Ascorbate in Aqueous Humor Augments Nitric Oxide Production by Macrophages

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Ascorbate in Aqueous Humor Augments Nitric Oxide Production by Macrophages

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Immunosuppressive molecules within the aqueous humor (AqH) are thought to preserve ocular immune privilege by inhibiting proinflammatory NO production by macrophages (Mφs). Consistent with previous observations, we observed that although Mφs stimulated in the presence of AqH expressed NO synthase 2 (NOS2) protein, nitrite concentrations in culture supernatants, an indirect measure of NO production, did not increase. Interestingly, NOS2 enzymatic activity, as measured by the conversion of l-arginine (l-Arg) into l-citrulline, was augmented in lysates of Mφs stimulated in the presence of AqH. These data suggested that intracellular l-Arg may have been limited by AqH. However, we observed increased mRNA expression of the l-Arg transporter, cationic amino acid transporter 2B, and increased l-Arg uptake in Mφs stimulated in the presence of AqH. Arginases were expressed by stimulated Mφs, but competition for l-Arg with NOS2 was excluded. Expression of GTP cyclohydrolase, which produces tetrahydrobiopterin (H4B), an essential cofactor for NOS2 homodimerization, increased after Mφ stimulation in the presence or absence of AqH and NOS2 homodimers formed. Taken together, these data provided no evidence for inhibited NOS2 enzymatic activity by AqH, suggesting that a factor within AqH may have interfered with the measurement of nitrite. Indeed, we observed that nitrite standards were not measurable in the presence of AqH, and this effect was due to ascorbate in AqH. Controlling for interference by ascorbate revealed that AqH augmented NO production in Mφs via ascorbate, which limited degradation of H2B. Therefore, AqH may augment NO production in macrophages by stabilizing H2B and increasing intracellular l-Arg.

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We have recently reported that rejection of E.G7-OVA tumors transplanted in the skin required the induction of tumoricidal NO production in intratumoral Mφs by cytotoxic CD8+ T cells (CTL) (5). In contrast, progressive E.G7-OVA growth in the a.c. of the eye occurred despite tumor infiltration by CTL and was associated with impaired NO production by intratumoral macrophages (Mφ) (5), a potential consequence of mechanisms that preserve ocular immune privilege. The a.c. is filled with aqueous humor (AqH) that contains multiple immunosuppressive factors that can inhibit NO production by Mφ (reviewed in Ref. 6). For example, TGF-β2 and α-melanocyte-stimulating hormone (α-MSH) have been shown to decrease NO synthase (NOS)2 protein levels by interfering with NOS2 mRNA transcription/stability, limiting NOS2 translation, and/or accelerating NOS2 protein degradation (7, 8). However, we observed that Mφs within ocular tumors expressed NOS2 protein in vivo, although they produced low nontumoricidal concentrations of NO (5). Similarly, Taylor et al. (9) showed that Mφs stimulated in the presence of AqH expressed NOS2, but a corresponding increase in nitrite in culture supernatants, an indirect measure of NO production, was not observed. In combination, these data suggested that posttranslational regulation of NOS2 may primarily occur within the ocular microenvironment.

There are several posttranslational mechanisms that could inhibit NOS2 enzymatic activity. NOS2 generates NO and l-citrulline by metabolizing l-arginine (l-Arg). Therefore, limiting l-Arg availability within the cell by preventing entry via the cationic amino acid transporter 2B (CAT2B) (10) or by increasing arginase activity that has similar Vmax/Km values to NOS2 (11) would decrease NO production. NOS2 enzymatic activity is also dependent on the formation of NOS2 homodimers, which may fail to form if indispensible cofactors, including tetrahydrobiopterin (H2B) (12) and 1 iron protoporphyrin IX (HEME) (13) are scarce. Alternatively, certain proteins, for example NAP110 (14) and Kalirin-7 (15), have been shown to bind to NOS2 monomers in a manner...
that prevents homodimerization. The cellular location of NOS2 is also important to its enzymatic activity. For example, NOS2 normally interacts with the cytoskeletal protein α-actinin 4 to localize NOS2 to the plasma membrane and/or submembranal zone (16). When this interaction is disrupted, NOS2 is dispersed throughout the cytoplasm and enzymatic activity is compromised (16).

To identify mechanisms of posttranslational regulation of NOS2 within the ocular microenvironment, we characterized NOS2 enzymatic activity in Møs stimulated in the presence or absence of AqH. Interestingly, we observed increased NOS2 enzymatic activity in cell lysates of Møs stimulated in the presence of AqH, although nitrite was profoundly reduced in culture supernatants. These incongruent observations were not explained by limited intracellular concentrations of L-Arg, abrogated expression of the enzyme GTP cyclohydrolase (GTP) that produces H4B, or inhibited formation of NOS2 homodimers. Rather, we demonstrate that high concentrations of ascorbate in AqH interfered with the Griess assay used to measure nitrite. After controlling for interference by ascorbate, we observed that AqH augmented NO production in Møs via ascorbate. AqH increased intracellular concentrations of L-Arg and limited degradation of H4B, suggesting two potential mechanisms for augmented NO production by Møs.

