# The effect of vanadate of arachidonic acid metabolism in human amnion cells

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*Summary:* This investigation was designed to investigate the effects of vanadate on arachidonic acid metabolism in human amnion cell in primary monolayer culture that served as a model system. The secretion of prostaglandin $E_2$ ($PGE_2$) 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. A maximal stimulation of $PGE_2$ production and $PGE_2$ synthase activity was determined with vanadate at a concentration of 32 μM) was effective maximally after 4 h of treatment, i.e., the production of $PGE_2$ was stimulated 2,3-fold, and the specific activity of $PGE_2$ synthase 2,1-fold compared with control incubations, respectively. We suggest that vanadate acts to increase the rate of conversion of arachidonic acid to $PGE_2$ by a mechanism 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; Human amnion; Prostaglandin $E_2$.

## INTRODUCTION

Vanadate is known to have a wide range of biological activities. In vitro, vanadate inhibits a number of phosphate transfer reactions, e.g., ATPase ([1]), phosphatases ([2, 3]), and kinases ([4]) while adenyl cyclase is activated ([5]). In NRK-1 cells, vanadate inhibited specifically protein-tyrosine phosphate dephosphorylation ([4]), and in A-431 membrane preparations (derived from a human vulvar epidermoid carcinoma) inhibited dephosphorylation of phosphotyrosine histones ([6]). In cell cultures, arachidonic acid metabolism is stimulated by a variety of compounds and some of these agonists act by way of deesterification of cellular phospholipids and/or by modification of the phosphorylation state of proteins ([7]). Evidence has been provided that vanadate stimulates the release of prostaglandin (PG) $E_2$ in rat renal cortical slices and it has been speculated that this was due to an activation of phospholipase $A_2$ ([8]). In addition, evidence has accumulated that is indicative, however, that de novo synthesis of cyclooxygenase is crucial in the regulation of PG production ([9]). Vanadate enhances the stimulatory effects of several agonists on cyclooxygenase products in different cell cutures, but inhibits the lipoxygenase pathway in rat basophil leukemia cells in the presence of the $Ca^{2+}$ ionophor A-23187 ([10]). It has been suggested that, at least in part, deesterification of lipids is

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positively or negatively regulated by phosphorylation reactions.

This study was designed to investigate the effects of vanadate on prostaglandin $E_2$ production in human amnion cells in primary monolayer culture. In addition, we determined the effects of treatment with vanadate on the rate of the conversion of arachidonic acid to $PGE_2$, viz., the combined activities of prostaglandin endoperoxide synthase (cyclooxygenase/peroxidase, E.C. 1. 14. 99. 1.) and prostaglandin endoperoxide E-isomerase (E.C. 5. 3. 99. 3.). Amnion cell cultures are suited ideally for investigations of the regulation of arachidonic acid metabolism since only one PG, viz., $PGE_2$ is biosynthesized, and the $PGE_2$ formed is not metobilized ([11, 12]). For brevity, we use the term "$PGE_2$ synthase" to denote the enzymatic conversion of arachidonic acid to $PGE_2$.

## 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 ($Na_3VO_4$) was purchased from Sigma, St. Louis, MO, 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 tissue was obtained aseptically from normal pregnancies at the time of elective cesarean sections conducted before the onset of labor. Amnion cells were dispersed enzymatically ([11]), 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), fungizone (0.5 μg/ml), kanamycin (200 μg/ml) and gentamycin (200 μg/ml). Confluent cells were incubated with vanadate modification of $PGE_2$ by radioimmunoassay ([13]) and the cells were collected for assay of enzyme activities.

### *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 method described previously ([14]) with modifications. Intact amnion cells in monolayer culture were incubated for various times with vanadate. The culture media were removed and aliquots were assayed for $PGE_2$ by radioimmunossay. The cells were scraped from the dishes and sonicated in potassium phosphate (50 mM, pH 7,4) buffer that contained EDTA (2 mM).

The sonicates were centrifuged at 750×g, and the supernatant fraction was used as the enzyme source. Assays were conduced by incubation of aliquots of the cell preparation with [1-$^{14}$C] arachidonic acid (10 μM), L-tryptophan (4.2 mM), reduced glutathion (5.1 mM), and hematin (1.75 μM) at 37° C for 10 min. in 1 ml (total vol.). 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 ([15]).

