Increased COX-2 Expression in Human Vaginal Epithelial Cells Exposed to Nonoxynol-9, a Vaginal Contraceptive Microbicide that Failed to Protect Women from HIV-1 Infection

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Problem

Despite displaying virucidal activity *in vitro*, nonoxynol-9 (N-9), a vaginal contraceptive microbicide candidate, failed to reduce the rate of human immunodeficiency virus (HIV) transmission in clinical trials. With frequent use, it even increased the risk of HIV acquisition. Such outcome was postulated to be because of N-9-induced mucosal inflammation, which resulted in recruitment of HIV-target immune cells to the sites of virus entry. Understanding the mechanism underlying the response of the vaginal epithelium to N-9 is critical to properly evaluate the safety of prospective vaginal microbicides and contraceptives.

Methods and results

Using DNA microarray and quantitative RT-PCR techniques, we observed that N-9 initiated a strong transcriptional upregulation of cyclooxygenase-2 (COX-2) in immortalized human vaginal epithelial cells (VK2/E6E7 cell line). Increased COX-2 protein expression evaluated by immunoblotting was dose- and time-dependent. The level of prostaglandin E_2 (PGE_2) increased subsequently to COX-2 elevation. This upregulation was in part because of NF-kB activation.

Conclusion

Expression of COX-2, a potent inflammation-related enzyme, as well as increased secretion of PGE_2, an important local mediator of mucosal immunoinflammatory responses, by human vaginal epithelial cells exposed to vaginal microbicide and contraceptive candidates may be used as a biomarker of undesirable compound properties.

Introduction

Nonoxynol-9 [nonylphenoxypolyethoxyethanol (N-9)] is the active component of most commercial formulations of female vaginal contraceptives. It is available as over-the-counter products in the form of contraceptive creams, gels, sponges, and films. N-9 is also used in combination with diaphragms and other barrier contraceptive methods including lubricated condoms. N-9 is a membrane disrupting detergent and its spermicidal activity results from solubilization of the sperm plasma membrane and consequent sperm immobilization.[1] In addition to being spermicidal, N-9 demonstrated *in vitro* antiviral activity,[2] which made it a candidate microbicide for preventing sexual transmission of human immunodeficiency virus (HIV). However, N-9 was ineffective in preventing HIV transmission in clinical trials, and when used

frequently, it even increased the rate of HIV acquisition.[3] Women who used N-9 frequently showed vaginal epithelial disruption more often, and a significant association was found between HIV seroconversion and occurrence of N-9-induced epithelial breaches.[4]

Susceptibility to HIV may also be increased by mucosal inflammation and immune activation, which would recruit additional HIV-target cells to the genital mucosa and/or increase the receptivity of those cells to HIV.[5,6] Indeed, it has been found that repeated use of N-9 can cause irritation and inflammation of the cervicovaginal mucosa.[7–9] A short exposure of the rabbit vagina to N-9 and other surface-active vaginal products was reported to induce significant mucosal inflammation with increased levels of IL-1, IL-6 and IL-8 and increased number of immune cells in cervicovaginal secretions.[8,10] Furthermore, cervicovaginal lavages collected from women who had used N-9 for 3 days enhanced HIV expression *in vitro*.[9] Therefore, it has been postulated that N-9 failure in HIV prevention trials may have been caused by a mucosal inflammatory reaction induced by N-9 that counterbalanced its antiviral effect.[9,10]

The N-9 clinical findings, as well as similar examples showing lack of effectiveness and possible enhancement of HIV transmission with SAVVY™ [11] and Ushercell™,[12] are a clear reminder of the need for developing new biomarkers and models to evaluate microbicide cervicovaginal safety. N-9 was selected for this study because it is the only microbicide candidate that has been proved to be harmful to the vaginal mucosa and enhance HIV transmission. Elucidating the molecular mechanisms underlying the vaginal cell response to N-9 that may have facilitated HIV transmission is critical to improve the evaluation of new microbicide candidates. In this study, we present data showing for the first time that N-9 causes a strong transcriptional and translational increase in the potent inflammation-related enzyme cyclooxygenase-2 (COX-2) in human vaginal cells. COX-2 upregulation in turn results in significantly elevated levels of prostaglandin E_2 (PGE_2), an important local mediator of mucosal immunoinflammatory responses.

