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Original research article

Evaluation of the prostaglandin F synthase activity of human and bovine aldo-keto reductases: AKR1A1s complement AKR1B1s as potent PGF synthases

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ABSTRACT

AKR1B1 of the polyol pathway was identified as a prostaglandin F2α synthase (PGFS). Using a genomic approach we have identified in the endometrium five bovine and three human AKRs with putative PGFS activity and generated the corresponding recombinant enzymes. The PGFS activity of the recombinant proteins was evaluated using a novel assay based on in situ generation of the precursor of PG biosynthesis PGH2. PGF2α was measured by ELISA and the relative potencies of the different enzymes were compared. We identified AKR1A1 and confirmed AKR1B1 as the most potent PGFS expressing characteristic inhibition patterns in presence of methylglyoxal, ponalrestat and glucose.

1. Introduction

Prostaglandins (PGs) represent a family of lipid mediators acting locally in the vascular, reproductive and other systems to maintain homeostasis through complementary and sometimes opposite actions [1–6]. Prostaglandin formation is a key component of PG action and the specific target of non-steroidal anti-inflammatory drugs.

PGs are synthesized in virtually all nucleated cells of the body from essential fatty acids (EFAs), primarily arachidonic acid (AA) in the western diet. The first step in PG formation is the conversion of AA from the cell membrane to PGG2 and then PGH2 by one of two prostaglandin G/H synthases (PTGS-1 or -2) better known as cyclooxygenases (COX-1 or -2). After PGH2 formation, terminal synthases generate the bioactive prostaglandins [6]. Specific terminal synthases required for enzymatic production of PGs were identified for PGE2 [7], thromboxane A2 (TxA2) [8], prostacyclin (PGI2) [9], PGD2 [10] and more recently PGF2α [11].

While PGF2α has not been studied as extensively as other PGs, it nevertheless plays important roles in the regulation of ocular pressure, renal absorption, cardio-vascular function, adipocyte differentiation and female reproductive function [12]. In the vascular system PGF2α exhibits vasoconstrictive and pro-thrombotic responses comparable to thromboxane with the additional feature that its molecule is chemically stable and remains active until catabolism by prostaglandin dehydrogenase, usually through a single passage in the lung.

Most prostaglandin F2α synthases (PGFS) identified to date are AKRs of the 1C family and their identification goes back and forth between human and bovine since the 80s. The discovery of the bovine lung PGFS [13] previously classed as AKR1C7 (NCBI-GI: 129896, EC: 1.1.1.188) [6] led to the attribution of PGFS function to its human homologue AKR1C3 [14]. Because the bovine endometrium produces huge amounts of PGF2α but does not express the bovine lung PGFS, our search for an alternate enzyme led to our identification of the role of bovine AKR1B1 (previously known as AKR1B5) in the formation of PGF2α from PGH2 in the bovine endometrium [15]. We have then extended this finding to the human where we have demonstrated that in addition to AKR1C3, AKR1B1 was able to produce PGF2α in the endometrium [11]. This was a major finding because the PGFS activity of AKR1B1 proved to be much higher than that of AKR1C3 [16]. In human, increased PGF2α production in response to IL-1β is associated with upregulation of AKR1B1, cPLA2 and COX-2 proteins [11,17]. In the bovine, we did not find any contribution of AKR1Cs [15] and

Abbreviations: AA, arachidonic acid; AKR, aldo-keto reductases; BLOTTO, fat-free dry milk in PBS-T; COX, cyclooxygenase; DD3, dihydrodiol dehydrogenase 3; IL-1β, interleukine-1 beta; PBS-T, PBS with Tween 20; PGs, prostaglandins; PGD2, prostaglandin D2; PGE2, prostaglandin E2; PGF2α, prostaglandin F2α; PGFS, PGF2α synthase; PGG2, prostaglandin G2; PGH2, prostaglandin H2.

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stimulation or inhibition of AKR1B1 does not explain entirely the variation in PGF2α biosynthesis [18]. Since abrogation of PGF2α release in response to AKR1B1 inhibitors was not as complete in bovine as in human endometrial cells [12], we hypothesized that another PGFS enzyme could be present.

In the present study, we have investigated if AKRs sharing at least 60% similarity with bovine AKR1B1 protein sequence were expressed in the bovine and human endometrium and if among those some exhibited PGFS activity. In order to estimate PGFS activity, we have adapted existing methods to implement a procedure whereby the PGH2 substrate was generated in situ in a cell free system. For clarity purposes, since many AKR have the same name between species while not meaning to be necessarily orthologues, "b" for bovine or "h" for human has been used throughout the text to specify the source and alleviate confusion.

2. Materials and methods

2.1. Materials

Reagents were purchased from the following suppliers: Superscript III reverse transcriptase, 1 kB DNA ladder, dithiothreitol, 5× first strand buffer and TRIzol from Invitrogen Life Technologies Inc. (Burlington, ON, Canada). Random primer-pd(N)6, deoxy-NTPs, RNA Guard, rTaq DNA polymerase, PCR 10× buffer from GE healthcare Canada (Baie d'Urfé, QC, Canada). Plasmid pDrive (TA cloning kit), DNA purification kits and QuantiTect SYBR Green PCR Kit for quantitative real time PCR were from Qiagen (Mississauga, ON, Canada) using LightCycler® System (Roche Diagnostics, Laval, QC, Canada). All oligonucleotide primers were chemically synthesized using ABT 394 synthase (Perkin-Elmer, Foster City, CA, USA). The other chemicals used were of molecular biology grade available from Laboratoire Mat or Fisher Biotech (Québec, QC, Canada). NADPH, methylglyoxal and 9,10-phenanthrenequinone, hematin was from Sigma–Aldrich (Oakville, ON, Canada) and arachidonic acid, sc-560, recombinant human COX-2 and ovine COX-1 proteins were from Cayman Chemical (Ann Arbor, MI, USA). Ponalrestat (Statil) was from Tocris Bioscience (Ellisville, MO, USA).

