IGF-I promotes a shift in metabolic flux in vascular smooth muscle cells

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Hall, Jennifer L., Gary H. Gibbons, and John C. Chatham. IGF-I promotes a shift in metabolic flux in vascular smooth muscle cells. Am J Physiol Endocrinol Metab 283: E465–E471, 2002. First published April 30, 2002; 10.1152/ajpendo.00072.2002.—Insulin-like growth factor I (IGF-I) stimulates glucose flux into both nonoxidative and oxidative pathways in vascular smooth muscle cells (VSMC). Rat VSMC were exposed to uniformly labeled [13C]glucose ([U-13C]glucose; 5.5 mM) and [3-13C]pyruvate (1 mM) in the presence and absence of IGF-I (100 ng/ml). IGF-I increased glucose flux through glycolysis and the tricarboxylic acid (TCA) cycle as well as total anaplerotic flux into the TCA cycle. Previous work in our laboratory identified an increase in GLUT1 content and glucose metabolism in neointimal VSMC that was sufficient to promote proliferation and inhibit apoptosis. To test whether IGF-I could potentiate the GLUT1-induced increased flux in the neointima, we utilized VSMC harboring constitutive overexpression of GLUT1. Indeed, IGF-I markedly potentiated the GLUT1-induced increase in glucose flux through glycolysis and the TCA cycle. Taken together, these findings demonstrate that upregulation of the glucose transporter protein GLUT1, modeling the established conditions (1, 21, 25, 28, 29). IGF-I is a potent stimulator of glucose transport in VSMC (13, 47) in addition to being a well established mediator of VSMC proliferation and survival in the process of vascular remodeling (2, 6, 17, 24, 50). However, its impact on glucose flux in the vasculature has not been defined.

We postulated that the ability of insulin-like growth factor (IGF) I to stimulate glucose transport would result in increased glucose flux into nutrient-signaling pathways similar to the effect of upregulated GLUT1 content (19). To test this hypothesis, we utilized an in vitro model system to systematically discern the role of IGF-I on metabolic flux in VSMC. Furthermore, we assessed the effect of IGF-I on VSMC with constitutive upregulation of GLUT1, modeling the established GLUT1 upregulation in intimal VSMC, to further determine the role of IGF-I on glucose flux in the intimal lesion. In accord with our hypothesis, IGF-I significantly increased glucose flux through glycolysis and the tricarboxylic acid (TCA) cycle. Moreover, IGF-I markedly potentiated GLUT1-induced increases in glucose flux through glycolysis and the TCA cycle. Finally, IGF-I led to an unexpected, marked increase in anaplerotic flux into the TCA cycle.

METHODS

Cell Culture

A7r5 rat aortic VSMC were purchased from American Type Culture Collection (Manassas, VA) and subcultured in...
DMEM (catalog no. 11855, Gibco Life Technologies, Grand Island, NY) plus 10% fetal bovine serum (Gibco), and 1% penicillin-streptomycin (Gibco). Concentrations of D-glucose, sodium pyruvate, and L-glutamine in the media were 5.5, 1, and 4 mM, respectively.

**Stable Transfection**

Stable VSMC lines overexpressing GLUT1 were constructed utilizing a retroviral transfection technique, as previously described (19). A retroviral expression plasmid (pDOJ-GT1) containing the rat GLUT1 cDNA encoding the entire coding region as well as 17 and 97 bp of 5′ and 3′ nontranslated sequences (M. Birnbaum, University of Pennsylvania) was transfected into a packaging cell line (293T cells; G. Nolan, Stanford University). A retroviral expression plasmid, pLEIN, encoding the reporter gene green fluorescent protein (GFP; Clonetech, Palo Alto, CA) was similarly transfected into the packaging cells to create control transfected VSMC lines. After transfection (24–72 h), viral supernatant was harvested, passed through a 2-μm filter, and directly applied to A7r5 VSMC, after which G418 (500 μg/ml; Gibco) was added. Upregulation of GLUT1 was confirmed by Western blotting, and GFP expression was tracked in living cells by fluorescent microscopy as previously described (19).

