

Calcium citrate improves the epithelial-to-mesenchymal transition induced by acidosis in proximal tubular cells

Citrato de cálcio melhora a transição epitélio-mesenquimal induzida por acidose em células do túbulo proximal

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ABSTRACT

Introduction: Epithelial-to-mesenchymal transition (EMT) is a key event in renal fibrosis. The aims of the study were to evaluate acidosis induced EMT, transforming-growth-factor (TGF) β 1 role and citrate effect on it. **Methods:** HK2 cells (ATCC 2290) were cultured in DMEM/HAM F12 medium, pH 7.4. At 80% confluence, after 24 hr under serum free conditions, cells were distributed in three groups (24 hours): A) Control: pH 7.4, B) Acidosis: pH 7.0 and C) Calcium citrate (0.2 mmol/L) + pH 7.0. Change (Δ) of intracellular calcium concentration, basal and after Angiotensin II (10⁻⁶M) exposition, were measured to evaluate cellular performance. EMT was evaluated by the expression of α -smooth muscle actin (α -SMA) and E-cadherin by immunocytochemistry and/or Western blot. TGF- β 1 secretion was determined by ELISA in cell supernatant. **Results:** At pH 7.0 HK2 cells significantly reduced E-cadherin and increased α -SMA expression (EMT). Supernatant TGF- β 1 levels were higher than in control group. Calcium citrate decreased acidosis induced EMT and improved cells performance, without reduction of TGF- β production. **Conclusions:** Acidosis induces EMT and secretion of TGF- β 1 in tubular proximal cells in culture and citrate improves cellular performance and ameliorates acidosis induced EMT.

Keywords: acidosis, citrates, epithelium.

RESUMO

Introdução: A transição epitélio-mesenquimal (TEM) é um evento chave na fibrose renal. Os objetivos do estudo foram avaliar se o citrato seria capaz de reverter a TEM induzida por acidose, e qual seria o papel do fator de crescimento transformador (TGF) β 1 neste evento. **Métodos:** Células de túbulo proximal (HK2) foram cultivadas em meio DMEM-F12, pH 7,4. Após confluência, as células foram distribuídas em três grupos A) controle: pH 7,4, B) Acidose: pH 7,0 e C) Acidose: pH 7,0 + citrato de cálcio (0,2 mmol/L). A variação na concentração de cálcio intracelular, antes e após a adição de angiotensina II (10⁻⁶M) foi medida para avaliar o desempenho celular. TEM foi avaliada pela expressão de α -actina de músculo liso (α -SMA) e E-caderina por imunocitoquímica e/ou de Western blot. A secreção de TGF- β 1 foi determinada por ELISA no sobrenadante. **Resultados:** Em pH 7,0, houve redução significativa na expressão de E-caderina e aumento de α -SMA indicando a presença de TEM e a concentração de TGF- β 1 foi maior do que no grupo controle. O citrato de cálcio melhorou TEM induzida pela acidose e a resposta das células à angiotensina II, sem redução do TGF- β . **Conclusões:** Acidose induz TEM e secreção de TGF- β 1 em células tubulares proximais em cultura e o citrato melhora o desempenho celular e a TEM induzida por acidose.

Palavras-chave: acidose, citratos, epitélio.

INTRODUCTION

Epithelial-to-mesenchymal transition (EMT) is a central mechanism in tubulointerstitial fibrosis¹⁻⁴ the final common pathway to chronic kidney disease. The phenotypic conversion of epithelial cells to

myofibroblast (with expression of α -SMA and less expression of E-Cadherin) is the main feature of this process that plays a key role in excessive matrix deposition in renal fibrosis.⁵ EMT develops in response to environmental stresses and cytokine/growth factors stimuli (eg. TGF- β 1).⁶⁻⁸

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Strategies to block any EMT step would have major impact on attenuating renal fibrosis.

