AMP-activated protein kinase influences metabolic remodeling in H9c2 cells hypertrophied by arginine vasopressin


1James Hogg ICAPTURE Centre for Cardiovascular and Pulmonary Research, Department of Pathology and Laboratory Medicine, University of British Columbia-St. Paul’s Hospital, Vancouver, British Columbia; 2Cardiovascular Research Group, Department of Pediatrics, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta; and 3Department of Biochemistry and Molecular Biology, Diabetes Research Group, Life Sciences Institute, University of British Columbia, Vancouver, British Columbia, Canada

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Saeedi R, Saran VV, Wu SS, Kume ES, Paulson K, Chan AP, Parsons HL, Wambolt RB, Dyck JR, Brownsey RW, Allard MF. AMP-activated protein kinase influences metabolic remodeling in H9c2 cells hypertrophied by arginine vasopressin. Am J Physiol Heart Circ Physiol 296: H1822–H1832, 2009. First published April 17, 2009; doi:10.1152/ajpheart.00396.2008.—Substrate use switches from fatty acids toward glucose in pressure overload-induced cardiac hypertrophy with an acceleration of glycolysis being characteristic. The activation of AMP-activated protein kinase (AMPK) observed in hypertrophied hearts provides one potential mechanism for the acceleration of glycolysis. Here, we directly tested the hypothesis that activation of AMPK causes the acceleration of glycolysis in hypertrophied heart muscle cells. The H9c2 cell line, derived from the embryonic rat heart, was treated with arginine vasopressin (AVP; 1 μM) to induce a cellular model of hypertrophy. Rates of glycolysis and oxidation of glucose and palmitate were measured in nonhypertrophied and hypertrophied H9c2 cells, and the effects of inhibition of AMPK were determined. AMPK activity was inhibited by 6-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrazolo-[1,5-a]pyrimidine (compound C) or by adenosine-mediated transfer of dominant negative AMPK. Compared with nonhypertrophied cells, glycolysis was accelerated and palmitate oxidation was reduced with no significant alteration in glucose oxidation in hypertrophied cells, a metabolic profile similar to that of intact hypertrophied hearts. Inhibition of AMPK resulted in the partial reduction of glycolysis in AVP-treated hypertrophied H9c2 cells. Acute exposure of H9c2 cells to AVP also activated AMPK and accelerated glycolysis. These elevated rates of glycolysis were not altered by AMPK inhibition but were blocked by agents that interfere with Ca2+ signaling, including extracellular EGTA, dantrolene, and 2-aminoethoxydiphenyl borate. We conclude that the acceleration of glycolysis in AVP-treated hypertrophied heart muscle cells is partially dependent on AMPK, whereas the acute glycolytic effects of AVP are AMPK independent and at least partially Ca2+ dependent.

cardiac hypertrophy; energy metabolism; glucose utilization

AMP-activated protein kinase (AMPK) is a heterotrimeric complex composed of catalytic (α) and regulatory subunits (β and γ) and is the mammalian representative of a highly conserved family of protein kinases with major roles in the control and regulation of energy metabolism (3, 14). AMPK is activated by physiological or pathological stresses that deplete cellular high-energy phosphates, including ATP and phosphocreatine (3, 14). Additionally, it has been shown that AMPK can be activated by hormones acting through Gα-linked G protein-coupled receptors and a Ca2+-dependent pathway independent of any changes in the energy status of the cells (14, 26). Once activated, AMPK mediates the inhibition of energy-consuming pathways and stimulation of energy-producing pathways, such as fatty acid oxidation, glucose uptake, and glycolysis (3, 14).

The activation of AMPK stimulates fatty acid oxidation in muscle by increasing the uptake of fatty acids across cellular and mitochondrial membranes (3, 14, 33). Key actions of AMPK include the translocation of fatty acid transport proteins to the sarcolemma, increased recruitment of lipoprotein lipase to the capillary endothelium (2, 10, 14), and lowering of the concentration of malonyl-CoA, a key allosteric inhibitor of carnitine palmitoyltransferase-1 (14, 37). Malonyl-CoA levels are reduced through the phosphorylation and inactivation of acetyl-CoA carboxylase, which synthesizes malonyl-CoA, and, possibly, through the phosphorylation and activation of malonyl-CoA decarboxylase (14, 55). The effects of AMPK on glucose metabolism in the heart are accounted for by the enhanced translocation of glucose transport proteins to the sarcolemma (14, 43) and by the stimulation of glycolysis through the phosphorylation and activation of 6-phosphofructo-2-kinase (PFK-2) (36). The consequent increase in fructose-2,6-diphosphate (F-2,6-P2) leads to the allosteric activation of PFK-1, a major determinant of glycolytic rates in the heart and other tissues (36, 53).

