

Nitric Oxide–Independent Vasodilator Rescues Heme-Oxidized Soluble Guanylate Cyclase From Proteasomal Degradation

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Abstract—Nitric oxide (NO) is an essential vasodilator. In vascular diseases, oxidative stress attenuates NO signaling by both chemical scavenging of free NO and oxidation and downregulation of its major intracellular receptor, the $\alpha\beta$ heterodimeric heme-containing soluble guanylate cyclase (sGC). Oxidation can also induce loss of the heme of sGC, as well as the responsiveness of sGC to NO. sGC activators such as BAY 58-2667 bind to oxidized/heme-free sGC and reactivate the enzyme to exert disease-specific vasodilation. Here, we show that oxidation-induced downregulation of sGC protein extends to isolated blood vessels. Mechanistically, degradation was triggered through sGC ubiquitination and proteasomal degradation. The heme-binding site ligand BAY 58-2667 prevented sGC ubiquitination and stabilized both α and β subunits. Collectively, our data establish oxidation–ubiquitination of sGC as a modulator of NO/cGMP signaling and point to a new mechanism of action for sGC activating vasodilators by stabilizing their receptor, oxidized/heme-free sGC. (*Circ Res.* 2009;105:33-41.)

Key Words: soluble guanylate cyclase ■ nitric oxide ■ ubiquitination ■ proteasome ■ BAY 58-2667

One major risk factor for the development of cardiovascular diseases, such as coronary heart disease, stroke, and myocardial infarction, is an imbalance of the production and elimination of reactive oxygen species, also termed as oxidative stress.^{1–3} As a consequence, the nitric oxide (NO)/cGMP signaling cascade is impaired, eg, through the excessive production of superoxide, which reacts with NO in a diffusion-limited reaction, yielding peroxynitrite.⁴ The biological impact of NO scavenging is further aggravated by the progressive inhibition and downregulation of the NO receptor soluble guanylate cyclase (sGC).^{5–9} Circumstantial evidence has implicated proteasomal pathways in this downregulation of sGC.^{10–12} Conversely, a novel class of sGC activators, represented by BAY 58-2667, are potentiated under oxidative stress conditions and represent thus an entirely new disease-specific vasodilator class.¹³

sGC is a heterodimer consisting of an α and a Fe²⁺/heme-containing β subunit that complexes NO with high affinity and specificity.¹⁴ Binding of NO to the Fe²⁺/heme results in allosteric activation of the enzyme and enhanced conversion

of GTP into the vasorelaxant and antiproliferative second messenger cGMP.^{14,15} In vitro experiments have demonstrated that oxidation of sGC heme to its ferric (Fe³⁺) form by the sGC inhibitor 1*H*-[1,2,4]-oxadiazolo [3,4-*a*]quinoxalin-1-one (ODQ) attenuates NO-mediated cGMP production, suggesting that the ferro (Fe²⁺) form of sGC is crucial for activation by NO.^{16–18} In addition, studies with primary endothelial and smooth muscle cells have revealed that, within 24 hours, ODQ causes a dramatic decrease in sGC protein levels.¹³ Similar results were obtained with other oxidizing compounds such as methylene blue or the peroxynitrite donor 1,3-morpholino-sydnominine hydrochloride (SIN-1), indicating that oxidative stress triggers downregulation of sGC protein levels.¹³

At present, the molecular mechanisms underlying the degradation of heme-oxidized sGC remain unknown. It has been speculated that heme oxidation destabilizes sGC, rendering it susceptible to the ubiquitin–proteasome machinery.¹³ Interestingly, the NO-independent sGC activator and vasodilator compound 4-[[[(4-carboxybutyl){2-[(4-phenethylbenzyl)oxy]phenethyl}

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amino)methyl] benzoic acid (BAY 58-2667)^{19,20} binds with high affinity to the heme pocket of the enzyme and subsequently abrogates the effects of heme-oxidizing compounds on sGC protein levels.¹³ This may point to a novel mechanism of action for this new compound.

In the present work, we therefore aimed to elucidate the molecular mechanism underlying the effects of heme oxidation-induced protein degradation of the $\alpha_1\beta_1$ sGC isoform. Specifically, we wanted to address 3 main questions: (1) whether ODQ-induced sGC downregulation occurs via the ubiquitin–proteasome pathway; (2) whether CHIP (carboxyl terminus of heat shock protein (Hsp)70 interacting protein) is involved as an E3 ligase for sGC ubiquitination; and (3) whether any stabilizing effects of BAY 58-2667 on sGC protein levels may provide new insights into the mode of action of this important new pharmacological vasodilator principle.

Materials and Methods

An Materials and Methods section is available in the Online Data Supplement at <http://circres.ahajournals.org>. Care and use of animals was in accordance with institutional guidelines.

Antibody Production

Antisera to human α_1 and β_1 subunits were raised in rabbits (α_1 : AS587, AS558; β_1 : AS566, AS556) or mice (α_1 : AS613; β_1 : AS614). Monoclonal antibody to β_1 (clone 5A5) was produced in mouse. Alternatively, antisera to human α_1 (SA3093) and β_1 (SA2934) were used.²¹

Construction of Expression Plasmids

Expression plasmids coding for human α_1 (pSG8- α_1) or β_1 (pEDmtr- β_1) were generated as described.²² The cDNAs for myc-tagged CHIP (myc-CHIP) and hemagglutinin-tagged ubiquitin (HA-Ub) were kindly provided by Dr Ivan Dikic (Institute of Biochemistry II, University of Frankfurt, Germany). For the expression of myc-tagged red fluorescent protein (myc-RFP), the RFP-cDNA was subcloned into pcDNA3myc.

Transfection and Cell Culture

HEK293, COS-1, and HEK293 cells stably expressing human $\alpha_1\beta_1$ (HEK-sGC)¹³ were cultured in DMEM with 10% FCS. Transient transfection of COS-1 cells was performed with DEAE-dextrane.²² HEK293 and HEK-sGC cells were transfected using Metafectene (Biontex Laboratories, Martinsried, Germany) according to the instructions of the manufacturer. Incubations of cells with 10 $\mu\text{mol/L}$ ODQ, 10 $\mu\text{mol/L}$ BAY 58-2667, or 0.5 $\mu\text{mol/L}$ MG132 were done 6 to 24 hours after transfection for the indicated time points. Nontransfected cells were treated with 1 to 5 $\mu\text{mol/L}$ ODQ, 5 $\mu\text{mol/L}$ BAY 58-2667, 5 $\mu\text{mol/L}$ NS2028, 0.2 $\mu\text{mol/L}$ epoxomicin, 0.5 $\mu\text{mol/L}$ MG132, or 5 $\mu\text{mol/L}$ lactacystin 24 hours after being plated.

Tissue Preparation

Aortae from 16- to 20-week-old Wistar Kyoto (WKY) (8 to 10 rats) or spontaneously hypertensive rats (SHR) (3 to 5 rats) were excised, cut into rings, and placed in DMEM supplemented with 10% FCS with or without 10 $\mu\text{mol/L}$ ODQ or NS2028 and/or 10 $\mu\text{mol/L}$ BAY 58-2667 for 20 hours. Prior incubation with ODQ/NS2028 and BAY 58-2667, aortic rings were incubated for 30 minutes with 10 $\mu\text{mol/L}$ BAY 58-2667 alone. Medium was aspirated, and tissues were frozen in liquid nitrogen and minced. Tissue powder was added to 0.3 mL of sample buffer (5% glycerol, 2.5% mercaptoethanol, 1.5% SDS, 50 mmol/L Tris-HCl [pH 8.0], 0.05 mg/mL bromophenol blue), sonicated and heated (10 minutes, 95°C). Samples were centrifuged (15,000g, 10 minutes) and subjected to SDS-PAGE (40 $\mu\text{g/lane}$). Protein concentrations were determined using the RC DC protein assay (Bio-Rad, Hercules, Calif).

