FEBS Letters 581 (2007) 4891–4898

A novel ovary-specific and ovulation-associated variant of epoxide hydrolase 2

Ketty Shkolnik[a], Shifra Ben-Dor[b], Dalia Galiani[a], Ariel Hourvitz[c], Nava Dekel[a,*]

[a] *Department of Biological Regulation, Weizmann Institute of Science, Rehovot 76100, Israel*
[b] *Department of Biological Services, Weizmann Institute of Science, Rehovot 76100, Israel*
[c] *IVF Unit, Department of Obstetrics and Gynecology, Chaim Sheba Medical Centre, Tel-Hashomer, Israel*

Received 14 May 2007; revised 6 September 2007; accepted 10 September 2007

Available online 19 September 2007

Edited by Laszlo Nagy

Abstract **Ovulation is a complex process initiated by the surge of the pituitary luteinizing hormone (LH) that provokes the expression of specific genes. We report herein the isolation and characterization of an ovulation-associated, ovary-specific novel isoform of epoxide hydrolase 2 (Ephx2), Ephx2C. This variant is exclusively expressed in the granulosa cells of preovulatory mouse ovarian follicles. The LH-induced expression of Ephx2C is mediated by the protein kinase A and partially by the protein kinase C signaling pathways. The involvement of p38 kinase has also been demonstrated.**

Keywords: Ovulation; Ovary; Epoxide hydrolase 2

1. Introduction

The release of the female gamete from the ovary, known as ovulation, is a key event in mammalian reproduction. Successful ovulation is a complex process by which mature ovarian follicles respond to the surge of the pituitary luteinizing hormone (LH) and rupture to release fertilizable oocytes. LH acts on mature follicles to down-regulate the expression of genes associated with folliculogenesis and stimulates the up-regulation of other specific genes involved in ovulation. Genes known to be induced by LH during the ovulatory process include the progesterone receptor (PR), cycloosygenase-2 (COX-2), CAAT enhancer binding protein β (reviewed at [1]), amphiregulin [2], receptor-interacting protein 140 [3], endothelin-2 [4], and others recently documented by microarray analysis [5,6] and differential display reverse transcriptase polymerase chain reaction (RT-PCR) [7].

The function of some of the above mentioned genes as essential players in the ovulatory cascade has been identified, whereas, the role of others, the expression of which is up-regulated following the LH surge, awaits further investigation. Furthermore, identification of new crucial participants in this highly complex cascade leading to ovulation is of major significance. Along this line, we have previously applied the SSH method [8] in order to systematically isolate genes with an ovulation-selective pattern of expression [9]. In the present work, we employed bioinformatics search tools to select cDNA clones with a preference for those representing putative novel genes.

During preliminary characterization of the ovulatory-dependent novel cDNAs, one such clone, 4E4, was identified as a novel splice variant of epoxide hydrolase 2 (Ephx2), predicted to encode for a yet unknown isoform of the protein. Epoxide hydrolases are enzymes that convert epoxide substrates to their corresponding less potent vicinal diols [10]. One class of such substrates for the EPHX2 protein includes epoxyeicosatrienoic acids (EETs), molecules generated by cytochrome P450 epoxidation of arachidonic acid. EETs and/or EET diols have been shown to induce changes in a variety of events associated with ovulation, such as theca internal blood flow [11], prostaglandin E2 production [12], COX-2 expression [13], estradiol biosynthesis [14], and plasminogen activator expression [15]. For that reason, one could anticipate dynamic changes in EET production in the hours preceding ovulation. Moreover, a recently published paper has reported the existence of an additional variant of Ephx2, Ephx2B, whose expression is also regulated by LH/hCG [16].

We herein report of identification of an ovary-restricted ovulation-associated novel isoform of Ephx2, Ephx2C.

2. Materials and methods

2.1. Animals

Sexually immature 24-day-old female C57BL/6 mice were purchased from Harlan (Harlan Laboratories, Rehovot, Israel) and handled at the Animal Breeding Center of the Weizmann Institute of Science. All experiments were conducted in accordance with the NIH Guidelines. The animals were subcutaneously injected with 5 IU pregnant mare's serum gonadotropin (PMSG) (Chrono-gest Intervest), followed by 5 IU human chorionic gonadotropin (hCG) (N.V. Organon Oss), intraperitoneally injected 48 h later. The mice were killed by cervical dislocation; the ovaries were removed at different time points before and after hCG administration and incubated in RNA Later (Ambion) for RNA purification.

*Corresponding author. Fax: +972 8 9344186.
E-mail address: nava.dekel@weizmann.ac.il (N. Dekel).

Abbreviations: LH, luteinizing hormone; RT-PCR, reverse transcriptase polymerase chain reaction; SSH, suppression subtractive hybridization method; EETs, epoxyeicosatrienoic acids; hCG, human chorionic gonadotropin; PMSG, pregnant mare's serum gonadotropin; MML-V, moloney murine leukemia virus; FSK, forskolin; TPA, tetradecanoyl phorbol acetate; BLASTn, basic local alignment search tool nucleotide; BLAT, BLAST-like alignment tool; RACE, rapid amplification of the cDNA ends; ORF, open reading frame; PKC, protein kinase C; PKA, protein kinase A; PLC, phospholipase C; DAG, diacyl glycerol

0014-5793/$32.00
doi:10.1016/j.febslet.2007.09.016

2.2. RNA extraction and cDNA preparation

Standard protocols were used, and details are provided in Supplemental data.

2.3. Relative Real-time PCR

4E4 primers were designed using *Beacon designer* software (Bio-Rad) and analyzed by BLAST-like alignment tool (BLAT) for their specificity (Table 1, Supplemental data). All Real-time PCRs were carried out using the iCycler iQ Detection System (Bio-Rad) and the DyNAmo SYBR Green qPCR Kit (Finnzymes) according to the manufacturer's instructions. Details of the reaction protocols are provided in Supplemental data.

2.4. Full-length cDNA sequence determination (RACE)

The 5^0 and 3^0 ends of the 4E4 cDNA were determined by the SMART rapid amplification of the cDNA ends (RACE) cDNA Amplification Kit (Clontech), according to the manufacturer's instructions. PCR products were purified, cloned and sequenced as detailed in Supplemental data.