Oxidation of long-chain fatty acids, the major source of energy in the myocardium, is low in hypertrophied hearts studied as isolated preparations or in vivo (1, 45), and it has been argued that this is explained by the reduced expression of oxidative enzymes and fatty acid uptake/transport proteins as well as low levels of myocardial carnitine (4, 44, 46). As a result, energy production in hypertrophied hearts is more dependent on carbohydrates with an acceleration of glycolysis being characteristic (1, 40, 45, 52).

Activation of AMPK in hypertrophied hearts has been observed in a number of studies (1, 40, 52). Furthermore, we (1) have recently shown that accelerated rates of glycolysis are normalized when fatty acid oxidation is elevated to rates similar to those in nonhypertrophied hearts. At the same time, elevated activity of AMPK in hypertrophied hearts is correspondingly reduced to values observed in nonhypertrophied...
hearts. Although these data provide correlative support for the view that AMPK is responsible for the acceleration of glycolysis in hypertrophied hearts, other mechanisms remain possible. The present investigation was designed to directly test the hypothesis that AMPK is responsible for the acceleration of glycolysis in hypertrophied heart muscle cells. Cultured H9c2 cells, a cell line derived from the embryonic rat heart (21), were used as an in vitro model of cardiac myocyte hypertrophy in which AMPK activity was selectively altered by pharmacological and molecular approaches. H9c2 cells were hypertrophied by exposure to arginine vasopressin (AVP), which is a peptide neurohormone that is significantly and chronically elevated in the plasma of heart failure patients (18, 32) and experimental animals (15) and has effects on processes that participate in the pathogenesis of heart failure, including body fluid regulation, vascular tone, and cardiac contractile function and remodeling (32). AVP may contribute to structural remodeling in heart failure (18) as it has been shown to cause hypertrophy of heart muscle cells in culture (8, 39) and stimulate protein synthesis in isolated hearts (16). Moreover, Hupf and colleagues (23) have demonstrated that the heart possesses a local AVP system that is activated in response to pressure overload, suggesting that, in pathological settings, the heart may not only respond to circulating AVP but also to locally produced AVP in a paracrine or autocrine fashion.

MATERIALS AND METHODS

Culture and treatment of H9c2 cells. H9c2 (2-1) cells (passage 12, American Type Culture Collection, Manassas, VA) were cultured in DMEM containing 10% FBS and 100 U/ml penicillin-streptomycin at 37°C in a humified atmosphere of 95% O2-5% CO2 (8). Differentiation toward a cardiac phenotype was induced by culture in DMEM containing 1% horse serum and 0.1 M all-trans-retinoic acid for 4 days (8, 38), with the medium being changed daily. Retinoic acid was prepared in the dark in DMSO and stored at −20°C until use, and the concentration of DMSO in the culture media was <0.2%.

Hypertrophy of H9c2 cells was induced by exposure of differentiated H9c2 cells to DMEM-F-12 media containing 0.5% horse serum with or without 1 μM AVP for 48 h, as previously described (8). After 48 h, AVP-containing media were replaced with Krebs-Henseleit (KH) solution containing 0.4 mM palmitate prebound to 3% BSA, 5.5 mM glucose, and 10−7 M insulin. Cells were studied over 8 h under these conditions with tracer amounts of labeled [5-3H]glucose (1.0 μCi/ml), [U-14C]glucose (1.0 μCi/ml), or [U-14C]palmitate (0.4 μCi/ml) added during the last hour of study to measure glycolysis, glucose oxidation, and fatty acid oxidation, respectively, in separate experiments. A delay of 7–8 h before measurements of substrate use was chosen to minimize residual direct effects of AVP.

Another set of experiments was performed to investigate the acute metabolic actions of AVP on H9c2 cells. Rates of glycolysis were measured in experiments conducted over 2 h in KH solution containing 0.4 mM palmitate prebound to 3% fatty acid-free BSA, 5.5 mM [5-3H]glucose (1.0 μCi/ml), and 10−7 M insulin with or without AVP. Slightly different conditions were used in selected experiments as shown in the figures. The lowest concentration of AVP capable of stimulating glycolysis was assessed by exposing H9c2 cells to a range of AVP concentrations, including concentrations observed in the setting of heart failure in humans and in rodents (7, 9, 15).