Aortic cGMP

For determination of cGMP content, aortic rings from WKY or SHR rats were used and treated for 20 hours with 10 $\mu\text{mol/L}$ ODQ and/or 10 $\mu\text{mol/L}$ BAY 58-2667 in DMEM including 10% FCS, followed by stimulation for 10 minutes with or without 30 $\mu\text{mol/L}$ DEA/NO in the presence of phosphodiesterase inhibitor 3'-isobutylmethylxanthine (IBMX) (1 mmol/L). Determination of vascular cGMP content was performed as described.²³

Immunoprecipitation and Western Blotting

For immunoprecipitation, cells from a 60-mm dish were lysed with 0.5 mL immunoprecipitation buffer (50 mmol/L Hepes [pH 7.5], 150 mmol/L NaCl, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 5 mmol/L *N*-ethylmaleimide, 1 mmol/L phenyl methyl sulfonyl fluoride [PMSF], supplemented with complete protease inhibitor cocktail). Cellular debris was pelleted (20,000g, 15 minutes), and the lysate was incubated with corresponding antisera for 4 to 14 hours at 4°C under rotation. Antibodies were precipitated with protein A/G PLUS-agarose. To detect ubiquitinated α_1 or β_1 , beads were washed with 1 mL each of buffer A (10 mmol/L Tris-HCl [pH 8.5], 600 mmol/L NaCl, 0.1% SDS, 0.05% NP-40), buffer B (0.5% Na-deoxycholate in PBS, 1% Triton X-100), buffer C (buffer B containing 2 mol/L KCl), and twice with 1 mL of 0.1× PBS. All buffers included 5 mmol/L *N*-ethylmaleimide and 1 mmol/L PMSF. Under these stringent washing conditions, α_1 or β_1 subunit but not the holoenzyme was present in the immunoprecipitate using anti- α_1 or anti- β_1 . To monitor interaction of sGC with CHIP, agarose beads were washed 3× with 1 mL each of immunoprecipitation buffer. Under these less stringent conditions, the holoenzyme was retained in the immunoprecipitate using antibodies to α_1 or β_1 , as evidenced by SDS-PAGE and Western blotting (WB).²⁴ To prepare total cell lysates, cells from a 60 mm dish were lysed in 0.5 mL sample buffer, sonicated and heated (10 minutes, 95°C). Before loading to the gel, samples were centrifuged (15,000g, 5 minutes).

Data Analysis and Statistics

Data are expressed as means±SEM of *n* independent experiments. Differences were assessed by 1-way ANOVA, and statistical significance was set at $P<0.001$. Statistical calculations were performed with Origin 6.1 software (OriginLab Corp, Northampton, Mass).

Results

sGC Heme Oxidation Decreases sGC Protein Levels in Rat Aorta

First, we wanted to extend our previous *in vitro* observations¹³ of a modulating effect of the well characterized heme oxidant ODQ and of BAY 58-2667 on sGC protein levels to isolated blood vessels. Rat aortic rings from WKY or SHR rats were incubated for 20 hours with 10 $\mu\text{mol/L}$ ODQ and BAY 58-2667, alone or in combination. DMSO was used as control. In the presence of ODQ, sGC protein levels in WKY and SHR aortic rings were drastically reduced, whereas endothelial nitric oxide synthase (eNOS) and GAPDH levels remained unchanged (Figure 1A and 1B). Importantly, exposure of aortic rings to equimolar concentrations of both ODQ and BAY 58-2667 attenuated sGC downregulation. Hence, ODQ-induced decrease of sGC protein levels and its attenuation by BAY 58-2667 can be consistently observed in vascular cells,¹³ as well as in intact aorta.

To investigate whether these findings at the protein level relate to sGC activity, we performed cGMP assays. Aortic rings from WKY or SHR rats were again treated for 20 hours with ODQ (10 $\mu\text{mol/L}$) and/or BAY 58-2667 (10 $\mu\text{mol/L}$) but now followed by a 10 minutes stimulation with or without 30 $\mu\text{mol/L}$ DEA/NO in the presence of PDE inhibitor IBMX

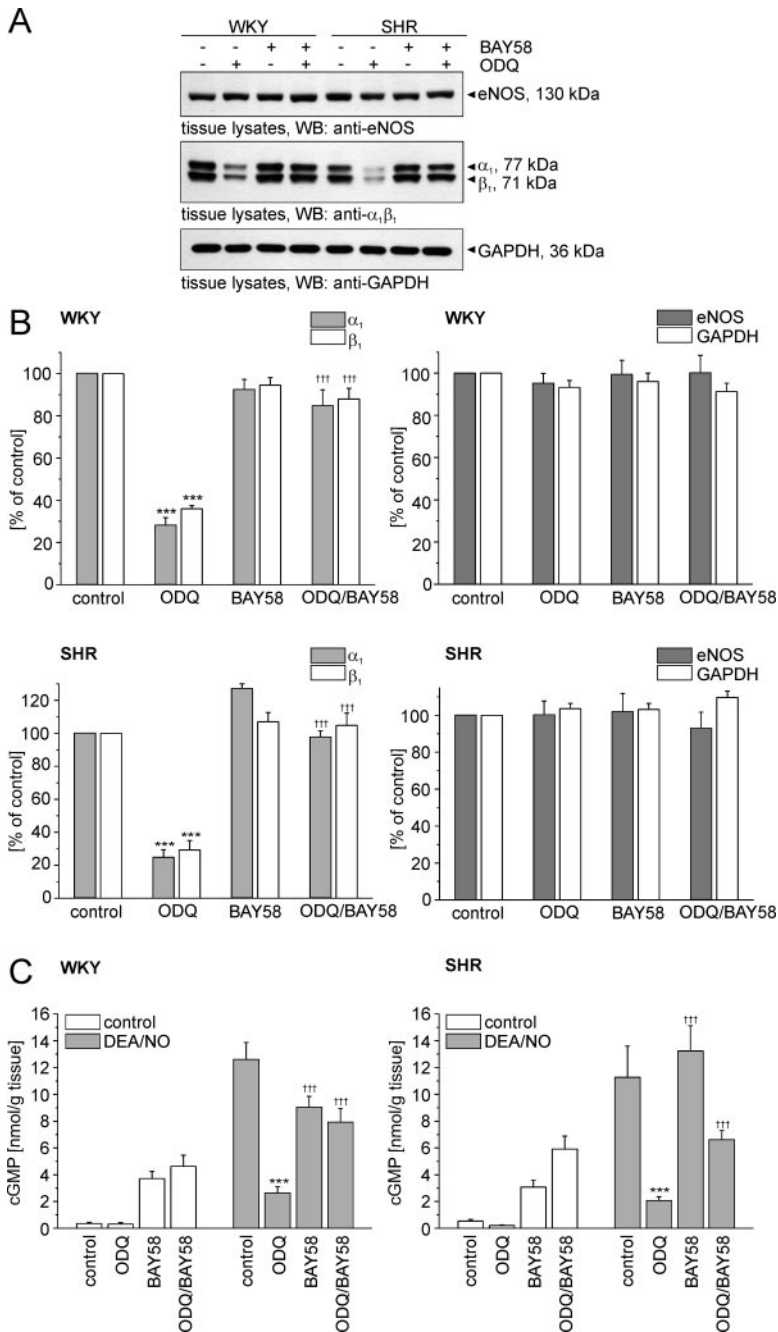


Figure 1. Effects of ODQ and BAY 58-2667 on rat sGC. Aortic rings from WKY and SHR were incubated for 20 hours with 10 $\mu\text{mol/L}$ ODQ and/or 10 $\mu\text{mol/L}$ BAY 58-2667. Tissue lysates were analyzed by WB with antibodies to eNOS, α_1 (SA3093), β_1 (SA2934), or to GAPDH (A), and protein levels were quantified by optical densitometry (B). Data are expressed as percentages of control (means \pm SEM of 8 to 10 [WKY] or 3 to 5 [SHR] independent experiments). *** P < 0.001 vs control; ††† P < 0.001 vs ODQ. C, Aortic rings were stimulated for 10 minutes with or without 30 $\mu\text{mol/L}$ DEA/NO in the presence of IBMX (1 mmol/L). cGMP levels are expressed as means \pm SEM of $n=7$ to 9. *** P < 0.001 vs control stimulated with DEA/NO; ††† P < 0.001 vs ODQ stimulated with DEA/NO.

