Author's response to reviews

Title: Estrogen-Induced DNA Synthesis in Vascular Endothelial Cells is Mediated by ROS Signaling

Authors:

Quentin Felty (Feltyq@fiu.edu)

Version: 2 Date: 9 December 2005

Author's response to reviews: see over
Dear Biomed Central Editorial Team:

Thank you very much for the critical review of our manuscript “Estrogen-Induced DNA Synthesis in Vascular Endothelial Cells is Mediated by ROS Signaling” MS# 2079002372829839. Please find enclosed our revised manuscript. We have incorporated all the suggestions of the reviewers in the text. All changes in the revised manuscript are highlighted in yellow.

Reviewer: Ying-Tung Lau
Reviewer’s report:

--------------------------------------------------------------------------------------------------

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

Major Comments:

1. E2 is known to exert vascular protection (AJP 286:R233-R249; 2004 and Science 308:1583-1587; 2005 for recent review) and angiogenic actions (e.g., Circulation 91:755-763; 1995) with ROS implicated as a downstream mediators (e.g., JBC 277:3101-3108, 2002). The Introduction, however, only address the potential damaging effect of E2 (mostly in in vivo situations) without making a clear connection with the current in vitro study.

Response: We agree with the reviewer that most of the previous findings indicated cardioprotective action by estrogen [1]. We have included this sentence in the first line of the Introduction. And in Paragraph 1 of Introduction line 19-21 and Paragraph 2 of lines 13-15 we have added text to make a clear connection with the current in vitro study.

2. Under very high concentrations of NAC (10 mM) and ebselen (40 uM), ROS formation became less than control level (Figs. 2 and 3) in the presence of E2 suggesting that these antioxidants may exert other effects or may influence the control (without E2) level of ROS. Considering that survival of HUVECs are easily compromised, various drugs and antioxidants should be first tested on the survival, ROS formation, and DNA synthesis of HUVECs.

Response: We have shown previously that these levels of antioxidants are not cytotoxic [2]. Moreover, the data from 1mM NAC cotreatment with E2, which is equal to the basal control, level did not inhibit ROS production or DNA synthesis. The 1mM NAC completely counteracted the E2-induced ROS and DNA synthesis without affecting the basal levels of ROS and DNA synthesis. This clearly indicates that this dose of NAC does not influence the cell death or new DNA synthesis.

--------------------------------------------------------------------------------------------------
Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

Minor Comments:
1. The effects of E2 was relatively small, was there significant stimulation of E2 on DNA synthesis at all (Fig. 4 and 5)? It was not clearly stated in either the Results or the Legends.

   Response: In the results section (p. 5, 2\textsuperscript{nd} paragraph, line 4-5), we did include that E2 effect seen in figure 4 was significant. Lines stating the significant E2 stimulation of DNA synthesis were added to figure legends 4 and 5 (last line).

2. Results section in the Abstract read more like conclusion, specific findings should be presented instead.

   Response: We have revised the results section of the abstract as per suggestion of the reviewer. Also, elaborated on specific findings in results section (p. 4, last paragraph, line 3; p.5, 1\textsuperscript{st} paragraph, lines 1-3, 6-7, 9-12, 18-20; p. 5, 2\textsuperscript{nd} paragraph, line 6, 8-10, 11-15).

Discretionary Revisions (which the author can choose to ignore)
Reviewer: Evangelia Papadimitriou
Reviewer's report:

The manuscript by Dr Felty discusses the involvement of ROS in estrogen-induced DNA synthesis in endothelial cells. The possible involvement of ROS in estrogen signalling is interesting, however, this manuscript needs further experiments to support their findings.

First of all, the authors do not discuss at all a possible mechanism as to how estrogens increase the production of ROS. Could a membrane estrogen receptor be involved? They should check this possibility or at least discuss it.

Response: In the discussion (p. 7, 1st paragraph, line 1-2), we did describe that the source of the ROS was from mitochondria and xanthine oxidase because both rotenone and allopurinol inhibited ROS formation. We also have added a similar statement to results section (p. 5, 1st paragraph, lines 18-20). As requested, we discussed the possible mechanism of how ROS is produced (p. 7, 1st paragraph, line 3-10).

Since the authors claim that the effect of estrogen on DNA synthesis is not mediated by nuclear receptors, they should present data, having used an ER antagonist.

Response: Both NAC and Ebselen which counteracted the E2-induced DNA synthesis are not estrogen antagonists of estrogen receptor. This clearly indicates that the nonreceptor signaling is involved with the E2-induced DNA synthesis. Furthermore, we had described in the Discussion (please see p.6, lines 12-15) that estrogen receptor antagonists such as tamoxifen could not be used in this study because tamoxifen induces cellular oxidative stress [3] which would make it difficult to discern the source of the ROS. Estrogen receptor antagonists such as ICI182,780 and raloxifene act as antioxidants [4,5] therefore these compounds would not discern whether a reduction in E2-induced ROS generation is due to the blockage of the estrogen receptor or by the antioxidant activity of these antagonists.

What is the effect of allopurinol or rotenone on DNA synthesis?

Response: We did not test in this study however we have previously shown that rotenone inhibits estrogen-induced DNA synthesis [6].

The DCFH probe is not selective for hydrogen peroxide.

