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Hyperosmotic stress contributes to mouse colonic inflammation through the methylation of protein phosphatase 2A

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Schwartz L, Abolhassani M, Pooya M, Steyaert JM, Wertz X, Israël M, Guais A, Chaumet-Riffaud P. Hyperosmotic stress contributes to mouse colonic inflammation through the methylation of protein phosphatase 2A. *Am J Physiol Gastrointest Liver Physiol* 295: G934–G941, 2008. First published August 28, 2008; doi:10.1152/ajpgi.90296.2008.—There are several reports suggesting hyperosmotic contents in the feces of patients suffering from inflammatory bowel disease (IBD). Previous works have documented that hyperosmolarity can cause inflammation attributable to methylation of the catalytic subunit of protein phosphatase 2A (PP2A) and subsequent NF-κB activation resulting in cytokine secretion. In this study, we demonstrate that dextran sulfate sodium (DSS) induces colitis due to hyperosmolarity and subsequent PP2A activation. Mice were randomized and fed with increased concentrations of DSS (0 mOsm, 175 mOsm, 300 mOsm, and 627 mOsm) for a duration of 3 wk or with hyperosmotic concentrations of DSS (627 mOsm) or mannitol (450 mOsm) for a duration of 12 wk. Long-term oral administration of hyposmotic DSS or mannitol had no demonstrable effect. Hyperosmotic DSS or mannitol produced a significant increase in colonic inflammation, as well as an increase in the weight of sacral lymph nodes and in serum amyloid A protein levels. Similar results were obtained through the ingestion of comparable osmolarities of mannitol. Hyperosmolarity induces the methylation of PP2A, nuclear p65 NF-κB activation. and cytokine secretion. The rectal instillation of okadaic acid, a well-known PP2A inhibitor, reverses the IBD. Short inhibiting RNAs (siRNAs) targeted toward PP2Ac reverse the effect of hyperosmotic DSS. The present study strongly suggests that DSS-induced chronic colitis is a consequence of the methylation of PP2Ac induced by hyperosmolarity.

inflammatory bowel disease; dextran sulfate sodium; osmolarity; NF-κB

INFLAMMATORY BOWEL DISEASE (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), represents a chronic relapsing and remitting inflammatory condition that affects individuals throughout life (28). No completely effective therapeutic strategy has been established because the etiology of IBD remains largely unknown although there has been extensive research on its pathogenesis. However, recent advances in the understanding of the pathophysiology of IBD have provided some clues for developing potentially helpful therapeutic tools.

Within the past three decades, several models of experimental colitis have been reported that demonstrate various pathophysiological aspects of human IBD. One of these models is based on oral administration of dextran sulfate sodium (DSS) mixed with the drinking water of rodents (24, 26).

Studies have shown that both acute and chronic colitis can be caused by administration of DSS (26, 22). DSS results in acute and chronic colitis with morphological changes that are similar to human UC. Long-term DSS administration first causes inflammation, followed by colorectal carcinoma, which is similar to the dysplasia-carcinoma sequence seen in the course of cancer development in human UC (42). The pathogenic mechanism of DSS-induced colitis remains undefined.

We recently published an article showing that any chemical, providing sufficient osmolarity, can induce an inflammation (1). The same osmolarity of chemically unrelated molecules, such as alanine, sodium chloride, or mannitol, induce similar cytokine secretion.

In epithelial cells such as normal colorectal epithelial cells, the induction of proinflammatory cytokine secretion through hyperosmolarity is mediated by the methylation of the catalytic subunit of protein phosphatase 2A (PP2Ac) (1). The methylation of PP2Ac, in turn, translocates NF-κB, a well-known transcription factor, which controls the synthesis of multiple cytokines. Xylitol, a sugar responsible for the methylation of PP2Ac, mimics the inflammatory effect of hyperosmolarity. Okadaic acid (OA), which removes the methyl from PP2Ac, alleviates the effect of hyperosmolarity.

Most investigators have focused on the role of osmolarity in the kidney where osmolarity levels as high as 1,500 mOsm have been reported (12, 31).

The idea that hyperosmolarity also regulates intestinal epithelial cell production of inflammatory cytokines is strengthened by the fact that hyperosmolarity has been reported in several inflammatory bowel diseases. These include CD and UC, as well as the IBD of the newborn and neonatal necrotizing enterocolitis (5, 32, 40).

It is our hypothesis that episodes of hyperosmolarity may contribute to (or even explain) the exacerbation of intestinal inflammatory disease via upregulation of the epithelial cytokine response. Here, we prove that the changes in osmolarity,

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which, in turn, result in PP2A methylation, have a role in colitis induced by DSS.

MATERIALS AND METHODS

Animals. The animals were treated in accordance with the European Community's guidelines concerning the care and use of laboratory animals. All aspects of the protocol conformed with the requirements of the laboratory's approval for animal research (1987 regulation) and were approved by a research ethic board.

For long-term DSS or mannitol exposure, male BALB/c mice 4–5 wk old were administered with DSS 2% (627 ± 124 mOsm) or mannitol 3.8% (450 ± 18 mOsm) for 12 wk. DSS (30–40 kDa; ICN Biomedicals, Costa Mesa, CA) or mannitol (Sigma, St. Louis, MO) were diluted in water, and osmolarity values were measured with a cryoscopic osmometer (Osmomat 030; GONOTEC, Berlin, Germany).

Eight mice received a saline solution as control condition. Animals were weighed daily, and the presence of blood in the stools or diarrhea was monitored (Hemoccult strips; Smith Kline Diagnostics, San Jose, CA). Four week before euthanasia, when stated, the mice were treated rectally with OA (10 nM in 0.2 ml) every 2 days after 12-h fasting.

For one experiment (Fig. 4), the animals were exposed to DSS or mannitol for a shorter duration. Eight male BALB/c mice in each group (4–5 wk old, four groups: control, DSS 0.5%, DSS 1.5%, and DSS 2% wt/vol) were treated with DSS for 3 wk. Eight mice received a saline solution as control condition.

Inflammatory scores. Inflammation was assessed on the basis of criteria described in Table 1 (36, 37). The final number was the sum of the diarrhea score, the visible fecal blood score, and the examination of the anus.

MPO assay. Tissue-associated myeloperoxidase (MPO) activity was determined using the standard enzymatic assay, and 1 U of MPO activity was defined as that degrading 1 μmol of hydrogen peroxide per minute (1).

Histology. To evaluate microscopic changes, mouse optimal cutting temperature Tissue-tek (OCT)-frozen (SAKURA, Zoeterwoude, Netherlands) colons were cut in a cryostat chamber into 5-μm sections. The slices were fixed with acetone for 10 min and stained with hematoxylin and eosin.

Sacral lymph nodes. The localization of the sacral lymph nodes was performed by footpad injections of 1% Evans blue. The day after, the animals were euthanized, and the sacral lymph nodes were collected, cleaned out of fat debris, and weighted.

