Nitration of Annexin II Tetramer†

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ABSTRACT: Annexin II tetramer (AII_t) is a member of the Ca^{2+}- and phospholipid-binding protein family and is implicated in membrane fusion during surfactant secretion. It had previously been shown that high concentrations of nitric oxide (NO) inhibit surfactant secretion from lung type II cells. NO reacts with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$), a tyrosine nitrating agent, which is found in lungs under certain pathological conditions. It is therefore hypothesized that nitration of AII_t by $ONOO^-$ may be a mechanism for the NO inhibition of regulated exocytosis. We therefore performed in vitro studies to test effects of $ONOO^-$ on AII_t. Western blot analysis using anti-nitrotyrosine antibodies showed a dose-dependent nitration of tyrosine residues in AII_t treated with $ONOO^-$. Nitration occurred on the core domain of the p36 subunit, as well as on the p11 subunit. $ONOO^-$ also caused the formation of dimers between p36 and p11 subunits which were stable in the presence of heating, SDS, and β-mercaptoethanol. AII_t-mediated liposome aggregation was inhibited by $ONOO^-$ with an IC_{50} of $\sim$30 μM. The inhibition was abolished by urate (a scavenger of $ONOO^-$ and $^\bullet OH$), but not by mannitol (a scavenger of $^\bullet OH$) or superoxide dismutase (a scavenger of O_2^-) and appeared to be specific to AII_t, since $ONOO^-$ only slightly influenced annexin I-mediated liposome aggregation. The conformational change of AII_t induced by Ca^{2+} had no effect on the inhibition. Furthermore, $ONOO^-$ only partially inhibited the binding of AII_t to membranes. Nitration of AII_t also occurred in intact A549 cells, a lung epithelial cell line, treated with $ONOO^-$. The results of this study suggest that AII_t-mediated liposome aggregation was inhibited by nitration of the protein.

Annexins are a large multigene family of proteins which are expressed in the majority of mammalian cells as well as molds and plants (*1*−*4*). Each member of the family has a conserved core domain consisting of either four or eight 70-amino acid repeats and a unique N-terminus. A notable characteristic of all the members is that they bind to phospholipids in a Ca^{2+}-dependent manner. Annexin II, which is abundant in the lung, exists as a monomer (p36) or a heterotetramer [$(p36)_2(p11)_2$ or AII_t].[1] Annexin II has been shown to aggregate and fuse biological membranes (*5*−*7*), leading to the idea that it is involved in regulated membrane trafficking events. In vitro, AII_t forms a complex with plasma membrane vesicles and chromaffin granules within the incubation milieu (*8*). AII_t has been immunocytochemically localized at the contact sites between chromaffin granules and the plasma membrane (*9*, *10*). Furthermore, addition of exogenous AII_t retards the secretory rundown, characterized by a decreased sensitivity to stimulation, from permeabilized chromaffin cells (*11*, *12*).

Lung surfactant is a complex of 90% phospholipids and 10% proteins secreted by alveolar type II cells in the lung. The major function of surfactant is to lower the collapsing pressure imposed on the alveoli caused by surface tension at the air−liquid interface. Surfactant also plays a role in host defense mechanisms in the lung. The secretion of lung surfactant is via exocytosis of lamellar bodies and is stimulated by various stimuli, including agonists for β-adrenergic receptors and purionoreceptors, Ca^{2+} ionophores, and protein kinase activators (*13*). There is accumulating evidence to show that AII_t also mediates lamellar body fusion in lung type II cells. AII_t promotes in vitro fusion of lamellar bodies with liposomes at micromolar concentrations of Ca^{2+} (*7*). The lung surfactant secretagogue, arachidonic acid, stimulates AII_t-mediated fusion, and the inhibitor of lung surfactant secretion, DIDS, inhibits it (*7*). Similarly, phenothiazines inhibit surfactant secretion as well as the AII_t-mediated fusion of lamellar bodies and liposomes (*14*). AII_t partially restores surfactant secretion from permeabilized type II cells (*15*). AII_t is also able to translocate from the

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[1] Abbreviations: AII_t, annexin II tetramer; AI, annexin I; NO, nitric oxide; $ONOO^-$, peroxynitrite; O_2^-, superoxide; SOD, superoxide dismutase; DPTA, diethylenetriaminepentaacetic acid; GSNO, *S*-nitrosoglutathione; PS, phosphatidylserine; PC, phosphatidylcholine; ECL, enhanced chemiluminescence; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBARS, thiobarbituric acid reactive substance; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; TNM, tetranitromethane; SE, standard error.

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cytosplasm to the plasma membrane of type II cells in response to stimulation (*16*). A fraction of annexin II was tightly associated with lung type II cells (*16*, *17*). Calpain, a neutral Ca^{2+}-dependent protease, has been shown to participate in lung surfactant secretion (*18*) and may do so by regulating annexin II activity (*19*).

The functional characteristics of AII_t can be regulated by post-translational modifications. There are single tyrosine and several serine residues, which are targets for phosphorylation on the N-terminal region of AII_t. Serine 25 is specifically phoshorylated by protein kinase C (PKC), and tyrosine 23 is phosphorylated by the tyrosine kinase $pp60^{c-src}$. Original data demonstrated that PKC activity was required for AII_t activity to sustain secretion in permeabilized cells (*12*). More recently, it has been shown that phosphorylation by PKC or $pp60^{c-src}$ inhibited the membrane aggregating activity of AII_t in vitro, by significantly increasing the calcium requirement of the protein (*8*, *20*, *21*). The same modifications however had no effect on binding of AII_t to membranes. This is interesting that bridging of the membrane occurs at the N-terminal end of the protein while binding to phospholipid vesicles occurs on the C-terminal end. Hence, the phosphorylation is regulatory with respect to the bridging activity of the protein but not for its ability to bind to the membrane. Proteolysis of the N-terminal region restores aggregation activity previously inhibited by phosphorylation (*5*). Since the activity of some isoforms of PKC is enhanced by increased calcium concentrations, AII_t activity could be initially inhibited by agents which increase intracellular calcium concentrations. In that case, AII_t-mediated membrane fusion might involve a removal of the inhibition by dephosphorylation or N-terminal cleavage.

The many roles that the multifaceted nitric oxide (NO) molecule plays in the body have been under intense scrutiny over the past several years. On one hand, it is an important signaling molecule and, on the other, a potentially toxic free radical, especially in the presence of superoxide (O_2^-). O_2^- can react rapidly with NO yielding peroxynitrite ($ONOO^-$). Since both NO and O_2^- are free radicals with unpaired electrons, there is a large release of free energy, approximately 22 kcal/mol or the equivalent to the hydrolysis of two ATPs, when they combine. Hence, the reaction is essentially irreversible. The rate constant of this reaction is near the diffusion-controlled limit ($4-6.9 \times 10^9\ M^{-1}\ s^{-1}$) (*22*, *23*). This is approximately 6 times faster than the scavenging of O_2^- with copper, zinc superoxide dismutase (SOD) at physiological strength. The concentration of SOD, however, is much higher than that of NO under normal conditions, approximately $4-10\ \mu M$ in the brain and liver. Tissues are well prepared to remove O_2^- and keep low the likelihood of it reacting with NO. However, under conditions where the majority of the cells in a specific region are stimulated, the concentration of NO can become high enough to compete with SOD for O_2^-.

$ONOO^-$ has been described as a binary toxin assembled by cells under conditions where the NO concentration approaches that of SOD (*24*). $ONOO^-$, and the protonated peroxynitrous acid, are potent oxidants which react with many cellular components. $ONOO^-$ oxidizes membrane lipids (*25*), tissue sulfhydryls (*26*), and guanine residues in DNA strands (*27*). It is especially adept at oxidizing iron−sulfur centers (*28*, *29*), zinc fingers (*30*), and protein thiols (*31*), all of which cause damage to proteins (*32*, *33*). Another modification caused by $ONOO^-$ is the nitration of tyrosine residues in proteins to generate 3-nitrotyrosine. Formation of 3-nitrotyrosine is also used as an indication of the presence of $ONOO^-$ in vivo. This modification has been shown to alter protein function (*34*−*37*). $ONOO^-$ has been implicated in a number of pathological conditions, including ischemia/reperfusion injury (*38*), atherosclerosis (*39*), acute lung injury (*40*, *41*), acute endotoxemia (*42*), and influenza-induced pneumonia (*43*).

