Calcium Regulates S-Nitrosylation, Denitrosylation, and Activity of Tissue Transglutaminase †

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Calcium Regulates S-Nitrosylation, Denitrosylation, and Activity of Tissue Transglutaminase†

T. S. Lai,‡ A. Hausladen,‡ T. F. Slaughter,§ J. P. Eu,‡ J. S. Stamler,‡,|| and C. S. Greenberg*,‡,⊥

Department of Medicine, Pathology, and Anesthesiology, Duke University Medical Center, and Howard Hughes Medical Institute, Durham, North Carolina 27710

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ABSTRACT: Nitric oxide (NO) and related molecules play important roles in vascular biology. NO modifies proteins through nitrosylation of free cysteine residues, and such modifications are important in mediating NO's biologic activity. Tissue transglutaminase (tTG) is a sulfhydryl rich protein that is expressed by endothelial cells and secreted into the extracellular matrix (ECM) where it is bound to fibronectin. Tissue TG exhibits a Ca^{2+}-dependent transglutaminase activity (TGase) that cross-links proteins involved in wound healing, tissue remodeling, and ECM stabilization. Since tTG is in proximity to sites of NO production, has 18 free cysteine residues, and utilizes a cysteine for catalysis, we investigated the factors that regulated NO binding and tTG activity. We report that TGase activity is regulated by NO through a unique Ca^{2+}-dependent mechanism. Tissue TG can be poly-S-nitrosylated by the NO carrier, *S*-nitrosocysteine (CysNO). In the absence of Ca^{2+}, up to eight cysteines were nitrosylated without modifying TGase activity. In the presence of Ca^{2+}, up to 15 cysteines were found to be nitrosylated and this modification resulted in an inhibition of TGase activity. The addition of Ca^{2+} to nitrosylated tTG was able to trigger the release of NO groups (i.e. denitrosylation). tTG nitrosylated in the absence of Ca^{2+} was 6-fold more susceptible to inhibition by Mg-GTP. When endothelial cells in culture were incubated with tTG and stimulated to produce NO, the exogenous tTG was S-nitrosylated. Furthermore, S-nitrosylated tTG inhibited platelet aggregation induced by ADP. In conclusion, we provide evidence that Ca^{2+} regulates the S-nitrosylation and denitrosylation of tTG and thereby TGase activity. These data suggest a novel allosteric role for Ca^{2+} in regulating the inhibition of tTG by NO and a novel function for tTG in dispensing NO bioactivity.

Nitric oxide (NO) plays an important role in many different biological processes (*1*, *2*). NO is synthesized by a family of enzymes called NO synthases (NOS) (*1*, *2*). Once generated, NO is extremely susceptible to both oxidation and reduction, resulting in the formation of surrogates that retain NO bioactivity (*3*). Nitric oxide can modulate vasodilation, smooth muscle proliferation, platelet aggregation, and apoptosis (*4*−*6*). In addition to elevating intracellular levels of cGMP, NO covalently modifies cysteine residues in proteins via S-nitrosylation (*7*). An increasing number of intra- and extracellular proteins such as albumin, glyceraldehyde-3-phosphate dehydrogenase, caspase, hemoglobin, ryanodine receptor/calcium release channel, and P21ras are S-nitrosylated in vivo (*8*−*12*). In this study, we examined how Ca^{2+} and NO regulate tissue transglutaminase (tTG) function, an enzyme that is highly expressed by endothelial cells and found in the extracellular matrix.

Tissue TG exhibits two distinct enzymatic activities (*13*−*15*). The first, a Ca^{2+}-dependent transglutaminase activity (TGase) involved in protein cross-linking, requires active site Cys277. The active site Cys277 is exposed in the presence of Ca^{2+} (*13*, *14*). TGase catalyzes the covalent modification of proteins by the formation of γ-glutamyl-ϵ-lysine bonds between proteins or polyamines (*13*, *14*). Tissue TG forms a Ca^{2+}-dependent thioester bond at Cys277 with select protein-bound glutamines, releasing ammonia (*13*, *14*). Then a reactive enzyme−substrate intermediate binds a primary amine group of either a lysine within a protein or a polyamine (*13*, *14*). The final reaction product contains an isopeptide bond that stabilizes inter- and intramolecular protein structure or generates a protein−polyamine conjugate (*13*, *14*). The TGase activity plays a role in wound healing, apoptosis, cell morphology, cell adhesion, and tumor growth and metastasis

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* To whom correspondence should be addressed: Duke University Medical Center, Box 2603, Durham, NC 27710. Fax: (919) 684-4670. E-mail: green032@mc.duke.edu.

‡ Department of Medicine, Duke University Medical Center.

