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Effects of prilocaine and articaine on human leucocytes and reactive oxygen species *in vitro*

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Background: The aim of this study was to investigate the ability of local anaesthetics to inhibit reactive oxygen and nitrogen species generated by either stimulated human leucocytes or cell-free systems using luminol chemiluminescence (CL).
Methods: Free radical generation was stimulated in leucocyte assay by formyl-methionyl-leucyl-phenylalanine (FMLP, 2 μM). In cell-free experiments, hydrogen peroxide (H_2O_2) 3.5 mM, sodium hypochloride 5 μM, ferrous sulphate ($FeSO_4$) 40 nM, peroxynitrite 50 nM and xanthine 0.1 mmol l^{-1} plus xanthine oxidase 0.25 U ml^{-1} were used to produce H_2O_2, hypochlorous acid (HOCl), hydroxyl radical, peroxynitrite and superoxide-induced CL, respectively.
Results: Prilocaine inhibited FMLP-induced CL in leucocytes (94±1%, at 1 mM), whereas articaine showed an activation (59±7%) at high concentration (1 mM) and inhibition (13±6%) at low concentration (0.1 mM). In cell-free experiments, prilocaine (22±6%, at 1 mM) and articaine (85±1%, at 1 mM) caused concentration-dependent inhibition in xanthine-xanthine oxidase-induced CL. Although articaine had no effect on H_2O_2-induced CL, prilocaine significantly attenuated the H_2O_2 signal (97±0.3%, at 1 mM). Prilocaine (99±0.04%, 1 mM) and articaine (70±6%, 1 mM) markedly inhibited HOCl-induced CL, whereas these drugs had no effect on $FeSO_4$-induced CL. Articaine inhibited peroxynitrite CL (63±6%, 1 mM), but prilocaine did not produce any depression on this signal.
Conclusion: Prilocaine interacted with superoxide, HOCl and H_2O_2, whereas articaine reacted with superoxide, HOCl, and peroxynitrite. The direct scavenging properties of these drugs might be involved in the inhibition observed in leucocyte assay and could provide experimental support for investigating the potential benefit of using these local anaesthetics in patients presenting pathologies associated with free radical reactions.

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Key words: Chemiluminescence; leucocytes; scavenging activity; prilocaine; articaine.

THE FORMATION of reactive oxygen species (ROS) and their metabolites appear to play a significant role in many pathological states (1). Free radical scavengers or antioxidants represent an important component of the body defences against such free radical mediated injury. Administration of a drug possessing certain antioxidant activity might be ameliorating since some of the local anaesthetics are known to have antioxidant properties, impair leucocyte functions and inhibit lysosomal enzyme release, which might be clinically important (2–4).

It is well established that inflammatory cells such as polymorphonuclear leucocytes (PMN) stimulated by a variety of both soluble and particulate agonists represent a major source of oxygen radicals and metabolites. The initial product of oxygen reduction is superoxide, generated during respiratory burst. Following this superoxide generation, other oxygen metabolites may then be formed including hydrogen peroxide (H_2O_2), singlet oxygen, hydroxyl radical and hypochlorous acid (HOCl), the latter being formed by a reaction catalysed by myeloperoxidase released from neutrophil granules (5, 6). Although prilocaine and articaine are widely used amide-type local anaesthetics having similar chemical structure, there is only little information about the effects of these drugs on leucocyte function. In one study, prilocaine has been shown to inhibit chemotaxis in response to formyl-methionyl-leucyl-phenylalanine (FMLP) (3). Since there is also no information on the direct interaction of prilocaine and articaine with free radicals and reactive metabolites, we tested the hypothesis that both prilocaine and articaine might produce similar effects on leucocyte- and reactive oxygen species-induced chemiluminescence (CL). Therefore, we investigated the ability of prilocaine and articaine to interact with ROS generated by either

stimulated human leucocytes or cell-free systems via CL produced either chemically or enzymatically.

