

EFFECTS OF ESTRADIOL METABOLITES ON cAMP PRODUCTION AND DEGRADATION

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Abstract: 17 β -Estradiol is metabolized to 2-hydroxyestradiol, and 2-hydroxyestradiol is converted to 2-methoxyestradiol. These steroids reduce proliferation of, migration of and collagen production by vascular smooth muscle cells with an order of potency of 2-methoxyestradiol > 2-hydroxyestradiol > 17 β -estradiol, i.e. the converse of their estrogenic potency. Since cAMP also inhibits cell growth, it is conceivable that these steroids alter cAMP synthesis or degradation, and this hypothesis was addressed in this study. In cultured preglomerular vascular smooth muscle cells pretreated with a high concentration of a broad spectrum phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine, 1 mM) to eliminate phosphodiesterase activity as a confounding variable, high (100 μ M), but not low (10 μ M), concentrations of 17 β -estradiol, 2-hydroxyestradiol and 2-methoxyestradiol attenuated isoproterenol-induced cAMP by 15%, 19% and 55%, respectively. 2-Hydroxyestradiol (100 μ M) also attenuated forskolin-induced cAMP by 31% in cells treated with 3-isobutyl-1-methylxanthine. In cells not pretreated with 3-isobutyl-1-methylxanthine and using concentrations of steroids (10 μ M) that did not interfere with adenylyl cyclase, 17 β -estradiol decreased by 34%, 2-hydroxyestradiol did not alter and 2-methoxyestradiol increased by 60% isoproterenol-induced cAMP. These results indicate that in preglomerular vascular smooth muscle cells, high concentrations of 2-hydroxyestradiol markedly inhibit adenylyl cyclase whereas 17 β -estradiol and 2-methoxyestradiol only modestly inhibit adenylyl cyclase even at high concentrations. Our results also indicate that lower concentrations of 17 β -estradiol, 2-hydroxyestradiol and 2-methoxyestradiol activate, have no effect on and inhibit, respectively, phosphodiesterase activity. The effects of these compounds on phosphodiesterase activity may

account in part for the greater potency of 2-methoxyestradiol as an anti-growth compound compared with 17 β -estradiol and 2-hydroxyestradiol.

Key words: Estradiol, Estradiol metabolites, 2-Methoxyestradiol, Cyclic adenosine monophosphate

Introduction

17 β -Estradiol is the primary estrogen made by the human ovary, and is metabolized in part to 2-hydroxyestradiol by cytochrome P450s [1]. 2-Hydroxyestradiol is then rapidly converted to 2-methoxyestradiol by catechol-O-methyltransferase [2–4]. *In vitro* 17 β -estradiol, 2-hydroxyestradiol and 2-methoxyestradiol inhibit proliferation of, migration of and collagen production by vascular smooth muscle cells [5–7], cardiac fibroblasts [8] and glomerular mesangial cells [9,10] and attenuate endothelin production by endothelial cells *in vitro* [11, 12]. The order of potency for these effects is opposite to the order of potency for binding to and activating estrogen receptors.

Since vascular smooth muscle cells, cardiac fibroblasts, mesangial cells and endothelial cells participate in vascular and renal diseases, the *in vitro* studies suggest that 2-hydroxyestradiol or 2-methoxyestradiol may exert protective effects on the cardiovascular and renal systems. In support of this hypothesis, *in vivo* studies demonstrate that 2-hydroxyestradiol and 2-methoxyestradiol and its synthetic analogue 2-ethoxyestradiol attenuate vascular and renal dysfunction in obese ZSF1 rats [13, 14], an animal model of the metabolic syndrome [15]. Moreover, 2-hydroxyestradiol attenuates renal disease in chronic puromycin aminonucleoside nephropathy [16], and both 2-hydroxyestradiol and 2-methoxyestradiol reduce renal and cardiovascular injury induced by chronic nitric oxide synthase inhibition [17]. 2-Methoxyestradiol, its metabolic precursor 2-hydroxyestradiol and its synthetic analogue 2-ethoxyestradiol also inhibit cardiac remodeling and fibrosis in isoproterenol-induced cardiac hypertrophy model in rats [18], and 2-methoxyestradiol inhibits injury-induced neointimal proliferation and smooth muscle cell growth [19]. Furthermore, 2-methoxyestradiol attenuates the elevated pulmonary artery pressure and inhibits vascular remodeling in small size pulmonary arteries in male and female rats with monocrotaline-induced pulmonary hypertension [18, 20, 21] and 2-methoxyestradiol also attenuates bleomycin-induced pulmonary hypertension and lung fibrosis and vascular remodeling in estrogen deficient female rats [22]. Finally, in intact and ovariectomized female rats with severe occlusive ("human like") pulmonary hypertension, 2-methoxyestradiol, but not estradiol, exhibits preventive and therapeutic effects [23].

The mechanisms by which 2-hydroxyestradiol and 2-methoxyestradiol exert their beneficial effects on the cardiovascular and renal systems are not well defined. Importantly, cAMP, like estradiol metabolites, inhibits vascular smooth muscle cell [24] and cardiac fibroblast [25] proliferation. cAMP and its downstream target protein kinase A block cellular proliferation by inhibition of cyclin-dependent kinase [cdk]4 and extracellular signal-regulated kinase [ERK] and by upregulation of the cdk2 inhibitor p27^{kip1} [26, 27]. Notably, 2-methoxyestradiol exhibits its antiproliferative effects in vascular cells, in part by inhibiting ERK1/2 and up-regulating the p27^{kip1} [1, 19]. Therefore, it is conceivable that the vascular, cardiac and renal protective effects of 2-hydroxyestradiol and 2-methoxyestradiol are mediated in part by cAMP.

The hypothesis that cAMP mediates in part the vasculoprotective and renoprotective effects of 2-hydroxyestradiol and 2-methoxyestradiol presupposes that these metabolites of 17 β -estradiol affect the synthesis or degradation of cAMP. Accordingly, the purpose of this study was to investigate the ability of 2-hydroxyestradiol and 2-methoxyestradiol to affect cAMP production and degradation in intact smooth muscle cells in culture. Since these metabolites are both vasculoprotective and renoprotective, we selected to study the effects of 2-hydroxyestradiol and 2-methoxyestradiol on preglomerular vascular smooth muscle cells (which are both a vascular and a renal cell).

