

The transport of cationic amino acids in human airway cells: expression of system y^+L activity and transepithelial delivery of NOS inhibitors

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SPECIFIC AIMS

1. To define the mechanisms responsible for arginine transport in airway epithelial cells we have used tight Calu-3 cell monolayers. In this model we have characterized the 1) influx routes of arginine through the apical and the basolateral membranes in the presence or absence of sodium and/or of L-leucine; 2) effects of L-leucine on the basolateral efflux of the cationic amino acid; 3) transepithelial (apical to basolateral) transport of arginine in the absence of sodium and/or of L-leucine.

2. To assess whether Calu-3 cell monolayers perform the transepithelial transfer of NOS inhibitors in pharmacologically active form, we evaluated 1) the effects of three NOS inhibitors (NMMA, NIL, and NAME) on the unidirectional fluxes and the transepithelial transport of arginine; 2) the NOS-inhibiting activity of basolateral medium conditioned by Calu-3 cell monolayers, incubated with NMMA, NIL, or NAME at the apical side.

PRINCIPAL FINDINGS

1. Calu-3 cells express the genes for transport systems y^+ , y^+L , and $B^{0,+}$

RT-PCR analysis of gene for cationic amino acid (CAA) transporters, expressed in Calu-3 cells, detected amplicons of the expected size for *SLC7A1* (coding for hCAT1 transporter), *SLC7A2* (for transcripts hCAT2B and hCAT2A), *SLC7A4*, *SLC7A6* (for y^+LAT2), *SLC7A7* (for y^+LAT1), *SLC3A2* (for 4F2hc/CD98), and *SLC6A14* (for ATB⁰⁺).

2. Apical transport of arginine is due to the operation of system $B^{0,+}$

Influx of arginine ([Arg]=100 μ M) at the apical membrane was due to a sodium-dependent transport mechanism that accounts for nearly 50% of the total influx of

the amino acid. This component was completely inhibited by leucine and therefore can be attributed to the activity of system $B^{0,+}$. The Na⁺-independent portion of apical arginine transport was not significantly inhibited by L-leucine and L-lysine. These results exclude the contribution of other high-affinity systems, such as $b^{0,+}$ and y^+ , to the apical transport of L-arginine.

3. The basolateral transport of arginine is due to the additive operations of systems y^+ and y^+L

At the basolateral side, arginine uptake was fully sodium independent. A partial (60%) inhibition by L-leucine was detected in the presence, but not in the absence, of extracellular sodium. This finding is a characteristic marker of system y^+L transport activity. The leucine-resistant portion of basolateral arginine transport was partially inhibited by L-lysine, indicating a component referable to the activity of system y^+ . Arginine efflux was markedly trans-stimulated by extracellular leucine (+170% after 5 min of incubation), indicating that system y^+L significantly contributes to CAA efflux from the basolateral membrane of Calu-3 cells.

4. Systems $B^{0,+}$ and y^+L contribute to the transepithelial transport of L-arginine

In the presence of sodium, transepithelial arginine flux was linear for at least 60 min. In the presence of sodium, transepithelial arginine flux was almost completely inhibited if leucine was present in the apical medium, but in the absence of sodium it was very low

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both in the presence and absence of leucine. If leucine was added to the basolateral medium, significant acceleration of the transepithelial flux of arginine was observed provided that extracellular sodium was present.

5. NOS inhibitors with amino acid structural features suppress the transepithelial arginine transport at the apical side and accelerate arginine efflux at the basolateral side

When added at the apical side of Calu-3 cell monolayers, NMMA and NIL almost abolished the transepithelial flux of the cationic amino acid, but NAME had a negligible effect. When added at the basolateral side, NMMA and NIL markedly trans-stimulated arginine efflux; NAME was completely ineffective. These results suggest that NMMA and NIL, but not NAME, interact with the apical system B^{0,+} and the basolateral system y^{+L}.

6. Calu-3 cell monolayers transport amino acid-like NOS inhibitors from the apical to the basolateral compartment

To ascertain whether NOS inhibitors were transported in pharmacologically active form across Calu-3 cell monolayers, we developed a bioassay based on the inhibition of NO production by murine RAW264.7 cells, a well-characterized model of macrophages that develop sustained, iNOS-dependent NO production when stimulated with LPS. NO production was assessed from the concentration of nitrites in the extracellular medium.

