Anti-inflammatory effects of Na⁺/H⁺ exchanger inhibitors

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In macrophages, Na⁺/H⁺ exchangers (NHEs) are activated by various stimuli and regulate the functions of macrophages. The NHE inhibitors amiloride, 5-(N,N-dimethyl)-amiloride (DMA) and 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) inhibited the lipopolysaccharide (LPS)-induced production of prostaglandin (PG) E₂ in the mouse macrophage-like cell line RAW 264. They inhibited both the LPS-induced release of arachidonic acid from membrane phospholipids at 4 h and the LPS-induced increase in the level of cyclooxygenase (COX)-2 protein at 6 h, but did not directly inhibit the COX activity. The vacuolar-type (H⁺)-ATPase (V-ATPase) inhibitor, bafilomycin A₁, which activates NHE via reducing intracellular pH, also increased the level of COX-2 protein. The bafilomycin A₁-induced expression of COX-2 was inhibited by the NHE inhibitors and by the Na⁺/Ca²⁺ exchanger (NCX) inhibitor SN-6, indicating that the activation of NHE leads to COX-2 expression via, in part, functional coupling with NCX. In an air pouch-type LPS-induced inflammation model in mice, amiloride and EIPA, as well as the COX inhibitor indomethacin, as well as the COX inhibitor indomethacin, significantly reduced the level of PGE₂ in the pouch fluid collected at 8 h and the vascular permeability during 4 to 8 h. The accumulation of leukocytes in the pouch fluid collected at 8 h was significantly inhibited by amiloride and EIPA but not by indomethacin. Thus, NHE inhibitors showed more effective anti-inflammatory activity than indomethacin. Therefore, NHEs might be a novel target for developing anti-inflammatory drugs that inhibit the expression of COX-2 in the activated inflammatory cells.


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Introduction

Na\textsuperscript{+}/H\textsuperscript{+} exchangers (NHEs) are transmembrane proteins that exchange intracellular H\textsuperscript{+} and extracellular Na\textsuperscript{+}. So far, nine mammalian isoforms of NHE have been identified (NHE1-9, Table 1), which differ in their distribution, regulatory properties, and physiological functions\textsuperscript{[2,3]}. NHE-1, an ubiquitously expressed NHE in the plasma membrane of nonpolarized cells, regulates functions of macrophages. Vacuolar-type (H\textsuperscript{+})-ATPase (V-ATPase) transports H\textsuperscript{+} from cytoplasmic compartment to the opposite side\textsuperscript{[4,5]}. Both NHE and V-ATPase are involved in cytoplasmic pH homeostasis in macrophages\textsuperscript{[6]}. Various stimuli including lipopolysaccharide (LPS), TNF-\textalpha\textsuperscript{[7]}

and IFN-\gamma\textsuperscript{[8]} also activate NHE in macrophages. The increased activity of NHE contributes to the production of cytokines\textsuperscript{[9]}, upregulation of I-A gene expression\textsuperscript{[9]}, expression of the Fc\gamma receptor\textsuperscript{[10]}, and CSF-1-induced proliferation\textsuperscript{[11]} in macrophages. In addition, NHE is activated by the reduction of cytoplasmic pH to maintain the cytoplasmic pH. Since extracellular pH is decreased in the chronic inflammatory site, resulting in the decrease in cytoplasmic pH of the infiltrated leukocytes, NHE in the leukocytes might be activated and play roles in the production of cytokines and prostaglandins (PGs). However, little is known about the involvement of NHE in the production of PGE\textsubscript{2} by macrophages and its mechanism. To clarify the involvement of NHE in PGE\textsubscript{2} production in the stimulated macrophages, RAW 264 cells, a macrophage-like cell line, were stimulated with LPS, which induces the activation of NHE, or the V-ATPase inhibitor bafilomycin A\textsubscript{1}, which activates NHE via reducing cytoplasmic pH, and effects of NHE inhibitors on PGE\textsubscript{2} production were examined. In addition, effects of NHE inhibitors on LPS-induced inflammation in air pouch-type inflammation model in mice were examined.

Table 1. Isoforms of NHE (modified from De Vito\textsuperscript{[10]})

<table>
<thead>
<tr>
<th>Isoforms</th>
<th>Membrane</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHE1</td>
<td>Plasma Membrane</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>NHE2</td>
<td>Plasma Membrane</td>
<td>Stomach, Colon, Kidney</td>
</tr>
<tr>
<td>NHE3</td>
<td>Plasma Membrane</td>
<td>Colon, Stomach, Intestine</td>
</tr>
<tr>
<td>NHE4</td>
<td>Plasma Membrane</td>
<td>Stomach, Colon, Kidney</td>
</tr>
<tr>
<td>NHE5</td>
<td>Organelle Membrane</td>
<td>Brain, Muscle, Spleen</td>
</tr>
<tr>
<td>NHE6</td>
<td>Organelle Membrane</td>
<td>Muscle, Heart, Brain</td>
</tr>
<tr>
<td>NHE7</td>
<td>Organelle Membrane</td>
<td>Brain, Pancreas, Thyroid</td>
</tr>
<tr>
<td>NHE8</td>
<td>Organelle Membrane</td>
<td>Kidney</td>
</tr>
<tr>
<td>NHE9</td>
<td>Organelle Membrane</td>
<td>Stomach, Intestine</td>
</tr>
</tbody>
</table>

Involvement of NHE in LPS-induced PGE\textsubscript{2} production\textsuperscript{[10]}

