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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 (5mM) and high (10 and 20mM) glucose concentrations without chemokines. We observed augmented ($p < 0.01$) monocyte adhesion to HUVEC monolayer at 10mM than 5mM glucose with no further increase at 20mM glucose concentration ($p < 0.03$ vs. 10mM; $p < 0.01$ vs. 5mM). But incremental increase in monocyte migration ($p < 0.01$), transmigration ($p < 0.01$) and stress fibre response ($p < 0.01$) were observed at 10 and 20mM glucose concentrations in comparison to 5mM glucose concentrations. We found gradational increase ($p < 0.01$) in phosphorylation of Akt ^{S473} and glycogen synthase kinase (GSK3 β ^{S9}) in hyperglycemia (10 and 20mM) when compared to 5mM glucose. Furthermore hyperglycemia (both 10 mM and 20 mM) treated monocyte showed upregulated phosphorylation of p101 and p110 γ subunits of PI-3 kinase in comparison to 5mM 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.

Keywords

Hyperglycemia; THP-1; monocyte; p101; p110 γ

Introduction

Diabetes Mellitus (DM) is a vascular risk with the prevalence of atherosclerotic cardiovascular events increased 2-4 folds when compared to those without diabetes (1). However, the cellular/molecular mechanism whereby atherosclerosis is accelerated in DM is poorly understood. While 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 post prandial 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

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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 since 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 trans-endothelial migration by congruent changes through enhanced pseudopodia formation. Our results show that hyperglycemia induced monocyte activity upregulate AKT-GSK phosphorylation by activation of unique class 1B PI3K isoform, p101 and p110 γ subunits. Inhibition of PI3K pathway abolishes hyperglycemia induced monocyte activity.

Materials and Methods

Cell Culture

We chose THP-1 cells from American Type Culture Collection (ATCC) as a model. These cells were cultured in RPMI 1640 (Mediatech) as described (8) and cell viability was assessed by trypan blue exclusion in every experiment. Hyperglycemic (10mM and 20mM) 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 hrs in regular (10% FBS containing) media and then for another 24 hours in serum-starved media (no FBS) before performing experiments. Osmotic control was achieved by using equi-molar concentration of mannitol corresponding to different glycemic media (5mM, 10mM and 20mM 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 μ g in 5 μ L DMSO/ml media) (Molecular Probes), washed and migration assays performed with or without the specific PI3K inhibitor (LY) and AKT inhibitor AKT-IV (both from Calbiochem) (9). Euglycemia (5mM glucose) was maintained in the upper chamber irrespective of glucose concentration. On the contrary corresponding euglycemic (5mM) and hyperglycemic (10mM and 20mM glucose) media were added in the bottom chamber of the well.

Western Blot analysis

Primed cells were lysed in lysis buffer (Radio immuno Precipitation Assay (RIPA) supplemented with protease inhibitor cocktail, phosphatase inhibitor and sodium orthovanadate (10). Protein concentrations were measured by BCA reagents. Equal amounts of protein were loaded onto SDS polyacrylamide gels electro-blotted on nitrocellulose (Bio-Rad) membranes. After blocking for 1 hour with 5% nonfat-dry milk in TBS-T (1% Tween-20), proteins were probed with appropriate antibody (anti-GSK-3 β ^{S9}:Cell Signaling; GSK3 β :BD Transduction; anti-pAKT1/2/3 serine 473:Santa Cruz 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). For immunoblot analysis, the x-ray bands were scanned; pixel density measured by using a densitometer and NIH image analysis program.

Immunoprecipitation Assay

Equal amount of protein from each lysed sample was incubated overnight with phosphoserine antibody (Millipore) and protein A sepharose beads at 4°C rotator in microfuge tubes (11). Overnight incubated samples were washed three times with the same lysis buffer at 4500 rpm for 5 minutes in 4°C. Same amount of Laemmli buffer was added to the pellet after removing the supernatant. Protein loaded gels were electro blotted on nitrocellulose membrane and probed with p101 (Upstate) and p110 γ (Santa Cruz) antibody and with their corresponding secondary antibody. The membranes were developed with Super Signal West chemiluminescence (Pierce) and image densitometry were measured by NIH image analysis software.

THP-1 Adhesion Assay to HUVEC

Adhesion studies were performed under HUVEC static condition with euglycemia (5 mM) 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) with Delta Soft 3 software at an excitation wavelength of 485nm and emission at 530nm as described previously (8).

Trans-endothelial migration

Thick rat tail collagen (BD Bio sciences) was prepared as described (12). HUVEC cells were seeded in 96 well microplate and grown in M199 supplemented media. Trans-endothelial 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 μ L) was added. At 1.5 hr. media containing THP-1 cells were removed and washed with PBS prior to adding 100 μ L RPMI media. Photomicrograph was performed by differential phase microscopy and data was quantified accordingly.

