



High Glucose downregulates the number of caveolae in monocytes through oxidative stress from NADPH oxidase: Implications for atherosclerosis

Toshio Hayashi, Packiasamy A.R. Juliet, Asaka Miyazaki, Louis J. Ignarro, Akihisa Iguchi

► To cite this version:

Toshio Hayashi, Packiasamy A.R. Juliet, Asaka Miyazaki, Louis J. Ignarro, Akihisa Iguchi. High Glucose downregulates the number of caveolae in monocytes through oxidative stress from NADPH oxidase: Implications for atherosclerosis. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2007, 1772 (3), pp.364. <10.1016/j.bbadis.2006.11.011>. <hal-00562745>

HAL Id: hal-00562745

<https://hal.science/hal-00562745v1>

Submitted on 4 Feb 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Accepted Manuscript

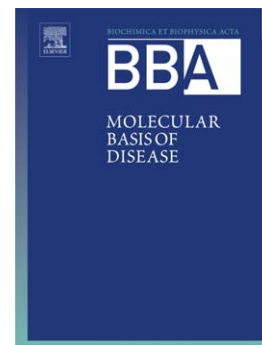
High Glucose downregulates the number of caveolae in monocytes through oxidative stress from NADPH oxidase: Implications for atherosclerosis

Toshio Hayashi, Packiasamy A.R. Juliet, Asaka Miyazaki, Louis J. Ignarro, Akihisa Iguchi

PII: S0925-4439(06)00264-X
DOI: doi: [10.1016/j.bbadis.2006.11.011](https://doi.org/10.1016/j.bbadis.2006.11.011)
Reference: BBADIS 62668

To appear in: *BBA - Molecular Basis of Disease*

Received date: 26 September 2006
Revised date: 24 November 2006
Accepted date: 28 November 2006



Please cite this article as: Toshio Hayashi, Packiasamy A.R. Juliet, Asaka Miyazaki, Louis J. Ignarro, Akihisa Iguchi, High Glucose downregulates the number of caveolae in monocytes through oxidative stress from NADPH oxidase: Implications for atherosclerosis, *BBA - Molecular Basis of Disease* (2006), doi: [10.1016/j.bbadis.2006.11.011](https://doi.org/10.1016/j.bbadis.2006.11.011)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

High Glucose downregulates the number of caveolae in monocytes through oxidative stress from NADPH oxidase: Implications for atherosclerosis

Toshio Hayashi ^{1,2} M.D, Ph.D., Packiasamy AR Juliet ²Ph.D., Asaka Miyazaki MSc., Louis J Ignarro Ph.D.* and Akihisa Iguchi M.D, Ph.D.

Dept of Geriatrics, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-Ku, 466-8550, Nagoya, Japan, Tel.+81 52 744 2364; Fax. + 81 52 744 2371, Email:hayashi@med.nagoya-u.ac.jp

* Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, 650 Charles E. Young Drive South. Los Angeles CA 90095

Key words: caveolae, glucose, nitric oxide, macrophage, NADPH oxidase

¹ Correspondence to:

Toshio Hayashi,

Dept of Geriatrics, Nagoya University Graduate School of Medicine,
65 Tsuruma-cho, Showa-ku, 466-8550, Nagoya, Japan,
Tel. +81 52 7442364; Fax. + 81 52 744 2371,
Email: hayashi@med.nagoya-u.ac.jp

² These authors contributed equally to this work

Abstract:

Atherosclerosis, an inflammatory disease, is closely associated with hyperglycemia, major sign of diabetes mellitus. Caveolae are vesicular invaginations of the plasma membrane that mediate the intracellular transport of lipids such as cholesterol. We evaluated the relationship between the expression of caveolin-1 and the number of caveolae in macrophages under conditions of high glucose concentration. Increased superoxide production, induction of inducible nitric oxide synthase (iNOS), and decreased caveolin-1 were observed in a concentration-dependent manner in THP-1 derived macrophages with high glucose concentrations. Mannitol, used as an osmotic control, showed no effects. Furthermore, co-localization of the NADPH oxidase component, p47^{phox}, and caveolin was confirmed by confocal microscopy. An atomic force microscopy (AFM) study showed that high glucose concentrations reduced the number and size of the caveolae. The percentage of cells with fragmented DNA was increased in cells grown in hyperglycemic media. Taken together, high glucose concentrations suppress the levels of caveolin-1 expression and reduce the number of caveolae. This might be due to the actions of superoxide via the activation of NADPH oxidase by translocation of its component and uncoupling of induced iNOS in macrophages. Furthermore, the apoptosis of macrophages might occur with high glucose concentrations, leading to the spreading of lipids from macrophages into intracellular spaces in the vessel wall.

1. Introduction

Diabetes mellitus is generally associated with a two- to four-fold increased risk of coronary heart disease and stroke [1,2]. Case-fatality rates for myocardial infarction and stroke are also increased [3,4]. Oxidative stress has an important role [5], and monocyte-derived macrophages are the inflammatory cells that are localized in human atherosclerotic plaques [6]. Under oxidative stress, macrophages generate reactive oxygen species (ROS), leading to LDL oxidation [7]. Monocytes from patients with type 2 diabetes produce increased levels of superoxide anions compared with control subjects [8]. Macrophages release O_2^- after cytokine stimulation [9]. The NADPH oxidase pathway is the main source of O_2^- generation in monocytes/macrophages [10,11]. Activation of the NADPH oxidase complex in the plasma membrane can lead to cell-mediated oxidation of low-density lipoprotein (LDL) in macrophages [12, 13]. Activation of the NADPH oxidase complex from a resting state to full superoxide-generating activity requires chemical modification and translocation of additional subunits from the cytosol to the oxidase complex on the cell membrane. Two of these important subunits are the polypeptides $p47^{phox}$ and $p67^{phox}$ [14, 15]. The level of $p47^{phox}$ translocation to the membrane has been shown to be increased with high glucose concentrations [16, 17]. We have previously shown that the THP- $p47^{phox}$ component of NADPH has a particularly important role in superoxide production compared with other subunits [9].

Caveolae are small, functionally important membrane invaginations found on the surface of many different cell types [18]. Caveolin is a cholesterol-binding protein and can form stable oligomers in the membrane [19]. Caveolin-1 (Cav-1) has been suggested to have a critical role in the development of atherosclerosis [20]. It has been reported that caveolin-1 is associated with cholesteryl ester uptake from LDL (but not oxidized LDL) and with cholesterol reverse transport by high-density lipoprotein (HDL) in THP-1 macrophages [21]. Insulin signaling for metabolic control depends on intact

caveolae, and destruction of caveolae disrupts insulin-stimulated phosphorylation of insulin receptor substrate-1 [22]. Although NO from endothelial nitric oxide synthase (eNOS) has an atheroprotective role, the role of NO from inducible NOS (iNOS) in macrophages is controversial, with some reports suggesting that it is atherogenic and others that it is atheroprotective [23, 24]; the former has been suggested because macrophages usually release O_2^- simultaneously.

Hence, the present study was designed to gain new insights into the co-localization and role of Cav-1 and $p47^{phox}$, a component of NADPH oxidase in atherogenesis, under conditions of high-glucose concentrations.

2. Methods

2.1. Materials

Phorbol myristate acetate (PMA) was purchased from Wako Co., Ltd., (Osaka, Japan). Lipopolysaccharide (LPS), tumor necrosis factor (TNF)- α , interferon (IFN)- γ , RPMI-1640, streptomycin, penicillin, apocynin (4-hydroxy-3-methoxyacetophenone), insulin and DCFH-DA (dichlorofluorescein diacetate) were from Sigma (St.Louis, MO). The PCR and RNA extraction kits were from Takara Co., Ltd. (Ohtsu, Japan). The Human iNOS Quantikine Kit was from R&D Systems Inc. (Minneapolis, MN). Polyclonal anti-caveolin and monoclonal anti- $p47^{phox}$ antibodies were purchased from Transduction Laboratories (San Diego, CA). The goat anti-rabbit antibody was from Medical and Biological Laboratories Co., Ltd. (Tokyo, Japan).

