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Regulation of polyamine synthesis and transport by retinoic acid and epidermal growth factor in cultured adult rat type II pneumocytes

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During injury of lung epithelial cells, the type II pneumocyte proliferates and differentiates into a type I pneumocyte to restore the epithelium. Polyamines, which constitute a family of small organic polycations, are required for this process of cell repair. Because retinoic acid (RA) and epidermal growth factor (EGF) also are involved, the purpose of this research was to determine their effect on polyamine transport and synthesis in cultured type II pneumocytes. Rat type II pneumocytes were isolated, cultured overnight, and treated with RA and/or EGF for 24 hours. Polyamine transport was determined by [^{3}H]spermidine uptake, and polyamine synthesis was assessed by the activity of the initial rate-limiting enzyme ornithine decarboxylase. EGF (100 ng/mL) significantly increased spermidine transport, but RA did not. At low concentrations of spermidine (2 μM), the combined effect of RA and EGF on spermidine transport was additive. Both EGF (25 ng/mL) and RA (1 μM) increased polyamine synthesis, and cotreatment resulted in an additive effect (a fourfold increase over the control). We also found that ornithine decarboxylase activity is greatly diminished in the presence of tyrphostin B56, which is a specific inhibitor for the tyrosine kinase of the EGF receptor, suggesting that polyamine synthesis within the type II pneumocyte may depend on activation of tyrosine kinase of the EGF receptor. These results indicate that RA and EGF increase the availability of polyamines, which may be important in the lung cell repair process. (J. Nutr. Biochem. 10:518–524, 1999) *© Elsevier Science Inc. 1999. All rights reserved.*

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Introduction

The lungs consist of two primary parenchymal cells: type I and type II pneumocytes. The type I pneumocyte is a squamous epithelial cell that functions in gas exchange. The type II pneumocyte is a cuboidal epithelial cell that has several important functions. It produces the alveolar surfactant, which lowers the surface tension of the water lining the alveoli and enables respiration. It also serves as a progenitor cell for the type I pneumocyte in the event of cell turnover or injury.[1] Type II pneumocytes play a crucial role in the functional and structural integrity of the pulmonary alveolus. During lung injury, type II pneumocytes proliferate and then differentiate into type I pneumocytes to repair the alveolar wall and restore the lung epithelium.[1] The precise mechanisms governing type II cell proliferation and differentiation have yet to be defined.

Vitamin A is known to play an important role in restoration of all types of epithelial tissues. In particular, retinoic acid (RA), which is an active metabolite of vitamin A, is essential in the process of cellular repair in the setting of lung injury.[2] The mechanism responsible for maintaining alveolar integrity stems partly from vitamin A-directed proliferation of type II pneumocytes in response to injury.[2] Massaro and Massaro[3] reported that postnatal treatment with RA increased the number of alveoli in rats by 50% compared with controls. This increase was likely the result of increased proliferation of the type II pneumocytes.[4] However, whether the RA-induced cell proliferation involves polyamines is unknown.

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Polyamines constitute a family of small organic polycations that are necessary for cell proliferation.[5] An increased content of polyamines in the cell precedes cell division, but the mechanism of their action is not known. Three positively charged polyamines are putrescine, spermidine, and spermine. These are thought to orient and stabilize the negatively charged DNA, facilitate replication, and promote synthesis of DNA, RNA, and protein.[5–7] Two general pathways to increase cell polyamine content are de novo polyamine synthesis,[8] which is regulated by the initial rate-limiting enzyme ornithine decarboxylase (ODC), and transmembrane polyamine transport.[9]

In type II pneumocytes, polyamine transport has been characterized extensively,[10–12] but relatively little information is available concerning factors that regulate the activities of the enzymes involved in polyamine synthesis for this particular cell type.[12] It is well established that the significant anticarcinogenic properties of RA are due to its ability to inhibit ODC activity. However, the effects of RA inhibition[13] or stimulation[14] of ODC activity are specific to certain cell types. The effect of RA on polyamine synthesis in type II pneumocytes is not known.

