Epidermal Growth Factor Receptor Mediates the Vascular Dysfunction But Not the Remodeling Induced by Aldosterone/Salt

Violaïne Griot-Charhbili, Céline Fassot, Smail Messaoudi, Claudine Perret, Vincent Agrapart, Frederic Jaisser

Abstract—Pathophysiological aldosterone (aldo)/mineralocorticoid receptor signaling has a major impact on the cardiovascular system, resulting in hypertension and vascular remodeling. Mineralocorticoids induce endothelial dysfunction, decreasing vasorelaxation in response to acetylcholine and increasing the response to vasoconstrictors. Activation of the epidermal growth factor receptor (EGFR) is thought to mediate the vascular effects of aldo, but this has yet to be demonstrated in vivo. In this study, we analyzed the molecular and functional vascular consequences of aldol-salt challenge in the waved 2 mouse, a genetic model with a partial loss of EGFR tyrosine kinase activity. Deficient EGFR activity is associated with global oxidative stress and endothelial dysfunction. A decrease in EGFR activity did not affect the arterial wall remodeling process induced by aldo-salt. By contrast, normal EGFR activity was required for the aldo-induced enhancement of phenylephrine- and angiotensin II–mediated vasoconstriction. In conclusion, this in vivo study demonstrates that EGFR plays a key role in aldosterone-mediated vascular reactivity. (Hypertension. 2011;57:238-244.) ● Online Data Supplement

Key Words: mineralocorticoid ■ cross-talk ■ in vivo ■ EGFR deficiency ■ vascular

Cardiovascular diseases are a major cause of death. Clinical trials (Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study and Randomized Aldactone Evaluation Study) have demonstrated the beneficial effects of administering low doses of pharmacological antagonists of the mineralocorticoid receptor (MR; the receptor of the mineralocorticoid hormone aldosterone [aldo]) to patients with heart failure, with improvements in survival rate and morbidity.1,2 These clinical benefits may partly result from improved vascular function.3 The MR is expressed in both endothelium and smooth muscle,4 and the vessels are now considered to be direct targets of aldo.5 In the mouse, the 11β-hydroxysteroid dehydrogenase type II enzyme is expressed in the endothelium but not in the smooth muscle,4,6 at variance with humans.7 Therefore, in the mouse, aldo is the specific ligand of the MR in the endothelium but not in the smooth muscle, where glucocorticoids could also activate MR. MR activation affects endothelial function and vascular tone: aldo infusion induces endothelial dysfunction, decreasing vasorelaxation in response to acetylcholine (Ach), and increasing the response to various vasoconstrictors.5,8,9

We showed recently that an increase in MR signaling in the endothelium is associated with an increase in blood pressure and altered vascular tone.4 Moreover, patients with primary aldosteronism have a higher aortic wall thickness than those with primary hypertension.10 Aldo is, thus, recognized as a cardiovascular risk factor that exacerbates vascular injury,3 although the underlying mechanisms remain unclear.

It has been suggested that the epidermal growth factor receptor (EGFR) could play a key role in the cardiovascular effects of aldo.11 In cultured cells, downstream EGFR signaling cascades are induced by aldo or prevented by MR blockade on aldo stimulation.12 Increases in EGFR expression and/or activation have been demonstrated after aldo stimulation, ex vivo after the incubation of rat aorta and renal, endothelial, and vascular smooth muscle cultured cells with aldo and in vivo in the aorta of adrenalectomized rats treated with aldo.13–16 However, the consequences of EGFR activation to the vascular effects of aldo have not yet been demonstrated in vivo.

We hypothesized that EGFR activation might contribute to the damaging effects of aldo in the vasculature in vivo. We analyzed the molecular and functional vascular consequences of
an aldosterone challenge in the wave 2 mouse (wa-2); this genetic model is characterized by a mutation in the Egfr gene (V743G), altering the receptor kinase domain and reducing the functional activity of EGFR to <10% of wild-type levels. We show that EGFR activity is dispensable for remodeling but required for the functional vascular effects of aldosterone.

Materials and Methods

Animals

wa-2 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Male wa-2 mice and age-matched wild-type littermate mice (used as controls [CTs]) were used for all of the experiments, which were conducted in accordance with the institutional guidelines and recommendations for the care and use of laboratory animals of the French Ministry of Agriculture and with the approval of our institutional animal care and use committee. All of the animals were housed at the Centre d’Exploration Fonctionnelle, Institut National de la Santé et de la Recherche Médicale U872.

