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Signal transduction pathways involved in non-genomic action of estrone on vascular tissue

Virginia Massheimer[a,b,*], *Nélida Polini*[a], *Cristina Alvarez*[a], *Silvia Benozzi*[a], *María Belén Rauschemberger*[a,b], *Juana Sellés*[a]

[a] *Cátedra de Análisis Clínicos II, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur., San Juan 670, B8000ICN Bahía Blanca, Argentina*
[b] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina*

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ABSTRACT

Previously we demonstrated that estrone non-genomically regulates rat aortic NOS and COX activity and that this effect depends on ovarian activity. The purpose of the present study was to characterize this effect and investigate the participation of phospholipase C and phophatidylinositol-3-kinase system in the intracellular transduction pathway involved in the response. Using aortic strips isolated from female fertile rats we showed that estrone stimulate nitric oxide synthase and cyclooxygenase in a short time interval (5–20 min), and that NO production was dependent in part on PGI_2 production since 1 μM indomethacin significantly reduced this free radical production. Injection of 17-β-estradiol to ovariectomized rats restored tissue capacity to rapidly increase NO production in response to "in vitro" treatment with 1 nM estrone. We also demonstrated that in aortic strips isolated from intact animals estrone elicited a rapid phospholipase C activation, inducing a biphasic increase in diacylglycerol generation (peaking at 45 s and 5 min). The presence of protein kinase C inhibitor chelerythrine did not prevent the increase of NO released in response to hormone treatment. We proved that PI_3K–Akt system does not mediate NOS and COX activation. However, PLC activation was dependent on PI_3K since presence of LY 294002 in the incubation medium abolished estrone-induced DAG increment. We concluded that, estrone rapid action on vascular tissue involves a cross talk between NOS and COX system, and the activation of PLC/DAG/PKC transduction pathways.

1. Introduction

It has been well established by epidemiological studies the relative low incidence of cardiovascular disease in premenopausal women, compared with men, and that this is reversed following menopause [1]. This has been attributed to a protective effect of estrogen on cardiovascular system [2]. Estrogen directly regulates vasomotor tone through both genomic and non-genomic action by means of estrogen receptors (ERs)-dependent and independent pathways [3–5]. Long-term effect of estrogen administration is associated with decreased Endothelin-1 circulating levels, and its endothelial cell release [6,7], reduced vascular expression of endothelial adhesion molecules [8], and decreased vascular smooth muscle cells (VSMC) growth [9]. It has been reported that estradiol (E_2) produce a rapid non-genomic effect on endothelial cells and vascular tissue, increasing NO release [10,11] and prostacyclin synthesis [12] resulting in vasorelaxation and inhibition

* *Corresponding author*. Tel.: +54 291 4595100; fax: +54 291 4595130.
E-mail address: massheim@uns.edu.ar (V. Massheimer).

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of platelet aggregation [13,14]. In previous studies we found that in aortic tissue isolated from estrogen deprived rats rapid NOS activation in response to "in vitro" 17-β-E_2 treatment is impaired [15].

Estrogen exerts its non-genomic action binding to ERs at membrane level activating different intracellular signal transduction cascades. It has been proved that in vascular tissue PI_3K/Akt and PLC–PKC participate in both genomic and non-genomic estrogen effects [4].

Hormonal replacement therapy (HRT) with estrogen has been proposed to be cardioprotective but, recent clinical trials concerning to HRT outcomes afforded controversial results in reference to the risk of heart disease [16]. This fact makes it relevant to investigate the vascular effects of other estrogen metabolites.

Beyond menopause the major estrogen remaining in circulation is estrone (E_1). Peripheral aromatization of androstenedione and testosterone is the main source of circulating E_1 [17,18]. Aromatase is the enzyme involved in this conversion which has been found in different tissues including VSMC [19]. It has been reported that estriol (E_3) and E_1 similarly to E_2 might have atheroprotective effects by regulating cytokines that modulate growth factors expression in VSMC [20]. Also other authors have shown, using human endothelial cells, that E_1 and other E_2 metabolites stimulate prostacyclin [12]. Little is known concerning metabolic pathways involved in E_1 action on vascular tissue. Morley et al. [21] have reported that E_1 as well us 17-β-estradiol induce a rapid and transient increase of intracellular calcium as a result of phosphoinositide breakdown.

Previously in our laboratory we demonstrated that rat aorta treated "in vitro" with E_1 produces a rapid non-genomic stimulation of COX and NOS, and this action is dependent on ovarian activity [22].

