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Caveolae and the Organization of Carbohydrate Metabolism in Vascular Smooth Muscle

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Abstract We have previously found that glycolysis and gluconeogenesis occur in separate ''compartments'' of the VSM cell. These compartments may result from spatial separation of glycolytic and gluconeogenic enzymes (Lloyd and Hardin [1999] Am J Physiol Cell Physiol. 277:C1250-C1262). We have also found that an intact plasma membrane is essential for compartmentation to exist (Lloyd and Hardin [2000] Am J Physiol Cell Physiol. 278:C803-C811), suggesting that glycolysis and gluconeogenesis may be associated with distinct plasma membrane microdomains. Caveolae are one such microdomain, in which proteins of related function colocalize. Thus, we hypothesized that membrane-associated glycolysis occurs in association with caveolae, while gluconeogenesis is localized to non-caveolae domains. To test this hypothesis, we disrupted caveolae in vascular smooth muscle (VSM) of pig cerebral microvessels (PCMV) with β methyl-cyclodextrin (CD) and examined the metabolism of [2-^{13}C]glucose (a glycolytic substrate) and [1-^{13}C]fructose 1,6-bisphosphate (FBP, a gluconeogenic substrate in PCMV) using ^{13}C nuclear magnetic resonance spectroscopy. Caveolar disruption reduced flux of [2-^{13}C]glucose to [2-^{13}C]lactate, suggesting that caveolar disruption partially disrupted the glycolytic pathway. Caveolae disruption may also have resulted in a breakdown of compartmentation, since conversion of [1-^{13}C]FBP to [3-^{13}C]lactate was increased by CD treatment. Alternatively, the increased [3-^{13}C]lactate production may reflect changes in FBP uptake, since conversion of [1-^{13}C]FBP to [3-^{13}C]glucose was also elevated in CD-treated cells. Thus, a link between caveolar organization and metabolic organization may exist. J. Cell. Biochem. 82: 399–408, 2001.

Key words: glycolysis; gluconeogenesis; plasma membrane; microvessels; ^{13}C-NMR

Early studies of glucose metabolism failed to identify a "glycolytic organelle" corresponding to the mitochondrion. The lack of such as organelle, coupled with the presence of glycolytic enzymes in "cytosol" fractions of cells, led to the conclusion that glycolysis operates via free diffusion of enzymes and intermediates in the cytoplasm.

Although this model of the glycolytic pathway was widely accepted until recently, it fails to account for considerable evidence which suggests that glycolytic enzymes are localized within cells, and are thus not freely diffusible. For example, glycolytic enzymes bind to F-actin [Arnold and Pette, 1968, 1970; Arnold et al., 1971; Clarke and Masters, 1975; Knull et al., 1980; Bronstein and Knull, 1981; Masters et al., 1981] and to microtubules [Karkhoff-Schweizer and Knull, 1987; Walsh et al., 1989; Lehotzky et al., 1993; Marmillot et al., 1994; Muronetz et al., 1994]. Glycolytic enzyme activity has also been found in association with the plasma membrane [Green et al., 1965; Weiss and Lamp, 1987, 1989; Hardin et al., 1992; Uyeda, 1992].

Colocalization of the enzymes of a metabolic pathway may allow them to engage in metabolite channeling, in which intermediates are passed from one enzyme to the next without release into the cytoplasm [Srere and Ovadi, 1990; Ovadi and Srere, 1992]. In the case of glycolysis and gluconeogenesis (pathways which share many enzymes and intermediates),

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channeling of intermediates could increase the efficiency of both pathways and allow them to be regulated independently.

We are currently studying the organization of carbohydrate metabolism in vascular smooth muscle (VSM). VSM is a useful system for the study of carbohydrate metabolism, since it is a highly glycolytic tissue [Hardin and Paul, 1995]. In addition to glycolysis, VSM can also resynthesize glucose from glycolytic intermediates under some conditions (gluconeogenesis) [Hardin and Roberts, 1995a,b; Lloyd and Hardin, 1999, 2000].

Glycolysis and gluconeogenesis share the same chemical intermediates, and utilize eight of the same enzymes. If glycolytic enzymes and intermediates are free to diffuse in the cytoplasm, then the intermediates of glycolysis and gluconeogenesis should mix freely. However, our group has found that glycolytic and gluconeogenic intermediates do not mix freely in VSM (compartmentation) [Hardin and Roberts, 1995a; Lloyd and Hardin, 1999]. This lack of mixing must reflect a spatial separation of glycolysis and gluconeogenesis. Thus, we hypothesized that glycolytic and gluconeogenic enzymes are differentially localized within the cell, and that disrupting enzyme localization would disrupt compartmentation.

