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Treatment of vascular smooth muscle cells with estradiol and beta-adrenergic agonists has an additive effect on cAMP levels, but no additive effect on inhibition of collagen synthesis

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Abstract

Several studies have suggested that increased cell levels of cAMP result in decreased rates of collagen synthesis. Oestrogen treatment of vascular smooth muscle cells (VSMCs) has been shown to cause increased levels of cAMP and decreased rates of collagen synthesis. Beta-adrenergic agonists are also known to increase cellular levels of cAMP in VSMCs, although the effect of beta-adrenergic agonists on collagen synthetic rates in VSMCs is unknown. Since beta-agonists and oestrogens are commonly used clinical agents these studies were conducted to determine the potential of these agents to have an additive effect on cell cAMP levels and inhibition of collagen synthetic rates. When VSMCs were treated with both oestrogen and isoproterenol there was an additive effect on cellular cAMP levels although the observed decrease in collagen synthetic rates was the same as observed in cells treated with just oestrogen. Treatment of VSMCs with propranolol inhibited isoproterenol-induced changes in cAMP but had no effect on either oestrogen-induced increases in cAMP levels or inhibition of collagen synthesis. The cellular location of cAMP following beta-adrenergic agonist treatment was different from the distribution of cAMP in control or oestrogen-treated VSMCs. This difference in cellular distribution of cAMP may partially explain the absence of collagen synthesis inhibition following beta-adrenergic agonist treatment of VSMCs.

Introduction

Oestrogen replacement therapy has been used for many years to slow the progression of atherosclerosis. Recent epidemiological studies have questioned this use of oestrogen since they have shown that postmenopausal women on oestrogen replacement therapy may actually experience an increased incidence of cardiovascular disease (Farquhar 2002; Lemaitre et al 2002). Prior to these studies it was generally believed that the administration of oestrogen to postmenopausal women decreased the risk of cardiovascular disease (for review see Bittner et al 2000). The mechanism by which oestrogen causes an increase in the complications associated with cardiovascular disease is not known.

A recent review (Rabbani & Topol 1999) suggested that a reduced collagen content in atherosclerotic lesions may result in a thinning and weakening of the fibrous covering of the lesion, which could result in rupture of the lesion resulting in rapid occlusion of the blood vessel due to blood clot formation at the site of lesion rupture. Several in-vivo and in-vitro studies have shown that oestrogen inhibits collagen synthesis in vascular smooth muscle cells (VSMCs), resulting in a decreased accumulation of collagen (Henneman 1968; Wolinsky 1972; Fischer et al 1981). The exact mechanism by which oestrogen treatment reduces collagen synthesis is unknown, although it appears to be related to oestrogen's ability to increase cellular cAMP levels (Dubey et al 2000). cAMP has been recognized as an inhibitor of collagen synthesis for many years (Baum et al 1978). Isoproterenol, a non-specific adrenergic beta-agonist, has been shown to increase cAMP levels and inhibit collagen synthesis in lung fibroblasts (Berg et al 1981) but has not been fully investigated in VSMCs. O'Callaghan &

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Wiliams (2002) concluded that incubation of human VSMCs with norepinephrine (noradrenaline) and the alpha-adrenergic blocker prazosin caused a decrease in VSMC collagen synthesis. They speculated that this inhibition was a result of unopposed beta-agonist stimulation. Stimulation of beta-adrenergic receptors has been shown to increase cAMP levels by activating G_s, which in turn stimulates adenylate cyclase activity. Oestrogen induced increases in cAMP through a different mechanism, which is reported to involve a non-genomic pathway mediated by the interaction of oestrogen with the α- or β-oestrogen receptor (Farhat et al 1996; Dubey et al 2000). The relationship of oestrogen-induced changes in cAMP levels and collagen synthesis by VSMCs in the presence of beta-agonists that would increase cAMP levels is not known. Since inhibition of collagen synthesis may be important to the stability of the vascular wall, these studies were initiated to investigate the potential of isoproterenol to act in an additive manner with oestrogens to promote inhibition of collagen synthesis in VSMCs.