It is known that E_1 also binds to ERs [23], but there is little information about the down stream signal systems involved in vascular tissue response to E_1. In our study we intent to further characterise rat aorta NOS and COX rapid stimulation elicited by "in vitro" E_1 treatment and the participation of PLC and PI_3K/Akt systems in the response observed.

2. Experimental

2.1. Materials

^{3}[H]-arginine and ^{3}[H]-arachidonic acid were purchased from New England Nuclear (Chicago), and Dowex AG 50WX8 was purchased from BioRad Laboratories, California. TLC silica gel plates were obtained from Merck KgaA, Germany. Estrone and all other reagents were from Sigma Chemical Co. (St. Louis, MO). Chelerythrine and Ly 294004, were kindly donated by Alomone. ICI 182780 was purchased from Tocris Bioscience (Park Ellisville, USA).

2.2. Animals

Female Wistar rats were fed standard rat food, given water ad libitum, and maintained on a 12 h light, 12 h dark cycle. The animals were divided in the three following experimental groups.

FR(+). Female rats with normal oestrous cycles, sexually mature (3–12 months old) that had progressed through at least three consecutive oestrous cycles. The animals chosen for the experimental design were those with similar oestrous cycle length and were sacrificed 24 h after estrus phase determined by microscopic observation of vaginal smears.

OVX. Four-months old female rats were bilaterally ovariectomized under anaesthesia (ketamine 25 mg/kg) and used after 2 months of oestrous deprivation.

OVX-IP. Two months after ovariectomy OVX rats were treated with 50 μg/kg of 17-β-E_2 or placebo intraperitoneally, for 5 days. Rats were sacrificed 24 h after the last injection. In all cases, the presence or absence of oestrous cycle activity was evaluated by optical microscope examination of vaginal smears.

All animal work was performed at the Unit of Animal Care belonging to the Biology, Biochemistry and Pharmaceutics' Department of the University. The Animal Care Use committee approved the protocol used.

2.3. Rat aortic strip (RAS) preparations

RAS were obtained as previously described [14]. Briefly, animals were killed by cervical dislocation and the thoracic aorta was immediately removed and placed in cold DPBS solution, cleaned of adherent connective tissue, and cut into 5-mm strips. Special care was taken to preserve the integrity of the endothelial layer. The strips were placed in the incubation medium (buffer D, in mM): 145 NaCl; 5.0 KCl; 1.2 $MgSO_4$; 1.0 $CaCl_2$; 10 glucose; 10 Hepes, pH 7.35 and pre-equilibrated for 15 min at 37 °C in a shaking water bath.

2.4. Determination of nitric oxide (NO) production

2.4.1. Measurement of NO by the conversion of ^{3}H-arginine to ^{3}H-citrulline

As previously described [14] RAS were pre-incubated in buffer D for 15 min at 37 °C, labeled with 10 μM ^{3}H-arginine (5 μCi) for 10 additional minutes, prior to E_1 treatment. Respective controls with the same vehicle in which E_1 was dissolved (ethanol less than 0.1%) were also processed. After treatment, the aortic tissue was washed in ice cold, unlabeled medium and immediately frozen in liquid nitrogen. The strips were homogenized for 45 s in ice-cold 20 mM Hepes–2 mM EDTA, pH 5.5–6.0 and then centrifuged at 2500 × *g* for 20 min. The supernatant was applied to a 1 ml Dowex AG 50WX8 column, and citrulline was eluted with 2 ml water. ^{3}H-citrulline was quantified by liquid scintillation [24]. The pellet was dissolved with 1N NaOH for protein determination by the Lowry method [25].

2.4.2. Nitrite assay

RAS were treated as indicated previously and nitrite was measured in the incubation media as an end product of NO released, using the spectrometric Griess reaction [26]. An aliquot of 700 μl incubation medium from each condition tested was mixed with 70 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine dihydrochloride in 2% phosphoric acid). After 20 min incubation at room

temperature, absorbance was read at 580 nm in a Hitachi U-1000 spectrophotometer. Lower limit of detection in the assay was 0.078 nmol NO_2^-/mg protein.