2.2. Preparation of endometrial tissues

Bovine uteri were collected at a local abattoir immediately after exsanguination, placed on ice and brought to the laboratory within 90 min. The cyclic stage of endometrial samples was determined as described previously based on gross morphology and examination of ovaries [19]. Endometrial strips were cut into small pieces, snap-frozen in liquid nitrogen, and stored at −80 °C until used for analysis. For human endometrial tissues, preparation was done as described previously [11,12] from women aged between 25 and 50 with normal cycle length (28–30 days) and no hormonal treatment. Informed consent for donation of anonymous endometrial samples was obtained before tissue collection. The research protocol was approved by the "Ethics Committee on Human Research of Centre Hospitalier Universitaire de Queĭbec."

2.3. Molecular cloning of bovine potential PGFS

Following blasting of the genomic DNA with bovine AKR1B1 mRNA sequence, 20 homolog proteins possessing at least 60% amino acids similarity were tested using specific forward and reverse primers (supplementary Table 1). These genes were synthesized and used for RT-PCR in bovine endometrial tissues. Consequently, the genes found to be expressed in the endometrium were cloned in a pDrive vector (Qiaquick PCR cloning kit, Qiagen) and then confirmed by sequencing. Finally, five bovine amplicons identified in the partial gene cloning protocol were selected based on expression level and amplified from endometrial RNA using primers listed in supplementary Table 2 to generate the full length cDNA coding for complete proteins listed in supplementary Tables 3–7.

2.4. Production of bovine and human His-tagged recombinant proteins

Five bovine genes identified as AKR1B1, 1A1, 1C1, 1C4 and DD3 were produced as recombinant proteins in BL21 DE3 pLysS. The human genes AKR1B1, 1C3 and 1A1 were produced in bacteria as for the bovine genes counterpart. Recombinant proteins including restriction enzyme sequences (supplementary Table 2) were produced as described previously for AKR1B1 using a pET-16b vector that included a 8× His at the N-terminal of the coding frame [15]. In summary, molecular cloning of the full-length enzyme was performed as described above for the partial fragments with the primers found in Table 2. Sequencing of both strands with T7 and SP6 primers validated the identity of the cloned gene. Once in the pDrive (Qiagen), the correct full-length fragment was digested using restriction enzymes. *Nde* I/*Bam*H I was used for bovine AKR1A1, bovine AKR1B1, human AKR1A1 and human AKR1C3 and *Nde* I/*Bgl* II for bovine AKR1C1, bovine AKR1C4, bovine dihydrodiol dehydrogenase 3 (DD3) and human AKR1B1. All fragments were purified using Qiagen PCR Purification Kit. Ligation was carried out at 13 °C overnight in presence of T4 DNA ligase (NEB). Sequencing both strands with T7 promoter and T7 terminator primers validated the identity and correct orientation of the cloned gene. The eight recombinant plasmids 8× His-AKRs in the prokaryotic expression vector pET-16b (Novagen) have been transformed in BL21 DE3 pLysS and grown overnight at 37 °C on agar plates containing ampicillin (150 μg/ml) and chloramphenicol (34 μg/ml). The next day, three to five colonies of each recombinant proteins were seeded separately in 25 ml of LB containing ampicillin (150 μg/ml) and chloramphenicol (34 μg/ml) and cultured under agitation overnight at 37 °C. Ten milliliters of the overnight culture was then seeded in 200 ml of LB medium containing only ampicillin (150 μg/ml). When the culture reached an absorbance (at A600 nm) between 0.5 and 0.7, induction was done with 1 mM IPTG for 4 h under vigorous shaking at 37 °C. The culture mixture was then centrifuged at 6000 × *g* 15 min and bacteria pellets were kept frozen at −80 °C until lysis with 10 ml of CelLytic B (Sigma) diluted 1/10 in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole pH8.0), containing benzonase (50 Units/ml). Lysozyme (1 mg/ml) was added and the lysate was shaken vigorously at room temperature during 20 min. The lysate was then centrifuged at 16,000 × *g* for 20 min and the supernatant (10 ml) was passed through a Ni-NTA column (Superflow, size 25 ml, Qiagen). The column was washed with three volumes (30 ml) of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole pH8.0) and elution was performed with 10 ml of the same buffer. The first two elution fractions (2 ml) were collected and recombinant proteins were dialyzed twice against 2 L of PBS at 4 °C for 12 and 6 h. Proteins were then loaded on a SDS polyacrylamide gel (SDS-PAGE) and stained with Coomassie Blue. Protein quantification was then done by the method of Bradford with average yields of 0.05–0.9 mg/ml.

2.5. Analysis of AKR1A1 and AKR1B1 by Western blot

Protein extraction, quantification and Western blot analysis were performed as described previously [19]. For each sample, aliquots of 5–10 μg protein were separated on 10% sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis. Proteins were then transferred onto nitrocellulose membranes (Bio-RaD Laboratories Canada Ltd., Mississauga, ON, Canada). The membranes were blocked overnight at 4 °C in phosphate-buffered saline (PBS)