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*J Immunol* 2010; 185:6535-6544; Prepublished online 22 October 2010;
doi: 10.4049/jimmunol.1002009
http://www.jimmunol.org/content/185/11/6535

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**Supplementary Material**  
http://www.jimmunol.org/content/suppl/2010/10/22/jimmunol.1002009.DC1

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Jamie J. Bernard and Richard L. Gallo

Antimicrobial peptides such as human β-defensins (hBDs) and cathelicidins are critical for protection against infection and can be induced by activation of TLRs, a pathway that also activates cyclooxygenase(Cox)-2 expression. We hypothesized that Cox-2 is induced by TLR activation and is necessary for optimal AMP production, and that inhibitors of Cox-2 may therefore inhibit antimicrobial action. Normal human keratinocytes (NHEKs) stimulated with a TLR2/6 ligand, macrophage-activating lipopeptide-2, or a TLR3 ligand, polyinosinic-polycytidylic acid, increased Cox-2 mRNA and protein and increased PGE2, a product of Cox-2. Treatment with a Cox-2 selective inhibitor (SC-58125) or Cox-2 small interfering RNA attenuated hBD2 and hBD3 production in NHEKs when stimulated with macrophage-activating lipopeptide-2, polyinosinic-polycytidylic acid, or UVB (15 mJ/cm²), but it did not attenuate vitamin D3-induced cathelicidin. SC-58125 also inhibited TLR-dependent NF-κB activation. Conversely, treatment with Cox-derived prostanooids PGD₂ or 15-deoxy-Δ12,14-PGJ₂ induced hBD3 or hBD2 and hBD3, respectively. The functional significance of these observations was seen in NHEKs that showed reduced anti-staphylococcal activity when treated with a Cox-2 inhibitor. These findings demonstrate a critical role for Cox-2 in hBD production and suggest that the use of Cox-2 inhibitors may adversely influence the risk for bacterial infection.

Thus, despite some suggestive evidence, the role of Cox-2 in the antimicrobial response, or the role of NSAIDs in promoting infectious disease in the skin, remains unclear.

In the present study, we investigated the in vitro effects of Cox inhibitors on AMP expression and bactericidal activity in normal human keratinocytes. We found that TLR3 and TLR2/6 ligands induce Cox-2, and that inhibiting Cox-2 abrogates TLR ligand-induced hBD2 and hBD3 induction and attenuates anti-staphylococcal activity. Cox-2 small interfering RNA (siRNA) also attenuated TLR ligand- and UVB-induced hBD2 and hBD3. Furthermore, we demonstrated that two Cox-derived lipid mediators, PGD2 and 15-deoxy-

Delta(12,14)-PGJ2 (15d-PGJ2), stimulate hBD expression in keratinocytes, suggesting a new role for PGs in enhancing AMP production and the subsequent innate immune response.

Materials and Methods

Reagents

The following reagents were purchased from Cayman Chemical (Ann Arbor, MI): PGE2, PGF2a, iloprost, U-46619, PGD2, 15d-PGJ2, aspirin, SC-560, SC-58125, NS-398, BAY-11-7082 (BAY), and CAY10470 (CAY). Polyinosinic-polycytidylic acid [poly(I:C)] (a synthetic analog of dsRNA) was purchased from InvivoGen (San Diego, CA). Macrophage-activating lipopeptide-2 (MALP-2) was purchased from Enzo Life Sciences (Plymouth Meeting, PA). 1α,25-dihydroxyvitamin D3 (1,25-D3) was purchased from Sigma-Aldrich (St. Louis, MO). Cox-2 ON-TARGETplus SMARTpool siRNA constructs were purchased from Thermo Fisher Scientific (Chicago, IL).

Cell culture conditions

Normal human epidermal keratinocytes (NHEKs) were grown in serum-free EpiLife cell culture media (Cascade Biologics, Portland, OR) containing 0.06 mM Ca2+ and EpiLife defined growth supplement at 37˚C under standard tissue culture conditions. The cultures were maintained for up to four passages in this media with the addition of 50 U/ml penicillin and 50 µg/ml streptomycin. Cells were treated at 70–80% confluence.

