NHE-1 and β1 integrin-dependent monocyte adhesion and migration after glucose, insulin or PPARγ stimulation

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Key words: monocytes, atherosclerosis-related functions, cell adhesion, cell migration, integrins, rosiglitazone, glucose, insulin, laminin-1, collagen type IV, endothelial cells

In the present study the effect of high glucose concentrations, insulin, PPARγ activators (rosiglitazone) and NHE-1 inhibitors (cariporide) in atherosclerosis-related functions of human monocytes was investigated. Monocyte adhesion to laminin-1, collagen type IV and endothelial cells, as well as monocyte migration through the same substrates were studied. Incubation of the monocyte suspension with high glucose concentrations, insulin and rosiglitazone induced all the studied atherosclerosis-related functions of the monocytes. In all these functions the addition of cariporide counteracted the activity of glucose, insulin and rosiglitazone. The use of antigen for β1 integrin also counteracted the activity of the above in monocyte adhesion in all three substrates. The data of the present study suggests that PPARγ activation in monocytes induces atherosclerosis, and that NHE-1 and β1 integrin play an important role in the beginning of atherosclerosis.

Hormones and factors related to obesity and metabolic syndrome, like glucose, insulin, leptin and oxidative stress, can act directly to monocytes inducing their atherosclerosis-related functions such as monocyte adhesion and migration through extracellular matrix proteins and vassal endothelium, CD36 expression and oxidized LDL phagocytosis.8,11,12 In atherosclerotic inflammation sites PPARγ (peroxisome proliferator-activated receptor γ) regulates the production of the cytokines TNFα and Interleukin-1β,13 NO synthase activity and expression of adhesion molecules VCAM-1 and ICAM-1, which induce monocyte adhesion.14 Monocytes bind to VCAM-1 and ICAM-1 via P-selectin glycoprotein ligand 1 (PSGL1) and integrins.15 Monocyte adhesion to vassal endothelium induces the expression and release of cytokines and chemotactic factors, like monocyte chemotactic protein-1 (MCP-1), interleukin-2 (IL-2) and interferon γ (IFNγ), which attract more monocytes and lead to monocyte migration to subendothelial space.15-17 These functions are regulated by monocyte integrin receptors.18

Monocytes interact with extracellular matrix and cause alterations in proteins of subendothelial basement membranes, like laminin-1 and collagen type IV. Monocytes can also adhere to and migrate through laminin-1 and laminin-8.19 Laminin-1 interacts with itself and with other extracellular matrix molecules, leading to the formation of a network that contributes to the basement membrane architecture.20 Collagen type IV, which is found in high concentrations in endothelial basilar

Introduction

The ubiquitously expressed sodium hydrogen exchanger (NHE-1) plays a central housekeeping role in all cells regulating cell volume and internal pH (pHi). NHE-1 is a transmembrane protein that exchanges intracellular H+ for extracellular Na+ across plasma membrane and consists of a N-terminal hydrophobic part of 10 transmembranic domains that catalyzes the ion exchanging process and a C-terminal hydrophilic part that is responsible for the regulation of physiological pHi.1-3 NHE-1 inhibitor cariporide binds to Na+-binding site of the antiport.4 NHE-1 activation is stimulated by cell surface receptors, including integrin receptors.5 The signals are regulating the affinity of the internal hydrogen-binding site of the exchanger. This antiport is also a plasma membrane anchoring protein for the cytoskeleton and specifically actin fibers.5,6 This function is important for cell adhesion to extracellular matrix proteins and cell migration. NHE-1 activity has been related to adhesion and spreading of fibroblasts in extracellular matrix.5 Stimulation of NHE-1 is related with the monocyte activation. NHE-1 activity has been also implicated in vascular smooth muscle cell proliferation as related to diabetes and atherosclerosis.7 High glucose concentration can induce atherosclerosis-related functions, such as cell adhesion and migration, via NHE-1 activation.8 It has been reported that hyperglycemia stimulates NHE-1 activity in vassal smooth muscle and in mesangial cells of kidney glomerulus.8,10

