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Inflammation Research

Hyperosmolarity causes inflammation through the methylation of protein phosphatase 2A

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Abstract. *Objective and Design:* We evaluated the role of the osmolarity in the pro-inflammatory responses of epithelial cells.
Material: Twenty-five female Wistar rats and colorectal (HT-29) and bladder (T24) cell lines were used.
Treatments: Rats and cells were exposed for 48 hours to hyperosmotic solutions.
Methods: Interleukin-8 (IL-8) production was measured by Enzyme Linked ImmunoSorbent Assay, mRNA transcription of pro-inflammatory cytokines by microarrays or RNase Protection Assay. Nuclear factor-kappa B (NF-κB) pathway and Protein Phosphatase 2A (PP2A) activations were measured. Myeloperoxydase (MPO) activation and Macrophage-Inflammatory Protein-2 (MIP-2) transcription were monitored.
Results: The exposure to hyperosmotic solutions enhanced the production of IL-8 and induced pro-inflammatory cytokines transcription. *In vivo*, MPO enhanced activity accompanied by an increased MIP-2 transcription was observed. *In vitro*, NF-κB activation is accompanied by an inhibitor of kappa B-alpha degradation and inhibitor of kappa B kinase (IKKγ) activation. We demonstrated the induction of IKKγ after methylation and activation of PP2A. Cytokine induction was inhibited by okadaic acid and calyculin A and stimulated by xylitol.
Conclusion: Hyperosmolarity can induce pro-inflammatory cytokine responses in colorectal and bladder epithelial cells. Inflammation appears to be the simple consequence of a shift of methylation of PP2A which in turn activates NF-κB.

Key words: Inflammatory mediators – Cytokines – In vivo inflammation – Intracellular signalling – NFκB.

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Introduction

Recent evidence indicates that epithelial cells (ECs) act as an immunologically important organ. They play a central role in orchestrating the immune responses to both exogenous and endogenous antigens by initiating a very efficient machinery of cytokine and chemokine production [1–5]. For example, human bladder ECs produce inflammatory chemokines such as interleukine-8 (IL-8) and Monocyte Chemoattractant Protein-1 (MCP-1) after intravesical administration of Bacillus Calmette-Guérin (BCG) [6–8] and colonic cells secrete pro-inflammatory chemokines during inflammatory bowel disease [9, 10].

In addition to classic inflammatory signals represented by bacteria, bacterial by-products or immune cell-derived cytokines, many non-immune factors are known to elicit the upregulation of pro-inflammatory molecules by ECs. The secretion of pro-inflammatory chemokines can be induced by burn [11], hypoxia [12], acidosis or hyperosmolarity [13–15]. Németh demonstrated that exposure of HT-29 and Caco-2 cells to hyperosmolarity (mannitol and NaCl) resulted in an increased secretion of IL-8 and Nuclear factor-kappa B (NF-κB) activity [16]. These results were confirmed by Hubert who reported that hyperosmolarity induced up to a fivefold increase in the production of IL-8 in Caco-2 cells [17]. Similarly, Loitsch and Hashimoto showed IL-8 production in bronchial epithelial cell cultures in response to hyperosmolarity [18, 19]. The idea that hyperosmolarity may regulate the ECs' production of pro-inflammatory cytokines is based on evidence that this stimulus has been shown to contribute to the inflammation in Crohn's disease, inflammatory bowel disease and neonatal necrotizing enterocolitis [20, 21]. The precise mechanism of action of hyperosmolarity and its clinical relevance remain however unclear.

Up to now, there have not been any reports published on the mechanistic effects of osmotic shock on the activation of NF-κB. Interestingly, a regulator of NF-κB has been recently reported that plays an important role in cancer development: Protein Phosphatase 2A (PP2A) [22–24] which is activated by methylation [25, 26].

In the present study, we evaluated the role of hyperosmotic stresses in the pro-inflammatory responses of colorectal and bladder epithelial cells both *in vitro* and *in vivo*. We also analyzed the NF-κB and PP2A pathways potentially involved.

Materials and Methods

Hyperosmolar Solutions

To test the effect of osmolarity on mucosal surfaces, mannitol, NaCl or L-alanine (all from Sigma-Aldrich, Saint-Quentin Fallavier, France) were dissolved in a Krebs-Henseleit solution. Final osmolar solutions of 300, 600, and 900 mOsm were prepared in complete DMEM medium (Invitrogen, Cergy Pontoise, France) supplemented with 10 % decomplemented FBS (Eurobio, Les Ulis, France) and 1 % non-essential amino acids. The osmolarity was measured using a cryoscopic osmometer (Osmomat 030**,** GONOTEC GmbH, Berlin, Germany).

Animal Treatment

The animals were treated in accordance with the European Community's guidelines concerning the care and use of laboratory animals. Female Wistar rats, 15 weeks of age (5 rats per group) were used to analyze the effect of increased osmolarity on the rectal epithelium. Animals had been deprived of food 24 h before rectal administration. Just before each administration, animals were anaesthetized intramuscularly by a combination of xylazine hydrochloride (Rompun, Bayer; 4.5 mg/kg) and ketamine hydrochloride (Imalgene, Merial; 90 mg/kg) as described previously [27]. The rats were instilled rectally with 500 µl of NaCL 0,09 % (isosmotic), hyperosmolar mannitol or NaCl solutions to final concentrations of 300 or 600. Twenty-four hours after administration, rats were sacrificed by intraperitoneal injection of 1 ml urethane (0,625 mg/ml, Sigma-Aldrich) and proximal and distal colon segments were removed, opened and scraped in 1000 µl ice cold phosphate buffer for whole colonic cells. Scraped suspensions were centrifuged (1000 rpm for 3 min) and supernatants were frozen at –20 °C. Cell pellets were stocked or lyzed by Ripa buffer (Sigma).

Cell Lines and Reagents

The human colon cancer cell line, HT-29 (ATCC, LGC Promochem, Molsheim, France) and the human bladder cancer cell line T24 (ATCC) were cultured in DMEM supplemented with 10 % decomplemented FBS and 1 % non-essential amino acids, in a humid atmosphere with 5 % CO_2 at 37 °C. All experiments were performed on cells that had reached confluence with a seed number of one million cells for HT-29 cells and of 4 x 10^5 cells for T24 cells per 25 cm^2 flask. After confluence, the media were replaced by hyperosmolar solutions. After 6, 12, 24 and 48 h exposure, the supernatants were centrifuged and frozen at –20 °C. Where applicable, the first 40 minutes of incubation, DMSO-dissolved calyculin A (100 nM) (Cell Signaling Technology, Danvers, MA) or Okadaic acid potassium salt (200 nM) (Calbiochem, VWR, Fontenay sous Bois, France) were diluted in the culture medium. Adequate controls were performed in non-treated cells. Where applicable, xylitol 100 µM (Sigma) was added to the culture medium for the same duration.

