INTRODUCTION

Cigarette smoke (CS) is associated with a variety of human pathologies including cardiovascular disease and cancer. Oral squamous cell carcinoma (OSCC) is the most common malignancy of the head and neck. The major inducer of OSCC is exposure to tobacco. Recent studies demonstrated that oxidative and nitrosative stress contributes to the development of oral carcinogenesis through DNA damage. All salivary reactive nitrogen species (RNS) analyzed from OSCC patients are significantly higher in comparison with healthy subjects. Our findings show that CS and external RNS addition induced reduction in $\alpha$-amylase activity and produced some excited carbonyl formation, but to a much less extent than CS. The addition of epigallocatechin-3-gallate (EGCG) to saliva produced no protective effect against damage to $\alpha$-amylase activity. Our proposed mechanism for the decrease in $\alpha$-amylase activity is the formation of adducts at SH groups of the $\alpha$-amylase active site. In this case, EGCG was unable to counteract this phenomenon, as it does not reduce the concentration of disulfides, and does not alter the amount of protein-SH moieties. However, EGCG did reduce the levels of excited carbonyl formation. Our results indicate that although RNS are abundant in CS, a significant decrease in amylase activity is due to other components in CS, probably aldehydes, reacting with the thiol group of proteins by the Michael addition reaction.

Key words: $\alpha$-amylase, epigallocatechin-3-gallate, cigarette smoke, reactive nitrogen species
study the effects of CS components, especially RNS, on salivary damage through oxidation and nitration of enzymes and proteins. In our study, the noxious effects of CS were evaluated in vitro directly in fresh human saliva samples.

Our working hypothesis was that when CS encounters saliva, there is a massive increase in reactive nitrogen species (RNS), which in turn cause protein alterations in the form of carbonyl formation and decreased enzyme activities. As a result, there is a decline in the defense mechanisms of saliva especially in its antioxidative properties, which can lead to the development of oral pathologies that are known to occur more frequently in smokers than in non-smokers. Thus, external addition of antioxidants may neutralize the effects of CS originated oxidants and could act as an effective means to neutralize the toxic effects of smoking. Our aims in this study were: 1) To determine interactions of RNS with human saliva and its effects on protein modifications and α-amylase (AMY) activity. 2) To elucidate the mechanisms by which antioxidants, like glutathione and epigallocatechine-3-gallate (EGCG), may reduce the interactions of RNS with human saliva.

MATERIAL AND METHODS

Collection of saliva

The study was approved by a local Ethics Committee. Whole saliva, which is basically total oral fluid, was collected from healthy male and female nonsmokers under non-stimulatory conditions in the morning. For this collection, which was performed at least 1 h after eating, volunteers were asked to generate saliva in their mouths and to spit into a wide test tube for 10-15 min. Following the collection, saliva was immediately centrifuged (1000 x g 3 min) to remove squamous cells and cell debris. The fresh supernatant was immediately used for CS and RNS studies, and α-amylase activity and oxidation studies were performed at the end of the CS and RNS exposure studies.

Cigarettes and RNS donors

The cigarettes used in this study were popular commercial cigarettes ("Time" cigarettes; Dubek Ltd., Tel Aviv, Israel) containing 14 mg of tar and 0.9 mg of nicotine per cigarette. NO donor S-nitroso-N-acetylpenicillamine (SNAP) and peroxynitrite donor 3-morpholine-sydnonimine (SIN-1) were obtained from Sigma-Aldrich (St. Louis, MA). Authentic peroxynitrite was purchased from Cayman Chemical Company (Ann Arbor, MI). RNS donors were added to saliva samples at final concentrations of 10 M ONOO−, 100 M SIN-1, 30 M SNAP.

Exposure of saliva to CS

In vitro study was carried out using "Time" cigarettes combined with a vacuum system, as described previously (17). Saliva (4-5 ml) was placed in 250 ml flasks with a sidearm to which the cigarettes were attached. A reproducible vacuum was created in the flask, and after opening the vacuum to the lighted cigarette for 5 s, 80 to 100 ml of CS puffs were drawn into the flask. Flasks were incubated in a metabolic shaker for a total of 3 h at 37°C. In a given experiment, puffs were administered to the flask over a 3-h time period at 20-min intervals, for a total of nine times. Samples for biochemical analysis were removed from the flasks at zero time and at 1, 2, and 3 h. Flasks were incubated in a metabolic shaker for a total of 3 h at 37°C.

Exposure of saliva to RNS

4-5 ml of human saliva was treated with RNS donors similar to that found to be present in a mainstream smoke of a single cigarette (results not shown), to give a final concentration of 10 µM ONOO−, 100 µM SIN-1, 30 µM SNAP. RNS donors were administered to the flask over a 3-h time period at 20-min intervals, for a total of nine times. Samples for biochemical analysis were removed from the flasks at zero time and at 1, 2, and 3 h. Flasks were incubated in a metabolic shaker for a total of 3 h at 37°C.

