EPIGENETIC MODIFICATION FOR THE FUTURE TREATMENT OF INFLAMMATORY DISEASE

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THERAPEUTIC POTENTIAL OF EPIGENETICS

EPIGENETICS: non genetic changes in chromatin structure resulting in changes in gene expression
- DNA methylation- long-term changes, developmental
- Histone modification

DNA methylation
- DNA methyltransferase inhibitors (e.g. azacytidine):
  reverse silencing of good genes
- Stimulate methylation: silence bad genes
- Applicable to lung cancer, inflammation?
- Problems of specificity and targeting

Histone modification
- Involved in cancer, fibrosis, inflammation
- Small molecule modifiers now identified (including existing therapies)
Repressive chromatin
Decreased transcription
Inflammatory gene repression

Active chromatin
Increased transcription
Inflammatory gene expression

Multiple transcription factors
HAT = histone acetyltransferase

INFLAMMATORY GENE TRANSCRIPTION

Core histones
Histone acetylation

Histone acetylation
HAT = histone acetyltransferase

mRNA

CHROMATIN STRUCTURE

* = Acetylation sites: Lysine residues

H2A
H2B
H3
H4
DNA

H3AC
H2AC
**HISTONE ACETYLATION**

- Histone octamer: H3, H4, H2A, H2B
- Histone 4
- Histone acetylation: \(-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}_3^+\) → \(-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}-\text{CO}-\text{CH}_3\)
- Lysine → ε-acetylated Lysine

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**HISTONE ACETYLATION AND GENE TRANSCRIPTION**

- Gene repression
- Histone deacetylation: HDAC 1-11
- Corepressors

- Gene transcription
- Histone acetylation: HATs: CBP, p300, pCAF etc
- Coactivators

- DNA
  - Nucleosome (histone octamers)
  - RNA polymerase II
  - Transcription factor
  - Acetylation of Lys
**HISTONE ACETYLATION**

**Histone acetyltransferase**

Anti-acetylated histone H4

A549 cells

**IL-1β (ng/mL)**

HAT activity (dpm/µg protein)

IL-1β (ng/mL)

0 0.01 0.1 1

**Histone acetyltransferase**

Ito K et al: Mol Cell Biol 2000

**HISTONE ACETYLATION AND GENE EXPRESSION**

NF-κB regulated genes

- Chemokines: CXCL1, CXCL8, CCL2, CCL5, CCL11
- Cytokines: GM-CSF, TNF-α, IL-1β, IL-6
- Enzymes: iNOS, cPLA₂, COX-2, MMP-9
- Receptors: NK₁, NK₂, bradykinin B₁, B₂
- Peptides: endothelin-1
- Adhesion mols: ICAM-1

Inflammatory stimuli (e.g. IL-1β, TNF-α)

Inflammatory transcription
ACTIVATION OF INFLAMMATORY GENES

Coactivators e.g. CBP

Repressed chromatin CLOSED

INFLAMMATORY GENE REGULATION

Transcription factors e.g. NF-κB

Histone acetylation

NF-κB

INFLAMMATION

Corticosteroids

↑ HDAC

HAT

INFLAMMATORY PROTEINS e.g. GM-CSF, IL-8

Histone deacetylation

mRNA ↓

INFLAMMATION

Repressed chromatin CLOSED

Activated chromatin OPEN

Core histones

Chromatin transcription factors

Coactivators e.g. CBP

Histone acetylation

mRNA ↓

HAT

HAT

Histone deacetylation

HAT
### EFFECT OF CORTICOSTEROID ON HDAC

**A549 cells** p65 (NF-κB) immunoprecipitates

<table>
<thead>
<tr>
<th></th>
<th><strong>HDAC activity (dpm)</strong></th>
<th><strong>HDAC2 protein</strong></th>
</tr>
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<tbody>
<tr>
<td></td>
<td><strong>IL-1β</strong></td>
<td><strong>Dex</strong> (10⁻¹⁰M)</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>*</td>
<td>Anti-HDAC2</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>Anti-p65</td>
</tr>
<tr>
<td>+</td>
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**Acetylation of lysine 8 on histone H4**

### CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

**GM-CSF promoter (-70 to +32bp)**

<table>
<thead>
<tr>
<th></th>
<th><strong>AcK8 IP</strong></th>
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<tbody>
<tr>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
</tr>
<tr>
<td>Dex</td>
<td>10 8 6 6 (-log M)</td>
</tr>
</tbody>
</table>

**Acetylation of lysine 8 on histone H4**
CORTICOSTEROID SUPPRESSION OF INFLAMMATORY GENES

Inflammatory stimuli
- e.g. IL-1β, TNF-α

Activated GR: highly specific for
activated inflammatory gene complex
(recognition of histone acetylation signature)

Corticosteroids

Inflammatory protein
(e.g. GM-CSF)

CBP
HAT

Gene activation

Gene repression

Deacetylation

Co-repressors

Recruitment

GR

HDAC2

EFFECT OF STEROID ON INFLAMMATORY GENES
Histone deacetylases (HDAC1-11):
- reverse histone acetylation
- switch off gene transcription
- HDAC2 switches off inflammatory genes
- HDAC2 recruited by glucocorticoid receptors to activated inflammatory genes: mediates suppression of inflammation by steroids

Ito K et al: FASEB J 2001

CORRELATION OF HDAC TO STEROID RESPONSE

Alveolar macrophage: normal smokers and non-smokers

Inhibitory effect of Dex on TNF-α (%)

Inhibitory effect of Dex on TNF-α (%)

Ito K et al: FASEB J 2001
**HDAC2 KNOCK-DOWN: RNAi**

*Alveolar/sputum macrophages*

<table>
<thead>
<tr>
<th></th>
<th>H1</th>
<th>H2</th>
<th>Sc</th>
<th>H2</th>
<th>NT</th>
</tr>
</thead>
<tbody>
<tr>
<td>αHDAC2</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

![Graph showing GM-CSF levels](image)

*Non-treated* | *Scrambled* | *HDAC2 KD* |
---|---|---|
| Non-treated | LPS | LPS + Dex (10⁻⁶M) |

*Ito K et al.: J Exp Med 2006*

**HDAC2 IN COPD LUNG**

*Peripheral lung (surgical resection)*

<table>
<thead>
<tr>
<th></th>
<th>H4 acetylation of κB binding site on IL-8 promoter (ChIP)</th>
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<tbody>
<tr>
<td>HDAC2</td>
<td></td>
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</table>

*↑ Histone acetylation of IL-8 gene correlated with ↓ HDAC2 → Neutrophilic inflammation*

![Graph showing HDAC2 expression and IL-8 mRNA](image)

*Non-smokers* | *Normal smokers* | *COPD* |
---|---|---|
| HDAC2 expression (ratio vs histone-1) | IL-8 mRNA (RT-PCR) |

*Ito K et al: N Engl J Med 2005*
HDAC2 AND STEROID RESPONSIVENESS IN COPD

Alveolar macrophages

HDAC activity

Plasmid vector with HDAC2 Restores HDAC2 to normal

COPD macrophages

GM-CSF secretion

Ito K et al: J Exp Med 2006

LPS + dexamethasone (1μM)

GM-CSF secretion

Empty vector HDAC2 vector HDAC1 vector

HDAC activity (ΔAFU)

n=6

Control

LPS

Ito K et al: BBRC 2004

NITRATION AND HDAC2 ACTIVITY

HDAC2 Immunoprecipitates

Anti-NT

Anti-HDAC2

C Sm COPD

Exhaled Peroxynitrite

Nitro-Tyr/HDAC2 ratio

N COPD

Osoata G et al: Chest 2009

HDAC2 activity (dpm/HDAC2)

p<0.001

p<0.001

Ito K et al: BBRC 2004
PEROXYNITRITE INDUCES STEROID RESISTANCE

Human airway epithelial cells

IL-1β + SIN-1 (500µM)

SIN-1: peroxynitrite generator

GM-CSF (% of control)

[0 - 100]

[Dexamethasone (-log M)]

