Protein kinase C-dependent phosphorylation and mitochondrial translocation of aldose reductase

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Abstract Although aldose reductase (AR) is a critical participant in osmoregulation, and the metabolism of glucose and aldehydes derived from lipid peroxidation, post-translational mechanisms regulating its activity have not been identified. In this paper, we report that stimulation of protein kinase C (PKC) in several cell types induces phosphorylation of AR and translocation of the phosphorylated protein to the mitochondria. In vitro, recombinant AR was directly phosphorylated by activated PKC, suggesting that AR may be an in vivo PKC substrate. Together, these observations reveal a novel link between PKC activation and the regulation of glucose and aldehyde metabolism.

Key words: Aldose reductase; Protein kinase C; Smooth muscle cell; HL60; REH; Phorbol ester; Bryostatin-1

1. Introduction

Aldose reductase (AR), a member of the aldo-keto reductase superfamily [1–3], catalyzes the reduction of a wide range of aldehydes, including glucose [1]. Reduction of glucose by AR generates sorbitol, which used for fructose synthesis, or as an inert osmolyte in the renal medulla [4]. Increased sorbitol generation and accumulation has also been linked to the development of secondary diabetic complications [1,3], and inhibition of AR has been shown to prevent diabetic cataracts, nephropathy, and neuropathy [1,3]. Recent structural and kinetic studies [5–7] indicate that aldehydes more hydrophobic than glucose are preferred AR substrates. Because several of these aldehydes are toxic and derived from lipid peroxidation [8], it has been suggested that AR may have a protective antioxidant function [9,10]. Consistent with this role, AR is upregulated by oxidants such as H_2O_2 [11], lipid peroxidation-derived aldehydes [12], and advanced glycosylation end products [13]. Nevertheless, the acute regulation of the enzyme by post-translational modifications has not been examined.

Members of the protein kinase C (PKC) family play a variety of regulatory roles in signaling events related to growth, differentiation, and cell death [14,15]. These enzymes are stimulated by oxidative stress and mediate the generation of reactive oxygen species. Multiple lines of circumstantial evidence suggest an interrelationship between AR and the PKC. Stimulation of AR during inflammation or growth is preceded by PKC activation, and both AR and PKCα are coordinately up regulated in cells overexpressing GLUT1 [16]. Moreover, PKCβ-selective inhibitors prevent sorbitol accumulation [17], and it has been suggested that the hyperglycemia-induced increase in endothelial AR and PKC is mediated by common signaling pathways activated by oxidative stress [18]. Additionally, it has been shown that AR is physically associated with the PKCε signaling module in transgenic mice overexpressing PKCε in the heart [19]. Nonetheless, the relationship between AR and PKC has not been directly investigated. Herein, we report for the first time that AR is phosphorylated upon stimulation of PKC in cells and in vitro, and that phosphorylation increases the association of AR with mitochondrial membranes.

2. Materials and methods

Bryostatin-1 (bryo-1) was a kind gift of G.R. Pettit (Arizona State University, Tempe, AZ, USA). The REH, HL60, and COS-7 cells were obtained from the ATCC (Rockville, MD, USA) and maintained in RPMI 1640 containing 10% bovine calf serum at 37°C in 5% CO_2. The murine IL-3-dependent cell line, NSF.N1H7 (H7) was a kind gift of H. Scott Boswell. The vascular smooth muscle cells (VSMC) were isolated from rat aorta and cultured as described before [20]. To measure phosphorylation, cells were serum-starved for 48 h and were incubated with 0.5 mCi ml^{-1} [^{32}P]orthophosphoric acid for 4 or 6 h, after which 1 μM okadaic acid was added to the medium. The cells were incubated for an additional 5 min and then stimulated with PKC agonists. After 10 or 30 min, the cells were frozen over dry ice, and pelleted at 5000×*g* for 5 min at 4°C and resuspended in 0. 5 ml of the lysis buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 50 mM NaF, 0.5 mM $NaVO_3$, 10 mM EDTA, 5 mM EGTA, 50 μM leupeptin, 50 μM pepstatin, 1 mM PMSF, 50 nM okadaic acid, 1% Nonidet P-40 and 1 mM iodoacetate. The cell extracts were centrifuged at 14 000×*g* for 15 min, and the supernatant was recovered and mixed with 0.2% sodium dodecyl sulfate (SDS). The AR protein was immunoprecipitated as described previously [21,22] using polyclonal antibodies raised against human recombinant AR in rabbits [12]. Samples were electrophoresed and Western blots were developed as described before [21]. Two-dimensional electrophoresis (2DE) was performed using immobilized pH gradient strips (IPG; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA), rehydrated overnight

