Hyperosmolar Stress Induces Neutrophil Extracellular Trap Formation: Implications for Dry Eye Disease

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Purpose. To determine if hyperosmolar stress can stimulate human neutrophils to form neutrophil extracellular traps (NETs) and to investigate potential strategies to reduce formation of NETs (NETosis) in a hyperosmolar environment.

Methods. Neutrophils were isolated from peripheral venous blood of healthy subjects and incubated in iso-osmolar (280 mOsM) or hyperosmolar (420 mOsM) media for 4 hours. Neutrophil extracellular traps were quantified using a PicoGreen dye assay to measure extracellular DNA. Two known inhibitors of NETosis, staurosporine and anti-β2 integrin blocking antibody, and two proresolution formyl peptide receptor 2 (FPR2) agonists, annexin/lipocortin-1 mimetic peptide and 15-epi-lipoxin A_4, were evaluated as possible strategies to reduce hyperosmolarity-induced NETosis.

Results. The amount of NETs induced by hyperosmolar medium (420 mOsM) increased linearly over time to 3.2 ± 0.3 times that induced by iso-osmolar medium at 4 hours ($P < 0.05$). NETosis increased exponentially with increasing osmolarity and was independent of the stimulus used to increase osmolarity. Upon neutrophil exposure to hyperosmolar stress, restoration of iso-osmolar conditions decreased NET formation by $52.7\% \pm 5\%$ ($P < 0.05$) but did not completely abrogate it. Among the strategies tested to reduce NETosis in a hyperosmolar environment, annexin-1 peptide was the most efficacious.

Conclusions. Hyperosmolarity induces formation of NETs by neutrophils. This NETosis mechanism may explain the presence of excessive NETs on the ocular surface of patients with dry eye disease. Because they reduce hyperosmolarity-induced NETosis, FPR2 agonists may have therapeutic potential in these patients.

Keywords: dry eye, hyperosmolar stress, extracellular DNA, neutrophil extracellular traps

O ur understanding of neutrophil functions has fundamentally changed in the last 10 years. For example, contrary to the previous belief that neutrophils were short-lived cells, it is now believed that the circulatory half-life of neutrophils in humans is more than 10 times longer than previously thought.[1] Another discovery about neutrophil functions that has gained much attention in the last decade is the recognition that neutrophils are able to expel their nuclear DNA in a final act of defense, as a sticky and toxic net that binds and kills pathogens.[2,3] These extracellular DNA (eDNA) webs, termed neutrophil extracellular traps (NETs), contain decondensed chromatin, histones, neutrophil elastase, and antimicrobial peptides such as cathelicidin (LL-37).[4] This unique type of neutrophil cell death, recently described as NETosis, is distinct from apoptosis and necrosis and is characterized by the active release of nuclear chromatin fibers.[3,5] Although NET formation is an important defense mechanism of the innate immune system, it has also been implicated in the pathobiology of chronic inflammatory conditions.[3,6–8]

We have recently reported the role of NETs and eDNA as possible sources of inflammation in dry eye disease (DED), a chronic inflammatory disorder of the ocular surface.[9–11] We reported the presence of excessive amounts of eDNA and molecular components of NETs on the ocular surface of patients with DED, along with a deficiency of tear fluid nucleases, and we showed that eDNA abundance was highest in patients with severe DED. Also, we showed that treatment with recombinant human DNase I eye drops (rhDNase I; 0.1% four times a day) in patients with severe refractory DED and excessive tear fluid eDNA resulted in symptomatic improvement, resolution of corneal rose bengal staining, and a reduction in tear fluid eDNA abundance.[10]

NETosis is triggered by a variety of stimuli, including microorganisms, bacterial components such as lipopolysaccharide (LPS), proinflammatory cytokines (IL-8), activated platelets, and endothelial cells (ECs).[5,11] Stimulation of neutrophils with microorganisms is much more efficient at inducing NET formation than stimulation with single bacterial components such as LPS, suggesting that multiple signals are required to achieve optimal NET production by neutrophils.[12,13]

Although NETs play an important role in the pathophysiology of dry eyes, the stimuli that induce NET formation in these patients are unknown. One of the important and well-known stressors of the ocular surface in dry eyes is hyperosmolarity. The 2007 Dry Eye WorkShop (DEWS) report considered both tear instability and hyperosmolarity to be core mechanisms of

dry eye, suggesting that tear instability leads to hyperosmolar conditions and inflammation, resulting in the dry eye symptoms of ocular irritation.[14] Hyperosmolarity stimulates death of the epithelial surface cells and a cascade of inflammatory events, which lead to loss of mucin-producing goblet cells. This exacerbates tear film instability and contributes to the circle of events that perpetuate DED.[15,16] Higher tear osmolarity is associated with increasing severity of dry eyes. Because we have found a high abundance of eDNA in patients with severe dry eyes, we hypothesized that hyperosmolar stress itself could stimulate NETosis.

Here, we describe the effect of hyperosmolar stress on human neutrophils and demonstrate that hyperosmolar stress, alone, can induce NETosis in healthy neutrophils, even in the absence of other NETosis inducers. Also, we investigated potential strategies to reduce NETosis in a hyperosmolar environment. Two strategies for reducing NETosis that have received attention are treatment with staurosporine or β2 integrin antagonists, which are inducers of apoptosis. However, both of these have been tested using preincubation of neutrophils with the respective agents, which is clinically impractical. Neutrophils on the surface of the eye are present in a hyperosmolar environment, so we tested whether these agents had the ability to reduce NETosis in a hyperosmolar environment.

We thus hypothesize that hyperosmolar stress promotes NETosis in emigrating neutrophils on the ocular surface of DED patients. This may be an additional mechanism by which hyperosmolar stress induces ocular surface inflammation.

METHODS

Study Population

Study approval was obtained from the Institutional Review Board of the University of Illinois at Chicago. Asymptomatic healthy individuals were enrolled, and informed consent was obtained from all participants after the nature and possible consequences of the study were explained. Research was conducted in accordance with the requirements of the Health Insurance Portability and Accountability Act and the tenets of the Declaration of Helsinki.

