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The Effect of Cigarette Smoke on the Translocator Protein (TSPO) in Cultured Lung Cancer Cells

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ABSTRACT

Lung cancer is prevalent in cigarette smokers. The mitochondrial membrane translocator protein (TSPO), is thought to protect cells from free radical damage. We examined the effect of cigarette smoke (CS) (containing free radicals) alone and in the presence of saliva (containing redox active free iron), on survival of H1299 lung cancer cells and on their mitochondrial characteristics, and whether TSPO binding was influenced by CS and by saliva. We exposed H1299 cells to CS in the presence/absence of saliva and also characterized TSPO binding in the cells using [3H] PK 11195 as a radioligand. CS induced a significant drop in mitochondrial potential ($\Delta\Psi m$), while addition of saliva did not lead to further loss of $\Delta\Psi m$ (42.5% vs. 39.85%). Scatchard analysis of the saturation curve of [3H]PK 11195 binding (0.2–6 nM final concentration) yielded a straight-line plot ($R = 0.9$). Average Bmax value was 3274 ± 787 fmol/mg of protein, and average Kd value was 9.2 ± 1.3 nM. Benzodiazepine diazepam partially prevented decrease in cell survival following exposure to CS and redox active iron containing media (saliva) while benzodiazepine clonazepam did not, indicating that this effect is TSPO-specific. Exposure of cells to CS resulted in alternation of biomolecules expressed by CLs peroxidation, reduction of TSPO binding, and depletion of the mitochondrial potential. This irreversible damage was enhanced in the presence of saliva. All these modulations may result in cellular death increase following CS exposure, enhanced in the presence of saliva. J. Cell. Biochem. 116: 2786–2792, 2015.

KEY WORDS: LUNG CANCER CELLS; TSPO; CIGARETTE SMOKE; CELL SURVIVAL

Cigarette smoking is responsible for approximately 90% of lung cancer cases [Alberg and Samet, 2003]. Lung cancer is prevalent in chronic cigarette smokers and, in the United States, it is the second major cause of death [Kung et al., 2008]. Since no effective treatment for this disease has become available to date, new thinking and exploration of novel mechanistic studies are necessary, aimed at fully understanding the development of this disease. A puff of cigarette smoke contains about 5,000 toxic compounds, including free radicals [Halliwell and Gutteridge, 1990]. These toxic compounds contribute to the adverse effects of cigarette smoke (CS), playing key roles in lung cancer [Houghton et al., 2008; Khan et al., 2008; Mouded et al., 2009].

Cigarette smoke is normally inhaled into the lungs and can affect the integrity of lung cells. Several studies indicate the presence of significant amounts of redox active iron and copper in pleural fluids and in the lung parenchyma [Dines et al., 1974; Gutteridge et al., 1996; Apostolidou et al., 2011], just as it is found in salivary glands and saliva [Reznick et al., 2004]. We have previously found a synergistic effect of CS and saliva [Hasnis et al., 2004], based on the reaction between salivary redox active iron and copper and low reactive free radicals in CS, and resulting in production of highly active hydroxyl free radicals. We also demonstrated this synergistic mechanism in an in vitro study where we enhanced cellular killing by adding exogenous iron to the cellular medium, which did not contain saliva, prior to the exposure of the cells to CS. Furthermore, we succeeded in preventing this cellular death by adding to the medium not only iron but also a potent iron chelator, desferal, prior to the CS exposure [Hasnis et al., 2004]. This synergistic induced attack of active free radicals seems to explain the high prevalence of oral and pharyngeal cancer as we have previously reported [Reznick et al., 2004]. Interestingly, another synergistic mechanism in which CS is involved was documented by Izzotti et al. [1998] who reported a synergism between CS and ethanol leading to the formation of DNA adducts in the upper digestive tract.