$PGE_2$ was purified further by thin-layer chromatography ([16]), 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 conducted in the absence of cell protein (<1% of total) or with preparations of cell sonicates that previously were heated at 100° C for 10 min. (<1% of total). Assays were conducted in quadruplicate.

Protein was quantified by the method of Lowry *et al.* with bovine serum albumin as the standard ([17]). Statistical analyses were conducted by use of the Student t test.

## RESULTS

First, we established optimal conditions for the determination of the rate of conversion of arachidonic acid to $PGE_2$. The formation of [$^{14}$C] $PGE_2$ from [$^{14}$] arachidonic acid (10 μM) at 37° C was linear with time up to 10 min. In incubations conducted for 10 min. the reaction was linear with protein concentrations between 0.08 and 0.9 mg/ml. By Lineweaver-

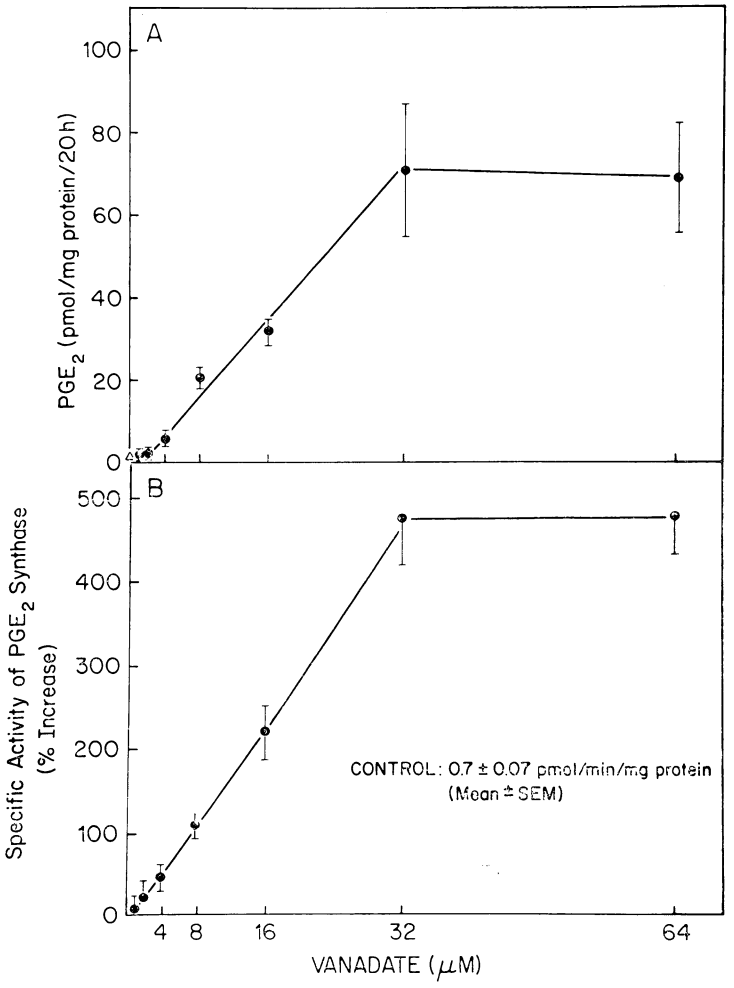


Fig. 1. — The production of $PGE_2$ (A) and the specific activity of $PGE_2$ synthase (B, expressed as percent increase over control cells) as a function of treatment with vanadate for 20 h (mean ± SEM, n = 4). Assays were conducted with preparations of human amnion cells as described in « Material and Methods ».

Burk analysis of the data obtained with [$^{14}$C] arachidonic acid in various concentrations (1-12 μM), the apparent Km was computed to be 1.6μM. In all subsequent assays 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. $PGE_2$ production and $PGE_2$ synthase specific activity were attenuated by simultaneous treatment of amnion cells with cycloheximide (35 μM).

