Transglutaminase 2 (TGase 2) expression is increased in inflammatory diseases. We demonstrated previously that inhibitors of TGase 2 reduce nitric oxide (NO) generation in a lipopolysaccharide (LPS)-treated microglial cell line. However, the precise mechanism by which TGase 2 promotes inflammation remains unclear. We found that TGase 2 activates the transcriptional activator nuclear factor (NF)-κB and thereby enhances LPS-induced expression of inducible nitric-oxide synthase. TGase 2 activates NF-κB via a novel pathway. Rather than stimulating phosphorylation and degradation of the inhibitory subunit α of NF-κB (I-κBα), TGase2 induces its polymerization. This polymerization results in dissociation of NF-κB and its translocation to the nucleus, where it is capable of up-regulating a host of inflammatory genes, including inducible nitric-oxide synthase and tumor necrosis factor α (TNF-α). Indeed, TGase inhibitors prevent depletion of monomeric I-κBα in the cytosol of cells overexpressing TGase 2. In an LPS-induced rat brain injury model, TGase inhibitors significantly reduced TNF-α synthesis. The findings are consistent with a model in which LPS-induced NF-κB activation is the result of phosphorylation of I-κBα by I-κB kinase as well as I-κBα polymerization by TGase 2. Safe and stable TGase2 inhibitors may be effective agents in diseases associated with inflammation.

Transglutaminase 2 (TGase 2; E.C. 2.3.2.13, protein-glutaminyltransferase) belongs to a family of Ca^{2+}-dependent enzymes that catalyzes N^{2}-(γ-L-glutamyl)-L-lysine isopeptide bond formation between peptide bound lysine and glutamine residues (1). N^{2}-(γ-L-Glutamyl)-L-lysine cross-linking stabilizes intra- and extracellular proteins as macromolecular assemblies that are used for a variety of essential physiological purposes, such as barrier function in epithelia, apoptosis, and extracellular matrix formation (2). TGase 2 is normally expressed at low levels in many different tissues and inappropriately activated in a variety of pathological conditions (3). However, the role TGase 2 specifically plays in disease etiology is unclear.

Autoimmune diseases are strongly associated with aberrant activation of macrophage and T cells, which cause serious inflammation. Abnormal increase of TGase 2 expression in autoimmune inflammatory myopathies and celiac disease has been reported (4–6). TGase 2 is increased in autoimmune diseases as a result of macrophage activation (7–9). The increase of TGase 2 expression seems to be closely associated with autoantibody formation. Autoantibodies against TGase 2 are present in the blood of patients with such autoimmune diseases as celiac disease (10), dermatitis herpetiformis (11), type 1 diabetes (12), lupus (13), and rheumatoid arthritis (14). TGase 2 has been detected in the synovial fluid of patients with arthritis (15) and in the serum and cerebral spinal fluid of patients with amyotrophic lateral sclerosis (16). These reports suggest that the inappropriate expression and/or presentation of TGase 2 to T cells might contribute to these diseases in genetically predisposed individuals.

Activation of glia, a process termed reactive gliosis, has been observed during the pathogenesis of neurodegenerative diseases. A hallmark of brain inflammation is the activation of microglia cells that produce neurotoxic factors such as nitric oxide (NO) (17) and TNF-α (18). Synthesis and release of these factors constitute a part of the innate immunity that enables the host to destroy invading pathogens. However, excessive production and accumulation of NO seems to contribute neurodegeneration (19). TGase 2 expression in rat brain astrocytes is induced by glutamate (20) or by the inflammation-associated cytokines such as interleukin-1β and TNF-α (21). Microarray analysis in a monkey model of neuro-AIDS has recently revealed that TGase 2 expression is specifically increased in the infected brain (22). Immunohistochemical staining in the infected brain shows clear increase of TGase 2 in the cells with astrocytic morphology. Multiple factors must contribute to the activation of TGase 2 in oxidative stress and in the elevation of intracellular calcium. This suggests that induced-TGase 2 in the activated astroglial cells may participate in the pathogenesis of neurodegenerative diseases. Neurodegenerative diseases, such as Parkinson’s disease (23, 24) and Alzheimer’s disease (25, 26), and neuro-AIDS brains (22) are closely associated with increased brain TGase 2 expression. Inflammatory markers also occur in Parkinson’s disease (27), Alzheimer’s disease (28), and AIDS dementia (17).

