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Leptomycin B, an inhibitor of the nuclear export receptor CRM1, inhibits COX-2 expression

Byeong-Churl Jang\textsuperscript{a}, Ursula Munoz-Najar\textsuperscript{a}, Ji-Hye Paik\textsuperscript{a}, Kevin Claffey\textsuperscript{a}, Minoru Yoshida\textsuperscript{b} and Timothy Hla\textsuperscript{a}\textsuperscript{*}

\textsuperscript{a}Center for Vascular Biology, Department of Physiology, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT06030-3501, \textsuperscript{b}Chemical Genetics Laboratory, RIKEN, Hirosawa 2-1, Wako, Saitama 351-0198, Japan.

\textbf{Running Title:} Leptomycin B inhibits COX-2 mRNA expression

\textbf{*Corresponding author:}

Timothy Hla, Ph.D.
Center for Vascular Biology
Department of Physiology
University of Connecticut Health Center
263 Farmington Avenue
Farmington, CT06030-3501
Tel: 860-679-4128
Fax: 860-679-1201
E-mail:hla@sun.uchc.edu
Summary

Cyclooxygenase (Cox)-2, the inducible prostaglandin synthase, is overexpressed in cancer and chronic inflammatory diseases. Post-transcriptional regulation of COX-2 mRNA is important in controlling the expression of the COX-2 gene. Here, we report that leptomycin B (LMB), a specific inhibitor of the nuclear export factor CRM1 potently inhibits the stabilization of COX-2 mRNA in MDA-MB-231 human mammary cancer cells. However, COX-2 promoter-driven reporter gene expression is not inhibited by LMB, suggesting that LMB acts at the post-transcriptional level. Subcellular fractionation experiments indicate that LMB inhibited the time-dependent export of COX-2 mRNA into the membrane-bound polysomal compartment at the endoplasmic reticulum. LMB suppressed COX-2 expression by IL-1β in HT-29 human colon cancer cells and in human umbilical vein endothelial cells (HUVEC) but had no effect on COX-2 expression induced by E. coli lipopolysaccharide (LPS) in monocytic THP-1 cells.

These data suggest that the nuclear export of COX-2 mRNA may be rate-limiting in a cell-specific manner. LMB may be useful to control COX-2 expression in various human diseases in which COX-2 plays a pathogenetic role.
Introduction

Cyclooxygenase (COX), also known as prostaglandin (PG) H synthase, is the rate-limiting enzyme in the biosynthesis of prostaglandins (PG) from arachidonic acid(1). PGs are implicated in various physiological and pathophysiological events, including inflammatory and neoplastic diseases(2-4).

Two isoforms of COX have been identified and cloned in eukaryotic cells. COX-1, the constitutively expressed isoform, is involved in the maintenance of physiological functions. In contrast, COX-2 is inducible by extracellular stimuli such as tumor promoters, pro-inflammatory cytokines, mitogens, oncogenes, and growth factors in a variety of cells(1). Overexpression of COX-2 is observed in human cancers such as colon and breast (4). Gene deletion of COX-2 suppressed the developmental of intestinal polyps in APC gene deleted mice, suggesting that COX-2 expression is critical for tumorigenesis(5). In addition, overexpression of COX-2 in the mammary glands of transgenic mice results in enhanced tumorigenesis in multiparous mice(6). These data strongly suggest that unregulated expression of COX-2 is an important event in tumorigenesis. The control of COX-2 gene expression is regulated at the level of transcriptional and post-transcriptional mechanisms(1,3,7). However, the mechanisms by which COX-2 expression is controlled are not fully understood.

Leptomycin B (LMB) was originally discovered as a potent anti-fungal antibiotic from Streptomyces sp.(8). However, recent data showed that LMB causes G1 cell cycle arrest in mammalian cells and is a potent anti-tumor agent against murine experimental tumors (9,10). Studies in Scizosaccharomyces pombe identified the cellular target of LMB as the CRM1 (chromosomal region maintenance)/exportin 1 protein(11), which is
critical for the export of RNA and proteins containing a nuclear export sequence (NES).
In the case of RNA export, CRM1 binds to ribonuclear proteins containing the NES motif (12-15). For example, the export of Rev-response element-containing human immunodeficiency virus (HIV) RNA is inhibited by LMB(13). Studies from Steitz and co-workers indicated that LMB disrupted the interaction of the RNA binding protein HuR with its protein partners, resulting in blockage of nuclear export of c-fos and perhaps other immediate-early gene mRNAs that bind to HuR (16,17). We and others have recently showed that the COX-2 mRNA binds specifically to HuR at the 3'-UTR (Sengupta, Jang and Hla, unpublished observations)(18). Based on these observations, we speculated that LMB might regulate COX-2 gene expression by controlling nuclear export of its mRNA.

