously described (Hardin & Roberts, 1994). Briefly, [1-^{13}C]fructose and custom synthesized [1,6-^{13}C]fructose were obtained from Cambridge Isotope Laboratories and received no further purification prior to use in the synthesis. [1,6-^{13}C]fructose 1,6-bisphosphate and [1-^{13}C]fructose 1,6-bisphosphate were synthesized enzymatically from [1,6-^{13}C]fructose and [1-^{13}C]fructose, respectively, using hexokinase and phosphofructokinase with the ATP supplied by an ATP regenerating system consisting of creatine kinase and phosphocreatine. The reactions were carried out at 30 °C in a solution, pH 8.0, consisting of the following: 50 mM Tris, 20 mM ATP, 25 mM $MgCl_2$, 24 mM PCr, 1 unit/mL phosphofructokinase, 5 units/mL hexokinase, and 1 unit/mL creatine kinase. The reaction was started by addition of [1,6-^{13}C]fructose or [1-^{13}C]fructose at a final concentration of 20 mM. After 5 h of incubation at 30 °C, perchloric acid was added to achieve a final concentration of 1 N. Samples were centrifuged at 15000*g* for 30 min at 4 °C. The supernatant was neutralized with 5 M K_2CO_3, and the resulting supernatant was frozen and stored at −20 °C. The recovery of fructose 1,6-bisphosphate from fructose was approximately 90% (Hardin & Roberts, 1994).

Tissue Handling. Hog carotid arteries were obtained from local abattoirs within ~30 min of slaughter. Arteries were placed in a cold (~5 °C) physiological saline solution (PSS),[1] pH 7.4, preequilibrated by bubbling with a gas mixture of 95% O_2 and 5% CO_2. PSS was composed of (mM): NaCl (116), KCl (4.6), KH_2PO_4 (1.16), $NaHCO_3$ (25.3), $CaCl_2$ (2.5), and $MgSO_4$ (1.16). At the laboratory, the arteries were placed into fresh PSS equilibrated with the gas mixture at ambient temperature. Segments were dissected free of loose fat, connective tissue, and adventitia. Tubular segments of artery were cut to obtain the desired tissue mass. When tissues were isometrically mounted, carotid segments were slid over glass capillary tubes so that artery segments were stretched approximately 10%.

Tissue Incubations with ^{13}C-Labeled Exogenous Substrates. All tissues were mounted, incubated at 37 °C, and contracted and relaxed twice in the presence of glucose and once in the absence of glucose. Contraction was elicited for 20 min by switching to PSS with 5 mM glucose with 80 mM KCl added. Tissues were relaxed for 20 min by switching the superfusate back to PSS with 5 mM glucose. Approximately 250−400 mg of tissue was added (isometrically mounted) into capped tissue incubation chambers filled with new superfusate consisting of either (a) 2.4 mL of concentrated PSS (1.25× concentration of PSS of all components) and 0.6 mL of [1,6-^{13}C]fructose 1,6-bisphosphate or [1-^{13}C]fructose 1,6-bisphosphate solution (see above) either with or without 5 mM [2-^{13}C]glucose or (b) PSS with 5 mM [2-^{13}C]glucose. The final concentration of [1,6-^{13}C]-fructose 1,6-bisphosphate or [1-^{13}C]fructose 1,6-bisphosphate was 2.1 mM. The superfusate was bubbled in 95% O_2 and 5% CO_2 throughout the incubations. After 180 min of incubation, superfusate and tissues were rapidly frozen in liquid N_2 and stored at −80 °C for further analysis. Tissues were blotted dry and weighed at the end of all incubations. All measurements are normalized to the weight of the tissue after blotting. Aliquots of the solutions used to superfuse the tissues were also sampled for NMR analysis to serve as a comparison for superfusates after incubation with tissues.

Incubations of hog carotid artery with [1,6-^{13}C]fructose were also performed to further verify that metabolism of exogenously applied fructose 1,6-bisphosphate was by uptake and metabolism of fructose 1,6-bisphosphate rather than by exogenously applied [1,6-^{13}C]fructose 1,6-bisphosphate having been converted to [1,6-^{13}C]fructose by ectophosphatases with subsequent spontaneous conversion to [1,6-^{13}C]glucose. Unmounted segments of hog carotid artery with a mean total mass per incubation chamber of 815 mg (range = 757−839, n = 4) were incubated in PSS equilibrated with 95% O_2/5% CO_2 and contracted and relaxed twice in the presence of glucose and once in the absence of glucose as described above. Carotid segments were then switched to 3 mL of PSS containing 2.1 mM [1,6-^{13}C]fructose and equilibrated with 95% N_2/5% CO_2. At the end of a 120 min incubation at 37 °C, 2.5 mL of superfusate was rapidly frozen in liquid N_2 and stored at −80 °C for further analysis. An aliquot of the solution used to superfuse the tissues was also sampled for NMR analysis to serve as a comparison for superfusates after incubation with tissues.

NMR Spectroscopy. Frozen superfusates (2.5 mL aliquots) containing ^{13}C-labeled substrates and metabolites of ^{13}C-labeled substrates were lyophilized in a Speed Vac (Savant Instruments, Inc.). The powder was resuspended in 1 mL 99% D_2O with 25 mM 3-(trimethylsilyl)-1-propanesulfonic acid (DSS) as a chemical shift reference. A 700 μL aliquot was transferred to a 5 mm NMR tube. ^{13}C NMR spectroscopy was performed on a Bruker AMX 500 spectrometer with the following acquisition parameters: 300 scans with 16 dummy scans, 30° pulse angle at 125.77 MHz, 33 333 Hz sweep width, and a 1 s predelay. A total of 32K points were acquired and processed with 1 Hz line broadening prior to Fourier transform. All spectra were broad-band proton decoupled and referenced with DSS at 0 ppm. NMR data were processed using Bruker software for peak magnitude determination. All peaks are expressed relative to the DSS peak at 0 ppm and no corrections were made for nuclear Overhauser effects which were assumed to be unchanged for all experiments. ^{13}C NMR signal intensities were scaled for the tissue mass in each organ chamber.

