Influence of glycogen storage on vascular smooth muscle metabolism

TARA J. ALLEN AND CHRISTOPHER D. HARDIN
Department of Physiology, University of Missouri, Columbia, Missouri 65212

Allen, Tara J., and Christopher D. Hardin. Influence of glycogen storage on vascular smooth muscle metabolism. Am J Physiol Heart Circ Physiol 278: H1993–H2002, 2000.—The role of glycogen as an oxidative substrate for vascular smooth muscle (VSM) remains controversial. To elucidate the importance of glycogen as an oxidative substrate and the influence of glycogen flux on VSM substrate selection, we systematically altered glycogen levels and measured metabolism of glucose, acetate, and glycogen. Hog carotid arteries with glycogen contents ranging from 1 to 11 µmol/g were isometrically contracted in physiological salt solution containing 5 mM [1-13C]glucose and 1 mM [1,2-13C]acetate at 37°C for 6 h. [1-13C]glucose, [1,2-13C]acetate, and glycogen oxidation were simultaneously measured with the use of a 13C-labeled isotopomer analysis of glutamate. Although oxidation of glycogen increased with the glycogen content of the tissue, glycogen oxidation contributed only ~10% of the substrate oxidized by VSM. Whereas [1-13C]glucose flux, [3-13C]acetate production from [1-13C]glucose, and [1,2-13C]acetate oxidation were not regulated by glycogen content, [1-13C]glucose oxidation was significantly affected by the glycogen content of VSM. However, [1-13C]glucose remained the primary (~40–50%) contributor to substrate oxidation. Therefore, we conclude that glucose is the predominate substrate oxidized by VSM, and glycogen oxidation contributes minimally to substrate oxidation.

tricarboxylic acid cycle; glycolysis; glucose; acetate; nuclear magnetic resonance

EARLY STUDIES indicated that vascular smooth muscle (VSM) oxidized exclusively carbohydrate, as indicated by a respiratory quotient of 1 (18). However, these results may not be indicative of the true physiological state, because glucose was provided at supraphysiologic concentrations. A later study by Chace and Odessy (9) determined that glucose accounts for only 5% of oxygen consumption in the presence of (0.5 mM each) palmitate, β-hydroxybutyrate, leucine, isoleucine, and glutamine (9). Therefore, it was concluded that glucose was a minor oxidative substrate for VSM under physiological substrate conditions (9). However, in that study, substrate oxidation was measured by radioisotope techniques, which measure substrate oxidation indirectly by liberation of 14CO2 from 14C-labeled substrates. Although radioisotope analysis is a very sensitive technique, it suffers from difficulty in measuring the oxidation of several substrates simultaneously.

The low entry of pyruvate derived from glucose into the tricarboxylic acid (TCA) cycle (5% of total entry) (9) is consistent with the fact that VSM is known to be a highly glycolytic tissue with a large production of lactate, even under fully oxygenated conditions (27). It is thought that this high degree of lactate production serves to provide energy for membrane ion pumps, whereas oxidative metabolism provides the energy required for contractile activation and maintenance (22, 29). The role of oxidative metabolism to support the energy requirements of force production is supported by the fact that a linear relationship exists between oxygen consumption and force production in VSM (28).

Although oxidative metabolism provides 70% of the energy requirements of the cell (29), considerable controversy surrounds the question of which substrates are oxidized physiologically. It has been proposed that glycogen may be one of the substrates that supports oxidative metabolism, because it has been shown that glycogen phosphorylase activity increases in direct relation to the activation state of the muscle (4, 21). However, the importance of glycogen as an oxidative substrate and the role of glycogen in altering oxidative substrate selection remain unclear.

The physiological significance of glycogen as an oxidative substrate for VSM may be questionable on the basis of the reported values for glycogen content of VSM ranging from 3.9 µmol/g in the human peripheral artery to as high as 13.9 µmol/g in the cow mesenteric artery (27). The resting glycogen content of hog carotid artery has been reported by two independent labs to be 2.4 µmol glucosyl units/g (21) and 2.8 µmol glucosyl units/g (7). With the assumption of an ATP consumption rate of ~1 µmol ATP·g−1·min−1 for contracting VSM and complete oxidation of glycogen, this level of glycogen would be depleted in ~38 min in the absence of additional substrates and thus would not provide a prolonged energy source for oxidative metabolism. This rapid depletion of glycogen substrate reserves raises speculation regarding the role of glycogen as an important oxidative substrate for VSM. However, it has been shown that VSM glycogen stores can accumulate approximately fivefold higher glycogen contents on incubation in 5 mM glucose for 12–16 h at 37°C (13). In addition, it has been shown that the glycogen utilization is directly proportional to the tissue glycogen content (15), suggesting that glycogen may play a much larger role in VSM metabolism than previously thought.
In the current study, to more clearly define the role of glycogen in VSM metabolism, hog carotid arteries were allowed to synthesize variable amounts of glycogen before isometric contraction. This enabled the systematic analysis of VSM metabolism at predetermined levels of glycogen and evaluation of the importance of glycogen as an oxidative substrate and its influence on VSM substrate selection.

**MATERIALS AND METHODS**

**Tissue preparation.** Hog carotid arteries were obtained from the local abattoir within ~30 min of slaughter. Arteries were placed in cold (~5–10°C) physiological saline solution (PSS), pH 7.4, preequilibrated by bubbling with a gas mixture of 95% O2–5% CO2. PSS contained penicillin (303 mg/l) and streptomycin (100 mg/l) in addition to the following (in mM): 116 NaCl, 4.6 KCl, 1.16 KH2PO4, 25.3 NaHCO3, 2.5 CaCl2, and 1.16 MgSO4. On reaching the laboratory, the arteries were dissected free of loose fat, connective tissue, and adventitia in fresh PSS. During all experimental manipulations, the PSS was continually equilibrated with a gas mixture of 95% O2–5% CO2.

**Tissue selection and experimental groups.** Hog carotid arteries were selected on the basis of their uniform diameter along the length of the artery as observed by gross inspection. By controlling the diameter, each artery was stretched to approximately optimum length on the length-tension curve, and thus tension maintenance and energy expenditure were equivalent.

