Influence of Aldosterone and Salt or Ouabain in A10 Rat Aorta Smooth Muscle Cells

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Key Words

Smooth muscle cell • Apoptosis • Fibronectin • Collagen III • Rat aorta • Vascular remodeling

Abstract

Background/Aims: It is currently under debate whether aldosterone is able to induce fibrosis or whether it acts only as a cofactor under pathological conditions, e.g. as an elevated salt (NaCl) load. **Methods:** We tested the interaction of 10 nM aldosterone, 15 mM NaCl and 1 μ M ouabain using rat aorta smooth muscle cells (A10) with respect to the following parameters: necrosis, apoptosis, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase activity, glutathione (GSH) content, collagen and fibronectin homeostasis and intracellular calcium distribution. **Results:** Necrosis rates were increased after 48 h of incubation with aldosterone, salt or ouabain and in the combination of aldosterone and salt or ouabain. Apoptosis rates were decreased. A reduced defense capacity against oxidative stress was mirrored in the decreased G6PD activity and GSH content. Collagen III or fibronectin synthesis rates were unchanged, but gelatinase activity was increased resulting in a decreased media collagen III and fibronectin content. Calcium stores were increased by aldosterone in combination

with ouabain. **Conclusion:** Aldosterone and salt per se can lead to cell injury that is aggravated in combination or with cardiotonic steroids. In cooperation with other vascular cells, this can generate a permissive milieu enabling aldosterone or salt to promote more extensive vascular injury.

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Introduction

Extracellular sodium content plays an important role in blood pressure regulation. Salt (NaCl) homeostasis is controlled by the mineralocorticoid aldosterone which thereby participates in long-term blood pressure regulation. Besides this well- and long-known action on volume and electrolyte homeostasis, aldosterone plays an increasingly recognized role for vessel structure and function with clinical relevance [1, 2]. The interaction between salt and aldosterone concerning vessel function or possible remodeling gains rising importance. Up to now, the interplay between salt and aldosterone on vessel function has been studied predominantly in whole animals [3–5]. Other studies suggest that aldosterone can generate an inflammatory milieu with the formation of reactive oxygen species (ROS) and matrix proteins [6, 7]. Vascular

function is based on different cell types (e.g. fibroblasts, endothelial and smooth muscle cells), but studies using whole animal models cannot distinguish between the individual contribution of these cell types to altered vascular function. There is a lack of studies investigating the effect of an increased salt load, aldosterone and ouabain (as a representative for cardiotonic steroids) on vascular smooth muscle cell (VSMC) function.

It is known that besides its role in blood pressure regulation, salt also seems to interact with the functionality of vascular endothelial cells [8, 9]. During the last years, it has become more and more evident that an increased salt load leads to the liberation of so-called cardiotonic steroids (CTS). Ouabain, a prominent member of CTS, plays an important role in sodium homeostasis and thereby is involved in blood pressure regulation [10]. The level of CTS is markedly increased in hypertensive patients and in congestive heart failure [11–13] as well as after 3 days of a high-salt diet in normal men [14]. Some studies also show an increased release of ouabain-like compounds from the brain of salt-sensitive rats after a high-salt diet [15]. Different CTS bind to different subunits of the Na^+ - K^+ -ATPase, thereby impeding its function. This can lead to an increase of intracellular sodium, followed by a reversed activity of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and elevated calcium concentrations in the cytosol or the endoplasmic reticulum [16, 17]. The resulting augmented intracellular calcium concentration leads to an enhanced contractility of VSMC, which in turn leads to an elevated vascular resistance followed by a rise in blood pressure. This scenario is also called the 'sodium lag' hypothesis [8].

Besides being affected by the mechanism involving CTS described above, the transport rate of the Na^+ - K^+ -ATPase is directly influenced by the extracellular sodium concentration. Elevated extracellular sodium concentrations lead to reduced export rates of sodium. It was also shown that even a minimal rise in extracellular sodium concentration leads to increased sodium transport rates via ENaC in endothelial cells, followed by an elevation of intracellular volume and decreased nitric oxide formation, which would result in an increased vascular tone [9].

