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The hemolytic toxin α-hemolysin (HlyA) is a virulence factor produced by several strains of Escherichia coli. It is involved in urinary tract infections, peritonitis, meningitis and septicemia. HlyA causes lysis of mammalian cells, including human erythrocytes (RBCs).

In this work, we studied the extent to which changes in cell volume kinetics and the concentration of intracellular diffusible ions affect cell volume regulation of RBCs exposed to HlyA. RBCs from healthy donors and from aquaporin 1 KOs (AQP1KO) individuals were used.

Cells at hematocrit 10% were treated with 0.1 ng/µl HlyA or vehicle at 37 °C for 1, 5 and 10 min. Cell volume was measured using the coulter counter principle, while cell volume changes at short periods of time were assessed by light scattering using a stopped flow rapid mixing equipment. For stopped flow experiments exponential curves were fitted to data and best fit exponential coefficients (k) were used to compare treatments. Intracellular concentrations of sodium and potassium were evaluated by flame photometry of lysed RBCs. HlyA induced changes in volume and shape of RBCs, which shrunk during the first min followed by continuous swelling, reaching 10% over control values after 10 min. Under a similar treatment, volume changes correlated with a 5-8 fold increase of intracellular Na+ and 5-7 fold decrease of intracellular K+ concentrations.

In wild type RBCs, cell volume kinetics was affected by toxin treatment. Values of k were 8.5 ± 0.5 seg-1 for untreated cells vs 6.5 ± 0.4, 4.1 ±0.4 and 3.4 ±0.3 seg-1 for cells treated with the toxin for 1, 5 and 10 min, respectively. In the absence of toxin, k values were 5 fold lower for AQP1KO cells, as compared to wild type cells. Nevertheless, the relative decrease of k values upon HlyA exposure was similar for both types of cells.

HlyA dependent decrease of volume, followed by swelling, correlated with acute and simultaneous Na+ uptake and K+ efflux. Values of the kinetic constant k decrease with time of exposure to the toxin, irrespective of the presence of aquaporin 1 in the cells.

FC3. Bioassay standardization to assess exosomes anti-inflammatory activity in vitro.

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Exosomes (Exo) are small sized-extracellular vesicles (40-150 nm), released by almost all kind of cells, and play a major role in cell-to-cell communication. In the last years, they have drawn attention due to its potential application in clinical diagnostics and therapeutics. However, determining their biological activity and potency has proven difficult, owing to the lack of biological assays. Here, we standardized an in vitro assay to assess the anti-inflammatory potential of mesenchymal stem cells (MSCs)-derived exosomes based on their ability to prevent the acquisition of the M1 phenotype in LPS-stimulated RAW264.7 macrophages. M1 phenotype was characterized by the induction of IL-1β, IL-6 and iNOS as determined using qRT-PCR. Nitric oxide (NO) released by iNOS turns into NO2-, that can be easily quantitated in the culture media by Griess reaction. Moreover, phenol red present in culture media did not interfere in the spectrophotometric detection of NO2-. Thus, we first tested different assay conditions in 96-well-plates, including two seeding densities (2x104 and 4x104 cells), four LPS doses (1, 10, 100 and 1000 ng/ml) and two time-points (16 and 24 h), in order to determine the best set-up to accurately measure NO2- production as a marker of M1 macrophage polarization. We found that seeding 2x104 cells/well and stimulating with 10 ng/ml LPS for 16 h allowed us to inhibit the inflammatory response by 60% using Dexamethasone (1 ug/ml). Using these established conditions, we were able to test different exosomes preparations in sextuplicate (5 µg protein/well) and to rank then by their anti-inflammatory activity. Finally, conditioned media containing NO2- can be processed immediately or stored at -20°C, as we found that NO2- is stable in culture media. In summary, we standardized a quick, cheap and reproducible in vitro macrophage assay that allows evaluating and estimating the anti-inflammatory activity of MSCs-derived exosomes. The assay is convenient for comparing multiple samples and, therefore, should be useful in developing protocols to improve the purification and characterization of anti-inflammatory exosomes.