Materials and methods

CL generated from human leucocytes

Isolation and separation of leucocytes

Human venous blood was obtained from 20 healthy non-smoking volunteers (9 female, 11 male between 25 and 55 years) after obtaining their informed consent. Leucocytes were isolated according to methods described previously (7, 8). Following collection of 9 ml of blood into tubes containing 3.8% sodium citrate (1 ml), dextran was added and allowed for sedimentation at room temperature for 60 min. The leucocyte-rich supernatant was removed and centrifuged at 900 r.p.m. for 20 min. Erythrocyte lysis was performed by washing cells with 0.2% NaCl for 30 s and mixing immediately with double volume of 1.6% NaCl. Then, leucocytes were centrifuged at 900 r.p.m. for 15 min and the pellet was resuspended in Hank's buffered salt solution (HBSS) containing 1 mM calcium (pH= 7.4). Leucocytes were washed with HBSS three times. After leucocyte count in a cell counter (Contraves, Digicell 300, Zurich, Switzerland), cell yield was adjusted to 10^7 cells ml^{-1} (stock cell suspension) by adding HBSS. Cell viability was assessed by a trypan blue exclusion test and more than 98% of the cells were found to be viable leucocytes (n=6).

FMLP-induced CL from human leucocytes

Stock leucocyte cell suspension (0.1 ml) was diluted with HBSS in a cuvette (total volume of 1 ml) and 20 µl luminol (50 µM; final cuvette concentration) was added, producing a final cell yield of 10^6 cells ml^{-1}. Then a stimulant, FMLP, was added to yield a final cuvette concentration of 2 µM. Luminol-CL was measured at 37°C using a chemiluminometer (Bio-Orbit 1250 Luminometer, Turku, Finland). The produced CL was measured continuously and recorded on a computer by using the Luminometer 1250 programme (version 1.12, BioOrbit) for 10 min (7, 8).

CL generated in cell-free systems

Xanthine-xanthine oxidase-induced CL

In order to characterize xanthine-xanthine oxidase-induced CL, 0.9 ml of phosphate-buffered saline (PBS: KH_2PO_4 10 mM and NaCl 150 mM, pH 7.4) were mixed with luminol 0.1 ml (250 µM, final cuvette concentration) in a cuvette. After further addition of 10 µl of xanthine (0.1 mM), 25 µl of xanthine oxidase (0.25 U ml^{-1}) was injected into the cuvette, and the produced CL was measured continuously for 5 min (8).

H_2O_2-induced CL

H_2O_2 (3.5 mM) was injected into the PBS and luminol (250 µM) mixture, and generated CL at 37°C was measured continuously for 10 min (7).

HOCl-induced CL

HOCl was prepared as described by Vissers and colleagues (9). NaOCl was diluted with PBS and the pH of the solution readjusted to 7.4 immediately before the addition to the CL cuvette. At this pH, the solution contains approximately 1:1 HOCl and OCl^- and is subsequently referred to as HOCl. HOCl (5 µM) was injected into the PBS and luminol (250 µM) mixture to induce CL, which was measured continuously for 3 min as described previously (7, 8).

Ferrous iron-induced CL

Hydroxyl radical was generated by addition of ferrous iron into the buffer solution as described by Yıldız and Demiryürek (10). Freshly prepared $FeSO_4$ (40 nM) was added to the PBS plus luminol (250 µM) mixture and CL was recorded continuously for 3 min.

Peroxynitrite synthesis and CL

Peroxynitrite was prepared using a quenched flow reaction as previously described (11). Briefly, an aqueous solution of 0.6 M sodium nitrite was rapidly mixed with an equal volume of 0.6 M H_2O_2 containing 0.7 M HCl and immediately quenched with the same volume of 1.2 M NaOH. All reactions were performed on ice. Excess H_2O_2 was removed by adding mangenese dioxide (MnO_2) powder to the peroxynitrite solution. The mixture was shaken for 5 min and then MnO_2 was removed by passage over a cellulose acetate disposable filter. The solution was used freshly or frozen at −20°C for as long as a week. The final concentration of peroxynitrite was determined spectrophotometrically in 1.2 M NaOH ($\in_{302}$=1670 M^{-1} cm^{-1}). Dilutions of this peroxynitrite stock solution were made in 1.2 M NaOH with the final dilution in 0.1 M NaOH before use. Luminol CL were measured at 37°C as described previously (12). PBS was mixed with luminol (250 µM) in a cuvette. After adding catalase (50 U ml^{-1}) into the cuvette to remove H_2O_2 left after MnO_2 treatment, peroxynitrite at 20 nM was injected and the CL produced was measured continuously and recorded for 3 min.

