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Triclosan Blocks *Mmp 13* Expression in Hormone-Stimulated Osteoblasts

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Abstract

Background—Matrix metalloproteinase-13 (*Mmp-13*) is an important enzyme for the modulation of bone turnover and gingival recession. Elevated levels of *Mmp-13* are associated with alveolar bone resorption, periodontal ligament destruction, and gingival attachment loss, which are the clinical symptoms of periodontal disease. Continued evidence suggests periodontal disease contributes to oral tissue destruction and is linked to numerous systemic conditions. Triclosan is a long standing, proven antibacterial and anti-inflammatory agent found in the only FDA-approved dentifrice for the treatment of plaque and gingivitis.

Methods—This study examined the inhibitory effects of triclosan on lipopolysaccharide (LPS), parathyroid hormone (PTH) and prostaglandin E_2 (PGE_2) induced expression of *Mmp-13* in UMR 106-01 cells, an osteoblastic osteosarcoma cell line. The cells were stimulated with PTH or PGE_2 to induce *Mmp-13* mRNA expression and Real Time RT-PCR was performed to determine gene expression levels. Western blot analysis assessed the presence or absence of protein degradation or inhibition of protein synthesis. *Mmp-13* Promoter Reporter Assay was utilized to explore possible direct effects of triclosan on the *Mmp-13* promoter.

Results—Triclosan significantly reduced PTH or PGE_2 elevated expression of *Mmp-13* in osteoblastic cells without affecting basal levels of the mRNA. Surprisingly, triclosan enhanced the expression of c-fos and amphiregulin mRNA. A promoter assay indicated triclosan directly inhibits the activation of the PTH-responsive minimal promoter of *Mmp-13*.

Conclusion—Our data appear to have identified a nuclear mechanism of action of triclosan which accounts for triclosan's ability to inhibit PTH or PGE_2 induced *Mmp-13* expression in osteoblastic cells.

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Keywords

Osteoblasts; Periodontal Diseases; Triclosan; Matrix Metalloproteinase 13

INTRODUCTION

Periodontal disease is characterized by a bacterially induced gingival inflammation, loss of gingival attachment, bone loss, and ultimately tooth loss[1]. Periodontal disease is quite complex involving the bacterial infection, the host immune response, and bone metabolism.[2,3]. periodontal disease has been linked to numerous systemic diseases[4].

Triclosan (2,4,4′-tricloro-2′-hydroxydiphenyl ether) is a widely used broad spectrum antibacterial/anti-inflammatory agent[5]. Metalloproteinases, when out of balance, play a major role in connective tissue destruction associated with periodontal disease, arthritis, osteoporosis as well as other diseases[6]. The use of non-antibiotic tetracycline, which inhibits collagenases at the protein level, has become an effective adjunct treatment for the management of excess metalloproteinase activity in arthritis and periodontitis[7,8].

Bone is a dynamic connective tissue consisting of a variety of cell types. Although considerably complex, osteoblasts are generally considered to be bone forming cells while osteoclasts are considered to be bone resorbing cells. Osteoblasts express receptors for parathyroid hormone (PTH) and prostaglandins as well as transmembrane cytokines including RANKL, which activates osteoclastogenesis in a paracrine manner. The secretion of type I collagen and specialized bone matrix proteins is a critical bone forming function of osteoblasts. [9,10] Osteoblasts also have a role in bone remodeling and are capable of contributing directly to the resorption of bone though the secretion of proteinases, in particular collagenase-3 (*Mmp-13*). Absence of *Mmp-13* in knockout mice resulted in significant interstitial collagen accumulation and an increase in trabecular bone indicating its essential role in bone turnover.[11,12].

A body of evidence has shown that MMP-13 is important in bone. The gene was shown to be expressed predominantly in ossifying centers during *in vivo* development of mice. [13] In postnatal rat calvariae, we have found that ample amounts of MMP-13 are detectable by immunohistochemistry from 1–14 days after birth[14,15] which subsequently decline to become undetectable in normal adult bone. The staining is always in select areas, mostly associated with sites of active modeling. At the cellular level, it is associated with osteocytes and bone lining cells which have the appearance of osteoblasts. By *in situ* hybridization, *Mmp-13* is expressed by the mineralizing hypertrophic chondrocytes and by trabecular osteoblasts in long bones of immature mice[16]. Similarly, in the developing human, MMP-13 appears to be specific for the skeleton and is expressed in hypertrophic chondrocytes, osteoblasts and periosteal cells. [17]

The rat osteoblastic osteosarcoma line, UMR 106-01, responds to all bone resorbing hormones by synthesizing collagenase-3 (*Mmp-13*)[18]. PTH is the most effective bone resorbing agent for stimulating *Mmp-13* production in these cells from the agents tested. PTH binds to its membrane receptor, which activates the G protein Gs. Activated Gs induces

the conversion of ATP to cAMP, which activates the protein kinase A (PKA) signaling cascade and leads to the phosphorylation of the CREB transcription factor. CREB then stimulates *c-fos* transcription by binding the cAMP Response Element (CRE), and in turn, c-Fos, together with Runx2 at the RD site, bound to the AP-1 promoter site induces *Mmp-13* transcription.