2.5. Multiple tissue array

Different tissues were isolated from two immature PMSG/hCG-treated (5 h after hCG administration) as well as from two untreated female mice. Additional tissues were isolated from two sexually mature and two immature male C57BL/6 mice. Animals were perfused with cold PBS solution (Invitrogen life technologies) prior to tissue dissection. Total RNA was isolated and semi-quantitative RT-PCR amplification of the designated splice variants was performed (35 cycles for each variant, 24 cycles for β-actin). The resultant PCR products were separated on a 1.5% agarose gel stained with ethidium bromide.

2.6. In situ hybridization

Protocol for in situ hybridization is provided in the Supplemental data.

2.7. Culture of follicles

Isolated intact, large antral ovarian follicles were recovered from sexually immature PMSG-primed 25-day-old female C57BL mice and grown in suspension of Leibovitz's L-15 tissue culture medium containing 5% fetal bovine serum (Biological Industries, Kibbutz Beit Hemeek, Israel) in 25-ml flasks gassed with 50% O_2 + 50% N_2. Incubations were carried out at 37 °C in an oscillating water bath with or without 1 μg/ml ovine LH (National Institutes of Health LH S-24) in the presence or absence of one of the reagents designated in Fig. 5 and detailed in Supplemental data.

3. Results and discussion

3.1. The expression of 4E4 is transiently elevated in an ovulation-associated manner

We have previously reported that using the differential screening technique SSH [9] we isolated 252 cDNA clones that are either selectively or exclusively expressed during ovulation in the mouse ovary. In the present study, we employed a BLASTn analysis in combination with BLAT to select cDNA clones with a preference for those representing putative novel genes.

One such novel cDNA clone, 4E4, was selected for further validation of its ovulation-associated expression pattern. Real-time PCR analysis of ovarian cDNA demonstrated a 15-fold increase of the 4E4 transcript expression at 4 h after hCG administration. This elevation was sustained up to the time of ovulation (12–14 h after hCG treatment) and dropped to near baseline levels at 24–48 h after hCG injection (Fig. 1). This pattern of 4E4 expression suggests a possible function for this gene during the ovulatory process.

3.2. Ephx2C – a novel isoform of Ephx2

Initial characterization of the 4E4 clone by BLAT revealed a perfect match between the last 9 bp of 4E4 and the first 9 bp of exon 3 of the Ephx2 gene; the majority of the sequence has no homology to any sequence entries, however it aligned perfectly to the genome directly upstream of Ephx2 exon 3. This implied that 4E4 is part of a new exon of a novel Ephx2 splice variant.

Since SSH yields only partial cDNA fragments (393 bp for 4E4), RACE was employed on ovarian cDNA to isolate the full-length sequence. In response to hCG treatment, this genomic region is highly transcriptionally active, and several RACE products were detected. Four distinct new splice variants of the gene were isolated. We chose to characterize the major transcript, which also had the longest open reading frame, and the strongest response to hCG (data not shown). The 5^0 and 3^0 RACE products of the major isoform, 350 bp and 2000 bp, respectively (Fig. 2A) were isolated, cloned and sequenced. The full-length cDNA sequence (Fig. 2B) was sub-

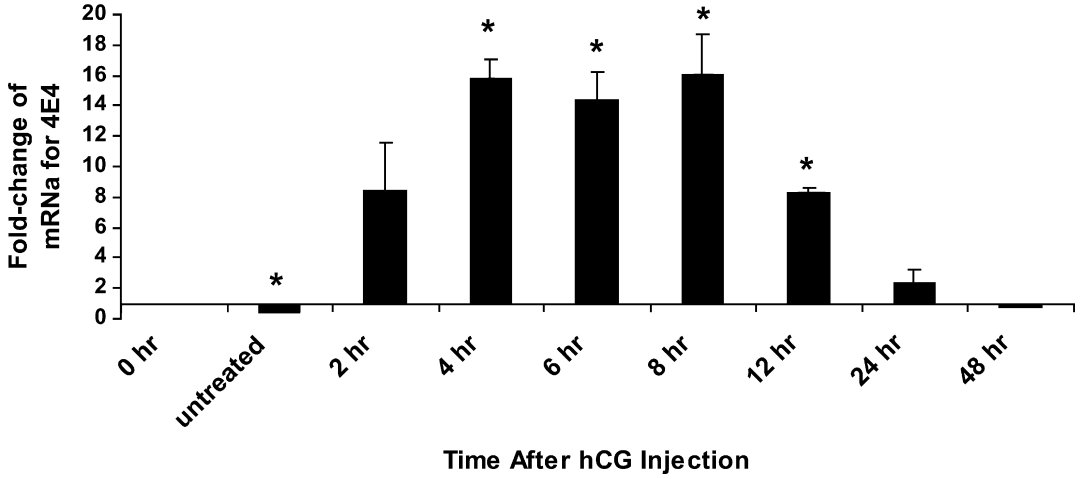
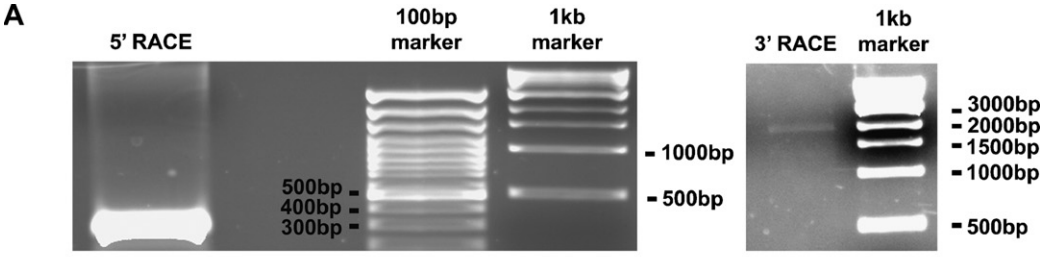


