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A role for phospholipase D in angiotensin II-induced protein kinase D activation in adrenal glomerulosa cell models

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ABSTRACT

The mineralocorticoid aldosterone plays an important role in regulating blood pressure, with excess causing hypertension and exacerbating cardiovascular disease. Previous studies have indicated a role for both phospholipase D (PLD) and protein kinase D (PKD) in angiotensin II (AngII)-regulated aldosterone production in adrenal glomerulosa cells. Therefore, the relationship between AngII-activated PLD and PKD was determined in two glomerulosa cell models, primary bovine zona glomerulosa (ZG) and HAC15 human adrenocortical carcinoma cells, using two inhibitors, 1-butanol and the reported PLD inhibitor, fluoro-2-indolyl des-chlorohalopemide (FIPI). FIPI was first confirmed to decrease PLD activation in response to AngII in the two glomerulosa cell models. Subsequently, it was shown that both 1-butanol and FIPI inhibited AngII-elicited PKD activation and aldosterone production. These results indicate that PKD is downstream of PLD and suggest that PKD is one of the mechanisms through which PLD promotes aldosterone production in response to AngII in adrenal glomerulosa cells.

1. Introduction

The mineralocorticoid aldosterone is important for normal sodium homeostasis and blood pressure control, with excess aldosterone resulting in hypertension and exacerbating congestive heart failure and cardiovascular disease. The production of this hormone is regulated by angiotensin II (AngII), which binds to the AngII type I receptor to activate phospholipase C. Phospholipase C-catalyzed phosphoinositide hydrolysis generates two second messengers: inositol 1,4,5-trisphosphate, which triggers calcium release from the endoplasmic reticulum, and diacylglycerol (DAG), which can activate DAG-responsive enzymes, such as members of the protein kinase C family. DAG can also be produced by the action of phospholipase D (PLD), which hydrolyzes phosphatidylcholine to yield phosphatidic acid (and choline). Phosphatidic acid is a lipid signal, activating such enzymes as Raf-1, mTOR and phosphatidylinositol 4-phosphate 5-kinase [reviewed in Cummings et al. (2002); Shapiro and Bollag (2008)]. Phosphatidic acid, in turn, is dephosphorylated by lipid phosphate phosphatases and phosphatidic acid phosphatases (or lipins) [reviewed in Brindley et al. (2009)] to generate the signal DAG.

Another DAG-responsive enzyme is the serine/threonine protein kinase, protein kinase D (PKD). PKD possesses two cysteine-rich C1 regions that bind DAG (or phorbol ester), and the enzyme can be activated by this binding. In addition, we have recently shown that in primary cultures of bovine zona glomerulosa (ZG) cells PKD activity can be stimulated by protein kinase C-mediated phosphorylation (manuscript in preparation), as has been demonstrated in other cell types [reviewed in Rozengurt (2011)]. Previous reports have shown that 1-butanol inhibited PKD activation stimulated by lysophosphatidic acid and platelet-derived growth factor in fibroblasts (Kam and Exton, 2004) and antibody ligation in a lymphocyte cell line (Caloca et al., 2008). Because 1-butanol can be used by PLD in place of water in a transphosphatidylation reaction to generate phosphatidylbutanol, thereby inhibiting the production of phosphatidic acid, this result suggests that PLD-derived lipid signals are important in PKD activation. However, a recent report indicates that 1-butanol can have effects that are not related to PLD inhibition in certain cell responses and in some cell types (Su et al., 2009) [although it should be noted that Caloca et al. (2008) did not rely solely on 1-butanol effects and also supported their conclusions with genetic manipulation of PLD and

Abbreviations: AngII, angiotensin II; DAG, diacylglycerol; FIPI, fluoro-2-indolyl des-chlorohalopemide; KRB+, bicarbonate-buffered Kreb's Ringer containing 2.5 mM sodium acetate; PKD, protein kinase D; PLD, phospholipase D; ZG, zona glomerulosa.

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PKD]. Other investigators have also obtained evidence of PKD activation mediated by PLD in response to the pharmacologic agents ilimaquinone (Sonoda et al., 2007) and reactive oxygen species (Cowell et al., 2009). Therefore, we investigated the effect of inhibiting PLD on AngII-induced PKD activation in primary cultures of bovine ZG cells and the human adrenocortical carcinoma cell line, HAC15, a clone of the H295R cell line (Parmar et al., 2008; Wang and Rainey, 2012; Wang et al., 2012). Since PLD activation has not been characterized in the HAC15 cells, we also determined the dose and time dependence of AngII-stimulated PLD activity.

