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Binding of Amadori glucose-modified albumin by the monocytic cell line MonoMac 6 activates protein kinase Cε protein tyrosine kinases and the transcription factors AP-1 and NF-κB

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An affinity purification procedure is employed for the isolation of FL-specific binding proteins from MM6 cell membranes using magnetobeads coated with glycated polylysine and elution with FL and glycated 6-aminocaproic acid. Two main binding proteins were identified as membrane-bound nucleolin and cellular myosin heavy chain, which are glycosylated. This study shows that in these cells binding of short-term glycated albumin leads to activation of PKC, especially its isoform ε and this is linked to translocation of AP-1 and NF-κB into the nucleus. Consequently, an increased formation of IL-1ß mRNA is observed. The PKC inhibitor GÖ6976 prevents all these effects. Glycated albumin also stimulates activation of PTK. The PTK inhibitor genistein prevents activation of AP-1 indicating that PTK is also involved in this process, whereas NF-κB translocation is only dependent on PKC activation.

Keywords: **glycation, receptor, fructoselysine, signal transduction, AP-1, NF-κB**

Abbreviations: **AGE, advanced glycation end product; AP-1, activator protein-1; BSA, bovine serum albumin; ECL, enhanced chemiluminiscence; EMSA, electrophoretic mobility shift assay; ERK, extracellular regulated protein kinase; FL, fructoselysine; IgG, immunglobulin G; IL, interleukin; MAPK, mitogen-activated protein kinase; MM6, MonoMac 6; NF-κB, nuclear factor-κB; PKC, protein kinase C; PTK, protein tyrosine kinase; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.**

Introduction

Unlike most post-translational modifications nonenzymatic glycation serves no useful function in the organism and is symptomatic of disturbed glucose metabolism. The increased reaction of proteins with glucose is likely to play a role in the development of late complications in diabetes. Unprotonated amino groups of proteins, especially the ε-amino groups of lysine residues, form a Schiff base with the carbonyl function of the open chain form of glucose, which rearranges into a ketoamine (Amadori product). Over time, Schiff bases and Amadori products undergo dehydration, fragmentation and rearrangements to produce stable, irreversibly bound advanced glycation end products (AGEs). Fructosyllysine (FL) is the main Amadori adduct in extracellular proteins [1]. This modification confers properties to the glycated proteins that are not possessed by the native ones. Amadori-modified proteins interact with specific receptors of several cell types resulting in different cell responses [2–9]. Binding proteins with molecular masses of 110 and 200 kDa in the monocytic cells U937 and MonoMac 6 (MM6) cell membranes have recently been described to interact with glycated albumin [3]. Binding of glycated albumin to MM6 cells induced secretion of the proinflammatory cytokines IL-1ß and TNF-α [3]. A differing individual expression of FL-specific sites on monocytes and macrophages was positively associated with indices of diabetic complications, such as microangiopathy, in diabetic patients and capillary basement membrane thickening in diabetic rats [4]. We report here the isolation and characterization of fructoselysine-specific binding proteins from MM6 cell membranes and the examination of the effects of glycated albumin on protein kinase C (PKC) and protein tyrosine kinase (PTK) activities. We show that PKC signaling, particularly through its ε-isoform, participates in the

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induction of transcription factors activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) binding activities and a stimulated transcription of the IL-1ß gene.

Materials and methods

Preparation of glycated albumin and amino acids

Short-term glycated albumin was prepared and isolated by boronate affinity chromatography as previously described [1,4]. Glycated albumin contained 1 mol FL/mol protein and did not bind specifically to a bovine aortic endothelial cell line (BkEz 7), which only recognizes AGE-moieties in glucose-modified proteins [4]. Native albumin was also eluted from a column of aminophenylboronic acid agarose (Sigma, Deisenhofen, Germany) to remove FL-modified protein, which is always present in small amounts in plasma proteins. N^{ε}-Fructoselysine and N^{ε}-fructosyl amino caproic acid were synthesized and purified according to Finot and Mauron [10] except that DEAE Sephadex A25 (Pharmacia, Uppsala, Sweden) was used as ion exchange resin.

Cell culture

MM6 cells were grown to logarithmic phase in RPMI 1640 supplemented with 10% fetal calf sera (Biochrom Seromed, Berlin, Germany), 10 μg/ml kanamycin, 1 mM sodium pyruvate, 2 mM glutamine and 1 $\times$ non-essential amino acids (Gibco Life Technologies, Karlsruhe, Germany). Only in experiments for estimations of protein tyrosine kinase activities the serum supplementation was reduced to 1% 18 h before addition of ligands.