C -12 -11 -10 -9 -8 -7 -6

Ito K et al: BBRC 2004

CORTICOSTEROID RESISTANCE IN COPD

COPD

Cigarette smoke

Inflammation

iNOS

ANTIOXIDANTS

iNOS INHIBITORS

Peroxynitrite scavengers

Peroxynitrite

THEOPHYLLINE

HDAC activator

Destruction by 28S proteasome

Proteasome inhibitors

Ub E3 ligase inhibitors

↑ Inflammatory genes

↓ Response to steroids

Barnes PJ: Ann Rev Physiol 2009

Osoata G et al: BBRC 2009

O2- → NO → Peroxynitrite

Tyr146

Tyr253

 Ub

Ub

Ub

HDAC2

THEOPHYLLINE

HDAC activator

Destruction by 28S proteasome

Proteasome inhibitors

Ub E3 ligase inhibitors

↑ Inflammatory genes

↓ Response to steroids

Barnes PJ: Ann Rev Physiol 2009

Osoata G et al: BBRC 2009
CORTICOSTEROID RESISTANCE

Oxidative stress  Nitrative stress

Cell membrane

Steroid resistance

\[
\uparrow \text{PI3K-δ} \rightarrow \text{Akt} \rightarrow \text{HDAC2}
\]

Oxidative stress

Peroxynitrite

Akt (PKB)

HDAC2

Peripheral lung

PI3K-Akt PATHWAY

Oxidative stress

\[
\begin{align*}
\text{PI3K} & \rightarrow \text{Akt} (PKB) \\
& \rightarrow \text{HDAC2}
\end{align*}
\]

PI3K activation

\[
\text{PI3K-δ mRNA}
\]

To Y et al: Am J Respir Crit Care Med 2010
THEOPHYLLINE AS HDAC ACTIVATOR

Theophylline in low therapeutic concentrations:
- activates HDAC
  - via a novel mechanism (not PDE/adenosine antagonist)
- markedly potentiates steroid effects
- reverses steroid resistance


COPD macrophages: nuclear lysates

![Graph showing HDAC activity (AFU/10µg) for B/L and Theo (10^-6 M) with significant increase at Theo (10^-4 M).]

THEOPHYLLINE RESTORES STEROID RESPONSE

Alveolar macrophages: smokers

![Graph showing IL-8 (ng/ml) for Cntrl, LPS, Theo (1µM), Dex (1nM), Theo + Dex, and TSA with significant decrease at Theo + Dex.]

THEOPHYLLINE EFFECT ON ChIP ANALYSIS

Histone acetylation of NF-κB site of IL-8 promoter

![Graph showing histone acetylation at κB site](image)

**Marwick J et al: BBRC 2008**

EFFECT OF THEOPHYLLINE IN SMOKING MICE

Theophylline 3 mg/kg p.o.

Lung Inflammation

Similar results with inhaled theophylline

Reversed by HDAC inhibitor

No detectable plasma levels (TSA)

![Graph showing lung HDAC activity and inflammation](image)

**Fox JC et al: ATS 2007**
REVERSAL OF SMOKE-INDUCED INFLAMMATION

**Dex+Theo**

Cigarette smoke (4%, 30 min)

**Drugs**

Air

**Dex**

**Theo**

**Dex+Theo**

Days: 1 2 3 4 5 6 7 8 9 10 11 12 13 14

**BAL Neutrophils**

Theophylline 10mg/kg orally (plasma conc 4.0±0.9mg/L)

**To Y et al: AJRCCM 2010**

COPD PATIENTS: CORTICOSTEROIDS + THEOPHYLLINE

Fluticasone

F+T combination

Placebo

Theophylline

Plasma theophylline~8mg/L

n=30

**Induced sputum**

Sputum neutrophils

Sputum neutrophil elastase

**HDAC activity**

PBMCs

Total HDAC activity (relative light units)

No difference in fluticasone or theophylline alone treatment

**Ford P et al: Chest 2010**
STEROID RESISTANCE IN SMOKING ASTHMATICS

NON-SMOKING ASTHMA

SMOKING ASTHMA

Inflammatory stimuli

Corticosteroids

Peroxynitrite

Histone acetylation

Cigarette smoke

Oxidative stress

HDAC2

Histone acetylation

Steroid resistance

GM-CSF

IL-8

eotaxin

THEOPHYLLINE + ICS IN SMOKING ASTHMATICS

Serum theophylline 5 mg/l

Change in PEF (L/min)

Duration (days)

Spears M et al: ERJ 2009
HOW DOES THEOPHYLLINE RESTORE HDAC?