**13C-Nuclear Magnetic Resonance Spectroscopy**

13C-nuclear magnetic resonance (NMR) spectroscopy was used to characterize alterations in glucose metabolism as a result of GLUT1 upregulation and treatment with IGF-I (100 ng/ml), as previously described (19). VSMC with constitutive upregulation of GLUT1 or the control transgene GFP was placed in DMEM (catalog no. 11966, Gibco) lacking D-glucose and sodium pyruvate and supplemented with 5.5 mM uniformly labeled [13C]glucose ([U-13C]glucose) and 1 mM [3-13C]pyruvate. VSMC (~1.0 × 10⁶ cells) were incubated in the labeled media for 24 h under serum-free conditions in the presence or absence of IGF-I (100 ng/ml). Medium was harvested and deproteinized and stored at −80°C before analysis. Cells were extracted with 6% perchloric acid and centrifuged at 4,000 g, and the protein-free supernatant was neutralized with KOH. The neutralized extract was stored at −80°C before analysis. Extracts were freeze dried and resuspended in deuterium oxide (D2O), and the pH was adjusted to ~7.0. There were no significant differences in total protein content of all samples as assessed by a Bradford protein assay performed as described by the manufacturer (Bio-Rad, Hercules, CA).

High-resolution 13C-NMR spectra were collected using a Bruker 500-MSL NMR spectrometer equipped with an 11.75T magnet and using a commercial 10-mm probe, as previously described (10, 19). Magnetic field homogeneity was optimized by observing the water signal using a 1H decoupling coil. Spectra were collected with a sweep width of 25 KHz, a 1-s relaxation delay, and a 60° pulse width. Bilevel WALTZ 1H decoupling was used to obtain full nuclear Overhauser enhancement and thus maximize signal to noise. The relative contributions of glucose and pyruvate to the total acetyl-CoA pool entering the TCA cycle were determined from glutamate isotopomer analysis (31) with software developed and kindly provided by Dr. Mark Jeffrey (TCAcal; University of Texas Southwestern Medical Center).

**Glutamate isotopomer analysis**

Metabolism of the exogenous [U-13C]glucose and [3-13C]pyruvate leads to formation of [1,2-13C2] and [2-13C]acetyl-CoA, respectively. From acetyl-CoA, the 13C label is transferred to the various TCA cycle intermediates. The concentrations of the TCA cycle intermediates are below the level of detection by 13C-NMR spectroscopy; however, glutamate is at a high enough concentration to be observed and is in exchange with the TCA cycle via α-ketoglutarate and aspartate aminotransferase. Thus measurement of the 13C label incorporation into glutamate can be used as an index of entry of [13C]acetyl-CoA into the TCA cycle. By use of the phenomenon of spin-spin coupling, it is possible to determine the relative contributions of different [13C]acetyl-CoA molecules to the overall TCA cycle flux, as described in detail by Malloy et al. (31). There are a variety of unlabeled substrates in the medium that can be oxidized by the VSMC, leading to the formation of unlabeled acetyl-CoA. With the use of glutamate isotopomer analysis, it is possible to assess the fraction of acetyl-CoA originating from unlabeled precursors; however, we cannot identify these substrates.

Substrates can also enter the TCA cycle other than via pathways leading to acetyl-CoA. These are so-called anaplerotic pathways, leading to the entry of 4-carbon units into the TCA cycle as opposed to the 2-carbon units from acetyl-CoA. One of these pathways is pyruvate carboxylase, which leads to the conversion of pyruvate to oxaloacetate. The 13C labeling pattern in glutamate is different when [13C]pyruvate is metabolized via pyruvate carboxylase compared with pyruvate dehydrogenase. Thus analysis of the glutamate isotopomer distribution enables us to determine not only the flux through pyruvate dehydrogenase but also the relative flux through pyruvate carboxylase. There are a number of other anaplerotic pathways that lead to the influx of unlabeled four-carbon units into the TCA cycle. This will result in decreased 13C enrichment of C2- and C3-glutamate relative to enrichment of C4-glutamate (31). Therefore, it is also possible, with the use of glutamate isotopomer analysis, to determine the relative contribution of unlabeled anaplerotic substrate to TCA cycle flux. We (19) and others (7, 21) have shown the feasibility of this technique in cultured cells.