(1 mmol/L). Consistent with our observations on sGC protein levels, ODQ drastically reduced NO-induced cGMP levels as compared to control (Figure 1C). In the presence of BAY 58-2667 alone or of both ODQ and BAY 58-2667, NO-induced cGMP levels were significantly elevated compared to aortic rings exclusively treated with ODQ. Hence, sGC protein levels correlate with functional activity of the enzyme, and both are redox-regulated in a BAY 58-2667 reversible manner.

HEK-sGC Cells Serve As a Model to Study sGC Turnover

To obtain a reliable cellular system to investigate the mechanisms of sGC turnover, we examined whether the same phe-

nomena occur in a transfectable human cell line overexpressing sGC. Using HEK293 cells producing high levels of human sGC (HEK-sGC), we observed drastic effects on sGC protein levels on treatment with 5 $\mu\text{mol/L}$ ODQ, 5 $\mu\text{mol/L}$ BAY 58-2667, or the combination of both for 16 hours, whereas GAPDH levels remained unchanged (Figure 2A and 2B). ODQ strongly down-regulated sGC protein under these conditions. In marked contrast, BAY 58-2667 increased sGC levels well above control. Exposure to equimolar concentrations of both ODQ and BAY 58-2667 fully inhibited the sGC downregulation induced by ODQ alone and further enhanced sGC protein levels almost to those seen with BAY 58-2667 alone. Thus HEK-sGC cells mimic native cells and ex vivo tissues with respect to heme oxidation-induced sGC downregulation.

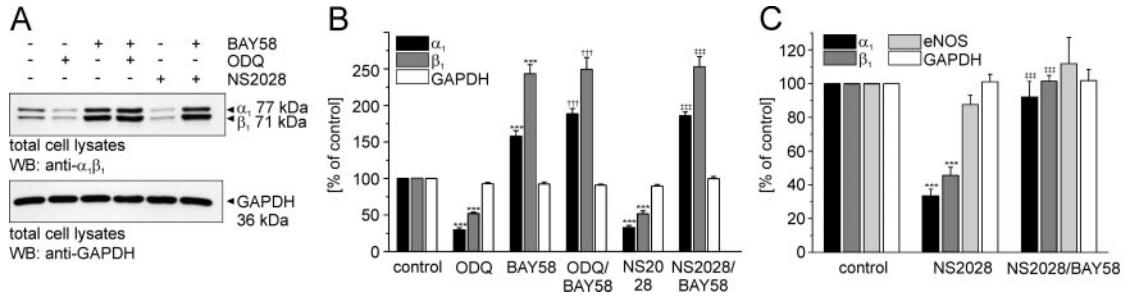


Figure 2. Effects of ODQ, NS2028, and BAY 58-2667 on sGC protein levels. HEK-sGC cells were incubated for 16 hours with 5 $\mu\text{mol/L}$ ODQ or NS2028 in the presence or absence of 5 $\mu\text{mol/L}$ BAY 58-2667. Total cell lysates were probed by WB with anti- $\alpha_1\beta_1$ (mixture of AS587 and AS566) or anti-GAPDH (A), and protein levels were quantified (B). Data are expressed as means \pm SEM of 10 independent experiments. C, Aortic rings from WKY (5 to 6 rats) were incubated for 20 hours with 10 $\mu\text{mol/L}$ NS2028 in the presence or absence of 10 $\mu\text{mol/L}$ BAY 58-2667. Tissue lysates were analyzed by WB with antibodies to eNOS, α_1 (SA3093), β_1 (SA2934), or to GAPDH, and protein levels were quantified. Data are expressed as percentages of control (means \pm SEM). *** P <0.001 vs control; ††† P <0.001 vs ODQ; ‡‡‡ P <0.001 vs NS2028.

ODQ-Induced Downregulation Is a Class Effect of sGC Heme Oxidants

To exclude that observed effects of ODQ on sGC protein levels are specific for this oxidant, we did similar experiments using the sGC inhibitor and heme oxidant 4*H*-8-bromo-1,2,4-oxadiazolo(3,4-*d*) benz(b)[1,4]oxazin-1-one (NS2028).^{25,26} Treatment of HEK-sGC cells with 5 $\mu\text{mol/L}$ NS2028 resulted in downregulation of sGC, which was inhibited by BAY 58-2667 (Figure 2A and 2B). Similar results were obtained when rat aortic rings were used. In the presence of NS2028, sGC protein levels were potently reduced, whereas eNOS and GAPDH levels remained nearly unaffected (Figure 2C). Addition of BAY 58-2667 again attenuated the NS2028-induced downregulation of sGC. These data suggest that ODQ-induced downregulation extends to the sGC oxidant, NS2028 and therefore appears to be a class effect of these reagents.

sGC Heme Oxidation Induces Proteasomal Degradation

We next examined whether proteasomal degradation is involved in oxidation-induced sGC degradation. HEK-sGC cells were incubated for 16 hours with ODQ in the absence or presence of proteasomal inhibitors epoxomicin, MG132, or lactacystin (Figure 3A and 3B). All inhibitors elevated sGC levels in the absence of ODQ, indicating that proteasomal turnover of sGC is physiological and operates under normal,

ie, nonoxidative conditions. Importantly, each of the inhibitors reversed the ODQ-induced decline in sGC protein, suggesting that the ODQ-mediated sGC downregulation was attributable to increased proteasomal degradation of both cyclase subunits (Figure 3A and 3B).

sGC Oxidation Leads to Ubiquitination in a BAY 58-2667-Reversible Manner

We next addressed whether sGC is prone to ubiquitination and whether this may serve as a trigger for sGC degradation. HEK-sGC cells overexpressing HA-Ub were incubated for 4 hours in the absence or presence of 0.5 $\mu\text{mol/L}$ MG132. After cell lysis, sGC β_1 (75 kDa) was immunoprecipitated using subunit-specific antibodies, followed by WB with anti-HA (Figure 4A). In the presence of HA-Ub, the immunoblot revealed a ladder of bands beyond 75 kDa, most likely representing different polyubiquitinated forms of sGC. Addition of the proteasomal inhibitor MG132 resulted in a further accumulation of ubiquitinated sGC forms (Figure 4A). Thus, under our experimental conditions, constitutive ubiquitination and subsequent degradation of sGC appears to be a major pathway of sGC turnover. To study the effects of ODQ and BAY 58-2667 on sGC ubiquitination, we incubated HEK-sGC cells overexpressing HA-Ub for 3 hours with 10 $\mu\text{mol/L}$ ODQ and/or BAY 58-2667 (Figure 4B). Controls using anti-HA showed similar expression levels of HA-Ub (Figure I in the Online Data Supplement). ODQ considerably en-

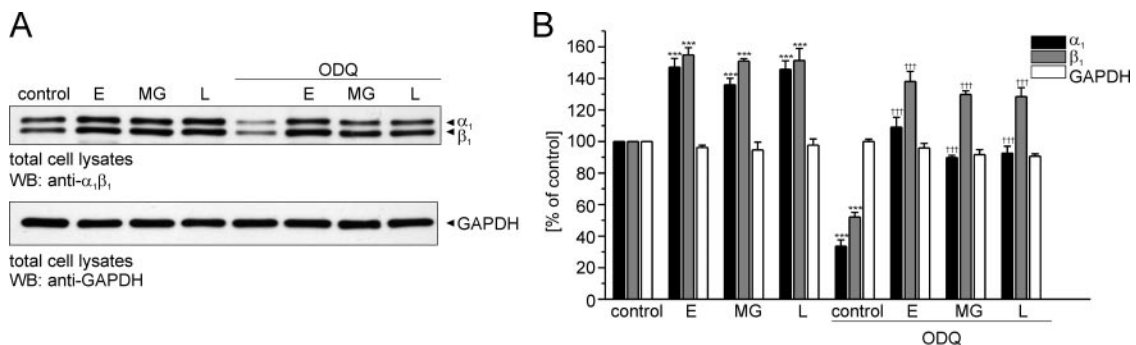


Figure 3. Proteasomal inhibitors block ODQ-induced sGC degradation. HEK-sGC cells were incubated with 0.2 $\mu\text{mol/L}$ epoxomicin, 0.5 $\mu\text{mol/L}$ MG132, or 5 $\mu\text{mol/L}$ lactacystin in the absence or presence of 1 $\mu\text{mol/L}$ ODQ for 16 hours. Total cell lysates were probed by WB with anti- $\alpha_1\beta_1$ (AS587/AS566) or anti-GAPDH (A), and protein levels were quantified (B). Data are expressed as means \pm SEM of 5 independent experiments. *** P <0.001 vs control; ††† P <0.001 vs ODQ.

