MECHANISMS OF STORAGE AND RELEASE OF S-NITROSOTHIOLS FROM THE VASCULAR ENDOTHELIUM

by

MALEKA PARVEEN HASHMI

(Under the Direction of Stephen J Lewis)

ABSTRACT

Evidence suggests that the vasodilator actions of S-nitrosothiols more closely resemble those of endothelium-dependent-relaxing factor (EDRF) than do those of nitric oxide (NO). S-nitrosothiols such as L-S-nitrosocysteine are thought to be stored within cytoplasmic vesicles in endothelial cells. Stimulation of endothelial cells with agonists or shear stress is thought to mobilize these vesicles by exocytotic mechanisms.

We have shown that cytoplasmic vesicles exists in the endothelium of rat cerebral and basilar arteries, and in small rat pulmonary, mesenteric and femoral arteries. The presence of these vesicles suggests that a ubiquitous process, namely, vesicular exocytosis, may be involved in the ability of the endothelium to regulate vascular tone. This study demonstrates the presence of NADPH diaphorase positive vesicles, which may be a histochemical marker for S-nitrosothiols, rather than for NO synthase. We have demonstrated that endothelial cells contain fusion proteins necessary for vesicular exocytosis at the light level and ultrastructurally.

Ultrastructurally, vesicle-associated membrane protein (VAMP) was shown to be closely associated with the vesicle membrane, while synaptosomal-associated protein of 25KDa (SNAP-25) and syntaxin were located in the endothelial cell membrane. The fusion proteins are therefore ideally located for core complex formation, which precedes exocytosis. We found that the endothelium-dependent agonist, acetylcholine, elicited a pronounced relaxation of small pulmonary, mesenteric and femoral arteries. This observation was paired with a significant increase in the number of fused vesicles at the endothelial cell membrane. In systemic arteries, hypoxia elicited relaxation and an increase in the numbers of fused vesicles at the endothelial cell membrane. In the small pulmonary artery, hypoxia elicited vasoconstriction and an increase in the number of fused vesicles.

These results support the possibility that endothelium-dependent relaxation in response to stimuli such as shear stress and hypoxia is achieved by (1) mobilization of S-nitrosothiol-containing vesicles to the endothelial plasma membrane, (2) fusion of the vesicle to the plasma membrane and then exocytosis (release), and (3) S-nitrosothiol-mediated relaxation of vascular smooth muscle.

Keywords: Endothelial cell, Endothelium-dependent-relaxing factor, Exocytosis, S-nitrosothiols,

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DEDICATION

To

My Parents

Prof. MSJ Hashmi and Dr Kashmeri Hashmi

My Husband

Dr Stephen Hill

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Words cannot begin to describe my thanks and appreciation for my major professor, Dr. Stephen Lewis. You always believed in me and I will never forget your constant support and encouragement. It has been a privilege working with and learning from you and I am honored to have been one of your students.

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CHAPTER 1

INTRODUCTION

Nitric Oxide as an Endothelium-Derived Relaxing Factor

Furchgott and Zawadski (1980) were the first to provide evidence that the endothelium is necessary for acetylcholine-induced vasodilation of rabbit aortic rings. Furchgott and Zawadski (1980) concluded that acetylcholine stimulated the endothelium to release a substance that was mediating this relaxation. Furchgott and Zawadski (1980) termed this substance endothelium-dependent-relaxing factor (EDRF). Subsequently, several groups attempted to identify EDRF, which resulted in a race to identify the factor(s) by which the endothelium governed relaxation. The identification of EDRF proved elusive for several reasons, primarily because its half life was extremely short (Furchgott and Zawadski, 1980). Ignarro et al (1987) and Palmer et al (1987) first provided evidence that nitric oxide was an EDRF. This suggestion initially drew skepticism since nitric oxide, a colorless, odorless gas that was discovered by Joseph Priestly in 1772, was not considered to be an endogenous substance but rather an atmospheric pollutant contributing to smog, acid rain and cigarette smoke (Culotta, 1992).

Since 1987, substantial evidence has accumulated that nitric oxide (NO) is formed by numerous types of mammalian cells including neurons and vascular endothelial cells (see Furchgott, 1999; Ignarro, 2002; Moncada and Higgs, 2006). Moreover, endogenous NO is thought to be involved in numerous physiological processes such as neurotransmission within the brain and autonomic nervous system, inhibition of platelet activity, immune cascades and endothelial control of vascular smooth muscle tone (Murad, 1996; Schmidt and Walter, 1994).

Nitric Oxide Synthases

The catalytically self-contained family of P_{450} enzymes, the NO synthases (NOS), oxidize the substrate, L-arginine, to the free radical, nitric oxide, and the by-product, L-citrulline. Catalytically-efficient NOS requires molecular oxygen, β-NADPH as the source of reducing equivalents (electrons), and cofactors such as heme iron and the electron-transfer molecules, flavin mononucleotide, flavin adenine dinucleotide, and tetrahydrobiopterin (Marletta, 1994). There are three isoforms of NOS. Two isoforms are constitutive and one isoform is inducible. The constitutive forms are termed neuronal NOS (NOS-I or nNOS) and endothelial NOS (NOS-III or eNOS), whereas the inducible form of NOS is known as NOS-II or iNOS (Lamas and Michel, 1997). Despite their trivial names, both constitutive forms of NOS are abundantly expressed in endothelial and epithelial cells, central, autonomic and sensory neurons and platelets (see Moncada et al., 1991; Ruggiero et al., 1996; Davisson et al., 1997).

Agonist-induced stimulation of acetylcholine and bradykinin receptors on the plasma membranes of endothelial cells causes a G-protein-mediated increase in intracellular Ca^{2+} levels in these cells (see Moncada et al., 1991). The increase in intracellular Ca^{2+} leads to the increase in the concentrations of Ca^{2+}-calmodulin complex. This complex binds to and “activates” eNOS and nNOS whereas iNOS does not have a Ca^{2+}-calmodulin binding domain and is insensitive to changes in intracellular Ca^{2+} (Forstermann et al, 1991). The application of acetylcholine or bradykinin to endothelial cells causes the rapid

release of femtomole to picomole concentrations of NO (Forstermann et al, 1991). iNOS expression in macrophages, hepatocytes, neutrophils and epithelial, endothelial, mesangial and vascular smooth muscle cells is activated by pro-inflammatory cytokines (Morris and Billiar, 1994) Upon expression, iNOS is constitutively active and does not have a Ca^{2+}-calmodulin binding domain and so is insensitive to changes in intracellular Ca^{2+} (Forstermann et al, 1991).

Nitric Oxide and Other EDRFs

The endothelium releases a variety of mediators that regulate vascular tone such as NO, prostacyclin, endothelin-1, thromboxane A_2, prostaglandin H_2 and superoxide anion (Palmer et al, 1987; Yanagisawa et al, 1988; Furchgott and Vanhoutte, 1989; Luscher and Vanhoutte, 1990; Moncada et al, 1991; Luscher et al, 1993; Vanhoutte, 1993). When NO is produced in the endothelium of arteries and veins it can diffuse into the adjacent vascular smooth muscle where it causes vasodilation primarily via the activation of soluble guanylate cyclase with subsequent production of cyclic GMP from GTP (Ignarro, 1990, 1999), which in turn activates cGMP-dependent protein kinase (see Moncada et al., 1991).

However, there is mounting evidence that NO itself may not be EDRF. For example, there is substantial evidence that NO does not fully account for agonist-induced relaxation in physiologically important resistance vasculature *in vivo* (see Rosenblum, 1992; Davisson et al., 1996a; Woodman et al., 2000). *In vitro* studies of resistance sized-arteries have demonstrated that endothelium-dependent

relaxation is not markedly affected when NO synthesis was inhibited, which suggested the existence of an additional endothelium-*dependent* but NO-*independent* vasodilatory mechanism (Komori et al, 1988; Huang et al, 1988; Komori and Vanhoutte, 1990). The portion of endothelium-dependent relaxation that was not blocked by inhibition of NO synthesis was associated with hyperpolarization of the vascular smooth muscle (Chen et al, 1988) and was abolished by potassium channel blockers or depolarizing concentrations of potassium (Adeagbo and Triggle, 1993). The unknown mediator responsible for this phenomenon was therefore termed endothelium-derived hyperpolarizing factor (EDHF) (Taylor and Weston, 1988).

EDHF is a diffusible substance and/or electrical signal that is generated by and released from the vascular endothelium. EDHF hyperpolarizes vascular smooth muscle and the subsequent relaxation occurs without an increase in intracellular levels of cyclic nucleotides and in particular cGMP (Fleming, 2000). Since NO is extremely labile and is easily inactivated by reactive biochemical species (Cocks et al, 1985; Griffith et al, 1984), it has been suggested that NO may exist in mammalian cells as NO-containing compounds such as *S*-nitrosothiols and dinitrosyl iron compounds (Zhang and Hogg, 2005, Vanin, 1998). Indeed, there is now substantial *in vitro* evidence (see Myers et al, 1990; Rubanyi et al, 1991; Rosenblum, 1992; Danser et al., 1998, 2000; Batenburg et al., 2004a,b) and *in vivo* evidence (see Davisson et al., 1996a; Woodman et al, 2000; Lewis et al., 2005, 2006a,b,c) that the pharmacological and physiological

properties of EDRF and EDHF more closely resemble those of the L-S-nitrosothiol, L-S-nitrosocysteine than those of NO. L-S-nitrosocysteine is highly polar and extremely lipophobic (i.e., it has no oil-to-water partition coefficient) (see Kowaluk and Fung, 1990). The question therefore arises as to how L-S-nitrosocysteine is secreted from endothelial cells.

Potential vesicle stores of L-S-nitrosocysteine

Endothelial cells contain cytoplasmic vesicles (Loesch et al, 1994) which may store S-nitrosothiols (Ignarro, 1990). The cytosol is not the most favorable environment in terms of S-nitrosothiol stability since they can be easily reduced by glutathione or thioredoxin (Haendeler et al, 2002; Freedman et al, 1995; Liu et al, 2001). In order to prevent degradation, it has been suggested that S-nitrosothiols may be stored in cytoplasmic and membranal vesicles, and in lipophilic protein folds and in interstitial spaces (Rafikova et al, 2002; Mannick et al, 2001). As mentioned, L-S-nitrosocysteine is extremely lipophobic (Kowaluk and Fung, 1990), and in order to pass through the cell membrane, it would need to be stored within a lipid structure, such as a vesicle that would be subject to Ca^{2+}-*dependent* and Ca^{2+}-*independent* exocytosis.

There is substantial indirect evidence that vesicular stores of nitric oxide containing factors (NOFs) exist in endothelial cells of resistance arteries. It has been demonstrated that inhibition of soluble guanylate cyclase blocks NO-mediated (Woodman et al, 2000; Olson et al, 1997) but not endothelium-

dependent relaxation (Woodman et al, 2000) in resistance arteries. NOS inhibitors abolish the vasorelaxant effects of endothelium-dependent agonists and Ca^{2+}-ionophore A23187 in large arteries (Garland et al, 1995; Hwa et al, 1994) but not in resistance arteries (Garland et al, 1995; Hwa et al, 1994; Edwards and Weston, 1998; Fisslthaler, 1999; Garland and McPherson, 1992; Illiano et al, 1992; Illiano et al, 1993; Nagao and Vanhoutte, 1991,1992; Adeagbo and Malik, 1990, Adeagbo and Triggle, 1993; Mantelli et al, 1995; Siegal et al, 1989). It has also been demonstrated that NOS inhibitors minimally reduce endothelium-dependent vasodilation *in vivo* (Mugge et al, 1991; Ross et al, 1991; Davisson et al, 1996a; Colombari et al, 1998; Danser et al, 1998, 2000; Tom et al, 2001). Moreover, studies have shown that when ATP production is inhibited, endothelium-dependent relaxation is reduced, which is analogous to the ATP-dependency of Ca^{2+}-*dependent* exocytosis in nerve terminals (Griffith et al, 1986; Weir et al, 1991; Richards et al, 1991).

The vasorelaxant responses elicited by endothelium-dependent agonists, such as acetylcholine and bradykinin, diminish rapidly upon repeated administration to resistance arteries treated with NOS inhibitors *in vivo* (Davisson et al, 1996a; Colombari et al, 1998) and *in vitro* (Danser et al, 1998, 2000; Tom et al, 2001). Since acetylcholine and bradykinin receptor function was not compromised, these findings raise the possibility that endothelium-dependent agonists elicit the Ca^{2+}-*dependent* exocytosis of vesicular stores of, for example, L-S-nitrosocysteine, and that these stores cannot be replenished in the absence

of NO synthesis. Indeed, Loesch and Burnstock (1994) demonstrated that NOS exists in the membranes of substantial numbers of cytoplasmic vesicles in vascular endothelial cells (see Fig. 1.1). The presence of NOS would provide a mechanism by which L-S-nitrosothiols are formed within the vesicles. NOS in endothelial cells translocates from plasma membranes to the cytosol after exposure to bradykinin (Michel et al, 1993). This is consistent with NOS-positive vesicles budding into the plasma membranes.

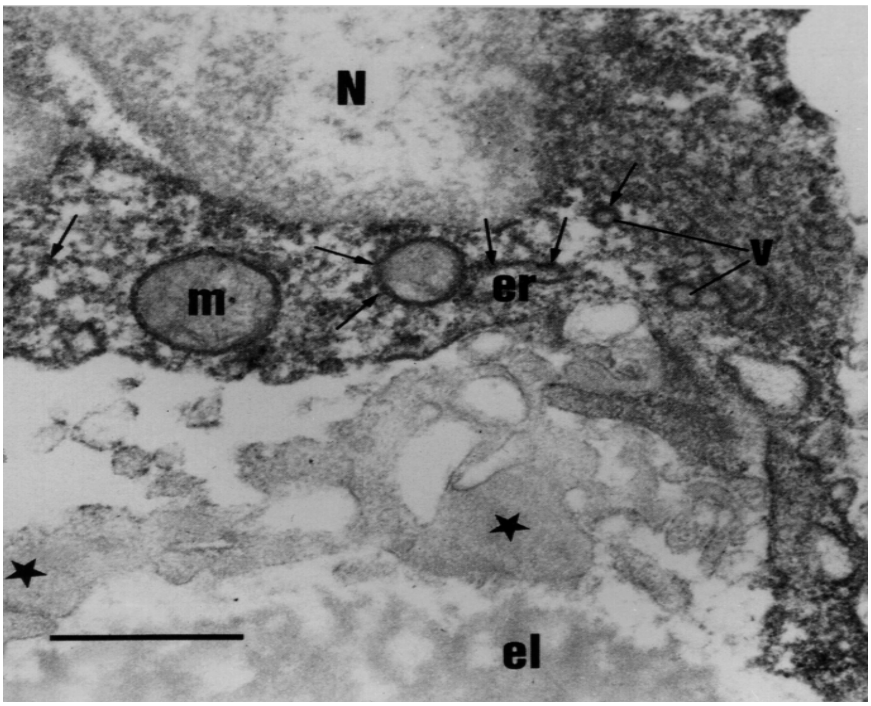

Fig. 1.1 *Ultrastructural distribution of NOS in an endothelial cell.*
NOS is localized to the membranes of vesicles (denoted v), mitochondria (m) and endoplasmic reticulum (er), nucleus (N), elastic lamina (el). Loesch et al (1994). Reprinted with kind permission of Springer Science and Business Media.

Vesicular exocytosis in endothelial cells may be analogous to that in nerve terminals in which some vesicles are subject to Ca^{2+}-*dependent* exocytosis whereas others are subject to Ca^{2+}-*independent* exocytosis (Greengard et al, 1993; Thomas-Reetz and de Camilli, 1994; Thureson-Klein and Klein, 1990).

Ca^{2+}-*dependent* exocytosis requires the presence of Ca^{2+}-calmodulin-*dependent* protein kinase II (CaMKII) and fusion proteins on vesicle membranes, is ATP-dependent, and mediates exocytosis under normoxia (Greengard et al, 1993; Thomas-Reetz and de Camilli, 1994; Thureson-Klein and Klein, 1990). It is possible that Ca^{2+}-*dependent* exocytosis of S-nitrosothiol-containing vesicles mediates endothelium-*dependent* vasodilation in response to agonists and sheer stress that increase intracellular Ca^{2+} (Furchgott and Vanhoutte, 1989; Ignarro, 1990; Luscher and Vanhoutte, 1990; Moncada et al, 1991; Nathan , 1992).

Ca^{2+}-*independent* exocytosis is disinhibited by low tissue O_2/ATP and requires a different array of fusion proteins on vesicle and plasma membranes (Greengard et al, 1993; Thomas-Reetz and de Camilli, 1994; Thureson-Klein and Klein, 1990). Hypoxia may stimulate exocytosis of Ca^{2+}-*insensitive* pools of NOFs and would be a vital defense mechanism against ischemia since the catalytic activity of NOS is reduced by hypoxia (Abu-Soud et al, 1996).

A key finding supporting the concepts that vesicles in endothelial cells may be subject to Ca^{2+}-*dependent* and Ca^{2+}-*independent* exocytosis is that the plasma membranes of these cells contain fusion proteins that support both types of exocytosis (Schnitzer et al, 1995). A key issue that remains unresolved is whether the membranes of endothelial vesicles contain relevant fusion proteins. In nerves terminals and adrenal medullary cells, Ca^{2+}-*dependent* exocytosis of neurotransmitters requires calmodulin (Greengard et al, 1993; Thomas-Reetz

and de Camilli, 1994; Thureson-Klein and Klein, 1990). Calmodulin binds to Ca^{2+} and this complex activates CaMKII in vesicle membranes, which initiates ATP-*dependent* exocytosis by rapid fusion of synaptophysin on vesicle membranes with syntaxins I and II in the plasma membrane (Greengard et al, 1993; Thomas-Reetz and de Camilli, 1994; Thureson-Klein and Klein, 1990).

Ultra-structural distribution of NADPH diaphorase

NADPH-diaphorase is used as a marker for identification of NOS (Dawson et al, 1991; Hope et al, 1991). NADPH-diaphorase activity is the process whereby nitroblue tetrazolium (NBT) is enzymatically reduced to diformazan (a blue precipitate) through the transfer of two electrons. It was assumed that the two electrons were donated by NOS. However, fixation of brain with 4% paraformaldehyde abolishes NOS activity in particulate and cytosolic fractions (Matsumoto et al, 1993). Although fixation abolishes NADPH diaphorase in the particulate fraction, 50-60% of NADPH diaphorase remains in the cytosol (Matsumoto et al, 1993) and is heavily localized in cytoplasmic vesicles (Loesch et al, 1993, 1994). Chayen *et al* (1994) provided a comprehensive series of arguments as to why NOS is not responsible for NADPH diaphorase in aldehyde-treated tissues. They concluded that a yet to be identified 'proteinaceous' factor promotes the NADPH-*dependent* reduction of NBT. One important point raised by Chayen et al (1994) was that α-NADPH is as effective as β-NADPH in promoting reduction of NBT. Since α-NADPH will not donate electrons to NOS, it is unlikely that diformazan arises from the catalytic activity of NOS.

Loesch and Burnstock (1993, 1994), demonstrated that NADPH-diaphorase and NOS do not co-localize in cytoplasmic vesicles in endothelial cells or autonomic nerve terminals. Whereas NOS is strictly localized to the vesicular membranes, NADPH-diaphorase is exclusively localized within the lumen of the cytoplasmic vesicles (see Fig. 1.2). These findings suggests that NADPH-diaphorase is not in fact a marker for NOS but rather may be a histochemical marker for factor(s) in the vesicle. Indeed, the ability of S-nitrosothiols such as L-S-nitrosocysteine and L-S-nitrosocysteamine to promote the NADPH-*dependent* reduction of nitroblue tetrazolium to diformazan (Lewis et al., unpublished findings) suggests that the presence of NADPH diaphorase in cytoplasmic vesicles may be due to the presence of preformed S-nitrosothiols.

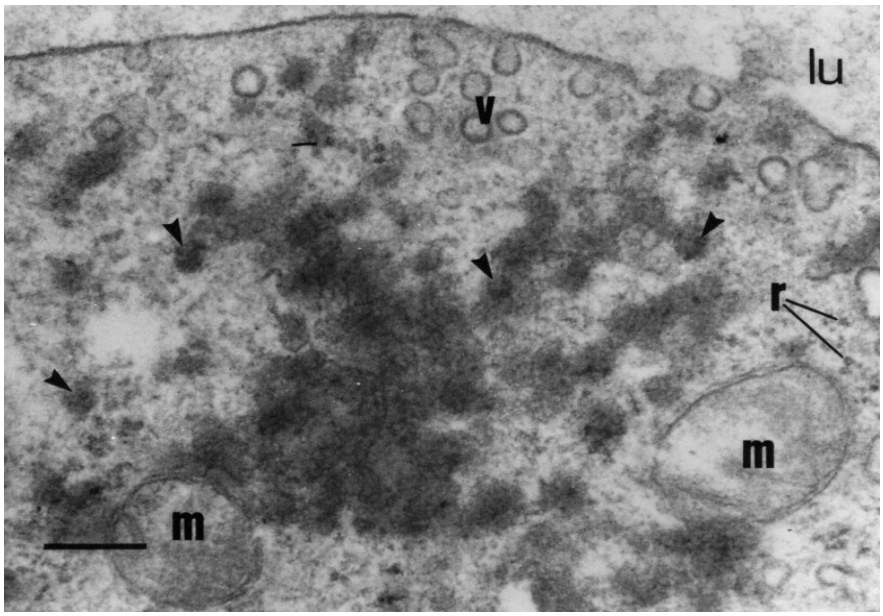

Fig. 1.2 *NADPH diaphorase in an endothelial cell*. NADPH diaphorase exists in the lumen of vesicles (**arrows**) but *not on vesicular membranes*. Several vesicles (**v**) close to the plasma membrane are devoid of staining, consistent with these vesicles having discharged their contents into the lumen (**lu**). Loesch et al (1994). Reprinted with kind permission of Springer Science and Business Media.

Direct detection of S-nitrosothiols in endothelial vesicles

We have directly detected S-nitrosothiols in endothelial vesicles collected from femoral and mesenteric arteries of rats, rabbits and dogs (Lewis et al, unpublished observations). For example, we collected vesicles from endothelial cells harvested from small (100-160 μm) femoral and mesenteric arteries from rats. Vesicle membranes were lysed by sonication and the luminal contents were examined spectrophotometrically. The observed spectra were characteristic of S-nitrosothiols (Oae and Shinhama, 1983; Bates, 1992) with a large absorption peak at 340 nm and a smaller peak at 545-550 nm (see Fig. 1.3).

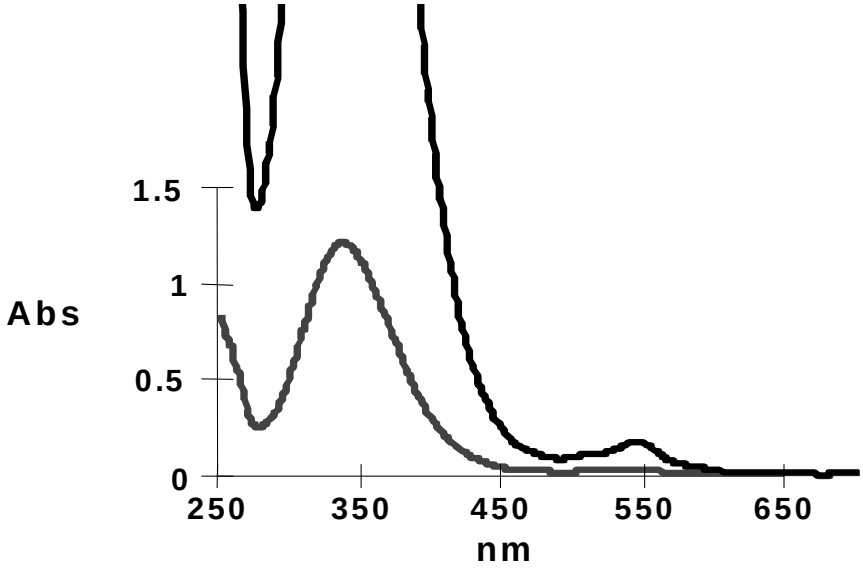

Fig. 1.3 *Spectrophotometric detection of S-nitrosothiols in endothelial vesicles*. Vesicle membranes were lysed by sonication and the luminal contents were examined. The absorption spectra of the luminal S-nitrosothiols contents is shown by the upper spectrum. The lower spectrum is the same sample as the upper spectrum diluted seven-fold in water.

The luminal contents were examined in a chamber where released NO was continuously measured by a chemiluminescence NO detector. There was an initial NO peak upon addition of the sample to the chamber, representing NO previously formed in the solution. Subsequent irradiation of the solution with long-wave UV light (340-360 nm) for 15 seconds caused a large volume of NO to be released. This is a classic sign that S-nitrosothiols were present in the solution (Oae and Shinhama, 1983; Bates, 1992).

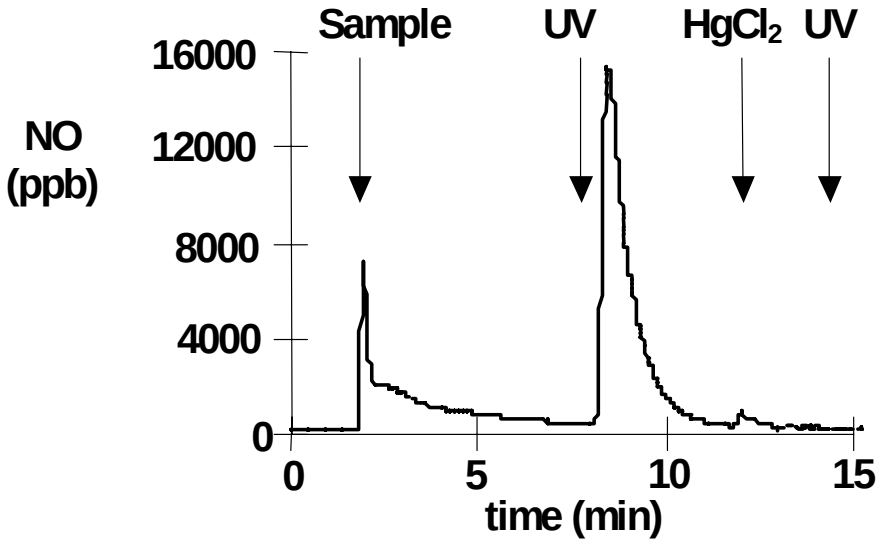

Fig. 1.4 *Production of NO from vesicular S-nitrosothiols*

The UV light-induced release of NO could be repeated many times as the decomposition of S-nitrosothiols by each brief exposure to UV light represented only a small portion of the S-nitrosothiols in solution. When the samples were treated with mercuric chloride ($HgCl_2$, 0.05 M), which causes S-nitrosothiols to decompose (Oae and Shinhama, 1983; Bates, 1992), no further NO could be generated by exposure to UV light. These findings provide fundamental evidence that vesicles in endothelial cells contain S-nitrosothiols.

Histochemical detection of S-nitrosothiols

Chayen et al, (1994) suggested that a “proteinaceous” factor may be responsible for NADPH-*dependent* reduction of NBT. We proposed that the proteinaceous factor is a preformed S-nitrosothiol and examined whether L-S-nitrosocysteine promotes the NADPH-*dependent* reduction of NBT. We first added test solutions together and examined diformazan production by UV-VIS spectrophotometry (see Table 1, diformazan denoted as DF). We observed that neither α- nor β-NADPH directly reduced NBT. Moreover, L-S-nitrosocysteine did not directly reduce NBT. However, L-S-nitrosocysteine but not NO directly promoted the α- or β-NADPH-*dependent* reduction of NBT and this readily took place in the presence of paraformaldehyde. The addition of L-S-nitrosocysteine and NBT to β- or α-NADP did not elicit diformazan.

Subsequent experiments demonstrated that S-nitrosothiols transferred NO^+ to (i.e., nitrosated) NBT, which altered the redox potential of NBT such that it can be directly reduced by either α- or β-NADPH (Lewis et al, unpublished observations). Interestingly, diformazan extracted from brain has a different absorption peak to authentic diformazan (Schmidt et al, 1992). Importantly, the absorption peak of diformazan from the reaction of S-nitrosocysteine with β-NADPH and NBT is identical to diformazan extracted from brain (Lewis et al, unpublished observations). This suggests that nitrosated NBT is converted to nitrosated diformazan in tissues and that NADPH diaphorase in aldehyde-perfused tissues is actually a marker for S-nitrosothiols.

Table 1.1 Production of diformazan

Solution	Solution	DF (Abs Units)
1.	NBT	≈ 0
2.	NBT + β-NADPH	≈ 0
3.	NBT + β-NADP	≈ 0
4.	NBT + β-NADPH + paraformaldehyde	≈ 0
5.	NBT + SNC	0.07 ± 0.03
6.	NBT + β-NADPH + SNC	0.94 ± 0.05*
7.	NBT + α-NADPH + SNC	0.86 ± 0.06*
8.	NBT + β -NADPH + paraformaldehyde + SNC	1.19 ± 0.08*
9.	NBT + β-NADP + SNC	≈ 0
10.	NBT + β-NADP + paraformaldehyde + SNC	≈ 0
11.	NBT + NO	0.06 ± 0.02
12.	NBT + β-NADPH + NO	0.05 ± 0.02
13.	NBT + β-NADPH + paraformaldehyde + NO	0.05 ± 0.02

The final concentrations of the S-nitrosothiols, NBT, NADPH and NADP were 0.5 mM. The final concentrations of cysteine, cystine and paraformaldehyde were 5 mM, 10 mM and 0.1%, respectively. Pure NO gas was bubbled directly into the described solutions for 20 min. Data presented are mean ± S.E.M. of absorbance units derived from 4-6 separate experiments.* $P < 0.05$, absorbance values for solutions 6, 7 and 8 versus absorbance values for solutions 1-4, 5, and 9-13.

We then sought evidence that NADPH diaphorase histochemistry detects S-nitrosothiols. S-nitrosothiols such as S-nitrosocysteine degrade upon exposure to UV light or alkaline pH (Oae and Shinhama, 1983). We exposed celiac ganglion sections (10 μm) to UV light for 15 min and then performed NADPH diaphorase histochemistry. NADPH diaphorase was present in the preganglionic terminals, which are wrapped around post-ganglionic cell bodies, which did not stain for NADPH diaphorase (see Fig. 1.5, left panel). UV light markedly diminished NADPH diaphorase staining in preganglionic terminals (see Fig. 1.5, right panel). Elevating tissue pH before NADPH diaphorase histochemistry also markedly reduced NADPH diaphorase staining (data not shown). These findings suggest that the factor responsible for NADPH-diaphorase in aldehyde-treated tissues is an S-nitrosothiol that is sensitive to UV light and alkaline pH.

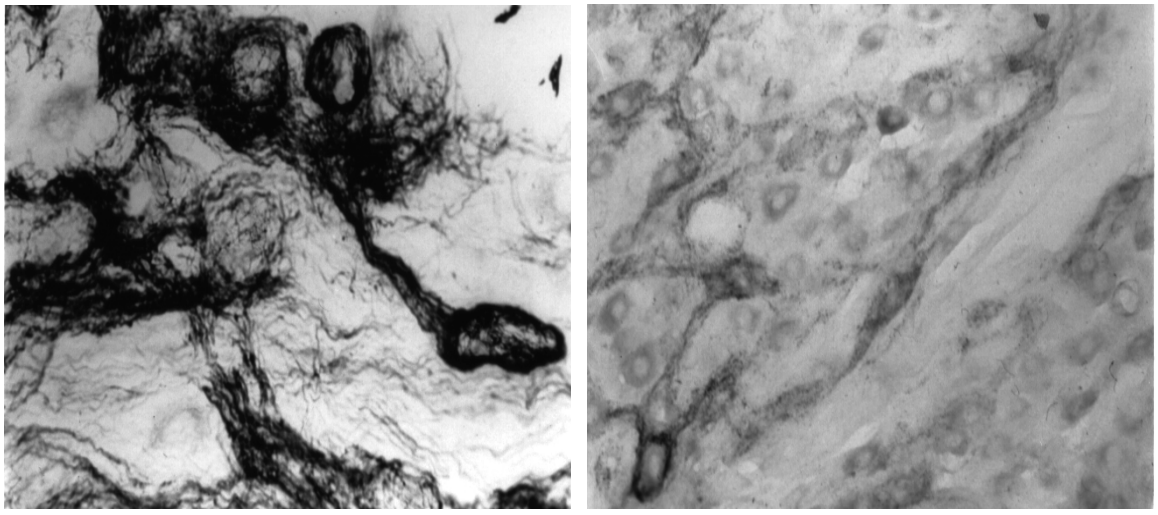

Fig. 1.5 *NADPH diaphorase staining in celiac ganglion sections*. The left-hand figure shows NADPH diaphorase in control preganglionic terminals, which are wrapped around post-ganglionic cell bodies. Note that cell bodies do not stain for NADPH diaphorase. The right hand-figure shows a celiac ganglion section that had been exposed to UV light. UV light markedly reduced NADPH diaphorase staining in the terminals.

Objectives

Acetylcholine and bradykinin may elicit exocytosis of S-nitrosothiol-containing vesicles in the endothelium of femoral and mesenteric arteries of rats (Davisson et al, 1996a; Colombari et al, 1998) and porcine coronary arteries (Danser et al, 1998, 2000; Tom et al, 2001). Moreover, autonomic nerves innervating femoral resistance arteries may use neurotransmitter pools of S-nitrosothiols (Davisson et al, 1994, 1996b,c, 1997; Possas and Lewis, 1997, 2006; Kregel et al, 1997; Crippa et al, 2000). Vascular smooth muscle (Matsunga and Furchgott, 1991; Chaudry et al, 1993; Venturini et al, 1993; Kubaszewski et al, 1994), macrophages (Uchizumi et al, 1993), blood (Stamler et al, 1992) and brain (Kluge et al, 1997) may also contain preformed S-nitrosothiols although the mechanisms of synthesis, storage and release have not been elucidated.

Endothelial cells in resistance arteries contain numerous cytoplasmic vesicles (Loesch et al, 1993, 1994; Bruns and Palade, 1968; Skutelsky and Danon, 1976; Bundgaard et al, 1979; Mazzone and Kornblau, 1980; Wagner and Casley-Smith, 1981; Huttner, 1983) that are subject to exocytosis (Bodin and Burnstock, 1981). The membranes of many of these vesicles contain NOS (Loesch et al, 1993, 1994). These findings led us to develop the **concepts** that (1) NOS in vesicle membranes allows for the synthesis and storage of L-S-nitrosocysteine in vesicles, (2) sub-populations of vesicles (those with CaMKII) undergo Ca^{2+}-*dependent* exocytosis in response to stimuli such as sheer stress and agonists such as acetylcholine, that are known to elevate intracellular Ca^{2+}

levels, (3) other vesicles (those with CaMKII) undergo Ca^{2+}-*independent* exocytosis in response to hypoxia, (4) vesicular stores of L-S-nitrosocysteine play vital roles in mediating the vasodilator responses to sheer stress and endothelium-*dependent* agonists *in vivo*, and (5) vesicular stores of L-S-nitrosocysteine mediate the vasodilator responses to hypoxia *in vivo*.

Our **objective** was to provide support for the **hypothesis** that vesicular stores of L-S-nitrosocysteine function as EDRFs and EDHFs and that these vesicular stores are subject to Ca^{2+}-*dependent* and Ca^{2+}-*independent* exocytosis. We performed light-level and ultra-structural studies on isolated arteries to address our **specific aims,** which were to determine:

1. The presence of vesicles in endothelial cells in arteries from mesenteric, femoral and pulmonary arteries of rats.
2. The presence of NOS in membrane of endothelial vesicles.
3. The presence of NADPH-diaphorase within endothelial vesicles.
4. The mobilization of endothelial vesicles to vesicular exocytosis upon stimulation with acetylcholine.
5. Mobilization of endothelial vesicles to exocytosis by hypoxia.
6. Whether acetylcholine mobilizes endothelial vesicles containing NADPH-diaphorase (S-nitrosothiols) within the lumen.
7. Presence of proteins such as CaMKII and fusion proteins on the membranes of vesicles in vascular endothelial cells that may be necessary for vesicular exocytosis.

The present studies are designed to complement our initial evidence that tissue NADPH diaphorase is a histochemical marker for S-nitrosothiols (ref 1), functional *in vivo* studies demonstrating the presence of preformed pools of S-nitrosothiols in vascular endothelial cells and nerve terminals (refs 2-4), and the mechanisms by which S-nitrosothiols act on vascular smooth muscle (refs 5-7).

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CHAPTER 2

LITERATURE REVIEW

GENERAL MORPHOLOGY BLOOD VESSELS

The circulatory system was first described in detail in 1628 by Sir William Harvey (see Harvey, 1978). Subsequently, Marcello Malphigi discovered that a physical separation between blood and tissue existed (Fishman, 1982). It was not until the 1800s that von Reckingausen provided evidence that blood vessels consisted of different layers of cells. The inner layer of most arteries and veins consist of squamous (flat, smooth) epithelial cells known as the ***endothelium***. In an adult human, the endothelial cell surface weighs approximately 1 kg, covers a surface area of approximately 1 to 7 m^2 and is composed of approximately 1 to 6 x 10^{13} cells (Augustin et al., 1994). Until recently, the endothelium was thought to act as an inert barrier, merely separating vascular smooth muscle from blood. Specifically, the primary role of the endothelium was thought to be maintenance of vessel wall permeability. As will be discussed below, it is now evident that the endothelium secretes numerous factors that play active roles in the maintenance of vessel diameter and also platelet aggregatory status via the release of many vasoactive and platelet-regulatory factors.

Tunica Intima

At the base of the epithelial layer is a thin layer of spongy connective tissue that secretes a relatively thin layer of elastic collagen. This stretchy layer forms the basement membrane for the endothelium. These two layers together are known as the ***tunica intima***, or inner sheath. In larger more distensible arteries, the tunica intima is often highly folded when the artery is relaxed.

Tunica Media

The number of layers of surrounding smooth muscle depends upon the type of artery. Large conduit arteries contain many layers of smooth muscle to maintain pressure gradient at high systolic levels of arterial pressure. The much smaller resistance arteries, which see considerably lower arterial pressures, have one or two layers of smooth muscle. These arteries, which are heavily innervated by sympathetic nerves, regulate blood flow to organs. Relatively small changes in the diameter of resistance arteries will have substantial effects on the rate of flow of blood cells into the capillaries and veins. Accordingly, minor changes in the diameter of these arteries can and do have pronounced effects on the levels of systolic and diastolic arterial blood pressure. The muscle layer is also underlain by a spongy layer of connective tissues that produces elastic collagen fibers. Together these two layers are known as the ***tunica media***, or middle sheath.

Tunica adventitia

Surrounding the tunica media is a layer of connective tissues that produces both elastic collagen fibers and more rigid collagen fibers. This layer will stretch, but only to a limit. This layer is called the *tunica adventitia*. This tougher fibrous layer prevents the ballooning of the blood vessel walls that could take place when systolic blood pressure is overly high. The adventitia of many and in particular resistance sized arteries contains a network of intrinsic nerve fibers. Despite extensive studies, the precise role of these nerves, which are not connected to the central or autonomic nervous systems, remain elusive.

THE ENDOTHELIUM

Tight junctions between endothelial cells

The integrity of the endothelial layer in blood vessels (i.e., relative ease by which large molecular weight blood-borne factors reach the underlying smooth muscle) varies within different vascular beds. Endothelial cells adhere to one another through junctional structures formed by transmembrane adhesive proteins that are responsible for homophilic cell-to-cell adhesion. These transmembrane proteins are linked to specific intracellular partners, which anchor the actin cytoskeleton and stabilize junctions (Dejana et al., 1999). Two major types of junctions have been described and are termed ***adherens junctions*** and ***tight junctions***. In addition to cell-to-cell adhesion, another type of junction, the ***gap junction***, mediates cell-to-cell communication. This structure is formed by connexins, and three of these proteins (i.e., Cx43, Cx40, and Cx37) are expressed in the endothelium. In turn, connexins are organized in connexons, which act as channels for the intercellular passage of ions and small-molecular-weight molecules (Simon and Goodenough, 1998).

In endothelial cells, the junctional architecture shows that *adherens junctions* are intermingled with *tight junctions* (Simionescu, 2000). In the brain, where a strict control of permeability between blood and the nervous system is required, junctions are well developed and rich in *tight junctions* (Rubin and Staddon, 1999; Wolburg and Lippoldt, 2002). In contrast, post-capillary venules, which allow for the dynamic trafficking of circulating cells and plasma proteins,

have poorly organized *tight junctions*. These morphological features are believed to account for the high sensitivity of post-capillary venules to permeability-increasing agents, such as histamine and bradykinin. In contrast, the endothelium of large arteries, which tightly controls permeability, has a well-developed system of *tight junctions*. Finally, lymphatic endothelial cells have unique junctional structures, the complexus adhaerentes (Schmelz and Franke, 1993; Schmelz et al., 1994), which are likely to be specialized in controlling the passage of lymphocytes to and from different tissue compartments.

Caveolae

Endothelial cells contain caveolae, which are invaginations in the cell membrane and mediate transcytosis within the endothelial cell (Minshall et al., 2003). Caveolae internalization is regulated by caveolin-1, which is a scaffolding protein that is inserted into the cytoplasmic face of the plasma membrane (Galley and Webster, 2004). Transcytosis of albumin via caveolae in the endothelial cells serves an important hemostatic function. Serum albumin has critical functions in maintaining the trans-endothelial oncotic pressure gradient (and hence in regulating tissue fluid balance) and in transporting a host of blood-borne substances, among them lipids, hormones, peptides, nucleic acids, and xenobiotics (Forker and Luxon, 1983; Partridge, 1979; Peters, 1979a,b). Albumin maintains endothelial barrier characteristics through interactions with extra-cellular matrix components (Curry and Michel, 1980; Huxley and Curry, 1985; Powers et al., 1989).

Endothelial function

It is now accepted that the endothelium is simply more than just a physical barrier between the blood and vascular smooth muscle. Study of endothelial function first became possible with the advent of new techniques that allowed endothelial cells to be cultured in vitro (Jaffe et al., 1973; Gimbrone et al., 1974; Lewis et al., 1973). The endothelium has a number of physiological roles and since it is strategically located at the interface between tissue and blood, it is in an ideal position to modulate the function of various organs. Endothelial cells maintain a non-thrombogenic blood-tissue interface and regulate thrombosis, thrombolysis, platelet adherence, blood flow and vascular tone.

Secretory functions

The vascular endothelium has paracrine, endocrine, and autocrine functions. Endothelial cells regulate vascular morphology by secreting (1) matrix products such as fibronectin, laminin, collagen, proteoglycans and proteases, and (2) growth factors including insulin-like growth factor, transforming growth factor and colony stimulating factor. Endothelial cells regulate blood aggregatory status by the secretion of anti-thrombotic factors such as prostacyclin, thrombomodulin, antithrombin, plasminogen factor and heparin. Conversely, under conditions of tissue injury, the endothelium also secretes pro-coagulant factors such as von Willebrand factor, thromboxane A2, thromboplastin, factor V, platelet activating factor and plasminogen activator inhibitor. The endothelium also directly participates in the progression of immune responses via the release

of inflammatory mediators such as leukotrienes and interleukins 1, 6, and 8. Endothelial cells also influence lipid metabolism by the secretion or production of LDL-receptors and lipoprotein lipase (Galley and Webster, 2004).

Endothelial cells control the passage of plasma proteins and circulating cells from blood to tissues. This function is finely regulated by transcellular and paracellular pathways (Dvorak et al., 1996). The transcellular pathway defines the passage of plasma components through the endothelial cytoplasm by the action of vesicular systems and fenestrae. The paracellular pathway is regulated by opening of cell-to-cell junctions and/or by rearrangement of their architecture. Endothelial cells are able to sense changes in blood pressure, oxygen tension and blood flow by as yet unknown mechanisms. In response to changes in local conditions they respond by secreting autocoids, namely prostacyclin and NO which regulate the tone of vascular smooth muscle (Pohl, 1990)

Regulation of vasomotor tone

As mentioned, the endothelium is crucial for the regulation of vascular tone. Endothelial cells produce/secrete a variety of (1) endothelium-derived relaxing factors (***EDRFs***) such as prostacyclin and the reactive free radical, nitric oxide ($NO^{\bullet}$) (Moncada, 2006), (2) endothelium-derived hyperpolarizing factors (***EDHFs***) whose identities are still controversial (de Wit et al., 2006), and (3) a variety of contracting factors (***EDCFs***), such as endothelin-1, thromboxane A2, leukotrienes, and free radicals (Vanhoutte 2000; Yanagisawa, 1988).

NITRIC OXIDE

Basic chemistry

$NO^{\bullet}$ is a free radical lipophilic diatomic gas that readily dissolves in biological fluids. Its small Stokes radius and neutral charge permits rapid membrane diffusion (Goretski and Hollocher, 1988; Stamler et al., 1992a). $NO^{\bullet}$ rapidly reacts with oxygen to produce a variety of nitrogen oxides. The stability and decay of $NO^{\bullet}$ depend upon a variety of factors including the redox status of the local environment and the presence of various metal ions (Henry et al., 1997). NO exists in three redox forms, namely (1) free radical nitric oxide ($NO^{\bullet}$), which has an unpaired electron in outer shell, (2) nitrosonium ion, NO^{+}, and (3) nitroxyl ion (NO^{-}) (Stamler et al., 1992a). These redox forms of NO have different chemical reactivities and properties. One notable feature of the free radical form of NO is that its unpaired electron allows it to readily react with superoxide anion, nitrogen derivatives and transition metals (Stamler et al., 1992a).

Potential endothelium-derived relaxing factor

Furchgott and Zawadzki (1980) first discovered that an unknown factor emanating from the vascular endothelium was responsible for the relaxation of the smooth muscle cells elicited by acetylcholine. More specifically, Furchgott and Zawadzki (1980) found that acetylcholine elicited pronounced concentration-dependent dilator responses in endothelium-*intact* rabbit artery rings whereas acetylcholine elicited minor contractions in endothelium-*denuded* rings. Furchgott and Zawadzki (1980) referred to this unknown vasodilator substance as

endothelium-dependent-relaxing factor (EDRF). A few years before this ground breaking discovery, Murad was attempting to identify factors that activated guanylate cyclase. After much research, they discovered that azide activated this enzyme indirectly via its generation of the free radical, $NO^{\bullet}$ (see Murad, 2004). In further experiments, Murad's group found that (1) bubbling $NO^{\bullet}$ gas through smooth muscle cells resulted in large increases in cGMP levels presumably via the activation of soluble guanylate cyclase, and (2) $NO^{\bullet}$ activated soluble guanylate cyclase via interactions with the heme moiety of the enzyme (Arnold et al., 1977; Murad, 2004).

Murad hypothesized that hormones may influence smooth muscle tone via the release of $NO^{\bullet}$ from vascular endothelial cells. This was revolutionary hypothesis since $NO^{\bullet}$ production in animals had not even been reported at that point in time (Hibbs and Bastian, 1999). Some years later, Ignarro independently showed that $NO^{\bullet}$ was chemically identical to EDRF (see Ignarro, 1990). The realization that EDRF and $NO^{\bullet}$ may be one and the same substance took six years of intense research. From 1980 to 1986 reports of similarities between the two gradually mounted. In hindsight this may seem to have been an inevitable accumulation of data, but at the time the picture was quite confusing.

The comparative pharmacology of EDRF and $NO^{\bullet}$ on vascular strips was enough to convince most of the scientific community that EDRF was $NO^{\bullet}$ (see Moncada, 2006). Nonetheless, Moncada and colleagues rightfully thought it

necessary to demonstrate that stimulation of the endothelium resulted in the direct release $NO^{\bullet}$ from these cells. Even though there were chemical methods for measuring the breakdown products of NO such as NO_2^- and NO_3^-, they tried to directly measure $NO^{\bullet}$ released from endothelial cells. Based on a specific chemiluminescent signal technique modified to detect very low quantities of $NO^{\bullet}$, they demonstrated that $NO^{\bullet}$ was generated from vascular endothelial cells after stimulation with bradykinin. Even more compelling was the fact that the quantities of $NO^{\bullet}$ that were being released were sufficient to account for the actions of EDRF (Palmer et al., 1987). The question that arose later was whether this $NO^{\bullet}$ may have actually originated from an S-nitrosothiol (see Myers et al., 1990).

Platelet studies were integral in confirming the nature of EDRF. It had been known for some years that $NO^{\bullet}$ inhibited platelet aggregation and in 1986 EDRF was shown to exhibit platelet anti-aggregation properties (Azuma et al., 1986). The actions of EDRF from vascular tissues and authentic $NO^{\bullet}$ on platelets were similar in their pharmacological profiles (Radomski et al., 1987). Even more compelling was the observation that the antiaggregating and the disaggregating effects of both EDRF and authentic $NO^{\bullet}$ were potentiated by sub-threshold concentrations of prostacyclin and *vice versa*. This effect was blocked by indomethacin and partially reversed by hemoglobin. The anti-aggregatory action of $NO^{\bullet}$ but not prostacyclin, was potentiated in the presence of superoxide dismutase. Moreover, it was observed that $NO^{\bullet}$ acted as a potent inhibitor of platelet aggregation in the same way that EDRF did (Radomski et al., 1987).

Nitric oxide synthase

The next question that arose was exactly how $NO^{\bullet}$ was being formed in mammalian tissues. Outside the body, $NO^{\bullet}$ is an unstable, toxic gas that is produced by internal combustion engines, electrical generating stations and lightning strikes. $NO^{\bullet}$ has been implicated in depletion of the ozone layer, formation of photochemical smog, and acid rain. It was astonishing to think that this gas not only was present in the body but that there was a system for generating it naturally. There were a number of possibilities regarding the origin of $NO^{\bullet}$, including the suggestion that NO_2^- or NO_3^- were enzymatically reduced to $NO^{\bullet}$ or that ammonia or an amino acid was the biological precursor. However, the most promising possibility was from the studies of Hibbs and those of Lyengar, who independently demonstrated that NO_2^- or NO_3^- were produced from the conversion of L-arginine to L-citrulline in activated macrophages (Hibbs et al., 1987; Lyengar et al., 1987). It was concluded that $NO^{\bullet}$ might be an unstable intermediate in the synthesis of the more stable NO_2^- or NO_3^- from L-arginine.

Using endothelial cells cultures, $NO^{\bullet}$ was detected not only biologically but chemically using specific chemiluminescence techniques (Palmer et al., 1988). Moncada's laboratory went on to provide compelling evidence that an enzyme, which they called $NO^{\bullet}$ synthase (NOS), was directly involved in the production of $NO^{\bullet}$ in endothelial cells. This synthase was clearly demonstrated to generate $NO^{\bullet}$ from L-arginine, with L-citrulline as the catalytic by-product, and thus the L-arginine: $NO^{\bullet}$ signaling pathway was born (Moncada et al., 1989).

NOS is a unique heme-containing P_{450} enzyme in that it is catalytically self-contained because of the presence of its own cytochrome P-450 reductase. Three isoforms of NOS are known to exist, two of which are constitutive and one whose expression is induced by inflammatory stimuli (Knowles and Moncada, 1994; Morris and Billiar, 1994; Nathan and Xie, 1994; Sessa, 1994; Stuehr and Griffith, 1992). The constitutive NOS that was first discovered in the vascular endothelium was designated as eNOS, whereas that present in the brain, spinal cord and peripheral nervous system is termed nNOS. The form of NOS induced by immunological or inflammatory stimuli is known as iNOS. The complementary deoxyribonucleic acids for all three NOS isoforms have been cloned from a variety of mammalian species including humans, as well as many plant and bacterial species (Charles et al., 1993; Geller et al., 1993; Marsden et al., 1992; Nakane et al., 1993). These studies have found important species differences in the NOS isoforms. In addition, knockout mice have been generated for each of the three NOS isoforms and *in vivo* and *in vitro* studies have provided useful if often conflicting information concerning the role of each isoform and the effects of its deletion in whole animals (Huang and Fishman, 1996).

NOS isoenzymes also exist in many other cell types. For example, eNOS is also located in platelets (Radomski et al., 1990) and neurons (Dinerman et al., 1994). nNOS exists in skeletal muscle (Kobzik et al., 1994) and in the epithelium of the bronchi and trachea (Kobzik et al., 1993). Expression levels of constitutive eNOS can be increased in certain situations such as chronic exercise (Sessa et

al., 1994) or pregnancy, when both eNOS and iNOS are induced (Weiner et al., 1994). Clear differences in the expression levels of iNOS have been identified in different tissues in the same species (Mohaupt et al., 1994). For example, iNOS is present constitutively in human bronchial epithelium (Kobzik et al., 1993), rat kidney (Mohaupt et al., 1994) and some fetal tissues (Baylis et al., 1994) and can be induced during pregnancy (Weiner et al., 1994).

Functional NOS proteins are dimers formed of two identical sub-units. There are three functionally distinct domains in eNOS and nNOS, a reductase domain, a Ca^{2+}-calmodulin-binding domain, and an oxygenase domain. iNOS does not contain the Ca^{2+}-calmodulin-binding domain and is unaffected by changes in intracellular Ca^{2+} levels (unlike eNOS and nNOS, iNOS is fully active in the absence of a Ca^{2+} signal). The reductase domain contains the β-NADPH binding site, and flavin adenine dinucleotide and flavin mononucleotide moieties, which act to transfer the 2 electrons obtained from β-NADPH to the oxygenase domain. It should be noted that the reductase domain transfers electrons to the oxygenase domain of the *opposite* sub-unit of the dimer. The binding of Ca^{2+}-calmodulin is the primary stimulus that increases eNOS and nNOS activity. The oxygenase domain contains the binding sites for tetrahydrobiopterin, heme and L-arginine. The oxygenase domain catalyzes the conversion of L-arginine into citrulline and NO. Tetrahydrobiopterin impacts greatly on the structure of NOS. It causes the heme iron of NOS to shift to a high spin state, it increases arginine binding and stabilizes the active dimeric form of the enzyme (Gross et al., 2000).

Physiological roles of nitric oxide

In physiological conditions, $NO^{\bullet}$ is produced by both eNOS and nNOS in the endothelium of arteries and veins. Endothelial eNOS and nNOS produce $NO^{\bullet}$ in response to phasic increases in shear stress elicited by the pulsatile arterial blood pressure wave form, and in response to endothelium-dependent agonists (both stimuli elicit Ca^{2+}-dependent increases in NOS activity) (see Moncada and Higgs, 1993). Released $NO^{\bullet}$ diffuses to the adjacent vascular smooth muscle and binds to the ferrous heme moiety of soluble guanylate cyclase (sGC), thereby activating the enzyme. Activated sGC converts guanosine triphosphate to the intracellular second messenger cGMP, which relaxes vascular smooth muscle cells (Ignarro, 1989). Most of the effects of cGMP are mediated by stimulation of the cGMP-dependent protein kinase (Lincoln and Corwell, 1993).

Vascular tone is determined at least in part by the balance of constrictor (e.g., prostaglandins and endothelin-1) and dilator factors (e.g., $NO^{\bullet}$, prostacyclin, and other EDRFs and EDHFs (see below) released by the vascular endothelium. Modifications to the guanidino group of L-arginine have yielded compounds that are competitive NOS inhibitors, and N^{G}-monomethyl-L-arginine (L-NMMA) and N^{G}-nitro-L-arginine methyl ester (L-NAME) have been shown to diminish $NO^{\bullet}$ release from endothelial cells and aortic rings. The acute systemic administration of these NOS inhibitors elicit a sustained hypertension in humans and experimental animals (Moncada and Higgs, 1991). This suggests that the basal release of $NO^{\bullet}$ is important in maintaining vascular tone *in vivo*.

$NO^{\bullet}$ has now been identified as a critical signaling molecule in maintaining blood pressure in the cardiovascular system, stimulating host defenses in the immune system, regulating neural transmission in the brain, regulating gene expression, platelet aggregation, learning and memory, male sexual dysfunction, cytotoxicity and cytoprotection and development of arteriosclerosis (Jeffrey and Snyder, 1995; Lipton et al., 1993; Lloyd-Jones and Bloch, 1996; Moncada et al., 1991; Palevitz and Lewis, 1998).

Nitric oxide in pathology

Since the endothelium is a major source of $NO^{\bullet}$ in the vasculature, it is obvious that damage to endothelial cells would result in a perturbation of $NO^{\bullet}$ production and therefore changes in vascular tone. Indeed, Several patho-physiological states induce endothelial dysfunction, which results in reduced endothelium-dependent vasodilation (see Ludmer et al., 1986; Moncada, 2006). When the endothelium is severely compromised, agonists that normally elicit endothelium-dependent vasodilation, produce a vasoconstriction via direct actions on the vascular smooth muscle (Okamura et al., 1996). In other words, in the presence of a functionally healthy endothelium, the vasoconstrictor effects of many compounds are negated by the concomitant release of EDRF.

The endothelium is vital for maintaining body hemostasis. Endothelial injury, dysfunction and activation are the underlying causes for many disease processes such as atherosclerosis (Anderson 2003), hypertension (Kojda and

Harrison, 1999; Taddei and Salvetti, 2002), sepsis (Symeonides and Balk, 1999), inflammatory responses (Ross, 1999), diabetes (Beckman et al., 2003; Rizzoni et al., 2001; Schofield et al., 2002; Makimattila and Yki-Jarvinen 2002; Endemann et al., 2004), chronic renal failure (Bolton et al., 2001), congestive heart failure (Monnick et al., 2002; Landmesser et al., 2002). Pre-eclampsia (Page, 2002), heart failure (Linke et al., 2003), coronary artery disease (Schachinger et al., 2000), nitrate tolerance (Munzel et al., 1995) and clinical conditions linked to ischemia followed by reperfusion (Sotnikova et al., 1998; Pagliaro et al., 2003).

Vascular diseases such as coronary artery, cerebrovascular and peripheral vascular diseases are major causes of mortality/morbidity in humans in the Western world. Many common risk factors for vascular disease, such as hypertension and diabetes, remain prevalent in Western and other populations, suggesting that vascular disease will continue to impose a substantial burden on health care resources throughout the next generation (Burt et al., 1995a; Burt et al., 1995b; *National High Blood Pressure Education Program Working Group*, 1994). The earliest detectable changes in the development phase of vascular disease are abnormalities of the endothelium, and specifically those resulting in loss of the endothelium's hemostatic functions that normally act to inhibit disease-related processes such as inflammation and thrombosis.

In addition to modulating blood flow (Umans, 1995), $NO^{\bullet}$ has important anti-atherogenic effects on platelets, vascular smooth muscle and endothelial

cells. $NO^{\bullet}$-mediated signaling is deficient in pre-atherosclerotic states such as hypercholesterolemia, diabetes mellitus, hypertension and smoking (Cooke et al., 1997; Harrison et al., 1998) and correlates with risk factor profile (Vita et al., 1990). More importantly, studies have identified deficient $NO^{\bullet}$-mediated endothelial function as a quantitative, independent predictor of adverse cardiac events (Schachinger et al., 2000; Heitzer et al., 2001).

