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Mechanisms of tolerance to sodium nitroprusside in rat cultured aortic smooth muscle cells

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1 While exposure of smooth muscle cells to sodium nitroprusside (SNP) leads to the development of tolerance to soluble guanylate cyclase (sGC) activation, the mechanisms responsible for this phenomenon in intact cells remain unclear. In the present study, possible mechanisms of tolerance were investigated in a cell culture model where sGC activity was estimated from the accumulation of cyclic GMP in response to 10 μM SNP over a 15 min period in the presence of a phosphodiesterase (PDE) inhibitor.

2 Pretreatment of rat aortic smooth muscle cells with 10-500 μM SNP led to a dose-dependent downregulation of cyclic GMP accumulation upon subsequent SNP stimulation. This effect was evident as early as 2 h following incubation with 10 μM SNP, reached a plateau at 4 h and was blocked by co-incubation with 30 μM oxyhaemoglobin.

3 Pretreatment of smooth muscle cells with the PDE inhibitor, zaprinast, resulted in downregulation of the SNP-induced cyclic GMP accumulation in a time- and concentration-dependent manner, that was first evident after 12 h. Moreover, while the zaprinast-induced downregulation of cyclic GMP accumulation was completely inhibited by the protein kinase A (PKA) inhibitor, H89, tolerance to SNP was partially reversed by H89.

4 β_1 sGC steady state mRNA levels of S-nitroso N-acetylpenicillamine (SNAP)- or 8Br-cyclic GMP-pretreated cells were unchanged, as indicated by Northern blot analysis. However, Western blot analysis revealed that α_1 protein levels were decreased in zaprinast, but not in SNP, SNAP or 8Br-cyclic GMP pretreated cells.

5 While thiol depletion did not prevent the development of tolerance, pretreatment of cells with SNP in the presence of reducing agents partially or completely restored the ability of cells to respond to SNP.

6 We conclude that tolerance to SNP results from two distinct mechanisms: an early onset, NO-mediated event that is reversed by reducing agents and a more delayed, PKA-sensitive process that is mediated through increases in cyclic GMP and a decrease in sGC protein levels.

Keywords: Sodium nitroprusside; tolerance; cyclic GMP; protein kinase A; soluble guanylate cyclase

Introduction

Nitrovasodilators are a class of nitric oxide (NO) generating compounds that exert their effects through activation of the soluble isoform of guanylate cyclase (sGC) that leads to increases in intracellular guanosine 3':5'-cyclic monophosphate (cyclic GMP) and smooth muscle relaxation (Katsuki *et al.*, 1977; Gruetter *et al.*, 1979; Ignarro & Kadowitz, 1985; Waldman & Murad, 1987). sGC is a heterodimer composed of a large (α) and a small (β) subunit. Three cDNA sequences for the α ($\alpha_1-\alpha_3$) and β ($\beta_1-\beta_3$) subunits have already been cloned and sequenced (Nakane *et al.*, 1988; 1990; Yuen *et al.*, 1990; Harteneck *et al.*, 1991; Giuili *et al.*, 1992). Rat smooth muscle cells have been shown to express the α_1, α_2 and β_1 subunits (Ujiie *et al.*, 1993). Although α and β subunits both appear to possess a catalytic domain, expression of enzymatic activity requires the presence of both a large and a small subunit (Buechler *et al.*, 1991). Interestingly, substitution of α_1 with α_2 in the α_1,β_1 complex yields a functional α_2,β_1 enzyme (Harteneck *et al.*, 1991).

Use of nitrovasodilators in both clinical and experimental settings results in the development of tolerance. Although tolerance to NO generating compounds has been extensively studied, the mechanisms by which it occurs remain unclear. It is generally accepted that tolerance to nitrovasodilators does not affect the pathway downstream of sGC activation, since responses to atrial natriuretic peptide and cell membrane permeable analogues of cyclic GMP are not impaired (Axelsson *et al.*, 1986; Papapetropoulos *et al.*, 1993). It is not clear, however, if tolerance to organic nitrates occurs at a site upstream of sGC or affects the enzyme itself. Several hypotheses exist attributing tolerance to impaired biotransformation of organic nitrates, depletion of intracellular thiols, desensitization of sGC to the stimulatory effect of NO or overproduction of superoxide anions (Needleman & Johnson, 1973; Axelsson *et al.*, 1986; Henry *et al.*, 1989a; Romanin & Kukovetz, 1989; Münzel *et al.*, 1995). While tolerance to GTN may be explained by impaired biotransformation to NO, tolerance to sodium nitroprusside (SNP), S-nitroso N-acetylpenicillamine (SNAP) or 3-morpholino-sydnonimine (SIN-1), all agents that generate NO non-enzymatically, cannot be accounted for by this mechanism. It should be noted that a phenomenon that can be thought as the reverse of tolerance has already been described by several investigators: endothelial denudation or inhibition of the endogenously produced endothelium-derived NO leads to the development of supersensitivity to the action of nitrovasodilators (Shirasaki & Su, 1985; Moncada *et al.*, 1991; Kodja *et al.*, 1994). It is thus possible that continuous exposure of smooth muscle *in vivo* to low amounts of NO produced by the endothelium renders the smooth muscle cells reversibly tolerant. This hypothesis is further supported by the finding that SNP-induced cyclic GMP accumulation in endothelium-smooth muscle co-cultures is lower than in single smooth muscle cell cultures (Papapetropoulos *et al.*, 1993).

Development of tolerance to the action of SNP has been documented in both vascular and non vascular cells in culture

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(Zhang *et al.*, 1993; Ujiie *et al.*, 1994). However, it is still unclear whether tolerance is mediated by NO itself or by the accompanying increase in cyclic GMP. Moreover it is still unknown whether tolerance results from desensitization of sGC to SNP or from reduction in gene expression of sGC. The aim of the present study was to explore some of the possible mechanisms by which tolerance to the action of SNP develops in culture.