Intraepithelial lymphocyte and epithelial cell preparation. Control and DSS-treated mice were euthanized after the short-term DSS exposure. Epithelial cells and intraepithelial lymphocytes (IELs) were isolated using the method of Todd et al. (38). Viable cells exhibiting lymphoid morphology were quantified by the Trypan blue method. Isolated IELs were then purified to obtain a $CD4^+$ population in an AutoMACS (Miltenyi Biotech, Bergisch Gladbach, Germany).

NF-κB p65 activation. Nuclear extraction was performed on $CD4^+$ IELs (1). Five micrograms of proteic extracts were tested for the NF-κB activation by using the NF-κB p65 TransAM transcription factor assay kit (Active Motif, Rixensart, Belgium), which is a nonradioactive transcription factor DNA-based ELISA, according to the manufacturer's instructions. In brief, activated NF-κB transcription factor in the nuclear cell extract binds to the consensus-binding site on the oligo immobilized in the well. Incubation with the supplied primary and secondary antibodies specifically quantifies the amount of activated transcription factor.

Foxp3 expression in $CD4^+$ T cells. RNA was extracted from an aliquot of the purified $CD4^+$ IELs with an RNeasy Kit (Qiagen, Valencia, CA). Quantitative differences in the levels of *Foxp3* were determined using semiquantitative RT-PCR on cDNA made with Superscript (Life Technologies, Gaithersburg, MD). ^{32}P-labeled dCTP was added to the PCRs, which were then run on standard sequencing gels (Gel-Mix 6, Life Technologies). Primers specific for *Foxp3* were 5′-GCTTGTTTGCTGTGCGGAGAC-3′ and 5′-GTTTCTGAAGTAGGCGAACAT-3′ (from GenBank accession no. XM 228771), and primers specific for the housekeeping gene *G6pd* were 5′-GACCTGCAGAGCTCCAATCAAC-3′ and 5′-CACGACCCTCAGTACCAAAGGG-3′ (from GenBank accession no. NM 017006) and were used as an internal control.

Determination of cytokine levels. Mouse colonic epithelial cells were withdrawn from control or DSS-treated mice and then cultured in DMEM supplemented with 10% decomplemented fetal bovine serum for 24 h. Cytokine secretion into the supernatant cells were quantified in triplicate after a 24-h ex vivo culture using the DuoSet ELISA development kit (R&D Systems, Minneapolis, MN) for mouse IL-6 and TGF-β1. The same kit was used for human cytokines in HT-29 cell supernatants: IL-8, IL-6, and monocyte chemoattractant protein (MCP)-1. For more technical specifications, see Abolhassani (1).

PP2A activity assay. At the end of administrations, mice were euthanized, and distal colons were removed to measure the total PP2A activity in fresh cells. Technical details have already been described in Abolhassani (1).

RT-PCR chemokine expression. Total RNA was isolated from cell suspensions by an RNeasy Kit (Qiagen). cDNAs were generated by incubating 2 μg of total RNA, 2,000 pmol Oligo dT, 1.0 mM dNTP, 200 U Moloney murine leukemia virus reverse transcriptase, and 5× RT buffer (Promega, Madison, WI). PCR primers (Qiagen) contained the following sequences: macrophage inflammatory protein (MIP)-2 sense (5′-CAGAGCTTGAGTGTGACG-3′) and antisense (5′-TCGTACCTGATGTGCCTC-3′); MCP-1 sense (5′-TCCACCACTATGCAGGTCTC-3′) and antisense (5′-TGGACCCATTCCTTATTGGG-3′); and GAPDH sense (5′-AATGGTGAAGGTCGGTGTGAAC-3′) and antisense (5′-GAAGATGGTGATGGGCTTCC-3′) (35). The PCR products were subjected to electrophoresis through 2% agarose gel (Invitrogen, Carlsbad, CA) and stained with a 0.5 mg/ml ethidium bromide Tris/borate/EDTA buffer.

Serum amyloid A expression. Serum amyloid A (SAA) concentrations were analyzed in EDTA plasma by using the solid phase sandwich immunoassay kit of mouse SAA (BioSource International, Camarillo, CA).

PP2Ac siRNA. The human colon cancer cell line, HT-29 (LGC Promochem, Molsheim, France) was cultured in a standard medium or containing DSS to reach an osmolarity of 600 mOsm (1). Combinations of three short inhibiting RNAs (siRNAs) targeting different positions within the β isoform of human *PP2Ac* mRNA (PP2Ac-siRNA) were used (Qiagen) (17). The sense strands were as follows: AUGUGCAAGAGGUUCGUUG, UGUCUGCGAAAGUAUGGGA, and UUGGUGUCAUGAUCGGAAU. A nonsilencing siRNA (non-PP2A siRNA) was included as control (sense strand: AATTCTCCGAACGTGTCACGT). Transfection of cells was carried out by electroporation using the Lipofectamine 2000 (Stratagene, La Jolla, CA). Twenty hours after transfection, cells were exposed to hyperosmotic stress (600 vs. 300 mOsm) for 12 h (cell growth not affected) before analyses.

Statistical analysis. Nonparametric tests were used. Median and confidence intervals (CI) of each sample and medians and CI for differences between unpaired samples were calculated according to

Table 1. *Scoring criteria of colonic inflammation*

Score	Diarrhea Score	Visible Fecal Blood	Examination of the Anus
0	Normal	Normal	Normal
1	Slightly loose feces	Slightly bloody	Slight inflammation
2	Loose feces	Bloody	Moderate inflammation
3	Watery diarrhea	Blood in whole colon	Ulceration

The final score is the sum of the diarrhea score, the visible fecal blood score, and the examination of the anus.

Gardner and Altman (11). The associated exact level of confidence was given at 96.9% for each sample size equaling 6. For differences between population medians of two unpaired samples, the associated level of confidence was based on the Wilcoxon two-sample rank sum distribution.

The nonparametric distribution-free Kruskal-Wallis test was used to compare three or more independent groups of sampled data. If significant differences were found, multiple-comparison tests (Tukey test) were carried out to identify which groups are different.

All statistical analyses were done with R software (30). Values were considered statistically significant when $P < 0.05$.

RESULTS

Clinical evaluation of long-term DSS administration. To gain insights into the effect of hyperosmolarity on the inflammatory response in DSS-treated mice, we compared DSS and mannitol (Man). Mice were fed for 12 wk with 627 mOsm DSS (2% solution) or 450 mOsm mannitol (3.8% solution) diluted in water. In a first experiment, mannitol-treated mice were exposed to a 627 mOsm solution, but this treatment was lethal in a few days.

Four weeks before euthanasia, the mice were treated rectally with OA, a well-known PP2A inhibitor. Oral administration of OA revealed itself to be too toxic.

