Vasopressin triggers senescence in K-ras transformed cells via RhoA-dependent downregulation of cyclin D1

Fabio L Forti and Hugo A Armelin

Departamento de Bioquimica, Instituto de Quimica, Universidade de Sao Paulo, Sao Paulo, Brazil

(Correspondence should be addressed to H A Armelin, Departamento de Bioquimica, Instituto de Quimica, Universidade de Sao Paulo, Av. Prof. Lineu Prestes, 748-Blo 09 Inf., S1 926, Cidade Universitaria, CEP 05508-900, CP 26077, Sao Paulo-SP, Brazil; Email: haarmeli@iq.usp.br)

Abstract

Arginine vasopressin (AVP), a vasoactive peptide hormone that binds to three G-protein coupled receptors (V1R, V2R, and V3R), has long been known to activate V1R and elicit mitogenesis in several cell types, including adrenal glomerulosa cells. However, in the mouse Y1 adrenocortical malignant cell line, AVP triggers not only a canonical mitogenic response but also novel RhoA-GTP-dependent mechanisms which downregulate cyclin D1, irreversibly inhibiting K-ras oncogene-driven proliferation. In Y1 cells, AVP blocks cyclin D1 expression, induces senescence-associated β-galactosidase (SAβ-Gal) and inhibits proliferation. However, ectopic expression of cyclin D1 renders Y1 cells resistant to both SAβ-Gal induction and proliferation inhibition by AVP. In addition, ectopic expression of the dominant negative RhoAN19 mutant blocks RhoA activation, yielding Y1 cell sub-lines which are no longer susceptible to cyclin D1 downregulation, SAβ-Gal induction, or proliferation inhibition by AVP. Furthermore, inhibiting RhoA with C3 exoenzyme protects Y1 cells from AVP proliferation inhibition and SAβ-Gal induction. On the other hand, AVP treatment does not activate caspases 3 and 7, and the caspase inhibitor Ac-DEVD-CMK does not protect Y1 cells from proliferation inhibition by AVP, implying that AVP does not trigger apoptosis. These results underline a pivotal survival activity of cyclin D1 that protects K-ras oncogene-dependent malignant cells from senescence.

Endocrine-Related Cancer (2007) 14 1117–1125

Introduction

The neurohypophysial hormone arginine vasopressin (AVP) is a vasoactive peptide hormone that binds to three G-protein coupled receptors (V1R, V2R, and V3R) in order to regulate several related physiological functions namely, body fluid osmolality, blood volume, vascular tone, and blood pressure (Michell et al. 1979, Thibonnier et al. 1993). In addition, AVP is also a mitogen that activates V1R, leading to a mitogenic response in vascular smooth muscle cells, 3T3 fibroblasts, renal mesangial cells, adrenal glomerulosa cells, and hepatocytes (Van Biesen et al. 1996). Here we report that AVP activates V1R in mouse Y1 adrenocortical malignant cells (Yasumura et al. 1966), eliciting a novel anti-mitogenic response that is dependent on RhoA-GTP and involves drastic cellular morphological alterations.

Amplification and overexpression of the K-ras oncogene is a major oncogenic lesion underlying the malignant state of the Y1 adrenocortical cell line (Schwab et al. 1983, Kimura & Armelin 1988). The phenotype of the malignant state includes evasion from apoptosis and limitless replicative potential. We have previously shown that AVP blocks cyclin D1 induction in G0/G1-cell cycle-arrested Y1 cells which were mitogenically stimulated with fetal calf serum (FCS) or fibroblast growth factor 2 (FGF2) (Schwindt et al. 2003). We now demonstrate that AVP blocks cyclin D1 induction by a process dependent on RhoA activity to trigger senescence and irreversibly inhibit Y1 cell proliferation. However, blocking cyclin D1 induction did not impair G1 phase progression, because cyclin D1 is not involved in the regulation of the Y1 cell cycle. These results emphasize an essential role of cyclin D1 that complements the strong oncogenic activity of overexpressed K-ras to maintain survivability and the malignant phenotype of cancer cells.
Materials and methods