Assessment of cellular hypertrophy. Cellular hypertrophy was evaluated by measuring changes in protein synthesis and content as well as DNA synthesis and the expression of atrial natriuretic factor (ANF), a well-recognized marker of pathological cardiac hypertrophy (13, 35). Protein synthesis induced by AVP treatment was quantitated by measuring the incorporation of phenylalanine into cells exposed to [14C]phenylalanine (1.0 μCi/ml), as previously described (47). [14C]phenylalanine was present in culture media during the first 24 h in either the presence or absence of AVP. DNA synthesis was determined by measuring the incorporation of [14C]thymidine (1.0 μCi/ml) into cells during the first 24 h in either the absence or presence of AVP, as previously described (8). Experiments were terminated by removing media, washing with ice-cold phosphate buffer, and adding ice-cold 10% trichloroacetic acid to precipitate proteins. After an overnight incubation at 4°C, precipitates were dissolved in 1 M NaOH-0.01% SDS for 2 h at 37°C. Aliquots of the resulting solution were counted in a liquid scintillation counter or used to determine protein content. Protein content of the cultures was determined using a commercial bicinchoninic acid protein assay kit (procedure no. TPRO-562, Sigma Chemical, St. Louis, MO).

ANF expression was measured in H9c2 cell lysate using a commercially available ELISA kit (R&D Systems, Mississauga, ON, Canada). Lysis of cells was performed using homogenization buffer (pH 7.5) containing MOPS (20 mM), sucrose (250 mM), KCl (150 mM), EDTA (1 mM), benzamidine (2.5 mM), pepstatin A (0.2%), leupeptin (5 μM), glutathione (2.5 mM), PMSF (0.5 mM), and microcystin-LR (0.14 μg/ml). The cell suspension was sonicated and then centrifuged for 10 min at 13,000 g at 4°C. The supernatant, which contained 10 μg total protein, was then used to measure ANF content.

Measurement of substrate utilization. Oxidation of palmitate and glucose was measured using modifications of previously described methods (22, 45). Briefly, preoxygenated KH solution containing [U-14C]palmitate (0.4 μCi/μmol) complexed to BSA or [U-14C]glucose (1.0 μCi/ml) was added to cells in a final volume of 2 ml. The flasks were then sealed with a rubber stopper fitted with a scintillation vial containing cellulose filter paper soaked with 0.3 ml of 1 M hyamine hydroxide. Rates of fatty acid and glucose oxidation were measured by quantitative collection of 14CO2 released as a gas and dissolved in the cell media as [14C]bicarbonate, respectively. After an incubation for 1 h at 37°C, the reaction was stopped by the injection of 5 M H2SO4 (0.5 ml), which also liberates 14CO2 dissolved in the KH solution. The filter papers were taken for scintillation counting after 2 h of gentle shaking at room temperature. Glycolysis was determined by measuring the rate of production of 3H2O from trace amounts of [5-3H]glucose (1.0 μCi/ml) added to the media (45). In the glycolytic pathway, 3H2O is completely liberated from [5-3H]glucose at the enolase and triosephosphate isomerase steps. To measure glycolysis, 3H2O was separated from [3H]glucose in the media with the use of screening columns (Fisher Scientific) containing Dowex 1-X4 anion exchange resin (200–400 mesh) suspended in potassium tetraborate. Metabolic rates are expressed as nanomoles of substrate per hour per milligram of protein.

Expression of metabolic proteins. Expression of key proteins involved in control of glucose catabolism, including glucose transporter (GLUT)-4, hexokinase (HK)-2, the Elc-subunit of the pyruvate dehydrogenase complex (PDC-Elcα), GLUT-1, phosphorylation of PFK-2 at Ser466, and the α-catalytic subunit of AMPK (α-pan AMPK), was determined in control and hypertrophied H9c2 cells by immunoblot analysis, as previously described (1, 45). Briefly, 30 μg of H9c2 cell lysate were separated by electrophoresis on 5–12.5% SDS-polyacrylamide gels and transferred by electroblotting to a nitrocellulose membrane. Membranes were probed with antibodies against GLUT-4 (1:1,000 dilution, Cell Signaling Technology, Mississauga, ON, Canada), GLUT-1 (10 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA), HK-2 (1:500 dilution, Santa Cruz Biotechnology), p-PFK-2 (1:200 dilution, Santa Cruz Biotechnology), PDC-Elcα (1:100,000 dilution, Santa Cruz Biotechnology), and α-pan AMPK (1:1,000 dilution, Upstate Biotechnologies, Lake Placid, NY). After an incubation with the appropriate secondary antibody, the signal was detected by ECL, and band intensities for each individual protein were quantified by densitometry, corrected for background staining, and

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normalized to the signal for GAPDH (1:15,000 dilution, Molecular Probes, Eugene, OR) or total PFK-2 (1:200 dilution, Santa Cruz Biotechnology).

**AMPK activity and its inhibition.** Total AMPK activity was measured in H9c2 cell homogenates after immunoprecipitation or precipitation with saturated ammonium sulfate by determining the incorporation of $^{32}$P into the synthetic AMARA peptide, AMARAASAAALARRR, as previously described (1). The extent of phosphorylation of AMPK was also determined by immunoblot analysis using a previously described method (1).

