Beyond the Physico-Chemical Barrier: Glycerol and Xylitol Markedly yet Differentially Alter Gene Expression Profiles and Modify Signaling Pathways in Human Epidermal Keratinocytes

Judit Szabó-Papp[1,2], Erika Herczeg-Lisztes[1], Lídia Ambrus[1], Attila Oláh[1], Attila Gábor Szöllősi[1], Mónika Andrási[3], Shabtay Dikstein[4], Lajos Kemény[5], Tamás Bíró[1], István Juhász[2]

[1]DE-MTA "Lendület" Cellular Physiology Research Group, Department of Physiology and Department of [2]Dermatology and [3]Surgery, University of Debrecen, H-4032 Debrecen, Hungary

[4]School of Pharmacy, The Hebrew University, POB 12065, Jerusalem, Israel

[5]MTA-SZTE Dermatological Research Group, H-6720 Szeged, Hungary

Running title: Polyols modify gene expression in human epidermal keratinocytes

Correspondence should be addressed to:

Tamás Bíró (University of Debrecen, Nagyerdei krt. 98. Debrecen, H-4032, Hungary; e-mail: biro.tamas@med.unideb.hu; phone: +3652-255-575; fax: +3652-255-116).

Conflict of interest:

The authors have declared that no conflict of interest exists.

Abstract

Polyols (such as e.g. glycerol, xylitol) are implicated as moisturizers of the skin and other epithelial tissues. However, we lack information about their exact cellular mechanisms and their effects on the gene expression profiles of the cells. Therefore, in the current study, we aimed at investigating the effects of glycerol and xylitol on normal human epidermal keratinocytes (NHEK), used as a model epithelial cellular system. The polyols (identical osmolarities; xylitol: 0.0045-0.45%; glycerol: 0.0027-0.27%) did not alter cellular viability or intracellular calcium concentration. However, they exerted differential effects on the expression of certain genes and signaling pathways. Indeed, although both polyols up-regulated the expression of such differentiation markers as filaggrin, loricrin, and involucrin, the efficiency of xylitol was superior; likewise, only xylitol was able to increase the expression of a “barrier” marker occludin. Moreover, whereas both polyols stimulated the MAPK pathway, only xylitol induced the activation-dependent translocation of protein kinase Cδ, a key molecule involved in promoting epidermal differentiation. Finally, in various cellular inflammation models, xylitol (by suppressing the expressions of multiple interleukins) exerted a remarkable anti-inflammatory effect whereas glycerol was more effective in decreasing the up-regulated expression of HLA-DR, a marker of keratinocyte immunoactivation. Taken together, these data strongly suggest the marked, yet differential, effects of glycerol and xylitol on multiple genes and signaling pathways in epidermal keratinocytes. Of further importance, our findings also invite clinical trials to explore the applicability and the impact of a combined glycerol-xylitol therapy in the management of various skin conditions. It is envisaged that such formulations will have multiple beneficial effects by promoting stratum corneum hydration and

epidermal differentiation, accelerating barrier junction recovery, preventing and/or treating skin irritation and inflammation, and inhibiting bacterial biofilm formation.

Introduction

Glycerol, a trihydroxy polyol (1,2,3-trihydroxypropane), is extensively used in a wide-array of topical dermatological and cosmeceuticals preparations since decades due to its beneficial effects on the skin. Indeed, glycerol improves hydration of the epidermis (moisturizing effect) by preventing water loss due to its hygroscopicity and by inhibiting stratum corneum (SC) lipid phase transition [1,2], exerts keratolytic actions by enhancing desmosomal degradation [3], and promotes wound healing [4,5]. In human studies, glycerol application was shown to increase stratum corneum hydration, accelerated skin barrier repair, exerted anti-irritant and penetration enhancing effects, and improved mechanical properties of the skin (see reviewed in [6]).

Importantly, glucose-derived glycerol is endogenously synthesized in the skin (mostly in the sebaceous glands) and was suggested to play a crucial role in the homeostatic regulation of the skin barrier [4,6,7,8]. Indeed, in asebia mice with impaired sebaceous gland functions and hence a dramatically suppressed (by 85%) endogenous glycerol production, decreased SC hydration was observed which was completely reverted by application of exogenous glycerol [7]. Likewise, hydration in the human skin was also found to be correlated with endogenous glycerol levels in the SC [9]. Therefore, it is proposed that the highly beneficial effects of topically applied glycerol in a multitude of skin conditions (e.g. dry skin syndromes such as winter xerosis, irritative skin syndromes, dermatoses with impaired barrier, atopic dermatitis [AD], etc.) is due to its “replacement actions” by normalizing endogenous glycerol-controlled homeostatic cutaneous mechanisms.

The cutaneous effects of glycerol were mostly (if not exclusively) attributed to its physico-chemical properties. Endogenously produced or exogenously applied glycerol penetrates to keratinocytes located in the basal layer(s) of the epidermis via aquaglyceroporin AQP3 pores and, due to its humectancy or hygroscopicity, promotes the establishment and maintenance of the epidermal barrier [4,5,6,8]. Indeed, in AQP3-deficient mice with decreased SC hydration and pathological barrier [4,10], application of glycerol improved skin hydration and accelerated barrier recovery [8].

In contrast to glycerol, we possess much more limited data on the cutaneous effects of xylitol, a closely related polyol which can also be synthesized in the human body. In multiple studies, xylitol was found to inhibit *Staphylococcus aureus* (SA) biofilm formation characteristic for AD [11,12,13,14,15] and augment skin collagen formation [16,17]. Moreover, in a human study, we have recently shown that xylitol (similar to glycerol) markedly suppressed sodiumlaurylsulphate-induced transepidermal water loss (TEWL) and exerted anti-irritant actions [18]. Interestingly, albeit xylitol containing hydrogel formulations was found to increase skin hydration [19], xylitol failed to correct the impaired hydration and epidermal barrier of AQP3-deficient mice [8].

The complexity of the epidermal barrier and the versatile, highly beneficial effects of these polyols suggest a multifaceted, yet mostly unknown, mechanism of action. Therefore, we hypothesized that their actions at the cellular level cannot be exclusively attributed to their physico-chemical properties, and propose that glycerol and xylitol may modulate the expressions of certain genes and the activities of signal

pathways that are involved the regulation of the fine-tuned proliferation and differentiation program of the epidermis. To test the validity of this hypothesis, we employed cultured normal human epidermal keratinocytes (NHEKs) and assessed the effects of polyols (applied at therapeutically relevant concentrations and at identical osmolarity) on multiple cellular functions. We report here for the first time that glycerol and xylitol markedly, yet differentially, alter the gene expression profile and modify various signaling pathways in human epidermal keratinocytes.

Materials and Methods

Cell culturing

Human skin samples were obtained after written informed consent from healthy individuals, undergoing dermatosurgery, adhering to Helsinki guidelines and after Institutional Research Ethics Committee permission. NHEKs were isolated after overnight dermo-epidermal separation in 2.4 U/ml dispase (Roche Diagnostics, Berlin, Germany) by short trypsin (0.05%, Sigma-Aldrich, St. Louis, MO) digestion. Cells were cultured in EpiLife serum free medium (Invitrogen, Paisley, UK) supplemented with 1 μM insulin, 1 μM cortisol (both from Sigma-Aldrich), 100 μg/ml streptomycin, 100 U/ml penicillin, 50 ng/ml amphotericin B, 0.4% bovine pituitary extract (all from Invitrogen) and 0.06 mM $CaCl_2$ (Sigma-Aldrich) [20].

Determination of viable cell numbers and proliferation

The number of viable cells (hence the rate of proliferation) was determined by measuring the conversion of the tetrazolium salt MTT (Methylthiazolyldiphenyl-tetrazolium bromide, Sigma-Aldrich) to formazan by mitochondrial dehydrogenases [20,21]. Cells were plated in 96-well multiter plates (10,000 cells/well density) in quadruplicates and were treated with xylitol (0.0045-0.45%) or glycerol (0.0027-0.27%) for various time intervals. Cells were then incubated with 0.5 mg/ml MTT for 2 hrs, and concentration of formazan crystals was determined colorimetrically according to the manufacturer's protocol.