In Wistar rats with 5/6 nephrectomy calcium citrate improves metabolic acidosis, decreases cell proliferation and glomerular/tubular α -SMA expression and interstitial fibrosis.⁹ Citrate is a major component of the tricarboxylic acid cycle that is freely filtered at the glomerulus and reabsorbed in the proximal tubule by a sodium/dicarboxylate cotransporter at the apical membrane and acidosis enhances its reabsorption. Citrate enters the proximal tubular cells and is taken up by the mitochondria and contributes to renal oxidative metabolism.¹⁰⁻¹³ In previous experiment, in HK2 cells in culture, we observed that medium acidification (pH 6.8) induces α -SMA expression (EMT) that was not observed with medium pH 7.4 nor 7.8, and if calcium or sodium citrate were added to culture medium, its pH increased and EMT diminished. We wonder if this change is mediated only by its alkalizing effect or it also depends on another intrinsic citrate cellular action.¹⁴ Cell cultures experiments are widely used as they have many advantages to study specific changes at a unique cellular type, but are acute models and to extrapolate their results to whole organism with chronic pathology have limitations.

The aims of the study were to confirm the effects of acidosis on EMT in human tubular cells HK2, the role of TGF- β as its mediator, and the effect of citrate on this process.

METHODS

CELL CULTURE

Human proximal tubular HK2 cells (ATCC 2290) were cultured in 75 cm² culture flasks (Corning), and 4 well plates slide chambers for immunocytochemistry (Labtek, Nunc) in DMEM/HAM F12 Medium (Sigma) supplemented with 10% bovine fetal serum, 20 mmol/L HEPES and antibiotics (Penicillin-Streptomycin solution, Sigma). At 80% confluence cells were synchronized by 24 hours on serum-free medium, distributed in three groups and kept for 24 hours on experimental conditions: **A) Control:** pH 7.4, **B) Acidosis:** pH 7.0, and **C) Citrate:** calcium citrate (0.2 mmol/L) + pH 7.0. Acidosis (pH 7.0) was obtained by adding HCl to DMEM/HAM F12 without (Group B) or with (Group C) Calcium citrate supplementation (0.2 mmol/L). pH was measured with a pHmeter (Jenco Elec. Ltd. Model 6201). During the assay cell supernatant pH was controlled at 12

and 24 hours in pair flasks and they were 7 ± 0.07 in Groups B and C. Institution Ethics Committee approval was obtained.

IMMUNOCYTOCHEMISTRY (ICC)

HK2 cells were grown on chamber slides and fixed with 96% alcohol after 24 hours in experimental conditions. After washing with PBS (NaCl 0.14M, KCl 0.004M, Na₂ HPO₄ 12H₂O 0.01M), cells were incubated with primary mouse monoclonal antibodies against α -SMA (Dako, N1584) overnight at room temperature, then with the biotinyne-conjugated goat anti-mouse secondary antibody (Dako), and exposed to streptavidine-peroxidase and diaminobenzidine (DAB) following manufacturer's Guide instructions (www.dako.com). Positive and negative controls were made with normal kidney tissue. Positive EMT cells were defined as those with α -SMA expression and fibroblast-like shape. Data are expressed as positive cells/100 cells. Software Image ProPlus was used to count cells/field by optic microscopy (20x) (Nikon, Japan). Score was calculated as the average of 3 chambers (10 fields/chamber). Data are shown as mean \pm SD.

WESTERN BLOT (WB)

Cells were centrifuged, the pellet washed twice (PBS, pH 7.4) and then suspended on lysis buffer containing protease inhibitors before sonication and stored at -20°C until used. Previously protein concentration was determined by Bradford's method. Standard procedures were used for SDS-PAGE electrophoresis and western blot. Protein bands were stained (Ponceau S) and used as loading control. Mouse monoclonal anti- α -SMA antibody (1:1000, Dako) or anti-E-cadherine antibody (1:2000, Dako) were used as primary, and HRP-conjugated goat anti-mouse as a secondary antibody (1:5000, Dako). The membrane was developed with the chromogenic substrate (Supersignal, Pierce). The intensity of bands was compared using software Image J.

