Korean J Physiol Pharmacol
Vol 7: 125–130, June, 2003

Effects of Chlorhexidine Digluconate on Rotational Rate of *n*-(9-Anthroyloxy)stearic Acid in *Porphyromonas ginginvalis* Outer Membranes

Hye-Ock Jang, Seong-Kweon Cha, Chang Lee, Min-Gak Choi, Sung-Ryul Huh, Sang-Hun Shin[1], In-Kyo Chung[1], and Il Yun

Department of Dental Pharmacology and Biophysics and [1]Oral and Maxillofacial Surgery and Clinical Pharmacology, College of Dentistry and Research Institute for Oral Biotechnology, Pusan National University, Busan 602–739, Korea

The aim of this study was to provide a basis for studying the molecular mechanism of pharmacological action of chlorhexidine digluconate. Fluorescence polarization of *n*-(9-anthroyloxy)stearic acid was used to examine the effect of chlorhexidine digluconate on differential rotational mobility of different positions of the number of membrane bilayer phospholipid carbon atoms. The six membrane components differed with respect to 2, 3, 6, 9, 12, and 16-(9-anthroyloxy)stearic acid (2-AS, 3-AS, 6-AS, 9-AS, 12-AS and 16-AP) probes, indicating different membrane fluidity. Chlorhexidine digluconate increased the rate of rotational mobility of hydrocarbon interior of the cultured *Porphyromonas gingivalis* outer membranes (OPG) in a dose-dependent manner, but decreased the mobility of surface region (membrane interface) of the OPG. Disordering or ordering effects of chlorhexidine digluconate on membrane lipids may be responsible for some, but not all of its bacteriostatic and bactericidal actions.

Key Words: Bacterial outer membranes, Chlorhexidine digulconate, Fluorescence probe technique, Membrane hydrocarbon interior, Membrane surface, Rotational rate, *n*-(9-Anthroyloxy) stearic acid

INTRODUCTION

It is known that chlorhexidine is antiplaque (Löe & Schiott, 1970; Newbrun, 1989), antigingivitis (Löe & Schiott, 1970; Newbrun, 1989), and fungicidal (Newbrun, 1989), and also effective in both the prevention and treatment of oral candidiasis (Ferretti et al, 1987a,b). It is bacteriostatic in low concentrations to many gram-positive and gram-negative bacteria, whereas much higher concentrations are bactericidal (Russell, 1986).

It is known that chlorhexidine is a potent membrane-active agent against both gram-positive and gram-negative bacteria (Hugo, 1978), causes membrane damage to neutrophils (Kenney et al, 1972; Knuutilla & Södering, 1981), produces deleterious membrane perturbations in blood cells (Gabler et al, 1987) and has a decreasing effect on the epithelial-cell-lipid packing order (Audus et al, 1992). Also, Tsuchiya (1999) reported that the antiplaque effect of chlorhexidine is due to the reduction in membrane fluidity of both hydrophilic and hydrophobic regions. However, such results were not obtained when the membranes of gingivitis causative microorganisms were used and, specifically, the results of Tsuchiya (1999) were obtained through the measurements that used phospholipid liposomes as samples.

The difficulty with the membrane fluidity concept is that any physical parameter chosen will be a property of the spectroscopic method employed, specifically its particular time window (from $\sim 10^{-5}$ sec for NMR to $\sim 10^{-9}$ sec for fluorescence and ESR) and the properties of the probe (shape, charge, location etc) (Stubbs & Rubin, 1993). It is highly likely that bulk or average spectroscopic properties of cell membranes may not be useful in building a hypothesis for the mechanism of pharmacological action of chlorhexidine on bacteriostatic or bactericidal effects. However, little attention has been given to the effects of chlorhexidine on the fluidity of special domain of anaerobic bacterial membranes. Furthermore, no studies have been carried out on the effects of chlorhexidine on the fluidity of bulk or special domain of membranes of *Porphyromonas gingivalis* (PG) of ginginvitis causative microorganisms.

