Acute Effects of 17ß-Estradiol and ATP
on Endothelial Cells; Effects of Genistein
and PMA on Calcium Response

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ABSTRACT

Various studies have shown that 17ß-estradiol (E2), has acute effects on cardiovascular system in addition to its genomic effect. Acute administration of E2 had been shown to increase intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)) in human umbilical vein endothelial cells (HUVEC). The present study investigates the signalling pathway responsible for Ca\(^{2+}\) response to E2. In the study, the effect of E2 on phosphoinositide turnover was investigated by use of Dowex-1 anion-exchange columns after labeling cells with myo\(^{3H}\)inositol. Additionally, the effects of tyrosine phosphorylation inhibitor genistein and protein kinase C activator 4ß-phorbol-12ß-myristate-13-acetate (PMA) on the Ca\(^{2+}\) response to E2 and ATP were investigated and compared in fura-2 loaded HUVEC. The data demonstrates that E2 treatment causes 45% increase in inositol phosphate production in parallel to increases in [Ca\(^{2+}\)]\(_i\). Genestein and PMA inhibit the Ca\(^{2+}\) response to E2 ~75%, 49%, while they inhibit the response to ATP ~62%, 73% respectively. Our data suggests the involvement of PLC in the signaling stimulated by E2 and indicates the involvement of tyrosine phosphorylation and PKC. Differences in the effect of the inhibitors on E2- and ATP-induced responses suggest that there may be differences in the upstream signaling initiated by E2 and ATP, such as different roles for tyrosine phosphorylation.

Key Words: 17ß-estradiol, ATP, [Ca\(^{2+}\)]\(_i\), Signal transduction, Inositol phosphates, Phospholipase C, Genistein, PMA, HUVEC.


INTRODUCTION

There is increasing evidence of estrogen-induced rapid nongenomic effects in various tissues including cardiovascular system\(^{(1,2)}\). Various in vivo and in vitro studies have shown that acute administration of 17ß-estradiol influence the vascular tone and reactivity. Although, the underlying mechanism is still unclear, acute administration of E2 has been shown to effect calcium homeostasis in vascular cells. It was shown that E2 has calcium antagonistic effects and inhibits voltage operated Ca\(^{2+}\) channels in vascular smooth muscle cells while stimulates elevations in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in endothelial cells\(^{(3-5)}\).

Since elevations in [Ca\(^{2+}\)]\(_i\) in endothelial cells triggers the synthesis and release of vasoactive compounds such as prostacyclin and nitric oxide (NO), some of the rapid effects of estrogen on vascular function may be explained by E2-induced elevations in [Ca\(^{2+}\)]\(_i\)\(^{(6)}\). In fact, numerous studies suggest that endothelium-deri-
ved NO mediates some of the effect of estrogen and responsible for the beneficial effects on cardiovascular system[7]. According to our previous study, E2 induced elevations in (Ca+2)i were caused by Ca+2 release from intracellular stores and by Ca+2 entry via ion channels in the plasma membrane[5]. These effects can be mediated through various signal transduction cascades which may involve receptor-tyrosine kinases and G-protein coupled receptor stimulation of phospholipase C (PLC). The PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate to inositol 1, 4, 5-trisphosphate which releases Ca+2 from intracellular stores, and 1, 2-diacylglycerol (DAG), which activates protein kinase C (PKC). There are at least 10 PLC isozymes defined and these are be divided into three types: PLC-β, PLC-g, PLC-d. But there are two basic mechanisms by which various agonist activate these PLCs. Many hormones and neurotransmitters activate PLC-β through receptors coupled to G proteins while growth factors or some cytokines activate PLC-g through receptors which have direct or indirect tyrosine kinase activity. However, PLC can also be activated directly by several lipid-derived second messengers produced by receptor-mediated activation of other phospholipases[8-10].

In this study, the signaling pathway responsible for elevations in (Ca+2)i induced by 17ß-estradiol in endothelial cells was investigated. The involvement of PLC was examined by the effect of E2 on inositol lipid breakdown and especially on inositoltrisphosphate (IP3) production. Additionally, the role of tyrosine phosphorylation and PKC in the signal transduction cascade was investigated by use of genistein (tyrosine phosphorylation inhibitor) and phorbol 12-myristate 13-acetate (PMA) (PKC activator). In the experiments, also the Ca+2 response to ATP and the effect of genistein, and PMA on the response was explored and compared with that of E2. ATP was chosen because, in endothelial cells, ATP increases (Ca+2)i with the involvement of formation of IP3, most probably via binding P2Y-purinoreceptors (P2Y2)[11-13].

