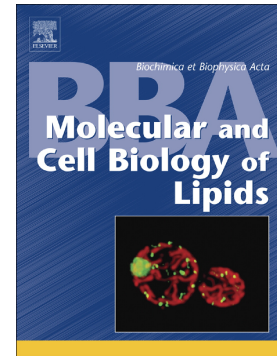# Accepted Manuscript

TAG synthesis and storage under osmotic stress. A requirement for preserving membrane homeostasis in renal cells

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TAG synthesis and storage under osmotic stress. A requirement for preserving membrane homeostasis in renal cells.

Running Title: ***Lipid metabolism regulation by hyperosmolarity.***

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Abbreviations:
ACC: Acetyl CoA Carboxylase; FASN: Fatty acid synthase; DGAT: diacylglycerol acyltransferase; FACoA: Fatty acyl CoA; G3P: *sn*-glycerol 3P; LD: Lipid Droplet, PA: phosphatidic acid; PAP: Phosphatidic acid phosphatase; PL: Phospholipid; Pi: orthophosphate; TAG: triglyceride; DAG: diacylglycerol; SREBP: Sterol regulatory element binding protein.

Abstract

Hyperosmolarity is a controversial signal for renal cells. It can induce cell stress or differentiation and both require an active lipid metabolism. We showed that hyperosmolarity upregulates phospholipid (PL) *de novo* synthesis in renal cells. PL synthesis requires fatty acids (FA), usually stored as triglycerides (TAG). PL and TAG *de novo* synthesis utilize the same initial biosynthetic route: *sn*-glycerol 3P (G3P) → phosphatidic acid (PA) → diacylglycerol (DAG). In the present work, we evaluate how such pathway contributes to PL and TAG synthesis in renal cells subjected to hyperosmolarity. Our results show an increase in PA and DAG formation under hyperosmotic conditions; augmented DAG production, due to lipin enzyme activity, lead to the increase of both TAG and PL. However, at early stages (24 and 48 h), most of the *de novo* synthesized DAG was directed to PL synthesis; longer treatments downregulated PL synthesis and the DAG formed was mainly driven to TAG synthesis. Hyperosmolarity induced ACC and FASN transcription which mediated FA *de novo* synthesis. New FA molecules were stored in TAG. Silencing experiments revealed that hyperosmotic-induction of lipin-1 and -2 was mediated by SREBP1. Interestingly, SREBP1 knockdown also dropped SREBP2, indicating a modulatory action between both isoforms. Impairing SREBP activity leads to a decline in TAG levels but not PL. Membrane homeostasis is maintained through the adequate PL synthesis and renewal and constitute a protective mechanism against hyperosmolarity. The present data reveal the relevance of TAG synthesis and storage for PL synthesis in renal cells.

Key Words. Triglyceride storage, phospholipid synthesis, lipin, DGAT, SREBP, hyperosmolarity.

1. Introduction

Phospholipids (PLs) are key molecules for life. Apart of being the main constituents of the lipid bilayer of all eukaryotic cell membranes, PL fatty acid tails govern membrane physicochemistry, modulate protein activity, and store lipid mediators and second messenger precursors'. The polar head of the molecule acts as docking sites for a variety of proteins and can also deliver aqueous soluble messengers [1-3]. Thus, the adequate synthesis and turnover of PLs in biomembranes are crucial events to maintain cellular structure and physiology.

PL *de novo* synthesis takes place in two well-defined stages. The first one leads to phosphatidic acid (PA) formation by the successive acylation of *sn*-glycerol 3P (G3P) in position 1 and 2 by specific acyltransferases. In a second stage, PA is dephosphorylated by the action of phosphatidic acid phosphatase (PAP1) activity, also known as lipin, to form diacylglycerol (DAG), which constitutes a branch point in the pathway since it can be condensed with CDP-choline or CDP-ethanolamine to form phosphatidylcholine (PC) or phosphatidylethanolamine (PE), respectively, or it can be acylated in position 3 to form triglyceride (TAG) by the action of diacylglycerol acyltransferase (DGAT) [4, 5]. Together with stearoyl esters and retinyl esters, TAG constitute the cellular pool of neutral lipids which are stored within the cell in lipid droplets (LDs) [6]. When fatty acids are needed they are mobilized from LDs and used to generate energy, membrane components, and/or signaling lipids [6, 7]. The accretion of TAG in LDs occurs by different reasons as increase of exogenous lipids availability such as serum lipoproteins or free fatty acids (FFAs) [6] or even in the absence of exogenous sources of FFAs apparently by recycling membrane phospholipids into TAG [7, 8]. It has been demonstrated that different pathological situations as oxidative stress, inflammation or viral infection induce LDs formation and that LDs may play a cytoprotective role against cellular stress [9, 10].

Various works from our laboratory indicate that osmotic stress triggers phospholipid synthesis to maintain membrane integrity in renal tissue. In the kidney, interstitial tissue is unique in having an osmolarity gradient along the corticomedullary axis; cortical interstitium is isosmolar respect to the plasma while inner medullary interstitial osmolarity can overpass several times plasma values depending on the hydration state of the organism [11]. This distinctive feature is closely related to the urinary concentrating mechanism [12]. However, when osmolarity increases over such a high physiological values, as occur in dehydration or other pathological situations, it becomes a stress factor and cells have

to trigger adaptive responses to survive [13, 14]. In addition, hyperosmolarity is a main signal for renal tubular cells differentiation [15-17]. Former works from our laboratory showed that the renal inner medullary cells possess the most dynamic phospholipid synthesis and membrane turnover among the various kidney zones [18, 19]. We have also demonstrated that the high-sodium enriched environment is responsible for regulation of renal phospholipid *de novo* synthesis [20]; these experiments showed that PL content and biosynthesis increased as a function of sodium chloride concentration reaching the maximal activity before epithelial cells reach their differentiated characteristics [20].

It is evident that osmotic-induced membrane biogenesis requires an adequate neutral lipid metabolism to provide enough quantity of fatty acids. As mentioned before, PL and TAG *de novo* synthesis utilize the same initial biosynthetic route: *sn*-glycerol 3P (G3P) → phosphatidic acid (PA) → diacylglycerol (DAG). Therefore, we were interested in understanding how this initial stage in glycerolipid synthesis was contributing to PL and TAG in renal cells subjected to hyperosmolarity. The data presented herein show that renal cell prioritizes PL synthesis even in the absence of external or de novo synthesized FA; in these situations, TAG stores are likely to be essential for preserving PL synthesis and renewal. These results may explain our previous observations in renal papillary tissue.

2. Materials and Methods

2.1. Culture conditions. Madin Darby canine kidney (MDCK) cells (American Type Culture Collection, passages 45–50) were grown in a mixture containing DMEM and Ham's- F12 (1:1), 10% FBS, and 1% antibiotic mixture (GIBCO®). After reaching 70–80% confluence, cells were placed in low-serum medium (0.5% FBS) for 24 h and then subjected to hyperosmolarity for different periods of time. Hyperosmolar media were made by adding aliquots of sterile 5 M NaCl to commercial medium to achieve desired final osmolalities that were determined by using an osmometer (µOSMETTE, Precision Systems; Sudsbury, MA)[16, 20]. After treatments, media containing dead cells and debris was discarded, and cells were washed twice with sterile PBS and treated with 0.25% trypsin-EDTA (GIBCO®) for 3 min. When cells were detached from the culture support, 20% of FBS was added to stop trypsin action. Cells were counted in a hemocytometer chamber (Neubauer´s chamber) in the presence of trypan blue to obtain the number of total and viable cells. Viability was calculated from these data as the percentage of non-trypan blue-stained cells of total counted cells. Aliquots of cell suspensions containing an adequate number of cells were used for the different experimental protocols. Although nonviable cells were included in the cell population used in the experiments, the number of trypan blue-stained cells was lower than 10%. Under these experimental conditions the viability of collected cells was over 90%.

