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ARTICLE ADDENDUM

The cardiac electrogenic sodium/bicarbonate cotransporter (NBCe1) is activated by aldosterone through the G protein-coupled receptor 30 (GPR 30)

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ABSTRACT

The sodium/bicarbonate cotransporter (NBC) transports extracellular Na^+ and HCO_3^- into the cytoplasm upon intracellular acidosis, restoring the acidic pH_i to near neutral values. Two different NBC isoforms have been described in the heart, the electroneutral NBCn1 ($1Na^+$:$1HCO_3^-$) and the electrogenic NBCe1 ($1Na^+$:$2HCO_3^-$). Certain non-genomic effects of aldosterone (Ald) were due to an orphan G protein-couple receptor 30 (GPR30). We have recently demonstrated that Ald activates GPR30 in adult rat ventricular myocytes, which transactivates the epidermal growth factor receptor (EGFR) and in turn triggers a reactive oxygen species (ROS)- and PI3K/AKT-dependent pathway, leading to the stimulation of NBC. The aim of this study was to investigate the NBC isoform involved in the Ald/GPR30-induced NBC activation. Using specific NBCe1 inhibitory antibodies (a-L3) we demonstrated that Ald does not affect NBCn1 activity. Ald was able to increase NBCe1 activity recorded in isolation. Using immunofluorescence and confocal microscopy analysis we showed in this work that both NBCe1 and GPR30 are localized in t-tubules. In conclusion, we have demonstrated that NBCe1 is the NBC isoform activated by Ald in the heart.

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Introduction

The NBC membrane family proteins are sodium/bicarbonate cotransporters localized in all sarcolemmal membrane zones of the cardiac ventricular myocytes, most notably in the transverse tubules (t-tubules).[1] NBC transports extracellular Na^+ and HCO_3^- into the cytoplasm upon intracellular acidosis, restoring the acidic pH_i to near neutral values. Two different NBC isoforms, the electroneutral NBCn1 ($1Na^+$:$1HCO_3^-$) and the electrogenic NBCe1 ($1Na^+$:$2HCO_3^-$)[2], have been described in the heart. We have previously demonstrated that these NBC isoforms can be independently regulated by angiotensin II, which stimulates the NBCn1 and inhibits the NBCe1.[3,4]

It is important to note that NBCe1 mediates the movement of one Na^+ and 2 HCO_3^- in the same direction, resulting in the influx of one negative charge across the plasma membrane in each complete cycle of transport activity. The NBCe1 current (I_{NBC}) has been characterized in myocardium as an anionic bicarbonate and sodium-dependent current which reversed at around −85 mV.[5,6] Furthermore, the I_{NBC} modulates the shape and duration of the cardiac action potential.

The steroid hormone aldosterone (Ald) plays a classic role acting through mineralocorticoids receptors (MR) located in the cytosol, which act as ligand-induced transcription factors. However, activated MR can also elicit additional non-genomic effects. It has been recently proposed that certain non-genomic effects of Ald were due to the activation of an orphan G protein-coupled receptor 30 (GPR30).[7,8] Consistently, we have recently demonstrated that Ald activates GPR30 in adult rat ventricular myocytes, which transactivates the epidermal growth factor receptor (EGFR) and in turn triggers a reactive oxygen species (ROS)- and PI3K/AKT-dependent pathway, leading to the stimulation of NBC.[9] Nevertheless, the NBC

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isoform involved in the stimulatory effect of Ald remains unknown. Thus, in this addendum to that previous study,[9] we investigated which was the NBC isoform involved in the Ald/GPR30-induced NBC activation.

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 intra-peritoneal injection of sodium pentobarbital (35 mg/kg body weight) and hearts rapidly excised when plane 3 of phase III of anesthesia was reached.