MATERIALS and METHODS

Isolation, Culture and Treatments of Human Umbilical Vein Endothelial Cells (HUVEC)

HUVEC were harvested by collagenase treatment (type IA, C-2674 Sigma) according to the method of Jaffe and cultured on coverslips placed in multiwelled plates with medium 199 (M-2520 Sigma) supplemented with 10% fetal calf serum (FCS, 04-001-1B, Biol. Indus., Kibbutz Beth Haemak, Israel) and antibiotics (penicillin-streptomycin, P-3539, Amphotericin B, A-9528 Sigma)[14]. Before the cells reached confluency they were incubated with estrogen-free medium for 48 hours. Hormone free medium was prepared by charcoal treatment of FCS and did not contain phenol red (M-3769 Sigma)[15]. Estrogen levels of charcoal stripped FCS were measured by immunoassay and found to be negligible. Stock solution of E2 was prepared in 99.9% ethanol and ATP in HEPES buffer, pH 7.4, genistein and PMA solutions in 100% dimethyl sulfoxide (DMSO). All the experiments were performed on primary HUVEC cultures before they were post confluent.

Intracellular Free Calcium Measurements

Free cytosolic calcium concentrations [(Ca+2)i] were measured using the fluorescent Ca+2 indicator dye fura-2. Membrane permeable acetoxymethyl ester form of fura-2 (fura-2 AM, F-0888 Sigma) was dissolved in dimethyl sulfoxide. Confluent endothelial monolayers on coverslips were incubated with 5 µmol/L of fura-2 AM at 37°C in HEPES buffered saline containing 0.1% BSA: 137 mmol/L NaCl, 4 mmol/L KCl, 10 mmol/L HEPES, 11 mmol/L glucose, 1.8 mmol/L CaCl2, 1 mmol/L MgCl2 and 0.1% BSA, pH 7.4) for 45 minutes. After the incubation period, extracellular fura 2 AM was removed by washing the coverslips with the buffer. This was followed by a 20 minute postincubation period in the buffer at 37°C for hydrolysis of fura-2 AM to the active form of dye, fura-2. The coverglasses were then inserted in the cuvette of a double beam spectrophuorimeter (Schimadzu RF 5000) and the excitation wavelength was alternated between 340 nm and 380 nm and emission fluorescence was recorded at 510 nm. The fluorescence ratio (R) was calculated as F340/F380, where F340 and F380 are the emission intensities at 340 and 380 nm excitation respectively. Sequential addition of 10 µM ionomycin (I-0634 Sigma) and EGTA (2 mM, pH8) to the cuvette provided the maximum and minimum fluorescence ratio (Rmax, Rmin). (Ca+2)i was calculated according to to the equation: (Ca+2)i = Kd X β X (R-
R_{\text{min}}/(R_{\text{max}}-R)$, where $K_d$, dissociation constant of fura-2, is 224 nM, and $\beta$ is the emission fluorescence values at 380 nm excitation in the presence of EGTA and ionomycin$^{16}$. The effects of E2 and ATP on $(Ca^{2+})_i$ of HUVEC cultures were investigated by addition of these agents into cuvettes in which fura-2 loaded culture cells were placed. To determine the contribution of extracellular Ca$^{2+}$ to changes in $(Ca^{2+})_i$, EGTA was added to the buffer at concentration of 0.1 mmol/L.

**Phosphoinositide Turnover Determination**

Endothelial cells were seeded in 6-well culture dishes or in 25 cm$^2$ flasks. On the second day after seeding, cells were incubated with myo-[2-$^{3}$H] inositol (10 µCi/mL) for 48 hours. At the end of incubation the monolayers were washed three times with Medium 199 (Sigma, 3769) and incubated with medium 199 containing 50 mM LiCl and 10% FCS for 45 minutes at 37°C. Then the medium was discarded and cells were incubated with HBS containing 50 mM LiCl, and 0.1% BSA for 15 minutes more before the addition of compounds like E2, genistein and E2. To control cells only ethanol was applied. Incubations were terminated after 5 minutes by discarding the HBS and addition of 1 mL boiling 1% (w/v) SDS/30 mM EDTA. Dishes were heated for 5 minutes at 96°C and 3 mL distilled water was added to each well. Total lysates were applied to columns containing 0.5 mL Dowex 1 (200-400 mesh, formate form). Columns were first washed with 14 mL water to wash out myo-[2-$^{3}$H] inositol. Subsequently the inositolphosphates were eluted by stepwise addition of solutions containing increasing amounts of formate in the order of 5 mM sodiumtetraborate/60 mM ammonium formate, 200 mM sodium formate/0.1 M formic acid, 400 mM sodium formate/0.1 M formic acid, 750 mM sodium formate/0.1 M formic acid, 1 M sodiumformate/0.1 M formic acid. In some experiments the total increase in inositolphosphates was determined by elution with 1M ammonium formate$^{17}$.

