Advancements in renal protection

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CHAPTER 9

PREPARATION OF ADVANCED GLYCATION END PRODUCTS *IN VITRO*

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Enhancement of epithelial sodium channel expression in renal cortical collecting ducts cells by advanced glycation end products

Chang CT, Wu MS, Tian YC, Chen KH, Yu CC, Liao CH, Hung CC, Yang CW.

Background. The epithelial sodium channel (ENaC) is a complex, and the alphaENaC subunit has a crucial role in sodium uptake induced by aldosterone in the distal nephron. Although experimental animal models of diabetes have demonstrated up-regulation of alphaENaC expression in renal cortical collecting duct (CCD) cells, the molecular mechanism remains unclear. Advanced glycation end products (AGEs) are by-products of long-term hyperglycaemia and comprise a significant pathogenic factor in diabetic nephropathy. We hypothesize that AGEs play a role in regulating alphaENaC gene expression.

Methods. Mouse CCD cells (mpkCCDcl(4)) were cultured with AGE to determine the effects of AGE on alphaENaC expression and sodium uptake. Gene expressions of ENaC were measured by real-time PCR and sodium uptake was measured with fluorescent dye as a sodium indicator (SBFI-AM). This study analysed mitogen-activated protein kinases signaling pathways by western blotting. Cells co-transfected with plasmids of the alphaENaC promoter carrying a luciferase reporter and plasmids expressing wild-type or mutant serum- and glucocorticoid-induced kinase 1 (Sgk1) mRNA were stimulated with AGE to identify the signaling pathway.

Results. The AGEs, stimulated in a time- and dose-dependent manner, enhanced alphaENaC mRNA expression and sodium uptake in mpkCCDcl(4) cells. The AGEs also significantly stimulated Sgk1 mRNA and Sgk1 activity in a time- and dose-dependent manner. Co-transfected with plasmid expressing mutant Sgk1 significantly limited stimulated alphaENaC promoter-driven luciferase activity by AGEs in mpkCCDcl(4) cells.

Conclusion. Experimental results indicate that AGEs induced alphaENaC expression and increased sodium uptake in renal CCD cells. The mechanism through which AGEs activate alphaENaC expression may be via activation of Sgk1 in mpkCCDcl(4) cells.

Preparation of advanced glycation end products *in vitro*

With great interest we read the article by Chang et al.1 on the enhancement of epithelial sodium channel mRNA expression and protein level in renal cortical collecting duct (CCD) cells by advanced glycation end products (AGEs). The authors show that AGEs can increase sodium uptake in CCD cells in a dose dependent way, providing a novel mechanism for the dysregulation of sodium balance in diabetic nephropathy.

However, we have concerns on their *in vitro* preparation of AGEs. Since their results are dependent on the stimulation of renal CCD cells with AGEs prepared *in vitro*, it is very important that they prepared their AGEs properly. For instance, Valencia et al. showed that AGE preparations which were free of significant levels of endotoxin contamination, failed to induce proinflammatory cellular responses, whereas endotoxin induced cell surface VCAM-1 on HMEC-4 endothelial cells and tumour necrosis factor-α secretion by primary human PBMCs.[2] Therefore, to exclude the possibility that the effects of AGEs on epithelial sodium channel expression in renal CCD are mediated by endotoxin contamination, the authors should have used endotoxin-free bovine serum albumin (BSA) and assayed the control and AGE preparation for endotoxin contamination, for example by using the *Limulus amebocyte* lysate assay.

The authors added azide to prevent contamination by live bacteria under these pro-biotic conditions (a high glucose concentration at 37 °C). They do not describe the use of sterilization filters before the 6 week incubation period nor after dialysis, which is a procedure with a risk of contamination. In our opinion using sterilization filters is important in the process of AGE-preparation. But as mentioned earlier, the main problem is contamination by endotoxin.