Materials and Methods

Drugs, cell culture reagents, isotopes and other chemicals

17β-Estradiol, isoproterenol, propranolol, terbutaline, pentobarbital, elastase, collagenase and trichloroacetic acid (TCA) were purchased from Sigma Chemical (St Louis, MO, USA). Hanks Balanced Salt Solution, Medium 199, fetal bovine serum, penicillin–streptomycin, kanamycin and amphotericin B were purchased from BRL Gibco Life Technologies (Rockville, MD, USA). [^{14}C]-L-proline was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Isolation of VSMCs

Aortic smooth muscle cells (SMCs) were isolated by elastase and collagenase digestion of the dorsal aorta, from 24-h fasted, adult male Sprague–Dawley rats (Charles River Laboratories), weighing 190–220 g. The aorta, from the aortic arch to the femoral bifurcation, was quickly removed and the adventitia was removed by blunt dissection and placed in pH 7.5 sterile Hanks Balanced Salt Solution (HBSS) containing 2.7 μM amphotericin B, 250 U mL^{-1} penicillin–streptomycin and 170 μM kanamycin. The intima from each aorta was removed and the remaining medias were minced into small pieces. The minced medias were then digested in sterile Medium 199 (M-199), pH 7.5, containing 2 mg mL^{-1} of collagenase and 0.4 mg mL^{-1} of elastase for 3 h in a shaking water bath at 37°C. After digestion, cells were plated in 25-cm flasks and grown in M-199 medium containing 15% fetal bovine serum albumin (FBSA) under 95% O_2–5% CO_2 for 8 days (Thyberg et al 1983). VSMC purity was characterized by immunofluorescence staining with smooth-muscle-specific anti-smooth-muscle α-actin monoclonal antibodies (Signet Laboratories, Dedham, MA) and by morphological criteria specific for smooth muscle (Chamley-Campbell et al 1979). After 8 days, VSMCs were passaged by trypsinization, and cells between passages 8 and 20 were used in all experiments.

In all experiments, smooth muscle cells were plated on 6-well plates in M-199 medium containing 15% FBSA at a density of approximately 10^4 cells per well. After approximately two days in culture, confluent cells covered 70–80% of each well. Each well was then washed twice with M-199 medium, and then incubated with M-199 medium containing the appropriate agents for one hour at 37°C under 95% O_2–5% CO_2. The values obtained from two or three wells were averaged and reported as a single experimental value. In all experiments, an equal number of VSMCs of the same passage was treated with the appropriate volume of vehicle and was used as a control.

Determination of collagen and non-collagen protein synthetic rates

Collagen and non-collagen protein synthetic rates were estimated by the hot TCA extraction method previously described (Langner & Newman 1972). Briefly, SMCs were incubated with M-199 medium containing 2.5 μCi mL^{-1} of [^{14}C]-L-proline for 1 h at 37°C under 95% O_2–5% CO_2. After 1 h the medium was removed and replaced with an equal volume of ice-cold 100 mM EDTA. The cells were loosened, sonicated and a sample was taken for protein analysis. The remaining sonicate was then combined with the incubation medium and sufficient 10% TCA was added to a final 5% concentration to precipitate proteins. Following centrifugation the supernatant was discarded and the pellets were washed six times in cold 5% TCA to ensure removal of free [^{14}C]-L-proline. Collagen was extracted from the resulting pellet by suspending the pellet in 5 mL of 5% TCA, which was then heated for 60 min at 90°C. The samples were then cooled, centrifuged and the supernatant, which contained the solubilized collagen, was collected. A sample was then counted in a liquid scintillation counter and was used as a measure of the collagen synthetic rate. The remaining pellet was dissolved in 5 mL of 0.5 M NaOH and a sample was counted in a liquid scintillation counter and was used to estimate the rate of non-collagen protein synthesis.

Total cyclic AMP levels

After incubation for 1 h in the presence of experimental agent or vehicle, VSMCs were placed on ice and the incubation medium was quickly removed and placed into separate tubes. The cells were then re-suspended in 10 mL of 100 mM EDTA, scraped from the well plate and were then transferred into tubes and sonicated. A portion of each sample was taken for determination of protein concentration. The incubation medium and remaining cell homogenate were then combined, boiled for 7 min to coagulate proteins, centrifuged and the resulting supernatant was assayed for cAMP content using a [^{3}H]-cAMP assay kit (Amersham Pharmacia Biotech, Piscatataway, NJ, USA); samples were counted using a liquid scintillation counter.

Determination of intracellular and extracellular cyclic AMP levels

After incubation for 1 h in the presence of either vehicle only or drug plus vehicle, the incubation medium was collected and assayed for extracellular cAMP. The remaining cells were then re-suspended in 10 mL of 100 mM EDTA, transferred into tubes and sonicated to release the intracellular cAMP. All samples in these experiments were assayed for cAMP content using a cAMP enzyme immunoassay kit (EIA) (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using a Dynex fluorimeter (Dynex Corporation) at an optical density of 450 nm. This assay system was used in these studies since it has a higher sensitivity for cAMP compared with the ^{3}H-cAMP competition assay system used above to estimated total cAMP levels.

