Vol. 205, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1994 Pages 765-771

VASCULAR ACTIONS OF C-TYPE NATRIURETIC PEPTIDE IN ISOLATED PORCINE CORONARY ARTERIES AND CORONARY VASCULAR SMOOTH MUSCLE CELLS

Chi-Ming WEI[1], Shiling HU[3], Virginia M. MILLER[2], and John C. BURNETT,Jr.[1]

[1]Cardiorenal Research Laboratory, Divisions of [1]Cardiovascular Diseases and [2]Cardiovascular Surgery, Mayo Clinic and Foundation, Rochester, MN 55905

[3]Research Division, CIBA- GEIGY Corp., Summit, NJ 07901

Received October 7, 1994

SUMMARY: C-type natriuretic (CNP) caused concentration-dependent relaxations in porcine coronary arteries with a maximal relaxation (10^{-6}M) of 46%. Relaxations to CNP in isolated coronary arteries were significantly attenuated with potassium channel antagonists charybdotoxin (10^{-7}M) and glibenclamide (10^{-7}M). Membrane potential and K^+ currents were measured in enzymatically dissociated smooth muscle cells from porcine coronary arteries with patch-clamp techniques in a whole-cell mode (n=5). CNP caused K^+ channel activation and membrane hyperpolarization in a dose-dependent manner. This hyperpolarization was markedly suppressed by the potassium channel inhibitor tetraethylammonium (TEA, 5 mM). These results demonstrate that CNP relaxes porcine coronary arterial smooth muscle by hyperpolarization of vascular smooth muscle through potassium channel stimulation.

C-type natriuretic peptide (CNP) is a 22-amino acid peptide genetically distinct from atrial natriuretic peptide (ANP). Like ANP, CNP has a 17-amino acid ring formed by a disulfide bond (1) but lacks the COOH-terminus amino acid extension from the ring structure. CNP is expressed in brain and in bovine and human endothelial cells (2-4). Studies of cells transfected for natriuretic peptide receptors have reported that CNP is the ligand for a CNP specific and selective receptor (NPR-B) (5). This receptor is expressed in vascular smooth muscle cells and in the kidney (6) and is linked to particulate guanylyl cyclase. CNP is a potent venodilator and selective arterial vasodilator with minimal natriuretic action (7-9). Thus, CNP, which is released from endothelial cells (2-4), may function as a paracrine factor in the control of vascular tone (8). In preliminary studies, we have reported that CNP in vivo is a coronary vasodilator in the canine coronary circulation (10) and precontraction in isolated canine coronary arteries with KCl blocks CNP vasorelaxation suggesting that CNP may be a hyperpolarzing factor (11).

The objective of the current study was to determine the vascular actions of CNP in isolated porcine coronary arteries and upon membrane potential in porcine coronary vascular smooth muscle cells and in the presence and absence of potassium channels inhibitors.

0006-291X/94 $5.00

MATERIALS AND METHODS

Organ Chamber Experiments: Rings were cut from coronary arteries of pigs (anesthetized with 30 mg/kg pentobarbital sodium intravenously). These were suspended for the measurement of isometric force in organ chambers filled with aerated (95% O_2 and 5% CO_2) modified Krebs-Ringer bicarbonate solution (composition in mM: 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 0.026 calcium sodium EDTA, and 11.1 dextrose; control solution) at 37°C. In one-half of the rings, the endothelium was removed by gently rubbing the internal surface with a cotton swab saturated with control solution. Each ring was stretched to the optimal point on its length-tension curve as determined by the tension developed to KCl (20 mM) at each level of stretch. The presence of endothelium was determined at the beginning of the experiment by relaxation to acetylcholine (10^{-6} M) during a contraction to prostaglandin $F_{2\alpha}$ (2×10^{-6} M) at optimal length. The maximal contractile tension of each ring was determined by KCl (60 mM). To study responses to CNP, the rings were initially contracted with prostaglandin $F_{2\alpha}$ (2×10^{-6} M). These contractions averaged 30-40% of the maximal contraction to KCl. The CNP was added cumulatively once the contraction had stabilized. To test whether potassium channels are activated by CNP, we used the potassium channel antagonists: charybdotoxin (10^{-7} M) which is a inhibitor of high conductance calcium-dependent potassium channels (12), apamine (10^{-7}M) which is a inhibitor of low conductance calcium-dependent potassium channels, or glibenclamide (10^{-7}M) which is a adenosine triphosphate (ATP)-sensitive potassium channels inhibitor (12).