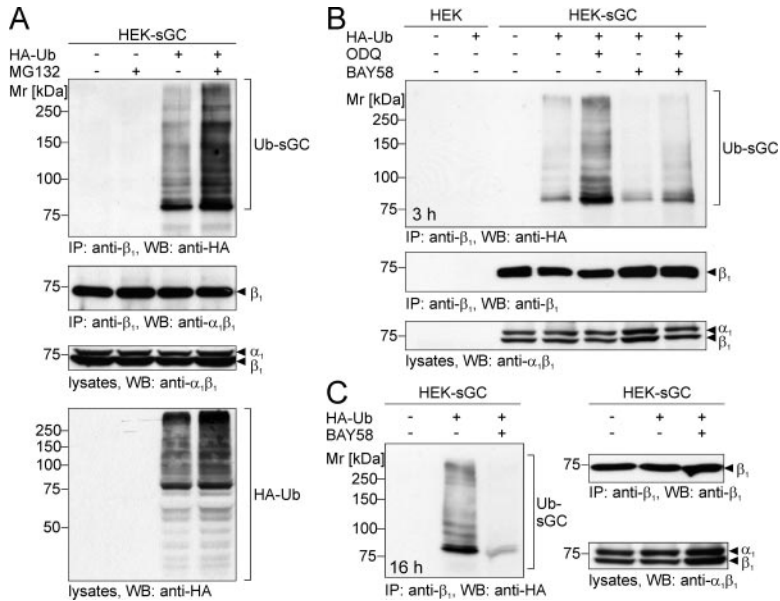


Figure 4. Oxidized sGC is prone to ubiquitination. A, HEK-sGC cells were transfected with HA-tagged ubiquitin (HA-Ub) and incubated 24 hours after transfection for 4 hours in the absence or presence of 0.5 μ mol/L MG132. Immunoprecipitation (IP) was performed with anti- β_1 (AS556), followed by WB with anti-HA or anti- $\alpha_1\beta_1$ (AS587/AS566; top gels). For control, cell lysates were probed with anti- $\alpha_1\beta_1$ or anti-HA (bottom gels). HEK293 (HEK) and HEK-sGC cells transfected with HA-Ub were treated 24 hours after transfection for 3 hours with 10 μ mol/L ODQ and/or BAY 58-2667 (B) or 6 hours after transfection for 16 hours with 10 μ mol/L BAY 58-2667 (C). Immunoprecipitation was performed with anti- β_1 (AS556), followed by WB with anti-HA or anti- β_1 (5A5). For control, cell lysates were probed with anti- $\alpha_1\beta_1$ (AS587/AS566). Lysates were also probed with anti-HA or anti-GAPDH (Online Figure I). Representative blots of at least 3 independent experiments are shown.

hanced sGC ubiquitination, implying that targeting of sGC to ubiquitination and proteasomal degradation is indeed the dominant route of degradation under heme-oxidizing conditions. By contrast, BAY 58-2667 almost abolished ODQ-induced ubiquitination (Figure 4B). This inhibitory effect of BAY 58-2667 on sGC ubiquitination was even more prominent when cells were incubated for extended periods (16 hours) with this compound (Figure 4C). Thus, BAY 58-2667 seems to stabilize sGC protein by preventing its ubiquitination and subsequent proteasomal degradation.

Both sGC Subunits Undergo Polyubiquitination

Next, we wanted to investigate in more detail whether both sGC subunits are targeted for degradation by ubiquitination. Thus, we used COS-1 cells transfected with cDNAs encoding human α_1 or β_1 , in the presence or absence of HA-Ub (Figure 5). Following immunoprecipitation with anti- α_1 or anti- β_1 , WB analyses revealed a strong poly-ubiquitination of β_1 and to a lesser extent of α_1 (Figure 5). Similar results were obtained when both sGC subunits were coexpressed with HA-Ub; again, ubiquitination of β_1 was more prominent than that of α_1 (Figure 5). To verify ubiquitination of β_1 by mass spectrometry (MS), we used COS-1 cells overexpressing β_1 in the absence or presence of HA-Ub. Immunoprecipitation with anti- β_1 followed by WB with the same antibody revealed β_1 and its monoubiquitinated forms, β_1 -Ub and β_1 -HA-Ub (Online Figure II, A). Excision of the major band at 71 kDa from the gel and analysis by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS identified β_1 (Online Figure II, A and C; Online Table I). MS analysis of the 77-kDa protein band revealed the presence of both β_1 and ubiquitin (Online Figure II, B and C; Online Table I). Two peptides of m/z 1523.82 and 2130.19 of the 77-kDa protein were assigned to ubiquitin by MALDI-TOF/TOF MS/MS with a protein score of 53. Hence, β_1 is the predominant ubiquitination target of sGC that undergoes monoubiquitination and possibly also polyubiquitination, as implied by the characteristic protein ladder.

E3 Ligase CHIP Interacts With and Ubiquitinates sGC

Finally, we wanted to identify a candidate E3 ubiquitin ligase mediating the observed sGC ubiquitination. The molecular chaperones Hsp90 and Hsp70 are well-established binding partners of both sGC^{10,27,28} and the ubiquitin-protein ligase CHIP.^{29,30} We therefore considered CHIP a prime candidate for an sGC E3 ligase. To test whether cochaperone CHIP physically interacts with sGC in mammalian cells, we co-transfected COS-1 cells with constructs encoding myc-tagged CHIP and human sGC. Immunoprecipitation with anti-myc followed by WB with anti- α_1 and anti- β_1 revealed the association of CHIP with sGC (Figure 6A). Similarly, immunoprecipitation with anti- β_1 followed by WB with anti-myc revealed a complex between CHIP and sGC (Online Figure III, A). The interaction of myc-CHIP with sGC was also observed in HEK-sGC cells that had been transfected with myc-CHIP (data not shown).

Having established that sGC directly or indirectly associates with CHIP, we tested whether CHIP also impacts on sGC protein levels. Indeed, coexpression of human $\alpha_1\beta_1$ with increasing amounts of myc-tagged CHIP and decreasing amounts of control protein myc-RFP in COS-1 cells revealed a gradual decline in sGC protein levels (Figure 6B). To test whether overexpression of CHIP enhances sGC ubiquitination, we used lysates of COS-1 cells coexpressing sGC and myc-tagged CHIP, alone or in combination, and in the absence or presence of HA-Ub. Immunoprecipitation was performed with anti- β_1 , followed by WB with anti-HA (Figure 6C; Online Figure III, B). In the presence of myc-CHIP, sGC ubiquitination was enhanced. Similar results were obtained with HEK-sGC cells coexpressing HA-Ub and myc-CHIP (data not shown), suggesting that CHIP may indeed serve as a major E3 ligase for sGC.