2.2. Cell culture

The human monocytic cell line (THP-1) was maintained in RPMI-1640 media. For experiments, cells were treated as follows. Briefly, THP-1 cells (2×10^6) were treated with 10 ng/ml of PMA for three days until the cells were adhered to the bottom of culture plates and exhibited macrophage-like morphology (confirmed by scanning electron microscopy). The differentiated cells were then incubated in serum and phenol-red-free media (THP-1 cells derived macrophages) with PMA. Freshly prepared glucose

solution of different final concentrations (5, 12.5, 25mM), mannitol (as a control to exclude the effects of osmotic pressure), or cytokines as cytokine mixture (1 µg/ml LPS, 1 ng/ml TNF-α, and 100-U/ml IFN-γ) were added to the medium. The cells were grown for 24 hours, after which time the media was collected and cells were used for further analysis.

2.3. Flow-cytometry analysis for ROS generation

Fluorescence-activated cell sorter (FACS, Becton Dickinson and Company, San Jose, CA) analysis using DCFH-DA was performed as previously described [25]. Each sample was evaluated using a FACS caliber flow cytometer (Becton Dickinson). The fluorescence of 10^4 cells was measured by gating the cytokine-stimulated THP-1 cells and results were analyzed by the Cell Quest program. During the process, cell lysis rates were less than 0.5%.

2.4. Measurement of nitrite and nitrate (NO_x)

The NO_x content of the media was measured using an automated NO detector high-pressure liquid chromatography (HPLC) system (ENO10; Eicom Co., Kyoto, Japan) as previously described [26]. Nitrate was converted to nitrite in an in-line copper-coated cadmium-reduction column (NO-RED), and the nitrite content was detected using the Griess reaction.

2.5. Measurement of iNOS protein

The levels of iNOS protein in THP-1 cells were measured using an iNOS Quantikine Kit (R&D Systems Inc., Minneapolis, MN). The values were expressed as units/ml.

2.6. Evaluation of iNOS mRNA

Total RNA was isolated from THP-1 cells with TRIZOL reagent according to the manufacturer's protocol (GIBCO BRL, Life Technologies, Carlsbad, CA). Primers for amplification of iNOS were designed from published human phagocyte sequences [9] and used to amplify fragments of 741 bp. The primer sequences were: iNOS sense, 5'-GGCCTGGAAACGCACAAGCTG-3' and anti-sense, 3'-

TTGGGGTTGAAGGCACAGCTG-5'.

2.7. Immunoprecipitation of p47^{phox} and caveolin

100 µg of protein was transferred to microcentrifuge tubes and incubated for 60 minutes at 4°C with either anti p47^{phox} antibody or anti-caveolin antibody. Immunoprecipitated complexes were then recovered and separated by 12% SDS-PAGE. Monoclonal antibodies directed against p47^{phox} or caveolin-1 (Transduction Laboratories) was then used to detect p47^{phox} and caveolin-1.

2.8. Immunoblotting of caveolin and p47^{phox}

Cells in a detergent-free buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, and protease-inhibitor mixture) were sonicated on ice by four 30-second bursts with 30-second intervals and centrifuged at 100,000 x g for 1 hour at 4° C in a TLA55 rotor (Beckman Instruments, Palo Alto, CA). The protein content of the resulting supernatant (cytosolic fraction) was compared with the pellet (membrane fraction). Equal amounts of protein were boiled in loading buffer and subjected to SDS-PAGE and Western blotting. Ten µg of the membranous portion of protein samples were electrophoresed, transferred to a polyvinylidene fluoride (PVDF) membrane, blocked with 2% skimmed milk powder and then incubated with the primary antibodies (against p47^{phox} and caveolin) for 1 hour. After incubation with the secondary antibody for 1 hour, the band density was measured using an NIH image analyzer.

2.9. Confocal fluorescence microscopy

Cells grown on 0.2% gelatin-coated glass cover slips were fixed with paraformaldehyde. After incubation with cold methanol at -20°C, the cells were incubated overnight with the primary antibodies at a 1:200 dilution in a wet chamber, and then for 2 hours with the secondary antibody. The slides were mounted using Fluoroguard anti-fade (Bio Rad Laboratories, Hercules, CA). The subcellular localization was observed using a BioRad Radiance 2100 confocal microscope.

2.10. Atomic force microscopy (AFM) and scanning electron microscopy (SEM)

examination of caveolae

THP-1 cells were seeded on glass cover slips and treated as described previously [27]. After the cells had attached and flattened, they were fixed with paraformaldehyde and stored at 4°C until analyzed [27-29]. Randomly selected regions of the membrane of THP-1 cells were chosen and scanned at high and low resolution by AFM (Fig.5). We have applied scanning AFM, to visualize caveolae on the surface of living and fixed cells. By scanning the membranes of cells, using the tapping mode of the AFM in fluid, we could visualize small membrane pits on the cell membranes of living and fixed THP-1 cells. The populations of pits with mean diameters of around 50-80 nm was present. In addition, the location of many pits visualized with the AFM was coincident with membrane spots in scanning electron microscopy mentioned as below[27-29]. The diameter and depth of the pit were measured with the aim of the section-analysis tool of the digital instrument software. Scanning electron microscopy (SEM) was used as described previously [30]. In brief, samples were dispersed in ultrafiltered ethanol. Droplets of the resulting suspension were applied to aluminum specimen holders, dried-down, sputter-coated with gold, and examined using an ISI DS130 scanning electron microscope.

2.11. Measurement of DNA fragmentation by flow cytometry Cells were harvested with trypsin and fixed with 70% ethanol, treated with 0.2 mg/ml of RNase for 30 minutes at 37°C, and then stained with 25 mg/ml propidium iodide (PI). Apoptotic cells were counted by flow cytometry.

2.12. Apoptosis-TUNEL (TdT; Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) Cells were fixed using 4% formalin and treated with proteinase K and endogenous peroxidases that had been inactivated with 3% H₂O₂. Samples were incubated with terminal deoxynucleotidyl transferase (TdT) for 1.5 hours at 37°C, and then stained by the streptavidin-biotin peroxidase method. Apoptotic cells were visualized after diaminobenzidine color development.

The TUNEL method was used to detect apoptotic cells. Paraffin-embedded sections were deparaffinized with xylene, incubated for 20 minutes with 16.2 µg/ml proteinase K (in 10 mM Tris-HCl buffer, pH 7.4; Boehringer Mannheim, Mannheim Germany) at room temperature, and then washed in distilled water. Endogenous peroxidase was inactivated in TdT buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride; Gibco BRL) for 2 minutes at room temperature. Slides were then incubated in TdT (0.3 equivalent units/ml) and biotin-dUTP (0.04 nmol/µl; Gibco BRL) in TdT buffer at 37°C for 90 minutes. Reactions were terminated by transferring the slides to a mixture of 300 mM sodium chloride and 30 mM sodium citrate (TB buffer), and then washing them in phosphate-buffered saline (PBS) for 10 minutes at room temperature three times. After washing in PBS, the slides were incubated with 10% (v/v) normal rabbit serum for 10 minutes to block nonspecific reactions. The slides were again washed in PBS, and were then incubated with peroxidase-conjugated streptavidin (Histofine SAB-PO kit; Nichirei) for 30 minutes at room temperature. After further washing in PBS, the slides were incubated in 3,3'-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin.

2.13. Data Analysis. The results were expressed as the mean \pm SEM and represent unpaired data. Data were compared by analysis of variance with repeated measurements. If a significant F value was found, Scheffe's test for multiple comparisons was used to identify differences among groups. Values of $p < 0.05$ were considered to indicate statistical significance.