NO alters surfactant metabolism in isolated alveolar type II cells by inhibiting disaturated phosphatidylcholine (DSPC) synthesis and decreasing cellular ATP levels (*44*, *45*). Our previous studies have shown that low levels of NO stimulate surfactant secretion in type II cells, but high levels of NO inhibit it (*46*). In vivo, high levels of NO signify the potential to form $ONOO^-$. Since AII_t is a fusogenic protein abundant in the lung and is important for surfactant secretion (*7*, *14*−*17*), it was hypothesized that formation of $ONOO^-$ by high concentrations of NO inhibits surfactant secretion by inactivating AII_t via nitration of the protein. As a first step to test our hypothesis, this study investigated in vitro modifications of AII_t by $ONOO^-$. We examined (i) whether nitration occurs in purified AII_t treated with $ONOO^-$, (ii) whether AII_t activity is affected by $ONOO^-$ or other nitrating agents, and (iii) whether the nitration of AII_t occurs in lung epithelial cells treated with $ONOO^-$.

MATERIALS AND METHODS

Materials. Diethylenetriaminepentaacetic acid (DPTA), sodium nitrate, hydrogen peroxide, thiobarbituric acid, superoxide dismutase (SOD), urate, and mannitol were purchased from Sigma (St. Louis, MO). Manganese dioxide was from Aldrich (Milwaukee, WI). *S*-Nitrosoglutathione (GSNO) was from Cayman (Ann Arbor, MI). Phosphatidylserine (PS) and phosphatidylcholine (PC) were from Avanti Polar Lipids (Alabaster, AL). Anti-nitrotyrosne antibodies were from Upstate (Lake Placid, NY). Enhanced chemiluminescence (ECL) reagents were from Amersham (Arlington Heights, IL).

Purification of Annexins. Lung annexins were purified from bovine lung tissue according to the method of Khanna and co-workers (*47*) with minor modifications (*15*).

$ONOO^-$ Preparation and Concentration Determination. $ONOO^-$ was synthesized in batches (*48*), and aliquots were kept frozen at −80 °C. Under these conditions, $ONOO^-$ remains stable for several months. Highly pure reagents were used to avoid trace metal contamination. A reaction device was made by connecting a tubing Y piece with two 10 mL syringes. The long arm of the Y piece forms the reaction chamber. All stock solutions were cooled on ice for 30 min before the reaction. A volume of 6.7 mL of 2 M sodium nitrate was added to one syringe, and 6.7 mL of acidified 8.2 M hydrogen peroxide was added to another. The syringes were then immersed in ice for 30 min. The cooled syringes were then held directly above 6 mL of 4.2 N NaOH in an ice bath beaker with continuous stirring. Both plungers were depressed rapidly with equal force using one hand. To remove the unreacted hydrogen peroxide, the yellow $ONOO^-$ solution was passed through a small column of granular manganese dioxide at 4 °C. The column was made by placing

a plug of glass wool in the bottom of a 5 mL syringe and pouring 2−3 cm of manganese dioxide.

To measure the $ONOO^-$ concentration, the sample was collected and diluted 200 times with 0.1 N NaOH. The UV−visible spectrum was measured (245−400 nm) in a quartz cuvette previously blanked with 0.1 N NaOH alone. The absorbance at 302 nm was read. The $ONOO^-$ was then decomposed by the addition of 20 μL of 6 N HCl, and the absorption spectrum was recorded again. The peak at 302 nm was no longer visible. The absorbance at 302 nm after the addition of the acid was subtracted from the absorbance at 302 nm before the addition of the acid. The $ONOO^-$ concentration was calculated using an extinction coefficient of 1670 M^{-1} cm^{-1}.

Annexin Treatment. In general, 5 μg of annexin was mixed in 50 μL of buffer [0.1 M phosphate (pH 7.4) and 0.1 mM DTPA] with varying concentrations of $ONOO^-$. Incubations were carried out at room temperature for 30 min. Since $ONOO^-$ decomposes rapidly in a buffer at physiological pH, special precautions must be taken to ensure that the largest possible amount of protein is exposed to $ONOO^-$ prior to this spontaneous decomposition. This is accomplished by placing a drop of concentrated $ONOO^-$ (1 μL) on the edge of the tube just above the level of the annexin solution and then vortexing. In this way, the $ONOO^-$ is rapidly and thoroughly mixed with the solution containing the protein. The concentration of the $ONOO^-$ stock solution was checked before use.

Liposome Preparation. Liposomes were prepared by the extrusion method as previously described (*7*). Typically, 375 μL of PS (10 mg/mL) was placed into a test tube and dried under a stream of N_2 gas until a barely visible film was evident at the bottom of the test tube. Next, 1.5 mL of liposome buffer [40 mM Hepes (pH 7.0) and 100 mM KCl] was added, and the mixture was vortexed until all of the lipids were thoroughly dissolved. The resulting large multilamellar vesicles were converted to small unilameller vesicles by being passed through a 25 mm polycarbonate filter with a pore size of 0.1 μm three times using an extruder (Lipex Biomembrane Inc., Vancouver, BC). The unilamellar vesicles thus obtained were between 60 and 100 nm in size (*49*).

Liposome Aggregation and Binding Assays. Annexin-mediated aggregation of PS liposomes was assessed by following the absorbance changes at 540 nm (*7*). The assay was carried out in 1.5 mL plastic cuvettes at a final volume of 1 mL. EGTA-Ca^{2+} buffer [40 mM Hepes (pH 7.0), 100 mM KCl, 1 mM EGTA, 2 mM Mg^{2+}, and 2 mM Ca^{2+}] in a volume of 910 μL was added to each cuvette. Subsequently, 40 μL of PS liposomes (2.5 μg/μL) was added to each numbered cuvette, and the contents were mixed by inversion. The turbidity was recorded as the zero time for each sample. Protein samples (50 μL) treated under various conditions were added to each cuvette and mixed. The aggregation assay was run for 30 min at room temperature, followed by another absorbance reading. Liposome aggregation activity was calculated as the difference in absorbance between the 0 and 30 min readings. At the end of the liposome aggregation assay, the sample was centrifuged at 100000*g* for 1 h to determine the amounts of AII_t bound to liposomes. The pellet was analyzed via 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) according to the method of Laemmli (*50*). The bands were quantitated by densitometry (GS-710 Calibrated Imaging Densitometer, Bio-Rad, Hercules, CA).

TBARS Assay. To determine the extent of lipid peroxidation in vitro, the TBARS (thiobarbituric acid reactive substance) assay was used (*51*). Malondialdehyde, a product formed by lipid peroxidation, reacts with thiobarbituric acid to give a red species which absorbs light at 535 nm. A stock solution of TCA, TBA, and HCl was made by mixing 15% (w/v) trichloroacetic acid (TCA), 0.375% (w/v) thiobarbituric acid (TBA), and 0.25 N hydrochloric acid (HCl) and then heating to dissolve the thiobarbituric acid. One milliliter of the biological sample was combined with 2.0 mL of the TCA/TBA/HCl solution and mixed thoroughly. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000*g* for 10 min. The absorbance of the sample was determined at 535 nm against a blank with all the reagents minus the lipid. The malondialdehyde concentration of the sample was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Western Blot. Protein samples were separated via 10% SDS−PAGE and transferred electrophoretically to nitrocellulose membranes in transfer buffer [25 mM Tris (pH 8.3), 192 mM glycine, and 20% (v/v) methanol]. The transfer was carried out at 100 mA for 2 h or at 25 mA overnight. The quality of the transfer was checked by staining the membrane in Ponceu S. Blots were blocked with 2% gelatin in Tris-buffered saline (TBS) for at least 1 h. The blots were then incubated with anti-annexin II, anti-p11, or anti-nitrotyrosine antibodies (1:1000 dilution) in antibody buffer [1% gelatin in TTBS (TBS with 0.05% Tween 20)] for a minimum of 3 h or overnight. The blots were washed three times in TTBS for 5 min each. Subsequently, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:5000 dilution) for a minimum of 1 h. The blots were washed three times in TTBS for 5 min each and developed by either ECL or horseradish peroxidase color development reagents.

Culture of A549 Cells. A lung epithelium-derived cell line (A549 cells), with some characteristics of alveolar type II cells, was used to investigate the nitration of annexin II. A549 cells were cultured in a 75 mm^2 tissue culture flask with 10 mL of MEM and 10% fetal bovine serum placed in an incubator containing a mixture of humidified air and 5% CO_2 at 37 °C. For the nitration experiments, cells were replated on 100 mm tissue culture dishes and viewed daily under a light microscope and used when the monolayer was estimated to be 80−90% confluent.