Previously, it was suggested that the Translocator Protein (TSPO), located mainly in the outer mitochondrial membrane, may protect

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cells from damage induced by free radicals [de Tassigny et al., 2013]. TSPO is involved in essential anti-carcinogenic/pro-apoptotic functions and regulation of the mitochondrial membrane potential, which are in turn related to tumorigenicity. Indeed, it has also been found that TSPO levels are enhanced in cancer cells and tissues [Carmel et al., 1999; Dimitrova-Shumkovska et al., 2010; Veenman et al., 2010; Xiao et al., 2010; Choi et al., 2011; ; Gatliff and Campanella, 2012; Joo et al., 2012; Lehtonen et al., 2012; Maulik and Kumar, 2012 Seneviratne et al., 2012; Veenman and Gavish, 2012; Yarana et al., 2012; Zeno et al., 2012; Caballero et al., 2013; de Tassigny et al., 2013; Thompson et al., 2013; Mitra et al., 2014; Veenman et al., 2014]. Furthermore, our group demonstrated a strong indication that CS adversely affects TSPO binding in oral cancer and salivary cells, and may thus reduce its related anti-carcinogenic activity [Nagler et al., 2010a,b]. Accordingly, we postulated that perhaps the presence of redox active iron ions in the lungs may explain the well known high sensitivity of lungs to CS, which leads to the development of lung cancer. This process may be mediated via CS-induced alteration of the TSPO binding and damaging of its anti cancer potential in the lung epithelial cells. Further credence to this suggestion may be found in the study published by Balansky et al. [1996] who showed that the mitochondria where the TSPO is located is specifically targeted by CS. These researchers found that adducts to mitochondrial DNA were detected following exposure of rats to CS.

The purpose of the current study was to examine the effect of CS alone and of the synergistic effect of CS and redox active iron containing media on survival of epithelial H1299 lung cancer cells and on their mitochondrial characteristics. We also examined whether TSPO binding was influenced by CS and by the synergistic effect of CS and the redox active iron containing media (saliva). In order to do so, we exposed the H1299 cells to CS in both the presence and the absence of saliva.

METHODS AND MATERIALS

CELLS

The human non-small lung cancer cell-line NCI-H1299 (H1299), was maintained as described by the American Type Culture Collection (ATCC). Culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine, supplemented with fetal bovine serum (10%), and penicillin–streptomycin solution (1%). The cells were grown at 37°C in 5% CO_2. Culture medium ingredients were acquired from Beit HaEmek - Biological Industries, Israel. Phosphate buffered saline (PBS): for all assays, we used Dulbecco's PBS, with $CaCl_2$ and $MgCl_2$, purchased from Sigma (St Louis, USA), unless otherwise indicated.

SALIVA COLLECTION

Whole saliva was pooled following collection from five volunteers (20–60 years, 3 females, and 2 males) who do not smoke or consume alcohol habitually, who were healthy according to their responses to an examining questionnaire, and who were free of caries or gingival disease according to an intra oral examination. The saliva was collected under non-stimulatory conditions, at least 1 h after food intake, between 8:00 and 12:00 AM, during up to 20 min. Subsequently, the saliva was centrifuged (1,200×*g*, 15 min) to remove cell debris and the supernatant was used for further applications or stored at −20°C for further analysis.

EXPOSURE OF H1299 LUNG CANCER CELLS TO CIGARETTE SMOKE (CS) WITH OR WITHOUT SALIVA

For cigarette smoke exposure, the cells were grown in dishes and used for the smoking procedure only once the dish was confluent. Depending on the protocol, cells were added to 30% saliva supernatant. Dishes containing cells were placed into a sealed smoking chamber. Cells that were used for controls were subjected to the same procedure, but exposed to fresh air. The cigarettes used in this study were commercial cigarettes containing 14 mg of tar and 0.9 mg of nicotine (Time Cigarettes, Dubek Ltd., Tel Aviv, Israel). To expose the cells to CS, a cigarette from which the filter tip had been removed was attached to a Cambridge filter (capable of removing particles >0.1 mm in diameter) which was combined with a vacuum system to enable the inhalation of gas-phase CS inside sealed 250 ml flasks containing the cells in 12–15 ml PBS with/without saliva as previously described. A reproducible vacuum, with a 0.3 bar pressure, was created in the flask and, upon opening the vacuum to the lighted cigarette for 5 s, 80–100 ml of CS "puffs" were drawn into the flask. After half the cigarette had been "inhaled," the flasks were incubated for 20 min at 37°C in a metabolic shaker, and then taken for further inhalation.