Methods

Cell Culture. All cell culture reagents were obtained from Invitrogen/GibcoBRL. Six 13 to 15 week-old spontaneously hypertensive rats (Taconic Farms, Germantown, NY) were used to acquire the preglomerular smooth muscle cells (PGSMCs) as previously described by us [28]. We selected to use PGSMCs from spontaneously hypertensive rats because our past experience has been that PGSMCs from this strain of rats grow much more quickly and robustly compared to PGSMCs obtained from other rat strains. Briefly, 1% Fe₂O₃ DMEM was forcefully injected into isolated kidneys through the renal artery. The iron-loaded kidney was removed from the rat, the cortex was minced, the microvessels (accurate, interlobular and afferent arteriolar) were obtained using a magnet, and the microvessels were incubated in collagenase type IV (0.6 mg/ml). PGSMVs were obtained by tissue culture and purified using the method of Aviv *et al.* [29]. All experiments were conducted between passage 4 and 10, and the PGSMCs were grown in DMEM/F12 supplemented with 10% FBS and 1x penicillin/streptomycin/amphotericin B.

Protocol I: Effects of 17 β -estradiol, 2-hydroxyestradiol and 2-methoxyestradiol on isoproterenol-induced cAMP in PGSMCs pretreated with 3-isobutyl-1-methylxanthine. Confluent cells were washed twice with 1 mL of phosphate-buffered saline and then incubated at 37°C with 0.5 mL of phosphate-

buffered saline containing 1 mM of 3-isobutyl-1-methylxanthine (IBMX, a broad spectrum phosphodiesterase inhibitor that blocks all families of phosphodiesterases; [30]) with or without 1 μ M of isoproterenol (ISO, a beta-adrenoceptor agonist that activates adenylyl cyclase via stimulatory G proteins). Some cells were treated also with 17 β -estradiol (10 or 100 μ M), 2-hydroxyestradiol (10 or 100 μ M) or 2-methoxyestradiol (10 or 100 μ M). Twenty minutes after adding the appropriate treatments, the medium was aspirated. The cAMP was extracted from the cells with 1 mL of propanol (4°C, overnight). The propanol was collected, centrifuged, and then taken to dryness in a Savant sample concentrator. The cell extracts were reconstituted and then assayed for cAMP concentrations by the method described below.

Protocol II: Effects of 2-hydroxyestradiol on forskolin-induced cAMP in PGSMCs. Confluent cells were washed twice with 1 mL of phosphate-buffered saline and then incubated for 20 minutes at 37°C with 0.5 mL of phosphate-buffered saline containing 1 mM IBMX with or without 1 μ M of forskolin (a direct activator of adenylyl cyclase; [31]). Some cells were treated also with 2-hydroxyestradiol (100 μ M). The cAMP in cell extracts was determined as described in Protocol I.

Protocol III: Effects of 17 β -estradiol, 2-hydroxyestradiol and 2-methoxyestradiol on isoproterenol-induced cAMP in PGSMCs in the absence and presence of 3-isobutyl-1-methylxanthine. Confluent cells were washed twice with 1 mL of phosphate-buffered saline and then incubated for 20 minutes at 37°C with 0.5 mL of phosphate-buffered saline without or with ISO (1 μ M) plus without or with 17 β -estradiol (10 μ M), 2-hydroxyestradiol (10 μ M) or 2-methoxyestradiol (10 μ M). The cAMP in cell extracts was determined as described in Protocol I.

Analysis of cAMP. cAMP was determined as previously described [32]. Briefly, 10 μ L of 1 μ M 9-D-arabinofuranosyladenine (internal standard), 10 μ L of 0.5 M acetate buffer (pH 4.8) and 10 μ L of 50% chloroacetaldehyde in water were added to each sample (200 μ L), and samples were heated for 1 hr at 80°C. Next, samples were centrifuged (14,000 rpm) in a microcentrifuge for 4 minutes, and 80 μ L of each sample was injected into an Isco (Lincoln, NE) high-pressure liquid chromatographic (HPLC) system (pump model 2,350, gradient programmer model 2,360, 4.6 \times 250 mm C₁₈ column, ChemResearch Data Management System), and the effluent was monitored with a Waters model 470 scanning fluorescent detector (wavelengths for excitation and emission were set at 275 nm and 420 nm, respectively). The flow rate was 1.2 ml/min, and the mobile phase was 95.5% citrate-phosphate buffer (8 g/4L citric acid and 9 g/4L K₂HPO₄) and 4.5% acetonitrile. The peak area for cAMP and internal standard were calculated. The concentration of cAMP was quantitated by comparing the ratio of peak areas to a standard curve.

Statistical Analysis. Statistical significance was evaluated by Fisher's Least Significant Difference test. All values in text and figures refer to as means \pm SEM. A value of $p < 0.05$ was considered statistically significant.

Results

Protocol I: Effects of 17 β -estradiol, 2-hydroxyestradiol and 2-methoxyestradiol on isoproterenol-induced cAMP in PGSMCs pretreated with 3-isobutyl-1-methylxanthine. The objective of the first protocol was to determine whether 17 β -estradiol, 2-hydroxyestradiol and 2-methoxyestradiol modify the signal transduction cascade: beta-adrenoreceptor \rightarrow Gs protein \rightarrow adenylyl cyclase. Accordingly, these experiments were performed in the presence of a maximally effective [33] concentration of a broad spectrum phosphodiesterase inhibitor IBMX so as to eliminate any influences of the test agents on phosphodiesterase activity. In this paradigm, ISO caused a 30- to 40-fold increase in cAMP levels (Figures 1–6). In the presence of IBMX, low concentrations (10 μ M) of 17 β -estradiol (Figure 1), 2-hydroxyestradiol (Figure 3) and 2-methoxyestradiol (Figure 5) had little, if any, effect on ISO-induced cAMP. Also, high concentrations (100 μ M) of 17 β -estradiol (Figure 2) and 2-methoxyestradiol (Figure 6) only slightly (15% and 19%, respectively) attenuated ISO-induced cAMP. In marked contrast, high concentrations (100 μ M) of 2-hydroxyestradiol reduced ISO-induced cAMP by 55% (Figure 4). These data indicate that high concentrations of 2-hydroxyestradiol, but not 17 β -estradiol or 2-methoxyestradiol, markedly inhibited either the beta-adrenoreceptor, the Gs-protein or adenylyl cyclase.