Materials and Methods

Reagents

Murine rIFN-γ (BD Pharmingen, San Diego, CA), ultrapure LPS from Escherichia coli (Invivogen, San Diego, CA), porcine TGF-β2 (R&D Systems, Minneapolis, MN), t-Arg (Sigma-Aldrich, St. Louis, MO), calcitonin gene-related peptide (CGRP; Sigma-Aldrich), α-MSH (Sigma-Aldrich), pigment epithelium-derived factor (PEDF; BioproducTech, Middletown, MD), ascorbate (Sigma-Aldrich), ascorbate oxidase (Sigma-Aldrich), and neutralizing mAb against TGF-β2 (R&D Systems) were used in these studies.

Mice

Male and female C57BL/6J (B6) or B6;129P2-No2tm1Lau/J (NOS2−/−) mice on a B6 background from The Jackson Laboratory (Bar Harbor, ME) were used. All experimental animals were bred and maintained in the animal facilities at the University of Pittsburgh under specific pathogen-free conditions. All experimental procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and were in adherence to the Association for Research in Vision and Ophthalmology guidelines for use of experimental animals in ophthalmology research.

Aqueous humor

Rabbit AqH, obtained by paracentesis of the a.c., was generously provided by the Charles T. Campbell Ophthalmic Microbiology Laboratory (University of Pittsburgh), or was purchased from Pel-Freeze Biologicals (Rogers, AR). Rabbit AqH was centrifuged, and the supernatant was filtered (100 kDa), and then washed extensively to remove nonadherent cells. The remaining cells were predominantly mature F4/80+ Møs, and then washed extensively to remove nonadherent cells. The remaining cells were predominantly mature F4/80+ Møs, and then washed extensively to remove nonadherent cells. The remaining cells were predominantly mature F4/80+ Møs, and then washed extensively to remove nonadherent cells. The remaining cells were predominantly mature F4/80+ Møs, and then washed extensively to remove nonadherent cells. The remaining cells were predominantly mature F4/80+ Møs, and then washed extensively to remove nonadherent cells. The remaining cells were predominantly mature F4/80+ Møs, and then washed extensively to remove nonadherent cells. The remaining cells were predominantly mature F4/80+ Møs, and then washed extensively to remove nonadherent cells.

Mø culture

Møs were obtained from B6 or NOS2−/− mice by peritoneal lavage 3–4 d after i.p. injection of 3 ml thioglycollate broth (BD Biosciences, San Diego, CA). Peritoneal exudate cells were plated overnight in standard growth medium (SGM; RPMI 1640 medium supplemented with 10% FBS; 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-ME, gentamicin, penicillin, and streptomycin) in serum-free X-Vivo 10 medium (Lonza) and then washed extensively to remove nonadherent cells. The remaining cells were predominantly mature F4/80+ Møs (Supplemental Fig. 1). RAW 264.7 Møs were obtained from American Type Culture Collection (Manassas, VA). Møs maintained in SGM or X-Vivo 10 medium were stimulated with a combination of IFN-γ (100 U/ml) and LPS (100 ng/ml) for indicated periods of time, at 37°C and 5% CO2 atmosphere in a humidified incubator. To stimulate Møs in the presence of AqH, the media was diluted with AqH at a 1:1 ratio. In some experiments, AqH and SGM were pretreated with neutralizing anti–TGF-β2 Abs, or AqH was treated with ascorbate oxidase (10 U/ml) for 1 h at room temperature.

RNA extraction and RT-PCR

Møs activated in the presence or absence of AqH were harvested at indicated time points utilizing a cell scraper in the presence of RLT buffer (Qiagen, Germantown, MD). Total RNA was then extracted using the RNeasy minikit from Qiagen following the manufacturer’s protocol. cDNA was prepared using a high-capacity cDNA reverse-transcription kit (Applied Biosystems, Carlsbad, CA), and expression of arginase 1 (ARG1; primer Mm00475798_m1), arginase 2 (ARG2; primer Mm00477592_m1), CAT2B (primer Mm00432032_m1), GTP cyclohydrolase 1 (GTPCH1; primer Mm00514993_m1), NOS2 (primerMm00440485_m1), and pyruvate carboxylase (primer Mm00500992_m1) was determined by quantitative real-time PCR using TaqMan PCR universal mix (Applied Biosystems) and an ABI StepOne Plus thermocycler (Applied Biosystems). All primers were obtained from Applied Biosystems. Relative expression was determined using the 2−ΔΔCT method. First, cycle threshold (CT) values for experimental gene expression were normalized to the CT values of the housekeeping gene pyruvate carboxylase within the same sample (ΔCT). The ΔCT from Mø-stimulated cultures was then subtracted from the ΔCT of nonstimulated Mø cultures (ΔΔCT) to determine fold differences from controls = 2−ΔΔCT.

