Lipid Rafts and the Oxidative Stress Hypothesis

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TO THE EDITOR

Epidermal keratinocytes undergo a complex differentiation program during which their cholesterol synthesis is consistently increased, cholesterol being a crucial element for the elaboration of the epidermal lipid barrier. Cholesterol is also an essential component of all animal plasma membranes and is required for the formation of membrane rafts, which are also named lipid rafts (Valencia *et al.*, 2006). Lipid rafts regroup numerous signaling molecules, and their disruption after cholesterol depletion induces drastic changes in cellular signal transduction (Simons and Toomre, 2000). In previous studies published in this journal, we investigated lipid rafts in keratinocytes by using the cholesterol chelator methyl-beta-cyclodextrin (MBCD) and found that lipid raft perturbation is responsible for an altered epidermal phenotype characterized by accelerated involucrin and delayed keratin 10 expression (Jans *et al.*, 2004; Mathay *et al.*, 2008). The activation of p38 mitogen-activated protein kinase was found to be a prerequisite for this alteration. Intriguing parallels in the response of keratinocytes that were either depleted in cholesterol or treated with H_2O_2 were identified; that is, both treatments, in a very similar time-course manner, increased the phosphorylation of p38 mitogen-activated protein kinase and induced the expression of heparin-binding EGF-like growth factor, a growth factor involved in epidermal homeostasis, at messenger RNA and protein levels. These data led us to hypothesize a role of oxidative mechanisms behind MBCD-induced effects (Mathay *et al.*, 2008). In support of this hypothesis, it was shown that oxidative stress signaling can transit through lipid rafts making them efficient redox signaling platforms (Jin and Zhou, 2009), and that UVA irradiation of keratinocytes generates oxidative stress and reduces the cholesterol content of lipid rafts by 60% (Grether-Beck *et al.*, 2008). Moreover, lack of cholesterol in plasma membranes of keratinocytes enhances UVA-induced oxidative stress (Valencia *et al.*, 2006); thus, cholesterol itself could act as an antioxidant, as previously suggested by Smith (1991). However, there seem to be cell type-specific redox implications of lipid rafts, as lipid raft disruption inhibits the synthesis of reactive oxygen species (ROS) in endothelial cells (Li *et al.*, 2007) and immortalized HaCaT keratinocytes (Gniadecki *et al.*, 2002), whereas augmented ROS production is reported in renal proximal tubule cells (Han *et al.*, 2008).

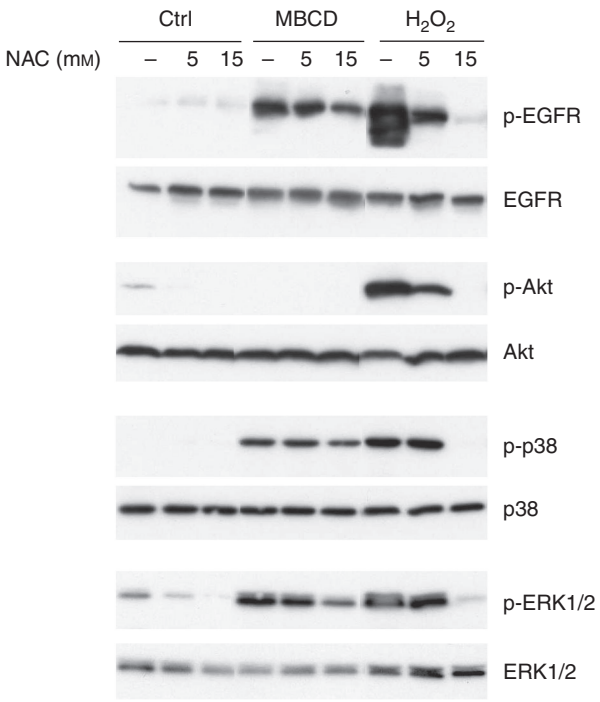

Figure 1. Cholesterol depletion and oxidative stress induce different signaling pathways in human epidermal keratinocytes. Autocrine confluent cultures (Minner *et al.*, 2010) were either pretreated or not with 5 or 15 mM *N*-acetyl-L-cysteine (NAC; pH 7.4) before treatments with 7.5 mM methyl-beta-cyclodextrin (MBCD; 1 hour) (Sigma-Aldrich) or 1 mM H_2O_2 (20 minutes). After the treatments, cell lysates were prepared and analyzed by western blotting with antibodies against phospho-EGFR (p-EGFR; Tyr1173) (Invitrogen, Merelbeke, Belgium), phospho-Akt (p-Akt; Ser473), phospho-p38 (p-p38; Thr180/Tyr182), and phospho extracellular signal-regulated kinase 1/2 (p-ERK1/2; Thr202/Tyr204) (Cell Signaling, Bioké, Leiden, The Netherlands). Data are representative of repeated experiments.

