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Full Length Research Paper

Matrix metalloproteinases 2 and 9 reduce epithelial sodium channel (ENaC) activity at pleural mesothelium

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The aim of the study was to investigate if Matrix metalloproteinase 2 (MMP2) and Matrix metalloproteinase 9 (MMP9) influence epithelial sodium channel (ENaC) current. Pleural membranes from sheep were mounted at Ussing chambers and were voltage-clamped. MMP2 and MMP9 were applied apically to the pleural membranes and amiloride was added to all experiments. All values are expressed as mean ± standard error of mean (SEM). In control experiments, the short circuit current (Isc) amiloride-sensitive was 7.6 ± 1.6 μA/cm^2. At concentrations 0.0001 and 0.1 ng/ml, non-statistical significant differences occurred whereas at concentration 10 ng/ml, MMP2 and MMP9 increased the Isc amiloride-sensitive to 15 ± 2.8 and 18 ± 3.1 μA/cm^2, respectively. This increase was statistically significant (p = 0.0016 and 0.0021, respectively). At concentration 20 ng/ml, MMP2 and MMP9 decreased significantly the Isc amiloride-sensitive to 2.8 ± 0.7 and 3 ± 0.7 μA/cm^2, respectively. These results demonstrated that MMPs act biphasically; at lower concentrations, they increase ENaC activity and thus they enhance sodium and water absorption from pleural cavity whereas at higher concentrations they hinder ENaC activity and thus aggregate pleural fluid formation.

Key words: Matrix metalloproteinases, sodium absorption, pleural fluid, epithelial sodium channel (ENaC).

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that break down the protein components of the extracellular matrix. In pleural effusions, MMP2 and MMP9 have been measured and found to be elevated in pleural exudates of different origin (parapneumonic, malignant, tuberculous) (Eickelberg et al., 1997; Iglesias et al., 2005). These MMPs may originate from the mesothelial cells or inflammatory cells that accumulate in the pleural cavity during an inflammatory response (Eickelberg et al, 1997). MMPs may cleave not only extracellular matrix (ECM) components, such as collagen and elastin, but also non- ECM protein substrates, such as cell surface molecules and ECM-bound growth factors (Arribas et al., 1996). ENaC resides at the apical membrane of mesothelial cells, participates in sodium and liquid reabsorption and is a major route for sodium removal from the pleural space (Agostoni and Zocchi, 2007; Ji and Nie, 2008).

No study has been so far conducted in order to investigate if MMPs affect epithelial sodium channel (ENaC) activity, although several studies have shown an increase in ENaC function by endogenous serine proteases, which is a different enzyme from metalloproteases family of proteases.

The aim of this study was to investigate if MMP2 and MMP9 influence ENaC activity that is if MMPs alter the current which is produced by ENaC.

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Abbreviations: MMPs, Matrix metalloproteinases; **ENaC,** epithelial sodium channel; **SEM,** standard error of mean.

METHODOLOGY

Sheets of visceral and parietal pleural membranes were obtained

Table 1. Effects of amiloride on the electrophysiological parameters of sheep visceral pleura.

Parameter	Before amiloride	After amiloride	Percent change
$R_{tm\ (\Omega cm^2)}$	11.7±2.3	12.5±2.5	6.2±2 (p>0.05)
$Isc_{\ (\mu A/cm^2)}$	-6.5±2.5	-9.2±4.6	33±11 (p>0.05)

Results represent means ± SEM, n = 6. The application of amiloride on the apical side of the visceral pleura increased R_{tm} by 6.2 ± 2% and the Isc by 33 ± 11%. None of the increases was statistically significant (p > 0.05).

from an adult sheep. The samples were collected from Girtoni Slaughterhause A.E, Larissa, Greece; the slaughterhouse immediately after slaughter and opening up of the carcass. The samples were immediately placed in oxygenated Krebs-Ringer bicarbonate (KRB) solution at 4°C and transferred to the laboratory within 30 min. The KRB solution contained (in mM) 117.5 NaCl, 1.15 NaH_2PO_4, 24.99 $NaHCO_3$, 5.65 KCl, 1.18 $MgSO_4$, 2.52 $CaCl_2$, and 5.55 glucose.