Nephrectomy-Aldo-Salt Treatment

Mice underwent unilateral nephrectomy and implantation of osmotic minipumps (model 2006, ALZET, Charles River Laboratories) filled with aldosterone (60 µg/kg per day) or vehicle (10% ethanol). Aldo-treated mice were supplied the following day with drinking water containing 1% NaCl for 4 weeks. Mice undergoing unilateral nephrectomy/aldosterone salt treatment are referred to as nephrectomy-aldosterone-salt treatment (NAS) mice, and vehicle-infused mice are referred to as sham mice.

Organ Weight and Vascular Histomorphometry

After euthanization, the kidney and the heart were weighed and tibia length measured. The thoracic aorta was frozen in liquid nitrogen and stored at −80°C. Morphological studies were performed on carotid artery fixed in 10% buffered formalin at the mean arterial pressure of 80 mmHg. The adventitial structure in CT, as well as in wa-2 mice, was visualized by immunostaining with the antibody against EIIIA-Fn (clon KRO), an endothelial cell marker (Abcam). The sections were stained with DAPI to visualize cell nuclei. The adventitia was defined as the area between the internal elastic lamina and the internal surface of the media. The area of the adventitia was measured by computer-directed color analysis (NIH-Elements AR2.3, Nikon).

Immunohistochemistry

Immunohistochemistry was performed using an antibody directed against collagen type I (Col1a1, Col1a2) and EIIIA-Fn (clone KRO, Santa Cruz Biotechnology) or against 4-hydroxy-2-enal (Alpha Diagnostic International), a marker of lipid peroxidation. Antigen immunolabeling was revealed by the indirect immunoperoxidase method.

Gene Expression Analysis

Real-time quantitative PCR was performed on total RNA extracted from thoracic aortas, as described previously. Details are given as Supplementary Methods in the online Data Supplement (please see http://hyper.ahajournals.org). The primers used are listed in Table S1 (also available in the online Data Supplement).

Western Blot

Western blot analysis was performed on protein extracted from aortas as described previously. Details are given as Supplementary Methods. Endothelial NO synthase (eNOS) antibody (Abcam) was used for immunoblotting. Actin was used as a protein-loading control and was detected with an antibody directed against total actin (Santa Cruz Biotechnology).

Ex Vivo Vascular Reactivity of Isolated Arteries

Vascular contractility and dilation were assessed in infraaortic aortic segments, as described previously. Abdominal aortas, mounted in a Myo Technology (Danish Myo Technology) recording system, were first allowed to stabilize for 20-40 minutes and were then stimulated with 60 mM/L of KCl. Endothelial integrity was assessed by evaluating the vasodilator effect of 10⁻⁶ mol/L of Ach (Sigma-Aldrich), after preconstriction treatment with 10⁻⁶ mol/L of phenylephrine (Phe; Sigma-Aldrich). Cumulative dose-response curves for Ach, sodium nitroprusside, Phe, and angiotensin II (Ang II; Sigma-Aldrich) were then plotted.

Carbonyl Groups Detection

To detect carbonyl groups on aortic proteins extracts, we used the OxyELISA Oxidized Protein Quantitation kit as described by the manufacturer (Millipore).

Statistics

The results are presented as mean±SEM. Differences between groups were assessed by 2-way ANOVA. Dose-response curves were analyzed by repeated-measures ANOVA (Statview software). Values of P<0.05 were considered significant.

Results

NAS Treatment Has Similar Consequences on Hemodynamic Parameters in CT and wa-2 Mice

Body weight was similar in CT and wa-2 mice, but wa-2 mice displayed mild cardiac hypertrophy, as shown by comparison with CT mice (Table S2). Systolic blood pressure and heart rate were also similar in mice of the 2 genotypes (Table S2). The NAS treatment was used to evaluate the response to chronic aldosterone challenge of mice with defective EGFR signaling. Fluid intake was checked throughout the protocol, and no difference was observed between CT and wa-2 mice on the NAS treatment (data not shown), confirming that all of the animals ingested similar amounts of salt. NAS treatment affected the systolic blood pressure of CT and wa-2 mice similarly, with increases of equivalent magnitude observed in both genotypes (CT-NAS: +15% versus CT-Sham; wa-2-NAS: +16% versus wa-2-Sham; n=6 mice per group), with no change in heart rate (Table S2). NAS induced similar degrees of cardiac and renal hypertrophy in CT and wa-2 mice, as estimated by calculating the ratio of cardiac or remaining kidney weight to tibial length (Table S2).