Determination of cell death

A decrease in the mitochondrial membrane potential is one of the earliest markers of apoptosis. Therefore, to assess the process, mitochondrial membrane potential of

NHEKs was determined using a MitoProbe™ DiIC1(5) Assay Kit (Life Technologies, Carlsbad, CA, USA). Cells (20,000 cells/well) were cultured in 96-well black-well/clear-bottom plates (Greiner Bio One, Frickenhausen, Germany) in quadruplicates and were treated with various compounds. After removal of supernatants, cells were incubated for 30 minutes with DilC1(5) working solution (50 µl/well), then washed with PBS (115 mM NaCl, 206 mM Na_2PO_4, pH 7.4; all from Sigma-Aldrich), and the fluorescence of DilC1(5) was measured at 630 nm excitation and 670 nm emission wavelengths using a FlexStation 3 FLuorescence Image MicroPlate Reader (FLIPR; Molecular Devices, San Francisco, CA).

Necrotic processes were determined by SYTOX Green staining (Life Technologies). The dye is able to penetrate (and then bind to the nucleic acids) only to necrotic cells with ruptured plasma membranes, whereas healthy cells with intact surface membranes show negligible SYTOX Green staining. Cells were cultured in 96-well black-well/clear-bottom plates (Greiner Bio One), and treated with polyols. Supernatants were then discarded, and the cells were incubated for 30 minutes with 1 µM SYTOX Green dye. Following incubation, cells were washed with PBS, the culture medium was replaced, and fluorescence of SYTOX Green was measured at 490 nm excitation and 520 nm emission wavelengths using FLIPR (Molecular Devices) [20,21].

Quantitative Real-Time PCR (Q-PCR)

To determine the quantitative expressions of various markers at the mRNA level, Q-PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) using the 5’ nuclease assay as detailed in our previous

report [22]. In brief, total RNA was isolated using TRIzol (Invitrogen) and 3 µg of total RNA were reverse-transcribed into cDNA by using 15 U of AMV reverse transcriptase (Promega, Madison, WI, USA) and 0.025 µg/µl random primers (Promega). PCR amplification was performed by using TaqMan primers and probes (Applied Biosystems). As internal controls, transcripts of cyclophilin A (PPIA) were determined [20,21,23].

Western blotting

To determine the activation of the MAPK Erk1/2, the Western blot technique was applied [21,24]. Cell lysates of keratinocytes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% gels were loaded with 30 µg protein per lane), transferred to BioBond nitrocellulose membranes (Whatman, Maidstone, UK), and then probed with primary antibodies against Erk1/2 and pshopho-Erk1/2 (1:500, both from Sigma-Aldrich). A horseradish peroxidase-conjugated IgG antibody (1:1000, Bio-Rad, Hercules CA, USA) was used as a secondary antibody, and the immunoreactive bands were visualized by a SuperSignal® West Pico Chemiluminescent Substrate enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) using LAS-3000 Intelligent Dark Box (Fuji, Tokyo, Japan). To assess equal loading, membranes were re-probed with an anti-β-actin antibody (1:2000, Santa Cruz, CA, USA) and visualized as described above. Immunoblots were then subjected to densitometric analysis using an Intelligent Dark Box (Fuji, Tokyo, Japan) and the Image Pro Plus 4.5.0 software (Media Cybernetics, Silver Spring, MD, USA).

Ca^{2+}-imaging

Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) upon applications of the polyols were detected by fluorimetric Ca^{2+}-imaging [20,25,26]. Cells were seeded in 96-well black-well/clear-bottom plates (Greiner Bio One) at a density of 10,000 cells/well and then were incubated with culturing medium containing the cytoplasmic calcium indicator 2 μM Fluo-4 AM (Invitrogen) at 37°C for 40 min. The cells were washed four times with and finally cultured in Hank's solution containing 1% bovine serum albumin and 2.5 mM Probenecid (both from (Sigma-Aldrich) for 30 min at 37 °C. The plates were then placed to FLIPR (Molecular Devices) and changes in $[Ca^{2+}]_i$ (reflected by changes fluorescence; lEX=494 nm, lEM=516 nm) induced by various concentrations of the drugs were recorded in each well.

Immunocytochemistry, Confocal microscopy

Human keratinocytes, seeded and cultured on sterile coverslips in 24-well plates, were fixed in acetone, permeabilized by 0.1% Triton-X-100 (Sigma-Aldrich), and then incubated with rabbit primary antibodies against PKCα (Sigma-Aldrich) or PKCδ (Santa Cruz) (1:200 dilution in both cases). For fluorescence staining, slides were then incubated with fluorescein-isothiocytanate (FITC) conjugated secondary antibodies (dilution 1:200, Vector Laboratories, Burlingame, CA, USA) and the nuclei were visualized using DAPI (Vector Laboratories). As negative controls, the appropriate antibody was either omitted from the procedure or was pre-incubated with synthetic blocking peptides [20,21,23,25].

To assess the translocation of the PKC isoforms (reflecting activation of the enzymes), the above labeling was performed on cells which were previously treated by 0.27% glycerol or 0.45% xylitol for various time intervals. FITC-labeled cells were

then subjected to a visualization procedure using a Zeiss LSM 510 confocal microscopy (Oberkochen, Germany) [24].

Statistical analysis

When applicable, data were analyzed using a two-tailed un-paired *t*-test and $P<0.05$ values were regarded as significant differences.

Results

Polyols do not affect proliferation and viability of cultured NHEKs

We first assessed the effects polyols on the growth and survival rate of cultured NHEKs. Pre-confluent (i.e. proliferating) NHEKs were treated with various concentrations of glycerol (0.0027-0.27%) and xylitol (0.0045-0.45%), establishing identical osmolarities, for up to 72 hrs and the viable cell number was assessed by colorimetric MTT assay. As presented in **Supplementary Figure 1** (which shows only the highest concentration) neither polyol affected the growth rate and the viable cell number of the cultures. The lack of cytotoxicity was also verified by fluorimetric DilC1(5) and SYTOX Green labeling (data not shown). Therefore, in the further experiments, the highest polyol concentrations were employed.

Polyols differentially alter expressions of various differentiation/barrier markers

Next, we studied the effects of polyols on the expression of such molecules (filaggrin, involucrin and loricrin) which play key roles in the differentiation program in epidermal keratinocytes hence participate in the establishment of the epidermal barrier (extensively reviewed in [27,28]). Since the differentiation program of cultured NHEKs is automatically initiated upon reaching confluence (high-cell density-induced differentiation) [29,30,31], we first investigated the putative pro-differentiating effects of polyols on pre-confluent cultures of NHEKs. Importantly, as revealed by Q-PCR analyses (**Figure 1A-C**), xylitol significantly increased the levels of specific mRNA transcripts of all differentiation/barrier markers after 24 and/or 48 hrs treatments. Likewise, glycerol also significantly elevated the expressions of involucrin and loricrin (mostly after 48 hrs); yet, only tendencies of up-regulation were observed (without reaching significant differences) in the case of filaggrin.

We then assessed the effects of polyols on post-confluent (hence already differentiating) NHEKs (**Figures 1A-C**). Again, xylitol significantly increased the expressions of all molecules investigated (albeit its efficacy appeared to be somewhat lower). However, on differentiating NHEKs, glycerol (after 48 hrs) was able up-regulate the levels of filaggrin and involucrin but not of loricrin.

We also studied the effects of polyols of the expression of occludin, a key molecule of epidermal junctions also involved in barrier formation (reviewed in [32]). Importantly, xylitol, unlike not glycerol, markedly increased the expression of occludin in the pre-confluent cultures (**Figures 1D**); interestingly, this effect was not observed in the pro-confluent cultures. Taken together, these results collectively suggest that glycerol and xylitol (although exhibiting differential effects at the molecular level) promote the expression of multiple differentiation and barrier markers thereby may enhance the processes of epidermal differentiation and barrier establishment/repair.