FC4. Aquaporin-4 facilitates cell proliferation in retinal Müller cells: Implications in Neuromyelitis Optica.

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Müller cells are involved in controlling extracellular homeostasis in the retina, regulating cell swelling by a regulatory volume decrease (RVD) mechanism that depends on the efflux of solutes and water through Aquaporin-4 (AQP4). Müller cells are also important for retinal integrity, as they respond to injury by re-entering the cell cycle for tissue repair. Since AQP4 was reported to modulate cell volume during cell cycle progression and facilitate proliferation in astrocytes, the aim of this study was to evaluate, using the novel inhibitor TGN-020, if AQP4 was involved in human Müller cells' proliferation in physiological conditions. Considering that AQP4 is the target of autoantibody IgG-NMO present in the sera of patients with Neuromyelitis Optica (NMO), we also evaluated if cell proliferation was altered in the presence of IgG-NMO. MIO-M1 human Müller cells were exposed to 100 nM TNG-020 or vehicle or to 1/50 dilution of IgG-NMO positive or control sera. Cell volume (videomicroscopy) and cell proliferation (cell count, cell cycle analysis by flow cytometry and BrdU incorporation by immunofluorescence) were measured. AQP4 inhibition with TGN-020 reduced osmotic water permeability (Pf, µm/s) from 20.3±1.2 to 12.2±0.4 (n=5, p<0.001) and %RVD 15min from 54±4 to 17±3 (n=5, p<0.001). MIO-M1 cell proliferation was decreased by TGN-020 (doubling time in hours, control vs. TGN-020: 31±1 vs. 40±3, n=4, p<0.05) without affecting cell viability. TGN-020 also increased the % of cells in G1/G0 phase, decreased the S phase of cell cycle and reduced BrdU incorporation by 20%. IgG-NMO positive sera decreased AQP4 plasma membrane expression in MIO-M1 cells, reducing Pf from 22.4±1.5 to 15.9±0.6 µm/s (n=6, p<0.001) and %RVD 15min from 66±5 to 48±4 (n=6, p<0.005), as well as cell proliferation (doubling time in hours, control vs. IgG-NMO: 59±5 vs. 86±4, n=3, p<0.05) in comparison to control sera. We

propose that inhibition or removal of AQP4 from the plasma membrane reduces AQP4-mediated water permeability altering cell proliferation. This is of particular importance in NMO, as the decreased ability of Müller cells to proliferate may affect retinal tissue repair.

FC5. Regulation of oxidative stress on the activity of Nrf-2 factor and the expression of antioxidant enzymes in lymphoid cells of a murine model of hyperthyroidism.

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Introduction: Hyperthyroidism is an endocrine disorder characterized by excessive secretion of T3 and T4 and low levels of TSH. Thyroid hormones exert pleiotropic actions on numerous tissues and induce an overall increase in metabolism, with an increase in energy demand and oxygen consumption. Objectives: Study the effect of oxidative stress on the activation of the nuclear factor Nrf-2 and the transcription of antioxidant enzymes in lymph node and spleen cells of euthyroid and hyperthyroid mice. Methodology: Hyperthyroidism was induced in Balb/c mice by treatment with 12 mg/l of T4 in drinking water for 30 days. Reactive oxygen species (ROS) were evaluated using the fluorescent probe DCFH-DA and flow cytometry. The expression of catalase (CAT), glutathione peroxidase-1 (GPx-1) and superoxide dismutase (SOD) was determined by PCR and western blot. Nrf-2 phosphorylation and its translocation to the nucleus were evaluated by western blot and confocal microscopy, respectively. Protein kinase C (PKC) activity was evaluated by measuring the incorporation of 32P into histone H1. PKC isoenzyme expression and the extracellular signal-regulated kinase (ERK) phosphorylation was evaluated by western blot. Results: We found a significant increase in the genomic and protein expression of CAT and GPx-1 in lymph node and spleen cells of hyperthyroid mice that was correlated with the increase in the ROS production. Lymphoid cells of euthyroid mice treated in vitro with H2O2 (250 μM) increased the expression of antioxidant enzymes. The hyperthyroidism increased the phosphorylation levels of Nrf-2 and ERK and the kinase activity of the classic isoenzymes of PKC (α, β and γ). Nrf-2 phosphorylation was decreased by preincubation of lymphoid cells with staurosporine (PKC inhibitor; 5 nM) or PD 98059 (ERK inhibitor; 20 μM). Conclusions: Hyperthyroidism increases ROS and kinase activity of PKC and ERK. The activation of these transduction signals leads to phosphorylation and translocation of Nfr-2 to the nucleus where it induces the transcription of antioxidant enzymes. These results indicate the modulation of hyperthyroidism of the cellular antioxidant system.

