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Signaling pathways involving the sodium pump stimulate NO production in endothelial cells

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Abstract

The cardiac steroid ouabain, a known inhibitor of the sodium pump (Na^+,K^+-ATPase), has been shown to release endothelin from endothelial cells when used at concentrations below those that inhibit the pump. The present study addresses the question of which signaling pathways are activated by ouabain in endothelial cells. Our findings indicate that ouabain, applied at low concentrations to human umbilical cord endothelial cells (HUAECs), induces a reaction cascade that leads to translocation of endothelial nitric oxide synthase (eNOS) and to activation of phosphatidylinositol 3-kinase (PI3K). These events are followed by phosphorylation of Akt (also known as protein kinase B, or PKB) and activation of eNOS by phosphorylation. This signaling pathway, which results in increased nitric oxide (NO) production in HUAECs, is inhibited by the PI3K-specific inhibitor LY294002. Activation of the reaction cascade is not due to endothelin-1 (ET-1) binding to the ET-1 receptor B (ET_B), since application of the ET_B-specific antagonist BQ-788 did not have any effect on Akt or eNOS phosphorylation. The results shown here indicate that ouabain binding to the sodium pump results in the activation of the proliferation and survival pathways involving PI3K, Akt activation, stimulation of eNOS, and production of NO in HUAECs. Together with results from previous publications, the current investigation implies that the sodium pump is involved in vascular tone regulation.
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Keywords: Na^+,K^+-ATPase; Ouabain; Signaling cascade; Akt; eNOs; PI3K

1. Introduction

The Na^+,K^+-ATPase has been known for almost 50 years as the enzyme that transports 3 sodium ions out of the cell and 2 potassium ions into the cell for each ATP hydrolyzed. This activity, however, which is specifically inhibited by cardiac glycosides like ouabain, does not seem to be the only property of the enzyme. Strong evidence has accumulated in recent years pointing towards a receptor–hormone interaction between the enzyme and its cardiac glycoside inhibitors when these are used at rather low concentrations [1,2]. These interactions result in the stimulation of various intracellular signaling cascades that include extracellular signal-regulated kinase (ERK) 1/2 activation [3–6], Ca^{2+} oscillations [6,7], translocation of nuclear factors to the nucleus [7], or stimulation of cell proliferation [6,8].

In a recent study, we demonstrated that ouabain, used at concentrations below those needed for global sodium pump inhibition, stimulates the release of endothelin-1 (ET-1) from human umbilical artery endothelial cells (HUAECs) [6]. Nevertheless, endothelial cells not only produce ET-1 but also the vasorelaxant nitric oxide (NO). Therefore, the focus of the work presented here was to investigate whether NO production by HUAECs might be stimulated by low concentrations of ouabain and, if so, to identify the signaling cascade involved in this stimulation.

2. Materials and methods

2.1. Isolation and culture of HUAECs

Human umbilical cords were collected within 2 h of birth and kept on ice in wash buffer [Hanks buffered salt solution (HBSS) containing 20 mm *N*-2-hydroxypiperazin-*N*′-2-ethanesulfonic acid (HEPES)] until ready for cell

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isolation. An artery was canulated, washed with wash buffer, filled with collagenase (CLS 2, Worthington, UK) in Pucks saline solution (Seromed, Berlin, Germany) containing 20 mM HEPES, and incubated at 37 °C for 20 min to detach the endothelium. The cells were washed out using 20–50 ml wash buffer containing 10% fetal calf serum (FCS) and centrifuged at 50×*g*, 4 °C for 10 min. The cell pellet was suspended in 10 ml endothelial cell growth medium (ECGM; Promocell, Heidelberg, Germany) and transferred to a gelatin-coated, 94 mm cell culture dish. After 4 h, the medium containing any nonadherent cells was aspirated and the medium was replaced. During culture at 37 °C under 5% CO_2, the medium was replaced every 48 h. After the first passage, ECGM was mixed with M199 (Gibco, Eggenstein, Germany) to give a 2:1 ratio, respectively, with an additional 2% FCS supplement (ECGM-2).

2.2. Preparation of cell lysates

HUAECs were plated at a density of 5×10^6 cells in culture dishes of 10 cm diameter and were grown to confluence as described above. The cells were then serum-starved (i.e. 0.5% serum) in a 2:1 M199:ECGM (basal medium) mixture (ECGM-SF) for 24 h before commencing the experiment.

