REGULATION OF THE NITRIC OXIDE RECEPTOR, SOLUBLE GUANYLYL CYCLASE

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1. Introduction

1.1 Historical overview and functions of NO and cGMP

cGMP is the second identified member of an important class of signaling molecules, the cyclic nucleotides. Discovered in 1963 by Ashman and co-worker (Ashman et. al., 1963), cGMP has been found to be involved in the regulation of vision, vasodilatation, platelets aggregation, smooth cell proliferation, cellular ion homeostasis, synaptic plasticity and other important physiological processes (McDonald and Murad 1995; Carvajal et. al., 2000; Hofmann et. al., 2000).

cGMP synthesizing enzymes, guanylyl cyclases, were found to be present in membranes as well as in cytosolic cell fractions. Enzymatic properties of the enzymes from various subcellular fractions are different. Therefore, guanylyl cyclases were divided into two subfamilies: particulate and soluble guanylyl cyclases (sGC) (Chrisman et. al., 1975; Garbers 1990). While a number of peptide hormones that stimulate particulate guanylyl cyclase were known for some time, the nature of physiological regulator of sGC remained for long time enigmatic. Finally in 1987, two independent groups showed the existence of a principally novel messenger molecule, nitric oxide (NO), which is the most important (if not the only) physiological activator of sGC. This small, fairly long-lived radical seems to be involved in the regulation of a plethora of physiological functions and many diseases. A lot of these effects are mediated by the activation of sGC. Consequently, the field of “NO” research grew exponentially in the last about 15 years, and the importance of this molecule in physiology was confirmed by the Nobel Prize in 1998. However, only minor efforts were made in investigating the regulation of the main NO receptor, sGC, during this time. A number of principal questions of its regulation are still unanswered.

One of the most important and perhaps one of the most unclear points in this field is the existence of additional regulation mechanism for sGC (in addition to direct activation of sGC by NO).

1.2 Structure of soluble guanylyl cyclase

Soluble guanylyl cyclase (EC 4.6.1.2) catalyses the conversion of GTP to sGMP and pyrophosphate. The enzyme is a member of the family of nucleotide cyclases which includes also the adenylyl cyclases and particulate guanylyl cyclases. All of these enzymes share very significant homology in the catalytic domain (Garbers 1992; Garbers and Lowe 1994). Thus, it has been demonstrated that three point mutations in the catalytic domain of sGC can change its specifity from GTP to ATP, converting the enzyme to an NO-sensitive adenylyl cyclase (Beuve 1999).

However, all cyclases are very different in their activation mechanisms. Particulate guanylyl cyclase and adenylyl cyclases are membrane proteins that are activated by the binding of peptide hormones or G-proteins, respectively. Unlike particulate guanylyl cyclase and adelylyl cyclases, sGC does not have a transmembrane domain and thus cannot be directly activated by G-proteins or hormones.

sGC is a heme-containing protein (Stone and Marletta 1994) and heme plays the essential role for sGC activation by NO or NO-releasing compounds (Ignarro et. al., 1986; Wedel et. al., 1994). It is now known that heme plays the central role in regulation site of sGC. In the early 90s it was determined that sGC is a heterodimer consisting of a larger α and β subunits, both of which are required for catalytic activity (Harteneck et. al., 1990). By homology screening, four isoforms of these subunits were found to be present in mammalian genomes. sGCα_1 and sGCβ_1 subunits are ubiquitously distributed and seem to be expressed in

virtually all cells and tissues showing sGC activity (Schmidt et al., 1992; Budworth et al., 1999; Theilig et al., 2001). sGCα_2 was found at the protein level in placenta (Russwurm, Behrends et al. 1998) and brain (Russwurm et al., 2001). sGCβ_2 has been found never at the protein level; its mRNA was detected in kidney and in liver (Yuen et al., 1990). Homologs of mammalian sGCα_1 and sGCβ_1 have been found in Drosophila (Yoshikawa et al., 1993; Liu et al., 1995; Shah and Hyde 1995).

sGCα and sGCβ subunits have a great degree of homology (Koesling et al., 1990). The catalytic domain of sGC is formed by heterodimerization of one sGCα and one sGCβ subunit, which are both necessary for catalytic activity (Wedel et al., 1995). Homodimerization of sGCα_1 or sGCβ_1 subunits has also been demonstrated, although the homodimers were inactive (Zabel et al., 1999). An intriguing exception to this rule seems to be sGCβ_2, which has been found to be active as a homodimer as determined in expression experiments in Sf9 cells (Koglin et al., 2001). An additional fascinating feature of sGCα_2 and sGCβ_2 is the presence of amino acid sequences that allow membrane association of proteins. While sGCβ_2 has an 86-amino acid C-terminal region containing the motif for isoprenylation/ carboxymethylation (Yuen et al., 1990), sGCα_2 was found to have C-teminal motif for interaction with PDZ-domain-containing proteins. The PDZ-domain-containing proteins (such as PSD95, Dlg, ZO-1 and SAP97) play an important role in the architecture of postsynaptic density and function of synapses (Hata et al., 1998; Sheng 2001). For instance, PSD95 has been shown to be the key participant in the attachment of NOS-I to the NMDA receptor (Brenman et al., 1996). Since Ca^{2+} influx through NMDA receptor is considered to be the main activating stimulus for NOS-I in neurons, the proximity of NOS-I to the NMDA receptor should amplify this effect. As the culmination of this very elegant hypothesis, the interaction

of sGCα_2 with PSD95 via a free PDZ-domain has been recently shown (Russwurm et al., 2001).

It has been known for quite some time that heme is an essential cofactor required for normal sGC activation; nevertheless, the stoichiometry of heme binding to sGC was until recently controversial (Waldman and Murad 1987; Stone and Marletta 1995). Now it is accepted that sGC contains one heme molecule per heterodimer (Brandish et al., 1998). Heme is ligated to the N-terminus of sGCβ via an axial histidine (histidine-105) (Wedel et al., 1994). Two cysteine residues were also found to be necessary for heme binding to sGC (Friebe et al., 1997). Heme serves as the NO receptor moiety of sGC and allows activation of the enzyme only in the ferrous state (Stone et al., 1996). Interestingly, in contrast to this it has been demonstrated that activation of sGC by arachidonic acid can be inhibited by heme (Ignarro and Wood 1987).

As the main NO receptor, sGC mediates many of the effects of this molecule. However, there are also other possibilities for signal transduction by NO via "redox regulation". This might be evolutionarily the earliest NO-mediated signaling pathways in organisms, the further development of which might have resulted in the formation of the NO-sGC-cGMP transduction machinery. The question about the onset of this signal transduction tandem is yet not clarified. NO synthesizing activities have been found in such invertebrates like Hydra vulgaris (Colasanti et al., 1997) and tapeworm (Gustafsson et al., 1996). cDNA of sGC has been found in Drosophila melanogaster (Shah and Hyde 1995) and mosquito Anopheles gambiae (Caccone et al., 1999). Biological effects of the sGC inhibitor, ODQ, have been described even in the myxomycete, Physarum polycephalum (Golderer et al., 2001).

1.3 Regulation of sGC activity

1.3.1 Activators of sGC

Beginning in the early 70s, scientists tried to identify the physiological sGC regulator. Although many of substances are able to modulate sGC activity, no significant insights into their physiological relevance could be obtained. In 1975, Murad and co-workers showed that the ability of sodium azide to stimulate sGC strongly depends on the presence of tissue homogenates (Kimura et al., 1975). Later, the ability of other nitrogen-containing substances such as nitroglycerine and sodium nitroprusside to stimulate sGC was demonstrated (Katsuki et al., 1977). In 1977, the same group showed that nitric oxide (NO) stimulated sGC without additional compounds or tissue extracts (Arnold et al., 1977). It was postulated that nitric compounds (in particular therapeutically used organic nitrates) activate sGC due to enzymatic (as well as spontaneous) NO release. However, these studies seemed to have little impact on the identification of the physiological sGC regulator, since no evidence for the existence of NO (a very short-lived free radical) in organisms was available at that time. The situation changed dramatically as two groups independently showed that NO is produced by endothelial cells and also mediates such physiological reactions as vasodilatation and stimulation of cGMP formation (Ignarro et al., 1987; Ignarro et al., 1987; Palmer et al., 1987). Thus, the explosive growth of nitric oxide research in life and medical sciences began.

NO is the strongest and up to now the only well-established, physiologically relevant sGC activator; it is able to stimulate sGC up to several hundred fold (Stone and Marletta 1998; Hoenicka et al., 1999; Lee et al., 2000) at nanomolar concentrations (Bellamy et al., 2000; Bon and Garthwaite 2001). Activation occurs via the binding of NO to the prosthetic heme group of sGC. It is sassumed that in the NO-free state, iron is pentavalent and bound to histidine-105 of sGCβ_1. After NO binding, the iron-histidine bond breaks, which produces

conformational changes in the sGC molecule and subsequently enzyme activation (Koesling 1999). Indeed, by recording of spectral profiles of sGC before and after NO addition, the changes in iron coordination and the break of the iron-histidine bond were confirmed (Schelvis et al., 1998; Sharma and Magde 1999). The essential role of this bond break was confirmed by the observation that another heme-binding molecule, CO, is a weak sGC activator. Unlike NO, CO does not break the iron-histidine-105 bond but evokes hexavalent ligation of iron (Stone and Marletta 1994; Burstyn et al., 1995). It is assumed that the failure of CO to break the iron-histidine bond is the reason for its weak sGC-activating potency. However, combined action of CO and another NO-independent sGC activator, YC-1 (see below), results in activation of sGC comparable to that of NO without loss of 6-coordinated state of heme (Stone and Marletta 1998; Denninger et al., 2000).

Additional evidence for the role of heme interaction with the protein for sGC activation is the ability of protoporphyrin IX (PPIX), an iron-free heme, to stimulate hemedeficient sGC (Ohlstein et al., 1982). NO or CO do not stimulate heme-deficient sGC. Interestingly, YC-1 could potentiate the PPIX-stimulated sGC activity, but no shift of the concentration-response curve as seen with NO or CO was observed (Friebe et al., 1996; Friebe and Koesling 1998).

An interesting observation was made by the group of Marletta. They found that just after NO binding, a 6-coordinated iron-complex is formed (Zhao et al., 1999). In this conformation, no activation of sGC is produced. The following step – release of axial histidine heme ligands, leading to the enzyme activation - is also dependent on the NO concentration. On the basis of these observations, the authors concluded that another NO binding site on sGC should be exist to modulate the ligation of heme and subsequently activation of sGC.

In this context, the role of SH groups for sGC regulation can be very interesting. It has been known for some time that SH-reactive reagents possess the ability to modulate sGC

activity (Ignarro et al., 1980; Ignarro and Gruetter 1980). The mechanism of this effect remains to be understood. Both inhibiting (Braughler 1983) and stimulating (Niroomand et al., 1989) effects of SH-reducing agents have been demonstrated in different studies. Moreover, it has been demonstrated that under some conditions, SH-oxidants are able to stimulate sGC in the presence of excess of glutathione (Wu et al., 1992). It has been speculated that under these conditions posttranslational modification of the enzyme could be responsible for the effect. Since NO (or some of its redox species) reacts very rapidly with SH groups, it cannot be excluded that some SH group-containing sites on sGC might participate in the regulation of heme-dependent activation of sGC by NO.

Another interesting substance modulating the heme-dependent activation of sGC is YC-1 (3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole). YC-1 is a recently described sGC activator whose effect is due to neither NO release nor to ligation of heme iron (Friebe and Koesling 1998). YC-1 itself is a weak sGC stimulator, producing up to 12–fold activation of sGC (Friebe et al., 1996). This compounds sensitizes sGC to NO and CO (Friebe and Koesling 1998). Interestingly, the joint action of two weak sGC activators, YC-1 and CO, produces a positively synergistic enzyme activation, which is comparable to the effect of NO (Stone and Marletta 1998; Kharitonov et al., 1999; Denninger et al., 2000). It remains to be investigated whether any YC-1-like compounds exist in animals, as has been speculated (McLaughlin et al., 2000). This is also important because CO is supposedly a physiological sGC regulator, at least in some brain regions (Verma et al., 1993; Hawkins et al., 1994; Leinders-Zufall et al., 1995). It has also been demonstrated that intestinal cGMP levels are markedly reduced in mice with deletion of heme oxygenase 2, an CO-producing enzyme (Zakhary et al., 1997). Existence of an enhancer of CO could help to explain how a weak sGC activator, CO, can significantly affect cGMP production in living cells. An YC-1-like

substance is speculated to play this role (McLaughlin et al., 2000).

Another unsolved question in the regulation of sGC is the rate and mechanism of the enzyme deactivation. Maintenance of the activated state is dependent on NO dissociation from sGC heme. In experiments with purified enzyme, the half-life of NO-sGC complexes was determined to be in the minute range (Kharitonov et al., 1997; Brandish et al., 1998), which is very long for one signal transduction protein that participates in the regulation of short-term processes. Another study established that addition of the sGC substrates GTP and Mg^{2+} reduces the half-life to about 5 sec (Kharitonov et al., 1997). Garthwaite and co-workers have found that in intact cells, deactivation (desensitisation) of sGC is even more rapid (within seconds or less) and is followed by slow recovery (half-life about 1.5 min). Lysis of cells completely disrupted this mechanism of sGC regulation (Bellamy and Garthwaite 2000). Interestingly, YC-1 has been found to slow dissociation of NO from sGC (Bellamy and Garthwaite 2002), which can be a mechanism involved in the compound's effect on sGC.

YC-1 received much attention as the first member of principally novel class of sGC activators as well as possible therapeutically important agents for sGC modulation. Unfortunately, the mechanisms of action of this compound are not completely understood. Additionally, this substance is not free from all pharmacological disadvantages of NO donors (YC-1 as well as other cGMP-elevating agents down-regulates PKG-1 levels in different cell types) (Ibarra et al., 2001) and is very hydrophobic, which is a serious limitation for the therapeutic use of the compound.

Interesting sGC-modulating compounds were developed recently by some pharmaceutical companies, such as Bayer and Aventis. Bayer identified a new YC-1-like activator of sGC, BAY41-2272 (Stasch et al., 2001). Using this drug, the binding site for YC-1 (which seems to be the same as for BAY41-2272) was identified (Stasch et al., 2001).

Very recently, an even more intriguing activator was reported by the same group. BAY58-2667 is a substance that activates heme-deficient or oxidized sGC even more potent than native (reduced) one (Stasch et al., 2002). Oxidized (or heme-free) sGC seems to be the primary target for this compound. Thus, BAY58-2667 represents a new class of sGC activators that activate that pool of sGC which is insensitive to NO-releasing compounds or YC-1. Compounds with similar ability to activate oxidized sGC were also identified by the research group of another company, Aventis.

A great number of other substances have been considered as possible physiological sGC regulators. However, after discovery of the role of NO in sGC activation, many (if not all) of these effects could be explained via effects on NO bioavailability or stability.

1.3.2 Inhibitors of sGC

To study functions and regulation of an enzyme, the existence of specific inhibitors is especially important. Different substances have been used as sGC inhibitors. One of the first sGC inhibitors was methylene blue. Currently, this compound is no longer considered as an sGC-specific inhibitor, since inhibition of other enzymes such as NOS (Mayer et al., 1993a; Mayer et al., 1993b) as well as NO scavenging by superoxide production (Marczin et al., 1992) can interfere with sGC inhibition and complicate the interpretation of results obtained. It has been also demonstrated that in contrast to another sGC inhibitor, ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), methylene blue was not able to completely block cGMP synthesis and relaxation in response to SNP in guinea pig trachea (Hwang et al., 1998). The ability of methylene blue to reduce cGMP accumulation in cultured rabbit pulmonary arterial smooth muscle cells in response to different NO donors has been found to vary and directly correlate with the ability to scavenge NO (Marczin et al., 1992). However, none of the known sGC inhibitors is free of disadvantages. It has been recently shown that the most

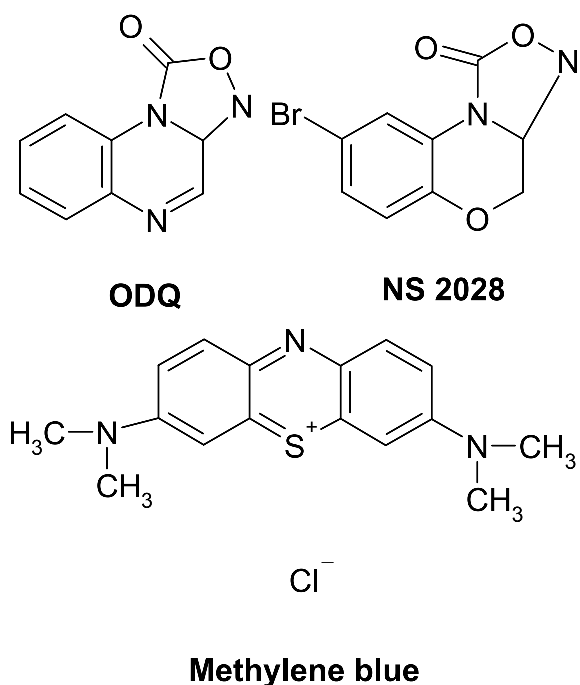

Fig. 1.1 Inhibitors of sGC.

frequently used sGC inhibitor, ODQ, also inhibits NOS (Feelisch et al., 1999). The reason for this interference is the mechanism of action of the compounds used. All of these reagents inhibit sGC by oxidation of the heme group of the enzyme (Schrammel et al., 1996; Dierks and Burstyn 1998). Of course, other heme-containing proteins (such as NOS) can be also affected. Thus,although, these substances can be successfully used for investigation of sGC function on purified sGC, their effects in complex systems (for instance, in cells or tissue homogenates) should be interpreted with great care. Despite these limitations, sGC inhibitors have been used in many studies and have resulted in a great deal of interesting and important information about the enzyme. Recently, another ODQ-related compounds, NS2028 (4H-8-bromo-1,2,4-oxadiazolo[3,4-d]-benz(b) [1,4]oxazin-1one), has been described as a novel, potent, and specific inhibitor of sGC (Olesen et al., 1998).

An interesting observation regarding interaction of the sGC activator, YC-1, and the sGC inhibitor, ODQ, has been made by Bellamy and Garthwaite (Bellamy and Garthwaite 2002). They showed that YC-1 both reverses and prevents inhibition of sGC by ODQ in cerebellar cells. This has been corroborated using purified sGC, indicating that the effect is exerted on the enzyme itself. The mechanisms involved in this effect of YC-1 are stillunknown.

In Figure 1.1, the chemical structures of the most frequently used sGC inhibitors are

shown.

1.3.3 Desensitization of the NO-dependent cGMP response

Modulation of the NO/cGMP signaling pathway has already been used for some time in therapeutic practice (however, the mechanisms of action of the drugs used were for long time unknown). The best example of this is organic nitrates, which found broad application for the treatment of angina pectoris and some other coronary diseases. Organic nitrates were used for clinical purposes as early as the 19th century, about 100 years before the molecular mechanisms of their action were clarified.

It has also been known for a long time that nitrates lose their protective action under prolonged application. This phenomenon is known as nitrate tolerance. Despite much investigation the in last decades, the mechanism of nitrate tolerance is not understood. It has been proposed that desensitisation to nitrate action is due to impaired biotransformation of these compounds and reduced production of NO (Feelisch and Kelm 1991), which possibly occurs by desensitisation of the enzymatic component of nitrate degradation. It has also been found that response to other NO donors is not affected in nitrate-tolerant animals (Forster et al., 1991; Unger et al., 1993). This contradicts, however, other studies that suggest impaired response to other NO donors after treatment with nitrates (Molina et al., 1987; Schroder et al., 1988). Additionally, not only organic nitrates, but also other NO-releasing compounds, such as nitrosothiols and SNP, were found to produce desensitisation (Davis et al., 1997; Hamad et al., 1999). Since predominantly spontaneous, non-enzymatic NO release from these compounds has been proposed, reduction of any enzyme activity leading to decreased NO production would be a very unlikely explanation for this phaenomenon. The demonstration that nitrates were not able to impair PPIX-stimulated sGC activity (Waldman et al., 1986) suggests that decreased sensitivity of sGC to NO might be responsible for the desensitisation.

Interestingly, not only exogenously added NO donors but also endogenously produced NO has been shown to desensitise the cGMP response (Brandes et al., 2000). In this study, it was demonstrated that the cGMP response to SNP in aortic rings can be increased by preinhibition of eNOS or genetic disruption of the eNOS gene. Since no differences in sGC protein were found between eNOS-knock-out or with N-nitro-L-arginine (an NOS inhibitor)-treated and control mice, the authors speculated that desensitisation of sGC was responsible for decreased sGC activity. It should be noted here that a study in our laboratory has reproduced these results showing increased sGC activity in eNOS-knock-out mice, but this was accompanied by an increase in the protein level of both sGC subunits (Ibarra et al., 2001).

Desensitization of sGC has been also observed during some pathological states such as renal endotoxemia (Knotek et al., 2000) and hypertension (Kojda et al., 1998). Except in cases where a reduction in the amount of sGC protein was found, the mechanism of sGC desensitisation remains to be elucidated.

1.3.4. Role of divalent cations in the regulation of sGC

sGC requires divalent metal cations for catalytic activity. Both Mg^{2+} and Mn^{2+} can maintain catalytic activity of sGC probably via binding to the catalytic site or forming a complex with nucleotide substrate (Me-GTP) (Frey et al., 1977; Mittal et al., 1977). Interestingly, these two metals differ in their effects on basal and stimulated sGC activity. While Mg^{2+} is required for maximal stimulation of sGC with NO, basal activity of the enzyme is higher in the presence of Mn^{2+} (Craven and DeRubertis 1976; Kimura et al., 1976). Exact mechanisms of these different actions of both cations are unclear. It has been suggested that divalent cations bind to other unknown sites of sGC and can modulate its activity in allosteric fashion (Kimura et al., 1976; Wang and Kimura 1976).

Another interesting point in the regulation of sGC by divalent cations is the role of

calcium in the regulation of this enzyme. It has been demonstrated that Ca^{2+} inhibits recombinant sGC (Parkinson et al., 1999) and sGC purified from bovine lung (Kazerounian et al., 2002). Inhibition of sGC by Ca^{2+} in rat anterior pituitary cells has been also demonstrated (Andric et al., 2001). The mechanism of the calcium effect on sGC activity is not completely elucidated. Recently, sGC has been found to specifically bind Ca^{2+}, and the existence of two different binding sites for divalent cations on the enzyme has been postulated (Kazerounian et al., 2002). Ca^{2+} is able to inhibit purified sGC via binding to a high affinity binding site in the concentration range that is in the physiological range of intracellular Ca^{2+} concentration in activated cells (100-500nM). At a Ca^{2+} concentration of about 10 μM the inhibition of sGC was complete, which was explained by the binding of Ca^{2+} to a low affinity site (Kazerounian et al., 2002). It has been also demonstrated that Mg^{2+} was not able to prevent the binding of Ca^{2+} to a high affinity binding site and thus also partly reversed Ca^{2+}-dependent sGC inhibition via displacement of Ca^{2+} from a low affinity binding site. Interestingly, Mn^{2+} was completely able to prevent the inhibitory effect of Ca^{2+} on sGC (Kazerounian et al., 2002). Demonstration of Ca^{2+}-dependent inhibition of sGC is an intriguing observation, since cGMP serves in many cases as a negative regulator of the Ca^{2+} concentration and the effects of these substances (Ca^{2+} and cGMP) are very often opposite.

1.3.5 Evidence for posttranslational regulation of sGC

Extremely little is known about the role of posttranslational mechanisms in the regulation of sGC. Although, in 1993 the modulation of sGC activity by enzyme phosphorylation was reported (Louis et al., 1993), no other studies confirmed or refuted the role of sGC phosphorylation until the year 2000. In 2000, Ferrero and co-workers demonstrated sGC phosphorylation by PKG in PC12 cells and showed a decrease in its activity due to phosphorylation. These two studies are the only reports concerning the

phosphorylation of sGC to date and nothing is known about sGC phosphorylation *in vivo*. An explanation for the lack of such information may be that there is no commercial anti-sGC antibody available that can be used for immunoprecipitation.

