

Aldosterone Induces Oxidative Stress via NADPH Oxidase and Downregulates the Endothelial NO Synthase in Human Endothelial Cells

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Received July 16, 2009; Revised February 3, 2010; Accepted April 11, 2010

This paper is available online at <http://ijpt.iums.ac.ir>

ABSTRACT

Aldosterone (Aldo) is traditionally viewed as a hormone regulating electrolyte homeostasis and blood pressure. Recent studies suggest that Aldo can cause microvascular damage, oxidative stress and endothelial dysfunction. However, its exact cellular mechanism in vascular complications remains unclearly defined. This study was conducted to determine the mechanisms responsible for Aldo-induced oxidative stress and the possible involvement of Aldo in the expression regulation of the endothelial NO synthase (eNOS) in human umbilical artery endothelial cells (HUAECs). HUAECs were incubated for 24 h with Aldo (100 nmol/L) in the presence and absence of Spiro (1 μ mol/L). NADPH oxidase protein expression and its activity were estimated. Moreover, eNOS protein level and 3-nitrotyrosine (3-NT) contents were also determined. Results showed that stimulation of HUAECs with Aldo (100 nmol/L) resulted in a significant upregulation in NADPH oxidase subunits (Nox2, p47^{phox} and p22^{phox} protein levels), and these alterations were significantly abolished on pre-incubation with the MR antagonist, Spiro. However, Aldo did not exhibit any significant change in Nox4 protein level. Using MR-reactive antibodies, we investigated the MR protein expression in HUAECs in response to Aldo. Of note, MR protein expression is upregulated by Aldo and downregulated by Spiro. Functionally, these effects were reflected on increased oxidative stress markers as evident by increased NADPH oxidase activity and 3-NT. Moreover, Aldo significantly inhibited eNOS protein expression and pretreatment with Spiro restored eNOS to the normal level. Taken together, the present results demonstrate that Aldo stimulates NADPH oxidase-mediated oxidative stress thereby reducing eNOS expression and Nox2 appears to be the most relevant isoform in this setting. Moreover, the MR antagonist, Spiro efficiently inhibited these changes.

Keywords: Aldosterone, Oxidative stress, NADPH oxidase, Endothelium NO synthase

Aldosterone (Aldo) is a mineralocorticoid hormone that plays an important role in regulating electrolyte balance and blood pressure [1,2]. An increasing number of studies have suggested that Aldo contributes to endothelial dysfunction [3]. The endothelium, acting in an autocrine and paracrine manner, plays a central role in vascular function control by regulating, among other actions, vascular tone and inhibiting monocyte and platelet adhesion and maintaining fibrinolytic balance. This function is altered in the presence of different cardiovascular risk factors and this situation is known as endothelial dysfunction. It is mainly characterized by impaired endothelium-dependent relaxations [4].

Several mechanisms account for the deleterious effects of Aldo on endothelium with one important effect being a decreased NO availability. This decrease

can involve not only a reduction in NO production but also an increase in NO inactivation by reactive oxygen species [5,6]. Aldo can increase oxidative stress by both increasing reactive oxygen species (ROS) production and reducing ROS scavenging capacity of the cells. Aldo administration in uni-nephrectomized rats treated for 4 weeks with dietary 1% NaCl increased H₂O₂ production by monocytes and lymphocytes [7]. Similarly, Aldo administration increased vascular superoxide production in normal rats [8]. Aldo-induced ROS has been observed in different pathological situations since in hypertensive animals [9] as well as in two different models of atherosclerosis [10].

Several molecular sources of endothelial ROS formation have been suggested [11,12]. An increasing body of evidence supports NADPH oxidase complexes

as a major source of superoxide anions in endothelial cells [13-15]. The NADPH oxidase complexes contain different catalytic NADPH oxidase subunits [16]. Up to seven NADPH oxidase isoforms have been described in different cell types [17]. The classical NADPH [18] oxidase complex is composed of a membrane-bound flavocytochrome b_{558} consisting of gp91^{phox} (Nox2) and p22^{phox} as well as cytosolic subunits [13,14]. Besides the gp91^{phox}/Nox2-containing complex, Nox4 is the prominent Nox isoform in endothelial cell [14,19]. Nox2 is a subunit of NADPH oxidase that is principally located in the endothelium and adventitia and is important for activation of this enzyme in response to Ang II [14]. Although several *in vivo* studies have shown that Aldo induces oxidative stress in the vasculature, the pathophysiological relevance of NADPH oxidase or its specific isoform that might be predominantly involved as well as the consequences of these events in endothelial cells remain to be defined. In addition, the possible underlying mechanism involved in this setting, waiting for clarification. The present study was conducted to examine effects of Aldo on the NADPH oxidase-derived oxidative stress and its specific isoform that could be involved in endothelial cells. In addition, the current study investigates the underlying mechanism involved in this scenario.