In our previous studies on the structural basis of compartmentation, we found that glycolysis is partially regulated by associations of glycolytic enzymes with microtubules, but that gluconeogenesis is not [Lloyd and Hardin, 1999]. As discussed above, the plasma membrane is another potential site of glycolytic and/or gluconeogenic enzyme localization. Thus, we next investigated the role of the plasma membrane in compartmentation by using β-escin to disrupt its integrity. These studies revealed that the plasma membrane plays a key role in directing glycolytic and gluconeogenic substrates into the appropriate pathways [Lloyd and Hardin, 2000], suggesting that glycolytic and gluconeogenic enzymes may be localized to separate plasma membrane microdomains.

Caveolae represent one specialized plasma membrane microdomain. These small, bulb-shaped indentations of the plasma membrane are found in many cell types, and are abundant in VSM [Rothberg et al., 1992]. The smooth muscle cell plasma membrane is clearly divided into caveolar and non-caveolar domains [Severs and Simons, 1986; North et al., 1993].

Recently, caveolae have been found to be enriched in a wide variety of proteins, relative to the plasma membrane as a whole. Many signal transduction proteins are localized in caveolae, including G-protein coupled receptors [Chun et al., 1994], G-protein subunits [Li et al., 1995, 1996], receptor tyrosine kinases [Couet et al., 1997], Ras [Li et al., 1996; Song et al., 1996], protein kinase C [Oka et al., 1997], nitric oxide synthase [Feron et al., 1996; Ju et al., 1997], the Ca^{2+}-ATPase [Nasu and Inomata, 1990; Schnitzer et al., 1995], and the inositol 1,4,5-trisphosphate receptor [Schnitzer et al., 1995]. Localization of interacting proteins within caveolae may function to increase the efficiency and specificity of cellular signal transduction [for reviews see Anderson, 1993; Neubig, 1994; Lisanti et al., 1995]. Indeed, caveolae-disrupting agents have been shown to interfere with normal function in some caveolae-associated signaling pathways, presumably by interfering with organization [Liu et al., 1997; Furuchi and Anderson, 1998].

Caveolae have also been reported to contain proteins related to glucose metabolism, including the glycolytic enzyme phosphofructokinase [Scherer and Lisanti, 1997] and GLUT-4 glucose transporters [Scherer et al., 1994; Gustavsson et al., 1996], although some controversy exists regarding GLUT-4 [see for example, Kandror et al., 1995]. Glycogen particles have also been observed in association with caveolae [Tanuma et al., 1982]. Therefore, we hypothesized that glycolytic enzymes are localized to caveolae in VSM, while gluconeogenic enzymes are localized to non-caveolar membrane domains. To test this hypothesis, we disrupted caveolae using β-methyl-cyclodextrin and studied the metabolism of both glycolytic and gluconeogenic substrates in VSM of pig cerebral microvessels. The results of these studies suggest that membrane-associated glycolysis occurs in association with caveolae.

METHODS

Tissue Collection

Pig brains were collected at a local abbatoir shortly after the animals were slaughtered. Tissues were immersed in physiological saline solution (PSS, 4°C) and transported to the laboratory on ice. PSS contained NaCl (116 mM) KCl (4.6 mM), KH_2PO_4 (1.16 mM), $NaHCO_3$ (25.3 mM), $CaCl_2$ (2.5 mM), $MgSO_4$

(1.16 mM), and glucose (5 mM), pH 7.4. Penicillin G (303 mg/l) and streptomycin sulfate (100 mg/l) were added to PSS to inhibit bacterial growth. PSS was oxygenated and pH-equilibrated by gassing with 95% O_2/5% CO_2 and was filtered through a 0.22 μM filter (Micron Separations, Inc., Westboro, MA) to remove potential contaminating organisms. Brains were stored in fresh PSS at 4°C until use.

Microvessel Isolation

Cerebral microvessels were isolated in HEPES-buffered PSS (HBPSS, 21°C) consisting of NaCl (118 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), $MgSO_4$ (1.0 mM), *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES, 28 mM), NaH_2PO_4 (1.0 mM), bovine serum albumin (0.2% w:v), heparin (1 U/ml), and isoproterenol (10 μM), pH 7.4. HBPSS was supplemented with antibiotics and filtered as described above. Microvessels were isolated by modification of the method of Sussman et al. [1988] derived from Brendel's method [Brendel et al., 1974], as we have previously described [Lloyd and Hardin, 1999, 2000]. Briefly, the outer layer of the brain was peeled away. The "gray matter" of the cerebral cortex was dissociated by aspiration into a plastic vacuum flask, then further homogenized in a Dounce-type homogenizer. The homogenate was poured over a 210-μm nylon mesh to catch large vessels and debris. Material trapped by this mesh was discarded. Material that passed through the 210-μm mesh was collected and poured over a 105-μm nylon mesh. Smaller vessels were retained on this mesh, while vessel fragments and other material passed through freely and were discarded. The trapped vessels were rinsed thoroughly with HBPSS. Finally, the mesh was inverted, and the vessels were rinsed off the mesh into a clean container with HBPSS.