In addition, it was demonstrated that the interaction between CTS and Na^+ - K^+ -ATPase influences intracellular signaling pathways associated with apoptosis, the generation of ROS (possibly leading to cell death) and nuclear gene activation via NF κ B and other transcription factors [8, 18, 19]. Linking CTS with aldosterone action, it has also been shown that both share common intracellular signaling pathways [20, 21].

An effective defense mechanism against oxidative stress is the maintenance of a sufficient amount of NADPH. For example, NADPH is needed to regenerate reduced glutathione (GSH) from its oxidized form. NADPH is amongst others supplied by two enzymes of the pentose-phosphate cycle, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGDH). In endothelial cells, a lack of G6PD activity leads to a decreased GSH content and G6PD overexpression leads to a higher resistance against oxidative stress and impaired vascular reactivity [22]. The overall activity of G6PD is the result of 3 events: synthesis rate, degradation rate and phosphorylation status. Phosphorylation by protein kinase A leads to a decrease of enzyme activity. Synthesis rate is enhanced by binding of the cAMP-response-element-binding protein, CREB, to the promoter region of G6PD [23]. Aldosterone can block the activation of CREB [24]. Furthermore, aldosterone has been suggested to increase the cAMP level [25, 26] so that it can possibly lead to a decrease of G6PD activity by two mechanisms: the inhibition of CREB and PKA-dependent phosphorylation.

Aldosterone can enhance fibrosis and the formation of ROS in rat heart, and also collagen metabolism in cultured rat cardiac and renal fibroblasts [27–31]. Marinobufagenin, another member of CTS, also interplays with collagen homeostasis in fibroblasts, leading to increased collagen production [32, 33]. In human aortic smooth muscle cells, 1 or 10 nM aldosterone administered for 24 h has no effect on collagen synthesis rates per se, whereas the effect of low concentrations of H_2O_2 was potentiated by aldosterone [19]. This leads to the assumption that aldosterone may not constitute a major threat to cells but that aldosterone in combination with an already existing threat, such as an increased formation of ROS, salt or CTS, leads to cellular alterations.

The proper function of vessels additionally depends in part on smooth muscle cell contractility which is influenced by the cytoplasmic calcium concentration and the sarcoplasmic calcium filling state. Besides its role in contraction behavior calcium also plays a role as signaling molecule in smooth muscle cells [34] and may thereby support cellular remodeling processes.

In this study, we investigated the effect of aldosterone on rat aorta smooth muscle cells in culture in the presence of salt or CTS (ouabain) with respect to their possible role in the remodeling events of vessels. Cell survival indicated by necrosis and apoptosis rates as well as the activities of the two NADPH-regenerating enzymes, G6PD and 6PDGH, were measured and compared with

the cellular GSH content. As remodeling is based on altered matrix formation, the homeostasis of the extracellular matrix proteins collagen III and fibronectin was also investigated. To elucidate a possible role of calcium as a signaling molecule in remodeling effects, the intracellular calcium distribution after incubation with aldosterone and/or ouabain was determined.

Methods

Cell culture was performed as described previously [35, 36]. We used A10 cells (ATCC, Rockville, Md., USA) cultured in serum-containing DMEM media (300 mosm/kg). Serum was removed 24 h prior to the experiments. Equal amounts of cells were cultivated in serum-free DMEM media enriched with the respective substances either on Petri dishes (Becton Dickinson GmbH, Heidelberg, Germany) or in 24-well plates. Cells were incubated in the presence of the various substances for 48 h if not stated otherwise. To exclude the possibility that a change in osmolality could be responsible for the observed effects of NaCl, we also incubated cells with 30 mM mannitol (= 330 mosm/kg, corresponding to the 15 mM NaCl added). This maneuver had negligible effects on the measured parameters.

Transfection of the cells was performed as previously described [35, 37] with Effectene reagent (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Western Blot Analysis

For detection of the mineralocorticoid receptor (MR), cells were lysed in ice-cold Triton X-100 lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA, 200 μ M sodium orthovanadate, 0.1 mM phenylmethylsulfonylfluoride, 1 μ g/ml leupeptin, 1 μ M pepstatin A, 40 mg/l bestatin, 2 mg/l aprotinin, 1% Triton X-100) at 4°C. Cell lysates were matched for protein content, separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were then blocked with TBS/Tween and 5% non-fat dry milk (137 mM NaCl, 20 mM TRIS, pH 7.6, 0.1% Tween-20). The bound primary antibody (1:100 diluted) was visualized using horseradish peroxidase-conjugated secondary IgG (1:2,000) and a detection system (Thermo Scientific, Rockford, Ill., USA).