In the present study, we investigated whether LMB can regulate the expression of COX-2 by modulating the nuclear export of COX-2 mRNA.
Experimental Procedures

**Cell Culture** – MDA-MB-231 cells (ATCC HTB-26) were cultured in Leibovitz’s L-15 medium supplemented with 10% fetal bovine serum (FBS, Hyclone). HT-29 cells (ATCC HTB38), a human colon cancer cell line, and THP-1 (ATCC TIB202), a human monocytic cell line, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS. Human umbilical vein endothelial cells (HUVEC, Clonetics) were cultured in M199 medium (Cellgro, Inc.) supplemented with 10% FBS and heparin-stabilized endothelial cell growth factor as described previously(19).

**Preparation of Whole Cell Lysates** - Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in modified RIPA lysis buffer [50mM Tris-Cl (pH 7.4), 150mM NaCl, 1% Triton X-100, 1% NP-40, 1mM EDTA, 1mM PMSF, and protease inhibitor mixtures (1X, Boehringer Mannheim)]. After 10 min incubation on ice, cells were collected by cell scraper and centrifuged at 14,000 x g for 15 min at 4°C.

**Western Blot Analysis** – Whole cell lysates (40µg/lane) were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transblotted onto nitrocellulose membrane (Protran; Schleicher&Schuell). Western blot analysis using the anti-COX-2 and β-actin mouse monoclonal antibodies were conducted as described previously(20).

**Northern Blot Analysis** - Total RNA was isolated using the RNA-STAT60 reagent (Tel-test) according to the instructions provided by the manufacturer. Northern blot analysis was done as described previously (20).

**In Situ Hybridization** – MDA-MB-231 cells were fixed in 4%(w/v) formaldehyde, 5%(v/v) acetic acid, and 0.9%(w/v) NaCl and dehydrated in 70%, 90%,
and 100% ethanol (EtOH). For in situ hybridization, deoxygenin (DIG)-labelled DNA probes (5ng/µl) (DIG-HIGH primer labeling system, Boehringer-Manheim) were added to the hybridization solution [60% de-ionized formamide, 300mM NaCl, 30mM Sodium Citrate, 10mM EDTA, 25mM NaH$_2$PO$_4$ (pH7.4), 5% dextran sulfate, and 250ng/µl sheared salmon DNA]. Fifty microliters of the hybridization mixture were added to the fixed cells. After 16h-hybridization, cells were washed under high stringency at 37°C.

For immunofluorescence detection, cells were blocked in a blocking solution [100mM Tris-HCl (pH 7.5), 150mM NaCl, 0.5% (w/v) blocking reagent (Boehringer-Manheim)]. After blocking, the slides were incubated with anti-DIG fluorescein (1:500). Slides were washed [100mM Tris-HCl (pH 7.5), 150mM NaCl, 0.05% Tween 20] and dehydrated in 70%, 90%, and 100% EtOH. Finally, slides were embedded in the anti-fading solution with DAPI (50ng/µl) as a counter-staining and cells were amaged by a Zeiss Axiovert fluorescence microscope or a Zeiss 410 Confocal microscope.

**Luciferase Assay** – MDA-MB-231 cells grown in 24-well plates were transfected for 4h with luciferase DNA construct containing a COX-2 promoter(21,22). After 48h-post-transfection, cells were grown for 6h in the presence or absence of serum along with or without LMB. Cell lysates were assayed with the Luciferase Assay System (Promega) using a TD 20/20 luminometer (Turner Designs Instruments, Sunnyvale, CA).

**RNA isolation from subcellular Fractions** – Cells were then washed in PBS and successively extracted as described. First, cells were extracted with the NP40 buffer [10mM Tris-Cl, (pH 7.5), 10mM NaCl, 3mM MgCl$_2$, 0.5%(v/v) NP40, 40U/ml RNasin, 1mM DTT]. The remaining pellet (rough endoplasmic reticulum (RER) and nucleus) was washed in the same buffer and extracted in buffer B [10mM Tris-Cl, (pH 7.5), 10mM
NaCl, 0.5%(v/v) NP40, 40mM EDTA, 40U/ml RNasin, 1mM DTT]. The remaining nuclear pellet was washed in the same buffer and extracted with high salt buffer [10mM Tris-Cl, (pH 7.5), 0.5M NaCl, 3mM MgCl₂, 0.5%(v/v) NP40, 40U/ml RNasin, 1mM DTT]. RNA was purified from each fraction by the RNA STAT solution as described above.