CELL SURVIVAL ASSAYS

Trypan blue exclusion dye assay. Cell counts were preformed after CS exposure with/without saliva using the Trypan Blue exclusion test. Trypan blue was purchased from Biological Industries (Beit Haemek, Israel). We have previously published various studies in which we used the trypan blue analysis for evaluating cell survival following CS exposure in different cellular models [Hasnis et al., 2004; Reznick et al., 2004; Nagler et al., 2010a,c]. Furthermore, we found that the results obtained by using the trypan blue survival assay correlated with the results obtained by another cell survival assay used, the propidium iodide assay [Nagler et al., 2010c]. The medium covering the dish was collected, the cells were trypsinized, and centrifuged at 1200×*g* for 10 min. Cell pellets were re-suspended in 1 ml of medium, and a sample was collected for cell counting. Cells were stained with the vital dye Trypan blue at a final concentration of 0.25% within a hemocytometer. Visual counting was performed using an inverted microscope. The survival rate was also measured following addition of Clonazepam and Diazepam which were purchased from Sigma–Aldrich, Israel.

As the survival rates were not altered at later time points, we chose to examine the CS and redox iron effects up to two hours following CS exposure. Previous published studies by our groups, in various cellular models, looking at various parameters including survival rates, revealed similar results, that is, that there were no further alterations at time points later than 2 h following exposure to CS [Hasnis et al., 2004; Reznick et al., 2004; Nagler et al., 2010a,c].

CARDIOLIPIN PEROXIDATION ANALYSIS

Cardiolipin (CL) in mitochondria contains a substantial proportion of highly unsaturated fatty acids and is therefore sensitive to oxidation.

It is known that reactive oxygen species (ROS) cause a decrease and a variation in CL content. 10-N-Nonyl-Acridine Orange (NAO) can bind specifically to unoxidizable cardiolipin phospholipids. NAO lost its affinity for CL with a hydroperoxide fatty acid (CL-OOH). A decrease in the cellular fluorescence of NAO is thus thought to reflect peroxidation or other modifications of CL. In our experiment, we used NAO flow cytometry to measure the cardiolipin peroxidation in the mitochondria in order to indicate the degree of the oxidative injury to the mitochondria. Briefly, samples of confluent cells were collected and centrifuged at 1,200×*g* for 10 min, 4°C. Cell pellets were re-suspended in 0.5 ml of 10 μg/ml NAO and incubated for 30 min at 37°C in dark conditions. Then, the cell suspensions were transferred into 5 ml FALCON® FACS tubes and analyzed with the flow cytometer using CellQuest software.

MITOCHONDRIAL TRANSMEMBRANE POTENTIAL ANALYSIS

The specific stain JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) was used to assay changes in the $\Delta\psi_m$, as described previously [Chelli et al., 2004]. The negative charge established by the intact $\Delta\psi_m$ allows the lipophilic dye JC-1, bearing a delocalized positive charge, to enter the mitochondrial matrix. In healthy cells, the JC-1 molecules form J-aggregates in the mitochondria. When the mitochondrial membrane potential collapses, the JC-1 does not accumulate within the mitochondria and remains in the cytoplasm in the monomeric form. When excited by the 490 nm wave length, JC-1-aggregates emit at 590 nm (orange–red fluorescence) and the monomers form emits at 527 nm (green fluorescence). The ratio red/green represents the incidence of the depolarization of the mitochondria. The proton ionophor Carbonyl Cyanide m-Chlorophenyl Hydrazone (CCCP) was used as a positive control. It is well established that CCCP causes depolarization of the $\Delta\psi_m$ [Chelli et al., 2004]. Briefly, for our assays, samples of confluent cells were collected and centrifuged at 1,200×*g* for 10 min. Cell pellets were re-suspended in 1 μg/ml JC-1 solution in PBS and incubated at 37°C for 30 min in dark conditions. After incubation, the cells were centrifuged at 1,200×*g* for 10 min, and re-suspended in 0.5 ml PBS. Then, the cell suspensions were transferred into 5 ml FALCON® FACS tubes and analyzed with the flow cytometer using CellQuest software.

