Supplemental data to Manuscript 28371

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Targeting the heme-oxidized nitric oxide receptor for selective vasodilation of diseased blood vessels

Supplemental Results

To address by which mechanism BAY 58-2667 protects the sGC protein, HEK-293 cells stably expressing human sGC (HEK-sGC) were incubated with 10 µM ODQ, which considerably decreased sGC protein levels (Supplemental Figure 2), matching the results obtained with porcine endothelial and smooth muscle cells (Figure 4A, D). Presence of 10 µM BAY 58-2667 completely reversed the ODQ-induced decrease and further increased the level of sGC beyond control (Supplemental Figure 2). To test whether down-regulation of sGC is associated with ubiquitination and proteasomal degradation of the enzyme, we overexpressed HA-tagged ubiquitin (HA-Ub) in HEK-sGC cells and incubated them for 14 h in the absence or presence of 10 µM of ODQ, BAY 58-2667 or both. Following cell lysis, immunoprecipitation was done using subunit-specific antibodies to β_1, followed by Western blotting with anti-HA (Supplemental Figure 3). Our results demonstrate that sGC undergoes ubiquitination under these conditions: a major band of 77 kDa was present which most likely represents the mono-ubiquitinated form of β_1, while a ladder of minor bands with increasing molecular masses likely reflects poly-ubiquitinated and/or multiply mono-ubiquitinated β_1. Presence of ODQ in the incubation medium further enhanced the level of ubiquitination, while BAY 58-2667 alone or in combination with ODQ significantly reduced the level of Ub-tagged sGC. Thus heme oxidation induced by ODQ appears to promote ubiquitin-dependent degradation of sGC, whereas BAY 58-2667 completely rescues this effect, most likely through stabilization of the heme-free cyclase. Hence we provide for the first time experimental evidence for the ubiquitination of sGC, which may easily explain

the considerable loss of sGC protein in the presence of heme-oxidizing ODQ, which is rescued by BAY 58-2667.

Supplemental Discussion

BAY 58-2667 is the first tool to functionally analyse the oxidation state of sGC in intact cells, organs and in vivo under physiological and pathophysiological conditions. In addition, it would clearly be desirable to directly measure the intracellular molar ratio of reduced, oxidized and heme-free sGC. However, even under well defined and controlled experimental settings it is currently technically not possible to determine this ratio given small amounts of oxidized/heme-free sGC and the major population of reduced sGC within a given enzyme preparation. UV/VIS-spectra of purified sGC preparations are not sensitive enough to detect a shift of the Soret peak caused by only small amounts of oxidized sGC, and possible heme-free sGC in the enzyme preparation cannot be detected by spectroscopy at all (Supplemental Figure 1). The (in contrast to other heme-binding proteins) non-covalent coupling of the heme moiety to sGC makes it impossible to obtain 100% heme-containing enzyme even within an optimized preparation. The different approaches in the past to quantify the exact amount of bound heme resulted in heme:sGC ratios from 0.9 to 1.5 underlining that an absolutely exact quantification of oxidized or heme-free sGC within the enzyme preparations is not possible (3, 4). Even more problematic is the fact that it is not possible to conserve the oxidation state of sGC during the process of cell lysis and purification. The omission of reductants results in a massive oxidation of sGC, the addition of strong reductants such as DTT or mercaptoethanol leads to a reduction of the heme moiety (5-9). While the latter is desired for an enzyme preparation, it is not useful to conserve and quantify the oxidation state of sGC.

The only way to overcome these obstacles would be the non-invasive measurement of the heme oxidation state of sGC within intact cells. One possibility could be spectroscopic measurements of cells after incubation with increasing concentrations of various oxidants. However, beside that various oxidants such as ODQ interferes with spectroscopic measurements, the total heme content of cells was estimated to about 1.5 nmol heme per mg protein (10) distributed over all cellular heme proteins (e.g. sGC, heme oxygenase, cytochromes, NADPH-oxidases, NO-synthases, transcription factors, like NPAS or BACH). sGC represents just a small fraction of this intracellular pool of heme proteins and therefore it is, up to now, not possible to determine the sGC heme content or its oxidation state by a non-invasive spectroscopic method within intact cells. This obstacle of the missing specificity could be in principle overcome, e.g. by immunoprecipitation with specific sGC-antibodies and subsequent recording of spectra. However, this approach would require the lysis of the cells resulting in the same problems described above concerning the conservation of the sGC oxidation state that would not reflect the situation in intact cells anymore. In addition, the amount of material that would have to be applied in an immunoprecipitation is in a range that one also would have to use a cellular in vitro system (such as transfected Sf9 cells) with artificial oxidants to render a subsequent detection of oxidized sGC possible. Therefore, a direct measurement of the sGC oxidation state ex vivo by using e.g. aortic tissue from an animal disease model would not be possible (beside the problematic aspect of conserving the intracellular sGC oxidation state) due to the lack of material.