Modification of protein thiols is another type of posttranslational modification of sGC. This mechanism could be involved in the effects of NO-producing substances. Some NO donors, such as SNP or nitrosothiols, produce NO in the form of the nitrosonium cation, NO^+, which is a perfect agent for nitrosylation of thiols. It is proposed that oxidation (or nitrosylation) of SH groups can modulate sGC activity (Ignarro and Gruetter 1980). To clarify this question, the group of Prof. Koesling produced mutated sGC in which cysteines were replaced by other amino acids. It was found that two cysteines (Cys-78 and Cys-214 in the sGCβ_1) are extremely important for the affinity of sGC for heme (Friebe et al., 1997). It remains to be clarified whether posttranslational modification of these cysteines can participate in the regulation of sGC activation and its sensitivity to NO.

In contrast to sGCα_1 and sGCβ_1, sGCα_2 and sGCβ_2 have additional possible means of for posttranslation modulation. sGCβ_2 has a motif for isoprenylation (Yuen et al., 1990) that suggests there may be regulated membrane association of the protein. The physiological relevance of this isoprenylation remains to be determined as well as the presence of sGCβ_2 protein.

sGCα_2 also has the ability to be attached to the membrane, although, the mechanism of the membrane attachment is completely different. sGCα_2 has no site for acetylation, but the C-terminus of sGCα_2 can interact with PDZ domain-containing proteins (Hoffmüller et al., 1999). Indeed, it was demonstrated that in brain sGCα_2 (and via it also sGCβ_1) is associated with PSD-95 and some other proteins of the postsynaptic membrane (Russwurm et al., 2001).

Because the same PSD-95 protein serves as a coupler between NOS-I and NMDA receptors (Brenman et al., 1996), such close spatial organization of signaling chain NMDA receptor/ NOS-I/sGC could dramatically increase the efficacy of signal transduction between the members of this cascade.

1.4 Regulation of sGC expression

In the last few years several studies have shown that sGC expression can be finely regulated in different cell types. Changes in sGC mRNA and protein levels during some pathological states were also demonstrated (Ruetten et al., 1999; Gupta et al., 1997).

Certainly, one of the first questions in direction of sGC expression regulation was the question about the role of the sGC activator NO. It was demonstrated that NO donors reduce sGC levels in medullary interstitial (Ujiie et al., 1994) and smooth muscle cells (Filippov et al., 1997). Recently, in our laboratory a similar effect of the NO donor, DETA/NO, on the levels of sGCα_1 and sGCβ_1 protein in pulmonary endothelial cells was determined (Ibarra et al., 2001). It was also demonstrated that agents inducing the expression of the constitutively active form of NOS, NOS-II, produce downregulation of sGC mRNA, protein, and activity levels (Papapetropoulos et al., 1996; Takata et al., 2001).

The question about the mechanisms of NO effects on sGC regulation and participation of cGMP in this process seems to be not completely resolved. Filippov and co-worker have investigated the mechanism of this down-regulation of sGC. It has been demonstrated that reduction of sGC mRNA is mediated by a decrease in its stability, an effect that is cGMP-dependent in smooth muscle cells (Filippov et al., 1997). cGMP analogues or cGMP-elevating agents down-regulate sGC mRNA levels also in other cell types (Ujiie et al., 1994; Papapetropoulos et al., 1996; Filippov et al., 1997). However, we have observed cGMP-

independent regulation of sGC protein levels in porcine endothelial and smooth muscle cells(Ibarra et al., 2001).

cAMP has been found to down-regulate sGC levels in all studies reported (Shimouchi et al., 1993; Papapetropoulos et al., 1995; Papapetropoulos et al., 1996). At least some of these effects were blocked by the inhibition of PKA (Papapetropoulos et al., 1996). Another possible explanation for cAMP effects is cross-talk between cAMP and cGMP, resulting in the elevation of cGMP. By using high concentrations of cyclic nucleotide analogues, the reciprocal modulation of endogenously produced cAMP and cGMP could not be excluded. In endothelial cells, however, where cGMP was without any effect, the cAMP-elevating agent forskolin was also able to decrease sGC protein levels, suggesting a more complex action mechanism (Ibarra et al., 2001).

Effects on sGC levels can be a factor contributing to several pathological states. Indeed, reduced sGC protein levels have been demonstrated in hypertension in aorta and heart (Ruetten et al., 1999). In agreement with this, impaired vasodilatation in response to sodium nitroprusside has been reported in hypertensive rats (Kloss et al., 2000). Reduced sGCβ_1 and sGCβ_2 mRNA levels have been found in kidneys of Dahl salt-sensitive rats (Gupta et al., 1997). In contrast, increased sGC protein levels were observed for hyperholesterolemia (Laber et al., 2002), which was considered by the authors as a compensatory mechanism for reduced (probably via oxidative damage) specific sGC activity. Similarly, increased sGC protein levels have been found in rat heart after myocardial infarction (Bauersachs et al., 1999) and in rat lung under hypoxic condition (Li et al., 1999). However, in the former case the activity of sGC was significantly reduced (Bauersachs et al., 1998).

It should be noted that in many cases the exact mechanism of sGC protein reduction are

unknown. Moreover, in many studies sGC mRNA and not protein levels have been used to determine sGC regulation. It is speculated that changes in translation or transcription rate or destabilization of mRNA might play an important role for regulation of the protein amount. However, there are very few studies concerning another pathway for protein level control, protein degradation.

Another interesting but poorly investigated aspect in the regulation of sGC is protein-protein interactions. It is well-known that protein-protein interactions play important role in regulation of different signalling proteins. Exiting example is NOS with more than 20 proteins regulating the enzyme (for review see Nedvetsky et al., 2002). Very recently, the first sGC-interacting protein, heat shock protein 90 (Hsp90), was identified (Venema et al., 2001). However, to date, extremely little is known about the role of this protein in the regulation of sGC.

2. Aims of the study

In the present study we tried to identify and to characterize factors which can modulate sGC activity and protein level and also its sensitivity to its main regulator, NO. Within this framework we would like to answer following questions:

1. Study the distribution of sGC protein and activity and correlation between both parameters in brain. We have assumed that if multiple factors are participating in the regulation of sGC activity and/or protein levels, a lack of correlation between both parameters may be found under some conditions. Brain was used as a suitable tissue for this study since (i) the complex organization of brain allows preparation of different regions with different functions and anatomical organization; (ii) sGC has been shown to play an important role in regulation of brain function; (iii) there is region-specific regulation of the NO/sGC/cGMP signal transduction pathway.

2. Study the role and mechanisms of long-term regulation of sGC activity and protein level by NO. Desensitization of sGC by NO is a well-known but poorly understood phenomenon. In our work we would like to further characterize the recently described down-regulation of sGC protein in endothelial cells by studying the effect of NO on sGC activity as well as mechanisms involved in these effects.

3. Study the role of intracellular localization of sGC on its activity and sensitivity to NO. Since NO is a very lipophilic molecule, its local concentration in (or near to) the membrane should be higher than in a more hydrophilic environment. Thus, translocation of sGC to the membrane or away from it could be a mechanism to regulate activation of the enzyme. In this regard, we tried to further characterize mechanisms of membrane attachment

of sGC, and study the effects of intracellular localization of sGc on its sensitivity to NO.

4. Role of heat shock protein 90 (Hsp90) in the regulation of sGC activity and protein level. Recently, it has been demonstrated that Hsp90 binds to sGC and can serve as a coupler between the NO receptor and the NO producer, NOS, facilitating cGMP producation in response to physiological stimuli (). We would like to investigate this finding under our experimental conditions. Additionally, we would like to study the role of Hsp90, a chaperone that is well known to be important for stabilization of a number of signalling proteins, in the regulation of sGC protein level.

3. Methods and Materials

3.1 Materials

3.1.1 Chemicals

Acrylamide (30%) with 0,8% Bisacrilamid	Carl Roth Gmbh (Karlsruhe)
APS	Merck (Darmstadt)
β-Mercaptoethanol	Carl Roth Gmbh (Karlsruhe)
Bovine serum albumin (BSA)	Sigma (Deisenhofen)
$CaCl_2$	Merck (Darmstadt)
Fetal calf serum	Biochrom (Berlin)
cGMP Detection Kit	Biotrend (Cologne)
Complete EDTA free (Protease inhibitor Set)	Roche Molecular Biochemicals (Mannheim)
Coomassie brilliant blue G 250	Serva (Heidelberg)
Creatine phosphate	Calbiochem (Bad Söden)
Creatinekinase	Calbiochem (Bad Söden)
Cycloheximide	Alexis Corporation (Lausen, Switzerland)
Cysteine	Sigma (Deisenhofen)
DEA/NO	Alexis Corporation (Lausen, Switzerland)
DETA/NO	Alexis Corporation (Lausen, Switzerland)
DMEM medium	Sigma (Deisenhofen)
DRB	Alexis Corporation (Lausen, Switzerland)
DTNB	Merck (Darmstadt)
DTT	Serva (Heidelberg)
EDTA	Sigma (Deisenhofen)
EGTA	Sigma (Deisenhofen)
Ethanol	Merck (Darmstadt)
Folin-Chiocalteu’s phenol reagent	Merck (Darmstadt)
Geldanamycin	Alexis Corporation (Lausen, Switzerland)
Glutathione reduced; GSH	Carl Roth Gmbh (Karlsruhe)
Glycine	Carl Roth Gmbh (Karlsruhe)
HEPES	Sigma (Deisenhofen)
HPR-Immunoglobulin (anti-mouse)	DAKO (Hamburg)
HPR-Immunoglobulin (anti-rabbit)	DAKO (Hamburg)
IBMX	Alexis Corporation (Lausen, Switzerland)
IPL41 medium	Gibco (Karlsruhe)
K_2HPO_4	Carl Roth Gmbh (Karlsruhe)
KCl	Carl Roth Gmbh (Karlsruhe)
KH_2PO_4	Carl Roth Gmbh (Karlsruhe)
Lactacystin	Alexis Corporation (Lausen, Switzerland)
M199 medium	Sigma (Deisenhofen)

Methanol	Merck (Darmstadt)
MG132	Sigma (Deisenhofen)
$MgCl_2$	Merck (Darmstadt)
NaCl	Carl Roth Gmbh (Karlsruhe)
$NaNO_2$	Merck (Darmstadt)
NaOH	Carl Roth Gmbh (Karlsruhe)
ODQ	Alexis Corporation (Lausen, Switzerland)
PAPA/NO	Alexis Corporation (Lausen, Switzerland)
Penycillin	Sigma (Deisenhofen)
Purified bovine lung sGC	Alexis Corporation (Lausen, Switzerland)
Radicicol	Alexis Corporation (Lausen, Switzerland)
SDS	Carl Roth Gmbh (Karlsruhe)
sGCα_1-antibody, rabbit	produced in our lab
sGCβ_1-antibody, rabbit	produced in our lab
Sodium acetate	Merck (Darmstadt)
Sodium nitroprusside; SNP	Merck (Darmstadt)
Spermine/NO	Alexis Corporation (Lausen, Switzerland)
Streptomycin	Sigma (Deisenhofen)
TEA	Carl Roth Gmbh (Karlsruhe)
TEMED	Sigma (Deisenhofen)
Tris	Carl Roth Gmbh (Karlsruhe)
DMSO	Merck (Darmstadt)
Zaprinast	Alexis Corporation (Lausen, Switzerland)

Water was deionized to 18 MΩ cm (Milli-Q; Millipore, Eschborn, Germany)

For experiments the substances were dissolved in the following solvents: Cycloheximide; DRB; geldanamycin; MG132; IBMX; ODQ; radicicol and zaprinast were diluted in DMSO. DETA/NO and DEA/NO were dissolved in 10 mM NaOH. SNP was diluted in 1 mM sodium acetate (pH 5.0). All other chemicals were dissolved in water.

3.1.2 Buffers and media

The following buffers were used in the present work:

Brain homogenisation buffer, pH 7.5

50 mM	TEA/HCl
0.5 mM	EDTA
7 mM	glutathione

0.2 mM	phenylmethylsulfonyl fluoride
1 µM	pepstatin A
1 µM	leupeptin

EC homogenisation buffer, pH 7.4

25 mM	TEA/HCl
1 mM	EDTA
5 mM	DTT
50 mM	NaCl
10% (v/v)	Glycerol
1 Tabl/50 ml	Complete (EDTA free)

Mammalian Ringers solution, pH 7.4

114 mM	NaCl
4.5 mM	KCl
1 mM	$MgSO_4$
11 mM	glucose
1 mM	NaH_2PO_4
25 mM	$NaHCO_3$

Rotiload sample buffer:

62.5 mM	phosphate buffer (pH 6.8)

10 % (v/v)	Glycerol
2 % (w/v)	SDS
0,01 % (w/v)	Bromphenol blue
5 % (v/v)	2-Mercaptoethanol

Phosphate-buffered saline (PBS) (pH 7.4):

137 mM	NaCl
2.7 mM	KCl
6.5 mM	Na_2HPO_4
1.5 mM	KH_2PO_4

Electrophoresis buffer (pH 8.9):

25 mM	Tris
192 mM	Glycine
0,1 % (w/v)	SDS

Blotting buffer (pH 10.0):

25 mM	Tris
192 mM	Glycine
20 % (v/v)	Methanol

Washing buffer (TBST) (pH 7.5):

20 mM	Tris
150 mM	NaCl
0.1%	Tween 20

sGC reaction buffer (pH. 7.5)

50 mM	TEA
5 mM	Creatinphosphate
100 U/ml	Creatinkinase
1 mM	IBMX
100 µM	Zaprinast
3 mM	$MgCl_2$
1 mM	GTP
3 mM	glutathione

sGC reaction buffer (pH. 7.5)

50 mM	TEA
5 mM	Creatinphosphate
100 U/ml	Creatinkinase
1 mM	IBMX
100 µM	Zaprinast

Buffers for preparation of subcellular fraction from rat heart:

Buffer A:

10 mM	MOPS, pH 7.4
0.6 M	sucrose
2.5 µg/ml	aprotinin
2.5 µg/ml	leupeptin
0.1 mM	PMSF

Buffer B:

20 mM	MOPS, pH 7.4
160 mM	NaCl
2.5 µg/ml	aprotinin
2.5 µg/ml	leupeptin
0.1 mM	PMSF

Buffer C:

20 mM	MOPS, pH 7.4
54 mM	LiCl
6 mM	KCl
100 mM	NaCl
2.5 µg/ml	aprotinin
2.5 µg/ml	leupeptin

0.1 mM	PMSF

Reagents for protein determination according to Lowry (Peterson, 1977)

A: 0.15 %	desoxycholic acid in water
B: 72 % (w/v)	trichloracetic acid in water
C: 1 %	$CuSO_4$ in water
D: 2 %	Na/K tartrate in water
E: 3.4 %	Na_2CO_3 in 0.2 M NaOH
F: 10 %	SDS in water

G: mix C, D, E and F in proportions of 1:1:28:10

H: Folin-Ciocalteu's phenol reagent diluted 1/4 with water

3.2 Devices and software

Apparatus used for experimental work were as follows.

Apparatus	Type	Producer
Analytical balance	Analytic AC 120 S	Sartorius (Göttingen, Germany)
Centrifuge	Heraeus Sepatech Biofuge 15	Heraeus Instruments (Hanau, Germany)
Computer	PowerMac G3 iMac	Apple Computer Inc. (Cupertino, CA, USA)
Homogenizer	Ultra Turrax	Janke &Kunkel (Staufen, Germany)
Mini SDS-PAGE system	Mini-Protean II	Bio-Rad (Hercules, CA, USA)
Semi-Dry Transfer Cell	Trans-Blot SD	Bio-Rad (Hercules, CA, USA)
Shaker	Vortex	Janke &Kunkel (Staufen, Germany)
Image Station	Kodak Digital Science 440	Eastman Kodak Company (New Haven, USA)
Thermomixer	Eppendorf 5436	Eppendorf-Netheler-Hinz (Hamburg, Germany)
UV/VIS Microplate reader	SpectraMax 340	Molecular Devices (Sunnyvale, USA)
SpeedVac Concentrator	Savant	Savant Instruments (Hicksville, USA)
Power supply	Biometra Power Pack 25	Biomedizinische Analytik (Göttingen, Germany)

Software used for either statistical analysis of data, drawing of figures or chemical structures were as follows:

Software	Version	Producer
Adobe Photoshop	3.0	Adobe Systems Inc. (San Jose, CA, USA)
Adobe Acrobat	4.0	Adobe Systems Inc. (San Jose, CA, USA)
AssayZap	1.0	Biosoft (Cambridge, UK)
Claris Draw	1.0v4	Claris Corporation (Santa Clara, CA, USA)
EndNote	5.0	ISI ResearchSoft (Berkeley, CA, USA)
GraphicConverter	4.0.8	Lemke Software (Peine, Germany)
GraphPad Prism	2.0/3.0	Graph Pad Software (San Diego, CA, USA)
Kodak 1D Image Analysis Software		Eastman Kodak Company (New Haven, USA)
Mac OS	9.2	Apple Computer Inc. (Cupertino, CA, USA)
Microsoft Office for Mac	2001	Microsoft Corporation (Redmond, WA, USA)
NIH Image Software	1.59	National Institutes of Health (Maryland, USA)
Ragtime	5.6 Privat	Ragtime Gmbh (Germany)

3.3 Preparation of human and rat brain samples

To investigate the distribution of sGCα_1 and sGCβ_1 distribution in human brain, post-mortem adult brain specimen of 12 autopsy cases (7 males and 5 females) were used. The samples were provided by the Austro-German brain bank (Würzburg, Germany). All cases were from deceased individuals who had no history of neurological or psychiatric disorders and lacked any neuropathological abnormalities. Ages ranged from 61 to 90 years with a mean age of 72.1 ± 2.7 years. The post-mortem delay time varied between 7 and 48 h (mean 21.3 ± 3.5 h). The following regions were obtained: prefrontal, frontal, temporal, parietal and occipital cortex, cingulate gyrus, corpus callosum, hypothalamus, habenula, mammillary bodies, raphe nucleus, locus coeruleus, dentate nucleus, caudate nucleus, putamen, nucleus accumbens, globus pallidus, substantia innominata, nuclei septii, amygdala, entorhinal cortex, hippocampus, substantia nigra, red nucleus, subthalamic nucleus, thalamus, cerebellar cortex, and ventral tegmental area. The dissection protocols and sample preparations were described earlier (Gsell et al., 1993; Blum-Degen et al., 1999) and performed at the Department of Psychiatry and Psychotherapy, Julius-Maximillian-University, Würzburg (Blum-Degen et al., 1999).

Preparation of rat brain samples was performed according to the procedure of Glowinski and Iversen (Glowinski and Iversen, 1966). Male Wistar rats (200-250g) were anaesthetized by CO_2, sacrificed, and six brain regions were prepared. All procedures with brain were performed on ice. Rhombencephalon was separated from other brain parts through one diagonal section and dissected into cerebellum and medulla oblongata with pons. Fore part of cortex cerebri was obtained through another diagonal section at the level of the chiasma

opticum. Hypothalamus was dissected from the middle part, and the commisura anterior was used as horizontal and the convolution between the corpora mammillaria and the ventral hypothalamus as caudal margin. For separation of striatum (putamen, nucleus caudatum and globus pallidus) the external wall of lateral ventricle as inner and boundary of corpus callosum as extern margin were used. The fore part of the striatum was separated from the cortex and combined with the rest of the striatum. The midbrain (including thalamus and subthalamus) was prepared from the middle part of dissected brain. The remaining parts of the cortex were combined with the fore part. Prepared brain regions were immediately frozen with liquid nitrogen and stored at –80°C. The preparation of rat brain from animal decapitation to freezing of tissues took not more than 10 min. These regions are referred to in text and figures by the following simplified names: cerebellum, medulla (corresponding to the medulla oblongata and pons), hypothalamus, midbrain (corresponding to the midbrain, thalamus, and subthalamus), striatum, and cortex (including white and grey matter of cerebral cortex). Frozen tissue sections were pulverized in liquid nitrogen and homogenized in brain homogenisation buffer. Protein concentrations were determined according to Bradford using ovalbumin as standard (Bradford, 1976).

3.4 Preparation of endothelial cells

Porcine pulmonary aortas were obtained fresh from a local slaughterhouse. Immediately after removal, aortas were transported to the laboratory on ice and then maintained in phosphate-buffered saline (PBS; 10 mmol/L Na_2HPO_4, 1.8 mmol/L KH_2PO_4, 140 mmol/L NaCl, 2.7 mmol/L KCl, pH 7.4) at 37°C. EC were isolated enzymatically by incubation of the aorta inner surface with collagenase type CLS II (0.5 mg/mL for 10 min at room temperature)

and then collected in Hepes-buffered medium 199 (Sigma, Deisenhofen, Germany). The resulting cell suspension was centrifuged at 250*g* for 10 min and washed twice with the same medium. The cell pellet was resuspended in growth medium [medium 199 supplemented with 15% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin] and cells were propagated in plastic flasks previously coated with 0.05% gelatin and maintained in an incubator at 37°C under an atmosphere of 5% CO_2 and saturated humidity. Upon confluence, EC monolayers were washed twice and detached by treatment with 0.05% trypsin/0.02% EDTA, subcultured (1:4) in 35-mm dishes previously coated with 0.05% gelatin, and grown again to confluence. Confluent EC monolayers from this second passage were used for experiments.

The effects of different substances on sGC or actin expression was examined at various times at 37°C. When the total incubation time was more than one day, the growth medium was replaced every 24 h by fresh solution that also contained the test substances. At the end of the incubation, cell monolayers were rapidly washed three times with ice-cold PBS and immediately thereafter homogenized in EC homogenisation buffer for determination of maximal sGC activity. Alternatively, for Western blot analysis, cells were lysed in 250 µL Roti-Load sample buffer preheated to 95°C and then boiled for additional 10 min.

3.5 Isolation of subcellular fractions from rat heart

Crude plasma membranes of rat heart were obtained by sequential centrifugation as described previously (Pitts, 1979), with slight modifications, according to the scheme depicted in Fig. 3.1. Three adult male Wistar rats (200 g) were sacrificed and the hearts were quickly removed, rinsed with deionized water, frozen in liquid nitrogen, and stored at -80°C. Frozen

hearts were pulverised in a liquid nitrogen-cooled stainless steel mortar and resuspended in 5 ml ice-cold buffer A. All following procedures were carried out at 4°C. The homogenate was centrifuged for 5 min at 500g to remove cell debris, and the resulting supernatant (S1) was centrifuged for 30 min at 12,000g. The supernatant (S2) was diluted to 40 ml with buffer B. Subsequently, 10 ml 1 M sucrose were added, and the resulting solution (S2') was centrifuged for 1 h at 160,000g. The resulting pellet (M, membranes) was resuspended in buffer C. Membranes and aliquots of all other fractions were stored at -80°C.

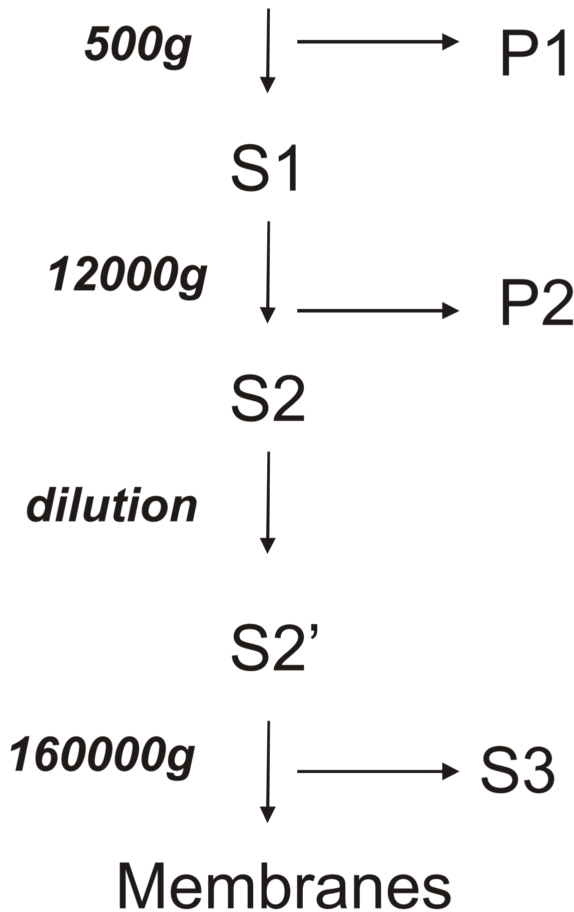

***Fig. 3.1.Schematic illustration of isolation of membrane fraction from rat heart**. Membrane fraction was preparated from homogenate of heart via 3 centrifugation steps (indicated on the figure. P - pelet; S - supernatant*

3.6 Preparation of rat lung endothelial plasma membrane and in situ perfusion with VEGF

Rat lungs were perfused via the pulmonary artery at 6-8 mm Hg (4 ml min^{-1}) for 5 min at 37°C with mammalian Ringers solution to flush the lungs, and then with either 3 ml of VEGF (100 ng ml^{-1}) in Ringers, or with Ringers alone. The flow was stopped for 10 min and lungs were then flushed with cold Ringers solution and perfused with a colloidal silica solution for endothelial stripping by a shearing procedure, as described previously (Schnitzer et al., 1995). These experiments were performed by Dr. P.

