

Research Article

Experimental data using candesartan and captopril indicate no double-edged sword effect in COVID-19

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The key link between renin–angiotensin system (RAS) and COVID-19 is ACE2 (angiotensin-converting enzyme 2), which acts as a double-edged sword, because ACE2 increases the tissue anti-inflammatory response but it is also the entry receptor for the virus. There is an important controversy on several drugs that regulate RAS activity and possibly ACE2, and are widely used, particularly by patients most vulnerable to severe COVID-19. In the lung of healthy rats, we observed that candesartan (an angiotensin type-1, AT1, receptor blocker; ARB) and captopril (an ACE inhibitor; ACEI) up-regulated expression of tissue ACE2 and RAS anti-inflammatory axis receptors (AT2 and Mas receptors). This effect was particularly pronounced in rats with metabolic syndrome (obesity, increased blood pressure and hyperglycemia) and aged rats. Treatment of cultures of human type-II pneumocytes with candesartan or captopril induced up-regulation of ACE2 expression in cells. Treatment with viral spike protein induced a decrease in full-length (i.e. transmembrane) ACE2, an increase in levels of a short intracellular ACE2 polypeptide and an increase in ADAM17 activity in cells, together with an increase in levels of soluble ACE2 and major proinflammatory cytokines in the culture medium. Spike protein-induced changes and levels of spike protein internalization in cells were inhibited by pretreatment with the above-mentioned drugs. The results suggest that these drugs increase ACE2 levels and promote the anti-inflammatory RAS axis in the lung. Furthermore, possible up-regulation of viral entry by the drug-induced increase in expression of transmembrane ACE2 is counteracted by additional mechanisms, particularly by drug-induced inhibition of ADAM17 activity.

Introduction

The renin–angiotensin system (RAS) was initially considered as a circulating humoral system, with functions in regulating blood pressure and sodium and water homeostasis, and its role in the hypertension and several cardiovascular and renal diseases is classically known. However, more recent studies have shown that RAS dysregulation is also involved in the inflammatory response associated with many other diseases [1], including the proinflammatory state associated with obesity and metabolic syndrome [2,3] and aging-related processes [4,5]. In addition to the classic or circulating (i.e. endocrine) RAS, there are tissue (i.e. paracrine) RAS in most organs [6,7], and also an intracellular or intracrine RAS [8]. The RAS is basically organized into two arms that counteract each other and must be correctly balanced [9,10]: a proinflammatory and pro-oxidative axis mainly constituted by angiotensin II (AngII) and angiotensin type 1 (AT1) receptors, and an anti-inflammatory antioxidative axis formed by AngII/AT2 receptors, and particularly Ang1-7 /Mas receptors (MasR) (see diagram in Supplementary Figure S1). Angiotensin-converting enzyme 2 (ACE2) plays a key role in the balance, because ACE2 transforms components of the pro-inflammatory axis (AngI and particularly AngII) into components of the anti-inflammatory axis

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(Ang1-9, and particularly Ang1-7). ACE inhibitors (ACEIs) and antagonists of AT1 receptors (ARBs) are two of the most widely used antihypertensive drugs. However, they have also been suggested for treatment of several of the above-mentioned diseases mediated by inflammatory processes [1]. The potential anti-inflammatory antioxidative mechanism involved in their effects is related to inhibition of the proinflammatory axis activity by inhibition of AngII synthesis (ACEIs) or blockage of AT1 receptors (see Supplementary Figure S1 for details).

Many recent studies have highlighted the major role of tissue RAS in severity of COVID-19 [11–13]. ACE2, the receptor for SARS-CoV-2, arises as the key link between RAS and COVID-19 [14]. ACE2 is a protease that plays its key role as a double-edged sword [15–17]. An increase in ACE2 activity is essential to balance the RAS against the anti-inflammatory response. Consistent with this, several previous studies have shown the protective effects of ACE2 and its product Ang1-7 against experimental lung injuries [10,18,19]. However, ACE2 is also the entry receptor for the virus [20–22] and an increase in ACE2 expression may increase cell infection. Furthermore, viral binding decreases the levels of ACE2 at the cell surface [23,24], thus shifting the balance toward inflammation, fibrosis and progression of disease severity. Therefore, a major question that has been insistently raised is whether we should promote the increase in ACE2 levels in tissues to reduce inflammation, or to decrease in ACE2 levels to reduce viral entry and replication. The lung is obviously a major target of research. The controversy has been particularly focused on several drugs that regulate RAS activity and are used by millions of patients of chronic diseases such as cardiovascular diseases, hypertension or diabetes. These drugs are ARBs (i.e. sartans) and ACEIs. Moreover, groups of patients that appear particularly vulnerable to COVID-19 such as elders, diabetics, obese and cardiovascular and hypertensive patients [25,26] are the most frequent users of these drugs.

Over the last few months, a large number of articles have focused on whether these drugs increase or decrease tissue levels (particularly lung levels) of ACE2 and whether this may increase or decrease the severity of COVID-19. However, most of them are review or opinion articles reporting that experimental data on this question are scarce and controversial [27–29]. Furthermore, in addition to ACE2, other RAS components may play a major role in COVID-19 severity, and the above-mentioned RAS modulatory drugs may also affect viral entry mechanisms by acting on different factors [30].

In the work reported here, we studied the effects of candesartan (an AT1 receptor blocker) and captopril (an ACEI) on lung levels of ACE2 and major components of lung RAS in young adult rats and in rat models of individuals vulnerable to severe COVID-19, such as rats with metabolic syndrome (obesity, increased blood pressure, hyperglycemia) and aged rats. *In vitro*, we used human alveolar type-II pneumocyte cells to study the effects of these drugs on changes induced by viral spike protein on ACE2 levels, on levels of spike protein internalization, and on the release of major proinflammatory cytokines such as IL-6, TNF- α and CCL-2.

Materials and methods

Experimental design

In a first set of experiments, we tested the effects of the AT1 receptor blocker candesartan and the ACE inhibitor captopril on lung levels of ACE2 and major components of lung RAS (i.e. AT1, AT2 and Mas receptors) using RT-PCR in young adult rats (10 weeks old; $n=30$; $n=6$ per group/treatment). Protein expression of ACE2 and AT1, AT2, and Mas receptors were confirmed by WB. In addition, we compared ACE2 activity levels in lung homogenates of control and treated young adult rats. In a second set of experiments, we used models of vulnerable groups including rats with metabolic syndrome (obesity, increased blood pressure, hyperglycemia; $n=20$; $n=5$ per group/treatment) and aged rats (18–20 months old; $n=18$; $n=6$ per group/treatment). We studied the effects of candesartan and captopril on ACE2 and major RAS receptor expression in lung tissue, relative to young adult controls. In a third set of experiments, we used the human alveolar type-II pneumocyte A549 cell line to study the effects of the above-mentioned drugs on changes induced by SARS-CoV-2 Spike RBD-Fc protein. All data were obtained from at least three independent experiments with at least $n=5$. First, we measured the effect of drugs on ACE2 mRNA expression in A549 cells in culture. Then, we studied the changes of both membrane-bound or intracellular forms of ACE2 (cellular lysate) and soluble released ACE2 (culture medium) after treatment with the viral spike protein with or without 24 or 48 h of preincubation with candesartan or captopril. Next, we investigated the effects of candesartan and captopril on SARS-CoV-2 Spike RBD-Fc internalization rate by measuring cytoplasmic fluorescence intensities of spike protein using confocal laser microscopy. In order to minimize the possibility that changes in levels of internalized spike protein could be related to differences in cell levels of ACE2 receptor, we used ACE2-GFP transiently transfected A549 cells and values of intracellular spike were expressed relative ACE2 levels. Finally, we quantified the effect of viral spike protein, preincubated or not preincubated with candesartan or captopril, on the release of major proinflammatory cytokines such as IL-6, TNF- α and CCL-2 to culture medium.

Cell cultures

Human alveolar type-II pneumocyte cell line A549 (CSC-C6236X, Creative Bioarray) was cultured in DMEM/F12 medium supplemented with 10% FBS, 2 mM L-glutamine (Sigma), 100 U/ml penicillin and 100 µg/ml Streptomycin. Cultures were maintained at 37°C and 5% CO₂ in humidified incubator. Cells were cultured until maximal confluence was reached in order to get high levels of ACE2 in cells [31]. In order to study the interaction of RAS modulators or anti-inflammatory compounds with SARS-CoV-2 Spike RBD-Fc protein, cells were pretreated or not pretreated with the AT1 receptor blocker Candesartan (1 µM; 4791, Tocris) or the ACE inhibitor Captopril (50 ng/ml; C4042, Sigma) for 24 h. Then, 1 µg/ml of SARS-CoV-2 Spike RBD-Fc protein (40592-V02H, Sino Biological) was added to the cells for 3 h at 37°C. Doses were established based on preliminary experiments and previous *in vitro* studies [32,33].

Animal models

Lung tissues from male young adult rats, aged rats and adult rats with Metabolic Syndrome (obesity, increased blood pressure and hyperglycemia) were used. Young adult rats used for metabolic syndrome received a high fat diet with 60% calories fat supplemented with 4% of NaCl (D18042603; Research Diets, U.S.A.) for a period of 18 weeks. In these rats, serum triglycerides (TG), cholesterol and glucose were measured using commercially colorimetric enzymatic kits (1001093; 1001314; 1001191; Spinreact, Spain), according to the manufacturer's instructions. Blood pressure was measured using a noninvasive pressure system MRBP (IITC Life Science, California, U.S.A.). The rats were orally treated or not treated with the AT1 receptor blocker Candesartan (1 or 10 mg/Kg; AstraZeneca) or the ACE inhibitor Captopril (5 or 40 mg/Kg; C4042, Sigma) for 3 weeks. The powered drug was administered orally mixed with "Nocilla" hazelnut cream (Nutrexpa, Barcelona, Spain). Doses were established based on preliminary experiments and previous *in vivo* studies [34–37]. Animal handling was performed in accordance with the Directive 2010/63/EU, European Council Directive 86/609/EEC and the Spanish legislation (RD53/2013). Animal experiments were approved by the corresponding committee at the University of Santiago de Compostela (15005/15/002) and were carried out in the Experimental Biomedicine Centre (CEBEGA; University of Santiago de Compostela). Rats were housed at constant room temperature (RT) (21–22°C) and 12-h light/dark cycle. The animals were euthanised using an overdose (800 mg/Kg) of intraperitoneal sodium pentobarbital and then the tissue samples were obtained.