Fractions were collected in 2.0 mL aliquots. Radioactivity in eluates was measured by liquid scintillation counter.

**Statistical Analysis**

Student’s unpaired t-test and One-way Analysis of Variance (ANOVA) were used, and if signifiancy was found Tukey-Kramer Multiple Comparison test was used to test pairwise differences.

**RESULTS**

**Effect of 17ß-Estradiol and ATP on (Ca$^{2+}$)$_i$ of HUVEC**

Application of 1µM E2 to fura-loaded endothelial cells caused elevations in $(Ca^{2+})_i$, which returned to a steady state slowly$^5$. The mean of E2-induced increases in $(Ca^{2+})_i$ compared to the basal values was 138.14 ± 11.3% (n= 17) (Figure 1a).

Similarly, ATP (100 µM) induced rapid increases in $(Ca^{2+})_i$ with a mean of 656.4 ± 63.3% (n= 6) compared to basal levels (Figure 1b).

Removing extracellular Ca$^{2+}$ decreased the response to both agonists slightly but did not prevent the elevations significantly. In the absence of extracellular Ca$^{2+}$, in EGTA/HEPES buffer, the mean of increases in 340/380 florescence ratio decreased from 17.52 ± 1.11% (n= 18) to 14.42 ± 1.94% (n= 7) for E2 and from 86.9 ± 9.1%, (n= 6) to 84.8 ± 8.87% (n= 8) for ATP.

**Effect of 17ß-Estradiol on Phosphoinositide Metabolism**

To investigate the effect of E2 on inositol lipid breakdown and especially on IP3 production, endothelial cells were labeled with myo-[3$^{3}$H]inositol and after stimulation with E2 for 5 minutes, inositolphosphates were measured. Anion exchange chromatography of the lysate obtained from labelled endothelial cells exhibited 5 distinct $^{3}$H-containing peaks. On the basis of previous studies, the peaks corresponds to: (a) inositol, (b) glycerophosphoinositol, (c) inositol-1-phosphate (IP), (d) inositolbisphosphate (IP2), (e) inositoltrisphosphate (IP3)$^{17}$. Figure 2 demonstrates anion-exchange elution profiles of lysates from prelabelled endothelial cells after stimulation with E2 in the presence and absence of genistein and vehicle ethanol (basal). The results demonstrates that E2 treatment produces increases in IP, IP2 and IP3 levels, while presence of genistein significantly inhibits the effect of E2 on phosphoinositide metabolism. The elution profile demonstrates that increases in IP2 were higher than that of IP3 and IP. Increase in IP3 levels were significant and 0.66 fold over control. Elution of total inositol phosphates by ammonium formate demonstrated that E2-induced to-
The increase was 45% and this increase was attenuated by genistein (Figure 3).

**Effect of Tyrosine Kinase Inhibition on 17ß-Estradiol and ATP-Induced Increases in (Ca++2) i**

The role of tyrosine phosphorylation in E2-induced Ca++2 signaling was investigated by use of tyrosine kinase inhibitor genistein. Genistein inhibits tyrosine phosphorylation by blocking ATP binding to the kinase. Genistein (100 µM) had nonsignificant effects on basal levels of (Ca++2) i, resting (Ca++2) i was 46.2 ± 4.7 nM (n= 20) in control cells and 55.4 ± 4.4 nM (n= 13) in genistein-treated cells. However, after genistein treatment application of E2 vehicle ethanol caused 20-30% elevations in (Ca++2) i.

Preincubation of endothelial cells with genistein reduced the peak increase in (Ca++2) i stimulated by E2. Without considering the effects of vehicle ethanol, E2-stimulated increase in (Ca++2) i was 36.33%, and compared to controls inhibition was approximately 74.7%, p<0.0001 (n= 8), (Figure 4). In the absence of extracellular Ca++2, E2-induced elevations were nearly completely inhibited (90%), p< 0.0001 (n= 6) by genistein.

Genistein inhibited the ATP-induced calcium peak 62% (n= 8) and markedly decreased the plateau phase of calcium response (Figure 4). In the absence of extracellular Ca++2 inhibition was in significantly less.

