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COVID-19 and Smoking. Is Nicotine the Hidden Link?

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Leung *et al.* have recently published in the *European Respiratory Journal* a paper on the expression of ACE-2 in the small airway epithelia of smokers and COPD Patients, discussing its effects on the risk of severe COVID-19 [1]. The authors found an increased expression of the ACE-2 gene in the airways of subjects with COPD and in current smokers. Indeed, a recent systematic review reporting data on thesmoking habit of patients infected with Severe Acute Respiratory Syndrome Coronavirus (SARS CoV)-2, concluded that smoking may be likely associated with a negative progression of the disease and with the adverse outcome [2]. These conclusions were challenged in a correspondence published by *Hua Cai* on the basis that a reliable mechanism explaining this association was missing [3]. The need for these results to be supported by additional studies is quite clear, but we believe that a robust mechanistic explanation exists. Nicotine has a known influence on the homeostasis of the renin-angiotensin system (RAS) up-regulating the angiotensin-converting enzyme (ACE)/angiotensin (ANG)-II/ANG II type 1 receptor axis, and down-regulating the compensatory ACE2/ANG-(1–7)/Mas receptor axis, contributing in turn to the development of cardiovascular and pulmonary diseases [4]. Different airway cells, such as bronchial epithelial cells, type II alveolar epithelial cells, and interstitial fibroblasts lung, express nicotinic acetylcholine receptors (nAChR), specifically the α7subtype [5]. All these cells express components of the RAS[4].In addition, Nicotine increases the expression and/or activity of ACE in the lung[4], an increase which have been found also in the serum of smokers, and that required at least 20 minutes to return to control level[4]. ACE2 serves as a physiologically relevant cellular entry receptor for SARSCoV, for the human respiratory Coronavirus NL63, and probably

for the (SARSCoV)-2[6].The ACE binds the SARS CoV-2 S protein, and through its tissutal expression mediates the localization and the efficiency of the infection[6].Moreover, Nicotine induces the epithelial-mesenchymal transition (EMT)[5,7], a mechanism sufficient to allow "normal" differentiated cells to acquire the stem cell-like characteristics and properties. We planned experiments on human bronchial epithelial cells (HBEpC), obtained from Cell Applications Inc. (www.cellapplications.com/product no. 502K-05a).Cells were maintained as adherent monolayer in complete bronchial/tracheal epithelial cell growth medium (www.cellapplications.com/product) at 37°C in a 95% air/5% CO_2, seeded at an initial density of 7.5×10^4 cells/cm^2, and sub-cultured with a 0.25% trypsin–1mM EDTA solution (Sigma-Aldrich, Milan, Italy) when cultures reached 80% confluence. HBEpC are derived from the surface epithelium of normal human bronchi non-diseased (i.e. Asthma, COPD, or Type 2 Diabetes). The morphology is consistent with epithelial origin, and is positive for epithelial cell marker cytokeratin 18. Semi-confluent HBEpC at 4^{th} passage (7.5×10^4 cells/cm^2) were treated:*(a)* for 1 h with zero or 1.0×10^{-7}M Nicotine (Sigma–Aldrich, Milan, Italy) dissolved in saline in complete medium; *(b)* with 1.0×10^{-6} M α-Bungarotoxin (α-BTX, Sigma–Aldrich, Milan, Italy)dissolved in saline, in the continued presence of nicotine at zero or 1.0×10^{-7}M for 1 h; *(c)* treated continuously with nicotine for additional passages, 1 passage every 48 h for a total of 16 passages. We showed, for the first time, that nicotine at 1×10^{-7} M (the concentration present on the alveolar lining fluids after one cigarette is in the range 6×10^{-6} to 6×10^{-5} M [5]is able to increase ACE2 (Figure 1A) in HBEpC. Treatment with nicotine induces phospho-S6 ribosomal protein (Ser235/236), Akt1, phospho-Akt (Ser473), phospho-Akt(Thr308) and phospho-p44/42 MAPK (Thr202/Tyr204) (Fig 1B).To verify the hypothesis that ACE2 is induced by nicotine through α7-nAChR, HBEpC, at 4^{th} passage,