2.2. Lipid extraction, separation and quantification. Total lipids were extracted by the method of Bligh and Dyer [21]. Briefly, MDCK cell pellets (~3×10^6 cells) were suspended in 800 µl of PBS and mixed with 2 ml of methanol and 1 ml of chloroform, vortexed gently for 30 s, and incubated on ice for 15 min. To yield two phases, 1 ml of chloroform and 1 ml of water were added, vortexed for 30 s and centrifuged at 800 g for 5 min. The lower organic phase containing total cell lipids was collected, dried under a nitrogen stream, and kept at -80°C for further analysis. Lipid species were separated by TLC. Dried extracts were applied drop by drop onto a 1 cm lane of thin-layer silica gel chromatoplates (Merck) and developed in a solvent mix containing petroleum ether / hexane / ethylic ether / acetic acid (40:40:20:1, v/v), dried and exposed to iodine vapors to reveal lipid spots. The different lipids were identified by comparison with the corresponding standards and the retention factors (Rfs): 0.06, 0.13, 0.18, 0.27 and 0.60 for polar lipids, DAG, cholesterol, FFA and TAG, respectively. To determine PA, TLC plates were developed in a solvent mix containing chloroform / methanol/ acetic acid / water (55:43:3:4, v/v) and phospholipid species identified according to Medth and Weigel procedure [22]. The quantification of phospholipids was carried out by measuring the quantity of free orthophosphate (Pi) according to the

Bartlett procedure [23]. Briefly, the zones of the plates containing phospholipid mass were scraped into a Kjeldhal tube, mixed with 70% perchloric acid in the presence of 0.5% ammonium molybdate, and heated at 200°C for 30 min to complete mineralization to release Pi from phospholipid polar head. Pi concentration was determined by using Fiske-Subarrow reagent [23]. The zones of the plates containing TAG were scraped and extracted from the silica with chloroform twice, dried under a nitrogen stream and quantified using a commercial enzymatic kit (TAG color, Wiener lab.), based on the determination of the glycerol released by triglyceride lipase activity.

2.3. Labeling experiments. To evaluate whether hyperosmolarity affects lipid biosynthesis, we studied the incorporation of the precursor [U-^{14}C]-glycerol into PL, TAG, DAG and PA. To do that, cells were grown to 70–80% confluence, placed in low-serum medium (0.5% FBS) for 24 h, and then subjected to hyperosmolarity (125 mM NaCl) for different periods of time (24, 48, and 72 h). In one set of experiments (Fig. 2), control and treated cells were incubated with 2 μCi/ml of [U-^{14}C]-glycerol (PerkinElmer ®) for 2, 4 and 6 h before cell harvesting. In the rest of the labeling experiments, cells were incubated for 4 h before they were collected. When metabolic inhibitors were used, cells were treated with them for 30 min before radioactive were added (different concentrations of TOFA and cerulenin, 20 μM fatostatin, all from Sigma-Aldrich). After labeling, lipids were extracted and separated by TLC as described above. The radioactivity incorporated into each lipid was visualized by radioautography and quantified by liquid scintillation counting.

2.4. PAP1/Lipin and LPP assays. Preparation of radioactive 1,2-diacyl-sn-glycerol-3-phosphate (PA): Radioactive PA was obtained from [2-^{3}H]glycerol-PC synthesized from bovine retinas incubated with [2-^{3}H]glycerol (200mCi/mmol) as previously described [24].

PAP1/Lipin activity was determined in an assay containing 50 mM Tris-maleate buffer, pH 6.5, 1 mM DTT, 1 mM EDTA and 1 mM EGTA, 0.2 mM Mg^{2+}, and 50 μg cellular protein in a volume of 100μl. The reaction was started by adding 80 μM of [^{3}H]-PA plus 55 μM PC. Parallel incubations were carried out after preincubating the enzyme with 4.2 mM NEM for 10 min. The difference between these two activities was labeled as PAP1/Lipin activity [25, 26]. PAP1/Lipin enzymatic reactions were stopped by adding chloroform:methanol (2:1, v/v). Blanks were prepared identically to each enzymatic assay except that the membrane fraction was either boiled for 5 min or inactivated by the addition of chloroform:methanol (2:1,v/v) before use. All assays for determination of PAP1/lipin activities were conducted for 30 min at 37 °C. Lipids were extracted with chloroform:methanol (2:1, v/v) and washed

with 0.2 volume of $CaCl_2$ (0.05%). DAG was separated by gradient-thickness thin-layer chromatography on silica gel G [12] and developed with hexane:diethyl ether:acetic acid (35:65:1, v/v). In this solvent, DAG migrates to the three fourths of the plate and PA stays in the origin. Radiolabeled samples were counted after the addition of 0.4 ml water and 10 ml 5% Omnifluor in toluene/Triton X-100 (4/1, v/v).

2.5. Transfections. SREBP1 and 2 were silenced using siRNA duplexes from Invitrogen (siRNA SREBP1 5´-CCACUUCAUCAAGGCUGACUCUUUG-3´ 5´-CAAAGAGUCAGCCUUGAUGAAGUGG-3´ and siRNA SREBP2 5´-CGCCCCAGCUUUCAAGUCCUUCA-3´ 5´-UGAAGGACUUGAAAGCUGGUGGCUG-3´). Cells were seeded (with or without glass cover slips) and grown until 70% confluence and transfected with siRNA or Scramble by means of Lipofectamine® 3000 (Invitrogen) following the manufacturer's instructions. This procedure was repeated 24 hours later. After 24 h, cells were subjected to hyperosmolar medium for 24 hours, and harvested for mRNA analyses or prepared for immunostaining.

2.6. RT-PCR and qPCR. MDCK cells were grown, treated, and collected as described above, then 2×10^6 cells were used for total RNA extraction using the SV Total RNA Isolation System (Promega) in accordance with the manufacturer's instructions. First-strand cDNA was synthesized from total RNA using the reverse transcription system (Promega) using oligo-dT (Biodynamics) as primer. To evaluate the levels of the different mRNA encoding lipogenic enzymes, RT-PCRs were performed using GoTaq® Polymerase and dNTPs from Promega. Quantitative PCR (qPCR) was performed on a Rotor Gene Q (Quiagen) by using the master mix qPCR PB-L-SYBR Green I (Bio-Lógicos® products) according to the protocol provided by the manufacturer. Primers used:

Protein	Forward	Reverse
Lipin-1	5'-CGCAAGTCCTTCAGGTTCTC-3'	5'-TGTGGAGATGACTTTGCAGC-3'
Lipin-2	5'-AAGACCAAAATGCTTCCCCT-3'	5'-TGATCCCCAGAATGGAAGAG-3'
DGAT-1	5'-TGGATAGTGAGCCGCTTCTT-3'	5'-AGGAGCCTCATAGTGGAGCA-3'
DGAT-2	5'-AGTGGCTCAGGCAGGTTAGA-3	5'-GATGCTCTTCAAAATGGGGA-3'
ACC	5'-GTGGCTTTGAAGGAGCTGTC-3'	5'-CCAGCCGGTGTCAATTCTAT-3'
FASN	5'-TCATCCCCCTGATGAAGAAG-3'	5'-GCAGTGGTCCACCAGGTAGT-3'
SREBP1	5'-AGACATGGCAACCACTGTGA-3	5'-GATAGTTCCTCCGCTCACCA-3'
SREBP2	5'-GATGTCATCTGTCGGTGGTG-3'	5'-GGGGGCTCTCTGTTACTTCC-3'
HMGCoAR	5'-GAAACGTGGGCATTGGGTTC-3'	5'-TCCTCAACACAACACCGCAT-3'
β-Actin	5'-CAAAGCCAACCGTGAGAAG-3'	5'-CAGAGTCCATGACAATACCAG-3'

2.7. Microscopy. Cells were cultured as described above but on glass cover slips. After treatment, cells were washed twice with sterile PBS, fixed with 3.7% paraformaldehyde in PBS for 30 min, and permeabilized with 0.1% Triton-X100 in PBS for 15 min. Fixed cells were stained with Oil red O for 5

min, and washed with water and then with PBS. Next, the staining of actin filaments was done with 1µg/ml phalloidin-FITC (Sigma Aldrich) for 5 min, washed with PBS, and mounted with a drop of Vectashield mounting medium (Vector Laboratories). For immunofluorescence, cells were fixed and permeabilized, blocked with 3% BSA in PBS for 1 h and incubated with primary antibodies (SREBP1 mouse monoclonal sc-365513 or SREBP2 rabbit polyclonal ab28482) at 4ºC overnight. Then, cells were washed with PBS and incubated 1 h with fluorescent secondary antibodies (anti-Mouse IgG Alexa Fluor® 488 conjugate, Invitrogen, or anti-Rabbit FITC conjugate, Vector) and 2.5 µM Hoechst 33258 (Sigma) to visualize nuclei. After labeling, samples were exhaustively washed with PBS and mounted with a drop of Vectashield mounting medium (Vector Laboratories). Differential Interference Contrast (DIC) and fluorescence images were obtained with a Nikon Eclipse Ti (with an objective Plan apo VC 60×, 1.4 DIC 1/2) with acquisition software Micrometrics SE Premium (Accu-Scope). Images were processed using ImageJ.

2.8. Statistical analysis. The results were expressed as mean ± SEM. Data from controls and different treatments were analyzed by ANOVA, and significant differences were assessed by a posteriori Bonferroni test ($p < 0.05$).

