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Short communication

Cigarette smoke-induced reduction in binding of the salivary translocator protein is not mediated by free radicals

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Oral cancer is the most common malignancy of the head and neck and its main inducer is exposure to cigarette smoke (CS) in the presence of saliva. It is commonly accepted that CS contributes to the pathogenesis of oral cancer via reactive free radicals and volatile aldehydes. The 18 kDa translocator protein (TSPO) is an intracellular receptor involved in proliferation and apoptosis, and has been linked to various types of cancer. The presence of TSPO in human saliva has been linked to oral cancer, and its binding affinity to its ligand is reduced following exposure to CS. In the present study we wished to further investigate the mechanism behind the CS-induced reduction of TSPO binding by exploring the possible mediatory role of reactive oxygen species (ROS) and volatile aldehydes in this process. We first analyzed TSPO binding in control saliva and in saliva exposed to CS in the presence and absence of various antioxidants. These experiments found that TSPO binding ability was not reversed by any of the antioxidants added, suggesting that CS exerts its effect on TSPO via mechanisms that do not involve volatile aldehydes and free radicals tested. Next, we analyzed TSPO binding in saliva following addition of exogenous ROS in the form of H_2O_2. These experiments found that TSPO binding was enhanced due to the treatment, once again showing that the CS-induced TSPO binding reduction is not mediated by this common form of ROS. However, the previously reported CS-induced reduction in salivary TSPO binding together with the role of TSPO in cells and its link to cancer strongly suggest that TSPO has a critical role in the pathogenesis of CS-induced oral cancer. The importance of further elucidating the mechanisms behind it should be emphasized.

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1. Introduction

Limited research has been done to reveal the pathogenesis of oral cancer in general, and the possible involvement of TSPO in particular. The main inducer for this type of cancer is cigarette smoke (CS), likely due to the creation of reactive oxygen species (ROS) [1,2]. TSPO has been reported to be significantly affected by ROS [3,4], studies suggesting that it plays a protective role against ROS cellular damage [5,6]. TSPO levels have been shown to correlate to resistance to H_2O_2 toxicity in hematopoietic cells, which is significantly increased by transfection of a TSPO-encoding vector [7]. Additionally, it has been found that TSPO complexes (covalent TSPO polymers) are produced in response to ultra-violet irradiation-induced ROS in cancer cells *in vitro* and *in vivo* [8]. TSPO is involved in major cellular processes such as proliferation and apoptosis [9,10], and has been found to positively correlate with tumorigenicity in various cancer tissues [11,12].

Our group has previously reported the presence of TSPO in human saliva, and found that its expression is enhanced in oral cancer tissues [13]. Additionally, we found that following exposure to CS, the TSPO binding affinity is reduced both *in vivo* and *in vitro* [13]. Our findings, together with the previously reported correlation between TSPO and free radicals, have led us to explore the possible role of free radicals in the CS-induced reduction in TSPO binding affinity.

For this purpose we performed two sets of experiments. In the first set we tested TSPO binding following addition of various

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antioxidants to saliva prior to CS exposure. Antioxidants act as free radical scavengers [14] and we investigated whether they were able to prevent, at least in a partial manner, the TSPO binding reduction caused by CS. Such outcome would have indicated that the specific form of free radical targeted by the specific antioxidant mediates the CS effect on TSPO. Each antioxidant used in our assay targets a different form of free radicals previously reported to be present in CS, as follows: the aldehyde scavenger, glutathione (GSH) [15,16], the cyanide chelator, hydroxocobalamin [17,18], the iron chelator, deferoxamine (desferal) [16], and the copper chelator, penicillamine [19]. Our second set of experiments was based on a more direct approach, which consisted of addition of exogenous ROS in the form of H_2O_2 to saliva, followed by TSPO binding and cell viability analyses. Our decision to use H_2O_2 was based on the fact that it is a common type of ROS that has been shown to induce oxidative stress in cells [20].

2. Methods

2.1. Saliva collection

Saliva samples were collected as previously described [13]. Following collection, saliva samples were transferred to polypropylene tubes and stored at −70 °C for further analysis.

2.2. Exposure of saliva to cigarette smoke in vitro

In order to expose saliva to CS, a cigarette was combined with a vacuum system to enable the inhalation of gas-phase cigarette smoke into a sealed apparatus, as previously described [13].