§ Department of Anesthesiology, Duke University Medical Center.

|| Howard Hughes Medical Institute.

⊥ Department of Pathology, Duke University Medical Center.

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(*13*, *14*, *16*, *17*). The second activity of tTG is GTP binding and hydrolysis, which is involved in transmembrane signal transduction and plays a role in cell cycle progression (*15*, *17*−*19*).

Tissue TG is expressed in all tissues with the highest expression level occurring in endothelial cells (*20*). Recently, both tTG and factor XIII were shown to be targets for S-nitrosylation (*21*, *22*). Nitrosylation of the tTG was correlated with inhibition of TGase activity and a decrease in the level of apoptosis (*21*). Inhibition of factor XIIIa activity was shown to occur in vitro after exposure to the NO donor, and this reaction enhances the fibrinolytic process (*22*). Since tTG is in proximity to sites of NO production, has 18 free cysteine residues, and utilizes Cys277 for catalysis, we investigated whether Ca^{2+} and other cofactors could regulate NO binding and release, and the function of tTG.

In this paper, we report that tTG binds and releases NO groups through a Ca^{2+}-dependent mechanism. Tissue TG can be polynitrosylated by the NO carrier, *S*-nitrosocysteine (CysNO), and by activated endothelial cells. Nitrosylation of tTG inhibits TGase activity in a Ca^{2+}-dependent manner. In addition, Ca^{2+} triggers the release of NO from nitrosylated tTG. The polynitrosylated tTG is biologically active and can inhibit ADP-induced platelet aggregation. The potential physiologic significance of this reaction in vascular biology is discussed.

MATERIALS AND METHODS

Materials. Sodium salts of ATP and GTP were purchased from Sigma (St. Louis, MO). [^{3}H]Putrescine dihydrochloride (35.5 Ci/mmol) and [γ-^{32}P]GTP or [γ-^{32}P]ATP (30 Ci/mmol) were purchased from New England Nuclear (Boston, MA). The $MgCl_2$ stock solution (1 M) was purchased from Sigma. The monoclonal antibody against guinea pig liver tTG (CUB 7401) was kindly provided by P. Birckbichler (*23*). All ATP and GTP solutions were prepared in 50 mM Tris-HCl (pH 7.0) and stored in aliquots at −80 °C. L-Cysteine (hydrochloride monohydrate) and reduced glutathione were purchased from Sigma. All other reagents used in this investigation were purchased from Sigma unless stated otherwise.

Purification of Recombinant tTG. The glutathione *S*-transferase (GST) fusion protein was purified as previously described (*24*). During the entire purification, 1 mM DTT was included in all solutions to maintain cysteine residues in the reduced state (*24*). In this study, the purified GST−tTG fusion protein was further dialyzed extensively against buffer containing 50 mM Tris-acetate (pH 7.5), 10% glycerol, and 1 mM EDTA to remove DTT. Protein concentrations were determined using the extinction coefficient for the GST−tTG fusion protein (see below).

Preparation of S-Nitrosocysteine. Solutions of *S*-nitrosocysteine (CysNO) were freshly prepared by mixing an equal volume of 200 mM L-cysteine in a 1 M HCl/0.1 mM EDTA mixture and 200 mM $NaNO_2$ in water at room temperature (*25*). The solutions were neutralized by adding equal volumes of 200 mM Tris-acetate (pH 7.5), 0.1 mM EDTA, and 0.5 M NaOH and were used immediately for S-nitrosylation of tTG as described below.

Nitrosylation of Recombinant tTG. The nitrosylation of the GST−tTG fusion protein was performed by incubating the protein in 0.1 mM Tris-acetate (pH 7.5) and 0.1 mM EDTA with the freshly prepared CysNO with ratios from 1:50 to 1:1000 at room temperature for 10 min. After nitrosylation, free CysNO was removed by dialyzing against 4 × 1 L of buffer containing 50 mM Tris-acetate (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, and 10% glycerol at 4 °C. A control experiment was also performed by dialyzing the same concentration of *S*-nitrosocysteine (without the GST−tTG fusion protein). We found no *S*-nitrosocysteine inside the dialysis bag after dialysis using the chemiluminescence method described in the following section.

