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European Journal of Pharmacology 344 (1998) 313–321

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Cross-tolerance between endogenous nitric oxide and exogenous nitric oxide donors

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Received 30 October 1997; accepted 12 December 1997

Abstract

It is still unclear whether cross-tolerance develops between endogenously produced nitric oxide and exogenous nitric oxide donors. Thus, cGMP accumulation was determined in cultured aortic smooth muscle cells exposed to a nitric oxide source. Exposure of human, rat, rabbit, porcine or bovine smooth muscle cells to sodium nitroprusside led to a time- and concentration-dependent development of tolerance. In rat aortic smooth muscle cells, cross-tolerance developed between the sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine, but not between sodium nitroprusside and atriopeptin. In addition, when rat aortic smooth muscle cells were treated with endotoxin or interleukin-1β, they displayed lower sodium nitroprusside-induced cGMP accumulation as compared to control cells. When rat aortic smooth muscle cells were exposed to sodium nitroprusside for 12 h they displayed a decreased ability to accumulate cGMP in response to endothelium-derived nitric oxide released from bovine aortic endothelial cells. In addition, co-cultures of rat aortic smooth muscle cells with bovine aortic endothelial cells showed an L-nitroarginine methylester-sensitive decrease in sodium nitroprusside-induced cGMP accumulation compared to single rat aortic smooth muscle cell cultures. We conclude that cross-tolerance between endothelium-derived nitric oxide and exogenously applied nitric oxide donors occurs in vitro. © 1998 Elsevier Science B.V.

Keywords: Sodium nitroprusside; Tolerance; cGMP; Nitric oxide (NO)

1. Introduction

Nitric oxide is a short lived radical implicated in cardiovascular homeostasis, host defense mechanisms and neurotransmission (Moncada et al., 1991a) that is generated during the five electron oxidation of L-arginine by nitric oxide synthase (Palmer et al., 1988). So far, three isoforms of this enzyme have been discovered in a variety of cell types (Schmidt et al., 1993). Type I nitric oxide synthase (or neuronal nitric oxide synthase) is constitutively expressed in neurons and skeletal muscle and has a molecular mass of 155 kDa (Bredt et al., 1991; Forstermann et al., 1995). Endothelial cells, platelets and cardiomyocytes constitutively express a particulate isoform (type III or endothelial nitric oxide synthase) which is shorter (135 kDa) than the one expressed by neuronal cells (Sessa et al., 1992; Forstermann et al., 1995). On the other hand most nucleated cells, including smooth muscle cells, express an inducible isoform, (type II nitric oxide synthase or inducible nitric oxide synthase) when exposed to cytokines or bacterial lipopolysaccharide (LPS; Xie et al., 1992). Under physiological conditions, nitric oxide synthase activity in the vessel wall is restricted to the endothelial lining. Stimuli that increase the release of nitric oxide above basal levels include agonists (such as acetylcholine, bradykinin, substance P or calcium ionophores) and shear stress (Moncada et al., 1991a; Kuchan and Frangos, 1994). Nitric oxide plays a key role in vascular homeostasis by contributing to the regulation of vascular tone, the inhibition of smooth muscle proliferation and inhibition of platelet aggregation (Moncada et al., 1991a).

Nitric oxide donors are nitric oxide liberating compounds that act by activating the soluble isoform of guanylate cyclase leading to increased intracellular levels of cGMP and vasorelaxation (Katsuki et al., 1977; Ignarro and Kadowitz, 1985; Waldman and Murad, 1987). Nitric oxide donors can be divided into nitrates (such as nitroglycerin) that require enzymatic biotransformation to liberate nitric oxide and non-nitrates (such as sodium nitroprus-

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side and *S*-nitroso-*N*-acetylpenicillamine) that generate nitric oxide non-enzymatically (Harrison and Bates, 1993; Anderson et al., 1994). Both nitrate and non-nitrate nitric oxide donors are used in clinical practice for the treatment of cardiovascular diseases.

Endothelial dysfunction is a prominent feature in atherosclerosis diabetes and hypertension. Large conduit arteries as well as resistance arterioles of animals with atherosclerosis are unresponsive to endothelium-dependent vasodilators such as acetylcholine or substance P (Harrison et al., 1991). Similarly, endothelium-dependent responses are altered in animal models of diabetes (Bucala et al., 1991; Pieper and Gross, 1991). Generation of free radicals and endothelium-derived nitric oxide inactivation has been confirmed in acute hypertension (Kontos et al., 1981), while endothelium-dependent responses are also altered in many cases of chronic hypertension (Konishi and Su, 1983; Luscher et al., 1991). It should be noted that smooth muscle cell responsiveness to the relaxing effects of nitric oxide donors in many of the above mentioned cases remains unaltered or is even augmented (Luscher et al., 1991; Papapetropoulos et al., 1994). On the other hand, overproduction of nitric oxide through the inducible nitric oxide synthase has been demonstrated to lead to reduced responsiveness of rat aortas to nitric oxide donors (Tsuchida et al., 1994).

