Sugar-Induced Premature Aging and Altered Differentiation in Human Epidermal Keratinocytes

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ABSTRACT: Normal human epidermal keratinocytes (NHEK) show both the Hayflick phenomenon and differentiation *in vitro*. The aim of this study was to induce senescence in keratinocytes using two sugars, glucose and glyoxal. Induction of senescence in early-passage NHEK was characterized by monitoring cell morphology, short-term growth characteristics, cell proliferation, and viability assay. In addition, apoptosis, senescence-associated (SA) β-gal activity, proteasomal activity and glycation, and glycoxidation of total proteins were determined. Our results show that a 3-day treatment with 100 mM glucose or 0.1 mM glyoxal induces in early-passage NHEK various cellular and biochemical characteristics comparable to those observed in serially subcultured late passage NHEK. Furthermore, sugar-treated prematurely aged NHEK showed impaired differentiation, as measured by the quantification of involucrin. There is preliminary evidence that a preexposure of NHEK to mild heat shock (41°C, 1 h, 6 h in advance) can abrogate some of the sugar-induced negative effects, which is an example of mild stress-induced hormesis. This experimental model can be useful to study the effects of potential antiaging interventions.

KEYWORDS: sugars; premature aging; skin; epidermal cells; differentiation; hormesis

INTRODUCTION

Normal human fibroblasts develop a premature senescence phenotype following exposure to various damaging agents, such as oxidants,[1] by the expression of oncogenes[2,3] and by the inhibition of vital cellular functions, such as the proteasome activity.[4] Other sources of molecular damage are sugars, which act either directly by promoting the formation of advanced glycation end products (AGEs) or indirectly by metabolizing to reactive carbonyl species, which

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modify various amino acids in proteins.[5,6] Accumulation of AGEs and carbonylated proteins is a well-established molecular phenotype during aging.[7,8] In this study, we have tested the effects of glucose and glyoxal on normal human epidermal keratinocytes (NHEK) cell number, proliferation, viability, apoptosis, senescence-associated (SA) β-gal activity, proteasome activity and glycation, and the levels of glycoxidatively damaged proteins. Furthermore, we have also checked for the effects of glucose and glyoxal on NHEK differentiation.

EXPERIMENTAL METHODS

Primary culture of NHEK was established and maintained in proliferative mode-keeping, low calcium EpiLife medium (Cascade Biologics, Mansfield, UK) as described previously.[9,10] Immortal keratinocyte cell line HaCaT was maintained in the same way. SA β-galactosidase staining was performed as described,[11] and apoptotic cells were identified by DAPI staining. A combined assay for cell proliferation and viability was performed as a modified BrdU enzyme-linked immunosorbent assay (ELISA) (BrdU Cell Proliferation ELISA (colorimetric); Roche Applied Science, Hvidovre, Denmark) with an integrated MTT reduction step. Proteasome assay was performed as described.[9] Protein content was determined by Lowry test (D_c Protein Assay Reagent A, S, and B; Bio-Rad Laboratories, Hercules, Canada) with BSA as a standard. To quantify glycoxidatively damaged proteins, the OxyBlot Kit (OxyBlot Protein Oxidation Detection Kit: S7150; Chemicon, Temecula, CA) was modified to the ELISA system: 1 μg derivatized total protein per well was coated and immunodetected with the same antibody concentrations as recommended. The negative controls were derivatized BSA and nonderivatized cell extract, and as the positive controls, CML-BSA, and DNP-containing OxyBlot marker were used.