Transglutaminase Assay of tTG. Transglutaminase activity (TGase) was determined by quantitating the incorporation of [^{3}H]putrescine (*26*) or 5-biotinamidopentylamine (BP) into *N*,*N*′-dimethylcasein as previously described (*27*). For the inhibition of TGase by CysNO, the purified GST−tTG fusion protein (1 μM) was incubated with 0−1 mM CysNO and dialyzed as described above. After dialysis, the TGase activity of the S-nitrosylated GST−tTG fusion protein was measured in the presence of 1 mM $CaCl_2$ using the BP incorporation assay as described previously (*27*). For the kinetic analysis, recombinant tTG was nitrosylated with 500 μM CysNO in the presence of Ca^{2+}, and free CysNOs were removed by dialysis. To determine the K_m for the glutamine substrate, the [^{3}H]putrescine incorporation was used and the concentration of *N*,*N*′-dimethylcasein was varied from 1.1 to 24.4 μM. To determine the K_m for the primary amine substrate, the BP incorporation assay was used and the concentration of BP was varied from 6 to 500 μM. Kinetic analysis of the data was performed by the Eadie−Hofstee or Lineweaver−Burk method (*28*), and the results were representative of at least two independent duplicate experiments.

Measurement of the S-Nitroso Content of Recombinant tTG. The *S*-nitroso content was determined by a photolysis/chemiluminescence detection system (Nitrolite and TEA model 543 Analyzer, Thermedics, Woburn, MA) as described previously (*9*). Briefly, nitrosylated samples were injected into a water stream (1 mL/min) combined with He as a carrier gas, and passed through a glass coil illuminated by a mercury vapor lamp. NO released by photolysis from nitrosylated proteins was carried by the He gas, and the signal was detected by a chemiluminescence nitrogen analyzer (TEA model 543). *S*-Nitrosoglutathione (GSNO) was used as a standard, and the signals were linear in the range of 2.7−90 pmol. The *S*-nitroso content of proteins was measured as the mercury-displaceable NO content of duplicate samples (*9*). The *S*-nitroso contents of tTG were calculated by subtracting the *S*-nitroso content of GST from that of the GST−tTG fusion protein.

In the NO release experiment, Ca^{2+} was added to the samples and incubated on ice for at least 5 min before injection into the instrument for SNO analysis.

S-Nitrosylation of tTG by NO Released from Endothelial Cells. Bovine aortic endothelial cells (passage 2, Hyclone Laboratories) were grown on T-150 flasks and maintained in CO_2 and a moisture incubator until they were ready for experiments. Right before the experiments, cells were washed with PBS and trypsinized. Trypsized cells were washed three times with PBS to remove traces of medium and trypsin and resuspended in PBS. Recombinant tTG (1 μM) was incubated with cells (∼3 × 10^7 cells in total) in a glass cylinder (1 cm

in diameter) with 1 μM calcium ionphore (A23187) in a final volume of 200 μL. After incubation at room temperature for 10 min, recombinant tTG was recovered from the mixture by low-speed centrifugation and stored at −80 °C until analysis was carried out. The *S*-nitroso content of recombinant tTG was analyzed using the chemiluminecence method as described above.

[γ-^{32}P]GTP or [γ-^{32}P]ATP Hydrolysis Assay. The assay was performed as described with some modifications (*19*). For the determination of the effects of nitrosylation on GTP (or ATP) hydrolysis of tTG, the GST−tTG fusion protein (1 μM) was nitrosylated with 0−1 mM CysNO at room temperature for 10 min and dialyzed to remove free CysNO as described above. For the hydrolysis reaction, the reaction mixture (50 μL) contained 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 2 μCi of [γ-^{32}P]G(A)TP (30 Ci/mmol), 250 μM unlabeled GTP (or ATP), 2 mM Mg^{2+}, and 50 pmol of nitrosylated tTG. The reactions were initiated by addition of the nitrosylated GST−tTG fusion protein and allowed to proceed at 37 °C for 30 min.

Trypsin Proteolysis of Recombinant tTG for Examining Conformational Changes. Control and nitrosylated recombinant tTG (1 μg) were incubated with 0.1 μg of trypsin (TPCK-treated, HPLC-purified, Calbiochem, La Jolla, CA) in the presence of either 5 mM $CaCl_2$, Mg-GTP (5 μM), or Mg-ATP (1000 μM) and incubated at 37 °C for 30 min. The reaction was stopped by adding SDS−PAGE loading buffer. Samples were analyzed by SDS−PAGE and immunoblotting using monoclonal antibody CUB7401.

Circular Dichroism. CD studies were performed on an Aviv circular dichroism spectrometer, model 62 DS. Scanning was performed in a 0.1 cm path length cuvette using recombinant tTG (or nitrosylated tTG) dialyzed into 10 mM sodium phosphate (pH 7.4) and 0.1 mM EDTA. Five scans were averaged for each measurement. The buffer containing 10 mM sodium phosphate (pH 7.4) and 0.1 mM EDTA did not interfere with the CD spectra.