Polyols do not elevate the in $[Ca^{2+}]_i$ of NHEKs

We then intended to assess the effects of polyols on such signaling pathways which were shown to be involved in controlling epidermal differentiation. First, we measured the effects of polyols on the level of in $[Ca^{2+}]_i$, whose elevation is one of the key events in initiating the cellular differentiation process [33]. Importantly, neither polyol elevated the $[Ca^{2+}]_i$ of the cultured NHEKs (**Supplementary Figure 2**).

Xylitol selectively translocates PKCδ in NHEKs

Protein kinase C (PKC) isoforms are key intracellular signaling molecules which are fundamentally involved the regulating cellular proliferation, differentiation, and survival (reviewed in [34]). In human epidermal keratinocytes, the activation of PKCα and δ isoforms was shown to promote differentiation [35,36]; therefore, we measured the effects of polyols on these isoenzymes. Since the activation of PKCs can be followed by assessing their translocation (i.e. transition from a given cellular compartment to another one) [24,36], we employed confocal microscopy following immunolabeling of the isoforms after treatment. As shown in **Supplementary Figure 3**, the polyols did not alter the subcellular localization of the Ca^{2+}-sensitive PKCα. However, xylitol (but not glycerol) induced the translocation of the Ca^{2+}-insensitive PKCδ from the cytosol mostly to the cytoskeletal fraction (**Figure 2A**); this suggests that xylitol activates PKCδ.

Polyols activate the MAPK pathway in NHEKs

Another important signal transduction system that controls multiple keratinocyte functions is the mitogen-activated kinase (MAPK) system [37,38]; therefore, we also measured the effects of polyols on the activity of this signaling pathway. As shown by Western blotting (**Figure 2B**), both xylitol and glycerol transiently activated the MAPK cascade, as reflected by the increased phosphorylation of one of the key MAPK, Erk1/2 (p44/48).

Polyols exert differential anti-inflammatory effects in NHEKs

In the final phase of our study, we assessed whether glycerol and xylitol can modulate the inflammatory response of keratinocytes. For this, NHEKs were treated with activators of the toll-like receptor (TLR) pathways (reviewed in [39]) – i.e. the

lipoteichoic acid (LTA) for TLR2; polyinosinic:polycytidylic acid (poly-IC) for TLR3; and lipopolysaccharide (LPS) for TLR4 – for 24 hrs and the effects of the polyols on the TLR-mediated up-regulation of various interleukins were determined. In addition, we also measured the expression changes of HLA-DR whose up-regulation in the skin was shown to be related to keratinocyte immune activation, inflammation, and TLR signaling [40,41].

Importantly, glycerol almost completely prevented the TLR induced up-regulation of HLA-DR in all three TLR-induced inflammation models (**Figure 3-5**). In addition, it effectively suppressed the poly-IC induced expressional elevations of IL1α, IL1β, IL6 and IL8 but was ineffective in preventing the pro-inflammatory response initiated by TLR2 or TLR4 activation (in addition, glycerol was unable to significantly modulate the up-regulated expression of IL18 in all TLR models).

Interestingly, xylitol exerted a markedly different pattern of action (**Figure 3-5**). Namely, in contrast to glycerol, it could not significantly normalize the up-regulated level of HLA-DR in either TLR model. However, it exerted a robust anti-inflammatory action in the TLR4 model (as it significantly suppressed the effect of LPS to elevate expressions of IL1β, IL6, IL8, and IL18) and a modest anti-inflammatory effect in the other 2 models (i.e. significantly prevented the effects of LTA on IL1α and of poly-IC on IL6 and IL8).

Discussion

As elegantly review by Fluhr et al [6], the highly beneficial effects of topically applied glycerol (e.g. promoting epidermal hydration and barrier repair, exerting anti-irritant and anti-inflammatory actions, accelerating wound healing) in a multitude of dry, irritant, and inflammatory skin condition, characterized by barrier defects, are exclusively attributed to the physico-chemical characteristics (i.e. humectancy and water sorption capacity) of the polyol. Likewise, a similar mode of action was implicated for xylitol-containing topical formulations. In this study, we challenged this paradigm and assessed the possibility that glycerol and xylitol may directly act on various cellular processes of cultured epidermal keratinocytes. Indeed, here we provide the first evidence that glycerol and xylitol exert profound effects on gene expressions and the activities of various signaling pathways of the cells. The observed differential effects (detailed below) of xylitol and glycerol found by us are astounding, since both polyols have high water sorption capacity [42]. However, since these two polyols exerted markedly different effects when applied at identical osmolarities, we propose that their actions are most probably not (or not exclusively) mediated by their physico-chemical properties.

First, we showed that both polyols (without affecting growth rate and viability of the cells) significantly up-regulated the expression of various differentiation marker. However, of great importance, marked differences in their efficacies were observed. Indeed, only xylitol was able to elevate the level of filaggrin, a key molecule of the epidermal barrier [27,43], in the pre-confluent keratinocyte cultures where, under control conditions, the differentiation program is not yet initiated (in the post-confluent, differentiating NHEKs both polyols were effective). In addition, glycerol up-

regulated the expression of the differentiation marker loricrin only in the pre-confluent cultures (and only after 48 hrs treatment) whereas xylitol was effective in both types of NHEKs (such differential effects of the polyols were not observed in the elevation of levels of involucrin, another marker of differentiation). Furthermore, xylitol (but, again, not glycerol) also up-regulated the expression of occludin, an important molecule in the formation epidermal barrier junctions. These intriguing findings collectively implicate that the cutaneous barrier-repairing and differentiation-promoting effects of xylitol may be superior to those of glycerol.

Interestingly, similarities and differences were also observed when we attempted to uncover the effects of the polyols on the cellular signaling pathways known to be involved in the regulation of epidermal growth and differentiation. As revealed by functional imaging, neither polyol altered the intracellular calcium homeostasis of the cells. This implies that their pro-differentiating effects are most probably not mediated by the elevation of $[Ca^{2+}]_i$, which is one of the key events in the induction of terminal differentiation of epidermal keratinocytes [33]. The lack of the involvement of $[Ca^{2+}]_i$ was also suggested by that neither polyol affected the subcellular localization of the calcium-dependent PKCα (reflecting lack of activation), which enzyme was shown to be involved in the $[Ca^{2+}]_i$-mediated differentiation of NHEKs [35]. However, xylitol (but not glycerol) was able to induce the translocation of the Ca-independent PKCδ. Since we have previously shown that (similar to PKCα) PKCδ promotes differentiation of human epidermal keratinocytes [36], this intriguing finding suggests that the more pronounced pro-differentiating and barrier-promoting effect of xylitol over glycerol might be mediated by the additional involvement of PKCδ-coupled signaling. Finally,

we have also presented that both polyols activated the MAPK pathway, another molecular system controlling keratinocyte growth and survival [37,38].

Of further importance, the differential effects of the two polyols could also be identified when we assessed their actions in various TLR-mediated inflammation and irritation keratinocyte models. In fact, glycerol was dramatically effective in preventing the up-regulation of HLA-DR, a marker of immune activation [40,41], induced by TLR2-4 stimulation. Interestingly, xylitol exerted only insignificant effect on the TLR-induced elevated HLA-DR expression. On the other hand, xylitol showed a marked anti-inflammatory potential whereas such an action of glycerol was rather modest.

In conclusion, based on our detailed findings and previously published data on the in vivo human effects of glycerol and polyols, we propose a novel, therapeutically important concept. According to this, we suggest the production and application of such topical formulations which contain the combination of glycerol and xylitol. It is envisaged that such formulations will have multiple beneficial effects in i) promoting SC hydration and epidermal differentiation (synergistic actions of the two polyols); ii) accelerating barrier junction recovery (action of xylitol); iii) preventing and/or treating skin irritation (action of glycerol) and skin inflammation (synergistic actions of the two polyols); and iv) inhibiting bacterial biofilm formation (action of xylitol). Indeed, in a human study, we have recently shown that a topical formulation containing combination of glycerol and xylitol exerted remarkable antibacterial and skin hydrating effects [45]. Therefore, future extensive clinical studies are now warranted to explore the applicability and the impact of combined glycerol-xylitol therapy in the management of various skin conditions.

