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SPHINGOSINE-1-PHOSPHATE AS A POTENTIAL REGULATOR OF RHO GTPASES IN KIDNEY PROXIMAL TUBULE CELLS DURING ISCHEMIA

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ABSTRACT

Kidneys play a crucial role in body homeostasis, and impairing their functions can lead to kidney diseases, including acute kidney injury. There are no effective treatments for these patients; thus, new therapies and the identification of early kidney injury biomarkers are needed to reduce morbidity and mortality. Among the possible biomarkers, bioactive lipids emerge due to their function as important mediators of diverse cellular responses. Sphingosine 1-phosphate (S1P), a bioactive lipid (sphingolipid), acts as an extracellular signaling molecule and an intracellular second messenger that plays a role in several important biological functions related to tissue repair. In this work, we verified the ability of S1P to modulate Rho GTPases, indicating a potential role in the maintenance of cellular polarity through the activation of signaling pathways involved in cytoskeleton assembly/remodeling. In an in vitro ATP depletion injury model, we demonstrated that the Rho GTPases (RhoA, Rac1, and Cdc42) genes and proteins were differentially expressed, affecting their activity in the renal cells used in this study. In addition, the pretreatment of the cells with S1P prevented most of the changes observed for the studied Rho GTPases, which suggests that this bioactive lipid preserved the cell polarity machinery control in vitro. In future studies, we would like to evaluate the amount of S1P and other bioactive lipids that potentially participate in the progression of kidney injury or repair, which would potentially identify new lipidic kidney injury and repair biomarkers, a vital tool for the early detection of prevalent nephropathies.

KEYWORDS: Sphingosine 1-phosphate, Rho GTPases, bioactive lipids, renal disease, sphingolipids.

INTRODUCTION

Despite their relatively small volume, kidneys play a crucial role in body homeostasis by regulating salt and water excretion to maintain the volume and composition of the extracellular fluid. The kidneys are constituted by different cell types, including epithelial cells, arranged in an ordered monolayer that present distinct domains: the luminal facing the nephron lumen and the basolateral facing the renal interstitium.[1,2] Due to this polarity, given by the asymmetrical distribution of proteins, transporters, lipids, receptors, etc., coordinated reabsorption and secretion occur, ensuring normal renal physiology. In the epithelium, adherent junctions are essential for developing and maintaining tissue structure and correct cell polarization. The most important component of epithelial adherent junctions is E-cadherin (E-cad) forms a clustering on the surface of epithelial cells to determine cell shape and tissue integrity.[3] The establishment of opposing domains results from a complex network of interactions between evolutionarily conserved proteins.[4,5] A wide range of signaling pathways have been implicated in regulating cell polarity, among them, the Rho GTPases: RhoA, Rac1, and Cdc42. Rho GTPases alternate between two conformations: i. one linked to GDP in its inactive state, where they are sequestered and kept in the cytoplasm, and ii. an active GTP-bound state.[4,6]

The impairment of renal functions gives rise to renal diseases, among them acute kidney injury (AKI), for which there is still no effective treatment.[7] AKI can be triggered by different causes, such as ischemia, and involves loss of cell polarity, implying the involvement of RhoA, Rac1, and Cdc42 in the pathogenesis of kidney diseases. Proximal tubule cells have a high energy demand and an intense supply of oxygen under normal conditions due to greater vascularization of the cortical region of the kidneys.[8,9] Therefore, proximal tubule cells are more severely affected in episodes of ischemic injury, which allowed us to consider an excellent model for studying the mechanisms involved in the progression of this lesion. Among the different lineages of renal tubule epithelial cells available, HK-2 cells, derived from human renal proximal tubules[10] from healthy human kidneys, are among the most commonly used and well-characterized.

Sphingolipids (SLs) are one of the major lipid classes and can exert a myriad of signaling functions, regulating cellular responses crucial for physiological and pathophysiological processes.[11–14] Among the bioactive SLs, sphingosine 1-phosphate (S1P) is a promising molecule for new therapeutic protocols for different diseases, acting either as an extracellular ligand or as an intracellular second messenger in diverse biological functions in health and disease.[15–17] The biological action of S1P is mainly determined by different G-protein coupled receptors (GPCRs) divided into five subtypes (S1PR1–S1PR5), which are coupled to their specific G-protein within the various tissues and cell types.[18] The ability to selectively activate or inhibit S1PR could be a promising approach for treating different diseases. S1P biosynthesis occurs by the de novo sphingolipid pathway or sphingomyelin hydrolysis and plasma sphingosine uptake.[11,15,16] Ceramide, a common S1P precursor either in de novo biosynthesis or during sphingomyelin hydrolysis, is subsequently converted by ceramidase into sphingosine and then to S1P through ATP-dependent phosphorylation in a reaction catalyzed by sphingosine kinases (SKs).[11,15,16] In a previous paper, we showed the ability of isolated kidney basolateral membranes to produce S1P, thus showing the presence of a basolateral membrane-bound SK.[19] Intracellular S1P can also trigger different signaling cascades and thus be converted back to sphingosine by S1P phosphatase or irreversibly metabolized to hexadecenal and phosphoethanolamine through endoplasmic sphingosine 1-phosphate lyase (S1PL). Thus, S1PL represents a key enzyme for regulating cellular S1P levels.[13,18,20]

Some of the S1P receptors are directly coupled to the modulation of Rho GTPases, which are well-known cell polarity regulators. Our group and others had already demonstrated the ability of S1P to protect renal cells from injury, mainly inhibiting cell death, augmenting cell proliferation, and modulating epithelial–mesenchymal transition (EMT).[21–24] There are still few reports in the literature linking S1P benefits to the modulation of Rho GTPases. Thus, the present study evaluated Rho GTPases modulation by S1P in human kidney cells subjected to an in vitro ischemia model. Our work provides new evidence that allows us to include renal cell polarity preservation in the hall of positive hallmarks elicited by S1P treatment.

MATERIALS AND METHODS

Material

K-SFM (Keratinocyte Serum Free Medium), trypsin, penicillin, and streptomycin were from Gibco, and fetal bovine serum (FBS) from Cultilab. Human renal proximal tubule cells of the HK-2 lineage were from ATCC. Sphingosine-1 phosphate, buffers, bovine serum albumin-fatty acids free (BSA-FAF), protease inhibitors and phosphatase inhibitors were from Sigma Chemical Co. Distilled water, deionized using the Milli-Q system of resins (Millipore Corp.), was used to prepare all solutions. Anti-RhoA, anti-Rac-1, and anti-Cdc42 primary antibodies were from Cell Signaling Technology (CAT# 2117S, 2465S, and 2466S, respectively). Anti-rabbit IgG secondary antibody (NA 934) and anti-β-actin (A5441) were from Sigma Chemical Co. All other reagents were of the highest purity available. Nitrocellulose membranes (Hybond) and the ECLTM system were from GE Healthcare.

Cell cultures

HK-2 cells were cultured in K-SFM medium with 1% penicillin and streptomycin and 2% FBS. Replacing was carried out every 4 days, with the culture showing 80% confluence, using 0.25% trypsin. Cells were maintained in a stove with atmospheric air and 5% CO_2 at 37 °C.

In vitro ischemia model (ATP depletion) and S1P treatment

Our group has been using for more than a decade the well-established in vitro ATP chemical depletion, also referred to as the best in vitro model to study ischemic injury [35,43,44], which is based on the use of Antimycin A, an inhibitor of complex III of the electrons transport chain, leading to ATP depletion [45]. This is a protocol widely used and indicated to promote injury to the proximal tubule kidney cells that are quite reliable to the cellular effects observed in acute renal ischemia *in vivo*. Therefore, we used the same ischemic protocol that was 10 μM AA incubation for 1.5 h, as recently submitted for publication (Assis et al., 2024, in press).