Endothelial dysfunction was first described in humans in a study that involved measuring changes in blood vessel diameter in response to increases in flow and shear stress in the brachial artery of hypertensive patients (Panza et al., 1990). Many studies in animals have also demonstrated that a loss of endothelium-derived $NO^{\bullet}$ is a critical factor in the pathogenesis of vascular disease states. Reduced $NO^{\bullet}$ has often been reported in the presence of impaired endothelial function. It is thought that reduced $NO^{\bullet}$ is due to a reduction in endothelial NOS activity. Whether this is due to the presence of endogenous NOS inhibitors or reduced bioavailability of L-arginine is not yet determined. Targeted deletion of the eNOS gene in mice results in hypertension (Huang et al., 1995) and impaired vascular remodeling, (Rudic et al., 1998), whereas augmenting $NO^{\bullet}$ by local gene delivery of NOS improves endothelial function, limits neointimal proliferation, and induces regression of atherosclerotic lesions (Channon et al., 2000).

It is now evident that reactive oxygen species (ROS) such as superoxide anion are important signaling molecules in the cardiovascular system. ROS alter

the activity of target proteins by oxidizing cysteine residues (e.g. transcription factors, protein phosphatases) and induce redox-sensitive gene transcription, smooth muscle cell growth and motility (Chiarugi and Cirri 2003). More recently, compelling evidence has arisen that superoxide anion is an endothelium-derived vasoconstrictor factor (Vanhoutte, 2000; Rey et al., 2001). However, enhanced ROS production is implicated in the pathogenesis of cardiovascular diseases including atherosclerosis, coronary artery disease, hypertension and diabetes mellitus (Cai and Harrison 2000). Increased ROS production is also involved in the expression of ischemia-reperfusion injury. Ischemia-reperfusion tissue injury is a frequent complication of surgical procedures, transplantation, stroke, circulatory shock, coronary artery disease, etc. (Li and Jackson 2002; Carlucci et al., 2002). Myocardial ischemia (i.e., lack of tissue oxygen) manifests as angina pectoris, the clinical sign of ischemic heart disease.

Superoxide anion directly interacts with $NO^{\bullet}$ to form peroxynitrite (Koppenol, 1992). Peroxynitrite is a cytotoxic oxidant and affects protein function and hence endothelial function through protein nitration. Peroxynitrite is a key mediator of LDL oxidation, thus highlighting its pro-atherogenic role (Griendling et al., 2003). Furthermore, peroxynitrite degrades tetrahydrobiopterin, a cofactor essential to eNOS (Milstien and Katusic, 1999). Under physiological conditions, the production of ROS is low and the endogenous antioxidant systems maintain the balance between superoxide anion production/elimination, thereby limiting the superoxide anion-mediated breakdown of $NO^{\bullet}$. In disease states in which

ROS production is increased or the antioxidant capacity is decreased, NO is transformed to peroxynitrite, thereby inhibiting endothelial-dependent relaxation (O'Donnell et al., 1997). Prior to the identification of $NO^{\bullet}$ as an EDRF, Rubanyi and Vanhoutte (1986) found that superoxide dismutase augmented endothelium-dependent relaxation. Their observation was confirmed by studies demonstrating improved vascular responses to vasodilators after superoxide dismutase administration (d'Uscio et al., 2001; Steinhorn et al., 2001; Jung et al., 2003). Under certain inflammatory conditions, iNOS is expressed in endothelial and vascular smooth muscle cells (Kibbe et al., 1999). The markedly increased levels of $NO^{\bullet}$ contribute to excessive vasodilation and hypotension, and promote peroxynitrite formation via interaction with enhanced superoxide anion levels observed during these conditions (see Moncada, 2006).

Impaired endothelium-dependent vasodilation is an early warning sign of adverse cardiovascular events and predicts long-term outcome (Schachinger et al., 2000; Perticone et al., 2001). Altered endothelium function is associated with arterial hypertension (Taddei and Salvetti 2002), pre-eclampsia (Page, 2002), diabetes (Makimattila and Yki-Jarvinen 2002), atherosclerosis (Anderson 2003), heart failure (Linke et al., 2003), coronary artery disease (Schachinger et al., 2000), nitrate tolerance (Munzel et al., 1995) and ischemia-reperfusion injury (Sotnikova et al., 1998; Pagliaro et al., 2003). The increased production of ROS and the beneficial effect of antioxidants in these disease states supports evidence that ROS cause endothelial dysfunction (Cai and Harrison 2000).

S-NITROSOTHIOLS

Basic chemistry

S-nitrosothiols are endogenous $NO^{\bullet}$-containing compounds that spontaneously release NO and nitrosonium ion (NO^{+}) (Zai et al., 1999). S-nitrosothiols are thio esters of nitrite with the generic structure, R-S-N=O (Oae and Shinhama, 1983). As will be described below, $NO^{\bullet}$ indirectly reacts with cysteine (thiol) residues to form L-S-nitrosothiols such as L-S-nitrosocysteine and S-nitrosoglutathione. The biological (e.g., vasorelaxant) actions of L-S-nitrosothiols were noted long before it was discovered that $NO^{\bullet}$ was an EDRF (Ignarro et al., 1980, 1981). Interest in S-nitrosothiols was understandably increased by the discovery that these compounds occur naturally in many cell types and fluids in humans and animals (see Stamler et al., 1992b).

S-nitrosothiols are formed from peptide or protein cysteine residues in the presence of $NO^{\bullet}$ and an electron acceptor such as copper, iron or oxygen (Inoue et al., 1999; Stamler et al., 2001; Zhang and Hogg, 2004a). Under physiological conditions, cellular S-nitrosothiol synthesis increases markedly in association with enhanced NOS activation (Zhang and Hogg, 2004b). S-nitrosothiols have been detected in human and rabbit plasma (Stamler et al., 1992b), human airway lining fluid (Gaston et al., 1998), in neutrophils (Clancy et al., 1994) and the brain (Do et al., 1996; Kluge et al., 1997). These studies have led to the conclusion that naturally occurring S-nitrosothiols act as endogenous stores of $NO^{\bullet}$ that are released when required (Stamler et al., 1992c).

Functional activities of S-nitrosothiols

S-nitrosothiols were originally thought to act merely as $NO^{\bullet}$ donors. However, it is now clear that S-nitrosothiols such as the putative EDRF, L-*S*-nitrosocysteine (Myers et al., 1990; Rosenblum et al., 1992), exhibit biological activity that is not dependent on its decomposition to $NO^{\bullet}$ (Kowaluk et al., 1990, Ceron et al., 2001, Travis et al., 2000). For example, $NO^{\bullet}$ is not detected from lower vasorelaxant concentrations of L-S-nitrosocysteine by chemiluminescence techniques, electron paramagnetic spectroscopy or agarose-hemoglobin trapping (Myers et al., 1990; Rubanyi et al., 1991), The $NO^{\bullet}$-independent actions of L-S-nitrosocysteine involve the S-nitrosylation of cysteine residues in functional proteins such as receptors and ion channels (Stamler et al., 1992c,1992d, 1997).

L-S-nitrosocysteine may exert its effects by activation of stereoselective *S*-nitrosothiol recognition sites (Lewis et al., 1996; Davisson et al., 1996, 1997; Ohta et al., 1997; Li et al., 2000; Chen et al., 2000; Lipton et al., 2001), which may recognize L-*S*-nitrosothiols of similar structure, such as L-*S*-nitroso-β,β-dimethylcysteine, (Travis et al., 1996, 1997), but not apply to larger L-*S*-nitrosothiols, such as L-*S*-nitrosoglutathione (Lewis et al., 1996; Davisson et al., 1997; Ohta et al., 1997) or *S*-nitroso-*N*-acetylpenicillamine (Chen et al., 2000). These stereoselective *S*-nitrosothiol recognition sites may be plasma membrane-bound receptors, which specifically recognize L-S-nitrosocysteine and similar lipophobic *S*-nitrosothiols. *In vitro*, L-SNC mediates vasodilation via the activation of calcium-activated potassium channels (Lang et al., 2003; Batenburg et al.,

2004). Activation of these channels promotes vasodilation via hyperpolarization of the smooth muscle cell and subsequent closure of voltage-sensitive calcium channels (Batenburg et al., 2004), thus functioning as an EDHF. Interestingly, Liu *et al* (2002) demonstrated that peroxynitrite reduced the activity of Ca^{2+}-activated K^+-channels in isolated human coronary arteries most likely by nitration rather than oxidation events. Moreover, there is *in vivo* evidence that peroxynitrite attenuates the vasodilator actions of L-S-nitrosocysteine whereas it does not affect those of NO (Graves et al., 2005; Lewis et al., 2005a). Whether this demonstrates that Ca^{2+}-activated K^+-channels are an important recognition site for L-S-nitrosocysteine *in vivo* is yet to be determined although there is compelling evidence that L-S-nitrosocysteine interacts with recognition sites that cannot be these K^+-channels (see Travis et al., 2000; Lewis et al., 2005b).

Decomposition of S-nitrosothiols

S-nitrosothiols decompose by both homolytic and heterolytic cleavage pathways. Their decomposition is accelerated by metal ions such as copper (Cu). The one-electron reduction of Cu^{2+} by thiolate anion moiety of S-nitrosothiols forms Cu^+, presumably by the formation of an intermediate species, $RSCu^+$ (Butler et al., 1998). The release of $NO^{\bullet}$ from the S-nitrosothiol is then facilitated by Cu^+. Transnitrosation involves transfer of NO^+ from the cysteine moiety of S-nitrosothiols to free and protein-associated cysteine residues. Transnitrosation from a stable S-nitrosothiol such as S-nitrosoglutathione to the relatively abundant L-cysteine would form the relatively unstable but potent S-nitrosothiol,

L-S-nitrosocysteine (Butler et al., 1998). Cu^{+} would then presumably decompose this S-nitrosothiol to release $NO^{\bullet}$ (Butler et al., 1998; Barnett et al., 1995; Patel and Williams 1990).

S-nitrosoglutathione is also degraded enzymatically by γ-glutamyl transpeptidase (Askew et al., 1995). γ-Glutamyl transpeptidase destabilizes S-nitrosoglutathione, which releases $NO^{\bullet}$ in the presence of Cu^{+}. S-nitrosothiols can also be decomposed by photochemical means. UV radiation (wavelength 365nm) of S-nitrosotoluene has been shown to be sufficient to cause fission of the S-N bond resulting in release of $NO^{\bullet}$. The resulting thiyl radical can combine with another molecule of S-nitrosothiol, thereby generating the disulphide and a further $NO^{\bullet}$ molecule (Barnett et al., 1995). Others have suggested that hemolytic cleavage at the S-N bond takes place to produce $NO^{\bullet}$ and an alkyl thiyl radical, followed by combination of thiyl radicals to give the corresponding disulphide (Josephy et al., 1984; Mile et al., 1992). A mechanism similar to photochemical decomposition is thermal decomposition, which hemolytically cleaves the S-N bond to release $NO^{\bullet}$ and an alkyl thiyl radical. This thiyl radical then reacts with another S-nitrosothiol to generate a disulphide and another molecule of $NO^{\bullet}$ (Josephy et al., 1984). Ascorbate (vitamin C) decomposes S-nitrosothiols by two mechanisms, at low concentrations, ascorbate reduces Cu^{2+} to Cu^{+}. Release of $NO^{\bullet}$ from the S-nitrosothiol is facilitated by Cu^{+}. At high concentrations, ascorbate acts as a nucleophile, attacking the nitroso group and thereby increasing $NO^{\bullet}$ release directly (Holmes and Williams, 1998).

VESICULAR EXOCYTOSIS

Exocytosis is the process involving fusion of membranes of intracellular vesicles with the plasma membrane of eukaryotic cells. This process has a two-fold purpose, 1) addition of vesicle membrane constituents into the plasma membrane, and 2) the release of vesicular contents such as neurotransmitters into the extracellular environment. There are two forms of exocytosis; constitutive and regulated exocytosis (Kelly, 1985). Constitutive exocytosis, the process by which vesicles fuse with the plasma membrane in the absence of external signals, is important for continuous maintenance of plasma membrane lipids and proteins and the extracellular environment. Regulated exocytosis, the process by which vesicles undergo exocytosis in response to specific stimuli such as an increase in intracellular Ca^{2+}, serves many diverse roles such as the release of neurotransmitters, hormones, enzymes and cytokines.

The best understood form of regulated exocytosis is synaptic transmission, the nervous system's main form of cell-to-cell communication. Fatt and Katz (1952) first introduced the concept of synaptic vesicles in order to explain the quantal release of acetylcholine from the neuromuscular junction. The presence and importance of cytoplasmic vesicles in neurotransmission has been established and numerous proteins that participate in and facilitate synaptic vesicle fusion and exocytosis have now been identified. Neurotransmitter release depends on fusion of the vesicle with the plasma membrane. Neuronal SNARE proteins such as VAMP2 (vesicle-associated membrane protein, also known as

synaptobrevin), syntaxin1 and SNAP-25 (synaptosomal-associated protein of 25KDa) are essential for membrane fusion. Due to anchoring in the vesicle membrane, VAMP (Trimble et al 1988) was originally classified as a vesicular (v)-SNARE (Sollner et al., 1993). Syntaxin (Bennett et al., 1992) and SNAP-25 (Oyler et al., 1989) were originally classified as target (t)-SNARE proteins because they were located in the target membranes. Syntaxin and VAMP possess carboxy-terminal trans-domains, whereas SNAP-25 is held in the membrane by palmitoylation on several cysteine residues.

SNARE proteins were originally classified v- or t-SNARE proteins due to the preferential localization of the founding members of this protein family on secretory vesicles or the plasma membrane (Söllner et al., 1993). However, it became apparent that both v- and t-SNARE proteins coexisted on vesicles and target membranes (Cao and Barlowe 2000; Nichols et al., 1997; Rowe et al., 1998; Walch-Solimema et al., 1995). Resolving the crystal structures of SNARE proteins (Sutton et al., 1998) led to the reclassification of SNARE proteins as Q- and R-SNARE proteins according to the presence of conserved glutamine or arginine residues in their cytoplasmic domains (Fasshauer et al., 1998b). It is now known that Q- and R-SNARE proteins form complexes (Bennett et al., 1992; Calakos et al., 1994; Chapman et al., 1994; Söllner et al., 1993). It was predicted that SNARE proteins would bind to each other via hydrophobic interactions, based on their appearance of coiled-coil or heptad repeat domains in their amino acid sequences (Hardwick and Pelham, 1992). Ternary complex formation

causes induction of helical structure in the VAMP and SNAP-25 coiled-coil domains, which are unstructured in their monomeric forms (Fasshauer et al., 1998; Canaves and Montal 1998).

Evidence also exists that exocytotic mechanisms reside in endothelial cells. When endothelial cells are activated during inflammation, there is a rapid translocation of P-selectin to the endothelial cell surface, followed by a slower synthesis and expression of adhesion molecules such as ICAM-1. P-selectin is stored in endothelial storage granules (i.e., Weibel-Palade bodies), which also contain von Willebrand factor (VWF), interleukin -8 (IL-8) and tissue plasminogen activator (Bonfanti et al., 1989; Huber et al., 2002; McEver et al., 1989; Qian et al., 2001; Vischer et al., 1994; Wagner et al., 1982; Wagner et al., 1991; Wagner et al., 1993; Wolff et al., 1998; Utgaard et al., 1998).

This rapid exocytosis of Weibel-Palade bodies is activated by vasopressin, hypoxia, histamine and thrombin (McEver et al., 1989; Kaufman et al., 2000; Pinsky et al., 1996; Sporn et al., 1986) and mediated by NSF and SNAREs (Jahn and Sudhof, 1999; Jahn et al., 2003; Mellman and Warren, 2000). Exocytosis of Weibel-Palade bodies causes rapid translocation of P-selectin from within the granule to the endothelial surface whereupon P-selectin interacts with P-selectin glycoprotein ligand-1 (PSGL-1) on the surface of leukocytes, thereby triggering leukocyte rolling (Mayadas et al., 1993). As such, it would seem that exocytosis of Weibel-Palade bodies is an integral step in vascular inflammation.

Transcytosis of albumin, insulin and transferritin is another example of vesicular transport in endothelial cells (Schnitzer and Oh, 1994; Schnitzer, 1993). Caveolae also contain the molecular transport machinery for vesicle budding, docking and fusion, such as VAMP, NSF and SNAP (Schnitzer et al., 1995).

Ignarro (1990) postulated that S-nitrosothiols may exist in the form of pre-formed pools in specialized storage vesicles in endothelial cells and that endothelium-dependent agonists may initiate the Ca^{2+}-dependent exocytotic release of these vesicular stores. Loesch et al (1994) demonstrated the ultra-structural distribution of NOS in an endothelial cell. They observed NOS localized to membranes of many small cytoplasmic vesicles that were distinct from caveolae. It is known that NOS in endothelial cells translocates from plasma membranes to the cytosol after exposure to bradykinin (Michel et al., 1993). This is consistent with NOS-positive vesicles budding into the plasma membranes and then returning to the cytoplasm. Taken together, this background data provided the rationale for the studies described in this thesis dissertation.

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CHAPTER 3

CYTOPLASMIC VESICLES IN ENDOTHELIAL CELLS:

A UBIQUITOUS PHENEMENON[1]

[1]**Hashmi-Hill MP**, Shields JP, Lewis THJ, Robertson TP, Lewis SJ. Light and ultrastructural investigation of cytoplasmic vesicles in endothelial cells of the rat. To be submitted for publication in the *American Journal of Physiology*, 2006

ABSTRACT

The vasorelaxant properties of the S-nitrosothiol, L-S-nitrosocysteine more closely resemble those of endothelium-dependent-relaxing factor (EDRF) than do those of nitric oxide (NO). The ***hypothesis*** driving this study is that preformed pools of S-nitrosothiols exist in cytoplasmic vesicles within vascular endothelial cells of resistance arteries (i.e., those that regulate arterial blood pressure). For this to be true then endothelial cells must contain vesicles that are subject to mobilization and fusion to the plasma membranes of cell (i.e., exocytosis).

The ***objective*** of this study was to determine the presence of cytoplasmic vesicles in the endothelium of cerebral, basilar, small pulmonary, mesenteric and femoral arteries. The above mentioned arteries were fixed and processed for transmission electron microscopy and the presence of cytoplasmic vesicles within the endothelium of these arteries was determined.

Cytoplasmic vesicles of approximately 75 nm in mean diameter (range of 50-100 nm) were observed in all arteries investigated. Subsequent evidence that these cytoplasmic vesicles contain vasoactive factors and are subject to Ca^{2+}-*dependent* and/or Ca^{2+}-*independent* exocytosis would represent a ubiquitous and fundamental mechanism by which the endothelium regulates blood flow.

Keywords: Endothelial cells, Nitric oxide, S-nitrosothiols, Vesicles

INTRODUCTION

The vascular endothelium plays a number of important physiological roles and since it is strategically located at the interface between tissue and blood, it is in an ideal position to modulate the function of various organs (see Moncada et al., 1991; Moncada and Higgs, 2006). Endothelial cells play a major role in regulating the functional status of vascular tone due to their capacity to release numerous endothelium-derived relaxing (EDRF), hyperpolarization (EDHF) and contracting (EDCF) factors (see Ignarro, 1990; Luscher and Vanhoutte, 1990; Batenburg et al., 2004a,b; Moncada and Higgs, 2006).

S-nitrosothiols are endogenous nitric oxide (NO)-containing compounds (Oae and Shinhama, 1983; Stamler et al., 1992a,b, 1997; Zai et al., 1999). L-S-nitrosothiols, such as the putative EDRF/EDHF, L-S-nitrosocysteine (Bates et al., 1991; Rubanyi et al., 1991; Myers et al., 1990; Rosenblum et al., 1992; Batenburg et al., 2004a,b) have biological activity that is independent of their decomposition to NO (Kowaluk et al., 1990; Myers et al., 1990; Rubanyi et al., 1991; Rosenblum et al., 1992; Travis et al., 2000; Ceron et al., 2001, Batenburg et al., 2004a,b). This NO-independent action of S-nitrosothiols involve the S-nitrosylation of cysteine residues in functional proteins, such as receptors, ion channels and enzymes (Stamler et al., 1992,b, 1997; Lang et al., 2003) and the activation of stereoselective recognition sites that may be a unique family of cell-surface receptors (see Lewis et al., 1996; Davisson et al., 1996a, 1997a; Lewis et al., 2005a,b, 2006a,b; Lipton et al., 2001).

Ignarro (1990) postulated that pre-formed S-nitrosothiols may exist in cytoplasmic vesicles within endothelial cells and that endothelium-dependent agonists initiate Ca^{2+}-dependent exocytotic release of these vesicular stores of S-nitrosothiols. Substantial ***indirect*** evidence has accumulated in support of this postulation (Weir et al., 1991; Davisson et al., 1996b; Danser et al., 1998, 2000; Kakayuma et al., 1998) and that such cytoplasmic vesicles may exist in nerve terminals (Davisson et al., 1996c,d, 1997b; Possas and Lewis, 1997; Buyukafsar *et al*., 1999a; Possas et al., 2006). Moreover, vascular smooth muscle (Matsunga & Furchgott, 1991; Chaudrey *et al*., 1993; Venturini *et al*., 1993; Kubaszewski et al., 1994), gastric fundus (Buyukafsar *et al*., 1999b), and corpus cavernosum (Buyukafsar *et al*., 1999b) contain photosensitive stores of these factors.

There is considerable ***direct*** evidence that preformed pools of S-nitrosothiols and dinitrosyl iron (II) cysteine complexes exist in tissues including the vasculature (Muller *et al*., 1996, 2002; Vanin *et al*., 1998; Malyshev *et al*., 1999; Manukhina *et al*., 1999; Smirin *et al*., 1999, 2000; Pshennikova *et al*., 2000), brain (Kluge *et al*., 1997), and red blood cells (Jia *et al*., 1996). However, there is no ***definitive*** evidence that such pools exist in vesicles in endothelial cells or nerves. Although the subcellular localization of putative stores of nitrosyl factors in endothelial cells has not been determined, it would seem reasonable to suggest that these factors exist in cytoplasmic vesicles in these cells that are mobilized to exocytosis by increases in intracellular Ca^{2+} elicited by stimuli such as sheer stress and the exposure to G protein-coupled receptor agonists.

Vascular endothelial cells and especially those in smaller arteries contain large numbers of cytoplasmic vesicles (Bruns et al., 1968a,b; Bundgaard et al., 1979; Mazzone & Kornblau, 1980; Wagner Casey-Smith, 1981; Huttner & Gabbiani, 1983; Loesch, 1993, 1994, 1996; Lane et al., 1995; Moldovan et al., 1995). Moreover, large numbers of these vesicles contain NO synthase (NOS) in their cytoplasmic membranes (Loesch, 1993, 1994). The presence of NOS would provide a mechanism by which S-nitrosothiols could be generated and ultimately stored in vesicles. The finding that bradykinin causes NOS to translocate to the plasma membranes of endothelial cells (Michel et al., 1993) is consistent with a Ca^{2+}-dependent mobilization and fusion of NOS-positive vesicles to the plasma membranes. Moreover, the plasma membranes of endothelial cells contain fusion proteins that are known to support vesicular exocytosis (Schnitzer et al., 1995). Moreover, studies in this thesis dissertation have provided evidence that fusion proteins exist on the membranes of cytoplasmic vesicles.

Calmodulin antagonists inhibit endothelium-dependent relaxations in arteries treated with NOS inhibitors by reducing the release rather than through inhibition of EDHFs/EDRFs (Illiano et al., 1992, 1993; Nagao et al., 1992). This suggests that, similar to other secretory cells (Thureson-Klein & Klein, 1990; Greengard et al., 1993; Thomas-Reetz and Camili, 1994; Sudhof, 1995), a Ca^{2+}-calmodulin complex may mobilize S-nitrosothiol-containing vesicles in endothelial cells. Inhibitors of mitochondrial electron transport, F1-ATPase or oxidative phosphorylation markedly inhibit endothelium-dependent relaxations (Griffith et al., 1986; Richards et al., 1991; Wei et al., 1991). This further supports the

contention that vesicular exocytosis of S-nitrosothiols occurs in endothelial cells since these inhibitors block vesicular exocytosis in nerve terminals and adrenal chromaffin cells (Thureson-Klein & Klein, 1990).

Loesch et al (1994) demonstrated the ultrastructural distribution of NOS in an endothelial cell from a rat basilar artery. They observed NOS localized to membranes of many small cytoplasmic vesicles that were distinct from caveolae. It is known that NOS in endothelial cells translocates from plasma membranes to the cytosol after exposure to bradykinin (Michel et al., 1993). This is consistent with NOS-positive vesicles fusing with or budding from plasma membranes and then returning to the cytoplasm. Taken together, the above data provide compelling support to the concept that cytoplasmic vesicles in endothelial cells contain preformed S-nitrosothiols and that these vesicles are subject to exocytosis and endocytosis.

We provided direct evidence that preformed pools of S-nitrosothiols exist in cytoplasmic vesicles within vascular endothelial cells (Lewis et al., 2006c). At present there is no evidence that small pulmonary, mesenteric and femoral arteries of the rat contain cytoplasmic vesicles that may be subject to vesicular exocytosis. Accordingly, the specific ***objective*** of the ultra-structural studies in this chapter is to determine whether cytoplasmic vesicles exist in the endothelium of the above arteries. The presence of such vesicles, if proven to be subject to Ca^{2+}-*dependent* and Ca^{2+}-*independent* mechanisms, would therefore represent

fundamental basis for a ubiquitous physiological phenomenon for the regulation of blood flow.

MATERIALS AND METHODS

Animals

All studies were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) revised in 1996. The protocols pertaining to the use of animals were approved by the Animal Care and Use Committee of the University of Georgia. Adult male Sprague-Dawley rats (n=4, ≈350g) were used in these studies.

Preparation of arteries

The rats were killed by decapitation and small (approximately 200-250 μm in diameter) basilar, cerebral, pulmonary, mesenteric and femoral arteries were dissected, rapidly rinsed in 0.1M phosphate-buffered saline to remove as much blood as possible, and immersion-fixed for 3h at 4°C with 4% paraformaldehyde and 0.25% glutaraldehyde (in 0.1M phosphate buffer, pH7.4). Arteries were then transferred to phosphate buffer and stored overnight at 4°C. The arteries were then prepared for transmission electron microscopy as described below.

Transmission Electron Microscopy

Arteries were post-fixed in 1% osmium tetroxide (OsO4) for 1h at room temperature and dehydrated in a graded ethanol series, and then a graded

acetone series. Arteries were infiltrated, embedded, and polymerized for 24h at 60°C in Embed 812 epoxy resin (Polysciences, Warrington, PA, USA) and silver (60nm) sections were cut with a diamond knife on an ultra-microtome (RMC MT-X, Tucson, AZ, USA). The sections were post-stained with uranyl acetate and lead citrate and viewed on a transmission electron microscope (model 100CX II, JEOL USA, Peabody, MA) in the *Center for Advanced Ultrastructural Research* at the University of Georgia. The electron microscope was set to operate at 80 KeV.

Analysis

The general shapes of cytoplasmic vesicles were qualitatively assessed. Average vesicle diameter was measured under the transmission electron microscope by measuring the diameter of 100 vesicles in each endothelial slice. At least 60 cell slices for each cerebral, basilar, pulmonary, mesenteric and femoral artery were taken from each of the 4 rats for quantitative analyses.

Statistics

The data are presented as mean ± SEM. All data were initially submitted to tests of normality and homogeneity of variances as described by Winer (1971). All data proved to be normal and so the data were analyzed by repeated-measures analysis of variance (Winer, 1971) followed by Student's modified t-test with the Bonferroni correction for multiple comparisons between means using the error mean square terms from the repeated measures analysis of

variance (see Wallenstein *et al*., 1980). A value of $P < 0.05$ was taken to denote statistical significance between the means.

RESULTS

The results of this study show that small cytoplasmic vesicles are present within the endothelium of small cerebral, basilar, pulmonary, mesenteric and femoral arteries of rats. Vesicular characteristics including the general shape (i.e., circular, elliptical and/or irregular forms) and specific numbers and diameters of the vesicles for each individual endothelial bed will be described first. Statistical comparisons for the quantified data will then be presented.

Cerebral Artery

Small cytoplasmic vesicles were readily observable in the endothelial cells of cerebral arteries (see Fig. 3.1A, magnification x 19,000). The majority of the vesicular structures were circular, but elliptical and irregular forms were also apparent. The general shapes of the cytoplasmic vesicles can be better seen in panel B of Fig. 3.1 which is an enlargement of the cell shown in Fig 3.1A (magnification x 28,500). The average number of vesicles present in each endothelial cell was 47.1 ± 1.6 (n=58 cell slices). Although the average diameter of these vesicles was ranged from 50-100 nm, the majority of the vesicles had very similar diameters that centered around 70-80 nm (mean diameter = 73.6 ± 1.8 nm, n=100 vesicles). Only a relatively small number of vesicles were found to be fused with the endothelial cell membrane.

Basilar Artery

Small cytoplasmic vesicles were readily observable in endothelial cells of basilar arteries (see Fig. 3.2, magnification x 48,000). The majority of the structures were circular, however elliptical and irregular forms were also observed. The average number of vesicles present in each endothelial cell was 57.9 ± 1.5 (n=57 cell slices). The diameter of these vesicles ranged from 50-100 nm but most had diameters that centered around 70-80 nm (mean = 76.5 ± 1.4, n=100 vesicles). A small number of vesicles were found to be fused with the endothelial cell plasma membrane.

Pulmonary Artery

Small cytoplasmic vesicles were readily observable in endothelial cells of pulmonary arteries (see Fig. 3.3, magnification x 58,000). The majority of the structures were circular, but elliptical and irregular forms were also apparent. The average number of vesicles present in each endothelial cell was 95.0 ± 1.9 (n=66 cell slices). The diameter of these vesicles ranged from 50-100 nm but most had diameters that centered around 70-80 nm (mean = 73.4 ± 1.8, n=100 vesicles).

Mesenteric Artery

Small cytoplasmic vesicles were readily observable in endothelial cells of mesenteric arteries (see Fig. 3.4, magnification x 48,000). The majority of the structures were circular, but elliptical and irregular forms were also apparent. The average number of vesicles present in each endothelial cell was 80.0 ± 1.8 (n=70

cell slices). The diameter of these vesicles ranged from 50-100 nm but most had diameters that centered around 70-80 nm (mean = 71.4 ± 1.9, n=100 vesicles).

Femoral Artery

Small cytoplasmic vesicles were readily observable in endothelial cells of femoral arteries (see Fig. 3.5, magnification x 58,000).Small cytoplasmic vesicles were readily observable. The majority of the structures were circular, but elliptical and irregular forms were also apparent. The average number of vesicles present in each endothelial cell was 67.4 ± 1.6 (n=73 cell slices). The diameter of these vesicles ranged from 50-100 nm but most had diameters that centered around 70-80 nm (mean = 77.0 ± 1.5, n=100 vesicles).

Between artery comparisons

A summary of the numbers of vesicles observed in endothelial cells from cerebral (CER), basilar (BAS), pulmonary (PUL), mesenteric (MES) and femoral (FEM) arteries are summarized in the top panel of Fig. 3.6. The numbers 1-5 indicate that there were significant differences in vesicle numbers between each endothelial bed. Pulmonary endothelial cells had the largest numbers of vesicles whereas cerebral endothelial cells had the least. The descending order of vesicle numbers was pulmonary (5) $>$ mesenteric (4) $>$ femoral (3) $>$ basilar (2) $>$ cerebral (1) endothelial cells ($P < 0.05$ for all comparisons).

A summary of the average vesicle diameters observed in endothelial cells from cerebral (CER), basilar (BAS), pulmonary (PUL), mesenteric (MES) and femoral (FEM) arteries are summarized in the bottom panel of Fig. 3.6. The average diameters of these vesicles were similar in each endothelial bed.

DISCUSSION

A principal finding of this study was that endothelial cells of rat basilar, cerebral, pulmonary, mesenteric and femoral arteries contain substantial numbers of cytoplasmic vesicles. The majority of the vesicular structures were circular in shape, but elliptical and irregular forms were also present in all endothelial cells. The diameters of the cytoplasmic vesicles in all endothelial cells ranged from 50-100 nm although most vesicles had a diameter between 70-80 nm. Mean vesicle diameters of endothelial cells were; cerebral artery (73.6 $\pm$ 1.8 nm), basilar artery (76.5 $\pm$ 1.4 nm), pulmonary artery (73.4 $\pm$ 1.8 nm), mesenteric artery (71.4 $\pm$ 1.9 nm), and femoral artery (77.0 $\pm$ 1.5 nm). The average diameters of these vesicles are consistent with reported vesicle diameters in endothelial cells of rats (60-80 nm) and in larger species including man (80-120 nm) (Bundgaard et al., 1979; Mazzone and Kornblau, 1980; Wagner Casey-Smith, 1981; Huttner and Gabbiani, 1983; Bruns et al., 1968a,b; Loesch, 1993, 1994, 1996; Harrison et al., 1995; Lane et al., 1995; Moldovan et al., 1995). In addition, the average diameters of the cytoplasmic vesicles in the endothelial cells of the various rat arteries we examined lie between those of synaptically-

active (i.e., exocytotic) small light/medium core vesicles (40-60nm) and large dense core vesicles (80-120 nm) in rat nerve terminals (Nirenberg et al., 1995).

There were striking differences in the numbers of cytoplasmic vesicles in endothelial cells of rat arteries. The average numbers of vesicles in endothelial cells were; cerebral artery (47.1 ± 1.6), basilar artery (57.9 ± 1.5), pulmonary artery (95.0 ± 1.9), mesenteric artery (80.0 ± 1.8 nm), and femoral artery (67.4 ± 1.5 nm). As such, pulmonary endothelial cells contained the highest numbers of vesicles whereas cerebral arteries contained the fewest. However, it is evident that each endothelial cell type contains more than enough vesicles to support dynamic exocytotic processes. The relatively greater numbers of cytoplasmic vesicles in pulmonary artery endothelial cells may be a function of their special role in lung physiology. Increases in shear stress or application of endothelium-dependent agonists elicits the release of EDRFs and EDHFs from pulmonary endothelial cells (Robertson et al., 1995, 2000a,b,c; 2001, 2003). As such, sub-populations of cytoplasmic vesicles in endothelial cells of pulmonary arteries may sub-serve Ca^{2+}-*dependent* release of vasodilator factors including, S-nitrosothiols (Lewis et al., 2006c), adenosine (Bodin and Burnstock, 2001) and/or prostacyclin (Spisni et al., 2001). However, pulmonary arteries are unique in that they are the only arteries that ***constrict*** rather than dilate in response to hypoxia, a phenomenon that is referred to as *hypoxic pulmonary vasoconstriction* (Robertson et al., 1995, 2000a,b,c; 2001, 2003).

The sustained phase of hypoxia-driven pulmonary artery vasoconstriction is abolished by removal of the endothelium and is therefore likely to be due to the rapid and sustained release of an (as yet unidentified) endothelium-derived contracting factor (Robertson et al., 1995, 2000a,b,c; 2001, 2003). Accordingly, sub-populations of cytoplasmic vesicles in pulmonary artery endothelial cells may sub-serve Ca^{2+}-*independent* release of this vasoconstrictor factor.

Endothelial cells of all arteries under investigation displayed a relatively small number of cytoplasmic vesicles that were obviously fused to the plasma membrane. These fused vesicles appeared as omega shapes when fused with the plasma membrane, reminiscent of the omega-shaped vesicles found in nerve terminals during vesicular exocytosis (Heuser, 1989; Heuser & Reese, 1981). Indeed, electron microscopic detection of omega-shaped images at active zones when neurotransmitter/neuromodulator release is occurring is the established hallmark of exocytosis (Heuser, 1989; Heuser & Reese, 1981). Accordingly, our observations suggest that exocytosis takes place in endothelial cells of rat basilar and cerebral arteries and rat small pulmonary, mesenteric and femoral arteries. The relatively small proportion of fused vesicles that were observed in the arteries probably reflects that at the time of immersion fixation, there would be little stimulus for exocytosis. This possibility is supported by our evidence that endothelium-dependent agonists and hypoxia elicit a marked increase in the numbers of cytoplasmic vesicles fused to the plasma membranes of endothelial cells (see Chapter 5).

Our findings are in agreement with evidence that vascular endothelial cells and especially those in smaller arteries contain large numbers of cytoplasmic vesicles (Bruns et al., 1968a,b; Bundgaard et al., 1979; Mazzone and Kornblau, 1980; Wagner Casey-Smith, 1981; Huttner and Gabbiani, 1983; Loesch, 1993, 1994, 1996; Lane et al., 1995; Moldovan et al., 1995). Moreover, large numbers of these vesicles contain NOS in their cytoplasmic membranes (Loesch, 1993, 1994, 1996). The presence of NOS would provide the mechanism by which S-nitrosothiols could be generated and ultimately stored in vesicles. Indeed, our recent studies have demonstrated that S-nitrosothiols exist in vesicles isolated from vascular endothelial cells (Lewis et al., 2006c). A variety of tissues including neurons, and vascular and gastric smooth muscle contain preformed pools of S-nitrosothiols and dinitrosyl iron complexes (Muller et al., 1996, 2002; Jia et al., 1996; Kluge et al., 1997; Vanin et al., 1998; Malyshev et al., 1999; Manukhina et al., 1999; Smirin et al., 1999, 2000; Pshennikova et al., 2000). Although it is unlikely that S-nitrosothiols are stored in vesicles in non-excitable tissues such as muscle, it would seem likely that S-nitrosothiols also exist in cytoplasmic vesicles in neurons and that some of these vesicles in endothelial cells and neurons are subject to Ca^{2+}-*dependent* exocytosis whereas other vesicles may be subject to Ca^{2+}-*independent* exocytosis (see Huttner and Gabbiani, 1983).

The cytosol is an unfavorable environment for S-nitrosothiols since they can be readily reduced (homolytically cleaved) by glutathione or thioredoxin (Haendeler et al., 2002; Freedman et al., 1995; Liu et al., 2001). In order to

prevent degradation of S-nitrosothiols, it has been suggested that S-nitrosothiols may be stored and/or protected in vesicles, in membranes, in lipophilic protein folds and in interstitial spaces (Ignarro, 1990; Rafikova et al., 2002; Lewis et al., 2002; Mannick et al., 2001). Since L-S-nitrosocysteine is extremely lipophobic (Kowaluk and Fung, 1990), some mechanism of facilitated diffusion must exist for this S-nitrosothiol to pass through the cell membrane and into extracellular space between the endothelial cell and the vascular smooth muscle. Storage of S-nitrosothiols in NOS-containing cytoplasmic vesicles that are subject to regulated exocytosis would be one such mechanism of facilitated diffusion.

Molecular biological studies provided evidence that the endothelium-dependent agonist, bradykinin, causes a marked translocation of intracellular NOS to the plasma membranes of endothelial cells (Michel et al., 1993). This finding is consistent with bradykinin eliciting a Ca^{2+}-*dependent* mobilization and fusion of NOS-positive vesicles to the plasma membranes. Evidence that the plasma membranes of endothelial cells contain fusion proteins that promote vesicular exocytosis in nerve terminals supports a wealth of functional evidence that exocytotic mechanisms exist in endothelial cells (see Schnitzer et al., 1995). Evidence presented in this dissertation show that fusion proteins known to exist on vesicles in nerve terminals are also present in endothelial cells certainly strengthens the overall conclusion that exocytotic mechanisms involving vesicle-cell membrane fusion events take place in endothelial cells.

Calmodulin antagonists inhibit endothelium-dependent relaxations in arteries treated with NOS inhibitors by antagonizing the release rather than the mechanisms of action of EDHFs/EDRFs (Illiano et al., 1992, 1993; Nagao et al., 1992). This raises the possibility that, similar to other secretory cells (Thureson-Klein and Klein, 1990; Greengard et al., 1993; Thomas-Reetz and Camili, 1994; Sudhof, 1995), a Ca^{2+}-calmodulin complex initiates the mobilization of S-nitrosothiol-containing vesicles in endothelial cells. Moreover, the findings that inhibitors of mitochondrial electron transport, F1-ATPase or oxidative phosphorylation markedly inhibit endothelium-dependent relaxations via release of EDRF (Griffith et al., 1986; Richards et al., 1991; Wei et al., 1991) further support the possibility that exocytosis of vesicular stores of nitrosyl factors occurs in endothelial cells since these inhibitors block vesicular exocytosis in nerve terminals and adrenal chromaffin cells (Thureson-Klein & Klein, 1990).

CONCLUSIONS

The results of this ultra-structural study show that cytoplasmic vesicles are common to the endothelium of cerebral, basilar, pulmonary, mesenteric and femoral arteries of the rat. These vesicles provide the basic structural requirement to support the facilitated secretion of compounds via exocytosis. The following chapters will extend these basic findings.

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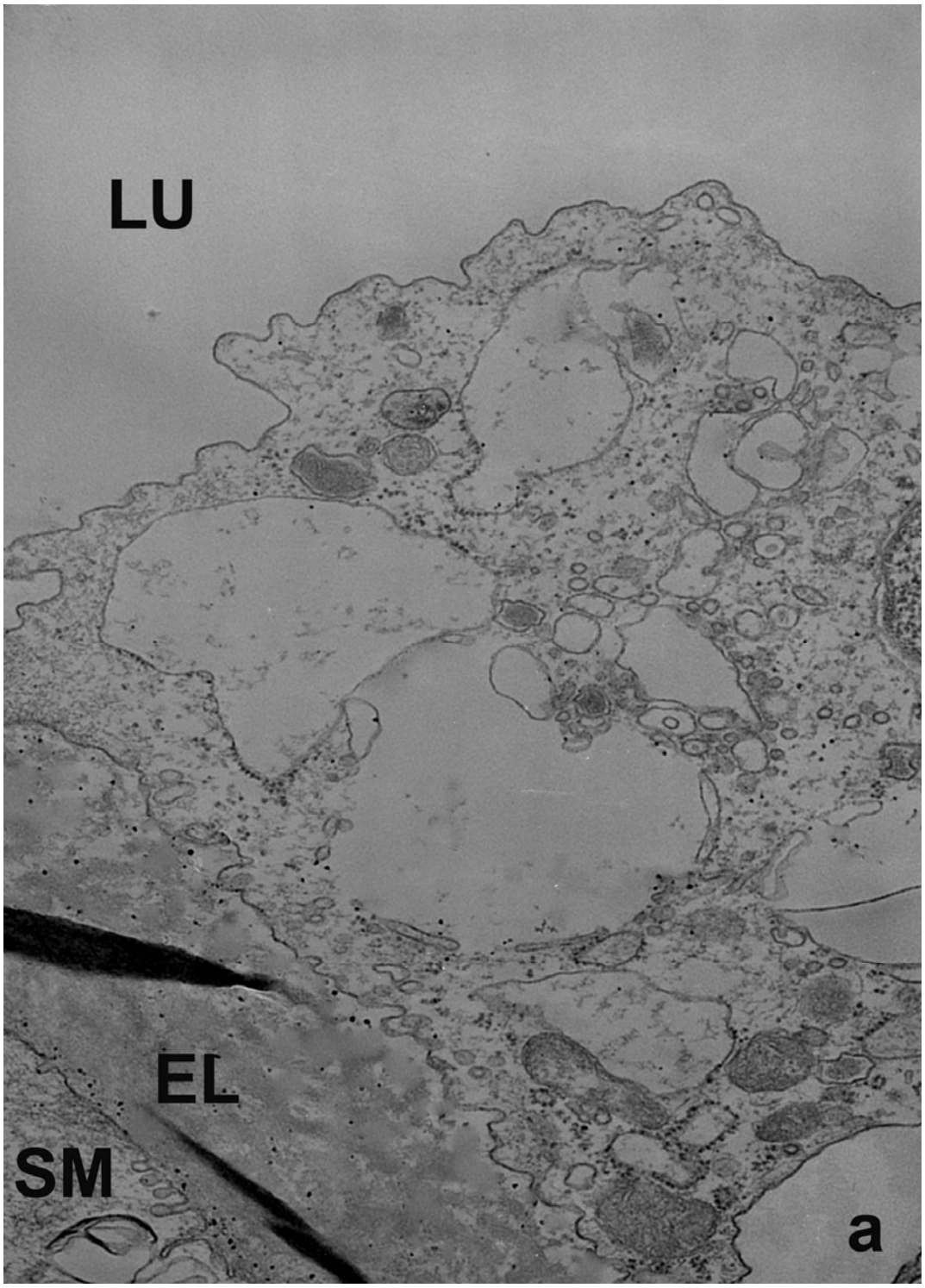

Fig. 3.1a Electron micrograph of a cerebral artery endothelial cell. Magnification x 19,000. EL=elastic lamina, SM=smooth muscle, LU=lumen

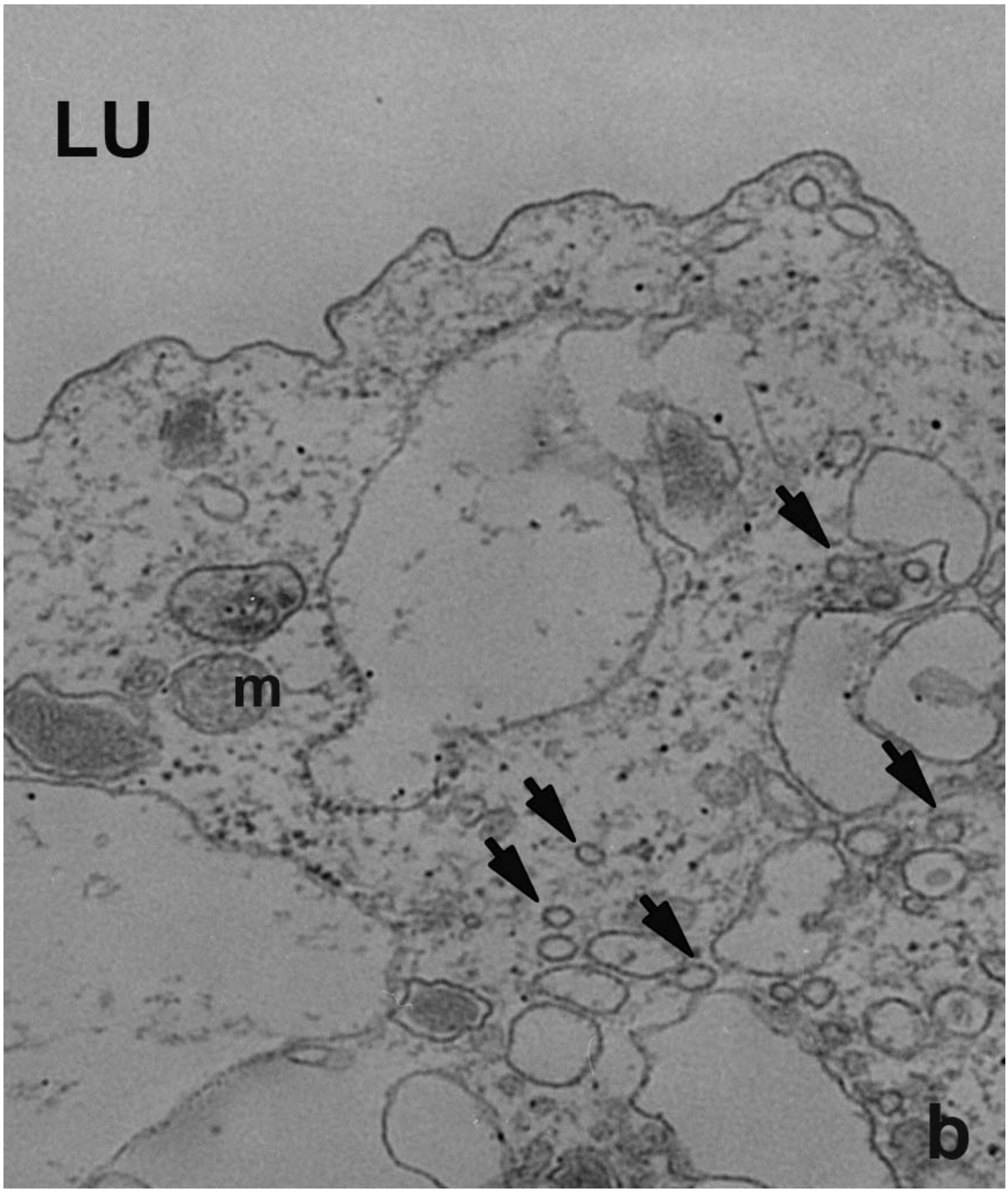

Fig. 3.1b Enlargement of part of the cerebral artery endothelial cell illustrated in Fig. 3.1a showing small cytoplasmic vesicles (arrows). Magnification x 28,500. EL=elastic lamina, SM=smooth muscle, LU=lumen

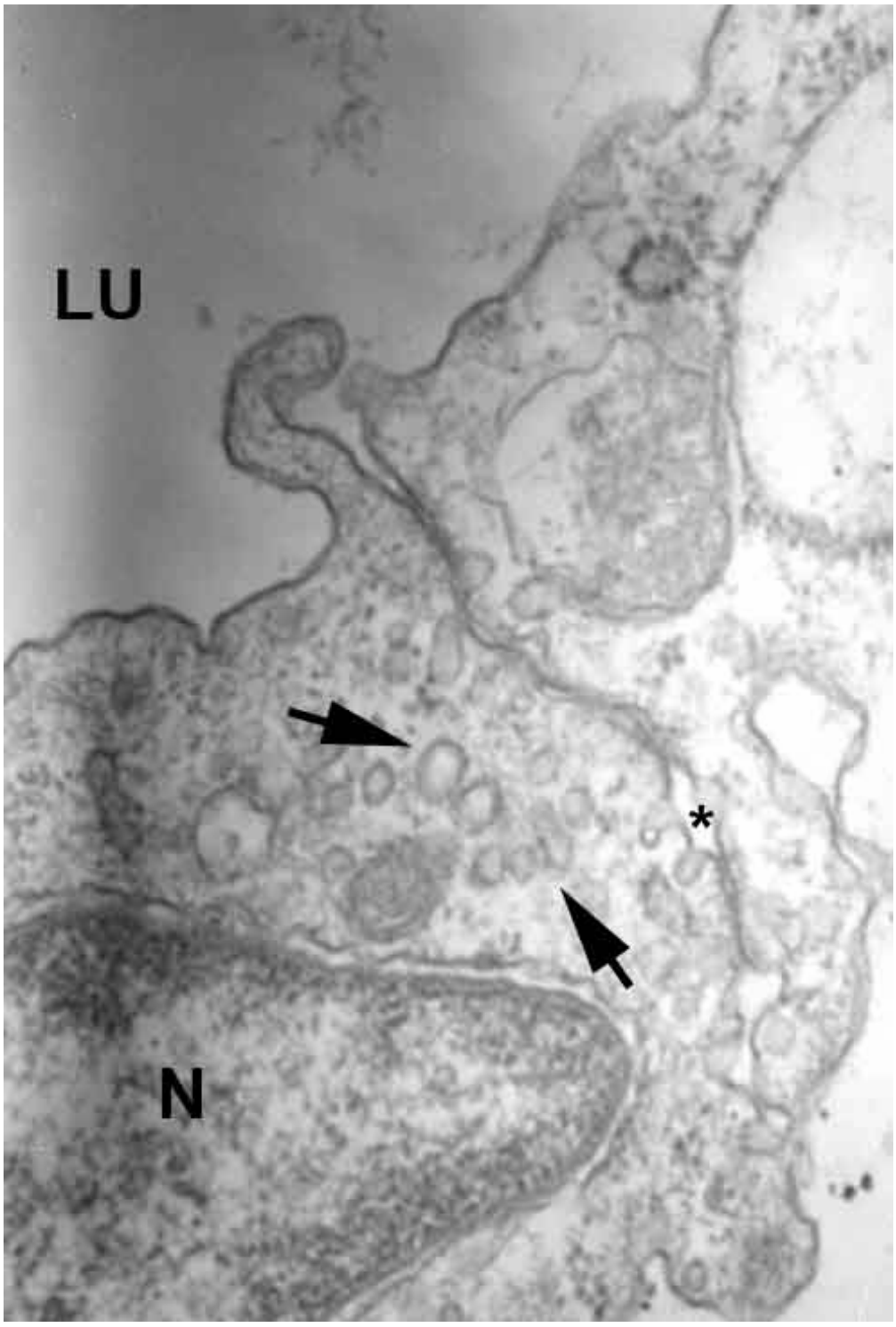

Fig. 3.2 Electron micrograph of basilar artery endothelial cells displaying cytoplsmic vesicles (arrows). Magnification x 48,000. Note the fused vesicles (stars). LU=lumen, N=nucleus.

