PODOTOXICITY OF GLUCOSE DEGRADATION PRODUCTS

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BACKGROUND

Hyperglycemia is the key driver of diabetic complications and increased concentrations of glucose degradation products. The study of peritoneal dialysis solution biocompatibility has highlighted the adverse biological effects of glucose degradation products. Recently, 3,4-dideoxyglucosone-3-ene (3,4-DGE) was identified as the most toxic glucose degradation product in peritoneal dialysis fluids. In addition, 3,4-DGE is present in high-fructose corn syrup and its precursor 3-deoxyglucosone is increased in diabetes. The role of 3,4-DGE in glomerular injury had not been addressed.

We studied the effects of 3,4-DGE on cultured human podocytes and in vivo in mice. 3,4-DGE induced apoptosis in podocytes in a dose- and time-dependent manner. 3,4-DGE promoted the release of cytochrome c from mitochondria and activation of caspase-3. While high glucose concentrations increased the levels of the podocyte intracellular antiapoptotic protein HSP27/HSPB1, 3,4-DGE decreased the expression of podocyte HSP27/HSPB1. Apoptosis induced by 3,4-DGE was caspase-dependent and could be prevented by the broad-spectrum caspase inhibitor zVAD-fmk. Antagonism of Bax by a Ku-70-derived peptide also prevented apoptosis. Intravenous administration of 3,4-DGE to healthy mice resulted in a decreased expression of HSP27/HSPB1 and caspase-3 activation in whole kidney and in podocytes in vivo.

In conclusion, 3,4-DGE promotes apoptosis of cultured human podocytes by a Bax- and caspase-dependent mechanism, suggesting a potential role in glomerular injury resulting from metabolic disorders.

AIM

Study the role of 3,4-DGE in glomerular injury

METHODS

Cell culture → Human podocytes are a previously described cell line transfected with a temperature-sensitive SV40 gene construct and a gene encoding the catalytic domain of human telomerase. At a permissive temperature of 33ºC, the cells remain in an undifferentiated proliferative state, whereas raising the temperature to 37ºC results in growth arrest and differentiation to the parental podocyte phenotype. Undifferentiated podocyte cultures were maintained at 33ºC in RPMI 1640 medium with penicillin, streptomycin, ITS (Insulin, transferrin, selenite), and 10% FCS. Once cells had reached 70 to 80% confluence, they were cultured at 37ºC for at least 14 days before use, when full differentiation and nephrin expression had taken place.

3,4-DGE and caspase and Bax inhibitors → 3,4-DGE (LC Scientific, Toronto, Canada) was directly dissolved in cell culture medium. Endotoxin levels were below the detection limit as assessed by a limulus amebocyte assay (Sigma). The pancaspase inhibitor Z-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk, Bachem, Bubendorf, Switzerland) and the caspase-8 inhibitor Z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone (IETD-fmk, Calbiochem, San Diego, CA, USA) were dissolved in DMSO and used at 200 µM, concentrations previously shown to provide optimal protection from apoptosis in cultured epithelial cells and other cell systems. Final concentration of DMSO was 0.05% and did not influence podocyte apoptosis. The Bax inhibitory peptides P5 and V5 and a negative control peptide were from Tocris (Ellisville, MO). P5 and V5 are cell-permeable synthetic peptides designed from Ku-70 that inhibit Bax translocation to mitochondria and Bax-mediated apoptosis in vitro. Peptides (200 µmol/l) or vehicle were added to the cell cultures 1 h before addition of 3,4-DGE.

Cell death and apoptosis → Cells were cultured to subconfluence in twelve-well plates. Apoptosis was assessed by functional and morphological studies. Cells were rested in serum-free medium for 24h before addition of stimuli. For assessment of apoptosis by flow cytometry adherent cells were pooled with spontaneously detached cells, and incubated in 100 µg/mL propidium iodide (PI), 0.05% NP-40, 10 µg/mL RNAse A in PBS at 4ºC for >1 h. This assay permeabilizes the cells, thus PI stains both live and dead cells. The percentage of apoptotic cells with decreased DNA staining (hypodiploid cells) was counted by flow cytometry using BD CellQuest Software (BD Biosciences).

