Peroxynitrite-dependent Zinc Release and Inactivation of Guanosine 5'-triphosphate Cyclohydrolase 1 instigates its Ubiquitination in Diabetes

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ABSTRACT

Aberrant degradation of guanosine 5'-triphosphate cyclohydrolase 1 (GTPCH1) with consequent deficiency of tetrahydrobiopterin (BH4) is considered as the primary cause for endothelial dysfunction in diabetes. How GTPCH1 becomes susceptible to the degradation remains unknown. We hypothesized that oxidation and release of the zinc ion by peroxynitrite (ONOO−), a potent oxidant generated by nitric oxide and superoxide anions, instigates GTPCH1 ubiquitination and degradation. Zinc contents, GTPCH1 ubiquitination, and GTPCH1 activity were assayed in purified GTPCH1, endothelial cells, and hearts from diabetic mice. Exogenous ONOO− dose-dependently released zinc, inhibited its activity, and increased the ubiquitin binding affinity of GTPCH1 in vitro and in endothelial cells. Consistently, high-glucose (30 mM) inhibited GTPCH1 activity with increased ubiquitination, which were inhibited by anti-oxidants. Furthermore, mutation of the zinc-binding cysteine (141) (C141R or C141A) significantly reduced GTPCH1 activity and reduced its half-life, but increased GTPCH1 ubiquitination, indicating an essential role of the zinc ion in maintaining the catalytic activity and stability of GTPCH1. Finally, GTPCH1 ubiquitination and degradation markedly increased in parallel with decreased GTPCH1 activity in the aortas and hearts of diabetic mice, both of which was attenuated by the inhibitors of ONOO− in mice in vivo. Taken together, we conclude that ONOO− releases zinc and inhibits GTPCH1 resulting in its ubiquitination and degradation of the enzyme.
INTRODUCTION

An adequate supply of tetrahydrobiopterin (BH4) in the endothelium is critical for maintaining the “coupled” status of endothelial nitric oxide synthase (eNOS) in healthy subjects. In contrast, BH4 deficiency, which is widely found in diseased vessels, is considered responsible for eNOS uncoupling in diabetes mellitus (1-4) or hypertension (5,6). Guanosine 5'-triphosphate cyclohydrolase 1 (GTPCH1) is the first enzyme in the de novo biosynthetic pathway of tetrahydrobiopterin (BH4). GTPCH1 inhibition leads to a rapid decrease of BH4 and consequent eNOS uncoupling (7). GTPCH1 is regulated by several mechanisms, including transcription, posttranslational modifications (7), and association with the GTPCH feedback regulatory protein (GFRP), which inhibits GTPCH1 activity (8).

Zinc binds to a large number of proteins, including numerous metalloenzymes, structural proteins, and transcription factors (9). In the human GTPCH1 crystal structure, zinc binds to two cysteines and one histidine (C141-H144-C212) (10) and is reported to be important in maintaining GTPCH1 structure and function (10). Because zinc has the highest charge-to-atomic radius ratio of any element and maintains a partial cationic character (11,12), zinc ion is reported to react fast with anionic oxidants such as ONOO⁻ and HOCl. For example, ONOO⁻ reacts rapidly with zinc-containing proteins such as eNOS, protein kinase C (PKC), and yeast alcohol dehydrogenase, because the zinc-thiolate cluster represents a selective target for ONOO⁻ (11-14).

Recent evidence indicates that loss or inactivation of the GTPCH1 protein and the consequent BH4 deficiency causes eNOS uncoupling in diabetes (1,7,15). Although the increased proteasome activity in diabetic mice might be an important cause for GTPCH1 degradation (1), the mechanism that caused the preferential degradation of GTPCH1 by proteasome is unknown yet. Diabetes-induced GTPCH1 loss is likely not caused by transcription because there is no change in GTPCH1 mRNA levels in streptozotocin (STZ)-induced diabetic mice (1). Diabetes does not affect the association of GTPCH1 with GFRP, which impairs GTPCH1 activity (1). Therefore, the modification of GTPCH1 protein might explain accelerated destruction in diabetes. Here, we provide evidence that ONOO⁻ releases zinc, inhibits GTPCH1 activity, and increases GTPCH1 ubiquitination.
RESEARCH DESIGN AND METHODS

Streptozotocin-induced diabetes in mice. C57BL/6J mice, aged 8–12 weeks, were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in temperature controlled cages with a 12-hour light/dark cycle and given free access to water and food. Mice were divided into four groups and received an injection of STZ (50 mg/kg body weight daily) for 5 consecutive days to induce diabetes mellitus (17). Diabetes mellitus is defined as random blood glucose levels of >450 mg/dL for >2 weeks after injection. One additional group of STZ-treated mice were subsequently treated with 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (Tempo; in drinking water, 1 mmol/kg body weight) for 4 weeks. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center.

Cells. Bovine aortic endothelial cells (BAECs), used between passages 3 and 10, were grown in endothelial basal medium (Lonza, Basel, Switzerland) supplemented with 2% fetal bovine serum, 1% (v/v) penicillin-streptomycin. Human embryonic kidney 293 (HEK293) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 1% (v/v) penicillin-streptomycin and 10% (v/v) fetal calf serum.

Plasmids construction and mutagenesis. Full-length human GTPCH1 cDNA (GCH1) was synthesized by PCR amplification. The amplified DNA with a C-Terminal FLAG tag was directionally cloned into plasmid pCI-neo (Promega, Madison, WI) for mammalian protein expression. For E. coli (BL21) protein expression, GCH1 was inserted into pGEX-4T-2 (Amersham Pharmacia Biotech). The zinc-deletion mutant of GTPCH1 (C141R) was generated using the Stratagene QuikChange site-directed mutagenesis kit. Plasmid pRK5-HA-Ubiquitin (HA-Ub, Addgene ID 17608) was obtained from Addgene Inc (Cambridge, MA). All constructed plasmids were sequenced entirely before use.