The following drugs were used: acetylcholine chloride (Sigma Chemical, St. Louis, MO), human CNP (Peninsula Laboratories, Belmont, CA), charybdotoxin (Sigma Chemical, St. Louis, MO), apamine (Sigma Chemical, St. Louis, MO), glibenclamide (Sigma Chemical, St. Louis, MO). All drugs were dissolved in distilled water immediately prior to study, and the concentrations are reported as the final molar concentration (M) in the organ chamber.

Electrophysiological Experiments: Smooth muscle cells from porcine coronary arteries were prepared by enzymatic dissociation method. The enzymes used were 0.2% (weight/volume) collagenase, 0.04% DL-dithiothritol, and 0.2% papain. After enzymatic treatment, fragments of tissue were gently agitated with a blunt-tipped glass pipette. The supernatant containing large quantity of single cells were filtered through a nylon mesh and stored at 4-6 °C. Experiments were started one hour after cell harvest.

The potassium currents and membrane potential in single smooth muscle cells from porcine coronary arteries were measured with the patch-clamp technique in a whole-cell configuration in the absence (n=4) and presence of CNP (0.01 μM, 0.03 μM, and 0.1 μM, n=4 in each group). Patch-clamp recording system has been described elsewhere (13, 14). Briefly, the system includes a List EPC-7 amplifier, a lowpass 8-pole Bessel filter (Frequency Devices 902LPF) and a DAS 900 Digital Data Adapter with built-in VCR (Toshiba Corp.). Data acquisition and analysis were made with a NEC 80386 personal computer loaded with P-clamp software, version 5.5 (Axon Instrument Inc.). Resting membrane potentials and the potassium currents were recorded when the amplifier was set, respectively, in current-clamp and voltage-clamp modes. The patch electrodes were filled with a pipette solution containing (mM) 140 KCl, 5 NaCl, 1 $MgCl_2$, 0.1 $CaCl_2$, 0.6 EGTA, 0.5 K_2ATP, and 10 HEPES. The bath solution had a composition of 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 2 glucose, and 2 HEPES. All patch-clamp experiments were carried out at 22 °C. With the patch-clamp method in whole-cell configuration, the membrane depolarization elicited outward currents were studied by presence of tetraethylammonium (TEA, 5 mM), which is a blocker of the potassium channel.

Statistical Analysis: The results are expressed as the means ± SEM. In organ chamber studies, n equals the number of dogs from which coronary arteries were taken. Rings with and without endothelium were studied in parallel, and Student's t-test for paired observations was used to determine statistical significance among the responses of rings with and without endothelium. Statistical significance was determined at $p<0.05$.

RESULTS AND DISCUSSION

Organ Chamber Experiments: CNP caused concentration-dependent relaxation of porcine (Fig. 1) coronary arterial rings contracted with $PGF_{2\alpha}$. CNP caused relaxation which averaged 46% maximal relaxation which were significantly attenuated by the presence of endothelium (Fig 1).

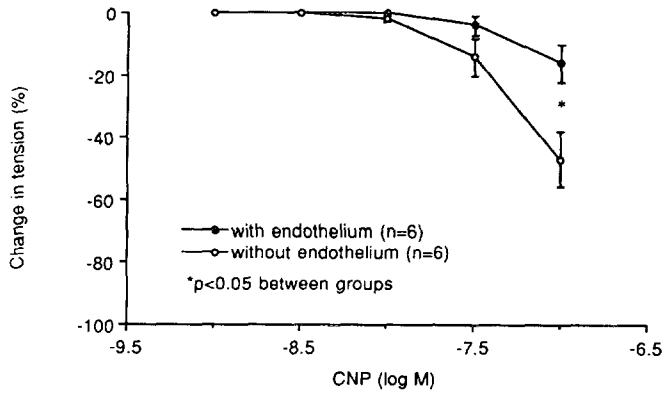

Figure 1.
Relaxations to C-type natriuretic peptide (CNP) in porcine coronary arteries with (closed circle) and without (open circle) endothelium. Data are shown as mean±SEM (n=6) and are expressed as a percent change in tension from contractions to prostaglandin $F_{2\alpha}$ (2×10^{-6} M; 30-40% of maximal contractions to 60 mM KCl). A significant difference between maximal relaxations of rings with and without endothelium was noted. (Student's t-test for paired observations, $p<0.05$.)

