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Inhibitory action of 10-HDA on the growth of *Escherichia coli*: Role of cell membrane integrity

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ABSTRACT

10-HDA (10-hydroxy-2-decenoic acid), an unsaturated medium-chain fatty acid in royal jelly, has been shown to possess biological activity in many fields, including the prevention of cardiovascular disease, cancer, bacteria and radiation. Inthis study, the antibacterial activity of 10-HDA against*E.coli* was evaluated. Meanwhile, the acting mechanism of 10-HDA on *E. coli* was investigated by analyzing the growth, permeability, and morphology of the bacterial cells following treatment with 10-HDA. The experimental results indicated 0.25 mg/ml 10-HDA could completely inhibit the growth of *E.coli*which dissolves in liquid Luria-Bertani (LB) medium. Meanwhile, 10-HDA resulted in the leakage of alkaline phosphatase, changedionic conductivity, and induced the respiratory chain dehydrogenases into inactive state, When *E. coli*were exposed to 1.0 mg/ml 10-HDA, the surface of the cells were roughand shrank obviouslyobserved by atomic force microscope (AFM), indicating the bacterial membranous structure was damaged severely. In conclusion, the combined results suggested that 10-HDA may damage the structure of bacterial cell membrane, which cause E.coli bacteria to die eventually.

KEYWORDS

10-HDA;
Escherichia coli;
Antibacterial mechanism;
Permeability;
Cell membrane.

INTRODUCTION

10-hydroxy-2-decenoic acid (10-HDA), a major fatty acid component of royal jellyincluding amounts of bioactive substances, has been shown to have antitumor[1-3], antioxidative action[4], collagen production promoting[5], MMP-inhibitory[6], antimicrobial[7] activities.

The presence of antimicrobial activity in RJ secreted from the pharyngeal glands of the honeybee has been documented for more than 60 years[8,9]. The inhibitory activity of RJ against both gram-positive and gram-negative bacteriahas been demonstrated. Antibacterial activity of ether-soluble fraction of RJ was rather stronger than that of raw RJ (containing 10-HDA). It was point out that the main component of ether-soluble fraction was 10-HDA occupying 38% of total acids[8]. The antibacterial activity of 10-HDA against Bacillus subtilis, Staphylococcus aureus, *E. coli* was stronger than that of the other C10 fatty acids. Extracts of royal jelly as well as its isolated compound were studied for their antimicrobial activity against *Escherichia coli*. The results of these tests showed the antimicrobial activity of 10-HDA against above strains[7].

The studies of 10-HDA against *E. coli*have been

reported, however, none of the investigation was related to the antibacterial mechanism. In this paper, to understand the acting mechanism of 10-HDA deeply, we studied the mechanism of inhibition to *E. coli* by 10-HDA in the cellular levels. For the first time, we offer evidences to indicate that 10-HDA can inhibit bacterial growth and even kill the cells through destroying bacterial membranous structure and permeability.

MATERIALS AND METHODS

Materials

10-HDA was purchased from Shanghai Gongshuo Biotechnology Company limited (Shanghai, China). The 10-HDA was dissolved in ethanol, and kept frozen at -20°C. The strains of *Escherichia coli* (CICC23657) conserved in our laboratory. LB medium contained the following: 5.0 g yeast extract, 10 gtryptone, and 10 g sodium chloride. The solid medium was supplemented with 2% agar in LB medium.

Assay for minimum inhibitory concentration of 10-HDA

The minimum inhibitory concentration (MIC) of 10-HDA was determined for*E.coli* by tube dilution method. Different volume of LB medium10-HDA solution, and strain suspension were added separately to tubes, resulting in final concentration of 10-HDA of 0, 0.062,0.125, 0.25, 0.5, and 1.0 mg/ml, respectively, and concentration of 10^7cfu/ml strains cells. The cultures were incubated at37±0.5 °C and shaken at 150 rpm for 18h. Then, 200 µl bacterial suspensions were coated on the solid-medium, and incubated at 37±0.5 °C for 12h, the number of colonies were calculated. The MIC was read as the lowest antimicrobial concentration exhibiting no growth. The experiments were repeated in triplicate on one occasion.