MATERIALS AND METHODS

Cell Culture

All cell culture reagents and chemicals were purchased from Sigma Chemical Co., unless indicated otherwise. Primary cultures of HUAECs were isolated with collagenase IV and cultured in M199 medium (Life Technologies) supplemented with 20 % (v/v) calf serum, as described previously [19]. Confluent cell cultures were incubated endothelium medium (EM) with low serum 0.5 % (v/v) calf serum for 24 h and were subsequently treated with Aldo (100 nmol/L) and the MR antagonist, Spiro (1 μ mol/L).

Estimation of NADPH oxidase activity in HUAEC

NADPH oxidase activity was measured in cells by using lucigenin-derived chemiluminescence as described previously [20]. The formation of reactive oxygen species in response to Aldo (100 nmol/L) was analyzed in HUAEC. The endothelial cells were detached, and adjusted and then incubated in white 96-well (10^6 cells per well) using trypsin. Then, they were transferred into Krebs–Henseleit solution (10 mmol/L glucose, 0.02 mmol/L Ca-Tritriplex, 25 mmol/L NaHCO₃, 1.2 mmol/L KH₂PO₄, 120 mmol/L NaCl, 1.6 mmol/L CaCl₂·2H₂O, 1.2 mmol/L MgSO₄·7H₂O, and 5 mmol/L KCl, pH 7.4). Oxygen radical production was measured in the presence of 5 μ mol/L lucigenin, with or without NADPH (100 μ mol/L) for 20 min. The reaction was started by the addition of NADPH (100 μ mol/L), and the relative light units (RLU) of chemiluminescence were measured over a period of 30 min in a FLUOstar OPTIMA multi-well reader (BMG, Offenburg,

Germany). Results were given as measured by lucigenin-enhanced in percent of control. Results are expressed as counts per minute per 10^5 cells. Lucigenin-mediated chemiluminescence was measured in the presence of different enzyme inhibitors. For instance, apocynin (10 μ mol/L) as NADPH oxidase inhibitor, L-NAME (*N*^o-nitro-L-arginine methyl ester; 1 μ mol/L) as uncoupled eNOS inhibitor and allopurinol (10 μ mol/L) as xanthine oxidase inhibitor. On the other hand, we examined the scavenging effect of Spiro using xanthine/xanthine oxidase generating ROS system. We added 500 μ M of xanthine and 10 mU/ml of xanthine oxidase are added to Western lightening reagent and measure chemiluminescence in the presence and absence of a concentration-dependent (0.1,1,10 μ mol/L) of Spiro. Lucigenin-mediated chemiluminescence was measured over a period of 30 min in a FLUOstar OPTIMA multi-well reader (BMG, Offenburg, Germany). Further studies to characterize the source or type of reactive species were performed in the presence of specific inhibitor superoxide anion the membrane-permeable superoxide dismutase (SOD) mimetic.

Western blot analysis

Cells were lysed in ice-cold lysis buffer that contained the following: 20 mmol/L Tris-HCl, 140 mmol/L NaCl, 1 mmol/L EDTA, complete miniprotease inhibitor cocktail, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mmol/L NaF, and 1 mmol/L orthovanadate, pH 7.8. The protein concentration was determined with BCA protein assay reagent (Perbio Science, Bonn, Germany). Equal amounts of membrane protein (20 μ g/lane) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Roth, Karlsruhe, Germany). After incubation in blocking solution (4% nonfat milk, Sigma), membranes were incubated with 1: 1000 Nox4, p22^{phox}, p47^{phox} and rabbit polyclonal antibody against MR (1:300) (Santa Cruz), Nox2 (upstate Lab), eNOS and 3NT antibodies (Transduction Lab) for 2 hours at room temperature. Membranes were washed and then incubated with a 1:3000 dilution of second antibody (Amersham Life Science) for 1 h, and the membranes were detected with the enhanced chemiluminescence system (Amersham Life Science). To correct for differences in protein loading, the membranes were washed and re-probed with 1:1000 dilution monoclonal antibody to human β -actin (Abcam). Relative intensities of protein bands were analyzed by scanner (model Scanmaker 8700, Microtek Laboratory) [21].

