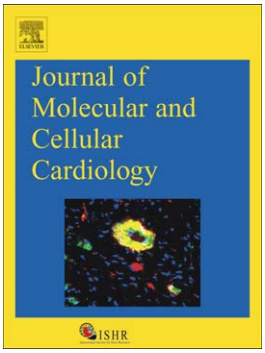

Aldosterone stimulates the cardiac sodium/bicarbonate cotransporter via activation of the g protein-coupled receptor gpr30

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ALDOSTERONE STIMULATES THE CARDIAC SODIUM/BICARBONATE COTRANSPORTER VIA ACTIVATION OF THE G PROTEIN-COUPLED RECEPTOR GPR30.

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ABSTRACT

Some cardiac *non-genomic* effects of aldosterone (Ald) are reported to be mediated through activation of the classic mineralocorticoid receptor (MR). However, in the last years, it was proposed that activation of the novel G protein-coupled receptor GPR30 mediates certain *non-genomic* effects of Ald. The aim of this study was to elucidate if the sodium/bicarbonate cotransporter (NBC) is stimulated by Ald and if the activation of GPR30 mediates this effect. NBC activity was evaluated in rat cardiomyocytes perfused with HCO_3^-/CO_2 solution in the continuous presence of HOE642 (sodium/hydrogen exchanger blocker) during recovery from acidosis using intracellular fluorescence measurements. Ald enhanced NBC activity (% of ΔJ_{HCO3}^-; control: 100±5.82 %, n=7 vs Ald: 151.88±11.02 %, n=5; P<0.05), which was prevented by G15 (GPR30 blocker, 90.53±7.81 %, n=7). Further evidence for the involvement of GPR30 was provided by G1 (GPR30 agonist), which stimulated NBC (185.13±18.28 %, n=6; P<0.05) and this effect was abrogated by G15 (124.19±10.96 %, n=5). Ald- and G1-induced NBC stimulation was abolished by the reactive oxygen species (ROS) scavenger MPG and by the NADPH oxidase inhibitor apocynin. In addition, G15 prevented Ald- and G1-induced ROS production. Pre-incubation of myocytes with wortmannin (PI3K-AKT pathway blocker) prevented Ald- or G1-induced NBC stimulation. In summary, Ald stimulates NBC by GPR30 activation, ROS production and AKT stimulation.

Key Words: aldosterone, GPR30, sodium/bicarbonate cotransporter, cardiomyocytes.

Non-standard Abbreviations and Acronyms

Aldosterone: Ald

Na^+/H^+ exchanger: NHE-1

Epidermal growth factor: EGF

Epidermal growth factor receptor: EGFR

N-(2-Mercapto-propionyl)glycine: MPG

Reactive oxygen species: ROS

Na^+/HCO_3^- cotransporter: NBC

Apocynin: Apo

Wortmannin: Wort

Eplerenone: eple

1. Introduction

Aldosterone (Ald) is an important regulator of pathophysiological cardiovascular function. The traditional and widely accepted pathway exerted by this hormone is based on the regulation of transcription via activation of their cytosolic mineralocorticoid receptors (MR). Classically, Ald enters the cells and binds to the MR. This binding translocates the MR to the nucleus, where it acts as a ligand-induced transcription factor. However, it has been proposed that activated MR can elicit additional non-classical effects, which do not require transcription or translation of genes and which implicate an increase production of reactive oxygen species (ROS) [1], phosphorylation of ERK 1/2 kinase [2] and activation of ionic membrane transporters [1, 3-7]. However, the lack of response to MR-antagonists of certain rapid Ald-induced actions has required additional explanations for these mechanisms [8, 9]. Moreover, the controversy about the receptor involved in the *non-genomic* effects of Ald remains unresolved [10].

In the last years the GPR30, a surface membrane G protein–coupled receptor, was proposed as the new candidate for the rapid *non-genomic* effects of Ald. GPR30 was first described as an orphan receptor. Some years later estrogen was proposed as its natural ligand [11]. Interestingly, it was reported that GPR30 is expressed in the heart [12]. The stimulation of this receptor by estradiol [13] or G1, a specific GPR30 agonist, [11, 12] mediates protection against ischemia-reperfusion injury [11]. G1 is a highly selective GPR30 agonist described for first time in 2006 by *Bologa et al* [14]. This non-steroidal compound activates multiple cellular signaling pathways via GPR30 and has been used to examine the cellular and physiological actions of GPR30. Interestingly, G1 shows no detectable activity towards the classical estrogen receptors [14] or the MR [15]. Cardiotropic effects of estradiol and G1 have also been recently reported [16, 17]. Furthermore, Gros *et al.* [15, 18] demonstrated that certain *non-genomic* effects of Ald in vascular smooth muscle were due to simultaneous

activation of MR and GPR30. In addition, these investigators reported that the effects of Ald to mediate its effects through GPR30 have also been depicted in a model without detectable MR [16].

In the heart two major alkalinizing mechanisms exist: the sodium/proton antiporter (NHE-1), which transports Na^+ into and H^+ out of the myocyte, and the sodium/bicarbonate cotransporter (NBC), which transports HCO_3^- and Na^+ into the myocyte. The NBC regulates both, intracellular pH (pH_i) and intracellular sodium ($[Na^+]_i$) concentration [19, 20]. In the heart, at least, two functional NBC isoforms exists, the electroneutral (NBCn1; $1HCO_3^-/1Na^+$) and the electrogenic (NBCe1; $2HCO_3^-/1Na^+$) [21]. In our laboratory, we have determined the influence of the anionic repolarizing current generated by NBCe1 in the configuration of the rat and cat ventricular action potential (AP) [22, 23]. We have also demonstrated that Angiotensin II (Ang II) stimulates NBCn1 via reactive oxygen species (ROS) production and ERK 1/2 kinase activation, and inhibits NBCe1 in a p38 kinase-dependent pathway [24, 25]. In addition, we have also reported that Ald stimulates the NHE-1, in an MR and non-genomic dependent pathway [3]. However, the potential regulation of NBC by Ald remains unknown. Thus, the aim of the present research is to investigate if Ald regulates NBC activity, elucidating which receptors and intracellular pathways are involved in such effect.

2. Material and Methods

All procedures followed during this investigation conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the experimental protocol was approved by the Animal Welfare Committee of La Plata School of Medicine. Male rats (body weight 300–400 g) were anaesthetized by intraperitoneal injection of sodium pentobarbital (35 mg/kg body weight) and hearts rapidly excised when plane three of phase III of anaesthesia was reached.