The pleural membranes were mounted carefully in Ussing chambers (K Mussler Scientific Instruments, Aachen, Germany) with an opening surface area of 1 cm^2. Tissues were bathed with 4 ml KRB solution on each side of the membrane and were continuously supplied with air comprising 95%O_2 and 5%CO_2. The mesothelial cell membrane facing the fluid side is hereby called the apical membrane, while that facing the blood side is called the basolateral membrane. The membrane was voltage-clamped (Vt fixed to zero) and the short circuit current (Isc) was measured and continuously recorded. After establishing a stable Isc, phosphate-buffered saline (PBS) (for control experiments) or MMP2 or MMP9 (0.0001, 0.1, 10 or 20 ng/ml) were added on the apical solution.

Experiments on the basolateral solution were not performed because ENaC channels are only expressed at the luminal side of mesothelial cells as has been previously revealed by patch clamp recordings and Ussing chambers experiments (Hatzoglou et al., 2001; Nie et al., 2009). After a 40 min incubation, amiloride (10^{-4} M) was added to the apical compartment of all experiments in order to calculate the ENaC-mediated current. This current was defined as the difference between the Isc value just before amiloride addition and the Isc value 5 min after amiloride addition. The amiloride-sensitive Isc was compared between control tissues (having received only PBS) and tissues treated with MMP2 or MMP9.

Dimethyl sulfoxide (DMSO) was used for the dissolution of amiloride and it had no effect on the electrophysiological properties of the sheep pleura, as shown previously in the literature (Nie et al., 2009). More particularly, the R_{TM} increased by 12 ± 2% and the Isc amiloride-sensitive increased by 5.5 ± 1.2 μA / cm^2 but these changes were not statistically significant (p > 0.05). The previous values express mean ± standard error of mean (SEM).

Statistical analysis was performed using SPSS for Windows (SPSS, Chicago, USA). The comparison between control experiments and experiments with application of MMPs was performed with unpaired t-test. P < 0.05 was regarded as significant.

RESULTS AND DISCUSSION

In control tissues (treated with PBS) of parietal pleura amiloride reduced immediately the Isc current by 53% (p < 0.05) and the transmesothelial resistance (Rtm) increased by 10% (p < 0.001). This verifies that apical Na^+ channels are responsible for the development of Isc, which means that at parietal pleural mesothelium, the short circuit current (Isc) is predominantly mediated by ENaC.

On the apical side of the visceral pleura, amiloride had no statistical significant effect neither on the Isc current nor on the transmesothelial resistance (Table 1) and this is the reason why no further experiments with the application of MMPs at visceral pleura were conducted.

In control tissues of parietal pleura, the ENaC-mediated current was 7.6 ± 1.6 μA/cm^2. When 0.0001 or 0.1 ng/ml MMP2 were added at the pleura, nonstatistically significant changes on the Isc amiloride-sensitive occurred. The incubation of the pleura with 10 ng/ml MMP2 increased significantly the Isc amiloride sensitive whereas the application of 20 ng/ml MMP2 on the pleura resulted in a significant decline in the Isc amiloride-sensitive (Figure 1).

The incubation of the pleura with MMP9 resulted in non-significant changes in the Isc amiloride-sensitive at the lower concentrations (0.0001 and 0.1 ng/ml). When the pleura was incubated with 10 ng/ml MMP9, the Isc amiloride-sensitive increased significantly whereas a significant reduction current occurred at concentration 20 ng/ml (Figure 1).

The present study indicates that MMP2 and MMP9 augment the ENaC-mediated current at concentration 10 ng/ml whereas they reduce the previous current at concentration 20 ng/ml. Under physiological conditions, ENaC contributes to sodium and water reabsorption from the pleural space to the interstitial tissue of the mesothelium (Agostoni and Zocchi, 2007).

In the present study, the upregulation of ENaC current by MMP2 and MMP9 implies enhanced absorption of the pleural liquid. In pleural exudates, where MMPs have been found elevated (Eickelberg et al., 1997), the increase in ENaC activity suggests possibly a protective role from the accumulation of pleural fluid. On the contrary, the decline in ENaC activity, which occurred at higher concentration, implies that MMPs inhibit sodium and liquid reabsorption from the pleural space and thus MMPs aggregate pleural fluid formation.