[^{3}H]PK 11195 BINDING ASSAYS

Binding assays with the TSPO specific tritiated ligand [^{3}H]PK 11195, were conducted as previously described [Awad and Gavish, 1987; Kelly-Hershkowitz et al., 1998; Carmel et al., 1999]. [^{3}H]PK 11195 was obtained from New England Nuclear (Boston, MA); unlabeled PK 11195 was purchased from Sigma–Aldrich, Israel. Briefly, cells were scraped from the flasks, collected in their culture medium, and centrifuged at 1,000×*g* for 10 min, 4°C. Then, the pellet was homogenized in 1 ml of PBS using a Kinematika Polytron (setting 6) for 15 s. Protein content was determined by the method of Bradford [1976]. Binding assays contained 400 μl of cell membrane (0.4 mg of protein/ml) and 25 μl of [^{3}H]PK 11195 solution (final concentration, 6 nM) in the absence (total binding) or presence (nonspecific binding) of 75 μl unlabeled PK 11195 (final concentration, 10 μM), up to a final volume of 500 μl. After incubation for 90 min at 4°C, the samples were filtered under vacuum over Whatman GF/B filters and washed three times with phosphate buffer. Filters were placed in vials containing 4 ml of Opti-Fluor and counted for radioactivity in a scintillation counter after 12 h with a 1600CA Tri-Carb liquid scintillation analyzer. The maximal number of binding sites (Bmax) and the equilibrium dissociation constants were calculated from the saturation curve of [^{3}H] PK 11195 binding, using Scatchard analysis.

STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA), followed by Tukey–Kramer post hoc analysis carried out as appropriate (unless mentioned otherwise). The criterion for statistical significance was $P < 0.05$. Results are expressed as means ± SD (unless mentioned otherwise).

RESULTS

THE EFFECT OF DIFFERENT SALIVARY CONCENTRATIONS ON LUNG CANCER CELL VIABILITY FOLLOWING CS EXPOSURE

Addition of saliva induced a viability reduction of the H1299 cells in a time-dependent fashion. When exposed to CS in the absence of saliva, the cell survival rate was reduced to 42%. Addition of saliva to the medium prior to exposure to CS resulted in a synergistic lethal effect, in which increased salivary concentration in the incubation medium resulted in decreased viability. When the culture medium contained 20%, 25%, and 30% saliva concentrations, survival rate decreased to 26%, 21%, and 18%, respectively. We chose to use a 30% concentration of saliva for further experiments.

TIME-DEPENDENCE EFFECT OF CS AND REDOX ACTIVE IRON CONTAINING MEDIA (SALIVA) ON CELL SURVIVAL

In order to evaluate the time-dependence effect of CS and saliva on cell viability, H1299 cells were incubated for different time periods in the presence or in the absence of saliva while exposed to CS. Cell viability was evaluated by the trypan blue exclusion dye assay. The

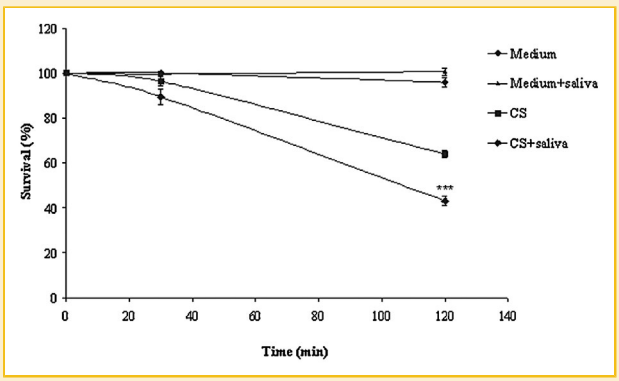

Fig. 1. Time-dependence effect of CS exposure and saliva on the H1299 cell survival rate. Cells were incubated in medium without saliva (●), medium with saliva (▲), medium without saliva and exposed to CS (■), medium with saliva and exposed to CS (◆). Cell viability was performed following 30 min (n = 5) and 120 min (n = 11) of CS exposure in the presence and the absence of saliva. ***$P < 0.001$ designates a comparison to cells that were exposed to CS without saliva, n = 5 in each group.

addition of saliva to the medium during exposure to CS resulted in a synergistic lethal effect and significant reduction in the survival rate (Fig. 1). CS exposure for 120 min without saliva resulted in 37% cell death, while in the presence of saliva the reduction in cell survival was 57% ($P < 0.001$, n = 11). A similar effect was already observed after 30 min of exposure. After 30 min, however, this trend was not yet statistically significant. No reduction in survival rate was found in the control cells.