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with immunoprecipitant in rehydration buffer containing 8 M urea, 2% CHAPS, 0.01 M dithiothreitol (DTT), 2% ampholytes and bromophenol blue. The strips were then focused with maximal 5000 V and 80 mA at 17°C for 24 h to reach 100 000 V h^{-1}. After focusing, the samples were equilibrated with buffer containing 6 M urea, 130 mM DTT, 30% glycerol, 112 mM Tris base, 4% SDS, 0.002% bromophenol blue and acetic acid, then with buffer containing 6 M urea, 135 mM iodoacetamide, 30% glycerol, 112 mM Tris base, 4% SDS, 0.002% bromophenol blue and acetic acid. The strips were loaded onto pre-cast 10% homogeneous, 22×22 cm slab gels (Genomic Solutions Inc., Ann Arbor, MI, USA). The gels were stained by SYPRO ruby stain (Bio-Rad Laboratory, Hercules, CA, USA). Time-of-flight mass spectrometry (MALDI-TOF MS), and peptide mass fingerprinting (PMF) was performed after in-gel tryptic digestion and protein identification was performed by using PROFOUND (http://129.85.19.192/profound_bin/WebProFound.exe) as described previously [23].

Subcellular fractions were obtained using previously published procedures [24]. The purity of the fractions was confirmed by assessing localization of fraction-specific proteins, including prohibitin (heavy membrane, HM) and CPP32 (cytosolic). The cell surface transferrin receptor (TrR) was localized exclusively to the light membrane fraction (LM; data not shown). The AR protein was purified and assayed for activity as described previously [6,7]. Recombinant AR was phosphorylated by the rat brain pan-PKC or human PKC isoforms (Panvera, Madison, WI, USA) at 30°C in 50 μl reaction mixtures containing 1 μg ml^{-1} AR, 50 mM Tris (pH 7.2), 50 mM NaCl, 1% glycerol, 10 mM $MgCl_2$, 2 mM DTT, 2 mM $CaCl_2$, 100 μM ATP, 1 μCi [γ-^{32}P]ATP, 40 μg ml^{-1} phosphatidylserine, 10 nM bryo-1, and 0.4 μg PKC. The proteins were separated on SDS–PAGE and ^{32}P incorporation was detected by autoradiography.

3. Results and discussion

To examine the effect of PKC on AR, we measured the incorporation of ^{32}P into the AR protein in three different types of cells stimulated by PKC agonists. The REH, HL-60, and VSMC cells were metabolically labeled with ^{32}P and incubated with 10 nM bryo-1, which is a potent stimulator of the PKC family of kinases [24]. After 15 min of incubation, the cells were lysed and AR was immunoprecipated from the extract using anti-AR antibodies. The immunoprecipitates were separated on SDS–PAGE and AR was identified by Western analysis using anti-AR antibody and ^{32}P incorporation was visualized by autoradiography. In all cases, stimulation with bryo-1 induced ^{32}P incorporation into AR (Fig. 1A), indicating that stimulation of PKC leads to the phos-

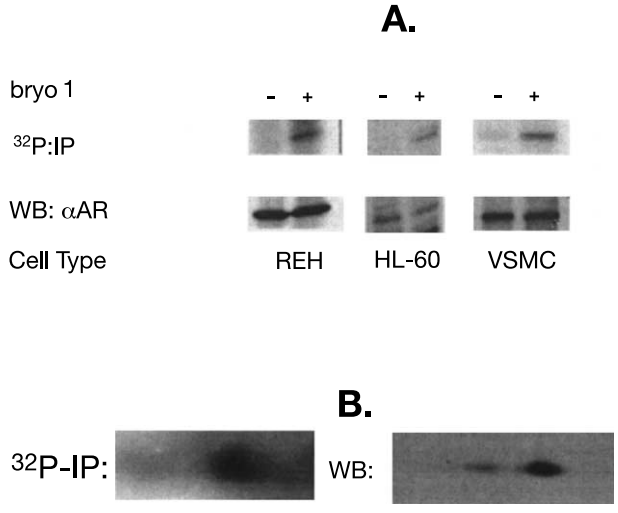

Fig. 1. Stimulation of PKC phosphorylates AR in REH, HL-60, smooth muscle, and COS-7 cells. The cells were labeled with [^{32}P]orthophosphoric acid for 1 h and then stimulated with either the vehicle or 10 nM bryo-1 for 15 min. Immunoprecipitated AR was separated on SDS–PAGE, and after autoradiography (panel A, top) the same filter was reprobed with AR-antisera for developing Western blots (panel A, bottom). Precipitates of the cell lysates with pre-immune serum were negative for AR. Panel B shows the autoradiogram and Western Blot of COS-7 cell AR labeled with [^{32}P]orthophosphoric acid and stimulated with 100 nM PMA for 30 min. The cells were lysed and AR was immunoprecipitated, and then subjected to 2D electrophoresis, Western blot analysis, and autoradiography.

