

Article

Food enrichment with *Glycyrrhiza glabra* extract suppresses ACE2 mRNA and protein expression in rats – possible implications for COVID-19

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Abstract: Angiotensin converting enzyme 2 (ACE2) is a key entry point of SARS-CoV-2 virus known to induce COVID-19. We have recently outlined the concept to reduce ACE2 expression by the administration of glycyrrhizin, a component of *Glycyrrhiza glabra* extract, via its inhibitory activity on 11beta hydroxysteroid dehydrogenase type 2 (11betaHSD2) and resulting activation of mineralocorticoid receptor (MR). We hypothesized that in organs, such as the intestine, which co-express 11betaHSD2, MR and ACE2, the expression of ACE2 would be suppressed. We studied organ tissues from an experiment originally designed to address the effects of *Glycyrrhiza glabra* extract on the stress response. Male Sprague Dawley rats were left undisturbed or exposed to chronic mild stress for five weeks. For the last two weeks, animals continued with a placebo diet or received a diet containing extract of *Glycyrrhiza glabra* root at a dose of 150 mg/kg of body weight/day. Quantitative PCR measurements showed a significant decrease in gene expression of ACE2 in the small intestine of rats fed with diet containing *Glycyrrhiza glabra* extract. This effect was independent of the stress condition and failed to be observed in non-target tissues, namely the heart and the brain cortex. In the small intestine we confirmed the reduction of ACE2 also at the protein level. Present findings provide first evidence to support the hypothesis that *Glycyrrhiza glabra* extract may reduce an entry point of SARS-CoV-2. Whether this phenomenon, when confirmed in additional studies, is linked to the susceptibility of cells to the virus requires further studies.

Keywords: COVID-19; glycyrrhizin; mineralocorticoid receptor; toll like receptor 4; angiotensin converting enzyme; aldosterone

1. Introduction

The pandemics of coronavirus disease 2019 (COVID-19) has clearly revealed the need to search for new therapeutic options including natural products as food supplements (Cawood et al., 2020; Hensel et al., 2020). The angiotensin converting enzyme 2 (ACE2) serves as an entry point for the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which leads to COVID-19. Therefore, reducing ACE2 expression would reduce the number of access points of the virus to the body during the primary infection and potentially the spread inside the body. Cells, which are susceptible for the infection with SARS-CoV-2 appear to be primarily type II pneumocytes, intestinal absorptive enterocytes, and nasal goblet secretory cells (Ziegler et al., 2020). Identification of mechanism to reduce membrane ACE2 expression at these cells may be valuable.

We have recently proposed that glycyrrhizin, a key component of an extract from *Glycyrrhiza glabra*, may have such an effect (Murck, 2020). Glycyrrhizin is metabolized into the systemically active metabolite glycyrrhetic acid. Via this metabolite glycyrrhizin

inhibits an enzyme called 11-beta-hydroxysteroid dehydrogenase type 2 (11betaHSD2) (Cosmetic Ingredient Review Expert, 2007). Its inhibition allows cortisol to access mineralocorticoid receptors (MR) in aldosterone specific peripheral tissues, including the kidney, lung, intestinal, nasal and endothelial cells, in which it would be otherwise prevented to do so. In other words, an inhibition of this enzyme leads to an aldosterone like activation at the MR by cortisol and may resemble the effects of high aldosterone levels in these organs. This may be relevant, as compounds, which reduce plasma aldosterone, including angiotensin-converting enzyme inhibitors and angiotensin receptor antagonists, increase the expression of ACE2 (Esler and Esler, 2020). Conversely, MR activation leads to a downregulation of ACE2, as demonstrated in the kidney (Fukuda et al., 2011).

