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Chapter 24

Vascular Tissue Preservation Techniques

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Introduction

Pharmacological studies on isolated blood vessels generally require freshly obtained tissues. These are usually taken from various animals, although results obtained from human tissue would be the most accurate and predictable for human pharmacology. Human tissue can be obtained from surgery and autopsy, but besides obtaining human material, the main problem is the diversity of tissue types available and the irregularity of supply. However, when available, often much more material is supplied than can be used within a few hours. Isolated tissues have a very short life-span, and experiments should commence as soon as possible, if not immediately, after removal from the body, but this is not always convenient. Hence, the advantages of a simple and reliable storage method for ensuring the supply of adequate vascular preparations for pharmacological studies are readily apparent.

Cold Storage of Vascular Tissue

Isolated vascular tissue can be stored for several days in physiologic salt solution at 2–8°C, and be used in pharmacological experiments. However, storage of blood vessels in the cold produces several species- and time-dependent progressive changes in their physiologic characteristics and their responses to drugs.

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Cold Storage-Induced Changes

The most consistent changes observed in blood vessels after cold storage for 5–9 d is a degeneration of nerve endings as assessed by attenuated responses to transmural electric stimulation of coronary arteries from cattle[1] and guinea pig,[2] and/or by reduced norepinephrine (NE) content and ^{3}H-NE uptake in rabbit aorta[3,4] and portal veins.[5,6] Apparently controversial results on the contractile responsiveness to receptor-mediated agonists of various blood vessels reflect both species differences and time dependency of changes occurring during prolonged cold storage. Considerable species differences have been observed when the NE sensitivity of portal veins from rabbit, guinea pig, and rat was tested after 5–9 d of storage at 2–4°C.[5,6] Perfused branches of the femoral artery from dogs were sensitized to the stimulant activity of α-adrenoceptor agonists and 5-HT when stored for 24 h at 6°C,[7] whereas the sensitivity to these agonists appeared unchanged when the same vessels from dogs[8,9] or monkeys[10] had been stored for 3–7 d at 4°C. The same applies for cold-stored canine auricular arteries[11] and cattle coronary arteries,[1] which had been stored for 5–6 d at 4°C. Naturally, the phenomenon of cold storage-induced sensitization of vascular smooth muscle is transient in nature. Hence, rabbit aortae develop increased sensitivity to NE and epinephrine when stored for up to 7 d at 2–6°C,[3,12,13] but this phenomenon is reversed when the storage time is extended to 10–14 d.[4,12]

Contrary to that observed with receptor-mediated contractile responses, cold storage of skeletal muscle arteries from both dog[8,9] and monkey[10] and of rabbit aortae[4,12] leads consistently to significant suppression of the contractile responses to potassium chloride (KCl). Furthermore, it has been observed that cold-stored rabbit aortae, although supersensitive to NE, are subsensitive to calcium, i.e., they require a higher extracellular calcium concentration to develop the same tension as fresh aortae.[13] Several observations support the contention that cold storage of vascular smooth muscle induces depolarization of the cell membrane. Evidence for this comes from the finding that in cold-stored vascular smooth muscle, the tissue Na^+ and Ca^{2+} content is markedly

increased, whereas the K^+ content is significantly decreased.[6,7,13] Even after 2 h of equilibration in normal Ringer solution at 37°C, when Na^+ content is normalized, the K^+ content is still significantly diminished.[13] Moreover, administration of external K^+ decreases the $^{45}Ca^{2+}$ influx into cold-stored portal veins, suggesting that cold storage alters the Na^+-K^+ balance by inhibiting the electrogenic Na^+ pump.[6] Recently, direct evidence for cold storage-induced depolarization of smooth muscle cells has been presented by the finding that in cold-stored guinea pig coronary arteries the resting membrane potential in the smooth muscle cells increases from –60 to –47 mV.[2]

In conclusion, cold storage is a simple method of storing isolated blood vessels for a short time period. However, both degeneration of nerve terminals and depolarization of the cell membrane will modify progressively their physiologic and functional characteristics.

