

mean-square error if multiplied with a given time series (say, movement velocity) and subtracted from the original signal at each time point. This pattern is given by $P_i = \langle v(t)H_i(t) \rangle / \langle v^2(t) \rangle$, where P_i is the pattern at the location of the i th sensor, $H_i(t)$ is the activity in this sensor, $v(t)$ is the time series (here, movement velocity) and $\langle \dots \rangle$ denotes a temporal average.

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Lipids are required for directional pollen-tube growth

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Successful pollination and fertilization are absolute requirements for sexual reproduction in higher plants. Pollen hydration, germination and penetration of the stigma by pollen tubes are influenced by the exudate on wet stigmas¹ and by the pollen coat in species with dry stigmas^{2–5}. The exudate allows pollen tubes to grow directly into the stigma, whereas the pollen coat establishes the contact with the stigma. Pollen tubes then grow into the papillae, which are covered by a cuticle. The components of the exudate or pollen coat that are responsible for pollen tube penetration are not known. To discover the role of the exudate, we tested selected compounds for their ability to act as functional substitutes for exudate in the initial stages of pollen-tube growth on transgenic stigmaless tobacco plants¹ that did not produce exudate. Here we show that lipids are the essential factor needed for pollen tubes to penetrate the stigma, and that, in the presence of these lipids, pollen tubes will also penetrate leaves. We propose

that lipids direct pollen-tube growth by controlling the flow of water to pollen in species with dry and wet stigmas.

In transgenic, female-sterile tobacco plants in which the secretory zone of the stigma is ablated by expression of the cytotoxic gene *STIG 1-barnase*, pollen tubes only penetrate the stigma and successfully fertilize ovules when stigma exudate of wild-type plants is applied to the ablated stigmas^{1,6}. To determine whether stigma function is also restored by exudates of other plant species, we applied the lipid-rich⁷ exudate of *Petunia* (a close relative of tobacco), and the carbohydrate-rich⁸ exudate of lily (a distantly related species) to the stigmas. With *Petunia* exudate, pollen tube penetration and seed setting occurred (Fig. 1a). Application of lily exudate resulted in pollen hydration and germination, but not penetration (Fig. 1b), indicating that lipids in the exudate may play a role in pollen tube penetration.

The lipid fraction of the exudate contains many saturated and unsaturated fatty acids, which usually occur as triacylglycerides^{9,10}. To determine whether triacylglycerides are involved in pollen tube penetration, we applied a purified oil with a fatty-acid composition similar to that of exudate to stigmaless pistils. Application of this oil restored stigma function and resulted in seed production. Addition of the same oil in a 1:1 mixture of oil and lily exudate also restored the ability of pollen tubes to penetrate the stigma (see Methods). To investigate the effect of individual components of the purified oil, we applied the unsaturated triacylglycerides triolein, trilinolein and trilinolenin. Only application of trilinolenin allowed penetration of enough pollen tubes into the style (Fig. 1c) to result in seed production. In contrast, a liquid mixture of saturated triacylglycerides did not result in pollen tube penetration (Fig. 1d; see Methods).

We investigated the effects of the products of breakdown of triacylglycerides by applying glycerol, free fatty acids (oleic, linoleic or linolenic acid), monolinolein or dilinolenin. None of these products allowed penetration of enough pollen tubes into the style (see Methods). In the presence of trilinolenin, the *trans* form of trilinolenin, pollen tubes penetrated the stylar tissue, but were immediately arrested and exhibited severe deformations. Oils that are not based on triacylglycerides, such as silicon oils, did not restore stigma function (see Methods). These data indicate that *cis*-unsaturated triacylglycerides are sufficient and essential for the normal functioning of the exudate.