We demonstrated previously that the recombinant peptide R2, which had dual inhibitory effects on purified TGase 2 and soluble phospholipase A2, reversed the inflammation of allergic conjunctivitis to ragweed in a guinea pig model (29). Recombinant peptides were constructed with the sequences from pro-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to advertise payment of page charges. This article must therefore be hereby marked.

† To whom correspondence should be addressed. Tel.: 914-597-2500, ext. 3041; Fax: 914-597-2757; E-mail: tgase@hanmail.net.

‡ The abbreviations used are: TGase 2, transglutaminase 2; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; iNOS, inducible nitric-oxide synthase; NMAA, N_{2}-(γ-L-glutamyl)-L-lysine; RT, reverse transcription; LDH, lactate dehydrogenase; IKK, I-κB kinase; I-κBα, inhibitory subunit α of nuclear factor-κB; NIK, nuclear factor-κB inducing kinase; SEAP, secreted alkaline phosphatase; RA, retinoic acid.
elafin (for inhibition of TGase 2), and antiflammagens (for inhibition of soluble phospholipase A2). It is interesting that we found that the only pro-elafin sequence, E2 (a part of elafin, a TGase inhibitor), itself also has dramatic anti-inflammatory effects (29). This strongly suggests that TGase activity may play a key role in macrophase activation resulting from inflammatory stress. We observed recently that TGase 2 expression is increased by LPS treatment in BV-2 microglia, and NO release is dramatically reduced by TGase inhibitors (30). During the LPS-induced microglia activation, TGase activity is increased about 5-fold in microglia after 24-h exposure to LPS in a time-dependent manner (30). The increase of NO synthesis is correlated with increase of TGase 2 expression. Furthermore, we observed that transient transfection of TGase 2 in BV-2 microglia increases NF-κB activity (30). This suggests that TGase 2 may aggravate inflammation by activating the NF-κB cascade in microglia. To test this hypothesis, we determined the identity of the major in vivo target of TGase 2 in the NF-κB cascade. We also tested whether TGase inhibition might be effective at reducing NF-κB activation in the LPS-induced rat brain injury model.

MATERIALS AND METHODS

Microglia Activation by LPS—The immortalized murine BV-2 cell line exhibits phenotypic and functional properties of reactive microglial cells (31). The cells are grown and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum, penicillin/streptomycin, and 1% glutamine, and by guest on August 16, 2017 http://www.jbc.org/ Downloaded from
HEPES-buffered saline was prepared. The plasmid and calcium mix were added slowly to the 2×/H11003 HEPES-buffered saline buffer, and the resulting mixture was incubated for 20 min at room temperature. The mixture was gently vortexed and added drop-wise to the culture medium.

**NF-κB Activity Assay**—We measured NF-κB activity using the secreted alkaline phosphatase (SEAP) reporter system 3 (pNFκB-SEAP; BD Biosciences Clontech). The culture medium was replaced with fresh medium at 12 h after transient transfection. The medium was collected for SEAP assay after 24 h and the cells were harvested for β-galactosidase assay. The vehicle vector pSG5 (Stratagene) was used as a control. Cells treated with pGAL plasmid (1 μg) were co-transfected with expression vectors to normalize expression to β-galactosidase activity (39). The SEAP assay was performed according to the supplier’s instructions (BD Biosciences Clontech). Values are the means of three determinations (S.D. < 10%).

**Measurement of NF-κB Activation by Electrophoretic Mobility Shift Assay**—Nuclear extracts of BV-2 microglia and SH-SY5Y were prepared from non-transfected control, vehicle control (pSG5; Stratagene), and TGase 2 transfected (pSG5/TG) cells using a nuclear extract kit (Sigma). A double-stranded consensus oligonucleotide for NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG C-3') was end-labeled with [32P]ATP. Binding reactions containing equal amounts of nuclear extract protein (16 μg) and 10 fmol (~10,000 cpm; Cherenkov counting) of oligonucleotide were performed for 30 min in binding buffer (10 mM HEPES, pH 7.9, 50 mM KCl, 2 mM EDTA, 0.3 mg/ml bovine serum albumin, 6 mM MgCl2, 10% glycerol, 1 mM dithiothreitol, and 2 μg of poly dI-dC). Total reaction volumes were held at 20 μl. Reaction products were separated in 6% polyacrylamide gels and analyzed by a bioimaging analyzer (Fujifilm).