Briefly, HK-2 cells were plated ($1x10^8$ cells) for experiments and cultured under standard conditions. We used cultures with 80-90% confluence in the experiments. Kidney cells received serum-free K-SFM medium. The following day, to analyze the effect of S1P, this lipid, solubilized previously in BSA-FAF, was added to serum-free K-SFM at the concentration of 1 μM for 1 hour before treatment with antimycin A. Then, all the culture medium containing S1P was removed, and the cells were washed twice with PBS (Phosphate-buffered saline). Posteriorly, they were incubated with the ischemic buffer, which consisted of: HBSS (Hank's Balanced Salt Solution), antimycin A 10 μM and DOG 1 μM (2-Deoxy-D-glucose, a glucose analog, to inhibit glycolysis); for 90 minutes in an stove at 37 °C with 5% CO_2. This combination of inhibitors prevents substrate oxidation, leading to almost complete exhaustion of ATP. Control cells were maintained in serum-free K-SFM during the treatment period of cells with antimycin A. Immediately after the incubation time in ischemic buffer, HK-2 cells were processed according to the protocol of interest.

Protein determination

Protein determination assays were performed using the Folin phenol method.[46] Bovine serum albumin was used as the standard.

SDS-PAGE and Western Blotting

Protein extraction was performed using 300 µL of RIPA buffer (1% NP-40, 1% Triton, 1% Sodium Deoxycholate, 10 mM Tris-HCl pH 7.5, 100 mM NaCl, and 0.1% SDS in deionized water) plus 10% protease and phosphatase inhibitors for each 75 cm^2 bottle. The cell homogenates were aliquoted and stored at -20 °C. SDS-PAGE was performed on a 12% polyacrylamide gel at 60 mA according to the Laemmli protocol (1970). The protein homogenate (60 µg) was separated and transferred to nitrocellulose membranes that were blocked in 5% milk skimmed with 0.1% Tween 20 for 2 hours. Then, the membranes were incubated with anti-RhoA, anti-Rac-1, and anti-Cdc42 primary antibodies (all diluted 1:1000) for 90 min. The membranes were washed three times with TBS containing 0.1% Tween 20 for 5 min each and subsequently incubated with anti-rabbit IgG secondary fluorescent antibodies (diluted 1:5000) for 90 min. The membranes were revealed in the Bio-Rad ChemiDoc XRS. β-actin was used as loading control, according the manufacturer's instructions.

Total RNA extraction

For RNA extraction, the DirectzolTM RNA MiniPrep Plus kit (Zymo) was used following the instructions provided by the manufacturer. The concentration of RNA extracted was evaluated by reading the absorbance at 260 nm in a NanoDrop Microvolume Spectrophotometer (Thermo Fisher Scientific). The purity was assessed by the ratio of the OD readings at 260/280 nm. RNA was stored at −80 °C until use.

First-strand cDNA synthesis

The first strand of cDNA was synthesized using the enzyme Improm-II Reverse Transcriptase (Promega) in the presence of oligodT (Promega), following the manufacturer's instructions.

Quantitative RT-PCR (qRT-PCR)

The qRT-PCR reactions were carried out on Quantitative Studio Real-Time PCR System (Applied Biosystems), using previously generated cDNA for the first strand. The reactions were carried out using the GoTaq RT-PCR Master Mix kit (Promega), with primer concentrations listed in Table I, 2.7.5 µL of SYBR green PCR master mix, 1 µL of cDNA, and nuclease-free water (Promega), with a final volume of 15 µL. Initially, the amplification efficiency between the target genes and the endogenous genes, GAPDH, β- actin, and RPL22 - ribosomal protein L22 (all the presented data normalized by GAPDH), was satisfactorily verified through an efficiency curve. The specificity of the amplicon was evaluated by the presence of a single dissociation curve after the RT-PCR run. All RT-PCR experiments were performed in triplicate. Relative gene expression analysis was performed using the program Quant Studio (Applied Biosys-tems) using the ΔΔCT method.

Immunoprecipitation Assay for Active Rho GTPases

To evaluate the activation of Rho GTPases, Cdc42, Rac1, and RhoA, we used the commercial kit from Cell Biolabs Inc. in accordance with the manufacturer's instructions. This kit uses colored agarose beads attached to the Rotekin RBD and PAK PBD to selectively isolate and immunoprecipitate the activated RhoA, Rac1, and Cdc42 from HK-2

cells (RhoA specifically binds to the RBD domain of Rotekin and Rac1 or Cdc42 to the PBD domain of PAK). The immunoprecipitate only contains active GTP-bound RhoA.

Confocal Microscopy and Immunofluorescence

Cytoskeleton morphology was evaluated using confocal fluorescence microscopy. For actin imaging, a 1:200 v/v solution of Alexa Fluor 555 Phalloidin was used (2h incubation time). Cell nuclei stained with DAPI solution added for 15 minutes.Image stacks were obtained through Z-Stack and later edited in ImageJ.

Statistical Analysis

SDS-PAGE and Western Blotting experiments were performed in duplicates (n = 5), using the one-way ANOVA test for statistical analysis with the Dunnett post-test. The results are presented as mean ± standard error. Molecular biology data were analyzed by one-way ANOVA using the Prism 5 software (GraphPad, San Diego, CA, USA), with Bonferroni post-test. Data are expressed as the mean of three independent experiments, and the significance level was set at $p < 0.05$.

RESULTS

HK-2 cells submitted to ischemia presented an altered shape and loss of cell polarity

Ischemic acute renal failure has been shown to induce apical membrane rupture by a collapse of microvilli, leading to a loss of cell polarity.[25] In addition, renal ischemia reduces cell adhesion, leading to reorganization of the actin cytoskeleton, being the actin network an important regulator of cell morphology. Thus, we decided to investigate whether Rho GTPases would be affected in cells submitted to ATP depletion, and if the pretreatment of the cells with S1P would preserve its expression and activity. The experimental approach was to study alterations in gene expression, cellular levels, and activity of the three main Rho GTPases, RhoA, Rac1, and Cdc42. RT-PCR, SDS-PAGE immunodetection, and immunoprecipitation for each of the Rho GTPases were performed.

ATP depletion and S1P pretreatment effects on RhoA

RhoA is one of the main controllers of cytoskeleton stability in epithelial cells, which implies its involvement in establishing and maintaining cell polarity.[26,27] Figure 1 clearly shows that HK-2 cells subjected to ATP depletion injury significantly increased RhoA gene expression by almost 100%, which leads us to speculate that this Rho GTPase could be implicated in the very early stages of the lesion. Interestingly, control cells pre-incubated with S1P showed an even more robust increase in RhoA expression (3× greater, $p<0.05$). In contrast, those cells pretreated with S1P and subjected to ATP depletion injury displayed RhoA expression levels similar to control cells (Figure 1). According to Figure 1, even though the relatively short incubation period with AA (1.5 h), it elicited a cellular response to prevent the loss of polarity and adhesiveness, which is, at least in part, prevented by S1P. An increase or decrease in mRNA levels of a given protein in a given time interval does not guarantee that the levels of the studied protein have been changed accordingly. To access it, cells were processed for SDS-PAGE and immunodetection using the anti-RhoA antibody. The result presented in Figure 2 reflects the cellular levels, or availability of RhoA in HK-2 cells, through the different treatments, being the data consistent with those in Figure 1. There is an increase in RhoA levels in cells undergoing ATP depletion injury, which does not occur when cells are pre-incubated with S1P (compare the second with the fifth bar). It is also evident that there is a greater availability of RhoA in cells pretreated with S1P in control conditions. This tendency for RhoA to increase under the same experimental conditions where an increase in mRNA

for this Rho GTPase was observed may reflect that the phenomenon has not reached a maximum effect yet. We suppose that these differences in RhoA availability would be statistically different at longer times after injury.