Response: We agree with the reviewer that DCFH probe is not specific for H2O2, however, it has been shown that DCFH is more sensitive to oxidation by H2O2 than superoxide anion. We have made the following revision to the text (p. 7, 2nd paragraph, lines 3-7). “Given that the DCFH probe is more sensitive toward oxidation by H2O2 than superoxide anion [7] and based on our results which show an increase in E2-induced ROS production (Figure 1) that can be blocked with H2O2 scavenging compounds NAC (Figure 2) and ebselen (Figure 3); the identity of the E2-induced oxidant appears to be H2O2.” Moreover, a rate constant of 68 M-1s-1 at pH=7.4 for the reaction of superoxide with NAC, suggest that the reaction of superoxide with NAC is insignificant at physiologic concentrations of superoxide [8].
The results should be described in more detail in the abstract.

Response: We have revised the results section of the abstract as per suggestion of the reviewer.
Reviewer: shampa chatterjee
Reviewer's report:

This paper reports on the increased proliferation of vascular endothelial cells treated with estrogen. Using the fluorescence dye H2DCF-DA for the detection of reactive oxygen species (ROS), the authors show that estrogen treatment causes ROS generation which then triggers DNA synthesis. In an earlier report this group (Felty et al. Biochemistry 2005, 44: 6900-9) had shown that the estrogen induced ROS acts as a signaling molecule activating oxidant sensitive transcription factors. This report concludes that endothelial cell growth or proliferation is not estrogen receptor dependent signaling but is ROS mediated. Although this is interesting there seems to be relatively little that is new in the present form. Some additional experiments need to be performed.

Major revisions

1. Where is the ROS produced? If it is mitochondrial as reported in their earlier paper how do the authors explain the inhibition by allopurinol? Is this ROS induced ROS generation? Also microscopic imaging of the cells after estrogen treatment by dihydrorhodamine 123 might show the localization of the ROS.

Response: Before we quantified the ROS levels in the treated cells, we did confocal imaging of cells with both DCFH (for ROS) and Mitotracker Red (for mitochondria). ROS was mostly localized (data not shown) in the mitochondria and cytoplasm. To measure superoxide radical formation we did use dihydroethidium and did not find any increase with the E2 treatment. Because of the very short life of superoxide we could not detect a change in the steady-state intracellular level of the superoxide radical. And this is in agreement with our previous findings [2].

2. The identity of the ROS involved in cell proliferation is not discussed. Data showing how the ROS production and DNA synthesis is affected in the presence of catalase and SOD should be included.

Response: Please see the response for reviewer #1. Our ongoing experiments using adenoviral vectors containing MnSOD and catalase supports our conclusion however we are not adding the data here because this is beyond the scope of this study.

3. Can the DNA synthesis be observed long after the estrogen dose? If it is a ROS signaling effect, a time course would indicate proliferation long after ROS production is stopped.

Response: We have reported that E2-induced DNA synthesis can be detected at 24h and 48h after treatment [6]. We performed a time course that showed an increase in the level of ROS production that was sustained for 90min (data not shown). Along with our observations of DNA synthesis after 18 h of E2 exposure, it indicates that the ROS signal is sustained long enough to stimulate DNA synthesis.
4. The authors observe increased DNA synthesis after estrogen treatment. However this observation by itself is not of high priority as it is well established that ROS triggers proliferation in most cell types. In addition to DNA synthesis how does ROS affect the transcription factors that control cell proliferation.

Response: We disagree with the reviewer that E2-induced ROS mediated stimulation of cell growth is well established. We were the first to show that physiological concentrations of E2 produced ROS signaling molecules that control G1/S phase transition in epithelial cells [2,6,7]. Before our paper there was only one other paper showing that ROS mediates cell cycle progression in fibroblast cells [9]. The present manuscript for the first time demonstrates that 17β-estradiol (E2)-induced ROS signaling is involved in the DNA synthesis of HUVEC cells.

5. Several papers point to the role of estrogen as an antioxidant. While the treatment time, concentrations etc. might differ between these studies this needs to be mentioned in the discussion section.

Response: The author is correct in pointing out that estrogen can act as an antioxidant however estrogen antioxidant activity occurs at high (µM) concentrations. We have added this to the Discussion please see Discussion (p. 5, lines 2-4).

“High concentrations of E2 (10 µM) have been shown to act as an antioxidants in vitro [10]. In contrast, our study used physiological concentrations of E2 (367 fmol and 3.67 pmol per ml medium) which do not act as antioxidants.”

Minor:
1. In the first part of the Introduction it should be stated that E2 is 17beta-estradiol-

Response: Made change see Introduction (last line)
Reviewer: Hua "Linda" Cai
Reviewer's report:

General
The author found that estrodiol induced DNA synthesis of endothelial cells is dependent on ROS signaling. The key thing is that is this ROS production transient? This is imporant as prolonged production of ROS is tissue damaging while transient production is often required for signaling. Thus time course data are required.

Response: Please see responses to Reviewers 1 and 2.

Also, DCF assay does not differentiate superoxide vs hydrogen peroxide. Amplex Red assay can be used for more specific detection of hydrogen peroxide to double verify major findings.

Response: Please see responses to Reviewers 1 and 2.

Also regarding time course again, nitric oxide along production of superoxide, whether simutateously produced or not, is importan in affecting the overall impact of estrogen on endothelial function. At lease discussion should be included though ideally some data should be included

Response: Made the following revision to Discussion (p. 6, 1st paragraph, line 10 )

Reference List