Epidermal growth factor (EGF) is a 6 kDa peptide that is expressed on the cell membrane of type II pneumocytes.[15] The levels of EGF are elevated in lung injury.[16] The precise role of EGF in type II pneumocyte function is not known, but in intestinal epithelial cells, it elevates polyamine levels. Subcutaneous administration of EGF increased ODC activity in mouse duodenum[17] and intraperitoneally administered EGF increased its activity in isolated enterocytes.[18] Because both EGF and RA have altered polyamine levels in other cell types and appear to play important roles in cell injury and repair, we hypothesized that EGF and RA would increase the availability of polyamines by increasing the activity of ODC and/or by enhancing transmembrane transport in the adult rat type II pneumocyte.

Methods and materials

Animal care

Male Sprague-Dawley rats (Sprague-Dawley, Indianapolis, IN, USA) were housed individually in stainless steel cages with a 12-hour light/dark cycle (6:00 AM to 6:00 PM) and allowed ad libitum access to tap water and rat chow. Animal care and use were approved by the Institutional Animal Care and Use Committee of Kansas State University. Animals were cared for in an animal facility approved by the American Association for the Advancement of Laboratory Animal Care.

Isolation of type II pneumocytes

Type II pulmonary epithelial cells were isolated from the lungs of adult male Sprague-Dawley rats weighing between 250 and 350 g by the elastase digestion and immunoglobulin G (IgG) panning method of Dobbs et al.[19] The freshly isolated cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (100 units/mL), streptomycin (10 mg/mL), and 5% fetal bovine serum (FBS) for 24 hours at 37°C and 5% carbon dioxide (CO_2). Viability and purity were ascertained by tannic acid staining and by trypan blue exclusion. Purity and viability were typically approximately 90%. After cells adhered to the culture dish and were washed, the experiments were carried out using serum-free medium. Cell count was determined using an ocular grid.

Spermidine transport

The protocol for transport was slightly modified from previously published methods.[12,20] After a 24-hour incubation (adherence of pneumocytes), cells were washed and exposed to either all-trans RA (Sigma Chemical Co., St. Louis, MO, USA); EGF, (Calbiochem, La Jolla, CA, USA); and/or a combination of the two compounds. The RA was dissolved in 0.1% dimethlysulfoxide (DMSO), which had no significant effect on spermidine transport. Cells were washed with DMEM and acclimated for 30 minutes at 37°C and 4°C in fresh medium prior to addition of [^{3}H]spermidine (Amersham, St. Louis, MO, USA) in concentrations of 0.1 to 20 μM. The experiments were carried out at both 4°C and 37°C to determine nonspecific and total transport, respectively. Specific transport was calculated as the total transport minus the nonspecific transport. After 3 hours, the residual [^{3}H]spermidine in media was aspirated, and cells were washed with cold saline (4°C). The cells were lysed with 2 mL of Scintiverse II (Fisher Scientific, St. Louis, MO, USA), which also was used to harvest the cells. The cocktail was transferred to a counting vial and counted using a Beckman LS 8000 scintillation counter (Beckman Instruments, Inc., Fullerton, CA, USA). Spermidine uptake rate was normalized to cell number and expressed as fmol per minute per million cells.