NAME, NMMA, or NIL were added to the apical medium of Calu-3 cell monolayers; after 48 h, the

basolateral medium was used to culture RAW264.7 cells. Nitrite production by RAW264.7 cells under basal or LPS-stimulated conditions was not significantly changed if macrophages were incubated in basolateral Calu-3-conditioned medium or in fresh unconditioned medium. However, basolateral media conditioned by Calu-3 cell monolayers preincubated with NMMA, NIL, or NAME at the apical side exerted a significant inhibitory effect on NO production. The inhibition was significantly higher ($P < 0.01$) if Calu-3 cell monolayers had been exposed to NIL (60%) or NMMA (58%) vs. NAME (25%).

The inhibition of NO production by RAW264.7 cells was dependent on the period of exposure of Calu-3 cell monolayers to the NOS inhibitors. Moreover, for NMMA- and NIL-conditioned media, but not with NAME, the inhibition was significantly higher if leucine had been present in the basolateral medium of Calu-3 cell monolayers during conditioning.

The three inhibitors completely suppressed NO production by RAW264.7 cells if added directly to the extracellular medium of macrophagic cells.

CONCLUSIONS AND SIGNIFICANCE

We have characterized the transepithelial transport of L-arginine through tight monolayers of Calu-3 cells as a model of human airway epithelium. A major finding of this contribution consists in the demonstration that Calu-3 monolayers are endowed with a functional system y^{+L}, supported by the following evidence: 1) Calu-3 cells express all the genes that code for system y^{+L} subunits; 2) the basolateral arginine influx is inhibited by L-leucine in the presence, but not the absence, of sodium; 3) leucine markedly trans-stimulates basolateral arginine efflux in the presence of sodium.

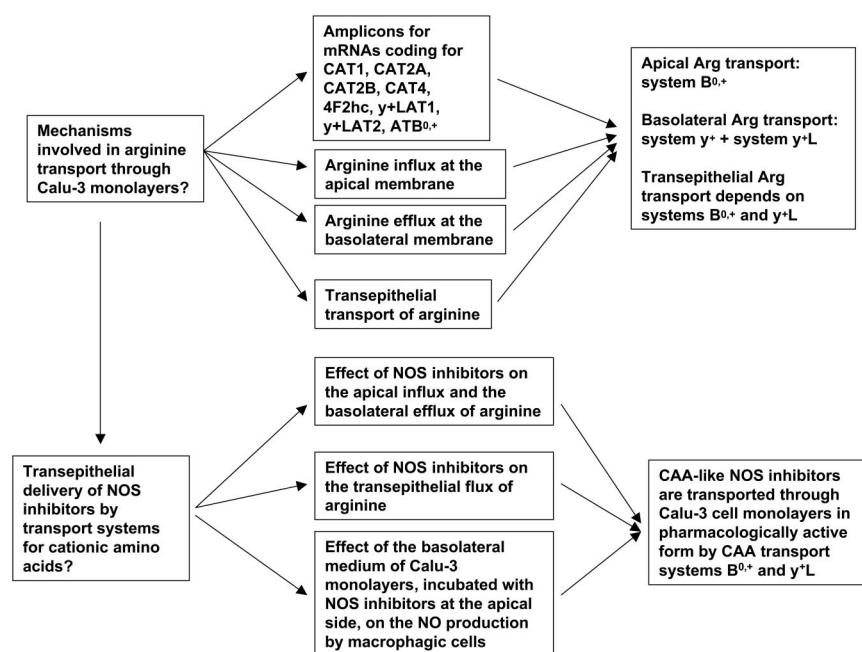


Figure 1. Schematic diagram.

The results also indicate that high-affinity arginine influx at the apical membrane is due only to system $B^{0,+}$. Indeed, neither at the apical nor at the basolateral membrane does leucine inhibit arginine influx in the absence of sodium, a result that excludes a significant contribution of system $b^{0,+}$ to the transport of the cationic amino acid in airway epithelial cells. Moreover, leucine and lysine additively inhibit basolateral, but not apical, arginine influx, indicating that the activity of system y^+ is restricted to the basolateral membrane. Like other epithelial models, Calu-3 cells express system y^+L activity at the basolateral but not at the apical membrane. Conversely, system $B^{0,+}$ is present at the apical but not the basolateral membrane. The polarized and mutually exclusive activities of systems $B^{0,+}$ and y^+L contribute to the unidirectional transepithelial transport of arginine from the apical to the basolateral compartments, although a component of basolateral efflux due to system y^+ cannot be excluded. The dependence of CAA transepithelial flux on the activity of system $B^{0,+}$ is clearly demonstrated by its substantial suppression in the absence of sodium as well as by its marked inhibition by apical leucine. Significant stimulation of the transepithelial flux of arginine is instead observed if leucine is added to the basolateral medium in the presence of sodium, thus demonstrating the intervention of system y^+L . This result indicates that in the absence of amino acids at the basolateral side, efflux of arginine through the basolateral membrane is rate-limiting for the overall transepithelial flux of the cationic amino acid.