2. Materials and methods

2.1. Materials

DMEM/F12 was purchased from Gibco (Grand Island, NY). Penicillin-streptomycin-fungizone and penicillin-streptomycin were purchased from Biologos, Inc. (Naperville, IL). Cosmic calf serum was obtained from Thermo Scientific (South Logan, UT). Horse serum and fetal bovine serum (FBS) were obtained from Atlanta Biologicals (Norcross, GA). ITS+ (insulin–transferrin–selenous acid with linoleic acid and bovine serum albumin) was from Becton–Dickinson Labware (Franklin Lakes, NJ). Phosphatidylethanol (PEt) and phosphatidic acid were purchased from Avanti Polar Lipids (Alabaster, AL). Butylated hydroxyanisole (BHA), α-tocopherol, ascorbic acid, gentamycin and 22(R)-hydroxycholesterol were obtained from Sigma (St. Louis, MO). The solid-phase radioimmunoassay kit was from Siemens (Tarrytown, NY). [^{3}H]Oleic acid and En^{3}Hance were purchased from Dupont NEN (Boston, MA). The rabbit anti-phosphoserine^{916}PKD and anti-PKD (total) antibodies were obtained from Cell Signaling Technology (Danvers, MA). The goat anti-rabbit (phosphoserine^{916}PKD and total PKD secondary antibody), and rabbit anti-goat (actin secondary antibody) were from Sigma. Immobilon P-FL was obtained from Millipore (Billerica, MA).

2.2. Cell culture

Bovine adrenal glomerulosa cells were prepared from near-term fetal calf adrenal glands obtained from a local meat-packing plant. Zona glomerulosa-enriched tissue slices were dissected from the adrenal glands. Cells were released from collagenase-digested slices using mechanical agitation, collected by centrifugation and cultured in a serum-containing DMEM/F12 medium as previously described (Shapiro et al., 2010).

The human adrenocortical carcinoma HAC15 cell line (Parmar et al., 2008), which is actually a clone of the H295R cell line (Wang and Rainey, 2012; Wang et al., 2012), was grown in DMEM/F12 medium containing 1% ITS, 1% penicillin–streptomycin and 10% Cosmic calf serum to approximately 70–75% confluence. The cells were then incubated for 20–24 h in low-serum medium containing 1% Cosmic calf serum prior to experimentation.

2.3. Measurement of aldosterone production

Primary cultures of bovine ZG cells were cultured overnight in serum-containing medium and then refed with serum-free medium as in Shapiro et al. (2010). After 20–24 h the cells were placed into bicarbonate-buffered Kreb's Ringer containing 2.5 mM sodium acetate (KRB^+) equilibrated with 5% CO_2 at 37 °C. Cells were then incubated with KRB^+ alone or KRB^+ containing 10 μM 22(R)-hydroxycholesterol in the presence and absence of 750 nM FIPI (or the DMSO vehicle control) for 60 min at 37 °C in an atmosphere of 5% CO_2. Supernatants were collected and stored frozen until assayed.

Cultured HAC15 cells were incubated with equilibrated low-serum DMEM/F12 medium containing the appropriate agents for the indicated time. The supernatants were collected and stored frozen −20 °C until aldosterone was assayed using a solid phase radioimmunoassay kit (Diagnostic Products, Los Angeles, CA).

2.4. Phopholipase D activity assay

PLD activity was monitored as the production of radiolabeled phosphatidylethanol (PEt) in [^{3}H]oleate-prelabelled cells. Cells were incubated for 20–24 h in low-serum medium containing 5 μCi/mL [^{3}H]oleate, equilibrated in KRB^+ and stimulated with appropriate agents in the presence of 0.5% ethanol. Cells were solubilized in 0.2% SDS containing 5 mM EDTA, and lipids were extracted into chloroform/methanol. Phospholipids were separated by thin-layer chromatography, visualized with autoflourography using En^{3}Hance, identified by co-migration with authentic standards and cut out and quantified by liquid scintillation spectrometry.

2.5. Western analysis

Cells incubated for 20–24 h in low-serum medium were treated with the appropriate agents as indicated. Cells were lysed, protein levels in the lysates determined and equal amounts of protein analyzed by western blotting using an anti-phosphoserine^{916}PKD-specific or anti-actin primary antibodies and alkaline phosphatase- or IRDye-conjugated secondary antibodies. Immunoreactive proteins were visualized using an ECF system (Amersham, Piscataway, NJ) with imaging on a Typhoon imager (Molecular Dynamics, Sunnyvale, CA). Alternatively, an Odyssey imaging system (Licor Biosciences, Lincoln, NE) was utilized and immunoreactivity quantified with Odyssey application software (version 2.1). Proteins were normalized to actin as indicated. Actin was used for normalization because both the total PKD and phosphoserine^{910}PKD antibodies were raised in rabbit, necessitating stripping and reprobing of the blot, with possibly nonhomogeneous loss of immunoreactivity, if total PKD were to be used for normalization. Nevertheless, in several instances we compared values using actin versus total PKD as the normalization control and observed essentially no differences.

2.6. Viability assay

HAC15 cells were incubated with or without FIPI (750 nM) in the presence or absence of AngII (10 nM) for 24 h. The pretreated cells were trypsinized and incubated with 0.4% trypan blue. The number of trypan blue-excluding cells, expressed as a percentage relative to the total cell number, was determined using a T10™ Automated Cell Counter (Biorad, Hercules, CA).

2.7. Statistical analysis

Experiments were performed a minimum of three times and the values obtained expressed as the means ± SEM. Significant differences were statistically determined with ANOVA followed by a Newman–Keuls post-hoc test using Graphpad Prism or Instat (La Jolla, CA).

3. Results

3.1. 1-Butanol inhibits AngII-induced PKD activation and aldosterone production in primary cultures of bovine adrenal glomerulosa cells

PLD hydrolyzes phosphatidylcholine to yield phosphatidic acid; however, in the presence of small amounts of a primary alcohol,