Hypoxia Upregulates Glucose Transport Activity Through an Adenosine-Mediated Increase of GLUT1 Expression in Retinal Capillary Endothelial Cells

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Elevation of intracellular glucose within retinal vascular cells is believed to be an important causal factor in the development of diabetic retinopathy. The intracellular glucose concentration is regulated by both the rate of glucose metabolism and glucose transport. Because retinal hypoxia often precedes proliferative diabetic retinopathy, we have studied the regulation of the glucose transport system by hypoxia in cultured bovine retinal endothelial cells (BRECs). Because retinal ischemia is known to increase intracellular adenosine levels, which subsequently regulate hypoxia-inducible genes, such as vascular endothelial growth factor and erythropoietin, the role of adenosine and its receptor-mediated pathways has also been evaluated. Hypoxia (0.5% O_2, 5% CO_2, and 94.5% N_2) stimulated GLUT1 mRNA expression in BRECs in a time-dependent manner with an 8.9 ± 1.5-fold ($P < 0.01$) increase observed after 12 h. GLUT1 mRNA expression returned to baseline (1.4 ± 0.3-fold of control) within 12 h after reinstitution of normoxia. N^6-Cyclopentyl adenosine (adenosine A_1 receptor agonist, K_d = 1 nmol/l) did not affect GLUT1 mRNA expression at concentrations up to 1 µmol/l, while 2-*p*-(2-carboxyethyl)-phenethyl-amino-5′-*N*-ethylcarboxamidoadenosine and 5′-(*N*-ethylcalboxamido)-adenosine (adenosine A_2 receptor [A_2R] agonists, K_d = 15 and 16 nmol/l, respectively) increased mRNA levels at concentrations as low as 10 nmol/l. Maximal stimulation was 2.3 ± 0.2- and 2.1 ± 0.2-fold, respectively ($P < 0.01$). The adenosine A_{2a} receptor antagonist 8-(3-chlorostyryl)caffeine (CSC) (K_d = 100 nmol/l for A_2R) inhibited hypoxia-stimulated GLUT1 mRNA expression by 40 ± 8% at 100 nmol/l. Hypoxia upregulated GLUT1 protein expression by 3.0 ± 0.3-fold after 12 h ($P < 0.01$), but this response was attenuated by CSC ($P < 0.05$). Hypoxia increased glucose transport activity by 2.1 ± 0.3-fold ($P < 0.001$) after 12 h, a response inhibited 65% by CSC ($P < 0.01$). A protein kinase A (PKA) inhibitor (H89, 20 µmol/l) suppressed hypoxia-induced GLUT1 mRNA expression by 42 ± 9% ($P < 0.01$). These data suggest that hypoxia in BRECs upregulates glucose transport activity through an increase of GLUT1 expression that is partially mediated by adenosine, A_2R, and the cAMP-PKA pathway. *Diabetes* 47:1480–1488, 1998

Retinopathy is a major complication of diabetes mellitus often resulting in visual loss. Previous epidemiological studies (1,2) and clinical trials (3) have demonstrated that hyperglycemia is an important causal factor for the initiation and progression of microvascular dysfunction, including diabetic retinopathy. Multiple hypotheses, including nonenzymatic protein glycosylation, changes in polyol pathway activity, and alterations in diacylglycerol and protein kinase C (PKC) levels, have been suggested as common mechanisms underling the diverse vascular complications of diabetes (4–7). Two of the classic complications of diabetic retinopathy involve proliferation of endothelial cells and increased retinal vascular permeability. Because intracellular glucose concentration is at least partially regulated by glucose transport activity, and because retinal endothelial cells play a central role in maintaining retinal blood barrier function (8,9), alterations of the glucose transport system in these cells might be involved in the pathogenesis of diabetic retinopathy.

Retinal hypoxia due to progressive retinal nonperfusion is a hallmark of diabetic retinopathy and is at least partially responsible for the development of intraocular neovascularization and increased retinal vascular permeability. Although cellular hypoxia has been reported to correlate with increased glucose uptake due to elevated glucose transporter mRNA and posttranslational alterations (10–14), the mechanisms responsible for the hypoxia-induced regulation of the glucose transport system in retinal vascular cells is incompletely understood.

Adenosine concentrations markedly increase under hypoxic conditions, mainly as a result of decreased recycling of adenosine to AMP by adenosine kinase (15). Indeed, retinal ischemia increases retinal adenosine concentrations in a porcine model (16). Previously, we have reported that hypoxia regulates the expression of vascular endothelial growth factor (VEGF) and its receptor (kinase insert domain-containing receptor [KDR]) through an increase in adenosine and subse-

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A_1R, $A_{2a}R$, $A_{2b}R$, A_2R, adenosine receptors; ARE, adenylate-uridylate–rich RNA element; BREC, bovine retinal endothelial cell; BSA, bovine serum albumin; CGS21680, 2-*p*-(2-carboxyethyl)-phenethyl-amino-5′-*N*-ethylcarbox-amidoadenosine; CPA, N^6-cyclopentyl adenosine; CSC, 8-(3-chlorostyryl)caffeine; DSS, disuccinimidylsuberate; GFX, GF 109203X; H89, *N*-[2((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide; IC_{50}, inhibitory concentration 50; KDR, kinase insert domain-containing receptor; KRPB, Krebs-Ringer phosphate buffer; NECA, 5′-(*N*-ethylcalboxamido)-adenosine; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline with 0.1% Tween-20; PKA, protein kinase A; PKC, protein kinase C; UTR, untranslated region; VEGF, vascular endothelial growth factor.