To suppress AMPK pharmacologically after the induction of hypertrophy by AVP, H9c2 cells were exposed to 40 $\mu$M of 6-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrrazolo-[1,5-alpyrimidine (compound C; EMD Chemicals, San Diego CA) (59) for 8 h in KH solution containing 0.4 mM palmitate prebound to 3% BSA, 5.5 mM glucose, and $10^{-7}$ M insulin.

As an alternative to pharmacological inhibition by compound C, experiments were carried out with a dominant negative (DN) form of AMPK (Ad-DN-AMPK). Recombinant, replication-deficient adenovirus containing mutant DN AMPK was constructed as previously described (25). Briefly, cDNA containing a DN catalytic $\alpha_2$-subunit carrying the D157A mutation (48, 56) was subcloned into a pAdTrack shuttle vector, linearized with Pmel, and inserted into adenovirus using the pAdEasy-1 system for homologous recombination in Escherichia coli. The recombinant adenovirus vector also contains the gene for green fluorescent protein (Ad-GFP) to serve as a reporter of the extent of viral infection and protein expression.

For these experiments, H9c2 cells were infected with adenovirus containing Ad-GFP [multiplicity of infection (MOI): 150] or Ad-DN-AMPK plus GFP (MOI: 150) by incubation in DMEM media containing 1% horse serum for 24 h after the removal of AVP. Media were replaced with KH solution containing 0.4 mM palmitate prebound to 3% BSA, 5.5 mM glucose, and $10^{-7}$ M insulin, and rates of glycolysis were measured by the addition of tracer amounts of [5-$^3$H]glucose 7 h later, as described above.

Similarly, the potential role of AMPK in the acute metabolic actions of AVP was assessed using pharmacological and molecular means to inhibit AMPK in H9c2 cells. Specifically, H9c2 cells were exposed to 40 $\mu$M compound C for 30 min before the switch to KH solution containing fatty acid, glucose, and insulin with or without 1 $\mu$M AVP. When used, compound C was present for the entire study period. H9c2 cells exposed to vehicle served as controls for these experiments. For inhibition by Ad-DN-AMPK, cells were exposed to Ad-GFP or Ad-DN-AMPK plus GFP for 24 h before an acute exposure to AVP.

**Determination of the role of Ca$^{2+}$ in the acute metabolic actions of AVP.** Binding of AVP to the V1a receptor, the receptor subtype shown to be involved with the hypertrophic response to AVP (16), leads to the activation of phospholipase C, production of inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG), mobilization of intracellular Ca$^{2+}$, and influx of extracellular Ca$^{2+}$ (8, 51). By selectively modulating intracellular Ca$^{2+}$ mobilization and extracellular Ca$^{2+}$ influx, we evaluated the role of Ca$^{2+}$ in mediating the metabolic response of H9c2 cells to AVP.
In one series of experiments, H9c2 cells were pretreated for 30 min with 25 μM dantrolene, an inhibitor of Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) via the ryanodine receptor (57). To evaluate the involvement of the IP\(_3\) receptor, whose activation also leads to Ca\(^{2+}\) release from the SR, cells were pretreated for 5 min with 100 μM 2-aminoethoxydiphenyl borate (2-APB), a noncompetitive IP\(_3\) receptor antagonist (34). The role of extracellular Ca\(^{2+}\) was assessed by preincubating H9c2 cells with 3 mM EGTA (to chelate extracellular Ca\(^{2+}\)) for 5 min before the challenge with AVP (42).

Data analysis. Results are expressed as means ± SE. Differences among groups were compared by ANOVA using NCSS (version 2000, Statistical Solutions, Saugus, MA). The Newman-Keuls test was used as a post hoc test to identify significant differences when ANOVA yielded a significant F-ratio. P values of <0.05 were considered significant.

RESULTS

AVP causes hypertrophy and metabolic remodeling of H9c2 cells. Chronic exposure of H9c2 cells to AVP did not significantly alter \([^{14}C]\)thymidine incorporation (Fig. 1A) or overall cell numbers \([51.5 \times 10^4 \pm 0.8 \text{ vs. } 52.3 \times 10^4 \pm 0.5 \text{ cells/60-mm dish, } n = 12 \text{ per group, } P = \text{ not significant (NS)}\], indicating that cell proliferation was not enhanced. In contrast, chronic AVP treatment of H9c2 cells significantly increased protein content (Fig. 1B), \([^{14}C]\)phenylalanine incorporation (Fig. 1C), and ANF expression (Fig. 1D), indicating that H9c2 cells exposed chronically to AVP exhibit several major characteristics of hypertrophy. Furthermore, rates of glycolysis were accelerated by ~80% in H9c2 cells after AVP treatment for 48 h (Fig. 2A). There were no significant differences in glucose oxidation between hypertrophied H9c2 cells and nonhypertrophied cells (Fig. 2B). However, rates of fatty acid oxidation were significantly decreased in hypertrophied H9c2 cells compared with nonhypertrophied cells (Fig. 2C). Notably, when H9c2 cells were treated with AVP for 48 h and AVP was then removed, the accelerated rates of glycolysis persisted for at least a further 48 h after AVP removal (data not shown).