Expression and purification of recombinant GTPCH1 in bacteria and mammalian cells. GST-tagged GTPCH1 or C141R were expressed in the E. coli BL21 (DE3). The cells were resuspended in lysis buffer [100 mM Tris-HCl pH 8.5, 100 mM NaCl, 10%
glycerol, 1% Triton X-100, and protease inhibitors (Calbiochem), then lysed by brief sonication. The GST fusion proteins were purified with GSH beads in accordance with the manufacturer’s protocol. For the expression of recombinant GTPCH1 in mammalian cells, FLAG-tagged WT or C141R GTPCH1 plasmids were transfected into HEK293 cells. The expressed FLAG-GTPCH1 or C141R proteins were purified using anti-FLAG resin (Sigma), following the protocol provided by the manufacturer.

**Detection of protein S-nitrosylation with the biotin-switch:** S-nitrosylated GTPCH1 was monitored by using the kits from Cayman Chemicals, according to the method provided by the supplier.

**Assay of GTPCH1 activity.** GTPCH1 activity was measured as described previously (1,16). This assay was based on the quantification of D-erythro-neopterin by high-performance liquid chromatography (HPLC).

**Statistics analysis.** Results are expressed as mean ± S.D. All values are normalized to control and the means of the control are calculated. Statistical significance for comparisons between 2 groups was calculated using the 2-tailed paired student t test. To assess comparisons between multiple groups, ANOVA followed by the Bonferroni procedure was performed using the GraphPad Prism 4 program (GraphPad Software, Inc, San Diego, CA). p <0.05 was considered to be statistically significant.

Materials and other methods including *in vitro* pull-down assays, enrichments of ubiquitinated proteins, determination of GTPCH1 half-life assays, and detection of superoxide anion can be found in on-line supplemental material.

**RESULTS**

**ONOO⁻ inhibits whereas NO activates GTPCH1 activity in vitro.** Bacterially expressed GTPCH1 was first purified as described in Methods. Purified GTPCH1 was seen as a single band at 30 KDa in silver staining of the gel (**Fig. 1A**). To determine the effects of ONOO⁻ or NO on GTPCH1 activity, purified recombinant GTPCH1 proteins
(0.5 µg) were exposed to chemically synthesized ONOO\(^-\) (0-100 µM) or vehicle (0.1 M NaOH). As shown in **Fig. 1B and Suppl. Fig 1**, ONOO\(^-\) (1 to 100 µM) inhibited GTPCH1 activity in a dose-dependent manner, causing an approximately 70% reduction of GTPCH1 activity at the highest concentration (100 µM). In contrast, Exposure of GTPCH1 activity to DETA-NONOate (DETA, 200 µM) significantly increased GTPCH1 activity (**Fig. 1C**). Similarly, exposure of GTPCH1 to spermine NONOate (SperNO, 1mM or 5mM) activated the enzyme (data not shown).

**NO but not ONOO\(^-\) increase the s-nitrosylation of GTPCH1 in vitro.** We next determined if ONOO\(^-\) inhibition on GTPCH1 was via S-nitrosylation of the enzyme. Recombinant FLAG-GTPCH1 were treated with vehicle or ONOO\(^-\) (100 µM), and then applied for biotin-switch assay, a commonly used method for the detection of s-nitrosylation. As expected, s-nitrosylation of GTPCH1 was barely detectable in basal condition and was not altered by 100 µM of ONOO\(^-\) treatment (**Fig. 1D**). In addition, exposure of GTPCH1 to ONOO\(^-\) didn’t have any obvious effect on the s-nitrosylation of GTPCH1 with or without N-Ethylmaleimide (NEM), an organic compound that irreversibly reacts thiol (**Fig. 1D**). In contrast, DETANO markedly increased the detection of s-nitrosylated GTPCH1 (**Fig. 1D**). Further, pretreatment of GTPCH1 with NEM ablated NO-increased s-nitrosylation of GTPCH1 (**Fig. 1D**). Similar effect was also observed with sperNO (data not shown). Taken together, NO but not ONOO\(^-\) increased the s-nitrosylation of GTPCH1.

**ONOO\(^-\) releases zinc from GTPCH1.** Zinc is important for maintaining the structure and function of GTPCH1 (10). Because ONOO\(^-\) reacts fast with the positively charged zinc ion resulting in its release from zinc-containing proteins (11), we next assayed whether or not ONOO\(^-\) released zinc from recombinant GTPCH1. As depicted in **Fig. 1E**, ONOO\(^-\) increased zinc release from GTPCH1 in a concentration-dependent manner. 50 µM ONOO\(^-\) caused approximately 60% zinc release, whereas a higher concentration of ONOO\(^-\) (100 µM) increased zinc release to approximately 80% (**Fig. 1E**). In contrast, NO had no effects on zinc release from GTPCH1 (**Fig. 1E**).
Inhibition of GTPCH1 activity by zinc chelator TPEN *in vitro*. To further support the concept that zinc release is responsible for ONOO−-induced GTPCH1 inhibition, we next determined if chemical zinc chelation mimicked the effects of ONOO−. To this end, recombinant GTPCH1 proteins were incubated with N,N,N′,N′-tetrakis(2-pyridinylmethyl)-1,2-ethanediane (TPEN), a potent zinc-chelating agent (11). Because TPEN was found to interfere with the GTPCH1 activity assay at concentrations ≥500 µM, TPEN (40 and 200 µM) were used in this study. As shown in Fig. 1F, the addition of 40 or 200 µM TPEN reduced GTPCH1 activity by 15% and 35%, respectively. Importantly, the inhibitory effects of TPEN (200 µM) were abolished by the addition of 10 µM zinc chlorite (Fig. 1F), indicating that the inhibitory effect of TPEN on GTPCH1 was likely due to zinc chelation.