Immunoprecipitation. A549 cells were washed three times with 10 mL of ice-cold phosphate-buffered saline (PBS). Five milliliters of modified PBS [50 mM Na_2HPO_4, 90 mM NaCl, 5 mM KCl, 0.8 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM glucose (pH 7.4)] was added to the monolayers and incubated for 10 min. Eighteen microliters of $ONOO^-$ (416 mM) or vehicle control was added to cells by tipping the plate gently down and then applying the $ONOO^-$ to the higher edge of the plate away from the solution. The solutions were immediately mixed by gently swirling the dish around. This ensures that the $ONOO^-$ contacts the maximum amount of cells possible prior to its decomposition. As an additional control, the same amount of $ONOO^-$ was allowed to decompose by incubating

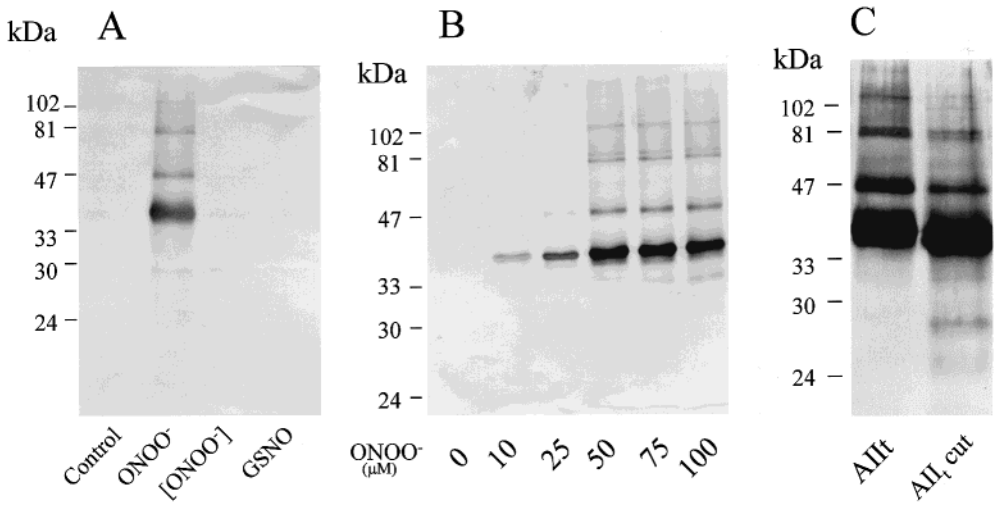

FIGURE 1: Nitration of AII_t in vitro by $ONOO^-$. (A) AII_t (5 μg) in 50 μL of buffer [0.1 M phosphate (pH 7.4) and 0.1 mM DTPA] was incubated for 30 min at room temperature with the following agents: no additions (control), 100 μM $ONOO^-$, 100 μM decomposed $ONOO^-$ ([$ONOO^-$]), or 1 mM GSNO. $ONOO^-$ was decomposed by incubating it with buffer for 30 min at room temperature prior to the addition of AII_t. (B) Dose response of AII_t nitration. AII_t (5 μg) in 50 μL of buffer [0.1 M phosphate (pH 7.4) and 0.1 mM DTPA] was incubated with various concentrations of $ONOO^-$ for 30 min at room temperature. (C) Nitration occurs on the core domain of AII_t. AII_t (5 μg) was incubated with or without calpain (1 μg) and Ca^{2+} (1 mM) in deionized water for 2 h at room temperature. $ONOO^-$ (100 μM) was added to the mixture for 30 min at room temperature. All samples were analyzed by 10% SDS−PAGE and Western blot analysis using anti-nitrotyrosine antibodies. The blot was developed by horseradish peroxidase color development reagents.

it for 2 h at room temperature in 5 mL of modified PBS before adding it to the cells. After incubation for 10 min, cells were scraped with a policeman rubber and collected by centrfugation. The cell pellet was lysed with 0.5 mL of lysis buffer [0.1 M phosphate (pH 7.4), 5 mM EGTA, 10 μg/mL leupetin, 1 μg/mL pepstatin A, 1 μg/mL aprotinin, and 0.1 mM DPTA] by a brief sonication. The particulate fraction was removed by centrifugation at 16000*g* at 4 °C for 30 min. The supernatant was precleared by incubation with 20 μL of protein A−Sepharose beads at 4 °C for 30 min. The beads were pelleted by centrifugation at 1000*g* and 4 °C for 5 min. Polyclonal anti-annexin II antibodies (2 μg) were added to the tube containing the supernatant and incubated for 1 h at 4 °C, followed by overnight incubation with 20 μL of protein A−Sepharose beads at 4 °C by end-to-end rotation. Immunoprecipitates were collected by centrifugation at 1000*g* for 5 min and washed three times with 1.0 mL of lysis buffer. The final pellet was resuspended in 50 μL of electrophoresis sample buffer and boiled for 5 min. The beads were removed by centrifugation, and the supernatant was separated by SDS−PAGE and transferred to a nitrocellulose membrane. The blot was first probed with polyclonal anti-annexin II antibodies (1:1000) and HRP-conjugated anti-rabbit IgG (1:5000). After ECL development, the blot was stripped off the antibodies and reprobed with monclonal anti-nitrotyrosine antibodies (1:1000) and HRP-conjugated anti-mouse IgG (1:5000), followed by ECL development.

Protein Concentration Determination. Protein concentrations were determined according to the method of Bradford (*52*) using the Bio-Rad protein assay kit using IgG as a standard.

RESULTS

Nitration of AII_t in Vitro by $ONOO^-$. To test whether AII_t can be nitrated by $ONOO^-$, purified AII_t was incubated with $ONOO^-$ for 30 min at room temperature in phosphate buffer [0.1 M phosphate (pH 7.4) and 100 μM DTPA]. DTPA is a metal chelator which functions to remove metals such as iron or copper from solution. This is important since these metals are known to catalyze many nonspecific reactions involving free radicals. At the end of the incubation, the proteins were separated by SDS−PAGE and Western blot analysis was performed using specific antibodies against nitrotyrosine. The results are presented in Figure 1. When the protein was treated with 100 μM $ONOO^-$, there was a distinct band at ∼36 kDa, the molecular mass of the heavy chain (p36) of AII_t (Figure 1A, $ONOO^-$), indicating the formation of nitrotyrosine in p36. Additional high-molecular mass bands were also apparent in the $ONOO^-$-treated sample which may represent cross-linked products of p36 and p11 (see below). In contrast, in the control, no bands were visible in the Western blot (Figure 1A, control). The same amount of AII_t protein was seen in the control and the $ONOO^-$-treated samples when the blot was stained with Ponceau S (data not shown), excluding the possibility that the observed difference was due to loading or transfer efficiency.

The nitrating species, under physiological conditions, remains controversial. It may be $ONOO^-$, peroxynitrous acid, or a nitrated intermediate such as a carbonyl formed from reaction with CO_2 or HCO_3^-. $ONOO^-$ rapidly decomposes at room temperature and physiological pH. To examine if nitration occurs via the decomposition products of $ONOO^-$, it was first decomposed in phosphate buffer for 30 min at room temperature prior to incubating it with AII_t. No nitrotyrosines were detected under these conditions (Figure 1A, [$ONOO^-$]). To investigate if NO alone caused nitration, AII_t was incubated with GSNO, a NO donor. Again, no nitration was detected (Figure 1A, GSNO).

A dose response was also determined to see approximately what level of bolus $ONOO^-$ added to AII_t would be required for the nitration. Nitrotyrosine formation was initially detected at concentrations of 10 μM, and it appears to saturate at 50−100 μM (Figure 1B).