**Fig. 2.** Effects of AVP-induced hypertrophy of H9c2 cells on glycolysis (A), glucose oxidation (B), and fatty acid oxidation (C). Values are means ± SE. *Significantly different from control (P < 0.05). n = 5–18 per group.
Accompanying the changes in substrate use, expression of several key proteins involved in the control of glucose metabolism was altered in hypertrophied H9c2 cells (Fig. 3). Specifically, the expression of HK-2 was significantly increased, whereas those of GLUT-4 and PDC-E1α were slightly but significantly decreased (Fig. 3). There were no significant changes in GLUT-1 expression (Fig. 3). In hypertrophied H9c2 cells, phosphorylation of PFK-2 was in-

![Image of protein expression and densitometry graphs](attachment:image.png)

**Fig. 3.** Effects of AVP-induced hypertrophy of H9c2 cells on the expression of proteins involved in glucose metabolism. GLUT-4, glucose transporter-4; GLUT-1, glucose transporter-1; HK-2, p-PFK-2, phospho-phosphofructokinase-2; PDH-E1α, E1α-subunit of pyruvate dehydrogenase. The expression of GAPDH and total PFK-2 were used as internal standards to calculate relative expressions. Each lane represents a separate dish of cells. Values are means ± SE.

*Significantly different from control (P < 0.05).
increased at Ser\textsuperscript{466}, a site that is responsible for the activation of PFK-2 in heart muscle (5). 

**Inhibition of AMPK reduces glycolysis in hypertrophied H9c2 cells.** AMPK activity was significantly elevated in hypertrophied H9c2 cells (control: 153.7 ± 19.2 pmol·min\textsuperscript{-1}·mg protein\textsuperscript{-1} vs. AVP: 222.4 ± 27.6 pmol·min\textsuperscript{-1}·mg protein\textsuperscript{-1}, n = 31–32 per group, P < 0.05), whereas the expression of the catalytic subunits of AMPK did not differ significantly between the two groups (data not shown).

To determine if AMPK is responsible for the accelerated rates of glycolysis observed in H9c2 cells hypertrophied by AVP, two approaches were used to suppress AMPK activity. In one approach, AMPK was inhibited by the expression of Ad-DN-AMPK, and in the alternate pharmacological approach, AMPK was inhibited by incubating cells with compound C. Exposure of H9c2 cells to Ad-DN-AMPK (MOI: 150) for 24 h resulted in nearly 100% gene transfer as demonstrated by the expression of GFP (Fig. 4A). AMPK activity was reduced in control and hypertrophied H9c2 cells infected with Ad-DN-AMPK (Fig. 4B). Data from cells exposed to Ad-GFP were comparable with corresponding control and hypertrophied cells (data not shown). The effectiveness of inhibition of AMPK was confirmed by the substantial abrogation of the acceleration of glycolysis caused by mannitol and oligomycin, two potent activators of AMPK (Table 1) (19, 24). Likewise, compound C treatment resulted in a substantial reduction in AMPK activity in hypertrophied and nonhypertrophied H9c2 cells (Fig. 4C), a reduction that was greater than that caused by Ad-DN-AMPK.

It should be noted that the extent of hypertrophy, as assessed by total protein content, was comparable in the presence (21.8 ± 1.8% of control values, n = 4) or absence of Ad-DN-AMPK (24.2 ± 3.0% of control values, n = 5, P = NS), indicating that exposure of cells to DN-AMPK in did not lead to the stimulation or regression of hypertrophy.
Inhibition of AMPK after induction of hypertrophy in H9c2 cells by AVP had only a partial effect on rates of glycolysis (Fig. 5 and Table 2). This effect was most evident when the variability of glycolytic rates caused by the inhibitors themselves was accounted for (Table 2). Interestingly, suppression of glycolytic rates was relatively greater for compound C than for Ad-DN-AMPK, a finding that likely reflects the greater reduction in AMPK activity caused by compound C (Fig. 4).

Glycolytic rates in Ad-GFP-treated cells were comparable with those in the corresponding control and hypertrophied H9c2 cells (data not shown).