In genera with dry stigmas, such as *Brassica* and *Arabidopsis*, the

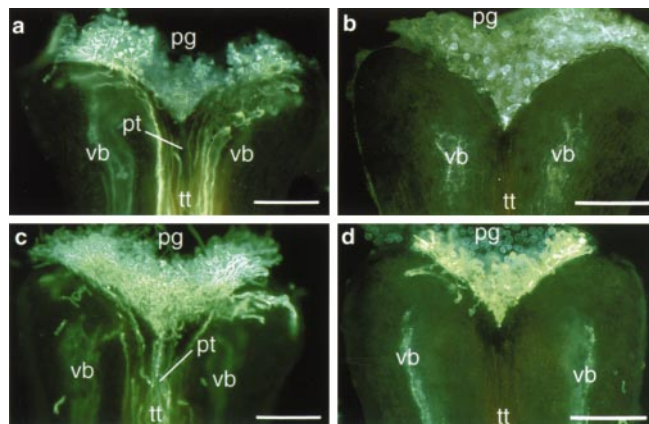


Figure 1 Fluorescence micrographs of longitudinal sections of stigmaless pistils 24 h after pollination. **a**, After application of *Petunia* exudate, pollen grains on the stigmaless surface become hydrated and germinate and the pollen tubes penetrate the stylar tissue. The effects of application of **b**, *Lilium* exudate, **c**, trilinolenin, and **d**, saturated triacylglycerides are also shown (see Methods). Bars represent 100 μ m. Abbreviations are as follows: pg, pollen grain; pt, pollen tube; tt, transmitting tissue; vb, vascular bundle.

pollen coat flows out from the exine to form a 'foot' (contact zone) between the grain and the stigma surface^{11,12}. The pollen coat contains lipids that are required for pollen hydration^{2,3,13,14}. It has been suggested that, during the evolution of dry stigmas, the pollen coat assumed the role of the exudate of wet stigmas^{15,16}. Our transgenic tobacco plants have lost the stigma's secretory zone and thereby the capacity to secrete exudate¹, but have retained the presence of a thin cuticle¹⁷ (Fig. 2c). Therefore, one could infer that stigmaless pistils may function as dry stigmas. Pollen of *Brassica napus*, *B. oleracea* and *Arabidopsis thaliana*, when placed on the ablated stigmas, formed a foot (Fig. 2a,b), and pollen tubes penetrated the pistil, but they were arrested at a length of $\sim 100\ \mu\text{m}$ (Fig. 2c). This supports the view that the pollen coat is functionally equivalent to the exudate, and suggests that lipids may also be required for pollen-tube penetration in species with dry stigmas.

The *Arabidopsis* mutants *cer1*, *cer3*, *cer6* and *pop1* are defective in synthesis of long-chain lipids and their pollen does not become hydrated on stigmas^{2,18} (Fig. 2d). Application of trilinolein enabled mutant pollen grains to produce tubes that penetrated the stigma

(Fig. 2e) and silique developed. Thus, triacylglycerides restore hydraulic contact between pollen grain and stigma in these mutants, allowing pollen to become hydrated.

The flower is the only part of the plant in which fluid or semifluid lipids are secreted at maturity on external surfaces, such as the stigma and pollen exine^{9,14,19}. To determine whether exudate can replace the stigmatoid tissue, we removed the cuticle of a mature leaf, applied *Petunia* exudate or trilinolein and added pollen. Tobacco pollen applied alone to the stripped leaf surface did not germinate, but, in the presence of exudate or trilinolein, pollen tubes penetrated the intercellular spaces of the parenchyma cells of the leaf and grew towards the abaxial side (Fig. 3a). Application of *Brassica* pollen grains (in the absence of exudate or oil) also resulted in penetration of the leaf by pollen tubes (Fig. 3b). No penetration occurred when pollen-germination medium, saturated triacylglycerides, dilinolein or silicon oils were applied to the leaf surface. Germination does not occur on a mature leaf when the cuticle is still present. However, pollen grains of tobacco in the presence of trilinolein or pollen grains of *Brassica* (in the absence of oil) were able to germinate and penetrate a young leaf with a thin cuticle (data