Methods

Cell culture

Rat aortic smooth muscle cells were isolated from 325–350 g Wistar rats (Harlan, Sprague-Dawley, Inc. Indianapolis, IN, U.S.A.) by previously published procedures (Geisterfer *et al.*, 1988). Animal handling and euthanasia were in accordance with guidelines from the Institutional Committee on Animal Use for Research and Education. Cells were positively identified as smooth muscle cells by indirect immunofluorescent staining for α-actin, using a mouse anti-α-actin antibody and anti-mouse IgG FITC conjugate, and grown in T-75 tissue culture flasks (Corning Glass Inc., Corning, N.Y., U.S.A.) in 50% F12 and 50% Dulbecco's Modified Eagle Medium (GIBCO Laboratories, Grand Island, N.Y., U.S.A.) supplemented with 10% foetal bovine serum (Hyclone Laboratories Inc., Logan, Utah, U.S.A.), 0.2 g l^{-1} L-glutamine, penicillin (100 u ml^{-1}) and streptomycin (0.1 mg ml^{-1}; Sigma Chemical Co, St. Louis, MO, U.S.A.). For the present study, cells between passage 3–7 were used.

RIA for cyclic GMP

The radioligand ([^{125}I]-succinyl cyclic GMP - tyrosine methyl ester) was prepared in our laboratory by the method of Hunter & Greenwood (1962) using carrier-free ^{125}I (Du Pont, NEN, Boston, MA, U.S.A.). The iodination reaction products were separated by reverse-phase high-performance liquid chromatography (Patel & Linden, 1988). Using a monoclonal antibody for cyclic GMP, radioimmunoassay was performed in the Gamma-flo automated RIA system (Brooker *et al.*, 1976). Standard stock solutions of cyclic GMP (20 μM) were prepared in 0.1 N HCl, and the absorbance of the solution was routinely monitored spectrophotometrically (Shimadzu, UV 160U). Standard dilutions (0.63–80 nM) were made from the stock solution.

Determination of intracellular cyclic GMP levels

Rat aortic smooth muscle cells were pretreated with varying concentrations of a nitric oxide donor (SNP; Sigma or SNAP; RBI, Natick, MA, U.S.A.) or a cyclic GMP-elevating agent (zaprinast; Rhone-Poulenc Rorer, Dagenham UK or atriopeptin II; Sigma) for 0–24 h in normal growth medium in the presence of serum. SNP, SNAP and other agents were added once at the beginning of the treatment unless otherwise indicated. To determine the development of tolerance, cells were washed with Earles' balanced salt solution (ES) at the end of the pretreatment period; cyclic GMP accumulation was then determined after exposure to 10 μM SNP for 15 min in the presence of 3-isobutyl-1-methylxanthine (IBMX, 0.3 mM; Sigma), to prevent cyclic GMP breakdown. After the 15 min incubation with SNP, medium was rapidly aspirated, and 500 μl of 0.1 N HCl was added to each well to stop enzymatic reactions and to extract cyclic GMP. When it was desirable to compare the cyclic GMP-elevating ability of various pretreatment protocols (so that correlations between intracellular cyclic GMP levels and tolerance development could be made; Table 1), cells were exposed to SNP, zaprinast, IBMX or IBMX + atriopeptin (ANF) for the indicated time, washed with ES and extracted in HCl. The HCl extract cyclic GMP was collected after 30 min and cell remnant was removed from the wells by adding boiling 1.0 N NaOH and scraping the well with a rubber policeman. The HCl extracts were analysed for cyclic GMP by radioimmunoassay and NaOH-solubilized samples were used for protein determination. When H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline sulphonamide, LC Laboratories, Woburn, MA, U.S.A.) was used to block cyclic AMP-dependent protein kinase (PKA; Chijiwa *et al.*, 1990), cells were pretreated for 1 h with 30 μM H89 before the 12 h incubation with zaprinast or SNP. To deplete soluble thiols or soluble as well as protein bound thiols, cells were incubated with 0.1–1 mM maleic acid diethylester (DEM) or 10–100 μM N-ethylmaleimide (NEM) for 30 min in ES before being exposed to SNP (100 μM, 2 h). To prevent tolerance to SNP, cells were simultaneously exposed to SNP and 30 μM oxyhaemoglobin or reducing agents (N-acetylcysteine, cysteine, dithiothreitol or ascorbic acid). Cyclic GMP accumulation in response to 10 μM SNP was then determined in the presence of IBMX as described above.

Protein determination

Protein content of the supernatant of the centrifuged (500 *g* for 5 min at room temperature), NaOH solubilized samples was measured by the Bradford (1976) method. Sample aliquots were combined with the protein binding dye (Bio-Rad, Richmond, CA, U.S.A.) and optical density was determined at 630 nm using a multiwell plate reader (Dynetech Laboratories Inc.). Bovine albumin, fraction V (Sigma) was used as standard.

Determination of protein bound and non protein bound sulphydryls

Sulphydryl concentration was determined by the Ellmann method (Sedlak & Lindsay, 1968). Briefly, vehicle or SNP (100 μM, 24 h) pretreated cells were trypsinized, pelleted and extracted with 250 μl 6% trichloroacetic acid (TCA). TCA insoluble material was sedimented by microcentrifugation and solubilized by sonication in 0.5% sodium dodecyl sulphate (SDS) containing 0.2 M EDTA. Separate aliquots of TCA and SDS-EDTA supernatants were combined with tris(hydroxymethyl)aminomethane (Tris)-EDTA buffer and 5,5′dithio(2-nitrobenzoic acid) and optical density was determined at 410 nm. Reduced glutathione, in TCA or SDS-EDTA, was used to construct standard curves for nonprotein and protein thiols, respectively.