Collectively, our results demonstrate that heme oxidation via ODQ results in a drastic decrease of sGC protein, most likely through CHIP-mediated ubiquitination and subsequent proteasomal degradation of sGC. Importantly, the sGC acti-

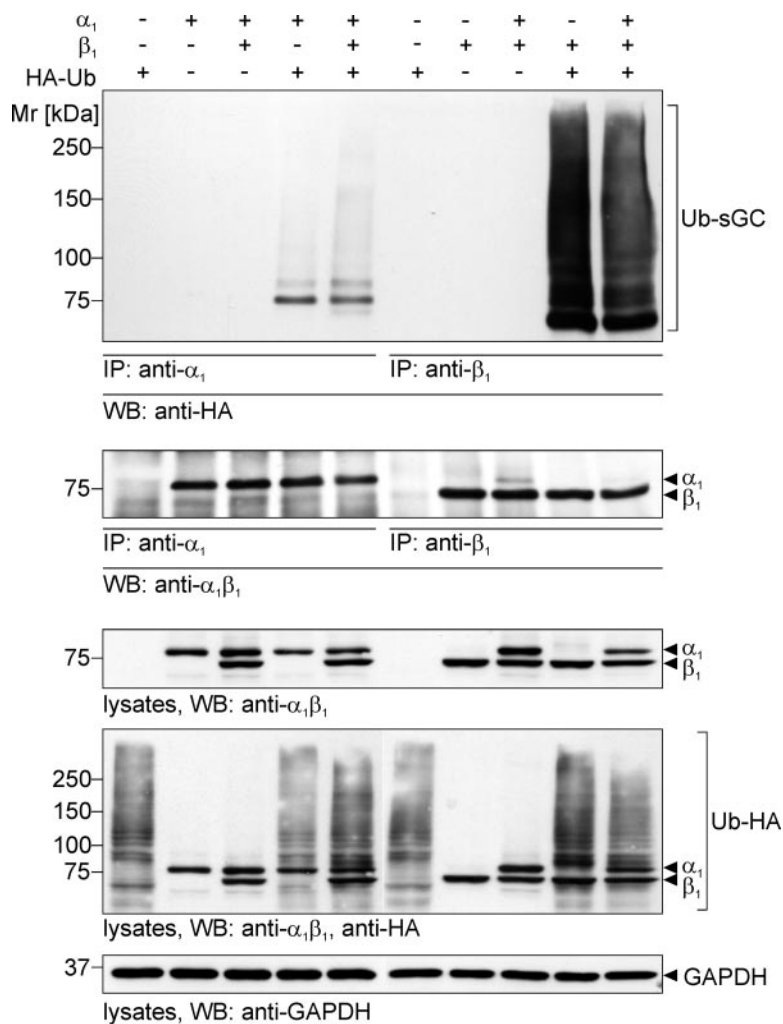


Figure 5. Both subunits of sGC are prone to ubiquitination. COS-1 cells expressing α_1 and/or β_1 , as well as HA-Ub, were lysed and immunoprecipitation was performed with anti- α_1 (AS558) or anti- β_1 (AS556), as indicated. For WB anti-HA, anti- $\alpha_1\beta_1$ (AS587/5A5), or anti-GAPDH were used. Representative blots of at least 3 independent experiments are shown.

vator and disease-specific vasodilator BAY 58-2667 can rescue this effect through stabilization of sGC, thereby protecting a key player of the NO/cGMP signaling under adverse oxidative conditions.

Discussion

Oxidative stress and enhanced generation of reactive oxygen species are major pathogenic factors in cardiovascular disease states associated with an attenuated NO/cGMP signaling pathway.¹⁻³ At present, 3 major mechanisms have been demonstrated to cause downregulation of NO-mediated cGMP production: (1) reduced bioavailability of NO attributable to the reaction of superoxide with NO⁴; (2) oxidation of the sGC heme moiety to its NO-insensitive ferric state, a reaction that can be reversed by reducing agents such as dithionite¹⁷; and (3) irreversible loss of the enzymatic capacity by downregulation of sGC protein levels.⁵⁻⁸ Here, we provide evidence that the latter 2 processes are related in that sGC heme oxidation causes ubiquitination and subsequent proteolytic degradation of sGC.

Downregulation of sGC in response to oxidative stress and heme oxidation appears to be a rather uniform response¹³ that may be physiologically and pathophysiologically relevant,³¹ eg, in hypertension. For example, in aortas from 16-month-

old SHR, superoxide production is elevated and sGC protein levels are decreased compared to age-matched WKY.⁵ From a therapeutic perspective, it is important that this sequence of events is prevented by the novel vasodilator class of sGC activators, represented by BAY 58-2667.³² BAY 58-2667 effectively protects sGC from heme oxidation-induced ubiquitination and proteasomal degradation. Most likely, this compound acts by binding to the unoccupied heme pocket of sGC, thereby stabilizing the heterodimer and preventing its degradation.^{13,19,33} Given its enormous therapeutic potential as an acute vasodilator and cardioprotective agent, a detailed understanding of its mode of action including chronic effects is of high relevance for cardiovascular medicine.

sGC ubiquitination is a key and rate-limiting step of sGC degradation. The relatively low amounts of sGC expressed in primary cells and aorta and the well-known lack of highly sensitive anti-ubiquitin antibodies in the field^{34,35} foil any detailed mechanistic studies addressing the modification of sGC by endogenous ubiquitin. However, our data using sGC-overexpressing cells clearly establish the mechanistic link between oxidation, ubiquitination, and proteasomal degradation.

In the sGC-overexpressing cells, application of BAY 58-2667 completely abolished ODQ-induced sGC ubiquitination and degradation, and potentially upregulated sGC levels

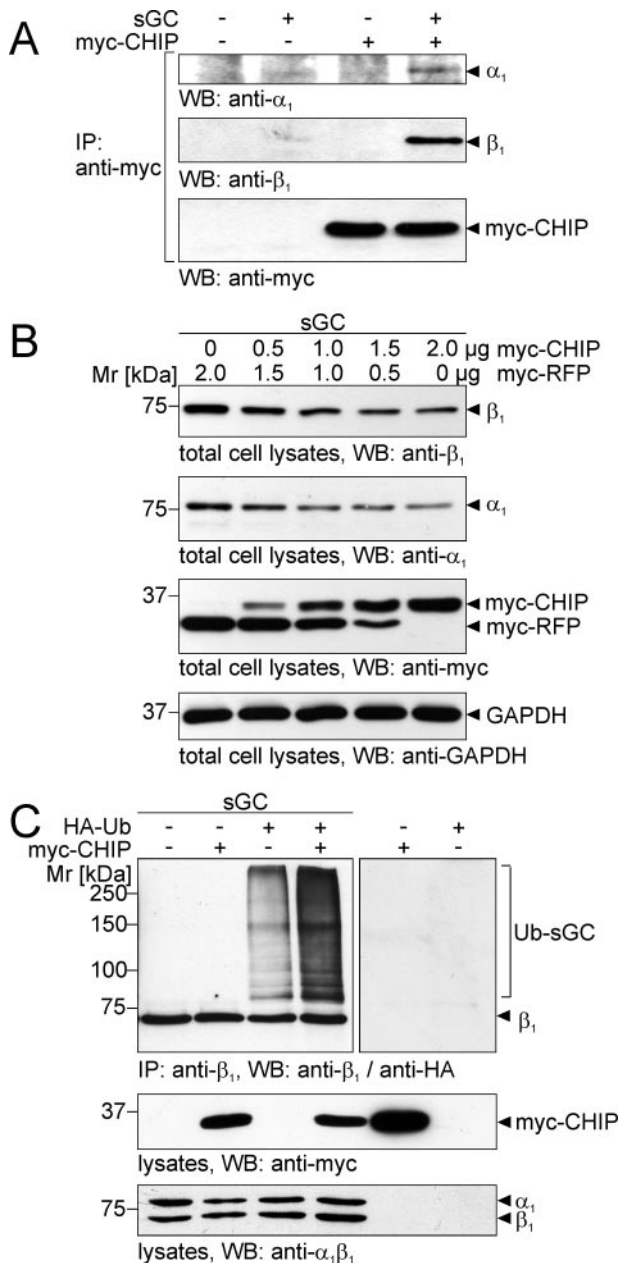


Figure 6. E3 ligase CHIP interacts with and ubiquitinates sGC. A, Lysates from COS-1 cells overexpressing sGC and myc-tagged CHIP were used for immunoprecipitation with anti-myc. Immunoprecipitates and cell lysates were analyzed by WB with anti- α_1 (AS587), anti- β_1 (5A5), or anti-myc. B, COS-1 cells were cotransfected with cDNAs encoding $\alpha_1\beta_1$, increasing amounts of myc-CHIP (2 to 0 μ g), and decreasing amounts of myc-RFP (0 to 2 μ g; control protein). Total cell lysates were probed by WB with anti- β_1 (AS566), anti- α_1 (AS587), anti-myc, or anti-GAPDH. C, Lysates from COS-1 cells overexpressing $\alpha_1\beta_1$ in the absence or presence of HA-Ub and/or myc-CHIP were used for immunoprecipitation with anti- β_1 (5A5). Immunoprecipitates were analyzed by WB with anti-HA and anti- β_1 (5A5). Cell lysates were probed with anti-myc, anti- $\alpha_1\beta_1$ (AS613/5A5), or anti-HA (Online Figure III, B). Representative blots of at least 3 independent experiments are shown.