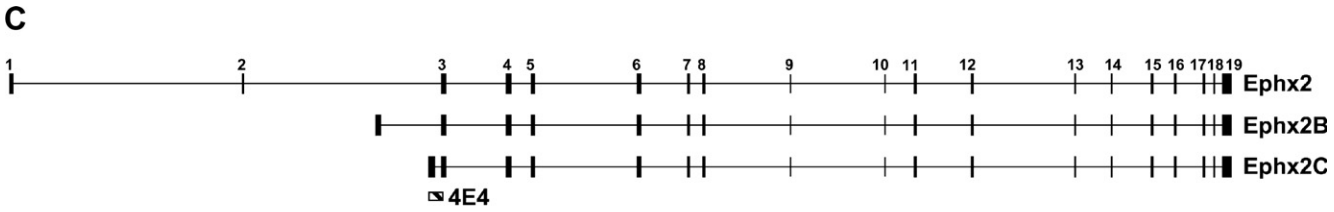
Fig. 1. Ovulation-associated expression pattern of the novel 4E4 clone by Real-time PCR. Ovarian RNA was isolated from untreated, control (0 h, PMSG-primed, no hCG), and PMSG-primed mice at the designated time points after hCG administration. The mRNA expression is relative to the control. Error bars represent the standard error of the mean ($n = 3$ independent in vivo experiments). Columns with asterisks are significantly different ($P < 0.05$) relative to control.



B

```
   1 GGGTCTCTCCTCTGGCCTGCATGCTGAGAGGGGAGTCCCCAACATGTTGGATCAATAAAA
  61 AATCCTCATGTGGTTTGCAGCGATGGCAGCGGTCTCTGTGTTCTTTGTGAGTGAGGGTCT
 121 TTTGACAAACTCCAACACAGCAACTCTGTCTGCATCTTGACTTTGGTCTTCACAGACTTC
 181 GGAATTGTGGGAAAGGAATTTACCCCGCTTCTGGGCTCCCCAGCTTCTGTTACTTTGGTG
 241 CAGCCCAGTGGGTACCACTCATGGATGAAAGCTACAGGAAGTCCTCCAAAGCCTGTGGAG
                         M  D  E  S  Y  R  K  S  S  K  A  C  G
 301 CCAATCTACCTGAGAATTTCTCCATAAGTCAAATATTCAGCCAAGCTATGGCAGCAAGAA
     A  N  L  P  E  N  F  S  I  S  Q  I  F  S  Q  A  M  A  A  R
 361 GCATCAACCGCCCCATGCTTCAGGCAGCCATTGCTCTCAAAAAGAAAGGATTCACAACAT
     S  I  N  R  P  M  L  Q  A  A  I  A  L  K  K  K  G  F  T  T
 421 GCATTGTCACCAACAACTGGCTGGACGACGGAGACAAGAGAGACAGCCTGGCCCAGATGA
     C  I  V  T  N  N  W  L  D  D  G  D  K  R  D  S  L  A  Q  M
 481 TGTGTGAGCTGAGCCAACACTTTGACTTCCTGATAGAGTCCTGTCAGGTTGGGATGATCA
     M  C  E  L  S  Q  H  F  D  F  L  I  E  S  C  Q  V  G  M  I
 541 AGCCTGAGCCTCAGATCTACAATTTTTTACTGGATACCCTGAAGGCAAAACCCAATGAGG
     K  P  E  P  Q  I  Y  N  F  L  L  D  T  L  K  A  K  P  N  E
 601 TTGTTTTCCTAGATGACTTTGGAAGTAATCTGAAGCCAGCCCGTGACATGGGGATGGTTA
     V  V  F  L  D  D  F  G  S  N  L  K  P  A  R  D  M  G  M  V
 661 CCATCCTGGTCCACAACACAGCCTCCGCTCTGAGAGAACTGGAGAAGGTCACAGGGACAC
     T  I  L  V  H  N  T  A  S  A  L  R  E  L  E  K  V  T  G  T
 721 AGTTTCCTGAGGCCCCACTGCCAGTCCCATGCAATCCAAATGACGTCAGCCATGGATATG
     Q  F  P  E  A  P  L  P  V  P  C  N  P  N  D  V  S  H  G  Y
 781 TGACAGTGAAGCCAGGGATCCGCCTGCATTTTGTGGAGATGGGCTCTGGCCCTGCCCTAT
     V  T  V  K  P  G  I  R  L  H  F  V  E  M  G  S  G  P  A  L
 841 GCCTTTGCCATGGGTTTCCTGAGAGCTGGTTTTCTTGGAGGTACCAGATCCCTGCTCTGG
     C  L  C  H  G  F  P  E  S  W  F  S  W  R  Y  Q  I  P  A  L
 901 CCCAGGCAGGCTTTCGTGTTCTGGCTATAGACATGAAAGGCTATGGAGACTCATCTTCTC
     A  Q  A  G  F  R  V  L  A  I  D  M  K  G  Y  G  D  S  S  S
 961 CTCCTGAAATAGAAGAATATGCCATGGAATTGCTGTGTAAGGAGATGGTGACATTCCTGG
     P  P  E  I  E  E  Y  A  M  E  L  L  C  K  E  M  V  T  F  L
1021 ATAAGCTGGGAATCCCTCAAGCAGTGTTCATTGGCCATGACTGGGCTGGTGTGATGGTGT
     D  K  L  G  I  P  Q  A  V  F  I  G  H  D  W  A  G  V  M  V
1081 GGAACATGGCTCTCTTCTACCCTGAGAGAGTGAGGGCTGTGGCCAGTTTGAACACGCCGT
     W  N  M  A  L  F  Y  P  E  R  V  R  A  V  A  S  L  N  T  P
1141 TTATGCCACCAGATCCTGATGTGTCTCCCATGAAAGTTATCCGATCTATCCCAGTTTTCA
     F  M  P  P  D  P  D  V  S  P  M  K  V  I  R  S  I  P  V  F
1201 ATTATCAGCTGTACTTTCAAGAACCAGGAGTGGCCGAGGCTGAACTGGAGAAGAACATGA
     N  Y  Q  L  Y  F  Q  E  P  G  V  A  E  A  E  L  E  K  N  M
1261 GTCGGACTTTCAAAAGCTTCTTCCGAGCCAGTGATGAGACAGGTTTCATCGCTGTGCATA
     S  R  T  F  K  S  F  F  R  A  S  D  E  T  G  F  I  A  V  H
1321 AAGCCACTGAAATAGGGGGAATCCTTGTGAATACTCCAGAAGATCCCAACCTCAGCAAAA
     K  A  T  E  I  G  G  I  L  V  N  T  P  E  D  P  N  L  S  K
1381 TTACTACTGAGGAAGAAATAGAGTTTTACATACAGCAGTTCAAGAAGACTGGCTTCAGAG
     I  T  T  E  E  E  I  E  F  Y  I  Q  Q  F  K  K  T  G  F  R
1441 GTCCTCTGAACTGGTACCGGAACACAGAAAGAAACTGGAAGTGGAGCTGTAAAGGGTTGG
     G  P  L  N  W  Y  R  N  T  E  R  N  W  K  W  S  C  K  G  L
1501 GACGAAAGATCTTGGTCCCAGCCCTGATGGTCACAGCTGAGAAGGACATTGTACTCCGTC
     G  R  K  I  L  V  P  A  L  M  V  T  A  E  K  D  I  V  L  R
1561 CTGAAATGTCCAAGAACATGGAAAAGTGGATCCCTTTCCTGAAAAGGGGACACATTGAAG
     P  E  M  S  K  N  M  E  K  W  I  P  F  L  K  R  G  H  I  E
1621 ACTGTGGTCACTGGACACAGATAGAGAAACCAACTGAGGTGAACCAGATTCTCATCAAGT
     D  C  G  H  W  T  Q  I  E  K  P  T  E  V  N  Q  I  L  I  K
1681 GGCTGCAGACTGAAGTCCAGAACCCATCAGTGACCTCCAAGATTTAGCCACTGGGGACAC
     W  L  Q  T  E  V  Q  N  P  S  V  T  S  K  I  *
1741 ATTTTAGTTTCTGGAACACAGCCTGATCTACAAGTACCAGCATCGTTCTTTTGCCAGCCA
1801 GTGATTTTNTTTTAAATGAAAATGATGGGATGAGATGTAATTTTAGATCGGGAAGAGAGT
1861 GTGTGTCTAATTCTTTTGAGTATGCCTGTGCCATCAGAGAAGAGATCCCACCCCAGTAGG
1921 AAGGTATGGGGCAGTCCAGTTTATAACTTTGCAACCAAACCCAAGCCTGCTCTTTTTGAA
1981 GCAGCTGATTGGAGAGTAAAGATTTTCATTCAATAAAGCTAAACCTCAGGGCAAAAAAAA
2041 AAAAAAAAAAAAAAAAAAA
```