2.5. Measurment of eicosanoids

PGI_2, was measured as previously described [27]. RAS were labeled with ^{3}[H]-arachidonic acid (0.3 μCi/ml) for 90 min, extensively washed with solution D, and then transferred to fresh incubation medium in order to perform hormonal treatment with the correspondent experimental conditions. Then, RAS were removed, and the incubation medium was acidified to pH 3.0–3.5 with citric acid (1 M) and extracted four times with 0.5 volumes of ethyl acetate. Pooled acetate extracts were dried under nitrogen. The eicosanoids were isolated by one dimensional thin layer chromatography (TLC) [28] on silica gel plates using ethylacetate:isooctane:acetic acid:water (66:30:12:60) as solvent. Iodine staining was used for detection, and the compounds were identified by comparison with standards. Labeled 6-keto-PGF1α (stable metabolite of prostacyclin) was scraped off and radioactivity measured by liquid scintillation. The results were expressed as a percentage of the total [^{3}H]-lipids.

2.6. Determination of DAG production

RAS were pre-labeled with [^{3}H]-arachidonic acid (0.3 μCi/ml) at 37 °C for 90 min under 95% O_2/5% CO_2, followed by extensive washing with buffer D, exposed to the hormone or vehicle for the indicated times, and immediately frozen in liquid nitrogen. Lipids were extracted with chloroform/methanol (2:1 v/v), as described by Folch et al. [29]. DAG was isolated by one-dimensional TLC on silica gel G plates developed with hexane/diethyl ether/acetic acid (75:25:4 v/v/v) as solvent. Lipids were detected by iodine staining and identified by comparison with standards. Labeled DAG was scraped off and radioactivity measured by liquid scintillation. Results were expressed as a percentage of the total ^{3}[H]-lipids.

2.7. Assay of PKC activity

PKC activity was determined in RAS homogenates using a histone phosphorylation assay as described previously [30]. RAS were mechanically homogenized in a buffer containing 20 mM Tris–HCl, pH 7.4, 0.33 M sucrose, 1 mM EGTA, 20 mM NaF, 1 mM DTT, 20 μg/ml aprotinine. To perform the reaction an aliquot with 100 μg protein was suspended in 100 μl assay buffer containing 5 μg histone (type III-S) with or without 1 mM $CaCl_2$, 10 μg phosphatidylserine, 0.75 μg DAG. Treatment was done by incubation for 5 min with PKC activator 12-O-tetradecanoylphorbol-13-acetate (TPA) in presence or absence of chelerythrine. Immediately after, phosphorylation reactions were started by addition of 10 μl, 100 μM, ATP-γ, ^{32}P-ATP (0.2 μCi) and incubated for 5 additional minutes. Reaction was stopped separating phosphorylated substract using a phosphocellulose disk (Whatmann P-81) and radioactivity quantified by liquid scintillation. Results were expressed in pmol of Pi/min per mg protein.

2.8. Statistical analysis

Data are presented as mean ± S.E.M. Differences between treated and control values were analysed for statistical significance using one-way analysis of variance (ANOVA), and Student's *t*-test using SSPS Statistical software version 10.0 for Windows. $P < 0.05$ was taken as significance level. Each experimental condition was reproduced in at least three independent experiments.

3. Results

3.1. Characterization of E_1 NOS and COX non-genomic activation

Previously we have demonstrated that E_1 exerts a direct non-genomic action on rat aortic metabolism, which involves NOS and COX activation.

To further characterize rat aortic tissue rapid response to E_1 treatment, we studied the time course of NOS and COX stimulation-induced by the hormone. First, we determined in RAS isolated from FR(+) rats NO producing when they were treated with 1 nM E_1 for different time intervals. In Fig. 1 it can be observed that NOS is significantly activated by the hormone between 5 and 20 min treatment, having the maximal effect at 10 min, remaining significant after 20 min treatment.

The time course profile of the prostacycline synthesis stimulation-induced by 1 nM E_1 shows a statistically significant increment of PGI_2 released to the incubation media as soon as after 1 min treatment reaching a plateau after 5–15 min exposure to the hormone (Fig. 2). Regarding the possibility that E_1-induced NOS activation could be modulated by COX enzymatic system we measured NO production elicited by 1 nM E_1 in the presence or absence of 1 μM indomethacin in the incubation media. As shown in Fig. 3, COX inhibition resulted in a significant reduction of NO released by FR(+) RAS in response to hormone treatment, suggesting that COX