Isolation of Neutrophils

Peripheral venous blood samples from healthy volunteers ($n = 9$ subjects) were collected in heparinized vacuum containers and processed immediately after phlebotomy. Neutrophils were isolated with Histopaque and dextran sedimentation, using a modification of previously described methodology.[17,18] Briefly, blood diluted 1:1 with Roswell Park Memorial Institute medium (RPMI-1640, cat. no. 11835-030; Life Technologies, Carlsbad, CA, USA) was carefully layered on a Histopaque column (cat. no. 10771; Sigma-Aldrich Corp., St. Louis, MO, USA) and centrifuged at $649g$ without brakes for 30 minutes, at 25°C. The pellet containing red blood cells (RBCs) and white blood cells was then treated with 2.5% dextran to promote RBC rouleaux formation, leading to the formation of a leukocyte-rich, RBC-poor layer above the RBC-rich sediment. This supernatant layer was collected and centrifuged to pellet the cells. After two washes with 1× PBS, the cells were treated with 1× RBC lysing buffer (cat. no. 555899; BD Biosciences, San Jose, CA, USA) to remove the residual RBCs. The final cell pellet was washed three times with RPMI medium supplemented with 2% fetal bovine serum (FBS). Neutrophils were counted using an automated cell counter (Cellometer K2; Nexcelom Bioscience, Lawrence, MA, USA). Acridine orange/ propidium iodide (AO/PI) staining solution (cat. no. CS2-0106; Nexcelom Bioscience) was used to stain live and dead cells, respectively. Isolated neutrophils, if ≥95% viable, were used for experiments.

Incubation of Neutrophils for NETosis Induction

RPMI medium containing 2% FBS (complete medium; CM) was used for all cell cultures. Freshly isolated neutrophils were resuspended in CM, plated in 48-well tissue culture-treated plates (0.5×10^6/well), and incubated at 37°C in the presence of 5% CO_2. After a 15-minute incubation to allow cells to adhere to the culture plate, iso-osmolar (280 mOsM; CM) or hyperosmolar (420 mOsM, prepared by addition of 80 mM NaCl to CM) media were added. Phorbol myristate acetate (PMA, 10 nM) stimulation was used as a positive control. In order to investigate whether formation of NETs increases with increasing osmolarity, neutrophils were incubated in media of intermediate osmolarity, 316, 354, and 390 mOsM, prepared by addition of 20, 40, and 60 mM NaCl, respectively. Neutrophils were incubated for 4 hours before quantification of NETs. In order to investigate whether formation of NETs increases over time, NETosis was measured at 1, 2, 3, and 4 hours. To confirm that the effect seen with hyperosmolar medium was due to hyperosmolarity and not due to NaCl itself, we also measured NET formation in sucrose-rich hyperosmolar medium (423 mOsM), prepared by adding 4.5% sucrose to CM. The actual osmolarity of all media was confirmed by freezing-point depression using an Advanced DigiMatic Osmometer (model 3D2; Advanced Instruments, Norwood, MA, USA).

We also performed experiments to evaluate whether in neutrophils that have been exposed to hyperosmolar stress, NETosis decreases when iso-osmolarity is restored. For these experiments, supernatant hyperosmolar culture medium was gently removed at 2 hours of incubation, new culture medium (iso-osmolar or hyperosmolar) was added, and neutrophils were cultured for another 2 hours.

At the end of the respective incubation periods, the supernatant medium was discarded; wells were washed gently with 1× PBS to remove any non-NET–associated DNA released into the medium from dying neutrophils and then treated with pulmozyme (200 IU/mL, diluted in serum-free RPMI medium) for 5 minutes with shaking at 37°C. After disruption of NETs with pulmozyme, EDTA (0.5 mM) was added to stop the reaction. The sample of NETs thus obtained was used for measurement of DNA using the PicoGreen dye assay, or for neutrophil elastase activity using the NET assay kit. Experiments were performed in triplicate for all collected blood samples. To assess test repeatability, neutrophils were isolated three times over 3 weeks and NETosis measurements were performed ($n = 3$ subjects).

Role of Possible Inhibitors of NETosis

We investigated the usefulness of possible therapeutic strategies for reducing NETosis in a hyperosmolar environment. We tested two known inhibitors of NETosis, staurosporine[19] (STS; cat. no. S6942, Sigma-Aldrich Corp.) and anti-β2 integrin blocking antibody[20] (clone TS1/18; BioLegend, San Diego, CA, USA), and two proresolution formyl peptide receptor 2 (FPR2) agonists, annexin/lipocortin-1 mimetic peptide (Ac2-26; R&D Systems, Minneapolis, MN, USA) and 15-epi-lipoxin A₄ (LXA₄; Cayman Chemical, Ann Arbor, MI, USA). Neutrophils were incubated with STS (1 μM), anti-β2 antibody (10 μg/mL), Ac2-26 (0.1 mg/mL), or LXA₄ (10 nM) in the respective media after they had been allowed to adhere for 15 minutes in iso-osmolar medium (CM). Neutrophil extracellular traps were measured after 4 hours of incubation in a manner similar to

that described above. Osmolarity was measured for all media after the addition of each agent to eliminate any change in osmolarity that could influence the results. Also, to eliminate any influence of dead/dying cells on the results, cell viability was measured in the collected NET samples using an AO/PI staining solution and the automated cell counter.

We incubated neutrophils with the agents under study in the presence of hyperosmolar stress because this is the situation most likely to be encountered in a clinical setting. If a patient uses eye drops that decrease NETosis, they would be required to act in the hyperosmolar milieu already present on the ocular surface. Because prior studies have shown a decrease in NETosis after preincubation with STS and anti-β2 integrin,[19,20] we measured NETosis after preincubation with these agents. Neutrophils were first incubated with STS for 15 minutes, or with anti-β2 integrin for 30 minutes (to replicate the experiments as mentioned in previous studies), after which the medium was removed and iso-osmolar medium with 10 nM PMA was added. NETosis was then measured after 4 hours of incubation with PMA.