Statistical analysis

Data were expressed as means \pm SEM and were analyzed using the unpaired Student's *t*-test for comparisons between two groups and one-way ANOVA followed by the Tukey test for multiple comparisons. A probability value $p < 0.05$ was considered statistically significant.

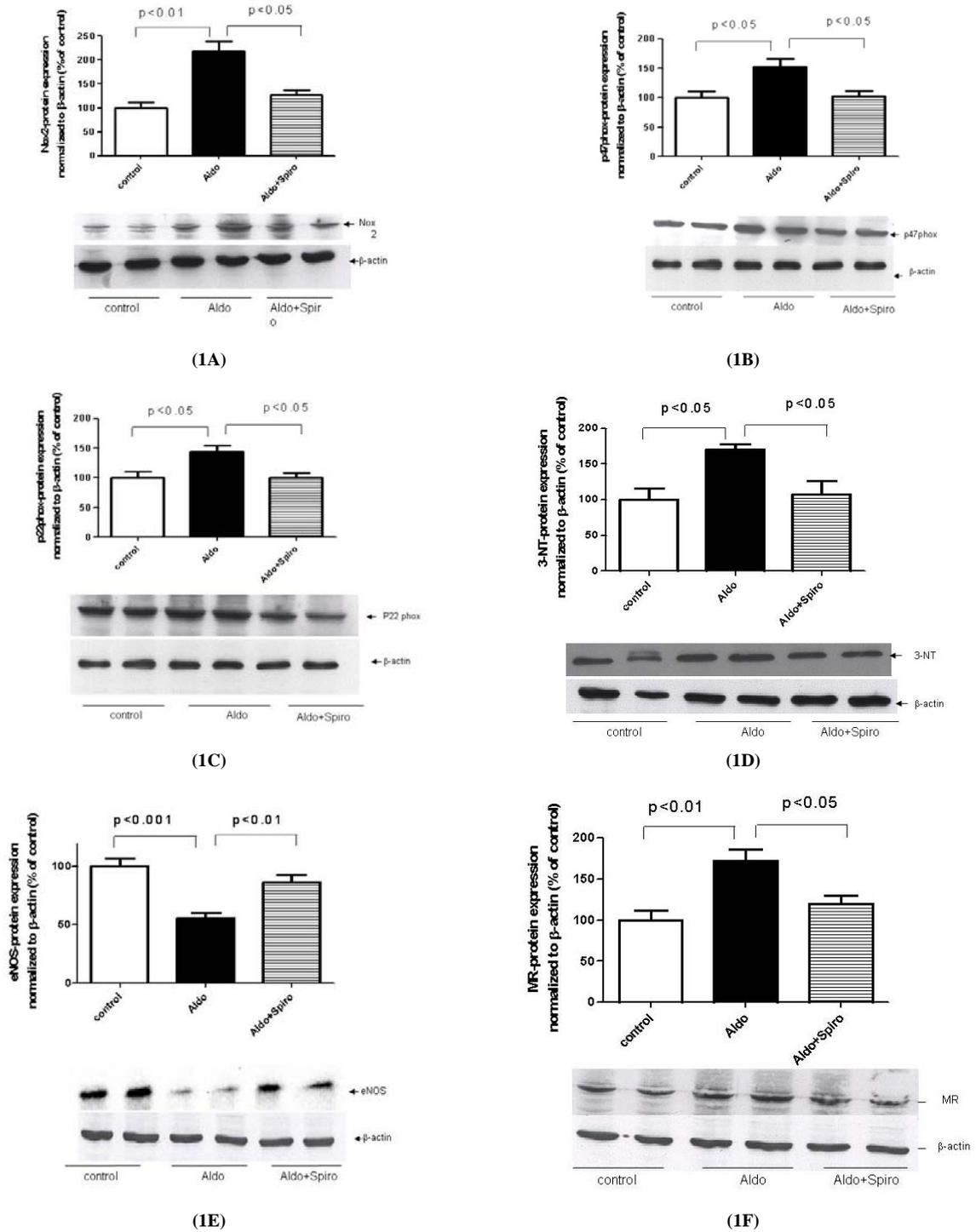


Fig 1. Effects of Aldo (100 nmol/L) on Nox2(1A), p47phox (1B), p22phox(1C), 3-NT (1D), eNOS (1E) and MR protein (1F) levels in HUAECs incubated for 24 h in the presence or absence of Spiro (1 μ mol/L). Quantitative analysis of proteins expression, normalized with β -actin, by scanning densitometry. Values for each bar are mean \pm SEM from 4 separate experiments and expressed as % of control; p values are expressed as indicated bars.