α-amylase activity determination (AMY assay)

The AMY assay method using Dimension® clinical chemistry system has been employed in this project. The AMY method utilizes a chromogenic substrate, 2-chloro-4-nitrophenol linked with maltotriose. The direct reaction of α-amylase with the substrate results in the formation of 2-chloro-4 nitrophenol, which is monitored spectrophotometrically. The AMY method responds to both pancreatic and salivary amylase isoenzymes. Enzyme activities were expressed as IU/L.

Thermochemiluminescence analysis (TCL)

Photon emission during heating was measured by TCL Photometer (manufactured by Lumitest Ltd., Israel) in the saliva as previously described using a photomultiplier model R265P (Hamamatsu Photonics, Japan) with a spectral response range of 300-650 nm. The TCL device measures the level of photon emission from excited carbonyls in biological macromolecules, i.e., oxidative modifications of lipids and proteins. The fluid examined is distributed over the surface of an aluminum tray (a type of miniature Petri dish) inside the sample preparation block and is then vacuum dried. The dish is then mounted on a constant temperature heater kept at 80° in the analysis block and the photon emission is measured. The TCL curve obtained is described mathematically by means of the two main parameters examined in the analysis: (1) the amplitude of the kinetic curve of the photon emission, and (2) its slopes. The TCL amplitudes both at 50 s (H1) and 280 s (H3) as well as the oxygenation potential and preincubation values are recorded, all representing the level of oxidative stress for which the saliva had been exposed to. H1 and H3 are base line and end point parameters of the TCL kinetic curve respectively that reflect electronically excited species formation due to heat induced oxidative modifications (18).

Addition of epigallocatechine-3-gallate (EGCG) to saliva

EGCG was obtained from Sigma-Aldrich (St. Louis, MA) and dissolved in water. It was added to the saliva at the final concentration of 100 µM (19), 1 h prior to the beginning of the experiment. Subsequently, the saliva was incubated with CS and RNS donors as described above. In addition, in all experiments, a control without the compounds was run with no difference in the assay conditions.

Statistical analysis

Statistical analysis was performed using an unpaired t-test and one way ANOVA. To determine statistical significance, the ranges and means±SD were computed. Statistical significance was set at P<0.05.

RESULTS

Fig. 1 shows the loss of α-amylase activity after exposure of saliva to CS and RNS donors at the end of 3-h periods of exposure (as described in the methods section). The initial
mean±SD of amylase activity from three volunteers was 45±8 x10^3 IU/L. Subsequently, 8 different experiments were performed on the saliva of these 3 volunteers. There was a statistical difference between CS and ONOO⁻ in reducing α-amylase activity (33±11% vs. 24±6%, P<0.05). SNAP also reduced α-amylase activity in a significant manner compared with control and in the same manner as ONOO⁻ (26±6%). SIN-1 had a significantly higher effect in decreasing α-amylase activity after 3 h in comparison with all treatments (45±9.3% vs. 33±11% of CS and 24±6% of ONOO⁻, P<0.001). All treatments significantly decreased α-amylase activity in comparison with control (P<0.001).

Fig. 2 shows changes in the saliva TCL ratio. Exposure of saliva to CS and RNS produced a varied decrease in the TCL ratio after 3 h (42±8% for CS, 18±2% for ONOO⁻, 27±3% for SIN-1, and 20±2% for SNAP, P<0.001). All treatments were
significantly different from control at 2 h (P<0.001). The rate of loss of \(\alpha\)-amylase activity was not significantly influenced by the presence of EGCG (33±11% for CS vs. 27±6% for CS+EGCG (Fig. 3A) and 24±6% for ONOO\(^{-}\) vs. 26±6% for ONOO\(^{-}\)+EGCG (Fig. 3B); NS in either) after 3 h; nor was it influenced at 1 and 2 h. The rate of loss of \(\alpha\)-amylase activity was not influenced by the presence EGCG in the case of SIN-1 and SNAP, either (data not shown).

A summary of all the experiments on \(\alpha\)-amylase activity in the presence of CS, RNS, and EGCG (Fig. 4) shows that there was no significant protective effect of EGCG against the damage to \(\alpha\)-amylase activity induced by CS and RNS. EGCG had no significant influence on its damage to \(\alpha\)-amylase activity, as opposed to GSH significant protective effect against the toxicity of CS and unsaturated aldehydes, as shown in our previous study (20).
Fig. 5 shows changes in the saliva TCL ratio with and without 100 μM EGCG. Exposure of EGCG-preincubated saliva to both CS (Panel A) and NOONO⁻ (Panel B) produced significant increases in the TCL ratio after 1, 2, and 3 h compared with those without EGCG; the increases were 42±8% vs. 27±4% (P<0.001) and 18±2% vs. 12±2% (P<0.05) at 3 h, respectively. Thus, excited carbonyl formation was less. Exposure of EGCG-preincubated saliva to SIN-1 produced a significant increase in the TCL ratio only after 3 h (27±3% vs. 8±1%, P<0.001 ) and that to SNAP produced a significant increase in this ratio only after 2 h (20 ±2% vs. 8±1% P<0.001); data not shown.