Western blot analysis

Homogenates from rat lung tissue or A549 cells were lysed in RIPA buffer containing PMSF (Sigma) and protease inhibitor cocktail (Sigma). Total proteins were quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific). An equal amount of protein lysates or cell culture medium was separated on 8–10% Bis-Tris polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were incubated overnight at 4°C with primary antibodies against the extracellular domain of ACE2 (ab108252; Abcam; 1:1000), AT1 (sc-31181; Santa Cruz; 1:200), AT2 (sc-9040; Santa Cruz; 1:200) and Mas (AAR-013; Alomone labs; 1:1000) receptors. Membranes were reincubated with loading controls: anti- α -tubulin (T5168; 1:50,000; Sigma) or GAPDH (G9545; 1:25,000; Sigma), or ponceau (Sigma, P7170-1L). The following horseradish peroxidase (HRP)-conjugated secondary antibodies were used: goat anti-rabbit-HRP and goat anti-mouse-HRP (Santa Cruz Biotechnology; 1:2500). Bound antibody was detected with an Immun-Star HRP Chemiluminescent Kit (Bio-Rad; 170-5044) and visualized with a chemiluminescence detection system (Bio-Rad; Molecular Imager ChemiDoc XRS System). The data were then expressed relative to the value obtained for the control to counteract possible variability among batches.

Specificity of antibodies

The specificity of antibodies against ACE2, AT1, AT2 and Mas receptors was assessed in our laboratory by WB analysis of lysates from HEK293 cells transiently transfected with the corresponding GPCR tagged to fusion tail DDK (i. e. a C-terminal DDK epitope tag DYKDDDDK) or GFP (green fluorescent protein), or preabsorption with the corresponding synthetic peptide antigen (see [9,38]).

RNA extraction and real-time quantitative polymerase chain reaction

Total RNA from rat lung tissues or A549 cell homogenate was extracted with TRIzol (Invitrogen, Paisley, U.K.) following the manufacturer's protocol. Total RNA (2 µg) was reversed transcribed to complementary DNA (cDNA) using nucleoside triphosphate containing deoxyribose, random primers and Moloney murine leukaemia virus (MMLV; Invitrogen, Thermo Fisher Scientific; 200U) reverse transcriptase. The RT-PCR analysis was performed using a QuantStudio 3 platform (Applied Biosystems, Foster City, CA, U.S.A.), the EvaGreen qPCR MasterMix (Applied Biological Materials Inc., Vancouver, Canada), and the corresponding primer sequences (see below) were

used to examine the relative levels of ACE2, AT1, AT2 and Mas receptors. β -Actin was used as a housekeeping gene and was amplified in parallel with the genes of interest. We used the comparative cycle threshold values (cycle threshold (Ct)) method ($2^{-\Delta\Delta Ct}$) to examine the relative messenger RNA (mRNA) expression. A normalized value was obtained by subtracting the Ct of β -actin from the Ct of interest (ΔCt). As it is uncommon to use ΔCt as a relative expression data due to this logarithmic characteristic, the $2^{-\Delta\Delta Ct}$ parameter was used to express the relative expression data. Primer sequences were as follows: for Mas receptor, forward 5'-CTTTGTGGAGAACGGGAT-3'; reverse 5'-GGAGATGTCAGCAATGGA-3' (NM_012757.2 *Rattus norvegicus* Mas1 proto-oncogene, G protein-coupled receptor (Mas1), mRNA); for ACE2, forward 5'-GTGGAGGTGGATGGTCTTTCAGG-3'; reverse 5'-CACCAACGATCTCCCGCTTCA-3' (NM_001012006.1 *Rattus norvegicus* angiotensin I converting enzyme 2 (Ace2), mRNA); for AT1, forward 5'-TTGTCTGGATAAATCACACAACCC-3'; reverse 5'-GTTAAGGGCCATTTTGTCTTCTGG-3' (NM_030985.4 *Rattus norvegicus* angiotensin II receptor, type 1a (Agtr1a), mRNA); for AT2, forward 5'-AACATCTCGCTGAAGACCAATAG-3'; reverse 5'-AGAAGGTCAGAACATGGAAGG-3' (NM_012494.3 *Rattus norvegicus* angiotensin II receptor, type 2 (Agtr2), mRNA); for human ACE2, forward 5'-TTCCATGCTAACGGACCCAGGA-3'; reverse 5'-TTTGTGCACATAAGGATCCTGAAGT-3' (NM_021804.3 *Homo sapiens* angiotensin I converting enzyme 2 (ACE2), transcript variant 2, mRNA).

Transfection of ACE2

Human epithelial lung cell line A549 were seeded at a density of 0.35×10^6 /well on to 12-well plates with glass cover and maintained at 37°C in a humidified CO₂ incubator (5% CO₂, 95% air). Cells were transiently transfected with 2 μ g of ACE2 cDNA (ACE2 tGFP-tagged, RG208442, Origene) using a commercial transfection reagent, Turbofect (R0533, Thermo Scientific). Forty-eight hours after transfection, cells were treated with different compounds, fixed and processed for confocal studies.

SARS-CoV-2 Spike RBD-Fc protein internalization assay

Human alveolar type-II pneumocyte cell lines, A549, transiently transfected with tGFP ACE2 were treated or not treated with Candesartan or Captopril for 24 h. Then, 1 μ g/ml of SARS-CoV-2 Spike RBD-Fc protein (40592-V02H, Sino Biological) was added to the cells for 3 h at 37°C. Cells were fixed and incubated overnight at 4°C with a mouse monoclonal antibody against human IgG-Fc (ab99757, abcam, 1:500) diluted in DPBS containing 1% BSA, 2% normal goat serum and 0.05% Triton X-100. Then, cells were incubated with the fluorescent secondary antibody Alexa Fluor 568- conjugated goat anti-mouse IgG (Molecular Probes; 1:200) for 2.5 h at RT. Finally, mounting was performed with Immu-mount (Thermo-Shandon). Internalization was measured using confocal laser microscopy (AOBS-SP5X; Leica Microsystems Heidelberg GmbH, Mannheim, Germany), performing sequential scan to avoid any potential overlap with the LAS AF software (Leica Microsystems GmbH). For each sample, over 25–30 optical fields were chosen randomly using a 63 \times objective. Images of cells at a unique plane were used for the analysis. RBD Spike protein internalization rate was expressed as the ratio of the fluorescence intensities measured at 568 nm (human Fc signal) and 488 nm (ACE2-GFP signal) excitation wavelengths. Fluorescence intensity was measured at the level of cellular cytoplasm and the background of each image was subtracted before calculating the ratio using the Software Leica LAS AF. The same conditions of laser intensities/exposure times were used for the entire experiment.

ACE2 activity

ACE2 activity from 20 μ g of rat lung tissue or 5 μ g of human A549 cells lysate was measured using a commercial ACE2 Activity Assay Kit (AnaSpec, AS-72086) following the manufacturer's specifications. The kit is based on the Mca/Dnp fluorescence resonance energy transfer (FRET) peptide (10 μ M). In the FRET peptide, the fluorescence of Mca is quenched by Dnp but a cleavage of the substrate produces a separation into two fragments by the enzyme so that the fluorescence of Mca is measured at excitation/emission = 330/390 nm using an Infinite M200 multiwell plate reader (TECAN). ACE2 activity was confirmed with the specific ACE2 inhibitor DX600 1 μ M, included as a control in the same kit (AnaSpec, AS-72086).

ADAM17 activity assay

ADAM17 activity from 20 μ g of human A549 cells lysate was measured using a commercial TACE activity assay kit (AnaSpec, AS-72085) following the manufacturer's specifications. The kit is based on the 5-FAM/QXL[®] 520 based fluorescence resonance energy transfer (FRET) peptide (1 μ M). In the FRET peptide, the fluorescence of 5-FAM is quenched by QXL[®] 520, but a cleavage of the substrate produces a separation into two fragments by the enzyme so

that the fluorescence of 5-FAM is measured at excitation/emission = 490/520 nm using an Infinite M200 multiwell plate reader (TECAN). ADAM17 activity was confirmed with the TACE inhibitor TAPI-0 (10 μ M), included as a control in the same kit (data not shown).

Enzyme immunoassays (EIA)

To analyze the release of proinflammatory markers, with or without the different treatments, A549 cell culture supernatants were collected and centrifuged at $2000 \times g$ for 10 min to eliminate cell's debris. Proinflammatory chemokine CCL-2 (MPC-1) (ab179886; Abcam) and proinflammatory cytokines IL-6 (BMS213HS, Invitrogen) and TNF- α (BMS223HS, Invitrogen) from A549 cells culture medium were measured using commercially available specific EIA Kits according to the manufacturer's instructions. Proinflammatory cytokines concentration was quantified using specific Standard curve from each cytokine (4PL curve fit).

Statistical analysis

All statistical analyses were performed using SigmaPlot 11.0 (Systat Software, Inc., CA, U.S.A.). All datasets were tested for normality with the Kolmogorov–Smirnov test. If the dataset passed the normality test, parametric tests were used: Student's *t* test for two group comparisons and one-way ANOVA followed by the Student–Newman–Keuls Method for multiple comparisons. For nonparametric data, two group comparisons were carried out by Mann–Whitney rank sum test and multiple comparisons by Kruskal–Wallis one-way analysis of variance on ranks test followed by Student–Newman–Keuls method or Dunn's method were used. All data were expressed as means \pm SEM. Differences were considered statistically significant at $P < 0.05$. GraphPad Prism 8 software (GraphPad Inc., San Diego, CA, USA) was used to create scatter dot plot graphs.

Results

Effects of candesartan and captopril in young adult rats

Candesartan induced a significant increase in lung levels of ACE2 both at low (1 mg/kg) and higher doses (10 mg/kg). RAS receptors of the anti-inflammatory axis were also up-regulated, particularly Mas receptors, while AT2 receptors were particularly up-regulated with the dose of 10 mg/kg (Figure 1A,B and Supplementary Figure S2a,b). Treatment with captopril (5 or 40 mg/kg) induced an increase in levels of ACE2 only with the higher dose, and a significant increase in Mas receptor expression was observed with both doses (Figure 1D,E and Supplementary Figure S2c,d). Candesartan and captopril also induced significant increases in lung ACE2 enzymatic activity (Figure 1C,F).