Earlier studies have shown that the fluorophores of anthroyloxy derivatives located at a graded series of levels from the surface to the center of the lipid bilayer structure (or a series of anthroyloxy fatty acids indicates that the depth of the group is almost linearly related to the number of carbon atoms between it and the carboxyl group (Tilley et al, 1979; Villala n & Prieto, 1991; Abrams et al, 1992;

Corresponding to: Il Yun, Department of Dental Pharmacology and Biophysics, College of Dentistry and Research Institute for Oral Biotechnology, Pusan National University, Busan 602-739, Korea. (Tel) 051-240-7813, (Fax) 051-254-0576, (E-mail) iyun@pusan.ac.kr

ABBREVIATIONS: 2-AS, 3-AS, 6-AS, 9-AS, 12-AS and 16-AP, 2, 3, 6, 9, 12, and 16-(9-Anthroyloxy)stearic acid; OPG, *Porphyromonas gingivalis* outer membranes; PG, *Porphyromonas gingivalis*; BSA, bovine serum albumin.

Abrams & London, 1993; Mason, 1994). The anthroyloxy stearate (AS) probes can be used to differentiate whether the bilayer has a fluidity gradient across it, as the anthroyloxy group can be positioned at different positions of the stearic acid moiety (Thulborn et al, 1979; Molitoris & Hoilien, 1987; Villalaín & Prieto, 1991; Abrams & London, 1993). These probes have been suggested to be primarily the dynamic component of membrane fluidity (Vincent et al, 1982; Schachter, 1984; Molitoris & Hoilien, 1987).

The aim of this study was to provide a basis for studying the molecular mechanism of pharmacological action of chlorhexidine, through investigation of the effect of chlorhexidine digluconate on differential rotational rate among the number of carbon atoms in the *Porphyromonas gingivalis* (PG) outer membrane phospholipids which differ in fluidity. Employing fluorescence polarization of 16-(9-anthroyloxy)palmitic acid (16-AP), 12-(9-anthroyloxy) stearic acid (12-AS), 9-(9-anthroyloxy)stearic acid (9-AS), 6-(9-anthroyloxy)stearic acid (6-AS), 3-(9-anthroyloxy)stearic acid (3-AS) and 2-(9-anthroyloxy)stearic acid (2-AS), we investigated the effect of chlorhexidine digluconate on rotational mobility of outer membranes (OPG) of cultured PG.

METHODS

Materials

The fluorescent probes, 16-AP, 12-AS, 9-AS, 6-AS, 3-AS and 2-AS, were purchased from Molecular Probes, Inc. (Junction City, OR, USA). Chlorhexidine digluconate, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were purchased commercially and were of the highest quality available. Water was deionized.

Bacterial growth conditions

ATCC 33277 (PG) was obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured as described previously (Lamont et al, 1995). PG was maintained as frozen stock cultures and grown anaerobically (Bactron IV Anaerobic Chamber, Sheldon Manufacturing Inc., OR, USA) in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 1% (wt/vol) yeast extract, 0.0005% (wt/vol) hemin, and 0.0001% (wt/vol) menadione at 37°C for 3 days in an atmosphere of 80% N_2, 10% CO_2, and 10% H_2.

Preparation of outer membranes

The procedure was essentially as described by Smalley et al. (1993). Briefly, freeze-dried whole cells (200 mg) were suspended in 60 ml of 0.14 M NaCl containing 10 mg of EDTA (pH 7.3) and the suspension was incubated with stirring for 30 min at 37°C (Smalley & Birss, 1993). This serves to dissociate OPG and to inhibit any possible cell associated protease activity (Tsutsui et al, 1987) during preparation. After passing twice through a 25 gauge needle, the cells were pelleted by centrifugation (20,000×g for 30 min, 4°C) leaving the supernatant which was crude OPG preparation. Residual EDTA and buffer salts were removed by dialysis against distilled water for 4 h at 4°C, and OPG fraction was then freeze-dried. The protein concentration was determined by the method of Lowry et al. (1951), using BSA as a standard.

Fluorescence measurements

The fluorescence measurements were taken using a modified method of earlier studies (Mollitoris et al, 1985; Mollitoris & Hoilien, 1987). The OPG was suspended in phosphate buffered saline (PBS: 8 g/l NaCl, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 1.15 g/l $Na_2HPO_4 \cdot 7H_2O$, 0.48 g/l Hepes, pH 7.4) to 50 μg protein/ml concentration. Stock solutions of the probes in methanol (2×10^{-5} M) were prepared and kept in a cold dark place. Aliquots were added to the suspensions of the outer membranes, so that the final concentrations of the probes were 4×10^{-8} M probes incorporated. The mixture was stirred for 20 min at room temperature in order to reduce the concentration of methanol that might alter the rotational rate of the outer monolayer of the outer membranes. Also, the mixtures were bubbled with dry nitrogen for 1 min with 20 min intervals in order to eliminate oxygen that might act as a quencher. Concentrated solutions of chlorhexidine digluconate were prepared in PBS and added to the labeled membrane suspension to give the desired concentration of the drug. The pH of the buffered sample was not changed significantly by addition of the drug.

