

Organic phosphates as a new class of soluble guanylate cyclase inhibitors

Tsuneharu Suzuki^{a,1}, Makoto Suematsu^{b,*}, Ryu Makino^c

^aResearch Laboratory, Minophagen Pharmaceutical Co., 2-2-3 Komatsubara, Zama, Kanagawa 228-0021, Japan

^bDepartment of Biochemistry and Integrative Medical Biology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

^cDepartment of Chemistry, College of Science, Rikkyo University, 3-34-1 Nishi-Ikebukuro, Toshima-ku, Tokyo 171-0021, Japan

Received 5 July 2001; revised 17 September 2001; accepted 17 September 2001

First published online 1 October 2001

Edited by Guido Tettamanti

Abstract This study aimed to examine effects of varied organic phosphates on activities of soluble guanylate cyclase (sGC). The enzyme was purified from bovine lung. Physiologically relevant concentrations of ATP, 2,3-bisphosphoglyceric acid and inositol hexakisphosphate inhibited its enzyme activities under steady-state conditions as well as those determined under stimulation with *S*-nitroso-*N*-acetylpenicillamine, a nitric oxide donor, carbon monoxide or YC-1. Lineweaver–Burk plot analyses revealed that these three organic phosphates act as competitive inhibitors. Other organic phosphates such as cardiolipin and sphingomyelin but not inorganic phosphates exhibited such inhibitory actions. These results suggest that organic phosphates serve as inhibitors for sGC-dependent signaling events. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Soluble guanylate cyclase; Nitric oxide; Carbon monoxide; Adenosine triphosphate; Organic phosphate; 2,3-Bisphosphoglyceric acid

1. Introduction

Guanylate cyclase (GTP pyrophosphate lyase (cyclizing), EC 4.6.1.2) constitutes a family of enzymes that catalyze the cyclization of GTP to cGMP [1,2]. There are two general classes of the cyclase, particulate (pGC) and soluble guanylate cyclases (sGC). pGC is membrane-bound and activated by several peptide ligands such as atrial natriuretic peptide [3–6], while sGC is an important receptor for nitric oxide (NO) which activates the enzyme to execute signal transduction in neurovascular systems [7,8]. Other known activators are carbon monoxide (CO), another gaseous mediator that binds to the prosthetic heme of the enzyme [9–11], and YC-1, a synthetic anti-platelet reagent which activates the enzyme through heme-independent mechanisms [12–14]. On the other hand, several synthetic inhibitors of the enzyme, such as 1*H*-(1,2,4)-oxadiazolo-(4,3-*a*)-quinoxalin-1-one, have been provided [15]. However, little information has been available as

to whether endogenous substances occurring in cells could modulate the sGC activities, except for a previous study showing that adenosine phosphates such as ATP inhibit the cGMP formation by the enzyme [16]. Considering that cellular contents of organic phosphates have been shown to be altered dramatically in response to redox changes, it is not unreasonable to hypothesize that the biologically active organic phosphates could regulate the sGC activities and alter the sensitivity of the enzyme to NO. We here examine actions of various organic phosphates on the sGC activities and provide evidence that some of them have the ability to inhibit the activities at their physiologically relevant concentrations.

2. Materials and methods

2.1. Purification of sGC from bovine lung

sGC was purified from bovine lung as described previously [17]. sGC-containing fractions were collected by judging the ability to generate cGMP in response to the NO application. The concentrated NO-sensitive fractions were further purified with a Superdex 200 pg HPLC column (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). The fractions with a specific activity over 8000 nmol/min/mg protein measured in the presence of NO were pooled. Then, the sample was finally purified to apparent homogeneity with a Protein Pak G-DEAE HPLC column (Waters, Milford, MA, USA). Protein contents of the resultant homogeneous enzyme were determined by the method of Bradford [18]. In order to determine the purity of the enzyme preparations, the purified sGC sample was subjected to SDS-PAGE as described elsewhere [19]. The sample was stored at –80°C until use.