In the presence of a physiological transmembrane sodium gradient and of neutral amino acids in the extracellular compartment, system y^+L operation favors arginine efflux from epithelial cells. Therefore, the activity of the system may be particularly important when the extracellular amino acid concentration at the apical side is increased. Examples of such conditions are acute respiratory distress syndrome and pulmonary alveolar phospholipoproteinosis (PAP). Patients affected by lysinuric protein intolerance (LPI), a severe autosomal recessive condition due to mutations of *SLC7A7/y+LAT1*, present a relatively high frequency of PAP that contributes significantly to the morbidity and mortality associated with the disease. The demonstration that airway epithelium is endowed with system y^+L activity and express *SLC7A7/y+LAT1* suggests this tissue may be a target of LPI mutation. This hypothesis deserves further investigation as the pathogenesis of lung alterations in LPI is still obscure.

The results presented here indicate that NOS inhibitors endowed with structural features of cationic amino acids are transported across Calu-3 cell monolayers in a pharmacologically active form. The basolateral medium of Calu-3 cell monolayers incubated with NMMA, NAME, or NIL added at the apical side significantly inhibits NO production by LPS-stimulated RAW264.7 murine macrophages. The basolateral medium condi-

tioned by Calu-3 cells in the absence of the inhibitors has no effect. The inhibitory effect is proportional to the period of conditioning in the presence of the NOS inhibitors.

Medium conditioned in the presence of apical NAME has a lower inhibitory effect than media conditioned in the presence of NMMA or NIL, suggesting the concentration attained by the former inhibitor in the basolateral compartment is lower. This behavior may be referable to the different transport pathways used by NMMA, NIL, or NAME to cross the airway epithelial monolayer. In fact, both NMMA and NIL, added at the apical side of the monolayer, inhibit in a competitive manner transepithelial arginine flux or the unidirectional apical influx of the amino acid to values comparable to those detected in the absence of sodium. NAME has no effect in either assay. These findings indicate that NMMA and NIL, but not NAME, interact with system $B^{0,+}$ in human airway cells. Moreover, NMMA and NIL, but not NAME, significantly stimulate the basolateral efflux of arginine to values similar to those obtained with leucine. Consistently, the presence of leucine in the basolateral compartment of Calu-3 cell monolayers, incubated with apical NIL or NMMA but not with NAME, significantly increases the NOS inhibitory effect of the conditioned medium. These findings are consistent with an interaction of NMMA and NIL with system y^+L . The poor interaction of NAME with systems $B^{0,+}$ or y^+L and the consequent difficult transepithelial delivery of this inhibitor may be due to the esterification of the α -carboxyl group, a unique structural feature of this NOS inhibitor.

The NO concentration in the exhaled air is raised in asthma, active fibrotic alveolitis, and other inflammatory airway pathologies. In several models of airway inflammation, the exaggerated NO production has been referred to iNOS induction in epithelial and inflammatory, subepithelial cells. Although the importance of these changes in the pathogenesis of the various conditions remains to be elucidated, the possibility of modulating NO production is of great interest. Exhaled NO levels have been lowered using NOS inhibitors or their prodrugs through inhalation and intravenous administration. Since NO production by iNOS has usually been associated with significant tissue damage, the finding that an iNOS-specific inhibitor such as NIL is transported across Calu-3 cells monolayer in pharmacologically active form is of interest in view of possible practical applications. Calu-3 cells have been repeatedly used in studies of drug transport as a reliable model of the behavior of airway epithelium. Thus, the results presented here suggest that NIL, and possibly other isoform-specific NOS inhibitors endowed with CAA structural characteristics, can selectively inhibit iNOS-dependent NO synthesis not only in airway epithelium, but also in subepithelial inflammatory cells. [F]