Oh (Sydney Kimmel Cancer Center, San Diego, California, USA).

3.7 Expression of sGC in Sf9/baculovirus system

To express recombinant human sGC, the Sf9/baculovirus-expressing system was used. The cell suspension was defrosted and cells were transferred to a plastic bottle (T75) containing IPL 41 medium. The cells adhered rapidly to the plastic surface and were incubated at 27°C for 2 days. After this, cells were removed from the plastic by thorough washing with IPL 41 medium and transferred to a glass bottle. The cells, which do not adhere to glass, were grown as a suspension culture. Each 24-48 h (depending on cell density), double volume of IPL 41 medium was added to the incubation bottle.

For transfection of Sf9 cells with baculovirus, cells were transferred to a glass bottle and grown to a density of 2-4*10^6 cells/ml. After reaching this density, the cells were centrifuged and transferred into fresh medium at a density of 2*10^6 cells/ml. Recombinant baculovirus was added (5pfu/cell) and cells were incubated for a further 72 h. After this, cells were centrifuged (20 min 400 g), medium was removed, and the pellet was washed with PBS and centrifuged again (20 min 400 g). Cells were lysed by incubation in a 10-fold volume (to volume of pellet) of the homogenization buffer (without NaCl and glycerol). Cells lysed were used as a positive control for Western blotting or sGC activity assay or were further used for purification of sGC.

sGC was purified using affinity binding of GST-tagged sGC on glutathione sepharose. Glutathione-Sepharose 4B (Amersham Pharmacia Biotech, Freiburg, Germany) was equilibrated with lysis buffer (see above) containing 75 mM NaCl, incubated with crude supernatant fractions of rhsGC-containing Sf9 cells for 1 h at 25 °C in a rotation mixer, and

washed two or three times with lysis buffer containing 75 mM NaCl. For GSH elution, GSH-Sepharose was incubated with 5 mM GSH in 50 mM Tris-HCl, pH 8.0, for 5 min at 25 °C. Fractions were brought to a final concentration of 10% (v/v) glycerol and stored at 20 °C.

3.8 Western blotting

SDS-PAGE and Western blotting were performed as previously described (Zabel et al., 1998; Ibarra et al., 2001). Proteins were denatured by boiling in Roti Load sample buffer and separated according to their molecular weight via SDS-PAGE under reducing conditions with 4% stacking and 8% running gels. Electrophoresis was performed at room temperature in a Protean Minigel box (BioRad, Munich). Current was maintained at 15-20 mA per gel during the full procedure. As protein molecular weight standards, the SDS-High Molecular Weight Marker Kit (glutamine dehydrogenase, 53 kDa; transferring, 76 kDa; β-galactosidase, 116 kDA; macroglobin, 170 kDa and myosin, 212 kDa) and Low Molecular Weight Marker Kit (Phosphorylase b, 97 kDa; albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa) from Pharmacia Biotech (Freiburg) were used.

Transfer of proteins from the gel to an ECL Hybond nitrocellulose membrane was performed by semi-dry blotting (1-1.5 mA/cm^2 for 1h). Unspecific binding to the membrane was blocked with 3% (w/v) dry milk (Carnation, USA) in TBST at room temperature for 1 h. The detection of sGCα_1 and sGCβ_1 proteins was carried out using polyclonal anti-sGCα_1 or anti-sGCβ_1 antibodies. Antibodies were diluted in 3% dry milk in TBST and incubated with nitrocellulose membranes at 4°C overnight. For sGC detection in brain homogenates, anti-sGCα_1 antibody was diluted 1:2000 and anti-sGCβ_1 1:1000. For endothelial cells, dilution of

antibody was 1:4000 for anti-sGCα_1 and 1:2000 for anti-sGCβ_1 antibodies. Because no additional bands were recognized with either antibody in endothelial cells, simultaneous incubation of blots with both antibodies was performed. After 3 washing steps with TBST (each for 15 min), the membranes were incubated with the secondary antibody. As the secondary antibody, goat anti-rabbit antibody conjugated to horseradish peroxidase (Dako A/S, Denmark) diluted 1:2000 in 3% milk in TBST was used. After incubation with secondary antibody for 1h the membranes were washed 4 times with TBST (each time for 15 min). Incubation with second antibody and washing took place at room temperature. Immunocomplexes were visualized using an enhanced chemilumunescence kit (Amersham Pharmacia Biotech, Freiburg).

3.9 Densitometry

Two different methods for quantification of immunoreactive signals in Western blots were used. One part of our study (determination of sGC protein distribution in rat and human brain) was performed using Kodak BioMax films for detection of chemiluminescent-labeled samples. After developing, films were scanned and digital images were quantified using NIH1.5 software (http://rsb.info.nih.gov/nih-image/) (Sutherland et al., 1991). Because films have a very low saturation level, the linear relationship between the chemiluminescent signals and the darkness of the image can be obtained only in a relatively narrow range. Since we have used Western blot data to quantitatively characterize distribution of sGC proteins in different brain regions, it is very important to verify if the signals quantified are in the linear range. To do this verification, we loaded different amounts of recombinant human sGC on each gel and used them to plot a calibration curve (see Fig. 4.1). For all films used for

densitometrical quantification, the following two conditions were required: (i) a linear relationship between immmunoreactive signals and the amount of recombinant sGC was maintained for the calibration range; (ii) chemiluminescent signals from samples were within this linear range.

All other Western blot experimental data were quantified with an Kodak Imager Station 440 CF. The advantage of this device is a much higher saturation level (about 64000 gray levels vs. 256 of film). This allows quantification of chemiluminescent signals over a much broader range. Since saturation is recognized and reported by the software, saturating conditions can be avoided. This was verified in preliminary experiments.

3.10 Determination of cGMP levels and sGC activity assay

For the determination of cGMP levels in endothelial cells, porcine pulmonary artery endothelial cells were incubated with the indicated substances for the indicated times. At the end of the incubation, cells were washed once with PBS (room temperature). To determine basal cGMP levels, cells were incubated for 3 min in PBS at 37°C. To stimulate sGC, 250 µM DEA/NO was added to the cells for 3 min. PBS was removed and cells were lysed in 80% ethanol (considering the buffer that remained on the dishes, the final concentration of ethanol was about 70%) and then scraped off. Ethanol was evaporated by centrifugation under vacuum (SpeedVac Concentrator; Savant). Samples were diluted in 300 µl Assay Buffer und sonicated to dissolve the pellet. Subsequently, samples were used for cGMP level measurement using cGMP-EIA kit.

To determine sGC activity, 10 µl of tissue homogenates or cell extracts (10-150 µg total protein) were incubated at 37°C in 100 µl sGC reaction buffer (see “Buffer and incubation

media”). Reactions were started by the addition of the sGC-containing samples to the incubation medium and, in the case of stimulatation, simultaneous addition of a stimulating compound. cGMP accumulation was measured for 10 min and the reaction was stopped by boiling the reaction mixture at 95°C for 10 min.

In experiments in which the role of SH agents were investigated, 5 mM DTT instead 7 mM GSH was used. Where applicable, this has been noted in the figure legends.

cGMP was measured using an enzyme-immunoassay kit from Biotrend (Cologne, Germany). cGMP-containing samples were diluted in the assay buffer (Biotrend kit) and 100 µl were added to each well of microtitre plates coated with anti-rabbit antibody. To each sample, 50 µl cGMP-alkaline phosphatase conjugate and 50 µl of rabbit anti-cGMP antibody were added. The plates were shaken at 350 rpm at room temperature for 2 h. After 4 washing steps with 200 µl wash buffer (from the Biotrend kit), 200 µl of substrate for alkaline phosphatase (solution of p-nitrophenyl phosphate) was added. Plates were incubated at room temperature for 1 h and the reaction was stopped by addition of 50 µl of 5 M NaOH. Optic density of samples was measured at 405 and 650 nm. Calculation of the amount of cGMP was performed according to the enzyme-immunoassay kit protocol using the AssayZap Software and a cGMP calibration curve (Biosoft, Cambridge, UK).

3.11 Use of NO donors

The following NO-generating compounds were used in the present study:

DEA/NO (2-(N,N-Diethylamino)-diazenolate-2-oxide) – an NO donor from the NONOates family. NONOates are the group of novel NO-producing substances with different chemical structures but the same mechanism of NO release. NONOates seem to have a

number of advantages compared to the "classical" NO donors such as glycerol trinitrate, nitrosothiols, and sodium nitroprusside: (i) NO release in pH-dependent mode with well-described kinetics; (ii) relatively high resistance to the presence of thiols, transient metals, and light irradiation; (iii) the free radical form of NO is proposed to be the main NO redox form released (Feelisch 1998). The half-life of NO release from DEA/NO is about 2 min at 37°C (pH 7.4). DEA/NO was used in the experiments where short (1-10 min) NO production was desired.

DETA/NO (2,2'-(Hydroxynitrosohydrazino)*bis*-ethanamine) is another NONOate with a very long half-life of NO release (about 20 h at 37°C and pH 7.4). This compound was used for long periods (1 to 24 h) of NO exposure.

Sodium (SNP; sodium pentacyanonirtosyl ferrate (II)) is one of the most popular NO donors used in many studies of sGC. SNP is an inorganic complex where NO is formally bound to iron as NO^+. Although this substance is used clinically to reduce blood pressure, the exact mechanism of its action as well as that of NO release is not completely understood. It is proposed that the main NO-releasing form is the nitrosyl cation (NO^+), which is very reactive and has chemical properties different from those of the NO radical. The disruption of SNP is heavily affected by thiols, transient metals, or light irradiation (Feelisch and Stamler 1996; Feelisch, Kotsonis et al. 1999). In our study SNP was used as an NO^+ donor to compare the sGC stimulation by this compounds with the novel NO donor DEA/NO.

Spermine/NO ((Z)-1-{N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino}-diazen-1-ium-1,2-diolate) and PAPA/NO ((Z)-1-[N-(3-ammoniopropyl)-N-(n-propyl)amino]-diazen-1-ium-1,2-diolate) have been used in control experiments to compare

their effects with effects of other NO donors (SNP and DEA/NO).

Nitrosocysteine is a member of the nitrosothiols. The stability of the compound in different solutions was tested. Nitrosocysteine was not used as an sGC activator because of extreme variations in its stability under different conditions (see 4.1.3).

3.12 Protein determination

In samples diluted with Rotiload sample buffer for SDS-PAGE, protein was determined according to the Lowry method after precipitation of protein by trichloroacetic acid (Peterson, 1977). Samples (20-50 µl) were diluted with 1 ml water and 100 µl of 0.15% deoxycholic acid was added. After 10 min incubation at room temperature on the Eppendorf shaker; 100 µl of 72% trichloracetic acid was added. After 15 min incubation at room temperature with shaking, samples were centrifugated (10min x 14000g). After aspiration of the supernatant, pellets were diluted with 300 µl water and 300 µl of Reagent G (see Buffer and Media) were added. Samples were incubated at room temperature with shaking for 10 min. Subsequently, 150 µl of Reagent H (see Buffer and Media) was added and samples were incubated for a further 30 min. The protein concentration was then measured at 595 nm using a SpectraMax 340 (Molecular Devices). BSA protein standards were used to calculate the concentration of protein from its optical density.

The protein concentration in samples without detergent was determined according to the methods of Bradford (Bradford, 1976) with minor modification of the methods for use with microtitre plates. Protein samples (50 µl) were added to the 96-well plates, and immediately after addition of 250 µl of Bradford reagent the optical density of samples was measured at 595 nm.

3.13 Statistical calculations

Statistical calculations were performed using GraphPad Prism 3.0 statistical package (GraphPad Software, San Diego, California, USA). Statistical differences between the means were analysed by Student's unpaired t-test. For multiple comparisons, one-way analysis of variance (ANOVA) followed by Bonferroni's test was employed. Spearmen correlation test was used to calculate the correlation between the distribution of sGC subunits and between protein and activity distributions in rat brain.

4. Results

4.1 Lack of correlation between sGC protein and activity in the rat brain

4.1.1 Quantification of sGC protein

Western blotting was used as a protein detection and quantification method. To validate the correctness of the results obtained, two questions were considered:

1. Is the protein/signal relationship linear?

2. Does the distribution of individual sGC subunits reflect the distribution of active heterodimers?

Anti-sGCα_1 and anti-sGCβ_1 antibodies were used for Western blotting. To correlate the protein/signal ratio, purified sGC as internal standard was loaded onto each gel. Fig. 4.1 clearly shows that up to 400 ng of purified protein per lane, the sGC signal is in linear proportion to the amount of protein. All signals from samples under investigation were within this range.

Both sGCα_1 and sGCβ_1 subunits were shown to be widely distributed in the body and for most tissues seem to be the only expressed sGC proteins. In rat brain both sGCα_1 and

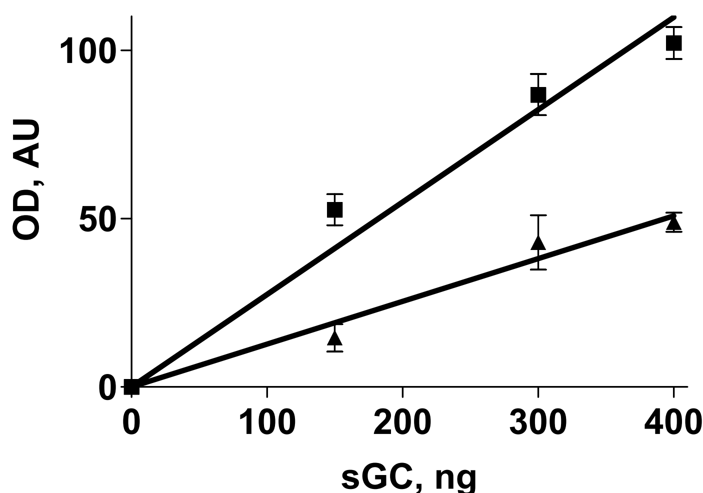

Fig. 4.1. Relationship between sGC protein amount and luminescent signal*. For both sGCα_1 (triangles) and sGCβ_1 (squares), three different known amounts of purified recombinant human sGC were loaded on SDS-gels. After Western blotting, immunoreactive signals were detected using the ECL reagent. Linear correlations were calculated using the Prism 3.0 software. Correlation parameters were $r = 0.987$, $r^2 = 0.974$, $p = 0.013$ for sGCα_1 and $r = 0.986$, $r^2 = 0.972$, $p = 0.014$ for sGCβ_1. Data points represent means ± SEM of three independent experiments.*

sGCβ_1 proteins were found in all regions tested (Fig.4.2). Distribution of both sGC proteins in different brain regions correlated well (correlation parameters were $r = 0.883$; $r^2 = 0.780$; $n = 6$; $p < 0.05$). To further corroborate the correlation in distribution of both sGC subunits in brain, we investigated the sGC distribution in 28 human brain regions to determine whether the same correlation can also be found. Indeed, the distribution of sGC subunits likewise correlated well in human brain ($r = 0.780$; $r^2 = 0.608$; $n = 28$; $p < 0.001$) (data not shown; published in Ibarra et al., 2001). Moreover, in frontal, temporal and parietal cortex, parallel age-dependent changes in sGC expression for both subunits were found (Ibarra et al., 2001). This suggested that the regulation of expression both sGC proteins is in general co-regulated. It should be noted, however, that in some human brain regions age-dependent changes of sGCα_1- and sGCβ_1- were not in parallel (Ibarra et al., 2001). Recently, it was demonstrated that even in sGCα_1 and sGCβ_1 overproducing cells heterodimers were preferentially formed and only minimal formation of inactive homodimers occurred (Zabel et al., 1999). On the basis of these data it can be proposed that the distribution of heterodimers should be similar to

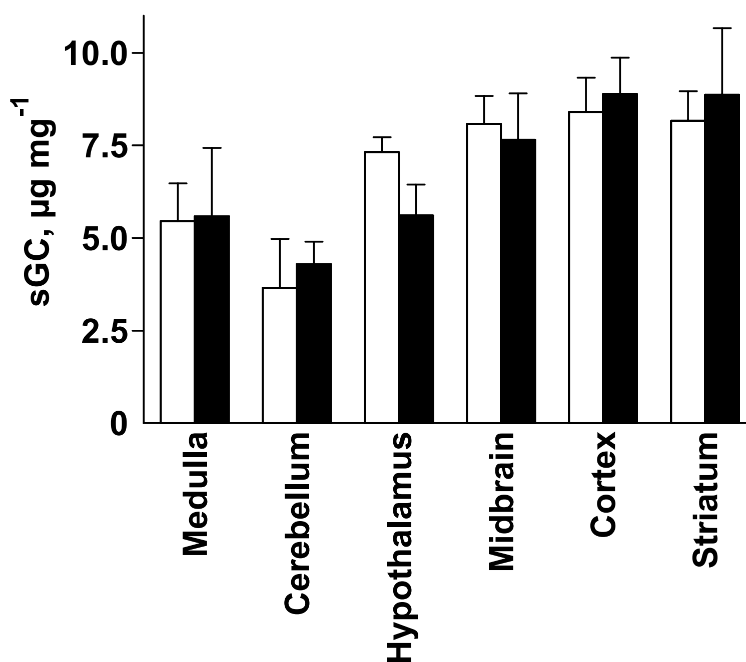

Fig. 4.2. Distribution of sGCα_1 (open bars) and sGCβ_1 (closed bars) proteins in rat brain. *Crude homogenates from six brain regions (30 µg protein) were separated by SDS-PAGE (10%), transferred to nitrocellulose membranes, and analyzed by immunoblotting with the two anti-sGC antibodies. Data represent means ± SEM of three animals. Known amounts of purified sGC were used as in-gel standards to quantify sGC amounts in the samples. All immunoreactive signals from the samples were within calibration curves.*

the distribution of individual subunits.

4.1.2 sGCα_1-immunocrossreactive protein (p160)

In all brain regions tested, the anti-sGCα_1 antibody recognized an immunoreactive protein with electrophoretic mobility in SDS-PAGE of about 160 kDa (p160; Fig. 4.3). The nature of this protein is unknown. Preincubation of the antibody with immunogenic sGCα_1-peptide completely blocked the recognition of both sGCα_1 and p160 (Fig. 4.3). Because anti-sGCβ_1 antibody did not recognize this high molecular weight band, the presence of non-disrupted sGC heterodimers is unlikely. Interestingly, p160 was not detected in other tissues tested (lung, heart, spleen and kidney) (data not shown; see also Zabel et al., 1998). Furthermore, there was no correlation between the distribution of sGC proteins and p160.

4.1.3 Detection of sGC activity: dependance on NO-generating compound

Different classes of NO donors are used to stimulate sGC activity. In all these cases, only the concentration of NO donor but not of NO is known. However, dependence of NO release on the experimental conditions and buffer composition, different redox states of released NO species, and production of by-products may be critical for determining maximal

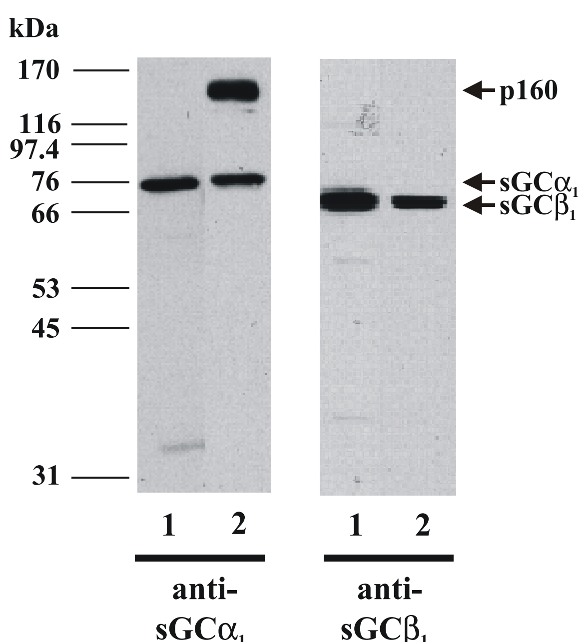

Fig. 4.3. sGCα_1 and sGCβ_1 immunoreactive pattern of rat cortex. *anti-sGCα_1 and anti-sGCβ_1 antibody were used to detect sGC-immunoreactive proteins in rat cortex (lane 2). Purified recombinant human sGC (lane 1) was used as a positive standard. Both antibodies detected immunoreactive proteins co-migrating with sGC standards. Additionally, one immunoreactive protein with electrophoretic mobility of about 160 kDa (p160) was detected by anti-sGCα_1 antibody. Immunoreactive signals from both sGC and p160 could be blocked by preincubation of the antibody with immunogenic peptide (data not shown).*

NO-stimulated sGC activity. In the present study NO donors from 3 different chemical classes were compared to determine whether their degradation rate depends on factors such as thiols or transition metals. As compounds for this investigation, nitrosocysteine, a member of the nitrosothiols, SNP, a member of nitrosometal complexes, and DEA/NO, one of the NONOates, were chosen.

The half-life of nitrosocysteine, measured in TBS (20 mM Tris; 150 mM NaCl; pH 7.4) at room temperature, is about 2 min (Fig. 4.4). It is well known that transition metals, particularly Cu ions, can drastically potentiate the disruption of nitrosothiols (Feelisch, 1998). Indeed, the presence of 50 nM (about 3 μg/L) Cu^{2+} decreased the half-life of nitrosocysteine to about 30 s (Fig. 4.4). This concentration is in the range of free copper ion concentration in water or biological samples (see “Copper in the environment” by Dave Brown; http://www.science.mcmaster.ca/Biology/4S03/COPPER.HTM#one). As shown in Figure 4.4, addition of non-distilled water even in the amount of 1% from the total volume of solution drastically accelerated the degradation of nitrosocysteine. Thus, it is very difficult to control the concentration of transition metals and their oxidative state in different tissues. It is likely that the degradation rate of nitrosocysteine as well as of other nitrosothiols (and production of NO) is significantly affected by using different biological samples. Therefore, nitrosothiols would appear to be inappropriate NO donors for determining sGC activity. An additional complication of nitrosothiols as NO donors is the ability of these compounds to participate in S-nitrosylation reactions. In these reactions, NO will not be released as a free radical but transferred from the nitrosothiol to another thiol as NO^+ (Stamler et al., 1992). This mechanism of nitrosothiol action was suggested to be responsible for many of its effects (Stamler et al., 1992; Stamler et al., 1992), but cannot be considered as a mechanism for NO donation.