Cryopreservation of Vascular Tissue

Mechanisms of Freezing Injury

Freezing of living mammalian cells without cryoprotective additives generally induces severe cell damage, and few if any cells survive.[14–18] During cooling to subzero temperatures, water tends to flow out of the cell and freeze externally. Microscopic observations have shown that cells do indeed shrink during freezing. If cooled too fast, cells are injured by the formation of intracellular ice crystals; if cooled too slowly, however, cells will be injured by the "solution effect," i.e., by changes in the composition of extra- and intracellular solutions, since the concentration of extracellular salts in the residual unfrozen medium increases as ice is formed.[15] As a consequence of changes in both temperature and concentration, cell membranes become leaky to cations and permeable to substances (e.g., to sucrose) that normally do not enter the cell.[16] In addition, evidence has been presented that the fraction of water that remains unfrozen is damaging, since it gives rise to crushing of the cells within the ice masses.[19]

Cryoprotective Additives

It is assumed that permeating cryoprotective agents such as dimethyl sulfoxide (DMSO) and glycerol, by entering the cell, replace some water, thereby protecting the cell from damage during freezing.[17] The cryoprotecting activities of various agents, such as DMSO, glycerol, *N*-methylacetamide, dimethylacetamide, *N*-methylformamide, dimethylformamide, and polyethylene glycol 400, have been tested in canine saphenous veins taking the maximal response to NE as a parameter for the postthaw recovery. The best postthaw recovery, about 60% of that produced by unfrozen veins, was obtained with venous segments that had been frozen slowly in fetal calf serum containing 1.8*M* DMSO.[20–22]

Nonpermeating cryoprotectants, such as sucrose and hydroxyethyl starch, function at the outer surface of the cell. They are suggested to stabilize the cell volume by retaining more liquid water at low temperatures, thereby reducing the external electrolyte concentration.[15] Both types of cryoprotecting agents act directly at the level of cell membrane, yet their protective action may be synergistic. Indeed, addition of 0.1*M* sucrose to a DMSO-containing cryomedium promotes the postthaw recovery of blood vessels, such as canine saphenous veins and porcine coronary arteries, although sucrose alone does not exhibit any noticeable cryoprotecting activity.[23]

Cryomedia

For many cell types, it is important to have serum or other high-molecular-weight polymers in the cryomedium. This has been demonstrated recently for endothelial cells in canine coronary arteries that had been frozen in Krebs-Henseleit solution (KH) containing 20% FCS and 1.8*M* DMSO. Omission of FCS from the cryomedium resulted in significant morphological changes and functional attenuation of the endothelial cells, although both the contractile and the endothelium-independent relaxant responses of the smooth muscle cells were unchanged.[24] However, considerable species differences appear to exist. Figure 1 shows concentration–response curves to substance P, the effect of which is strictly endothelium-dependent,[25] in pig circumflex coronary arteries that

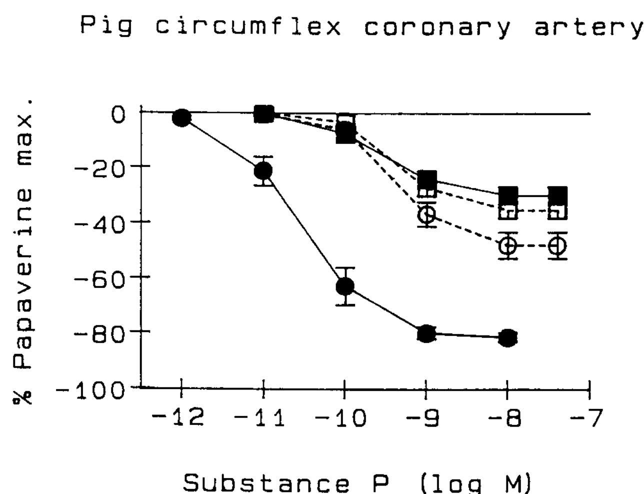

Fig. 1 Relaxant responses to substance P of rings from porcine circumflex coronary arteries stimulated with 3 μ*M* $PGF_{2\alpha}$ without (●) and after cryopreservation at –196°C in fetal calf serum (FCS, ■), Krebs-Henseleit solution (KH, O), and in KH with 50% FCS (□), containing 1.8*M* DMSO and 0.1*M* sucrose as cryoprotectants. The effects are expressed as percentages of the maximum response to papaverine, and the bars represent means ± SEM. For each point, *n* = 14. Method according to ref. 23.