**Effect of TGase Inhibitors on Reduced IκBα in SH-SY5Y Cells**—Cystamine is known to inhibit TGase activity by blocking access of the

---

**Fig. 1. The induction of TGase 2 in LPS-induced BV-2 microglia.** A, after 24-h LPS treatment with BV-2 microglia, TGase activity was increased about 5-fold concomitant with a 10-fold increase of nitrite release. B, RT-PCR analysis of iNOS and TGase 2 after LPS treatment in BV-2 cells. TGase 2 expression is increased about 3-fold concomitant with a 10-fold increase of iNOS expression. C, to test whether iNOS affects TGase activity, BV-2 was treated with LPS and NMMA (iNOS inhibitor). NMMA did not affect TGase activity. Data are presented as mean ± S.D. of three separate experiments (Ct, non-treated BV-2; LPS, LPS treated BV-2).
glutamine residue in substrate proteins to the TGase active site (23, 40, 41). Iodoacetamide (Sigma) is also known to inhibit TGase as a strong competitive irreversible inhibitor. The effectiveness of this TGase inhibitor has been demonstrated in many studies (e.g. Refs. 42, 43). E2 (DPVKG) was designed to contain the pro-elafin sequence (44, 45). R2 (KVLDGQDP) was designed to contain pro-elafin and anti-fibrinogen sequence.

**Fig. 2.** The in vivo target of TGase 2 in the NF-κB cascade. To identify major targets of TGase 2 in the NF-κB cascade, a series of Western blottings was done using SH-SY5Y cells stably transfected with TGase 2 (A–C). A, TGase activity is about 8-fold increased in the cytosolic fraction of SH-SY5Y/TG cells. B, Western blotting of I-κBα and NF-κB showed about a 50% decrease in the cytosol and a 30% increase in the nucleus by densitometry, respectively. P-I-κBα was not clearly detected in SH-SY5Y cells or SH-SY5Y/TG cells. Data are presented as mean ± S.D. of three separate experiments (Ct, SH-SY5Y/FRT; TG, SH-SY5Y/TG; Cyt, cytosolic fraction; Nuc, nucleic fraction). C, Western blotting analyses found no change of p-I-κBα between SH-SY5Y and SH-SY5Y/TG cell lines with or without SC-514 (IKK-2 inhibitor) treatment. BV-2 cells with LPS treatment were used as a positive control for I-κBα phosphorylation.
The polymerization of I-\(\kappa\)Bα by TGase 2 depletes free I-\(\kappa\)Bα without ubiquitination. A, SH-SYSY/TG cells were incubated for 6 h with proteasome inhibitors including MG 132, lactacystin, or carboxenzoxy-I-isoleucyl-\(\gamma\)-butyl-I-glutamyl-I-alanyl-I-leucinal (PSI). The cytosol was extracted from cells and analyzed by Western blotting of I-\(\kappa\)Bα and ubiquitin. LDH activity in the medium and caspase-9 expression by Western blotting in the treated cells were not detected (data not shown). The level of I-\(\kappa\)Bα (arrowhead) in the SH-SYSY/TG cells treated by the proteasome inhibition remained low whereas ubiquitinated high molecular weight proteins were increased in a dose-dependent manner. Ubiquitinated I-\(\kappa\)Bα (asterisk) was not increased. Western blotting of LDH was used for loading control. (Ct, SH-SYSY/FRT cells; TG, SH-SYSY/TG cells). B, in SH-SYSY/TG cells, Western blotting of I-\(\kappa\)Bα showed formation of polymerized I-\(\kappa\)Bα (asterisk) concomitant with a reduced level of I-\(\kappa\)Bα (arrowhead) compared with the controls (W, SH-SYSY; Ct, SH-SYSY/FRT; TG, SH-SYSY/TG cells). C, in \(\text{in vitro}\) incubation of 2 \(\mu\)g purified I-\(\kappa\)Bα (arrowhead) with 0.001 units of TGase 2 for 30 min resulted in the complete formation of high molecular weight polymers of I-\(\kappa\)Bα (asterisk) as detected by Western blotting (TGase 2, guinea pig liver TGase 2). D, in \(\text{in vitro}\) incubation of NF-\(\kappa\)B (p52) with TGase 2 did not result in formation of polymers. Data are representative of three repeated experiments in each group.