Fig. 2. The full-length cDNA sequence of clone 4E4, as determined by RACE, corresponds to a novel isoform of soluble epoxide hydrolase. (A) The PCR products from the 5^0 and 3^0 RACE reactions. (B) Nucleotide and deduced amino acid sequence of Ephx2C. Marked nucleotide sequences represent primer sequences used for RACE analysis. (C) Genomic structure of the Ephx2 isoforms. Ephx2 is located on the mouse genome (NCBI version 36) chr14:65,038,487–65,078,610. The location of 4E4 is also shown. (D) Amino acid alignment of Ephx2, Ephx2B and Ephx2C proteins. Symbols: ( ) indicate the key catalytic Asp residue required for lipid phosphatase activity, (d) divalent cation or phosphate binding amino acids, and (r) catalytic triad necessary for epoxide hydrolase activity. Arrow indicates approximate junction between phosphatase and epoxide hydrolase domains (as detailed in [16]).



D

```
Ephx2     MALRVAAFDLDGVLALPSIAGAFRRSEEALALPRDFLLGAYQTEFPEGPT
Ephx2B    ..................MRFAAMAAFSVFFVSKGLLMNSNIWCVGQEGP
Ephx2C    ..................................................

Ephx2     EQLMKGKITFSQWVPLMDESYRKSSKACGANLPENFSISQIFSQAMAARS
Ephx2B    SQEDTDTIHTSEWVPLMDESYRKSSKACGANLPENFSISQIFSQAMAARS
Ephx2C    ................MDESYRKSSKACGANLPENFSISQIFSQAMAARS

Ephx2     INRPMLQAAIALKKKGFTTCIVTNNWLDDGDKRDSLAQMMCELSQHFDFL
Ephx2B    INRPMLQAAIALKKKGFTTCIVTNNWLDDGDKRDSLAQMMCELSQHFDFL
Ephx2C    INRPMLQAAIALKKKGFTTCIVTNNWLDDGDKRDSLAQMMCELSQHFDFL

Ephx2     IESCQVGMIKPEPQIYNFLLDTLKAKPNEVVFLDDFGSNLKPARDMGMVT
Ephx2B    IESCQVGMIKPEPQIYNFLLDTLKAKPNEVVFLDDFGSNLKPARDMGMVT
Ephx2C    IESCQVGMIKPEPQIYNFLLDTLKAKPNEVVFLDDFGSNLKPARDMGMVT

Ephx2     ILVHNTASALRELEKVTGTQFPEAPLPVPCNPNDVSHGYVTVKPGIRLHF
Ephx2B    ILVHNTASALRELEKVTGTQFPEAPLPVPCNPNDVSHGYVTVKPGIRLHF
Ephx2C    ILVHNTASALRELEKVTGTQFPEAPLPVPCNPNDVSHGYVTVKPGIRLHF

Ephx2     VEMGSGPALCLCHGFPESWFSWRYQIPALAQAGFRVLAIDMKGYGDSSSP
Ephx2B    VEMGSGPALCLCHGFPESWFSWRYQIPALAQAGFRVLAIDMKGYGDSSSP
Ephx2C    VEMGSGPALCLCHGFPESWFSWRYQIPALAQAGFRVLAIDMKGYGDSSSP

Ephx2     PEIEEYAMELLCKEMVTFLDKLGIPQAVFIGHDWAGVMVWNMALFYPERV
Ephx2B    PEIEEYAMELLCKEMVTFLDKLGIPQAVFIGHDWAGVMVWNMALFYPERV
Ephx2C    PEIEEYAMELLCKEMVTFLDKLGIPQAVFIGHDWAGVMVWNMALFYPERV

Ephx2     RAVASLNTPFMPPDPDVSPMKVIRSIPVFNYQLYFQEPGVAEAELEKNMS
Ephx2B    RAVASLNTPFMPPDPDVSPMKVIRSIPVFNYQLYFQEPGVAEAELEKNMS
Ephx2C    RAVASLNTPFMPPDPDVSPMKVIRSIPVFNYQLYFQEPGVAEAELEKNMS

Ephx2     RTFKSFFRASDETGFIAVHKATEIGGILVNTPEDPNLSKITTEEEIEFYI
Ephx2B    RTFKSFFRASDETGFIAVHKATEIGGILVNTPEDPNLSKITTEEEIEFYI
Ephx2C    RTFKSFFRASDETGFIAVHKATEIGGILVNTPEDPNLSKITTEEEIEFYI

Ephx2     QQFKKTGFRGPLNWYRNTERNWKWSCKGLGRKILVPALMVTAEKDIVLRP
Ephx2B    QQFKKTGFRGPLNWYRNTERNWKWSCKGLGRKILVPALMVTAEKDIVLRP
Ephx2C    QQFKKTGFRGPLNWYRNTERNWKWSCKGLGRKILVPALMVTAEKDIVLRP

Ephx2     EMSKNMEKWIPFLKRGHIEDCGHWTQIEKPTEVNQILIKWLQTEVQNPSV
Ephx2B    EMSKNMEKWIPFLKRGHIEDCGHWTQIEKPTEVNQILIKWLQTEVQNPSV
Ephx2C    EMSKNMEKWIPFLKRGHIEDCGHWTQIEKPTEVNQILIKWLQTEVQNPSV

Ephx2     TSKI
Ephx2B    TSKI
Ephx25    TSKI
```

