Effect of Hyperglycemia on Human Monocyte Activation

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Abstract: Our recent study defined the chemokine-induced human monocyte signaling under normoglycemic condition. To explore the hyperglycemia-induced monocyte signaling, we performed adhesion, migration, and transmigration assays on human monocytes obtained from THP-1 cell line in the presence of normal (5 mM) and high (10 and 20 mM) glucose concentrations without chemokines. We observed augmented ($P < 0.01$) monocyte adhesion to human umbilical vein endothelial cell monolayer at 10 than 5 mM glucose with no further increase at 20-mM glucose concentration ($P < 0.07$ vs 10 mM; $P < 0.01$ vs 5 mM). But incremental increases in monocyte migration ($P < 0.01$), transmigration ($P < 0.01$), and stress fiber response ($P < 0.01$) were observed at 10- and 20-mM glucose concentrations in comparison to 5-mM glucose concentrations. We found gradational increase ($P < 0.01$) in phosphorylation of Akt ^{S473} and glycogen synthase kinase (GSK3β^{S9}) in hyperglycemia (10 and 20 mM) when compared with 5 mM glucose. Furthermore, hyperglycemia (both 10 and 20 mM)–treated monocyte showed up-regulated phosphorylation of p101 and p110γ subunits of PI-3 kinase in comparison to 5 mM glucose. Hyperglycemia-induced monocyte migration was restored to basal levels in the presence of PI-3 kinase inhibitor, LY. These observations imply that modest hyperglycemia per se, as is commonly observed in diabetic individuals, is a potent stimulator of monocyte activity even without chemokines.

Key Words: hyperglycemia, THP-1, monocyte, p101, p110γ

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Diabetes mellitus (DM) is a vascular risk with the prevalence of atherosclerotic cardiovascular events increased 2 to 4 folds when compared with those without diabetes.[1] However, the cellular/molecular mechanism whereby atherosclerosis is accelerated in DM is poorly understood. Although dyslipidemia, which commonly accompanies DM, is a recognized risk for cardiovascular disease, it is becoming increasingly apparent that suboptimal glycemic control may accelerate cardiovascular disease in DM.[2] Epidemiological studies have hinted at the independent role of postprandial hyperglycemia on cardiovascular disease.[3] However, the direct influence, if any, of hyperglycemia on atherogenesis remains unclear. Increased expression of vascular cellular adhesion molecules in the diabetic endothelial cell has been shown in in vitro[4] and in vivo studies.[5] Furthermore, short-term hyperglycemia promotes monocyte adhesion to rat thoracic aorta[6] and to human aortic endothelial cells.[7]

We have recently demonstrated the role of Akt–glycogen synthase kinase (GSK) on chemokine-induced monocyte activation in humans during normoglycemic conditions.[8] In this study, we sought to explore the role of glucose per se (without chemokine activation) on the phenotypic characteristics and molecular signaling of human monocytes. We reasoned that because monocytes are the trigger cells for the atherosclerotic process, we should start by examining the effects of both normoglycemia and hyperglycemia, within the ranges observed in patients with diabetes, on monocyte functions and signaling. We show that such hyperglycemia greatly increases monocyte adhesion to endothelium, migration, and transendothelial migration by congruent changes through enhanced pseudopodia formation. Our results show that hyperglycemia-induced monocyte activity up-regulate AKT-GSK phosphorylation by activation of unique class 1B PI-3 kinase (PI3K) isoform, p101 and p110γ subunits. Inhibition of PI3K pathway abolishes hyperglycemia-induced monocyte activity.

MATERIALS AND METHODS

Cell Culture

We chose THP-1 cells from ATCC (American Type Culture Collection) as a model. These cells were cultured in RPMI 1640 (Mediatech, Manassas, VA) as described,[8] and cell viability was assessed by trypan blue exclusion in every experiment. Hyperglycemic (10 and 20 mM) condition was maintained by adding appropriate amounts of glucose to the media. To examine signaling in euglycemia and hyperglycemia, THP-1 cells were cultured in corresponding glycemic states for 48 hours in regular (10% fetal bovine serum containing) media and then for another 24 hours in serum-starved media (no fetal bovine serum) before performing experiments. Osmotic control was achieved by using equimolar concentration of mannitol corresponding to different glycemic media (5, 10, and 20 mM glucose) in every experiment.