ODC assay

Activity of ODC enzyme was determined using methods previously described.[12,21] The ODC assay buffer was prepared using 50 mM Tris buffer + 0.1 mM EDTA, 100 mM DTT, 2 mM PLP, 500 μM PMSF, 30% solution Brij 35, and 500 mM NaF. After the initial adherence period of approximately 24 hours at 37°C, cultured cells were exposed to either RA, EGF, or the combination of the two in DMEM for an additional 24 hours. Tyrphostin B56 (Calbiochem), which is a specific inhibitor for EGF receptor tyrosine kinase, was also incubated with the cells to evaluate the role of the EGF receptor tyrosine kinase activity on ODC activity. The treated cells were washed with cold saline (4°C) and harvested with 0.6 mL of ODC assay buffer. The cell suspension was vortexed, the extract was centrifuged at 45,000 *g*, and the resulting supernatant of soluble protein was assayed for ODC activity. Each sample was tested in triplicate. The extract was incubated with 20 μL of [^{14}C]ornithine cocktail (0.25 nCi[^{14}C]ornithine, 5 mM ornithine, distilled water). During the 2-hour incubation at 37°C, the released $^{14}CO_2$ was captured by a filter paper spotted with 2 M NaOH. The filter paper was located in a center well suspended above the enzyme reaction mixture. Released $^{14}CO_2$ was measured after placing the filter paper in 2 mL of Scintiverse II and counted in a Beckman LS 8000 scintillation counter. Enzyme activity was expressed as fmol of CO_2 released per minute per million cells.

Statistical analysis

Data were expressed as means ± SE. Treatment-dependent changes were analyzed by analysis of variance (ANOVA) and Duncan's multiple range test. Differences between two means were determined by Student's *t*-test. Data were considered statistically significant at a *P* value of less than 0.05.

Results

Effects of RA and EGF on spermidine transport

The effects of RA and EGF on specific spermidine uptake were determined using 10^{-6} M RA or 100 ng/mL EGF or the combination of the two with increasing concentrations of spermidine (*Figure 1*). The concentrations for RA and EGF used in this study were based on previous work of

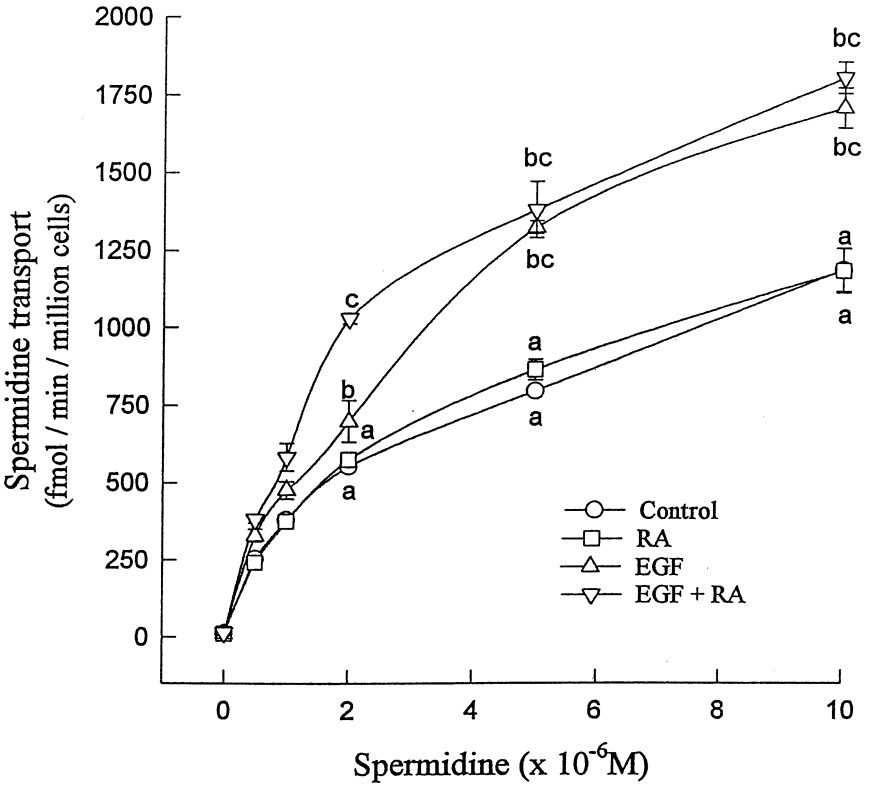

Figure 1 The effects of epidermal growth factor (EGF), retinoic acid (RA), and EGF + RA on specific spermidine transport (uptake) in cultured adult rat type II pneumocytes. Adhered cells were treated for 24 hours with 100 ng/mL EGF, 1 μM RA, or the combination of the two. Cells then were incubated with [^{3}H]spermidine at various concentrations for 3 hours. Values are means ± SE. Each point represents three observations. This figure illustrates one representative experiment of three. Means not sharing a common vertical letter are significantly different at $P < 0.05$.