Ouabain was added to the cells at various concentrations in ECGM-SF and the cells were further incubated at 37 °C for 30 min. The cells were then lysed using a commercially available cell lysis buffer (Cell Signaling Technology, Frankfurt, Germany) according to the protocol of the provider. The lysis buffer contained 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, μg/ml leupeptin. Immediately before use, 1 μM PMSF was added to the lysis buffer. All lysis steps were carried out on ice. After centrifugation of the lysates at 4 °C for 15 min at 13,000×*g*, the protein in the supernatant was determined using the bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL) by including the lysis buffer also in the standard. Aliquots were stored at −20 °C.

2.3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransfer of isolated proteins

Proteins of the cell lysates were separated by SDS-PAGE using slab gels of 10% acrylamide and 0.3% *N*,*N*′-methylene-bis-acrylamide [9]. A total of 10 μg of protein was run on each lane. Biotinylated and pre-stained molecular weight markers (Cell Signaling Technology, Frankfurt, Germany) were run in parallel. After the electrophoretic separation, proteins were electro-blotted onto nitrocellulose membranes (Schleicher and Shull, Dassel, Germany) at 10 V for 30 min.

2.4. Immunodetection of Akt

Phosphorylated Akt was detected in a Western blot using a polyclonal antibody raised in rabbit (Cell Signaling Technology) specifically recognizing the phosphorylated Ser473 of the enzyme. In all cases, the protocols of the providers were followed. Overall Akt protein was detected by a specific polyclonal immunoglobulin (Cell Signaling Technology).

After electrotransfer, incubation with either of the antibodies was carried out by following the protocol recommended by the provider. The visualization of the phosphorylated or nonphosphorylated Akt was performed by using the appropriate horseradish peroxidase-conjugated IgG (1:2000 in TBS-T containing 5% nonfat milk) provided by the enhanced chemiluminescence (ECL) kit (Amersham-Pharmacia). A horseradish peroxidase-conjugated anti-biotin IgG (Cell Signaling Technology) to detect the molecular weight marker was also included in the incubation medium at a dilution of 1:2000. After film exposure, bands were relatively quantified using a digital documentation system (Biostep, Jahnsdorf, Germany) and the Phoretix TotalLab gel image analysis software (Biostep).

2.5. Ouabain-induced translocation of endothelial nitric-oxide synthase (eNOS)

HUAECs were grown as described above and were serum-starved for 24 h before commencing the experiment. Ouabain (10 nM) was added to each dish and the incubation continued for various times. The first time point was taken at 1 min, since this is the time required for removing the medium and washing the cells. Afterwards cell lysates were prepared as described above. Proteins were separated by electrophoresis and subsequently blotted onto a nitrocellulose membrane as described above. Membranes were then incubated overnight at 4 °C in TBS-T/5% BSA. A polyclonal antibody against eNOS raised in rabbits (Cell Signaling Technology) was added at a 1:1500 dilution to the solution and incubation was continued for another 90 min. The immunoreactive bands were detected by the by ECL kit (Amersham-Pharmacia) as described above.

2.6. Immunodetection of phosphorylated eNOS

The conditions for SDS-PAGE and for electrotransfer of the proteins are the same as above. The primary polyclonal antibody (Cell Signaling Technology) raised in rabbits recognizes the phosphorylated Ser1177 of eNOS. The dilution used was 1:1000 in TBS-T containing 5% nonfat dry milk. All further incubation steps followed the protocol of the provider. Detection of the immunoreactive proteins was accomplished using the ECL kit (Amersham-Pharmacia). The abundance of phosphorylated eNOS was measured by the digital documentation system and the Phoretix TotalLab gel image analysis software mentioned above (Biostep).

2.7. Nitric oxide (NO) detection

HUAECs were grown as described above. After 24 h in serum-free medium (i.e. 0.5% FCS), cells were washed twice with phosphate-buffered saline (PBS). Thereafter, cells were incubated at 37 °C, 5% CO_2 for 60 min in 2 ml of PBS containing 100 μM L-arginine and 1 μM 4,5-diaminofluorescein diacetate (DAF-2 DA; Merck Biosciences, Schwalbach, Germany). The latter compound is taken up by the cell and becomes converted to DAF-2 by intracellular esterases. At physiological pH, DAF-2 is relatively nonfluorescent. In the presence of NO and O_2, however, DAF-2 is converted to the fluorescent DAF-2 triazole (DAF-2T), which displays a 180-fold increase in fluorescence quantum efficiency over DAF-2 [10,11]. Ouabain (10 nM) was then added and incubation was continued for a further 30 min. For the negative control, ouabain was omitted. In order to inhibit NO production, some samples contained besides 10 nM ouabain also 10 μM N^G-nitro-L-arginine methyl ester (L-NAME; Sigma, Dassel, Germany). The effect of L-NAME on basal NO production was also measured in the absence of ouabain.