Acknowledgement

The research was supported by Hungarian research grants “Lendület” LP2011-003/2014, OTKA 101761, OTKA 105369, and TÁMOP-4.2.2./A-11/1/KONV-2012-0025.

References

1. Batt MD, Fairhurst E: Hydration of the stratum corneum. Int J Cosmet Sci 1986;8:253-264.
2. Froebe CL, Simion FA, Ohlmeyer H, Rhein LD, Mattai J, Cagan RH, Friberg SE: Prevention of stratum corneum lipid phase transition in vitro by glycerol – an alternative mechanism for skin moisturization. J Soc Cosmet Chem 1990;41:51–65.
3. Rawlings A, Harding C, Watkinson A, Banks J, Ackerman C, Sabin R: The effect of glycerol and humidity on desmosome degradation in stratum corneum. Arch Dermatol Res 1995;287:457-464.
4. Hara M, Ma T, Verkman AS: Selectively reduced glycerol in skin of aquaporin-3-deficient mice may account for impaired skin hydration, elasticity, and barrier recovery. J Biol Chem 2002;277:46616-46621.
5. Hara-Chikuma M, Verkman AS: Aquaporin-3 facilitates epidermal cell migration and proliferation during wound healing. J Mol Med 2008;86:221-231.
6. Fluhr JW, Darlenski R, Surber C: Glycerol and the skin: Holistic approach to its origin and functions. Br J Dermatol 2008;159:23-34.
7. Fluhr JW, Mao-Qiang M, Brown BE, Wertz PW, Crumrine D, Sundberg JP, Feingold KR, Elias PM: Glycerol regulates stratum corneum hydration in sebaceous gland deficient (asebia) mice. J Invest Dermatol 2003;120:728-737.
8. Hara M, Verkman AS: Glycerol replacement corrects defective skin hydration, elasticity, and barrier function in aquaporin-3-deficient mice. Proc Natl Acad Sci U S A 2003;100:7360-7365.

9. Choi EH, Man MQ, Wang F, Zhang X, Brown BE, Feingold KR, Elias PM: Is endogenous glycerol a determinant of stratum corneum hydration in humans? J Invest Dermatol 2005;125:288-293.
10. Ma T, Hara M, Sougrat R, Verbavatz JM, Verkman AS: Impaired stratum corneum hydration in mice lacking epidermal water channel aquaporin-3. J Biol Chem 2002;277:17147-17153.
11. Akiyama H, Oono T, Huh WK, Yamasaki O, Ogawa S, Katsuyama M, Ichikawa H, Iwatsuki K: Actions of farnesol and xylitol against staphylococcus aureus. Chemotherapy 2002;48:122-128.
12. Katsuyama M, Ichikawa H, Ogawa S, Ikezawa Z: A novel method to control the balance of skin microflora. Part 1. Attack on biofilm of staphylococcus aureus without antibiotics. J Dermatol Sci 2005;38:197-205.
13. Katsuyama M, Kobayashi Y, Ichikawa H, Mizuno A, Miyachi Y, Matsunaga K, Kawashima M: A novel method to control the balance of skin microflora. Part 2. A study to assess the effect of a cream containing farnesol and xylitol on atopic dry skin. J Dermatol Sci 2005;38:207-213.
14. Dowd SE, Sun Y, Smith E, Kennedy JP, Jones CE, Wolcott R: Effects of biofilm treatments on the multi-species lubbock chronic wound biofilm model. J Wound Care 2009;18:508, 510-512.
15. Ikezawa Z, Komori J, Ikezawa Y, Inoue Y, Kirino M, Katsuyama M, Aihara M: A role of staphyococcus aureus, interleukin-18, nerve growth factor and semaphorin 3a, an axon guidance molecule, in pathogenesis and treatment of atopic dermatitis. Allergy Asthma Immunol Res 2010;2:235-246.

16. Knuuttila ML, Kuoksa TH, Svanberg MJ, Mattila PT, Karjalainen KM, Kolehmainen E: Effects of dietary xylitol on collagen content and glycosylation in healthy and diabetic rats. Life Sci 2000;67:283-290.
17. Mattila PT, Pelkonen P, Knuuttila ML: Effects of a long-term dietary xylitol supplementation on collagen content and fluorescence of the skin in aged rats. Gerontology 2005;51:166-169.
18. Korponyai C, Kovács RK, Erős G, Dikstein S, Kemény L: Antiirritant properties of polyols and amino acids. Dermatitis 2011;22:141-146.
19. Leite e Silva VR, Schulman MA, Ferelli C, Gimenis JM, Ruas GW, Baby AR, Velasco MV, Taqueda ME, Kaneko TM: Hydrating effects of moisturizer active compounds incorporated into hydrogels: In vivo assessment and comparison between devices. J Cosmet Dermaol 2009;8:32-39.
20. Tóth BI, Dobrosi N, Dajnoki A, Czifra G, Oláh A, Szöllősi AG, Juhász I, Sugawara K, Paus R, Bíró T: Endocannabinoids modulate human epidermal keratinocyte proliferation and survival via the sequential engagement of cannabinoid receptor-1 and transient receptor potential vanilloid-1. J Invest Dermatol 2011;131:1095-1104.
21. Dobrosi N, Tóth BI, Nagy G, Dózsa A, Géczy T, Nagy L, Zouboulis CC, Paus R, Kovács L, Bíró T: Endocannabinoids enhance lipid synthesis and apoptosis of human sebocytes via cannabinoid receptor-2-mediated signaling. FASEB J 2008;22:3685-3695.
22. Bodó E, Bíró T, Telek A, Czifra G, Tóth BI, Mescalchin A, Ito T, Bettermann A, Kovács L, Paus R: A hot new twist to hair biology - involvement of vanilloid receptor-1 (VR1/TRPV1) signaling in human hair growth control. Am J Pathol 2005;166:985-998.

23. Telek A, Bíró T, Bodó E, Tóth BI, Borbíró I, Kunos G, Paus R: Inhibition of human hair follicle growth by endo- and exocannabinoids. FASEB J 2007;21:3534-3541.
24. Czifra G, Tóth IB, Marincsák R, Juhász I, Kovács I, Ács P, Kovács L, Blumberg PM, Bíró T: Insulin-like growth factor-I-coupled mitogenic signaling in primary cultured human skeletal muscle cells and in C2C12 myoblasts. A central role of protein kinase Cdelta. Cell Signal 2006;18:1461-1472.
25. Borbíró I, Lisztes E, Tóth BI, Czifra G, Oláh A, Szöllősi AG, Szentandrássy N, Nánási PP, Péter Z, Paus R, Kovács L, Bíró T: Activation of transient receptor potential vanilloid-3 inhibits human hair growth. J Invest Dermatol 2011;131:1605-1614.
26. Czifra G, Szöllősi AG, Tóth BI, Demaude J, Bouez C, Breton L, Bíró T: Endocannabinoids regulate growth and survival of human eccrine sweat gland-derived epithelial cells. J Invest Dermatol 2012;132:1967-1976.
27. Elias PM, Feingold KR: Skin barrier. Taylor & Francis 2006, New York
28. Oláh A, Szöllősi AG, Bíró T: The channel physiology of the skin. Rev Physiol Biochem Pharmacol 2012;163:65-131.
29. Fuchs E: Epidermal differentiation: The bare essentials. J Cell Biol 1990;111:2807-2814.
30. Lee YS, Yuspa SH, Dlugosz AA: Differentiation of cultured human epidermal keratinocytes at high cell densities is mediated by endogenous activation of the protein kinase C signaling pathway. J Invest Dermatol 1998;111:762-766.
31. Papp H, Czifra G, Lázár J, Gönczi M, Csernoch L, Kovács L, Bíró T: Protein kinase C isozymes regulate proliferation and high cell density-mediated differentiation in HaCaT keratinocytes. Exp Dermatol 2003;12:811-824.