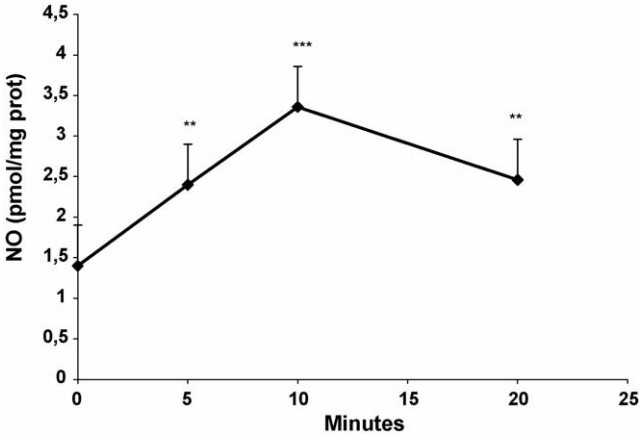

Fig. 1 – Effect of E_1 on NO production—time course profile: FR(+) RAS were labeled with 10 μM ^{3}H-arginine (5 μCi) and exposed to 1 nM E_1 for the time indicated. ^{3}H-citruline was measured as described in Section 2. Results are the average ± S.D. of three independent experiments ($n = 4$). $^{**}P < 0.01$, $^{***}P < 0.001$, respect to each control value.

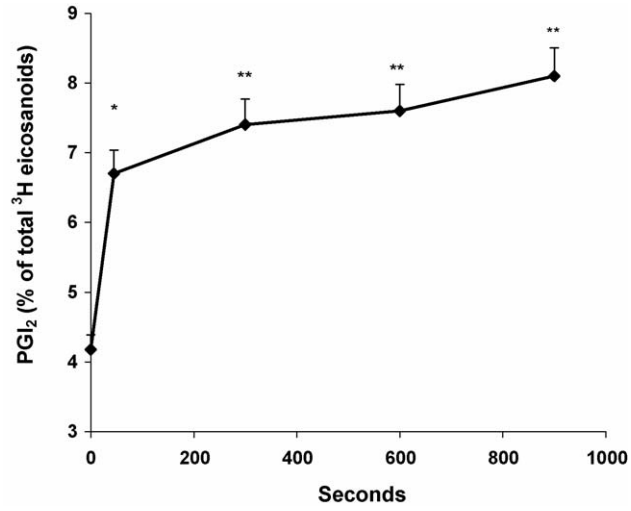

Fig. 2 – Time course of the stimulation of PGI_2 production by E_1: FR(+) RAS were labeled with ^{3}H-arachidonic acid (0.3 μCi/ml) for 90 min and then exposed to 1 nM E_1 for the indicated time interval. Immediately after treatment the incubation medium was collected and PGI_2 was measured as described in Section 2. Results are the average ± S.D. of four independent experiments performed in triplicate. $^*P < 0.05$, $^{**}P < 0.01$, respect to each control value.

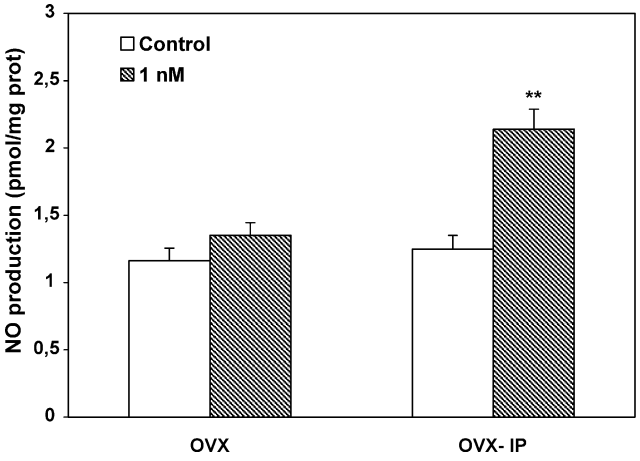

Fig. 4 – 17-β-E_2 administration to OVX rats restores NOS rapid stimulation-induced by "in vitro" E_1 treatment: RAS isolated from OVX rats injected with vehicle or OVX-IP were labeled with 10 μM ^{3}H-arginine (5 μCi) and exposed to 1 nM E_1 for 5 min. ^{3}H-citruline was measured as described in Section 2. Results are the average ± S.D. of three independent experiments ($n = 3$). $^{**}P < 0.01$, respect to each control value.

messenger pathway is involved, at least partially, in NOS stimulation-induced by E_1.

3.2. *In vivo effect of 17-β-estradiol on rat aorta non-genomic response to E_1 treatment*

In previous studies we found that E_1 non-genomic action on rat aortic tissue metabolism was dependent on ovarian function since RAS isolated from OVX rats did not increase NO production when treated "in vitro" with physiological concentrations of E_1 [19]. We checked the possibility that 17-β-E_2 administration to OVX could restore NOS rapid activation elicited by "in vitro" E_1 exposure. To that end RAS isolated from OVX-IP (OVX rats which were injected with 17-β-E_2) were treated with 1 nM E_1. As shown in Fig. 4. E_2 supplementation restores tissue capacity to increase NO production after 10 min treatment with E_1, implicating that circulating E_2 levels are relevant for E_1 rapid non-genomic NOS activation. Pre-incubation of RAS from both OVX-IP and FR(+) rats with ICI 182780 abolished E_1-induced rapid NOS stimulation, implying that ERs are involved in the response (Fig. 5).