Platelet Aggregation. Blood was drawn from normal individuals, and platelet rich plasma was isolated by centrifugation at 150*g* for 10 min at room temperature in a tabletop centrifuge (Beckman, model GS-6) and used within 3 h. Cleaved tTG was prepared from the GST−tTG fusion protein using factor Xa as described previously (*19*). Recombinant tTG (1 μM) was S-nitrosylated by CysNO (500 μM) and dialyzed to remove free CysNO as described above. Different concentrations of SNO-tTG were applied to a test tube containing 450 μL of platelet rich plasma (PRP) which was held in an aggregometer (BIO/DATA Corp.) and incubated for 5 min at 37 °C. The degree of platelet aggregation was measured after 5 μM ADP was added. The maximum level of platelet aggregation using 5 μM ADP was used as the 100% standard.

Protein Concentration Determination. The extinction coefficients of the GST−tTG fusion protein and GST at 280 nm were determined by the protein microsequencing facility at Duke University and are 30.8 ($A_{1\%}$) for the GST−tTG fusion protein and 16.6 ($A_{1\%}$) for GST.

Molecular Modeling. The homologous three-dimensional model of tTG was constructed on the basis of the three-dimensional coordinates of blood coagulation factor XIII A chain (Brookhaven Protein Data Bank entry 1ggt) and was also published by Casadio et al. (*29*). The three-dimensional coordinates are available from the Brookhaven Protein Data Bank (entry 1FAU).

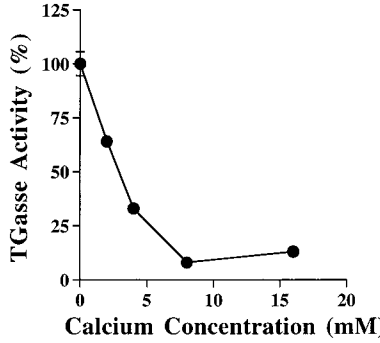

FIGURE 1: Effects of Ca^{2+} on inhibition of TGase activity by CysNO. Recombinant tTG (1 μM) was nitrosylated with 500 μM CysNO in buffer containing 100 mM Tris-acetate (pH 7.5), 50 mM NaCl, and 0.1 mM EDTA at room temperature for 10 min in the presence of 0−16 mM calcium ions. Unbound CysNOs was removed by dialysis as described in Materials and Methods. After dialysis, tTG was diluted to a concentration of 2 μg/mL and asssayed for TGase activity using the BP incorporation assay as described in Materials and Methods.

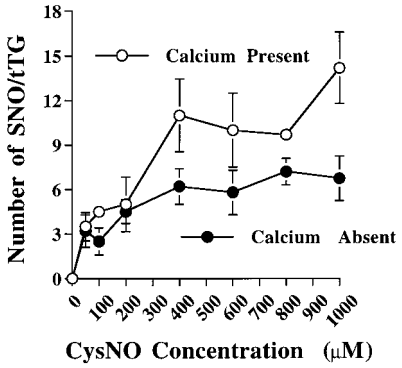

FIGURE 2: *S*-Nitrosothiol contents of tTG after being treated with 0−1 mM CysNO. Recombinant tTG (1 μM) was nitrosylated with 0−1 mM CysNO in the absence (●) or presence of 8 mM Ca^{2+} (○), and free CysNOs were removed by dialysis. After dialysis, the *S*-nitrosothiol contents were determined using the chemiluminescence method as described in Materials and Methods. Results are the averages of three independent experiments.

RESULTS

Effects of S-Nitrosylation on the Enzymatic Activities of tTG. In the absence of calcium ions, 1 mM CysNO had no effect on the TGase activity. In contrast, there was a Ca^{2+} concentration-dependent inhibition of TGase by CysNO (Figure 1). The inhibition of TGase activity by CysNO was reversed by DTT (1 mM). In contrast, the GTP/ATPase activity of tTG was not affected by CysNO in the presence or absence of calcium. Similar findings were found when *S*-nitroso glutathione (GSNO) was used as the NO donor. However, the time required to inhibit tTG with GSNO was longer. All subsequent experiments were therefore performed with CysNO.

Quantitation of the S-Nitrosothiol Content of tTG. When tTG was nitrosylated in the absence of calcium ions, the *S*-nitrosothiol content of tTG peaked at 7.2 ± 0.9 mol/mol of tTG (Figure 2). When nitrosylation was performed in the presence of calcium ions, the maximal *S*-nitrosothiol content of tTG increased to 14.5 ± 2 mol/mol of tTG (Figure 2).