**Fig. 3.** The polymerization of I-\(\kappa\)Bα by TGase 2 depletes free I-\(\kappa\)Bα without ubiquitination. A, SH-SYSY/TG cells were incubated for 6 h with proteasome inhibitors including MG 132, lactacystin, or carboxenzoxy-I-isoleucyl-\(\gamma\)-butyl-I-glutamyl-I-alanyl-I-leucinal (PSI). The cytosol was extracted from cells and analyzed by Western blotting of I-\(\kappa\)Bα and ubiquitin. LDH activity in the medium and caspase-9 expression by Western blotting in the treated cells were not detected (data not shown). The level of I-\(\kappa\)Bα (arrowhead) in the SH-SYSY/TG cells treated by the proteasome inhibition remained low whereas ubiquitinated high molecular weight proteins were increased in a dose-dependent manner. Ubiquitinated I-\(\kappa\)Bα (asterisk) was not increased. Western blotting of LDH was used for loading control. (Ct, SH-SYSY/FRT cells; TG, SH-SYSY/TG cells). B, in SH-SYSY/TG cells, Western blotting of I-\(\kappa\)Bα showed formation of polymerized I-\(\kappa\)Bα (asterisk) concomitant with a reduced level of I-\(\kappa\)Bα (arrowhead) compared with the controls (W, SH-SYSY; Ct, SH-SYSY/FRT; TG, SH-SYSY/TG cells). C, in \(\text{in vitro}\) incubation of 2 \(\mu\)g purified I-\(\kappa\)Bα (arrowhead) with 0.001 units of TGase 2 for 30 min resulted in the complete formation of high molecular weight polymers of I-\(\kappa\)Bα (asterisk) as detected by Western blotting (TGase 2, guinea pig liver TGase 2). D, in \(\text{in vitro}\) incubation of NF-\(\kappa\)B (p52) with TGase 2 did not result in formation of polymers. Data are representative of three repeated experiments in each group.

**Fig. 3.** The polymerization of I-\(\kappa\)Bα by TGase 2 depletes free I-\(\kappa\)Bα without ubiquitination. A, SH-SYSY/TG cells were incubated for 6 h with proteasome inhibitors including MG 132, lactacystin, or carboxenzoxy-I-isoleucyl-\(\gamma\)-butyl-I-glutamyl-I-alanyl-I-leucinal (PSI). The cytosol was extracted from cells and analyzed by Western blotting of I-\(\kappa\)Bα and ubiquitin. LDH activity in the medium and caspase-9 expression by Western blotting in the treated cells were not detected (data not shown). The level of I-\(\kappa\)Bα (arrowhead) in the SH-SYSY/TG cells treated by the proteasome inhibition remained low whereas ubiquitinated high molecular weight proteins were increased in a dose-dependent manner. Ubiquitinated I-\(\kappa\)Bα (asterisk) was not increased. Western blotting of LDH was used for loading control. (Ct, SH-SYSY/FRT cells; TG, SH-SYSY/TG cells). B, in SH-SYSY/TG cells, Western blotting of I-\(\kappa\)Bα showed formation of polymerized I-\(\kappa\)Bα (asterisk) concomitant with a reduced level of I-\(\kappa\)Bα (arrowhead) compared with the controls (W, SH-SYSY; Ct, SH-SYSY/FRT; TG, SH-SYSY/TG cells). C, in \(\text{in vitro}\) incubation of 2 \(\mu\)g purified I-\(\kappa\)Bα (arrowhead) with 0.001 units of TGase 2 for 30 min resulted in the complete formation of high molecular weight polymers of I-\(\kappa\)Bα (asterisk) as detected by Western blotting (TGase 2, guinea pig liver TGase 2). D, in \(\text{in vitro}\) incubation of NF-\(\kappa\)B (p52) with TGase 2 did not result in formation of polymers. Data are representative of three repeated experiments in each group.

**Fig. 3.** The polymerization of I-\(\kappa\)Bα by TGase 2 depletes free I-\(\kappa\)Bα without ubiquitination. A, SH-SYSY/TG cells were incubated for 6 h with proteasome inhibitors including MG 132, lactacystin, or carboxenzoxy-I-isoleucyl-\(\gamma\)-butyl-I-glutamyl-I-alanyl-I-leucinal (PSI). The cytosol was extracted from cells and analyzed by Western blotting of I-\(\kappa\)Bα and ubiquitin. LDH activity in the medium and caspase-9 expression by Western blotting in the treated cells were not detected (data not shown). The level of I-\(\kappa\)Bα (arrowhead) in the SH-SYSY/TG cells treated by the proteasome inhibition remained low whereas ubiquitinated high molecular weight proteins were increased in a dose-dependent manner. Ubiquitinated I-\(\kappa\)Bα (asterisk) was not increased. Western blotting of LDH was used for loading control. (Ct, SH-SYSY/FRT cells; TG, SH-SYSY/TG cells). B, in SH-SYSY/TG cells, Western blotting of I-\(\kappa\)Bα showed formation of polymerized I-\(\kappa\)Bα (asterisk) concomitant with a reduced level of I-\(\kappa\)Bα (arrowhead) compared with the controls (W, SH-SYSY; Ct, SH-SYSY/FRT; TG, SH-SYSY/TG cells). C, in \(\text{in vitro}\) incubation of 2 \(\mu\)g purified I-\(\kappa\)Bα (arrowhead) with 0.001 units of TGase 2 for 30 min resulted in the complete formation of high molecular weight polymers of I-\(\kappa\)Bα (asterisk) as detected by Western blotting (TGase 2, guinea pig liver TGase 2). D, in \(\text{in vitro}\) incubation of NF-\(\kappa\)B (p52) with TGase 2 did not result in formation of polymers. Data are representative of three repeated experiments in each group.
RESULTS