Fluorescence measurements were carried out with a Multi Frequency Cross-Correlation Phase and Modulation Fluorometer (ISS K2-003), equipped with a thermostated cell holder and performed at pH 7.4 (37±0.1°C). The fluorescent probes were excited at 360 nm (4 nm slit width) and emissions were recorded at 445 nm (8 nm slit width) through a sharp cut-off filter (Schott KV418). Corrections for light scattering (membrane suspensions without fluorescent probes) and for fluorescence in the ambient medium (quantified by pelleting the membranes after each estimation) were routinely made, and the combined corrections were less than 9% of the total fluorescence intensity observed for anthroyloxystearate-loaded suspensions. Blanks (membrane suspensions without fluorescent probes), prepared under identical conditions, served as controls for the fluorometric measurements. The intensity of the components of the fluorescence which were parallel ($I_{//}$) and perpendicular ($I_{\perp}$) to the direction of the vertically polarized excitation light was determined by measuring the emitted light through polarizers oriented vertically and horizontally. Polarization (P) was obtained from intensity measurements using $P = (I_{//} - GI_{\perp})/(I_{//} + GI_{\perp})$, where G is a grating correction factor for the monochromator's transmission efficiency for vertically and horizontally polarized light. This value is given by the ratio of the fluorescence intensities of the vertical to horizontal components, when the exciting light is polarized in the horizontal direction. The polarization was expressed as anisotropy [$\gamma = 2P/(3-P)$] of the probes.

Data analysis

Each value was expressed as mean±SEM (n=5 for all experiments). Differences in anisotropy (r) were statistically analyzed by the Student's t-test.

Table 1. Fluorescence parameter of 16-(9-anthroyloxy)palmitic acid (16-AP), 12-(9-anthroyloxy)stearic acid (12-AS), 9-(9-anthroyloxy)stearic acid (9-AS), 6-(9-anthroyloxy)stearic acid (6-AS), 3-(9-anthroyloxy)stearic acid (3-AS) and 2-(9-anthroyloxy)stearic acid (2-AS) in cultured *Porphyromonas gingivalis* outer membranes (OPG)

Membrane	Parameter	16-AP	12-AS	9-AS	6-AS	3-AS	2-AS
OPG	anisotropy	0.104±0.002	0.110±0.003	0.125±0.003	0.133±0.003	0.142±0.002	0.145±0.004

Fluorescence measurements were performed at 37°C (pH 7.4). Values are represented as mean±SEM of 5 determinations.

RESULTS

In the present study, using the fluorescence probe technique, we examined differential effects of dicationic chlorhexidine digluconate on the rotational mobility among the number of carbon atoms in the OPG phospholipids which differ in fluidity. In order to determine the effects of the chlorhexidine digluconate on the aforementioned rotational mobility rate, it is first necessary to demonstrate that the drug does not interact directly with fluorescent probes, thereby quenching its fluorescence. Quenching of absorbance-corrected fluorescence intensity of 16-AP, 12-AS, 9-AS, 6-AS, 3-AS and 2-AS in OPG by the chlorhexidine digluconate was not observed at all the concentration levels at which the drug was tested. Hence, the possibility of direct quenching of fluorescence of the probes by the drug was ruled out.

The anisotropy (r) values of 16-AP, 12-AS, 9-AS, 6-AS, 3-AS and 2-AS in the outer monolayer of intact OPG at 37°C (pH 7.4) are shown in Table 1. Table 1 shows that the rate of rotational mobility of hydrocarbon interior is faster than that of the surface region. The rotational mobility's degrees of the number of phospholipid carbon atoms differed, depending on the carbon numbers, in the descending order of the 16-AP, 12-AS, 9-AS, 6-AS, 3-AS and 2-AS.