Results

3.1. The effect of high glucose on superoxide and NO production in differentiated THP-1 cells

THP-1 cells stimulated with PMA (THP-1-derived macrophages) for 72 hours showed an increase in superoxide production compared with untreated cells, but this increase

was not statistically significant. Addition of mannitol as an osmotic control did not affect the levels of superoxide production. THP-1-derived macrophages cultured in glucose for 24 hours showed increased levels of superoxide production. Of the various concentrations of glucose used (5–25 mM), high glucose concentrations (12.5 and 25 mM) induced significant increases in the superoxide production compared with control cells that received mannitol (Fig. 1). This result indicates that addition of glucose induces oxidative stress in differentiated THP-1 cells. The NO_x production was determined by HPLC. Glucose increased the levels of NO_x production in macrophages in a dose-dependent manner (5–25 mM) ($P < 0.01$ at 12.5 and 25 mM) compared with the levels in monocytes (Fig. 2a).

3.2. The induction of iNOS by high glucose in differentiated THP-1 cells

Measurement of the levels of iNOS mRNA showed that glucose increased the expression of iNOS mRNA in a concentration-dependent manner (Fig. 2b), and Quantikine ELISA was used to confirm the induction of iNOS protein expression in THP-1 cells (Fig. 2c). These results were consistent with the HPLC NO_x data (Fig. 2a). A cytokine mixture was used as a positive control to induce iNOS, which increased mRNA, however the protein production might tend to be a little bit lower than the expected amount without any statistical significant decrement.

3.3. The reduction in caveolin-1 levels with high glucose in differentiated THP-1 cells

The expression of caveolin-1 protein in THP-1 cells was determined by Western-blot analysis. The protein extracted from cells grown in media supplemented with the cytokine mixture was collected and used as a positive control. Stimulation of THP-1 cells with PMA increased the levels of caveolin-1 expression. THP-1 cells did not express other caveolin isoforms. The addition of glucose at 12.5 and 25 mM and PMA reduced the levels of caveolin expression in THP-1-derived macrophages to half the level seen with stimulation by PMA only (Fig. 3a). Addition of insulin (7 mM) alone

did not result in any noticeable change in the levels of Cav-1 (caveolin 1) expression (data not shown), whereas simultaneous addition of insulin and glucose increased the levels of caveolin-1 expression up to the control level (Fig.3b). The cells were incubated in glucose-containing (1, 5, 12.5, and 25 mM) media for 7 hours, and then in media containing both glucose and insulin; however, insulin had no effect on caveolin-1 expression when added after 7 hours (Fig. 3.b). Preincubation with insulin for 7 hours, followed by incubation for 7 hours in fresh media containing glucose, further showed that insulin has no effect (data not shown).

3.4. The induction of NADPH oxidase with high glucose in differentiated THP-1 cells

Confocal microscopy analysis was used to examine the co-localization of caveolin and p47^{phox}, a component of NADPH oxidase, in the plasma membrane. Monocytes (as control) and THP-1-derived macrophages treated with PMA were immunolabeled with fluorescent antibodies against caveolin and p47^{phox} (Fig. 4a-c). In macrophages, p47^{phox} was shown to be translocated from the cytosol to the membrane region and associated with caveolin. The co-localization of caveolin and p47^{phox} in caveolae of the plasma membrane is shown by the yellow area in Fig. 4d. The co-localization of caveolin and p47^{phox} was also shown by immunoprecipitation (Fig. 4e).

3.5. The effect of differentiation and high glucose on caveolae of THP-1 cells

Randomly selected regions of the membrane of THP-1 cells were chosen and scanned at high and low resolution by AFM (Fig. 5). Pits of various dimensions were observed on the surface of the THP-1-derived macrophages, but not significantly in THP-1 cells. A population of small membrane pits with a mean diameter of about 70 nm was observed on the THP-1 cell membrane (Figs 5 and 6). This is similar to the diameter of caveolae estimated from scanning electron microscopy experiments (Fig. 5 a). The data obtained from AFM show that addition of glucose slightly altered the size of caveolae (40–120 nm) and significantly reduced the number of caveolae on the membrane. Significantly

fewer caveolae were counted in cells treated with 25 mM glucose than in cells treated with 5 mM glucose (Fig. 6).

3.6. The effect of high glucose on apoptosis in differentiated THP-1 cells.

THP-1 cells were cultured with PMA alone, with glucose with PMA or with media containing mannitol and PMA. We measured the DNA fragmentation using PI staining and flow cytometry (Fig. 7 upper panel). Superoxide dismutase and ascorbic acid (negative control) significantly reduced the levels of apoptotic cell death. The level of cell death was 8.5% in control monocytes, but it was about 24% in cells grown in media containing 12.5 or 25 mM glucose. The results were confirmed by data obtained from TUNEL assays (Fig. 7 lower); 12.5 and 25 mM glucose significantly increased the number of Tunnel-positive cells compared with control or THP-1-derived macrophages treated with 5 mM glucose.

Discussion

Cardiovascular disorders are much more frequent and severe in patients with diabetes than in healthy subjects [31]. Macrophages are significant cellular participants in the development of atherosclerosis in patients with diabetes, and show features of inflammatory disease [31]. In this study, we used THP-1 cells because they have many characteristics of human monocytes/macrophages [32]. Macrophages contain NADPH oxidase, which is the main source of superoxide (O_2^-) generation after cytokine stimulation [16,17]. We detected iNOS and activation of NADPH oxidase in THP-1-derived macrophages after treatment with high glucose, as well as with treatment with a cytokine mixture. O_2^- from NADPH oxidase was released continuously and the levels were much higher than that from iNOS with high glucose concentrations [23, 33 and our preliminary observation]. O_2^- and NO react with each other to generate peroxynitrite, which is considered to be a key component in the pathogenesis of atherosclerosis [33]. Activation of NADPH oxidase requires the translocation of additional subunits from the cytosol to the oxidase complex at the cell membrane. Two

such important subunits for activation are the polypeptides p47^{phox} and p67^{phox}, although NADPH oxidase has 4 subunits. The present study aimed to gain new insights into the roles of Cav-1 and NADPH oxidase in the development of atherosclerosis in patients with diabetes because previous results had suggested that there is an increased p47^{phox} translocation to membranes under high-glucose conditions.

Caveolin-1 is a structural component of caveolae, and has an important role in the function of enzymes such as eNOS and NADPH oxidase [34]. Measurement of the superoxide levels shows that the extent of O₂⁻ release is significantly higher in cells grown in media containing 25 mM glucose. This may be because high-glucose conditions may induce protein kinase C (PKC)-α, which in turn activates p47^{phox} translocation to membranes and induces O₂⁻ release.

Cardiovascular disease is an important complication in patients with diabetes mellitus, and it is responsible for increased morbidity in these patients. The etiology of the diabetic macro-angiopathy has been speculated to be due to O₂⁻ release from vessels. Experiments in animal models such as rats have indicated the release of O₂⁻ to be due to NADPH oxidase and eNOS uncoupling in the endothelium [35]. However, intimal thickening is seen in human adult coronary arteries and the aortae because plasma LDL-cholesterol levels are 3–4 times higher in humans than in other animals (100 vs. 25 mg/dl). The amount of O₂⁻ from macrophages was several times greater than that from endothelial cells (data not shown). Even in animal diabetes models, apocynin, which is an NADPH oxidase inhibitor, suppressed the progression of diabetes-associated vascular change [36]. We speculated that high-glucose-induced O₂⁻ release from macrophages is important in determining the character of diabetic vessels as well as for foam-cell formation.