The induction of TGase 2 in LPS-induced BV-2 microglia is shown in Fig. 1. TGase activity was increased about 5-fold concomitant with a 10-fold increase of NO release after 24-h LPS treatment (Fig. 1A). RT-PCR analysis of iNOS and TGase 2 was performed in BV-2 cells after LPS treatment (Fig. 1B). TGase 2 is increased about 3-fold concomitant with a 10-fold increase of iNOS. Furthermore, we observed that transient transfection of TGase 2 in the BV-2 microglia increases NF-κB activity (30). iNOS is triggered by NF-κB activation. Therefore, the data suggest that TGase 2 is probably involved in the regulation of the NF-κB cascade. To test whether NO induces TGase 2 expression, BV-2 was treated with LPS and NMMA (iNOS inhibitor). NMMA did not affect TGase activity, but it did not reduce NO secretion in a dose-dependent manner (Fig. 1C).

To identify major targets of TGase 2 in the NF-κB cascade, a series of Western blotting experiments was done using SH-SY5Y cells stably transfected with TGase 2 (Fig. 2). TGase activity is increased about 8-fold in the cytosolic fraction of the SH-SY5Y/TG cells (Fig. 2A). Western blotting analyses found no change of NF-κB activating kinase, NIK, IKKα, and p-IKK [p-IKKα (Ser-180) and p-IKKα (Ser-181)] (data not shown). Western blotting of I-κBα and NF-κB followed by densitometry showed a ~50% decrease in the cytosol and a ~30% increase in the nucleus, respectively (Fig. 2B). No change of p-I-κBα was observed between SH-SY5Y and SH-SY5Y/TG cells (Fig. 2B). TGase 2 transfection in the BV-2 microglia, which resulted in a ~5-fold increase of TGase activity (30), was associated with a decrease of I-κBα level as assessed by Western blotting (Fig. 2A). To test whether the decrease of free I-κBα by TGase 2 transfection is IKK-dependent or not, SC-514 treatment (IKK-2 inhibitor) was employed (Fig. 2C). We found no change of p-I-κBα between SH-SY5Y and SH-SY5Y/TG cells with or without SC-514, whereas LPS-treated BV-2 showed a decrease of p-I-κBα with SC-514 (Fig. 2C).

To test whether TGase 2 reduces I-κBα level via the ubiquitin-proteasome system, SH-SY5Y/TG cells were incubated for 6 h with proteasome inhibitors including MG 132 (0, 10, 100 μM), lactacystin (0, 1, 10 μM), or carbobenzoxy-l-isoleucyl-γ-butylyl-l-glutamyl-l-alanyl-l-leucinal (0, 1, 10 μM) (Fig. 3A). The cytosol was extracted from cells and analyzed by Western blotting of I-κBα and ubiquitin. LDH activity in the medium and caspase-9 expression by Western blotting in the treated cells were not detected during course of the experiment (data not shown).