Effect of 10-HDA on the growth of *E. coli*

Growth curves of *E. coli* exposed to 10-HDA were determined based on the absorbing value of OD_{600}. *E. coli*were grown in LB medium and incubated overnight at 37 °C. Then, 200 µl *E. coli* bacterial suspension in a logarithmic growth phasewas prepared for an antibacterial test. 10-HDA solution was added to the cultures with the final concentration of 10-HDA for 4×MIC of *E. coli*. The cultures were incubated at 37 °C for 24h. The ethanol solution has no 10-HDA was used as control. The final concentration of ethanol added to the liquid medium was less than 3% (v/v). The antibacterial activity was estimated periodically by measuring the turbidity of the culture medium at 600nm with UV-vis spectrophotometer (Rayleigh, UV-9200, Beijing, China). Each batch experiment was carried out in triplicate, and the results were reported as an average of three replicates.

Examination of cell morphology by atomic force microscopy(AFM)

The *E. coli*were cultivated in sterilized LB medium at 37！ with shaking at 150 rpm. Then the cells were harvested in the logarithmic phase at a concentration equivalent to an optical density at 600 nm (OD_{600} nm) value of 0.1. These bacterial cells were then used for further experiments immediately. Different volume of LB medium, 10-HDA solutions, and E.coli cells were added to 10ml cultures resulting in final concentration is 1.0 mg/ml of 10-HDA (4×MIC) and 10^7cfu/ml of *E. coli*. Control experiment was conducted in absence of 10-HDA. The cultures were incubated at 37±0.5 °C with shaking at 150rpm for 12h. The cultures were centrifuged and the precipitumwere used for observation by atomic force microscopy (Asylum Research, America).

Effect of 10-HDA on the leakage of alkaline phosphatase of *E. coli*

To determine the effect of 10-HDA on the integrity of the outer membrane of *E. coli*, the leakage of alkaline phosphatase (AKP) in the suspension liquid after 10-HDA treament were detected. Aftercentrifugating the *E. coli* suspensionsof the late logarithmic phase, 0.85% saline washing, different amounts of fresh sterile LB medium,10-HDA and cells were added into 10ml sterile test tube. To make the final concentration of 10-HAD is 1.0 mg/ml and final concentration of *E.coli* is 10^8cfu/ml. Control experiments were conducted without 10-HDA. The cultures were incubated at 37± 0.5 °C with shaking at 150 rpm for different time. Then the cultures were centrifuged at 8000 rpm for 5 min, and the supernatants were used to detect alkaline phosphatase. The AKP levels were analysed by disodium

phenyl orthophosphate method.

Effect of 10-HDA on the electrical conductivity of *E. coli* medium

E. colisuspensions in the middle of logarithmic phase werecentrifugated. *E. coli* cells were washed twice with 0.5% sterile glucose isotonic solution, and were suspended by isotonic solution. 10-HDA solution of 4×MIC was added into the suspensions. Meanwhile, the blank control and the negative control were set up. The cultures were incubated at 37±0.5 °C with shaking at 150rpm. At different time, 5 ml of these cultures were centrifuged at 8000rpm for 5min, and the supernatants were used to detect the electrical conductivity by conductivity meter.