MPO Assay

Tissue-associated Myeloperoxydase (MPO) activity was determined using the standard enzymatic assay. The cell stock pellets were resuspended in phosphate buffer. After three washes, the pellets were then solubilised in 10 volumes of ice-cold 0.5 % hexadecyltrimethylammonium bromide in 50 mM phosphate buffer and sonified to solubilise the enzyme. The sonicated extracts were allowed to stand at 4 °C for 20 min and then centrifuged at 10,000 g for 15 min at 4 °C. MPO activity in the supernatants were then assayed by mixing 0.1 ml of the supernatant with 2.9 ml of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml of s-dianisidine dihydrochloride and 0.0005 % hydrogen peroxide. The change in absorbance at 460 nm was measured spectrophotometrically over 3 min, and one unit of MPO activity was defined as that degrading 1 µmol of hydrogen peroxide per minute.

MIP-2 RT-PCR

Methods for real-time RT-PCR have been described in detail by Heid [28] and especially for rats by Isowa [29]. Total RNA was prepared from scraped cell suspensions by using the RNeasy Qiagen Kit according to the manufacturer's instructions (Qiagen, Hamburg, Germany). For gene specific PCR, equal amounts of cDNA samples were amplified in 20 µl reaction volumes containing 200 µM dNTP, 1 U Taq DNA polymerase (Qiagen) and 1 µM primers during 35 cycles (30s denaturation, 94 °C; 30s annealing, 55 °C and 30 s polymerization, 72 °C) with an Omnigene temperature cycler (Hybaid, Ashford, UK). The primers were purchased from Proligo Primers & Probes (Paris, France). The forward primer for β-actin was 5'-GTGGGCCGCTCTAGGCAC CAA-3', and the reverse primer was 5'-CTCTTTGATGTCACGCAGGATTTC-3'. The forward PCR primer for MIP-2 was 5'-ATGCTGTACTGGTCCTGCTCCT-3', and the reverse primer was 5'-CTTCAGGGTTGAGACAAACTTCA-3'. Relative mRNA levels were calculated after normalizing to β-actin.

IL-8 Quantification

IL-8 secretion into the supernatants of control or treated HT-29 or T24 cells was quantified by Enzyme Linked ImmunoSorbent Assay (ELISA) by using DuoSet ELISA kit (R&D Systems, Minneapolis, MN) for human IL-8, according to the manufacturer's instructions. Colorimetric results were read in a MRX Dynatech Microplate reader (Dynatech, Chantilly, VA) at a wavelength of 450 nm in 96-well high-binding Stripwell Costar EIA microplates (Costar 2592). Substrate Reagent Pack (Catalog # DY999, R&D Systems, Minneapolis, MN) was used for all Streptavidin-HRP reactions. Each sample was assayed in triplicate. The sensitivity limit of the assay was less than 3.5 pg/ml.

RNase Protection Assays (RPA)

HT29 or T24 cells were exposed for 24 h to 900 mOsm mannitol or control media. RNA was extracted by RNeasy Qiagen Kit. The mRNA expression was measured by the RiboQuant multiprobe RNase protection assay (BD Biosciences Pharmingen, San Diego, CA), following the manufacturer's instructions. Briefly, antisense RNA probes were transcribed using the cDNA template sets: Human cytokine kit 5 (hCK-5) and Human cytokine kit 2b (hCK-2b). For transcription, except for Biotin-16-UTP (Roche Diagnostics, GmbH, Mannheim Germany), all reagents were supplied by the manufacturer in RiboQuant Non-Rad In Vitro Transcription Kit (BD Biosciences Pharmingen). For hybridization, 20 µg RNA samples precipitated by ethanol and dried using a vacuum evaporator centrifuge, were resuspended in hybridization buffer (BD Biosciences Pharmingen) at 56 °C, mixed with 30 ng of probe prepared as previously described, heated to 90 °C, and then incubated at 56 °C for 18 h. After RNase digestion by RiboQuant Non-Rad RPA Kit (BD Biosciences Pharmingen) the samples were then air-dried and size-separated using PAGE. The bands were electrotransferred to a positively charged nylon membrane (BD Bi-

osciences Pharmingen) by a Semi-dry Electroblotter (100 mA for 20 min) and crosslinked by UV. For chemiluminescent probe detection, we used BD RiboQuant Non-Rad Detection kit (BD Biosciences Pharmingen) based on Streptavidin-HRP and luminol reactions. Revealed membranes were exposed to Hyperfilm ECL (Amersham) and nucleotide lengths vs. migration distances were compared with the standards (30 ng transcribed biotin-labeled probes) on a logarithmic grid.

Microarray RNA Analysis

The HT29 or T24 cells were exposed for 24 h to increased mannitol concentrations. The cells were harvested after cold PBS washes and RNA was extracted. Human inflammatory cytokines and receptors gene array kit (GEArray™ Q Series HS-015.2) was obtained from SuperArray Bioscience Corp. (Frederick, MD, USA). For probe synthesis and probe biotin labeling, we used GEArray AmpoLabeling-LPR Kit (SuperArray Bioscience Corp.) and Biotin-16-dUTP (Roche Diagnostics, GmbH, Mannheim Germany) as described in the manufacturer's instructions. Biotinylated and amplified cDNA probes were hybridized overnight at 60 °C with different array membranes and AP-streptavidin chemiluminescent detection was performed by SuperArray Detection Kit (SuperArray Bioscience Corp.). Membranes were exposed on X-ray films (Hyperfilm™, Amersham Biosciences, Piscataway, NJ) and image acquisition was performed with a desk scanner at 200 dpi. For data acquisition, we used ScanAlyze software version 2.50 developed by Dr. Michael Eisen (http://rana.lbl.gov/EisenSoftware.htm). Data analysis was carried out with GEArray Analyzer software (www.superarray.com). Raw data was subtracted from the mean signals of three negative controls as areas without spotted gene sequences (blanks) or areas spotted by genes not expressed in human cells (pUC18). We normalized the subtracted data by its ratio to averaged signals of two positive controls (housekeeping genes β-actin or GAPDH) for adjusting the loading. Each experiment was performed at least twice to ensure the reproducibility of results.

NF-κB p65 Activation

Cells were stimulated with hyperosmotic or isosomotic medium for varying time intervals. We measured p65 nuclear translocation, 6 or 24 h after media replacement of confluent T24 or HT-29 cell cultures by hyperosmolar 600 or 900 mOsm mannitol treated media in the presence or absence of 100 μM xylitol. Before replacement of media, the culture plates were treated for 40 min with DMSO vehicle (final concentration < 0.1 %), or with 200 nM okadaic acid dissolved in DMSO. Human recombinant TNF-α [210-TA] (R&D systems) was added to media in a concentration of 200 ng/ml 60 min prior to harvesting. The cells were washed with ice-cold PBS and homogenized by simple syringe aspirations and after washing and centrifuging two time, the homogenates were resuspended in 1 ml hypotonic lysis buffer (20 mM HEPES, pH 7.5, 5 mM NaF, 10 mM Na2MoO4, 0.1 mM EDTA and 250 mM p-nitrophenyl phosphate). After this step, 0.1 ml 10 % Nonidet P-40 was added and after 10 min the suspension was centrifuged and the nuclear pellet was resuspended in 0.2 ml complete lysis buffer (Active Motif, Carlsbad, CA, USA). After resuspending the nuclear pellet in lysis buffer for 30 min with shaking, the lysates were centrifuged for 10 min at 14,000 g at 4 °C and the supernatant (nuclear cell extract) stored at –80 °C. The protein concentration was determined by a MicroBCA protein assay (PIERCE, Rockford, IL). Five μg of nuclear extracts were tested for the NF-κB activation by using the NF-κB p65 TransAM™ transcription factor assay kit (Active Motif) according to the manufacturer's instruction.