Cox inhibitor experiments

NHEKs were preincubated with the Cox inhibitors aspirin (100 µM), SC-58125 (10 µM), NS-398 (10 µM), or SC-560 (10 µM) for 0.5–1 h prior to stimulation. NHEKs were stimulated with poly(I:C) (10 µg/ml), MALP-2 (500 ng/ml), or 1,25-D3 (100 nM) or were treated with prosstanooids, PGE2 (10 µM), PGF2a (10 µM), iloprost (10 µM), U-46619 (10 µM), PGD2 (1 µM), or 15d-PGJ2 (500 nM) for 24 h. NHEKs were irradiated by UVB at 15 mJ/cm².

FIGURE 1. Cox inhibitors attenuate poly(I:C)-induced β-defensins. A. NHEKs were pretreated with aspirin (100 µM), SC-58125 (10 µM), NS-398 (10 µM), or SC-560 (10 µM) for 30 min and then treated with poly(I:C) (10 µg/ml) for 24 h. Poly(I:C) induces hBD2 mRNA expression ∼75-fold over untreated when normalized to GAPDH levels (⁎p < 0.05). Aspirin, SC-58125, and NS-398 significantly attenuated poly(I:C)-induced hBD2 mRNA expression (⁎p < 0.05). SC-560 failed to attenuate poly(I:C)-induced hBD2 mRNA expression. B. Poly(I:C)-induced hBD2 protein expression was attenuated by pretreating NHEKs with SC-58125 (10 µM) as demonstrated by fluorescence microscopy (original magnification ×400): vehicle (MFI = 0.3), poly(I:C) (MFI = 13.0), SC-58125 (MFI = 0.8), SC-58125 plus poly(I:C) (MFI = 13.9). C. NHEKs were pretreated with aspirin (100 µM), SC-58125 (10 µM), NS-398 (10 µM), or SC-560 (10 µM) for 30 min and then treated with poly(I:C) (10 µg/ml) for 24 h. Poly(I:C)-induced hBD3 mRNA expression ∼10-fold over untreated when normalized to GAPDH levels (⁎p < 0.05). Aspirin, SC-58125, and NS-398 significantly attenuated poly(I:C)-induced hBD3 mRNA expression (⁎p < 0.05). SC-560 failed to attenuate poly(I:C)-induced hBD3 mRNA expression. D. hBD3 protein expression was attenuated by pretreating NHEKs with SC-58125 (10 µM) as demonstrated by fluorescence microscopy (original magnification ×400): vehicle (MFI = 106), poly(I:C) (MFI = 104), SC-58125 (MFI = 34), SC-58125 plus poly(I:C) (MFI = 54). A single NHEK stained for hBD3 is highlighted by the white box in the poly(I:C) panel and amplified to demonstrate hBD3 localization. Cells in B and D were stained with defensin primary Abs and an Alexa Fluor 568 secondary Ab.
Cox-2 siRNA
NHEKs were transfected with 3 μM Cox-2 siRNA constructs using DharmaFECT (Thermo Fisher Scientific) transfection reagent. Cells were incubated for 24 h and the transfection was repeated. Twenty-four hours after the last transfection, cells were treated with TLR ligands or UVB as previously described.

Real-time quantitative RT-PCR
Total RNA was extracted by using TRizol Reagent (Invitrogen, Carlsbad, CA). One microgram of total RNA was used for cDNA synthesis by the iSCRIPT cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Real-time RT-PCR was conducted in an ABI PRISM 7000 sequence detector (Applied Biosystems, Carlsbad, CA). The primers and probes used for real-time RT-PCR were purchased from Applied Biosystems. RNA analysis was performed using the TaqMan Master Mix reagents kit (Applied Biosystems). The quantification of gene expression was determined by the comparative ΔΔCt method. The target gene expression in the test samples was normalized to the endogenous reference GAPDH level and was reported as the fold difference relative to the GAPDH gene expression. All of the assays were performed in triplicate and repeated at least three times.

Western blotting
NHEKs were stimulated with poly(I:C) (10 μg/ml), MALP-2 (500 ng/ml), or MALP-2 (500 ng/ml) plus 1,25-D3 (100 nM) for 24 h to analyze Cox-1 and Cox-2 protein. Cells were lysed in 1× RIPA buffer with protease inhibitors. NHEKs were preincubated with SC-58125 (10 μM) and then treated with poly(I:C) or MALP-2 for 60 min to analyze RelA/p65 and lamin B1 protein. Nuclear lysates were separated from cytoplasmic lysates using a PARIS kit (Ambion, Austin, TX). Ten percent gels were run and transferred onto polyvinylidene difluoride transfer membranes (Millipore, Bedford, MA). Membranes were blocked with the Odyssey infrared imaging system blocking buffer (LI-COR, Lincoln, NE) then incubated with anti–Cox-1 (Cayman Chemical), anti–Cox-2 (Cayman Chemical), anti–RelA/p65 (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-lamin B1 (Abcam, Cambridge, MA) primary Abs in 5% blocking buffer for 2 h at room temperature. Membranes were washed and incubated with goat anti-rabbit IRDye 680 or goat anti-mouse IRDye 800CW secondary Abs (LI-COR) for 30 min at room temperature. Membranes were washed and fluorescence was detected using the Odyssey infrared imaging system (LI-COR). Western blotting for each protein was repeated at least three times.

