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Signal Transduction in Rat Vascular Smooth Muscle Cells: Control of Osmotically Induced Aldose Reductase Expression by Cell Kinases and Phosphatases

Agata Fazzio,*,† Stefan E. Spycher,* and Angelo Azzi*,[1]
**Institut für Biochemie und Molekularbiologie, Universität Bern, CH-3012 Bern, Switzerland; and*
†Istituto di Scienze Biochimiche e Farmacologiche, Università di Catania, Catania, Italy

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We have studied the osmotically induced gene expression (measured as chloramphenicol acetyl transferase (CAT) reporter gene expression) in rat smooth muscle cell primary cultures (rSMC), under the control of osmotic response elements (ORE). It was found that osmotically induced gene expression is sensitive to signal transduction inhibitors and activators. In particular, protein kinase C inhibition by calphostin C prevented gene expression by osmotic response. On the other hand, receptor tyrosine kinase inhibition has been shown to produce an enhancement of gene expression. This suggests that tyrosine kinase receptor activation exerts an inhibitory action on ORE induced gene expression. Gene expression was also induced by treating cells with PD098059, a specific inhibitor of mitogen-activated protein kinase kinase. Moreover, the same inhibitors and activators have been shown to affect the hyperosmosis induced expression of aldose reductase gene.

Key Words: **osmotic response; aldose reductase; signal transduction; protein kinase C; MAP-kinase; protein phosphatase; smooth muscle cells; gene expression.**

Cells react to increased osmolarity with changes in gene expression mediated by a membrane sensor or by changes in intracellular solutes (1). One of the mechanisms of cell osmoregulation is based on sorbitol accumulation. This organic molecule maintains osmotic cell integrity by balancing the extracellular hyperosmotic stress (2). Although the cellular responses to hyperosmotic stress are very important in cell pathophysiology, the corresponding molecular mechanisms have only recently begun to be addressed. Thus, relatively little is known about the cascade of signals between the initial extracellular stimulus (hyperosmolarity) and the ultimate adaptive response. Several recent reports demonstrate induction of a number of genes on exposure of cells to hyperosmotic stress (3–7). Recently, in rat vascular smooth muscle cells, we found that the aldose reductase gene is induced by hydrogen peroxide (H_2O_2) and 4-hydroxy-2,3-*trans*-nonenal (HNE), an aldehyde formed by peroxidation of ωw-6-polyunsaturated fatty acids, such as linoleic acid and arachidonic acid (8, 9).

Hypertonicity greatly increases aldose reductase transcription (7). The best-characterised metabolic activity of the aldose reductase enzyme, the NADPH mediated reduction of glucose to sorbitol, is thought to play a central role in cellular osmoregulation. The promoter region of the aldose reductase gene has been partially characterised (5, 8–11): it contains several elements that control the basal expression of the enzyme. A sequence of 132-base pairs has been described containing three very similar repeats of 10-11 base pairs (12). These sequences have strong homology with the canine betaine transporter gene and the osmotic response element of the rabbit. These so called osmotic response elements (ORE A, B and C) are able to confer hyperosmotic response on a downstream gene (12). However, the mechanism by which osmotic pressure changes trigger events at the level of gene expression has not been clarified.

In the present study we have chosen aortic smooth muscle cells, in which an osmotic stress may produce events relevant in the progression of atherosclerosis (13). We have shown that osmotically induced gene expression, under the control of ORE elements (10) is sensitive to signal transduction inhibitors and activators. In particular, protein kinase C activation and inhibition, as well as receptor tyrosine kinase inhibition, have been shown to produce profound effects in the expressions of chloramphenicol acetyl transferase

[1] To whom correspondence should be addressed at Institute of Biochemistry and Molecular Biology, University of Bern, Bühlstrasse 28, 3012 Bern, Switzerland. Fax: +4131 6313737. E-mail: angelo.azzi@mci.unibe.ch.