2.3. TSPO binding analysis

For assays of the TSPO binding characteristics in saliva, samples were thawed and immediately centrifuged (800× g, 10 min, 4 °C). The pellets, containing the cellular fractions, were suspended in ice cold PBS and then homogenized using a Kinematika Polytron (setting 6) for 10 s. Protein concentrations were determined and binding of $[^3H]$PK 11195 to cell membranes was conducted, as previously described [13]. The reaction mixture contained 400 μl of the homogenized cell membranes (25 μg protein) and 25 μl of $[^3H]$ PK 11195 (0.2–12 nM final concentration), in the absence (total binding) or in the presence (non-specific binding) of 10 μM unlabeled PK 11195. After 90 min of incubation at 4 °C, the cell membranes were filtered through Whatman CF/C filters using a vacuum system, washed three times with 4 ml of 5 mM ice cold phosphate buffer and placed in vials containing 5 ml of Opti-Fluor (Packard Bioscience, Groningen, The Netherlands). Radioactivity was counted after 12 h with a 1600 CA Tri-Carb liquid scintillation analyzer (Packard – Global Medical Instrumentation, Ramsey, Minnesota). Specific binding was obtained by subtracting non-specific binding from total binding. The binding values were calculated from the saturation curve of $[^3H]$PK 11195 binding, using Scatchard analysis.

2.4. Protein measurements

Protein concentration required for the binding assay was determined using the method of Bradford et al. [21]. Bradford solution was used for determination of protein concentration and bovine serum albumin (BSA) was used for the standard curve.

2.5. Addition of antioxidants to saliva

The following antioxidant compounds were tested: glutathione (2 mM), hydroxocobalamine (1 mM), desferal (5 mM) and penicillamine (1 mM and 5 mM). All compounds were purchased from Sigma-Aldrich, Israel, and diluted according to the manufacturer's instructions. They were added separately to saliva samples several minutes prior to exposure to CS. Control samples contained the same volume of buffer used to solubilize the antioxidants, and were used for standardization. With the addition of each compound, the saliva samples were mixed well and exposed to CS for 60 min. Following CS-exposure, the saliva samples were washed three times with phosphate buffer saline (PBS) in order to dismiss all possible compound remains. Next, we used these samples for binding analysis.

2.6. Exposure of saliva to H_2O_2

H_2O_2 was added to saliva samples in order to examine the effect of ROS on TSPO binding. The concentration applied was based on previous studies performed by our group with the human neuroblastoma SHSY-5Y cell line (not published). In these studies, the cells were exposed to increasing concentrations of H_2O_2 (ranging from 50 to 250 μM) for two hours. The TSPO binding was tested and found to be significantly increased in response to 200 μM H_2O_2. Here we prepared a 200 μM H_2O_2 solution from a stock solution (purchased from Sigma–Aldrich Israel) according to manufacturer's instructions on the day of the experiment. Following exposure to H_2O_2 for a period of 60 min, saliva samples were washed three times with phosphate buffer saline (PBS) and immediately used them for cell-viability and binding analysis.

2.7. Statistical analysis

Results are expressed as means ± SE. The n values for the experimental and control groups were larger than 5. Statistical significance was determined by a Student's *t*-test. The criterion for statistical significance was $p < 0.05$.

3. Results

3.1. Antioxidants tested are not capable of reversing the negative effect of CS on TSPO binding

In order to examine whether the reduction in TSPO binding following CS exposure is due to ROS and/or aldehydes, we tested TSPO binding following CS exposure in the presence of the following antioxidants, as described in the introduction section: penicillamine (1 mM and 5 mM), hydroxocobalamine (1 mM), desferal (5 mM), and glutathione (2 mM). Our assumption was that when added to saliva samples prior to CS exposure, the antioxidants would react with CS-borne reactive free radicals, therefore preventing any effect that these radicals might have on TSPO binding. Following addition of antioxidants and CS-exposure, the samples were used in binding assays with $[^3H]$PK 11195 (final concentration 6 nM). The results showed a ~30% reduction in binding in all CS-exposed samples as compared to their controls, with no regard to presence of any antioxidant tested. The mean binding values, as calculated from at least three independent experiments, each containing at least three saliva samples and tested in duplicates, are presented in Fig. 1.

3.2. The addition of H_2O_2 leads to an increase in TSPO binding

In order to further investigate this finding, we applied H_2O_2 to saliva samples in order to examine the specific effect of ROS on TSPO binding. Following exposure of saliva samples to 200 μM of H_2O_2 for a period of 60 min we tested TSPO binding with the $[^3H]$PK 11195 ligand (final concentration 6 nM). The mean ± SD binding, as

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