The high conductance Ca^{++}-activated potassium channel antagonist charybdotoxin (10^{-7} M) and the ATP-sensitive potassium channel antagonist glibenclamide (10^{-7}M) significantly inhibited relaxations to CNP (Fig 2-A and 2-B). However, the low conductance calcium-dependent potassium channel antagonist apamine (10^{-7}M) had no effect on CNP relaxations (Fig 2-C).

Electrophysiological Experiments: To identify possible ionic mechanisms for the vasodilator activity of CNP, its effect on the whole-cell K^+ currents and membrane potential were examined. Figure 3 (upper part) demonstrates that CNP augmented whole-cell currents in a concentration-dependent manner. The result is also demonstrated in the current-voltage relationship in the lower part of the figure. As shown in Figure 3, CNP increases the whole cell current in a dose dependent way at depolarizing voltages but its effect at the resting voltage is small if any.

Extracellular TEA at low millimolar concentrations has long been known to effectively block the large conductance Ca^{++}-activated K^+ channel (15-19). Figure 4 demonstrated that the whole cell currents of smooth muscle cells from porcine coronary artery are largely blocked by TEA (5 mM). This data suggests contribution of large conductance Ca^{++}-activated K^+ channels to the whole-cell currents at depolarizing voltages.

The present studies demonstrate for the first time that CNP, which is an endothelium-derived peptide, causes relaxation of isolated porcine epicardial coronary arterial rings and that this action is partially blocked by an inhibitor of high conductance Ca^{++}-activated and ATP-sensitive K channels. Secondly, these studies also demonstrate in porcine coronary vascular smooth muscle cells that CNP hyperpolarizes the cell membrane via K^+ channel opening at depolarizing voltages.

The present study documents that CNP relaxes isolated coronary arteries thus extending previous reports that CNP also is a potent venodilator of peripheral veins (7). Of interest is that while this vasorelaxation is not dependent upon the endothelium, the presence of the endothelium limits the full vasorelaxing action of CNP. The mechanism of this unique attenuation is undefined but could evolve degradation or clearance of CNP by neutral