Northern and Western blot analyses

Total RNA was isolated from smooth muscle cells with a commercially available kit (RNAzol, Biotecx Laboratories Inc, Houston, TX, U.S.A.), quantified by absorbance at 260 nm and stored at −70°C in a mercaptoethanol/ethanol/ammonium acetate solution. To generate the probe for the northern blot hybridization, a set of primers for the β_1 sGC subunit (forward, base position 1450 5′GGTTTGCCAGAACCTTGTATCCACC3′ and reverse, base position 1733 5′GAGTTTTCTGGGGACATGAGACACC3′; Nakane *et al.*, 1988) was used to amplify a 284 bp fragment in a reverse transcription-polymerase chain reaction as previously described (Papapetropoulos *et al.*, 1995). The probe was then labelled with [α-^{33}P]dCTP (NEN Research Products, Boston, MA, U.S.A.) using a random primer labelling kit (Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.) to a specific activity of 10^8 cpm μg^{-1} and used in Northern blot analysis (Papapetropoulos *et al.*, 1994). For the Western blot analysis after the pretreatment, cells were lysed in lysis buffer (1%NP40, 150 mM NaCl, 20 mM HEPES pH 7.0, 1 mM EDTA, 10 μg ml^{-1} aprotinin and 1 mM phenylmethyl sulphonylfluoride (PMSF); all reagents from Sigma). Cell lysates were centrifuged at 7,500 *g*, the supernatant fraction was collected and protein concentration measured by the Bradford method: 50 μg protein per lane were electrophoresed in SDS-7.5% polyacrylamide gels and transferred to a PVDF (Bio-Rad) membrane at 60 V for 1.5 h at 4°C in a buffer containing

25 mM Tris and 700 mM glycine. Membranes were incubated overnight at 4°C with 5% dry milk (Bio-Rad) in buffer containing 0.1% (v/v) Tween 20 (Bio-Rad) in Tris buffered saline (TTBS) to block nonspecific binding. The following day membranes were incubated with 1:750 of a monoclonal antibody (H_6) against the α_1 subunit of sGC (Lewicki *et al.*, 1983) in 5% milk in TTBS for 1 h at room temperature, washed 3 times with TTBS for 20 min each time, blocked for an additional hour with 5% milk in TTBS and finally incubated for 1 h with a horseradish peroxidase conjugated anti-mouse IgG (1:10,000 dilution, Bio-Rad). Immunoreactive protein bands were visualized using the ECL system (Amersham), after 15 min exposure to X-ray film. To check for consistency in loading and transfer, membranes were subsequently incubated with a monoclonal antibody against smooth muscle α-actin and immunoreactive bands visualized after exposure to X-ray film for 30 s.

Data analysis

Data are presented as means ± s.e.mean of the indicated number of individual observations. Data are expressed either as pmol cyclic GMP mg^{-1} protein 15 min^{-1} or as a percentage of the control value. Statistical comparisons between groups were performed by one way ANOVA or Student's *t* test, as appropriate. Differences among means were considered significant when $P<0.05$.

Results

Baseline cyclic GMP levels of untreated cells ranged between 2–8 pmol mg^{-1} protein 15 min^{-1}. Exposure of cells to 10 μM SNP for 15 min in the presence of phosphodiesterase (PDE) inhibition (0.3 mM IBMX) led to at least a 50 fold increase in intracellular cyclic GMP levels. Pretreatment of rat aortic smooth muscle cells with 10 μM SNP for 12 h led to the development of tolerance to subsequent stimulation of sGC by SNP as indicated by the lower SNP-induced cyclic GMP accumulation in these cells (24 ± 1% of vehicle pretreated cells). When cells were pretreated with 10 μM SNAP, cross-tolerance developed as cyclic GMP accumulation in response to SNP was 39 ± 3% of control. On the other hand, pretreatment of the cells with 30 μM of the stable degradation product of NO, nitrite ($NaNO_2$) did not alter sGC activity (89 ± 4% of control). Oxyhaemoglobin (30 μM) added during the pretreatment period prevented the development of tolerance (97 ± 15 and 107 ± 10% of control for SNP and SNAP, respectively).

Cells exposed to 10 μM SNP for 12 h showed a similar decrease in SNP-induced cyclic GMP accumulation upon subsequent stimulation of sGC, irrespective of the level of PDE inhibition. When no IBMX was used to inhibit the PDE during the 15 min incubation prior to HCl extraction of cyclic GMP, intracellular levels of cyclic GMP were 23 ± 3% of control; when 0.3 or 1 mM IBMX was used to inhibit PDE, SNP-induced cyclic GMP accumulation was 24 ± 1% and 23 ± 5% of control, respectively, for the SNP pretreated cells. In all subsequent experiments 0.3 mM IBMX was used to inhibit PDE and potentiate the SNP responses.

Pretreatment of cells with 20 μM SNP for as little as 2 h lowered the cyclic GMP accumulation upon subsequent exposure to SNP from 805 ± 61 for the control to 490 ± 24 pmol mg^{-1} protein 15 min^{-1}; a plateau was reached after 4 h of pretreatment (Figure 1a).Development of tolerance was also concentration-dependent, reaching a maximum at 50–100 μM SNP, following 12 h of pretreatment (Figure 1b). The lowest concentration of a single dose of SNP that reproducibly resulted in the development of tolerance was 10 μM. However, when cells were exposed to 75 nM SNP every 90 min for 12 h (total SNP added 0.6 μM) tolerance developed. Tolerance in this case was of comparable magnitude to that developed after a single 10 μM dose of SNP (data not shown).

To investigate whether the action of SNP is mediated by NO itself or the concomitant rise in cyclic GMP levels, cells were pretreated with the cyclic GMP-specific PDE inhibitor, zaprinast (Bergstrand *et al.*, 1977). Exposure of cells to zaprinast induced a time- and concentration-dependent decrease in SNP-induced cyclic GMP accumulation that was 17 ± 4% of control after 12 h pretreatment with 1 mM zaprinast (Figure 2). However, a marked difference in the time course of the effects of zaprinast and SNP was noted, since downregulation of cyclic GMP accumulation by zaprinast required at least 12 h. To investigate whether the difference between the time courses of SNP and zaprinast is due to different mechanisms of action, intracellular cyclic GMP levels were monitored following exposure of cells to SNP or zaprinast (Table 1). Exposure of cells to 10 μM SNP (in the absence of IBMX) increased cyclic GMP levels for up to 1.5 h. Acute exposure to higher SNP concentrations (up to 500 μM) increased cyclic GMP levels further. On the other hand, a 9 h exposure to zaprinast (500 μM) was required to increase cyclic GMP levels significantly, although a tendency to increase cyclic GMP was evident at 3–6 h. To mimic the rapid elevation in cyclic GMP brought about by SNP, ANF was used as a cyclic GMP-elevating agent that does not release NO (Table 1). Since 0.1 μM ANF consistently failed to elevate cyclic GMP levels to values similar to those achieved with 100 μM SNP, IBMX was used to inhibit PDE and potentiate the cyclic GMP-elevating effect of ANF. Cyclic GMP levels in the ANF + IBMX group increased and remained elevated throughout the 2 h pretreatment period. SNP-induced cyclic GMP levels (Table 1) decreased in SNP, but not ANF(+IBMX) pretreated cells suggesting that the action of SNP, at least at early time points, is mediated directly by NO.