To evaluate a third possible action point for S1P, we studied the activation of RhoA using the well-described "pull-down" assay (see Methods). Figure 3 shows that the cells subjected to ATP depletion have less than half the activated RhoA content and that the pre-treatment of HK-2 cells with S1P prevented this drop in RhoA activity. The visualization of the 21 kDa band in the Ponceau red stain was quite clear and specific, as there are no other proteins that complex with rotekin, as specified by the kit's manufacturer. Appropriate positive controls supplied by the manufacturer worked as expected (CT P in the “pull down” images. Negative controls also worked as expected but these data are not shown).

ATP depletion and S1P pretreatment effects on Rac1

Continuing the study, we started to focus on Rac1. Figure 4 shows that during the experimental period (1.5 h), there was no significant effect on Rac1 mRNA expression when HK-2 cells were subjected to ATP depletion. The only change in the expression pattern for Rac1 mRNA was a 2-fold increase in control cells pretreated with S1P. It is evident that in the experimental times tested here, no cell response led to a change in the Rac1 mRNA expression profile.

Then, we check the availability of Rac1 in HK-2 cells through the different treatments. Figure 5 shows almost a 50% increase in Rac1 levels in the injured cells compared to control. Interestingly, this effect was absent when HK-2 cells are pretreated with S1P. Here, it should be remembered that, in the same period of the experiment, there was no significant change in mRNA levels for Rac1 (Fig. 4).

Lastly, we evaluated whether Rac1 activity was altered in the different experimental conditions using the same commercial kit (Fig. 3). Figure 6 shows that closer to that observed for RhoA, the ATP depletion injury causes a reduction in Rac1 activity, which is completely prevented by the pretreatment of HK-2 cells with S1P. Interestingly, control cells incubated with S1P also show reduced Rac1 activity. This result suggests that these Rho GTPases are inhibited at the early stages of the injury and have their activity fully preserved when the cells are preincubated with S1P.

Effect of ATP depletion injury and S1P pretreatment in Cdc42

Cdc42 plays important roles in regulating different cell processes such as adhesion, remodeling of the actin cytoskeleton, and cell polarity.[29,32]

Here we show that the ATP depletion injury does not alter Cdc42 expression, while S1P pretreatment significantly increased Cdc42 mRNA content either in control or injured cells (Fig. 7). On the other hand, when we assessed the availability of this Rho GTPase under the different experimental conditions, we observed a 1.5-fold increase in the cells subjected to injury (Fig. 8). These increased levels of Cdc42 were equivalent to those in the cells pre-treated with S1P, with or without the ATP depletion injury. Using the same protocol described for RhoA and Rac1, Cdc42 activity was assessed. Figure 9 clearly shows no changes in Cdc42 activity in the different experimental conditions tested.

The results presented here show that the three Rho GTPases studied are differentially affected in the injury process by ATP depletion and also in the protective cell effort driven by the S1P pretreatment. This short period for lesion

establishment was sufficient to include the Rho GTPases in the early events of the ischemic lesion and exposes how complex this network of events can be, with the Rho GTPases in a key role for modulating the dynamics of the actin cytoskeleton, the stability, and maintenance of intercellular junctions, therefore accounting for the maintenance of proximal tubule cells architecture, polarity and thus, physiology.

Confocal immunofluorescence detection of cytoskeletal changes

To directly assess morphological alterations in HK-2 cells submitted to the different experimental conditions, focusing on cytoskeletal remodeling, we used immunofluorescence for actin fibers, which allowed us to easily detect altered patterns of the cytoskeleton under injury comparing to those in the cells pretreated with S1P. Figure 10 shows the normal actin fibers distribution (red), with a peripheral presence that seems more intense in points that probably refer to focal adhesions. It is also possible to see a perinuclear net of actin fibers supporting the characteristic epithelial cell shape. On the other hand, cells submitted to injury are not as numerous as expected and presented a round shape and small volume when compared to the control. The actin fibers appear condensed in the perinuclear site, which correlates with the round-shape morphological alteration and strongly suggests a complete loss of normal cell polarity. S1P pretreatment of the cells preserved, at least in part, the assembly of the actin cytoskeleton. Interestingly, control cells pretreated with S1P displayed a significative cytoskeleton rearrangement mainly in the cell periphery, suggesting an intensification in the cell-to-cell contact, an important fate in the correct establishment of normal cell polarity and physiology.

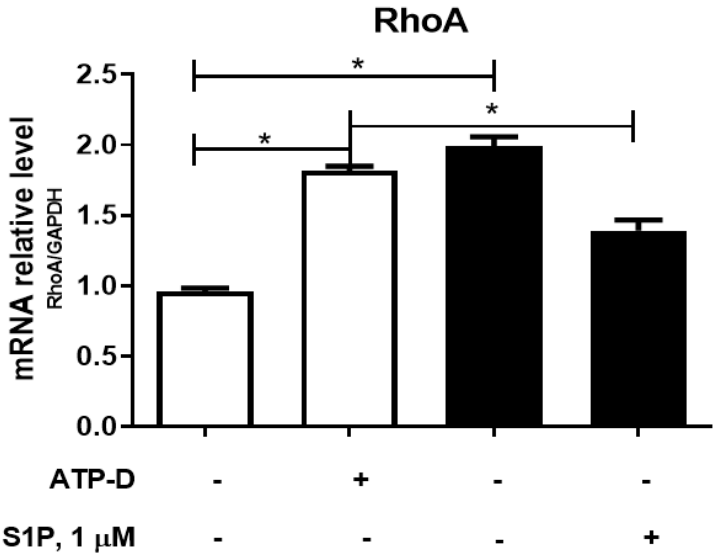

Figure 1: S1P prevents the upregulation of RhoA expression in HK-2 cells subjected to in vitro ischemia model (ATP depletion). HK-2 cells (90% confluence) were cultured in the different experimental conditions depicted in the abscissa, as described in Material and Methods. RhoA transcript levels were measured by qRT-PCR and normalized to GAPDH transcript levels. After the experiments, mRNA was extracted and processed for RT-PCR analysis using the primers for RhoA and GAPDH (as endogenous control). The data were analyzed by One-way ANOVA using Prism 5 software, with Bonferroni post-test. Results are expressed as mean ± standard error of three independent experiments (n=3) done in triplicate.