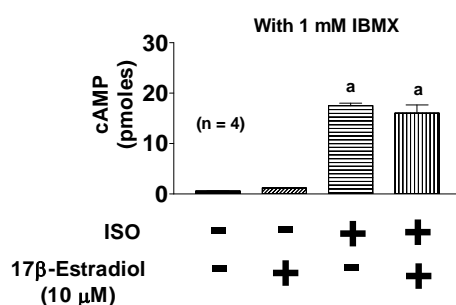


Figure 1 – Effects of low concentrations (10 μ M) of 17 β -estradiol on isoproterenol (ISO) induced cAMP in the presence of IBMX (1 mM). ^a $p < 0.05$ compared with no ISO groups

Слика 1 – Дејство на ниски концентрации (10 μ M) на 17 β -естрадиол врз изопротеренол (ISO) индуцираниот cAMP во присуство на IBMX (1 mM). ^a $p < 0.05$ во споредба со групата без ISO

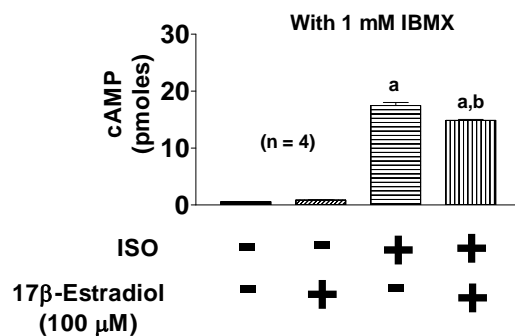


Figure 2 – Effects of high concentrations (100 μM) of 17β-estradiol on isoproterenol (ISO) induced cAMP in the presence of IBMX (1 mM). ^a*p* < 0.05 compared with no ISO groups. ^b*p* < 0.05 compared with ISO in the absence of 17β-estradiol

Слика 2 – Дејство на високи концентрации (100 μM) на 17β-естрадиол врз изопротеренол (ISO) индуцираниот cAMP во присуство на IBMX (1 mM). ^a*p* < 0.05 во споредба со групата без ISO. ^b*p* < 0.05 во споредба со ISO во отсуство на 17β-естрадиол

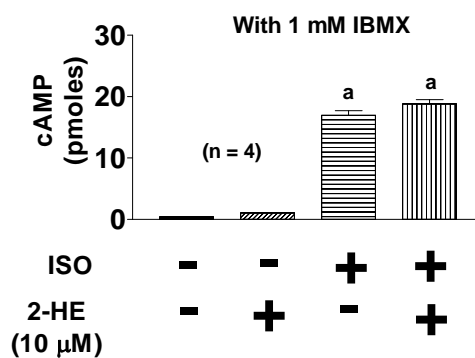


Figure 3 – Effects of low concentrations (10 μM) of 2-hydroxyestradiol (2-HE) on isoproterenol (ISO) induced cAMP in the presence of IBMX (1 mM). ^a*p* < 0.05 compared with no ISO groups

Слика 3 – Дејство на ниски концентрации (10 μM) на 2-хидроксестрадиол (2-HE) врз изопротеренол (ISO) индуцираниот cAMP во присуство на IBMX (1 mM). ^a*p* < 0.05 во споредба со групата без ISO

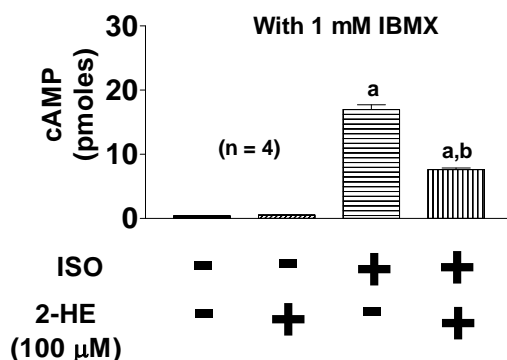


Figure 4 – Effects of high concentrations (100 μ M) of 2-hydroxyestradiol (2-HE) on isoproterenol (ISO) induced cAMP in the presence of IBMX (1 mM). ^a $p < 0.05$ compared with no ISO groups. ^b $p < 0.05$ compared with ISO in the absence of 2-HE

Слика 4 – Дејство на високи концентрации (100 μ M) на 2-хидроксиестрадиол (2-HE) врз изопротеренол (ISO) индуцираниот cAMP во присуство на IBMX (1 mM). ^a $p < 0.05$ во споредба со групата без ISO. ^b $p < 0.05$ во споредба со ISO во отсуство на 2-HE

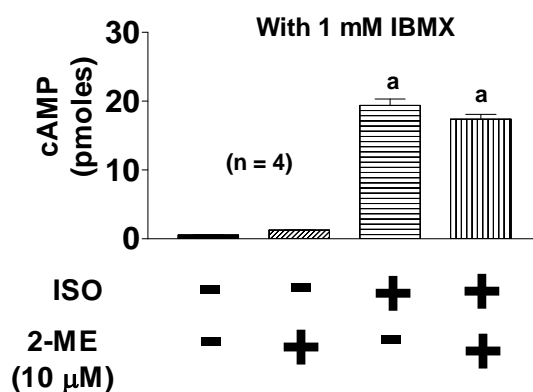


Figure 5 – Effects of low concentrations (10 μ M) of 2-methoxyestradiol (2-ME) on isoproterenol (ISO) induced cAMP in the presence of IBMX (1 mM).