Materials and methods

Materials

N-9 was a gift from OrthoMcNeil Corporation. Rabbit anti-human COX-2 polyclonal antibody was purchased from Abcam Inc (Cambridge, MA, USA). Goat anti-human COX-1 polyclonal antibody was from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Rabbit polyclonal anti-histone antibodies were a gift from Dr. E. Bers (Biological Institute, St Petersburg University, Russia). All secondary antibodies were purchased from Zymed (SanFrancisco, CA, USA). The secondary antibodies used in immunoblotting were horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Ig)G and goat anti-mouse IgG. Those used for immunofluorescence were goat anti-rabbit IgG conjugated with fluorescein isothicyonate (FITC). The polyvinylidene fluoride (PVDF) transfer membrane was Immobilon-P (Millipore Corporation, Bedford, MA, USA). Enhanced luminescence (ECL) immunodetection was performed using Western Lightning Chemiluminescence reagent (PerkinElmer Life and Analytical Sciences, Boston, MA, USA). The inhibitors SB 202190, SB 203580, U 0126, and BAY 11-7082 were purchased from Calbiochem (San Diego, CA, USA).

Cell Culture and Treatment

The vaginal keratinocyte cell line VK2/E6E7 was a gift from Dr. Raina Fichorova (Brigham and Women's Hospital). Cells were maintained in keratinocyte serum-free medium (Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with bovine pituitary extract (50 μg/mL), epidermal growth factor (0.1 ng/mL), penicillin–streptomycin (1%), and $CaCl_2$ (0.4 mM). Cells were grown to ~70–80% confluence. For dose-dependence analysis, cells were grown in culture medium containing N-9 at indicated concentrations for 6 hr. The concentration of N-9 for the time-dependence study was 12.5 μg/mL. In some experiments, Bay 11-7082 (an inhibitor of IKK–IκB kinase), SB 203580 and SB 202190 [inhibitors of p38 mitogen-activated protein kinase (MAPK)], and U 0126 (inhibitor of MEK1/2 kinase) were added to the cells at indicated concentrations 30 min before N-9 application. The inhibitors at the concentrations used in the experiments were not cytotoxic, as evaluated by cytotoxicity assay (data not shown).

Cytotoxicity Assay

Cells grown in 96-well cell culture plates until confluence were incubated for 6 hr with N-9 at twofold

serial concentrations or with N-9 at 12.5 μg/mL for different periods of time. The number of viable cells was estimated using CellTiter 96 AQueous One solution Cell Proliferation assay (Promega, Madison, WI, USA), which is based on assessment of mitochondrial activity.

Preparation of Whole Cell Lysates

Cells grown in 100-mm Petri dishes were washed three times with cold phosphate-buffered saline (PBS), after which they were scraped and mixed well in 0.25 mL of PBS containing 1 mM phenylmethylsulphonyl fluoride (PMSF). At this step, protein concentration was determined using RC DC protein assay kit (BioRad, Hercules, CA, USA). Then, 0.25 mL of 2× Laemmli sample buffer was added, and the samples were boiled for 5 min.

Electrophoresis and Immunoblotting

Cell proteins (20 μg/lane) were separated by 10–15% step gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto PVDF membrane (Immobilon-P). Non-specific binding was blocked for 1 hr in PBS containing 0.1% Tween-20 (PBST) and 5% non-fat dry milk. The membrane was further incubated overnight at 4°C with the indicated antibodies diluted in PBST containing 0.35 M NaCl and 3% milk. Antibody dilutions were as follows: rabbit polyclonal COX-2 – 1:4000, mouse monoclonal COX-1 – 1:500, rabbit polyclonal histones –1:2000. Secondary antibodies were HRP-conjugated goat anti-rabbit IgG (1:6000) and goat anti-mouse IgG (1:8000). Immunodetection was conducted using an ECL reagent. Immunoblots were visualized using Kodak image station 440 CF.