even beyond control. These data clearly point to the existence of both a basal and an ODQ-augmented turnover of sGC. Similar results were obtained when another sGC inhibitor and heme oxidant, NS2028,^{25,26} was used. Although ligands often

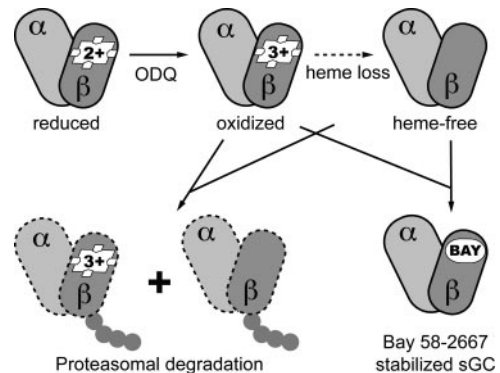


Figure 7. Regulation of sGC by the ubiquitin-proteasome pathway and rescue by BAY 58-2667. Reduced sGC functions as the physiological NO receptor. On heme oxidation, sGC is targeted to ubiquitination and proteasomal degradation. BAY 58-2667 selectively binds to heme-deficient and/or oxidized sGC and stabilizes it, thereby preventing attenuation of the NO/cGMP signaling cascade.

lead to downregulation and desensitization of their pharmacological targets,^{36,37} the novel sGC activator apparently stabilizes its receptor enzyme. The precise mechanism by which BAY 58-2667 prevents ubiquitin-mediated sGC degradation is presently unclear. Given that BAY 58-2667 and zinc-protoporphyrin IX bind with high affinity to the heme pocket of sGC,^{13,19} it is tempting to speculate that occupation of the heme-binding site by an oxidation-resistant ligand masks a crucial lysine residue in the β subunit serving as the major acceptor site for ubiquitination. In this context, a recent study is of great interest showing that the effects of BAY 58-2667 on sGC activity and stability depend on its unique structural ability to reassemble the spatial structure of the heme moiety within sGC.³⁸ Interestingly, 2 residues of the β_1 subunit, Y135 and R139, known to be crucial for heme binding also seem to be essential for the stabilizing effects of BAY 58-2667.

The ubiquitin-proteasome pathway is responsible for protein quality control in cells and has emerged as an important player in many intracellular processes.³⁹ Our experiments show that both sGC subunits are prone to ubiquitination and are degraded via the proteasomal pathway. In this context, it is important to mention that we have identified by MS the monoubiquitinated form of β_1 , however, the typical ladder of protein bands seen with overexpressed HA-Ub clearly points to polyubiquitination of β_1 . Under our experimental conditions, ubiquitination of β_1 was much stronger than that of α_1 , which could be attributable to differences in the avidity of the antibodies used for immunoprecipitation. However, given the tight association between the sGC subunits, it is conceivable that α_1 is cotargeted to the proteasome via ubiquitinated β_1 . Alternatively, α_1 may undergo ubiquitin-independent proteasomal degradation.^{40,41} The former notion is supported by a recent study demonstrating that a splice variant of α_1 lacking its 240 N-terminal amino acids ("C- α_1 ") forms C- $\alpha_1\beta_1$ heterodimers, which are enzymatically active and appear to be protected against ODQ-induced downregulation.⁴² The authors speculate that "stable" C- $\alpha_1\beta_1$ may explain the positive vasodilatory effects of BAY 58-2667 in diseased

blood vessels.²⁴ It will be intriguing to test for the differential expression of α_1 versus C- α_1 under pathological conditions.

Finally, we have identified CHIP as a likely candidate for an E3 ligase mediating sGC ubiquitination. These findings are in line with the recent observation that sGC is a CHIP target⁴³ and that Hsp90-bound CHIP targets glucocorticoid receptor via ubiquitination to the proteasomal machinery.³⁰ Thus, it appears that simultaneous (and probably independent) binding of CHIP and its targets to molecular chaperones, such as Hsp70 and Hsp90, may allow CHIP to tilt the delicate balance between protein folding and protein degradation, thereby changing the fate of its targets.

Taken together, our results show that physiological sGC protein quality control occurs via the ubiquitin-proteasome machinery. Under heme-oxidizing conditions, where the response of sGC to its physiological activator NO is impaired through oxidation of the central heme iron, sGC is targeted to ubiquitination and proteasomal degradation (Figure 7). The impairment of the NO/cGMP cascade, eg, during progression of cardiovascular diseases, thus may reflect, in part, degradation of the major intracellular NO receptor sGC. Moreover, our present studies shed new light on the molecular mechanisms by which novel type of sGC activators such as BAY 58-2667 exert their beneficial effects in cardiovascular diseases by stabilizing their receptor, ie, oxidized/heme-free sGC.

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Disclosures

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Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and penicillin/streptomycin were obtained from PAA (Pasching, Austria); collagenase from Worthington (Lakewood, NJ); ECLTM detection reagents from Amersham Biosciences (Buckinghamshire, UK); monoclonal antibody to c-Myc (clone 9E10) and protein A/G PLUS-agarose from Santa Cruz Biotechnology (Santa Cruz, CA); antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Abcam (Cambridge, MA); antibody to hemagglutinin (anti-HA) from BAbCO (Richmond, CA); monoclonal antibody to eNOS from BD Biosciences (Franklin Lakes, NY); MG132, epoxomicin, lactacystin and N-ethylmaleimide from Calbiochem (San Diego, CA); ODQ and NS2028 from Alexis Biochemicals (Axxora; San Diego, CA); complete protease inhibitor cocktail from Roche (Mannheim, Germany). All other reagents including porcine pancreatic elastase and phenylmethylsulfonyl fluoride (PMSF) were from Sigma-Aldrich (St. Louis, MO). BAY 58-2667 was obtained from Bayer HealthCare.

Antibody Production. Antisera to the human α_1 (AS587) and β_1 subunits (AS566) were raised in rabbits using synthetic peptides corresponding to positions 94-121 (α_1) and 593-614 (β_1), respectively. Antisera to the regulatory domain of human α_1 (1-419) and to the catalytic domain of human β_1 (404-619) were produced in rabbits (AS558 and AS556, respectively) or mice (AS613 and AS614, respectively) using the corresponding purified glutathione S-transferase (GST) fusion proteins. Monoclonal antibody to human β_1 (clone 5A5) was raised in mouse using the β_1 regulatory domain (1-134) fused to GST as the antigen. Alternatively, antisera to the human α_1 (SA3093) and β_1 (SA2934) subunits were used.¹

Construction of Expression Plasmids. Expression plasmids coding for human α_1 (pSG8- α_1) or β_1 (pEDmtx- β_1) were generated as described previously.^{2,3} The cDNAs for myc-tagged CHIP (myc-CHIP) and hemagglutinin-tagged ubiquitin (HA-Ub) were kindly provided by Dr. Ivan Dikic (Institute of Biochemistry II, University of Frankfurt,

Germany). For the expression of myc-tagged red fluorescent protein (myc-RFP), we amplified the corresponding cDNA by PCR using the vector pDsRed-Monomer-N1 from Clontech Laboratories (Mountain View, CA) as a template and subcloned the product into the *EcoRI-XhoI* sites of pcDNA3myc. All constructs were verified by sequencing prior to use.