It has been previously shown that PTH-induced expression of *Mmp-13* in osteoblasts requires the cooperative interaction between c-Fos, c-Jun, and Runx2 transcription factors on their cognate AP-1 and runt domain (RD) binding sites, respectively. These three transcription factors interact physically and cooperatively bind the AP-1 and RD binding sites within the *Mmp-13* promoter [19,20]. Our objective was to examine how triclosan may affect the expression of *Mmp-13* in bone cells. Triclosan reduced expression of PTH and PGE_2 induced *Mmp-13* and we subsequently explored the mechanism of action.

MATERIALS AND METHODS

Materials

Rat PTH (1–34), 8-Bromo cAMP, Tri-Reagent™, RNA isolation reagents[#] Triclosan (TCN)* and PGE_2 ** were used in this study. Primers used were *Mmp-13*,[¥] *β-actin*, [¥] *c-fos*, [¥] and *amphiregulin*[¥]. Standard tissue culture media and reagents were used throughout experiments.[£] Taq Man® Reverse Transcriptase reagents[£££] were used to prepare cDNA. SYBR Green PCR Core Reagents[€] were used for cDNA amplification on a PTC-200 Real time PCR – DNA Engine Opticon.™ ***

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Sequence – rat B-actin F 5′-TCC TGA GCG CAA GTA CTC TGT G –3′

rat B-actin R 5′ CGG ACTCAT CGT ACT CCT GCT T -3′

rat MMP-13 F (845) 5′-GCC CTA TCC CTT GAT GCC ATT -3′

rat MMP–13 R (947) 5′-ACA GTT CAG GCT CAA CCT GCT G –3′

Other primers are listed in the supplementary materials of Qin L, Tamasi J, Raggatt L, Li X, Feyen JH, Lee DC, Dicicco-Bloom E, **Partridge NC**. J Biol Chem. 2005 Feb 4;280(5): 3974–81. Amphiregulin is a novel growth factor involved in normal bone development and in the cellular response to parathyroid hormone stimulation.

Cell Culture

UMR 106-01 rat osteoblastic osteosarcoma cells were grown in medium containing Minimum Essential Medium (MEM), 5% FBS, 10 units penicillin/10 μg streptomycin, 1% non-essential amino acids (NEAA), and 25 mM HEPES. Cells were grown to 80% confluence in 10 cm^2 wells. Cells were serum starved for 24 hours prior to collection and treated with the indicated agents at time zero. After 4 h of exposure, the cells were harvested from the plates with Tri-Reagent™ for the mRNA assays.

Isolation and Analysis of mRNA

RNA was isolated from the cells by adding 1 ml of Tri-Reagent™ per well. In addition, 0.2 ml of chloroform was added per ml of Tri-Reagent™ to ensure complete dissociation of nucleoprotein complexes. Following centrifugation, the layer containing RNA was transferred to a new RNase free tube. RNA was precipitated with 0.5 ml of isopropanol. The pellet was then washed with 75% ethanol, dried, and resuspended in 100 μl of RNase free water. The RNA concentration was determined on a spectrophotometer at 260 nm.

The cDNA was reverse transcribed from mRNA using the reagents in the Taq Man® Reverse Transcription kit. Real Time RT-PCR was performed to determine gene expression levels using the indicated primers. All samples were normalized to their own *β*-actin mRNA level.

Western blot analysis

UMR 106-01 cells were plated at 1.6×10^6 cells/100 mm dish. Cells were pre-treated with 0.003% triclosan or ethyl alcohol (ETOH) and then treated with 10^{-8} M PTH or control medium for 4 h. Cell lysates were then prepared in 500 μl lysis buffer (RECIPE?) and resolved on 10% SDS-PAGE, transferred to a PVDF membrane, and blotted with antibodies (Santa Cruz, CA) against c-Fos (sc-52), c-Jun(sc-45), Runx2 (PEBP2, sc-10758), and cdk2 (sc-163; loading control) at a dilution of 1:300. The second antibody (Santa Cruz, CA) was a goat-anti-rabbit polyclonal antibody (sc-2004) conjugated to horseradish peroxidase at a dilution of 1:10,000. The blots were developed with a commercial electrochemiluminescence (ECL) detection kit.