Measurement of PGE_2 Production

VK2 cells were grown on 12-well plates and treated with N-9 as indicated for each experiment. PGE_2 levels in the culture medium were measured by competitive immunoassay using Assay Designs' Correlate-EIA Prostaglandin kit.

Immunofluorescence

Cells grown on glass coverslips were fixed in methanol at −20°C for 15 min and washed with PBS three times. Antibodies against COX-2 (1:100) were applied, and the cells were incubated at 37°C for 1 hr. The cells were then rinsed with PBS and incubated with anti-rabbit IgG labeled with FITC diluted 1:100 in PBS for 1 hr at 37°C.

RNA extraction, Microarray, and Quantitative PCR Analysis

Total RNA was isolated from cultured cells using RNeasy mini kit from Qiagen Sciences (MD, USA), according to the manufacturer's instructions.

For microarray analysis, RNA was converted into Cy5- and Cy3 -labeled DNA by reverse transcriptase. Labeled DNA was hybridized to oligonucleotide arrays (Pan human cancer set from Eurofin MWG Operon that were custom printed on epoxy-coated glass slides ~2000 genes in triplicates). The slides were scanned on microarray scanner using Scan Array Express software (PerkinElmer Life and Analytical Sciences, Waltham, MA).

For quantitative polymerase chain reaction (PCR) analysis, RNA (1 μg) was converted to cDNA using RT^2 First strand kit from SABioscience (Frederick, MD, USA), which contains a combination of random primers and oligo dT primers.

PCR amplification was performed on Applied Biosystems 7900 using RT^2 Real-Time™ SYBR Green PCR master mix (PA-012) from SABioscience. The primers for COX-2 were forward 5′-TGGATGCTTCGTTAATTTGTTC-3′ and reverse 5′-ACCCACAGTGCTTGACAC-3′; for GAPDH: forward 5-AGAGCACAAGAGGAAGAGAGAG-3, reverse 5′-GGTTGAGCACAGGGTACTTTATT-3′.

The thermocycler parameters were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Expression of COX-2 mRNA was normalized using GAPDH as an internal reference. Relative gene expression in N-9-treated cells compared to control cells was calculated by dividing the normalized expression in the treated cells by the normalized expression in the control cells.

Results

N-9 is Cytotoxic to Vaginal Cells

To study the molecular basis of the vaginal epithelial response to N-9, we used the well characterized human vaginal epithelial cell line VK2/E6E7. These

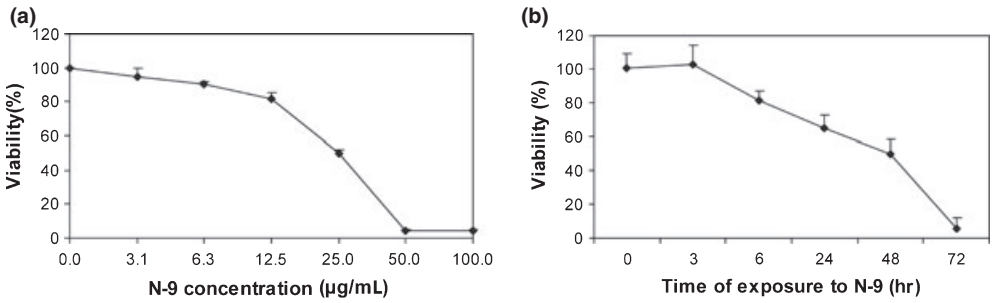

Fig. 1 Dose- and time-dependent nonoxynol-9 (N-9) cytotoxicity. The number of viable cells was estimated using CellTiter 96 AQueous One solution Cell Proliferation assay (Promega, Madison, WI, USA), which is based on assessment of mitochondrial activity. VK2/E6E7 cells were exposed to N-9 at different concentrations for 6 hr (a) or for different time at N-9 concentration of 12.5 μg/mL (b). Data are expressed as means ± SD of three replicates. Results have been confirmed in several independent experiments.

cells are similar in characteristics to the cells of the tissue of origin and have proved to be an adequate model to study vaginal epithelial responses to topical agents.[8,9,13]

N-9-induced cytotoxicity showed a clear dose- and time-dependent response (Fig. 1). For RNA expression analysis, we selected a 6 hr exposure to N-9 at a concentration of 12.5 μg/mL, which induced around 20% cell death, allowing for the evaluation of early gene expression in absence of significant cytotoxicity.