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 μM 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 (mM) 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 2-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.[10]

Ammonium pulse

As described above, the experiments were performed in HCO_3^- buffered solution. Under these conditions, both alkalizing pH_i regulatory systems are operative, the Na^+/H^+ exchanger (NHE1) and NBC. Thus, all the experiments were performed in the presence of the NHE1 inhibitor HOE642 (10 μM) in order to examine the NBC activity in isolation. The total NBC activity was assessed by evaluating the pH_i recovery from a double ammonium pre-pulse (the first was the control of the second pulse). The dpH_i/dt at each pH_i, obtained from an exponential fit of the recovery phase, was analyzed to calculate the net HCO_3^- influx (J_{HCO3-}), then $J_{HCO3-}=\beta_{tot}\, dpH_i/dt$, where βtot is total intracellular buffering capacity. β_{tot} was calculated by the sum of the intracellular buffering due to CO_2 (βCO_2) plus the intrinsic buffering capacity (β_i). βCO_2 was calculated as, $\beta CO_2=2.3\ [HCO_3^-]_i$, where $[HCO_3^-]_i = [\ HCO_3^-]_o\ 10\ pH_i\text{-}pH$.[11,12] 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.

Potassium pulse

To investigate the NBCe1 activity in isolation we performed a potassium pulse as previously described.[2] Increasing isotonically extracellular K^+ $[K^+]_o$ from 5 to 45 mM produced a depolarization of approximately 60 mV that enhanced the electrogenic NBC activity and in turn increased pH_i. The high K^+ was applied for 14 minutes and during this period the pH_i was recorded. The data were expressed as increase of pH_i units in comparison to the zero time point in high K^+ solution. The HCO_3^--buffered solution used in the K^+-induced depolarization experiments contained (mM): 118 NaCl, 5 KCl, 1 $MgSO_4$, 0.35 NaH_2PO_4, 10 glucose, 40 choline chloride, 20 $NaHCO_3$, pH 7.4 after continuous bubbling with 5% CO_2 and 95% O_2. K^+-induced depolarization was assessed by replacing 40 mM choline chloride with 40 mM KCl, maintaining ionic strength.

Immunostaining of cardiac myocytes and analysis by confocal microscopy

Isolated adult rat cardiomyocytes plated on laminin-coated coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline solution, permeabilised with 0.1% Triton X-100 and blocked with 2 % bovine

serum albumin. Myocytes were then incubated with 1:50 dilution of the primary antibody followed by incubation with 1:200 dilution of secondary antibodies coupled to Alexa Fluor 488 donkey anti-rabbit (Invitrogen). In control experiments, samples were incubated with primary or secondary antibody alone. Coverslips were mounted on slides with fluorescent mounting medium (ProLong Gold Antifade, Invitrogen). Images were acquired using an inverted laser scanning microscope (Olympus Bx61). Images were collected with an oil immersion ×60 1.4 objective (numerical aperture 0.2, plan Apochromat, Zoom 1.5×). Excitation/emission wavelengths were 488/nm (argon laser)/500–600 nm.

Statistics

Data were expressed as means ± SEM 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 (2-tailed test).

Results

NBCe1 is the NBC isoform stimulated by Ald and GPR30 activation

We have previously demonstrated that Ald stimulates NBC in adult rat ventricular myocytes, accelerating pH_i recovery during acidosis,[9] as also shown herein in Figure 1A. We performed a double ammonium pulse in HCO_3^- buffered solution in the presence of the blocker of the NHE1 HOE642 (10 μM). Ald (10 nM) was applied to the extracellular solution 10 min before the second pulse. As observed in the representative recordings (Fig. 1A upper panel), the average HCO_3^- influx (J_{HCO3-}) (Fig. 1A lower panel) or the percentage effect of Ald on J_{HCO3-} corrected by the normal attenuation of the recovery rate of the second pulse[9] (Fig. 1C), Ald was able to enhance NBC mediated pH_i recovery from acidosis in a clear significant manner. In order to study if the effect of Ald is due to the stimulation of NBCe1, NBCn1 or both isoforms, we next performed a double ammonium pulse in the presence of a specific and inhibitory antibody against NBCe1 isoform, called a-L3.[2] Figure 1B–C illustrates that the pre-incubation of the myocytes with a-L3 (dilution 1/500) prevented the stimulatory effect of Ald during the pH_i recovery, suggesting that Ald has no effect on NBCn1 and exclusively stimulates the NBCe1 isoform.

