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Dihydrolipoic but not alpha-lipoic acid affects susceptibility of eukaryotic cells to bacterial invasion

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ABSTRACT

Sensitivity of eukaryotic cells to facultative pathogens can depend on physiological state of host cells. Previously we have shown that pretreatment of HeLa cells with N-acetylcysteine (NAC) makes the cells 2–3-fold more sensitive to invasion by the wild-type *Serratia grimesii* and recombinant *Escherichia coli* expressing gene of actin-specific metalloprotease grimelysin [1]. To evaluate the impact of chemically different antioxidants, in the present work we studied the effects of α-Lipoic acid (LA) and dihydrolipoic acid (DHLA) on efficiency of *S. grimesii* and recombinant *E. coli* expressing grimelysin gene to penetrate into HeLa and CaCo cells. Similarly to the effect of NAC, pretreatment of HeLa and CaCo cells with 0.6 or 1.25 mM DHLA increased the entry of grimelysin producing bacteria by a factor of 2.5 and 3 for the wild-type *S. grimesii* and recombinant *E. coli*, respectively. In contrast, pretreatment of the cells with 0.6 or 1.25 mM LA did not affect the bacteria uptake. The increased invasion of HeLa and CaCo cells correlated with the enhanced expression of E-cadherin and β-catenin genes, whereas expression of these genes in the LA-treated cells was not changed. Comparison of these results suggests that it is sulfhydryl group of DHLA that promotes efficient modification of cell properties assisting bacterial uptake. We assume that the NAC- and DHLA-induced stimulation of the E-cadherin-catenin pathway contributes to the increased internalization of the grimelysin producing bacteria within transformed cells.

1. Introduction

α-Lipoic acid (LA) known also as thioctic acid is a fatty acid widely distributed in cellular membranes, cytosol, and extracellular spaces. It is synthesized within the cells but mostly absorbed from food and transported to cytoplasm via fatty acid-, Na^+ -dependent vitamin- or H^+ -linked monocarboxylate-transporter systems [2–4]. Being a naturally occurring cofactor for the mitochondrial dehydrogenases LA is also involved in many other biological functions [5]. In cells LA reduces sulfhydryl groups with NADPH-dependent reductase producing the reduced form, dihydrolipoic acid (DHLA). Both the oxidized and reduced forms of LA show antioxidant properties. These compounds are able to chelate metals inhibiting hydroxyl radical production, act as a scavenger of reactive oxygen and nitrogen species. DHLA as a more powerful antioxidant is able to restore superoxide anion radical, regenerate endogenous antioxidants (vitamins C, E, glutathione) and restore oxidative injures [6,7], thus being deeply involved in the maintenance of the redox status of the cells.

Besides their direct antioxidant effects, the thiol-containing antioxidants interact with regulatory and signaling proteins modulating signal transduction and gene expression [8,9]. LA was shown to down-regulate expression of genes encoding redox-sensitive pro-inflammatory proteins and to induce expression of antioxidant genes [10]. It also inhibited expression of intercellular adhesion molecule-1 ICAM-1 [11] and alpha4beta1 integrin [12] and beta1-integrin [13]. The effects of other well-known thiol-containing antioxidant, N-acetylcysteine (NAC) on up- and down-regulation of genes responsible for cell proliferation and differentiation were studied in detail [14,15]. These data indicate that despite the difference of their chemical properties LA/DHLA and NAC can induce similar responses. To address this issue, LA-, DHLA- and NAC-induced effects should be compared in the same biological system.

We have previously shown that pre-treatment of HeLa cells with N-acetylcysteine increases their susceptibility to invasion by facultative pathogen *Serratia grimesii* producing actin-specific protease grimelysin and recombinant *Escherichia coli* expressing

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grimelysin gene [1]. This effect did not correlate with changes in glutathione level [16] or cytoskeleton rearrangements [1] but was accompanied by an increased expression of E-cadherin, cell surface receptor playing a role in cell adhesion and cell–cell junctions [1]. Taking these effects as a reference, in the present work we have tried to modify susceptibility of HeLa and CaCo cells to bacterial invasion by LA and DHLA in order to evaluate the impact of these chemically different compounds on the mechanisms of bacteria-host cell interaction.