PicoGreen Assay for Measurement of eDNA

The Quant-iT PicoGreen dsDNA assay kit (cat. no. P7589; Life Technologies) was used to quantify eDNA as a measure of the amount of NETs produced, as previously described.[10] Pico-Green is a cell-impermeant dye that binds to extracellular double stranded Deoxyribonucleic acid (dsDNA) without staining live cells. Fluorescence signal intensity was measured at excitation, 480 nm, and emission, 520 nm, using a microplate reader (Synergy H1; BioTek, Winooski, VT, USA). Neutrophil extracellular traps collected from each well were measured in triplicate.

Neutrophil Elastase Activity Assay

To confirm the presence of NETs in our samples, we also measured the neutrophil elastase activity in some samples. The Neutrophil Extracellular Trap assay kit (cat. no. 601010; Cayman Chemical) was used per manufacturer's instructions. After 2 hours of incubation with test substrate, absorbance was measured at 405 nm using a microplate reader. The substrate absorbs light only after it has been cleaved by neutrophil elastase. Thus, the absorbance indicates neutrophil elastase activity. A standard curve was plotted (linear, $r^2 = 0.999$) with known concentrations of neutrophil elastase enzyme, and the equation thus derived was used to convert the absorbance of test samples to neutrophil elastase activity.

Immunofluorescence Staining and Microscopy

Isolated neutrophils were plated on tissue culture-treated compartment slides and incubated for 4 hours in the presence of iso-osmolar or hyperosmolar culture media. The glass slides were fixed with 4% paraformaldehyde for 30 minutes, followed by immunofluorescence staining and microscopy, as described previously.[9] Slides were analyzed using an inverted Zeiss Axio Observer microscope (Zeiss, Thornwood, NY, USA) to visualize neutrophil morphology and identify NETs. Neutrophil extracellular traps were identified as extracellular strands positive for both neutrophil elastase and 4′,6-diamidino-2-phenylindole (DAPI). The primary antibody used was mouse monoclonal anti-human neutrophil elastase (clone NP570; DAKO, Glostrup, Denmark), while the secondary antibody used was Dylight 594-conjugated anti-mouse IgG (1:1000; Jackson Immunoresearch Laboratories, West Grove, PA, USA). The specificity of the anti-

neutrophil elastase antibody has been previously validated.[9,21,22]

Statistical Analysis

Following compilation of data using Microsoft Excel Office statistics software (Redmond, WA, USA), the arithmetic means and standard errors (SEs) of means were calculated for all quantitative parameters. Quantitative variables between different groups were compared using Student's t-tests (SPSS Statistics, version 22; IBM Corporation, Armonk, NY, USA). $P < 0.05$ was considered statistically significant.

RESULTS

All experiments were performed with freshly isolated human neutrophils that had a viability $> 95\%$ (range, 95.2%–97.7%). The experiments were performed within 1 hour of isolation.

We determined the effect of hyperosmolarity on formation of NETs (NETosis) ($n = 9$ subjects). NETosis was measured using two methods: a PicoGreen dye assay for eDNA and a Neutrophil Extracellular Trap assay for neutrophil elastase activity. After 4 hours of incubation, fluorescence due to released DNA in hyperosmolar (420 mOsM) medium was 3.2 ± 0.3 times that released in iso-osmolar (280 mOsM) medium (range, 2.3–6.6). The amount of NETs released was significantly greater in hyperosmolar medium than in iso-osmolar medium ($P < 0.05$; $n = 9$ subjects, 16 samples) (Fig. 1A1). NETosis induced by 420 mOsM medium was 57.3% ± 4.45% (range, 30.5%–81.0%) of NETosis induced by 10 nM PMA (a known stimulator of NETosis). NETosis induced by PMA was similar in hyperosmolar medium as compared to iso-osmolar medium ($P = 0.48$; $n = 4$). To assess the test–retest variability of our assay method, NETosis measurements were repeated in some subjects ($n = 3$ subjects, three measurements over 3 weeks). The reproducibility for the increase in NETosis in hyperosmolar medium was ±10% for each subject. NETosis data using the NET assay for neutrophil elastase activity were similar to data obtained with the PicoGreen dye assay (Fig. 1A2). To confirm whether the increase in NETosis was due to hyperosmolarity and not due to the presence of excess NaCl, we tested NETosis in medium made hyperosmolar (423 mOsM) with the addition of sucrose. NETosis was significantly greater in sucrose-rich hyperosmolar medium than in iso-osmolar medium ($P < 0.05$).