Western Blot Analysis of NF-κB Regulators and PP2Ac

Nuclear and cytoplasmic protein extracts (25 μg) of HT-29 and T24 cell cultures treated by different hyperosmolar solutions of mannitol-based media at different points in time were separated on an 11 % reducing polyacrylamide gel and blotted to a nitrocellulose Hybond-polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ). Equal protein laid down on the gel was verified by red Ponceau-S (Sigma). The membrane was blocked overnight with a 5 % (w/v) non fat dried milk solution containing 10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1.5 mM $MgCl_2$, and 0.1 % Tween 20 prior to incubating with primary antibodies: mouse monoclonal IgG1 IKKγ (B-3 sc-8032) or mouse monoclonal IgG1 IκBα (H-4 sc-1643) or mouse monoclonal IgG2b anti-P-IκBα (B-9 sc-8404) or rabbit polyclonal IgG anti-P-IKKγ (SER 376 sc-31721R) or goat polyclonal IgG anti-PP2A-B56-alpha (C-18 sc-6116) or goat polyclonal IgG anti-P-PP2A (TYR 307 sc-12615) (all from Santa Cruz Biotechnologies, CA) or mouse monoclonal IgG1 anti-methylated-PP2Ac (2A10 05-546) (Upstate, Charlottesville, VA) or mouse monoclonal IgG1 anti-β-actin (C4 MAB1501) (Chemicon, France) for 4 h. After washing, the membranes were incubated with horseradish peroxidase-labeled secondary antibodies: goat anti-mouse IgG1 sc-2060 or goat anti-mouse IgG (sc-2031) or goat anti-rabbit IgG (sc-2030) or donkey anti-goat IgG (sc-2033) (all from Santa Cruz Biotechnologies) for 2 h and the labeled proteins were detected using enhanced chemiluminescence reagents (Pierce Biotechnology).

PP2A Activity Assay

Cells were harvested after cold-PBS washes and PP2A activity was measured by R&D systems PP2A DuoSet_ IC activity assay kit according to the manufacturer's description. An immobilized capture antibody specific to the catalytic subunit of PP2A binds both active and inactive PP2A. After washing away unbound material, a synthetic phosphopeptide substrate is dephosphorylated by active PP2A to generate free phosphate and unphosphorylated peptide. The free phosphate is detected by a sensitive dye-binding assay using malachite green and molybdic acid. Calculating the rate of phosphate release makes it possible to determine the activity of PP2A.

Co-Immunoprecipitation of mPP2A and IKK Gamma

HT-29 cells were cultured to confluence in 100 mm tissue culture plates. Twenty-four hours before harvesting, the medium was replaced by a hyperosmolar mannitol-based medium (900 mOsm). Harvested treated and non-treated (control) cells were washed by ice-cold PBS/Phosphatase Inhibitors (Active Motif). Cell pellets were gently resuspended in 500 μl Hypotonic Buffer (Active Motif) and transferred to the pre-chilled microcentrifuge tube, incubated for 15 min on ice, 25 μl Nonidet P40 was added and gently pipeted up and down. After centrifugation for 30 sec at 14,000 g, the supernatants were separated. The cytoplasmic fraction was kept on ice. Immunoprecipitation (IP) incubation buffer, IP wash buffer and samples were prepared as described in Nuclear Complex Co-IP kit (Active Motif); 200 μg of extract and 2 μg of mouse monoclonal IgG1 anti-methylated-PP2Ac (2A10 05-546, Upstate, Charlottesville, VA) were mixed and this antibody/extract mixture was incubated overnight at 4 °C on a rotator. Protein G-Sepharose beads (Sigma) were washed thoroughly (three times after centrifugation at 4000 rpm for 30 sec at 4 °C) and added to antibody/extract mixture and incubated for 3 h at 4 °C. 50 μl of resuspended antibody binding beads was used for each IP reaction (approx. 50 % beads/volume). Bound proteins were eluted by centrifugation at 4000 rpm for 30 sec at 4 °C. Each bead pellet was resuspended in 8 μl of 2X Reducing Loading Buffer (130 mM Tris pH 6.8, 4 % SDS, 0.02 % bromophenol blue, 20 % glycerol, 100 mM DTT) and was boiled at 95–100 °C for 3–5 min prior to applying on an 11 % SDS-PAGE gel. Western blot was revealed by anti-methylated PP2A or IKKγ as described above.

Statistical Analysis

All data is expressed as the mean ± SEM. A One-way ANOVA test was performed using the GraphPad InStat version 3.0 software for Windows, (GraphPad Software Inc., San Diego, CA). Values were considered statistically significant when p was less than 0.05.

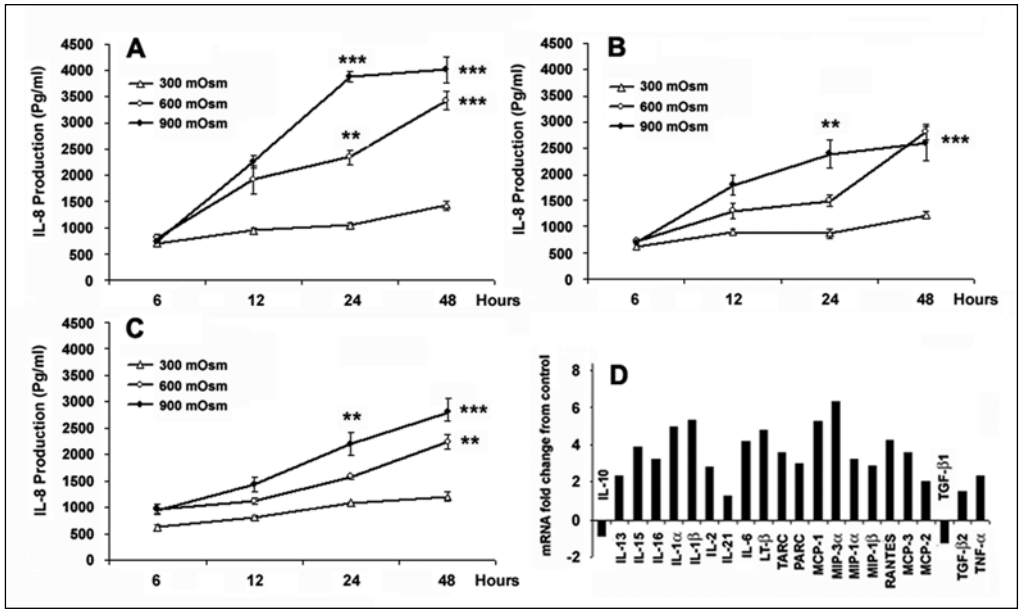

Fig. 1: Pro-inflammatory Cytokines after Hyperosmotic Stimulation

IL-8 production was measured by ELISA in the culture supernatants of stimulated HT-29 cells at different points in time within 48 h. Hyperosmotic treatments were performed by different solutes: mannitol (A), NaCl (B) and L-alanine (C). Data is the product of 4 separate experiments (n = 4). ** p <0.01; *** p <0.001. Similar results were obtained using T24 cells (data not shown). D) HT-29 cells were incubated with either an isoosmolar medium or mannitol medium (600 mOsm). Human inflammatory cytokines and receptors gene array membranes were probed with cDNA derived from 1 µg total RNA from HT-29 control cells and from hyperosmolar HT-29 cells treated with mannitol for 24 h. Raw data was subtracted from the mean signals of three negative controls as areas without spotted gene sequences (blanks) or areas spotted by the genes not expressed in human cells (pUC18). We normalized this subtracted data by its ratio to averaged signals for two positive controls (β-actin and GAPDH). Each experiment was performed at least twice to ensure the reproducibility of results. Data is expressed as a ratio of stimulated to control cells on the vertical axis of these figure panel.