Enzyme immunoassay
Supernatants were assayed by the PGE2 or PGD2 enzyme immunoassay (EIA) from Cayman Chemical according to the manufacturer’s instructions. This assay was performed in triplicate and repeated three times.

Fluorescence microscopy
NHEKs were grown on chamber slides and treated as described in Results. Cells were fixed in 2% formaldehyde for 15 min, washed with 1× PBS, and stained for β-defensins.
blocked in 3% BSA for 30 min, and stained with a rabbit anti-p65 Ab (Santa Cruz Biotechnology) (1/200) or rabbit IgG for 2 h at room temperature. Cells were then washed with 1× PBS stained with Alexa Fluor 568 anti-rabbit IgG (1/1000) for 1 h at room temperature. Cells were washed with 1× PBS, mounted in ProLong anti-fade reagent containing DAPI (Molecular Probes, Eugene, OR) and evaluated with an Olympus BX41 microscope (Olympus, Mellville, NY) at original magnification of ×400. Levels of mean fluorescence intensity (MFI) were measured with ImageJ (National Institutes of Health, Bethesda, MD).

**Bacterial extract preparation and bacterial killing assay**

*Staphylococcus aureus* strain ΔmpF was a gift from A. Peschel (Microbial Genetics, University of Tubingen, Tubingen, Germany), and strain Sa113 was a gift from Dr. Victor Nizet (University of California, San Diego, CA). Bacteria were grown in tryptic soy broth at 37˚C for 15–16 h and collected in the log phase. The number of *S. aureus* was enumerated by applying a conversion factor (2.0 × 10^8 bacteria/ml = 0.6 OD U at 600 nm). Bacteria (CFU) was diluted in 3% tryptic soy broth in DMEM (Invitrogen) and sonicated keratinocytes were added at a multiplicity of infection (MOI) of 20 and incubated at 37˚C for 1, 3, and 5 h. After incubation, 10-fold dilutions were prepared and plated on tryptic soy broth agar and the plates were incubated for 1 d at 37˚C. All assays were performed in duplicate and repeated at least three times.

**Statistical analysis**

To determine statistical significance between groups, comparisons were made using two-tailed *t* tests. Analyses of multiple groups were done by one-way or two-way ANOVA using GraphPad Prism version 4 (GraphPad Software, San Diego, CA). For all statistical tests, a *p* value of <0.05 was accepted for statistical significance.

**Results**

**Cox-2 inhibitors attenuate β-defensin production**

The essential role of keratinocytes in innate immune responses (27), and prior observations that Cox-2 influences immunity (17, 28), led us to the hypothesis that Cox-2 plays a role in hBD production by keratinocytes. NHEKs were preincubated for 30 min with inhibitors of either Cox-1, Cox-2, or Cox-1 and Cox-2, then treated with the TLR3 ligand poly(I:C), a potent stimulus of hBD2 and hBD3 (29). After 24 h, hBD2 and hBD3 mRNA expression was analyzed by quantitative RT-PCR. Aspirin (a Cox-1/ Cox-2 dual inhibitor), NS-398 (a Cox-2 selective inhibitor), and SC-58125 (a Cox-2 selective inhibitor) attenuated poly(I:C)-induced hBD2 mRNA expression, whereas SC-560 (a Cox-1 selective inhibitor) failed to attenuate hBD2 (Fig. 1A). NHEKs treated with the Cox-2 selective inhibitor SC-58125 also showed decreased poly(I:C)-induced hBD2 protein expression as evaluated by fluorescence microscopy at 24 h (Fig. 1B). A similar dependence on Cox-2 activity was also seen for hBD3 mRNA and protein expression induced by poly(I:C) (Fig. 1C, 1D).