Up to now BAY 58-2667 represents the first and only reliable biochemical tool that targets specific oxidized sGC within the whole cellular collectivity of heme proteins in a non-invasive manner. This methodical advancement in specificity was

rendered possible by two points: The unique sGC heme-binding motif (11-15) in combination with a highly optimized compound leading to an affinity to the oxidized enzyme in the subnanomolar range. This advance allows, based on the measured enzymatic activity, for the first time a non-invasive quantification of the intracellular sGC redox state.

Supplemental Methods

Materials. DMEM, FCS and penicillin/streptomycin were obtained from PAA; ECL detection reagents from Amersham Biosciences; protein A/G PLUS-Agarose from Santa Cruz Biotechnology; complete protease inhibitor cocktail from Roche; N-ethylmaleimide from Calbiochem; antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Abcam; and antibody to hemagglutinin (anti-HA.11) from BAbCO. All other reagents including phenylmethylsulfonyl fluoride were from Sigma-Aldrich.

Antibody production. Antisera to human sGC α_1 (AS587) and β_1 subunits (AS566) were raised in rabbits using synthetic peptides covering amino acid positions 94-121 and 593-614, respectively; antisera to the catalytic domain of β_1 (404-619) were produced in rabbit (AS556) or mice (AS614) with the corresponding GST fusion protein, as described (1, 2).

Expression plasmids. The cDNA for hemagglutinin-tagged ubiquitin was kindly provided by Dr. Ivan Dikic (Institute of Biochemistry II, University of Frankfurt, Germany).

Cell culture. HEK-293 cells stably transfected with human sGC (HEK-sGC) were cultured in DMEM supplemented with 10% FCS. Transient transfections were done with Metafectene (Biontex Laboratories) according to the manufacturer's instructions. Incubation of cells with BAY 58-2667 or ODQ were done 26 h post-transfection.

Immunoprecipitation and western blotting. Cells from a 60 mm dish were lysed with 0.5 ml buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 5 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, supplemented with protease inhibitor cocktail) and the cleared lysate was incubated with AS556 for 4 h at 4°C under rotation. Antibodies were precipitated with protein A/G PLUS-agarose. Beads were washed with 1 ml each of buffer A (10 mM Tris, pH 8.5, 600 mM NaCl, 0.1% SDS, 0.05% NP-40), buffer B (0.5% Na-deoxycholat in PBS, 1% Triton X-100), buffer C (buffer B containing 2 M KCl) and twice with 0.1 x PBS, all supplemented with 5 mM N-ethylmaleimide and 1 mM phenylmethylsulfonyl fluoride. Following immunoprecipitation samples were subjected to SDS-PAGE and analyzed by western blotting.