^a $p < 0.05$ compared with no ISO groups

Слика 5 – Дејство на ниски концентрации (10 μ M) на 2-метоксиестрадиол (2-ME) врз изопротеренол (ISO) индуцираниот cAMP во присуство на IBMX (1 mM). ^a $p < 0.05$ во споредба со групата без ISO

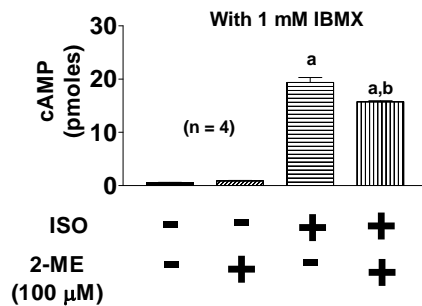


Figure 6 – Effects of high concentrations (100 μM) of 2-methoxyestradiol (2-ME) on isoproterenol (ISO) induced cAMP in the presence of IBMX (1 mM). ^a*p* < 0.05 compared with no ISO groups. ^b*p* < 0.05 compared with ISO in the absence of 2-ME

Слика 6 – Дејство на високи концентрации (100 μM) на 2-метоксиестрадиол (2-ME) врз изопротеренол (ISO) индуцираниот cAMP во присуство на IBMX (1 mM). ^a*p* < 0.05 во споредба со групата без ISO. ^b*p* < 0.05 во споредба со ISO во отсуство на 2-ME

Protocol II: Effects of 2-hydroxyestradiol on forskolin-induced cAMP in PGSMCs. The objective of the second protocol was to determine whether high concentrations of 2-hydroxyestradiol attenuate cAMP production by directly inhibiting adenylyl cyclase. Accordingly, the effects of 100 μM of 2-hydroxyestradiol on forskolin-induced cAMP were examined in PGSMCs treated with a high concentration of IBMX. As shown in Figure 7, a high concentration of 2-hydroxyestradiol reduced forskolin-induced cAMP by 31%, indicating that 2-hydroxyestradiol was a direct inhibitor of adenylyl cyclase as high concentrations.

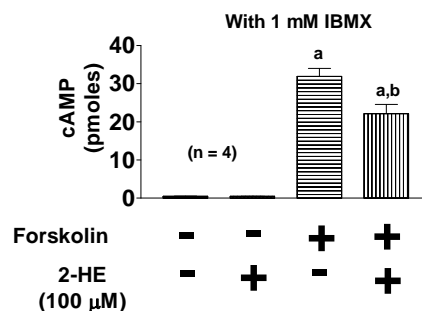


Figure 7 – Effects of high concentrations (100 μM) of 2-hydroxyestradiol (2-HE) on forskolin induced cAMP in the presence of IBMX (1 mM). ^a*p* < 0.05 compared with no forskolin groups. ^b*p* < 0.05 compared with forskolin in the absence of 2-HE

Слика 7 – Дејство на високи концентрации (100 μM) на 2-хидроксиестрадиол (2-HE) врз форсколин индуцираниот cAMP во присуство на IBMX (1 mM). ^a*p* < 0.05 во споредба со групата без форсколин. ^b*p* < 0.05 во споредба со форсколин во отсуство на 2-HE

Protocol III: Effects of 17 β -estradiol, 2-hydroxyestradiol and 2-methoxyestradiol on isoproterenol-induced cAMP in PGSMCs in the absence and presence of 3-isobutyl-1-methylxanthine. The objective of the third protocol was to determine whether 17 β -estradiol, 2-hydroxyestradiol and 2-methoxyestradiol modify phosphodiesterase activity. These experiments were conducted with concentrations of 17 β -estradiol, 2-hydroxyestradiol and 2-methoxyestradiol that did not alter ISO-induced production of cAMP as determined in Protocol I. 17 β -estradiol (Figure 8), 2-hydroxyestradiol (Figure 9) and 2-methoxyestradiol (Figure 10) decreased, did not change and increased, respectively, ISO-induced cAMP. Importantly, the effects of 17 β -estradiol and 2-methoxyestradiol on ISO-induced cAMP were abolished in the presence of IBMX (Figures 1 and 5, respectively). These results indicate that 17 β -estradiol stimulated and 2-methoxyestradiol inhibited phosphodiesterase activity.

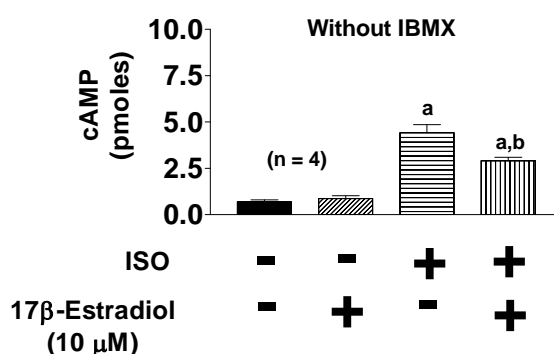


Figure 8 – Effects of low concentrations (10 μ M) of 17 β -estradiol on isoproterenol (ISO) induced cAMP in the absence of IBMX. ^a $p < 0.05$ compared with no ISO groups. ^b $p < 0.05$ compared with ISO in the absence of 17 β -estradiol

Слика 8 – Дејство на ниски концентрации (10 μ M) на 17 β -естрадиол врз изопротеренол (ISO) индуцираниот cAMP во отсуство на IBMX. ^a $p < 0.05$ во споредба со групата без ISO. ^b $p < 0.05$ во споредба со ISO во отсуство на 17 β -естрадиол

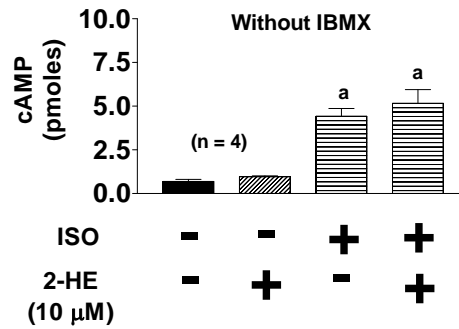


Figure 9 – Effects of low concentrations (10 μM) of 2-hydroxyestradiol (2-HE) on isoproterenol (ISO) induced cAMP in the absence of IBMX.