We performed immunofluorescence staining to visualize NETs (Figs. 1B1–1B3) and to determine the cellular changes that occurred upon exposure to hyperosmolarity (Figs. 1C–1F). We measured the nuclear area of neutrophils (demarcated by DAPI-stained areas) under different conditions. In iso-osmolar medium, the neutrophils had multilobed nuclei; mean nuclear area was 29.4 ± 1.6 μm², and neutrophil elastase staining did not colocalize with the DAPI nuclear staining (Figs. 1C1–1C3). After 4 hours of incubation in hyperosmolar medium, the nuclear area had increased significantly to 32.8 ± 1.1 μm² ($P < 0.05$). Also, in contrast to those in iso-osmolar medium, the nuclei in hyperosmolar medium were round (lobes were indistinct or absent) and occupied an eccentric position, apposed to the cell membrane (Figs. 1D1–1D3). With further incubation in hyperosmolar medium (18 hours), the nuclear area increased to 73.3 ± 2.1 μm², which was significantly greater than that at 4 hours ($P < 0.05$). The neutrophil elastase staining colocalized with DAPI nuclear staining, a finding that is typically seen in neutrophils undergoing NETosis, suggesting admixing of neutrophil elastase with decondensed nuclear chromatin (Figs. 1E1–1E3).[5,12,23,24] In some neutrophils, extracellular release of fine strands of chromatin and neutro-
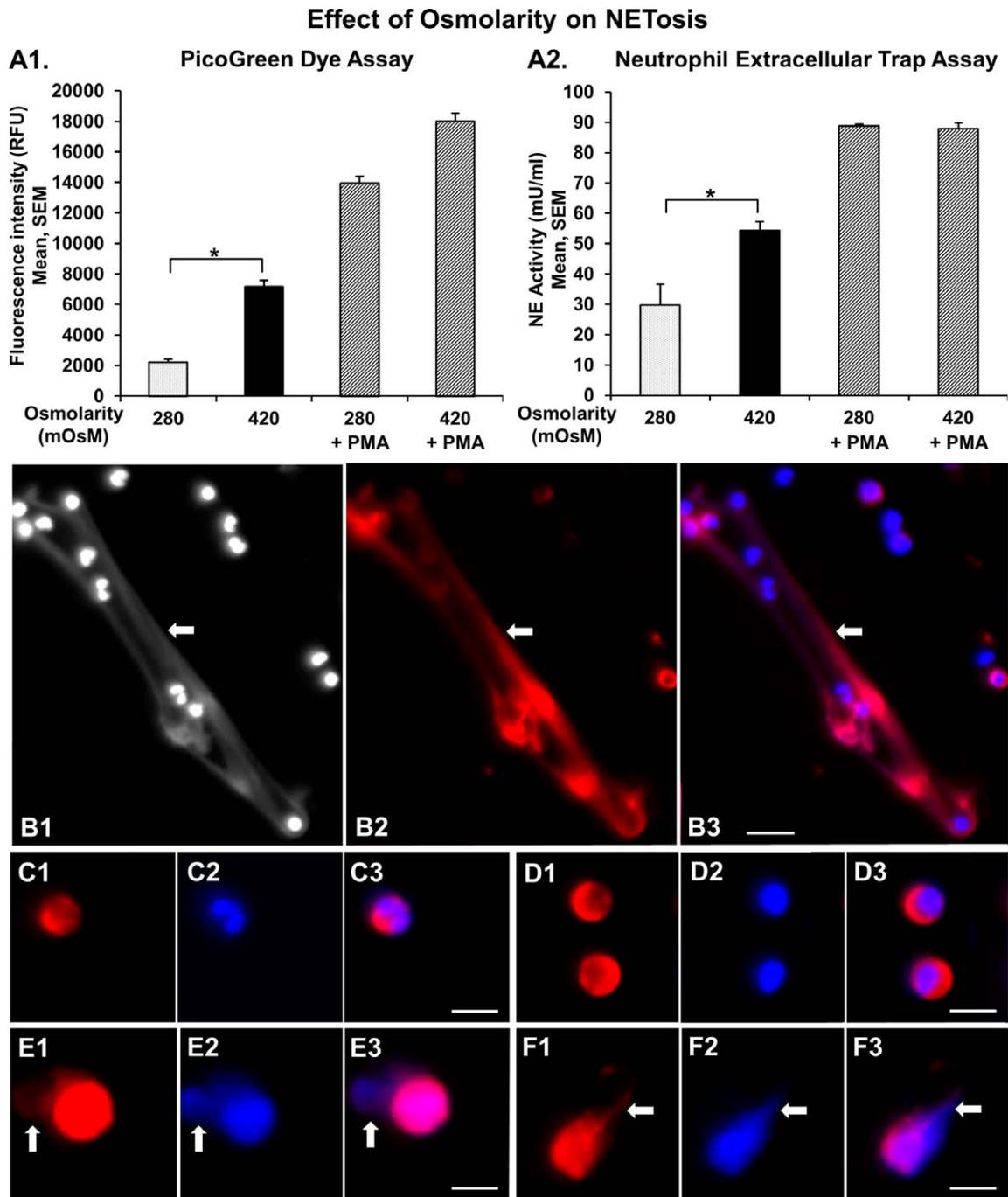
FIGURE 1. Hyperosmolarity-induced formation of NETs (NETosis). Hyperosmolarity was achieved by addition of NaCl. Representative data from neutrophils isolated from a subject are shown; each data point was performed in triplicate. (**A1**) PicoGreen dye assay for eDNA shows increased NET formation in hyperosmolar (420 mOsM) medium as compared to that in iso-osmolar medium (280 mOsM). NETosis induced by PMA was similar in hyperosmolar and iso-osmolar media. (**A2**) NET assay for neutrophil elastase activity shows similar findings. (**B1–B3**) Immunofluorescence staining image showing NETs (*arrows*) formed from neutrophils incubated in hyperosmolar medium. NETS stained positive for DAPI ([**B1**], *grayscale*) and neutrophil elastase ([**B2**], *red*). (**B3**) Overlay (*blue*, DAPI; *red*, neutrophil elastase). (**C–F**) Change in neutrophil morphology with increasing duration of exposure to hyperosmolarity. (**C1–C3**) Freshly isolated neutrophils show multilobed nuclei ([**C2**], DAPI, *blue*), and neutrophil elastase staining ([**C1**], *red*) does not colocalize with DAPI staining. (**D1–D3**) After 4 hours of incubation, the lobes are no longer distinct; nuclei have become rounded and are positioned eccentrically in the cell. (**E1–E3**) After incubation for 18 hours in hyperosmolar medium, nuclei are significantly larger in size and occupy almost the entire cell, and neutrophil elastase ([**E1**], *red*) staining colocalizes with DAPI nuclear staining ([**E2**], *blue*). Confluent NETs (stained with neutrophil elastase and DAPI) are seen adjacent to neutrophils (**E1–E3**), and NET strands (stained with neutrophil elastase and DAPI) are seen to extrude from neutrophils (**F1–F3**). NETs, neutrophil extracellular traps; NaCl, sodium chloride; eDNA, extracellular DNA; PMA, phorbol myristate acetate; DAPI, 4′,6-diamidino-2-phenylindole; RFU, relative fluorescence units; NE, neutrophil elastase; SEM, standard error of the mean. $^*P < 0.05$. *Scale bars*: 20 μm.