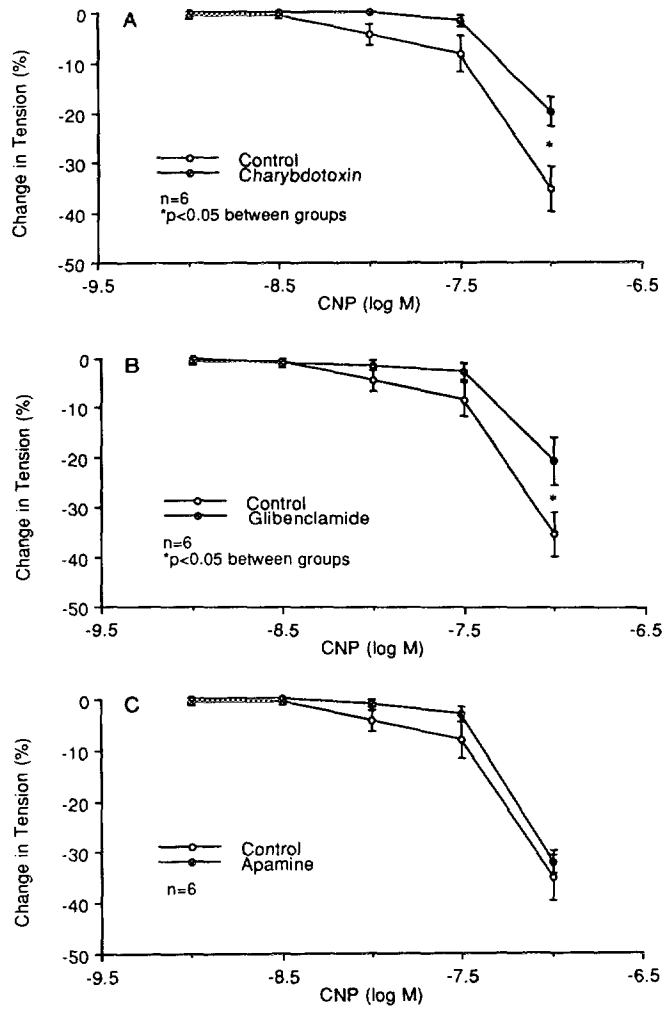

Figure 2.
Relaxations to CNP in rings of porcine left circumflex coronary artery without endothelium in the absence and presence of charybdotoxin (10^{-7}M) (2-A), glibenclamide(10^{-7}M) (2-B) and apamine(10^{-7}M) (2-C). Relaxations are expressed as a percent change in tension from a contraction to prostaglandin $F_{2\alpha}$ (2 x 10^{-6} M). Data are shown as mean±SEM. Asterisk denotes statistically significant difference in maximal relaxation and area under the curve in the absence and presence of inhibitors; Student's t-test for paired observations, $p<0.05$.

endopeptidase or the natriuretic peptide clearance receptor respectively or release of an endothelial-derived contracting factor.

Hyperpolarization of the vascular smooth muscle may be necessary for the natriuretic peptide-mediated vasorelaxation to occur (20). In support of this suggestion, the present studies demonstrated that CNP caused an increase in K^+ currents and concomitant concentration-dependent hyperpolarization of coronary artery vascular smooth muscle cells, although the CNP increase in current was more pronounced at higher membrane potential. These data suggest that CNP activates potassium channels in coronary arterial smooth muscle cell membranes which may lead to relaxation.

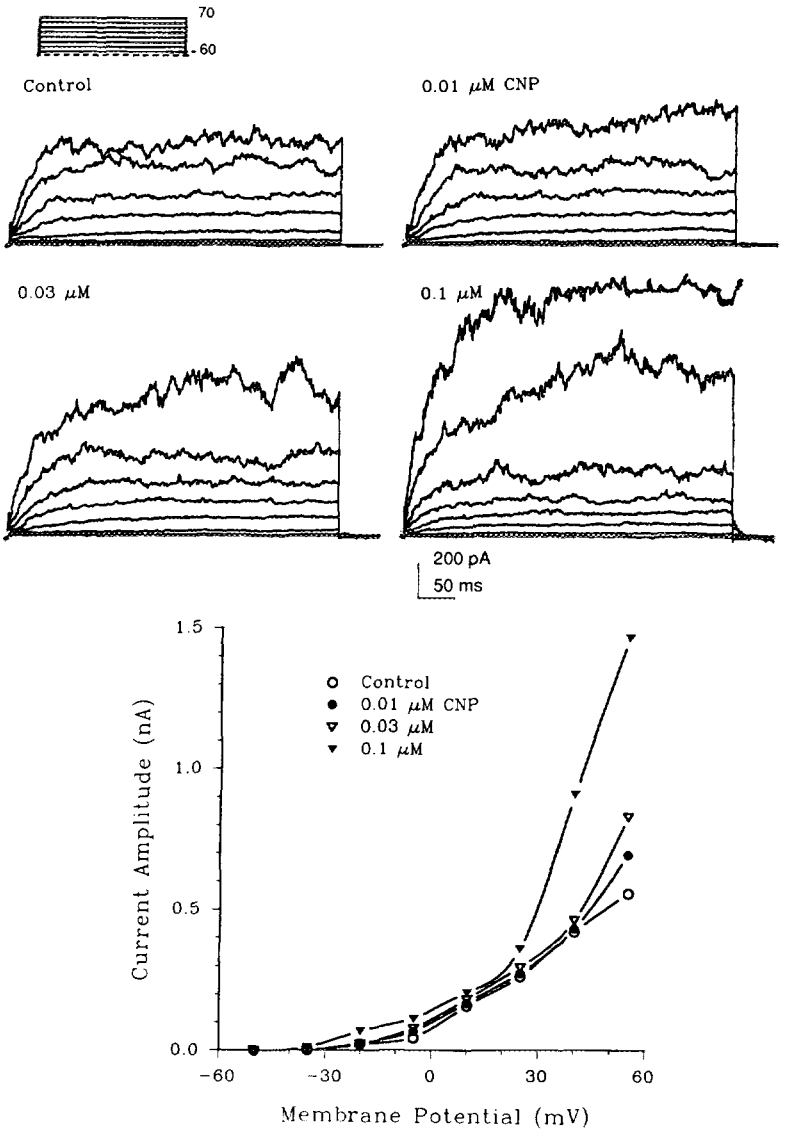

Figure 3.
Augmentation of whole-cell K^+ currents by C-type natriuretic peptide (CNP) in smooth muscle cells from porcine coronary arteries. A family of K^+ currents elicited by depolarizing pulses of a duration of 500 ms and an increment of 15 mV from -50 mV to 55 mV were recorded in control and in CNP at 0.01, 0.03 and 0.1 μM, respectively. Holding potential was -60 mV, see pulse protocol in the top left corner. Lower panel shows the relationship between the amplitude of steady-state K^+ currents and membrane potentials in the absence and presence of CNP at 0.01, 0.03, and 0.1 μM.