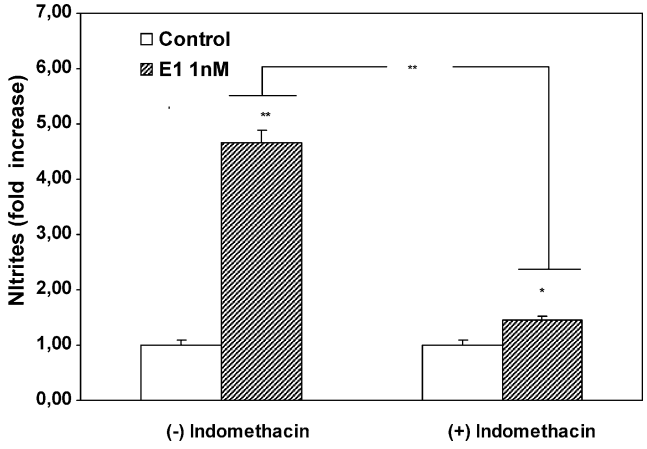

Fig. 3 – Effect of indomethacin in NOS stimulation-induced by E_1: FR(+) RAS were pre-incubated in the presence or absence of 10 μM indomethacin and then exposed to 1 nM E_1 for 5 min. NO production was measured by Griess reaction as described in Section 2. Results are the average ± S.D. of three independent experiments ($n = 4$). $^*P < 0.05$, $^{**}P < 0.01$, respect to each control value.

In order to evaluate if the non-genomic action of E_1 in rat aortic tissue shares common features with E_2 signaling, which would account, at least in part, for the dependence of E_1 non-genomic action on E_2, we studied if PLC and PI_3K/AKT signal transduction pathways are involved in E_1 rapid NOS stimulation.

3.3. *PLC system participation on rat aorta non-genomic response to E_1 treatment*

It is known that PLC activation produces inositol 4,5-biphosphate (PIP_2) breakdown producing DAG and Inositol 1,4,5-triphosphate (IP_3) as second messengers. Using FR(+) rats we investigated whether this signal transduction pathways is involved in aortic tissue rapid response to E_1. To that end, DAG production was measured. The time course of E_1-induced DAG production by rat aorta is shown in Fig. 6. The hormone-induced a biphasic increase of DAG, observing the first increment at 45 s (55% above control), and the second after 5 min treatment (75% above control).

DAG is a natural activator of PKC, consequently we evaluated the participation of this later kinase in E_1-induced NOS

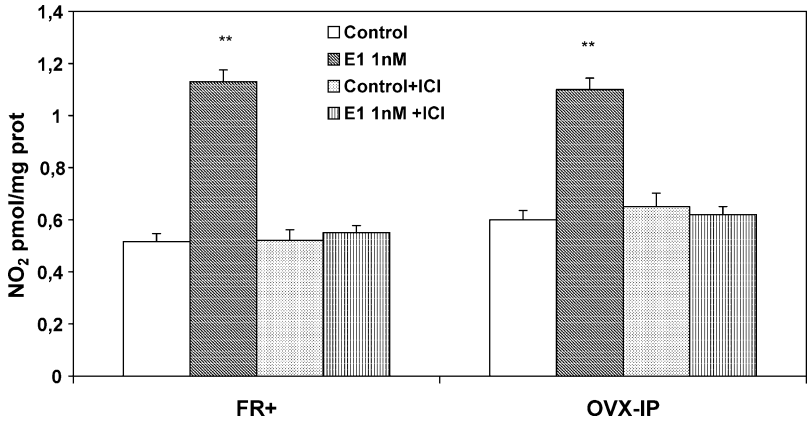

Fig. 5 – ERs antagonist ICI 182780 suppresses E_1 NOS rapid stimulation: RAS isolated from OVX-IP and FR(+) rats were treated with 1 nM E_1 for 5 min in the presence or absence of 1 μM ICI 182780. NO production was determined using Griess reaction as described in Section 2. Results are the average ± S.D. of four independent experiments. $^{**}P < 0.01$, respect to each control value.