N-9 Induces Upregulation of COX-2 Gene Expression

Changes in the RNA transcription pattern of human vaginal epithelial cells after N-9 treatment (12.5 μg/mL for 6 hr) were analyzed using a DNA microarray technique. With the microarray platform employed in this study, prostaglandin-endoperoxidase synthase 2, or *COX-2*, was the most significantly upregulated gene (19.4 ± 7.6-fold increase). Potent upregulation of COX-2 was confirmed with the Affymetrix® platform (data not shown). Quantification of COX-2 mRNA in VK2 cells using real-time PCR showed an 18-fold (18.0 ± 2.8) increase in COX-2 mRNA in the N-9-treated cells compared to control cells, thus confirming DNA microarray results (Fig. 2a).

Data on COX-2 mRNA induction by N-9 were further corroborated by immunofluorescence, showing a high level of COX-2 protein in N-9-treated cells. COX-2 was localized to the cytoplasm, predominantly in the perinuclear region (Fig. 2b). Control (medium treated) vaginal cells were negative for COX-2.

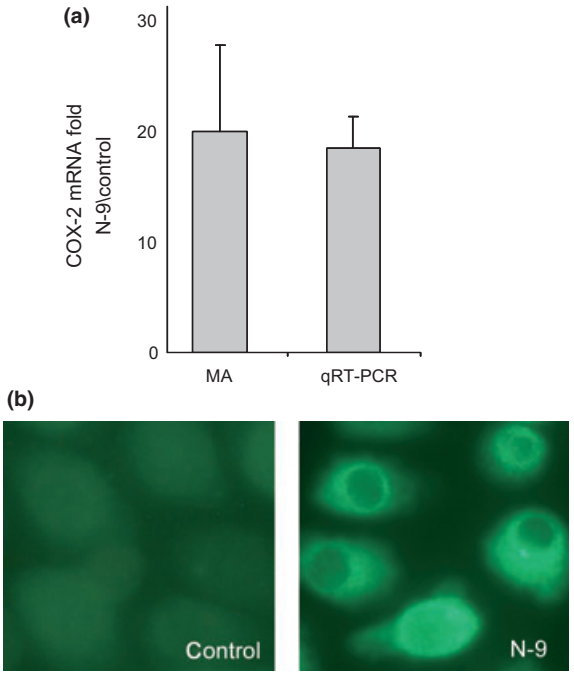

Fig. 2 Cyclooxygenase-2 (COX-2) expression in VK2/E6E7 cells exposed to nonoxynol-9 (N-9) at 12.5 μg/mL for 6 hr. (a) COX-2 mRNA upregulation estimated by DNA microarray technique (MA) and confirmed by real-time quantitative RT-PCR (qRT-PCR). Values represent means ± SD of COX-2 mRNA expression relative to control. Data were pooled from four MA and three qRT-PCR independent experiments, each in triplicate. (b) Immunofluorescence staining for COX-2. VK2/E6E7 cells (control and N-9 treated for 6 hr) were grown on coverslips and fixed with methanol, COX-2 was detected using anti-COX-2 antibodies (Abcam, 1:100).

Preliminary data indicate that COX-2 is also upregulated in cervicovaginal tissue constructs (EpiVag; MatTek Corporation, Ashland, MA, USA) and hysterectomy-derived explants.