Protein assays

Protein concentrations were determined by using either the Bicinchoninic Acid Protein Assay Kit (BSA) (Pierce, Rockville, IL) or the Coomassie Plus Protein Assay Kit (Pierce, Rockford, IL). Bovine serum albumin samples at a range of 25–240 μg mL^{-1} for the BSA assay or 1–25 μg mL^{-1} for the Coomassie assay were used as standards. Similar results were obtained using either assay system.

Statistical analysis

The statistical significance of the data from each study was analysed by use of a one-way analysis of variance to test for the overall treatment effect. If a significant difference was indicated, the data was further analysed using Tukey's procedure. Data that had a P value of < 0.05 was considered to be statistically significant in all experiments.

Results

The ability of oestrogen to stimulate the production of cAMP and inhibit collagen synthesis in VSMCs is shown in Figure 1. When cells were incubated for 1 h with 10^{-6} to 10^{-4} M estradiol, cAMP increased from a control level of approximately 10–40 pmol (mg protein)$^{-1}$. As cAMP levels increased, there was a related decrease in VSMC collagen synthesis (Figure 1). A similar relationship was seen when oestrogen-induced cAMP levels were plotted against changes in non-collagen protein synthesis (data not shown).

Treatment of VSMCs with either isoproterenol or oestrogen for 1 h significantly increased cAMP levels compared with control cells (Figure 2). There were no significant changes in cAMP levels in VSMCs that were treated with only with propranolol. Propranolol, however, significantly reduced cAMP levels in isoproterenol-treated cells but had no significant effect on cAMP levels in oestrogen-treated cells (Figure 2).

Treatment of VSMCs with isoproterenol had no effect on collagen or non-collagen protein synthesis in VSMCs.

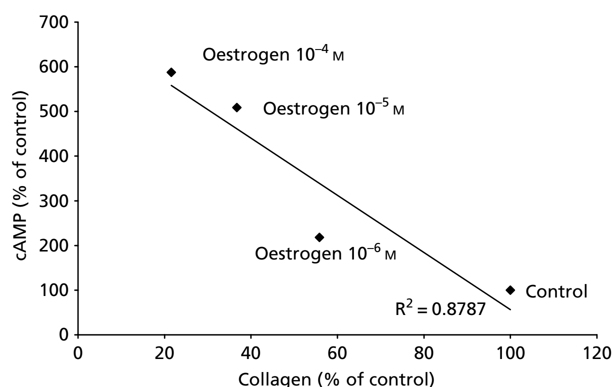

Figure 1 The relationship of estradiol-induced changes in cellular levels of cAMP and collagen synthesis in VSMCs. Each point represents the average of two observations. The relationship between changes in VSMC cAMP levels and rates of collagen synthesis were evaluated by linear regression analysis. An R value of 0.8787 was statistically significant at $P < 0.1$.

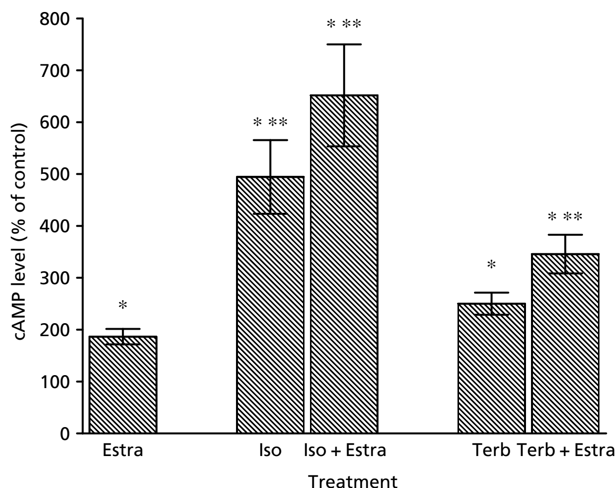

Figure 2 The effect of estradiol 10^{-5} M (Estra), isoproterenol 10^{-6} M (Iso) and propranolol 10^{-6} M (Pro) on cAMP levels in VSMCs. Each value represents the mean of at least four observations; bars represent s.e.m. *$P < 0.05$ compared with control cells; **$P < 0.05$ compared with isoproterenol-treated cells.