For the determination of cellular (pro)collagen and fibronectin, cells were lysed in ice-cold RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 1% (w/w) Nonidet P-40, 0.1% SDS, 1% (w/v) Na-deoxycholate, 0.1% Triton X-100, protease inhibitor cocktail I from Calbiochem, 1 mM EDTA, 184 mg/l sodium orthovanadate) for 25 min at 4°C. Membranes were blotted with either a rabbit anti-collagen type III or fibronectin antibody (1:1,000, Biotrend, Cologne, Germany). The primary antibody was detected using horseradish peroxidase-conjugated secondary IgG (1:25,000) visualized by the Amersham ECL system.

Protein Determination

Protein content was determined using the bicinchoninic acid assay (Thermo Scientific) with bovine serum albumin as standard [38].

Determination of G6PD and 6PGDH Activities

Activities of G6PD and 6PGDH were determined according to Matsui et al. [39]. Cells were lysed with morpholinoethanesulfonic acid (MOPS)-Triton buffer (20 mM MOPS, 0.1% Triton X-100, pH 7.4) and supernatants were used. In one reaction batch both activities were determined and in another batch only the activity of 6PGDH was quantified. To measure the activities of both enzymes, an aliquot of cell lysate was incubated with 0.4 mM glucose-6-phosphate, 0.4 mM 6-phosphogluconate and 0.2 mM NADP⁺ in a total of 200 μ l buffer (50 mM TRIS, 1 mM MgCl₂, pH 8.1). The increase of NADPH-based absorbance was monitored using a multiwell-multilabel reader at a wavelength of 340 nm over 60 min. The slope represented the enzyme activity. Another aliquot of cell lysate was incubated solely in the presence of 0.4 mM 6-phosphogluconate and 0.2 mM NADP⁺ to determine the 6PGDH activity. The activity of G6PD was calculated by the difference of these two activities.

Determination of Lactate Dehydrogenase Release

Lactate dehydrogenase (LDH) activity in media and in cell lysates was measured using standard protocol [40] adapted to lower scale (200 μ l) in a multiwell-multilabel reader (Victor², Wallac, Turku, Finland).

Determination of Caspase-3 Activity

Caspase-3 activity was measured as described by Schwerdt et al. [41]. Cells were washed once with PBS buffer (4°C) and incubated with 100 μ l cell lysis buffer (10 mM TRIS, 100 mM NaCl, 1 mM EDTA, 0.01% Triton X-100, pH 7.5) for 10 min on ice, harvested and centrifuged at 16,000 g for 10 min at 4°C. Sixty microliters of the supernatant were incubated with 65 μ l reaction buffer [20 mM piperazine-1,4-bis(2-ethanesulfonic acid), 4 mM EDTA, 0.2% CHAPS, 10 mM DTT, pH 7.4] containing 42 μ M DEVD-AFC (Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin, end concentration) at 37°C, and the fluorescence of the cleaved product, 7-amino-4-trifluoromethylcoumarin (AFC), was measured after 60 min using a multiwell-multilabel counter (Tecan, Männedorf, Switzerland) at 400-nm excitation and 505-nm emission. Cleaved AFC was quantified by a calibration curve using known AFC concentrations.

Determination of GSH Content

GSH was measured according to Kamencic et al. [42]. Cells were lysed in MOPS-Triton buffer as described above and 100 μ l of cell lysate was incubated with 1 mM monochlorobimane and 0.1 U glutathione-S-transferase at 30°C. A fluorescence of 380-nm excitation and 470-nm emission wavelength was determined in the samples after 30 min of incubation in a multiwell-multilabel counter (Tecan). GSH content was determined by a GSH standard (0–125 μ M) incubated in parallel. As a control, cells were pretreated for 15 min with 2 mM diethyl maleate, which depletes GSH.