2.1. *pH_i measurements*

pH_i was measured in single myocytes with an epi-fluorescence system (Ion Optix, Milton, MA). Myocytes were incubated at room temperature for 10 min with 10 μmol/L BCECF-AM followed by 30 min washout. Dye-loaded cells were placed in a chamber on the stage of an inverted microscope (Nikon.TE 2000-U) and continuously superfused with a solution containing (mmol/L) 5 KCl, 118 NaCl, 1.2 $MgSO_4$, 0.8 Cl_2Mg, 1.35 Cl_2Ca, 10 glucose, 20 $NaHCO_3$, pH 7.4 after continuous bubbling with 5% CO_2 and 95% O_2. The myocytes were stimulated via two-platinum electrodes on either side of the bath at 0.5 Hz. Dual excitation (440 and 495 nm) was provided by a 75-watt Xenon arc lamp and transmitted to the myocytes. Emitted fluorescence was collected with a photomultiplier tube equipped with a band-pass filter centered at 535 nm. The 495-to-440 nm fluorescence ratio was digitized at 10 kHz (ION WIZARD fluorescence analysis software). At the end of each experiment, the fluorescence ratio was converted to pH by in vivo calibrations using the high K^+-nigericin method [26]. As described above, the experiments were performed in HCO_3^- buffered solution. Under these conditions, both pH_i regulatory systems are operative, NHE and NBC. In order to examine the NBC activity in isolation all the experiments were performed in the

presence of 10 μmol/L HOE642. The total NBC activity was assessed by evaluating the pH_i recovery from a double ammonium pre-pulse (“the first acting as control for the second pulse”). All the inhibitors were added 10 minutes before the beginning of the first ammonium pulse and were present throughout the experiment.”

The dpH_i/dt at each pH_i, obtained from an exponential fit of the recovery phase, was analyzed to calculate the net H^+ efflux (J_H), then $J_H=\beta_{tot}\ dpH_i/dt$, where β_{tot} is total intracellular buffering capacity. β_{tot} was calculated by the sum of the intracellular buffering due to CO_2 (β_{CO2}) plus the intrinsic buffering capacity (β_i). β_{CO2} was calculated as, $\beta_{CO2}=2.3\ [HCO_3^-]_i$, where $[HCO_3^-]_i = [HCO_3^-]_o\ 10^{pHi-pHo}$ [27, 28]. β_i of the myocytes was measured by exposing cells to varying concentrations of NH_4Cl in Na^+-free HEPES bathing solution. pH_i was allowed to stabilize in Na^+-free solution before application of NH_4Cl. β_i was calculated from the equation $\beta_i = \Delta[NH_4^+]_i/\Delta pH_i$ and referred to the mid-point values of the measured changes in pH_i. β_i at different levels of pH_i were estimated from the least squares regression lines β_i vs. pH_i plots. The average was illustrated as the percentage of increase in J_H at pH_i 6.8 during the second pulse in comparison with the first pulse.

2.2. *Determination of extracellular signal-regulated protein kinases (AKT)*

Left and right ventricular myocardial slices (1 x 5 mm) were obtained from anesthetized 4-month old male Wistar rats and incubated in buffer HCO_3^- under aeration (95% O_2–5% CO_2) at 37°C during 10 min, and then incubated 15 min with G1 (1 μmol/L). Other drugs (G15 1 μmol/L and Apo 300 μmol/L) were added to the buffer 10 min before G1. At the end of the experimental protocols cardiac slices were frozen and homogenized in lysis buffer (300 mmol/l sacarose; 1 mmol/L DTT; 4 mmol/L EGTA, protease inhibitors cocktail (Complete Mini Roche); 20 mmol/L Tris-HCl, pH 7.4). After a brief centrifugation the supernatant was kept and protein concentration determined by the Bradford method. Samples were denatured

and equal amounts of protein were subjected to PAGE and electrotransferred to PVDF membranes. Membranes were then blocked with non-fat-dry milk and incubated overnight with anti-phospho-AKT (Cell Signaling, 4060S). To normalize the amount of the phosphorylated proteins the membranes were stripped and probed with an antibody that specifically detects GAPDH (Millipore, MAB374). Peroxidase conjugated anti-rabbit (NA934, GE Healthcare Life Sciences) or anti-mouse IgG (NA931, GE Healthcare Life Sciences) were used as secondary antibodies and bands were visualized using the ECL-Plus chemiluminescence detection system (Amersham). Blots were visualized using a Chemidoc Image Station (Bio-Rad) and quantified by densitometric analysis (Scion Image) [21].

2.3. *Intracellular ROS*

a) In isolated myocytes

Intracellular ROS production was measured on a Zeiss 410 microscope using rat ventricular myocytes loaded with 5 μmol/L 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCF DA, Molecular Probes) for 30 min at 37 °C as previously described [29]. CM-H2DCF DA (DCF) was excited at 488 nm and the emitted fluorescence was recorded at 510–560 nm. As DCF can produce artifactual signal amplification upon continuous light exposure, cells were imaged at extremely low intensity and images were taken every 180 seconds to minimize light exposure during the duration of the experiment. Nevertheless, in order to avoid misinterpretation of the recordings, the slight spontaneous increase in the fluorescence signals were fitted with regression lines before and after the corresponding treatments and the ratio between both slopes were used to express the data.

b) In left ventricular slices

We used lucigenin-enhanced chemiluminescence to measure O_2^- production by rat cardiac tissue with 5 μmol/L lucigenin. The chemiluminescence in arbitrary units (AU) was recorded with a luminometer (Chameleon; Hidex, Turku, Finland) during 30 s, each with a 4.5 min

interval during 30 min. O_2^- production was normalized to milligrams dry weight tissue per minute. Control tissue slices without any pharmacological intervention produced low levels of O_2^- that were only slightly detected above background. Although the lucigenin-enhanced chemiluminescence method does not allow accurate detection of the basal O_2^- production [30], its sensitivity was enough to detect an increase in O_2^- production above background after Ald and G1. The increase in O_2^- production was expressed as a percentage of basal value after 15 min of each intervention.

2.4. *Chemicals*

All drugs used in the present study were of analytical reagent and the concentrations used were selected according to previously reported efficacy; G1 (1μmol/L) [14, 31-33], G15 (1μmol/L) [34], Aldosterone (10 nmol/L) [3], cycloheximide (10 μmol/L) [35-38], U0126 (10 μmol/L) [39], wortmannin (100 nmol/L) [40], H89 (10 μmol/L) [41], KN93 (2.5 μmol/L) [42], EGF (0.2 μg/mL) [3] and MPG (2 mmol/L) [43] were purchased from Sigma–Aldrich. Eplerenone (10 μmol/L) [44] was kindly donated by Gador SA, Argentina. Apocynin (300 μmol/L) [44] was purchased from FLUKA. AG 1478 (1 μmol/L) [45] was obtained from Calbiochem. HOE642 (10 μmol/L) [24, 46, 47] was kindly donated by Sanofi-Aventis.

2.5. *Statistics*

Data were expressed as means ± S.E.M. and were compared with Students's t test or One-way ANOVA followed by Student-Newman-Keuls post-hoc test. A value of $P<0.05$ was considered statistically significant (two-tailed test).