The chlorhexidine digluconate may induce disordering or ordering in their host lipids, including lipopolysaccharide. Ordering takes place in the head group or lipopolysaccharide region (surface region), whereas disordering occurs deep within the acyl chains. If the degree of disordering or ordering effects on the membranes' outer monolayer by chlorhexidine digluconate is expressed in percentage, the magnitude of ordering effects induced by chlorhexidine digluconate on the membrane surface region (in the case of 2-AS) is smaller than that of disordering effects (in the cases of 16-AP, 12-AS, 9-AS and 6-AS) induced by the drug on the membrane hydrocarbon interior. However, we found that, when the altered anisotropy (r) values were converted to temperature, the magnitude of ordering effects induced by chlorhexidine digluconate on the membrane surface region was greater than that of disordering effects induced by the drug on membrane hydrocarbon interior (As to the method to convert the anisotropy (r) values to temperature, refer to the following section).

Disordering effects of chlorhexidine digluconate on rotational rate in the monolayer hydrocarbon interior

It is found that the disordering effects of chlorhexidine digluconate on the hydrocarbon interior of bacterial outer membrane increases roughly in proportion with its depth of site of action (the more effective penetration into hydrocarbon interior could result in higher disordering effects on the outer membrane). The effects of increasing concentrations of chlorhexidine digluconate on the anisotropy (r) of the probes in hydrocarbon interior of the monolayer of OPG are shown in Fig. 1. The chlorhexidine digluconate decreased the anisotropy (r) of the probes (increased rate of rotational mobility) in the monolayer of the OPG in a concentration-dependent manner. The significant decreases in the anisotropy (r) values of 16-AP and 12-AS by the drug were observed at all the concentration levels (0.01, 0.10, 1.00, 2.00, 3.00, 4.00 and 5.00 mM) experimented in this study except at the 0.001 mM level (Fig. 1).

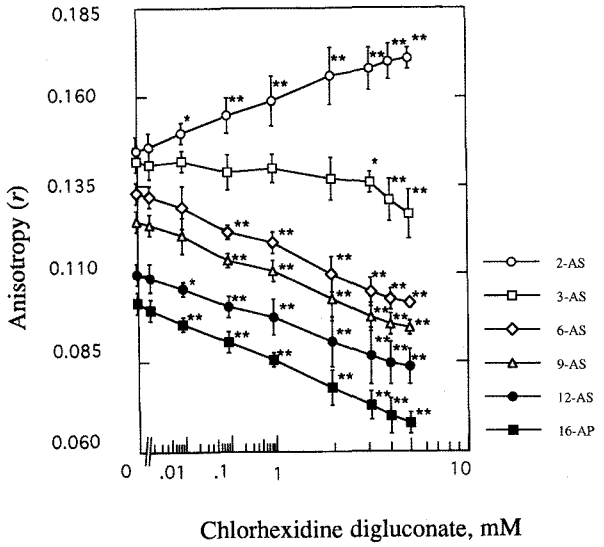

Fig. 1. Effects of chlorhexidine digluconate on the anisotropy (r) of 16-(9-anthroyloxy)palmitic acid (16-AP), 12-(9-anthroyloxy) stearic acid (12-AS), 9-(9-anthroyloxy)stearic acid (9-AS), 6-(9-anthroyloxy)stearic acid (6-AS), 3-(9-anthroyloxy)stearic acid (3-AS) and 2-(9-anthroyloxy)stearic acid (2-AS) in cultured *Porphyromonas gingivalis* outer membranes (OPG). Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents mean± SEM of 5 determinations. An asterisk and double asterisk signify $P<0.05$ and $P<0.01$, respectively, compared to control according to Student's t-test.

The difference in the anisotropy (r) value of 12-AS found in hydrocarbon interior of the monolayer of OPG before and after adding 0.10 mM chlorhexidine digluconate was 0.009. This can be illustrated by comparing the effects of temperature on this parameter. The anisotropy (r) of 12-AS in hydrocarbon interior of the monolayer of OPG was 0.110± 0.003 at 37°C (pH 7.4) and 0.140±0.005 at 25°C (pH 7.4). Thus, the difference in the anisotropy (r) values of 12-AS in hydrocarbon interior of the monolayer of OPG before and

after adding 0.10 mM chlorhexidine digluconate was 0.009, which were the same difference value as produced by raise of approximate 3.6°C temperature.