Determination of Extracellular Collagen and Fibronectin Abundance by ELISA

For all experiments leading to the detection of collagen or fibronectin, cells were also incubated in the presence of ascorbic acid and beta-aminopropionitrile as described previously [43]. Collagen III or fibronectin abundance was determined by ELISA [43]. We tested the cross-reactivity of the primary antibodies

Fig. 1. Western blot analysis of A10 cells showing the expression of the MR. **a** Protein (10.2 μ g) was administered. **b** GRE-SEAP activity after aldosterone administration. * Indicates significant difference from control (Vehicle).

(1:1,000) using the collagen standards and did not observe any significant cross-reactivity.

Determination of Gelatinase Activity

Activity of gelatinases in media was measured as described in Gekle et al. [19] using the ENZ®Gelatinase/Collagenase assay kit from Molecular Probes (Leiden, The Netherlands). The increase of fluorescence emitted by cleaved fluorescein-conjugated gelatin was measured at 480 nm excitation and 520 nm emission. Bacterial collagenase activity (Sigma) was used as positive control.

Reporter Gene Assays

Transactivation was assessed by the MercuryT Pathway Profiling (Reporter Gene Assay) Systems from Clontech Inc., using secretory alkaline phosphatase (SEAP) as a reporter, essentially as described earlier [44]. In brief, A10 cells were cotransfected with p glucocorticoid response element (pGRE)-SEAP and LacZ. SEAP activity in the media was determined with the AttoPhos® System from Promega (Mannheim, Germany) and normalized to a transfection control (β -galactosidase).

Determination of Cytosolic Ca^{2+}

Cytosolic free calcium was determined using the Ca^{2+} -sensitive dye fura-2 (Molecular Probes, Leiden, The Netherlands) according to Grynkiewicz et al. [45] with an inverted Axiovert 100 TV microscope ($\times 400$; oil immersion, Zeiss, Oberkochen, Germany) and an automatic filter change device (Hamamatsu, Herrsching, Germany). The fluorescence signal was monitored at 510 nm with the excitation wavelength alternating between 334 and 380 nm using a 100-W xenon lamp. The sampling rate was one ratio every 2 s. $[Ca^{2+}]_i$ was calculated using a K_D of 225 nM after subtraction of background fluorescence.

Materials

Protease inhibitors were obtained from Calbiochem (Bad Soden, Germany). Unless stated otherwise, all other materials were from Sigma, Munich, Germany. Control Ringer solution was composed of (mM): NaCl 130.0, KCl 5.4, $CaCl_2$ 1.0, $MgCl_2$ 1.0, NaH_2PO_4 1.0, HEPES 10 and glucose 5 (pH 7.4 at 37°C) and the respective vehicles (ethanol or DMSO $\leq 1\%$). An antibody against the MR was created by Gomez-Sanchez et al. [46]. Antibodies against collagen III or fibronectin were obtained from Rockland (Gilbertsville, Pa., USA). Horseradish-coupled second antibody was from Cell Signaling (Danvers, Mass., USA).

Statistics

The data are presented as mean values $\pm$ SEM. Significance of difference to respective controls was tested by paired or unpaired Student's t test or ANOVA as applicable. Differences were considered significant if $p < 0.05$. Cells from at least two different passages were used for each experimental series. The number of cells or tissue culture dishes investigated is represented by n.

Results

A10 Cells

To assure that the MR pathway used by aldosterone is functionally active in A10 cells, we first determined the presence of the MR. As shown in figure 1a, its presence is clearly visible. To further assure the functionality of aldosterone-induced signaling pathways, we next analyzed the promoter-activating capacity of aldosterone by measuring the activity of SEAP, the expression of which was under the control of a GRE-containing promoter transfected into A10 cells (fig. 1b). In cells transfected with GRE-SEAP, 1 nM of aldosterone led to a clearly increased reporter activity (1.92 ± 0.42 -fold, $n = 6$). This confirms that the MR pathway is functional in A10 cells.

Cell Survival

To analyze the possible cell-damaging effects of aldosterone in combination with salt or ouabain, we first studied apoptotic and necrotic cell death. As shown in figure 2a, apoptosis rates measured by caspase-3 activity were decreased in all conditions. The largest decrease was inducible in cells incubated with a combination of aldosterone and salt or ouabain. In contrast, necrosis rates were increased in all experimental setups after 48-hour exposure (fig. 2b). The combination of aldosterone and ouabain led to the highest increase of LDH release. This indicates that cells do not undergo apoptotic but necrotic cell death when exposed to aldosterone and/or salt or ouabain. This behavior is mirrored by reduced protein contents (fig. 2c).