A. Effect of Increasing Osmolarity on NETosis

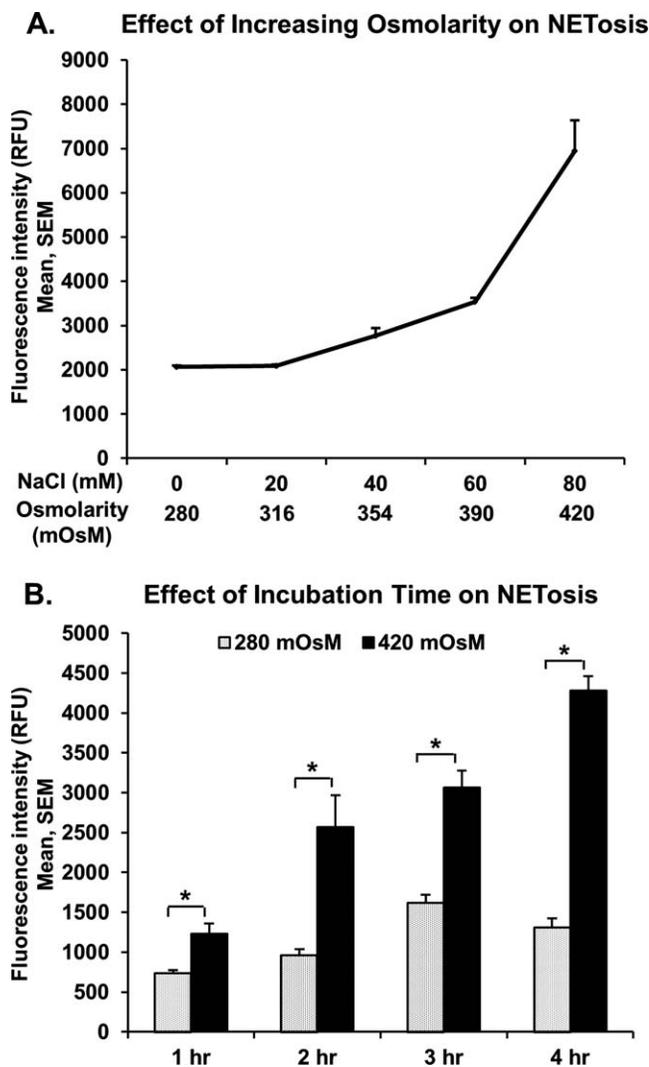## B. Effect of Incubation Time on NETosis

FIGURE 2. Effects of increasing osmolarity and increasing incubation time on NETosis. Hyperosmolarity was achieved by addition of NaCl. Representative data from neutrophils isolated from a subject are shown; each data point was performed in triplicate. (**A**) *Graph* shows nonlinear increase in NETosis with increasing osmolarity and larger increases in NETosis at higher osmolarities. (**B**) *Bar diagram* shows that NETosis increases linearly with increasing incubation times in hyperosmolar medium but not in iso-osmolar medium. NETosis in hyperosmolar medium is significantly greater than in iso-osmolar medium at all time points. NETs, neutrophil extracellular traps; NaCl, sodium chloride; RFU, relative fluorescence units; SEM, standard error of the mean. *$P < 0.05$.

phil elastase was visible (Figs. 1F1–1F3). Neutrophils stimulated with PMA also had significantly enlarged nuclei (37.9 ± 0.7 μm²) than those in iso-osmolar medium ($P < 0.05$), and nuclear material filled the entire cell. The chromatin stained with neutrophil elastase, and abundant strands of NETs were visualized. In contrast to PMA-stimulated NETs, hyperosmolarity-induced NETs tended to be confluent and were present in the vicinity of NETosing neutrophils. Also, fewer long strands were present.

Because tear fluid osmolarity increases with increasing severity of DED, we determined whether NETosis also increased with increasing osmolarity. There was a nonlinear (exponential) increase in NETosis with increasing osmolarity (Fig. 2A); larger increases in NETosis were observed at higher osmolarities. NETosis in medium with fixed hyperosmolarity (420 mOsM),

which corresponds to the tear fluid osmolarity of severe DED,[25-27] showed a linear increase over 4 hours (Fig. 2B).

Because patients with severe dry eye and tear fluid hyperosmolarity use iso-osmolar artificial tears, we performed experiments to determine whether normalizing osmolarity affected subsequent NETosis. Neutrophils were preincubated for 2 hours in hyperosmolar medium, followed by incubation for 2 hours in iso-osmolar or hyperosmolar medium (Fig. 3). Neutrophils preincubated in hyperosmolar medium, when subsequently incubated in iso-osmolar medium, produced significantly less NETs as compared to those that underwent incubation in hyperosmolar medium only ($P < 0.05$, $n = 5$). The mean reduction was 52.7% ± 5%. However, NETosis was not completely abrogated by normalizing osmolarity. After preincubation in hyperosmolar medium for 2 hours, neutrophils continued to form NETs during subsequent incubation in iso-osmolar medium, although the increase (46.4% ± 19.4%, $P > 0.05$) was not statistically significant.

Next, we investigated strategies to reduce NETosis in a hyperosmolar environment. We used STS (a known inducer of apoptosis),[19] β2 integrin antagonist (a master switch for NETosis),[20] and proresolution FPR2 agonists (annexin-1 mimetic peptide [Ac2-26] and LXA₄) to evaluate their potential for reducing hyperosmolarity-induced NETosis (Fig. 4). Media osmolarities were unchanged with the addition of Ac2-26, LXA₄, or anti-β2 integrin. The addition of STS caused a 5% increase in medium osmolarity. Because patients with dry eyes have hyperosmolar tears, any therapeutic agent applied to the ocular surface would encounter a hyperosmolar milieu. Therefore, we first replicated the published results on STS and anti-β2 integrin preincubation to reduce NETosis (Figs. 4A, 4B) and then investigated whether similar results would be obtained in a hyperosmolar medium (Fig. 4C). With preincubation, STS and anti-β2 integrin caused a reduction of PMA-induced NETosis by 35.2% ± 4.7% and 22% ± 6%, respectively ($P \le 0.05$; Figs. 4A, 4B). However, STS caused a significant increase in NETosis in hyperosmolar medium (91.1% ± 19%, $P \le 0.05$, $n = 8$), while anti-β2 integrin did not affect NETosis significantly ($P > 0.05$, $n = 4$; Fig. 4C). Coincubation of neutrophils with proresolution FPR2 agonists caused a decrease in NETosis in all subjects ($n = 8$); however, the difference was statistically significant in only six subjects for Ac2-26 and three subjects for LXA₄ (Fig. 4D). The mean reduction of NETosis was 20.9% ± 3.4% with Ac2-26 and 16.0% ± 2.5% with LXA₄. To exclude differences in neutrophil viability in different experimental conditions, we analyzed cell viability using AO/PI staining. Viability of neutrophils in presence of hyperosmolar medium and/or the FPR2 agonists was within ± 8% of that in iso-osmolar medium; however, STS caused greater decline in neutrophil viability (20%).