EGFR Functional Deficiency Does Not Affect the Vascular Remodeling Induced by NAS

Aortic EGFR expression level was not affected by NAS (Figure S1). In basal conditions, CT-sham and wa-2-sham mice had similar arterial wall structures (Figure 1B) and morphological parameters (Figure 1A and Table). CT-NAS and wa-2-NAS mice displayed vascular remodeling, characterized by an increase in intima-media thickness (Figure 1A and 1B) and medial cross-sectional area, but with no differences in elastin and collagen densities between CT and wa-2 mice (Table).

Genes encoding structural proteins (Col1a1, Col3a1, Col4a1, Fn, fibrillin 1, fibrillin 1, and fibrillin 5) were expressed at similar levels in CT-sham and wa-2-sham mice (Figure 1C). The NAS treatment doubled Col4a1, fibrillin 5, fibrillin 1, and Fn mRNA levels in both CT and wa-2 mice (Figure 1C). Accordingly, immunohistochemistry of cellular Fn revealed that NAS treatment increased EIIIA-Fn staining, mainly in the adventitial structure in CT, as well as in wa-2 mice whereas EIIIA-Fn is not detectable in the aorta of sham mice (Figure 1D). NAS treatment enhanced Col1a1 and Col3a1 mRNAs in wa-2 mice, not in CT mice; fibrillin 1 transcripts were not modified by NAS in CT or wa-2 mice (Figure 1C).
Defective EGFR Activity Induces Endothelial Dysfunction But Does Not Alter Agonist-Induced Vasoconstriction

The maximum vascular contraction (expressed as force, in milliNewtons [mN]) in response to 60 mmol/L of KCl was similar in CT and wa-2 mice, with or without NAS treatment (CT-sham: 9.44±0.42 mN; CT-NAS: 8.52±0.46 mN; wa-2-sham: 9.71±0.69 mN; wa-2-NAS: 10.9±1.11 mN; *P value not significant [NS]). Ph-e-mediated vasoconstriction was similar in CT-sham and wa-2-sham mice (Figure 2A), indicating that EGFR activity is not involved in Phe signal transduction in basal conditions (EC50: CT-sham: 3.06±0.07 μmol/L; wa-2-sham: 0.47±0.10 μmol/L; n=5 per group; *P value NS). Interestingly, aorta relaxation in response to Ach was weaker in wa-2 mice than in CT mice (Figure 2B) with a decrease in Ach sensitivity (EC50: CT-sham: 0.04±0.01 μmol/L; wa-2-sham: 0.07±0.01 μmol/L; n=5 per group; *P<0.05). The response to sodium nitroprusside (an NO donor) was similar in CT-sham and wa-2-sham mice (Figure 2C), indicating that vasodilation in response to exogenous NO is not altered (EC50: CT-sham: 0.04±0.01 μmol/L; wa-2-sham: 0.04±0.02 μmol/L; n=5 per group; *P value NS).

Defective EGFR activity increases oxidative stress and reduces eNOS expression in the aorta. The expression of the eNOS is strongly decreased in the aorta of wa-2-sham mice as compared with CT-sham (Figure 3A), suggesting a decrease in NO bioavailability. These results are associated with an increase in protein carbonylation (Figure 3B) and lipid peroxidation (Figure 3C) indicating oxidative stress in wa-2 mice in the basal state. Plasma 8-isoprostane level was moderately increased in wa-2 mice as compared with CT (Figure S2). The contribution of the NADPH oxidases was difficult to access because of the lack of coordination between mRNA and protein expression levels of the NOX2 and

### Table. Histomorphometric Parameters of the Carotid Artery in CT and wa-2 Mice With NAS or Without NAS (Sham)

<table>
<thead>
<tr>
<th>Histomorphometric Parameters</th>
<th>Control</th>
<th>NAS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CT-Sham (n=6)</td>
<td>wa-2-Sham (n=4)</td>
</tr>
<tr>
<td>MCSA, mm²</td>
<td>0.0276±0.0050</td>
<td>0.0237±0.0020</td>
</tr>
<tr>
<td>Elastin density</td>
<td>29.7±4.1</td>
<td>24.9±3.8</td>
</tr>
<tr>
<td>Collagen density, %</td>
<td>23.3±4.6</td>
<td>20.7±4.1</td>
</tr>
</tbody>
</table>

MCSA indicates medial cross-sectional area. Values are mean±SEM. *P<0.05 NAS vs sham.
NOX4 subunits and their regulatory subunits (p22phox and p47phox; Figures S3 and S4). Taken together, these results indicated that, in basal condition, defective EGFR activity affects vasorelaxation in response to Ach probably because of decreased eNOS expression and increased oxidative stress.

