Visinin-Like 1 Is Upregulated in Aldosterone-Producing Adenomas With KCNJ5 Mutations and Protects From Calcium-Induced Apoptosis

Tracy Ann Williams, Silvia Monticone, Valentina Crudo, Richard Warth, Franco Veglio, Paolo Mulatero

Abstract—Visinin-like 1 (VSNL1) is upregulated in aldosterone-producing adenomas (APAs) compared with normal adrenals. We demonstrate that VSNL1 overexpression in adrenocortical carcinoma cells (NCI H295R) upregulates basal and angiotensin II–stimulated CYP11B2 gene expression 3.2- and 1.5-fold, respectively. Conversely, silencing VSNL1 by RNA interference decreases angiotensin II–stimulated CYP11B2 expression and aldosterone secretion by 41.0% and 34.5%, respectively. Mutations in the potassium channel KCNJ5 have been identified in APAs that result in sodium influx and membrane depolarization and are postulated to result in calcium influx in adrenal glomerulosa cells. VSNL1 and CYP11B2 are 8.1- and 6.0-fold more highly expressed, respectively, in APAs harboring KCNJ5 mutations compared with those without, and the upregulation of VSNL1 in these APAs accounts for the overexpression of VSNL1 in the total APA sample set compared with normal adrenals. Silencing VSNL1 in H295R cells renders them sensitive to ionomycin-induced apoptosis, indicating that VSNL1 protects these cells against calcium-induced cell death. Concomitant expression of mutated KCNJ5 (G151R) and silencing VSNL1 results in apoptosis of H295R cells, an effect that is blocked by nifedipine and is absent using a control small-interfering RNA or when wild-type KCNJ5 is expressed and VSNL1 is silenced. These data demonstrate that VSNL1 plays a dual function in vitro in the regulation of CYP11B2 gene expression and in the inhibition of calcium-induced apoptosis. In addition, VSNL1 may play a role in the pathophysiology of APAs harboring mutations in the potassium channel KCNJ5 via its antiapoptotic function in response to calcium cytotoxicity and its effect on aldosterone production. (Hypertension. 2012;59:00-00.)

Key Words: VSNL1 □ KCNJ5 □ aldosterone-producing adenoma □ primary aldosteronism □ aldosterone

Primary aldosteronism (PA) is the most frequent form of endocrine hypertension and is characterized by the excessive production of the mineralocorticoid hormone aldosterone by the adrenal glands.1 A common underlying cause of PA is the presence of an aldosterone-producing adenoma (APA), a benign tumor, also known as Conn syndrome, that is found in 30% to 40% of PA patients. Unilateral adrenalectomy often normalizes or markedly improves the blood pressure in PA patients, and, therefore, APA is the most common, specifically treatable, and potentially curable form of hypertension.1,2

Three forms of familial hyperaldosteronism (FH) have been described (FH-I, -II, and -III). FH-I (or glucocorticoid-remediable aldosteronism) accounts for <1% of PA patients.3 The molecular basis of FH-I is an unequal crossing over between the genes encoding 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) to produce a chimeric gene transcribed under the control of adrenocorticotropic hormone.4,5 FH-II is a nonglucocorticoid-remediable form of hyperaldosteronism that displays linkage with chromosomal region 7p226 but is of unknown etiology. FH-II is the most frequent form of FH (≈6% of PA patients)3 and its diagnosis requires the confirmation of PA in ≥2 family members and FH-I and -III exclusion.1,2 Finally, FH-III is an autosomal-dominant form of PA that has been described in only 2 families so far3,8 and is caused by germline mutations in the KCNJ5 gene that encodes the potassium channel Kir3.4 (potassium inwardly rectifying channel, subfamily J, member 4).9 To date, 2 mutations causing FH-III have been identified, T158A9 and G151E.8 Somatic KCNJ5 mutations in APA, G151R and L168R, have also been described,9 and all of the mutations, both germline and somatic, are located in or near the selectivity filter of the potassium channel. The mutations result in increased sodium conductance and cell depolarization, and in adrenal glomerulosa cells, this is proposed to result in calcium influx.9