Supplementation of nitric oxide in the form of nitric oxide donors can, thus, be used to correct the nitric oxide deficiency encountered in many disease states. It is important to determine whether cross-tolerance between endogenously produced nitric oxide and nitric oxide donors exists. Previous studies investigating the endothelium-dependent responses in both nitrate and non nitrate nitric oxide donor tolerant animals or vessels have yielded conflicting results (Henry et al., 1989a; Mülsch et al., 1989; Berkenboom et al., 1990; Bult et al., 1991; Anderson et al., 1994; Murohara et al., 1996). To help clarify this question the present study was undertaken. We chose to investigate the development of cross-tolerance between endogenous nitric oxide and exogenous nitric oxide donors using agents that release nitric oxide non-enzymatically (Feelisch, 1991). The use of nitrovasodilators that generate nitric oxide non-enzymatically simplifies the interpretation of our findings since the observed cross-tolerance cannot result from impaired biotransformation, depletion of intracellular thiols or overproduction of superoxide anions, all of which have been implicated in the development of tolerance to nitrate vasodilators (Needleman and Johnson, 1973; Henry et al., 1989a; Münzel et al., 1995), but rather to decreased soluble guanylate cyclase activation. We employed sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine; they both rapidly generate nitric oxide in a spontaneous manner in the presence of light or at alkaline pH and in the presence of reducing agents. Tolerance and cross-tolerance is defined as the reduced ability of cells exposed to endogenous or exogenous nitric oxide to generate cGMP following subsequent challenge with endothelium-derived or nitrovasodilator-generated nitric oxide.

2. Materials and methods

2.1. Materials

Rats were purchased from Harlan/Sprague–Dawley, Indianapolis, IN. Tissue culture plastic ware was obtained from Corning Glass, Corning, NY, growth medium was obtained from GIBCO Laboratories, Grand Island, NY, and fetal calf serum from Hyclone Laboratories, Logan, UT. ^{125}I was purchased from Du Pont, NEN, Boston, MA. The protein binding dye was purchased from Bio-Rad, Richmond, CA. *S*-Nitroso-acetylpenicillamine was purchased from Research Biochemicals International, Natick, MA and interleukin-1β from Boehringer Mannheim, Indianapolis, IN. All other chemicals, including penicillin, streptomycin, bradykinin, rat atriopeptin II, bacterial lipopolysaccharide, 3-isobutyl-1-methylxanthine, sodium nitroprusside, bovine serum albumin were obtained from Sigma Chemical Co, St. Louis, MO.

2.2. Cell cultures

Rat aortic smooth muscle cells were isolated and cultured as previously described (Papapetropoulos et al., 1995). Rabbit and porcine smooth muscle cells were isolated using a protocol similar to that for rat smooth muscle. Human renal arterial and venous smooth muscle cells were isolated by the explant method from victims of sudden death within 6 h. Cells were positively identified as smooth muscle cells by indirect immunofluorescent staining for α-actin, using mouse anti-α-actin antibody and anti-mouse IgG FITC conjugate. Smooth muscle cells between passages 3–10 were used. Bovine aortic endothelial cells were harvested non-enzymatically from aortas obtained at the local abattoir, using previously published procedures (Ryan and Maxwell, 1986). Bovine aortic endothelial cells between passages 1–3 were used.

2.3. Determination of intracellular cGMP

2.3.1. Incubation protocols

Cells were pretreated with vehicle (dimethylsulfoxide or water), sodium nitroprusside, *S*-nitroso-*N*-acetylpenicillamine, bacterial lipopolysaccharide or interleukin-1β (for pretreatment details, see individual experiments). At the end of the pretreatment, cells were washed with Earle's balanced salt solution and incubated with Earle's balanced salt solution containing a nitric oxide donor for 15 min in the presence of 3-isobutyl-1-methylxanthine (0.3 mM) to prevent cGMP breakdown. When bacterial lipopolysaccharide or interleukin-1β were used for the pretreatment, cells were incubated for 30 min with 100 μM L-nitroarginine