The effect of zaprinast was completely abolished by the PKA-specific inhibitor, H89, whereas that of SNP was partially reduced (Figure 3). To determine if the development of tolerance to SNP is due to decreases in the amount of sGC mRNA and/or protein, northern and western blot analyses were performed for the β_1 and α_1 sGC subunits, respectively.

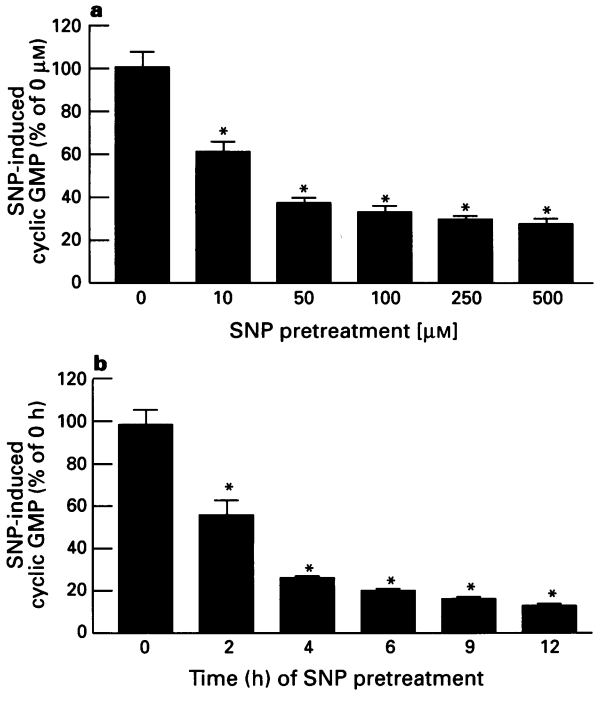

Figure 1 Time and concentration-dependence of tolerance development to SNP. Smooth muscle cells were incubated with 20 μM SNP (b) for the indicated time or with 10–500 μM SNP (a) for 12 h and then stimulated with 10 μM SNP for 15 min. Means ± s.e.mean, $n=4$ wells $^*P<0.05$ from control (0 h or 0 μM).

No change in the amount of mRNA was observed in SNAP or 8Br-cyclic GMP pretreated cells (Figure 4). Western blot analyses revealed unchanged α_1 sGC levels in cells pretreated with 100 μM SNP for 2 or 12 h, 10 μM SNAP or 1 mM 8Br-cyclic GMP for 12 h (Figure 5a). SNP-induced cyclic GMP accumulation in parallel processed cultures was 381 ± 15 pmol mg^{-1} protein 15 min^{-1} for control and 179 ± 13, 117 ± 3 and 264 ± 20 for 2 h SNP, 12 h SNP and SNAP, respectively. However, α_1 sGC protein levels were found to be decreased in cells pretreated with 300 μM zaprinast for 24 h (50 ± 2% of control cyclic GMP accumulation in parallel treated cultures; Figure 5b) suggesting that the zaprinast-induced downregulation of cyclic GMP accumulation was due to decreased amounts of sGC protein.

Development of tolerance to SNP was a rapidly reversible phenomenon, as sGC activity returned to control levels within 2 h after washing out the SNP (Figure 6). When cells were treated with 10 μM SNP for 12 h, responses to SNP stimulation were 71.2 ± 1.2% of control 4 h after the SNP was washed out and returned to control levels within 12 h. Since thiols are known to be involved in the regulation of sGC activity, we tested the hypothesis that thiol depletion may prevent the de-

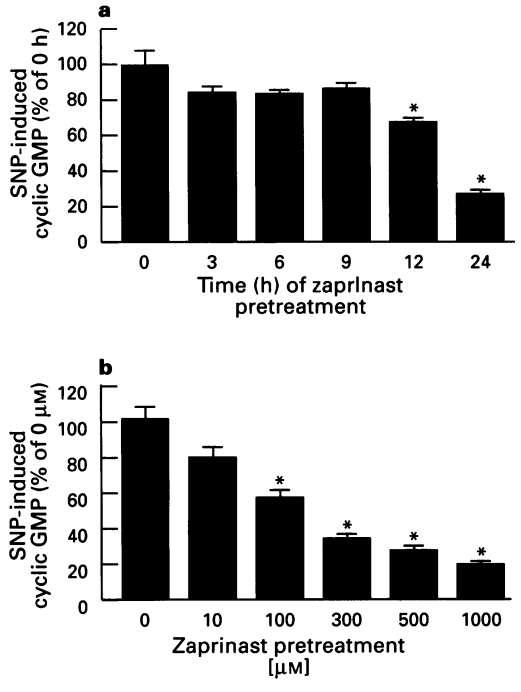

Figure 2 Time and concentration-dependence of zaprinast-induced downregulation of cyclic GMP accumulation. Smooth muscle cells were pretreated with 300 μM zaprinast (a) for the indicated time or with 10–1000 μM zaprinast (b) for 12 h. After the pretreatment period agents were washed out and cells were stimulated with 10 μM SNP. Means ± s.e.mean, $n=4$ wells $^*P<0.05$ from control (0 h or 0 μM).