Fig. 2 (*continued*)

jected to BLAT analysis and found to possess significant similarity to the Ephx2 gene (NM_007940), as well as to a recently identified isoform of this gene, Ephx2B (AY098585) [16] (Fig. 2C). Based on the significant level of overlap between these sequences, the ovulation-selective cDNA sequence has been referred to as Ephx2C (EF597241). It is a new splice variant, with a unique 5^0 exon, distinct from the first exons of the previously identified isoforms (Fig. 2C). From the full-length Ephx2C cDNA sequence, a single open reading frame was identified that can encode for a protein of 488 amino acids (Fig. 2D). The putative protein products of Ephx2, Ephx2B and Ephx2C overlap through most of their length and differ only within the N-terminus (Fig. 2D). The segment of the protein conserved between the three isoforms contains the catalytic domain responsible for epoxide hydrolase activity [17].

3.3. Tissue expression profile of Ephx2, Ephx2B and Ephx2C

To assess the specificity of expression of the various Ephx2 isoforms in diverse mouse tissues, RNA was extracted and subjected to semi-quantitative RT-PCR analysis using specific primers for each splice variant. The forward primer for each PCR product corresponded to the unique first exon of each

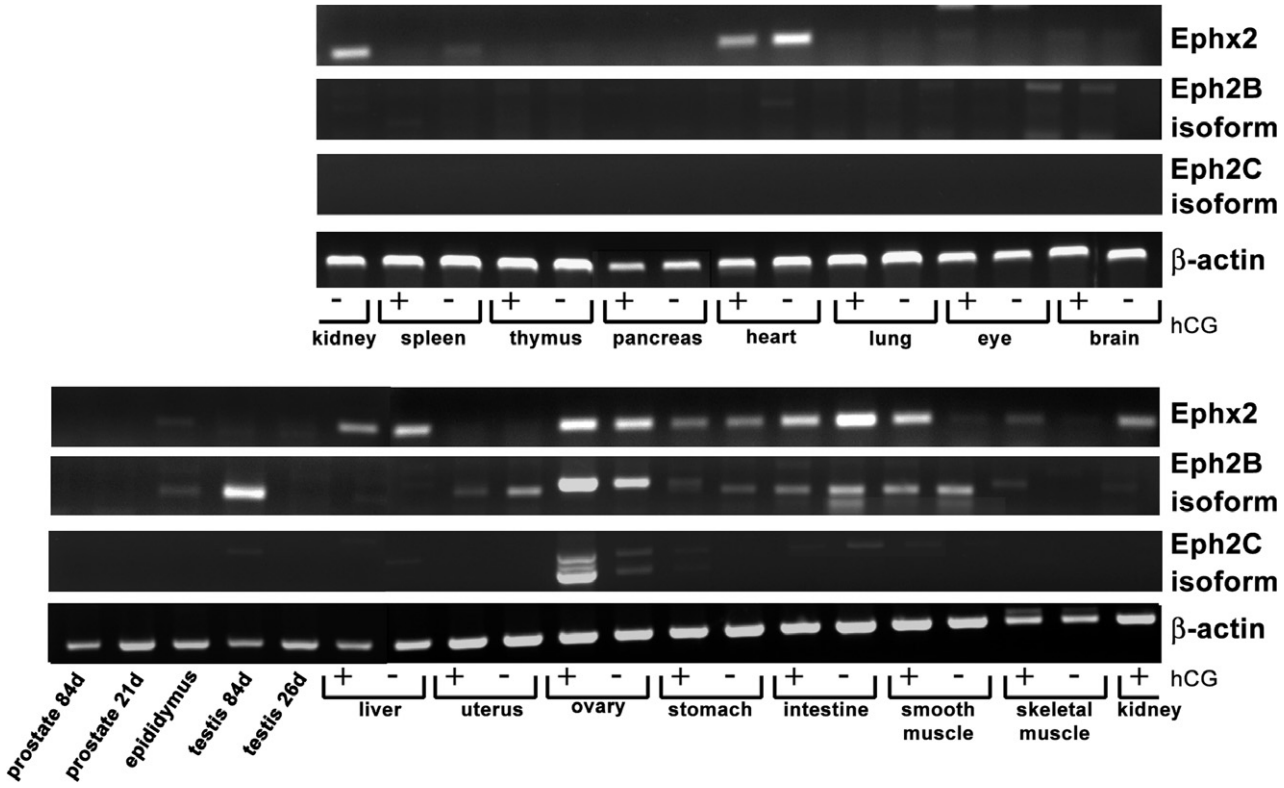


Fig. 3. Ovary-specific and ovulation-associated expression profile of the novel Ephx2C isoform. Tissue expression profile of Ephx2, Ephx2B and the novel Ephx2C isoform in female and male mouse tissues. A representative experiment is shown ($n = 3$). In the Ephx2B panel, the upper band is the published isoform, and in the Ephx2C panel, the lower band is the published isoform.