**Alanine and lactate isotopomer analysis**

All of the relative fluxes described above are determined via glutamate isotopomer analysis, which reflects the oxidative metabolism of the exogenously supplied [13C]labeled substrates entering the TCA cycle via pyruvate dehydrogenase or pyruvate carboxylase. However, pyruvate can also be converted to alanine via alanine aminotransferase and lactate via lactate dehydrogenase. [3-13C]pyruvate will be metabolized to [3-13C]lactate, and [U-13C]glucose metabolism will result in [U-13C]lactate, generating singlet and doublet C5 resonances of lactate, respectively. Thus the ratio of the C5 doublet of lactate to the total intensity of the C5 resonance represents the contribution of exogenous glucose to lactate formation relative to exogenously supplied pyruvate. Similar isotopomer analysis of the C5-alanine resonance enabled us to assess the relative contribution of glucose to alanine formation. Thus isotopomer analysis of the C5 resonances of alanine and lactate provides an index of the metabolism of [U-13C]glucose via glycolysis.

**Statistical Analysis**

Comparisons between two groups were analyzed via a Student's t-test (P < 0.05), whereas experiments involving three or more treatments/groups were analyzed by an analysis of variance (ANOVA) with a Student-Newman-Keuls post hoc test (P < 0.05). If data exhibited unequal variance, a Mann-Whitney rank sum test (2 groups) or Kruskal-Wallis
One-Way Analysis of Variance on Ranks, in conjunction with Tukey's post hoc test, was performed (3 or more groups). Data are presented as means ± SE.

RESULTS

The aim of this study was to test our hypothesis that IGF-I increased glucose flux in VSMC. A secondary objective was to further elucidate a potential relationship between metabolic control and the regulation of vascular remodeling. We employed two parallel models of established metabolic alterations within a vascular lesion: upregulation of IGF-I and GLUT1. These models were based on studies from our laboratory and others, defining a significant upregulation of GLUT1 and IGF-I in VSMC in the context of vascular remodeling (17, 19). Data are presented from cultured VSMC harboring constitutive upregulation of GLUT1 or the control transgene GFP in the absence or presence of IGF-I. An emphasis is placed on IGF-I treatment, given that we have partially presented glucose flux data from GLUT1 cells in the absence of IGF-I (19).

An example of a typical spectrum generated after incubation of VSMC with [U-13C]glucose and [3-13C]-pyruvate for 24 h is shown in Fig. 1A. Resonances from glucose, aspartate, lactate, and alanine are all clearly evident. Incorporation of 13C label into glutamate and aspartate from transamination of the TCA cycle intermediates α-ketoglutarate and oxaloacetate, respectively, indicates the metabolism of [13C]glucose and pyruvate into the TCA cycle. In all of the samples, resonances from [13C]glycogen were below the limit of detection. This suggests that under the conditions of these experiments VSMC convert very little, if any, glucose to glycogen.

The C4-glutamate resonances from control cells incubated with and without IGF-I are shown in Fig. 1B. The expansion of the resonance reveals a complex multiplet structure that arises due to the incorporation of 13C label from glucose and pyruvate via acetyl-CoA into glutamate. The resonances identified as C4D45 and C4Q result exclusively from the metabolism of [U-13C]glucose and the C4D34 and C4S from the metabolism of [3-13C]pyruvate. Thus changes in the intensities of the C4D45 and C4Q resonances relative to the C4D34 and C4S resonances provide an indication of changes in the amount of glucose entering the TCA cycle compared with pyruvate. A comparison between the C4-glutamate resonances demonstrates an increase in the C4D45 resonances relative to the C4S with IGF-I, consistent with an increase in glucose oxidation.

Table 1 summarizes C4-glutamate isotopomer data from control and GLUT1-transfected cells at baseline [serum-free (SF) conditions] and in the presence of IGF-I. It can be seen that, in both groups of cells, the addition of IGF-I increases the contribution of resonances resulting from the metabolism of [U-13C]glucose relative to those resulting from metabolism of [3-13C]pyruvate.

Detailed analysis of the C4-glutamate multiplet structure, combined with that from the C2- and C3-glutamate resonances, yields the relative contributions of glucose and pyruvate and unlabeled substrates to glucose flux.
the TCA cycle via acetyl-CoA. Figure 2 demonstrates that IGF-I significantly increases glucose flux through the TCA cycle in both control and GLUT1-transfected VSMC. In accordance with the increase in glucose flux through the TCA cycle, the percent contribution of pyruvate to the TCA cycle was significantly decreased in both groups with IGF-I. As we have previously reported (19), the upregulation of GLUT1 resulted in a small but significant increase in the contribution of glucose to the TCA cycle at baseline.