Cellular NO production is mediated by NOS, which use O₂⁻ and NADPH as substrates to convert the amino acid L-arginine into L-citrulline and release NO. iNOS is not usually expressed in healthy quiescent cells, but it is rapidly induced in response

to stimulation with cytokines such as IFN- γ in inflammatory diseases, including atherosclerosis, and it is reported to downregulate caveolin levels [37]. In our study, cells expressed iNOS after stimulation with a cytokine mixture and glucose. High glucose concentrations also increased the expression of iNOS protein and NO $_x$ production in a concentration-dependent manner. There are two possible pathways. First, it is unclear whether certain post-translational modifications on iNOS cause it to be redistributed to membranous subdomains, where the interaction with caveolin is speculated to take place. Second, the availability of NO (*in vivo*) is regulated by a combination of NO synthesis and breakdown [38]. Although 5mM glucose can induce iNOS in monocytes/macrophages, higher concentration is necessary to down-regulate caveolin level. There are several reports about the relation between glucose and caveolin in smooth muscle cells and adipocytes; no reports are available in monocytes/macrophages as much as we know [39, 40]. Under high-glucose conditions, the increased levels of superoxide produced by NADPH oxidase react with NO and lead to the formation of peroxynitrite, which in turn is converted to the pro-atherogenic compound.

In the THP-1 monocyte cell line, caveolin-1 expression is induced with phorbol-ester-mediated differentiation [41]. Increased levels of caveolin-1 have been shown to induce the formation of caveolae [42, 43]. In general, many caveolin molecules are required to form a single caveolae, and a large increase in caveolin expression would be expected to result in a moderate increase in the number of caveolae [42, 43]. Increased Cav-1 expression is associated with increased formation of morphologically distinguishable caveolae invaginations. Addition of glucose to the media reduced the numbers of caveolae because it decreased the levels of caveolin-1, which is a structural component of caveolae. The co-localization of p47^{phox} with caveolin in the differentiated THP-1 cells indicates that caveolin expression is affected by the increased translocation of p47^{phox} from the cytosol to the membrane, where it can

bind to caveolin and inhibit its synthesis. This, in turn, decreased the caveolae count. These findings indicate that the formation and stability of caveolae are affected by ROS. Monocyte-derived macrophages have a critical role in the development of atherosclerosis and are intimately involved in the formation of arterial lesions. NADPH oxidase, the source of superoxide production in monocytes/macrophages, is partially responsible for defective caveolae formation, which in turn affects cholesterol-ester transport and reverse transport. It has been recognized that caveolae act in signaling pathways, acting as a platform to which numerous signaling molecules can bind, as well as regulating flux through many distinct signaling cascades [44]. Cholesterol metabolism in macrophages may be a central determinant in maintaining cholesterol homeostasis in the arterial wall and for foam-cell formation. The reduced numbers of caveolae with high-glucose conditions may be responsible for the defective cholesterol reverse transport by HDL without inhibition of oxidized LDL transport in macrophages, which could lead to foam-cell formation. Furthermore, high glucose concentrations might cause increased apoptosis of macrophages, which would lead to the spread of lipids from macrophages into intracellular spaces in vessel wall. In the present study, insulin was only observed to have an effect when co-incubated with high glucose. Glucose itself might affect the production of ROS and therefore NO release. However, insulin has various effects in many organs such as the liver and skeletal muscles, and the data in the present study are not sufficient to evaluate the role of insulin and further investigation is required.

The findings of this study may be useful for developing therapeutic interventions for vascular disease. For example, apocynin, which is an NADPH oxidase inhibitor, may have potential as a therapeutic tool. When THP-1-derived macrophages were incubated with apocynin, which inhibits the movement of p47^{phox} to the membrane, before AFM analysis, there was no decrease in caveolae count with high glucose (data not shown).

Our results conclusively demonstrate that high glucose suppresses the expression of caveolin-1 and reduces the number of caveolae via translocation of p47^{phox}, a component of NADPH oxidase. This may be responsible for the defective regular cholesterol transport in macrophages, which could lead to foam-cell formation.

References:

- [1] J.Stamler, O.Vaccaro, J.D.Neaton, D.Wentworth, Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. *Diabetes Care* 16 (1993) 434-444.
- [2] P.McCarron, R Greenwood, P. Elwood, The incidence and etiology of stroke in the Caerphilly and Speedwell Collaborative Studies II: risk factors for ischemic stroke. *Public Health* 15 (2001) 12-20.
- [3] B.Y. Chun, A.J. Dobson, R.F. Heller, The impact of diabetes on survival among patients with first myocardial infarction. *Diabetes Care* 20 (1997) 704-708.
- [4] G. Wannamethee, P.H.Whincup, A.G.Shaper, M.Walker, P.W.MacFarlane, Factors determining case fatality in myocardial infarction “who dies in a heart attack”? *Br Heart J* 74(1995) 324-31.
- [5] P.Rosen, P.P. Nawroth, G. King, The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a Congress Series sponsored by UNESCO-MCBN, the American Diabetes Association and the German Diabetes Society. *Diabetes Metab Res Rev* 17 (2001) 189-212.
- [6] R.Ross, Atherosclerosis—an inflammatory disease. *N. Engl. J. Med.* 340(1999) 115-126.
- [7] D.Steinberg, Low density lipoprotein oxidation and its pathobiological significance. *J Biol Chem* 272 (1997) 20963-20966.
- [8] F.Y.Chang, M.F.Shaio, Respiratory burst activity of monocytes from patients with non-insulin-dependent diabetes mellitus. *Diabetes Res. Clin. Pract.* 29 (1995) 121-127.
- [9] P.A.Juliet, T.Hayashi, A.Iguchi, L.J.Ignarro, Concomitant production of nitric oxide and superoxide in human macrophages. *Biochem. Biophys. Res. Commun.* 310 (2003) 367-370.
- [10] M.K.Cathcart, Regulation of superoxide anion production by NADPH oxidase in monocytes/macrophages: contributions to atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 24 (2004) 23-28.
- [11] M.Aviram, Macrophage foam cell formation during early atherogenesis is determined by the balance between pro-oxidants and anti-oxidants in arterial cells and blood lipoproteins. *Antioxid. Redox Signal* 1 (1999) 585-594.
- [12] Y.Groemping, K.Lapouge, S.J.Smerdon, K.Rittinger, Molecular basis of phosphorylation-induced activation of the NADPH oxidase. *Cell* 113(2003) 343-355.
- [13] S.Parthasarathy, N.Santanam, Mechanisms of oxidation, antioxidants, and atherosclerosis. *Curr Opin. Lipidol.* 5 (1994) 371-375.
- [14] A.W.Segal, A.Abo, The biochemical basis of the NADPH oxidase of phagocytes. *Trends Biochem. Sci.* 18(1993)43-47.