Thompson and Rosner.[22] RA did not significantly alter spermidine transport. However, EGF significantly increased transport of spermidine relative to the control. When EGF was added in combination with RA, the effects at low concentrations of spermidine (2 μM) were additive. At higher concentrations of spermidine (>5 μM) the results were similar to EGF alone.

Effects of RA and EGF on ODC activity

ODC activity was determined with RA ranging from 10^{-9} M to 10^{-6} M (*Figure 2*). A fourfold increase in ODC activity was noted at 10^{-6} M RA when compared with the vehicle control. The greatest increase in activity was shown between concentrations of 10^{-7} M and 10^{-6} M RA. At low doses of RA within its physiologic range, it had no effect on ODC activity and only significantly increased the activity when the levels of RA approached pharmacologic levels.

EGF also significantly stimulated ODC activity (*Figure 3*). A fourfold increase in ODC activity was observed at the lowest concentration of 25 ng/mL and resulted in an approximately fivefold increase at 100 ng/mL.

The combined effect of RA and EGF on ODC activity also was investigated (*Figure 4*). Type II cells were co-treated with 10^{-6} M RA and 100 ng/mL EGF for 24 hours. As previously observed, treatment of EGF alone resulted in a significant increase in ODC activity, as did treatment of RA alone. The combined effect of EGF and RA on ODC activity was additive, with a fourfold increase in ODC activity compared with the control, suggesting that each compound acts independently of the other.

Effects of tyrphostin on RA-induced ODC activity

To determine a possible mechanism for the action of RA on type II pneumocyte ODC activity, we used tyrphostin B56, which is a specific inhibitor of EGF receptor tyrosine kinase activity,[23] with and without RA. Tyrphostin not only blocked the RA-induced increase in ODC activity, but also decreased the activity of ODC in the vehicle control (*Figure 5*), indicating that the effect of RA may be mediated by an EGF receptor-dependent pathway.

Discussion

The results of the present study demonstrated that: (1) EGF significantly increased spermidine transport, whereas RA did not; (2) both RA and EGF increased the activity of ODC, showing an additive effect when combined; and (3) the RA-induced increase in ODC activity was prevented by an inhibitor of EGF receptor tyrosine kinase activity.

The effects of EGF on polyamine transport have been investigated in other cell types[24] but not in the type II pneumocyte. The EGF-induced increase in spermidine transport observed in the present study appears to play an important role in lung cell repair. In the setting of monocrotaline-induced lung injury, Gillespie et al.[16] reported that exogenous EGF increased lung polyamine content. In bleomycin-injured lungs, expression of the EGF receptor mRNA is increased.[25] Furthermore, an increase in transport of spermidine occurs in the remaining lung after a unilateral pneumonectomy.[26] EGF and its receptor are localized in the region of the alveolar epithelial cells.[27] In cell repair, EGF