had been frozen in various cryomedia, i.e., FCS, KH, and KH with 50% FCS, containing 1.8*M* DMSO and 0.1*M* sucrose. In these experiments, optimal postthaw recovery was obtained with pig coronary arteries that had been frozen in a medium without FCS. The same applies for smooth muscle contractile responses to $PGF_{2\alpha}$ and endothelium-independent relaxant responses to isoprenaline of pig circumflex coronary arteries that were better preserved in tissues that had been frozen in a medium without FCS (Fig. 2). However, here again considerable species differences exist. In contrast to that observed with pig coronary arteries, optimal postthaw recovery of both contractile responses to NE and relaxant responses to aminophylline of human pulmonary arteries were obtained with tissues that had been frozen in media containing at least 50% FCS (unpublished).

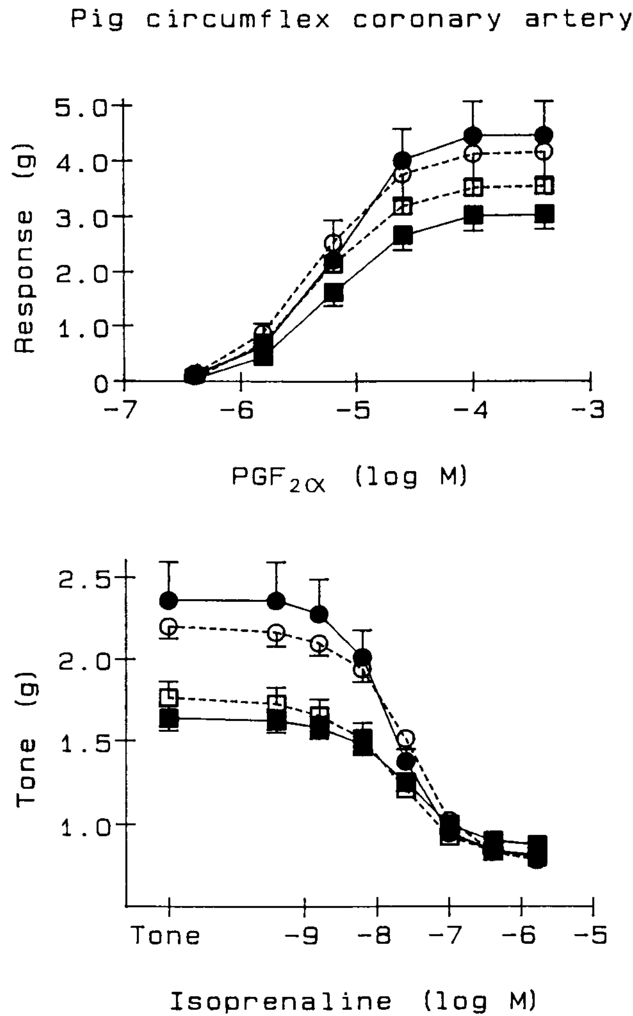

Fig. 2. Contractile responses to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, top) and relaxant responses to isoprenaline during stimulation with 3 μ*M* $PGF_{2\alpha}$ in the presence of 10 n*M* ketanserin (bottom), of rings from porcine circumflex coronary arteries without (●) and after cryopreservation at –196°C in fetal calf serum (FCS, ■), Krebs-Henseleit solution (KH, ○), and in KH with 50% FCS (□), containing 1.8*M* DMSO and 0.1*M* sucrose as cryoprotectants. The effects are expressed in grams. Tone indicates the $PGF_{2\alpha}$-induced active tone in addition to the existing passive preload. The bars represent means ± SEM. For each point, *n* = 8–20. Method according to ref. *23*.