To elucidate the role of glycogen in oxidative metabolism, hog carotid arteries were incubated under conditions to give a wide range of glycogen concentrations. Before the glycogen synthesis incubations, the hog carotid arteries were incubated in PSS in the absence of exogenous substrate for 30 min. The arteries were then incubated in PSS containing 80 mM KCl for an additional 30 min, which has been shown to deplete endogenous glycogen stores to ~1 µmol/g wet wt (14). Hog carotid arteries were then incubated in PSS containing 5 mM glucose for 12–16 h at 37°C before isometric contraction, which resulted in glycogen concentrations ranging from 7 to 15 µmol glucosyl units glycogen/g wet wt of tissue (~1–3 µmol/g). This range was selected on the basis of the observation that hog carotid artery can synthesize at least 0.93 µmol glucosyl units of glycogen/g·h−1·h−1 in the first 6 h of incubation under these conditions (13). Therefore, arteries that did not synthesize at least 6 µmol/g of new glycogen were considered nonviable and were excluded, and only tissues with glycogen contents above 7 µmol/g (1 µmol/g limit dextrin and 6 µmol/g newly synthesized glycogen) were used in further analysis. In addition, a subset of arteries were not incubated to synthesize glycogen, which enabled the analysis of glycogen utilization and the role of glycogen in altering substrate selection at lower glycogen concentrations (mean ± SE; 1.1 ± 0.1 µmol/g).

**Isometric contraction of glycogen-repleted VSM.** After the replenishment of endogenous glycogen stores, three sections of each artery (~30 mg each) were removed, blotted dry, weighed, and frozen in liquid nitrogen for subsequent analysis of glycogen content (initial glycogen content). The remaining portion of each artery (~300 mg) was isometrically mounted and placed in a separate incubation chamber with 15 ml of PSS containing 5 mM [1-13C]glucose, 1 mM [1,2-13C]acetate, and 80 mM KCl for 6 h at 37°C. After 6 h, three sections from each artery (~30 mg each) were removed, blotted dry, weighed, and frozen in liquid nitrogen for subsequent analysis of glycogen content (final glycogen content). The remaining portion of the artery (~200 mg) was blotted dry, weighed, and frozen in liquid nitrogen for subsequent extraction and 13C nuclear magnetic resonance (NMR) analysis. Aliquots (1.5 and 4.0 ml) of the superfusate for each tissue were collected both before and after isometric contraction to assay for [1-13C]glucose flux, total lactate production, and 1H NMR spectroscopy.

**Isometric contraction of arteries not repleted with glycogen.** Arteries obtained from the abattoir were incubated in PSS in the absence of substrate for 30 min at 37°C. Three sections from each artery (~30 mg each) were removed, blotted dry, weighed, and rapidly frozen in liquid nitrogen for subsequent analysis of glycogen content (initial glycogen content). An additional three sections from each artery (~30 mg each) were placed in separate incubation chambers in PSS containing 5 mM glucose for 6 h. Tissues that did not synthesize at least 6 µmol/g glucosyl units of glycogen during this incubation were excluded from further analysis. This was done to ensure that tissues used for subsequent 13C NMR analysis were viable and therefore appropriate representatives for measurement of substrate metabolism of VSM with low levels of glycogen.

The remaining portion of each artery (~300 mg) was isometrically mounted and placed in PSS containing 5 mM [1-13C]glucose, 1 mM [1,2-13C]acetate, and 80 mM KCl for 6 h at 37°C and, after the incubation, was treated as described in the isometric contraction of glycogen-repleted VSM.

**Measurement of glycogen flux.** The glycogen content of the tissues was determined with the use of an enzyme-linked assay (21). Triplicate measurements were taken for each artery, and the glycogen content for each tissue was expressed as the mean of the three measurements. Two criteria were established before initiation of the experiments, either of which excluded arteries from further analysis. Tissues were only included in further analysis if their mean initial glycogen content (on the basis of triplicate measurements) was at or above 7 µmol/g of glycogen and the individual measurements of glycogen did not deviate more than 50% from the mean glycogen content for that tissue (of 518 total arteries examined, 235 met both inclusion criteria). Glycogen flux was calculated as the difference between the glycogen content immediately after the glycogen synthesis incubation (initial glycogen content) and glycogen content after the 6-h isometric contraction (final glycogen content).

**Measurement of [1-13C]glucose flux and total lactate production.** Aliquots of the starting superfusate and the superfusate after isometric contraction from each tissue were assayed to determine [1-13C]glucose flux and total lactate production with the use of enzyme-linked assays as described by Lowry and Passonneau (19). The absorbance was measured at 340 nm with the use of a Hitachi U2000 dual beam spectrophotometer. The metabolites [1-13C]glucose and lactate concentrations (in mM) were calculated on the basis of the least squares regression line for standard samples of known concentrations. [1-13C]glucose flux (expressed as µmol/g) was determined by subtracting the concentration of [1-13C]glucose remaining in the superfusate after isometric contraction from the starting superfusate concentration determined by enzymatic assay (~5 mM) and normalizing for the mass of tissue in the incubation chamber during the 6-h isometric contraction. Total lactate production was calculated as the lactate concentration in the superfusate sample after the 6-h isometric contraction normalized for the mass of tissue present during the isometric contraction.

**Perchloric acid-methanol extracts.** To determine the effect of increasing glycogen content on substrate oxidation in VSM, arteries for extraction were grouped based on the initial...
glycogen load of the tissues. The arbitrary groupings began at a glycogen range of 7–8 µmol/g and increased by 1 µmol/g increments up to 15 µmol/g glycogen. Tissues that had similar glycogen contents (within 1 µmol/g range) were combined in order by experimental date and tissue number until 1.5–2.0 g of total tissue mass were reached for each extract.

Frozen tissues were pulverized in liquid nitrogen, extracted in 15 ml of 8% perchloric acid (PCA)-40% methanol, and placed in the freezer (−20°C) for at least 1 h. The pulverized tissue was homogenized with a Polytron tissue homogenizer (Brinkmann; 5× 30 s), and the homogenate was centrifuged at 10,000 g for 30 min at 4°C. The supernatant was neutralized with KOH and HCl, after which point it was placed in the freezer to cool and then centrifuged at 2,800 g for 15 min. The supernatant was transferred to a new centrifuge tube and lyophilized in a Speed Vac (Savant Instruments) for 13C NMR analysis.