MITOCHONDRIAL POTENTIAL ($\Delta\Psi_M$)

H1299 lung cancer cells were exposed for 120 min to CS, in the presence as well as in the absence of saliva. JC-1 incorporation was measured by the flow cytometry method (FACS). CS induced a significant drop in $\Delta\Psi_m$ by 42.5 ± 4%, as compared to the level prior to the beginning of CS exposure (time point 0). Addition of saliva did not lead to further loss of $\Delta\Psi_m$ (by 39.85% ± 4%), (Table I). Thus, $\Delta\Psi_m$ disruption did not relate to the synergistic effect between CS exposure and saliva.

MITOCHONDRIAL OXIDATIVE DAMAGE—CARDIOLIPIN PEROXIDATION ANALYSIS

The extent of NAO binding to mitochondrial CL was significantly reduced by 59.7 ± 5% following 120 min of CS exposure per se ($P < 0.01$, n = 3), as compared to the NAO binding level prior to the beginning of CS exposure (time point 0). An additional decrease in the extent of NAO staining was observed in cells that were exposed to CS in the presence of saliva in a moderate yet significant manner ($P < 0.05$), (Table I). The extent of NAO binding to mitochondrial CL was significantly reduced by 68 ± 4% following 120 min of CS exposure in the presence of saliva ($P < 0.01$, n = 3).

TSPO [^{3}H]PK 11195 BINDING IN H1299 CELLS AND THE EFFECT OF CS ON TSPO BINDING

We characterized TSPO binding in H1299 lung cancer membranes using [^{3}H]PK 11195 as a radioligand. Scatchard analysis of saturation curve of [^{3}H]PK 11195 binding (0.2–6 nM final concentration) yielded a straight-line plot (R = 0.9) (Fig. 2). The average B_{max} value was 3274 ± 787 fmol/mg of protein, and the average K_d value was 9.2 ± 1.3 nM.

TSPO BINDING CHARACTERISTICS FOLLOWING CS EXPOSURE

In order to examine the possible role of TSPO regarding the damage induced by free radicals originating from CS, H1299 lung cancer cells were exposed to CS in the presence or absence of saliva for 60 min. TSPO binding in H1299 homogenates was detected using a single concentration of [3H]PK 11195 (6 nM final concentration) and is presented in Figure 3. More than a twofold reduction ($P < 0.01$, n = 3) of TSPO ligand binding was observed in cells that were exposed to CS for 60 min. Addition of saliva to the medium prior to CS exposure for 60 min revealed a similar TSPO binding reduction ($P < 0.01$, n = 3).

TABLE I. Mean Levels of Mitochondrial Potential ($\Delta\Psi_m$) and Cardiolipin Peroxidation Following CS Exposure in the Presence or Absence of Saliva as Compared With the Levels Prior to CS Exposure

Analysis	CS	CS + Saliva	*P*-value
Mitochondrial potential ($\Delta\Psi_m$)	−42.5%	−39.5%	NS
Cardiolipin peroxidation	−59.7	−68%	<0.05

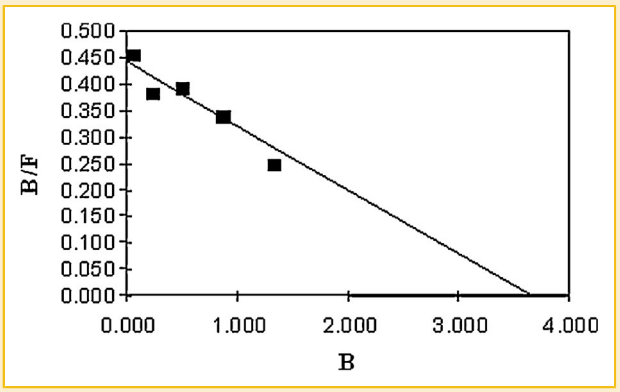

Fig. 2. A representative Scatchard plot analysis of the saturation curve of [3H]PK11195 specific binding of control H1299 cells. B; bound (fmol/mg protein). F; free (nM). R = 0.9.