QUANTIFICATION OF TGF- β 1 IN CELL SUPERNATANT

The quantification of TGF- β 1 in cell supernatant was done using the R&D Systems, TGF- β 1 Quantikine ELISA kit (cat. N° DB 100) according to the manufacturer's instructions, on a multi-detection microplate reader (Multiskan Ex, Labsystems). We used a correction factor of 0.2 (10 ml of supernatant/50 μ l of cell lysate).

INTRACELLULAR CALCIUM DETERMINATION

[Ca²⁺] measurements were performed in a spectrofluorometer according to Grynkiewicz *et al.*¹⁵ with FURA-2 AM. Intracellular calcium concentration was measured after 24 hs in culture in all groups before (basal) and after Angiotensin II exposition (10⁻⁶ M) (as an evaluation of cell performance). Δ[Ca²⁺] was the difference in calcium concentration between basal and after Angiotensin II exposure.

STATISTICAL ANALYSIS

All data are expressed as mean ± standard deviations. Groups were compared using one-way analysis of variance (ANOVA) with post-test of Newman-Keules. *p* values < 0.05 was considered statistically significant.

RESULTS

MORPHOLOGICAL ALTERATIONS AND ACIDOSIS INDUCED EMT

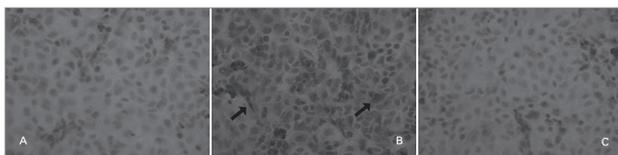
After 24 hours exposure to pH 7.0 in the medium many HK 2 tubular cells acquire a fibroblast-like shape (black arrow in Figure 1B), elongated and spindle shaped. Acidosis induced α-SMA expression, which was significantly higher *versus* Control (A) and Citrate (C) Groups (ANOVA, *p* value < 0.05) (Table 1 and Figure 1 A-C).

TABLE 1 α-SMA STAINING BY ICC AND TGF-β SUPERNATANT CONCENTRATION

Groups	α-SMA(+)/100 Cells	TGF-β (pg/μl)
A- pH 7.4 (Control)	1.71 ± 0.71	16.52 ± 0.43
B- pH 7.0 (Acidosis)	5 ± 0.98 ^a	31.17 ± 4.59 ^a
C- Cit Ca + pH 7.0 (Citrate)	1.12 ± 0.44 ^b	26.78 ± 4.18 ^c

ANOVA: ^a *p* < 0.05 Acidosis vs. Control (αSMA and TGF-β). ^b *p* < 0.05 Citrate vs. Acidosis (α-SMA). ^c *p* < 0.05 Citrate vs. Control (TGF-β). Data are shown as media ± SD (n = 3). ICC: Immunocytochemistry; Cells (+) were defined as those with morphological changes plus α-SMA(+) staining. TGF-β: Transforming growth factor beta. α-SMA: α Smooth Muscle Actin.

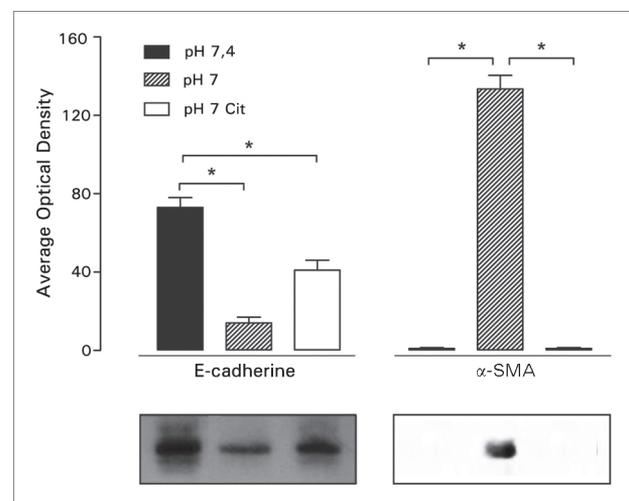
Figure 1. HK-2 cells immunostaining for αSMA (20x). A) Control group (pH7.4); B) Acidosis group (pH 7.0) showed EMT: fibroblast-like shape and expression of α-SMA (black arrow), that diminished with calcium citrate; C) Citrate group. The representative pictures are shown (n = 3).



