Inversion of the Intracellular Na\(^+\)/K\(^+\) Ratio Blocks Apoptosis in Vascular Smooth Muscle Cells by Induction of RNA Synthesis

Sergei N. Orlov, Sebastien Taurin, Nathalie Thorin-Trescases, Nickolai O. Dulin, Johanne Tremblay, Pavel Hamet

Abstract—This study examines the involvement of RNA and protein synthesis in the modulation of apoptosis in vascular smooth muscle cells (VSMC) by intracellular monovalent cations. In VSMC transfected with E1A adenovirus (VSMC-E1A), inversion of the \([\text{Na}^+] / [\text{K}^+]\) ratio by an inhibitor of the Na\(^+-\)K\(^+\) pump, ouabain, prevented the development of apoptosis triggered by serum withdrawal. Inhibition of apoptosis by ouabain was abolished by inhibitors of RNA and protein synthesis, actinomycin D, and cycloheximide, respectively. In VSMC-E1A, incubation with ouabain for 4 and 24 hours augmented RNA synthesis by 20% to 50% and 3-fold to 4-fold, respectively. In quiescent VSMC, the effect of ouabain and serum on RNA synthesis was additive. Ouabain did not affect the level of phosphorylation of ERK, JNK, and p38 MAP kinases and blocked apoptosis independent of the presence of the MAPK kinase inhibitors PD98059 and SB 202190. Equimolar substitution of NaCl with KCl in the incubation medium abolished the effect of ouabain on intracellular Na\(^+\) and K\(^+\) concentration, apoptosis, and RNA synthesis. Thus, our results demonstrate that the antiapoptotic effect of the inverted \([\text{Na}^+] / [\text{K}^+]\) ratio is mediated by MAPK-independent induction of de novo synthesis of RNA species encoding inhibitor(s) of programmed cell death. (Hypertension. 2000;35:1062-1068.)

Key Words: muscle, smooth, vascular — apoptosis — potassium — sodium — RNA

Both vascular smooth muscle cell (VSMC) growth and programmed cell death contribute to the altered geometry of vessels in hypertension.\(^1\)-\(^3\) Much is known about the pharmacological regulation of VSMC replication (for review see References 4 and 5), which is in contrast with the lack of systematic studies on the pharmacology of VSMC apoptosis. As in the majority of cells investigated so far, apoptosis of VSMC can be triggered by serum withdrawal\(^2,6\) or by treatment with antisense oligodeoxynucleotides against VSMC-derived growth factors.\(^7\) Apoptosis in serum-deprived VSMC is poteniated by overexpression of c-myc or its functional analogue, E1A adenovirus, and coexpression of p53 with c-myc/E1A and is inhibited by overexpression of \(bcl-2\) (for recent review, see Reference 8). Several lines of evidence suggest that the antiapoptotic action of serum in VSMC is caused by the presence of growth factors such as IGF and EGF and is mainly mediated by receptor-coupled polyphosphoinositide-3-kinase.\(^8\) However, the intracellular mechanisms controlling the expression and functional activity of proapoptotic and antiapoptotic gene products in VSMC are poorly understood.

Recently, we reported that inversion of the intracellular \([\text{Na}^+] / [\text{K}^+]\) ratio caused by sustained inhibition of the Na\(^+-\)K\(^+\) pump with ouabain delays development of the apoptotic machinery in serum-deprived VSMC as well as in VSMC transfected with c-myc or E1A-adenovirus.\(^9\) This study examines whether inhibition of programmed cell death by ouabain is caused by de novo synthesis of inhibitors of apoptosis or by posttranslational modification of preexisting intermediates of the apoptotic machinery.