The production of $PGE_2$ over a period of 20 h was determined by radioimmunoassay of $PGE_2$ in the culture medium as a function of the concentration of vanadate. In nontreated cells, $PGE_2$ production (mean ± SEM, n = 4) was 0.93 ± 0.3 pmol/mg protein/20 h and maximal

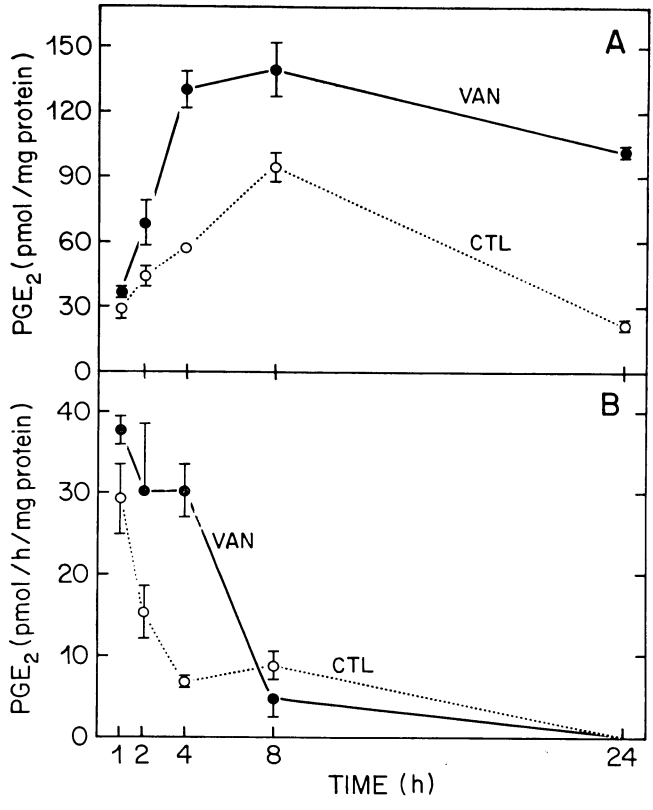


Fig. 2. — A, the accumulation of $PGE_2$ in the culture medium, and, B, the rate of production of $PGE_2$ as determined by radioimmunoassay. Amnion cell cultures were not (CTL) or were treated with vanadate (32 μM) for different times. Assays were conducted as described in « Materials and Methods » and data are expressed as the mean ± SEM (n = 4).

stimulation to 70.8±24 pmol/mg protein/20 h was effected by treatment with vanadate at a concentration of 32 μM, and was maintained at this level with higher concentrations of vanadate (Fig. 1A). Similarly, a maximal increase (5.8 fold) of the specific activity of $PGE_2$ synthase over control cell culturas that had not been treated with vanadate was observed at a concentration of vanadate of 32 μM (Fig. 1B). Treatment of cell cultures with vanadate up to 64 μM did not compromise the viability of the cells as judged by trypan blue exclusion.

As a function of time, the accumulation of $PGE_2$ in the culture medium (mean ±SEM, n=4, as determined by radioimmunoassay) reached a maximum between 4 and 8 h of treatment with vanadate at a concentration of 32 μM (Fig. 2A). The rate of production (mean ± SEM, n=4) of $PGE_2$ of cells that were treated with vanadate was maximal at 1 h, 37.4±1.3 pmol/mg protein/h, and declinel precipitously to 4.6±2 pmol/mg protein/h by 8 h (Fig. 2B). In cell cultures that had not (CTL) or that had been treated with vanadate (32 μM) for various ti-

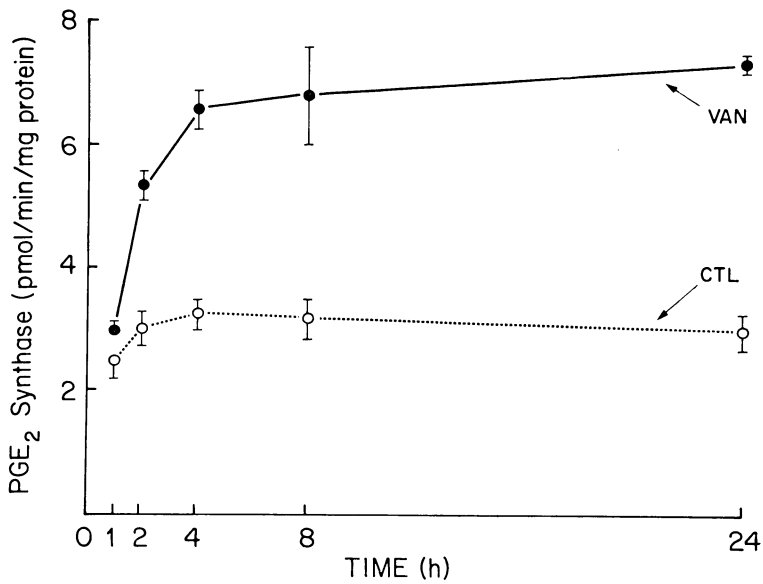


Fig. 3. — The specific activity of $PGE_2$ synthase in amnion cell cultures that were not (CTL) or that were treated with vanadate (32 μM) for various times. $PGE_2$ synthase was determined as described in « Materials and Methods » and the data are expressed as the mean ± SEM (n = 4).