AVP acutely accelerates glycolysis in H9c2 cells. Treatment of H9c2 cells with AVP for as little as 2 h led to a dose-dependent stimulation of glycolysis (Fig. 6). The lowest effective dose of AVP, 10 nM, was comparable to concentrations of circulating AVP seen in the setting of heart failure (7, 9). To determine the receptor involved in AVP-induced stimulation of glycolysis, H9c2 cells were pretreated with either d(CH2)5[Tyr(Me)2]AVP, a potent and selective V1a receptor antagonist (30), or d(CH2)5[ε-Ile2,ε-Ile4,Tyr-NH(2)9]AVP, a potent and selective V2 receptor antagonist (30). Cells were exposed to these receptor antagonists for 30 min before the addition of 1 μM AVP. The V1a receptor antagonist completely abolished the acceleration of glycolysis (Fig. 6), whereas the V2 receptor antagonist had no effect.

AMPK, Ca2+, and acute stimulation of glycolysis by AVP. Acute exposure of H9c2 cells to AVP resulted in a significant twofold increase in the measured activity of AMPK (Fig. 7A) that was significantly and markedly reduced by both Ad-DN-AMPK and compound C (Fig. 7A). However, inhibition of AMPK by either Ad-DN-AMPK or compound C did not cause a significant reduction in glycolysis in H9c2 cells treated for this short duration with AVP (Fig. 7B and C) even when inhibitor effects were taken into account (Table 2); this is a result differing from that seen in H9c2 cells hypertrophied by longer-term (48 h) exposure to AVP (Fig. 5 and Table 2). Given the well-known effects of AVP on cellular Ca2+ (41, 50) and the potential for Ca2+ to accelerate glucose use (28, 58), pharmacological agents that interfere with Ca2+ entry or intracellular release from Ca2+ stores were used to assess the role of Ca2+ in the acute metabolic actions of AVP. In contrast to AMPK inhibition, treatment of H9c2 cells with 2-APB completely abolished the effect of AVP on glycolysis (Fig. 8A), whereas incubation with dantrolene (Fig. 8B) or EGTA (Fig. 8C) partially attenuated the elevation in glycolysis caused by AVP. These data indicate that the acute stimulation of glycolysis by AVP is mediated by changes in cellular Ca2+, especially Ca2+ release from the SR by stimulation of the 2-APB receptor.

**DISCUSSION**

In the present study, we demonstrate that AVP induces cellular hypertrophy in H9c2 cells with parallel metabolic remodeling of heart muscle cells characterized by an acceler-

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**Table 1. Effect of Ad-DN-AMPK on glycolysis stimulated by oligomycin and mannitol**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No Ad-DN-AMPK</th>
<th>Ad-DN-AMPK</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>4.2±0.3</td>
<td>4.7±0.3</td>
</tr>
<tr>
<td>Oligomycin (0.5 μM)</td>
<td>9.7±2.2*</td>
<td>6.1±0.1*†</td>
</tr>
<tr>
<td>Mannitol (0.5 mM)</td>
<td>10.1±1.0*</td>
<td>4.94±1.4†</td>
</tr>
</tbody>
</table>

Values are means ± SE (in nmol glucose·h⁻¹·mg protein⁻¹); n = 3–14 per group. Ad-DN-AMPK, adenovirus-mediated transfer of dominant negative AMP-activated protein kinase. *Significantly different from the corresponding no treatment group (P < 0.05); †significantly different from the corresponding no Ad-DN-AMPK group (P < 0.05).

**Fig. 5. Effects of AMPK inhibition on glycolysis in hypertrophied H9c2 cells.**

A: rates of glycolysis in hypertrophied and nonhypertrophied H9c2 cells treated with Ad-DN-AMPK. B: rates of glycolysis in hypertrophied and nonhypertrophied H9c2 cells treated with compound C. Values are means ± SE. *Significantly different from control (P < 0.05); †significantly different from Ad-DN-AMPK (P < 0.05). n = 8–25 per group.
ation of glycolysis and a reduction in long-chain fatty acid oxidation. The metabolic changes observed in hypertrophied heart muscle cells resemble those observed in intact hypertrophied hearts and include alterations in the expression of metabolic proteins and activation of AMPK. We demonstrate, for the first time, that the acceleration of glycolysis in hypertrophied heart muscle cells is only partially dependent on AMPK, a finding that suggests that factors in addition to AMPK are involved. Our results also indicate that the rapid acceleration of glycolysis caused by the acute administration of AVP is not dependent on AMPK activation, even though AMPK is activated. Rather, AVP acutely stimulates glycolysis by a Ca\(^{2+}\)-dependent mechanism.

Hypertrophy, as assessed by increased protein content and synthesis as well as elevation of ANF (Fig. 1), was produced in the heart muscle cell line H\(\text{9c2}\) by prolonged exposure to AVP for 48 h, in keeping with results from previous studies (8, 16, 39). AVP is released from the posterior pituitary and affects body fluid balance and vascular tone (17, 32). Levels of AVP are significantly and chronically elevated in the plasma of patients and experimental animals with heart failure (7, 9, 15). Moreover, the heart possesses a local AVP system that is activated in response to pressure overload and may act in a paracrine or autocrine fashion (23). Systemic and myocardial AVP may therefore contribute to structural remodeling of the failing heart.