3. Results

3.1 *Effect of aldosterone on NBC-activity after intracellular acidification.*

In order to study NBC activity in isolation, we performed a double NH_4Cl pulse in HCO_3^- buffered solution in the presence of 10 μmol/L HOE642 (NHE-1 blocker). As the procedure of exposing the myocyte to two consecutives NH_4Cl pulses might decrease the ability of the cell to recover from the second acidosis, we first performed the double NH_4Cl pulse without any treatment. *Figure 1A upper panel* shows a representative double *NH_4Cl pulse.* As illustrated in *Figure 1A lower panel*, which depicts the average bicarbonate influx (J_{HCO3}^-) during the recovery from acidosis, the J_{HCO3}^- of the second pulse is approximately 20 % lower in comparison to that of the first pulse in the range of pH_i from 6.8 to 7.

It has been demonstrated that Ald stimulates NHE-1 in adult rat ventricular myocytes, accelerating pH_i recovery during acidosis [3]. No data are available concerning the effect of Ald on NBC activity. *Figure 1B* illustrates the effect of exposing myocytes to Ald 10 nmol/L. This hormone was added to the bath solution 10 minutes before the beginning of the second *NH_4Cl pulse*. Ald significantly increases J_{HCO3}^- at acidic pH_i (*Figure 1B lower panel*). In *Figure 1D* (and also in *Figure 1E* and other Figures of this work) the results are expressed as the percentage increase in the J_{HCO3}^- (at pH_i 6.8) during the second pulse in comparison to the first pulse (% of ΔJ_{HCO3}^-). Considering the control experiments of *Figure 1A* and in order to avoid underestimation of the effects of the different treatments, we applied, to control and every treatment, a 20 % correction to the second pulse in all the data presented as % of ΔJ_{HCO3}^-. After this correction, as shown in *Figure 1D*, Ald induced a J_{HCO3}^- increase of about 50 %. This positive effect of Ald was not blocked by the protein synthesis inhibitor cyclohexamide (10 μmol/L; *Figure 1D*), indicating that the mechanism involved is *non-genomic*. Since it was recently reported that certain cardiac *non-genomic* effects of Ald were

induced by the stimulation of the calcium-calmodulin dependent type II kinase (CaMKII) [2], we performed experiments in the presence of the CaMKII inhibitor, KN93 (2.5 μmol/L; *Figure 1D*). The Ald-induced increase in J_{HCO3}^- mediated by the NBC was not affected by this blocker, suggesting that under our experimental conditions CaMKII does not participate in this effect of the hormone.

3.2 *GPR30 is involved in the rapid effect of Ald on NBC- activity.*

The effect of Ald on NBC activity was partially blocked by the MR inhibitor eplerenone (eple, 1 μmol/L; *Figure 1D*). Interestingly, this effect of Ald was totally prevented by pretreatment of the cells with the selective blocker of the GPR30, G15 (1 μmol/L; *Figure 1D*), indicating that this novel Ald receptor is involved in this *non-genomic* effect of this hormone. In order to confirm the participation of GPR30 on NBC stimulation, myocytes were pre-incubated 10 minutes before the beginning of the second pulse with G1 (1 μmol/L). Consistently, G1 significantly increased the J_{HCO3}^- mediated by NBC (*Figure 1C*). On average, the percentage increase in J_{HCO3}^- at pH_i 6.8 in the presence of G1 was about two fold, effect inhibited by the pre-incubation with G15 or eple (*Figure 1E*). The fact that eple partially blocked this effect could be explained by off-target effects of this MR antagonist on GPR30, as was previously described in vascular smooth muscle [15, 18].

3.3 *Role of the epidermal growth factor receptor (EGFR) transactivation in Ald-induced NBC activation through GPR30.*

Since GPR30 is coupled to Gs we next assessed the potential role of PKA in the intracellular pathway triggered by the activation of this receptor. However, the effect of G1 was not

affected by the PKA blocker H89 (10 μmol/L; *Figure 1E*). On the other hand, it has been described that the GPR30 activation can transactivate the epidermal growth factor receptor (EGFR) through its βγ subunit [48]. We therefore investigated if the stimulation of NBC activity by G1 is mediated by EGFR transactivation. *Figure 1E* shows that G1-induced NBC stimulation was abrogated by AG1478 (EGFR blocker, 1 μmol/L), indicating the involvement of EGFR transactivation after GPR30 stimulation. Consistently, exogenous EGF (0.2 μg/ml) was able to enhance the J_{HCO3}^- mediated by NBC (*Figure 2*).

EGFR activation could lead to stimulation of the ERK1/2 and/or PI3K/AKT pathways. The blocker of AKT pathway wortmannin (100 nmol/L), but not that of the ERK1/2 pathway U0126 (10 μmol/L), cancelled the Ald- and G1-induced NBC stimulation (*Figure 1D and 1E*), suggesting that only PI3K/AKT signaling is required to induce NBC stimulation after GPR30 activation. In order to confirm the involvement of this mechanism, phosphorylation of AKT after treatment of the cells with G1 was performed. Accordingly, *Figure 3* shows that G1 increases the phosphorylation level of AKT, effect that was prevented by G15 and the NADPH oxidase blocker apocynin (Apo; 300 μmol/L), suggesting the involvement of ROS in AKT phosphorylation induced by GPR30 activation.

3.4 *Role of ROS in Ald-induced NBC stimulation induced by GPR30 activation.*

In the last years, several studies positioned ROS as important molecules involved in the intracellular signaling of different extracellular stimulus, such as Ang II, endothelin, EGF and Ald [2, 3, 49]. Moreover, these molecules were shown to induce the increase in ROS production in the heart. In addition, we have demonstrated that ROS are involved in Ang II-induced NBC stimulation in cat ventricular myocytes [24]. To determine whether ROS are also implicated in the NBC activation generated by GPR30 activation induced by either Ald

or G1, we examined the effect of the ROS scavenger MPG (2 mmol/L) and Apo (300 μmol/L) on NBC-mediated pH_i recovery. As shown in *Figure 1D and 1E*, the effect of Ald and G1 was abolished by pre-treatment of the cells with MPG or Apo. "These drugs showed no significant effect on the control NBC-mediated J_{HCO3} during the recovery of acidosis (in mmoles/min at pH_i 6.8; Control: 1.24±0.05; n=16, MPG: 1.02±0.07; n= 8, Apo: 1.04±0.10; n=8)."

In order to give further support to these results we measured ROS production after Ald or G1 pre-incubation. The increase in ROS production induced by Ald, both in isolated myocytes (*Figure 4A*) or in slices of myocardium (*Figure 4C*), was partially prevented with G15, suggesting that Ald might be increasing ROS production by both MR and GPR30 activation. In concordance, parallel experiments performed in the presence of G1 confirmed that GPR30 activation is involved in ROS production in cardiac myocytes and tissue (*Figure 4B and 4D*). In addition, the production of ROS induced by G1 was blocked by AG1478 (1 μmol/L; *Figure 4D*), indicating that ROS are generated downstream of the transactivation of EGFR. On the other hand, Apo abrogated the increase in the phosphorylation level of AKT induced by G1 (*Figure 2*), suggesting that superoxide anion production by NADPH oxidase is required for AKT activation.