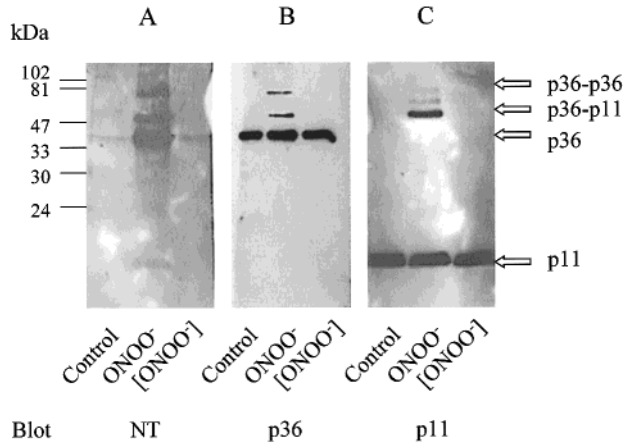

FIGURE 2: Subunits of AII_t are cross-linked by $ONOO^-$ treatment. AII_t (5 μg) was incubated at room temperature in 0.1 M phospate buffer (pH 7.4) with 0.1 mM DTPA for 30 min in a final volume of 50 μL with the following agents: no other agents added (control), 100 μM $ONOO^-$ ($ONOO^-$), and 100 μM $ONOO^-$ decomposed at room temperature for 30 min prior to the addition of AII_t ([$ONOO^-$]). The samples were analyzed by 15% SDS−PAGE and Western blot analysis using specific antibodies against nitrotyrosine (A), p36 (B), and p11 (C). All blots were developed by ECL.

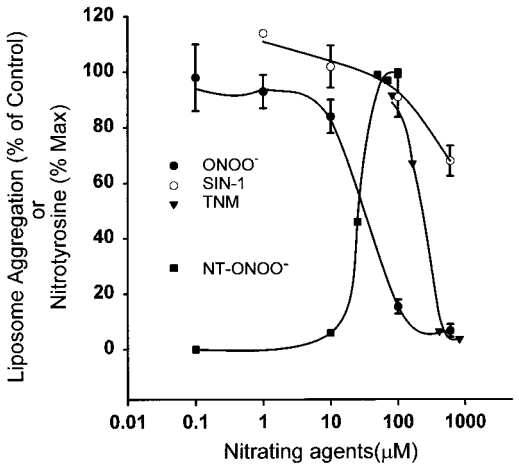

FIGURE 3: Nitrating agents inhibit AII_t-mediated liposome aggregation. AII_t (5 μg) was incubated with various concentrations of nitrating agents in buffer [0.1 M phosphate (pH 7.4) and 0.1 mM DTPA] for 30 min at room temperature. The incubation mixtures were added to PS liposomes (100 μg) in 1 mL of Ca^{2+}-EGTA buffer containing 1 mM free Ca^{2+}, and the turbidity was monitored. Liposome aggregation activity was expressed as the increase in absorbance at 540 nm over the initial value after a 30 min incubation. Liposome aggregation data are means ± SE from three experiments for $ONOO^-$ (●) and SIN-1 (○) or means from two experiments for TNM (▼). Data for the nitrotyrosine level (■, NT-$ONOO^-$) were derived from Figure 1B by quantitating the p36 band using density scanning. Data were means from two experiments and expressed as a percentage of the density from 100 μM $ONOO^-$-treated AII_t. A similar result was obtained by scanning all bands.

Nitration Occurs in the Core Domain of the Protein. Some tyrosine residues would be more vulnerable to $ONOO^-$ attack than others on the basis of their location in the three-dimensional structure of the protein. The annexin family of proteins is structurally distinct by virtue of their N-terminal regulatory domain. The N-terminus contains a proteolytic site for cleavage by calpain, a neutral Ca^{2+}-dependent protease (*19*). This protease selectively cleaves the N-terminal portion of the protein into two fragments of 33 and 3 kDa. This property allows separation of the N-terminal domain from the core domain. To investigate if tyrosine residues in the core domain were susceptible to nitration by $ONOO^-$, AII_t was cleaved by calpain prior to incubation with $ONOO^-$. Removal of the N-terminus of AII_t caused a slight shift in mobility on SDS−PAGE (data not shown). Western blot analysis revealed nitrotyrosine formation in both intact and calpain-cleaved AII_t (Figure 1C), suggesting that the core domain is nitrated when it is treated with $ONOO^-$. However, whether the N-terminus of AII_t is also nitrated remains to be determined.

Cross-Linking of Intersubunits in $ONOO^-$-Treated AII_t. Throughout our experiments, it was observed that bolus addition of $ONOO^-$ to purified AII_t led to the formation of additional high-molecular mass bands on the nitrotyrosine blot. The molecular masses of these bands corresponded to those of p36−p11 dimers, and p36−p36 dimers. To confirm that the high-molecular mass bands are dimerization products between subunits, Western blot analysis using specific antibodies to p11 and p36 subunits as well as to nitrotyrosine on the samples treated with $ONOO^-$ was performed. A 15% acrylamide gel was used for this experiment to better resolve the p11 subunit. All three blots were run on the same gel under the same conditions. For the nitrotyrosine blot, the $ONOO^-$-treated AII_t exhibited the characteristic bands seen in previous experiments with negligible detection in control or decomposed samples (Figure 2A). In addition, the p11 subunit not observed previously was also evident possibly due to higher resolution of low-molecular mass proteins with a higher-percentage gel and increased sensitivity using ECL. As expected, in the p36 blot, a clear band was seen in all lanes at the molecular mass corresponding to 36 kDa. However, in the $ONOO^-$-treated sample, two additional bands were shown with molecular masses of ∼47 and ∼72 kDa (Figure 2B). Similarly in the p11 blot, an additional band with a molecular mass of ∼47 kDa was detected, in addition to the p11 band (Figure 2C). Furthermore, the magnitudes of these higher-molecular mass bands were greatly reduced in calpain-cleaved AII_t in which the p11 binding sites are removed (Figure 1C). These results suggest that the higher-molecular mass bands may represent p36−p11 and p36−p36 dimers, formed in the $ONOO^-$-treated samples. These putative dimerization products are stable against boiling, SDS, and reducing agents. These new proteins also retain their epitope for specific antibody reactions for nitrotyrsoine, p11, and p36. The combined results demonstrate that $ONOO^-$ treatment leads to the formation of high-molecular mass bands in AII_t which may be due to the dimerization of p11 and p36 subunits.

Inhibition of AII_t-Mediated Liposome Aggregation by $ONOO^-$. $ONOO^-$ is a strong oxidant and nitrating agent. Nitration of tyrosine residues in proteins by $ONOO^-$ has been shown to inhibit the function of a number of proteins, including enzymes important for respiration (*28*, *29*). Our results show that AII_t is nitrated in a dose-dependent manner by $ONOO^-$. To investigate the functional significance of this modification, the ability of nitrated AII_t to aggregate PS liposomes was measured by determining the absorbance change at 540 nm. As shown in Figure 3, $ONOO^-$ caused a dose-dependent inhibition of AII_t-mediated liposome aggregation. The concentration which caused a 50% inhibition

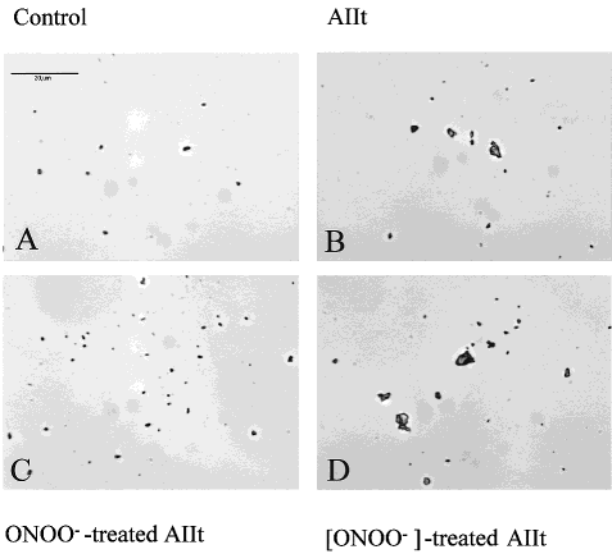

FIGURE 4: Light micrographs of AII_t-mediated liposome aggregation. AII_t (5 μg) was incubated with 100 μM $ONOO^-$ or decomposed $ONOO^-$ ([$ONOO^-$]) for 30 min. The mixtures were added to PS liposomes (100 μg) in 50 μL of Ca^{2+}-EGTA buffer containing 1 mM free Ca^{2+} and incubated for 30 min. Photographs were then taken: (A) liposomes alone, (B) liposomes with AII_t, (C) liposomes with $ONOO^-$-treated AII_t, and (D) liposomes with decomposed $ONOO^-$-treated AII_t.