DISCUSSION

The present study reports several important findings. First, hyperosmolarity induced formation of NETs by neutrophils, and hyperosmolarity-induced NETosis was independent of the stimulus used to increase osmolarity. NETosis increased exponentially with increasing hyperosmolarity but showed a linear rise with increasing incubation time. Second, in neutrophils that had been primed by exposure to hyperosmolar stress, normalization of osmolarity significantly decreased NET formation but did not completely abrogate it. Finally, among the strategies tested to reduce NETosis in hyperosmolar medium, annexin-1 peptide was the most efficacious. To the best of our knowledge, this is the first published report to document that hyperosmolarity induces NETosis and that
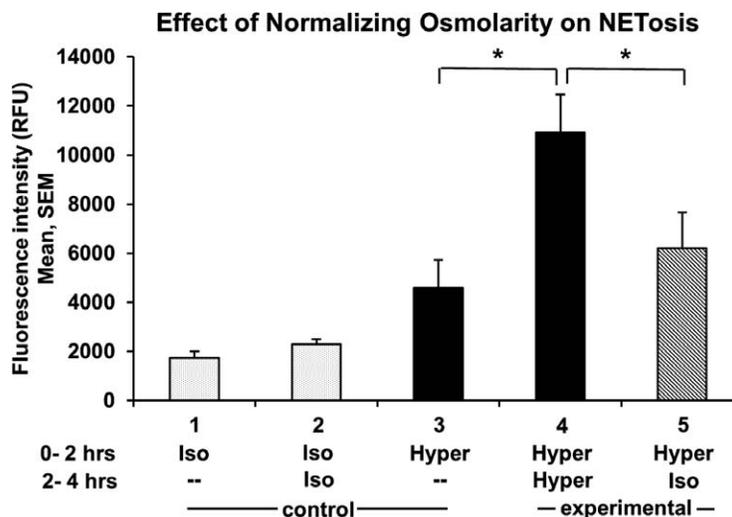
Figure 3. Effect of normalizing osmolarity on subsequent NETosis. Hyperosmolarity was achieved by addition of NaCl. Representative data from neutrophils isolated from a subject are shown; each data point was performed in triplicate. After initial incubation in hyperosmolar culture medium for 0 to 2 hours, neutrophils were cultured for 2 to 4 hours in either hyperosmolar (*bar 4*) or iso-osmolar medium (*bar 5*). NETosis was significantly less with 2- to 4-hour incubation in iso-osmolar medium (*bar 5*) as compared to hyperosmolar medium (*bar 4*). Control conditions were (1) iso-osmolar incubation for 0 to 2 hours only (*bar 1*); (2) iso-osmolar incubation for 0 to 2 hours followed by iso-osmolar incubation for 2 to 4 hours (*bar 2*); and (3) hyperosmolar incubation for 0 to 2 hours only (*bar 3*). NETs, neutrophil extracellular traps; NaCl, sodium chloride; Iso, iso-osmolar medium; Hyper, hyperosmolar medium; RFU, relative fluorescence units; SEM, standard error of the mean. *$P < 0.05$.

annexin-1 peptide reduces NETosis induced by hyperosmolarity. Our findings have important clinical implications.

Hyperosmotic stress is an often overlooked process that potentially contributes to a number of human diseases (reviewed in Ref. 28). Recent research supports a significant role for hyperosmotic stress in the release of proinflammatory cytokines and inflammation. In a healthy state, the osmolarity of the tear film is 296 to 302 mOsm/L.[15,25–27,29,30] However, in patients with DED, this value is approximately 316 to 360 mOsm/L.[15,25,26,30–32] A strong correlation exists between tear hyperosmolarity and the severity of dry eye syndrome. Tear hyperosmolarity is one of the central events in the development of DED pathology.[14,15] It results from aqueous tear deficiency and/or increased evaporation of the aqueous tear phase from the exposed ocular surface. Tear hyperosmolarity stimulates apoptosis of the epithelial surface cells and also stimulates a cascade of inflammatory events that leads to ocular surface disease. Our data suggest that stimulation of NET formation by neutrophils is another potential mechanism by which tear hyperosmolarity causes ocular surface disease. It is known that neutrophils undergo a low level of recruitment on the ocular surface,[33] and numerous neutrophils are present in the tear film during ocular surface inflammation.[34] We have reported the presence of numerous neutrophils and NETs on the ocular surface of patients with severe tear-deficient dry eyes.[9,10] As neutrophils egress from the circulation (where osmolarity is 285–295 mOsm/L) onto the ocular surface of patients with dry eyes, they encounter a hyperosmolar milieu. Our data show that neutrophils release NETs when exposed to hyperosmolar stress. Although NET formation is an important innate immune system defense mechanism and provides health benefits, it has been implicated in the pathobiology of inflammatory conditions, such as systemic lupus erythematosus (SLE).[3,6–8] We have reported that NETs on the ocular surface of patients with severe tear deficiency are associated with expression of type I interferon, plus inflammatory cytokines like interleukin-6 and tumor necrosis factor-α in ocular surface cells.[9] Thus, we propose that neutrophils that egress onto the ocular surface of patients with DED release NETs in the hyperosmolar mileu, which contributes to ocular

surface disease. Our results show that the amount of NETs released by neutrophils increased exponentially as hyperosmolarity increased. Therefore, the contribution of NETs to ocular surface disease is likely to be more relevant to severe DED associated with greater hyperosmolarity (>350 mOsm/L) as opposed to mild dry eye with minimally elevated osmolarity. This conclusion is consistent with our previous report demonstrating that markedly symptomatic patients with severe tear-deficient DED and extensive ocular surface disease had the greatest abundance of eDNA in their tear fluid.[10]