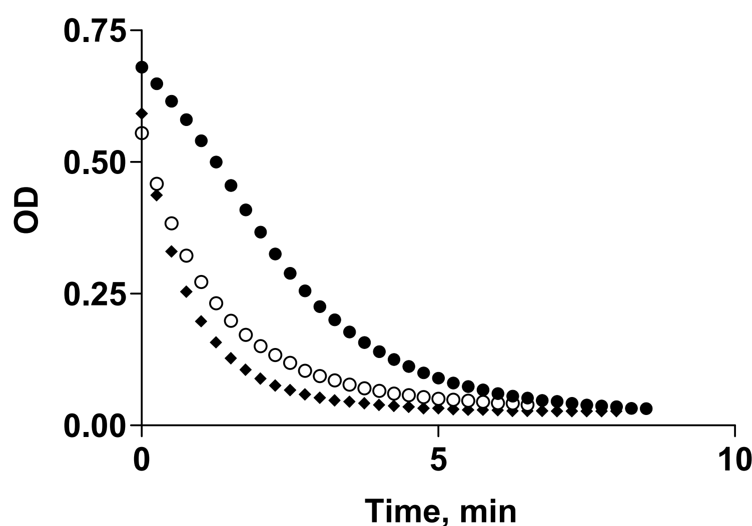

Fig. 4.4. Decomposition of S-nitroso-L-cysteine (NC) in TBS buffer at room temperature. *NC was added to TBS buffer (closed circles) to a final concentration of 100 µM and optical density at 340 nm was measured every 15 s. Addition of non-distilled water up to a final concentration of 1% (open circles) or 50 nM Cu^{2+} ions (closed rhombus) dramatically accelerated decomposition of NC. Data presented are means of two independent measurements.*

SNP has a very strong optical absorption at wavelengths up to 320 nm (Fig. 4.5) but not at longer wavelengths. To determine the SNP degradation rate spectrophotometrically, changes in the SNP spectrum after degradation of the compound by storing the solution for several days at neutral pH, room temperature, and in the presence of daylight were measured. Fig 4.5 shows a subsequent increase in optical density at 325 and 396 nm after 3 days. Unfortunately, these peaks were at the bottom of a much stronger peak which may affect their quantification. Taken together, the unknown nature of chemical changes which are responsible for such spectral changes of SNP in solution makes correct interpretation of results very difficult. Interestingly, addition of 1 mM DTT to the SNP solution resulted in complex changes in its spectral properties (Fig. 4.6), suggesting formation of some other products compared with SNP degradation in thiol-free solution. A rapid and strong increase at 325nm was observed. Another thiol, GSH, was not able to produce similar changes in the SNP spectrum, suggesting some other chemical reactions in the solution of SNP in the presence of this thiol reagent. Together these data suggested a very complex process of SNP degradation in the presence or absence of thiols. Additional disadvantages of this compound

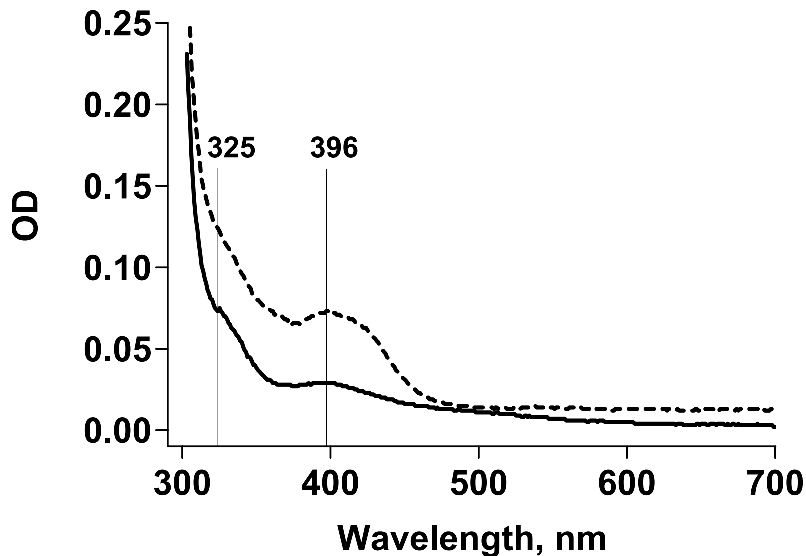

Fig. 4.5. Spectral changes of SNP solution after decomposition. *Optical density of freshly dissolved 1 mM SNP solution in TBS (solid line) and the same solution after 7 days (room temperature, normal room illumination) (dashed line) was measured. After decomposition of SNP optical density at 325 and 396 nm increased.*
Note: *During decomposition the color of the SNP solution changed from light red to deep green.*

include (i) its ability to release NO in much lower amounts than other NO donors (Wink et al., 1996; Gordge et al., 1998); (ii) the fact that its decomposition is affected by several other factors which are difficult to control (such as transition metals, reducing agents, light radiation, or enzymatic metabolism) (Feelisch, 1998; Feelisch et al., 1999); (iii) the release of NO in the form of the nitrosyl cation NO^+ (Stamler et al., 1992), which is much more reactive than NO itself and thus may have effects different from those of NO (Stamler et al., 1992). This makes SNP not a very suitable NO donor for measuring NO-stimulated sGC activity. Despite these disadvantages, we have used this compound in our study and have compared its effects with those of DEA/NO, since SNP is still a very popular NO donor and sGC stimulator that is frequently used for different applications.

The third NO donor tested was DEA/NO. DEA/NO is a member of a novel class of NO donors, NONOates (diazeniumdiolates). In comparison to other classes of NO-producing compounds, these substances have a number of advantages, such as a relative independence of their decomposition rates with respect to thiols and transition metal concentrations and well-documented and reproducible half-life values of NO release (Wink et al., 1996; Feelisch,

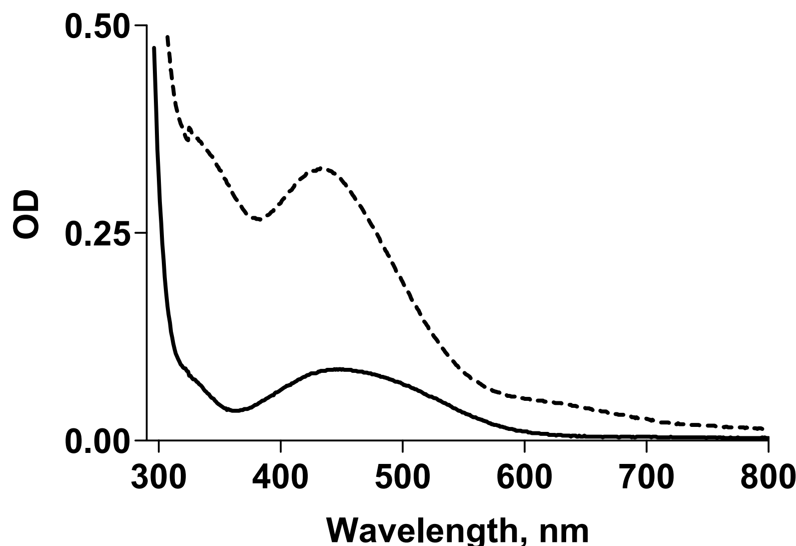

Fig. 4.6. Spectral changes of SNP solution in the presence of DTT. *1 mM DTT was added to 1 mM solution of SNP. The spectrum of the solution before (closed line) and 9 min after addition of DTT (dashed line) was recorded. Spectral changes in SNP solution in the presence of DTT had different characteristics than changes of SNP solution without the thiol. GSH could not produced changes in SNP like DTT.*
Note*: Immediately after DTT addition SNP solution changed its color from light red to strong purple, and after some minutes this coloration was gone.*

1998). To verify this, the decomposition rate of DEA/NO was measured by optical density at 240 nm. Interestingly, it was found that the half-life of this compound is shorter than it has been reported (3.5 min vs. 16 min reported at room temperature, pH 7.4). Addition of 50 nM Cu^{2+} ions or DTT up to 7 mM did not affect the decomposition rate (Fig. 4.7). GSH, at concentrations above 1 mM, slowed the decomposition of DEA/NO (half-life with 7 mM GSH was about 6.5 min).

4.1.4 Distribution of sGC activity in rat brain

Both SNP and DEA/NO produced a concentration-dependent stimulation of sGC in rat brain homogenates. The IC_{50} values for DEA/NO and SNP were about 6 and 23 μM, respectively (Fig. 4.8). In all regions maximal effects was observed with 300 μM DEA/NO or 1000 μM SNP (Fig. 4.8). The ability to stimulate sGC was greater for DEA/NO. This was true for all regions tested, since in no region could SNP produce sGC stimulation comparable to that of DEA/NO (Fig. 4.9). The most profound differences in SNP and DEA/NO effects were

observed in midbrain, striatum, and cortex. In these regions, DEA/NO stimulated sGC up to 20-fold greater than SNP. The highest DEA/NO-stimulated sGC activities were found in cortex (529.5 ± 9.9 pmol mg^{-1} min^{-1}), midbrain (467.8 ± 7.9 pmol mg^{-1} min^{-1}), and striatum (277.7 ± 5.3 pmol mg^{-1} min^{-1}). The sGC activities in hypothalamus, cerebellum, and medulla were 4-8-fold lower than in cortex (Fig. 4.9). To corroborate the effect of DEA/NO, other NONOates (compounds of different chemical structure but with the same mechanism of NO release), PAPA/NO and spermine/NO, were used. Both substances produced sGC stimulation to the same extent as DEA/NO (Fig. 4.10). To investigate whether the acceleration of SNP degradation by DTT could potentiate its effects on sGC activity, DTT instead of GSH was used. When using DTT as a SH-reducing agent, SNP-stimulated sGC activity was increased 2.5-3 fold (Fig. 4.10). Replacement of GSH by DTT also produced an increase of DEA/NO-stimulated sGC activity, although the extent of this increase was much lower (about 60%).

4.1.5 Lack of correlation between sGC protein and activity distribution in rat brain

The present data regarding the distribution of sGC proteins and activities in rat brain demonstrate a great difference in distribution of both the parameters. Although an even

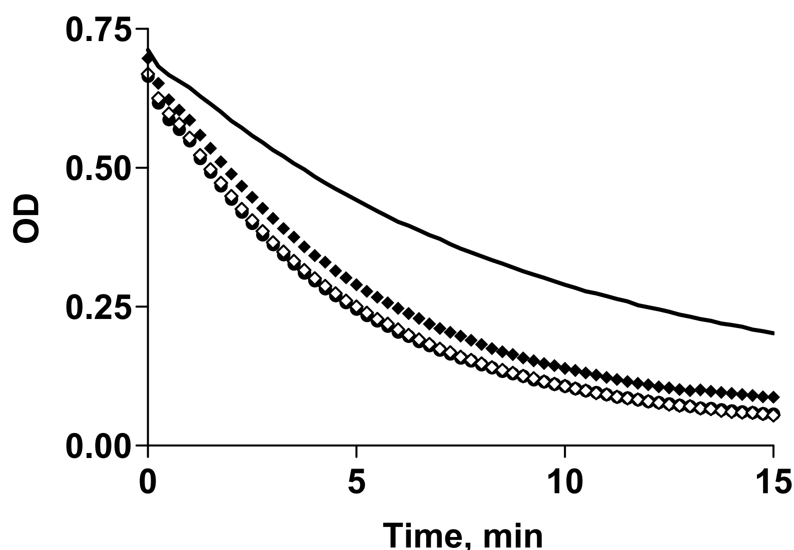

***Fig. 4.7. Decomposition of DEA/NO**. To analyse the decomposition of DEA/NO, optical absorbtion at 260 nm was measured. Decomposition of 1 mM DEA/NO was measured in 20 mM TEA buffer (pH 7.4) at room temperature in the absence (open rhombus), or presence of 50 nM Cu^{2+} ion (closed circles), 7 mM DTT (closed rhombus) or 7 mM GSH (solid line). The experiment was repeated twice with the same results.*

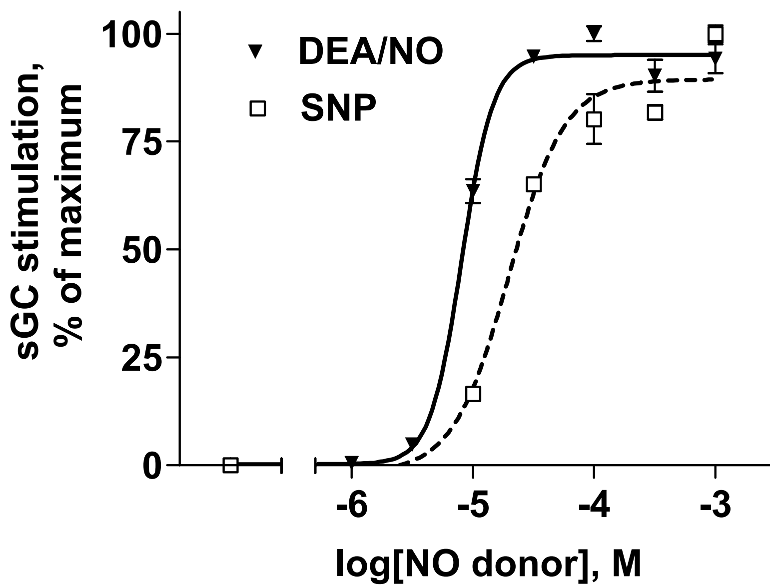

Fig. 4.8. Concentration-response curves for rat cortical sGC activation by SNP or DEA/NO. *Basal and NO-stimulated cGMP formation in 60-100 µg rat cortex homogenate was measured for 10 min. cGMP was determined by enzyme immunoassay (Biotrend, Cologne). Data represent means ± SEM of three independent experiments and the difference between NO-stimulated and basal cGMP formation (NO-stimulated sGC activity.*

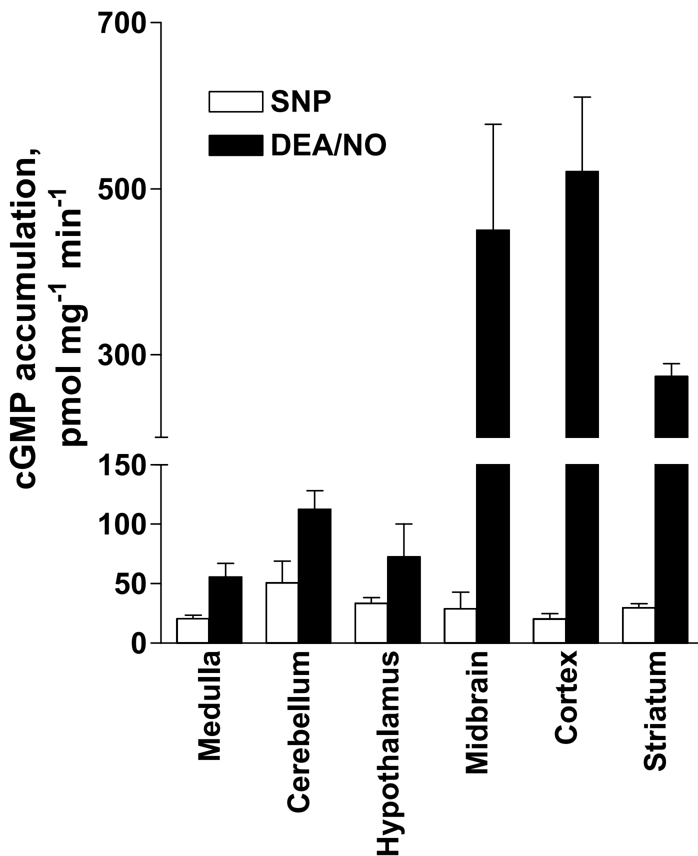

Fig. 4.9. Distribution of SNP- and DEA/NO-stimulated sGC activity in rat brain. *Basal and NO-stimulated cGMP formation was measured in homogenates of different rat brain regions (30-100 µg protein) and cGMP was determined by enzyme immunoassay (Biotrend, Cologne). Data represent the difference between NO-stimulated and basal cGMP formation (NO-stimulated sGC activity). Means ± SEM of three independent experiments each performed in triplicate are shown.*

distribution of both sGC proteins has been found, the two different NO donors used have produced significant differences in the stimulation of sGC in different rat brain regions. To illustrate this clearly, statistical analysis of data was performed and the correlation between sGC protein and activity distribution investigated. As demonstrated in Fig. 4.12, neither the distribution of SNP nor of DEA/NO-stimulated activities correlated with the distribution of sGC proteins.

4.2 DETA/NO-produced changes in sGC regulation

4.2.1 Effects of long-term exposure of endothelial cells to DETA/NO on the cGMP level in the cells

Besides having an activating effect on sGC, NO is known to produce a desensitization of the cGMP response when cells are subjected to prolonged exposure to NO or NO donors. The exact mechanism of this phenomenon is still not understood. Different factors, such as reduced bioavailability of NO (Mulsch et al., 1988; Feelisch and Kelm, 1991; Forster et al., 1991), desensitization of sGC (Davis et al., 1997; Knotek et al., 2000), and up-regulation of

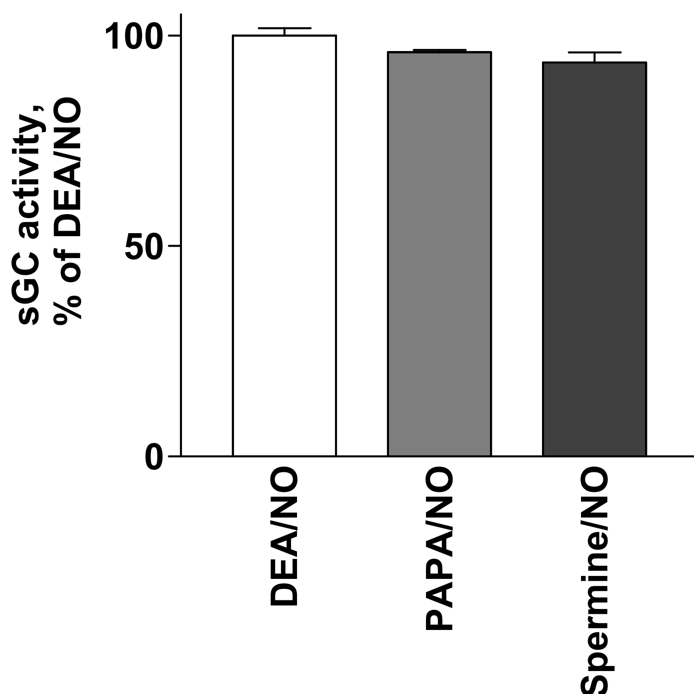

Fig. 4.10. Effects of different NONOates on the sGC activity in rat brain. *300 µM DEA/NO, 1 mM PAPA/NO and 1 mM spermine/NO were used to stimulate sGC. All three NO donors produced similar stimulation of sGC activity. Data are present as percent of sGC activity in the presence of DEA/NO (mean ± SD of 3 independent experiments performed in triplicate).*

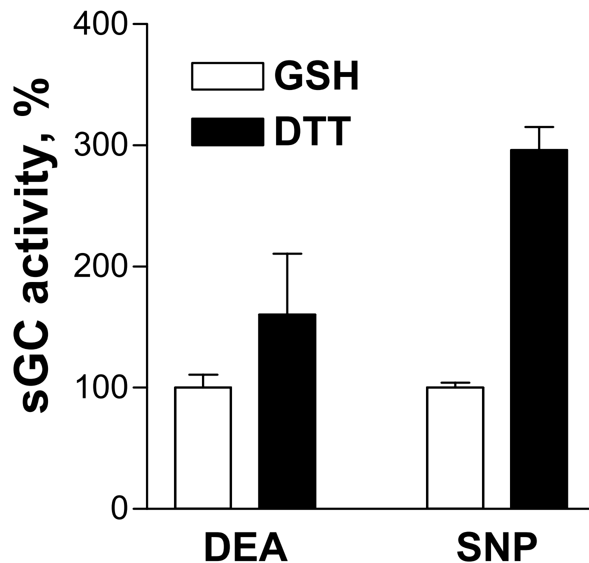

Fig. 4.11. Effects of two different SH-reagents, GSH and DTT on the sGC activation by SNP and DEA/NO. *Basal and NO-stimulated cGMP formation in 60-100 µg of rat cortex homogenate was measured for 10 min and the amount of cGMP was determined by enzyme immunoassay (Biotrend, Cologne). The data represent means ± SEM of two independent experiments each perfomed in triplicates and expressed as % of sGC activity in the presence of GSH by the stimulation of the respective NO donor.*

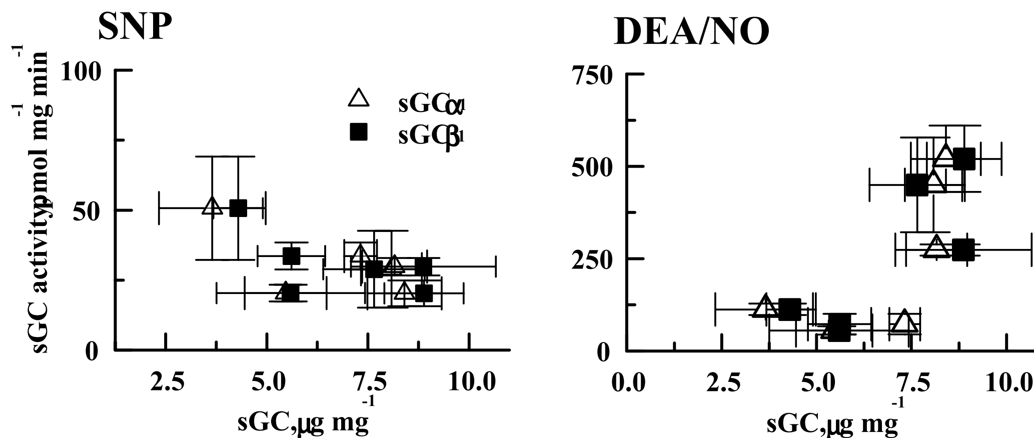

Fig. 4.12. Distribution of sGC proteins in the rat brain correlate neither with DEA/NO- nor with SNP-stimulated sGC activity. *Spearman nonparametric correlation test was used for the calculation. All calculations were performed using the GraphPad Prism 3.0 software (GraphPad Software, San Diego, USA)*

phosphodiesterases (Mullershausen et al., 2001) have been considered to be responsible for this. However, desensitization of sGC seems to be a quantitatively relevant mechanism regulating the NO/cGMP response. Moreover, studies in several labs demonstrated that sGC protein or mRNA levels could also be negatively regulated by NO (Ujiie et al., 1994; Filippov et al., 1997; Ferrero and Torres, 2002).

A recent study in our laboratory has demonstrated that long-term exposure of endothelial

cells to an NO donor, DETA/NO, produced down-regulation of the NO/sGC/cGMP signaling pathway at the protein level (decrease of sGC and PKG-I proteins) (Ibarra et al., 2001). Thus, it was of interest to investigate sGC down-regulation by NO. In the present study, this model was extended and sGC activity desensitization by NO was examined.

Endothelial cells were exposured to 100 µM DETA/NO for the different periods of time (from 5 min to 72 h) and cGMP levels were measured by enzyme immunoassay. Very short stimulation (5 min) of endothelial cells with DETA/NO resulted in significant elevation of cGMP levels from 0.76 ± 0.09 to 3.66 ± 0.15 pmol/106 cells. However, cGMP levels rapidly decreased and no elevation was observed compared to the basal level after longer exposure times (2 or 72 h) (Fig 4.13). Since DETA/NO is an NO donor with a very long half-life (about 20 h in experimental conditions), it is very unlikely that this is because of a decrease in the NO concentration.

Next, the short-term cGMP response after long-term exposure to DETA/NO was studied. For this purpose, endothelial cells were incubated for 2 and 72 h with 100 µM DETA/NO and then were stimulated with 250µM DEA/NO (an NO donor with a half-life under the experimental conditions of about 2-4 min) for 3 min. DEA/NO should produce a rapid but marked increase in the NO concentration in the incubation medium, which should lead to maximal stimulation of sGC. After 2 h of exposure to low NO concentrations (produced by 100 µM DETA/NO), the cGMP response of cells to DEA/NO was dramatically reduced (Fig. 4.14). Further slight down-regulation of the response after 72 h treatment with DETA/NO was observed (Fig. 4.14).

4.2.2 Effects of exposure of endothelial cells to DETA/NO on the sGC activity in cell homogenates

Experiments performed in our laboratory recently showed that long-term exposure of porcine endothelial cells to DETA/NO produces time- and concentration-dependent loss of sGC proteins (Ibarra et al., 2001). The first effects of the NO donor were seen after 24 h of exposure. In the present study the changes in sGC activity upon long-term exposure to DETA/NO were investigated. Endothelial cells treated with DETA/NO were homogenized and sGC activity in cell homogenates was measured in the presence of 100 µM DEA/NO. After 2 h of treatment with DETA/NO, NO-stimulated sGC activity was reduced by about 40% and remained at this level for 72 h (Fig.4.15).