Rhodamine- Phalloidin Staining of THP- 1 cells

Being non-adherent 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 μ L of THP-1 cell suspension (15×10^4 cells per ml) was added. For homogenous sedimentation of cells the plates were spun at 2000 RPM for 30 seconds. The sedimented cells on the cover slips were fixed with 1-2% paraformaldehyde (pH= 7.36) for 15 min at room temperature and gently washed with 1 $\times$ PBS. Subsequently the cells were permeabilized in 0.1% Triton-X100 in 1 $\times$ PBS (pH= 7.4) for 1 min. 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 chemo-attractant induced monocyte activity (adhesion, migration, and transmigration) is up-regulated through phosphorylation of AKT^{S9} and GSK3 β ^{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 (Fig 1a, 1b)

To mimic conditions in people with DM, we maintained THP-1 cells in the presence of moderate hyperglycemia (10 or 20mM) for 72 hours prior to conducting our experiments.

We compared our results to monocyte adhesion at normoglycemia (5 mM). Monocyte adhesion to HUVEC monolayer was significantly ($p<0.01$) increased during hyperglycemia (10mM and 20mM glucose) than normoglycemia (5mM glucose) at 90 minutes. Interestingly there was no augmentation of monocyte adhesion when glucose concentration was increased from 10 to 20mM.

Effect of Hyperglycemia on trans-endothelial migration of THP-1 cells (Fig 1c, 1d)

Monocyte transmigration through the endothelium is an essential step before transformation to macrophages (13). Hyperglycemia (both 10mM and 20mM glucose) significantly increased ($p<0.01$) transendothelial migration of monocytes compared to normoglycemia (5mM 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 (Figs 2a- 2d)

We observed upregulated ($p<0.02$) phosphorylation of AKT^{S473} at both 10mM and 20 mM glucose concentrations when compared to normoglycemia (5mM glucose) (Fig 2a, 2b). On further evaluation of downstream signaling cascades, we observed increased ($p<0.01$) phosphorylation of GSK3 β ^{S9} in both hyperglycemic (10mM and 20mM) concentrations when compared to normoglycemia (5mM) (Fig 2c, 2d).

Hyperglycemia upregulates PI3K class IB pathway in THP-1 cells (Figs 3a- 3d)

The mechanism of activation of AKT-GSK axis by hyperglycemia in human monocytes is relatively unknown. PI3K isoforms 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 3a, 3b) and p110 γ (catalytic molecule) (Fig 3c, 3d) are up-regulated by increased ($p<0.05$) phosphorylation during hyperglycemia than normoglycemia.

Role of AKT on THP-1 migration (Fig 4a)

THP-1 cells were serum starved overnight, migration capabilities tested in the Boyden Chamber following 72 hours exposure to normo/hyperglycemia and readings obtained at 1 and 2 hrs. There was significant ($p<0.05$) increase in monocyte migration at 10mM and 20mM glucose compared to 5mM glucose. There was also dose dependent increase ($p<0.04$) in monocyte migration when glucose was increased from 10mM to 20mM concentration. AKT inhibitor (AKT-IV from Calbiochem) treated monocytes showed reversal of hyperglycemia induced monocyte migration at both 10mM and 20mM glucose concentrations implying a major role of AKT on glucose induced monocyte activation.

Role of PI3K on THP-1 migration (Fig 4b)

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 hrs. There was significant ($p<0.02$) increase in monocyte migration at 10mM and 20mM glucose compared to 5mM glucose. There was also dose dependent increase ($p<0.03$) in monocyte migration when glucose was increased from 10mM to 20mM concentration in both 1hr and 2hrs. PI3K inhibitor (LY) treated monocytes showed reversal of hyperglycemia induced monocyte migration at both 10mM and 20mM glucose concentrations implying a major role of PI3K as an up stream of AKT on glucose induced monocyte activation.

Effect of Hyperglycemia on Filopodia (Fig 4c)

Hyperglycemia (both 10mM and 20mM glucose) treated monocytes demonstrated 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).

Discussion

People with type 2 diabetes (DM) are prone to accelerated atherosclerosis. Factors that contribute to this process include co-existent 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 is 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 intra-vascular plaque formation and augmented atherosclerosis (17). We have demonstrated that moderate hyperglycemia (10mM and 20mM glucose), that is commonly observed in people with DM, leads to monocyte activation, at least in part, through a unique class of PI3K isoforms, p101 and p110 γ 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 10mM and 20mM) increased adhesion (4-6 fold) to HUVEC monolayer when compared to 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 20mM glucose concentrations. Furthermore and in contrast to our observations in the adhesion experiments, there were incremental increases in monocyte transmigration as glucose concentrations were increased from 10 to 20mM. It is noteworthy that we designed these experiments under moderate hyperglycemia that is not uncommonly observed in people with DM both in the post prandial and post absorptive 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, e.g., dyslipidemia, inflammatory mediators, 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 fibre 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.