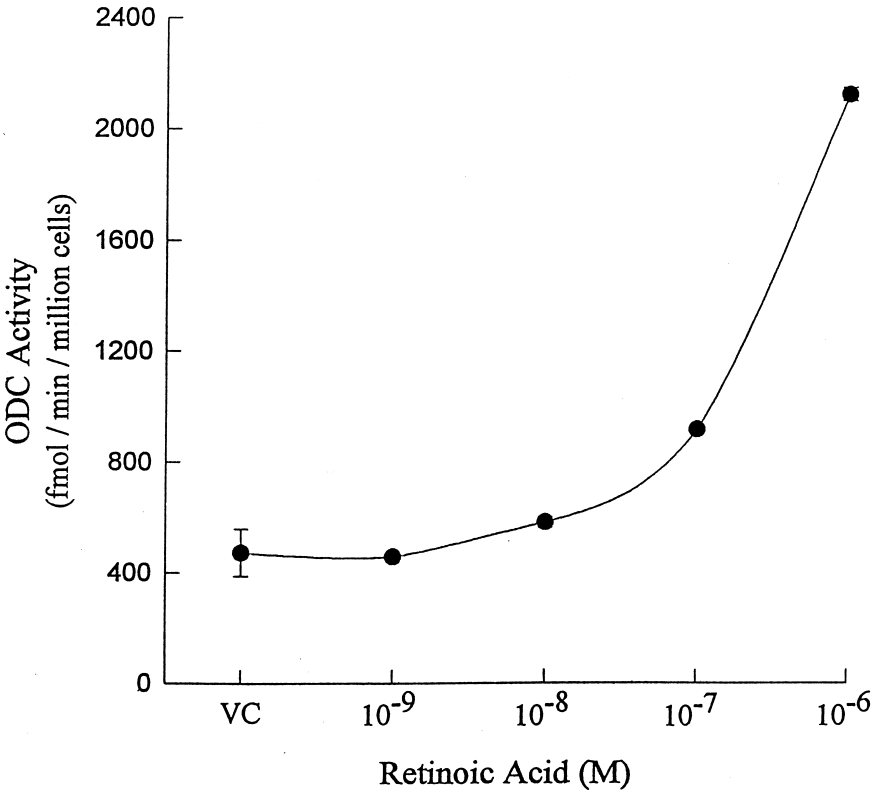

Figure 2 The effect of retinoic acid on activity of ornithine decarboxylase (ODC) in cultured adult rat type II pneumocytes. Adhered cells were treated for 24 hours with the vehicle control or with various concentrations of RA. The cells were harvested, and the soluble protein of the cell suspension was incubated with [^{14}C]ornithine for 2 hours to determine ODC activity. Values are means ± SE of two independent experiments. Three observations were made for each treatment group in an individual experiment. The error bar shown is the only one that extended past the symbol.

increases thymidine incorporation and cell number[28] and stimulates polyamine transport and synthesis, as was observed in the present study. The EGF-induced increase in polyamine availability is consistent with its role in promoting cell proliferation during lung cell repair, and it is the first to be reported here for the type II pneumocyte.