After incubation, the supernatant was removed and cells were washed twice with phosphate buffered saline (PBS) and subsequently suspended in 2 ml of the commercially available lysis buffer under the conditions mentioned above. Following centrifugation at 13,000×*g* for 15 min at 4 °C, supernatants were collected for fluorescence spectroscopy. The pellets were used for the BCA protein assay (Pierce, Rockford, IL).

Fluorescence was measured in a Hitachi spectral fluorimeter at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The fluorescence obtained in the absence of ouabain was set to account for 100%.

2.8. Effect of an endothelin B (ETB) receptor antagonist and a phosphatidylinositol 3-kinase (PI3K) inhibitor on Akt and eNOS phosphorylation

In order to investigate the involvement of the ET_B receptor in the cascade leading to Akt and eNOS activation, HUAECs were used which had been grown and serum-starved for 24 h as described above. Either at the beginning of serum starvation or just for the last 2 h of this period, the highly specific ET_B antagonist BQ-788 (Alexis Biochemicals, Grünberg/Germany) was added to the medium at a concentration of 10 μM. Ouabain was added afterwards to a final concentration of 10 nM and incubation continued for an additional 30 min. Thereafter, cell lysates were collected as described above and were investigated for phospho-Akt and phospho-eNOS immunoreactivity.

To assess the potential role of PI3K in this pathway, the same experiment was performed with the exception that the PI3K inhibitor LY294002 (50 μM; Cell Signaling Technology) was used in place of BQ-788.

3. Results

3.1. Immunodetection of activated (phosphorylated) and total Akt

Akt (also called PKB) is a protein kinase that plays a crucial role in controlling the balance between cell survival and apoptosis [7,12]. After 30 min of incubation, ouabain at low concentrations ranging between 0.1 and 10 nM stimulated Akt phosphorylation as detected by the use of an antibody that specifically recognizes the phosphorylated Ser473 of the enzyme (Fig. 1A). The molecular weight of the detected phosphorylated protein is 60 kDa, which corresponds to the expected relative molecular mass of Akt. In the absence of ouabain, no phosphorylated Akt was detected. The difference in the amount of phosphorylated Akt detected was not due to variation in the total Akt protein (Fig. 1B). The experiment also shows that the presence of ouabain does not influence the total amount of Akt in the cells.

The time course of the Akt phosphorylation was examined by incubating HUAECs for various times with 10 nM ouabain. While the total Akt in the supernatant of the cell lysates was not influenced by ouabain (Fig. 2A), ouabain stimulated Akt phosphorylation in a time-dependent manner (Fig. 2B). In the experiment shown here, after 30 min the phosphorylation reached a maximum that was 3-fold higher than the phosphorylation level after 1 min. On the average, phosphorylation was more than 2-fold higher after 30 min (Fig. 2C). After another 30 min, the phosphorylation level was somewhat reduced, but still 2.5-fold higher than the phosphorylation at 1 min.