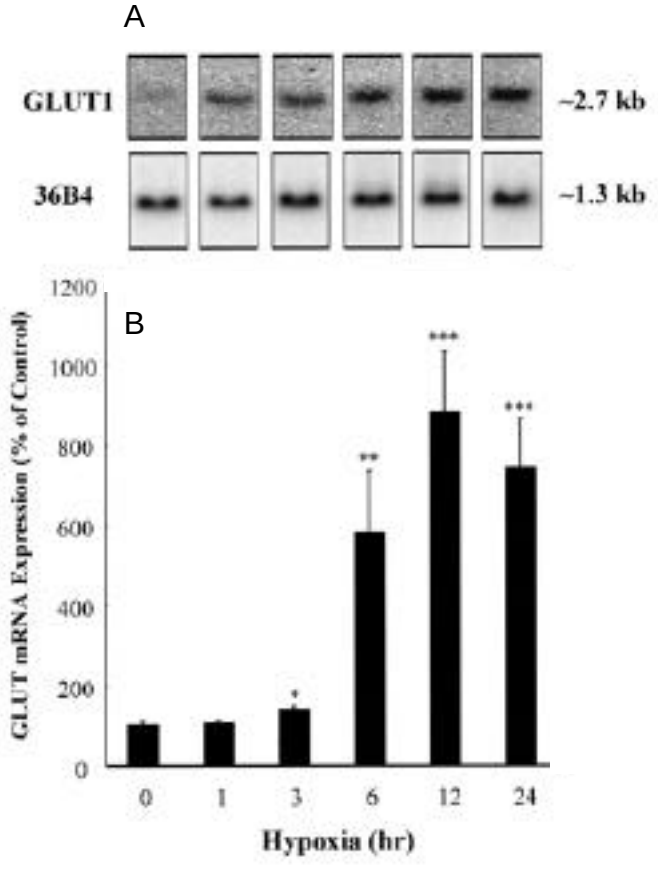

FIG. 1. Hypoxia upregulates GLUT1 mRNA expression. BRECs were exposed to 0.5% oxygen, 5% CO_2, and 94.5% N_2 for the duration indicated. A representative Northern blot using GLUT1 cDNA and control 36B4 probe (*A*) and quantitation of multiple experiments after normalization to the control signal (*B*) are shown. Results are expressed as normalized GLUT1 mRNA expression (percent of normoxic control ± SD). *P < 0.05, **P < 0.01, ***P < 0.001.

quent activation of the cAMP–protein kinase A (PKA) pathway by the adenosine A_2 receptor (A_2R) (17,18). Erythropoietin, whose expression is regulated by hypoxia in a similar manner (19), is also induced by adenosine through the A_2R (20).

In the present study, we have examined the hypothesis that hypoxia can upregulate the glucose transport system in microvascular bovine retinal endothelial cells (BRECs). Because retinal hypoxia is a major component of diabetic retinopathy, and because adenosine and its receptors have been shown to mediate hypoxic effects in other systems, we have investigated the hypoxia-induced regulation of GLUT1 and evaluated the role of adenosine, its receptors, and PKA in mediating this response. We find that hypoxia increases GLUT1 mRNA concentration, protein expression, and activity through an adenosine-mediated mechanism involving the A_2R and the cAMP-PKA pathway.

RESEARCH DESIGN AND METHODS

Materials. Plasma-derived horse serum was obtained from HyClone (Logan, UT). N^6-Cyclopentyl adenosine (CPA), 5′-(*N*-ethylcalboxamido)-adenosine (NECA), 2-*p*-(2-carboxyethyl)-phenethyl-amino-5′-*N*-ethylcarbox-amidoadenosine (CGS21680), and 8-(3-chlorostyryl)caffeine (CSC) were purchased from Research International Biochemicals (Natick, MA). GF 109203X (GFX) and *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H89) were purchased from Calbiochem (La Jolla, CA). Adenosine deaminase was purchased from Worthington Biochemicals (Freehold, NJ). ^{3}H-Deoxyglucose and [^{32}P]dATP were obtained from Amersham (Arlington Heights, IL). Human GLUT1 cDNA was provided by Dr. Barbara M. Kern. Rabbit polyclonal anti-GLUT1 antibody was purchased from Chemicon (Temecula, CA).

Cell cultures. Primary cultures of BRECs were isolated by homogenization and a series of filtration steps as previously described (21). Primary BRECs were grown on fibronectin- (NY Ben Reagents, New York Blood Center, NY) coated dishes (Costar, Cambridge, MA) containing Dulbecco's modified Eagle's medium with 5.5 mmol/l glucose, 10% plasma-derived horse serum, 50 mg/l heparin, and 50 U/l VEGF (Boehringer Mannheim, Indianapolis, IN). Cells were cultured in 5% CO_2 at 37°C, and media were changed every 3 days. Endothelial cell homogeneity was confirmed by immunoreactivity with anti–factor VIII antibodies analyzed by confocal microscopy. After the cells reached confluence, the medium was changed every 3 days, and only cells from passage 7–11 were used for experiments.

Hypoxia studies. Confluent cell monolayers were exposed to 0.5% O_2, 5% CO_2, and 94.5% N_2 using a Lab-Line Advanced Computer-Controlled Infrared Water-Jacked CO_2 Incubator with reduced oxygen control (model 480; Melrose Park, IL). All cells were maintained at 37°C in a constant 5% CO_2 atmosphere with oxygen deficit induced by nitrogen replacement. Cells cultured under these conditions showed no morphological changes by light microscopy after exposures exceeding 72 h, excluded Trypan Blue dye (>98%), and can be subsequently passaged normally. These conditions have been previously shown to invoke hypoxia-mediated responses in these cells and to correspond to in vivo hypoxic conditions (31). Cells incubated under standard normoxic conditions from the same batch and passage were used as controls (95% air, 5% CO_2).

Northern blot analysis. Total RNA was isolated from individual P-100 tissue culture plates using guanidium thiocyanate (22). Northern blot analysis was performed on 20–25 µg total RNA after 1% agarose-2 mol/l; formaldehyde gel electrophoresis and subsequent capillary transfer to Biotrans nylon membranes (ICN Biochemicals, Irvine, CA) and ultraviolet crosslinking using a Stratagene UV Stratalinker 2400 (La Jolla, CA). Radioactive probes were generated using Amersham Multiprime labeling kits and [^{32}P]dATP. Blots were prehybridized, hybridized, and washed in 0.5 × sodium chloride–sodium citrate and 5% SDS at 65°C with four changes over 1 h in a rotating hybridization oven (Robbins Scientific Model 400, Sunnyvale, CA). All signals were analyzed using Molecular Dynamics Computing Densitometer and PhosphoImager (Mountain View, CA), and lane loading differences were normalized using 36B4 cDNA probe.

Western blot analysis. After incubation, cells were solubilized in 100 µl of lysis buffer (1% Triton X-100, 50 mmol/l HEPES, 10 mmol/l EDTA, 10 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 1 mmol/l sodium orthovanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 2 mmol/l phenylmethylsulfonyl fluoride) and clarified by 3 min of centrifugation at 12,000 rpm. After determining protein concentration using the Bio-Rad Protein Assay (Richmond, CA), equal amounts of protein were subjected to SDS electrophoresis under reduced conditions (15 mg/ml dithiothreitol) and electrically transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH). We used 10% gels to separate proteins. After blocking with phosphate-buffered saline (PBS) with 0.1% Tween-20 (PBS-T; Bio-Rad), including 3% bovine serum albumin (BSA) (Arnel Products, New York, NY) for 2 h at room temperature, filters were incubated with anti-GLUT1 antibody for 1.5 h at room temperature. After washing with PBS-T, filters were incubated with ^{125}I-labeled protein A for 1 h. The signals were detected using Molecular Dynamics Computing Densitometer and PhosphorImager.

