Published in final edited form as:

Free Radic Biol Med. 2009 March 15; 46(6): 828. doi:10.1016/j.freeradbiomed.2008.12.021.

Characterization of oxidized guanosine 5'-triphosphate (oxo⁸GTP) as a viable inhibitor of soluble guanylyl cyclase (sGC)

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Abstract

The guanine base is liable for oxidation by free radicals regardless of the cellular moiety it is bound to. However, under conditions of oxidative stress, oxo⁸GTP formation has been shown to occur without oxidation to the guanine base in DNA. In vitro studies have suggested that oxo⁸GTP could impact G-protein signaling and RNA synthesis. Whether increased levels of oxo⁸GTP translate into cellular malfunction is unknown. Data presented herein shows that oxo⁸GTP is formed in cell-free preparations as well as in PC12 cells after exposure to physiologically relevant oxidative conditions generated with 10 μ M copper sulphate and 1 mM L-Ascorbic Acid (Cu/Asc). We also determined that oxo⁸GTP has biological activity as a potent inhibitor of nitric-oxide stimulated soluble guanylyl cyclase (sGC). The increase in oxo⁸GTP formation in purified GTP and PC12 cells exposed to Cu/Asc caused a significant reduction in the product of sGC activity, cGMP. This oxidation of GTP was attenuated by addition of reduced glutathione under these same Cu/Asc conditions thus preventing the decrease in sGC activity. This suggests that oxo⁸GTP is produced by free radicals *in vivo* and could have significant impact on cell functions regulated by sGC activity such as synaptic plasticity in the central nervous system.

Keywords

Oxidative stress; guanosine triphosphate; GTP; 8-oxoguanosine triphosphate; oxo⁸GTP; soluble guanylyl cyclase; sGC; guanosine; 8-oxoguanosine; oxo⁸G

Introduction

Oxidative stress has been implicated in the onset and development of several pathological processes including cancer and age-related neurodegenerative diseases such as Alzheimer's Disease (AD) and Parkinson's Disease [1–5]. Identifying the role of oxidative stress in pathological conditions is often accomplished by probing cellular DNA to quantify increased levels of oxidized guanine, reported as 8-oxo-2'-deoxyguanosine (oxo⁸2dG), employed as a cellular biomarker [6–8]. However, the percent of oxo⁸2dG relative to unmodified 2'-deoxyguanosine (2-dG) in DNA in tissues from pathologies such as Alzheimer's Disease and Parkinson's Disease is quite low, ranging from 0.002% to 1% [1,2,9]. This implies that nuclear guanosine (G) moieties found in DNA may not be the best place to quantify oxidative insult.

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Increases in oxo⁸dG levels in DNA can also occur after hydroxyl radical (OH[•]) attack to the cytosolic pool of guanines present in 2-dG 5'-triphosphate (dGTP). This oxidative modification produces the respective oxidized adduct oxo⁸dGTP, which can be incorporated into DNA during replication or repair [10]. Despite evidence of the oxidation to G in GTP, forming oxo⁸GTP, and the fact that GTP levels are 50 to 100 times larger in cells than dGTP, little attention has been given to the biological consequences of its formation [11,12]. Using a recently developed HPLC method, it has been shown that under basal conditions the percent of cytosolic oxo⁸GTP relative to GTP in human embryo kidney 293T (HEK 293T) cells was more than 400 times greater than the percent of oxo⁸2dG relative to 2-dG in nuclear DNA. Additionally, after exposure to an oxidative challenge, there was significantly increased levels of oxo⁸GTP formed without a change in the levels of oxo⁸2dG in DNA [13]. These findings identify oxo⁸GTP as a more sensitive indicator of oxidative stress than oxo⁸2dG and a likely candidate for understanding the consequences of oxidative damage to cell function.