FIG. 4. Binding test of free or polymerized I-κBα to NF-κB. A, a flowchart of the experiment. B, NF-κB(p65) (2.0 μM) was incubated with I-κBα at various concentrations (0.25, 0.5, 1.0, and 2.0 μM) with or without TGase 2 treatment (0.001 units) for 30 min at 37 °C. Incubation of 2.0 μM I-κBα shows complete polymerization under these conditions (Fig. 3C). The mixture of I-κBα and NF-κB(p65) was immunoprecipitated (IP) against NF-κB(p65) and subjected to Western blotting against I-κBα. The free I-κBα is very efficiently bound to NF-κB(p65) in a dose-dependent manner (arrow). However, the polymerized I-κBα (asterisk) lost almost all binding capacity to NF-κB(p65). Densitometry analysis revealed that the binding efficiency of polymerized I-κBα to NF-κB is reduced to less than 10% the level of free I-κBα. Data are presented as mean ± S.D. from three repeats.
shown). If TGase 2-induced NF-κB activation depends on the IKK/ubiquitin/proteasome pathway, the I-κBa and ubiquitinated I-κBa should be increased. The level of I-κBa (arrowhead) in SH-SY5Y/TG cells was decreased by proteasome inhibition, whereas ubiquitinated high molecular weight proteins were increased in a dose-dependent manner (Fig. 3A). Increased ubiquitinated I-κBa (asterisk) was not detected by Western blotting (Fig. 3A). In SH-SY5Y/TG cells, Western blotting of I-κBa showed a reduced level of I-κBa (Fig. 3B, arrowhead) accompanied by highly polymerized I-κBa (Fig. 3B, asterisk). In vitro incubation of purified I-κBa (Fig. 3C, arrowhead) with 0.001 units of purified guinea pig liver TGase 2 for 30 min resulted in completely polymerized I-κBa (Fig. 3C, asterisk) by Western blotting. In vitro incubation of NF-κB (p52) with TGase 2 did not result in polymerization of this protein (Fig. 3D).

We determined whether the polymerized I-κBa no longer interacts with NF-κB in vitro (Fig. 4). Under the condition of TGase treatment shown in Fig. 3C, free I-κBa (0.25–2 μM) is completely cross-linked to a high molecular weight polymer. After free I-κBa was treated with or without TGase 2, the mixture was incubated with or without TGase 2, and the precipitates were subjected to Western blot analysis against I-κBa. The free form of I-κBa was able to bind to NF-κB very efficiently in a dose-dependent manner (Fig. 4B, arrow). However, the polymerized I-κBa (Fig. 4B, asterisk) lost binding capacity. Densitometry analysis revealed that the binding efficiency of polymerized I-κBa to NF-κB is reduced to less than 10% of the level of free I-κBa (Fig. 4B).

NF-κB activation analyses were done by NF-κB/SEAP reporter assay normalized to β-galactosidase activity (Fig. 5, A and B, lower bar graph) and by NF-κB (Fig. 5B, top bar graph) and by NF-κB (Fig. 5B, top inset) (A and B). Electrophoretic mobility shift assay was also performed with nuclear fractions of BV-2 and SH-SY5Y after transfection with TGase 2 (C). A, transiently transfected TGase 2 in BV-2 cells reduced I-κBa in the cytosol, which resulted in a 2-fold increase of NF-κB activity. The data are presented as mean ± S.D. from three samples. (W, non-transfected; Ct, transfected with empty vector, pSG5; TG, transfected with TGase 2, pSG5/TG). B, stable transfection of TGase 2 in SH-SY5Y cells reduced I-κBa in the cytosol, which resulted in a 3-fold increase of NF-κB activity. The data are presented as mean ± S.D. from three samples. (W, SH-SY5Y; Ct, SH-SY5Y/FRT, TG, SH-SY5Y/TG cells). C, binding reactions were performed with nuclear extracts from BV-2 and SH-SY5Y cells with or without TGase 2 transfection using a double-stranded consensus oligonucleotide for NF-κB end-labeled with [32P]ATP. A gel-shift assay showed 3- and 2-fold increases of NF-κB in BV-2 and SH-SY5Y after TGase 2 transfection, respectively. The data are presented as mean ± S.D. from three repeats.
The transcriptional and post-transcriptional levels. In addition to Tgase 2 induction by LPS treatment, Tgase 2 can be induced by various stresses, including oxidative stress (58), UV (50, 51), calcium influx brought about by treatment with a calcium ionophore or maito toxin (52, 53), retinoic acid (RA) (54, 55), inflammatory cytokines (56, 57), glutamate (20), and virus infection (22). Oxidative stress and reactive oxygen intermediates have been shown to increase Tgase 2 expression (58). UV radiation generates singlet oxygen and superoxide in the eye. Superoxide rapidly dismutates to hydrogen peroxide, which elevates Ca\(^{2+}\) (59). Therefore, it is possible that changes in Ca\(^{2+}\) activate Tgase 2. Tgase 2 is significantly increased and translocated to the nucleus by maito toxin treatment in neuronblasta cells (53). Maito toxin activates both voltage-sensitive and ligand-gated calcium channels, which increases intracellular calcium concentrations. RA increases Tgase activity biphosphorically in Chinese hamster ovary cells (54). A recent report showed that phosphoinositide 3-kinase activity was required for RA to increase Tgase 2 protein levels in NIH3T3 cells (55).