2.2. Determination of enzyme activity

Reaction mixtures contained 5 mM MgCl₂, 3 mM dithiothreitol, 1 mM 3-isobutyl-1-methylxanthine, 1 mM GTP, and 2.5 nM sGC in 40 mM TEA–HCl buffer at pH 7.4. Enzyme reactions were conducted at 37°C for 10 min and terminated by addition of 30% acetic acid in a final volume of 1 ml. The guanylate cyclase activity was determined by measuring the cGMP contents using enzyme-linked immunosorbent assay (Amersham Pharmacia Biotech). *S*-Nitroso-*N*-acetylpenicillamine (SNAP, Inter Medical, Tokyo, Japan), an NO donor, was dissolved in phosphate-buffered saline at pH 7.4, at 30 min prior to the start of experiments. Desired concentrations of CO were applied to the enzyme as described previously [11]. YC-1 (Alexis Biochemicals, San Diego, CA, USA) was dissolved in *N,N*-dimethylformamide (DMF). The final DMF concentration in all samples was 1% (v/v). All measurements were performed in duplicate and were repeated three times. Data in the present study are expressed as mean ± S.D. of measurements. Differences in statistical significance among groups were analyzed by one-way ANOVA with Fisher's multiple comparison test, unless indicated otherwise. *P* < 0.05 was considered statistically significant.

2.3. Reagents studied

GTP, adenosine, AMP, ADP, ATP, 2,3-bisphosphoglyceric acid (2,3-BPG), inositol hexakisphosphate (IHP), phosphoribosyl pyro-

*Corresponding author. Fax: (81)-3-3358 8138.

¹ Present address: Department of Biochemistry and Integrative Medical Biology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

E-mail addresses: t-suzuki@sc.itc.keio.ac.jp (T. Suzuki), msuem@sc.itc.keio.ac.jp (M. Suematsu), rmakino@rikkyo.ac.jp (R. Makino).

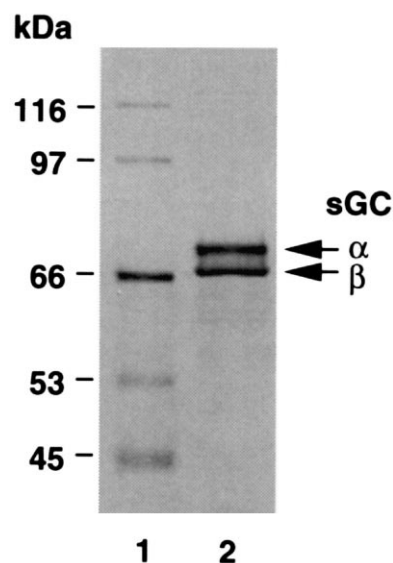


Fig. 1. A representative picture of SDS-PAGE for purified samples of sGC. Lane 1 denotes molecular markers. Lane 2 indicates a pattern of purified sGC (0.2 μ g), displaying two bands with distinct molecular weights.

phosphate (PRPP), phosphatidic acid, phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, phosphatidylinositol biphosphate, sphingosine, sphingosine 1-phosphate, sphingomyelin, ceramide, and cardiolipin were from Sigma Chemical Co. (St. Louis, MO, USA). GTP, adenosine, AMP, ADP, ATP, 2,3-BPG, IHP and PRPP were dissolved in buffer to give desired concentrations. Other organic phosphates were dissolved with 1% ethanol and then added to the reaction mixture. In these experiments, the same concentration of ethanol was added as a vehicle in the control experiments.