^a*p* < 0.05 compared with no ISO groups

Слика 9 – Дејство на ниски концентрации (10 μM) на 2-хидроксиестрадиол (2-HE) врз изопротеренол (ISO) индуцираниот cAMP во отсуство на IBMX. (1 mM). ^a*p* < 0.05 во споредба со групата без ISO

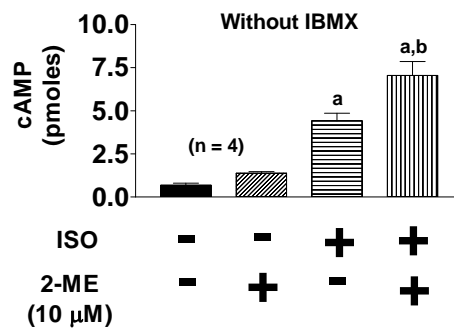


Figure 10 – Effects of low concentrations (10 μM) of 2-methoxyestradiol (2-ME) on isoproterenol (ISO) induced cAMP in the absence of IBMX. ^a*p* < 0.05 compared with no ISO groups. ^b*p* < 0.05 compared with ISO in the absence of 2-ME

Слика 10 – Дејство на ниски концентрации (10 μM) на 2-метоксиестрадиол (2-ME) врз изопротеренол (ISO) индуцираниот cAMP во отсуство на IBMX (1 mM). ^a*p* < 0.05 во споредба со групата без ISO. ^b*p* < 0.05 во споредба со ISO во отсуство на 2-ME

Discussion

The purpose of the first protocol was to determine whether 17β-estradiol, 2-hydroxyestradiol or 2-methoxyestradiol have any effects on the signal

transduction pathway of beta-adrenoceptor → Gs protein → adenylyl cyclase. To address this issue, we chose to stimulate PGSMCs with ISO, a potent beta-adrenoceptor agonist, in the presence of a high concentration of IBMX to maximally block phosphodiesterase. By conducting the experiments in cells incubated with maximally effective concentrations of IBMX, any changes in cAMP levels induced by the steroids could not have been due to alterations in phosphodiesterase activity since this family of enzymes was rendered non-functional by IBMX.

In the present study, 17β-estradiol and 2-methoxyestradiol had no (low concentrations) or little (high concentrations) effect on ISO-induced cAMP in IBMX-treated cells. These results imply that 17β-estradiol and 2-methoxyestradiol have limited ability to alter the signal transduction pathway of beta-adrenoceptor → Gs protein → adenylyl cyclase. In contrast to 17β-estradiol and 2-methoxyestradiol, high concentrations of 2-hydroxyestradiol attenuated ISO-induced AMP levels by 55% in IBMX-treated cells. These data indicate that high concentrations of 2-hydroxyestradiol either inhibit the beta-adrenoreceptor, the Gs protein, or adenylyl cyclase.

The purpose of the second protocol was to determine whether high concentrations of 2-hydroxyestradiol inhibit adenylyl cyclase or interfere with the signal transduction from receptor to adenylyl cyclase. In this regard, we examined the effects of high concentrations of 2-hydroxyestradiol on forskolin-induced cAMP in IBMX-treated cells. Since forskolin directly stimulates adenylyl cyclase without the participation of G-proteins or G-protein coupled receptors [31], agents that act proximal to adenylyl cyclase would not interfere with forskolin-induced cAMP, whereas agents that directly inhibit adenylyl cyclase would attenuate forskolin-induced cAMP. In the current study, high concentrations 2-hydroxyestradiol attenuated forskolin-induced cAMP to approximately the same degree as the effect of ISO was attenuated. These results imply that 2-hydroxyestradiol, unlike 17β-estradiol and 2-methoxyestradiol, can markedly attenuate adenylyl cyclase activity. This conclusion is consistent with the findings of Braun [34] who reported that catechol estrogens, but not 17β-estradiol nor 2-methoxyestradiol, inhibited the soluble form of rat germ cell adenylyl cyclase.

The purpose of the third protocol was to determine whether 17β-estradiol, 2-hydroxyestradiol or 2-methoxyestradiol have any effects on phosphodiesterase activity. To address this issue, we examined the effects of each steroid on ISO-induced cAMP in the presence and absence of IBMX. In this protocol, we employed concentrations of each steroid that were demonstrated in the first protocol not to inhibit the signal transduction of beta-adrenoceptor → Gs protein → adenylyl cyclase. Therefore, any changes in ISO-induced cAMP in the absence of IBMX would most likely be due to alterations in the activity of phosphodiesterase.

In the third protocol, 17β -estradiol decreased ISO-induced cAMP, 2-hydroxyestradiol had no effect on ISO-induced cAMP and 2-methoxyestradiol markedly increased ISO-induced cAMP. These data imply that 17β -estradiol augments phosphodiesterase activity, 2-hydroxyestradiol has no effect on phosphodiesterase activity and 2-methoxyestradiol inhibits phosphodiesterase activity. To confirm that the changes in ISO-induced cAMP caused by 17β -estradiol and 2-methoxyestradiol were indeed mediated by alterations in the activity of phosphodiesterase, we also performed the experiments in the presence of IBMX. As anticipated, in the presence of IBMX, neither 17β -estradiol nor 2-methoxyestradiol affected ISO-induced cAMP. These data support our conclusion that the effects of 17β -estradiol and 2-methoxyestradiol on ISO-induced cAMP in the absence of IBMX were mediated by alterations in phosphodiesterase activity.

In the current study, we used as our experimental model system cultured PGSMCs. The advantage of this approach is that the signal transduction system of beta-adrenoreceptor \rightarrow Gs protein \rightarrow adenylyl cyclase and the endogenous phosphodiesterase system were physiologically intact. Since each component of the cAMP system is regulated by multiple processes [35–37] that would only be reconstituted in intact cells, this approach allowed us to examine the global effects of 17β -estradiol, 2-hydroxyestradiol or 2-methoxyestradiol on the cAMP system. An alternative approach would have been to study each component of cAMP regulation in isolation (for example purified enzymes); however, such an approach does not address the ability of 17β -estradiol, 2-hydroxyestradiol or 2-methoxyestradiol to alter cAMP regulation by signal transduction systems in their native configuration.

There are multiple families of phosphodiesterases [30]. Which phosphodiesterase family is stimulated by 17β -estradiol and inhibited by 2-methoxyestradiol? We previously investigated the role of different phosphodiesterase families in the regulation of endogenous cAMP in cultured PGSMCs [33]. Importantly, our previous results indicate that phosphodiesterase type 4 (PDE4) accounts for practically all the metabolism of cAMP in PGSMCs. The implication of this past study for the current study is that 17β -estradiol and 2-methoxyestradiol activate and block, respectively, PDE4. However, we cannot conclude from the present study that 17β -estradiol and 2-methoxyestradiol affect PDE4 specifically. Whether 17β -estradiol and 2-methoxyestradiol have effects on other phosphodiesterase families would have to be determined by examining the effects of 2-methoxyestradiol on cAMP metabolism in cells expressing other phosphodiesterase isozymes.