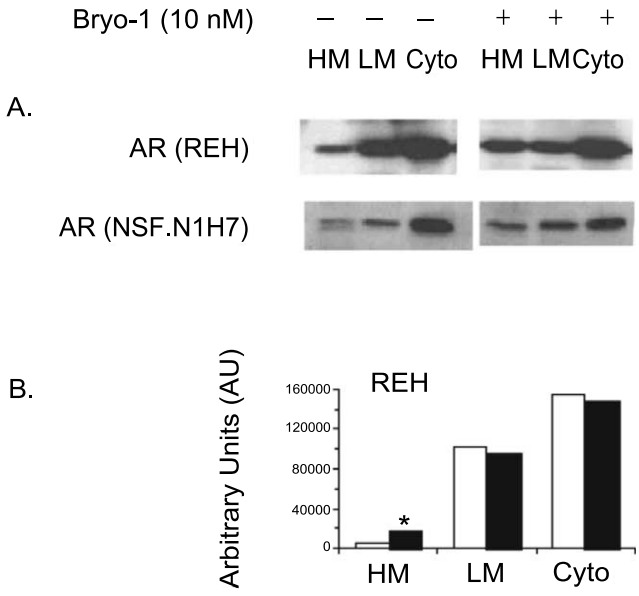

Fig. 2. Stimulation of PKC selectively enriches the mitochondrial membrane fraction with AR. The REH or the NSF.N1H7 cells (10^7) were stimulated with 10 nM byrostatin-1, and the following fractions were isolated: HM, LM, and cytoplasm (Cyto) and separated on SDS–PAGE. The Western blots are shown in panel A. Panel B shows a graphical representation of the densitometric analysis of three independent experiments.

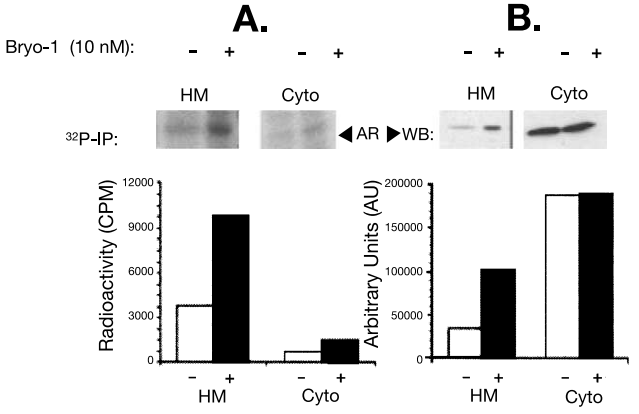

Fig. 3. Preferential phosphorylation of AR associated with the mitochondrial fraction. The REH cells (2×10^7) were labeled with [^{32}P] and stimulated with 10 nM bryo-1. Following subcellular fractionation, 50 μg protein from each fraction was separated on SDS–PAGE. After autoradiography, the same membrane was subjected to Western blotting with AR-antisera. The autoradiographs and the extent of radioactivity incorporated is shown in panel A. Panel B shows Western blots of AR in the HM and cytosolic (Cyto) fraction. Bars in panels A and B are the mean values obtained from three independent experiments.

phorylation of AR. Similar results were obtained with the three cell types examined, suggesting that phosphorylation of AR upon stimulation of PKC may be a phenomenon common to cells of diverse origins. Incubation of VSMC with 100 nM PMA for 10 min also led to the phosphorylation of AR (data not shown), indicating that the phosphorylation of AR is independent of the nature of the agonist used to activate PKC.

To determine whether the phosphorylated band was identical to AR protein, the ^{32}P-labeled COS-7 cells were stimulated with PMA and then subjected to 2DE. AR was identified by Western analysis as a 36 kDa protein focusing between 6.9 and 7.8 pH units (Fig. 1B), which corresponded to the position of the protein spot (in p*I* and molecular weight) detected by autoradiography. To further confirm the identity of this protein, immunoprecipitates from PMA-stimulated VSMC was separated by 2DE and the corresponding spot was excised and subjected to in-gel tryptic digestion followed by MALDI-TOF MS and PMF. Peptide masses obtained from MALDI-TOF MS confirmed the identity of the immunoprecipitated protein as AR.