Metabolic Incubations

Microvessels were isolated from three brains for each metabolic experiment. The suspension containing rinsed vessels was brought to a total volume of 100 ml with HBPSS, then divided into two equal parts. One aliquot was poured over a 105-μm mesh, then resuspended in 25 ml HBPSS containing 5 mM glucose and 4 mM fructose 1,6-bisphosphate (FBP) (Esafosfina, Biomedica Foscama, Italy; gift from Dr. Guiseppe Lazzarino), pH 7.4. The second aliquot was treated identically, except that HBPSS also contained 10 mM β-methyl-cyclodextrin to disrupt caveolae. Both aliquots were incubated in a shaking bath at 37°C for 2 h. After this incubation, each aliquot was again poured over a 105-μm mesh, rinsed with substrate- and drug-free HBPSS, and resuspended in 9 ml of HBPSS containing 5 mM [1-^{13}C] FBP and 5 mM [2-^{13}C]glucose, pH 7.4. An 8 ml sample of each vessel suspension was placed in a culture flask and incubated in a shaking bath for 3 h at 37°C. At the conclusion of the incubation, a 5.5 ml sample was removed from each flask and centrifuged for 5 min at 1,000*g* to pellet the vessels. A 4-ml aliquot of the supernatant solution was frozen in liquid N_2 for NMR analysis. A 4-ml sample of the starting solution (containing labeled substrates) was also collected and frozen.

NMR Spectroscopy

Supernatant samples were lyophilized to powder in a Speed Vac (Savant Instruments, Farmingdale, NY), then resuspended in 800 μl of 99.9% D_2O (Cambridge Isotope Laboratories, Inc., Andover, MA). D_2O contained 25 mM 3 (trimethylsilyl)-1-propanesulfonic acid (TMS-PS) as a chemical shift reference. Immediately after resuspension, 650 μl of the solution were transferred to a 5-mm NMR tube for analysis.

^{13}C NMR was performed using a Bruker DRX 300 spectrometer. All data were acquired with the following parameters: 900 scans plus 64 dummy scans, 30° pulse angle at 125.77 MHz, 33,333 Hz sweep width, and 1 sec predelay. 32,768 points were acquired and processed with line broadening of 1 Hz before Fourier transform of the data. Spectra were broad-band proton decoupled and peaks were referenced to the signal of TMSPS, set to 0 ppm. Peak intensity was determined using Bruker software. No corrections for nuclear Overhauser effects were made, since these were assumed to be the same for all samples. Intensity of the peaks of interest was normalized to the intensity of the TMSPS peak.

Transmission Electron Microscopy (TEM)

Microvessels were isolated and treated with cyclodextrin as described above, then examined using TEM to verify that cyclodextrin disrupted caveolar structure. All incubations and drug treatments were performed exactly as for metabolic experiments, except that unlabeled

FBP and glucose were used during the 3 h incubation period. Samples of both control and cyclodextrin-treated microvessels were collected following the initial incubation with cyclodextrin and again following the 3 h "metabolic" incubation.

Immediately after collection, microvessel samples were fixed in 2% glutaraldehyde/2% paraformaldehyde (in 0.1 M cacodylate buffer, pH 7.35) for 2–4 h. Samples were rinsed in 0.1 M cacodylate buffer (3×10 min) before secondary fixation in 1% osmium tetroxide in 0.1 M cacodylate buffer containing 1.5% potassium ferricyanide (2 h). The tissue was rinsed in distilled H_2O (5×5 min), then stained in 2% aqueous uranyl acetate (30 min). The samples were rinsed again in distilled H_2O (3×15 min). Next, the tissue was dehydrated in a graded ethanol series: 20% (30 min), 50% (30 min), 70% (overnight), 90% (30 min), and 100% (2×30 min and 1×1 h). After dehydration, the samples were incubated in propylene oxide (PO) (2×30 min and 1×1 h) to prepare them for resin infiltration. Epon-Araldite (EA) was used as the embedding resin. Samples were infiltrated with resin by incubation in the following EA:PO mixtures: 1:2 (4 h), 1:1 (4 h), 2:1 (overnight). Finally, the tissue was incubated in pure EA (overnight), then embedded in fresh resin and cured at 60°C for three days.