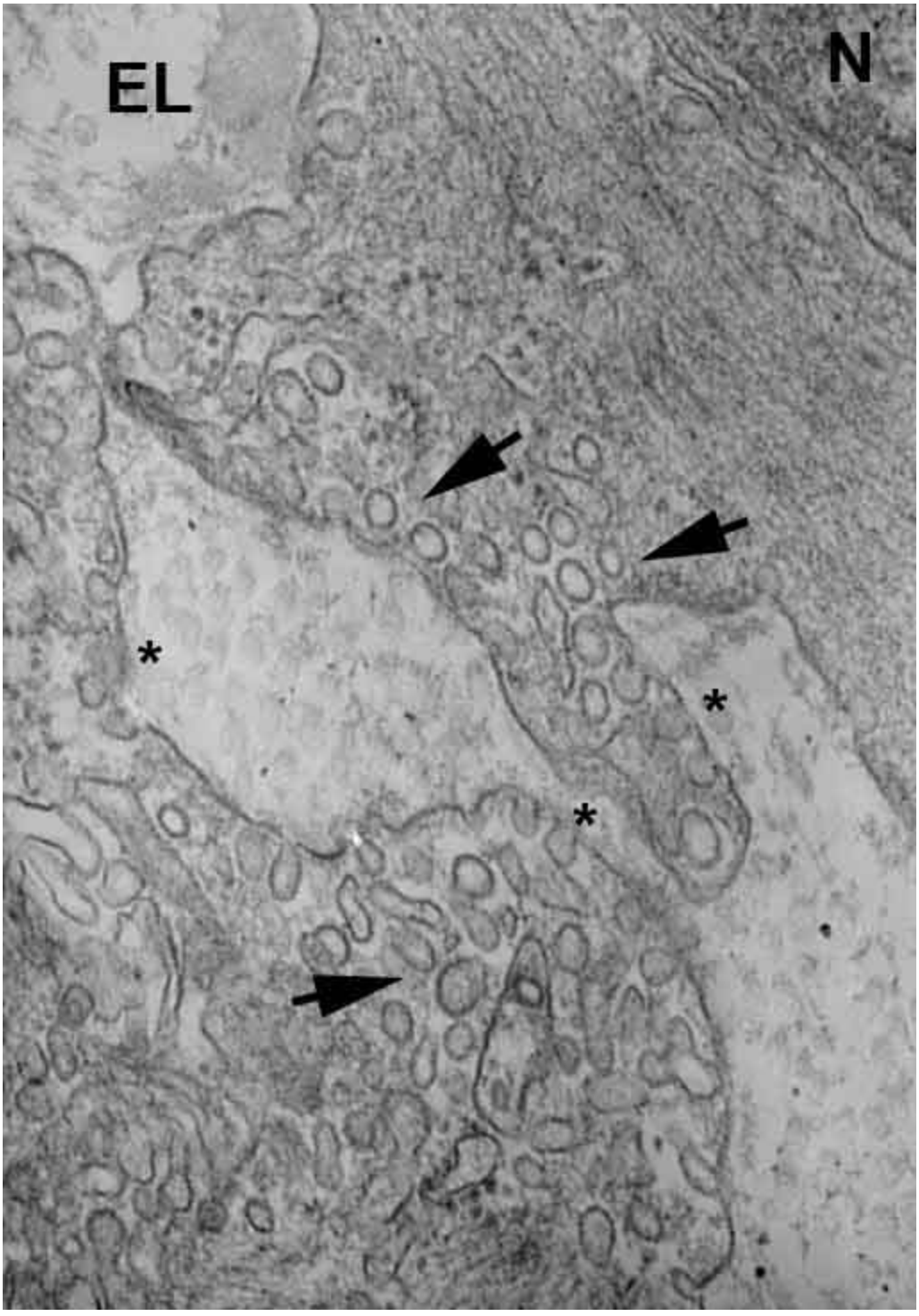

Fig. 3.3 Electron micrograph of a pulmonary artery endothelial cell displaying cytoplasmic vesicles (arrows). Magnification x 58,000. Note the fused vesicles (stars). LU=lumen, N=nucleus

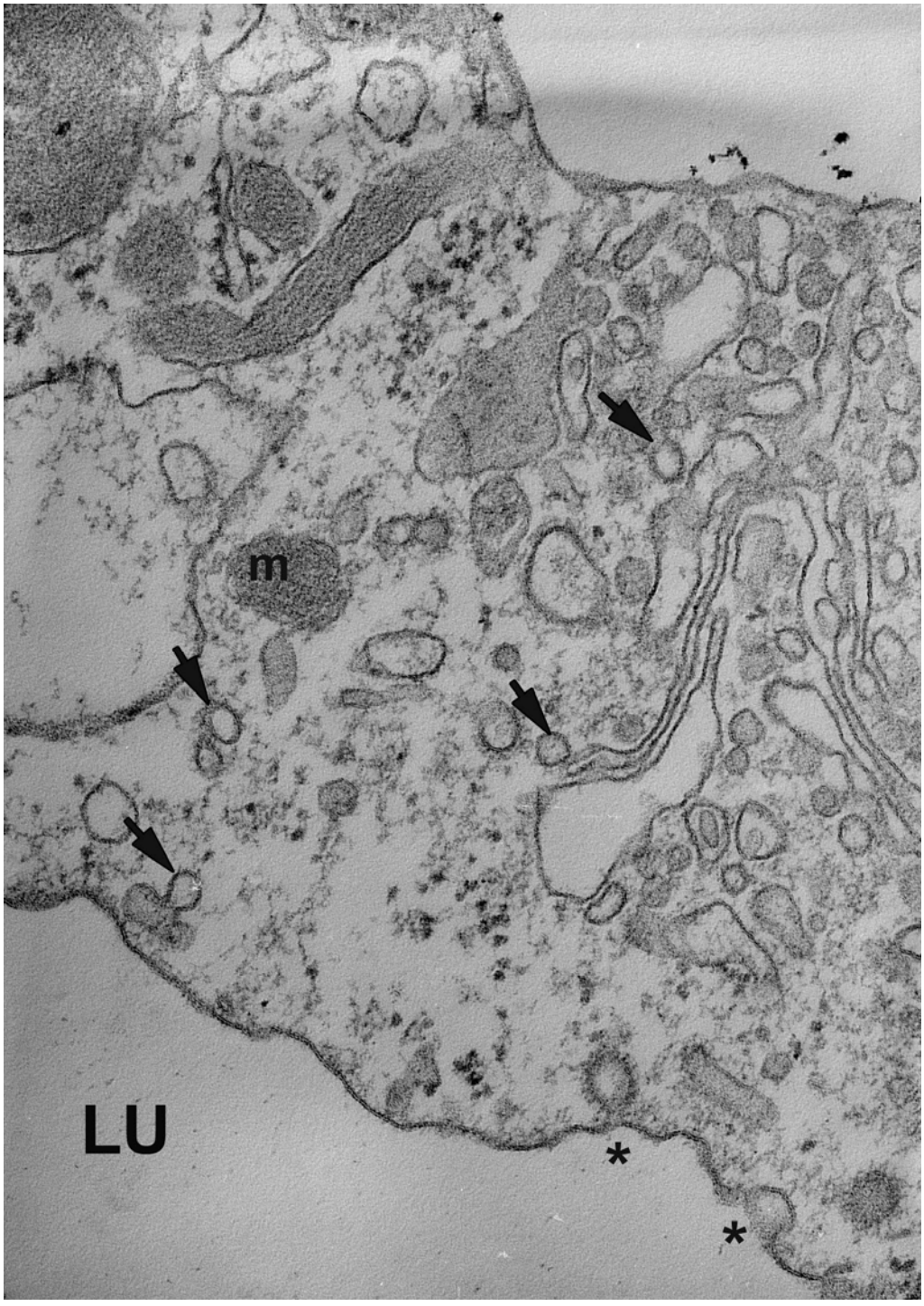

Fig. 3.4 Electron micrograph of a mesenteric artery endothelial cell displaying cytoplasmic vesicles (arrows). Magnification x 48,000. Note the fused vesicles (stars). LU=lumen, m=mitochondria

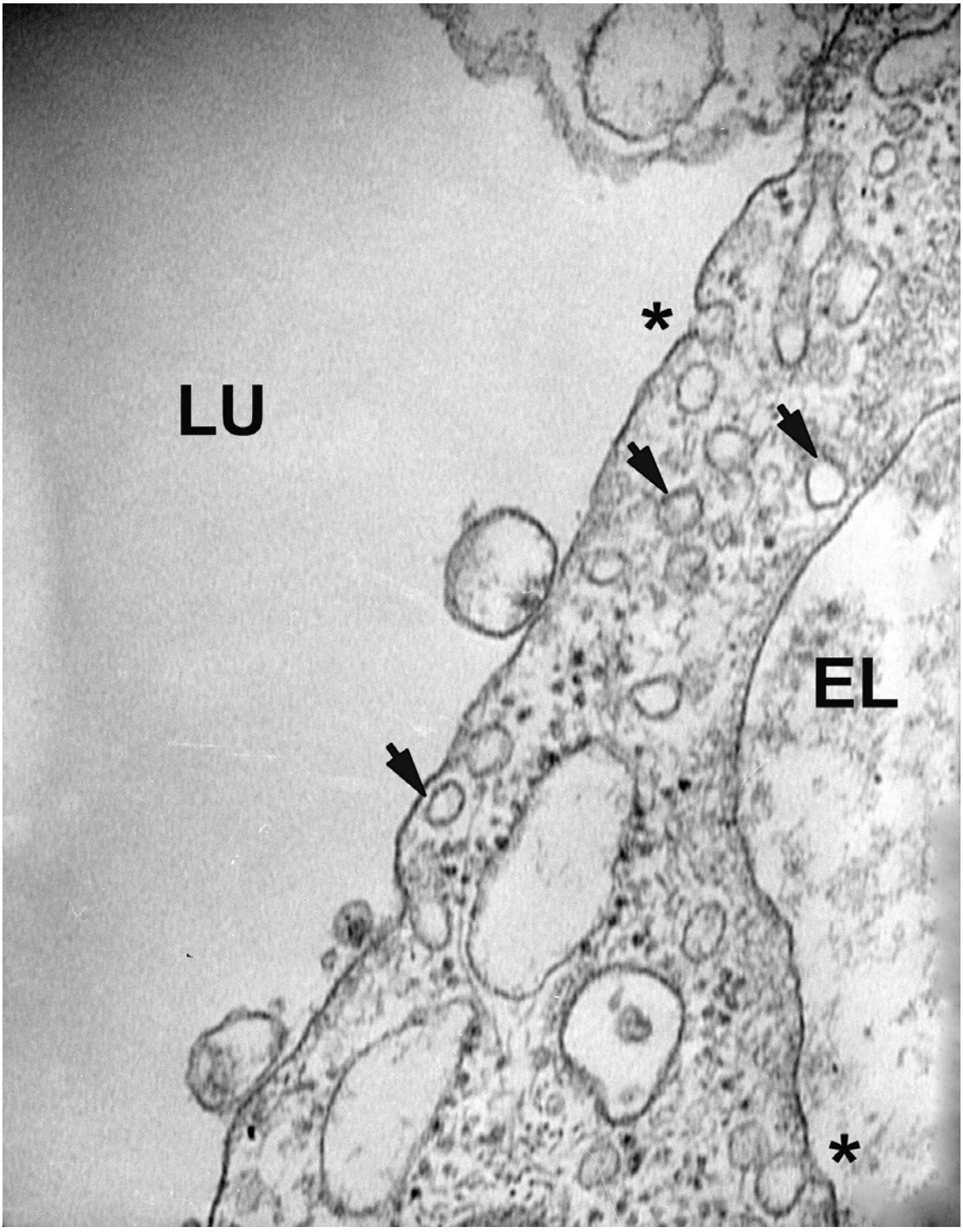

Fig. 3.5 Electron micrograph of a femoral artery endothelial cell displaying cytoplasmic vesicles (arrows). Magnification x 58,000. Note the fused vesicles (stars). LU=lumen, EL=elastic lamina

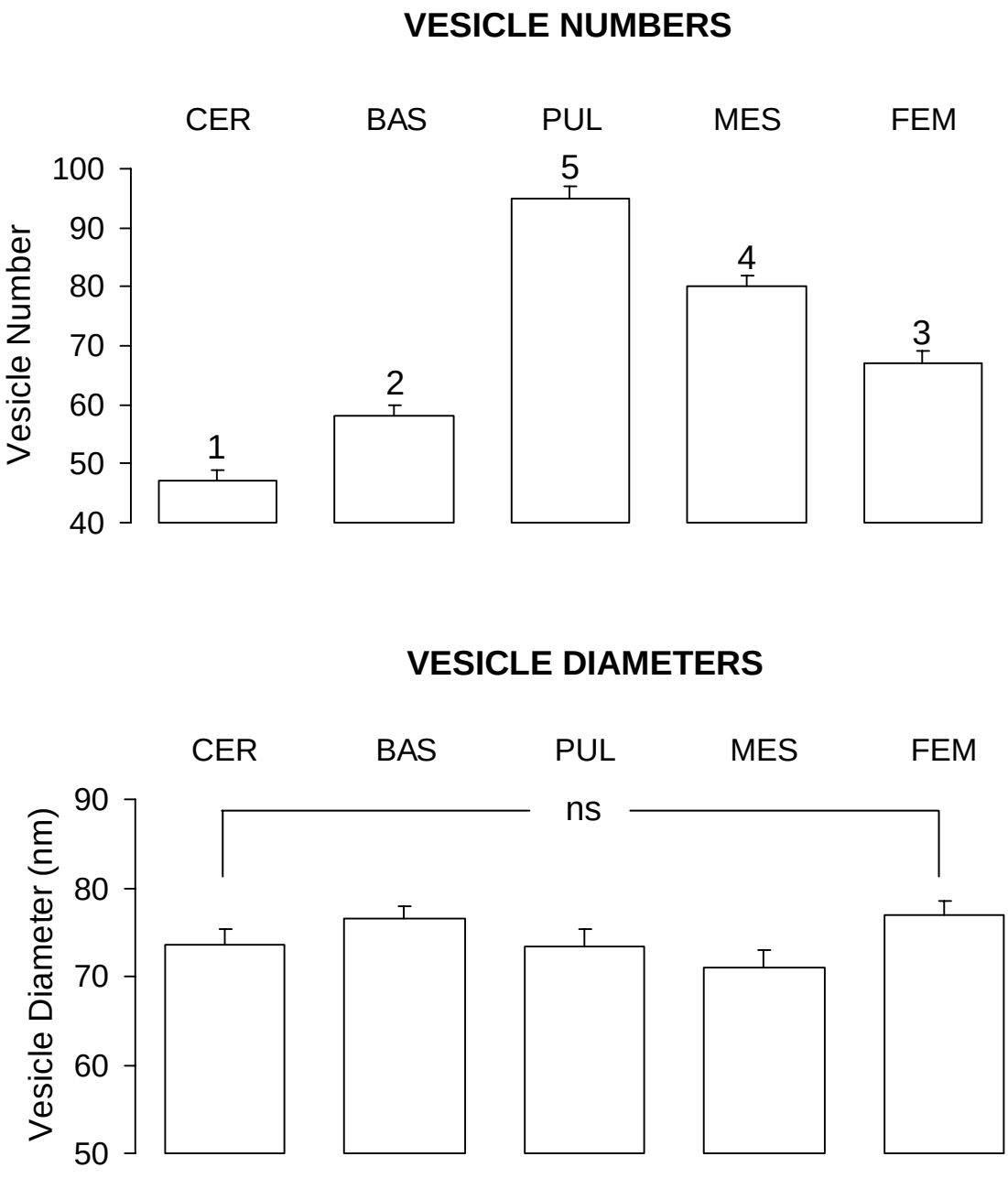

Fig. 3.6. Average number and diameter of vesicles in endothelial cells from small cerebral (CER), basilar (BAS), pulmonary (PUL), mesenteric (MES) and femoral (FEM) arteries of rats. The data are mean ± SEM. The descending order in terms of number of vesicles was pulmonary (5) > mesenteric (4) > femoral (3) > basilar (2) > cerebral (1) endothelial cells ($P < 0.05$ for all comparisons). ns = non-significant.

CHAPTER 4

LIGHT MICROSCOPIC AND ULTRASTRUCTURAL INVESTIGATION OF PROTEINS NECESSARY FOR VESICULAR EXOCYTOSIS IN ENDOTHELIAL CELLS FROM DIFFERENT VASCULAR BEDS[1]

[1]**Hashmi-Hill MP**, Shields JP, Lewis THJ, Robertson TP, Lewis SJ. Light and ultrastructural investigation of proteins necessary for vesicular exocytosis in endothelial cells in rat mesenteric, femoral and pulmonary arteries. To be submitted for publication in the *American Journal of Physiology*, 2006

ABSTRACT

S-nitrosothiols are naturally occurring nitric oxide (NO)-containing factors. The finding that the vasodilator actions of the S-nitrosothiol, L-S-nitrosocysteine, more closely resembled those of the endothelium-derived relaxing factor (EDRF) released by acetylcholine than NO in isolated arteries raises the possibility that this S-nitrosothiol is synthesized and released by vascular endothelial cells. S-nitrosothiols are stored in cytoplasmic vesicles within endothelial cells. The question arises as to whether these vesicles are subject to exocytosis. The ***objective*** of the studies in this chapter was to perform immunohistochemical studies at the light microscopic and ultrastructural levels to determine whether the proteins that support active vesicular exocytosis are present in the endothelium of rat small pulmonary, mesenteric and femoral arteries. These arteries were selected because they are involved in the minute-to-minute regulation of blood flow and hence arterial blood pressure. The results provide compelling evidence that the endothelium of these arteries contain numerous proteins that are known to support vesicular exocytosis.

Light microscopic investigation demonstrated the presence of vesicle-associated membrane protein (VAMP), synaptosomal-associated protein of 25KDa (SNAP-25), syntaxin, neurexin, synaptotagmin, synaptophysin, *N*-ethylmaleimide-sensitive factor (NSF), rab3A, rabphilin, calcium-calmodulin-dependent protein kinase II (CAMKII), endothelial NOS, (eNOS), neuronal NOS (nNOS), within the endothelium of small pulmonary, mesenteric and femoral arteries. Ultrastructurally, VAMP, CAMKII and eNOS were observed in

association with cytosolic vesicles. SNAP-25 and syntaxin were associated with the endothelial cell membrane.

These results provide compelling evidence that the endothelium of these arteries contain proteins that support vesicular exocytosis.

Keywords: Endothelial cell, Exocytosis, SNAREs, S-nitrosothiols, Vesicles

INTRODUCTION

Furchgott and Zawadzski (1980) provided evidence that acetylcholine relaxed isolated large conduit arteries via the release of a non-prostanoid endothelium-derived relaxing factor (EDRF). Subsequent studies have provided substantial evidence that the EDRF in these conduit arteries is the free radical, nitric oxide (NO) (Ignarro et al., 1987; Palmer et al., 1987). NO synthase (NOS) inhibitors such as N^G-nitro-L-arginine markedly attenuate the vasorelaxant responses elicited by endothelium-dependent agonists in isolated large conduit arteries (Moncada et al., 1991; Moncada, 2006). However, NOS inhibition only minimally attenuates the vasorelaxant responses to endothelium-dependent agonists in smaller arteries (see Danser et al., 1998; Batenburg et al., 2004a,b) or resistance arteries *in vivo* (see Davisson et al., 1996; Woodman et al., 2000). This suggests that endothelium-dependent agonists may release an EDRF and/or an endothelium-derived hyperpolarizing factor (EDHF) other than NO.

S-nitrosothiols are endogenous NO-containing compounds (see Stamler et al., 1992, 1997; Arnelle and Stamler, 1995). The finding that the vasodilator actions of the S-nitrosothiol, L-S-nitrosocysteine, more closely resembled those of the EDRF released by acetylcholine than NO in isolated arteries (Myers et al., 1990), raises the possibility that this S-nitrosothiol is synthesized and released by vascular endothelial cells. Indeed, there is now substantial evidence that L-S-nitrosocysteine is an EDRF and an EDHF (see Rosenblum, 1992; Batenburg et al., 2004a,b). Although S-nitrosothiols can decompose to NO, the vasorelaxant

properties of these compounds cannot be explained by their decomposition to the free radical (see Kowaluk and Fung, 1990). S-nitrosothiols possess extensive biological activity via their capacity to S-nitrosylate (i.e., transfer NO^+ to) cysteine residues in functional proteins such as receptors and ion-channels (Stamler et al., 1992, 1997; Stamler, 1994; Zai et al., 1999; Lang et al., 2003). In addition, S-nitrosothiols exert their effects via stereoselective recognition sites on vascular smooth muscle (Davisson et al., 1996b; Batenburg 2004a) and the central nervous system (Davisson et al., 1997; Lewis et al., 1996; Lipton et al., 2001).

L-S-nitrosocysteine is highly polar and lipophobic (see Kowaluk and Fung, 1990). The question therefore arises as to how L-S-nitrosocysteine is able to be secreted by endothelial cells. Ignarro (1990) postulated that S-nitrosothiols may exist in preformed pools in specialized storage vesicles in endothelial cells and that endothelium-dependent agonists may initiate Ca^{2+}-*dependent* exocytotic release of these vesicular stores. Subsequent ultra-structural studies have demonstrated that endothelial (eNOS) and neuronal (nNOS) forms of NOS are present on the membranes of large numbers of cytoplasmic vesicles in vascular endothelial cells and autonomic nerve terminals (Loesch and Burnstock, 1993, 1994). The presence of NOS would allow for the synthesis of S-nitrosothiols prior to their storage in vesicles. We have recently obtained direct evidence that cytoplasmic vesicles in rat, rabbit and canine vascular endothelial cells contain high concentrations of L-S-nitrosocysteine and lower but still substantial concentrations of L-S-nitrosocysteamine (Lewis et al., 2006).

Loesch and Burnstock (1993, 1994) also demonstrated that contrary to NOS, NADPH diaphorase was localized in the ***lumen*** but not in the membranes of cytoplasmic vesicles in endothelial cells and autonomic nerve terminals. NADPH-diaphorase is used as a histochemical marker for NOS (Dawson et al., 1991; Hope et al., 1991). However, Chayen et al (1994) raised several compelling arguments that NADPH diaphorase is due to the presence/biological activity of another proteinaceous material. With this in mind, we performed studies that demonstrated that NADPH diaphorase is a sensitive marker for S-nitrosothiols in tissues including cytoplasmic vesicles (Lewis et al., 2006).

The question arises as to whether S-nitrosothiol-containing cytoplasmic vesicles in endothelial cells undergo Ca^{2+}-*dependent* or Ca^{2+}-*independent* exocytosis (see Minshall et al., 2002). Schnitzer et al (1995) demonstrated that caveolae in the plasma membranes of endothelial cells contain molecular machinery, including vesicle-associated membrane protein (VAMP), N-ethylmaleimide sensitive factor (NSF) and synaptosomal-associated protein of 25KDa (SNAP), that supports vesicle budding, docking and fusion processes. Bodin and Burnstock (2001) demonstrated that adenosine-containing vesicles in endothelial cells undergo Ca^{2+}-*dependent* exocytosis. In addition, endothelial cells contain Weibel-Palade bodies that are specialized secretory granules (Weibel and Palade, 1964). Upon tissue damage or injury, endothelial cells are activated by vasopressin, hypoxia, histamine or thrombin (Sporn et al., 1986; McEver et al., 1989; Kaufmann et al., 2000) causing Weibel-Palade bodies to

fuse to plasma membranes (Silverstein, 1999) to release von Willebrand factor. Exocytosis of von Willebrand factor is mediated by soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) proteins and NSF (Jahn and Sudhof, 1999; Mellman and Warren, 2000). At present, the fusion proteins in cytoplasmic vesicles of endothelial cells that are subject to Ca^{2+}-*dependent* or Ca^{2+}-*independent* exocytosis have not been identified.

The ***concepts*** addressed in this dissertation are that (1) S-nitrosothiols are formed and stored in endothelial vesicles, (2) sub-sets of these vesicles, and specifically those that contain Ca^{2+}-calmodulin-sensing proteins such as Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII), undergo Ca^{2+}-*dependent* exocytosis as elicited by sheer stress and endothelium-dependent agonists, and (3) other sub-sets of vesicles, and specifically those devoid of Ca^{2+}-calmodulin-sensing proteins, undergo Ca^{2+}-*independent* exocytosis in response to hypoxia.

The specific **objective** of the studies in this chapter was to perform immuno-histochemical studies at the light microscopic and ultrastructural levels to determine whether the proteins that support vesicular exocytosis are present in the endothelium of small pulmonary, mesenteric and femoral arteries. These arteries were selected because they are involved in the minute to minute regulation of blood flow and hence arterial blood pressure. The results provide compelling evidence that the endothelium of these arteries contain numerous proteins that are known to support vesicular exocytosis.

MATERIALS AND METHODS

Preparation of arterial sections for immunohistochemistry

All studies were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) revised in 1996. The protocols pertaining to the use of animals were approved by the Animal Care and Use Committee of the University of Georgia.

Small (≈ 200 μm diameter) pulmonary, mesenteric and femoral arteries were dissected from male Sprague Dawley rats (14-16 weeks of age) and fixed overnight in 4% paraformaldehyde at 4° C. The fixed arteries were washed three times in 0.1% phosphate buffered saline (pH 7.5) for 5 min at a time. The arteries were then embedded in Histogel (Fisher Scientific) and frozen at - 20° C for 10 min. The frozen blocks containing the fixed arteries were sectioned into 5 μm thick slices on a cryostat (LEICA CM 1900, Vashaw Scientific Inc.). The sections were then placed on Superfrost glass slides (Fisher Scientific) and allowed to dry at room temperature for 30 min before immunohistochemistry was performed.

Immunohistochemistry

Tissue sections were treated with 1% hydrogen peroxide to block endogenous peroxidase activity, and treated with protein blocking solution (1.5% goat serum diluted in phosphate-buffered saline). Tissue sections were immunostained with primary antibody diluted in phosphate-buffered saline and 1.5% bovine serum albumin and incubated for 30 minutes at room temperature.

The tissue sections were then developed with an avidin-biotin kit (Santa Cruz, Santa Cruz, CA), using 3,3'-diaminobenzidine as the chromogen. Slides were cover-slipped with Permount. For control slides, the primary antibody was omitted. CD4 was used as a negative control.

Immunogold Labeling

Adult male Sprague-Dawley rats were killed by decapitation and the pulmonary, mesenteric and femoral arteries were dissected and immersion-fixed for 3 h at 4°C with fixative containing 4% paraformaldehyde and 0.25% glutaraldehyde (in 0.1M phosphate buffer, pH7.4). Arteries were then transferred to phosphate buffer and stored overnight at 4°C. The arteries were then prepared for transmission electron microscopy.

Transmission electron microscopic analyses

The collected arteries were first dehydrated in a graded ethanol series, followed by a graded acetone series. The arteries were infiltrated, embedded, and polymerized for 24h at 50°C in LR White solution. Silver sections were cut with a diamond knife on an ultramicrotome (RMC MT-X, Tucson, AZ, USA) and collected on nickel grids. The sections were incubated with the antibodies overnight at 4°C at various dilutions and then labeled with a goat anti-mouse immunoglobulin G serum-coated colloidal gold probe, coupled to 15-nm gold particle (AuroProbe EM, Amersham International, Amersham, UK) at a dilution of 1:40. The sections were post-stained with a mixture of uranyl acetate and lead

citrate and then viewed on a transmission electron microscope in the *Center for Advanced Ultrastructural Research* of the University of Georgia (model 100CX II, JEOL USA, Peabody, MA) operating at 80 KeV.

Measurements

In order to calculate the percentage coverage of immunoreactivity at the light level, the arteries were divided into quadrants and the staining in each quadrant was designated as high, medium, low or absent. These analyses were done in at least three adjacent sections of each artery from at least 5 rats. In order to establish the percentage of endothelial cells positive for immunogold labeling, the cells were counted in ultra-thin sections taken from different levels of specimens. The cells were counted under the electron microscope exclusively. At least 10 cells from each artery from at least 5 rats were examined.

Antibodies

VAMP-1, dilution 1:200 (Santa Cruz, Santa Cruz, CA, USA and Calbiochem, San Diego, CA USA), CaMKII, dilution 10µg/ml (BIOMOL, PA USA), synaptotagmin, dilution 1:1000, synaptophysin, dilution 1:1000, syntaxin-4, dilution 1:1000, NSF, dilution 1:1000, neurexin I, dilution 1:200, synapsin-I, dilution 1:1000, Rab3a, dilution 1:1000, SNAP-25, dilution 1:200, Rabphilin-3A, dilution 1:1000, neurexin I, dilution 1:200 (all BD Transduction Laboratories, San Diego, CA, USA), eNOS, dilution 2µg/ml (BIOMOL, PA, USA), nNOS, dilution 1:3000 (Sigma, USA)

RESULTS

A. Immunohistochemistry

Pulmonary artery

Heavy eNOS immunoreactivity was observed in many areas of the endothelium, whereas staining was patchy in other parts (Fig. 4.1). Approximately 60% of the endothelium displayed eNOS immunoreactivity. The smooth muscle was free of staining. Approximately 40% of the endothelium labeled heavily for nNOS (Fig. 4.2) while the rest of the endothelium and smooth muscle was devoid of staining. Approximately 80% of the endothelium displayed CaMKII immunoreactivity (Fig. 4.3). Staining was heavy in parts, while light and patchy in others. The smooth muscle was devoid of staining.

VAMP-1 immunoreactivity was observed in 50-60% of the endothelium (Fig. 4.4) although staining was not uniform (heavy to light). Smooth muscle was devoid of staining. Approximately 70-80% of the endothelium displayed SNAP-25 immunoreactivity, although the staining was relatively light (Fig. 4.5). The smooth muscle was devoid of staining. Positive immunoreactivity for syntaxin-4 was observed in 60-70% of endothelium (Fig. 4.6). The distribution was light but uniform. The smooth muscle was devoid of positive staining.

Neurexin-1 immunoreactivity was observed in 80-90% of the endothelium (Fig. 4.7). Although areas of heavy staining were observed, most neurexin-1 staining was light and uniform. The smooth muscle was free of neurexin-1.

Synaptotagmin immunoreactivity in the endothelium was patchy and relatively light (Fig. 4.8). Approximately 40% of the endothelium was devoid of staining. Smooth muscle was free of staining. Synaptophysin immunoreactivity was seen in 75-80% of the endothelium (Fig. 4.9). The distribution was light and patchy. Smooth muscle was devoid of staining.

Light and patchy NSF immunoreactivity was observed in 25-30% of the endothelium (Fig. 4.10). Smooth muscle was free of staining. Rab3a staining was uniformly light in 75-85% of the endothelium (Fig. 4.11). Smooth muscle was free of staining. Approximately 85% of endothelium displayed rabphilin-3A immunoreactivity (Fig. 4.12). Rabphilin-3A staining was heavy in parts and light in others. Smooth muscle was not stained.

No positive staining was observed for CD4 in any artery (Fig. 4.13). Moreover, no staining was observed in arteries in which the above primary antibodies were omitted (Fig. 4.14).

Mesenteric artery

Moderately heavy eNOS immunoreactivity was observed in some parts of the endothelium, but staining was patchy in other parts (Fig. 4.1). Approximately 95% of the endothelium was positively labeled for eNOS. The smooth muscle was free of staining. Approximately 95% of the endothelium displayed relatively heavy nNOS immunoreactivity (Fig. 4.2). The remainder of the endothelium and

all smooth muscle was free of staining. Approximately 95% of the endothelium displayed heavy and uniform CaMKII immunoreactivity (Fig. 4.3). The smooth muscle was devoid of staining.

VAMP-1 immunoreactivity was observed in 90-95% of the endothelium (Fig. 4.4). The staining was heavy and uniform. The smooth muscle was devoid of immunoreactivity. SNAP-25 immunoreactivity was observed in 60-70% of the endothelium (Fig. 4.5). This staining was relatively light. There was no staining in the smooth muscle. Syntaxin-4 immunoreactivity for was observed in 90-95% of the endothelium (Fig. 4.6). The distribution was moderately heavy and uniform. The smooth muscle was devoid of staining.

Neurexin-1 immunoreactivity was observed in 90-95% of the endothelium (Fig. 4.7). Most neurexin-1 staining was heavy and uniform. Unexpectedly, 50-60% of smooth muscle displayed neurexin-1 immunoreactivity. Synaptotagmin immunoreactivity for was observed in 90-95% of the endothelium (Fig. 4.8). Synaptotagmin staining was heavy and uniform. Synaptophysin immunoreactivity was observed in 90-95% of the endothelium (Fig. 4.9). The distribution of synaptophysin was light and uniform. Again, quite unexpectedly, approximately 20% of the smooth muscle was positively stained for synaptophysin.

NSF immunoreactivity was observed in 90-95% of the endothelium (Fig. 4.10). The staining was light and uniform in distribution. The smooth muscle was

free of staining. Rab3a immunoreactivity for was observed in approximately 95% of the endothelium (Fig. 4.11). Rab3a immunoreactivity was moderately heavy and relatively uniform throughout the endothelium. The smooth muscle was free of staining. Rabphilin-3A immunoreactivity was observed in 90-95% of the endothelium (Fig. 4.12). Staining was uniformly heavy and even. The smooth muscle was free of staining.

No positive staining was observed for CD4 in any artery (Fig. 4.13, Fig. 4.14). Moreover, no positive staining was observed in arteries in which the above primary antibodies were omitted.

Femoral artery

Relatively moderate eNOS immunoreactivity was observed in 70-75% of the endothelium (Fig. 4.1). The smooth muscle was free of positive staining. Light and patchy nNOS immunoreactivity for was observed in 60-70% of the endothelium (Fig. 4.2). The smooth muscle was free of positive staining. CaMKII immunoreactivity was observed in 90-95% of the endothelium (Fig. 4.3). The staining was heavy and uniform. The smooth muscle was devoid of staining.

VAMP-1 immunoreactivity was observed in 30-40% of the endothelium (Fig. 4.4). This staining was not uniform and many endothelial invaginations were devoid of any staining. The smooth muscle was devoid of immunoreactivity. SNAP-25 immunoreactivity was observed in 70-80% of the endothelium (Fig.

4.5). The staining was heavy and uniform. There was no SNAP-25 staining in the smooth muscle. Syntaxin-4 immunoreactivity was observed in 60-70% of the endothelium (Fig. 4.6). The distribution of syntaxin-4 was light but uniform. In addition, approximately 15% of smooth muscle stained positively for syntaxin-4.

Neurexin-1 immunoreactivity was observed in 80-90% of the endothelium (Fig. 4.7). Most of the positive staining was heavy and uniform. The smooth muscle was free of immunoreactivity. Moderately heavy synaptotagmin immunoreactivity was observed in 80-90% of the endothelium (Fig. 4.8). The smooth muscle was free of staining. Synaptophysin immunoreactivity was observed in 90-95% of the endothelium (Fig. 4.9). Staining was moderately heavy and uniform. The smooth muscle was devoid of staining.

NSF immunoreactivity was observed in 95-100% of the endothelium (Fig. 4.10). Staining was heavy but somewhat uneven. The smooth muscle was free of staining. Rab3a immunoreactivity was observed in 85-90% of the endothelium (Fig. 4.11). Staining was relatively light but uniform. The smooth muscle was free of staining. Heavy Rabphilin-3A immunoreactivity was observed in 90-95% of the endothelium (Fig. 4.12). The smooth muscle was free of staining.

No positive staining was observed for CD4 in any artery (Fig. 4.13, Fig. 4.14). Moreover, no positive staining was observed in arteries in which the above primary antibodies were omitted.

B. Immunogold labeling

Pulmonary Artery

Numerous eNOS-positive gold particles were observed in endothelial cells (Fig. 4.15A). The gold particles appeared as single particles and often in clusters of three or more particles. The labeling was mostly confined to the cytoplasm and was in close proximity to vesicles or ribosome rich areas. However, labeling was also observed at the cell membrane. Approximately 50% of examined cells displayed eNOS gold labeling. CaMKII-positive gold labeling was observed in endothelial cells (Fig. 4.16A). The distribution of CaMKII was not uniform and appeared in patches. The gold particles were distributed within close proximity to cytosolic vesicles and also appeared in the cytoplasm, often as single gold particles. Approximately 16% of examined cells displayed CaMKII gold labeling.

VAMP-1-positive gold labeling was observed in endothelial cells (Fig. 4.17A). The distribution was patchy and in close proximity to vesicles and ribosome rich areas. VAMP-1-positive gold labeling occurred as single particles. Approximately 10% of examined cells displayed positive gold labeling to VAMP-1. SNAP-25-positive gold labeling was observed in endothelial cells (Fig. 4.18A). The distribution of SNAP-25-positive gold particles was patchy. The gold particles were mainly localized to the cell membrane, although some labeling was seen in the cell nucleus. Approximately 20% of examined cells displayed positive gold labeling to SNAP-25. Syntaxin-4-positive gold labeling was observed in endothelial cells (Fig. 4.19A). Syntaxin-4-positive gold particles were

located close to the plasma membrane and between the membrane and elastic lamina. Vesicles were free of gold particles. Approximately 12% of examined cells displayed syntaxin-4 gold labeling.

Mesenteric Artery

eNOS-positive particles were found throughout endothelial cells (Fig. 4.15B). The gold particles appeared as single particles and clusters and were localized close to vesicles and ribosomes. Gold particles were occasionally observed at the cell membrane. Approximately 44% of examined cells displayed gold eNOS labeling. CaMKII-positive particles were found throughout endothelial cells (Fig. 4.16B) and were located close to or on the vesicle membrane. Approximately 12% of examined cells displayed CaMKII gold labeling.

VAMP-1-positive particles were found throughout endothelial cells although this labeling was not profuse (Fig. 4.17B). VAMP-1 was found in the cytoplasm but not on cell membranes (Fig. 4.17B). Approximately 12% of examined cells displayed positive labeling. SNAP-25-positive particles were found throughout endothelial cells in close proximity to or on the cell membrane (Fig. 4.18B). Vesicles were devoid of labeling. Approximately 8% of cells displayed positive labeling to SNAP-25. Syntaxin-4-positive particles were found in endothelial cells (Fig. 4.19B). Syntaxin-4 was observed mainly in close proximity to or on the cell membrane. Occasional labeling was observed in the cytoplasm. Approximately 16% of examined cells displayed syntaxin-4 labeling.

Femoral Artery

eNOS-positive gold labeling was observed throughout endothelial cells (Fig. 4.15C). Labeling often appeared in clusters of 4 or more particles and were frequently observed in ribosome rich areas. Approximately 36% of examined cells displayed positive gold labeling to eNOS. CaMKII-positive labeling was observed in endothelial cells (Fig. 4.16C). Gold particles were mainly localized in close proximity to vesicles and appeared as single gold particles. Approximately 10% of examined cells displayed CaMKII gold labeling.

VAMP-1-positive gold labeling was distributed throughout endothelial cells (Fig. 4.17C). The labeling was uniform but not heavy. Labeling was localized to the cytoplasm and often on membranes of vesicles. Approximately 6% of examined cells displayed positive gold labeling to VAMP-1. Relatively sparse SNAP-25-positive gold labeling was found in endothelial cells (Fig. 4.18C). Distribution was patchy, often appearing as couplets, and was localized within close proximity of the cell membrane. Vesicles were devoid of gold labeling for SNAP-25. Approximately 6% of examined cells displayed positive gold labeling to SNAP-25. Syntaxin-4-positive gold labeling in endothelial cells was observed mainly at the cell membrane (Fig. 4.19C). Occasional labeling of cytoplasm and vesicles was seen. The gold particles appeared as singles particles and at times as couplets. Approximately 8% of examined cells displayed positive gold labeling to syntaxin-4.

DISCUSSION

This study provides light-level evidence that endothelial cells of rat small pulmonary, mesenteric and femoral arteries stain for eNOS, nNOS CaMKII, VAMP-1, SNAP-25, syntaxin-4, neurexin-1, synaptotagmin, synaptophysin, NSF, Rab3a and Rabphilin-3A (see Table 4.1). This study also provides ultra-structural evidence as to the subcellular distribution of eNOS, CaMKII, VAMP-1, SNAP-25 and syntaxin-4 in these endothelia.

eNOS

eNOS plays a fundamental role in endothelium-dependent relaxation via its production of $NO^{\bullet}$ (Moncada et al., 1991, 1996; Moncada and Higgs, 2006), and by subsequent formation of S-nitrosothiols by iron/copper-catalyzed interaction of $NO^{\bullet}$ with cysteine-containing residues (Stamler et al., 1992; Zhang and Hogg, 2005). At the light level, eNOS immunoreactivity was present in approximately 60%, 95% and 75% of the endothelium of pulmonary, mesenteric and femoral arteries, respectively. At the ultra-structural level, eNOS immunoreactivity was observed in approximately 50%, 44% and 36% of pulmonary, mesenteric and femoral endothelial cells, respectively. In our ultra-structural studies, we used the post-embedding colloidal gold method. Using pre- and post-embedding methods would likely have yielded similar values to those at the light level. Nonetheless, it is evident that a substantial percentage of vascular endothelial cells contain eNOS and this enzyme is likely to participate in endothelial regulation of vascular tone. We did not perform co-labeling studies

with eNOS and nNOS to examine whether these isoforms are strictly localized to the same cell (see below). The possibility certainly exists that eNOS and nNOS are expressed in different cells such that the total numbers of NOS positive endothelial cells are greater than those expressing eNOS or nNOS only. The functional significance of such segregation of NOS isoforms is open to question.

nNOS

The products of nNOS including $NO^{\bullet}$ and S-nitrosothiols play fundamental roles as neurotransmitters/neuromodulators in central, afferent and autonomic neurons (Ruggiero et al., 1996; Possas and Lewis, 2000; Possas et al., 2006). The presence of nNOS in endothelial cells suggests that nNOS may participate in endothelium-dependent relaxation. At the light level, nNOS immunoreactivity was found in ≈40%, 95% and 60% of the endothelium of pulmonary, mesenteric and femoral arteries, respectively. We have not examined the distribution of nNOS or co-labeling of nNOS and eNOS ultrastructurally. The relatively high percentage of mesenteric endothelial cells expressing nNOS or eNOS suggests that the two isoforms exist in the same cell. The relatively lower expressions of nNOS or eNOS in pulmonary and femoral endothelial cells suggests that some endothelial cells may express one or other of the NOS isoforms.

In contrast to our findings in smaller arteries, Loesch and Burnstock (1993) found that only 6% of endothelial cells of the rabbit aorta displayed NOS-immunoreactivity at the ultra-structural level. The discrepancy in these findings

may relate to the type of artery under investigation. More specifically, the rabbit thoracic aorta is a true conduit artery and may therefore not be as dependent on endothelium-control (NOS-dependent)-mechanisms as smaller (pre-resistance) sized arteries that play a role in regulating systemic arterial blood pressure.

In addition to endothelial cells, eNOS and nNOS exist in numerous other cell types (Förstermann et al., 1991; Pollock et al., 1991; Kobzik et al., 1993, 1994; Loesch, 1993, 1994; Ruggiero et al., 1996). Our studies support the contention that both eNOS and nNOS often serve important functional roles in the same cell type (see Loesch, 1993, 1994; Ruggierro et al., 1996).

SNARE proteins: VAMP-1, syntaxin-4 and SNAP-25

SNARE proteins such as VAMP (also known as synaptobrevin), syntaxins and SNAP-25 are essential for membrane fusion of cytoplasmic vesicles (McMahon and Südhof, 1995). VAMP-1 is a synaptic vesicle protein that binds only weakly to syntaxins or SNAP25 separately but very tightly when SNAP-25 and syntaxins are complexed together, resulting in the formation of a stable SDS-resistant complex (Hayashi et al., 1994). We examined VAMP-1 because synaptic and exocytotic vesicles contain members of the VAMP family (vSNARE) for specific docking and/or fusion (Cain et al., 1992; Trimble et al., 1988; Braun et al., 1994; Baumert et al., 1989). VAMP was first identified as an 18-kDa protein expressed in brain tissue and enriched in purified synaptic vesicles (Trimble et al., 1988; Baumert et al., 1989). Vesicle fusion/neurotransmitter release are

inhibited when VAMP is removed from synaptic vesicles by zinc protease neurotoxins (Schiavo et al., 1992). VAMPs interact with distinct isoforms of the tSNARE syntaxins, a family of proteins residing on membranes with which vesicles dock and fuse (Bennett et al., 1993). α,β SNAP and NSF (see below) bind together and modulate vesicle formation and/or vesicle fusion through direct interactions with SNARE proteins (Clary et al., 1990; Whiteheart et al., 1993).

The endothelium of rat small pulmonary, mesenteric and femoral arteries stained for VAMP-1 at the light-level. VAMP-1 was observed in approximately 60%, 95% and 40% of pulmonary, mesenteric and femoral endothelium, respectively. Staining was heavy in pulmonary and mesenteric endothelium but moderate in femoral endothelium. Approximately 70% endothelial cells of small pulmonary, mesenteric and femoral arteries stained for SNAP-25 at the light-level. Staining was dense in femoral artery endothelium but moderate in pulmonary and mesenteric endothelium. Over 70% of endothelial cells of small pulmonary, mesenteric and femoral arteries stained for syntaxin-4. Staining was moderately heavy in the mesenteric endothelium but relatively light in the pulmonary and femoral endothelium. It is evident that VAMP-1, SNAP-25 and syntaxin-4 may play a significant roles if somewhat differentially important roles in vesicular exocytosis in the endothelium of all three vascular beds.

Our ultra-structural studies detected VAMP-1, SNAP-25 and syntaxin-4 in endothelial cells. VAMP-1 was associated with cytosolic vesicles whereas SNAP-

25 and syntaxin-4 were associated with the endothelial cell plasma membrane. Schnitzer et al (1995) demonstrated that plasma membrane caveolae of endothelial cells contain virtually all pertinent proteins necessary for exocytosis including VAMP. Taken together, the present findings and those findings of Schnitzer et al (1995) suggest that SNAP-25 and syntaxin-4 are associated with caveolae whereas VAMP-1 is associated with caveolae and cytoplasmic vesicles in endothelial cells of small pulmonary, mesenteric and femoral arteries.

NSF

The ATPase, NSF was first discovered in yeast as an essential factor in endoplasmic reticulum to Golgi vesicle transport (Novick et al. 1980, 1981). NSF binds to the synaptic SNARE complex via a cytosolic factor called α-SNAP (soluble NSF attachment protein) (Clary et al. 1990), which forms a 20S complex. Upon hydrolysis of ATP by NSF, the 20S complex dissociates into its component subunits (Sollner et al. 1993). Synaptic vesicle membrane fusion results in *cis* SNARE complexes in the plasma membrane. These complexes must be disassembled for VAMP to be selectively recycled into the vesicle membrane, whereas syntaxin and SNAP-25 remain in the plasma membrane. It has been suggested that the main action of NSF most likely occurs just after exocytosis of synaptic vesicles, prior to recycling (Sollner et al. 1993).

Endothelial cells of rat small pulmonary, mesenteric and femoral arteries displayed NSF immunoreactivity at the light-level. NSF immunoreactivity was

observed in approximately 30% of pulmonary endothelium and 95% of mesenteric and femoral endothelium. Staining was relatively light in pulmonary and mesenteric endothelium but was heavy in femoral endothelium. This would suggest that NSF plays a relatively more important role in the femoral endothelium although it would appear that the expression levels of NSF are abundant enough to be involved in exocytotic processes in all endothelia. Plasma membrane caveolae are rich in NSF (Schnitzer et al., 1995). It still remains to be determined as to whether the positive NSF immunoreactivity observed in our studies represents NSF in caveolae or other membrane or cytosolic structures.

Neurexin-1

The neurexins are a family of highly polymorphic proteins, the first of which was identified as the Ca^{2+}-dependent α-latrotoxin receptor (Ushkaryov et al., 1992, Ushkaryov and Südhof, 1993). These proteins exist on the plasma membranes of synaptic structures. The α-latrotoxin from black widow spider venom induces massive exocytosis of synaptic vesicles from presynaptic neurons (Gorio et al., 1978). There is *in vitro* evidence that the cytoplasmic domain of neurexin interacts with the synaptic vesicle protein synaptotagmin (Petrenko et al., 1991; Hata et al., 1993; Perin, 1994), which will be discussed below.

Our finding that approximately 80-90% of rat small pulmonary, mesenteric and femoral endothelium displayed dense staining for neurexin-1 suggests that this protein plays an important role in regulating vesicular exocytosis in these

endothelia. Future ultra-structural studies will be needed to confirm the subcellular distribution and likely membrane-disposition of this protein.

Synaptotagmin I

The dependence of exocytosis on Ca^{2+} has been long recognized, however, the identity of the Ca^{2+} sensor or sensors has remained elusive. We chose to look for the presence of synaptotagmin I, the first protein to be purified from synaptic vesicles (Matthew et al., 1981), because of evidence that this protein is a Ca^{2+} sensor (Brose et al., 1992). However, the precise action of synaptotagmin is unclear since there is evidence that it has inhibitory (Bommert *et al*., 1993; Martin *et al*., 1995) and facilitatory (Mikoshiba et al 1995) roles in transmission. There is substantial evidence that acidic phospholipids and Ca^{2+}-calmodulin bind to synaptotagmin (Perin et al., 1990).

Endothelial cells of rat small pulmonary, mesenteric and femoral arteries were positive for synaptotagmin. At the light-level, 40% of pulmonary endothelium displayed relatively light synaptotagmin staining whereas approximately 90% of mesenteric and pulmonary endothelium displayed heavy to moderate staining. Although this suggests that synaptotagmin has a greater role in the mesenteric and femoral endothelium, the expression levels of synaptotagmin would appear to be abundant enough to drive exocytosis in pulmonary endothelium. Future ultra-structural studies will be needed to confirm the subcellular distribution and likely vesicle-disposition of this protein.

CaMKII

As mentioned, although the dependence of exocytosis on Ca^{2+} has been long recognized, the identities of the precise Ca^{2+} sensors have not been fully established. We chose to look for the presence of CaMKII because this protein is a major component of synaptic vesicles and acts as a binding protein for Ca^{2+}, calmodulin and synapsin I (Benfenati et al., 1992). The complex formed between synapsin I and CaMKII is thought to regulate the efficiency of neurotransmitter release (Greengard et al., 1993).

We found that at least 80% of rat small pulmonary, mesenteric and femoral endothelium was positive for CaMKII at the light level. Moreover, our ultra-structural studies found that CaMKII was closely associated with cytoplasmic vesicles in the three endothelia. These data provide evidence that CaMKII on cytoplasmic vesicles plays an important role in Ca^{2+}-*dependent* exocytosis in endothelial cells of small arteries. It is probable that future ultra-structural studies will confirm the likely vesicular disposition of this protein.

Rab3a and Rabphilin-3a

Rab proteins are involved in targeting and regulating vesicular membrane traffic (Ferro-Novick and Novick,1993; Simons and Zerial, 1993; Fischer von Mollard et al., 1994). Rab3a is one of approximately 40 Rab proteins. In mammals, they all have specific subcellular localizations (Pfeffer 1994, Novick & Zerial 1997). Like other GTPases, Rab cycles between a cytosolic GDP-bound

inactive state and a vesicular GTP-bound active state with the aid of many accessory proteins. Rab3a in particular has been strongly implicated in synaptic vesicle dynamics. Rab3a and Rab3C are the most abundant Rab proteins in neurons and are predominantly localized to synaptic vesicles (Fischer von Mollard et al., 1994). We chose to look at Rab3a and Rabphilin-3A, its effector protein for downstream targets (Stahl et al., 1996).

We found that approximately 80-90% of pulmonary, mesenteric and femoral endothelium stained for Rab3a at the light-level (Rab5 was not detected). Staining was relatively light in pulmonary and femoral endothelium but heavy in mesenteric endothelium. Rabphillin-3A staining was observed in approximately 90% of pulmonary, mesenteric and femoral endothelium at the light-level. Staining was uniformly heavy in the three endothelia. These findings provide evidence that Rab3a and Rabphilin-3A may participate in exocytotic mechanisms in endothelial cells of small arteries. We have not yet looked at Rab3a or Rabphilin-3A at the ultra-structural level. Similar to other GTPases, Rab3a is likely to exist in plasma membrane caveolae (Schnitzer et al., 1995).

Synaptophysin

Synaptophysin is one of the most abundant integral membrane proteins in synaptic vesicles and comprises approximately 10% of total synaptic vesicle proteins (see McMahon, 1996). Synaptophysin was one of the first synaptic vesicles proteins to be identified (Navone et al., 1986) and sequenced (Sudhof et

al., 1987). Despite this, the role of synaptophysin in the synaptic vesicles life cycle is still relatively unknown. It has been suggested that synaptophysin participates in the formation of an exocytotic fusion pore, which leads to neurotransmitter release (Thomas et al., 1988; Leube et al., 1987). Neurons of synaptophysin knockout mice do not display obvious deficits in neurotransmitter release (Eshkind & Leube, 1995; McMahon, 1996). Nevertheless the positioning of synaptophysin on the synaptic vesicle implies a functional role in exocytosis. Some studies have shown that synaptophysin is localized to small synaptic-like vesicles and not to large dense-core vesicles in PC12 cells (Clift-O'Grady et al., 1990; Winkler, 1997). However, James and Richard (1993) found synaptophysin to be present on both types of vesicles in PC12 cells.

We found that approximately 80-90% of pulmonary, mesenteric and femoral endothelium stained for synaptophysin at the light-level. Staining was relatively light in pulmonary and mesenteric endothelium but heavy in femoral endothelium. Again, it is probable that future ultra-structural studies will confirm the likely vesicular disposition of this protein.

CONCLUSIONS

The basic machinery necessary for exocytosis (i.e., those that form the core complex) are present in the endothelium of the pulmonary, mesenteric and femoral artery. These studies suggest that vesicular exocytosis may take place in these endothelia. This possibility will be further explored in the next chapters.

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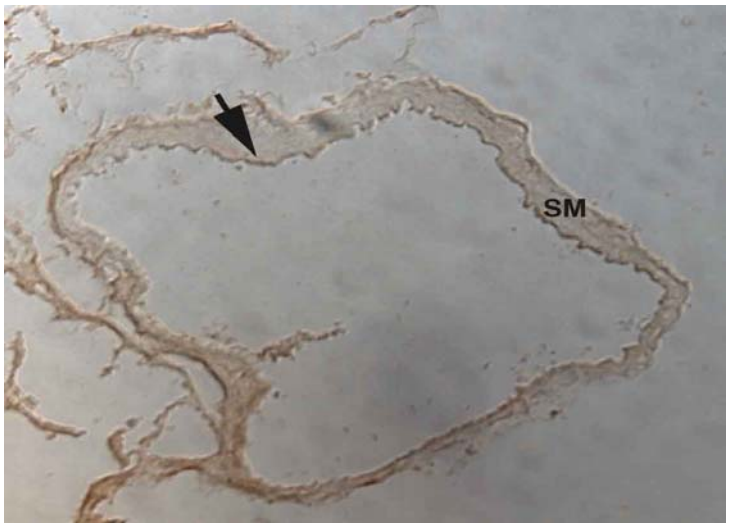
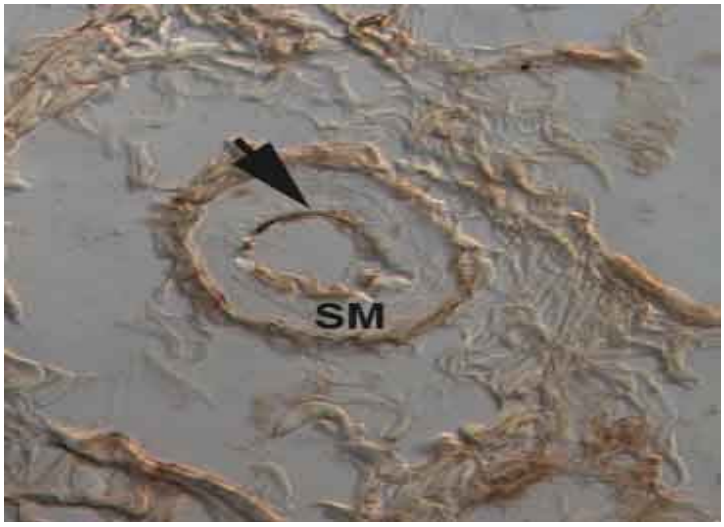
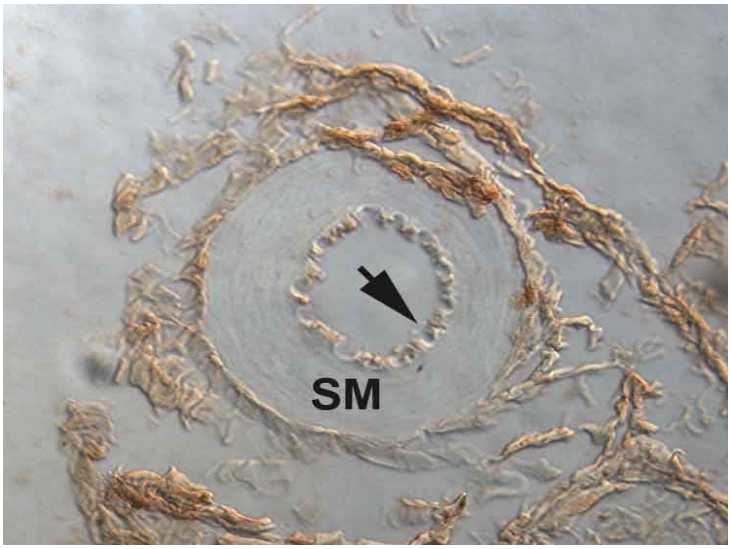
Fig 4.1 eNOS immunoreactivity. Light micrographs of 5μm transverse sections of pulmonary, mesenteric and femoral artery displaying positive immunoreactivity in the endothelium for eNOS. Arrow denotes the endothelium. SM = smooth muscle. Magnification x 10.



Pulmonary artery

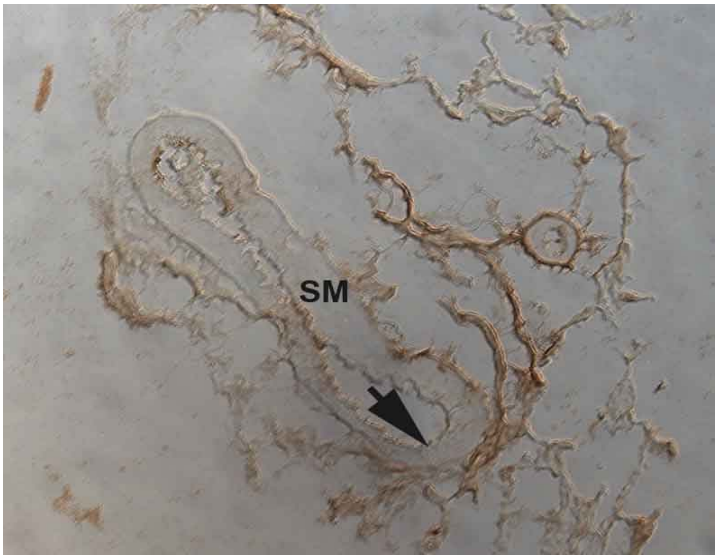


Mesenteric artery



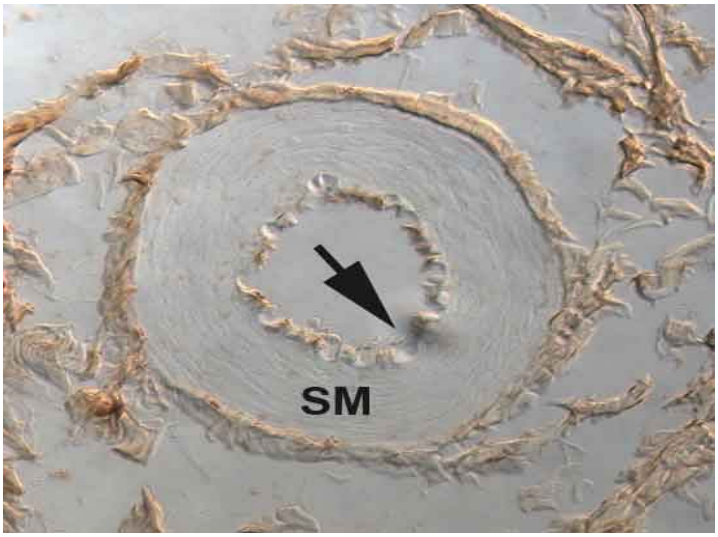
Femoral artery

Fig 4.2 nNOS immunoreactivity. Light micrographs of 5μm transverse sections of pulmonary, mesenteric and femoral artery displaying positive immunoreactivity in the endothelium for nNOS. Arrow denotes the endothelium. SM = smooth muscle. Magnification x 10.



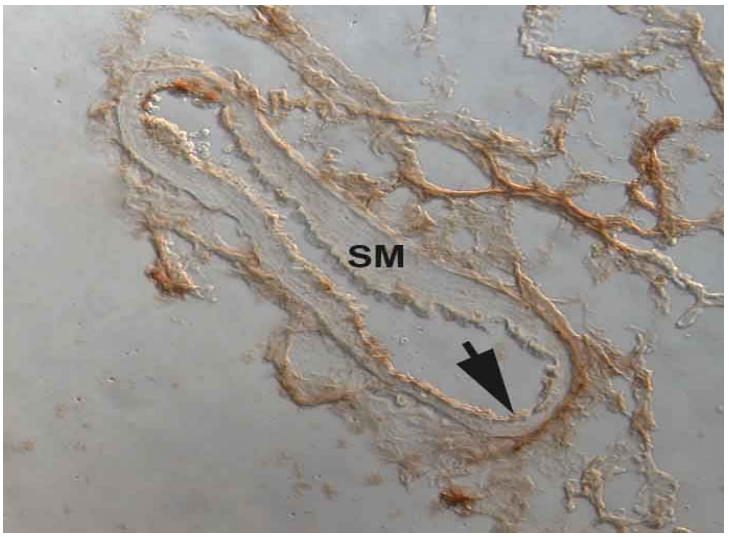
Pulmonary artery

Mesenteric artery

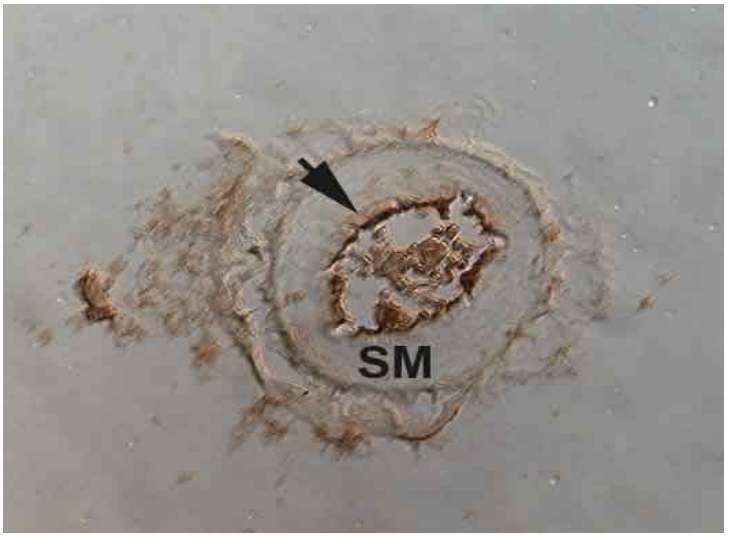


Femoral artery

Fig 4.3 CaMKII immunoreactivity. Light micrographs of 5μm transverse sections of pulmonary, mesenteric and femoral artery displaying positive immunoreactivity in the endothelium for CaMKII. Arrow denotes the endothelium. SM = smooth muscle. Magnification x 10.