Experimental protocol

The effects of various concentrations of prilocaine and articaine were examined by adding them into the mixture immediately prior to the stimulant administration. Duplicate assays were performed in all experi-

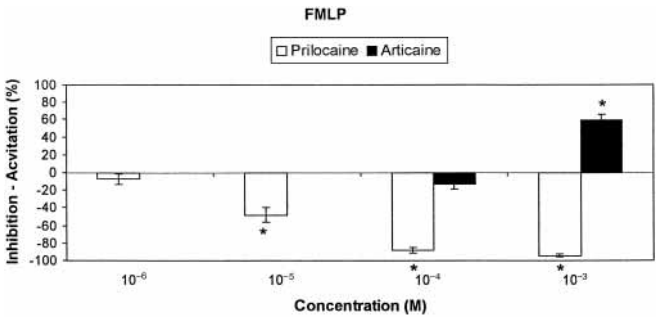

*Fig. 1. The concentration-dependent inhibitory effects of prilocaine (n=3–7) and articaine (n=5–6) on FMLP (2 μM) induced luminol chemiluminescence. Data are shown as mean±SEM. *P<0.05 significantly less than its control value.*

ments. Results were calculated as peak CL or percent of the peak CL and expressed as mean±SEM. *n* refers to the number of individual volunteers (for leucocyte experiments) and number of experiments (for cell-free assays).

Statistical analysis

Analysis of variance (ANOVA) was used to assess the observed differences in the CL among the concentrations. Student-Newman-Keul's test was used in cases of detecting significant differences by ANOVA. Differences were considered to be statistically significant when the *P* value was less than 0.05.

Drugs

Prilocaine (Citanest 2%, Eczacıbaşı, İstanbul, Turkey) and articaine (Ultracaine 2%, Hoechst, İstanbul, Turkey) (both preparations not mixed with epinephrine or any other drug) were diluted with PBS. Luminol was prepared daily in 2 M NaOH (2.5%) and diluted with PBS before use. Luminol sodium, ferrous sulphate heptahydrate, catalase (from bovine liver), sodium hypochlorite, FMLP, xanthine (sodium salt) and xanthine oxidase (grade I, from butter milk) were purchased from Sigma chemical Company (St. Louis, MO, U.S.A.). H_2O_2 was purchased from Merck (Darmstadt, Germany).

Results

Effects of prilocaine and articaine on luminol-CL in the isolated human leucocytes

FMLP (2 μM) produced 4079±978 mV (n=20) CL signal in the leucocyte assay. Prilocaine produced a concentration-dependent inhibition in the FMLP-stimulated leucocytes CL and the most marked inhibition was observed at 1 mM (94±1%, n=5) as shown in Fig. 1. Although articaine produced a significant activation in the leucocyte assay at 1 mM (59±7%, n=6), an inhibition (13±6%, n=5) was observed at low concentration (0.1 mM).

Effects of prilocaine and articaine on luminol-CL in the cell-free systems

Concentrations of 0.25 U ml^{-1}, 3.5 mM, 5 μM, 40 nM, and 50 nM were selected for xanthine oxidase, H_2O_2, HOCl, $FeSO_4$, and peroxynitrite, respectively and gen-