Thick sections of cured blocks were cut with a glass knife using an ultramicrotome (LKB 8800 Ultrotome III) and viewed under a light microscope to evaluate the tissue content of the section. Once a suitable section of the block was identified, ultrathin (70–90 nm) sections were cut using a diamond knife (Diatome). Sections were picked up on 200-mesh copper grids. The sections were then stained with 3% aqueous urany1 acetate (10 min), rinsed 60× in distilled water, contrasted with Reynold's lead citrate (7 min), and rinsed a final 60× in water. Grids were air-dried, then viewed in a Hitachi H-600 transmission electron microscope at a accelerating voltage of 75 keV. Cells were photographed at an original magnification of 60,000×.

Statistical Analysis

Significant differences in NMR peak intensities of control and cyclodextrin-treated samples were identified using a two-tailed t-test for paired samples. P values less than 0.05 were considered significant.

Reagents

Na_2HPO_4 and NaH_2PO_4 were obtained from Aldrich Chemical Co., Milwaukee, WI. Electron microscopy reagents and supplies were obtained from Electron Microscopy Sciences (Fort Washington, PA). All other reagents (unless stated) were obtained from Sigma Chemical Co., St. Louis, MO.

RESULTS

Treatment With β-Methyl-Cyclodextrin Disrupts Caveolar Organization in VSM of PCMV

There are numerous reports of caveolar disruption induced by cyclodextrins [see for example Furuchi and Anderson, 1998; Hailstones et al., 1998; Park et al., 1998]. These compounds appear to disorganize caveolae by removing cholesterol from the plasma membrane [Hailstones et al., 1998; Park et al., 1998].

TEM of both control and cyclodextrin-treated PCMV demonstrated that a 2-h treatment with 10 mM cyclodextrin was sufficient to disrupt caveolar organization. This is consistent with studies by other groups, in which 5–10 mM cyclodextrin disrupted caveolae within 1 h [Park et al., 1998]. The plasma membranes of control (untreated) cells contained numerous caveolae both before and after the metabolic incubation (Fig. 1). Caveolae in control cells had narrow necks and were bulb-shaped. In contrast, treated cells contained large numbers of flattened caveolae with wide openings (although some normal caveolae were also evident in cyclodextrin-treated cells) (Fig. 2). The altered caveolar morphology was present both before and after the metabolic incubation. Thus, cyclodextrin treatment produced clear alterations in caveolar morphology which persisted throughout the period of study. No other obvious structural changes were observed in cyclodextrin-treated cells. Short (1 h) exposures to cyclodextrin were less effective at altering caveolar structure, while higher concentrations and longer incubations appeared to cause cell damage (data not shown).

Glycolysis From Glucose is Reduced in Cyclodextrin-Treated PCMV

When untreated (control) PCMV ($n = 8$) were incubated for 3 h with 5 mM [1-^{13}C]FBP and 5 mM [2-^{13}C]glucose, they utilized [1-^{13}C]FBP mainly for gluconeogenesis but utilized

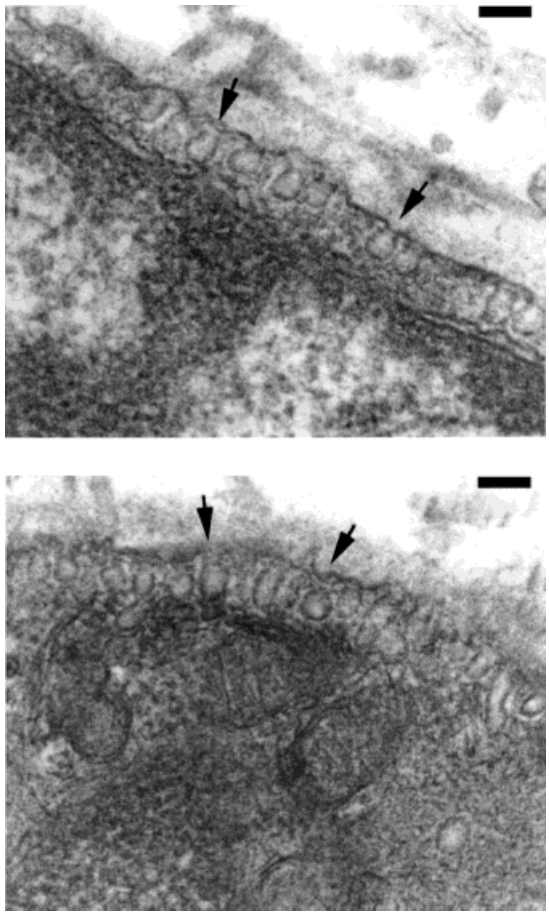

Fig. 1. Electron micrograph of caveolae in untreated cells. Cells were incubated with FBP and glucose for 2 h prior to beginning the 3-h metabolic incubation period. **Top**, cells prior to beginning of 3-h metabolic incubation. **Bottom**, cells after the 3-h metabolic incubation. Cells contain numerous, narrow-necked caveolae both before and after the metabolic incubation (arrows), showing that caveolae remain intact throughout the period of study. Bar = 100 nm.