t-Arg uptake assay

Triplicate primary Mø cultures (5.0 × 105 cells in 2 ml SGM contained within a 60-mm petri dish) were untreated or stimulated with IFN-γ and LPS in the presence or absence of AqH. Eighteen hours later, plates were washed with warm (37°C) PBS and then incubated with 0.1 μM 1-[15N]L-Arg (Perkin Elmer, Boston, MA) in warm PBS for 2 min. Plates were then washed twice with warm PBS, and cells were scraped and homogenized in lysis buffer and transferred to a tube containing scintillation fluid in which radioactive cpm were measured.

NOS2 protein expression

Stimulated and nonstimulated Mø cultures (5.0 × 105 cells in 2 ml SGM contained within a 60-mm culture-treated petri dish) were washed with PBS, and then cells were scraped from plates in Complete Lysis M buffer (Roche, Manheim, Germany) to prepare cell lysates. Cell lysates were homogenized by pipetting and sonication. Protein concentration was determined by a Bradford assay (Bio-Rad, Hercules, CA), and 10–30 μg total protein was loaded onto a 6% polyacrylamide gel. Following electrophoresis, separated proteins were transferred to a polyvinylidene difluoride membrane that was blocked with Odyssey Blocking Buffer (LI-COR, Lincoln, NB), and then primary Abs were added at the following dilutions: anti-NOS2, 1:1000; anti-ARG1, 1:1000; and anti-β-tubulin 1:20,000 in 3% nonfat dried milk in PBS-Tween-20. Secondary Abs were then used at the following dilutions: goat anti-rabbit IRDye 680 1:2,000; and goat anti-Mouse IRDye 800CW 1:20,000. Protein expression was resolved by imaging on the Odyssey Infrared Imager (LI-COR).

NOS2 activity measurements

NO production was measured indirectly by measuring nitrite levels in supernatants from triplicate cultures (5.0 × 105 cells in 0.2 ml medium contained within an individual well of a 96-well plate, or 2.5–5.0 × 105 cells in 2.0 ml medium contained with a 60-mm petri dish) by the Griess assay (Promega, Madison, WI), according to the manufacturer’s instructions. In some experiments, culture supernatants were incubated with 10 U/ml ascorbate oxidase for 1 h at room temperature prior to use in the Griess assay. To measure NOS2 enzymatic activity, the conversion of supplemented L-Arg into L-citrulline was measured using a NOS activity assay kit, according to the manufacturer’s instructions (Cayman Chemical, Ann Arbor, MI).

Determination of the presence of NOS2 monomers and dimers

Size exclusion chromatography of Mø lysates was performed, as described previously (17), at 4°C. A column prepacked with Superdex 200 (200; Amersham, Pharmacia Biotech) was equilibrated at 0.5 ml/min with 40 mM EPPS buffer (3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid) (pKa of 8, pH 7.6–8.6), containing 3 mM DTT, 10% glycerol, and 150 mM NaCl. Equal amounts of lysates were diluted to 200 μl prior to injections. The proteins in the column effluent were detected at 280 nm using a flow-through detector. Aliquots of each column fraction were subjected to SDS-PAGE and Western blotted to detect NOS2. The m.w. of the protein fractions were estimated relative to gel filtration m.w. stand-
ars. Band intensities on NOS2 Western blots from column fractions were measured using Image J quantification software. Values from fractions containing monomers or dimers were summed, and then the monomer:dimer ratio was determined by simple division.

Ascorbate measurement
Ascorbate was measured in AqH using the Ascorbate Assay Kit (Cayman Chemical), according to the manufacturer’s instructions.