The difference in the anisotropy (*r*) value of 12-AS in hydrocarbon interior of the monolayer of OPG before and after adding 3.00 mM chlorhexidine digluconate was 0.023, which were the same difference value as produced by raise of approximate 9.2°C temperature.

Ordering effects of chlorhexidine digluconate on rotational rate of the monolayer surface region

The effects of increasing concentrations of chlorhexidine digluconate on the anisotropy (*r*) of 2-AS in surface region of the monolayer of OPG are shown in Fig. 1. Chlorhexidine digluconate increased the anisotropy (*r*) of 2-AS (decreased rate of rotational mobility) in a concentration-dependent manner. The significant increases in the anisotropy (*r*) values by the drug were observed at all the concentration levels (0.01, 0.10, 1.00, 2.00, 3.00, 4.00 and 5.00 mM) experimented in this study, except at the 0.001 mM level (Fig. 1).

The difference in the anisotropy (*r*) values of 2-AS found in surface region of the monolayer of OPG before and after adding 0.10 mM chlorhexidine digluconate was 0.010. This can be illustrated by comparing the effects of temperature on this parameter. The anisotropy (*r*) of 2-AS in surface region of the monolayer of OPG was 0.145 ± 0.004 at 37°C (pH 7.4), and 0.172 ± 0.006 at 25°C (pH 7.4). Thus, the difference in the anisotropy (*r*) value of 2-AS in surface region of the monolayer of OPG before and after adding 0.10 mM chlorhexidine digluconate was 0.010, which was the same difference value as produced by the fall of approximate 4.4°C temperature.

The difference in the anisotropy (*r*) value of 2-AS found in surface region of the monolayer of OPG before and after adding 3.00 mM chlorhexidine digluconate was 0.023. Variation in the anisotropy (*r*) value was also noticed by the change in temperature as mentioned earlier. The difference in the anisotropy (*r*) values of 2-AS in surface region of the monolayer of OPG before and after adding 3.00 mM chlorhexidine digluconate was 0.023, which was the same difference value as produced by the fall of approximate 10.2°C temperature.

Tsuchiya (1999) reported that chlorhexidine increased fluorescence polarization of both 1-anilinonaphthalene-8-sulfonic acid (ANS) and *N*-phenyl-1-naphthylamine (PNA) in both liposomes of dipalmitoylphosphatidylcholine (DPPC) and dioleoylphosphatidylcholine (DOPC). Although the polarization of PNA in the DPPC liposomes was exceptionally decreased, chlorhexidine at $1 \sim 100\,\mu M/l$ increased the polarization of ANS and PNA in the 50 mol% DPPC and 50 mol% DOPC liposomes. The results of Tsuchiya (1999) are not consistent with our present results. However, Tsuchiya's results are concordant with the results of the study about the effects of chlorhexidine on fluidity of the DPPC hydrocarbon interior, employing the florescent prove PNA. These differences cannot be fully explained.

DISCUSSION

The precise location of molecular mechanism of pharmacological action of chlorhexidine has been a continuously controversial subject. The current consensus is that chlorhexidine has a site(s) of action, located within the cell membrane, presumably on the inner and outer membranes. Therfore, in order to provide important groundwork for the research on molecular mechanism of pharmacological action of chlorhexidine, comprehensive studies encompassing all of the following essential questions should be carried out;

Does chlorhexidine act on the bacterial membrane proteins or on lipids? Does chlorhexidine act on both proteins and lipids, or on the lipid-protein interface? If it acts on proteins, does it act specifically on certain protein or non-specifically on various proteins? What kinds of actions does chlorhexidine impart on proteins?

If chlorhexidine affects the fluidity of lipids, where does it show its predominant effects between inner and outer membranes? If chlorhexidine acts on either of the inner membrane and outer membrane of bacteria, where does it exert its predominant effects between the inner and outer monolayers of the membrane? If chlorhexidine acts on a specific monolayer of the membrane, where does it exert its predominant effects between the hydrophillic and hydrophobic regions, and what kind of effects does it display?

If chlorhexidine affects non-specifically on both proteins and lipids, what kind of protein-lipid interactions does it display? Also, if chlorhexidine acts on the protein-lipid interface, what kind of actions does it have on the interface and what kind of effects does it display?