RESULTS

Effect of Aldo on NADPH Oxidase, 3-Nitrotyrosine 3-NT and eNOS expressions

To investigate the contributing role of NADPH oxidase isoforms in Aldo-mediated oxidative stress, expression of NADPH oxidase was determined by Western blotting analysis. HUAECs stimulated by Aldo

(100 nmol/L) for 24 h showed a significant ($p < 0.01$) upregulation in Nox2 protein level (Fig 1A). In addition, Aldo markedly up-regulated protein expression levels of the cytosolic p47^{phox} subunit ($p < 0.05$) (Fig 1B) as well as the membrane subunit p22^{phox} protein levels ($p < 0.05$) (Fig 1C) compared to the control. Pretreatment with Spiro (1 μ mol/L) significantly ($p < 0.05$) inhibited the upregulation in Nox2, p47^{phox} and p22^{phox} protein

levels—induced by Aldo. However, Aldo did not exhibit any significant change in Nox4 protein level. Moreover, stimulation of HUAECs with Aldo significantly ($p < 0.01$) increased 3-NT protein level as a marker of oxidative stress and this elevation was significantly ($p < 0.05$) inhibited on pretreatment these cells with Spiro (1 $\mu\text{mol/L}$) (Fig 1D). On the other hand, HUAECs treated with Aldo showed a marked ($p < 0.001$) down regulation in eNOS protein expression and Spiro significantly ($p < 0.01$) restored eNOS protein level to the normal (Fig 1E). To verify that all the previous effects of Aldo are related to MR, effects of Aldo and Spiro on MR expressional regulation were examined. Interestingly, Aldo (100 nmol/L, for 24 h) was found to up-regulate MR receptor in HUACEs and Spiro significantly inhibited these response induced by Ald (Fig 1F).

Effect of Aldo on superoxide production

Stimulation of HUAECs with Aldo (100 nmol/L) for 24 h resulted in a 2-fold increase in superoxide production as measured by lucigenin-derived chemiluminescence compared with control ($p < 0.001$). However, pretreatment with Spiro ($\mu\text{mol/L}$) significantly ($p < 0.01$) inhibited the Aldo-induced increase in superoxide production (Fig 2A). To distinguish and identify the source of superoxide generating enzymes, Aldo-mediated superoxide production was measured in the presence and absence of NADPH oxidase inhibitor, apocynin or xanthine oxidase inhibitor, allopurinol or uncoupled eNOS inhibitor, L-NAME. It is noteworthy that the increase in superoxide production measured by lucigenin-mediated chemiluminescence was significantly ($p < 0.01$) inhibited by apocynin rather than xanthine oxidase or uncoupled eNOS inhibitors. Neither allopurinol nor L-

NAME was able to inhibit Aldo-induced increased superoxide formation. SOD was able to completely inhibit the lucigenin-enhanced chemiluminescence, indicating that these chemiluminescences are superoxide anions-dependent. On the other hand, using xanthine and xanthine oxidase generating ROS system, we examined the scavenging or antioxidant properties of Spiro (Fig 2B).

DISCUSSION

The present study showed that stimulation of HUAECs with Aldo (100 nmol/L) resulted in a significant elevation of NADPH oxidase expression and activity. These changes were inhibited by the MR antagonist, Spiro. We used a 100 nmol/L dose of Aldo which is a close approximation to the *in vivo* situation, particularly under hyperaldosteronism conditions. An accumulating body of evidence indicates that ROS are implicated in many pathophysiological processes [22] including scavenging of endothelium-derived nitric oxide (NO), and prevention of its protective signaling functions [23,24]. Although ROS may derive from mitochondria, xanthine oxidase, cyclooxygenase, uncoupled NO synthase, heme oxygenases, or peroxidases, it has been frequently shown that NADPH oxidases are the primary producers of ROS in vascular tissues [25,26]. Previous studies have suggested that ROS produced by NADPH oxidase mediate many angiotensin II effects in the cardiovascular system [14,25]. Several reports support the potential role of Aldo in the regulation of NADPH oxidase. Recently, it has been reported that Aldo increases NADPH oxidase expression in the vasculature [27]. Systemic administration of Aldo increases oxidative stress in the heart, vasculature, and kidney increases macrophage