WESTERN BLOT: INDUCTION OF EMT MARKERS BY ACIDOSIS

Western blot analysis showed overexpression of α-SMA proteins and a decreased E cadherin expression in the cells exposed to pH 7.0. In the Citrate group, the α-SMA expression significantly decreased and E-Cadherin increased when compared to the group exposed to acidosis alone as evidence of less EMT due to citrate action (ANOVA, *p* < 0.05) (Figure 2).

Figure 2. Western Blot analysis of αSMA and E-Cadherin proteins in HK2 cells. A) Control group (pH 7.4); B) Acidosis group (pH 7.0); C) Citrate group (Calcium citrate + pH 7.0) * Anova *p* < 0.05 vs. Control group.



TGF-β1 INSUPERNATANT OF HK2 CELL CULTURE.

TGF-β is described as a major inductor of EMT.¹⁶⁻²³ We observed higher concentrations of TGF-β1 in cell supernatant of HK2 cells exposed to pH 7.0 (Groups B and C) *versus* control group (ANOVA *p* < 0.05). Calcium citrate had no effect on acidosis induced TGF-β1 secretion (NS) (Table 1).

INTRACELLULAR CALCIUM CONCENTRATION

- Basal intracellular calcium concentration did not show differences between groups (ANOVA, NS).
- After Angiotensin II exposure, calcium concentration showed lesser increase in the Acidosis group *versus* Control and Citrate groups. (ANOVA *p* < 0.05) (Table 2 and Figure 3).

DISCUSSION

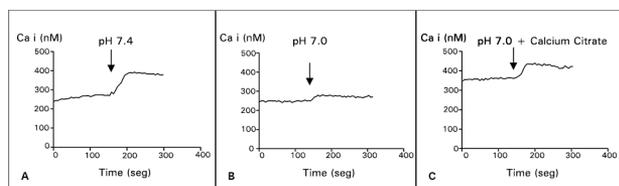
Tubulointerstitial fibrosis is a hallmark of chronic kidney disease (CKD) progression and EMT is a key event on its production. The proximal tubular

TABLE 2 INTRACELLULAR CALCIUM CONCENTRATION WITH FURA-2

[Ca _i] (nM)	A: pH 7.4	B: pH 7.0	C: Cit Ca + pH 7.0
Basal	283 ± 57	255 ± 22.5	251 ± 115
A II (1 × 10 ⁻⁶)	447 ± 91	300 ± 43	380 ± 165
Δ [Ca _i]	144 ± 67	45 ± 22*	129 ± 57#

ANOVA, * $p < 0.05$ Acidosis vs. Control pH 7.4; # $p < 0.05$ Citrate vs. Acidosis pH 7. p NS Citrate vs. Control pH 7.4. Data are shown as media ± SD (n = 3). [Ca_i]: intracellular calcium concentration. All: Angiotensin II.

Figure 3. Intracellular calcium concentration [Ca_i] was determined using FURA 2 in basal conditions, and showed no difference between groups. After Angiotensin II (1 × 10⁻⁶) was added (black arrow at 100-150 msec) cells exposed to pH 7.0 (B) showed a diminished response on increasing calcium concentration *versus* (A) Control group (7.4) or (C) Citrate group (citrate + pH 7).



cell (PTC) is a major potential source of pro-fibrotic growth factors such as TGF-β1^{16,17} and it may acquire a fibroblastic phenotype (myfibroblast), as assessed by the expression of specific markers *in vitro* and *in vivo*,^{18,19} following persistent injury.²⁰⁻²⁹

It has been demonstrated⁹ that citrate ameliorates CKD progression and decreases glomerular/tubular α-SMA expression, so our hypotheses were: a) acidosis induces EMT, b) TGF-β1 may mediate this effect and c) citrate prevents acidosis induced EMT by its alkalizing effect and/or by other mechanisms, such as inhibition of TGF-β1 secretion or direct intracellular actions. We use a cell culture model, in spite of the above mentioned limitations, as it allows to study the effects of acidosis, as a single factor, on proximal tubular cells.