32. Cummins PM: Occludin: One protein, many forms. Mol Cell Biol 2012;32:242-250.
33. Hennings H, Michael D, Cheng C, Steinert P, Holbrook K, Yuspa SH: Calcium regulation of growth and differentiation of mouse epidermal cells in culture. Cell 1980;19:245-254.
34. Mackay HJ, Twelves CJ: Targeting the protein kinase C family: Are we there yet? Nat Rev Cancer 2007;7:554-562.
35. Lee YS, Dlugosz AA, McKay R, Dean NM, Yuspa SH: Definition by specific antisense oligonucleotides of a role for protein kinase Calpha in expression of differentiation markers in normal and neoplastic mouse epidermal keratinocytes. Mol Carcinog 1997;18:44-53.
36. Papp H, Czifra G, Bodó E, Lázár J, Kovács I, Aleksza M, Juhász I, Ács P, Sipka S, Kovács L, Blumberg PM, Bíró T: Opposite roles of protein kinase C isoforms in proliferation, differentiation, apoptosis, and tumorigenicity of human HaCaT keratinocytes. Cell Mol Life Sci 2004;61:1095-1105.
37. Gazel A, Nijhawan RI, Walsh R, Blumenberg M: Transcriptional profiling defines the roles of erk and p38 kinases in epidermal keratinocytes. J Cell Physiol 2008;215:292-308.
38. Harmon RM, Simpson CL, Johnson JL, Koetsier JL, Dubash AD, Najor NA, Sarig O, Sprecher E, Green KJ: Desmoglein-1/erbin interaction suppresses erk activation to support epidermal differentiation. J Clin Invest 2013;123:1556-1570.
39. O'Neill LA, Golenbock D, Bowie AG: The history of toll-like receptors - redefining innate immunity. Nat Rev Immunol 2013;13:453-460.

40. Travers JB, Hamid QA, Norris DA, Kuhn C, Giorno RC, Schlievert PM, Farmer ER, Leung DY: Epidermal HLA-DR and the enhancement of cutaneous reactivity to superantigenic toxins in psoriasis. J Clin Invest 1999;104:1181-1189.
41. Rozis G, Benlahrech A, Duraisingham S, Gotch F, Patterson S: Human langerhans' cells and dermal-type dendritic cells generated from CD34 stem cells express different toll-like receptors and secrete different cytokines in response to toll-like receptor ligands. Immunology 2008;124:329-338.
42. Cohen S, Marcus Y, Migron Y, Dikstein S, Shafran A: Water sorption, binding and solubility of polyols. J Chem Soc, Faraday Trans 1993;89:3271-3275.
43. McAleer MA, Irvine AD: The multifunctional role of filaggrin in allergic skin disease. J Allergy Clin Immunol 2013;131:280-291.
44. Bíró T, Szabó I, Kovács L, Hunyadi J, Csernoch L: Distinct subpopulations in hacat cells as revealed by the characteristics of intracellular calcium release induced by phosphoinositide-coupled agonists. Arch Dermatol Res 1998;290:270-276.
45. Erős G, Korponyai C, Szabó K, Behány Z, Szél E, Dikstein S, Kemény L: Antibacterial and skin hydrating effects of the glycerol- and xylitol containing Xylinep® gel. Bőrgyógy Vener Szle 2014;90:152-155.

Figure legends

Figure 1. *Effect of polyols on the expression of various differentiation and barrier markers in human epidermal keratinocytes*

Pre-confluent (i.e. proliferating) and post-confluent (i.e. differentiating) NHEKs were treated with glycerol (0.27%) and xylitol (0.45%) for the time indicated and then the mRNA expressions of filaggrin (**A**), involucrin (**B**), loricrin (**C**), and occludin (**D**) were assessed by Q-PCR. Data are presented using the ΔΔCT method; PPIA-normalized mRNA expressions of the vehicle controls (in the respective 24 or 48 hrs treated samples) were set as 1. Data are expressed as mean±SD of 3 independent determinations. Two additional experiments yielded similar results. *P<0.05 compared to the control.

Figure 2. *Xylitol, but not glycerol, induces the translocation of PKCδ whereas both polyols activate the MAPK pathway in NHEKs*

A) Confocal microscopy. NHEKs were treated with vehicle (Control) or with Glycerol (0.27%) and xylitol (0.45%) for the time indicated, then immunofluorescent staining of PKCδ was performed. Images were acquired by a laser scanning confocal microscope using the “z-stack mode” with strictly the same antibody staining and visualization procedure (the second image from the level of the coverslips). Arrows indicate the translocation of PKCδ upon xylitol treatment. Two additional experiments yielded similar results. **B**) Western blotting. NHEKs were treated with vehicle (Control), glycerol (Gly, 0.27%) and xylitol (Xyl, 0.45%) for 10, 30 or 60 min, and then Western blotting was performed to reveal expressions of the MAPK Erk1/2 (42 kDa/44 kDa) and its phosphorylated form (P-Erk1/2; 42 kDa/44 kDa). As positive

control, the PKC activator of phorbol 12-myristate 13-acetate (PMA) was employed. Two additional experiments yielded similar results.

Figure 3. *Effect of polyols on gene expression in various models of inflammation – TLR2 activation*

Keratinocytes were treated with 10 µg/ml lipoteichoic acid (LTA, TLR2 activator with or without glycerol (0.27%, **A**) and xylitol (0.45%, **B**) for 24 hrs. The mRNA expression of multiple pro-inflammatory interleukins (ILs) as well as the keratinocyte immunoactivation marker HLA-DR was then assessed by Q-PCR. Data are presented using the ΔΔCT method; PPIA-normalized mRNA expression of the vehicle control was set as 1 (solid line). Data are expressed as mean±SD of 3 independent determinations. *P<0.05 compared to the vehicle control whereas $^{\#}$P<0.05 compared to the LTA-treated group. Two-three additional experiments yielded similar results.

Figure 4. *Effect of polyols on gene expression in various models of inflammation – TLR3 activation*

Keratinocytes were treated with 20 µg/ml polyinosinic:polycytidylic acid (poly-IC, TLR3 activator) with or without glycerol (0.27%, **A**) and xylitol (0.45%, **B**) for 24 hrs. The mRNA expression of multiple pro-inflammatory interleukins (ILs) as well as the keratinocyte immunoactivation marker HLA-DR was then assessed by Q-PCR. Data are presented using the ΔΔCT method; PPIA-normalized mRNA expression of the vehicle control was set as 1 (solid line). Data are expressed as mean±SD of 3 independent determinations. *P<0.05 compared to the vehicle control whereas

[#]P<0.05 compared to the poly-IC-treated group. Two-three additional experiments yielded similar results.

Figure 5. *Effect of polyols on gene expression in various models of inflammation – TLR4 activation*

Keratinocytes were treated with 5 µg/ml lipopolysaccharide (LPS, TLR4 activator) with or without glycerol (0.27%, **A**) and xylitol (0.45%, **B**) for 24 hrs. The mRNA expression of multiple pro-inflammatory interleukins (ILs) as well as the keratinocyte immunoactivation marker HLA-DR was then assessed by Q-PCR. Data are presented using the ΔΔCT method; PPIA-normalized mRNA expression of the vehicle control was set as 1 (solid line). Data are expressed as mean±SD of 3 independent determinations. *P<0.05 compared to the vehicle control whereas [#]P<0.05 compared to the LPS-treated group. Two-three additional experiments yielded similar results.