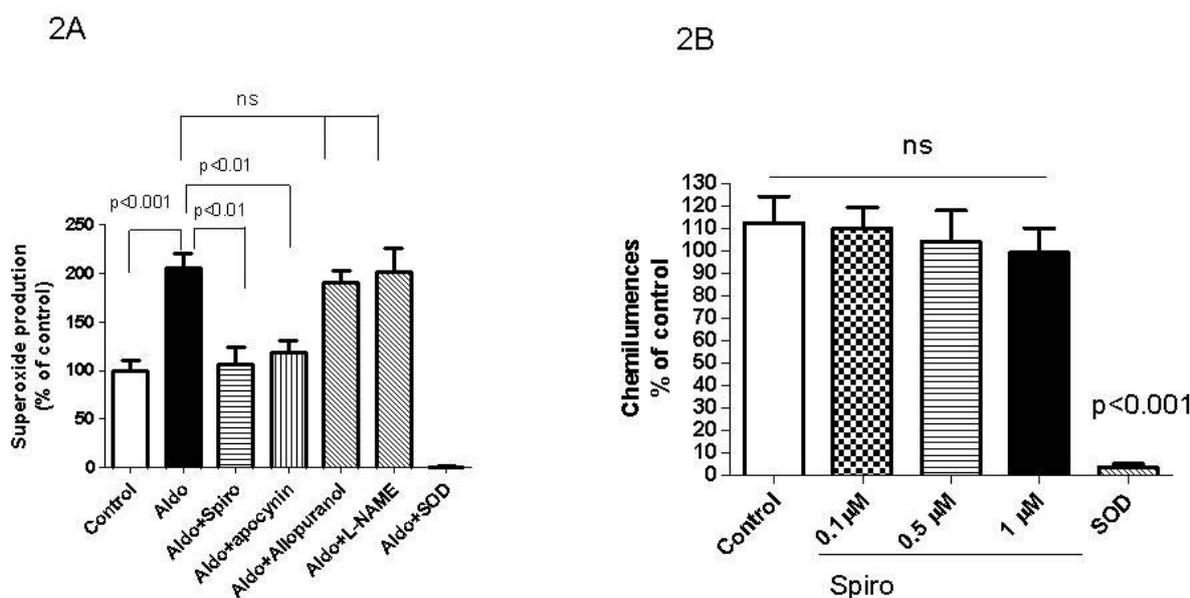


Fig 2. Effect of Aldo on superoxide production in HUAECs. **(2A)** HUAECs were pre-incubated for 24 h with vehicle or Aldo (100 nmol/L) in the presence and absence of the MR antagonist, Spiro (1 $\mu\text{mol/L}$). Some dishes were co-incubated with superoxide-generating enzyme inhibitors such as apocynin, allopurinol or L-NAME. **(2B)** Antioxidant properties of Spiro. Values for each bar are mean \pm SEM from 8 separate experiments and expressed as % of control; p values are expressed as indicated bars.

NADPH oxidase [28]. MR activation contributes to Ang II-mediated activation of NADPH oxidase in the heart and aorta [29]. In addition, exogenous Aldo stimulates aortic expression of p22^{phox} and Nox2 through an MR-dependent mechanism and of p47^{phox} mRNA through both AT₁ receptor and MR-dependent mechanisms [30]. In agreement with these *in vivo* findings, the present study demonstrates a marked up-regulation of Nox2 in HUAECs in contrast to Nox4 that did not exhibit any change. Accordingly, Nox2 might be considered as the relevant isoform involved in Aldo-NADPH oxidase pathway via MR activation.