GTP participates in several critical physiological functions including RNA synthesis, cell signaling through activation of GTP-binding proteins, as well as the production of the second messenger cyclic guanosine monophosphate (cGMP) [14–16]. Recent studies have shown that addition of oxo⁸GTP to *in vitro* transcription reactions reduces the total amount of mRNA synthesized as well as increases the number of mutations in the mRNA produced [17]. Additionally, oxo⁸GTP was shown to increase the activity of the GTP-binding protein Ras as well as downstream activators of the Ras-ERK pathway in HEK293T cells, while the opposite effect was observed with the GTP-binding protein Rac1 [18]. Given these reported effects of oxo⁸GTP and our verification of its formation in cells, we opted to determine the role of oxo⁸GTP on the activity of soluble guanylyl cyclase (sGC) [E.C. 4.6.1.2.]. In the presence of NO, sGC drives the conversion of GTP to cGMP, a pathway important for a range of physiological processes such as vasodilation, platelet aggregation, and neurotransmission [19]. Alterations in the capacity of sGC to respond to NO, level of expression of sGC, as well as sGC activity have been associated with physiological deficiencies that may play a role in the pathologies of a variety of disorders including cardiovascular disease and neurological diseases such as Alzheimer's Disease (AD) and Creutzfeldt-Jakob disease [20–23]. Despite evidence of oxidative stress as a major player in these pathologies and evidence of GTP being a target for free radical attack, there have been no studies aimed at identifying the consequences of oxo⁸GTP on sGC activity [24].

We hypothesize that the GTP pool is vulnerable to conditions of oxidative stress and the formation of oxo⁸GTP will impact cellular function, specifically the activity of sGC. Our results support this hypothesis, identifying oxo⁸GTP as an inhibitor of purified and endogenous sGC in PC12 cells. Our results also make evident the fact that oxo⁸GTP can be used as cellular biomarker of oxidative stress with deleterious biological consequences.

Materials and Methods

Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated. 8-oxoguanosine-5'-triphosphate (oxo⁸GTP) was purchased from TriLink Biotechnologies (San Diego, CA). 8-hydroxy guanosine (oxo⁸G) was obtained from Cayman Chemical (Ann Arbor, MI). Soluble guanylyl cyclase (sGC) isolated from bovine lung, DEA NONOate (DEA/NO), and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Alexis Biochemicals (Lausen, Switzerland). Ultrapure water was obtained from a Milli-Q UF-Plus apparatus (Millipore, Billerica, MA).

cGMP Determination by HPLC-EC

cGMP was resolved by HPLC with a reverse phase YMC basic column (4.6×150 mm; particle size 3-micron) (YMC Inc., Wilmington, NC) and quantified using a CoulArray electrochemical detection system (ESA, Inc., Chelmsford, MA). An isocratic mobile phase consisting of 100 mM sodium acetate, pH 5.2, 4% Methanol (HPLC Grade) diluted in water polished with C18 Sep-Pak cartridges (Waters Corp., Milford, MA) was utilized to elute cGMP from the column. The mobile phase was filtered using $0.2 \mu\text{m}$ nylon filters and degassed by sonication before use with the HPLC. Potentials of the twelve coulometric analytical cells of the CoulArray system, placed in series, were as follows: 50, 125, 175, 200, 250, 380, 500, 700, 785, 850, 890, 900 mV. Data were recorded, analyzed, and stored using CoulArray for Windows data analysis software (ESA Inc., Chelmsford, MA). A calibration curve was generated from standards of cGMP ranging from 5 nanograms (13.6 picomoles) to 1 milligram (2.7 nanomoles). Elution of the cGMP analyte was monitored in the 700, 785, and 850 mV channels and injected amounts were plotted relative to the sum of the peak areas.

Measurement sGC activity in cell-free and PC12 extract samples

5 ng of purified sGC from bovine lung was incubated with 1 mM GTP and stimulated with increasing concentrations of DEA/NO ($0.001 \mu\text{M}$ to $100 \mu\text{M}$ diluted in ice-cold 10 mM NaOH) in buffer containing 3 mM MgCl_2 , 0.5 g/L BSA, 50 mM TEA, 3 mM DTT, pH 7.4 at 37°C for 10 minutes. The reaction was stopped by placing on ice and immediately adding 0.5 N HClO_4 to a final concentration of 0.05 N HClO_4 . Samples were mixed and centrifuged at $14,000 \times g$ at 4°C for 15 minutes. Supernatants were then subjected to $0.45 \mu\text{m}$ filtration and $10 \mu\text{L}$ was then immediately injected into the HPLC-EC.