Low osmolarity DSS or mannitol have no demonstrable effect on colonic inflammation. However, high osmolarity DSS and mannitol induce inflammation (Fig. 1). High osmolarity, either caused by DSS or mannitol, is responsible for diarrhea, fecal blood, and inflammation of the anus. Hyperosmotic concentration of DSS [CI 95.9% for difference between medians DSS and control (3.5–5.0)] led to a high increase in clinical and macroscopic scores in a similar way to mannitol [CI 95.9% for difference between medians Man and control (3.0–4.5)] (Fig. 1*A*).

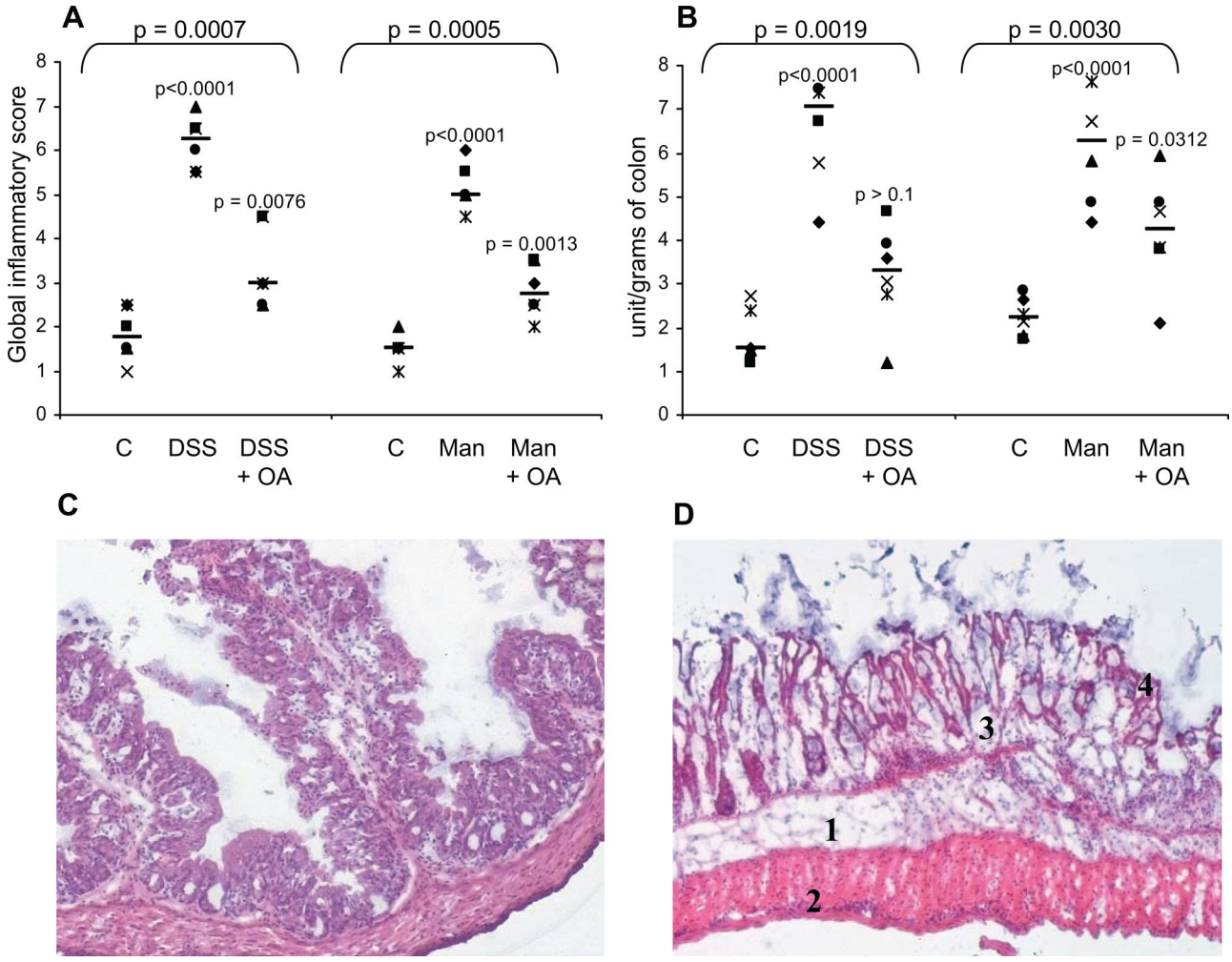

Fig. 1. Colonic inflammation in response to long-term dextran sulfate sodium (DSS) exposure. *A*: global inflammatory score established from 3 independent scores (diarrhea, visible fecal blood, and inflammation of the anus; detailed in Table 1). Hyperosmotic DSS (median 6.25) and mannitol (Man) (median 5) induce inflammation of the colon compared with control (C) group ($n = 8$). This inflammation is partly reversed by administration of okadaic acid (OA). *B*: myeloperoxidase (MPO) activity, measured by enzymatic assay in distal colonic cells, is increased more than twice between control (median 1.51) and hyperosmotic DSS (median 7.07) or mannitol (median 6.25 vs. 2.23). This MPO activity is partly reversed by the rectal administration of OA. For each group, the horizontal bar represents the median value. The result of Kruskal-Wallis test (*P* value) is given at the top of the figure. The *P* value given by the Tukey test above a stripchart indicates the result of the comparison of this group vs. control. *C* and *D*: representative photomicrographs of optimal cutting temperature (OCT)-frozen longitudinal sections of proximal mouse colons treated with 175 mOsm (*C*) or 627 mOsm (*D*) of DSS. The colon microstructure and epithelial cell microvilli are disorganized by hyperosmotic DSS treatment resulting in enhanced lamina propria thickness (1), inflammatory cell infiltration (2), crypt (3), and surface epithelial cell (4) loss. Slides were stained by hematoxylin standard staining as described above (30 × 100 magnification).

The slides (Fig. 1, *C* and *D*) confirm the clinical findings, namely colonic inflammation in response to hyperosmotic treatments. The low concentrations of DSS (Fig. 1*C*) have no noticeable effect. Higher osmolarities (Fig. 1*D*) are responsible for inflammatory features, such as epithelial cell death, as well as proliferation, recruitment of inflammatory cells, swelling of the colon, and disappearance of the intestinal crypts, all common features of inflammation.

As shown in Fig. 1*B*, both hyperosmotic DSS [CI 95.9% for difference between medians DSS and control (3.18–6.27)] and mannitol [CI 95.9% for difference between medians Man and control (2.26–5.80)] increase MPO activity of colonic cells. MPO activity is widely accepted as an enzyme marker to quantify the degree of inflammation and estimate the accumulation of inflammatory cells such as neutrophils. This is confirmed by the presence of immune cells, such as macrophages and neutrophils, as can be seen on the slides.

Long-term exposure to DSS and mannitol increases the expression of inflammatory chemokines in sacral lymph node cells. It has been reported that the sacral lymph nodes of mice with colitis are enlarged since the lymphoid cells undergo proliferation and recruitment (19, 41). Low osmolarity DSS or mannitol had no effect on sacral nodes. The weight of sacral lymph nodes was higher in animals treated with hyperosmotic DSS [CI 95.9% for difference between medians DSS and control (5.96–9.96)] and mannitol [CI 95.9% for difference between medians Man and control (5.47–8.64)] than in the isotonic controls (Fig. 2*A*). This strongly confirms the presence of colonic inflammation.