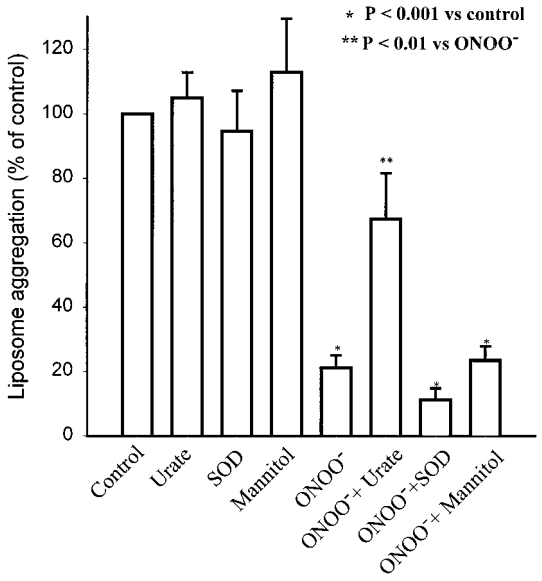

FIGURE 5: Free radical scavenger effects on $ONOO^-$-mediated inhibition of liposome aggregation. AII_t (5 μg) was incubated with or without $ONOO^-$ (100 μM) for 30 min at room temperature in buffer [0.1 M phosphate (pH 7.4) and 0.1 mM DTPA] in the absence and presence of the following free radical scavengers: 100 μM urate, 2400 units/mL SOD, and 50 μM mannitol. The incubation mixtures were added to PS liposomes (100 μg) in 1 mL of 1 mM Ca^{2+}-EGTA buffer, and the turbidity was monitored. Liposome aggregation activity was defined as the increase in absorbance at 540 nm over the initial value after a 30 min incubation. The results were expressed as the percentage of control (no additions). Data are means ± SE from four experiments.

(IC_{50}) was ~30 μM. For comparison, nitrotyrosine formation in AII_t treated with $ONOO^-$ as detected by Western blot (Figure 1B) was quantitated by densitometry and presented in Figure 3. The nitrotyrosine formation corresponds well to the loss of AII_t's activity. The concentration of $ONOO^-$ that caused 50% of the maximal nitrotyrosine formation was similar to the IC_{50} of aggregation activity. When $ONOO^-$ (100 μM) was decomposed, much less inhibition (30 ± 13%) was observed compared to that of undecomposed $ONOO^-$ (80 ± 4%), suggesting that inhibition was not due to the decomposed products of $ONOO^-$. Furthermore, $ONOO^-$ alone had no effects on liposome aggregation (data not shown). SIN-1, a donor of $ONOO^-$, also inhibited AII_t-mediated liposome aggregation, although it was much less potent than authentic $ONOO^-$. The IC_{50} was greater than 600 μM. Tetranitromethane (TNM) is a known chemical nitrating agent, and it also inhibited AII_t-mediated liposome aggregation with an IC_{50} of ~180 μM.

Using a light microscope, the results presented above were confirmed by visualizing liposome aggregation. In the absence of AII_t, small dots representing individual liposomes were seen (Figure 4A). The addition of AII_t led to the formation of large aggregates (Figure 4B). However, when AII_t was pretreated with $ONOO^-$, there was no visible aggregation (Figure 4C), whereas decomposed $ONOO^-$-treated AII_t caused aggregation again (Figure 4D).

Effects of a Free Radical Scavenger on $ONOO^-$ Inhibition of AII_t-Mediated Liposome Aggregation. $ONOO^-$ has a relatively short half-life in biological systems and is thus capable of forming intermediates such as the hydroxy radical or superoxide. To determine which species is responsible for inhibition of AII_t's activity, various specific free radical scavengers were added to the incubation mixtures prior to the addition of $ONOO^-$. As shown in Figure 5, once again, 100 μM $ONOO^-$ caused an 80% inhibition of liposome aggregation. In the presence of urate, a scavenger of $ONOO^-$ and OH^-, a major part of the AII_t activity was recovered. SOD, which removes O_2^-, or the OH^- scavenger, mannitol, was also tested. The results showed that these two agents gave no protection against $ONOO^-$ inhibition of AII_t-mediated liposome aggregation. Urate, SOD, or mannitol alone had no effect on AII_t's activity. It was concluded that $ONOO^-$ rather than an intermediate is responsible for inhibiting AII_t activity.

Effects of Ca^{2+} and Membrane on $ONOO^-$-Mediated Inhibition. To further characterize the mechanism by which $ONOO^-$ inhibits liposome aggregation mediated by AII_t, the vulnerability of AII_t to $ONOO^-$ was investigated under four different conditions. Ca^{2+} has been shown to induce protein conformational changes in AII_t (*53*, *54*), and may alter the environment around tyrosine residues. AII_t was incubated with $ONOO^-$ in the presence or absence of Ca^{2+}, and liposome aggregation activity was measured. As shown in Figure 6, a similar inhibition (~80%) was observed under both conditions. The results suggest that the conformational change in AII_t induced by Ca^{2+} has no effect on $ONOO^-$ inhibition. In contrast, when AII_t was preincubated with liposome and Ca^{2+} to allow AII_t binding to membrane and then treated with $ONOO^-$, only a slight inhibition (~10%) was observed. This could be due to one of two reasons: (i) binding of AII_t to membrane or (ii) preaggregation which hides the nitration sites. However, when preincubation was carried out in the presence of EGTA, in which the protein does not bind to the membrane during the preincubation, still less inhibition (40%) was seen than without preincubation (80%). Therefore, another possibility is that $ONOO^-$

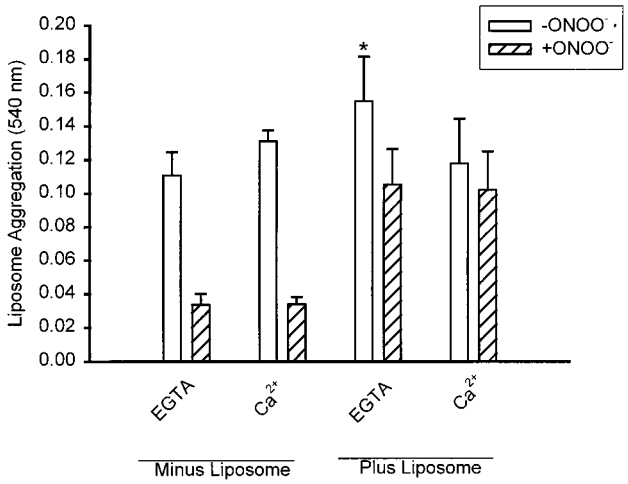

FIGURE 6: Effect of Ca^{2+} and liposome on $ONOO^-$ inhibition. For Minus Liposome, AII_t (5 μg) was incubated with $ONOO^-$ (100 μM) in the presence of 1 mM Ca^{2+} or 1 mM EGTA in 50 μL of buffer [0.1 M phosphate (pH 7.4) and 0.1 mM DTPA] at room temperature for 30 min. At the end of the incubation, the incubation mixtures were added to PS liposomes (100 μg) in 1 mL of Ca^{2+}-EGTA buffer containing 1 mM free Ca^{2+}, and the turbidity was monitored. Liposome aggregation activity was defined as the increase in absorbance at 540 nm over the initial value after a 30 min incubation. For Plus Liposome, AII_t (5 μg) was mixed with 50 μg of liposomes in 50 μL of buffer containing 1 mM Ca^{2+} (allowing the binding of AII_t to membranes) or 1 mM EGTA (no binding of AII_t to membranes). In one group, 100 μM $ONOO^-$ was added ($+ONOO^-$), and in another group, no $ONOO^-$ was added ($-ONOO^-$). After a 30 min preincubation, the mixture was added to 1 mL of Ca^{2+}-EGTA buffer containing 50 μg of liposomes and 1 mM free Ca^{2+} for the aggregation assay, and the turbidity was monitored for 30 min. Liposome aggregation was defined as the difference in absorbance at 540 nm between 30 min and time zero. Since some aggregation occurs during the preincubation period in the presence of Ca^{2+}, the absorbance at 540 nm for 100 μg of liposmes in 1 mL of Ca^{2+}-EGTA buffer was used as the time zero value (in the range of 0.02−0.03). Data are means ± SE from four experiments. An asterisk indicates $P < 0.05$ vs $ONOO^-$ (Student's *t* test).