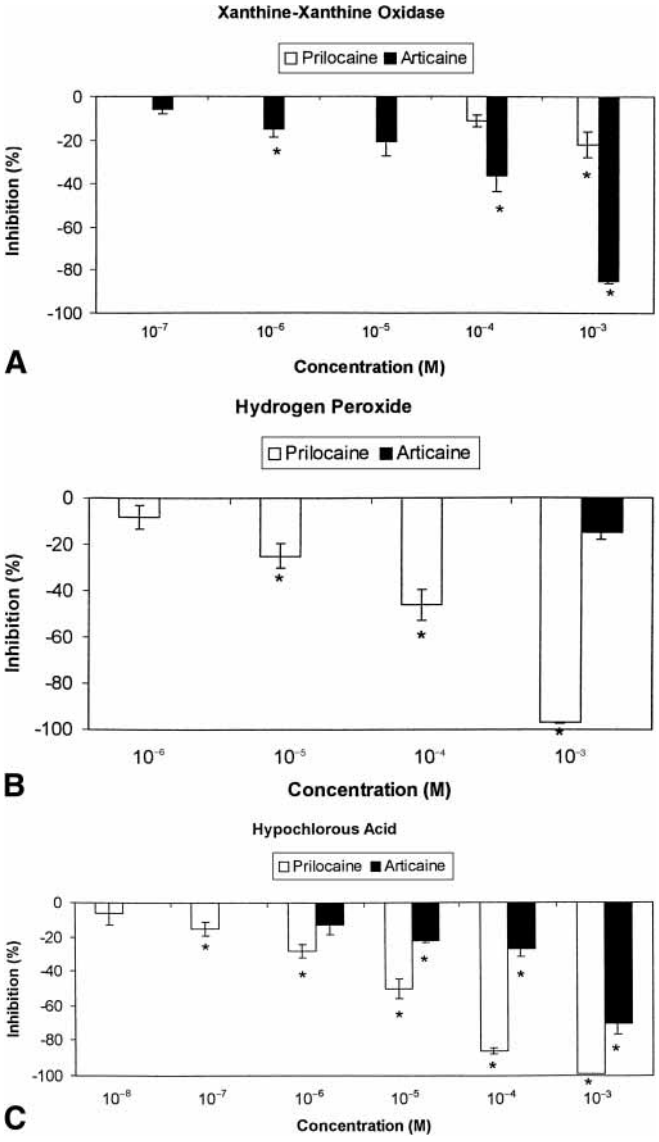

*Fig. 2. The concentration-dependent inhibitory effects of prilocaine (n=6) and articaine (n=4–6) in cell-free systems on xanthine-xanthine oxidase-induced luminol chemiluminescence (**A**), effects of prilocaine (n=4–9) and articaine (n=3) on H_2O_2-induced luminol chemiluminescence (**B**), and effects of prilocaine (n=6–8) and articaine (n=3–6) on hypochlorous acid-induced chemiluminescence (**C**). Data are shown as mean±SEM. *P<0.05 significantly less than its control value.*

erated CL peaks 2500±244 mV (n=56), 3641±444 mV (n=61), 4711±164 mV (n=72), 5308±256 mV (n=32), and 3969±514 mV (n=76), were found to be comparable with that produced by FMLP in leucocyte experiments.

Prilocaine (1 μM–1 mM) and articaine (0.1 μM–1 mM) caused a concentration-dependent inhibition in the xanthine-xanthine oxidase-induced luminol CL. There were 22±6% (n=6) and 85±1% (n=6) suppression by prilocaine and articaine at 1 mM, respectively (Fig. 2A). Prilocaine significantly inhibited H_2O_2-induced CL in a concentration-dependent manner (97±0.3%, n=7, at 1 mM) (Fig. 2B), whereas articaine had no effect on this signal. HOCl-induced luminol CL was also attenuated by prilocaine (0.01 μM–1 mM) and articaine (1 μM–1 mM) in a concentration-dependent manner (Fig. 2C). In the presence of prilocaine, there was almost complete inhibition at 1 mM (99±0.04%, n=6). Articaine at 1 mM produced 70±6% (n=7) inhibition (Fig. 2C). Articaine, but not prilocaine, produced a significant depression on peroxynitrite-induced luminol CL. This inhibition was marked at high (63±6%, n=8, 1 mM), but not low concentration (9±6%, n=8, 0.1 mM). There was also no significant inhibition on ferrous iron-induced luminol CL either by prilocaine (1±4%, n=6, 1 mM) or by articaine (6±3%, n=6, 1 mM).