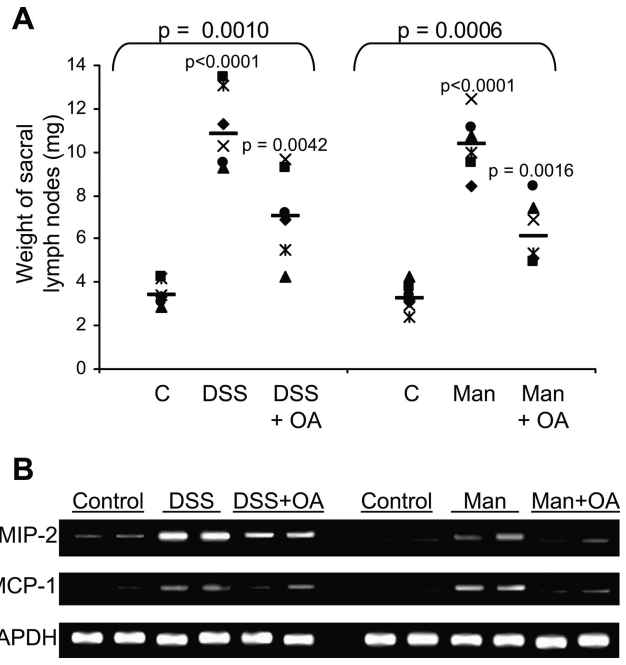

Fig. 2. Response of sacral lymph nodes to long-term DSS exposure. Confidence intervals (CI) at 96.9% are given in parentheses. Sacral lymph node weight (*A*) is increased by hyperosmotic DSS [control (2.88–4.28), DSS (9.27–13.44)] and mannitol exposure [control (2.38–4.26), Man (8.41–12.44)]. Chemokine mRNA expression in sacral lymph nodes (RT-PCR) after DSS (*left*) or mannitol (*right*) treatments +/− rectal administration of OA are displayed in *B*. For each group, the horizontal bar represents the median value. The result of Kruskal-Wallis test (*P* value) is given at the top of the figure. The *P* value given by the Tukey test above a stripchart indicates the result of the comparison of this group vs. control. MIP-2, macrophage inflammatory protein 2; MCP-1, monocyte chemoattractant protein 1.

Plasmatic SAA is increased after hyperosmotic administration of DSS or mannitol. SAA is an acute-phase protein whose levels positively correlate with disease activity in IBDs (25). Here again, exposure to low osmolarity had no demonstrable effect, but hyperosmotic DSS [CI 95.9% for difference between medians DSS and control (208.0–399.8)] or mannitol [CI 95.9% for difference between medians Man and control (158.2–209.9)] result in an inflammatory syndrome (Fig. 3).

Exposure to hyperosmotic DSS results in cytokine secretion and transcription factor activation. Using $CD4^+$ intraepithelial lymphocytes, we demonstrate that hyperosmotic DSS results in NF-κB activation and inhibition of Foxp3 expression (Fig. 4). Only a DSS solution with an osmolarity value of 627 mOsm significantly increases p65 NF-κB activation (Fig. 4*A*) and decreases Foxp3 mRNA expression (Fig. 4*B*). Similarly, the proinflammatory IL-6 cytokine and the antiinflammatory factor TGF-β1 were measured in a 24-h ex vivo culture of intestinal epithelial cells. A significant increase in IL-6 (Fig. 4*C*) and a significant decrease in TGF-β1 (Fig. 4*D*) were observed in the cells exposed to 627 mOsm DSS but not at lower osmolarity.

Chemokine expression in sacral lymph nodes was measured by RT-PCR after long-term DSS or mannitol exposure as shown in Fig. 2*B*. MIP-2 expression is much higher in DSS-treated mice than in control mice. Animals exposed to mannitol also showed a higher expression of MIP-2 than control mice.

Similarly, MCP-1 expression was higher in hyperosmotic DSS- or mannitol-treated animals compared with isotonic control (Fig. 2*B*).

Hyperosmolarity results in PP2A activation. Recently, we have demonstrated that hyperosmolarity causes inflammation through the methylation of PP2A. As shown in Fig. 5, PP2A activity is upregulated in inflamed mucosa in both DSS and mannitol models, indicating that DSS acts by hyperosmolarity in the same way that mannitol does. OA is a nonspecific inhibitor of PP2A methylation.

Colonic inflammation caused either by hyperosmotic DSS [CI 95.9% for difference between medians DSS and control (1.67–6.47)] or mannitol [CI 95.9% for difference between medians Man and control (2.19–4.65)] was reversed by OA, suggesting an involvement of the PP2A pathway (Fig. 5). Similarly the increased MPO activity was altered in the presence of OA (Fig. 1*B*). The increase in SAA concentration was also blocked by OA administration, suggesting again an implication of the PP2A pathway (Fig. 3).

The role of PP2A is not limited to the gastrointestinal tract. The MIP-2 expression in the sacral lymph node is partially blocked by OA (Fig. 2*B*). The MCP-1 expression in the same nodes was decreased by the OA administration in both DSS and mannitol models (Fig. 2*B*).

PP2A siRNA inhibits NF-κB activation, PP2A activity, and proinflammatory cytokine secretion induced by hypertonic medium. OA inhibits both PP2A and PP1. To confirm the specific implication of PP2A in the hyperosmotic-induced inflammatory response, we performed an in vitro PP2A RNA interference experiment. We used the human colon cancer cell line HT-29, which was transfected with a combination of 3 siRNAs, targeting different positions within the β isoform of *PP2Ac* mRNA (PP2Ac-siRNA). As shown in Fig. 6*A*, hyperosmotic-induced NF-κB nuclear translocation (median value:

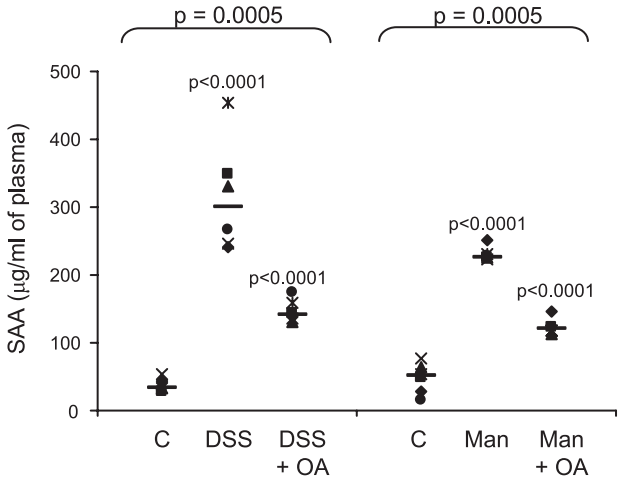

Fig. 3. Serum amyloid A (SAA) protein level is increased in plasma after long-term DSS exposure. CI at 96.9% are given in parentheses. SAA was measured by ELISA after hyperosmotic DSS [control (28.9–53.7), DSS (240.2–453.5)] or mannitol exposure [control (14.9–76.9), Man (221.8–252.4)] during 12 wk compared with control and hyperosmotic treatment plus OA administration (+OA). For each group, the horizontal bar represents the median value. The result of Kruskal-Wallis test (*P* value) is given at the top of the figure. The *P* value given by the Tukey test above a stripchart indicates the result of the comparison of this group vs. control.