Methods

Materials

VSMC were obtained by explant methods from the aortas of 10- to 13-week-old male Brown Norway (BNJx) rats in accordance with previously described methods.\(^1\) VSMC transfected with E1A adenovirus (VSMC-E1A), prepared as outlined in detail elsewhere,\(^6,10\) were kindly provided by Dr M.R. Bennett (University of Cambridge, UK). Chromatin cleavage assay and caspase-3 activity were used for the measurement of apoptosis in accordance with previously described methods.\(^9,11,12\) RNA and protein synthesis were estimated by incorporation of \([\text{H}]\)-uridine and \([\text{H}]\)-leucine, respectively.\(^13,14\) The intracellular concentration of exchangeable Na\(^+\) and K\(^+\) was measured as the steady-state distribution of extracellular and intracellular \(^22\)Na and \(^86\)Rb, as described previously in detail.\(^15\) The volume of intracellular water was determined as \([^{14}\text{C}]\)-urea available space.\(^16\) MAPK phosphorylation was determined by Western blot analysis.
with antibodies against phospho-ERK, phospho-JNK, and phospho-p38, according to recently described protocol.\textsuperscript{17} Protein content in the cell lysate was measured by the Bradford method.

**Chemicals**

\(^{[3]}H\)-thymidine, \(^{[3]}H\)-uridine, \(^{[3]}H\)-leucine, and \(^{14}C\)-urea were from Amersham; \(^{86}RbCl\) and \(^{22}NaCl\) were from Dupont; PD98059 and SB202190 were from Calbiochem Novabiochem; ouabain, cycloheximide, and actinomycin D were from Sigma; anti–phospho-ERK and anti–phospho-p38 antibodies were from New England Biolab Inc; anti–phospho-JNK was from Santa Cruz Biotechnology; 7-amino-4-methylcoumarin (AMC) was from Molecular Probes; and DEVD-AMC (\(^N\)-acetyl-Asp-Glu-Val-Asp-AMC) and DEVD-CHO were from BIOMOL Research Laboratories. The remaining chemicals were obtained from Sigma, Gibco BRL, and Anachemia.

**Results**

**Kinetics of Apoptosis Modulation by Ouabain**

Figure 1 shows that serum withdrawal resulted in rapid accumulation of chromatin fragments in VSMC-E1A. Indeed, after 2 hours of serum deprivation, the amount of intracellular chromatin fragments was increased from \(2.2\pm0.2\%\) to \(16.3\pm2.4\%\) of total \(^{[3]}H\)-labeled DNA and reached maximal values (25% to 27%) in 4 to 6 hours.

Recently, we reported that 2-hour preincubation of VSMC-E1A with 1 mmol/L ouabain led to a 10-fold elevation of the intracellular content of exchangeable Na\(^+\) and decreased the content of intracellular K\(^+\) by a 4-fold.\textsuperscript{9} A 2-hour preincubation with ouabain completely abolished the effect of serum deprivation on the accumulation of chromatin fragments for the next 12 hours; after 24 hours of serum deprivation, the amount of intracellular chromatin fragments was still 2-fold less than in serum-deprived control cells (Figure 1). Previously, we confirmed the antiapoptotic effect of ouabain by analysis of DNA laddering, caspase-3 activity, and phase-contrast microscopy.\textsuperscript{9}

**Effects of Actinomycin D and Cycloheximide on Apoptosis Modulation by Ouabain**

We did not observe any effect of 4-hour incubation with 2 \(\mu\)g/mL of actinomycin D or cycloheximide on the baseline intracellular content of Na\(^+\) and K\(^+\) and its modulation by ouabain, whereas after 24 hours of incubation with actinomycin D, baseline [K\(^+\)], was declined by 2-fold, accompanied by 3- to 4-fold elevation of [Na\(^+\)]. (data not shown). Considering these results, we took advantage of the rapid induction of apoptosis in serum-deprived VSMC-E1A to study the effect of inhibitors of RNA and protein synthesis.
Figure 2a shows that 30-minute preincubation with actinomycin D completely blocked RNA synthesis and decreased protein synthesis by 65%, whereas cycloheximide reduced RNA and protein synthesis by 85% and 80%, respectively. Neither actinomycin D nor cycloheximide significantly affected the increment of chromatin fragmentation triggered by 3-hour incubation of VSMC-E1A in serum-deprived medium (Figure 2b). This observation is in accordance with the lack of effect of actinomycin D and cycloheximide on the development of apoptosis in human VSMC transfected with p53.18,19 In contrast to baseline apoptosis, the antiapoptotic action of ouabain was completely blocked by actinomycin D and sharply attenuated by cycloheximide.