Measurement of relative substrate oxidation 13C NMR spectroscopy. The lyophilized powder from the frozen PCA-methanol extracts was resuspended in 1 ml of 99% D2O with 25 mM 3-(trimethylsilyl)-1-propane-sulfonic acid (TMSPS) and 5 mM EDTA. The resuspension was centrifuged for 15 min, the supernatant was filtered (0.45-µm syringe filter), and 650 µl of filtrate were transferred to a 5-mm NMR tube. A Bruker DRX 300 spectrometer was used to perform 13C NMR analysis with the following acquisition parameters: 20,480 scans with 64 dummy scans, sweep width 19,960 Hz, 30° pulse angle at 75.477 MHz, and a 1-s predelay with broad-band proton decoupling. The 32,768 points acquired were processed with 1-Hz line broadening before Fourier transform. The TMSPS peak at 0 parts per million (ppm) was used as a chemical shift and peak intensity reference. The data were processed with the use of Bruker software for peak intensity determination. The relative oxidation of [1-13C]glucose, [1-13C]acetate, and unlabeled substrates was calculated by use of 13C-isotopomer analysis of glutamate.

13C-isotopomer analysis of glutamate. 13C-isotopomer analysis of glutamate has been used extensively in the heart to characterize the substrates preferentially oxidized by cardiac muscle (24). In addition, we have previously used 13C-isotopomer analysis to examine the utilization of exogenous substrates by noncardiac smooth muscle (2). The advantage of this technique is that it allows for direct measurement of the oxidation of several substrates simultaneously (for example [1-13C]glucose, [1-12C]acetate, and glycogen). Briefly, the two 13C-labeled substrates and unlabeled endogenous substrates will result in three patterns of labeling of acetyl-CoA ([1,2-13C]acetoy-CoA, [2-13C]acetoy-CoA, and unlabeled acetyl-CoA). The contribution of [1-13C]acetoy-CoA, [2-13C]acetoy-CoA, and acetyl-CoA to the labeling pattern of glutamate is a measure of the relative entry into the TCA cycle of each of the substrates producing the differentially labeled acetyl-CoA pools ([1,2-13C]acetate, [1-13C]glucose, and unlabeled substrates, respectively). The calculation involves the quantitation of the relative peak intensities of the third (−32.5 ppm) and fourth (−37 ppm) carbon resonances of glutamate. Each substrate has a different 13C-labeling pattern that gives rise to predictable splitting patterns in the spectrum of the fourth carbon of glutamate, which are specific for each substrate. The 13C NMR spectrum of the fourth carbon of glutamate is deconvoluted into the respective peaks arising from the oxidation of the 13C-labeled substrates. Because the individual peaks are characteristic of the substrates based on the position of the 13C-labeled carbon in each substrate, these peaks can be used to calculate the extent to which each substrate was oxidized. For example, the intensity of the peaks that are characteristic of [1-13C]glucose oxidation (singlet and doublet) can be used to determine the extent of [1-13C]glucose oxidation. Calculation of substrate oxidation with the use of 13C-isotopomer analysis of glutamate is described in detail elsewhere (see Refs. 2 and 24).

In addition, 13C-isotopomer analysis allows calculation of anaplerosis or input of intermediates into the TCA cycle via pathways other than citrate synthase. Anaplerosis is calculated by determining the ratio of the overall intensity of the third carbon of glutamate to the fourth carbon of glutamate. If there is no anaplerosis, the ratio should be 1, because the 13C resonances of the third and fourth carbons of glutamate should evolve simultaneously. However, if anaplerosis provides a significant source of carbon to the TCA cycle, it will dilute the 13C signal from the third carbon of glutamate relative to the fourth carbon of glutamate.

Validation of 1H NMR spectroscopy to measure percentage of lactate derived from [1-13C]glucose and glycogen. For the validation of 1H NMR analysis for determining the percentage of total lactate production derived specifically from [1-13C]glucose ([3-13C]lactate) and unlabeled glycogen ([3-12C]lactate) in the superfusate samples, it was necessary to run standards containing known percentages of [3-13C]lactate and [3-12C]lactate. [3-12C]Acetate stock solution with 99% purity and 1.1% natural abundance for [3-13C]lactate. The lyophilized powder was resuspended in 1.5 ml of 99% D2O with 25 mM TMSPS, and 650 µl were transferred to a 5-mm NMR tube. A Bruker DRX 500 spectrometer was used to obtain 1H NMR spectra under fully relaxed conditions (5.5 s total interpulse delay, 90° pulse) with a 6-kHz sweep width stored in 32-K blocks. The TMSPS peak at 0 ppm was used as a chemical shift and peak intensity reference. Lactate fractional enrichment was determined by calculating the ratio of the intensity of the 1H and 13C-labeled satellites (due to [3-13C]lactate) relative to the total resonance intensity, which includes both [3-13C]lactate and [3-12C]lactate resonances. The calculated percentages of [3-12C]lactate and [3-13C]lactate by 1H NMR spectroscopy for each sample were compared with the actual percentage added to each sample.

Measurement of lactate production specifically from [1-13C]glucose and glycogen with the use of 1H NMR spectroscopy. Because the two substrates that produce lactate, [1-13C]glucose and glycogen, were differentially labeled, they resulted in two different populations of lactate ([3-13C]lactate from [1-13C]glucose and [3-12C]lactate from unlabeled glycogen), which were distinguished and quantified by 1H NMR spectroscopy.