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Submitted: 08/13/10; Accepted: 12/18/10
DOI: 10.4161/cam.5.3.14534
membranes, reassembles leading to the formation of a sheet-like network that is mainly responsible for the structure and the morphology of the basement membrane and plays major role in biological functions such as cell adhesion, cell migration and differentiation of many types of cells. Laminin-1 and collagen type IV, which is known to interact with several types of cells, are recognized from integrin receptors. Monocyte integrin receptors are mainly alpha2beta1 (α2β1), alpha1beta1 (α1β1), and alpha6beta1 (α6β1). α2β1 integrin receptor is involved in hyperglycemia, diabetes mellitus and atherosclerosis. In monocyte adhesion α6β1 and β2 integrins are involved.

Therefore we studied the role of NHE-1 activity monocyte adhesion and migration through the basilar membrane proteins laminin-1 and collagen type IV as well as through endothelial cells, induced by glucose, insulin and PPARγ activation by rosiglitazone, a thiazolidinedione PPARγ-agonist. We also studied the role of β1 integrin receptor subunit in monocyte adhesion to the above substrates induced by glucose, insulin and rosiglitazone.

Results

Monocyte adhesion either to laminin or to collagen type IV was significantly increased in the samples where either glucose, or insulin or rosiglitazone was added. Increase was observed also in monocyte adhesion to endothelial cells. The use of cariporide, the NHE-1 inhibitor, together with glucose, insulin or rosiglitazone inhibited monocyte adhesion to all the above substrates, laminin, endothelial cells or collagen type IV (Fig. 1). The use of GF109203X (Gf), the inhibitor of all PKCs, and Go6976 (Go), an inhibitor of PKCoa and PKCB (PKCB, and PKCBp), decreased the monocyte adhesion to collagen type IV caused by glucose or insulin (Fig. 2). Addition of wortmanin, a PI3K inhibitor, had the same effect as cariporide and the PKC inhibitors (Fig. 2). Monocyte adhesion to BSA (Fig. 3) and plastic-polystyrene well (Fig. 4) substrate was also investigated. Adhesion to BSA and plastic is lower than the level of control and the use of the antibody for β1 integrin decrease the adhesion capacity below the control levels. Control value in adhesion to laminin-1 is 0.304 ± 0.067, in adhesion to collagen type IV 0.297 ± 0.009, in adhesion to endothelial cells 0.295 ± 0.013, while in adhesion to BSA and plastic these values are 0.246 ± 0.007 and 0.167 ± 0.034 respectively. Addition of glucose or insulin did not significantly increase the amount of adhered monocytes to these substrates.

The role of β1 integrin receptor subunit in monocyte adhesion to the above substrates was studied by using the antibody for β1 integrin (anti-β1). The antibody was added together with glucose, insulin or rosiglitazone. The addition of anti-β1 cause a decrease in the amount of adhering monocytes in control samples (the antibody lowers the control levels in adhesion to laminin-1, collagen type IV and to endothelial cells to 0.229 ± 0.004, 0.228 ± 0.006 and 0.241 ± 0.005 respectively) as well as in samples where glucose, insulin or rosiglitazone was added in laminin, collagen type IV or endothelial cell substrate (Fig. 5), indicating the involvement of β-1 integrin in the signaling pathway of glucose, insulin or rosiglitazone leading to the adhesion of monocytes to all the above substrates.

Monocyte migration was observed through laminin, collagen type IV, as well as through endothelial cells as substrates, under the influence of glucose, insulin, rosiglitazone and NHE-1 inhibitor, cariporide. The addition of glucose, insulin and rosiglitazone increased monocyte migration, whereas the addition of cariporide significantly reversed the effect of these activators in migration through laminin-1, endothelial cells or collagen type IV (Fig. 6). Moreover, use of rosiglitazone together with glucose had not any statistically significant difference in monocyte adhesion (Fig. 1) or migration (Fig. 6) compared to the use of glucose.
or rosiglitazone alone. The use of the PKC inhibitors Gf and Go decreased the transmigration of the monocytes through collagen type IV substrate caused by glucose (Fig. 7). Besides, inhibition of monocyte migration was observed when PI3K was inhibited (Fig. 7). Glucose-induced monocyte adhesion and migration through laminin-1 and endothelial cells under the influence of PKC and PI3K inhibitors have been also tested. PKC and PI3K inhibitors appeared to counteract the effect of glucose in adhesion and migration through these substrates as well (data not shown).