Effects of Ouabain on RNA and Protein Synthesis

In light of the suppression of the antiapoptotic action of ouabain by actinomycin D and cycloheximide, we examined the effects of ouabain on RNA and protein synthesis in serum-supplied VSMC-E1A. After 2 and 6 hours of application, ouabain augmented RNA synthesis by 40% to 50% and increased it up to 3- to 4-fold in 24 hours (Figure 3a). In contrast to RNA, protein synthesis was attenuated by 40% and 30% after 2 and 6 hours of ouabain addition but recovered in 24 hours (Figure 3b).

The low intensity of apoptosis in serum-deprived VSMC compared with cells transfected with E1A allows us to establish quiescence and to compare the effect of ouabain on RNA synthesis in the presence of growth factors. Figure 4a shows that 4 hours of incubation of quiescent VSMC with ouabain slightly augmented RNA synthesis, whereas after 24-hour incubation in serum-deprived medium, RNA synthesis was increased in ouabain-treated cells by 3- to 4-fold. Approximately the same increment of RNA synthesis was observed after 24-hour incubation of VSMC with 10% calf serum (CS); at this time point, the combined addition of serum and ouabain augmented RNA synthesis by 7-fold. Four-hour incubation with ouabain did not affect protein synthesis in serum-deprived VSMC.

Table 1. Effect of Ouabain on Intracellular Concentration of Na⁺ and K⁺ in VSMC-E1A Incubated in Media With Different Concentrations of Monovalent Cations

<table>
<thead>
<tr>
<th>Composition of Incubation Medium, mmol/L</th>
<th>Intracellular Concentration, mmol/L</th>
<th>Intracellular Water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na⁺</td>
<td>K⁺</td>
</tr>
<tr>
<td>Medium 1, NaCl 130; KCl 5</td>
<td>Control 14.1±1.0</td>
<td>179.1±16.2*</td>
</tr>
<tr>
<td></td>
<td>+ Ouabain 16.2*</td>
<td>207.6±15.5</td>
</tr>
<tr>
<td>Medium 2, NaCl 14; KCl 123</td>
<td>Control 3.6±0.6</td>
<td>5.1±1.0</td>
</tr>
<tr>
<td></td>
<td>+ Ouabain 5.0±1.0</td>
<td>8.1±1.0</td>
</tr>
<tr>
<td>Medium 3, NaCl 14; KCl 5, choline chloride 120</td>
<td>Control 6.4±0.9</td>
<td>21.8±2.8*</td>
</tr>
<tr>
<td></td>
<td>+ Ouabain 8.3±0.9</td>
<td>24.0±2.8</td>
</tr>
</tbody>
</table>

*P<0.001 compared with control cells.

Figure 4. Effect of ouabain on RNA synthesis (a) and apoptosis (b) in VSMC. a, To establish quiescence, VSMC growing in DMEM containing 10% CS were incubated for 24 hours in presence of 0.2% CS and for next 4 or 24 hours in presence (+) or absence (−) of 10% CS without or with 1 mmol/L ouabain. [3H]-uridine then was added for additional 2 hours. [3H]-uridine incorporation in absence of CS and ouabain (12 103 and 13 645 cpm/mg protein for 4 and 24 hours, respectively) was taken as 100%. Mean±SE from experiments performed with 6 repeats are given. b, Growing VSMC were incubated for next 4 or 24 hours in presence (+) or absence (−) of CS without or with 1 mmol/L ouabain; content of intracellular chromatin fragments was measured as indicated in Methods section. Mean±SE from 3 experiments performed in quadruplicate are given. *, **P<0.05 and 0.001 compared with control cells.
with 10% CS augmented \[^{14}H\]-leucine uptake by 94\(\pm\)12\%, and this increment was completely abolished by ouabain. Neither baseline nor serum-induced protein synthesis was affected after 24-hour incubation of VSMC with ouabain (data not shown).

