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Original article

Synergistic anticancer effects of combined γ-tocotrienol and celecoxib treatment are associated with suppression in Akt and NFκB signaling

Amit B. Shirode, Paul W. Sylvester *

College of Pharmacy, University of Louisiana, 700, University Avenue, Monroe, LA 71209-0470, United States

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ABSTRACT

The selective cyclooxygenase (COX)-2 inhibitor, celecoxib, and the vitamin E isoform, γ-tocotrienol, both display potent anticancer activity. However, high dose clinical use of selective COX-2 inhibitors has been limited by gastrointestinal and cardiovascular toxicity, whereas limited absorption and transport of γ-tocotrienol by the body has made it difficult to obtain and sustain therapeutic levels in the blood and target tissues. Studies were conducted to characterize the synergistic anticancer antiproliferative effects of combined low dose celecoxib and γ-tocotrienol treatment on mammary tumor cells in culture. The highly malignant mouse +SA mammary epithelial cells were maintained in culture on serum-free defined control or treatment media. Treatment effects on COX-1, COX-2, Akt, NFκB and prostaglandin E_2 (PGE_2) synthesis were assessed following a 3- or 4-day culture period. Treatment with 3–4 μM γ-tocotrienol or 7.5–10 μM celecoxib alone significantly inhibited +SA cell growth in a dose-responsive manner. However, combined treatment with subeffective doses of γ-tocotrienol (0.25 μM) and celecoxib (2.5 μM) resulted in a synergistic antiproliferative effect, as determined by isobologram analysis, and this growth inhibitory effect was associated with a reduction in PGE_2 synthesis, and decrease in COX-2, phospho-Akt (active), and phospho-NFκB (active) levels. These results demonstrate that the synergistic anticancer effects of combined celecoxib and γ-tocotrienol therapy are mediated by COX-2 dependent and independent mechanisms. These findings also suggest that combination therapy with these agents may provide enhanced therapeutic response in breast cancer patients, while avoiding the toxicity associated with high-dose COX-2 inhibitor monotherapy.

1. Introduction

Over the past 30 years, there has been increasing interest in the role of the cyclooxygenase (COX) family of prostaglandin synthases in the growth and progression of various types of cancer [1,2]. Cyclooxygenase-2 (COX-2) is an inducible form of COX that catalyzes the conversion of arachidonic acid to prostaglandins and plays a major role in the inflammatory response [3,4]. It is now clearly established that overexpression of COX-2 plays a major role in nearly all stages of tumor development, growth and progression [4,5]. As such, a great deal of effort has been focused on the development of agents that target and inhibit COX-2 activity for use in cancer chemotherapy. Unfortunately, use of high therapeutic doses of selective COX-2 inhibitors is associated with a variety of gastrointestinal and cardiovascular toxicities and these adverse effects have greatly limited their clinical use in cancer chemoprevention and treatment [6,7].

Vitamin E is a general term representing a family of compounds that is further divided into two subgroups called tocopherols and tocotrienols. Tocopherols are commonly found in high concentrations in a wide variety of foods, whereas tocotrienols are relatively rare and found in appreciable levels only in a few specific vegetable fats, such as palm oil [8]. Although chemically very similar, tocopherols have a saturated, whereas tocotrienols have an unsaturated phytyl chain attached to a chromane ring structure. Previous investigations have clearly established that tocotrienols, but not tocopherols, display potent antiproliferative and apoptotic activity against neoplastic mammary epithelial cells at treatment doses that have little or no effect on normal cell growth and function [9,10]. However, studies have also established that it is very difficult to obtain and/or sustain therapeutic levels of γ-tocotrienol in the blood and target tissues by simple oral administration because absorption and transport mechanisms within the body display significant preference for α-tocopherol [11].

The intracellular mechanism mediating the anticancer effects of γ-tocotrienol has been shown to be associated with the attenuation of EGF receptor dependent mitogenic signaling,

* Corresponding author. Tel.: +318 342 1958; fax: +318 342 1737.
E-mail Address: sylvester@ulm.edu (P.W. Sylvester).

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particularly the phosphatidylinositol 3-kinase (PI3K)/PI3K-dependent kinase (PDK)/Akt, and NFκB signaling pathways [11]. PI3K is a lipid signaling kinase that subsequently activates PDK-1, which then phosphorylates and activates Akt. Activated Akt phosphorylates target various proteins associated with cell proliferation and survival [12]. NFκB signaling is a complex multistep process, and activation of NFκB can be initiated by several different kinases, such as Akt and NFκB-inducing kinase. Activated NFκB translocates to the nucleus, binds to DNA, and initiates gene transcription associated with enhanced cell proliferation and survival [13].

Recent studies have shown that combined treatment of γ-tocotrienol with other anticancer agents, such as statins, resulted in synergistic antiproliferative effects [14]. Therefore, it was hypothesized that combined treatment with low doses of γ-tocotrienol and a COX-2 inhibitor might also result in an enhanced therapeutic response. Studies were conducted to characterize the effects of combined low dose treatment of celecoxib, a selective COX-2 inhibitor, with low doses of γ-tocotrienol on the growth of the highly malignant +SA mouse mammary epithelial cells *in vitro*. Additional studies were conducted to determine the intracellular signaling mechanisms involved in mediating the inhibitory effects of combined low dose γ-tocotrienol and celecoxib treatment on EGF-dependent mitogenesis in these cells.