3.5 *GPR30 is the only receptor involved in Ald-induced NBC stimulation.*

Gros et al was the first work which implicated GPR30 in some of Ald effects, as vascular relaxation [18]. In addition, these authors demonstrated that eple could prevent G1-induced ERK 1/2 phosphorylation, suggesting a non-specific effect of the MR blocker on GPR30. As shown in *Figure 1E*, eple partially inhibited G1-induced NBC stimulation, indicating that this compound also exerts non-specific effects under our conditions. These results allow us to

suggest that the inhibitory effect of eple on Ald-induced NBC stimulation depicted in *Figure 1D* might be mediated through GPR30 and not MR inhibition. We next corroborated the specificity of G15 and G1 for GPR30, in order to discard the possibility that they might have been exerting effects on MR. Experiments were performed in HEPES-buffered solution (bicarbonate-free solution) and NHE-1 activity was evaluated in the presence of Ald plus the absence or presence of eple or G15. *Figure 5* shows that Ald-induced NHE-1 stimulation was prevented by eple, as was previously reported by us [3], but was not prevented by G15. Accordingly, G1 did not modify NHE-1 activity (*Figure 5B*). These results ruled out non-specific effects of G15 or G1 on MR and allow us to propose two parallel pathways triggered by Ald: one that involves MR activation inducing NHE-1 stimulation, and the other that implicates GPR30 activation that lead to NBC stimulation.

4. Discussion

The main finding of this study is that GPR30 appears to be a novel cardiac Ald receptor involved in some of the *non-genomic* effects of the hormone in adult cardiac myocytes. Supporting our data, Ashton et al. [50] working with a cell line of cardiac myoblasts (H9c2), have just recently suggested that the *non-genomic* effects triggered by a non-permeable Ald molecule (Ald-PEG) were mediated by GPR30 activation. In contrast to previously published data that demonstrated MR-dependent cardiovascular *non-genomic* effects [3, 15, 18], Ashton et al. [50] proposed that the Ald-induced *non-genomic* effects were exclusive mediated by GPR30 (i.e. ERK1/2 activation), without participation of MR. In the present work, in agreement with the studies of the group of Feldman [15, 18], we demonstrated that cardiovascular *non-genomic* effect of Ald are shared by MR and GPR30 activation.

It has been previously demonstrated that Ald, in addition to exerting the classic genomic response, also triggers *non-genomic* effects, which do not involve protein synthesis. Previous studies have discussed about which could be the receptor responsible for such *non-genomic* effects, proposing in some cases the classic MR, situated in the cytosol and also along the sarcolemma, where it might be co-localized with the EGFR [51]. On the other hand, there is evidence showing that some *non-genomic* effects of Ald cannot be prevented by MR blockers, supporting the existence of "other Ald receptor" [52]. Accordingly, *Gros et al* demonstrated in vascular muscle cells, that GPR30 participates in Ald *non-genomic* effects. We showed herein for the first time that Ald activates GPR30 in the heart, which transactivates the EGFR and in turn triggers a ROS- and PI3K/AKT-dependent pathway, leading to NBC stimulation (*Figure 6*).

It is well-known that Ald increases the intracellular level of ROS, which act as physiological signaling molecules. Our results allow us to suggest that under our conditions, GPR30 is

involved in Ald-induced ROS production. In the presence of Ald and G15, some ROS production still remains present, suggesting that Ald-induced ROS production might be triggered by both MR and GPR30. It is possible that these divergent ROS sources might act in distinct intracellular compartments, mediating different effects of the hormone. Consistent with this proposal, some authors proposed the existence of redoxosomes, managing the production of ROS and their local effects [53, 54]. In the present study we showed that Apo, a NADPH oxidase inhibitor, abrogated Ald-induced NBC stimulation, indicating that this enzyme is involved in the GPR30 intracellular pathway. Since exogenous EGF was reported to induce cardiac NADPH oxidase stimulation [53] and in the present work we showed that AG1478 blocked Apo-sensitive ROS production, and Apo inhibited AKT phosphorylation, the idea that NADPH oxidase-induced superoxide anion production occurs downstream of EGFR transactivation and upstream of AKT activation can be suggested (*Figure 6*). However, the exact sites where these ROS are acting were not elucidated in the present work. It was recently suggested that enhanced NADPH oxidase ROS production after EGFR activation is able to block the phosphatases PTEN and SHP2, which normally dephosphorylates PIP3 (inhibiting AKT signaling) and EGFR, respectively [55]. Thus, it is possible to speculate that ROS produced after GPR30 activation might be activating the EGFR in a feed-forward manner.

We have previously demonstrated that Ald-induced NHE-1 stimulation via MR activation is dependent on ROS production and ERK 1/2 phosphorylation [3]. Experiments performed in this study, showing that neither G1 is able to activate NHE-1, nor G15 is able to prevent Ald-induced NHE-1 activation during the recovery of acidosis in HEPES solution, support the hypothesis that Ald might trigger two parallels pathways: one via MR activation and NHE stimulation and the other via GPR30 activation and NBC stimulation. The explanation for the existence of these two divergent pathways is not apparent to us and is not the focus of the

present study. It is possible that the differential cellular localization of these two alkalinizing transporters and/or receptors can account for these results. In support of this proposal, it was recently reported that NBC is localized in t-tubules while NHE-1 is only confined to the intercalated disks [56]. The identification of the NBC isoform stimulated by Ald was not included among the aims of this study. However, it might be interesting to investigate if Ald, as we have demonstrated for Ang II [24, 25] exerts differential effect on NBC isoforms.

One limitation of the present study is the exclusive use of pharmacological compounds to elucidate the MR-independent actions of Ald. However, it has been previously demonstrated that G15 did not affect the eple-sensitive increase in ERK1/2 phosphorylation induced by Ald in vascular smooth muscle cells lacking GPR30 expression [15]. In addition, in these cells G1 failed to enhance ERK1/2 phosphorylation [15]. Consistently, in the present study we showed that G15 did not alter the MR-mediated increase in NHE activity induced by Ald, whereas G1 did not mimic the effect of Ald on this transporter. Overall, these results emphasized the specificity of these agents for, blocking or evoking, GPR30-mediated events.

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Disclosures

None.