ONOO− increases GTPCH1 binding to ubiquitin *in vitro*. Protein oxidation and the consequent conformational changes usually result in ubiquitination and degradation (19,20). As zinc is important not only for GTPCH1 activity but also in maintaining its structure (10), we reasoned that zinc release from GTPCH1 by ONOO− might increase ubiquitin binding. To test this hypothesis, FLAG-GTPCH1 was expressed in HEK293 cells and purified using anti-FLAG resin. The purity of FLAG-GTPCH1 was analyzed by SDS-PAGE using silver staining (data not shown). Purified proteins were further identified by immunoblotting (Fig. 2A). To examine whether ONOO− increased GTPCH1 ubiquitination, purified FLAG-GTPCH1 immobilized on anti-FLAG resin was first treated with ONOO− (10 to 100 µM) and then incubated with tetraubiquitin (Ub4). The Ub4 bound to FLAG-GTPCH1 (ONOO− or vehicle treated) was further analyzed by immunoblot. As shown in Fig. 2B&D, the recombinant FLAG-GTPCH1 for each experimental group is equal and ONOO− did not significantly change the FLAG-GTPCH1 amount on anti-FLAG resin. FLAG-GTPCH1, but not the negative control (anti-FLAG resin only), directly interacts with Ub4. Importantly, ONOO− increased the binding of FLAG-GTPCH1 to Ub4 in a dose-dependent manner (Fig. 2B, C). 100 µM ONOO− increased GTPCH1 binding to Ub4 by approximately 4-fold (Fig. 2C).
Zinc supplementation doesn’t affect ONOO-induced GTPCH1 ubiquitination. To test if zinc supplement prevented the effect of ONOO on GTPCH1, 10 µM of ZnCl2 was added prior or after ONOO⁻ addition. As shown in Fig. 2D, zinc supplementation failed to reverse ONOO⁻-induced GTPCH1 ubiquitination.

**NO but not ONOO⁻ increases the s-nitrosylation of GTPCH1 in intact cells.** GTPCH1-overexpressing HEK293 cells were exposed with DETANO or ONOO⁻ (100 µM). As depicted in Fig. 2E, DETANO but not ONOO⁻ markedly increased the detection of s-nitrosylated GTPCH1. As expected, pretreatment with NEM ablated NO-increased s-nitrosylation of GTPCH1 (Fig. 2E). In contrast, ONOO⁻ didn’t have any obvious effect on the s-nitrosylation of GTPCH1 in intact cells (Fig. 2F).

**Genetic zinc deletion from GTPCH1 affects its activity.** Because mutation of one of the amino acids in the GTPCH1 zinc binding site abolishes its zinc binding (21), we generated zinc-free C141R and C141A mutants. As shown in Fig. 3A, the zinc-free C141R and C141A mutants had barely detectable activity when compared to WT GTPCH1 (Fig. 3A). Although ONOO⁻ significantly inhibited GTPCH1 activity in WT, ONOO⁻ didn’t alter the activity of C141A and C141R GTPCH1 mutants (Fig. 3B).

**The zinc-deletion GTPCH1 mutant (C141R) exhibits reduced half-life.** To determine the stability of C141R, FLAG-GCH1 or C141R were transfected into HEK293 cells. After the transfection for 48 hours, the cells were treated with CHX (100 µg/mL). At the times indicated, cells were harvested to assay GTPCH1 levels. As shown in Fig. 3C, the half-life of C141R GTPCH1 was less than 1 hour, whereas WT GTPCH1 exhibited no significant change within 12 hours, suggesting that zinc deletion markedly reduces GTPCH1 stability.

**Increased ubiquitination in cells overexpressing GTPCH1 C141R mutants.** We next determined whether zinc affects GTPCH1 ubiquitination. To this end, FLAG-GTPCH1 or C141R were transfected into HEK293 cells with or without the co-expression of HA-Ub. HA-Ub chains bound to FLAG-GTPCH1 or C141R were detected
by co-immunoprecipitation (IP) assays using anti-FLAG resin. As shown in Fig. 3D, in the absence of HA-Ub, there was no detectable signal in the blot (Lane 1 and 3, Fig. 3D). When HA-Ub and FLAG-GTPCH1 were simultaneously overexpressed in HEK293 cells, HA-ubiquitin chains were found in the immunoprecipitates of FLAG-GTPCH1 (Lane 2, Fig. 3D), which indicates that GTPCH1 specifically associates with polyubiquitin chains. Importantly, genetic zinc deletion in GTPCH1 (FLAG-C141R) dramatically increased its association with HA-ubiquitin chains when compared to the co-expression of HA-Ub and WT FLAG-GTPCH1 (Fig. 3D).

**GTPCH1 C141R exhibits increased binding with tetraubiquitin in vitro.** Next, we determined how zinc removal affects the GTPCH1/Ubiquitin association. We assayed the affinity of purified GST-tagged WT GTPCH1 and the C141R mutant with Ub4 in pull down assays *in vitro*. Ub4 were pulled down by GST-GTPCH1 but not GST beads (Fig. 3E). In addition, when compared to WT GST-GTPCH1, GST-C141R dramatically increased binding to Ub4 *in vitro* (Fig. 3E), suggesting that zinc deletion in GTPCH1 increased its degradation, likely via increased binding to ubiquitin chains.

**ONOO¯ inhibits GTPCH1 activity in intact endothelial cells.** We next determined the effects of ONOO¯ on GTPCH1 in cultured bovine aortic endothelial cells (BAEC). As depicted in Fig. 4A, exposure of BAECs to ONOO¯ (50 µM) for 30 minutes caused approximately 60% inhibition of GTPCH1. Similarly, short exposure (1 hour) of BAEC to 3-morpholinosydnonimine N-ethylcarbamide (SIN-1), an ONOO¯ donor, lowered GTPCH1 activity by approximately 50% (Fig. 4B).

**ONOO¯ reduces GTPCH1 stability in endothelial cells.** To determine whether ONOO¯ affects the stability of GTPCH1, confluent endothelial cells were exposed to ONOO¯ (50 µM) or vehicle. After the treatment, cycloheximide (CHX, 100 µg/ml) was added to block *de novo* protein synthesis. The levels of GTPCH1 were unchanged in vehicle-treated BAECs during 4 hours of incubation. However, the levels of GTPCH1 in ONOO¯-treated BAECs were only approximately 20% of those in vehicle-treated cells (Fig. 4C). The half-life of GTPCH1 in ONOO¯-treated BAECs was reduced to
approximately 1.6 hours (Fig. 4D).