To determine whether Cox-2 activity was necessary for optimal AMP production in response to other AMP-inducing stimuli, we next tested the effect of Cox-2 inhibition on AMP expression by 1,25-D3, a potent inducer of cathelicidin (CAMP), a TLR2/6 ligand MALP-2, and UVB. UVB induces hBD2 and hBD3 (30, 31) and was previously shown to induce Cox-2 and PGE2 in human keratinocytes (32, 33). Twenty-four hours following stimulation, hBD2, hBD3, and cathelicidin mRNA expression were analyzed by quantitative RT-PCR. Inhibition of Cox-2 by SC-58125 attenuated hBD2 and hBD3 mRNA induced by MALP-2 or by MALP-2 plus 1,25-D3 (Fig. 2A, 2B). hBD2 and hBD3 mRNA induced in NHEKs stimulated with 15 mj/cm2 UVB was suppressed by either Cox-2 inhibitor NS-398 or SC-58125 (Fig. 2C, 2D). In contrast to hBD-inducing stimuli, SC-58125 failed to inhibit 1,25-D3–induced cathelicidin mRNA expression (Fig. 2E).
In addition to pharmacological inhibition of Cox-2, Cox-2 was reduced by targeted knockdown by RNA interference (Fig. 3A). NHEKs that were thus made deficient in Cox-2 protein demonstrated a reduced ability to induce hBD2 (Fig. 3B) and hBD3 (Fig. 3C) in response to poly(I:C). Consistent with these findings, Cox-2-deficient NHEKs also demonstrated a reduced ability to induce hBD2 (Fig. 3D) and hBD3 (Fig. 3E) in response to UVB.

TLR ligands induce Cox-2 mRNA protein and activity

Because inhibition of Cox-2 suppressed optimal hBD2 and hBD3 production, we hypothesized that TLR ligands could influence Cox-2 mRNA and protein expression in keratinocytes. Cox-2 and Cox-1 mRNA expression was measured by quantitative RT-PCR after 24 h of MALP-2 or poly(I:C) stimulation. MALP-2 induced Cox-2 mRNA ~4-fold and poly(I:C) induced Cox-2 mRNA ~6-fold (Fig. 4A). Cox-1 mRNA was not induced by MALP-2 or poly(I:C) treatment (Fig. 4B). The increase in Cox-2 mRNA expression corresponded with an increase in Cox-2 protein abundance as observed by Western blotting, while Cox-1 protein levels remained unchanged under these conditions (Fig. 4C).

We next evaluated PGE2, a product of Cox-2 enzymatic activity, in NHEKs activated by ligands that enhanced Cox-2 mRNA and protein abundance. Unstimulated NHEKs released ~225 pg/ml PGE2. MALP-2, MALP-2 plus 1,25-D3, and poly(I:C), but not 1,25-D3, all significantly induced PGE2 release when compared with both untreated and vehicle-treated NHEKs, and this induction was abrogated upon preincubation with SC-58125 (Fig. 4D). Collectively, these data suggest that Cox-2 is necessary for keratinocytes to respond to UVB, TLR3, and TLR2/6 ligands by increasing β-defensin expression, but not cathelicidin expression.

Cox-2–derived PGs induce β-defensins

Because Cox-2 inhibitors significantly attenuate hBD2 and hBD3 induction, we next investigated whether prostanoid products of Cox-2 could induce AMP expression. NHEKs were stimulated with PGE2, PGF2α, iloprost (a stable analog of PGI2), U-46619 (a thromboxane A2 receptor agonist), PGD2, or 15d-PGJ2. Concentrations of these prostanoids were selected that had no apparent effect on cell viability as evaluated by visual observation of growth in culture. PGE2, PGF2α, iloprost, and U-46619 failed to induce hBD2 and hBD3 at concentrations of 100 nM, 500 nM, 1 μM (data not shown), and 10 μM (Fig. 5A, 5B). 15d-PGJ2 induced hBD2 mRNA expression ~100-fold at 24 h (Fig. 5A). PGD2 and 15d-PGJ2 induced hBD3 mRNA expression ~40-fold and 300-fold at 24 h (Fig. 5B). A time course experiment demonstrated that 15d-PGJ2 significantly induced hBD2 (Fig. 5C) and hBD3 (Fig. 5D) mRNA between 2 and 6 h. Additionally, 15d-PGJ2 increased the expression of hBD2 (Fig. 5E) and hBD3 (Fig. 5F) protein after 24 h.