**RNase Protection Assay** - The RNase protection assay was performed according to the instructions provided by the manufacturer (Ambion) with some modifications. Briefly, RNA (10µg) was hybridized with P³²-labeled antisense riboprobes of human COX-2 (500bp) and human GAPDH (100bp). The hybridization reaction was then treated with RNAses A and T to remove unhybridized probes. The protected and hybridized mRNA was resolved in 6% polyacrylamide gel containing 8M Urea. The gel was dried and exposed on a Kodak film at -80°C. The bands were visualized by autoradiography or PhosphoImager (Molecular Dynamics).
RESULTS AND DISCUSSION:

**Leptomycin B potently inhibits the expression of COX-2 mRNA and protein induced by serum withdrawal in MDA-MB-231 cells:** Previously we showed that COX-2 mRNA and protein were induced by serum withdrawal in MDA-MB-231 human breast carcinoma cells(20). We recently found that the interaction of COX-2 mRNA with the RNA binding protein HuR is critical for stabilization of COX-2 mRNA (unpublished observation). Steitz and co-workers have shown that one of the functions of HuR is to facilitate the nuclear export of immediate-early mRNAs into the cytoplasm(16). Furthermore, they showed that HuR ligands bind to CRM1, and that HuR-binding mRNA export is inhibited by LMB, a specific inhibitor of CRM1-dependent nuclear export(16). We determined the effect of LMB to see if COX-2 mRNA export is critical in the regulation of expression. Northern and Western analysis showed that LMB potently and effectively suppressed the expression of COX-2 mRNA and protein in a concentration-dependent manner (Fig. 1A,B). Extremely low concentrations of LMB -10ng/ml (18 nM) - was sufficient to completely inhibit the serum withdrawal-induced COX-2 mRNA (Fig. 1A).

**Leptomycin B does not inhibit the transcription of COX-2 gene:** Serum-withdrawal induced expression of COX-2 gene in MDA-MB-231 cells were previously characterized to require the stabilization of the COX-2 mRNA via signaling of the p38 stress-activated kinase pathway(20). Nuclear run-on analysis did not indicate changes in transcription rate of the COX-2 gene(20). Thus, the effect of LMB most-likely is at the post-
transcriptional level. To confirm this point, we measured the expression of COX-2-promoter-driven luciferase expression in transiently-transfected cells. As shown in figure 2, LMB had no effect on COX-2 transcription. Neither serum addition nor withdrawal modulated COX-2 transcription, suggesting that LMB inhibits COX-2 expression by an effect at the post-transcriptional level.

Subcellular localization of COX-2 mRNA: To better define the subcellular localization of the COX-2 mRNA, we conducted biochemical fractionation and in situ hybridization experiments. Cells were extracted with NP40 buffer to isolate the cytosolic fraction. The remaining nuclear / rough endoplasmic reticulum (RER) pellet was extracted with EDTA to remove the membrane-bound polysomal RNA. EDTA-resistant fraction was extracted with high salt to isolate nuclear RNA. As shown in figure 3A, U6 RNA was found predominantly in the nuclear fraction and GAPDH RNA was found in the cytosolic and RER compartments. This validates the biochemical extraction scheme that was employed. As expected, COX-2 mRNA was induced only when cells were serum deprived. In contrast to the fractionation of U6 and GAPDH mRNAs, COX-2 mRNA showed a predominantly RER/ polysomal distribution, consistent with the fact that COX-2 is a protein located in the lumen of the ER. A significant proportion of the COX-2 mRNA was also found in the nuclear fraction. Since transcription rates are similar in 10% and 0% serum treated cells, these data suggest that both nuclear and ER-associated COX-2 mRNA species accumulate under serum-deprivation induced condition.

To further validate the biochemical fractionation data, we conducted an in situ hybridization experiment with COX-2 and GAPDH probes to detect the respective
transcripts. As shown in figure 3B, GAPDH signals were diffusely localized throughout the cytoplasm of MDA-MB-231 cells regardless of treatment of the cells with 10% or 0% serum. However, COX-2 mRNA showed a dramatic change when cells were treated with 0% serum for 24 h. A strong signal around the nuclear membrane and ER was observed only under 0% serum treatment whereas background signals were seen in cells cultured with 10% serum. These data confirm the biochemical fractionation experiments that COX-2 mRNA accumulates in membrane bound polysomal (ER-associated) and nuclear compartments. Predominant accumulation of the COX-2 mRNA at the nuclear membrane (both EDTA extractable and high-salt extractable) suggest that nuclear transport of COX-2 mRNA may be rate-limiting.