Mmp-13 Promoter Assay (CAT assay)

The −148 bp sequence of the rat collagenase-3 (*Mmp-13*) promoter was subcloned upstream of a CAT reporter gene in pSV0 (Promega, Madison, WI). The empty pSV0 plasmid was used as the negative control. The pSV2 plasmid was used as a positive control.

UMR 106-01 cells were plated at 4×10^5 cells/well in a 6-well plate in EMEM containing 5% fetal bovine serum. The following day, cells were transfected with 2 μg of DNA and 6 μl of GeneJammer (Agilent Technologies, La Jolla, CA) per well. After 48 h, the cells were treated with either control (ETOH), TCN (0.0007% or 7 ppm), or TCN + PTH-containing media for 12 h. CAT activity was measured by incubating 50 μl of cell lysate in duplicate in a 100 μl reaction volume consisting of 250 μM *n*-butyryl-coenzyme A and 23 mM [^{14}C] chloramphenicol (0.125 μCi/assay). The values were normalized according to the protein concentration as determined by the Bradford method (Bio-Rad, Hercules, CA). A standard

curve using purified CAT was performed in every experiment to determine the linear range of the enzyme assay.

Statistics

All experiments were performed in triplicate and the quantitative data are shown as means ± SD. A one way ANOVA was performed to determine significance and when found in experiments with more than two products a multiple comparison (Tukeys) test was also conducted. A significant separation was noted with p values less than or equal to 0.05.

RESULTS

Gene Expression

Preliminary experiments were needed to determine the optimal conditions for cell treatment and to confirm the response and behavior of the UMR cell line. To determine the best experimental design, method development initiated with the stimulation of UMR cells with 10^{-8} M PTH or 25 mg/ml LPS. At 4 h post –treatment, *Mmp*-13 expression was measured. PTH stimulation resulted in a 20-fold change from the ethanol (ETOH) control, consistent with published data from Partridge and colleagues, while LPS caused less than a 2-fold increase in the expression of *Mmp*-13. Therefore, we determined that LPS, while an important factor in periodontal disease, would not be useful for this research as a stimulating agent for this osteoblastic cell line (Fig 1).

Triclosan had little effect on *Mmp-13* expression in unstimulated cells. Cells treated with PTH or PGE_2 together with triclosan had a lesser increase in *Mmp-13* expression, showing that triclosan could block the expression of *Mmp-13* by two different agents operating though two different G-protein-coupled receptors (Fig. 2a)

In a recent patent from Barnes et al., Anti-bone loss and anti-attachment loss effects of an oral composition, US 2012/0107843 A1, 5/3/2012, a dose response of triclosan demonstrated similar findings in the same osteoblastic cell line regardless of the dosage of triclosan. In all cases the addition of triclosan to unstimulated cells resulted in no increase of *Mmp-13* expression as compared to the negative control. PTH stimulation produced a nearly 80 fold increase of *Mmp-13* expression while the addition of triclosan from 1 ppm to 10 ppm significantly muted the *Mmp-13* expression. (fig 2b)

We next tested 8-Bromo cAMP, a cell permeable cAMP analog, to investigate if the inhibitory effect of triclosan was at a post-receptor level. Results showed that 8-Bromo cAMP alone induced a robust increase in *Mmp-13* expression while triclosan alone did not. Triclosan decreased the stimulation of *Mmp-13* expression by 8-Bromo cAMP showing that triclosan's effect was downstream of cAMP.

We compared the response to triclosan on the PTH primary response genes *amphiregulin* (AR) and *c-fos.* Cells were stimulated with 10^{-3}M 8-Bromo cAMP, triclosan, or 8-Bromo cAMP + 0.003% triclosan in combination and the mRNA was extracted 4h post-treatment. The expression of *c-fos* increased 9.5, 5, and 21-fold compared to control with the respective treatments. Expression of *Mmp-13* increased 2, 17, and 4-fold compared to control with the

same treatments. Finally, AR expression paralleled the effects on *c-fos* and increased by 7, 5, and 12-fold compared to control with the three treatments. These experiments confirm our previous results showing that triclosan can reduce *Mmp-13* expression in stimulated cells. In contrast, triclosan stimulated the expression of both *c-fos* and AR (Fig. 3).