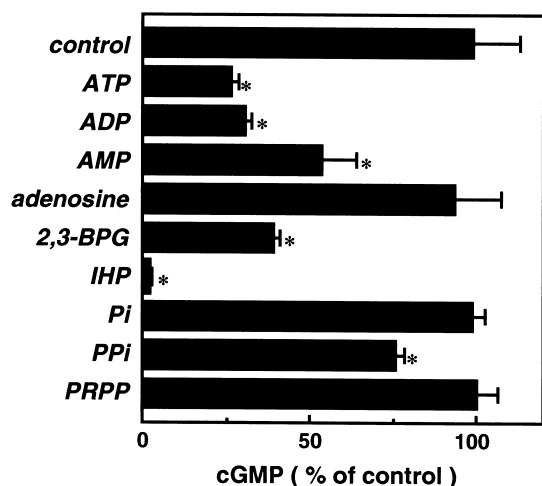
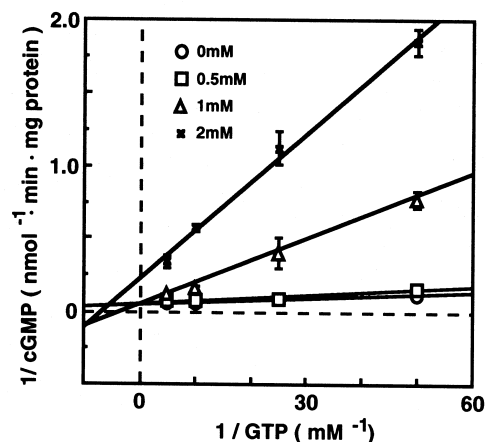
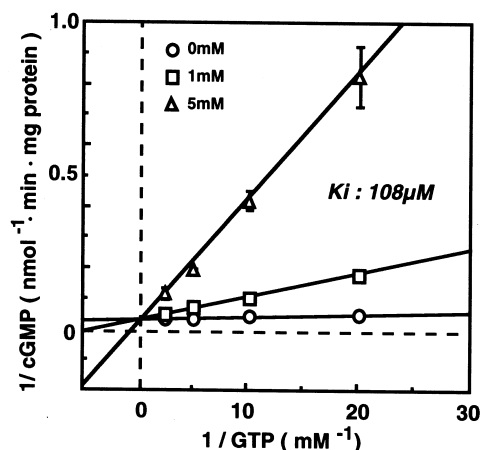


Fig. 2. Effects of organic and inorganic phosphates on activities of sGC. The sGC activity was measured under conditions in the absence of NO, being 77.5 ± 5.5 nmol/min/mg protein, and designated the control activity. Concentrations of compounds studied were 1 mM in all experiments. Data are means \pm S.D. of measurements from three separate experiments. * $P < 0.05$ as compared with the control values. Note that tetrapotassium pyrophosphate (Ppi) but not dipotassium hydrogen phosphate (Pi) significantly attenuates the control activity.

A: ATP



B: 2,3-BPG



C: IHP

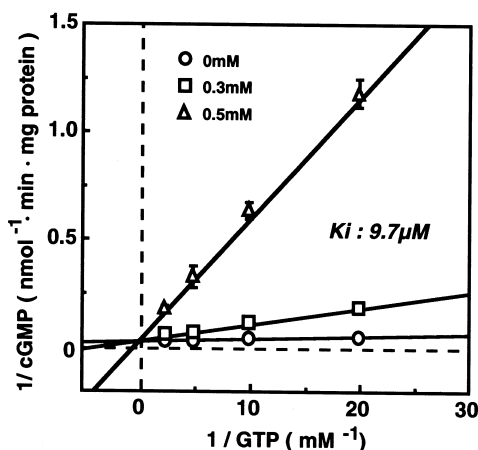


Fig. 3. Competitive inhibition of the sGC activities by (A) ATP, (B) 2,3-BPG and (C) IHP revealed by Lineweaver-Burk plot analyses. Data plots denote means \pm S.D. from three independent experiments. Note that ATP exhibits competitive inhibition with its lower concentrations less than 1 mM, while it shows mixed competition patterns at 2 mM.

3. Results

3.1. Inhibitory actions of ATP, 2,3-BPG and IHP on the basal activities of sGC

Fig. 1 illustrates a representative picture of SDS-PAGE of purified sGC samples. As seen, two subunits of sGC were noted as judged by distinct molecular weights of 74 and 71 kDa for α and β subunits, respectively. These results indicated a quality of the enzyme purification, being in good agreement with a previous report [17]. Considering that the molecular weight of bovine sGC is 145 kDa, 1 mg of the sample contained approximately 6.9 nmol of sGC. Using the purified sGC sample, effects of ATP and its related compounds on the enzyme activities were examined. The activities were determined in the absence of effectors such as NO, being designated the baseline activity. Effects of these reagents were examined at a final concentration of 1 mM. As seen in Fig. 2, ATP significantly attenuated the baseline activity. ADP and AMP also inhibited the activities but with smaller extents of the inhibition than ATP, indicating that their inhibitory actions were reduced in parallel with the decreasing number of