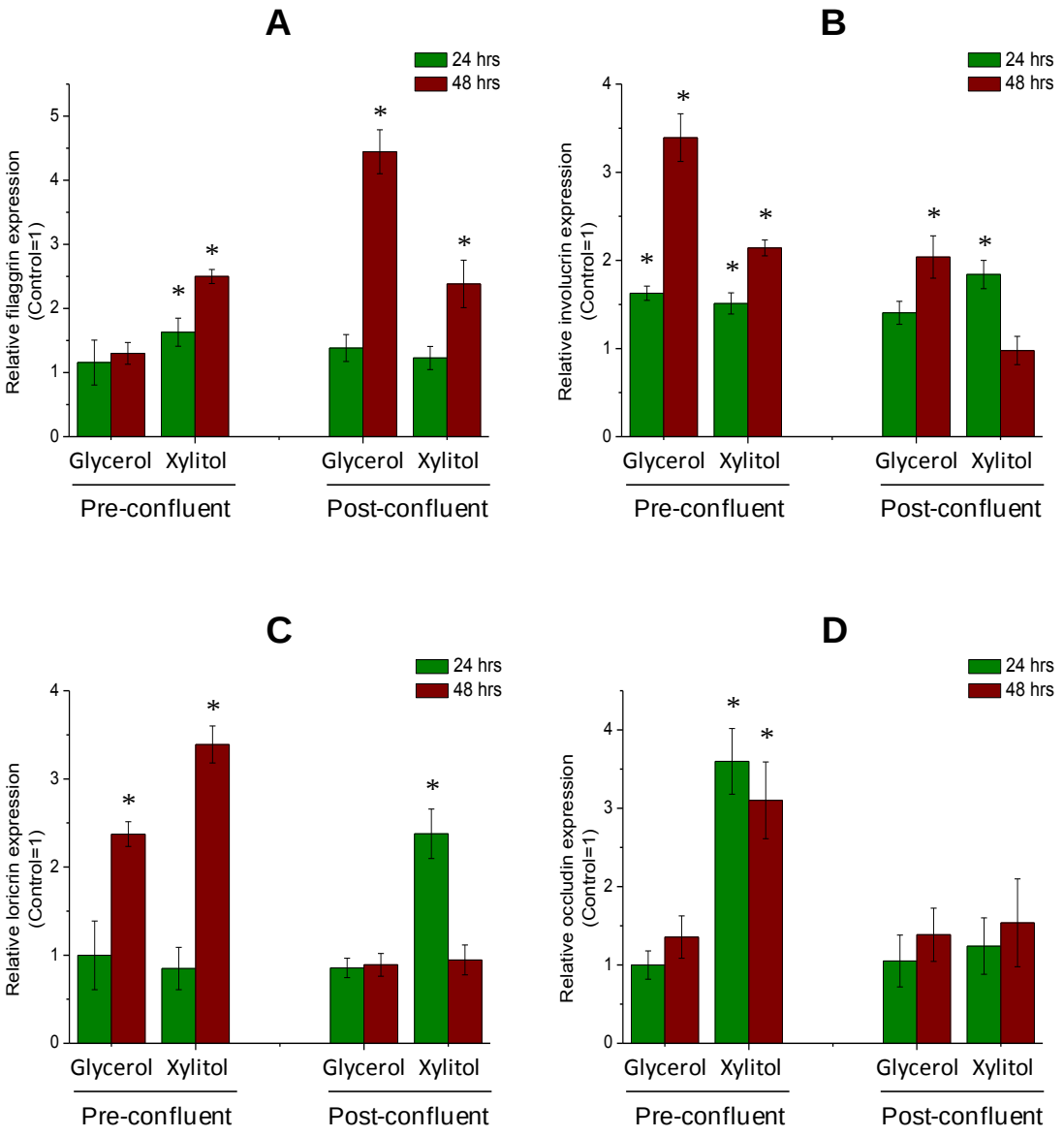

A
B
C
D
24 hrs
48 hrs
Relative filaggrin expression (Control=1)
Relative involucrin expression (Control=1)
Relative loricrin expression (Control=1)
Relative occludin expression (Control=1)
Glycerol
Xylitol
Pre-confluent
Post-confluent

Figure 1.

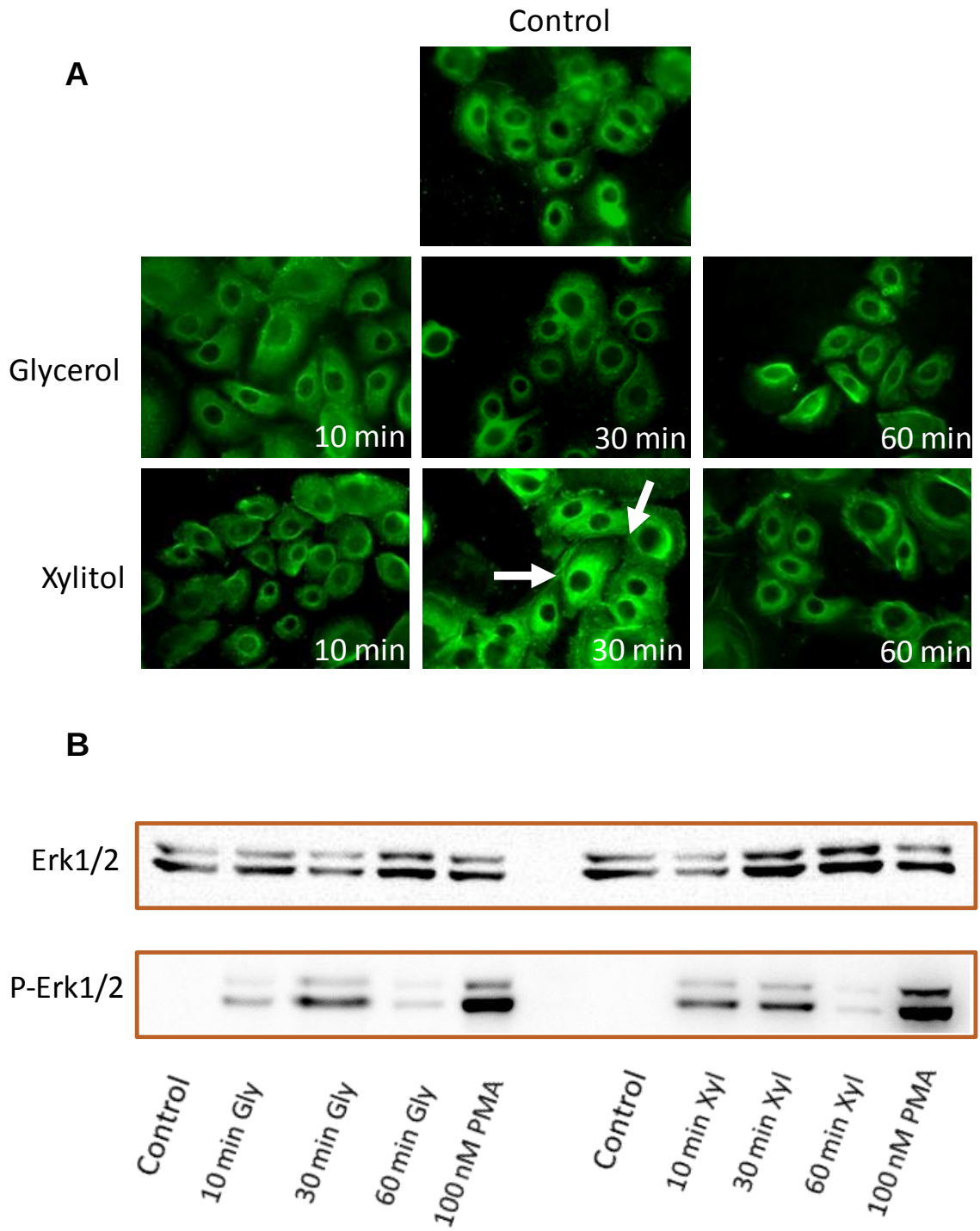
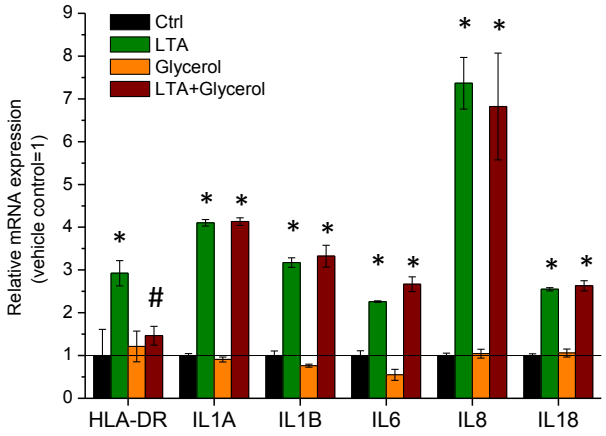

A
Control
Glycerol
10 min
30 min
60 min
Xylitol
10 min
30 min
60 min
B
Erk1/2
P-Erk1/2
Control
10 min Gly
30 min Gly
60 min Gly
100 nM PMA
Control
10 min Xyl
30 min Xyl
60 min Xyl
100 nM PMA

Figure 2.