Preparation and characterization of receptor proteins

MM6 binding proteins were prepared as earlier described [11], Shortly, uncoated magnetobeads (dynabeads M-280 tosylactivated, Deutsche Dynal, Hamburg, Germany) were incubated with poly-L-lysine (m.w. 5,000 to 15,000, Sigma). The polylysine coated beads were glycated with glucose. 1×10^9 MM6 cells were harvested by centrifugation, washed twice with 0.01 M Na_2HPO_4, 0.14 M NaCl (pH 7.4) (PBS), once with PBS containing 10 mM EDTA and resuspended in homogenization buffer 10 mM Tris/HCl (pH 7.4), 300 mM sucrose, 0.1 mM EDTA containing an excess of proteinase inhibitors (10 mM PMSF, 20 mM benzamidine, 10 μg/ml antipain, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml bacitracin, 50 μg/ml aprotinin (Sigma)). After repeated freezing and thawing cells were homogenized using a Turrax stirrer and centrifuged at 800$\times$ g for 9 min. The supernatant was collected and recentrifuged at 20,000$\times$ g for 30 min. To eliminate membrane associated proteins pelleted membranes were washed once with a tenfold volume of sucrose free homogenization buffer and once with 10 mM Tris/HCl, 0.1 mM EDTA (pH 7.4). Finally pelleted cell membranes were solubilized in 57 mM ß-octylglucoside, 0.02 M Tris/HCl (pH 7.4), 0.23 M NaCl, 10 mM EDTA and proteinase inhibitors as described above for 30 min at 4°C. The lysates were centrifuged for 1 h at 100,000$\times$ g and 4°C and incubated with 20 mg of prepared magnetic beads for 2 h at 4°C. After five stringent washes of the beads with 57 mM ß-octylglucoside, 0.02 M Tris/HCl, 0.23 M NaCl, 10 mM EDTA (pH 7.4) bound proteins were eluted with 200 μl 57 mM ß-octylglucoside, 10 mM Tris/, 0.4 M N^{ε}-fructosyl amino caproic acid, 0.2 M N^{ε}-fructosyllysine HCl (pH 7.4). Eluted proteins were separated by SDS-PAGE using 7.5% acrylamide gels as described [3]. After electrophoresis proteins were stained with Coomassie blue. The identity of the isolated proteins was checked using ligand blots with glycated albumin [3] in combination with immunochemical detection of bound albumin with suitable antibodies.

After SDS-PAGE proteins were blotted on nitrocellulose membranes (Sigma) and characterized for carbohydrates by staining with a glycan detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturers' recommendations. Western blots were performed with rabbit polyclonal antisera against human cellular myosin II heavy chain (Eurogentec, Amsterdam, The Netherlands) and nucleolin (generous gift from Dr. D. Kübler, Heidelberg, Germany) as well as an alkaline phosphatase conjugated second anti-rabbit IgG antibody (Sigma). For ligand blotting with glycated albumin an anti-albumin polyclonal rabbit antibody (Sigma) and the alkaline phosphatase conjugated second antibody were used. Antigen antibody reactions were vizualized with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt (NBT/BCIP, Roche) as a substrate for alkaline phosphatase.

PKC activity assay

1×10^7/ml MM6 cells were incubated in the presence of ligands as indicated in the figures (500 μg/ml) for 2 h at 37°C. The endotoxin level of the incubations was estimated using an endotoxin kit (Sigma, Saint Louis, MO, USA) and was lower than 0.4 ng/ml. For positive control cells were incubated for 30 min with 50 ng/ml phorbol-12-myristate-13-acetate (PMA, Calbiochem, Bad Soden, Germany). Thereafter, cells were washed with PBS. Cell lysates were prepared in 25 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 10 mM 2-mercaptoethanol, 1 μg/ml leupeptin and aprotinin, 0.5 mM PMSF with a Dounce homogenizer. Lysates were centrifuged for 5 min at 4°C and 14,000$\times$ g. PKC total activity in the lysates was determined using an assay kit from Promega (Mannheim, Germany) according to the manufacturers' instructions and ^{32}P-γATP (Amersham) for phosphorylation of the biotinylated model peptide neurogranin, which was estimated in a Phosphorimager after its binding to the SAM^{TM} biotin capture membrane. PKC activity measurements were predetermined to be in the linear range of the assay. Data are expressed as pmoles phosphate incorporated/ min/μg protein. Protein was estimated using a modified Coomassie method (BioRad, München, Germany).