To test the oxidative stress hypothesis in the model of autocrine human epidermal keratinocyte cultures, we first analyzed whether the antioxidant *N*-acetyl-L-cysteine (NAC, Sigma-Aldrich, Bornem, Belgium) could protect cell viability in MBCD- or H_2O_2-treated keratinocytes. As expected, H_2O_2 treatment (1 mM for 20 minutes) induced a 64% cell mortality rate, but the presence of the antioxidant protected keratinocytes against this H_2O_2-induced cell mortality (3%) (data not shown). Conversely, cholesterol-depleted keratinocytes presented a limited 30% cell

Abbreviations: MBCD, methyl-beta-cyclodextrin; NAC, N*-acetyl-*L*-cysteine; ROS, reactive oxygen species*

mortality; however, this mortality could not be avoided by the addition of NAC. The phosphorylation status of signaling molecules known to be redox-sensitive was also analyzed (Figure 1). H_2O_2 and MBCD induced phosphorylations of the EGFR and of the mitogen-activated protein kinases p38 and extracellular signal-regulated kinase 1/2, but only the signaling induced by H_2O_2 could be inhibited by NAC. We also found another discrepancy between the effects induced by H_2O_2 and MBCD, as MBCD provoked a complete dephosphorylation of Akt conversely with H_2O_2, which induced a strong Akt phosphorylation. In addition, MBCD-induced mitogen-activated protein kinase phosphorylations were only minimally reduced in the presence of the NAC antioxidant. This observation can likely be explained by the fact that NAC increases the intracellular reduced state and thereby protects protein tyrosine phosphatase activity (Chiarugi and Cirri, 2003), resulting in modest dephosphorylation of EGFR and extracellular signal-regulated kinase 1/2. These first data suggested that the proposed hypothesis was not valid and further experiments were performed to ascertain the noninvolvement of oxidative mechanisms after lipid raft disruption by MBCD. For instance, we investigated the activity levels of protein tyrosine phosphatases (Figure 2a), as these enzymes are known primary targets of cellular oxidation (Finkel, 2003). Although in H_2O_2-treated keratinocytes an important decrease in protein tyrosine