3. Results

3.1. TAG metabolism in renal cells is upregulated by high NaCl media. In a previous report we showed that hyperosmolarity activates phospholipids (PLs) biosynthesis in MDCK cultures [20]. As it was mentioned before, in a first stage PLs *de novo* synthesis requires a sequential fatty acid (FA) esterification. For this process, cells can obtain FA from cellular stores, that means, from triglycerides (TAG) or from cell environment (diet or culture medium). Considering that MDCK were cultured in low serum media (0.5% FBS), it is probable that cells obtain FA from stored TAG or from FA *de novo* synthesis. Thus, we first evaluated whether hyperosmotic medium affects TAG content (Fig. 1). MDCK cells were cultured with commercial medium alone (isosmolar condition, 298 mOsmol/Kg H_2O equivalent to 0 mM NaCl) or supplemented with different concentrations of sterile NaCl to reach osmolalities between 300 and 600 mOsmol/Kg H_2O (50 to 150 mM NaCl) like those found in renal inner interstitium. Similar to that observed with phospholipids [20], TAG content increased as a function of NaCl concentration in culture medium (Fig. 1A). Media containing 125 and 150 mM NaCl (equivalent to 512 ± 12 and 590 ± 22 mOsmol/Kg H_2O, respectively) significantly increased TAG content by 122 and 96 %, respectively, over isosmolar condition (no NaCl added). Such an effect was also dependent on the time of treatment, reaching the maximal variation respect to isosmolar value after 48 h of incubation with 125 mM NaCl (Fig. 1B). Figure 1C shows cellular TAG stored in lipid droplets (LDs). As it is seen, hyperosmotic treatment by 72 h almost doubled oil red O staining due to the increase in the number and the size of LDs (Fig. 1D). These results are in accordance with those found in renal tissue (not shown) confirming the dependence on osmolality gradient of renal glycerolipid metabolism [18, 19].

The rise in TAG content after NaCl treatment can be attributed to an increase in its synthesis and/or a decrease in its breakdown. TAG *de novo* synthesis begins when a molecule of *sn*-glycerol 3P is successively acylated to form phosphatidic acid (PA) which is after converted to diacylglycerol (DAG) by PA phosphatase (PAP1, also known as lipin). DAG can be converted either into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) through the Kennedy pathway, or into TAG by the action of diacylglycerol acyltransferase (DGAT) (See scheme A in figure 2) [27]. To study *de novo* biosynthetic pathway, and the relationship between TAG and PL, we monitored the incorporation of [U^{14}C]-glycerol (^{14}C-Gly) into these lipids during 2, 4 and 6 h after hyperosmotic treatment for 24, 48 and 72 h (Fig. 2). As it is observed, at every time evaluated, the incorporation of ^{14}C-Gly into both TAG and PL was significantly higher in hyperosmolarity than the corresponding value in isosmolality (Fig. 2B and C).

Thus, saline treatment visibly modifies biosynthetic process. The number of picomole of PL formed per cell at 24 and 48 h of NaCl treatment doubled the picomole of TAG synthesized in the same periods of time (Fig. 2B and 2C). This observation goes along with the fact that, under hyperosmotic conditions, MDCK cells increase their volume and size and reach a polarized-epithelial cell phenotype [16, 20]. So, they must generate new membranes and most DAG formed through the common biosynthetic pathway (Fig. 2A) is driven to PL synthesis. After membrane replenishment, cells have to redirect DAG to TAG synthesis in order to avoid PL accumulation; this fact may explain the abrupt fall in PL synthesis after 72 h (Fig. 2C). Despite the number of picomole of PL synthesized per cell is higher than the picomole of TAG that reflects the physiological needs of the cells, the change in the rate of the whole process (estimated as the slope of ^{14}C-Gly incorporation curve and expressed as picomole of ^{14}C-Gly-lipid formed per hour by 10^6 cells, Fig. 2D and E), was significant higher for TAG synthesis (5, 19 and 10-fold increase for TAG synthesis respect to control values after 24, 48 and 72 h, figure 2D, versus 2, 9 and 6-fold increase for PL synthesis compared to control values, figure 2E). This last observation may be related to the physiological relevance of TAG synthesis in renal cells against changes in environmental osmolality.

3.2. *sn*-**glycerol 3P (G3P)** → **phosphatidic acid (PA)** → **diacylglycerol (DAG): a key metabolic route against osmotic stress**. To get a better understanding on how hyperosmolarity modulates these biosynthetic pathways, we evaluated PA and DAG formation, common intermediary molecules for both PL and TAG synthesis (Fig. 3). After 24 h, both PA and DAG synthesis increased 6 and 5 times, respectively, over control values. PA levels remained constant after 48 h of NaCl, but DAG significantly decreased by 48 % compared to 24 h (Fig. 3A). This observation goes along with the fall in lipin activity that after 48 h of treatment returned to 24 h isosmolar value (Fig. 3B). The decrease in DAG steady state level from 24 h to 48 h could be due to: 1) a lower lipin activity (Fig 3B), even if DAG decrease was much more pronounced than enzyme activity reduction; 2) the action of a diacylglycerol lipase (DAGL); 3) a higher activity in PL synthesis (Fig. 2C) and/or 4) a greater activity of DGAT. Regarding DGAT activity, it is worth to point out that a high DGAT mRNA level does not mean a higher enzyme expression or activity. In addition, the formation of TAG (Fig. 2B) greatly exceeds the decrease in DAG (Fig. 3B). Thus, we could speculate that after 48 h of treatment DAG production is delivered to TAG biosynthetic pathway. This is reflected in TAG synthesis at 72 h of treatment but not in PLs biosynthetic activity.

The level of lipin mRNA expression was evaluated by qPCR (Fig. 3C and D). Despite three lipin isoforms have been reported [28], only lipin-1 and lipin-2 were detected in our experimental conditions. As it is seen, hyperosmolarity significantly increased the mRNA expression of both isoforms after 24 and 48 h, but the rise of lipin-2 was higher than that observed for lipin-1. After 24 h of NaCl, lipin-1 mRNA expression doubled its basal expression while lipin-2 mRNA level was 10 times higher than basal values (Fig. 3C and D). Even though a rise in mRNA expression does not mean an increase in the level of protein and/or enzyme activity, we could speculate that 24 h increased lipin activity could be attributed to lipin-2 isoform; however, it should be proven.

We previously showed that hyperosmolarity upregulated the expression of the whole set of Kennedy pathway PC-synthesizing enzymes, in particular, cholinephosphotransferase that catalyzes the condensation of DAG and CDP-choline to form PC accompanying the increase in PC synthesis [20, 29]. Now we determine whether diacylglycerol acyltransferase (DGAT), involved at the final step of TAG synthesis is affected by hyperosmolarity. As it is shown in figures 3E and F, mRNA levels of both DGAT isoforms, DGAT-1 and DGAT-2, were significantly increased after 48 h of treatment. As mentioned above, although mRNA increase does not mean protein level and/or enzyme activity increase, this finding coincides with the elevated rate of TAG synthesis still observed after 72 h of NaCl (Fig. 2D) that may contribute to drain the DAG not used in PL synthesis. As it was observed for lipin enzyme, the increase in DGAT-2 levels was significantly higher than that observed for DAGT-1 (12 times for DGAT-2 and 5.5 times for DGAT-1 respect to their isosmolar values).

3.3. Fatty acids required for hyperosmotic-induced TAG synthesis are *de novo* synthesized. The fatty acids (FAs) that cells need for biosynthetic processes can be obtained from cell environment (diet or culture medium), TAG stores, and/or can be *de novo* synthesized. Considering that MDCK cultures were maintained in low serum media (0.5 % FBS), the possibility of obtaining FA from culture medium is limited. As previously mentioned, NaCl-triggered PL synthesis probably utilizes LDs-stored FA. Then, to replenish LDs, TAG synthesis was activated (Fig. 2B). In this case, the FA required for PA formation should be *de novo* synthesized. To test this possibility, we evaluated the expression of FA-synthesizing enzymes, acetyl CoA carboxylase (ACC) and fatty acid synthase (FASN), and evidenced their activity by using different concentrations of TOFA, ACC inhibitor, or cerulenin, FASN inhibitor (Fig. 4). The analysis of q- and RT-PCR showed that the expression of both enzymes significantly increased between

12 and 24 h after treatment, thus preceding the highest FA demand for PA synthesis (Fig. 3A) occurred after 24 and 48 h of hyperosmotic challenge (Fig. 4A, B and C). As it is seen in figure 4D and 4E, both inhibitors blocked hyperosmotic-induced TAG synthesis in a concentration dependent fashion. Similarly, TOFA and cerulenin, decreased the endogenous TAG content (Insets in Fig. 4D and E). These results clearly indicate that FA *de novo* synthesis is required for hyperosmotic-induced PA synthesis directed to TAG synthesis and clearly reveal that stored-TAG is being hydrolyzed since its content is also affected by FA synthesis inhibitors. The fall in TAG content indicates that cells utilize stored-FA to face the demand of hyperosmotic-induced PL synthesis that it was not hindered neither by TOFA nor by cerulenin affected PL biosynthesis (Fig. 4F and 4G). Therefore, when osmolality changes in environment, MDCK cells increase PL synthesis and content by using stored FA at LDs; as the stimulus continues, cells activate TAG synthesis to restore TAG content and refill LD stores.