isoform (details and primer sequence in Supplementary data). The primers for Ephx2C can also identify one of the additional splice variants isolated by us, which is 136 bp longer. To ensure the specificity of the correct band, all the PCR products were sequenced. As shown in Fig. 3, Ephx2C expression was detected exclusively in ovarian tissue with a significant increase following hCG administration, whereas the Ephx2, as expected, had a wide range of tissue expression. Ephx2B was highly abundant in ovarian tissue; however, its expression is detected in other tissues as well (Fig. 3). This is inconsistent with the previously reported ovary-selective expression [16]. Importantly, the mRNA level of the Ephx2B isoform was elevated to a much lesser extent as compared to the Ephx2C isoform that showed a dramatic increase in expression following hCG administration.

These results indicate that we have identified a new ovary-specific and ovulation-associated isoform of Ephx2. Overall, the variability in tissue expression of the three isoforms suggests a distinct role of each isoform in the particular tissues.

3.4. Spatial characterization of Ephx2C expression pattern in the mouse ovary

Using a specific riboprobe for the Ephx2C variant, which cannot detect any of the other splice variants, in situ hybridization was performed. These experiments revealed that the message encoding Ephx2C is expressed in both granulosa and cumulus cells of the large antral follicles, with no detectable signal in either the oocyte or the theca cells (Figs. 4B and D). Time course analysis confirmed that Ephx2C mRNA expression increases from undetectable levels at 48 h after PMSG (Fig. 4E) to high levels at 8 h after hCG (Figs. 4B and D) followed by a drop to undetectable levels at 24 h after hCG administration (Fig. 4F). To note, it was previously shown that the Ephx2 and Ephx2B isoforms are also expressed in the granulosa cell compartment of the ovary prior to ovulation but at non-overlapping phases [16].

3.5. Characterization of the signaling cascade leading to up-regulation of Ephx2 isoforms

It was previously shown that LH activates both the protein kinase A (PKA) and the protein kinase C (PKC) signaling pathways to induce the expression of preovulatory genes, such as COX-2 [18] and PR [19]. To investigate which intracellular signaling pathways are involved in the LH-induced up-regulation of Ephx2C mRNA, we by-passed the hormone receptor interaction by activating down-stream regulators of the LH signaling cascade. In addition, in a set of complementary experiments, we employed specific inhibitors of these signaling pathways. These experiments were performed in vitro on isolated intact large antral ovarian follicles. Real-time PCR analysis was performed with primers that were specific to each isoform.

As shown in Fig. 5A, LH stimulated a substantial increase in Ephx2B and Ephx2C mRNA expression in these follicles ($P < 0.05$). Forskolin (FSK), an activator of adenylate cyclase

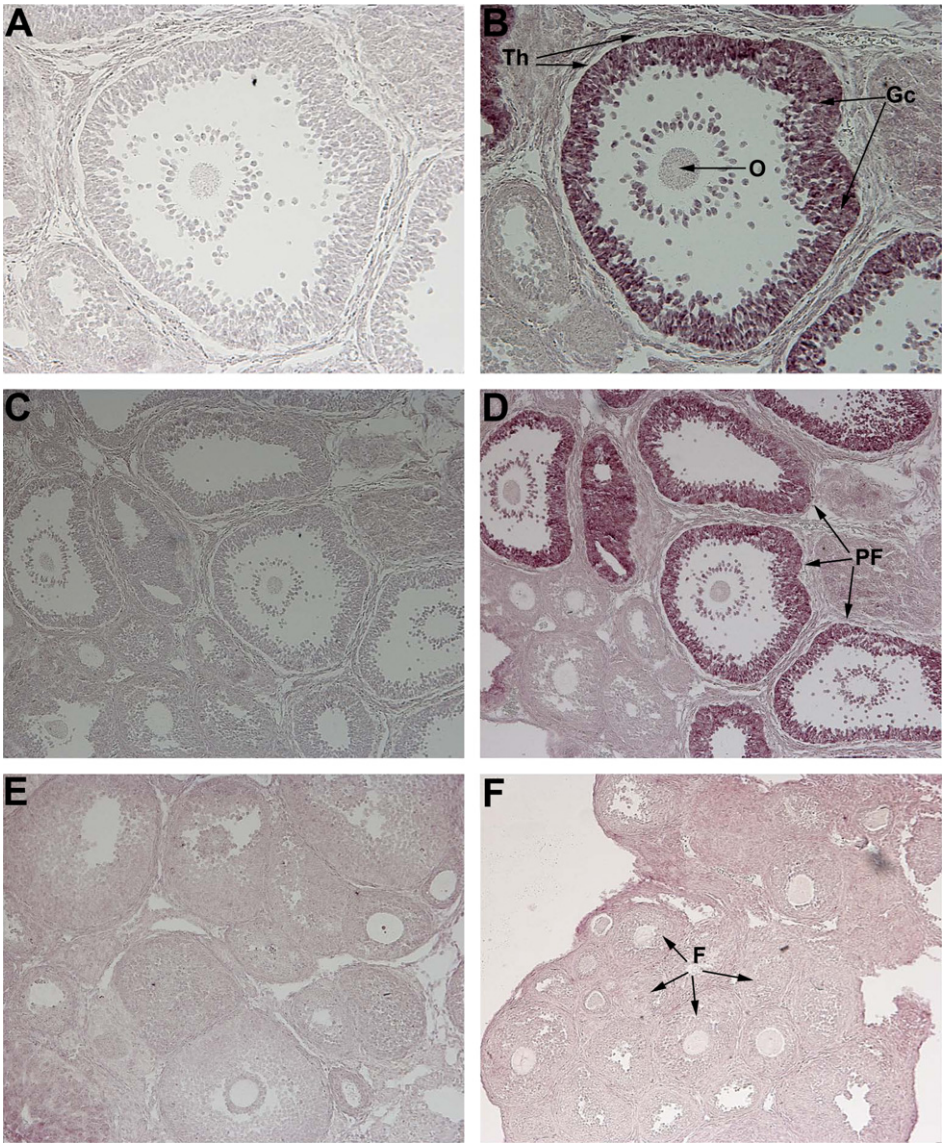


Fig. 4. Localization of Ephx2C mRNA in the ovary by in situ hybridization. (A+C) Sense probe hybridization (B+D) Antisense probe hybridization. (A–D): tissues were isolated 8 h after hCG administration. E, no hCG treatment; F, 24 h after hCG administration. Gc, granulosa cell layer; Th, theca cell layer; O, oocyte; PF, preovulatory follicles; F, follicles.