What are the physiological implications of the current findings? From a physiological perspective, it is conceivable that catechol-O-methyltransferase plays an important role in determining the effects of endogenously produced 2-hyd-

roxyestradiol on the metabolism of cAMP. In tissues with low catechol-O-methyltransferase activity, 2-hydroxyestradiol would not be effectively converted to 2-methoxyestradiol, and 2-hydroxyestradiol would accumulate, whereas 2-methoxyestradiol levels would be depleted. In such a situation, inhibition of adenylyl cyclase would be favoured over inhibition of phosphodiesterase, and this would diminish cellular levels of cAMP. Conversely, in tissues rich in catechol-O-methyltransferase activity, 2-hydroxyestradiol would be effectively converted to 2-methoxyestradiol, and 2-hydroxyestradiol would be depleted with the concomitant accumulation of 2-methoxyestradiol. In this case, inhibition of phosphodiesterase would be favoured over inhibition of adenylyl cyclase and this would augment cellular levels of cAMP. Our previous studies demonstrate that different tissues have markedly different capacities to convert 2-hydroxyestradiol to 2-methoxyestradiol [2, 4], so this mechanism could allow 2-hydroxyestradiol to regulate cellular cAMP in a tissue-specific manner. An important caveat, however, is that the concentrations of 2-hydroxyestradiol and 2-methoxyestradiol used in the current study were very high and it is unknown whether such concentrations would be achieved *in vivo* in relevant tissue compartments. However, in the present study, a large stimulus to adenylyl cyclase activity (30-to 40-fold increase in cAMP) was employed to provide a large cAMP signal and thus facilitate the HPLC-based analysis. It is conceivable that lesser concentrations of 2-hydroxyestradiol or 2-methoxyestradiol would be required to alter cAMP regulation when the input to the signal transduction system is lower.

What are the pharmacological implications of the current findings? From a pharmacological perspective, the results of the present study have several implications. Currently, there are only a few inhibitors of adenylyl cyclase, and these are mainly P-site inhibitors [38] that have multiple effects. Clinically, there are no useful inhibitors of adenylyl cyclase that are useful for treating diseases. Although 2-hydroxyestradiol is an inhibitor of adenylyl cyclase only at high concentrations, the molecular structure of 2-hydroxyestradiol could be used as a starting-point for development of more potent adenylyl cyclase inhibitors. In this regard, in drug development a weak inhibitor is often discovered first and then optimized by chemical modification to generate a more potent and clinically useful inhibitor. If an analogue of 2-hydroxyestradiol were developed that potently inhibits adenylyl cyclase, this could be useful for treatment of diseases in which adenylyl cyclase activity is too high. For example, inhibitors of adenylyl cyclase could be used to regulate the immune system [39]. Also, too much cAMP in the gastrointestinal tract leads to profound diarrhoea, for example cholera [40].

What is the pharmacological relevance of phosphodiesterase inhibition by 2-methoxyestradiol in the present study? 2-Methoxyestradiol is an anti-mitogenic compound [5–7] which exhibits antiproliferative effects in part by

inhibiting cyclin-dependent kinase and extracellular signal-regulated kinase [ERK] and by up-regulating the cdk2 inhibitor p27^{kip1} [1, 19], effects which at cellular levels are also produced by cAMP [26, 27]. Our recent studies demonstrate that 2-methoxyestradiol is renoprotective in rats with nephropathy induced by chronic inhibition of nitric oxide synthase [16]. Importantly, PDE4 inhibitors are also anti-mitogenic [41], and renoprotective [42, 43]. Likewise, enhanced activity of phosphodiesterase was observed in experimental pulmonary hypertension [44], phosphodiesterase inhibition improves agonist-induced relaxation and hypertensive pulmonary arteries and partially reverses experimental pulmonary hypertension [45, 46], and 2-methoxyestradiol inhibits vascular remodeling of pulmonary arteries and has preventive and therapeutic effects in experimental pulmonary hypertension [19–22]. Thus, it is possible that the PDE4 inhibiting activity of 2-methoxyestradiol importantly contributes to the pharmacological effects of 2-methoxyestradiol.

In summary, 2-hydroxyestradiol inhibits adenylyl cyclase activity, whereas its "downstream" metabolite, 2-methoxyestradiol, inhibits phosphodiesterase activity. Thus, the balance of 2-hydroxyestradiol versus 2-methoxyestradiol, a ratio determined by tissue catechol-O-methyltransferase activity, may determine whether 2-hydroxyestradiol decreases or increases tissue levels of cAMP. Finally, our results suggest new avenues for developing specific inhibitors of adenylyl cyclase and PDE4.

Footnote

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REFERENCES

1. Dubey RK., Tofovic SP., Jackson EK. (2004): Cardiovascular pharmacology of estradiol metabolites. *J Pharm Exp Ther*; 308: 403–409.
2. Zacharia LC., Jackson EK., Gillespie DG. and Dubey RK. (2001a): Increased 2-methoxyestradiol production in human coronary versus aortic vascular smooth muscle cells. *Hypertension*; 37: 658–662.
3. Zacharia LC., Jackson EK., Gillespie DG. and Dubey RK. (2001b): Catecholamines abrogate the anti-mitogenic effects of 2-hydroxyestradiol on human aortic vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*; 21: 1745–1750.
4. Zacharia LC., Dubey RK., Mi Z. and Jackson EK (2003): Methylation of 2-hydroxyestradiol in isolated organs. *Hypertension*. 42(1): 82–7.
5. Dubey RK., Gillespie DG., Zacharia LC., *et al.* (2000): Methoxyestradiols mediate the antimitogenic effects of estradiol on vascular smooth muscle cells via estrogen receptor-independent mechanisms. *Biochem Biophys Res Commun*; 278: 27–33.