In CT mice, NAS treatment is associated with decreased eNOS expression (Figure 3A) and increased expression of several markers of oxidative stress (Figure 3B and 3C and Figure S2). eNOS expression and oxidative stress markers were not further altered in wa-2-NAS mice (Figure 3B and 3C and Figure S2). Expression and phosphorylation of extracellular signal-regulated kinase, a potential target of MR activation, were similar in CT and wa-2 mice and were not affected by NAS treatment (Figure S5).

Defective EGFR Activity Attenuates the Functional Consequences of NAS Treatment In Vivo

In CT mice, 1 month of NAS treatment resulted in impaired Ach-mediated vasorelaxation (comparison with CT-sham mice; Figure 4A) with a decrease in Ach sensitivity (EC50: CT-sham: 0.05±0.01 μmol/L; CT-NAS: 0.14±0.02 μmol/L; n=9 to 10 per group; P<0.005). By contrast, NAS treatment did not affect Ach-induced relaxation in wa-2 mice (comparison with wa-2-sham mice; Figure 4B; EC50: wa-2-sham: 0.17±0.07 μmol/L; wa-2-NAS: 0.21±0.06 μmol/L; n=9 to 10 per group; P value NS). NAS treatment sensitized the arteries to Phe in CT mice (Figure 4C), resulting in higher levels of Phe-induced contractility (EC50: CT-sham: 0.45±0.05 μmol/L; CT-NAS: 0.25±0.03 μmol/L; n=9 to 10 per group; P<0.005). NAS treatment had no such effect on Phe sensitivity in wa-2 mice (Figure 4D): wa-2-sham and wa-2-NAS mice displayed similar levels of Phe-induced contractility (EC50: wa-2-sham: 0.34±0.05 μmol/L; wa-2-NAS: 0.42±0.09 μmol/L; n=7 to 9 per group; P value NS). Thus, the NAS-induced hyperconstriction to Phe is attenuated in mice with deficient EGFR activity.

EGFR transactivation is involved in Ang II signaling20 and is thought to play a key role in MR-Ang II type 1 receptor cross-talk.21,22 NAS treatment potentiated the response to Ang II in CT mice (Figure 5A) but not in wa-2 mice (Figure 5B). The vasoconstrictive response to Ang II was similar in CT and wa-2 mice in basal conditions (Figure 5C, top), but the response to NAS was blunted in wa-2 as compared with CT mice (Figure 5C, bottom). This effect was not attributable to differences in aortic Ang II type 1 receptor expression,

![Figure 2](image-url) **Figure 2.** Vasoactive responses in the aortas of CT and wa-2 mice. A, Cumulative dose-response curve for Phe-mediated vasoconstriction (expressed as force, in milliNewtons) in CT and wa-2 mice. B, Cumulative dose-response curve for the vasodilation induced by Ach in CT and wa-2 mice. C, Cumulative dose-response curve for the vasodilation induced by sodium nitroprusside (SNP) in CT and wa-2 mice. n=5 mice per group. Values are mean±SEM; *P<0.05.

![Figure 3](image-url) **Figure 3.** Expression of eNOS and oxidative stress markers in the aorta. A, Aortic eNOS expression (Western blot) in CT-sham, CT-NAS, wa-2-sham, and wa-2-NAS. B, Aortic protein carbonylation (quantification by OxyELISA) in CT-sham, CT-NAS, wa-2-sham, and wa-2-NAS. n=5 mice per group. Values are mean±SEM; *P<0.05. C, Immunohistological staining of 4-hydroxy-2-nonenal (HNE), target of lipid peroxidation, in the thoracic aorta of CT and wa-2 mice under sham and NAS treatment. a, CT-sham; b, CT-NAS; c, wa2-Sham; d, wa2-NAS. Magnification: ×20.
because no differences were observed among the 4 experimental groups (relative mRNA levels: CT-sham: 1.00±0.14, CT-NAS: 0.93±0.17, wa-2-sham: 0.84±0.09, and wa-2-NAS: 0.93±0.19; n=5 to 10 mice per group; P value NS). The effect of NAS treatment on Ang II–mediated constriction is, therefore, affected by the partial loss of EGFR function in vivo.