methylester to inhibit cGMP accumulation, due to induction of inducible nitric oxide synthase, prior to sodium nitroprusside stimulation. To stimulate cGMP formation during the post-treatment period, 100 μM sodium nitroprusside were used for all experiments involving rabbit, pig, bovine and human cells. When rat cells were used, 10 μM sodium nitroprusside was used, since sodium nitroprusside-induced cGMP accumulation in rat cells reaches a plateau at this concentration. To determine if rat aortic smooth muscle cells exposed to sodium nitroprusside developed tolerance to endothelium-derived nitric oxide, short-term co-culture experiments were performed. For these experiments, rat aortic smooth muscle cells were grown in the presence or absence of sodium nitroprusside (10 μM, 12 h) in 24 multiwell plates and the bovine aortic endothelial cells were grown on glass coverslips. The short-term co-cultures were established by gently transferring, with the aid of fine forceps, the coverslips with the endothelial cells into wells containing untreated or sodium nitroprusside-pretreated rat aortic smooth muscle cells in Earle's balanced salt solution containing 3-isobutyl-1-methylxanthine. After 15 min, the supernatant solution was aspirated, the coverslips with bovine aortic endothelial cells removed from the wells, and intracellular cGMP content quantified as described below. To determine if rat aortic smooth muscle cells exposed to endothelium-derived nitric oxide become tolerant to sodium nitroprusside or *S*-nitroso-acetylpenicillamine, rat aortic smooth muscle cells were co-cultured for 24 h with bovine aortic endothelial cells (300 000 cells/cm^2). Co-cultures were pretreated for 30 min with 100 μM L-nitroarginine methylester to inhibit cGMP accumulation in response to basally released nitric oxide from the bovine aortic endothelial cells and were then stimulated with 10 μM sodium nitroprusside or *S*-nitroso-acetylpenicillamine.

2.3.2. cGMP radioimmunoassay

After the cGMP accumulation period (15 min), medium was rapidly aspirated and 500 μl of 0.1 M HCl was added to each well to stop enzymatic reactions and to extract cGMP. 30 min later, the HCl extract was collected and cell remnants removed from the wells by adding hot 1.0 M NaOH and scraping the well with a rubber policeman. The HCl extract was analyzed for cGMP by radioimmunoassay (Papapetropoulos et al., 1995) and NaOH-solubilized samples were used to determine protein concentration (Bradford, 1976).

2.4. Data analysis and statistics

Data are presented as means ± standard error of the mean of the indicated number of individual observations. cGMP values are expressed either as pmol/mg protein per 15 min or as percent of the control value. Statistical comparisons between groups were performed using the one way ANOVA. Differences among means were considered significant when $P < 0.05$.

3. Results

3.1. Development of tolerance and cross-tolerance to exogenously applied nitric oxide

Baseline vehicle-treated cGMP levels of untreated smooth muscle cells from different species were between 1–4 pmol/mg protein per 15 min. Pre-treatment of aortic smooth muscle cells from different species with 10 μM sodium nitroprusside for 12–48 h (sodium nitroprusside was replenished every 12 h) led to the development of tolerance to subsequent stimulation of soluble guanylate cyclase in the rat and rabbit cells, but not in the bovine and porcine, as indicated by the lower sodium nitroprusside-induced cGMP accumulation in these cells, compared to vehicle pretreated cells (Fig. 1A). Exposure of porcine

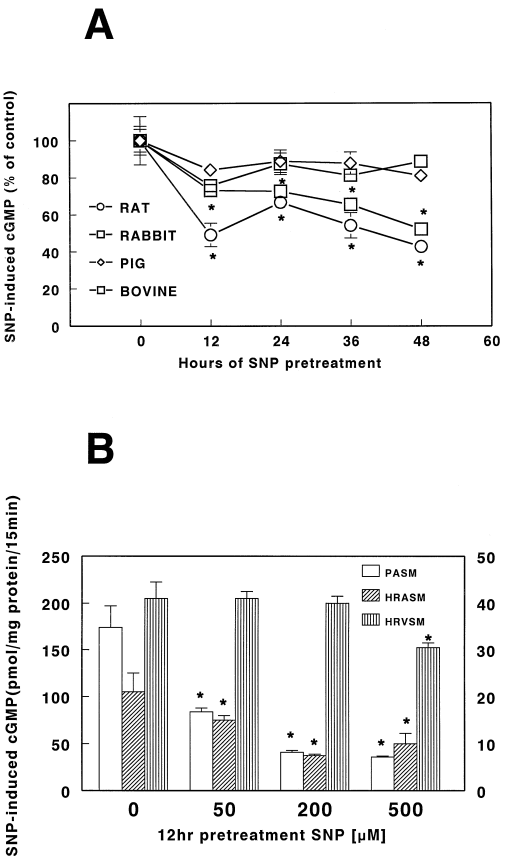

Fig. 1. Time and concentration dependence of tolerance development to sodium nitroprusside (SNP) in smooth muscle cells. (A) Aortic smooth muscle cells were incubated with sodium nitroprusside (10 μM) for the indicated time. Cells were then washed and stimulated with sodium nitroprusside to determine the development of tolerance. (B) Smooth muscle cells were incubated with the indicated concentration of sodium nitroprusside for 12 h before determining cGMP levels in response to acute sodium nitroprusside stimulation. Values for the porcine aortic smooth muscle cells (PASM) are depicted in the left axis whereas values for human renal vein smooth muscle cells (HRVSM) and human renal artery smooth muscle cells (HRASM) are in right axis. Means ± S.E.M, $n = 4$ wells. $^{*}P < 0.05$ from control (0 h of sodium nitroprusside pretreatment or 0 μM sodium nitroprusside).