3.4. NaCl induced-transcriptional regulation of lipin but not DGAT involves SREBP. qPCRs shown in figures 3 and 4 indicate that hyperosmolarity activates the transcription of lipid biosynthetic enzymes. These observations were confirmed by performing PCR and TAG synthesis experiments in the presence of the transcriptional and translational inhibitors actinomycin D and cycloheximide; both inhibitors blocked the expression of lipin and DGAT and the synthesis of TAG (not shown). It is widely reported that lipogenic gene expression is mainly regulated by the transcription factor sterol regulatory element-binding protein (SREBP) [30, 31]. In order to evaluate whether SREBP was mediating the transcription of lipogenic enzymes, we studied the content and the synthesis of lipids in the presence of fatostatin that blocks SREBP activation [32]. Figure 5A shows a typical chromatogram obtained from MDCK cells treated with hyperosmotic media in the absence or presence fatostatin for 24 and 48 h. As it is seen, fatostatin (Fato) markedly decreased TAG content (Fig. 5B) which was reflected in LDs size reduction but not in changes in their number (Fig. 5C and D). Similar to those results showed in Fig. 4 and discussed in section 3.3, LDs TAG-emptying was probably due to TAG decreased-synthesis (Fig. 5E) and TAG utilization for phospholipid synthesis which was not affected by fatostatin (Fig. 5G). Figures 5H and 5I show the reduction of lipin-1 and -2 mRNA expression by fatostatin treatment. Despite we observed that actinomycin D blocked all lipogenic enzyme transcription (not shown), fatostatin did not affect DGAT isoenzymes expression. Thus, the fall in TAG seems to be a consequence of decreased-lipin expression which in turn causes a reduction in DAG *de novo* synthesis (Fig. 5F).

To confirm the participation of SREBP in transcriptional regulation of lipogenic enzymes, we performed experiments using specific small interference RNAs (siRNA). Both SREBP1 and 2 mRNAs were expressed in isosmotic conditions in MDCK cells. SREBP1 and 2 mRNAs expression showed an early non-significant decrease (4 h of NaCl treatment) followed by a steadily induction that reached a maximal level after 24 h of treatment (Fig. 6A and B). Protein expression and location were studied by immunofluorescence microscopy. After 24 h, hyperosmotic treatment caused an increase in nuclear-fluorescence associated to SREBP1 and a fine but evident redistribution of SREBP1 labeling within nuclear compartment, which was found nearby nucleolar structures (Fig. 6C, upper panel). It is worth pointing out that after 24 h of treatment, both lipin-1 and lipin-2 significantly augmented their expression. A different pattern was found for SREBP2 protein labeling that was found inside nucleoli and associated to nuclear envelope and to supra-nuclear vesicle-like structures showing a strong labeling after 24 h of NaCl treatment (Fig. 6C, lower panel). MDCK transfection with SREBP1 and SREBP2 siRNAs (S1 and S2 siRNAs, respectively), modified SREBPs mRNA (Fig. 6D and E) and protein levels (Fig. 6F). S1-siRNA decreased SREBP1 mRNA levels. Unexpectedly, the expression of SREBP2 mRNA was also blocked by S1-siRNA. In contrast, S2-siRNA, only caused the fall of its own mRNA and of SREBP2-target gene HMG-CoA reductase (not shown). These experiments confirm fatostatin assays and also reveal that hyperosmotic transcriptional activation of both lipin-1 and lipin-2 was mediated by SREBP1. Similar to that found with fatostatin, DGAT-1 and DAGT-2 expression were not affected by silencing treatment (Fig. 6G and H).

4. Discussion.

The aim of the present work was to establish the relationship between PL and TAG *de novo* synthesis dynamics in renal cells subjected to hyperosmotic environment. Since both metabolic pathways utilize the same initial biosynthetic route: sn-glycerol 3P (G3P) $\rightarrow$ phosphatidic acid (PA) $\rightarrow$ diacylglycerol (DAG), we were interested in understanding how this initial stage in glycerolipid synthesis was contributing to PL and TAG accumulation.

Hyperosmolarity is a dual and controversial signal for renal cells. Abrupt changes in environmental osmolality induce cell stress that can lead to death. However, under physiological conditions, hyperosmolarity induces renal cell differentiation and the maturation of urine concentrating system. Moreover, variation of interstitial osmolality is necessary to concentrate urine in mature kidneys [14, 17, 33]. Interestingly, both situations require an active lipid metabolism: as a protective mechanism against osmotic stress for preserving membrane structure and as a physiological tool for constructing cell structure and tissue architecture of differentiated cell state.

The fact that hyperosmolarity induces cell differentiation has been demonstrated in different cell types. In the last years, a growing body of evidence has been supporting the idea that the tonicity-responsive enhancer binding protein (TonEBP), also known as NFAT5 (nuclear factor of activated T cells 5) is involved in differentiation process. TonEBP/NFAT5 is a transcription factor considered a master regulator of adaptive and protective responses against hyperosmolarity [34, 35]. In the kidney, TonEBP/NFAT5 activation is dependent on sodium increase but not on potassium salts or urea variations [34], and its activity mediates the expression of several proteins present in differentiated cell state [14]. Moreover, Han and col. demonstrated that TonEBP contributes to the maturation of urinary concentrating system in developing rat kidney [33], accompanying the idea that presence of high sodium concentrations surrounding immature renal cells activates TonEBP which in turn induces cell differentiation [34]. In addition, the involvement of TonEBP/NFAT5 has been shown in skeletal muscle differentiation [36], P19CL6 cells differentiation to cardiomyocytes [37], CACO-2 cell differentiation [38] and human bone marrow stem cells (hBMSCs) differentiation to chondrogenic cells [39]. Madonna et al. have demonstrated that TonEBP activity regulates vascular development of iPS cells under glucose-hypertonic conditions [40]. The fact that cell differentiation activates a specific lipid synthesis program have been extensively reported [41-46]. Even though all these differentiation processes should be accompanied with

the synthesis and maturation of new membranes, no reports about hypertonicy-induced lipid metabolism have been found neither in human renal cells nor in stem cells.

The effect observed on lipid metabolism seems to be ion specific. Since the renal interstitial solutes that contribute to hyperosmolarity are mainly NaCl and Urea, we performed our experiments in the presence of both solutes. Between these solutes only NaCl (not urea) is able to modulate lipid metabolism. Such an effect was shown in a previous manuscript where we showed how hyperosmolarity regulates membrane biogenesis and phospholipid profile [20]. We also performed TAG synthesis and content determinations in the presence of urea (not shown) and similar to that previously found for phospholipids, urea did not cause any change. In addition, we tested the effect of hyperosmolarity made of different concentrations of KCl and glucose on lipid metabolism and no effect was found in any case. The fact that high NaCl-hyperosmolarity modulates lipid metabolism was also observed in glycosphingolipids [16] and sulfoglycosphingolipids SM4 and SM3 [47] synthesis in renal cells. In addition, sodium-hyperosmotic medium modulates lipid droplet formation in human corneal epithelial cells [48]. Apart from that, numerous studies evaluating the effect of hyperosmolarity on other MDCK parameters are consistent with the fact that NaCl is the responsible for the observed effects [13, 14, 16, 34, 47-51].

We previously showed that high NaCl-hyperosmotic medium induces phospholipid synthesis activity and phospholipid mass accretion in MDCK cells. Such an effect, occurring within the first 48-72 h of hypertonic treatment, contributes to the enrichment of the apical and basolateral membranes in sphingomyelin (SM) and phosphatidylcholine (PC) content, respectively, and is needed for membrane biogenesis during cell polarization-differentiation process [20]. We also demonstrated that hyperosmotic-mediated MDCK membrane synthesis involves the rise in the expression and activity of PC synthesis rate-limiting enzyme, CTP:P-choline cytidylyltransferase (CCT). CCT activity regulation requires protein redistribution between hypertonic-induced intranucleoplasmic compartments, lamin A/C-speckles and paraspeckles [15, 29]. In the present work, we demonstrated that hyperosmolarity induces the synthesis and accretion of another lipid class, the triglycerides; the present data also show that under osmotic stress MDCK cells prioritize PL synthesis even in the absence of their main structural constituent: fatty acids. Thus, under hyperosmolar environment, TAG stores become essential for preserving PL synthesis and renewal in cell membranes. Despite it was shown that hyperosmolarity regulates differentiation through

TonEBP/NFAT5 and we demonstrated that hyperosmotic environment triggers PL synthesis which is necessary for membrane biogenesis in differentiation process, we were not able to demonstrate that PL biosynthetic enzymes expression was mediated by TonEBP/NFAT5 since its silencing did not cause PL synthesis inhibition [20]. Whether or not TonEBP were mediating TAG synthesis is now under study in our laboratory.