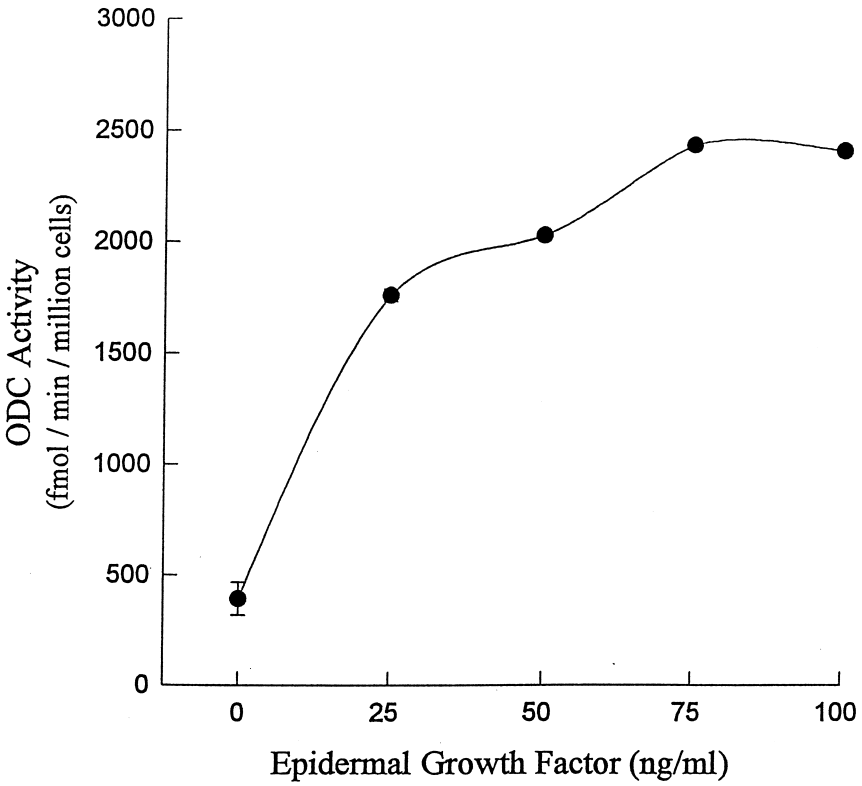

Figure 3 The effect of epidermal growth factor on activity of ornithine decarboxylase (ODC) in cultured adult rat type II pneumocytes. Adhered cells were treated for 24 hours with various concentrations of EGF. The cells were harvested, and the soluble protein of the cell suspension was incubated with [^{14}C]ornithine for 2 hours to determine ODC activity. Values are means ± SE of two independent experiments. Three observations were made for each treatment group in an individual experiment. The error bar shown is the only one that extended past the symbol.

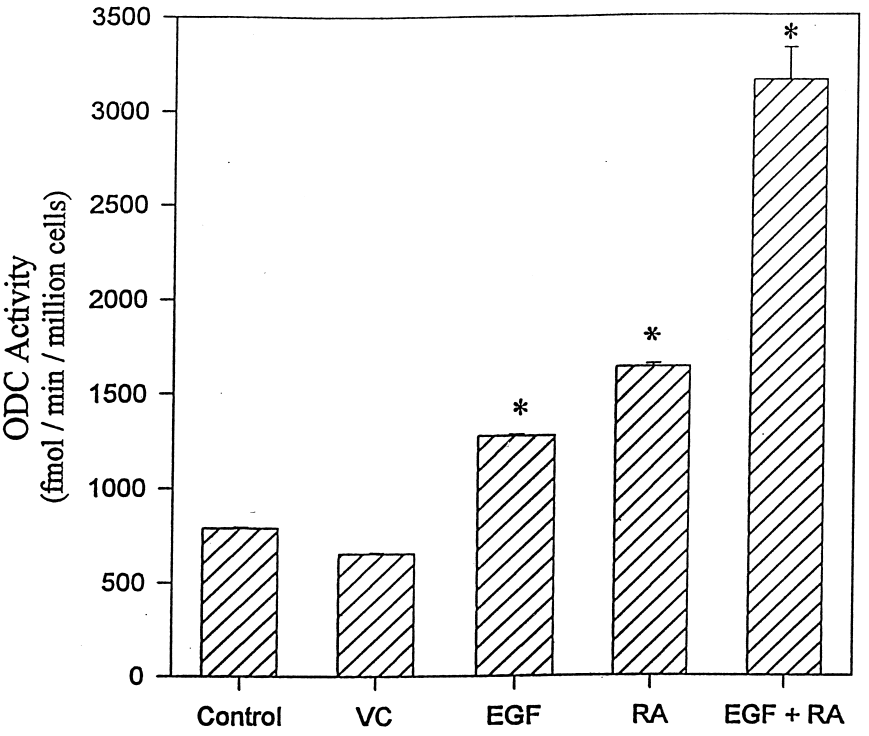

Figure 4 The effects of epidermal growth factor (EGF) and retinoic acid (RA) on activity of ornithine decarboxylase (ODC) in cultured adult rat type II pneumocytes. Adhered cells were treated for 24 hours with 100 ng/mL EGF and/or 1 μM RA or the vehicle control (VC). The cells were harvested, and the soluble protein of the cell suspension was incubated with [^{14}C]ornithine for 2 hours to determine ODC activity. Values are means ± SE of two independent experiments. Three observations were made for each treatment group in an individual experiment. The error bars shown are the only ones that extended past the symbol. *Different from VC, $P < 0.05$.