Activation of the Ras-ERK pathway by epidermal growth factor was sufficient to elicit this effect, because continuous Ras signaling mimicked the actions and inhibited RA-induced Tgase 2 expression, whereas blocking ERK activity in these same cells restored the ability of RA to up-regulate Tgase 2 expression (60). This is a good example of signal-regulated Tgase 2 induction. Exposure of astrocytes in primary culture to gluta mates also increases Tgase 2 expression and translocation of Tgase 2 into the nucleus (20). Glutamate exposure of astroglial cells causes ligand-gated channel receptor activation, associated with an excitotoxic cell response. In the glutamate-exposed astroglial cells, an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate receptor inhibitor reduced Tgase 2 expression (20). Tgase 2 is induced by inflammatory cytokines, such as TNF-α in liver cells (56) and interferon-γ in intestinal cells (57). Tgase 2 also can be induced directly by NF-κB activation in liver cells because the Tgase 2 promoter has an NF-κB binding motif (61). It is interesting that Tgase 2 seems to control NF-κB activation. This is an intriguing finding because many stimulations triggering NF-κB activation overlap with stimulations of Tgase 2 such as nontypeable Haemophilus influenza infection (62), UV (63), reactive oxygen intermediates (peroxan date) (64), interleukin-1α, and TNF-α signaling (65) in addition to LPS. This implies that Tgase 2 and NF-κB may induce each other (Fig. 8). Several pathways for NF-κB activation do not rely on IKK activation. These pathways up-regulate inflammatory mediators. An IKK-independent NF-κB activation pathway via activation of the Mkk3/6-p38 mitogen-activated protein kinase pathway occurs in epithelial cells challenged by nontypeable Haemophilus influenza (62). Therefore, nontypeable Haemophilus influenza seems to activate NF-κB in human epithelial cells via two distinct signaling pathways, including TLR2-TAK1-dependent NIK-IKKβ/β-IκBα and Mkk3/6-p38 mitogen-activated protein kinase pathways. UV-induced IκBα degradation is IKK-independent (63). Even an IκBα mutant containing alanines at positions 32 and 36 is also susceptible to UV-induced degradation (75). UV-induced NF-κB activation depends on phosphorylation of IκBα at C-terminal sites (PEST domain, explained below) that are recognized by CK2 (caseine kinase II) (66). Furthermore, CK2 activity toward IκBα is UV-inducible through a mechanism that depends on activation of p38 mitogen-activated protein kinase. Inhibition of this pathway prevents UV-induced IκBα degradation and increases UV-induced cell death (66). However, this mechanism of IκBα degradation remains an enigma. Ionizing radiation induces tyrosine phosphorylation in human B-lymphocyte precursors by stimulation of tyrosine kinases (64).
tyrosine kinase inhibitor herbimycin A and the free radical scavenger N-acetyl-cysteine inhibit both radiation- and H$_2$O$_2$-induced NF-$\kappa$B activation, indicating that activation triggered by reactive oxygen intermediates is dependent on tyrosine kinase activity (64). Pervanadate-induced tyrosine phosphorylation leads to degradation of I-$\kappa$B, and this degradation is required for NF-$\kappa$B activation in human myeloid U937 and HeLa cells (67). A proteosome inhibitor blocked pervanadate-induced degradation without blocking tyrosine phosphorylation of I-$\kappa$B, indicating that phosphorylation alone is insufficient to induce degradation (67). The accumulated evidence suggests that NF-$\kappa$B activation may occur through different pathways depending on the stress. However, a major NF-$\kappa$B regulatory mechanism depends on signal-dependent I-$\kappa$B degradation. In addition to the ubiquitination-mediated degradation, I-$\kappa$B can be degraded also by a lysosomal system or by a proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST) sequence pathway. The lysosomal degradation of I-$\kappa$B is activated under conditions of nutrient deprivation (68). In the Chinese hamster ovary cells, the half-life of I-$\kappa$B is 4.4 days in normal medium but it is reduced to 0.9 day in serum-deprived medium (68). This report also showed that increase of I-$\kappa$B degradation is completely blocked by lysosomal inhibitors (68). Rogers et al. found that the amino acid sequences of proteins with intracellular half-lives of less than 2 h contain one or more regions rich in PEST (69). A PEST region is found in the I-$\kappa$B carboxyl terminus. Deletion of this region in I-$\kappa$B results in increased half-life, suggesting that the PEST sequence may play a direct role in the degradation of I-$\kappa$B (70). I-$\kappa$B is readily converted to homopolymers catalyzed cross-
linking by TGase 2 in vitro (Fig. 3C). Furthermore, polymerized I-κBα lost binding capacity to NF-κB (Fig. 4B). Densitometry analysis shows that polymerized I-κBα lost more than 90% binding capacity of free I-κBα to NF-κB. This implies that I-κBα is both an acyl donor and an acyl acceptor for TGase cross-linking. We found that putative acyl donors (glutamine residues) are clustered at the end of the carboxyl terminus before PEST sequences, and putative acyl-acceptors (lysine residues) are clustered at the amino terminus. This resembles many other TGase substrate structures as building blocks found in barrier tissues. We suggest that antiparallel homopolymerization of I-κBα may lead to inhibition of binding to NF-κB, or concentrate PEST sequences on one side, which could accelerate degradation of I-κBα. Therefore, TGase 2-induced I-κBα polymerization may be a major pathway for rapid depletion of free I-κBα during stress induced by UV, radiation, and nontypeable Haemophilus influenza (Fig. 8).