- [15] T.L.Leto, K.J.Lomax, B.D.Volpp, Cloning of a 67-kD neutrophil oxidase factor with similarity to a noncatalytic region of p60c-src. *Science* 248(1990) 727-730.
- [16] N.Kadri-Hassani, C.L.Leger, B. Descomps, The fatty acid bimodal action on superoxide anion production by human adherent monocytes under phorbol 12-myristate 13-acetate or diacylglycerol activation can be explained by the modulation of protein kinase C and p47phox translocation. *J. Biol. Chem.* 270(1995) 15111-15118.
- [17] O.Cachia, J.E.Benna, E.Pedruzzi, B.Descomps, M.A.Gougerot-Pocidalo, C.L.Leger, alpha-tocopherol inhibits the respiratory burst in human monocytes. Attenuation of p47(phox) membrane translocation and phosphorylation. *J Biol Chem* 273(1998) 32801-32805.
- [18] Y.Yanagitani, H.Rakugi, A.Okamura, Angiotensin II type 1 receptor-mediated peroxide production in human macrophages. *Hypertension* 33(1999) 335-339.
- [19] S.Monier, D.J.Dietzen, W.R.Hastings, D.M.Lublin, T.V.Kurzchalia, Oligomerization of VIP21-caveolin in vitro is stabilized by long chain fatty acylation or cholesterol. *FEBS. Lett.* 388(1996)143-149.
- [20] P.G.Frank, S.E.Woodman, D.S.Park, M.P.Lisanti, Caveolin, caveolae, and endothelial cell function. *Arterioscler. Thromb. Vasc. Biol.* 23(2003)1161-1168.
- [21] S.Matveev, D.R.van der Westhuyzen, E.J.Smart, Co-expression of scavenger receptor-BI and caveolin-1 is associated with enhanced selective cholesteryl ester uptake in THP-1 macrophages. *J. Lipid Res.* 40(1999)1647-1654.
- [22] S.Parpal, M.Karlsson, H.Thorn, P.Stralfors, Cholesterol depletion disrupts caveolae and insulin receptor signaling for metabolic control via insulin receptor substrate-1, but not for mitogen-activated protein kinase control. *J. Biol. Chem.* 276(2001)9670-9678.
- [23] T.Hayashi, D.Sumi, P.A.Juliet, Gene transfer of endothelial NO synthase, but not eNOS plus inducible NOS, regressed atherosclerosis in rabbits. *Cardiovasc. Res.* 61(2004) 339-351.
- [24] X.L.Niu, X.Yang, Y.Hoshiai, Inducible nitric oxide synthase deficiency does not affect the susceptibility of mice to atherosclerosis but increases collagen content in lesions, *Circulation* 103(2001)1115-1120.
- [25] T. Hayashi, T. Esaki, E.Mu-to, A.Iguchi, Dehydroepiandrosterone retards atherosclerosis formation through its conversion to estrogen: the possible role of nitric oxide. *Arterioscler. Thromb. Vasc. Biol.* 20 (2000) 782-792.
- [26] T.Hayashi, K.Yamada, T.Esaki, E.Muto, G.Chaudhuri, A.Iguchi, Physiological concentrations of 17beta-estradiol inhibit the synthesis of nitric oxide synthase in macrophages via a receptor-mediated system. *J. Cardiovasc. Pharmacol.* 31(1998) 292-298.
- [27] H.Lucius, T.Friedrichson, T.V.Kurzchalia, G.R.Lewin, Identification of caveolae-like structures on the surface of intact cells using scanning force microscopy. *J. Membr. Biol.* 194 (2003) 97-108.
- [28] X. Zhang, A. Chen, D. De Leon, H. Li, E. Noin, V.T. Moy, M.S. Goligorsky. Atomic force microscopy measurement of leukocyte-endothelial interaction. *Am J Physiol Heart Circ Physiol* 286 (2004) H359-367
- [29] H.Xu, D.Dinsdale, B.Nemery, P.H.Hoet, Role of residual additives in the cytotoxicity and cytokine release caused by polyvinyl chloride particles in pulmonary cell cultures. *Toxicol. Sci.* 72 (2003) 92-102.
- [30] G.Pogatsa, Altered responsiveness of vascular smooth muscle to drug in diabetes.

- In: L.Szekeres, J.G.Papp, eds, Pharmacology of smooth muscle. New York: Springer-Verlag. (1994) 693-712,
- [31] A.P.Burke, F.D.Kolodgie, A.Zieske, Morphologic findings of coronary atherosclerotic plaques in diabetics: a postmortem study. *Arterioscler. Thromb. Vasc. Biol.* 24(2004)1266-1271.
- [32] M.Abrink, A.E.Gobl, R.Huang, K.Nilsson, L.Hellman, Human cell lines U-937, THP-1 and Mono Mac 6 represent relatively immature cells of the monocyte-macrophage cell lineage. *Leukemia* 8(1994)1579-1584.
- [33] C.C.White, T.A.Brock, L.Y.Chang. Superoxide and peroxynitrite in atherosclerosis. *Proc. Natl. Acad. Sci. USA.* 91(1994)1044-1048,
- [34] T.Okamoto, A.Schlegel, P.E.Scherer, M.P.Lisanti, Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. *J Biol Chem* 273(1998)5419-5422.
- [35] K.Shinozaki, A.Kashiwagi, Y.Nishio, Abnormal biopterin metabolism is a major cause of impaired endothelium-dependent relaxation through nitric oxide/O₂-imbalance in insulin-resistant rat aorta. *Diabetes* 48(1999)2437-2445.
- [36] T. Hayashi, P.A. Juliet, H. Kano-Hayashi, T. Tsunekawa, D. Dingqunfang, D. Sumi, et al., NADPH oxidase inhibitor, apocynin, restores the impaired endothelial-dependent and -independent responses and scavenges superoxide anion in rats with type 2 diabetes complicated by NO dysfunction. *Diabetes. Obes. Metab.* 7 (2005) 334-343.
- [37] G. Llaverias, M. Vazquez-Carrera, M.R. Sanchez, V Noe, L.C. Ciudad, C.J. Laguna, M. Alegret Rosiglitazone upregulates caveolin-1 expression in THP-1 cells through a PPAR-dependent mechanism. *J Lipid Res.* 45(2004)2015-2024.
- [38] I. Navarro-Lerida, M.T. Portoles, A.A. Barrientos, F. Gavilanes, L. Bosca, I. Rodriguez-Crespo, Induction of nitric oxide synthase-2 proceeds with the concomitant downregulation of the endogenous caveolin levels. *J. Cell Sci.* 117 (2004) 1687-1697.
- [39] S.Shigematsu, R.T. Watson, A.H. Khan, J.E.Pessin, The adipocyte plasma membrane caveolin functional/structural organization is necessary for the efficient endocytosis of GLUT4. *J Biol Chem.* 278 (2003) 10683-10690.
- [40] A.W. Cohen, T.P. Combs, P.E. Scherer, M.P. Lisanti, Role of caveolin and caveolae in insulin signaling and diabetes. *Am J Physiol Endocrinol Metab* 285 (2003) E1151-1160.
- [41] A.M. Fra, E. Williamson, K. Simons, R.G. Parton, De novo formation of caveolae in lymphocytes by expression of VIP21-caveolin. *Proc. Natl. Acad. Sci. U S A* 92 (1995) 8655-9.
- [42] E.J. Smart, Y. Ying, W.C. Donzell, R.G. Anderson, A role for caveolin in transport of cholesterol from endoplasmic reticulum to plasma membrane. *J. Biol. Chem.* 271(1996) 29427-29435.
- [43] M. Jayachandran, T. Hayashi, D. Sumi, A. Iguchi, V.M. Miller, Temporal effects of 17beta-estradiol on caveolin-1 mRNA and protein in bovine aortic endothelial cells. *Am J Physiol Heart Circ Physiol* 281 (2001) H1327-1333.
- [44] R.G. Anderson, K. Jacobson, A role for lipid shells in targeting proteins to caveolae, rafts, and other lipid domains. *Science* 296 (2002) 1821-1825.

Acknowledgement: This work was supported by grants from Japanese Society for

Promotion of Science 16406001

The abbreviations used are: PMA, phorbol myristate acetate; NO, nitric oxide; LDL, low-density lipoprotein; INF- γ , human interferon gamma; TNF- α , tumor necrosis factor alpha; SOD, superoxide dismutase.

Figure Legends

Fig.1.

Superoxide production in THP-1 cells under various conditions. **Monocyte [MO]**: control, unstimulated monocyte. **Macrophage [MP]**: macrophage-like THP-1 cells that were differentiated into macrophages by PMA treatment for 72 hours. **Mannitol treated macrophage [Man]**: mannitol-treated macrophage-like cells (used as an osmotic control; the osmolarity was the same as that by treatment with 25 mM glucose). **High-glucose-treated macrophage [Glucose]**: macrophage-like cells treated with different concentrations of glucose (5–25 mM). Superoxide levels were measured by FACS Caliber assays as described in the Methods section. ** $P < 0.01$, *** $p < 0.001$ vs. MO

Fig.2.

(a) NO metabolites [$\text{NO}_2^- + \text{NO}_3^-$] production, (b) mRNA of iNOS, and (c) iNOS activity from THP-1 cells under various conditions. **Monocyte [MO]**: control, unstimulated monocyte. **Macrophage [MP]**: macrophage-like THP-1 cells that were differentiated into macrophages by PMA treatment for 72 hours. **Mannitol-treated macrophage [Man]**: mannitol-treated macrophage-like cells (used as an osmotic control; the osmolarity was the same as that by treatment with 25 mM glucose). **High-glucose-treated macrophage [Glucose]**: macrophage-like cells treated with different concentrations of glucose (Glucose 5–25 mM). The level of NO metabolite in the media was measured after a 24-hour incubation. **Cytokine-mixture-treated macrophage [CYT]**: macrophage-like cells treated with a mixture of cytokines [LPS, IFN- γ and

TNF- α). RNA was extracted for RT-PCR. Protein was used for the iNOS Quantikine ELISA and values were expressed as U/ml. The details of all procedures are described in the Methods section. * $P < 0.05$, ** $p < 0.01$ vs. MO.