0.511) is inhibited by PP2Ac-siRNA (median value: 0.133; $P = 0.00013$ Tukey test). Similarly, PP2A phosphatase-enhanced activity caused by hyperosmolarity (median value: 1.021) is reduced by PP2Ac-siRNA (median value: 0.562; $P = 0.0023$) (Fig. 6*B*). The secretion of the proinflammatory cytokines was also reduced in the presence of PP2Ac-siRNA (Fig. 6, *C*, *D*, *E*, and *F*) [Tukey tests IL-6: mean difference = 103.3 and CI (20.3–186.3), $P = 0.012$; MCP-1: mean difference = 108 and CI (62.0–154.0), $P = 0.00002$]. Interestingly, Fig. 6, *E* and *F*, shows that siRNA inhibits the proinflammatory IL-8 secretion induced by both hyperosmotic DSS [Tukey test: mean difference = 233.9 and CI (130.4–337.4), $P = 0.00004$] and mannitol [Tukey test: mean difference = 298.4 and CI (176.0–420.6), $P = 0.00001$] media, thus confirming the role of PP2A in hyperosmolarity-induced inflammation.

DISCUSSION

In most tissues, with the notable exception of the kidney, the extracellular osmolarity is lower than the osmolarity of the plasma. It is thought to be around 300 mOsmol (6). There are scant data on its evaluation during inflammation (9, 13, 32, 40). We assessed the osmolarity of the inflammatory fluid in other models and found an osmolarity of 425–450 mOsmol (data not shown) markedly above the normal osmolarity of the extracellular fluid. This increased osmolarity is due to protein breakdown (14).

We have previously shown both in vivo and in vitro that hyperosmolarity can induce proinflammatory cytokine responses in epithelial cells (1). It is our hypothesis that the role of hyperosmolarity has been underestimated and this factor may be a common feature in inflammation. Inflammation is characterized by tumor, dolor, rubor, and calor as stated by Galen 2000 years ago. Inflammation can be caused by factors as diverse as heat, freezing temperature, trauma, or chemicals.

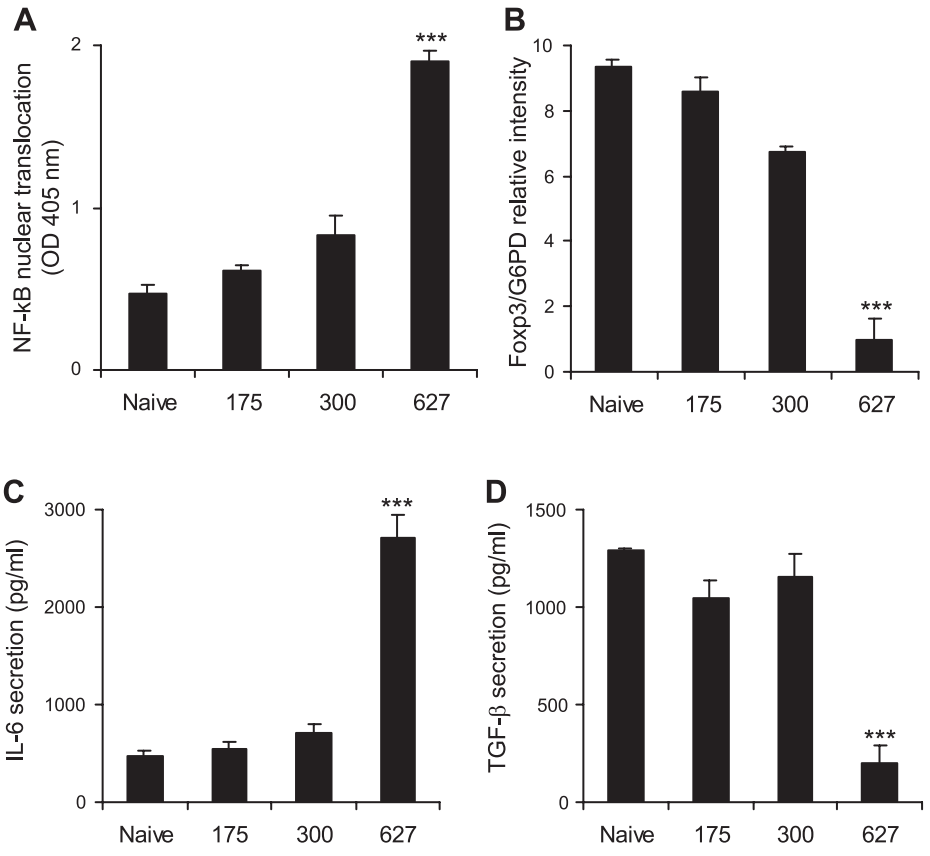

Fig. 4. Effect of different osmolarities of DSS on NF-κB nuclear translocation and cytokine expression by epithelial cells. Mice were exposed for 3 wk to various osmolarities of DSS (175, 300, and 627 mOsm), and $CD4^+$ intraepithelial lymphocytes (IELs) (*A* and *B*) or epithelial cells (*C* and *D*) were purified from colon and cultured ex vivo during 24 h. In $CD4^+$ IELs, p65 NF-κB nuclear translocation (*A*) is induced, and, on the contrary, Foxp3 expression (*B*) (semi-quantitative RT-PCR) is downregulated by hyperosmolarity. In intestinal epithelial cells, IL-6 (*C*) proinflammatory cytokine secretion (ELISA) is enhanced and TGF-β1 (*D*) secretion (ELISA) is reduced by hyperosmotic treatment. Data are means ± SE, $n = 8$; samples were pooled by 2. ***$P < 0.001$ vs. naive condition.