Discussion

In the present study, we demonstrated that prilocaine suppressed respiratory burst of leucocytes in a concentration-dependent manner. This is in agreement with the observation showing that prilocaine caused an inhibition in leucocyte superoxide anion production induced by FMLP or phorbol myristate acetate (3). Although superoxide generation was measured by the reduction of ferricytochrome c in that study, inhibition by prilocaine was observed at concentrations ranging from 1 mM to 10 mM (3). The maximum plasma concentration of prilocaine during intravenous regional anaesthesia has been reported to be 4.4 μg ml^{-1} (2×10^{-5} M) (13). Our results showed that prilocaine significantly depressed FMLP-stimulated leucocyte chemiluminescence. Human neutrophils stimulated by phorbol ester and incubated with prilocaine and a *Cypridina luciferin* analogue as a superoxide-specific chemiluminescence probe in another study (14) and prilocaine has been shown to suppress superoxide generation in a concentration-dependent manner.

Articaine is one of the most widely used local anaesthetic agents in dentistry. The amide structure of articaine is similar to that of other local anaesthetics, but it contains an additional ester group which is quickly hydrolysed by esterases. The mean maximum plasma drug concentration after submucosal injection in dentistry is about 580 μg l^{-1} (2×10^{-6} M) for articaine without epinephrine (15). The concentration of articaine in the alveolus of a tooth in the upper jaw after extraction has been reported to be about 100 times higher (2×10^{-4} M) than that in systemic circulation (15). In our experiment, articaine showed an activation at high concentration. The reason for this activation in the presence of articaine or the mechanism for the inhibition of the leucocyte CL by prilocaine or articaine are unknown, since they are not examined in the present study. But they may be related to the direct interaction with the cell components, as observed only in the leucocyte assay. However, it appears that luminol is not involved in this activation since high concentration of articaine did not induce any effect in ferrous iron-induced CL and in other cell-free experiments. It has been reported that local anaesthetics can cause an inhibition of the phosphorylation of cytoplasmic protein, which is essential for the activation of NADPH oxidase in neutrophils by protein kinase C (16), produce an impairment of the myeloperoxidase enzyme release after stimulation in human neutrophils (17), or suppress the functions of cells by inhibition of phospholipase D activation (18). Therefore, in addition to the direct interactions, these effects are likely to be involved in the inhibitory effects of prilocaine and articaine in leucocyte assay.

To our knowledge, this is the first study reporting the interaction of prilocaine or articaine with ROS and their metabolites. In the cell-free experiments, prilocaine and articaine reacted with HOCl and superoxide in a concentration-dependent manner. However, interaction with superoxide was marked at high concentrations of prilocaine. Our results provide the first experimental evidence that prilocaine and articaine are potent scavengers of HOCl. H_2O_2-induced luminol CL was markedly inhibited by prilocaine, but articaine had no scavenging activity in these experiments. Our results showed that articaine was able to scavenge peroxynitrite at high concentration. This is also the first experiment demonstrating that articaine can interact with peroxynitrite. However, prilocaine and articaine failed to inhibit ferrous iron-induced CL, indicating that these drugs have no scavenging activity against hydroxyl radical. These results might contribute to the development of new molecules possessing antioxidant activities in accordance with the chemical structure of the local anaesthetics.

In conclusion, these results suggested that prilo-

caine and articaine are capable of interacting directly with the reactive oxygen and nitrogen species and their metabolites, except hydroxyl radical. Our results indicated that articaine has no marked effect on leucocytes at therapeutic plasma concentrations. These direct interactions of prilocaine with free radicals might explain the inhibition observed in the leucocyte assay at high concentrations that may be reached at or near the site of injection in local anaesthetic use. Our results provide experimental support for investigating the potential benefit of using prilocaine or articaine as a local anaesthetic agent in patients presenting pathologies associated with free radicals.

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