The response of the cell to changes in environmental osmolality involves three stages. In the first hours, renal cells in culture suffer a decrease in their volume, a rise in ionic strength, aggregation of macromolecules and a delay in transcription and translation processes, among others. Afterwards, cells activate adaptive mechanisms such as accumulation of organic osmolytes and transcription of osmoprotective genes. After 20 h, cells are adapted and activate the transcription of specific genes for renal function or differentiation [14, 17, 52]. Hence, the kinetic of renal cell adaption and differentiation goes along with the kinetic of glycerolipids synthesis presented herein. Regarding the mechanism by which high environmental NaCl concentration is sensed and triggers the activation of these pathways is still controversial [53]. However, several reports provide experimental data supporting the role of cytoskeleton and integrin proteins in such action. In nervous cells, extracellular matrix (ECM) hydration, ECM-integrin interaction and cytoskeleton rearrangement are involved in osmosensing process [54]. Thus, hyperosmotic stimulus changes the interaction of ECM proteins with water and cations. Such status activates transient receptor potential (TRP) protein that interacts the transmembrane protein integrin. The full opening of TRPV channel draws water outflow by from intracellular compartment, decreases cell volume and increases the pushing force of actin cytoskeletal network causing cell shrinkage. Following cell shrinkage, a regulatory volume increase (RVI) occurs that includes the increase in cytosolic Ca^{2+} (activated-TRPV channels), the activation of mitogen-activated kinases, and the mobilization of aquaporins from vesicles to the membrane. RVI, reduce microtubule-associated TRPV and hyperosmotic activation of osmosensory neurons occurs [54].

We previously showed that hyperosmolarity reduces PL biosynthetic activity during the first 12 h; but between 12 and 24 h of treatment, PL synthesis is recovered and maintained elevated for 48 h (Fig. 2C and E) [20]. This evidence agrees the fact that high NaCl media induce differentiation of MDCK by 48 h of treatment [15, 16, 20]. Hence, subconfluent cultures subjected to high NaCl are committed to produce new PL molecules to provide membranes to the cells undergoing differentiation. To achieve this

requirement, PL synthesis is activated and remained elevated by the first 48 h. Later, after 72 h, PL synthesis comes down (Fig. 2C and E). The high level of PL *de novo* synthesis was accompanied with the early generation of precursor molecules, PA and DAG (Fig. 3). These molecules are also biosynthetic precursors of TAG whose synthesis become significantly evident after 48 h of hyperosmotic treatment (Fig. 2B and D). Therefore, the DAG formed when NaCl activates *de novo* glyceride biosynthetic process is prioritized for PL more than for TAG synthesis. These findings agree with previous works demonstrating that the destiny of DAG depends on the needs of the cell for synthesizing PC or PE, the main phospholipids in bilayer structure; but when PC or PE synthesis are inhibited, DAG is converted to TAG [55-57]. In our experimental system, PL biosynthetic activity decreased after 72 h of treatment. It is worth to point out that the reduction in DAG synthesis preceded the fall in PL biosynthetic activity. Even though osmotic-induced DAG production fall at 48 h, it is still significantly higher than DAG generation in control cells (Fig. 3A). Therefore, such an excess of DAG could be redirected to TAG synthesis which remains high active at 72 h (Fig. 2B and 2D).

We did not determine the reason for the decrease in PL synthesis but it is probably related to PL biosynthetic enzymes downregulation. It is widely accepted that, CCT, the rate-limiting enzyme of PC synthesis, is activated by high levels of DAG, fatty acids or anionic phospholipid as PA and inactivated by the decrease in these lipids or the increase in PC concentration in ER membranes; CCT is also regulated by the physicochemical state of the bilayers [58, 59]. Therefore, it could be possible that the early production of hyperosmotic-activated PA and DAG synthesis (Fig. 3A) activates CCT and, in consequence, CDP-choline synthesis increases, resulting in PC content augment [20]. When cells reach the adequate PL concentration in endoplasmic reticulum membranes, DAG synthesis declines (Fig. 3A), and CCT activity falls. However, further experiments are necessary to prove this hypothesis. The decrease in DAG (48 h) occurs before PL synthesis decrease (72 h of treatment) (Fig. 2E); however, the level of TAG at 72 h of hyperosmolarity is similar to that observed at 48 h (Fig. 2D). These observations would reinforce the idea that a decreased availability of DAG, due to a diminished lipin activity, would affect the formation of PLs requiring DAG in their biosynthesis like PC and PE; as consequence, a high PA availability could be delivered for phosphatidylinositol (PI) and/or cardiolipin (CL) formation [5]. The fact that TAG levels are not influenced by the availability of DAG could suggest a greater expression and/or affinity of DGAT activity. Concerning to PA metabolism, apart from our previous experiments also performed in MDCK cells [20], the fact that high-NaCl causes the increases glycerolipid synthesis

has been reported by Robciuc et al. [48], who demonstrated that hypertonic medium induces TAG accumulation in human corneal epithelial cells. Thus, we can say that we are demonstrating the effect of high NaCl on the de novo synthesis of phosphatidic acid formation by the first time.

The production of DAG destined to membrane biogenesis is catalyzed by the enzyme phosphatidic acid phosphatase 1 (PAP1), also known as lipin. A growing piece of evidence reveal that lipin proteins are important determinants of lipid homeostasis since they catalyze the conversion of phosphatidic acid (PA) into diacylglycerol (DAG) [60-62], being a branch-point for the synthesis of triglyceride, zwitterionic phospholipids or anionic phospholipids [28]. Apart from regulating both triglyceride and glycerophospholipid synthesis through PAP activity, lipin proteins can regulate fatty acid oxidation enzymes through transcriptional co-activation [63]. Three lipin isoforms have been reported: lipin-1, lipin-2 and lipin-3 [27, 28, 64]. Lipin-1 is highly expressed in white/brown adipose tissue and skeletal muscle where is responsible for PAP activity contributing to TAG synthesis. Despite lipin-1 is also expressed in liver, kidney and brain, its contribution to PAP activity remains unclear. Lipin-2 isoform is highly expressed in kidney, liver and lung, and it is considered the main protagonist of PAP activity in these tissues. In our experimental conditions, lipin-1 as well as lipin-2 are expressed but not lipin-3 (Fig. 3C and D). Even though both lipin-1 and lipin-2 mRNA levels were increased by hyperosmolarity, the rise in the expression of lipin-2 was several times higher than that observed for lipin-1. Moreover, after 48 h of hypertonic treatment lipin-2 increase was almost 20 times of its basal value (Fig. 3D). From our results, we are not able to assert the function of each one of lipin isoforms because the enzyme assay does not discriminate between one or other isoform. Both, lipin-1 and/or lipin-2 could be involved in the formation of DAG for TAG and PL synthesis. It is interesting to mention that it has been reported that among the three mammalian lipin proteins with PAP activity, lipin-1 specific activity is higher than lipin-2 and -3 activities [62]; Knockdown of lipin-2 in isolated hepatocytes reduces PAP activity and TAG synthesis [4], these observations agree with our suggestion that DAG directed to TAG synthesis is mainly produced by lipin-2. In addition, although different isoforms are present in most of the tissues their levels depend on tissue type [65]. In agreement with our results, a major expression of lipin-2 respect to lipin-1 is observed in kidney [66, 67].

After 48 h, TAG synthesis is activated for two reasons. On one hand, when PL needs have been reached, cells must drive the excess of DAG to cellular stores, the lipid droplets (Fig. 1); apart from that, cells must refill pre-existing TAG stores which have probably been depleted in the early PL synthesis. The

present experiments were performed in low serum conditions. Hence, the early synthesis of PL should use stored FA. This fact was clearly evidenced in the experiment showed in Fig. 4; even though FA synthesis was inhibited, PL synthesis continued. However, TAG synthesis dropped demonstrating its requirement for newly synthesized FA. FA synthesis enzymes were upregulated by hyperosmotic media being their expression maximal at 48 h of treatment.