The present research attempted to elucidate only a fraction of those aforementioned essential studies, employing PG (causative germ for gingivitis and periodontitis) and outer membranes (OPG) as samples.

In regard to 2-AS distribution region, we paid special attention to the study by Villalaín & Prieto (1991) among several studies (Thulborn et al, 1979; Vincent et al, 1982; Schachter, 1984; Molitoris & Hoilien, 1987). It was reported that 2-AS is a peculiar member of the family of probes, since the chromophore is adsorbed on the membrane interface (Villalaín & Prieto, 1991). Membrane interface is difficult to precisely define, and it certainly includes the polar region near the phospholipid head groups or even the carbonyl groups which are largely involved in hydrogen bonds. 16-AP, 12-AS, 9-AS, 6-AS and 3-AS are distributed in the hydrophobic interior of the cell membrane outer monolayer (Thulborn et al, 1979; Vincent et al, 1982; Schachter, 1984; Molitoris & Hoilien, 1987). The anthroyloxy stearate (AS) probes can be used to differentiate whether the bilayer has a fluidity gradient across it, as the anthroyloxy group can be positioned at different positions of the stearic acid moiety (Thulborn et al, 1979; Vincent et al, 1982; Schachter, 1984; Molitoris & Hoilien, 1987; Villalaín & Prieto, 1991). These probes have been suggested to primarily prove the dynamic component of membrane fluidity (Vincent et al, 1982; Schachter, 1984; Molitoris & Hoilien, 1987). Since rotational mobility rate of membrane lipid bilayers can be measured by a rather simple method without measuring life time of a fluorescent probe, it is economical.

Our data presented herein have shown that, even at physiologically relevant concentrations (Buck et al, 2001; Spratt et al, 2001; Gerlach & White, 2001), chlorhexidine digluconate increases or decreases the rate of hydrocarbon interior and surface region of the monolayer of OPG. Using the membrane interface probe 2-AS, we found that chlorhexidine digluconate decreased the rotational mobility of lipids in the interface of OPG, whereas chlorhexidine

digluconate increased the rotational mobility of the probes (16-AP, 12-AS, 9-AS, 6-AS and 3-AS) in the hydrocarbon interior of the outer monolayer of the outer membrane. This is due to differences in the intrinsic component and/or the structure in surface and hydrocarbon region of the monolayer of bacterial outer membranes. The clear mechanism of the action(s) of the chlorhexidine digluconate as to its ordering and disordering effects on the monolayer of bacterial outer membranes is not yet known, however, the following presumption might be possible.

Chlorhexidine binds to anionic groups on the bacterial surface, probably the phosphate groups of teichoic acid in gram-positive bacteria and phosphate groups of lipopolysaccharides in gram-negative bacteria (Newbrun, 1989). The chlorhexidine digluconate binds (the competitive binding of the chlorhexidine digluconate and water) strongly to the phosphate moiety of phospholipids and lipopolysaccharides in the outer monolayer surface, and weakly to the carbonyl group in competition with water in the monolayer and effectively establish formation of hydrogen bonds with the carbonyl moiety, which is associated with a significant change in hydration of the chlorhexidine digluconate molecule itself. The incorporation of the chlorhexidine digluconate into the bacterial membranes causes alterations of the surface charge density of the membrane's outer monolayer and then a conformational change in the phospholipid head groups. At the same time, they may exert a significant influence on hydration of the lipid bilayer. Fisher et al. (1975) and Fisher & Quintana (1975) have demonstrated that ion-ion interactions take place between the dicationic chlorhexidine molecules and anionic carboxylate groups in stearic acid monolayers at pH of 5.0~6.0 (but not at pH 3), and that the hexamethylene hydrophobic chain of the biguanide is constrained at the cell surface. They further demonstrated the ability of chlorhexidine to anchor to the polar head groups of the film-forming molecules. These results help explain the hydrophobicity-increasing effect of chlorhexidine on the bacterial cell surface (Fisher et al, 1975; Fisher & Quintina, 1975). Consequently, such competitive binding decreases the rate of rotational mobility and increases hydrophobicity. The interaction of the dicationic chlorhexidine digluconate with the outer monolayer's hydrocarbon region will rearrange the intermolecular hydrogen-bonded network among phospholipid molecules and/or protein molecules, liberating hydrated water molecules on the monolayer of the bacterial outer membranes. The interaction will also change the orientation of the P-N dipole of phospholipid molecules. These changes should cause disordering of the hydrocarbon interior of the monolayer. Thus, disordering and ordering effects of the chlorhexidine digluconate could affect the transport of small molecules in bacterial outer membranes, leading to the bacteriostatic or bactericidal action.