Frozen aliquots (4.0 ml) of the superfusates were lyophilized in a Speed Vac (Savant Instruments) and resuspended in 1.5 ml of 99% D2O with 25 mM TMSPS, and 650 µl were transferred to a 5-mm NMR tube. A Bruker DRX 500 spectrometer was used as described above for the standard samples. [3-13C]lactate fractional enrichment (from [1-13C]glucose) was determined by integrating the area of the 1H- and 13C-satellites (due to [3-13C]lactate) and dividing by the total resonance area of the third carbon of lactate. Because the maximum 13C fractional enrichment at this carbon is 50% (due to the production of [3-13C]lactate and 1 molecule from [3-13C]lactate from [1-13C]glucose), the calculated lactate production from glucose is doubled. The remaining contribution to [12C]lactate must therefore be derived from unlabeled glycogen.
The absolute flux of lactate derived from [1-13C]glucose and glycogen can be determined by using the percentage of total lactate derived from either [1-13C]glucose or glycogen (1H NMR spectroscopy) and by knowing the total amount of lactate produced by that particular tissue (enzyme-linked assay). The absolute flux of lactate for [1-13C]glucose and of glycogen is expressed in micromoles per gram.

Statistical methods. Statistics and curve fits were calculated with the use of Microsoft Excel (for the personal computer) for least squares regression analysis and a two-tailed Student’s t-test for two samples, assuming unequal variance. P values of ≤ 0.05 were accepted as indicating significant differences in the means.

RESULTS

The role of glycogen in VSM metabolism and the interaction between the metabolism of glycogen and other physiological substrates were assessed in isometrically contracted hog carotid artery segments containing varied glycogen contents (1–15 µmol/g). Because the arteries were incubated individually for the isometric contractions, we were able to measure several parameters (including [1-13C]glucose flux, total lactate production, lactate production specifically from [1-13C]glucose and glycogen) for each artery and relate these to the particular glycogen content for that tissue. In addition, arteries with similar glycogen contents were pooled together in extracts to determine the contribution of [1-13C]glucose, [1,2-13C]acetate, and unlabeled substrates to the TCA cycle as oxidative substrates. The analysis of the multiple fates of the substrates simultaneously enabled detailed analysis of the role of glycogen levels on VSM metabolism.

When hog carotid artery segments were isometrically mounted and contracted in PSS containing 5 mM [1-13C]glucose, 1 mM [1,2-13C]acetate, and 80 mM KCl at 37°C, the glycogen flux was shown to increase linearly in proportion to the glycogen content of the tissue before contraction ($r^2 = 0.8874, n = 235$; Fig. 1). In addition, the slope (0.7812) of the linear trend line provides an estimate of the percentage of glycogen utilized during the 6-h isometric contraction. This indicates that regardless of the precontracted glycogen content, 78% of glycogen was utilized during the 6-h isometric contraction in the presence of the exogenous substrates 5 mM [1-13C]glucose and 1 mM [1,2-13C]acetate.

The pathways for metabolism of glucose and glycogen are traditionally thought to be identical from glucose 6-phosphate to pyruvate; therefore, we examined whether the increase in glycogen flux concomitant with an increased glycogen content would decrease [1-13C]glucose utilization, as would be expected for two inputs to a single regulated pathway. However, [1-13C]glucose flux was not a function of glycogen content ($r^2 = 0.0065$; Fig. 2). Therefore, with increases in glycogen content of VSM, there is an increase in glycogen flux but no measurable effect on [1-13C]glucose flux during contraction.

The data in Figs. 1 and 2 depict the total utilization of either [1-13C]glucose or glycogen regardless of the fate of their metabolism. However, it is possible to distinguish between the two possible fates (lactate production and oxidation) of these carbohydrate substrates by selecting substrates that can be differentiated on the basis of their 13C label. For example, because the glucose provided was labeled with 13C at the first carbon, it would produce lactate with the 13C label incorporated into the third carbon ([3-13C]lactate). However, the glycogen was synthesized from glucosyl units that did not contain a 13C label; therefore, metabolism of glycogen to lactate formed [3-13C]lactate. These two differentially labeled pools of lactate were measured with the use of 1H NMR spectroscopy to determine the relative percentages of lactate derived from both [1-13C]glucose and glycogen. Because the 1H NMR spectra were obtained under fully relaxed conditions, the intensities of the [3-13C]lactate and [3-13C]lactate peaks were directly proportional to their concentration in the superfusate. This is verified by the data in Fig. 3 showing the linear relationship ($r^2 = 0.9976$) between the percentage of [3-13C]lactate measured in the stan-
consistent with the data showing that [1-13C]glucose content of the tissue (Fig. 4) did not change with increasing glycogen isometric contraction. The production of [3-13C]lactate function of the glycogen content of the tissue before derived specifically from [1-13C]glucose (solid squares). Measurement of [1-13C]glucose flux and total lactate production from both [1-13C]glucose and glycogen as a function of the glycogen content of tissue. Shown in Fig. 4 is the magnitude of lactate production from both [1-13C]glucose and glycogen as a function of the glycogen content of the tissue before isometric contraction. The production of [3-13C]lactate derived specifically from [1-13C]glucose (solid squares in Fig. 4) did not change with increasing glycogen content of the tissue \( (r^2 = 0.0263) \). These data are consistent with the data showing that [1-13C]glucose flux does not change with increases in glycogen content (Fig. 2). However, there was a substantial increase in glycogen conversion to [3-12C]lactate with increases in precontraction glycogen content \( (r^2 = 0.6102) \) (Fig. 4). Figure 5 shows total lactate production (derived from both [1-13C]glucose and glycogen) plotted as a function of precontracted glycogen content. Although not significant, there is a slight increase in total lactate production as the glycogen content increases, which is due to increased conversion of glycogen to [3-12C]lactate.

Although [1-13C]glucose flux (Fig. 2) and [1-13C]glucose conversion to [3-12C]lactate (Fig. 4) were unchanged as the glycogen content of VSM was increased, it is possible that the increase in glycogen content affects the substrates oxidized by the TCA cycle. To examine this, tissues were combined for extraction and subsequent 13C NMR analysis on the basis of their glycogen content after the 12- to 16-h glycogen synthesis incubation. For each extract, the oxidation of [1-13C]glucose, [1,2-13C]acetate, and unlabeled substrates was determined and related to the mean glycogen content for the tissues included in the extract. As the precontraction glycogen content increased, there was a decrease in the labeled substrate oxidation. In these experiments, because no unlabeled exogenous substrate was provided, the unlabeled substrates in these experiments could include glycogen, endogenous fatty acids, and amino acids. However, because the endogenous glycogen substrate pool was repleted before incubation, it is likely that this primarily accounts for the unlabeled substrate oxidation.