To assess whether triclosan was inhibiting the expression of c-Fos protein either by degradation or inhibition of protein synthesis, western blot analysis was performed. As shown in Figure 4, c-Fos, c-Jun, Runx2, and cdk2 protein were present at 4 h post-treatment, indicating that triclosan did not have an effect on any of their protein levels. Therefore, we hypothesized that triclosan may be preventing the interaction between Fos/Jun and the AP-1 site, in the *Mmp-13* promoter and its own promoter. This scenario would prevent PTH-mediated stimulation of *Mmp-13* transcription and enhance *c-fos* transcription, since it would prevent feedback inhibition of the *c-fos* promoter by its own protein. To explore this hypothesis, a CAT assay was performed using −148 bp of the *Mmp-13* promoter that contained the AP-1 and Runx2 binding sites. The promoter without stimulation showed basal activity (94 ± 24). The negative control plasmid with and without stimulation had minimal activity (17.4 ± 3.9 and 17.7 ± 4.0, respectively). Stimulation with PTH induced activity over baseline as expected (200 ± 24.6 vs. 94 ± 24.0, respectively). However, addition of triclosan with PTH reduced the activity to baseline levels (200 ± 24.6 vs. 97.5 ± 2.7, respectively). Stimulation with triclosan alone showed similar activity as the basal control (135 ± 3.7 vs 94) (Fig. 5).

DISCUSSION

In this study, we found that the UMR 106-01 rat osteosarcoma cell line was responsive to both PTH and PGE_2 with respect to stimulation of *Mmp-13* expression and was not responsive to LPS. Expression levels reach a robust level at 4 h post-treatment. Triclosan significantly abrogated both the PTH and PGE_2-mediated stimulation of *Mmp-13* expression.

Both PTH and PGE_2 stimulate *Mmp-13* expression though the cAMP and PKA pathway. Since CREB mediates the transcription of c-Fos in the nucleus and the mRNA is translated in the cytoplasm before re-entering the nucleus to induce *Mmp-13* transcription, *c-fos* expression is a primary response and *Mmp-13* expression is a secondary response. Additionally, PTH and PGE_2-mediated induction of AR expression is also a primary response though the PKA pathway.[21]

Since the PKA pathway involves several factors, there are a number of opportunities where triclosan can modulate the expression of *Mmp-13*. Our data suggest that triclosan does not exert its effect on the receptor or at the level of the membrane, since triclosan reduced the expression of *Mmp-13* in both PTH and PGE_2 stimulated osteoblasts. Additionally, it is unlikely that triclosan imparted an effect at the level of the receptor, since it would have to exert the same effect on two different receptors. Similarly, triclosan inhibited 8-Bromo cAMP-mediated stimulation, suggesting an intracellular effect. Using a systematic approach, key steps along the transduction pathway were investigated. We determined that triclosan had an effect downstream of cAMP by using 8-Bromo cAMP, a cell-permeable cAMP

analog that is more resistant to phosphodiesterases than cAMP and preferentially activates cAMP-dependent protein kinase[21]. Addition of 8-Bromo cAMP significantly increased the expression of *Mmp-13* as expected. Importantly, addition of triclosan significantly reduced 8-Bromo cAMP-mediated expression of *Mmp-13*, suggesting that triclosan functions at the level of cAMP or further downstream.

Since the free catalytic subunit of PKA translocates to the nucleus and exerts an effect on gene expression, several experiments were conducted to assess if triclosan acted within the nucleus. A previous study had shown that ^{14}C-labeled triclosan was taken up by fibroblast cells and translocated to the nucleus. [23] To further investigate this observation, two primary response genes of the PKA regulated pathway, *amphiregulin* (AR) and *c-fos*, were investigated. Triclosan upregulated AR, which is a member of the epidermal growth factor (EGF) family and a potent growth factor for pre-osteoblasts. AR null mice display significantly less tibial trabecular bone than wild type mice[21]. AR is also known to lower *Mmp-13* expression, similar to triclosan. Qin et al. reported that AR null mice have increased numbers of mature osteoblasts and osteoclasts, with a greater number of osteoclasts. Therefore, it is possible that AR exerts an inhibitory effect on osteoclastogenesis. Further exploration into the effects of AR as well as the role triclosan may play in the upregulation of AR is required.