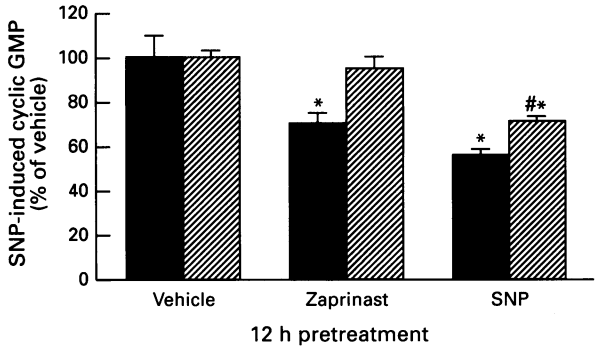

Figure 3 The protein kinase A-selective inhibitor, H89, completely prevents the zaprinast-, but not the SNP-induced downregulation in sGC activity. Smooth muscle cells were pretreated with vehicle, 300 μM zaprinast or 10 μM SNP for 12 h. When H89 was used to inhibit protein kinase A, cells were preincubated with 30 μM H89 for 60 min before the addition of zaprinast or SNP. Means ± s.e.mean; $n=4$ wells. Solid columns, without H89, hatched columns, +H89, $^*P<0.05$ from respective vehicle; $\#P<0.05$ from without H89.

Table 1 Correlation of cyclic GMP levels with the development of tolerance

Cyclic GMP (pmol mg^{-1} protein 15 min^{-1})	*Time* 0	15 min	20 min	1.5 h	2 h	3 h	6 h	9 h	12 h
Experiment 1									
SNP, 0 μM	7.9 ± 0.5								
10 μM			12.8 ± 0.8*	11.9 ± 1.5*		8.7 ± 0.6	9.8 ± 0.8	9.5 ± 0.5	8.2 ± 0.1
50 μM		45.1 ± 2.1*							
100 μM		99.7 ± 6.5*							
250 μM		241.8 ± 17.6*							
500 μM		297.5 ± 13.6*							
Zaprinast (500 μM)	8.0 ± 0.6		8.0 ± 0.2	10.0 ± 1.0		11.6 ± 1.0	12.3 ± 0.6	20.8 ± 2.1*	8.5 ± 0.7
Experiment 2									
Vehicle		5.0 ± 0.2			4.3 ± 0.3				
SNP (100 μM)		16.0 ± 1.5*			8.0 ± 0.5*				
IBMX (300 μM)		5.4 ± 0.4			3.4 ± 0.2				
+ANF (0.1 μM)		18.8 ± 1.9*			16.6 ± 0.7*				
Tolerance (% of control)									
SNP (100 μM)					47.5 ± 4.6*				
IBMX (300 μM)					84.7 ± 5.9				
+ANF (0.1 μM)					78.4 ± 5.8				
Zaprinast (500 μM)									65.9 ± 1.9*

Cyclic GMP-elevating agents were added at time 0 and cyclic GMP levels or the development of tolerance determined at the indicated time. In experiments 1 and 2, cyclic GMP levels were determined in the absence of any additional phosphodiesterase inhibition. To determine the development of tolerance, 10 μM SNP-induced cyclic GMP levels were estimated in the presence of 0.3 mM IBMX (see Methods). IBMX: isobutylmethylxanthine; SNP: sodium nitroprusside; ANF: atriopeptin; Data are mean ± s.e.mean; $n=4$ wells; $^*P<0.05$ from vehicle or control.

velopment of tolerance to SNP. Pretreatment of cells with NEM for 30 min led to a complete loss (0.1 mM NEM) or a marked reduction (10 μM NEM) of sGC activity (189±19 and 46±3 pmol mg^{-1} protein 15 min^{-1} for control and 10 μM NEM, respectively) when assayed 2 h later (Figure 7). However, when cells were exposed to 100 μM SNP for 2 h following the NEM exposure tolerance still developed to a similar degree (30±1 and 13±17% of control for vehicle and 10 μM NEM pretreated cells, respectively). Similar results were obtained with DEM, a soluble thiol depleting agent. To determine whether pretreatment of cells with SNP results in decreased soluble and/or protein bound thiols, cells were pretreated for 24 h with vehicle or 10 μM SNP. No evidence for thiol depletion was found since soluble thiols were 18±4 and 13±1 nmol mg^{-1} protein for vehicle and SNP pretreated cells, respectively and protein bound thiols were 48±12 and 38±2 nmol mg^{-1} protein for vehicle and SNP pretreated cells, respectively. Parallel processed cultures, pretreated with SNP, showed a 40% lower cyclic GMP accumulation as compared to control. On the other hand, addition of reducing agents in the medium bathing the cells during the SNP pretreatment partially (10 mM N-acetylcysteine, 10 mM cysteine, 2 mM dithiothreitol, 0.5 mM L-ascorbic acid) or completely (2 mM L-ascorbic acid) reversed the development of tolerance to SNP (Figure 8).

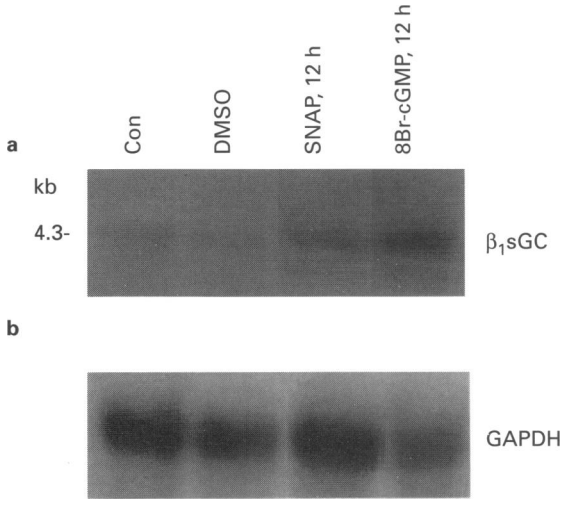

Figure 4 Steady state β_1 sGC mRNA levels do not change after pretreatment with SNAP or 8Br-cyclic GMP, as indicated by northern blot analysis. (a) Total RNA (10 μg) was electrophoresed, transferred to a nylon membrane and hybridized with the 284 bp β_1 RT-PCR product as a probe. (b) Blots were stripped and reprobed with a GAPDH probe to check for consistency in loading and transfer. Lambda phage DNA digested with *Hind*III was used as a size marker.