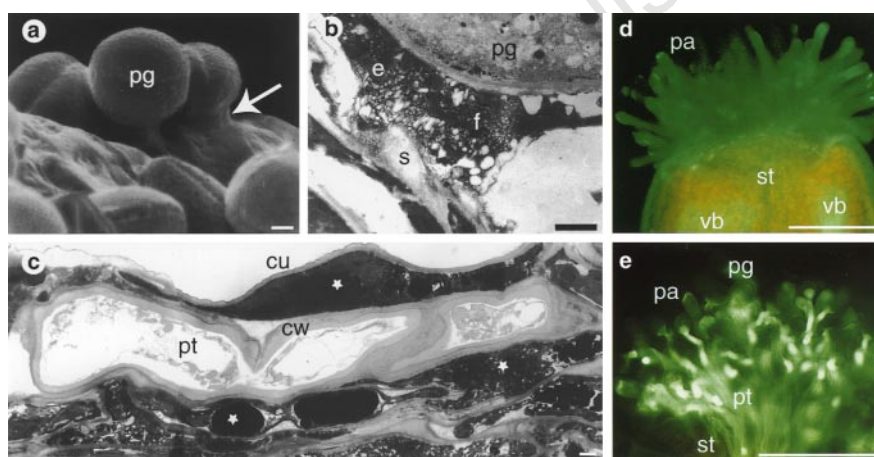


Figure 2 Growth of *Brassica* pollen on stigmaless pistils, and lack of growth of *Arabidopsis pop1* mutant pollen after self-pollination of *Arabidopsis*. **a-c**, Germination and tube growth of *Brassica* pollen on stigmaless pistils 24 h after pollination. **a**, SEM image showing the foot between the pollen grain and the stigmaless surface (arrow). **b**, TEM image showing the electron-opaque appearance in the foot characteristic of compatible pollen-stigma interactions. **c**, Pollen tubes penetrated the stigmaless pistil and grew between the

compressed, degenerated cells (asterisk) of the ablated stigma. Bar represents $1\ \mu\text{m}$. **d,e**, Effect of lipids on *Arabidopsis pop1* mutants 24 h after self-pollination. **d**, Pollen grains did not adhere to the papillae and were washed away during the preparation. **e**, After application of trilinolein to the stigma, pollen tubes penetrated the style. Bars represent $50\ \mu\text{m}$. Abbreviations are as follows: cu, cuticle; cw, cell wall; e, exine; f, foot; pa, papilla; pg, pollen grain; pt, pollen tube; s, surface; st, style; vb, vascular bundle.

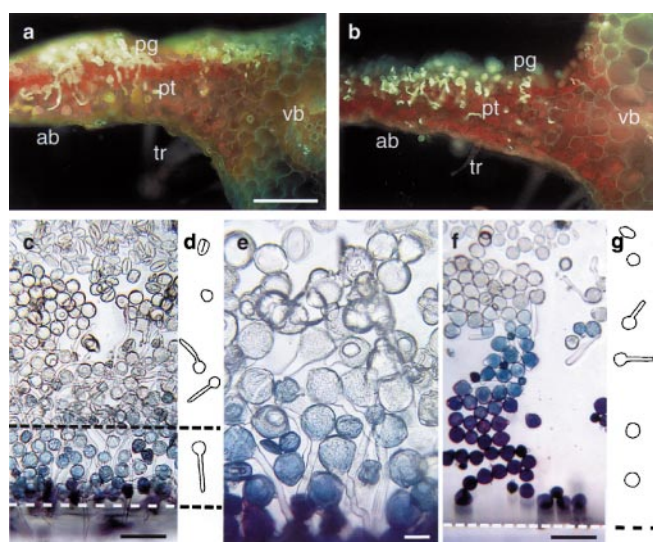


Figure 3 Growth of pollen on leaves and in oil bordering gelled germination medium. **a,b**, Germination and tube growth of pollen on the abaxial side of tobacco leaves from which the cuticle was removed (transverse sections, 24 h after pollen application). **a**, When trilinolein was applied to leaves, tobacco pollen became hydrated and germinated, and pollen tubes penetrated into the leaf. **b**, *Brassica* pollen applied to leaves also germinated and tubes penetrated the tissue. Bar represents $100\ \mu\text{m}$. **c-g**, Germination and tube growth of tobacco pollen in oil bordering gelled germination medium (3 h after establishment of cultures). The gradient of blue indicates hydration of the pollen (toluidine blue was incorporated in the medium). **c**, The pollen grains in trilinolein that are nearest to the gel (white dashed line) produce tubes that grew towards and into the agarose. Further away from the boundary, growth is non-directional (black dashed line); some pollen grains became hydrated but did not germinate and the furthest away did not hydrate. Bar represents $5\ \mu\text{m}$. **d**, Diagrammatic representation of the events shown in **c**. **e**, Close-up view of **c**. Bars represent $10\ \mu\text{m}$. **f**, Pollen grains in saturated triacylglycerides. Bar represents $5\ \mu\text{m}$. **g**, Diagrammatic representation of the events shown in **f**. Abbreviations are as follows: ab, abaxial; pg, pollen grain; pt, pollen tube; tr, trichome; vb, vascular bundle.

not shown). This indicates that the state of the cuticle is also crucial for pollen tube penetration^{12,20,21}. Our results show that penetration of tissues by pollen tubes depends on the presence of lipid carried either by the coat on the pollen of *Brassica* or by the exudate, and it can occur regardless of the cell type that the pollen encounters.