Effects of Adding Calcium Ions after Nitrosylation. When tTG was nitrosylated in the absence of Ca^{2+}, there was no

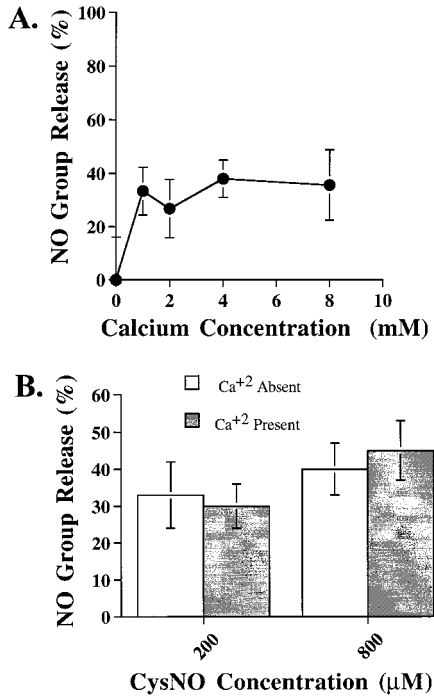

FIGURE 3: Nitrosylated tTG releases nitric oxide in the presence of calcium ions. (A) Recombinant tTG (1 μM) was nitrosylated with 200 μM CysNO, and free CysNOs was removed by dialysis as described in Materials and Methods. Ca^{2+} (1−8 mM) was added to the dialyzed samples and immediately analyzed for *S*-nitrosothiol content as described in Materials and Methods. (B) Recombinant tTG (1 μM) was nitrosylated with 200 or 800 μM CysNO in the presence or absence of Ca^{2+}. After removal of free CysNO by dialysis, samples were analyzed for *S*-nitrosothiol content after 1 mM Ca^{2+} was added. All determinations have *P* values of <0.05.

inhibition of TGase or GTP/ATPase activity when Ca^{2+} was subsequently added. However, the addition of ≥1 mM Ca^{2+} to the nitrosylated tTG resulted in the release of NO groups (Figure 3A,B). Additional experiments demonstrated that other divalent ions, including Mg^{2+} and Sr^{2+} at concentrations of ≥1 mM, also caused NO groups release from nitrosylated tTG (data not shown). We also examined whether there is any difference in the release of NO groups when tTG was nitrosylated with a higher concentration of CysNO (800 μM) in the presence or absence of Ca^{2+}. We added 1 mM Ca^{2+} immediately before analyzing the *S*-nitrosothiol content of nitrosylated tTG. When nitrosylation was performed in the absence of Ca^{2+}, we found the level of release of SNO was increased from 33 ± 9 to 40 ± 7% for 200 and 800 μM CysNO, respectively (Figure 3B). The SNO content (moles of SNO per mole of tTG) was decreased from 4.5 ± 0.8 to 3 ± 0.5 for 200 μM CysNO and from 7.2 ± 0.9 to 4.3 ± 0.4 for 800 μM CysNO (Figure 3). When tTG was nitrosylated in the presence of Ca^{2+}, we found the level of release of SNO was increased from 30 ± 6 to 45 ± 8% for 200 and 800 μM CysNO, respectively (Figure 3B). The SNO content was decreased from 5 ± 1.9 to 3.5 ± 1.5 for 200 μM CysNO and from 9.7 ± 0.3 to 5.3 ± 0.1 for 800 μM CysNO.

Effects of S-Nitrosylation on GTP (or ATP) Inhibition of TGase Activity. We found nitrosylated tTG was 6-fold more susceptible to inhibition by Mg-GTP. The IC_{50} for inhibition of TGase activity was reduced from 6 to 1 μM Mg-GTP (Figure 4). In contrast, nitrosylation had no effect on the inhibition of TGase activity by Mg-ATP.

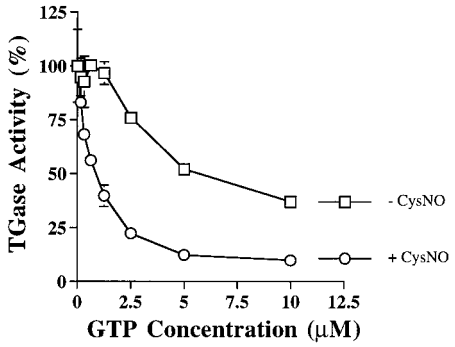

FIGURE 4: Nitrosylation of tTG enhances the inhibition by GTP. Recombinant tTG (1 μM) was nitrosylated in the presence of 200 μM CysNO, and free CysNOs was removed by dialysis as described in Materials and Methods. After dialysis, nitrosylated tTG (2 μg/mL) was analyzed for TGase activity by the BP incorporation assay in the presence of 0−10 μM GTP (○). Recombinant tTG that was not nitrosylated was used as a control (□).