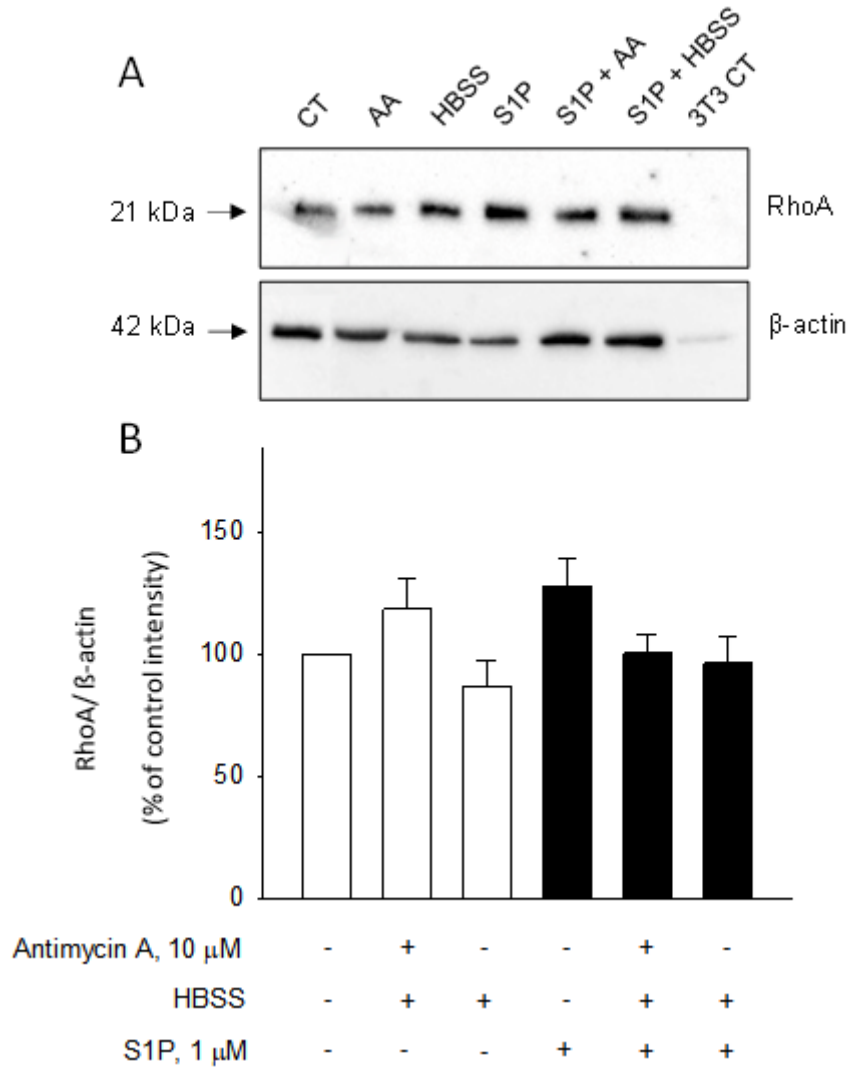

Figure 2: RhoA protein levels in HK-2 cells are similar to the pattern observed for the mRNA levels following injury/protection. HK-2 cells were cultured under the different experimental conditions described in Material and Methods. Cells were processed for protein analysis by SDS-PAGE and immunodetection for Rho A. **A.** Upper panel: detection of the 21 kDa band referring to RhoA in the different experimental conditions indicated at the top of the image. 3T3 fibroblast homogenates were used as a positive control. Bottom panel: β-actin immunodetection, used as a loading control for each sample tested. **B.** Graph represents the mean ± SEM of the ratio between the densitometric intensity of the RhoA band and the β-actin band. SDS-PAGE and Western Blotting experiments were performed in duplicate (n = 5), and a one-way ANOVA test with Dunnett's post-test was utilized for the statistical analysis (performed by the SigmaPlot program). No significative difference was observed.

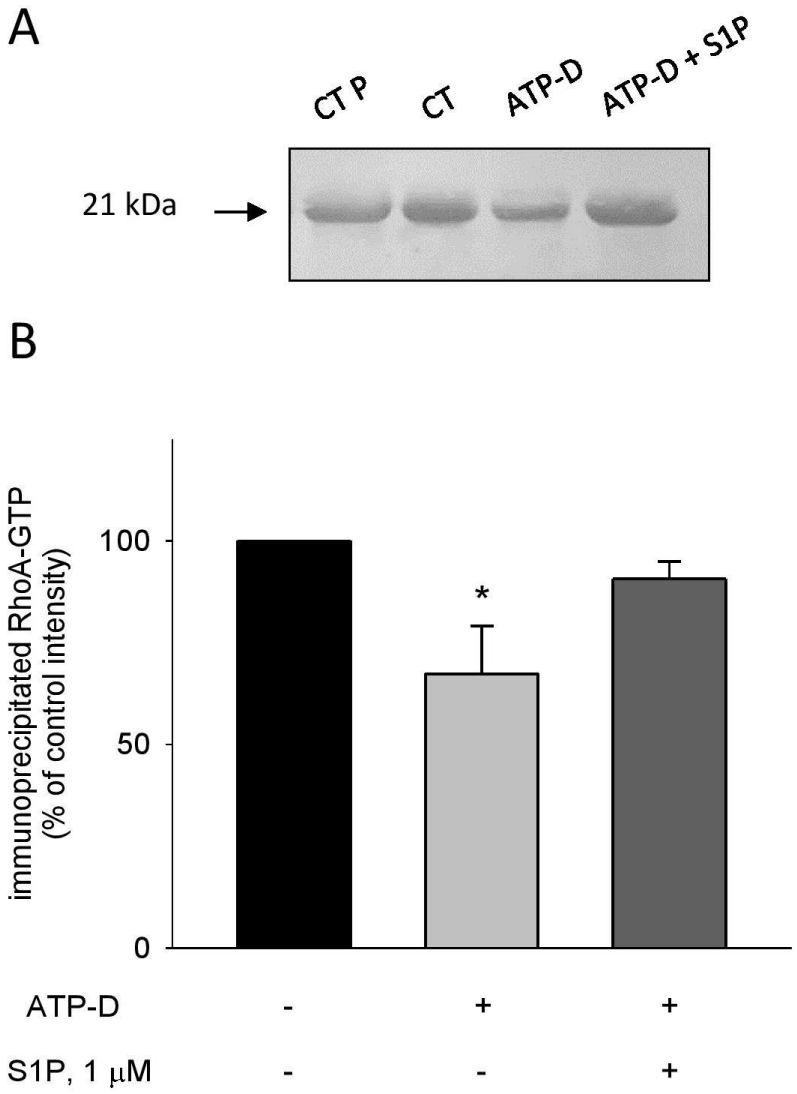

Figure 3: Pretreatment of HK-2 cells with S1P prevents the reduction of RhoA activity observed after ATP depletion. HK-2 cells were cultured under different experimental conditions to 90% confluence. Whole-cell homogenates were individually submitted to a RhoA "pull down" assay with the specific kit (Cell BioLabs), as also described under Material and Methods. The assay explores the affinity of GTP-bind-activated RhoA to interact with its cellular partner, rotekin, being this protein immobilized in agarose spheres. Once the RhoA/rotekin immunoprecipitation is done, it is followed by a step of discarding the beads before resuspending the immunoprecipitate in sample buffer for SDS-PAGE. **A**. Representative image of the detection of RhoA (21 kDa) by staining the nitrocellulose membrane with Ponceau red. CT P, positive control provided by the manufacturer. **B**. Densitometric analysis of the bands visualized with Ponceau red staining. Graph represents the mean ± SEM of the densitometric intensity of the RhoA band. SDS-PAGE and Western Blotting were obtained from three independent experiments (n = 3), and a one-way ANOVA test with Dunnett's post-test was utilized for the statistical analysis (performed by the SigmaPlot program). * Statistically different, $p<0,01$.