Summarized in Table 1 are the mean values for the percent oxidation of [1-13C]glucose, [1,2-13C]acetate, and unlabeled substrates of hog carotid artery extracts grouped by their glycogen content. The values for substrate oxidation in Table 1 are the average values for the extracts for that particular glycogen range. The

![Fig. 3. Validation of 1H nuclear magnetic resonance (NMR) technique used to measure percentage of total lactate production derived specifically from [1-13C]glucose and glycogen. Known percentages of [3-13C]lactate and [3-12C]lactate were combined (3 mM lactate total, 2.5 ml total volume) and analyzed by 1H NMR. Shown is a plot of actual [3-13C]lactate added to each sample vs. measured percentage of [3-13C]lactate by 1H NMR analysis. A significant correlation \( (r^2 = 0.9976) \) exists between actual and measured values for percentage of [3-13C]lactate and [3-12C]lactate in superfusate samples. Values are expressed as means ± SE of 3 measurements.](image)

![Fig. 4. Lactate production derived specifically from [1-13C]glucose ([3-13C]lactate) and unlabeled glycogen ([3-12C]lactate) plotted as a function of glycogen content of tissue. Percentages of glycogen content of tissue derived from [1-13C]glucose (■) and glycogen (▲), as determined by 1H NMR, was used to calculate absolute flux of lactate derived specifically from [1-13C]glucose and glycogen by use of measurement of total lactate production measured by enzymatic assay. Although lactate production from glycogen ([3-12C]lactate) did not change with increases in glycogen content \( (r^2 = 0.6102) \), there was a significant increase in lactate derived from glycogen ([3-13C]lactate) with increases in glycogen content \( (r^2 = 0.0263) \). These data are consistent with the data showing that [1-13C]glucose flux does not change with increases in glycogen content (Fig. 2). However, there was a substantial increase in glycogen conversion to [3-12C]lactate with increases in precontraction glycogen content \( (r^2 = 0.6102) \) (Fig. 4).](image)

![Fig. 5. Total lactate production (derived from both [1-13C]glucose and glycogen) is plotted as a function of glycogen content of VSM before isometric contraction \( (n = 235) \). Total lactate production was measured by enzymatic assay of the superfusate after 6-h isometric contraction. Least squares regression analysis reveals no correlation between total lactate production and precontraction glycogen content; however, there is a slight increase in total lactate production as glycogen content of tissue increases.](image)
The reduction in [1-13C]glucose oxidation was only observed in the 7 to 8 µmol/g glycogen range (Table 1). When analyzed by glycogen content level in increments of 1 µmol/g loads, the [1-13C]glucose oxidation was reduced in every group with a glycogen content above 7 µmol/g compared with the lowest glycogen content examined (1.14 ± 0.08 µmol/g). However, a significant reduction in [1-13C]glucose oxidation was only observed in the 7 to 8 µmol/g glycogen range compared with the 0.3 to 3 µmol/g glycogen range (Table 1). The reduction in [1-13C]glucose oxidation occurred concomitant with an increase in unlabeled substrate oxidation with increases in glycogen content. However, as with [1-13C]glucose oxidation, unlabeled substrate oxidation was significantly increased (P ≤ 0.05) only in the 7 to 8 µmol/g glycogen range compared with the 0.3 to 3 µmol/g glycogen range (Table 1). It might be expected that an increase in unlabeled substrate oxidation concomitant to increases in glycogen content might attenuate the oxidation of [1,2-13C]acetate. However, [1,2-13C]acetate oxidation was remarkably constant despite glycogen content ranging from 1–11 µmol/g (Table 1).

Figure 6 shows the small decrease in [1-13C]glucose oxidation concomitant with an increase in unlabeled substrate oxidation (glycogen, endogenous lipids, and/or amino acids). However, a significant trend did not exist between the glycogen content of the tissue and [1-13C]glucose, [1,2-13C]acetate, and unlabeled substrates as a function of glycogen content of vascular smooth muscles, measured by 13C-isotopomer analysis of glutamate from extracts of hog carotid arteries; n = no. of measurements. Significance is considered at P = 0.05. *Significant compared with a range of 0.3–3-µmol/g.

Values are means ± SE for anaplerosis (percent input, scaled to citrate synthase flux) and substrate oxidation of [1-13C]glucose, [1,2-13C]acetate, and unlabeled substrates as a function of glycogen content of vascular smooth muscles, measured by 13C-isotopomer analysis of glutamate from extracts of hog carotid arteries; n = no. of measurements. Significance is considered at P = 0.05. *Significant compared with a range of 0.3–3-µmol/g.