Results

Hyperosmolarity induces inflammatory cytokine and chemokine expression

Hyperosmolar media incubations of HT-29 (Figure 1) or T24 (data not shown) epithelial cells induce a highly significant increase in IL-8 production in a dose-dependant manner up to 48 h (Figure 1A, B, C). We noticed a difference in the amplitude of the reaction depending on the nature of the solution (for review, see [30]). Furthermore, exposition to hyperosmotic solutions resulted in a large expression of numerous pro-inflammatory cytokines and chemokines. Figure 1D shows the array-measured effect of a 24-hour exposure to 600 mOsm media on HT-29 cells that over-transcribed numerous pro-inflammatory mediators. This overexpression is massive amongst the following cytokines: IL-13, IL-15, IL-16, IL-1α, IL-1β, IL-2, IL-6, Lymphotoxin beta (LT-β) and TNF-α and amongst the chemokines TARC (Thymus and activation-regulated chemokine), MCP-1 (Monocyte chemoattractant protein-1), MIP-3α (Macrophage inflammatory protein-3 alpha), MIP-1α,β, RANTES (Regulated upon activation, normal T cell expressed and secreted) and MCP-3. On the contrary, the analysis of two mediators known to be anti-inflammatory highlighted a net down-regulation of these molecules: IL-10 and TGF-β. In all the cells exposed to hyperosmotic stress, we performed an MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and trypan blue coloration and did not notice any alteration of cellular viability up to 72 h of incubation (data not shown).

Hyperosmolarity increases tissue Myeloperoxidase activity and MIP-2 expression

In order to verify these inflammatory effects in normal cells, we used two *in vivo* models already set up in our laboratory [27]. Twenty four hours after rectal administration of the increasing hyperosmolar solutions in rats, there was a significant dose-dependent increase in the colonic cells' Myeloperoxydase (MPO) activity, as observed in Figure 2A. These activities were extremely significant when comparing control (PBS) or isotonic (300 mOsm) administrations versus 600 mOsm hyperosmotic stress, regardless of the chemical nature of osmoles. MPO activity is widely accepted as an enzyme marker to quantify the degree of inflammation and estimate the accumulation of inflammatory cells (neutrophils) in tissues. Thus, this enhanced MPO activity reflects the recrutement of neutrophils to the inflammation site. Similarly, there is a dose-dependent increase in Macrophage-Inflammatory Protein-2 (MIP-2) transcription, as shown by RT-PCR assay in rat colonic cells (Figure 2B). Noticeably, MPO activity and MIP-2 transcription are also dependant on the nature of the solution, presumably because of the different biochemical mechanisms initiated by the stresses (for review see [30]).

NF-κB is activated in epithelial cells after hyperosmolar stimulation

We studied the activation of nuclear factor NF-κB to get a better knowledge of the molecular pathways involved in the

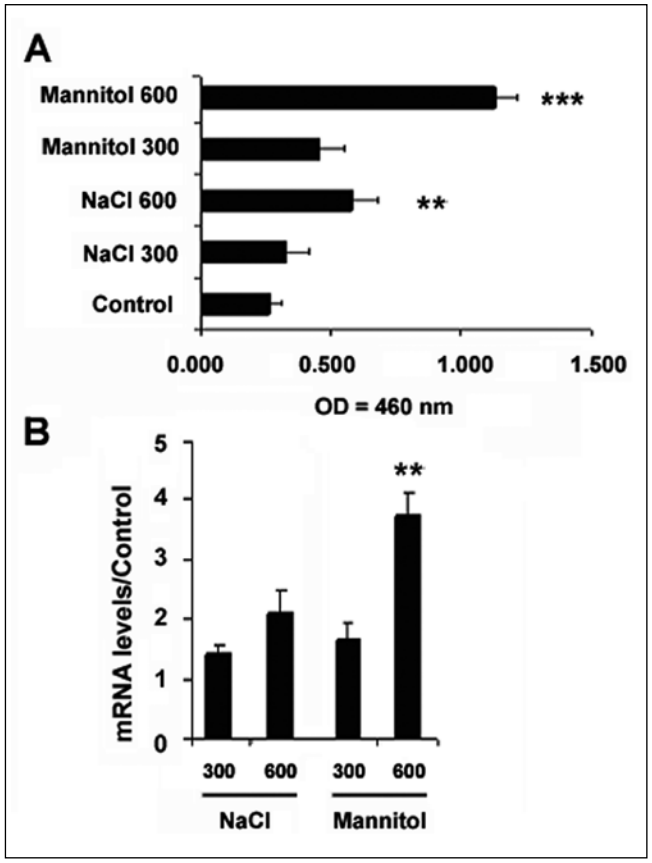

Fig. 2: MPO Assay and Mucosal MIP-2 Overexpression
A) MPO activity was measured in colon segments of rats 24 h after rectal administration of different isosmotic and hyperosmotic solutions. The change in absorbance at 460 nm was registered spectrophotometrically over 3 min and one unit of MPO activity was defined as that degrading 1 µmol of hydrogen peroxide per minute. Data was expressed as mean ± SEM for n = 5. B) Colon scraped cells were lyzed and the transcription of MIP-2 mRNA was measured by RT-PCR. One µL of each cDNA sample was used for each 20 µL PCR reaction. Real-time measurements were analyzed in duplicate in three independent runs. Relative mRNA levels were calculated after normalizing to β-actin. ** p <0.01; ***p <0.001.

