Vanadate, epidermal growth factor and prostaglandin E_2 production in human amnion cells

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Summary: We have investigated the effects on vanadate (VAN) and mouse epidermal growth factor (mEGF) on prostaglandin (PG) E_2 production in human amnion cells in monolayer culture that served as a model system. The secretion of PGE_2 into the culture medium was quantified by radioimmunoassay. The rate of conversion of [^{14}C] arachidonic acid to [^{14}C] PGE_2 (PGE_2 synthase) was determined in cell sonicates under optimal in vitro conditions. After a treatment period of 4 h we observed an increase in PGE_2 production in the presence of mEGF (4.7-fold), VAN (1.8-fold) or both agents (6.8-fold) over control samples. Similarly, the specific activity of PGE_2 synthase was stimulated maximally after 4 h with mEGF (2.1-fold), VAN (1.7-fold) or with both (4,8-fold) compared with untreated tissue samples. In the presence of cycloheximide those effects were abolished. We suggest, that VAN and mEGF act by a mechanism(s) that involves de novo protein synthesis or that alters the phosphorylation state of enzymes that are requisite for the conversion of arachidonic acid to PGE_2.

Key words: Vanadate; EGF; Human amnion; Prostaglandin E_2.

INTRODUCTION

A number of mitogenic agents have been identified and a great deal of knowledge has accumulated with respect to the mechanism of action of these growth factors, particularly epidermal growth fator (EGF). Moreover, the mitogenic or comitogenic properties of vanadate (VAN) have attracted much attention over the past years. VAN is known to have a wide range of biological activities. Micromolar concentrations of VAN have been found to elicit a mitogenic response in quiescent cultures of human fibroblast ([1]), mouse mammary glands ([2]), and 3T3 and 3T6 cell lines ([3]), and it effects a number of phosphate transfer reactions ([4]). VAN has carcinogenic properties ([5]), and in cultured human and mouse fibroblasts stimulates DNA synthesis and acts synergistically with EGF and insulin ([1, 2]). Although an association between cell growth and prostaglandin (PG) production has been demonstrated for nonmalignant ([6]) and ma-

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lignant ([7]) cells, the role of PGs in mitogenic processes remains unknown.

Previously, we found that VAN stimulated PGE_2 production in human amnion cells in a time- and dose-dependent fashion ([8]). In this study, we evaluated the effect of VAN and mEGF on PG production and PGE_2 synthase specific activity in human amnion cells. For brevity, we use the term "PGE_2 synthase" to denote the combined activities of PG endoperoxide synthase (EC 1.14.99.1) and PG endoperoxide E-isomerase (EC 5.3.99.3.)

MATERIALS AND METHODS

Materials

[1-^{14}C] arachidonic acid (59.6 mCi/mmol), and [1-^{14}C] PGE_2 (58.4 mCi/mmol) were purchased from Amersham, Arlington Heights, IL, USA. Nonradiolabeled PGE_2 was from Cayman Chemical Company, Ann Arbor, MI, USA. Sodium orthovanadate and cycloheximide were purchased from Sigma, St. Louis, MO, USA. Mouse epidermal growth factor (mEGF) was from Collaborative Research Inc., Lexington, MA, USA. Culture media and supplies were obtained from Gibco, Grand Island, NY, USA.

Preparation and maintenance of amnion cells in primary monolayer culture and determination of PGE_2 production.

Human amnion tissues were obtained from normal pregnancies at the time of elective cesarean section conducted before the onset of labor. Amnion cells were dispersed enzymatically ([9]), placed in culture dishes (60 mm diameter), and allowed to replicate to confluence in Ham F12: Dulbecco Minimal Essential Medium (1:1, v/v) that contained heat inactivated fetal calf serum (10%, v/v), penicillin (200 units/ml), streptomycin (100 μg/ml) and gentamycin (200 μg/ml). Confluent cells were incubated with VAN (32 μM), and/or mEGF (15 ng/ml) in the presence or absence of cycloheximide (35 μM). Thereafter, the culture media were collected for quantification of PGE_2 by radioimmunoassay ([10]), and the cells were removed from the dishes for assay of enzyme activities. Previously, we found that VAN at 32 μM and mEGF at 15 ng/ml were maximally effective in stimulating PGE_2 synthesis in human amnion cell cultures after a treatment period of 4 h ([6, 8]).

Assay of the rate of conversion of arachidonic Acid to PGE_2.

The rate of conversion of arachidonic acid to PGE_2, viz., the specific activity of PGE_2 synthase, was determined by use of the methods described previously ([11]) with modifications. Intact amnion cells in monolayer culture were incubated for 4 h with the agents to be tested. The culture media were removed and aliquots were assayed for PGE_2 by radioimmunoassay. The cells were scraped from the dishes and sonicated in potassium phosphate buffer (50 mM, pH 7.4) that contained EDTA (2 mM). The sonicates were centrifuged at 750×g, and the supernatant fraction was used as the enzyme source.