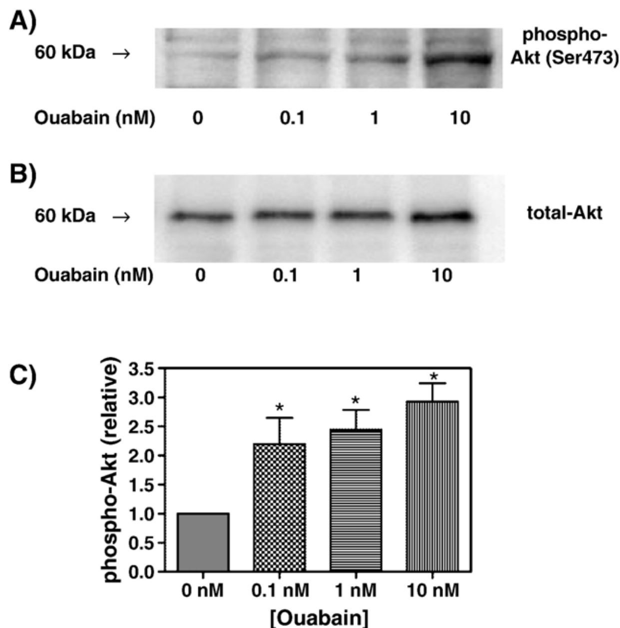

Fig. 1. Activation of Akt by ouabain. (A) Proteins from cell lysates from HUAECs were isolated after ouabain incubation for 30 min and blotted after SDS-PAGE onto nitrocellulose. The activated Akt was visualized by using a polyclonal antibody raised in rabbit against the phosphorylated Akt and, as a secondary antibody, a horseradish peroxide-conjugated anti-IgG of the ECL kit. (B) Visualization of total Akt. Blots shown are representative of results shown in several separate experiments. (C) Densitometric analysis of the bands shown in A and from 3 other experiments (n=4; ±SEM) with density in corresponding control bands (total Akt) set to 100. Data were analyzed by one-way ANOVA using the GraphPad-Prism software (GraphPad Software Inc, San Diego, CA) (*p<0.05 versus control).

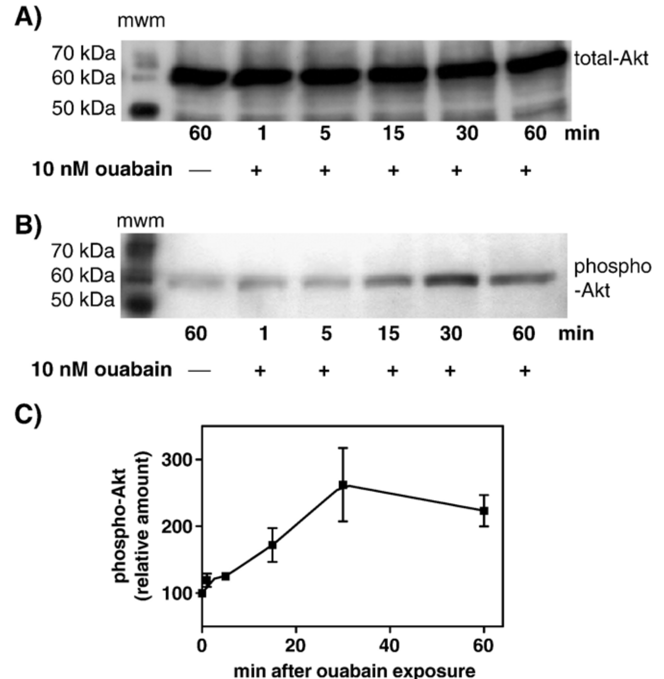

Fig. 2. Time course of Akt activation. (A) The abundance of total Akt in the cell lysates is not influenced by 10 nM ouabain. (B) Under the same conditions, ouabain stimulates Akt phosphorylation over a period of 60 min. Control proteins were isolated after 60 min from HUAECs that were not incubated with ouabain but otherwise treated identically. (C) Densitometric analysis of the bands shown in panel B and from 2 other experiments (n=3; ±SEM) with density in control band set to 100. (mwm=molecular weight markers).

3.2. Akt-catalyzed phosphorylation of eNOS

Endothelial NO synthase (eNOS) is directly regulated by Akt, which phosphorylates the enzyme at Ser1177 [13]. Since ouabain activates Akt by phosphorylation, it was important to determine whether ouabain treatment would also result in eNOS phosphorylation in the HUAECs.

Treatment of HUAECs for 30 min with various concentrations of ouabain resulted in eNOS phosphorylation as revealed by SDS-PAGE and Western blotting (Fig. 3). Since very little phosphorylated eNOS was found in the absence of ouabain, the stronger signals obtained at 0.1, 1, and 10 nM ouabain can be attributed to ouabain action (Fig. 3B). At 0.1 nM ouabain, activation was 118±8% above control and reached a maximum of 139±7% above control at 10 nM ouabain (n=3; ±SEM). The total amount of eNOS was the same in all samples (Fig. 3A).