The last step in TAG synthesis is catalyzed by diacylglycerol-acyltransferase (DGAT). Two isoforms, DGAT-1 and DAGT-2 has been reported [4, 68]. Both proteins are expressed in MDCK cells in our experimental conditions, and both seemed to be transcriptionally regulated by hyperosmolarity, showing the maximal induction after 48 h (Fig. 3E and F). Although both isoforms are involved in TAG synthesis in different tissues it has been reported that they use different substrates [68]; thus, DGAT-1 is involved in esterifying exogenous FA taken up by cells from the environment or in re-esterifying exogenous FA into DAG and MAG after lipases action [68, 69]. In contrast, DGAT-2 would be the responsible for incorporating endogenous *de novo* synthetized FA into TAG [68, 70, 71]. These reports support our present results. Thus, early after NaCl addition, PLs synthesis proceeds by using FA from stored TAG. The requirements for membrane biogenesis and the decrease of TAG concentration probably triggers FA *de novo* synthesis with the concomitant increase of DGAT-2 expression which in turn activates the *de novo* synthesis of TAG (Fig. 1, 2 and 3). It is interesting that the maximal expression of DGAT-2 precedes the highest accretion of TAG at 72 h. Although DGAT mRNA level was not measured after 72 h of treatment, we could infer that its activity remains elevated since TAG content is still elevated after 96 h of treatment (not shown).

Transcriptional regulation of lipogenic enzymes could be mediated by several transcription factors. However, SREBP is considered as the lipid master regulator [30, 31]. So, we studied its participation in hyperosmolarity-induced transcriptional activation (Fig. 5 and 6). Fatostatin, a known inhibitor of SREBPs activation [72], blocked the expression of ACC and FASN (known targets of SREBP, not shown) and lipins; however, DGAT expression was not affected (Fig. 5). The fall in lipogenic enzymes was reflected in the decrease in TAG content (Fig. 5B and D) which was probably due to two main events: the fall in TAG synthesis (Fig. 5E) because of an impaired *de novo* FA synthesis, and the utilization of preexisting TAG pool for PL synthesis which was not affected by fatostatin (Fig. 5G), reconfirming the relevance of PL biosynthetic process. Thus, if cells are not able to obtain DAG by *de novo* synthesis they will obtain it from their storage to maintain PL synthesis and membrane renewal.

This fact can be evidenced by the reduction in the size (not the number) of LDs after fatostatin treatment (Fig. 5D). Two main SREBP isoforms have been reported, SREBP1 and SREBP2 [30, 31]. SREBP1c and SREBP2 are subjected to distinct forms of transcriptional regulation, whereas SREBP1a appears to be constitutively expressed at low levels in liver and most other tissues of adult animals [30]. Thus, the increased SREBP1 mRNA levels after 24 h of NaCl treatment could be attributed to the SREBP1c isoform (Fig. 6A and B). Despite the regulation of glycerolipids is classically associated to SREBP1 and that of cholesterol to SREBP2, nowadays it is known that both isoforms can regulate all lipogenic genes depending on the cell type and metabolic state [30, 73]. The analysis of lipid enzyme expression after silencing experiments revealed that only SREBP1 modulates the transcriptional activation of Lipin-1 and -2, and confirm the fact that is not involved in DGAT regulation. The silencing experiments also showed that SREBP1 and SREBP2 regulates their own expression. In addition, our findings showed that the silencing of SREBP1 gene abrogates SREBP2 (Fig. 6), indicating that SREBP1 protein is necessary for SREBP2 expression. The transcriptional regulation of the SREBPs genes is a complex process that involves several transcription factors including their own proteins since they have SREs present in the enhancer/promoters of each gene   [30, 74, 75]. Thus, it is possible that hyperosmolarity activates SREBP1 to provide cells with the adequate set of lipogenic enzymes that carry on the synthesis of glycerolipids. But, the generation of new membranes also requires cholesterol to balance lipid composition at the endoplasmic reticulum; therefore, an extra activity of SREBP2 could be needed, being its synthesis induced by SREBP1. However, more experiments are needed to prove this theory.

The increase in SREBP activity can be evidenced by an increase in its expression (Fig. 6A and B), an increase in its redistribution from cytoplasmic to nuclear compartment (Fig. 6C) and an increase in the expression of targets genes (Fig. 6G and H). The SREBP is synthetized as a 128 kDa precursor protein which is bound to the endoplasmic reticulum and to the nuclear envelope by SCAP protein (SREBP cleavage-activating protein). Upon activation, the SREBP/SCAP complex migrates to the Golgi and there, the active 68 kDa mature SREBP is released by site 1 protease (S1P) and site 2 protease (S2P) in a sequential cleavage process. Mature SREBP translocates from cytoplasm to nucleoplasm and promotes the transcription of many lipogenic genes [31]. The activity of SREBP could be modulated by post-translational modifications such as phosphorylation by different kinases. It has been reported that SREBP1 is negatively regulated by GSK3-β, AMPK and protein kinase A phosphorylation [76, 77] and it

is activated by p38, ERK and JNK [78, 79]. Also, focal adhesion kinases, like FAK and c-Scr, have been found to activate SREBP by phosphorylation [80].

In renal cells, it has been demonstrated that cell shrinkage activates p38 and ERK MAPKs [81]. Modifications of cellular architecture related to hypertonicity-induced cell shrinkage are associated with a reorganization of the architecture of the actin cytoskeleton and with changes in the F-actin-G-actin equilibrium. Specific cytoskeleton components may sense cell volume decrease and initiate signaling cascades leading to RVI. In addition, signal transduction cascades leading to remodeling of the actin cytoskeleton and to MAPK activation share some common elements [82]. In MDCK cells, hyperosmolarity induces cell shrinkage with actin cytoskeleton reorganization involving AMPK activity [83]. Recently, Neuhofer et al. reported that focal adhesion kinase (FAK), a widely expressed non-receptor protein tyrosine kinase intimately involved in integrin-mediated signaling, transduces mechanical forces, such as are present during cell shrinkage in cells exposed to osmotic stress, into intracellular signals to elicit adaptive cellular responses. In MDCK we demonstrated that ERK1/2 MAPK is activated by hypertonicity and such activation is required for Kennedy pathway enzyme expression [20]. Thus, we can hypothesize that hyperosmolarity causes rearrangements in actin cortical cytoskeleton and its associated proteins. These events in turn activates different protein kinases-mediated signaling pathways leading to SREBP activation and the expression of its target genes which could be involved in renal glycerolipid synthesis and cell differentiation. However, such a sequence should be proven.

Summarizing, in this work we studied the molecular mechanisms underlying membrane biogenesis in cells subjected to hyperosmolarity. The relationship between PL and TAG *de novo* synthesis dynamics was evaluated. In particular, we were interested in understanding the initial stage in glycerolipid synthesis since both utilize the same biosynthetic route: *sn*-glycerol 3P (G3P)$\rightarrow$ phosphatidic acid (PA) $\rightarrow$ diacylglycerol (DAG). The results presented herein show that renal cells activate a synchronized program to coordinate glycerolipid synthesis to provide themselves an adequate membrane extension, and to guarantee the provision of substrates. Our results clearly demonstrate the relevance of an adequate store of TAG for maintaining membrane homeostasis even in the absence of substrates.

Conflict of interest.

The authors declare that they have no conflicts of interest with the contents of this article.

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Figure Legends

Figure 1. *Hyperosmolarity increases triglyceride (TAG) cell content.* MDCK cells were grown in a mixture containing DMEM/ Ham´s-F12 (1:1), 10 % FBS and 1 % antibiotic mixture. After reaching 70-80 % of confluence, cells were placed in low serum medium (0.5 % FBS) for 24 h and then subjected to different NaCl concentrations (**A**), or incubated for different periods of time in a medium in the absence (Iso) or in the presence of additional 125 mM NaCl (**B**). After treatment, cells were collected and lipids were analyzed as described in Methods. (**C**) In other set of experiments, cells were grown on cover slips and subjected to 125 mM NaCl for 72 h. Then, cells were fixed and *stained* with Oil red O (ORO) as was described in Methods. Differential Interference Contrast (DIC) and fluorescence images were obtained with a Nikon Eclipse Ti with acquisition software Micrometrics SE Premium (Accu-Scope). (**D**) The area and the number of lipid droplets/cell were estimated by using ImageJ software. The results are expressed as the mean ± SEM of 5 independent experiments.