A


Ctrl
LTA
Glycerol
LTA+Glycerol
Relative mRNA expression
(vehicle control=1)
HLA-DR
IL1A
IL1B
IL6
IL8
IL18

B

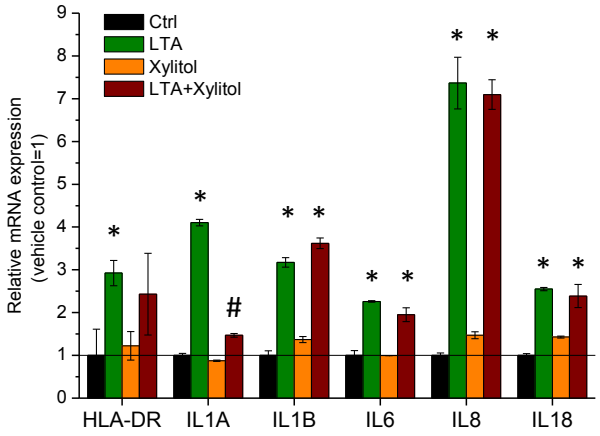
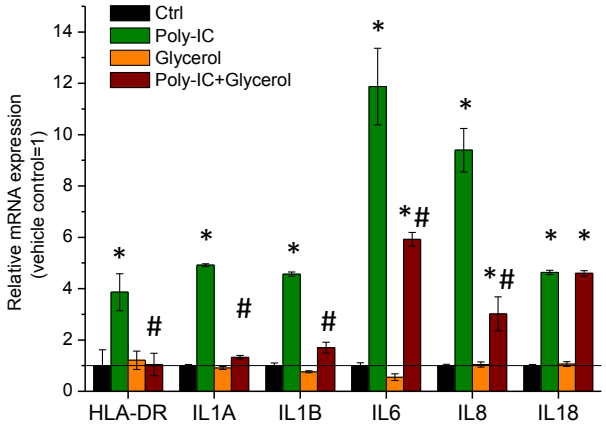

Ctrl
LTA
Xylitol
LTA+Xylitol
Relative mRNA expression
(vehicle control=1)
HLA-DR
IL1A
IL1B
IL6
IL8
IL18

Figure 3.

A


Ctrl
Poly-IC
Glycerol
Poly-IC+Glycerol
Relative mRNA expression
(vehicle control=1)
0
2
4
6
8
10
12
14
HLA-DR
IL1A
IL1B
IL6
IL8
IL18

B

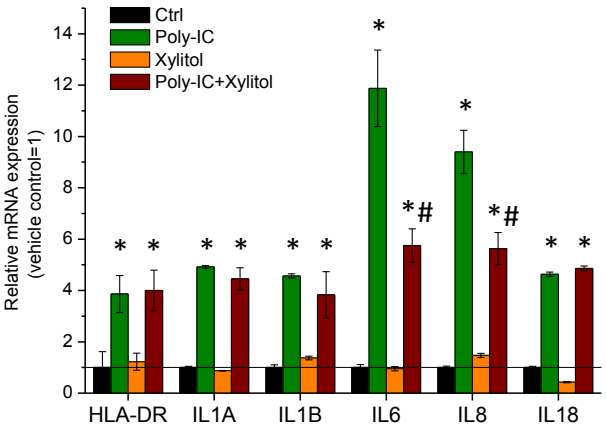
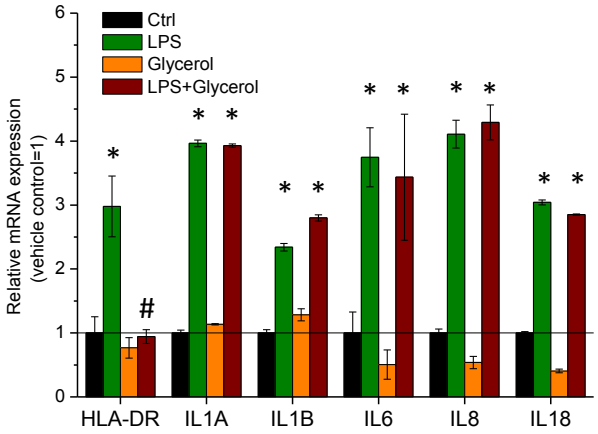

Ctrl
Poly-IC
Xylitol
Poly-IC+Xylitol
Relative mRNA expression
(vehicle control=1)
0
2
4
6
8
10
12
14
HLA-DR
IL1A
IL1B
IL6
IL8
IL18

Figure 4.

A


Ctrl
LPS
Glycerol
LPS+Glycerol
Relative mRNA expression
(vehicle control=1)
HLA-DR
IL1A
IL1B
IL6
IL8
IL18

B

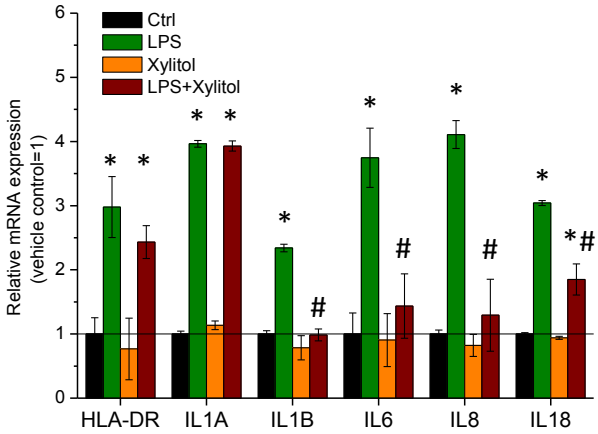

Ctrl
LPS
Xylitol
LPS+Xylitol
Relative mRNA expression
(vehicle control=1)
HLA-DR
IL1A
IL1B
IL6
IL8
IL18

Figure 5.

Supplementary data

Supplementary figure legends

Supplementary Figure 1. *Polyols do not affect viability and proliferation of NHEKs*

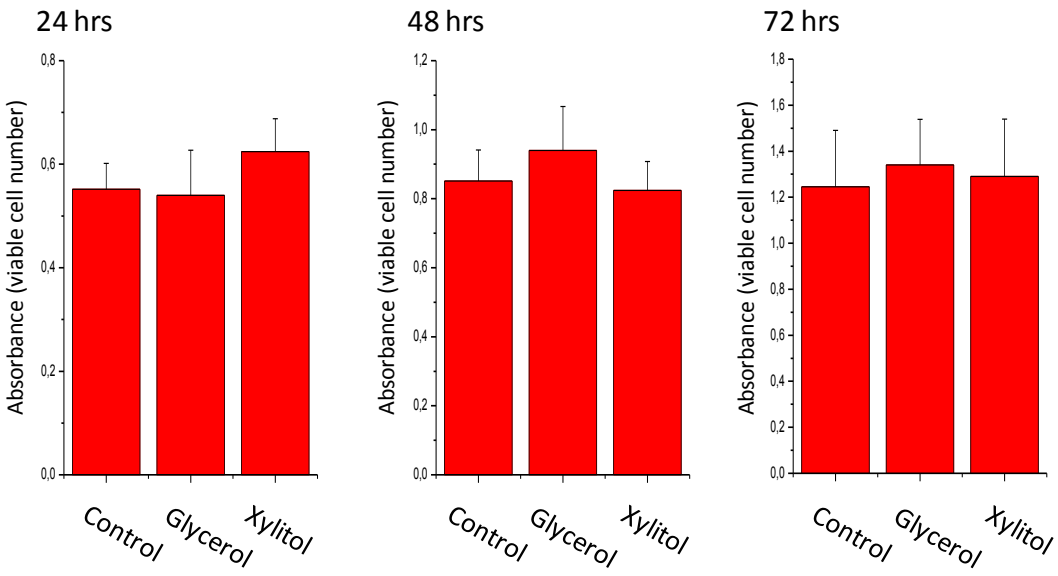
Pre-confluent (i.e. proliferating) NHEKs were treated with glycerol (0.27%) and xylitol (0.45%) in quadruplicates for the time indicated and then MTT assays were performed to assess viability and growth rate of the cells. Data are expressed as mean±SEM. Three repeated experiments yielded similar results.