Previous studies report that serineproteases, which are different from MMPs family of proteases, play an important role in regulating ion transport by ENaC (Planes and Caughey, 2007). Indeed, in airway cells prostasin, a membrane-bound serine peptidase has been recognized to regulate ENaC activity by stimulating ENaC-mediated current (Donaldson et al., 2002). Similar to the results of the current study, a biphasic dose-dependent phenomenon is also true for serine proteases;

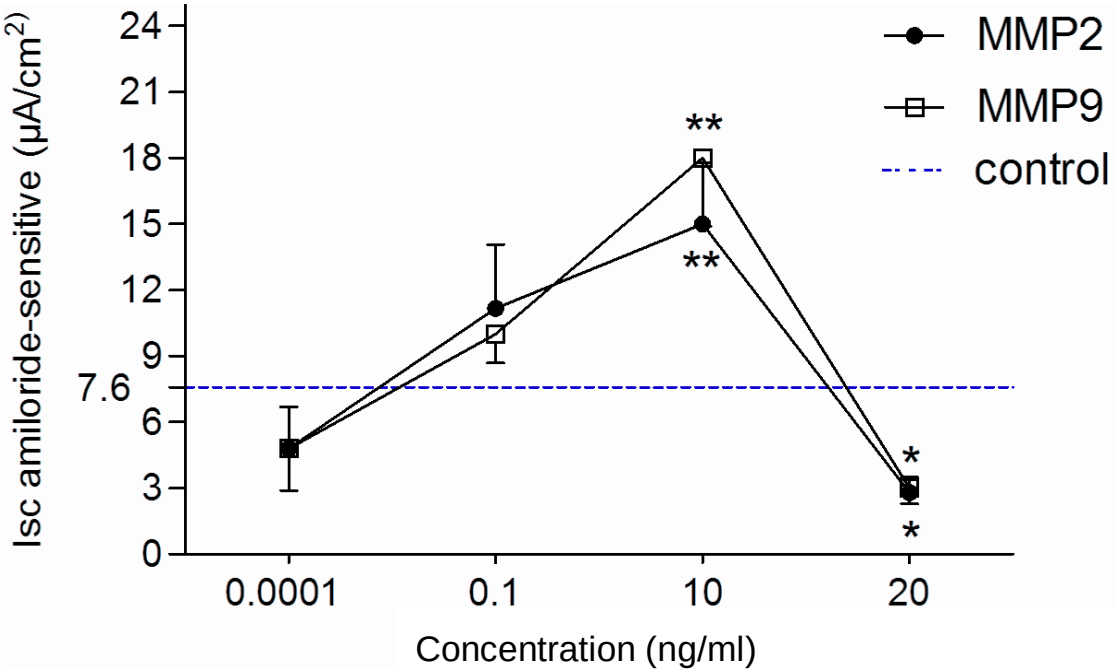

Figure 1. Effect of MMP2 and MMP9 added apically on the sheep parietal pleura. Values are means ± SEM. control n = 9, MMP2 and MMP9 n = 6; *p < 0.05 versus control experiments. When 0.0001 ng/ml MMP2 and MMP9 were incubated with the pleura, the Isc amiloride-sensitive was 4.8 ± 1.9 for both MMPs studied and this did not differ significantly from the control value. The incubation of the pleura with 0.1 ng/ml MMPs increased the Isc amiloride-sensitive to 11.17 ± 2.9 and 10 ± 4.3 for MMP2 and MMP9, respectively, but these changes were not statistically significant (p > 0.05). At concentration 10 ng/ml, MMP2 increased the Isc amiloride-sensitive to 15 ± 2.8 μA/cm^2 and MMP9 to 18 ± 3.1 μA/cm^2 (p = 0.0016 and 0.0021, respectively). At concentration 20 ng/ml, MMP2 decreased the Isc amiloride-sensitive from the value of 7.6 to 2.8 ± 0.7 μA/cm^2 and this decrease was statistically significant (p = 0.028). A significant decrease occurred also for MMP9 at concentration 20 ng/ml (from the value of 7.6 to 3 ± 0.7, p = 0.048).

high levels of serine proteases, including trypsin and kallikrein, are known since several years to degrade ENaC channel and inhibit sodium transport in toad bladder (Garty and Edelman, 1983) whereas lower levels of serine proteases are known to stimulate ENaC current constrictively (Planes and Caughey, 2007).

A possible mechanism through which MMP2 and MMP9 act on ENaC current is the proteolytic cleavage of ENaC by MMPs, although indirect channel inactivation via receptors or a protease cascade cannot be excluded. Certainly, our results should be further confirmed with substrate studies and the performance in *in vivo* experiments.

To conclude, this study is the first to indicate an effect of MMPs on ENaC current. At initial stages of inflammatory proceedings when concentrations of MMPs at pleural exudates are relatively low, MMPs may contribute to pleural fluid removal from the pleural space by enhancing sodium absorption. As the inflammatory response at pleural cavity proceeds and subsequently enzyme concentrations increase, MMPs ameliorate pleural fluid aggregation by impairing ENaC-mediated sodium absorption.

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