4.2.3 Inhibition of PDE did not prevent DETA/NO-induced desensitization of cGMP response in endothelial cells

Subsequently, it was investigated whether up-regulation of phosphodiesterases (PDE) could be responsible for the observed desensitization of the cGMP response, as it has been recently described for platelets and aortic tissue (Mullershausen et al., 2001). For this purpose, the DEA/NO-induced cGMP response was measured in the presence of PDE inhibitors. The

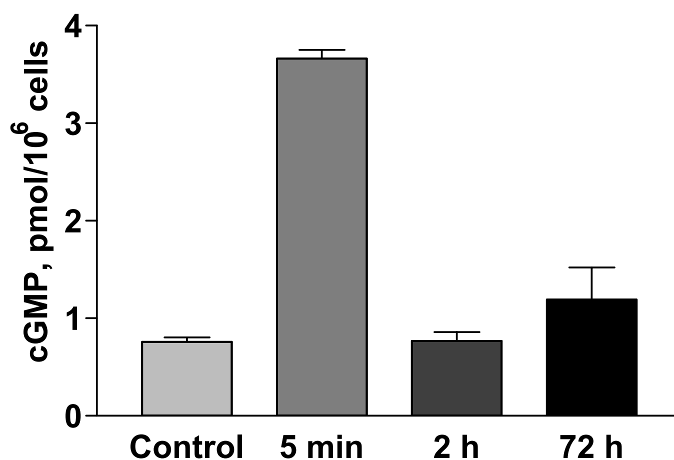

Fig. 4.13. Changes in cGMP level in endothelial cells after treatment with DETA/NO. *100 µM DETA/NO was added to endothelial cell culture and after indicated time cells were lysed in 70% ethanol. Ethanol was evaporated and the pellet was diluted in assay buffer. cGMP determination was performed with enzyme immunoassay kit (Biotrend, Cologne) according to the protocol of the manufacturer. The data are means ± SEM of three independent determinations.*

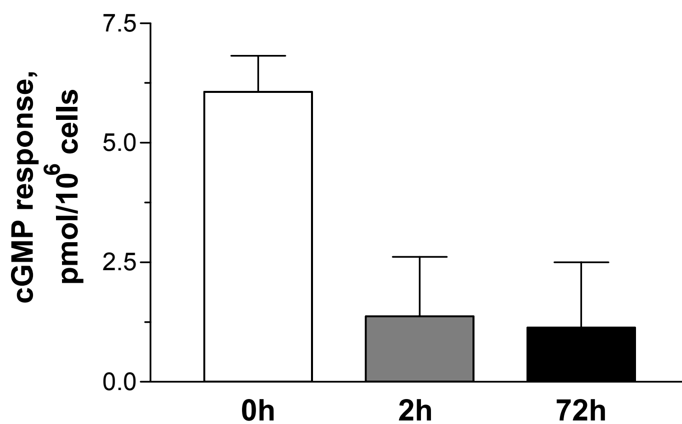

Fig. 4.14. Pretreatment of endothelial cells with DETA/NO down-regulate maximal cGMP response. *Endothelial cells were treated with 100 µM DETA/NO for the times indicated in the figure and then sGC was stimulated with 250 µM DEA/NO for 5 min. Stimulation was stopped by lysis of the cells with 70% ethanol. Data represent means ± SEM of three independent experiments.*

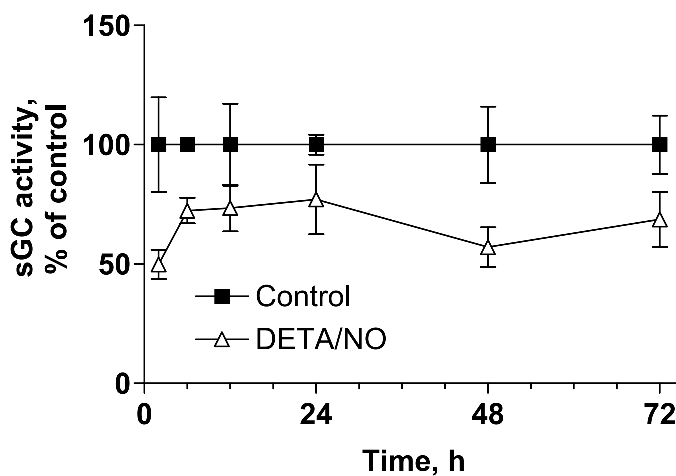

Fig. 4.15. Pretreatment of endothelial cells with DETA/NO reduced maximal sGC activity measured in cell homogenates. *Endothelial cells were treated with 100 µM DETA/NO (open triangles) for the times indicated in the figure. Matched untreated samples were used as control (closed squares) After homogenization of cells, sGC activity was determined in an in vitro assay. Data represent means ± SEM of three independent experiments and are expressed as % of DEA/NO-induced cGMP level in endothelial cells which were not treated with DETA/NO.*

non-selective PDE inhibitor, 1 mmol/L IBMX, and selective inhibitor of cGMP-hydrolysing PDEs, 100 µmol/L zaprinast, were used to inhibit PDEs in endothelial cells. Endothelial cells were exposed to 100 µM DETA/NO for 2 h and a maximal cGMP response was stimulated with 250 µM DEA/NO. Thirty min before stimulation with DEA/NO, PDE inhibitors were added to the incubation medium. Treatment of unstimulated cells with PDE inhibitors resulted in only a slight and insignificant increase in cGMP levels. Stimulation of these cells with DEA-NO produced more than a 13-fold increase in cGMP over the basal level (Fig. 4.16). In cells treated with DETA-NO, inhibition of PDEs resulted in a 9-fold increase in cGMP levels, although, further stimulation of the cells with DEA-NO resulted in only an insignificant

increase in cGMP levels, suggesting an impaired cGMP response under prolonged exposure to the NO donor. To exclude the possibility that the effects observed were due to consumption of cellular GTP, the cells were stimulated with combination of DEA-NO and YC-1. Under these conditions, an increase in cGMP levels of more than 100-fold over the basal level was observed, suggesting that a deficit of GTP is unlikely to limit cGMP formation during stimulation of cells with DEA-NO only.

4.2.4 Effects of thiol reagents on NO donor-induced sGC down-regulation

Thiol oxidation has been considered for some time to be involved in the mechanism of sGC regulation (Ignarro and Gruetter, 1980; Braughler, 1983; Wu et al., 1992). In the present study, the role of thiol oxidation for short- and long-term effects of DETA/NO on sGC regulation was investigated. For this purpose, SH-reducing compounds were used to modulate DETA/NO produced sGC activity and protein down-regulation.

N-acetylcysteine (NAC), a membrane-permeable SH-reducing agent, was not able to

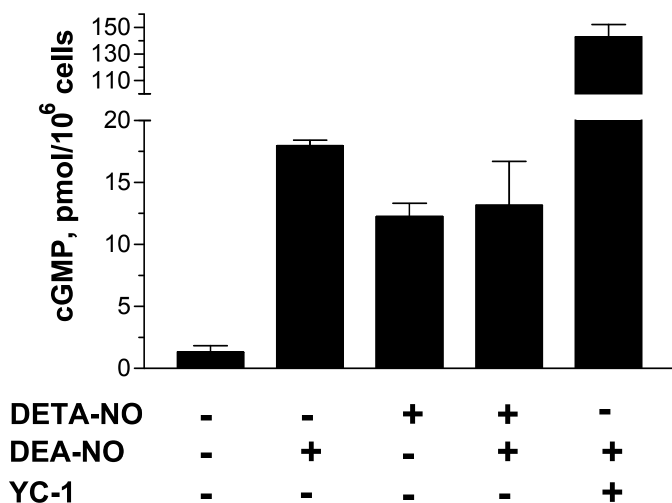

Fig. 4.16. Pretreatment of endothelial cells with PDE inhibitors did not prevent DETA/NO-induced desensitization of cGMP response in endothelial cells. *Endothelial cells were treated with 100 µM DETA/NO for 2h. 30 min before stimulation with DEA/NO, 1 mM IBMX and 100 µM zaprinast were added to the incubation medium to inhibit PDEs. Maximal cGMP response was stimulated with 250 µM DEA/NO or combination of 250 µM DEA and 50 µM YC-1 for 3 min. Medium was removed and cells were washed once with PBS and lysed in 70% ethanol. After evaporation of ethanol and resolving of pellet, the cGMP content was measured with commercial kit (Biotrend, Cologne) according to the protocol of the manufacturer). DEA/NO-produced increase in cGMP levels (difference between cGMP level in cells without and with stimulation with DEA/NO) was 16.6 in untreated cells and 1.0 pmol/10⁶ cells in cells pretreated with DETA/NO. Data are means ± SEM of three independent experiments performed in triplicate.*

significantly affect basal cGMP levels in endothelial cells (Fig. 4.17). Similarly, this substance did not affect the DEA/NO-stimulated cGMP response and could not reduce the desensitization of the cGMP response produced by prolonged treatment of the cells with DETA/NO (Fig 4.17).

Additionally, it was tested whether NAC can abolish the DETA/NO-induced down-regulation of sGC protein recently observed in our laboratory (Ibarra et al., 2001) and also reported by others (Papapetropoulos et al., 1996; Filippov et al., 1997). NAC alone had no significant effect on sGC protein levels and did not affect the DETA/NO response (Fig. 4.18).

To validate the results obtained for NAC, another SH-reducing agent, dithiothreitol (DTT), was used. It has been found that 1 mM DTT alone produced a significant decrease in the amount of sGC protein in endothelial cells. Simultaneous action of DTT and DETA/NO resulted in an even more dramatic decrease in sGC protein levels than DETA/NO alone (Fig. 4.19). For positive control, N-ethylmaleimide (NEM) was used as a compound whose biological effects are mediated by oxidation of SH-group. NEM produced a marked decrease in the amount of sGC protein in endothelial cells. This decrease, as well as the toxicity of the substance (data not shown), could be partially reversed by DTT (Fig. 4.19).

4.2.5 cGMP accumulation induced by the newly described activator of oxidized sGC, BAY58-2667, is facilitated by pretreatment of the cells with DETA/NO

Another possible mechanism of long-term regulation of sGC by NO is oxidation of sGC heme. Indeed, it is well known that oxidation of heme makes sGC insensitive to NO, consequently inhibiting the enzyme. It is also the mechanism of action of sGC inhibitors like ODQ and NS2028.

Very recently, a new sGC activator was described. The substance, BAY58-2667, is a weak activator of sGC (Stasch et al., 2002). If used together with an NO donor, effects of both

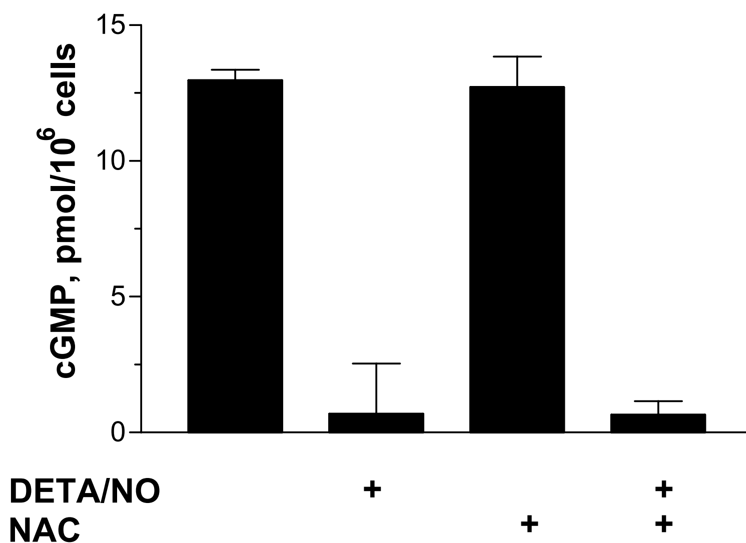

Fig. 4.17. NAC was not able to prevent DETA/NO-induced desensitization of the cGMP response in endothelial cells. *Endothelial cells were treated with 100 µM DETA/NO, 1 mM NAC or a combination of both substances for 2 h. Thirty min before stimulation with DEA/NO, 1 mM IBMX and 100 µM zaprinast were added to the incubation medium to inhibit PDEs. Maximal cGMP response was stimulated with 250 µM DEA/NO for 3 min. Data are DEA/NO-induced cGMP accumulation (means ± SEM of three independent experiments).*

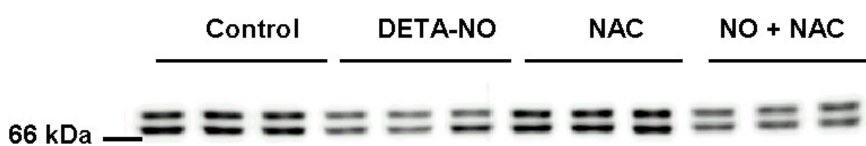

Fig. 4.19. Effects of NAC on DETA/NO-produced down-regulation of sGC protein. *Porcine endothelial cells were incubated with 1 mM NAC, 100 µM DETA/NO or with both substances simultaneously. After 72 h cells were lysed with sample buffer for electrophoresis and the amount of sGC protein was determined via Western blotting. A representative blot is shown.*

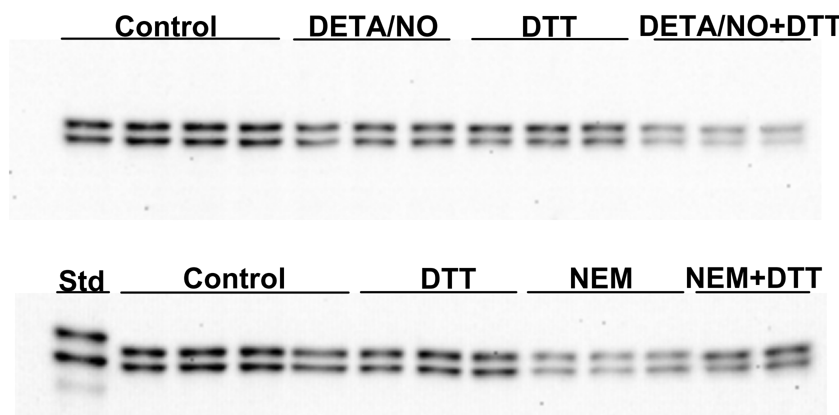

Fig. 4.18. Effects of DTT on the DETA/NO-produced down-regulation of sGC protein. *Porcine endothelial cells were incubated with 1 mM DTT, 100 µM DETA/NO (or 10 µM N-etylendiamide) or with both substaces simultaneously. After 72 h cells were lysed with sample buffer for electrophoresis and the amount of sGC protein t was determined via Western blotting. A representative blot is shown*

activators are additive. Interestingly, BAY58-2667 produced a stronger activation of the heme-deficient enzyme, and its effect on heme-containing sGC could be dramatically potentiated by oxidation of sGC by ODQ (Stasch et al., 2002). Thus, BAY58-2667 is a unique pharmacological tool allowing discrimination between oxidized (heme-deficient) and native sGC.

Treatment of endothelial cells with ODQ resulted in a higher cGMP response to subsequent stimulation with BAY58-2667 (Fig. X), indicating that the sGC-stimulating potential of BAY58-2667 increases with oxidation of sGC heme. Furthermore, it was studied whether treatment of the cells with DETA/NO for 2 h affects cGMP accumulation in response to BAY58-2667. Pre-incubation of endothelial cells with DETA/NO resulted in potentiation of the cGMP response to subsequent BAY58-2667 stimulation, suggesting that heme oxidation during the treatment of the cells with NO donor occurs.

4.2.6 BAY58-2667 prevents sGC protein level decrease produced by ODQ

Effect of sGC heme oxidation on the sGC protein level has not been well studied. ODQ produces a concentration-dependent loss of sGC proteins in endothelial cells within 24 h of treatment (Fig. 4.21), suggesting that oxidation of sGC heme results in the decrease of enzyme protein. Effects of BAY58-2667 on the sGC protein level in endothelial cells were also studied. Interestingly, 10 μmol/L BAY58-2667 increased sGCα_1 and especially sGCβ_1 protein amounts if the cells were incubated with the substance for 24 hours. Moreover, BAY58-2667 prevented the decrease in sGC protein produced by ODQ (Fig.4.21).

4.3 Effect of intracellular localization of sGC on its regulation

4.3.1 Membrane association of sGC in endothelial cells

sGC is a cytosolic protein. Cytoplasmic localization of sGC was the first parameter for

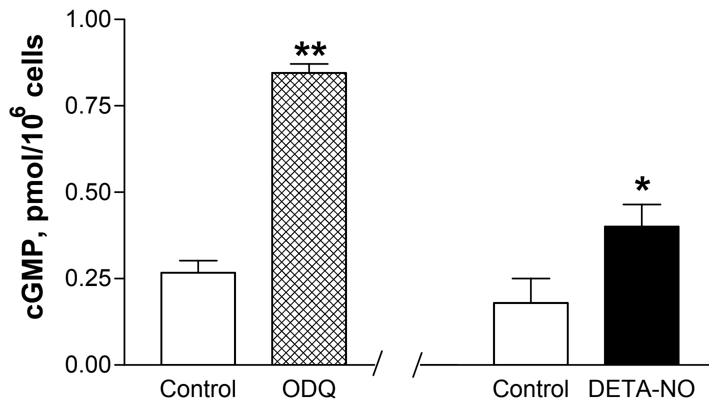

Fig. 4.20. BAY58-2667-stimulated cGMP accumulation is increased under treatment of endothelial cells with heme oxidizing compound, ODQ, or DETA/NO. *Cells were treated with 20 µM ODQ for 30 min. cGMP production in these cells (ODQ) and untreated cells (control) was stimulated with 10 µM BAY58-2667 for 3 min. Untreated cells (control) or cells treated with 100 µM DETA-NO for 2 h (DETA-NO) were washed twice with incubation medium and incubated for 10 min. Subsequently, cells were treated with 10 µM BAY58-2667 for 3 min and lysed with 80 % ethanol. Data are means ± SEM of three independent experiments. Asterisks indicate statistically significant differences (p<0.05 vs. control).*

differentiation of this protein from particulate guanylyl cyclase. Recently, however, membrane association of sGC in different cells and tissues was demonstrated in our laboratory (Zabel et al., 2002). In the present study, membrane association of sGC in endothelial cells as well as the physiological importance of sGC membrane association were investigated.

In experiments with whole isolated rat lung, it was demonstrated that membrane association of sGC in endothelial cells was stimulated by treatment of lung with VEGF (Fig. 4.22; Zabel et al., 2002). This was Ca^{2+}-dependent, because perfusion of lung with BAPTA/

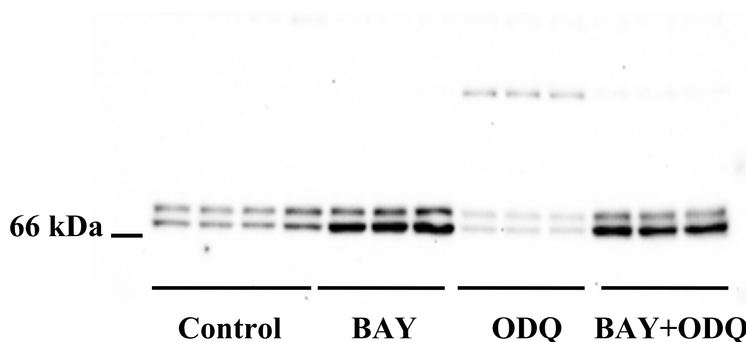

Fig. 4.21. BAY58-2667 prevented ODQ-induced decrease in sGC protein level in endothelial cells. *Endothelial cells were treated with 10 µM ODQ, 10 µM BAY58-2667 or a combination of the substances for 24 h. ODQ produced a dramatic decrease in protein level of both sGC subunits and induced appearence of high-molecular weight sGC-cross-reacting protein. BAY58-2667 alone increased sGC protein levels and prevented ODQ effects. A representative blot is shown.*

AM-containing buffer prior to VEGF stimulation completely blocked VEGF effect (Fig. 4.22; Zabel et al., 2002). To establish an appropriate model for convenient investigation of sGC membrane association in endothelial cells, cultured primary porcine endothelial cells were used. After subcellular fractionation by ultracentrifugation, sGC protein was measured by Western blotting of cytosolic and membrane fractions of endothelial cells. As previously shown for other cells and tissues, both sGCα_1 and sGCβ were found in membrane fractions of endothelial cells (Fig. 4.23). Surprisingly, in contrast to the experiments with lung, stimulation of the cells with VEGF was not able to stimulate sGC translocation to the membrane. Short (1-10 min) incubation of endothelial cells with a calcium ionophore, A23187, also did not produce any effect on sGC membrane association (Fig. 4.24). The difference between in vivo and in vitro data suggests that regulation of sGC membrane association during VEGF treatment may be a complex process requiring intercellular communication or the presence of blood flow.

4.3.2 Intracellular localization of sGC affects its sensitivity to NO in heart homogenates

The function of membrane association of sGC is unknown. Since NO is a very lipophilic molecule, its concentration in the membrane should be much higher than in the cytosol. This implies that at the same overall NO concentration, membrane-associated sGC has a greater potential to be activated. To test this hypothesis, membrane and cytosolic fractions of rat heart were used (we could not use membrane fractions from endothelial cells for this purpose, since the yield from endothelial cells was not great enough for an activity assay). It was previously demonstrated that both fractions contain sGCα_1 and sGCβ_1 proteins. cGMP accumulation rate at different concentrations of the NO donor, DEA/NO, was measured. In both fractions, DEA/NO produced the maximal stimulation of sGC in the same

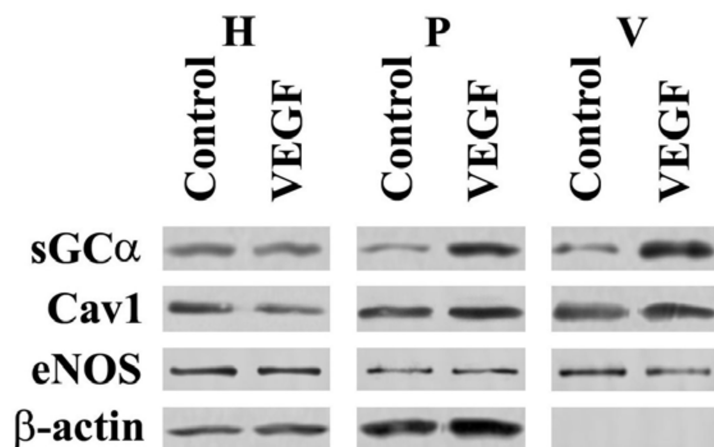

Fig. 4.22. VEGF-induced translocation of sGC to the membrane in endothelial cell in lung.
The rat lung vasculature was treated in situ with VEGF (100 ng/ml). The lungs were subfractionated to yield homogenate (H), fraction of plasma membrane (P), and caveolae (V). Protein from each fraction (15 µg H, 5 µg P and 1 µg V) was analysed by Western blotting for the presence of sGCα_1, caveolin-1, eNOS, and β-actin (data from Zabel et al., 2002; performed by Dr. Phil Oh,Sydney Kimmel Cancer Center, San Diego, California, USA)

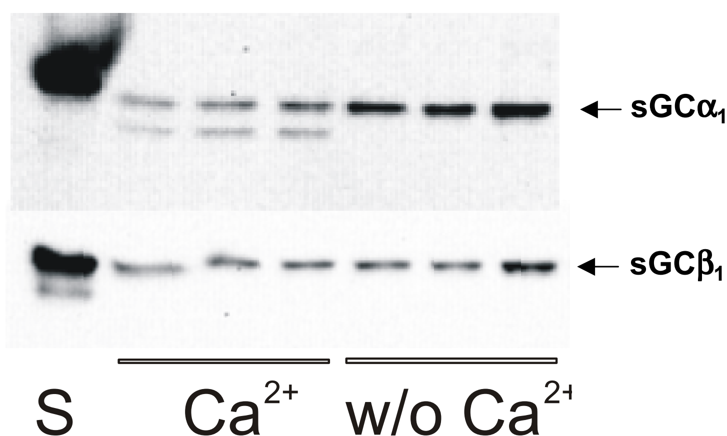

Fig. 4.23. sGC was present in membrane fraction of cultured endothelial cells.
Membrane fractions were isolated from cultured confluent endothelial cells. Both sGCα_1 and sGCβ_1 were detected in membrane fractions of endothelial cells. If 1 mM Ca^{2+} was present in solutions during membrane preparation, proteolytic degradation of sGCα_1 seemed to take place. The protease(s) was resistent towards a commercial cocktail of protease inhibitors (Complete EGTA-free; Roche).

concentration range, 3-10 µM (Fig. 4.25). However, in the membrane fraction, DEA/NO was able to produce sGC stimulation at a lower concentration than in the cytosolic fraction. To exclude possible nonspecific NO trapping by proteins, the sGC assay was always performed with the identical protein amount for both subcellular fractions.