activation. Previously to hormone treatment, FR(+) RAS were incubated with 1 μM chelerythrine, a PKC activity inhibitor [31]. This compound did not prevent the increment of NO release in response to 1 nM E_1 exposure (Table 1). Since no inhibition was obtained, to exclude the possibility that chelerythrine was inactive, we measured its activity using a PKC phosphorylation assay. PKC activity was determinate in RAS homogenate exposed to the forbol ester TPA, as specific agonist of PKC, in the presence or absence of 1 μM chelerythrine. We found that the presence of chelerythrine completely suppressed the four-fold increase in PKC activity-induced by TPA (0.11 ± 0.02 vs 0.59 ± 0.08; 0.07 ± 0.03 vs 0.12 ± 0.04 pmol of Pi/mg prot/min, control vs TPA in the absence/presence of chelerythrine, respectively). In summary, these results provide evidence that in rat aorta even though PLC is activated by E_1 treatment, PKC is not involved in NOS activation. Further studies will be conducted in order to establish the physiological ultimate role of this system in E_1 action.

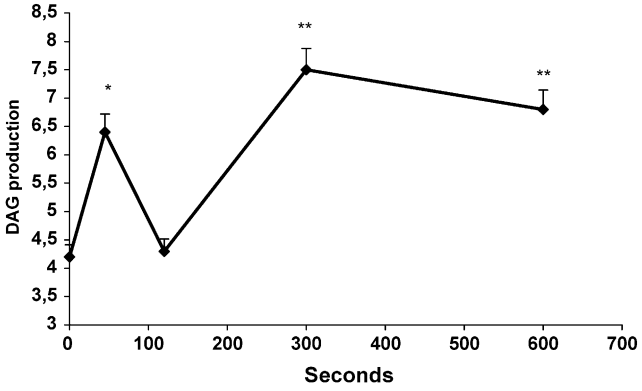

Fig. 6 – Time course profile of DAG synthesis: FR(+) RAS were labeled with ^{3}H-arachidonic acid (0.3 μCi/ml) for 90 min and then exposed to 1 nM E_1 for the time indicated. Immediately after treatment, DAG was measured as indicated in Section 2. Results are the average ± S.D. of three independent experiments performed in quadruplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, respect to each control value.

3.4. *Participation of PI_3K/Akt pathways in E_1 non-genomic action*

It has been well established that the PI_3K/Akt cascade is involved in NOS rapid stimulation-induced by E_2 in vascular tissue, effect which is mediated by ERs [5].

Therefore, we studied the participation of this signal transduction pathway in rat aorta E_1 non-genomic action.

First, we evaluated the role of PI_3K/Akt system on E_1 NOS activation. To that end we determined RAS NO production in response to E_1 treatment incubating FR(+) RAS in the presence or absence of 1 μM LY 294002 (an specific PI_3K inhibitor) previously to be exposed to 1 nM E_1. As can be observed in Fig. 7 inhibition of PI_3K does not modified NOS stimulation elicited after 5 min treatment with E_1. Similar results were obtained using 5 μM LY 294002 or extending to 20 min the time of treatment with the E_1 (data not shown). Wortmannin, another PI_3K inhibitor, also was ineffective in abolish NOS activation by E_1 (pmol NO/mg protein: 1.54 versus 2.41 and 1.61 versus 2.53, control versus E_1 with and without Wortmannin pre-incubation, respectively).

Considering the partial participation of COX pathway in NOS stimulation as described above, the effect of PI_3K in COX stimulation by E_1 treatment was investigated. Pre-incubation

Table 1 – Effect of PKC antagonist chelerythrine in E_1-induced NOS stimulation

	NO (pmol/mg protein)	
	Chelerythrine (–)	Chelerythrine (+)
Control	1.01 ± 0.15[a]	1.24 ± 0.18[a]
Estrone (1 nM)	1.67 ± 0.12*	2.45 ± 0.02***

*P < 0.05; ***P < 0.001, respect to each control value. RAS were labeled with 10 μM ^{3}H-arginine (5 (Ci/ml) in the presence or absence of 1 μM chelerythrine and then exposed to 1 nM E_1 or vehicle for 5 min. ^{3}H-citruline was measured as described in Section 2. Results are the average ± S.D. of three independent experiments ($n = 4$).

[a] n.s. control vs. control plus chelerythrine.