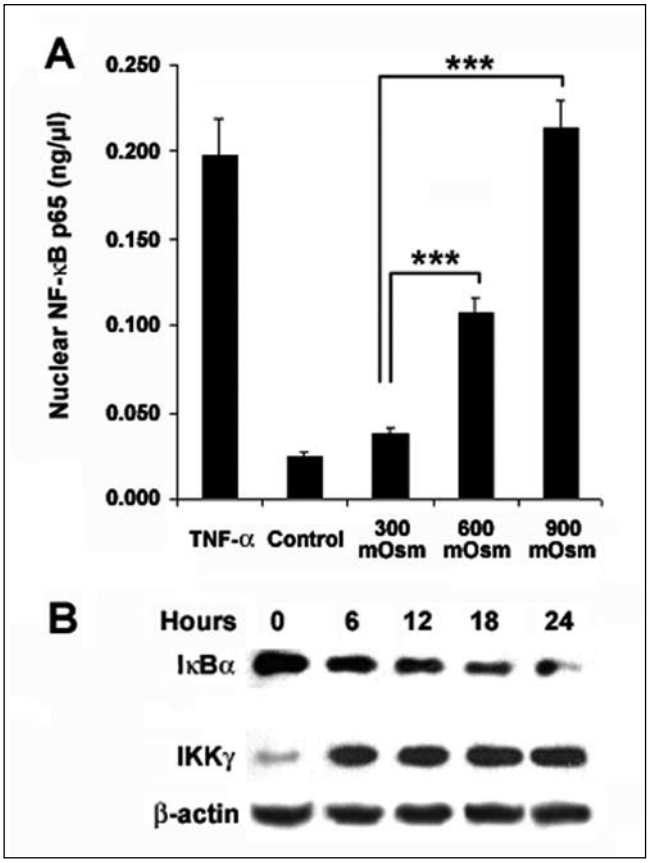

Fig. 3: NF-κB Activation after Osmotic Stimulation
A) HT-29 cells were incubated during 6 h with the culture medium (Control) or mannitol was added to increase osmolarity. NF-κB nuclear translocation was measured by binding NF-κB p65 subunit in nuclear extracted proteins to an immobilized consensus oligonucleotide (5'-GGGACTTTCC-3') in an ELISA-based assay. TNF-α (200 ng/ml, 60 min prior to harvesting) was added as a positive control. Data was compared as optical density reads in 450 nm spectrophotometer and NF-κB concentration was calculated on the basis of a standard curve of serial dilutions to the concentrations 0.008–0.5 ng/µl. The test sensitivity is 5 pg/µl. The assay reproducibility is from 0.039 to 2.5 µg of nuclear extract/well (n = 4). ***p <0.001. B) HT-29 cells were stimulated by addition of 600 mOsm mannitol media at different periods of times. Cytoplasmic protein extracts (20 µg) were separated by SDS-PAGE and labeled for IκBα, IKKγ and β-actin. The experiment was performed twice. Similar results were obtained using T24 cells (data not shown).

mechanism of hyperosmolarity-induced inflammatory responses. Using a DNA-based ELISA method, we measured quantitatively the translocation of p65 NF-κB to nuclear compartments of HT-29 and T24 cell lines after mannitol based hyperosmolar treatment. There was a dose-dependant NF-κB translocation in response to hyperosmolarity (Figure 3A): 600 and 900 mOsm mannitol treatments caused an extremely significant NF-κB activation in HT-29 cells. Tumor Necrosis Factor alpha (TNFα) was used as a control. This translocation, which demonstrates that the activation of NF-κB is a consequence of the cytoplasmic IκB degradation after 600 mOsm stimulation (as shown in Figure 3B line 1). This very interesting activation effect was maintained for at least 24 h after hyperosmolar shocks. Obviously, the feedback effect of Inhibitor of kappa B alpha (IκBα) phosphorylation and consequent translocation of p65 NF-κB has been abolished during 24 h and, furthermore, consecutive culture of cells in a hyperosmolar medium has perturbed the IκBα regulation and balance in cytoplasm. To explain this experimental result, we measured the presence of Inhibitor of kappa B Kinase (IKKγ) in cytoplasmic fractions. As presented in Figure 3B line 2, levels of IKKγ were maintained very higher in stressed cells than in untreated ones up to 24 h.

Hyperosmotic shock activates PP2Ac

In order to study the mechanism leading to high a cytoplasmic level of IKKγ, we studied the activity of serine-threonine phosphatase PP2A – the only documented complex that coprecipitates with IKKγ [31]. The curves shown in Figure 4A reveal a concentration-dependent and specific activation

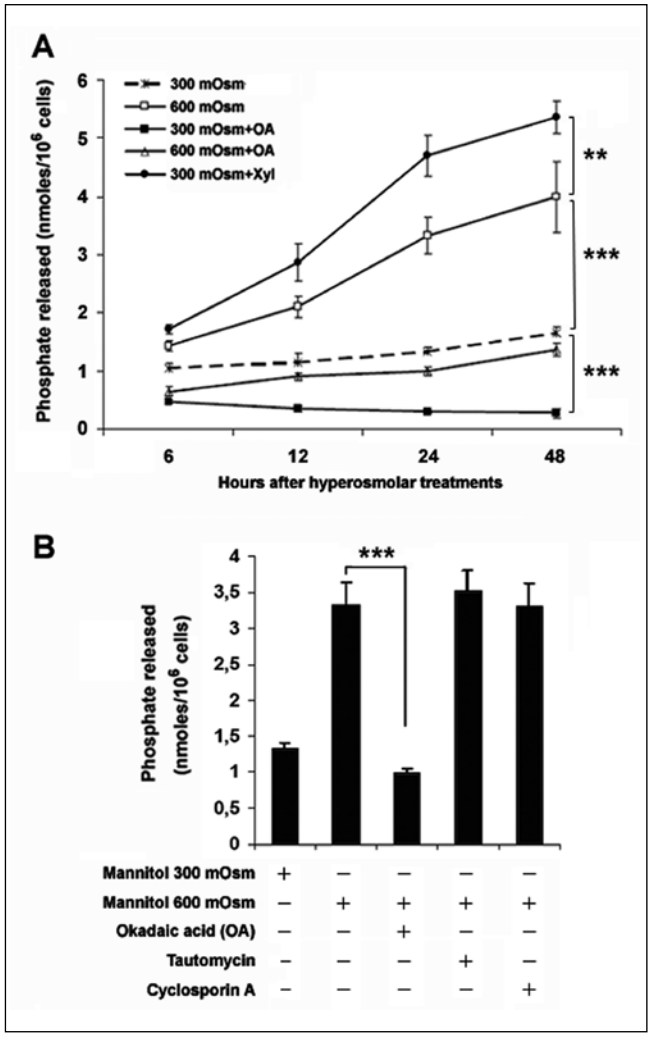

Fig. 4. Osmotic Stimulation Increases PP2A Activity
A) HT-29 cells were treated by increasing hyperosmolar solutions during 48 h in presence or absence of 100 µM xylitol (Xyl). Where indicated, okadaic acid (OA) (200 nM) was added to the medium during the first 40 min of treatment. PP2A activity was monitored before a standard curve of 0.078–5000 nmoles in terms of phosphate quantity released from a synthetic phosphopeptide substrate at different points in time. The free phosphate is detected by a sensitive dye-binding assay using malachite green and molybdic acid (n = 4). ** $p < 0.01$; *** $p < 0.001$. B) HT-29 cells were treated during 24 h of either isoosmolar or hyperosmolar (600 mOsm) mannitol solutions of HT-29 cells in the presence of different phosphatase inhibitors of PP2A (OA 200 nM), PP1 (Tautomycin 200 nM) or PP2B (Cyclosporin A 200 nM). (n = 4). *** $p < 0.001$.

of a phosphatase in cytoplasm up to 48 h after hyperosmotic stimulus. Released phosphate reached a fourfold increase after exposure of HT-29 cells to 600 mOsm mannitol solution compared with an isotonic control solution (300 mOsm). This activity was clearly altered in the presence of okadaic acid (OA), a well-known PP2A inhibitor. In fact, after 48 h, OA completely abolished the hyperosmolarity-induced phosphate release. However, xylitol (Xyl) a PP2A inducer intensified very significantly the phosphate release after 24 and 48 h of treatment (Figure 4A). These findings demonstrate a clear role of PP2A in hyperosmolarity-induced events. To confirm the phosphatase type implicated, we measured phosphate release in the presence of different specific inhibitors. Figure 4B represents the effects of two specific phosphatase inhibitors tautomycin (Protein Phosphatase 1 inhibitor) and cyclosporine A (Protein Phosphatase 2B inhibitor) on hyperosmolarity-induced phosphate release in HT-29 cells. The lack of inhibition confirmed the specificity of PP2A activation in response to hyperosmotic stress.