In unstimulated cells, AR was localized primarily to the cytosolic fraction, and $<10\%$ of this protein was associated with the LM or HM fractions. However, upon bryo-1 stimulation, AR in the cytosolic and the LM fraction was decreased, whereas AR associated with HM was increased more than three-fold (Fig. 2). Similar changes in intracellular distribution of AR were observed in NSF.N1H7 cells. As shown in Fig. 2, in unstimulated NSF.N1H7 cells only trace levels of AR were associated with the HM fraction. However, stimulation of these cells by bryo-1 led to an increase in AR associated with this fraction.

To examine whether the phosphorylation of AR upon PKC activation displays site-specificity, metabolically labeled REH cells were stimulated with bryo-1 for 15 min, after which the cells were harvested and their subcellular fractions were pre-

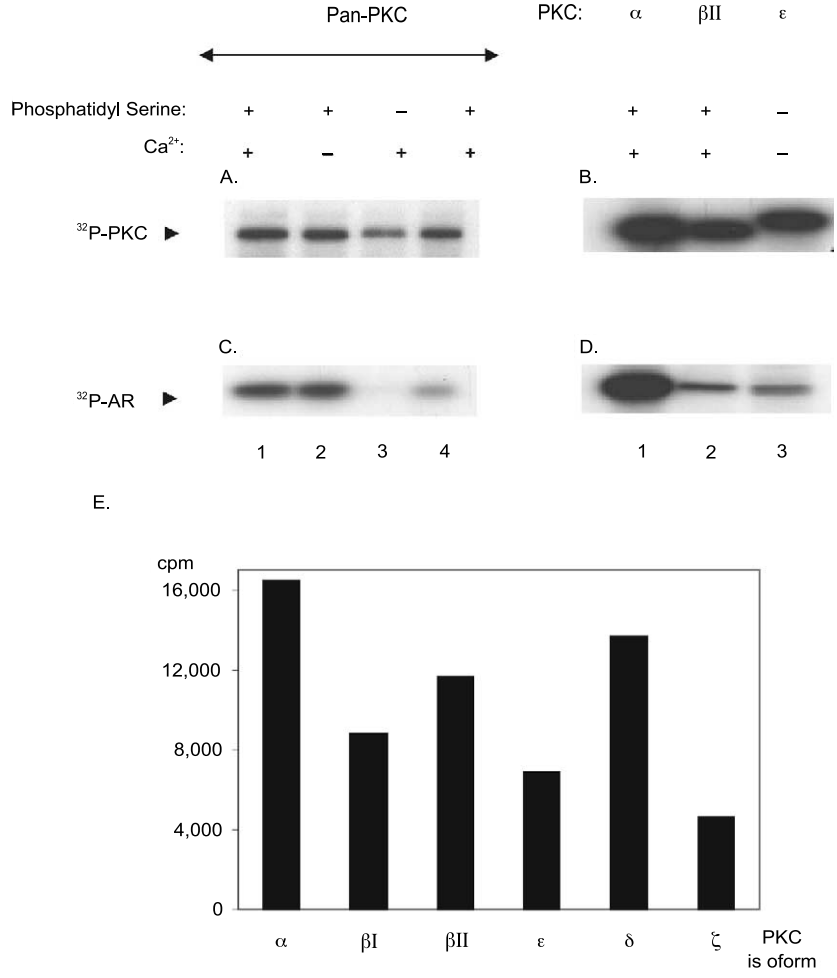

Fig. 4. Activated PKC isoforms phosphorylate AR. Recombinant AR was incubated with the indicated PKC isoforms and the reaction mixture was separated on SDS–PAGE and the extent of phosphorylation was measured by autoradiography. Panel A shows autophosphorylated pan-PKC and panel B shows the autophosphorylated PKCα, βII, and ε. The presence or absence of phosphatidylserine and Ca^{2+} in the phosphorylation mixture is also indicated. The PKCε phosphorylation did not require either Ca^{2+} or phosphatidylserine, although the autophosphorylation of pan-PKC was greatly reduced in absence of phosphatidylserine (panel A, lane 3), but was less affected by the nominal absence of Ca^{2+} in the incubation mixture (lane 2). Panel C shows phosphorylation of AR by activated pan-PKC in the presence (lane 1) or the absence (lane 3) of phosphatidylserine or the absence of Ca^{2+} (lane 2). Lane 4 of panel C shows pan-PKC-induced phosphorylation of denatured AR (heated at 70°C for 15 min). Panel D shows the phosphorylation of AR when incubated with α, βII, and ε, in lanes 1–3, respectively. Panel E shows the extent of ^{32}P incorporation into AR protein in the presence of indicated PKC isoforms.