**U937 cells**

**Immunoprecipitated PI3K-δ**

A549 cells

Intact (IC_{50}=134μM)

H_{2}O_{2} stimulated (IC_{50}=2.1μM)

LY: LY 294002, non-selective PI3K inhibitor

**PI3K-δ INHIBITION IN VIVO**

A/J Mice

Cigarette smoke (4%, 30 min)

Drugs

IC87114: PI3K-δ inhibitor

LY294002: pan PI3K inhibitor

*** NS

Neutrophils (x10^4 cells/ml)

Air  ▶  Smoke  IC  Dex+IC  Dex+LY

Dex

** NS

BÅL
Marwick J et al: AJRCCM 2009

### PI3K-δ NULL MICE

![Bar graph showing BAL neutrophils/ml x 10^3](image)

- **Sham**
- **Smoke**
- **Smoke + budesonide**

**Steroid-resistant**

**Steroid-responsive**

**Steroid-resistant**

Wild type (balb/c)  
PI3K-δ null  
PI3K-γ null

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Borisy AA et al: PNAS 2003

### UNEXPECTED SYNERGY

![Bar graph showing cells x10^6](image)

- **Brown Norway rats: inhaled ovalbumin challenge**
- **Inhaled administration**

- **B/L**  
- **Vehicle**  
- **Bud**  
- **NT**  
- **Bud+NT**

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![Combinator](image)
**NORTRIPTYLENE AND HDAC REVERSAL**

Effect of nortriptylene hydrochloride

<table>
<thead>
<tr>
<th>HDAC activity</th>
<th>U937 cells</th>
<th>PI3K activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>H₂O₂</td>
<td>H₂O₂ + NH (1μM)</td>
</tr>
</tbody>
</table>

**PI3Kδ inhibition**

- **IC₅₀=0.82μM**
- (No effect on PI3Kα, PI3Kγ)

% Inhibition

- [Nortriptylene (μM)]

**PI3Kδ inhibition**

- Imminoprecipitated enzyme

**REVERSAL OF CORTICOSTEROID RESISTANCE**

- Oxidative stress
- Antioxidants
  - Nrf2 activators (sulforaphane)
- THEOPHYLLINE
- Nortriptyline
- PI3Kδ inhibitors
- Akt inhibitors
- HDAC2 activators?
- Macrolides (non-antibiotic)

Reversal of steroid resistance
Macrolides prevent decrease in promoter activity

Relative luminescence

HDAC2 promoter activity

Erythromycin

Non-antibiotic macrolide

HDAC2

Promoter activity

Normoxia

Hypoxia

EM

EM703

Erythromycin

Non-antibiotic macrolide

Epigenetic modification of histones

Phosphorylation

Nitration

Ubiquitination

SUMOylation

Methylation

Inflammatory genes
METHYLATION AND STEROID ACTION

5-aza-dC: 5-aza-2'-deoxycytidine: Methytransferase inhibitor


EFFECT OF STEROID ON HISTONE METHYLATION

TGF-β1 promoter

HMT associates with GR

FP: fluticasone propionate
CONCLUSIONS

- Multiple histone modifications regulated by enzymes involved in regulation of inflammatory genes
  acetylation, methylation, phosphorylation, nitration, ubiquitination, sumoylation
- HDAC2 recruitment mediates antiinfl effects of corticosteroids ↓ due to oxidative/nitrative stress
- HDAC2 activity restored by gene transfer, theophylline reverse corticosteroid resistance in COPD cells
- Theophylline ↑ in HDAC2 mediated by PI3Kδ inhibition
- Histone methylation (H3K9) ↑ by corticosteroids HMT (SUV39H1) recruited by corticosteroids
- New therapeutic approaches targeting epigenetic changes now possible

Lee K et al: J Immunol 2006
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