(AC) elevated the expression of these isoforms at levels similar to that of LH, whereas the PKC activator, tetradecanoyl phorbol acetate, stimulated the Ephx2C expression to a much lesser extent, with no effect on Ephx2B expression (Fig. 5A). In agreement with the above findings the effect of LH on Ephx2B and Ephx2C mRNA expression was prevented by H89, a specific inhibitor of PKA, whereas the inhibitor of PKC, GF109203, exhibited only a partial effect on LH action (Figs. 5A and B). Ephx2 mRNA expression was not stimulated by LH, and seems not to be regulated by any of these pathways.

It was previously published [20] that in preovulatory granulosa cells hCG induces the activation of MEK and p42/44 MAPK through the cAMP/PKA-dependent pathway, but also stimulates p38-kinase phosphorylation independently of PKA and PKC. Indeed, UO126, a specific inhibitor of MEK, reduced the mRNA expression of Ephx2B and Ephx2C almost completely. SB202190, a specific inhibitor of the p38 MAPK, inhibited Ephx2C almost completely, while showing a much lesser effect on Ephx2B.

Taken together, our results indicate that the LH-induced mRNA expression of Ephx2 isoforms is mediated by various intracellular signaling molecules, including AC, PKA, MEK, PKC and p38 kinase. However, there are differences between the various isoforms: Ephx2 expression is not stimulated at all by LH; Ephx2B expression is stimulated by the PKA pathway, and to a much lesser extent by p38 MAPK; Ephx2C expression is stimulated equally by PKA and p38 MAPK, and in addition, it is partially stimulated by PKC (Fig. 5B).

To conclude, Ephx2C is a novel ovary specific isoform of Ephx2 that is distinct from both Ephx2 and Ephx2B and is highly expressed in response to the ovulatory stimulus. Its LH-induced mRNA expression involves the PKA and p38 MAPK pathways, and to a lesser extent PKC. More studies

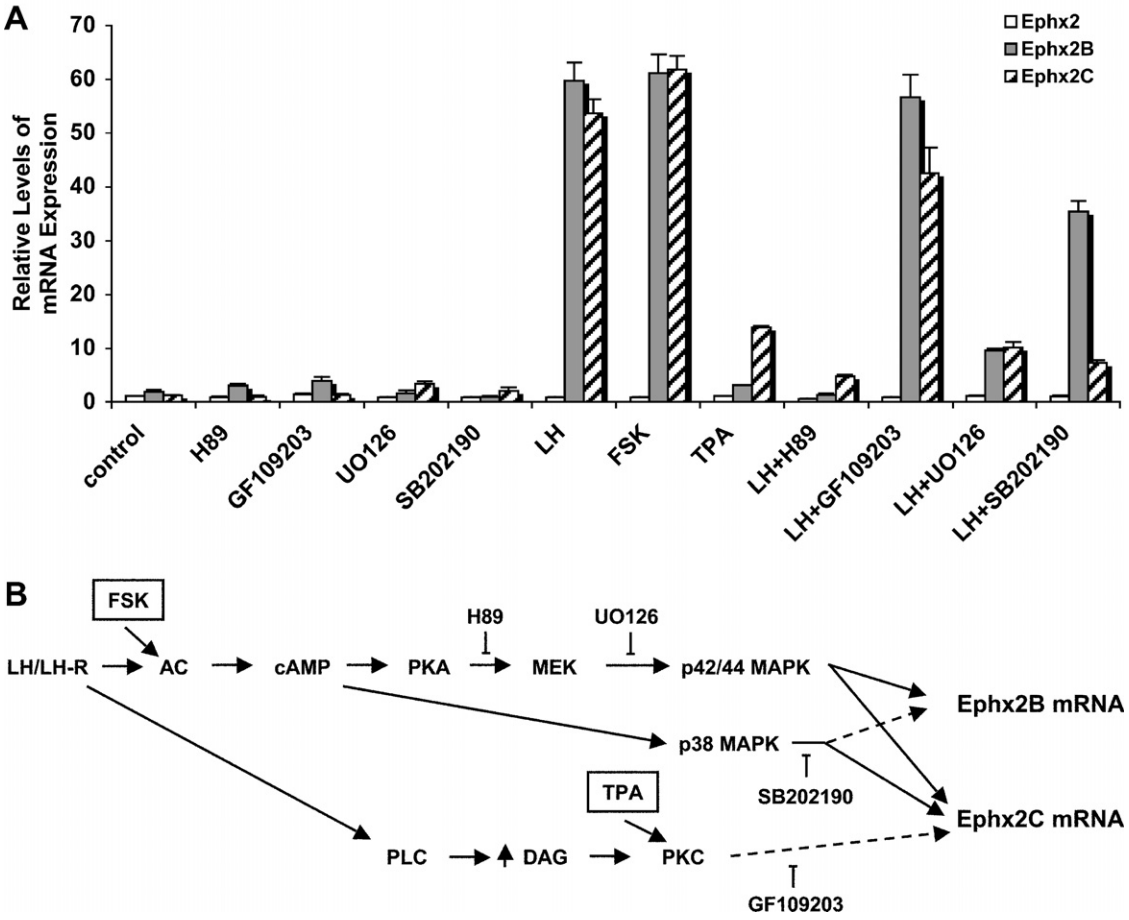


Fig. 5. Regulation of Ephx2C mRNA expression in murine follicles in vitro. (A) Follicles were incubated with various activators or inhibitors of either PKA, PKC or MAPK signaling pathways, and expression levels of Ephx2, Ephx2B, and Ephx2C mRNA were assessed by Real-time PCR. Error bars represent the standard error of the mean (n = 3–6 independent culture experiments; each sample included follicles from 3 to 4 mice). (B) Schematic presentation of the results. Full arrows – full activation/suppression, dashed arrows – partial activation/suppression.

are needed to elucidate the exact role of this novel isoform in ovulation.