Nuclei of formalin-fixed cells were stained with DAPI (Sigma) to observe the typical morphological changes, as previously described.

In vivo administration of 3,4-DGE → All animal studies were conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals. C57/BL6 mice (12- to 14-week-old) (IFFA-CREDO, Barcelona, Spain) were intravenously injected with 200 ul saline containing 0,75 µmols of 3,4-DGE or saline vehicle (n=5) and killed after 24 h. The dose of 3,4-DGE was calculated based on cell culture experiments and an extracellular volume of 7.5 ml/mouse. Under general anesthesia kidneys were perfused with cold saline. One kidney from each mouse was frozen in OCT and used for immunofluorescence. The other kidney was snap-frozen in liquid nitrogen for protein studies.

Immunostaining of mouse tissue→ Immunofluorescence was carried out in OCT frozen mouse kidney sections 5 µm thick fixed in 4% paraformaldehyde for 10 min and permeabilized in 0.2% Triton X-100/PBS for additional 10 min, washed in PBS and incubated with goat polyclonal anti-HSP27/HSPB1 (1:60, Santa Cruz Biotechnology), rabbit polyclonal anti-cleaved caspase-3 (1:50, Cell Signaling, Hertfordshire, U.K.), and FITC-mouse anti-synaptopodin (1:10, Progen, Heidelberg, Germany) followed by incubation with anti-rabbit Alexa Fluor 633 (1:300, Invitrogen) or anti-goat Alexa Fluor 633 (1:300, Invitrogen). After washing, slides were mounted in 70% glycerol in PBS, and analyzed with a DM-IRB confocal microscope (Leica DM, Bannockburn, IL). Negative controls included incubation with a non-specific immunoglobulin of the same isotype as the primary antibody.

Western blot → Western blots were performed as described previously. Membranes were incubated overnight at 4ºC with rabbit polyclonal anti-HSP27/HSPB1 antibody (1:500; Santa Cruz Biotechnology), rabbit polyclonal anti–cleaved caspase-3 (1:1,000; Cell Signaling), or mouse anti-tubulin monoclonal antibody (1:5000, Sigma, St. Louis, MO) followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:2,000, Amersham, Aylesbury, UK). Blots were developed with the enhanced chemiluminescence method (ECL) following the manufacturer's instructions (Amersham).

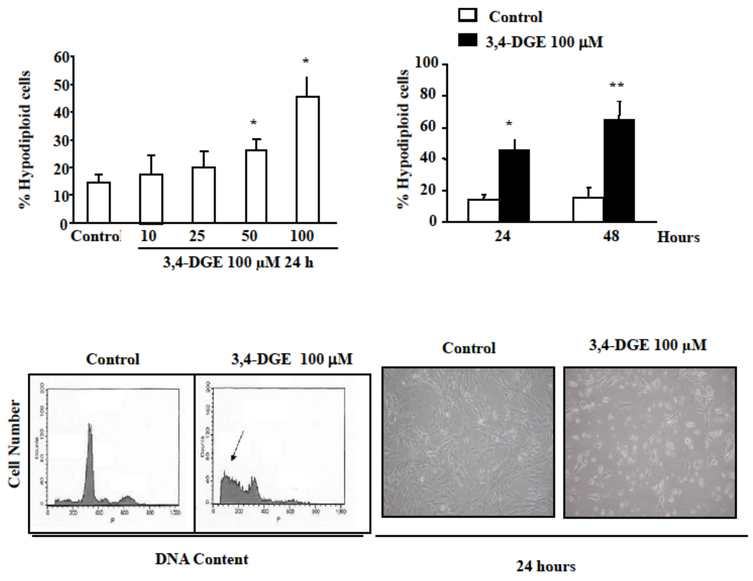
Confocal microscopy → Cells plated onto Labtek™ slides were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100 in PBS for 10 min each. After washing in PBS cells were incubated overnight at 4ºC with rabbit polyclonal anti–cleaved caspase-3 (1:100, Cell Signaling) or rabbit polyclonal anti–cytochrome c antibody (1:100; Santa Cruz Biotechnology) followed by incubation with anti-rabbit Alexa Fluor 488 (1:300, Invitrogen) and TRITC-phalloidin (Sigma). Cell nuclei were counterstained with DAPI (Vector Laboratories, Inc., Burlingame, CA). After washing, cells were mounted in 70% glycerol in PBS, and analyzed with a DM-IRB confocal microscope (Leica DM, Bannockburn, IL).