Glucose uptake studies. BRECs cultured on 12-well cluster dishes were washed with Krebs-Ringer phosphate buffer (KRPB) (136 mmol/l NaCl, 4.7 mmol/l KCl, 10 mmol/l $NaPO_4$, 0.5 mmol/l $MgCl_2$, and 1 mmol/l $CaCl_2$) with 0.2% BSA two times and incubated in KRPB for 20 min. Cells were then incubated in KRPB containing 0.5 µCi/ml ^{3}H-deoxyglucose and cold 1 mmol/l 2-deoxyglucose for 5 min. The reaction was terminated by placing the plates on ice and adding ice-cold PBS. After three washes with PBS, the cells were dissolved in 0.1% SDS. Aliquots of each sample were mixed with scintillator (Universal zol; ICN) and counted by a scintillation counter. Protein measurement was performed using the BCA protein assay kit (Bio-Rad, Hercules, CA).

Statistical analysis. Determinations were performed in triplicate, and experiments were repeated at least three times. Results are expressed as means ± SD unless otherwise indicated. Statistical analysis used the unpaired Student's t test to compare quantitative data populations with normal distributions and equal variance. Data were analyzed using the Mann-Whitney rank-sum test for populations with nonnormal distributions or unequal variance. A P value of <0.05 was considered statistically significant.

RESULTS

Hypoxia upregulates GLUT1 mRNA expression. To investigate the effect of hypoxia on GLUT1 mRNA expression in BRECs, Northern blot analysis was performed on BRECs

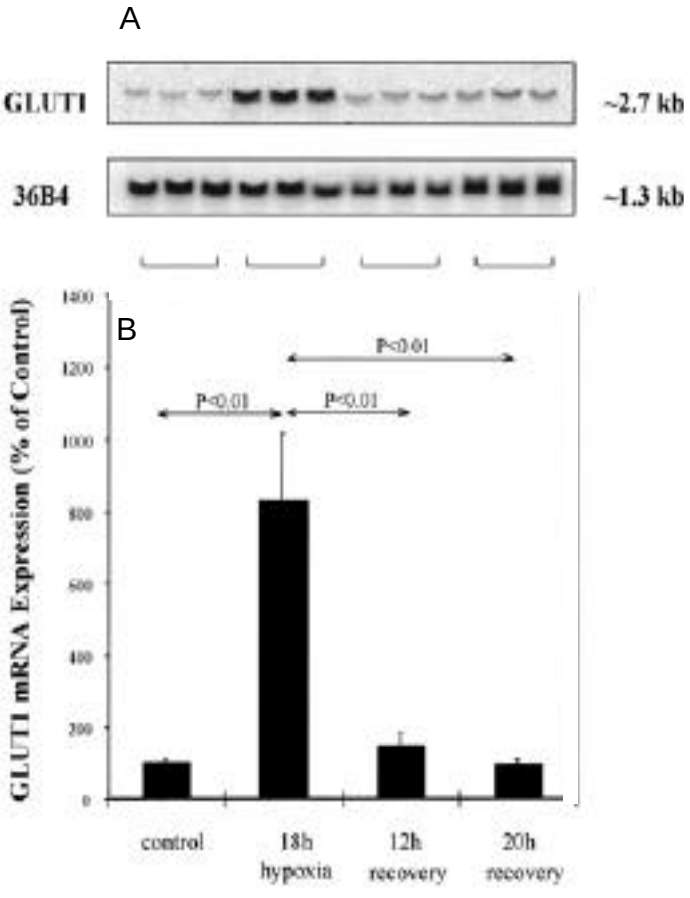

FIG. 2. Hypoxia-induced GLUT1 mRNA expression is reversed by reinstitution of normoxia. Cells were exposed to 18 h of hypoxia followed by the reinstitution normoxia (95% air, 5% CO_2) for the duration indicated. A representative Northern blot using GLUT1 cDNA and control 36B4 probe (*A*) and quantitation of multiple experiments after normalization to the control signal (*B*) are shown.

exposed to a mixture of 0.5% O_2, 5% CO_2, and 94.5% N_2 (Fig. 1). Hypoxia stimulated GLUT1 mRNA expression in BRECs in a time-dependent manner with an initial statistically significant increase observed after 3 h ($P < 0.05$) and elevated expression maintained for over 24 h. Maximal hypoxia-induced GLUT1 mRNA expression of 8.9 ± 1.5-fold ($P < 0.01$) was observed after 12 h. Elevated expression was maintained over 36 h (data not shown).

Hypoxia-induced GLUT1 mRNA expression is reversed by reinstitution of normoxia. The response of hypoxia-induced GLUT1 mRNA expression to reinstitution of normoxia was assessed by returning cells to normal oxygen conditions after 18 h of hypoxic exposure (Fig. 2). Hypoxia increased GLUT1 mRNA 8.3 ± 1.9-fold ($P < 0.01$). Reinstitution of normoxia for 12 and 24 h reduced GLUT1 mRNA levels to 1.4 ± 0.3- and 1.0 ± 0.1-fold of control, respectively ($P < 0.01$). Neither of these two conditions were significantly different from control levels.

Adenosine receptor agonists increase GLUT1 mRNA expression. Because it is well documented that hypoxia increases intracellular adenosine levels (15,16), we investigated whether adenosine analogs could mimic the hypoxia-induced increase of GLUT1 mRNA. CPA is a stable adenosine A_1 receptor (A_1R) agonist whose inhibitory concentration 50 (IC_{50}) for A_1R and for A_2R binding is ~1.0 and 600 nmol/l, respectively (23,24). Exposure to CPA for 24 h did not affect GLUT1 mRNA expression at concentrations up to 1,000 nmol/l, but it did increase mRNA expression at higher concentrations (Fig. 3*A*). Stimulation of cells with 50 µmol/l CPA increased mRNA levels 160 ± 36% ($P < 0.01$). Induction of GLUT1 mRNA expression by high-dose CPA (50 µmol/l) was initially observed after 12 h (data not shown).