Activated PKC isoenzymes

1×10^7/ml MM6 cells were incubated in the presence of ligands as indicated in the figures (500 μg/ml) for 2 h at 37°C. For positive control cells were incubated for 30 min with 50 ng/ml phorbol-12-myristate-13-acetate. After washing with Tris-EDTA (Tris-HCl, 110 mM NaCl, 5 mM KCl, 4 mM EDTA (pH 7.5)) cells were suspended in 400 μl cold hypotonic lysis buffer (20 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, 5 mM dithiothreitol, 1 mM PMSF, 20 μg/ml leupeptin, 100 μg/ml aprotinin, 10 mM benzamidine (pH 7.5)) and homogenized in a Dounce homogenizor using 8 strokes. Cell lysates were centrifuged at 100,000 × g for 1 h at 4°C. The supernatant (cytosolic fraction) was quickly frozen and stored at −70°C. The remaining pellet was solubilized in lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 10 mM EGTA, 5 mM dithiothreitol, 1 mM PMSF, 20 μg/ml leupeptin, 100 μg/ml aprotinin, 10 mM benzamidine, 1% Triton X-100 (pH 7.5)) and allowed to stand on ice for 20 min. The lysate was centrifuged for at 100,000× g for 1 h at 4°C. The supernatant (membrane fraction) was snap-frozen and stored at −70°C.

20 μg protein from membrane and cytosolic fractions, prepared as described from stimulated and non-stimulated MM6 cells were separated on 8% SDS polyacrylamide gels and transferred to Hybond ECL nitrocellulose (Amersham Pharmacia Biotech, Buckinghamshire, England). The nitrocellulose sheets were blocked with 2% BSA in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% Tween 20 (pH 7.5) for at least 2 h. The PKC isoenzymes α, β, γ, δ, ε and ζ were detected with isoenzyme-specific polyclonal rabbit antibodies (Gibco Life Technologies) and anti-rabbit horse radish peroxidase conjugated second antibody. Each set of blots with samples to be compared were developed together using the Enhanced Chemiluminiscence (ECL) detection kit and exposed to Hyperfilm (Amersham). The images of the autograms were captured using the gel documentation system Phase Gelcam CCD camera (Phase, Lübeck, Germany) and stored in the bmp file formate. The files were reopened to analyze the intensity of the fractions using the Gel-Pro Analyzer software, version 2.0 (Media Cybernetics, L.P.).

PTK activity assay

MM6 cells were cultured as described to 75% confluence. Thereafter, cells were washed twice with PBS and grown in medium containing 1% fetal calf serum for 18 h. 1×10^7 cells/ml were then incubated with 500 μg/ml of the indicated ligands for 2 h. For positive controls cells were incubated in the presence of 9 μg/ml insulin (Gibco Life Technologies). After the incubations cells were washed carefully with PBS to remove the incubation medium completely and suspended in 2 ml of 20 mM HEPES, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 30 μM 2-glycerophosphate, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 1% Triton X-100 (pH 7.4). After 10 min on ice the suspension was rocked for 15 min at 4°C. The cell lysates were centrifuged for 1 h at 100,000× g. PTK activity was determined using the PTK assay kit from Promega with ^{32}P-γ ATP as phosphorylating agent and a biotinylated PTK specific peptide as substrate. After passage over a SAM^{TM} biotin capture membrane bound ^{32}P was estimated using a Phosphorimager. PTK measurements were predetermined to be in the linear range of the assay. Data are expressed as pmoles phosphate incorporated/min/μg protein.

Detection of activated transcription factor AP-1 and NF−κB by electrophoretic mobility shift assay (EMSA)

1×10^7/ml MM6 cells were incubated in the presence of the indicated ligands and 2 mM α-lipoic acid [12] for 2 h at 37°C. For inhibition experiments cells were pretreated for 60 min with either 40 μM of the PTK inhibitor genistein (Calbiochem-Novabiochem, Bad Soden, Germany) or 100 nM of the PKC inhibitor GÖ6976 (Gibco Life Technologies) in 0.1% dimethylsulfoxide. Cells were collected and washed twice with ice-cold PBS. The cell pellet was resuspended in 1 ml 10 mM HEPES (pH 7.6), 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% NP-40, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 2 μg/ml leupeptin and incubated on ice for 10 min. The samples were then centrifuged at 1,000× g for 5 min at 4°C. The nuclear pellet was resuspended in 50 μl 20 mM HEPES (pH 7.9), 0.42 M NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol and protease inhibitors as above and mixed on ice for 15 min. The nuclear extract was centrifuged at 15,000× g for 10 min at 4°C. The supernatant (nuclear proteins) was collected and stored at −70°C. Protein content was quantified using BioRad protein assay. NF-κB consensus oligonucleotide (5′- AGT TGA GGG GAC TTT CCC AGG C-3′) and AP-1 consensus oligo-nucleotide (5′-CGC TTG AGT CAG CCG GAA- 3′) were 5′ end labeled with ^{32}P γ-ATP (Amersham) using T4 poly-nucleotide kinase (Promega) to specific activity of 5,000 to 20,000 cpm/100 fmole. Non-incorporated radioactive nucleotide was removed using Microspin G25 columns (Amersham). 2 to 6 μg of nuclear proteins were incubated with 0.5 ng of labeled oligonucleotide in binding buffer (10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% glycerol, 0.05 mg/ml poly(dI-dC) for 20 min at room temperature in a final volume of 10 μl. Free oligonucleotide and oligonucleotide protein complexes were separated by electrophoresis on a native 6% polyacrylamide gel. The gel was dried and exposed to a X-ray film with intensifying screens overnight at −70°C. Specificity of binding was ascertained by competition with a 100-fold excess of unlabeled consensus oligonucleotides. Nuclear extracts were also incubated with radiolabeled OCT-specific oligonucleotide to verify that the amount of protein used in each fraction was the same.