**Effect of LMB treatment on subcellular localization of COX-2 mRNA:** To determine if LMB effect on the suppression of the COX-2 mRNA occurs in the nuclear compartment or ER-compartment or both, MDA-MB-231 cells were treated with LMB and at various times thereafter COX-2 and GAPDH mRNAs were analyzed by biochemical fractionation followed by RNAse protection experiments. As shown in figure 4A, 24 h serum starvation induced significant levels of COX-2 mRNA in both ER-associated and nuclear compartments (lanes 3 and 4). Continued treatment with 0% FBS further induced COX-2 mRNA, and at 24 h thereafter, maximal levels of COX-2 mRNA accumulated at the membrane-bound ER fraction whereas a smaller fraction was found in the nucleus. Treatment with LMB from 0-24 h resulted in pronounced suppression of COX-2 mRNA (but not the GAPDH mRNA), especially at 24 h time point (lanes 15 and 16). Interestingly, both membrane-bound ER and nuclear-COX-2 mRNA species were suppressed.
To further confirm the time-dependent effect of LMB, cells were treated with 0% FBS at time 0 h and subsequently treated or not with LMB from 8-24 h (figure 4B). As it can be seen, LMB treatment from 8-24 h suppressed both nuclear and RER–localized COX-2 transcript levels. However, membrane-bound ER–localized COX-2 transcripts were profoundly suppressed by LMB. Although nuclear COX-2 mRNA was suppressed by LMB, it was not suppressed to the same degree as the cytosolic counterpart. These data suggest that COX-2 mRNAs accumulate at the nuclear pore complex in MDA-MB-231 cells and that inhibition of the CRM1 pathway with LMB results in degradation of COX-2 mRNA in both the intranuclear and extranuclear (membrane-bound ER) compartments of the nuclear pore complex. Indeed, time-course analysis of COX-2 mRNA in membrane-bound ER and nuclear compartments after LMB treatment indicates that COX-2 mRNAs in both compartments decay with similar rates (data not shown). Thus, LMB appears to decouple the COX-2 mRNA from the nuclear export pathway and target it for degradation in both cytosolic and membrane-bound ER compartments.

**Cell-type and stimuli-specific inhibition of COX-2 expression by LMB:** We also tested whether LMB can inhibit the expression of COX-2 by known COX-2 inducers such as IL-1β or LPS in other cell types including HT-29 a human colon carcinoma cell line, HUVEC (human umbilical vein endothelial cells), or THP1 a human monocytic cell line. The data showed that the expression of COX-2 induced by IL-1β in HT-29 cells is inhibited by LMB in a concentration-dependent manner (Fig. 5A). The COX-2 expression induced by IL-1 in HUVEC was also partially inhibited by LMB at 10ng/ml concentration (Fig. 4B). However, LPS-stimulated COX-2 expression in THP1 cells was
not inhibited by LMB at even 20ng/ml (Fig. 4C). COX-2 regulation in various cell systems is regulated at both transcriptional as well as post-transcriptional levels(1,3,7). Since LMB is a specific regulator of CRM1-dependent nuclear export, it is likely that it would only block expression of genes that are controlled by CRM1-dependent export of mRNA. These data suggest that LMB inhibits COX-2 expression in a cell-type and agonist-specific manner.

In summary the data presented here demonstrate for the first time that LMB potently inhibits COX-2 expression induced by serum withdrawal in a human breast cancer cell line MDA-MB-231. Furthermore, our data suggest a possible mechanism by which LMB inhibits COX-2 expression by showing that LMB blocks nuclear export of COX-2 mRNA, which may be an important control point of COX-2 gene expression. Finally we provide some evidence that LMB may be a new and potent inhibitor of COX-2 expression with a cell and/or agonist type specificity.
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Footnotes

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1. The abbreviations used are: COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LMB, leptomycin B; PG, prostaglandin; MAPK, mitogen-activated protein kinase; HUVEC, human umbilical vein endothelial cells; CRM1, chromosomal region maintenance; NES, a nuclear export sequence; IL-1β, interleukin-1β; LPS, lipopolysaccharide; PBS, phosphate-buffered saline, RER, rough endoplasmic reticulum.
Figure Legends

Fig. 1. Leptomycin B strongly inhibits the expression of COX-2 mRNA and protein induced by serum withdrawal in MDA-MB-231 cells. A, MDA-MB-231 cells were grown in culture medium containing 10% FBS without LMB [none or vehicle (v)] or in culture medium containing 0% FBS along with LMB in different concentrations (0, 2.5, 5, 10, 20ng/ml) for 24h. Total RNA was isolated and analyzed for the COX-2 mRNA expression by Northern blot analysis. GAPDH mRNA was used as an internal control. B, MDA-MB-231 cells were treated the same as in A. Whole cell lysates were prepared and analyzed for expression of the COX-2 protein by Western blot analysis.