Migration Assay Using the Boyden Chamber

Overnight serum starved cells were kept under euglycemia (5 mM) or hyperglycemia (10 or 20 mM), stained with calcein-AM (25 μg in 5 μL DMSO/mL media) (Molecular Probes, Carlsbad, CA), and washed, and migration assays performed with or without the specific PI3K inhibitor (LY) and AKT inhibitor AKT-IV (both from Calbiochem, Gibbstown, NJ).[9] Euglycemia (5 mM glucose) was maintained in the upper chamber irrespective of glucose concentration. On the contrary, corresponding euglycemic (5 mM) and hyperglycemic (10 and 20 mM glucose) media were added in the bottom chamber of the well.

Western Blot Analysis

Primed cells were lysed in lysis buffer (RIPA [radioimmunoprecipitation assay]) supplemented with protease inhibitor cocktail, phosphatase inhibitor, and sodium orthovanadate.[10]

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Protein concentrations were measured by BCA reagents. Equal amounts of protein were loaded onto sodium dodecyl sulfate–polyacrylamide gels electroblotted on nitrocellulose (Bio-Rad, Hercules, CA) membranes. After blocking for 1 hour with 5% nonfat dry milk in TBS-T (1% Tween-20), proteins were probed with appropriate antibody (anti-GSK3βS^{9} [Cell Signaling, Danvers, MA], GSK3β [BD Transduction, San Diego, CA], anti-pAKT1/2/3 serine 473 [Santa Cruz, Santa Cruz, CA], and total AKT [Santa Cruz]). After overnight incubation with the primary antibody, the blot was washed 3 times in TBS-T, incubated for an hour with the specific secondary antibody, and developed with Super Signal West chemiluminescence (Pierce ThermoScientific, Rockford, IL). For immunoblot analysis, the x-ray bands were scanned; pixel density was measured by using a densitometer and National Institutes of Health (NIH) image analysis program.

Immunoprecipitation Assay

Equal amount of protein from each lysed sample was incubated overnight with phosphoserine antibody (Millipore, Billerica, MA) and protein A sepharose beads at 4°C rotator in microfuge tubes.[11] Overnight incubated samples were washed 3 times with the same lysis buffer at 4500 revolutions per minute for 5 minutes in 4°C. Same amount of Laemmli buffer was added to the pellet after removing the supernatant. Protein loaded gels were electroblotted on nitrocellulose membrane and probed with p101 (Upstate [Millipore], Temecula, CA) and p110γ (Santa Cruz) antibody and with their corresponding secondary antibody. The membranes were developed with Super Signal West chemiluminescence (Pierce ThermoScientific), and image densitometry was measured by NIH image analysis software.

THP-1 Adhesion Assay to Human Umbilical Vein Endothelial Cell

Adhesion studies were performed under human umbilical vein endothelial cell (HUVEC) static condition with euglycemia (5 mM)–treated or hyperglycemia (10 or 20 mM)–treated THP-1 cells. Overnight-starved THP-1 cells were stained with calcein-AM and allowed to adhere to the compact monolayer. Adherent monocytes were measured in a spectrofluorometer (Spectrafluor; TECAN, Mississauga, Ontario, Canada) with Delta Soft 3 software at an excitation wavelength of 485 ηm and emission at 530 ηm as described previously.[8]

Transendothelial Migration

Thick rat tail collagen (BD Biosciences, Bedford, MA) was prepared as described.[12] Human umbilical vein endothelial cells were seeded in 96-well microplate and grown in M199 supplemented media. Transendothelial migration of THP-1 cells was performed within a week after a compact HUVEC monolayer on the collagen base was established as described.[13] Media from the HUVEC monolayer was removed, and THP-1 cell suspension (100 μL) was added. At 1.5 hours, media containing THP-1 cells were removed and washed with phosphate-buffered saline (PBS) before adding 100 μL RPMI media. Photomicrograph was performed by differential phase microscopy, and data were quantified accordingly.