Supplementary Figure 2. *Polyols do not elevate $[Ca^{2+}]_i$ of NHEKs*

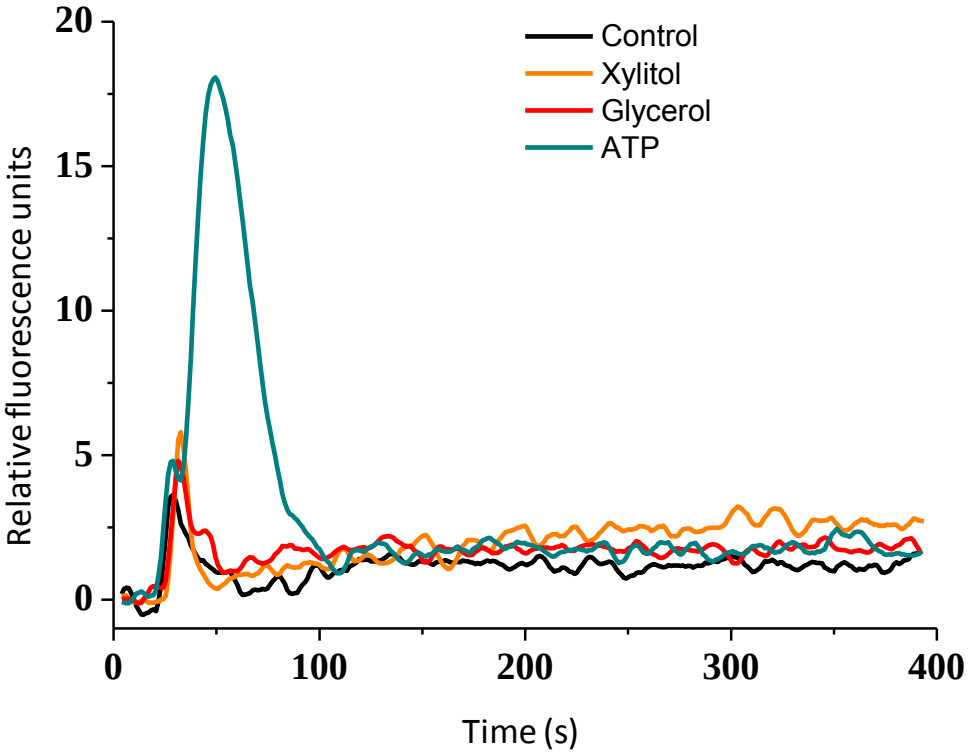
Fluorimetric Ca^{2+} imaging using Fluo-4. Glycerol (0.27%) and xylitol (0.45%) were applied as indicated by the arrow. As a positive control, ATP (180 μM) was applied [44]. Fluorescence (measured in relative fluorescence units) was normalized to the baseline. Two additional series of experiments yielded similar results.

Supplementary Figure 3. *Neither polyol induce the translocation of PKCα in NHEKs*

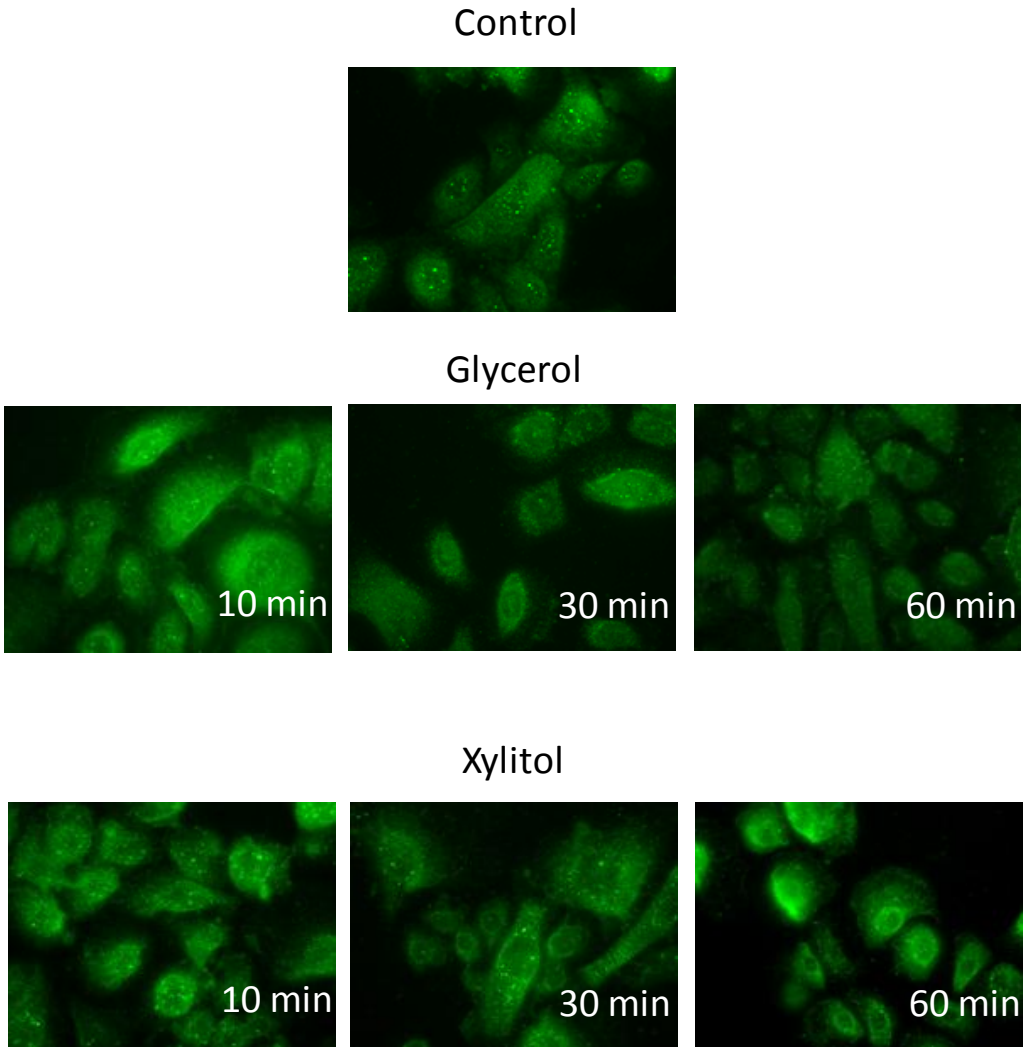
Confocal microscopy. NHEKs were treated with vehicle (Control) or with Glycerol (0.27%) and xylitol (0.45%) for the time indicated, then immunofluorescent staining of PKCα was performed. Images were acquired by a laser scanning confocal microscope using the "z-stack mode" with strictly the same antibody staining and visualization procedure (the second image from the level of the coverslips). Note the lack of translocation after treatment with polyols. Two additional experiments yielded similar results.



Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Figure 3.