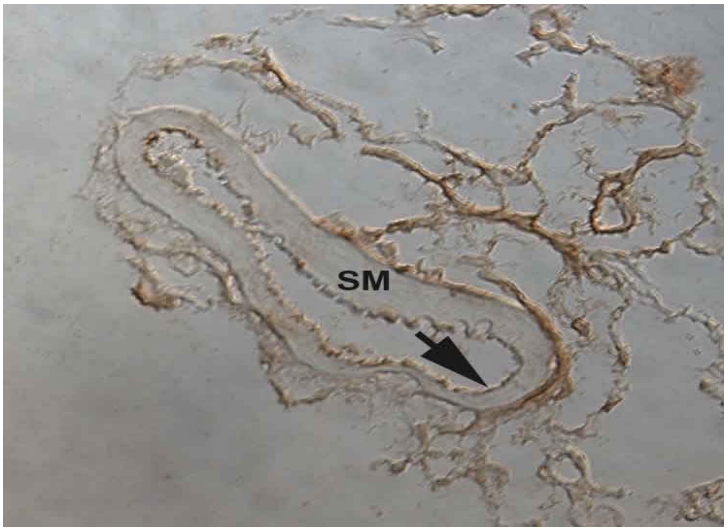
Pulmonary artery



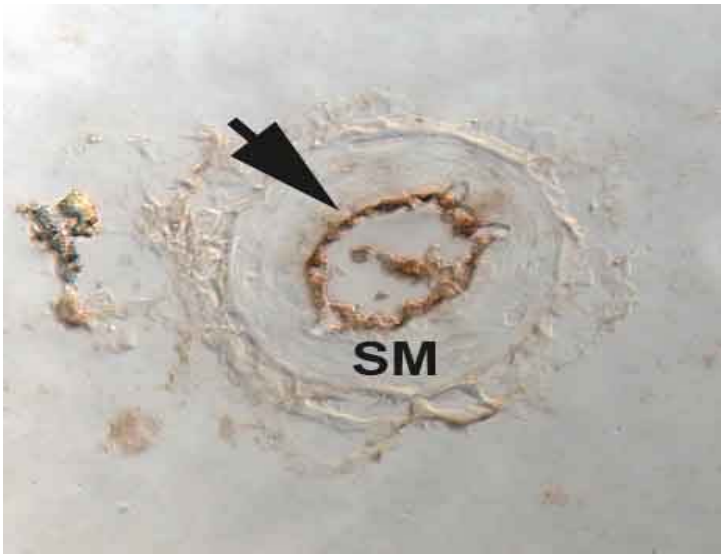
Mesenteric artery

Femoral artery

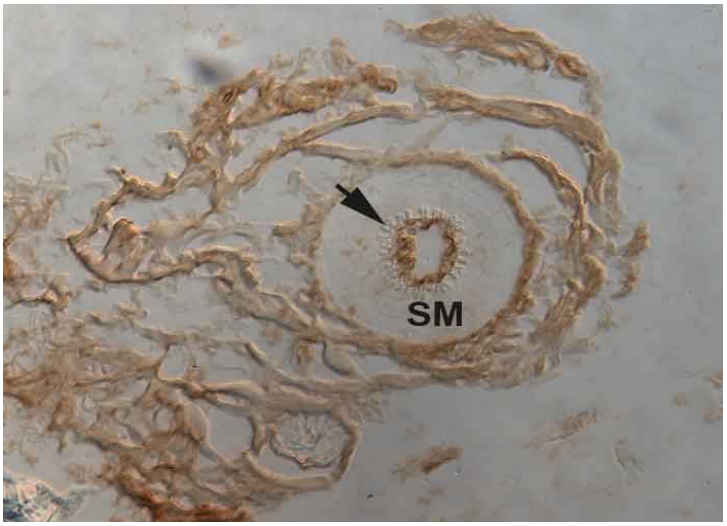
Fig 4.4 VAMP-1 immunoreactivity. Light micrographs of 5μm transverse sections of pulmonary, mesenteric and femoral artery displaying positive immunoreactivity in the endothelium for VAMP-1. Arrow denotes the endothelium. SM = smooth muscle. Magnification x 10.



Pulmonary artery

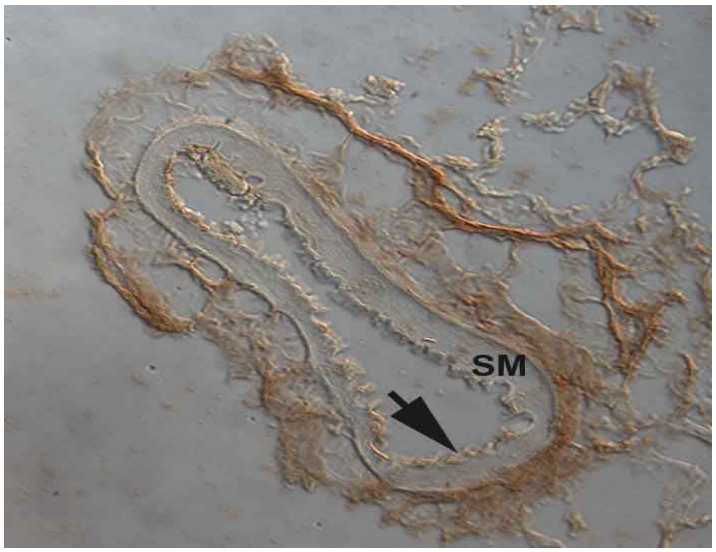


Mesenteric artery

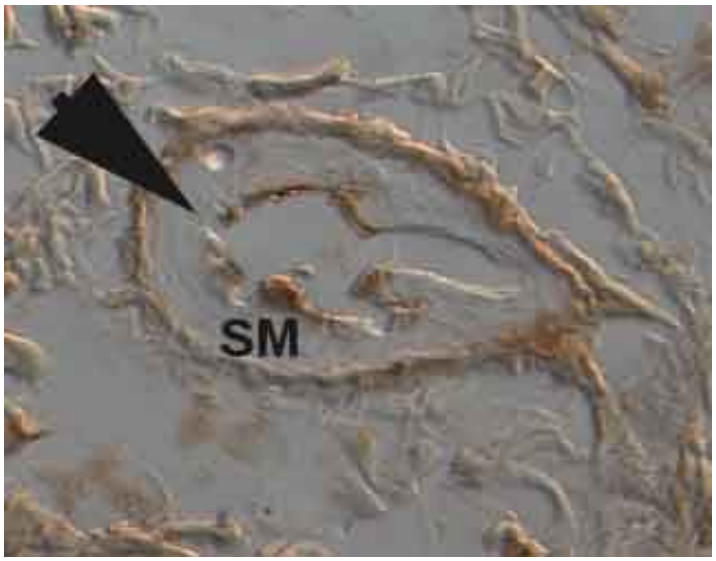


Femoral artery

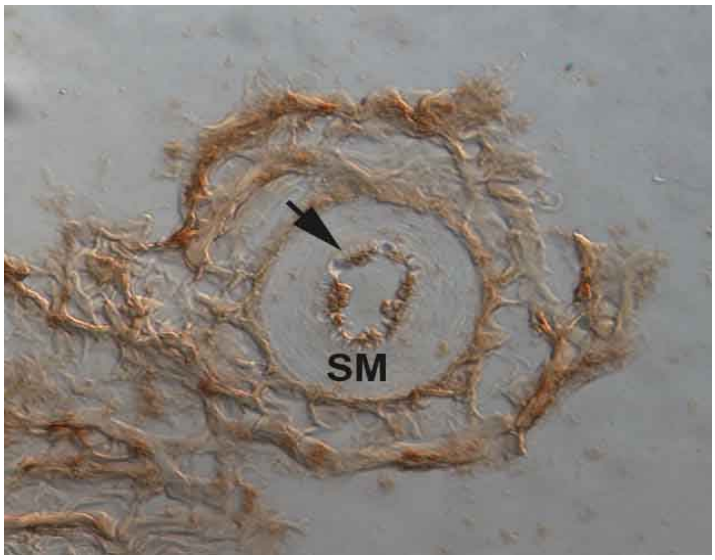
Fig 4.5 SNAP-25 immunoreactivity. Light micrographs of 5μm transverse sections of pulmonary, mesenteric and femoral artery displaying positive immunoreactivity in the endothelium for SNAP-25. Arrow denotes the endothelium. SM = smooth muscle. Magnification x 10



Pulmonary artery

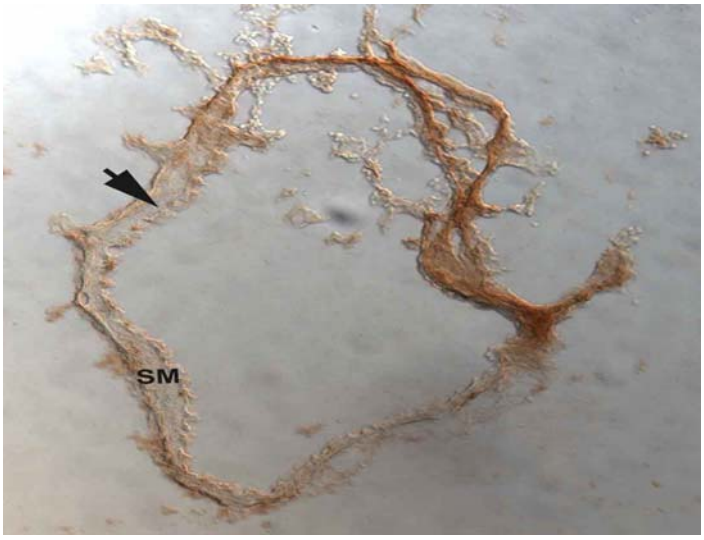


Mesenteric artery

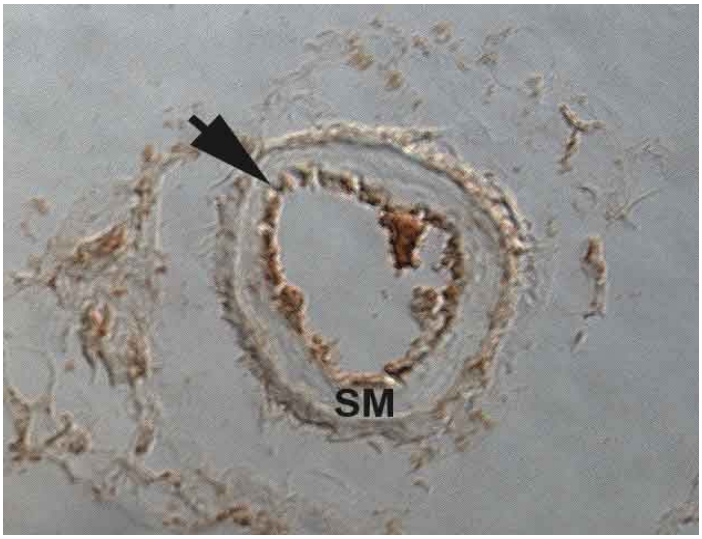


Femoral artery

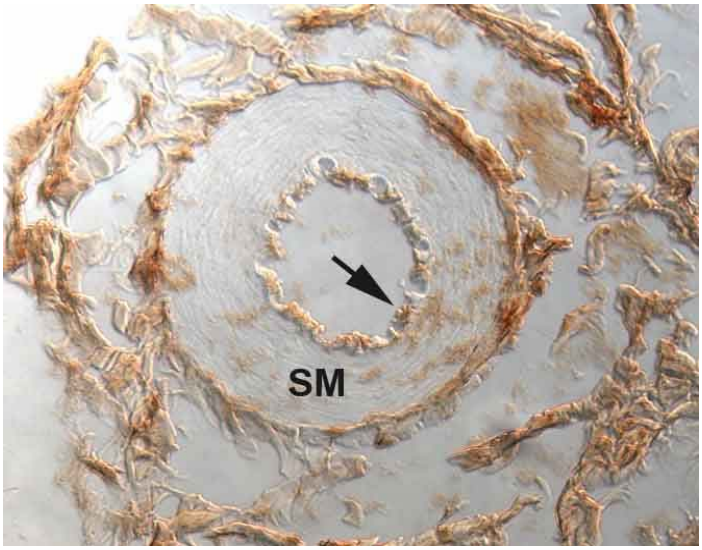
Fig 4.6 Syntaxin immunoreactivity. Light micrographs of 5μm transverse sections of pulmonary, mesenteric and femoral artery displaying positive immunoreactivity in the endothelium for Syntaxin-4. Arrow denotes the endothelium. SM = smooth muscle. Magnification x -10



Pulmonary artery

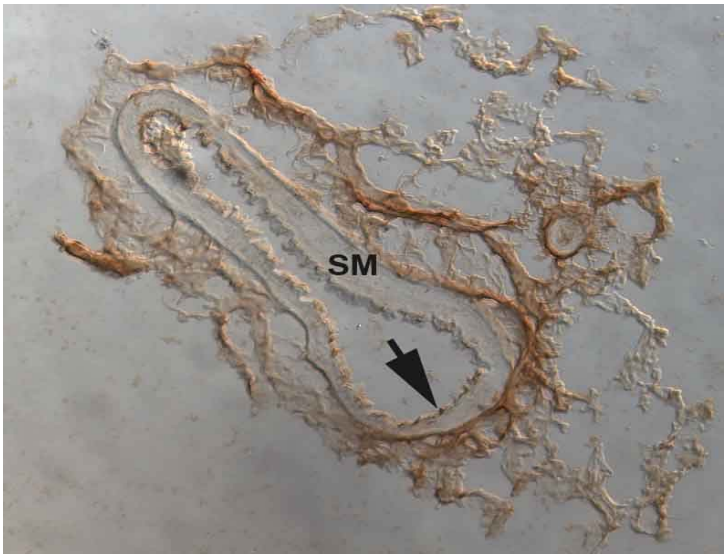


Mesenteric artery



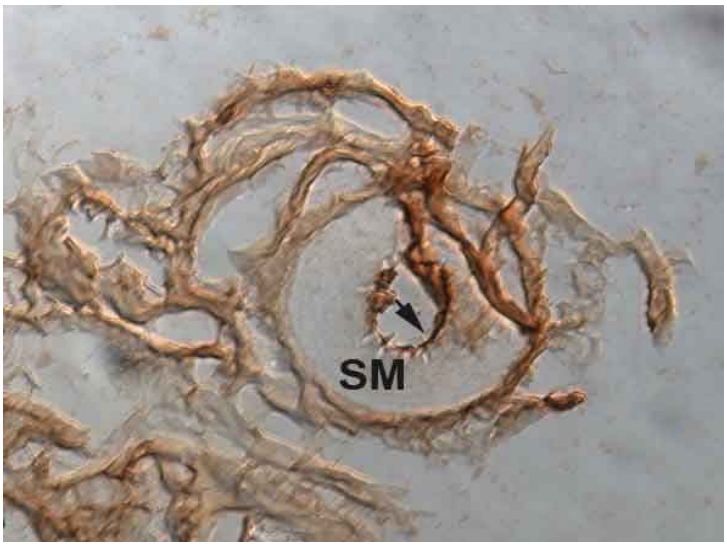
Femoral artery

Fig 4.7 Neurexin immunoreactivity. Light micrographs of 5μm transverse sections of pulmonary, mesenteric and femoral artery displaying positive immunoreactivity in the endothelium for Neurexin. Arrow denotes the endothelium. SM = smooth muscle. Magnification x 10.



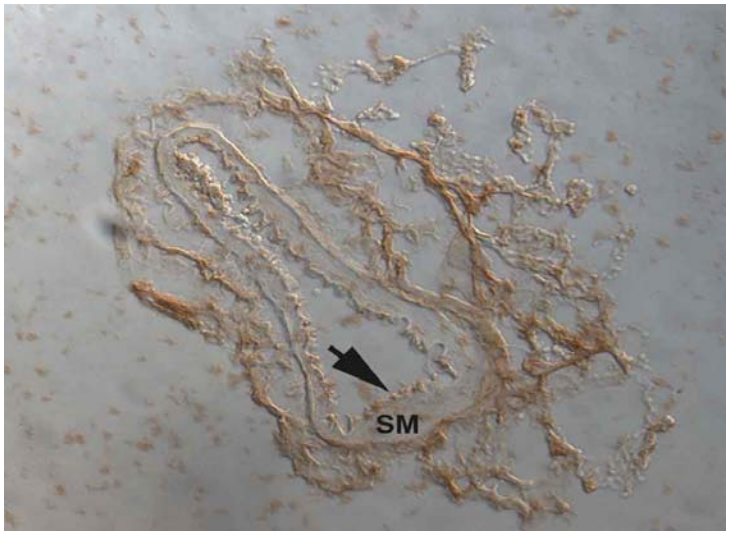
Pulmonary artery

Mesenteric artery



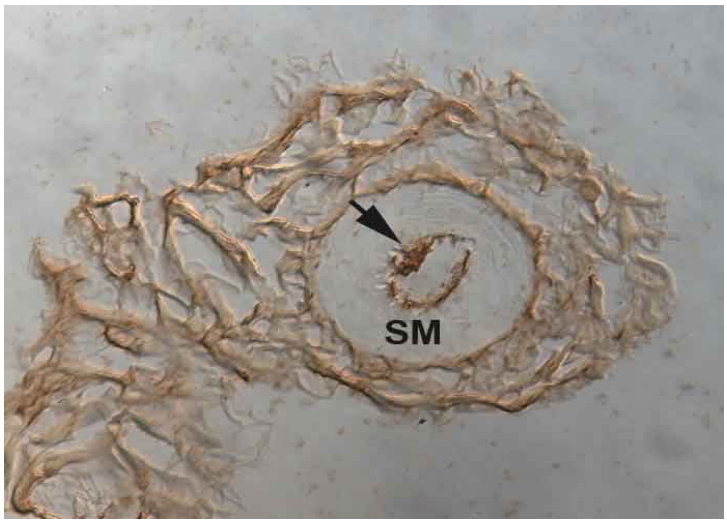
Femoral artery

Fig 4.8 Synaptotagmin immunoreactivity. Light micrographs of 5μm transverse sections of pulmonary, mesenteric and femoral artery displaying positive immunoreactivity in the endothelium for Synaptotagmin. Arrow denotes the endothelium. SM = smooth muscle. Magnification x10.



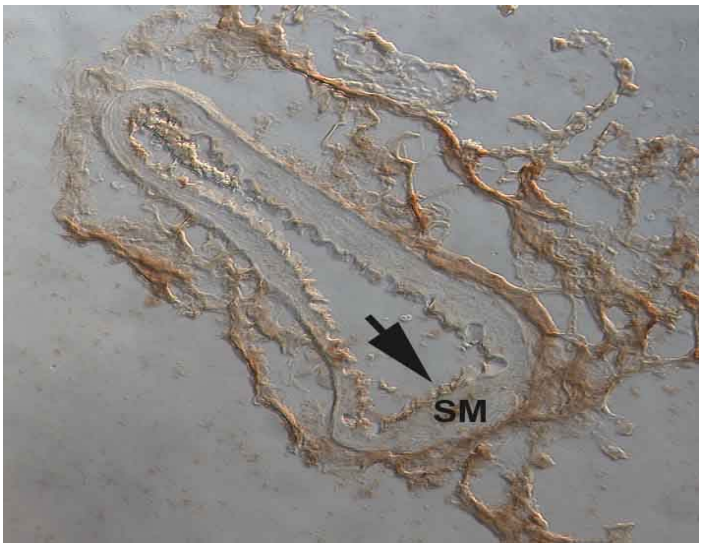
Pulmonary artery

Mesenteric artery

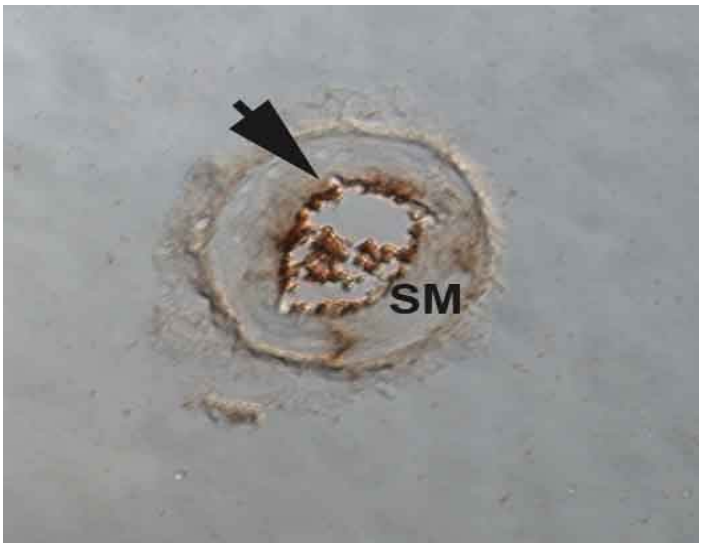


Femoral artery

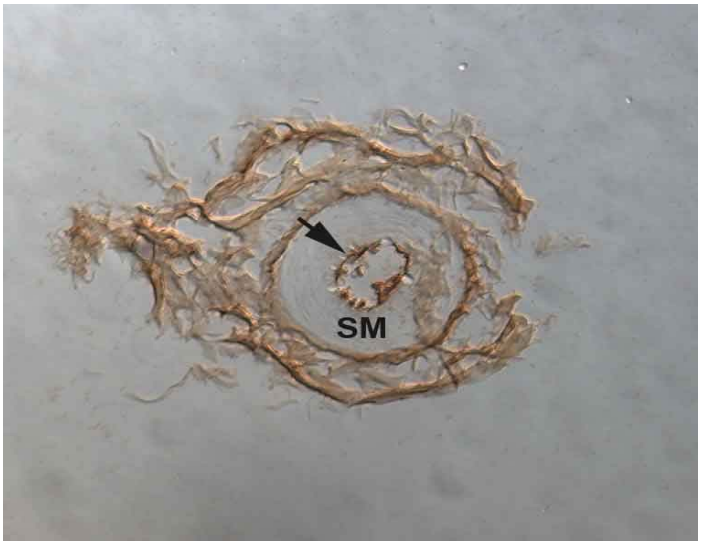
Fig 4.9 Synaptophysin immunoreactivity. Light micrographs of 5μm transverse sections of pulmonary, mesenteric and femoral artery displaying positive immunoreactivity in the endothelium for Synaptophysin. Arrow denotes the endothelium. SM = smooth muscle. Magnification x 10.



Pulmonary artery

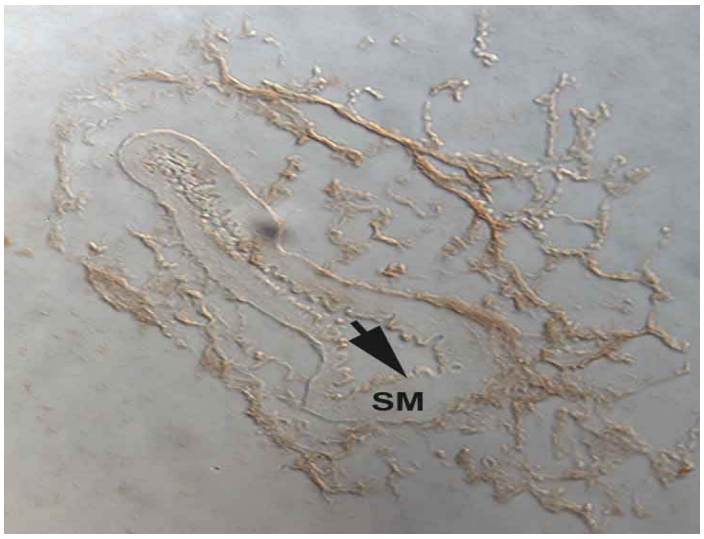


Mesenteric artery

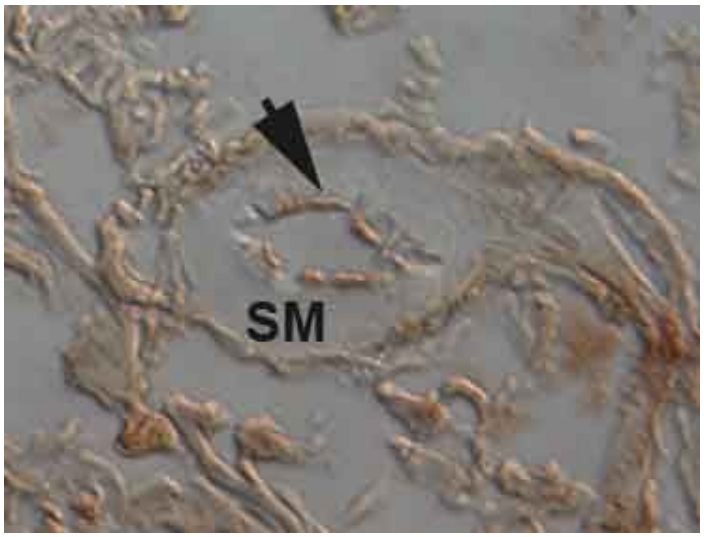


Femoral artery

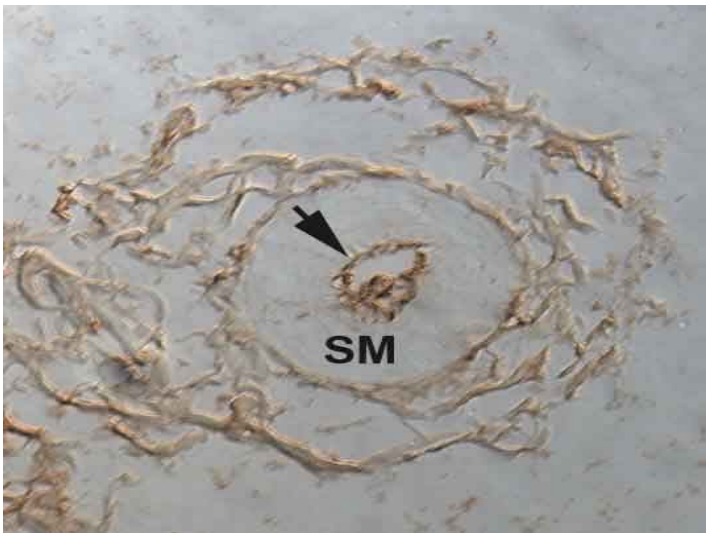
Fig 4.10 NSF immunoreactivity. Light micrographs of 5μm transverse sections of pulmonary, mesenteric and femoral artery displaying positive immunoreactivity in the endothelium for NSF. Arrow denotes the endothelium. SM = smooth muscle. Magnification x 10.



Pulmonary artery

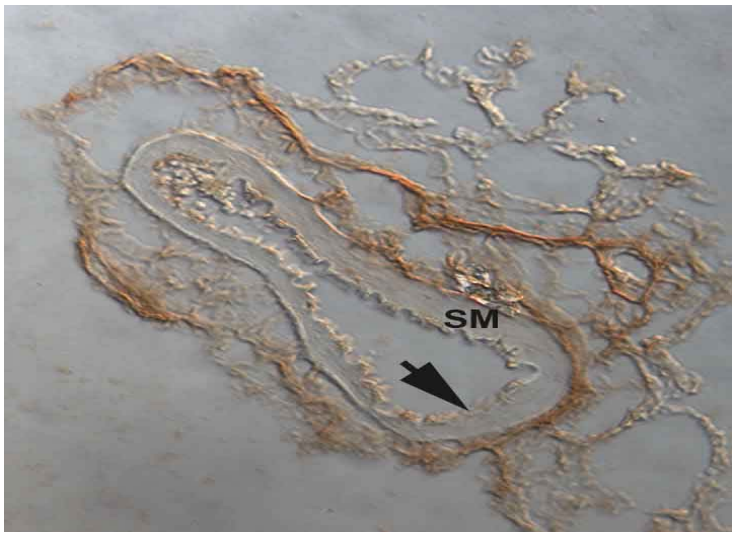


Mesenteric artery

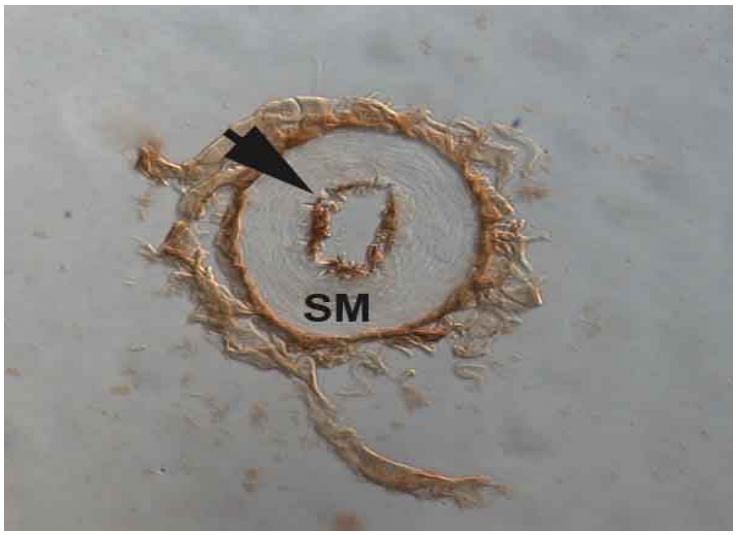


Femoral artery

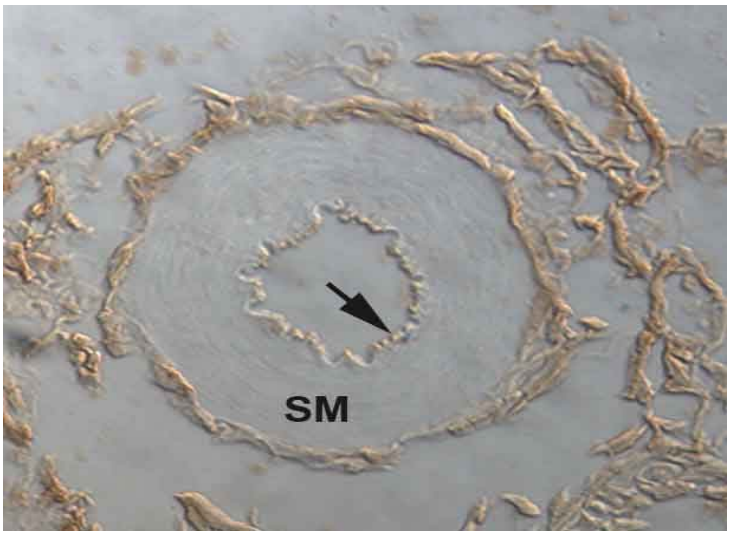
Fig 4.11 Rab3A immunoreactivity. Light micrographs of 5μm transverse sections of pulmonary, mesenteric and femoral artery displaying positive immunoreactivity in the endothelium for Rab3A. Arrow denotes the endothelium. SM = smooth muscle. Magnification x 10



Pulmonary artery



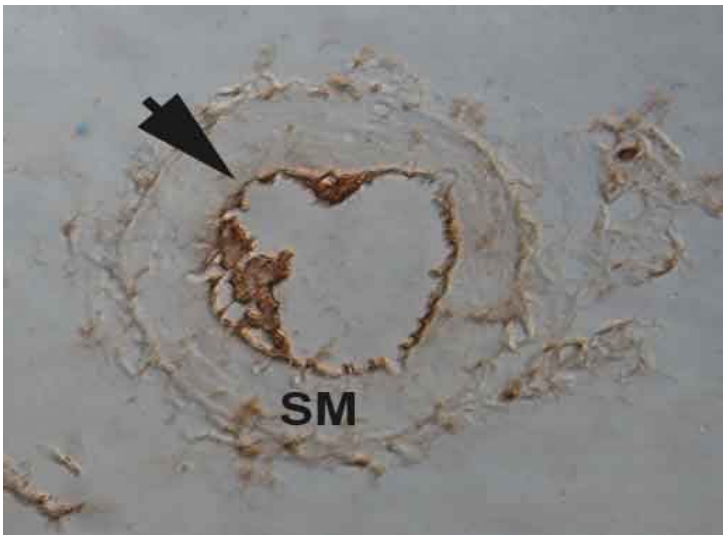
Mesenteric artery



Femoral artery

Fig 4.12 Rabphilin immunoreactivity. Light micrographs of 5μm transverse sections of pulmonary, mesenteric and femoral artery displaying positive immunoreactivity in the endothelium for Rabphilin. Arrow denotes the endothelium. SM = smooth muscle. Magnification x 10.

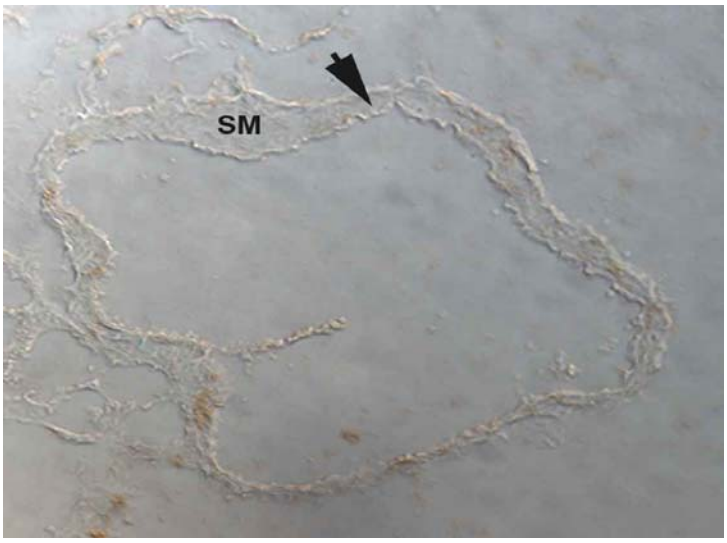
Pulmonary artery



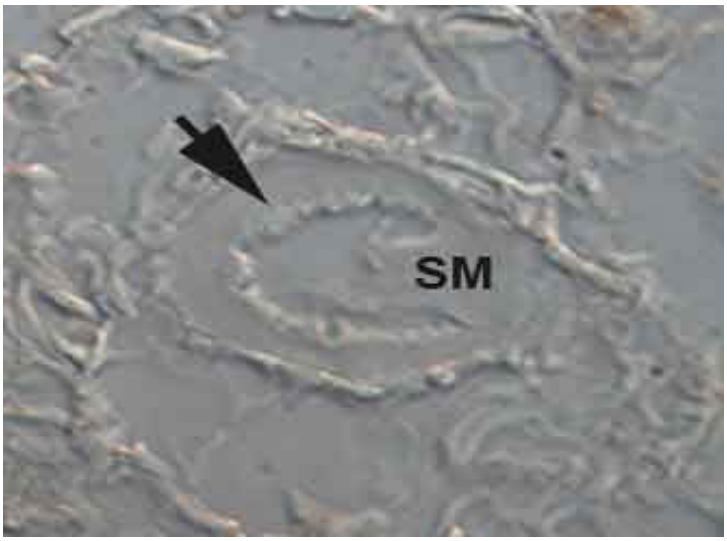
Mesenteric artery

Femoral artery

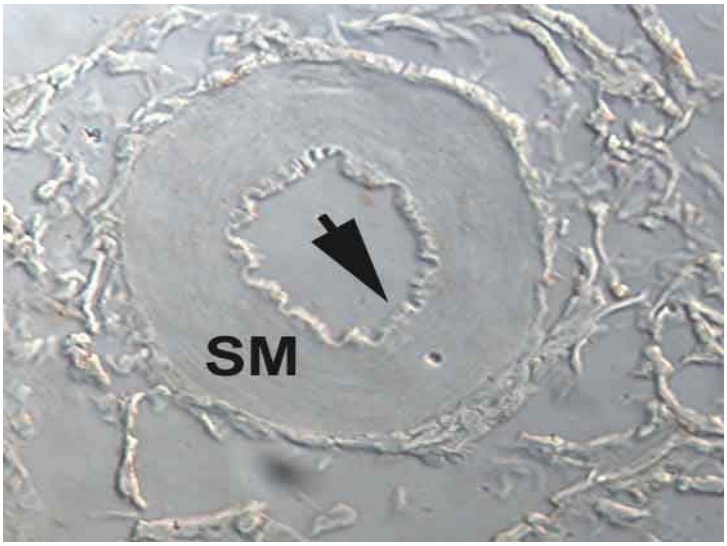
Fig 4.13 CD4 immunoreactivity. Light micrographs of 5μm transverse sections of pulmonary, mesenteric and femoral artery displaying negative immunoreactivity in the endothelium for CD4. Arrow denotes the endothelium. SM = smooth muscle. Magnification x 10.



Pulmonary artery

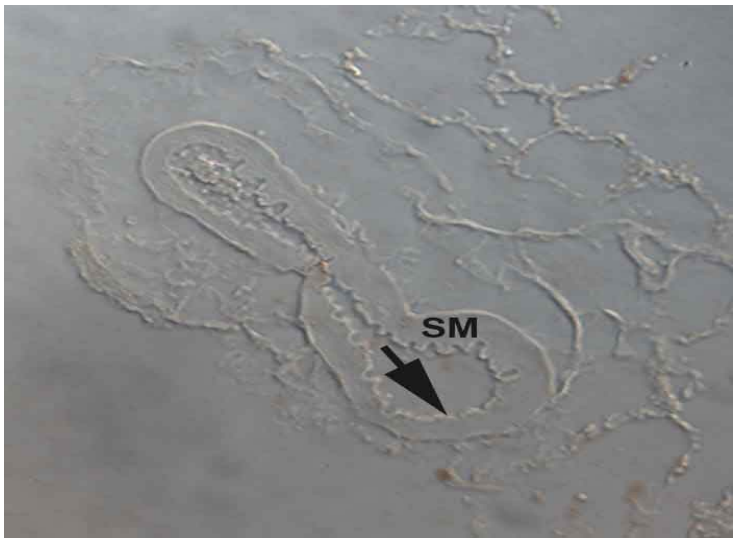


Mesenteric artery

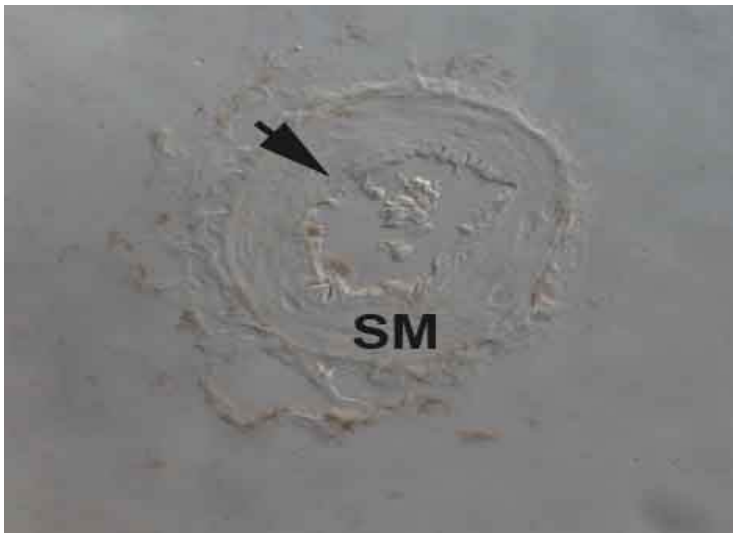


Femoral artery

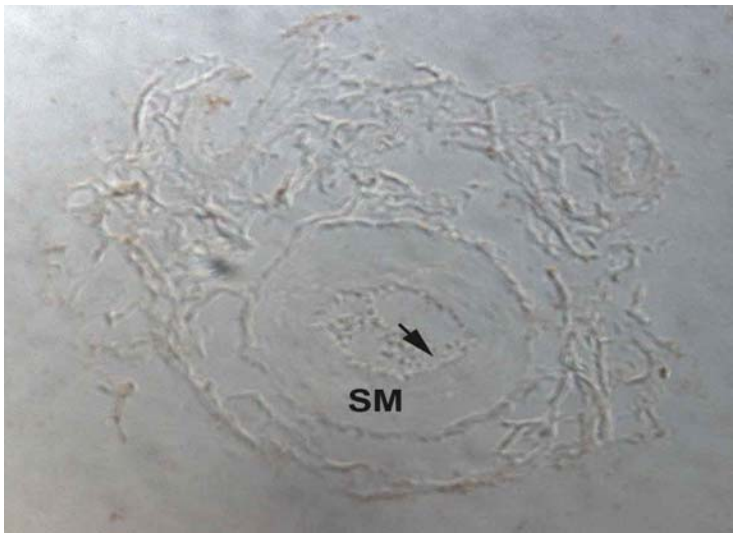
Fig 4.14 Absence of immunoreactivity. Light micrographs of 5μm transverse sections of pulmonary, mesenteric and femoral artery displaying no immunoreactivity in the endothelium due to omission of primary antiserum. Arrow denotes the endothelium. SM = smooth muscle. Magnification x10.



Pulmonary artery



Mesenteric artery



Femoral artery

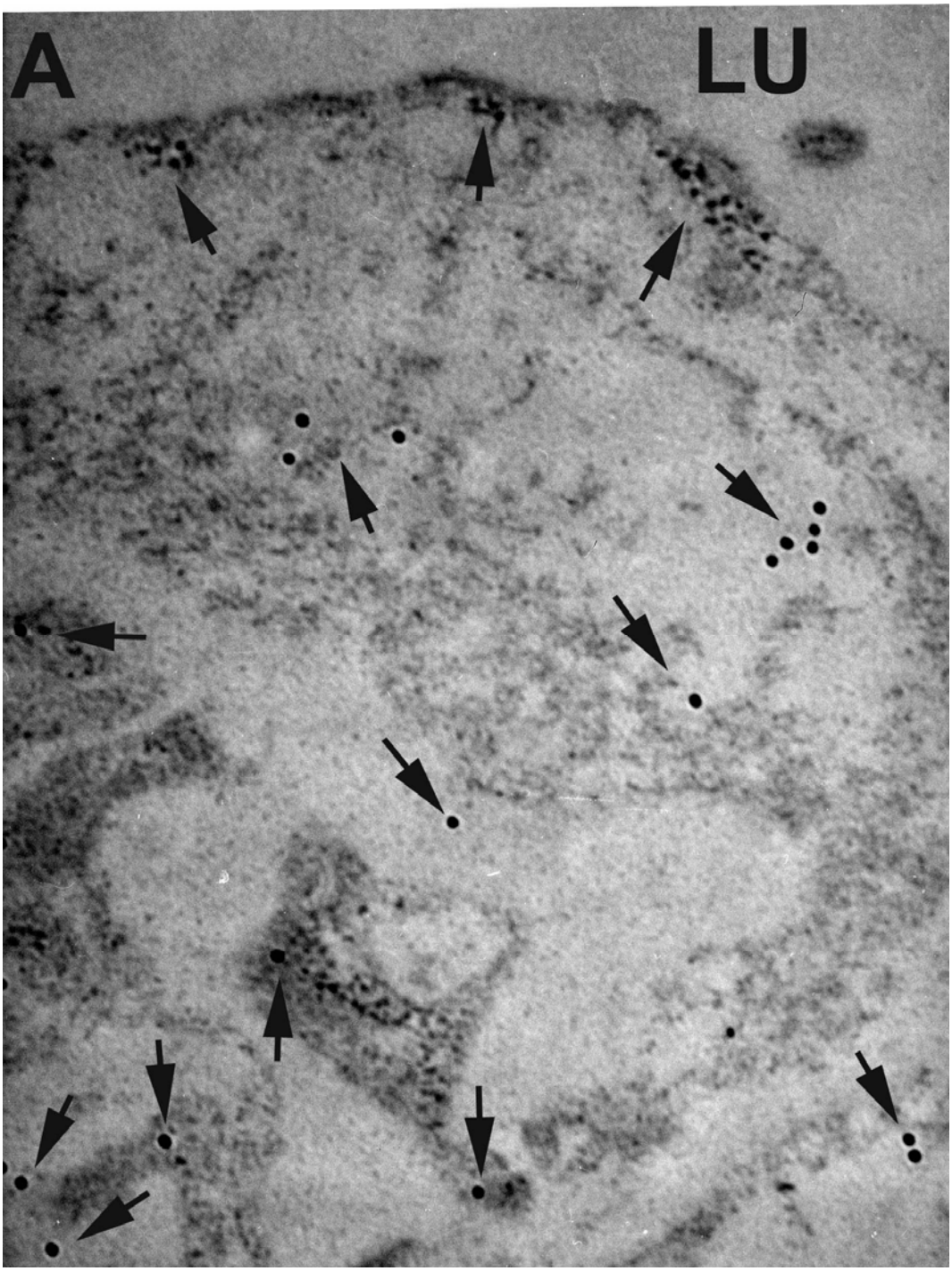


Fig 4.15A Immunogold labeling of eNOS in a pulmonary artery endothelial cell. Arrows denote position of gold particles. LU = lumen. Magnification x 48,000.

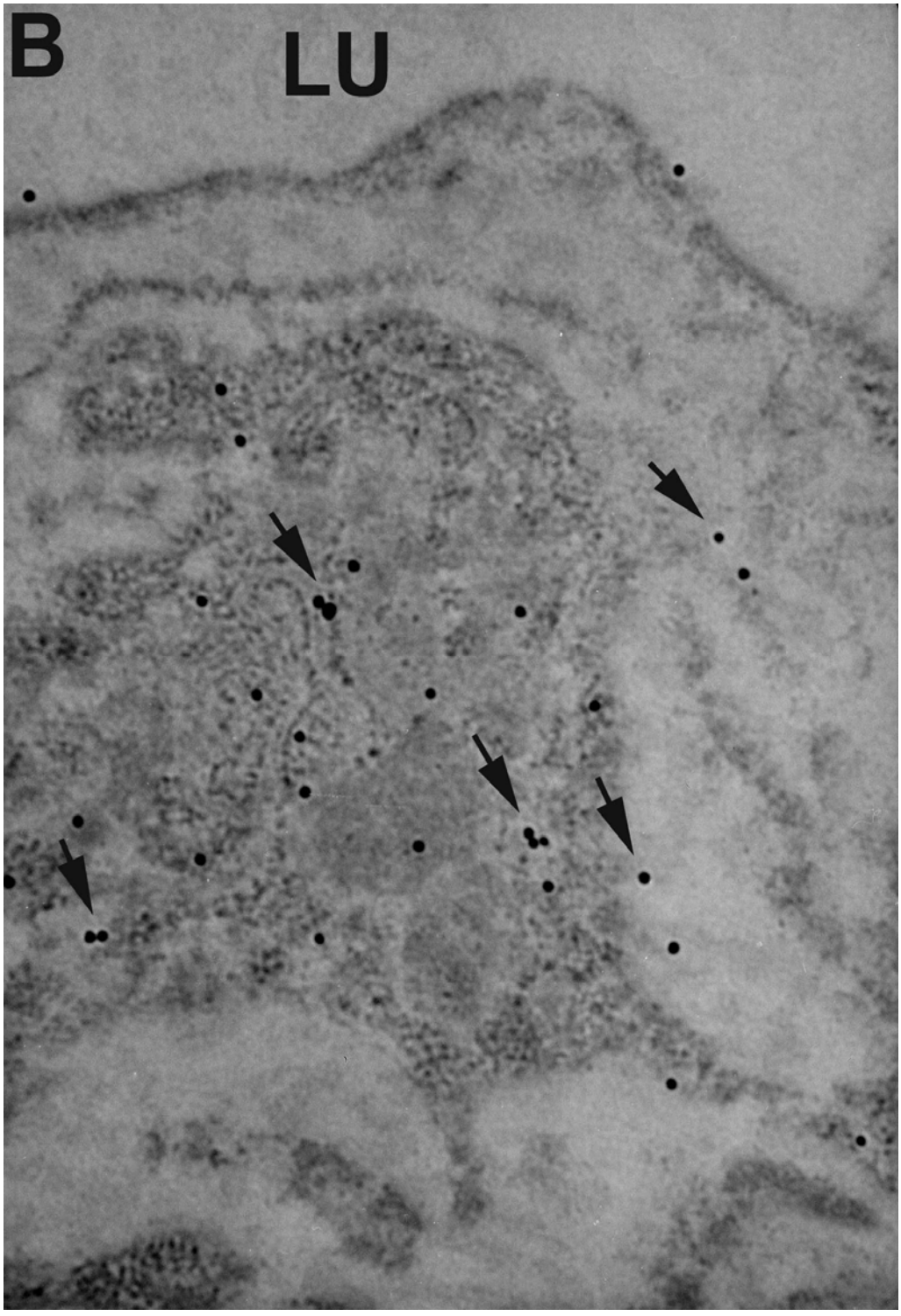


Fig 4.15B Immunogold labeling of eNOS in endothelial cell of mesenteric artery. Arrows denote position of gold particles. LU = lumen. Magnification x 48,000.

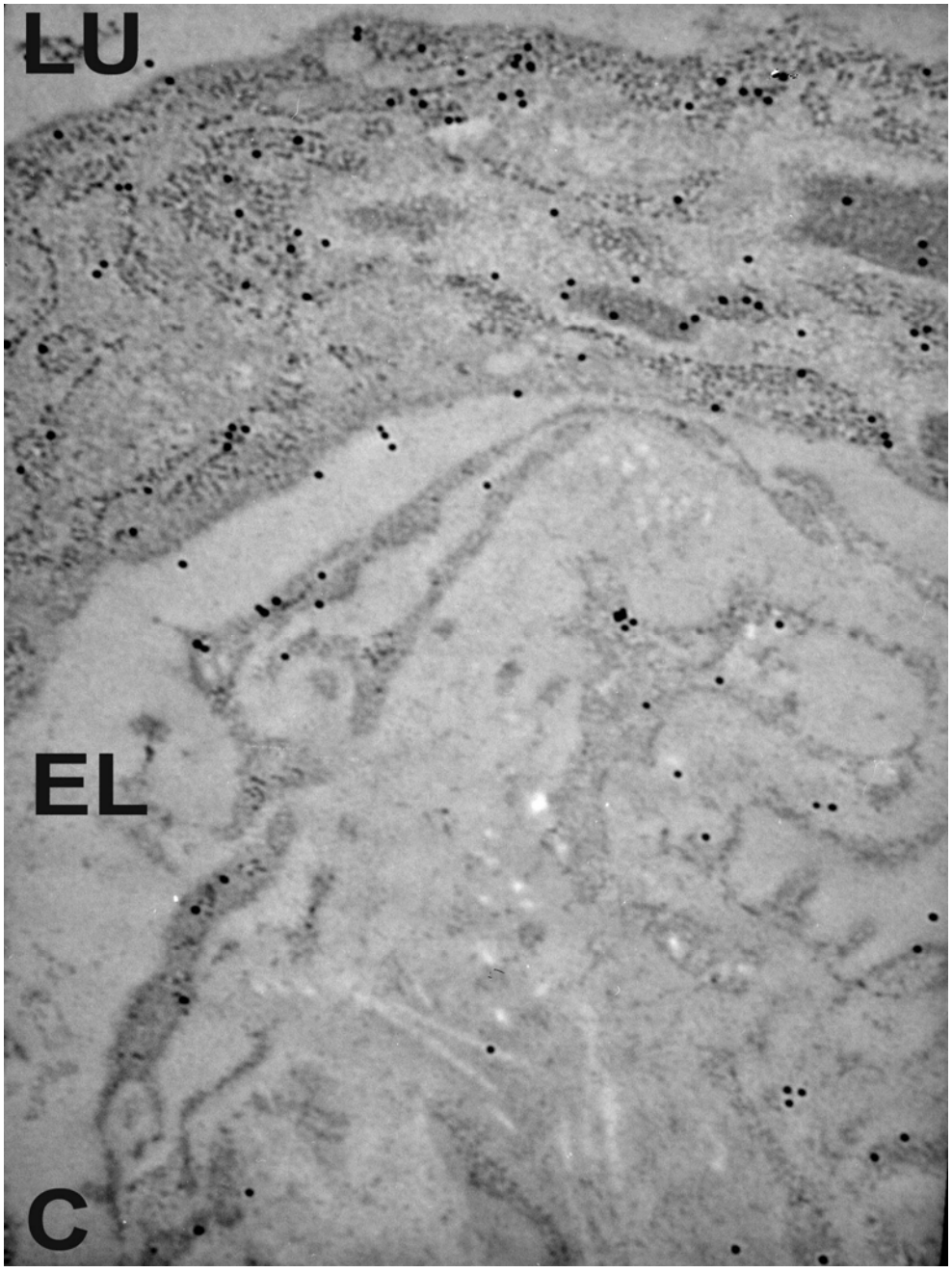


Fig 4.15C Immunogold labeling of eNOS in endothelial cell of femoral artery. Electron micrograph of fragments of two endothelial cells from the femoral artery positively labeled for eNOS using immunogold particles. Arrows denote position of gold particles. LU = lumen, EL = elastic lamina. Magnification x 29,000.

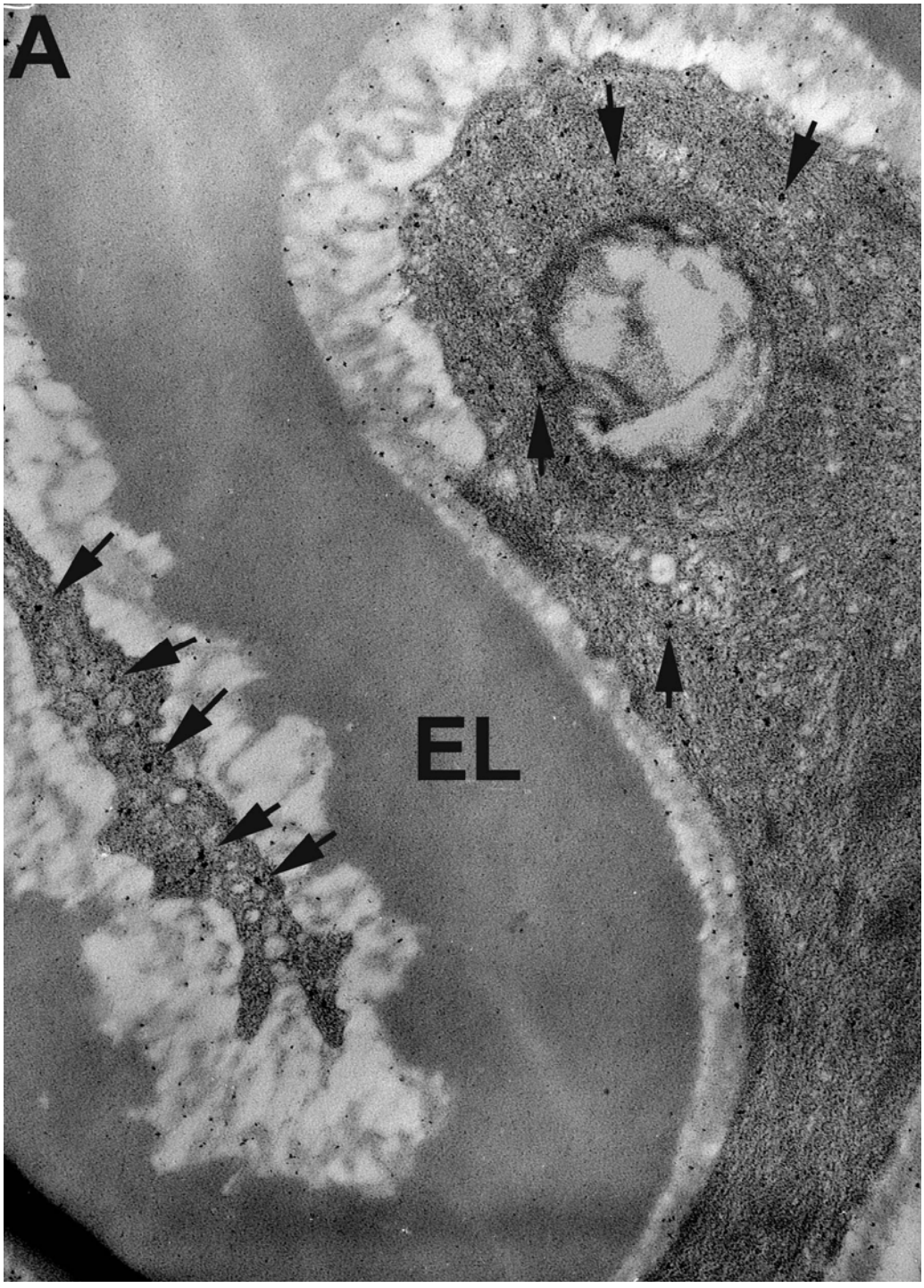


Fig 4.16A Immunogold labeling of CaMKII in a pulmonary artery endothelial cell. Arrows denote position of gold particles. EL = elastic lamina.. Magnification x 29,000.

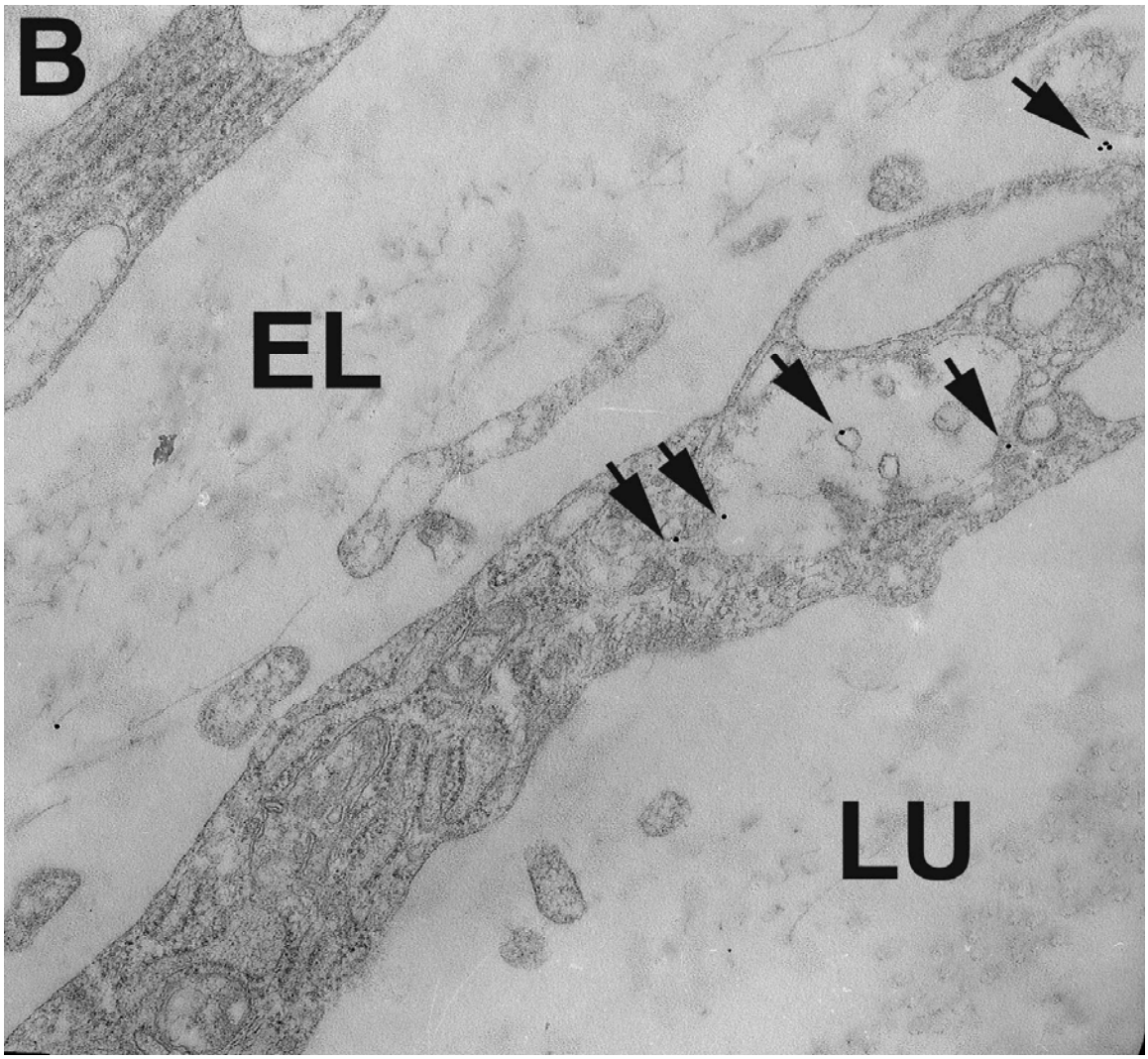


Fig 4.16B Immunogold labeling of CaMKII in endothelial cell of mesenteric artery. Electron micrograph of part of an endothelial cell from the mesenteric artery positively labeled for CaMKII using immunogold particles. Arrows denote position of gold particles. EL = elastic lamina. LU = lumen. Magnification x 19,000.