Table 1. Values for anaplerosis and substrate oxidation

<table>
<thead>
<tr>
<th>Glycogen Content, µmol/g wet wt</th>
<th>[1-13C]glucose</th>
<th>[1,2-13C]acetate</th>
<th>Unlabeled</th>
<th>Anaplerosis, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low glycogen: range 0.3–3, mean 1.14 ± 0.08 (n = 27)</td>
<td>50.48 ± 0.85 (n = 3)</td>
<td>27.59 ± 3.11 (n = 3)</td>
<td>21.93 ± 2.41 (n = 3)</td>
<td>31.6 ± 3.49 (n = 3)</td>
</tr>
<tr>
<td>High glycogen: range 7–12, mean 9.16 ± 0.14 (n = 105)</td>
<td>40.6 ± 1.7 (n = 12)*</td>
<td>29.8 ± 1.8 (n = 12)</td>
<td>29.6 ± 3.2 (n = 12)</td>
<td>36.3 ± 2.4 (n = 12)</td>
</tr>
<tr>
<td>Glycogen: range 7–8, mean 7.40 ± 0.06 (n = 24)</td>
<td>36.16 ± 1.25 (n = 3)*</td>
<td>25.66 ± 1.20 (n = 3)</td>
<td>38.17 ± 2.18 (n = 3)*</td>
<td>27.93 ± 2.30 (n = 3)</td>
</tr>
<tr>
<td>Glycogen: range 8–9, mean 8.55 ± 0.04 (n = 33)</td>
<td>44.39 ± 3.95 (n = 4)</td>
<td>33.54 ± 4.64 (n = 4)</td>
<td>22.08 ± 7.77 (n = 4)</td>
<td>42.66 ± 4.92 (n = 4)</td>
</tr>
<tr>
<td>Glycogen: range 9–10, mean 9.45 ± 0.07 (n = 19)</td>
<td>40.74 ± 2.39 (n = 2)</td>
<td>32.81 ± 2.99 (n = 2)</td>
<td>26.45 ± 5.38 (n = 2)</td>
<td>36.46 ± 0.80 (n = 2)</td>
</tr>
<tr>
<td>Glycogen: range 10–11, mean 10.53 ± 0.09 (n = 10)</td>
<td>40.14 (n = 1)</td>
<td>25.78 (n = 1)</td>
<td>34.08 (n = 1)</td>
<td>33.06 (n = 1)</td>
</tr>
<tr>
<td>Glycogen: range 11–12, mean 11.42 ± 0.06 (n = 19)</td>
<td>39.92 ± 4.25 (n = 2)</td>
<td>27.48 ± 0.90 (n = 2)</td>
<td>32.60 ± 5.15 (n = 2)</td>
<td>37.47 ± 3.94 (n = 2)</td>
</tr>
</tbody>
</table>

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In addition to determining the relative input of substrates into the TCA cycle at the level of acetyl-CoA, 13C-isotopomer analysis provides a means to calculate anaplerosis or the entry of substrates into the TCA cycle other than through citrate synthase. If there is no anaplerosis, then the C-3 and C-4 regions of the 13C spectra of glutamate should evolve simultaneously and to the same extent. However, if anaplerosis occurs, it will dilute incorporation of the 13C label into C-3 peaks.
Fig. 7. Percent oxidation of [1-13C]glucose and unlabeled substrates as determined by 13C-isotopomer analysis of glutamate. Data are taken from Fig. 6. As oxidation of unlabeled substrates increases, there is a significant decrease in [1-13C]glucose oxidation ($r^2 = 0.7227$).

of glutamate versus C-4 peaks of glutamate. Examples of possible anaplerotic substrates include pyruvate, propionate, aspartate, glutamate, and the branched-chain amino acids. Studies in VSM have indicated that pyruvate carboxylation may be the primary anaplerotic pathway (5), although it has not been determined whether anaplerosis plays a significant role in VSM metabolism. The average value for anaplerosis was calculated to be 35 ± 2% of the flux through citrate synthase ($n = 15$) and did not significantly change with the glycogen content of the tissue (Table 1). Therefore, glycogen is not likely to be a source of anaplerotic substrates in VSM.

**DISCUSSION**

The uncertainty of the role of glycogen in VSM metabolism may result from the fact that the glycogen content of VSM in previous studies (3, 18, 26) is low and variable. In fact, the glycogen content of VSM has been shown to range from 3.9 µmol/g in the human peripheral artery to as high as 13.9 µmol/g in the cow mesenteric artery (27). The wide range and many times low values of glycogen content reported in the literature raise questions as to the physiological importance of this endogenous substrate. However, our lab has shown that hog carotid artery is capable of synthesizing much higher levels of glycogen than previously reported (13). By analyzing the glycogen utilization in the hog carotid arterial VSM with predetermined levels of glycogen, the current study was aimed at clarifying the role of glycogen in VSM metabolism.

The importance of glycogen as an oxidative substrate and the role of glycogen in altering oxidative substrate selection were studied with 13C-isotopomer analysis of glutamate. 13C-isotopomer analysis of glutamate enables simultaneous analysis of oxidation of three different classes of substrates. Using 13C-isotopomer analysis in conjunction with enzymatic methods allows detailed analysis of the role of glycogen in regulating the utilization of exogenous [1-13C]glucose and [1,2-13C]acetate.

Regulation of glycogen flux but not [1-13C]glucose flux by glycogen content. Regulation of glycogenolysis by glycogen content in hog carotid arterial VSM has been examined previously in our lab (15). The previous data in addition to our current data (Fig. 1) indicate that glycogenolysis increases in proportion to the glycogen content of the tissue. Regardless of the precontraction glycogen content of VSM, 28% of the glycogen remained after the 6-h isometric contraction in the presence of the exogenous substrates 5 mM [1-13C]glucose and 1 mM [1,2-13C]acetate on the basis of the slope of the regression line (Fig. 1). This value is consistent with that predicted by our previous study (15). We have previously discussed possible processes involved in the regulation of glycogenolysis by glycogen content (15), but briefly, glycogen degradation may release phosphorylase from the glycogen macromolecule, uncoupling the actions of phosphorylase b kinase and thereby diminish glycogen breakdown.

It might be expected that, because glycogenolysis and lactate derived specifically from glycogen increase in proportion to tissue glycogen content, glycogen flux would inhibit [1-13C]glucose flux. This would be consistent with classical theories of carbohydrate metabolism regulation in which increases in glucose 6-phosphate, an intermediate in glucose metabolism, inhibits hexokinase and thus decreases [1-13C]glucose flux. In addition, increased glycogen flux could elevate levels of ATP and citrate, both of which inhibit phosphofructokinase. However, as the glycogen content of the tissue increased, there was no change in [1-13C]glucose flux (Fig. 2).

The inability of glycogen content to regulate [1-13C]glucose utilization may be a function of metabolic compartmentation within the VSM cell. Although the catabolism of glucose and glycogen share nine common enzymatic steps, it is possible that they utilize spatially separate enzymatic pathways (12, 22). This organization would support the observed inability of enhanced glycogen flux to regulate the glycolytic pathway of glucose. This also would be consistent with the thought that these two carbohydrate sources serve different functions within the cell. It has been demonstrated that glucose via aerobic glycolysis is preferentially used to support the Na\(^+\)-K\(^+\) pump in VSM (8, 16, 17, 20). This could result from a coupling between the ADP produced due to Na\(^+\)-K\(^+\) pump activity and glycolytic activity (29). It has been shown that ATPase-driven ion transport is increased after addition of glucose, an effect abolished by permeabilization of the cells. It was concluded that glycolysis localized to the cell membrane may serve to buffer local ADP levels and thus relieve its inhibition of ATPase ion transport (20).