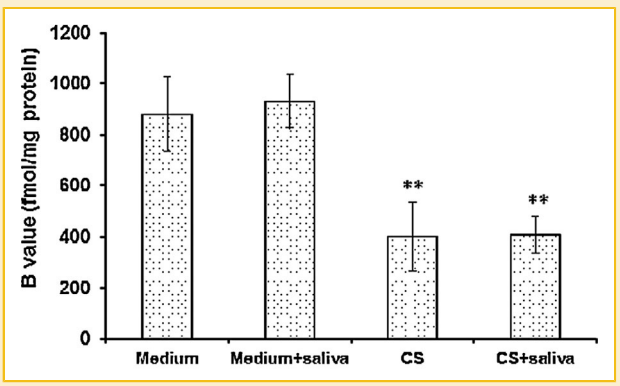

Fig. 3. TSPO binding (B) values (means ± SD) detected at a single concentration of [3H]PK 11195 (6 nM final concentration) in H1299 cells following exposure to CS and saliva for 60 min. **$P < 0.01$ designates a

THE MODULATORY ROLE OF DIAZEPAM AND CLONAZEPAM ON THE SYNERGISTIC EFFECT OF CS AND REDOX ACTIVE IRON CONTAINING MEDIA (SALIVA) ON H1299 CELLS SURVIVAL

Following CS exposure per se, addition of diazepam (a TSPO specific ligand in peripheral tissues) to the medium resulted in a further cellular loss. The survival loss following CS without diazepam was of 32% while following CS exposure in the presence of diazepam it was of 50% ($P < 0.05$, n = 5), (Fig. 4). Interestingly, application of diazepam to cells exposed to CS in the presence of saliva resulted in an enhanced protective effect. While the survival loss following CS exposure in the presence of saliva without diazepam was 56%, it dropped to 37% following the addition of diazepam prior to CS exposure ($P < 0.05$, n = 5), (Fig. 4).

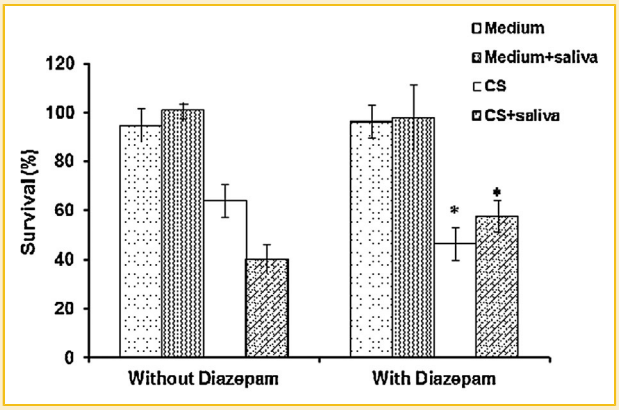

Fig. 4. The effect of diazepam on H1299 survival rates (means ± SD) following exposure to CS and saliva. Cells were incubated in the presence of medium without saliva (diagonal stripes), medium with saliva (stripes), medium without saliva and exposed to CS (inlaid), and medium with saliva and exposed to CS (rhomb). The experimental groups of cells were incubated with 10^{-8} M diazepam prior to the exposure of CS and saliva. *$P < 0.05$ as compared to cells that were exposed to CS with or without saliva, but not incubated with diazepam, n = 5 in each group.

Following CS exposure *per se*, addition of clonazepam (a central benzodiazepine receptor ligand which is not specific to TSPO) to the medium resulted in a further cellular loss. Hence the survival loss following CS without clonazepam was of 31% while following CS exposure in the presence of clonazepam it was of 47% ($p<0.05$, n=5), (Fig. 5). However, application of clonazepam to cells exposed to CS in the presence of saliva, did not demonstrate a protective effect. Thus, while the survival loss following CS exposure in the presence of saliva without clonazepam was of 52%, it was of 62% following the addition of clonazepam prior to CS exposure, (Fig. 5).