Such action could therefore be a mechanism, which could be employed to reduce ACE2 expression and therefore access of the virus to specific cells. A relevant tissue overexpresses, besides ACE2, both 11betaHSD2 and the MR. This includes lung and nasal, as well as intestinal epithelial cells. From a mechanistic perspective it is important that small intestine, in particular ileum cells, co-express MR, 11betaHSD2 and ACE2, implying that the ileum could serve as an entry point for Cov-SARS-2 and be a target for 11betaHSD2 inhibition; it is at least a model organ to test for the effect of glycyrrhizin on ACE2. .

The downstream consequences of reduced ACE2 expression are somewhat controversial. ACE2 activity is generally protective, including for lung tissue (Kaparianos and Argyropoulou, 2011). It does so by converting angiotensin II to angiotensin1-7 (Tikellis and Thomas, 2012) as well as by suppressing the consequences of the activation of the receptor for endotoxin (LPS), i.e. the toll-like receptor 4 (TLR4) and as a consequence related inflammation in the lung (endotoxin storm) (Ye and Liu, 2020): ACE2 overexpression inhibited the LPS induced inflammation in the mentioned study. Therefore, the reduced expression of ACE2 could be regarded as concerning. The anti-inflammatory ACE2-system is, however, balanced against the pro-inflammatory classical ACE (Ingraham et al., 2020), which leads to an increase in the pro-inflammatory mediator angiotensin II. Inhibition of 11betaHSD2 by glycyrrhizin or glycyrrhetic acid suppresses the classical renin-angiotensin-aldosterone system (RAAS), i.e. reduces the plasma concentrations of renin, angiotensin and aldosterone (Armanini et al., 1996; Cosmetic Ingredient Review Expert, 2007) and increases cortisol/corticosterone locally (MacKenzie et al., 1990). This inhibition of the classical RAAS and activation of glucocorticoid receptors may therefore add to a potential beneficial effect of glycyrrhizin via the reduction of the pro-inflammatory angiotensin II (Benicky et al., 2011). Furthermore, a direct anti-inflammatory effect of *Glycyrrhiza glabra* extract and glycyrrhizin, via inhibition of TLR4 and inhibition of the release of high mobility group box 1 (HMGB1), has been described (Seo et al., 2017; Yu et al., 2005). Such actions would counteract the consequences of ACE2 suppression on inflammation. In accordance, glycyrrhizin has protective effects in acute respiratory distress syndrome induced by the TLR4 activator LPS in mice (Yu et al., 2005).

The objective of this retrospective analysis is to explore the capability of *Glycyrrhiza glabra extract* to reduce ACE2 expression in the small intestine (ileum), as a target tissue with active 11betaHSD2 and MR expression, in comparison to non-target tissues (brain and heart), to provide first mechanistical evidence that *Glycyrrhiza glabra* extract may have clinical benefits via reduced expression of ACE2. The tissues were obtained in an already performed study in rats, which was designed to identify the effects of *Glycyrrhiza glabra* extract on the stress response.

2. Materials and Methods

2.1. Animals

Forty-eight male Sprague-Dawley rats (Velaz, Prague, Czech Republic), weighing 225-250 g at the beginning of the experiments were used. The rats were allowed to habituate to the housing facility for 5 days. The animals were housed under standard laboratory conditions with free access to food and water. A constant 12:12 h light-dark cycle was maintained with light on at 07.00 h and off at 19.00 h. Temperature was maintained at 22±2 °C and humidity at 55±10%. All experimental procedures were approved by the

Animal Health and Animal Welfare Division of the State Veterinary and Food Administration of the Slovak Republic (permission No. Ro 2291/18-221/3) and conformed to the NIH Guidelines for Care and Use of Laboratory Animals.

2.2. Study design

This study was not originally designed to study the effects of *Glycyrrhiza glabra* root extract on ACE2 expression, but instead, it was based on data from an already performed study in rats, which addressed the effects of *Glycyrrhiza glabra* extract on the stress response (report in preparation). However, given the urgent need to identify treatments for COVID-19, we were motivated to address a hypothesis, which was formulated before (Murck, 2020). With respect to the nature of this study, obvious limitations had to be accepted, in particular the unavailability of lung tissue at the time of raising the here answered questions.

