# Cyclic GMP Formation in Rat Cerebellar Slices Is Stimulated by Endothelins via Nitric Oxide Formation and By Sarafotoxins via Formation of Carbon Monoxide

Zurit Shraga-Levine, Ronit Galron, and Mordechai Sokolovsky*

*Laboratory of Neurobiochemistry, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel*

*Received August 12, 1994; Revised Manuscript Received October 21, 1994*[⊗]

ABSTRACT: Involvement of a cyclic GMP pathway in signal transduction stimulated by endothelins (ETs) and sarafotoxins (SRTXs) was explored using rat cerebellar slices. These peptides activated the same receptor binding sites (ET-1 and SRTX-b at the picomolar sites; ET-3 and SRTX-c at the nanomolar sites) to produce cyclic GMP, but their signaling pathways differed. The endothelins (ET-1 and ET-3) were found to signal via nitric oxide formation and to involve pertussis toxin-sensitive G-protein(s). The SRTXs (b and c), while also stimulating cyclic GMP production, did so via a pathway which is not L-arginine-dependent, i.e., carbon monoxide formation, and did not involve pertussis-toxin-sensitive G-protein(s). This is the first demonstration that the signaling pathways of endothelins and sarafotoxins may differ, even though they share the same binding sites.

Several hormones and neurotransmitters stimulate the synthesis of guanosine 3′,5′-cyclic monophosphate (cGMP) in a variety of cells and tissues [reviewed in Garbers (1989)]. In many cases the agonist-induced increase in cGMP is mediated by nitric oxide, which binds to the heme moiety of soluble guanylyl cyclase, thereby activating it to synthesize cGMP (Ignarro et al., 1984; Garthwaite, 1991; Moncada et al., 1991). Previous studies have indicated that ET-1 and ET-3 mediate cGMP signaling in various cells, such as cultured bovine endothelial cells (Hirata et al., 1993), porcine kidney epithelial cells (Ishii et al., 1991), rat aorta (Fujitani et al., 1993; Moritoki et al., 1993), and intact rat glomeruli (Edwards et al., 1992).

Previous studies revealed the existence of at least two distinct endothelin receptor subtypes, $ET_A$-R and $ET_B$-R, that mediate the stimulation of phospholipases C and D in various tissues and cell type [reviewed in Sokolovsky (1992)]. These receptor subtypes are characterized by affinities in the nanomolar range. We recently described a novel subtype of endothelin receptor (ET-R), which is activated at picomolar ligand concentrations, whereas the "conventional" receptor is stimulated at nanomolar ligand concentrations (Sokolovsky et al., 1992). We were therefore interested in determining whether the signal transduction pathway of $ET_B$-R [the predominant receptor subtype in the rat cerebellum (Ambar et al., 1989; Sokolovsky et al., 1992; Sokolovsky, 1993)] is coupled to cGMP activation and, if so, whether this activation is ligand-specific. To this end, we employed four ligands: endothelins 1 and 3 and sarafotoxins b and c. We show here that ET-1 and SRTX-b induce either stimulation or inhibition of cGMP production, depending on ligand concentration. Moreover, we show that cGMP production induced by the ETs occurs via nitric oxide formation whereas SRTX-induced cGMP production occurs via an L-arginine-independent pathway involving the formation of carbon monoxide. These findings indicate that the endothelins and the sarafotoxins stimulate different signal transduction pathways, though they share the same receptors.

## MATERIALS AND METHODS

ET-1, ET-3, SRTX-b, and SRTX-c were purchased from American Peptide Company (Santa Clara, CA). cGMP was assayed by a radioimmunoassay kit purchased from Du Pont−New England Nuclear (NEX-133). Pertussis toxin was purchased from List Biochemicals (Campbell, CA) and BQ-123 from Novabiochem (Switzerland).