Tissue Superfusions in the NMR Spectrometer. Experiments involving intact tissue in the spectrometer were also performed in a Bruker AMX 500 spectrometer using the following acquisition parameters: 800 scans with 8 dummy scans, 30° pulse angle at 125.77 MHz, 30 303 Hz sweep

[1] Abbreviations: DSS, 3-(trimethylsilyl)-1-propanesulfonic acid; PSS physiological saline solution.

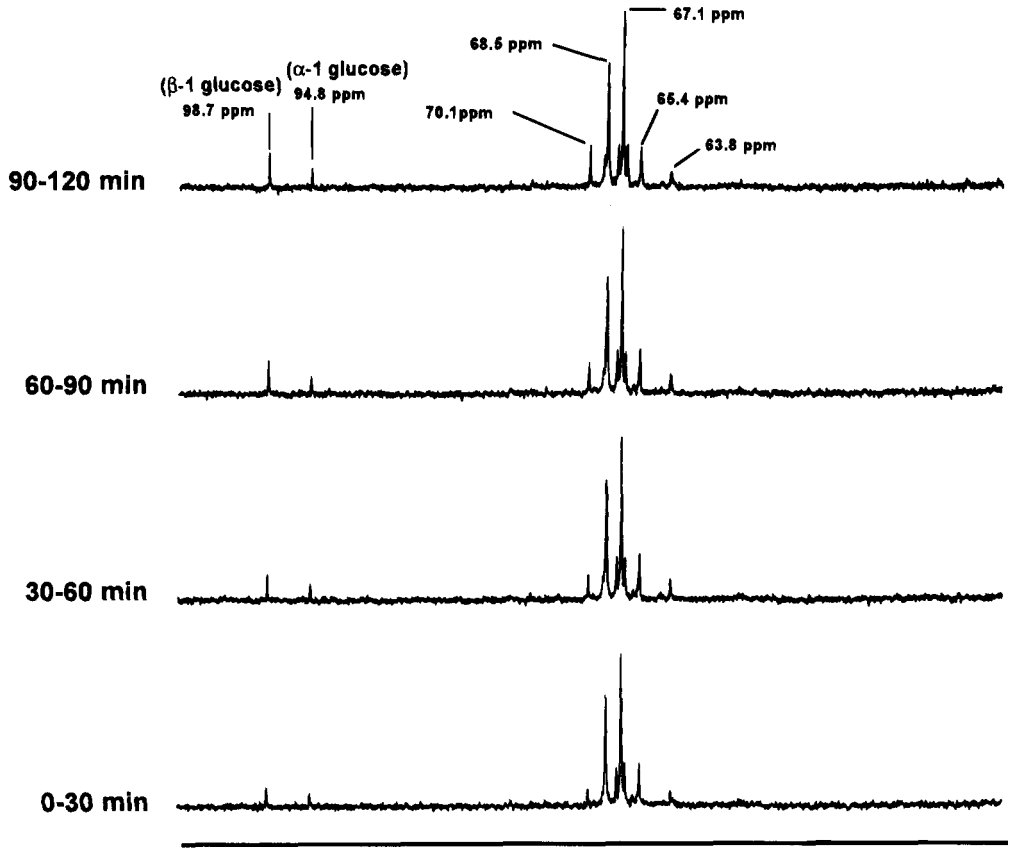

FIGURE 1: Stacked plot of sequentially acquired ^{13}C NMR spectra obtained from unmounted segments of hog carotid artery superfused in the NMR spectrometer at 2.5 mL/min. See text for peak identifications.

width, and a 1 s predelay. A total of 32K points were acquired and processed with 3 Hz line broadening prior to Fourier transform. All spectra were broad-band proton decoupled, and chemical shifts were assigned by comparison to those obtained by ^{13}C NMR of lyophilized superfusates referenced to DSS (above). NMR data were processed using Bruker software for peak magnitude determination. No corrections were made for nuclear Overhauser effects which were assumed to be unchanged for all experiments. Approximately 0.5 g of unmounted tissues were superfused with a solution consisting of 2.4 mL of concentrated PSS (1.25× concentration of PSS of all components) and 0.6 mL of [1,6-^{13}C]fructose 1,6-bisphosphate solution. Concentrated PSS is thus diluted by the [1,6-^{13}C]fructose 1,6-bisphosphate solution so that the tissue is superfused in a solution having the same concentration of the components of PSS plus the components of the [1,6-^{13}C]fructose 1,6-bisphosphate solution. Well oxygenated conditions were obtained by superfusing the tissues at a flow rate of 2.5 mL/min with superfusate continuously equilibrated with 95% O_2 and 5% CO_2 as described previously (Hardin & Kushmerick, 1994). Superfusions in the NMR spectrometer were done at ambient temperature (~22 °C).

Statistics. Statistical significance was determined using an unpaired one sided Student's *t*-test with $\alpha = 0.05$ assuming unequal variances.

RESULTS

When unmounted segments of hog carotid artery are superfused with well-oxygenated PSS in the NMR spectrometer in the presence of 2.1 mM [1,6-^{13}C]fructose 1,6-bisphosphate as the only exogenous substrate, new resonances appear at 63.8, 68.7, 70.1, 94.8, and 98.7 ppm (Figure 1). The peak intensity of all five peaks progressively increased during 120 min of superfusion. Three peaks have been identified on the basis of chemical shift. The peaks have been identified as follows: the β-anomer of [1-^{13}C]-glucose (98.7 ppm), α-anomer of [1-^{13}C]glucose (94.8 ppm), and [6-^{13}C]glucose (63.8 ppm). Therefore, well-oxygenated and well-superfused hog carotid arteries were capable of metabolizing [1,6-^{13}C]fructose 1,6-bisphosphate with [1,6-^{13}C]glucose as a major product indicating gluconeogenic flux.