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 cells proliferation (22) through PI3K and AKT phosphorylation. Extreme hyperglycemia

(33mM glucose) has been shown to stimulate endothelial cell activity via several different signaling cascades that include eNOS (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 are 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 IB PI3K subunits (p101 and p110 γ) in hyperglycemia induced monocyte activation. These subunits are abundantly expressed in hematopoietic cells (25). Migration of human and mouse endothelial cells have been shown to be regulated by PI3K β and PI3K γ signaling related to angiogenic modulation (26). Genetic manipulation of PI3K γ (PI3K γ -/-) in mice leads to downregulation of inflammatory responses (27) with reduced macrophage migratory response to chemotactic stimuli (28).

To date, four PI3K subclasses class IA, IB, III and IV have been described (29), (30). Three of them (class IA, III and IV) are primarily activated via tyrosine kinase receptor signaling while class IB is activated through a unique receptor PI3K γ . 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 two isoforms, p101 and p110 γ that work as regulatory and catalytic subunits respectively. The interaction between the two 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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Figure 1.

1a: Photomicrographs show adhesion of THP-1 cells with compact monolayer of HUVEC in normoglycemia (Glu 5mM) and hyperglycemia (Glu10 and 20 mM). **1b:** Quantitative data analysis reveals significant increased (as fold changes) ($p < 0.01^{**}$) in adhesion of THP-1 cells with compact monolayer of HUVEC in hyperglycemia (both 10mM and 20mM) in comparison to normoglycemia. No significant differences in adhesion between the hyperglycemic conditions (10mM and 20mM) are observed. (The data is representative of 3 or more experiments and shows mean $\pm$ sem). **1c:** Photomicrographs show transendothelial migration of THP-1 cells through the compact monolayer of HUVEC seeded on thick collagen base in different glycemic conditions (Glucose 5mM, 10mM and 20mM). **1d:** Quantitative analysis show increased ($p < 0.01^{**}$) transendothelial migration of THP-1 cells during hyperglycemia (Glu 10 and 2 mM) compared to normoglycemia (glu 5mM). There was also enhanced ($p < 0.03^{**}$, Glu 10 vs 20mM) transendothelial migration when glucose concentration was increased (data shown as mean $\pm$ sem, $N \geq 3$).

Figure 2.

2a: Immunoblot shows phosphorylation of AKT at serine 473 in different glycemic conditions (Glu 5, 10 and 20mM). **2b:** NIH image analysis of the immunoblot demonstrates that hyperglycemia (both glucose 10mM and 20mM) treated THP-1 cells have significant upregulation ($p < 0.02^{**}$) of AKT phosphorylation at serine 473. **2c:** Western blot shows phosphorylation of GSK3 β s9 at normoglycemia (Glu 5mM) and hyperglycemia (Glu 10 and 20mM). **2d:** Image densitometry of GSK 3 β phosphorylation shows significant upregulation ($p < 0.01^{**}$) of GSK3 β s9 phosphorylation in hyperglycemia (both glu 10 and 20mM) in comparison to normoglycemia (glu 5mM). (Quantitative data shown as mean $\pm$ sem, $N \geq 3$).

Figure 3.

3a: Western blot reveals phosphorylation of p101 in different glycemic conditions (Glu 5, 10 and 20mM). **3b:** Image densitometry analysis of the immunoblot exhibits that hyperglycemia (both glu 10 and 20mM) treated THP-1 cells have significant upregulation ($p < 0.05^{**}$) of p101 phosphorylation in comparison to normoglycemia. **3c:** Immunoblot shows phosphorylation of p110γ at normoglycemia (glu 5mM) and hyperglycemia (glu 10 and 20mM). **3d:** Image densitometry of p110γ phosphorylation shows significant upregulation ($p < 0.05^{**}$) of p110γ phosphorylation in hyperglycemia (both glu 10 and 20mM) treated THP-1 cells in comparison to normoglycemia (glu 5mM). (Quantitative data shown as mean $\pm$ sem, $N \geq 3$).

Figure 4.

4a: Boyden chamber migration assay shows fold changes in monocyte migration in different glycaemic media with or without AKT inhibitor (AKT-IV). Hyperglycemia (both glu 10 and 20mM) induced migration ($p < 0.05^{**}$) of THP-1 cells at both 1 and 2 hr (glucose 5mM) 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 20mM. **4b:** Boyden chamber assay shows comparison of fold changes in migration of THP-1 cells at normoglycemia (glu 5mM) and hyperglycemia (glu 10 and 20mM) 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 significant ($p < 0.03^{**}$) increase in THP-1 cell migration when glucose was increased from 10mM to 20mM. (Quantitative data shown as mean $\pm$ sem, $N \geq 3$). **4c:** Rhodamine Phalloidin stain reveals fold changes in filopodia in response to changes in ambient glucose concentrations. Hyperglycemia (both glu 10 and 20mM) treated THP-1 cells show significant upregulation ($p < 0.001^{**}$) of filopodia formation when compared to normoglycemia (glu 5mM) (quantitative data is representative of 3 or more experiments and shows mean $\pm$ sem).