Acknowledgements: The authors would like to thank Dr. N. Nevo for animal handling for the tissue array assay. This study was supported in part by grants from the Center for Women's Health Research, WIS Chief Scientist Office, Israel ministry of Health; the Israel Science foundation (682/04); and the Dwek Fund for Biomedical Research. N. Dekel is the incumbent of the Philip M. Klutznick Professional Chair in Developmental Biology.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.09.016.

References

[1] Richards, J.S., Russell, D.L., Ochsner, S. and Espey, L.L. (2002) Ovulation: new dimensions and new regulators of the inflammatory-like response. Annu. Rev. Physiol. 64, 69–92.
[2] Park, J.Y., Su, Y.Q., Ariga, M., Law, E., Jin, S.L. and Conti, M. (2004) EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science 303, 682–684.
[3] Tullet, J.M., Pocock, V., Steel, J.H., White, R., Milligan, S. and Parker, M.G. (2005) Multiple signaling defects in the absence of RIP140 impair both cumulus expansion and follicle rupture. Endocrinology 146, 4127–4137.
[4] Ko, C., Gieske, M.C., Al-Alem, L., Hahn, Y., Su, W., Gong, M.C., Iglarz, M. and Koo, Y. (2006) Endothelin-2 in ovarian follicle rupture. Endocrinology 147, 1770–1779.
[5] Jo, M., Gieske, M.C., Payne, C.E., Wheeler-Price, S.E., Gieske, J.B., Ignatius, I.V., Curry Jr., T.E. and Ko, C. (2004) Development and application of a rat ovarian gene expression database. Endocrinology 145, 5384–5396.
[6] Leo, C.P., Pisarska, M.D. and Hsueh, A.J. (2001) DNA array analysis of changes in preovulatory gene expression in the rat ovary. Biol. Reprod. 65, 269–276.
[7] Espey, L.L. and Richards, J.S. (2002) Temporal and spatial patterns of ovarian gene transcription following an ovulatory dose of gonadotropin in the rat. Biol. Reprod. 67, 1662–1670.
[8] Diatchenko, L., Lau, Y.F., Campbell, A.P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E.D. and Siebert, P.D. (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc. Natl. Acad. Sci. USA 93, 6025–6030.
[9] Hourvitz, A., Gershon, E., Hennebold, J.D., Elizur, S., Maman, E., Brendle, C., Adashi, E.Y. and Dekel, N. (2006) Ovulation-selective genes: the generation and characterization of an ovulatory-selective cDNA library. J. Endocrinol. 188, 531–548.
[10] Newman, J.W., Morisseau, C. and Hammock, B.D. (2005) Epoxide hydrolases: their roles and interactions with lipid metabolism. Prog. Lipid. Res. 44, 1–51.
[11] Brannstrom, M., Zackrisson, U., Hagstrom, H.G., Josefsson, B., Hellberg, P., Granberg, S., Collins, W.P. and Bourne, T. (1998)

Preovulatory changes of blood flow in different regions of the human follicle. Fertil. Steril. 69, 435–442.
[12] Fang, X., Moore, S.A., Stoll, L.L., Rich, G., Kaduce, T.L., Weintraub, N.L. and Spector, A.A. (1998) 14,15-Epoxyeicosatrienoic acid inhibits prostaglandin E2 production in vascular smooth muscle cells. Am. J. Physiol. 275, H2113–H2121.
[13] Peri, K.G., Varma, D.R. and Chemtob, S. (1997) Stimulation of prostaglandin G/H synthase-2 expression by arachidonic acid monoxygenase product, 14,15-epoxyeicosatrienoic acid. FEBS Lett. 416, 269–272.
[14] Van Voorhis, B.J., Dunn, M.S., Falck, J.R., Bhatt, R.K., VanRollins, M. and Snyder, G.D. (1993) Metabolism of arachidonic acid to epoxyeicosatrienoic acids by human granulosa cells may mediate steroidogenesis. J. Clin. Endocrinol. Metab. 76, 1555–1559.
[15] Reinthaller, A., Kirchheimer, J.C., Deutinger, J., Bieglmayer, C., Christ, G. and Binder, B.R. (1990) Plasminogen activators, plasminogen activator inhibitor, and fibronectin in human granulosa cells and follicular fluid related to oocyte maturation and intrafollicular gonadotropin levels. Fertil. Steril. 54, 1045–1051.
[16] Hennebold, J.D., Mah, K., Perez, W., Vance, J.E., Stouffer, R.L., Morisseau, C., Hammock, B.D. and Adashi, E.Y. (2005) Identification and characterization of an ovary-selective isoform of epoxide hydrolase. Biol. Reprod. 72, 968–975.
[17] Grant, D.F., Storms, D.H. and Hammock, B.D. (1993) Molecular cloning and expression of murine liver soluble epoxide hydrolase. J. Biol. Chem. 268, 17628–17633.
[18] Morris, J.K. and Richards, J.S. (1995) Luteinizing hormone induces prostaglandin endoperoxide synthase-2 and luteinization in vitro by A-kinase and C-kinase pathways. Endocrinology 136, 1549–1558.
[19] Natraj, U. and Richards, J.S. (1993) Hormonal regulation, localization, and functional activity of the progesterone receptor in granulosa cells of rat preovulatory follicles. Endocrinology 133, 761–769.
[20] Salvador, L.M., Maizels, E., Hales, D.B., Miyamoto, E., Yamamoto, H. and Hunzicker-Dunn, M. (2002) Acute signaling by the LH receptor is independent of protein kinase C activation. Endocrinology 143, 2986–2994.