Rhodamine-Phalloidin Staining of THP-1 Cells

Being nonadherent cells, THP-1 monocytes are difficult to fix and stain on a slide. Hence, we followed the protocol as published,[14,15] with minor modifications. With adequate aseptic precaution, coverslips were placed in a 6-well plate on which 200 μL of THP-1 cell suspension (15×10^4 cells/mL) was added. For homogenous sedimentation of cells, the plates were spun at 2000 revolutions per minute for 30 seconds. The sedimented cells on the cover slips were fixed with 1% to 2% paraformaldehyde (pH 7.36) for 15 minutes at room temperature and gently washed with 1× PBS. Subsequently, the cells were permeabilized in 0.1% Triton-X100 in 1× PBS (pH 7.4) for 1 minute. Finally, the cells were treated with rhodamine-phalloidin (Molecular Probes) stain for 15 minutes and processed for confocal microscopy.

RESULTS

Our recent report[8] demonstrated that chemoattractant-induced monocyte activity (adhesion, migration, and transmigration) is up-regulated through phosphorylation of AKT^{S9} and GSK3β^{S479} in the presence of normoglycemia. The current sets of experiments were designed to evaluate the role of hyperglycemia on phenotypic characteristics of monocytes and the signaling pathways involved in monocyte activation.

Effect of Hyperglycemia on Monocyte Adhesion

To mimic conditions in people with DM, we maintained THP-1 cells in the presence of moderate hyperglycemia (10 or 20 mM) for 72 hours before conducting our experiments (Fig. 1, A and B). We compared our results to monocyte adhesion at normoglycemia (5 mM). Monocyte adhesion to HUVEC monolayer was significantly ($P < 0.01$) increased during hyperglycemia (10 and 20 mM glucose) than normoglycemia (5 mM glucose) at 90 minutes. Interestingly, there was no augmentation of monocyte adhesion when glucose concentration was increased from 10 to 20 mM.

Effect of Hyperglycemia on Transendothelial Migration of THP-1 Cells

Monocyte transmigration through the endothelium is an essential step before transformation to macrophages (Fig. 1, C and D).[13] Hyperglycemia (both 10 and 20 mM glucose) significantly increased ($P < 0.01$) transendothelial migration of monocytes compared with normoglycemia (5 mM glucose). In contrast to adhesion results, there was dose-dependent increase ($P < 0.03$) of monocyte transmigration when glucose was increased from 10 to 20-mM concentrations.

Role of AKT-GSK Axis in Hyperglycemia Induced THP-1 Cells Activation (Fig. 2, A–D)

We observed up-regulated ($P < 0.02$) phosphorylation of AKT^{S473} at both 10- and 20-mM glucose concentrations when compared with normoglycemia (5 mM glucose) (Fig. 2, A and B). On further evaluation of downstream signaling cascades, we observed increased ($P < 0.01$) phosphorylation of GSK3ß^{S9} in both hyperglycemic (10 and 20 mM) concentrations when compared with normoglycemia (5 mM) (Fig. 2, C and D).

Hyperglycemia Up-Regulates PI3K Class IB Pathway in THP-1 Cells (Fig. 3, A–D)

The mechanism of activation of AKT-GSK axis by hyperglycemia in human monocytes is relatively unknown. PI-3 kinase misinforms and its subclasses are key signaling molecules that regulate various cellular activities including motility. We found that unique isoforms of PI3K, p101 (regulatory molecule) (Fig. 3, A and B) and p110γ (catalytic molecule) (Fig. 3, C and D), are up-regulated by increased ($P < 0.05$) phosphorylation during hyperglycemia than normoglycemia.

Role of AKT on THP-1 Migration

THP-1 cells were serum starved overnight, migration capabilities tested in the Boyden chamber after 72-hour exposure to normoglycemia/hyperglycemia, and readings obtained at 1 and