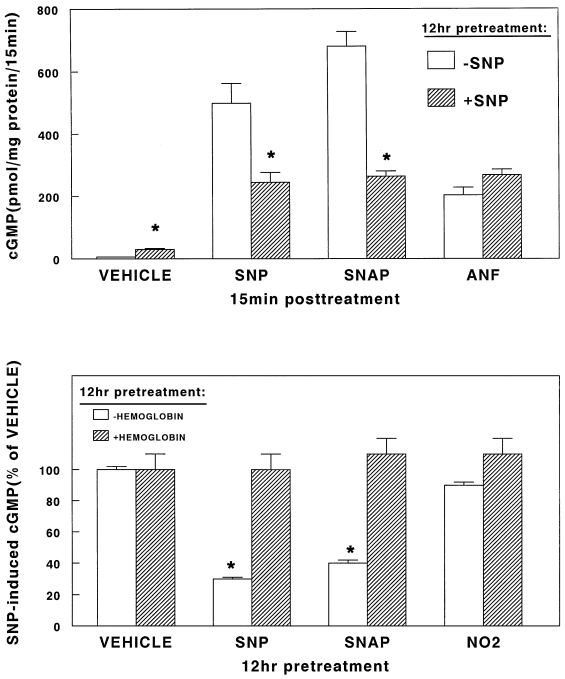

Fig. 2. Development of cross-tolerance between sodium nitroprusside (SNP) and *S*-nitroso-acetylpenicillamine (SNAP), but not atriopeptin II (ANF), in rat aortic smooth muscle cells (RASM). (A) Rat aortic smooth muscle cells were pretreated for 12 h with 10 μM sodium nitroprusside before being washed and stimulated with sodium nitroprusside (10 μM), *S*-nitroso-*N*-acetylpenicillamine (10 μM) or rat atriopeptin II (1 μM). Means $\pm$ S.E.M., $n = 4$ wells. $^{*}P < 0.05$ from -sodium nitroprusside. (B) Rat aortic smooth muscle cells were exposed to sodium nitroprusside (10 μM), *S*-nitroso-acetylpenicillamine (10 μM) or sodium nitrite (NO_2; 30 μM) in the presence or absence of oxyhemoglobin (30 μM) for 12 h. At the end of the pretreatment period, cGMP accumulation was determined during an acute exposure to 10 μM sodium nitroprusside, in the presence of 3-isobutyl-1-methylxanthine, to inhibit cGMP breakdown. Means $\pm$ S.E.M., $n = 4$ wells. $^{*}P < 0.05$ from vehicle.

cells to higher sodium nitroprusside concentrations for 12 h led to the development of tolerance (Fig. 1B). Exposure of human renal artery smooth muscle cells to sodium nitroprusside for 12 h also led to the development of tolerance, whereas renal vein smooth muscle cells were less sensitive to sodium nitroprusside as only a small degree of tolerance developed at 500 μM sodium nitroprusside. When rat aortic smooth muscle cells were pretreated with 10 μM sodium nitroprusside for up to 48 h, cGMP levels increased 2–5 fold over baseline. Acute exposure of cells to 10 μM sodium nitroprusside for 15 min in the presence of phosphodiesterase inhibition (0.3 mM 3-isobutyl-1-methylxanthine) led at least a 60-fold increase in intracellular cGMP levels in rat aortic smooth muscle cells, depending on the passage and confluence state of the cells. Pretreatment of rat aortic smooth muscle cells with 10 μM sodium nitroprusside for 12 h (Fig. 2A) led to the development of cross-tolerance to sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine, but not to atriopeptin II (ANF; activator of the particulate guanylate cyclase). Tolerance to sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine was inhibited by the nitric oxide scavenger oxyhemoglobin (30 μM). On the other hand, pretreatment of the cells with 30 μM of the stable degradation product of nitric oxide sodium nitrite did not alter cGMP accumulation in response to sodium nitroprusside (Fig. 2B).

3.2. Development of cross-tolerance between exogenous nitric oxide donors and endogenously produced endothelium-derived nitric oxide

Rat aortic smooth muscle cells were exposed to interleukin-1β or endotoxin to stimulate nitric oxide production within the smooth muscle cells and were then evaluated for the development of tolerance to the action of sodium nitroprusside. We have previously shown that under these conditions de novo synthesis of the inducible nitric oxide synthase leads to nitric oxide generation within the smooth muscle cell that is first evident after 4 h (Marczin et al., 1996; Papapetropoulos et al., 1996b). To evaluate sodium nitroprusside-induced cGMP accumulation in rat aortic smooth muscle cells exposed for 12 hr to interleukin-1β (0–100 U/ml) or bacterial lipopolysaccharide (0–1000 ng/ml), endogenous nitric oxide production was inhibited by treating the cells with 100 μM L-nitroarginine methylester for 30 min prior to stimulation with 10 μM sodium nitroprusside. Smooth muscle cells stimulated with bacterial lipopolysaccharide or interleukin-1β showed a 30–70% decrease in sodium nitroprusside-induced cGMP accumulation (Fig. 3).