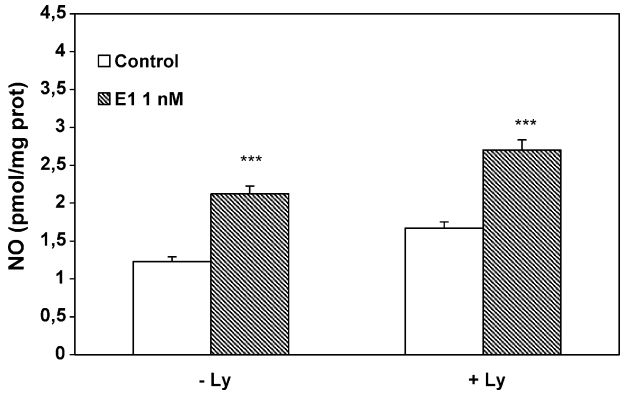

Fig. 7 – Role of PI_3K signaling pathway on E_1 NOS activation: FR(+) RAS were labeled with 10 μM ^{3}H-arginine (5 μCi) in the presence or absence of 1 μM Ly 294002 and then exposed to 1 nM E_1 for 5 min treatment. ^{3}H-citruline was measured as described in Section 2. Results are the average ± S.D. of three independent experiments ($n = 4$). $^{***}P < 0.001$. respect to each control value.

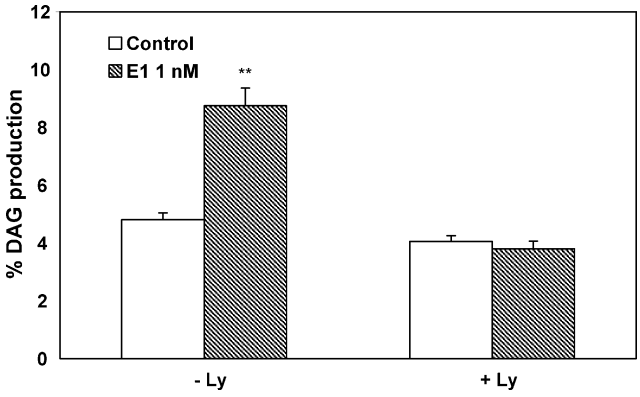

Fig. 9 – Suppression by Ly 294002 of PLC activation-induced by E_1: FR(+) RAS were labeled with ^{3}H-arachidonic acid (0.3 μCi/ml) for 90 min in the presence or absence of 10 μM Ly 294002 and then exposed to 1 nM E_1 for 10 min. Immediately after treatment, DAG was measured as indicated in Section 2. Results are the average ± S.D. of three independent experiments performed in quadruplicate. $^{**}P < 0.01$, respect to each control value.

of FR(+) RAS with 1 μM LY 294004 did not prevent the increase of PGI_2 liberated into incubation medium after 10 min E_1 treatment (Fig. 8).

The involvement PI_3K pathway in E_1 PLC activation was also considered. Pre-incubation of FR(+) RAS with 1 μM LY 294004 followed by 1 nM E_1 exposure showed that the inhibition of PI_3K completely abolished the increment of DAG production produced by the hormone, implying that this kinase system mediates E_1 PLC activation (Fig. 9).

4. Discussion

This study shows that, in aortic tissue isolated from rat with normal estrogen level, NOS activation-induced by E_1 is partially dependent on COX activation and that the administration of 17-β-E_2 to OVX rats restores tissue capacity to elicit a rapid increment in NO production in response to E_1 "in vitro" treatment.

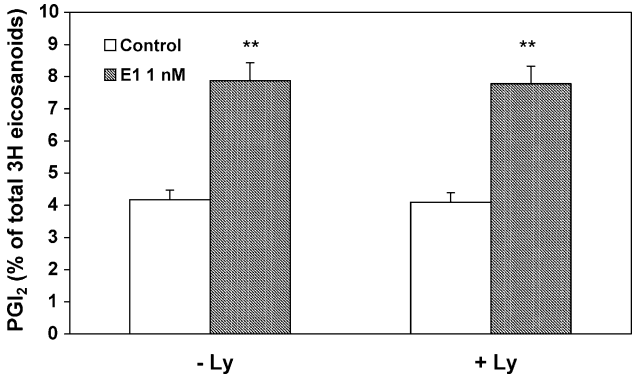

Fig. 8 – Effect of PI_3K inhibitor Ly 294002 on COX activation by E_1: FR(+) RAS were labeled with ^{3}H-arachidonic acid (0.3 μCi/ml) for 90 min in the presence or absence of 1 μ M Ly 294002 and then exposed to 1 nM E_1 for 10 min. Immediately after treatment the incubation medium was collected and PGI_2 was measured as described in Section 2. Results are the average ± S.D. of three independent experiments performed in quadruplicate. $^{**}P < 0.01$, respect to each control value.