PP2Ac activation is linked to cytokine and chemokine production after hyperosmotic stresses

Exposure of HT-29 cells to different hyperosmolar mannitol-based solutions increases IL-8 production with time, as observed in Figure 1, whereas OA (200 nM) suppresses this induction totally and reverses the chemokine production to control values (Figure 5A). These results confirmed the dependency of hyperosmolarity-induced chemokine production on PP2A activity. When we assayed the expression of IL-8 and MCP-1 by T24 cells on an RNase protection gel, chemokine transcriptions stimulated by hyperosmolarity were repressed in cells treated with OA in a dose-dependent manner (10–150 nM) (Figure 5B). IL-10 expression assessed on another gel indicated a reverse manifestation and after 150 nM OA, demonstrated a net over-expression (Figure 5B). We found the same profile of RNA transcription in HT-29 cells (data not shown).

PP2A activity is necessary for mRNA cytokines and chemokines transcription after osmotic stresses

In Figure 6A, array results are shown using another cell line T24, which confirm the array data in HT-29 cells (Figure 1D). In this experiment, array membranes were hybridized with cDNA from T24 cells treated during 24 h by 600 mOsm mannitol hyperosmolar solution in the presence or absence of calyculin A, which is another PP2A inhibitor. Similar to HT-29 cell responses, there is an over-expression of IL-13, IL-15, IL-16, IL-1α, IL-1β, IL-2, IL-6, LT-β, TNF-α, TARC, MCP-1, MIP-3α (~7.3 fold), MIP-1, RANTES and MCP-2 besides the suppression of IL-10 and TGF-β1. In the presence of calyculin A (Figure 6B), the transcription pattern of T24 cells has been reversed entirely and returned to basal conditions decreasing nearly all expression ratios of pro-inflammatory elements to less than two.

NF-κB activity after osmotic shocks depends on PP2A induction

Western blot analysis of NF-κB cascade elements in HT-29 and T24 cells after OA or Xyl treatments demonstrated a very clear relationship between these elements and PP2A activity. In Figure 7A, 900 mOsm mannitol-based media completely suppressed IκBα in HT-29 and T24 cells only in the absence of OA, while in presence of this PP2A inhibitor, the dose-dependent effect of hyperosmotic stress disappeared within 24 h. Conversely, western blotting of IKKγ shows its stabilization (Figure 7B) notably in the presence of Xyl (PP2A

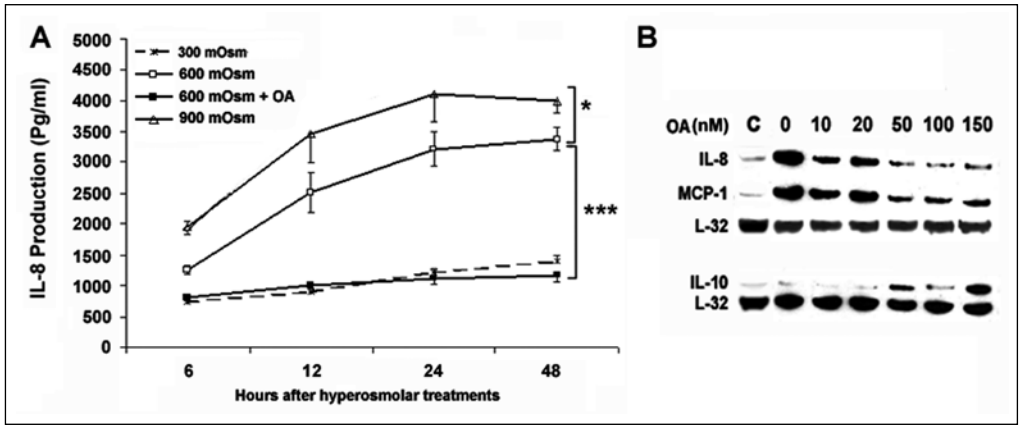

Fig. 5: Interleukin-8 Secretion after Osmotic Stimulation is Mediated by PP2A
A) HT-29 cells were treated by hyperosmolar solutions (300, 600 or 900 mOsm) within 48 h in the presence of DMSO or okadaic acid potassium salt (200 nM) (OA). IL-8 production was measured by using ELISA on the basis of a standard curve of 62.5–4,000 pg/ml at different points in time (n = 4). Each sample was assayed in triplicate. The sensitivity limit of the assay was less than 3.5 pg/ml. B) The expression of IL-8, MCP-1 and IL-10 were measured by RNase protection assay in T24 cells of normal control culture (C) or treated with 900 mOsm hyperosmotic media in the presence of different concentrations of okadaic acid (OA: 0–150 nM). L-32 is shown as a loading control. Similar results were obtained in HT-29 cells (data not shown).

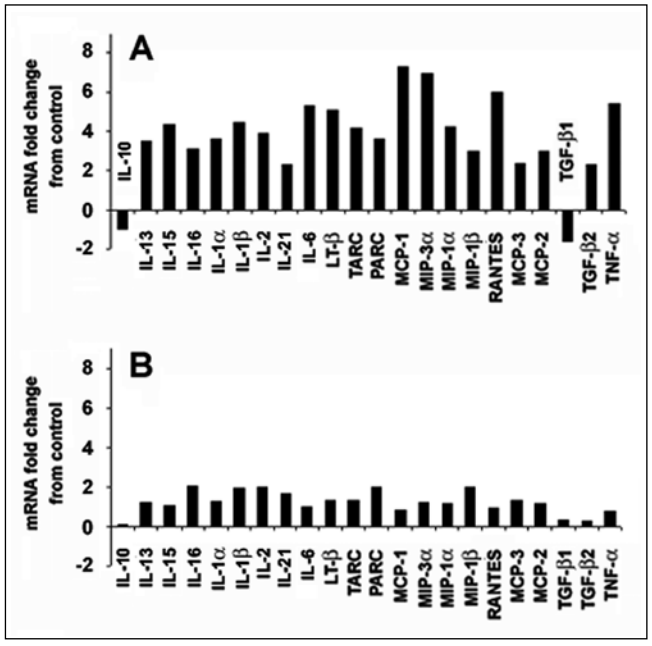

Fig. 6: Increased Pro-inflammatory Cytokine Transcription after Hyperosmotic Stimulation is Mediated by PP2A
Hyperosmolar mannitol (600 mOsm) treatment of T24 cells in the presence of DMSO as control (A) or DMSO-dissolved calyculin A (100 nM) (B) was monitored by oligo membranes. The osmolarity of non-treated medium was measured about 310 mOsm. Cell treatments by hyperosmolar media were prolonged up to 24 h and calyculin A was added within the first 40 min of treatment. Human inflammatory cytokines and receptors gene array membranes were hybridized with cDNA derived from T24 control cells and from hyperosmolar T24 cells treated with mannitol for 24 h. We normalized the signals by their ratio to averaged signals of two positive controls (β-actin and GAPDH). Each experiment was performed at least twice to ensure the reproducibility of results. Data is expressed as a ratio of stimulated to control cells on vertical axis of these figure panels.