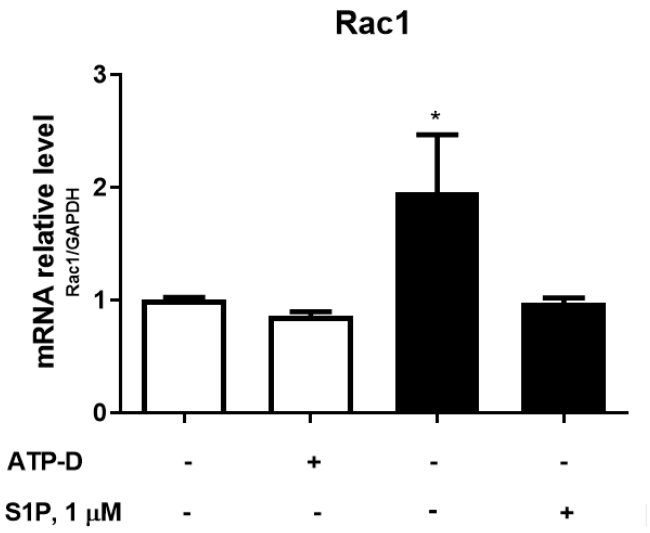

Figure 4: ATP depletion injury does not alter Rac1 expression. HK-2 cells (90% of confluence) were prepared as described in the Material and Methods. qRT-PCR measured Rac1 transcript levels normalized to GAPDH transcript levels. Results are expressed as mean ± SEM of three independent experiments (n = 3). Statistical data analysis was obtained by one-way ANOVA with Bonferroni post-test, using Prism 5 software. *Statistically different, p<0,01.

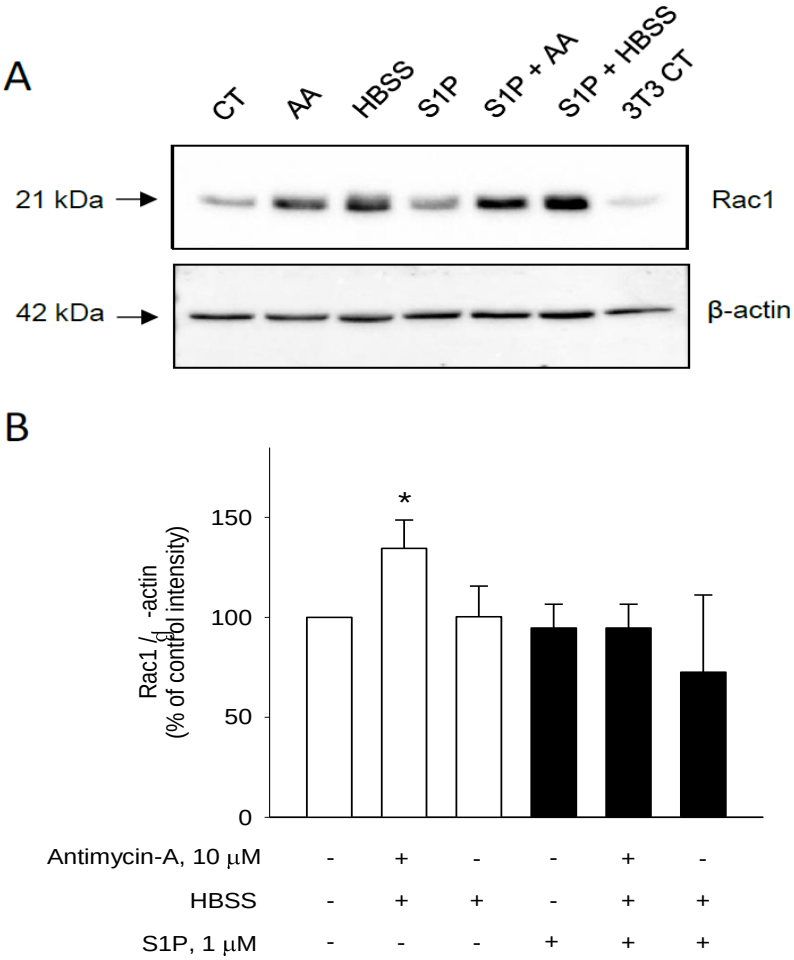

Figure 5: S1P pretreatment prevents increased Rac1 protein levels in HK-2 cells submitted to ATP depletion injury. HK-2 cells were cultured under the different experimental conditions de-scribed in Material and Methods. Afterward, the cells were processed for protein analysis by SDS-PAGE and immunodetection for Rac1 and β-actin. **A.**

Top panel: detection of the 21 kDa band referring to Rac1 in the different experimental conditions indicated at the top of the image. 3T3 fibroblasts were used as positive control. Bottom panel: β-actin immunodetection, used as a loading control for each sample used. **B.** Graph represents the mean ± SEM ratio between the densitometric intensity of the Rac1 band and the β-actin band. The SDS-PAGE and Western Blotting experiments were carried out in duplicates (n = 5), using the one-way ANOVA test followed by Dunnett's post-test for the statistical analysis (performed using SigmaPlot software). * Statistically different, p<0,01.

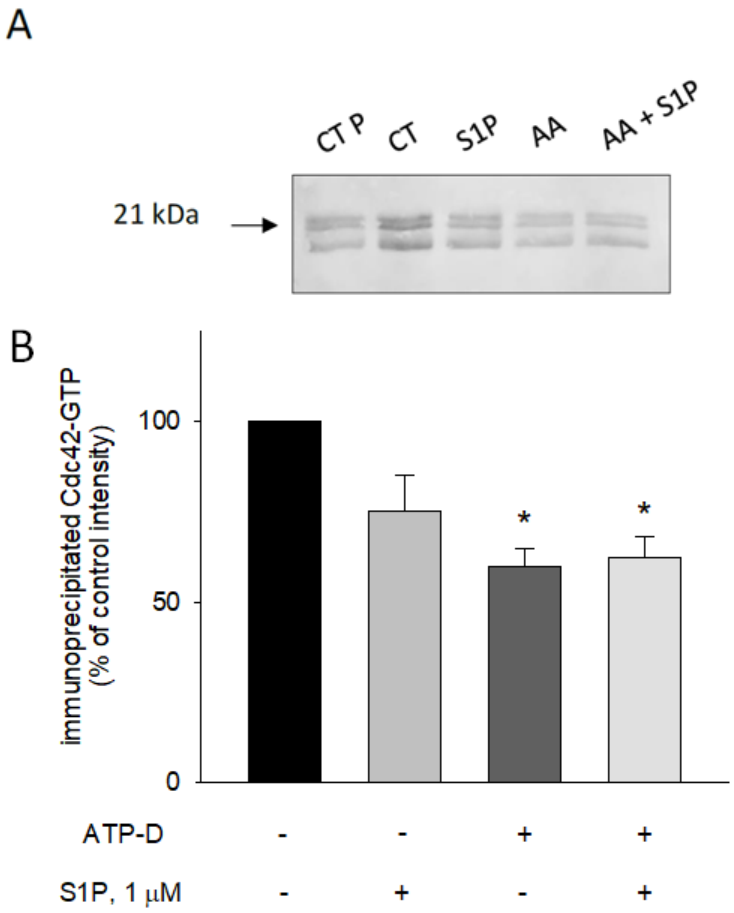

Figure 6: Pretreatment of HK-2 cells with S1P prevents the reduction of Rac1 activity observed in ATP depletion assay. HK-2 cells were cultured in different experimental conditions at 90% confluence. Whole-cell homogenates were submitted to a "pull down" assay to identify GTP-bind-Rac1 activated using a specific kit (Cell BioLabs), as described under Material and Methods. The commercial kit explores the affinity of GTP-bound activated Rac1 to interact with its cellular partner, the PAK protein, which is immobilized in agarose spheres. After immunoprecipitation of Rac1/PAK, the agarose spheres are washed away, and the immunoprecipitated solubilized in sample buffer for SDS-PAGE. **A.** Representative image of the detection of Rac1 (21KDa) by staining the nitrocellulose membrane with Ponceau red. CT P, positive control provided by the manufacturer. **B.** Densitometric analysis of the bands visualized with Ponceau red staining. Graph represents the mean ± SEM of the densitometric intensity of the Rac1 band. SDS-PAGE and Western Blotting were obtained from three independent experiments (n = 3), and a one-way ANOVA test with Dunnett's post-test was utilized for the statistical analysis (performed by the SigmaPlot program). *Statistically different, p<0,01.