Discussion
Using wa-2 mice carrying a spontaneous mutation in the Egfr gene that reduces the kinase activity of this receptor to 10% normal levels,17,18 we show that the functional (but not structural) consequences of aldo-salt administration were attenuated by the impairment of EGFR kinase activity. These findings thus demonstrated the crucial role of EGFR in vivo in aldo signaling in the vasculature.

Aldo infusion, together with salt intake, induces endothelial dysfunction in association with an increase in oxidative stress.23 MR blockade increases NO bioavailability and improves endothelial function by decreasing oxidative stress.9 Mineralocorticoids are also known to modulate vascular tone by increasing the response to various vasopressors, such as catecholamine and Ang II.24,25 Moreover, aldo enhances Ang II–induced remodeling in vascular smooth muscle by increasing signaling through the Ang II pathways.26–28 In vascular smooth muscle cells, aldo enhances Ang II–induced extracellular signal–regulated kinase and JNK phosphorylation26–29 and signaling through the RhoA/Rho kinase pathway.29,30 Pharmacological antagonism of EGFR activity with AG1478 prevents these effects, highlighting the potential role of EGFR activation. In nonvascular cells, such as Madin-Darby canine kidney cells overexpressing the MR, mitogen-activated protein kinase activation by aldo is also EGFR dependent.31 Activation of the c-Scr kinase by either aldo or aldo+Ang II appears to be involved in RhoA/Rho pathway

Figure 4. Effect of NAS treatment on aortic vasoactive responses in the aorta of CT and wa-2 mice. A, Cumulative dose-response curve for the vasodilation induced by Ach in CT mice with (n=9) or without (n=9) NAS treatment. B, Cumulative dose-response curve for the vasodilation induced by Ach in wa-2 mice with (n=9) or without (n=7) NAS treatment. C, Cumulative dose-response for Phenylephrine–mediated vasoconstriction (expressed as force, in milliNewtons) in CT mice with (n=9) or without (n=10) NAS treatment. D, Cumulative dose-response curve for Phenylephrine–mediated vasoconstriction (expressed as force, in milliNewtons) in wa-2 mice with (n=9) or without (n=7) NAS treatment. Values are mean±SEM; *P<0.05 vs the corresponding sham mice.

Figure 5. Ang II–induced contractility of the aorta. A, Cumulative dose-response curve for Ang II–mediated vasoconstriction in CT mice with (n=8) or without (n=10) NAS treatment. B, Cumulative dose-response curve for Ang II–mediated vasoconstriction in wa-2 mice with (n=9) or without (n=6) NAS treatment. *P<0.05 sham versus NAS. C, D, Dose-dependent effect of Ang II on CT (□) and wa-2 (■) in control conditions (sham, C) or after nephrectomy/aldosterone/salt treatment (NAS, D). Data are expressed as force, in milliNewtons. Values are mean±SEM; †P<0.05 CT vs wa-2 mice.
activation and NADPH-oxidase superoxide generation through EGFR transactivation.29 Altogether, these data suggest that the deleterious effects of aldo in the vessels might be mediated by EGFR activation.11 However, this hypothesis has never been tested in vivo.

Under basal conditions, EGFR deficiency (wa-2 mice) did not change the structure of the carotid artery, whereas NAS treatment induced vascular remodeling. As reported previously in the rat,32,33 we found that NAS modulated vascular Fn expression but had no effect on expression in the aorta of the genes encoding Col1 and Col3 in CT mice. On the opposite, NAS upregulated the mRNA levels of Col1 and Col3 in the aorta of wa-2 mice. The significance of this observation is unclear, because it was not associated with an increase in arterial fibrosis. Fibulin 5 and fibrillin 1 have been implicated in elastic fiber assembly. The significance of this observation is unclear, because it was not associated with an increase in arterial fibrosis. Fibulin 5 and fibrillin 1 have been implicated in elastic fiber assembly.

As reported previously,9 NAS induces endothelial dysfunction, with impairment of the vasodilatory response to Ach in CT mice. Such effect was not observed in wa-2 mice. However, it is difficult to conclude from this result that EGFR activation is not involved in the endothelial dysfunction caused by aldo-salt treatment because, in basal conditions, EGFR deficiency (wa-2 mice) was already associated with an endothelial dysfunction, probably related to the decreased eNOS expression and increased local oxidative stress. Based on data from cultured cells, it has been suggested that EGFR transactivation is a key process in acute Phe and Ang II signaling in vascular smooth muscle cells.34,35 Our study indicates that, in vivo, under basal condition, CT and wa-2 mice have similar vasoconstrictive responses to Phe and Ang II. The initial hypothesis, therefore, appears to be incorrect: EGFR activity does not seem to be essential for this process in basal conditions in vivo. However, it should be noted that an acute blockade of EGFR in cultured cells may have different consequences from those of chronic changes to EGFR activity in vivo and wa-2 mice have reduced EGFR activity from birth, and, therefore, compensatory mechanisms may occur.