High conductance calcium-dependent potassium channels may mediate relaxations to CNP in the porcine tissue as charybdotoxin a inhibitor of these channels (12) decreased the relaxations. The adenosine triphosphate (ATP)-sensitive potassium channels may also be activated by CNP as the inhibitor of these channels glibenclamide (12) significantly attenuated these relaxations. Differential expression of the different calcium-dependent potassium channels or their modulation by other neurohumoral factors may affect the response to CNP in different vascular beds (7,9) or under pathophysiological conditions such as heart failure.

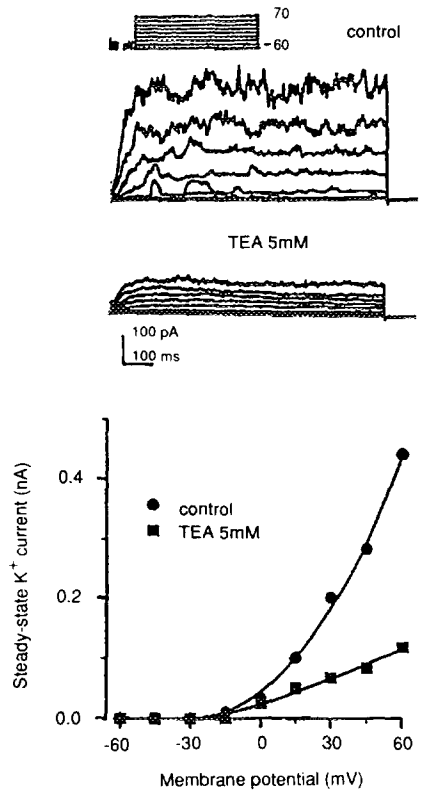

Figure 4.
Whole-cell currents elicited by depolarization steps from -60 mV to +60 mV with an increment of 15 mV in control (top), in the presence of 5 mM TEA (middle), and their respective current-voltage relationship (bottom). Holding potential was -60 mV.

Previous electrophysiological studies suggest the existence of an endothelium-derived hyperpolarizing factor (EDHF) which results in hyperpolarization and relaxation of vascular smooth muscle (21-23). The nature of EDHF remains to be clarified. EDHF may be a labile metabolite of arachidonic acid formed through the P450 pathway (24). More recently, the K^+ channel involved in the EDHF-induced hyperpolarization in the guinea pig coronary artery was largely the Ca^{2+}-activated K^+ channel (25). The results of the present study both in isolated coronary arteries and in coronary vascular smooth muscle cells suggest that endothelium-derived natriuretic peptide (CNP) may be a type of EDHF in the coronary circulation.

Two important observations in the current study and recent reports (26) suggest a link between activation of natriuretic peptide-particulate guanylyl cyclase system and stimulation of K^+ channels. In the current studies, CNP functions via activation of K^+ channels in the coronary artery. Studies by White and co-workers (26) demonstrated that in intact pituitary tumor cells, ANP resulted in cGMP stimulated K^+ channel activity through activation of a cGMP-dependent protein kinase. While the current investigation did not address the signaling mechanism between CNP, cGMP and K^+ channels, the present studies are consistent with a mechanism by which CNP could cause it's biological action via a functional interaction between the CNP-receptor and K^+ currents.

In conclusion, the current studies establish in both intact coronary arteries and in isolated coronary vascular smooth muscle cells that CNP causes relaxation of coronary arterial smooth muscle in part by hyperpolarization of smooth muscle through potassium channel stimulation. We conclude that CNP therefore may be a type of endothelial derived hyperpolarizing factor in the control of coronary vascular tone.

Acknowledgments: This work was supported in part by grants from the Minnesota Affiliate of the American Heart Association (MHA-103), the National Kidney Foundation Midupper Grant, the Grant from Merck Co., the National Heart, Lung, and Blood Institute (HL-36634, HL-07111 and HL42614) and the Mayo Foundation.