One possible interpretation of the results depicted in Figure 1 is that the exogenous [1,6-^{13}C]fructose 1,6-bisphosphate is not directly taken up by the vascular smooth muscle cells but rather is metabolized by nonspecific ectophosphatases resulting in formation of [1,6-^{13}C]fructose outside the cell. Since fructose can slowly spontaneously convert to glucose, [1,6-^{13}C]fructose outside the cell may be converted to [1,6-^{13}C]glucose outside the cell. To determine if [1,6-^{13}C]-fructose is either metabolized directly by hog carotid artery or undergoes spontaneous conversion to [1,6-^{13}C]glucose, we incubated unmounted hog carotid artery segments in tissue incubation chambers (mean tissue mass = 815 mg) with 2.1 mM [1,6-^{13}C]fructose for 120 min under hypoxic conditions (PSS equilibrated with 95% N_2/5% CO_2). If [1,6-^{13}C]glucose is formed by conversion of [1,6-^{13}C]fructose, then either [1,6-^{13}C]glucose should be observable in the superfusate or products of [1,6-^{13}C]glucose utilization, such as [3-^{13}C]lactate, should be observable since hypoxia substantially stimulates glycolytic metabolism. Shown in Figure 2 (bottom) is the ^{13}C NMR spectrum from 2.5 mL of the

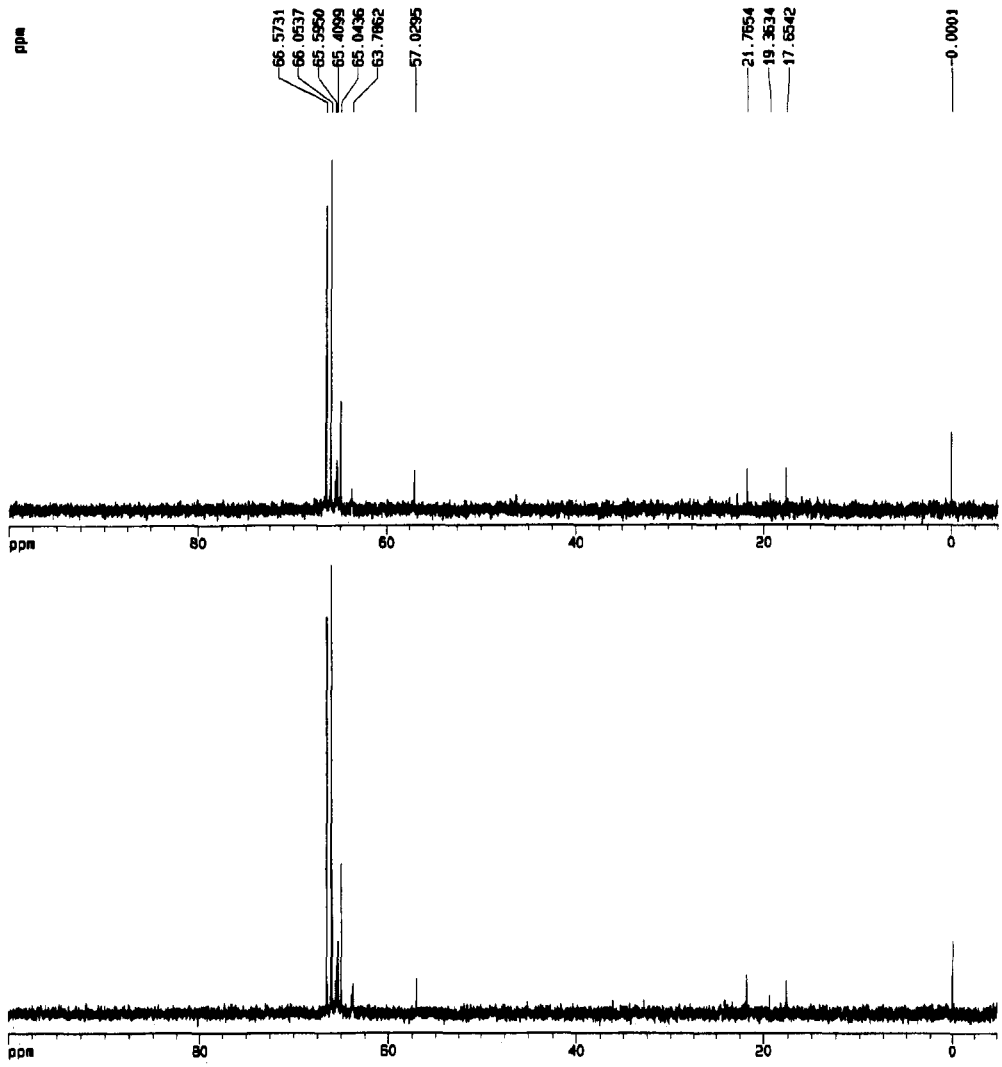

FIGURE 2: ^{13}C NMR spectra of an initial incubation solution containing PSS and 2.1 mM [1,6-^{13}C]fructose (bottom) and of the incubation solution sampled at the end of a 120 min incubation under hypoxic conditions (see text for details). No new resonances appear after incubation of the incubation solution with hog carotid artery segments. Shown (top) is a representative spectrum from four incubations.

superfusate containing PSS and 2.1 mM [1,6-^{13}C]fructose after lyophilization and resuspension in D_2O and DSS. The resonances observed from [1,6-^{13}C]fructose correspond with those previously reported from our laboratory (Hardin & Roberts, 1994). Shown in Figure 2 (top) is a typical ^{13}C NMR spectrum from 2.5 mL of the superfusate containing PSS and 2.1 mM [1,6-^{13}C]fructose taken after 120 min of incubation in the presence of hog carotid artery. No new resonances appear, indicating that fructose is not spontaneously converted to glucose and that hog carotid artery cannot metabolize fructose. A total of four incubations with [1,6-^{13}C]fructose were performed, and no new resonances were observed in any of the superfusates.

Line widths are broader in NMR analysis of living tissue compared to tissue or superfusate extracts thereby resulting in decreased sensitivity and resolution. To increase sensitivity and resolution of resonances, we carried out incubations of hog carotid artery segments well superfused with PSS equilibrated with 95% O_2/5% CO_2 in the presence of either [2-^{13}C]glucose, ^{13}C-labeled fructose 1,6-bisphosphate, or both and sampled the superfusate after 180 min of incubation at 37 °C. Shown in Figure 3 is an example of a ^{13}C NMR spectrum of a superfusate sampled at the end of a 180 min incubation in the presence of 5 mM [2-^{13}C]glucose and 2.1 mM [1,6-^{13}C]fructose 1,6-bisphosphate. The superfusate was lyophilized and resuspended in 99% D_2O and 25 mM DSS. There are a few notable differences between the spectrum shown in Figure 3 compared to those shown in Figure 1. In Figure 3 resonances representing [2-^{13}C]glucose and [2-^{13}C]-lactate are present. In addition, [3-^{13}C]lactate is also detectable (22.7 ppm) indicating lactate production from fructose 1,6-bisphosphate. This production of lactate from fructose 1,6-bisphosphate was not observed in the experiment depicted in Figure 1 and generally was not observed in incubations of tissues with only ^{13}C-labeled fructose 1,6-bisphosphate as the sole exogenous substrate (see below).