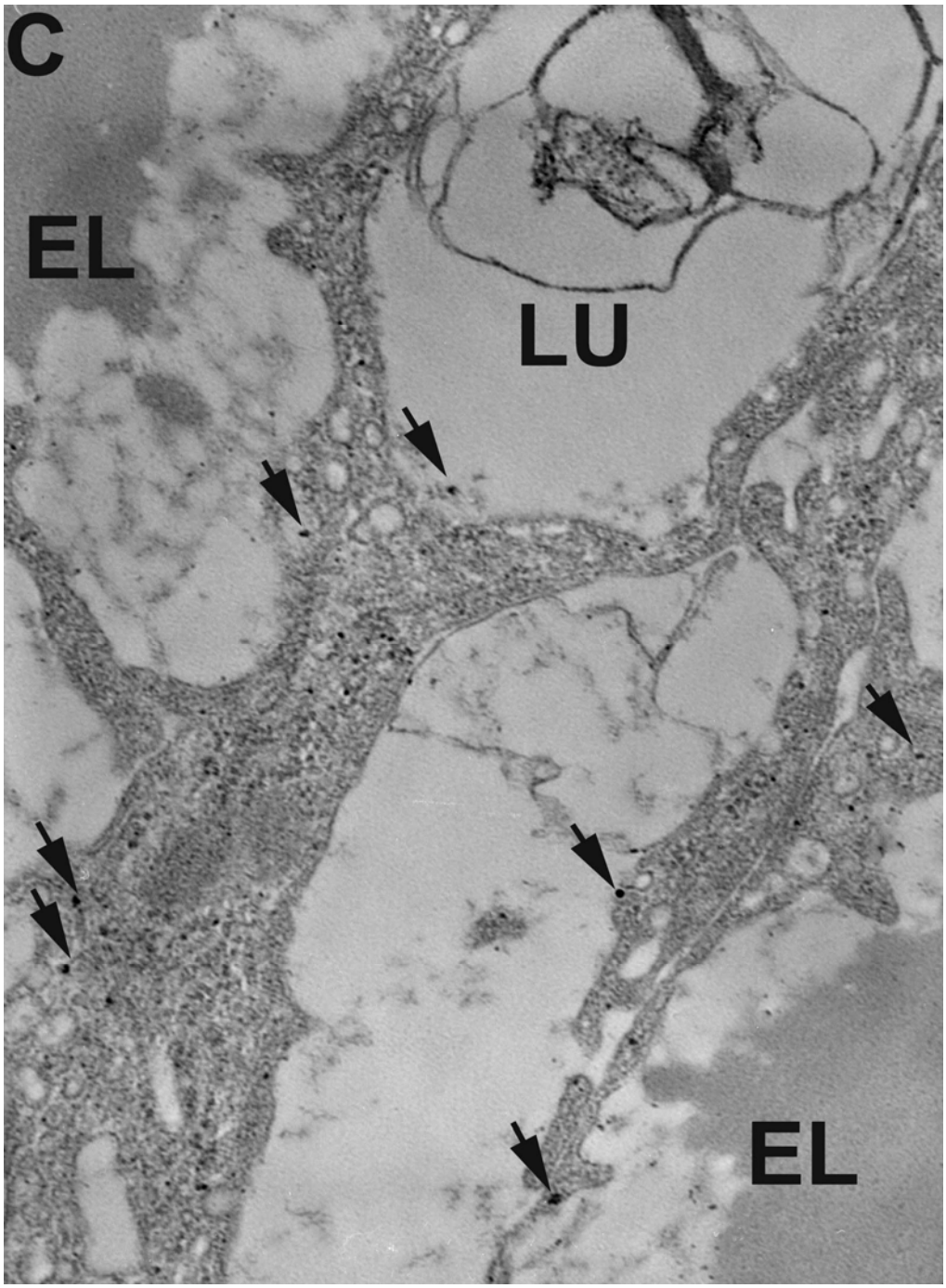


Fig 4.16C Immunogold labeling of CaMKII in endothelial cell of femoral artery. Electron micrograph of part of an endothelial cell from the femoral artery positively labeled for CaMKII using immunogold particles. Arrows denote position of gold particles. LU=lumen. EL = elastic lamina. Magnification x 36,000.

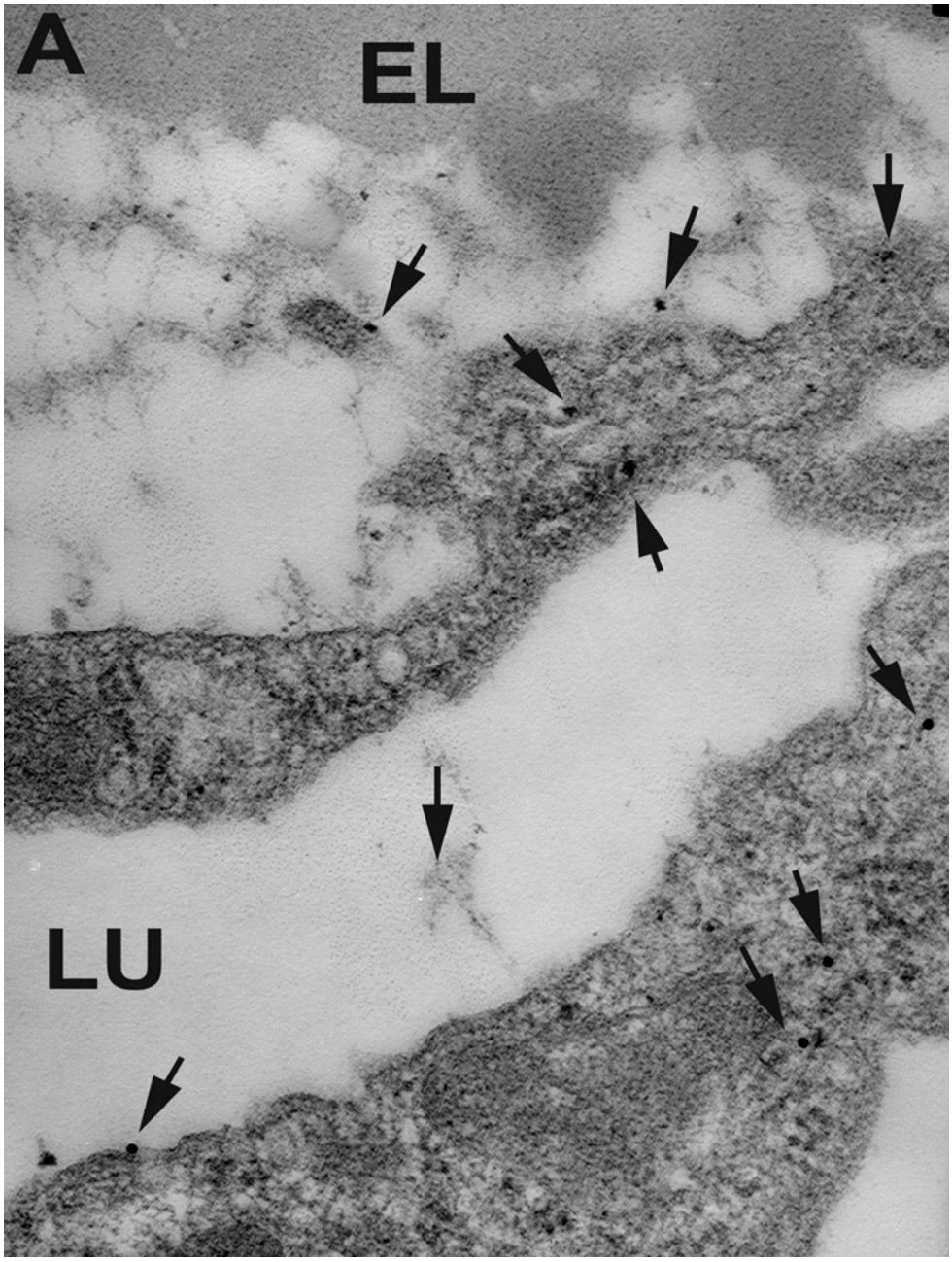


Fig 4.17A Immunogold labeling of VAMP-1 in endothelial cell of pulmonary artery. Electron micrograph of part of an endothelial cell from the pulmonary artery positively labeled for VAMP-1 using immunogold particles. Arrows denote position of gold particles. LU = lumen, EL = elastic lamina. Magnification x 58,000.

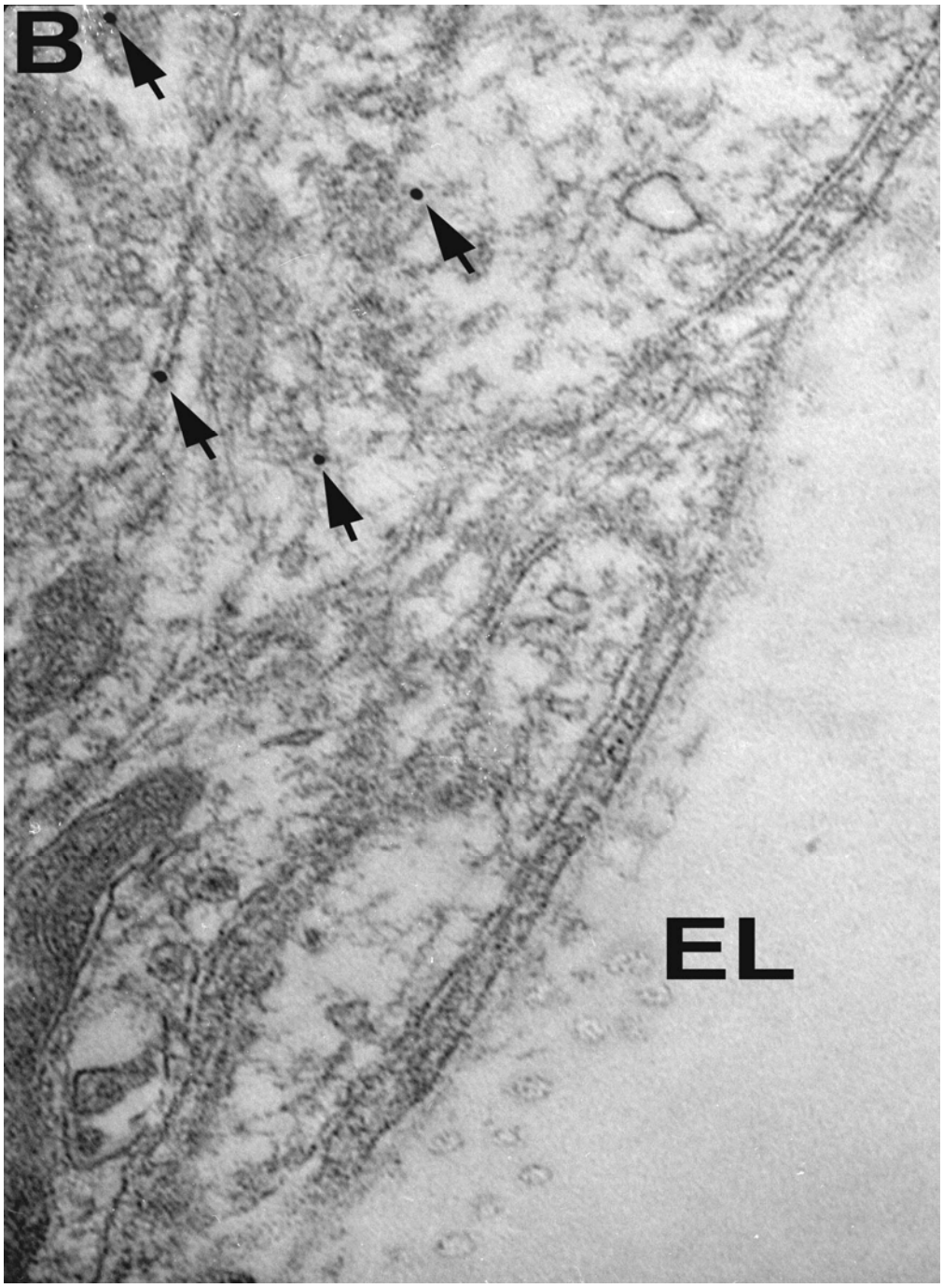


Fig 4.17B Immunogold labeling of VAMP-1 in endothelial cell of mesenteric artery. Electron micrograph of part of an endothelial cell from the mesenteric artery positively labeled for VAMP-1 using immunogold particles. Arrows denote position of gold particles. EL = elastic lamina. Magnification x 48,000.

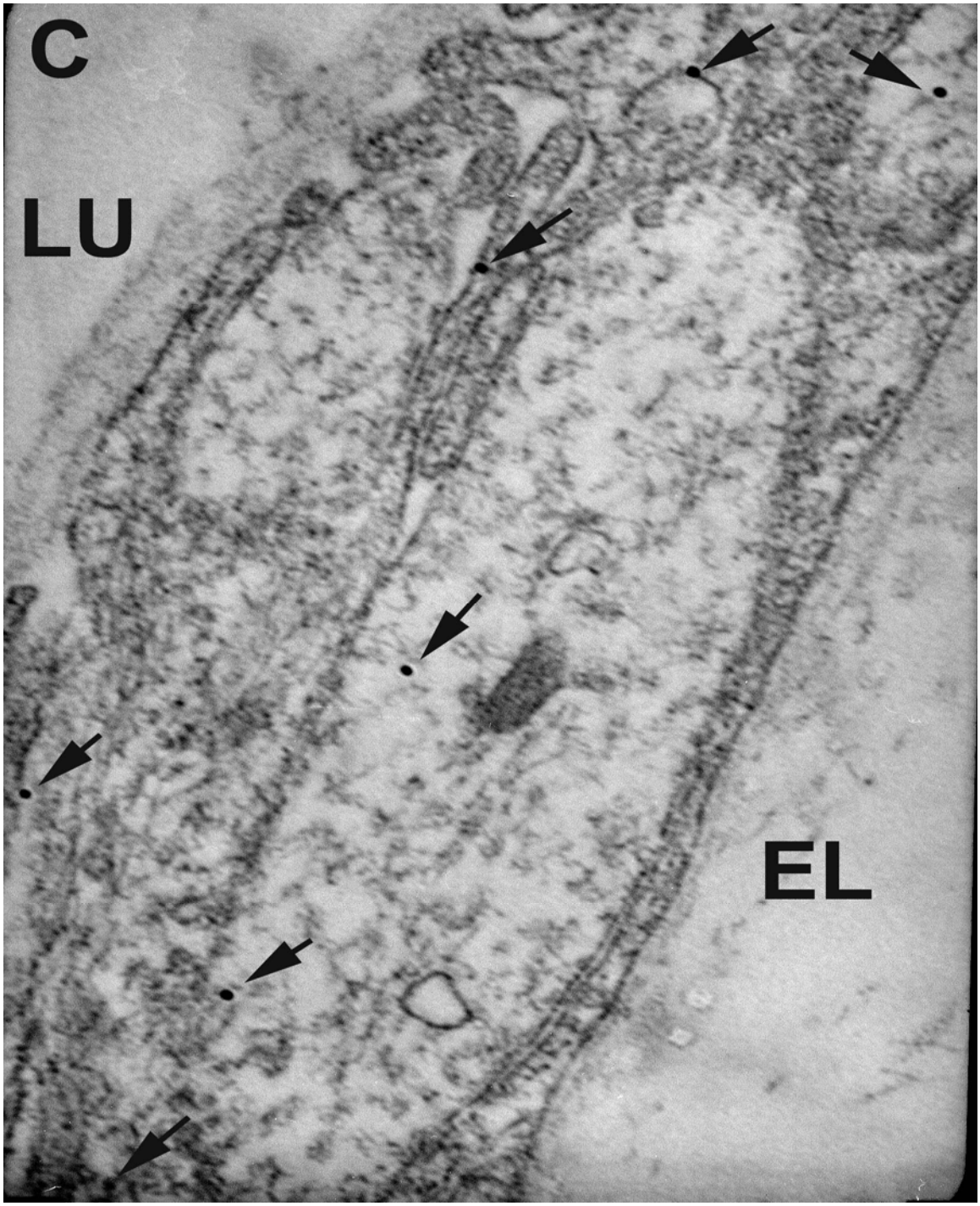


Fig 4.17C Immunogold labeling of VAMP-1 in a femoral artery endothelial cell. Arrows denote position of gold particles. LU = lumen, EL = elastic lamina. Magnification x 48,000.

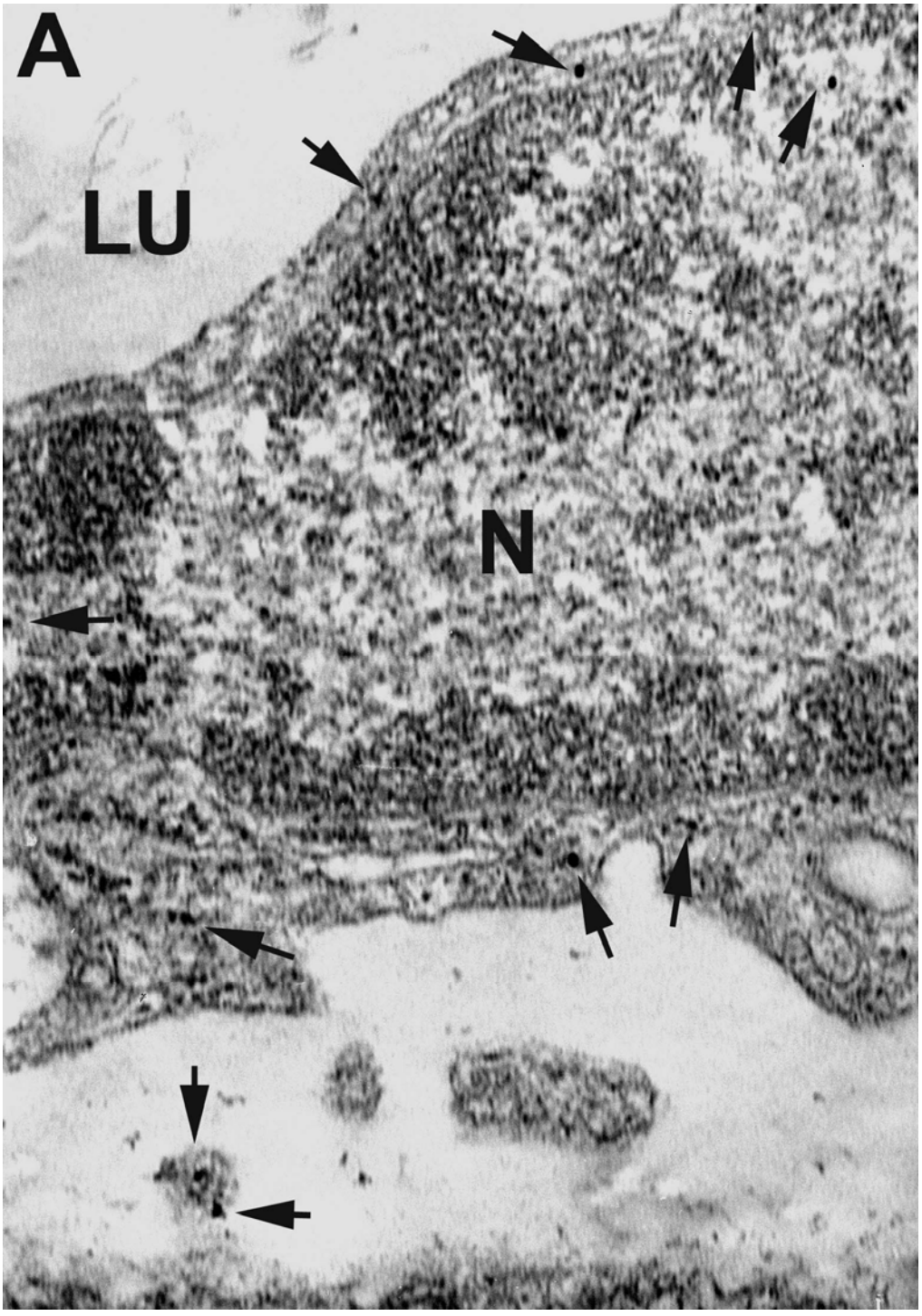


Fig 4.18A Immunogold labeling of SNAP-25 in a pulmonary artery endothelial cell. Arrows denote position of gold particles. LU = lumen, N = nucleus. Magnification x 48,000.

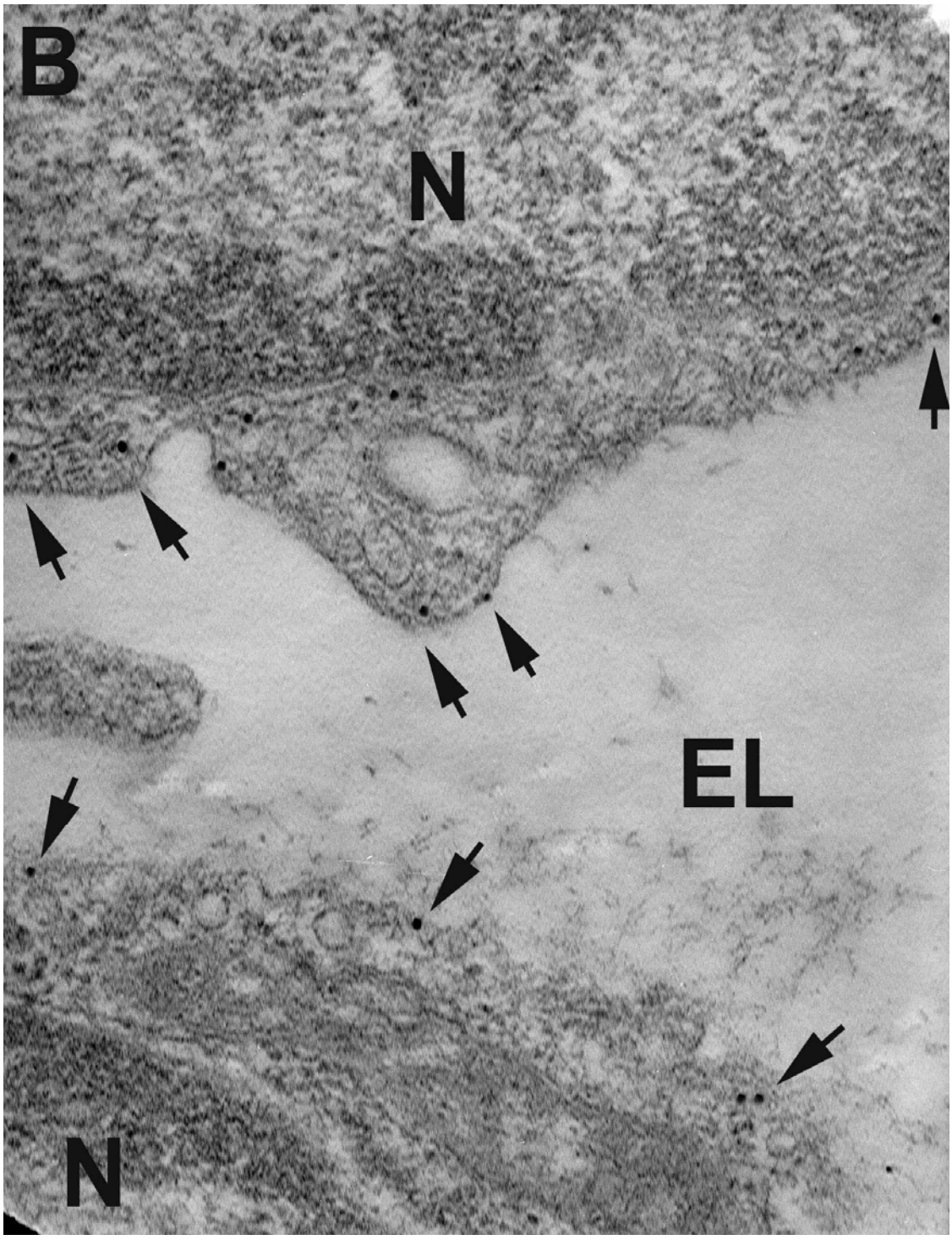


Fig 4.18B Immunogold labeling of SNAP-25 in a mesenteric artery endothelial cell. Arrows denote position of gold particles. N = nucleus, EL = elastic lamina. Magnification x 48,000.

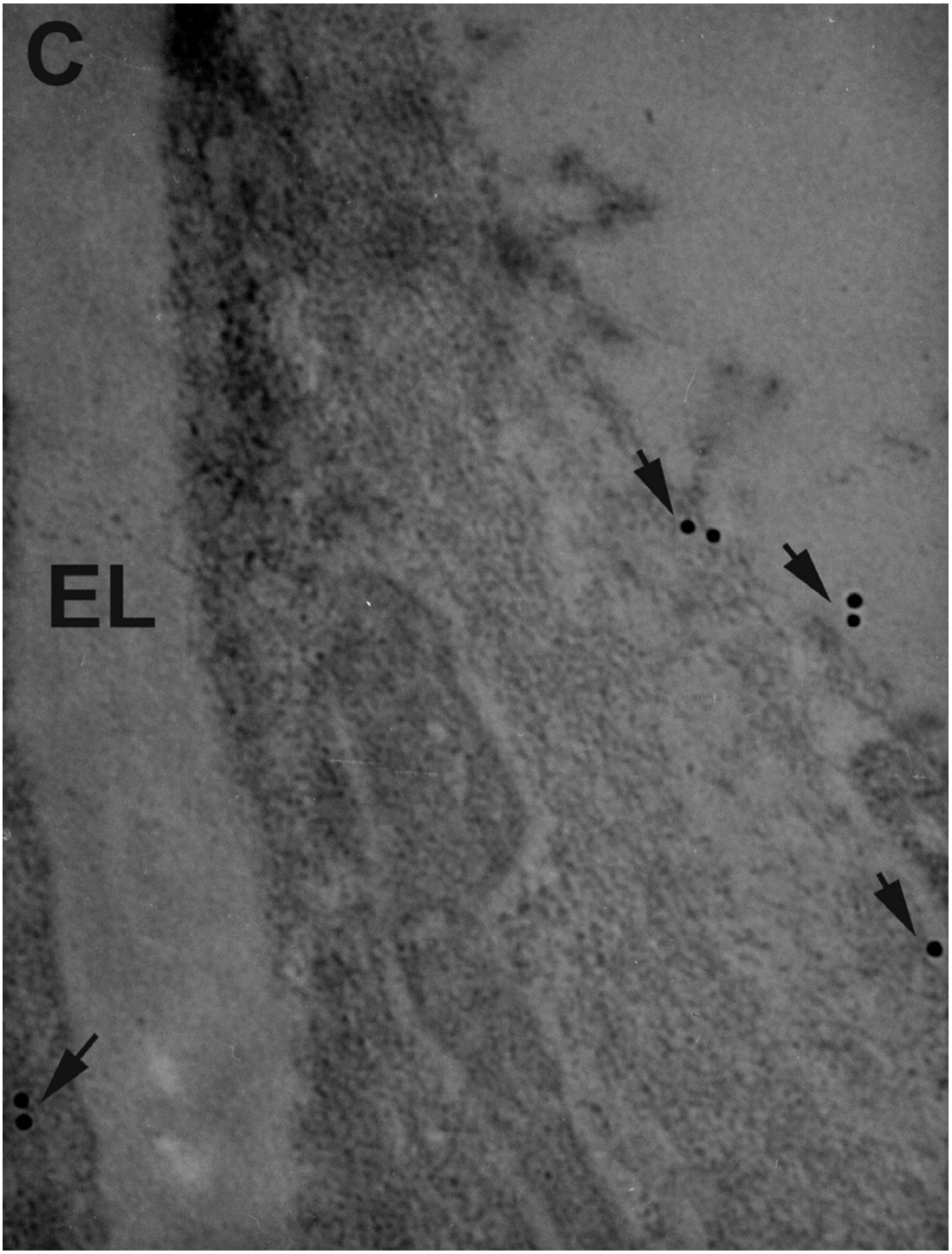


Fig 4.18C Immunogold labeling of SNAP-25 in a femoral artery endothelial cell. Arrows denote position of gold particles. EL=elastic lamina. Magnification x 58,000.

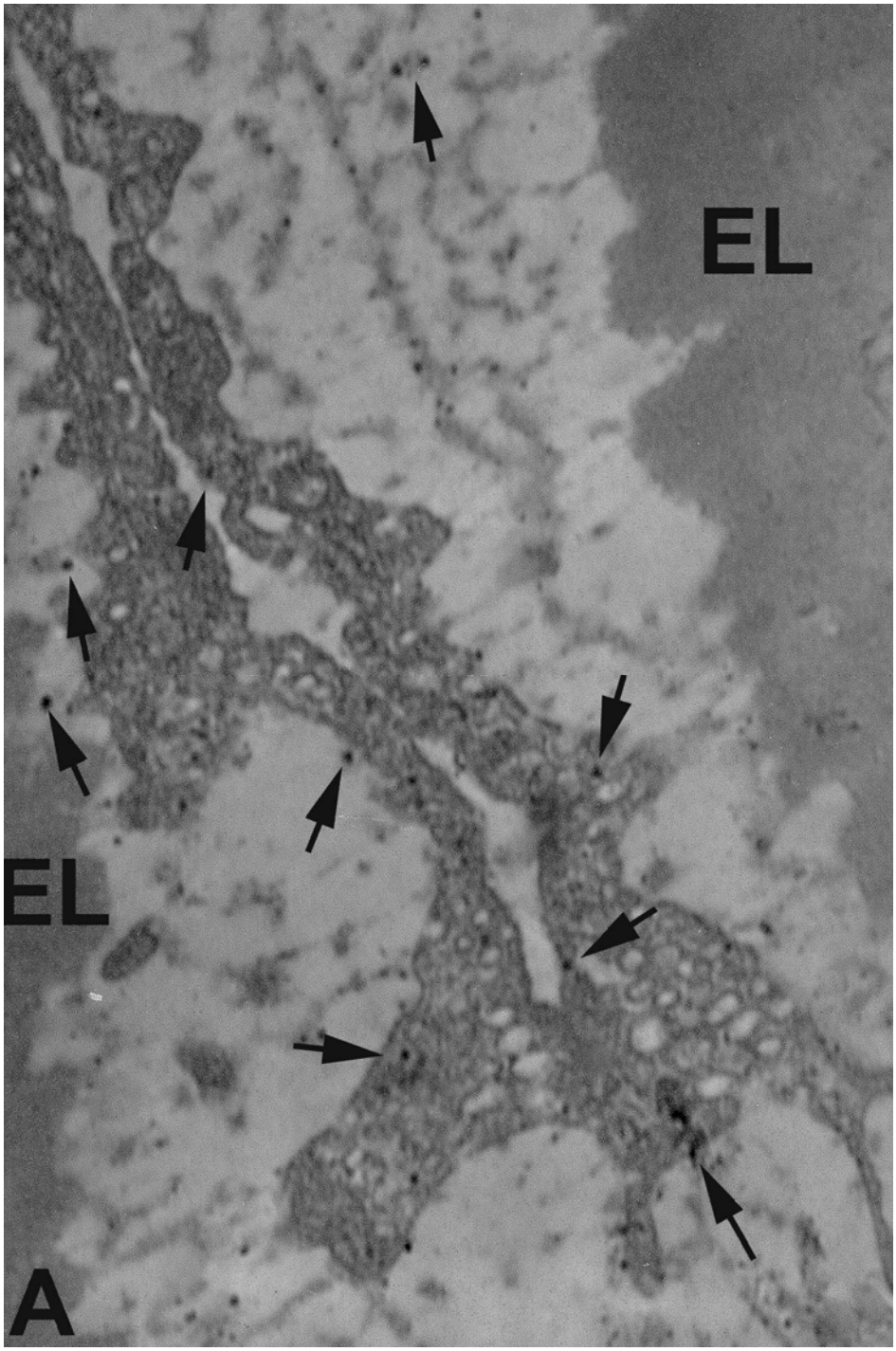


Fig 4.19A Immunogold labeling of syntaxin-4 in a pulmonary artery endothelial cell. Arrows denote position of gold particles. EL = elastic lamina. Magnification x 36,000.

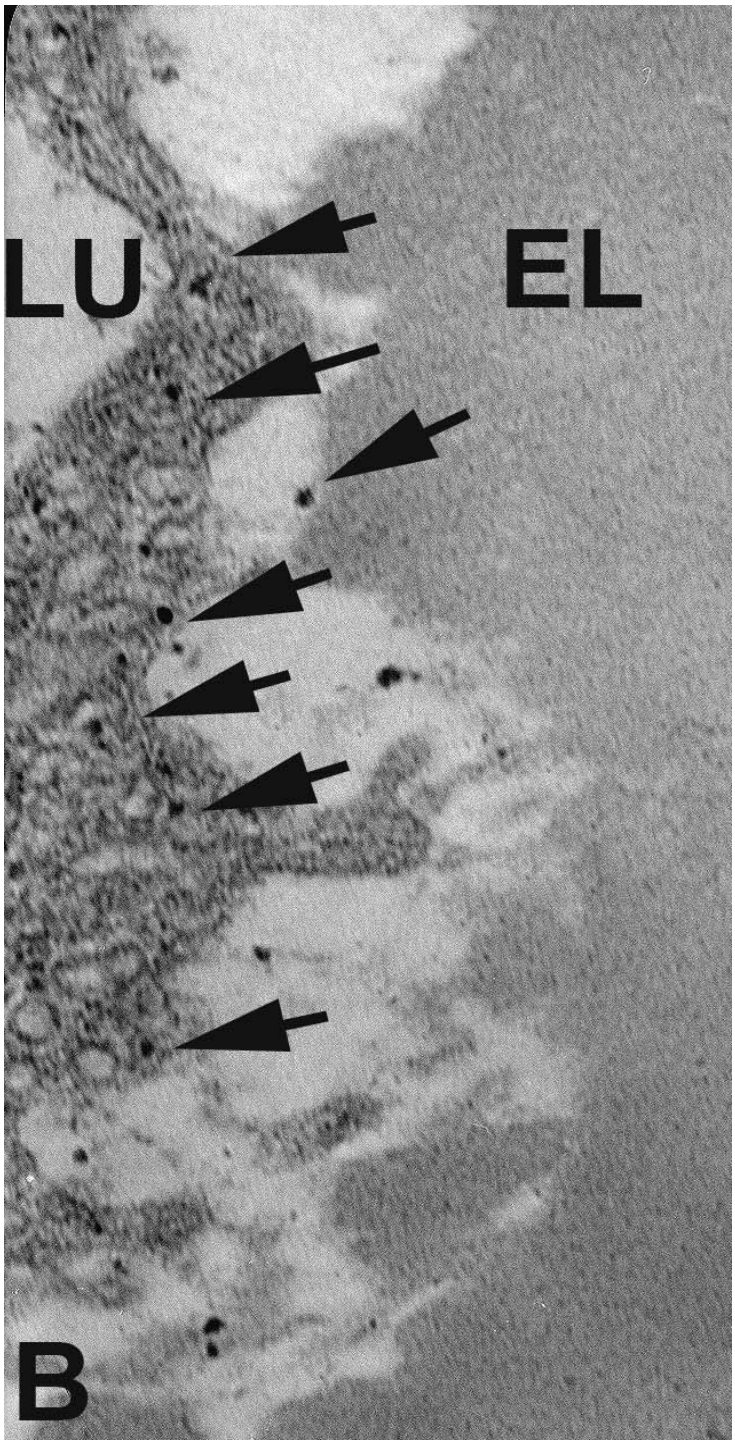


Fig 4.19B Immunogold labeling of syntaxin-4 in a mesenteric artery endothelial cell. Arrows denote position of gold particles. EL = elastic lamina, LU = lumen. Magnification x 48,000.

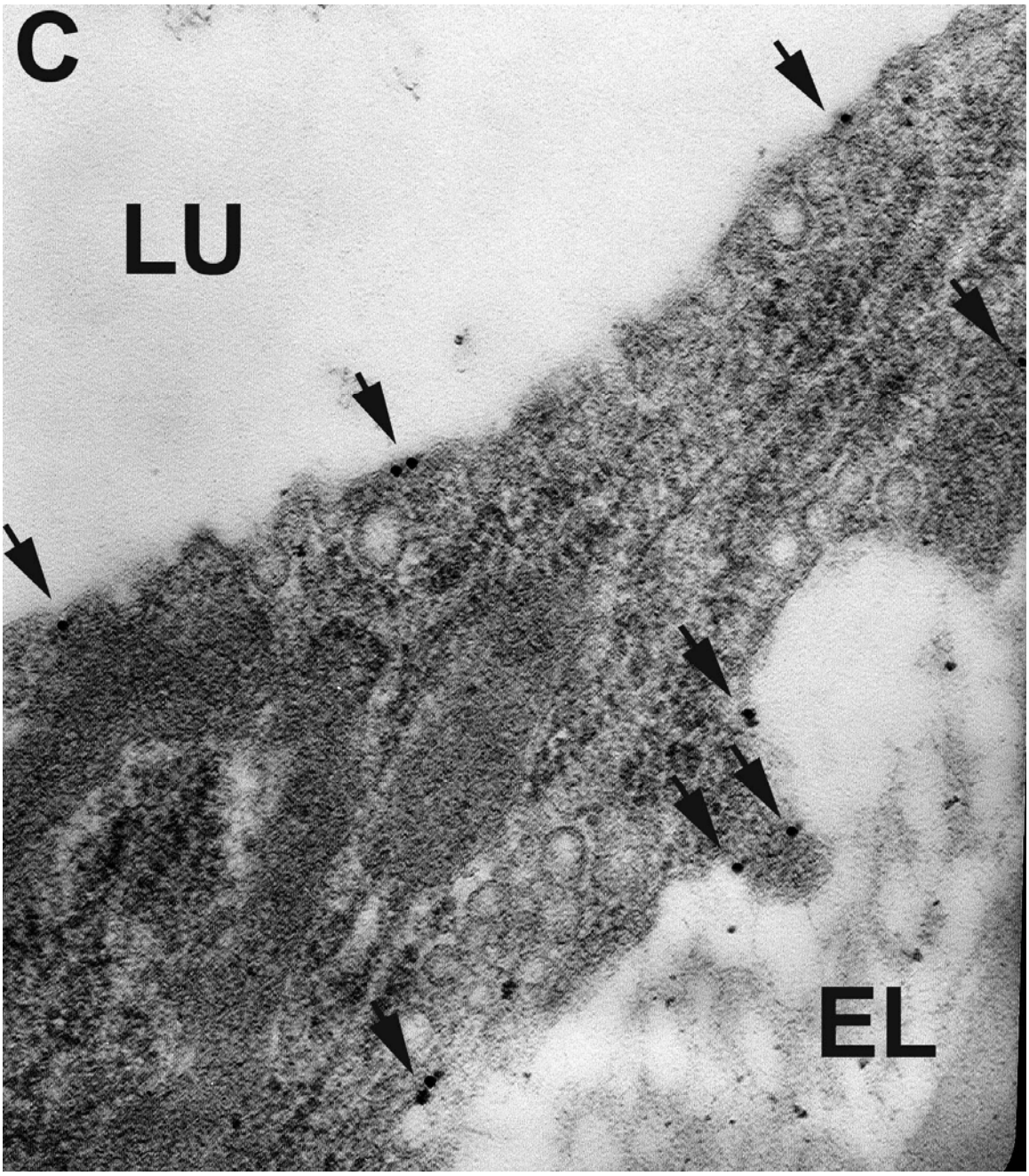


Fig 4.19C Immunogold labeling of syntaxin-4 in a femoral artery endothelial cell Arrows denote position of gold particles. LU = lumen, EL = elastic lamina. Magnification x 58,000.

Table 4.1. Summary of immunoreactivities at the light-level.

	Arteries		
Protein	Pulmonary	Mesenteric	Femoral
eNOS	heavy, 60%,	heavy, 95%	moderate, 75%
nNOS	heavy, 40%	heavy, 95%	light, 70%
CaMKII	heavy, 80%	heavy, 95%	heavy, 90-95%
VAMP-1	heavy, 60%	heavy, 95%	moderate, 40%
SNAP-25	light, 80%	light, 70%	dense, 80%
Syntaxin-4	light, 70%	heavy, 95%	light, 70%
Neurexin-1	heavy, 90%	heavy, 95%	heavy, 90%
Synaptotagmin	light, 40%	heavy, 95%	moderate, 90%
Synaptophysin	light, 80%	light, 95%	heavy, 95%
NSF	light, 30%	light, 95%	heavy, 95%
Rab3	light, 80%	moderate, 95%	moderate, 90%
Rabphillin-3A	heavy, 85%	heavy, 95%	heavy, 95%
Cd4	none	none	none

See text for abbreviations. The % signs denote the maximal percentage of endothelium in which immunoreactivity was detected.

CHAPTER 5

ACETYLCHOLINE AND HYPOXIA CAUSE MOBILIZATION OF CYTOPLASMIC VESICLES WITHIN ENDOTHELIAL CELLS OF PULMONARY, MESENTERIC AND FEMORAL ARTERIES[1]

[1]**Hashmi-Hill MP**, Shields JP, Lewis THJ, Robertson TP, Lewis SJ. Acetylcholine and hypoxia elicit vesicular exocytosis in endothelial cells of rat mesenteric, femoral and pulmonary arteries: An ultra-structural study. To be submitted for publication in *Circulation Research*, 2006

ABSTRACT

The endothelium is necessary for acetylcholine-mediated relaxation of blood vessels. Acetylcholine-induced stimulation of G protein-coupled muscarinic receptors on the plasma membrane of endothelial cells causes a marked increase in intracellular Ca^{2+} levels in these cells. This increase in intracellular Ca^{2+} would mobilize vesicles to fuse to the plasma membrane thereby releasing vasoactive factors stored within these vesicles. Hypoxia is another stimulus that elicits vesicular exocytosis although it is highly controversial as to whether this exocytosis is Ca^{2+}-*dependent* or Ca^{2+}-*independent* exocytosis.

The first ***objective*** of this ultrastructural study was to demonstrate that the application of acetylcholine to isolated rat small pulmonary, mesenteric and femoral arteries mobilizes cytoplasmic vesicles in endothelial cells of these arteries. The second ***objective*** was to determine whether the exposure of the above arteries to hypoxia mobilizes cytoplasmic vesicles in endothelial cells. In order to achieve these objectives we used small vessel wire myography followed by transmission electron microscopy.

Acetylcholine elicited a pronounced relaxation that was associated with a substantial increase in the numbers of vesicles fused with the endothelial cell membranes of pulmonary, mesenteric and femoral arteries. Hypoxia elicited a pronounced vasoconstriction in isolated pulmonary arteries and a substantial dilation in mesenteric and femoral arteries. These responses were associated

with a substantial increase in the numbers of vesicles fused with the endothelial cell membranes of all arteries.

These novel findings demonstrate that acetylcholine and hypoxia elicit pronounced mobilization and fusion of cytoplasmic vesicles to the plasma membranes of vascular endothelial cells.

Keywords: Endothelial cells, Exocytosis, Vesicles

INTRODUCTION

Furchgott and Zawadzski (1980) were the first to demonstrate that acetylcholine-induced relaxation was dependent on an intact endothelium. In large conduit arteries, there is substantial evidence that the non-prostanoid endothelium-derived relaxing factor (EDRF) is nitric oxide (NO) (Ignarro et al., 1987; Palmer et al., 1987; Moncada et al., 1991; Moncada and Higgs, 2006). Subsequent studies identified a catalytically-self contained P_{450} enzyme, NO synthase (NOS), which converts L-arginine to NO and the by-product, L-citrulline (see Moncada et al., 1991; Moncada and Higgs, 2006). Pretreatment of large conduit arteries with competitive NOS inhibitors such as N^G-L-arginine methyl ester have been consistently found to markedly attenuate endothelium-dependent relaxation (see Moncada et al., 1991; Moncada and Higgs, 2006). Moreover, pretreatment of these arteries with compounds that interfere with the intracellular mechanisms by which NO relaxes vascular smooth muscle, including inhibitors of soluble guanylate cyclase or cGMP-dependent protein kinase (PKG), markedly attenuate endothelium-dependent relaxation elicited by agonists such as acetylcholine or bradykinin (see Moncada et al., 1991; Moncada and Higgs, 2006).

Unlike large arteries, the pre-treatment of smaller and especially-resistance sized arteries with competitive NOS inhibitors or compounds that interfere with the intracellular mechanisms by which NO relaxes vascular smooth muscle, only ***minimally*** affect endothelium-*dependent* relaxation *in vivo* (see

Davisson et al., 1996; Travis et al., 2000; Woodman et al., 2000; Lewis et al., 2005a,b,c, 2006a,b,c) or *in vitro* (see Danser et al., 1998, 2000; Tom *et al*., 2001; Kakuyama et al., 1998; Batenburg et al., 2004a,b). These findings clearly suggest that the endothelium of small arteries synthesize and release an EDRF and/or an endothelium-derived hyperpolarizing factor (EDHF) (see Vanhoutte, 1993; McGuire et al., 2001; Busse et al., 2002) that is ***not*** NO *per se*.

Vascular endothelial cells and especially those in smaller arteries contain large numbers of cytoplasmic vesicles (Bruns et al., 1968a,b; Bundgaard et al., 1979; Mazzone & Kornblau, 1980; Wagner Casey-Smith, 1981; Huttner and Gabbiani, 1983; Loesch, 1993, 1994, 1996; Lane et al., 1995; Moldovan et al., 1995). Moreover, large numbers of these vesicles contain NOS in their cytoplasmic membranes (Loesch, 1993, 1994). The presence of NOS would provide a mechanism by which NO could be generated. However, the presence of NOS on vesicular membranes would also provide the mechanism by which NO-containing factors could be generated and ultimately stored in vesicles.

S-nitrosothiols are endogenous NO-containing factors in cardiovascular tissues (Muller *et al*., 1996, 2002; Vanin *et al*., 1998; Malyshev *et al*., 1999; Manukhina *et al*., 1999; Smirin *et al*., 1999, 2000; Pshennikova *et al*., 2000), brain (Kluge *et al*., 1997), and red blood cells (Jia *et al*., 1996). L-S-nitrosocysteine, which is a highly polar and lipophobic S-nitrosothiol, (Kowaluk and Fung, 1990), is a putative EDRF (Myers et al., 1990; Rubanyi et al., 1991;

Rosenblum, 1992) and EDHF (Batenburg et al., 2004a,b). Recent findings that this S-nitrosothiol exists in high concentrations in cytoplasmic vesicles in endothelial cells (Lewis et al., 2006a) supports evidence that endothelium-dependent vasodilation involves Ca^{2+}-*dependent* exocytosis (i.e., mobilization and fusion of cytoplasmic vesicles to plasma membranes) of preformed vesicular pools of S-nitrosothiols (Ignarro, 1990; Loesch et al., 1993, 1994; Davisson et al., 1996; Danser et al., 1998, 2000; Tom *et al*., 2001). In addition, repetitive applications of endothelium-dependent agonists elicit progressively smaller vasodilator responses *in vivo* and in isolated artery preparations in the presence of NOS inhibitors (Davisson *et al*., 1996; Danser *et al*., 1998; Tom *et al*., 2001). These findings are consistent with these agonists eliciting a use-dependent depletion of vesicular stores of S-nitrosothiols that cannot be replenished in the absence of NO synthesis.

NADPH-diaphorase is used as a marker for identification of NOS (Dawson et al., 1991; Hope et al., 1991). NADPH-diaphorase is the process whereby nitroblue tetrazolium is enzymatically reduced to diformazan (a blue precipitation) through the transfer of two electrons. It was assumed that the two electrons were donated by NOS. However, fixation of brain with 4% paraformaldehyde abolishes NOS activity in both particulate and cytosolic fractions (Matsumoto et al., 1993). Moreover, although fixation abolishes NADPH diaphorase in the particulate fraction, 50-60% of NADPH diaphorase remains in the cytosol (Matsumoto et al., 1993) and is heavily localized in cytoplasmic vesicles (Loesch et al., 1993, 1994).

Chayen *et al* (1994) provided a comprehensive series of arguments as to why NOS is not responsible for NADPH diaphorase in aldehyde-treated tissues. One important point raised by Chayen et al (1994) was that α-NADPH is as effective as β-NADPH in promoting reduction of NBT. Since α-NADPH will not donate electrons to NOS, it is unlikely that diformazan arises from the catalytic activity of NOS. We have recently demonstrated that NADPH diaphorase histochemistry actually detects preformed pools of S-nitrosothiols including L-S-nitrosocysteine.

The possibility that endothelium-dependent vasodilation is due to the release of vesicular stores of S-nitrosothiols is also supported by evidence that (1) inhibition of ATP synthesis, which reduces Ca^{2+}-*dependent* exocytosis in nerve terminals, markedly reduces endothelium-dependent relaxation (Griffith et al., 1986; Weir et al., 1991; Richards et al., 1991), (2) bradykinin causes NOS to translocate to the plasma membranes of endothelial cells (Michel et al., 1993), consistent with fusion of NOS-positive cytoplasmic vesicles to plasma membranes, (3) plasma membranes of endothelial cells contain fusion proteins that promote vesicular exocytosis (Schnitzer et al., 1995; Chapter 4), (4) the membranes of cytoplasmic vesicles in endothelial cells have fusion proteins and Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII), which support Ca^{2+}-*dependent* vesicular exocytosis (Chapter 4), (5) calmodulin antagonists inhibit endothelium-dependent relaxation by reducing the release of rather than the mechanisms of action of EDHFs (Illiano et al., 1992, 1993; Nagao et al., 1992), suggesting that, similar to other secretory cells (Thureson-Klein & Klein, 1990;

Greengard et al., 1993; Thomas-Reetz and Camili, 1994; Sudhof, 1995), a Ca^{2+}-calmodulin complex may mobilize S-nitrosothiol-containing vesicles in endothelial cells, and (6) known inhibitors of mitochondrial electron transport, F1-ATPase or oxidative phosphorylation, which block vesicular exocytosis in nerve terminals and adrenal chromaffin cells (Thureson-Klein and Klein, 1990), also markedly inhibit endothelium-dependent relaxations (Griffith et al., 1986; Richards et al., 1991; Wei et al., 1991).

Because of the potential importance of vesicular stores of S-nitrosothiols in the regulation of vascular tone (Myers et al., 1990; Davisson et al., 1996; Danser et al., 1998, 2000; Tom *et al*., 2001; Kakuyama et al., 1998; Batenburg et al., 2004a,b), our first ***objective*** was to determine whether acetylcholine elicits the exocytotic mobilization of cytoplasmic vesicles in endothelial cells of isolated rat small pulmonary, mesenteric and femoral arteries. Acetylcholine exerts its effects on endothelial cells via G protein-coupled muscarinic receptors, which elicit phospholipase C-mediated increases in intracellular Ca^{2+} (see Luscher and Vanhoutte, 1990). The ***concept*** to be addressed is that acetylcholine elicits Ca^{2+}-dependent exocytosis of vesicles that express Ca^{2+}-signaling proteins such as CaMKII. As will be described below, the arteries were mounted on small vessel myographs and exposed to acetylcholine (or hypoxia) and then immersion-fixed *in situ* during the sustained vasorelaxation phase. Time-matched control arteries (i.e., those that did not receive acetylcholine) were also immersion fixed.

Hypoxia elicits a pronounced endothelium-dependent vasodilation in systemic arteries (see Minkes et al., 1995) whereas it elicits pronounced vasoconstriction in pulmonary arteries (Von Euler and Liljestrand, 1946). This hypoxic pulmonary vasoconstriction (HPV), allows the pulmonary circulation to divert blood flow away from hypoxic regions to normoxic areas thereby preserving ventilation-perfusion. The vasoconstriction is characterized by two phases (Kovitz et al., 1993; Leach et al., 1994, 2000; Robertson et al., 1995; 2000a,b,c, 2001, 2003; Lazor et al., 1996; Dipp et al., 2001; Dipp and Evans, 2001). Phase I consists of a transient rise in tension, while phase II consists of a more slowly developing and sustained constriction, which is due to the release of an unidentified endothelium-derived contracting factor (EDCF) (Robertson et al., 2003).

ATP is essential for Ca^{2+}-dependent exocytosis since it is required by CaMKII (Greengard et al., 1993). ATP production decreases during hypoxia due to the O_2-dependency of the respiratory chain (see Moro et al., 2005). Although hypoxia reduces ATP/Ca^{2+}-*dependent* exocytosis in nerve terminals, the decrease in ATP elicits the mobilization of another pool of cytoplasmic vesicles (see Greengard et al., 1993). Moreover, relatively minor falls in tissue O_2 markedly reduces NOS activity (Abu-Soud et al., 1996). The second ***objective*** of this study was to determine whether hypoxia elicits the exocytotic mobilization of cytoplasmic vesicles in endothelial cells of isolated rat small pulmonary, mesenteric and femoral arteries mounted on wire mographs. The ***concept*** to be addressed is that hypoxia elicits Ca^{2+}-*independent* exocytosis of (1) S-

nitrosothiol-containing vesicles in the endothelium of mesenteric and femoral arteries, and (2) vesicles that contain the HPV factor and those that contain S-nitrosothiols (to limit the expression of HPV) in the endothelium of pulmonary arteries.

MATERIALS AND METHODS

Animals

All studies were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) revised in 1996. The protocols pertaining to the use of animals were approved by the Animal Care and Use Committee of the University of Georgia. Adult male Sprague-Dawley rats (≈300g) were used in these studies.

Acetylcholine–induced mobilization studies

Male Sprague-Dawley rats were decapitated and small femoral, mesenteric and pulmonary arteries were dissected and mounted as ring preparations on small vessel myographs in physiological saline solution at 37°C (Robertson et al., 1995, 2000a,b,c; 2001, 2003). Maximum tension was determined by applying a series of stretches and depolarizing concentrations of K^+ (Robertson et al., 1995, 2000a,b,c; 2001, 2003). The arteries were then preconstricted to 50% maximum by the addition of the α_1-adrenoceptor agonist, phenylephrine (1 μM). Once a stable baseline had been established, acetylcholine (1 μM) was applied to the baths to elicit a sustained endothelium-

dependent relaxation. At the maximum fall in tension, which occurred within 30-60 sec, the rings were immersion fixed *in situ* with 4% paraformaldehyde and 0.2% glutaraldehyde (in 0.1M phosphate buffer, pH7.4). The arteries were transferred to phosphate buffer and stored overnight at 4°C. In order to establish the resting status of vesicles under these conditions, other arteries did not receive acetylcholine. These control arteries were immersion fixed at the point corresponding to that when the arteries exposed to acetylcholine were immersion fixed.

Hypoxia-induced mobilization

Male Sprague-Dawley rats were decapitated and small femoral, mesenteric and pulmonary arteries (200µm diameter) were dissected and mounted as ring preparations on small vessel myographs in physiological saline solution (PSS) at 37°C. The arteries were then preconstricted to 50% max with phenylephrine (1µM) under normoxic conditions (21% O_2). Hypoxia was induced by changing the oxygen gas supply to low oxygen (1% O_2). At the point of maximum fall in tension in mesenteric and femoral arteries, the rings were immersion fixed *in situ.* In order to establish the resting status of vesicles under these conditions, other arteries maintained under normoxic conditions were immersion fixed at the point corresponding to that when the arteries exposed to hypoxia were immersion fixed. At the time the Phase II increases in tension in pulmonary arteries (HPV) reached plateau levels, the rings were immersion fixed *in situ*. Again, other arteries maintained under normoxia were immersion fixed at

the time corresponding to that when the arteries exposed to hypoxia were immersion fixed.

Transmission electron microscopy

Arteries were post-fixed in 1% osmium tetroxide (OsO_4) for 1h at room temperature and dehydrated in a graded ethanol series, followed by a graded acetone series. The arteries were infiltrated, embedded, and polymerized for 24h at 60°C in Embed 812 epoxy resin (Polysciences, Warrington, PA, USA) and silver sections were cut on an ultramicrotome (RMC MT-X, Tucson, AZ, USA). The sections were post-stained with uranyl acetate and lead citrate and viewed on a transmission electron microscope (model 100CX II, JEOL USA, Peabody, MA) in the *Ultra-Structural Research Facility* at the University of Georgia. The transmission electron microscope was set to operate at 80 KeV.

Measurements and Statistical Analysis

Each experiment was repeated on artery sections from three rats. The total numbers of cytoplasmic vesicles and those fused to the plasma membranes were counted in all sections of each artery (50 sections) under the transmission electron microscope. The %mobilization of vesicles elicited by acetylcholine or hypoxia was evaluated by expressing the number of fused vesicles as a percentage of the overall number of cytoplasmic vesicles.

The data are presented as mean ± SEM. All data were initially submitted to tests of normality and homogeneity of variances as described by Winer (1971). All data proved to be normal and so the data were analyzed by repeated-measures analysis of variance (Winer, 1971) followed by Student's modified t-test with the Bonferroni correction for multiple comparisons between means using the error mean square terms from the repeated measures analysis of variance (see Wallenstein *et al*., 1980). A value of $P < 0.05$ was taken to denote statistical significance between the means.

RESULTS

A. Acetylcholine-induced changes in resting tension

A typical example of the vasorelaxant effects of acetylcholine in an endothelium-intact femoral artery ring that was pre-constricted by phenylephrine (1 μM), is shown in the bottom trace of Fig 5.1. The addition of phenylephrine elicited a rapid and sustained increase in tension (i.e., vasocontraction). Subsequent application of acetylcholine (1 μM) elicited an immediate reduction in resting tension (i.e., vasorelaxation) that reached plateau levels within 30-60 seconds. The application of phenylephrine to another femoral artery ring elicited a very similar increase in tension (see Fig. 5.1, top trace). The time points at which this femoral artery ring and the femoral artery ring that was exposed to acetylcholine, was immersion fixed *in situ* with 4% paraformaldehyde and 0.2% glutaraldehyde is denoted by the dotted line. As can be seen, the immersion fixation was performed at exactly the same time-point in both arteries. The

fixation procedure elicited a minimal change in tension in the artery exposed to acetylcholine and a greater but still minimal increase in tension in the control artery.

B. Acetylcholine-induced mobilization of vesicles

Pulmonary Artery

Typical examples of electron micrographs of pulmonary endothelial cells from control arteries and those exposed to acetylcholine are shown in Fig 5.2A and Fig 5.2B, respectively. Under control conditions, fused vesicles (i.e., those fused with the plasma membrane of the endothelial cell) were located on both the luminal and abluminal membrane of the cell (Fig. 5.2A). Acetylcholine elicited a substantial increase in the number of fused vesicles (Fig. 5.2B). Fused vesicles were observed on the luminal and abluminal membranes of the endothelial cell and also on the membranes facing adjacent endothelial cells. A summary of the %mobilization of cytoplasmic vesicles (number of fused vesicles expressed as a percentage of the total numbers of cytoplasmic vesicles) in control arteries and in those exposed to acetylcholine are summarized in Fig. 5.3. The %mobilization of vesicles in control endothelial cells was 4.4 ± 0.5%. As can be seen, acetylcholine elicited a pronounced increase in the numbers of vesicles fused to plasma membranes. The %mobilization of vesicles in endothelial cells exposed to acetylcholine was 38.2 ± 1.7% ($P < 0.05$, compared to control cells).