phosphates. On the other hand, adenosine did not alter the baseline activity. These results were in agreement with a previous observation in experiments using crude sGC preparation from rat lung [16]. Other types of organic phosphates such as 2,3-BPG and IHP exhibited potent inhibitory actions on the enzyme activities. On the other hand, neither PRPP nor dipotassium hydrogen phosphate (Pi) showed such inhibitory action, though tetrapotassium pyrophosphate (PPi) displayed a mild but significant inhibition on the activities. We next attempted to examine mechanisms for inhibitory actions of ATP, 2,3-BPG and IHP on the basal enzyme activities. As seen in Lineweaver–Burk plot analyses in Fig. 3, at concentrations less than 1 mM, ATP displayed a competitive inhibition pattern, showing the intersection on the y -axis. With higher concentrations, however, the mechanisms appeared to involve a mixed inhibition pattern, as judged by the intersection in the left side of the y -axis. On the other hand, both 2,3-BPG and IHP exhibited a competitive inhibition pattern over their concentrations examined, and the K_i values were 108 μ M and 9.7 μ M, respectively. Considering physiological concentrations of these reagents in cells such as human or avian

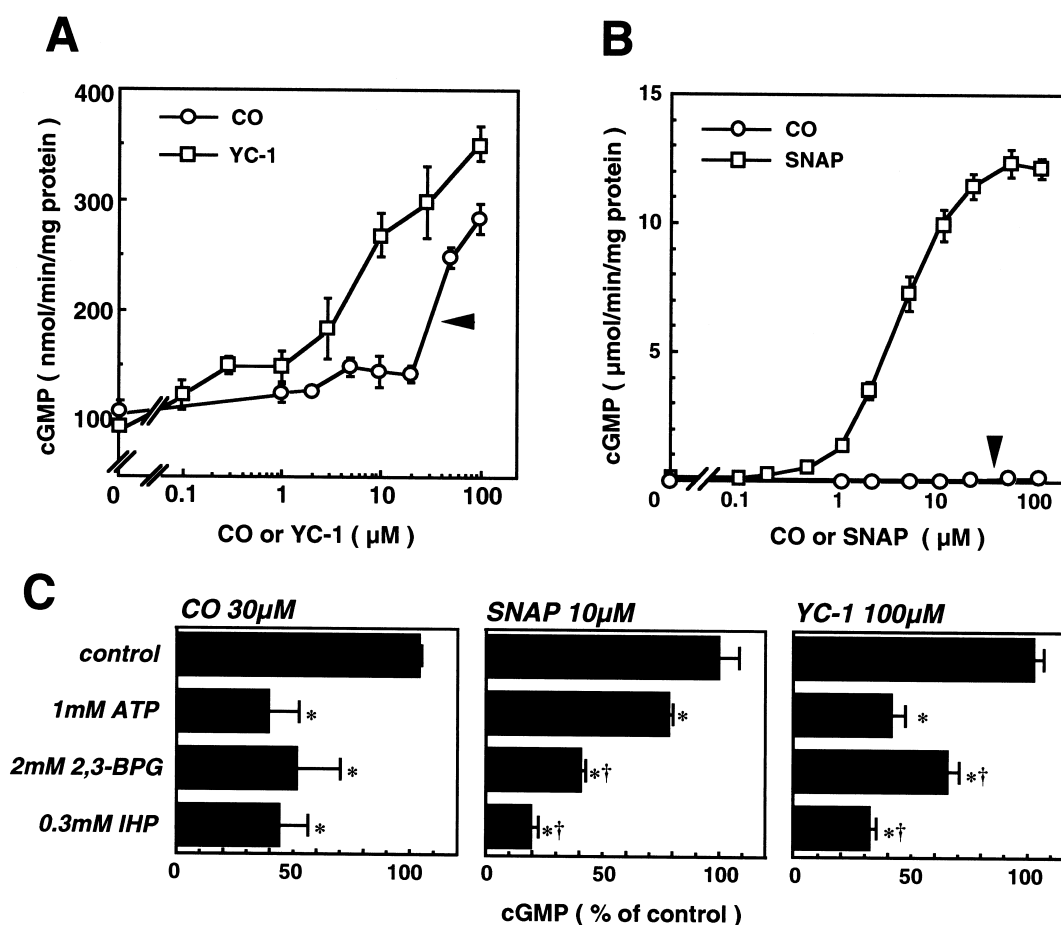


Fig. 4. Effects of ATP, 2,3-BPG and IHP on the allosteric effector-dependent activation of sGC. A: Dose-dependent activation of the sGC activities by CO and YC-1. B: Comparison of dose-dependent sGC activation between CO and NO. SNAP was used as a donor of NO. Curves with arrows in A and B indicate identical data showing CO-dependent activation. Data are means \pm S.D. of measurements from three separate experiments. C: Effects of 1 mM ATP, 2 mM 2,3-BPG and 0.3 mM IHP on activities of sGC treated with CO, SNAP and YC-1. Note that these concentrations of the reagents comparably inhibit the CO-elicited activation of the enzyme. Data indicate mean \pm S.D. of measurements from three independent experiments. * P < 0.05 as compared with the control values, † P < 0.05 as compared with the data showing effects of ATP.