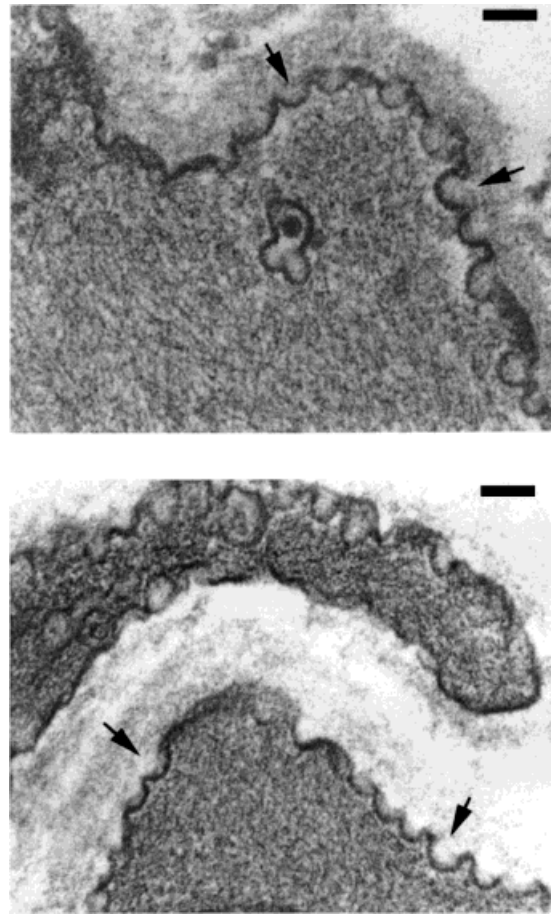

Fig. 2. Electron micrograph of caveolae in cyclodextrin-treated cells. Cells were incubated with FBP, glucose, and cyclodextrin for 2 h prior to beginning the 3-h metabolic incubation period. **Top**, cells prior to beginning of 3-h metabolic incubation. **Bottom**, cells after the 3-h metabolic incubation. Caveolae in treated cells are wider and flatter than caveolae in control cells (arrows) (compare with Fig. 1). Caveolar morphology is altered at both time points examined, showing that the effects of cyclodextrin on caveolar structure were maintained throughout the metabolic incubation. Bar = 100 nm.

[2-^{13}C]glucose for glycolysis (Fig. 3). The majority of [1-^{13}C]FBP metabolism could be accounted for by the production of [1-^{13}C]glucose. Measurable [3-^{13}C]lactate production was observed in only three of eight experiments, and [3-^{13}C]lactate peak intensity was much less than [1-^{13}C]glucose peak intensity. However, PCMV actively metabolized [2-^{13}C]glucose to [2-^{13}C]lactate, demonstrating that the glycolytic pathway is active in PCMV. Thus, glycolysis and gluconeogensis are compartmented in control PCMV, in agreement with our previous findings [Lloyd and Hardin, 1999].

The intensity of the [2-^{13}C]lactate peak (lactate from glucose, glycolysis) was significantly ($P = 0.001$) reduced in PCMV which were treated with 10 mM cyclodextrin for 2 h ($n = 8$), to 73.9±3.7% of the peak intensity of self-paired controls (mean±SEM) (Fig. 4). Thus, disrupting caveolar organization decreased the flux of glucose through the glycolytic pathway, suggesting that a functional association between glycolysis and caveolae exists.

Glycolysis From FBP is Increased in Cyclodextrin-Treated PCMV

As discussed above, measurable production of [3-^{13}C]lactate from [1-^{13}C]FBP was observed in control samples in only three of eight experiments. In contrast, [3-^{13}C]lactate was detected