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reporter gene expression under the control of ORE's. Moreover, the same inhibitors and activators have been shown to affect the expression of aldose reductase gene activated by an osmotic response. Isolation and identification of the transcription factor(s) involved in ORE responses and the characterisation of their ORE interaction will be the object of a forthcoming study. It will help in elucidating the mechanisms by which membrane sensors transduce external osmotic signals to chemical events, competent to regulate the aldose reductase gene in vascular smooth muscle cells.

MATERIALS AND METHODS

Tissue culture materials were purchased from Falcon Labware (Becton Dickinson & Co.); growth media and serum were from Gibco Laboratories (Grand Island, NY, USA). Rat vascular smooth muscle cells were isolated from the aortic media by explanting the rat aortic wall. Phorbol 12-myristate 13-acetate (PMA) was from Sigma Chemical Company, St. Louis, MO. $\gamma[^{32}P]$ATP and d-*threo*-[dichloroacetyl-1-^{14}C]chloramphenicol were from Amersham International. Calphostin C, Tyrphostin, Genistein, amino-3′-methoxyflavone (PD098059) and Okadaic acid were from Alexis Corporation. All nucleotides were obtained from Microsynth (Balgach, Switzerland). The permeabilising reagent used in transfection experiment (DOTAP) was from Boehringer Mannheim.

Cell culture. Primary rat vascular smooth muscle cells rSMC (a kind gift of Dr. T. Resink (Kantonspital, Basel)) were cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10% heat-inactivated foetal calf serum (FCS) at 37°C/5% CO_2. Cells were used between passages 24 and 30 throughout these experiments. Cells were made quiescent by incubation for 24 h in low serum media (DMEM/0.2% FCS).

Nuclear protein extraction. Nuclear protein extracts were prepared from cells grown to 90% confluence in 75-cm^2 flasks. All nuclear extraction procedures were performed on ice, with ice-cold reagents according to Schreiber et al (14). Cells were washed twice with phosphate buffer (PBS), harvested by scraping into 1.5 ml of PBS and pelleted at 1500g × 5 min. The pellet was washed twice with PBS, resuspended in 400 μl of lysis buffer (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 1 mM PMSF) and incubated for 15 min on ice. Than 25 ml of 10% v/v of Nonidet NP-40 (Fluka) was added and the samples were vigorously vortexed for 10 sec. After centrifugation at 14,000 g × 30 sec. The nuclear pellet was resuspended in 50 μl of extraction buffer (20 mM HEPES pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF), and vigorously rocked at 4°C for 15 min. Nuclear proteins were isolated by centrifugation at 14,000 g × 5 min. Protein was determined by Bradford assay (15) and stored at −70°C until used for EMSA.

Electrophoretic mobility shift assay (EMSA). Nuclear protein extracts were prepared from rSMC cells subjected to iso-osmotic (DMEM, 0.2% FCS), hyperosmotic (DMEM, 0.2% FCS plus 220 mM NaCl), or PMA treatment (100 nM) for 30 min at 37°C. 7 pmol of a double-stranded ORE oligonucleotide (1 copy of the ORE sequence, see below) was labelled with 50 μCi $\alpha[^{32}P]$dATP, 25 μM of each cold deoxinucleotide (dCTP, dGTP and dTTP) using 1 μl Klenow enzyme (2 unit, Boehringer) and incubated at 37°C for 30 min. Unincorporated label was removed using QIAquick columns (QIAGEN). Competitor oligonucleotide with filled-in 5′ overhang sequence was prepared in the same way but using 700 pmol oligonucleotide and replacing $\alpha[^{32}P]$dATP with cold dATP. For EMSA 10 μg of nuclear proteins were incubated with the ^{32}P-end labelled double-stranded ORE oligonucleotide in binding buffer for 30 min at room temperature (binding buffer: 10 μg/ml BSA, 2.5 μg/ml poly[(d-I)-(d-C)], 1x Ficoll buffer, 1x buffer D, 5 mM $MgCl_2$; 5x Ficoll buffer contains 20% Ficoll 400, 100 mM HEPES, 300 mM KCl, 10 mM DTT, 0.1 mM PMSF; 5x buffer D contains 20 mM HEPES, pH 7.9, 20% v/v glycerine, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40 (v/v), 2 mM DTT, 0.1 mM PMSF). Incubating samples with 100-fold molar excess of the unlabelled oligonucleotide probe tested the specificity of the binding reaction. Protein-nucleic acid complexes were resolved using a non-denaturing polyacrylamide gel consisting of 6% acrylamide (30:0.8 ratio of acrylamide: bisacrylamide) and run in 0.5 × TBE buffer (45 mM Tris-HCl; 45 mM boric acid; 1 mM EDTA, pH 8.0) for the first hour at 50 V and for the next 2.5 h at 200 V, using a water cooling system. Gels were transferred to Watmann 3M paper, dried under vacuum at 80°C for 2 h and exposed to photographic film at −80°C.