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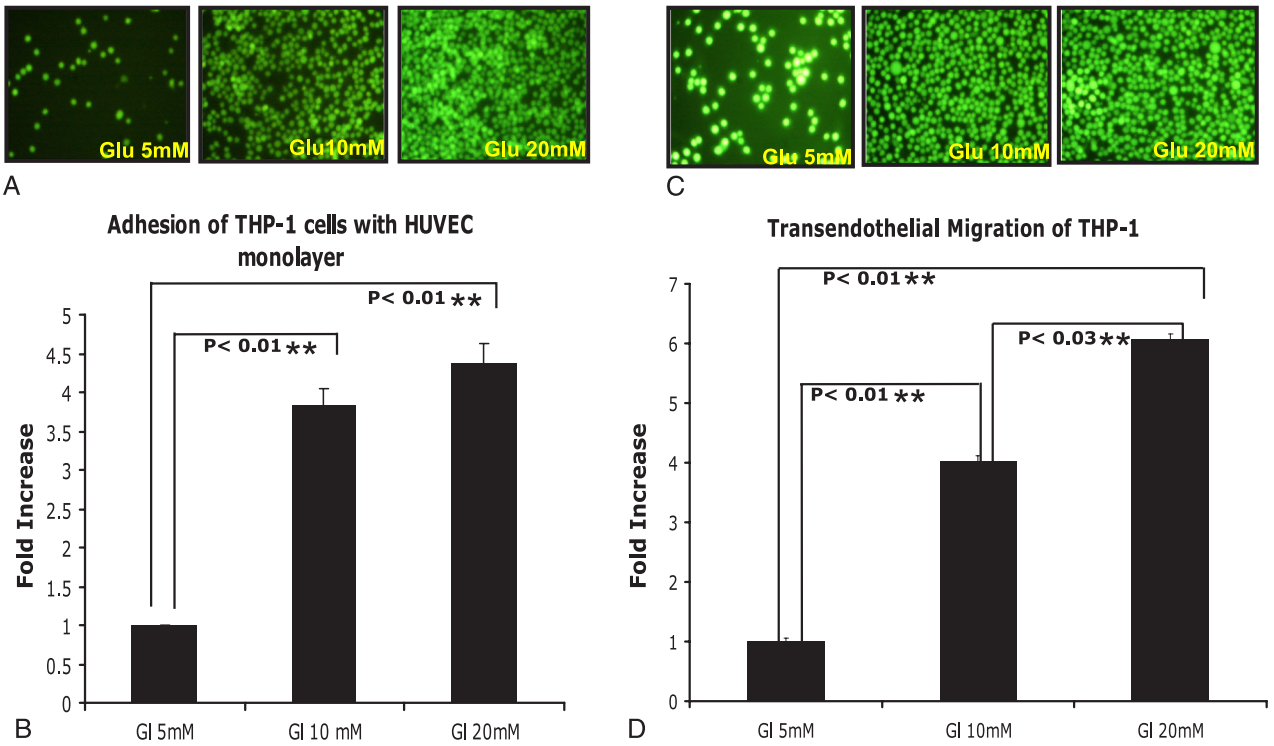

FIGURE 1. A, Photomicrographs show adhesion of THP-1 cells with compact monolayer of HUVEC in normoglycemia (Glu 5 mM) and hyperglycemia (Glu10 and 20 mM). B, Quantitative data analysis reveals significant increases (as fold changes) ($P < 0.01$**) in adhesion of THP-1 cells with compact monolayer of HUVECs in hyperglycemia (both 10 and 20 mM) in comparison to normoglycemia. No significant differences in adhesion between the hyperglycemic conditions (10 and 20 mM) are observed. The data are representative of 3 or more experiments and show mean ± SEM. C, Photomicrographs show transendothelial migration of THP-1 cells through the compact monolayer of HUVECs seeded on thick collagen base in different glycemic conditions (Glu 5, 10, and 20 mM). D, Quantitative analysis show increased ($P < 0.01$**) transendothelial migration of THP-1 cells during hyperglycemia (Gl 10 and 20 mM) compared with normoglycemia (Gl 5 mM). There was also enhanced ($P < 0.03$**, Gl 10 vs 20 mM) transendothelial migration when glucose concentration was increased (data shown as mean ± SEM, n ≥ 3).

