Activation of the receptor for advanced glycation end products induces nuclear inhibitor of protein phosphatase-1 suppression

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The activation of the receptor for advanced glycation end products (RAGE) is involved in the development of diabetic nephropathy. Analysis of protein phosphatase-1 indicated that advanced glycation end products did not affect its expression, but increased its phosphatase activity. Using differential display analysis we previously demonstrated that stimulation of RAGE in podocytes modulates the expression of numerous genes, among others nuclear inhibitor of protein phosphatase-1 (NIPP1). Here we found that silencing of NIPP1 induced podocyte hypertrophy, cell cycle arrest, and significantly increased protein phosphatase-1 activity. NIPP1 downregulation was associated with increased $p27^{Kip1}$ protein expression. Reporter assays revealed a transcriptional activation of nuclear factor-κB in podocytes after suppression of NIPP1. The protein level of NIPP1 was also significantly reduced in podocytes of diabetic mice. Blocking the RAGE *in vivo* by a soluble analog elevated the NIPP1 protein in podocytes of diabetic mice. Thus, activation of the RAGE by advanced glycation end products or other ligands suppresses NIPP1 expression in diabetic nephropathy, contributes to podocyte hypertrophy, and glomerular inflammation.

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Podocytopathy with foot process effacement, podocyte hypertrophy, and detachment is characteristic of early diabetic nephropathy (DN).[1–6] The development and progression of DN is intensified by continuing hyperglycemia, contributing to the formation of advanced glycation end products (AGEs). The receptor for AGEs (RAGE) is a multiligand pattern-recognition receptor, but it binds AGEs with high affinity.[7–10] RAGE activation induces intracellular signaling cascades resulting in the activation of the transcription factor nuclear factor-κB (NF-κB).[11–15] Podocyte AGE-bovine serum albumin (AGE-BSA) treatment increased $p27^{Kip1}$ expression, inducing cell cycle arrest and cellular hypertrophy without apoptosis.[11,16,17]

NIPP1, the nuclear inhibitor of protein phosphatase-1 (PP1), was originally isolated as a specific inhibitor of the Ser/Thr phosphatase PP1.[18–22] NIPP1 is a ubiquitously expressed protein preferentially located in the cell nucleus and interacts with about 50% of all PP1 molecules.[19,20,23,24] NIPP1 contains three nuclear localization signals[25–27] and is involved in the translocation and nuclear retention of the PP1 catalytic subunit.[24] The NIPP1/PP1 holoenzyme is inactive in nuclear extracts, but it can be activated through multiple NIPP1 phosphorylations by protein kinase A or casein kinase 2.[28–30] The PP1 phosphatase is involved in a variety of cellular processes such as metabolism and cell cycle regulation.[24] It has also been shown that PP1 is involved in the regulation of the cell cycle progression through different mechanisms.[31–34]

Using differential display analysis, we previously demonstrated that stimulation of the receptor for advanced glycation end products (RAGE) by AGE-BSA treatment in podocytes modulates the expression of numerous genes, for example, NIPP1.[35] The aim of this study was to investigate to what extent NIPP1 expression is altered in AGE-BSA–treated podocytes and to identify its potential role in the pathophysiology of DN.

RESULTS

Influence of AGE-BSA on NIPP1 expression in podocytes

We studied the changes in NIPP1 expression by real-time polymerase chain reaction (PCR) in podocytes treated for

24 h with AGE-BSA and found a significant decrease in NIPP1 mRNA expression compared with the control-BSA (co-BSA)-treated cells (Figure 1a). Western blot analysis revealed that NIPP1 protein levels are also significantly inhibited by AGE-BSA (Figure 1b and c). We examined the cellular distribution of NIPP1 and observed that NIPP1 was found mostly in the cell nucleus and only to a small extent in the cytoplasm of co-BSA–treated podocytes. However, AGE-BSA addition significantly reduced NIPP1 protein expression in both the cytoplasm and the nucleus (Figure 1d and e).

AGE-BSA treatment increased PP1 activity

As NIPP1 is a regulatory subunit of PP1 phosphatase,[19,22,23] we investigated the expression and enzymatic activity of PP1 in AGE-BSA–treated podocytes. Real-time PCR and protein analyses revealed no significant differences in either PP1 mRNA (Figure 2a) or protein expression (Figure 2b and c) upon AGE-BSA stimulation. We observed a moderate translocation of the PP1 protein from the nucleus to the cytoplasm and accumulation in the regions near the nucleus (Figure 2d, arrow) when AGE-BSA was added (Figure 2d and e). As it is known that NIPP1 affects PP1 activity,[19,24,27,36] we analyzed PP1 activity using Ser/Thr phosphatase assay. We detected significantly higher PP1 activity in the AGE-BSA–treated cells compared with the co-BSA (Figure 2f).