The transport pathway for spermidine has been characterized in primary cultures of type II pneumocytes.[10] Spermidine is taken up by the cell in a temperature-, sodium-, and concentration-dependent saturable pathway. Uptake of spermidine was inhibited by the other polyamines, putrescene and spermine, indicating a common pathway for transport. Little is known about the effects of RA on polyamine transport, and the results vary depending on cell type. RA significantly inhibited putrescene transport in hepatocytes,[29] but had no effect on spermidine transport by type II pneumocytes in the present study. However, RA enhanced the EGF-induced increase in spermidine transport at low concentrations of spermidine. The enhanced EGF response that resulted from RA treatment may be explained by an RA-induced increase in EGF receptors on the type II pneumocytes, which was observed in our laboratory (data not shown) as well as in others.[30,31]

ODC is an enzyme that has been used to determine the de

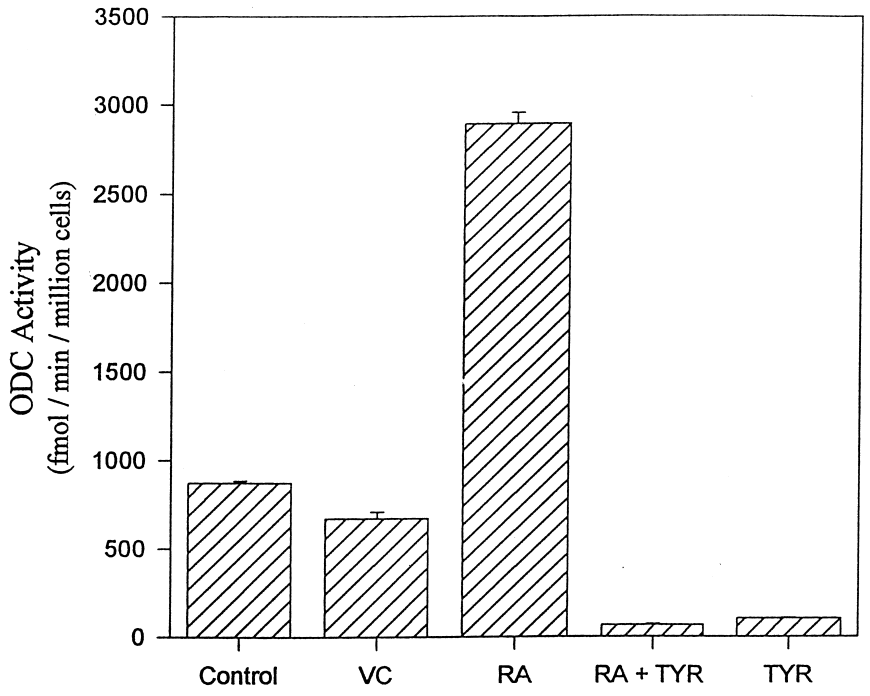

Figure 5 The effects of retinoic acid (RA) and tyrphostin (TYR) on activity of ornithine decarboxylase (ODC) in cultured adult rat type II pneumocytes. Adhered cells were treated for 24 hours with 1 μM RA and/or 30 μM tyrphostin or the vehicle control (VC). The cells were harvested, and the soluble protein of the cell suspension was incubated with [^{14}C]ornithine for 2 hours to determine ODC activity. Values are means ± SE of two independent experiments. Three observations were made for each treatment group in an individual experiment. The error bars shown are the only ones that extended past the symbol.

novo synthesis of polyamines. The effect of EGF on ODC activity has been investigated in many cell types, but not in type II pneumocytes. In both SV40-immortalized human keratinocytes and in A431 epidermoid carcinoma cells, EGF increases ODC activity.[32,33] We also found that EGF increased ODC activity in type II pneumocytes, which is consistent with the growth-promoting response of EGF in other epithelial cells, specifically the enterocytes.[18]