Plasmid construction. To investigate the effects of the osmotic regulatory element we made a HindIII/BamHI synthetic oligonucleotide corresponding to three copies of the double-stranded ORE sequence. A three-fold repeat of the following sequence (ORE-C: TGG AAAA TCA) was linked by two repeats of the following nucleotides: ACC TCC ACA. The 57 bp double-stranded oligonucleotide (5′AGGCTTGGAAAATCACCTCCACATGGAAAATCACCTCCACATGGAAAATCACCTCCAG-3′) was cloned into the HindIII/BamHI site of the pBL-tk-CAT plasmid (a kind gift from Dr. P. Bäuerle), containing the herpes simplex thymidine kinase (TK) promoter upstream of the CAT reporter gene. This plasmid called ORE_3-TK-CAT was used to transiently transfect rSMC cells.

Transient transfection. Functional analyses of the ORE elements were performed by transiently transfecting rSMC cells with a plasmid construct mentioned above. Cells were transfected (8 μg CAT plasmid) in duplicates in 60 mm culture dishes by incubation with cationic liposomes (DOTAP) for 6 h in DMEM 0.2% FCS. The liposome to DNA ratio was 10:1. After transfection, cells were allowed to recover overnight in DMEM 0.2% FCS, exposed to either iso-osmotic medium (DMEM 0.2% FCS) or hyperosmotic medium (DMEM 0.2% FCS supplemented with 220 mM NaCl) for 30 min, and than differently treated for 18 h. After exposure to experimental conditions, cellular proteins were extracted. Cells were collected in 60 μl per dish of 0.25 M Tris-HCl, pH 7.8, and 1 mM DTT; the crude extracts were prepared by 4 cycles of freezing, thawing. 4 μl of cell extract were subjected to Bradford protein assay (15). Chloramphenicol acetyl transferase activity was assayed as described by Gorman et al (16) except that extracts were heated to 60°C for 10 min before the enzymatic test was done according to Crabb and Dixon (17) with 0.1 μCi [^{14}C]Chloramphenicol in each reaction. Two hours incubation at 37°C followed. The acetylated chloramphenicol products were separated by thin-layer chromatography (TLC), exposed in a BioRad GS-250 Molecular Imager for 18 h. CAT activity is reported as fold induction over control cells (transfected and treated with medium alone at 37°C) and corrected for total cellular protein.

RESULTS AND DISCUSSION

Electrophoretic mobility shift assay of the rat aldose reductase ORE element in the presence of specific competitor. The interaction of ORE with putative osmotically induced transcription factors was studied by electrophoretic mobility shift assay (EMSA). Cells were subjected to hyperosmotic or iso-osmotic treatment as well as supplemented with 100 nM phorbol-myristate acetate (PMA). As shown in Fig. 1, radioactive ORE bound less protein from iso-osmotically treated cell extracts (lane 1) relative to that from hyperosmotic and PMA treated cells (lanes 2 and 3). The specificity of

