*cis*-9,*trans*-11-Conjugated linoleic acid down-regulates phorbol ester-induced NF-κB activation and subsequent COX-2 expression in hairless mouse skin by targeting IκB kinase and PI3K-Akt
Introduction
Inflammation & Cancer

- A close association between inflammation and cancer has long been suspected. *Nature*, 2004

- There is now growing evidence supporting that chronic inflammation may lead to malignancies of different organs including stomach, colon, breast, skin, prostate, pancreas, etc. *Trends Immunol.*, 2005; *Nature Rev. Immunol.*, 2005

- It has been estimated that 15% of all cancers are somehow linked to inflammation. *Science*, 2005
Arachidonic acid & cyclooxygenase-2 (COX-2)

- Phospholipase A2 (cPLA2, sPLA2)
- Stimulus
- Glycerophospholipids
- Glycerol
- Arachidonic acid
- COX-1, COX-2
- Prostaglandin
  - TXA2, PGD2, PGE2, PGF2, PDI2
- Carcinogenesis
- Tumorigenesis
- Cancer development

Molecular Medicine, 2003
Cyclooxygenase-2 (COX-2) and Cancer

In response to diverse pro-inflammatory stimuli, such as cytokines, endotoxins, growth factors, and tumor promoters including phorbol ester, prostaglandins are produced in abundance through metabolic conversion of arachidonic acid by the enzyme cyclooxygenase-2 (COX-2), which is inappropriately up-regulated in various premalignant and malignant tissues.

Cyclooxygenase-2 (COX-2) and Cancer

- Enhanced Cox-2 activity and synthesis of prostaglandins (PGs) stimulate proliferation, angiogenesis, invasiveness and inhibit apoptosis.  
  Tsuji et al., 2001

- Moreover, genetically engineered COX-2 overexpressing transgenic mice are highly susceptible to spontaneous tumor formation, while COX-2 knock out animals are less prone to experimentally induced tumorigenesis.  

- Therefore, targeting COX-2 is a rational approach for cancer chemoprevention.
Signaling pathways of COX-2 gene induction

Human COX-2 Promoter

Pro-inflammatory stimuli

TNFα, β  IL-1α, β  LPS  
TNF-R  IL-1 R  TLR4  
Cytokines  Growth factors

Nuclear factor interleukin 6  c-AMP response element

Sp1  NF-κB  AP2  NF-IL6  CRE/E box  TATA box

-270/-265  -223/-214  -132/-124  -59/-53  -31/-25  1

J Biol Chem., 1995

Prostaglandins & other Lipid Mediators, 2002
**Transcription factor:**

**Nuclear Factor-κB (NF-κB)**

- **NF-κB**, predominantly as a heterodimer of p65 and p50, binds with the kappaB consensus sequence located in the cox-2 gene promoter, thereby regulating COX-2 protein expression.

*The Journal of Clinical Investigation, 2005*

*J. Biol. Chem., 1998*
NF-κB activation and IKK (IκB kinase) complex

- IκB kinase play central role in regulation NF-κB, is a key signaling molecule involved in controlling cell proliferation, survival, anti-apoptosis, and tumorigenesis.

  J. Biol. Chem., 1998

- Recent studies provide genetic evidence that IKKβ-dependent NF-κB activation creates an essential link between inflammation and cancer.

  Cell, 2004; Nature, 2004
Signaling pathways of COX-2 gene induction

- Besides IKKs, several other protein kinases are also reported to regulate NF-κB activation.
  

- Of these upstream kinases, the role of mitogen-activated protein kinases (MAPKs), such as extracellular signal regulated protein kinase (ERK) and p38 MAPK, in regulating NF-κB activation has been well-documented.
  
  *Carcinogenesis*, 2003; *Oncogene*, 2005

- Another serine/threonine kinase, Akt that promotes cell survival by preventing apoptosis, has also been reported to regulate COX-2 expression through the NF-κB/IκB pathway.
  
  *Mol. Cancer*, 2004
linoleic acid & Conjugated linoleic acid (CLA)

**linoleic acid**

**cis-9, trans-11**
Conjugated linoleic acid
Conjugated linoleic acid (CLA)

**Natural products**

- **cis-9, trans-11**
- **trans-7, cis-9**
- **trans-11, cis-13**
- **trans-10, cis-12**

(98-73%)

**Butyrivibrio fibrisolvens**

Rumen

- **Dietary fat**
  - e.g. Linoleic acid (cis-9, cis-12 C₁₈:₂)

- **Conjugated linoleic acid, CLA**
  - (cis-9, trans-11 C₁₈:₂)

Meat & milk

- **Vaccenic acid**
  - (trans-11 C₁₈:₁)

- **Stearic acid**
  - (trans-11 C₁₈:₁)
Conjugated linoleic acid (CLA)

- In a pioneering study, Ha and colleagues demonstrated the inhibitory effect of CLA on chemically induced mouse skin carcinogenesis. *Carcinogenesis*, 1987

- Since then, there has been an increasing body of data on the anti-carcinogenic effects of CLA in various tumor models. *Anticancer Res.*, 1998; *Cancer Res.*, 1990
Objective

- Recently, it has been reported that 9Z,11E-CLA significantly inhibits mouse skin tumorigenesis. *Mol. Carcinog.*, 2000

- But underlying molecular mechanisms remain to be elucidated.