Assays were conducted by incubation of aliquots of the cell preparation with [1-^{14}C] arachidonic acid (10 μM), L-tryptophan (4.2 mM), reduced glutathione (5.1 mM), and hematin (1.75 μM) at 37° C for 10 min. in 1 ml (total volume).

Reactions were terminated by the addition of acetic acid; nonradiolabeled PGE_2 (15 μg) was added and PGE_2 was extracted into ethyl acetate. The solvent was removed by evaporation under nitrogen and radiolabeled arachidonic acid was separated from radiolabeled PGE_2 by silicic acid column chromatography as described ([12]). PGE_2 was purified further by thinlayer chromatography ([13]), and radioactivity was quantified by liquid scintillation spectrometry.

To account for losses during the extraction and purification procedure, [^{14}C] PGE_2 was used as a recovery marker; on average, the recovery ranged from 70 - 80%. All experimental values were corrected for incorporation of radioactivity into PGE_2 in incubations in the absence of cell protein (<1% of total). Assay were conducted with replicates of three tissue specimens analysed in quadruplicate. Protein was quantified by the method of Lowry and associates ([14]), and statistical analyses were performed by use of the unpaired Student t test.

RESULTS

The rate of conversion of [^{14}C] arachidonic acid to [^{14}C] PGE_2 (PGE_2 synthase) was linear with time up to 10 min. and was linear with protein concentrations between 0.08 and 0.9 mg/ml. By Lineweaver-Burk analysis, the apparent Km for arachidonic acid was computed to be 1.6 μM. In all subsequent assays of PGE_2 synthase, the incubation time was 10 min. at 37° C with protein concentrations of

0.4-0.6 mg/ml and arachidonic acid at a concentration of 10 μM.

The effect of VAN (32 μM) and/or mEGF (15 ng/ml) in the absence or presence of cycloheximide (35 μM) on PGE_2 production and PGE_2 synthase specific activity was investigated. Amnion cell cultures were not (CTL) or were treated with these agents for 4 h. The data are presented as the mean ± SEM of three different tissue preparations that were analysed in quadruplicate. The production of PGE_2 was increased significantly in cells that had been treated with mEGF (145 ± 18, $p<0.01$), VAN (53 ± 7, $p<0.05$) or both (204 ± 12 pmol/mg protein/4h, $p<0.001$) compared with cells that had not been treated (Fig. 1). Similarly, the specific activity of PGE_2 synthase was significantly higher in cells that had been maintained in the presence of mEGF (3.7 ± 0.5, $p<0.02$), VAN (3 ± 0.3, $p<0.02$) or with both agents (8.6 ± 2 pmol/min/mg protein, $p<0.01$) compared with cell cultures that did not contain these factors (Fig. 2).

Importantly, the stimulatory effects of mEGF and/or VAN on PGE_2 production and PGE_2 synthase specific activity were almost completely abolished by simultaneous treatment of amnion cells with cyclohexamide at a concentration of 35 μM (Figs. 1, 2). The viability of amnion cells after the incubation period with the agents to be tested was not compromised as judged by trypan blue exclusion, and was > 90%.

DISCUSSION

Despite the fact that an association between cell growth and PG biosynthesis has been established in some cell systems ([6, 7]), little is known about the mechanism of action by which mitogenic agents effect several possible pathways of arachidonic acid metabolism. Free arachidonic acid is the obligate precursor of products that are formed by way of the cyclooxygenase- and lipoxygenase pathway. The majority of investigations have been directed towards, a definition of the mechanisms that serve to effect the release of arachidonic acid from lipid storage forms. However, more recent studies demonstrated that PG production is dependent on protein synthesis ([6, 15]), and it is believed that a continuous resynthesis of PGH_2 synthase is necessary because of the irreversible autoinactivation that occurs during the enzymatic reduction of PGG_2 to PGH_2 ([16]).

Previously, we found that VAN stimulates PGE_2 production in human amnion cells in primary monolayer cultures in a time-and dose-dependent fashion by a mechanism(s) that involves an increase in the rate of conversion of arachidonic acid to PGE_2 ([8]). The possibility exists, therefore, that VAN acts to stimulate the specific activity of PGH_2 synthase (cyclooxygenase/peroxidase) or PG endoperoxide-E isomerase, or both. In this investigation we present evidence that mEGF has a similar stimulatory potency as VAN with respect to a stimulation of PGE_2 production and PGE_2 synthase specific activity. The effects of both agents were at least additive.