in the exponential growth phase, plated at a density of 1 *x* 10^6 cells/ml, were incubated withα7-nAChR siRNA (0.1 μg) diluted in 100 μl of siRNA transfection medium. Transfection was performed as described by Li *et al.* [8], who transfected, successfully, HBE16 human airway epithelial cell line (unaffected cells).A clone of transfected HBEpC that did not express α7-nAChR proteins, also after treatment with nicotine, (Figure 1C) and is not able to induce phospho-S6 ribosomal protein (Ser235/236), Akt1, phospho-Akt (Ser473), phospho-Akt(Thr308) and phospho-p44/42 MAPK (Thr202/Tyr204) after nicotine treatment (Fig 1D), was selected for further experiments. Nicotine did not induce ACE2 in this clone (si-mRNA-α7-HBEpC) (Figure 1A). This observation supports the hypothesis that ACE2 increase is specifically mediated by α7-nAChR. Moreover, when HBEpC were incubated simultaneously with nicotine and α-BTX, an α7 nicotine antagonist [9],no induction of ACE2 was observed (Fig 1D). Importantly, treatment with Nicotine, α-BTX or with the combination is not cytotoxic (data not shown).On these bases, we suggest that smoking may promote cellular uptake mechanisms of SARS CoV-2 through α7-nAChR signaling.A possible α7-nAChR down-stream mechanism may be the induction of phospho-Akt and phospho-p44/42 MAPK. This mechanism was hypothesized, partially, by *Olds and Kabbani* on their schematic model explaining how nicotine exposure increases the risk of COVID-19 entry into lung cells [10].α7-nAChRis present both in neuronal and non-neuronal cells (i.e. lung, endothelial, lymphocyte) consequently smoking may impact COVID-19 pathophysiology and clinical outcome in several organ systems including brain.