2. Materials and methods

2.1. Reagents and chemicals

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. Isolated γ-tocotrienol (> 98% pure) was provided by Carotech Bhd. (Ipoh, Malaysia). Celecoxib was a gift from Pfizer Inc. (New York). Antibodies for COX-2, Akt, phospho-Akt (Ser473), phospho-NFκB were purchased from Cell Signaling Technology (Beverly, MA). COX-1 primary antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Goat antirabbit secondary antibody was purchased from PerkinElmer Biosciences (Boston, MA). Mouse anti-actin and peroxidase goat antimouse antibody were purchased from Calbiochem (San Diego, CA). The PGE_2 EIA-Monoclonal assay kit was purchased from Cayman Chemical Co. (Ann Arbor, MI).

2.2. Cell line and culture conditions

Experiments conducted in the present study represent a logical continuation of previous studies that have extensively characterized the antiproliferative and apoptotic effects of γ-tocotrienol in the highly malignant +SA mammary epithelial cell line [15]. The highly malignant +SA mammary epithelial cell line was derived from an adenocarcinoma that developed spontaneously in a BALB/c female mouse [16]. The +SA cell line is characterized as being highly malignant, estrogen-independent, and displays anchorage-independent growth when cultured in soft agarose gels. When +SA cells are injected back into the mammary gland fat pad of syngeneic female mice, they form rapidly growing anaplastic adenocarcinomas that are highly invasive and metastasize to the lung [16]. Cell culture and the experimental procedures used in this present study have been previously described in detail [9]. Briefly, cells were grown and maintained in serum-free Dulbecco's modified Eagle's medium (DMEM)/F12 control media containing 5 mg/ml bovine serum albumin (BSA), 10 μg/ml transferrin, 100 μg/ml soybean trypsin inhibitor, and 100 U/ml penicillin and 100 μg/ml streptomycin, 10 μg/ml insulin, and 10 ng/ml EGF as a mitogen. Cells were maintained at 37.0 °C in a humidified atmosphere of 95.0% air and 5.0% CO_2.

2.3. Measurement of viable cell number

Viable cell number was determined using the 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) colorimetric assay as previously described in detail [9]. Briefly, at the end of the treatment period, media in all treatment groups was removed and replaced with fresh control media containing 0.42 mg/mL MTT, and the cells were returned to the incubator for a period of 4 h. At the end of the incubation period, media was removed, the MTT crystals were dissolved in 1 ml of isopropanol, and the optical density of each sample was read at 570 nm on a microplate reader (SpectraCount, Packard BioScience Company, Meriden, CT). Cell number was calculated against a standard curve prepared by plating known concentrations of cells, as determined by the hemocytometer, at the start of each experiment.

2.4. Experimental treatments

For all experiments, an aqueous stock solution of highly lipophilic γ-tocotrienol was prepared as previously described [9]. Briefly, an appropriate amount of γ-tocotrienol was first dissolved in 100 μL of 100% ethanol, then added to a small volume of sterile 10% BSA in water and incubated overnight at 37 °C with continuous shaking. This stock solution was then used to prepare various concentrations of 0–7 μM γ-tocotrienol-supplemented treatment media. A stock solution of celecoxib was prepared by dissolving a known amount in sterile dimethyl sulfoxide (DMSO) at the start of each experiment. This stock solution was then used to prepare various concentrations of 0–20 μM celecoxib-supplemented treatment media. Final concentration of DMSO and/or ethanol was maintained as the same in all treatments groups within a given experiment and never exceeded 0.1%.

2.5. Growth studies

+SA cells were initially plated at a density of 5×10^4 cells/well (6 wells/group) in serum-free defined control media in 24-well culture plates and allowed to adhere overnight. The following day, cells were divided into different treatment groups and media was removed and replaced with fresh control or treatment media, and then returned to the incubator. Cells were treated with celecoxib (0–20 μM) or γ-tocotrienol (0–7 μM) alone and in combination. Cells in their respective treatment groups were fed fresh media every day throughout experimentation.

2.6. Measurement of prostaglandin E_2 (PGE_2) levels

+SA cells were initially plated at a density of 5×10^4 cells/well (six wells/group) in serum-free defined control media in 24-well culture plates and allowed to adhere overnight. The following day, cells were divided into different treatment groups and media was removed and replaced with fresh control or treatment media, and then returned to the incubator for a 72 h culture period. Cells were treated alone or in combination with vehicle, celecoxib (2.5 μM or 20 μM) or γ-tocotrienol (0.25 μM or 3 μM). In these particular experiments, media was not replaced at any time after the start of treatment exposure. At the end of the 72 h treatment period, media was collected and assayed for PGE_2 according to the methods described in the EIA kit provided by the manufacturer (Cayman Chemical Co. Ann Arbor, MI). Optical density was measured at 420 nm on a Synergy-2 Multi Mode Microplate Reader (BioTek Instruments Inc., Winooski, VT).