Figure 2. *Triglyceride (TAG) and Phospholipid (PL) synthesis (A) is upregulated by hyperosmolarity.* MDCK cells were grown in a mixture containing DMEM/ Ham´s-F12 (1:1), 10 % FBS and 1 % antibiotic mixture. After reaching 70-80 % of confluence, cells were placed in low serum medium (0.5 % FBS) and then subjected to 125 mM NaCl for 24, 48 and 72 h; 2, 4 or 6 hours before the treatment finished, 2 μCi/ml [U-^{14}C]-glycerol was added to each well. After labeling, cells were collected and lipids were analyzed as described in Methods. The radioactivity incorporated to each lipid was visualized by radioautography and quantified by liquid scintillation counting. The results, expressed as pmol of ^{14}C-gly-TAG (**B**) or ^{14}C-gly-PL (**C**) produced per 10^6 cells, represent the mean ± SEM of 5 independent experiments. Panels **D** and **E** represent the rates of TAG and PL biosynthesis in isosmolar and hyperosmolar conditions. Abbreviations: G3P, *sn*-glycerol 3P; FACoA, fatty acylCoA; PA, phosphatidic acid; Pi, orthophosphate.

Figure 3. *Lipid metabolic intermediaries PA and DAG are upregulated by hyperosmolarity.* MDCK cells were grown as described above and then subjected to 125 mM NaCl for 24 and 48 h; 4 h before the treatment finished, 2 μCi/ml [U-^{14}C]-glycerol was added. After labeling, cells were collected and lipids analyzed as described in Methods. The radioactivity incorporated to each lipid was quantified by liquid

scintillation counting. The results, expressed as pmol of ^{14}C-gly-PA (**A**) or ^{14}C-gly-DAG (**A**) produced per 10^6 cells, represent the mean ± SEM of 5 independent experiments. In other set of experiments, after hyperosmotic treatment, cells were collected and used for the lipin activity assay (**B**) or for RNA extraction and qPCR assays (**C, D, E** and **F**). The graphs are representative of 3 independent experiments.

Figure 4. *Hyperosmotic induced-lipid synthesis requires fatty acid de novo synthesis.* MDCK cells were grown as described above and then subjected to 125 mM NaCl for different periods of time. In one set of experiments, the expression of fatty acid synthesizing enzymes, ACC and FASN was determined by q- and RT-PCR (panels **A**, **B** and **C**). In other set of experiments, 30 min before adding NaCl, cells were treated with different concentrations of TOFA (ACC inhibitor, panels **D** and **F**) or cerulenin (FASN inhibitor, panels **E** and **G**); 3 h before the treatment finished, 2 µCi/ml [U-^{14}C]-glycerol was added. After labeling, cells were collected and lipids analyzed as described in Methods. The results, expressed as pmol of ^{14}C-gly-TAG (**D** an**d E**) or ^{14}C-gly-PL (**F** and **G**) produced per 10^6 cells, represent the mean ± SEM of 3 independent experiments. In the *inserts* of figures D and E are represented the effect of inhibitors on endogenous content of TAG. The images are representative of 3 independent experiments. The cartoon represents the *de novo* biosynthetic pathway where the molecules or enzymes analyzed in these studies are highlighted.
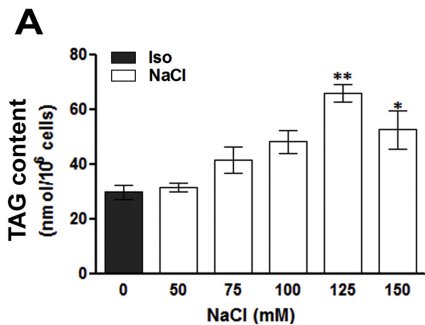
Figure 5. *Effect of fatostatin on hyperosmotic induced-lipid synthesis.* MDCK cells were grown as described above and then subjected to 125 mM NaCl for 24 and 48 h. In one set of experiments, 30 min before adding NaCl, cells were treated with 20 µM fatostatin (Fato) and determined endogenous content (panel **A** and **B)** or 3 h before the treatment finished, 2 µCi/ml [U-^{14}C]-glycerol was added. After labeling, cells were collected and lipids analyzed as described in Methods. The results, expressed as pmol of ^{14}C-gly-TAG, ^{14}C-gly-DAG or ^{14}C-gly-PL produced per 10^6 cells, represent the mean ± SEM of 3 independent experiments (panels **E, F** and **G**). In other set of experiments, after hyperosmotic treatment, cells were collected and used for RNA extraction and qPCR assays. The images are representative of 3 independent experiments (panel **H, I, J** and **K**). Panels **C** and **D** shows phalloidin-FITC-oil red O staining of cells treated with fatostatin (**C**) and the quantitative analysis of LDs by using Image J software.

Figure 6. *SREBP1 and SREBP2 mediate hyperosmotic induced-transcriptional activation of lipid genes.* MDCK cells were grown as described above and then subjected to 125 mM NaCl for 4, 8 and 24 h. After treatment, cells were collected and used for RNA extraction and PCR analysis of SREBP1 and SREBP2 (**A** and **B**). Cells grown on cover slips that received similar treatment were used for microscopy studies (**C**). In other set of experiments, cells were transfected with SREBP small interference RNA (siRNA) before to the addition of hyperosmotic media. After treatment, cells were collected and used for RNA extraction and PCR analysis of SREBP1 and 2 (**D** and **E**) or lipogenic enzymes (**G** and **H**). Cells grown on cover slips that received same treatment were used for microscopy studies (**F**). The results represent the mean ± SEM of 3 independent experiments. *p < 0.05, vs. NaCl values.

Highlights

- Hyperosmolality activates glycerolipid metabolism.

- Hyperosmotic renal cell cultures prioritize phospholipid (PL) synthesis.

- The fate of the common substrate diacylglycerol (DAG) depends on cell membrane demand.

- Triglycerides (TAG) are main actors in maintaining phospholipid synthesis.

Figure 1

Figure 2

A

[^{14}C-Gly]-Lipids
(pmol/10^6 cells)

24 h   48 h

** ** ** **

\#   p<0.001, DAG, NaCl values: 48 vs. 24 h.
** p<0.001, NaCl vs Iso values.

☐ PA: Iso
■ PA: NaCl
▨ DAG: Iso
▨ DAG: NaCl

B

Lipin Activity
(nmol DAG·mg protein^{-1}·h^{-1})