In a different series of experiments exposure of cells to 10 μM sodium nitroprusside for 12 h led to the develop-

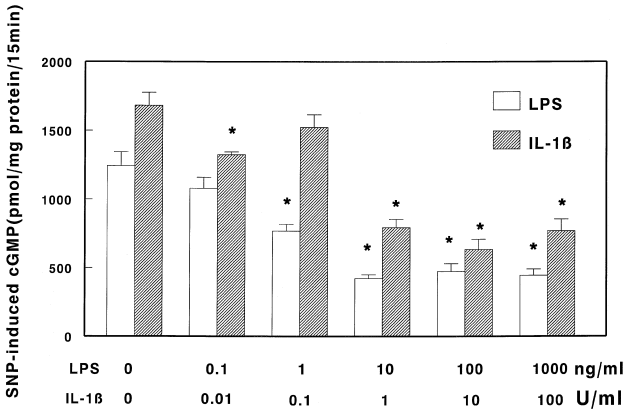

Fig. 3. Concentration-dependence of lipopolysaccharide (LPS)- and interleukin-1β(IL-1β)-induced downregulation of cGMP accumulation in response to sodium nitroprusside. Rat aortic smooth muscle cells (RASM) were pretreated with 0–1000 ng/ml bacterial lipopolysaccharide or 0–100 U/ml interleukin-1β for 12 h. Cells were treated with 100 μM L-nitroarginine methylester for 30 min to inhibit inducible nitric oxide synthase, prior to sodium nitroprusside stimulation. cGMP accumulation was determined during a 15 min exposure to 10 μM sodium nitroprusside. Means $\pm$ S.E.M., $n = 4$ wells. $^{*}P < 0.05$ from 0 ng or 0 U/ml.

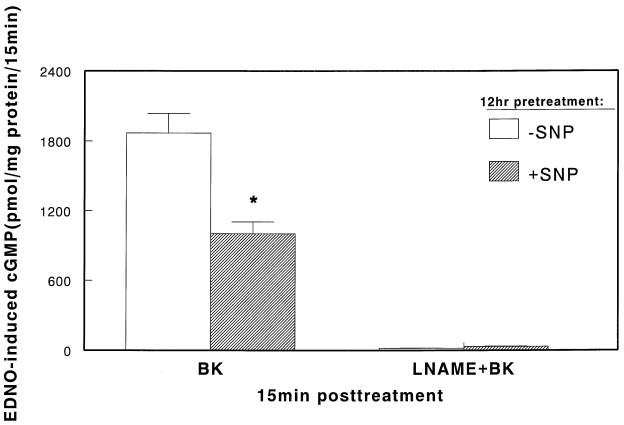

Fig. 4. Pretreatment of rat aortic smooth muscle cells (RASM) with sodium nitroprusside (SNP) downregulates the cGMP accumulation in response to endothelium-derived nitric oxide (EDNO). Rat aortic smooth muscle cells were pretreated with sodium nitroprusside (10 μM) for 12 h and subsequently evaluated for their responsiveness to bradykinin (10 μM)-induced endothelium-derived nitric oxide release from bovine aortic endothelial cells. Bovine aortic endothelial cells (BAE) were seeded on glass coverslips and transferred onto the smooth muscle cells for 15 min in the presence of 3-isobutyl-1-methylxanthine to allow for cGMP accumulation and then carefully removed. Means $\pm$ S.E.M., $n = 4$ wells. $^{*}P < 0.05$ from -sodium nitroprusside.

ment of cross-tolerance between sodium nitroprusside and the endogenously released nitric oxide from bovine aortic endothelial cells (Fig. 4). The decrease in endothelium-derived nitric oxide-induced cGMP accumulation in sodium nitroprusside-tolerant cells occurred for both basally released nitric oxide and for nitric oxide following agonist stimulation (36 ± 4 and 9 ± 2 pmol/mg protein/15 min for the basal $-$sodium nitroprusside and $+$sodium nitroprusside, respectively). To investigate whether prolonged exposure to endogenously produced endothelium-derived nitric oxide, at low physiological concentrations, leads to suppression of nitric oxide donor-induced cGMP accumulation and cross-tolerance, rat aortic smooth muscle cells were co-cultured with bovine aortic endothelial cells for 24 h (Fig. 5). After the 24 h, smooth muscle cells and co-cultures were incubated for 30 min with 100 μM L-nitroarginine methylester to inhibit cGMP accumulation in response to endothelium-derived nitric oxide from the bovine aortic endothelial cells and were then stimulated with 10 μM sodium nitroprusside or *S*-nitroso-acetylpenicillamine. Bovine aortic endothelial cells under the present cell culture conditions did not express soluble guanylate cyclase activity, so any increases in cGMP were due to smooth muscle soluble guanylate cyclase. Smooth muscle cells in the co-cultures responded by accumulating lower amounts of cGMP than smooth muscle cells cultured alone. To confirm that the downregulation in cGMP accumulation in response to sodium nitroprusside that is observed in the co-culture is due to endothelium-derived nitric oxide, smooth muscle cells or smooth muscle/bovine aortic endothelial cell co-cultures were incubated with 1