PC12 cells were seeded at a density of $5\text{--}7 \times 10^7$ cells per plate in 10 mL of RPMI 1640 medium containing 5% fetal bovine serum (FBS), 10% horse serum (HS), 2 mM L-glutamine, 1mM sodium pyruvate, 25 units penicillin G, and $25 \mu\text{L/mL}$ of streptomycin. Cells were grown until 80–90% confluent (about 4 days) on collagen coated 100 mm petri dishes maintained at 37°C , 5% CO_2 , and 95% air atmosphere. Following a 10 minute incubation at 37°C with media containing 1mM IBMX, cells are incubated with increasing concentrations of DEA/NO ($0.001 \mu\text{M}$ to $100 \mu\text{M}$) at 37°C for 10 minutes. Prior to cGMP extraction, these PC12 cells were assessed for viability using the trypan blue exclusion method. The media was then replaced with 5 mL of ice-cold phosphate-buffered saline (PBS) containing 1mM IBMX prior to collecting cells and centrifuging at 1500 rpm at 4°C for 5 minutes. The cells were then washed with $300 \mu\text{L}$ of ice-cold PBS, sonicated, mixed, and centrifuged at $14,000 \times g$ at 4°C for 15 minutes. A $10 \mu\text{L}$ aliquot was set aside for Bradford protein analysis and $30 \mu\text{L}$ of 0.5 N HClO_4 was then added to remaining supernatant and centrifuged at $14,000 \times g$, 4°C for 15 minutes. After $0.45 \mu\text{m}$ filtering, the $27 \mu\text{L}$ of supernatant was immediately injected into the HPLC-EC. cGMP was quantified in each sample based on calibration curves generated from known quantities of cGMP.

Measurement of GTP and Oxo⁸GTP

GTP and Oxo⁸GTP were isolated and quantified using HPLC-EC with preparative dephosphorylation as described previously [13]. Briefly, PC12 cells grown under standard conditions described above were exposed to normal media (control) or media containing 1mM L-ascorbic acid and $10 \mu\text{M}$ copper sulfate (Cu/Asc) for 4 hours. After washing three times with ice-cold PBS, cells were collected in $300 \mu\text{L}$ of PBS. Following centrifugation, protein was precipitated using $30 \mu\text{L}$ of 60% Trichloroacetic Acid (TCA), vortex-mixed for 20s, placed in an ice-bath for 10 minutes, and vortex-mixed again for 20s. Cell extracts were then centrifuged at 13,000 rpm for 10 minutes at 4°C . The resulting supernatant was neutralized to pH 6.0 with a solution containing 3 M KOH and 3 M KHCO_3 . GTP and oxo⁸GTP were then dephosphorylated to their respective nucleosides guanosine (G) and 8-oxoguanosine (oxo⁸G).

for detection via HPLC-EC. On ice, 25 Units of alkaline phosphatase (dissolved in Tris-HCl pH 8.0), 1.8 mM sodium acetate, and 100mM Tris-HCl, were added to 30 μ L of sample in total volume of 60 μ L. After incubation at 37°C for 1 hour, the dephosphorylation reaction was stopped by placing on ice. This was followed by filtering through Ultrafree-MC (30-kD) tubes (Millipore Corp., Bedford, MA). Detection of the generated nucleosides (G and oxo⁸G) was carried out by injecting 27 μ L of the filtrate into the HPLC-EC using exactly the same HPLC-EC conditions described above. Guanosine elution was monitored in the 700, 785, and 850 mV channels and injected amounts were graphed relative to the sum of peak areas. Oxo⁸G was monitored in the 250 mV channel and injected amounts were graphed relative to peak area. Calibration curves for G and Oxo⁸G were generated from known quantities of each analyte ranging from 120 pmoles to 1.92 nmoles for G and 2.4 pmoles and 38.4 pmoles for oxo⁸G. G and oxo⁸G quantified in samples were reported as GTP and oxo⁸GTP respectively.