**ONOО^- destabilizes GTPCH1 via increased ubiquitination.** We next determined if the reduced GTPCH1 stability was due to the increased GTPCH1 ubiquitination. Ubiquitinated GTPCH1 was monitored in the presence or absence of MG132, a potent proteasome inhibitor. MG132 alone caused a slight but clear increase in ubiquitinated GTPCH1 (Fig. 5A). In the absence of MG132, ONOO^- (50 µM) did not alter the level of ubiquitinated GTPCH1 (Fig. 5A). In presence of MG132, ONOO^- (50 µM) markedly increased ubiquitinated GTPCH1 compared to the level in vehicle-treated cells (~6-fold, Fig. 5B). Importantly, ONOO^- induced ubiquitination disappeared in the absence of MG132, a potent proteasome inhibitor (Fig. 5A).

We also tested the effects of SIN-1 (1 mM) on GTPCH1 ubiquitination. In the absence of MG132, SIN-1 did not alter the levels of ubiquitinated GTPCH1 when compared to those in vehicle-treated cells (Fig. 5C). MG132 alone slightly increased the levels of ubiquitinated GTPCH1 (~2-fold, Fig. 5D). In the presence of MG132, SIN-1 markedly increased the levels of ubiquitinated GTPCH1 (~6-fold, Fig. 5D). Taken together, these data suggest that ONOO^- enhanced the GTPCH1 ubiquitination in endothelial cells, which caused its destruction and reduced half-life.

**High glucose increased GTPCH1 ubiquitination in endothelial cells.** There is evidence that exposure of endothelial cells to high glucose enhances the levels of both O_2^- and ONOO^-, which increased proteasome activity and caused GTPCH1 degradation in HUVECs (1). We first confirmed the high glucose effects on GTPCH1 protein level. High glucose (30 mM), but not an osmotic control (OG) dramatically reduced GTPCH1 protein levels in BAECs (Fig. 6A).

Next, we investigated the high glucose effects on GTPCH1 ubiquitination. In order to exclude the possible effects of proteasome, the high-glucose induced GTPCH1 ubiquitination was determined in the presence of MG132 (10 µM), which concentration almost completely inhibited proteasome activity in BAECs (data not shown), and blocked high glucose-induced GTPCH1 reduction (Fig. 6B). Importantly, high glucose markedly increased the levels of ubiquitinated GTPCH1 (~3-fold, Fig. 6C).
Anti-oxidant and ONOO⁻ scavenger attenuates high-glucose destabilized GTPCH1. Because the formation of ONOO⁻ requires the simultaneous formation of O₂⁻ and NO from NOS, inhibition of either O₂⁻ or NO production abolishes the formation of ONOO⁻ (1). It was important to further determine whether the high-glucose induced ONOO⁻ reduced GTPCH1 stability. As expected, high glucose markedly reduced both GTPCH1 protein levels (Fig. 7A, C & D) and GTPCH1 activity (Fig. 7B). Importantly, Tempo, a potent anti-oxidant, abolished the reduction of both GTPCH1 protein (Fig. 7A) and GTPCH1 activity (Fig. 7B). Overexpression of Mn-SOD (Fig. 7C) or Cu-Zn SOD (not shown) abolished this high glucose-induced GTPCH1 reduction, excluding the potential effects of hydrogen peroxide.

We next determined if ONOO⁻ was required for high glucose-induced GTPCH1 reduction and inhibition. L-NG-nitroarginine methyl ester (L-NAME) is a non-selective competitive NOS inhibitor. We tested if L-NAME abolished high glucose-induced GTPCH1 degradation. As shown in Fig. 7D, pharmacological inhibition of ONOO⁻ formation with L-NAME abolishes the GTPCH1 degradation induced by high glucose. Consistently, uric acid (UA), a potent scavenger of ONOO⁻, abolished the effects of high glucose on GTPCH1 protein (Fig. 7A) and GTPCH1 activity (Fig. 7B). Since uric acid (UA) or Tempo (Fig. 7A) or L-NAME (Fig. 7D) alone did not alter the levels of GTPCH1, these data suggest that high glucose-induced ONOO⁻ caused GTPCH1 inhibition and instability.

Diabetes triggers both ONOO⁻ and endothelial dysfunction in vivo. To further determine the effects of ONOO⁻ on GTPCH1 ubiquitination and degradation in vivo, C57BL6 mice were made diabetic by STZ injection. Diabetic mice were subsequently treated with vehicle, Tempo (in drinking water, 1 mmol/kg body weight) for 4 weeks. Injection of STZ significantly increased serum glucose whereas Tempo treatment did not alter the blood glucose levels in control mice or STZ-induced diabetic mice (Suppl. Table). Body weights in diabetic groups were 15% lower than non-diabetic mice but Tempo treatment didn’t alter body weight reduction in STZ-injected group (Suppl. Table).
We first determined acetylcholine-triggered endothelium-dependent vaso-relaxation. As expected, the maximal endothelial-dependent relaxation in STZ-injected diabetic mice was significantly reduced when compared to non-diabetic control mice (Fig. 8A). In contrast, the endothelium-independent relaxation in response to sodium nitroprusside (SNP) was not changed in STZ-injected diabetic mice with control non-diabetic mice (data not shown). Although Tempo alone had no effect on the endothelial dependent maximal relaxation (Fig. 8A), Tempo administration in STZ-injected diabetic mice normalized the maximal relaxation in STZ-injected mice (Fig. 8A).

Since eNOS is the main source of NO, it was important to determine if diabetes altered eNOS expression and the serine1177 phosphorylation of eNOS in isolated aortas. The expression of eNOS was markedly increased in STZ-injected aortas when compared to those from non-STZ injected mice (Fig. 8B). Tempo treatment abolished the increase of eNOS in STZ-injected mice (Fig. 8B). In contrast, despite increased eNOS expression in STZ-injected mice, the level of p-eNOS (serine 1177) was reduced in STZ-injected diabetic mice. However, Tempo had no effects on reduced levels of p-eNOS (Fig. 8B) in diabetic mice. Taken together, improved maximal relaxation by Tempo in diabetic mice was unlikely due to improved levels of p-eNOS or eNOS reduction.