Analysis of cytokine mRNA by semiquantitative RT-PCR

Confluent MM6 cells were incubated in the presence of ligands as indicated in Figure 6 (500 μg/ml, 2 mg/ml) for 2 h at 37°C

in multiwell dishes. The PKC and PTK inhibitors GÖ6976 and genistein were added 1 h before incubation with glycated or non-glycated albumin. To test the specificity of binding glycated albumin to its receptor proteins cells were preincubated with anti-nucleolin and anti-myosine antisera, final dilution 1:1000, for 1 h.

Total RNA was isolated from monocytic cells using the RNeasy extraction kit (Quiagen, Karlsruhe, Germany) according to the manufacturers' protocol. Reagents for reverse transcription were purchased from Promega. cDNA was synthesized as follows: 1 μl oligo(dT)primer (500 ng/μl) was added to 2 μg total RNA in 12.5 μl of diethylpyrocarbonate treated water. The sample was heated to 65°C for 5 min, cooled on ice for 2 min, mixed sequentially with 4 μl 5x first-strand buffer, 1 μl deoxynucleoside triphosphates (dNTPs, 10 mM each), 0.5 μl RNAsin (40 U/μl) and 1 μl Maloney murine leukemia virus reverse transcriptase (200 U/μl). Incubation was performed for 1 h at 42°C and 10 min at 70°C. PCR amplification was carried out in a Personal Cycler (Biometra, Göttingen, Germany). Reagents were from Amersham Pharmacia Biotech. The cytokine specific primers are IL-1ß-for 5′-GGC AGA AGT ACC TGA GCT CG-3′ and IL-1ß-rev 5'-TTC AAC ACG CAG GAC AGG TA-3′. For control, PCR amplification of ß-actin DNA was performed in each estimate.

2.5 μl cDNA solution were amplified using 2.5 U Taq DNA polymerase, 1 μl dNTP (10 mM each) and 20 pM of primer in 1× PCR manufacturer's buffer in a total volume of 50 μl. The thermoamplification program consisted of an initial denaturation (30 s at 94°C), followed by 32 cycles of 30 s denaturation (94°C), 30 s annealing (58°C), 1 min elongation (72°C) and a final extension period of 5 min at 72°C. Products were separated on a 2% agarose gel containing 3% (v/v) ethidium bromide and detected by trans-illumination with UV light. Gels were photographed using a digital camera system, bands representing amplified products were scanned. Scans of cytokine-specific PCR products were related to ß-actin PCR products (standard) and quantified using Gel-Pro Analyzer software (Media Cybernetics, L.P.). Values were corrected for ß-actin expression.

Statistical analysis

Data in the Figures 6 and 7 are expressed as means and standard deviations. Results were tested for significance by a paired t-test (SigmaPlot). Significance level was $p < 0.05$.

Results

Preparation and characterization of FL-specific receptor proteins

Two proteins of 200 and 100 kDa were obtained after affinity purification of MM6 membrane proteins on glycated dynabeads in a Coomassie stained SDS polyacrylamide gel (Figure 1, lane 1). High detergent concentrations and ionic strengthes in the washing buffer suppressed non-specific interactions of membrane proteins with the glycated polylysine matrix. The use of FL and glycated 6-amino caproic acid as eluents increased the specificity of the purification procedure (see Materials and methods). The proteins were identified by ligand blotting with glycated albumin as Amadori product binding proteins (Figure 1, lane 2), which did not bind native albumin [2–4]. In Western blots antisera against cellular myosin heavy chain reacted with the 200 kDa protein fraction (Figure 2, lane 1), whereas antibodies against nucleolin stained the 100 kDa protein (Figure 2, lane 2). Both binding proteins stained for carbohydrates with a glycan detection kit (Figure 2, lane 3).