Statistical Analysis

All data were analyzed using Prism GraphPad 4.0 software (Graph pad Software Inc., San Diego, CA). Calibration curve parameters for each of the analytes were obtained by linear regression analysis. Double reciprocal kinetic plots (Lineweaver-Burke) were analyzed using linear regression analysis. An unpaired t-test was performed on GTP, oxo⁸GTP, and cGMP values collected from PC12 cells exposed to Cu/Asc; values denoted with an asterisk are significantly different from their corresponding controls ($P < 0.05$). A one-way ANOVA with Newman-Keuls Multiple Comparison Test post-hoc analysis was performed on *in vitro* GTP oxidation experiments with and without GSH; values denoted with an asterisk are significantly different from their corresponding controls ($P < 0.05$).

Results

Assay for sGC Activity Using HPLC-EC

In order to measure sGC activity with the highest specificity and sensitivity, we developed a chromatographic analysis protocol employing HPLC-EC to detect cGMP and determine sGC activity in cell-free preparations with purified sGC and in cell culture, using PC12 cells. sGC activity was defined as the amount of cGMP formed in a 10 minute reaction at 37°C after the addition of DEA/NO to activate sGC. Figure 1A is representative of a typical injection of cGMP standard (40 ng) into the HPLC system; Figure 1B is representative of cGMP detection in an acid extract from DEA/NO stimulated PC12 cells. For validation of the linear response of this system, calibration curves ranging from 1 ng to 1 μ g are presented (Figure 1C). The response was linear up to 4 milligrams (10.9 nanomoles) of injected cGMP with a minimum detection limit of 5 picograms (13.6 femtomoles).

To test the validity of this approach to determine sGC activity, we stimulated purified sGC and PC12 cells with the NO donor DEA/NO. Figure 2A demonstrates that, after stimulation of purified sGC, levels of cGMP increased with a typical sigmoidal response to increasing concentrations of DEA/NO. cGMP was produced in a dose-dependent manner with maximal stimulation at 10 μ M DEA/NO and an EC₅₀ of 266 nM, values similar to previously reported values of sGC activity measured in cell-free preparations by radioimmunoassay [25]. Figure 2B illustrates the response of endogenous sGC in PC12 cells, which yielded a similar concentration-response with increasing amounts of DEA/NO. Maximal stimulation was achieved by 50 μ M DEA/NO and an EC₅₀ was determined to be 5.7 μ M. Unstimulated, basal levels of cGMP were below detection limits. These results with purified sGC and endogenous PC12 cell sGC demonstrate that under our experimental conditions, HPLC-EC is a valid method for detecting changes in sGC activity.

Kinetic Characterization of Oxo⁸GTP as an Inhibitor of sGC

In order to determine the role of oxo⁸GTP in modulating the activity of sGC, we carried-out kinetic studies using purified sGC in the presence of increasing levels of oxo⁸GTP. Oxo⁸GTP was found to inhibit the conversion of GTP to cGMP at low (50 μ M) and high (500 μ M) initial concentrations of GTP. Figure 3A shows cGMP levels produced by purified sGC after stimulation with DEA/NO (10 μ M) and concentrations of 50 μ M or 500 μ M GTP in the presence of increasing levels of oxo⁸GTP (0.1 μ M to 2 mM). Values for IC₅₀ were calculated to be 1.8 mM with 500 μ M GTP and 288 μ M with 50 μ M GTP. Despite these high IC₅₀ values, inhibition of sGC incubated in the presence of 50 μ M GTP was significant at levels of oxo⁸GTP as low as 100 nM (Figure 3A, inset). All kinetic experiments were carried out by stimulation of purified sGC with 10 μ M DEA/NO. Addition of 100 μ M DEA/NO to our cell-free sGC preparations with oxo⁸GTP was ineffective at overcoming inhibition of the enzyme (data not shown). To define the nature of the inhibition of sGC by oxo⁸GTP, we calculated Michaelis-Menten kinetic parameters of the enzyme by plotting the substrate (GTP) versus the initial velocity (V_o). The percent conversion of substrate (GTP) to product (cGMP) was found to be between 0.1 and 16%, the production of cGMP was linear with time, and purified sGC was stable during the 10 minute reaction period. Therefore, the Michaelis-Menten model is assumed [26]. We found the Michaelis constant (K_m) for GTP with purified sGC stimulated with 10 μ M DEA/NO to be 24 μ M, similar to previously published studies using cell-free human sGC and synthetic NO donors (Table 1, Figure 3B) [27]. The addition of 50 μ M or 250 μ M oxo⁸GTP resulted in a very small change in K_m values calculated using sGC stimulated with 10 μ M DEA/NO (Table 1, Figure 3B). However, the maximum reaction velocity (V_{max}) of sGC decreased from 683.06 μ M GTP without inhibitor to 346.14 μ M GTP in presence of 250 μ M oxo⁸GTP (Table 1, Figure 3B). The transformation of the same data into double-reciprocal plots at increasing concentrations of oxo⁸GTP indicate that oxo⁸GTP behaves as a noncompetitive, mixed type inhibitor of sGC (Figure 3B, inset).