Statistics → Data are expressed as mean ± standard deviation. Mann-Whitney, 2-sided t-test or one-way ANOVA were applied to assess differences between groups. A p value < 0.05 was considered statistically significant.

RESULTS

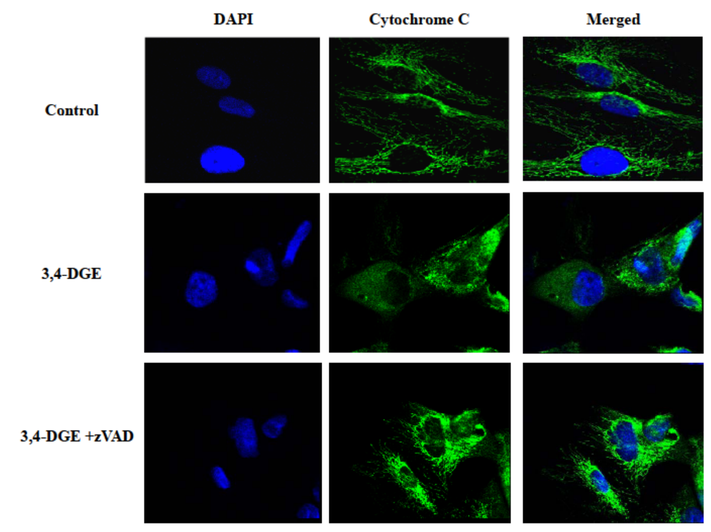
3,4-DGE promotes apoptosis in cultured human podocytes

3,4-DGE-induced apoptosis was dose- and time-dependent. Cell death was assessed by flow cytometry of DNA content (hypodiploid cells). Mean±SD of 3 independent experiments. *p<0.05 versus control 24h, **p<0.02 vs control 48h. Presence of hypodiploid apoptotic cells among cells exposed to 3,4-DGE for 24 h. Flow cytometry of DNA content. Phase contrast microscopic photographs (original magnification x200). Note numerous detached cells among those exposed to 100 µM 3,4-DGE for 24h.