Effects of treatments in rats with metabolic syndrome and aged rats

Rats with metabolic syndrome (MetS) showed a significant increase in weight relative to control rats, as well as significant increase in blood levels of cholesterol, triglycerides and glucose, and a significant increase in blood pressure (Supplementary Figure S3). Rats with MetS showed a decrease in ACE2 and AT2 receptor expression that were significantly increased by candesartan and captopril (Figure 2 and Supplementary Figure S4). Both drugs increased the expression of receptors of the anti-inflammatory axis (Mas and AT2). Interestingly, MetS rats also showed increased expression of receptors of the proinflammatory axis (i.e. AT1 receptors) that was significantly decreased by treatment with candesartan and captopril.

A similar picture was observed in aged rats, which showed a significant decrease in expression ACE2 and receptors of the RAS anti-inflammatory axis (Mas and AT2), and a significant increase in AT1 receptor levels. Candesartan significantly increased expression of ACE2, AT2 and Mas receptors, and decreased AT1 receptor expression (Figure 2C and Supplementary Figure S4e)

Effects on human alveolar type-II pneumocyte cultures

As indicated in methods, high level of cell confluence correlated with higher levels of ACE2 expression in cells (Figure 3A). First, we checked the effects of candesartan and captopril, which induced a significant increase in the expression of ACE2 in pneumocytes, particularly 24 h after treatment (Figure 3B). Treatment with viral spike protein (SARS-CoV-2 spike-RBD; 1 μ g/ml) induced a significant decrease in cell levels of full-length ACE2 protein (120 kDa), which was inhibited by pretreatment with candesartan or captopril (Figure 4A). We also observed that treatment with spike protein induced a decrease in ACE2 activity in cells, which was inhibited by pretreatment with candesartan and captopril (Figure 4B,C). Interestingly, a short ACE2 isoform (60 kDa) increased in cells after treatment with spike protein and was significantly reduced by pretreatment with candesartan or captopril (Figure 4D). Treatment with

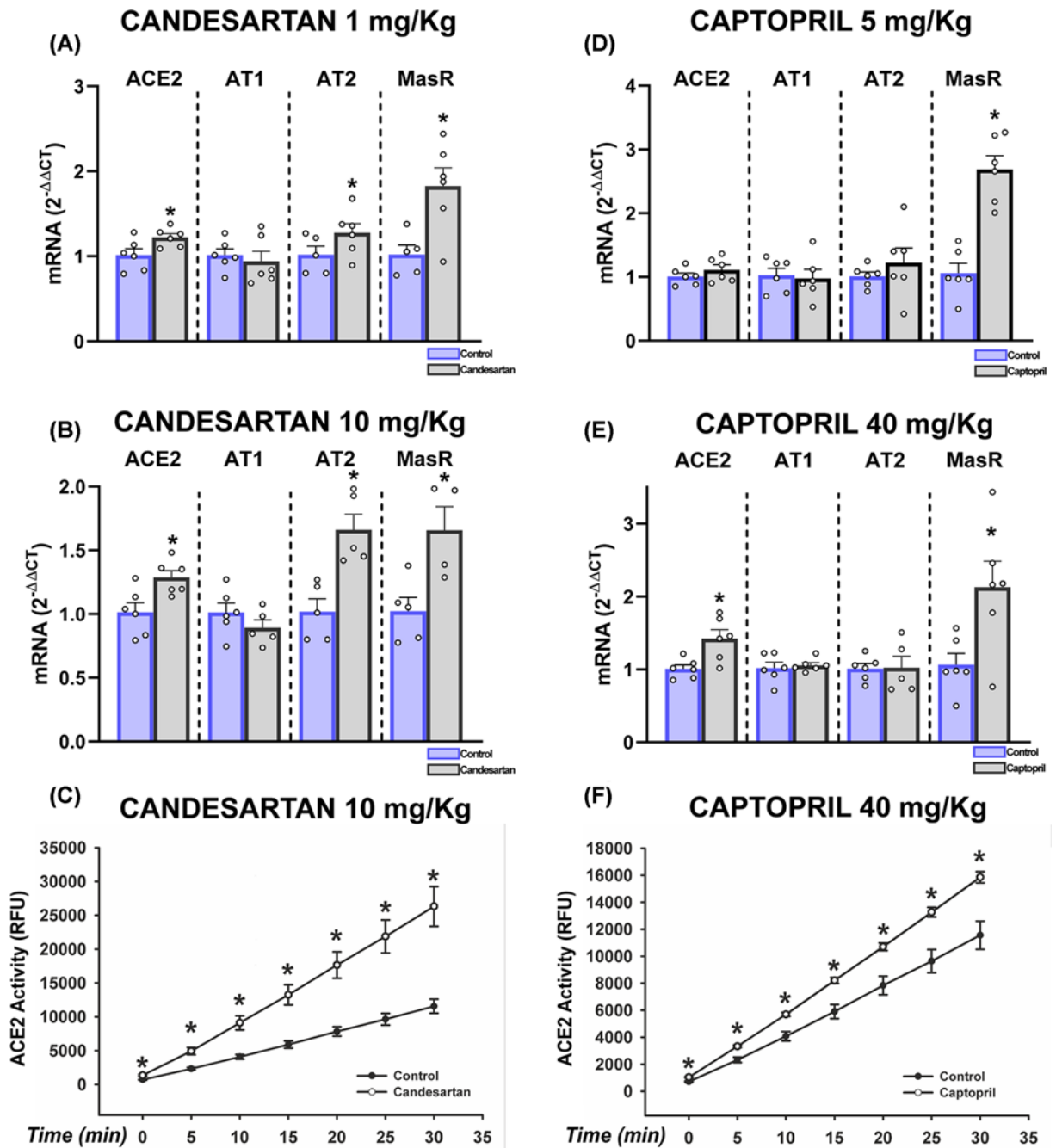


Figure 1. Effect of candesartan and captopril in the lung of young rats

Effects of candesartan (A–C) and captopril (D–F) on mRNA expression of ACE2 and AT1, AT2 and Mas receptors (A,B,D,E) and ACE2 enzymatic activity (C,F) in the adult rat lung. Data are mean \pm SEMs. * $P < 0.05$ relative to control group (Student's test and Mann–Whitney rank sum test).

spike protein induced an increase in levels of soluble ACE2 (105 kDa) in culture medium, which was reduced by pretreatment with the drugs (Figure 4F).

We also studied possible effects of treatments on spike protein internalization using confocal microscopy. First, we confirmed that spike protein was internalized by cultured pneumocytes expressing ACE2 (Figure 4F–H). Then, we investigated the effects of candesartan and captopril on SARS-CoV-2 Spike RBD-Fc internalization rate by measuring cytoplasmic fluorescence intensities of spike protein using confocal laser microscopy (Figure 5). To minimize the

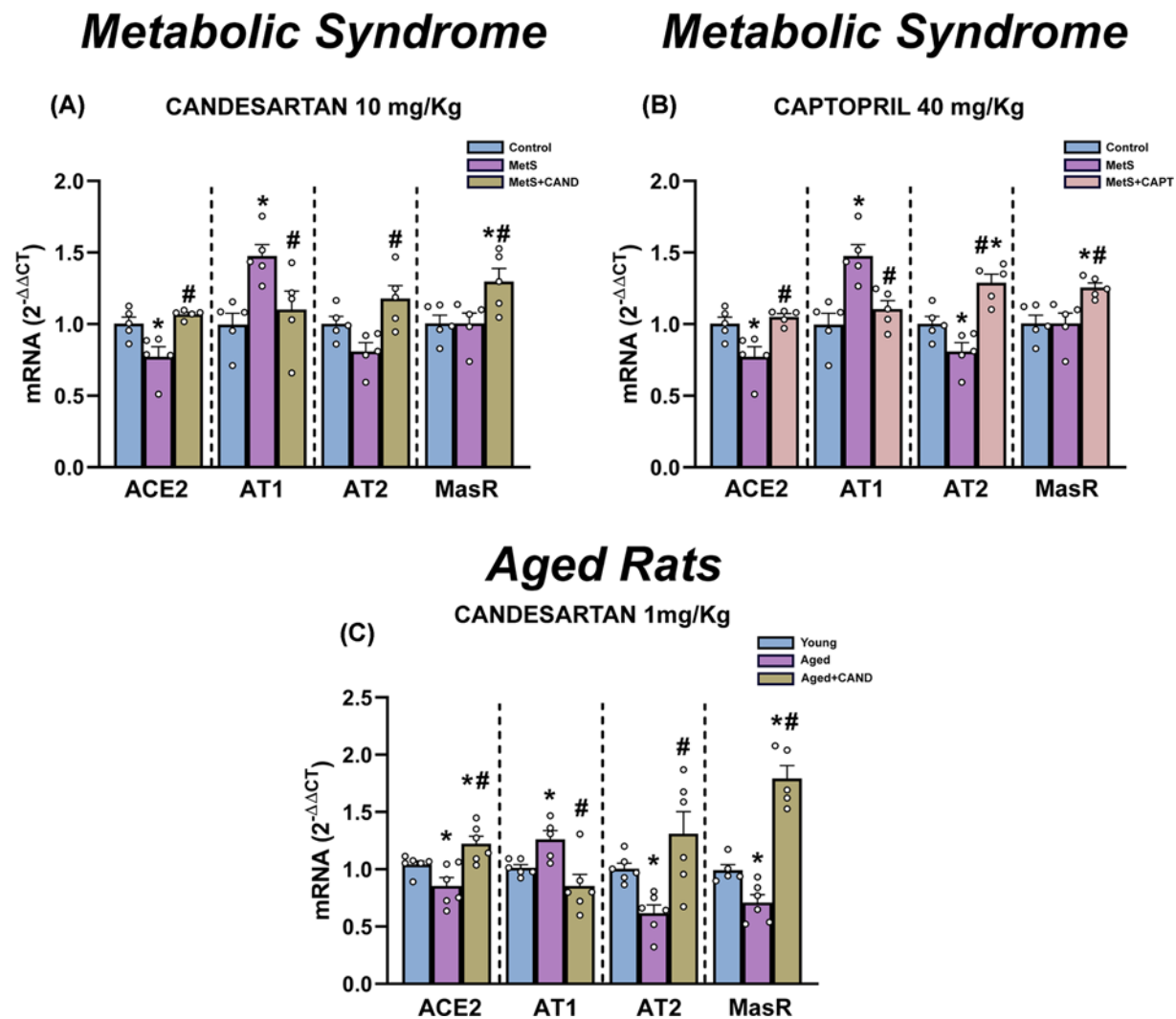


Figure 2. Effect of candesartan and captopril in the lung of rats with metabolic syndrome and aged rats