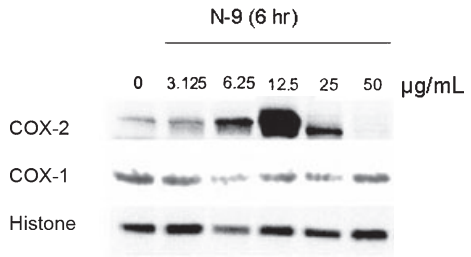

Fig. 3 Induction of cyclooxygenase-2 (COX-2) by nonoxynol-9 (N-9) in VK2/E6E7 cells is dose dependent. Cells were incubated in the presence of N-9 at indicated concentrations for 6 hr. Representative picture of COX-2, COX-1, and histones detected on immunoblots using specific antibodies.

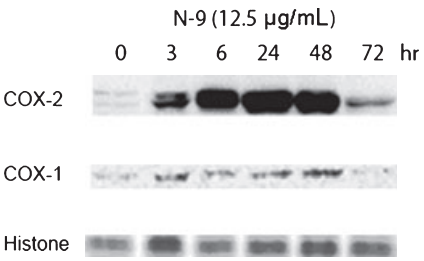

Fig. 4 Induction of cyclooxygenase-2 (COX-2) by nonoxynol-9 (N-9) in VK2/E6E7 cells is time dependent. Cells were incubated in the presence of N-9 (12.5 µg/mL) for indicated time. COX-2, COX-1, and histones were detected on immunoblots using specific antibodies.

N-9 Induces COX-2 Protein Synthesis and PGE_2 Production in a Dose- and Time-Dependent Manner

Induction of COX-2 protein was further studied by immunoblotting incubating VK2 cells with N-9 at different concentrations for 6 hr. N-9 stimulated COX-2 protein synthesis in a dose-dependent manner (Fig. 3). Although COX-2 typically is not constitutively expressed, a very low amount of the protein was found in untreated cells in some experiments. This was most likely due to the presence of EGF in the recommended cell culture medium. EGF has been reported to induce COX-2 synthesis.[14] A slight increase in COX-2 level was seen at 3.125 µg/mL of N-9, when almost no cytotoxicity was observed (Fig. 1a). An increase in COX-2 and slight decrease in cell viability (7–10%) was observed at 6.25 µg/mL. A sharp peak of COX-2 protein synthesis consistently occurred at 12.5 µg/mL, an N-9 concentration that induced 15–20% cell death. This peak was followed by a decrease in COX-2 levels at 25 µg/mL (~50% cytotoxicity). At 50 µg/mL, when practically no viable cells were present, COX-2 was not detected.

COX-2 induction was also tested in human ectocervical (Ect1/E6E7) and endocervical (End1/E6E7) cell lines. In Ect1 cells, COX-2 upregulation followed a dose-dependent response, although overall it was weaker than in VK-2 cells. In End1 cells, COX-2 levels were consistently very low, almost undetectable (Supplementary material, Fig. S1). These findings demonstrate a differential response to N-9 by epithelial cells originated from the different parts of the female lower genital tract.

COX-1 was not induced by N-9. It was seen at a low level in both control and N-9-treated cells.

To evaluate COX-2 protein expression as a function of time of exposure to N-9, VK2 cells were treated with N-9 at 12.5 µg/mL for 3, 6, 24, 48, and 72 hr. COX-2 protein production continuously increased up to 24 hr (Fig. 4), in parallel with an increase in N-9-induced cytotoxicity (Fig. 1b). At 48 hr, when cell viability was ~50%, a slight decrease in COX-2 level was observed. At 72 hr, when only ~5% cells were alive, COX-2 protein level detected by immunoblot was low. COX-1 expression was very low at all times, and at 72 hr the protein was not detectable by immunoblot. Absence/low levels of COX-2 and COX-1 at 72 hr were most likely due to cell death–induced protein degradation. Removal of N-9 after 6 hr of incubation led to a return to baseline levels for COX-2.