Fig. 2. Caspase-3 activity (a), LDH release (b) and protein content (c) of A10 cells after 48-hour exposure to 10 nM aldosterone (aldo), 15 mM NaCl and/or 1 μ M ouabain; n = at least 12 [for ouabain and aldo/ouabain (a) and for aldo and aldo/NaCl (b) n = 4]. * Indicates significant difference from control.

GSH Content

The observed decreased cell survival was probably based on diminished protection against oxidative stress. An indicator of cellular defense capacity against oxidative stress is its content of reduced GSH, which we determined in the cells after their exposure to aldosterone and/or salt as well as to ouabain. As shown in figure 3, a slight reduction of GSH content was evident compared to the control (not significant) when aldosterone alone was administered. Salt or ouabain alone already led to a significant reduction of GSH content and the combination of aldosterone and either salt or ouabain also led to a distinctly diminished content. This shows that where the GSH content is concerned, salt, ouabain or the combination of one of these with aldosterone constitutes an elevated risk factor for these cells.

G6PD and 6PDGH Activities

The diminished GSH content may be the consequence of the reduced capacity of the cells to produce sufficient amounts of NADPH and to protect themselves against oxidative stress. Enzymes involved in the regeneration of NADPH are, among others, G6PD and 6PDGH. As demonstrated in figure 4, the exposure to 10 nM aldosterone already led to a slight decrease in G6PD activity but to almost no alteration in 6PDGH activity. The incubation with salt led to a comparable decrease in G6PD or 6PDGH activity. These activity-decreasing effects on G6PD were potentiated when both substances were added together.

Fig. 3. GSH content of A10 cells after 48-hour exposure to 10 nM aldosterone (aldo), 15 mM NaCl and/or 1 μ M ouabain; n = at least 8. * Indicates significant difference from control.

When administered alone, ouabain had no effect on both enzyme activities, but it could significantly enhance the effect of aldosterone. This indicates that the effect of aldosterone on G6PD activity can be aggravated when either salt is present or endogenous ouabain is liberated.

Fig. 4. Activities of G6PD (**a**) and 6PGDH (**b**) after 48-hour exposure of A10 cells to 10 nM aldosterone (aldo), 15 mM NaCl and/or 1 μ M ouabain; n = at least 14. * Indicates significant difference from control.

Fig. 5. Collagen III (**a**) and fibronectin (**b**) secretion of A10 cells after 48-hour exposure to 10 nM aldosterone (aldo), 15 mM NaCl and 1 μ M ouabain; n = at least 12. * Indicates significant difference from control.

Matrix Deposition

Altered cell matrix composition is assumed to be partly responsible for the effects of aldosterone and/or salt on blood vessels. Therefore, we determined the formation of collagen III and fibronectin in the presence of aldosterone, salt, ouabain or a combination of aldosterone and salt or ouabain. As shown in figure 5, the abundance of collagen III in media was clearly decreased when aldosterone, salt or ouabain was administered. The combination of these substances also led to a clearly decreased collagen III content in media. Similar to collagen III, fibronectin secretion was reduced, except when aldosterone alone was administered. These data suggest that VSMC per se are not responsible for enhanced vascular matrix deposition under these conditions.

Collagen III and Fibronectin Homeostasis

The reduced extracellular collagen III and fibronectin concentrations can be the result of reduced synthesis

or enhanced degradation rates due to an increased gelatinase activity. Ouabain alone or in combination with aldosterone showed the largest decrease of extracellular collagen III or fibronectin. Figure 6 shows that the intracellular content of collagen III or fibronectin is not altered after incubation with aldosterone, ouabain or the two combined, which indicates that reduced synthesis rates of collagen III and fibronectin are not responsible for the observed reduced extracellular concentrations.

Gelatinase Activity

To test whether the reduced extracellular amount of matrix proteins may be the result of elevated degradation, we determined the activity of gelatinase in the media. As demonstrated in figure 7, the activity of gelatinases was clearly elevated when cells were exposed to salt or ouabain or a combination of these with aldosterone. Exposure to aldosterone alone had no significant effect.