The addition of rosiglitazone caused an increase in intracellular pH of monocytes. Inhibition of NHE-1 activity, using cariporide, reversed this effect of rosiglitazone, as well as the effect of glucose or insulin action (Table 1). Furthermore, addition of cariporide alone had no statistically significant difference compared to the control samples.

**Discussion**

It has been reported that glucose can stimulate cell functions of NHE-1 associated with the cytoskeleton and that high glucose concentrations induce changes in integrin expression in cultured proximal tubular and glomerular epithelia. In addition NHE-1 plays an important role as a plasma membrane-cytoskeleton anchoring protein and in cell migration by influencing cell volume, intracellular pH and connection between plasma membrane and cytoskeleton. It is also known that the signal for the activation of NHE-1 is not dependent on glucose metabolism but rather on glucose binding to glucose transporters. Previous data presented by our group indicated that glucose binding at the outer domain of the glucose transporter affects the activation of NHE-1. High glucose concentration can influence atherosclerosis-related monocyte functions, via NHE-1 activation. The results of the present study corroborate previous observations indicating that inhibition of NHE-1 counteracts the effect of insulin as well and indicate for the first time a similar protective effect of cariporide treatment on rosiglitazone action in all the studied atherosclerosis-related functions of monocytes.

Glucose can induce atherosclerosis by non-enzymatic glycosylation of proteins and lipids, oxidative stress or Protein Kinase C (PKC) activation. PKC is determinant in mediating the NHE-1 activation in response to growth factors and other stimuli, while PKC agonists can stimulate NHE-1. In red blood cells glucose via Glut-1 activates PKC and p42/44 MAPK, which can activate NHE-1. The results of the present study indicate that PKC, and in particular PKCc, PKCB and PKCβII isoforms, plays an important role in the signaling pathway from glucose that leads to monocyte adhesion and transmigration.

It is reported that PI3K is responsible for Glut-4 receptor translocation and is involved in intracellular glucose metabolism. According to the data of the present study, inhibition of phosphatidylinositol-3-kinase (PI3K) can also counteract the effect of glucose in atherosclerosis-related functions, indicating that PI3K mediates atherosclerosis-related signal transduction by glucose.

Insulin can act through PI3K stimulating PKC and Akt, which causes apoptosis or through Ras/c-Raf/Erk inducing cell growth. Increased insulin concentrations act through a pro-atherogenic pathway mediated by p38 MAPK increasing VCAM-1 adhesion molecule expression, which is involved in monocyte adhesion.

Results of the present study show that
The data of the present study indicate that the activator of peroxisome proliferators-activated receptors γ (PPARγ), rosiglitazone has similar effect with glucose and insulin and induces monocyte adhesion to laminin, collagen type IV and endothelial cells and also induces monocyte migration through these substrates. The combined use of rosiglitazone and glucose does not seem to have any additional inductive effect on monocyte adhesion and migration compared to the effect of glucose or rosiglitazone alone, thus rosiglitazone at the concentration administered can increase cell adhesion and migration of monocytes to the highest degree possible. Furthermore, rosiglitazone action affects monocytes in a similar way to glucose or insulin, increasing intracellular pH at the level of glucose or insulin samples by activating NHE-1.

The data of the present study indicate that glucose, insulin or rosiglitazone induce monocyte adhesion and migration through laminin-1, collagen type IV and endothelial cells substrates as well. Monocytes interact with extracellular matrix and cause alterations such as phosphorylation to proteins of the subendothelial basement membranes, like laminin-1 and collagen type IV. In human vasculature the most common laminins are laminin-8 and -10. It is known that laminin-1 and collagen type IV are recognized by monocyte integrin receptors. Experiments of monocyte adhesion to BSA or plastic showed that glucose, insulin or rosiglitazone cannot affect monocyte adhesion to these substrates, indicating that integrin receptors play a crucial role in signal transduction that leads to increased monocyte adhesion. It has been reported that monocyte adhesion is mediated by α6β1 and β2 integrins. Moreover, laminin-1 binds to α1β1, α2β1, α6β1, α6β4, α7β1 integrins, laminin-8 binds to α6β1 and laminin-10 binds to α3β1 and α6β1, which suggests

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![Figure 4](image-url) Figure 4. Number of monocytes attached to plastic (polystyrene well) after a 30 min incubation at 37°C in the presence of either glucose 20 mM, glucose 20 mM + cariporide 20 nM, insulin 50 μU/ml or insulin 50 μU/ml + cariporide 20 nM. The mean values of five independent experiments are presented. Error bars indicate standard deviation (SD).