Effects of candesartan (A and C) and captopril (B) on mRNA expression of ACE2 and AT1, AT2 and Mas receptors in the lung of rats with metabolic syndrome (A and B) and aged rats (C). Rats treated with candesartan (A) or captopril (B) were compared with the same group of untreated animals in (A) and (B). Data are mean \pm SEMs. * $P < 0.05$ relative to control adult rats; # $P < 0.05$ relative to untreated rats with metabolic syndrome (A and B) or aged rats (C) (one-way ANOVA with Student–Newman–Keuls method post hoc test or Kruskal–Wallis one-way analysis of variance on ranks with Student–Newman–Keuls method post hoc test).

possibility that changes in levels of internalized spike protein could be related to differences in cell levels of ACE2 receptor, we used ACE2-GFP transiently transfected A549 cells and values of intracellular spike were expressed relative to ACE2 levels (Figure 5A). We observed high levels of spike protein internalization relative to controls not treated with spike protein (Figure 5A, and 5B–D relative to 5E–G). In cultures treated with spike protein and candesartan (Figure 5A,H–J) or captopril (Figure 5A,K–M), spike protein fluorescence in cells decreased around 50% relative to cells treated with spike protein alone (Figure 5A,E–G). Consistent with the above-mentioned observations, we also observed that treatment with spike protein induced a significant increase in levels of the pro-inflammatory cytokines IL-6, TNF- α , and CCL2 in the culture medium, which were significantly reduced in cultures treated candesartan or captopril prior to exposure to spike protein (Figure 6A–C)

As several of the drug-induced effects cannot be explained by drug-induced increase in ACE2 expression, we investigated whether other mechanisms involved in spike protein internalization may contribute to the effects of drugs. After observing that drugs induced changes in the levels of soluble ACE2 in the culture medium, we investigated possible effects on ADAM17 activity. Interestingly, ADAM17 activity significantly increased in cells treated with spike

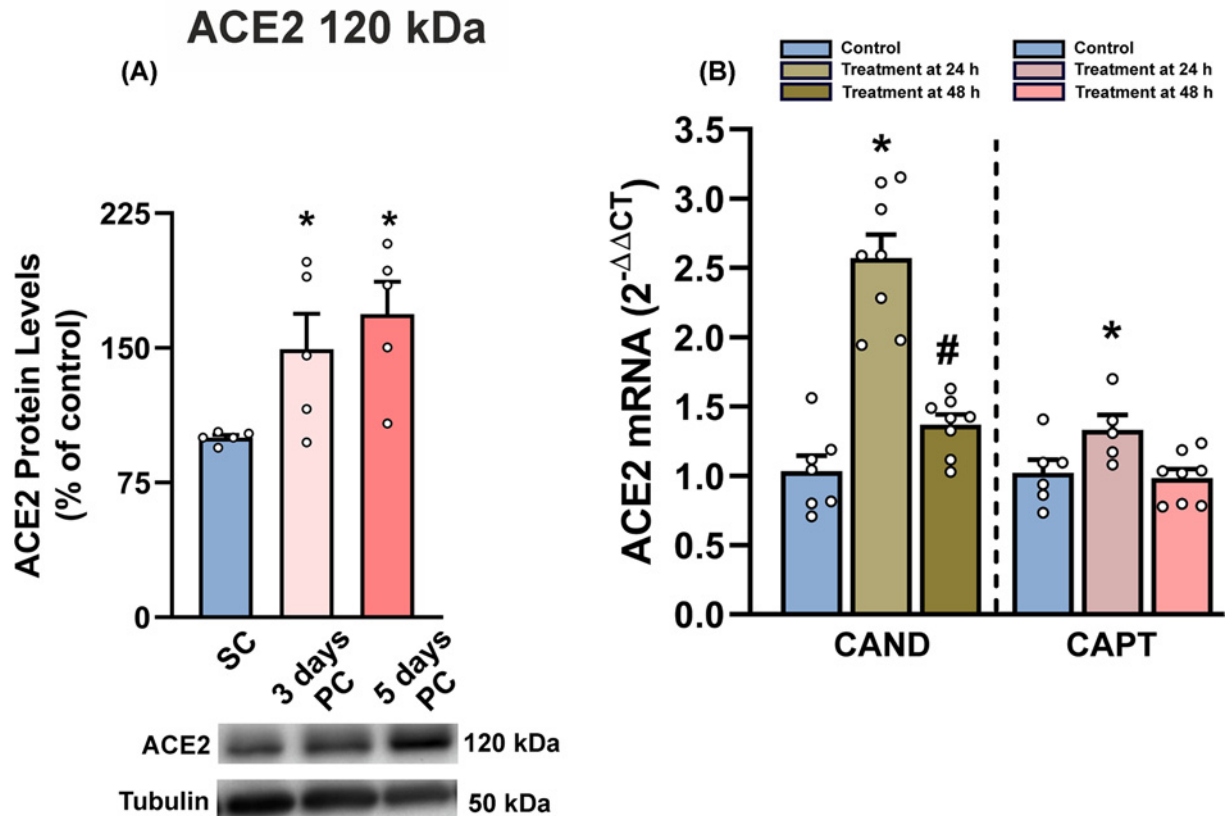


Figure 3. ACE2 levels in cultures of treated and untreated human alveolar type-II A549 cells

Cell confluence increased cell levels of ACE2 protein (A). Effects of 24 or 48 h treatment with candesartan and captopril on mRNA expression of ACE2 enzyme (B). Data are mean ± SEMs. **P*<0.05 relative to sub-confluence (A) or untreated controls (B), #*P*<0.05 relative to 24 h treatment (one-way ANOVA with Student–Newman–Keuls method post hoc test or Kruskal–Wallis one-way analysis of variance on ranks with Student–Newman–Keuls Method post hoc test); PC, post-confluence; SC, sub-confluence.

protein relative to untreated control cells. However, the increase was inhibited by pretreatment with candesartan or captopril (Figure 6D,E)

Discussion

The effect of AT1 receptor blockers and ACEIs on lung levels of ACE2 is controversial, and several experimental or clinical trials have suggested that these drugs increase, decrease, or do not modify ACE2 levels [28,39–41]. In addition, the beneficial or detrimental effects of changes in ACE2 cell levels are also controversial, as an increase in cell-surface ACE2 may result in up-regulation of the anti-inflammatory and antioxidative RAS arm, but also in ACE2-mediated increase in viral entry [17,21,42]. The controversy appears particularly relevant for groups of patients vulnerable to severe COVID-19, such as aged people and people with hypertension, diabetes or obesity [24,25], many of them taking the above-mentioned drugs.

The present *in vivo* experiments show that an AT1 receptor blocker and an ACEI up-regulate ACE2 expression in the lung of control healthy rats, together with up-regulation of other major components of the RAS anti-inflammatory antioxidative axis such as AT2 and Mas receptors. Altogether suggests that the use of captopril, and probably other ACEIs, or candesartan, and probably other ARBs, enhances the anti-inflammatory RAS arm in the lung promoting anti-inflammatory, antifibrotic and antithrombotic responses (see Supplementary Figure S1) that may contribute to a better outcome of COVID-19 and other lung diseases. This effect was pronounced in rats with metabolic syndrome (obesity, hyperglycemia and increased blood pressure) and in aged rats. In these models of most vulnerable individuals, we observed a significant decrease in lung levels of ACE2 together with a decrease in the expression of receptors of the anti-inflammatory axis (AT2 in MetS rats and AT2 and Mas receptors in aged rats), and a significant increase in the expression of AT1 receptors, which revealed a clear unbalance toward the proinflammatory RAS in