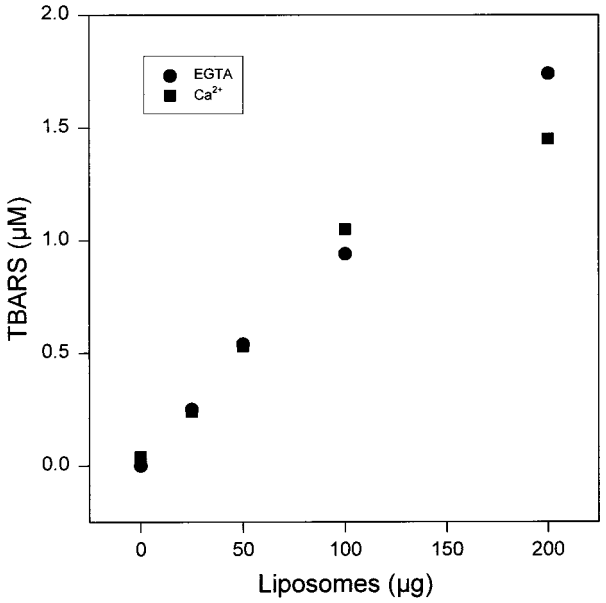

FIGURE 7: $ONOO^-$ causes lipid peroxidation in liposomes. Various amounts of liposomes (PS) were incubated with 100 μM $ONOO^-$ in 50 μL of buffer [0.1 M phosphate (pH 7.4) and 0.1 mM DTPA] with 1 mM Ca^{2+} or 1 mM EGTA at room temperature for 30 min. Samples were mixed with 2 mL of a TCA/TBA/HCl solution [15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid, and 0.25 N hydrochloric acid] and boiled for 15 min. The absorbance was read at 535 nm, and the TBARS of the sample was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

reacts with lipids. This effectively lowers the $ONOO^-$ concentration that is available to react with the protein. $ONOO^-$ has been shown to cause the peroxidation of lipids (*25*). The TBARS assay was used to determine whether $ONOO^-$ causes peroxidation of liposomes in our system. Indeed, in the presence of EGTA or Ca^{2+}, the TBARS value increased linearly with increasing an lipid concentration (Figure 7).

Binding Is Partially Affected by Nitration. The membrane binding of AII_t treated with $ONOO^-$ was examined using a centrifugation assay. AII_t was treated with $ONOO^-$ or decomposed $ONOO^-$ for 30 min and incubated with liposomes in the presence of Ca^{2+}. The AII_t associated with liposomes was pelleted by centrifugation and analyzed by SDS−PAGE. Figure 8A shows that AII_t's ability to bind liposomes was partially affected by $ONOO^-$, but not by decomposed $ONOO^-$. This experiment was then repeated with various concentrations of $ONOO^-$, and the gel was quantitated by densitometry and compared with the aggregation activity (Figure 8B). Although the IC_{50}s were similar in both cases, the binding activity was less inhibited by $ONOO^-$ than the aggregation activity. To exclude the possibility that a small reduction in the amount of AII_t bound may have a large effect on the aggregation activity, we varied the starting AII_t amount and assessed the binding and aggregation in the same assay as described in Materials and Methods. As shown in Figure 8C, $ONOO^-$ at 1 mM inhibited liposome aggregation at all AII_t concentrations. However, the binding of AII_t to membrane was only partially affected at the low concentrations of AII_t, but not at the high concentration of AII_t.

Annexin I Is Nitrated, but Is Inhibited Much Less by ONOO⁻. To determine whether annexin I (AI) is also nitrated, we repeated the experimental approach which was used with AII_t on AI. When purified AI was incubated with 100 μM $ONOO^-$ for 30 min and analyzed by Western blot analysis using specific antibodies against nitrotyrosine, a band was seen at the molecular mass corresponding to that of AI, indicating that the protein was nitrated (Figure 9A). A faint band at 76 kDa may represent a dimer as seen in AII_t. A lower-molecular mass band was a proteolytic product in the AI preparation which was confirmed by Western blot using anti-annexin I antibodies (data not shown), suggesting that tyrosine residues in the core domain are susceptible to nitration by $ONOO^-$. To confirm this, AI was cleaved by calpain prior to incubation with $ONOO^-$ (*55*). Western blot analysis revealed nitrotyrosine formation in both intact and calpain-cleaved AI (Figure 9B), suggesting that the core domain is nitrated. Whether the treatment of AI with $ONOO^-$ lost its ability to aggregate liposomes was subsequently examined. As shown in Figure 9C, much less inhibition of AI-mediated liposome aggregation was observed compared to that of AII_t. To rule out the possibility that the AI concentration that was used might be above the saturation point and the effect of nitration on its aggragation activity might be masked, we plotted a AI dose−resposne curve of liposome aggregation (Figure 9D). At all concentrations of

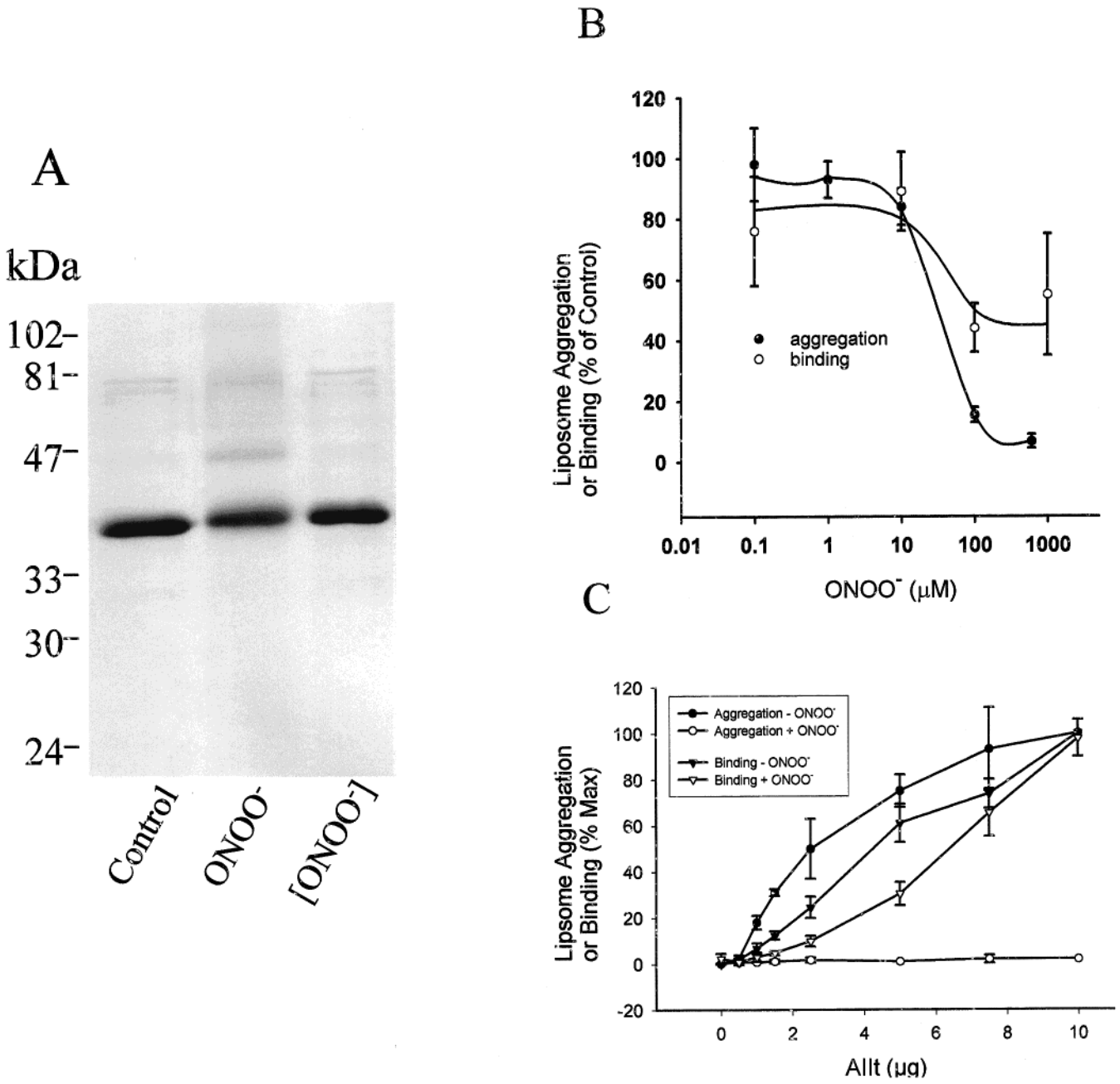

FIGURE 8: $ONOO^-$ only partially prevents binding of AII_t to liposomes. (A) AII_t (5 μg) was incubated in the absence (control) or presence of 100 μM $ONOO^-$ or decomposed $ONOO^-$ ([$ONOO^-$]) in buffer [0.1 M phosphate (pH 7.4) and 0.1 mM DTPA] for 30 min at room temperature. The protein samples were incubated with liposomes in Ca^{2+}-EGTA buffer containing 1 mM free Ca^{2+} for 20 min. AII_t associated with liposomes was analyzed by SDS−PAGE and stained with Coomassie blue. (B) AII_t (5 μg) was incubated with various concentrations of $ONOO^-$ for 30 min. The aggregation (●) and binding activities (○) were determined as described in Materials and Methods. The results were expressed as the percentage of control (no $ONOO^-$). Data are means ± SE from four experiments. (C) AII_t (0−10 μg) was inucbated with 1 mM $ONOO^-$ for 30 min, and the aggregation and binding activities were determined. For the comparison, the results were expressed as percentages of the maxmal activity (i.e., 10 μg of AII_t without $ONOO^-$ treatment). Data are means ± SE from three experiments.