- In this study, the authors examined the effect of 9Z,11E-CLA on TPA-induced COX-2 expression in HR-1 hairless mouse skin *in vivo*, and explored possible underlying molecular mechanisms.
Materials & Methods
Animal treatment

9Z,11E-CLA/TPA

Western blot analysis

COX-2, p-ERK, p-p38, p-IKKα/β, pp65(Ser 536)

Nuclear extracts

Preparation of cytosolic and nuclear extracts from mouse skin

Electrophoretic mobility shift assay (EMSA)

In vitro kinase assay (radioactive)

NF-κB DNA binding activity

IκB kinaseα/β (IKKα/β) activity

6-7 weeks of age (HR-1Hairless mice)
Results & Discussions
9Z,11E-CLA inhibited TPA-induced COX-2 expression in hairless mouse skin

Fig. 1. Inhibitory effects of 9Z,11E-CLA on TPA-induced COX-2 expression in mouse skin. (a) Dorsal skins of male HR-1 mice were treated topically with acetone alone or TPA (10 nmol in 0.2 ml acetone) for indicated time periods. Data are representative of two different sets of animals showing similar trend of COX-2 expression.
9Z,11E-CLA inhibited TPA-induced COX-2 expression in hairless mouse skin

- Topical application of 9Z,11E-CLA (0.25 or 1 mg) to mouse skin immediately after TPA treatment for 6 h diminished COX-2 expression in a dose-dependent manner.
TPA-induced NF-κB activation was negated by 9Z,11E-CLA in mouse skin

Fig. 2. The inhibitory effect of 9Z,11E-CLA on TPA-induced NF-κB activation in hairless mouse skin in vivo. (b) Inhibitory effects of 9Z,11E-CLA on TPA-induced NF-κB activation. Dorsal skin of male HR-1 mice was treated topically with 10 µmol TPA for different time periods. Epidermal nuclear extract (10 µg) was probed with radiolabeled NF-κB oligonucleotide.
TPA-induced NF-κB activation was negated by 9Z,11E-CLA in mouse skin
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- Treatment of mouse skin with 9Z,11E-CLA reduced TPA induced DNA binding as well as nuclear translocation of NF-κB by blocking phosphorylation and subsequent degradation of IκBα.
9Z, 11E-CLA suppressed phosphorylation and catalytic activity of IKK in TPA-treated mouse skin

Fig. 3. Effect of topically applied 9Z,11E-CLA on TPA-induced activation of IKK in hairless mouse skin. Dorsal skins of mice were treated with TPA (10 nmol) with or without 9Z,11E-CLA (0.25 or 1 mg). Control animals were treated with acetone only. Animals were killed 3 h after TPA treatment. Whole epidermal tissue lysates were subjected to western blot analysis and in vitro kinase assay. (a) Inhibitory effect of 9Z,11E-CLA on TPA-induced phosphorylation of IKKα/β (serine 181). Quantification of pIKKα/β immunoblot was normalized to that of actin. N=3 per treatment group; *P < 0.032 (control versus TPA alone), **P < 0.05 (TPA alone versus 9Z,11E-CLA plus TPA).
9Z, 11E-CLA suppressed phosphorylation and catalytic activity of IKK in TPA-treated mouse skin

- The authors also performed an *in vitro* radioactive kinase assay to measure the catalytic activities of IKKα and IKKβ.

- Topical application of TPA resulted in increased phosphorylation of IKK (Ser181), which was decreased by 9Z,11E-CLA treatment.

- Moreover, co-treatment of 9Z,11E-CLA suppressed TPA-induced catalytic activity of IKKβ.

and methods.
IKKβ regulates TPA-induced COX-2 expression and NF-κB DNA binding in hairless mouse skin *in vivo*

- As mentioned above, release of NF-κB from its inhibitory protein IκBα is essential for the activation of NF-κB.
- Phosphorylation of IκBα is a precedent step for its degradation, which is mainly dependent on the activation of the upstream kinase IKKβ.