Effect of 10-HDA on enzymatic activity of respiratory chain dehydrogenases in *E. coli*

The dehydrogenase activity was determined according to triphenyltetrazolium chloride (TTC) method. Under physiological conditions, colorless TTC is reduced by the bacterial respiratory chain dehydrogenase to a red formazan; thus, the dehydrogenase activity can be determined by the change of the spectrophotometric value of TF. In this text, 1ml *E. coli* suspensionsof the late logarithmic phase with 2ml Tris-HCl buffer (0.05mol/L pH 8.6), 2ml glucose solution (0.1mol/L), 2ml triphenyltetrazolium chloride (TTC,1mg/ml) solution were added into sterile test tube. 10-HDA solutions of different concentration were mixed with above. Experiments were conducted in absence of 10-HDA as the control. Cultivations were performed at 37±0.5！for 5 h, and added 0.1 ml H2SO4 in each tube to stop the reaction. Toluene was used to extract the product from each tube. The dehydrogenase activity was then calculated according to the maximum absorbance of triphenylformazan (TF) at 490 nm by UV spectrophotometer.

RESULTS

Assay for minimum inhibitory concentration of 10-HDA

We used tube dilution method to detect the minimum inhibitory concentration (MIC) of 10-HDA for E.coli.As shown in TABLE 1, when the concentration of 10-HDA was 0.25 mg/ml, no growth of *E. coli* could be detected, indicating the minimum inhibitory concentration (MIC) of 10-HDA to *E. coli* was 0.25 mg/ml.

TABLE 1 : The minimum inhibitory concentration of 10-HDA(MIC)

strains	10-HDA concentration {mg.ml^{-1}}					
	1.0	0.5	0.25	0.125	0.062	0
E. coli CICC23657	/	/	/	+	++	+++

Notes: "/" showed no colony growth; "+" small amount of colonies growth; "++" a large number of colonies growth; "+++" showed that colonies densely covered the plate

Growth curve of *E. coli* exposed to 10-HDA

The growth curve of *E. coli* treated with 10-HDA was shown in Figure 1 by measuring optical density at 600 nm. The growth curve of *E. coli* included three phases: lag phase, exponential phase, and stabilization phase. However, decline phases in each growth curve could not be revealed because we only assayed the total numbers of bacteria, including live and dead ones, based on the value of OD_{600}. Under absence of 10-HDA, *E. coli* reached exponential phase rapidly. But exposed to 1.0 mg/ml of 10-HDA, *E. coli* cells growth were lagged to 15h, which prolonged the lag phase of *E. coli*, and the concentration of *E. coli*was lower than blank control and ethanol control in the stabilization phase. This result indicated that 10-HDA could effectively inhibit the proliferation and growth of *E. coli*.

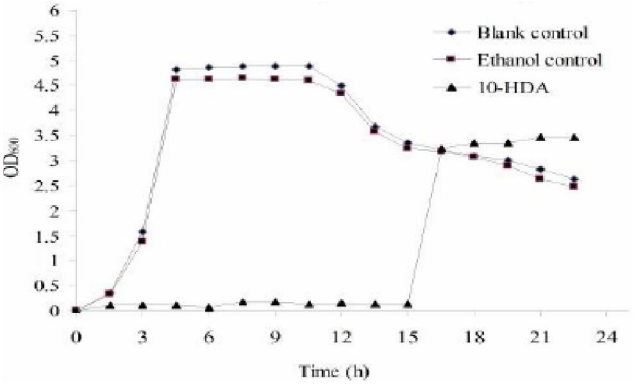

Figure 1 : Growth curve of *E. coli* exposed to 10-HDA

Effect of 10-HDA on the cell morphology of *E. coli*

The micrographs of *E. coli* cells treated and untreated with 10-HDA by AFM were displayed in Fig. 2. Micrograph by AFM showed the surface of normal *E. coli* cells and that with ethanol treated control were smooth and showed typical characters of rod shape,

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and its cell walls were compact and intact (Figure 2A, a and B, b). After treated with 1.0 mg/ml 10-HDA for 5 h, *E. coli* cells membrane components became disorganized and scattered from their original ordered, and the whole cells turned thin and shriveled, suggesting cells were damaged severely (Figure 2C, c). It is postulated that the debris originates from the periplasm. The above result indicates that, after acting with 10-HDA, the cell membrane or cell wall of bacteria was destroyed, permeability of the cell was increased and the inner contents were much leaked, thus leading to death of bacteria.