Discussion

To investigate the mechanism of tolerance to NO in intact cells, rat cultured aortic smooth muscle cells were pretreated with SNP or SNAP. These agents were chosen as inducers of tolerance over the organic nitrates, in order to avoid the possibility that impaired biotransformation may account for the development of tolerance. Exposure of rat cultured aortic smooth muscle cells to SNP or SNAP led to the development of tolerance, as indicated by the diminished cyclic GMP accumulation upon subsequent stimulation of sGC with SNP. This observation is consistent with previously published reports that exposure of crude or purified sGC to NO leads to the development of tolerance to the action of NO (Braughler,

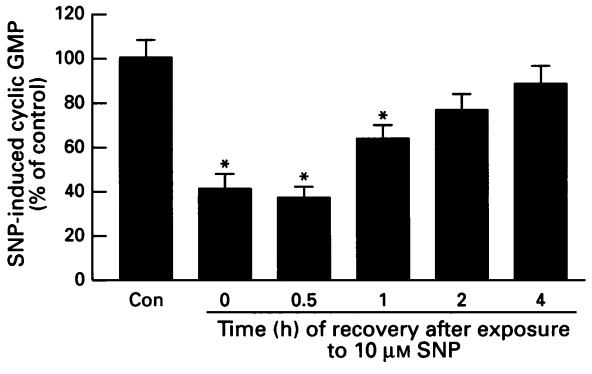

Figure 6 Development of tolerance to SNP is rapidly reversible. Cells were incubated with SNP (100 μM) for 4 h, washed extensively, allowed to recover for the indicated time and stimulated with 10 μM SNP. Means±s.e.mean, $n=4$ wells $^*P<0.05$ from control.

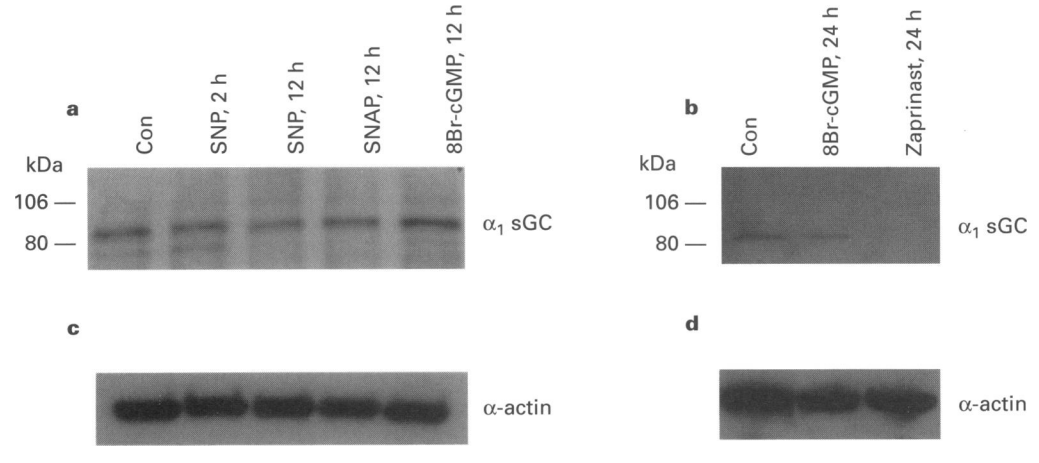

Figure 5 Protein levels of α_1 sGC are not altered following pretreatment with NO donors or 8Br-cyclic GMP but are reduced after 24 h exposure to zaprinast; 50 μg protein from each group was subjected to SDS-PAGE and transferred to a PVDF membrane. Membranes were incubated with 1:750 dilution of a monoclonal antibody (H_6) for the α_1 subunit (82 kDa). Prestained protein standards were used as molecular size markers. (b) Membranes were incubated with a monoclonal antibody against smooth muscle α-actin to check for consistency in loading and transfer.

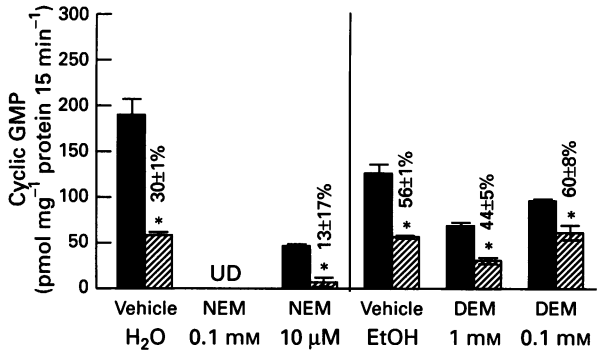

Figure 7 Thiol depletion does not prevent the development of tolerance to SNP. Rat aortic smooth muscle cells were incubated with vehicle, NEM or DEM for 30 min; agents were then washed out and cells incubated for 2 h in the absence (solid columns) or presence (hatched columns) of 100 μM SNP. After the pretreatment, cells were stimulated with 10 μM SNP. Numbers represent the % of (−)SNP-induced cyclic GMP levels. UD: undetectable. Means ± s.e.mean, $n=4$ wells $^*P<0.05$ from −SNP.

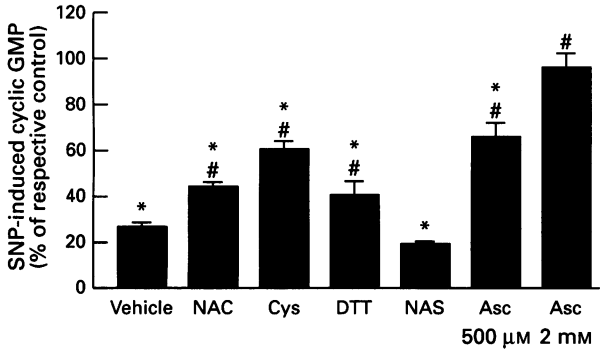

Figure 8 Reducing agents prevent the development of tolerance to SNP. Cells were exposed to 10 μM for 2 h in the absence or presence of N-acetylcysteine (NAC; 10 mM), cysteine (Cys; 10 mM), dithiothreitol (DTT; 2 mM), N-acetylserine (NAS; 10 mM) or L-ascorbic acid (Asc; 500 μM or 2 mM). At the end of the 2 h period, agents were washed out and sGC activity was determined from stimulation of cyclic GMP formation with 10 μM SNP. Means ± s.e.mean, $n=4$ wells $^*P<0.05$ from control (not shown), $\#P<0.05$ from vehicle.