ACIDOSIS INDUCES EMT AND TGF-β1 SECRETION

Metabolic acidosis is frequent in patients with chronic kidney disease and has adverse consequences such as muscle wasting, bone disease and progression of renal failure.³⁰⁻³³ Clinical research has recently demonstrated that bicarbonate or sodium citrate supplementation slows CKD progression.³⁴⁻³⁶ Our data confirm that acidosis induces EMT on tubular renal cell culture: phenotypic changes with α-SMA expression by ICC and enhanced α-SMA and diminished E-cadherin expression by WB. Calcium citrate attenuates it

(Figures 1 and 2). As far as we know, it is the first description that acidosis induces EMT on HK2 cells and that citrate prevents it, on an acute cell injury model.

In order to study the mechanism involved, we measured TGF-β1 supernatant concentration. We found that groups exposed to pH 7.0 secreted more TGF-β1 to cell supernatant than those with pH 7.4 (Table 2). Tian *et al.*¹⁹ had previously demonstrated that addition of recombinant TGF-β1 (10 ng/ml for 2 days) to serum deprived confluent monolayers HK2 cells induced EMT. Acidosis induced TGF-β1 secretion, so it would be a “mediator” in acidosis induced EMT. We expected minor TGF-β1 supernatant concentration in Citrate group, but data did not show a difference. Thus, the attenuation of EMT due to calcium citrate administration, evidenced by lower expression of EMT markers, could not be exclusively explained by a TGF-β dependent mechanism. Additional mediators must be involved as the attenuation of Reactive Oxygen Species (ROS) mitochondrial production by citrate effect on cell energy metabolism.³⁷⁻⁴⁰

CALCIUM CITRATE EFFECTS

As calcium is an important second messenger implicated in multiple intracellular pathways, basal intracellular calcium concentration was measured. No differences were observed between groups, so we ruled out a significant role of calcium. Besides calcium and sodium citrate produced similar results as mentioned before¹⁵, we decided to evaluate only calcium citrate in these experiments, because we considered that it may have more clinical applicability (avoid sodium overload and is “more palatable” considering quality of life of patients).³⁸

As calcium citrate is an alkalizing agent, when it was added to culture medium, pH increased and EMT was not observed. In order to analyze if its beneficial effects on EMT were only mediated by medium alkalization, in the present experiments the Citrate medium was prepared with HCl addition after citrate supplementation in order to titrated final medium pH to 7. As EMT was diminished despite of pH 7 maintenance we conclude that the effect was not only mediated by an alkalizing effect and there may be other pathway involved.

TGF-β1 supernatant concentration was higher in both groups with pH 7 (B and C), without difference between them (Table 1). These data suggest that citrate could antagonize the intracellular effects of acidosis without changes on the TGF-β1 secretion induced by it.

Zhang *et al.*³⁹ have shown in HK2 cells that aldosterone induces EMT via ROS of mitochondrial origin. Citrate is a major component of the tricarboxylic acid cycle and has beneficial effects on oxidative metabolism.⁴⁰ As release of calcium is a cellular energy dependent mechanism, we considered that the improvement in $\Delta[\text{Ca}^{2+}]$ observed in citrate group was a marker of better cellular performance. The effect of citrate on EMT could be associated to an improved cellular energetic metabolism, less production of reactive oxidative species (ROS) and then less EMT. Further experiments directed to determine ROS production by acidosis and its modulation by citrate are on going.

CONCLUSIONS

We conclude that 1) acidosis induces epithelial-to-mesenchymal transition and secretion of TGF- β 1 to the cellular supernatant of HK2 cells; and 2) that citrate improves cellular performance and ameliorates acidosis induced EMT by an action independent of its alkalinizing effect and TGF β 1. So, citrate would be an ideal multipurpose drug to improve CKD prognosis.

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