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Figure Legends

Figure 1. Aldosterone stimulates NBC via GPR30 activation, EGFR transactivation and subsequent stimulation of ROS production and AKT pathway. (A-C, upper panels): Representative traces of pH_i during the application of two consecutive NH_4Cl pulses (20 mmol/L NH_4Cl), in control conditions **(A)**, in the presence of vehicle alone (first pulse) or plus 10 nmol/L aldosterone (Ald, second pulse) **(B)** and in the presence of vehicle alone (first pulse) or plus 1 μmol/L G1 (second pulse, GPR agonist) **(C)**. **(A-C, lower panels):** average bicarbonate influx J_{HCO3}^-, carried by NBC, under the conditions established in the upper panels. * indicates P<0.05 vs. control. **(D):** Percentage average increase in J_{HCO3}^- at pH_i 6.8 expressed as the difference between the second and the first pulse, in the presence of 10 nmol/L Ald, and in the presence of Ald plus eplerenone (MR antagonist, Eple 10 μmol/L), plus G15 (GPR30 antagonist, 1μmol/L), plus cycloheximide (protein synthesis inhibitor, Cyclo, 10 μmol/L), plus KN93 (CaMKII inhibitor, 2.5 μmol/L), plus MPG (ROS scavenger, 2 mmol/L), plus apocynin (NADPH oxidase blocker, Apo, 300 μmol/L), plus wortmannin (PI3K-AKT pathway inhibitor, Wort, 100 nmol/L) or plus U0126 (ERK1/2 pathway blocker, 10 μmol/L). * indicate P<0.05 vs. control. The n value for each treatment is shown inside the bars. **(E):** Percentage average increase in J_{HCO3}^- at pH_i 6.8 expressed as the difference between the second and the first pulse, in the presence of 1 μmol/L G1, and in the presence of G1 plus G15 (1μmol/L), plus Eple (10 μmol/L), plus MPG (2 mmol/L), plus Apo (300 μmol/L), plus AG1478 (EGFR blocker, AG, 1μmol/L), plus H89 (PKA blocker, 10 μmol/L), plus Wort (100 nmol/L) or plus U0126 (10 μmol/L). * indicate P<0.05 vs. control. The n value for each treatment is shown inside the bars.

Figure 2. EGF activates NBC-mediated pH recovery. (A): Representative traces of intracellular pH (pH_i) during the application of 2 consecutive ammonium pulses (20 mmol/L of NH_4Cl), in the absence (first pulse) and presence of 0.2 mg/ml of EGF. **(B):** Average increase in J_{HCO3}^- at pH_i 6.8 expressed as the percentage of increase in the J_{HCO3}^- (at pH_i 6.8) during the second pulse in comparison to the first pulse (% of Δ_{JHCO3}^-) in the presence of 0.2 mg/ml of EGF. * indicates P<0.05 vs. control. The n value for each treatment is shown inside the bars.

Figure 3. GPR30 activation promotes AKT phosphorylation. (A): Representative immunoblot showing the increases of AKT phosphorylation after 15 min of incubation with the specific agonist of GPR30, G1 (1 μmol/L). The effect of G1 was prevented with by G15 (1 μmol/L) and Apo (300 μmol/L). **(B):** Average data are depicted in the bar graphs as percentage of pAKT/GAPDH respect to control. * indicates P<0.05 vs. control. The n value for each treatment is shown inside the bars.

Figure 4. GPR30 stimulates ROS production. (A-B): Representative fluorescence images of three independent experiments in control condition or in the presence of Ald or G1 on DCF fluorescence. This effect was abrogated in the presence of 1 μmol/L of G15, GPR30 inhibitor. The normalized slope (treatment slope/pre-treatment slope, see Methods) of DCF fluorescence significantly increased with 10 nmol/L Ald and 1 μmol/L G1, and prevented in the presence of GPR30 inhibition (G15, 1 μmol/L). * indicates P<0.05 vs. control. The n value for each treatment is shown inside the bars. **(C-D):** Superoxide production in tissue slices detected by lucigenin-chemiluminescence method. Ald (10 nmol/L) significantly increased $\cdot O_2^-$ production, effect that was partially prevented by the GPR30 inhibitor, G15 (1 μmol/L). In the same way, G1 also increased $\cdot O_2^-$ production, effect that was abrogated by the

NADPH oxidase inhibitor Apo (300 μmol/L), the antagonist of EGFR AG1478 (1 μmol/L) and by the specific GPR30 inhibitor G15. * indicates P<0.05 vs. control. ** indicates P<0.05 vs Ald. The n value for each treatment is shown inside the bars.

Figure 5. Aldosterone stimulates NHE-1 activity exclusively through MR activation. Average proton efflux (J_H), carried by the Na^+/H^+ exchanger (NHE-1) in HEPES buffer solution expressed as the percentage of increase in the J_H (at pH_i 6.8) during the second pulse in comparison to the first pulse (% of ΔJ_H) in the presence of Ald (10 nmol/L), Ald plus G15 (1 mmol/L) and Ald plus Eple (1 μmol/L) **(A)**, or in the presence of G1 (1 μmol/L) **(B)**. * indicates P<0.05 vs. control. The n value for each treatment is shown inside the bars.

Figure 6. Scheme of the proposed actions triggered by GPR30 activations by Ald. Activation of GPR30 by Ald or G1 transactivates the EGFR via βγ subunits. Activation of EGFR by transactivation or by exogenous EGF leads to stimulation of NADPH oxidase (NOX) and the PI3K-AKT pathway. Hydrogen peroxide (H_2O_2) produced by dismutation of superoxide anions (O_2^-) generated by NOX might inhibit the phosphatase PTEN, avoiding the dephosphorylation of PIP3 and the blockade of AKT phosphorylation and activation. At the same time H_2O_2 might increase the phosphorylation level of the EGFR and the continuous activation of NOX, in a feed-forward mechanism. NBC is the final target of the AKT pathway, either by direct phosphorylation or by activation of unidentified regulatory proteins. Note that GPR30 and NBC are shown co-localized in t-tubules.