3.3. Ouabain-induced eNOS translocation

eNOS is localized at the inner surface of the plasma membrane in an inactive, caveolin-associated form [14]. Increased intracellular [Ca^{2+}] causes Ca^{2+}/calmodulin to bind

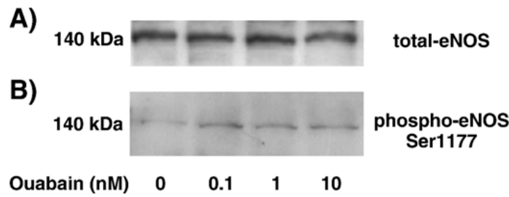

Fig. 3. Ouabain-induced stimulation of eNOS phosphorylation. A) Total eNOS. B) Preincubation of HUAECs for 30 min with various concentrations of ouabain stimulates eNOS, as determined using a rabbit antibody that recognizes the phosphorylated Ser1177 of eNOS. Under these conditions, no apparent differences were seen in phosphorylation levels obtained by ouabain at 0.1, 1, or 10 nM. All three concentrations, however, clearly stimulated eNOS phosphorylation above control levels (0 nM ouabain). Blots shown are representative of three experiments conducted similarly.

to eNOS, which in turn dissociates from caveolin-1 into intracellular compartments and initiates NO production [14,15]. Since ouabain has been shown to cause intracellular [Ca^{2+}] elevation in various cells including HUAECs [6,7,16], we were interested to investigate whether the cardiac glycoside would induce any changes in the content of eNOS protein in the cell lysates prepared from HUAECs. In contrast to the previous experiment shown in Fig. 3, where various concentrations of ouabain were used for the same time of 30 min, here we incubated the cells with 10 nM ouabain for 0, 1, 5, 15, 30 and 60 min. As determined by SDS-PAGE of proteins from cell lysates of HUAECs followed by Western blotting for eNOS, there was a clear ouabain-induced, time-dependent translocation of eNOS from the Triton-insoluble (caveolar) to a Triton-soluble fraction (Fig. 4A). Most of the protein translocated within the first 15 min (Fig. 4B).

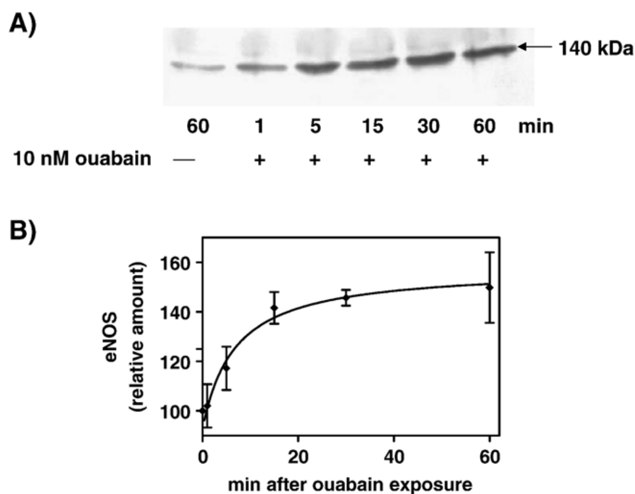

Fig. 4. Ouabain-induced translocation of eNOS. Membranes were probed with anti-eNOS antibodies as described in Materials and methods. (A) A total of 10 nM ouabain added to HUAECs caused the time-dependent translocation of eNOS. Control proteins were isolated after 60 min from HUAECs that had not been incubated with ouabain. (B) Densitometric analysis of the results in panel A and from 2 other experiments ($n=3$; ±SEM) with density set in control band to 100.

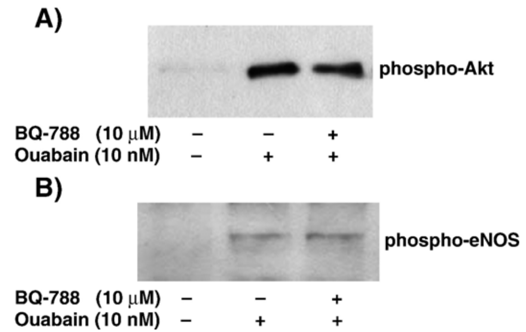

Fig. 5. Effect of the ET_B receptor antagonist BQ-788 on Akt and eNOS phosphorylation. (A) A total of 10 μM BQ-788 applied to HUAECs 2 h before the addition of 10 nM ouabain did not influence ouabain-induced Akt phosphorylation. A 24-h incubation with BQ-788 did not alter the result (not shown). (B) Similarly, ouabain-induced eNOS phosphorylation was not inhibited by BQ-788 under the same conditions. Blots shown are representative of two experiments conducted similarly.