In another set of experiments, isolated myocytes were exposed to an isosmotic high extracellular K^+ solution. This hyperkalemic solution induced a depolarization of the membrane potential which selectively stimulated NBCe1 and therefore resulted in cellular alkalization. Figure 2A shows representative traces of continuous pH_i recordings of myocytes exposed to a-L3 (1/500), Ald (10 nM), Ald and G15 (1 μM; antagonist of GPR30) or G1 (1 μM; agonist of GRP30). As observed in the average results (Fig. 2B), Ald increased NBCe1 activity, which was abrogated by the addition of G15. Furthermore, G1 significantly stimulates NBCe1 activity. These results confirm that Ald specifically stimulates NBCe1 isoform through GPR30. As expected, a-L3 canceled the alkalization produced by the membrane depolarization, indicating that the increase in pH_i was due to the stimulation of NBCe1 and confirming that this strategy is useful to study the function of this NBC isoform in isolation.

Co-localization of NBCe1 and GPR30

The spatial distribution of NBCe1 and GPR30 was studied with immunofluorescence and confocal microscopy. Fixed and permeabilised isolated rat ventricular myocytes were incubated with antibodies against the cytoplasmatic domain of NBCe1 (Millipore) or against GPR30 (Abcam) coupled to anti-rabbit Alexa Fluor 488 and confocally imaged. The immunostaining for both proteins showed strong transverse striated pattern (Fig. 3A–B). The normalized longitudinal line-scans (right of panels A-B) displayed oscillatory patterns for NBCe1 and GPR30 with an average in-phase periodicity of ~1.7 μm (Fig. 3C) (Auto TT analysis program[13]). These results strongly suggest a co-localization of NBCe1 and GPR30 in the t-tubules. No labeling was detected in control experiments in which primary or secondary antibodies were omitted (not shown). Figure 3D shows the specificity of NBCe1 and GPR30 antibodies by Western blot of homogenates of rat ventricular myocytes. NBCe1 antibody showed a band at approximately 120 kDa as previously described.[2] On the other hand, GPR30 antibody recognized a predicted major band at ~42 kDa.[14]

Discussion

In our previous study we found that Ald induced stimulation of cardiac NBC via activation of the novel receptor GPR30.[9] Herein, we have identified that the