Oxidative stress induces oxo⁸GTP formation and sGC inhibition reversible by GSH

The direct addition of oxo⁸GTP tells us very little about the consequences linked to its formation in biological systems. We have previously shown that a mixture of L-ascorbic acid (1 mM) and copper sulfate (10 μ M) are sufficient to induce oxo⁸GTP levels in cell-free reactions of GTP as well as in cell culture [13]. To further investigate if the free radical-induced formation of oxo⁸GTP can result in biological levels that decrease sGC activity, we exposed GTP to the Cu/As mixture before assessment of sGC activity. Figure 4A indicates that a four hour exposure of pure GTP to Cu/Asc results in an increase in oxo⁸GTP that is three orders of magnitude higher than control. It should be noted that incubations of GTP with L-ascorbic acid alone also showed a statistically significant, yet smaller, increase in oxo⁸GTP (107.197 ± 5.445 picomoles). Pre-treatment of all buffers with Chelex resin had no effect on oxo⁸GTP formation by Asc, indicating a direct effect and not due to oxidation by trace metal contaminants (data not shown). On the other hand, four-hour incubations of copper sulfate with 1mM GTP at concentrations as high as 200 μ M yields 0.395 ± 0.009 picomoles of oxo⁸GTP, which is not statistically different from the small amounts produced in a four-hour incubation of pure GTP alone at 37°C (0.333 ± 0.010 picomoles). The formation of oxo⁸GTP by the Cu/Asc system led to a significant reduction of recovered GTP, from 11.295 ± 0.102 to 8.518 ± 0.135 nanomoles (Figure 4A, inset). Preincubation of the GTP substrate (1mM) with Cu/As lead to a significant reduction in the levels of cGMP produced by sGC after stimulation with DEA/NO (Figure 4B). It has been previously demonstrated that either Cu or ascorbate alone can inhibit sGC and this inhibition is prevented by the antioxidant reduced glutathione (GSH) [28,29]. To validate this finding using our experimental paradigm, we exposed GTP to Cu/Asc in the presence of 1 mM GSH. Presence of GSH in the reaction mixture lead to a significant reduction in the levels of oxo⁸GTP formed by Cu/Asc and prevented the inhibition of sGC

activity (Figure 4B). GSH also prevented the reduction in the levels of GTP after Cu/Asc exposure (Figure 4A, inset).

It was suggested that inhibition of sGC by Cu and Asc alone could be due to direct oxidative damage to the enzyme [28,29]. To test this, we preincubated purified sGC with the Cu/Asc mixture before stimulation with NO. Using concentrations of NO ranging from 1nM to 1 μ M, we found that direct exposure of sGC to the Cu/Asc mixture does not lead to a reduction in the production of cGMP by untreated GTP (Figure 5). This indicates that there is no direct effect on the enzyme by these oxidation conditions, and rather the reduction of cGMP production is due to elevations in cellular oxo⁸GTP and its inhibitory effect on the enzyme.

Increase in Oxo⁸GTP in PC12 Cells Exposed to Cu/Asc: Effect on cellular sGC

The data presented and other reported effects of oxo⁸GTP interfering with the role of GTP in *in vitro* systems, prompted us to evaluate oxo⁸GTP in a cellular system. In order to determine whether a similar challenge can affect the GTP pool in PC12 cells, we exposed PC12 cells to similar oxidizing conditions. Figures 6A and 6B are representative chromatograms of cytosolic extracts from PC12 cells under control and Cu/Asc-exposed, oxidizing conditions. We found that a four-hour incubation with Cu/Asc significantly increased cellular oxo⁸GTP, two-fold from basal levels, in PC12 cells (Figure 6C). Levels of GTP were unchanged between control and Cu/Asc-exposed cell populations (Figure 6D). GTP levels in untreated PC12 cells (1.5 nmoles GTP/ 10⁶ cells) were similar to basal levels we previously reported in untreated HEK 293T cells (2.3 nmoles GTP/ 10⁶ cells) using this method [13].

