Advanced glycation endproducts stimulate the MAP-kinase pathway in tubulus cell line LLC-PK$_1$

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Received 17 May 1997

Abstract Advanced glycation endproducts (AGEs) are suggested to play an important role in diabetic nephropathy. They induce specific cellular responses such as the release of cytokines in different cell lines. The effect of AGEs on signal transduction pathways was investigated in the renal tubulus cell line LLC-PK$_1$. Using a serine-phosphate-specific antibody AGE-induced cellular responses associated with phosphorylation/dephosphorylation events were demonstrated. In particular, the p42^{MAP} kinase and its downstream target, the AP-1 complex, are shown to be activated by AGE-BSA but not by BSA. In contrast, only partial phosphorylation is observed for the p70^{S6}-kinase. Thus, AGEs appear to induce specific signal transduction pathways.

Key words: Advanced glycation endproducts; MAP-Kinase; Tubulus

1. Introduction

The amino groups of proteins react non-enzymatically with reducing monosaccharides, including glucose, fructose, hexose-phosphates, trioses and triose-phosphates. This 'non-enzymatic glycosylation' or 'glycation' reaction leads through subsequent oxidation, cyclisation and dehydration reactions to a broad range of heterogeneous fluorescent and yellow-brown, substances called 'Advanced Glycation Endproducts' or 'AGEs'. AGE formation is irreversible and causes protease-resistant cross-linking of long-lived peptides and proteins, leading to protein deposition and amyloidosis. Recent progress in the understanding of this process has confirmed that AGEs play a significant role in the pathogenesis of vascular complications in ageing and degenerative diseases, particularly in Alzheimer's disease, diabetes and end stage renal disease [1–3]. The pathological effects of AGEs were originally attributed to their physicochemical properties including protein cross-linking but more recent studies increasingly emphasise the role of AGEs in cellular signalling events by oxygen free radicals [4,5].

Among the cellular actions of AGEs, release of cytokines such as interleukin 1 and 6 and TNF-α has been described in different cell types such as macrophages, endothelial cells and even neurones [4,6]. These responses are most likely mediated by AGE receptors, among which the receptor for AGEs (RAGE) is the best studied member in term of signal transduction [7]. Binding of AGEs to RAGE results in production of superoxide radicals which subsequently activates transcription factor NF-κ-B, with consequences for a range of cellular functions [5]. However, intermediate signalling pathways from RAGE to the superoxide producing enzyme and the transcriptional activation are yet to be identified.

To demonstrate specific effects of AGEs on signal transduction changes in the phosphorylation pattern of serine kinases by AGEs as well as the effects on the p70^{S6}-kinase and p42^{MAP}-kinase are shown in this study .

The AGE-level in the serum is closely dependent on kidney function. On one hand circulating low molecular weight AGEs are eliminated by the kidney, on the other hand AGEs are suggested to be one of the major causes of diabetic nephropathy [8,9]. While AGEs have been shown to be involved in glomerulosclerosis and to effect mesangial cells [9,10], little is known about effects of AGEs on the tubulus. Therefore a further objective of this study was to demonstrate that, in addition to the mesangial cells also tubular cells respond to AGEs at the level of gene expression and signal transduction.

2. Material and methods

LLC-PK$_1$ renal epithelial cells (ATCC CRL 1392) were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal calf serum as described [11]. Cells were grown for 4 days until confluent monolayers were observed. Before treatment the cells were deprived for 24 h in DMEM containing 0.2% foetal calf serum.

Bovine serum albumin (1 mM BSA Fraction V, Sigma) was glycated by incubation with 1 M glucose in 50 mM potassium phosphate, pH 7.3, 1 mM EDTA under sterile conditions at 37°C for 40 days following a modified protocol of Makita et al. [26]. The AGE-BSA solution was lyophilized and extensively dialysed against bidestilled water. Residual glucose was less than 0.1 mM after this procedure. The preparation showed an E_{350} of 28 mM^{-1} albumin.