24 h   48 h

** * \#

** p<0.001, NaCl vs. Iso values.
*   p<0.001, Iso 48 h vs. Iso 24 h.
\#   p<0.001, NaCl 48 h vs. NaCl 24 h.

■ Iso
☐ NaCl

*G*3P
↓
PA
LIPIN →Pi
↓
DAG
DGAT   CPT/EPT
↓   ↓
TAG   PC/PE

C

Lipin-1/β-Actin

■ Iso
☐ NaCl

Iso 6 12 24 48 h

NaCl

*p<0.003 vs Iso

D

Lipin-2/β-Actin

■ Iso
☐ NaCl

*   *

Iso 6 12 24 48 h

NaCl

*p<0.02 vs Iso

E

DGAT-1/β-Actin

■ Iso
☐ NaCl

*

Iso 6 12 24 48 h

NaCl

*p<0.01 vs Iso
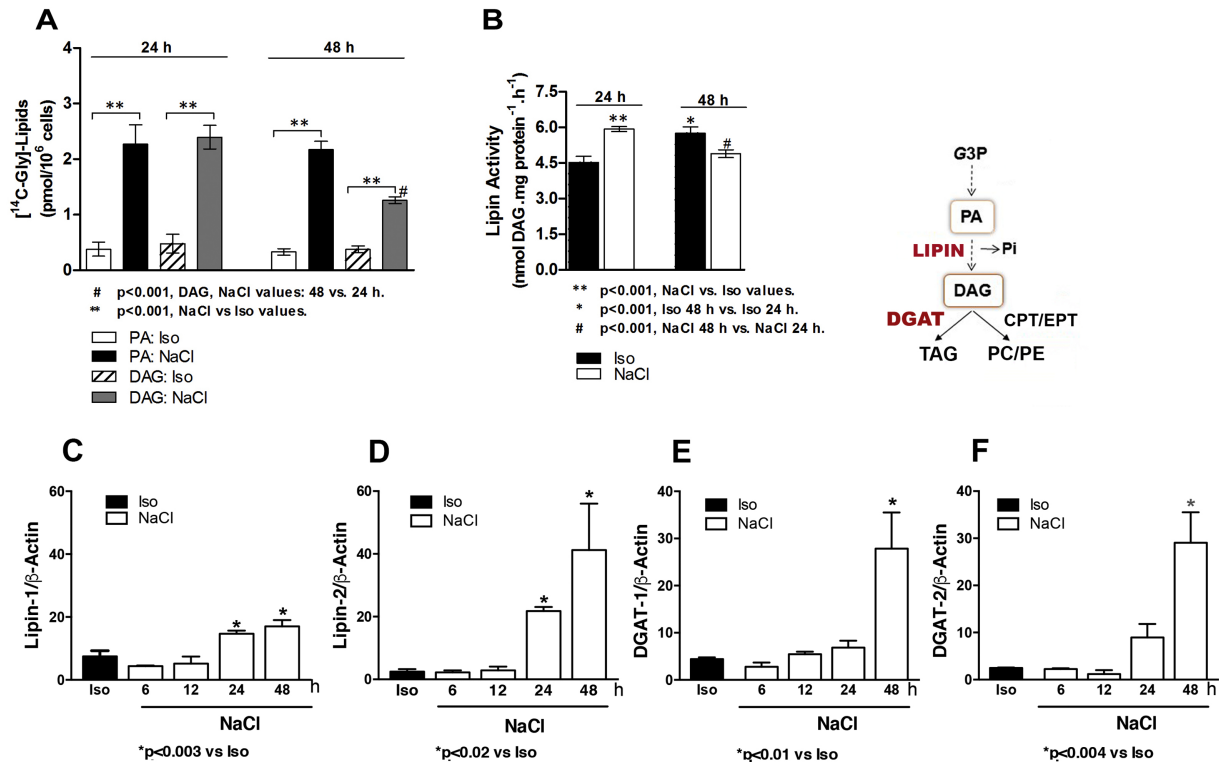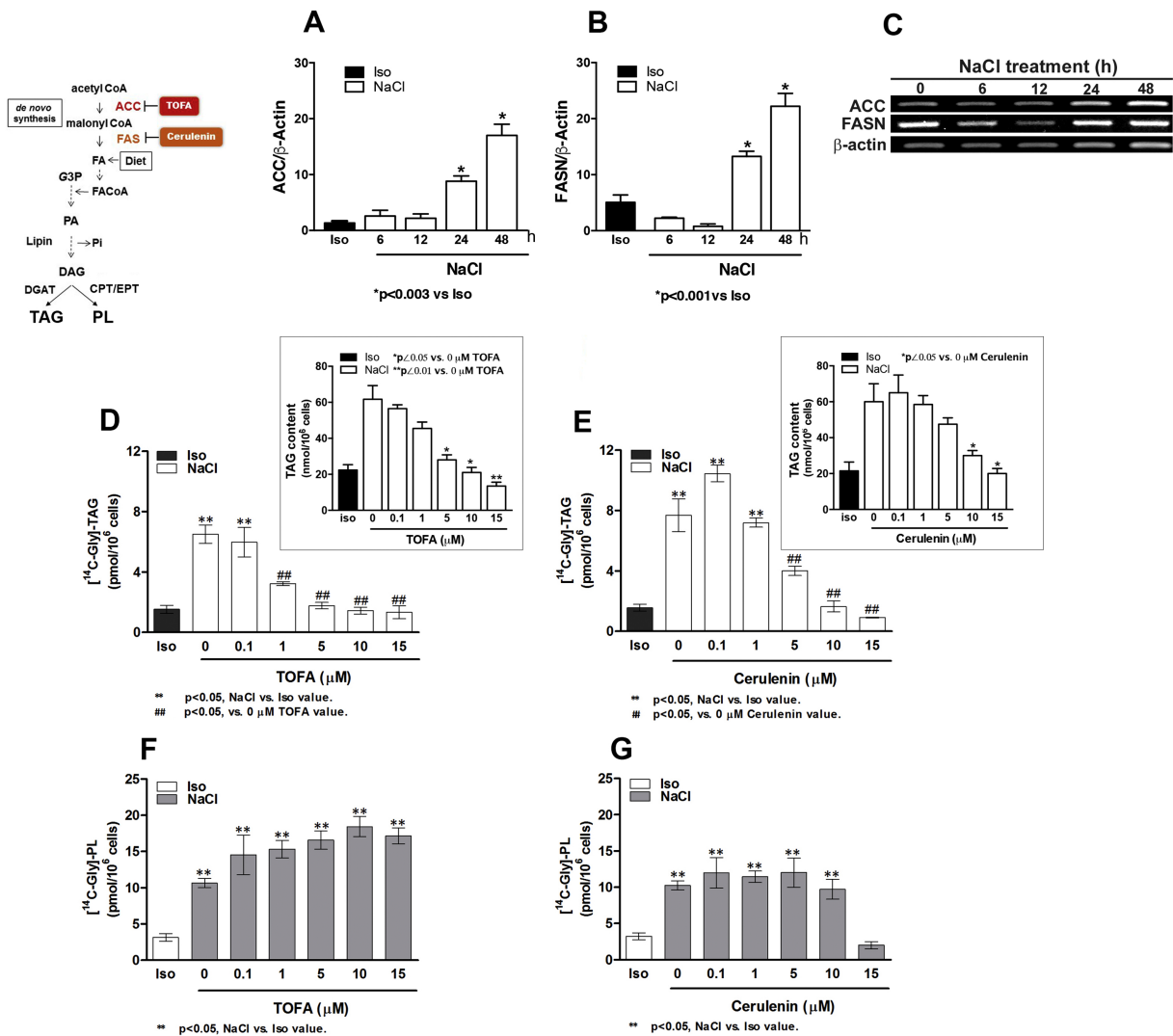
F

DGAT-2/β-Actin

■ Iso
☐ NaCl

*

Iso 6 12 24 48 h

NaCl

*p<0.004 vs Iso
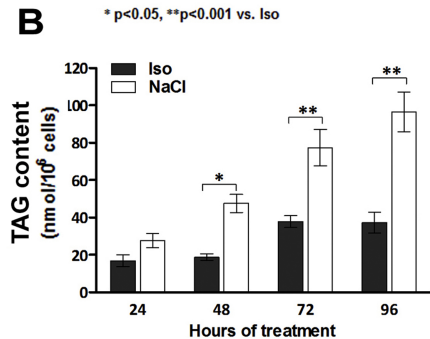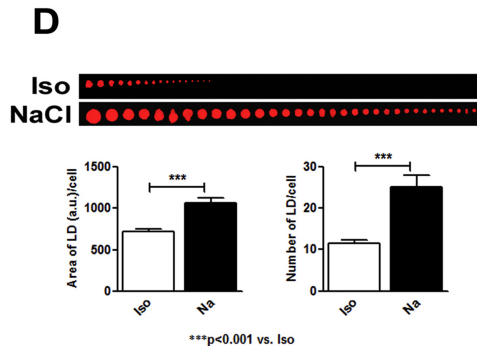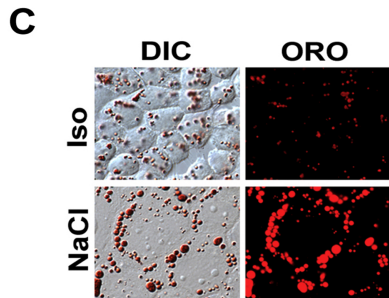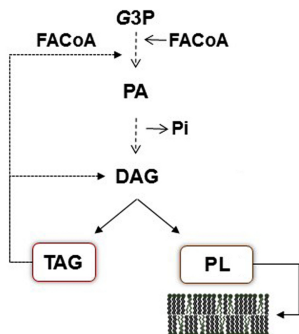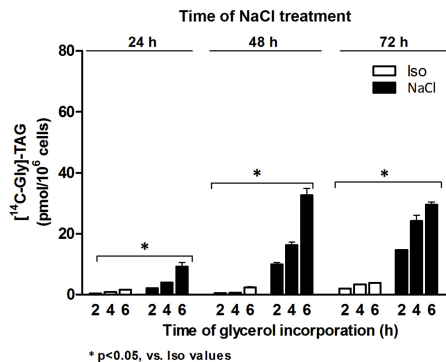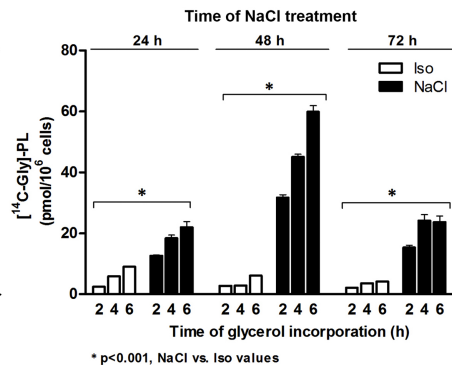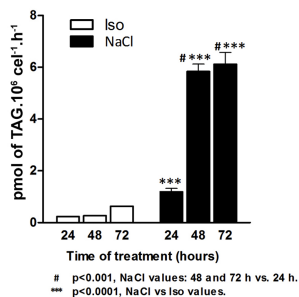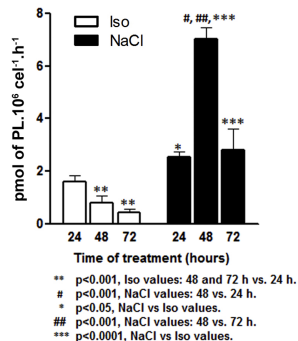
Figure 3

Figure 4

Figure 5

Figure 6