*Tissue Preparation.* Cerebella dissected from decapitated adult male rats were sliced with a Sorvall TC-2 tissue sectioner (200 × 200 μM) and dispersed in Krebs buffer (123 mM NaCl, 5 mM KCl, 1.4 mM $KH_2PO_4$, 1.3 mM $MgSO_4$, 0.8 mM $CaCl_2$, 10 mM glucose, and 20 mM HEPES, bubbled with 5% $CO_2$ and 95% $O_2$ to achieve pH 7.4).

*cGMP Assay.* Slices were preincubated with 0.5 mM IBMX for 10 min in Krebs buffer, transferred to vials containing 0.5 mM IBMX and the indicated ligands for 5 min, and then inactivated by boiling for 5 min in 4 volumes of Tris-HCl, pH 8.4, and 5 mM EDTA. The slices were homogenized in a Brinkmann polytron PT-10 (setting 7, 0.5 min) and their cGMP levels determined by radioimmunoassay. Protein concentrations were determined with bovine serum albumin used as a standard. Results (means ± SE) are expressed as the amount of cGMP (pmol) per mg of protein in the assay. Student's *t*-test was used for statistical analysis; $p < 0.01$ was considered statistically significant.

## RESULTS AND DISCUSSION

ET-1 ($10^{-12}$ M) produced a rapid increase of cGMP in rat cerebellar slices. The effect was maximal within 0.5−1 min and then slowly decreased. Similar results were obtained with ET-3 and SRTX-b (not shown). For technical convenience we chose an incubation period of 5 min (after which the effect of the ligand was about 90% of that obtained after 1 min) in all subsequent experiments, which were carried out in the presence of 0.5 mM IBMX. As shown in Figure 1, ET-1 and SRTX-b at picomolar concentrations each

* Author to whom correspondence should be addressed. Telephone: 972-3-6413612. Fax: 972-3-6415053.

[⊗] Abstract published in *Advance ACS Abstracts,* November 15, 1994.

0006-2960/94/0433-14656$04.50/0 

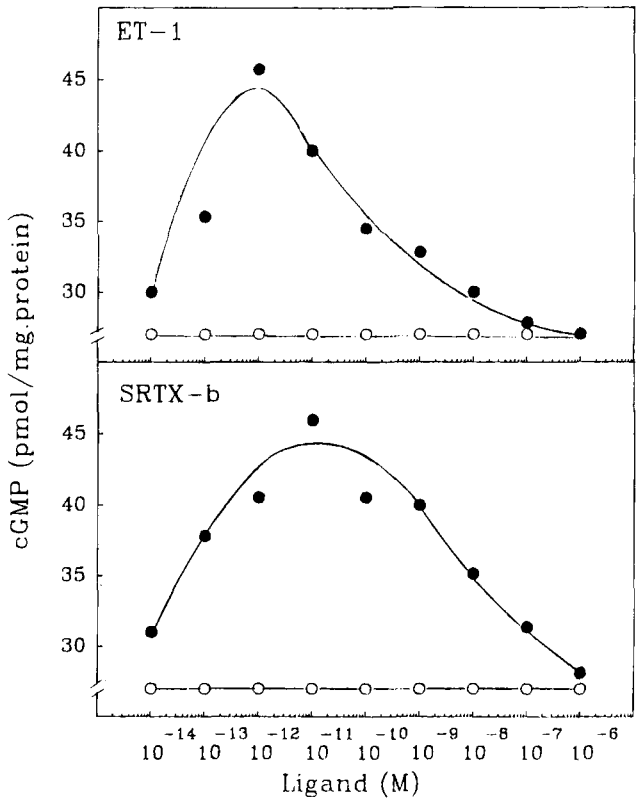



FIGURE 1: Dose–response curve of cGMP production stimulated by ET-1 and by SRTX-b. Data are from a representative experiment that was repeated 5–7 times and varied by only 5–10%. (●) ET-1 and SRTX-b-induced; (○) basal.