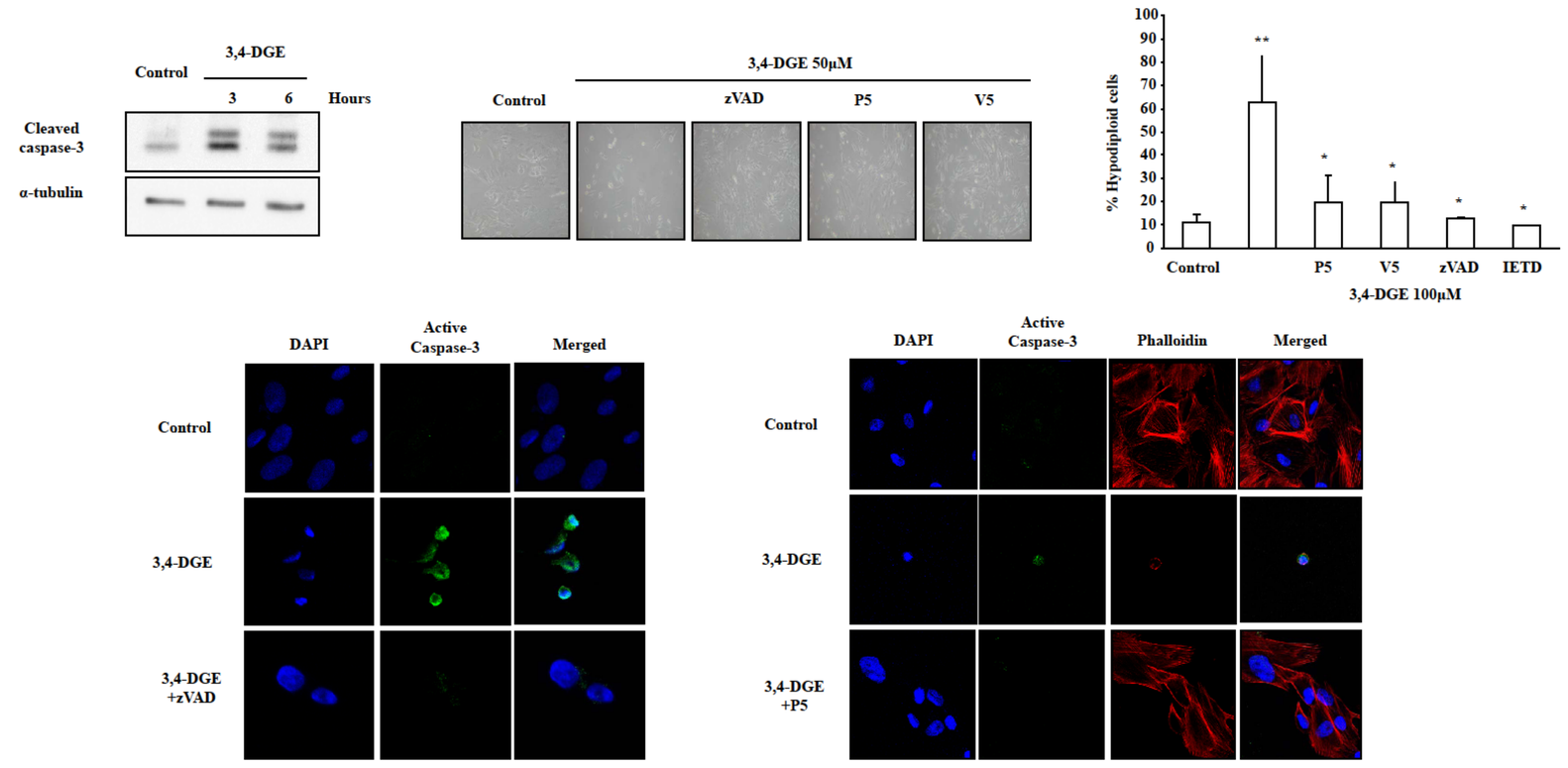
3,4-DGE promotes cytochrome c release from mitochondria in cultured human podocytes

Cytochrome c is released from mitochondria in human podocytes exposed to 3,4-DGE (100 µM) for 6 hours. Note the punctuate mitochondrial pattern in control cells and diffuse labeling indicating cytochrome c release in cells exposed to 3,4-DGE (arrow). Confocal microscopy (original magnification x200).



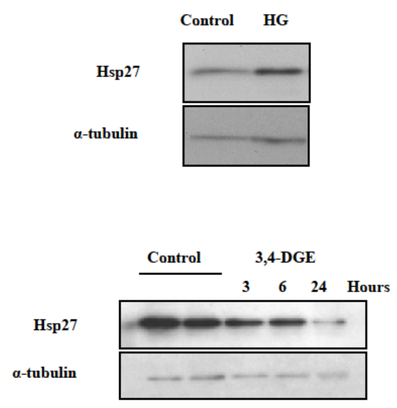
3,4-DGE promotes caspase-3 activation in cultured human podocytes. Caspase and Bax inhibitors prevent 3,4-DGE-induced apoptosis

Incubation of human podocytes with 100 µM 3,4-DGE for 6 hours resulted in the appearance of cleaved caspase-3 fragments that correspond to active caspase-3. The Ku-70-derived P5 and V5 Bax inhibitor peptides and caspase inhibitors zVAD and IETD prevented cell death induced by 3,4-DGE for 24h. Mean±SD of 3 independent experiments **p<0.005 vs control, * p<0.05 vs 3,4-DGE alone. Both Bax inhibitors and zVAD prevented eventual cell death, as can be observed by the decrease in detached cells as compared to cells exposed to 3,4-DGE only. Contrast phase microscopy (original magnification ×200). Cleaved caspase-3 was observed in cells with pyknotic nuclei characteristic of apoptosis. The use caspase inhibitor zVAD prior to exposure to 100 µM 3,4-DGE for 6 hours prevented cleavage of caspase-3. Confocal microscopy (original magnification x200). The Ku-70-derived P5 Bax inhibitor peptide prevented cleavage of caspase-3 induced by 100 µM 3,4-DGE for 6 hours in human podocytes. Confocal microscopy (original magnification ×200).