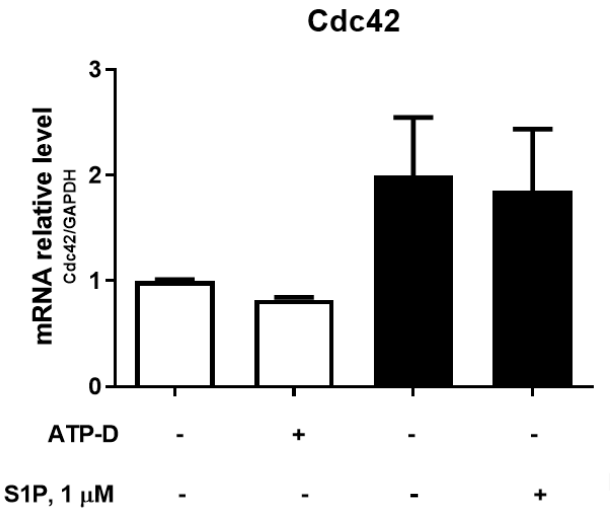

Figure 7: ATP depletion injury does not alter Cdc42 expression, while S1P pretreatment significantly increased Cdc42 mRNA content. HK-2 cells (90% of confluence) were cultured as de-scribed in Material and Methods. Cdc42 transcript levels were measured by qRT-PCR normalized to GAPDH transcript levels. The results are expressed as mean ± SEM of three independent experiments (n = 3) performed in triplicate. Statistical analysis was performed by one-way ANOVA, with Bonferroni post-test using Prism 5 software. No significant difference was observed.

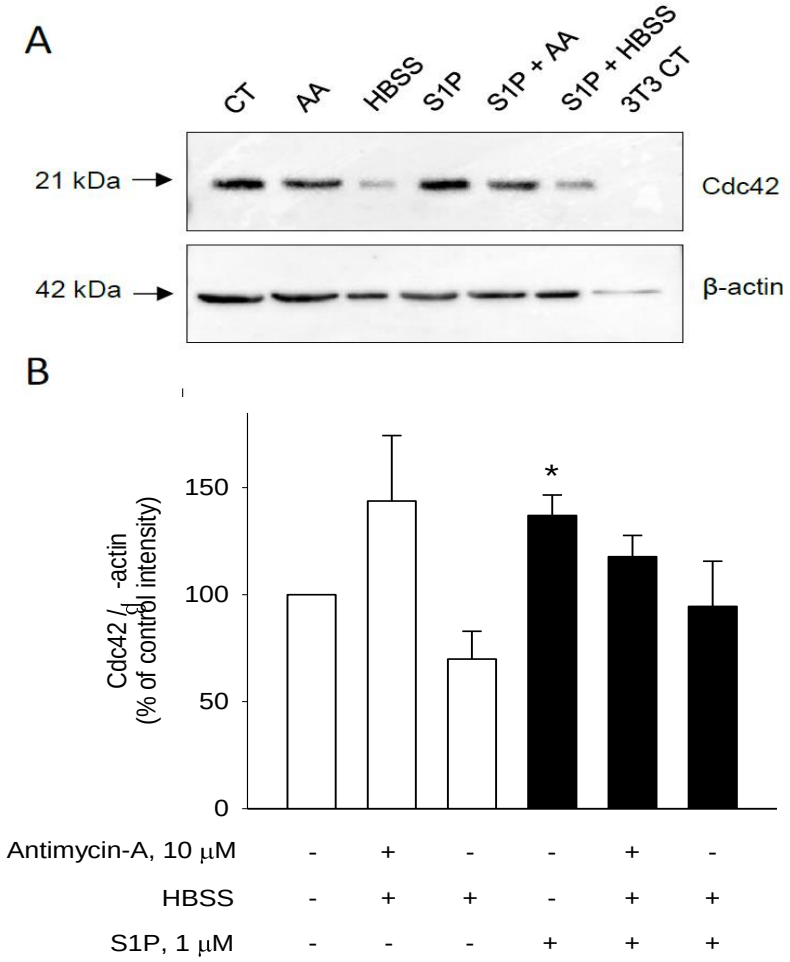

Figure 8: S1P pretreatment does not prevent the increase in Cdc42 levels in HK-2 cells subjected to ATP depletion injury while increasing the levels of this Rho GTPase in cells cultured in control or injury conditions.

HK-2 cells were cultured under the different experimental conditions described in Material and Methods. After the experiments, whole-cell homogenates were processed for protein analysis by SDS-PAGE and immunodetection for Cdc42 and β-actin. **A.** Upper panel: detection of the 21 kDa band referring to Cdc42 under the different experimental conditions indi-cated at the top of the image; bottom panel: β-actin immunodetection, used as a loading control for each sample. **B.** Graph represents the mean ± SEM of the ratio between the densitometric intensity of the Cdc42 band and the actin band from five different experiments performed in duplicate (n = 5). Statistical analysis was performed using the one-way ANOVA test with the Dunnett post-test (performed in SigmaPlot software).

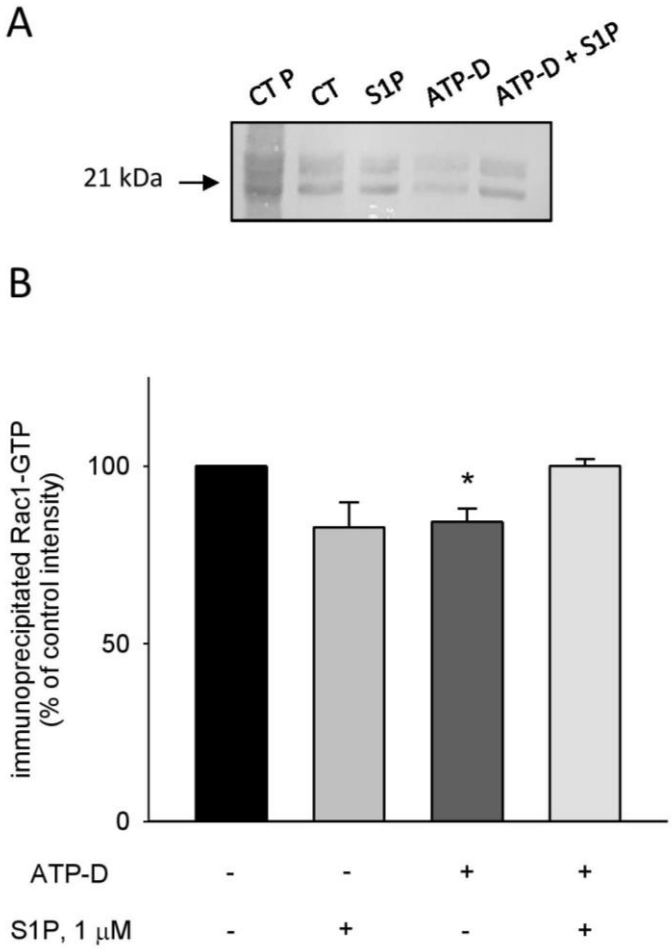

Figure 9: Cdc42 activity is inhibited after ATP depletion and not prevented by S1P pretreatment. HK-2 cells (90% confluence) were cultured in the different experimental conditions described in Material and Methods. Whole-cell homogenates were submitted to a "pull down" assay using a specific commercial kit (Cell BioLabs) to immunoprecipitate GTP-Binding activated Cdc42. The kit explores the affinity of activated Cdc42 to its intracellular partner, the PAK protein, which is immobilized on agarose spheres. After the pull-down, the agarose spheres are washed away, and the immunoprecipitated is solubilized in sample buffer for SDS-PAGE. **A.** Representative image of the detection of Cdc42 (21KDa) by staining the nitrocellulose membrane with Ponceau red. CT P, positive control provided by the manufacturer. **B.** Densitometric analysis of the bands visualized with Ponceau red staining. Graph represents the mean ± SEM of the densitometric intensity of the Cdc42 band. SDS-PAGE and Western Blotting were obtained from three independent experiments (n = 3), and a one-way ANOVA test with Dunnett's post-test was utilized for the statistical analysis (performed by the SigmaPlot program). *Statistically different, p<0,01.