Freezing Procedure

The optimal cooling rate not only depends on the type of cryoprotecting agent, but it also differs from cell to cell and is primarily dependent on (1) the water permeability of the cell membrane and (2) the surface-area-to-volume ratio of the cell. The presence of many different cell types within a tissue or organ implies, therefore, that no one freezing/thawing procedure can satisfy them all.[17] Moreover, in many peripheral arteries, cells are quite densely packed together, which may also reduce cell survival. Veins and pulmonary vessels are considerably less compact and often show better postthaw recovery than peripheral arteries. To minimize the effects of freezing damage, tissues are generally equilibrated with the cryoprotective medium before being slowly frozen. For most mammalian cells, the optimal cooling rate lies between 0.3 and 10°C/min. Once a sample is cooled to about –70°C, it can be transferred directly into liquid nitrogen (–196°C) and stored there indefinitely until use.

Thawing Procedure

In general, a rapid rate of warming that limits the growth of ice crystals in the frozen samples is desirable. As ice is converted into free water, cells are exposed to an extracellular hypotonic solution. Moreover, the dehydrated cells must now rehydrate in order to remain in osmotic equilibrium. Serum or other high-molecular-weight polymers in the medium may reduce the damaging effect of the dilution shock.[15] Before use, the frozen samples are thawed rapidly by placing the ampules for 2.5 min in a 37°C water bath. The vascular tissues are then rinsed in a dish containing Krebs-Henseleit solution to dilute the cryoprotective agent. With isolated mammalian cells, often a stepwise dilution protocol is used in order to avoid osmotic shock, which may kill cells when returned to isotonic solution. With isolated blood vessels, such as canine saphenous veins and rat portal veins, that had been frozen in fetal calf serum containing 1.8*M* DMSO, this procedure, did not improve the postthaw recovery (unpublished).

Hence, many factors during the freezing/thawing process, such as cryoprotective additives, cryomedium, temperature, and

rate of addition and removal of the cryoprotectant(s), as well as the rate of cooling and rewarming, may modify the postthaw recovery of cryopreserved tissues. Optimal combination between cryoprotectants and cooling/thawing rates has to be derived largely by a process of trial and error for each tissue type.

Postthaw Recovery

Effects of Ion Transport

When frozen/thawed vessels are suspended in Krebs-Henseleit solution, in most instances, the initial preload is followed by a transient contraction before the smooth muscle relaxes, suggesting a transient depolarization. On the other hand, in frozen/thawed canine saphenous veins, contractile responses and $^{45}Ca^{2+}$ uptake during both depolarization or α_2-adrenoceptor stimulation are well preserved and similar to that observed with unfrozen veins. Moreover, a significant correlation between $^{45}Ca^{2+}$ uptake and contractile responses during stimulation by KCl or guanfacine in the absence and presence of various calcium channel blockers suggests that cryopreservation of smooth muscle cells from dog saphenous veins does not alter the Ca^{2+} transport.[26] By contrast, in frozen/thawed human pulmonary arteries, a potassium channel opener, such as cromakalim, SDZ PCO400, or RP 49356, proved to be significantly less potent than in unfrozen vessels,[27] suggesting that in certain vascular tissues, the freezing/thawing process may induce some depolarization of the smooth muscle cell membranes.

Enzyme Activity and Biomechanical Properties

After cryopreservation of human and canine veins, both the monoamine oxidase activity[28,29] and the endogenous prostaglandin synthesis[28,30–32] are well preserved. However, despite unchanged collagen synthesis and elastic properties of venous tissue,[33,34] the evidence suggests that under arterial hemodynamic conditions, the compliance of cryopreserved canine veins is significantly reduced.[30]

Adrenergic Nerve Endings

Adrenergic nerve endings appear to be well preserved after cryopreservation. Evidence for this comes from experiments with rabbit ear arteries that had been immersed in newborn calf serum containing 1.8*M* DMSO and stored at –70°C. After thawing, the contractile responses to electric field stimulation of these arteries are similar to those elicited in unfrozen tissues.[35] In addition, determination of basal and stimulation-induced tritium overflow after preincubation with ^{3}H-NE of canine saphenous vein strips has shown that cryopreservation does not alter the absolute tritium overflow during sustained electrical stimulation, yet the basal outflow from frozen/thawed vein strips is slightly higher than that observed with strips from fresh veins.[22]