Supplemental References

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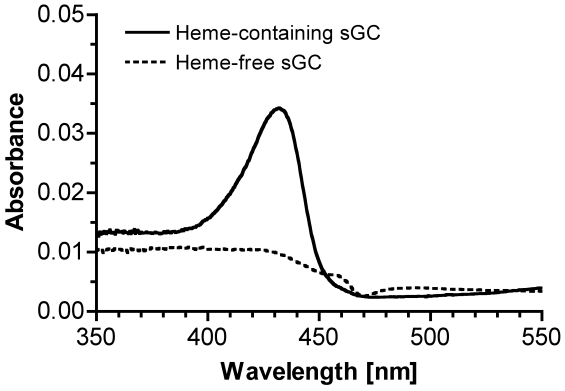
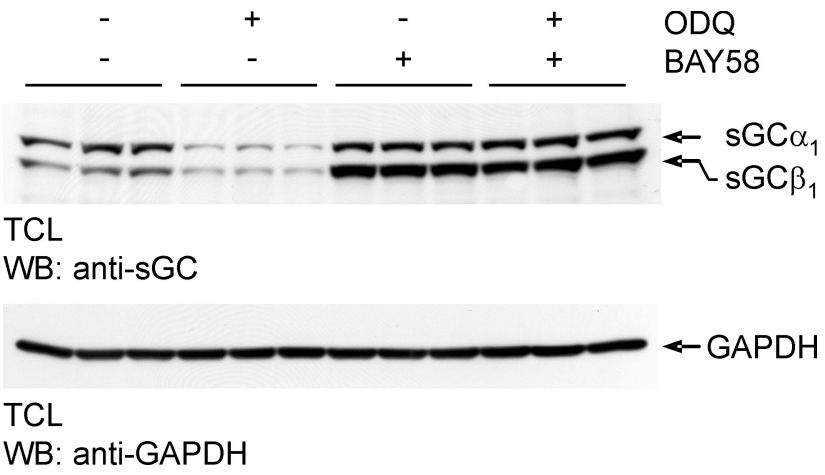
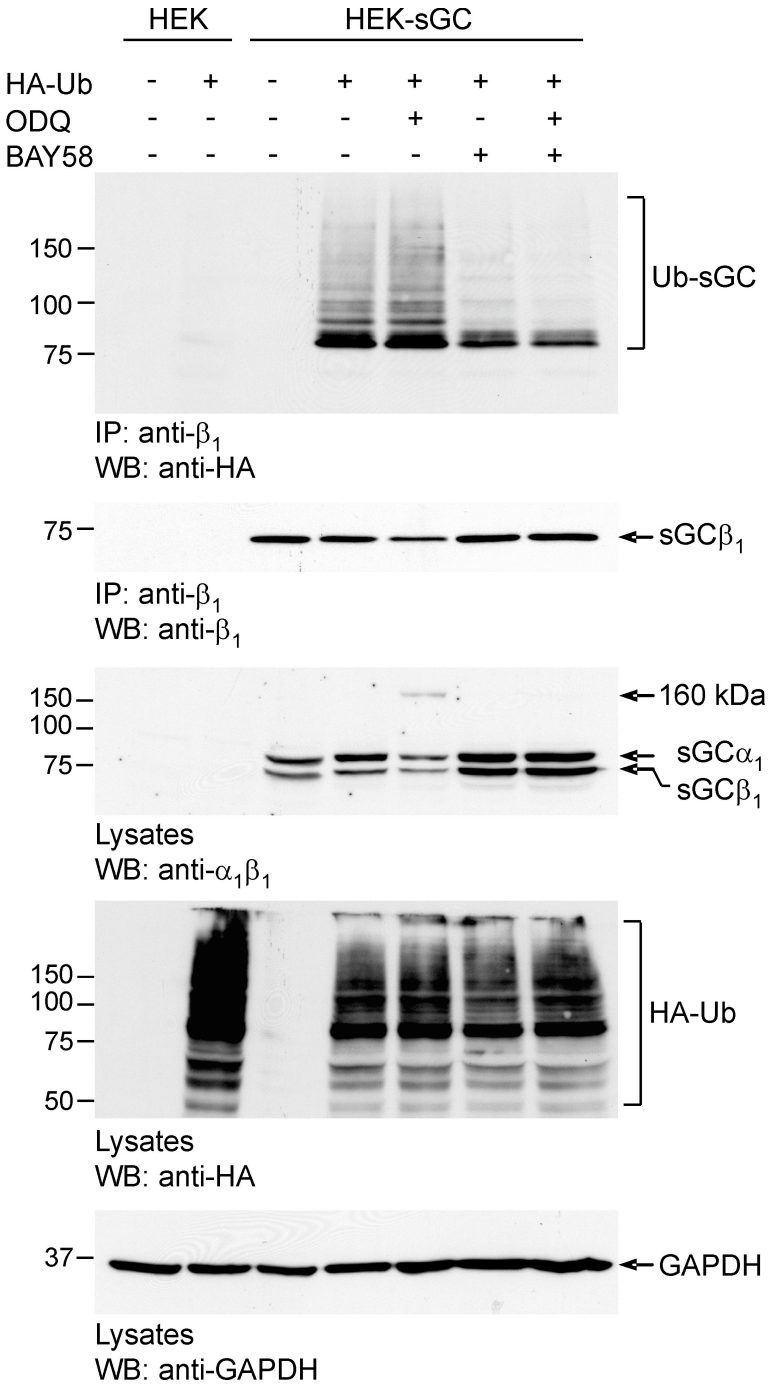
Supplemental Figures



Supplemental Figure 1. UV/VIS spectra recorded from sGC (20 µg) after incubation in the absence or presence of Tween-20 and subsequent separation of the enzyme from detergent and unbound heme by ion exchange chromatography. The measured enzyme preparations were subsequently used in parallel for the sGC activity assays and BAY 58-2667 binding studies shown in Figure 2 E-J.



Supplemental Figure 2. Effects of BAY 58-2667 and ODQ on sGC protein levels. HEK-sGC cells were incubated for 24 h with 10 µM BAY 58-2667, 10 µM ODQ, or both. Total cell lysates (TLC) were probed by Western blotting (WB) with antibodies to sGC $\alpha_1\beta_1$ (anti-sGC; mixture of AS566 and AS587) or to GAPDH (anti-GAPDH; loading control).



Supplemental Figure 3. Effects of BAY 58-2667 and ODQ on sGC ubiquitination. HEK-sGC were transfected with HA-Ub and incubated for 14 h with 10 μM BAY 58-2667, 10 μM ODQ, or both. Immunoprecipitation (IP) was done with anti-β_1 (AS556), followed by western blotting with anti-HA or anti-sGCβ_1 (AS614) (*upper panels*). For control, total cell lysates were probed with antibodies to sGC$\alpha_1\beta_1$ (AS566, AS587), HA or GAPDH (*center and bottom panels*). HEK-293 cells served as control.