We previously identified differentially expressed genes in APAs compared with normal adrenals (NA) by microarray...
analysis. One of the upregulated genes was visinin-like 1 (VSNL1), and this observation was validated by real-time PCR using a wider set of adrenal samples. VSNL1 belongs to the visinin-like protein subfamily of neuronal calcium sensor proteins that includes 4 other members, VSNL2, VSNL3, hippocalcin, and neurocalcin. As for other neuronal calcium sensors, the VSNLs function in the transduction of calcium signals and act as modulators of multiple intracellular targets, much like the prototype calcium sensor calmodulin. In contrast to the restricted neuronal expression of hippocalcin, VSNL1 is extensively expressed in peripheral human tissues, possibly reflecting its diverse functions (for review, see Reference 1). A recent study identified VSNL1 as a positively regulated target of the nuclear receptor steroidogenic factor 1 (NR5A1) in the human adrenocortical carcinoma cell line NCI H295R. Because steroidogenic factor 1 plays a pivotal role in the regulation of adrenal development and steroidogenesis, the study by Ferraz-de-Souza et al raises the possibility that VSNL1 could be involved in mediating these functions.

To define a role for VSNL1 in the pathophysiology of APA, we studied its effect on CYP11B2 expression and regulating cell growth in NCI H295R cells. We show the following: (1) VSNL1 overexpression increases basal and angiotensin II (Ang II)–stimulated CYP11B2 transcription, whereas silencing VSNL1 decreases Ang II–stimulated CYP11B2 expression with a concomitant reduction in aldosterone secretion; (2) VSNL1 and CYP11B2 but not CYP11B1 display increased expression in APA that harbor mutations in KCNJ5 compared with those that express wild-type KCNJ5; and (3) VSNL1 protects H295R cells against calcium-induced apoptosis and promotes survival from the calcium toxicity induced by the expression of the G151R mutation in KCNJ5.

Methods

Patient Selection

Patients were studied and selected as described previously.

Semiquantitative RT-PCR

VSNL1 expression levels were compared in H295R cells and in pooled samples of cDNA produced from APA or normal adrenals by semiquantitative RT-PCR and agarose gel electrophoresis (Figure 1A). A 503-bp cDNA fragment of VSNL1 was amplified using the PCR conditions as described before, as the following primers: VSNL1 forward primer 5′aggtgagagcaggtggtgtg3′ and reverse primer 5′tgctctttgaggtggtcgt3′. GAPDH was used as the endogenous reference gene: forward primer 5′tagctgtggatgctgtctgt3′ and reverse primer 5′ttgctctttgcagcttctt3′.

RNA Extraction, Reverse Transcription, and TaqMan Gene Expression Assays

All of the gene expression assays used to describe fold changes in this study were performed using TaqMan real-time PCR assays. Total RNA was extracted from adrenal tissues or from NCI H295R cells and reverse transcribed as described previously. Real-time PCR was performed in triplicate using TaqMan gene expression assays (Applied Biosystems, Foster City, CA), and expression levels were analyzed using the 2−ΔΔCt relative quantification method, using ACTB as the endogenous reference gene.

VSNL1 and KCNJ5 Expression Vectors

VSNL1 cDNA was purchased from the National Institutes of Health mammalian gene collection (Invitrogen, Carlsbad, CA) and subcloned into the pcDNA3.1/V5His6 vector (Invitrogen). A control plasmid contained the VSNL1 cDNA in the antisense direction to the VSNL1, and overexpressed VSNL1 was detected by Western blotting using an anti-V5 antibody (Figure 1B, top). H295R cells were transfected with either control plasmid (Ctrl) or pcDNA3.1/VSNL1 with a C-terminal V5 tag (VSNL1), and overexpressed VSNL1 was detected by Western blotting using an anti-V5 antibody (Figure 1B, top). H295R cells were transfected with either control plasmid (Ctrl) or pcDNA3.1/VSNL1 or pcDNA3.1/V5His6 (VSNL1), and total RNA was extracted 72 hours posttransfection. TaqMan gene expression assays were used to determine the fold change in CYP11B2 expression level using ACTB as the endogenous reference gene. Values are the mean±SD of 4 independent experiments, *P<0.001 (B, bottom). Cells were transfected as before, but cells were stimulated for the last 24 hours with angiotensin II (Ang II; 10 nM/mL). Values are the mean±SD of 3 independent experiments, *P<0.01. (C).