It was also interesting to determine if improved maximal relaxation was due to increased production of superoxide anions. As shown in Fig. 8C and Suppl Fig 2, STZ significantly increased superoxide anions. Importantly, inhibition of eNOS with L-NAME significantly attenuated STZ-induced superoxide, suggesting that eNOS was the main source of superoxide. Consistently, in STZ-injected diabetic mice, 3-nitrotyrosine (NT)-positive protein, a foot-print of ONOO⁻ in tissues, was markedly increased (Fig. 8D), which was ablated in Tempo-treated STZ mice (Fig. 8D). Increased 3-NT was also confirmed in diabetic heart tissues. STZ-injected diabetes increased the levels of 3-NT and Tempo treatment lowered its levels of 3-NT in diabetic hearts (Suppl. Fig. 3A).

ONOO⁻ accelerates GTPCH1 ubiquitination and degradation in diabetic aortas and hearts in vivo. Next, we assayed GTPCH1 activity. As depicted in Fig. 8E and Suppl. Fig. 3B, GTPCH1 activity was inhibited by in both diabetic aortas and hearts.
Importantly, the administration of Tempo restored GTPCH1 activation in both aortas and hearts (Fig. 8E and Suppl. Fig. 3B).

Finally, we determined whether diabetes mellitus accelerated GTPCH1 ubiquitination. To this end, both aortic and heart tissue homogenates of STZ-induced diabetic mice or control mice were used for ubiquitin affinity precipitation (AP), and the co-precipitated proteins with ubiquitin chains were analyzed by western blot. Compared to control non-diabetic group, diabetic mouse aortas (Fig. 8F) and hearts (Suppl. Fig. 3C,3D,3E) exhibited higher levels of ubiquitinated GTPCH1 (~4-fold; Suppl. Fig. 3D). As a result, total levels of GTPCH1 in STZ-induced diabetic aortas and hearts had significantly lower levels of GTPCH1 in the homogenates of diabetic aortas (Fig. 8F) and diabetic hearts (Suppl. Fig. E). Taken together, the effects of Tempo in suppressing diabetes-enhanced acceleration of GTPCH1 ubiquitination and degradation and in improving maximal endothelial relaxation in diabetic mice is likely due to its inhibition of GTPCH1 activity in diabetes.

DISCUSSION

In this study, we have for the first time demonstrated that ONOO− releases zinc from GTPCH1 and that zinc removal lowers GTPCH1 activity. Further, zinc-deleted GTPCH1 exhibits increased ubiquitination and reduced stability. We found that ONOO− generated by high glucose suppresses GTPCH1 activity along with increased ubiquitination and destruction of this enzyme. Finally, GTPCH1 ubiquitination and destruction is markedly increased in parallel with enhanced ONOO− in STZ-induced diabetic mice in vivo. Overall, our results suggest that ONOO− releases zinc, inhibits GTPCH1 activity, and increases GTPCH1 ubiquitination.

The major finding of this study is that ONOO− removes the zinc in GTPCH1, resulting in enzyme inhibition. Several lines of evidence are consistent with the hypothesis that loss of zinc by ONOO− oxidation underlies the inactivation of GTPCH1. First, ONOO− dose-dependently releases zinc from GTPCH1 (Fig. 1B). Second, the effects of ONOO− are mimicked by TPEN, a selective zinc-chelating agent. Finally, GTPCH1 (C141R), a common genetic variant in humans (22) which lacks the essential zinc binding ability due to the substitution of one cysteine, is inactivated. Our results
further demonstrate that GTPCH1 zinc is also essential in preventing its ubiquitination and destruction. The ubiquitination-proteasome system is usually considered to degrade unneeded or damaged protein (23,24). Zinc removal resulted in GTPCH1 structure change (10). Modified proteins with the changed conformation may lead to rapid degradation by facilitating their ubiquitination (19,20). In the present study, our results suggest that GTPCH1 zinc removal by ONOO\textsuperscript{−} increased its ubiquitination via direct polyubiquitin binding. We further provided the evidence that ONOO\textsuperscript{−} dramatically increased GTPCH1 ubiquitination and the ubiquitinated GTPCH1 shows accelerated destruction. Importantly, C141R, mimicked zinc loss of GTPCH1, showed dramatically increased ubiquitination, reduced GTPCH1 protein stability, and accelerated degradation.

Zinc-containing proteins or transcription factors might be important targets for anionic oxidants such as ONOO\textsuperscript{−}. In addition to ONOO\textsuperscript{−}-mediated GTPCH1 oxidation described above, zinc-containing eNOS is also a target of ONOO\textsuperscript{−} (11). Although both eNOS and GTPCH1 contains zinc, zinc in eNOS binds to 4 cysteines (defined as zinc-thiolate cluster) whereas zinc in GTPCH1 binds to two cysteines, and one histidine (11). In addition, zinc atom has no catalytic activity in eNOS in which the zinc atom bound two eNOS monomers at dimer interface whereas zinc in GTPCH1 is essential in guanine ring opening of GTP. Because of these structural and functional differences, ONOO\textsuperscript{−}-induced modifications have different impacts in the enzymes. For example, ONOO\textsuperscript{−}-induced zinc release in eNOS doesn’t lead to its ubiquitination or degradation (11) whereas zinc release from GTPCH1 dramatically increases ubiquitination-dependent degradation, as shown in this study. This reaction of ONOO\textsuperscript{−} appears to be unique because NO alone did not affect zinc-thiolate cluster. Conversely, NO but not ONOO\textsuperscript{−} markedly increased the detection of s-nitrosylated GTPCH1 whereas ONOO\textsuperscript{−} didn’t have any obvious effect on the s-nitrosylation of GTPCH1 in intact cells. Thus, the mechanism by which ONOO\textsuperscript{−}-induced zinc release results in GTPCH1 ubiquitination represents a novel post-translational modification of zinc-containing proteins.