Our data show that upon exposure to hyperosmolar stress, neutrophils continued to release NETs, albeit in reduced amounts, even if the iso-osmolar milieu was restored. The clinical implication of this finding is that although pulsed application of iso-osmolar or hypotonic artificial tear eye drops to the ocular surface will intermittently reduce osmolarity, NETosis will likely not be completely abrogated because neutrophils will inevitably be exposed to hyperosmolarity and, once exposed to hyperosmolar stress, would then continue releasing NETs. The residence time of eye drops on the ocular surface is quite limited; therefore a hyperosmolar mileu is likely to be again established soon after the residence time of the artificial tear eye drops, especially in areas of tear film instability. The ocular residence time for emulsion eye drops (e.g., Restasis; Allergan, Irvine, CA, USA) has been estimated as approximately 2 hours.[35] Artificial tears with lower viscosity will have even shorter ocular residence time. We expect that the therapeutic agent will be applied to the ocular surface as a topical formulation; therefore it will encounter the hyperosmolar milieu of the ocular surface. We unexpectedly found that therapeutic strategies known to reduce NETosis fail to do so in a hyperosmolar medium. For example, preincubation of neutrophils with STS, a proapoptotic agent, for 15 minutes is known to significantly reduce NETosis induced by PMA,[19] and we were able to replicate these data. However, a significant increase in NETosis occurred when neutrophils were incubated with STS in hyperosmolar medium. Similarly, we were able to replicate a significant reduction in NETosis after preincubation with β2 integrin antibody for 30 minutes and subsequent incubation with
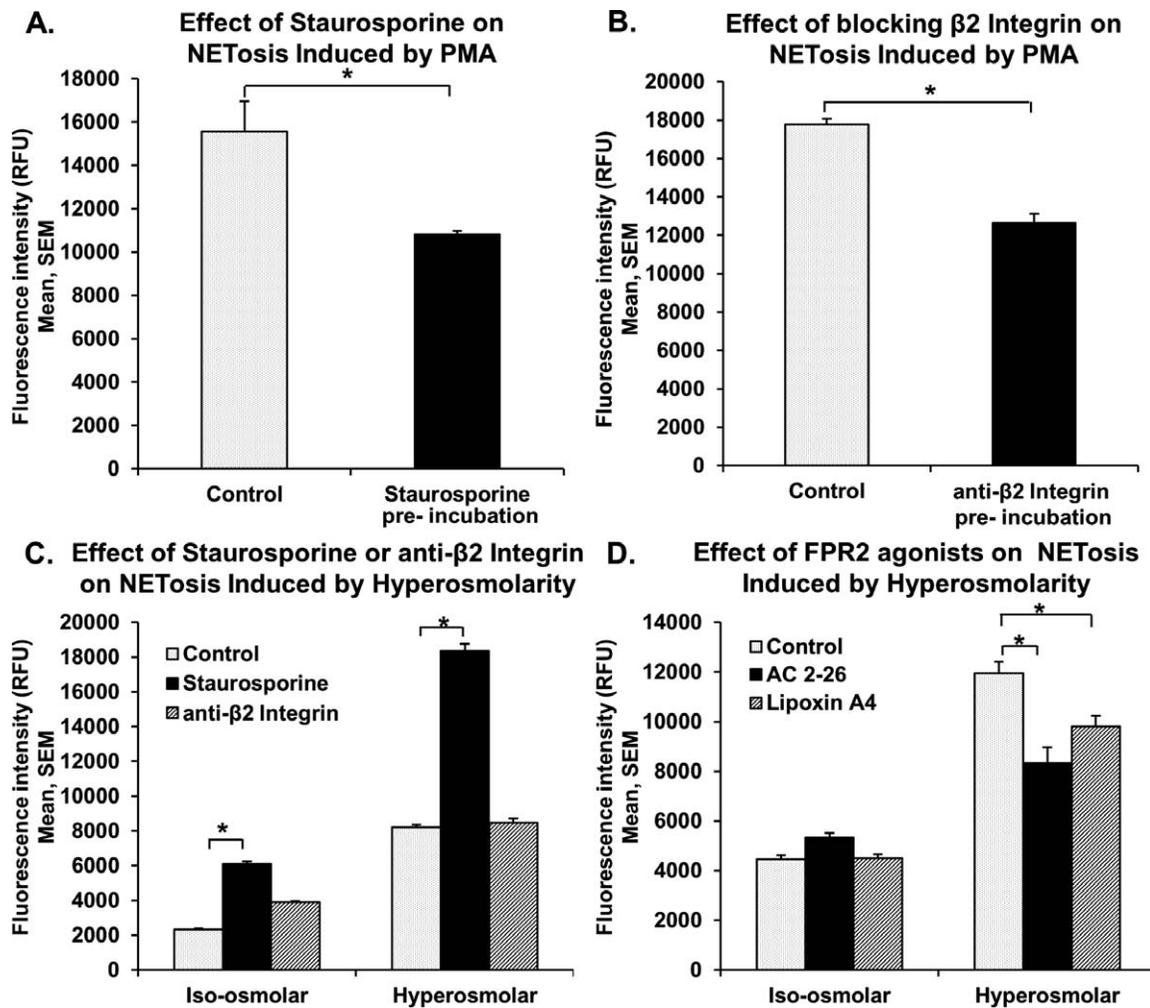
FIGURE 4. Role of anti-inflammatory/proapoptotic agents in decreasing NETosis. Representative data from neutrophils isolated from a subject are shown; each data point was performed in triplicate. (**A**, **B**) The effects of staurosporine (**A**) or anti-β2 integrin antibody (**B**) on PMA-induced NETosis in iso-osmolar medium. When the neutrophils were preincubated with staurosporine or anti-β2 integrin antibody, there was a significant decrease in NETosis stimulated by subsequent PMA incubation as compared to neutrophils that were not preincubated with these compounds prior to PMA stimulation (control). (**C**) When neutrophils were coincubated with staurosporine or anti-β2 integrin antibody in hyperosmolar medium (no preincubation), NETosis did not decrease as compared to incubation of neutrophils in medium without these compounds (control). Rather, there was a significant increase in NETosis with staurosporine coincubation. (**D**) Effect of proresolution FPR2 agonists on hyperosmolarity-induced NETosis. Both Ac2-26 and lipoxin A_4 caused a significant decrease in hyperosmolarity-induced NETosis. NETs, neutrophil extracellular traps; PMA, phorbol myristate acetate; Ac2-26, annexin-1 mimetic peptide; RFU, relative fluorescence units; SEM, standard error of the mean. *$P < 0.05$.

