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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 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 1-arginine (1-Arg) into 1-citrulline, was augmented in lysates of Mφs stimulated in the presence of AqH. These data suggested that intracellular 1-Arg may have been limited by AqH. However, we observed increased mRNA expression of the 1-Arg transporter, cationic amino acid transporter 2B, and increased L-Arg uptake in Mφs expressed by stimulated Mφs. Therefore, AqH may augment NO production in macrophages by stabilizing H4B and increasing intracellular 1-Arg. The Journal of Immunology, 2013, 190: 000–000.

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-B2 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 1-citrulline by metabolizing 1-arginine (1-Arg). Therefore, limiting 1-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 indispensable cofactors, including tetrahydrobiopterin (H4B) (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 Mψs stimulated in the presence or absence of AqH. Interestingly, we observed increased NOS2 enzymatic activity in cell lysates of Mψs 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 Mψs via ascorbate. AqH increased intracellular concentrations of l-Arg and limited degradation of H4B, suggesting two potential mechanisms for augmented NO production by Mψs.

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), l-Arg (Sigma-Aldrich, St. Louis, MO), calci-citonin gene-related peptide (CGRP; Sigma-Aldrich), α-MSH (Sigma-Aldrich), pigment epithelium-derived factor (PEDF; BioproductsMD, Lincon, NB), and then primary Abs were added at the following dilutions: goat anti-rabbit IRDye680 1:20,000 and goat anti- mouse IRDye800CW 1:20,000. Secondary Abs were then used at the following dilutions: goat anti-rabbit IgG (Bio-Rad, Hercules, CA), and 10–30 μg total protein was loaded on a 6% polyacrylamide gel. Following electrophoresis, separated proteins were transferred to a polyvinylidene difluoride membrane that was blocked with Odyssey Blocking Buffer (LI-COR, Ann Arbor, MI). An Odyssey Infrared Imager (LI-COR).

NO production was measured indirectly by measuring nitrite levels in culture supernatants utilizing a cell scraper in the presence of RLT buffer (Qiagen, Germantown, MD). Total RNA was then extracted using the TRIzol method. cDNA was prepared using a high-capacity cDNA reverse-transcription kit (Applied Biosystems, Carlsbad, CA), and expression of arginase 1 (ARG1; primer M004757989_m1), arginase 2 (ARG2; primer M00477592_m1), CAT2B (primer M00432032_m1), GTP cyclohydrolase 1 (GTPCH1; primer M00514993_m1), NOS2 (primerM00440485_m1), and pyru- vate carboxylase (primer M00500992_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 exper- imental gene expression were normalized to the CT values of the housekeeping gene pyruvate carboxylase within the same sample (∆ΔCT). The ∆ΔCT from Mψ-stimulated cultures was then subtracted from the ∆ΔCT of nonstimulated Mψ cultures (∆ΔCT) to determine fold differences from controls = 2ΔΔCT.

l-Arg uptake assay

Triplicate primary Mψ 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 [3H]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 Mψ cultures (5.0 × 105 cells in 2 ml SGM contained within a 60-mm tissue 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 on 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 IRDye680 1:20,000 and goat anti-Mouse IRDye 800CW 1:20,000. Protein expression was resolved by im- aging 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 instruc- tions. 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 Mψ 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-
ard. 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$_4$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 dihydroxythritol (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$_2$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$_4$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$_2$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; Alttech 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$_4$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 postest comparison of selected groups 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 cell 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) 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 Mφs (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$_4$B. Therefore, our incongruent measurements of NO production made by Griess assays and NOS2 enzymatic activity assays could have been due to AqH

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.
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 independent experiments (Fig. 3A). The addition of AqH further increased 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 expression (Fig. 3A). These data indicated that the concentration of intracellular L-Arg was increased rather than inhibited by AqH treatment.

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

Intracellular L-Arg can be depleted by competition with arginases (ARG1 and ARG2) that have similar Vmax/Km 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 expression increased dramatically relative to unstimulated Mφs in two independent experiments (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 stimulated with or without AqH. As Mφ cultures stimulated in the absence of AqH produced NO despite ARG1 expression, 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 stimulated with or without AqH. As Mφ cultures stimulated in the absence of AqH produced NO despite ARG1 expression, which was equivalent to Mφ cultures stimulated 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 competition 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 H4B and L-Arg (13, 26). H4B is produced by

![Graph A](http://www.jimmunol.org/)  
**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.
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.