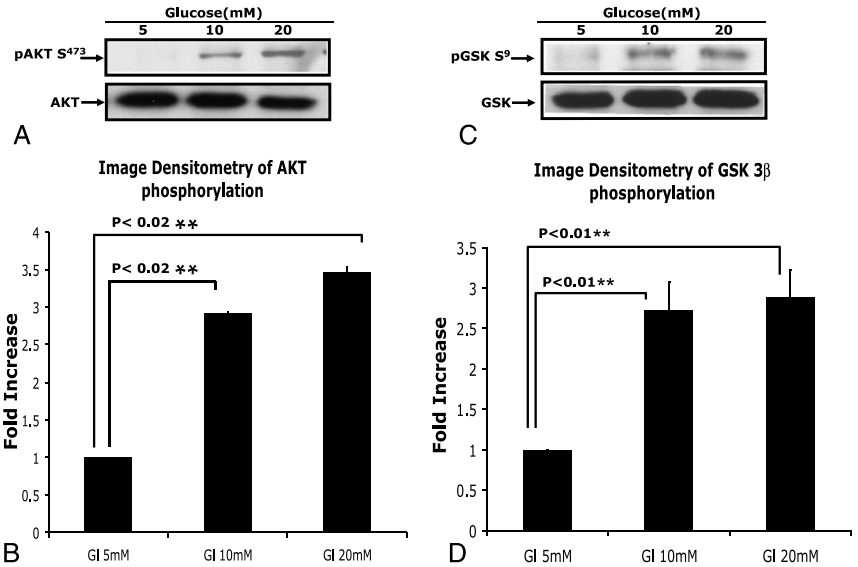

FIGURE 2. A, Immunoblot shows phosphorylation of AKT at serine 473 in different glycemic conditions (glucose 5, 10, and 20 mM). B, NIH image analysis of the immunoblot demonstrates that hyperglycemia (both Gl 10 and 20 mM)–treated THP-1 cells have significant up-regulation ($P < 0.02$**) of AKT phosphorylation at serine 473. C, Western blot shows phosphorylation of GSK3βs9 at normoglycemia (glucose 5 mM) and hyperglycemia (glucose 10 and 20 mM). D, Image densitometry of GSK 3β phosphorylation shows significant up-regulation ($P < 0.01$**) of GSK3βs9 phosphorylation in hyperglycemia (both Gl 10 and 20 mM) in comparison to normoglycemia (Gl 5 mM). Quantitative data shown as mean ± SEM, N ≥ 3.

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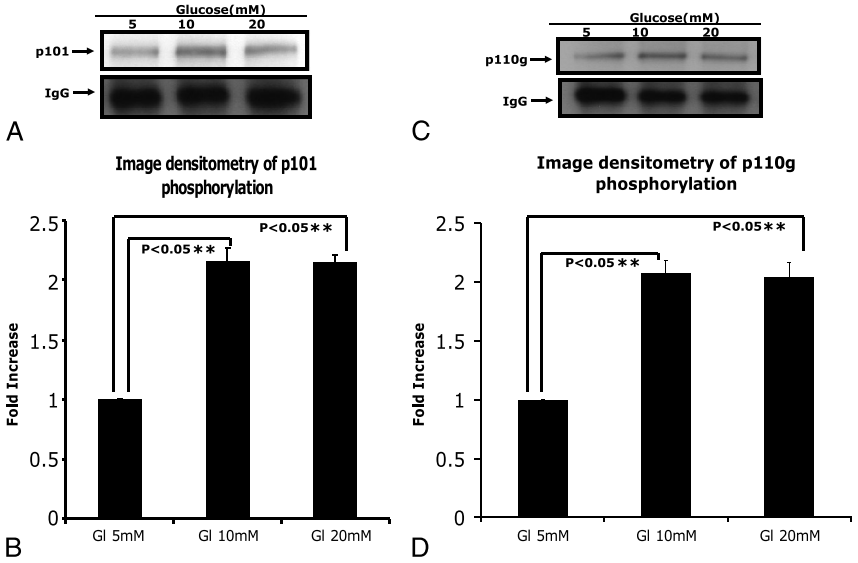

FIGURE 3. A, Western blot reveals phosphorylation of p101 in different glycemic conditions (glucose 5, 10, and 20 mM). B, Image densitometry analysis of the immunoblot exhibits that hyperglycemia (both Gl 10 and 20 mM)–treated THP-1 cells have significant up-regulation ($P < 0.05$**) of p101 phosphorylation in comparison to normoglycemia. C, Immunoblot shows phosphorylation of p110γ at normoglycemia (glucose 5 mM) and hyperglycemia (glucose 10 and 20 mM). D, Image densitometry of p110γ phosphorylation shows significant up-regulation ($P < 0.05$**) of p110γ phosphorylation in hyperglycemia (both Gl 10 and 20 mM) treated THP-1 cells in comparison to normoglycemia (Gl 5 mM). Quantitative data shown as mean ± SEM, N ≥ 3.