The results in Figure 3 demonstrate that hog carotid artery segments, well superfused with well-oxygenated PSS, are capable of metabolizing both fructose 1,6-bisphosphate and glucose with gluconeogenesis being the major metabolic fate of fructose 1,6-bisphosphate while glycolysis is the fate of glucose uptake and metabolism. Since glucose labeled at both the first and the sixth carbons is expected to be synthesized if [1,6-^{13}C]fructose 1,6-bisphosphate is participating in gluconeogenesis, it is impossible to determine whether fructose 1,6-bisphosphate is metabolized directly through fructose 1,6-bisphosphatase or if some fructose 1,6-bisphosphate reacts with aldolase and is isomerized by

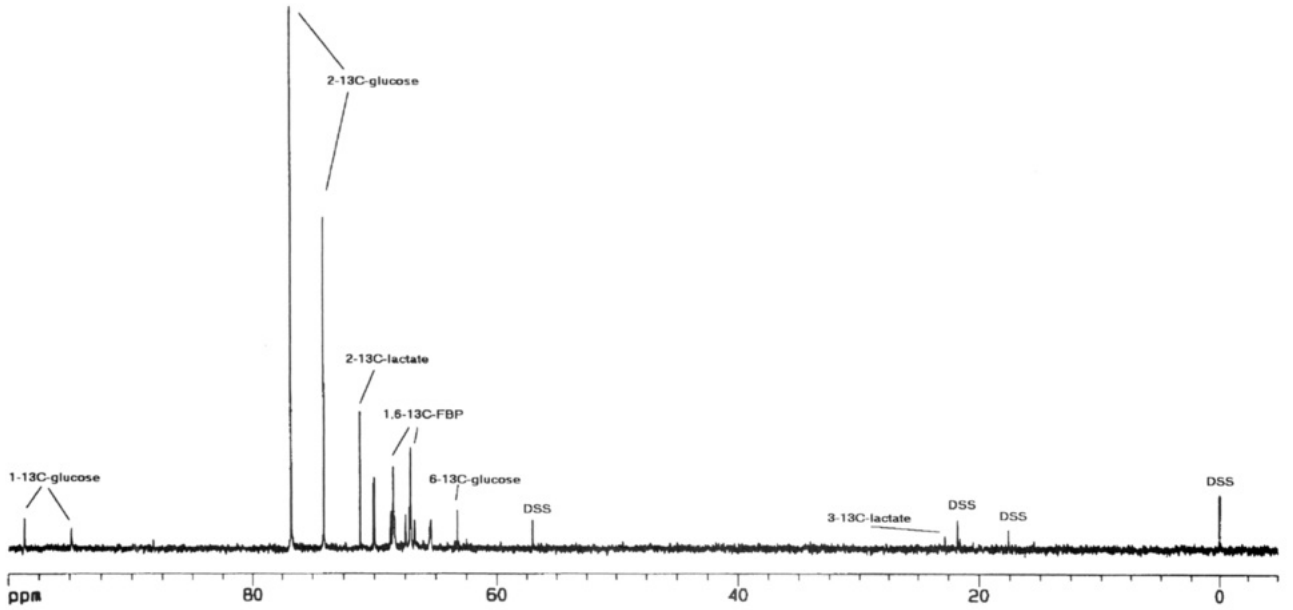

FIGURE 3: ^{13}C NMR spectrum of a superfusate sampled at the end of a 180 min incubation of mounted hog carotid artery in the presence of 2.1 mM [1,6-^{13}C]fructose 1,6-bisphosphate and 5 mM [2-^{13}C]glucose.

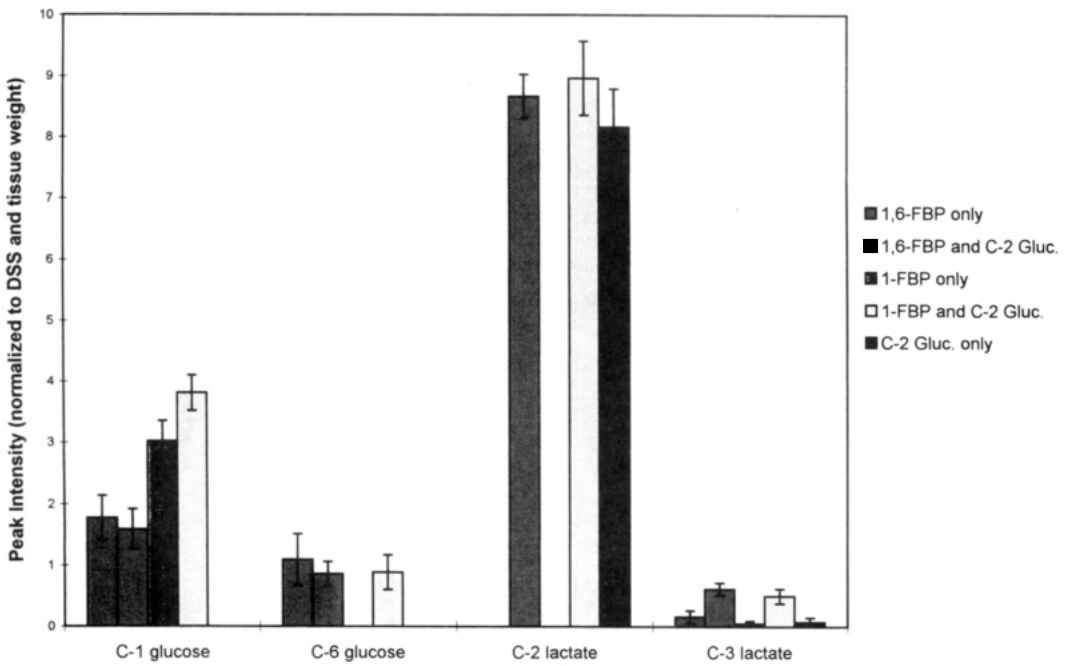

FIGURE 4: ^{13}C NMR peak intensities normalized to DSS-methyl peak intensity and to tissue mass for five experimental groups (see Materials and Methods for details).