AI that were tested (0−10 μg), $ONOO^-$, even at a high concentration (1 mM), had no major effects on AI-mediated liposome aggregation, although AII_t-mediated liposome aggregation was completely abolished under the same conditions (Figure 8C).

Nitration of AII_t in A549 Cells. The studies described above provide evidence that AII_t is nitrated in vitro. An important question is whether this modification also occurs in cells. To answer this question, nitration of AII_t in A549 cells was examined. The A549 cells are from a lung epithelium-derived cell line, which has some characteristics similar to those of lung type II cells. A549 cells grown to ~90% confluence in 100 mm dishes were incubated with 1.5 mM $ONOO^-$ or decomposed $ONOO^-$ for 10 min. At the end of the incubation, cells were washed three times with PBS and collected. Annexin II was immunoprecipitated with anti-annexin II antibodies and subsequently analyzed by Western blotting using anti-annexin II or anti-nitrotyrosine antibodies. As shown in Figure 10, a comparable amount of annexin II was present in the immunoprecipitate under all conditions. However, the nitration of annexin II was observed only in $ONOO^-$-treated cells, but not in the control or

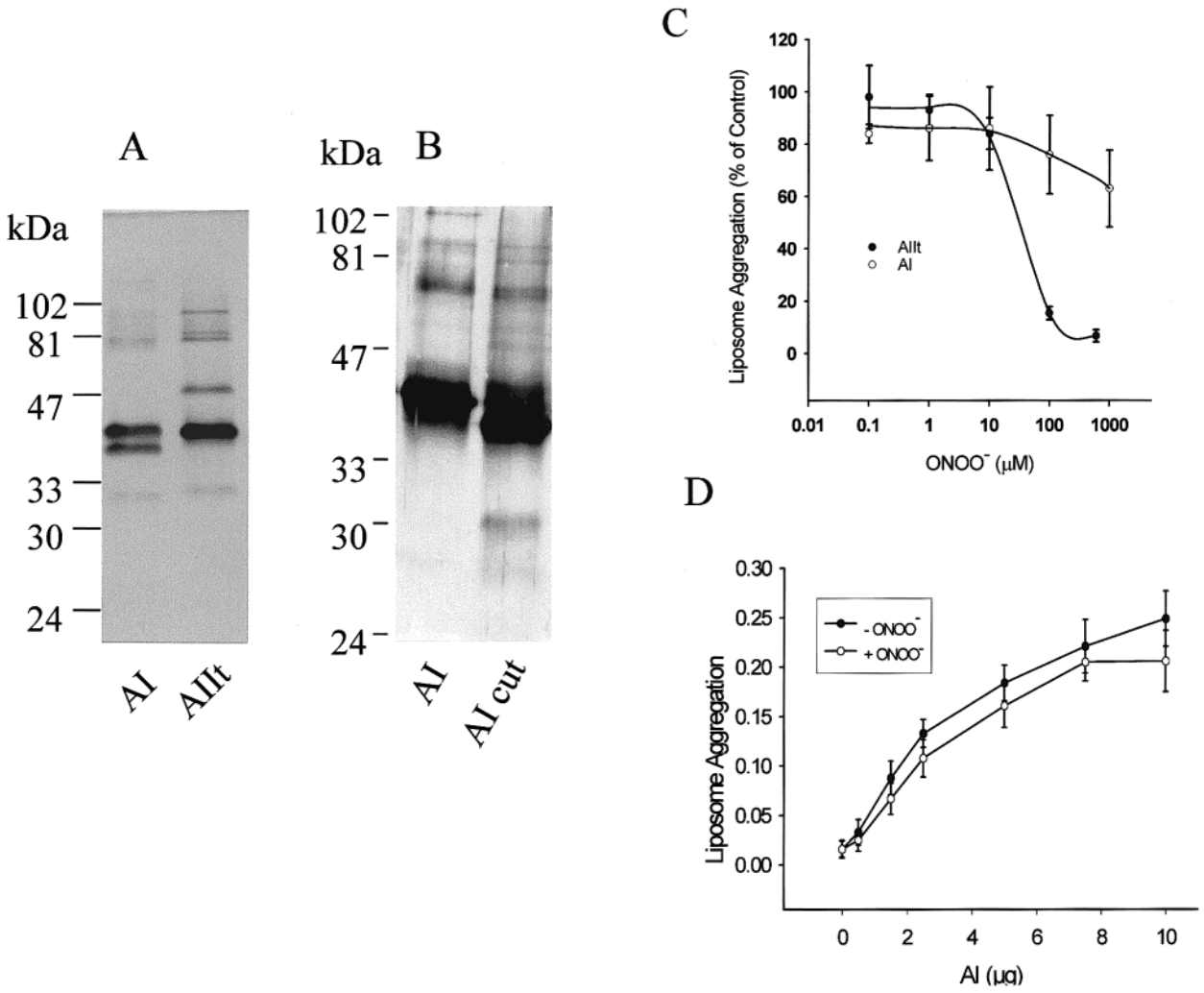

FIGURE 9: AI and AII_t are nitrated by $ONOO^-$, but $ONOO^-$ inhibits liposome aggregation mediated by AII_t, more than by AI. (A) AII_t (5 μg) and AI (5 μg) were incubated with $ONOO^-$ (100 μM) in buffer [0.1 M phosphate (pH 7.4) and 0.1 mM DTPA] for 30 min at room temperature and analyzed by 10% SDS−PAGE and Western blotting using anti-nitrotyrosine antibodies. (B) AI (5 μg) was incubated with or without calpain (1 μg) and Ca^{2+} (1 mM) in deionized water for 2 h at room temperature, and then treated and analyzed as described for panel A. (C) AII_t (5 μg) or AI (5 μg) was incubated with various concentrations of $ONOO^-$ in buffer [0.1 M phosphate (pH 7.4) and 0.1 mM DTPA] for 30 min at room temperature. The incubation mixtures were added to PS liposomes (100 μg) in Ca^{2+}-EGTA buffer containing 1 mM free Ca^{2+}, and the turbidity was monitored. The results were expressed as the percentage of control (no $ONOO^-$). Data are means ± SE from three or four experiments. (D) AI (0−10 μg) was incubated with 1 mM $ONOO^-$ for 30 min. The level of liposome aggregation was determined and defined as the increase in absorbance at 540 nm over the initial value after a 30 min incubation. Data are means ± SE from five experiments.

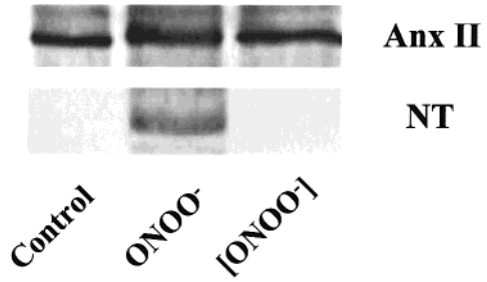

FIGURE 10: $ONOO^-$ causes nitration of AII_t in A549 cells. A549 cells were treated with no additions (control), 1.5 mM $ONOO^-$, or 1.5 mM decomposed $ONOO^-$ ([$ONOO^-$]) for 20 min. At the end of the incubation, cells were washed three times with PBS and lysed. Annexin II was immunoprecipitated with anti-annexin II antibodies and analyzed by SDS−PAGE and Western blotting using antibodies against nitrotyrosine (NT) or annexin II (Anx II). The blots were developed by ECL.

decomposed $ONOO^-$-treated cells. The result indicates that the nitration does occur in intact cells treated with $ONOO^-$.