We focused our attention on E_1 transduction system, obtaining evidence that the mechanism of action by which this hormone regulates NOS activity differs from that reported for E_2 [4]. We found that E_1 increases PLC activity but PKC is not involved in NOS stimulation. Moreover, PI_3K/AKT system does not mediate E_1 action on COX and NOS, but it seems to be involved in PLC activation.

In agreement with our observations about the existence of a link between COX and NOS systems, different reports in the literature inform about cross talk between these enzymes in a variety of cells types. In cultured human umbilical vein cells (HUVEC) exposed to shear stress, NO appears to exert an inhibitory action on prostacyclin production [32]. In human pulmonary artery smooth muscle cells which were exposed to lipopolysacaride it was shown that NO enhances PGI_2 production [33]. Besides, previous studies in our laboratory with rat aortic tissue treated with progesterone, also showed the existence of a cross talk between NOS and COX systems [27]. Recently it has been informed studies on adenosine-induced NO release by rat aorta endothelium, which propose that PLA_2-COX activation and PGI_2 release produces PKA activation, eNOS phosphorylation, and the consequent increment in NO production [34]. Further studies to elucidate the metabolic pathway involved in the NOS/COX cross talk induced by E_1 are under current investigation.

In the present study we observed that 17-β-E_2 injection to OVX rats restores the tissue capacity to elicit a rapid increase of NO released in response to E_1 treatment. This recovery observed in OVX-IP was mediated by ERs, since NOS activation elicited by E_1 was abolished by ICI 182780. Rapid E_1 NOS stimulation in FR(+) was also dependent on ERs.

The fact that 17-β-E_2 administration to OVX rats restores tissue capacity to increase NO production in response to "in vitro" E_1 treatment made us to speculate that, endogenous E_2 levels could regulate the expression of NOS [35] or some key protein involved in E_1 NOS stimulation signal transduction. Reports in the literature indicate that E_2 upregulates eNOS in endothelial cells from different origins as described for human endothelial cell lines [36] and rat cerebral microvessels [37]. In mice cerebral microvessels, it was shown that E_2-induced eNOS upregulation mediated by ERs, observing also changes in COX abundance [38]. Otherwise, it has been demonstrated that in OVX as well as fertile rats, low doses of the selective ERs modulator EM-652, increased the expression and enzymatic activity of aortic eNOS [35].

In the present study we explored other metabolic signal transduction pathways involved in E_1 non-genomic action on rat aorta. Calcium influx and PKC activation has been related in vascular smooth muscle cells contraction [39]. Reports in literature inform that in RAS obtained from female OVX rats, or males treated with 17-β-E_2 implants showed a significant increase of vasorelaxation and PKC activity compared with untreated animals, that lead to the authors to conclude that endogenous estrogen would be responsible for the gender differences in vascular reactivity and PKC activity [40]. We studied if PLC–PKC signal transduction system is involved in rat aortic tissue response to E_1 treatment. We found that in RAS isolated from FR(+) rats, E_1 activates PLC system, and increase DAG production. Little is known about E_1 and PLC/PKC signal transduction cascade. Reports in literature show that PKC has a critical role in developing brain, mediating E_2 ERK phosphorylation [41]. In our experimental model E_1 stimulate PLC, but PKC activation seems not to affect NOS. We could not rule out the possibility that the activation of PLC/PKC could participate in a different signal cascade regulating gene expression at nuclear level.

Concerning to PI_3K/Akt signal transduction system, many reports in the literature inform about the participation of PI_3-kinase in response to E_2 treatment in different tissues, mediating either genomic or non-genomic effects of the hormone [42–47]. However, little is known about the responses to E_1. In the present study we show that neither COX nor NOS activation elicited by E_1 treatment depends on PI_3K–Akt. Concerning PI_3K and NOS, we previously informed, using the same experimental system, that PI_3K mediates the early phase of progesterone NOS activation, since pre-incubation of RAS with LY 294002 prevented the increment of NO production in response to 5 min progesterone treatment [48]. We found that, PI_3K participates in E_1 activation of PLC/PKC cascade. Our results show that aortic tissue response to E_1 treatment involves PI_3K only in PLC–PKC signal system and not in NOS activation (the classical mechanism described for E_2). This observation opens a new possibility that could relate the physiological role of PI_3K–PLC–PKC cascade in not only non-genomic, but also genomic effects of E_1 in vascular tissue.

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