triosephosphate isomerase prior to gluconeogenesis. By incubation of arterial segments with [1-^{13}C]fructose 1,6-bisphosphate, we were able to determine whether or not fructose 1,6-bisphosphate is isomerized prior to participation in gluconeogenic flux. Shown in Figure 4 are the normalized peak intensities of the metabolic products found in the superfusates of hog carotid artery segments incubated in the presence of [1,6-^{13}C]fructose 1,6-bisphosphate, [2-^{13}C]glucose, or [1-^{13}C]fructose 1,6-bisphosphate individually or in combinations of fructose 1,6-bisphosphate and glucose. As expected if fructose 1,6-bisphosphate participates in gluconeogenesis, incubations with both [1,6-^{13}C]fructose 1,6-bisphosphate and [1-^{13}C]fructose 1,6-bisphosphate result in formation of glucose labeled at the first carbon. However, no labeling of glucose at the sixth carbon was observed when [1-^{13}C]fructose 1,6-bisphosphate was used as the sole exogenous substrate. Therefore, fructose 1,6-bisphosphate appears to access fructose 1,6-bisphosphatase directly with no detectable flux through aldolase and triosephosphate isomerase prior to gluconeogenesis when fructose 1,6-bisphosphate is the sole exogenous substrate. When [2-^{13}C]-glucose and [1-^{13}C]fructose 1,6-bisphosphate are applied exogenously together, label appears in the sixth carbon of glucose indicating an isomerization of trioses derived from exogenous fructose 1,6-bisphosphate metabolism prior to gluconeogenic flux. However, the utilization of fructose 1,6-bisphosphate (either label configuration) does not appear to alter the lactate production from glucose.

There was little or no lactate production from fructose 1,6-bisphosphate utilization under these metabolic conditions. In the absence of [2-^{13}C]glucose, the [3-^{13}C]lactate (22.7 ppm) production, derived from glycolytic flux of [1,6-^{13}C]-

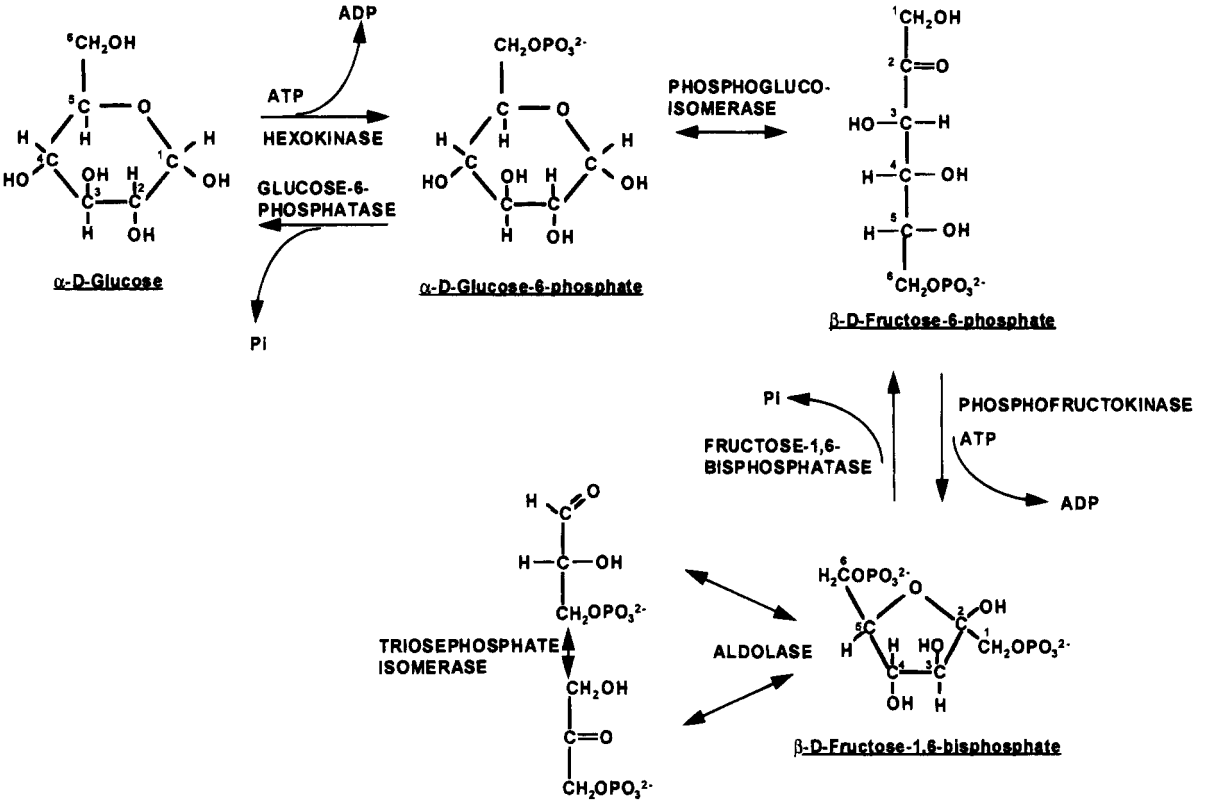

FIGURE 5: Schematic representation of label positions derived from [2-^{13}C]glucose, [1,6-^{13}C]fructose 1,6-bisphosphate, and [1-^{13}C]fructose 1,6-bisphosphate. If gluconeogenesis from [1-^{13}C]fructose 1,6-bisphosphate occurs without reaction through aldolase and triosephosphate isomerase, the glucose formed will have label in the same positions as the starting fructose 1,6-bisphosphate ([1-^{13}C]glucose). However, if isomerization occurs, then both [1-^{13}C]glucose and [6-^{13}C]glucose will be observed. Similarly, if glucose catabolism proceeds beyond the aldolase step and the resulting trioses participate in gluconeogenesis, then resonances from [5-^{13}C]glucose and [2-^{13}C]glucose will be observed.