Aerobic lactate production from [1-13C]glucose and glycogen. It has been shown that VSM has a high lactate production even under fully oxygenated conditions, and that this lactate is preferentially derived from glucose (22, 29). This idea is consistent with the fact that 77.3 ± 9.7% ($n = 235$) of [1-13C]glucose flux in
the current study was attributable to aerobic lactate production. In fact, lactate production from [1-13C]glucose accounted for ~80% of total lactate production within the VSM cell. Whereas the contribution of glycogen to lactate production ([3-13C]lactate) increases with increases in glycogen content of VSM, lactate production derived specifically from [1-13C]glucose ([3-13C]lactate) is remarkably constant despite a greater than 10-fold change in glycogen content (Fig. 3). This suggests that the glycolytic pathways for glucose and glycogen exist as separate entities within the VSM cell. This is consistent with previous work showing that the intermediates of glycolysis derived from glycogen and glucose do not mix in hog carotid artery (12). This is further supported by evidence indicating that the glycolytic intermediates do not freely diffuse within the cytoplasm of the VSM cell as indicated by their inability to be detected in bathing solution after permeabilization of the VSM with dextran sulfate (11). Therefore, the enzymatic pathways for glycolysis of both glucose and glycogen may be structurally organized in such a way that restricts regulation between the two pathways. However, this is not to suggest that lactate production from glucose is unregulated. A study by Barron et al. (5) showed that addition of octanoate (0.5 mM) to the incubation media inhibited lactate production derived from glucose by 64%. Whereas the regulatory mechanisms involved in octanoate induced inhibition of aerobic glycolysis of glucose is uncertain, it is likely that the inhibition occurred as a result of the increase in oxygen consumption (30% increase) due to oxidation of octanoate (5).

Substrate oxidation by VSM. Although VSM displays a high lactate production even under fully oxygenated conditions, VSM produces 70% of its energy via oxidative phosphorylation (29). The identity of the substrates preferentially oxidized by VSM has remained elusive on the basis of the shortcomings of the previous techniques used to assess substrate oxidation, including radioisotope techniques. Radioisotope techniques, although very sensitive, suffer from the disadvantage that they measure oxidation of single 14C-labeled substrate indirectly by measuring the liberation of 14CO2 derived from the 14C-labeled substrate. Although previous studies using radioisotope techniques have provided examples where substrates can be primarily oxidized, they have not determined the substrates preferentially oxidized by VSM in vivo. A distinct advantage of 13C-isotopomer analysis is that it allows for the direct measurement of the entry of multiple substrates into the TCA cycle simultaneously at the level of acetyl-CoA. Therefore, 13C-isotopomer analysis of glutamate can be used to measure the oxidation of three pools of substrates simultaneously and determine the importance of glycogen as an oxidative substrate and the role of glycogen in altering oxidative substrate selection. To determine the effect of glycogen content on substrate oxidation by the TCA cycle, 13C-isotopomer analysis of glutamate was performed on tissues with predetermined levels of glycogen.

Regulation of [1-13C]glucose oxidation by glycogen content. Oxidation of [1-13C]glucose was determined to comprise ~40–50% of the total substrates entering the TCA cycle, regardless of the glycogen content of the tissue (Fig. 6). This value is consistent with the level of [1-13C]glucose oxidation (~30–60%) previously reported by our lab for contracted VSM in the presence of exogenous acetate ranging from 0 to 5 mM (2). This indicates that although [1-13C]glucose oxidation is modestly affected by glycogen content of VSM, ranging from 1 to 11 µmol/g, and exhibits little change under quite variable substrate conditions, [1-13C]glucose is the primary substrate oxidized by hog carotid arterial VSM.

The primary oxidation of [1-13C]glucose is in direct contrast to what was previously thought concerning the oxidation of glucose. It was assumed that because glucose was primarily converted to lactate, it contributed minimally to oxygen consumption. However, our data suggest that while 77% of [1-13C]glucose is converted to lactate and 22% is oxidized by the TCA cycle, the 22% of [1-13C]glucose that is oxidized accounts for ~40–50% of all the substrates oxidized by the TCA cycle. Although the oxidation of [1-13C]glucose is attenuated with the increase in glycogen content and thus unlabeled substrate oxidation, [1-13C]glucose remains the predominant substrate oxidized by hog carotid arterial VSM, even in the presence of an alternate exogenous oxidative substrate in the form of 1 mM [1,2-13C]acetate (Fig. 7). Even at the highest glycogen content examined by 13C-isotopomer analysis of glutamate (glycogen range 11–12 µmol/g; mean ± SE, 11.4 ± 5.6 µmol/g), [1-13C]glucose comprised ~40% of all the substrates oxidized (Table 1). This indicates that under the substrate conditions of this study, [1-13C]glucose is the primary substrate utilized for both aerobic lactate production and oxidative phosphorylation and, in the absence of an alternate exogenous oxidative substrate, [1-13C]glucose is the primary substrate oxidized by hog arterial VSM, even in the presence of an alternate exogenous substrate (glycogen, lipid, and/or amino acids) for times of reduced exogenous substrate availability.

These data suggest that unlike aerobic lactate production from [1-13C]glucose, which does not display regulation by glycogen, the entry of pyruvate into the TCA cycle for oxidative phosphorylation is regulated by glycogen. Therefore, the regulation of glucose oxidation by glycogen must occur at the entry of pyruvate into the mitochondrial matrix. Although there is no direct experimental evidence to support this conclusion, it seems likely, based on the finding that with increased glycogen content, [1-13C]glucose flux and [1-13C]glucose conversion to lactate do not change, whereas [1-13C]glucose oxidation decreases (Figs. 4 and 6).

decreased. One possible explanation for this discrepancy could be an increase in TCA cycle turnover and thus a decrease in overall rate of input of substrates into the TCA cycle. A simple increase in absolute TCA cycle flux concurrent with the absolute entry of acetyl-CoA from glucose remaining constant would result in a decrease in the percent entry of glucose into the TCA cycle, which is the parameter measured in our experiments. However, TCA cycle turnover did not increase with increases in precontraction glycogen content (data not shown; analysis on the basis of the calculation of oxygen consumption discussed below). Another possible explanation for the decrease in [1-13C]glucose oxidation in the absence of changes in [1-13C]glucose flux or [3-13C]acetate production could be that the decreased oxidation of [1-13C]glucose increased the flux of [1-13C]glucose through other metabolic pathways like glycogen synthesis and/or the pentose phosphate pathway. It is likely that an increased proportion of glucose was shunted through the pentose phosphate pathway (25), because that has been shown to account for ~15% of glucose uptake, and glycogen synthesis does not occur in pig carotid artery during contraction (12).