Endothelial Cell Function

Relaxant responses to various hormones and neurotransmitters are known to be mediated by the release of endothelium-derived relaxing factor(s) (EDRF)[36] and represent a useful tool to assess the viability of vascular endothelial cells after cryopreservation. Using this method, it has been demonstrated that endothelium-mediated relaxant responses to acetylcholine of a rabbit central ear artery and its main side branch are well preserved after storage for several days at –70°C while suspended in newborn calf serum containing 1.8*M* DMSO.[35] The same applies for the endothelium-dependent relaxant responses of both human[37] and porcine[23,38] coronary arteries following cryostorage at –75 and –196°C. For some arteries, such as canine circumflex coronary, it appears to be important to have serum in the cryomedium, to provide protection for the endothelial cells.[24] However, as shown in Fig. 1, with pig circumflex coronary arteries, optimal postthaw endothelial cell function can be obtained when the arteries are frozen in KH containing 1.8*M* DMSO and 0.1*M* sucrose without FCS. Yet the substance P-induced endothelium-dependent relaxation[25] was considerably weaker in the frozen/thawed arteries as compared to unfrozen tissues. Canine veins may release both endothelium-derived relaxing and contracting factors,[39] and it has been

shown that even in autogenous venous grafts, the endothelium of cryopreserved veins can release both relaxing and contracting factors.[40]

Smooth Muscle Cell Function

Functional changes of smooth muscle cells following cryopreservation have been estimated by measuring responses to numerous contracting agonists and/or endothelium-independent vasodilators in isolated blood vessels from various species such as dogs,[21,23,24,26,28,40–42] pigs,[23,38] rabbits,[35] and humans.[21,27,29,37] Generally, a good functional postthaw recovery was obtained with vessels that had been immersed in media containing 1.8–2.1*M* DMSO, slowly frozen to –70°C, and stored at –70 to –196°C. After thawing, in most of these blood vessels, the maximum contractile force was found to be diminished when compared to that produced by unfrozen tissues. However, there was always a very good correlation when the affinities for different agonists on frozen/thawed and unfrozen vessels were compared. Thus, highly significant correlations of pD_2 values for various agonists on fresh and frozen/thawed canine basilar arteries,[28] pig circumflex coronary arteries,[38] human pulmonary vessels,[27] and human saphenous veins[29] have been demonstrated (Fig. 3). Furthermore, the affinity parameters (pA_2 and pD'_2 values) for all receptor antagonists investigated up to now proved to be unchanged after cryopreservation. The same is true for calcium channel blockers tested against KCl and guanfacine in canine saphenous veins.[26]

Summary

Despite the relevance of human isolated tissue to human pharmacology, its use is still very much the exception rather than the rule. The major reason for this is that the supply of fresh human material is both irregular and unpredictable, and once removed from the patient, it has a very short life-span.

Storage of isolated blood vessels in physiologic salt solution at 4°C induces rapid and progressive changes of physiologic and functional properties within a few days. Cryopreservation and storage at –196°C of isolated blood vessels offers the prospect of