DNA Sequencing of KCNJ5 in APA

DNA sequencing was performed as described in Boulkroun et al. The APA samples used in the present study correspond with those of the Torino group used in the study of Boulkroun et al in which 45% carry KCNJ5 mutations and were G151R (n=10), L168R (n=3), and T158A (n=1). All of the mutations described in this study are somatic mutations: the T158A mutation was first described by Choi.
et al \(^9\) as a germline mutation, but in our patient the mutation was absent in the peripheral DNA.\(^8\)

**Western Blotting**

Western blotting was as described previously using anti-V5 mouse monoclonal (1:1000 dilution; Invitrogen) and anti-actin polyclonal (1:500; Sigma-Aldrich) antibodies.

**Cell Transfection and Gene Silencing**

NCI H295R cells were transfected by Amaxa nucleofection in 100 μL of nucleofector R solution (program P20), as described previously.\(^10\) VSNL1 gene expression was silenced using small-interfering RNA (siRNA) by transfection of 1 × 10\(^6\) cells using 2 μL of a 100-μmol/L solution of Silencer Select predesigned siRNA (Applied Biosystems). Control silencing transfections used the silencer negative control siRNA #1 (Applied Biosystems).

In some experiments, cells (1 × 10\(^6\)) were co-transfected with pcDNA3.1/KCNJ5 (1 μg; wild-type or G151R) and the VSNL1 or control siRNA (2 μL of a 100-μmol/L solution). In incubations in the presence of nifedipine (10 μmol/L; Sigma-Aldrich), nucleofected cells were plated directly in 5-mL complete medium supplemented with nifedipine (10 μmol/L) added from 50 mg/mL of stock solutions in dimethyl sulfoxide. This resulted in a final concentration of 0.007% dimethyl sulfoxide that was maintained for all of the cell incubations.

**Functional Assays**

Apoptosis assays for TUNEL of DNA fragments were performed as described previously.\(^10\) Aldosterone in cell culture medium was measured by radioimmunoassay.\(^10\)

**Immunofluorescence**

H295R cells transfected with pcDNA3.1/KCNJ5\(_{WT}\) or pcDNA3.1/KCNJ5\(_{G151R}\) were incubated overnight at 4°C with an antihuman KCNJ5 rabbit polyclonal antibody (1:75 dilution, Sigma-Aldrich) and subsequently with an antirabbit Alexa Fluor 488 conjugate (1:1000 dilution, Cell Signaling Technology) for 1 to 2 hours in the dark before mounting in Vectashield with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and fluorescence microscopy.

**Statistical Analyses**

Data were analyzed with the Kolmogorov-Smirnov test to determine their distributions. Statistical significance between groups was calculated in normally distributed data by Student \(t\) test for independent samples and in nonnormally distributed data by Kruskal-Wallis and Mann-Whitney U tests. \(P<0.05\) was considered statistically significant.