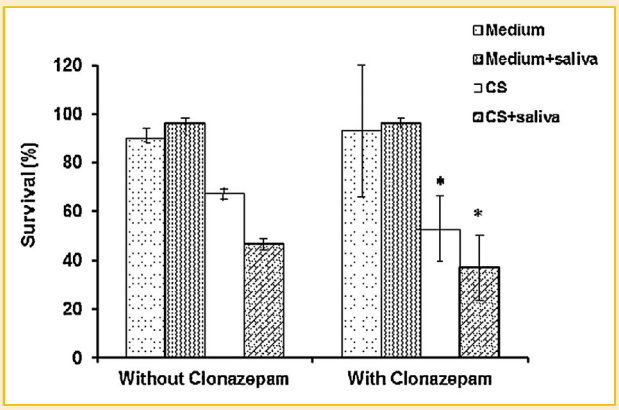

Fig. 5. The effect of clonazepam on H1299 survival rates (means ± SD) following exposure to CS and saliva. Cells were incubated in the presence of medium without saliva (diagonal stripes), medium with saliva (stripes), medium without saliva and exposed to CS (inlaid), and medium with saliva and exposed to CS (rhomb). The experimental groups of cells were incubated with 10^{-8} M clonazepam prior to the exposure of CS and saliva. *$P < 0.05$ as compared to cells that were exposed to CS with or without saliva, but not incubated with diazepam, n = 5 in each group.

DISCUSSION

A correlation between saliva concentration in the cell's medium and cell survival following CS exposure was found. Maximal lethal synergistic effect was demonstrated at a saliva concentration of 30%, and accordingly we used this concentration in our experiments. CS exposure (without saliva) induced a time-dependent reduction in cell survival, which was maximal following 120 min of exposure (37% survival loss). Addition of saliva to the cells' medium prior to CS exposure resulted in a lethal synergistic effect, demonstrated by a 1.6-fold increase in cellular death at 120 min (57% survival loss). We assumed that CS exposure in the presence of saliva results in extensively-induced oxidative stress, accompanied by an increased number of apoptotic cells. CS exposure of 120 min is expected to result in cells undergoing early apoptosis, and one expects the cells to be stained by trypan blue, as indeed was demonstrated.

The exact mechanism underlying the rapid reduction we observed in cell survival is as yet unknown. However, protein structural modification, which resulted in the increased level of carbonylation [Nagler et al., 2010b] [3], may be one possible explanation. Protein structural modifications in the lung matrix were suggested to be a key cause for their degradation, which leads to degenerative diseases such as emphysema [Banerjee et al., 2007].

Oxidative stress and ROS are important inducers of apoptosis, as they are accompanied by changes in $\Delta\Psi_m$. The $\Delta\Psi_m$ reduction is a general feature of cell death. Decrease of the $\Delta\Psi_m$ characterizes an early stage of apoptosis, preceding other manifestations of apoptosis as DNA fragmentation, ROS production, and increase in membrane permeability. The $\Delta\Psi_m$ is a key marker of mitochondrial function, and thus alteration in $\Delta\Psi_m$ is considered an early, irreversible, and universal event in the cell death process. We wanted to investigate a possible correlation between exposure to CS in the presence of saliva and $\Delta\Psi$m reduction. We found similar $\Delta\Psi$m reduction rates in cells following CS exposure both in the presence and the absence of saliva.

These results suggest that CS causes mitochondrial dysfunction in human lung cancer cells, H1299. According to our results, $\Delta\Psi_m$ reduction following CS exposure is a non-salivary mediated phenomenon, as shown by the fact that saliva presence did not cause any additional reduction induced by the CS in $\Delta\Psi_m$. The mechanism underlying CS-induced depolarization of the mitochondrial membrane is not yet elucidated. Yet, CS-borne derivatives may be responsible.

Lipid peroxidation is one of the destructive effects induced by ROS overproduction. It may lead to cell membrane disruption, which in turn results in a release of protein-catabolic enzymes and subsequent cell death. Lipid peroxidation in the mitochondrial membrane can be detected by the fluorescent probe NAO as previously explained. NAO binds specifically to the CL monolayer in the mitochondrial membrane, but not to oxidized CL's or to any other type of phospholipids. Cardiolipins, which are polyunsaturated acidic

phospholipids, are found exclusively on the mitochondrial membrane, where they are intimately associated with the enzyme complexes of the respiratory chain. Cardiolipins in mitochondrial membranes contain a substantial level of highly unsaturated fatty acids and are therefore sensitive to oxidation. Decrease in the cellular NAO fluorescence is thus considered a reflection of CL peroxidation [Petit et al., 1992]. Reduction in NAO fluorescence following CS exposure is probably a result of the mitochondrial CL peroxidation and enhanced in the presence of saliva. We conclude that a CL oxidation process took place.