Most of the research involving the effect of RA on ODC activity has focused primarily on the antiproliferative action of RA in transformed cells. Treatment of embryonal carcinoma F9 cells with RA caused a sharp reduction in ODC activity.[34] In human keratinocytes, RA markedly suppressed the synthesis of putrescine, the product of ODC.[35] This RA-induced inhibition of ODC, however, has not always been observed. After an intraperitoneal injection of RA, hepatic ODC activity increased, as did the putrescene and spermidine concentrations.[36] We also found an increase in ODC when cultured type II pneumocytes were incubated with RA. This is the first study to report this effect. The enhanced ODC activity in response to RA may present a mechanism by which RA stimulates cell proliferation in cultured type II pneumocytes[4] and increases the number of alveoli in rats.[3]

It is important to note that, when administered alone, the concentration of RA required to induce polyamine synthesis was in a pharmacologic range. To be of physiologic significance, RA at lower concentrations may require the interaction of other factors, particularly EGF, to enhance polyamine synthesis. In support of this view, we showed that the combination of EGF and RA resulted in additive effects. Furthermore, RA enhanced the proliferative response of EGF at much lower concentrations (10^{-9} M) in other cell types.[37] We did not test varying concentrations of RA combined with EGF, but it is an important avenue of future research.

Results from the present study suggest that RA may enhance the response and effectiveness of EGF. In Swiss mouse 3T3 cells, RA increased both EGF binding and EGF-induced ODC activity.[14] The additive effects of RA and EGF on ODC activity that we observed in the present study may be explained by an RA-induced increase in EGF receptors on the type II pneumocytes,[30,31] as mentioned previously. Whether the RA-enhanced ODC activity is related to the increase in EGF receptors is not known, but this study showed that the activity of ODC may depend on the tyrosine kinase activity of the EGF receptor. The tyrosine kinase inhibitor tyrphostin B56 that we used in our study tends to be specific for the EGF receptor. The specificity of tyrphostins, however, depends on concentration. Future studies will be necessary to establish that tyrphostin B56, at the concentration used in cultured type II pneumocytes in the present study, was specific to the tyrosine kinase of the EGF receptor.

One of the primary functions of the type II pneumocyte is to restore the lung epithelium by replacing type I pneumocytes after cell death or injury. Proliferation of the type II pneumocyte is necessary for this cell repair process, and the proliferation may depend on tyrosine kinase activity of the EGF receptor.[23] The tyrosine kinase dependency was demonstrated by using tyrphostin B56 and genistein, which are inhibitors of the enzyme. The specific transduction signals and/or mediators for the tyrosine kinase-dependent proliferation are not known. In the present study, we found that tyrphostin prevented the RA-induced increase in ODC activity. Because polyamines are necessary for cell proliferation, the mechanism for the tyrosine kinase-dependent proliferation is due partly to the activation of ODC. Furthermore, the dose of tyrphostin used in our study has been shown in other studies to decrease type II pneumocyte thymidine incorporation and inhibit cell proliferation while maintaining cell viability.[23] It is likely that polyamines may serve as one of the mediators in tyrosine kinase-dependent proliferation during the cell repair process. It is also important to note that polyamines also promote cell differentiation,[38] and migration[39] and help prevent apoptosis.[40] Thus, the polyamines may play important roles in various aspects of the type II pneumocyte cell repair process.

Our data suggest that phosphorylation was necessary to activate ODC in the type II pneumocyte. The phosphorylated form of ODC increases stability against intracellular proteolysis and increases catalytic capacity of the phosphorylated enzyme relative to the unphosphorylated form.[41] In jejunal mucosa in vivo, tyrosine kinase activity was correlated highly with ODC activity.[42] In contrast, both the serine and threonine residues of the ODC enzyme are phosphorylated in transformed macrophages.[41] Whether the EGF receptor tyrosine kinase enzyme phosphorylates the tyrosine residue of ODC of the type II pneumocyte presents an intriguing question for future studies.

In summary, EGF increased the transmembrane transport of spermidine in type II pneumocytes. Furthermore, EGF and RA alone or in combination significantly stimulated ODC activity. The present data also suggested that type II pneumocyte ODC activity may depend on tyrosine kinase activity of the EGF receptor. The increased availability of cellular polyamines in response to RA and EGF may be an important step in the type II pneumocyte cell repair process.

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