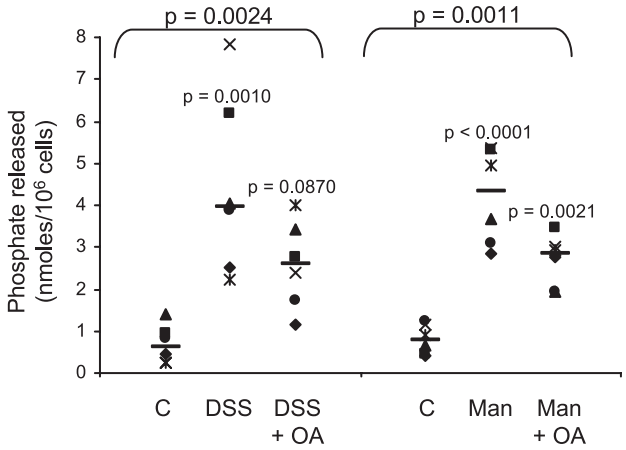

Fig. 5. Protein phosphatase 2A (PP2A) activation in response to long-term DSS exposure. CI at 96.9% are given in parentheses. The amount of phosphate released by PP2A in colonic cells after long-term exposure to DSS [control (0.254–1.387), DSS (2.242–7.854)] or mannitol [control (0.393–1.240), Man (2.86–5.362)] is 5 times higher compared with control. This is reduced by OA administration. For each group, the horizontal bar represents the median value. The result of Kruskal-Wallis test (*P* value) is given at the top of the figure. The *P* value given by the Tukey test above a stripchart indicates the result of the comparison of this group vs. control.

In this study, we show that hyperosmolarity explains most of the features of inflammation.

DSS-induced colitis is a well-rehearsed model of inflammation. Our results confirm that the inflamed colon shows signs of recruitment of neutrophils and macrophages, cell apoptosis and proliferation, cytokine secretion, and vascular leakage. These features are common to any form of inflammation.

The very mechanism of this polymer's toxicity remains to be elucidated. It is thought that it does not penetrate into the epithelial cell, and previous reports have postulated the importance of various factors (3). However, as this polysaccharide contains ~17% sulfur with up to three sulfate groups per glucose molecule, we do favor a key role for the negative charges of the DSS on the local osmolarity. When diluted into water, ions are attracted by the negative charges of DSS, markedly increasing the osmolarity of the solution (29).

In this study, we show that, in the DSS model of murine colitis, the inflammation response can be caused by osmotic stress, which stimulates the NF-κB activation through the PP2A pathway.

Drinking water has a very low osmolarity. The addition of the DSS as long as the osmolarity is below 300 mOsmol has little, if any, effect on the mice. However, higher osmolarity levels are responsible for a major inflammatory syndrome. For example, treatment with 627 mOsmol is responsible for vascular leakage resulting in the presence of fecal blood, recruitment of immune cells, and the secretion of multiple cytokines.

This inflammatory syndrome is not confined to the gastrointestinal tract. Hyperosmolarity caused by the presence of DSS in the lumen appears to be correlated with increased size of the sacral lymph nodes and the increased plasma level of SAA.

In that setting, inflammation appears to be the simple consequence of a shift in PP2A methylation, which in turn activates NF-κB. NF-κB is the key determinant of the epithelial inflammatory cascade and plays a central role in its regulation. Activation of NF-κB has been noted in IBD in humans and in DSS colitis in mice (10, 21, 23, 33).

The data presented here also provide evidence that inflammatory response, mediated by NF-κB, is regulated by PP2A in the context of a hyperosmotic stimulus caused by either DSS or mannitol.

PP2A is an endogenous regulator of inflammatory cell signaling and is the most common phosphatase accounting for about 1% of the total protein content of the cell (34). 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, whereas 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 PP2Ac must be methylated before it can complex with the B unit to form the active enzyme (2, 15, 18). One of the targets of methylated PP2A is inhibitor of κB kinase (IKK-γ), which in turn activates NF-κB. The activation of NF-κB results in the release of proinflammatory mediators and the inhibition of antiinflammatory cytokines. This is demonstrated by the fact that PP2A inhibitors decrease the secretion of proinflammatory cytokines after hyperosmotic stimulation (1).

OA is a toxin that accumulates in bivalves and causes diarrheic shellfish poisoning. OA inhibits the methylation of PP2A and therefore abolishes the effect of hyperosmolarity (1, 8, 16). It is not known whether the toxicity of OA is mediated by the methylation of PP2A. Because of its toxicity, oral administration was impossible (data not shown). Rectal administration had not been reported before.

Pharmacological inhibition of PP2A by OA resulted in a substantial decrease in global inflammatory scores, MPO activity, sacral lymph node weight, and SAA protein levels. Since OA inhibits both PP2A and PP1 (7), we used interfering RNA directed toward the catalytic subunit of PP2A to confirm the key role of that phosphatase. The addition of siRNAs directed against PP2Ac reverses the effect of DSS and mannitol, strongly suggesting that hyperosmotic shock is mediated by that phosphatase.

Long-term DSS administration can lead to dysplasia, often a precursor to tumorigenesis. Interestingly, PP2A has been reported to play an important role in cancer development (2, 15, 18). There are numerous other published examples that delineate the key role that PP2A could play with respect to the multi-stage process of carcinogenesis. These include but are not restricted to downregulation of Art kinase, which is involved in apoptosis (20), regulation of Ras signaling (27), stabilization of c-Myc, and regulation of apoptosis of neutrophils through inhibition of the JNK pathway (4, 43). It is important to point out that there is considerable evidence that PP2A can also inhibit the carcinogenic process in certain cell types and has even been affirmed to be a tumor-suppressor gene (39). The explanations for these apparently opposing effects are certainly not yet elucidated. However, the reason may be, as noted above, that PP2A is not a single protein but a multitask enzyme whose exact function may depend on the nature of the B subunit.

This study does not attempt to validate the use of DSS-treated mice as a model for the study of IBD. Rather, this study shows that a significant proportion of the DSS-induced inflammation is linked to hyperosmotic stress that activates NF-κB in the colonic epithelium via PP2A. Furthermore, our results