Effects of CysNO on the Kinetic Properties of tTG. To investigate whether nitrosylation by CysNO had any effect on the kinetic properties of tTG, we determined the K_M for the primary amine substrate, biotinylated pentylamine (BP), and the glutamylamine substrate, *N*,*N*′-dimethylcasein. When recombinant tTG was nitrosylated with 500 μM CysNO in the presence of 5 mM Ca^{2+} and free CysNO was removed by dialysis, we found the $K_{M,BP}$ and $K_{M,N,N'-dimethylcasein}$ for control tTG were 12.1 ± 5 μM (V_{max} = 158 ± 20 mOD/min) and 1.4 ± 1 μM (V_{max} = 43 ± 6 pmol of putrescine/40 min), respectively. The $K_{M,BP}$ and $K_{M,N,N'-dimethylcasein}$ for nitrosylated tTG were 15 ± 6 μM (V_{max} = 50 ± 8 mOD/min) and 2 ± 1.5 μM (V_{max} = 12 ± 4 pmol of putrescine/40 min).

Effects of CysNO on the Sphingosylphosphocholine (Lyso-SM)-Dependent Reduction in Calcium Ion Concentration. Lyso-SM can reduce the calcium ion concentration required to activate tTG (*30*). We found nitrosylation performed at 0−1 mM CysNO had no effect on lyso-SM, reducing the calcium ion requirement for activating the TGase activity (*30*).

Effects of Nitrosylated tTG on ADP-Induced Platelet Aggregation. We investigated whether S-nitrosylated tTG could serve as a NO donor to inhibit platelet aggregation. Tissue TG nitrosylated in the absence of Ca^{2+} inhibited ADP-induced platelet aggregation in a concentration-dependent manner (Figure 5). At 50 nM nitrosylated tTG, platelet aggregation induced by 5 μM ADP was inhibited by 50% (Figure 5).

Effects of Nitrosylation on the Conformation of tTG. To test whether there was any conformational change induced by S-nitrosylation, recombinant tTG was treated with 0−1 mM CysNO in the presence or absence of Ca^{2+}. After removal of free CysNO, trypsin digestion was performed. We found that S-nitrosylation had no effects on the patterns of trypsin digestion performed in the presence of Ca^{2+} (5 mM), ATP (1 mM), or GTP (5 μM) (*19*).

Circular dichroism (CD) was also performed to examine whether there was conformational change after S-nitrosylation. Recombinant tTG was nitrosylated with 500 μM and 1 mM CysNO in the presence of Ca^{2+} since TGase activity was significantly inhibited at these concentrations. After removal of free CysNO, control and nitrosylated tTGs were

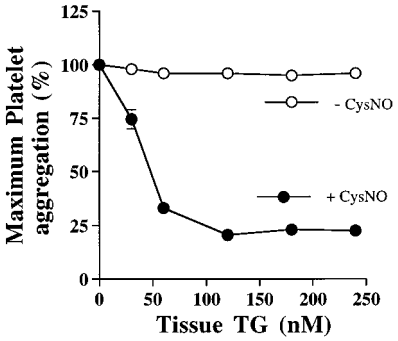

FIGURE 5: Nitrosylation of tTG inhibits platelet aggregation. Recombinant tTG (1 μM) was nitrosylated in the presence of 500 μM CysNO, and free CysNOs were removed by dialysis as described in Materials and Methods. After dialysis, different concentrations of nitrosylated tTG were added to platelet rich plasma (PRP) and incubated for 5 min at 37 °C before 5 μM ADP was added (●). Maximum platelet aggregation using 5 μM ADP was used as the 100% standard.

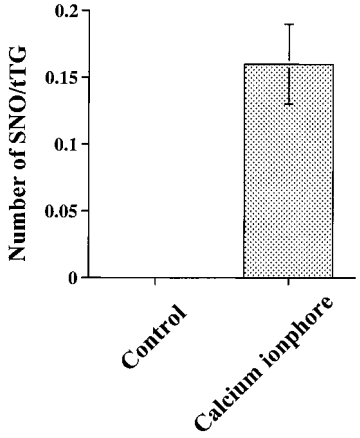

FIGURE 6: Recombinant tTG is nitrosylated by NO produced from activated endothelium. Recombinant tTG (1 μM) was added to bovine aortic endothelial cells, and 1 μM calcium ionphore (A23187) was added as described in Materials and Methods. Recombinant tTG was recovered and analyzed for *S*-nitroso content. tTG recovered from untreated cells was used as a control.

subjected to CD spectrum analysis. The CD spectrum was recorded at wavelengths from 290 to 190 nm. We found CD spectra of control and nitrosylated tTG are almost superimposable, demonstrating that there was no unfolding of the proteins after nitrosylation.