Mesenteric Artery

Typical examples of electron micrographs of mesenteric endothelial cells from control arteries and those exposed to acetylcholine are shown in Fig 5.4A and Fig 5.4B, respectively. Under control conditions, fused vesicles (i.e., those fused with the plasma membrane of the endothelial cell) were located on both the luminal and abluminal membrane of the cell (Fig. 5.4A). Acetylcholine elicited a substantial increase in the number of fused vesicles (Fig. 5.4B). Fused vesicles were observed on both the luminal and abluminal membranes of the endothelial cell and also on membranes facing adjacent endothelial cells. A summary of the %mobilization of cytoplasmic vesicles (number of fused vesicles expressed as a percentage of the total numbers of cytoplasmic vesicles) in control arteries and in those exposed to acetylcholine are summarized in Fig. 5.5. The %mobilization of vesicles in control endothelial cells was 6.3 ± 0.4%. Acetylcholine elicited a pronounced increase in the numbers of vesicles fused to plasma membranes. The %mobilization of vesicles in endothelial cells exposed to acetylcholine was 27.6 ± 2.4% ($P < 0.05$, compared to control cells).

Femoral Artery

Typical examples of electron micrographs of femoral endothelial cells from control arteries and those exposed to acetylcholine are shown in Fig 5.6A and Fig 5.6B, respectively. Under control conditions, fused vesicles (i.e., those fused with the plasma membrane of the endothelial cell) were located on both the luminal and abluminal membrane of the cell (Fig. 5.6A). Acetylcholine elicited a

substantial increase in the number of fused vesicles (Fig. 5.6B). Fused vesicles were observed on both the luminal and abluminal membranes of the endothelial cell and also on membranes facing adjacent endothelial cells. However, there was more of a tendency for fused vesicles to be located on luminal membranes. A summary of the %mobilization of cytoplasmic vesicles (number of fused vesicles expressed as a percentage of the total numbers of cytoplasmic vesicles) in control arteries and in those exposed to acetylcholine are summarized in Fig. 5.7. The %mobilization of vesicles in control endothelial cells was 5.7 ± 0.5%. Acetylcholine elicited a pronounced increase in the numbers of vesicles fused to plasma membranes. The %mobilization of vesicles in endothelial cells exposed to acetylcholine was 23.4 ± 1.7% ($P < 0.05$, compared to control cells).

C. Hypoxia-induced changes in resting tension

A typical example of the effects of hypoxia on resting tone of a pulmonary artery ring that was preconstricted with phenylephrine is shown in Fig. 5.8A. As can be seen, hypoxia elicited an initial transient ***increase*** in resting tension (Phase I vasocontraction) that was followed by what would have been a sustained Phase II *increase* in resting tension if the artery ring was not immersion fixed. Similar responses were observed in all pulmonary artery rings examined. Immersion fixation caused minor initial changes in resting tension in this and all pulmonary artery rings. Resting tone diminished as a result of the fixation due to the loss of contractility of the vascular smooth muscle.

A typical example of the effects of hypoxia on the resting tone of a femoral artery ring that was preconstricted with phenylephrine is shown in Fig. 5.8B. In contrast to the pulmonary artery rings, hypoxia elicited a sustained ***decrease*** in resting tension (i.e., vasorelaxation) in this and all other femoral artery rings. Immersion fixation elicited transient increases in tension in this and all other femoral arteries. Again, resting tone diminished as a result of the fixation due to the loss of contractility of the vascular smooth muscle.

In addition, the effects of hypoxia and immersion-fixation on the resting tension of all mesenteric artery rings that were pre-constricted with phenylephrine were virtually identical to those described for the femoral artery rings (data not shown).

D. Hypoxia-induced mobilization of vesicles

Pulmonary Artery

Typical examples of electron micrographs of pulmonary endothelial cells from control arteries and those exposed to hypoxia are shown in Fig 5.9A and Fig 5.9B, respectively. Under control conditions, fused vesicles (i.e., those fused with the plasma membrane of the endothelial cell) were located on both the luminal and abluminal membrane of the cell. Hypoxia elicited a substantial increase in the number of fused vesicles. Again, fused vesicles were observed on the luminal and abluminal membranes of the endothelial cells. A summary of the %mobilization of cytoplasmic vesicles in control arteries and in those

exposed to hypoxia are summarized in Fig. 5.10. The %mobilization of vesicles in control endothelial cells was 3.9 ± 0.3%. As can be seen, hypoxia elicited a pronounced increase in the numbers of vesicles fused to plasma membranes. The %mobilization of vesicles in endothelial cells exposed to hypoxia was 16.3 ± 0.6% ($P < 0.05$, compared to control cells).

Mesenteric Artery

Typical examples of electron micrographs of mesenteric endothelial cells from control arteries and those exposed to hypoxia are shown in Fig 5.11A and Fig 5.11B, respectively. Under control conditions, fused vesicles (i.e., those fused with the plasma membrane of the endothelial cell) were located on both the luminal and abluminal membrane of the cell. Hypoxia elicited a substantial increase in the number of fused vesicles. Fused vesicles were observed on both the luminal and abluminal membranes of the endothelial. A summary of the %mobilization of cytoplasmic vesicles in control arteries and in those exposed to hypoxia are summarized in Fig. 5.12. The %mobilization of vesicles in control endothelial cells was 6.1 ± 0.5%. Hypoxia elicited a pronounced increase in the numbers of vesicles fused to plasma membranes. The %mobilization of vesicles in endothelial cells exposed to hypoxia was 23.5 ± 1.9% ($P < 0.05$, compared to control cells).

Femoral Artery

Typical examples of electron micrographs of femoral endothelial cells from control arteries and those exposed to hypoxia are shown in Fig 5.13A and Fig 5.13B, respectively. Under control conditions, fused vesicles (i.e., those fused with the plasma membrane of the endothelial cell) were located on both the luminal and abluminal membrane of the cell (Fig. 5.13A). Hypoxia elicited a substantial increase in the number of fused vesicles (Fig. 5.13B). Fused vesicles were observed on both the luminal and abluminal membranes of the endothelial.

A summary of the %mobilization of cytoplasmic vesicles (number of fused vesicles expressed as a percentage of the total numbers of cytoplasmic vesicles) in control arteries and in those exposed to hypoxia are summarized in Fig. 5.14. The %mobilization of vesicles in control endothelial cells was 5.7 ± 0.4%. Hypoxia elicited a pronounced increase in the numbers of vesicles fused to plasma membranes. The %mobilization of vesicles in endothelial cells exposed to hypoxia was 23.9 ± 1.3 % ($P < 0.05$, compared to control cells).

DISCUSSION

Fusion of cytoplasmic vesicles under control conditions

The present study demonstrates that a small proportion of vesicles were fused to the plasma membranes of endothelial cells of control rat small pulmonary, mesenteric and femoral arteries (i.e., those maintained under normoxic conditions and in the absence of acetylcholine). The small numbers of fused vesicles suggests that Ca^{2+}-dependent exocytotic processes were minimal

in these artery rings in which a sustained preconstriction was established with the α_1-adrenoceptor agonist, phenylephrine. More specifically, these arteries were not subject to phasic sheer stress that is induced by changes in pulse pressure *in vivo* or endothelium-dependent agonists, which elicit increases in intracellular Ca^{2+} and Ca^{2+}-calmodulin-*dependent* exocytosis. Whether the fusion processes in the unstimulated rings is a form of uncontrolled release or a constitutive form of exocytosis remains to be determined. It should be noted that almost all secretory cells exhibit a basal exocytosis. In rat parotid cells (Castle et al., 1996; Huang et al., 2001) and insulin-secreting cells (Arvan et al., 1991; Turner et al., 2000), some secretory proteins are sequestered away from secretory granules during their biogenesis so that constitutive secretory vesicles can access them for basal release (Arvan and Castle, 1998).

The relative numbers of fused vesicles in endothelial cells from the control mesenteric and femoral arteries were similar to one another. Interestingly, the relative numbers of fused vesicles were smaller in endothelial cells from control pulmonary arteries. Whether this reflects a relatively smaller degree of uncontrolled release or constitutive exocytosis in these pulmonary endothelial cells remains to be determined. It is important to note that the occurrence of fused vesicles in endothelial cells is not a result of fixation. Specifically, Noguchi et al (1986) demonstrated that there were almost as many fused vesicles in rapid-frozen, freeze-substituted endothelial cells, as there were in aldehyde-fixed

cells. Noguchi et al (1986) concluded that aldehydes do not induce membrane fusion, and that the distribution of vesicles was preserved by such fixation.

Acetylcholine–induced mobilization of vesicles

The application of acetylcholine to pulmonary, mesenteric and femoral arteries resulted in pronounced and equivalent vasorelaxant responses and substantial increases in the number of cytoplasmic endothelial vesicles that were fused to the endothelial cell membrane. The increase in fused vesicles in the abluminal membrane would increase the availability of vasoactive vesicle contents such as S-nitrosothiols (see Lewis et al., 2006a) to the closely associated vascular smooth muscle. The number of fused vesicles was also increased in the luminal membranes of the endothelial cells, which suggests that vesicle contents of vasoactive factors are released into the blood to affect down-stream vascular structures. Interestingly, the number of fused vesicles was also increased in the membranes that were adjacent to other endothelial cells. This finding raises the intriguing possibility that the released vesicle contents may regulate the functional status (e.g., resting membrane potential, resting status of vesicular exocytosis) of adjacent endothelial cells. Acetylcholine elicited a relatively greater fusion of cytoplasmic vesicles in pulmonary endothelial cells than in mesenteric or femoral endothelial cells. This may be due to a multiplicity of mechanisms including the relatively greater sensitivity of pulmonary cytoplasmic vesicles to increases in intracellular levels of Ca^{2+} (see Greengard et al., 1993).

We previously demonstrated the presence of SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) proteins in endothelial cells of rat small pulmonary, mesenteric and femoral arteries (see Chapter 3). For example, we detected SNAP-25 (synaptosomal-associated protein of 25KDa) and syntaxin in the endothelial cell membrane and VAMP-1 (vesicle-associated membrane protein-1) on the membranes of cytoplasmic vesicles. Moreover, we have obtained evidence that acetylcholine increases the association of VAMP-1 with the endothelial cell membranes, which is strongly suggestive of stimulated vesicular exocytosis. Taken together, these findings support a variety of indirect evidence that endothelium-dependent vasorelaxation elicited by acetylcholine involves the exocytotic release of vasorelaxant factors (see Davisson et al., 1996; Danser et al., 1998, 2000; Tom *et al*., 2001; Kakuyama et al., 1998) which may include S-nitrosothiols (see Lewis et al., 2006a).

Loesch et al (1993, 1994) demonstrated NOS exists in the membranes large numbers of cytoplasmic vesicles in endothelial cells. This provides the mechanism by which S-nitrosothiols are synthesized and ultimately stored within the vesicles (see Lewis et al., 2006a). It would seem possible that S-nitrosothiol-containing vesicles are mobilized to exocytosis via the increases in intracellular Ca^{2+} levels elicited by shear stress and endothelium-dependent agonists (Furchgott and Vanhoutte, 1989; Ignarro, 1990; Luscher and Vanhoutte, 1990; Moncada et al., 1991). Numerous pathological states including hypertension are

associated with endothelial dysfunction and reduced endothelium-dependent vasodilation (see Ludmer et al., 1986; Kojda and Harrison, 1999; Taddei and Salvetti 2002). In addition, deletion of the endothelial NOS gene in mice results in hypertension (Huang et al., 1995) whereas long-term inhibition of NOS induces progressive hypertension and perivascular fibrosis in experimental animal models (Bayliss et al., 1992; Ribeiro et al., 1992; Zatz and Baylis, 1998).

On the basis of the above observations, one possible mechanism underlying reduced endothelium-dependent vasodilation in hypertensive states would be the down-regulation of NOS and/or reduced formation/storage of S-nitrosothiols in cytoplasmic vesicles in endothelial cells. However, it is known that endothelial NOS activity is actually ***increased*** in many forms of hypertension (see Leclercq et al., 2002; Sullivan et al., 2003; Forstermann and Munzel, 2006). This raises the intriguing and yet unexplored possibility that endothelium dysfunction in hypertension involves the ***down-regulation*** of CaMKII and/or fusion proteins that mediate Ca^{2+}-*dependent* exocytosis of S-nitrosothiol-containing vesicles in response to sheer stress and endothelium-dependent agonists.

Hypoxia-induced mobilization of vesicles

Systemic arteries such as the femoral and mesenteric arteries dilate in response to hypoxia in an attempt to increase blood flow and oxygen delivery to hypoxic tissues (see Minkes et al., 1995). In contrast, hypoxia elicits a pronounced

constriction in pulmonary arteries (Kovitz et al., 1993; Leach et al., 1994, 2000; Robertson et al., 1995; 2000a, 2000b, 2003; Lazor et al., 1996; Dipp et al., 2001; Dipp and Evans, 2001) to allow the pulmonary circulation to divert blood flow from hypoxic regions to normoxic areas thereby preserving ventilation-perfusion (Von Euler and Liljestrand, 1946). This HPV is characterized by two phases, namely an initial transient rise in tension (Phase I) and a more slowly developing and sustained constriction (Phase II), which is due to the release of an endothelium-derived contracting factor (EDCF) (Robertson et al., 2003). As expected, this study found that hypoxia elicited a substantial and equivalent vasorelaxation in isolated mesenteric and femoral arteries and a biphasic increase in tension in isolated pulmonary arteries.

Hypoxia caused a substantial increase in the number of cytoplasmic endothelial vesicles that were fused to the endothelial cell membrane of pulmonary, mesenteric and femoral arteries. The increase in fused vesicles in the abluminal membrane would increase the availability of vasoactive vesicle contents such as S-nitrosothiols (see Lewis et al., 2006a) to the closely associated vascular smooth muscle. The number of fused vesicles was also increased in the luminal membranes of the endothelial cells, which suggests that vesicle contents of vasoactive factors are released into the blood to affect down-stream vascular structures. In contrast, to the effects of acetylcholine, the number of fused vesicles was not increased in the membranes that were adjacent to other endothelial cells. This raises the intriguing possibility that the intracellular

signaling processes elicited by acetylcholine and hypoxia differentially regulate site-specific vesicular exocytosis. Hypoxia elicited a relatively ***smaller*** increase in the fusion of cytoplasmic vesicles within pulmonary endothelial cells than in mesenteric or femoral endothelial cells. This may be due to the relatively reduced sensitivity of pulmonary cytoplasmic vesicles to changes in intracellular status elicited by hypoxia (see Greengard et al., 1993).

Pohl and Busse (1989) demonstrated that hypoxia stimulated the release of EDRF from rabbit aorta and femoral artery and concluded that relatively lower partial pressures of O_2 that exist in small arteries and arterioles might therefore be a physiological stimulus for continuous release of EDRF. Using recombinant proteins, Meffert et al (1996) demonstrated that S-nitrosothiols increase the formation of the core complex (VAMP/SNAP-25/syntaxin) and proposed that this increased association contributes to Ca^{2+}-*independent* vesicle release elicited by nitrosyl factors. Accordingly, reductions in the intracellular concentrations of S-nitrosothiols in endothelial cells may diminish Ca^{2+}-*independent* vesicle release. This raises the important possibility that the increased vasoconstrictor tone elicited by NOS inhibitors may involve the loss of Ca^{2+}-*independent* mobilization of vesicular pools of EDRFS and/or EDHFs.

In these studies, we did not monitor the effects of hypoxia on intracellular Ca^{2+} levels in endothelial cells. Hypoxia may increase, decrease or have no effect on these Ca^{2+} levels. Although moderate hypoxia may not result in a

measurable depletion of ATP levels it has been established that ***less*** ATP is available for exocytotic mechanisms (Lovgren and Hellstrand, 1985; Scott and Coburn, 1985). In addition, severe hypoxia is associated with a moderate reduction of ATP and substantial loss of ATP available for exocytotic mechanisms (Namm and Zucker, 1973; Hellstrand et al. 1977). Franco-Obregon and Lopez-Barneo (1996) have proposed that the O_2 sensitivity of Ca^{2+} channels in smooth muscle of rabbit cerebral, celiac, femoral, and main pulmonary arteries, and porcine coronary arteries contributes to hypoxic dilatation of systemic arteries. In addition, hypoxia elicits the hyperpolarization of vascular smooth muscle in a variety of arteries (Lombard et al., 1986; Ekmehag, 1989; Daut et al., 1990; von Beckerath et al., 1991). This would directly elicit vascular relaxation via the hyperpolarization-induced closure of voltage-sensitive Ca^{2+}-channels (see von Beckerath et al., 1991). However, it should be noted that hyperpolarization of endothelial cells would ***increase*** the driving force for Ca^{2+} in these cells (see Lewis et al., 2006c).

Finally, Aalkjaer and Lombard (1995) demonstrated that the graded increases in intracellular Ca^{2+} levels elicited by increasing doses of arginine vasopressin in rat cerebral and mesenteric small arteries was not affected by moderate or severe hypoxia. This clearly suggests that hypoxia has ***no direct*** effects on intracellular Ca^{2+} levels in these arteries. Taken together, the above findings are consistent with the possibility that hypoxia elicits vasodilation in mesenteric and femoral arteries via exocytotic release of vesicular stores of

vasodilator factors. Whether this exocytosis is due to Ca^{2+}-*dependent* or Ca^{2+}-*independent* processes is an open question that we plan to address in future studies.

On the basis of findings that our hypoxic regimen does not elicit relaxation in endothelium-denuded mesenteric or femoral arteries (unpublished observations), we suggest that hypoxic vasodilation in these arteries is mediated by the release of preformed pools of EDRFs/EDHFs. However, the sustained relaxation elicited by hypoxia in rabbit thoracic aortic is endothelium-*independent* and associated with decreased intracellular concentrations of phosphocreatine and P_i in vascular smooth muscle (Scott et al., 1987, 1989; Coburn et al., 1986). The discrepancy in these findings may be directly related to the size of the arteries under question. Specifically, because resistance arteries contain 1-2 layers of vascular smooth muscle, the endothelium is well placed to regulate vascular tone. However, the aorta is a large conduit artery that contains multiple layers of smooth muscle. Accordingly, the response of this muscle to hypoxia may depend more on intrinsic signaling mechanisms since EDRFs/EDHFs would not gain access to all smooth muscle cells and therefore promote a coordinated relaxation.

The observation that acetylcholine-mediated ***dilation*** of pulmonary arteries was associated with increased fusion of cytoplasmic vesicles supports the concept that acetylcholine elicits the release of preformed vesicular pools of

vasodilator factors such as S-nitrosothiols (see Lewis et al., 2006c). As mentioned, hypoxia elicited a pronounced ***constriction*** of the pulmonary arteries. Robertson et al (2003) demonstrated that phase II of HPV in rat pulmonary arteries is due to the release of an as yet unidentified EDCF. Our findings raise the possibility that this EDCF may be stored in vesicular endothelial cells and released upon exposure of the endothelial cells to hypoxia. Interestingly, hypoxia was a substantially weaker stimulus of vesicular exocytosis in pulmonary endothelial cells than was acetylcholine. One interpretation of this finding is that hypoxia mobilizes vesicles that contain EDCF to elicit the sustained phase of HPV whereas it minimally mobilizes the vesicles that contain vasodilator factors, in order to allow HPV to be expressed as fully as possible.

Endothelin-1 (ET-1) exists in vesicles within endothelial cells (see Harrison et al., 1995). However, despite some evidence that endothelin-1 may be the EDCF released by hypoxia in pulmonary arteries, it appears that this peptide is not responsible for the sustained constriction of phase II of HPV (Robertson et al., 2003). The sustained endothelium-dependent constriction seen during phase II of HPV (Leach et al., 1994) involves Ca^{2+} sensitization of the contractile apparatus, which is mediated by the activation of Rho kinase (Robertson et al., 2000) rather than protein kinase C (Robertson et al., 1995). Whether the mobilization of endothelial vesicles observed in this study is involved in the expression of the sustained constriction during phase II HPV remains to be determined.

CONCLUSIONS

Our findings support the ***concept*** that endothelium-dependent agonists and hypoxia elicit the release of vesicular stores of vasoactive factors to subserve the physiological adjustments necessary for arteries to respond to increases in sheer stress or hypoxia. Future studies will address whether the ability of endothelium-dependent agonists and hypoxia to mobilize cytoplasmic vesicles in endothelial cells is modified during the development of genetic and humoral forms of hypertension.

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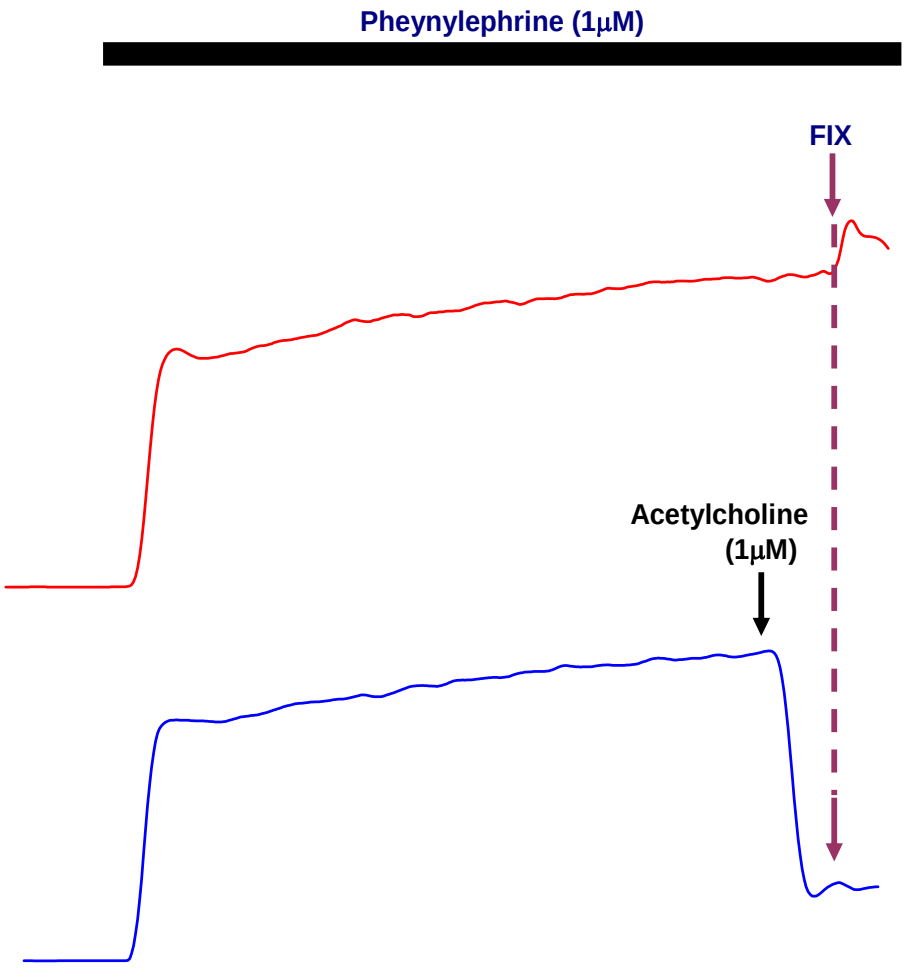

Fig. 5.1 Typical myograph trace illustrating application of acetylcholine (1μM) in the presence of pretone (1 μM phenylephrine). After application of acetylcholine (1 μM), there is a rapid fall in vessel tension, at which point arteries are fixed in situ with 4% paraformaldehyde and 0.2% glutaraldehyde and prepared for TEM. This was repeated three times in three independent experiments.

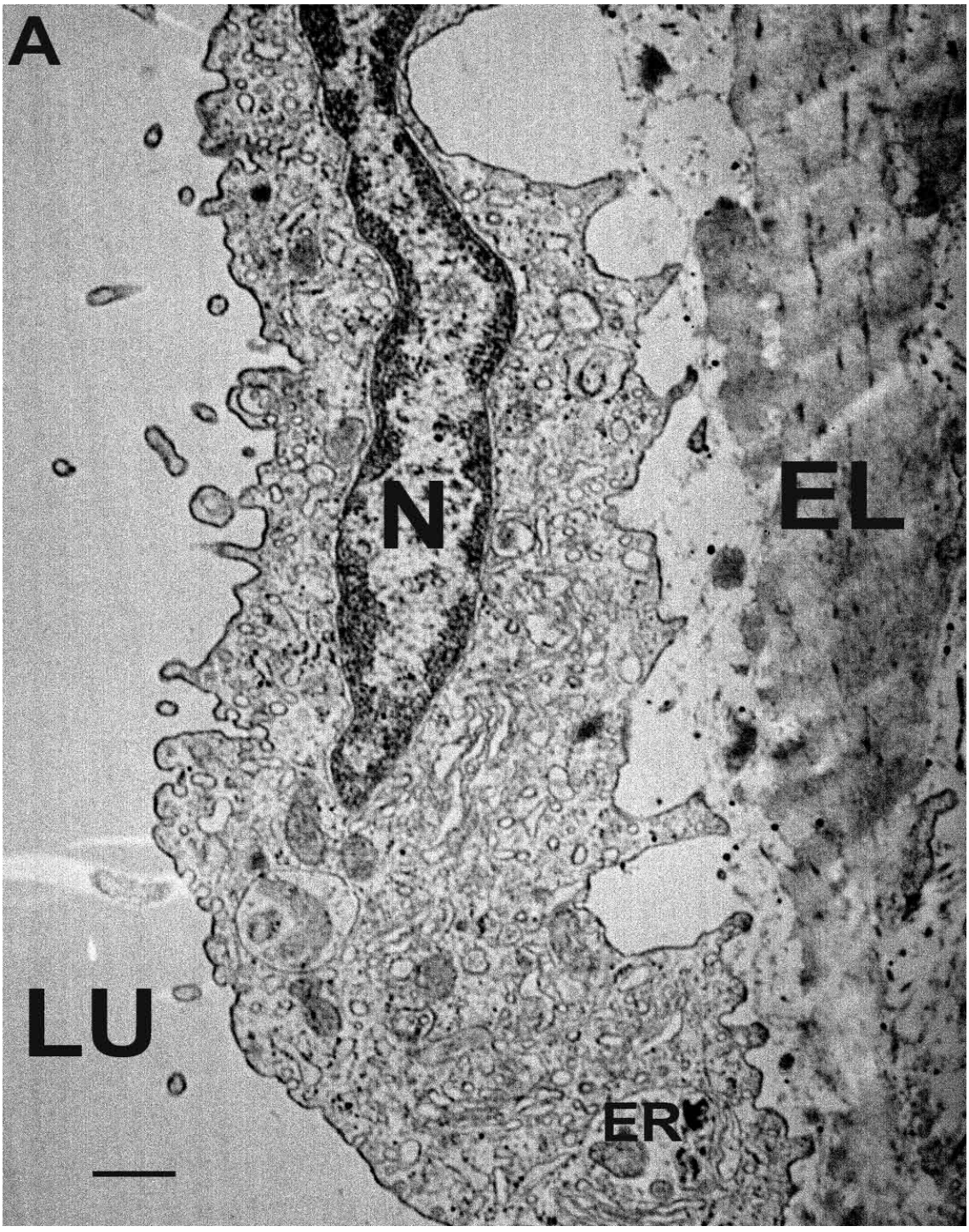

Fig. 5.2A Electron micrograph of an endothelial cell from the pulmonary artery under control conditions. LU = lumen, N = nucleus, EL = elastic lamina, ER = endoplasmic reticulum. Magnification x 10,000. Scale bar represents 0.4 µm.

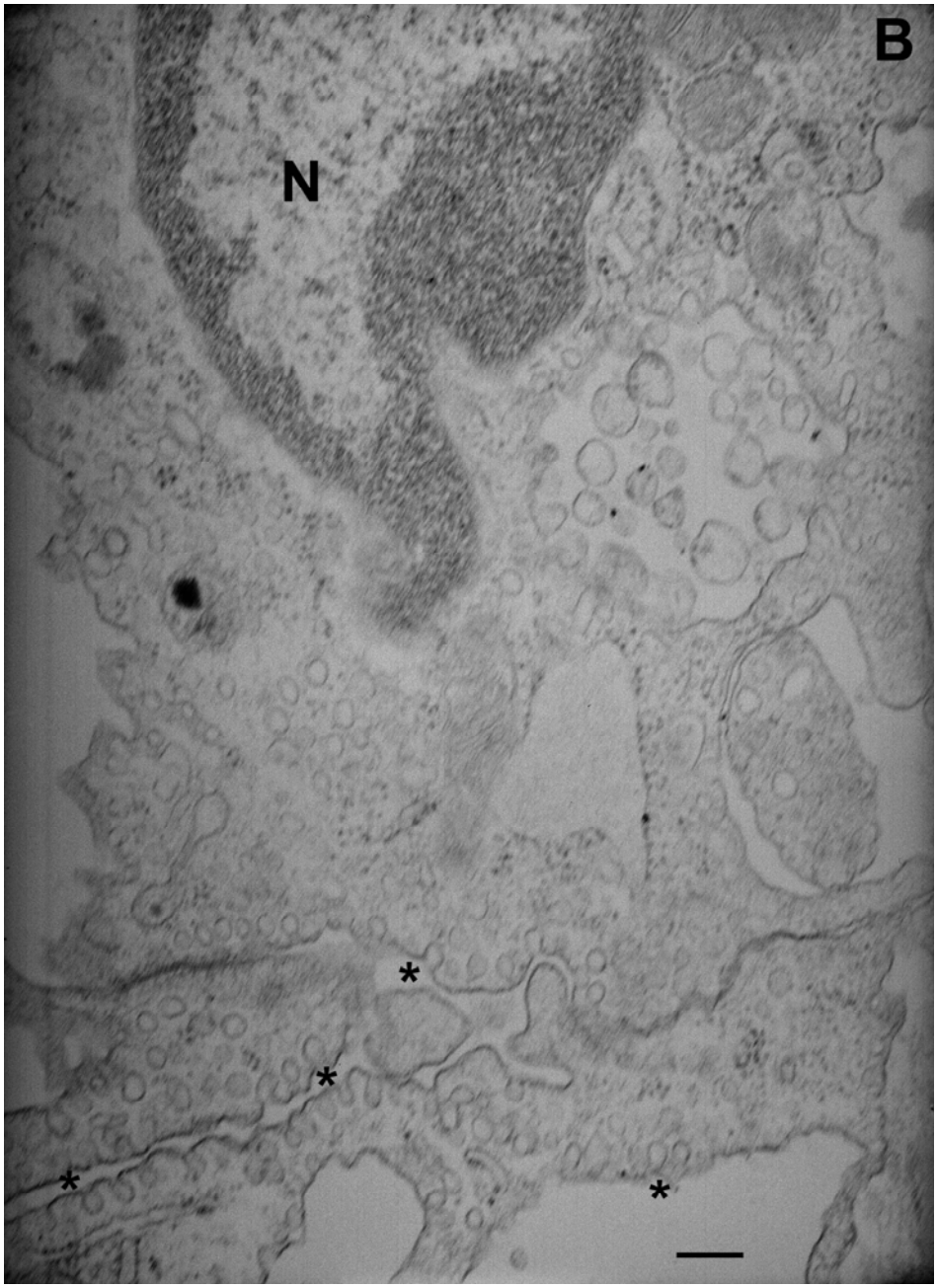

Fig. 5.2B Electron micrograph of multiple endothelial cells from the pulmonary artery after application of acetylcholine (1µM). Note the fused vesicles at the endothelial cell membrane (denoted by asterisks). Magnification x 29,000. N = nucleus. Scale bar represents 0.4 µm.

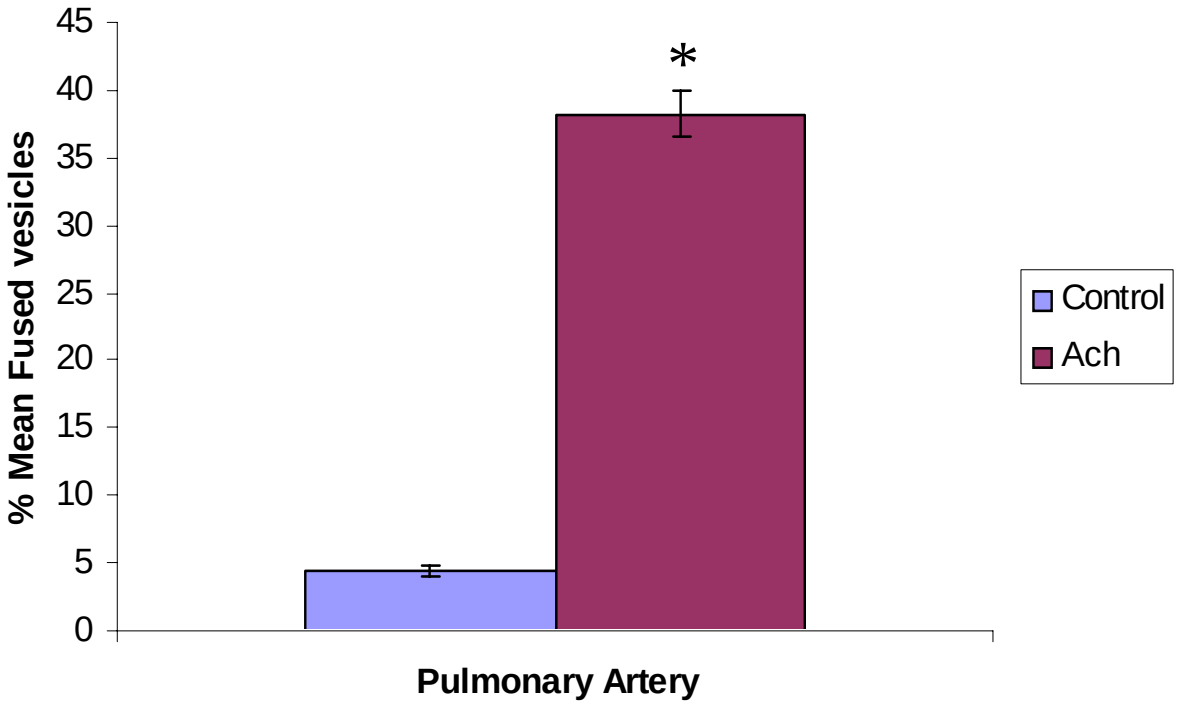

Fig. 5.3 Effects of acetylcholine on endothelial cells from pulmonary arteries. Comparison of fused vesicles at endothelial cell membranes under control conditions versus acetylcholine (1 µM) application. The numbers of fused vesicles are expressed as % of the total numbers of cytoplasmic vesicles. The data are presented as mean ± SEM (n=3). *P < 0.05, acetylcholine versus control.

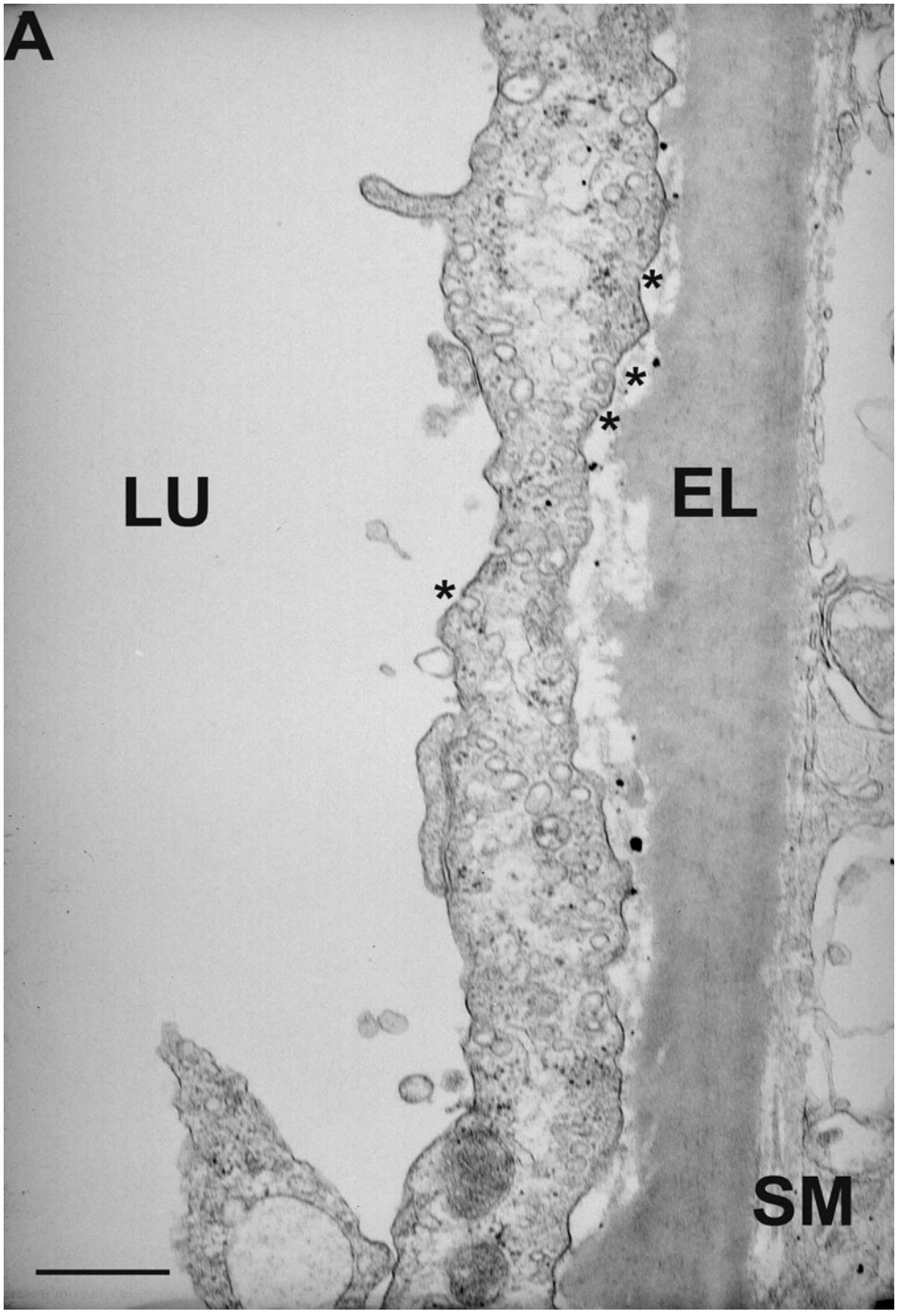

Fig. 5.4A Electron micrograph of an endothelial cell from the mesenteric artery under control conditions. LU = lumen, SM = smooth muscle, EL = elastic lamina. Magnification x 19,000. Scale bar represents 0.5 µm.

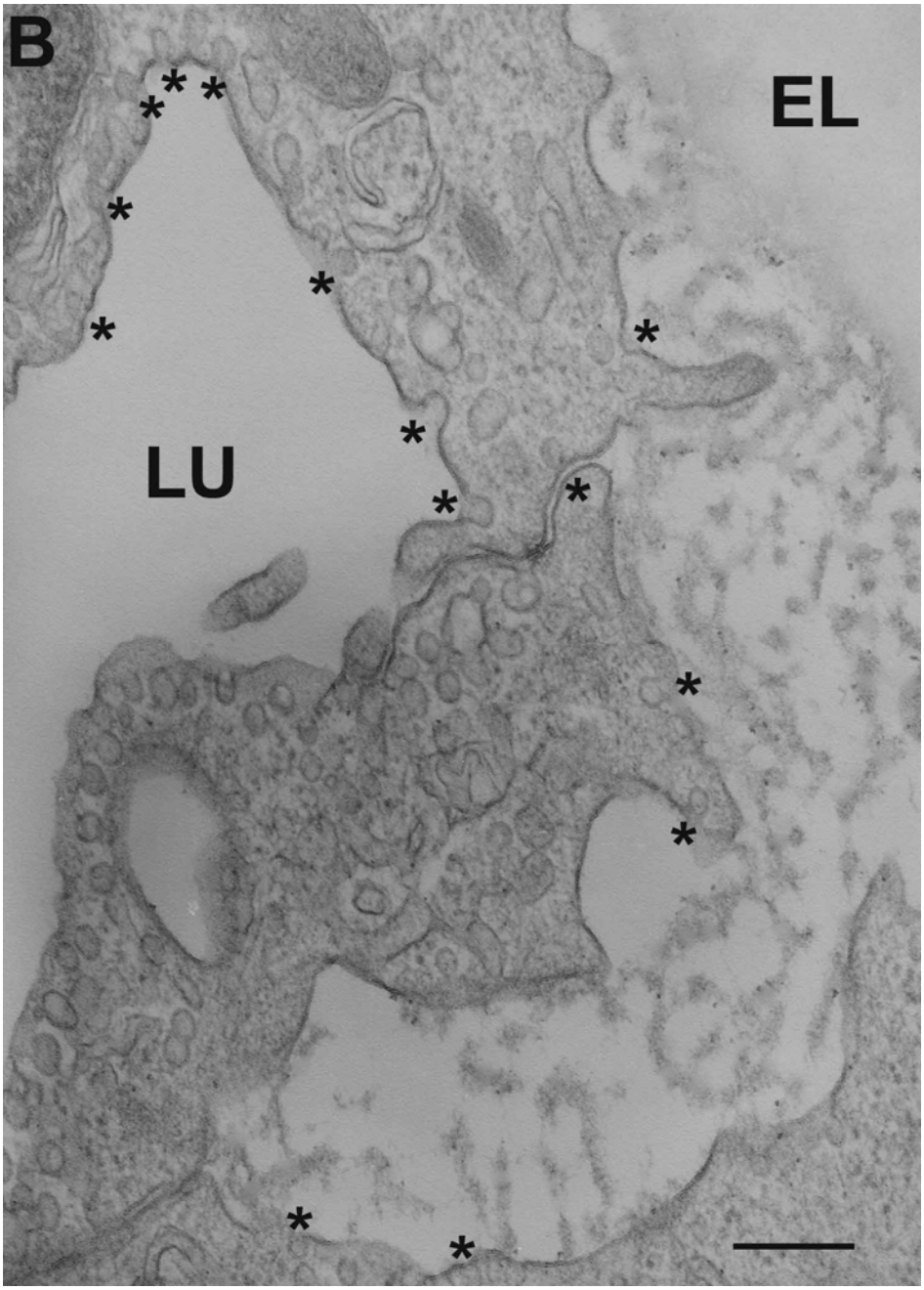

Fig. 5.4B Electron micrograph of multiple endothelial cells from the mesenteric artery after application of acetylcholine (1 µM). Note the fused vesicles at the endothelial cell membrane (denoted by asterisk). Magnification x 36,000. LU = lumen, EL = elastic lamina. Scale bar represents 0.4 µm.

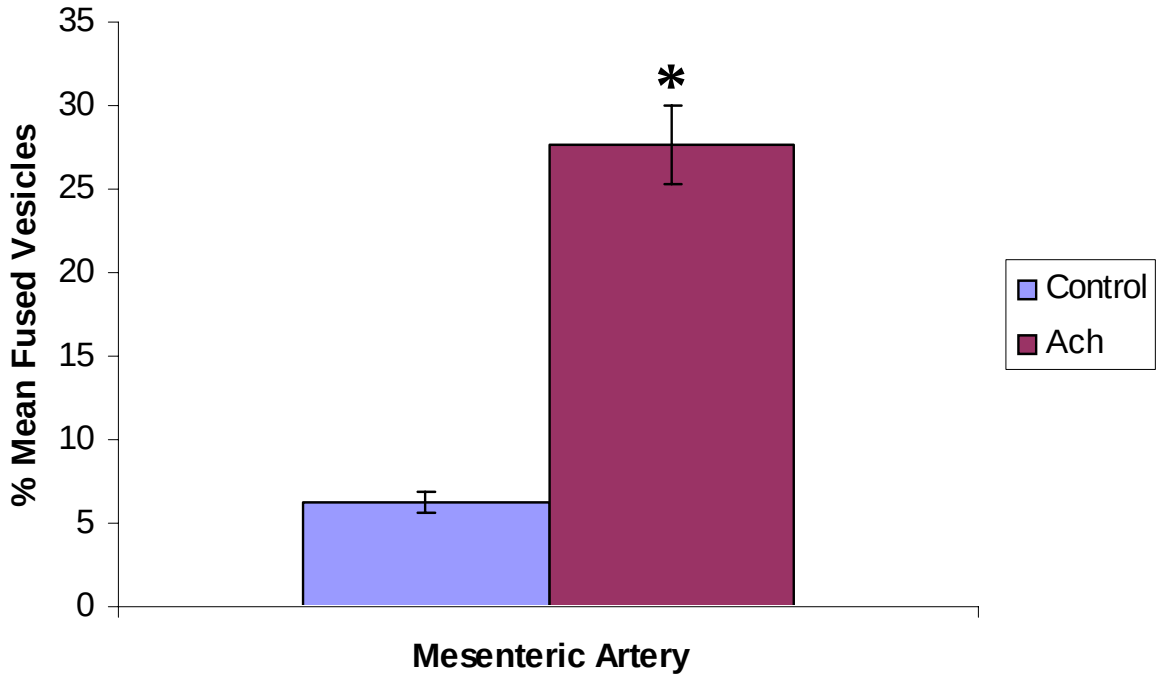

Fig. 5.5 Effects of acetylcholine on endothelial cells from mesenteric arteries. Comparison of fused vesicles at endothelial cell membranes under control conditions versus acetylcholine (1µM) application. The numbers of fused vesicles are expressed as % of the total numbers of cytoplasmic vesicles. The data are presented as mean ± SEM (n=3). *P < 0.05, acetylcholine versus control.

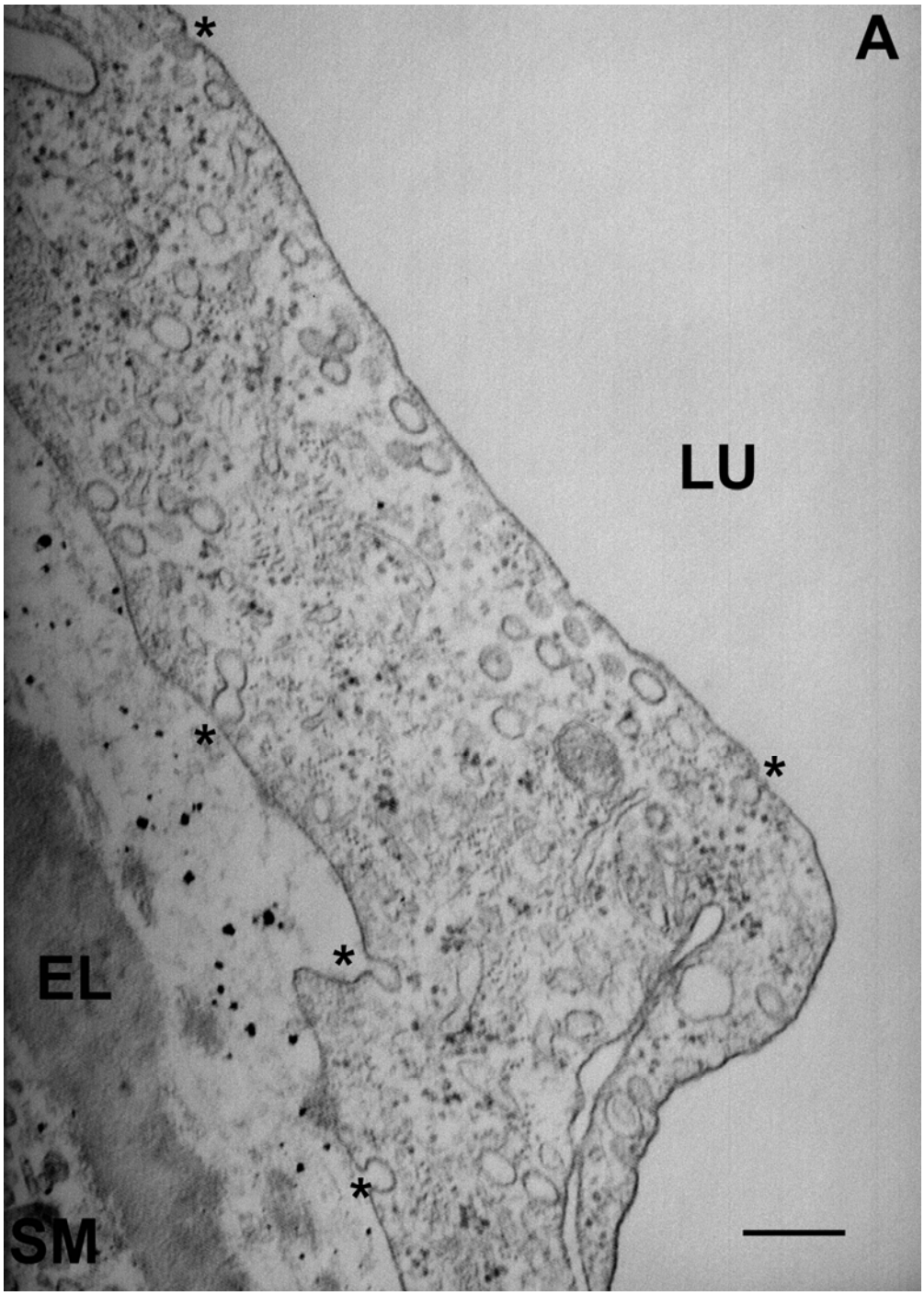

Fig. 5.6A Electron micrograph of an endothelial cell from the femoral artery under control conditions. LU = lumen, SM = smooth muscle, EL = elastic lamina, Magnification x 36,000. Scale bar represents 0.3 µm.

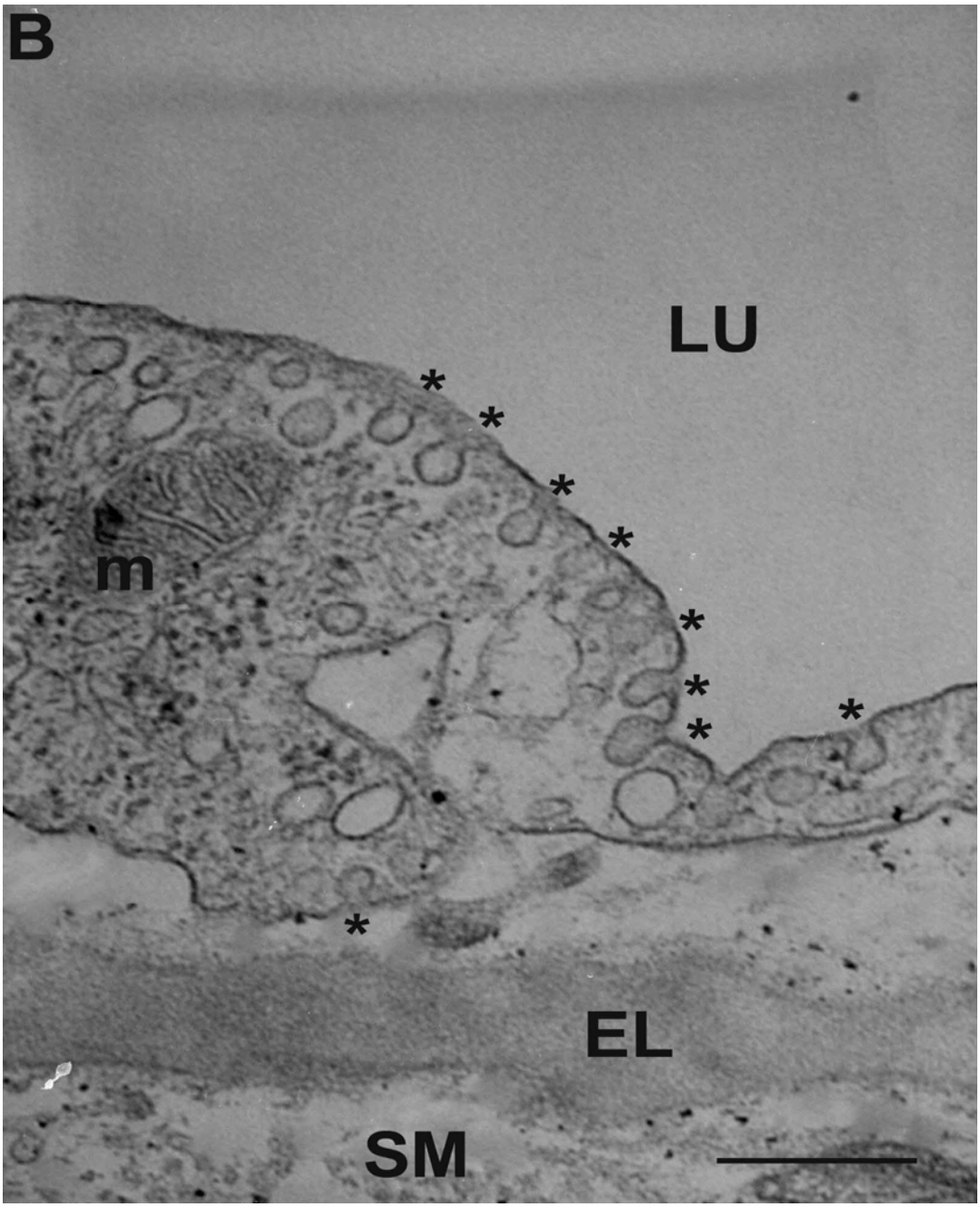

Fig. 5.6B Electron micrograph of an endothelial cell from the femoral artery after application of acetylcholine (1µM). Note the fused vesicles at the endothelial cell membrane (denoted by asterisk). Magnification x 58,000. LU = lumen, SM = smooth muscle, EL = elastic lamina. Scale bar represents 0.5 µm.

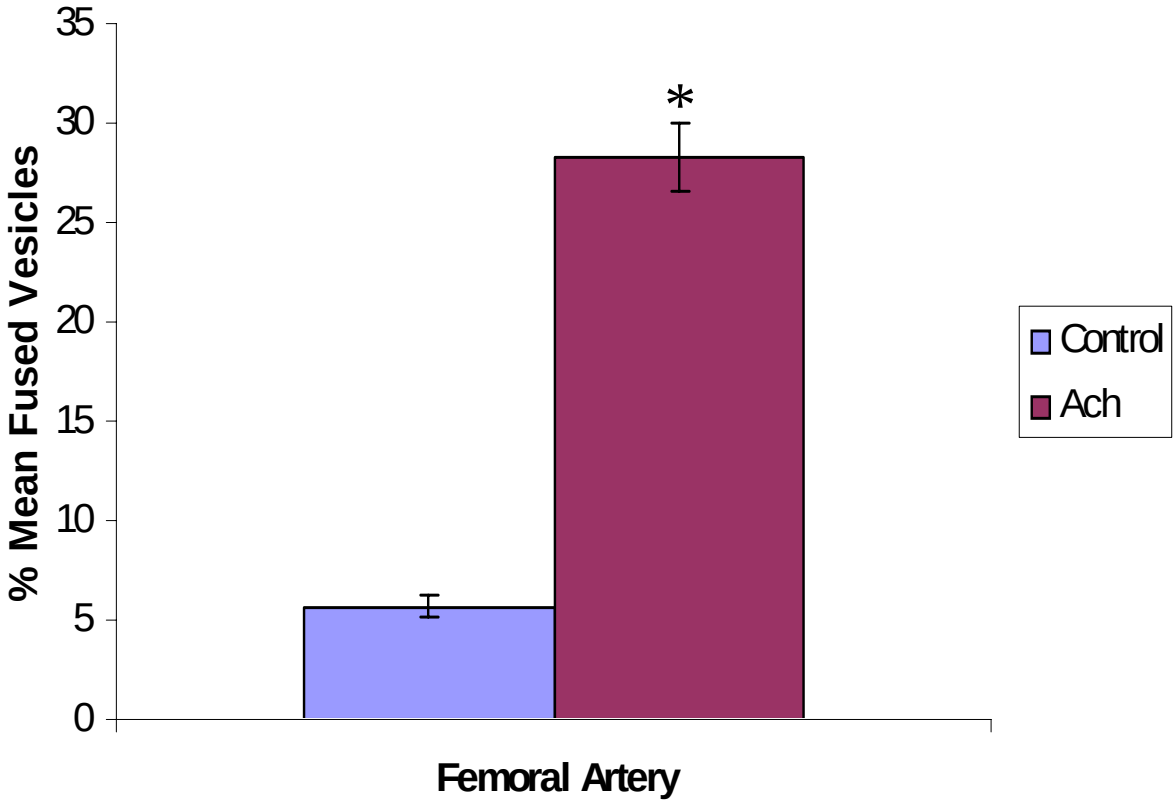

Fig. 5.7 Effects of acetylcholine on endothelial cells from femoral arteries. Comparison of fused vesicles at endothelial cell membranes under control conditions versus acetylcholine (1µM) application. The numbers of fused vesicles are expressed as % of the total numbers of cytoplasmic vesicles. The data are presented as mean ± SEM (n=3). *$P < 0.05$, acetylcholine versus control.

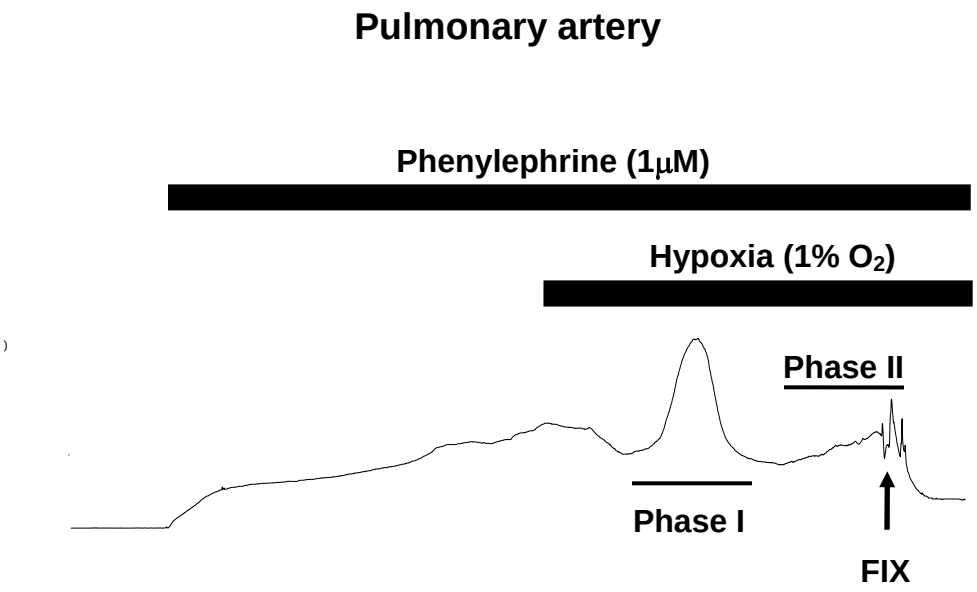

Fig 5.8A Typical example of the effects of hypoxia on the resting tone of a pulmonary artery. The trace illustrates the effect of hypoxia (1% O_2) in the presence of pre-tone (1μM phenylephrine). After onset of hypoxia, there is a rapid rise in vessel tension (Phase I), followed by a slower developing sustained constriction (Phase II). In this study, pulmonary arteries were fixed *in situ* at the beginning of Phase II and prepared for transmission electron microscopy.