Our results show that, similarly to NAC, DHLA increases susceptibility of HeLa cells to invasion by S. grimesii and up-regulates expression of E-cadherin gene. In contrast, no effect of LA on these properties was detected. These results show that the sulfhydryl activity of DHLA, like that of NAC, plays a role in the enhanced susceptibility of HeLa cells to bacteria. It is plausible that these reagents interact with cell surface receptors and either activate them directly or modulate signal transduction regulating the E-cadherin-β-catenin pathway.

2. Materials & methods

2.1. Reagents

Culture media αMEM and DMEM were obtained from Biolot LLC (Russia). Fetal bovine serum (FBS) was from Thermo Scientific (Thermo Fisher Scientific Inc.). Peptone and yeast extract were obtained from Difco (Franklin Lakes, NJ), nonessential amino acids (NEAA), NAC, PBS, sodium deoxycholate, Triton X-100, reagents for PCR, were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell cultures, bacterial strains, and growth conditions

Human cervical carcinoma HeLa M and colorectal adenocarcinoma CaCo-2 cells were obtained from the Russian Cell Culture Collection (Institute of Cytology, St. Petersburg, Russia). Cells were grown in the antibiotic-free αMEM supplemented with 10% FBS and 1% NEAA at 37 °C under 5% CO_2 atmosphere. *S. grimesii* strain 30063 was from the German Collection of Microorganisms and Cell Cultures (DSMZ). The recombinant *E. coli* SCS1, expressing grimelysin gene was obtained as described previously [17,18]. Bacteria were grown in Luria broth (LB medium) containing 1% peptone, 0.5% yeast extract, and 1% NaCl at pH 7.0 at 37 °C with aeration.

2.3. Incubation of cells with antioxidants

LA and DHLA were freshly dissolved in the culture medium. Cells were grown to get 60% confluents in 6-well plates for 24 h, and LA or DHLA was added to the medium to the final concentration of 0.6 or 1.25 mM for 22–24 h. After the medium was replaced by the fresh one without LA/DHLA the cells were used for quantitative invasion assay either immediately or after growing for 24 h more.

Cells were incubated with NAC as discribed previously [1].

2.4. Gentamicin invasion assay

Efficiency of invasion was evaluated by gentamycin invasion assay as discribed previously [19] with minor modifications [1].

2.5. RNA analysis

Isolation of RNA. For RNA isolation, the TRIZOL reagent (Invitrogen) was used following the manufacturer's instructions. Quality and quantity of isolated nucleic acids were estimated by spectrophotometry and gel electrophoresis.

cDNA preparation. For reverse transcription, 2 μg total RNA was reverse transcribed with oligo-dT (Invitrogen) and 200 U Moloney murine leukemia virus reverse transcription (Invitrogen) according to the manufacturer's instructions [20].

2.6. Real-time RT-PCR

Real-time RT-PCR was performed to examine the E-cadherin mRNA levels in Hela M, and CaCo-2 cells. Q-RT-PCR was performed using IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and an iCycler Thermal Cycler (Bio-Rad Laboratories). The quantity of mRNA was normalized to the housekeeping gene β-microglobulin.

2.7. Semi-quantitative RT-PCR

Expression analyses of E-cadherin gene was conducted by semiquantitative RT-PCR. Gene-specific primer pairs were designed using BLAST primer software and were as followed: β-Actin forward: 5′-CTG GGA CGA CAT GGA GAA AA-3′, β-Actin reverse: 5′-AAG GAA GGC TGG AAG AGT GC-3′, E-cadherin forward: 5′-CCC TGG CTT TGA CGC CGA GA-3′, E-cadherin reverse: 5′-CTC GGT CCA GCC CAG TGG TG-3′, β-microglobulin forward: 5′- GCT ATC CAG AAA ACC CCT CAA -3′, β-microglobulin reverse: 5′- CAT GTC TCG ATC CCA GTA GAC GGT -3′

PCR reactions were optimized to 94 °C for 3 min, 35 amplification cycles at 94 °C for 1 min, the appropriate annealing temperature (60 °C for β-actin and β-microglobulin and 64 °C for E-cadherin) for 1 min, 72 °C for 1 min, and a final extension of 10 min at 72 °C. Amplified products were resolved on 1.5% agarose gels and visualized by ethidium bromide staining.