We have shown that *cis*-unsaturated triacylglycerides and the pollen coat enable pollen-tube growth within specialized (dry or wet stigmas) or unspecialized (leaf) tissues. The hydraulic contact between pollen grain and the underlying tissue is established by the pollen coat or exudate^{2,3,5,7}. To investigate the role of triacylglycerides in water transfer between the underlying tissue and the pollen, we created artificial boundaries between oil and a pollen-germination medium. Pollen grains were placed in the oil and a gelled germination medium containing toluidine blue replaced the plant tissue. In *cis*-unsaturated triacylglycerides, pollen grains nearest to the interface became hydrated and germinated, and the pollen tubes grew towards and into the agarose (Fig. 3c–e). In contrast, in saturated triacylglycerides, fewer pollen grains germinated and their tubes did not grow directionally (Fig. 3f, g). Similar results were obtained when water replaced the germination medium in the gel. Differences in the distribution of toluidine blue stain indicate a difference in water supply when different oils were used. The results show that the two oils have different effects on pollen germination and pollen-tube growth.

To confirm that directional growth was a response to a directional supply of water, we disordered the oil phase by replacing it with an emulsion of unsaturated triacylglycerides and germination medium (ratio 1:1). Pollen-tube growth was no longer directional, and tobacco pollen tubes did not penetrate wild-type stigmas bearing exudate when the grains were pre-imbibed in germination medium (data not shown). Thus, directed pollen-tube growth *in vivo* and *in vitro* occurs only when there is a directional supply of water. The control that lipids exert on the movement of water depends on the physicochemical characteristics of the lipids. This control may be a direct consequence of the properties of the lipid as a matrix in which water is distributed²², or an indirect consequence resulting from effects of unsaturated lipids on the permeability of the plasma membrane of the pollen and/or stigmatic cells. The importance of controlled water flow concurs with findings that this flow plays a crucial role in pollen–pistil interactions^{5,23,24}.

Cis-unsaturated triacylglycerides are therefore required for penetration of the stigma by tobacco pollen tubes. These lipids can functionally replace both the exudate and the stigmatoid tissue, and even allow pollen tubes to penetrate leaf tissue. The pollen coat of species with dry stigmas has the same function as the exudate. We propose that the lipids present during pollen–stigma interactions are necessary to regulate water uptake by the pollen grain/tube, establishing an external water gradient. The strength of the gradient, which in pollen–stigma interactions is determined by the composition of the lipids, steers directional growth^{25–30}. □

Methods

Plant material. Transgenic *STIG 1-barnase* tobacco plants¹, *Nicotiana tabacum* cv. Petit Havana SR1, *Petunia hybrida* W166K, *Arabidopsis thaliana*, *Arabidopsis eceriferum* *cer1*, *cer3*, *cer6* and *pop1* mutant plants, *Brassica napus*, *Brassica oleracea* and *Lilium longiflorum* cv. Snow Queen were grown under growth-chamber conditions (day phase: 15 h 20 °C, 65% relative humidity; dark phase: 9 h 18 °C, 65% relative humidity).

Studies of pollen-tube growth *in vivo*. Flowers of transgenic *STIG 1-barnase* tobacco plants were emasculated before anthesis. Exudate or 2 µl selected medium was applied to the stigmaless surface before it was pollinated with excess fresh pollen. In general, when ≥15 pollen tubes penetrated the style, seed production occurred.

Applied compounds. Germination medium was 10% sucrose and 0.01% boric acid. The different oil and lipid compounds used were as follows: chromatographically purified olive oil (fatty acids 10% palmitic acid (16:0), 2% stearic acid (18:0), 83% oleic acid (18:1, [*cis*]-9), 4% linoleic acid (18:2, [*cis*]-