The C3-lactate resonances from control cells incubated with and without IGF-I are shown in Fig. 1C. The relative intensities of the doublet to singlet of the lactate resonance provide an index of the contribution of [U-13C]glucose to lactate formation. In Fig. 1C, it is evident that, after the addition of IGF-I, there is an increase in the doublet (C3D) relative to the singlet (C3S), consistent with an increased contribution of glucose to cellular lactate formation. The data for all groups are summarized in Table 2. In both control cells and VSMC overexpressing GLUT1, IGF-I increased the percent contribution of [U-13C]glucose to cellular lactate and alanine. In addition to collecting spectra from cell extracts, we also obtained 13C-NMR spectra from culture medium at the end of the incubation and were able to determine the relative contribution of [U-13C]glucose to the lactate accumulating in the medium. This was also increased with IGF-I treatment in both groups of cells (Table 2).

In addition to determining the relative contribution of glucose into the TCA cycle and to cell lactate and alanine formation, we were also able to determine the contribution of anaplerotic pathways to TCA cycle flux. The three main anaplerotic reactions include synthesis of oxaloacetate from pyruvate, synthesis of succinyl-CoA from propionyl-CoA, and synthesis of α-ketoglutarate from glutamate. In contrast to the relatively small changes (<20%) in the contribution of glucose to acetyl-CoA and lactate, IGF-I treatment almost doubled total anaplerotic flux in the control VSMC (Fig. 3A). This increase was split fairly evenly between flux through pyruvate carboxylase and other anaplerotic pathways. In VSMC with constitutive upregulation of GLUT1, IGF-I treatment had no significant effect on anaplerotic flux (Fig. 3B). However, at baseline, upregulation

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Table 1. Percent contribution of different isotopomers of the C4 resonance of glutamate in VSMC overexpressing the control transgene GFP or GLUT1 at baseline (serum free [SF]) or in the presence of IGF-I.

<table>
<thead>
<tr>
<th>Isotopomer</th>
<th>GFP VSMC</th>
<th>GLUT1 VSMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.028</td>
<td>0.0066</td>
</tr>
<tr>
<td>Values</td>
<td></td>
<td></td>
</tr>
</tbody>
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Table 2. Percent lactate and alanine in cell extracts resulting from metabolism of [U-13C]glucose and percent lactate in tissue culture media from VSMC overexpressing GFP or GLUT1 at baseline or in the presence of IGF-I.

<table>
<thead>
<tr>
<th>Isotopomer</th>
<th>% Cell Lactate from Glucose</th>
<th>% Cell Alanine from Glucose</th>
<th>% Medium Lactate from Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>74.3 ± 2.5</td>
<td>67.5 ± 1.5</td>
<td>63.9 ± 0.1</td>
</tr>
<tr>
<td>IGF-I</td>
<td>79.7 ± 1.2</td>
<td>75.5 ± 0.5</td>
<td>72.3 ± 0.8</td>
</tr>
<tr>
<td>P value</td>
<td>0.061</td>
<td>0.0003</td>
<td>0.001</td>
</tr>
<tr>
<td>GLUT1 VSMC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>70.4 ± 2.3</td>
<td>68.3 ± 2.2</td>
<td>65.4 ± 0.2</td>
</tr>
<tr>
<td>IGF-I</td>
<td>82.2 ± 0.7</td>
<td>78.7 ± 0.4</td>
<td>75.7 ± 0.8</td>
</tr>
<tr>
<td>P value</td>
<td>0.0004</td>
<td>0.0004</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as ng/ml.
of GLUT1 increased anaplerosis by >40% compared with controls. These findings demonstrate that upregulated glucose flux via either IGF-I or GLUT1 upregulation leads to a significant shift in anaplerotic flux into the TCA cycle. In contrast to glucose flux into glycolysis and the TCA cycle, the addition of IGF-I to GLUT1-overexpressing cells did not potentiate anaplerotic flux.

DISCUSSION

Previous work in our laboratory (19) identified a significant increase in GLUT1 expression in neointimal lesions within the vasculature compared with the normal medial wall. To determine the specific role of GLUT1 upregulation in vascular biology we established VSMC with constitutive upregulation of GLUT1 and demonstrated that GLUT1 upregulation resulted in a threefold increase in glucose transport and a small but statistically significant increase in the contribution of glucose flux as measured by 13C-NMR spectroscopy (19). The aim of this study was to extend these findings and define the impact of IGF-I on the regulation of glucose flux in VSMC and determine the ability of IGF-I to further amplify GLUT1-induced increases in glucose flux. We found that 1) IGF-I stimulates glucose flux into nonoxidative and oxidative pathways in VSMC; 2) IGF-I further potentiates GLUT1-induced increases in glucose flux in VSMC; and finally 3) IGF-I significantly amplified total anaplerotic flux into the TCA cycle in VSMC.