Increased eNOS-derived O\textsubscript{2}•\textsuperscript{−} and ONOO\textsuperscript{−} have been demonstrated in cultured endothelial cells exposed to high glucose, and in diabetic animals and human blood vessels (11,25,26). Studies have indicated that hyperglycemia impairs GTPCH1 activity
and reduces GTPCH1 protein levels without any change in mRNA levels (1,27). Our previous report also indicated that hyperglycemia-derived GTPCH1 inactivation is not caused by GFRP (1). Therefore, the posttranslational modification of GTPCH1 is likely the major cause for diabetes-induced GTPCH1 loss. We have previously reported that tyrosine nitration of the proteasome by ONOO− produced during hyperglycemia or by angiotensin II increased proteasome activity and accelerated GTPCH1 degradation (1,5). However, the mechanism that caused the preferential degradation of GTPCH1 by proteasome is unknown yet. In this study, we extend our early observations and found that GTPCH1 is a direct target for hyperglycemia-induced ONOO− in diabetes mice. Major evidence in support of this notion includes the following: first, we have identified GTPCH1 as a direct target of ONOO− in vitro and in cultured endothelial cells; second, exposure to high glucose increases GTPCH1 ubiquitination, and ONOO− scavenger attenuates hyperglycemia-induced GTPCH1 inactivation and degradation; third, STZ-induced hyperglycemia greatly accelerates GTPCH1 ubiquitination and associated with the increased ONOO− in diabetic mice, which was prevented by administration of Tempo in parallel with the impaired ONOO−; Finally, the effects of Tempo in suppressing diabetes-enhanced acceleration of GTPCH1 ubiquitination and degradation might be due to its inhibition of GTPCH1 oxidation in diabetes. Taken together, our results suggested that zinc in GTPCH1 renders it a direct target for ONOO−, resulting in zinc loss and the consequent inactivation and ubiquitination. Since the 26S proteasome is also a target of ONOO− and tyrosine nitration of the proteasome by ONOO− increased proteasome activity in HUVECs (1), the combinational effects of ONOO− on GTPCH1 ubiquitination and proteasome results in acceleration of GTPCH1 reduction in diabetes. Indeed, we found that GTPCH1 protein level is lowered in STZ-induced diabetic mice in parallel with increased ubiquitination. Importantly, Tempo, which prevents ONOO− production during hyperglycemia, not only prevents GTPCH1 ubiquitination and degradation, but also restores GTPCH1 activity.

GTPCH1 degradation or loss of activity results in a rapid decrease in BH4 (1), which is commonly observed in cardiovascular disorders (1,2,5,7,15). BH4 deficiency has been linked to many cardiovascular disorders, including diabetes mellitus (1-4), and hypertension (5-7,15). For example, diabetic rat (27) and mouse (1) models suggest
that reduced BH4 results from decreased GTPCH1 expression and activity without alterations in GTPCH1 mRNA levels (1). Administration of BH4 (28,29) or GTPCH1 gene transfer (15,30) increases BH4 bioavailability and restores endothelial function in diabetes. In deoxycorticosterone acetate (DOCA)-salt hypertensive rats, GTPCH1 activity is decreased during the late stages of hypertension. As a result, eNOS is uncoupled in DOCA-salt hypertension because of the reduced essential cofactor BH4. Arterial gene transfer of GTPCH1 restores GTPCH1 activity, restores BH4 levels, and normalizes eNOS function in these animals (31). Accumulating evidence has revealed that, during cardiovascular diseases, eNOS becomes "uncoupled," leading to production of superoxide anions (O_2^-) rather than NO. The transformation of eNOS from a protective enzyme to a contributor of oxidative stress has been observed in vitro and in vivo, including in patients with cardiovascular risk factors (32,33).

Increasing evidence suggest that loss of BH4 appears to be a common route for endothelial dysfunction in vivo in diabetes and oral supplementation of BH4 in human type II diabetes improves endothelial function (28,34) and insulin sensitivity (35). In type II diabetic rats, renal BH4 is considered to play a crucial role in the pathogenesis of diabetic nephropathy (36). Thus, oxidants-induced GTPCH1 ubiquitination and consequent proteasome-mediated degradation represents a common pathway for BH4 loss. Thus, ONOO^- scavengers, which might be effective in preserving GTPCH1 and BH4 in endothelial cells, might be effective in treating diabetic vascular diseases.

In conclusion, ONOO^- releases zinc, inhibits GTPCH1 activity, and increases GTPCH1 ubiquitination. Our data support that scavenging of ONOO^- might help prevent GTPCH1 inactivation and ubiquitination and endothelial dysfunction in diabetes.
Acknowledgements

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No potential conflicts of interest relevant to this article were reported.

Y.Z. and J.W. contributed to the study design, performed experiments, and wrote the manuscript. H.P.Z performed some experiments. P.S. performed experiments. M.H.Z. contributed to the study design and interpretation and wrote the manuscript. M.H.Z is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
REFERENCES

**Invest 117**, 2658-2671


FIGURE LEGENDS

Figure 1
ONOOGTPCH1 activity, but increases its ubiquitination associated with zinc release. **A.** Purification of recombinant FLAG-GTPCH1. The purity of FLAG-GTPCH1 was analyzed by SDS-PAGE using silver staining. **B.** Effect of ONOO- on purified recombinant GTPCH1 activity. The enzyme activity of GTPCH1 was determined by high-performance liquid chromatography (HPLC). Data are shown as mean±standard deviation (n=6). *P<0.05 vs. control. **C.** Effect of nitric oxide (NO) on recombinant GTPCH1 activity. **D.** NO but not ONOO- increases the S-nitrosylation of GTPCH1. **E.** ONOO- stimulated zinc release from recombinant GTPCH1. Zinc was assayed as described in Experimental Procedures and was expressed as percentage of maximal zinc release from GTPCH1 diluted in 7 mol/L guanidine HCl. n=3; *P<0.05 vs. control. **F.** The effect of the zinc chelator TPEN on GTPCH1 activity. n=3; *P<0.05 vs. control; #P<0.05 vs. +TPEN 200 µM.