S-Nitrosylation of Recombinant tTG by Activated Endothelium. To demonstrate that tTG can be nitrosylated in situ, recombinant tTG was incubated with bovine aortic endothelial cells in the presence of 1 μM calcium ionphore (A23187) as described in Materials and Methods. We found there is a dramatic increase in the SNO content of recombinant tTG treated with calcium ionphore (Figure 6). The results indicate that 16 ± 3% of the recombinant tTG was S-nitrosylated which was equivalent to 0.16 ± 0.03 mol of SNO/mol of tTG.

DISCUSSION

The TGase and GTP/ATPase activities of tTG are implicated in many different physiologic processes (*13*, *14*). The regulation of these activities is not well defined but is important for understanding the role of the tTG in cell biology. Recently, two transglutaminases, guinea pig liver tTG and blood coagulation factor XIII A chains, were reported to be inhibited by NO in vitro (*21*, *22*). However, the biochemical mechanism(s) regulating this process was not investigated. We provide evidence that (1) calcium ions regulate S-nitrosylation and release of NO groups from tTG, (2) nitrosylation in the presence of Ca^{2+} results in inhibition of TGase activity but not GTP/ATP hydrolysis activity, (3) Ca^{2+} causes partial NO release, and (4) S-nitrosylated tTG is more sensitive to inhibition by Mg-GTP. Furthermore, we established that nitrosylated tTG can inhibit platelet aggregation and that endothelial cell NO synthase can cause nitrosylation of tTG.

S-Nitrosylation reactions can be mediated through NO carriers such as nitrosothiols (SNO) (*7*), NO complexes with transition metals (*7*), and N_2O_3, or a direct reaction between NO and thiols in the presence of electron acceptors (*7*). Currently, it is still unclear which pathway is the major mechanism for nitrosylation in vivo. It is believed that the NO carrier(s) targets the free cysteine residues within specific local environment. Cysteine residues that are located on the exposed surface of proteins are obvious targets for nitrosylation. However, it has been shown recently that hydrophobic protein compartments are also candidate sites (*31*, *32*). The size and geometry of these interior compartments are critical for the generation of N_2O_3, and this may result in a more specific nitrosylation (*31*). Examination of the three-dimensional molecular model of tTG reveals 18 free cysteine residues with 9 cysteines being surface-exposed (*29*, *33*, *34*). The surface-exposed cysteines include Cys10, Cys27, Cys98, Cys143, Cys230, Cys269, Cys524, Cys545, and Cys620. Active site Cys277 is buried and only 2.9 Å away from Cys336, 8.6 Å away from Cys285, and <10 Å away from Cys370 and Cys371. In the absence of Ca^{2+}, we found up to seven cysteine residues were nitrosylated (Figure 2). It is likely that the surface-exposed cysteine residues are the initial targets for nitrosylation, although other cysteine residues are not excluded. S-Nitrosylation of these 7 cysteine residues did not affect the TGase activity, indicating that the critical cysteine residue(s), including active site Cys277, was not modified. The cryptic nature of the active site Cys277 accounts for the lack of inhibition of TGase activity by CysNO in the absence of Ca^{2+} (*35*). However, when tTG was nitrosylated in the presence of calcium ions, we found up to 15 cysteines were nitrosylated (Figure 2). Moreover, S-nitrosylation of 9 cysteines was sufficient to inhibit enzyme activity, suggesting that modification of only 1 or 2 cysteines is necessary and sufficient for this effect (Figure 1, 2). Tissue TG undergoes a change in conformation in the presence of calcium ions that can be detected by a change in susceptibility to proteolysis in the C-terminus in the vicinity of Cys524 (*29*). Using small-angle neutron and X-ray scattering, the gyration radius of tTG (30 Å) increases in the presence of Ca^{2+} (39 Å) (*29*). The increase in gyration radius (9 Å increase) causes massive widening of the protein, and more cysteine residues are likely to be exposed. The active site Cys277 is exposed in the presence of Ca^{2+} (*34*) and also became a target for nitrosylation. In addition, the nitrosylation on Cys285, Cys336, Cys370, and Cys371 could influence the catalysis efficiency since they are less than 10 Å from Cys277.