In addition to causing hypertrophy, AVP leads to metabolic remodeling of H\(\text{9c2}\) cells, activating glycolysis and reducing long-chain fatty acid oxidation (Fig. 2). Those metabolic alterations in hypertrophied H\(\text{9c2}\) cells persist for at least 2 days after the removal of AVP (data not shown), indicating that the changes are related to a chronic remodeling process. The metabolic alterations observed in hypertrophied H\(\text{9c2}\) cells recapitulate those seen in intact hearts from experimental animal models of cardiac hypertrophy induced by pressure overload (1, 40, 45, 52).

The changes observed here in the expression of HK-2, GLUT-4, and PDC-E1 (Fig. 3) are consistent with the changes in activity or expression of these proteins observed in some models of hypertrophy in the intact heart (11) and heart failure (49), but not all studies have given consistent results (31, 45). Collectively, these findings indicate that while changes commonly occur in the expression of proteins and enzymes directly controlling glucose catabolism in hypertro-

### Table 2. Effect of Ad-DN-AMPK or compound C on glycolysis in H\(\text{9c2}\) cells hypertrophied by AVP or exposed to AVP acutely

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chronic AVP</th>
<th>Acute AVP</th>
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<tbody>
<tr>
<td></td>
<td>Ad-DN-AMPK</td>
<td>Compound C</td>
</tr>
<tr>
<td>Control</td>
<td>100.0±3.7</td>
<td>100.0±5.7</td>
</tr>
<tr>
<td>AVP</td>
<td>194.1±12.1*</td>
<td>12.1*</td>
</tr>
<tr>
<td>Ad-DN-AMPK</td>
<td>100.0±3.6</td>
<td>15.2*</td>
</tr>
<tr>
<td>Ad-DN-AMPK + AVP</td>
<td>160.9±11.25*</td>
<td>146.5±5.4*</td>
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<tr>
<td>Compound C</td>
<td>100.0±5.6</td>
<td>100.0±4.1</td>
</tr>
<tr>
<td>Compound C + AVP</td>
<td>136.1±4.2*</td>
<td>187.4±18.8*</td>
</tr>
</tbody>
</table>

Values are means ± SE (expressed as percentages of the control value); n = 3-25 per group. AVP, arginine vasopressin. *Significantly different from control (P < 0.05); †significantly different from the corresponding group not exposed to AVP (P < 0.05); ‡significantly different from AVP (P < 0.05).

Fig. 6. Concentration dependency and the receptor subtype responsible for the acute effect of AVP on glycolysis. A: H\(\text{9c2}\) cells were incubated for 2 h in DMEM-F-12 base medium with the indicated concentrations of AVP, and rates of glycolysis were determined as described in MATERIALS AND METHODS. To test the role of specific AVP receptors, cells were treated (B) with the indicated antagonists in DMEM-F-12 base media for 30 min before the addition of 1 \(\mu\)M AVP. Control, no AVP. Values are means ± SE. *Significantly different from control or no AVP (P < 0.05). n = 6–9 per group.
phied heart muscle, the direction and extent of change vary between models. In addition, it was found that the phosphor-
lation of PFK-2 on Ser466 was increased (Fig. 3), indicating greater activation. AMPK is known to increase PFK-2 activity
by causing the phosphorylation of Ser466 (5). The activation of
PFK-2 leads to increased the production of F-2,6-P2, a potent
activator of the key glycolytic enzyme PFK-1, ultimately
leading to increased rates of glycolysis.

The reduction of glycolysis in hypertrophied H9c2 cells
observed in the present study after the inhibition of AMPK by
complementary pharmacological and molecular approaches is
a direct demonstration that AMPK contributes to the accel-
eration of glycolysis in hypertrophied heart muscle cells. How-
ever, inhibition of AMPK failed to completely normalize rates
of glycolysis (Fig. 5), suggesting that other factor(s) also
contribute to the increased glycolysis in this setting. In addition
to the effects related to hypertrophic and metabolic remodel-
ing, acute exposure of H9c2 cells to AVP (Fig. 6A) accelerated
glycolysis at concentrations of AVP comparable with those
reported in the plasma of patients and rodents with congestive
heart failure, which, therefore, is potentially clinically relevant

Fig. 7. AMPK activity in H9c2 cells exposed to AVP acutely and the effect of
AMPK inhibition on glycolysis. A: H9c2 cells were incubated for 2 h in
the absence (control) or presence of 1 μM AVP before measurements of AMPK
activity. Alternatively, cells were treated with Ad-DN-AMPK (MOI: 150) or
40 μM compound C as described in MATERIALS AND METHODS before the
incubation with 1 μM AVP for 2 h and measurements of AMPK activity (A)
or glycolysis (B and C). Values are means ± SE. *Significantly different from
control (P < 0.05); $significantly different from AVP (P < 0.05); #signifi-
cantly different from Ad-DN-AMPK or compound C (P < 0.05). n = 3–9 per
group.