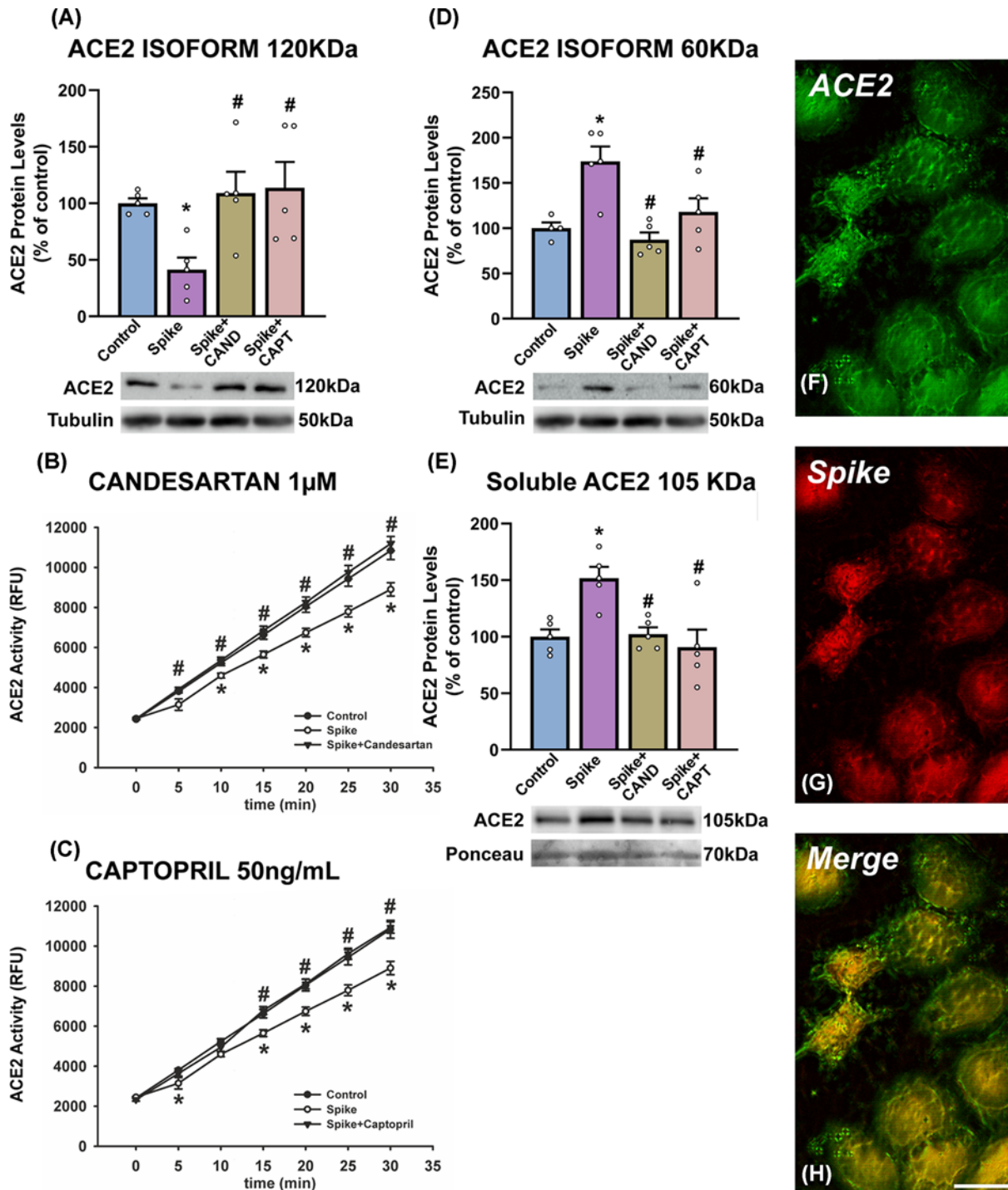


Figure 4. Levels of different ACE2 isoforms and ACE2 enzymatic activity in cells or culture medium after spike protein, candesartan and captopril treatments

Levels of ACE2 protein (A, D and E) and enzymatic activity (B and C) in cells (A and D) and culture medium (E) in cultures of human alveolar type-II A549 cells treated with spike protein alone or spike protein together with candesartan or captopril. Laser confocal microscopy showing colocalization (H, yellow) of ACE2 (green, F) and spike protein (red, G) in cells after treatment of cultures with spike protein. * $P < 0.05$ relative to untreated controls; # $P < 0.05$ relative to cells treated with spike alone (one-way ANOVA with Student–Newman–Keuls Method post hoc test or Kruskal–Wallis one-way analysis of variance on ranks with Student–Newman–Keuls method post hoc test); scale bar (for F–H): 25 μ m.

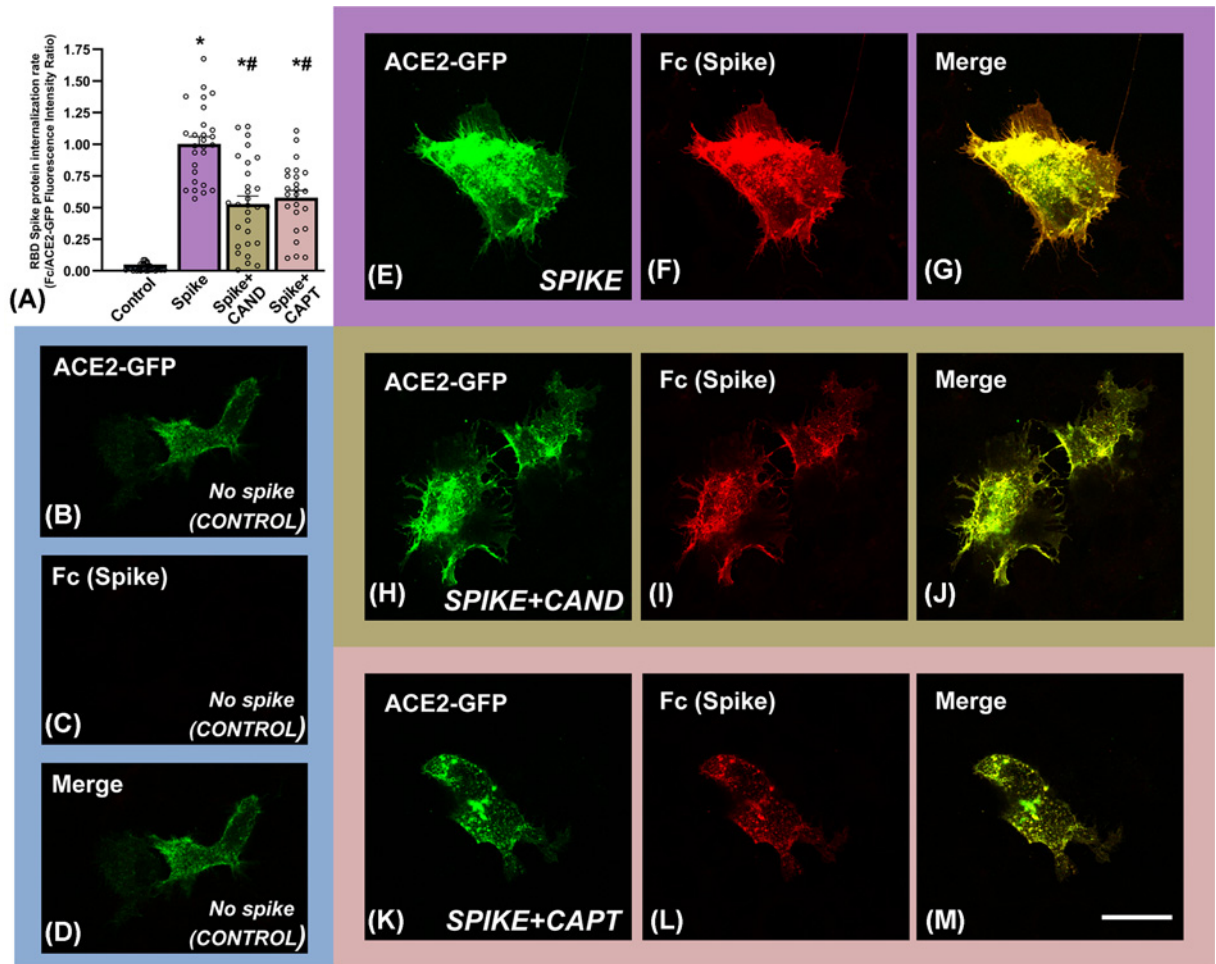
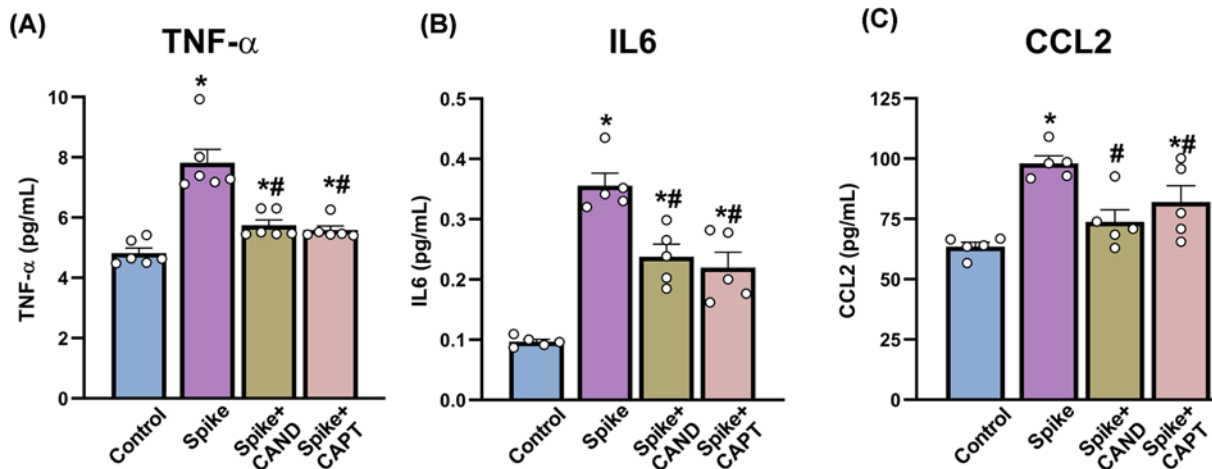


Figure 5. Effects of candesartan and captopril on spike protein internalization in cultures of human alveolar type-II A549 cells

Effects of candesartan and captopril on SARS-CoV-2 Spike RBD-Fc internalization rate by measuring cytoplasmic fluorescence intensities of spike protein using confocal laser microscopy (A). ACE2-GFP (green), spike protein (red) and merge (yellow) fluorescence in untreated controls (B–D), and cultures treated with spike protein alone (E–G) or spike protein and candesartan (H–J) or spike protein and captopril (K–M). * $P < 0.05$ relative to untreated controls, # $P < 0.05$ relative to cells treated with spike alone (Kruskal–Wallis one-way analysis of variance on ranks with Dunn’s method post hoc test); scale bar (for all photographs): 25 μm .

lungs. Interestingly, treatment with candesartan and captopril shifted the balance towards the anti-inflammatory axis by inducing a significant increase in lung levels of ACE2, AT2 and Mas receptors, and a decrease in AT1 receptor expression. Expression of receptors of the proinflammatory axis (AT1) was reduced to levels of young healthy controls, while the expression of receptors of the anti-inflammatory axis such as AT2 and, particularly Mas receptors was increased to levels higher than those of young healthy untreated rats. This suggests that treatment with these drugs may reduce vulnerability of aged patients and patients with MetS even below levels of healthy young patients, at least in that related to RAS dysregulation. It is frequently assumed that changes in ACE2 levels are responsible for the unbalance between both RAS axes, and this is the case for COVID-19. Further factors may impact on RAS unbalance in other diseases. In the case of MetS and aging, the decrease in levels of ACE2 may be the cause or, more probably, the consequence of dysregulation of other RAS components. For instance, in aged brains dysregulation of RAS components may be related to initial dysregulation of other factors such as sirtuins or IGF-1 [43,44]. In COVID-19, the dysregulation observed in MetS and aging is further increased (and probably more hardly compensated) by binding of SARS-CoV-2 to ACE2. In any case, the present results indicate that the dysregulation can be counteracted by the drugs here assayed.