AqH does not interfere with NOS2 homodimerization

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-

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 Mφ. The dashed line indicates no change over control. Data from two independent experiments are shown.
terference 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 Mφs 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 AqH stabilizes H₄B
Ascorbate has been shown to augment NO production in Mφs by limiting H₄B oxidation, which extends the t₁/₂ of this molecule (29). Therefore, we evaluated the degradation of H₄B added to medium, medium diluted in AqH, and medium diluted in AqH that was pretreated with ascorbate oxidase. As shown in Fig. 9, degradation

FIGURE 6. Influence of AqH on NOS2 homodimerization. Measurement of NOS2 monomers and dimers in cell lysates from RAW 264.7 Mφs stimulated in the absence (A) or presence (B) of AqH under TGF-β2 neutralizing conditions. Approximate m.w. of individual fractions shown based on elution of known standards. (C) Band intensities for individual fractions. Data presented are from an individual experiment of three performed. High m.w. NOS2 aggregates were observed in two of three experiments.
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-citruline 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 Mf cultures 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...
Ascorbate is a critical cofactor for optimal NOS2 enzymatic activity. In Fig. 1A with Supplemental Fig. 3, these data could suggest that ascorbate is a critical cofactor for optimal NOS2 enzymatic activity under normal physiological conditions.

Figs 1A: Treatment
none
AqH
AqH (AO bx)

% H4B
Time (hours)
0 1 2 3 4

FIGURE 9. Ascorbate in AqH stabilizes H4B. Percentage of H4B spiked in SGM medium, SGM supplemented 1:1 with AqH that was treated with vehicle, or SGM supplemented 1:1 with AqH that was treated with ascorbate oxidase (AO) after incubation at 37°C at 5% CO2 atmosphere for indicated periods of time. This experiment was performed twice with equivalent results.

Frozen before use and Mφs were cultured in serum-containing medium. In contrast, Taylor et al. (9) used fresh AqH and cultured Mφs in serum-free medium. It has been suggested that the suppressive activity of AqH toward T cells is destroyed by freezing AqH, and that proteases in serum degrade immunosuppressive peptides (19, 31). However, we also observed augmented NO production by Mφs that were stimulated in serum-free medium in the presence of fresh AqH (Supplemental Fig. 3). These data argue against the loss of immunosuppressive activity in AqH due to freezing of AqH or our culture conditions. In fact, the augmentation of NO production by Mφs stimulated in the presence of AqH was greater when fresh AqH and serum-free medium were used. Therefore, we find no evidence of an immunosuppressive factor within AqH that inhibits NO production in Mφs.

Why Mφs stimulated with fresh AqH in serum-free conditions produced even more NO is not completely understood. One simple explanation is that freezing AqH decreased the concentration of ascorbate, thereby minimizing its enhancing effect on NO production. However, we directly compared fresh and frozen AqH, and, although the concentration of ascorbate was slightly lower in frozen AqH, mM concentrations were still observed (data not shown). Another potential explanation is that the serum-free media we used, which did not contain ascorbate, reduced the basal ascorbate concentration in Mφs. Hence, the effect of ascorbate supplementation was magnified. In support of that explanation, May et al. (32) showed that freshly prepared thioglycollate-elicited Mφs contained ~3 mM ascorbate. After overnight culture in ascorbate-free medium, this concentration was reduced to 1–2 mM. Our peritoneal Mφ cultures were allowed to adhere overnight in serum-free medium. Therefore, the intracellular ascorbate concentration would have been reduced. The nitrite concentrations in supernatants of control Mφs stimulated without AqH were noticeably lower than in similar cultures stimulated in serum-containing, and thus ascorbate-containing, RPMI 1640 (compare Fig. 1A with Supplemental Fig. 3). These data could suggest that ascorbate is a critical cofactor for optimal NOS2 enzymatic activity.

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 H4B does not directly correlate with increased NOS2 enzymatic activity. For example, Nakai et al. (29) artificially elevated intracellular H4B in Mφs by either ascorbate or sepiapterin supplementation. Sepiapterin generates H4B via a salvage pathway, whereas ascorbate is thought to reduce H4B oxidation and thereby limit its degradation. Although H4B 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 H4B may be more critical than the total H4B 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

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References