Cell lines
Stock cultures from the parental mouse Y1 adrenocortical tumor cell line (Yasumura et al. 1966) and Y1-transfectant clonal sub-lines were grown in 10% FCS–Dulbecco Modified Eagle Medium–DME ± 100 μg/ml G418 (Geneticin). To arrest the cell cycle at the G0/G1 boundary, phosphorylation was induced by Dr Garry Nolan, Stanford University, CA, USA) to yield stocks of retroviral particles kept at −80 °C. Y1 cells (at 50% confluence in 10% FCS medium) were infected at high viral multiplicity (5 μg/ml polybrene; 24 h), grown for 24 h in fresh 10% FCS–DME, split among three 100 mm dishes, neutrally selected with 500 μg/ml G418 and cloned. Large sets of Y1 transfectant clones, respectively, named Y1-RhoAW, Y1-RhoAV14, or Y1-RhoAN19, were stocked frozen in liquid nitrogen.

RhoA-GTP assay by reaction with RBD–GST fusion protein
One milligram protein aliquots of frozen stored cell lysates (50 mM Tris–HCl pH 8.0, 0.5% Nonidet P-40, 150 mM NaCl, 1% SDS, 0.5% sodium deoxycholate, 1 mM DTT, 10 μg/ml leupeptin, pepstatin, aprotinin, PMSF, and sodium orthovanadate) were incubated (60 min, 4 °C) on a rotating platform with RBD–GST fusion protein (RhoA binding domain of rhoteikin–glutathione S transferase (Reid et al. 1996)). DNA constructs kindly provided by Dr Martin A Schwartz, The Scripps Institute, La Jolla, CA, USA) bound to glutathione-agarose beads in order to bind RhoA-GTP. RhoA-GTP-bound beads were recovered by centrifugation, washed with lysis buffer, resuspended in sample buffer, loaded onto 12% SDS-PAGE gels and processed for western blotting. Untreated cell lysates of 100 μg protein aliquots (total RhoA) were also included to estimate percentage levels of RhoA-GTP. RhoA was detected with monospecific polyclonal mouse antibodies (Santa Cruz).

Levels of cyclin D1 protein expression
Cells were lysed in cold 62.5 mM Tris–HCl pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol (DTT), 1% w/v bromophenol blue, sonicated (2 min), boiled (5 min), clarified by centrifugation (10 000 g, 5 min, 4 °C), loaded on 10% SDS-PAGE gels (150 μg protein aliquots), and finally processed for western blotting. Total Erk1/2 or Thr202/Tyr204-phosphorylated ERK1/2, total Akt or Ser473-phosphorylated Akt, total GSK-3α/β, and total GSK-3α/β isoforms were detected with specific polyclonal rabbit antibodies (Cell Signaling), followed by a secondary peroxidase-conjugated anti-rabbit polyclonal antibody for chemiluminescent detection (ECL, Amersham-Pharmacia).

Cleavage and expression analysis of caspase-3 and caspase-7 proteins
Caspases were analyzed according to the Apoptosis Sampler Kit protocol (Cell Signaling, Danvers, MA, USA).

Flow cytometry
G0/G1-arrested cells, after AVP and/or FCS stimulation, were trypsinized, centrifuged, resuspended (2 ml, 10% FCS–DME), spun down (1000 g, 5 min), washed twice with PBS, suspended and fixed in 500 μl 70% ethanol (30 min, 20 °C), re-centrifuged and washed twice (1% BSA/PBS), and finally resuspended in 150 μl PBS plus 0.1% sodium citrate, 50 μl of 1 mg/ml RNAse A (Sigma–Aldrich), and 100 μl of 100 μg/ml propidium iodide (PI, Sigma–Aldrich). Resuspended cells were incubated overnight at 4 °C. Fluorescence analysis was performed with a FACStar Plus flow cytometer (Becton-Dickinson Immunocytometry Systems Inc., San Jose, CA, USA) with 250 mW, 488 nm light from an argon-ion laser (Coherent Inc., Santa Clara, CA, USA); PI-red fluorescence was read at 560/580 nm.
Data from samples of 10,000 cells were collected and analyzed using a Macintosh G3 computer running CellQuest V3.1 software (Becton-Dickinson, Franklin Lakes, NJ, USA); DNA profiles were analyzed with MODFIT (Verity software, Topsham, ME, USA).