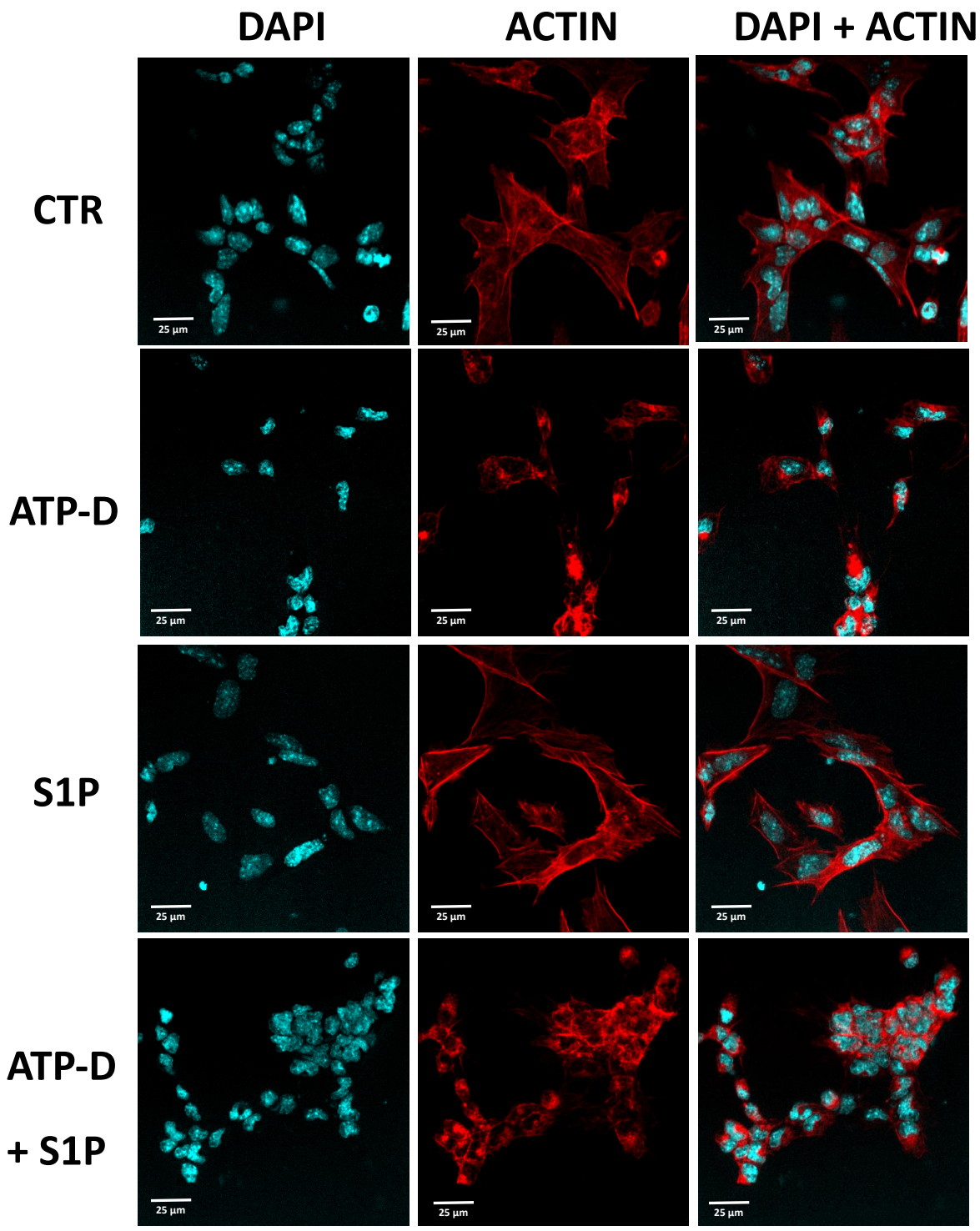

Figure 10: Cytoskeletal rearrangement and morphological alterations caused by ATP depletion are prevented upon S1P pretreatment of HK-2 cells. HK-2 cells (5×10^4) were cultivated on gelatin-covered (Attachment Factor, Sigma) glass coverslips and treated as previously described. Cells were processed for confocal microscopy as described under Material and Methods. For actin imaging (red), a 1:200 v/v solution of Alexa Fluor 555 Phalloidin was used (2 h incubation time). After a wash with PBS, the DAPI solution is added for 15 minutes. Images were acquired from the cells under the conditions depicted using a ZEISS Elyra PS1 for the confocal microscopy, and image stacks were obtained through Z-Stack. Representative images from three different experiments done in duplicate (n = 3). Calibration bar = 25 µm.

Table I: Primers concentration and specification used for quantitative RT-PCR (qRT-PCR).

Name	Specificity	Sequence 5' 3'	Concentration
GAPDH **F** GAPDH **R**	Human/ Murine	5’ TGCACCACCAACTGCTTAGC3’ 5’ GGCATGGACTGTGGTCATGAG3’	400 nM
CDC42 **F** CDC42**R**	Human	5’ ACCGACTGTTTTTGACAACTATGC3’ 5’ GTGGATAACTCAGCGGTCGT 3’	500 nM
RAC1 **F** RAC1 **R**	Human	5’ TGGCTAAGGAGATTGGTGCTG 3’ 5' CGGATCGCTTCGTCAAACAC 3'	500 nM
RhoA **F** RhoA **R**	Human	5' GCATTTCTGTCCCAACGTGC 3' 5’ AAAGCGCCAATCCTGTTTGC 3’	500 nM

DISCUSSION

Fluid transport across the renal tubular epithelium is certainly a key event that decisively contributes to whole-body homeostasis, being such events directly dependent on the correct cellular transporters distribution through the apical and basolateral membranes of the renal tubular cells.[2,4] Given the ability of Rho GTPases to orchestrate the correct polarization of epithelial cells, it was imperative to assess how the renal lesion would modulate these Rho GTPases and how the S1P pretreatment of the renal cells, would protect them from morphological and functional alterations derived from the loss of the correct polarization. The regeneration of the renal epithelium submitted to an acute injury or chronic disease would require i) inhibition of cell death and cell detachment, ii) activation of the proliferation of remaining cells, and iii) maintenance of the epithelial cell polarity, which is crucial for reabsorption and secretion processes along the nephron, therefore crucial for kidney functions.