Fig. 2. Lack of Effect of LMB on COX-2 transcription. MDA-MB-231 cells were transiently transfected with the COX-2 promoter luciferase DNA construct. At 48h post-transfection, cells were treated with or without LMB for 6h in the presence or absence of serum. Cell lysates were prepared and assayed for reporter gene activity. Data represent mean ± s.e. of triplicate determinations.

Fig 3. Subcellular localization of COX-2 mRNA in MDA-MB-231 cells. A, MDA-MB-231 cells were grown in the presence or absence of serum for 24h. Nuclear (high salt nuc), cytoplasmic (NP40 cyto) and RER (EDTA RER) RNA fractions were purified as described and analyzed for the presence of COX-2 and GAPDH mRNA by RNase protection assay or for U6 small nuclear RNA detection by Northern analysis. Lane 9 contains probe alone without extract. B. MDA-MB-231 cells were analyzed by in situ hybridization with DIG-labelled hCOX-2 or hGAPDH DNA probes as described.
Imaging was conducted by a Zeiss Axiovert fluorescent microscope (inset) or by a Zeiss 410 confocal microscope. The inset shows a high power (60x) magnification.

**Fig. 4. Effect of nuclear export of COX-2 mRNA by LMB.** A, MDA-MB-231 cells grown in 10% or 0% FBS for 24h were further incubated in the absence of serum with or without LMB (10ng/ml) for the additional times (4, 8, 24h). At each time point, cells were sequentially treated with NP40/ETDA buffer to extract the RNA from cytosolic and RER fractions (C) and high salt buffer (N) for extraction of nuclear RNA. Each RNA fraction was analyzed for COX-2 and GAPDH mRNA by RNase protection assay. B, MDA-MB-231 cells were grown in either 10% or 0% FBS for initial 8h and further incubated in the absence of serum with or without LMB (10ng/ml) for the additional 16 h. Cytosolic/RER (C) and nuclear (N) RNAs were analyzed for COX-2 and GAPDH as described above.

**Fig. 5. Effect of LMB on COX-2 expression in various cell types.** A, HT-29 cells were stimulated with 20ng/ml of IL-1β in the absence or presence of LMB in different concentrations (0, 10, 20, 40ng/ml) for 24h. B, HUVEC were stimulated with 20ng/ml of IL-1β in the absence or presence of LMB (10ng/ml) for 24h. C, THP-1 cells were treated with LPS (1µg/ml) in the absence or presence of LMB in different concentrations (0, 10, 20ng/ml) for 24h. In all cases (A, B, C), after 24h, whole cell lysates were prepared and analyzed for the expression of the COX-2 protein by Western blot analysis.
Figure 1

(A) COX-2 mRNA

(B) COX-2 protein

FBS (10%) + + - - - - -
LMB (ng/ml) - v - v 2.5 5 10 20

Actin protein
Figure 2
### A

|            | Total RNA | NP40 cyto | EDTA RER | high salt nuc |
|------------|-----------|-----------|----------|---------------|
| FBS (10%)  | +         | -         | +        | +             |

- COX-2 mRNA
- GAPDH mRNA

- U6 sn RNA

### B

**COX-2**

- 0%
- 10%

**GAP**

- 0%
- 10%

*Figure 3*
Figure 4
Figure 5

A

|          | IL-1β (20ng/ml) | LMB (ng/ml) |
|----------|-----------------|-------------|
|          | -               | 10          |
|          | +               | 20          |
|          | +               | 40          |

HT-29

COX-2

Actin

B

|          | IL-1β (20ng/ml) | LMB (10ng/ml) |
|----------|-----------------|---------------|
|          | -               | -             |
|          | +               | +             |

HUVEC

COX-2

Actin

C

|          | LPS (1ug/ml) | LMB (ng/ml) |
|----------|-------------|-------------|
|          | -           | 10          |
|          | +           | 20          |

THP-1

COX-2

Actin
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