Intriguingly, triclosan upregulated *c-fos* mRNA expression as well. This finding was surprising, since c-Fos is required for *Mmp-13* expression and yet triclosan caused a reduction in *Mmp-13* expression. Therefore, triclosan caused an upregulation of the primary response genes AR and *c-fos*. However, triclosan clearly inhibited the secondary response gene *Mmp-13*. This puzzling scenario led us to two questions. First, could triclosan be responsible for generalized protein degradation or the inhibition of c-Fos protein synthesis? To address this question, western blots were performed. The results indicated that triclosan did not inhibit c-Fos protein expression, and we concluded that triclosan does not cause generalized protein degradation or block c-Fos protein synthesis. Second, we assessed if triclosan could inhibit the binding of AP-1 to the *Mmp-13* promoter. The CAT promoter/reporter assay using the −148 bp promoter of rat *Mmp-13* that contained the AP-1 and Runx2 binding sites showed that triclosan may exert its effect through this region of the *Mmp-13* promoter. These data suggested that triclosan may interfere with AP-1 and explain the reduction in *Mmp-13* expression. Moreover, the ability of triclosan to upregulate the mRNA *c-fos* could also be due to preventing AP-1 binding to the negative feedback site in the *c-fos* promoter.

This study has potentially identified the specific mechanism of action that accounts for triclosan's ability to inhibit *Mmp-13* expression in an osteoblastic cell line. Other interesting findings were also observed and are worth noting. First, *Mmp-13* expression in PTH or PGE_2 stimulated cells can be reduced by triclosan, which is an effect similar to AR. Triclosan upregulates AR expression in this osteoblastic cell line. Moreover, the promoter/reporter assay suggests that triclosan may interfere with the *Mmp-13* promoter region, which would provide a logical explanation as to why *Mmp-13* transcription is reduced even in the presence of triclosan-enhanced *c-fos* transcription. It also suggests that triclosan inhibits the negative feedback at the AP-1 site of the *c-fos* gene.

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Key Finding

Triclosan blocks *Mmp 13* expression in PTH stimulated osteoblasts by preventing the interaction between Fos/Jun and the AP-1 site, in the *Mmp-13* promoter and its own promoter.

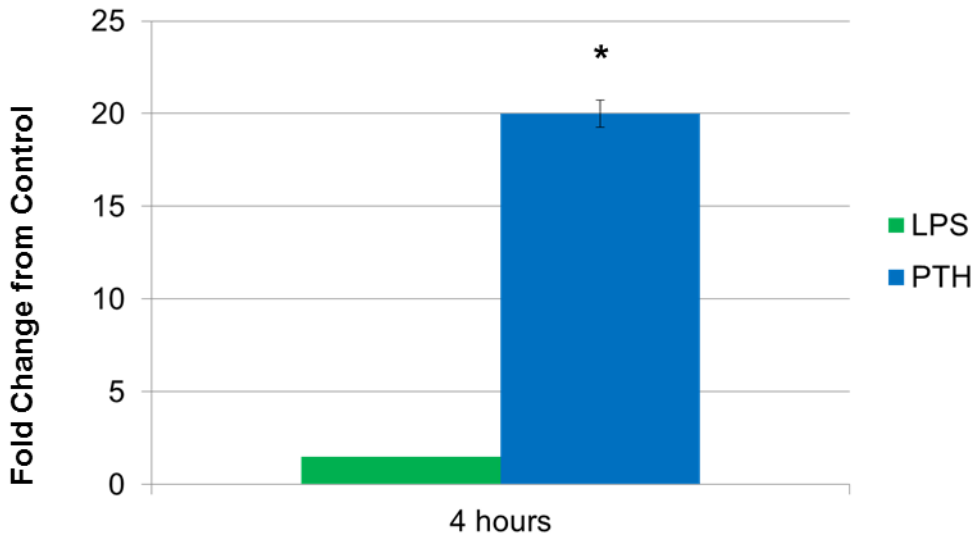

Figure 1. Expression of *Mmp*-13 by UMR Cells when Stimulated with LPS or PTH

LPS was not found to be statistically significantly different from the ETOH control while the difference in the fold change between PTH and ETOH was statistically significant. *(P<0.05).