This was true whether the cells were incubated with isoproterenol for 1 h (Figure 3) or for 6 h (6-h data not shown) even though cAMP levels were elevated. Propranolol alone, or in combination with either isoproterenol or estradiol, had no effect on collagen or non-collagen protein synthetic rates.

To further evaluate the effects of beta-stimulation on VSMC cAMP levels and collagen and non-collagen protein synthesis, cells were treated with terbutaline (10^{-6} M), a selective β_2-adrenergic agonist. Treatment of cells with terbutaline also significantly increased cAMP levels (Figure 4). When VSMCs were treated with estradiol plus terbutaline or estradiol plus isoproterenol there was an apparent additive effect on cell cAMP production (Figure 4). As

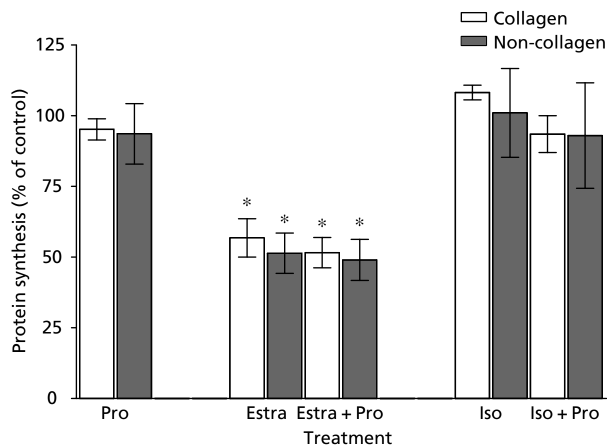

Figure 3 The effect of estradiol 10^{-5} M (Estra), isoproterenol 10^{-6} M (Iso) and propranolol 10^{-6} M (Pro) on collagen and non-collagen synthetic rates in VSMCs. Each value represents the mean of at least four observations; bars represent s.e.m. $*P < 0.05$ compared with control cells.

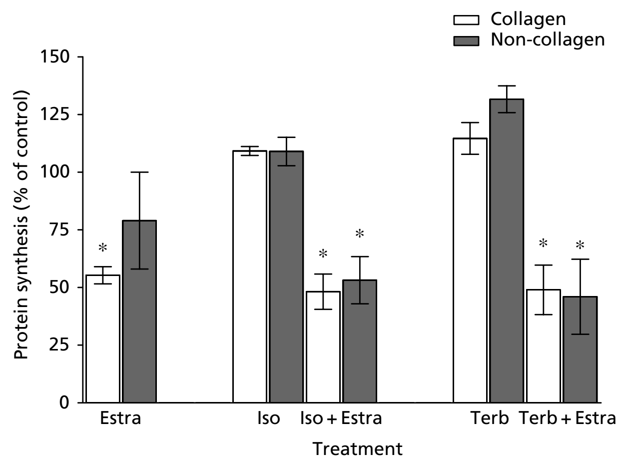

Figure 5 The effect of estradiol 10^{-5} M (Estra), isoproterenol 10^{-6} M (Iso) and terbutaline 10^{-6} M (Terb) on collagen and non-collagen synthetic rates in VSMCs. Each value represents the mean of at least four observations; bars represent s.e.m. $*P < 0.05$ compared with control cells.

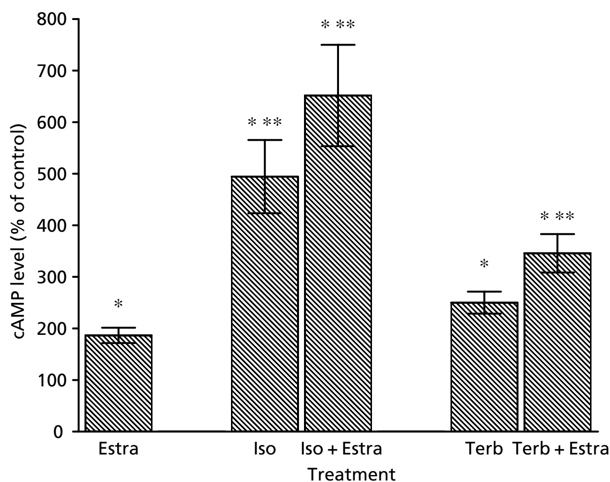

Figure 4 The effect of estradiol 10^{-5} M (Estra), isoproterenol 10^{-6} M (Iso) and terbutaline 10^{-6} M (Terb) on cAMP levels in VSMCs. Each value represents the mean of at least four observations; bars represent s.e.m. $*P < 0.05$ compared with control cells; $**P < 0.05$ compared with estradiol-treated cells.