DISCUSSION

Nitration of AII_t. This study provides the first evidence that AII_t is nitrated by $ONOO^-$. Calpain, a neutral Ca^{2+}-dependent protease, cleaves AII_t at Ala-29 to separate the regulatory N-terminus and the core domain of the protein (*19*). When calpain-cleaved AII_t was exposed to $ONOO^-$, no significant difference was detected in the amount of nitration between the protein with the N-terminus removed and the intact form. These results demonstrate that the core domain is nitrated and that cleavage of the N-terminal region does not significantly protect it from nitration. However, we cannot rule out the possibility that the N-terminus of AII_t is also nitrated.

There are 19 tyrosine residues in the p36 subunit and two in the p11 subunit. It was also demonstrated that the p11 subunit of AII_t is nitrated. The p11 subunit modulates the function of the AII_t protein by binding to the N-terminal region of the p36 subunit (*56*, *57*). Nitration of the p11

subunit would be likely to alter its binding characteristics to those of the p36 subunit. The idea that $ONOO^-$ might alter the interaction between p11 and p36 is demonstrated by the result showing the formation of dimers between subunits. Two clear bands are present at ~47 and 72 kDa on SDS−PAGE and a nitrotyrosine blot of AII_t treated with $ONOO^-$. The 47 kDa band can be detected by antibodies against p36 or p11, whereas the 72 kDa band can only be detected by antibodies against p36, indicating that they are the p11−p36 and p36−p36 dimers. These dimers are quite stable as indicated by their ability to resist SDS treatment, heat denaturation, and β-mercapthoethanol reduction.

Previously, it has been shown that $ONOO^-$ can oxidize tyrosine residues to tyrosyl radicals. Furthermore, these tyrosyl radicals have been shown to be stable for several minutes in biological systems. Hence, the formation of stable tyrosyl radicals and their subsequent reaction to form dityrosine bonds could represent the mechanism for $ONOO^-$-induced dimerization of AII_t. In a recent publication, investigators showed that treatment of activated epidermoid (A431) cells with $ONOO^-$ caused the formation of a homodimer of the activated EGF receptor (*58*). They presented indirect evidence that the dimers consisted of dityrosine cross-links. Most recently, dityrosine formation has been directly detected in $ONOO^-$-treated α-synuclein by HPLC (*59*).

Nitration and AII_t Activity. In general, nitration can disrupt protein function in three major ways. (i) It can cause conformational or stearic changes that disrupt the ability of the protein to bind substrates. Chemical nitration by TNM inactivates more than 100 mammalian proteins which depend on tyrosine residues for their activity (*60*). (ii) It can lead to improved degradation by proteases. For example, lysates of red blood cells dramatically increase the rate and extent of proteolytic cleavage of bovine serum albumin nitrated by $ONOO^-$ (*60*). (iii) Nitration can interfere with cellular signaling mediated by tyrosine kinases. For example, nitration of a single tyrosine residue by $ONOO^-$ of the cell cycle kinase cdc2 prevented tyrosine phosphorylation (*61*). A similar effect was also seen in endothelial cells exposed to $ONOO^-$ in which there was a 51% decrease in the level of phosphorylation by tyrosine kinases (*62*).

The in vitro results show that $ONOO^-$-mediated nitration of tyrosine residues on AII_t inhibits the protein's activity by a direct effect. The inhibition appears to be mediated by nitration from the $ONOO^-$ anion rather than by another free radical generated during the decomposition of $ONOO^-$ since neither the hydroxy radical scavenger, mannitol, nor the O_2^- scavenger, SOD, protects against $ONOO^-$-mediated inhibition of liposome aggregation by AII_t. The inhibition is prevented by urate, an $ONOO^-$ and OH^- scavenger.

A possible mechanism for the inhibition of AII_t lies in its quaternary structure. AII_t is a unique member of the annexin family since it exists as a monomer as well as a heterotetramer. The properties conferred on the protein by this structure display an especially high sensitivity to calcium. The N-terminal domain of AII_t plays an important regulatory function as illustrated by changes in the $K_d(Ca^{2+})$ for chromaffin granule aggregation under various conditions. The AII monomer (p36) can aggregate chromaffin granules with a $K_d(Ca^{2+})$ of ~1 mM. Removing the first 27 residues of the N-terminus by partial proteolysis reduces the $K_d(Ca^{2+})$ for chromaffin granule aggregation to ~141 μM. If the proteolysis continues to remove the first 43 residues of the amino terminus, the result is the further reduction of the $K_d(Ca^{2+})$ to ~28 μM (*5*). The effect of p36 binding to p11 and subsequent heterotetramer (AII_t) formation is even more dramatic. It results in a $K_d(Ca^{2+})$ for chromaffin granule aggregation of ~2 μM. These results show that the N-terminus inhibits chromaffin granule aggregation activity. The inhibition is removed by the noncovalent association between p11 and p36 or N-terminal cleavage. Therefore, nitration and subsequent disruption of the binding of two p11 subunits to two p36 subunits might be a critical factor with regard to inhibition of the phospholipid aggregating ability of the protein. However, it is unlikely that the formation of dimers in $ONOO^-$ -treated AII_t has a major effect on AII_t-mediated liposome aggregation unless they behave as an inhibitor because dimers only account for a small portion of the proteins. The physiological functions of those dimers remain to be determined.

Nitration of AI. When experiments in which the factors that are thought to lead to $ONOO^-$ formation in vivo (NO production and SOD reduction) are simulated, it is evident that $ONOO^-$-mediated nitration is a selective process in which not all tyrosine residues are potential targets (*63*−*65*). Several factors affect the efficiency of nitration by $ONOO^-$. Specifically, protein folding and other amino acids in the local environment seem to be important (*60*). The crystal structures for several annexin proteins have been determined, corroborating a family resemblance at the structural level. Given the homology of the annexin proteins, it is not surprising that AI is also nitrated by $ONOO^-$. However, the liposome aggregation by $ONOO^-$-treated AI was only slightly inhibited by less than 10%, as compared to ~80−100% for AII_t. This disparity could be explained by the different mechanisms of liposome aggregation mediated by AI and AII_t, since AII_t contains two p36 monomers and two p11 subunits whereas AI is a monomer.

Ca^{2+} and Membrane Effect on $ONOO^-$ Inhibition of AII_t. AII_t exists in at least three stable conformations. In the first conformation, the protein is located in the cytoplasm. The binding of calcium induces a second conformation which translocates annexin to negatively charge phospholipids. The association with the phospholipids induces a third conformation in which annexin can self-associate, dragging the attached phospholipids with them. One would expect that different conformations would have different vulnerabilities to inhibition by $ONOO^-$. We found that binding to calcium offered AII_t no protection from $ONOO^-$-mediated inhibition. When AII_t was preincuabted with Ca^{2+} and liposomes, the condition that allows binding of AII_t to membranes, AII_t-mediated lipsome aggregation was slightly inhibited (10%) by $ONOO^-$. Although this could be due to the hiding of nitration sites by the binding of AII_t to membranes or preaggregation, it may account for a portion of the observed effect, since $ONOO^-$ itself reacts with lipids (Figure 7) and only 40% inhibition was seen when AII_t was preincubated with liposomes in the presence of EGTA, the condition that does not allow binding of AII_t to membranes.

Nitration of AII_t in Cells. In vitro observations might not reflect what happens in vivo. An important question is whether nitration occurs in vivo. This question was addressed by immunoprecipitating annexin II from cell lysates of a type

II-like cell line, A549 cells, incubated with $ONOO^-$. The results show that AII_t was nitrated by $ONOO^-$ under these conditions but not when the cells were incubated with decomposed $ONOO^-$ or vehicle. A concern that one might have is that a high concentration of $ONOO^-$ was used in this experiment. However, $ONOO^-$ decomposes very fast. It has been estiamted that the bolus addition of 0.25 mM $ONOO^-$ is approximately equivalent to a steady-state level of 1.0 μM maintained for 7 min.

Another concern arises from the approach used in experiments on cultured cells; i.e., $ONOO^-$ is an anion, and one would assume it could not easily cross the membranes of cells. However, a recent study suggests that it indeed crosses the membrane with assistance from a transporter at a rate equivalent to that of water and approximately 400 times faster than O_2^- (*66*). It was shown that $ONOO^-$ can passively diffuse across the membranes of red blood cells when it is in its protonated form or it can be transported by an anion exchanger (*67*). In preliminary experiments, it was shown that cytosolic proteins are extensively nitrated after only treatment for 1 min with $ONOO^-$, providing indirect evidence that it can enter A549 cells (W. H. Rowan, III, and L. Liu, unpulished results).

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