Because CPA at concentrations (<1,000 nmol/l) that selectively stimulate A_1R (24) did not increase GLUT1 mRNA levels, and because higher doses can stimulate both A_1R and A_2R, we investigated whether adenosine could increase GLUT1 mRNA expression through selective effects on the A_2R. Cells were stimulated with the adenosine A_{2a} receptor ($A_{2a}R$)-selective agonist CGS21680, whose IC_{50} for $A_{2a}R$ binding is 15 nmol/l as opposed to 2,600 nmol/l for A_1R (23). CGS21680 increased GLUT1 mRNA levels in a dose-dependent manner (Fig. 3*B*) with significant increases observed as low as 10 nmol/l (168 ± 14%, $P < 0.01$) and maximal stimulation observed at 1,000 nmol/l (225 ± 20%, $P < 0.01$). In addition, the effects of NECA, an A_2R agonist whose IC_{50} for A_2R binding is 16 nmol/l (23), were also evaluated. NECA increased GLUT1 mRNA expression in a dose-dependent manner (Fig. 3*C*) with significant increases observed as low as 10 nmol/l (183 ± 13%, $P < 0.01$) and maximal response observed at 10 µmol/l (209 ± 20%, $P < 0.01$). NECA-induced GLUT1 mRNA expression was initially observed after 6 h (data not shown).

Adenosine receptor antagonists inhibit the hypoxic induction of GLUT1 mRNA. To determine whether the hypoxia-induced increase of GLUT1 mRNA could be prevented by blocking adenosine receptor activation, the effects of the $A_{2a}R$ antagonist CSC and adenosine-degrading enzyme, adenosine deaminase, were evaluated. The $A_{2a}R$ antagonist CSC, whose IC_{50} is 25 µmol/l for A_1R binding and 100 nmol/l for A_2R binding, inhibited the hypoxic response at 100 nmol/l, 1 µmol/l, and 10 µmol/l by 40 ± 8.1 ($P < 0.01$), 37 ± 13.3 ($P < 0.05$), and 56 ± 6.9% ($P < 0.01$), respectively. These are concentrations expected to be selective for blocking A_2R (Fig. 4). Although not as potent as CSC, adenosine deaminase (10 U/ml) inhibited the hypoxic induction of GLUT1 mRNA by 24 ± 8.1% ($P < 0.05$).

Hypoxia and adenosine agonists increase GLUT1 protein expression. Hypoxia increased GLUT1 protein concentration in a time-dependent manner with statistically significant increases observed after 1 h and maximal stimulation observed after 24 h (Fig. 5). Hypoxia of 12- and 24-h duration increased GLUT1 protein levels by 3.0 ± 0.3- ($P < 0.01$) and 6.3 ± 0.5-fold ($P < 0.01$), respectively.

The A_2R agonist NECA (100 nmol/l) increased GLUT1 protein in a time-dependent manner with statistically significant increases first observed after 6 h and a maximal stimulation of 209 ± 19.5% ($P < 0.01$) observed after 24 h (Fig. 6).

Adenosine receptor antagonists inhibit hypoxia-induced GLUT1 protein expression. To investigate whether hypoxia-induced increases of GLUT1 protein could be suppressed by blocking adenosine receptor activation, BRECs were exposed to the A_2R-selective inhibitor CSC under normoxic and hypoxic conditions. CSC (10 µmol/l) did not significantly effect GLUT1 protein levels under normoxic conditions (Fig. 7). Hypoxia induced a nearly twofold increase in GLUT1 protein ($P < 0.01$), and this response was inhibited 25 ± 8.7% ($P < 0.05$) by CSC.

Hypoxia-stimulated glucose transport activity is inhibited by an A_2R antagonist. Because hypoxia increases both GLUT1 mRNA and protein expression, the ability of hypoxic