H$_4$B measurement
H$_2$B (20 μM) was spiked into 2.0 ml solutions of SGM, SGM diluted 1:1 with AqH, or SGM diluted 1:1 with AqH that was pretreated with ascorbate oxidase (10 U/ml for 1 h). Solutions were then incubated at 37°C at 5% CO$_2$ atmosphere in a humidified incubator. Aliquots (50 μl), removed at multiple time points, were added to ph 3 HPLC grade water (450 μl Fisher Scientific) containing 100 μM dithioerythritol (Sigma-Aldrich) and 100 μM diethylenetriaminepentaacetic acid (Sigma-Aldrich). Samples were stored at -85°C, before analysis. For analysis, the thawed samples were measured by HPLC with fluorescence detection, as described previously (18). Briefly, protein was removed by adding 10 ml of a 1:1 mixture of 1.5 mol/l HClO$_4$ and 2 mol/l H$_3$PO$_4$ to 90 μl samples, followed by centrifugation at 13,000 × g for 5 min at 4°C. To determine total biopterin (H$_4$B, dihydropterin [H$_2$B], and oxidized biopterin) by acid oxidation, 10 μl 1% iodine in 2% potassium iodine solution was added to the protein-free supernatant (90 μl). To determine H$_2$B and oxidized biopterin by alkali oxidation, 10 μl 1 M NaOH was added to 80 μl sample, and then 10 μl 1% iodine in 2% potassium iodine solution was added. Samples were incubated at room temperature for 1 h in the dark. Alkaline-oxidation samples were then acidified with 20 μl 1 M H$_3$PO$_4$. Iodine was reduced by adding 5 μl fresh ascorbic acid (20 mg/ml). Samples of 90 μl were injected into a 250-mm–long, 4.6-mm inner-diameter Spherisorb ODS-1 column (5 μm particle size; Alltech Associates, Deerfield, IL) isocratically eluted with a methanol-water (5:95, v/v) mobile phase running at a flow rate of 1.0 ml/min. Fluorescence detection (350 nm excitation, 450 nm emission) was performed using a fluorescence detector (RF10AXL; Shimadzu). H$_2$B concentrations were calculated by subtracting H$_2$B plus oxidized biopterin from total biopterins.

Statistical analysis
Statistical analyses were performed using GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA). ANOVA with Tukey’s posttest was performed using GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA). ANOVA with Tukey’s posttest was used. The p values <0.05 were considered statistically significant: *p < 0.05, **p < 0.01, ***p < 0.001; ns, not statistically significant.

Results
Influence of aqueous humor on NOS2 enzymatic activity in Mφs
To identify mechanisms that inhibit NOS2 enzymatic activity within the ocular microenvironment, we used a relevant in vitro system in which Mφs were stimulated with LPS and IFN-γ in the presence of AqH (9). NO production was monitored indirectly by measurement of nitrite in Mφ culture supernatants (Griess assay) as nitrite is a stable breakdown product of NO. Nitrite increased when primary peritoneal Mφs (Fig. 1A) or the RAW 264.7 Mφ cell line (Fig. 1B) was stimulated. In contrast, nitrite did not accumulate in culture supernatants of stimulated Mφs from NOS2-deficient mice (Supplemental Fig. 2), which indicated that NO was produced by NOS2. The addition of AqH profoundly suppressed nitrite accumulation in supernatants of stimulated Mφs (Fig. 1A, 1B), despite NOS2 protein expression (Fig. 1C) that reproduced previous observations (9).

To directly evaluate NOS2 enzymatic activity, we monitored the conversion of l-Arg into l-citrulline in cell lysates of Mφs stimulated in the presence or absence of AqH. As shown in Fig. 1D, NOS2 enzymatic activity in Mφs increased upon stimulation, which was consistent with increased nitrite in culture supernatants. However, NOS2 enzymatic activity was even greater in lysates of Mφs stimulated in the presence of AqH (Fig. 1D), although nitrite levels in these culture supernatants were reduced (Fig. 1A).

FIGURE 1. Influence of aqueous humor on NOS2 activity. Nitrite concentrations in culture supernatants of primary peritoneal Mφs (A) or RAW 264.7 Mφ cells (B) that were untreated or stimulated with LPS and IFN-γ in the absence or presence of AqH. Nitrite was measured 18 h after treatment. Data shown are representative experiments of 2–11 that were performed with similar results. (C) NOS2 and α-tubulin (loading control) protein expression in cell lysates of primary Mφs cultured as indicated. Data are representative of two experiments performed. (D) NOS2 enzymatic activity in lysates of Mφs treated as indicated. Each line represents measurements from an independent experiment.

TGF-β2, α-MSH, CGRP, or PEDF does not inhibit NO production by Mφs
Certain molecules present in normal AqH, including TGF-β2, α-MSH, and CGRP, have been shown to inhibit NO production in activated Mφs (7–9). Therefore, we tested whether these molecules at concentrations reported to be present in normal AqH (9, 19, 20) were able to significantly decrease NO production by primary Mφs activated with IFN-γ and LPS, TGF-β2, CGRP, or α-MSH alone or in combination was not suppressive (Fig. 2A). Superphysiological concentrations of these molecules (10 ng/ml TGF-β2, 20 ng/ml CGRP, or 300 ng/ml α-MSH) were also incapable of suppressing nitrite accumulation by stimulated Mφ (percent inhibition: <20%) (data not shown). Neutralization of TGF-β2 in culture medium or in AqH increased NOS2 protein levels slightly; however, a corresponding increase in NO production was not observed (data not shown). Another factor present in the AqH that has been shown to inhibit NO production by Mφs is PEDF (21, 22). However, the addition of PEDF to stimulated Mφ cultures also did not inhibit NO production (Fig. 2B). Taken together, these data indicated that TGF-β2, CGRP, α-MSH, and PEDF within AqH did not mediate suppression of NO production in Mφs under our experimental conditions.