Cytokine Levels



ADAM 17 Activity

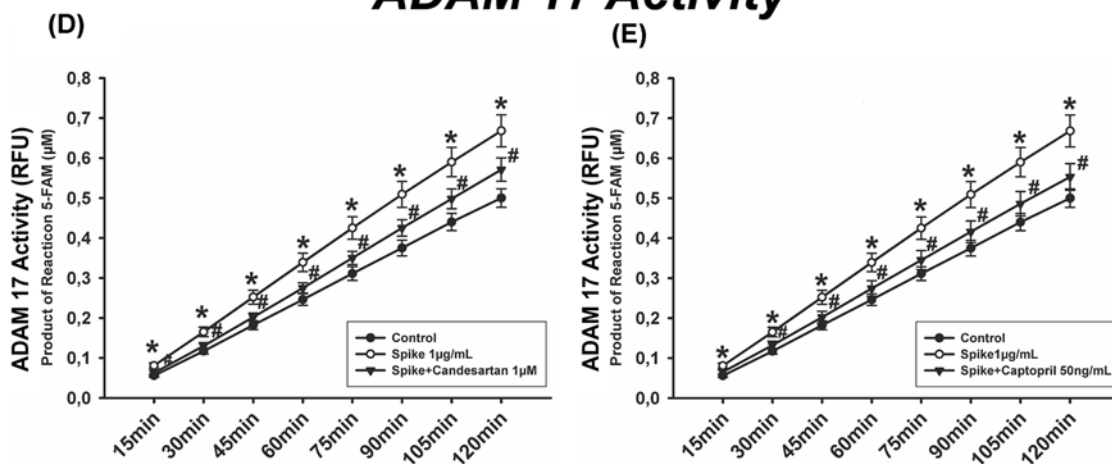


Figure 6. Levels of pro-inflammatory cytokines and ADAM17 enzymatic activity in cultures after treatments with spike protein, candesartan and captopril

Levels of proinflammatory cytokines (TNF- α , IL-6, CCL-2; **A–C**) and ADAM17 enzymatic activity (**D** and **E**) in culture medium (A–C) and cells (D and E) in cultures of human alveolar type-II A549 cells treated with spike protein alone or spike protein together with candesartan or captopril. * $P < 0.05$ relative to untreated controls; # $P < 0.05$ relative to cells treated with spike alone (one-way ANOVA with Student–Newman–Keuls method post hoc test or Kruskal–Wallis one-way analysis of variance on ranks with Student–Newman–Keuls method post hoc test).

Although the up-regulation of lung ACE2 and anti-inflammatory axis receptors appears beneficial for the outcome of COVID-19 patients, an up-regulation of the viral receptors may be deleterious so that ACE2 up-regulation has been considered as a double-edged sword [15–17]. However, the present *in vitro* observations treating human type-II pneumocytes with the viral spike protein and RAS modulators, suggest that the above-mentioned drugs may even decrease viral entry as suggested by our observations on spike protein inside cells using laser confocal microscopy. After SARS-CoV binds to ACE2, the virus enters the host cell using two possible mechanisms: endocytosis and cell membrane fusion. Receptor-mediated endocytosis is a major mechanism for viral entry [24,45,46]. Spike protein binding is the trigger for this entry pathway and induces translocation of ACE2 together with spike protein from the cell surface to endosomes [24,46]. This was observed both using spike protein and spike-bearing pseudoviruses [24], which suggests that treatment of cells with spike protein is a reliable model to study effects of drugs on SARS-CoV entry mechanisms.

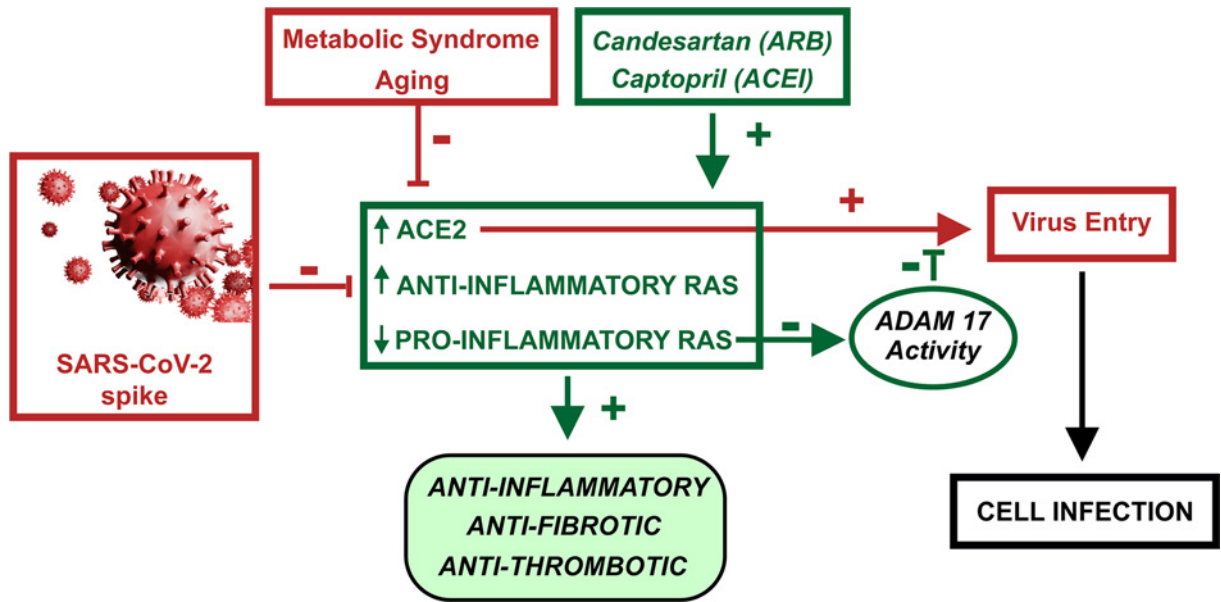


Figure 7. Diagram summarizing major results and conclusions

Candesartan and captopril increase ACE2 levels and anti-inflammatory RAS arm activity, and inhibit the proinflammatory RAS axis. This leads to anti-inflammatory, anti-fibrotic and anti-thrombotic effects in the lung, and counteracts the opposite effects of aging and metabolic syndrome (obesity, hyperglycemia and hypertension) and SARS-CoV-2/spike protein on the same RAS components. The candesartan/captopril-induced increase in ACE2 levels leads to an increase in levels of viral receptors. However, a simultaneous candesartan/captopril-induced reduction in ADAM17 activity reduces viral entry and cell infection.

Consistent with that observed in the rat model, treatment of cultures with candesartan or captopril induced up-regulation of ACE2 expression in lung cells. Treatment with spike protein induced a decrease in cell levels of full-length (i.e. transmembrane) ACE2, which was inhibited by pretreatment with candesartan or captopril. This may be explained by the observed drug-induced up-regulation of ACE2 expression. Additional mechanisms may also contribute to drug-induced increase in cell-surface ACE2. It has been suggested that ACE2 and AT1 receptors may be colocalized at the plasma membrane forming complexes, and that elevated AngII levels decrease ACE2 activity through AT1-dependent ACE2 internalization followed by lysosomal targeting for degradation, which may be prevented by AT1 receptor blockers [47]. In the present experiments, a captopril-induced reduction in AngII levels or candesartan blockage of AT1 receptors may reduce internalization of ACE2 together with virus/spike protein and increase cell-surface ACE2 activity. However, the results observed in the present experiments suggest that additional mechanisms of interaction between SARS-CoV-2/spike protein and RAS are also involved. We observed that spike protein treatment induced an increase in levels of soluble ACE2 in the culture medium. This is consistent with previous studies showing that binding the virus or spike protein to cell-surface ACE2 induces ACE2 ectodomain shedding, decreases cell-surface levels of ACE2 and promotes virus/spike protein internalization [30,48–50]. Interestingly we also detected a spike-induced increase in cell levels of a short ACE2 polypeptide (60 kDa), which may correspond to an internalized glycosylated ACE2 polypeptide.

ADAM17 is a metalloprotease and disintegrin located in the cell membrane and that can cleave a wide number of substrates and has been involved in several cardiovascular and neurological disorders [51]. Interestingly, ADAM17 also plays a major role in the entry mechanism of several viruses [52,53] and particularly SARS-CoV viruses. The mechanism used by ADAM17 to facilitate SARS-CoV entry has not been totally clarified; however, it is known that activation of ADAM17 plays a major role in ACE2 shedding [49–51] and enhances viral entry [30,48]. It has been shown that activation of ADAM17 by the viral spike protein depends on the cytoplasmic domain of ACE2 and that this is positively involved in viral entry [30,48]. In addition, the ACE2 cytoplasmic domain is also required for SARS-spike-induced ACE2 shedding [30,48]. Consistent with this, ADAM17 inhibitors attenuated entry of both pseudotyped virus expressing the SARS-spike protein and infectious strains of SARS-CoV, and have been suggested as antiviral compounds [48]. Interestingly, the present experiments showed that the investigated drugs act as ADAM17 inhibitors. Candesartan and captopril reduce levels of soluble ACE2 in the culture medium suggesting a reduction of

ACE2 shedding, and induce also a reduction in levels of intracellular short-length ACE2, which suggests reduction in spike/ACE2 internalization. This may be related with the observation of drug-induced decrease in ADAM17 activity in our cultures, which is consistent with previous observations in cardiomyocytes and neurons showing that AngII via AT1/Nox-derived ROS increases ADAM17 activity [54,55].