To examine whether keratinocytes induce PGD2 in response to TLR ligands, NHEKs were stimulated with poly(I:C) or MALP-2 for 24 h and PGD2 release was measure in the supernatant by ELA. Both poly(I:C) and MALP-2 induced PGD2 ~4-fold (Fig. 5G).

Inhibition of Cox-2 attenuates TLR ligand-induced NF-κB activation

NF-κB activity is involved in the regulation of hBDs (34, 35). To determine whether Cox-2 inhibition influences NF-κB activation in keratinocytes, NHEKs were preincubated with SC-58125, then treated with poly(I:C) or MALP-2 for 5, 15, 30, or 60 min, at which time cells were stained for RelA/p65. Nuclear localization of RelA/p65 was analyzed by both fluorescence microscopy and Western blotting. As expected, MALP-2 and poly(I:C) promoted the nuclear translocation of RelA/p65 by 30 min (data not shown) and RelA/p65 remained in the nucleus at 60 min (Fig. 6A). Preincubation with SC-58125 attenuated both MALP-2– and poly(I:C)-induced nuclear translocation of RelA/p65 at 60 min (Fig. 6A). Western blotting-purified nuclear lysates revealed a similar effect...
and demonstrated that preincubation with SC-58125 inhibited MALP-2– and poly(I:C)-induced RelA/p65 nuclear localization at 60 min (Fig. 6B).

Cox-2 inhibition reduced RelA/p65 nuclear localization and, therefore, we next wanted to test whether the ability of 15d-PGJ2 to induce hBD2 and hBD3 was dependent on NF-κB activity. 15d-PGJ2 induced RelA/p65 nuclear localization by 30 min in NHEKs (Supplemental Fig. 1). NHEKs were pretreated with the NF-κB inhibitors BAY (1 μM) or CAY (1 μM) for 30 min prior to 15d-PGJ2 addition. BAY and CAY both block IκB-α phosphorylation and inhibited 15d-PGJ2–induced RelA/p65 nuclear translocation (data not shown). Pretreatment with BAY attenuated 15d-PGJ2–induced hBD2 (Fig. 6C) and hBD3 (Fig. 6D) mRNA expression, and pretreatment with CAY attenuated 15d-PGJ2–induced hBD2 (Fig. 6E) and hBD3 (Fig. 6F) mRNA expression, suggesting that the mechanism of hBD induction was partially dependent on NF-κB activity.

Cox-2 enhances S. aureus killing
Staphylococci are prevalent bacterial skin inhabitants and are resistant to AMPs in part due to the expression of mprF, which modifies anionic membrane lipids (36). However, the expression of hBD3 is a critical determinant of the anti-staphylococcal activity of keratinocytes (37). Therefore, we next examined the anti-staphylococcal activity of keratinocytes with inhibited Cox-2 activity to determine whether the prior observations were functionally relevant to the capacity of keratinocytes to act as effectors of innate immune defense. Two different strains of S. aureus were used: strain DmprF, a well-defined strain selected for its sensitivity to cationic AMPs because it cannot modify the membrane, and the more resistant Sa113. NHEKs were preincubated with SC-58125, treated with poly(I:C) for 24 h, and then cells were harvested in 1× PBS and sonicated to isolate total soluble antimicrobial factors. These NHEK lysates were then incubated with S. aureus. Assays were done at an MOI of 20 for 1, 3, and 5 h. At 1 h, SC-58125 reduced the anti-staphylococcal activity of poly(I:C)-treated NHEKs by 3 logs in the AMP-sensitive DmprF strain, and at 5 h it inhibited the antimicrobial effect by 2 logs (Fig. 7A). A similar, but lesser, magnitude of effect was also seen on anti-staphylococcal activity against the more AMP-resistant Sa113 strain at 3 and 5 h (Fig. 7B).

Because 15d-PGJ2 stimulated hBD2 and hBD3 in keratinocytes, we next investigated whether 15d-PGJ2 treatment of NHEKs could enhance their bacteriocidal activity. NHEKs were treated with 15d-PGJ2 for 24 h, at which time cells were harvested in 1× PBS and sonicated. NHEK-sonicated lysates were then incubated with S. aureus strains DmprF and Sa113 at a MOI of 20 for 0.5, 1, and 3 h. 15d-PGJ2 enhanced the anti-staphylococcal activity of
NHEKs by >3 logs in the ΔmprF strain at 0.5 h and completely inhibited bacteria survival at 3 h (Fig. 7C). 15d-PGJ2 enhanced the anti-staphylococcal activity of NHEKs by >3 logs in the Sa113 strain at 0.5 and 1 h and by >5 logs at 3 h (Fig. 7D).