AqH increases l-Arg transport via CAT2B
The measurement of NOS2 enzymatic activity in cell lysates involved supplementation with l-Arg and H$_2$B. Therefore, our incongruent measurements of NO production made by Griess assays and NOS2 enzymatic activity assays could have been due to AqH...
limiting the concentration of intracellular L-Arg in intact Mφs. To
test this possibility, we first evaluated the mRNA expression of the
L-Arg transporter, CAT2B, in Mφs stimulated in the presence or
absence of AqH. In comparison with control unstimulated Mφs,
CAT2B expression increased after Mφ stimulation in two inde-
pendent experiments (Fig. 3A). The addition of AqH further in-
creased CAT2B expression at 18 h (experiment 1, 1.7-fold greater;
experiment 2, 1.3-fold greater in comparison with Mφs stimulated
without AqH), suggesting that L-Arg uptake via CAT2B was not
impaired in AqH-treated Mφs. Nevertheless, to evaluate the
functional activity of CAT2B, we directly measured L-Arg uptake
by Mφs 18 h after stimulation and 2 min after addition of [3H]-L-
Arg (Fig. 3B) when L-Arg transport was linear (23). L-Arg uptake
was significantly greater in stimulated Mφs than in unstimulated
Mφs, and greatest in Mφs stimulated in the presence of AqH,
which was consistent with their higher CAT2B mRNA expres-
sion (Fig. 3A). These data indicated that the concentration of intracel-
lular L-Arg was increased rather than inhibited by AqH treatment.

Arginase activity does not contribute to AqH-mediated
inhibition of NO production by Mφ

Intracellular L-Arg can be depleted by competition with arginases
(ARG1 and ARG2) that have similar V\text{max}/K\text{m} values to NOS2
(11). Therefore, NOS2, ARG1, and ARG2 mRNA expression
were measured by quantitative RT-PCR (Fig. 4A–C). Upon Mφ
stimulation in the presence or absence of AqH, NOS2 mRNA expres-
sion increased dramatically relative to unstimulated Mφs. Data from two independent experiments are shown (Fig. 4A). ARG1 and ARG2
mRNA expression also increased after stimulation, albeit to
a much lesser extent than NOS2, and were further increased by
AqH at 18 h (Fig. 4B, 4C) (Arg1: experiment 1, 5.7-fold greater;
experiment 2, 2.4-fold greater; Arg2: experiment 1, 2.0-fold
greater; experiment 2, 2.5-fold greater, in comparison with Mφs
stimulated without AqH). However, this analysis did not compare
copy number of NOS2, ARG1, and ARG2 mRNAs. In non-
stimulated Mφ cultures, the cycle titration number for NOS2 was
higher than ARG1 and ARG2 (data not shown). Assuming equal
efficiency of PCRs, these data suggested that the total mRNA copy
number for ARG1 and ARG2 exceeded NOS2 copies. Therefore,
we evaluated ARG1 and NOS2 protein expression to better
evaluate the absolute concentration of these enzymes in intact Mφs. As shown in Fig. 4D, resting primary Mφs expressed ARG1,
but not NOS2 protein, which was consistent with previous reports
(24). Upon stimulation, ARG1 expression was maintained and
NOS2 expression increased equivalently in Mφ cultures stimu-
lated with or without AqH. As Mφs stimulated in the absence of AqH produced NO despite ARG1 expression, which
was equivalent to Mφs cultured in the presence of AqH,
these data suggested that arginase activity did not contribute to
AqH-mediated suppression of NO production. In addition, Mφs
were cultured in RPMI 1640 medium that contains L-Arg at a
concentration (1.15 mM) that was too high to promote competi-
tion between arginases and NOS2 for L-Arg (25).

Effect of AqH on GTPCH1 expression

NOS2 enzymatic activity requires the formation of homodimers
that are stabilized by H\text{4}B and L-Arg (13, 26). H\text{4}B is produced by

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**FIGURE 2.** Influence of TGF-β2, α-MSH, CGRP, or PEDF on NOS2 activity. Nitrite concentration in culture supernatants of primary peritoneal Mφs stimulated in the presence of AqH, TGF-β2, α-MSH, and/or CGRP (A) or PEDF (B) for 18 h. Plots are individual experiments representative of two to three experiments performed.