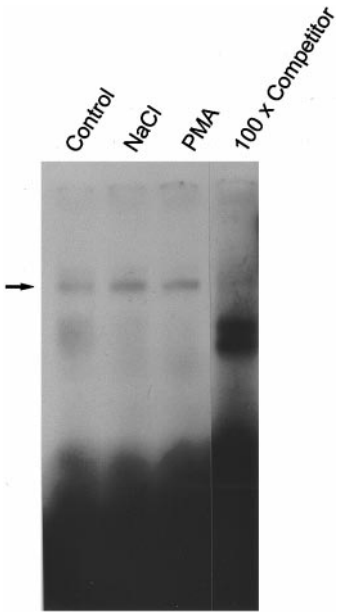

FIG. 1. Electrophoretic mobility shift assay of the hALR ORE in the presence of a specific competitor. Radio-labelled double-stranded ORE oligonucleotide was incubated with 10 μg of nuclear protein extract from rSMC cells maintained in iso-osmotic medium (lane 1) (DMEM 0.2% FCS) or exposed to hyperosmotic medium (lane 2) (DMEM 0.2% FCS + 220 mM NaCl), or treated with 100 nM PMA (lane 3). 100-fold molar excess of unlabelled specific competitor oligonucleotide (cold ORE) was used (lane 4). The ORE-specific signal is marked by the arrow.

DNA binding was demonstrated by competitive inhibition with 100-fold molar excess of unlabelled oligonucleotide (lane 4). This analysis suggests that not only hyperosmotic stress, but also phorbol ester result in the induction or activation of proteins that become associated with ORE. (18) v-Jun has been shown to contain multiple regions that participate in transcriptional activation in an interdependent manner and that binding of a protein or a transcription factor to a DNA responsive element may imply either induction or repression of a gene. Constructs containing a three-fold repeat of the osmotic responsive element (ORE_3) and a reporter gene Chloramphenicol acetyltransferase, (CAT) have therefore been utilised to establish the relationship between DNA protein binding and gene expression.

Induction of ORE-mediated CAT expression in response to PKC activation and inhibition. Phorbol 12-myristate 13-acetate (PMA) induced an even stronger protein binding to aldose reductase ORE than hyperosmotic treatment (cf. Fig. 1). The consequences of PMA on ORE controlled CAT gene-expression have been studied. rSMC cells were transfected with the ORE_3-TK CAT reporter plasmid and treated with 100 nM PMA for 18 h. PMA induction of ORE-mediated CAT expression was larger than that of hyperosmotic NaCl (Fig. 2). PMA, added in hyperosmotic condition, further increased gene expression. ORE appears thus to behave not only as an osmotic response element but also as a TPA responsive element (TRE-like element). Since no homology between the two consensus sequences is present (ORE sequence: TGG AAA ATT ACC TCC A; TRE sequence: TGA C/GTC/AA) it may be concluded that TRE is not the only TPA responsive element in these cells. Phorbol esters are well-established direct protein kinase C (PKC) activators, both *in vitro* and *in vivo*. Although there is no absolute evidence that PKC is the only phorbol ester "receptor" it is generally agreed that PKC is the major, if not the only, cellular site of action of phorbol ester (19). To verify if PMA action was mediated by PKC, cells were treated with calphostin C, a specific PKC inhibitor. As shown in Fig. 2 calphostin C strongly inhibited PMA induced ORE-mediated CAT expression in hyperosmotic conditions. This result indicates that protein kinase C is involved, via ORE, in the positive control of aldose reductase gene transcription. Calphostin C did not modify the basal CAT expression in iso-osmotic conditions (Fig. 2), but was able to prevent the osmotic induced CAT transcription, suggesting that also the osmotic response induced aldose reductase gene transcription is modulated by PKC.