Independence of acetate oxidation from glycogen content. Despite large alterations in glycogen content (1–11 µmol/g), [1,2-13C]acetate utilization was remarkably constant (Fig. 6). [1,2-13C]Acetate oxidation provided ~30% of the total substrate entry into the TCA cycle at the level of acetyl-CoA (Table 1, Fig. 6). The exogenous acetate concentration in vivo is near 0.1 mM in normal human subjects (10) and can approach ~1 mM in diabetic subjects (1). However, total fatty acid levels are ~0.5 mM in normal human individuals (30). The concentration of [1,2-13C]acetate used in the current study (1 mM) was chosen to mimic short-chain fatty acid levels (0.5 mM) in vivo (30). However, it is possible that this concentration of [1,2-13C]acetate (1 mM) resulted in underestimation of unlabeled substrate oxidation in the current study due to the greater ease at which acetate is utilized compared with the long-chain fatty acids in the plasma.

Unlabeled substrate oxidation—glycogen oxidation. Metabolized unlabeled substrates could include endogenous glycogen, lipids, and/or amino acids because no other unlabeled endogenous substrates were provided. Oxidation of these substrates cannot be distinguished from each other in the current study, and it is possible that all may contribute to unlabeled substrate oxidation. However, it is possible to estimate the contribution of glycogen to the unlabeled substrate oxidation by comparing the percentage of unlabeled substrate oxidation between the extracts with the lowest glycogen content (mean ± SE, 1.1 ± 0.1 µmol/g) and the highest glycogen content (mean ± SE, 11.4 ± 0.1 µmol/g). Measurement of glycogen flux of the tissues with the lowest glycogen content indicates that glycogen synthesis occurs rather than glycogen degradation; therefore, glycogen could not be a substrate for oxidative metabolism at this glycogen level. Unlabeled substrate oxidation increases from 21.9 ± 2.4% (glycogen content 1.1 ± 0.1 µmol/g) to 32.6 ± 5.2% (glycogen content 11.4 ± 0.1 µmol/g) (Table 1), and the difference between these measures (~11%) is an estimate of the contribution of glycogen to substrate oxidation. The remainder of the unlabeled substrate oxidation (~20%) can be attributed to the utilization of either endogenous lipid and/or amino acids. This raises the question of the physiological significance of glycogen to VSM metabolism in vivo. Therefore, it may be that glycogen is predominately utilized for a substrate in times of low exogenous substrate availability and thus cannot be accurately assessed in the current study.

Oxygen consumption. The average rate of oxygen consumption can be calculated from the entry of [1-13C]glucose, [1,2-13C]acetate, and unlabeled substrates into the TCA cycle and the measures of total substrate utilization and lactate production from both [1-13C]glucose and glycogen. Total TCA cycle flux was calculated to be 81 ± 16 nmol·g⁻¹·min⁻¹ (n = 14). Total TCA cycle flux is a function of the TCA cycle flux due to oxidation of [1-13C]glucose, [1,2-13C]acetate, and unlabeled glycogen. We can determine the contribution of [1-13C]glucose, [1,2-13C]acetate, and unlabeled glycogen to total TCA cycle flux by knowing the relative contribution of each substrate to the TCA cycle (13C-isotopomer analysis of glutamate). Taking this a step further, TCA cycle flux can be used to determine oxygen consumption by knowledge of the ratio between oxygen consumption and citric acid cycle flux for [1-13C]glucose (R = 3), [1,2-13C]acetate (R = 2), and unlabeled glycogen (R = 3); these values have been published previously (23). The following equation can be used to equate TCA cycle flux to oxygen consumption: VO₂ = TCA_ARA (ARA) + TCAGLUC(RGLUC) + TCAGLY(RGLY) + TCAAC(RAC) (23). In this equation, TCA cycle flux (TCA) and the ratio between citric acid cycle flux (R) for each substrate (anaplerosis, TCA_ARA and R_ARA), glucose (TCAGLUC, RGLUC), glycogen (TCAGLY, RGLY), and acetate (TCAAC, RAC) are utilized to determine total oxygen consumption. With the use of this equation, oxygen consumption was calculated to be 0.254 ± 0.049 µmol·g⁻¹·min⁻¹ (n = 14), which is consistent with the reported literature values. It has been shown that oxygen consumption of unstimulated hog carotid arterial VSM is 0.069 µmol·g⁻¹·min⁻¹ on contraction by KCl (27).

Conclusions. Glycogen oxidation accounts for ~10% of the substrates oxidized in hog carotid arterial VSM at high glycogen levels. Therefore, glycogen plays a minimal role in substrate oxidation of VSM during conditions of adequate substrate availability. Although the oxidation of the other endogenous substrates could not be directly assessed in our study, it is likely that they predominate compared with glycogen. Because the unlabeled endogenous substrates are likely to be lipids, the utilization of endogenous lipid will be important to investigate. The role of glycogen content in the regulation of other pathways such as glucose utilization, lactate production, and acetate utilization was minimal; however, glycogen levels did regulate glucose oxidation. We conclude that glucose predominates as
an oxidative substrate in VSM regardless of the glyco-
gen content of the tissue.

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Address for reprint requests and other correspondence: C. Hardin, Dept. of Physiology, MA-415 Medical Sciences Bldg., Univ. of Missouri, Columbia, MO 65212 (E-mail: HardinC@health.missouri.edu).

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