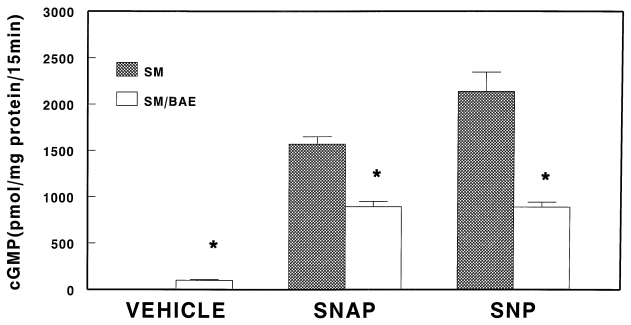

Fig. 5. Sodium nitroprusside (SNP)-induced cGMP accumulation is greater in single smooth muscle cell cultures (SM) than smooth muscle-endothelium co-cultures (SM/BAE). Rat aortic smooth muscle cells (RASM) were cultured alone or in co-culture with bovine aortic endothelial cells (BAE) for 24 h. cGMP accumulation was then determined following sodium nitroprusside (SNP, 10 μM) or *S*-nitroso-acetylpenicillamine (SNAP, 10 μM) stimulation for 15 min in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.3 mM). Co-cultures (and rat aortic smooth muscle cells) were pretreated with L-nitroarginine methylester for 30 min to inhibit cGMP accumulation due to endothelium-derived nitric oxide release. Means $\pm$ S.E.M., $n = 4$ wells. $^{*}P < 0.05$ from SM.

mM of the nitric oxide synthase inhibitor L-nitroarginine methylester for the duration of the co-culture (24 h). Treatment with 1 mM L-nitroarginine methylester was found to inhibit more than 90% or the endothelium-derived

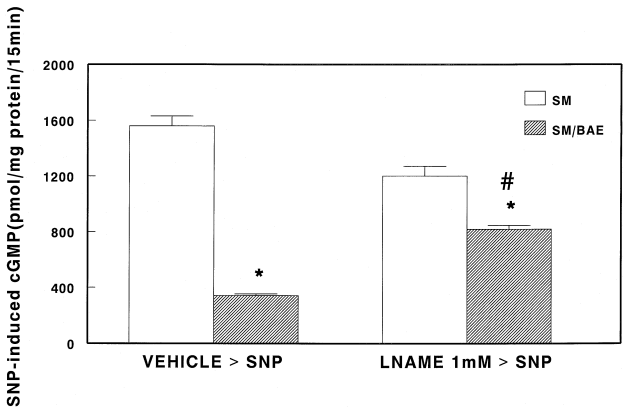

Fig. 6. Inhibition of nitric oxide synthesis prevents partially the reduction in sodium nitroprusside (SNP)-induced cGMP accumulation in endothelium-smooth muscle co-cultures. Rat aortic smooth muscle cells (RASM) and rat aortic smooth muscle cell/bovine aortic endothelial cell (BAE) co-cultures were incubated with vehicle or 1 mM L-nitroarginine methylester (L-NAME). After 24 h, rat aortic smooth muscle cells co-culture cGMP content was determined following stimulation with 10 μM sodium nitroprusside. Prior to sodium nitroprusside stimulation co-cultures (and rat aortic smooth muscle cells) were pretreated with L-nitroarginine methylester (100 μM) to inhibit cGMP accumulation due to nitric oxide synthesis. Vehicle > sodium nitroprusside: vehicle pretreatment for 24 h followed by 30 min posttreatment with L-nitroarginine methylester and 15 min exposure to sodium nitroprusside. L-Nitroarginine methylester 1 mM > sodium nitroprusside: L-nitroarginine methylester pretreatment for 24 h followed by 30 min posttreatment with L-nitroarginine methylester and 15 min exposure to sodium nitroprusside. Means $\pm$ S.E.M., $n = 4$ wells. $^{*}P < 0.05$ from SM, $^{\#}P < 0.05$ from vehicle.

nitric oxide release from the bovine aortic endothelial cells (as assessed by cGMP accumulation). Under these conditions, soluble guanylate cyclase responsiveness of the smooth muscle cells in the co-cultures was partially restored (Fig. 6).