Following the habituation to the animal facility, the rats were randomly assigned to the control groups (n=24) and to groups of animals exposed to chronic mild stress (n=24). The model of chronic mild stress was based on seven different stress stimuli (Duncko et al., 2001). They involved social isolation (animal alone in the cage), unknown cage mate (the animal shared the cage with a rat from another cage), stroboscopic light (light flashes with frequency of 5 flashes/s), cage tilt (cages were tilted to 45 degrees from the horizontal), wet cage (water surface reached 2 cm above the bottom of the cage), continuous lighting (lighting for 24 h) and water deprivation. These stimuli were applied for 12 h each, in a randomised order, twice a day for 5 weeks. Control animals were housed undisturbed in a different room under the same light and temperature conditions. They had free access to food and water.

2.3. Treatment

The control rats as well as rats exposed to chronic mild stress were randomly assigned to one of the two groups: animals fed a diet with extract of *Glycyrrhiza glabra* (n=12) and animals fed a placebo diet (n=12). The extract of *Glycyrrhiza glabra* roots (Gall-Pharma GmbH, Judenburg, Austria) (Batch. no. P17092209) contained 6.25% of glycyrrhizinic acid. Water was used as a solvent during the extraction. The extract was mixed into the placebo diet at a dose of 150 mg/kg/day (SSNIFF Specialdiäten GmbH, Soest, Germany). The dose was selected according to (Chakravarthi and Avadhani, 2013). In this study behavioral effects of *Glycyrrhiza glabra* extract were observed, ensuring that a relevant plasma level has been reached. Unfortunately, we were not able to measure plasma levels in this study directly. The placebo diet (SSNIFF Specialdiäten GmbH, Soest, Germany) consisted of carbohydrates (65%), protein (24%) and fat (11%).

As mentioned above, the experiments lasted 5 weeks. All animals received normal control diet for the first 3 weeks. The rats assigned for *Glycyrrhiza glabra* were fed the diet containing extract of *Glycyrrhiza glabra* for the next two weeks.

2.4. Organ collection

Following 5 weeks of experimental procedures, the animals were quickly decapitated with a guillotine between 08.00 and 10.30 h in the morning. The brain was quickly removed from the skull and the prefrontal cortex was dissected on an ice-cold plate. The heart was removed and rinsed in 0.9% NaCl solution. The left heart ventricle was cut from the whole heart. Subsequently, small intestine was removed from the body and all samples were frozen and stored at -70 °C until analyzed.

2.5. ACE2 mRNA and protein quantification

The gene expression of ACE2 was measured in the small intestine, the prefrontal cortex and the left heart ventricle by quantitative PCR. In the case of small intestine, its content was removed before tissue homogenisation. Total RNA extraction, transcription of mRNA into cDNA as well as gene expression quantification was performed as described

previously (Buzgoova et al., 2019). Primer BLAST NCBI software was used to design primers specific for the studied genes as well as reference genes (Tab. 1).

Table 1. Oligonucleotide sequences used in quantitative PCR.

Gene	Sense	Sequence 5' → 3'
ACE2	Forward	ACCCTTCTTACATCAGCCCTACTG
	Reverse	TGTCCAAAACCTACCCACATAT
UQCRFS1 - reference gene	Forward	ACAGTGGGCTGAATGTTCC
	Reverse	CACGGCGATAGTCAGAGAAGTC
TfR1 - reference gene	Forward	ATACGTTCCCCGTTGTTGAGG
	Reverse	GGCGGAAACTGAGTATGGTTGA
HPRT1 – reference gene	Forward	CGTCGTGATTAGTGATGATGAAC
	Reverse	CAAGTCTTTCAGTCCTGTCCATAA