Our observations on proinflammatory cytokine release also suggest an overall beneficial effect of candesartan and captopril. Consistent with previous studies [56,57], we observed a spike-induced increase in the release to culture medium of major proinflammatory cytokines, which are also increased in SARS-CoV-infected patients [58,59]. We analyzed TNF- α , IL-6 and CCL2. TNF- α [60] and IL-6 [32,61] have been identified as major inducers of the SARS-CoV-2 hyperinflammatory response. CCL2 is considered a fibrosis-associated chemokine involved in several lung inflammatory disorders including SARS, asthma and pulmonary fibrosis. CCL2 is up-regulated in the sera of SARS-CoV patients and the supernatant of a SARS-CoV-infected culture systems [56,57,59,62]. Several mechanisms may be involved in the changes in proinflammatory cytokine release observed in the present study. First, we observed a spike-induced down-regulation of the anti-inflammatory RAS axis and up-regulation of the proinflammatory AT1/NOX2 axis, which may lead to an increase in cytokine release [32,63,64] mediated by NOX2-derived ROS [54,65]. Second, ADAM17 is not only involved in shedding of ACE2 [49–51], but in the release several cytokines [66]. It is well-known that ADAM17 or TACE (TNF- α -converting enzyme) releases TNF- α from cells [67], and that ADAM17 promotes IL-6/soluble IL-6 receptor release [32]. In addition, ADAM17 may induce CCL2 release via an indirect mechanism involving acidic mammalian chitinase (AMCase) [68]. In summary, both an increase in the anti-inflammatory axis activity (together with inhibition of the proinflammatory arm) and a decrease in ADAM17 activity may explain a decrease in spike-induced proinflammatory cytokine release in our cultures after treatment with candesartan or captopril.

Conclusions

The present *in vivo* and *in vitro* studies show that candesartan, an AT1 receptor blocker, and captopril, an ACEI, up-regulate ACE2 expression and anti-inflammatory RAS activity in the lung. Furthermore, up-regulation of the SARS-CoV-2 main receptor, ACE2, is counteracted by drug-induced mechanisms that reduce SARS-CoV-2 spike protein entry, particularly by inhibition of ADAM17 activity (Figure 7).

Clinical perspectives

- There is an important controversy on effects of several drugs that regulate RAS activity, and possibly ACE2 levels, on COVID-19 outcome. This has been particularly focused on AT1 receptor blockers (ARBs) and ACE inhibitors that are widely used, particularly by patients most vulnerable to severe COVID-19.
- Our results with candesartan and captopril suggest that these drugs up-regulate ACE2 expression and anti-inflammatory RAS activity in the lung. Furthermore, up-regulation of the SARS-CoV-2 main receptor, ACE2, is counteracted by drug-induced mechanisms that reduce SARS-CoV-2 spike protein entry, particularly by inhibition of ADAM17 activity.
- Our observations indicate an overall beneficial effect of the here tested drugs in COVID-19 clinical outcome.

Data Availability

Data are available from the corresponding author upon reasonable request

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Maria A. Pedrosa: Investigation, Methodology. **Rita Valenzuela:** Conceptualization, Investigation, Methodology. **Pablo Garrido-Gil:** Investigation, Methodology. **Carmen M. Labandeira:** Conceptualization, Writing—review and editing. **Gemma Navarro:** Investigation, Methodology. **Rafael Franco:** Conceptualization, Writing—review and editing. **Jose L. Labandeira-Garcia:** Conceptualization, Formal analysis, Funding acquisition, Writing—original draft, Writing—review and editing. **Ana I. Rodriguez-Perez:** Conceptualization, Formal analysis, Funding acquisition, Writing—original draft, Writing—review and editing.

Ethics Approval

Animal handling was conducted in accordance with the Directive 2010/63/EU, European Council Directive 86/609/EEC and the Spanish legislation (RD53/2013). Rodent experiments were approved by the corresponding committee at the University of Santiago de Compostela. Animals were housed at constant room temperature (RT) (21–22°C) and 12-h light/dark cycle.

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Abbreviations

ACE2, angiotensin-converting enzyme 2; ACEI, angiotensin-converting enzyme inhibitor; AngII, angiotensin II; AT1, angiotensin type 1; AT2, angiotensin type 2; CCL2, Chemokine CC motif ligand 2; FRET, fluorescence resonance energy transfer; IL-6, interleukin 6; MasR, Mas receptor; MetS, metabolic syndrome; RAS, renin-angiotensin system; TG, triglyceride; TNF- α , tumor necrosis factor- α .

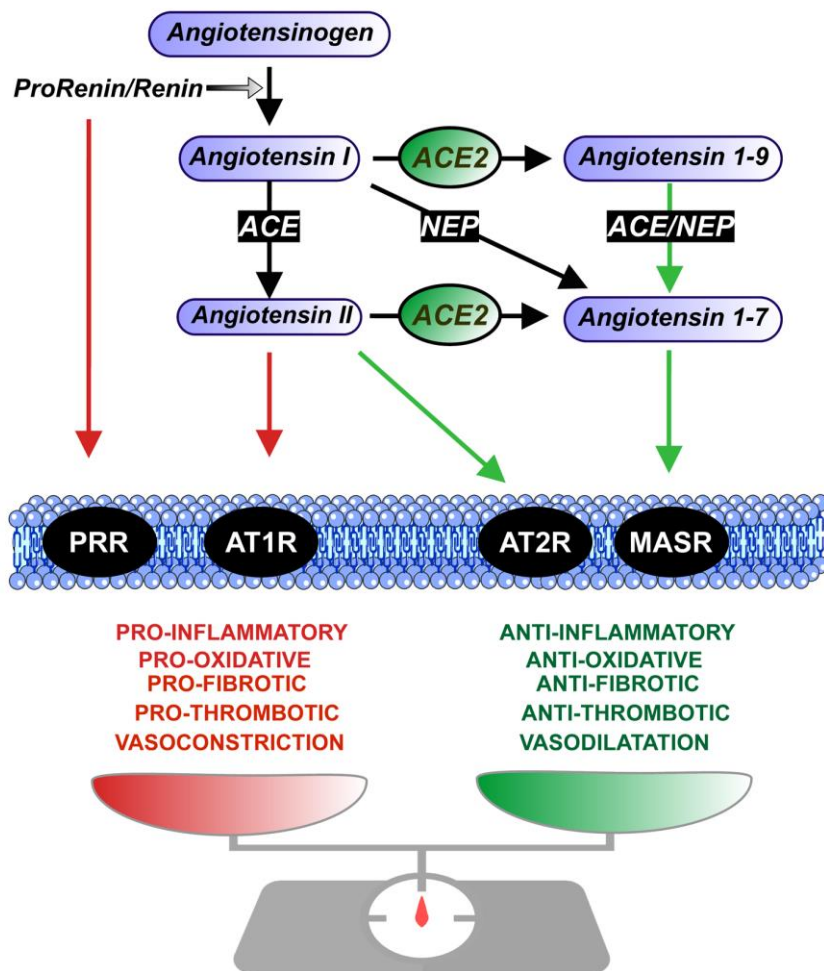
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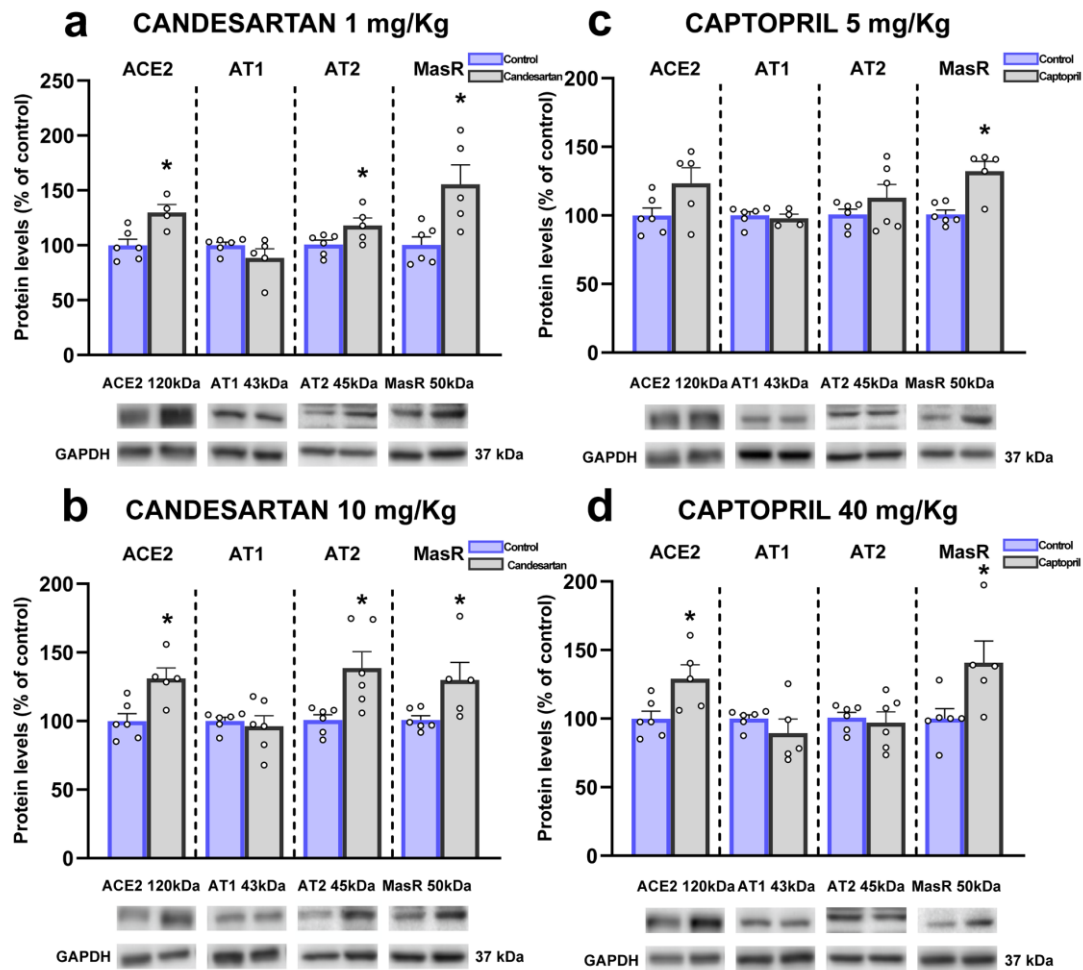
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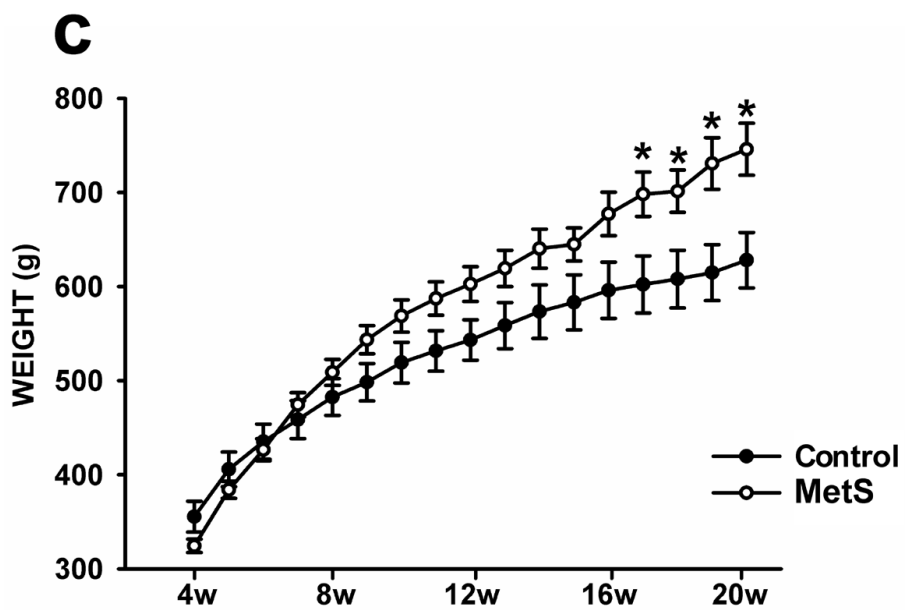
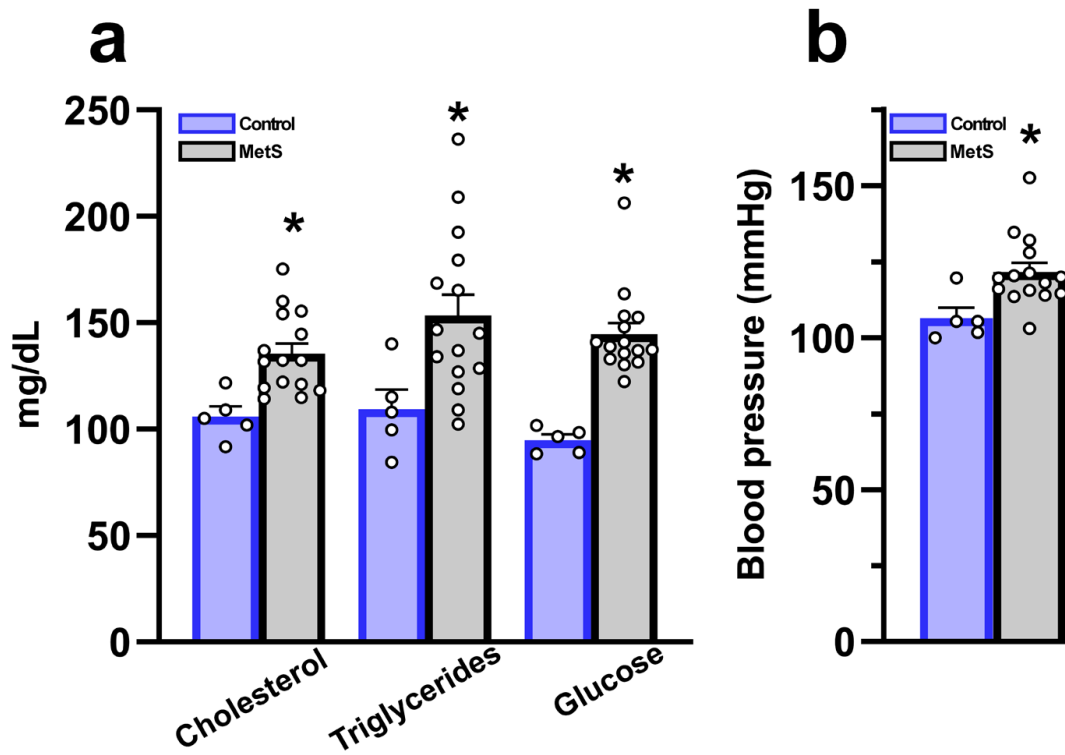
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Supplementary Figure 1. The renin–angiotensin system (RAS) is basically organized into two arms that counteract each other: a pro-inflammatory and pro-oxidative axis (red lines) mainly formed by angiotensin II and AT1 receptors (AT1R), and an anti-inflammatory anti-oxidative axis (green lines) formed by Angiotensin II/AT2R, and particularly Angiotensin 1-7 /MasR. Angiotensin II is formed by the sequential action of two enzymes prorenin/renin (that forms angiotensin I) and angiotensin converting enzyme (ACE) on the precursor protein angiotensinogen. Renin and its precursor prorenin (PR) also act on specific PR receptors. Angiotensin converting enzyme 2 (ACE2) plays a key role in the balance, because ACE2 (together with additional peptidases such as Nephilysin, NE) transforms components of the pro-inflammatory axis (i.e. Angiotensin I and particularly Angiotensin II) into components of the anti-inflammatory axis (i.e. Angiotensin 1-9, and particularly Angiotensin 1-7).