Figure 2
ONOOGTPCH1 ubiquitination. **A.** Purification and identification of FLAG-GTPCH1 proteins. FLAG-GTPCH1 plasmids were transfected to HEK293 for 2 days and the proteins were analyzed by immunoblotting using anti-FLAG antibody or antibody specific for GTPCH1. **B & C.** Recombinant FLAG-GTPCH1 bound to anti-FLAG resin was treated with ONOO- (10 to 100 µM, separately) and incubated with tetraubiquitin chains (Ub4). Bound proteins were detected by immunoblotting. **D.** Effect of zinc supplementation (zinc chloride 10 µM) on ONOO-induced GTPCH1 ubiquitination. **E.** Effects of NO and ONOO- on GTPCH1 s-nitrosylation in purified recombinant GTPCH1. CTR (-): No Biotin label; CTR (+): Vehicle (NaOH) treated and Biotin labeled; ONOO- (100uM), DETANO 200uM; SPERNO 5mM or 1mM, and then applied for Biotin-switch assay. **F.** Effects of NEM on NO- or ONOO- induced S-nitrosylation. The blot is a representative of three to five blots from three to five individual experiments.
Figure 3
Genetic deletion of zinc from GTPCH1 impairs its activity and accelerates GTPCH1 degradation and ubiquitination via direct ubiquitin binding. **A.** Enzyme activity of recombinant GTPCH1 and zinc-deleted GTPCH1 (C141A, C141R). FLAG-tagged WT or C141A- or C141R GTPCH1 plasmids were constructed and expressed in HEK293 cells as described in Experimental Procedures. Recombinant WT GTPCH1 or the C141A or C141R mutants were purified by anti-FLAG resin and subjected to activity assay by HPLC. n=3; *P<0.05 vs. control. **B.** Effects of ONOO• on GTPCH1 activity of GTPCH1 WT, C141A or C141R mutant. **C.** Representative blots of three independent experiments showing the protein stability of C141R. HEK293 cells were transfected with FLAG-C141R or -GTPCH1 plasmids. Twenty-four hours after transfection, cells were incubated with cycloheximide (CHX, 100 µg/mL) and, at the indicated times, cells were harvested, lysed, and analyzed for GTPCH1 levels by using an anti-FLAG antibody. In all cases, β-actin levels were measured as a loading control. **D.** C141R ubiquitination in cultured cells. HEK293 cells were co-transfected with FLAG-tagged WT or C141R GTPCH1, and HA-tagged ubiquitin plasmid or empty vector. Cell lysates were immunoprecipitated by anti-FLAG resin and immunoblotted using the indicated antibodies. **E.** Effects of C141R ubiquitination *in vitro*. Purified recombinant GST-GTPCH1 (WT) or GST-C141R proteins were incubated with tetraubiquitin chains. Bound ubiquitin chains were separated by SDS-PAGE and analyzed by immunoblotting. The blot shown is representative of three independent experiments. Data are shown as mean ± standard deviation (n=3). *P<0.05 vs. control.

Figure 4
**ONOO• inhibits the activity but increases the degradation of GTPCH1 in endothelial cells. A.** The effect of ONOO• on GTPCH1 activity in BAECs. Confluent BAECs were starved overnight and treated with ONOO• (1, 10, or 50 µM, separately) or equal volume of vehicle (0.1 M NaOH) for 15 minutes in 0.1 M HEPES buffer. HPLC was used to determine the GTPCH1 enzyme activity. n=3; *P<0.05 vs. control. **B.** BAECs were treated with SIN-1 for indicated time. n=3; *P<0.05 vs. control. **C & D.** Effect of ONOO• on GTPCH1 stability. BAECs were treated with 50 µM ONOO• or
vehicle for 15 minutes and then with CHX (100 µg/mL). Cells were harvested at the indicated time points in presence of CHX and the lysate were subjected to immunoblotting using anti-GTPCH1 and anti-β-actin antibodies. The blot shown is representative of three independent experiments.

Figure 5

**ONOÖ⁻ increases GTPCH1 Ubiquitination of in intact endothelial cells. A & B.** The effect of ONOO⁻ on GTPCH1 ubiquitination in cultured BAECs. Confluent BAECs were pretreated with the 26S proteasome inhibitor MG132 (0.5 µmol/L) or an equivalent volume of dimethyl sulfoxide (DMSO) (control) for 30 minutes, and treated with ONOO⁻ (50 µM) or vehicle in 0.1 M HEPES buffer. BAEC lysates were incubated with UIM-agarose. GTPCH1 that was affinity-precipitated with ubiquitin was analyzed by immunoblotting using anti-GTPCH1 and anti-β-actin antibodies. AP indicates affinity precipitation. Ub: ubiquitin; IB: immunoblot; C & D. SIN-1 on GTPCH1 ubiquitination. BAECs were pretreated with MG132 or DMSO for 30 minutes and treated with 1 mM SIN-1 (ONOÖ⁻ donor). The blot shown is a representative of three independent experiments.

Figure 6

**High glucose increases GTPCH1 ubiquitination and degradation. A.** High glucose increases GTPCH1 degradation. Confluent BAECs were treated with normal-glucose (NG) medium (D-glucose, 5 mmol/L), osmotic glucose (OG: D-glucose 5 mmol/L, L-glucose 25 mmol/L), or high glucose (HG; D-glucose, 30 mmol/L) and the cells were harvested, lysed, and analyzed for GTPCH1 levels by immunoblotting. **B & C.** High glucose increases GTPCH1 ubiquitination. BAECs were pre-treated with MG132 (0.5 µM) for 30 minutes and treated with NG medium (D-glucose, 5 mM) or HG (D-glucose, 30 mM) in the presence of MG132 (0.5 µmol/L) for 6 hours. Cell lysates were incubated with UIM-agarose for ubiquitin affinity precipitation (AP). Ubiquitinated proteins were analyzed by immunoblotting with anti-GTPCH1 and anti-β-actin antibodies. The blot shown is representative of three independent experiments.
Figure 7
ONOO$^-$ scavenger and anti-oxidant attenuates high-glucose-induced GTPCH1 degradation via endogenous ONOO$^-$ in endothelial cells. **A.** Uric acid (UA, 100 µM) or Tempo (10 µM) prevented GTPCH1 degradation. BAECs were pre-treated with UA or Tempo for 30 minutes and treated with NG medium (D-glucose, 5 mM) or HG (D-glucose, 30 mM) for 3 days. **B.** GTPCH1 activity. n=3; *P<0.05 vs. NG control; ***, #P<0.05 vs. HG only. **C.** Adenoviral overexpression of Mn-SOD prevents GTPCH1 degradation. BAECs were transformed with adenoviral vector GFP or Mn-SOD for 16 hours and treated as indicated for 3 days. **D.** L-NAME (1 mM) prevents GTPCH1 reduction. The blot shown is representative of three independent experiments.