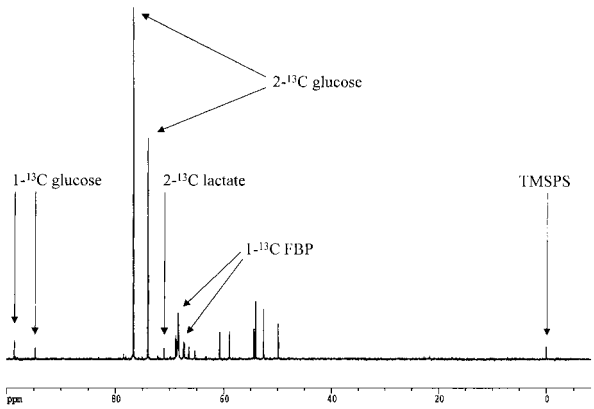

Fig. 3. Metabolism of [1-^{13}C]FBP and [2-^{13}C]glucose in intact cells. [2-^{13}C]glucose (α anomer, 76.7 ppm; β anomer, 74.0 ppm) is metabolized mainly to [2-^{13}C]lactate (71.1 ppm). In contrast, [1-^{13}C]FBP (α anomer, 68.4 ppm; β anomer, 67.4 ppm) is metabolized mainly to [1-^{13}C]glucose (α anomer, 98.6 ppm; β anomer, 94.8 ppm), with little or no [3-^{13}C]lactate production observed (22.7 ppm). The signal of TMSPS at 0 ppm is used as a reference.

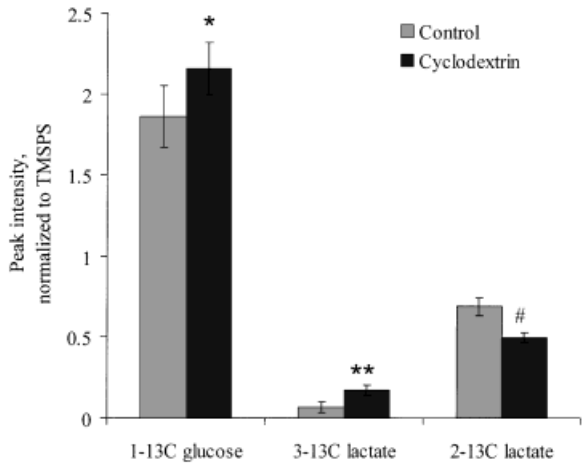

Fig. 4. Alterations in metabolism following cyclodextrin treatment. Untreated PCMV (gray) utilized [1-^{13}C]FBP mainly for gluconeogenesis, producing [1-^{13}C]glucose (left). Production of [3-^{13}C]lactate from [1-^{13}C]FBP via glycolysis was minor (center) and was not detected in all samples. In contrast, [2-^{13}C]glucose was effectively utilized for glycolysis, producing [2-^{13}C]lactate (right). In cyclodextrin-treated PCMV (black), [1-^{13}C]glucose production was increased to 120±6.6% of self-paired controls (left). Utilization of [1-^{13}C]FBP for glycolysis was also increased in cyclodextrin-treated PCMV (center). Most interestingly, glycolysis from [2-^{13}C]glucose in cyclodextrin-treated PCMV is reduced to 73.9±3.7% of self-paired controls (right). *$P = 0.011$; **$P = 0.001$; #$P = 0.016$.

in cyclodextrin-treated samples in seven of the eight experiments. Thus, caveolar disruption moderately, but significantly ($P = 0.02$) increased flux of [1-^{13}C]FBP through the glycolytic pathway (Fig. 4).

Gluconeogenesis From FBP is Increased in Cyclodextrin-Treated PCMV

In both control and cyclodextrin-treated PCMV, [1-^{13}C]glucose was the major product of [1-^{13}C]FBP metabolism. However, production of [1-^{13}C]glucose from [1-^{13}C]FBP via gluconeogenesis was significantly ($P = 0.01$) increased in cyclodextrin-treated PCMV, to 120±6.6% of self-paired controls (mean±SEM) (Fig. 4).

DISCUSSION

Glycolysis is Organized by Associations of Glycolytic Enzymes With Cytoplasmic Structures

We have previously shown that glycolysis and gluconeogenesis are compartmented in VSM of pig cerebral microvessels [Lloyd and Hardin, 1999, 2000], suggesting that glycolytic and gluconeogenic intermediates do not diffuse freely in the cytoplasm. It is likely that this restricted diffusion reflects differential localization of glycolytic and gluconeogenic enzymes. In agreement with this idea, we have found that disrupting the cytoplasmic structures that metabolic enzymes associate with also disrupts metabolic function [Lloyd and Hardin, 1999, 2000].

Our previous studies [Lloyd and Hardin, 2000] suggested that plasma membrane organization is linked to metabolic organization. Therefore, the goal of the present study was to explore the possibility that the glycolytic and gluconeogenic pathways are associated within distinct plasma membrane microdomains.

Functionally-Related Proteins Colocalize in Plasma Membrane Caveolae

Caveolae are becoming well-known as a unique plasma membrane microdomain. Organization of functionally-related proteins within caveolae appears to be essential for normal function in some signal-transduction pathways (see Introduction). Caveolae are a prominent feature of the smooth muscle plasma membrane, and at least one glycolytic enzyme is associated with caveolae [Scherer and Lisanti, 1997]. Thus, metabolic pathways may also be organized in caveolae.