fructose 1,6-bisphosphate or [1-^{13}C]fructose 1,6-bisphosphate, was not statistically different from zero (Figure 4). Indeed, [3-^{13}C]lactate was detected in two of eight samples incubated with [1,6-^{13}C]fructose 1,6-bisphosphate alone and in only one of eight samples incubated with [1-^{13}C]fructose 1,6-bisphosphate alone. However, when [2-^{13}C]glucose was included with the labeled fructose 1,6-bisphosphate during the incubations, there was a significant increase in [3-^{13}C]-lactate production from the labeled fructose 1,6-bisphosphate. Therefore, in the presence of exogenous glucose, there is a participation of fructose 1,6-bisphosphate in both gluconeogenic and glycolytic flux. However, in the presence or absence of fructose 1,6-bisphosphate, glucose is only metabolized to lactate with no trioses participating in gluconeogenesis (Figures 4 and 5).

DISCUSSION

Fructose 1,6-bisphosphate is not normally believed to be able to cross the cell membrane. However, we have recently shown that hypoxic hog carotid artery is capable of metabolizing exogenously applied fructose 1,6-bisphosphate to lactate (Hardin & Roberts, 1994) indicating that fructose 1,6-bisphosphate can cross the cell membrane and access intracellular glycolytic enzymes. Possible mechanisms by which fructose 1,6-bisphosphate may cross the plasma membrane have also been previously discussed (Hardin & Roberts, 1994). In the current study we examined the metabolic fate of exogenously applied ^{13}C-labeled fructose 1,6-bisphosphate and glucose to determine whether the intermediates of the pathway for utilization of exogenous fructose 1,6-bisphosphate mixed with the intermediates of the pathways for utilization of exogenous glucose.

Under the metabolic conditions studied, exogenously applied fructose 1,6-bisphosphate is metabolized to glucose while exogenously applied glucose is metabolized to lactate. In addition, the intermediates of these pathways do not appear to fully mix. In order for fructose 1,6-bisphosphate to be converted to glucose, exogenously applied fructose 1,6-bisphosphate must cross the plasma membrane and have access to enzymes with the activities of fructose 1,6-bisphosphatase, phosphoglucoisomerase, and glucose-6-phosphatase.

Metabolism of Fructose 1,6-Bisphosphate Occurs Inside the Cell. We have previously shown that fructose 1,6-bisphosphate can cross the cell membrane under hypoxic conditions in hog carotid artery (Hardin & Roberts, 1994). Under well-oxygenated conditions, the conversion of exogenously applied fructose 1,6-bisphosphate to glucose did not appear to occur outside the cell by the action of nonspecific ectophosphatases converting fructose 1,6-bisphosphate to fructose with subsequent spontaneous conversion to glucose. If ectophosphatases converted [1,6-^{13}C]fructose 1,6-bisphosphate to fructose, then [1,6-^{13}C]fructose should have been observable in the superfusate. When [1,6-^{13}C]fructose 1,6-bisphosphate was the sole exogenous substrate, the only new resonances that appeared were those of [6-^{13}C]glucose (63.8 ppm), [1-^{13}C]glucose (98.7 and 94.8 ppm), and two unidentified resonances at 70.1 and 68.7 ppm. No resonances corresponding to [1,6-^{13}C]fructose were observed [66.6, 65.9, 65.3, and 65.0 ppm, (Hardin & Roberts, 1994)] (see Figure 2). Therefore, if fructose 1,6-bisphosphate was converted to fructose, the levels of fructose formed were undetectable.

Since the spontaneous conversion of fructose to glucose is slow at physiological pH, such a mechanism of extracellular conversion of fructose 1,6-bisphosphate to glucose would be quite unlikely. Furthermore, in the presence of exogenous [2-^{13}C]glucose, [1-^{13}C]fructose 1,6-bisphosphate underwent isomerization prior to gluconeogenesis resulting in both [1-^{13}C]glucose and [6-^{13}C]glucose production. The production of [6-^{13}C]glucose from [1-^{13}C]fructose 1,6-bisphosphate could not have occurred from a simple dephosphorylation of fructose 1,6-bisphosphate with subsequent spontaneous conversion to glucose. Taken together with our previously reported evidence that exogenous fructose 1,6-bisphosphate is capable of crossing the cell membrane to produce lactate under hypoxic conditions, these results indicate that exogenously applied fructose 1,6-bisphosphate is capable of crossing the cell membrane and acting as a gluconeogenic substrate under well-oxygenated and well-superfused conditions in hog carotid artery.

Gluconeogenic Flux in Vascular Smooth Muscle. The results in Figure 1 demonstrate that under well-superfused and well-oxygenated conditions exogenously applied [1,6-^{13}C]fructose 1,6-bisphosphate is largely converted to [1,6-^{13}C]glucose. We have also previously shown that long term (12−16 h) superfusion of mounted hog carotid artery with [1-^{13}C]glucose results in a small but detectable isomerization of glucose with appearance of a resonance corresponding to [6-^{13}C]glucose or glycogen [Figure 2 of Hardin and Kushmerick (1994)]. This isomerization was likely the result of a breakdown of glucose to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate and isomerization by triosephosphate isomerase with subsequent gluconeogenesis. Although it was not determined whether glucose or glycogen was formed since those experiments examined tissue and superfusate simultaneously, those results are consistent with the existence of fructose 1,6-bisphosphatase and phosphoglucoisomerase activities in this tissue. It is commonly believed that glucose-6-phosphatase-phosphotransferase activity is found only in liver, kidney, and intestinal mucosa. However, glucose-6-phosphatase-phosphotransferase activity is known to exist in other tissues including pancreas, brain, adrenals, and testes and is localized to the endoplasmic reticulum, plasma membrane, and nuclei [for a review, see Nordlie (1975)]. Indeed, glucose-6-phosphatase activity has been reported in mouse soleus and gastrocnemius muscles with activity localized in the sarcoplasmic reticulum, lateral elements of the triads, and the myonuclear envelope (Watanabe et al., 1986). In addition, localization of glucose-6-phosphatase to the endoplasmic reticulum and nuclear envelope of endothelial cells was observed. In cardiac tissue and coronary vascular tissue from dogs, glucose-6-phosphatase was also localized to the endoplasmic reticulum and the nuclear envelope (Borgers et al., 1971). Therefore, our results are consistent with exogenously applied fructose 1,6-bisphosphate having the ability to cross the plasma membrane and having access to activities of fructose 1,6-bisphosphatase, phosphoglucoisomerase, and glucose-6-phosphatase-phosphotransferase. Although glucose-6-phosphatase has previously been shown to exist in vascular tissue (Borgers et al., 1971), our results indicate that the activity of this enzyme may be significant in intact vascular smooth muscle.