A summary of all the experiments of the TCL assay in the presence of CS or RNS in the presence or absence of EGCG is shown in Fig. 6. There was a significantly different effect of CS on changes in the TCL ratio in comparison with all RNS; the effects of RNS being smaller. In addition, changes in the TCL ratio due to exposure to RNS were influenced by the presence of EGCG. EGCG had a smaller influence on the TCL ratio when saliva was exposed to CS, as opposed to the effects of GSH, found a precious study (20), which provides significant protection against CS toxicity.

DISCUSSION

RNS might play a crucial part in the CS-associated damage to saliva components. Since CS is associated with increased nitration of tyrosine residues of plasma proteins, the production of 3-nitrotirosine is considered a marker of NO-dependent oxidative damage. Petruzzelli et al. (21) have shown higher levels of 3-nitrotirosine in plasma of healthy smokers compared with non-smokers. However, no research has so far checked this phenomenon in saliva.

As outlined above, in the human oral cavity, nitrate secreted as a salivary component is reduced to nitrite and NO by certain bacteria, and salivary nitrite may be transformed to NO, NO₂⁻, and N₂O₃ which can lead to tyrosine nitrination. The concentration of nitrate in saliva (0.2-2.5 mM) is dependent on the amount of nitrate ingested. The nitrate in saliva is reduced to nitrite (pKa of 3.3) and NO successively by bacteria such as Streptococcus salivarius, S. mitis, and S. bovis. Thus, it seems that oral bacteria are one of the main sources of RNS in the oral fluid. The concentration of nitrate in saliva (0.05-1 mM) is dependent on the concentration of nitrate. The nitrite and NO formed in the human oral cavity can be oxidized by molecular oxygen and by salivary peroxidase producing NO₂⁻ (14). This information was confirmed by us, when we used Griess reagent for detection of nitrite, and noticed high levels of nitrite at time 0 in two subjects. That has also been confirmed by Hasnis (22).

Our next step was to elucidate whether addition of different RNS donors will induce changes in saliva protein oxidation and α-amylase activity. Our findings show that RNS (ONOO⁻ and NO) addition indeed induced a reduction in α-amylase activity after 3 h of CS exposure, but to a less extent than CS alone. On the other hand, SIN-1, as a slow RNS donor, did not produce any effect in the first 2 hours and only after that we noticed a major reduction of 45% in α-amylase activity (Fig. 1). When we compared the RNS effect on α-amylase activity to those of CS and unsaturated aldehydes, we found that the latter had a much more significant reducing effect, with the exception of SIN-1.

With respect to photoemission of excited carbonyls measured by TCL, RNS did produce some excited carbonyl formation, but to a much less extent than CS and unsaturated aldehydes (15-25% for various RNS vs. 40% for CS and unsaturated aldehydes) (Fig. 2). It is known that the main reaction of peroxynitrite with thiols is a two electron oxidation leading to disulphides; this being a major mechanism in the ONOO⁻ mediated inactivation of various enzymes, in our case, α-amylase. Cysteines are considered the most sensitive targets for oxidation in proteins. ONOO⁻ oxidizes sulphhydryls about 10³ times faster than does H₂O₂ under the same conditions (23). These findings bring us to the conclusion that the major contributors to protein modifications and damage to salivary α-amylase activity by CS are unsaturated aldehydes and that the contribution of RNS is less significant.

To elucidate the mechanism of damage caused by RNS to saliva proteins and enzymes, we added epigallocatechine-3-gallate (EGCG) to saliva, an anti-nitrating agent, at a final concentration of 100 µM, 1 h prior to the beginning of the experiment. Subsequently, saliva was incubated with various RNS donors and CS. EGCG produced no protective effect against damage caused by CS and RNS to α-amylase activity, as can be seen in Fig. 3. EGCG directly reacts with tyrosyl radicals, thus preventing their transformation into nitrotyrosine (24). Therefore, our proposed mechanism for the decrease in α-amylase activity is the formation of adducts at SH groups. As mentioned before, ONOO⁻ is able to react with thiols leading ultimately to the formation of disulphides; this being a major mechanism in the ONOO⁻ mediated inactivation of various enzymes. In this case, as opposed to GSH, EGCG was unable to counteract this phenomenon, as it does not reduce the concentration of mixed disulphides and does not increase the amount of protein-SH moieties. However, when we examined the ability of EGCG as a known antioxidant, to reduce the levels of excited carbonyl formation after exposure to CS, using TCL measurements, we noted a significantly lower effect than that after GSH; 15% for EGCG and 25% for GSH (20). We also found out that EGCG significantly reduced formation of excited carbonyls after exposure to various RNS (Fig. 6).

Our results together with previous studies suggest an explanation for the involvement of RNS in damage to salivary components and a possible mechanism of CS induced damage to saliva, which may lead to progression of oral cavity associated diseases. Future in vitro studies should include using saliva from smokers and non-smokers before and after treatment with antioxidiant supplements, such as EGCG.

Acknowledgment: this work was supported by the Krol foundation of Barnegat, NJ, USA and a grant from the Vice President of Research of the Technion.

Conflict of interests: None declared.

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Received: July 27, 2009
Accepted: October 15, 2009

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