*Cell, 2004*
IKKβ regulates TPA-induced COX-2 expression and NF-κB DNA binding in hairless mouse skin in vivo

Fig. 4. Effects of SC-514, a specific inhibitor of IKKβ, on TPA-induced DNA binding of NF-kB and expression of COX-2. Dorsal skins of male HR-1 mice were treated topically with SC-514 (0, 0.2 or 1 μmol) dissolved in 0.2 ml acetone before TPA treatment. Control animals were treated only with acetone. (a) Mice were sacrificed 3 h after TPA treatment and 10 mg of protein from nuclear extract was incubated with the radiolabeled oligonucleotides containing the NF-κB consensus sequence for analysis by EMSA. (b) Mice were killed 6 h after TPA application, and whole lysate (30 μg) was analyzed for COX-2 expression by immunoblotting.
Signaling pathways of COX-2 gene induction

Of these upstream kinases, the role of mitogen-activated protein kinases (MAPKs), such as extracellular signal regulated protein kinase (ERK) and p38 MAPK, in regulating NF-κB activation has been well-documented. (Carcinogenesis, 2003; Oncogene, 2005)

Gene expression

Pro-inflammatory stimuli

& transcription factors
9Z, 11E-CLA inhibited activation of ERK and p38 MAPK in TPA-stimulated mouse skin

- 9Z, 11E-CLA attenuated TPA-induced NF-κB activation possibly through inhibition of these upstream kinases (ERK & p38), leading to eventual COX-2 suppression.

*Normalized to p38 followed by statistical analysis in comparison to control.
The role of Akt/PKB in mediating the inhibitory effects of 9Z,11E-CLA on TPA-induced NF-κB activation and COX-2 expression in mouse skin?

Another serine/threonine kinase, Akt that promotes cell survival by preventing apoptosis, has also been reported to regulate COX-2 expression through the NF-κB/IκB pathway.

*Mol. Cancer, 2004*
Akt appears to be a potential target of 9Z,11E-CLA in suppressing TPA-induced activation of COX-2 and NF-κB in mouse skin

Fig. 6. Inhibitory effects of 9Z,11E-CLA on TPA-induced phosphorylation of Akt and that of LY294002 on TPA induced COX-2 expression and NF-κB activation in hairless mouse skin in vivo. (a) Dorsal skins of hairless mice were treated topically with either acetone or 9Z,11E-CLA (0.25 or 1 mg) followed by TPA treatment. Control animals were treated with acetone in lieu of TPA. The expression of phospho-Akt was measured by western blot analysis of whole tissue lysates, and immunoblots were quantified and adjusted to Akt by statistical analysis of densitometric data.
Akt appears to be a potential target of 9Z,11E-CLA in suppressing TPA-induced activation of COX-2 and NF-κB in mouse skin

Fig. 6. Inhibitory effects of 9Z,11E-CLA on TPA-induced phosphorylation of Akt and that of LY294002 on TPA induced COX-2 and NF-κB activation in hairless mouse skin in vivo. (b) Dorsal skins of hairless mice were treated with LY294002 (0.5 or 5 nmol) immediately before TPA (10 nmol) treatment. Animals were killed at 3 h after TPA treatment and total protein was analyzed for pAkt expression by immunoblotting. (c) Animals were treated with LY294002 as indicated in Figure 6b. Nuclear extracts (10 μg) were incubated with radiolabeled oligonucleotides containing the NF-κB consensus sequence for analysis by the EMSA. Blots are representative of three independent experiments. (d) Animals were treated with LY294002 as indicated in Figure 6b, and sacrificed after 6 h. Whole tissue lysates were subjected to western blot analysis for measuring the expression of COX-2.

These findings suggest that the inhibitory effect of 9Z,11E-CLA on COX-2 expression in TPA-treated mouse skin may be mediated via blockage of the PI3K-Akt signaling.
IKK\(\beta\) regulates TPA-induced Akt activation in hairless mouse skin

This result suggest that IKK\(\beta\) regulates the activation of Akt in TPA-stimulated hairless mouse skin in vivo. }

Adjusted with actin by statistical analysis in comparison to control using a densitometer.
Conclusion
In conclusion, 9Z,11E-CLA inhibited TPA-induced NF-κB activation and subsequent COX-2 expression by blocking the IKK and Akt signaling in hairless mouse skin in vivo, which provides a mechanistic basis of the previously reported antitumor promoting activity of 9Z,11E-CLA.

In contrast, our present study reveals that co-treatment with the IKKβ selective inhibitor SC-514 abolishes TPA-induced phosphorylation of Akt, indicating that IKKβ is perhaps one of the upstream regulators of Akt activation in TPA-treated mouse skin in vivo.
Thanks for your attention!
Arachidonic acid, AA
Pro-inflammatory stimuli

Signal transduction & transcription factors

Gene expression