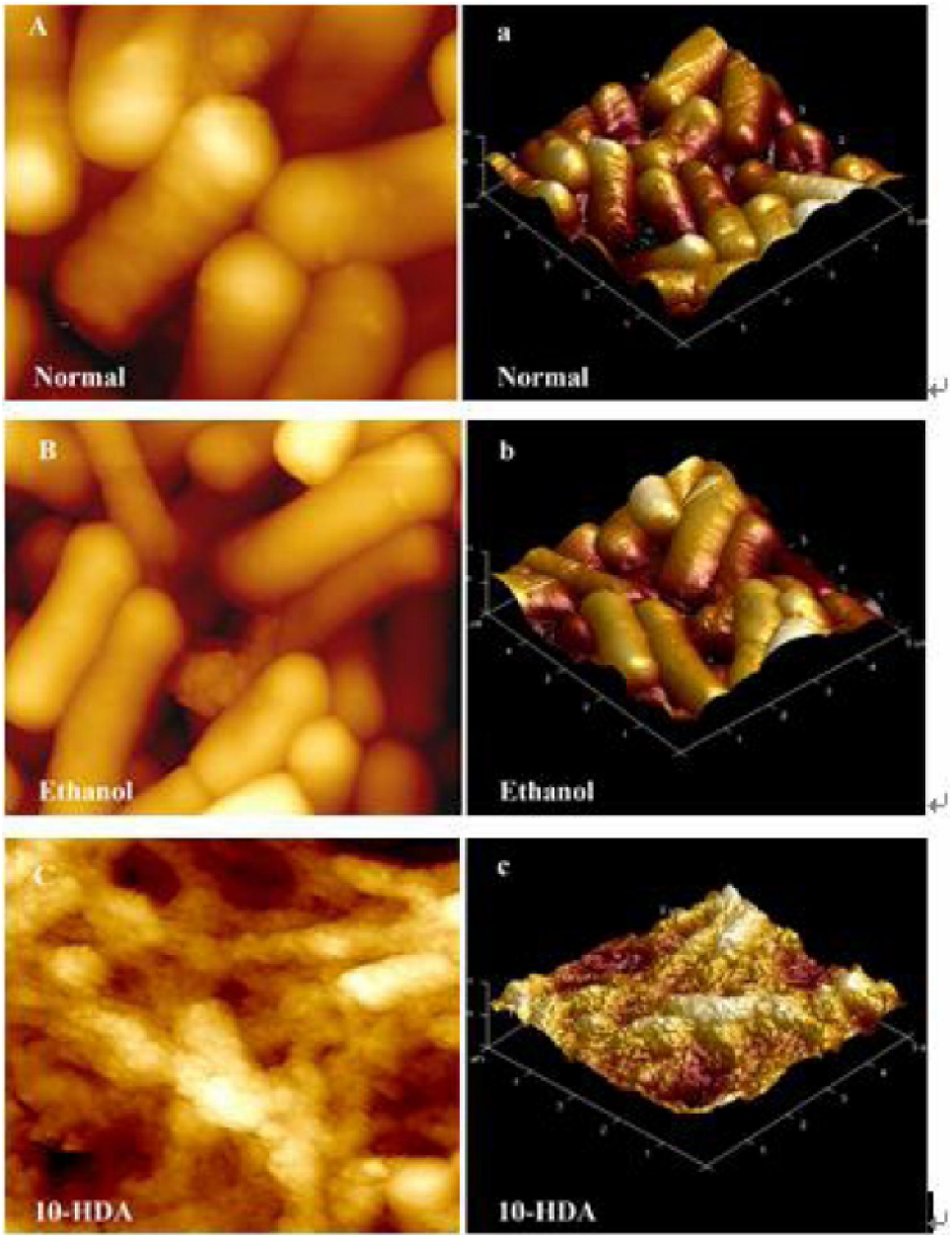

Figure 2 : Effect of 10-HDA on *E. coli* cells observed by atomic force microscopy(AFM). *E. coli* cells were treatedwith 1mg/ml 10-HDA for 5 h (C). A, B were normal *E. coli* cells and the cells treated with ethanol as control, respectively. a, b, c were three-dimension graphcorresponding to A, B, C, respectively (scanning area 5μm×5μm)