Fig.3.

(a) Amount of caveolin-1 in THP-1 cells under various conditions. **MO**: control, unstimulated monocytes. **MP**: macrophage-like THP-1 cells that were differentiated into macrophages by PMA treatment. **Man**: mannitol-treated macrophage-like cells (used as an osmotic control; the osmolarity was the same as that by treatment with 25 mM glucose) **CYT**: macrophage-like cells treated with a mixture of cytokines [LPS, IFN- γ , and TNF α]. **Glucose**: macrophage-like cells treated with different concentrations of glucose (glucose 5, 12.5, and 25 mM).

(b) Amount of caveolin-1 in THP-1 cells after insulin treatment and before or after glucose treatment. **Right 3 lanes**: Cells were differentiated into macrophages by PMA treatment and treated with glucose for 72 hours. The culture media was then washed off and 5–25 mM of insulin was added to the culture media for 24 hours. **Left 4 lanes**: To determine the relationship between insulin [7 mM] and Cav-1 expression, insulin was applied to the PMA-supplemented media, and various concentrations of glucose [5, 12.5, and 25 mM] were also added and to the cells were cultured for 24 hours. ** $P < 0.01$, *** $p < 0.001$ vs. MO.

Fig. 4

(a–d) Con focal microscopic examination of caveolin and p47^{phox} using the double immunostaining method. THP-1 cells stimulated with PMA and 25 mM glucose were double-labeled with antibodies to p47^{phox} (Alexa) and caveolin-1 (FITC). Green staining indicated caveolin-1, and red staining indicated p47^{phox}. 40X magnification. (a) **MA**: THP-1-derived macrophages stained for the p47^{phox} subunit of NADPH oxidase. The p47^{phox} subunit was distributed in the cytosol. (b) **Glucose [25 mM]**: p47^{phox} translocated towards the membrane after incubation with glucose. (c) **Glucose [25**

mM]: Glucose-treated macrophages were stained for caveolin. Caveolin was present in the Golgi apparatus and the membrane. (d) **Glucose [25 mM]**: The caveolin and p47^{phox} were co-localized in the plasma membrane (yellow). Immunoprecipitation of caveolin-1 and p47^{phox} in differentiated THP-1 cells. (e) **Glucose [25 mM]**: The existence of caveolin and antibody of p47^{phox} in the membranous portion of cells treated with PMA and 25 mM glucose.

Fig.5.

Scanning electron micrographs showing the surface morphology of glucose-treated, fixed THP-1-derived macrophages (a). Bar represents 1 μ m. *Membrane invaginations are referred to as “pits” (the *s in the upper photographs), indicating caveolae. Caveolae determination by AFM analysis (b–d). (b) THP-1-derived macrophages treated with 25 mM glucose after stimulation with PMA. (c) control THP-1 cells, (d) THP-1 cells that were differentiated into macrophages by PMA. Fewer pits are observed in (b) than in (c) and (d), when normalized for the size of the cell surface.

Fig.6.

Caveolae determination by AFM analysis. All membrane invaginations are referred to as “pits” (the Xs in the upper photograph). (a–c) Membrane topography from the surface of the fixed THP-1 cells; clusters of pits are indicated by asterisks. Five fields were scored for each cells and the figure shows the mean of five cells. (a) Control THP-1 cells, (b) THP-1 cells that were differentiated into macrophages by PMA. (c) THP-1-derived macrophages treated with 25 mM glucose after stimulation with PMA.

Fig. 7.

DNA fragmentation evaluated by FACScan and TUNEL methods. **Upper panel:** FACScan using PI staining. Nuclei were stained with PI and the percentages of cells with fragmented DNA were determined by laser-flow-cytometry analysis. **MO:** control, unstimulated monocytes. **Glucose:** macrophage-like cells treated with different concentrations of glucose (glucose 5, 12.5, and 25 mM for 72 hours). **AS:** ascorbic acid

(25 μ M), **SOD**: Cu/Zn superoxide dismutase (200 U/ml), **Man**: mannitol-treated macrophage-like cells (used as an osmotic control; the osmolarity was the same as that by treatment with 25 mM glucose).

Lower: TUNEL (TdT-mediated dUTP-biotin nick-end labeling)-stained cells. Only the condensed brown macronuclei are TUNEL- positive [positive control (b)].

(a): Negative control. (b): Positive control. (c): Control cells: THP-1-derived macrophages. (d) 5 mM glucose-treated macrophages (for 72 hours). (e) 12.5 mM glucose-treated macrophages. (f) 25 mM glucose-treated macrophages. Bar is 50 μ m.

p<0.001 vs. MO.

Figure 1

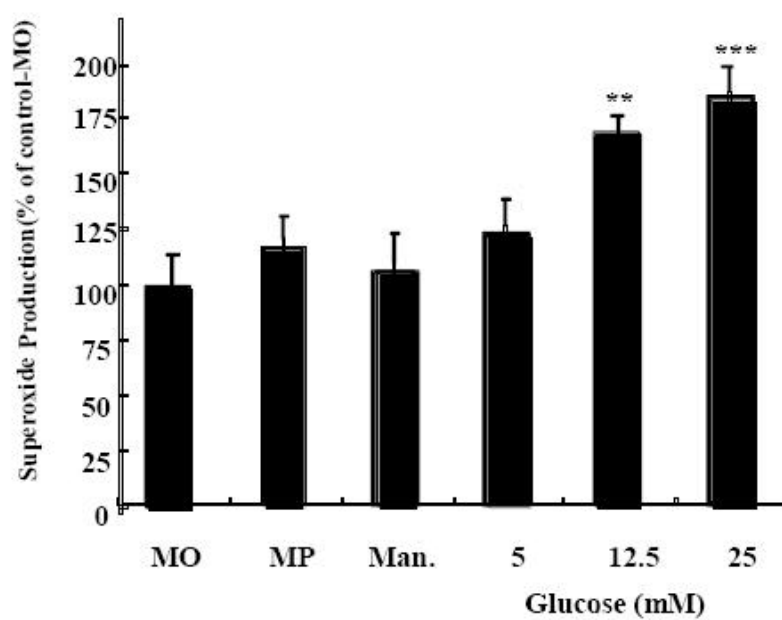
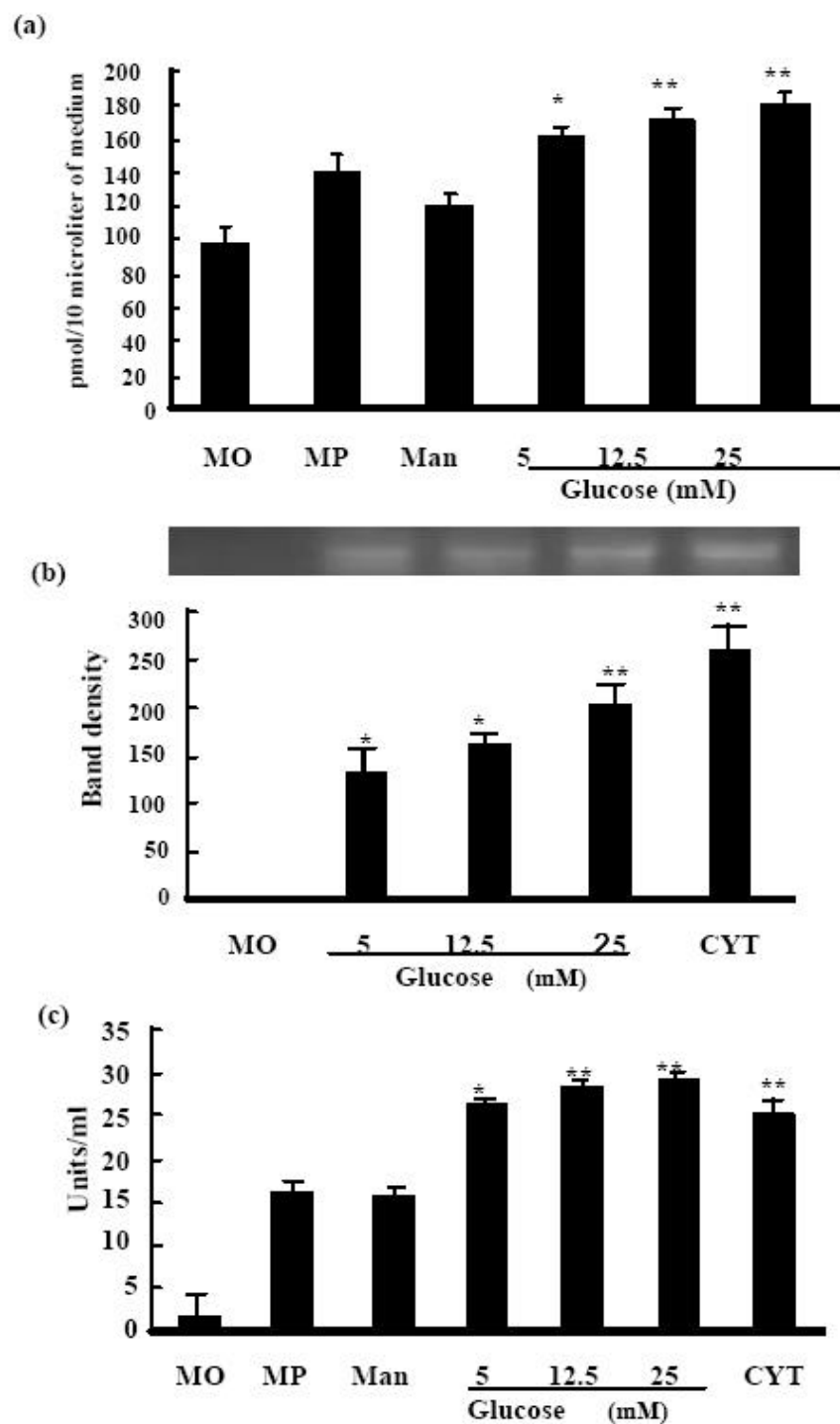