Figure 1

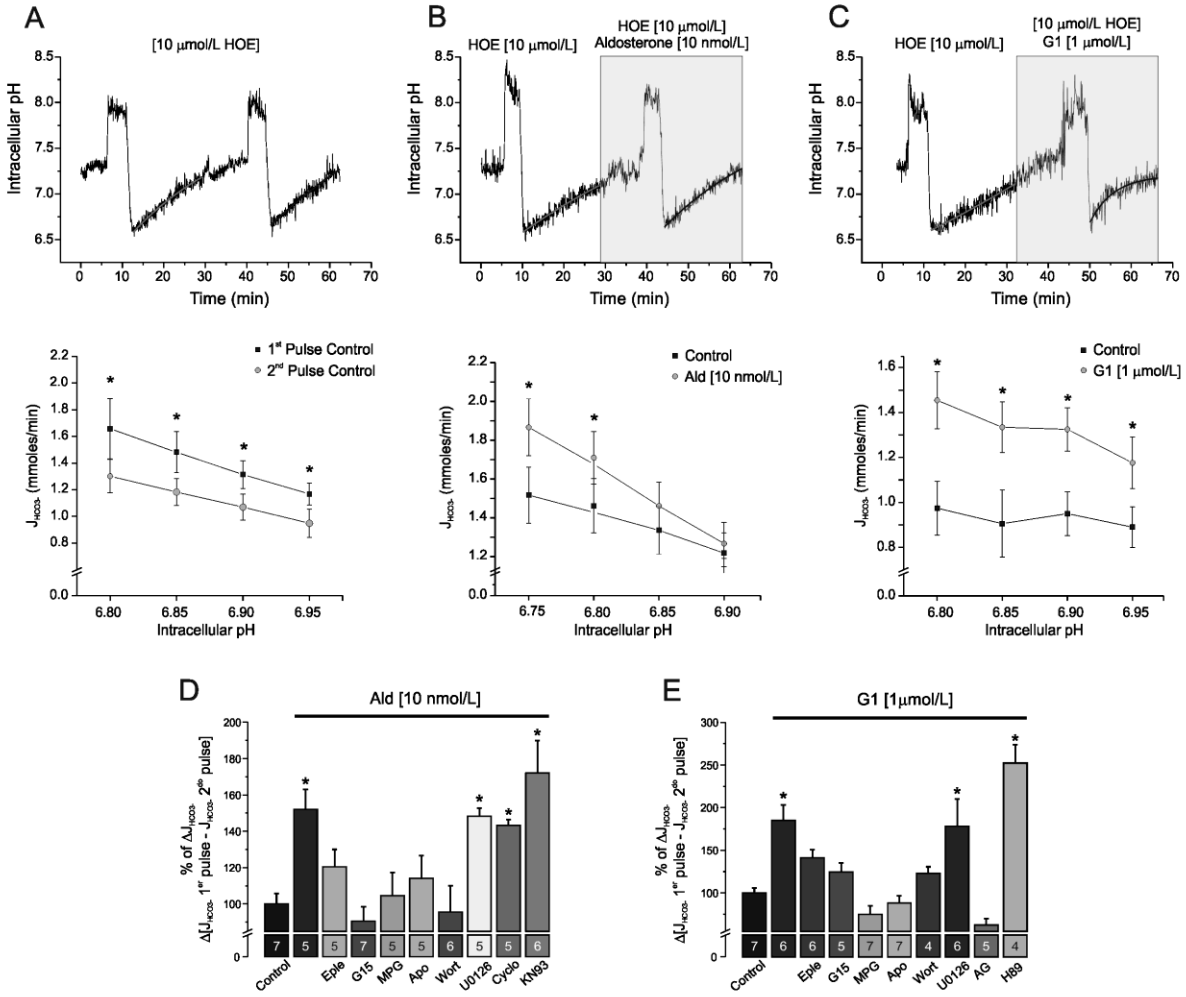


Figure 2

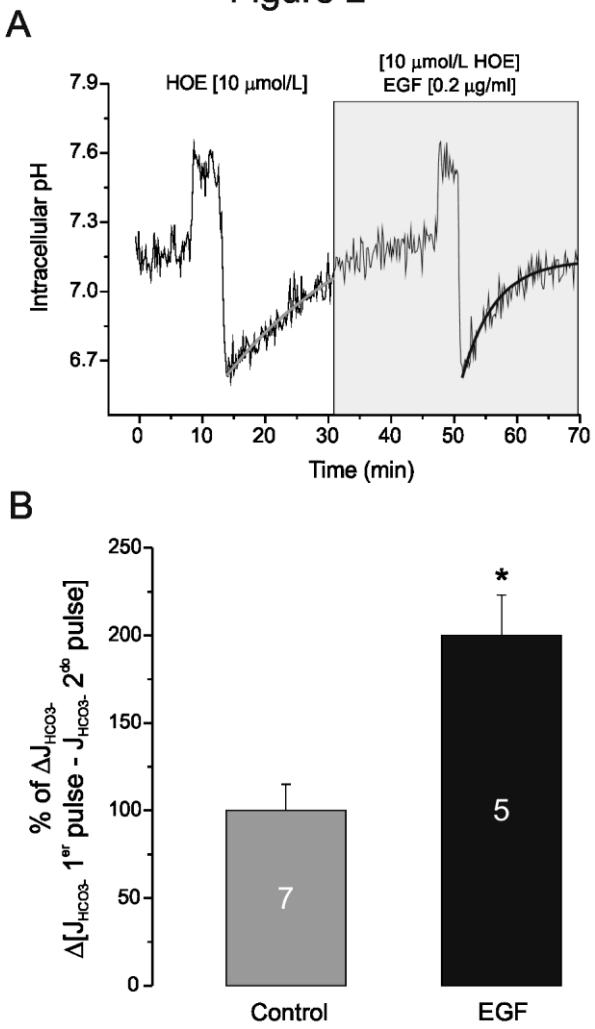


Figure 3

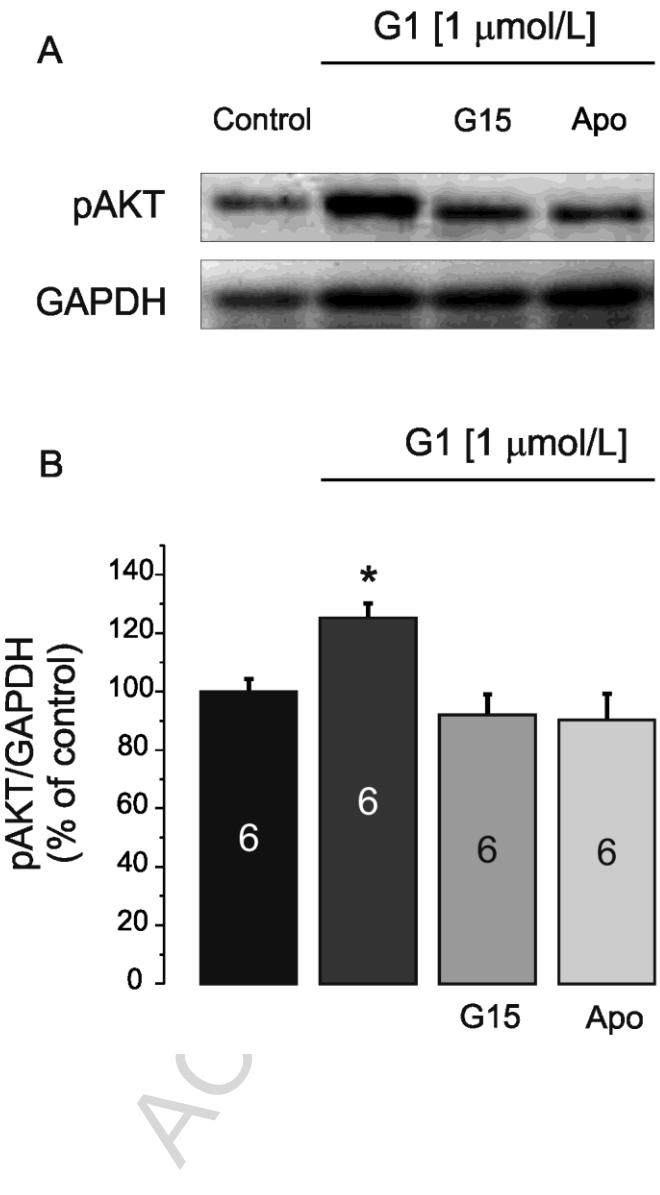


Figure 4

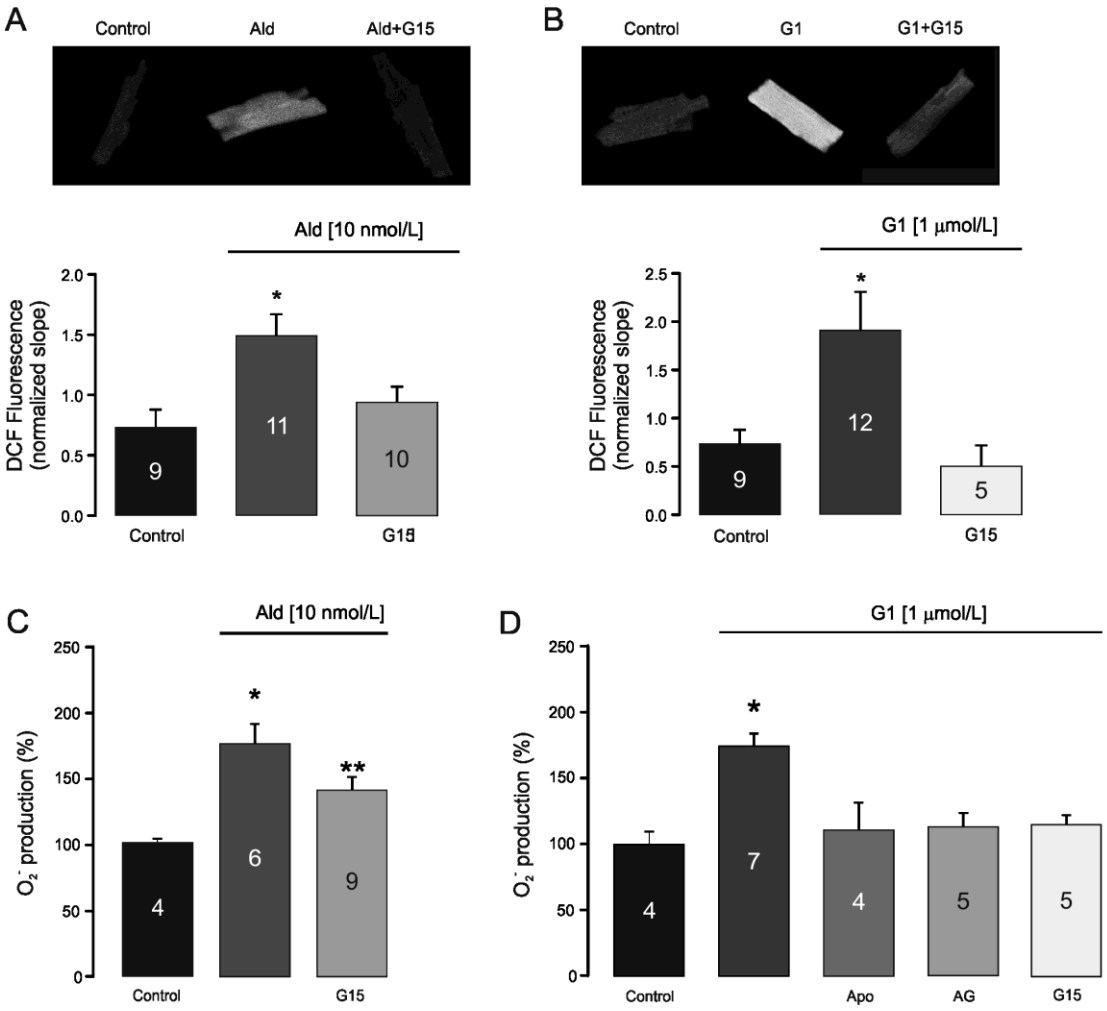


Figure 5

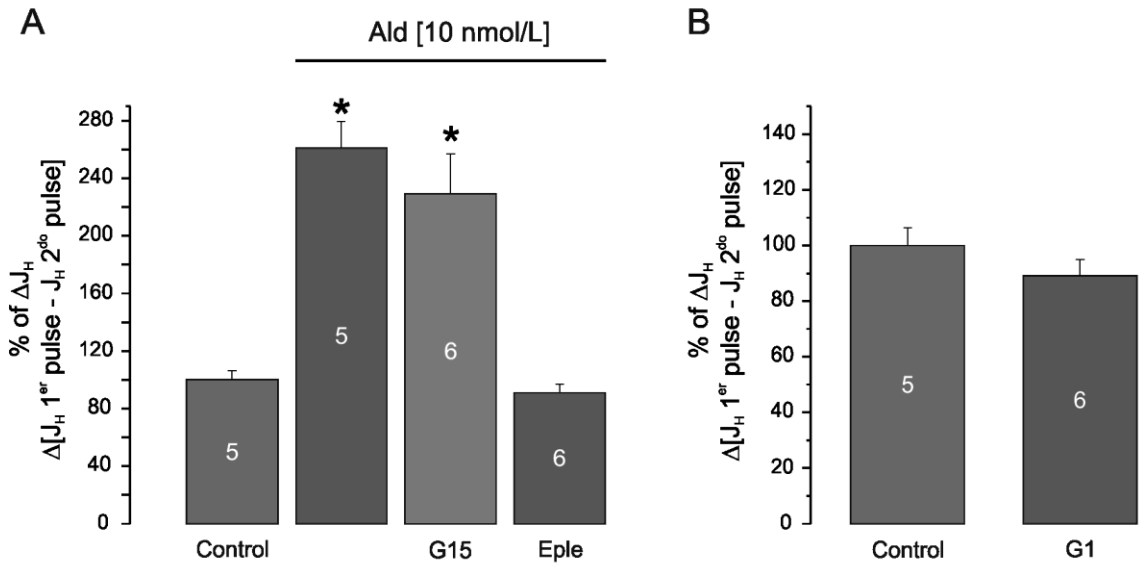