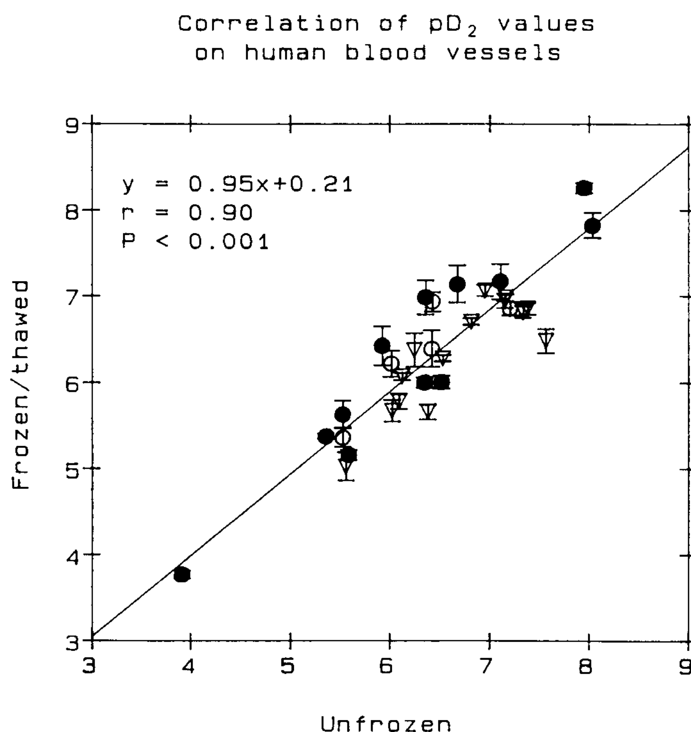

Fig. 3. Correlation between drug potencies as assessed by the pD_2 values, for various agonists determined on unfrozen (abscissa scale) and frozen/thawed (ordinate scale) human saphenous veins (▽), human basilar arteries (○), and human pulmonary arteries (■). Data were compared by linear regression analysis. The correlation coefficient and regression for the correlation are indicated.

indefinite storage of the material with only damage associated with the freezing and thawing process. Despite certain problems, such as maintenance of the endothelial function and some reduction in contractile force, the main biochemical properties, uptake mechanisms, and affinities of most agonists and antagonists have been shown to be well preserved after cryopreservation. Hence, this technique offers clear potential for ensuring the supply of vascular material for pharmacological studies.

References

[1] Kalsner, S. and Quillan, M. (1989) *J. Pharmacol. Exp. Ther.* **249,** 785–789.
[2] Keef, K. D. and Kreulen, D. (1988) *Circ. Res.* **62,** 585–595.
[3] Shibata, S., Hattori, K., Sakurai, I., Mori, J., and Fujiwara, M. (1971) *J. Pharmacol. Exp. Ther.* **177,** 621–632.
[4] Varma, D. R. and McCullough, H. N. (1969) *J. Pharmacol. Exp. Ther.* **166,** 26–34.
[5] Hughes, J. and Vane, J. R. (1970) *Br. J. Pharmacol.* **39,** 476–489.
[6] Kaiman, M. and Shibata, S. (1987) *Blood Vessels* **15,** 217–230.
[7] Murphy, J. C., Carrier, O., and Shadi, J. (1973) *Am. J. Physiol.* **225,** 1187–1191.
[8] Sinanovic, O. and Chiba, S. (1987) *Arch. Int. Pharmacodyn. Ther.* **287,** 146–157.
[9] Sinanovic, O. and Chiba, S. (1987) *Eur. J. Pharmacol.* **143,** 353–360.
[10] Sinanovic, O. and Chiba, S. (1988) *Jpn. J. Pharmacol.* **46,** 237–246.
[11] Ito, T. and Chiba, S. (1985) *Arch. Int. Pharmacodyn. Ther.* **275,** 13–21.
[12] Shibata, S. (1969) *Circ. Res.* **14,** 179–187.
[13] Carrier, O., Jr., Murphy, J. C., and Tenner, T. E. (1973) *Eur. J. Pharmacol.* **24,** 225–233.
[14] Litvan, G. G. (1972) *Cryobiology* **9,** 182–189.
[15] Mazur, P. (1977) *The Freezing of Mammalian Embryos* (Elliott, K. and Whelan, J., eds.), Elsevier, Amsterdam, pp. 19–42.
[16] Pegg, D. E. (1976) *J. Clin. Path.* **29,** 271–285.
[17] Pegg, D. E. (1985) *Progress in Transplantation* (Morris, P. J. and Tilney, N. L., eds.), Churchill Livingstone, Edinburgh, pp. 69–105.
[18] Pegg, D. E. (1987) *The Biophysics of Organ Cryopreservation* (Pegg, D. E., and Karow, A. M., Jr., eds.), Plenum, New York, pp. 117–140.
[19] Schneider, U. and Mazur, P. (1987) *Cryobiology* **24,** 17–17.
[20] Müller-Schweinitzer, E. (1988) *Folia Haematol, Leipzig* **115,** 405–410.
[21] Müller-Schweinitzer, E. (1988) *TIPS* **9,** 221–223.
[22] Müller-Schweinitzer, E. and Tapparelli, C. (1987) *Neuronal Messengers in Vascular Function* (Nobin, A., Owman, C., and Arneklo-Nobin, B., eds.), Elsevier (Biomedical Division), Amsterdam, pp. 105–110.
[23] Müller-Schweinitzer, E. and Ellis, P. (1992) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **345,** 594–597.
[24] Ku, D. D., Willis, W. L., and Caulfield, J. B. (1990) *Cryobiology* **27,** 511–520.
[25] Gulati, N., Mathison, R., Huggel, H., Regoli, D., and Beny, J. L. (1987) *Eur. J. Pharmacol.* **137,** 149–154.
[26] Ebeigbe, A. B., Müller-Schweinitzer, E., and Vogel, A. (1988) *Brit. J. Pharmacol.* **94,** 381–388.
[27] Ellis, P. and Müller-Schweinitzer, E. (1991) *Br. J. Pharmacol.* **103,** 1377–1380.