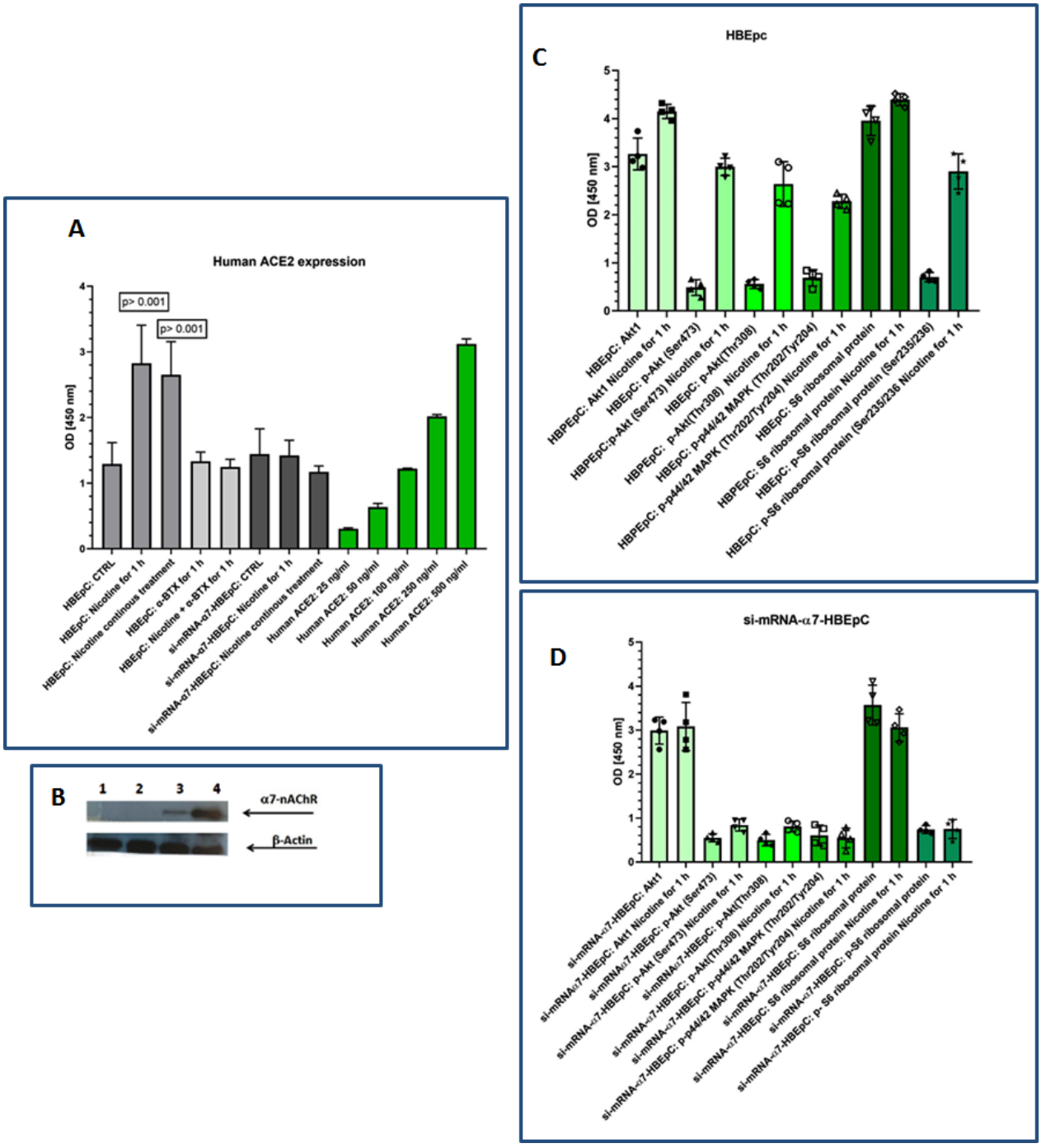

FIGURE LEGEND

Figure 1. Effect of nicotine on HBEpC or si-mRNA-α7-HBEpC

Panel A: ACE2 detection. ACE2 was measured with Human ACE2 ELISA Kit ab235649 (www.abcam.com/human-ace2-elisa-kit-ab235649.html) according to manufacture instructions. Data are mean ± SE; p was evaluated using *t* test. The green plots are the human ACE2 standard. Experiments were performed twice in triplicate.

Panel B: Induction of phospho-S6 ribosomal protein (Ser235/236), Akt1, phospho-Akt (Ser473), phospho-Akt(Thr308) and phospho-p44/42 MAPK (Thr202/Tyr204) in HBEpC. Data were obtained using PathScan® cell growth Multi-target Sandwich ELISA kit n.7239 (Cell signaling) PathScan® Cell Growth Multi-Target Sandwich ELISA Kit is a solid phase sandwich enzyme linked immunosorbent assay (ELISA) that combines the reagents necessary to detect endogenous levels of S6 ribosomal protein, phospho-S6 ribosomal protein (Ser235/236), Akt1, phospho-Akt (Ser473), phospho-Akt(Thr308) and phospho-p44/42 MAPK (Thr202/Tyr204).Data are mean ± SE; p was evaluated using t test.. Experiments were performed twice in duplicate.

Panel C: α7-nAChR protein detection.

Western Blotting was performed as described previously [11]. Human α7-nAchR antibody NBP1-49348 was purchased by Novus Biologicals [www.novusbio.com]

1-2 si-mRNA-α7-HBEpC treated with zero (lane 1) or 1.0 $x10^{-7}$M Nicotine (lane 2) for 1 h

3-4 HBEpC treated with zero (lane 3) or 1.0 $x10^{-7}$M Nicotine (lane 4) for 1 h

Experiments were performed twice.

Panel D: as in Panel B, treated cells are si-mRNA-α7-HBEpC

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