ELISA for quantification of differentiation markers was performed as described.[10] For the quantification of the glycated proteasome subunit α7, cells were collected in phosphate-buffered solution (PBS), sonicated, and total protein was applied on ELISA. Antibodies used were: Hsp27: spa800 (Nordic Biosite, Täby, Sweden), involucrin: ab14504 (Abcam, Cambridge, UK), p38-sc535 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal (LGC Promochem, Middlesex, UK), anti-CML-α7 antibody: 8.75×10^8 phages per mL of which 4.3% were displaying.[12]

RESULTS AND DISCUSSION

FIGURE 1 shows the dose–response curves for cell number, proliferation, and viability in NHEK treated for 3 days with glucose and glyoxal. Glucose treatment resulted in a decrease in cell number already from 5 mM glucose

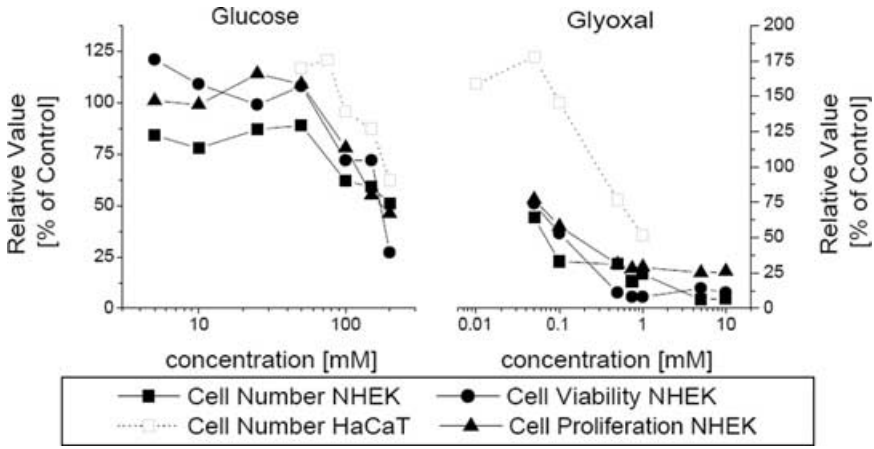

FIGURE 1. Dose–response curves displaying normalized cell numbers, cell proliferation, and cell viability at different concentrations of glucose and glyoxal on NHEK and HaCaT keratinocytes.

added to that of original concentration in the EpiLife medium, and did not change up to a concentration of 50 mM. Within this dose interval, cell viability and proliferation oscillated around the control levels and even increased values (by up to 21%) were observed, which might be explained by a higher energy supply. At glucose concentrations of 100 mM and higher, the cell number, the proliferation rate, and viability decreased in a dose-dependent manner.

In contrast to glucose, glyoxal treatment resulted in a dose-dependent decrease in NHEK cell number within the entire tested spectrum, and was accompanied by a decrease in cell proliferation and viability (FIG. 1). The LD50 for glyoxal was 50 μM and for glucose 200 mM. This difference may be due to a higher reactivity and/or an easier membrane penetration of glyoxal. Effects of several concentrations of sugars were also checked on immortalized HaCaT keratinocytes (FIG. 1). In contrast to NHEK, HaCaT keratinocytes were more tolerant to glucose (interpolated LD50: 230 mM) and glyoxal (LD50: 1 mM).

Based on the above results, 100 mM glucose and 0.1 mM glyoxal were chosen for further experiments of NHEK. Sugar-treated NHEK and HaCaT cells became much larger and vacuolated within 3 days of treatment and appeared similar as to serially passaged senescent cells (pictures not shown). TABLE 1 summarizes the results of this study. The decrease in cell number by sugar treatment was not due to increased apoptosis, which was lower than in the control. Furthermore, the proportion of SA β-gal-positive cells increased significantly in number by 52% in 100 mM glucose and by 44% in 0.1 mM glyoxal-treated NHEK. General glycoxidation of total proteins in NHEK after 3 days of sugar treatment was 58% and 68% higher in glucose- and glyoxal-treated cells, respectively.

TABLE 1. Effects of glucose and glyoxal on early-passage NHEK after 3 days[a]

Characteristics	Treatments and effects	
	100 mM glucose	0.1 mM glyoxal
Morphology	Senescent-like	Senescent-like
Apoptosis	33% decrease	56% decrease
SA β-gal	52% increase	44% increase
Total protein glycoxidation	58% increase	68% increase
Proteasome activity	11% increase	3% decrease
Proteasome glycation	5% decrease	16% decrease

[a]Normalized against the control.