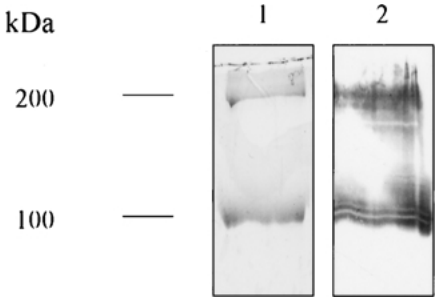

Figure 1. Lane 1: SDS-PAGE of MM6 membrane proteins after affinity purification with glycated dynabeads stained with Coomassie blue; lane 2: ligand blots with glycated albumin of the isolated membrane proteins.

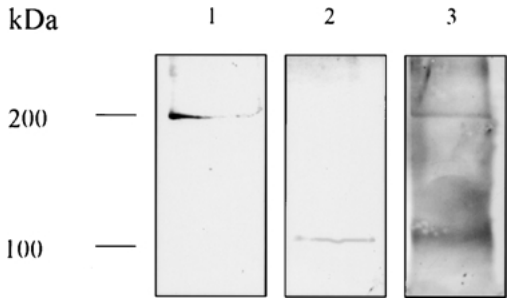

Figure 2. Lane 1: Immunochemical detection that the 200 kDa fructosyllysine-specific binding protein is highly homologous with cellular myosin heavy chain (reaction with an antibody for cellular myosin heavy chain); lane 2: the 100 kDa membrane protein reacted with an anti-nucleolin antibody; lane 3: the 100 and 200 kDa proteins (membrane-bound nucleolin and cellular myosin heavy chain) stained for carbohydrates using a glycan detection kit.

PKC and PTK activities

MM6 cells responded to incubation with glycated albumin with a trend toward activation of cellular PKC (0.91 ± 0.66 pmoles ^{32}P/min/μg protein). Non-glycated albumin was not able to stimulate PKC activity compared to basal values (0.7 ± 0.36 pmoles ^{32}P/min/μg protein versus 0.73 ± 0.4 pmoles/min/μg protein).

The presence of the PKC isoenzymes α, β, δ, ε and ζ mainly in the cytosol of MM6 cells was confirmed with immunoblotting

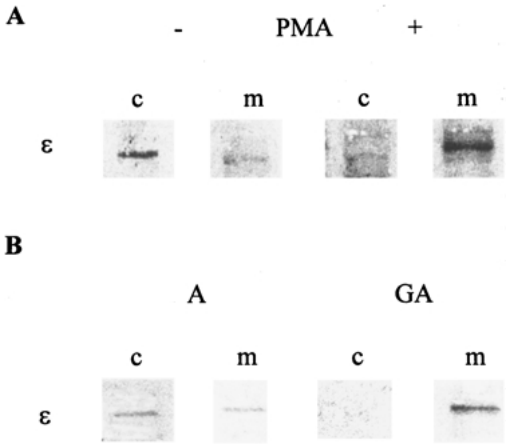

Figure 3. Translocation of PKCε isoenzyme in MM6 cells incubated with PMA in comparison to control (A) and incubated with glycated and non-glycated albumin (B). The cytosolic (c) and membrane (m) protein fractions were isolated from cells. 20 μg protein per lane were tested for the presence of PKCε with rabbit PKC isoenzyme-specific antibody, followed by incubation with goat anti-rabbit IgG conjugated with horse radish peroxidase. The protein bands were developed with ECL. Results were obtained from one of three similar experiments. A = non-glycated albumin; GA = glycated albumin.

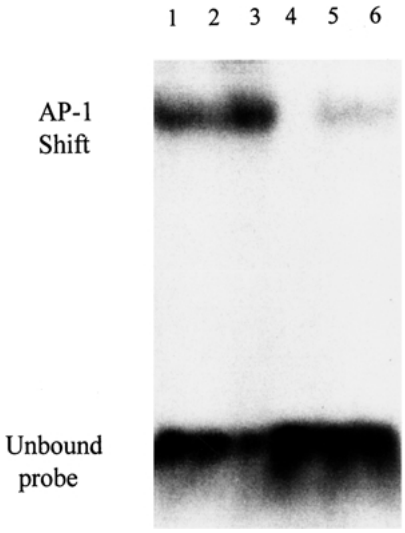

Figure 4. Activation of AP-1 by glycated albumin in MM6 cells. This activation is linked to PKC and PTK. Glycated albumin (lane 3) induced an increase in AP-1 DNA binding over untreated control or native albumin (lanes 1 and 2). 100-fold excess of unlabeled AP-1-specific oligonuleotide was able to compete for binding (lane 4). Pretreatment with 40 μM genistein diminished AP-1 activation (lane 5). 100 nM GÖ6976 attenuated glycated albumin-induced AP-1 translocation into the nuclear proteins (lane 6).

experiments. Because a cytosol-to-membrane shift of the isoenzymes indicates activation, the immunoreactivity of cytosolic and membrane fractions of these isoenzymes was investigated by western blots with isoenzyme-specific antibodies. Of these five isoforms only PKCε a minor variant of total PKC enzyme activity, was predominantly translocated into the membrane fraction after incubation with glycated albumin. PKCδ showed minimal translocation, whereas PKCα, β and ζ were primarily in the cytosol (Figure 3, Table 1).