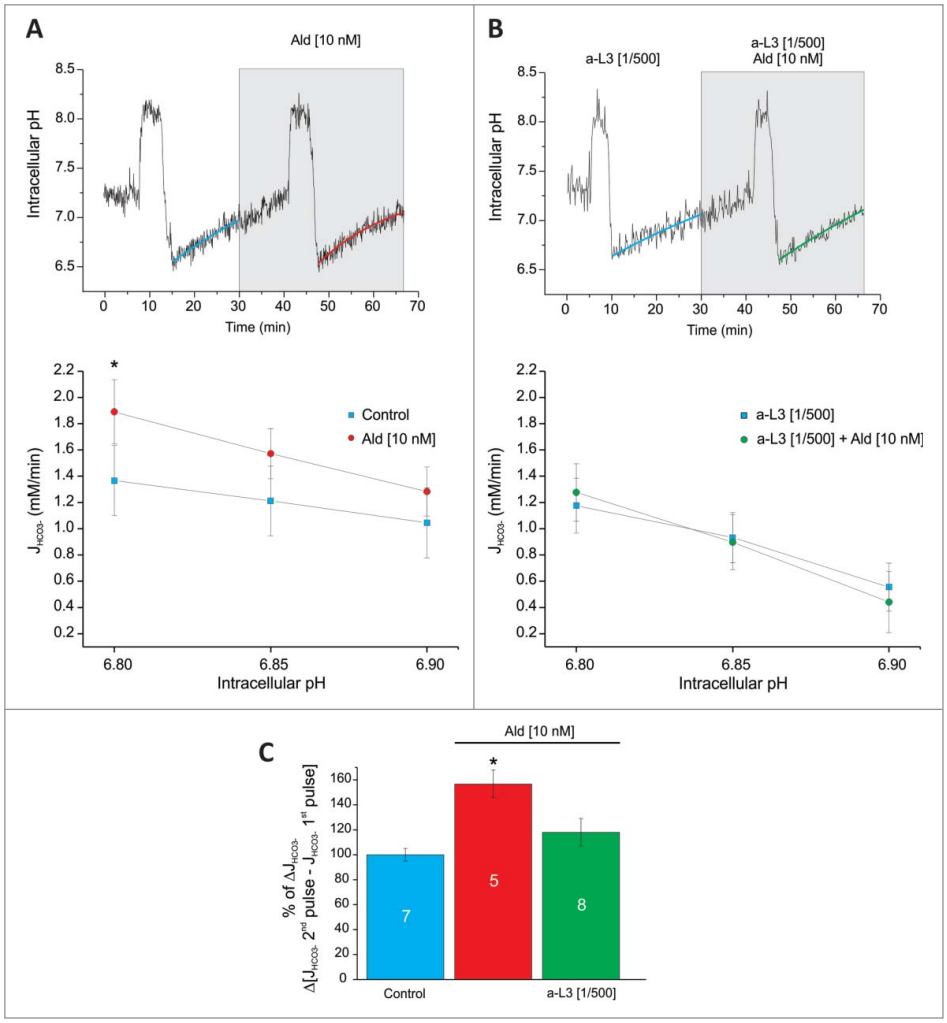

Figure 1. Aldosterone stimulates the electrogenic NBC isoform, NBCe1. (A) *upper panel*: Representative traces of intracellular pH (pH_i) during the application of 2 consecutive ammonium pulses (20 mM NH_4Cl), in the absence (first pulse) and presence of 10 nM aldosterone (Ald; second pulse). *A, lower panel*: Average bicarbonate influx (J_{HCO3-}), carried by the NBC, before and after application of 10 nM Ald. * indicates $P < 0.05$ vs. control. (B) *upper and lower panels*: Same as panel A but in the continuous presence of an inhibitory antibody of NBCe1; a-L3 (1/500). a-L3 was applied 10 minutes before the first pulse and maintained throughout the experiment. (C) Average J_{HCO3-} obtained at pH_i of 6.8 expressed as the percentage of increase in the J_{HCO3-} during the second pulse in comparison to the first pulse (% of ΔJ_{HCO3-}); n values are shown inside bars. * indicates $P<0.05$ vs. control.

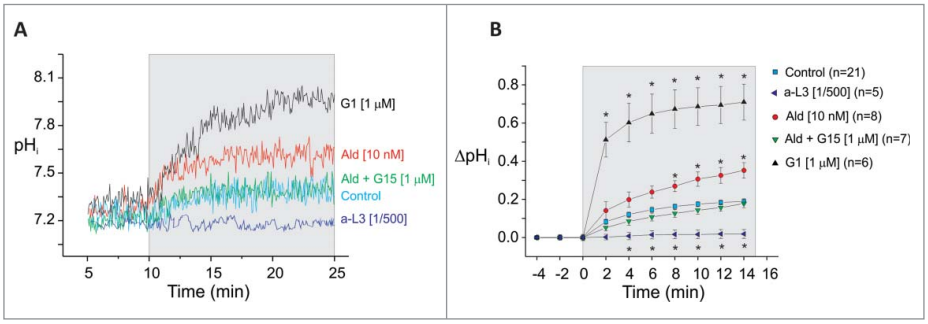

Figure 2. NBCe1 activity is mediated by GPR30. (A) Representative traces of pH_i recorded from isolated ventricular myocytes during exposure to the potassium pulse in the presence of GPR30 agonist (G1, 1 μM), Ald (10 nM), Ald with the GPR30 antagonist (G15, 1 μM) or in the presence of the inhibitory antibody of NBCe1; a-L3 (1/500). (B) Average data of the time course of the effect of pH_i alkalization induced by the hyperkalemic-induced depolarization of membrane potential in control and in the presence of 10 nM Ald, Ald plus the GPR30 blocker G15 (1 μM), G1 (1 μM) or in the presence of a-L3 (1/500). Data are expressed as an increase of pH_i units in comparison to the zero time point in high K^+ solution. n values are shown between brackets. * indicates $P < 0.05$ vs. control.