We then set out to determine if the increase in oxo⁸GTP in PC12 cells incubated with Cu/Asc would correlate with changes in sGC activity. To accomplish this, PC12 cells were stimulated for 10 minutes with DEA/NO (0.1, 0.5, or 1 μ M) and levels of cGMP were assessed by HPLC-EC following a four-hour exposure to untreated media (control) or media containing Cu/Asc. The activity of PC12 sGC was significantly reduced by exposure to the Cu/Asc system (Figure 7). The extent of this sGC inhibition was similar at all concentrations of DEA/NO used (0.1 μ M, 0.5 μ M, and 1 μ M) (Figure 7). A maximum stimulating concentration of DEA/NO (280 μ M) yielded no differences between control and Cu/Asc exposed PC 12 cells (Figure 7, inset). This suggests that inhibitory effects are only detected in the linear portion of sGC response to NO. Initial attempts to prevent oxidation of the GTP pool using a cell-permeable GSH analog, GSH-methyl ester, were unsuccessful (data not shown). Interestingly, high levels (5mM) of GSH-methyl ester caused a significant decrease in cGMP levels after DEA/NO stimulation (data not shown). Therefore, further investigation of the reversal of oxo⁸GTP production and subsequent sGC inhibition in PC12 cells needs to be evaluated with alternative antioxidants such as mannitol, previously shown to reduce oxidative stress in this cell type [30].

Discussion

We have found that under oxidizing conditions the guanine base incorporated into GTP is sensitive to free radical attack causing the formation of oxo⁸GTP. Accumulation of this damaged form of GTP can have deleterious effects on cell function as evidenced by a reduction in purified sGC activity as well as endogenous sGC in PC12 cells. Conditions of oxidative stress induced by 1 mM L-ascorbic acid and 10 μ M copper sulfate are sufficient to induce formation of oxo⁸GTP without oxidation of the Gua incorporated in DNA [13]. These results support a growing body of evidence that oxo⁸GTP is a modulator of cellular targets of GTP including GTP-binding proteins, GTP cyclohydrolase, and RNA production [17,18,31,32].

Employing our sGC assay in cell-free preparations, we were able to characterize oxo⁸GTP as an endogenous inhibitor of NO-stimulated sGC. The most characterized and widely used inhibitors of sGC to date are methylene blue, LY-83583, ODQ, and NS 2028. These inhibitors

act by either directly or indirectly interfering with NO binding to the ferrous heme iron which prevents the change in conformation of sGC and subsequent enzyme activation [33–35]. This type of synthetic inhibition is partially overcome upon competitive addition of NO donors such as DEA/NO [34]. Due to the fact that increasing concentrations of DEA/NO were not able to alter the degree of the inhibition induced by oxo⁸GTP, we suggest oxo⁸GTP is not acting at the NO-binding site (Figure 7). Analysis of double reciprocal plots of substrate (GTP) versus velocity of sGC showed a decrease in $V_{\max}$ with increasing oxo⁸GTP concentration with little effect on K_m (Table 1). This suggests a noncompetitive, mixed type of inhibition of sGC by oxo⁸GTP. However, further analysis of inhibition is necessary to accurately characterize the specifics of oxo⁸GTP inhibition on sGC activity. The extent of inhibition of purified sGC by oxo⁸GTP was dependent in the initial concentration of GTP (Figure 3A). Thus, changes in cellular levels of GTP by exposure to toxins or pathological conditions would change the dynamics of oxo⁸GTP inhibition.