Senescence-associated β-galactosidase (SAβ-Gal) assay

G0/G1-arrested cells (at 30% confluence in SFM, 35 mm dishes) were stimulated with AVP and/or FCS, rinsed with PBS, fixed (5 min, room temperature, 2% formaldehyde/0.2% glutaraldehyde), rinsed twice more (PBS), and incubated for 8 h at 37 °C with fresh X-Gal staining solution (1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside; stock solution: 20 mg/ml dimethylformamide), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl2, 40 mM citric acid, sodium phosphate, and pH 6.0). Stained cells were washed twice with PBS and kept at 4 °C until examination under a Nikon microscope. Photomicrographs were made by a coupled digital system using MetaMorph software. SAβ-Gal stained cells were estimated by counting ~500 cells per duplicate plates to yield the percent of senescent cells.

Results

AVP downregulates cyclin D1 induction in Y1 adrenocortical malignant cells to trigger senescence suppressing cell proliferation

In Y1 adrenocortical malignant cells, AVP blocks cyclin D1 induction (Fig. 1A), induces SAβ-Gal (Fig. 1B) and suppresses proliferation (Fig. 1C). On the other hand, constitutive expression of a cyclin D1 transgene (Fig. 1A) renders the sub-line Y1-D1G

![Figure 1](https://example.com/figure1.png)

**Figure 1** AVP blocks the induction of cyclin D1 by FCS, inducing senescence-associated β-galactosidase (SAβ-Gal) and suppressing proliferation of Y1 adrenocortical malignant parental cells, but not the Y1-D1G sub-line that constitutively expresses a cyclin D1 transgene. (A) Cyclin D1 expression detected by western blotting. (B) SAβ-Gal induction in Y1 parental cells by 24-h treatment of 1 nM AVP in both SF-DME and 10% FCS–DME. (C) Suppression of Y1 parental cell proliferation by 1 nM AVP. (D) 1 nM AVP does not induce SAβ-Gal, and (E) does not inhibit proliferation in Y1-D1G cells. These results are representative of at least three experiments.
resistant to both SAβ-Gal induction (Fig. 1D) and proliferation suppression (Fig. 1E) by AVP. In addition, clonogenic assays show that 24 h AVP treatment is sufficient to drastically reduce colony growth in cultures of Y1 cells (Fig. 2A), but not in cultures of Y1-D1G cells (Fig. 2B). This strong and irreversible proliferation block caused by AVP in Y1 cells is likely to be due to senescence rather than apoptosis. In Y1 cells, camptothecin or DMSO, but not AVP, FGF2, or both, promoted cleavage of the caspase 3 and 7 pro-enzymes (Fig. 2C). Furthermore, a strong and specific inhibitor of caspase-3, -6, -7, -8, and -10, namely, Ac-DEVD-CMK, does not protect Y1 cells from the noxious effect of AVP in clonogenic assays (Fig. 2D). Thus, these results strongly support the conclusion that AVP does not initiate apoptosis in Y1 cells.

**AVP requires RhoA activity to trigger senescence in Y1 adrenocortical malignant cells**

In Y1 cells, AVP causes morphological changes and stimulates migration (see Supplementary Figure 1, which can be viewed online at http://erc.endocrinology-journals.org/supplemental), two processes mediated by the Rho family of GTPases. In fact, poorly migrating G0/G1-arrested Y1 cells, in serum-free DME (Supplementary Figure 1), show undetectable levels of RhoA-GTP (Fig. 3A). When serum-depleted Y1 cells are stimulated with AVP, RhoA-GTP levels rapidly increase and remain elevated (Fig. 3A), while cells resume migration (Supplementary Figure 1). Thus, as expected, RhoA deactivation and reactivation correlate, respectively, with Inhibition and re-stimulation of migration in Y1 cells. However, abolishing RhoA activity in Y1 cells with ectopic expression of the dominant negative mutant RhoAN19 (Fig. 3B) severely inhibits cell migration (Supplementary data, Figure 1), but not cell proliferation (Fig. 3D). In addition, Y1-RhoAN19 sub-lines, typified by clone 2.1 of Fig. 3D and F, are not susceptible to proliferation inhibition (Fig. 3D) and SAβ-Gal induction by AVP (Fig. 3F). In contrast, Y1 cells stably infected with a viral vector expressing the constitutively active mutant RhoAV14 (Fig. 3B) display proliferation that is severely inhibited by AVP (Fig. 3C) and remain susceptible to SAβ-Gal induction by AVP (Fig. 3E).