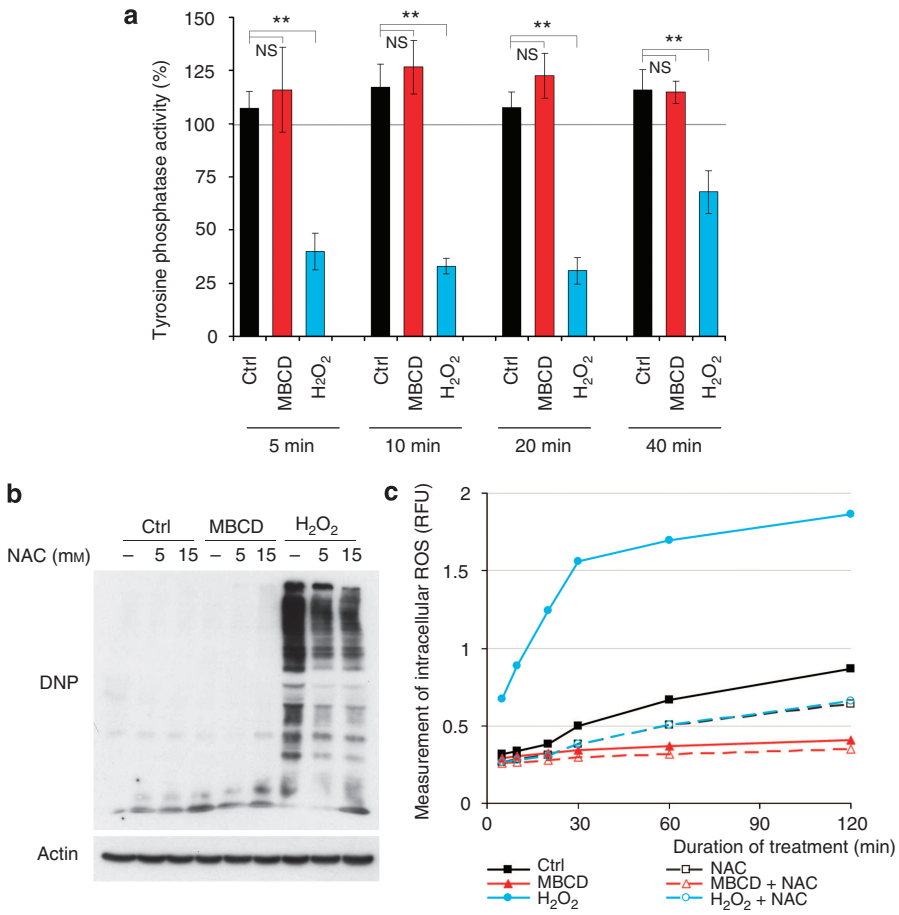

Figure 2. Tyrosine phosphatase activity, protein oxidation, and generation of reactive oxygen species after cholesterol depletion or H_2O_2 treatment of keratinocytes. (**a**) Protein tyrosine phosphatase (PTP) activity is not affected by cholesterol depletion, whereas its activity is inhibited by H_2O_2. Confluent cultures were left untreated or were treated with 7.5 mM methyl-beta-cyclodextrin (MBCD) or 1 mM H_2O_2 for 0, 5, 10, 20, or 40 minutes; then cultures were immediately lysed and tyrosine phosphatase activity was measured by the colorimetric assay ''Tyrosine Phosphatase Assay System'' (Promega, Leiden, The Netherlands). The PTP activity of the samples was analyzed by measuring the phosphate release from the phosphopeptide DADE(pY)LIPQQG. The represented data are means ± SEM from five independent experiments. The PTP activity of cells before treatments was arbitrarily set at 100%. Data have been analyzed by analysis of variance after testing the homogeneity of variance (Bartlett); *post hoc* comparisons have been performed by Dunnett's test (NS, nonsignificant; $^{**}P<0.01$, $n=5$). (**b**) Cholesterol depletion does not induce protein oxidation in keratinocytes. Confluent keratinocyte cultures were pretreated or not with the antioxidant *N*-acetyl-L-cysteine (NAC; 5 or 15 mM) for 1 hour before treatment with 7.5 mM MBCD or 1 mM H_2O_2 for 1 hour. Immediately after the treatments, proteins were extracted and derivatized with 2,4-dinitrophenylhydrazine using the OxyBlot kit (Chemicon, Millipore, Brussels, Belgium) for 15 minutes at room temperature. The dinitrophenylhydrazone-derivatized proteins were detected by western blotting using a primary antibody specific to the dinitrophenyl moiety of the proteins and horseradish peroxidase-conjugated secondary antibody. The illustrated OxyBlot data are representative of three independent experiments. (**c**) Cholesterol depletion reduces the generation of reactive oxygen species below the baseline level of untreated keratinocytes. Confluent keratinocyte cultures were loaded with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (Molecular Probes, Invitrogen) for 30 minutes in the presence or absence of 15 mM NAC in autocrine culture medium without phenol red; then, cultures were treated with 100 μM H_2O_2 or 7.5 mM MBCD in the presence or absence of 15 mM NAC. Intracellular reactive oxygen species production in living cells was measured at the indicated time points on a microplate fluorescence reader (λ_{exc} 585 nm; λ_{em} 520 nm).

phosphatase activity was observed and shown to be reversible, the protein tyrosine phosphatase activity measured in MBCD-treated keratinocytes was not significantly different at any time point when compared with the activity measured in untreated cells, indicating that disorganization of lipid rafts does not cause inactivation of protein tyrosine phosphatases. Similarly, detection of protein oxidation showed that cultures subjected to H_2O_2 treatment presented important protein oxidation, which was partially protected by preincubation with NAC (Figure 2b), but in untreated and in cholesterol-depleted cultures, protein oxidation was never detected. Finally, an ultimate experiment was performed in which intracellular ROS synthesis was directly measured by a fluorescent dye. Hydrogen peroxide induced a strong intracellular ROS production during the first 20 minutes, which was clearly decreased when preincubation with NAC was performed. ROS measurements showed that keratinocytes with a perturbed lipid raft organization presented only very low ROS levels, which were even below the baseline ROS levels measured in untreated cells (Figure 2c). In other words, all these results suggest that disruption of lipid rafts is rather able to inhibit the basal ROS production, an observation that is in total accordance with recent literature showing that MBCD treatment protects yeast cells from oxidative stress (Du and Ayscough, 2009). In conclusion, contrary to the hypothesis suggested by our previous study (Mathay *et al.*, 2008), the data presented hereby clearly point out that lipid raft disruption by cholesterol depletion does not modulate the cellular redox balance toward oxidation in normal keratinocytes. To pursue the identification of mechanisms involved in keratinocytes after cholesterol depletion, we have now undertaken transcriptional profiling. This unbiased approach should help to elucidate major actors, mechanisms, and pathways involved during and after lipid raft disruption in epidermal keratinocytes.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Tannic Acid and Quercetin Display a Therapeutic Effect in Atopic Dermatitis via Suppression of Angiogenesis and TARC Expression in Nc/Nga Mice

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TO THE EDITOR

The number of atopic dermatitis (AD) patients has been increasing steadily worldwide. AD is a dangerous disorder because it not only causes chronic inflammation but also leads to bacterial and viral skin infections. Although the pathogenesis of AD is not completely understood, inflammation-related angiogenesis (Groneberg *et al.*, 2005) and cytokine production associated with T helper type 2 (Th2) polarization

Abbreviations: AD, atopic dermatitis; TA, tannic acid; TARC, thymus and activation-regulated chemokine; TSLP, thymic stromal lymphopoietin