Sample Preparation for Mass Spectrometry. For mass spectrometry (MS) analyses, samples were subjected to SDS-PAGE. After silver staining of the gel, protein bands of interest were excised and subjected to in-gel digestion protocols that were adapted for a Microlab Star digestion robot (Bonaduz, Switzerland).^{4,5} Briefly, samples were reduced, alkylated and subsequently digested using proteomics grade trypsin from Sigma-Aldrich (St. Louis, MO). The peptide extracts were dried in a vacuum centrifuge and stored at -20°C.

MALDI-TOF MS and MALDI-TOF/TOF MS/MS. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) and MALDI-TOF/TOF MS experiments were done with a 4800 MALDI TOF/TOF™ Analyzer (Applied Biosystems, Foster City, CA). The samples were dissolved in 5 µL of water/acetonitrile/trifluoroacetic acid (water/ACN/TFA: 29.5/70/0.5). For the matrix, α -cyano-4-hydroxycinnamic acid (3 mg/ml; Bruker Daltonics Inc., Manning Park Billerica, MA) dissolved in the same mixture was used. Analyte and matrix were spotted consecutively in a 1:1 ratio on a stainless steel target and dried under ambient conditions. Prior to analysis, the dried sample was washed with ice-cold 5% formic acid to reduce salt contamination. All spectra acquired by MALDI-TOF MS were externally calibrated with a Sequazyme™ peptide mass standards kit (Applied Biosystems). Data analyses were done using the software Data Explorer version 4.9 (Applied Biosystems), and proteins were identified with the search engine Mascot (www.matrixscience.com; Matrix Science, Boston, MA) (peptide mass tolerance: 60 ppm; MS/MS tolerance: 0.3 Da; maximum missed cleavages: 1) using the protein database NCBI nr. Proteins identified by MALDI-TOF MS with a score of 79 or higher were considered significant ($p < 0.05$). Single peptides identified by MALDI-TOF/TOF MS/MS with individual ions scores greater than 47 indicate identity or extensive homology ($p < 0.05$).

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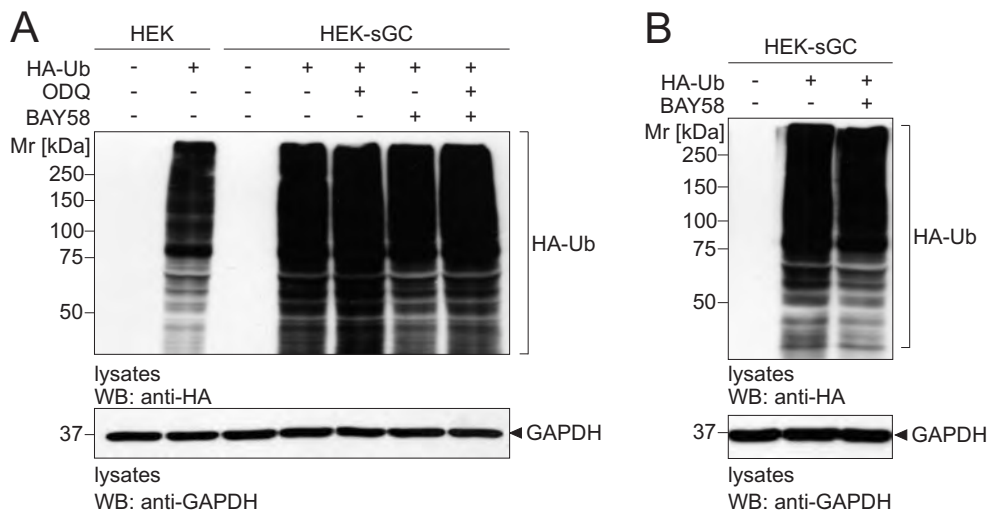
Figure Legends

Online Fig. I. Cell lysates from Fig. 4B (A) or 4C (B) were analyzed by Western blotting with anti-HA or anti-GAPDH.

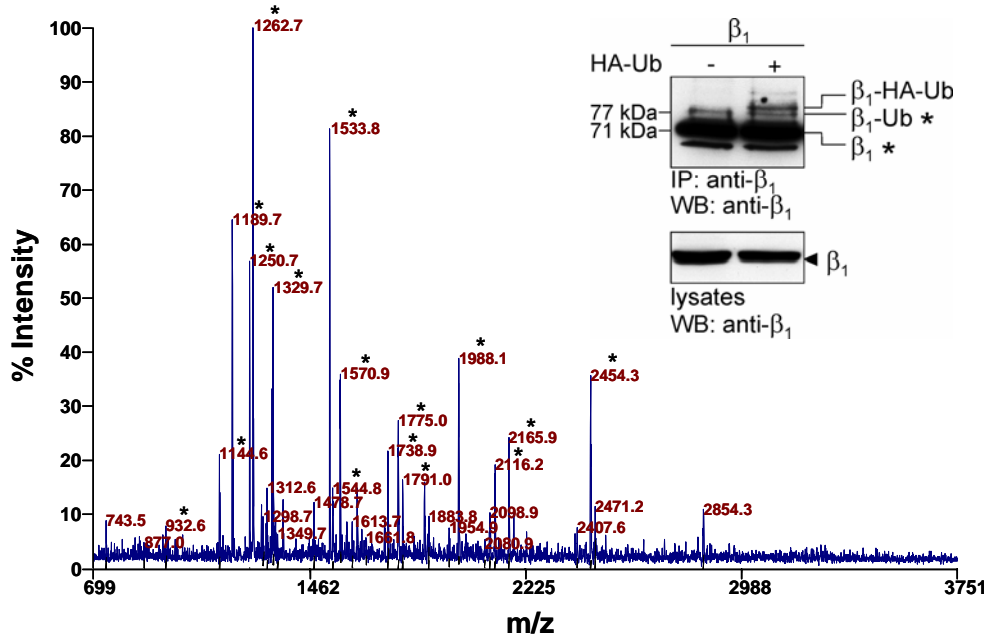
Online Fig. II. Mass spectrometry analysis of monoubiquitinated β_1 . (A, *inset*) Lysates from COS-1 cells overexpressing β_1 in the absence or presence of HA-Ub were used for immunoprecipitation with anti- β_1 (5A5). Immunoprecipitates were analyzed by SDS-electrophoresis and Western blotting with anti- β_1 (5A5). Cell lysates were probed with anti- β_1 (5A5). Protein bands marked with an asterisk (71 and 77 kDa) were excised from the corresponding SDS gel and used for MALDI-TOF analysis. (A) MALDI-TOF mass spectra of the 71 kDa protein; matched peptides that correspond to sGC β_1 are indicated with an asterisk (for sequence analysis, see Online Table I). (B) Magnifications of the MALDI-TOF mass spectra of the 77 kDa protein (1500-1600 m/z (*left panel*); 2000-2200 m/z (*right panel*)). Two peptides, indicated with an arrow, were assigned to ubiquitin and validated via MALDI-TOF/TOF MS/MS with a protein score of 53 (for sequence analysis, see Online Table I). Peptides that correspond to β_1 are indicated with an asterisk. (C) Summarized results obtained from the MALDI-TOF (A) and MALDI-TOF/TOF experiments (Online Table I).

Online Fig. III. (A) Lysates from COS-1 cells overexpressing sGC and myc-tagged CHIP were used for immunoprecipitation of sGC with anti- β_1 (AS556). Immunoprecipitates and cell lysates were analyzed by Western blotting with anti- $\alpha_1\beta_1$ (AS613/5A5) or anti-myc. (B) Cell lysates from Fig. 6C were probed with anti-HA and anti- $\alpha_1\beta_1$ (AS613/5A5).