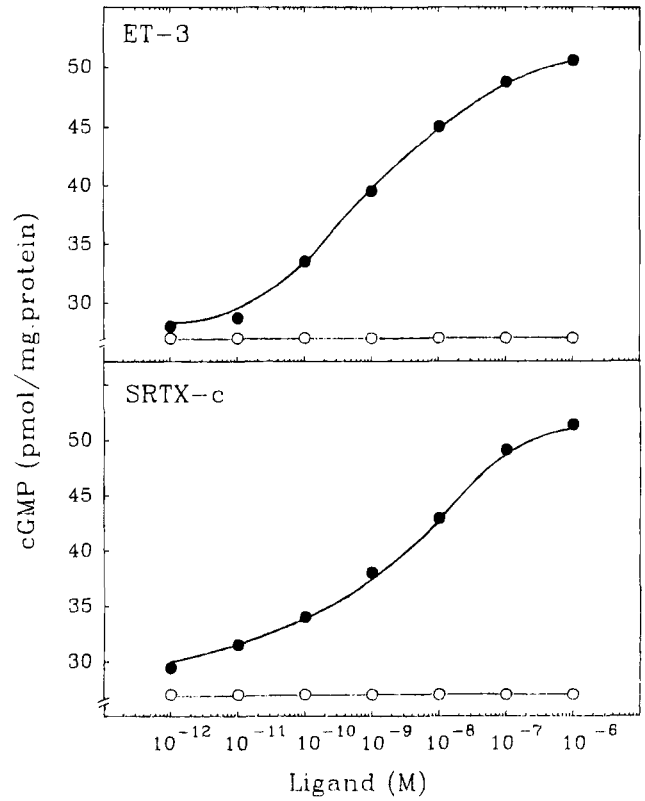



FIGURE 2: Dose–response curve of cGMP production stimulated by ET-3 and by SRTX-c. Data are from a representative experiment that was repeated 5–7 times and varied by only 5–10%. (●) ET-3 and SRTX-c-induced; (○) basal.

induced a dose-dependent increase in cGMP, with maximal stimulation in both ligands being similar and about 1.7–2-fold higher than basal cGMP levels. ET-1 or SRTX-b concentrations higher than $10^{-12}$ or $10^{-11}$ M, respectively, caused a dose-dependent inhibition of cGMP production, which decreased to basal levels at concentrations higher than $10^{-7}$ M. We recently reported a similar behavior pattern for cAMP production in rat atrial slices (Sokolovsky et al., 1994). This pattern of behavior may be indicative of a rapid desensitization process and/or the presence of more than one ET-R (as discussed below). If the former, the data in Figure 1 would indicate that desensitization induced by ET-1 or SRTX-b is rapid compared to that induced, if at all, by ET-3 or SRTX-c.

As shown in Figure 2, ET-3 and SRTX-c exhibited a different dose response from that of ET-1 and SRTX-b. No cGMP production was observed when ET-3 or SRTX-c concentrations were in the picomolar range, but cGMP was produced at the nanomolar range with maximal effect at $10^{-7}$–$10^{-6}$ M. It is interesting to note that the pharmacological rank order of ligands that bind to ET-R in the cerebellum (Ambar et al., 1989; Sokolovsky et al., 1992; Sokolovsky, 1993) is typical of $ET_B$-R, i.e., with similar binding affinities for ET-1, ET-3, SRTX-b, and SRTX-c. However, as shown in Figures 1 and 2, while the $EC_{50}$ values for ET-1 and SRTX-b were similar to their $K_d$ values, the $K_d$ values for ET-3 and SRTX-c (Ambar et al., 1989; Sokolovsky 1993) were lower than their corresponding $EC_{50}$ values. A likely explanation is that while the majority of the binding sites are of the $ET_B$-R subtype, there is also a minority population of sites of the $ET_A$-R subtype, and this minor population is responsible for the stimulation of cGMP