2 hours (Fig. 4A). There was a significant ($P < 0.05$) increase in monocyte migration at 10 and 20 mM glucose compared with 5 mM glucose. There was also a dose-dependent increase ($P < 0.04$) in monocyte migration when glucose was increased from 10- to 20-mM concentration. AKT inhibitor (AKT-IV from Calbiochem)–treated monocytes showed reversal of hyperglycemia-induced monocyte migration at both 10- and 20-mM glucose concentrations, implying a major role of AKT on glucose-induced monocyte activation.

Role of PI3K on THP-1 Migration

Normoglycemia- and hyperglycemia-treated THP-1 cells were serum starved overnight, migration capabilities tested in the Boyden chamber, and readings obtained at 1 and 2 hours (Fig. 4B). There was a significant ($P < 0.02$) increase in monocyte migration at 10 and 20 mM glucose compared with 5 mM glucose. There was also a dose-dependent increase ($P < 0.03$) in monocyte migration when glucose was increased from 10- to 20-mM concentration in both 1 and 2 hours. PI-3 kinase inhibitor (LY)–treated monocytes showed reversal of hyperglycemia-induced monocyte migration at both 10- and 20-mM glucose concentrations, implying a major role of PI3K as an upstream of AKT on glucose-induced monocyte activation.

Effect of Hyperglycemia on Filopodia

Hyperglycemia (both 10 and 20 mM glucose)–treated monocytes demonstrated a significant increase ($P < 0.001$) in pseudopodia formation in comparison to euglycemia (5 mM glucose) that was not observed under mannitol control (data not shown) (Fig. 4C).

DISCUSSION

People with type 2 diabetes (DM) are prone to accelerated atherosclerosis. Factors that contribute to this process include coexistent dyslipidemia, hypertension, hyperglycemia, and renal dysfunction, among others. However, the mechanism of accelerated atherosclerosis induced by hyperglycemia per se is under active investigation.[16] The cellular mechanisms and signaling pathways involved in hyperglycemia-induced atherosclerosis are the focus of our current investigations. In the present study, we have defined some of the initial steps of hyperglycemia-induced monocyte activation that could potentially set the stage for intravascular plaque formation and augmented atherosclerosis.[17] We have demonstrated that moderate hyperglycemia (10 and 20 mM glucose), which is commonly observed in people with DM, leads to monocyte activation, at least in part, through a unique class of PI3K isoforms, p101 and p110γ, even in the absence of chemokines. This distinctive PI3K isoform appears to be important in modulating downstream signaling of AKT-GSK axis, leading to increased monocyte activity. However, the relative contributions, if any, of other PI3K isoforms to hyperglycemia-induced monocyte activation need further study. We have recently shown that the chemokine platelet-activating factor increases human monocyte adhesion and migration via the AKT-GSK axis[8] under normoglycemic conditions. The current observations complement our published observations demonstrating that modest hyperglycemia is also a potent chemoattractant in its own right that leads to monocyte activation through largely common pathways.

We report that hyperglycemia (both 10 and 20 mM) increased adhesion (4- to 6-fold) to HUVEC monolayer when compared with normoglycemia. These results were of the same order of magnitude as observed with the chemokine platelet-activating factor[18,19] reported recently. Monocytes migrate through the vascular endothelium before transforming into macrophages. To quantitate this process of transmigration across endothelium, we performed transmigration experiments and observed augmented movement of monocytes across endothelial surfaces at both 10- and 20-mM glucose concentrations. Furthermore and in contrast to our observations in the adhesion experiments, there were incremental increases in monocyte