erythrocytes and muscle cells [20–22], these results suggest that physiologically relevant concentrations of ATP, 2,3-BPG and IHP could suppress the basal sGC activities.

3.2. ATP, 2,3-BPG and IHP attenuate the sGC activation by CO, NO and YC-1

The observation showing inhibitory actions of ATP, 2,3-BPG and IHP on the basal sGC activities led us to examine whether these reagents could also attenuate the enzyme activation by effectors such as CO, NO and YC-1. As seen in Fig. 4A, CO and YC-1 dose-dependently activated the sGC activities. At 30 μ M, CO increased the sGC activity approximately by two-fold. As reported previously [13,23], however, the potency of CO to activate sGC was far smaller than that of NO. As shown by the arrow in Fig. 4B, the dose-response curve for CO appeared almost flat as compared with that for SNAP. Actually, the ability of the NO donor SNAP to activate the enzyme was approximately 50-fold greater than that elicited by sufficient concentrations of CO. We then chose concentrations of ATP, 2,3-BPG and IHP at 1 mM, 2 mM and 0.3 mM, respectively, which inhibited the CO-elicited activation of the enzyme approximately by 50% (Fig. 4C). Under these experimental conditions, these organic phosphates significantly attenuated the SNAP- or YC-1-induced activation of sGC, but with different extents of inhibition: among these compounds, IHP displayed the most potent inhibitory action on the enzyme. These results suggest that ATP, 2,3-BPG and IHP inhibit the sGC activities irrespective of the presence or absence of these effectors.

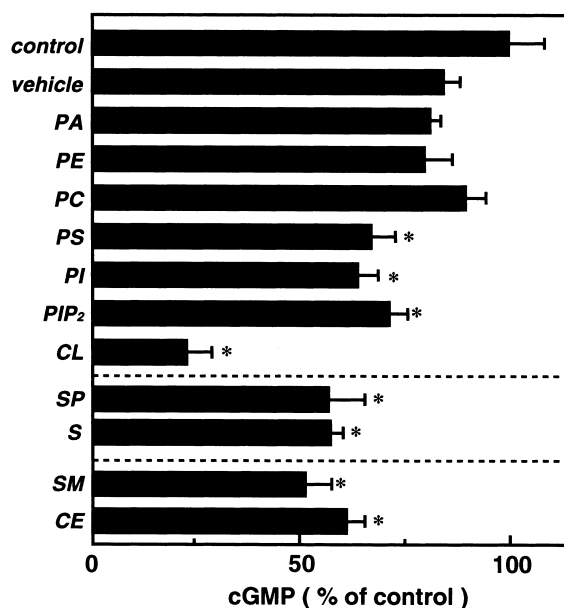


Fig. 5. Effects of different phospholipids on activities of sGC. PA: phosphatidic acid; PE: phosphatidylethanolamine; PC: phosphatidylcholine; PS: phosphatidylserine; PI: phosphatidylinositol; PIP₂: phosphatidylinositol bisphosphate; CL: cardiolipin; SP: sphingosine 1-phosphate; S: sphingosine; SM: sphingomyelin; CE: ceramide. These phospholipid concentrations were 1 μ M, dissolved in ethanol as a vehicle at a final concentration of 1% (v/v). Data were means \pm S.D. of measurements from three independent experiments. * P < 0.05 as compared with the values in the vehicle group.

3.3. Biologically active phospholipids inhibit the baseline sGC activities

The current observation raised a possibility that other organic compounds with phosphate might alter the sGC activities. Fig. 5 illustrates effects of varied phospholipids on the basal enzyme activities. At 1 μ M, phosphatidylserine, phosphatidylinositol, phosphatidylinositol bisphosphate and cardiolipin exhibited a significant inhibition on the activities. Among these compounds, cardiolipin had the most potent inhibitory action on the enzyme, displaying approximately a 75% inhibition. Other phospholipids such as sphingosine 1-phosphate also displayed a significant suppression. However, considering that sphingosine at the same concentration exhibited a comparable inhibition rate, its effect was unlikely to be ascribable to the action of the phosphate.