Based on a genetic approach, we provide the first demonstration that EGFR activity is a key component in the functional consequences of aldo in the vessel but is not involved in the structural remodeling of the vascular wall induced by aldo (Figure 6). The wa-2 mouse harbors a point mutation in the egfr gene leading to a global EGFR deficiency. It is, therefore, not possible to determine whether the in vivo vascular interaction between aldo-salt and EGFR occurs in the endothelium, in the smooth muscle, or in both. EGFR transactivation has been studied principally in smooth muscle cells, but several reports have indicated that EGFR activation is also important in endothelial cells.35,36 The use of murine models with endothelium- or smooth muscle-specific deletions of the EGFR gene should provide further insight into this issue.

The mechanisms underlying the differential implication of EGFR in vascular function and remodeling remain to be explored, but the phosphoinositide 3-kinase (PI3K) may be a good candidate (Figure 6). Indeed, PI3K, more specifically the PI3Kα isoform, has been recognized recently as a key player in smooth muscle contraction:37 PI3K affects calcium mobilization through opening of voltage-gated and receptor-coupled calcium channels. Ang II and endothelin 1 are among the main vasoconstrictors that have been shown to activate PI3K; moreover, increased PI3K activity has been reported to be crucial in modulating the arterial tone in deoxycorticosterone acetate salt hypertension.38 Finally, EGFR also controls PI3K activity both in the endothelium and smooth muscle cells as demonstrated previously using pharmacological agents and the wa-2 mice.39,40 Further studies should address this point.

**Clinical Perspectives**

EGFR transactivation appears crucial in the vascular aldo/Ang II cross-talk in vivo. Our results point on a role of EGFR in cardiovascular pathophysiology with potential adverse effects related to EGFR inhibition in human therapeutics. Tyrosine kinases inhibitors like gefitinib and erlotinib received regulatory approval for use in cancer patients.41 However, the long-term physiological consequences of suppressed EGFR activity are unknown, although cardiac toxicity is already a concern.42 From our results, we suggest that vascular consequences should also be taken into account.

**Acknowledgments**

We thank Nicolette Farman for stimulating scientific discussions and for editing the article.

**Sources of Funding**

V.G.-C. and S.M. held postdoctoral fellowships from Lefoulon-Delalande and Région Ile-de-France, respectively. This work was supported by grants from Institut National de la Santé et de la Recherche Médicale, the Agence Nationale pour la Recherche (ANR005-PCOD005 and ANR-09-BLAN-0156-01), the European Section of the Aldosterone Council, the Leducq Fondation (Transatlantic Network of
Excellence in Cardiovascular Disease, the Fondation de France, and the Centre de Recherche Industrielle et Technique.

Disclosures.

References


17. Geckel M, Gekle M. Aldosterone upregulates the EGFR in endothelial cells. Pflugers Arch. 2007;454:403–413.


References


Epidermal Growth Factor Receptor Mediates the Vascular Dysfunction But Not the Remodeling Induced by Aldosterone/Salt

Violaine Griol-Charhbili, Céline Fassot, Smail Messaoudi, Claudine Perret, Vincent Agrapart and Frederic Jaisser

Hypertension. 2011;57:238-244; originally published online January 3, 2011; doi: 10.1161/HYPERTENSIONAHA.110.153619

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/57/2/238

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2010/12/30/HYPERTENSIONAHA.110.153619.DC1

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/
The Epidermal Growth Factor Receptor mediates the vascular dysfunction but not the remodeling induced by aldosterone/salt

Violaine GRIOL-CHARHBILI1,2, Céline FASSOT1,2*, Smail MESSAOUDI1,2*, Claudine PERRET1,2, Vincent AGRAPART1,2, Frederic JAISSER1,2
* These authors contributed equally to this work

1 INSERM, U872, Centre de Recherche des Cordeliers, 75006 Paris, France.
2 Pierre et Marie Curie University, 75006 Paris, France.