The questions of whether chlorhexidine digluconate interfered with membrane protein function by directly binding to the proteins or whether the main modes of action occurred indirectly through a change in the physicochemical properties of the lipid membranes into which the chlorhexidine digluconate readily diffused have remained controversial. Because biological membranes are of highly complex composition, it has not been possible to monitor changes in the local lipid environment and to determine its effect on the membrane protein function at the same time.

It is possible to explain the multiplication effects by citing the increased or decreased mobility of protein triggered by lipids, however the reverse situation of protein triggering change in lipids is more likely. It is certain that chlorhexidine digluconate increases or decreases the mobility of the outer monolayer of bacterial outer membrane, but the direct effects of chlorhexidine digluconate on protein appear to have such effects magnified on the lipid. That is to say, before, during or even after the interaction of the chlorhexidine digluconate with proteins, the fluidization of membrane lipids may provide an ideal microenvironment for optimum bateriostatic or bactericidal effects. In conclusion, the present data suggest that chlorhexidine digluconate, in addition to its direct interaction with proteins, concurrently interact with membrane lipids, affects the fluidity of the membrane, and consequently induces conformational changes of proteins which are known to be tightly associated with membrane lipids.

ACKNOWLEDGEMENT

This paper was supported by research grant from the Korea Science and Engineering Foundation (KOSEF, R01-2002-000-00255-0, 2002-2005).

REFERENCES

Abrams FS, Chattopadhyay A, London E. Determination of the location of fluorescent probes attached to fatty acids using parallax analysis of fluorescence quenching: effect of carboxyl ionization state and environment on depth. *Biochemistry* 31: 5322–5327, 1992

Abrams FS, London E. Extension of parallax analysis of membrane penetration depth to the polar region of model membranes: Use of fluorescence quenching by a spin-label attached to the phospholipid polar headgroup. *Biochemistry* 32: 10826–10831, 1993

Audus KL, Tavakoli-Saberi MR, Zheng H, Boyce EN. Chlorhexidine effects on membrane lipid domains of human buccal epithelial cells. *J Dent Res* 71: 1298–1303, 1992

Buck RA, Eleazer PD, Staat RH, Scheetz JP. Effectiveness of three endodontic irrigants at various tubular depths in human dentin. *J Endod* 27: 206–208, 2001

Ferretti GS, Ash RC, Brown AT, Largent BM, Kaplan A, Lillich TT. Chlorhexidine for prophylaxis against oral infections and associated complications in patients receiving bone marrow transplants. *J Am Dent Assoc* 114: 461–467, 1987a

Ferretti GS, Hansen IA, Whittenburg K, Brown AT, Lillich TT, Ash RC. Therapeutic use of chlorhexidine in bone marrow transplant patients: case studies. *Oral Med Oral Surg Oral Pathol* 63: 683–687, 1987b

Fisher RG, Quintana RP, Boulware MA. Surface-chemical studies on chlorhexidine and related compounds. I. Effects at air-water, n-hexane-water, and hydroxyapatite-water interfaces. *J Dent Res* 54: 20–24, 1975

Fisher RG, Quintana RP. Surface-chemical studies on chlorhexidine and related compounds. II. Interaction with monomolecular-film systems. *J Dent Res* 54: 25–31, 1975

Gabler WL, Roberts D, Harold W. The effect of chlorhexidine on blood cells. *J Periodontal Res* 22: 150–155, 1987

Gerlach RW, White DJ. Removal of extrinsic using a tartar control whitening dentifrice: a randomizied clinical trial. *J Clin Dent* 12: 42–46, 2001

Hugo WB. Membrane-active antimicrobial compounds-a reappraisal of their mode of action in the light of the chemiosmotic theory. *Int J Pharm* 1: 127–131, 1978

Kenney EB, Saxe SR, Bosles RD. Effect of chlorhexidine on human polymorphonuclear leukocytes. *Arch Oral Biol* 17: 1633–1636, 1972