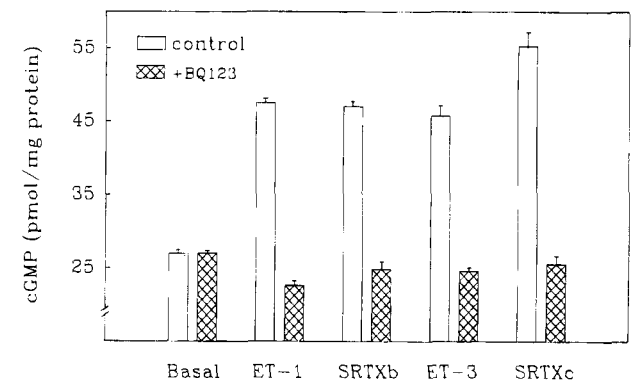



FIGURE 3: Effect of BQ-123 (100 nM) on cGMP production stimulated by the four ligands. Cerebellar slices were preincubated for 10 min with BQ-123. Results are expressed as the mean ± SEM of 3–4 experiments. Student's *t*-test was performed on each pair of stimulated vs basal values ($p < 0.01$).

production. Since the $ET_A$-R has a much lower affinity than the $ET_B$-R toward ET-3 and SRTX-c, these two ligands will induce cGMP production only at higher concentrations, sufficient for binding to the $ET_A$-R sites. If the percentage of the latter sites in cerebellar slices is low, binding studies might fail to detect the presence of $ET_A$-R. $K_d$ values lower than the $EC_{50}$ values of cGMP production (130–150 pM vs 15–30 nM) were also observed for the binding of ET-1 and ET-3 in intact rat glomeruli (Edwards et al., 1992).

To further examine the possible existence of $ET_A$-R in the cerebellar slices, we employed the $ET_A$-R-specific antagonist BQ-123. As shown in Figure 3, BQ-123 inhibited cGMP production induced by endothelins and by SRTXs. These findings support the notion that cGMP production in the cerebellar slices is mediated via a minor population of

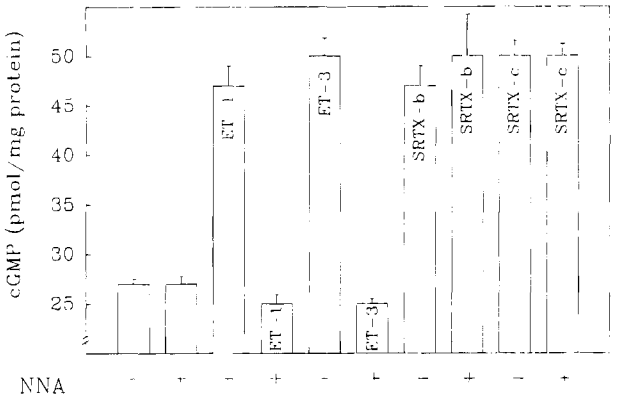



FIGURE 4: Effect of L-NNA (200 $\mu$M) on cGMP production stimulated by the four ligands. Cerebellar slices were preincubated for 20 min with L-NNA. Results are expressed as the mean ± SEM of 3−4 experiments. Student's *t*-test was performed on each pair of stimulated vs basal values ($p < 0.01$).

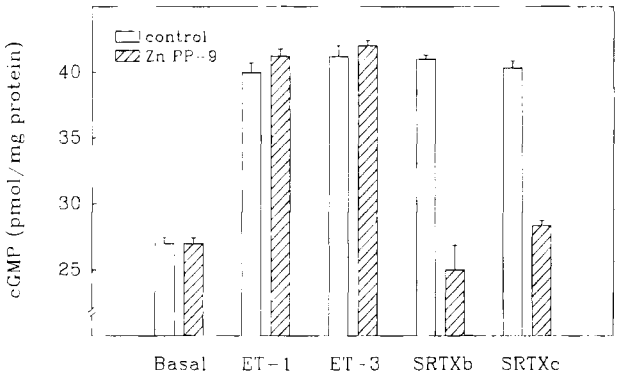



FIGURE 5: Effect of Zn PP-9 (0.1 $\mu$M) on cGMP production stimulated by ET-1 (1 pM), ET-3 (1 $\mu$M), SRTX-b (1 pM), or SRTX-c (1 $\mu$M). Slices were preincubated for 10 min with Zn PP-9, and then cGMP production was induced by each of the four ligands. Results are expressed as the mean ± SEM of 3−4 experiments. Student's *t*-test was performed on each pair of stimulated vs basal values ($p < 0.01$).