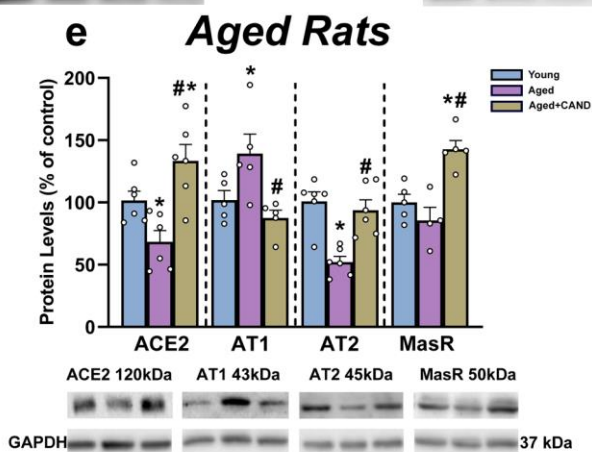
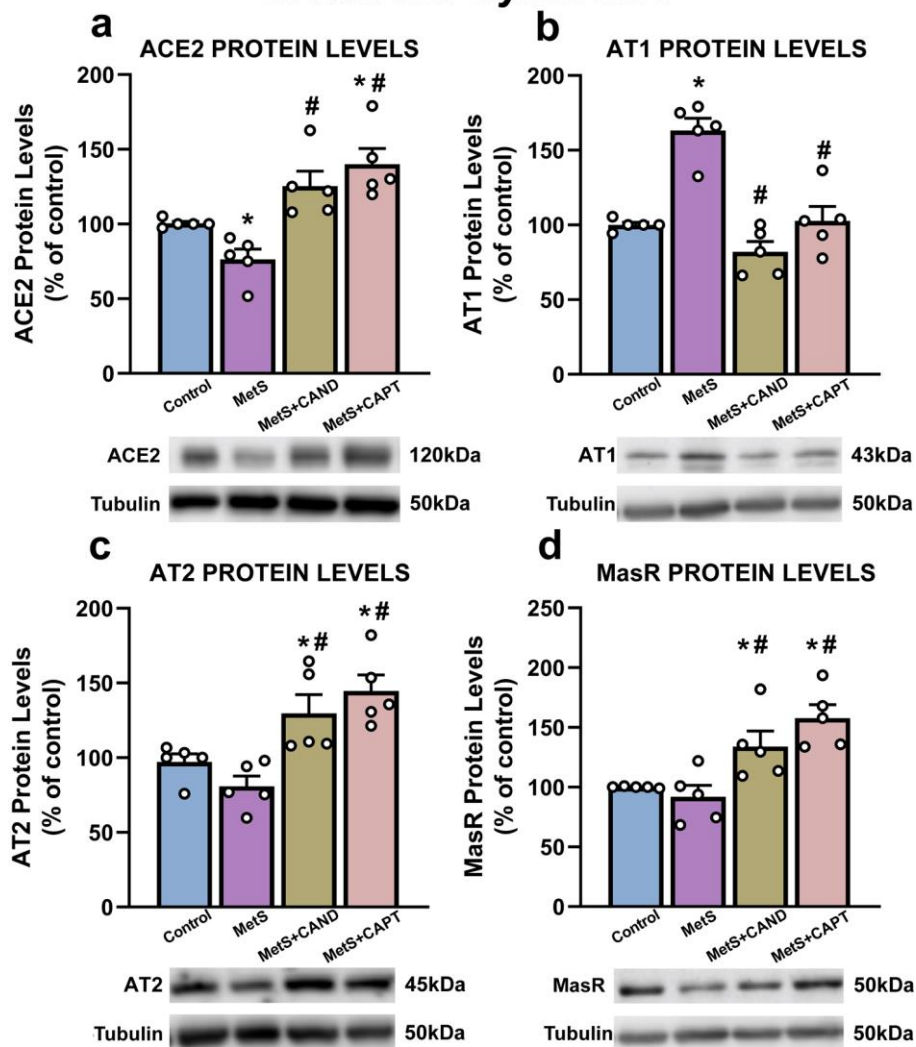


Supplementary Figure 2. Effects of candesartan (**a, b**) and captopril (**c, d**) on protein expression of ACE2 and AT1, AT2 and Mas receptors in the adult rat lung. Data are mean \pm SEMs. * $p < 0.05$ relative to control group (Student's test and Mann-Whitney Rank Sum Test)



Supplementary Figure 3. Rats with metabolic syndrome (MetS) showed a significant increase in blood levels of cholesterol, triglycerides and glucose (**a**), significant increase in blood pressure (**b**) and increase in weight relative to control rats (**c**) (Student's test and Mann-Whitney Rank Sum Test).

Metabolic Syndrome



Supplementary Figure 4. Protein levels of ACE2 (**a**) and AT1 (**b**), AT2 (**c**) and Mas (**d**) receptors in the lung of control rats and rats with metabolic syndrome (MetS) untreated or treated with candesartan and captopril, and aged rats untreated or treated with candesartan (**e**). Data are mean \pm SEMs. * $p < 0.05$ relative to control adult rats, # $p < 0.05$ relative to untreated rats with metabolic syndrome (**a-d**) or aged rats (**e**). (one-way ANOVA with Student-Newman-Keuls Method post hoc test or Kruskal-Wallis One Way Analysis of Variance on Ranks with Student-Newman-Keuls Method post hoc test). CAND, Candesartan; CAPT, Captopril.