Hypertonic Saline Inhibits GPCR Priming of the Neutrophil via Arrest of Receptor Endocytosis

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Neutrophil Priming for Cytotoxicity

Primer

activate

$\text{O}_2^-$
GPCR (PAF) priming requires CME

McLaughlin, et al. Journal of Immunology, in press
Postinjury Hypertonic Saline Resuscitation:

Clinical and animal studies: potential immunomodulatory effect

Intracellular mechanisms remain unclear...
Hypothesis:

HTS abrogates PAF priming of the PMN through arrest of PAF receptor endocytosis.
1) Receptor internalization and cytosolic Ca\(^{2+}\) flux
   - Flow cytometry
   - Immunomicroscopy
   - Ratiometric calcium measurement

2) β-arrestin recruitment to the PAFR complex
   - Immunomicroscopy

3) Recruitment of clathrin to the signalosome and activation of p38MAPK
   - Immunoprecipitation

4) Scission of the signalosome from the membrane via dynamin
   - Immunomicroscopy

5) Association of EEA-1 and RAB5A at the early endosome
   - Immunomicroscopy
   - Immunoprecipitation

6) p67 translocation
   - Subcellular fractionation and immunoblot
HTS inhibits PAF-induced translocation of p67phox to the plasma membrane

S.C. Membrane

Blot: P67phox

<table>
<thead>
<tr>
<th></th>
<th>PAF</th>
<th>HTS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Sheppard, *Journal of Trauma*, 2004
1) Receptor internalization and cytosolic Ca^{2+} flux
   - Flow cytometry
   - Immunofluorescence
   - Ratiometric calcium measurement

2) β-arrestin recruitment to the PAFR complex
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3) Recruitment of clathrin to the signalosome and activation of p38MAPK
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   - Immunofluorescence

5) Association of EEA-1 and RAB5A at the early endosome
   - Immunofluorescence
   - Immunoprecipitation

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Investigating protein-protein interactions:
- Immunoblot
- Immunoprecipitation
- Microscopy
Immunomicroscopy
FRET (fluorescent resonance energy transfer)
Hypertonic saline impairs RAB5A-EEA1 association

* = P<0.05
Hypertonic saline impairs RAB5A-EEA1 association

<table>
<thead>
<tr>
<th>IP: Rab5a</th>
<th>Blot:</th>
<th>Resting</th>
<th>1 min</th>
<th>3 min</th>
<th>140 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEA-1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PAF</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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Hypertonic saline abrogates dynamin-dependent scission of the PAFR from the membrane

![Images of PAFR, Dynamin-2, and Overlay under resting, +PAF, and HTS+PAF conditions]

% Co-localization

resting +PAF HTS+PAF

* = P<0.05
1) Receptor internalization and cytosolic Ca\(^{2+}\) flux
   - Flow cytometry
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Incubate with HTS (180mM) or buffer

Prime with PAF

Isolate human PMNs
Clathrin-dependent p38 MAPK activation

<table>
<thead>
<tr>
<th>IP: β-arrestin-1</th>
<th>Blot:</th>
<th>Resting</th>
<th>1 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clathrin HC</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>p-p38 MAPK</td>
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</tbody>
</table>

Clathrin HC: 200 kDa

p-p38 MAPK: 38 kDa

PAF: - - + + +
HTS: - + - + +
1) Receptor internalization and cytosolic Ca\(^{2+}\) flux
- Flow cytometry
- Immunomicroscopy
- Ratiometric calcium measurement

2) \(\beta\)-arrestin recruitment to the PAFR complex
- Immunomicroscopy

3) Recruitment of clathrin to the signalosome and activation of p38MAPK
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4) Scission of the signalosome from the membrane via dynamin
- Immunomicroscopy

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- Immunomicroscopy
- Immunoprecipitation

6) p67 translocation
- Subcellular fractionation and immunoblot

Incubate with HTS (180mM) or buffer
Prime with PAF
Isolate human PMNs
Hypertonic saline abrogates B-arrestin recruitment to the PAFR

**FRET efficiency**

* = P<0.05
1) Receptor internalization and cytosolic Ca^{2+} flux
   Flow cytometry
   Immunomicroscopy
   Ratiometric calcium measurement

2) β-arrestin recruitment to the PAFR complex
   Immunomicroscopy

3) Recruitment of clathrin to the signalosome and activation of p38MAPK
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5) Association of EEA-1 and RAB5A at the early endosome
   Immunomicroscopy
   Immunoprecipitation

6) p67 translocation
   Subcellular fractionation and immunoblot
Hypertonic saline inhibits PAFR internalization after PAF ligation

Green: PAFR

% control MFI

0m 1m 5m 10m

resting +PAF HTS+PAF

HTS+PAF

+PAF

+HTS
Hypertonic saline does not prevent PAFR ligation or G-protein induced calcium flux
Incubate with HTS (180mM) or buffer

Prime with PAF

Isolate human PMNs

1) Receptor internalization and cytosolic Ca²⁺ flux
   - Flow cytometry
   - Immunomicroscopy
   - Ratiometric calcium measurement

2) β-arrestin recruitment to the PAF complex
   - Immunomicroscopy

3) Recruitment of clathrin to the signalosome and activation of p38MAPK
   - Immunoprecipitation

4) Scission of the signalosome from the membrane via dynamin
   - Immunomicroscopy

5) Association of EEA-1 and RAB5 at the early endosome
   - Immunomicroscopy

6) p67 translocation
   - Subcellular fractionation and immunoblot
Conclusion:

• Clinically relevant concentrations [180mM] of hypertonic saline inhibit PAFR internalization via elimination of ß-arrestin recruitment to the PAFR

• Hypertonic saline does not effect receptor-ligand interaction demonstrated by a lack of effect on Ca^{2+} flux
Hypertonic Saline Inhibits GPCR Priming of the Neutrophil via Arrest of Receptor Endocytosis

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Ernest E. Moore, MD
Nathan McLaughlin, BS
Phillip Eckels, BS
Anirban Banerjee, PhD
Chris C. Silliman, MD, PhD

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Belle Bonfils Blood Center
The PAF receptor is not shed or degraded upon receptor ligation:
Acceptor Photobleaching FRET:

![Image of fluorescence microscopy results showing acceptor photobleaching and FRET changes with different treatments.](image-url)