![Figure 5](image-url) Figure 5. Number of monocytes attached to a laminin-1, endothelial cells and collagen type IV coated polystyrene well after a 30 min incubation at 37°C in the presence of glucose 20 mM, glucose 20 mM + cariporide 20 nM, insulin 50 μU/ml or rosiglitazone 10 μM and a 45 min incubation on ice at the presence of the monoclonal antibody for β1 integrin subunit. The mean values of at least six independent experiments are presented. Error bars indicate standard deviation (SD). *p < 0.05 versus control sample, **p < 0.05 versus glucose sample.
that α6β1 integrin is a common receptor for all the above types of laminin. The use of a β1 integrin subunit antibody inhibited the increased monocyte adhesion to laminin-1, to collagen type IV and to endothelial cells caused by glucose, insulin or rosiglitazone. These results indicate the essential role of β1 integrin subunit in the signal transduction that leads to increased monocyte adhesion to these substrates upon influence of glucose, insulin or rosiglitazone.

As it is previously referred, NHE-1 can bind to cytoskeleton proteins.⁵,³⁴ Integrins activate RhoA GTPase, which activates ROCK (Rho kinase). ROCK phosphorylates the myosin light chains and stimulates actin fibers reorganization.⁵⁶ Also, NHE-1 signal transduction towards actin fibers is mediated by RhoA and ROCK⁵,⁶ and NHE-1 activity acts downstream of RhoA to regulate activation of integrins.⁶ Therefore, inhibition of NHE-1 by the use of cariporide can inhibit the signal that stimulates integrin receptors.

Under physiological conditions, monocytes from the bloodstream adhere and migrate to tissues and differentiate into macrophages or dendritic cells. Monocyte adhesion and migration from the vessel lumen through basilar membrane or through tissues is a process that includes binding and unbinding to extracellular matrix substances.³⁷ Monocyte adhesion to vassal epithelial cells induces the expression and release of cytokines and chemotactic factors, like monocyte chemotactic protein-1 (MCP-1), interleukin-2 (IL-2) and interferon-γ (IFNγ), which attract more monocytes and lead to monocyte migration to subendothelial space.¹⁵,¹⁷ In subendothelial space monocytes uptake oxidized low density lipoproteins (oxidized LDL) and turn into foam cells leading to atherosclerotic plaque formation.⁵⁸ According to the above in combination with the data of the present study, hyperglycemia and hyperinsulinemia in pathological conditions like diabetes, obesity and metabolic syndrome can lead to increased monocyte adhesion and migration. Moreover, the use of rosiglitazone as an antidiabetic drug can also induce these atherosclerosis-related functions of monocytes.

In conclusion, glucose induces monocyte adhesion and migration through laminin-1, collagen type IV and endothelial cells, stimulating PKCo, PKCB and PKCB₁₁ isoforms, PI3K and NHE-1, while insulin appears to act through the same signaling pathway to glucose in order to induce the above functions. The data of the present study also indicate that there is a correlation between monocyte adhesion and migration through the studied substrates under the influence of glucose, insulin and rosiglitazone and inhibitors of NHE-1, PKC and PI3K. Furthermore, β1 subunit of integrin receptor appears to be the main responsible molecule for monocyte adhesion in laminin-1, collagen type IV and endothelial cells. Moreover, PPARγ activation in monocytes by the use of rosiglitazone also induces monocyte adhesion and migration via NHE-1, indicating the central role of NHE-1 in the signaling pathways that lead to atherogenic functions. Therefore, pharmacological inhibition of NHE-1 by cariporide treatment can counteract the effect of high glucose concentrations, insulin and PPARγ activation and result in an effective treatment of vascular complications in atherosclerosis.