Western blots were performed as previously described [12]. Briefly, cells were lysed in 50 mM Tris-Cl, pH 6.7, 2% SDS, 2% mercaptoethanol, 1 mM sodium orthovanadate followed by digestion of nucleic acids with benzonase (Merck, Darmstadt, FRG). After SDS-PAGE (20 μg protein/well), proteins were transferred onto reinforced nitrocellulose by semidry blotting. The sheets were saturated with 2% BSA and incubated for 1 h with either mouse monoclonal antiphosphoserine antibody clone 4A9 (1 μg/ml, Biomol, Germany), rabbit polyclonal anti MAP-kinase (1 μg/ml, Santa Cruz, Germany) or rabbit polyclonal anti rat S6-kinase (1 μg/ml, Biomol, Germany) antibodies. After washing, sheep anti-mouse IgM horseradish peroxidase-labelled (1:10 000, Sigma) in case of antiphosphoserine or goat anti rabbit IgG horseradish peroxidase-labelled antibody (1:5000, Dianova) in case of the MAP and S6-kinase was added for 1 h. The detection was done by chemiluminescence, using luminol (2.5 mM) and *p*-coumaric acid (400 μM) as enhancer.

The electrophoretic mobility shift assay was mainly performed according to the protocol of Lane et al. [13]. Nuclear cell extracts ol LLC PK$_1$ cells were prepared according to the protocol of Andrews

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PII S0014-5793(97)00644-3

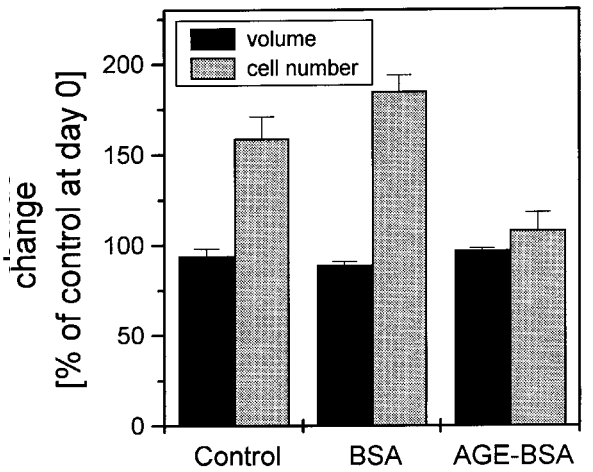

Fig. 1. AGEs inhibit proliferation of LLC-PK1 cells. Proliferating LLC-PK1 cells were treated with 50 μM AGE-BSA or 50 μM BSA. After 24 h, cell number and cell volume were determined with a Coulter-like cell counter (CASY-1 system). Results are means ±S.E.M. of independent triplicate experiments.

and Faller [14]. The AP-1 consensus sequence (CTAGAGCATGAGTCAGACACT) was synthesised on a Gene-Assembler Plus (Pharmacia, Freiburg, Germany) and purified by preparative denaturating gel electrophoresis. The oligonucleotide was labelled by filling the ends with Klenow polymerase (Boehringer Mannheim, Germany) according to manufacturer's instructions and subsequently purified from unincorporated nucleotides by gel filtration on Sephadex G-25 (Pharmacia, Freiburg). Binding was performed in 10 mM HEPES, pH 7.5, 0.5 mM EDTA, 50 mM KCl, 1 mM DTT, 5 mM spermidine, 2 mM Ficoll 400, 6% glycerol, 1 mg/ml DNAse-free BSA, 0.2 mM benzamidine, 0.1 mM PMSF and 1 pM poly-dI/dC. To a total of 20 μl 10 μg of nuclear extract and 1 μl of labelled oligonucleotide (0.1 pMol with approximately 25000 cpm/μl) were added and incubated for 30 min at 4°C. Protein-DNA-Complexes were analysed by native polyacrylamide electrophoresis (5%) in 0.5×TBE and 5% glycerol. After drying the gel was exposed to a Kodak X-ray film overnight at −70°C using an intensifying screen.