We hypothesized that glycolytic enzymes are associated with caveolae, while gluconeogenic

enzymes are found in non-caveolar membrane domains. We tested this hypothesis by disrupting caveolae in VSM of pig cerebral microvessels and examining how this treatment affected glycolysis and gluconeogenesis.

Glycolysis From Glucose is Reduced Following Caveolar Disruption

Electron microscopic examination of the tissue revealed that cyclodextrin treatment caused caveolae to flatten into shallow depressions, in contrast to the narrow-necked, bulb-shaped structures observed in untreated cells. Studies by other groups have shown that many proteins which are localized to caveolae are redistributed throughout the plasma membrane following cyclodextrin treatment [Furuchi and Anderson, 1998].

Production of [2-^{13}C]lactate from [2-^{13}C]glucose was significantly reduced in cyclodextrin-treated PCMV, relative to self-paired controls. If glycolytic enzymes are directly associated with caveolae (via electrostatic interactions, for example) then this decrease could be due to dispersal of the enzymes following caveolar disruption. If the enzymes are indirectly associated with caveolae (via interactions with caveolins, for example) then dispersal of these proteins could result in pathway disorganization. Disorganization of the pathway by either mechanism could reduce pathway efficiency.

The decrease in glycolysis from glucose was relatively modest in treated cells. However, glycolysis also occurs in association with microtubules and F-actin (see Introduction). Presumably, only the fraction of the glycolytic enzymes which is associated with caveolae will be affected by cyclodextrin treatment, leaving the remainder of the pathway fully functional.

FBP Utilization is Increased Following Caveolar Disruption

In contrast to glycolysis from glucose, glycolysis from [1-^{13}C]FBP was significantly increased following cyclodextrin treatment. Whereas [3-^{13}C]lactate was observed in only three control samples, [3-^{13}C]lactate was detected in seven of eight cyclodextrin-treated samples.

Why is glycolysis from exogenous FBP increased, when glycolysis from glucose is reduced? Although this result may appear to contradict our findings that glycolysis from glucose is decreased following cyclodextrin treatment, it could be consistent with differential membrane localization of glycolysis and gluconeogenesis, as outlined below.

In intact cells, FBP appears to have only limited access to glycolytic enzymes, since the major metabolic product of [1-^{13}C]FBP metabolism is [1-^{13}C]glucose rather than [3-^{13}C]lactate [Lloyd and Hardin, 1999]. We have previously provided evidence that FBP enters cells via dicarboxylate transporters [Hardin et al., 1998]. Thus, one possible mechanism by which access of FBP to glycolytic enzymes could be limited is colocalization of the dicarboxylate transporter with gluconeogenic enzymes [Lloyd and Hardin, 2000]. In this model, FBP never reaches glycolytic enzymes, since it is metabolized by gluconeogenic enzymes as soon as it crosses the membrane. Colocalization of a metabolic enzyme with a functionally-related transporter exactly analogous to this has recently been reported [McDonald et al., 1997]. When caveolae are disrupted, glycolytic enzymes localized there may be redistributed throughout the plasma membrane. Therefore, FBP entering cyclodextrin-treated cells could have greater access to glycolytic enzymes, resulting in increased production of [3-^{13}C]lactate (despite an overall reduction in pathway efficiency).

Another possible explanation for the increased [3-^{13}C]lactate production observed after cyclodextrin treatment is simply that the amount of FBP which enters the cell is increased by the treatment. This hypothesis is supported by the observation that utilization of FBP for gluconeogenesis was also increased in cyclodextrin-treated cells. Production of [1-^{13}C]glucose from [1-^{13}C]FBP was significantly increased in cyclodextrin-treated cells, relative to self-paired controls.

If cyclodextrin does increase FBP uptake, the mechanism behind the increase is unknown. It is unlikely that cells are permeabilized by cyclodextrin, since cyclodextrin is not membrane-permeable [Park et al., 1998], and a much longer treatment with cyclodextrin (overnight) than that used in the present study (2 h) does not permeabilize cells [Hailstones et al., 1998]. In addition, the alterations in metabolism which we observed following cyclodextrin treatment are not similar to those which we have previously observed in cells permeabilized with β-escin [Lloyd and Hardin, 2000]. It seems more likely that cyclodextrin increases transport of FBP across an intact plasma membrane by

altering the physical properties of the membrane or the activity of the transporter itself. Since cyclodextrin removes cholesterol from the entire membrane, not just caveolar domains [Furuchi and Anderson, 1998], FBP transport properties could well be affected by cyclodextrin, even if FBP transport does not occur in caveolae. Therefore, when examining FBP metabolism, we cannot separate the possible effect of cyclodextrin on FBP transport from the effect that it appears to have on metabolic organization.