4.3.3 Intracellular distribution of sGC in eNOS knock-out mice

Prior studies in our lab have demonstrated that the NO donor, SNP, produced a translocation of sGC to the membrane in platelets (Zabel et al., 2002). It is also known that stimulation of endothelial cells with VEGF results in NO release (Papapetropoulos et al.,

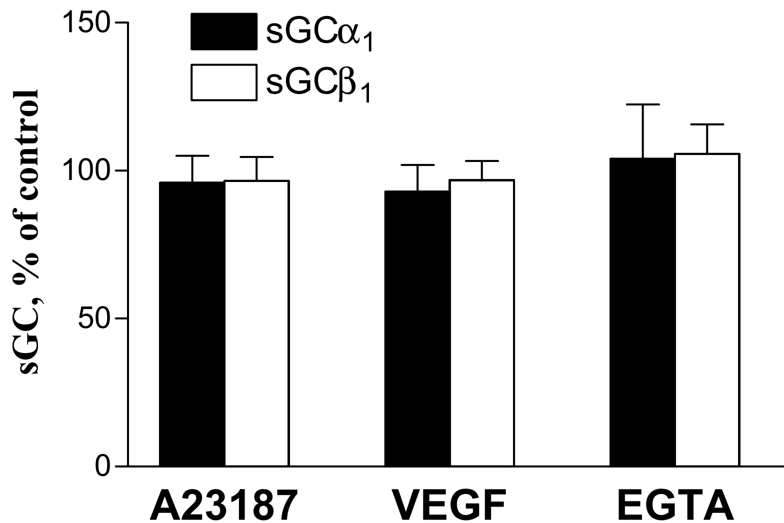

Fig. 4.24. Neither VEGF nor calcium ionophore were able to induce sGC translocation to the membrane in cultured endothelial cells.
Confluent endothelial cells were stimulated with 80 ng/ml VEGF or 500 nM calcium ionophore, A23187, for 5 min and immmediately washed with ice-cold PBS. Membrane fractions were isolated according to the protocoll described in Materials and Methods and sGC proteins were detected by Western blotting. Data are expressed as % of sGC content in the membrane fraction of untreated cells. Value are means ± SEM from 5 independent experiments performed in triplicate.

1997; van der Zee et al., 1997). Thus, an NO-dependent mechanism may be responsible for VEGF-induced sGC membrane translocation. Additionally, a possible interaction between nNOS and sGCα_2/sGCβ_1 has been demonstrated (Russwurm et al., 2001). Therefore, it was investigated in the present study whether the intracellular sGC distribution is different in eNOS knock-out mice.

For this purpose, various tissues of eNOS-/- mice were homogenized and membrane and cytosolic fractions were isolated. Distribution of both sGCα_1 and sGCβ_1 subunits in these tissues was measured by Western blotting and compared with that of wild-type mice. As Fig. 4.26 shows, no significant differences in intracellular distribution of sGC between knock-out and wild-type mice were observed.

4.4 Hsp90 activity is required to maintain sGC in endothelial cells

4.4.1 Effects of Hsp90 inhibitors on sGC protein levels in endothelial cells

The interaction of sGC with Hsp90 has been recently demonstrated (Venema et al.,

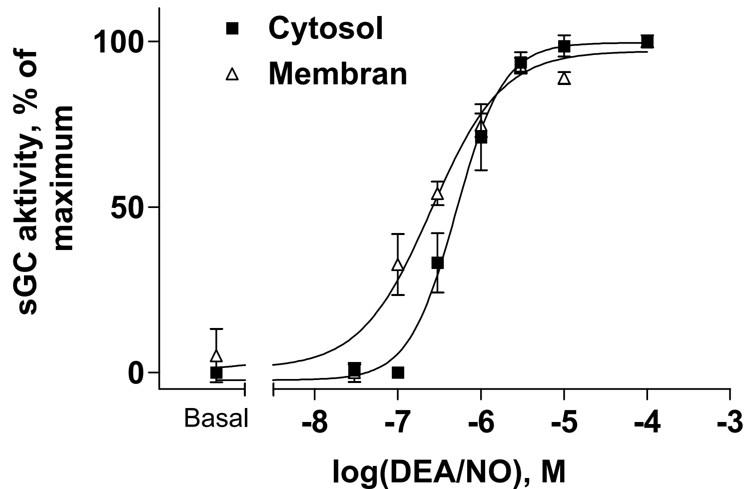

Fig. 4.25. Membrane-associated sGC was more sensitive to NO donor stimulation than cytosolic sGC.
Membrane and cytosolic fractions were isolated from rat heart. 1 µg of total protein was used for the sGC activity assay (see Materials and Methods). Different DEA/NO concentrations were used to stimulate sGC. Data are means ± SEM of 4 independent experiments expressed as % of the maximal activity in the membrane or cytosolic fraction, respectively.

2001). To clarify the role of this interaction, substances specifically inhibiting Hsp90, - geldanamycin and radicicol, were used.

Incubation of endothelial cells with geldanamycin produced concentration-dependent loss of sGC protein. The maximal effect was observed at 300 nM of the inhibitor (Fig. 4.27). The effect of the drug was also time-dependent, and after 24-h incubation, the sGC protein level was reduced by about 30-40% while the reduction was detected already after 2 h of incubation of the cells with geldanamycin (Fig. 4.28). Radicicol, a structurally different hsp90 inhibitor, had effects that were similar in amplitude and kinetics (Fig. 4.29 and 4.30).

4.4.2 Effects of an Hsp90 inhibitor, geldanamycin, on sGC protein levels in other cell types

It is also of interest to determine whether the Hsp90 inhibitor effects on sGC protein levels could be reproduced in other cell types. To analyze this, primary porcine smooth muscle cells (SMC) and the pheochromacytoma cell line PC12 were used. Incubation of both cell types with 300 nM geldanamycin resulted in a significant decrease in sGCα_1 and sGCβ_1 protein levels (Fig. 4.31), suggesting that proper functioning of Hsp90 is necessary to

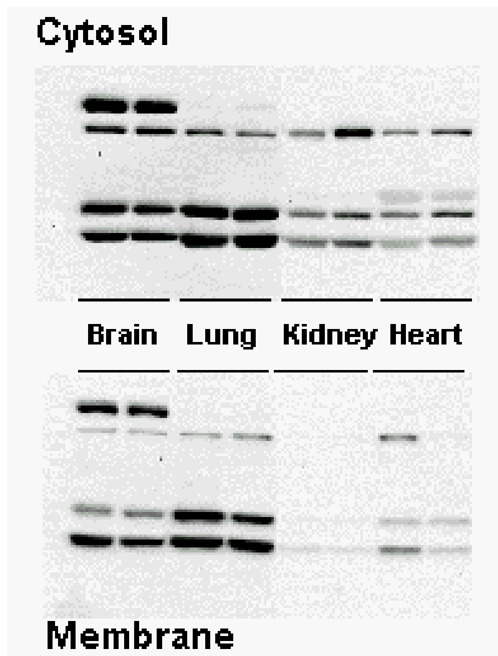

Fig. 4.26. Distribution of sGC between cytosolic and membrane fractions of NOS-III knock-out mice.
Organs from wild-type and NOS-III knock-out mice were prepared and immediately frozen in liquid nitrogen. Organs were pulverized and homogenized in ice-cold buffer. After separation of membrane and cytosolic fractions, sGC proteins were detected by Western blotting. The left lane in each pair of lanes represents sample from wild-type mouse and the right lane that of the knock-out animal. Representative blost are shown.

maintain sGC protein in different cell types.

4.4.3 No effect of geldanamycin on short-term sGC activity

Since it has been recently demonstrated that Hsp90 couples sGC to eNOS and regulates stimulation of sGC (Venema et al., 2001), we considered reproducing these results under our conditions. Endothelial cells were treated with geldanamycin for 30 min to 2 h (when no or only a small effect on sGC protein levels had been observed), and subsequently, NO donor-stimulated cGMP accumulation was measured. Surprisingly, no effect of the Hsp90 inhibition on cGMP accumulation was observed (Fig. 4.32). This is in contrast to the published data (Venema et al., 2001). To study if the signal transduction from eNOS to sGC is impaired in geldanamycin-treated cells, the calcium ionophore, A23187, was used. Under these conditions, the intracellular calcium concentration increases and a calcium-dependent enzyme, eNOS, should be stimulated. Indeed, treatment of endothelial cells with A23187 dramatically stimulates cGMP accumulation in these cells. This effect could be explained only by stimulation of NO production by eNOS, since a direct inhibitory effect of calcium on sGC activity has been reported (Kazerounian et al., 2002) and direct activation of sGC by Ca^{2+}

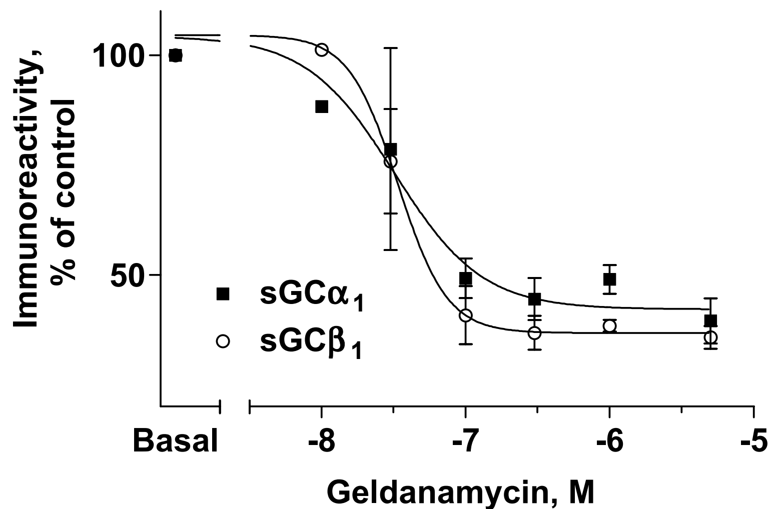

Fig. 4.27. Concentration-response curve of an Hsp90 inhibitor, geldanamycin, on the sGC protein levels in endothelial cells. *Confluent endothelial cells were treated with different concentrations of geldanamycin for 24 h. The sGC protein level was measured by Western blotting using sGCα_1- and sGCβ_1-specific antibodies. Data represent means ± SEM of 4 independent experiments performed in triplicate expressed as percent of control.*

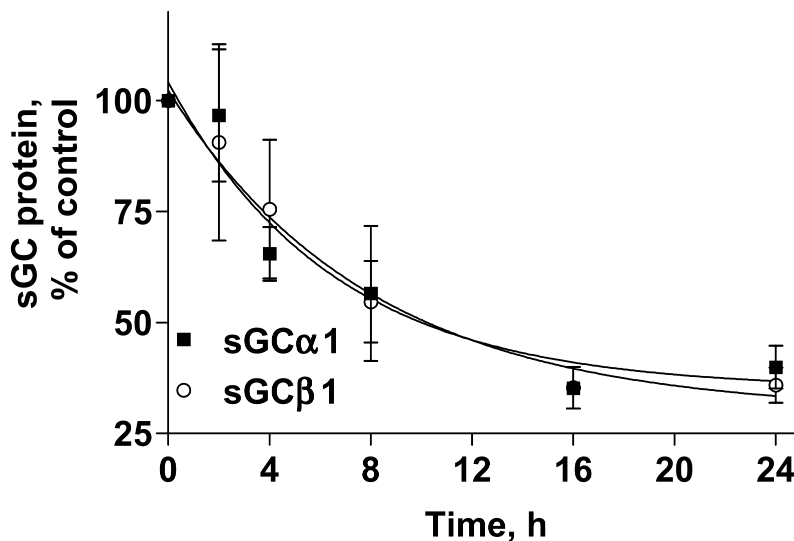

Fig. 4.28. Time dependence of an Hsp90 inhibitor, geldanamycin, on the sGC protein levels in endothelial cells. *Confluent endothelial cells were treated with 300 nM geldanamycin for different times. The sGC protein level was measured by Western blotting using sGCα_1- and sGCβ_1-specific antibodies. Data represent means ± SEM of 4 independent experiments performed in triplicate expressed as percent of control.*

would not be expected. Also, under these conditions no effect of Hsp90 inhibition on cGMP accumulation was found (Fig. 4.32).

4.4.4 Inhibition of transcription cannot explain the geldanamycin-induced decrease in sGC protein levels

Inhibition of Hsp90 has been shown to inhibit the function of some nonreceptor tyrosine kinases (Hartson et al., 1998; Xu et al., 1999). Changes in the activity of these protein kinases can result in disturbances in translation of different genes (Bedard et al., 1989; Scholz et al., 1996).

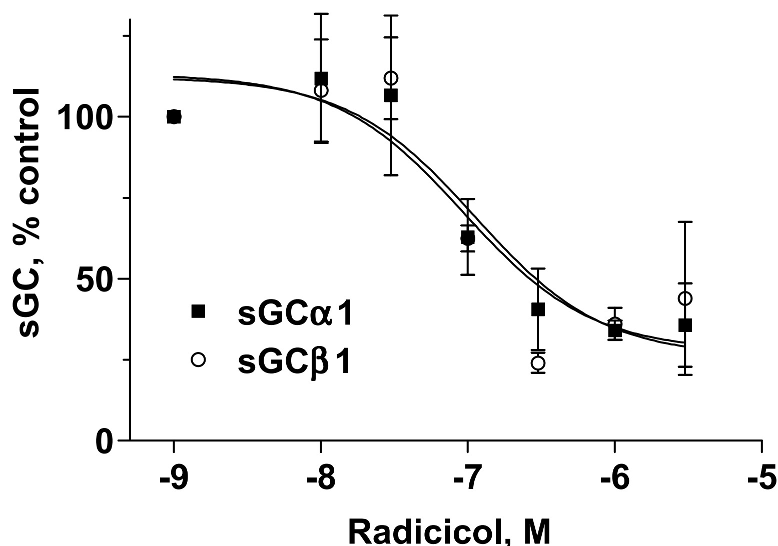

Fig. 4.29. Concentration-response curve of an Hsp90 inhibitor, radicicol, on the sGC protein levels in endothelial cells. *Confluent endothelial cells were treated with different concentrations of radicicol for 24 h. The sGC protein level was measured by Western blotting using sGCα1- and sGCβ1-specific antibodies. Data represent means ± SEM of 4 independent experiments performed in triplicate expressed as percent of control.*

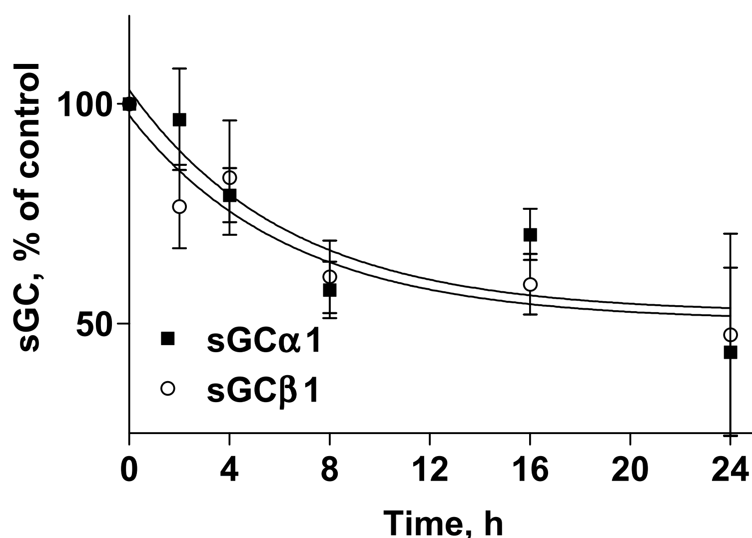

Fig. 4.30. Time dependence of an Hsp90 inhibitor, radicicol, on the sGC protein levels in endothelial cells. *Confluent endothelial cells were treated with 300 nM radicicol for different times. The sGC protein level was measured by Western blotting using sGCα1- and sGCβ1-specific antibodies. Data represent means ± SEM of 4 independent experiments performed in triplicate expressed as percent of control.*

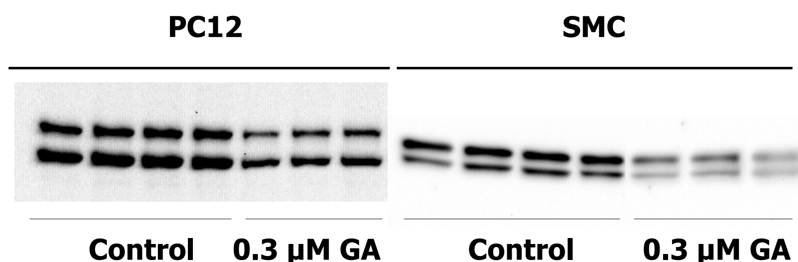

Fig. 4.31. Decrease in sGC protein levels in response to geldanamycin in different cell types. *Primary porcine smooth muscle cells or PC12 cells were treated with 300 nM geldanamycin for 24 h. sGC protein levels were detected by Western blotting. In both cell types, geldanamycin decreased sGC protein levels to an extent that was similar to that produced in endothelial cells. Representative blots are shown.*

Therefore, effects on sGC translation could be one of the possible mechanisms of action of Hsp90 inhibitors.

To investigate this possibility, an inhibitor of transcription, DRB (Sehgal et al., 1976; Harrold et al., 1991), was used. DRB alone (100 µM, up to 24 h of treatment) did not produce any significant decrease in sGC protein levels (Fig. 4.33). Moreover, DRB was not able to influence the effect of geldanamycin on sGC protein levels in endothelial cells.

4.4.5. Effects of proteasome inhibition on the decrease in sGC produced by geldanamycin

It is well known that inhibition of the interaction of hsp90 with certain proteins results in proteolytical degradation of these proteins by specific proteolytic structures, so-called proteasomes (Fujita et al., 2002). To investigate whether the same mechanism might be

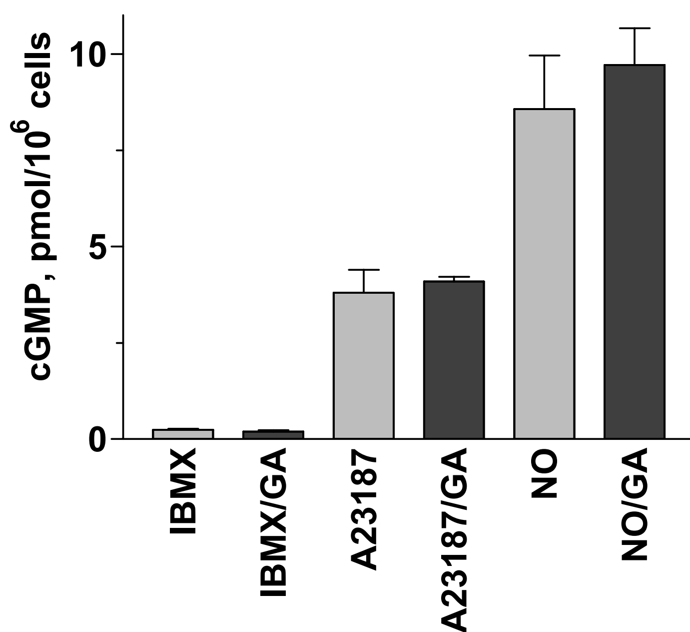

Fig. 4.32. Short-term effect of geldanamycin on cGMP accumulation in endothelial cells. *Confluent endothelial cells were treated with 300 nM geldanamycin for 2 h. During the last 30 min of incubation with geldanamycin, 1 mM IBMX and 100 µM zaprinast were added to the cell medium to inhibit cGMP hydrolysis by PDEs. cGMP levels were measured without additional stimulation of the cells (IBMX and IBMX/GA), after stimulation with 500 nM of calcium ionophore A23187 (A23187 and A23187/GA), and after stimulation with 250 µM DEA/NO (NO and NO/GA) for 3 min. After stimulation, cells were rapidly washed with PBS and lysed with 70 % ethanol. After evaporation of ethanol, cGMP levels were measured with the Biotrend EIA kit (Biotrend, Cologne). Data represent means ± SEM of 3 independent experiments performed in triplicate.*

responsible for sGC loss in hsp90 inhibitor-treated cells, two proteasome inhibitors, MG132 and lactacystin, were used. Treatment of endothelial cells with MG132 alone produced a decrease of sGCα_1 and sGCβ_1 protein levels (Fig. 4.34). However, geldanamycin could not produce further reduction of sGC protein levels in MG132-treated cells (Fig. 4.34). Similar results were obtained if another proteasome inhibitor, lactacystin, was used (Fig. 4.35). A marked and significant decrease in sGC protein levels in the presence of the proteasome inhibitor was observed, but geldanamycin was not able to produce any significant reduction in sGC protein levels in the presence of this compound (Fig. 4.35). The reason for the decrease in sGC protein levels in the presence of proteasome inhibitors is yet unknown. It can be speculated that a toxic side effect of proteasome inhibition may switch on some processes resulting in down-regulation of sGC protein levels.