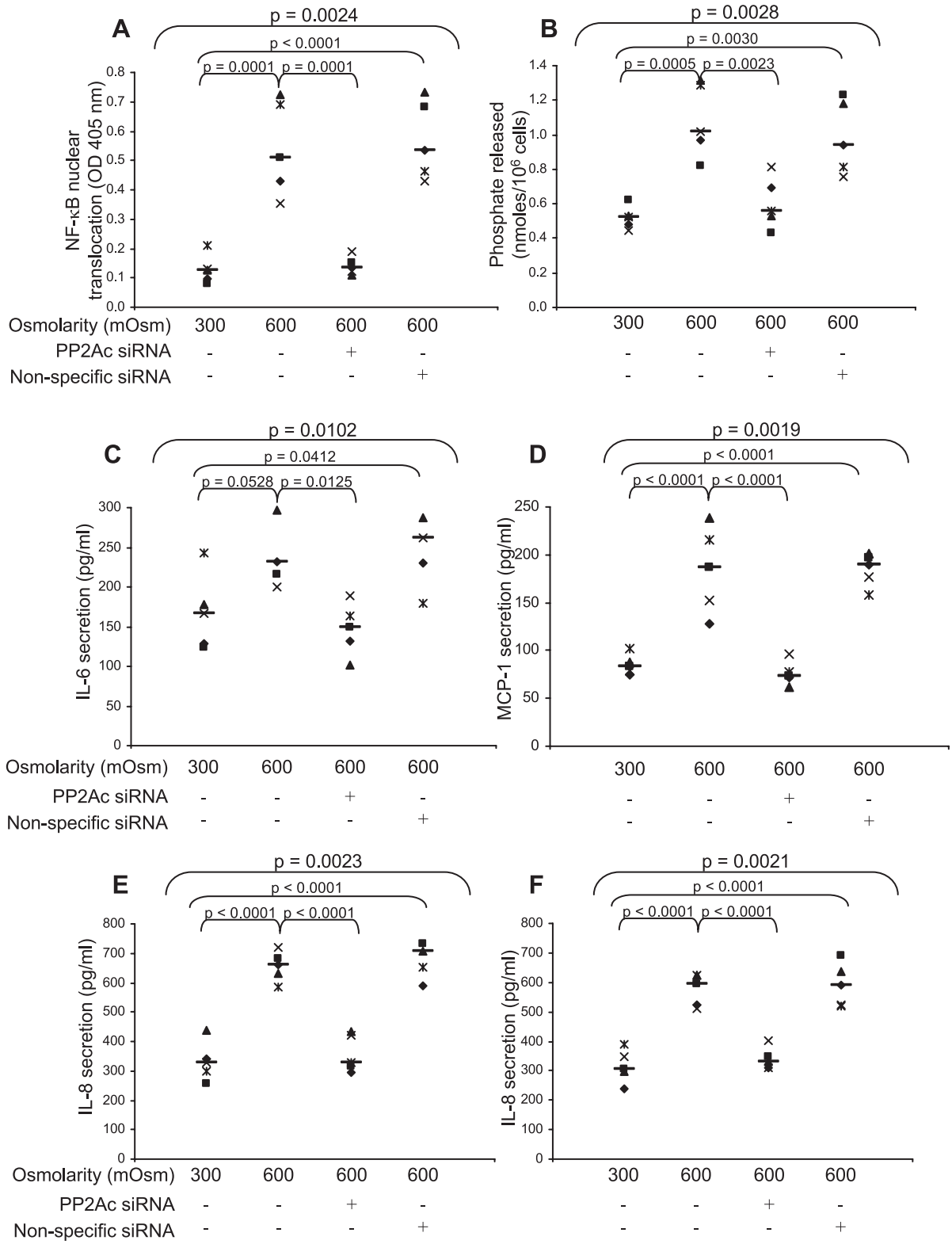

Fig. 6. PP2Ac short inhibiting (si)RNAs abolished the inflammatory reaction caused by DSS or mannitol hyperosmotic stimulation of HT-29 colonic cells. CI at 96.9% are given in parentheses. HT-29 cells were transfected with PP2Ac-specific and -nonspecific siRNAs and then incubated in isosmotic (300 mOsm) or DSS-hyperosmotic media (600 mOsm) for 12 h ($n = 5$). NF-κB nuclear translocation (*A*) [600 mOsm (0.352–0.724), 600 mOsm + PP2A siRNA (0.109–0.188)] and PP2A activity (*B*) [600 mOsm (0.822–1.314), 600 mOsm + PP2A siRNA (0.428–0.814)] are specifically inhibited by PP2Ac siRNAs. Consequently, IL-6 (*C*) [600 mOsm (199.42–308.14), 600 mOsm + PP2A siRNA (101.52–188.13)], MCP-1 (*D*) [600 mOsm (128.3–238.14), 600 mOsm + PP2A siRNA (101.52–188.13)], and IL-8 (*E* and *F*) proinflammatory cytokine productions (ELISA) are reduced in the presence of PP2Ac siRNAs. IL-8 secretion by HT-29 cells is similar in presence of mannitol (*E*) [600 mOsm (586.4–721.8), 600 mOsm + PP2A siRNA (295.3–432.8)] or DSS (*F*) [600 mOsm (509.8–624.7), 600 mOsm + PP2A siRNA (311.5–401.4)] in the presence or absence of PP2Ac siRNAs. For each group, the horizontal bar is the median. Upper values represent the global *P* values (Kruskal-Wallis test). Above each pair of points, the values represent the *P* values of the comparison between the two conditions (Tukey test).

support a possible role for hyperosmotic stress in the pathophysiology of IBD. It also suggests the potential involvement of hyperosmolarity in the carcinogenic process.