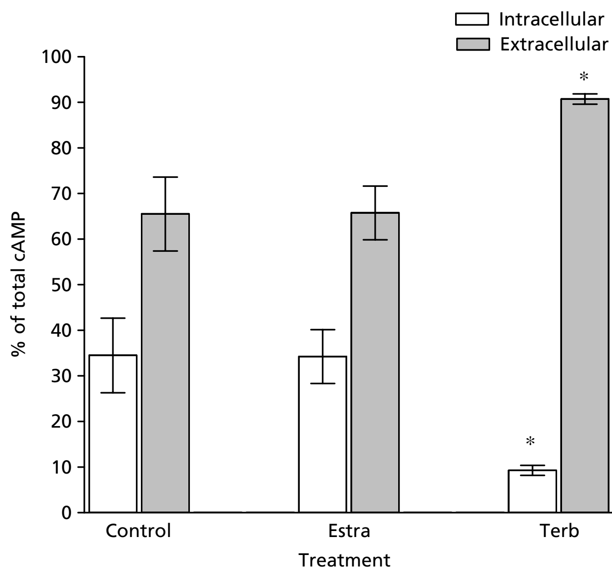

Figure 6 The distribution of intracellular and extracellular cAMP following estradiol 10^{-5} M (Estra) and terbutaline 10^{-6} M (Terb) treatment of VSMCs. Each value represents the mean of eight observations; bars represent s.e.m. $*P < 0.05$ compared with control cells.

previously seen with isoproterenol, incubation of VSMCs with the more selective beta-agonist, terbutaline, had no effect on collagen or non-collagen protein synthesis (Figure 5). When terbutaline was combined with estradiol, the rate of collagen and non-collagen protein synthesis was the same as observed when cells were treated with only estradiol (Figure 5).

The intracellular and extracellular distribution of cAMP following treatment of VSMCs with either vehicle, estradiol or terbutaline, is shown in Figure 6. In cells treated with estradiol, approximately 40% of cAMP is intracellular and 60% is extracellular, which is similar to the distribution of cAMP in control cells. In cells treated with terbutaline, only 10% of cAMP is located within the cell, while 90% of cAMP is located extracellularly.

Discussion

Baum et al (1978), using lung fibroblasts, was the first to report that elevation of cellular cAMP could reduce collagen production by as much as 40%. Several other studies have shown that increasing cAMP levels by adding

exogenous dibutyryl cAMP, stimulating its synthesis with choleral toxin or prostaglandins, decreasing its rate of breakdown by inhibiting phosphodiesterase with dipyridamole or stimulating beta-adrenergic receptors with isoproterenol all have the same effect of increasing cAMP and inhibiting collagen synthesis (Kollros et al 1987; Perr et al 1989). These data suggest that cAMP has a central role in controlling the rate of collagen synthesis in a variety of cell types.

In these studies, when we treated rat VSMCs with increasing concentrations of estradiol there was an apparent dose-related increase in cAMP, which was accompanied by a decreased rate of collagen synthesis (Figure 1). These observations are similar to the results previously reported by Farhat et al (1996) and Dubey et al (2000). The mechanism by which oestrogen-induced increases in cAMP inhibits collagen synthesis is not known. Dubey et al (2000) hypothesized that oestrogen-induced cAMP inhibits collagen synthesis by activating a cAMP-adenosine pathway, which results in the conversion of cAMP into adenosine. The newly formed adenosine then binds the adenosine subtype 2 receptor (A_2) causing inhibition of collagen synthesis. These effects of oestrogen on the cAMP-adenosine pathway were blocked by the oestrogen-receptor antagonist ICI 182780, but not by the protein-synthesis inhibitor cycloheximide, suggesting that the oestrogen's effects were mediated via a non-genomic pathway linked to oestrogen α- or β-receptor.

Treatment of VSMCs with isoproterenol significantly increased cAMP production although, as shown in Figure 3, the increase in cAMP production did not result in the expected decrease in either collagen or non-collagen protein synthesis. The addition of propranolol, a beta-adrenergic antagonist, to isoproterenol-treated VSMCs cells significantly reduced cAMP production in these cells, as expected, since cAMP production by isoproterenol requires activation of adrenergic beta-receptors. Propranolol had no effect on estradiol's ability to increase cAMP or inhibit collagen and non-collagen protein synthesis (Figures 2 and 3), suggesting that estradiol does not increase cell cAMP production or inhibit collagen and non-collagen protein synthesis by interacting with the adrenergic beta-receptor.