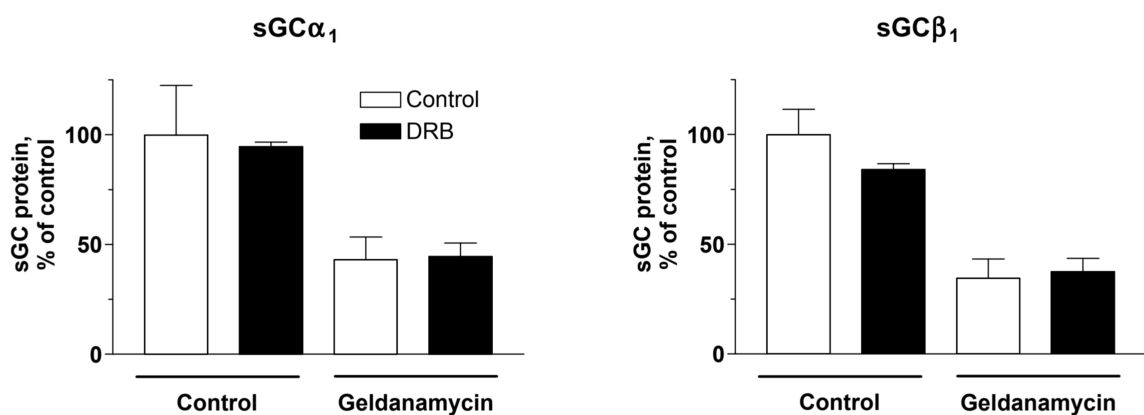

Fig. 4.33. Effect of transcription inhibitor, DRB, on the sGC protein level in endothelial cells. *Endothelial cells were treated with 300 nM geldanamycin, 100 µM DRB, or a combination of these substances for 24 h. sGC protein levels were determined by Western blotting. Data represent means ± SEM of 4 independent experiments performed in triplicate and are expressed as percent of control.*

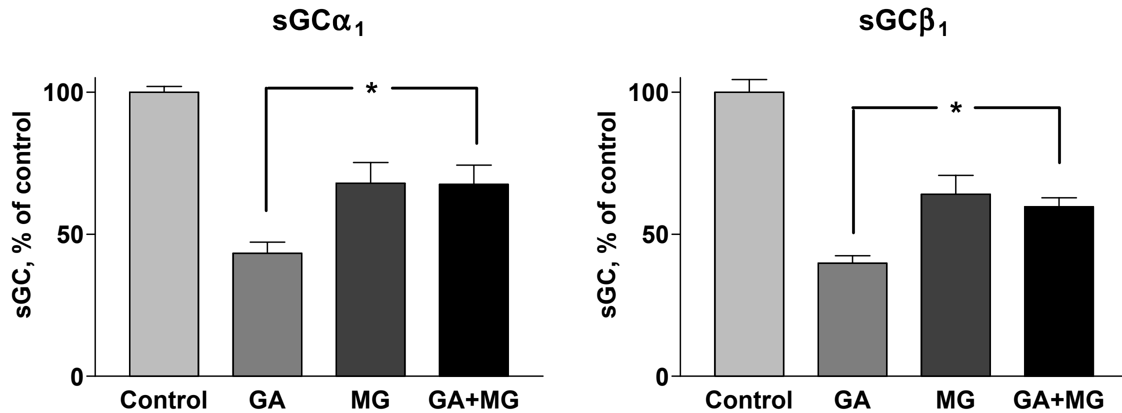

Fig. 4.34. A proteasome inhibitor, MG132, abolished geldanamycin-produced sGC protein level decrease in endothelial cells. *Endothelial cells were treated for 24 h with 300 nM geldanamycin (GA), 1 µM MG132 (MG), or with a combination of these substances. In the presence of the proteasome inhibitor, geldanamycin-induced sGC loss was prevented. Data represent means ± SEM of 3 independent experiments performed in triplicate and are expressed as percent of control.*

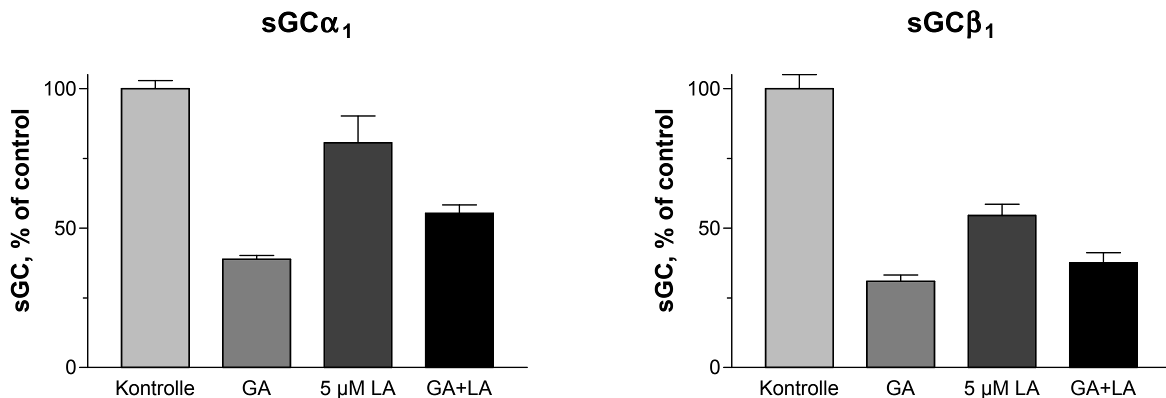

Fig. 4.35. Proteasome inhibitor, lactacystine (LA), abolished geldanamycin-produced sGC protein decrease in endothelial cells. *Endothelial cells were treated for 24 h with 300 nM geldanamycin (GA), 1 µM lactacystine (LA), or with a combination of these substances. In the presence of the proteasome inhibitor, geldanamycin-induced sGC loss was significantly reduced. Data represent means ± SEM of 4 independent experiments performed in triplicate and are expressed as percent of control.*

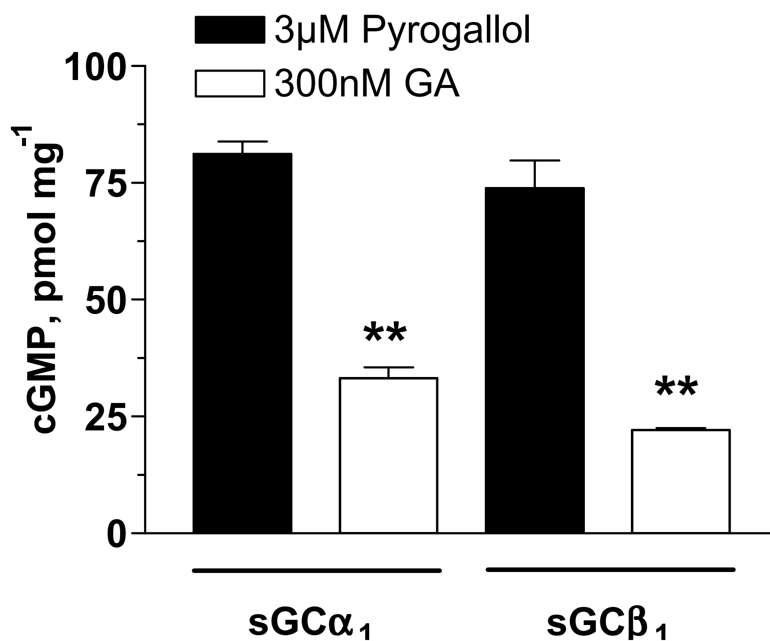

Fig. 4.34. Pyrogallol (3 µM) produced only a slight decrease in sGC protein levels. *Endothelial cells were treated with 3 µM pyrogallol for 24 h. For comparison, the effect of 300 nM geldanamycin (GA) is shown. Pyrogallol produced only a slight decrease in sGC protein levels in endothelial cells. Geldanamycin produced a significantly greater loss of sGC proteins in the cells. Means ± SEM of 3 independent experiments performed in triplicate are presented. Asterisks indicate statistically significant differences ($p < 0.01$) in comparison with 3 µM pyrogallol.*

4.4.6 Effects of quinones on sGC protein levels in endothelial cells

Geldanamycin is a chemical compound containing a quinone structural moiety. Quinones are known producers of oxygen radicals. It has already been demonstrated that some of the geldanamycin effects are due to generation of superoxide anion and not due to Hsp90 inhibition (Dikalov et al., 2002). However, in all these studies very high concentrations of geldanamycin (up to 20 µM) have been used. To investigate whether radical formation by geldanamycin could be responsible for the geldanamycin effects under our conditions, pyrogallol, a well-known radical generator (Lee and Lin, 1994), was used. In contrast to geldanamycin, pyrogallol (up to 3 µM) only slightly reduced of sGC protein level in endothelial cells (Fig. 4.36).

5. Discussion

5.1. Decomposition of different NO donors varies markedly depending on the experimental conditions

Because of the instability of the free radical NO, experimental handling of this gas is difficult. Therefore, in most experimental work not NO itself but compounds that are able to release NO in solution (NO donors) have been used to study the biological effects of NO. It is assumed that the physiological effects of NO donors are due to the release of NO and are reproducible under different experimental conditions. Unfortunately, this is not always the case.

NO-generating compounds differ in their chemical and biological properties (Feelisch, 1998). Effects that are independent of NO release or mediated by the release of alternative redox species of NO have been demonstrated for all classes of NO donors (Maragos et al., 1993; Mongin et al., 1998; Nedvetsky et al., 2000). The rate of NO release from different NO donors can vary drastically with different experimental conditions. The use of different NO donors to activate sGC can be a critical factor for proper determination of sGC activity. Three members of three different, most frequently used NO donor classes were used: nitrosothiols (nitrosocysteine), nitrosometal complexes (SNP), and NONOates (DEA/NO). Decomposition of nitrosocysteine was dramatically affected by contaminations present in water or biological samples. Since we do not have any possibility to control for the effects of all of these factors on the decomposition of nitrosocysteine, it is extremely difficult to interpret the results obtained. Additionally, nitrosothiols release NO in the form of NO^+ (Stamler et al., 1992; Feelisch, 1998), which has completely different effects on sGC than the radical form of NO

(Dierks and Burstyn, 1996). Thus, the rate and efficacy of NO^+ reduction to NO is an additional unknown factor that is extremely difficult to control.

SNP seems to have disadvantages that are similar to those of nitrosothiols. Although copper ions did not affect the decomposition rate of SNP, the use of different thiols resulted in dramatic changes in the rate and chemical character of SNP decomposition. The present data do not allow one to speculate about exact chemical events occurring during decomposition of SNP in the absence and in the presence of DTT, but changes in spectral characteristics suggest that not only is degradation of the compound accelerated but also another degradative pathway is activated in the presence of this thiol reagent. The reason that SNP was used in the present study is the extreme popularity of this compound as an sGC activator. Therefore, effects of this drug on sGC activity were compared to effects of another NO donor, DEA/NO.

DEA/NO is a member of the NONOates, a novel group of NO donors. Several laboratories have studied NONOates and have demonstrated a number of advantages of these compounds in comparison with other NO donors (Wink et al., 1996; Schmidt et al., 1997). Under our conditions as well, decomposition of DEA/NO and activation of sGC by this substance was affected by thiols, but to a much lower extent than the effects of SNP. Copper ions did not affect decomposition of DEA/NO. These data suggest that although, NONOate effects are not absolutely independent of buffer composition, these substances seem to be more suitable tools to study sGC activity. The differences in chemical properties of different NO donors should be taken into consideration in interpreting results. It is already accepted by the scientific community that in interpreting NO donor results, their different capacities for thiol nitrosylation and generation of oxygen radicals as well as the different effects of

degradation products should be taken into account. The same limitation could also be a reason for the different sGC-activating abilities of different NO-generating compounds.

5.2 Additional regulatory mechanisms must be involved in the regulation of sGC activity in rat brain

sGC is the main receptor for the important signaling molecule, NO. Participation of sGC in the regulation of blood vessel tone, modulation of neurotransmission, cell proliferation, and other fundamental processes makes sGC an interesting subject for basic and applied research. Paradoxically, despite more than 30 years of sGC research, very little is known about its regulation. To date, the only well-established physiological regulator of sGC is NO. Extremely little is known about other factors that modulate sGC action. However, it would be very unusual if a signalling protein would be involved in only a single mechanism of regulation. The present study provides some new insights into the regulation of sGC.

The first interesting observation of the present study was the lack of any correlation between sGC protein and activity distribution in rat brain. This may be viewed as indirect evidence for additional sGC regulatory mechanisms in rat brain.

Interestingly, the two NO donors used produced very different results with regard to sGC activation. SNP, one of the most frequently used NO donors, was obviously not able to produce maximal sGC stimulation under our assay conditions even in millimolar concentration. In cortex, midbrain, and striatum the effects of SNP were up to 20-fold lower than that of DEA/NO. In cerebellum, hypothalamus, and medulla the difference between DEA/NO and SNP effects reached only about 2-fold. Although the data do not provide any direct explanation for this phenomenon, it seems very likely that the degradation rate of SNP can vary significantly in different brain regions. Indeed, it has been demonstrated that

degradation of SNP is affected by different thiols (our data, see Section 4.1.2), oxygen concentration, and light irradiation (Feelisch, 1998). An additional complication of the use of SNP is the redox state of released NO species. Formally, SNP donates not NO but NO^+. NO^+ is a very reactive NO redox-related species of nitric oxide and rapidly reacts with buffer components, even with water (Koppenol, 1996). Therefore, it is extremely difficult to predict the real concentration of NO produced by SNP in solutions. It has also been demonstrated that NO^+ is not able to activate soluble guanylyl cyclase (Dierks and Burstyn, 1996) and an additional reduction of this compound to NO is necessary to produce an sGC-activating substance. Finally, CN^- ions are released by the degradation of SNP (Feelisch, 1998). It was demonstrated earlier that cyanide is able to inhibit NO-induced sGC activity (Ignarro et al., 1984; Wood and Ignarro, 1987). It seems plausible that with increasing SNP concentrations, the negative effect of cyanide on sGC prevails and prevents maximal activation of sGC. The effect of cyanide may be an explanation for the differences between SNP effects *in vivo* and *in vitro*. SNP is an effective vasodilator that is successfully used in clinical practice. Since *in vivo* cyanide produced during SNP degradation will be eliminated by detoxification enzymes and dilution, no inhibition of sGC may occur. All of these factors make the interpretation of SNP effects on sGC stimulation extremely difficult. It can be speculated that the frequent use of SNP as an sGC activator and its disadvantages mentioned above is the reason why the lack of correlation between sGC protein and activity distribution has been not reported earlier. In contrast to other NO donors tested, DEA/NO was the only donor whose stability seemed to be only slightly affected by thiols and not to be dependent on the presence of transition metals (in our case, copper). These properties make DEA/NO an attractive tool to investigate sGC

activation in different tissues and cell samples. Therefore, we have used DEA/NO-stimulated sGC activity as an indicator of maximal sGC activity.

Despite the relatively even distribution of sGC proteins in different rat brain regions, the distribution of the DEA/NO-stimulated sGC activity varied quitly between the regions tested. The activities in cortex, midbrain, and striatum were up to 10-fold higher than in medulla, cerebellum, and hypothalamus. The mechanisms that are responsible for this difference between protein and activity levels are unknown.

Because sGC is active only as a heterodimer, one explanation for this discrepancy is the unequal distribution of the two sGC subunits and the presence of sGC partially in inactive monomeric or homodimeric forms. Distribution of both sGCα_1 and sGCβ_1 subunits correlates in rat brain. The same has also been demonstrated for human brain (Ibarra et al., 2001). Although these data provide no direct information about the distribution of active heterodimers, they allow speculation that the dominant form of sGC in brain is the heterodimer. Indeed, heterodimerization is the prevalent process even in sGC over-expressing Sf9 cells, where only minor homodimer formation was found by co-expression of the two sGC subunits (Zabel et al., 1999). While it should be noted that from Western blotting data no conclusion about cellular distribution of both subunits can be made, such a possibility of differential expression of sGCα_1 and sGCβ_1 in different cells cannot be excluded.

An additional explanation for the lack of correlation between protein and activity distribution might be the presence of sGCα_2 and sGCβ_2 subunits, which can also contribute to NO-sensitive cGMP formation. Different regional expression patterns of sGCα_1 and sGCα_2 have been demonstrated in rat brain (Gibb and Garthwaite, 2001). On the other hand, sGCα_2/

$sGC\beta_1$ and $sGC\alpha_1/sGC\beta_1$ do not differ in their enzymatic characteristics (Russwurm et al., 1998). Thus, the distribution of sGC activity should correlate with the distribution of $sGC\beta_1$. $sGC\beta_2$ was found to form less active or inactive heterodimers with $sGC\alpha_1$ (Gupta et al., 1997) and has been recently shown to be active as a homodimer (Koglin et al., 2001). Although the presence of $sGC\beta_2$ could be a reason for the lack of correlation between sGC protein and activity distribution, no data about the presence of this isoform at the protein level in brain are available. To clarify the question of whether $sGC\beta_2$ indeed can contribute to sGC formation in brain, the expression of this subunit must be demonstrated.

Thus, at the present state of knowledge neither homodimerization nor the presence of additional sGC subunits constitute plausible explanations for the observed lack of correlation between sGC protein levels and activity. However, it may be that posttranslational modifications are involved. Very little known is about posttranslational modification of sGC. Only two studies have demonstrated sGC phosphorylation in cell culture (Louis et al., 1993; Ferrero et al., 2000), but the role of sGC phosphorylation *in vivo* remains unclear. It should be noted that one reason for this little success in investigation of posttranslational modifications of sGC is the lack of an sGC-specific antibody able to precipitate sGC. Although, immunoprecipitation of sGC with a commercially available antibody was shown recently (Ferrero et al., 2000), experiments in our lab have demonstrated that this antibody also recognizes protein(s) different from sGC that has similar electrophoretic mobility in SDS-PAGE (Kirsch et al., 2002). Ironically, preincubation of the antibody with the sGC-specific immunogenic peptide provided by the manufacturer blocks immunorecognition of this protein(s) (Kirsch et al., 2002). Our antibodies directed against $sGC\alpha_1$- or $sGC\beta_1$-specific

peptides showed much greater specifity in Western blotting, but we could not achieve immunoprecipitation of sGC with these antibodies from any sGC-immunopositive tissues.

5.3 Role of sGC desensitization in regulation of the cGMP level in porcine endothelial cells

It has been known for some time that long-term exposure of cells to NO desensitizes the cGMP response. One of the many possible mechanisms for this desensitization is via an effect on sGC activity. Indeed, in several studies a rapid deceleration of the cGMP level increase in response to NO donors has been demonstrated. However, the recently published study by Koesling and co-workers demonstrated that this decrease in the cGMP response was mediated not via desensitization of sGC but via accelerated PDE5 activity (Mullershausen et al., 2001). In several other studies, it was demonstrated that the reduced cGMP response observed during some pathological states could be explained by increased ROS production and NO scavenging by superoxide anion (Bauersachs et al., 1998; Mollnau et al., 2002). All these studies cast doubt on the role of sGC desensitization in regulation of the cGMP response. To clarify this question, porcine endothelial cells were exposed to DETA/NO to produce desensitization of the cGMP response. It was found that a after short increase in cGMP levels this parameter decreased to basal levels. Since DETA/NO is an NO donor with a very long half-life (about 20 h at 37°C and pH 7.4), this reduction in the cGMP response cannot be explained by a fall in the NO concentration. Additionally, short-term stimulation of cGMP synthesis by DEA/NO (an NO donor with a half-life of about 2 min under experimental conditions that a short but dramatic increase in NO level) was also reduced in DETA/NO-treated cells. In previous experiments, we found that long-term exposure of endothelial cells down-regulates sGC protein level (Ibarra et al., 2001). However, this effect first occurs after 24 h of DETA/NO

treatment. Down-regulation of cGMP response occurs within 2 h. Thus, the decrease in sGC protein cannot explain this rapid desensitization of the cGMP response.

It is very unlikely that reduction of the bioavailability of NO during short stimulation with DEA/NO is an explanation for the decreased cGMP response. sGC was stimulated with 250 μM DEA/NO, an NO donor with a very short half-life, and even under conditions of accelerated NO degradation (for instance, via elevated superoxide), NO concentrations should be enough to stimulate sGC. Using a mathematical model for calculation of NO concentrations in biological media (Schmidt et al., 1997), it was calculated that the NO concentration upon addition of 250 μM DEA/NO should reach 6-8 μM, which is much greater than the nanomolar K_m for sGC (Bellamy et al., 2000; Bon and Garthwaite, 2001). Thus, the reason of cGMP response desensitization should be the affected sGC regulation or increased cGMP breakdown and not decreased bioavailability of NO.

It has been recently demonstrated that desensitization of the cGMP response in platelets and aortic tissue is due to activation of PDE5 and not to desensitization of sGC (Mullershausen et al., 2001). In endothelial cells, PDE5 seems to be the main cGMP-hydrolysing PDE isoform (Ashikaga et al., 1997; Keravis et al., 2000). Demonstration that the PDE inhibitors, IBMX and zaprinast, did not prevented cGMP response desensitization after exposure of cells to DETA/NO suggests that up-regulation of PDE is not responsible for this effect of the NO donor. Different impacts of PDEs on regulation of the cGMP response may be due to different cell types used. Thus, Garthwaite and co-workers (2000) demonstrated that a PDE-dependent mechanism of cGMP response desensitization seems to be insignificant for cerebellar cells due to the low PDE activity in these cells (Bellamy et al., 2000). PDE5 has

been shown to be expressed only in spindle (angiogenic) endothelial cells and not in cobblestone (resting) endothelial cells (Keravis et al., 2000). The loss of PDE5 was accompanied by a 10-fold reduction in hydrolyzing activity towards cGMP in cobblestone endothelial cells (Keravis et al., 2000).

To further investigate the role of sGC desensitization in regulation of the cGMP response, sGC activity in homogenates of cells treated for different times with DETA/NO was measured. As with cGMP accumulation in intact cells, sGC activity was reduced in homogenates from cells exposed to DETA/NO.

It has been recently demonstrated in our laboratory that the NO-induced decrease in sGC protein level in endothelial cells is cGMP independent (Ibarra et al., 2001). It is likely that NO donor-induced desensitization of sGC is also cGMP independent. First, it has been demonstrated that cGMP has no direct inhibitory effect on sGC up to the millimolar concentration range (Lee et al., 2000). Second, it has been reported that in cells expressing both sGC and particulate GC, elevation of the cGMP level by activators of particulate GC had no effect on sGC activation (Hamad et al., 1999).

There are a couple of cGMP-independent mechanisms by which NO may affect protein function. One of the best studied is the nitrosylation of SH groups. It has been demonstrated that this mechanism is responsible for NO-mediated inactivation of NMDA receptors (Lipton et al., 1993), caspases (Kim et al., 1997), and glyceraldehyde-3-phosphate dehydrogenase (Molina y Vedia et al., 1992; Mohr et al., 1996). It has also been proposed for sGC that modification of SH groups can regulate the activity of the enzyme (Brandwein et al., 1981), although the exact mechanisms of action of SH reducing or oxidizing agents on sGC are not

completely understood. Neither DTT nor N-acetylcysteine could prevent the DETA/NO-induced decrease in sGC protein and activity level in our study. In contrast, if cells were treated with the well-known SH-modifying compound, N-ethylmaleimide, DTT was able to decrease the toxicity of this drug and also partially prevent the decrease in sGC protein levels. These data allowed the conclusion that modification of SH groups is unlikely to be involved in NO-induced desensitization of sGC.

Another possible mechanism for NO-induced desensitization of sGC is oxidation of sGC heme. Oxidation of heme will clearly result in inactivation of sGC, since it is a mechanism of action of sGC inhibitors like ODQ and NS2028. It has also been speculated that heme oxidation might be responsible for the NO-induced decrease in sGC activity and protein level in smooth muscle cells (Papapetropoulos et al., 1996). Perhaps the greatest problem preventing clarification of this question is the absence of an sGC-specific antibody that is able to precipitate sGC. Because of this, it is not known whether oxidation of sGC heme takes place during exposure of cells to NO. An indirect way was used in the present study to determine whether sGC heme oxidation takes place under our experimental conditions.

An interesting sGC activator has been recently described by Stasch and co-worker (Stasch et al., 2002). BAY58-2667 differs in its activation mechanism from other known sGC activators. BAY58-2667 activates heme-deficient or ODQ-oxidized sGC more potently than native enzyme (Stasch et al., 2002), suggesting that oxidized (or heme-deficient) sGC is the primary target for this compound. Because of this, BAY58-2667 is a unique tool allowing discrimination between reduced and oxidized (heme-deficient) forms of sGC. An additional advantage of BAY58-2667 is the lack of any effect on PDE activity (Stasch et al., 2002). It

was demonstrated here that treatment of intact cells with ODQ potentiates BAY58-2667-induced cGMP formation as was demonstrated earlier for purified enzyme (Stasch et al., 2002). In a similar way, pretreatment of the cells with DETA/NO for 2 h results in potentiation of the BAY58-2667-induced cGMP accumulation. This suggests that oxidation of the sGC heme occurs during treatment of the cells with the NO donor.

The effect of heme oxidation on sGC protein levels is controversial. Recently, it was demonstrated that the sGC inhibitor, ODQ, which oxidizes heme iron, induced sGC protein down-regulation in bovine chromaffin cells (Ferrero and Torres, 2002). However, these data are in contrast with an observed protective effect of ODQ on sGC protein reduction produced by NO in smooth muscle cells (Filippov et al., 1997). The discrepancies can be due to differences in experimental protocols. While permanent treatment of chromaffin cells with ODQ for full duration of experiments (up to 48 h) was used by Ferrero and Torres (Ferrero and Torres, 2002), Fillipov and co-worker (Filippov et al., 1997) treated the cells with the sGC inhibitor for only 30 minutes. In agreement with the report by Ferrero and Torres, in the present study ODQ decreased sGC protein levels in endothelial cells. This demonstrates that oxidation of sGC heme results not only in inhibition of the enzyme but also in down-regulation of the sGC protein.