Silencing of NIPP1 expression in podocytes elevated PP1 activity

We further silenced NIPP1 expression in podocytes by transient transfection of NIPP1-specific small interfering RNA (NIPP1 siRNA). Real-time PCR data showed a suppression of NIPP1 mRNA (Figure 3a) by NIPP1 siRNA, but not co-siRNA. NIPP1 protein level was also significantly reduced (Figure 3b). We also performed a real-time PCR

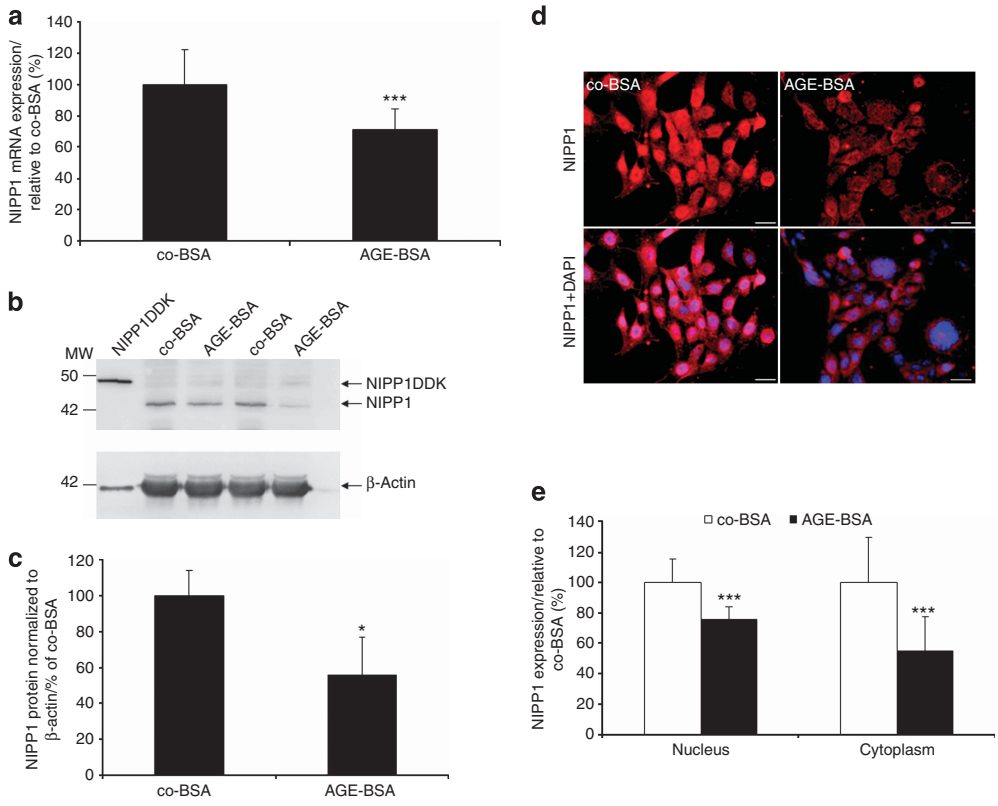

Figure 1 | Expression of nuclear inhibitor of protein phosphatase-1 (NIPP1) in podocytes treated with control-bovine serum albumin (co-BSA) and advanced glycation end product-BSA (AGE-BSA). (**a**) Real-time polymerase chain reaction (PCR) analysis of NIPP1 mRNA expression. Treatment of podocytes with AGE-BSA reduced the expression of NIPP1 mRNA, compared with co-BSA-treated cells. The data are quantified as described in the Materials and Methods section and expressed as the percentage of co-BSA. ***$P<0.001$ versus co-BSA, $n=18$. (**b**) Detection of NIPP1 protein by western blot analyses in lysates from co-BSA- and AGE-BSA-treated cells. The equal loading was monitored by β-actin protein expression. Left margins show the molecular weight of the proteins in kDa. Lysate expressing NIPP1DDK-tagged protein was used as a positive control for NIPP1 expression. Representative western blot analysis are shown. NIPP1 protein level was decreased after AGE-BSA addition. (**c**) Quantification of NIPP1 protein expression. The expression of NIPP1 protein was normalized to β-actin and presented in percentage relative to co-BSA. *$P<0.05$ versus co-BSA, $n=9$. (**d**) Immunological analysis of NIPP1 protein expression. Representative images of NIPP1 detection in co-BSA- and AGE-BSA-treated podocytes, as well as merge image of NIPP1 and 4′,6-diamidino-2-phenylindole (DAPI) (nuclear staining), are shown. The staining demonstrates mainly nuclear localization and only a weak staining for NIPP1 into the cytoplasm of the podocytes. Bars = 20 μm. (**e**) Densitometry quantification of NIPP1 immunoreactivity. The relative intensity of NIPP1 in the cell nucleus and cytoplasm is presented in percentage relative to co-BSA. ***$P<0.001$ versus co-BSA, $n=6$.