Effect of 10-HDA on the leakage of alkaline phosphatase (AKP) of *E. coli*

Alkaline phosphatase (AKP) exists between the outer membrane and inner membrane of bacteria, and the integrity of cell wall could be identified by detecting the leakage of AKP in the bacterial suspension liquid. Results showed that in starting time, almost no AKP

could be detected to leak from bacterial cells by 10-HDA. While the leakage amount of AKP from cells began to increase after treatment with 10-HDA for 180 min, and it was increased with the passage of time (Figure 3), suggesting that 10-HDA could destroy bacteria cell wall and enhance the membrane leakage of AKP.

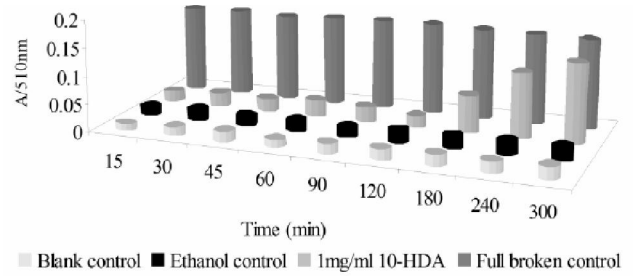

Figure 3 : Action of 10-HDA on the leakage of alkaline phosphatase of *E. coli*

Effect of 10-HDA on the electrical conductivity of *E. coli* medium

To determine the effect of 10-HDA on the permeability of bacterial cell membrane, we detected the electrical conductivity of *E. coli* medium. It was showed that the electrical conductivity was significantly increased after *E. coli* were treated with 10-HDA (1.0 mg/ml) for 15 min compared to the blank control and ethanol control, and there was no obviously changed with the passage of time (Figure 4). The results suggested that 10-HDA could enhance the permeability of bacterial cell membrane, which resulted in the leakage of the ions and small molecules to induce the cells death.

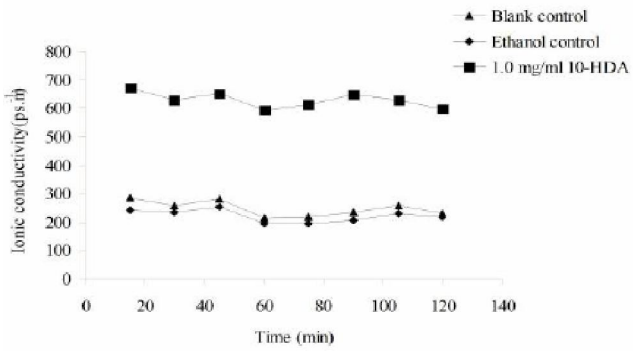

Figure 4 : Effect of 10-HDA on the electrical conductivity of *E. coli*

Effect of 10-HDA on respiration chain dehydrogenases of *E. coli*

The effect of 10-HDA on respiration chain dehydrogenase of *E. coli* was shown in Figure 5. Activity of respiratory chain dehydrogenases in normal *E. coli* cells and that with ethanol treated control was high (A/490nm >1.0) after incubation for 5 h. While the cells treated with 0.25mg/ml 10-HDA, the enzymatic activity reduced obviously compared to the control (A/490nm = 0.633). And the enzymatic activity of cells treated with 0.5mg/ml 10-HDA was even lower than that of cells treated with 0.25mg/ml 10-HDA. After being treated with 1.0 mg/ml 10-HDA, the enzymatic activity almost fell to the bottom, maintaining feeble activity during treatment for 5 h (A/490nm = 0.022). The results indicated that the activity of respiratory chain dehydrogenases of E.coli could be inhibited by 10-HDA, and the higher concentration of 10-HDA, the lower the activity of enzymes.