Taken together, this suggests that oxidation of the heme may be enough to explain the decrease in sGC protein and activity,and may be responsible for coupling these avents. Under conditions where the NO concentration is increased, the sGC heme oxidized by NO cannot be reduced rapidly enough. This results in elevation of the pool of oxidized (and thus NO-insensitive) sGC. This oxidized sGC is the main target for degradation. If it is true, this

selective degradation of inactive sGC could explain why no difference in sGC activity had been observed in endothelial cells after 2 h and 72 h of exposure to DETA/NO. However, it should be noted here that this speculation about accelerated degradation of oxidized (inactive) sGC has no evidence supporting it and was just a working hypothesis used to explain observed discrepancies in the kinetics of sGC protein and activity reduction.

In conclusion, sGC desensitization can be an explanation for the lack of correlation between sGC protein and activity distribution. Indeed, cells or tissues with relatively high NO production (or tissues where a transient increase in NO production occurs) should have desensitized sGC and show lower specific sGC activity compared to tissues where no desensitization occurred. This may explain why some rat brain regions showed only a slight increase in sGC production in response to NO, although they had about the same sGC protein level compared with other brain regions with greater sGC activity.

5.4 Membrane association of sGC and its possible physiological role

Very recently, another possible mechanism for the regulation of sGC was reported. sGCα_2/sGCβ_1 heterodimers can be attached to the membrane (Russwurm et al., 2001). sGCα_2 is a protein that can interact with so-called PDZ domain-containing proteins (Russwurm et al., 2001). Such interaction may be important for proteins requiring proximity to certain cellular structures. An example for such regulation is neuronal NOS (NOS-I). NOS-I is activated via Ca^{2+} influx through NMDA channels (Dawson, Dawson et al. 1993). The NMDA receptor (channel) has a C-terminus that can bind to PSD-95 or PSD-93 (Brenman et al., 1996). This can result in clustering of NMDA receptors and amplification of local Ca^{2+} influx in response to glutamate. Efficiency of down-stream signal transduction may be increased by close

localization of the calcium-sensitive protein NOS-I to NMDA clusters. This is achieved via interaction of PSD-95 proteins with an internal stretch of amino acid in NOS-I. Coupling of sGC to this multimolecular complex via free PDZ domains on PDS-95 is also possible. The localization of a member of the signaling chain may not only increase efficiency of signal transduction, but may also allow locally restricted signal transduction.

Recently, membrane association of sGCα_1 and sGCβ_1 in different cells and tissues was shown (Zabel et al., 2002). Since no PDZ binding domain for sGCα_1 or sGCβ_1 as well as other possible membrane-binding domains are known, the mechanism of this membrane association is unclear. While membrane attachment of sGC to endothelial cell membrane could be stimulated by VEGF in experiments with full lung, this was reproduced on cultured endothelial cells. Although some part of sGC in cultured endothelial cells is membrane bound, none of the stimuli used (VEGF, bradykinin, Ca ionophore or chelators) was able to affect this membrane association. We presently have no explanation why active regulation of sGC membrane association by VEGF observed in full lung could not be reproduced in cultured endothelial cells. Technical limitations might be one explanation for this. Preparation of membrane fractions by ultracentrifugation took over 2 h. It is very likely that membrane association of the cytosolic protein sGC is transient and labile and is disrupted under preparation conditions. Another possibility is a complex mechanism of regulation of sGC membrane association. For proper function of endothelial cells such factors as interaction with other cells types (for instance, smooth muscle cells), the presence of blood flow, and shear stress are very important. One could speculate that in cultured endothelial cells in the absence of these factors, some of the signaling mechanisms regulating membrane association of sGC

may be shut down.

It is well known that membrane association plays a crucial role in the activation or regulation of some signaling proteins (Huang, 1989; Dudler and Gelb, 1996; Garcia-Cardena et al., 1997). Membrane-associated sGC is more sensitive towards NO, since it was partially stimulated at NO donor concentrations that had no effect on cytosolic sGC. The simplest explanation for increased sensitivity of sGC towards NO would be asymmetrical distribution of a hydrophobic molecule, NO, between membrane and cytosolic fractions because of its lipophilicity. This should result in a higher local concentration of NO close to sGC in themembrane fraction and activation of the enzyme at lower NO donor concentrations. Thus, membrane-associated sGC might be responsible for the first response to the slightly increased overall NO concentration. Because only a minor part of sGC is membrane associated, it seems very unlikely that under these conditions membrane-associated sGC can significantly affect the overall cGMP concentration in the cell. However, a local increase in the cGMP level close to the membrane is possible. Such an elevation of cGMP might modulate activity of cGMP-dependent ion channels without increasing the overall cGMP concentration in the cell and could thus be one of the mechanisms of differential modulation of cGMP targets in different cell compartments.

5.5 Hsp90, a novel player in sGC regulation

Molecular chaperones play an important role in regulation of protein folding and maintainance of proper and stable protein conformation (Hendrick and Hartl, 1995; Boston et al., 1996; Hartl, 1996). Chaperones participate also in regulated degradation of proteins (Alberti et al., 2002). Hsp90, the best investigated chaperone with such specific functions, is

necessary to maintain the steroid hormone receptor in ligand-competent conformation (Picard et al., 1990), for maturation of protein kinases (Xu et al., 1999), and in regulation of NOS phosphorylation and activation (Garcia-Cardena et al., 1998; Fulton et al., 2001). Recently, Venema and co-workers demonstrated that Hsp90 binds to sGC, builds a complex between sGC and NOS in endothelial and smooth muscle cells, and augments cGMP accumulation in these cells (Venema et al., 2001). Therefore, acute and long-term effects of Hsp90 on sGC activity and protein levels were studied more intensively in the present study.

In contrast to a previous report (Venema et al., 2001), no acute effect of the Hsp90 inhibitor, geldanamycin, on basal, calcium ionophore- or NO donor-stimulated cGMP accumulation in endothelial cells was found. Exact reasons for the discrepancies between these two reports are not known. Venema and co-workers showed that Hsp90 builds a complex between sGC and NOS (Venema et al., 2001). This should result in an increase of efficacy of NO transfer from the source of its synthesis to its receptor, sGC. In this situation, activation of NOS may produce a greater increase in cGMP levels. However, the proximity of sGC to NOS cannot explain potentiation of the ability of an NO donor to elevate cGMP as demonstrated by the same group (Venema et al., 2001). If it is true and Hsp90 really potentiates the NO donor-induced cGMP accumulation, this effect must be due to another mechanism.

Effects of Hsp90 inhibitors on the sGC protein levels in endothelial cells clearly demonstrated that functional Hsp90 is necessary to maintain sGC proteins in these cells. Effects of Hsp90 inhibitors were not confined to endothelial cells, since in smooth muscle cells and PC12 cells, geldanamycin produced a similar decrease in sGC protein levels.

Inhibition of Hsp90 can result in a loss of a protein by different ways. The most likely explanation is unfolding, destabilization anddegradation of the proteins. However, effects on sGC expression cannot be excluded, since Hsp90 was shown to be involved in regulation of protein expression (Srethapakdi et al., 2000; Dias et al., 2002). Unfortunately, translation inhibitors could not be used to clarify the role of gene expression in Hsp90 inhibitor effects, since all translation inhibitors used (cycloheximide, emetine, puromycin, and anysomycin) were highly toxic during the long incubation times. Therefore, an inhibitor of transcription, DRB (Harrold et al., 1991), was used. Interestingly, DRB alone was not able to produce an sGC reduction comparable with that of the Hsp90 inhibitors geldanamycin and radicicol. This suggests that half-life values for sGC proteins and (or) mRNA in endothelial cells are enough long to maintain sGC protein levels under inhibition of transcription at least for 24 h. Taking into account the recent observations about the great stability of sGC protein (Ferrero and Torres, 2002), it can be supposed that inhibition of Hsp90 results in a decrease in sGC protein stability and accelerated protein degradation.

Some proteins need Hsp90 to escape degradation by proteolytic machinery (Fujita et al., 2002; Wang et al., 2002). Proteasome inhibitors, MG132 and lactacystin, were able to prevent Hsp90 inhibitor-induced sGC loss, indicating that degradation of sGC by proteasomes is involved in Hsp90 inhibitor-induced sGC loss. Importantly, MG132 or lactycystin alone do not elevate sGC content, suggesting that proteasomes do not participate in regulation of sGC protein amount under normal conditions. It is not known why the proteasome inhibitors produced a decrease in sGC protein levels. It may be that it is because of a side effect of the compounds. Perhaps inhibition of proteasomes switches some processes in the cells, which

result in a decrease in sGC protein levels.

Although the exact mechanisms of Hsp90 effects on sGC regulation are not yet known, stabilization of the enzyme and protection against proteosomal degradation seem to be most likely mechanism of action. Another possibility is the participation of Hsp90 in maturation and posttranslational modification of sGC as demonstrated for other proteins (Bender et al., 1999).

5.6 Possible physiological roles of previously unrecognized mechanisms of sGC regulation

It is rather unusual that a signaling protein is under control of only one regulatory mechanism. For sGC there is currently no other established regulatory concept beyond its activation by NO.

It could be speculated that the great variety of factors affecting NO production and stability and regulating in this way the concentration of the sGC activator makes additional regulation of sGC superfluous. However, such additional regulation might make NO/sGC/ cGMP signal transduction more efficient and precise. Under conditions of prolonged increased NO concentrations, over-stimulation of sGC can result in long-term elevation of cGMP levels. In the cardiovascular system, elevation of cGMP will result in a fall of blood pressure and blood vessel resistance. It has been demonstrated also that prolonged elevation of intracellular cGMP levels can induce apoptotic degeneration of cells (Li et al., 1997). Thus, mechanisms preventing over-stimulation of sGC and over-production of cGMP should be protective during conditions with elevated NO concentration. It is well known that prolonged stimulation with NO results in desensitization of sGC, which may represent such a protective mechanism. Unfortunately, this desensitization of sGC is a great complication in the use of

NO-dependent sGC activators to treat cardiovascular diseases.

In the present study, it was demonstrated that oxidation of sGC heme iron may be one of the mechanisms of sGC desensitization by NO. We speculate that oxidized sGC may be preferentially degraded. In this way, not only the activity of sGC but also its protein level could be tuned to the new steady-state level of NO. A short-term increase in the NO concentration results in reversible oxidation of sGC heme iron and a fall in NO-induced cGMP synthesis. If the NO concentration is increased for a prolonged period and oxidation of heme prevails over reduction, sGC protein levels will decrease. It should be noted here that this speculation presupposes existence of an sGC heme-reducing mechanism in cells. The existence of such a mechanism has not yet been documented.

Spatial vicinity of sGC to an NO source could reduce the amount of NO required to activate sGC and protect the cell from deleterious effects of high NO concentrations. Furthermore, depending on sGC localization, different cGMP targets could be preferentially activated. Membrane association of sGC demonstrated in our laboratory (and by others for the other isoform of sGC, sGCα_2) might be one of these mechanisms. Indeed, NO is a lipophilic molecule (Malinski et al., 1993) that will localize to the membrane fraction in higher concentration than in cytosol. Additionally, at least in endothelial cells, the NO-producing enzyme NOS-III is attached to the membrane via regulated protein-protein interactions (for review see Nedvetsky et al., 2002). Indeed, it has been demonstrated that attachment of NOS-III to membrane structures, caveolae, is required for activation of the enzyme by VEGF or bradykinin (Garcia-Cardena et al., 1997; Dimmeler et al., 1999; Sun and Liao, 2002). In this situation NO would concentrate in the membrane fraction because of its lipophilicitythe and

synthesis close to membrane. Thus, membrane-associated sGC could be better stimulated than sGC in the cytosol, as demonstrated in the present study by *in vitro* experiments. Although membrane-associated sGC constitutes less than 10% of total sGC (Zabel et al., 2002), cGMP formation close to the membrane could be important for discrimination between intracellular and membrane-bound targets of cGMP (protein kinase G, cGMP-regulated PDEs, and cGMP-operated ion channels). The exact physiological role of sGC membrane attachment needs to be identified. Indirect evidence for the physiologically important role of membrane association of sGC might be the active regulation of this association demonstrated for endothelial cells and platelets (Zabel et al., 2002).

Since sGCα_1/sGCβ_1 heterodimers have no membrane association domain or known sites for lipophilic modification, protein-protein interactions seem to be very likely mechanims of sGC membrane attachment. The first sGC-interacting protein was described recently (Venema et al., 2001). It is an important molecular chaperone, Hsp90, participating in the regulation of several signalling proteins. The exact role that Hsp90 plays in the regulation of sGC is unknown. Hsp90 not only participates in re-folding of unfolded proteins, but also performs a number of specific functions such as modulation of posttranscriptional modification, maintenance of active conformational states, and proper intracellular localization of client proteins. It can be (Venema et al., 2001) speculated that Hsp90 also plays some specific roles in regulation of sGC. Hsp90 is an attractive candidate for the role of a coupler between sGC and NOS (Venema et al., 2001). However, the present data and data provided by others (Venema et al., 2001) are controversial on this point and further studies are needed to clarify this.

It is also known that formation of sGC heterodimers occurs only upon co-expression of both sGC subunits in the same cell and cannot be performed by mixing of separately expressed proteins. This allows one to speculate that co-translational folding and heterodimerization may be responsible for this. It is known from the example of other proteins that a complex protein machinery is involved in these processes in cells. Also, Hsp90 plays an important role in these processes(Frydman, 2001). Therefore, the role of Hsp90 in the formation of sGC heterodimer should be further investigated.

In summary, the present study demonstrates that not only activation by its main regulator, NO, but also regulation of oxidative state, intracellular localization, and interactions with other proteins are important factors contributing to the regulation of sGC.

6. Summary

Soluble guanylyl cyclase (sGC) is the best established receptor for nitric oxide (NO) and regulates a great number of important physiological functions. Surprisingly, despite the well-appreciated roles of this enzyme in regulation of vascular tone, smooth muscle cell proliferation, platelet aggregation, renal sodium secretion, synaptic plasticity, and other functions, extremely little is known about the regulation of sGC activity and protein levels. To date, the only well-proven physiologically relevant sGC regulator is NO.

In the present study, some additional possibilities for sGC regulation were shown. Firstly, we evaluated the ability of different NO donors to stimulate sGC. Significant differences in the sGC stimulation by SNP and DEA/NO were found. DEA/NO stimulated sGC much stronger than did SNP. Interestingly, no correlation between the sGC protein and maximal activity distribution was found in rat brain regions tested, suggesting the existence of some additional regulatory mechanisms for sGC. The failure of SNP to stimulate sGC maximally might be one of the reasons why the lack of correlation between the distribution of sGC activity and proteins in brain was not detected earlier.

Prolonged exposure of endothelial cells to NO donors produced desensitization of the cGMP response. This desensitization cannot be explained by increased PDE activity, since PDE inhibitors were not able to prevent the NO donor-induced decrease of the maximal cGMP response in endothelial cells. The failure of SH-reducing agents to improve the cGMP response after its desensitization by NO suggests that a SH-independent mechanism mediates NO effects. Demonstration that the potency of the recently described activator of oxidized (heme-free) sGC, BAY58-2667, to stimulate sGC increases after prolonged exposure of the

cells to an NO donor, DETA/NO, suggests that oxidation of heme may be a reason for NO-induced desensitization of sGC and decrease in sGC protein level. Indeed, the well-known heme-oxidizing agent ODQ produces a dramatic decrease in sGC protein levels in endothelial cells and BAY58-2667 prevents this effect. Although the mechanism of sGC activation and stabilization by BAY58-2667 is unknown, this substance is an interesting candidate to modulate sGC under conditions where sGC heme iron is oxidized.

Very little is known about regulation of sGC by intracellular localization or translocation between different intracellular compartments. In the present study, an increase in sGC sensitivity to NO under membrane association was demonstrated. Treatment of isolated lung with VEGF markedly increased sGC in membrane fractions of endothelial cells. Failure of VEGF to stimulate sGC membrane association in cultured endothelial cells allows us to propose a complex mechanism of regulation of sGC membrane association and/or a transient character of sGC membrane attachment.

A very likely mechanism for the attachment of sGC to membranes is via sGC-interacting proteins. These proteins may participate also in other aspects of sGC regulation. The role of the recently described sGC interaction partner, Hsp90, was investigated. Short-term treatment of endothelial cells with an Hsp90 inhibitor does not affect NO donor or calcium ionophore-stimulated cGMP accumulation in the cells. However, inhibition of Hsp90 results in a rapid and dramatic decrease in sGC protein levels in endothelial cells. These effects were unrelated to changes in sGC transcription, since inhibition of transcription had much slower effect on sGC protein levels. In contrast, inhibitors of proteasomes abolished the reduction in sGC protein levels produced by an Hsp90 inhibitor, suggesting involvement of

proteolytic degradation of sGC proteins during inhibition of Hsp90. All these data together suggest that Hsp90 is required to maintain mature sGC proteins.

In conclusion, in the present study it was demonstrated that multiple mechanisms are involved in the regulation of sGC activity and its sensitivity to NO. Oxidation of sGC heme by NO seems to be one of the mechanisms for negative regulation of sGC in the presence of high or prolonged stimulation with NO. Another possible means of regulating sGC sensitivity to NO is via the intracellular translocation of the enzyme. It has been also demonstrated here that attachment of sGC to the membrane fraction results in an apparent increase in the enzyme sensitivity to NO. Additionally, Hsp90 was required to maintain sGC protein in endothelial and other cell types. However, we could not find any acute affect of Hsp90 on sGC activity, as reported recently. All these findings demonstrate that the regulation of sGC activity and protein level is a much more complex process than had been assumed earlier.

7. Zusammenfassung

Lösliche Guanylylcyclase (sGC) ist der Hauptrezeptor für Stickstoffmonooxid (NO), der sich an der Regulation zahlreicher physiologischer Funktionen beteiligt. Trotz ihrer sehr gut untersuchten Rolle in der Regulation der Blutgefässenrelaxation, synaptische Plastizität, Aggregation der Trombozyten, renale Sekretion und anderen wichtigen Funktionen, ist die Regulation der sGC selber noch nicht ausreichend verstanden. Der einzige, zur Zeit bekannte, physiologische Regulator der sGC ist NO.

In der vorgelegten Arbeit wurde die Existenz anderer Möglichkeiten der sGC Regulation gezeigt. Zuerst, wurde die Fähigkeit verschiedener NO Donoren sGC zu stimulieren untersucht. DEA/NO stimulierte sGC viel stärker als SNP. Interessanterweise, wurde keine Korrelation zwischen der Verteilung des sGC Proteins und der Enzymaktivität unter V_{max}-Bedingungen in verschiedenen Rattenhirnregionen gefunden. Das deutet auf zusätzliche Regulationsmechanismen hin. Die fehlende Fähigkeit von SNP sGC maximal zu stimulieren könnte ein Grund dafür sein, warum dieses Phänomen nicht schon früher gezeigt wurde.

Langfristige Behandlung von Endothelzellen mit NO Donoren produzierte eine Desensitisierung der nachfolgenden cGMP Antwort. Diese Desensitisierung kann nicht durch erhöhte Phosphodiesterase-Aktivität erklärt werden, da Phosphodiesterasenhemmer die durch NO Donor verursachte Abnahme der cGMP Antwort nicht rückgängig macht. SH-reduzierende Substanzen waren nicht in der Lage die cGMP Antwort zu verbessern, was zur Annahme führt, dass SH-Gruppenoxidation keine wichtige Rolle bei der Wirkung von NO auf sGC spielt. Es müssen daher andere Regulationsmechanismen vorhanden sein. Oxidation des Häms scheint ein möglicher Mechanismus der NO-induzierten sGC Desensitisierung.

Einkürzlich beschriebener Aktivator der oxidierten (bzw. Häm-freien) sGC, BAY58-2667, stimulierte sGC nach Vorbehandlung mit NO Donoreb stärker als ohne Vorbehandlung. Es wird vermutet, dass oxidierte sGC verstärkt abgebaut wird was die durch NO oder Häm oxidierende Substanzen induzierte sGC Proteinabnahme erklären würde. Tatsächlich, nahm sGC Proteinlevel nach der Behandlung mit der Häm oxidierenden Substanz, ODQ, ab. BAY58-2667 verhinderte diesen Effekt.

Ferner erhöht die Membranassoziation von sGC derer Empfindlichkeit gegenüber NO. Die Membranassoziation der sGC in Endothelzellen ist reguliert. Behandlung isolierter Lunge mit VEGF erhöht den Anteil an membrangebundener sGC in Endothelzellen dramatisch. In kultivierten Endothelzellen könnte VEGF die Membranassoziation jedoch nicht stimulieren, was einen komplexen Mechanismus der Membranassoziation der sGC *in vivo* vermuten lässt.

Wenig ist bekannt über die Interaktionen von sGC mit anderen Protein und der möglichen Rolle dieser Interaktionen bei der Regulation des Enzyms. Proteininteraktionen scheinen aber ein möglicher Mechanismus für die Membranassoziation der sGC zu sein. Aus diesem Grund wurde die Rolle eines vor kurzem beschriebenen sGC-bindenden Proteins, Hsp90, auf die sGC Regulation untersucht. Kurzfristige Behandlung der Endothelzellen mit Hsp90 Inhibitoren hat keine Auswirkung auf NO Donor- und Calciumionophore-stimulierte cGMP-Produktion. Langfristige Hemmung von Hsp90 führte dagegen zur schnellen und deutlichen Abnahme des sGC Proteins. Dieser Effekt ist nicht durch eine Veränderung der Translation zu erklären, weil Tranlationshemmer einen viel langsameren sGC Abfall verursachten. Im Gegenteil, konnte ein Proteasomeninhibitor, MG132, die Effekte von Hsp90 Hemmern rückgängig machen. Das lässt eine proteolytische Abbau der sGC für die Effekte

von Hsp90 Hemmer verantwortlich machen. Diese Daten deuten darauf hin, dass Hsp90 für Aufrechterhaltung des Enzyms notwendig ist.

Zusammenfassend, wurde in der vorliegenden Arbeit gezeigt, dass sGC Aktivität und ihre Empfindlichkeit gegenüber ihren Aktivator NO durch multiple Faktoren beeinflusst werden kann. Oxidation des Häms durch NO könnte ein Mechanismus der negativen Regulation der sGC bei dauernd erhöhter Konzentration von NO sein. Ein zusätzlicher Mechanismus der Regulation der Empfindlichkeit der sGC gegenüber NO scheint die intrazellulare Translokation zu sein. Wir konnten hier zeigen, das die Membranassoziation der sGC ihre Empfindlichkeit gegenüber NO erhöht. Auch dieProteinlevel der sGC scheinen unter Kontrolle verschiedener Faktoren zu sein. Einer davon ist Hsp90, der für die Aufrechterhaltung des sGC Proteins sowohl in Endothelzellen als auch in anderen Zelltypen notwendig ist. Alle diese Daten zeigen, dass Regulation der sGC ein viel komplexerer Vorgang ist als bis her angenommen wurde und eröffnen interessante neue Forschungsrichtungen innerhalb dieses wichtigen Signalweges.

8. References

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Member of German Society for Biochemistry and Molecular Biology (GBM)
Member of Federation of European Biochemical Societies (FEBS)
Member of International Union of Biochemistry and Molecular Biology (IUBMB)

PUBLICATIONS:

7 papers, 1 book chapter, 1 commentary, 1 book and 10 abstracts

LIST OF PUBLICATIONS
Experimental papers

Nedvetsky, P.I., Kleinschnitz, C., and Schmidt, H.H.H.W. (2002) Regional distribution of protein and activity of the nitric oxide receptor, soluble guanylyl cyclase, in rat brain suggests multiple mechanisms of regulation. *Brain Res.* ***950*** *(1-2),* 148-154.

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Wingler, K., Ibarra, C., **Nedvetsky, P.I.,** and Schmidt, H.H.H.W. (2001) Expressional regulation and subcellular localization of the nitric oxide receptor soluble guanylyl cyclase (sGC). In: J.D. Catravas, A.D. Callow, and U.S. Ryan (eds.) Vascular Endothelium. Source and Target of Inflammatory Mediators. IOS Press; Ohmsha. pp. 177-187.

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* **Nedvetsky, P.I.,** Müller, H., and Schmidt, H.H.H.W. (2002) Hsp90 stabilizes soluble guanylyl cyclase in endothelial cells. *In*: Abstract-book of the VIII. NO-Forum. October 3-5, 2002. Frankfurt am Main, Germany, V29.

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* - Oral presentations