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**Results**

**VSNL1 Modulates Ang II–Stimulated CYP11B2 Expression in H295R Cells**

VSNL1 transcription is upregulated in APAs compared with NAs.\(^10\) To elucidate a possible role for VSNL1 in APA pathophysiology, we studied its function using the NCI H295R cell line. VSNL1 is transcribed in H295R cells, demonstrated by semiquantitative RT-PCR, to a level comparable to a pooled set of APA samples (Figure 1A). H295R cells were transfected with a control plasmid or a construct encoding V5-tagged VSNL1 and VSNL1 expression was detected after 72 hours by Western blotting using an anti-V5 antibody (Figure 1B, top). VSNL1 overexpression resulted in a 3.18±0.55-fold (\(n=4; P<0.001\)) increase in the basal level of CYP11B2 mRNA (Figure 1B, bottom) and a 1.5-fold increase in Ang II–stimulated (10 nmol/L) CYP11B2 mRNA levels (77.97±6.27 and 117.54±12.93; \(n=3\); \(P<0.01\); increase over basal levels in control or VSNL1-transfected cells, respectively; Figure 1C). In contrast, VSNL1 overexpression had no significant effect on basal levels of CYP11B1 mRNA (1.02±0.15-fold; \(n=4\)).

Silencing VSNL1 expression by transfection of siRNA resulted in an 87% reduction in VSNL1 mRNA levels (fold change: 0.13±0.043; \(n=10\); \(P<0.001\)) 72 hours posttransfection measured by TaqMan real-time PCR (Figure 2A). Silencing VSNL1 decreased the stimulatory effect of Ang II (10 nmol/L) on CYP11B2 mRNA levels from a 64.36±12.60-fold increase over basal levels (\(n=4\)) with control siRNA transfection compared with a 37.77±6.33-fold increase over basal levels (\(n=4\)) when VSNL1 was silenced, corresponding with a 41.3% reduction (\(P=0.02\); Figure 2B). Accordingly, measurement of aldosterone secretion into cell medium by radioimmunoassay demonstrated a concomitant decrease in 10 nmol/L of Ang II–stimulated aldosterone secretion over basal levels when VSNL1 was silenced from 19.95±2.49-fold (\(n=5\)) when a control siRNA was used to 13.06±1.38-fold (\(n=5\)) when an siRNA to interfere with VSNL1 expression was used corresponding with a 34.5% decrease (\(P=0.01\); Figure 2C).

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Figure 2. Effect of silencing VSNL1 on CYP11B2 expression in H295R cells. Cells were transfected with either a control small-interfering RNA (siRNA; siCtrl) or an siRNA specific for VSNL1 (siVSNL1), and total RNA was extracted 72 hours posttransfection. TaqMan gene expression assays were used to determine the fold change in VSNL1 expression level using ACTB as the endogenous reference gene. Values are the mean±SD of 10 independent experiments, \(P<0.001\) (A). Cells were transfected as described in A, but cells were stimulated for the last 24 hours with angiotensin II (Ang II; 10 nmol/L), TaqMan gene expression assays were used to determine fold changes in CYP11B2 expression levels, and ACTB was the reference gene. Values are the mean±SD of 4 independent experiments, \(P=0.02\) (B). Cells were transfected as described under B, except aldosterone secretion in the culture medium was measured by radioimmunoassay. Values are the mean±SD of 5 independent experiments, \(P<0.01\) (C).
VSNL1 and CYP11B2 Display Increased Expression in APAs Harboring Mutations in KCNJ5