4. Discussion

The present study provides evidence that a variety of biologically active organic phosphates have the ability to suppress the catalytic activities of sGC in vitro. Among these sGC-inhibiting substances, 2,3-BPG and IHP appear to block the activity in a competitive manner, while ATP suppresses the activity in competitive and non-competitive manners at low and high concentrations, respectively. The inhibitory effect of this adenosine derivative was reported in previous experiments using a crude preparation of rat lung sGC [16] or human placental enzyme [24], but mechanisms for the inhibition were not fully addressed. The inhibitory effect of the reagent on sGC activity is reduced with a decreasing number of phosphates, indicating that ATP is a relatively potent inhibitor as compared with other derivatives such as AMP and adenosine. Furthermore, the present study first provides evidence that ATP at physiologically relevant concentrations is able to inhibit NO- or CO-dependent sGC activation in vitro.

Although whether the current results could be extrapolated to the in vivo system is quite unknown, the direct inhibitory action of ATP on sGC demonstrated in this study sheds light on a novel mechanism for regulation of blood flow under hypoxic conditions. Several lines of functional linkage of ATP to compensation of local blood flow in vivo have been demonstrated. First, upon ischemia, ATP is rapidly hydrolyzed to generate adenosine which accesses to vascular smooth muscle cells for vasorelaxation [25]. Second, recent studies suggest that hypoxia triggers the release of ATP from erythrocytes which in turn stimulates purinergic receptor on endothelial cells and evokes NO generation for vasorelaxation [26]. Since the local oxygen concentration is a determinant for the half-life of NO in situ [27], hypoxia could help NO-mediated vasodilation to guarantee ample blood supply to the hypoxic area at risk. Finally, considering the inhibitory action of ATP on sGC, stimulation of local NO generation could not only activate sGC directly but also help sGC activation indirectly through suppression of mitochondrial membrane potential, a determinant of ATP synthesis in cells [28]. In this context, the roles of ATP in the regulation of sGC sensitivity to NO deserve further study on in vivo evidence for ATP-mediated regulation of blood supply to hypoxic regions and its functional link to local sGC activities.

It should be noted that in this study cardiolipin possessed a potent inhibitory action on sGC activity. A recent investigation revealed the involvement of this phospholipid in the reg-

ulation of vasospasm [29]. Under normal conditions, cardiolipin binds tightly to the mitochondrial inner membrane. Its oxidative modification could cause the reduction of mitochondrial membrane potential and the release of cytochrome *c* into the cytoplasm to trigger apoptosis [30]. On the other hand, it is well known that patients with anti-phospholipid antibodies frequently demonstrate cardiovascular insufficiency or pre-eclampsia. Recent experimental data have provided evidence that the injection of cardiolipin into the circulation aggravates cerebrovascular spasm after subarachnoid hemorrhage in parallel with an increase in the titer of anti-cardiolipin antibody [29]. Although the anti-cardiolipin antibody rather than the antigen by itself appeared to contribute to the aggravation of the vascular dysfunction in the previous study, the present result showing the inhibitory action of cardiolipin on sGC raises the possibility that the release of this phospholipid into the circulation could inhibit sGC in and around vascular walls and/or platelets and thus increase the risk of vasospasm. The mechanisms for the inhibitory effects of phospholipids on sGC activities deserve further study on the pathophysiological implications of this observation at the cell and organ levels.

Acknowledgements: This work was supported by Grant-in-Aid for Creative Scientific Research by the Japan Society for the Promotion of Science 13GS0015.