Altogether, these results demonstrate that RhoA activity is not essential for the proliferation of Y1 cells. However, RhoA activity is required to initiate the AVP-induced senescence that irreversibly blocks proliferation. These conclusions are further supported by experiments.

![Figure 2](https://www.endocrinology-journals.org/1120.png)
showing that inhibition of RhoA activity with the C3 exoenzyme protects Y1 cells from AVP in clonogenic assays. (A) Western blotting showing activation of RhoA by AVP in G0/G1 arrested Y1 cells, kept in SF-DME. (B) Western blotting showing undetectable levels of RhoA-GTP in G0/G1 arrested cells of the Y1-RhoAN19-2.1 clonal sub-line and constitutively high levels of RhoA-GTP in the Y1-RhoAV14-1.1 clonal sub-line, irrespective of AVP and/or FGF2 treatment. Growth curves showing inhibition of the proliferation of (C) the Y1-RhoAV14-1.1 clone, but not of (D) the Y1-RhoAN19-2.1 clone. Cytochemical assays detecting SAβ-Gal induction in (E) Y1-RhoAV14-1.1, but not in (F) Y1-RhoAN19-2.1; figures in the micrographs are estimates of percent cells positive for SAβ-Gal. Three clonal sub-lines of each series, namely, Y1-RhoAV14 and Y1-RhoAN19 were tested with similar results. Exoenzyme C3 (G) inhibits SAβ-Gal induction by AVP and (H) protects Y1 parental cells from AVP in clonogenic assays; results of colonies/plate from a single experiment (unpaired Student t-test, two-sided p, n = 3).

Cyclin D1 downregulation by AVP is mediated by RhoA-GTP

In G0/G1-arrested Y1 parental cells, cyclin D1 protein induction by FCS is completely blocked by AVP (Figs 1A and 4A). On the other hand, in G0/G1-arrested Y1-RhoAN19-2.1 cells that are constitutively deficient in RhoA-GTP (Fig. 3B), 5 h of incubation with FCS induces cyclin D1 regardless of the presence of AVP (Fig. 4D). These results clearly show that cyclin D1 induction by FCS is independent of RhoA activity, whereas the blocking of cyclin D1 induction by AVP is dependent on RhoA-GTP. However, steady-state levels of cyclin D1 protein are subject to complex regulation. In the Y1-RhoV14-1.1 sub-line, chronically
high levels of RhoA-GTP (Fig. 3A and B) keep ERK1/2 permanently activated so that downstream, constitutive basal levels of cyclin D1 protein are maintained (Fig. 4C). Conversely, AVP completely blocks cyclin D1 induction by FCS in G0/G1-arrested Y1-RhoV14-1.1 cells. However, cyclin D1 protein basal levels decrease slowly irrespective of FCS presence, mainly because GSK3α/β are largely held inactive (Fig. 4C). Furthermore, in the transfectant sub-line Y1-D1G, cyclin D1 protein is overexpressed due to constitutive transcription from the cyclin D1 transgene, as well as the low rate of cyclin D1 protein degradation; this is found irrespective of AVP treatment (Fig. 4B). Thus, AVP is a strong inhibitor of endogenous cyclin D1 induction, but is a poor promoter of cyclin D1 protein degradation.