COX-2 is an inducible enzyme that catalyzes essential steps in prostanoid synthesis. PGE_2, a major COX-2 product, is considered to be a potent mediator of inflammatory processes. PGE_2 released by VK2 cells was measured in culture medium using a competitive immunoassay (ELISA). Fig. 5 shows that PGE_2 production increased following the elevation of COX-2 expression. Once increased, levels of PGE_2 released into the culture medium remained high throughout the experiment, even when COX-2 expression decreased. This may be because of lack of PGE2 degradation in culture medium.

Cell Signaling Pathways Involved in COX-2 Vaginal Epithelial Induction by N-9

Several intracellular signaling pathways have been reported to mediate COX-2 induction. Pathway activation depends on stimulus and cell type. The human *COX-2* gene promoter contains several

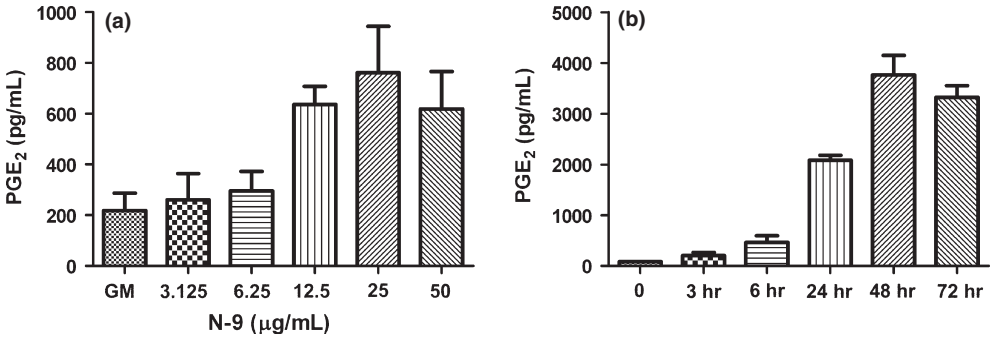

Fig. 5 Effect of nonoxynol-9 (N-9) on PGE_2 production. (a) VK2/E6E7 cells were treated for 6 hr with increasing concentrations of N-9. (b) VK2/E6E7 cells were treated with N-9 at a concentration of 12.5 μg/mL for increasing amounts of time. PGE_2 levels in the culture medium were measured by enzyme competitive immunoassay. Results are expressed as the means ± SD of at least three independent experiments.

sequences that have been shown to be binding sites for transcription factors promoting COX-2 induction. NF-κB (nuclear factor kappa B) has been proved to be of particular importance for COX-2 upregulation in many cell types.[15] BAY 11-7082 prevents NF-κB activation by blocking phosphorylation of the inhibitory protein IκB by IKK (IκB kinase). Pre-treatment of N-9-stimulated VK2 cells with BAY 11-7082 inhibited COX-2 expression, indicating involvement of NF-κB signaling pathway in COX-2 transcription in vaginal epithelial cells (Fig. 6). To explore other signaling pathways mediating N-9-induced COX-2 expression, VK2 cells were pre-treated with SB 202190 and SB 203580, inhibitors of the p38 MAPK pathway, and U0126, inhibitor of the ERK1/2 pathway activator–MEK1/2. SB 202190 and SB 203580 strongly inhibited COX-2 protein expression, while the inhibitory effect of U 0126 was less pronounced (Fig. 6).

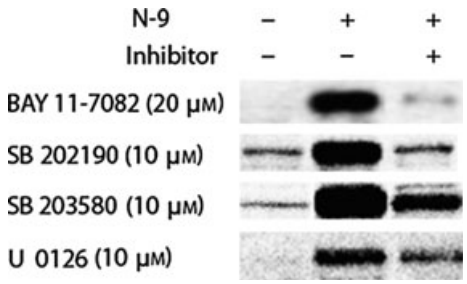

Fig. 6 Effect of signal transduction inhibitors (BAY 11-7082, SB 202190, SB 203580, and U 0126) on nonoxynol-9 (N-9)-induced cyclooxygenase-2 (COX-2) expression. VK2/E6E7 cells were pre-treated with inhibitors at the stated concentrations for 30 min before incubation with 12.5 μg/mL of N-9 for 6 hr. COX-2 was detected by immunoblotting.