The expression levels of VSNL1, CYP11B2, and CYP11B1 in APAs with mutations in KCNJ5 (n = 14) were determined by real-time PCR compared with APAs expressing wild-type KCNJ5 (n = 17). VSNL1 was 8.1-fold more highly expressed in APAs with KCNJ5 mutations compared with those with wild-type KCNJ5 (Figure 3A). We originally reported VSNL1 as an upregulated gene in APAs compared with NAs from a microarray analysis that used 8 APAs, of which 6 we now know carry mutations in KCNJ5. However, we show here that VSNL1 expression in APAs with wild-type KCNJ5 is not significantly different from that of NAs, whereas the APAs with mutated KCNJ5 display significantly higher levels of VSNL1 expression compared with both APA with wild-type KCNJ5 (P < 0.02) and NA (P < 0.05; Figure 3A). In fact, the increased transcription of VSNL1 in APAs with mutated KCNJ5 entirely accounts for the overexpression of VSNL1 observed in the total APA sample set compared with NAs. VSNL1 was upregulated in 8 (57%) of 14 APAs with mutated KCNJ5 compared with 3 (17.6%) of 17 APAs with wild-type KCNJ5 (when “upregulated” is defined as ≥2-fold the mean expression of VSNL1 in APAs with wild-type KCNJ5). CYP11B2 also displayed increased expression (6.0-fold) in APAs with KCNJ5 mutations compared with those without, consistent with the effect of an increase in intracellular calcium levels on the transcription of this gene (Figure 3B). CYP11B2 was significantly higher in APAs with mutated KCNJ5 compared with both APAs with wild-type KCNJ5 (P < 0.05) and NAs (P < 0.001). In contrast, there were no significant differences in CYP11B1 expression between APAs with and without KCNJ5 mutations and NAs (Figure 3C). Finally, VSNL1 expression was significantly lower in incidentalomas (n = 5) and cortisol-producing adenomas (n = 5) compared with APA with KCNJ5 mutations (P < 0.01), consistent with a specific involvement of VSNL1 in the pathophysiology of these APAs and not in other benign adrenocortical tumors.

VSNL1 Protects H295R Cells From Calcium-Induced Apoptosis

Untreated H295R cells were morphologically indistinguishable under phase-contrast microscopy from those incubated for 24 hours with 2 μmol/L of ionomycin (Figure 4A), an ionophore that raises intracellular calcium and can be used to induce apoptosis. Conversely, cells treated for 4 hours with 0.5 μmol/L of staurosporine, an inhibitor of protein kinases, exhibited characteristic signs of apoptosis, the most evident being cell shrinkage (Figure 4A). In contrast, apoptosis was induced by ionomycin (2 μmol/L) when cells were transfected with an siRNA designed to silence VSNL1 (siVSNL1), demonstrated by the TUNEL reaction, but apoptosis was undetectable when cells were transfected with a control siRNA (Figure 4B). Conversely, cells transfected with either control siRNA or siVSNL1 both displayed an apoptotic response to staurosporine (Figure 4B). Therefore, adrenocortical cells are resistant to calcium-induced apoptosis, and the calcium-sensor protein VSNL1 mediates this protective effect.

VSNL1 Protects H295R Cells From Apoptosis Induced by KCNJ5G151R Expression

DNA sequencing of H295R cell RT-PCR products showed that they carry wild-type KCNJ5. The wild-type and mutated forms of KCNJ5 were overexpressed in H295R cells and detected by immunofluorescence microscopy using a rabbit anti-KCNJ5 polyclonal antibody followed by an antirabbit Alexa fluor-488–conjugated secondary antibody: no differences in subcellular distribution were detected between the wild-type and mutated forms of this potassium channel (Figure 4C). We investigated a role for VSNL1 in protecting adrenocortical cells from potential increases in intracellular calcium-induced apoptosis.
calcium caused by the expression of KCNJ5<sub>G151R</sub>. Cotransfection of pcDNA3.1/KCNJ5<sub>G151R</sub> and an siVSNL1 in H295R cells resulted in a marked increase in fluorescent labeling by the TUNEL reaction (Figure 4D) compared with control transfections using either pcDNA3.1/KCNJ5WT and control siRNA or pcDNA3.1/KCNJ5WT and siVSNL1 or control siRNA. In addition, the apoptotic effect produced by transfection of KCNJ5<sub>G151R</sub> and siVSNL1 was ablated in the presence of nifedipine (10 μmol/L), a calcium channel blocker (Figure 4D). Therefore, expression of the G151R-mutated form of the KCNJ5 potassium channel potentially induces apoptosis in adrenocortical carcinoma cells via calcium entry, but they are protected from cell death by the calcium-sensor visinin-like protein 1.

**Discussion**

VSNL1 encodes the calcium-sensor protein visinin-like 1 and was identified previously as an upregulated gene in a sample
set of APAs compared with normal adrenals by microarray and real-time PCR analysis. The present study was undertaken to investigate the potential function of VSNL1 in mediating aldosterone synthesis and cell growth. We demonstrate that VSNL1 modulates basal transcription of CYP11B2 and in response to physiological levels of Ang II. Because Ang II exerts its effects by generating a cytoplasmic calcium signal, VSNL1 most likely acts as an effector for the transduction of this signal to indirectly modulate CYP11B2 transcription.