After 4 hours of serum withdrawal, the content of intracellular chromatin fragments in nontransfected VSMC was increased from 0.7\(\pm\)0.2\% to 1.7\(\pm\)0.4\% and 1.4\(\pm\)0.2\% in the absence and presence of ouabain, respectively (Figure 4b). In VSMC treated with serum-deprived medium for 24 hours, the content of chromatin fragments was elevated up to 11\% to 12\%. Similar to VSMC-E1A, the increment of chromatin cleavage triggered by serum-deprivation was completely blocked by ouabain.

Effects of Extracellular Na\(^+\) and K\(^+\)

The addition of ouabain to the control medium (medium 1) led to \(\approx\)13-fold elevation of intracellular Na\(^+\) concentration and to a 10-fold decrease of [K\(^+\)]\(\text{in}\), in VSMC-E1A (Table 1). As predicted, equimolar substitution of NaCl with KCl (medium 2) completely abolished the effect of ouabain on intracellular Na\(^+\) and K\(^+\) concentration. Neither apoptosis triggered by serum withdrawal nor RNA synthesis were affected by 6-hour equimolar substitution of NaCl with KCl (data not shown). Similar to the results reported in Figures 1 and 3, 6-hour incubation of VSMC-E1A in medium 1 with ouabain caused \(\approx\)10-fold decrease of chromatin fragmentation triggered by serum deprivation, augmented RNA synthesis by \(\approx\)50\%, and inhibited protein synthesis by 30\% (Figure 5a). We did not observe any effect of ouabain on chromatin cleavage and macromolecular synthesis in Na\(^+\)-depleted medium 2. The lack of antiapoptotic action of ouabain after substitution of NaCl with KCl was confirmed by phase-contrast microscopy (Figure 5b) and by analysis of caspase-3 activity (Table 2).

Role of MAP Kinase Signaling Cascade

An overwhelming number of stimuli triggering mRNA expression use signaling cascade, which is terminated by phosphorylation of 3 protein kinases from the MAPK family: extracellular signal-regulated protein kinases (ERK1/2 or p42/p44), JNK or stress-activated protein kinase (SAPK), and a mammalian homologue of Hog, p38 protein.\(^{21}\) Considering this, we explored the role of these kinases in the induction of RNA synthesis revealed in ouabain-treated VSMC. Figure 6 shows that neither 2-hour incubation of VSMC-E1A with ouabain in the presence of 10\% CS nor additional 6-hour incubation in the absence of growth factors affected the level of phosphorylation of ERK1/2, JNK, and p38. Quiescent VSMC treated with CS and anisomycin displayed drastic phosphorylation of ERK and JNK/p38, respectively, and were used as a positive control in this study.

In additional experiments, we studied the effect of inhibitors of ERK- and p38-directed MAP kinase cascade (PD98059 and SB 202190, respectively) on apoptosis. Table 3 shows that neither baseline apoptosis in serum-deprived VSMC-E1A nor its inhibition by ouabain was significantly modified by these compounds.