1983) and that exposure of bovine coronary arterial rings, porcine vena cordis magna, rat aorta or cultured vascular smooth muscle cells to NO or NO-donors (SNP, SNAP or SIN-1) leads to impaired relaxation and cyclic GMP accumulation (Henry *et al.*, 1989a,b; Zhang *et al.*, 1993; Kojda *et al.*, 1994). The effects of both SNP and SNAP were mediated by the release of NO since the presence of oxyhaemoglobin during the pretreatment period reversed the effects of SNP and SNAP. Development of tolerance to SNP was concentration-dependent and was established as early as 2 h after exposure to 10 μM SNP, reaching a plateau at 4–6 h. SNP-induced cyclic GMP levels were found to be suppressed in SNP pretreated cells as compared to control, irrespective of the level of PDE inhibition. This is in agreement with previous observations that tolerance to GTN affects PDE activity only minimally, if at all (Axelsson *et al.*, 1986).

In the present experiments 10–100 μM SNP was used to produce tolerance. These concentrations are well within the range used in the literature to produce tolerance in cultured cells (Zhang *et al.*, 1993; Ujiie *et al.*, 1994). SNP, when used in a clinical setting, is infused at a rate of 0.5–1.5 $\mu g\ kg^{-1}min^{-1}$ which would lead to a concentration of 80 nM in the blood (calculations based on a 70 kg person, estimating blood volume at 5 l and not taking into consideration the rate of SNP decomposition over 1 min). The lowest concentration of SNP that reproducibly caused tolerance when added once to rat cultured aortic smooth muscle cells was 10 μM; however, 75 nM SNP was added every 90 min for 12 h, a similar degree of tolerance to the single 10 μM SNP dose developed. Nevertheless, it is difficult to extrapolate the relevance of drug doses from *in vitro* to *in vivo* and between different species. The proposed pathways for the development of tolerance cannot be assumed to offer a universal explanation for tolerance to nitrovasodilators and may only be applicable to *in vitro* situations.

To investigate whether the effects of SNP were due to NO itself or to the concomitant rise in cyclic GMP, cells were pretreated with the cyclic GMP-specific PDE (class V) inhibitor, zaprinast. Zaprinast pretreatment led to a concentration- and time-dependent downregulation in SNP-induced cyclic GMP accumulation. However, the time course of the action of zaprinast was markedly different from that of SNP, as the effects of zaprinast were first evident after pretreatment for at least 12 h. Incubation of cells with 500 μM zaprinast significantly elevated cyclic GMP levels after 9 h, probably due to low basal sGC activity. On the other hand, SNP increased cyclic GMP only during the first 1.5 h after its addition. To ensure that the delayed action of zaprinast, as compared to SNP, is not due to different time course of cyclic GMP elevation, cells were incubated with the particulate GC (pGC) activator ANF in the presence of IBMX (combination of the two drugs was necessary to raise intracellular cyclic GMP to levels comparable to those achieved with SNP). Pretreatment of cells with ANF (plus IBMX) for 2 h failed to reproduce the effects of SNP, suggesting that the action of SNP, at least at early time points, is not due to increased intracellular cyclic GMP levels. It should be noted that exposure of cells to cyclic AMP-elevating agents, such as IBMX and forskolin, reduces sGC activity, mRNA and protein levels, through activation of PKA (Papapetropoulos *et al.*, 1995). However, a 6 h pretreatment with IBMX is required for the downregulation of SNP-induced cyclic GMP accumulation. Further evidence for the existence of a different mechanism between NO and cyclic GMP in the downregulation of SNP-induced cyclic GMP accumulation was provided by using the PKA-selective inhibitor, H89 (Chijiwa *et al.*, 1990). Inhibition of PKA fully prevented the effects of zaprinast, whereas it only partially reversed the action of SNP.

NO has been shown to alter gene expression in endothelial as well as non vascular cells (Magrinat *et al.*, 1992; Kourembanas *et al.*, 1993). To investigate if the development of tolerance to NO results from or is accompanied by a decrease in sGC subunit mRNA and/or protein levels, northern and western blot analyses were performed. Pretreatment of cells with 10 μM SNAP or the membrane permeable analogue of cyclic GMP, 8Br-cyclic GMP (1 mM) for 12 h did not change steady state mRNA levels for the β_1 sGC subunit. Similarly, exposure of cells to 100 μM SNP for 2 or 12 h, 10 μM SNAP or 1 mM 8Br-cyclic GMP for 12 h did not alter sGC α_1 protein levels. However, pretreatment of the cells with zaprinast led to decreased α_1 sGC protein levels, which provides further evidence for a different mechanism of action between NO and cyclic GMP in decreasing sGC activity. Figure 9 presents our current hypothesis. Tolerance to SNP can be attributed to at least two distinct mechanisms. The major one is rapid, PKA-insensitive and is directly mediated through NO, while the other one is mediated through a rise in cyclic GMP, is slower in onset and can be prevented by PKA-inhibition. Komas & collaborators (1991) have reported the presence of four PDE isozymes in the endothelium-denuded rat aorta: a calmodulin-activated (class I), a cyclic GMP-specific (class V), a cyclic AMP-specific (class IV) and a cyclic GMP-inhibitible cyclic AMP (class III) PDE. Increases in intracellular cyclic GMP, such as those followed by zaprinast pretreatment, would inhibit the class III PDE and increase cyclic AMP, which in turn would lead to decreased sGC activity through reduction in mRNA and/or protein levels (Shimouchi *et al.*, 1993; Papapetropoulos *et al.*, 1995). The fact that 8Br-cyclic GMP is