**Effect of PMA on 17ß-Estradiol and ATP Stimulated Increases in (Ca++2) i**

Treatment of endothelial cells with 100 nM phorbol 12-myristate 13-acetate (PMA) for 2.5 hours inhibited the E2- and ATP-stimulated Ca++2 response 49% and 73.2% respectively compared to control cells (Figure 5).

**DISCUSSION**

The present data shows that E2 treatment causes increases in turnover of inositolphosphates in endothelial cells in parallel to increases in intracellular Ca++2 levels. 17ß-estradiol treatment caused a significant increase in phosphatidylinositol breakdown with nearly 0.66 fold increase in IP3 levels in parallel to 2.3 fold increase in (Ca++2) i. Although the exact structure of these phosphoinositides were not determined in the present study, these lipids which rapidly accumulated after stimulation are most likely to act as second messengers. These kinds of second messengers can be derived by the action of PLC or phosphoinositol-3-hydroxy kinase (PI3 kinase) when a stimulatory agonist molecule binds at membrane receptors[18]. Therefore, E2-induced production of phospholipid derived second messengers demonstrate that E2 may generate a rapid intracellular signaling system beside the conventional genomic pathway.

During the last 2 years, mechanism of the acute ef-
fecteds of E2 on endothelial cells has been under investigation and it has been shown that E2 stimulates PI3 kinase which leads to the activation of Akt kinase and NO production\cite{19}. Also activation of mitogen-activated protein (MAP) kinase family by E2 has been reported\cite{20}. Yet the signaling mechanism (s) by which E2 change intracellular Ca\textsuperscript{2+} is not clear. Since the activation of phospholipase C may be the initiating event in the signal transduction pathway leading to the IP\textsubscript{3} formation and to (Ca\textsuperscript{2+})\textsubscript{i} increase, our data provides a hint for the involvement of phospholipase C in the signal transduction pathway stimulated by E2 in endothelial cells.

Comparison of the ability of E2 with other agonist such as bradykinin or ATP to stimulate Ca\textsuperscript{2+} mobilization and IP\textsubscript{3} production shows that E2 is less potent than these agonists. Bradykinin, and EGF is reported to produce 1.8-3 fold increase in IP\textsubscript{3} formation and 4-10 fold increase in intracellular Ca\textsuperscript{2+} concentration respectively\cite{21-23}. However, the ratio of increases in IP\textsubscript{3} levels to increases in (Ca\textsuperscript{2+})\textsubscript{i} induced by E2 appears similar to the ratio induced by other agonists while the latency of Ca\textsuperscript{2+} response is much longer.

The elution profile of phospholipids demonstrates a greater increase in IP\textsubscript{2} levels compared to IP\textsubscript{3}. This finding may be due to the fact that the levels of IP\textsubscript{3} iso- mers are limited by their metabolism. Although inositol phosphatase inhibitor LiCl was added to incubation medium, some enzymes may still participate in the metabolism of the isomers by phosphorylation and dephosphorlation during the 5 minute incubation time in our experiments\cite{24}.

E2-stimulated phosphoinositide production was inhibited by genistein. Genistein is a tyrosine kinase inhibitor which inhibits both receptor and nonreceptor tyrosine kinases but has little effect on ser-and thr-specific kinases\cite{25}. Protein tyrosine phosphorylation is a mechanism involved in initiation of signal transduction by growth factors. Growth factor receptors which are known to have inherent tyrosine kinase activity, interact with and activate PLC\textgreek{g} isoforms and MAP kinase, the Ras GTPase-activating protein. Therefore, it is accepted that tyrosine phosphorylation plays important role in cell proliferation and differentiation\cite{9,10}. However, tyrosine kinase activity is not limited to Growth factor receptors\cite{26}. Various agonists, which have receptors devoid of tyrosine kinase activity may initiate...
tyrosine phosphorylation and may further induce mitogenic effects\cite{27,28}. Moreover, there is accumulating evidence of involvement of tyrosine phosphorylation in the regulation of Ca\(^{2+}\) flux following cell stimulation with both receptor-dependent and independent agonists\cite{29}.