3.4. Effect of the ET_B receptor antagonist BQ-788 and of the PI3K inhibitor LY294002 on Akt or eNOS phosphorylation

Binding of ET-1 to the ET_B receptor of the endothelial cells stimulates Akt and eNOS phosphorylation [17]. Since ouabain induces the exocytosis of ET-1 from HUAECs [6], we investigated a possible involvement of this receptor in the ouabain-induced stimulation of Akt and eNOS phosphorylation seen here by using the ET_B receptor-specific antagonist BQ-788. Neither Akt (Fig. 5A) nor eNOS phosphorylation (Fig. 5B) was inhibited by 10 μM BQ-788 [18], suggesting that the observed ouabain-induced activation of Akt and eNOS is not mediated through the ET_B receptor. In contrast to BQ-788, the PI3K inhibitor LY294002 at 50 μM [19] fully suppressed the ouabain-induced eNOS or Akt phosphorylation (Fig. 6), pointing to an important role of PI3K in the activation of these enzymes by ouabain.

3.5. Stimulation of NO production by ouabain

Endothelial cells regulate vascular tone by producing either the vasoconstrictive ET-1 or the vasodilatory NO [20]. Treatment of HUAECs with ouabain resulted in an increase in NO production (Fig. 7). Although ouabain-induced stimulation

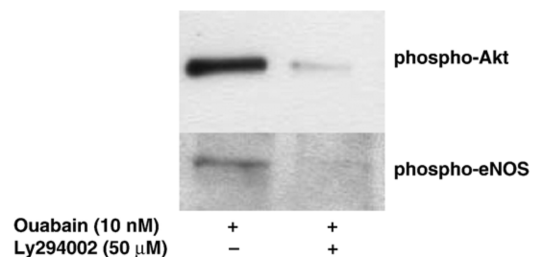

Fig. 6. Inhibition of ouabain-induced phospho-Akt and phospho-eNOS formation by the PI3K inhibitor LY294002. A total of 50 μM LY294002 was added to the HUAECs culture 2 h before the addition of 10 nM ouabain. This led to the inhibition of ouabain-induced phospho-Akt and phospho-eNOS formation. Blots shown are representative of two experiments conducted similarly.

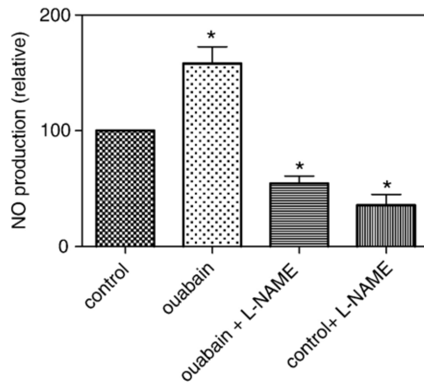

Fig. 7. Stimulation of NO production by ouabain. Ouabain stimulated NO formation by HUAECs as detected by the fluorogenic substrate DAF-2. For the control, ouabain was omitted. This value was taken to represent 100% NO formation. Ouabain-stimulated NO production was inhibited by 10 μM L-NAME to a level below the 100% value of the control ($n = 3$; ±SEM). Data were analyzed by one way ANOVA using the GraphPad-Prism software (*$p < 0.05$ versus control).

of NO production was clearly evident, NO production was also detected in unstimulated cells. The NO synthesis inhibitor L-NAME [21] at 10 μM inhibited the ouabain-stimulated NO production even below the level of that obtained in the absence of ouabain. Similarly, L-NAME reduced NO production in ouabain-untreated cells below the NO production of the control.

4. Discussion

Recent investigations demonstrate that the role of the mammalian sodium pump is not only associated with the ion pumping process that contributes to the maintenance of the membrane potential and sodium gradient; they clearly show the involvement of the enzyme in communicating its interactions with ouabain and other cardiac steroids into the cytosol and nucleus by activating a variety of tissue-specific cell signaling cascades [1,2].

In endothelial cells, at concentrations that do not cause a global inhibition of the sodium pump [6], ouabain was shown to induce Ca^{2+} oscillations and ET-1 release within minutes after application. Since ET-1-induced vasoconstriction is often counterbalanced by NO-induced vasodilatation [17,22], we investigated whether ouabain at low concentrations stimulates NO production in HUAECs. The results shown here support this hypothesis. The current investigation unveils that the reaction cascade induced by ouabain follows a known signal transduction pathway that includes activation of PI3K, Akt activation by phosphorylation at Ser473, eNOS phosphorylation at Ser 1177 and, finally, increased production of NO (Fig. 8). In a recent investigation it was shown that the bradykinin-stimulated increase of NO production in rat endothelial cells was augmented by ouabain [23]. It is likely that ouabain triggers the same reaction cascade in these cells as in the HUAECs, which then leads to elevated NO production.