activator) that increased IKKγ activation more than 50 times, based on intensity analyses (Figure 7B framed bands). NF-κB activation was followed in 300 and 900 mOsm mannitol-treated HT-29 cells in the presence of TNFα as a positive control. The profile of NF-κB nuclear translocation confirmed the changes in IκBα and IKKγ manifestations in cytoplasm (Figure 7C). OA inhibited very significantly the translocation of this nuclear factor after hyperosmotic stresses even in the presence of TNFα as a classic activator of NF-κB. It is interesting to notice that, even in the absence of hyperosmotic stress, PP2A contributes to TNFα- induced NF-κB activation, as already described in other systems [32–34]. Western blot analysis of HT-29 methylated PP2Ac indicated a methylation-mediated activation in carboxyl terminal residue of PP2A after hyperosmotic stresses (Figure 8A line 1). There also is a complete reverse relation between methylated PP2A and IκBα in the presence of increasing osmolality (Figure 8A line 2). In strong hyperosmolar conditions, Xyl fortified this reciprocal relation (Figure 8A). We confirmed this correlation by blotting of IKKγ and IκBα simultaneously with the presence of increasing OA concentrations. As shown in Figure 8B, with high OA concentrations of 150 and 200 nM, IKKγ is not stable and it leaves cytoplasmic roles whereas in these conditions, the presence of activated IκBα is remarkably clear. In order to validate the direct relationship and molecular interaction of IKKγ with PP2Ac after hyperosmotic stress, we coimmunoprecipitated IKKγ with PP2Ac. Only after hyperosmotic treatment of HT-29 cells did we find evidence of the presence of IKKγ following the immunoprecipitation of methylated PP2Ac from total cytoplasmic protein extracts, as shown on IKKγ blotted gel in Figure 8C (lane 4).

Discussion

In this study, we show that colonic epithelial cells and colon *in vivo* stimulated by hyperosmolar solutions produce IL-8 and a wide range of pro-inflammatory cytokines and chemokines. This is mediated by a hyperosmolarity-induced activation (methylation) of PP2A that stimulates the NF-κB pathway, i.e. IKKγ complex activation and IκBα degradation that leads to NF-κB nuclear translocation and pro-inflammatory mediators' transcription.

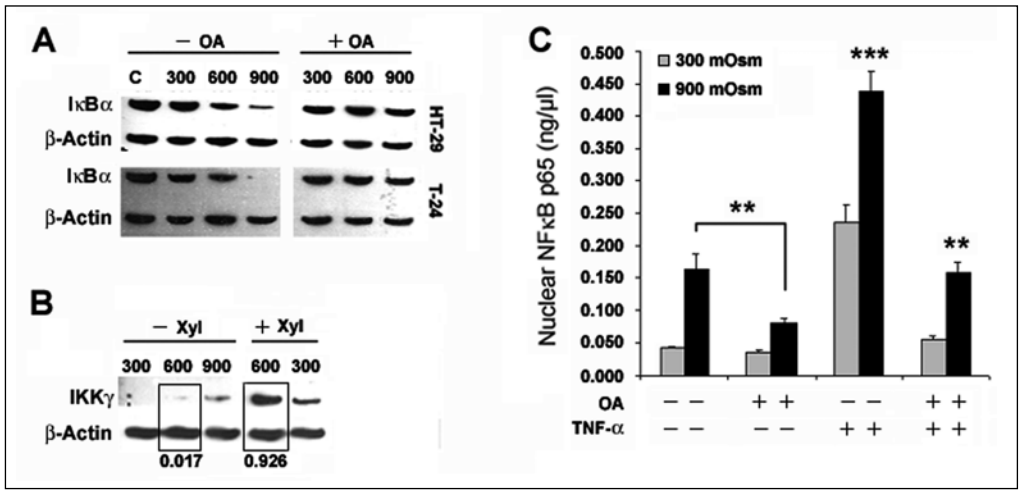

Fig. 7: NF-κB Pathway Activation in Response to Hyperosmolarity or TNFα is Mediated by PP2A

A) IkBα protein expression levels analyzed by western blot in HT-29 and T24 cells incubated in increasing mannitol-based hyperosmolar media during 24 h in the presence or absence of okadaic acid (OA, 200 nM) β-actin is shown as a loading control. B) IKKγ protein expression levels analyzed by western blot in T24 cells incubated during 24 h in different hyperosmolar media in the presence or absence of xylitol (Xyl, 100 µM). Framed bands were scanned and analyzed by an intensometer. The numbers under each frame represent the ratio of IKKγ/β-actin intensity. C) Nuclear translocation of NF-κB p65 was measured in T24 cells incubated in 900 mOsm mannitol during 6 h in the presence or absence of okadaic acid (OA 200 nM) or TNFα (200 ng/ml) (n = 4). ** $p < 0.01$; *** $p < 0.001$.

Modern biology has shown that during inflammation, whatever its cause, there is an enhanced secretion of pro- inflammatory agents such as TNF, interleukins, proteases such as metalloproteases or caspases [35]. Increased osmolarity appears as a good candidate for the enhanced secretion of numerous mediators of inflammation. It has been reported in the literature listed in the references that osmotic shock can induce the secretion of: prostaglandins [35], TNF and interleukins [36, 17, 19], leukotriene [35], histamine [15], growth factors such as Transforming Growth Factor beta (TGF-β) [37], or Platelet-Derived Growth factor (PDGF) [387], proteases such as metalloprotease [14], plasminogen activator [39] or caspases [13].

We demonstrate that osmotic stress increases the secretion of multiple pro-inflammatory cytokines and chemokines both *"in vivo"* and *"in vitro"*. These data generalize and support previous published studies [13, 14, 16, 17, 18, 19]. The inflammatory effects of osmotic stress are known to be mediated by the activation of NF-κB [16]. Furthermore, it is known that NF-κB hyperosmolarity-induced activation is crucial in the initiation of cyclooxygenase-2 gene expression in renal epithelial cells [40]. However, our findings provide complementary information on the molecular mechanisms linking together hyperosmotic stress, PP2A methylation and NF-κB activation in epithelial cells.

We show that hyperosmotic stress in epithelial cells activates the canonical (or classical) NF-κB pathway. NF-κB is the key determinant of the epithelial inflammatory cascade and plays a central role in its regulation. Lower cytoplasmic IκBα accumulation is known to be associated with the nuclear translocation of NF-κB and its increased activity. It has been reported that the IκBα degradation and subsequent NF-κB activation are controlled by phosphatases. Miskolci [41] demonstrated that okadaic acid induces sustained activation of NF-κB and degradation of the nuclear IκBα, and also increases interleukin expression. Similarly, inhibitors of protein kinase C-delta (PKCdelta) and IκB kinase (IKK) inhibit the okadaic acid-induced activation of NF-κB [41].