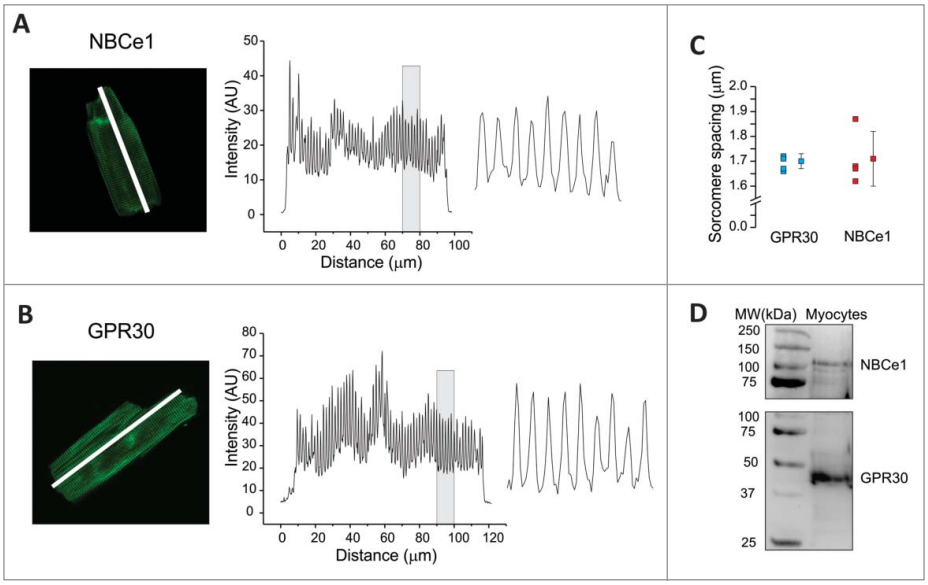

Figure 3. NBCe1 and GPR30 are co-localized in t-tubules. Confocal images of rat ventricular myocytes stained with the antibody against NBCe1 (A) or the antibody against GPR30 (B). Fluorescence intensity profiles were normalized to the peak (arbitrary units, AU) along the longitudinal white line depicted in the images. A fraction of each graph has been expanded on the right for better visualization. (C) Individual and average values of the fluorescent peak spacing, showing no differences in NBCe1 and GPR30 pattern. (D) Typical immunoblots of homogenates of rat ventricular cardiomyocytes showing bands of 120 kDa and 42 kDa, corresponding to NBCe1 and GPR30, respectively.

NBC isoform implicated in such effect is NBCe1. Using specific NBCe1 inhibitory antibodies we demonstrated that Ald does not affect NBCn1 activity. Moreover, Ald was able to increase NBCe1 activity recorded in isolation with the potassium pulse. It was recently reported that NBC is localized in t-tubules while NHE1 is only confined to the intercalated disks.[1] Using immunofluorescence and confocal microscopy analysis we show in this work that both NBCe1 and GPR30 are localized in t-tubules.

It has been reported that the activation of GPR30 is cardioprotective. The administration of G1 reduced the infarct size in ischemia-reperfusion,[15,16] diminished left ventricular wall thickness and myocyte hypertrophy,[17] attenuated heart failure[18] and induced a decrease in perivascular fibrosis.[19] Moreover, the activation of GPR30 inhibited angiotensin II-induced hypertrophy in H9c2 cardiomyocytes.[20]

The NBC is responsible for 30 % of Na^+ influx into the cells during the recovery from acidosis, being the other 70 % due to the NHE1. However, both transporters are equally operative at pH close to basal.[4,21-23] The increase in $[Na^+]_i$ is crucial for cardiac pathophysiology because, as it is well-known, it decreases the driving force of the forward mode (extruding Ca^{2+} mode) of the Na^+/Ca^{2+} exchanger (NCXf) or even favors the reverse mode of this transporter (NCXr), leading to an increase in $[Ca^{2+}]_i$.[24-26] Due to its stoichiometry, the NBCe1 acts as a Na^+-sparing transporter, because it needs half amount of Na^+ to mediate the influx of the same amount of HCO_3^- than the NBCn1. Thus, it could be possible to speculate that the activation of NBCe1 through GPR30 results in a decreased Na^+ uptake upon defending the cell against intracellular acidosis, explaining at least in part the cardioprotective properties of G1 commented above. Additional experiments are needed to elucidate this interesting possibility.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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