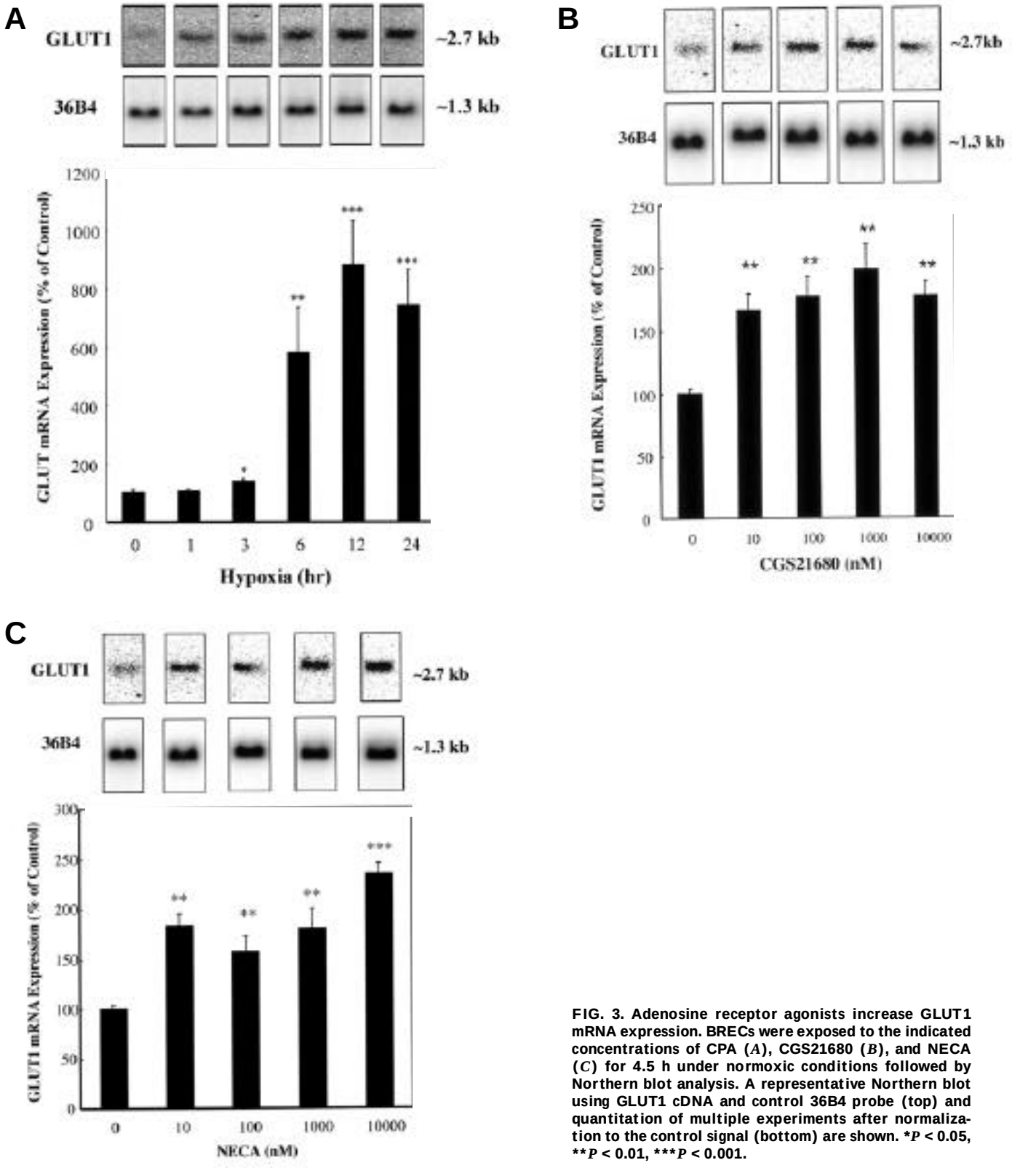

FIG. 3. Adenosine receptor agonists increase GLUT1 mRNA expression. BRECs were exposed to the indicated concentrations of CPA (*A*), CGS21680 (*B*), and NECA (*C*) for 4.5 h under normoxic conditions followed by Northern blot analysis. A representative Northern blot using GLUT1 cDNA and control 36B4 probe (top) and quantitation of multiple experiments after normalization to the control signal (bottom) are shown. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

conditions to alter glucose transport activity in BRECs was evaluated. Hypoxia increased 2-deoxyglucose uptake in a time-dependent manner with initial statistically significant increases observed after 6 h and a maximal 6.3 ± 0.6-fold ($P <$ 0.01) increase observed after 24 h (Fig. 8*A*).

Because A_2R antagonists inhibited hypoxia-induced GLUT1 mRNA (Fig. 4) and protein expression (Fig. 7), the effect of A_2R inhibition on hypoxia-induced glucose transport was evaluated (Fig. 8*B*). Hypoxia of 12-h duration increased glucose transport activity by 207 ± 31% ($P <$

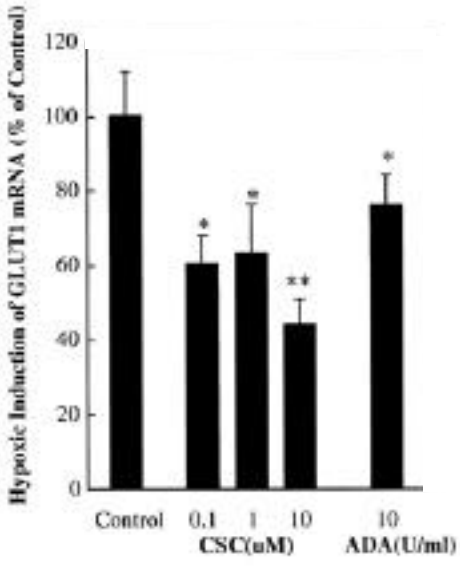

FIG. 4. Adenosine receptor antagonists inhibit the hypoxia-induced expression of GLUT1 mRNA. Immediately before the initiation of hypoxia, BRECs were treated with the indicated dose of CSC or adenosine deaminase (10 U/ml). After 12 h, Northern blot analysis was performed. Quantitation of multiple experiments after normalization to the control signal are shown. *P < 0.05, **P < 0.01.

0.001). CSC (10 µmol/l), a specific antagonist of A_2R, had no significant effect on basal glucose transport but suppressed the hypoxia-induced glucose transport activity by 65% (P < 0.01 vs. hypoxia alone).

Hypoxic induction of GLUT1 mRNA is partially mediated by the cAMP-PKA pathway. Stimulation of the A_1R inhibits adenylcyclase, whereas A_2R stimulation activates adenylcyclase, resulting in activation of cAMP-PKA pathway. To investigate whether adenylcyclase activation might induce GLUT1, cells were stimulated with the stable cAMP analog dibutyryl-cAMP, and GLUT1 mRNA expression was evaluated (Fig. 9*A*). Dibutyryl-cAMP (50 µmol/l) increased GLUT1 mRNA expression 50 ± 8.9% (P < 0.05) after 4 h. To determine whether cAMP activation of PKA was involved in the hypoxic induction of GLUT1 mRNA, cells were exposed to 12 h of hypoxia and various concentrations of H89, a selective inhibitor for PKA (Fig. 9*B*). H89 (20 µmol/l) inhibited the hypoxic induction of GLUT1 mRNA by 42 ± 8.5% (P < 0.01). A_1R activation stimulates phosphatidylinositol turnover leading to activation of PKC. However, 5 µmol/l of the PKC inhibitor GFX did not inhibit the hypoxia-induced increase of GLUT1 mRNA (data not shown).

DISCUSSION

Five distinct isoforms of mammalian facilitative glucose transporters have been cloned, each sharing 40–60% overall amino acid homology but differing in their distribution and functional properties (25). Among these glucose transporters, GLUT1 is the most widely distributed, regulates basal glucose uptake, and is reported to be concentrated in endothelial and epithelial cells that form occluding junctions (26). In the eye, the blood-retinal barrier is maintained by retinal capillary endothelial cells and retinal pigment epithelial cells. The predominant glucose transporter in these cells is GLUT1 (27,28).

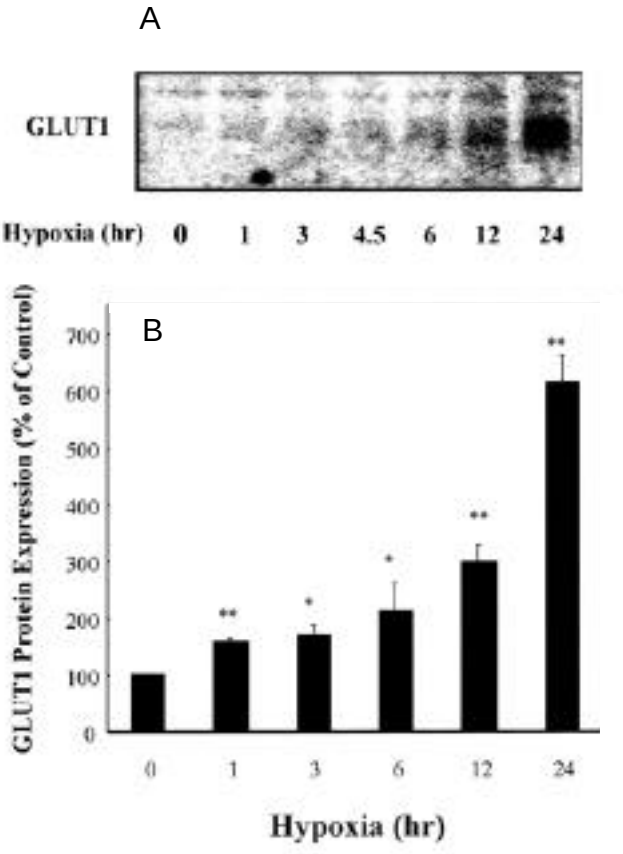

FIG. 5. Hypoxia increases GLUT1 protein expression. BRECs were exposed to hypoxia for the indicated periods. Solubilized cell lysates were subjected directly to Western blot analysis using anti-GLUT1 antibody. A representative Western blot (*A*) and quantitation of multiple experiments (*B*) are shown. Results are expressed as GLUT1 protein expression (percent of control ± SD). *P < 0.05, **P < 0.01.