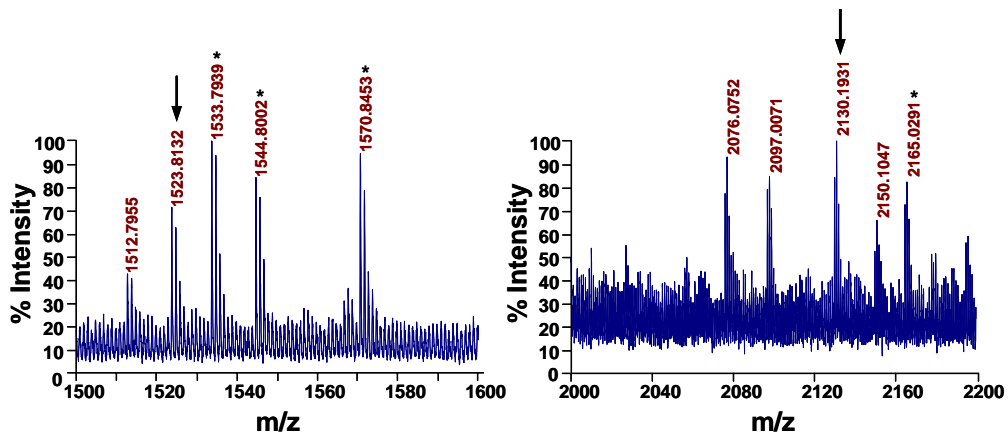
Online Table I. MALDI-TOF identification of trypsin-generated peptides derived from the 71 kDa protein (*upper table*). Two peptides (m/z 1523.82 and 2130.19) of the 77 kDa protein could be assigned to ubiquitin by MALDI-TOF/TOF MS/MS (*lower table*).



A

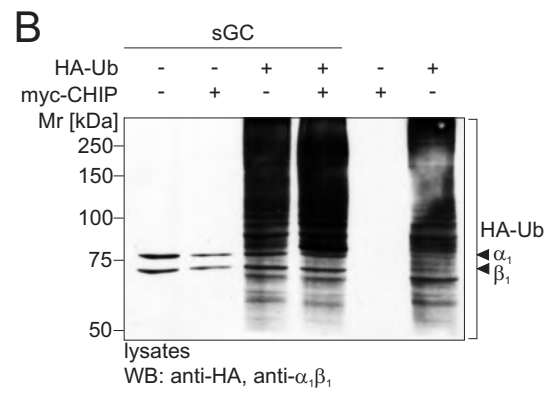
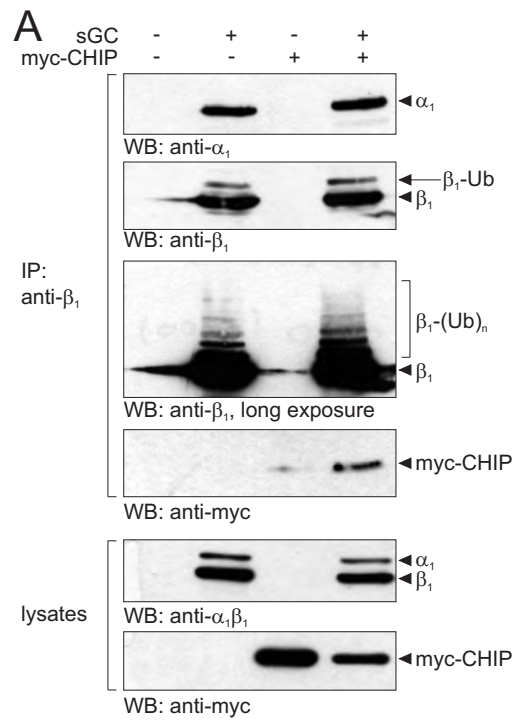


B



C

Identified Protein	Database Accession Number	Score	Sequence coverage	Number of assigned peptides
MALDI-TOF MS				
sGC β_1	gi 4504215	412	61%	39
MALDI-TOF/TOF MS/MS				
Ubiquitin	gi 229532	53	41%	2



MALDI-TOF MS						
Start	End	Observed mass m/z	Error [ppm]	Missed cleavages	Sequence	Modification
1	15	1775,0143	27	0	-.MYGFVNHALELLVIR.N	
1	15	1791,0046	24	0	-.MYGFVNHALELLVIR.N	Oxidation (M)
16	27	1477,759	18	1	R.NYGPEVWEDIKK.E	
28	40	1533,7897	23	0	K.EAQLDEEGQFLVR.I	
41	57	1872,9988	21	1	R.IYDDESKTYDLVAAASK.V	
58	72	1646,9254	19	0	K.VLNLNAGEILQMFGK.M	
73	88	2012,9791	27	0	K.MFFVFCQESGYDTILR.V	
96	116	2454,281	23	0	R.EFLQNLDALHDHLATIYPGMR.A	
130	139	1250,6823	24	0	K.GLILHYYSER.E	
130	151	2529,4648	23	1	K.GLILHYYSEREGLDIVIGIIK.T	
140	151	1297,7921	15	0	R.EGLQDIVIGIIK.T	
152	170	2195,2122	24	1	K.TVAQQIHGTEIDMKVIQQR.N	
208	215	1015,5061	14	0	R.ISPYTFCK.A	
216	225	1262,6964	23	0	K.AFPFHIFDR.D	
216	238	2752,4568	20	1	K.AFPFHIFDRDLVVTQCGNAIYR.V	
290	307	2165,9873	25	1	K.LECEDELGTGTEISCLRLK.G	
336	350	1726,9602	21	1	R.RGLYLSDIPLHDATR.D	
337	350	1570,85	18	0	R.GLYLSDIPLHDATR.D	
351	364	1738,943	24	1	R.DLVLLGEQFREEYK.L	
351	360	1189,6506	6	0	R.DLVLLGEQFR.E	
389	407	2116,2161	24	1	K.KTDTLLYSVLPPSVANELR.H	
390	407	1988,1217	26	0	K.TDTLLYSVLPPSVANELR.H	
408	415	932,5995	22	1	R.HKRPVPAK.R	
416	436	2408,2629	23	1	K.RYDNVTILFSGIVGFNAFCSK.H	
417	436	2252,2136	48	0	R.YDNVTILFSGIVGFNAFCSK.H	
446	456	1333,7708	18	0	K.IVNULLNDLYTR.F	
446	464	2269,2346	23	1	K.IVNULLNDLYTRFDTLTDSR.K	
472	494	2596,3196	21	1	K.VETVGDKYMTVSGLPEPCIHHAR.S	
479	494	1867,9386	24	0	K.YMTVSGLPEPCIHHAR.S	
540	552	1544,7872	22	0	R.YCLFGNTVNLTSR.T	
540	559	2291,1484	22	1	R.YCLFGNTVNLTSRTETTGEK.G	
560	570	1329,7042	18	1	K.GKINVSEYTYR.C	
562	570	1144,605	36	0	K.INVSEYTYR.C	
571	587	2096,9797	25	0	R.CLMSPENSDPQFHLEHR.G	
571	593	2696,3035	24	1	R.CLMSPENSDPQFHLEHRGPVSMK.G	
597	606	1292,6757	23	0	K.EPMQVWFLSR.K	
597	606	1308,666	20	0	K.EPMQVWFLSR.K	Oxidation (M)
597	607	1420,7733	23	1	K.EPMQVWFLSRK.N	
597	607	1436,7733	26	1	K.EPMQVWFLSRK.N	Oxidation (M)

MALDI-TOF/TOF MS/MS						
Start	End	Observed mass m/z	Error [ppm]	Missed cleavages	Sequence	Ion score
30	42	1523,823	27	1	K.IQDKEGIPPDQQR.L	33
55	72	2130,193	18	1	R.TLSDYNIQKESTLHLVLR.L	20

Nitric Oxide–Independent Vasodilator Rescues Heme-Oxidized Soluble Guanylate Cyclase From Proteasomal Degradation

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