Figure2



MO: Monocyte. MP: Macrophage. Man: Mannitol treated macrophage. Glucose: High glucose treated macrophage. CYT: Cytokine mixture treated macrophage

Figure 3.

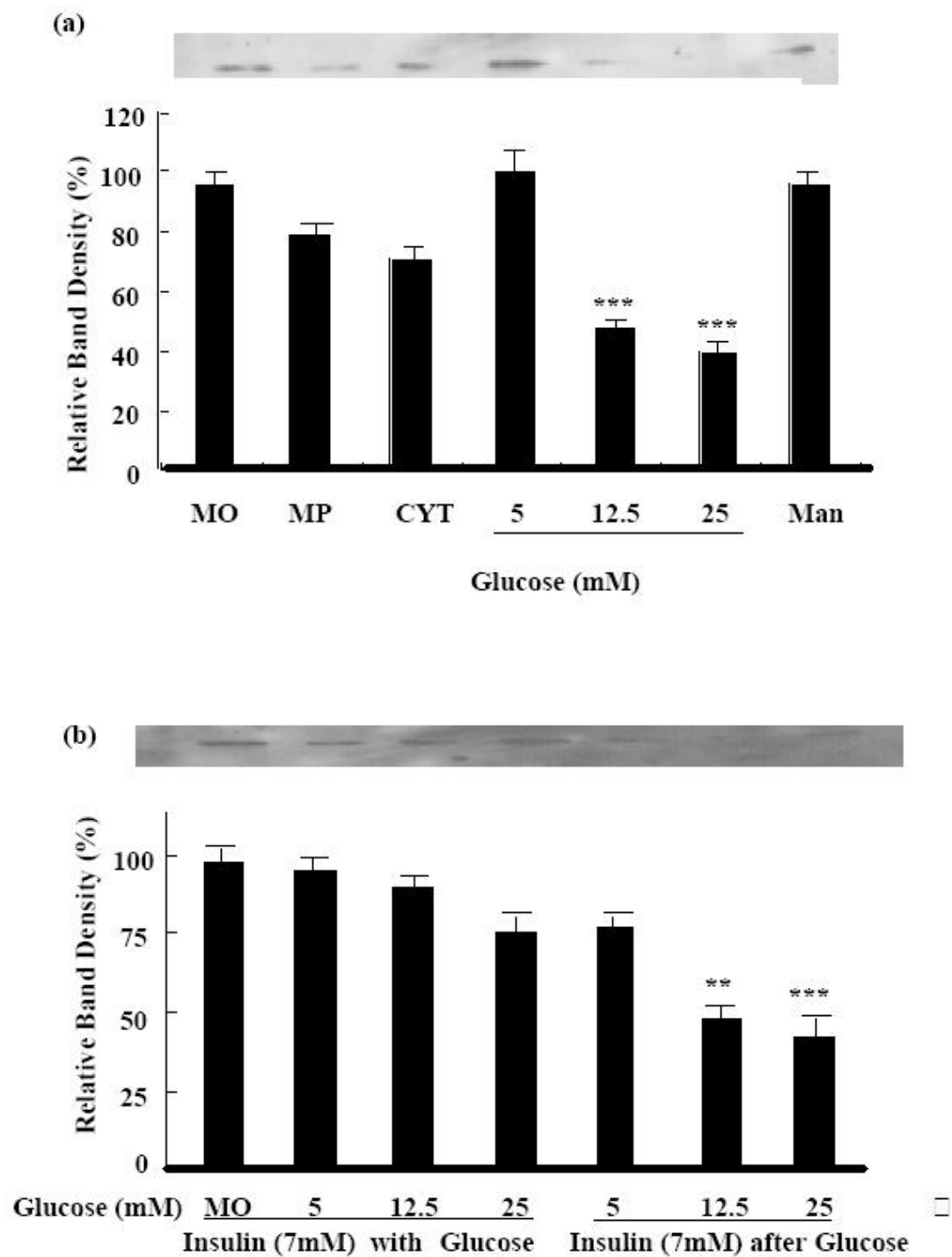


Figure 4

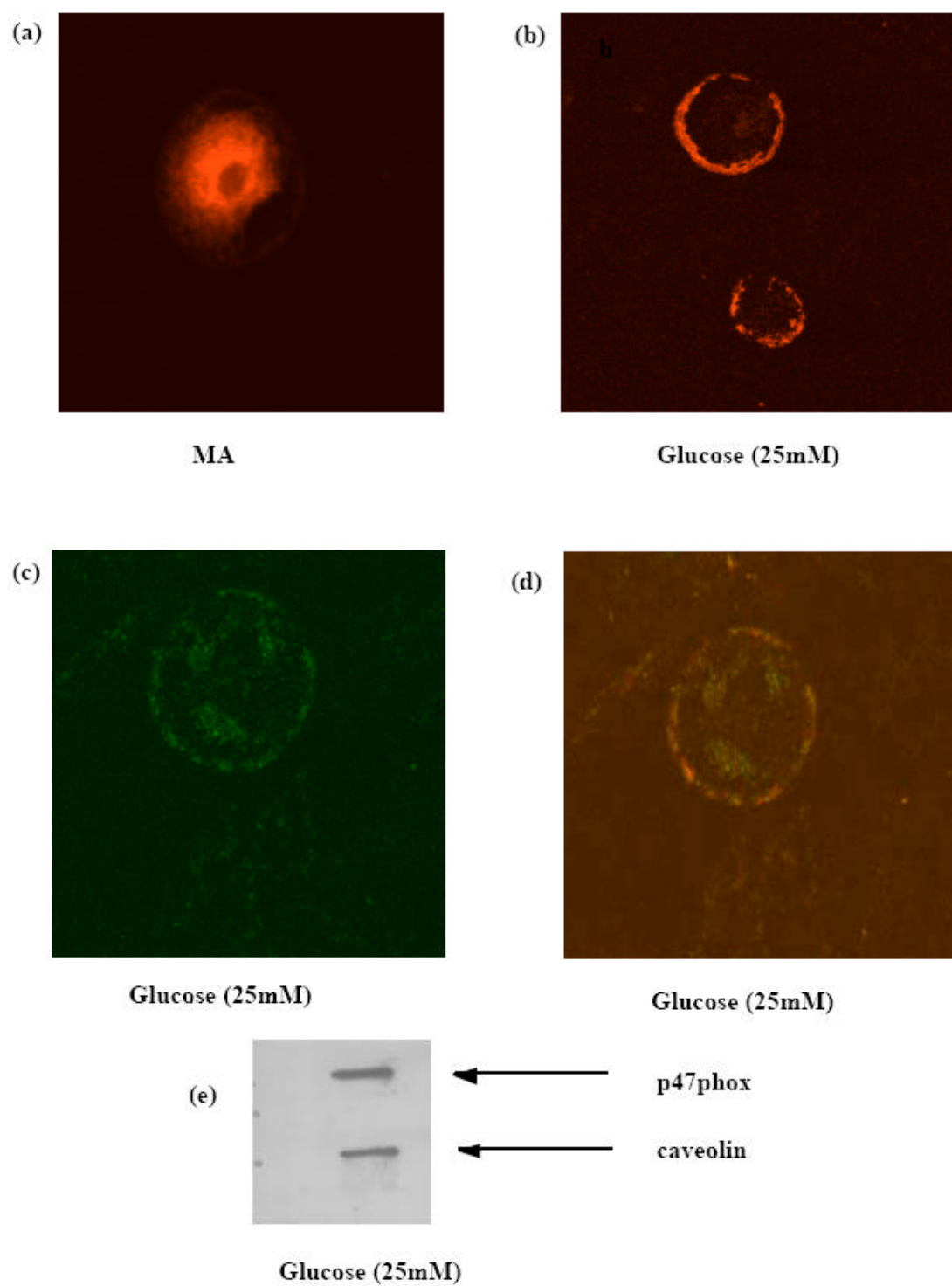