Fig. 2a

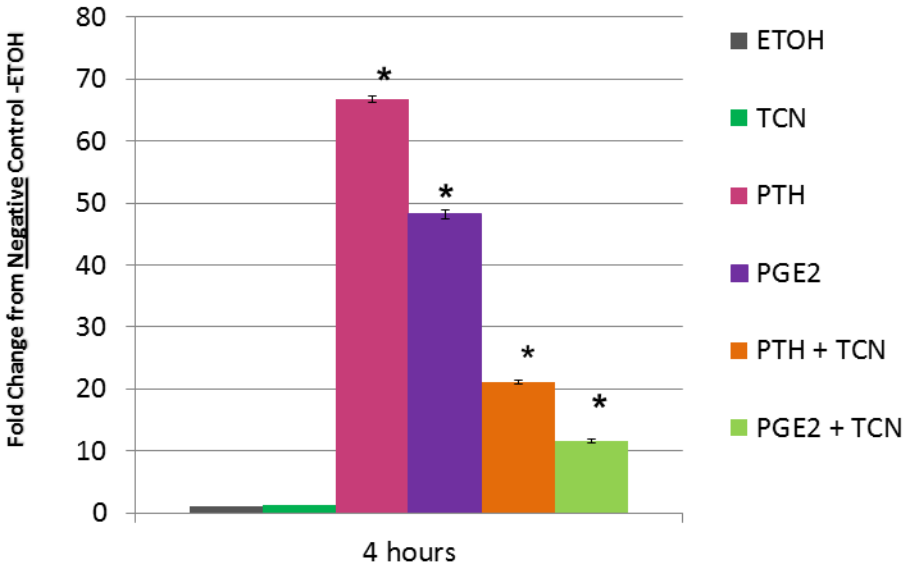

Fig 2b

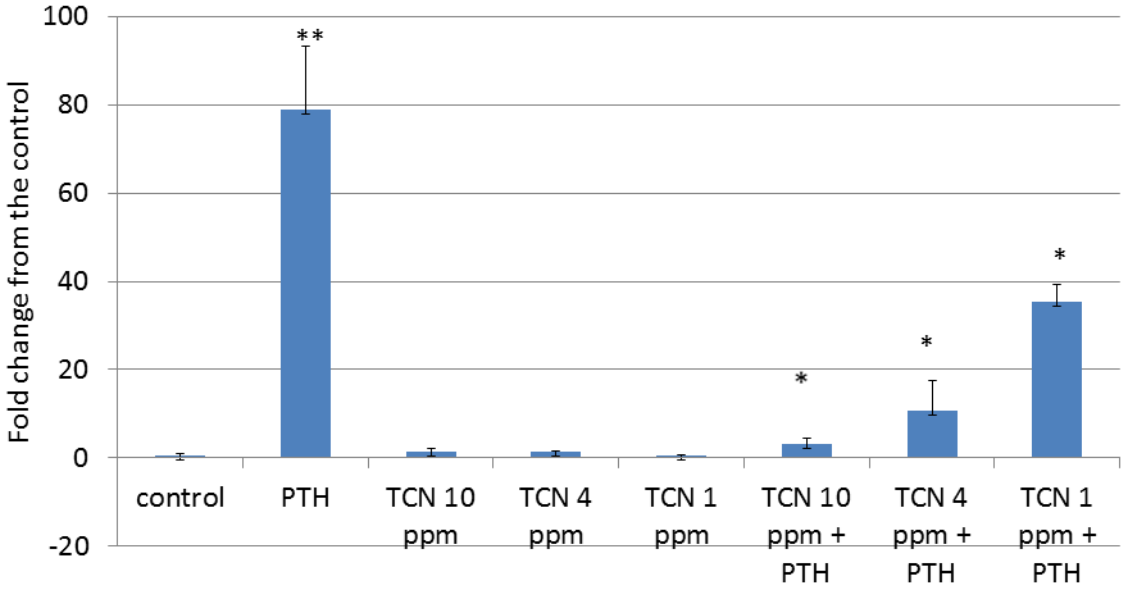

Figure 2. Expression of *Mmp-13* by UMR Cells stimulated with PTH or PGE_2 and TCN

a. UMR cells were treated with ethanol vehicle, 0.003% triclosan, 10^{-8} M PTH, 10^{-6} M PGE_2, PTH + triclosan or PGE_2 + triclosan. At a 4 h time point expression levels of *Mmp-13*

mRNA were measured as fold stimulation. Statistical analysis found no significant difference between TCN treated cells and ETOH. Cells stimulated with PTH, PGE2, PTH + TCN, and PGE_2 + TCN were significantly different from cells treated with ETOH or TCN alone.* ($p<0.05$) The addition of TCN to PTH stimulated cells produced a statistically significant decrease from the cells treated with PTH alone** ($p< 0.05$). The addition of TCN to PGE_2 stimulated cells produced a statistically significant decrease from cells treated with PGE_2 alone *** ($p<0.05$).