We analyzed the effect of the NHE inhibitors amiloride and its analogs, 5-(N,N-dimethyl)-amiloride (DMA) and 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), on the LPS-induced PGE\textsubscript{2} production in culture of RAW 264 cells. The stimulation with LPS increased the COX-2 expression at 4 h and the PGE\textsubscript{2} level in the conditioned medium collected at 8 h (Fig. 1). We revealed that amiloride (10, 30 and 100 \textmu M) suppresses the LPS-induced PGE\textsubscript{2} production in a concentration-dependent manner (Fig.1B). It is reported that amiloride inhibits Na\textsuperscript{+} channels and the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger\textsuperscript{[11]} in addition to NHE. However, the main mechanism by which amiloride inhibited the LPS-induced PGE\textsubscript{2} production might be due to the inhibition of NHE, because the selective inhibitors of NHE, DMA and EIPA\textsuperscript{[12]}, also significantly reduced the LPS-induced PGE\textsubscript{2} production (Fig.1D). These inhibitors also inhibited the LPS-induced increase in the level of COX-2 protein at 6 h (Fig. 1). In addition, amiloride (10, 30 and 100 \textmu M), DMA (10 \textmu M), and EIPA (3 \textmu M) inhibited partially the LPS-induced release of \textsuperscript{[1]}H-arachidonic acid from membrane phospholipids of \textsuperscript{[1]}H-arachidonic acid-labeled RAW 264 cells without affecting the basal release (data not shown). However, it was unlikely that amiloride directly inhibited COX activity because amiloride did not inhibit the arachidonic acid-induced production of PGE\textsubscript{2}.

![Fig.1 Effects of NHE inhibitors on LPS-induced expression of COX-2 and PGE\textsubscript{2} production](image-url)

RAW 264 cells were incubated for 6 h (for COX-2) or 8 h (for PGE\textsubscript{2}) in medium containing LPS with or without NHE inhibitors. COX-2 levels in the cells were detected by Western blotting (A and C) and PGE\textsubscript{2} levels in the conditioned medium were determined by RIA (B and D). Statistical significance: *p < 0.05, **p < 0.01. N.D., not detectable. (Modified from Kamachi et al.\textsuperscript{[14]})
Functional coupling of NHE with NCX in macrophages

The inhibition of V-ATPase by bafilomycin A₁ reduces cytosolic pH resulting in the activation of NHEs. On treatment of RAW264 cells with bafilomycin A₁ (100 nM), the level of COX-2 protein increased from 8 h, attained a maximum at 12 h, and then declined (data not shown). Amiloride (30 and 100 μM) significantly inhibited the bafilomycin A₁-induced expression of COX-2 protein in a concentration-dependent manner (Fig.2A). DMA (10 μM) and EIPA (3 μM) also inhibited the bafilomycin A₁-induced expression of COX-2 (Fig.2A). The activation of NHEs results in the accumulation of intracellular Na⁺, which, in turn, increases the intracellular concentration of Ca²⁺ via the Na⁺/Ca²⁺ exchanger (NCX). Therefore, to clarify the involvement of NCX in the bafilomycin A₁-induced COX-2 protein expression, we examined the effect of the NCX inhibitor SN-6. We found that SN-6 decreased the bafilomycin A₁-induced expression of COX-2 protein in a concentration-dependent manner (Fig.2B). These findings suggested that NHE was functionally coupled with NCX in induction of COX-2 protein expression.

Anti-inflammatory actions of NHE inhibitors

We confirmed that NHE inhibitors exhibited anti-inflammatory actions using an air pouch-type LPS-induced inflammation model in mice. Consistent with the analysis in vitro, amiloride and EIPA significantly reduced the level of PGE₂ in the pouch fluid collected 8 h after injection of LPS solution into the air pouch as indomethacin did (Fig.3A). As well as indomethacin, amiloride and EIPA significantly inhibited the increase in vascular permeability from 4 to 8 h (Fig.3B). In addition, amiloride and EIPA significantly lowered the number of leukocytes infiltrating the pouch fluid at 8 h, while indomethacin did not (Fig.3C). The reduction in vascular permeability caused by amiloride and EIPA is probably due to the inhibition of the production of PGs. Suppression of neutrophil infiltration into the pouch fluid by NHE inhibitors in this model might be due to the inhibition of chemokine production and leukocyte migration as it is reported that NHE inhibitors suppressed the production of several chemokines by macrophages and the migration of neutrophils. As a probable mechanism, we found that NHE inhibitors inhibited the bafilomycin A₁-induced activation of c-Jun N-terminal kinase.
which is involved in the production of several cytokines and chemokines as well as COX-2. In contrast, it is reported that PGE₂ negatively regulates the production of chemokines by various cells, such as macrophages and dendritic cells, and that indomethacin increases chemokine production. These findings also suggested that NHE inhibitors might exert the anti-inflammatory effects in a COX-independent manner. Further investigation is required to demonstrate this possibility.

**Conclusion**

In this study, we suggested that NHE plays a role in the LPS- and V-ATPase inhibitor-induced expression of COX-2 by coupling with NCX (Fig. 4). The NHE inhibitors reduced LPS-induced PGE₂ production *in vitro* and *in vivo* and showed anti-inflammatory actions more effectively than indomethacin. Although NHE is distributed in most tissues, it is virtually quiescent under resting conditions, and is activated by several inflammatory stimuli. Therefore, it is conceivable that NHE inhibitors selectively suppress PGE₂ production in the activated inflammatory cells without affecting the physiological production of PGs. Thus, NHEs might be a novel target for developing anti-inflammatory drugs that inhibit the expression of COX-2 and inhibit PGE₂ production in activated inflammatory cells.

**References**