We demonstrated recently that TGase inhibition using the peptide inhibitor R2, cystamine, and iodoacetamide in the LPS-induced microglia resulted in decreased NO synthesis (30). Herein, we showed that TGase inhibitors reverse the depletion of I-κBα in SH-SY5Y/TG cells (Fig. 6). Therefore the effect of TGase inhibition on NO synthesis in the microglia is probably caused by blocking the depletion of I-κBα. In the LPS-induced brain injury model, we found that increased TGase 2 expression is restricted to the subcomparal organ and choroid plexus (Fig. 7A) concomitantly with I-κBα expression (47). The increase of I-κBα mRNA parallels both I-κBα protein degradation and NF-κB activation, because transcription of I-κBα is regulated by NF-κB. Therefore, the TGase 2 expression pattern shows an early phase activation of inflammatory process (Fig. 7A). In this model, we failed to detect a change of I-κBα protein in the brain after TGase inhibitor treatment, because I-κBα is ubiquitously abundant in the brain. Therefore, we analyzed TNF-α expression, which is specifically regulated by NF-κB.

Our results are similar to those in another report (71), which showed that elafin, including the pro-elafin sequence (secretory leukoprotease inhibitor), prevents inflammation in an LPS-induced acute lung injury model. Elafin treatment before the lung injury greatly reduces NF-κB activation by inhibiting I-κBα degradation (71). Elafin prevents LPS-induced NF-κB activation by inhibiting degradation of I-κB without affecting the LPS-induced phosphorylation and ubiquitination of I-κB in U937 cells (72). In that study, a full-length elafin construct containing pre-elafin sequences was employed (72). It is interesting that the pre-elafin domain of SLPI, which is also known as a ‘cementoin,’ is an excellent TGase 2 substrate. We used the cementoin sequence to synthesize the strong competitive TGase inhibitors E2 and R2. This cementoin peptide contains four repeats of E2 and R2 sequences. It is possible that the cementoins in elafin may serve as in vivo TGase inhibitors (73), which could interfere with the prevention of NF-κB activation (Fig. 8). This hypothesis is supported by the finding that elafin does not affect 20S proteasome peptidase-related activity in the cytoplasmic extract of U937 cells (72).

In conclusion, TGase 2 induces NF-κB activation via two different pathways, an IKK-independent pathway and an IKK-dependent pathway. We propose here a model for the role of TGase 2 during immune cell activation (Fig. 8). TGase 2-induced NF-κB activation may be an important defense against infection, or conversely, a disease-associated mechanism in the inflammation process. This lends greater versatility to the innate immune system responding to various stimuli because TGase 2 is induced by various stresses. We demonstrated in this study that TGase inhibition is effective as a therapeutic approach to LPS-induced brain injury. This suggests that TGase inhibition may be beneficial in diseases associated with inflammation.

Acknowledgments—We thank Dr. Baker for technical advice regarding the immunohistochemical studies and Drs. Arthur J. L. Cooper and Rajiv R. Ratan for critical reading of the manuscript.
Transglutaminase 2 Induces Nuclear Factor-κB Activation via a Novel Pathway in BV-2 Microglia
Jongmin Lee, Yoon-Seong Kim, Dong-Hee Choi, Moon Suk Bang, Tai Ryoon Han, Tong H. Joh and Soo-Youl Kim

doi: 10.1074/jbc.M407627200 originally published online October 7, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407627200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 75 references, 35 of which can be accessed free at http://www.jbc.org/content/279/51/53725.full.html#ref-list-1