References

- [1] Waldman, S.A. and Murad, F. (1987) *Pharmacol. Rev.* 39, 163–196.
- [2] Denninger, J.W. and Marletta, M.A. (1999) *Biochim. Biophys. Acta* 1411, 334–350.
- [3] Gerzer, R. (1985) *Klin. Wochenschr.* 63, 529–536.
- [4] Gunning, M., Ballermann, B.J., Silva, P., Brenner, B.M. and Zeidel, M.L. (1990) *Am. J. Physiol.* 258, F467–F472.
- [5] Furuya, M., Takehisa, M., Minamitake, Y., Kitajima, Y., Hayashi, Y., Ohnuma, N., Ishihara, T., Minamino, N., Kangawa, K. and Matsuo, H. (1990) *Biochem. Biophys. Res. Commun.* 170, 201–208.
- [6] Savarino, S.J., Fasano, A., Watson, J., Martin, B.M., Levine, M.M., Guandalini, S. and Guerry, P. (1993) *Proc. Natl. Acad. Sci. USA* 90, 3093–3097.
- [7] Furchgott, R.F. and Zawadzki, J.V. (1980) *Nature* 288, 373–376.
- [8] Ignarro, L.J., Harbison, R.G., Wood, K.S. and Kadowitz, P.J. (1986) *J. Pharmacol. Exp. Ther.* 237, 893–900.
- [9] Verma, A., Hirsch, D.J., Glatt, C.E., Ronnett, G.V. and Snyder, S.H. (1993) *Science* 259, 381–384.
- [10] Suematsu, M. and Ishimura, Y. (2000) *Hepatology* 31, 3–6.
- [11] Suematsu, M., Goda, N., Sano, T., Kashiwagi, S., Egawa, T., Shinoda, Y. and Ishimura, Y. (1995) *J. Clin. Invest.* 96, 2431–2437.
- [12] Ko, F.N., Wu, C.C., Kuo, S.C., Lee, F.Y. and Teng, C.M. (1994) *Blood* 84, 4226–4233.
- [13] Friebe, A., Schultz, G. and Koesling, D. (1996) *EMBO J.* 15, 6863–6868.
- [14] Stasch, J.P., Becker, E.M., Alonso-Alija, C., Apeler, H., Dembowsky, K., Feurer, A., Gerzer, R., Minuth, T., Perzborn, E., Pleiß, B.U., Schröder, H., Schroeder, W., Stahl, E., Steinke, W., Straub, A. and Schramm, M. (2001) *Nature* 410, 212–215.
- [15] Celtek, S., Kasakov, L. and Moncada, S. (1996) *Br. J. Pharmacol.* 118, 137–140.
- [16] Ignarro, L.J., Gross, R.A. and Gross, D.M. (1976) *J. Cyclic Nucleotide Res.* 2, 337–346.
- [17] Makino, R., Matsuda, H., Obayashi, E., Shiro, Y., Iizuka, T. and Hori, H. (1999) *J. Biol. Chem.* 274, 7714–7723.
- [18] Bradford, M.M. (1976) *Anal. Biochem.* 72, 248–254.
- [19] Laemmli, U.K. (1970) *Nature* 227, 680–685.
- [20] Brewer, G.J., Kruckeberg, W.C., Westover, C.J. and Oberman, H.A. (1976) *Prog. Clin. Biol. Res.* 11, 5–19.
- [21] Isaacs, R.E., Harkness, D.R., Goldman, P.H., Adler, J.L. and Kim, C.Y. (1977) *Hemoglobin* 1, 577–593.
- [22] Blei, M.L., Conley, K.E. and Kusmerick, M.J. (1993) *J. Physiol.* 465, 203–222.
- [23] Stone, J.R. and Marletta, M.A. (1994) *Biochemistry* 33, 5636–5640.
- [24] Idriss, S.D., Pilz, R.B., Sharma, V.S. and Boss, G.R. (1992) *Biochem. Biophys. Res. Commun.* 183, 312–320.
- [25] Marshall, J.M. (2000) *Acta Physiol. Scand.* 168, 561–573.
- [26] Sprague, R.S., Ellsworth, M.L., Stephenson, A.H. and Lonigro, A.J. (1996) *Am. J. Physiol.* 271, H2717–H2722.
- [27] Nishikawa, M., Sato, E.F., Utsumi, K. and Inoue, M. (1996) *Cancer Res.* 56, 4535–4540.
- [28] Shiomi, M., Wakabayashi, Y., Sano, T., Shinoda, Y., Nimura, Y., Ishimura, Y. and Suematsu, M. (1998) *Hepatology* 27, 108–115.
- [29] Nomura, H., Hirashima, Y., Endo, S. and Takaku, A. (1998) *Stroke* 29, 1014–1018.
- [30] Nomura, K., Imai, H., Koumura, T., Kobayashi, T. and Nakagawa, Y. (2000) *Biochem. J.* 351, 183–193.