The concentration of ACE2 protein in the small intestine was determined by Rat Ace2 ELISA Kit (cat. no. ER0609, FineTest, China). The intestines were thawed and their contents were removed. The samples were then frozen in liquid nitrogen in a mortar and pulverized by a pestle. A pre-test with the mentioned ELISA kit was performed to determine the optimal amount of tissue to be used in the subsequent analysis. We found out that 12,5 mg of powdered tissue (1/8 of the recommended amount) was the most favorable amount that fit well into the kit's standard curve. The powdered tissue was suspended in 900 µl of PBS (according to the manufacturer's protocol) and was left to stay at room temperature for 30 minutes. The samples were centrifuged at 5,000 x g for 5 minutes at 4°C. From this point on, the analysis was performed according to the manufacturer's protocol with 100 µl of sample supernatant being put into the plate wells. The results are expressed as ng/mg of tissue.

2.6. Hormone measurements

The trunk blood was collected and the plasma was used for the analyses. Plasma corticosterone was measured by double-antibody radioimmunoassay (MP Biomedicals, USA). Both intra- and inter-assay coefficients of variation (CVs) were < 5%. Plasma renin activity was measured using angiotensin I radioimmunoassay kit (Immunotech, France). The intra- and inter-assay CVs were 11.3% and 20.9%, respectively. Serum aldosterone was analyzed by a coated-tube radioimmunoassay (RIAZENco Aldosterone kit, ZenTech, Belgium), according to the manufacturer's instructions. The intra- and inter-assay CVs were 3.8% and 6.2%, respectively.

2.7. Statistical analysis

The software package used for the statistical analysis was Statistica 7 (Statsoft, USA). The values were checked for the normality of distribution using the Shapiro-Wilks test. Data not normally distributed were winsorized to normalize the distributions before analyses. Data from the gene expression of ACE2 were analyzed by ANOVA with main factors of treatment (*Glycyrrhiza glabra* extract vs. placebo) and stress (chronic mild stress vs. control). Repeated measures ANOVA for factor time, treatment and stress was used for evaluation of food intake. Results are expressed as means ± SEM. The overall level of statistical significance was set as $p < 0.05$.

3. Results

In the small intestine, a tissue with known high activity of 11β-HSD2, the gene expression of ACE2 was significantly lower in rats fed the diet with *Glycyrrhiza glabra* extract compared to rats fed the placebo diet (Fig.1A). Two-way analysis of variance (ANOVA) revealed a significant main effect of treatment ($F_{(1,44)}=4.41$; $p=0.0415$) on concentrations of mRNA coding for ACE2 in the small intestine. The effect of stress was not statistically significant.