On the other hand, the cytosolic component of p47^{phox} component was shown to have a pivotal role in the regulation of enzymatic activity. In this regard, Landmesser et al [31] reported that the hypertensive response and production of vascular superoxide was markedly blunted in p47^{phox} knockout mice. Nishiyama et al [32] have also shown that Aldo induced NADPH oxidase activation and membranous translocation of p47^{phox} in HUAECs. In consistent with this concept, the current study shows that Aldo increased p47^{phox} protein level. Thus, Aldo can increase ROS production in HUAECs by activating NADPH oxidase, mainly via p47^{phox} translocational regulation. Furthermore, NADPH oxidase catalyzes the one-electron reduction of molecular oxygen to superoxide anion which can react with nitric oxide to form short-lived peroxynitrite. Peroxynitrite forms stable 3-NT-conjugated protein moieties [23]. In this context, Aldo was found to increase in the 3NT content as a biochemical marker of oxidative stress and this was significantly inhibited on pretreatment with Spiro, supporting the potential role MR-mediated oxidative stress. It is worth-mentioning that the origin of superoxide anions is likely attributed to NADPH oxidase activation as evident by its inhibition by the NADPH oxidase inhibitor, apocynin whereas neither xanthine oxidase inhibitor, nor uncoupled eNOS inhibitor could inhibit the increased in superoxide formation. Similarly, Spiro was able to inhibit Aldo-mediated NADPH oxidase activation, suggesting MR might be considered as upstream of NADPH oxidase. To verify this hypothesis, the antioxidant properties of Spiro were examined. Of interest, Spiro could not inhibit the superoxide generated by xanthine and xanthine oxidase system. Based on these findings, the present study demonstrates that Spiro can antagonize Aldo-mediated superoxide production and this is attributed to its inhibition to NADPH oxidase enzyme. It has been reported that superoxide production reduces nitric oxide bioactivity while reducing the expression of nitric oxide synthase [33]. Evidence of endothelial dysfunction was seen in isolated renal artery segments and aortic rings from rats exposed to a model of excessive MR stimulation. In animals treated with Spiro, normal endothelial function was restored [8,34]. Similarly, in rabbits fed a proatherosclerotic diet, treatment with Spiro normalized superoxide formation and improved endothelial function [35]. In healthy male volunteers, Aldo has been shown to cause acute endothelial dysfunction [36].

Notably, the MR-antagonist, Spiro, significantly antagonized the inhibitory effect of Aldo on eNOS expression. Importantly, using MR-reactive antibodies and Western blot, we investigated the MR protein expression in HUAECs in response to Aldo. This suggests that MR was the main receptor mediating the pro-oxidative effect of Aldo in the current investigations. The present findings are in agreement with the previous *in vivo* study demonstrating that eplerenone administration to hypercholesterolemic rabbits normalized superoxide generation, decreased NADPH oxidase activity to basal levels, and nearly normalized endothelium-dependent vasorelaxation.[10] A study by Keidar et al [37] showed similar inhibition of atherosclerosis when eplerenone reduced markers of oxidative stress, including the ability of macrophages to oxidize LDL, macrophage superoxide anion release, and the susceptibility of LDL to oxidation.

Regarding the mechanism of action Aldo, it has been reported that Aldo binds intracellular MR and translocates it to the nucleus, where it binds to its ligand and interacts with the regulatory region of target gene promoters [38]. By contrast, Aldo might have a non-genomic effect within minutes [39]. Of Note, we could not detect ROS production within 2 hours of stimulation by Aldo (data not shown); it took longer time in agreement with previous reports [27,34]. However, some reports showed that Aldo induces ROS production through activation of NADPH oxidase within 30 minutes [40,41]. Such a discrepancy might be attributed to using different cell types or different experimental conditions.

In conclusion, the present results demonstrate that Aldo stimulates NADPH oxidase-mediated oxidative stress thereby reducing eNOS expression and Nox2 appears to be predominately involved in this scenario. In addition, the MR, Spiro effectively inhibited these consequences. Nevertheless, the current study suggests that NADPH oxidase might act as key regulator in Aldo-mediated oxidative stress in HUAECs, thereby contributing to the development of Aldo-induced vascular injury. This study adds a new dimension to the understanding of the role of Aldo in activation of NADPH oxidase in endothelial cells and supports the notion that Aldo can induce the dysregulation of endothelial cells and that might be responsible for atherosclerosis. Further studies will be necessary to investigate the molecular mechanism underlying Aldo-induced dysregulation of the endothelial, because this knowledge may lead to novel strategies for the prevention of oxidative stress and improvement the endothelial function via blockade of MR.

ACKNOWLEDGMENT

I am grateful to Prof. H. Morawietz, Faculty of Medicine, Dresden, Germany, for his kind help to achieve this work. This study was supported by a grant from DAAD (German Academic for Scientific Exchange) and Ministry of Higher Education of Egypt.

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