3. Results and discussion

AGEs elicit a wide range of cell-mediated responses in different cell types which leads to a variety of effects, including anti-proliferative effects [15,16]. In the present study an anti-proliferative effect was also observed in the tubulus cell line LLC-PK$_1$. After incubation with 50 μM AGE modified BSA for 24 h AGE-BSA reduced significantly cell proliferation in comparison with control cells incubated with the same concentrations of BSA. In contrast there were neither significant changes in cell volume (Fig. 1) nor any significant cytotoxic effects to LLC-PK$_1$ cells (unpublished results), this is in accordance with observations of Chibber et al. [27]. In presence of non-modified BSA a slight increase of cell number could be observed (Fig. 1). This indicates that LLC-PK$_1$ cells respond to extracellular AGEs. In order to demonstrate whether these effects are receptor mediated, we looked for activation of fast signal transduction pathways by Western blot analysis. The influence of AGE modified BSA was measured on confluent monolayer cultures of serum deprived non-proliferating LLC-PK$_1$ cells to avoid additional effects of serum. Under these conditions, cell number remained constant after 24 h treatment with AGE-BSA.

Initially overall phosphorylation of proteins was examined using a phospho-serine-specific monoclonal IgM antibody. Western blots of crude protein extracts of AGE-BSA and BSA-treated cells were probed with this antibody and signals were visualised by using the chemiluminescence detection method. After short-term exposure of the blot (few seconds), only one single band with a molecular weight of about 75 to 80 kDa was visible (Fig. 2, upper panel). After prolonged exposure (5 min) many proteins carrying phospho-serine residues became visible (Fig. 2, lower panel). The most prominent effect is the dephosphorylation of a protein of about 100 kDa (Fig. 2, lower panel, marked with an arrow) after treatment with AGE-BSA, while this protein remains phosphorylated in cell extracts of BSA-treated cells. This result is in agreement with observations of Hasegawa and coworkers, who showed a marked decrease in tyrosine phosphorylation of paxillin and pp125^{FAK} after plating of endothelial cells on glycated basement matrix [17]. Our data show that fast phosphorylation/dephosphorylation events also occur in LLC-PK$_1$ cells upon administration of AGE-BSA indicating the involvement of a signal transduction mechanism in the development of the observed effects on cell physiology.

Subsequently we examined more specific effects of AGE-BSA treatment by monitoring the activation of two major

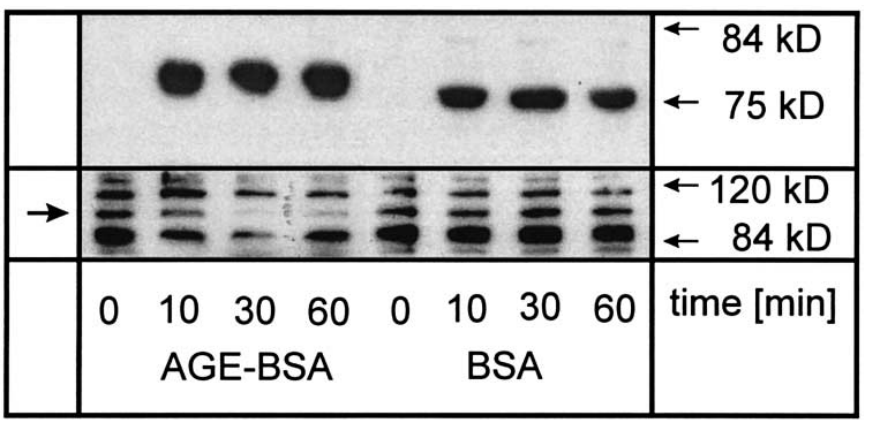

Fig. 2. AGEs induced serine phosphorylation/dephosphorylation. Serum deprived LLC-PK1 cells were treated for the indicated times in min with 50 μM AGE-BSA or BSA. After lysis, the lysates (20 μg protein) were subjected to SDS-PAGE. Proteins were transferred onto nitrocellulose and phosphoserine was detected by immuno-detection with antiphosphoserine antibody 4A9. Detection was done by chemiluminescence. Upper panel: short-term exposure (few seconds), lower panel: long-term exposure (min).