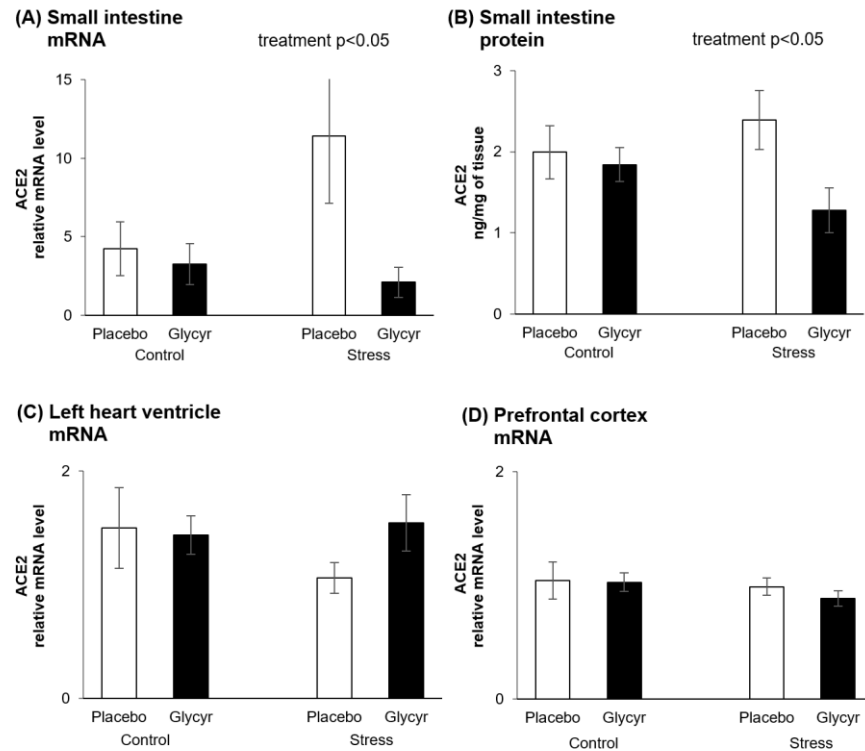


Figure 1. ACE2 gene expression in the small intestine (A), ACE2 protein expression in the small intestine (B), ACE2 gene expression in the left heart ventricle (C) and ACE2 gene expression in the prefrontal cortex (D) of rats treated with glycyrrhiza glabra extract or placebo with or without exposure to chronic mild stress. Each value represents mean \pm SEM (n = 12 rats/group). Statistical significance as revealed by two-way ANOVA.

To verify the observed changes in intestinal ACE2 mRNA production, ACE2 protein concentrations were measured by ELISA. The protein concentrations of ACE2 in the small intestine were significantly lower in rats fed the diet with *Glycyrrhiza glabra* extract compared to rats fed the placebo diet (Fig. 1B). Two way ANOVA revealed a significant main effect of treatment ($F_{(1,44)}=4.46$, $p=0.0403$) on concentrations of ACE2 protein in the small intestine. The effect of stress was not statistically significant.

Concentrations of mRNA coding for ACE2 in the left heart ventricle were not affected by *Glycyrrhiza glabra* extract treatment (Fig.1C). Similarly, the gene expression of ACE2 in the prefrontal cortex was unchanged (Fig.1D).

The statistical analysis of corticosterone concentrations in plasma revealed a significant interaction between the main factors treatment and stress ($F_{(1,44)}=10.23$; $p=0.0030$). The analysis by Tukey post hoc test showed that plasma corticosterone was significantly increased in the control group, which received *Glycyrrhiza glabra* extract vs. placebo ($p=0.0186$; Fig.2A). This was not found in the stressed group, which showed a numerical reduction of corticosterone with the administration of *Glycyrrhiza glabra* extract. Aldosterone concentrations were numerically, but not significantly reduced (Fig.2B). Plasma renin activity was unchanged (Fig.2C).