PP2A is the most common phosphatase accounting for about one per cent of the total protein content of the cell. It is a very complex molecule. PP2A is a heterotrimer consisting of three units - A, B and C. The A unit is a highly conserved regulatory scaffolding unit, while the C unit, also highly conserved, is the catalytic portion of the enzyme. The B unit is a regulatory unit that directs the enzyme to complex with the appropriate substrate. More than 20 different B units have been identified to date. The heterotrimer, $PP2A_C$, must be methylated before it can complex with the B unit to form the active enzyme [22, 23, 24]. The importance of PP2A with respect to the generation of inflammatory cytokines and chemokines is supported by very recent work that suggests that activation of NF-κB by IKKγ will not occur without prior formation of a PP2A/ IKKγ complex [30]. After formation of the PP2A/ IKKγ complex, IκBα is phosphorylated and NF-κB is translocated to the nucleus.

In this paper, we demonstrate that osmotic shock results in the activation of PP2A (defined by its methylation) [25 and 26]. One of the targets of methylated PP2A is IKKγ, which in turn activates NF-κB. The activation of NF-κB results in the release of pro-inflammatory mediators as well as the inhibition of anti-inflammatory cytokines. This is demonstrated by the fact that specific PP2A inhibitors decrease the secretion of pro-inflammatory cytokines after hyperosmotic stimulation. Okadaic acid inhibits the methylation of PP2A [42, 43] and therefore abolishes the effect of hyperosmolarity. Xylitol is a precursor of xylulose-5P, which is known to stimulate PP2A [44, 45, 37]. We show that xylitol stimulates the methylation of PP2A, reverses the inhibition of the methylation of PP2A induced by okadaic acid. The stimulation of the methylation of PP2A results in the translocation of the transcription factor NF-κB and the stimulation of the inflammatory cascade.

The kidney medulla is one of the normal tissue in mammals that is exposed to a hypertonic environment under physiological conditions, due to the operating urinary concentrating mechanism. The degree of hypertonicity in the renal medulla fluctuates widely depending on the hydration

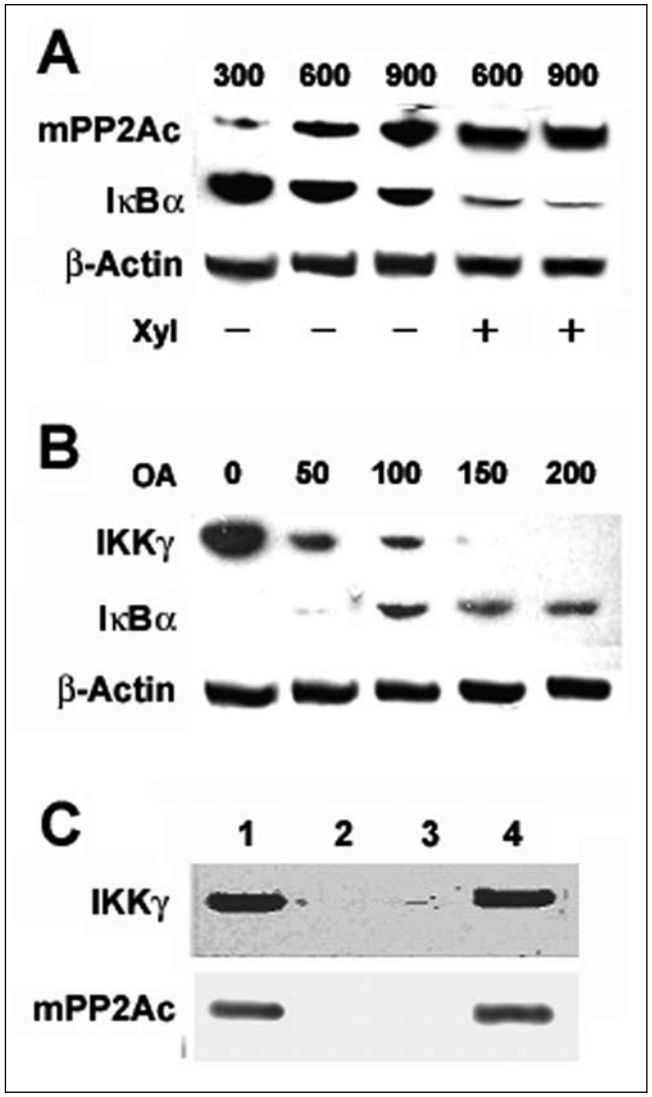

Fig. 8: Coimmunoprecipitation of IKKγ with the Methylated PP2Ac

A) Methylated PPA2 (mPP2Ac) and IκBα proteins were analyzed by western blot after 24-hour incubation of HT-29 cells in different hyperosmolar mannitol-based media in the presence or absence of xylitol (Xyl 100 μM). β-actin is shown as a loading control. B) IKKγ and IκBα proteins were analyzed by western blot after 24-hour incubation of HT-29 cells in 600 mOsm mannitol-based media in the presence of increasing concentrations of okadaic acid (in nM). β-actin is shown as a loading control. C) Western blot analysis of IKKγ and mPP2A proteins after immunoprecipitation of mPP2Ac from HT-29 cells treated by 900 mOsm mannitol-based hyperosmolar medium during 24 h: Lane 1) Treated cells total lysate, Lane 2) Non-treated cells total lysate, Lane 3) Treated cells cytoplasmic fraction immunoprecipitated without mPP2Ac antibody, Lane 4) Treated cells cytoplasmic fraction immunoprecipitated with mPP2Ac antibody.

status of the animal. In dehydrated mammals, osmolarity of the renal medulla exceeds 1,000 mOsm in humans and 3,000 mOsm in rats. Hyperosmotic (405 mOsm) exposure of monocytes and macrophages led to an upregulation of betaine/gamma-amino-n-butyric acid (GABA) transporter BGT-1 [46]. Similarly increased extracellular osmolarity results in the activation of methylase [47, 48]. Hyperosmotic stress is observed in several pathological situations. In the case of bronchial asthma, hyperosmolarity resulting from water loss contribute to exercise-induced airway bronchoconstriction [49, 50]. Similarly, hyperosmolarity has been shown to be associated with a variety of intestinal inflammatory conditions that are characterized by overproduction of cytokines of epithelial origins. For example, patients with Crohn's disease have dramatically higher colonic osmolarity than healthy patients (~100 to 110 mOsm/l) [51, 52].

We conclude that the importance of osmolarity contribution to inflammation may have been underestimated. There is a high protein content in every inflammatory exudate, whatever its cause. For example, in the case of pleural exudates, the pleural fluid is yellow in color and has a high concentration of protein. This large amount of protein is a diagnostic criterion for inflammation as opposed to a transudate. The increased protein content is present whatever the cause for inflammation (tuberculosis, foreign body, trauma, and autoimmune diseases).

Similarly, there is a large concentration of protein in ascites occurring during an inflammation, such as tuberculosis or pancreatitis [53]. The same is true for pericarditis [54], atherosclerosis [55], arthritis [56, 57] or asthma and pneumonia [58]. Each time there is inflammation, there appears to be an increase in the amount of protein in the extracellular space. It is our hypothesis that this increased protein concentration results in increased osmotic pressure, which in turn is a key pro-inflammatory pathway. Increased protein would then not be a consequence but a cause of inflammation.

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