To our knowledge, these experiments are the first to define the impact of GLUT1 upregulation and IGF-I on glucose flux in the vasculature. Similar increases in GLUT1 content in mesangial cells and skeletal muscle have resulted in increased glycolytic flux as measured by lactate output (22, 37). Furthermore, IGF-I has been shown to stimulate glycolysis in skeletal muscle and astrocytes (12, 44, 45). Thus our data demonstrating an increase in glycolytic flux in response to increased glucose transport agrees with previous work in other cell systems. To our knowledge, our findings demonstrating that increased glucose transport increases glucose flux into the TCA cycle is a new finding. Furthermore, the fact that IGF-I is able to further amplify a previously induced GLUT1-mediated increase in glucose flux is also novel.

Epidemiological, animal, and molecular biology studies have identified a role for metabolism in vascular reactivity and disease (3–5, 8, 14, 18–21, 27, 34, 38, 46). Given this precedent, it is somewhat surprising that the role of metabolic control in vascular remodeling is not better understood. However, the challenge of defining alterations in metabolic regulation in the process of vascular remodeling in the intact vessel is technically challenging and poses several issues with regard to diffusion distance of radiolabeled or 13C-labeled substrates within a developing lesion in the vascular wall. We chose to utilize an in vitro modeling system to circumvent these issues and begin to define the control of metabolic pathways in VSMC. Although this study demonstrates the usefulness of this model, we acknowledge the limitations of a cell culture system, including lack of VSMC contraction, coordinating molecular pathways between adventitia and endothelium, and the role of connective tissue. However, our data demonstrating a range of glucose contribution to glycolysis between 50 and 60% in cultured VSMC are in close agreement with previous work by Allen and Hardin (1) demonstrating that glucose accounts for 30–60% of the total substrate flux through glycolysis in isolated porcine arteries. In addition, our finding of ∼50–70% glucose contribution to the TCA cycle flux also falls in line with previous work in vascular smooth muscle (1). It is noteworthy that comprehensive studies have demonstrated that glucose flux into the TCA cycle is not significantly different between contracting and noncontracting vascular smooth muscle, providing increasing validation for our modeling approach to studying metabolic flux in the vascular lesion (1). Taken together, this lends support to our working in vitro model system as a suitable approach to begin to define the role of specific alterations in metabolic genes in the regulation of flux through nutrient-signaling pathways in the progression of vascular disease. Future studies are planned to incorporate measurements of metabolic regulation in vessels ex vivo to further validate our hypothesis.

The ability of IGF-I and GLUT1 to nearly double total anaplerotic flux was an unexpected finding. Previous work in vascular rings has shown relatively high anaplerotic flux in VSMC relative to the TCA cycle flux
(1). However, more studies are needed to better define specific pathways that are involved in the regulation of anaplerosis in the vasculature. This work will entail a more detailed analysis of carbon flux through the TCA cycle through the use of both NMR and gas chromatography-mass spectrometry (9) as well as defining gene expression and activity of key enzymes such as pyruvate dehydrogenase and pyruvate carboxylase. In cardiac muscle, anaplerotic flux is substantially lower than that seen here (35). It has been speculated that anaplerotic flux may serve as a biosensor between the cytoplasm and the mitochondria (11, 36, 39, 43, 48). Furthermore, evidence suggests a role for anaplerosis in the regulation of contractile function in the heart (15, 23, 30, 42, 49). Recent studies have also shown that flux through pyruvate carboxylase regulates amino acid synthesis (33, 34) and may be important in controlling cell growth (34, 43). These findings suggest that alterations in anaplerotic flux may regulate vascular cell function and proliferation. Future studies will be needed to address this.

In conclusion, our studies demonstrate that IGF-I stimulates glucose flux through nonoxidative and oxidative pathways and nearly doubles total anaplerotic flux. Furthermore, IGF-I is able to further potentiate GLUT1-induced increases in glucose flux in VSMC. The physiological implications of this finding may be important under conditions of vascular remodeling in which GLUT1 content is upregulated and IGF-I concentrations are altered.

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