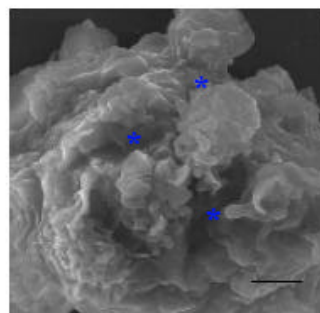
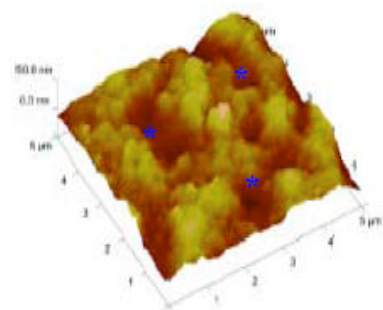
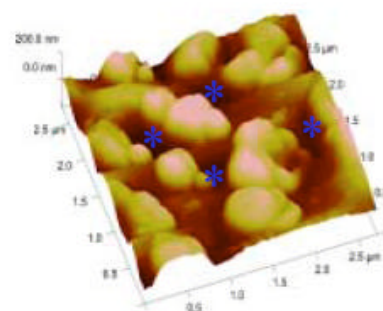
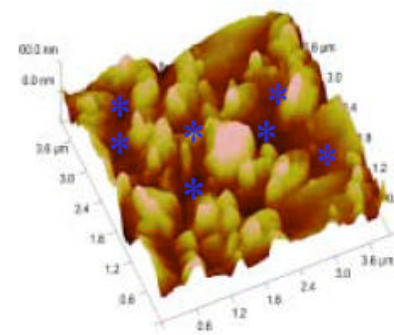
Figure 5**(a)****(b)****(c)****(d)**

Figure 6

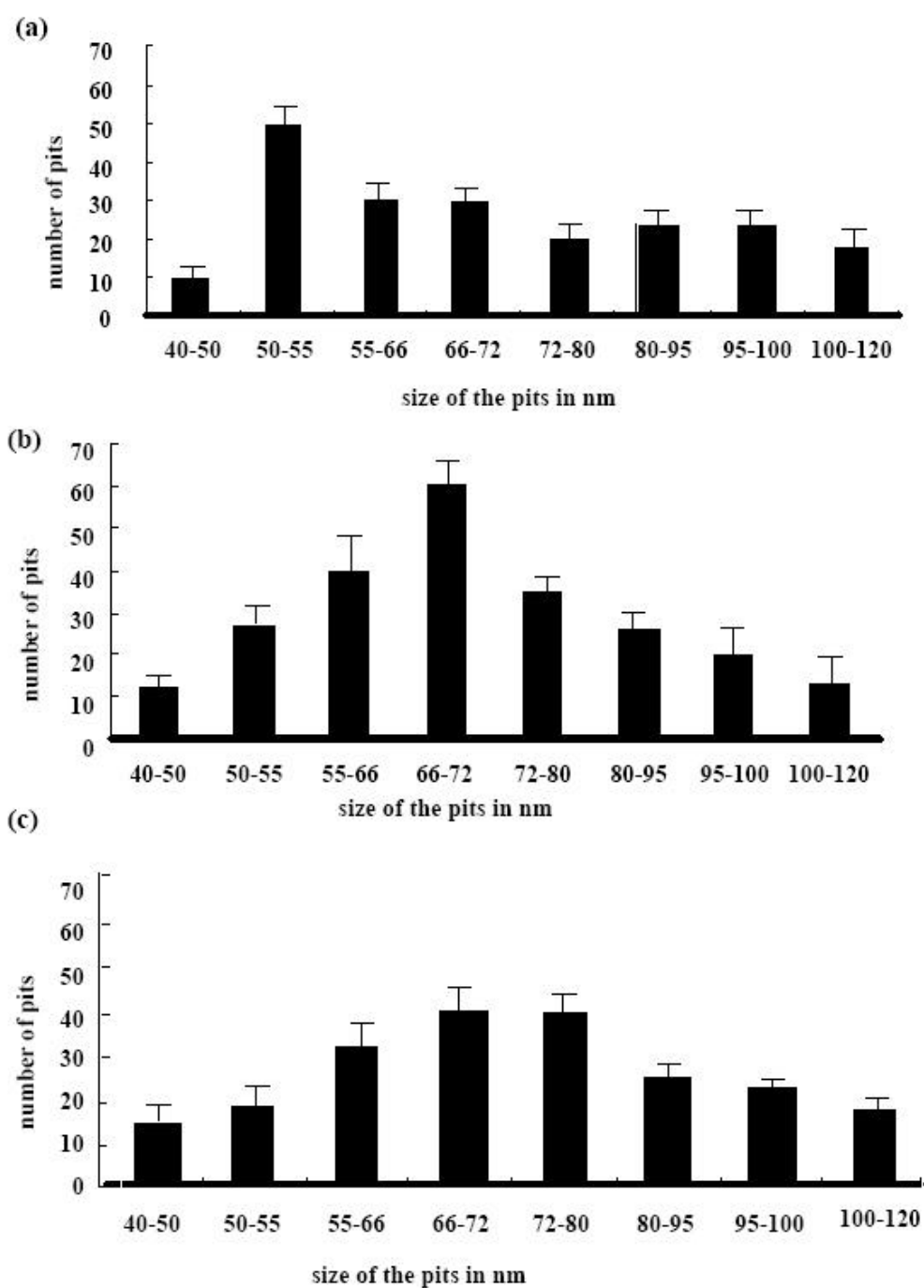


Figure 7