This is supportive of the proposition that there are more than one distinct mechanisms operative. Evidence has been provided by others that VAN stimulates synergistically the mitogenic actions of mitogens in quiescent human fibroblasts and is most effective with EGF ([1]). Moreover, VAN acts as a co-mitogen in insulin-stimulated mouse mammary glands ([2]), and exhibits insulin-like actions in rat adipocytes ([17]). The precise mechanisms of action of VAN are not well understood, partly because of the complex chemistry of VAN in aqueous solution and because of the number of metabolic pathways that are effected ([4]). Our findings that the stimulatory effect of mEGF and/or VAN on

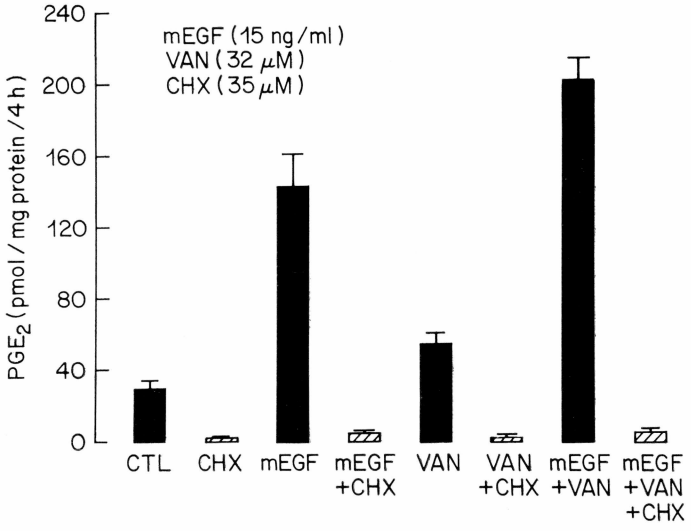

Fig. 1. — The accumulation of prostaglandin (PG) E_2 in the culture medium of cells that were not (CTL) or that were treated with mouse epidermal growth factor (mEGF) and/or vanadate (VAN) in the absence or presence of cyclohexamide (CHX) for 4 h. These is a statistically significant increase with mEGF ($p < 0.01$), VAN ($p < 0.05$), or both ($p < 0.001$) compared with untreated tissues. CHX almost completely abolished these effects. The data are presented as the mean ± SEM of three different tissues analyzed in quadruplicate.

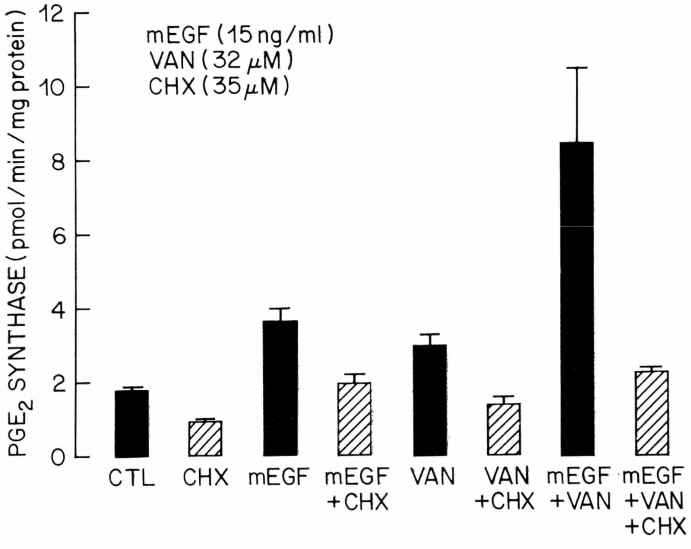

Fig. 2. — The specific activity of PGE_2 synthase. A statistically significant increase is effected in the presence of mEGF ($p < 0.02$), VAN ($p < 0.02$), or with both agents ($p < 0.01$) compared with CTL. The addition of CHX prevented these effects (for abbreviations and conditions see legend of fig. 1).

PGE_2 production and PGE_2 synthase specific activity was attenuated by simultaneous treatment of cell cultures with cyclohexamide is supportive of the view that de novo protein synthesis is required. On the other hand VAN may prevent rapid protein degradation as has been demonstrated in isolated rat hepatocytes ([18]), or else, may activate cyclooxygenase/peroxidase and/or PG endoperoxide E-isomerase by covalent modification of the phosphorylation state of these enzymes. Moreover, a novel aspect of the insulin-like effects of VAN has been introduced more recently, i.e., evidence has been provided that the effect of VAN is the result of the esterification of tyrosine residues by VAN, and it has been hypothesized that an enzyme that is activated by phosphorylation is similarly activated by spontaneous esterification with VAN ([19]).

Based on the results of this investigation we propose that the stimulation of PGE_2 production by treatment of amnion cells with VAN is due to an activation by way of phosphorylation-dephosphorylation or else de novo protein synthesis of enzymes that regulate the conversion of arachidonic acid to PGE_2. The interactions between VAN and mEGF on specific cell functions should prove to have important physiological implications. The at least additive effect of both compounds on arachidonic acid metabolism in human amnion cells may be an indication of an common pathway in the mechanism of action of the two factors.

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