Recently, a germline mutation (T158A) in a family with FH-III and 2 somatic mutations (G151R and L168R) in APAs was identified in the gene encoding the potassium channel KCNJ5, and a second germline KCNJ5 (G151E) mutation was identified shortly afterward in another kindred with familial hyperaldosteronism. These mutations alter channel selectivity, thus producing nonselective cation channels. The frequency of these somatic mutations in a large multicenter European study was determined as 34% (range: 14% to 50%, with the diversity probably related to differences in diagnostic strategies and patient selection for adrenalectomy). The APA samples used in the present study correspond with a subset used for this analysis. These are composed of 31 APAs, of which 14 carry KCNJ5 mutations with the G151R mutation predominating, accounting for 10 of the KCNJ5 mutations.

We show that the transcription of both VSNL1 and CYP11B2 is significantly increased in APAs with KCNJ5 mutations compared with those expressing wild-type KCNJ5. The increased transcription of CYP11B2 is consistent with the proposed calcium influx in adrenal glomerulosa cells resulting from mutations interfering with the selectivity filter of the KCNJ5 potassium channel. VSNL1 gene expression was highly upregulated in APAs with KCNJ5 mutations compared with those expressing wild-type KCNJ5; in fact, the increased transcription of VSNL1 in APAs with KCNJ5 mutations accounts for the upregulation of VSNL1 in the total APA sample set when compared with normal adrenal tissue.

Furthermore, the increased VSNL1 gene expression in APAs with KCNJ5 mutations was also significantly higher than that in a sample of incidentaloma and cortisol-producing adenomas. This indicates that VSNL1 is specifically involved in the pathophysiology of APAs harboring mutations in KCNJ5 and not in the development of other benign adrenocortical tumors.

Calcium signaling governs a plethora of cell functions vital for cell survival, but calcium overload can also cause cytotoxicity and trigger cell death. Cellular transformation is supported by calcium-stimulated proliferation but limited by calcium-dependent apoptosis, and calcium signaling pathways are often remodeled in tumor cells to sustain cell proliferation and avoid cell death. At least 2 other members of the neuronal calcium sensor family promote cell survival: neuronal calcium sensor-1 increases neuronal resistance to several stress factors, and the neuron-restricted visinin-like protein hippocalcin binds neuronal apoptosis-inhibitory protein in an interaction promoted by calcium that facilitates neuronal survival against calcium-induced death stimuli. In this study we show that VSNL1 protects adrenocortical carcinoma cells against calcium-induced apoptosis and promotes survival of these cells that express KCNJ5_{G151R}, an effect that is ablated in the presence of the calcium channel blocker nifedipine. This leads to the hypothesis that APAs with KCNJ5 mutations may harness the calcium influx to drive cellular proliferation while avoiding calcium-dependent cell death through a mechanism mediated at least in part by VSNL1.

Perspectives

The present study demonstrates that VSNL1 can modulate CYP11B2 gene expression and aldosterone secretion in vitro and can protect adrenocortical carcinoma cells from calcium-induced apoptosis. We show that VSNL1 and CYP11B2 expression is significantly increased in APAs with KCNJ5 mutations compared with those without and compared with incidentalomas and cortisol-producing adenomas. In addition, expression of the G151R-mutated form of KCNJ5 induces apoptosis in H295R cells when VSNL1 is silenced, but its apoptotic effect is ablated by the calcium channel blocker nifedipine. These data indicate that VSNL1 may play a key role in the pathophysiology of APAs harboring KCNJ5 mutations, but not in other benign tumors of the adrenal cortex, via its antiapoptotic function in response to calcium cytotoxicity and its role in the regulation of aldosterone production.

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Disclosures

None.

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