Activation of eNOS is described in the literature to occur by different pathways: Akt-dependent phosphorylation and Ca^{2+}-induced translocation of the protein from the membrane-bound state to intracellular compartments [14,24,25]. In our experiments, the involvement of the Akt-dependent pathway was demonstrated by the abolition of eNOS phosphorylation by the use of the PI3K-specific inhibitor LY294002. Nevertheless, we also observed a strong, ouabain-induced translocation of eNOS from a Triton-insoluble (caveolar) to Triton-soluble fraction (Fig. 4). As shown earlier, this might be due to ouabain-induced Ca^{2+} elevation in HUAECs [6]. Nevertheless, while translocation of eNOS from the plasma membrane to intracellular compartments has been described to be associated with activation [24], in our experiments the level of phosphorylated eNOS does not correspond to the total amount of eNOS detected (Fig. 4). The detection of phosphorylated eNOS is rather difficult since the enzyme seems to be a target of very active phosphatases [26]. Thus, in most investigations phosphorylated eNOS is demonstrated shortly after applying a substance that stimulates enzyme phosphorylation. We assume that the detected enzyme is probably transiently phosphorylated and active before it is rapidly dephosphorylated by phosphatases.

How does ouabain and its interaction with the sodium pump lead to the PI3K activation, Akt and eNOS phosphorylation, and NO production? Since ouabain induces ET-1 release from HUAECs [6], one possibility would be via ET-1 binding to the ET_B receptor that is localized in the membrane of HUAECs [17]. Although this pathway might have a small contribution to the effects seen, it cannot play a major role in the ouabain-induced stimulation of NO production because the addition of the ET_B receptor-specific antagonist BQ-788 did not influence Akt or eNOS phosphorylation. Thus, there must be a different mechanism by which the cardiac steroid confers the information to the intracellular milieu via its binding to the sodium pump.

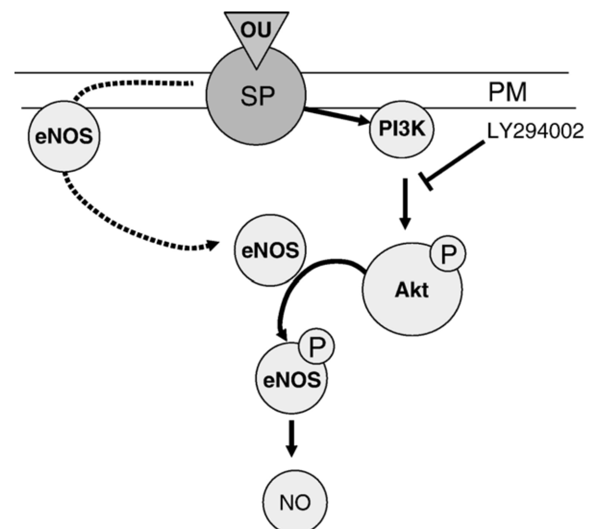

Fig. 8. A schematic summary of the ouabain effects observed in this investigation. Ouabain (OU) binding to the sodium pump (SP) leads to stimulation of phosphatidylinositol 3-kinase (PI3K), which activates Akt by phosphorylation. This enzyme then phosphorylates eNOS and stimulates the enzyme to produce NO. In nature, this reaction possibly counteracts the ouabain-induced ET-1 release observed in a previous investigation [6]. The mechanism by which ouabain stimulates eNOS translocation, possibly from caveolae, are not yet understood.

Most likely this mechanism involves a direct binding of PI3K to the sodium pump. PI3K has been previously shown to directly interact with the sodium pump by its binding to a proline-rich motif of the Na^+,K^+-ATPase α subunit [27].

At the current stage, our working model is that the binding of ouabain to the sodium pump confers a signal via PI3K that then turns on the proliferation and survival pathways involving Akt and stimulating eNOS and NO production in HUAECs. In a living organism, this reaction cascade might be a measure for counterbalancing the effects of endothelin-1, whose release from HUAECs was shown earlier to be stimulated by ouabain [6].

Acknowledgment

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