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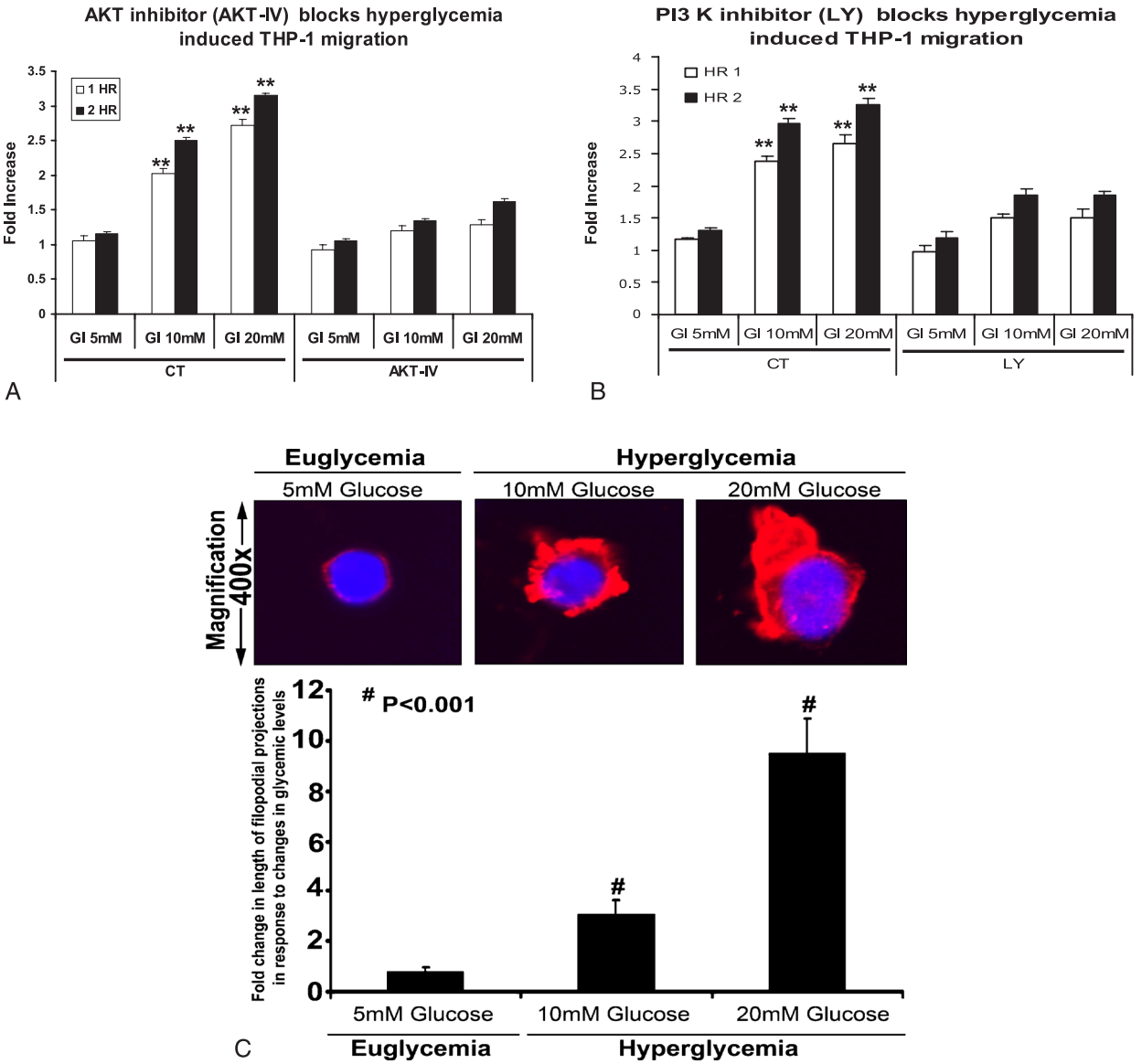