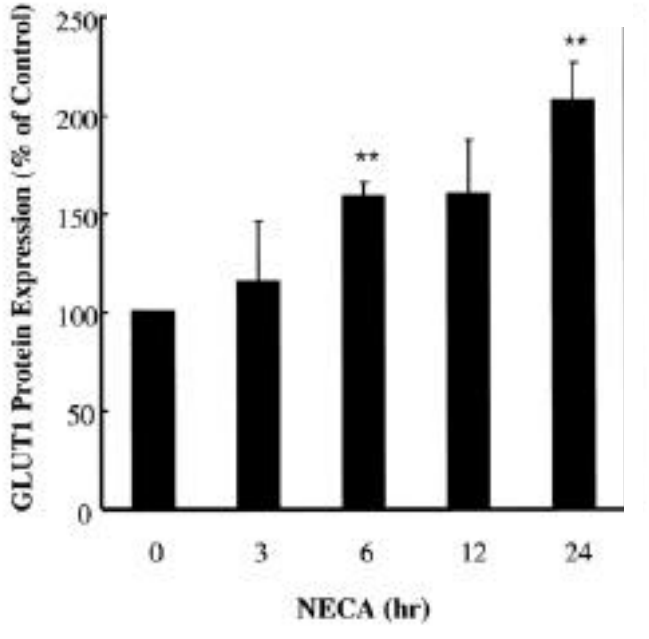

FIG. 6. Adenosine agonist increases GLUT1 protein expression. BRECs were stimulated with 100 nmol/l of NECA for the indicated periods under normoxic conditions. Quantitation of multiple experiments are shown. **P < 0.01.

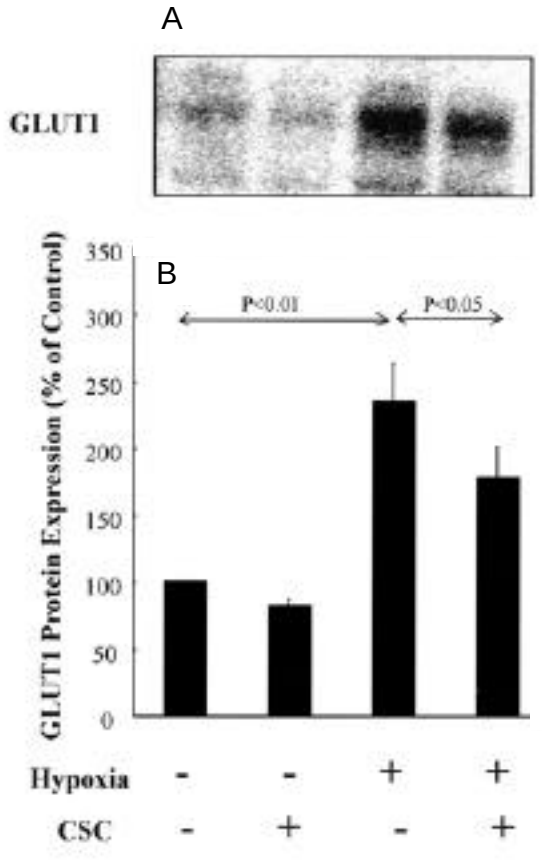

FIG. 7. Adenosine receptor antagonists inhibit the hypoxic induction GLUT1 protein. Immediately before the initiation of hypoxia, BRECs were treated with CSC (10 μmol/l), and Western blot analysis was performed after 12 h. A representative Western blot (*A*) and quantitation of multiple experiments (*B*) are shown.

In the present study, we present data suggesting that hypoxia upregulates GLUT1 mRNA and protein expression, resulting in increased glucose uptake into capillary microvascular BRECs. Furthermore, hypoxia partially mediates this response through elevated concentrations of adenosine that activate A_2R, resulting in increased concentrations of cAMP and activation of PKA.

Hypoxic induction of glucose uptake through increased expression of GLUT1 is supported by several findings. Cells exposed to 0.5% oxygen increased GLUT1 mRNA levels 8.9-fold after 12 h, and GLUT1 protein concentrations increased 6.3-fold after 24 h. There was a concurrent 6.3-fold increase in 2-deoxyglucose uptake after 24 h. Hypoxia-induced GLUT1 mRNA expression has been reported in bovine aortic endothelial cells (11) and smooth muscle cell (29). In the large vessel endothelial cells (11), stimulation (ninefold) was similar to that observed in this study for microvascular BRECs even though other receptor-mediated characteristics are known to differ (21,30). In contrast, the stimulation in L6 muscle cells was sixfold (29). Reinstitution of normoxia also reversed the hypoxia-induced expression of GLUT1 mRNA as has been demonstrated for other oxygen-sensitive adenosine-mediated genes such as VEGF (31).