Induction of ORE-mediated CAT expression in response to tyrosine kinase receptor inhibition. The regulatory pathway involved in the modulation of the aldose reductase gene expression through the ORE element was further investigated by using tyrosine kinase inhibitors. rSMC cells were transiently transfected with the ORE_3-TK-CAT and treated for 18 h with 20 μM Tyrphostin and 25 μM Genistein in isoosmotic or hyperosmotic conditions. The inhibitors induced an approximately 4-fold CAT expression in iso-osmotic conditions and 6-7 fold under hyperosmotic conditions (Fig. 3). Induction of CAT gene expression was also observed treating the cells with PD 098059 (Fig. 4), a specific inhibitor of mitogen-activated pro-

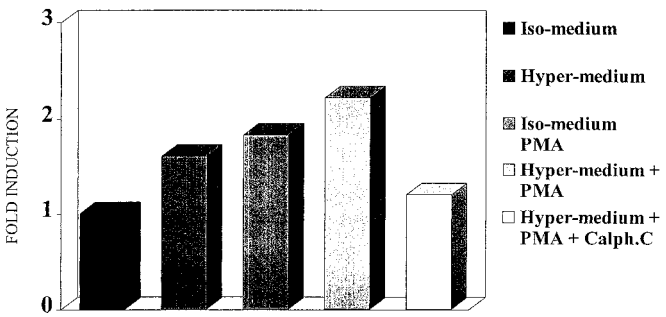

FIG. 2. Phorbol-myristate-acetate induces the ORE-mediated CAT expression in rSMC cells. Protein Kinase C inhibitor reduces this activation. Transfected rSMC cells were treated with 100 nM PMA for 18 h under iso- or hyperosmotic conditions. 300 nM Calphostin C was added to the cells for 18h prior to harvesting. Data represent 4 separate transfections.

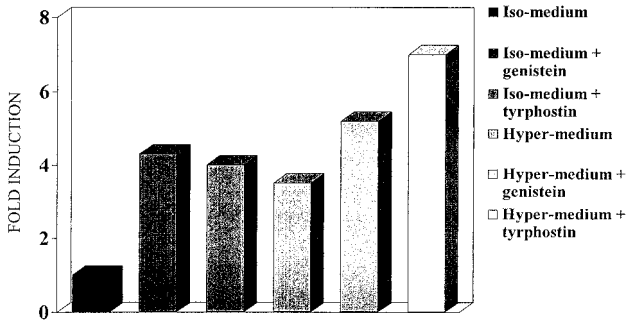

FIG. 3. Tyrosine kinase inhibitor tyrphostin activates the ORE-mediated CAT expression in rSMC cells. Treatment of the transfected cells with 20 μM tyrphostin for 18 h strongly induced ORE-gene expression in iso- and hyperosmotic condition. Data represent 3 separate experiments.

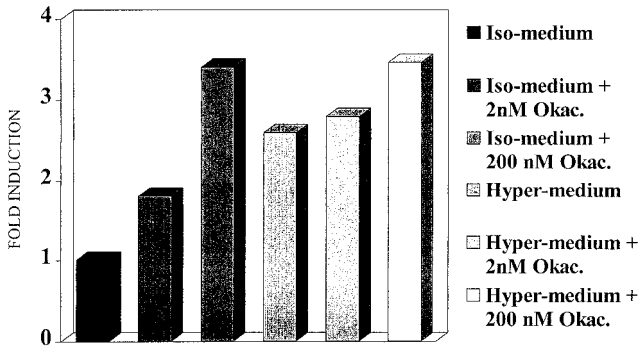

FIG. 5. Effect of okadaic acid on ORE mediated gene expression in rSMC cells. Transfected rSMC cells were treated with 2 nM or 200 nM okadaic acid for 18 h. Cells were then harvested and the amount of CAT activity was determined by thin layer chromatography.

tein kinase kinase in vitro and in vivo. The effect is observed in hyperosmotic medium. The experiment in Fig. 4 suggests therefore a negative modulation of the ORE-mediated CAT expression by the mitogen-activated protein kinase kinase. This is in line with the notion that MAP kinases are downstream in the gene activation pathways of tyrosine kinase receptors. The latter have been shown also to exert an inhibitory action on ORE induced reporter gene expression (cf. Fig. 3).