FIGURE 4. A, Boyden chamber migration assay shows fold changes in monocyte migration in different glycemic media with or without AKT inhibitor (AKT-IV). Hyperglycemia (both glucose 10 and 20 mM)–induced migrations ($P < 0.05$**) of THP-1 cells at both 1 and 2 hours (glucose 5 mM) are totally blocked on AKT inhibition. Dose-dependent increase ($P < 0.04$**) in THP-1 cells migration is also observed when glucose was increased from 10 to 20 mM. B, Boyden chamber assay shows comparison of fold changes in migration of THP-1cells at normoglycemia (Gl 5 mM) and hyperglycemia (Gl 10 and 20 mM) at 1 and 2 hours. Hyperglycemia-induced monocyte migration ($P < 0.02$**) of THP-1 cells was totally obstructed by PI3K inhibitor (LY). There was a significant ($P < 0.03$**) increase in THP-1 cell migration when glucose was increased from 10 mM to 20 mM. Quantitative data shown as mean ± SEM, N ≥ 3. C, Rhodamine-phalloidin stain reveals fold changes in filopodia in response to changes in ambient glucose concentrations. Hyperglycemia (both Gl 10 and 20 mM)–treated THP-1 cells show significant up-regulation ($P < 0.001$**) of filopodia formation when compared with normoglycemia (Gl 5 mM). Quantitative data are representative of 3 or more experiments and shows mean ± SEM.

transmigration as glucose concentrations were increased from 10 to 20 mM. It is noteworthy that we designed these experiments under moderate hyperglycemia that is not uncommonly observed in people with DM both in the postprandial and postabsorptive states, hence mimicking, at least in part, the metabolic milieu of type 2 diabetes. However, we also realize that the monocyte in a diabetic individual is subject to other modulating influences in vivo, for example, dyslipidemia, inflammatory mediators, and cytokines, to name a few. Hence, the present study was designed to tease out the effects of hyperglycemia per se on monocyte activation and was thus limited in its scope. Future studies will be necessary to evaluate the combined effects of hyperglycemia, chemokines, and other mediators on monocyte activity and functions.

The transmigration experiments as previously described[8] and the stress fiber assays were modified to test the hypotheses that moderate hyperglycemia would enhance the capabilities of activated monocytes to invade through the endothelial surface by inducing pseudopodia formation. Both these hypotheses were confirmed.

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Furthermore, PI3K-AKT signaling also plays a crucial role in modulating endothelial cell microvascular permeability and angiogenesis.[20,21] Prolonged and severe hyperglycemia (20 and 40 mM glucose for 8 days) has been shown to modulate endothelial cell proliferation[22] through PI3K and AKT phosphorylation. Extreme hyperglycemia (33 mM glucose) has been shown to stimulate endothelial cell activity via several different signaling cascades that include endothelial nitric oxide synthase[22] and PI3K/AKT/NO[23] through phosphorylation of PI3K and AKT that could lead to vascular dysfunction and atherosclerosis.[24] The translational relevance of these reports is limited because the degree of hyperglycemia (33 and 40 mM glucose) under which the experiments were conducted is incompatible with human existence beyond perhaps a few hours.

The PI3K subunits described above in hyperglycemia-induced activation of endothelial cell signaling have belonged to class IA (p85 and p110b). In contrast, our experiments have demonstrated an important role of class 1B PI3K subunits (p101 and p110γ) in hyperglycemia-induced monocyte activation. These subunits are abundantly expressed in hematopoietic cells.[25] Migration of human and mouse endothelial cells has been shown to be regulated by PI3Kβ and PI3Kγ signaling related to angiogenic modulation.[26] Genetic manipulation of PI3Kγ (PI3Kγ$^{-/-}$) in mice leads to down-regulation of inflammatory responses[27] with reduced macrophage migratory response to chemotactic stimuli.[28]

To date, 4 PI3K subclasses, classes IA, IB, III, and IV, have been described.[29,30] Three of them (classes IA, III, and IV) are primarily activated via tyrosine kinase receptor signaling, whereas class IB is activated through a unique receptor PI3Kγ. This receptor is a GPCR (G protein–coupled receptor) and appears to be present only in mammals, being abundant in white blood cells and myeloid tissue–derived cells.[31,32] The class IB PI3K pathway includes 2 isoforms, p101 and p110γ, which work as regulatory and catalytic subunits, respectively. The interaction between the 2 subunits has been unclear. Our current report is the first of its kind that has demonstrated the role of these PI3K class IB subunits in hyperglycemia-induced monocyte activation. Future studies are necessary to further define the modulatory role, if any, of these signaling molecules on human monocyte-macrophage activation and atherosclerosis in type 2 diabetes.

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