Mitochondria play a crucial role in the induction of apoptosis. Cytochrome complex (cyt c) release from mitochondria that triggers caspase activation appears to be largely mediated by reactive oxygen species. Cyt c is bound to the CL molecules in the outer surface of the inner mitochondrial membrane. It would be expected that oxidative damage to CL by ROS may disturb the interaction of cyt c with these phospholipids and result in a dissociation of cyt c from the membrane, enabling its release into the extramitochondrial space [Subramanian et al., 1998; Tuominen et al., 2002]. According to the present study, CS exposure induces a $\Delta\Psi_m$ depletion and increases lipid peroxidation of CL. All together, this contributes to our knowledge that CS causes mitochondrial-mediated ROS oxidative damage that finally leads to apoptosis. Our currently reported result of the reduction in NAO following CS exposure which is further intensified by a redox iron gains further significance with respect to lung cancer due to a review just recently published by Zhong and Yin [2015]. In their report, the authors summarize recent knowledge concerning the role of oxidative stress-induced lipid peroxidation products in the pathogenesis of cancer. They focus on the involvement of mitochondria with respect to the generation of 4-hydroxynonenal (4-HNE) from oxidation of mitochondria-specific phospholipid cardiolipin and its effects on proteins and DNA. Noteworthy, in two other recently published papers, the important role of both copper and iron in the promotion of lung carcinogenesis and metastasis in general are elucidated [MacDonald et al., 2014; Xiong et al., 2014]. However, both the Trypan blue exclusion dye assay and the Cardiolipin peroxidation assay are based on measuring membrane disintegration, and membrane permeability may be transiently affected by various experimental conditions. Therefore, we intend to study cell viability in the future with other assays which are based on other principles, such as the Alamar Blue assay or the MTT assay. Moreover, in this study we also intend to examine the CS effects on the salivary redox activity employing the ascorbate addition assay we have used in the past [Nagler et al., 1997]. Noteworthy is that in a preliminary study, we found that the total protein concentration in the saliva was not altered following its exposure to CS (data not shown). This supports the conclusion that the CS effect on saliva is specific and not general, as will be further explained.

Scatchard analysis in H1299 cells that have not been exposed to CS revealed a straight-line plot. The maximal number of binding sites (Bmax) was $3{,}274 \pm 787$ fmol/mg protein and the equilibrium dissociation constant (Kd) was 9.2 ± 1.3 nM. More than a twofold reduction in TSPO binding (B) value was demonstrated following CS exposure by 6 nM [^{3}H]PK 11195 specific ligand binding, irrespective of salivary presence in the culture medium.

Previous studies have shown that diazepam can modulate an antioxidant response and reduce oxidative phenomena during short-term administration in subcellular preparations of rat brain regions [Musavi and Kakkar, 2000]. It was also shown that a single dose of diazepam can cause free radical mediated changes and that the antioxidant defense response appears to be tissue-region specific [Musavi and Kakkar, 1998]. In the present study, the benzodiazepine diazepam, a TSPO specific ligand in peripheral tissues, partially prevented the decrease in cell survival following CS and redox active iron containing media (saliva) exposure. In contrast, the benzodiazepine clonazepam which is not TSPO specific but rather a central diazepine receptor ligand did not show a similar effect. These results indicate that this effect is a TSPO-specific effect.

The current study demonstrates that exposure of the epithelial lung cancer cells H1299 to CS, results in alternation of biomolecules expressed by structural (CLs peroxidation) damage. This irreversible type of damage is enhanced in the presence of saliva. Structural damage can be demonstrated by modulation of TSPO in its binding capacity and depletion of the mitochondrial potential. Taken together, all of the modulations listed above may result in cellular death increases following CS exposure, which seems to be enhanced in the presence of saliva.

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