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REFERENCES

1. **Abolhassani M, Wertz X, Pooya M, Chaumet-Riffaud P, Guais A, Schwartz S.** Hyperosmolarity causes inflammation through the methylation of protein phosphatase 2A. *Inflammation Research.* In press.
2. **Alberts AS, Deng T, Lin A, Meinkoth JL, Schönthal A, Mumby MC, Karin M, Feramisco JR.** Protein phosphatase 2A potentiates activity of promoters containing AP-1-binding elements. *Mol Cell Biol* 13: 2104–2112, 1993.
3. **Araki Y, Andoh A, Fujiyama Y, Hata K, Makino J, Okuno T, Nakanura F, Bamba T.** Application of 2-aminopyridine fluorescence labeling in the analysis of in vivo and in vitro metabolism of dextran sulfate sodium by size-exclusion high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl* 753: 209–215, 2001.
4. **Avdi NJ, Malcolm KC, Nick JA, Worthen GS.** A role for protein phosphatase-2A in p38 mitogen-activated protein kinase-mediated regulation of the c-Jun NH(2)-terminal kinase pathway in human neutrophils. *J Biol Chem* 277: 40687–40696, 2002.
5. **Cheromcha DP, Hyman PE.** Neonatal necrotizing enterocolitis. Inflammatory bowel disease of the newborn. *Dig Dis Sci* 33: 78S–84S, 1988.
6. **Debnam.** ES osmolarity and partitioning of fluids. *Surgery* 23: 190–194, 2005.
7. **Favre B, Turowski P, Hemmings BA.** Differential inhibition and post-translational modification of protein phosphatase 1 and 2A in MCF7 cells treated with calyculin-A, okadaic acid, and tautomycin. *J Biol Chem* 272: 13856–13863, 1997.
8. **Floer M, Stock J.** Carboxyl methylation of protein phosphatase 2A from Xenopus eggs is stimulated by cAMP and inhibited by okadaic acid. *Biochem Biophys Res Commun* 98: 372–379, 1994.
9. **Freed AN, Davis MS.** Hyperventilation with dry air increases airway surface fluid osmolality in canine peripheral airways. *Am J Respir Crit Care Med* 159: 1101–1107, 1999.
10. **Gan H, Ouyang Q, Chen Y, Xia Q.** Activation of nuclear factor-kappaB and effects of anti-inflammatory treatment thereon in intestinal mucosa of patients with ulcerative colitis. *Zhonghua Yi Xue Za Zhi* 82: 384–388, 2002.
11. **Gardner MJ, Altman DG.** *Statistics With Confidence*. London, UK: BMJ Books, 1989.
12. **Hao CM, Yull F, Blackwell T, Kömhoff M, Davis LS, Breyer MD.** Dehydration activates an NF-κB-driven, COX2-dependent survival mechanism in renal medullary interstitial cells. *J Clin Invest* 106: 973–982, 2000.
13. **Hashimoto S, Matsumoto K, Gon Y, Nakayama T, Takeshita I, Horie T.** Hyperosmolarity-induced interleukin-8 expression in human bronchial epithelial cells through p38 mitogen-activated protein kinase. *Am J Respir Crit Care Med* 159: 634–640, 1999.
14. **Israel M, Schwartz L.** *Cancer: A Dysmethylation Syndrome*. Paris, France: John Libbey Eurotext, 2005.
15. **Janssens V, Goris J, Van Hoof C.** PP2A: the expected tumor suppressor. *Curr Opin Genet Dev* 15: 34–41, 2005.
16. **Jessus C, Rime H, Haccard O, Van Lint J, Goris J, Merlevede W, Ozon R.** Tyrosine phosphorylation of p34cdc2 and p42 during meiotic maturation of Xenopus oocyte. Antagonistic action of okadaic acid and 6-DMAP. *Development* 3: 813–820, 1991.
17. **Katsiari CG, Kyttaris VC, Juang YT, Tsokos GC.** Protein phosphatase 2A is a negative regulator of IL-2 production in patients with systemic lupus erythematosus. *J Clin Invest* 115: 3193–3204, 2005.
18. **Lechward K, Awotunde OS, Swiatek W, Muszyñska G.** Protein phosphatase 2A: variety of forms and diversity of functions. *Acta Biochim Pol* 48: 921–933, 2001.
19. **Lim BO.** Efficacy of wogonin in the production of immunoglobulins and cytokines by mesenteric lymph node lymphocytes in mouse colitis induced with dextran sulfate sodium. *Biosci Biotechnol Biochem* 68: 2505–2511, 2004.
20. **Liu W, Akhand AA, Takeda K, Kawamoto Y, Itoigawa M, Kato M, Suzuki H, Ishikawa N, Nakashima I.** Protein phosphatase 2A-linked and -unlinked caspase-dependent pathways for downregulation of Akt kinase triggered by 4-hydroxynonenal. *Cell Death Differ* 10: 772–781, 2003.
21. **Marrero JA, Matkowskyj KA, Yung K, Hecht G, Benya RV.** Dextran sulfate sodium-induced murine colitis activates NF-kappaB and increases galanin-1 receptor expression. *Am J Physiol Gastrointest Liver Physiol* 278: G797–G804, 2000.
22. **Melgar S, Karlsson A, Michaëlsson E.** Acute colitis induced by dextran sulfate sodium progresses to chronicity in C57BL/6 but not in BALB/c mice: correlation between symptoms and inflammation. *Am J Physiol Gastrointest Liver Physiol* 288: G1328–G1338, 2005.
23. **Neurath MF, Pettersson S, Meyer zum Büschenfelde KH, Strober W.** Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice. *Nat Med* 2: 998–1004, 1996.
24. **Ni J, Chen SF, Hollander D.** Effects of dextran sulphate sodium on intestinal epithelial cells and intestinal lymphocytes. *Gut* 39: 234–241, 1996.
25. **Niederau C, Backmerhoff F, Schumacher B.** Inflammatory mediators and acute phase proteins in patients with Crohn's disease and ulcerative colitis. *Hepatogastroenterology* 44: 90–107, 1997.
26. **Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R.** A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* 98: 694–702, 1990.
27. **Ory S, Zhou M, Conrads TP, Veenstra TD, Morrison DK.** Protein phosphatase 2A positively regulates Ras signaling by dephosphorylating KSR1 and Raf-1 on critical 14-3-3 binding sites. *Curr Biol* 13: 1356–1364, 2003.
28. **Podolsky DK.** Inflammatory bowel disease. *N Engl J Med* 347: 417–429, 2002.
29. **Pollack GH.** *Cells, Gels and the Engine of Life*. Seattle, WA: Ebner & Sons, 2001.
30. **R Development Core Team.** *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing, 2006.
31. **Santos BC, Pullman JM, Chevaile A, Welch WJ, Gullans SR.** Chronic hyperosmolarity mediates constitutive expression of molecular chaperones and resistance to injury. *Am J Physiol Renal Physiol* 284: F564–F574, 2003.
32. **Schilli R, Breuer RI, Klein F, Dunn K, Gnaedinger A, Bernstein J, Paige M, Kaufman M.** Comparison of the composition of faecal fluid in Crohn's disease and ulcerative colitis. *Gut* 23: 326–32, 1982.
33. **Schreiber S, Nikolaus S, Hampe J.** Activation of nuclear factor kappaB in inflammatory bowel disease. *Gut* 42: 477–484, 1998.
34. **Shanley TP, Vasi N, Denenberg A, Wong HR.** The serine/threonine phosphatase, PP2A: endogenous regulator of inflammatory cell signaling. *J Immunol* 166: 966–972, 2001.
35. **Shibata M, Hariya T, Hatao M, Ashikaga T, Ichikawa H.** Quantitative polymerase chain reaction using an external control mRNA for determination of gene expression in a heterogenous cell population. *Toxicol Sci* 49: 290–296, 1999.
36. **Stucchi AF, Shofer S, Leeman S, Materne O, Beer E, McClung J, Shebani K, Moore F, O'Brien M, Becker JM.** NK-1 antagonist reduces colonic inflammation and oxidative stress in dextran sulfate-induced colitis in rats. *Am J Physiol Gastrointest Liver Physiol* 279: G1298–G1306, 2000.
37. **Sukumar P, Loo A, Adolphe R, Nandi J, Oler A, Levine RA.** Dietary nucleotides augment dextran sulfate sodium-induced distal colitis in rats. *J Nutr* 129: 1377–1381, 1999.
38. **Todd D, Singh AJ, Greiner DL, Mordes JP, Rossini AA, Bortell R.** A new isolation method for rat intraepithelial lymphocytes. *J Immunol Methods* 224: 111–127, 1999.
39. **Van Hoof C, Goris J.** PP2A fulfills its promises as tumor suppressor: which subunits are important? *Cancer Cell* 5: 105–106, 2004.
40. **Vernia P, Gnaedinger A, Hauck W, Breuer RI.** Organic anions and the diarrhea of inflammatory bowel disease. *Dig Dis Sci* 33: 1353–1358, 1988.
41. **Wu XF, Fei MJ, Shu RG, Tan RX, Xu Q.** Fumigaclavine C, an fungal metabolite, improves experimental colitis in mice via downregulating Th1 cytokine production and matrix metalloproteinase activity. *Int Immunopharmacol* 5: 1543–1553, 2005.
42. **Yamada Y, Marshall S, Specian RD, Grisham MB.** A comparative analysis of two models of colitis in rats. *Gastroenterology* 102: 1524–1534, 1992.
43. **Yeh E, Cunningham M, Arnold H, Chasse D, Monteith T, Ivaldi G, Hahn WC, Stukenberg PT, Shenolikar S, Uchida T, Counter CM, Nevins JR, Means AR, Sears R.** A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. *Nat Cell Biol* 6: 308–318, 2004.