6. Barchiesi F., Jackson EK., Gillespie DG., Fingerle J. and Dubey RK. (2002): Methoxyestradiols mediate the antimitogenic effects of estradiol on human aortic SMC growth. *Hypertension*; 39: 874–879.
7. Dubey RK., Gillespie DG., Zacharia L. *et al.* (2003): CYP450- and COMT-derived estradiol metabolites inhibit activity of human coronary artery SMCs. *Hypertension*; 41: 807–813.
8. Dubey RK., Gillespie DG., Zacharia LC. *et al.* (2002b): Methoxyestradiols mediate the antimitogenic effects of locally applied estradiol on cardiac fibroblast growth. *Hypertension*; 39: 412–417.
9. Xiao S., Gillespie DG., Baylis C., Jackson EK. and Dubey RK. (2001): Effects of estradiol and its metabolites on glomerular endothelial NO synthesis and mesangial cell growth. *Hypertension*; 37: 645–650.
10. Zacharia LC., Jackson EK., Gillespie DG. and Dubey RK. (2002): Catecholamines block 2-hydroxyestradiol-induced antimitogenesis in mesangial cells. *Hypertension*; 39: 854–859.
11. Dubey RK., Jackson EK., Keller PJ., Imthurn B. and Rosselli M. (2001): Estradiol metabolites inhibit endothelin synthesis by an estrogen receptor-independent mechanism. *Hypertension*; 37: 640–644.
12. Dubey RK., Gillespie DG., Keller PJ. *et al.* (2002a): Role of methoxyestradiols in the growth inhibitory effects of estradiol on human glomerular mesangial cells. *Hypertension*; 39: 418–424.
13. Tofovic SP., Dubey RK. and Jackson EK. (2001): 2-Hydroxyestradiol attenuates the development of obesity, the metabolic syndrome, and vascular and renal dysfunction in obese ZSF1 rats *J Pharmacol Exp Ther*; 299: 973–977.
14. Zhang X., Jia Y., Jackson EK., Tofovic SP. (2007): 2-Methoxyestradiol and 2-ethoxyestradiol retard the progression of renal disease in aged, obese, diabetic ZSF1 rats. *J Cardiovasc Pharmacol*; 49: 56–63.
15. Tofovic SP. and Jackson EK. (2003): Animal models of the metabolic syndrome, in *Methods in Molecular Medicine: Renal Disease: A Guide to Selecting and Using Technical Approaches* (Goligorsky MS ed) Humana Press, Totowa, pp 29–36.
16. Tofovic SP., Dubey R., Salah EM. and Jackson EK. (2002): 2-Hydroxyestradiol attenuates renal disease in chronic puromycin aminonucleoside nephropathy. *J Am Soc Nephrol*; 13: 2737–2747.
17. Tofovic SP., Salah EM. Dubey R., Melhem MF. and Jackson EK. (2005a): Estradiol metabolites attenuate renal and cardiovascular injury induced by chronic nitric oxide synthase inhibition. *J Cardiovasc Pharmacol*; 46: 25–35.
18. Tofovic SP., Zhang X., Zhu H. *et al.* (2008): 2-Ethoxyestradiol is antimitogenic and attenuates monocrotaline-induced pulmonary hypertension and vascular remodeling. *Vascular Pharmacology*; 48: 174–183.
19. Barchiesi F., Jackson EK., Fingerle J. *et al.* (2006): Methoxyestradiol, an estradiol metabolite, inhibits neointima formation and smooth muscle cell growth via double blockade of the cell cycle *Cir Res*; 99: 234–237.

20. Tofovic SP., Eman S., Mady H., Jackson EK., Melhem M. (2005b): Estradiol metabolites attenuates monocrotaline-induced pulmonary hypertension in rats. *J Cardiovasc Pharmacol*; 46: 430–437.
21. Tofovic SP., Zhang X., Jackson EK., Dacic S., Petrusevska G. (2006): 2-Methoxyestradiol mediates the protective effects of estradiol in monocrotaline-induced pulmonary hypertension. *Vascular Pharmacology*; 45: 358–367.
22. Tofovic SP, Zhang X, Jackson EK, Zhu H, Petrusevska G: 2-Methoxyestradiol attenuates bleomycin-induced pulmonary hypertension and fibrosis in estrogen-deficient rats. *Vascular Pharmacol*, 2009, in press.
23. Tofovic SP., Rafikova O. (2009): Preventive and Therapeutic Effects of 2-Methoxyestradiol, but not Estradiol, in Severe Occlusive Pulmonary Arterial Hypertension in Female Rats. *Am J Res Crit Care Med*; 179: A1802.
24. Hayashi S., Morishita R., Matsushita H., *et al.* (2000): Cyclic AMP inhibited proliferation of human aortic vascular smooth muscle cells, accompanied by induction of p53 and p21. *Hypertension*; 35: 237–243.
25. Marienfeld U., Walter U. and Simm A. (2001): Inhibition of rat cardiac fibroblast growth by cAMP--but not by cGMP-dependent protein kinase. *Basic Res Cardiol*; 96: 184–191.
26. Koyama H., Bornfeldt KE., Fukumoto S., Nishizawa Y. (2001): Molecular pathways of cyclic nucleotide-induced inhibition of arterial smooth muscle cell proliferation. *J Cell Physiol*; 186: 1–10.
27. Paternot S., Coulonval K., Dumont JE., Roger PP. (2003): Cyclic AMP-dependent phosphorylation of cyclin D3-bound CDK4 determines the passage through the cell cycle restriction point in thyroid epithelial cells. *J Biol Chem*; 278: 26533–26540.
28. Vyas SJ., Mokkapatti R., Dubey RK., Chinoy MR. and Jackson EK. (1998): Guanine nucleotide-binding inhibitory protein- mediated inhibition of adenylyl cyclase is enhanced in spontaneously hypertensive rat preglomerular vascular smooth muscle cells. *J Pharmacol Exp Ther*; 285: 828–834.
29. Aviv A., Higashino H., Hensten D., *et al.* (1983): Na⁺-K⁺-ATPase in rat vascular smooth muscle cell grown *in vitro*. *Am J Physiol*; 244: C227–C233.
30. Beavo JA. (1995): Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol Rev*; 75(4): 725–748.
31. Simonds WF. (1999): G protein regulation of adenylate cyclase. *Trends Pharmacol Sci*; 20: 66–73.
32. Jackson EK., Mi Z., Koehler MT., Carcillo JA Jr and Herzer WA. (1996): Injured erythrocytes release adenosine deaminase into the circulation. *J Pharmacol Exp Ther*; 279: 1250–1260.
33. Jackson EK., Mi Z., Carcillo JA., Gillespie DG. and Dubey RK. (1997): Phosphodiesterases in the rat renal vasculature. *J Cardiovasc Pharmacol*; 30: 798–801.
34. Braun T. (1990): Inhibition of the soluble form of testis adenylate cyclase by catechol estrogens and other catechols. *Proc Soc Exp Biol Med*; 194: 58–63.