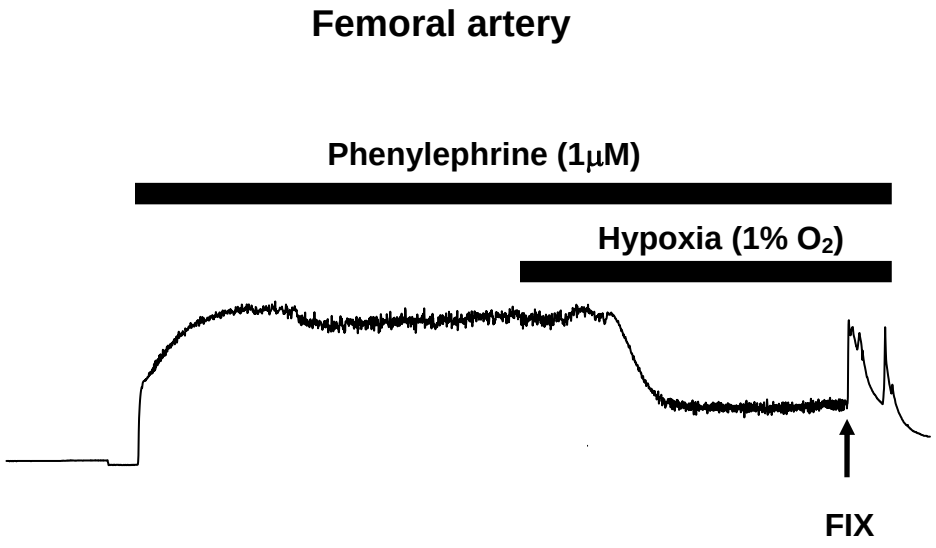

Fig 5.8B Typical example of the effects of hypoxia on the resting tone of a femoral artery. The trace illustrates the effect of hypoxia (1% O_2) in the presence of pre-tone (1μM phenylephrine). After onset of hypoxia, there is a rapid fall in vessel tension, at which point arteries are fixed *in situ* and prepared for transmission electron microscopy.

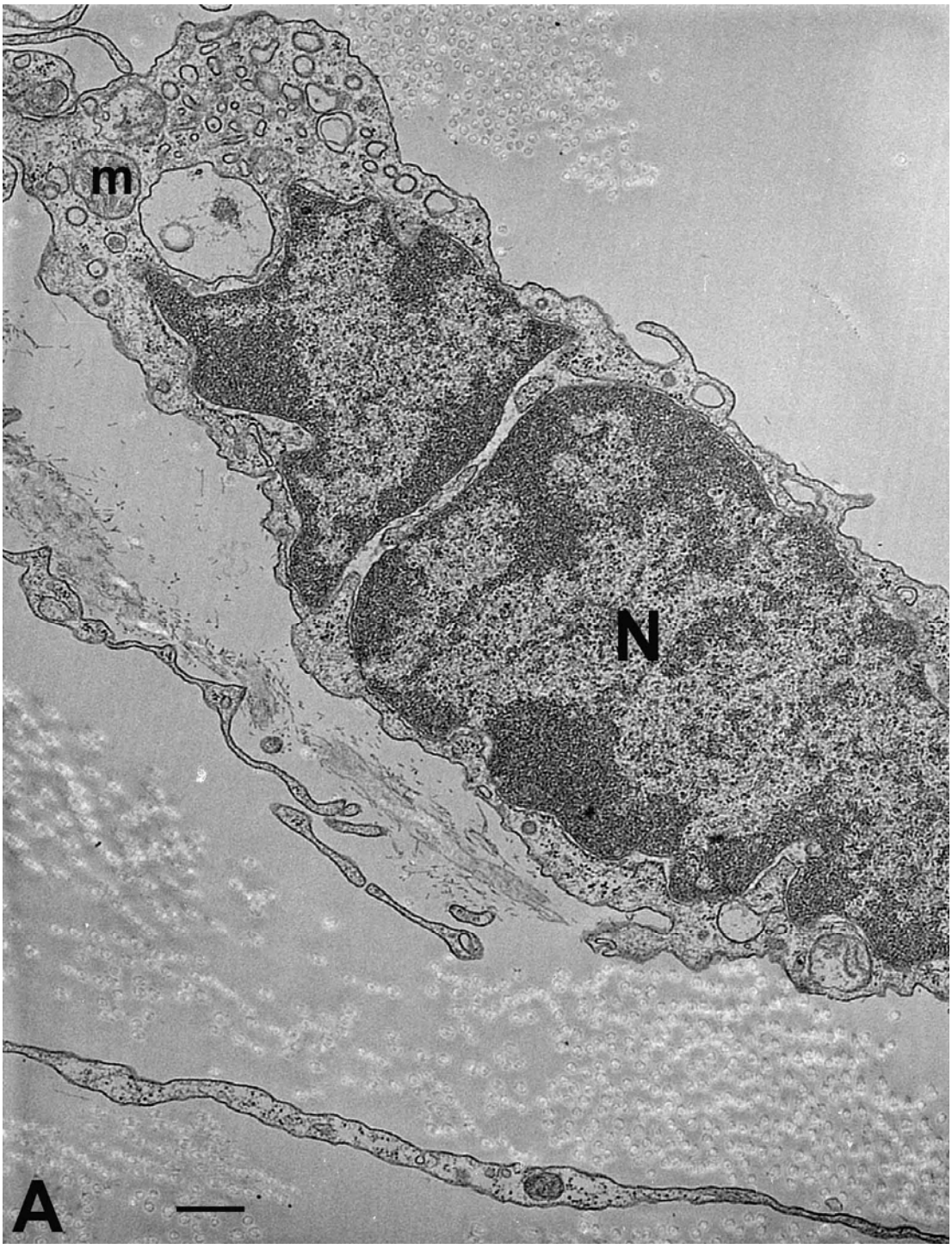

Fig. 5.9A Electron micrograph of an endothelial cell from a pulmonary artery under normoxic conditions. N = nucleus, m = mitochondria. Magnification x 10,000. Scale bar represents 0.4 µm.

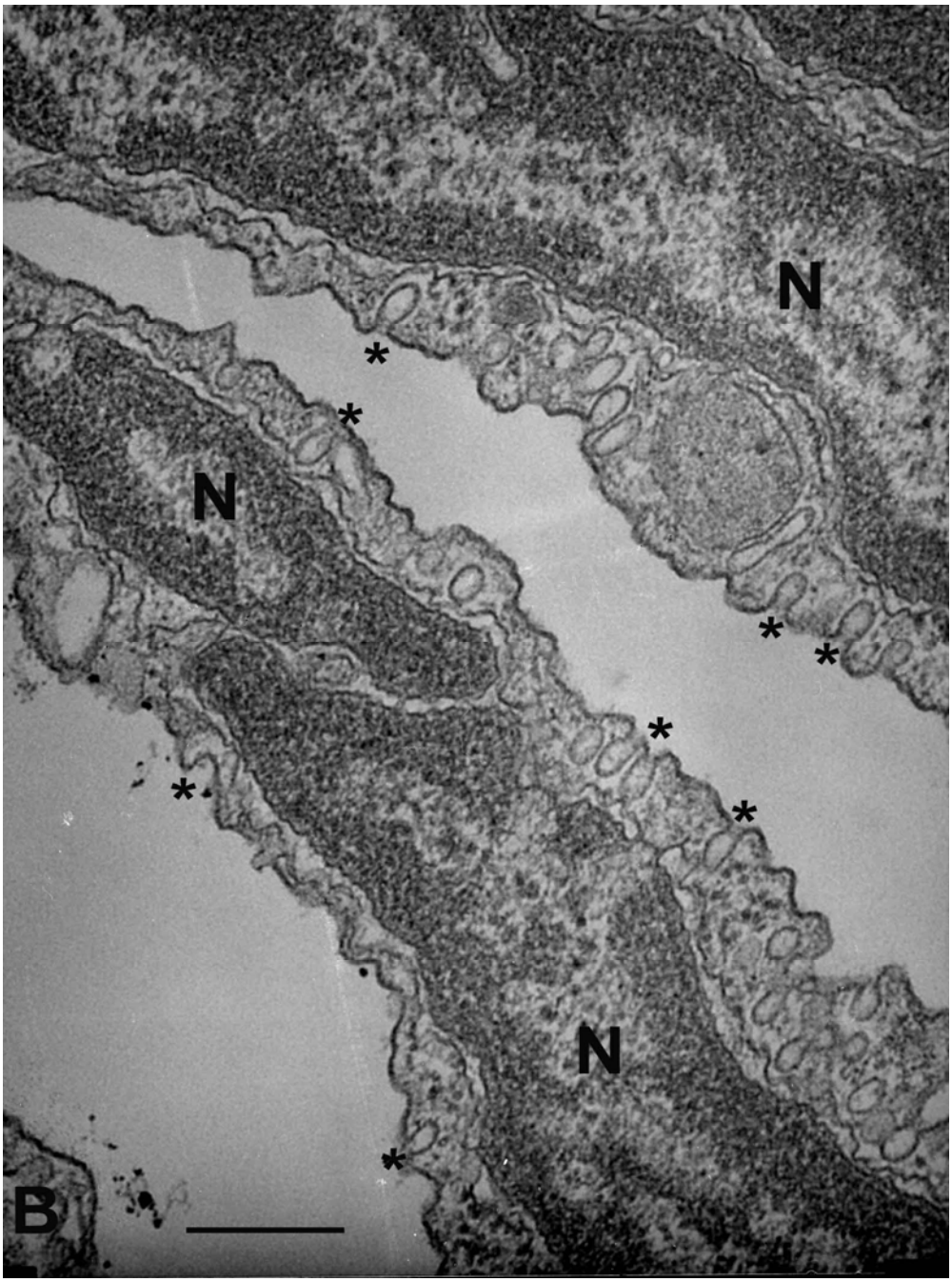

Fig. 5.9B Electron micrograph of an endothelial cell from the pulmonary artery after onset of hypoxia. Note the fused vesicles at the endothelial cell membrane (denoted by asterisk). Magnification x 48,000. LU = lumen, SM = smooth muscle, EL = elastic lamina. Scale bar represents 0.5 µm.

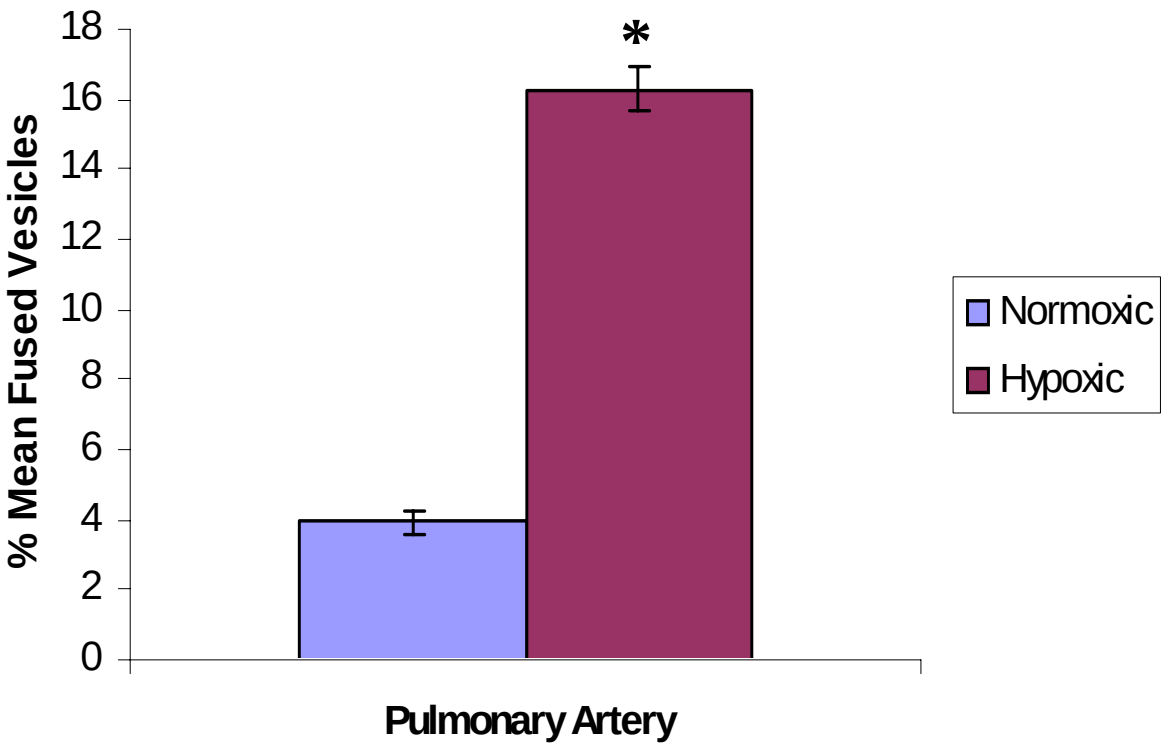

Fig. 5.10 Effects of hypoxia on endothelial cells from pulmonary arteries. Comparison of fused vesicles at endothelial cell membranes under normoxic and hypoxic conditions. The numbers of fused vesicles are expressed as % of the total numbers of cytoplasmic vesicles. The data are presented as mean ± SEM (n=3). *$P < 0.05$, hypoxia versus control.

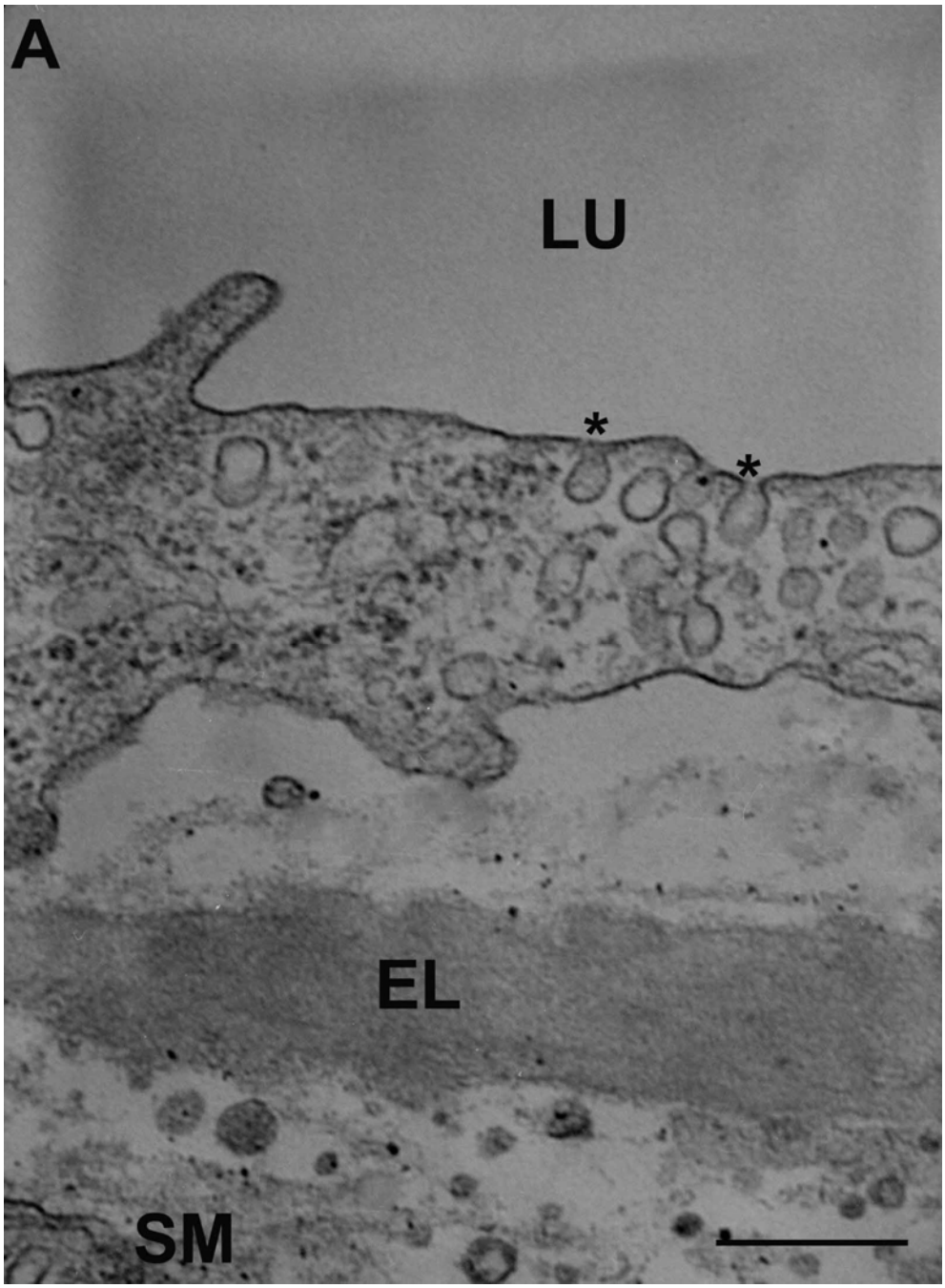

Fig. 5.11A Electron micrograph of an endothelial cell from the mesenteric artery under normoxic conditions. LU = lumen, SM = smooth muscle, EL = elastic lamina. Magnification x 58,000. Scale bar represents 0.5 µm.

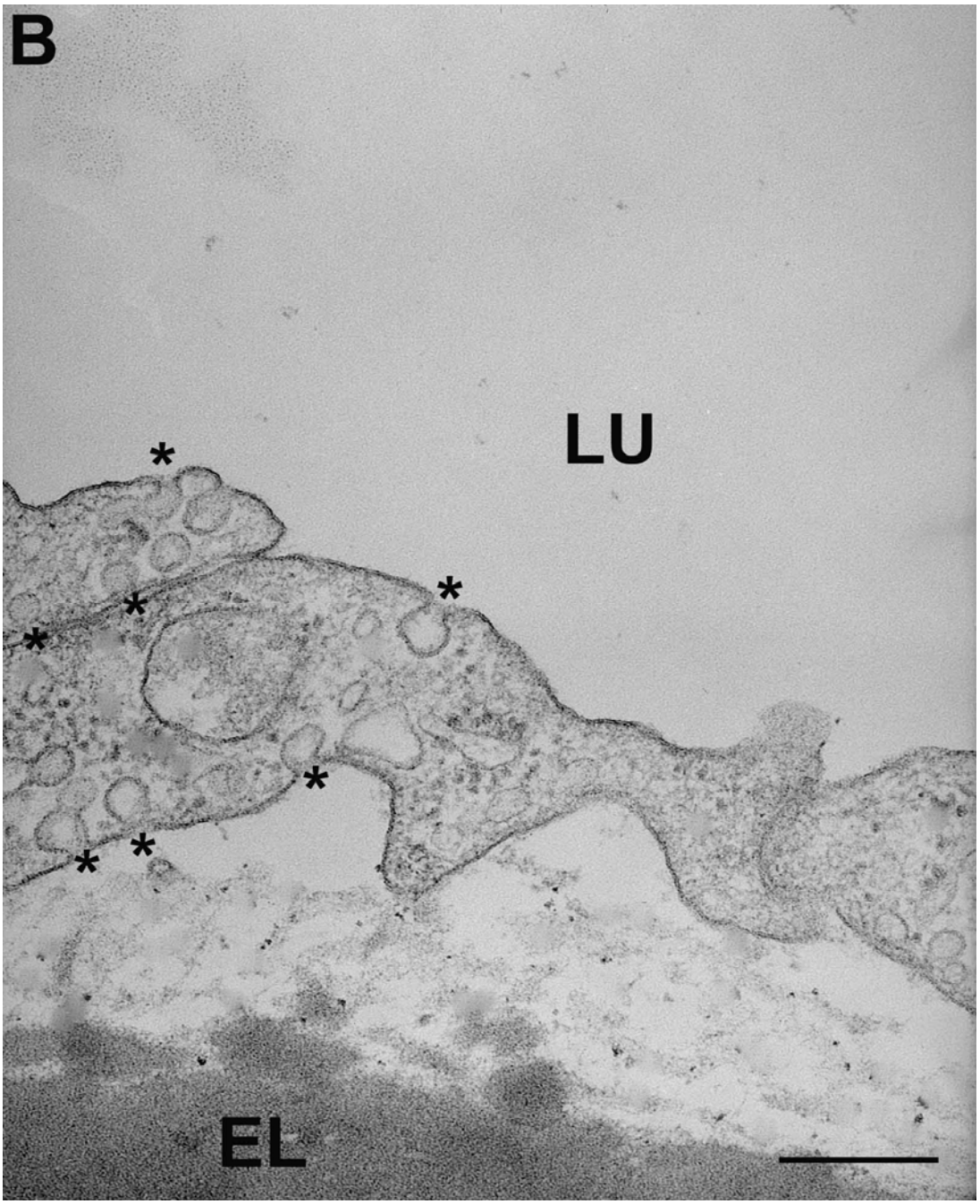

Fig. 5.11B Electron micrograph of an endothelial cell from a mesenteric artery after onset of hypoxia. Note the fused vesicles at the endothelial cell membrane (denoted by asterisks). Magnification x 48,000. LU = lumen, EL = elastic lamina. Scale bar represents 0.4 µm.

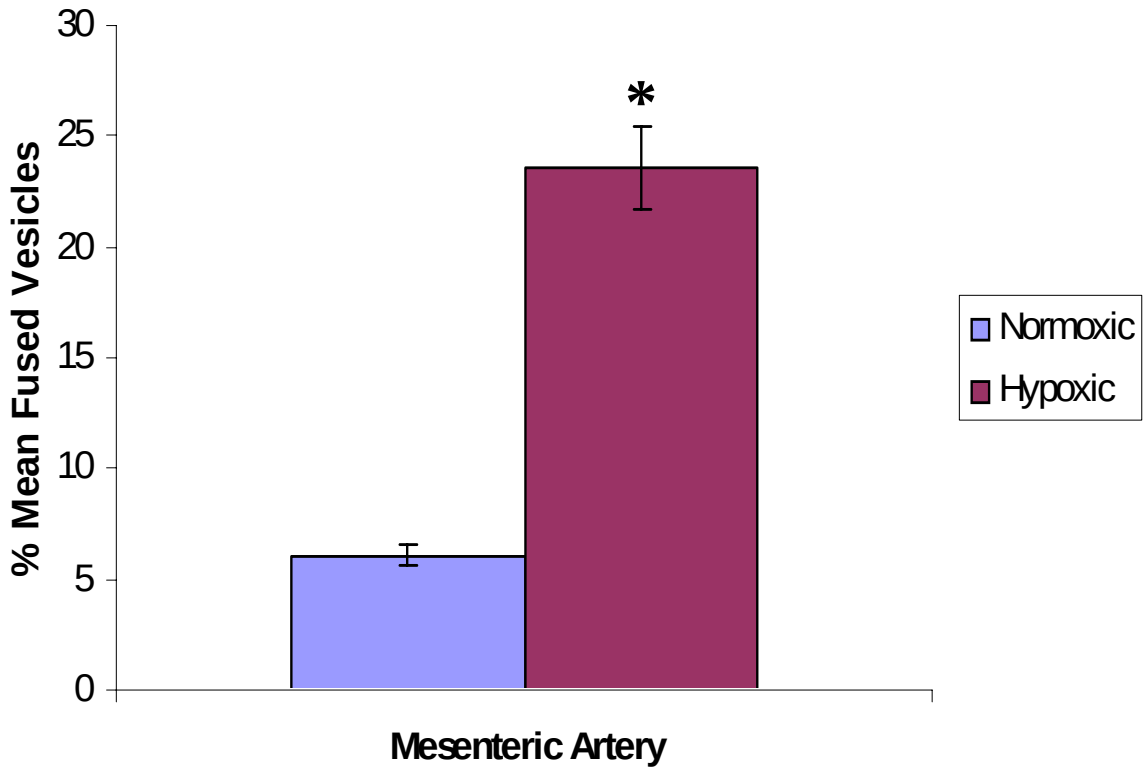

Fig 5.12 Effects of hypoxia on endothelial cells from mesenteric arteries. Comparison of fused vesicles at endothelial cell membranes under normoxic and hypoxic conditions. The numbers of fused vesicles are expressed as % of the total numbers of cytoplasmic vesicles. The data are presented as mean ± SEM (n=3). *$P < 0.05$, hypoxia versus control.

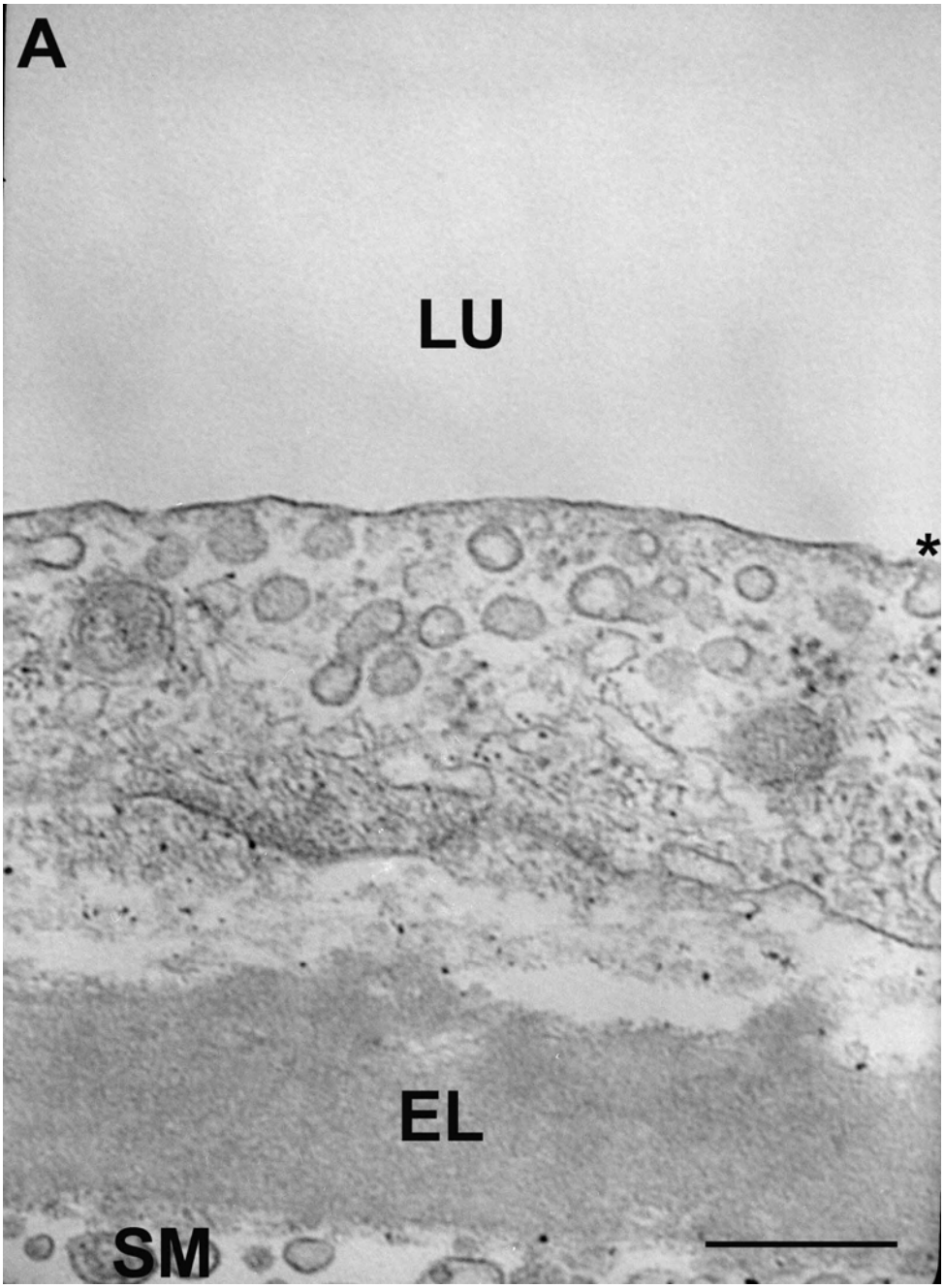

Fig. 5.13A Electron micrograph of an endothelial cell from a femoral artery under normoxic conditions. LU = lumen, SM = smooth muscle, EL = elastic lamina. Magnification x 58,000. Scale bar represents 0.5 µm.

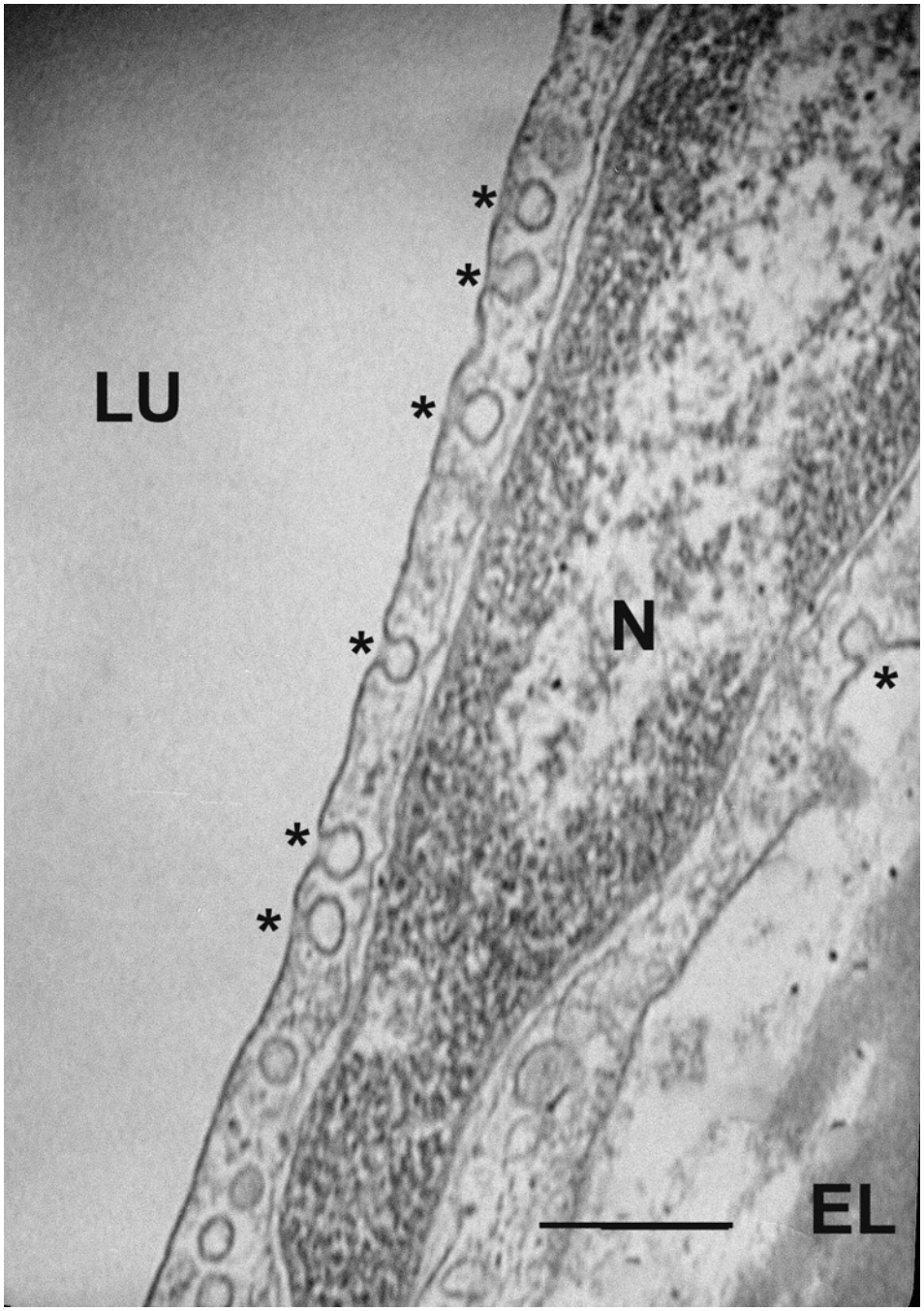

Fig. 5.13B Electron micrograph of an endothelial cell from a femoral artery after onset of hypoxia. Note the fused vesicles at the endothelial cell membrane (denoted by asterisks). Magnification x 58,000. LU = lumen, N = nucleus, EL = elastic lamina. Scale bar represents 0.5 µm.

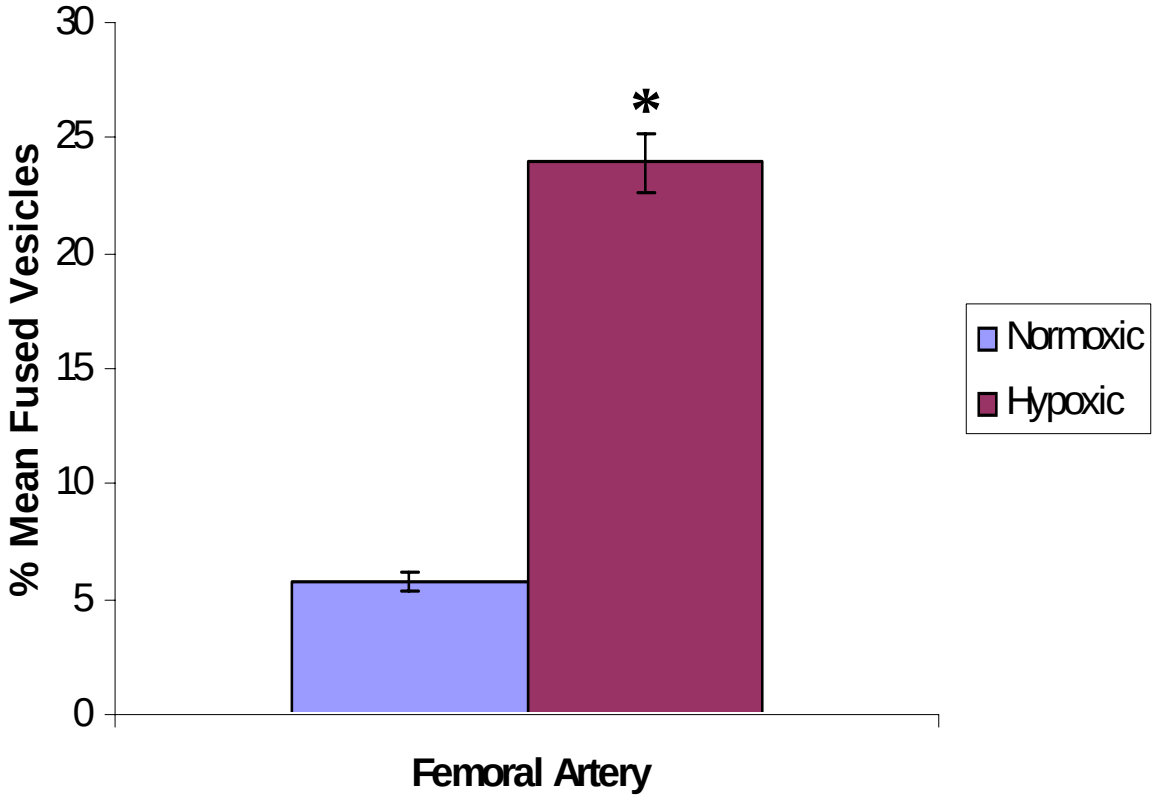

Fig. 5.14 Effects of hypoxia on endothelial cells from femoral arteries. Comparison of fused vesicles at endothelial cell membranes under normoxic and hypoxic conditions. The numbers of fused vesicles are expressed as % of the total numbers of cytoplasmic vesicles. The data are presented as mean ± SEM (n=3). *$P < 0.05$, hypoxia versus control.

CHAPTER 6

VISUALIZATION AND MOBILIZATION OF NADPH-DIAPHORASE-CONTAINING CYTOPLASMIC VESICLES FROM ENDOTHELIAL CELLS OF PULMONARY, MESENTERIC AND FEMORAL ARTERIES[1]

[1]**Hashmi-Hill MP**, Shields JP, Lewis THJ, Robertson TP, Lewis SJ. Ultra-structural evidence in endothelial cells that acetylcholine induces exocytosis of vesicular stores of S-nitrosothiols. To be submitted for publication in *Circulation Research*, 2006

ABSTRACT

S-nitrosothiols such as L-S-nitrosocysteine are stored in cytoplasmic vesicles in endothelial cells and their mobilization may be responsible for endothelium-dependent relaxation in small arteries. NADPH-diaphorase is a histochemical marker for S-nitrosothiols in tissues.

Because of the potential importance of vesicular stores of S-nitrosothiols in regulation of vascular tone the **first objective** of this ultra-structural study was to determine the distribution of NADPH diaphorase in the endothelium of small pulmonary, mesenteric and femoral arteries of the rat. Because of the potential importance of vesicular release of S-nitrosothiols in endothelium-dependent regulation of vascular tone, the **second *objective*** was to determine whether endothelial vesicles displaying NADPH diaphorase undergo Ca^{2+}-*dependent* exocytosis (i.e., movement of vesicles to and fusion with plasma membranes) upon exposure to the endothelium-dependent agonist, acetylcholine, which is known to elicit increases in intracellular Ca^{2+} levels.

In order to achieve the above objectives, we perfomed functional studies in which we used acetylcholine to induce relaxation of small pulmonary, mesenteric and femoral arteries of the rat and then immersion fixed these arteries for subsequent NADPH diaphorase histochemistry and transmission electron microscopy to determine the distribution of NADPH diaphorase in the endothelium of the above arteries.

Endothelial cells of small pulmonary, mesenteric and femoral arteries displayed very high numbers of NADPH diaphorase positive vesicles that often existed in clusters. Stimulation of endothelial cells with acetylcholine resulted in a marked accumulation of NADPH diaphorase-containing vesicles on or in close proximity to the endothelial cell membrane.

These results suggest that cytoplasmic vesicles within endothelial cells contain S-nitrosothiols and that acetylcholine stimulates these vesicles to undergo Ca^{2+}-*dependent* exocytosis. The exocytotic release of vesicular stores of S-nitrosothiols may be a major mechanism by which processes/compounds that increase intracellular levels of Ca^{2+} (e.g., shear stress, endothelium-dependent agonists) relax smooth muscle in resistance arteries.

Keywords: Endothelial cells, Vesicles, S-nitrosothiols, NADPH diaphorase

INTRODUCTION

The endothelium releases a variety of mediators that regulate vascular tone. Mediators such as nitric oxide (NO), prostacyclin, superoxide anion, endothelin-1, thromboxane A_2 and prostaglandin H_2 either dilate or constrict vascular smooth muscle (Furchgott and Zawadski, 1980; Luscher et al., 1993; Moncada et al., 1991, Palmer et al., 1987; Vanhoutte, 1993; Yanagisawa et al., 1988). Upon synthesis in the endothelium, NO diffuses to the adjacent smooth muscle of the blood vessel and cause vasodilation via activation of soluble guanylate cyclase. The subsequent production of cyclic GMP from GTP (Ignarro, 1989) activates cGMP-dependent protein kinase (PKG) (Lincoln and Cornwell, 1993), which relaxes vascular smooth muscle via numerous and often cell-specific mechanisms such as the closure of L-type voltage-sensitive Ca^{2+}-channels (Travis et al., 2000; Lewis et al., 2005a) or via the activation of Na^+/K^+-ATPase (see Batenburg et al., 2004a,b; Lewis et al., 2006a).

There is mounting evidence that NO may not be the major non-prostanoid EDRF in resistance arteries (i.e., those arteries that control arterial blood pressure). Specifically, endothelium-dependent relaxation in resistance beds is only minimally affected by inhibition of NO synthesis or by blockade of the intracellular mechanisms by which NO relaxes vascular smooth muscle (Komori et al., 1988; Huang et al., 1988; Komori and Vanhoutte, 1990; Travis et al., 2000; Woodman et al., 2000; Lewis et al., 2005a,b,c). These findings clearly suggest the existence of another endothelium-*dependent* but NO-*independent* vasodilator

mechanism in resistance arteries. Endothelium-*dependent*, NO-*independent* vasodilation is often associated with hyperpolarization of the vascular smooth muscle cell (Chen et al., 1988) is not cyclic GMP-dependent (Fleming, 2000) and is diminished by K^+-channel blockers or by depolarizing concentrations of K^+ (Adeagbo and Triggle, 1993). The as yet unidentified mediator(s) responsible for endothelium-*dependent*, NO-*independent* vasodilation was therefore termed endothelium-derived hyperpolarizing factor (EDHF) (Taylor and Weston, 1988).

Since NO is very labile and is easily inactivated by many reactive species (Cocks et al., 1985; Griffith et al., 1984), it has been suggested that NO may be released from vascular endothelial cells in the form of S-nitrosothiols (Myers et al., 1990; Rubanyi et al., 1991; Rosenblum, 1992) or dinitrosyl iron (II)-cysteine complexes (Vanin, 1998). The cytosol is not the most favorable environment for S-nitrosothiols because they are readily reduced (decomposed) by iron, glutathione or thioredoxin for example (Haendeler et al., 2002; Freedman et al., 1995; Liu et al., 2001). In order to prevent degradation of S-nitrosothiols, it has been suggested that these compounds may be stored or protected in cytoplasmic vesicles, in membranes, in lipophilic protein folds and in interstitial spaces (Ignarro, 1990; Rafikova et al., 2002; Lewis et al., 2002, 2006; Mannick et al., 2001).

The large number of cytoplasmic vesicles is a conspicuous feature of endothelial cells (Bruns and Palade, 1968a,b; Loesch et al., 1993, 1994). The

possibility that endothelium-dependent vasodilation involves exocytosis of vesicular stores of S-nitrosothiols is supported by evidence that (1) NO synthase (NOS) exists in the membranes of many cytoplasmic vesicles of endothelial cells (Loesch et al., 1993), (2) the S-nitrosothiol, L-S-nitrosocysteine, exists in high concentrations in cytoplasmic vesicles of endothelial cells (Lewis et al., 2006b), (3) inhibition of ATP synthesis, which reduces Ca^{2+}-*dependent* exocytosis in nerve terminals, reduces endothelium-dependent relaxation (Griffith et al., 1986; Weir et al., 1991; Richards et al., 1991), and (4) the vasorelaxant responses elicited by endothelium-dependent agonists rapidly diminish upon repeated administration to resistance arteries treated with NOS inhibitors *in vivo* (Davisson et al., 1996a; Colombari et al., 1998) and *in vitro* (Danser et al., 1998, 2000; Tom et al., 2001), which is consistent with the use-dependent depletion of these stores which cannot be replenished in the absence of NO synthesis.

NADPH-diaphorase is used as a histochemical marker for the presence and distribution of NOS in tissues (Dawson et al., 1991; Hope et al., 1991). NADPH-diaphorase is the process whereby nitroblue tetrazolium is enzymatically reduced to diformazan (a blue precipitation) through the transfer of two electrons. It was assumed that the two electrons were donated by NOS. However, fixation of brain with 4% paraformaldehyde abolishes NOS activity in both particulate and cytosolic fractions (Matsumoto et al., 1993). Although fixation abolishes NADPH diaphorase in the particulate fraction, 50-60% of NADPH diaphorase remains in

the cytosol (Matsumoto et al., 1993) and is heavily localized to cytoplasmic vesicles in both endothelial cells and nerve terminals (Loesch et al., 1993, 1994).

Chayen *et al* (1994) provided a comprehensive series of arguments as to why NOS is not responsible for NADPH diaphorase in aldehyde-treated tissues. They concluded that a yet to be identified 'proteinaceous' factor promotes the NADPH-dependent reduction of NBT. One important point raised by Chayen et al (1994) was that α-NADPH is as effective as β-NADPH in promoting reduction of NBT. Since α-NADPH will not donate electrons to NOS, it is unlikely that diformazan arises from the catalytic activity of NOS. Loesch and Burnstock (1993, 1994) demonstrated that whereas NOS exists in the membranes of cytoplasmic vesicles, NADPH-diaphorase is found exclusively within the lumen of these vesicles (Loesch et al., 1994). This suggests that NADPH-diaphorase is not a direct marker for NOS. We have provided evidence that NADPH-diaphorase in endothelial vesicles of aldehyde-treated tissues is due to the presence of preformed S-nitrosothiols, which promote NADPH-dependent reduction of nitroblue tetrazolium to diformazan (Lewis et al., 2006b).

Because of the potential importance of vesicular stores of S-nitrosothiols in regulation of vascular tone (Myers et al., 1990), the first ***objective*** of this ultra-structural study was to demonstrate the presence of NADPH-diaphorase in the ***endothelium*** of the small pulmonary, mesenteric and femoral arteries of the rat. The second ***objective*** was to determine whether endothelial vesicles displaying

NADPH-diaphorase are subject to Ca^{2+}-*dependent* exocytosis (i.e., movement of vesicles to, and fusion with, the plasma membrane) upon exposure to a vasorelaxant concentration of the endothelium-dependent agonist, acetylcholine.

MATERIALS AND METHODS

Animals

All studies were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) revised in 1996. The protocols pertaining to the use of animals were approved by the Animal Care and Use Committee of the University of Georgia. Adult male Sprague-Dawley rats (≈300g) were used in these studies.

Distribution of NADPH-diaphorase-containing vesicles in the endothelium

The ***objective*** of this study was to determine the distribution of NADPH diaphorase (diformazan)-positive vesicles in endothelial cells. Rats were killed by decapitation and small (approximately 200-250 μm in diameter) pulmonary, mesenteric and femoral arteries were dissected, rapidly rinsed in 0.1M phosphate-buffered saline to remove as much blood as possible, and immersion-fixed for 3h at 4°C with 4% paraformaldehyde and 0.25% glutaraldehyde (in 0.1M phosphate buffer, pH7.4). The arteries were transferred to phosphate buffer and stored overnight at 4°C. The arteries were then prepared for transmission electron microscopy and NADPH-diaphorase histochemistry as described below.

Mobilization of NADPH-diaphorase-containing vesicles

The ***objective*** of this study was to determine whether the vasorelaxation elicited by endothelium-dependent agonist, acetylcholine, in isolated small diameter arteries is associated with the mobilization of NADPH diaphorase-containing vesicles to, and fusion with, the plasma membranes in vascular endothelial cells, and whether the fusion of vesicles takes place specifically at the interface of endothelial cells with vascular smooth muscle. Male Sprague-Dawley rats were decapitated and small femoral, mesenteric and pulmonary arteries were dissected and mounted as ring preparations on small vessel myographs in physiological saline solution at 37°C (Robertson et al., 1995, 2000a,b,c; 2001, 2003). Maximum tension was determined by applying a series of stretches and depolarizing concentrations of K^+ (Robertson et al., 1995, 2000a,b,c; 2001, 2003). The arteries were then preconstricted to 50% maximum by the addition of the α_1-adrenoceptor agonist, phenylephrine (1μM).

Once a stable baseline had been established, acetylcholine (1μM) was applied to the baths to elicit a sustained endothelium-dependent relaxation. At the maximum fall in tension, which occurred within 30-60 sec, the rings were immersion fixed *in situ* with 4% paraformaldehyde and 0.2% glutaraldehyde (in 0.1M phosphate buffer, pH7.4). The arteries were transferred to phosphate buffer and stored overnight at 4°C. The fixed arteries were processed for NADPH-diaphorase histochemistry and transmission electron microscopy. To establish the resting status of vesicles under these conditions, other arteries did not

receive acetylcholine. These control arteries were immersion fixed at the point corresponding to that when the arteries exposed to acetylcholine were fixed.

Histochemistry of NADPH-diaphorase

Fixed arteries were rinsed in 0.1M Tris-HCl buffer (pH 7.4) and then incubated in the dark for 5h at 37°C in 0.1M Tris-HCl buffer (pH 7.4) containing 1.2mM β-NADPH, 0.24mM Nitroblue tetrazolium, 15.2mM L-malic acid (all Sigma, St Louis, MO, USA) and 0.1% Triton X-100. To control for non-specific NADPH-diaphorase activity, β-NADPH was omitted from the incubation medium. As will be seen omission of β-NADPH eliminated NADPH-diaphorase reaction product in endothelial cells.

Transmission electron microscopy

Arteries were post-fixed in 1% osmium tetroxide (OsO4) for 1h at room temperature and dehydrated in a graded ethanol series, followed by a graded acetone series. The arteries were infiltrated, embedded, and polymerized for 24h at 60°C in Embed 812 epoxy resin (Polysciences, Warrington, PA, USA) and silver sections were cut on an ultramicrotome (RMC MT-X, Tucson, AZ, USA). The sections were post-stained with uranyl acetate and lead citrate and viewed on a transmission electron microscope (model 100CX II, JEOL USA, Peabody, MA) in the *Center for Advanced Ultrastructural Research* at the University of Georgia. The transmission electron microscope was set to operate at 80 KeV.

Observations

All quantitative and qualitative observations were made while viewing the sections under the transmission electron microscope.

RESULTS

A. Visualization of NADPH-diaphorase activity in the endothelium of arteries taken directly from rats

Pulmonary Artery

A typical example of an electron micrograph of an ultra-thin section of a pulmonary artery endothelial cell in which β-NADPH was ***not included*** in the reaction mixture is shown in Fig. 6.1A. As can be seen, no NADPH-diaphorase (i.e., particles of diformazan) was present in this cell. A typical example of an electron micrograph of an ultra-thin section of a pulmonary artery endothelium cell in which β-NADPH ***was included*** in the reaction mixture is shown in Fig. 6.1B. As can be seen, high-density, amorphous and vesicular-shaped deposits of NADPH diaphorase were present in the cytoplasm of the endothelial cell and in close proximity to the endothelial cell plasma membrane (deposits denoted by arrows in Fig. 6.1B). Many vesicles were devoid of NADPH-diaphorase staining. Dense deposits of NADPH-diaphorase were found in every endothelial cell examined (n=25 cells from 5 different arteries).

Mesenteric Artery

A typical example of an electron micrograph of an ultra-thin section of a mesenteric artery endothelial cell in which β-NADPH was ***not included*** in the reaction mixture is shown in Fig. 6.2A. No NADPH-diaphorase staining was present in this cell. A typical example of an electron micrograph of an ultra-thin section of a pulmonary artery endothelium cell in which β-NADPH ***was included*** in the reaction mixture is shown in Fig. 6.2B. Numerous high-density vesicular shaped deposits throughout the cytoplasm were observed (deposits denoted by arrows in Fig. 6.2B). Some deposits were occasionally in close association with the plasma cell membrane. The richest accumulation of NADPH-diaphorase product was found in the perinuclear cytoplasm of the cell. Unlike the pulmonary artery and the femoral artery (see below), very ***few*** vesicles were devoid of NADPH diaphorase. Dense deposits of NADPH-diaphorase were found in every endothelial cell examined (n=25 cells from 5 different arteries).

Femoral Artery

A typical example of an electron micrograph of an ultra-thin section of a femoral artery endothelial cell in which β-NADPH was ***not included*** in the reaction mixture is shown in Fig. 6.3A. As can be seen, no NADPH-diaphorase was present in this cell. A typical example of an electron micrograph of an ultra-thin section of a pulmonary artery endothelium cell in which β-NADPH ***was included*** in the reaction mixture is shown in Fig. 6.3B. The endothelial cell displayed moderate to high-density, amorphous deposits which were patchily

distributed in the cytoplasm and occasionally in association with the endothelial cell membrane (deposits denoted by arrows in Fig. 6.3B). Similar to the pulmonary artery but unlike the mesenteric artery, many vesicles were devoid of NADPH-diaphorase staining. Dense deposits of NADPH-diaphorase were found in every endothelial cell examined (n=25 cells from 5 different arteries).

B. Visualization of NADPH-diaphorase activity in the endothelium of arteries exposed to acetylcholine

Pulmonary artery

A typical example of an electron micrograph of an ultra-thin section of a pulmonary artery endothelial cell that was exposed to acetylcholine and in which β-NADPH was ***not included*** in the reaction mixture during subsequent NADPH diaphorase histochemistry, is shown in Fig. 6.4A. Numerous vesicles (denoted by the asterisk symbols) were fused to the membrane. It is important to note that no NADPH diaphorase staining was present in the cell. Similar findings were obtained in every endothelial cell examined (n=25 cells from 5 different arteries).

A typical example of an electron micrograph of an ultra-thin section of a pulmonary artery endothelial cell that was exposed to acetylcholine and in which β-NADPH ***was included*** in the reaction mixture during subsequent NADPH diaphorase histochemistry, is shown in Fig. 6.4B. Large numbers of NADPH diaphorase-positive vesicular-like structures were observed in the cytoplasm and especially in close proximity to the plasma membrane ***adjacent*** to the vascular

smooth muscle. Upon qualitative analysis (quantitative analyses in progress) it was evident that many more NADPH diaphorase-positive vesicles were in close proximity to the plasma membrane of endothelial cells exposed to acetylcholine than control endothelial cells. Substantial numbers of vesicles devoid of NADPH-diaphorase were fused with the endothelial cell membrane. Similar findings were obtained in all endothelial cells examined (n=25 cells from 5 different arteries).

Mesenteric Artery

Typical examples of electron micrographs of ultra-thin sections of mesenteric arteries that were exposed to acetylcholine and subjected to NADPH diaphorase histochemistry with and without β-NADPH in the reaction mixture are shown in Fig 6.5A and 6.5B, respectively. Identical findings to those of pulmonary arteries were obtained. Specifically, (1) no NADPH diaphorase staining was seen when β-NADPH was left out of the reaction mixture, and (2) large numbers of NADPH diaphorase-positive vesicle-like structures were present in the cytoplasm of the endothelial cell and in close proximity to the cell membrane adjacent to vascular smooth muscle, and (3) acetylcholine increased the association of NADPH diaphorase-*positive* and NADPH diaphorase-*negative* vesicles to plasma membranes of the endothelial cell. We obtained such findings in all examined mesenteric artery endothelial cells (n=25 cells from 5 different arteries).

Femoral Artery

Typical examples of electron micrographs of ultra-thin sections of mesenteric arteries that were exposed to acetylcholine and subjected to NADPH diaphorase histochemistry with and without β-NADPH in the reaction mixture are shown in Fig 6.6A and 6.6B, respectively. The distribution of NADPH diaphorase-*positive* and NADPH diaphorase-*negative* vesicles and the pronounced effects of acetylcholine on the patterns of distribution (n=25 cells from 5 different arteries) were virtually identical to those described for pulmonary and mesenteric arteries described above.

DISCUSSION

Visualization of NADPH-diaphorase activity

We have provided evidence that NADPH diaphorase in aldehyde-treated tissues including endothelial cells and central and autonomic neurons is a specific histochemical marker for preformed vesicular stores of S-nitrosothiols (Lewis et al., 2002, 2006b). Moreover, we and others have provided evidence that these vesicular stores of S-nitrosothiols play vital roles in regulating arterial blood pressure (see Davisson et al., 1996a; Danser et al., 1998, 2000; Kakuyama et al., 1998), via their capacity to dilate arteries via the activation of cell-surface stereoselective recognition sites on vascular smooth muscle (see Davisson et al., 1996b; Batenburg et al., 2004a,b; Lewis et al., 2005b,c).

This study found that every rat small pulmonary, mesenteric and femoral artery endothelial cell examined displayed dense deposits of NADPH-diaphorase. In contrast, Loesch et al (1993) found that only 30% of the endothelial cells in rabbit thoracic aorta exhibited NADPH-diaphorase. The discrepancy in these findings is likely to be due to the size and function of small diameter (≈200 μm) pulmonary, mesenteric and femoral arteries as opposed to the thoracic aorta. The resting tone of the thoracic aorta regulates systolic arterial blood pressure (Safar et al., 2001). Endothelial nitrosyl factors regulate the distensibility of vascular smooth muscle in this artery *in vivo* and therefore systolic arterial blood pressure (see Fitch et al., 2001; Safar et al., 2001; Berry et al., 2004; Lopez et al., 2004). As such, the presence of NADPH diaphorase in 30% of endothelial cells in the rabbit thoracic aorta may reflect the relative amounts of preformed S-nitrosothiols that are required to control the distensibility of vascular smooth muscle in this artery.

Smaller arteries and especially those with diameters at and below ≈200 μm operate under a state of partial constriction (tone), and are the arteries generating peripheral vascular resistance to blood flow (Brody and Zimmerman, 1976; Jacob et al., 1988; Izzard et al., 1995). This intrinsic tone also provides a set point from which arteries can constrict or dilate to control blood flow (Izzard et al., 1995; Schubert and Mulvany, 1999). Thus resistance-sized arteries have a major influence on peripheral vascular resistance and both systolic and diastolic arterial blood pressures (Schubert and Mulvany, 1999). In addition, because

blood flow is dependent inversely on vessel diameter to the fourth power, small changes in luminal diameter lead to substantial changes in blood flow (Ku and Zhu, 1993). Accordingly, the presence of NADPH diaphorase in virtually 100% of endothelial cells in small pulmonary, mesenteric and femoral arteries of the rat may reflect the relative amounts of preformed S-nitrosothiols that are required to control the tone of vascular smooth muscle in these arteries.

In agreement with the findings of Loesch et al (2003, 2004) in rabbit thoracic aorta and rat basilar artery, we noted that some vesicles of rat small pulmonary, mesenteric and femoral arteries were devoid of NADPH-diaphorase activity. The larger vesicles devoid of NADPH diaphorase are likely to be Weibel-Palade bodies that secrete a variety of factors including von Willebrand factor (Tondaij et al., 2006), P-selectin (Cleator et al., 2006) and endothelin-1 (Harrison et al., 1995; Fukushige et al., 2000), a potent vasoactive agent (Yanagisawa et al., 1988). Endothelial cells contain endothelin ET_{B1} receptors (Warner et al., 1993), which elicit the release of nitrosyl factors (Gray and Clozel, 1994). The smaller vesicles lacking NADPH diaphorase may be those that store and secrete adenosine (Bodin and Burnstock, 2001) and/or chemokines (Oynebraten et al., 2005). These vesicles may also contain the potent EDRF, prostacyclin (Moncada et al., 1976), since prostacyclin synthase is abundant in cytoplasmic vesicles in human umbilical vein endothelial cells (Spisni et al, 2001).

Visualization of NADPH-diaphorase activity after mobilization of vesicles

Exposure of rat small pulmonary, mesenteric and femoral arteries cells to the endothelium-dependent agonist, acetylcholine, elicited a substantial increase in the number of vesicles fused to the plasma membrane of these cells, and in particular the membranes adjacent to the vascular smooth muscle. This was readily seen in the sections in which β-NADPH was not included in the reaction mixture. More specifically, the lack of NADPH diaphorase in these cells did not obscure the cytoplasmic vesicles and in particular those that were closely associated with the plasma membranes of the endothelial cells (see below). The ability of acetylcholine to elicit exocytotic fusion of cytoplasmic vesicles is a ***novel*** finding, which supports the concept that Ca^{2+}-*dependent* and Ca^{2+}-*independent* mobilization of vesicular stores of vasoactive factors plays a key role in the regulation of vascular tone (see Davisson et al., 1996a).

NADPH diaphorase in endothelial cells of rat small pulmonary, mesenteric and femoral arteries was found in large numbers of vesicular-like structures, many of which were closely associated with the abluminal membrane of the endothelial cell (i.e., that in close association with the vascular smooth muscle). Exposure of these cells to acetylcholine elicit a marked qualitative increase in the number of NADPH diaphorase-positive vesicles that were close to or actually associated with the plasma membrane of these cells. Whether these vesicles containing NADPH diaphorase reaction product were fusing with the cell membrane was difficult to ascertain due to the dark hue of the reaction product

obscuring the endothelial cell membrane. Nonetheless, we expect that on-going analyses based on quantifying the relative amounts of NADPH diaphorase associated with plasma membranes will confirm the qualitative assessments.