**FIGURE 3.** AqH does not inhibit L-Arg transport. (A) mRNA expression of the CAT2B at 6 and 18 h after LPS and IFN-γ stimulation of primary Mφs in the presence or absence of AqH, under TGF-β2 neutralizing conditions. Data are presented as fold change in comparison with unstimulated Mφs. Data from two independent experiments are shown. (B) [3H]-L-Arg uptake by primary Mφs cultured as indicated. One experiment, which is representative of two experiments performed, is shown.
As the coenzyme GTPCH1. Therefore, we measured GTPCH1 mRNA expression by quantitative RT-PCR to determine whether differences in expression of GTPCH1 might explain inhibited NO production in Mφs activated in the presence of AqH. In two independent experiments, GTPCH1 mRNA increased in Mφs stimulated in the presence or absence of AqH (Fig. 5), suggesting that inhibited coenzyme activity did not explain AqH-induced suppression of NO production in Mφ.

**FIGURE 4.** Influence of AqH on arginase and NOS2 expression. mRNA expression of NOS2 (A), arginase (ARG1) (B), and ARG2 (C) at 6 and 18 h after LPS and IFN-γ stimulation of primary Mφs in the presence or absence of AqH, under TGF-β2 neutralizing conditions. Data are presented as fold change in comparison with unstimulated Mφs. The dashed line indicates no change over control. Data from two independent experiments are shown. (D) NOS2, ARG1, and α-tubulin (loading control) protein expression in cell lysates from Mφ stimulated in the presence or absence of AqH. One representative experiment of two experiments performed is shown.

**FIGURE 5.** GTPCH1 expression in stimulated Mφs. mRNA expression of GTPCH1 was evaluated at 6 and 18 h after stimulation of primary Mφs in the presence or absence of AqH, under TGF-β2 neutralizing conditions, by quantitative RT-PCR relative to unstimulated Mφs. The dashed line indicates no change over control. Data from two independent experiments are shown.

As NOS2 enzymatic activity requires the formation of NOS2 homodimers (17), we examined whether AqH interfered with NOS2 homodimerization within cell lysates from RAW 264.7 cells stimulated in the presence of AqH. Cell lysates were fractionated by size exclusion chromatography and obtained fractions electrophoresed on a denaturing gel and immunoblotted with anti-NOS2 Abs (Fig. 6A, 6B). Consistent with previous observations (17), Mφs stimulated in the absence of AqH showed NOS2 protein molecules comprised of homodimers (eluting in fractions 7–13 at ~250 kDa) and monomers (eluting in fractions 14–20 at ~140 kDa) (Fig. 6A) at approximately a 1:1 ratio (Fig. 6C). Mφs activated in the presence of AqH presented a different pattern, showing high m.w. NOS2 aggregates (eluting in fractions 1–6 at ~700 kDa [Fig. 6B]). However, these aggregates did not interrupt the formation of NOS2 dimers (Fig. 6C). A decreased concentration of monomers was also observed.

**Ascorbate in AqH interferes with the Griess assay**

As our accumulated data provided no evidence of inhibited NOS2 activity by AqH, we tested whether a factor within AqH interfered with the Griess assay used to measure nitrite in culture supernatants. Titrated nitrite standards were prepared in media or media supplemented with AqH at the same concentration as was used in Mφ cultures. As shown in Fig. 7A, AqH completely inhibited measurement of nitrite by the Griess assay, confirming our hypothesis.

The Griess assay is very susceptible to reducing agents as it involves an oxidation reaction. Therefore, we tested whether the antioxidant ascorbate, which is present in very high concentrations within AqH (27), was responsible for interference with the Griess assay. Ascorbate was measured in four lots of rabbit AqH, and the average concentration (1.0 ± 0.6 mM) was comparable to concentrations observed in a previous report (28). For comparison, the concentration of ascorbate in rabbit serum was 30 μM (28). As shown in Fig. 7B, a 1 mM ascorbate solution also completely inhibited measurement of nitrite standards by the Griess assay. To determine the threshold concentration of ascorbate that would cause interference, titrated ascorbate solutions (1 mM–488 nM) were added to a 50 μM nitrite standard solution and the Griess assay was performed. Ascorbate concentrations as low as 0.1 mM completely inhibited nitrite measurements (Fig. 7D). We also tested the threshold of interference by AqH. Complete interference was observed with AqH diluted 1:16 in medium (Fig. 7C). The ascorbate concentration at this dilution would be ~0.1 mM based on a 1.0 mM concentration in AqH. To confirm that in-
Interference was due to ascorbate, ascorbate was oxidized by incubation with ascorbate oxidase. As shown in Fig. 7A and 7B, ascorbate oxidation restored measurements of nitrite standards. These data clearly indicated that ascorbate in AqH interfered with the Griess assay, resulting in artifactual low nitrite measurements.