Knuutilla M, Södering E. Effect of chlorhexidine on the release of lysosomal enzymes from cultured macrophages. *Acta Odontologica Scandinavica* 39: 285–289, 1981

Lamont RJ, Chan A, Belton CM, Izutsu KT, Vasel D, Weinbero A. *Porphyromonas gingivalis* invasion of gingival epithelial cells. *Infect Immun* 63: 3878–3885, 1995

Lowry OH, Rosebrough NR, Farr AL, Randall RJ. Protein measurement with the olin phenol reagent. *J Biol Chem* 193: 265–275, 1951

Löe H, Schiott Rindom C. The effect of mouthrinses and topical applications of chlorhexidine on the development of dental plaque and gingivitis in man. *J Periodontal Res* 5: 79–83, 1970

Mason JT. Properties of phosphatidylcholine bilayers as revealed by mixed-acyl phospholipid fluorescent probes containing *n*-(9-anthroyloxy) fatty acids. *Biochim Biophys Acta* 1194: 99–108, 1994

Molitoris BA, Alfery AC, Arris RA, Simon FR. Renal apical membrane cholesterol and fluidity in regulation of phosphate transport. *Am J Physiol* 249: 12–19, 1985

Molitoris BA, Hoilien C. Static and dynamic components of renal cortical brush border and basolateral membrane fluidity: Role of cholesterol. *J Membrane Biol* 99: 165–172, 1987

Newbrun E. Anticaries and antiplaque/anti-gingivitis agents. In Neidle EA, Yagiela JA ed, Pharmacology and Therapeutics for Dentistry. The C.V. Mosby Co., Philadelphia, pp 603–624, 1989

Russell AD. Chlorhexidine: Antibacterial action and bacterial resistance. *Infection* 14: 212–215, 1986

Schachter D. Fluidity and function of hepatocyte plasma membranes. *Hepatology* 4: 140–151, 1984

Smalley JW, Birss AJ, Mckee AS, Marsh PD. Haemin-binding proteins of *Porphyromonas gingivalis* W50 grown in a chemostat under haemin-limitation. *J Gen Microbiol* 139: 2145–2150, 1993

Smalley JW, Birss AJ. Trypsin-like enzyme activity of the extracellular vesicles of *Bacteroides gingivalis* W50. *J Gen Microbiol* 133: 2883–2894, 1987

Spratt DA, Pratten J, Wilson M, Gulablvala K. An in vitro evaluation of the antimicrobial efficacy of irrigants on biofilms of root canal isolates. *Int Endod J* 34: 300–307, 2001

Stubbs CD, Rubin E. Molecular mechanism of ethanol and anesthetic actions: lipid- and protein-based theories. In Alling C, Diamond I, Leslie SW, Sun GY, Wood WG ed. Alcohol, Cell Membranes, and Signal Transduction in Brain, Pleum Press, New York, pp 1–11, 1993

Thulborn KR, Tilley LM, Sawyer WH, Treloar FE. The use of *n*-(9-anthroyloxy) fatty acids to determine fluidity and polarity gradients in phospholipid bilayers. *Biochim Biophys Acta* 558: 166–178, 1979

Tilley L, Thulborn KR, Sawyer WH. As assessment of the fluidity gradient of the lipid bilayer as determined by a set of *n*-(9-anthroyloxy) fatty acids (*n*=2,6,9,12,16). *J Biol Chem* 254: 2592–2594, 1979

Tsuchiya H. Effects of green tea catechins on membrane fluidity. *Pharmacol* 59: 34–44, 1999

Tsutsui H, Kinouchi T, Wakano Y, Ohnishi Y. Purification and characterization of a protease from *Bacteroides gingivalis* 381. *Infect Immun* 55: 420–427, 1987

Villalaín J, Prieto M. Location and interaction of *N*-(9-anthroyloxy)-stearic acid probes incorporated in phosphatidylcholine vesicles. *Chem Phys Lipids* 59: 9–16, 1991

Vincent M, De Furesta D, Gallay J, Alfsen A. Fluorescence anisotropy decays of *n*-(9-anthroyloxy) fatty acids in dipalmitoyl phosphatidylcholine vesicles: Localization of the effects of cholesterol addition. *Biochim Biophys Res Commun* 107: 914–921, 1982