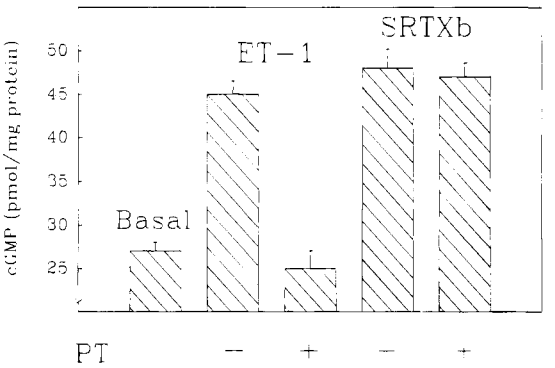



FIGURE 6: Effect of PT (1 $\mu$g/mL, 1 h, 36 °C) on cGMP production stimulated by ET-1 and SRTX-b. Results are expressed as the mean ± SEM of 3−4 experiments. Student's *t*-test was performed on each pair of stimulated vs basal values ($p < 0.01$).

ET receptors of the $ET_A$-R subtype.

To determine whether cGMP accumulation induced by ETs and SRTXs involves a nitric-oxide-dependent pathway, we examined the effect of $N^w$-nitro-L-Arg (NNA), a specific inhibitor of NO synthesis (Rees et al., 1989), on cGMP production, in the presence and absence of L-Arg (1 mM). As shown in Figure 4, preincubation with L-NNA (200 $\mu$M) decreased the ET-1- and ET-3-stimulated accumulation of cGMP in cerebellar slices. Addition of L-Arg (1 mM) reversed this inhibitory effect: cGMP (in pmol/mg of protein ± SD) increased from 23 ± 1 (+NNA) to 44 ± 3 (+NNA + L-Arg). Basal levels of cGMP were unaffected by these treatments. These results suggest that ET-1 and ET-3 increase cerebellar cGMP levels by stimulation of NO synthesis and subsequent activation of soluble guanylate cyclase. Methylene blue (10 $\mu$M, pretreatment 15 min), which inhibits the latter process, also blocked the induction of cGMP accumulation by 1 pM ET-1 and SRTX-b or 1 $\mu$M ET-3 and SRTX-c.

NNA did not inhibit cGMP accumulation induced by SRTX-b (1 pM) or SRTX-c (1 $\mu$M) (Figure 4), suggesting that cGMP production in the rat cerebellum is ligand-specific. Thus, the endothelins appear to induce cGMP accumulation via an L-Arg-dependent pathway, whereas the effect of the sarafotoxins, though also leading to cGMP production, is achieved via an L-Arg-independent pathway.