2.8. Western blot

Cells were grown to confluence in plastic dishes, treated with antioxidants and then disrupted by a freezing–thawing procedure at −70 °C, further incubated for 10 min with buffer contained 5 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.05% Nonidet P-40, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail (Complete™, Roche Molecular Biochemicals). The proteins were separated by SDS-PAGE, and then transferred onto PVDF membranes. After been blocked with 1% BSA for 2 h at room temperature (RT), the membranes were then incubated with antibodies against E-cadherin (1:250, Abcam), and β-actin (1:1000, Abcam) at 4 °C overnight. After washing 3 times with TBST, the membranes were incubated with alkaline phosphatase-conjugated secondary antibody (1:10,000, Sigma–Aldrich) at RT for 2 h. After washing 3 times with TBST, the membrane was imaged with a gel imaging system (BIO-RAD).

2.9. Statistical analysis

Data were analyzed statistically using one-way analysis of variance (ANOVA) with Excel Data Analysis Pack. A difference was considered significant at the $p < 0.05$ level.

3. Results

3.1. α-Lipoic acid does not change susceptibility of HeLa and CaCo-2 cells to invasion by S. grimesii and recombinant E. coli expressing grimelysin gene

Pre-treatment of HeLa cells with 10 mM N-acetylcysteine increased the entry of grimelysin producing bacteria by a factor of 1.5–2 and 3-3.5 for wild-type *S. grimesii* and recombinant *E. coli* expressing grimelysin gene, respectively [1]. To evaluate the

efficiency of the invasion into the cells pre-treated with LA, HeLa M cells were incubated with 0.6 mM or 1.25 mM LA. According to the literature, these concentrations lie in the range of 0.2 μM–2.0 mM LA/DHLA usually used in *in vitro* experiments. Then the cells were washed out and infected with the bacteria as described in Material & Methods. The number of intracellular bacteria was determined by the gentamycin invasion assay [19]. Fig. 1A shows that the number of intracellular *S. grimesii* in the HeLa cells pretreated with LA for 24 h was the same as in the control (non-treated) cells (Fig. 1A, boxes 1, 2). When the HeLa cells were cultivated for further 24 h after LA was removed from the culture medium the efficiency of the invasion did not change either (Fig. 1A, box 3). The number of intracellular *E.coli* SCS1 carrying grimelysin gene was similar to that of *S. grimesii* (Fig. 1A, box 4) and did not change when the cells were infected by the bacteria in 24 h after LA was removed from the culture medium (Fig. 1A, boxes 5, 6). Moreover, the susceptibility of HeLa cells to the invasion remained the same when the cells were pre-incubated with 1.25 mM LA (not shown).

Similar experiments were performed using epithelium colorectal adenocarcinoma cell line CaCo-2.