Discussion
Keratinocytes are sentinel to epidermal protection as they are the first barrier against invading organisms and possess potent antimicrobial activity by their release of several molecules, including AMPs such as cathelicidins and β-defensins. Prior work has shown that the production of AMPs by many tissues is essential to defense against infectious microbial pathogens (38). Thus, the mechanisms that regulate AMP expression are an important area of investigation that may impact human diseases such as skin infections, atopic dermatitis, rosacea, and psoriasis (6). In this study, we examined the role of Cox-2 in keratinocyte AMP expression. We found that Cox-2 activity is important for optimal hBD2 and hBD3 production after a variety of stimuli. Moreover, we demonstrated that 15d-PGJ2, a small lipid molecule derived from Cox activity, increases hBD2 and hBD3 production in keratinocytes and enhances antimicrobial action. These uncover a previously unknown role for Cox-2 in controlling the innate immune response of the epidermis and suggest that compounds that influence this pathway may be useful for controlling innate immunity.

Support for a role for Cox-2 in hBD2 and hBD3 expression comes from several independent experimental approaches. We demonstrated that Cox-2 selective inhibitors and knockdown of Cox-2 by siRNA attenuate TLR ligand-induced hBD2 and hBD3 expression (Figs. 1–3) and that TLR3 and TLR2/6 ligands induce Cox-2 to and its product PGE2 in human keratinocytes (Fig. 4). Moreover, we demonstrated that Cox-2 selective inhibitors and Cox-2 siRNA also attenuate UVB-induced hBD2 and hBD3 in human keratinocytes (Figs. 2, 3). UVB was known to induce Cox-2 activity and PGE2 (32, 33), and recently to induce β-defensins (30, 31). These data suggest that Cox-2 induction is necessary for optimal hBD expression after UVB and demonstrate that the suppressive effect of this Cox-2 inhibitor applies to more than TLR ligands. However, the suppressive effect of Cox-2 inhibitors on hBD production reaches a plateau between the 10 and 20 μM dose (data not shown), and Cox siRNA failed to maintain hBDs at the uninduced level, suggesting a partial Cox-2–independent mechanism for hBD induction. Furthermore,
we did not see an attenuation of vitamin D-induced human cathelicidin expression with Cox-2 inhibitors (Fig. 2E). This provides further evidence that human cathelicidin and β-defensins are differentially regulated and that the effects of Cox-2 cannot be generalized to all AMPs. These data also suggest that Cox-2 inhibition would not inhibit UVB-mediated induction of cathelicidin in the skin, as recent data suggests that this is mediated, at least in part, by local synthesis of hormonally active vitamin D.

The addition of the Cox-2 product 15d-PGJ2 dose- (Fig. 6C, 6D) and time-dependently (Fig. 5C, 5D) induced hBD2 and hBD3 expression. Recently, Kanda et al. (39) demonstrated that PGD2 induced a small increase in hBD2 in human keratinocytes. Interestingly, PGD2 and 15d-PGJ2 induce β-defensins more potently than do the TLR ligands, suggesting a differential role for exogenously added versus endogenously generated PGs. 15d-PGJ2 is a dehydration product of PGD2 that, in our experiments, induced hBD3 in human keratinocytes. In our studies, we demonstrated that 15d-PGJ2 induced hBD3 more potently than PGD2 and that PGD2 and 15d-PGJ2 induce β-defensins more potently than do the TLR ligands, suggesting a differential role for exogenously added versus endogenously generated PGs. 15d-PGJ2 has been shown to have both pro- and anti-inflammatory properties in keratinocytes (40) and is a well-established ligand for the peroxisome proliferator-activated receptor γ (PPARγ) and for inhibiting inflammation at nanomolar and low micromolar doses. However, rosiglitazone, a thiazolidinedione and potent PPARγ activator, failed to induce hBD2 and hBD3 in NHEKs (data not shown). PPARγ did not appear to be the mechanism by which 15d-PGJ2 mediated hBD induction. However, we demonstrated that 15d-PGJ2 induced RelA/p65 nuclear localization (Fig. 6A), suggesting that Cox-2 inhibitors abrogate NF-κB activation. We also found that SC-58125 had no effect on p44/42 MAPK (ERK1/2), p38 MAPK (MapK), or JNK activation (data not shown), as these signaling intermediates represent other reporter regulators of defensin expression (43–46). Our observations with NF-κB suggest this is the most likely canonical pathway from TLR activation that is influenced by Cox-2 products.