Short title: vascular Epidermal Growth Factor Receptor and aldosterone

Corresponding author: Dr Frederic JAISSER
Address: Centre de Recherche des Cordeliers, 15 rue de l’Ecole de Médecine, 75006 Paris, France
Phone: 331 44 27 8106; Fax: 331 44 27 64 21
E-mail: frederic.jaissier@erc.jussieu.fr
ONLINE SUPPLEMENT

SUPPLEMENTARY METHODS

**Blood pressure and heart rate measurement in conscious mice**
Systolic blood pressure (SBP) was measured in trained conscious mice by tail cuff plethysmography, with a BP2000 Visitech device (Bioseb) as described\(^1\). Heart rate (HR) was extracted from the pulse signal.

**mRNA and protein expression analysis**
Samples were homogenized in Trizol reagent, according to the manufacturer’s instructions (Nucleospin® RNA II kit, Macherey-Nagel). Total RNA samples were treated with DNase I. Reverse transcription was performed with the reverse transcriptase Superscript II (200 U/µl, Invitrogen). Real-time Q-PCR was carried out on an iCycler (Biorad Laboratories), with the use of gene-specific primers and SYBR Green I to determine the relative abundance of each transcript, as previously described\(^2\). The primers used are listed in Supplementary Table S1. The ubiquitin (UBC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were used as reference genes for normalization. The relative expression levels of the target genes were calculated with the \(2^{(-\Delta\Delta Ct)}\) method, as previously described\(^2\).

Aortas were lysed in SDS buffer (1% SDS, 10 mM Tris·HCl, pH 7.4, antiproteases (roche; ref 04693124001) and antiphosphatas (Sigma Aldrich). Lysates were clarified by centrifugation (11,000 g for 15 min at 15°C). Lysate protein concentrations were measured using the BCA protein assay kit with BSA as a standard. Supernatants were stored at –80°C. Proteins (20 µg) were separated by SDS-PAGE electrophoresis and electrotransferred onto nitrocellulose membranes. Proteins (20 µg) were separated by SDS-PAGE electrophoresis, and electrotransferred onto nitrocellulose membranes. Antibodies against NOX2 (Santa-Cruz Biotechnology), NOX 4 (Santa-Cruz Biotechnology), p22phox (Santa-Cruz Biotechnology), p47phox (Santa-Cruz Biotechnology); ERK (Cell signalling), pERK (Cell signalling) and EGFR (Cell signalling) were used for immunoblotting. Actin was used as a protein-loading control and was detected with an antibody directed against total actin (Santa-Cruz Biotechnology). Chemiluminescent signal was produced using ECL+ solution (Amersham) and detected with a LAS-3000 luminescent image analyzer (Fuji). Relative densitometry was determined using the computer software Multi Gauge V2.3 (Fuji).

**Plasma 8-isoprostane concentrations**
Plasma 8-epi-prostaglandin F2alpha (8-isoprostane) concentrations were determined on blood samples collected from the tail into heparin-containing microtubes (Microvette CB) according to the manufacturer's protocol (8-isoprostane enzyme immunoassay, Cayman).

**SUPPLEMENTARY REFERENCES**

## ONLINE SUPPLEMENT

### SUPPLEMENTARY TABLES

**Table S1**: Primers sequences.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>AATGGTGAAGGTCGGTG</td>
<td>GAAGATGGTGATGGGCTTCC</td>
</tr>
<tr>
<td>UBC</td>
<td>AGGTCAACAGGAGAACAGC</td>
<td>TCACACCCCAAGAACAAGCACA</td>
</tr>
<tr>
<td>AT1R</td>
<td>ATTCAACGCTCCCATAAGG</td>
<td>TGAATTCATAAGCCTTTAGAGC0</td>
</tr>
<tr>
<td>Col1a</td>
<td>CCCGGAGACTCTGGACCTT</td>
<td>GCTCCGACACGCCTCCTC</td>
</tr>
<tr>
<td>Col3a</td>
<td>CCTGGAGCCCTGGAACATTAG</td>
<td>GCCCATTTGCACCAGGTTCT</td>
</tr>
<tr>
<td>Col4a</td>
<td>ATTCTTTCGTAGTCACACC</td>
<td>GTGGGCTTTTGGACATCCT</td>
</tr>
<tr>
<td>Fibulin-1</td>
<td>GCAGACACCTCCTGCCAAGA</td>
<td>GGTTACAGCGGTTGAGACAG</td>
</tr>
<tr>
<td>Fibulin-5</td>
<td>GTGGAGACCCGAAAACCACACG</td>
<td>CTGTCTCTGCACTGGGTC</td>
</tr>
<tr>
<td>Fibrillin-1</td>
<td>TCTCGGAACACCACCAAGGGT</td>
<td>CAGCTGATGCATTGGCACAC</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>CTCAGGCGCAGCTTGTCAACC</td>
<td>AGCTGCGGTCACGCTTCT</td>
</tr>
<tr>
<td>Nox2</td>
<td>CGCCCCCTGTCCTCAATCACC</td>
<td>CTTGCCACCATGCTGGTCC</td>
</tr>
<tr>
<td>Nox4</td>
<td>GGCCCTAGGTATGTGTTTAGGC</td>
<td>GGGCGCTACATGCAAC</td>
</tr>
<tr>
<td>p22phox</td>
<td>TGCAGAGTGGACCAGGGGTG</td>
<td>GCCACAGATGCCACAGTGAT</td>
</tr>
<tr>
<td>p47phox</td>
<td>GTGTCCTGTGCAGGCCGAGG</td>
<td>ATCTCGCCGGCCCTCAATGGG</td>
</tr>
</tbody>
</table>