Interestingly, L-ascorbic acid and copper sulfate, independently, have been found to inhibit sGC in a similar manner in a cell-free system like the one used in our studies [28,29]. Schrammel et. al. presented data showing that both copper ions and L-ascorbic acid act as inhibitors of sGC via NO-independent mechanisms and the inhibition is prevented by the addition of GSH. They suggest a possible direct effect on the sGC protein by Cu and Asc. Our results with a combined Cu/Asc system replicate their findings of sGC inhibition but point to oxo⁸GTP as the target in this system instead of direct modification to sGC. Exposure of sGC to Cu/Asc 4 hours before addition of GTP and activation by DEA/NO yielded no difference in the amount of cGMP produced with unexposed sGC (Figure 5). Thus, in the *in vitro* system our data suggests a sole effect in GTP and not to sGC by Cu/Asc. This conclusion is supported by the decreased formation of oxo⁸GTP by Cu/Asc in the presence of GSH and consequent rescue of sGC activity (Figure 4). It could be possible that the reduction in sGC activity in PC12 cells follows the results from *in vitro* studies. However, Cu/Asc could impact redox sensitive aminoacids in sGC interfering with its activity. Future studies will address this by the use of antioxidant and evaluation of protein modification.

Comparative results using either cell-free sGC or endogenous sGC in the PC12 neuronal cell culture model demonstrate the physiological relevancy of our findings. Analysis of acid cellular extracts from PC12 cells show that after exposure to Cu/Asc the levels of oxo⁸GTP formed by Cu/Asc correspond to 24 nM in the cell extracts and GTP levels are approximately 50 μ M. Oxo⁸GTP was elevated from 0.048% to 0.2 % of the total cellular GTP pool and NO-stimulated activity of sGC was reduced to 50% after Cu/Asc (Figure 6 and Figure 7). The relationship between concentrations of 8-oxoGTP and GTP in acid cellular extracts is comparable to the data presented in Figure 3A (inset); where a significant reduction in cGMP generation with purified sGC when GTP levels are in the low range of the spectrum (Figure 3A, inset). Whether all of the inhibition of sGC in PC12 cells is due to oxo⁸GTP formation, as was established in cell-free preparations, can not be discerned from the current studies. Future studies are aimed to resolve the extent to which oxo⁸GTP is responsible for modifying the sGC system *in vivo*. However, our results do suggest that sGC has a higher affinity for oxo⁸GTP than the endogenous GTP substrate yielding less cGMP formation. This finding recapitulates a recent finding that another enzyme using GTP as a substrate, GTP cyclohydrolase (GTPCHI), has an affinity for oxo⁸GTP that is three orders of magnitude higher than for the native substrate [32]. Structural studies identifying binding sites of oxo⁸GTP with GTPCHI suggest coordination with the 8-oxoguanine group. A similar mechanism of inhibition due to coordination with 8-oxoguanine by the sGC enzyme also needs to be investigated.

Consequences of lowered cGMP levels in the CNS include reduced neuronal activation, impairment of long term potentiation (LTP), and the disruption of neuritogenesis [36–41]. Further investigation of oxo⁸GTP levels and resulting changes in sGC could provide key

mechanistic insights into the pathology of normal developmental, aging, and disease processes known to be highly affected by oxidative stress. The need to address the role of oxo⁸GTP in biological systems is emphasized by the fact that the MTH1 protein, responsible for hydrolyzing oxo⁸dGTP to oxo⁸dGMP and thus preventing its incorporation into DNA, has a rate of cleavage 50 times lower for oxo⁸GTP than for oxo⁸dGTP [42,43]. This coupled to the fact that GTP levels are 50–100 times larger than dGTP, make oxidation of GTP and understanding of its biological consequence an important subject of investigation [11,12]. No specific repair mechanism for sanitation of cellular oxo⁸GTP has been identified to compensate for the inefficiency of MTH1.

Concentrations of L-ascorbic acid and copper sulfate used in this study are comparable to concentrations found in brain, where copper reaches levels of 30 μ M during normal neurotransmission [44], and as high as 400 μ M in pathological conditions, such as AD [45–47]. Further studies evaluating the consequences of oxidative stress-induced, elevated oxo⁸GTP levels and resulting modulations in sGC activity in brain tissue are necessary to investigate physiological impacts of this oxidative damage.

In conclusion, our results show oxo⁸GTP to be an inhibitor of sGC, and that oxidative environments induced with Cu/Asc cause an elevation in oxo⁸GTP that significantly lowers available cGMP. The methodology outlined within this study to quantify oxo⁸GTP and sGC activity concomitantly can be applied to a variety of systems investigating oxidative stress as a component of pathologies including cardiovascular disease and neurodegenerative disease. Furthermore, investigating this previously overlooked biomarker and its cellular consequences may lead to novel therapeutic interventions for these diseases.