Ascorbate in aqueous humor augments NO production in Mφs

To determine the true concentration of nitrite in Mφ cultures stimulated in the presence of AqH, Mφ culture supernatants were treated with ascorbate oxidase prior to performing the Griess assay. As shown in Fig. 8, nitrite concentrations in supernatants from Mφs stimulated in the presence of AqH were greater in comparison with Mφ cultures stimulated without AqH. Importantly, this difference was only observed when ascorbate was oxidized in culture supernatants. These data indicated that AqH augmented NO production in Mφs, which was consistent with our observations made with NOS2 enzymatic activity assays (Fig. 1D).

Nakai et al. (29) previously demonstrated that ascorbate increases NO production in Mφs, and we reproduced this finding with macrophages stimulated with LPS and IFN-γ in the presence of 1 mM ascorbate (Fig. 8B). Hence, we reasoned that ascorbate in AqH was responsible for augmented NO production in Mφs. To test the influence of ascorbate in AqH on NO production, ascorbate was oxidized in AqH by incubation with ascorbate oxidase prior to addition to Mφ cultures. As shown in Fig. 8B, oxidation of ascorbate in AqH abrogated the enhancement of NO production by AqH, which confirmed that ascorbate in AqH was responsible for increasing NO production. Similar results were observed when ascorbate was removed from AqH by dialysis (data not shown).

Ascorbate in aqueous humor stabilizes H₂B

Ascorbate has been shown to augment NO production in Mφs by limiting H₂B oxidation, which extends the t½ of this molecule (29). Therefore, we evaluated the degradation of H₂B added to medium, medium diluted in AqH, and medium diluted in AqH that was pretreated with ascorbate oxidase. As shown in Fig. 9, degradation...
of H4B in medium was rapid and complete after 4 h at 37˚C in a 5% CO2 atmosphere. In contrast, AqH limited the degradation of H4B. The stabilization of H4B was due to ascorbate in AqH as pretreatment of AqH with ascorbate oxidase abrogated the stabilizing effect.

Discussion

In this study, we have demonstrated that ascorbate in AqH augments NOS2 enzymatic activity and NO production in stimulated Mfs. These data contradict the interpretations of a previous study by Taylor et al. (9), which indicated that AqH inhibited NO production by Mfs based on their observations that nitrite did not accumulate in culture supernatants of Mfs stimulated in the presence of AqH. We reproduced those observations, but show that the low nitrite levels in these cultures were erroneous. High concentrations of ascorbate in AqH interfered with nitrite measurements made by Griess assays. When interference by ascorbate was controlled for, we observed increased nitrite in culture supernatants of Mfs stimulated in the presence of AqH. The augmentation of NO production by Mfs was mediated by ascorbate, as treatment of AqH with ascorbate oxidase abrogated this augmentation. Our interpretation that AqH augments NO production in Mfs was further supported by direct measurement of NOS2 enzymatic activity, which showed greater conversion of L-Arg into L-citrulline in lysates of Mfs stimulated in the presence of AqH.

It is important to note that the previous study (9) relied entirely on nitrite measurements in culture supernatants and did not indicate that interference by ascorbate in AqH was controlled for. However, what remains puzzling is that this study also showed that nitrite levels were restored in AqH-treated Mfs culture supernatants if AqH was incubated with neutralizing Abs to CGRP (9). Ascorbate would still remain in this treated AqH sample, which should have interfered with the Griess assay and resulted in artifactual low nitrite readings. One potential explanation is that the addition of Abs produced false-positive readings. In support of that interpretation, certain molecules, including proteins, have been shown to cause both false-positive and false-negative readings in the Griess assay (30). False-negative readings could also explain other observations showing that CGRP or PEDF alone inhibited nitrite levels in stimulated Mf culture supernatants (9, 22). However, we were unable to reproduce inhibitory effects with these molecules. Regardless, the sensitivity of the Griess assay to interference by many different molecules requires careful controls in which nitrite standards are measured in the presence of tested aqueous solutions.

There were some methodological differences between our study and the previous study. For example, in our study, AqH was stored...
It is important to note that a previous study also demonstrated that ascorbate increases NO production in RAW 264.7 Mφs (29), which supports our observations. Interestingly, their automated method (33) for measuring nitrite in culture supernatants did not require the addition of ascorbate oxidase to eliminate interference by ascorbate in the Griess assay. This is most likely due to differences in sample preparation. In their procedure, culture supernatants were first deproteinized by precipitation with sulfosalicylic acid and centrifuged, and then an aliquot of the supernatant was treated with NH₄Cl and NaOH. These steps would dilute the original ascorbate concentration and potentially oxidize ascorbate. In our study, and in the study by Taylor et al. (9), which we refute, undiluted Mφ culture supernatants with high concentrations of ascorbate were analyzed. Therefore, treatment with ascorbate oxidase was necessary to prevent interference with the Griess assay, which requires an oxidation reaction.