GAPDH: glyceraldehyde-3-phosphate dehydrogenase, UBC: ubiquitin, AT1R: Angiotensin II type 1 Receptor, Col1a: collagen 1a, Col3a: collagen 3a, Col4a: collagen 4a, Nox2: NADPH oxidase 2, Nox4: NADPH oxidase 4, p22phox and p47phox: p22phox and p47phox regulatory subunits.

**Table S2**: Body, heart and kidney weights, Systolic Blood Pressure, and Heart Rate of CT and wa-2 mice under control conditions or after 4 weeks of nephrectomy/aldosterone/salt treatment (NAS).

<table>
<thead>
<tr>
<th>General characteristics</th>
<th>Control</th>
<th>NAS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CT-Sham</td>
<td>Wα-2-Sham</td>
</tr>
<tr>
<td>BW (g)</td>
<td>25.7±0.5</td>
<td>26.1±1.1</td>
</tr>
<tr>
<td>TL (mm)</td>
<td>17.56±0.3</td>
<td>16.0±1.3</td>
</tr>
<tr>
<td>KW/TL (mg/mm)</td>
<td>16.5±0.5</td>
<td>15.63±1.59</td>
</tr>
<tr>
<td>HW/TL (mg/mm)</td>
<td>6.92±0.38</td>
<td>8.42±0.41†</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>95±3</td>
<td>100±2</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>554±67</td>
<td>590±14</td>
</tr>
</tbody>
</table>

Heart and kidney weights are normalized by tibial length (TL). BW, body weight; TL, tibia length; KW, kidney weight; HW, heart weight (n=9-10 per/group); Systolic Blood Pressure; HR, Heart Rate (n=4-6 per group). Values are means ± SEM, * p<0.05 NAS vs Sham, † p<0.05 wa-2 vs CT.
**ONLINE SUPPLEMENT**

**SUPPLEMENTARY FIGURES**

**Fig S1: EGFR protein expression is not affected by NAS.** NAS treatment effect on aortic EGFR protein expression in Sham-operated and NAS mice. n=5 per group. Values are means ± SEM.

**Fig S2: Plasma levels of 8-isoprostane**
Plasma levels of 8-isoprostane, a global marker of oxidative stress. n=5-9 per group. Values are means ± SEM * p<0.05 NAS vs Sham.
Fig S3: NADPH subunits mRNA expression is not affected by NAS in wa-2 mice. Relative mRNA levels for NADPH oxidase subunits in CT-Sham (n=10), CT-NAS (n=5), wa-2-Sham (n=6) and wa-2-NAS mice (n=9). Values are means ± SEM * p<0.05 NAS vs Sham. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; UBC: ubiquitin; Nox 2 and Nox 4: NADPH oxidase types 2 and 4, respectively; p22 and p47: p22phox and p47phox regulatory subunits.
Fig S4: Effect of NAS on NADPH subunits protein expression. Effect of NAS treatment on aortic expression of NOX2, NOX4, p47phox and p27phox in CT (left column) and wa-2 (right column) mice (n=5 per group). Values are means ± SEM * p<0.05 NAS vs Sham.

Fig S5: Expression and phosphorylation of ERK are not affected by NAS. A-B: NAS treatment does not modify aortic ERK expression and phosphorylation in CT and wa-2 mice (n=5 per group). Values are means ± SEM.