Acetylcholine also elicited the mobilization of NADPH diaphorase-negative vesicles to the plasma membranes of rat small pulmonary, mesenteric and femoral arteries. Since the arteries were immersion fixed at the nadir of vessel tension, it appears that vesicles containing S-nitrosothiols (NADPH diaphorase positive vesicles) and those that are devoid of S-nitrosothiols contribute to the arterial relaxation. We are assuming that the lumen of the NADPH diaphorase-negative vesicles were devoid of S-nitrosothiols and therefore would not have contained NADPH diaphorase prior to exocytosis. However, it is possible that the lumen of vesicles contained S-nitrosothiols that were secreted prior to immersion fixation and so would have stained for NADPH diaphorase before exocytosis. Again, the vesicles that do not stain for S-nitrosothiols/NADPH diaphorase could contain any number of vasoactive factors including von Willebrand factor (Tondaij et al., 2006), P-selectin (Cleator et al., 2006), endothelin-1 (Harrison et al., 1995; Fukushige et al., 2000), adenosine (Bodin and Burnstock, 2001) chemokines (Oynebraten et al., 2005), and/or prostacyclin (Moncada et al., 1976), since prostacyclin synthase, is abundant in cytoplasmic vesicles in human umbilical vein endothelial cells (Spisni et al, 2001).

CONCLUSIONS

The present study provides compelling evidence that NADPH diaphorase/S-nitrosothiol-containing vesicles are mobilized to exocytosis by the endothelium-dependent agonist, acetylcholine, most likely by Ca^{2+}-dependent mechanisms. NOS inhibitors abolish the dilator effects of endothelium-*dependent* agonists and Ca^{2+}-ionophore A23187 in large vessels (Garland et al., 1995; Hwa et al., 1994) ***but not*** in resistance-sized arteries (Garland et al., 1995; Hwa et al., 1994; Edwards and Weston, 1998; Fisslthaler, 1999; Garland and McPherson, 1992; Illiano et al., 1992; Illiano et al., 1993; Nagao and Vanhoutte, 1991,1992; Adeagbo and Malik, 1990, Adeagbo and Triggle, 1993; Mantelli et al., 1995; Siegal et al., 1989). Moreover, NOS inhibitors ***minimally reduce*** endothelium-dependent vasodilation *in vivo* (Mugge et al., 1991; Ross et al., 1991; Davisson et al., 1996; Colombari et al., 1998; Danser et al., 1998, 2000; Tom et al., 2001). In addition, inhibition of soluble guanylate cyclase blocks NO-mediated (Woodman et al., 2000; Olson et al., 1997) but not endothelium-dependent relaxation (Woodman et al., 2000) in resistance arteries. Several studies have also found that inhibition of ATP production markedly diminishes endothelium-dependent relaxation, which is analogous to Ca^{2+}-*dependent* exocytosis in nerve terminals (Griffith et al., 1986; Weir et al., 1991; Richards et al., 1991). Taken together, these findings support the novel concept that endothelium-dependent vasodilation depends in part on exocytosis of vesicular pools of S-nitrosothiols (see Davisson et al., 1996b; Lewis et al., 2006b).

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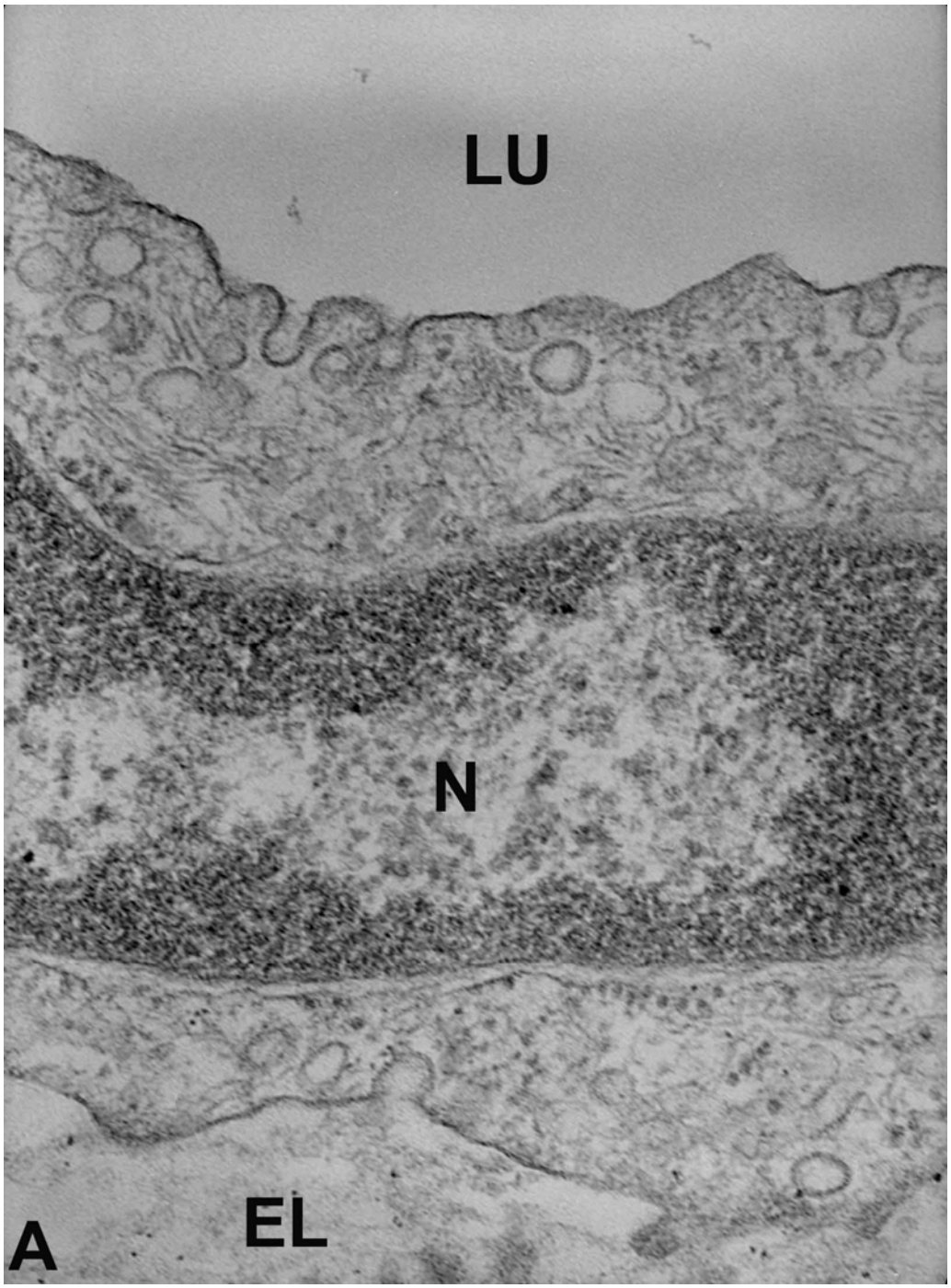

Fig. 6.1A Electron micrograph of a pulmonary artery endothelial cell lacking NADPH diaphorase. β-NADPH was omitted from the incubation medium. LU = lumen, N = nucleus, EL = elastic lamina. Magnification x 48,000.

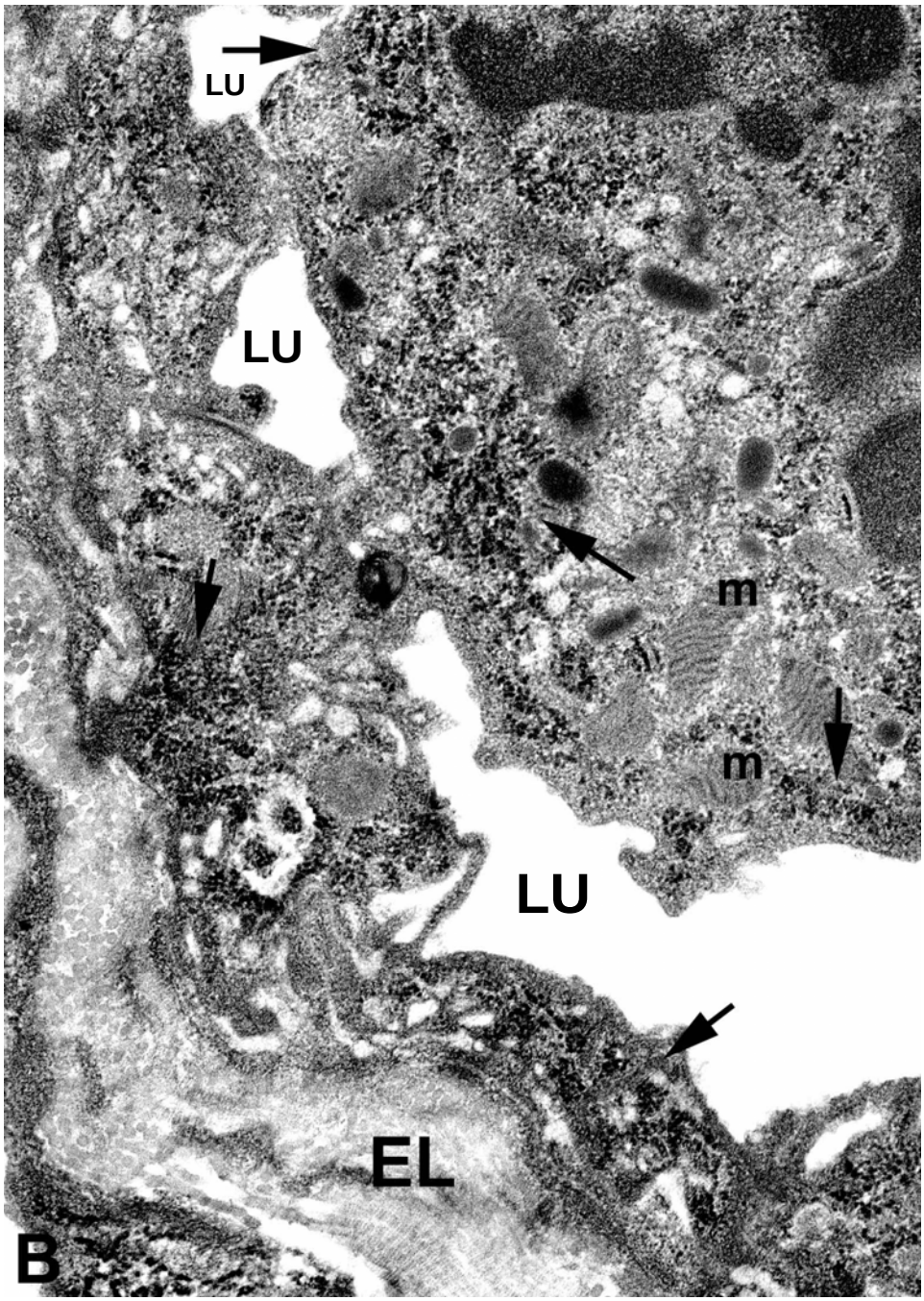

Fig. 6.1B Electron micrograph of multiple pulmonary artery endothelial cells displaying NADPH-diaphorase. Arrows indicate regions of cytoplasm displaying clustered NADPH diaphorase reaction product. LU = lumen, EL = elastic lamina, m = mitochondria. Magnification x 19,000.

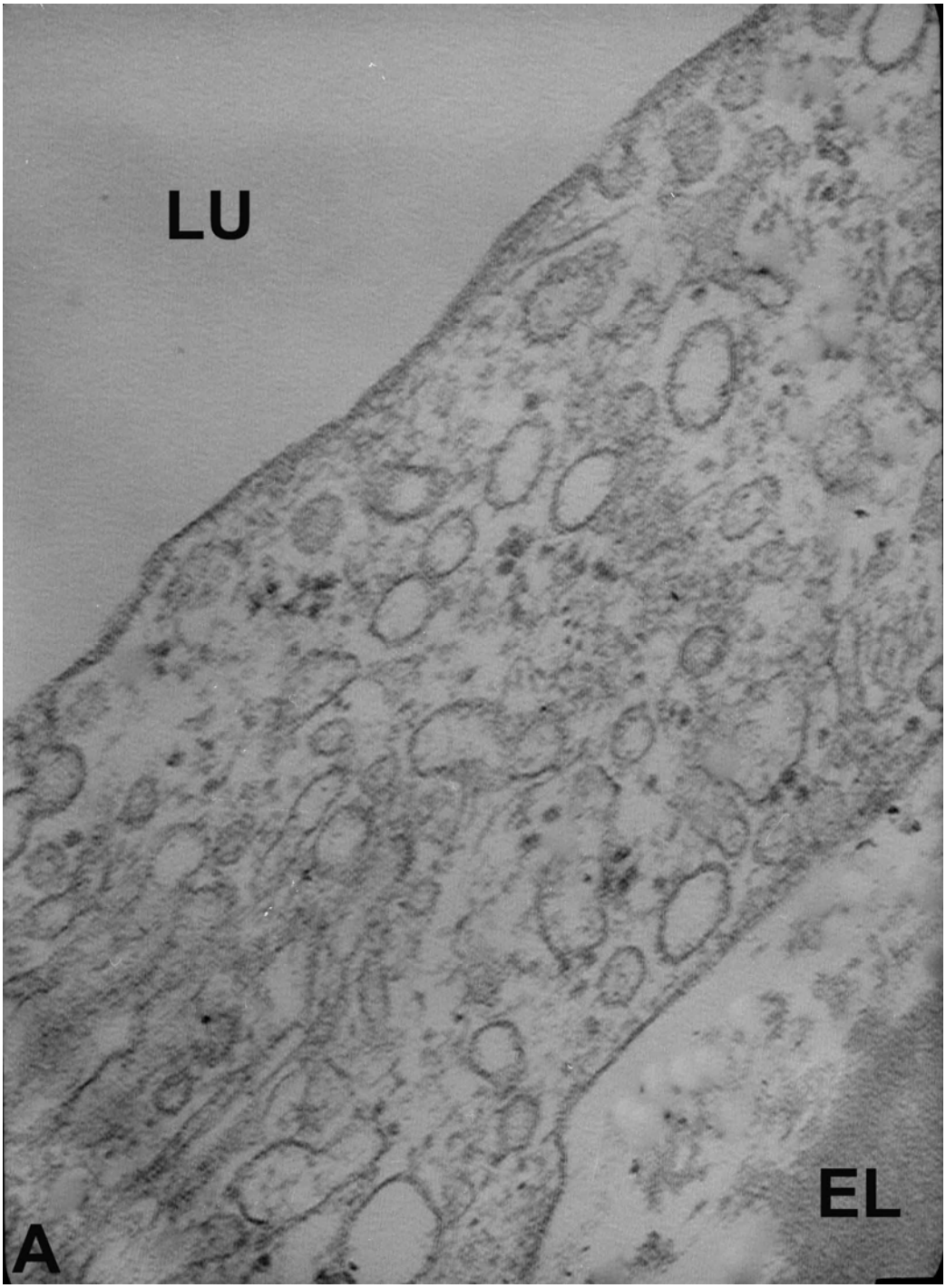

Fig.6.2A Electron micrograph of a mesenteric artery endothelial cell lacking NADPH diaphorase. β-NADPH was omitted from the incubation medium. LU= lumen, N=nucleus, EL=elastic lamina. Magnification x 72,000.

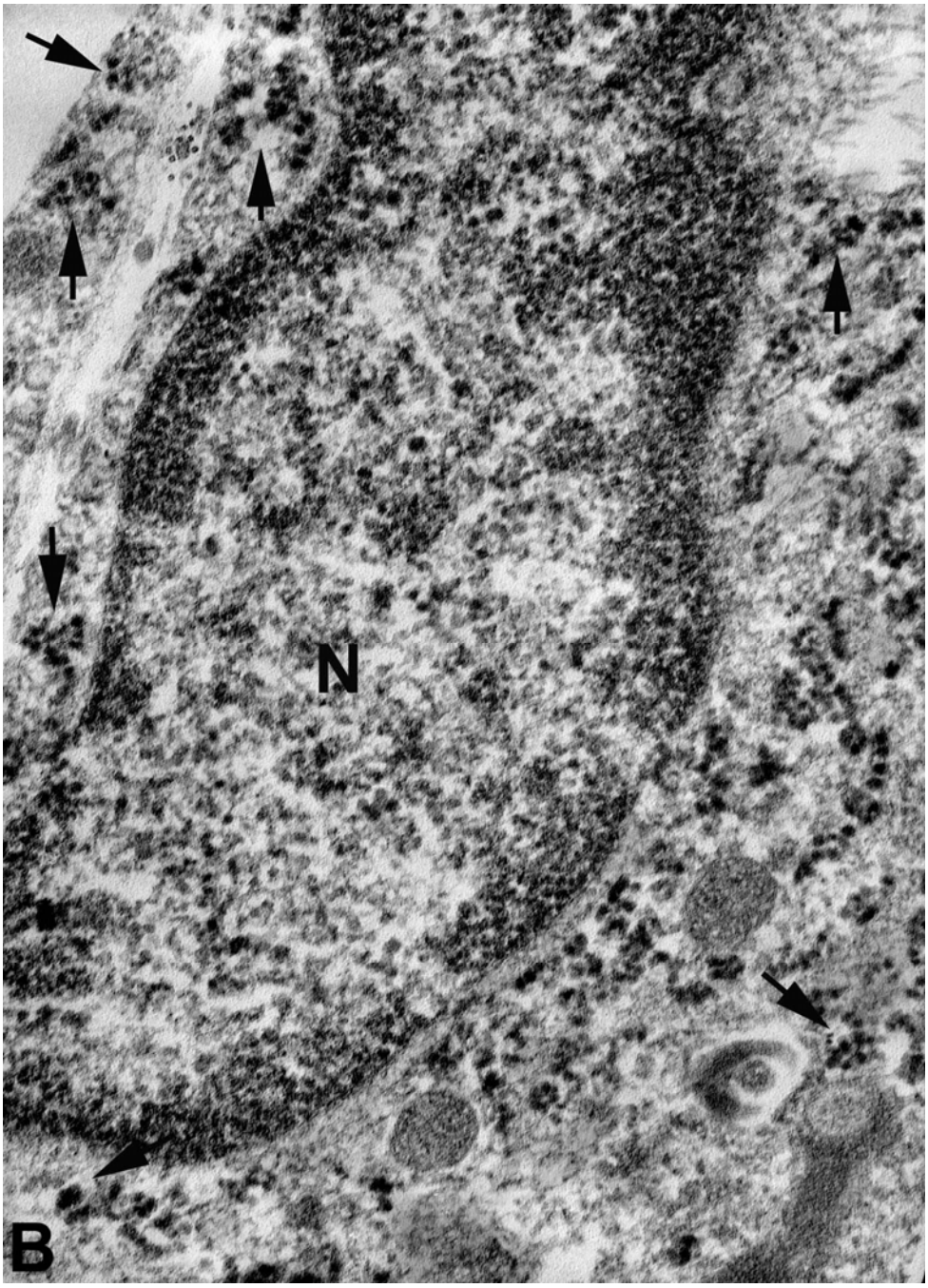

Fig. 6.2B Electron micrograph of a mesenteric artery endothelial cell displaying NADPH diaphorase. Arrows indicate regions of cytoplasm displaying clustered NADPH diaphorase reaction product. N=nucleus, Magnification x 48,000.

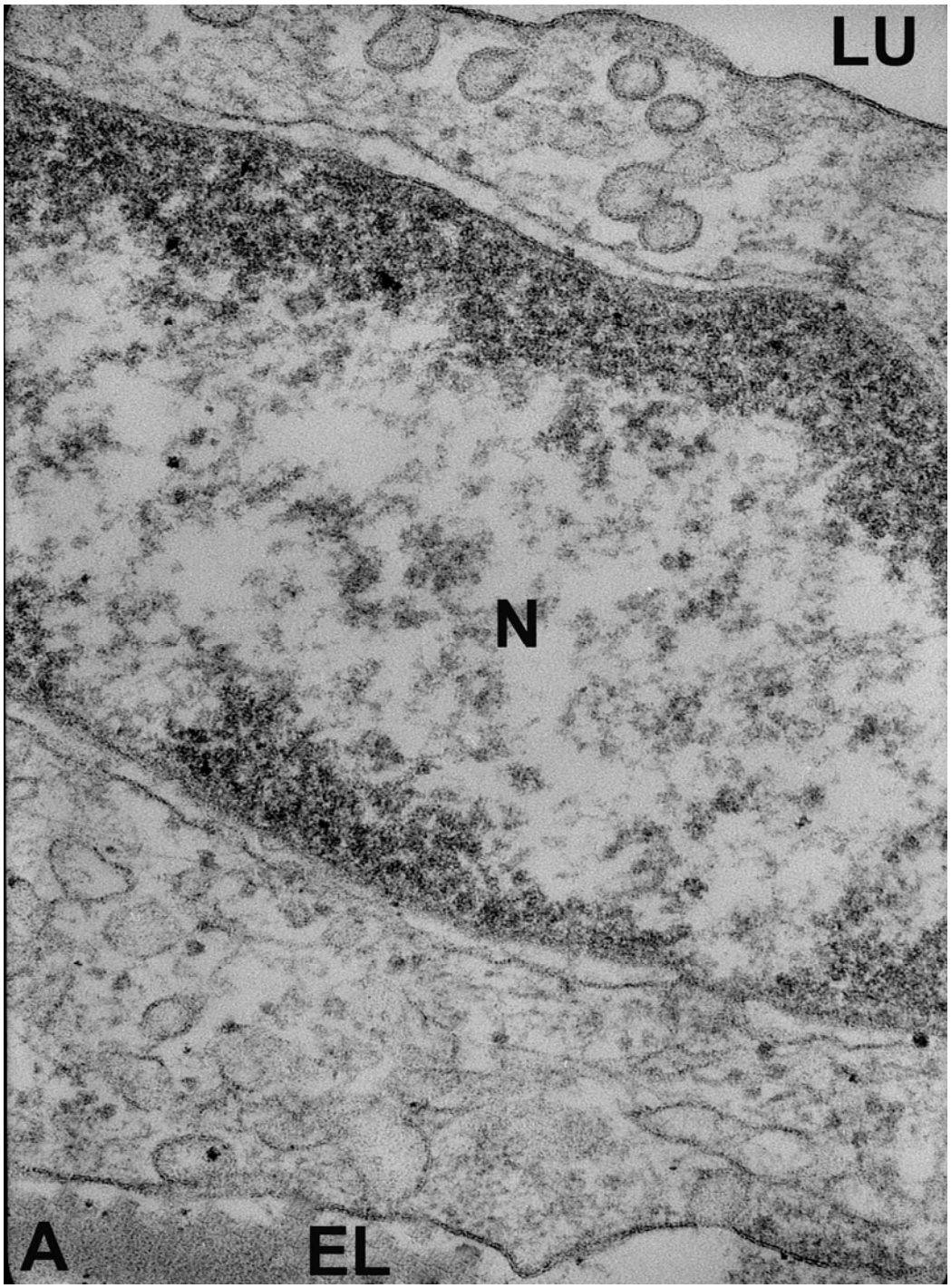

Fig. 6.3A Electron micrograph of a femoral artery endothelial cell lacking NADPH diaphorase. β-NADPH was omitted from the incubation medium. LU = lumen, N = nucleus, EL = elastic lamina. Magnification x 72,000.

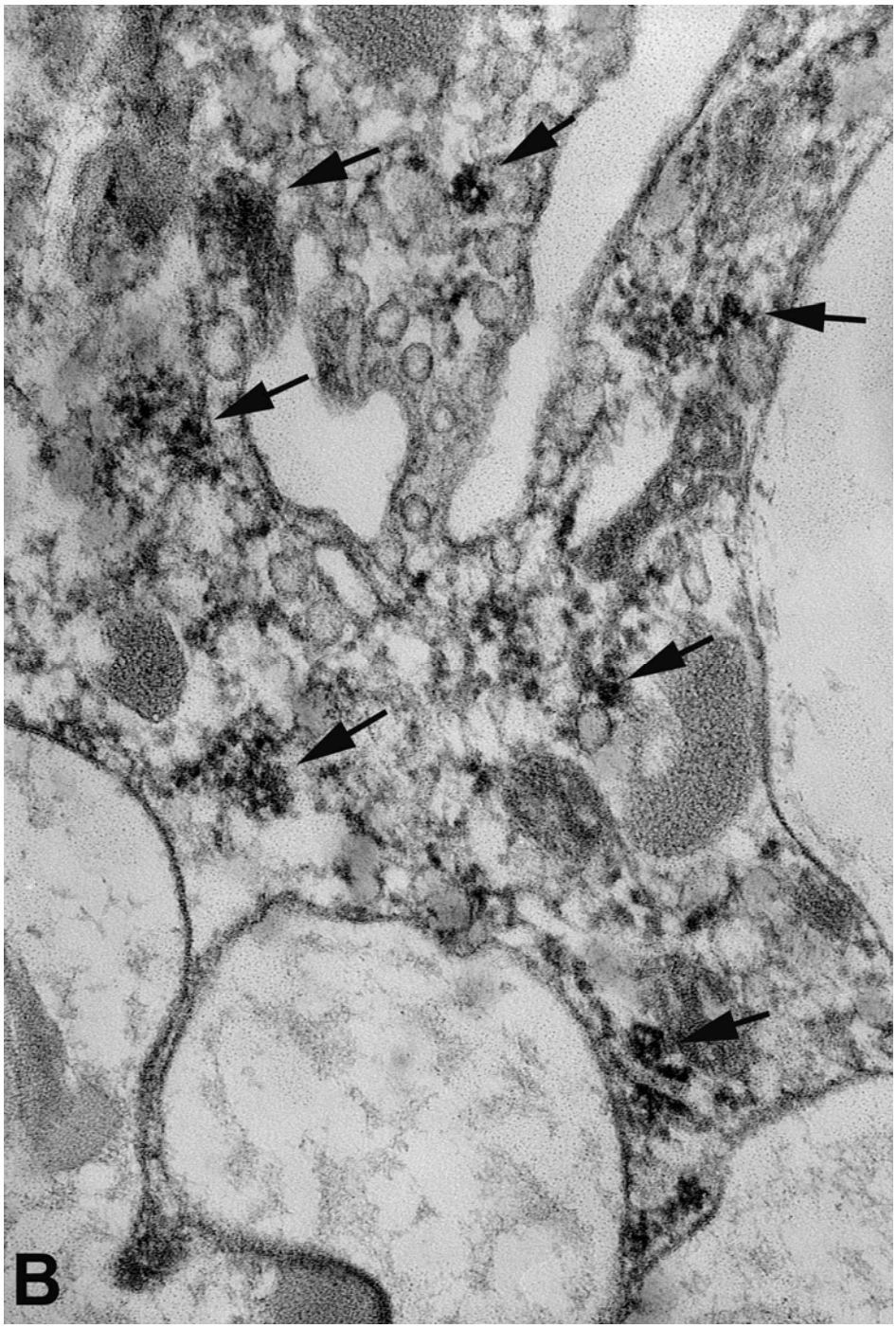

Fig. 6.3B Electron micrograph of a femoral artery endothelial cell displaying NADPH diaphorase. Arrows indicate regions of cytoplasm displaying clustered NADPH diaphorase reaction product. Magnification x 36,000.

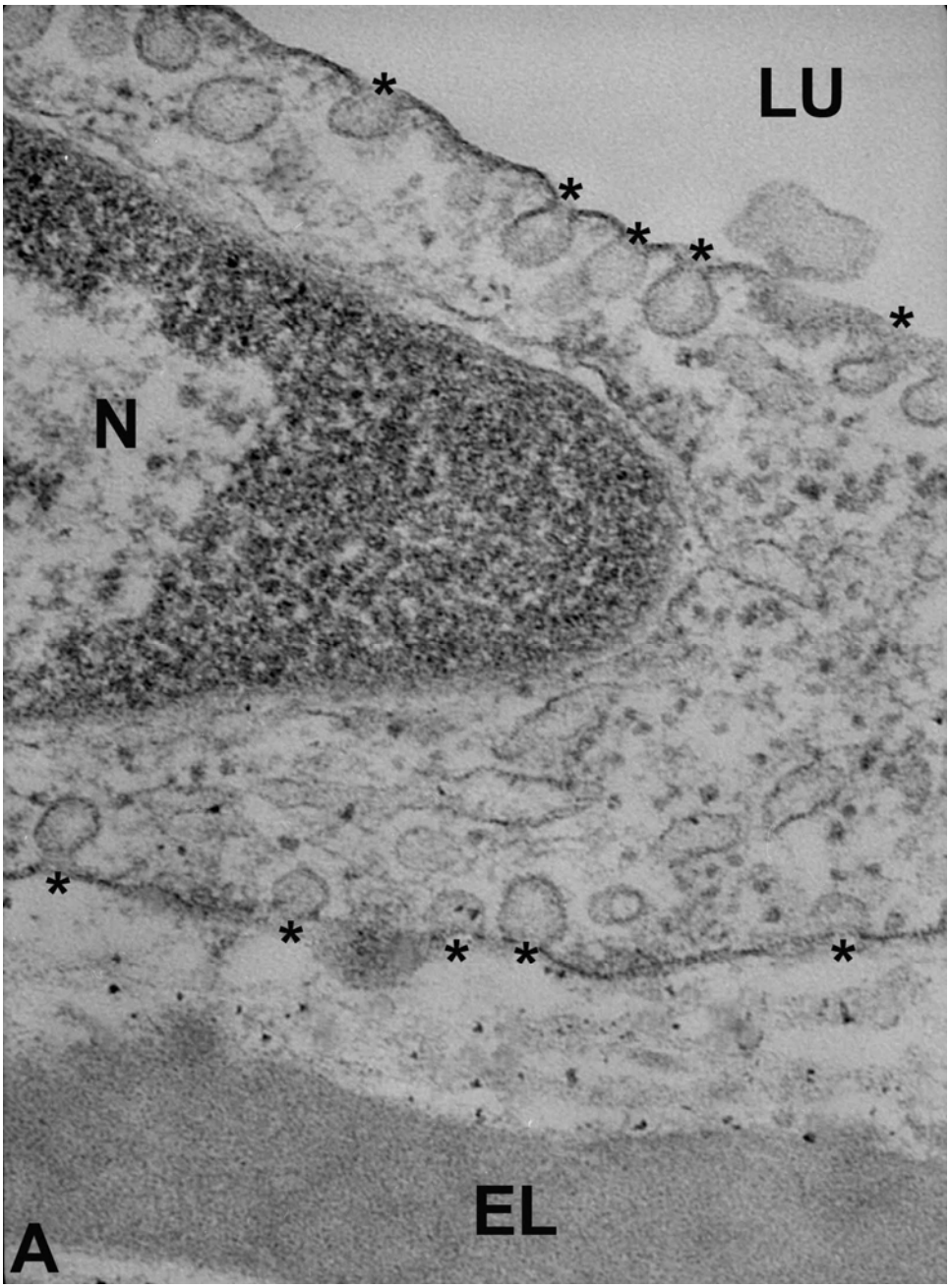

Fig. 6.4A Electron micrograph of an acetylcholine (1 μM) stimulated pulmonary artery endothelial cell lacking NADPH diaphorase. β-NADPH was omitted from the incubation medium. Note the vesicles fused with endothelial cell membranes (asterisks) due to agonist stimulation. LU = lumen, N = nucleus, EL = elastic lamina. Magnification x 72,000.

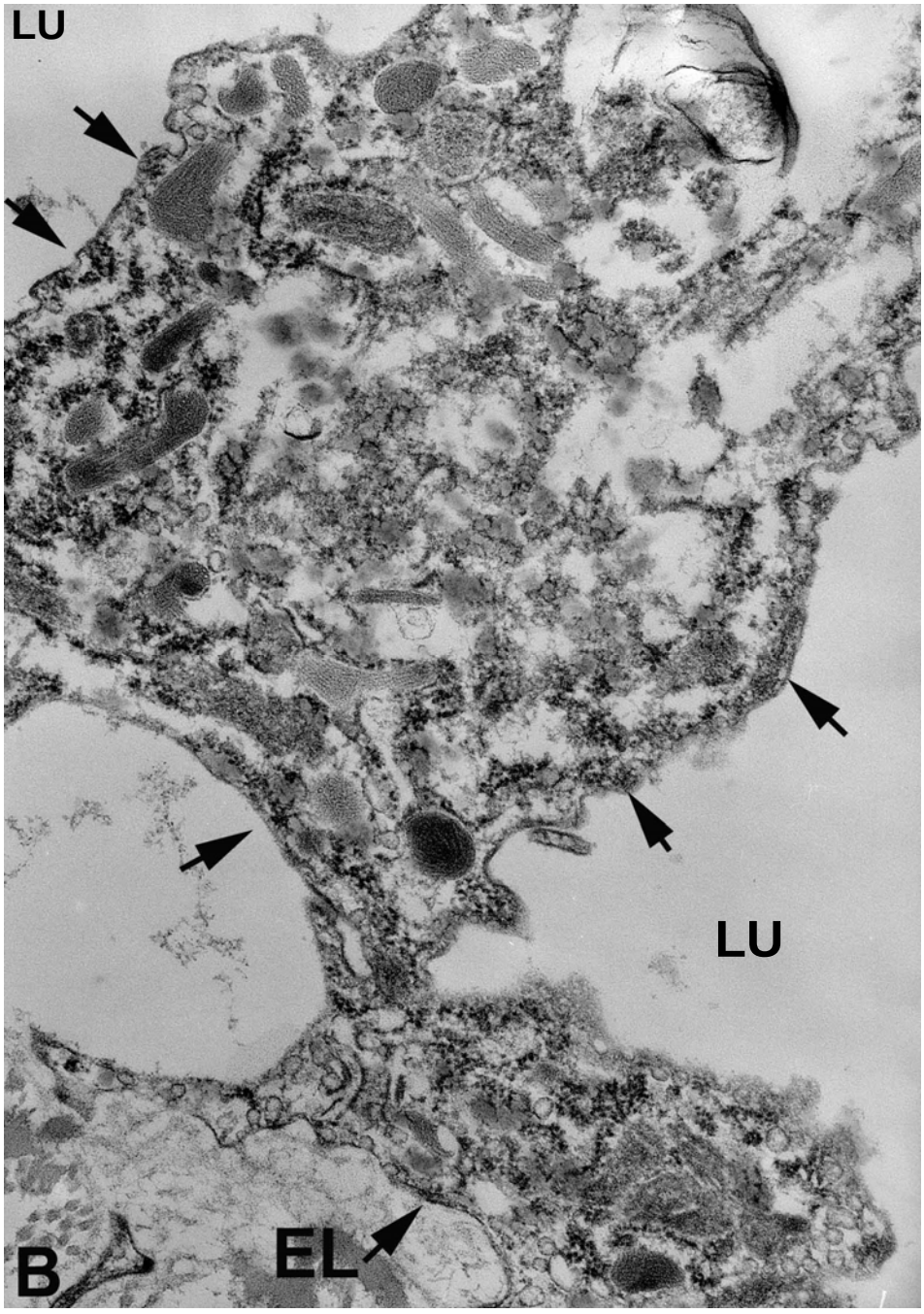

Fig. 6.4B Electron micrograph of an acetylcholine (1 μM) stimulated pulmonary artery endothelial cell displaying NADPH diaphorase activity. Arrows indicate regions of the plasma membrane displaying clustered NADPH diaphorase reaction product. EL = elastic lamina. LU = Lumen. Magnification x 19,000

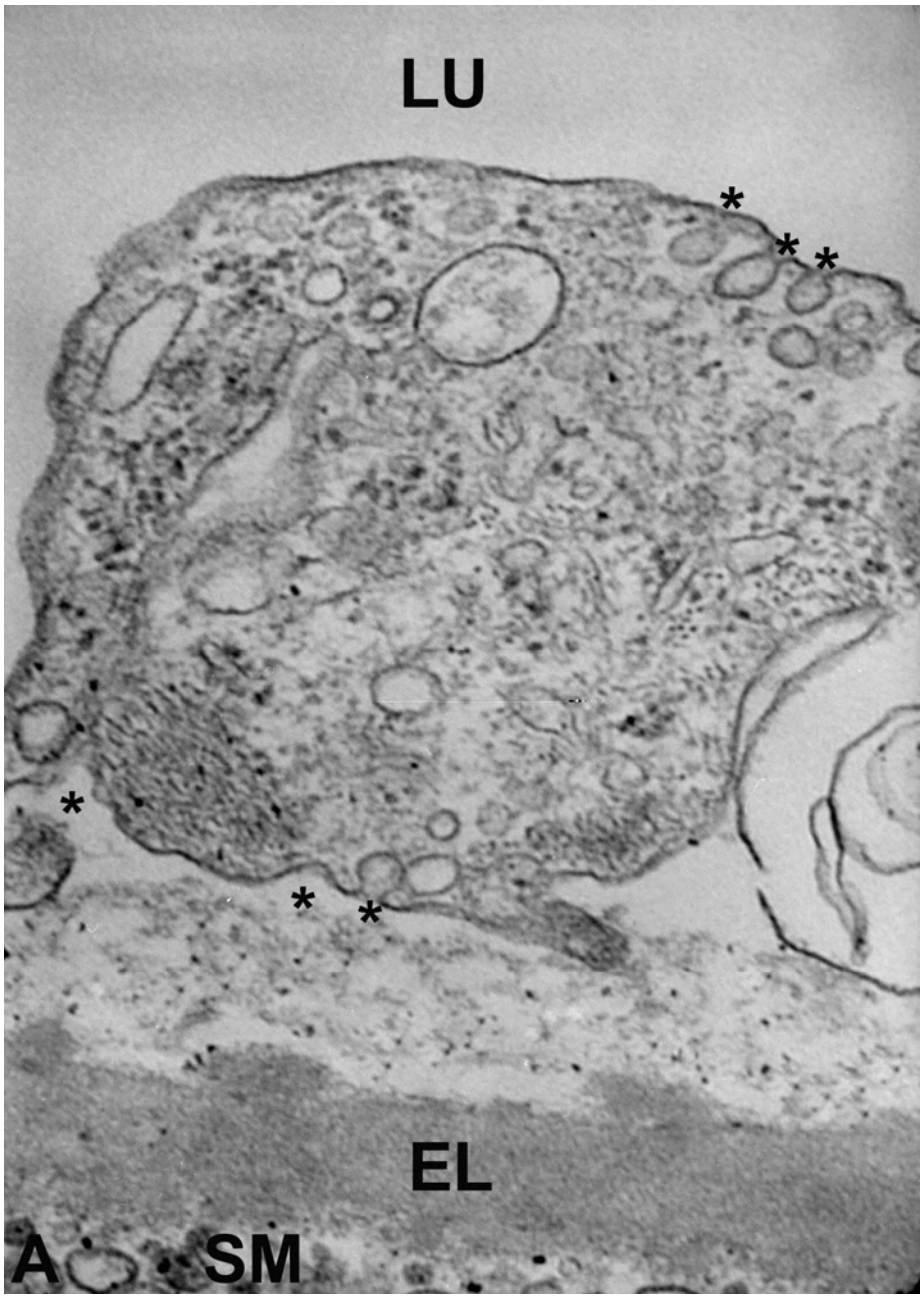

Fig. 6.5A Electron micrograph of an acetylcholine (1 μM) stimulated mesenteric artery endothelial cell lacking NADPH diaphorase. β-NADPH was omitted from the incubation medium. Note the vesicles fused with endothelial cell membranes (asterisks) due to agonist stimulation. LU= lumen, EL = elastic lamina, SM = smooth muscle. Magnification x 58,000.

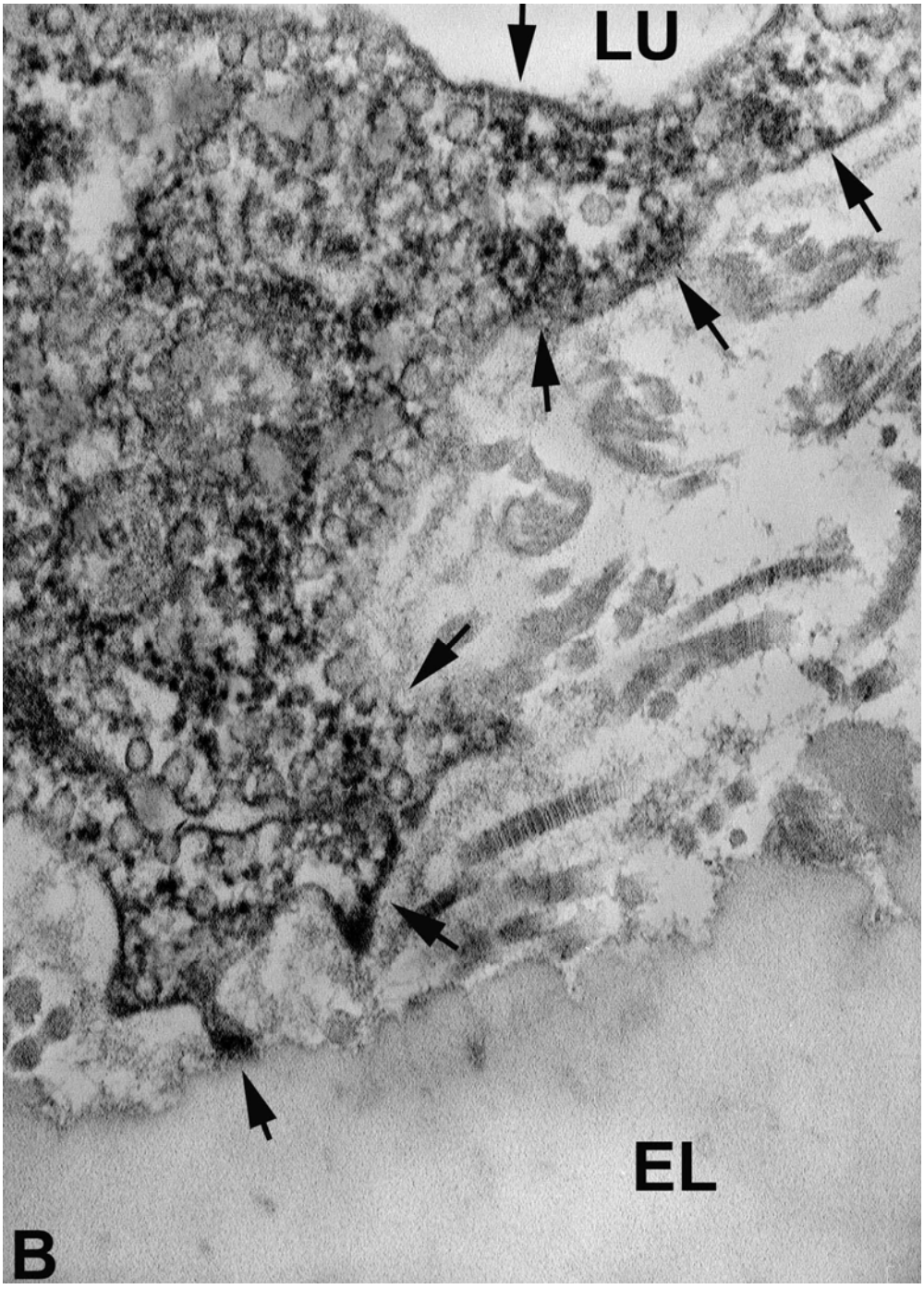

Fig. 6.5B Electron micrograph of an acetylcholine (1 μM) stimulated mesenteric artery endothelial cell displaying NADPH diaphorase. Arrows indicate regions of the plasma membrane displaying clustered NADPH diaphorase reaction product. EL = elastic lamina. LU = lumen. Magnification x 48,000

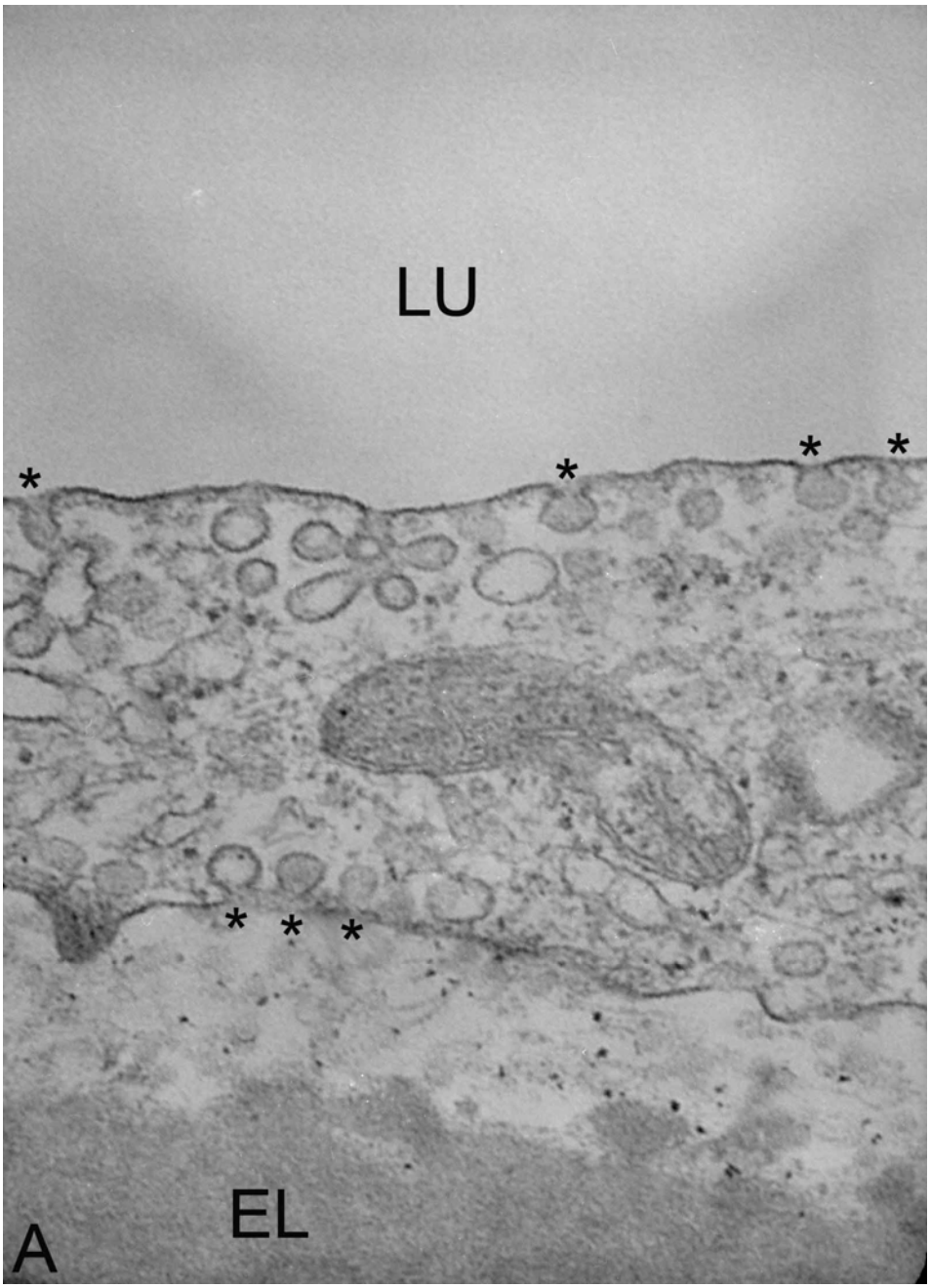

Fig. 6.6A Electron micrograph of an acetylcholine (1 μM) stimulated femoral artery endothelial cell lacking NADPH diaphorase. β-NADPH was omitted from the incubation medium. Note the vesicles fused with endothelial cell membranes (asterisks) due to agonist stimulation. LU = lumen, EL = elastic lamina. Magnification x 58,000.

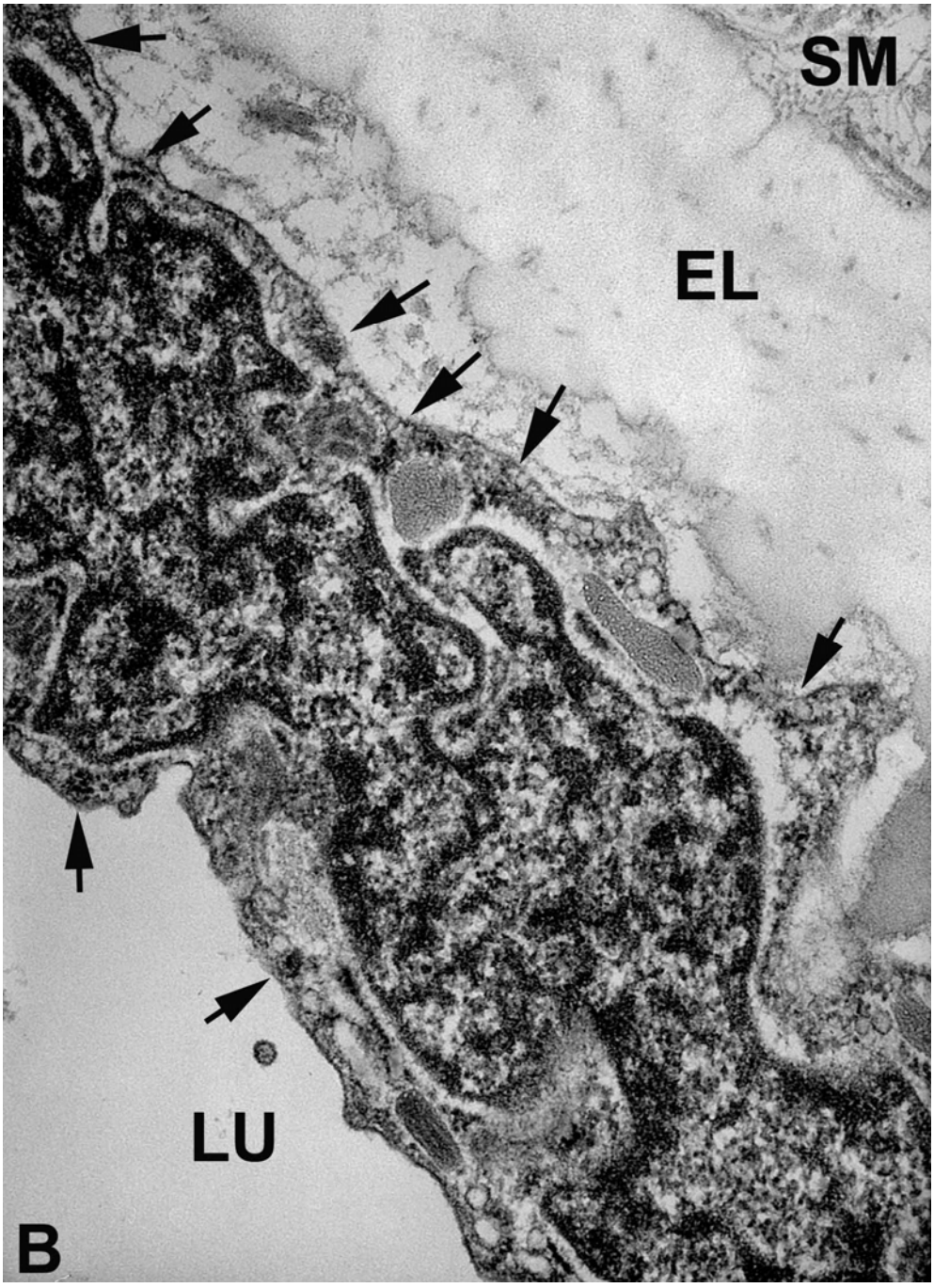

Fig. 6.6B Electron micrograph of an acetylcholine (1 μM) stimulated femoral artery endothelial cell displaying NADPH diaphorase. Arrows indicate regions of the plasma membrane displaying clustered NADPH diaphorase reaction product. EL = elastic lamina, LU = lumen, SM = smooth muscle. Magnification x 29,000

CHAPTER 7

SUMMARY AND CONCLUSIONS

The discovery of endothelium-dependent relaxation of rabbit aortic rings by Furchgott and Zawadski (1980) paved the way for new insights into the physiological regulation of the vascular smooth muscle tone and the etiology of vascular diseases that affect artery function including atherosclerosis (Anderson 2003), hypertension (Kojda and Harrison, 1999; Taddei and Salvetti 2002), sepsis (Symeonides and Balk, 1999), inflammatory responses (Ross, 1999), diabetes (Beckman et al., 2003; Rizzoni et al., 2001; Schofield et al., 2002; Makimattila and Yki-Jarvinen 2002; Endemann et al., 2004).

The initial discovery that endothelium-dependent relaxing factor (EDRF) in large arteries was nitric oxide (NO) (Ignarro et al., 1987 and Palmer et al., 1987) led to an explosion of research in this area. Over time it became apparent that NO did not fully account for agonist-induced endothelium-*dependent* relaxation in physiologically important resistance arteries (see Rosenblum, 1992; Davisson et al., 1996a; Woodman et al., 2000). The portion of endothelium-dependent relaxation that was not blocked by inhibition of NO synthesis was associated with hyperpolarization of the vascular smooth muscle (Chen et al, 1988) and was abolished by potassium channel blockers or depolarizing concentrations of potassium (Adeagbo and Triggle, 1993). The unknown mediator responsible for this phenomenon was therefore termed endothelium-derived hyperpolarizing factor (EDHF) (Taylor and Weston, 1988).

There is now substantial evidence that NO exists in mammalian cells as NO-containing *S*-nitrosothiols and dinitrosyl iron compounds (Zhang and Hogg, 2005, Vanin, 1998). Moreover, there is a wealth of *in vitro* (see Myers et al, 1990; Rubanyi et al, 1991; Rosenblum, 1992; Danser et al., 1998, 2000; Batenburg et al., 2004a,b) and *in vivo* (see Davisson et al., 1996a; Woodman et al, 2000; Lewis et al., 2005, 2006a,b,c) evidence that the L-S-nitrosothiol, L-S-nitrosocysteine, is a primary EDRF and EDHF in resistance arteries.

Since S-nitrosothiols are readily degraded by a variety of intracellular factors, has been suggested that S-nitrosothiols may be stored in cytoplasmic and membranal vesicles, and in lipophilic protein folds and in interstitial spaces (Rafikova et al, 2002; Mannick et al, 2001). Moreover, L-S-nitrosocysteine is highly polar and extremely lipophobic (i.e., it has no oil-to-water partition coefficient) (see Kowaluk and Fung, 1990) and in order for it to pass through the cell membrane, it would be need to be stored within a lipid structure, and especially a vesicle that would be subject to Ca^{2+}-*dependent* and Ca^{2+}-*independent* exocytosis. These observations led us to the present set of studies described in this dissertation. The ***objectives*** were to determine:

1. The presence of vesicles in endothelial cells in arteries from mesenteric, femoral and pulmonary arteries of rats.
2. The presence of NO synthase (NOS) in membrane of endothelial vesicles.
3. The presence of NADPH-diaphorase within endothelial vesicles.

4. The mobilization of endothelial vesicles to vesicular exocytosis upon stimulation with acetylcholine.
5. Mobilization of endothelial vesicles to exocytosis by hypoxia.
6. Whether acetylcholine mobilizes endothelial vesicles containing NADPH-diaphorase (S-nitrosothiols) within the lumen.
7. Presence of proteins such as Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) and fusion proteins on the membranes of vesicles in vascular endothelial cells that similar to neurons and adrenal chromaffin cells may be necessary for vesicular exocytosis.

Our first study addressed **aim 1**

Although Loesch et al (1994) demonstrated the presence of vesicles in endothelial cells of the basilar artery, we needed to determine that these vesicles were common to all endothelial cells, since this would provide evidence that these vesicles are physiologically relevant and that they carry out a function that is common to most/all arteries. We chose a range of different arteries such as cerebral, basilar, pulmonary, mesenteric and femoral. We found that all of the above arteries exhibited the vesicles described by Loesch et al (1994).

Our second study addressed **aims 2 and 7**

We needed to determine that the membranes of endothelial cells and cytoplasmic vesicles expressed fusion proteins that support vesicular exocytosis. Most importantly, we needed evidence that synaptosomal-associated protein of

25kD (SNAP-25) and syntaxin were located on the endothelial cell membrane and that vesicle-associated membrane protein (VAMP) was located on the vesicle membrane, since these three proteins constitute the core complex that is thought to mediate fusion (Bennett et al., 1992; Calakos et al., 1994; Chapman et al., 1994; Söllner et al., 1993). Once it was determined that these and other proteins necessary for fusion were visible at the light level, we proceeded to ultrastructural studies. VAMP, SNAP-25 and syntaxin and CaMKII were detected, however at low levels and we conclude that perhaps other isoforms of these proteins warrant further investigation.

Our third study addressed **aims 4 and 5**.

Acetylcholine-induced relaxation of arteries requires an intact endothelium (Furchgott and Zawadski, 1980). We wanted to see whether acetylcholine and hypoxia would mobilize endothelial vesicles to exocytosis. We used the appearance of omega shaped structures within the endothelial cell membrane as evidence of vesicular exocytosis. Our studies found clear evidence that vesicular exocytosis does take place in endothelial cells in response to the above stimuli.

Our final study addressed **aims 3 and 6**

Studies in our laboratory provided evidence that NADPH diaphorase histochemistry detects preformed pools of S-nitrosothiols. Loesch et al. (1994) demonstrated the presence of NADPH-diaphorase positive vesicles in the endothelium of the basilar artery, suggesting that the vesicular release of S-

nitrosothiols may take place. We wanted to ascertain whether NADPH diaphorase positive vesicles were also present in endothelial cells of small pulmonary, mesenteric and femoral arteries. In study 3, we determined that acetylcholine mobilized endothelial vesicles to fuse to the cell membrane. In this study, we wanted to determine whether NADPH diaphorase positive vesicles would be mobilized to exocytosis by acetylcholine. We found that small pulmonary, mesenteric and femoral arteries contain NADPH diaphorase-positive vesicles and that qualitative analyses suggest that acetylcholine increased the association of these vesicles with the plasma membranes.

In summary, the studies reported in this dissertation provide compelling evidence that endothelium-dependent relaxation of small arteries is mediated at least in part by the mobilization of S-nitrosothiols containing vesicles.

Points arising from PhD defense

Point 1. It should be noted that the average diameter of the vesicles observed in this study was approximately 70-80nm. However, this may not reflect the true diameter. Only the largest measurement of vesicle diameter observed truly reflects the actual diameter. In this study the largest diameter observed was 100nm. Hence, it may be suggested that the diameter of the endothelial vesicles of small pulmonary, mesenteric and femoral arteries observed in this study is higher than reported.

Point 2. We reported that the average diameter of the endothelial vesicles observed in this study was approximately 70-80 nm. This raises the possibility that the vesicles observed to be fused with the endothelial cell membrane may actually be caveolae, whose average diameter has been reported to be 50-80 nm (van Deurs et al, 1993). Immunogold labeling of endothelial cells with caveolin, a marker for caveolae (Smart et al, 1994) would clarify this issue.

Point 3. We observed exocytosis of endothelial vesicles. The possibility that these vesicles may be caveolae endocytosing and forming free endocytic vesicles (clathrin-coated pits), could be addressed by labeling for endocytic markers for clathrin. Experiments involving a labeling assay in order to distinguish between surface-connected and free vesicles would need to be performed. Also, double-labelling experiments with endocytic markers and antibodies against caveolin could be performed.

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