35. Vatner DE., Asai K., Iwase M. *et al.* (1999): Beta-adrenergic receptor-G protein-adenylyl cyclase signal transduction in the failing heart. *Am J Cardiol*; 83: 80H–85H.
36. Sunahara RK., Dessauer CW. and Gilman AG. (1996): Complexity and diversity of mammalian adenylyl cyclases. *Annu Rev Pharmacol Toxicol*; 36: 461–480.
37. Berman DM. and Gilman AG. (1998) Mammalian RGS proteins: barbarians at the gate *J Biol Chem*; 273: 1269–1272.
38. Dessauer CW., Tesmer JJ., Sprang SR. and Gilman AG. (1999): The interactions of adenylate cyclases with P-site inhibitors. *Trends Pharmacol Sci*; 20: 205–210.
39. Kaminski NE. (1996): Immune regulation by cannabinoid compounds through the inhibition of the cyclic AMP signaling cascade and altered gene expression. *Biochem Pharmacol*; 52: 1133–1140.
40. Raufman JP. (1998): Cholera. *Am J Med*; 104: 386–394.
41. Johnson-Mills K., Arauz E., Coffey RG., Krzanowski JJ. Jr and Polson JB. (1998): Effect of CI-930 [3-(2H)-pyridazinone-4,5-dihydro-6-[4-(1H-imidazolyl)phenyl]-5-methyl-monohydrochloride] and rolipram on human coronary artery smooth muscle cell proliferation. *Biochem Pharmacol*; 56: 1065–1073.
42. Begany DP., Carcillo JA., Herzer WA., Mi Z. and Jackson EK. (1996): Inhibition of type IV phosphodiesterase by Ro 20–1724 attenuates endotoxin-induced acute renal failure. *J Pharmacol Exp Ther*; 278: 37–41.
43. Carcillo JA., Herzer WA., Mi Z., Thomas NJ. and Jackson EK. (1996): Treatment with the type IV phosphodiesterase inhibitor Ro 20–1724 protects renal and mesenteric blood flow in endotoxemic rats treated with norepinephrine. *J Pharmacol Exp Ther*; 279: 1192–1204.
44. Maclean MR., Johnston ED., McCulloch KM. *et al.* (1997): Phosphodiesterase Isoforms in the Pulmonary Arterial Circulation of the Rat: Changes in Pulmonary Hypertension¹ *J Pharm Exp Ther* 283, 619–624.
45. Wagner RS., Smith CJ., Taylor AM., Rhoades RA. (1997): Phosphodiesterase inhibition improves agonist-induced relaxation of hypertensive pulmonary arteries. *J Pharmacol Exp Ther*; 327: 70–75.
46. Dony E., Lai Y-J., Dumitrascu R., Pullamsetti SS. *et al.* (2008): Partial reversal of experimental pulmonary hypertension by phosphodiesterase-3/4 inhibition. *Eur Respir J*; 31: 599–610.

Резиме

ДЕЈСТВО НА МЕТАБОЛИТИТЕ НА ЕСТРАДИОЛ ВРЗ СИНТЕЗАТА И РАЗГРАДБАТА НА ЦИКЛИЧНИОТ АДЕНОЗИН МОНОФОСФАТ

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Апстракт: 17 β -естрадиол се метаболизира во 2-хидроксиестрадиол, а 2-хидроксиестрадиол во 2-метоксиестрадиол. Овие стероиди ја намалуваат пролиферацијата и миграцијата на васкуларните мазни мускулни клетки и нивната синтеза на колаген со редослед на потенција 2-метоксиестрадиол > 2-хидроксиестрадиол > 17 β -естрадиол, што е обратно пропорционално на нивната естрогенска активност. Бидејќи цикличниот аденозин монофосфат (сАМР) исто така го инхибира клеточниот раст, можно е овие стероиди да влијаат врз синтезата и разградбата на сАМР. Оваа претпоставка беше тестирана во оваа студија. Клеточна култура на прегломеруларни васкуларни мазни мускулни клетки беше третирана со високи концентрации на 3-изобутил-1-метилксантин (IBMX; 1 mM) за да се елиминира активността на фосфодиестеразата како следечка варијабла. Високи (100 ЕМ), но не ниски (10 ЕМ) концентрации на 17 β -естрадиол, 2-хидроксиестрадиол и 2-метоксиестрадиол го намалија покажувањето на сАМР индуцирано со изопротеренол за 15%, 19% и 55%. Во клетките на култура претходно третирана со IBMX, 2-хидроксиестрадиол (100 ЕМ) исто така го намали за 31% зголемувањето на концентрациите на сАМР индуцирано со форсколин. Во клетките на култура која не беше претходно третирана со IBMX, користејќи концентрации на стероидите (10 ЕМ) кои не влијааа врз активността на аденил циклазата, 17 β -естрадиолот го намали за 34%, 2-хидроксиестрадиолот немаше никакво дејство, а 2-метоксиестрадиолот го зголеми за 60% покажувањето на концентрациите на сАМР индуцирано со изопротеренол. Овие резултати укажуваат дека во прегломеруларни васкуларни мазни мускулни клетки, високи концентрации на 2-хидроксиестрадиол сигнификантно, а 17 β -естрадиол и 2-метоксиестрадиол само умерено, ја инхибираат аденилната циклаза. Нашите резултати сугерираат дека ниски концентрации на 17 β -естрадиол ја активира, 2-хидроксиестрадиол нема ефект, а 2-метоксиестрадиол ја инхибира фосфодиестеразната активност. Ефектите на овие стероиди врз фосфодиестеразната активност може да ја објаснат

поголемата потентност на 2-мехоксиестрадиолот како антипролиферативен агенс во однос на 17 β -естрадиолот и 2-хидроксиестрадиолот.

Клучни зборови: естрадиол, метаболити на естрадиол, цикличен аденозин монофосфат.

Running Title: Effects of Estradiol Metabolites on cAMP.

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