The ability of glycated albumin to increase PTK activity was also examined. PTK activity was significantly stimulated by glycated albumin (1.11 ± 0.11 pmoles ^{32}P/min/ mg protein, $p < 0.05$, compared to non-glycated albumin) in comparison with non-glycated albumin (0.94 ± 0.09 pmoles/min/μg protein) and controls (0.56 ± 0.03 pmoles ^{32}P/min/μg protein).

Table 1. Ratio of membrane to cytosolic PKC isoenzymes of MM6 cells

Isoenzyme	*No ligands*	*PMA*	*Native albumin*	*Glycated albumin*
α	1.25	0.46	0.57	0.23
ß	0.79	1.07	0.56	0.30
δ	0.33	2.29	1.45	1.51
ε	2.08	4.01	1.62	4.71
ζ	0.42	1.27	0.47	0.48

Results of western blot analysis were determined by measuring the integrated optical density of the reactive bands. The ratios of membrane fraction to cytosolic fraction were calculated. Results were means from triple determinations of three independent incubations.

Activation of transcription factors AP-1 and NF-κB

Because oxidative stress activates the transcription factors AP-1 and NF-κB, reduced α-lipoic acid was added prior to the incubations with ligands [12]. Without preincubation with lipoic acid a high level of activated transcription factors was already observed without stimulation by ligands. MM6 cells reacted upon incubation with glycated albumin in contrast to the non-glycated protein with greater activation of AP-1 and NF-κB. In studies with AP-1 specific oligonucleotides a higher amount of AP-1 binding activity in nuclear extracts after incubation with glycated albumin was found. But there was not any shift upwards, consistent with no formation of heavier Fos/Jun heterodimers (Figure 4).

To link AP-1 binding to activation of PKC or PTK the specific inhibitors GÖ6976 and genistein were used. Pretreatment with these compounds prevented glycated albumin-induced activation of AP-1 (Figure 4). These experiments showed that activation of AP-1 with glycated albumin requires PKC and PTK.

Glycated albumin, but not native albumin, increased the amount of activated NF-κB in nuclear extracts. Glycated albumin-induced NF-κB activation was more effectively prevented by GÖ6976. The PTK inhibitor genistein did not show

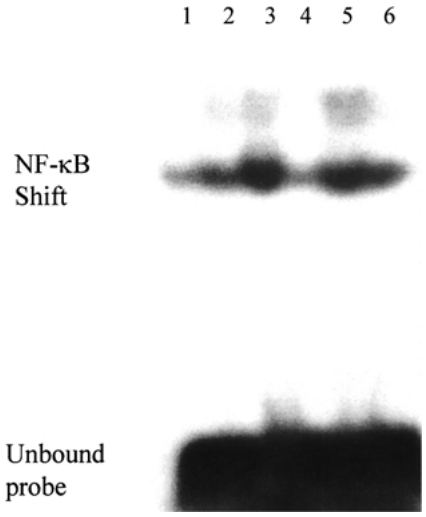

Figure 5. Glycated albumin causes activation of NF-κB in MM6 cells. This activation is linked to PKC. Glycated albumin (lane 3) increases NF-κB DNA binding over untreated control or native albumin (lanes 1 and 2). 100-fold excess of unlabeled NF-κB specific oligonucleotide was able to compete for binding (lane 4). Pretreatment with 100 nM GÖ6976 attenuated glycated albumin-induced NF-κB translocation (lane 6), whereas 40 μM genistein had no effect (lane 5).

significant effects (Figure 5). Therefore, this activation was only dependent on PKC activity.

From these results it is evident that PKC and PTK stimulated by binding of glycated albumin to its fructoselysine-specific receptor proteins play a role in activation of transcription.

Expression of IL-1ß mRNA

The influence of glycated albumin on the level of cytokine mRNA was investigated. The involvement of PKC and PTK signal transduction pathways in the transcriptional response mechanisms of MM6 cells after stimulation with glycated albumin was evaluated using the specific protein kinase inhibitors GÖ6976 and genistein.

As shown in Figure 6, IL-1ß specific mRNA expression was increased dose-dependent in response to glycated albumin. To facilitate the interpretation of the data obtained results were expressed as percentage of mRNA expression to the control (100%). 0.5 mg/ml glycated albumin raised the mRNA level to about 133% of the control with non-glycated albumin ($p < 0.02$). After stimulation with 1 and 2 mg/ml glycated albumin an increase to 147 and 215% was observed ($p < 0.042$).