Hypoxia is known to induce substantial increases in adenosine (15), mainly due to decreased recycling of adenosine to AMP by adenosine kinase (15). Adenosine can mediate multiple cellular functions through cell surface adenosine receptors. Indeed, the regulation of erythropoietin (19,32), VEGF, and its receptor KDR are regulated in this manner (17,18,33,34). Adenosine mediates its effects through cell surface receptors, which act via G proteins and couple to adenylcyclase and ion channels (35,36). Adenosine receptors possess seven transmembrane helical structures (37) and are categorized as A_1, A_{2a}, A_{2b}, A_3, and possibly other A_1

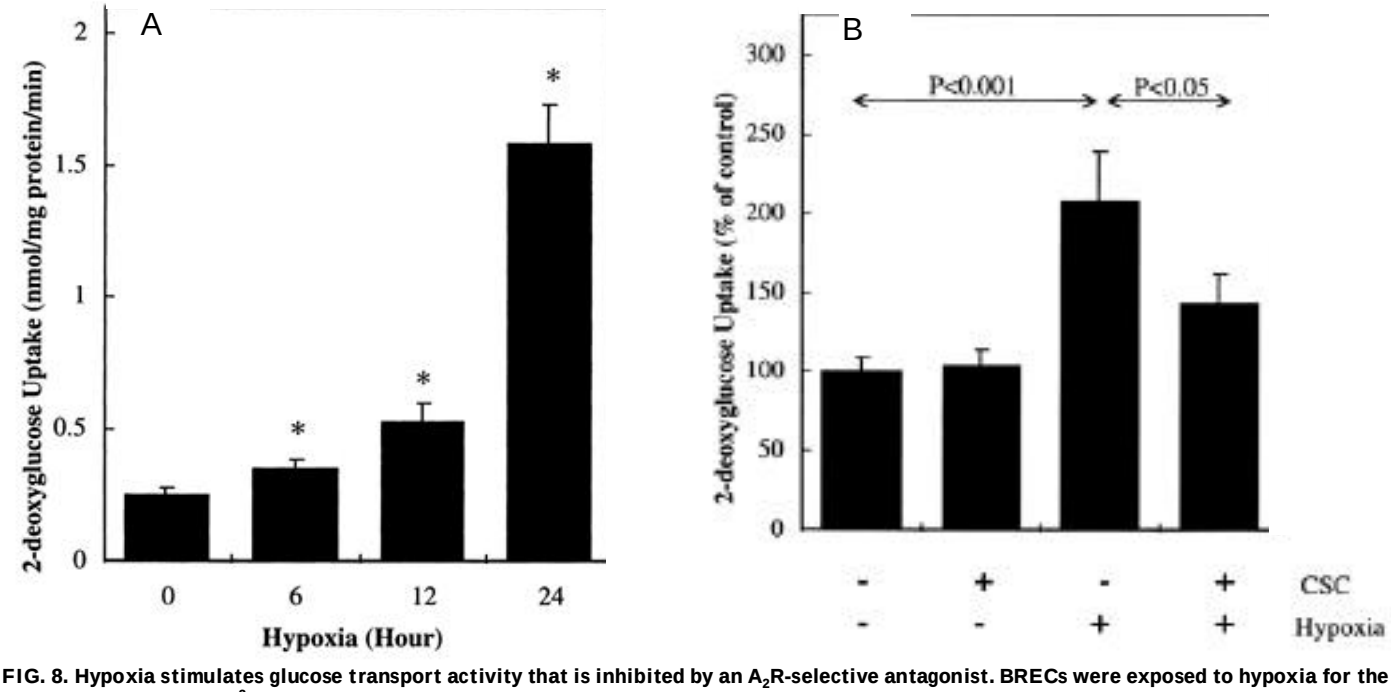

FIG. 8. Hypoxia stimulates glucose transport activity that is inhibited by an A_2R-selective antagonist. BRECs were exposed to hypoxia for the indicated duration, and ^{3}H-deoxyglucose uptake was measured (*A*). Immediately before the initiation of hypoxia, BRECs were exposed to 10 μmol/l CSC, and ^{3}H-deoxyglucose uptake was measured after 12 h (*B*).