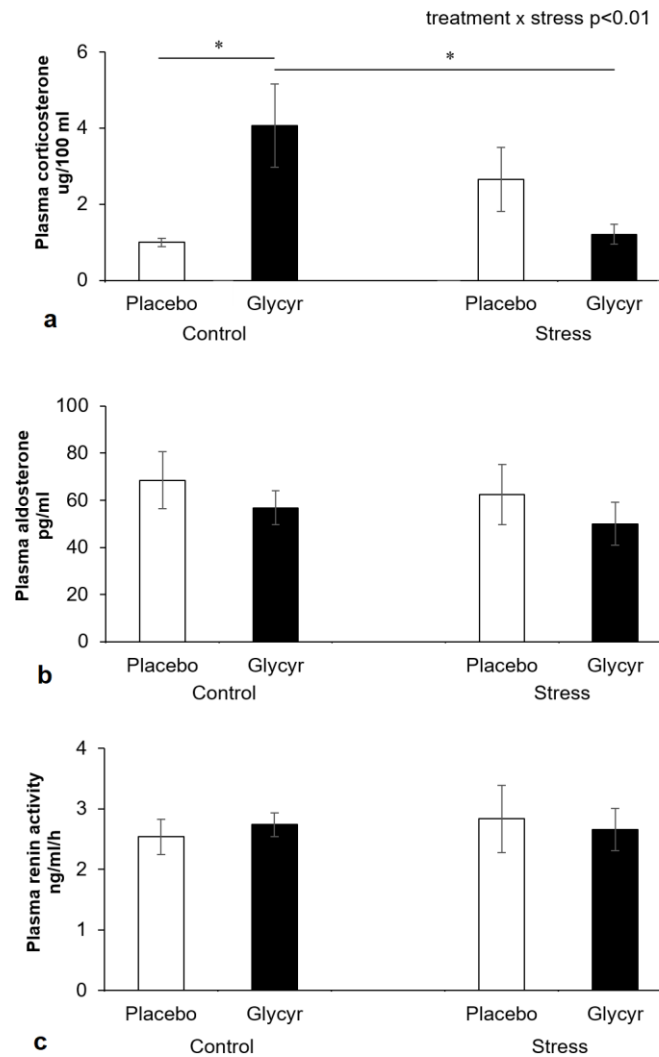


Figure 2. Concentrations of plasma corticosterone (A), concentrations of plasma aldosterone (B) and plasma renin activity (C) in rats treated with glycyrrhiza glabra extract or placebo with or without exposure to chronic mild stress. Each value represents the mean \pm SEM (n=12 rats/group). Statistical significance as revealed by two-way ANOVA followed by Tukey post-hoc test: * $p < 0.05$.

To check the potential influence of *Glycyrrhiza glabra* extract on food intake and thus on the dose of the drug ingested, we measured the food intake in two 2-day time intervals. The food intake was not significantly affected by the treatment (Tab. 2). Repeated measures ANOVA revealed a significant main effect of stress ($F_{(1,44)}=43.43$; $p < 0.001$), as well as time ($F_{(1,44)}=40.59$; $p < 0.001$) on food intake. Chronic mild stress induced a significant reduction of food intake. The values in rats exposed to chronic mild stress were lower compared to those in unstressed controls.

Table 2. The effect of treatment with *Glycyrrhiza glabra* extract on average food intake of stressed and non-stressed animals in selected two-day time intervals. Each value represents the mean \pm SEM (n = 12 rats/group). Statistical significance as revealed by repeated measures ANOVA.

Food intake (g)	Group				Statistical significance
	Control		Stress		
Treatment day	Placebo	Glycyr	Placebo	Glycyr	
1-2	62.6 \pm 1.2	65.2 \pm 0.9	56.3 \pm 0.6	59.8 \pm 1.1	treatment N.S.
8-9	62.7 \pm 0.9	61.7 \pm 1.1	57.3 \pm 0.8	53.8 \pm 1.2	stress p<0.001 time p<0.001

4. Discussion

The main finding of this study is the support for the hypothesis (Murck, 2020) that the treatment with *Glycyrrhiza glabra* reduces the expression of both gene and protein of ACE2 in a tissue, which co-expresses 11betaHSD2 and MR, and may therefore reduce the cellular uptake and spread of SARS-CoV-2. Mechanistically corticosterone acts as a mineralocorticoid to activate the MR in this situation and as a consequence reduces ACE2 expression. This effect was independent of the stress condition and failed to be observed in non-target tissues, such as the heart and the brain. Observed increase in plasma corticosterone in the control condition with the treatment of *Glycyrrhiza glabra* extract confirms target engagement.

The observed increase in glucocorticoid concentrations may also be partially responsible for an expected clinical benefit, as the synthetic glucocorticoid dexamethasone is clinically effective against COVID-19 symptoms (Johnson and Vinetz, 2020), which is considered to be mediated via its anti-inflammatory effect. An alternative pathway of glycyrrhizin to affect inflammatory processes is via its activity to modify gut microbiota (Xu et al., 2018). It has been reported that gut bacteria metabolize steroids into compounds, which modifies 11betaHSD2 (Morris and Ridlon, 2017) and may therefore have an impact on local ACE2 expression. Interestingly, ACE2 expression appears to affect the gut microbiome and in turn, changes in gut microbiota may lead to changes in ACE2 expression (Richards et al., 2017), which points to a complex interaction.