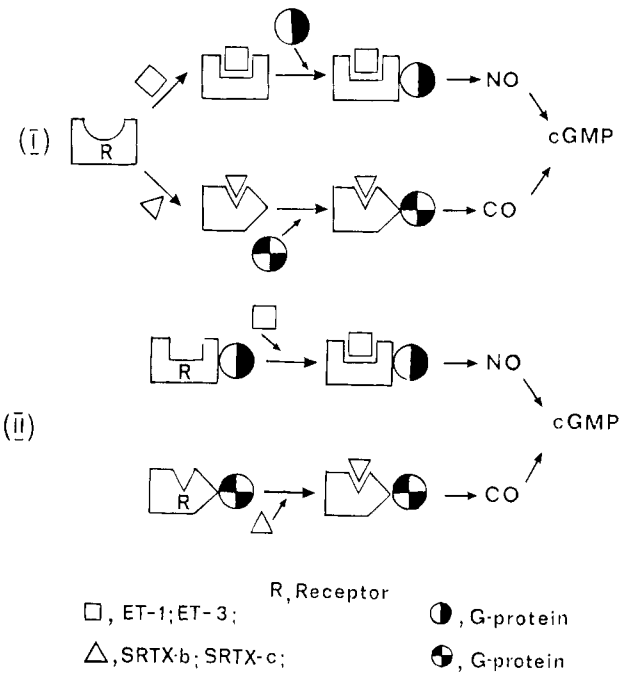
Guanylyl cyclase can also be activated by carbon monoxide (Verma et al., 1993). Since NNA had no effect on cGMP levels elevated by SRTX-b and SRTX-c, we were interested in examining the possible participation of CO in cGMP regulation by SRTXs. To this end, we used zinc protoporphyrin-9 (Zn PP-9), which inhibits the activity of heme oxygenase, the enzyme that degrades heme to biliverdin and releases carbon monoxide. To determine whether Zn PP-9 inhibits guanylyl cyclase directly, we added sodium nitroprusside [SNP (10 $\mu$M)], which generates NO, which in turn can stimulate guanylyl cyclase. Zn PP-9 (0.1 $\mu$M) had no effect on cGMP levels elevated by SNP, indicating the absence of direct guanylyl cyclase inhibition by Zn PP-9. As shown in Figure 5, Zn PP-9 had no effect on cGMP levels stimulated by ET-1 and ET-3; it did, however, inhibit cGMP levels elevated by SRTX-b and SRTX-c, indicating that the CO pathway is involved in signaling by SRTXs.

To determine whether signaling via cGMP cascades is mediated by G-proteins, we examined the effect of PT-catalyzed ADP-ribosylation of G-protein on cGMP production stimulated by ETs and SRTXs, using a previously described experimental protocol (Sokolovsky, 1993). Treatment of rat cerebellar slices with PT (1 $\mu$g/mL, 1 h, 36 °C) completely inhibited stimulation of cGMP production by ET-1 (1 pM) (Figure 6) but did not affect its stimulation by SRTX-b (Figure 6). Thus, it appears that PT-sensitive G-protein mediates only ET-stimulated cGMP formation, which, as shown above, apparently occurs via the nitric oxide pathway. On the other hand, the lack of involvement of PT-sensitive G-protein in the SRTX-b-induced cGMP pathway further supports the above results, suggesting that the induction of cGMP production in cerebellar slices by endothelins and by sarafotoxins occurs via different mechanisms.

Production of cGMP induced by ET-1 or ET-3 was not blocked by the extracellular $Ca^{2+}$ chelator EGTA (1 mM) (not shown).

Endothelins are involved in numerous physiological responses. The involvement of $ET_A$-R at the pM sites for ET-1 and at other sites, though with lower affinity for ET-3 (e.g., $ET_A$-R involvement in cGMP production, as demonstrated in this study), suggests the operation of a novel signaling pathway for such responses. We have also recently documented the stimulation of cAMP production at the pM sites via $ET_A$-R (Sokolovsky et al., 1994), which points to the existence of several signaling pathways induced by

Scheme 1




endothelins. Endothelins stimulate $ET_A$- and $ET_B$-R-mediated phosphoinositide hydrolysis in rat cerebellar slices via PT-insensitive G-protein (Sokolovsky, 1993), triggering the release of intracellular $Ca^{2+}$ and thereby activating $Ca^{2+}$/calmodulin-dependent NO-synthase. Since we show here that cGMP production induced by endothelins involves PT-sensitive G-protein, and because $ET_A$-R is not involved in stimulation of phospholipase C (Sokolovsky et al., 1992), the possible existence of another NO-dependent pathway should be considered.