Our data suggest two possible mechanisms by which AQH augments NO production in Mφs. First, we demonstrate that AQH increases L-Arg uptake in Mφs, providing more available substrate for NOS2 to generate NO and L-citrulline. Second, ascorbate in AQH stabilized H₄B. Therefore, increased intracellular concentrations of L-Arg and H₄B could increase the formation of functional NOS2 dimers that would thereby increase total NOS2 enzymatic activity. In support of that interpretation, we did observe fewer NOS2 monomers and more NOS2 dimers in lysates of Mφs stimulated in the presence of AQH (Fig. 6). However, it is important to note that increasing intracellular H₂B does not directly correlate with increased NOS2 enzymatic activity. For example, Nakai et al. (29) artificially elevated intracellular H₂B in Mφs by either ascorbate or sepiapterin supplementation. Sepiapterin generates H₂B via a salvage pathway, whereas ascorbate is thought to reduce H₂B oxidation and thereby limit its degradation. Although H₂B was concentrated 20-fold greater in sepiapterin-treated Mφs in comparison with ascorbate-treated Mφs, ascorbate-treated Mφs produced more NO. Supplementation of ascorbate and sepiapterin had a synergistic effect, producing even more NO. Hence, the redox state of H₂B may be more critical than the total H₂B concentration in terms of influencing NOS2 enzymatic activity.

The formation of high m.w. NOS2 aggregates in Mφs stimulated in the presence of AQH suggests unique interactions between NOS2 and other proteins that may inhibit or activate NOS2 enzymatic activity in these holoenzyme complexes. As our data show that AQH increased NOS2 enzymatic activity, this suggests that negative regulatory proteins, including NO-associated protein of 110 kDa (NAP-110) (14) and Kalirin-7 (15), which bind to the N terminus of NOS2 and prevent homodimerization, are not involved. However, we cannot exclude the possibility that AQH promoted association of NOS2 with proteins that enhanced its activity. Along those lines, Daniluc et al. (16) showed that NOS2 association with α-actinin 4 was critical for enzymatic activity. In addition to changing the cellular location of NOS2, α-actinin 4 appeared to be a critical cofactor for NOS2 function, as NOS2 dimers without α-actinin 4 were not enzymatically active. Future experimentation involving immunoprecipitation of proteins bound to NOS2 will be necessary to evaluate whether AQH influences NOS2 enzymatic activity by changing the proteins associated with NOS2.

At first glance, it is hard to understand why an immune-privileged site would contain a molecule that augments proinflammatory NO production in Mφs. However, ascorbate also minimizes inflammation by neutralizing reactive oxygen species. For example, Rosenbaum et al. (27) showed that ascorbate in AQH inhibited myeloperoxidase activity in neutrophils. NO combines
with reactive oxygen species to form peroxynitrite, which induces cell death (34). Therefore, ascorbate may limit the toxic effects of NO by limiting peroxynitrite formation.

In summary, our data overturn a long-standing paradigm of AqH-mediated immunosuppression of Mφ NO production by demonstrating that AqH augments proinflammatory NO production in Mφs via ascorbate. High concentrations of ascorbate in AqH interfere with Griess assays, highlighting the need to carefully control for interference when evaluating the influence of aqueous solutions on NO production in cultured cells.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure Legends

Supplemental Figure 1: Thioglycolate induced PECs are primarily F4/80+ Mφs
Flow cytometric analysis of PECs for expression of F4/80 and GR-1 (A.) after overnight culture in SGM and washing to remove nonadherent cells. Isotype control staining of PEC demonstrates the specificity of antibody staining (B.). Two experiments with similar results were performed.

Supplemental Figure 2: Mφ stimulation induces NOS2 dependent NO production
Primary Mφ from WT and NOS2 KO mice were stimulated with LPS (4 ng/ml) and IFNγ (100 U/ml) in the presence or absence of AqH. Eighteen hours later nitrite was measured in culture supernatants. ANOVA with Tukey’s post-test was used to compare bracketed groups. ***p<0.001. One experiment was performed.

Supplemental Figure 3: AqH augments NO production in Mφs
Nitrite concentrations in culture supernatants, treated with ascorbate oxidase, from Mφs cultured as indicated in serum free medium in the presence or absence of fresh AqH. ***p<0.001 by ANOVA and Tukey’s multiple comparison post-test of bracketed groups. Panel A and B. represent two independent experiments.
Supplemental Figure 2
Supplemental Figure 3