b. Dose Response with Triclosan UMR cells were treated with ethanol vehicle, 10^{-8} M PTH, and/or a dose response of TCN of 1, 4 or 10 ppm. Messenger RNA was analyzed after 4 h treatment. Results are depicted as the fold change from the ethanol vehicle. Means ± SD of triplicate measurements are indicated. PTH produced a 78.844 (± 14.45) as compared to negative control (ETOH). The addition of triclosan in the dose of 10 ppm, 4 ppm, and 1 ppm registered a 1.518 (± 0.702), 1.424 (± 0.162), 0.417 (± 0.239) fold change vs. ETOH respectively. The addition of triclosan (again at 10, 4 and 1 ppm) to PTH stimulated cells produced a 3.011 (± 1.498), 10.559 (± 6.869), 35.383 (± 3.934) fold change vs. ETOH respectively. PTH was found to be significantly different from the control **($p<0.05$). The addition of TCN at all doses to PTH stimulated cells were found to be significantly different from PTH stimulated cells. *($p<0.05$)

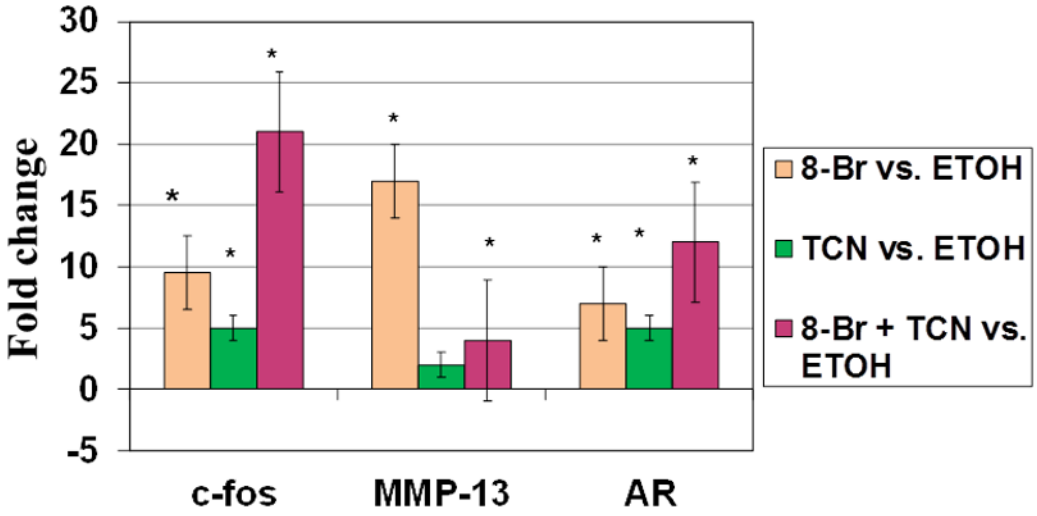

Figure 3. Effect of 8-Br-cAMP and Triclosan on *Mmp-13, c-fos,* and AR

UMR cells were treated with ethanol vehicle, 0.003% triclosan, and/or 10^{-3} M 8-Br-cAMP for 4 h. RNA was isolated and the fold-stimulation of *Mmp*-13, c-fos, and AR mRNAs determined. Results are depicted as the fold change from the ethanol vehicle. Means ± SD of triplicate measurements are indicated. All treatments that are significantly different from the cells treated with vehicle are shown by *($p<0.05$).