mes, the rate of conversion of arachidonic acid to $PGE_2$, i.e., $PGE_2$ synthase, was increased maximally by 4 h and was maintained up to 24 h (Fig. 3).

## DISCUSSION

Free arachidonic acid is the obligate precursor of products that are formed by way of the cyclooxygenase and lipoxygenase pathway. Heretofore, 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. In addition, it has been demonstrated that prostaglandin production is dependent on protein synthesis ([9]), 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 $PGH_2$ ([18]).

We found that vanadate stimulates $PGE_2$ production in human amnion cells in primary monolayer culture in a time- and dose dependent fashion by a mechanism(s) that involves an increase in the rate of conversion of arachidonic acid to $PGE_2$. The possibility exists, therefore, that vanadate acts to stimulate the specific activity of $PGH_2$ synthase (cyclooxygenase/peroxidase) or PG endoperoxide-E-isomerase, or both. Others have demonstrated that vanadate stimulated the release of $PGE_2$ from rat renal cortical slices, and it has been speculated that this might have been due to an activation of phospholipase $A_2$ ([8]). In various cell lines that had been treated by several agonists it has been shown that vanadate enhances the production of cyclooxygenase products in some, but not all, cell systems, and that vanadate inhibits the synthesis of lipoxygenase metabolites in two leukocyte cell lines when treated with the

$Ca^{2+}$ ionophor A23187 ([10]). It has been hypothesized that at least in some cells deesterification of phospholipids is regulated by phosphate transfer reactions. We cannot rule out the possibility that vanadate stimulates the activities of phospholipase $A_2$ and/or phosphatidylinositol-specific phospholipase C in the human in vivo. Both phospholipases are well characterized in human amnion tissue ([19]) and it has been proposed that they might be crucial for the provision of free arachidonic acid as the substrate for prostaglandin biosynthesis. However, it has been demonstrated that the effect of vanadate on the transformation of NRK-1 cells is not the consequence of a change in phosphatidylinositol metabolism ([4]). In addition, we find repeatedly that the presence of extracellular arachidonic acid, (that is) present in the fetal calf serum, is requisite for the biosynthesis of $PGE_2$ in human amnion cell cultures (unpublished observation). Others have presented evidence that exogenous arachidonic acid is utilized for prostaglandin biosynthesis in various systems ([9, 20, 21]). On the other hand, it may well be that other agents, e.g., $Ca^{2+}$, act to release arachidonic acid from intracellular glycerophospholipids, since phospholipase $A_2$ and phosphatidylinotisol-specific phospholipase C are $Ca^{2+}$ dependent enzymes ([19]).

In this study we found that the rate of production of $PGE_2$ declines over the periods of time while the specific activity of $PGE_2$ synthase remains elevated (Figs. 2, 3). This is suggestive that the availability of free arachidonic acid is rate-limiting. Moreover, treatment of amnion cells with vanadate did not alter the Km of $PGE_2$ synthase for arachidonic acid under optimal in vitro conditions. The stimulatory effect of vanadate on $PGE_2$ production and $PGE_2$ synthase specific activity was attenuated by simultaneous treatment of cell cultures with cycloheximide. This is supportive of the view that de novo protein synthesis is required. Alternatively, vanadate may prevent rapid protein degradation as has been demonstrated in isolated rate hepatocytes ([22]), or else, may activate cyclooxygenase/peroxidase and/or endoperoxide E-isomerase by covalent modification of the phosphorylation state of these enzymes. We propose, that the effect of vanadate on arachidonic acid metabolism in human amnion cells is due to an activation by way of phosphorylation-dephosphorylation, or else, de novo protein synthesis of enzymes that regulate the production of $PGE_2$.

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