PMA.[20] When neutrophils were incubated with β2 integrin antibody in hyperosmolar medium, NETosis was not reduced. Our data therefore suggest that therapeutic strategies to reduce NETosis must be evaluated in hyperosmolar medium.

There are several potential therapeutic strategies that may reduce the amount of NETs on the ocular surface of patients with DED. These potential strategies are (1) reducing formation of NETs with anti-inflammatory and proresolving mediators such as annexin-1 and LXA_4; (2) clearing NETs with nucleases such as DNase I; (3) osmoprotection using agents such as carboxymethylcellulose[28]; and (4) reducing neutrophil egress on to the ocular surface with drugs such as corticosteroids. Both annexin-1 and LXA_4 are proresolving mediators that serve as innate modulators of inflammation.[36–40] They attenuate leukocyte recruitment by inhibiting cell adhesion and transmigration and act via a common G_i protein-coupled receptor, namely, FPR2[41]; FPR2 is one of a family of pertussis toxin-sensitive FPR receptors that interact with structurally diverse pro- and anti-inflammatory ligands.[42,43] The 15-epi-LXA_4 was

more resistant to metabolic inactivation than native LXA_4.[44,45] The role of annexin-1 in resolution of ocular inflammation has been investigated in animal models of uveitis. Both annexin-1 and its mimetic peptide, Ac2-26, reduced leukocyte influx and promoted resolution of ocular inflammation.[46] Similarly, annexin-1$^{-/-}$ mice exhibited an exacerbated inflammatory response, characterized by increased neutrophil influx and COX-2 expression.[46] Thus, we chose to investigate if annexin-1 mimetic peptide Ac2-26 or 15-epi-LXA_4 had any influence on NETosis. We did observe a reduction in NETosis with annexin-1 peptide, suggesting that its therapeutic potential should be further investigated. Besides reducing NET formation, an alternative therapeutic strategy is to remove NETs from the ocular surface using DNase I. We have reported the use of DNase I to treat ocular surface disease in two patients with severe tear-deficient DED.[10] In theory, combinatorial strategies that include osmoprotective artificial tears to reduce hyperosmolarity, proresolution agents to reduce NETosis, and DNase I eye drops to remove NETs may have greater efficacy than single

agents. A representative dry eye animal model can provide the background inflammatory environment useful in investigating the effects of NETosis inhibition strategies. The most frequently used dry eye animal model is the murine model.[47] However, there are considerable differences in NETosis between human and murine neutrophils.[5,48] Murine neutrophils take much longer to form NETs.[48] Neutrophil extracellular traps released from human neutrophils appear as web-like structures,[3,5] whereas murine NETs have a compact structure.[48] Furthermore, human neutrophils represent 65% to 75% of all peripheral blood leukocytes, whereas in the murine model, only 10% to 25% of leukocytes are neutrophils.[48,49] These differences need to be considered while preclinical data are translated from animal models to humans.

NETosis induced by hyperosmolarity is not as robust as that induced by PMA. In hyperosmolar medium, we observed microscopic features that were characteristic of neutrophils undergoing NETosis. At 4 hours, the nucleus became rounded and appeared apposed to the cell membrane. With further incubation, the nuclear size almost doubled, signifying chromatin decondensation, and intracellular neutrophil elastase colocalized with chromatin. These features differentiated NETosis from apoptosis, in which chromatin material condensed and nuclear size decreased.[5,12,23,24] In addition, confluent NETs were observed in the vicinity of NETosing neutrophils. Strand-like NETs were also seen, but they were fewer and shorter than those induced by PMA. Joshi et al.[50] also found short and imperfect NETs in basal hyperglycemic condition of some diabetic subjects, similar to our observations.

Hyperosmolar stress also has an inhibitory effect on some critical neutrophil functions. It is known to inhibit neutrophil migration and degranulation of tertiary granules, as well as the respiratory burst.[51-54] It leads to defective phagocytosis and intracellular killing of bacteria.[51,54-56] Thus, in a hyperosmolar milieu, the classical neutrophil-related innate defense mechanisms seem to be compromised. Viewed in this context, the release of NETs from neutrophils in response to hyperosmolarity would seem to offset the loss of classical neutrophil-related innate defense mechanisms such as phagocytosis. Therefore, over a compromised ocular surface in severe DED, NETs may be an important innate defense mechanism. However, in the absence of clearance of NETs from the ocular surface, as may happen in tear-deficient DED due to absence of nucleases, NETs may accumulate on the ocular surface and cause surface inflammation and disease. Joshi et al.[50] have reported that if neutrophils residing in hyperglycemic conditions are further stimulated (e.g., by bacterial LPS), they are unable to efficiently produce NETs. Their findings may have clinical implication in the context of DED patients. The compromised ability of neutrophils to efficiently increase NETosis when exposed to further stress may make the ocular surface of these patients susceptible to infections. Our data showed a greater increase in NETosis in hyperosmolar culture media containing sucrose, as compared to the iso-osmolar condition (without further stimulation), than that reported by Joshi et al.[50] The reason for this difference could be that we used a much higher sucrose concentration in our experiments (to achieve osmolarity of 420 mOsM).

In summary, hyperosmolarity induced formation of NETs by neutrophils. Hyperosmolarity-induced NETosis may explain why excessive NETs are present on the ocular surface of patients with DED.

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