**Methods**

**Monocyte isolation.** Monocytes were isolated from whole blood from healthy individuals as previously described in reference 26. Thirty milliliters of whole blood heparinized (3.8 U/ml heparin) were placed in falcon tubes with 6 ml PBS (phosphate-buffered saline) plus 1 mM EDTA, pH 7.2. 10 ml Fiqoll-paque plus was placed under the blood with a gauge spinal needle. The falcon tubes were centrifuged at 400 g for 20 min at room temperature (RT) without break and at minimum acceleration. The Peripheral Blood Mononuclear Cells layer (PBMCs) formed above the Red Blood Cells layer was aspirated by a Pasteur pipette and placed in falcon tubes. Thirty-five milliliters of PBS (with 1 mM EDTA, pH 7.2) were added. The falcon tubes were centrifuged at 150 g at RT for 10 min and monocytes washed twice and resuspended in IMDM (Iscove’s Modified Dulbeco’s Medium) plus NaHCO₃ containing 1% L-glutamine, 25 mM Hepes, 10% FBS, 1% penicillin/streptomycin 100 U/ml. In falcon tubes were placed 25 ml of 46% iso-osmotic Percoll (92.5% Percoll, 75% DPBS 10x). Twenty-five milliliters of monocyte suspension was layered over the 46% iso-osmotic Percoll
and the falcon tubes were centrifuged for 30 min at 550 g. Fifteen milliliters of the formed white layer were aspirated by a Pasteur pipette and dissolved in 35 ml of freeze PBS (containing 1 mM EDTA, pH 7.2). Monocytes were used directly in experiments or centrifuged for 10 min at 4°C and then 10 x 10^6 monocytes/ml resuspended in Freezing Medium (75% IMDM plus 15% FCS, 10% DMSO-Dimethyl Sulfoxide) and placed in Biofreeze vials. The vials are placed in Cryo-1°C/min freezing container and then in liquid nitrogen.

**Endothelial cell isolation.** A part from umbilical cord was hung vertically and the vein washed with phosphate-buffered saline (PBS) pH 7.4 containing 0.9% NaCl. The one end of the umbilical cord was closed and the vein was filled with RPMI 199 medium plus 0.5 μM, glucose 20 mM, insulin 50 μU/ml, rosiglitazone 10 μM, cariporide (Sanofi-Aventis) 20 nM, GF109203X (Gf) (Alexis) 10 μM, Go6976 (Go) (Alexis) 0.5 μM, wortmanin (Fluca) 50 nM. In some experiments the same samples were pre-incubated with the β1 integrin monoclonal antibody antiCD11b-FITC. This antibody binds to β1 integrin receptor subunit, blocking its binding capacity.

### Table 1. Effect of glucose, insulin and cariporide on intracellular pH (pHi) of monocytes

|                      | pHi     | ΔpHi  |
|----------------------|---------|-------|
| **Control**          |         |       |
| Glucose 20 mM        | 6.816 ± 0.003 | 6.853 ± 0.001 | 0.036 |
| Glucose + Cariporide 20 mM | 6.815 ± 0.009 | 6.818 ± 0.007 | 0.002 |
| Insulin 50 μU/ml      | 6.812 ± 0.007 | 6.843 ± 0.006 | 0.03 |
| Insulin + Cariporide 20 mM | 6.817 ± 0.002 | 6.819 ± 0.009 | 0.002 |
| Rosiglitazone 10 μM   | 6.820 ± 0.003 | 6.848 ± 0.005 | 0.028 |
| Rosiglitazone + Cariporide 20 mM | 6.817 ± 0.003 | 6.818 ± 0.003 | 0.0007 |
| Cariporide 20 nM      | 6.818 ± 0.008 | 6.817 ± 0.003 | -0.0008 |

Statistical analysis with Dunett’s test. Values are means ± SD of at least six independent experiments (four samples each). *p < 0.01 versus control samples, **p < 0.05 versus glucose samples.