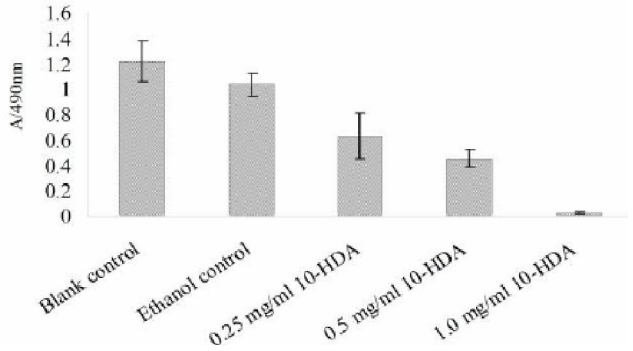

Figure 5 : Action of 10-HDA on respiration chain dehydrogenases of *E. coli*.Data are average from duplicate experiments. Error bars represent standard deviations of duplicate incubations

DISCUSSION

The growth curve of *E. coli* exposed to 10-HDA indicated that 10-HDA could inhibit the growth and reproduction of *E. coli*. A minor amount of 10-HDA could prolong the lag phase of *E. coli*. Until the concentration of 10-HDA was up to 0.25mg/ml, *E. coli* cells of 10^7cfu/ml were completely inhibited, and then the MIC of 10-HDA was 0.25 mg /ml in this condition.

To understand the antibacterial mechanism of 10-HDA, we selected *E. coli* as model to study the effect of 10-HDA on the permeability and the membrane structure of *E. coli* cells. It is well known that *E. coli*possess an outer membrane outside the peptidoglycan layer which is to serve as a selective permeability barrier, protecting bacteria from harmful agents. The lipid bilayer of outer membrane is asymmetric: the inner leaflet mostly con-

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tains close-packed phospholipid chains, while the outer leaflet is composed of the lipopolysaccharide (LPS) molecules. Evidences from genetic and chemical experiments have proved that the LPS layer of the outer membrane plays an essential role in providing a selective permeability barrier for *E. coli*. And LPS structures could increase permeability compared with that of native cells (Amro et al.2000). Our experimental results showed that 10-HDA apparently enhanced the permeability of membrane resulted in amounts of the ions and small molecule leakage in cells. So it could be conferred that turbulence of membranous permeability would be an important factor to inhibit the bacterial growth.

Additionally, our results of experiments showed that the activity of respiratory chain dehydrogenases in *E. coli* might be inhibited by 10-HDA; the higher concentration of 10-HDA, the lower the activity of enzymes. It is assumed that 10-HDA may break through barrier of outer membrane permeability, peptidoglycan and periplasm, and destroy respiratory chain dehydrogenases, furthermore inhibiting respiration of cells.

From the AFM, we found *E. coli*treated with 10-HDA were dissolved and dispersed, and their membrane components became disorganized and scattered from their original ordered and close arrangement. Cell membrane was disaggregated and lost their intrinsic function. These phenomena suggest possible antibacterial mechanisms by which 10-HDA inhibit bacterial growth, as well as cellular responses to the 10-HDA treatment.

Based on the present research, the action model of 10-HDA may be described as 10-HDA making a break through the permeability of outer membrane firstly, resulting in the leakage of cellular materials. Secondly, 10-HDA enters the inner membrane and inactivates respiratory chain dehydrogenases, thus inhibiting respiration and growth of cells. Simultaneously, 10-HDA may affect some proteins and phosphate lipids and induce collapse of membrane, resulting in cell decomposition and death eventually. Taking into account the mobility of 10-HDA into cells and their fate in a bioprocess or even in the environment, the risk aspects for the application in larger scales and in the environment should be strengthened in future study.

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