Figure 8
ONOO$^-$ is involved in GTPCH1 inhibition and GTPCH1 ubiquitination in diabetic mouse aortas in vivo. Control (11 mice) and STZ-induced diabetic mice (12 mice) were fed Tempo as described in Experimental Procedures. **A.** Tempo treatment normalizes the maximal endothelium-dependent vaso-relaxation in diabetes mice. **B.** Effects of Tempol treatment on total eNOS and the serine 1177 phosphorylation of eNOS in control and STZ-injected diabetic mice; **C.** Tempo treatment suppresses diabetes-enhanced superoxide production; **D.** Tempo ablates 3-NT formation in diabetic mouse aortas; **E.** Tempol abolishes diabetes-induced GTPCH1 inhibition in diabetic mouse aortas; **F.** Effects of diabetes and Tempo treatment on GTPCH1 ubiquitination in vivo. The total GTPCH1 and ubiquitinated GTPCH1 were assayed in isolated mouse aortas. The blot is a representative of six blots obtained from six independent experiments. n=6).
Diabetes
Diabetes
24x16mm (300 x 300 DPI)
Online Supplemental Material to

Peroxynitrite-dependent Zinc Release and Inactivation of Guanosine 5'-triphosphate Cyclohydrolase 1 instigates its Ubiquitination in Diabetes

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Materials. Antibodies against ubiquitin and the GST-tag were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). MG132 was purchased from BioMol (Plymouth Meeting, PA). UIM-agarose was obtained from Boston Biochem Inc. (Cambridge, MA). Glutathione (GSH) beads were from Amersham Pharmacia Biotech (Uppsala, Sweden). Full-length human GTPCH1 cDNA (GCH1) and lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA) and QuikChange site-directed mutagenesis kits were obtained from Stratagene (La Jolla, CA). The restriction enzymes EcoRI and SalI were from New England Biolabs (Ipswich, MA). Qiaquick DNA isolation kits were from Qiagen (Valencia, CA). The S-nitrosylation kits with the biotin-switch were from Cayman Chemicals (Ann Arbor, MI). All other chemicals, if not indicated, were purchased from Sigma-Aldrich.

Assays of zinc release. Release of zinc from GTPCH1 was measured by the 4-(2-pyridylazo)resorcinol (PAR) assay as described previously (1). All buffers used for the zinc assay were pretreated with Chelex 100 to remove background Zn^{2+}. To determine the total zinc content of untreated GTPCH1, maximal zinc release was determined by diluting GTPCH1 proteins in guanidine HCl (7 mol/L). The extinction coefficient for PAR\cdot Zn^{2+} was experimentally determined to be 70 mM^{-1}cm^{-1} using a known concentration of zinc chloride.
**Immunoprecipitation and immunoblotting.** Cells were harvested and re-suspended in lysis buffer, and immunoprecipitated using anti-FLAG resin (Sigma). Western blotting was carried out using standard techniques with specific antibodies.

**In vitro pull-down assay.** Tetraubiquitin (Ub4) was used in binding assays (2) with GST-GTPCH1 or mutant immobilized on GSH beads. Ub4 chains were also used in FLAG-GTPCH1 pull-down assays with recombinant FLAG-GTPCH1 immobilized on anti-FLAG resin.

**Enrichments of ubiquitinated proteins.** Ubiquitinated proteins were enriched by ubiquitin affinity beads (UIM-agarose). These beads are conjugated to an ubiquitin-associated domain to facilitate their binding to ubiquitinated proteins. Cell lysates or tissue homogenates were incubated with UIM-agarose overnight at 4°C.

**Determination of GTPCH1 half-life assay.** Cultured cells were treated with cycloheximide (CHX, 100 µg/mL), and lysed at the indicated time points in the presence of CHX. Total proteins were subjected to analysis by Western blotting.

**Assays of the endothelium-dependent and –independent relaxation.** Aortic rings isolated from the treated mice were subjected to organ chamber assay of endothelium-dependent and -independent vasodilation as described previously (3,4).

**Detection of reactive oxygen species.** Intracellular O$_2^-$ was assessed by the DHE fluorescence/HPLC assays, as described previously (3,4).

**References**


**Supplemental Table: Effects of Tempo on body weights and blood glucose in control and STZ-injected mice**

<table>
<thead>
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<th>Control</th>
<th>STZ</th>
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<tr>
<td></td>
<td>w/o Tempo</td>
<td>with Tempo</td>
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<tr>
<td>Body weight (g/dl)</td>
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<td>23.3 ± 0.2</td>
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<tr>
<td>Glucose (mg/dl)</td>
<td>153 ± 29</td>
<td>169 ± 25</td>
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</table>
**Supplemental Figure 1.** Representative chromatograms for HPLC measurement of GTPCH1 activity. A. Neopterin (Sigma, 1 µM) was used for standard for HPLC measurement of GTPCH1 activity. B. Buffer only as blank for negative control. C. GTPCH1 activity. 0.4 µM recombinant GTPCH1 plus 2 mM GTP was incubated in 37 °C for 2 hours. D. 0.4 µM recombinant GTPCH1 was pretreated 50 µM ONOO– on ice for 5 minutes and then plus 2 mM GTP was incubated in 37 °C for 2 hours.
Supplemental Figure 2
Representative chromatograms for HPLC measurement of superoxide production (2-hydroxyethidium and ethidium) in diabetes.
Supplemental Figure 3

ONO-O^-induced GTPCH1 modification and ubiquitination in diabetic mouse hearts in vivo Control and STZ-induced diabetic mice were given Tempo as described in Experimental Procedures. Mouse hearts from control and diabetic mice were isolated and assayed for A (right panel) to E. A. 3-NT-positive proteins; B. GTPCH1 activity; C, D, E. Effects of Tempol on GTPCH1 and ubiquitinated GTPCH1 in isolated mouse aortas from non-diabetic and diabetic mice in vivo. The blot is a representative of three blots obtained from 5 mice. Results were obtained from five mice. *P<0.05 vs. control. #P<0.05 vs. STZ. NS indicates P>0.05 vs. control.