Figure 7. Number of monocytes migrated on a collagen type IV coated Transwell membrane after a 30 min incubation at 37°C in the presence of either glucose 20 mM, glucose 20 mM + Gf 10 μM, glucose 20 mM + Go 0.5 μM or glucose 20 mM + wortmanin 50 nM. The mean values of six independent experiments are presented. Error bars indicate standard deviation (SD). *p < 0.05 versus control sample, **p < 0.05 versus glucose sample.

The falcon tubes were centrifuged at 1,200 rpm for 10 min at RT and endothelial cells were resuspended in RPMI 199 medium plus 20% FCS, 25 mM Hepes, 50 μU/ml penicillin/streptomycin, 0.25 μg/ml fungizone, 50 μg/ml gentamycin, 90 μg/ml heparin. The cell suspension was placed in cell culture flasks covered with gelatin 1% w/v in PBS pH 7. The cells were used in experiments after the first passage.

**Monocyte adhesion to laminin and collagen type IV.** Monocytes were isolated from whole blood from healthy individuals as previously described in reference 26. Experiments of monocyte adhesion were performed with both freshly isolated and freezeed monocytes. No significant difference in their adhesion ability was observed. Fifty microliters of laminin-1 37 μg/ml or collagen type IV 20 μg/ml placed in a 96-well polystyrene plate. The plate with laminin or collagen type IV left uncovered at room temperature overnight. Dry coated wells were filled with 200 μL of 2 mg/ml bovine serum albumin (BSA, Sigma) in 10 mM Tris-HCl-HEPES, 0.01% CaCl2, buffer pH 6.8 (blocking buffer) and incubated for 2 h at 37°C. Some wells were incubated in parallel only with 2% BSA and served as background. Then 10^4 monocyte were added to RPMI 1640 medium plus 10% FCS containing glucose 20 mM, insulin 50 μU/ml, rosiglitazone 10 μM, cariporide (Sanofi-Aventis) 20 nM, GF109203X (Gf) (Alexis) 10 μM, Go6976 (Go) (Alexis) 0.5 μM, wortmanin (Fluca) 50 nM. In some experiments the same samples were pre-incubated with the β1 integrin monoclonal antibody antiCD11b-FITC. This antibody binds to β1 integrin receptor subunit, blocking its binding capacity. Equal amounts of monocytes were incubated in eppendorf tubes under similar conditions. Non-adhered cells were removed by washing the plates with PBS pH 6. The monocytes incubated in the eppendorf tubes were added in the wells. The binding of monocytes was determined using the myeloperoxidase (MPO) assay as previously described in reference 27. In specific, the cells were lysed using 0.5% (w/v) hexadecyltrimethylammonium bromide (Sigma) in PBS (pH 6) for 30 min. One-fifth milligram per milliliter dianisidinedihydrochloride (Sigma) in PBS (pH 6) containing 0.4 mM H2O2 were added. After 15 min, the MPO activity in
the lysate was measured spectrophotometrically at 450 nm using ELISA reader. Reference tubes were centrifuged, the supernatant was removed and the sedimented cells were lysed transferred in empty wells of the plate and myeloperoxidase activity measured.

**Monocyte adhesion to endothelial cells.** Endothelial cells were isolated from umbilical cord vein as previously described. Fifty microliters of endothelial cell substrate placed in a 96-well polystyrene plate. The plate with endothelial cells left at 37°C, 5% CO₂ overnight. 10⁴ monocyte in RPMI 1640 medium plus 10% FCS containing glucose 20 mM, insulin 50 μU/ml, rosiglitazone 10 μM, Gf 10 μM, Go 0.5 μM, wortmanin 50 μM. Monocyte binding to endothelial cells was determined using the MPO assay.