In addition to the aforementioned concerns, the degree of glycation was only indirectly assessed as fluorescence measured by spectrophotometry. Although this indeed strongly suggests that AGEs have been formed, it would have been more elegant to show specific AGE levels such as N^{ε}-(carboxymethyl)lysine (CML) and N^{ε}-(carboxyethyl)lysine (CEL). The authors used the correct control preparation which was treated identically with the exception that glucose was omitted. In our lab we recently prepared AGEs by incubating endotoxin-free BSA 300 mg/mL at 37 °C for 6 weeks with D-glucose (90 g/L) in a 0.4 M phosphate buffer at pH 7.6. Interestingly, we saw an increase in CML and CEL in our con-

trol preparation which was treated identically but without the addition of glucose, when compared to non-incubated BSA (Figure). It would have been interesting to use non-incubated endotoxin-free BSA as a control as well.

We conclude that Chang et al. propose a novel mechanism that could be involved in disturbances of sodium balance in diabetic nephropathy, i.e an AGE-induced increase in expression of the epithelial sodium channel mRNA and protein, with enhanced sodium uptake in renal CCD cells. To substantiate this interesting hypothesis it would be important to exclude endotoxin mediated effects.

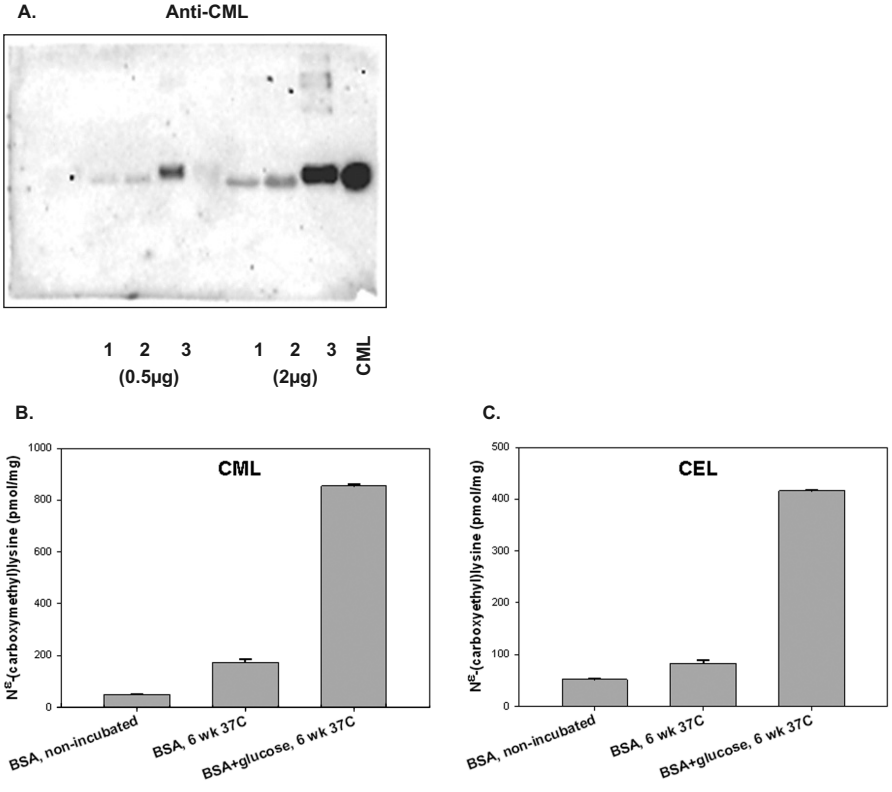

Figure 1.
A) N^{ε}-(carboxymethyl)lysine (CML) measured by western blot analysis: 1, BSA non-incubated; 2, BSA incubated for 6 weeks at 37°C; 3, BSA incubated for 6 weeks at 37°C with 0.5M glucose.
B) CML and C) N^{ε}-(carboxyethyl)lysine (CEL) levels measured by liquid chromatography-tandem mass spectrometry.

References

1. Chang CT, Wu MS, Tian YC et al. Enhancement of epithelial sodium channel expression in renal cortical collecting ducts cells by advanced glycation end products. Nephrol.Dial.Transplant. 2007; 22: 722-731

2. Valencia JV, Mone M, Koehne C, Rediske J, Hughes TE. Binding of receptor for advanced glycation end products (RAGE) ligands is not sufficient to induce inflammatory signals: lack of activity of endotoxin-free albumin-derived advanced glycation end products. Diabetologia 2004; 47: 844-852