Fig. 6. Western blot showing intracellular collagen III (a) and fibronectin (b) of A10 cells after 24-hour exposure to 10 nM aldosterone, 10 nM ouabain or both; 37 µg of protein was applied on each lane.

Thus, the reduced extracellular concentrations of matrix proteins can be explained by an increased activity of degrading enzymes.

Cellular Ca^{2+} Homeostasis

Cytoplasmic calcium concentration is responsible for the contraction force of smooth muscle cells. Furthermore, calcium is a signaling molecule which can influence cell fate. To determine the effect of the endogenous cardiotonic steroid ouabain and aldosterone on cellular calcium homeostasis, we measured the cytosolic calcium concentration of A10 cells using the calcium-sensitive dye fura-2. The addition of aldosterone or ouabain had no significant effect on basal cytosolic calcium concentration (fig. 8a), and also in combination they had no evident effect. Although the cytosolic calcium level remained constant, the filling state of intracellular calcium stores measured by thapsigargin-induced raise of cytosolic calcium was increased when aldosterone and ouabain were added simultaneously (fig. 8b).

Discussion

The importance of aldosterone was originally considered to be the maintenance of water and electrolyte balance. But its role in vessel structure and function becomes more and more established; it can exert disadvantageous effects on VSMC and endothelial cells. However, in most

Fig. 7. Gelatinase activity in media of A10 cells collected after 24-hour exposure to 10 nM aldosterone (aldo), 15 mM NaCl and/or 1 µM ouabain; n = at least 7. * Indicates significant difference from control.

in vivo studies, mineralocorticoids were administered combined with salt loading and even partial nephrectomy, resulting in the hypothesis of a pathological, i.e. para-inflammatory, milieu as a prerequisite for the deleterious actions of mineralocorticoids [6, 7]. The proper function of vessels is the result of a composite of cell types including endothelial, smooth muscle and even blood cells [47].

Fig. 8. Intracellular calcium in A10 cells after exposure to 10 nM aldosterone (aldo), 1 μ M ouabain or both. **a** Basal cytosolic calcium concentration. **b** Change of cytosolic calcium after addition of thapsigargin. * Indicates significant difference from control.

However, studies in whole animals cannot distinguish the impact of each cell type. The principal contribution of VSMC to vessel remodeling (altered matrix composition, parainflammation and cell loss) has been shown in the context of arteriosclerosis. Yet, so far, the question whether VSMC also influence or mediate remodeling in response to aldosterone and salt challenge has not been addressed. The aim of our study was to test the hypothesis of a predisposing milieu for the pathophysiological effects of aldosterone, using only VSMC in culture and to unravel their potential contribution to vascular injury.

A prerequisite for the action of aldosterone in A10 cells is the presence of its receptor and its intracellular signaling pathway which can end in activation of the GRE. The presence of the MR is confirmed by the Western blot shown. Also, the aldosterone-induced MR transcriptional activity is functionally present as shown by the elevated GRE-SEAP activity.

Mineralocorticoid- or salt-induced vascular remodeling may be in part based on altered cell survival rates expressed as altered apoptosis [48] or necrosis rates. Aldosterone was shown to induce apoptosis in endothelial cells, rat neonatal cardiomyocytes or podocytes [49–51]. We observed cell loss (mirrored in protein loss) after exposure to aldosterone, salt or ouabain, which is due to increased necrosis and not to elevated apoptotic cell

death. Although apoptosis rates were reduced, the elevated necrosis rates outbalanced the decreased apoptosis rates. Aldosterone alone had no effect on apoptosis rates, which is in contrast to previous studies (see above). But these studies were not performed in a VSMC line, which could account for this discrepancy. Another study showed that aldosterone even has antiapoptotic effects in human VSMC involving MDM2 p53-binding protein [52].

The elevated necrosis rates presumably result from a decreased capacity of A10 cells to defend themselves against oxidative stress. This is mirrored by the decreased activities of two important NADPH-regenerating enzymes, G6PD and 6PGDH, and by the diminished GSH content. In A10 cells, already 10 nM of aldosterone led to a slight decrease in G6PD activity. Similarly, 15 mM salt exerted a slight activity-decreasing effect, whereas ouabain alone had no effect. The combination of aldosterone and salt or ouabain led to a further decrease in G6PD activity. Thus, aldosterone or salt led to impaired G6PD activity; in combination, i.e. as released during increased salt loading [53], they exerted an even stronger impairment. The diminished GSH content detected in the cells after exposure to aldosterone and salt or ouabain supports this hypothesis.