4. Discussion

The major findings of the present study are that (1) exposure of cultured smooth muscle cells to nitric oxide donors results in the development of tolerance in a species, time- and concentration-dependent manner, (2) cross-tolerance is observed between nitric oxide donors (sodium nitroprusside, *S*-nitroso-acetylpenicillamine, endothelium-derived nitric oxide and inducible nitric oxide synthase-derived nitric oxide) but not between activators of the soluble and the particulate guanylate cyclase (sodium nitroprusside and atriopeptin) and 3) continuous exposure of smooth muscle cells to low amounts of nitric oxide release from endothelial cells results in decreased sodium nitroprusside-induced cGMP accumulation in response to exogenous nitric oxide donors (sodium nitroprusside and *S*-nitroso-acetylpenicillamine) in an L-nitroarginine methylester-reversible manner.

Exposure of cultured aortic smooth muscle cells from different species to 10 μM sodium nitroprusside led to the development of tolerance in the rat and rabbit, but not in the bovine and pig, as indicated by the diminished cGMP accumulation upon subsequent stimulation with sodium nitroprusside. The inability of sodium nitroprusside to induce tolerance in the bovine and porcine aortic smooth muscle cells after exposure to 10 μM sodium nitroprusside is probably associated with a difference in the sensitivity of various species to nitric oxide, since higher concentrations of sodium nitroprusside (50–500 μM) led to the development of tolerance in the porcine cells. Similarly to the cells from other mammalian species, human cells isolated from renal vessels become tolerant to sodium nitroprusside in a concentration-dependent manner after exposure to sodium nitroprusside for 12 h. The observation that cells isolated from the renal vein were more resistant to sodium nitroprusside tolerance is intriguing; however, further studies are required in order to elucidate the mechanism(s) responsible for the resistance of human renal vein smooth muscle cells to sodium nitroprusside tolerance. The above data taken collectively demonstrate that differences in the development of tolerance exist between different vascular beds and species. Human and bovine cells, consistently with previously published reports (Papapetropoulos et al., 1996a), exhibited low soluble guanylate cyclase activity even under control conditions (no sodium nitroprusside tolerance) and were, thus, not used in subsequent experiments. Instead, rat aortic smooth muscle cells were used to study the development of cross-tolerance due to the fact that they displayed consistently higher soluble guanylate cyclase activity than rabbit aortic smooth muscle cells and developed tolerance to sodium nitroprusside at a lower sodium nitroprusside concentration compared to the porcine cells.

The observation that pretreatment of cultured smooth muscle cells with sodium nitroprusside leads to the development of tolerance to the action of sodium nitroprusside and cross-tolerance to *S*-nitroso-acetylpenicillamine, is consistent with reports that exposure of smooth muscle (Zhang et al., 1993; Papapetropoulos et al., 1996c) and medullary interstitial cells (Ujiie et al., 1994) to NO-generating agents leads to decreased levels of cGMP accumulation after soluble guanylate cyclase activation. Similar observations have been made in vivo, in vitro in organ bath studies as well as with crude and purified soluble guanylate cyclase: exposure of bovine coronary arterial rings, porcine vena cordis magna or rat aorta to nitric oxide, sodium nitroprusside, *S*-nitroso-acetylpenicillamine or 3-morpholinosydnonimin leads to impaired relaxation (Henry et al., 1989a,b; Kojda et al., 1994) and incubation of purified soluble guanylate cyclase with, nitric oxide gas renders the enzyme less sensitive to nitric oxide stimulation (Braughler, 1983). However, the development of tolerance to non-nitrate nitric oxide donors is sometimes of smaller magnitude when compared to nitrate vasodilators (Kowaluk et al., 1987; Henry et al., 1989a; Zhang et al., 1993; Zhang et al., 1994). This can be explained by the fact that tolerance to the action of nitrates occurs both at the level of biotransformation and at the level of soluble guanylate cyclase activation (Henry et al., 1989a). The smaller degree of tolerance developed with the non-nitrate nitric oxide donors may make these agents more useful in clinical settings.

Pretreatment of rat aortic smooth muscle cells with *S*-nitroso-acetylpenicillamine led to the development of tolerance to sodium nitroprusside. Previous reports have indicated that there is no tolerance development to *S*-nitroso-acetylpenicillamine in vivo (Bauer and Fung, 1991; Shaffer et al., 1992). However, in vitro studies have demonstrated that under certain conditions tolerance does develop to this agent (Kowaluk et al., 1987). While cross-tolerance developed between sodium nitroprusside and *S*-nitroso-acetylpenicillamine, the responses to the particulate guanylate cyclase activator atriopeptin were increased in rat aortic smooth muscle cells pretreated with sodium nitroprusside. This is in agreement with previously published results in cultured porcine coronary artery smooth muscle cells (Zhang et al., 1993). It is also in line with our observations that rat aortic smooth muscle cells exposed to interleukin-1β (and thus producing large amounts of nitric oxide through the inducible nitric oxide synthase), did not show decreased responses to atriopeptin (Papapetropoulos et al., 1996b).