**Cyclin D1 is a survival factor and not a regulator of G1 phase progression, protecting Y1 adrenocortical malignant cells from senescence**

FACS analysis of cell cycle progression in G0/G1-arrested Y1 cells shows a small inhibition by AVP of FCS-stimulated G1→S→G2 transition (Fig. 5A), in spite of completely abolishing cyclin D1 induction (Figs 1A and 4A). In the Y1-RhoAN19-2.1 sub-line, G0/G1-arrested cells display a relatively smaller rate of G1 phase progression (Fig. 5B) regardless of a strong induction of cyclin D1 by FCS (Fig. 4D). In addition,
in the transfectant sub-line Y1-D1G, high constitutive expression of the cyclin D1 transgene (Figs 1A and 4B) is not sufficient to prevent exit from the cell cycle to quiescence upon serum depletion (Schwindt et al. 2003). However, cyclin D1 plays a critical survival role in Y1 cells and cyclin D1 depletion triggers cell senescence, ultimately leading to irreversible proliferation inhibition instead of a reversible cell cycle arrest in G1 phase.

Discussion

Amplification and mutations in ras oncogenes cause experimental transformation in animal cells and are also involved in the etiology of many human neoplasias (Barbacid 1987, Bos 1988). The mouse Y1 adrenocortical cell line (Yasumura et al. 1966) is an example of a cell line displaying a malignant phenotype dependent upon amplified and overexpressed K-ras (Schwab et al. 1983, Kimura & Armelin 1988, Forti et al. 2002). In Y1 cells, AVP triggers a canonical mitogenic response (Schwindt et al. 2003; this paper) and additionally initiates mechanisms of proliferation suppression via the downregulation of cyclin D1 mediated by RhoA-GTP. Thus, AVP is uncovering novel RhoA-dependent mechanisms which trigger irreversible inhibition of K-ras-driven proliferation in malignant cells; these results are of obvious interest for cancer biology and therapeutics.

The family of Rho GTPases belongs to the superfamily of Ras-related small GTPases, members of which are found in all eukaryotic cells. Twenty-two mammalian genes encode 22 Rho-GTPases, but only RhoA, Rac-1, and Cdc42 have, so far, been sufficiently studied (Aspenstrom et al. 2004; reviewed in Jaffe & Hall 2005). In normal cell lines, G1 phase progression, adhesion, and migration are coordinately controlled by RhoA, Rac1, and Cdc42; inhibition of RhoA, Rac1, or Cdc42 blocks both G1 phase progression and cell migration (Olson et al. 1995, Mammoto et al. 2004). In Y1, a malignant cell line, G1 phase progression is uncoupled from cell adhesion and migration. Y1 cell migration is dependent on RhoA activity (Supplementary Figure), but G1 phase progression and, ultimately, cell proliferation are not dependent (Figs 3 and 5). Mitogens, like FCS and/or AVP, activate RhoA in G0/G1-arrested Y1 cells (Fig. 3A) with different results: FCS promotes robust proliferation (Figs 1C) and migration whereas AVP promotes migration (Supplementary Figure), but triggers senescence (Fig. 1B), thus irreversibly blocking proliferation (Figs 1C and 2A) in spite of activating the ERK mitogenic pathway (Fig. 4A). On the other hand, blocking RhoA activity with the dominant negative mutant RhoA-N19 (Fig. 3B, D and F) or with a C3 inhibitor (Fig. 3G and H) protects Y1 cells from AVP-induced senescence. Our results apparently contradict other reports stating that RhoA activity is important in the initiation of malignant transformation (Coleman et al. 2006). At present, it is difficult to reconcile these disparate results mainly because there are no recognizable patterns of alteration in the organization and

---

**Figure 5** AVP does not inhibit the G0/G1 → S cell cycle transition induced by FCS in G0/G1-arrested Y1 parental cells and clonal sub-lines Y1-RhoAN19-2.1 and RhoAv14-1.1. Cells were plated in 10% FCS–DME and 12–24 h later underwent serum starvation for 48 h in SF-DME to arrest the cell cycle. G0/G1-arrested cells were stimulated with 10% FCS and/or 1 nM AVP for 24 h and processed for flow-cytometric analysis as detailed in the Materials and methods. These results are from three experiments.
function of RhoA, Rac1, and Cdc42 during cell transformation in fully malignant cells.