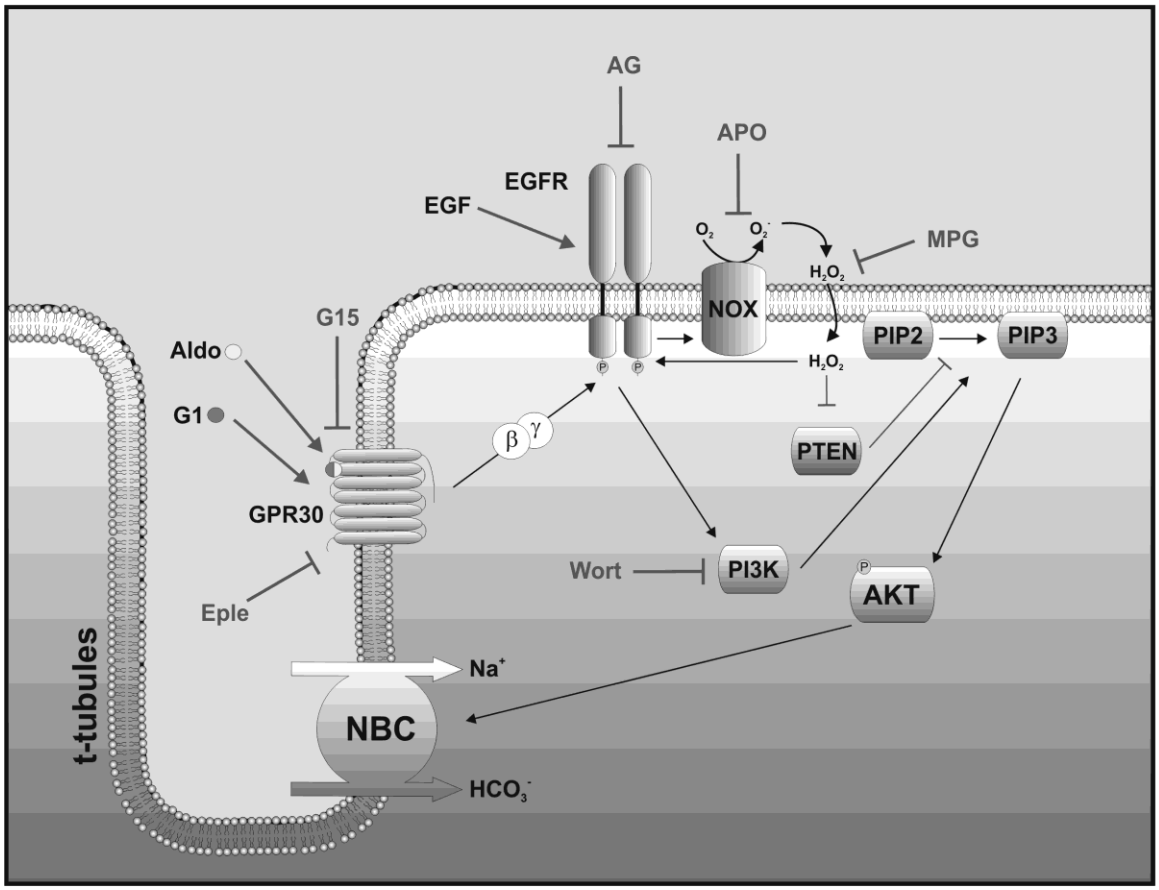
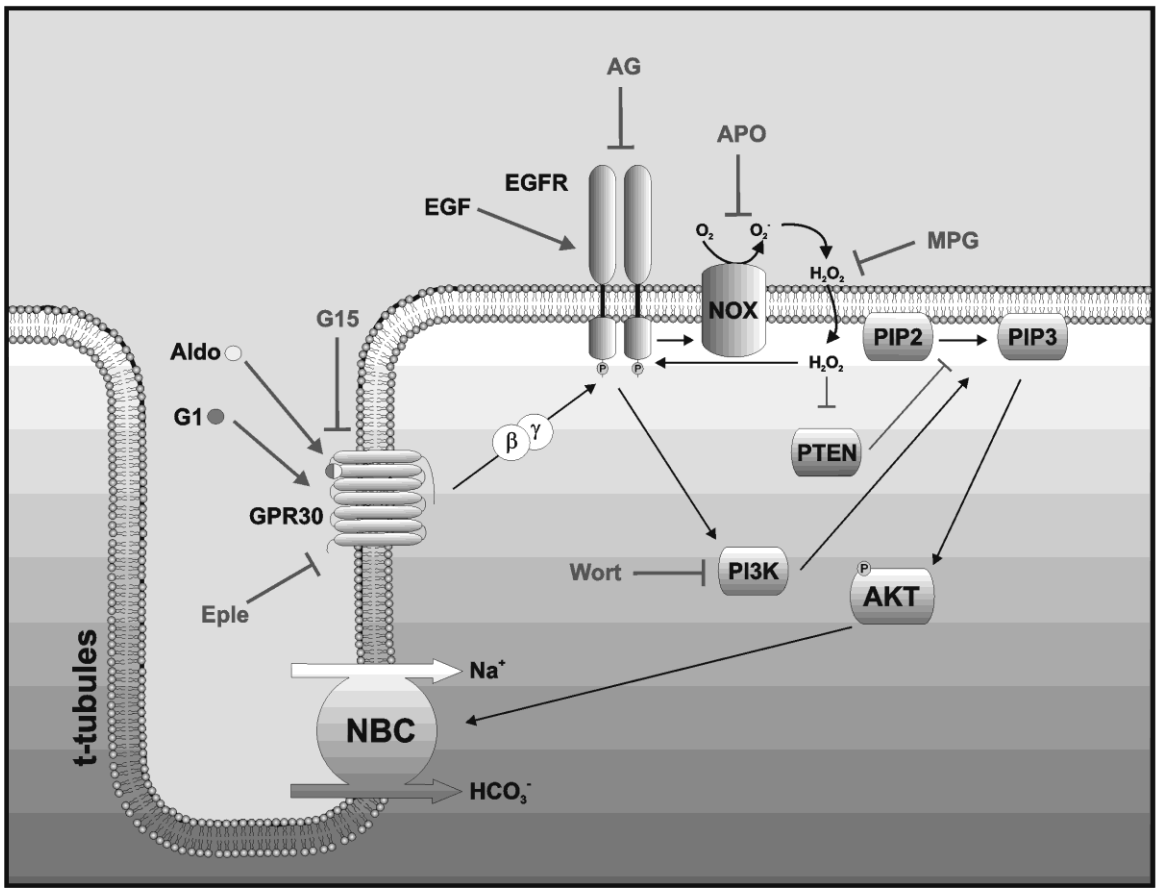


Figure 6



Graphical abstract



Highlights

- ✓ GPR30 appears to be a novel cardiac Aldosterone (Ald) receptor involved in some of the *non-genomic* effects of the hormone.
- ✓ Ald enhanced sodium/bicarbonate cotransporter (NBC) activity by GPR30 activation.
- ✓ GPR30 is involved in Ald-induced ROS production.
- ✓ GPR30 activation leads to the transactivation of the EGFR, which in turn triggers a ROS- and PI3K/AKT-dependent pathway.