3,4-DGE downregulates HSP27/HSPB1 in human podocytes

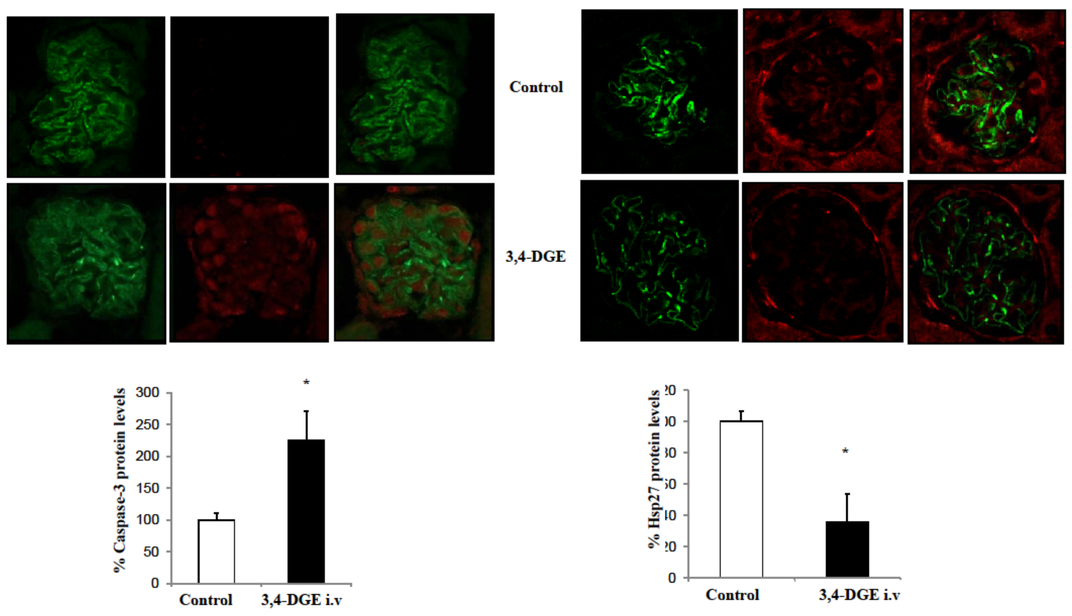
Representative Western blot of human podocytes exposed to low (5 mM, control) or high glucose (HG, 25 mM) for 24 hours. Note the increased HSP27/HSPB1 expression in cells exposed to high glucose. By contrast, 100 µM 3,4-DGE decreased HSP27/HSPB1 expression as assessed by Western blot. Quantification and representative Western blot. Mean±SD of 3 independent experiments *p<0.03 versus control, **p<0.001 vs control.



In vivo actions of 3,4-DGE

Representative immunofluorescence images of glomeruli from control and 3,4-DGE-injected mice in which podocytes are stained with synaptopodin (green). Note cleaved caspase-3 staining (red) in cell bodies co-localizing with synaptopodin in foot processes of cells with morphological features and location typical of podocytes. Original magnification x400. Quantification Western blot image of whole kidney cleaved caspase-3. Mean±SD of 5 mice per group. *p<0.05 vs control.

Representative immunofluorescence images of glomeruli from control and 3,4-DGE-injected mice in which podocytes are stained with synaptopodin (green). Note HSP27/HSPB1 (red) in some synaptopodin (green) positive cells in the control glomerulus, but absence of HSP27/HSPB1 in the glomerulus from a 3,4-DGE-injected mice. Original magnification x400. Quantification Western blot of whole kidney HSP27/HSPB1 protein. Note decreased expression in mice injected with 3,4-DGE when compared to controls. Mean±SD of 5 mice per group. *p<0.03 vs control.



CONCLUSIONS

In conclusion, 3,4-DGE is cytotoxic to podocytes and impairs their adaptive response to stress by downregulaitng HSP27/HSPB1. This may be relevant to glomerular injury in the course of diabetes and in diseases associated with high HFCS intake such as obesity.

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Diabetes - experimental models
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