Discussion

It has been speculated that the failure of N-9 to prevent HIV transmission in clinical effectiveness trials was because of an induced vaginal inflammatory reaction that caused disruption of the mucosal epithelial barrier and influx of immune HIV-target cells, which counterbalanced the antiviral effect of N-9.[9,10] However, what triggered that reaction remains unknown.

In this study, the effect of N-9 on the vaginal mucosa was analyzed using immortalized human vaginal epithelial cells, which have been shown to maintain morphological and immunocytochemical characteristics of the tissue of origin.[13] We demonstrate for the first time that N-9 induces expression of cyclooxygenase 2 (COX-2) in human vaginal epithelial cells, both at transcriptional and at translational levels. Enhanced expression of the active enzyme was confirmed by increased levels of PGE_2 being secreted into the culture medium, following a pattern linked to the increased expression of COX-2.

Increased COX-2 expression in vaginal cells is not a phenomenon specific to N-9 exposure. We observed a similar transcriptional and translational upregulation of COX-2 when VK2 cells were exposed to other anti-HIV microbicide candidates displaying cytotoxic surface-active properties such as C31G (Supplementary material, Fig. S2). In addition, COX-2 expression by vaginal epithelial cells has also been reported in response to *Trichomonas vaginalis*

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adherence, being postulated as a critical factor in the mucosal inflammatory response to the parasite.[16,17] Treatment of the vaginal cells with the polyanionic microbicide candidates, cellulose sulfate and PRO2000, and also with the main polymer of the 'universal placebo', hydroxyethyl cellulose, did not cause COX-2 upregulation (Supplementary material, Fig. S3), in agreement with the non-inflammatory nature of these compounds.

COX-2, or prostaglandin endoperoxide synthase 2, is one of the most prominent enzymes involved in inflammatory processes.[18,19] COX-2 belongs to a family of cyclooxygenases, which also includes COX-1 and COX-3, the latter being a splice variant of COX-1. Cyclooxygenases catalyze the first committed step of a cascade of reactions by which arachidonic acid is converted into prostanoids: prostaglandins, prostacyclins, and thromboxanes.[20] COX is a rate-limiting enzyme, and therefore, the levels of COX expression are critical determinants of prostanoid concentrations. COX isoforms differ in their pattern of expression. COX-1 is constitutively expressed in most tissues and is thought to be a housekeeping enzyme. COX-2 is most often induced by adverse stimuli related to inflammation or physiological imbalance. Although the original hypothesis describing COX-1 as a 'good' enzyme and COX-2 as a 'bad' one represents an oversimplification of their roles,[21] COX-2 and one of its major products, prostaglandin E_2 (PGE_2), are often found elevated in inflammation and are considered principal players in such process.[22,23]

Our results show that N-9 induces a dose- and time-dependent upregulation of COX-2 level and activity in VK2 cells. COX-2 expression in vaginal cells reached its peak level after a 6-hr incubation with 12.5 μg/mL of N-9 when cytotoxicity was relatively low (~20%). Lower concentrations of N-9 or shorter exposures also produced significantly elevated levels of COX-2. Based on these data, it can be suggested that COX-2 synthesis is an early reaction of vaginal cells to N-9, which precedes an apparent cytotoxic effect and sets off subsequent cellular processes leading to an immunoinflammatory response. It is, therefore, possible that COX-2 may be upregulated in vaginal cells as a mechanism of defense against the membrane disruptive effects of N-9.