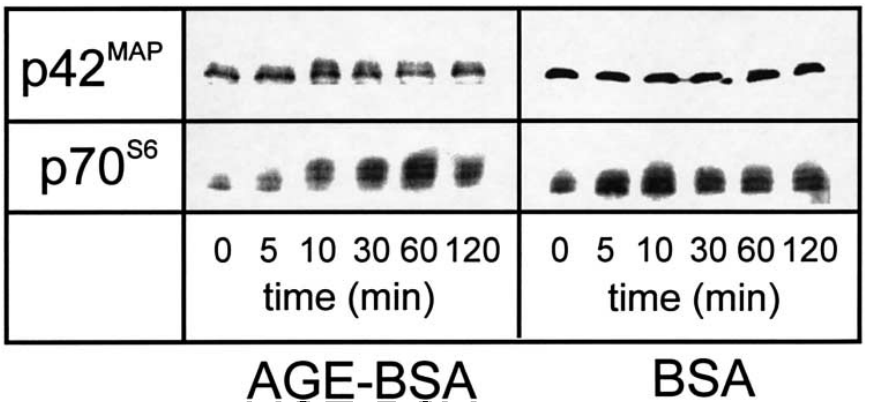

Fig. 3. AGEs activate the $p42^{MAP}$-kinase. Quiescent LLC-PK1 cells were treated with 50 μM AGE-BSA or BSA for the indicated times. According to Fig. 2, the $p42^{MAP}$ kinase and the $p70^{S6}$ kinase were detected by Western blotting.

protein kinases, the $p70^{S6}$ kinase and the $p42^{MAP}$-kinase. These two kinases represent two different signal transduction pathways.

The $p70^{S6}$ kinase phosphorylates the 40S ribosomal protein S6, which is an important factor for cell growth after stimulation with growth factors in mammalian cells [18,19]. The $p70^{S6}$ kinase is located downstream of the PI3-kinase [20] and is activated by phosphorylation. Full activation of $p70^{S6}$ kinase is achieved by multiple phosphorylations causing multiple band shifts in Western blots. However, AGE-BSA stimulates only a partial phosphorylation of $p70^{S6}$ kinase (Fig. 3) since not all possible band shifts are visible. As only the completely phosphorylated form of $p70^{S6}$ kinase can be correlated with an active kinase [21], AGE-BSA appears not to fully activate this kinase. In cell extracts of BSA-treated controls, no phosphorylation of $p70^{S6}$ kinase was detectable by the band shift assay. Therefore, AGE-induced phosphorylation may enhance activation of $p70^{S6}$ kinase in the presence of additional signals.

The $p2^{MAP}$-kinase (Erk) signalling cascade has been suggested to act as a signal integration mechanism for many external signals in eucaryotic cells [22]. The $p42^{MAP}$-kinase itself is activated by dual phosphorylation of conserved threonine and tyrosine residues within a characteristic TEY motif via the ras and raf pathway. The active (phosphorylated) form migrated slower and is identified as a shifted band in Western blots using a MAP-kinase-specific antibody (Fig. 3). Whereas in Western blots of BSA-treated control cells no gel shift was observed, in extracts of cells incubated for 10 min with AGE-BSA (50 μMol) a band shift could be detected indicating phosphorylation of MAP-kinase (Fig. 3). After 60 min, the shifted band disappeared indicating dephosphorylation and inactivation of $p42^{MAP}$-kinase. The time kinetic is similar to those observed after administration of growth factors to cells. These results indicate a direct or indirect stimulation of MAP-kinase phosphorylation by AGEs pointing to a specific response of LLC-PK_1 cells to non-enzymatic endproducts.

To further demonstrate activation of $p42^{MAP}$ kinase, we looked for downstream signals induced by this kinase. Activation of the AP-1 complex was chosen mainly for two reasons. Firstly, induction of the transcription factor c-*fos* is one prominent and well characterised downstream effect of $p42^{MAP}$-kinase activation [22]. Secondly, it has been reported that AGEs induce oxidative stress [6]. In addition to NF-κ-B activation, AP-1 activation has been shown to occur in the course of response to stress, especially oxidative stress [23,24]. Indeed, in nuclear extracts of LLC-PK_1 cells after 30 min of incubation with artificial AGE-BSA an activation of the AP-1 complex was detectable by the band shift assay when compared with the controls, e.g. cells incubated with the same concentration of unmodified BSA (Fig. 4). The observed signal was completely inhibited by an excess of unlabelled AP-1 oligonucleotide while the presence of excess SP-1 oligonucleotide-binding sequence had no effect on the observed shift (Fig. 4). Therefore, our results demonstrate that, as expected, treatment with AGEs enhances AP-1 activation. This supports the hypothesis that phosphorylation of $p42^{MAP}$ kinase is of physiological importance.