Since glycoxidatively damaged proteins are preferentially degraded by the proteasome, its activity and its own glycation were measured in sugar-treated NHEK. After 3 days of sugar treatment, the proteasome activity was slightly increased in glucose-treated or unchanged in glyoxal-treated NHEK. The level of glycated α7 subunit of the proteasome was decreased by 5% and 16% in glucose- and glyoxal-treated NHEK, respectively (TABLE 1). However, initially glucose had caused an increase of proteasome glycation after 1 day of treatment (+61%) with a synchronous decrease in its activity (–44%). This might be explained by an initial glycation of the proteasome followed by a preferential turnover of the glycated α7 subunit.[13]

Since keratinocytes undergo differentiation *in vivo* to maintain homeostasis of the skin, this property was investigated in sugar-induced premature NHEK by measuring involucrin levels. FIGURE 2 shows that involucrin levels are increased by 1.7-fold within 6 h of treatment with 1.2 mM calcium, which is a known differentiation marker.[10] Glyoxal pretreatment for 3 days resulted in an 18-h delay in involucrin synthesis but its final levels reached similar or higher levels above the control. Glucose pretreatment, however, resulted in a less pronounced delay of 6 h in involucrin synthesis, but final levels were lower than in the positive control.

As a potential antiaging intervention, hormetic mild heat shock (1 h, 41°C, 6 h in advance), was tested for its effects.[14] Preliminary results show a reduction in both the total protein glycoxidation by 6% and 29%, and the proteasome glycation by 19% and 4% in glucose- and glyoxal-treated NHEK, respectively. This was associated with a parallel increase in proteasome activity by 6% and 8% in glucose- and glyoxal-treated NHEK, respectively. Furthermore, in case of glyoxal, differentiation could be normalized by mild heat shock, which abrogated the glyoxal-induced delay in involucrin synthesis (FIG. 2).

In conclusion, our studies show that 100 mM glucose and 0.1 mM glyoxal can induce premature aging in NHEK and HaCaT keratinocytes. This model system can be useful to test the effects of potential antiaging and differentiation–modulatory agents, including natural and synthetic molecules, plant extracts, and hormetins.

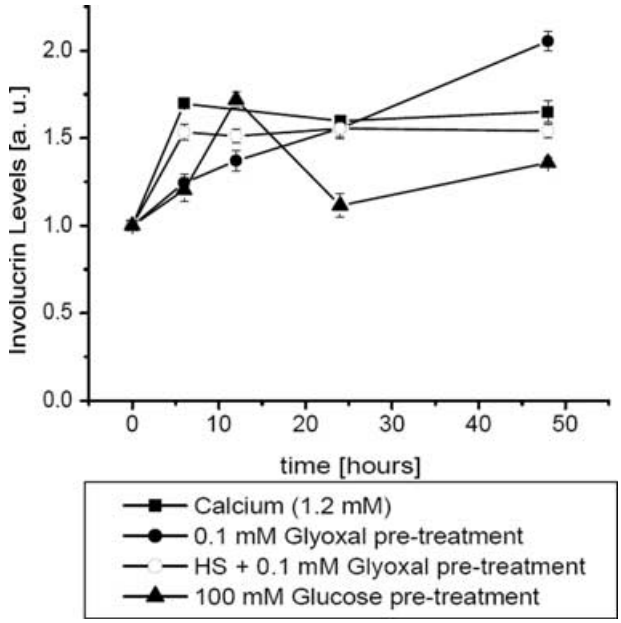

FIGURE 2. Effect of sugar-induced premature aging and mild heat shock (HS) pre-treatment on calcium-induced differentiation (involucrin levels) in NHEK.

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