According to our data genistein was the most potent inhibitor of E2-induced Ca\(^{2+}\) response in endothelial cells. Inhibition was 75\% in HEPES buffer, 90\% in Ca\(^{2+}\) free-EGTA/HEPES buffer. Since absence of extracellular Ca\(^{2+}\) did not significantly inhibited the E2 evoked Ca\(^{2+}\) peaks, this finding demonstrates that tyrosine phosphorylation is involved in the generation of

Figure 3. (\(^3\)H) Inositol labelled endothelial cells were stimulated for 5 minutes with E2 or E2 vehicle ethanol (basal) or with E2 after incubation with genistein. Cells were lysed and applied to Dowex-1 columns. After washing out (\(^3\)H) inositol with water, total inositolphosphates were eluted by addition of 1 M ammonium formate/0.1 M formic acid. Radioactivity in eluates was measured by liquid scintillation counting. The results (mean ± SEM of 4 separate experiments) show the percent increases in total inositolphosphates. The graph demonstrates that E2 causes 45\% increase in phosphoinositide turnover which can be significantly inhibited by genistein pretreatment in HUVEC.

Figure 4. The role of tyrosine phosphorylation in E2-stimulated signaling pathway in HUVEC. The tracing demonstrates that genistein attenuates the peak increase in (Ca\(^{2+}\)\(_i\)) evoked by E2 and ATP 75\% and 62\% respectively compared to controls and markedly decreases the second phase of ATP response. The tracing represent the mean of data obtained from 4 experiments. Cells were treated with 100 µM genistein for 12 minutes before stimulation with E2 (10 µM) and ATP (100 µM). After genistein treatmant E2 vehicle ethanol caused nearly 20\% increase in (Ca\(^{2+}\)\(_i\)). The extracellular Ca\(^{2+}\) concentration was 1.8 mM during the experiments.

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Figure 5. The role of PKC on E2-evoked Ca\(^{2+}\) response in HUVEC. Tracing demonstrates that PMA attenuates the peak increase in (Ca\(^{2+}\)\(_i\)) stimulated by E2 and ATP compared to controls as shown in Figure 1. E2-deprived HUVEC were incubated with 100 nM PMA for 2.5 hours before stimulation with E2 (10 µM) and ATP (100 µM). Tracing represent the mean of data obtained in 9 experiments.
the signal which mobilizes Ca\(^{2+}\) from the internal stores rather than inhibiting the Ca\(^{2+}\) influx by directly blocking Ca\(^{2+}\) entry pathways.

In addition to attenuation of E2-induced Ca\(^{2+}\) peak, inhibition of E2-induced IP\(_3\) production by genistein suggests that tyrosine phosphorylation is an early event in the pathway which results in Ca\(^{2+}\) release from internal stores. This finding further indicates that tyrosine phosphorylation may be involved in PLC activation, yet the identity, and exact position of this kinase in the signaling cascade remains to be elucidated.

On the other hand, genistein reduced the ATP-induced Ca\(^{2+}\) peak less compared to E2 peak but markedly decreased the plateau phase of the calcium response. This finding suggests that tyrosine phosphorylation is further involved in the influx of Ca\(^{2+}\) from the extracellular space in ATP-induced Ca\(^{2+}\) response pathway. Differences in the effect of genistein on E2- and ATP-induced responses suggest different roles for tyrosine phosphorylation in ATP-and E2-stimulated signal transduction pathways.

Diacylglycerol, generated by activation of PLC, is the endogenous activator of PKC. The involvement of PKC in regulation of PLC activity by various mechanisms such as receptor-desensitization or regulation of certain types of Na\(^+\) and Ca\(^{2+}\) channels has been shown in numerous studies\(^{[10,30,31]}\). The ability of PMA to inhibit inositol lipid breakdown and elevations in (Ca\(^{2+}\))\(_i\) has been also demonstrated for agonist which activate PLC\(^{[32]}\). In the present study, PMA treatment resulted in 62% inhibition of response of the cells to E2 and 73% inhibition of the response to ATP consistent with the data in literature\(^{[11]}\). The molecular mechanism by which PMA block hormone-stimulated Ca\(^{2+}\) response has not been clarified yet but this finding suggest that PKC is involved in the signaling and has a role in feedback regulation of E2-mediated activation.

In conclusion E2 induced formation of IP\(_3\), increase in intracellular calcium and the sensitivity of the response to PKC activation, tyrosine kinase inhibition indicates that PLC may be involved in the signaling pathway initiated by E2. Yet its complete role and identity has to be investigated further.

In various other cell types E2-induced Ca\(^{2+}\) response and PLC activation was reported\(^{[1,33-35]}\). Although PLC mediated Ca\(^{2+}\) mobilization from intracellular stores is the major event in the signaling cascade triggered by peptide hormones, it is becoming increasingly clear from both in vivo and in vitro studies that estrogens exert direct and rapid effects on vascular cells. These results, suggesting that E2 uses PLC to increase intracellular calcium concentration via inositol 1, 4, 5-trisphosphate formation may be an important step in understanding the mechanism of action and the membrane effects of the hormone.

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