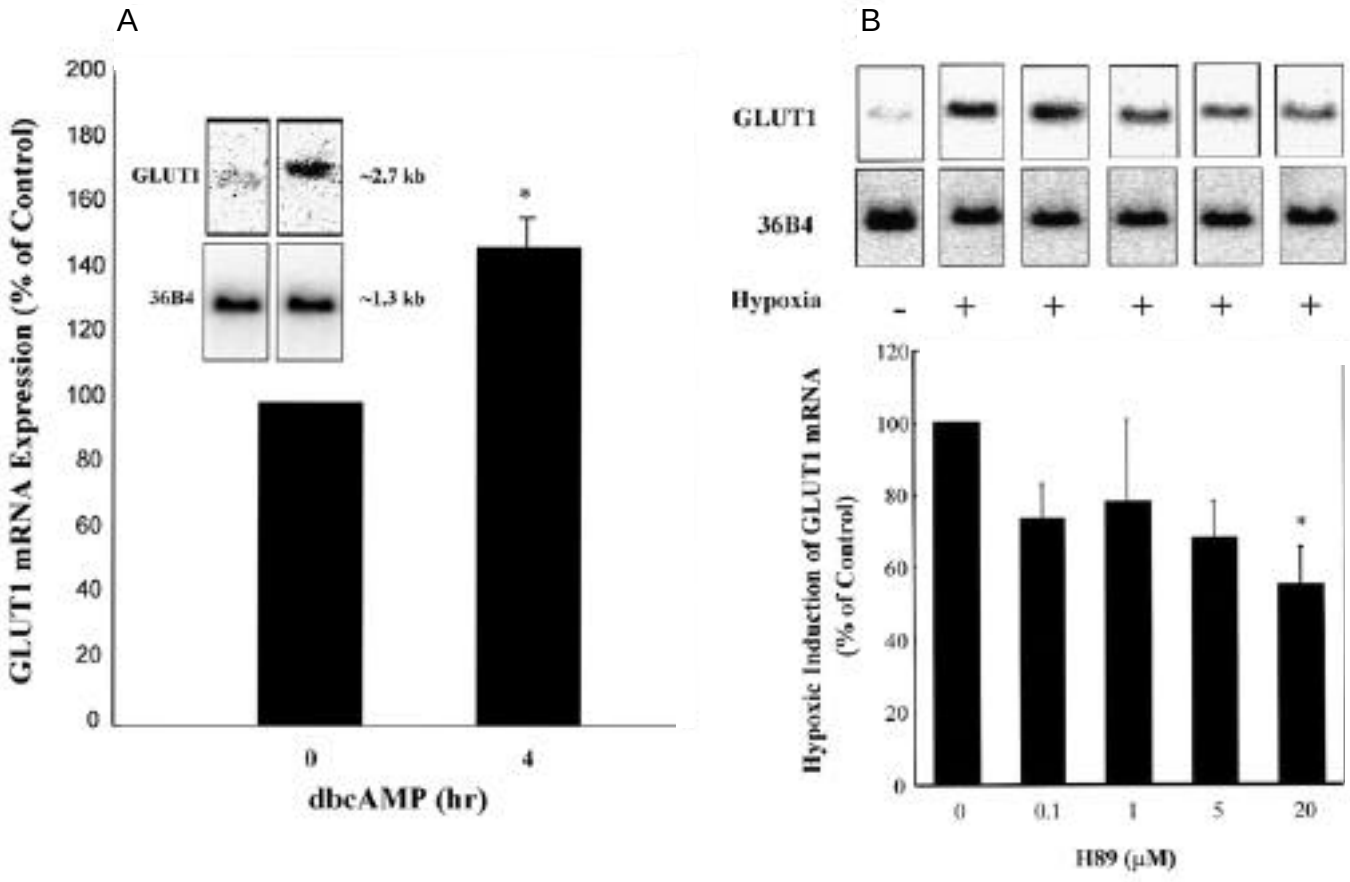

FIG. 9. cAMP stimulates GLUT1 mRNA expression, while inhibition of PKA suppresses the hypoxic induction of GLUT1 mRNA expression. BRECs were stimulated under normoxic conditions with 100 μmol/l dibutyryl-cAMP for 6 h, and GLUT1 mRNA expression was assessed by Northern blot analysis (*A*). Immediately before the initiation of hypoxia, BRECs were treated with 20 μmol/l H89 (a selective PKA inhibitor), and Northern blot analysis was performed after 12 h (*B*). Representative Northern blots using GLUT1 cDNA and control 36B4 probe (top and inset) and quantitation of multiple experiments after normalization to the control signal (bottom) are shown. *$P < 0.05$.

subtypes by pharmacological interaction with a variety of agonists and antagonists (36).

The selective involvement of A_2R in mediating the hypoxic induction of GLUT1 is supported by several findings. CPA, an A_1R agonist, showed little effect at concentrations up to 1,000 nmol/l, but it increased GLUT1 mRNA expression at higher concentrations (Fig. 3*A*). Because the IC_{50}s for A_1R and A_2R are 1.0 and 680 nmol/l, respectively (23,24), these data suggest that hypoxic induction of GLUT1 mRNA expression is partially mediated through A_2R. In contrast, the A_2R-selective agonist CGS21680 increased GLUT1 mRNA levels at concentrations as low as 10 nmol/l (Fig. 3*B*). Because the IC_{50} of this agonist are 15 and 2,600 nmol/l for A_2R and A_1R, respectively (23), these data again suggest that the hypoxic response is partially mediated by the A_2R.

There are two A_2R subtypes, $A_{2a}R$ and $A_{2b}R$. CGS21680 is a more potent agonist of $A_{2a}R$ than of $A_{2b}R$, while NECA stimulates both equally. Because both CGS21680 (Fig. 3*B*) and NECA (Fig. 3*A*) had very similar effects, a predominant role of $A_{2a}R$ is suggested.

The role of A_2R in mediating the hypoxic induction of GLUT1 mRNA is further supported by the ability of CSC (an $A_{2a}R$-selective antagonist) and adenosine desaminase (an enzyme that degrades adenosine) to inhibit this response (Fig. 4). Because adenosine desaminase does not enter intact cells, and because its effect was not greater than that observed for the $A_{2a}R$-selective antagonist CSC, the role of $A_{2a}R$ and extracellular adenosine stimulation is further supported. Finally, GLUT1 protein expression responded in a similar manner to GLUT1 mRNA expression with regard to hypoxia (Fig. 5), adenosine receptor stimulation (Fig. 6), and A_2R inhibition (Fig. 7).

The role of cAMP and PKA in the hypoxia-induced expression of GLUT1 is supported by several findings. Activation of A_2R increases cAMP concentrations, whereas activation of A_1R decreases them (35). Our previous studies have shown that in retinal vascular cells, hypoxia and A_2R agonists increase intracellular concentrations of cAMP, while A_1R agonists do not inhibit forskolin-induced cAMP expression (18). In the present study, the finding that cAMP increases GLUT1 mRNA supports the role of A_2R in the hypoxia-mediated induction of GLUT1. The involvement of PKA is supported by inhibition of hypoxia-induced GLUT1 expression by H89, a selective inhibitor of PKA (Fig. 9*B*). The manner in which PKA activates GLUT1 awaits further investigation.

Hypoxia also stimulates PKC (38,39) and *src*-related pathways (40,41). However, the PKC-selective inhibitor GFX did not inhibit the hypoxic stimulation of GLUT1 expression, suggesting that the PKC pathway does not have a major role in hypoxic regulation of this gene in BRECs. This finding again implicates mediation through A_2R rather than A_1R, since A_1R stimulation stimulates the PKC pathway (35).

Overall, in BRECs, GLUT1 regulation by hypoxia is similar to that of VEGF, as we have previously reported (18), in that adenosine, A_2R, and subsequent stimulation of the cAMP-PKA pathway are involved. Concerted regulation of VEGF and GLUT1 has been reported in response to ischemia and hypoxia due to mRNA stabilization (12). Three adenylate-uridylate-rich RNA elements (AREs) in the 3′-untranslated region (UTR) of VEGF mRNA form a hypoxia-inducible RNA-protein complex. An RNA-protein complex with similar sequence and protein binding characteristics has been identified for GLUT1 (14). The increase in mRNA stability of GLUT1 in response to agonists such as phorbol ester, cAMP, and tumor necrosis factor-α is correlated with increased binding of specific proteins to AREs in a region of the GLUT1 3′-UTR that normally directs rapid mRNA turnover (42). Thus, it is possible that hypoxia may increase protein binding to the AREs in the 3′-UTR of these genes by an adenosine-, A_2R-, and cAMP-PKA–mediated pathway. Hypoxia can also induce transcriptionally mediated regulation of genes, such as VEGF (43–45), GLUT1 (13), and glycolytic enzymes (46). Hypoxia inducible factor-1 and its binding elements mediate this response. Other factors such as AP1 (47), SP1 (48), and the nuclear factor-IL6 site (49) are also involved in hypoxic gene regulation. Further studies will be required to determine the detailed mechanisms by which adenosine-mediated activation of the cAMP-PKA signaling pathway increases GLUT1 mRNA.

In summary, we have demonstrated that hypoxia upregulates glucose transport activity in microvascular BRECs partially through an adenosine-mediated increase of GLUT1 mRNA and protein expression. Furthermore, the data suggest that adenosine is signaling through A_2R, resulting in increased cAMP levels and PKA activation. One could postulate that the retinal nonperfusion characteristic of diabetic retinopathy increases glucose transport, inducing elevated intracellular glucose concentrations and resulting in additional microvascular compromise that further worsens diabetic retinopathy.

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