The reduction in ACE2 mRNA levels as well as the ACE2 protein content in the small intestine revealed by feeding the rats with *Glycyrrhiza glabra* extract containing diet in the present study represents a novel original finding. Consistently with our hypothesis, the expression of ACE2 in tissues without evident 11betaHSD2 activity, such as the heart and brain cortex (Gray et al., 2017; Wyrwoll et al., 2011), remained unchanged. It may be suggested that reduced ACE2 expression induced by treatment with *Glycyrrhiza glabra* extract could have several beneficial implications for future clinical research. The most important future direction is to verify the protective effect of the extract and/or its main component glycyrrhizin against the entry of SARS-CoV-2 into the cells. Indeed, our preliminary results have shown a direct antiviral effect of glycyrrhizin on the replication of isolated human SARS-CoV-2 in a Vero E6 cell culture in a plaque-reduction inhibition test. Depending on the concentration of glycyrrhizin added to the cell culture media, an inhibition of SARS-CoV-2 replication down to the detection limit of the assay was observed. Glycyrrhizin exerted a stronger effect when it was present in the cell culture media during the infection and subsequent incubation than when it was added after the virus infection step. Thus, the whole extract from *Glycyrrhiza glabra* or glycyrrhizin, which are generally regarded as safe, are promising dietary supplements to help with prevention or early treatment of COVID-19 (Klempa et al., 2021). Preliminary clinical data on the positive effects of glycyrrhizin (Ding et al., 2020) or glycyrrhizin containing extracts (Luo et al., 2020) to treat patients with COVID-19 support the here outlined mechanistical data.

Next to suggested potential attenuation of the virus entry and replication, the extract may induce positive effects on the mood. We have shown recently that adjunct treatment with *Glycyrrhiza glabra* extract to the standard therapy with antidepressant drugs improved the treatment outcome in patients with depressive disorder (Murck et al., 2020).

To discuss the present findings in the context of pulmonary diseases, the primarily targeted cells for the glycyrrhizin treatment are lung epithelial cells (type II pneumocytes), which express 11betaHSD, both type 1 and 2 (Suzuki et al., 2003a; Suzuki et al., 1998). However, 11betaHSD2 appears to be upregulated in acute respiratory distress syndrome (Suzuki et al., 2003b). Aldosterone leads to an increase in alveolar clearance via an interaction with MR (Suzuki et al., 2001). These observations support a role of 11betaHSD2 inhibition and resultant MR activation in lung protection of inflammatory stimuli. An independent confirmation of the role of MR in lung protection comes from the clinical observations that the MR agonist fludrocortisone in combination with corticosteroids leads to a better clinical outcome in septic shock than corticosteroids alone (Archontakis Barakakis et al., 2019). It should, however, be noted that aldosterone via MR activation has in many situations pro-inflammatory effects, depending on tissue and other factors.

A limitation of the presented concept is its focus on ACE2 only as an entry point for SARS-CoV-2. It has to be noted that other proteins, including CD209L and CD147 may serve this purpose. An interaction between these proteins and either the renin-angiotensin-aldosterone system, ACE2 or the effect of glycyrrhizin has not been reported.

5. Conclusions

In conclusion, the treatment with *Glycyrrhiza glabra* root extract leads to a significant reduction in the expression of ACE2 in the small intestine, which may serve as an entry point of SARS CoV-2. An important aspect of the current study is to motivate additional work, which needs to be performed to provide more conclusive evidence. Whether a similar effect exists in the lungs needs to be further explored, but it is plausible, given a similar receptor constellation of ACE2, 11betaHSD2 and MR. Whether this phenomenon, when confirmed in additional studies, is linked to the susceptibility of cells to the virus requires further studies.

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