Necrosis as a consequence of exposure to aldosterone, salt and/or ouabain may lead to elevated inflammatory responses in the vascular wall with the risk of further loss of tissue function and fibrotic alterations. It was shown for the rat heart that the combination of aldosterone and salt load enhances NADPH oxidase activity in endothelial and inflammatory cells finally leading to a fibrogenic and proinflammatory phenotype [54]. In human aortic smooth muscle cells in primary culture, aldosterone alone had no effect on collagen secretion rates [19]. However, in the same study it was shown that aldosterone in combination with oxidative stress induced by H₂O₂ led to altered collagen homeostasis which was reversible by blockade of the MR, leading to the idea that aldosterone sensitizes the cells to oxidative stress-induced collagen secretion.

In contrast to these studies, in the A10 cells we used here, collagen III and fibronectin concentrations in media were not increased, but rather decreased when aldosterone, salt or ouabain alone or in combination were administered. These findings become explainable by the increased activity of gelatinases with concomitant unaltered collagen and fibronectin synthesis rates, so that the reduced extracellular collagen III and fibronectin content are based on elevated degradation rates. Although these extracellular matrix-reducing effects are in contrast to

other studies [55], the decrease of extracellular collagen or fibronectin secretion is clearly distinct so that augmented matrix deposition does not play an important role in aldosterone- or salt-induced cell disturbance in the A10 cells. However, it should be kept in mind that in the intact vessel, the situation may be different and an interaction between the different cell types leading to altered extracellular matrix homeostasis cannot be excluded.

Increased salt load leads to the liberation of cardiotonic steroids [56], like ouabain and others. Ouabain also participates in the interplay of increased salt load and hypertension [16]. In addition, at nanomolar concentrations, ouabain can induce signaling processes independently of an inhibition of $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity by a mechanism involving epidermal growth factor receptor [57, 58] which is also involved in the action of aldosterone [59], i.e. aldosterone and ouabain can use common signaling pathways. It is unclear to what extent aldosterone may play an additional role in ouabain-induced effects or vice versa and which influence their common presence may have on VSMC. In our experiments, the effect of ouabain when administered alone was not always consistent. For example, GSH content was reduced by ouabain, but G6PD activity was not altered, which suggests that there are additional mechanisms leading to this reduced GSH content. However, for all the parameters studied here, the effect of a combination of ouabain and aldosterone is clearly different from the control and sometimes even more distinct compared to the substances alone.

A possible link between the action of aldosterone and/or salt with CTS is calcium, because a blockade of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ will lead to an increase in cellular calcium content and contractility with influences on the blood pressure (sodium lag hypothesis [8]). Calcium entry via $\text{Na}^+/\text{Ca}^{2+}$ exchanger into arterial smooth muscle

cells was found to be responsible for salt-induced hypertension in mice [60]. Our results show that although the basal calcium level remained more or less constant, the filling state of intracellular calcium stores increases when aldosterone in combination with ouabain is administered. This provides an elevated reservoir of calcium which can participate in the generation of increased contraction with effects on vessel tone. So far, we have no indication whether the increased calcium level is involved in the observed cell damage or not. This remains to be elucidated.

In conclusion, we can show that the *in vivo* (i.e. in animals) observed adverse effects of high salt load, cardiotonic steroids and increased aldosterone levels can be at least partially reproduced in isolated VSMC, suggesting a direct action, most likely via decreased defense capacities against oxidative stress. This is similar to the behavior of vascular endothelial cells [9] and shows that besides possible systemic effects, there is a direct interaction at the level of VSMC. The underlying molecular mechanisms are not completely understood and have to be investigated in future studies. In addition, the question whether aldosterone aggravates the salt effect, or vice versa, has not been finally resolved. Our results do not allow us to distinguish to what extent the presence of aldosterone creates the unfavorable milieu or whether salt is responsible. Published results yield support for both hypotheses [7, 61, 62]. However, it is clear that the combination of aldosterone and salt or ouabain constitutes a risk factor for VSMC.

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