**Figure 5.** Effect of substitution of NaCl with KCl in incubation medium on chromatin cleavage, RNA, and protein synthesis in VSMC-E1A (a) and appearance of apoptotic cells (b). a, VSMC-E1A cells were incubated for 6 hours in medium 1 (NaCl 130 mmol/L; KCl 5 mmol/L) or medium 2 (NaCl 14 mmol/L; KCl 123 mmol/L) with or without 10\% CS and 1 mmol/L ouabain; content of chromatin fragments and RNA and protein synthesis were estimated as indicated in Methods section. To measure RNA and protein synthesis, cells were incubated in presence of 10\% CS only. Increment of chromatin fragments triggered by serum deprivation and level of RNA and protein synthesis in absence of ouabain were taken as 100\% and shown by broken line. For more details on composition of media 1 and 2, see Table 1. Data from experiments performed in quadruplicate are shown as mean\(\pm\)SE. **\(P<0.01\) and 0.0001 compared with control cells. b, Phase-contrast microscopy of VSMC-E1A after 6-hour incubation in serum-free medium 1 (A, B) or medium 2 (C, D) without (A, C) or with (B, D) 1 mmol/L ouabain. Apoptotic cells are shown by arrows.
TABLE 2. Effect of Extracellular Na\(^+\) and K\(^+\) on Caspase-3 Activity in Control and Ouabain-Treated VSMC-E1A

<table>
<thead>
<tr>
<th>Composition of Incubation</th>
<th>Caspase-3 Activity, nmol (mg prot(^{-1})·h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Cells</td>
</tr>
<tr>
<td></td>
<td>+10% CS</td>
</tr>
<tr>
<td>Medium 1, NaCl 130; KCl 5</td>
<td>0.78±0.12</td>
</tr>
<tr>
<td>Medium 2, NaCl 14; KCl 123</td>
<td>0.90±0.08</td>
</tr>
</tbody>
</table>

Cells were preincubated in the presence of 10% CS for 2 hours and then incubated for the next 5 hours in the presence or absence of ouabain and CS. For the composition of media 1 and 2, see Table 1. Data from experiments performed in quadruplicate are given as mean±SE.

*P<0.001 compared with control serum-deprived cells.

Discussion

Data obtained in the present study show that the recently discovered inhibition of apoptosis in VSMC by compounds that invert the intracellular [Na\(^+\)]/[K\(^+\)] ratio is abolished by RNA and protein synthesis inhibitors (Figure 2b). Moreover, prolonged incubation with ouabain sharply increased RNA synthesis (Figures 3 and 4a). Previously, it was shown that prolonged incubation with ouabain sharply increased RNA synthesis under long-term inhibition of the Na\(^+\),K\(^+\) pump, including that of the rat aorta. 32

In contrast to RNA synthesis, ouabain inhibited protein synthesis during the initial 4 to 6 hours, an effect that was not detectable at 24 hours (Figure 3b). This is in accordance with data obtained with other cell types studied so far. 24,33,34 With the use of cell-free systems, it was shown that equimolar substitution of K\(^+\) with Na\(^+\) only slightly affected RNA synthesis; in contrast, protein synthesis was completely blocked in K\(^+\)-free medium at a step of transfer of amino acid from aminoacyl tRNA to the polypeptide. 35 We speculate that rapid suppression of protein synthesis by Na\(^+\),K\(^+\)-pump inhibitors is mediated by the K\(^+\)-dependent step of the ribosomal protein synthesis machinery, whereas under sustained Na\(^+\),K\(^+\) pump inhibition, this inhibitory effect is compensated by increased synthesis of a few [Na\(^+\)]/[K\(^+\)]-sensitive mRNA species, which cannot be detected under estimation of total \([\text{H}]\)-leucine-labeled protein content.

In thymocytes and Jurkat cells, apoptosis can be partially inhibited by substitution of extracellular Na\(^+\) with K\(^+\). 9,36,37 That is probably caused by involvement of K\(^+\) loss in cell volume decrease and induction of apoptosis. 9,36,38 In contrast to immune system cells, 39 apoptosis in VSMC is insensitive

![Figure 6. Phosphorylation of MAP kinases (a, ERK; b, JNK; and c, p38) in VSMC-E1A treated with ouabain. VSMC-E1A were preincubated for 2 hours in presence of 10% CS without (1) or with 1 mmol/L ouabain (2), then for 6 hours in serum-free medium without (3) or with (4) ouabain. Quiescent VSMC treated for 10 minutes (a) or 30 minutes (b, c) with 5% CS (a) or 0.5 µg/mL anisomycin (b, c) were used as positive controls. 5, Control VSMC; 6, serum- or anisomycin-treated VSMC.](image)