Accumulation of cGMP in response to either basally or agonist stimulated endothelium-derived nitric oxide was also found to be decreased in sodium nitroprusside pre-

treated cells, indicating that cross-tolerance between exogenous nitric oxide donors and endothelium-derived nitric oxide develops. This observation is in agreement with the finding that aortic rings of rabbits chronically treated with molsidomine exhibited diminished relaxations to 3-morpholinosydnonimin and acetylcholine (Bult et al., 1991). However, in other reports development of tolerance to nitrate or non-nitrate nitric oxide donors did not affect endothelium-dependent responses. In these latter studies, endothelium-dependent responses were evaluated after 3-morpholinosydnonimin or nitroglycerin pretreatment (Henry et al., 1989a; Berkenboom et al., 1990) without inhibiting the activity of cyclooxygenase or the release of endothelium-derived hyperpolarizing factor, which may be secreted in amounts sufficient to fully relax the preconstricted vessels.

To investigate if tolerance to the action of sodium nitroprusside develops in cells exposed to endogenously produced nitric oxide, rat aortic smooth muscle cells were treated with bacterial lipopolysaccharide or interleukin-1β and then stimulated with sodium nitroprusside. We have previously demonstrated that exposure of rat aortic smooth muscle cells to either substance leads to the appearance of inducible nitric oxide synthase mRNA, protein and the releases of large amounts of nitric oxide (Marczin et al., 1996). Cells exposed to bacterial lipopolysaccharide or interleukin-1β displayed a concentration-dependent reduction in cGMP levels when stimulated with sodium nitroprusside indicating that cross-tolerance develops between endogenously produced nitric oxide and exogenous nitric oxide donors.

Co-culture of smooth muscle cells with bovine aortic endothelial cells, a means of exposing smooth muscle cells to low nitric oxide levels, led to impaired sodium nitroprusside- and *S*-nitroso-acetylpenicillamine-induced cGMP accumulation, suggesting the development of cross-tolerance between endothelium-derived nitric oxide and exogenous nitric oxide donors. When exogenous nitric oxide donors were used, 10 μM was the lowest concentration that reproducibly led to the development of tolerance after a single addition of the sodium nitroprusside. We have previously reported that as low as 75 nM sodium nitroprusside can lead to the development of tolerance in rat aortic smooth muscle cells if supplied every 90 min for 12 hr (Papapetropoulos et al., 1996c). This can explain how the continuous release of small amounts of endothelium-derived nitric oxide lead to a reduction of soluble guanylate cyclase responsiveness. The observation that cGMP accumulation in response to sodium nitroprusside or *S*-nitroso-acetylpenicillamine is greater in single rat aortic smooth muscle cells cultures than in rat aortic smooth muscle cells/bovine aortic endothelial cell co-cultures, is in line with previous reports that removal of the endothelium or inhibition of endothelium-derived nitric oxide production leads to supersensitivity to the action of sodium nitroprusside (Shirasaki and Su, 1985; Moncada et al., 1991b; Kojda et al., 1994). It is also in agreement with the observation that cGMP accumulation in rat aortic smooth muscle cells of spontaneously hypertensive rats is increased as compared to that of cells isolated from Wistar Kyoto rats and may relate to the lower levels of biologically active nitric oxide released from the endothelium of the hypertensive animals (Papapetropoulos et al., 1994).

Incubation of rat aortic smooth muscle cells/bovine aortic endothelial cell co-cultures with 1 mM L-nitroarginine methylester to block nitric oxide production from the bovine aortic endothelial cells, led to a partial recovery of the soluble guanylate cyclase responsiveness to sodium nitroprusside, confirming that impaired soluble guanylate cyclase responses are at least partly due to the production of endothelium-derived nitric oxide. The inability of L-nitroarginine methylester to completely reverse the endothelium-induced hyporesponsiveness of soluble guanylate cyclase to nitric oxide, suggests that other endothelium-derived substances contribute to the decrease in sodium nitroprusside-induced cGMP accumulation in endothelium-smooth muscle co-cultures. We have previously reported that cAMP-elevating agents cause a reduction in soluble guanylate cyclase activity, mRNA and protein levels in cultured rat aortic smooth muscle cells (Papapetropoulos et al., 1995). Thus, release of cAMP-elevating substances (such as prostacyclin) from the endothelium may contribute to the reduction of soluble guanylate cyclase activity seen in the co-cultures.

In conclusion, exposure of smooth muscle cells to either exogenously supplied non-nitrate nitric oxide donors (sodium nitroprusside, *S*-nitroso-acetylpenicillamine) or endogenously produced NO, by the constitutive or the inducible isoform of nitric oxide synthase, leads to reduced cGMP accumulation to subsequent exposure to nitric oxide. The observation that cross-tolerance develops between nitric oxide donors and endogenously produced nitric oxide is of significance in cases where nitric oxide donors are administered as substitutes to overcome the deficiency of endothelium-derived nitric oxide brought about by cardiovascular disease.

Acknowledgements

We are pleased to acknowledge the expert technical assistance of Ms. Connie Snead and Mr. Jim Parkerson. This study was supported by HL52958.

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