Currently activation of transcription factors by advanced

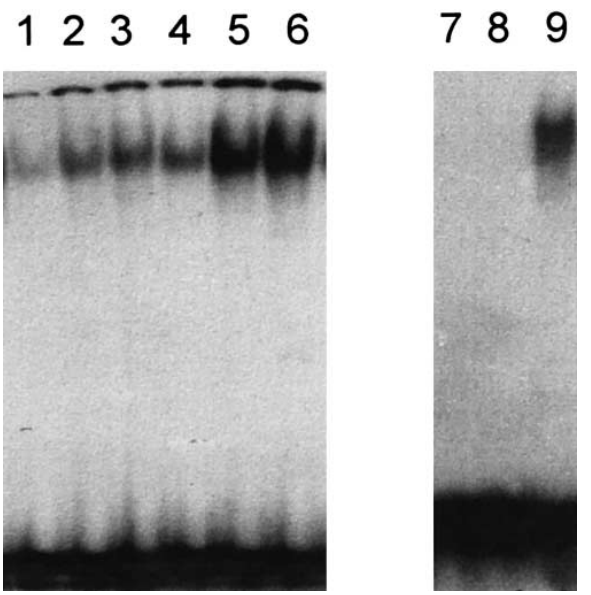

Fig. 4. Demonstration of AGE induced AP-1 activity by gel shift analysis. Nuclear extracts of LLC-PK1 incubated with 50 μM AGE-BSA (lanes 4, 5, 6) and BSA (lanes 1, 2, 3) for 30 min were prepared as described in Section 2 and analysed by gel shift assay using an AP-1 specific oligonucleotide. On lane 1, 2 and 3, 5 μg, 10 μg and 15 μg of the nuclear extracts from BSA-treated cells were loaded, on lane 4, 5 and 6 the same amounts of nuclear extracts from AGE-BSA-treated cells were loaded. Lane 7 is without extract, lane 8 shows competition with an excess of unlabelled AP-1 oligonucleotide, lane 9 is in presence of an excess of unlabelled SP-1 oligonucleotide.

glycation endproducts has been reported for the major inducible transcription factor in many immune and inflammatory processes, NF-κ-B via the RAGE-receptor [6]. For many types of stress a co-activation of the NF-κ-B complex and the activation protein (AP-1) was observed, although the signal transduction pathways differ for both complexes [23,24]. Therefore it is reasonable to assume that following treatment with AGEs co-activation of AP-1 and NF-κ-B can occur. Indeed, the promoter region of the tissue factor gene, whose expression is induced by AGEs contains both NF-κ-B and AP-1-binding sites [25].

In conclusion, our results confirm earlier observations that AGEs cause specific cellular response in different cell types. Furthermore it is shown that the cellular response to AGEs is associated with phosphorylation/dephosphorylation events. The $p42^{MAP}$ kinase was shown to be activated by AGE-BSA but not by BSA alone. The activation of the AP-1 complex supports the assumption that $p42^{MAP}$ kinase is activated specifically. In contrast, only partial phosphorylation was observed for the $p70^{S6}$-kinase. The present results do not allow to demonstrate whether AGEs alone are capable of causing the observed effects directly or whether AGE-induced caused oxidative stress is responsible for the described cellular effects. Future work will be aimed at characterising the complete signal transduction pathway of AGEs.

Acknowledgements: We thank Dieter Palm, Peter Riederer, Johannes Thome and Anne M. Cunningham for discussions. This work was supported by a fellowship of the Claussen-Stiftung. to G.M., by the SFB-355 and the Hirnliga e.V.

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