Compartmentation of Glucose and Fructose 1,6-Bisphosphate Metabolism. When both fructose 1,6-bisphosphate and glucose are provided simultaneously as exogenous substrates, there appears to be a partial but quite incomplete mixing of the intermediates of their metabolism. If the [1-^{13}C]fructose 1,6-bisphosphate derived from exogenous [1-^{13}C]fructose 1,6-bisphosphate uptake and the [2-^{13}C]fructose 1,6-bisphosphate derived from [2-^{13}C]glucose catabolism are converted by aldolase to the trioses and the trioses isomerized by triosephosphate isomerase, then the ratio of [3-^{13}C]lactate to [6-^{13}C]glucose would be equal to the ratio of [2-^{13}C]lactate to [5-^{13}C]glucose (see Figure 5) if the intermediates of the two pathways fully mixed. However, as shown in Figure 3, the [2-^{13}C]lactate production from [2-^{13}C]glucose is far greater than the [5-^{13}C]glucose production (none detected in any of the experiments) from the breakdown of [2-^{13}C]-glucose to [2-^{13}C]fructose 1,6-bisphosphate and reaction with aldolase and triosephosphate isomerase with subsequent gluconeogenesis. The resonance of β-[5-^{13}C]glucose would appear approximately 1.5 ppm to the right of the β-[2-^{13}C]-glucose. Therefore, despite simultaneous metabolism of both exogenous substrates, fructose 1,6-bisphosphate metabolism appears to be primarily gluconeogenic while glucose metabolism appears to be exclusively glycolytic. These results are inconsistent with a complete mixing of the intermediates of these pathways at the level of fructose 1,6-bisphosphate and the trioses.

The Observed Metabolic Compartmentation Is Intracellular and Not Intercellular. Since there appears to be a lack of mixing of the two pools of fructose 1,6-bisphosphate, these results can be interpreted as resulting from either an intracellular compartmentation or an intercellular compartmentation involving two cell types. Since endothelial cells have been shown to possess glucose-6-phosphatase activity (Watanabe et al., 1986), it might be speculated that the gluconeogenic activity was occurring in the endothelial cell layer and the glycolytic activity was occurring in the vascular smooth muscle layer. The procedure we use to isometrically mount hog carotid arteries on glass capillary tubes likely results in substantial damage to the endothelial cell layer. Since the endothelial cell layer is a monolayer of cells that average 0.2−0.5 μm thickness (Rhodin, 1980), and since the wall of the dissected hog carotid is approximately 0.5 mm thick, the percentage of the tissue mass occupied by endothelial cells is approximately 0.1%. Assuming we can compare peak intensities of glucose and lactate (even with differences in nuclear Overhauser effects and in T_1, the comparison is likely to be valid within a factor of two), then the production of lactate from fructose 1,6-bisphosphate is approximately 20% of the production of lactate from glucose. Since the lactate production rate of hog carotid artery is approximately 100 nmol/(min·g) (Paul, 1980), then if the production of glucose from fructose 1,6-bisphosphate was only from the endothelial cell layer, the production of glucose would be approximately 20 000 nmol/(min·g) since the endothelial cell mass is 1/1000th of the tissue mass. This enormous gluconeogenic flux assumes that all the endothelial cells are intact. If 90% are damaged, the flux increases by another factor of 10. Since that high of a flux is inconceivable, it stands to reason that the vast majority, if not all, of the gluconeogenic flux is not occurring in the endothelial cell layer. Therefore our results appear to be the result of an intracellular compartmentation rather than an intercellular compartmentation. However, we cannot currently rule out the possibility of an intercellular compartmentation between two populations of vascular smooth muscle cells in the

vascular wall. If our results can be explained by two populations of vascular smooth muscle cells, one cell population would account for all of the glucose utilization and none of the fructose 1,6-bisphosphate utilization. The other cell population would account for all of the fructose 1,6-bisphosphate utilization but be incapable of glucose utilization. Although we cannot currently eliminate this possibility, we consider it a highly unlikely scenario.

Exogenous Fructose 1,6-Bisphosphate Accesses Enzymes Poised for Gluconeogenic Flux. When only fructose 1,6-bisphosphate is available as an exogenous substrate, fructose 1,6-bisphosphate appears to be utilized for gluconeogenesis without any prior reaction with aldolase and triosephosphate isomerase. In Figure 4, when tissues were incubated with [1-^{13}C]fructose 1,6-bisphosphate only, resonances corresponding to [1-^{13}C]glucose yet not [6-^{13}C]glucose were observed. These results are inconsistent with intracellular [1-^{13}C]fructose 1,6-bisphosphate having access to aldolase assuming aldolase and triosephosphate isomerase are at equilibrium as they appear to be in skeletal muscle (Connett, 1985). In addition, when either [1,6-^{13}C]fructose 1,6-bisphosphate or [1-^{13}C]fructose 1,6-bisphosphate were applied alone exogenously, [3-^{13}C]lactate production was not significantly different from zero indicating insignificant glycolytic flux. Therefore, when fructose 1,6-bisphosphate is the sole exogenous substrate, fructose 1,6-bisphosphate appears to participate in gluconeogenic flux and not glycolytic flux. These results may indicate that the glycolytic enzymes to which exogenously applied fructose 1,6-bisphosphate has access are "poised" for gluconeogenesis rather than glycolysis.