FC6. Aquaporin-2 and Na+/H+ exchanger isoform 1 modulate the efficiency of renal cell migration.

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Aquaporin-2 (AQP2), in addition to its canonical role as a water channel, promotes renal cell migration by the modulation of integrin β1 trafficking and the turnover of focal adhesions. This novel role described for AQP2 opens the possibility to further investigate if AQP2 also works in concert with other components of the cell migration machinery. Na+/H+ exchanger isoform 1 (NHE1) is a well-known protein involved in the regulation of cell migration, which is proposed to act at the leading-edge membrane to direct migration. NHE1 activity is highly modulated by Ca2+ and we recently showed a physical interaction between AQP2 and the Ca2+ channel TRPV4. Then, the aim of our work was to investigate the possible crosstalk between AQP2, its mechanosensitive partner TRPV4, and NHE1 to regulate cell migration. We used two renal cell models: one not expressing AQPs and another one expressing AQP2. We performed wound healing and cell tracking assays to evaluate cell migration; immunofluorescence assays to evaluate lamellipodia volume, focal adhesions, and assembly of F-actin; and fluorescence videomicroscopy to measure lamellipodia pHi and NHE activity. Our results confirm that AQP2 promotes renal cell migration during wound closure, AQP2-expressing cells follow a less tortuous route compared with AQP2-null cells. Lamellipodia of AQP2-expressing cells exhibit significantly smaller volumes and size of focal adhesions and more alkaline pHi due to increased NHE1 activity than AQP2-null cells. The blockage of AQP2 or TRPV4 significantly reduced lamellipodia NHE1 activity. Also, the blockage of NHE1 significantly reduced the rate of cell migration, the number of lamellipodia and the assembly of F-actin only in AQP2-expressing cells. Altogether these results let us propose that during lamellipodia protrusion the presence of AQP2 activates its partner TRPV4, leading to Ca2+ entry and to the consequent activation of NHE1. It is likely that the interplay between AQP2, TRPV4, and NHE1 defines the pH dependent-actin polymerization, providing mechanical stability to delineate lamellipodia structure and consequently the speed and directionality of cells, promoting the migration.

FC7. Release of ATP by TRPV4 activation is dependent upon the expression of AQP2 in renal cells.

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The involvement of purinergic signalling in kidney physiology and pathophysiology is rapidly gaining recognition. Purinergic signalling influences water and electrolyte transport in all segments of the renal tubule. In several tissues, there is increasing evidence that ATP release is dependent upon activation of the transient receptor potential cation channel (TRPV4). Because we have recently found that TRPV4 physical and functional interacts with the water channel AQP2 in cortical collecting ducts cells (CCD) (1), the aim of this work was to examine the possibility that TRPV4/AQP2 interaction influences ATP release in these cells. We used two rat CCD cell lines expressing AQP2 (AQP2-RCCD1) or not (WT-RCCD1). Extracellular ATP (ATPe) measurements were carried out with cells laid on coverslips that were mounted in the assay chamber of a custom-built luminometer. Cells were stimulated with the specific TRPV4 activator GSK1016790A (GSK, 10 nM) and ATPe was measured using firefly luciferase. We found that GSK stimulate ATP release only in AQP2- expressing cells (MaxAQP2 = 222.9 ± 32 nM (n=10)). ATP release stimulated by GSK in AQP2-RCCD1 cells was inhibited by the TRPV4 specific antagonist HC-