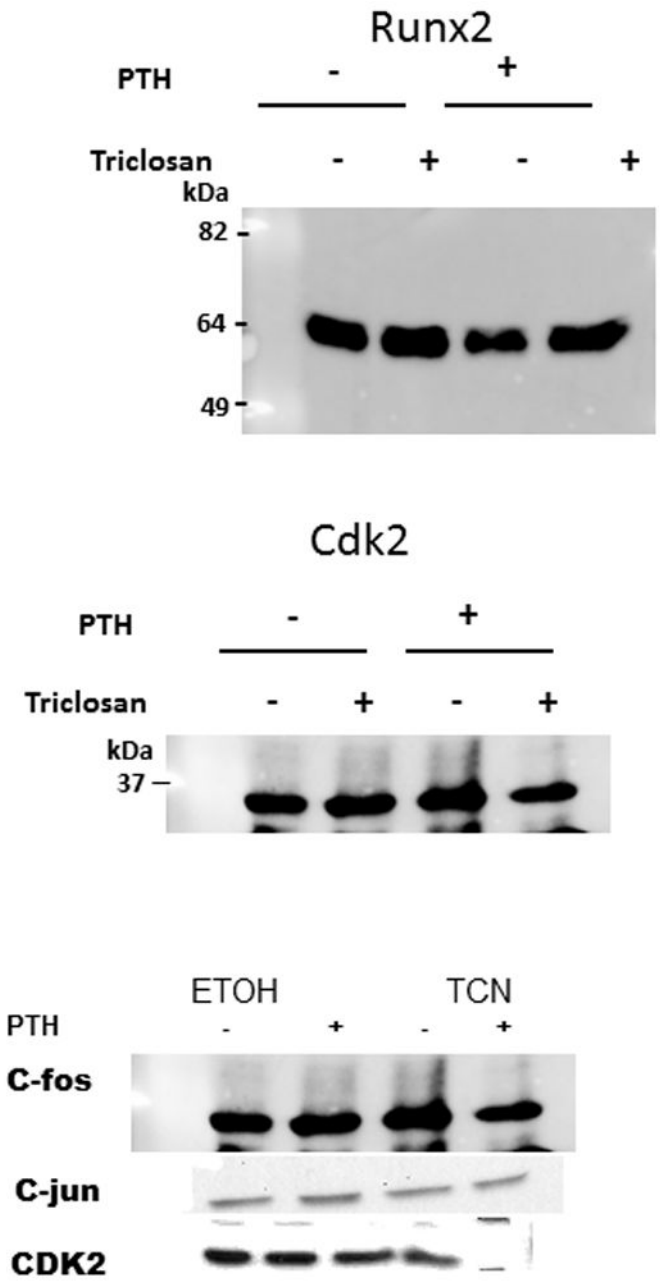

Figure 4. Effect of Triclosan on Fos, RUNX2 and Jun Proteins

Western blot of c-Fos, RUNX2 and c-Jun. Cells were treated with either ethanol or 0.003% triclosan followed by 10^{-8} M PTH and harvested at 4 h. Cdk2 was used as a loading control.

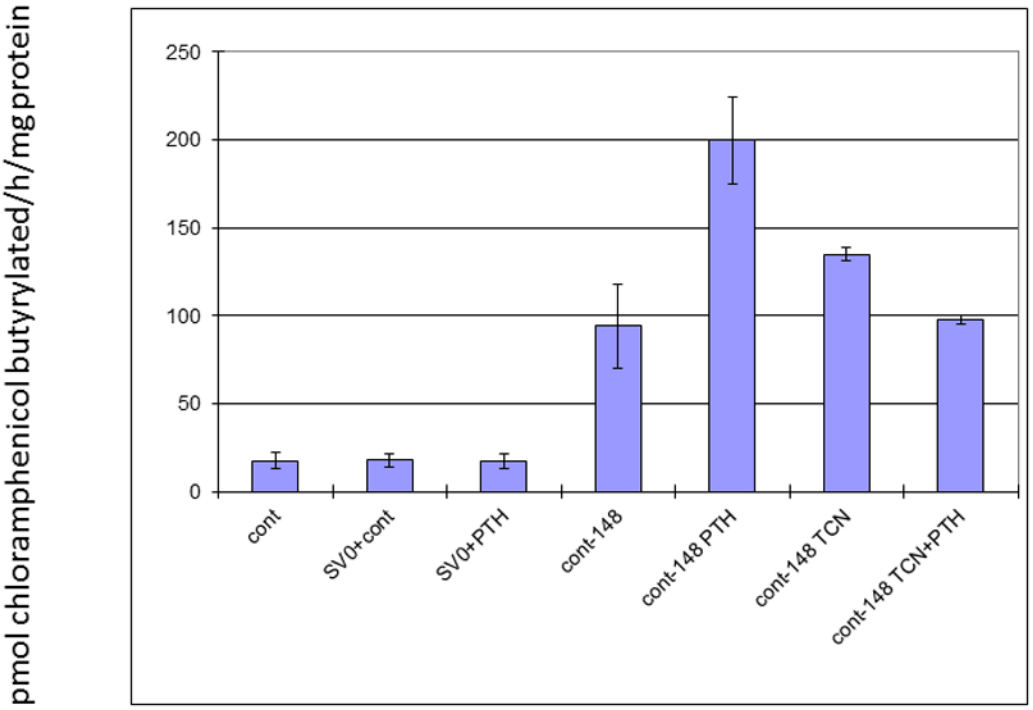

Figure 5. Inhibition of PTH-Stimulated *Mmp-13* Promoter by Triclosan

Cells were treated for 12 h with the indicated agents and then harvested for CAT activity. This was measured as pmol chloramphenicol butyrylated/h/mg protein[24]. n=3. The positive control at a level of 669 (±223) was too robust to be depicted on the y axis on this graph.