Pretreatment of MM6 cells with 100 nM GÖ6976 for 1 h decreased the mRNA level significantly to 17% ($p < 0.005$) measured in the presence of glycated albumin, whereas 40 μM genistein showed no significant effects (Figure 7). Therefore, mainly a PKC mediated signal transduction is involved in the increased transcription of the proinflammatory cytokine mRNA after binding of Amadori-modified albumin to monocytic MM6 cells.

Preincubation of MM6 cells with an anti-myosin antiserum reduced glycated albumin stimulated transcription of IL-1ß mRNA by 49.3% ($p < 0.01$) (Figure 7). Activation of transcription by non-glycated albumin was not affected. Anti-nucleolin antiserum diminished increased transcription with glycated albumin by less than 10% (data not shown).

Discussion

The diversity and the molecular mechanisms of the Maillard reaction in vivo and its significance in the pathophysiology of aging, in diabetic sequelae, arteriosclerosis and degenerative brain diseases are beginning to be unravelled. AGEs play a pivotal role for the development of these illnesses [13–15]. The importance of Amadori adducts as early products of the Maillard reaction in vivo for the pathogenesis of diabetic late complications has not yet been elucidated in such a comprehensive way.

Glycated Amadori-modified albumin is the main glycated protein in human plasma. Its concentration increases during

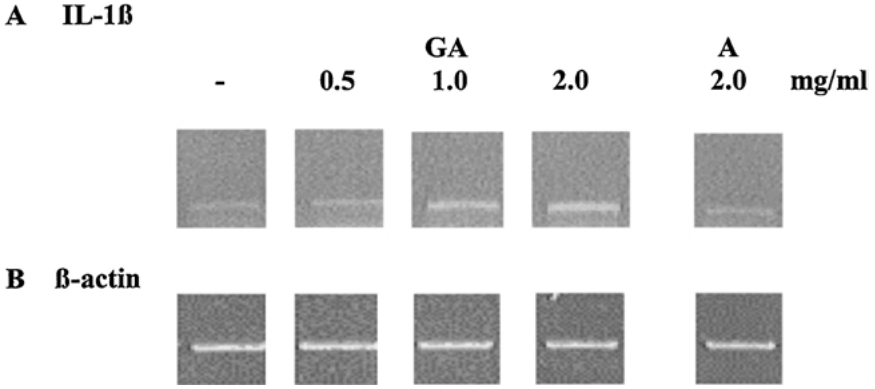

Figure 6. Effects of glycated albumin on transcription of IL-1ß mRNA in MM6 cells. Glycated albumin stimulated cytokine-specific mRNA synthesis dose-dependent. Differences in mRNA expression of cells incubated with glycated were statistically significant. GA = glycated albumin; A = fructoselysine-free albumin.

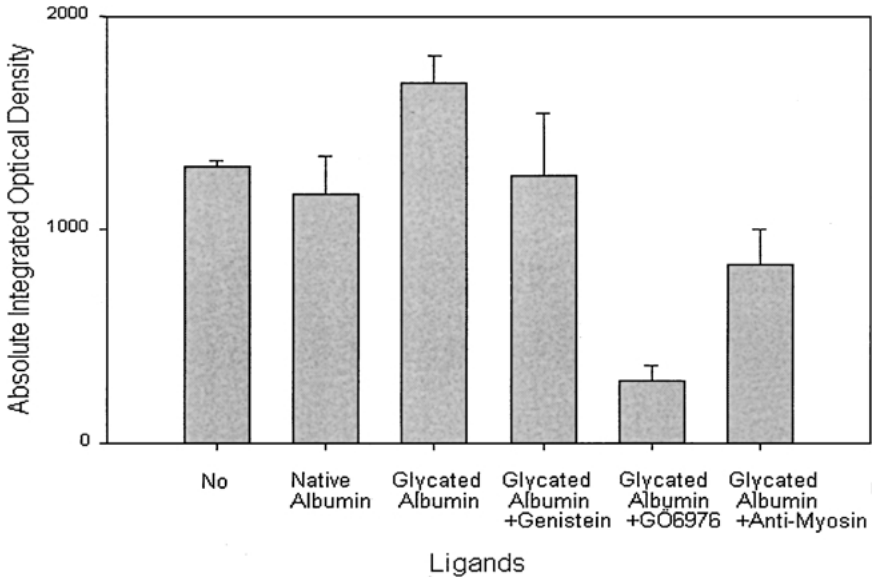

Figure 7. Inhibition of activation of IL-1ß mRNA transcription by the protein kinase inhibitors GÖ6976 and genestein as well as by an anti-myosin antiserum. Glycated albumin stimulated cytokine-specific mRNA synthesis was mainly blocked by PKC inhibitor GÖ6976 ($p < 0.02$) and anti-myosin antibody ($p < 0.01$). MM6 cells were stimulated with 0.5 mg/ml proteins as ligands. GA = glycated albumin, A = fructoselysine-free albumin.