pared. As shown in Fig. 3, the extent of AR phosphorylation was significantly higher in the HM fraction, indicating that stimulation of PKC not only leads to redistribution of AR, but also enhances the fraction of phosphorylated AR associated with the mitochondria.

To examine whether AR is a direct substrate of PKC, recombinant AR was incubated in the PKC assay buffer containing pan-PKC. After varying durations of incubation, the reaction mixture was separated on SDS–PAGE, transferred to a nitrocellulose membrane, and the phosphorylation of AR and PKC was visualized by autoradiography. As shown in Fig. 4, AR was intensely phosphorylated in the complete reaction mixture containing calcium, phosphatidylserine and pan-PKC. In the absence of PKC, no ^{32}P was incorporated into AR. For optimal phosphorylation, the native conformation of AR appeared to be necessary, because when denatured AR (prepared by heating the protein for 15 min at 70°C) was incubated with the pan-PKC, the extent of phosphorylation was diminished (Fig. 4C, lane 4). The phosphorylation of AR also required phosphatidylserine and calcium. Although omission of phosphatidylserine from the incubation mixture completely prevented phosphorylation, the presence of calcium was less critical (Fig. 4C). Because pan-PKC contains a mixture of PKC isozymes, it is likely that some of the non-classic PKC isoforms may have phosphorylated AR in the absence of calcium. Regardless, these observations clearly show that AR is phosphorylated by PKC and that this phosphorylation depends upon phosphatidylserine and calcium.

To establish the relative specificity of PKC isoforms for AR, AR was incubated with pure, recombinant human PKC isoforms. Among these isozymes, PKCα appeared to be most efficient in phosphorylating AR, whereas, less intense phosphorylation was observed with $PKC\beta_{II}$ and PKCε. The relative specificity of other PKC isoforms is shown in Fig. 4E. These values are expressed as the total cpm incorporated in AR when incubated with equimolar concentrations of the PKC isoforms. When normalized to the extent of incorporation induced by PKCα, the relative efficiencies of the other PKC isoforms with AR were: $(\alpha = 1)$ $\beta_I = 0.54$, $\beta_{II} = 0.71$, $\varepsilon = 0.42$, $\delta = 0.83$ and $\zeta = 0.28$. Moreover, with activated PKCα alone, AR was rapidly phosphorylated and the extent of phosphorylation was maximal in 30 min and half-maximal phosphorylation was observed in < 2 min. At maximal phosphorylation, 0.92–1.01 mol of inorganic phosphate were incorporated per mol of AR.

Collectively, these results suggest that phosphorylation of AR by PKC may be a phenomenon general to several cell types. At least for the cells examined, rapid and robust phosphorylation of AR was observed, which in the REH cells was associated with the migration of AR to the mitochondria. Although AR is considered a cytosolic protein, our data (Fig. 2) show that even in the non-stimulated cells, detectable levels of AR were associated with LM and HM fractions, and that the association of AR with the HM fraction was increased upon PKC stimulation. This is in agreement with the observation that brief episodes of ischemia lead to a PKC-dependent increase in the association of AR with the particulate fraction in the heart [25], and suggest that AR associates with cell membranes either directly or as a part of a signaling complex as described for the PKCε signaling module [19].

The physiological implications of PKC-dependent phosphorylation and translocation of AR remain unclear. However, given the role of AR in the metabolism of lipid-derived aldehydes, we speculate that AR may be required for the detoxification of aldehydes generated during the breakdown of membrane lipids, which is a necessary step in PKC activation. Thus, AR may be essential for removing lipid peroxidation products from the lipid microdomains that associate with, and are essential for, PKC activation. Additionally, phosphorylation by PKC may be a mechanism for regulating glucose metabolism or fructose synthesis. Although further experiments are required to test this view, our current results suggest that AR may be one of the downstream targets and ende-ffectors of PKC signaling. These data reveal a novel and unanticipated link between PKC and aldehyde or glucose metabolism and may be of significance in understanding the PKC-induced changes in the intermediary metabolism of cells stimulated by growth factors, mitogens, or cytokines.

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