Induction of ORE-mediated CAT expression in response to protein phosphatase inhibition. Protein phosphorylations, as a means for intracellular signalling, require continuous dephosphorylation, to permit efficient cell regulation. The use of okadaic acid at low nanomolar concentrations permits to distinguish between PP_2A, that is inhibited and PP_1 that becomes inhibited only at 100 fold higher concentrations. In Fig. 5 it is shown that at low osmotic strength 2 nM okadaic acid activates very little relative to the effect observed at 200 nM. This suggests that the ORE mediated CAT expression is under the control of PP_1 and to a lesser extent of PP_2A. At high osmotic strength both concentrations were almost ineffective, indicating lack of additivity between osmotic response and phosphatase control.

Control experiments with the basic pBL-tk-CAT plasmid yielded very low CAT activities identical to the activity obtained from mock-treated cells (data not shown). This proves that the observed activity changes were due to the presence of the osmotic responsive element (ORE_3) and were not mediated by the pBL-tk-CAT plasmid itself.

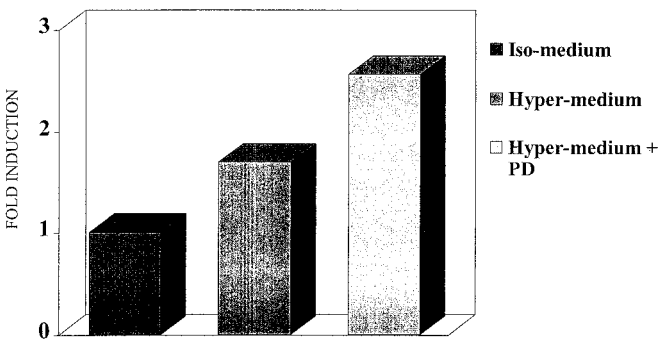

FIG. 4. Induction of the ORE gene expression by 2′-Amino-3′-methoxyflavone (PD), a MAP kinase kinase inhibitor. Transfected rSMC cells were treated with 25 μM PD for 18 h. Cells were then harvested and the amount of CAT activity was determined by thin layer chromatography.

CONCLUSIONS

The mechanisms by which cells respond to extracellular stimuli involves a series of signal transduction events across the cell membrane, within the cytosol and up to the nucleus. Signals are efficiently transferred by a cascade of phosphorylation-dephosphorylation events, involving tyrosine and serine/threonine kinases. The finding that PMA activates the expression of a reporter gene only under the control of ORE suggests a direct effect and not one mediated by JNK activation (20).

It has been shown that cell exposure to UV light or osmotic shock induced clustering and internalisation of surface receptors for epidermal growth factor (EGF), tumor necrosis factor (TNF), and interleukin-1 (IL-1). This event activates the c-Jun amino-terminal protein kinase (JNK) cascade, causing induction of many target genes (21). The involvement of the tyrosine-kinase and protein kinase C signal transduction cascade in the regulation of the ORE-mediated CAT expression, produced by a hyperosmotic stress, has been addressed in this study by inducing tyrosine kinase and protein kinase C inhibition and activation. At the same time a protein phosphatase inhibitor and a MAP kinase kinase inhibitor have been also employed, to permit to better understand the pathway of aldose reductase expression regulation.

The findings reported here can be summarised by the following considerations. Hyperosmotic stress acts, at least in part, by activating aldose reductase gene transcription *via* the osmotic responsive element. Osmotic response coupling to gene expression appears to be mediated by the phosphorylation/dephosphorylation cascade involving tyrosine kinases and protein kinase C. Hyperosmotic stress may primarily affect tyrosine kinase receptors followed by MAP kinase activation, which ultimately will produce an inhibition of aldose reductase gene expression by affecting ORE. On the other side a second pathway exists, consisting in a PKC-mediated activation. The existence of a steady state dephosphorylation affecting this pathway is indicated by the potentiating effect of okadaic acid. The two pathway responses, agonist and antagonist for hyperosmotic gene expression coexist. It appears thus that the positive response of aldose reductase expression to hyperosmotic stress is due to a dominating role of the PKC pathway over the tyrosine kinase receptor pathway.

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