In the cell cycle, regulation of G1 phase progression is characterized by a rigid sequential ordering of three gene transcription waves: first, immediate early genes; second, cyclin D genes; and third, cyclin E genes (Sherr 1993, Sherr & Roberts 1999, 2004). Only the first two waves are under the control of extracellular mitogens and anti-mitogens. At the mid-G1 phase, transcription of the cyclin D genes and expression of cyclin D proteins are rate-limiting steps (Sherr & Roberts 1999, 2004). This central role in the regulation of G1 phase progression makes D cyclins potentially strong oncoproteins (Quelle et al. 1993, Resnitzky et al. 1994). Actually, it is presently accepted that cyclin D1 is a causative oncogene in a large proportion of human mammary carcinomas (Roy & Thompson 2006). However, cyclin D1 pathways remain incompletely elucidated (Sherr & Roberts 2004), despite the incisive experimentation done in mouse models over the last few years (Yu et al. 2001, 2006, Landis et al. 2006). Cyclin D1^−/− knockout mice, but not D2^−/− and D3^−/− knockout mice, are viable and protected from mammary cancer driven by MMTV-ras or MMTV-neu oncogenes (Yu et al. 2001). In addition, a knock-in mouse for the cyclin D1-K112E point mutant deficient in CDK4 activation develops normal mammary glands (Landis et al. 2006), but remains resistant to MMTV-neu-driven mammary carcinogenesis (Yu et al. 2006). Thus, according to these highly elaborated experimental results, in mice, fully active wild-type cyclin D1/CDK4 must complement MMTV-neu to cause the development of mammary cancer (Landis et al. 2006, Yu et al. 2006). However, in this case, the exact function of cyclin D1/CDK4 is still an open question.

Cell cycle control in Y1 cells is partially deregulated by two independent oncogenic lesions. First, high chronic levels of K-ras-GTP maintain constitutively elevated basal activity of the PI3K/Akt pathway (Forti et al. 2002; this paper, Fig. 4A). Second, the G1 phase is relatively short (Lotfi & Armelin 2001) and, moreover, cyclin D1 and E are concomitantly induced instead of being induced sequentially (Rocha & Armelin, unpublished data). This last oncogenic lesion leaves cyclin D1/CDK4/6 without any role in the regulation of G1 phase progression (Fig. 5); this control is under the following signaling sequence: ERK1/2 → cyclin E-CDK2 → pRb-phosphorylation → DNA synthesis initiation. However, cyclin D1 plays a pivotal survival function protecting Y1 tumor cells from senescence (Figs 1A, D, E, and 2B). In conclusion, maintenance of the Y1 cell malignant phenotype depends on cyclin D1 survival function complementing the strong oncogenic activity of overexpressed K-ras, resembling mammary tumor development in MMTV-ras-mice (Yu et al. 2001, 2006, Landis et al. 2006). In human breast cancer, the cyclin D1 gene is frequently found to be amplified and overexpressed (Roy & Thompson 2006). However, our observations with Y1 adrenocortical malignant cells underline a more subtle oncogenic activity of cyclin D1 at physiological concentrations and under normal regulation, independent of its regulatory roles in G1 phase progression, which might pass unnoticed in many naturally occurring human cancers.

Acknowledgements

We wish to thank Dr Nigel Carter (The Salk Institute, La Jolla, CA, USA), Dr Martin A Schwartz (The Scripps Institute, La Jolla, CA, USA), and Dr Garry Nolan (Stanford University, CA, USA) for RhoA mutant clones, the RBD(Rothekin)-GST fusion clone, and FNX-Ampho (Phoenix) cells respectively. We also thank Jacqueline Salotti and Alexandre Dermagas Oliveira, from our laboratory, for reading the manuscript and help with preparing figures. This work was supported by grants and fellowships from FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil) and CNPq (Conselho Nacional do Desenvolvimento Científico e Tecnológico, Brazil). The authors declare no conflict of interest.

References

Forti FL, Schwindt TT, Moraes MS, Eichler CB & Armelin HA 2002 ACTH promotion of p27(Kip1) induction in mouse Y1 adrenocortical tumor cells is dependent on both PKA activation and Akt/PKB inactivation. Biochemistry 41 10133–10140.


Sherr CJ & Roberts JM 2004 Living with or without cyclins and cyclin-dependent kinases. *Genes and Development* **18** 2699–2711.