Exogenous Glucose Accesses Enzymes Poised for Glycolysis. When only [2-^{13}C]glucose is used as exogenous substrate, there is a large production of [2-^{13}C]lactate indicating a substantial glycolytic flux from glucose in this tissue. A high rate of glucose utilization and lactate production is a characteristic of this tissue. However, over the 180 min of incubation in the presence of [2-^{13}C]glucose, there was no detectable incorporation of label into [5-^{13}C]-glucose. Our previous results using mounted hog carotid artery demonstrated barely detectable glucose resonances arising from isomerization of gluconeogenic trioses after 12−16 hours of incubation (Hardin & Kushmerick, 1994). Therefore, when mounted hog carotid artery is incubated with glucose as the sole exogenous substrate, glucose appears to participate in almost exclusively glycolytic flux with production of lactate and almost no gluconeogenic flux from the trioses. Indeed, gluconeogenic flux from trioses derived from glucose breakdown was not observed at the end of the 3 h incubation used in the current study. In addition, we saw no evidence of exogenous glucose participating in flux through the pentose shunt. When only [2-^{13}C]glucose was the sole exogenous substrate, only [2-^{13}C]lactate was produced indicating the [2-^{13}C]glucose 6-phosphate did not measurably participate in the pentose shunt. These results are consistent with those of Morrison et al. (1972), who found that flux of glucose substrate through the pentose shunt was approximately 2% of the flux through glycolysis in swine aorta. Such a low rate of flux through the pentose shunt would have been unmeasurable in our studies. Therefore, exogenous glucose appears to be primarily converted to lactate indicating that exogenous glucose has access to glycolytic enzymes that are poised for glycolysis.

The Nature of the Compartments in Vascular Smooth Muscle. The incomplete mixing of glycolytic intermediates in hog carotid artery has been previously reported (Hardin & Kushmerick, 1994; Lynch & Paul, 1983; Lynch and Paul, 1986) for intermediates resulting from glucose and from glycogen breakdown. In the current studies we report a partitioning of glycolytic intermediates from exogenously applied fructose 1,6-bisphosphate and glucose metabolism. In addition, in the current study glucose synthesis occurred simultaneously with glucose breakdown to lactate without complete mixing of the intermediates at the level of fructose 1,6-bisphosphate. However, some limited mixing of the glycolytic intermediates did appear to occur in the current study. Although no labeled carbons derived from [2-^{13}C]-glucose participated in gluconeogenic flux (after conversion to the trioses), some carbons from fructose 1,6-bisphosphate metabolism were converted to lactate even though most participated in gluconeogenic flux. It appears that the enzymes to which exogenous glucose has access are always poised for glycolytic flux whether or not exogenous fructose 1,6-bisphosphate is present. In the absence of exogenous glucose, fructose 1,6-bisphosphate has access to glycolytic enzymes poised for gluconeogenic flux, but when exogenous glucose is present, exogenous fructose 1,6-bisphosphate has some limited access to the enzymes poised for glycolytic flux. Regardless of whether this speculative scenario is correct, the results depicted in Figure 4 are clearly inconsistent with a complete mixing of the two pools of fructose 1,6-bisphosphate.

Since the intermediates of the gluconeogenic metabolism of fructose 1,6-bisphosphate appear to mix somewhat with the intermediates of glycolytic metabolism of exogenous glucose, and not *vice versa*, the compartment for glucose utilization may not allow intermediates to readily leave the constraints of the compartment while the compartment for fructose 1,6-bisphosphate utilization appears to not be as strictly channeled. It has been hypothesized that the glycolytic flux in smooth muscle is carried out by glycolytic enzymes localized to the cell membrane (Hardin et al., 1992; Paul et al., 1989). However, the location of the gluconeogenic enzymes responsible for fructose 1,6-bisphosphate metabolism under well-oxygenated conditions remains unclear. When fructose 1,6-bisphosphate is the sole exogenous substrate, it appears that fructose 1,6-bisphosphate does have access to fructose 1,6-bisphosphatase but does not have access to aldolase. However when both fructose 1,6-bisphosphate and glucose are present exogenously, fructose 1,6-bisphosphate appears to have some limited access to aldolase which may be the same pool of aldolase to which fructose 1,6-bisphosphate derived from glucose metabolism has access. Therefore there may be some "spill over" of the gluconeogenic pathway (fructose 1,6-bisphosphate utilization) to the glycolytic pathway when both fructose 1,6-bisphosphate and glucose are exogenous substrates. Although there is a partitioning of the intermediates of gluconeogenesis and glycolysis, the partitioning is not always complete, and thus at least a part of the partitioning may be the result of a balance of local reaction rate within a set of colocalized enzyme and normal diffusion of the intermediates away from the set of colocalized enzymes as previously proposed (Hardin & Kushmerick, 1994).

Disequilibrium of Aldolase and Triosephosphate Isomerase. When fructose 1,6-bisphosphate was the sole exogenous

substrate, gluconeogenesis occurred without any detectable reaction with aldolase and isomerization prior to gluconeogenic flux. In addition, when both fructose 1,6-bisphosphate and glucose were provided as exogenous substrates, incomplete mixing of the intermediates derived from the metabolism of the two substrates occurred. Therefore, in addition to a compartmentation of carbohydrate metabolism in smooth muscle, our evidence suggests that aldolase and triosephosphate isomerase may not be in equilibrium in this vascular smooth muscle. In skeletal muscle these enzymes were found to be at equilibrium but at an apparently different equilibrium than determined in vitro (Connett, 1985). However, it has been proposed that glycolytic enzyme binding in striated muscle may result in an altered combined equilibrium of aldolase and triosephosphate isomerase since bound enzyme may provide a locus of altered free energy for fructose 1,6-bisphosphate in the cell (Hardin, 1987). Regardless of the mechanism, equilibrium of aldolase and triosephosphate isomerase in vascular smooth muscle cannot be assumed on the basis of the evidence provided.

Although exogenously applied fructose 1,6-bisphosphate is not a physiological substrate, its use in this study has helped to elucidate the nature of the compartmentation of carbohydrate metabolism in vascular smooth muscle. It is likely that fructose 1,6-bisphosphate can cross cell membranes of tissues other than vascular smooth muscle and may be an important tool in investigations of the organization of carbohydrate metabolism in other tissues.

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