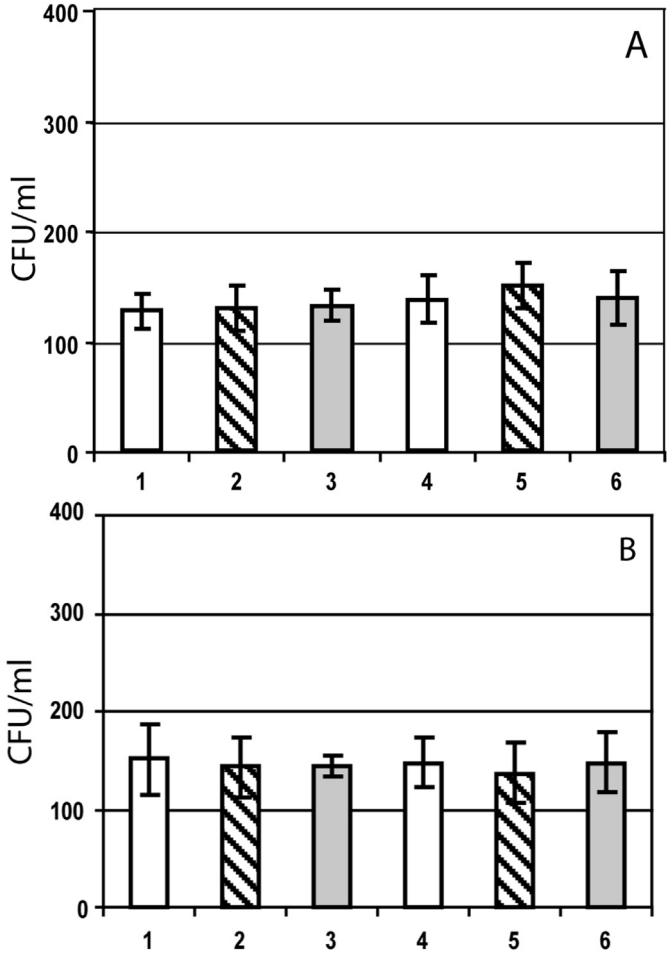

Fig. 1. Quantitative evaluation of the effect of LA on susceptibility of HeLa (A) and CaCo-2 (B) cells to invasion by the wild-type and recombinant bacteria. The cells were infected by *S. grimesii* (1–3) or recombinant *E. coli* SCS1 (4–6) as described in Materials & Methods. *White boxes*, the infected cells without LA treatment, *striped boxes,* the cells preincubated with 0.6 mM LA before the infection, and *grey boxes*, the cells pre-incubated with 0.6 mM LA and cultured for 24 h more before the infection. The values are expressed as mean ± S.D. (*error bars*). A difference was considered significant at the $p < 0.05$ level.

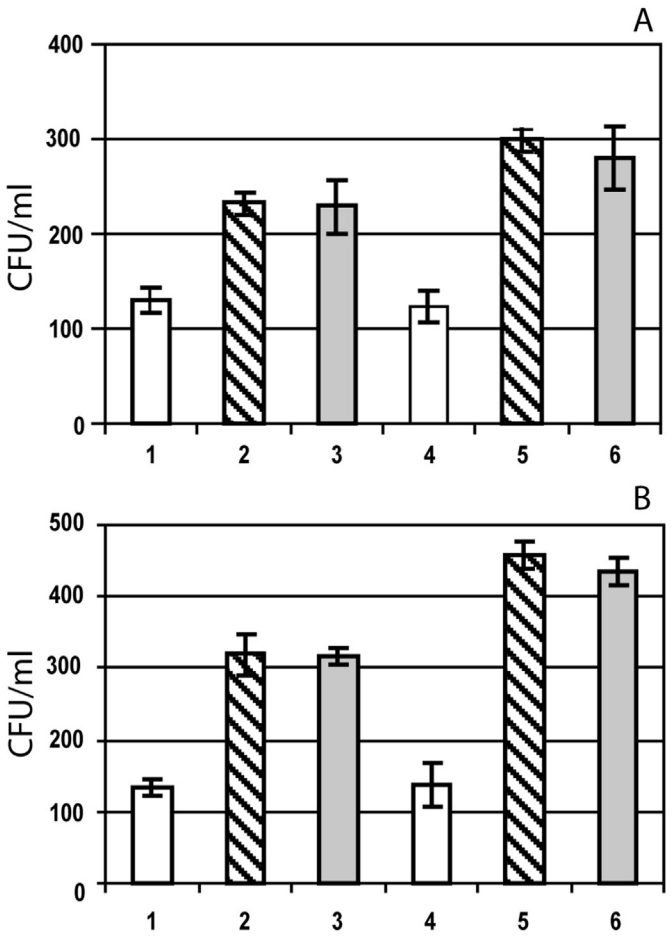

Fig. 2. Quantitative evaluation of the effect of DHLA on susceptibility of HeLa (A) and CaCo-2 (B) cells to invasion by the wild-type and recombinant bacteria. The cells were infected by *S. grimesii* (1–3) or recombinant *E. coli* SCS1 (4–6) as described in Materials & Methods. *White boxes*, infected cells without DHLA treatment, *striped boxes,* the cells preincubated with 1.25 mM DHLA before the infection, and *grey boxes*, the cells pre-incubated with 1.25 mM DHLA, and cultured for 24 h more before the infection. The values are expressed as mean ± S.D. (*error bars*). A difference was considered significant at the $p < 0.05$ level.

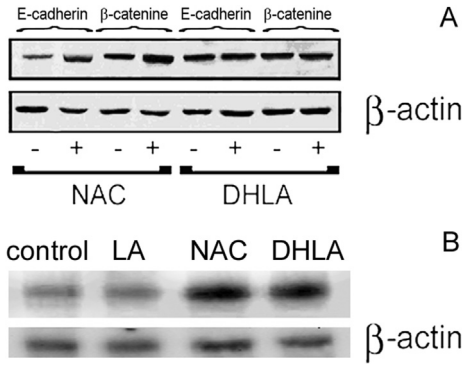

Fig. 3. Expression of E-cadherin and β-catenin gene determined by semi-quantitative RT-PCR *and Western blotting. A*: (−), non-treated cells, (+), cells treated with 10 mM NAC or 1.25 mM DHLA for 24 h β-actin was used as a standard. B: Western blot result showed that the level of E-cadherin proteins of the DHLA and NAC treated Hela cells was increased compared with that of wild type ones and pretreated with LA. Control stands for the Hela cells of wild type; LA, DHLA and NAC stand for those with LA, DHLA and NAC-pretreated cell samples.