REFERENCES

1. Komatsu, Y., Nakao, K., Suga, S., Ogawa, Y., Mukoyama, M., Arai, H., Shirakami, G., Hosoda, K., Nakagawa, O., & Hama, N. (1991) Endocrinology, 129, 1104-1106.
2. Stingo, A.J., Clavell, A.L., Heublein, D.M., Wei, C.M., Pittelkow, M.R., & Burnett, J.C.Jr. (1992) Am. J. Physiol., 263, H1318-H1321.
3. Heublein, D.M., Clavell, A.L., Stingo, A.J., Lerman, A., Wold, L., & Burnett, J.C.Jr. (1992) Peptides, 13, 1017-1019.
4. Suga, S., Nakao, K., Itoh, H., Komatsu, Y., Ogawa, Y., Hama, N., & Imura, H. (1992) J. Clin. Invest., 90, 1145-1149.
5. Koller, K.J., Lowe, D.G., Bunnett, G.L., Minamino, N., Kangawa, K., Matsuo, H., & Goeddel, D.V. (1991) Science, 252, 120-123.
6. Schulz, S., Singh, S., Bellet, R.A., Singh, G., Tubb, D.J., Chin, H., & Garbers, D.L. (1989) Cell, 58, 1155-1162.
7. Wei, C.M., Aarhus, L.L., Miller, V.M., & Burnett, J.C.Jr. (1993) Am. J. Physiol., 264, H71 H73.
8. Clavell, A.L., Stingo, A.J., Wei, C.M., Heublein, D.M., & Burnett, J.C.Jr. (1993) Am. J. Physiol., 264, R290-R295.
9. Wei, C.M., Kim, C.H., Miller, V.M., & Burnett, J.C.Jr. (1993) J. Clin. Invest., 92, 2048-2052.
10. Wright, R.S., Miller, W.L., Aarhus, L.L., & Burnett, J.C.Jr. (1993) Am. J. Hypertension., 6, 16A.
11. Wei, C.M., Hu, S., Stevens, T.L., Kim, C.H., Kinoshita, M., Matsuda, Y., Miller, V.M., & Burnett, J.C.Jr. (1994) J. Am. College Cardiol., 259A.
12. Brayden, J.E., & Nelson, M.T. (1992) Science, 256, 532-535.
13. Hu, S., & Kim, S.S. (1993) Eur. J. Pharmacol., 230, 215-221.
14. Hu, S., Kim, H.S., Okolie, P., & Weiss, G.B. (1990) J. Pharmacol. Exp. Ther., 253, 771-778.
15. Benham, C.D., Bolton, T.B., Lang, R.J., & Takewaki, T. (1985) Pflugers Arch., 403 (2), 120-127.
16. Singer, J.J., & Walsh, J.V.Jr. (1986) Mem. Biochem., 6 (2), 83-110.
17. Latorre, R., Oberhauser, A., Labarca, P., & Alvarez, O. (1989) Annu. Rev. Physiol., 51, 385-399.
18. Hu, S.L., Yamamoto, Y., & Kao, C.Y. (1989) J. Gen. Physiol., 94 (5), 849-862.
19. Akbarali, H., Nakajima, T., Wyse, D.G., & Giles, W. (1990) Can. J. Physiol. Pharmacol., 68, 1489-1494.
20. Kauser, K., & Rubanyi, G.M. (1992) Endothelial Regulation of Vascular Tone. (U. S. Ryan and G. M. Rubanyi, Ed.), pp. 341-354. Marcel Dekker, Inc.
21. Huang, A.H., Busse, R., & Bassenge, E. (1988) Arch Pharmacol, 338, 438-443.
22. Chen, G.F., & Suzuki, H. (1989) J. Physiol., 410, 91-106.
23. Nagao, T., & Vanhoutte, P.M. (1992) J. Physiol., 445, 355-367.
24. Komori, K., & Vanhoutte, P.M. (1990) Blood Vessels, 27, 238-245.
25. Chen, G.F., Yamamoto, Y., Miwa, K., & Suzuki, H. (1991) Am. J. Physiol., 260, H1888 H1892.
26. White, R.E., Lee, A.B., Shcherbatko, A.D., Lincoln, T.M., Schonburnn, A., & Armstrong, D.L. (1993) Nature, 361, 263-266.