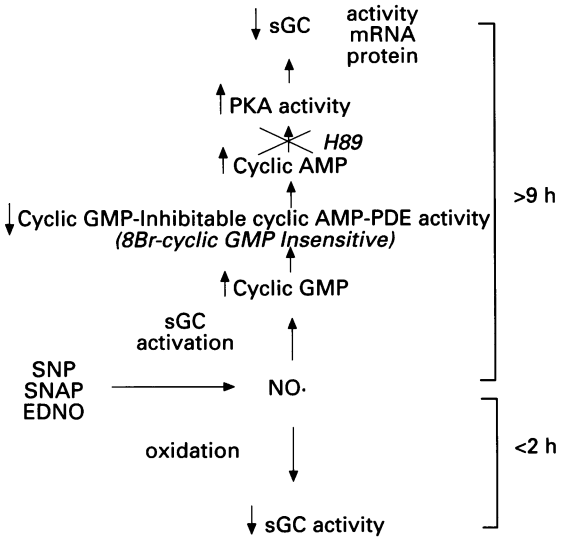

Figure 9 Schematic representation of the proposed mechanisms of tolerance to NO in intact cells.

unable to reproduce the effects of zaprinast on sGC is in line with observations that cyclic GMP-modulated PDEs are insensitive to 8-BR-cyclic GMP (Lincoln & Cornwell, 1993). In addition, Shimouchi *et al.* (1993) reported that 8Br-cyclic GMP did not decrease steady state mRNA of the β_1 sGC subunit in rat cultured foetal lung fibroblasts. The results obtained from experiments with 8Br-cyclic GMP provide evidence that the action of cyclic GMP is not mediated by a cyclic GMP-dependent protein kinase (cG-PK); cG-PK are readily activated by 8Br-cyclic GMP and their expression is reduced in passaged cells (Lincoln & Cornwell, 1993). Thus, tolerance to SNP at early time points is NO-mediated, while tolerance of cells after prolonged exposure to SNP is partly NO and partly cyclic GMP mediated. In this case mRNA and/or protein levels of SNP- and SNAP-pretreated cells would be expected to be reduced. The fact that northern and western blot analyses failed to demonstrate reduction in sGC subunit levels in SNAP-induced tolerance, suggests either a relative insensitivity of these methods, and/or the possibility that the action of SNP at the concentrations used here is mainly mediated through NO itself; this is further supported by the modest reversal of SNP action by H89.

Recently, Ujiie and collaborators (1994) reported that exposure of rat medullary interstitial cells to 300 μM SNP for 18 h resulted in decreased expression of both the α_1 and β_1 sGC subunits. Regulation of sGC gene expression by SNP may become increasingly important at higher SNP concentrations (that lead to higher cyclic GMP levels which in turn would further increase cyclic AMP levels), more prolonged incubation times or in cell types that the class III PDE is the major cyclic AMP degrading enzyme.

Development of tolerance to SNP was rapidly reversible. When cells were incubated with 10 μM SNP for 4 h, sGC activity returned to control levels as early as 2 h after the removal of SNP; longer recovery times were observed in cells incubated with SNP for 12 h. sGC activity is known to be redox regulated since both oxidizing and reducing agents can enhance or suppress enzyme activation depending on their concentration (Waldman & Murad, 1987; Wu *et al.*, 1992). Although the mechanism by which thiols are implicated in the expression of sGC activity remains unclear, it has been shown that NO activation of sCG leads to mixed disulphide formation that reversibly inactivates the enzyme (Brandwein *et al.*, 1981; Kamisaki *et al.*, 1986). To investigate further the rapid, PKA-insensitive component of tolerance to SNP, cells were exposed to thiol depleting agents and pretreated with SNP. Exposure of cells to NEM, an agent that depletes both soluble and protein bound thiols failed to prevent the development of tolerance to SNP, as cyclic GMP accumulation in response to subsequent SNP stimulation was reduced to a similar degree in both vehicle and NEM pretreated cells. Similar results were obtained when DEM was used to deplete soluble thiols. It should be noted that exposure of cells to NEM resulted in decreased sGC activity. This is in agreement with previous reports that massive oxidation of sGC results in loss of enzyme activity, (Waldman & Murad, 1987). Kojda *et al.* (1993) reported a reduction in free thiols and disulphides in porcine coronary artery rings following a 30 min exposure to 100 μM of various nitrovasodilators. In the present experiments, no change in soluble or protein bound thiols was observed in cells pretreated with 10 μM SNP, suggesting that tolerance to SNP does not result from thiol depletion. The reason for this difference may be due to the lower SNP concentration used in the present studies or to species differences. On the other hand, supplementation with N-acetylcysteine, dithiothreitol and cysteine, but not N-acetyl serine (which lacks an SH group), partially prevented the development of tolerance to SNP. Ascorbic acid, a non-thiol reducing agent, was able to reverse the development of tolerance to SNP in a concentration-dependent manner; complete reversal was observed at 2 mM of ascorbic acid. The mechanism by which reducing agents prevent SNP tolerance may be related to either maintenance of the haeme iron in a reduced state, thereby, facilitating the reaction between NO and haeme iron to form NO-haeme, or by preventing oxidation of critical thiol groups involved in expression of enzymatic activity, reinforcing the notion that the redox state of sGC is an important factor regulating its activity. In a recent report by Münzel *et al.* (1995), tolerance to nitroglycerin in rabbits could be at least partly attributed to increased formation of superoxide anions. While no experiments were performed to investigate directly the possibility that tolerance to SNP is also accompanied by increased free radical production, the lack of reduction in cellular thiols suggests that the present experimental conditions do not constitute a major oxidative stress.

In conclusion, tolerance to NO releasing agents involves at least two distinct mechanisms; a rapid, PKA-insensitive mechanism mediated by NO itself and prevented by reducing agents; and another mechanism of slower onset, dependent on increased cyclic GMP levels, which is PKA-sensitive and mediated through decreased sGC protein levels. The contribution of each pathway to the development of tolerance will depend on the amounts of NO and cyclic GMP present. The PKA-sensitive pathway would be of greater importance in cells with low cyclic GMP-PDE and high sGC activities. On the other hand, the PKA-sensitive pathway will not contribute to the development of tolerance in cells that lack the class III PDE or in cells that this PDE isozyme does not play a major role in cyclic AMP breakdown. Should the present observations hold true *in vivo*, tolerance to nitrovasodilators may be prevented or at least ameliorated by intermittent administration of these compounds in conjunction with reducing agents.

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