<table>
<thead>
<tr>
<th>Additions</th>
<th>10% CS</th>
<th>CS-Free</th>
<th>10% CS</th>
<th>CS-Free</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None (control)</td>
<td>3.4±0.6</td>
<td>28.9±3.9</td>
<td>3.0±0.7</td>
<td>5.0±0.6*</td>
</tr>
<tr>
<td>2. PD98059</td>
<td>3.6±0.4</td>
<td>27.7±4.0</td>
<td>2.9±0.5</td>
<td>4.7±0.8*</td>
</tr>
<tr>
<td>3. SB 202190</td>
<td>4.1±0.9</td>
<td>24.0±3.3</td>
<td>2.9±0.8</td>
<td>5.6±1.0*</td>
</tr>
</tbody>
</table>

VSMC were preincubated for 2 hours in DMEM containing 10% CS without or with 50 µmol/L PD98059 or SB 202190 and 1 mmol/L ouabain. Medium then was aspirated and cells were incubated for an additional 6 hours in the same media with or without CS. Mean±SE from experiments performed in quadruplicate are given.

*P<0.001 compared with control (ouabain-untreated), serum-deprived cells.
to cell volume modulation,11,9 and in these cells ouabain affects apoptosis and macromolecular synthesis through inversion of the [Na⁺]/[K⁺] ratio rather than by dissipation of transmembrane gradients of these cations, alteration of membrane potential, or by side effects unrelated to monovalent ion transport. Indeed, suppression of apoptosis in serum-deprived VSMC was observed under inhibition of the Na⁺,K⁺-pump by digoxin and digitoxin (unpublished data) or by incubation of cells in K⁺-depleted medium.9 Equimolar substitution of Na⁺ with K⁺ in the incubation medium abolished the effect of ouabain on the [Na⁺]/[K⁺] ratio (Table 1), on apoptosis (Figure 5, a and b, and Table 2), and on RNA and protein synthesis (Figure 5a). To estimate the relative contribution of [Na⁺], versus [K⁺], in these phenomena, we substituted NaCl with choline chloride, assuming that under these conditions ouabain does not significantly affect [Na⁺], but causes loss of [K⁺]. Indeed, as seen in Table 1, the intracellular Na⁺ concentration in control VSMC-E1A incubated in medium 1 and in ouabain-treated cells incubated in medium 3 is approximately the same. However, substitution of NaCl with choline chloride eventually increased baseline [K⁺], and decreased the loss of K⁺ triggered by ouabain. Thus, another approach should be designed to evaluate the role of [Na⁺], and [K⁺], in the regulation of apoptosis and macromolecule synthesis.

In conclusion, the results obtained in the present study demonstrate that inhibition of apoptosis in VSMC by an inverted intracellular [Na⁺]/[K⁺] ratio is mediated by de novo synthesis of the inhibitor of apoptosis, which we call [Na⁺]/[K⁺]-regulated inhibitor(s) of apoptosis (NKRIA). These results also raise several questions. What is the origin of the sensor triggering the antiapoptotic signal under inversion of the [Na⁺]/[K⁺] ratio? Which mechanisms are involved in transduction of the signal and the induction of NKRIA expression? Does this transduction pathway use ERG? Which step upstream of caspase-3 is affected by NKRIA? Is an enhanced level of ouabain-like substances involved in ouabain-like substances in vascular smooth muscle cells? For evidence of distinct modes of cell death. Hypertension. 1999;33:906–913.


Sergei N. Orlov, Sebastien Taurin, Nathalie Thorin-Trescases, Nickolai O. Dulin, Johanne Tremblay and Pavel Hamet

Hypertension. 2000;35:1062-1068
doi: 10.1161/01.HYP.35.5.1062

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/35/5/1062

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/