9,12) and 1% linolenic acid (18:3, [*cis,cis,cis*]-9,12,15)) (Larodan, Sweden); the triacylglycerides triolein (C18:1, [*cis*]-9), trilinolein (18:2, [*cis,cis*]-9,12), trilinolenin (18:3, [*cis,cis,cis*]-9,12,15) and trilinolelaidin (C18:2, [*trans,trans*]-9,12) (Sigma); a mix of saturated triacylglycerides (equal parts by mass of trihexanoin (C6:0), triheptanoin (C7:0), trioctanoin (C8:0), trinonanoin (C9:0) and tridecanoin (C10:0)) (Larodan, Sweden); 1,3 dilinolein (18:2, [*cis,cis*]-9,12) (Sigma); 1-monolinolein (18:2, [*cis,cis*]-9,12) (Sigma); the fatty acids oleic acid (C18:1, [*cis*]-9), linoleic acid (C18:2, [*cis,cis*]-9,12) and linolenic acid (18:3, [*cis,cis,cis*]-9,12,15) (Sigma); glycerol (Sigma); and the silicon oils AR20, AR200 (phenylmethyl-polysiloxane), CR350 (chlorphenylmethyl siloxane) (Wacker-Chemie, Germany), DC200 (60,000 mm² s⁻¹ dimethyl siloxane polymer) and DC344 (polydimethyl cyclosiloxane tetramer) (Dow Corning Europe, Belgium).

24 h after application of compounds and pollination, we collected pistils. Vibratome-cut sections of ~150 µm were placed in a drop of aniline blue stain (0.1% in 0.1M K₃PO₄) and examined with a Leitz Wetzlar orthoplan microscope equipped for ultraviolet epifluorescence, using the appropriate filters. Hand-cut sections of pollinated leaves and pistils of *Arabidopsis* were pretreated in 2M NaOH at 60 °C for 10 min to soften the tissue. Pistil squashes were used to count the number of pollen tubes that had penetrated the stigma. Pistils were fixed in ethanol/acetic acid (3:1) for 12 h or more at 4 °C and in 2M NaOH for 1 h at 60 °C, and were then rinsed with water and placed directly into a drop of aniline blue stain.

Statistical analysis. We used the Wilcoxon test (SAS system version 6.07). We counted pollen tubes in each pistil after treatment with the above compounds (20 pistils per treatment) and compared this number with the numbers of pollen tubes in pollinations with tobacco exudate. If the *P* value was ≤0.05, the difference between the two pollination experiments was taken to be significant. Using the following compounds, the average number of penetrated pollen tubes in the stigma and associated *P* values were: tobacco exudate, 21; *Petunia* exudate, 46 (*P* = 0.03); *Lilium* exudate, 0; purified oil, 25 (*P* = 0.86); purified oil:*Lilium* exudate 1:1, 24 (*P* = 0.96); trilinolein, 33 (*P* = 0.28); triolein, 15 (*P* = 0.33); trilinolenin, 11 (*P* = 0.02); saturated triacylglycerides, 1 (*P* = 0.0001); trilinolelaidin, 2 (*P* = 0.0001); glycerol, 0; oleic acid, 8 (*P* = 0.002); linoleic acid, 0; linolenic acid, 0; monolinolein, 0; dilinolein, 3 (*P* = 0.0001); AR20, 0; AR200, 3 (*P* = 0.0001); CR350, 0; DC344, 0; DC200, 1 (*P* = 0.0001).

Transmission electron microscopy (TEM) was performed as described¹⁷.

Scanning electron microscopy (SEM). Fresh pistils were mounted on stubs and sputtered with gold. The samples were examined with a JEOL JSM-T300.

***In vitro* germination.** An artificial boundary between oil and germination medium or between oil and water was created by applying 1 ml germination medium or water (agarose, 0.7%; toluidine blue, 0.01%) to a glass slide. After polymerization, the gel was cut with a razor blade to produce a sharp edge. A coverslip was placed on the gel with an overlap at the edges of 2–3 mm. After this, 50 µl of a suspension of pollen in oil was applied to the edge of the gel. Pollen germination and pollen-tube growth were viewed with a Leitz dialux 20 EB microscope.

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Dynamic activation of endothelial nitric oxide synthase by Hsp90

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Heat-shock protein 90 (Hsp90) coordinates the trafficking and regulation of diverse signalling proteins, but its precise role in regulating specific cellular targets is not known^{1,2}. Here we show that Hsp90 associates with endothelial nitric oxide synthase (eNOS) and is rapidly recruited to the eNOS complex by agonists that stimulate production of nitric oxide, namely vascular endothelial growth factor, histamine and fluid shear stress. Moreover, the binding of Hsp90 to eNOS enhances the activation of eNOS. Inhibition of signalling through Hsp90 attenuates both agonist-stimulated production of nitric oxide and endothelium-dependent relaxation of isolated blood vessels. Our results indicate that Hsp90 facilitates signalling mediated by growth-factor, G-protein and mechanotransduction pathways that lead to the activation of eNOS. These observations indicate that in addition

to its role as a molecular chaperone involved in protein folding and maturation, Hsp90 may also be recruited to cellular targets depending on the activation state of the cell.