hyperglycemia. Previous investigations have demonstrated that Amadori adducts can bind via specific receptors to monocytes and macrophages [2–4] as well as to endothelial and mesangial cells and to renal brush border membranes [5–7,16]. Two fructoselysine-specific binding proteins with molecular masses of 100 and 200 kDa were obtained by affinity purification of MM6 membrane proteins and characterized as nucleolin and cellular myosin heavy chain. These receptor proteins were also isolated from membranes of U937 cells [11, 17] and these amino acid sequence homologies were not found in any of the AGE-receptors, from which sequence data are described up to now.

Nucleolin is a multifunctional, major nuclear protein with RNA-binding properties, which cycles between cytoplasma and nucleus. Minor amounts of it are found in the plasma membranes of some cells, where it binds different ligands [18–22]. Cellular myosin heavy chain is a 224 kDa protein, which is also expressed in myeloid cell lines [23]. The molecular masses of 180 to 200 kDa of the fructosyllysine-specific binding protein indicate that a shortened product in comparison with the cytosolic protein may be inserted into the cell membrane. Cellular myosin has previously been shown also to be membrane-bound in neuronal plasma membranes [24].

Membrane-bound nucleolin and cellular myosin heavy chain are enzymatically glycosylated. Both proteins contain glycosylation sites in their amino acid sequence [23,25]. But the cytosolic forms of both proteins are free from carbohydrates and do not bind glycated albumin [17]. Their glycosylation may be a signal traffiking them into the plasma membrane and conferring ligand binding properties to them.

Binding of glycated albumin to MM6 cells causes an increased secretion of the proinflammatory cytokines IL-1ß and tumor necrosis factor α [3]. The results of these new experiments establish that glycated albumin showed a trend toward stimulation of total PKC and PTK activities in MM6 cells and demonstrate that activation of only the PKCε isoenzyme is required for the production of IL-1ß after stimulation with glycated albumin. This interpretation is supported by the observations that PKC inhibitor GÖ6976 prevents activation of the transcription factors AP-1 and NF-κB as well as the increased transcription of IL-1 mRNA by glycated albumin. Both transcription factors are involved in the expression control of the IL-1 gene, which possesses binding sites for these factors in the 5′ upstream regulatory region [26]. AP-1 activation was shown also to depend on PTK by inhibition experiments with genistein. It has also been demonstrated previously that AP-1 can be activated by PKC [27] and PTK [28,29]. Non-glycated albumin did not induce IL-1 secretion [3] and cannot activate protein kinases, the transcription factors AP-1 and NF-κB and the transcription of IL-1 mRNA in the same way as glycated albumin.

Cellular events after activation of PKCε are largely unknown. The main downstream effector pathway is the activation of the ERK MAPK cascade via Raf-1 kinase, which is either directly phosphorylated or activated by an autocrine growth factor dependent mechanism [30]. Further experiments are necessary to establish this signal transduction pathway mediating activation of NF-κB and AP-1.

The interactions of glycated albumin with its receptor proteins and the following cellular events can be inhibited by receptor-specific antibodies. The reduced activity of anti-nucleolin antiserum in comparison to anti-myosin may be interpreted that anti-nucleolin antibodies do not block sites for binding glycated albumin or sites inducing signal transduction.

The molecular events connecting receptor activation after binding of glycated albumin with the activation of intracellular protein kinases are still unknown. The present results support a' cross talk' between PKC and PTK in the activation process of transcription factors AP-1 and NF-κB by glycated albumin. On the other side, PKC can activate NF-κB directly by phosphorylation of IκB [31].

Glucose-modified proteins play a role in the development of diabetic nephropathy [8,9,32–36]. Glycated albumin induces PKCβ_1 activation in glomerular endothelial and mesangial cells, which was associated with a stimulated production of transforming growth factor ß and basement membrane proteins [34,35].

All these studies may have physiological significance, because an albumin Amadori-modified to an extent as seen *in vivo* and free from AGEs was used for the incubation experiments. Amadori glucose adducts are the principal forms, in which glycated albumin exists *in vivo* and may be involved in the pathogenesis of diabetic vascular diseases when formed in excess during chronic hyperglycemia.

During the preparation of the manuscript we became aware that Amadori-modified albumin also stimulated NF-κB and AP-1 activation in peritoneal mesothelial and vascular smooth muscle cells [37,38].

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