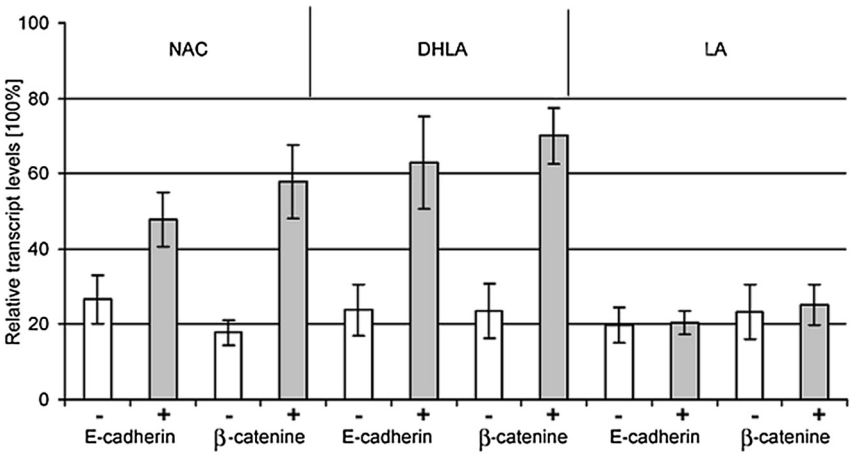

Fig. 4. Quantitative analysis of the relative transcript levels of E-cadherin and β-catenin gene in HeLa cells treated with 10 mM NAC, 1.25 mM DHLA and 1.25 mM ALA. β-mioglobin was used as a standard.

Under the same experimental conditions, both the wild-type and recombinant bacteria invaded CaCo-2 cells with the same efficiency as HeLa M cells (Fig. 1B) indicating that LA does not seem to affect the factors underlying sensitivity of HeLa and CaCo-2 cells to bacteria.

3.2. Dihydrolipoic acid (DHLA) increases susceptibility of HeLa cells to invasion by S. grimesii and recombinant E. coli expressing grimelysin gene

Upon entering the cell LA produces the sulfhydryl form, dihydrolipoic acid (DHLA) that acts as a direct antioxidant inside the cells. Moreover, DHLA can release into the culture medium [21]. It was natural therefore to elucidate whether DHLA affects susceptibility of eukaryotic cells to bacterial invasion.

Fig. 2A shows the number of intracellular S. grimesii and recombinant *E. coli* SCS1 in the HeLa M cells pretreated with 1.25 mM DHLA for 24 h. The efficiency of HeLa M invasion by both wild-type and recombinant bacteria was increased by a factor of 2 and 3, respectively (Fig. 2A, striped boxes). This effect is similar to the effect of NAC [1] both in its magnitude and in indicating that HeLa M cells were more sensitive to invasion with the recombinant than the wild-type bacteria. Incubation of HeLa cells for 24 h upon removal of DHLA from the culture medium did not increase the efficiency of the invasion keeping it close to the invasion of the cells infected directly after DHLA was washed out (Fig. 2A, grey boxes).

DHLA had the same effect on susceptibility of CaCo-2 cells to the invasion, with increasing the invasion efficiency by a factor of 2.2 and 3, respectively (Fig. 2B, striped boxes) indicating that CaCo-2 cells are also more sensitive to invasion by recombinant bacteria. When the cells were infected with bacteria in 24 h after removal of DHLA from the culture medium, the efficiency of invasion remained the same (Fig. 2B, grey boxes).

3.3. The effect of antioxidants on E-cadherin and beta-catenin expression

It has previously been shown that treatment with N-acetylcisteine (NAC) up-regulates expression of several genes including E-cadherin and increases focal adhesion, as a result of newly established adherence junctions containing E-cadherin/β-catenin complexes [15]. E-cadherin, via β- and α-catenins, can trigger dynamic events of actin polymerization and membrane extensions culminating in bacterial uptake [22]. Our previous work has also demonstrated the influence of NAC on E-cadherin gene expression [1]. Therefore we compared expression of E-cadherin gene in NAC, LA and DHLA treated cells by RT-PCR and qPCR.