Since VSMCs have primarily beta$_2$-adrenergic receptors (Vatner et al 1986), terbutaline, a beta$_2$-adrenergic agonist, was utilized to determine whether a more selective beta-agonist had the same effect on cAMP and collagen and non-collagen synthesis as observed with isoproterenol, a non-selective beta-agonist. As seen in Figures 4 and 5, the treatment of VSMCs with terbutaline also resulted in a significant increase in cAMP but no inhibition of collagen or non-collagen synthesis, as seen with isoproterenol. The increase in cAMP observed with terbutaline was similar in magnitude to that seen with estradiol but much less than the increase seen with isoproterenol, which is most likely related to the dose of terbutaline that was used in these studies.

Treatment of cells with isoproterenol and estradiol or terbutaline and estradiol had an additive effect on cAMP levels (Figure 4) but no additive effect on the inhibition of collagen or non-collagen protein synthesis (Figure 5). The level of inhibition of cellular protein synthesis by either combination treatment resulted in a level of inhibition seen with estradiol treatment alone, suggesting that only the estradiol-induced cAMP was responsible for inhibition of protein synthesis.

The observation that beta-adrenergic-induced elevation of cAMP production did not inhibit collagen or non-collagen protein synthesis in VSMCs differs from the data of Berg et al (1981), who reported that incubation of lung fibroblasts with isoproterenol resulted in an increase in cell cAMP levels and a decrease in collagen synthesis. In their studies, lung fibroblasts were incubated for 6 h compared with the 1-h incubation used in our studies. To determine whether a longer incubation would result in inhibition of collagen synthesis, isoproterenol and VSMCs were incubated for 6 h. The longer incubation period did not result in any significant alteration in collagen or total protein synthetic rates (data not shown). The lack of inhibition of collagen synthesis in VSMCs following a 6-h incubation suggests that a significant difference may exist between VSMCs and fibroblasts relative to the involvement of cAMP in the regulation of collagen synthesis. Other differences in experimental conditions, such as cell passage and density, should also be considered. Numerous studies have shown that activation of the beta-adrenergic receptor can mediate the export of cAMP from the cell (Mills et al 1994; Rosenberg & Li 1995). This is consistent with our observations that in VSMCs treated with terbutaline, 90% of the cAMP was located extracellularly and 10% was intracellular. This distribution of cAMP following beta-stimulation was significantly different from the normal distribution pattern observed in control and estradiol-treated cells (Figure 6).

O'Callaghan & Wiliams (2002) observed a decrease in collagen synthesis in VSMCs after incubating VSMCs in the presence of noradrenaline and prazosin and concluded that the decreased collagen synthesis was a result of unopposed beta-stimulation. It is difficult to compare their results to our data since they treated their cells for four days compared with the 1-h incubation used in these studies and used drugs that did not directly stimulate beta-receptors. Our observation that beta-induced increases in cAMP did not result in decreased collagen synthesis is difficult to explain based upon the existing literature, which strongly supports the hypothesis that increased cAMP levels decreases collagen synthesis. In addition, oestrogen-induced increases in cAMP appeared to be correlated with a decrease in collagen and non-collagen protein synthesis (Figure 1), which is consistent with the existing literature. The observation that beta-stimulation results in a distribution of cAMP that is significantly different from control or estradiol-treated cells suggests that beta-induced cAMP may not be involved with inhibiting collagen and non-collagen synthesis because of its different cellular location. Additional studies to determine the exact mechanisms involved in preventing beta-induced cAMP from modulating VSMC protein synthesis should be conducted.

Conclusion

Collagen is important in maintaining the integrity of the surface of atherosclerotic lesions. Agents that inhibit the synthesis of collagen or stimulate the breakdown of collagen could promote the rupture of atherosclerotic plaques. Since cAMP is believed to inhibit collagen synthesis and since there are several different agents that increase cell cAMP production the potential for interaction between cAMP levels and collagen synthesis may be clinically important and should be determined. In this study, the interaction between estradiol and beta-adrenergic agonists was investigated. The results clearly demonstrate that co-administration of estradiol and a beta-adrenergic agonist can have an additive effect on total cAMP levels in VSMCs but no additive effect on the inhibition of collagen or non-collagen protein synthesis. A possible explanation for this observation is that beta-induced increases in cAMP are transported out of the cell and therefore may not be available to inhibit collagen and non-collagen protein synthesis.

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