Several peripheral membrane signalling proteins, including Src and its family members, Raf, G-protein $\beta\gamma$ subunits and mitogen-activated protein kinase kinase (MEK) can exist in heterocomplexes with Hsp90 (ref. 3). However, the regulation and function of the binding of Hsp90 to its native cellular substrates in mammalian cells remains unknown. Genetic studies in *Saccharomyces cerevisiae* and *Drosophila melanogaster* have shown that *hsp90* homologues are essential genes. In *S. cerevisiae*, Hsp82 is an integral component of reconstituted steroid-receptor- and v-Src-dependent signal-transduction events, whereas in *Drosophila* Hsp83 is involved in mediating receptor tyrosine kinase signalling pathways⁴⁻¹⁰. Potential mechanisms to account for these effects include Hsp90-mediated effects on protein folding, stability and targeting of signalling proteins.

eNOS is the nitric oxide synthase (NOS) isoform that produces endothelium-derived nitric oxide (NO)¹¹. NO is a highly lipophilic gas that is not stored in cells, but is immediately synthesized and released upon stimulus-dependent activation of NOS. NO derived from the endothelium is important in regulating cardiovascular haemodynamics, proliferation of vascular smooth muscle cells, leukocyte adhesion and platelet aggregation¹². eNOS is a peripheral membrane protein in endothelial cells because of its co-translational *N*-myristoylation and post-translational cysteine palmitoylation¹³. Both fatty-acylation reactions are necessary for specific targeting of eNOS to the Golgi, and cysteine palmitoylation is necessary for movement from the Golgi into glycolipid- and cholesterol-rich plasmalemmal microdomains, caveolae^{14,15}. As eNOS coprecipitates with a protein having a relative molecular mass of 90K (refs 16, 17) and as Hsp90 may be involved in several signal-transduction cascades^{7,18}, we studied the possibility that eNOS may exist as a component of an Hsp90 heterocomplex in endothelial cells.

As seen in Fig. 1a, immunoprecipitation of eNOS from bovine microvascular endothelial cell lysates results in the coprecipitation of Hsp90. Immunoprecipitation with non-immune antisera does not result in the precipitation or coprecipitation of the proteins. Immunoprecipitation of Hsp90 also coprecipitates eNOS. As shown for other Hsp90-containing complexes, the eNOS-Hsp90 complex disassembles if immunocomplexes are washed with a high-ionic-strength buffer (0.5 M NaCl). To determine whether eNOS interacts

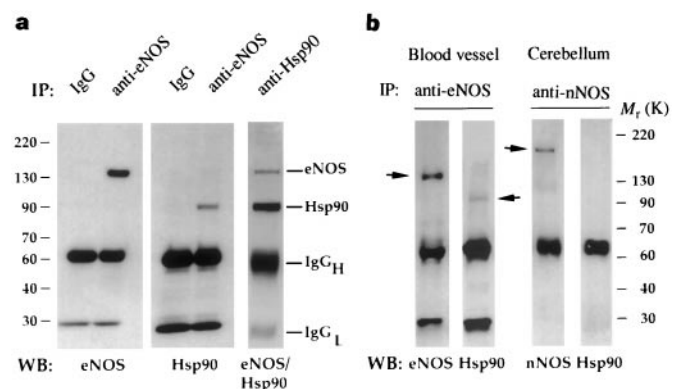


Figure 1 Association between eNOS and Hsp90. **a**, Lysates from BLMVECs were immunoprecipitated with non-immune mouse immunoglobulin (IgG) or with monoclonal antibody against eNOS and Hsp90. Immunoprecipitates (IPs) were western blotted (WB) for eNOS (left), Hsp90 (centre) or both proteins (right) IgG_H and IgG_L denote IgG heavy and light chains, respectively. **b**, Lysates from a single rat aorta or cerebellum were immunoprecipitated with antibodies against eNOS or nNOS. IPs were blotted for eNOS, nNOS and Hsp90. In **b**, the arrows (from left to right) denote eNOS, Hsp90 and nNOS, respectively.

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