Fig. 3A shows that treatment of Hela M cells with 10 mM NAC resulted in an increased expression of both E-cadherin and beta-catenin genes. On the other hand, no visible effect of 1.25 mM DHLA on expression of E-cadherin and β-catenin genes in the HeLa cells was revealed. To increase sensitivity of the RT-PCR method, we repeated the experiment using a ten-fold more sensitive quantitative qPCR method. Using this approach, we revealed the increased expression of E-cadherin and β-catenin genes in the cells treated both with NAC and DHLA (Fig. 4). Consistently with the lack of the effect of LA on bacterial invasion, we did not reveal any increase in expression of E-cadherin and β-catenin genes in the HeLa cells treated with LA (Fig. 4, last set of boxes).

These results were paralleled by the data of immunochemical analysis (Fig. 3B). The increased expression of E-cadherin gene resulted in a increase of E-cadherin recovered in the lysates of DHLA-treated cells, whereas no effects of LA on the amount of E-cadherin were observed.

4. Discussion

LA can function both as a coenzyme in mitochondrial energy metabolism and as a antioxidant and cell redox modulator. It is thereby a unique antioxidant since it exerts protective functions both in reduced and oxidized form [2,23]. The oxidized form is reduced to DHLA intracellularly. On the other hand, DHLA can release into the culture medium [21,24]. Therefore in the present work we used both LA and DHLA to evaluate their impact on susceptibility of transformed cells to bacterial invasion and compare it with the effects produced by NAC. Our results showed that incubation of HeLa or CaCo cells with LA did not change interaction of the cells with *S. grimesii* and recombinant *E. coli* producing grimelysin. In contrast, incubation of the cells with DHLA led to a 2–3-fold increase in the bacteria uptake. The increased susceptibility of the cells to the invasion was preserved at least for 24 h after DHLA was removed from the culture medium. These results are quantitatively similar to the effects of NAC observed earlier [1].

The antioxidant properties of the thiol-containing compounds are characterized by increasing production of intracellular glutathione and depletion of reactive oxygen species (ROS). However, in contrast to the ROS-scavenging activity of NAC, the influence of LA and DHLA is not so unequivocal. As studied in the range of

concentrations (0.2 μM–1 mM) and exposure time both LA and DHLA can also produce pro-oxidant effects [6,10,25,26]. In line with these data, our recent experiments demonstrated that treatment of transformed 3T3-SV40 fibroblasts with NAC diminished ROS concentration, whereas 0.1–1.25 mM LA or DHLA promoted ROS generation in a dose-dependent manner [27]. Therefore similar stimulation of bacterial invasion by NAC and DHLA, contrasted to a lack of LA-producing effect, cannot be explained by the changes in the redox status of the treated cells. On the other hand, specific interaction of these thiol-containing compounds with cellular substrates can be a factor affecting sensitivity of the cells to bacterial invasion. As the chemical difference between LA and DHLA reveals itself primarily at the cell surface, their interaction with such component of cell surface as matrix metalloproteases and cell surface receptors could be of a primary importance.

LA and DHLA inhibit activity of matrix metalloproteinases MMP-9 and MMP-2 in a dose-dependent fashion [28–30]. Moreover, LA reduced MMP-2 and MMP-9 specific mRNA expression [12,30]. Since MMP activity controls structure and functional rearrangements of extracellular matrix, the LA/DHLA-induced changes in the MMP activity might modify cell surface properties and thus increase sensitivity of the cells to bacteria. It is shown, however, that induction and activation of MMP can contribute to bacterial pathogenesis [31,32].

Previously we have found that the enhanced uptake of grimelysin-producing bacteria by HeLa cells treated with NAC correlates with up-regulation of E-cadherin gene [1]. In the present work similar correlation between the efficiency of invasion and expression of E-cadherin and β-catenin gene was revealed upon treatment of the cells with DHLA but not with LA. Moreover similarity between the effects produced by DHLA and NAC were confirmed by Western blot analysis with antibodies against E-cadherin. Comparison of these results suggests that it is sufhydryl group of DHLA and NAC that promotes efficient modification of cell properties assisting bacterial uptake. Both E-cadherin and β-catenin are known to participate in the initial contact of bacteria with eukaryotic cells [22]. We assume therefore that the NAC- and DHLA-induced stimulation of the E-cadherin-catenin pathway contributes to the increased internalization of the grimelysin producing bacteria within transformed cells. More studies are needed to reveal whether these sulfhydryl reagents directly activate cell surface receptors involved in bacterial recognition or they specifically modulate signal transduction regulating the E-cadherin-β-catenin pathway.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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