The results presented in this communication can be explained in terms of a number of possible mechanisms:

(1) Ligand-induced coupling of $ET_A$-R to specific G-proteins (see Scheme 1). In this case, the binding of endothelins to the $ET_A$-R induces its coupling to a PT-sensitive G-protein, leading to the production of cGMP via NO formation. On the other hand, the binding of SRTXs results in coupling of the $ET_A$-R to another G-protein, which is PT-insensitive. This leads to cGMP production via the CO pathway.

(2) Pre-existing functional coupling of $ET_A$-R with PT-sensitive and PT-insensitive G-proteins. This mechanism is similar to the one described above, except that the $ET_A$-R is coupled to a specific G-protein *prior* to ligand binding. In this case (see Scheme 1), the $ET_A$-R bound to the PT-sensitive G-protein interacts specifically with endothelins, while $ET_A$-R complexed with PT-insensitive G-protein(s) binds SRTXs.

(3) Two separate $ET_A$-R subtypes, one of them specific for endothelins and the other for SRTXs. The one that responds to endothelins operates via NO formation, whereas the one that interacts with SRTXs operates via the CO pathway.

In summary, we show here that endothelins and sarafotoxins activate the same receptor sites to produce cGMP but that their signaling pathways differ. The endothelin-induced signal operates via nitric oxide formation and is mediated by PT-sensitive G-protein. The sarafotoxins, while also stimulating cGMP production, operate via a pathway that involves CO formation and does not involve PT-sensitive G-protein.

## ACKNOWLEDGMENT

We thank Ms. Shirley Smith for excellent editorial assistance.

## REFERENCES

Ambar, I., Kloog, Y., Schvartz, I., Hazum, E., & Sokolovsky, M. (1989) *Biochem. Biophys. Res. Commun. 158*, 195−201.
Edwards, R. M., Pullen, M., & Nambi, P. (1992) *Am. J. Physiol. 263*, F1020−F1025.
Fujitani, Y., Udea, H., Okata, T., Urade, Y., & Karaki, H. (1993) *J. Pharmacol. Exp. Ther. 267*, 683−689.
Garbers, D. L. (1989) *J. Biol. Chem. 264*, 9103−9106.
Garthwaite, J. (1991) *Trends Neurosci. 14*, 60−67.
Hirata, Y., Emori, T., Eguchi, S., Kanno, K., Imai, T., Ohta, K., & Marmuro, F. (1993) *J. Clin. Invest. 91*, 1367−1373.
Ignarro, L. J., Ballot, B., & Wood, K. S. (1984) *J. Biol. Chem. 259*, 6201−6207.
Ishii, K., Warner, T. D., Sheng, H., & Murad, F. (1991) *J. Pharmacol. Exp. Ther. 259*, 1102−1107.
Martin, W., Villani, G. M., Jothianandan, D., & Furchgott, R. F. (1985) *J. Pharmacol. Exp. Ther. 232*, 708−716.
Moncada, S., Palmer, R. M. J., & Higgs, E. A. (1991) *Pharmacol. Rev. 43*, 109−142.
Moritoki, H., Miyano, H., Takeuchi, S., Yamaguchi, M., Hisayama, T., & Kondoh, W. (1993) *Br. J. Pharmacol. 108*, 1125−1130.
Rees, D. D., Palmer, R. M. J., Hodson, H. F., & Moncada, S. (1989) *Br. J. Pharmacol. 96*, 418−424.
Sokolovsky, M. (1992) *Pharmacol. Ther. 54*, 129−149.
Sokolovsky, M. (1993) *Cell. Signalling 5*, 473−483.
Sokolovsky, M., Ambar, I., & Galron, R. (1992) *J. Biol. Chem. 267*, 20551−20554.
Sokolovsky, M., Shraga-Levine, Z., & Galron, R. (1994) *Biochemistry 33*, 11417−11419.
Verma, A., Hirsch, D. J., Glatt, C. E., Ronnett, G. V., & Snyder, S. H. (1993) *Science 259*, 381−384.