The fact that nitrosylation did not significantly change the K_M values for primary amine (BP) and glutamyl (*N*,*N*′-

dimethylcasein) substrates indicates that S-nitrosylation did not modify the affinity of tTG for its substrates. The major kinetic effect of S-nitrosylation was the effect on the V_{max} value. Trypsin digestion experiments and CD spectra analysis also indicated that nitrosylation did not cause unfolding or major conformational changes of the protein. Since the formation of the isopeptide bond requires the proper alignment among the catalytic triad (*13*), primary amine, and glutamyl substrates, it is possible that nitrosylation causes inefficient transfer of glutamyl substrate to the primary amine substrate for the generation of the isopeptide bond (*36*). Alternatively, the inhibition of TGase activity by CysNOs could be due to the S-nitrosylation of active site Cys277, or the cysteine residues in the vicinity that are involved in the forming of the catalytic triad.

Protein modification mediated by an NO carrier could be due to S-nitrosylation or thiol oxidation with formation of a mixed disulfide bond, sulfenic acid, or sulfinic acid (*37*). The inhibition of TGase activity was not due to disulfide bond formation since nonreducing SDS gel electrophoresis of nitrosylated tTG followed by immunoblotting indicated that there was no change in mobility. The formation of sulfinic acid was also excluded since the inhibition was completely reversible with dithiothreitol.

The ability of tTG to bind NO groups indicates that NO can form a stable complex with tTG. The conformational change induced by Ca^{2+} might destabilize some of the *S*-nitrosothiols and caused the release of NO from nitrosylated tTG. We can only speculate that the Ca^{2+}-induced allosteric modification of tTG triggers the release of NO groups by analogy to the conformation-induced release from SNO-hemoglobin by allosteric effectors (*11*). When nitrosylation was performed in the absence of Ca^{2+}, the amount of NO that was released increased as a function of the degree of protein S-nitrosylation (Figure 3). We therefore anticipate that the cysteines dispensing NO upon the binding of calcium are different from those that are S-nitrosylated in its presence. That is, Ca^{+2}-induced S-nitrosylation of a group of cysteine residues distinct from those that release NO.

Modification of tTG by NO donors may have physiological relevance since the protein can bind a substantial number of NO molecules. We found immunoprecipitated tTG from rat kidney tissue was S-nitrosylated, indicating that tTG was a target for NO modification in vivo (C. S. Greenberg, unpublished observations). In addition, when recombinant tTG was incubated with bovine aortic endothelial cells, we found it was S-nitrosylated when NO production was induced by calcium ionphore. These observations demonstrate that tTG both in vivo and in situ is a target for NO modification.

NO may provide a novel mechanism for regulating the activity of tTG in vivo. When Ca^{2+} levels are low such as in the intracellular compartment, the tTG is nitrosylated and is made more sensitive to the inhibitory effects of Mg-GTP (Figure 4). The nitrosylated tTG acts as an NO carrier (or NO buffer) and releases NO when calcium levels increase. TG releases NO when tTG is secreted or after cellular injury releases the protein, exposing it to high extracellular calcium levels. Furthermore, when a significant amount of nitrosylated tTG accumulated in the vessel wall, tTG could store and release NO to modify platelet aggregation. Calcium has been previously shown to allosterically control the level of S-nitrosylation of the ryanodine receptor, however, the allosteric mechanism whereby calcium ions cause NO release from SNO-tTG is novel and reminiscent of NO release from hemoglobin (*11*). In hemoglobin an oxygen-dependent allosteric mechanism regulates the binding and release of the NO group (*11*). But there are 18 free thiols in tTG several of which can serve as a buffer to bind and release NO groups.

We previously reported that Mg-GTP is a potent inhibitor of tTG activity (*19*). In this study, we found that tTG nitrosylated in the absence of Ca^{2+} is 6-fold more susceptible to GTP inhibition. The affinity of nitrosylated tTG for GTP could be increased by NO and further studies are needed to define the mechanism. Inside the cells, the concentration of Mg-GTP is 100−150 μM. The high levels of NO generated in vivo could function to keep the TGase activity of tTG suppressed. The combined actions of NO and Mg-GTP would function to ensure that intracellular tTG remains inactive.

The mechanism by which a polynitrosylated protein transmits signals through the membrane to increase intracellular cGMP levels and inhibit platelet aggregation was recently reviewed by Stamler et al. (*38*). In this study, nitrosylated tTG which is located in several compartments within the vessel walls releases NO and inhibits platelet aggregation. This represents the first example of an extracellular matrix protein synthesized by the endothelium that can bind and release NO to inhibit platelet function. Additional studies are in progress to determine to what extent nitrosylated tTG functions to inhibit platelets at the vessel wall in vivo.

In conclusion, we report that tTG, a SH rich protein present in endothelial cells and the extracellular matrix, can bind and release NO groups in a calcium-dependent reaction. The nitrosylated tTG can also release NO to inhibit platelet aggregation. These results suggest that the nitrosylation of tTG may play an important role in modulating NO's function in the vessel wall.

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