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Figure 1. HPLC-ECD analysis of cGMP

Representative chromatograms from (A) 40 ng cGMP standard, and (B) an acid extract from NO-stimulated (0.5 μM DEA/NO) PC12 cells. cGMP retention time identified by the arrow. Figure 1C depicts a representative calibration curve using cGMP standards ranging 5 ng to 1 μg. The signal is representative of the area under the peak for three channels in the EC detector.

Figure 2. sGC activity with increasing NO concentrations
cGMP levels produced by purified sGC (A) or from PC12 cells (B) after stimulation with increasing concentrations of DEA/NO. Data are expressed as the mean amount of product (cGMP) $\pm$ SEM (n = 3–6) normalized to the maximal response observed in each experiment.

Figure 3. Pharmacological characterization of sGC inhibition by oxo^8GTP

(A) Initial velocity (V_0) assessment of purified sGC in presence of increase levels of Oxo^8GTP . V_0 were determined at two different initial concentrations of GTP (500 μM or 50 μM) for each level of oxo^8GTP tested. sGC stimulation was done with 10 μM DEA/NO. Inset: changes in V_0 in sGC stimulated with 10 μM DEA/NO in the presence of 50 μM GTP (left bar) or 50 μM GTP and 100 nM oxo^8GTP (right bar). (B) V_0 determination of sGC activity at fixed levels of oxo^8GTP with increasing levels of GTP. Inset: $1/V_0$ graphed vs. $1/[\text{GTP}]$. Data are expressed as the means $\pm$ SEM ($n = 3$, * $p < 0.05$).

Figure 4. Formation of Oxo⁸GTP with Cu/Asc and the effect of GSH

(A) Levels of oxo⁸GTP formed after incubation of GTP with Cu/Asc in absence or presence of GSH, as described in Methods. Inset: Level of GTP in control (white bar), or after Cu/Asc exposure (black bars), in absence of GSH. (B) Levels of cGMP produced by sGC after stimulation with DEA/NO using GTP (white bars) or GTP after incubation with Cu/Asc (black bars). The experiment was done in the absence or presence of GSH. Data expressed as the means $\pm$ SEM (n = 3, * p < 0.05 compared to control, # p < 0.05 compared to Cu/Asc without GSH).

Figure 5. sGC activity of purified sGC pre-incubated with Cu/Asc

Levels of cGMP produced by purified sGC pre-incubated with Cu/Asc (black bars) or PBS (white bars) before addition of substrate (GTP) and stimulation with 1, 100, or 1000 nM DEA/NO. Data are expressed as means $\pm$ SEM.

Figure 6. Analysis of GTP and oxo⁸GTP levels in PC12 cells after Cu/Asc exposure
 Typical elution profiles for oxo⁸G (A) and G (B) detected at 250mV and 785mV respectively. (C) Bar graphs represent cellular levels of oxo⁸G, in control (white bars) and in Cu/Asc – exposed PC12 cells (black bars). Data expressed as pmoles per mg of total protein in cellular extract. (D) Levels of GTP in control (white bars) or Cu/Asc-exposed PC12 cells. Data expressed as the nmoles of GTP per mg of total protein in cellular extract. * Indicates a significant difference from control (n =6, * p < 0.05).

Figure 7. Activity of sGC in DEA/NO-stimulated PC12 cells after Cu/Asc exposure

Levels of cGMP produced by PC12 after stimulation with DEA/NO. sGC activity was determined in control (white bars) and in Cu/Asc-exposed (black bars) PC12 cells. Exposure conditions are as described in methods. DEA/NO added to cell culture ranged from 0.1 µM to 1 µM. Also, a high level DEA/NO of 280 µM was used (inset). Data expressed as pmoles of cGMP produced in 10 minutes per mg of total protein in the cellular extract $\pm$ SEM. *Denotes significant difference from control; $p < 0.05$.

Table 1Inhibition of sGC by Oxo⁸GTP

	Oxo ⁸ GTP (μM)		
	0	50	250
K _m (μM)	24.07	19.24	18.9
V _{max}	683.06	385.20	346.14