Fig. 8. Role of Ca2+ in the metabolic actions of AVP in H9c2 cells. Cells were
first incubated in the absence or presence of either 100 μM 2-aminoethoxy-
diphenyl borate (2-APB) for 5 min (A), 25 μM dantrolene for 30 min (B), or
3 mM EGTA for 5 min (C). All experiments were performed in Ca2+ -free
DMEM-F-12 base media, and H9c2 cells exposed to the relevant vehicle
served as controls. After this initial period, control and antagonist-treated cells
were further incubated in the absence or presence of 1 μM AVP for 2 h to measure the rates of glycolysis. Values are
means ± SE. *Significantly different from control (P < 0.05); $significantly
different from AVP (P < 0.05); #significantly different from AVP (P < 0.05); $significantly different from the agent (P < 0.05). n = 6–8 per each group.
A stimulatory effect of AVP on glucose catabolism has been previously described in a variety cell types, tissues, and organs, including isolated perfused rat hearts (12, 27, 54). Our results clearly demonstrate that the acceleration of glycolysis in H9c2 cells is a direct effect of AVP on heart muscle cells.

AVP exerts its actions through binding to three specific receptor subtypes, including V1a, V2, and V3 receptors, all of which belong to the Gα-coupled receptor family (50). We show that AVP exerts its acute metabolic effects in H9c2 cells via the V1a receptor (Fig. 6B), the receptor subtype responsible for AVP-induced hypertrophy of heart muscle cells (39). The V1a receptor acts independently of the cAMP cascade and instead leads to the sequential activation of phospholipases, hydrolysis of membrane phosphoinositides, release of IP3 and DAG, and mobilization of SR Ca\(^{2+}\) stores, followed by the sustained entry of extracellular Ca\(^{2+}\) (50).

Acute exposure to AVP causes a substantial elevation of AMPK activity (Fig. 7A), but the acute AVP-induced acceleration of glycolysis in H9c2 cells is independent of AMPK because compound C or Ad-DN-AMPK failed to block the glycolytic response to AVP (Fig. 7, B and C). Our data strongly implicate intracellular and extracellular Ca\(^{2+}\)-dependent pathways (Fig. 8) in the acute AVP-induced stimulation of glycolysis in H9c2 cells, consistent with the effects of AVP in the liver (29). It should be recognized, however, that the agents used in our study are not necessarily fully selective. For example, 2-APB may also influence other aspects of Ca\(^{2+}\) homeostasis, such as inhibition of Ca\(^{2+}\)-ATPase activity and the store-operated Ca\(^{2+}\) entry channel (6). Further studies will be required to fully define the specific Ca\(^{2+}\)-dependent pathways involved in H9c2 cells.

The failure to observe an effect of AMPK inhibition on the acceleration of glycolysis by acute exposure to AVP was surprising and is also difficult to easily explain. Presumably, what our data indicate is that the control of glycolysis differs between acute and chronic exposure to AVP. In the acute setting, AVP-induced changes in intracellular Ca\(^{2+}\) are the primary factors responsible with no measurable contribution from AMPK. With chronic exposure, AMPK becomes only partially responsible for the acceleration in glycolysis observed. Taken together, our findings serve to emphasize that multiple factors participate in the control of rates of glycolysis. Why significant elevations in AMPK activity caused by acute AVP exposure does not participate in this control is unknown at this time and requires further investigation.

The functional significance of alterations in glycolysis caused by acute exposure to AVP is not immediately apparent. It is possible that AVP may participate in pathological remodeling of the heart at different levels and over different time frames. For instance, AVP may participate in altering the metabolic phenotype of hearts exposed to pressure overload over a short time frame and serve as an early adaptive and transient response. Over a longer time frame, continued exposure to AVP would lead to more permanent alterations in structure and metabolism, as observed in hypertrophied and failing hearts.

In the present study, H9c2 cells were used as an in vitro model system to investigate the effect of AVP on metabolic remodeling in the heart. These cells, which were originally derived from the rat embryonic ventricle, have morphological characteristics similar to those of immature embryonic cardiomyocytes and biochemical and electrophysiological properties similar to those of adult cardiac cells (21). Such cells, however, cannot be considered completely representative of cardiac myocytes. Thus, our findings must be confirmed using adult cardiac myocytes in culture and, ultimately, in intact hearts. Furthermore, future studies will be required to fully clarify the additional mechanisms responsible for increased glucose use in hypertrophied hearts.

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