Acute and chronic kidney diseases are characterized by changes in tubular epithelial cells. Such changes are mediated by F-actin cytoskeleton remodeling regulated by Rho GTPases. The role of the RhoA/ROCK pathway in renal and cardiovascular remodeling involves oxidative stress and its signaling through the induction of NADPH oxidase.[37] Interestingly, many of the reports in the literature that aim to modulate Rho GTPases as an important point in the regeneration process try to inhibit and not activate them, as it would be more plausible. It seems evident that the idea of maintenance of the Rho GTPases activity would ensure the remodeling of the cytoskeleton and the polarity of the renal epithelium cells.[26,32,33]

S1P receptors are among the most effective GPCRs that couple to Gα12/13, activating GEFs for RhoA. The S1P signaling pathway for Gα12/13 and RhoA is associated with the transcriptional regulation of different genes.[34] It has been reported that S1P protects the kidney from ischemic injury through several mechanisms involving immunological modulations and pro-survival compensations.[16]

Previously, we demonstrated that SphK activity was significantly increased in the kidneys of rats submitted to injury by unilateral ureteral obstruction, implying a greater availability of S1P in the injured kidney.[21,35]

Here we show for the very first time the alterations in the machinery involved in maintaining human kidney cell polarity, focusing on gene expression, protein levels, and activity of RhoA, Rac1, and Cdc42, and how S1P pretreatment was able to counteract the injury effects. These Rho GTPases were chosen due to their key role in cellular polarity determination and maintenance, cytoskeleton rearrangement, and cell adhesion, which are determinants of renal tissue.[5,36,37]

In the kidneys, S1P is released during apoptotic cell death and participates in coordinating anti-inflammatory responses. The selective S1PR1 agonist, SEW 2871, limited ischemic kidney damage when administered in the first 24 h. Furthermore, sphingosine kinase 2 (SK2)-deficient mice exhibited increased renal damage after ischemia/reperfusion (I/R).[23] Cellular transfection/overexpression studies suggest that coupling of S1P1 and S1P3 receptors to Gi act as typical chemotactic receptors by activation of the Rac pathway that controls cell polarization, formation and expansion of lamellipodium and the organization of focal complexes at the leading edge of the cell adhesion. S1P2 and S1P3 receptors coupled to G12/13 stimulate the RhoA/ROCK pathway.[38] It is already described that upon S1P administration, there is a cytoskeletal remodeling, which is characterized by (1) rapid and sustained activation of Rac1, (2) weak and later activation of RhoA, and (3) no action on Cdc42.[39] We showed a significant increase in RhoA gene expression upon injury, which is even higher in control cells exposed to S1P (Fig. 1). The preincubation of the cells with S1P resulted in normal expression of the RhoA gene. This result points to the early mobilization of RhoA upon injury, which is abolished by the S1P pretreatment. The huge increase in RhoA gene expression in the injured cells led us to imagine that the perception of the altered microenvironment was sufficient to trigger RhoA expression, probably an attempt to avoid the loss of cell polarity. The same picture was obtained when we tested the RhoA availability in the different experimental conditions. RhoA was augmented in the injury group and control cells pretreated with S1P. The S1P pretreatment abolished the increased availability of RhoA (Fig. 2). The S1P effect on RhoA was also observed when we studied the RhoA activity. S1P pretreatment prevents the reduction in RhoA activity upon injury (Fig. 3).

Rac1 also plays a role in ischemic injury, as demonstrated by Gao and colleagues[40], who showed the involvement of Rac1 in the increased NADPH oxidase activity in the ischemic kidney. Inhibition of Rac1 resulted in reduced NAPDH oxidase activity, less oxidative stress and apoptosis, and preservation of renal function, supporting the view that Rac1 contributes to the overall ischemic injury harmful effects. In the cardiac tissue, Rac1 depletion reduced ischemia-reperfusion injury, while its specific overexpression in cardiomyocytes from diabetic hearts resulted in heart injury with increased contractile dysfunction.[41] Rac1 also influences the maintenance of intercellular adhesions and tight junctions. Their activation has been associated with a loss of junctional integrity.[42] We showed (Fig. 4) that the ATP depletion did not alter Rac1 expression, while the preincubation of control cells with S1P significantly augmented it. Meanwhile, the Rac1 availability is significantly augmented in the injury group, being this effect completely abolished with the S1P pretreatment. The combined effect of S1P modulating the oxidative stress cited above[40] and protecting HK-2 cells from altered Rac1 availability and activity represents additional evidence for the benefits referred to S1P as a protective lipid mediator to renal tubular cells under ischemic conditions.

Cdc42 has also been shown essential to cytoskeleton remodeling, polarized distribution of proteins and lipids contributing to the correct polarization of epithelial cells, here included the renal epithelial cells[29], while some reports showed that it can also be involved in harmful effects. Here, it was possible to observe that there was no early alteration in Cdc42 expression. In contrast, the pretreatment of the cells with S1P, in the presence or the absence of antimycin A, promoted a significant increase in the Cdc42 gene expression. As we are dealing with cell cultures and S1P is a well-known cell proliferation activator, this augment may be related to the cellular division process induced by S1P. Also, we detected a 1.5-fold increase in the Cdc42 availability in renal cells subjected to injury. However, cells pre-exposed to S1P, with or without the induction of ATP depletion injury, presented increased levels of Cdc42, close to those found in cells submitted to the injury. This result allows us to postulate at least two divergent explanations: i) HK-2 cells subjected to injury preserve Cdc42 to go through the critical period of the injury, maintaining the correct assembly

of their adherent junctions and cytoskeleton polymerization that is crucial to preserve cell polarity; or ii) HK-2 cells pre-incubated with S1P would be better preserved and even stimulated to proliferate and establish new interactions with other adjacent cells, increasing the regulation of the cellular junction apparatus and also the maintenance of cell polarity.

According to the literature, the different Rho GTPases follow complex patterns of activation and mobilization at different times, in other words, the orchestrated events will require differential action of each Rho GTPase, which reflects the difficulty in correctly establishing the physiological and pathophysiological contribution of each Rho GTPase to the injury/repair process. As a limitation, we could not ascribe to any specific S1P receptor the modulatory and protective effects obtained. Based on the literature, we can suggest that S1P1 and S1P3 receptors are the major isoforms in the kidneys and were already related to kidney diseases.[16] We can also consider using just one renal cell lineage, although they are human cells, a limitation of the work.

From the results presented here, we can assume that S1P protective action can happen at different levels according to the Rho GTPase studied, which reinforces the hypothesis that these cytoskeletal and cell polarity regulators are required by cells at different times during the process of injury and even repair. Understanding these windows of action for each Rho GTPase could greatly contribute to regenerative medicine, suggesting developing a formulation containing S1P for future therapeutical protocols.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: The manuscript present here has full consent for publication by the other authors.

Availability of data and materials: The raw data are under the responsibility of the first author and in the laboratory's respective folders. Access is granted upon request.

Competing interests: Authors declare no competing or conflict of interest.

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Research involving Human Participants and/or Animals: Not applicable.

Author's Contributions

Statement: The authors declare that all data were generated in-house and that no paper mill was used

Gloria M. R. S. Grelle: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Roles/Writing - original draft; Writing -review & editing.

Teresa C. Calegari-Silva: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing -review & editing.

Pedro Pompeu: Data curation; Formal analysis; Investigation.

Rafael Garrett: Supervision; Roles/Writing - original draft; Writing -review & editing.

Rafael H. F. Valverde: Formal analysis, Roles/Writing - original draft; Writing -review & editing.

Marcelo Einicker-Lamas: Conceptualization; Formal analysis; Funding acquisition; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing -review & editing.

All authors read and approved the final version of the manuscript here submitted.

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