The expression of COX- 2, however, is also regulated by a broad spectrum of pro-inflammatory stimuli triggering several cell signaling pathways.[24,25] Which pathway is turned on depends on the stimulus and cell type involved. NF-κB pathway has been repeatedly shown to play a central role in inflammation. The promoter region of *COX-2* contains two putative NF-κB binding sites, and the importance of NF-κB activation in *COX-2* upregulation has been reported in many studies.[15] We found that N-9-stimulated COX-2 expression in VK2 cells was almost completely abolished when the cells were pre-treated with an NF-κB activation inhibitor. N-9-induced activation of NF-κB in cervicovaginal cells *in vivo* and *in vitro* has been reported earlier and postulated to have relevance in HIV-1 mucosal transmission.[9,26]

The existence of a crosstalk between various transcription factors and signaling pathways modulating *COX-2* expression has been previously described.[27] Preliminary data presented in this manuscript show that in addition to NF-κB, COX-2 induction by N-9 in VK2 cells is also mediated through p38 MAPK and MEK1/2 pathways. Furthermore, adherence of *Trichomonas vaginalis* to vaginal epithelial cells also appears to induce COX-2 via activation of p38 MAPK.[16]

The COX-2 product, PGE_2, is involved in many physiological and pathological processes. PGE_2 is a local mediator, which acts in an autocrine and paracrine fashion through four subtypes of G protein-coupled receptors (EPs 1–4) that are linked to different signaling pathways including those involved in inflammation and immune regulation.[22] PGE_2 is essential in the early inflammatory response. Along with other mediators that are substantially potentiated by PGE_2, it induces vasodilation and increases vascular permeability. This initiates migration of immune cells to inflammation sites, which is followed by a chain of events resulting in edema, erythema, and other signs of the acute inflammation including local epithelial lesions.[28] Infiltrating immune cells being recruited to these sites, especially those that are activated and bear CD4 and CCR5 receptors, serve as targets for HIV-1 initial mucosal infection. Disruption of epithelial integrity or a simple reduction in the number of epithelial cell layers as a result of the inflammatory process may further facilitate HIV tissue penetration and infection of target cells.[29]

PGE_2 is intimately involved in inflammation and immune modulation,[22,30,31] processes that enhance HIV infection. Direct connections between PGE_2 levels and HIV-1 infection have been established in several studies.[32–34] We suggest that increased PGE_2 production because of COX-2 upregulation in vaginal cells exposed to N-9 may have been a biological factor contributing to the increased rate of HIV-1

transmission observed in women who used N-9 frequently in the COL-1492 trial. N-9-induced upregulation of COX-2 by epithelial cells would produce additional amounts of PGE2, triggering vasodilation and enhancing leukocyte trafficking into the mucosal areas that are prime targets for HIV-1 infection. In addition, PGE2 stimulates LTR-driven gene expression and HIV-1 replication is enhanced by the LTR promoter. There is evidence that vaginal epithelial secretions of women treated with N-9 stimulate HIV replication via activation of LTR.[9]

Furthermore, the lack of clinical effectiveness of another candidate microbicide, C31G (SAVVY),[11] might also have been , at least in part, because of the same mechanism. We propose that COX-2 vaginal epithelial expression can serve as a new early pre-clinical biomarker of microbicide cervicovaginal safety. Although N-9, or for that matter, any other potent surfactant, is no longer being pursued as microbicide, we believe that the significance of these findings lies on the identification of COX-2 expression as an early sign of vaginal mucosal distress. Microbicide as well as contraceptive candidates inducing COX-2 should be carefully evaluated for genital mucosal inflammatory reactions.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Figure S1. COX-2 expression in Ect1/E6E7 (A) and End1/E6E7 (B) cells exposed to N-9 at indicated concentrations for 6 hr. COX-2 in VK2/E6E7 cells is shown as a reference. COX-2 was detected using anti-COX-2 antibody. Loading was monitored by β-actin.

Figure S2. C31G induced COX-2 expression in VK2/E6E7 cells. The cells were treated with increasing concentrations of C31G. COX-2 was detected using anti-COX-2 antibody. Loading was monitored by Ponceau S staining of the blot.

Figure S3. COX-2 expression in VK2 cells exposed to microbicide candidates – polyanions PRO2000 and cellulose sulfate (CS) and to placebo, hydroxyethyl cellulose (HEC) applied at a concentration of 1 mg/mL for 6 hr. N-9 is shown as a positive control. Loading was monitored by β-actin.

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