It is possible that uptake of glucose and FBP could have been altered due to changes in membrane fluidity influencing transport proteins. However, if there is an effect of altered membrane fluidity on membrane transporters, the effect should be similar for a variety of transport proteins. It appears rather unlikely that changes in membrane fluidity would result in increased transport of one metabolite (FBP) while decreasing transport of another (glucose). Since we observed increased gluconeogenesis from FBP and decreased glycolysis from glucose, the alterations in metabolism are best explained by altered distributions of transporters and metabolic enzymes. It is also likely that cyclodextrin treatment disrupts a variety of signal transduction pathways, since many signal transduction proteins are localized to caveolae including G-protein coupled receptors [Chun et al., 1994], G-protein subunits [Li et al., 1995, 1996], receptor tyrosine kinases [Couet et al., 1997], Ras [Li et al., 1996; Song et al., 1996], protein kinase C [Oka et al., 1997], nitric oxide synthase [Feron et al., 1996; Ju et al., 1997], the Ca^{2+}-ATPase [Nasu and Inomata, 1990; Schnitzer et al., 1995] and the inositol 1,4,5- trisphosphate receptor [Schnitzer et al., 1995]. It is possible that these alterations could potentially influence metabolic rate. However, since the cells are not contracting, we are measuring a basal (unstimulated) level of glucose metabolism. The basal metabolic rate of the cell should be relatively independent of signaling pathway activation, and thus would not be expected to be affected by disruption of signal transduction. However, activation of signaling pathways could possibly activate metabolic pathways. With activation of a quiescent signaling pathway, it is possible that glycolysis could be activated. However, we observed a decreased glycolysis from glucose with cyclodextrin treatment. In addition, the shift in the distribution of FBP metabolism cannot be explained by activation of signaling pathways. Therefore, although there could have been activation of some signaling pathways, such an activation likely does not account for our results. In addition, activation of apoptotic pathways also is not consistent with our results. We observed a decrease in glycolytic flux with cyclodextrin treatment which is inconsistent with an increase in cytoplasmic calcium and with inhibition of mitochondrial ATP production as would occur with apoptosis. Therefore, the best explanation for our results is that cyclodextrin treatment resulted in a redistribution of metabolic enzymes partly abolishing the separate membrane domains responsible for glycolysis and gluconeogenesis.

Physiological Function of Localized Metabolic Enzymes

Localized glycolytic enzymes may provide metabolic support for nearby ATP-consuming processes. For example, enzymes localized to F-actin may provide ATP for use by the contractile apparatus (in muscle cells) or for other F-actin-mediated processes such as cell motility (in non-muscle cells). Similarly, membrane-localized glycolytic enzymes may contribute to the production of ATP for use by ATP-consuming ion transport processes.

The function of localized gluconeogenic enzymes in vascular tissue in unknown. These enzymes could be part of a metabolite-scavenging pathway which converts intermediates that have "escaped" from the glycolytic pathway back to glucose. Alternatively, gluconeogenic enzymes in microvascular smooth muscle cells may represent a local glucose-recycling pathway which helps ensure a constant supply of glucose for the surrounding tissue. Gluconeogenesis does appear to be more pronounced in microvessels (relative to glycolysis) than in large conduit arteries, in agreement with this suggestion [Hardin and Roberts, 1995a; Lloyd and Hardin, 1999].

CONCLUSIONS

The most important finding of the present study is that glycolysis from glucose is inhibited by disruption of plasma membrane caveolae. These data suggest that membrane associated glycolysis occurs in association with caveolar domains of the plasma membrane, and that

disruption of caveolar organization disrupts pathway organization and function as well. Similar findings have been reported for cellular signal transduction pathways localized in caveolae. Thus, significant parallels between the organization of metabolism and the organization of signal transduction may exist.

Localization of glycolytic enzymes to caveolae may be relevant in some disease states, since the number and protein composition of caveolae reflect the phenotypic state of the cells. For example, expression of caveolin (a major protein component of caveolae) is decreased in cancer cells, and re-expression of caveolin inhibits tumor growth [Lee et al., 1998]. Similarly, cultured smooth muscle cells express few caveolae when in a synthetic (dedifferentiated) phenotype, and the numbers of caveolae increase when the cells redifferentiate [Thyberg et al., 1997]. One report suggests that smooth muscle cells of the synthetic phenotype do not exhibit compartmentation of glycolysis [Kemp et al., 1990]. Thus, glucose metabolism may be altered secondary to changes in caveolar organization in both cancer and atherosclerosis. The role of caveolae in metabolic organization should be further explored.

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