**Monocyte migration through laminin and collagen type IV.** Transmigration of monocytes through laminin-1- or collagen type IV-coated filters 6.5 mm diameter, 5 μm pore (Transwell culture inserts, Costar, Cambridge) was performed as previously described in references 19 and 28–30. Monocyte migration was studied using both freshly isolated and frozen monocytes. No significant difference in their migration ability was observed. Filters were coated with laminin 20 μg/ml or collagen type IV 12 μg/ml. The filters were blocked with 0.5% BSA by incubation 1 h at 37°C. 5 x 10⁴ monocytes in 250 μl RPMI 1640 medium with glucose 20 mM, insulin 50 μU/ml, rosiglitazone 10 μM, cariporide 20 nM, Gf 10 μM, Go 0.5 μM, wortmanin 50 μM (no FCS) were added in the filters, while the same media plus 10% FCS was added under the filters. FCS gradients monocytes migrate through the filters. The plates were incubated for 30 min at 37°C. Nonmigrated monocytes were removed by immersion of the top side of the filter in PBS and mechanical removal. The filters were dried and stained with Hemacolor staining kit (Merck). Then the cells were lysed by placing the filters in 200 μl of acetic acid 10%. The migrated monocytes were measured spectrophotometrically at 570 nm using ELISA reader.

**Monocyte migration through endothelial cells.** Monocyte transmigration was performed through endothelial cell-coated filters. Filters were coated with endothelial cells. Under the filters was added 500 μl of RPMI 1640 medium containing 10% FCS. The plates were incubated at 37°C, 5% CO₂ overnight. 5 x 10⁴ monocytes in 250 μl RPMI 1640 medium with glucose 20 mM, insulin 50 μU/ml, rosiglitazone 10 μM, cariporide 20 nM, Gf 10 μM, Go 0.5 μM, wortmanin 50 mM were added in the filters and the plates were incubated for 30 min at 37°C. Nonmigrated monocytes were removed by immersion of the top side of the filter and the filters washed twice with PBS. Then the filters were dried and stained with Hemacolor staining kit and the cells were lysed with 10% acetic acid. The transmigrated monocytes were determined spectrophotometrically at 570 nm.

**Measurement of pHi.** Intracellular pH (pHi) was measured fluorometrically using the fluorescent indicator 2',7' -bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM). Monocytes were washed four times with HCO₃⁻-free buffer (135 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, 20 mM HEPES pH 7.3). One millimolar iodoacetic Na (glycylglycine inhibitor), 0.125 mM DIDS (HCO₃⁻/Cl⁻ exchanger inhibitor) and 0.4 mM methazolamide (carbonic anhydrase inhibitor) were added to all samples (including the control) in order to avoid any interference from other pH-regulating systems. Monocytes were then loaded with the fluorescent indicator BCECF-AM (Applichem). A suspension of 10⁴ monocytes per ml of the above-mentioned buffer that contained 50 μM BCECF-AM was incubated for 30 min at 37°C in the dark. BCECF-AM is readily cleaved towards the fluorescent form BCECF by intracellular esterases. After the incubation with the fluorescent dye, monocytes were washed with the same buffer five times in order to remove the unbound fluorescent probe, and were resuspended in the same buffer. Cariporide was added and incubation took place at 37°C for 15 min in the dark. Fluorescence was measured immediately after the addition of glucose 20 mM, insulin 50 μU/ml or rosiglitazone 10 μM in a Perkin-Elmer LS55 fluorescence spectrometer. Data were obtained as the ratio of the pH-sensitive excitation wavelength (495 nm) to the pH-insensitive excitation wavelength (435 nm), with the emission wavelength set at 530 nm. Routinely, at each experiment, a similar amount of BCECF (Applichem) loaded monocytes were suspended in a series of calibration buffers (30 mM KCl, 1 mM MgCl₂ plus 100 mM potassium phosphate buffer for pH adjustment to about 6.8, 6.9, 7.0, 7.1 and 7.2). After three washes with the same buffer nigericin at a final concentration of 13 μM was added to each suspension. Fluorescence was measured 5 min after nigericin addition as stated above. The pH of each tube was measured by a pH meter (Accumet, Fisher Scientific).

**Statistical analysis.** The statistical software GraphPad Instat, version 3.00 (GraphPad Software, San Diego, California, www.graphpad.com) was used for the statistical evaluation. Values are expressed as means ± standard deviations. The two-tailed p < 0.05 was used as the significance level. The statistical significance of the differences between groups of data was estimated by one-way analysis of variance with Bonferroni multiple comparisons test.

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264 Cell Adhesion & Migration Volume 5 Issue 3
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