Interestingly, although keratinocytes have the capacity to produce both PGD2 and 15d-PGJ2, most of these PGs are released by mast cells in the skin. Mast cells are a potent source of AMPs and can provide protection against infection. Future studies will examine whether mast cell-derived PGs act to cooperate in the epidermal innate immune defense strategy and enhance skin antimicrobial activity.

To better understand the mechanism of Cox-2 on hBD expression, we examined downstream signaling events known to influence expression of these genes. TLR ligand-induced epithelial hBD gene expression has been reported to be mediated, in part, by NF-κB signaling, and NF-κB sites are active in the hBD2 promoter (34, 35). SC-58125 attenuated both poly(I:C)- and MALP-2–induced RelA/p65 nuclear localization (Fig. 6A), suggesting that Cox-2 inhibitors abrogate NF-κB activation. We also found that SC-58125 had no effect on p44/42 MAPK (ERK1/2), p38 MAPK (MapK), or JNK activation (data not shown), as these signaling intermediates represent other reporter regulators of defensin expression (43–46). Our observations with NF-κB suggest this is the most likely canonical pathway from TLR activation that is influenced by Cox-2 products.

We also demonstrated that a Cox-2 selective inhibitor significantly attenuated poly(I:C)-induced keratinocyte S. aureus killing (Fig. 7A, 7B), and that the addition of a prostaglandin product of Cox-2, 15d-PGJ2, increased functional antimicrobial action (Fig. 7C, 7D). These functional effects could be the result of multiple antimicrobial effector molecules that are produced by keratinocytes. However, the robust decrease in antimicrobial action after the inhibition of Cox-2 and the increase in antimicrobial action after the addition of a Cox-2 product are evidences consistent with the induction of hBD3, as Kisich et al. (47) showed that secreted keratinocyte hBD3 promotes significant bactericidal activity against S. aureus.
Prior to our study, little mechanistic information was known about how Cox-2 inhibitors might influence the innate immune response. With the present observations, there are broad potential clinical applications to consider. NSAIDs and Cox-2 selective inhibitors are widely used to treat both acute and chronic inflammatory disease, and some evidence suggests that NSAID use is linked to bacterial infection (21–25). For example, Factor et al. (23) observed a connection between new NSAID use and group A β-hemolytic streptococcal infection. However, no major effect has clearly been observed on enhanced susceptibility to infection. This lack of a clearly evident change in infection risk is consistent with a selective inhibition of some but not all of the AMPs produced by keratinocytes. Carefully controlled animal and in vitro observations have been necessary to uncover the essential role of AMPs in mammalian immunity (48), but their relative effects in mammals are less obvious compared with the profound defects seen when severe alterations in cellular and humoral immunity occur. Thus, although the anti-inflammatory benefits of Cox inhibition may outweigh risks, these data suggest that NSAID use must be carefully monitored for subtle influences on infection, or under circumstances of compromised immunity or bacterial infection.

Another implication of the present findings is that regulating alternative systems for increasing AMP responses. Infectious and inflammatory diseases are a major health care burden. Although pharmaceutically generated antibiotics can be an effective treatment for infections, the continual emergence of drug-resistant organisms has limited the effectiveness of this approach. Antibiotic resistance poses a tremendous threat to human health, which is best evidenced by methicillin-resistant S. aureus (49). Additionally, background levels of antibiotic-resistant genes continue to rise in soil (50). An alternative interventional strategy for treatment is to enhance native host cell antimicrobial defense. Our results reveal a new mechanism for the involvement of Cox-2 in hBD induction by TLR ligands and indicate that the induction of cyclooxygenase-2 in hBD expression by TLR ligands and indicate that the involvement in the immune system (48), but their relative effects in mammals are less obvious compared with the profound defects seen when severe alterations in cellular and humoral immunity occur. Thus, although the anti-inflammatory benefits of Cox inhibition may outweigh risks, these data suggest that NSAID use must be carefully monitored for subtle influences on infection, or under circumstances of compromised immunity or bacterial infection.

Disclosures

The authors have no financial conflicts of interest.

References


Supplementary figure 1