[28] Müller-Schweinitzer, E. and Tapparelli, C. (1986) *Naunyn Schmiedeberg's Arch. Pharmacol.* **332,** 74–78.
[29] Müller-Schweinitzer, E., Tapparelli, C., and Victorzon, M. (1986) *Brit. J. Pharmacol.* **88,** 685–687.
[30] Showalter, D., Durham, S., Sheppeck, R., Berceli, S., Greisler, H., Brockman, K., Makaroun, M., Webster, M., Steed, D., Siewers, R., and Borovetz, H. (1989) *Surgery (St. Louis)* **106,** 652–659.
[31] Louagie, Y. A., Legrand-Monsieur, A., Lavenne-Pardonge, E., Remacle, C., Delvaux, P., Maldague, P., Buche, M., Ponlot, R., and Schoevaerdts, J.-C. (1990) *J. Cardiovasc. Surg.* **31,** 92–100.
[32] Passani, S. L., Angelini, G. D., Breckenridge, I. M., and Newby, A. C. (1988) *Eur. J. Cardiothorac. Surg.* **2,** 233–236.
[33] Brockbank, K. G. M., Donovan, T. J., Ruby, S. T., Carpenter, J. F., Hagen, P.-O., and Woodley, M. A. (1990) *J. Vasc. Surg.* **11,** 94–102.
[34] L'ltalien, G. J., Maloney, R. D., and Abbott, W. M. (1979) *J. Surg. Res.* **27,** 239–243.
[35] Thompson, L., Duckworth, J., and Bevan, J. (1989) *Blood Vessels* **26,** 157–164.
[36] Furchgott, R. F. and Zawadzki, J. V. (1989) *Nature* **288,** 373–376.
[37] Ku, D. D., Winn, M. J., Grigsby, T., and Caulfield, J. B. (1992) *Cryobiology* **29,** 199–209.
[38] Schoeffter, P. and Müller-Schweinitzer, E. (1990) *J. Pharm. Pharmacol.* **42,** 646–651.
[39] Furchgott, R. F. and Vanhoutte, P. M. (1989) *FASEB J.* **3,** 2007–2018.
[40] Elmore, J. R., Gloviczki, P., Brockbank, K. G. M., and Miller, V. M. (1991) *J. Vasc. Surg.* **13,** 584–592.
[41] Weber, T. R., Dent, D. L., Lindenauer, S. M., Allen, E., Weatherbee, L., Spencer, H. H., and Gleich, P. (1975) *J. Surg. Res.* **18,** 247–255.
[42] Dent, T. L., Weber, T. R., Lindenauer, S. M., Ascher, N., Weatherbee, L., Allen, E., and Spencer, H. H. (1974) *Surg. Forum (Philadelphia)* **25,** 241–243.