Loss of Caveolae, Vascular Dysfunction, and Pulmonary Defects in Caveolin-1 Gene-Disrupted Mice

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Caveolae are plasma membrane invaginations that may play an important role in numerous cellular processes including transport, signaling, and tumor suppression. By targeted disruption of caveolin-1, the main protein component of caveolae, we generated mice that lacked caveolae. The absence of this organellar impaired nitric oxide and calcium signaling in the cardiovascular system, causing aberrations in endothelium-dependent relaxation, contractility, and maintenance of myogenic tone. In addition, the lungs of knockout animals displayed thickening of alveolar septa caused by uncontrolled endothelial cell proliferation and fibrosis, resulting in severe physical limitations in caveolin-1–disrupted mice. Thus, caveolin-1 and caveolae play a fundamental role in organizing multiple signaling pathways in the cell.

Caveolae are characteristic flask-shaped invaginations of the plasma membrane with a diameter of 50 to 100 nm. They are present on many cell types, including endothelial cells, smooth muscle cells, and adipocytes (1, 2). Fifty years after the discovery of caveolae (3), their function is still controversial and may include transcytosis of solutes through endothelial cells (4), cholesterol transport (5), potocytosis (6), signal transduction (2, 7), and tumor suppression (8). VIP21/caveolin-1 (cav-1), a protein of average size of 3.4 pups per litter, we randomly assigned half the pups to the experimental group and fed each of these pups with about 12 g of hard-boiled egg at the end of morning and evening activity periods. The other pups in the litter received no additional food. Sample sizes in these analyses were the number of fed and unfed pups, and tests shown are based on GLMs controlling for sex and repeated measures within litters. Pup feeding was maintained until the animals were 60 days old, and the frequency with which they were fed by helpers had started to fall (27).

27. P. N. M. Brotherton et al., Behav. Ecol. 12, 590 (2001).
30. GENSTAT 5.4.1. (Lawes Agricultural Trust, Rothamsted Experimental Station, Hertfordshire, UK, 1998).
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transcytosis occurs through, or is compensated for by, an alternative pathway. Caveolin also has been suggested to play a role in cholesterol transport (23). However, the blood lipoprotein composition and the cholesterol content of high-density lipoprotein were

scaffolding domain of the protein. The disruption of the cav-1 gene resulted in the complete absence of caveolae in endothelial and epithelial cells of lung tissue from knockout mice (Fig. 2, C and D). In wild-type animals, abundant caveolae were present in these cells (Fig. 2, A and B). The lack of caveolae was observed in all organs analyzed, i.e., lung, adipose tissue, diaphragm, kidney, and heart. Thus, cav-1 is essential for caveolae formation, in agreement with the observation that ectopic expression of cav-1 is sufficient to induce caveolae in cells lacking this organelle (14). Occasionally, we detected invaginations with an electron-dense diaphragm at their neck in the large vessels (Fig. 2E), suggesting a possible morphologically distinct minor variety of cav-1-independent invaginations generated perhaps by caveolin homologs or other proteins.

We analyzed the expression of cav-1 homologs cav-2 and cav-3 in knockout mice. Although the cav-2 transcript was present in cav-1−/− mice, the protein could not be detected in the lungs (Fig. 1D). Similar results were obtained with other tissues including adipose, where cav-2 is normally most abundant. Because cav-1 and cav-2 form hetero-oligomers (15), this suggests that cav-2 in the absence of cav-1 may be degraded. In contrast, the expression of the muscle-specific cav-3 was normal in cav-1−/− animals.

Cav-1 is one of the major protein constituents of the plasma membrane cholesterol-enriched microdomains (rafts) (16, 17). This prompted us to study whether the absence of cav-1 and cav-2 affected the contents of rafts isolated by an established procedure (18). Neither the pattern of protein expression (Fig. 1E) nor the lipid composition of rafts from knockout animals showed appreciable differences compared with that of the wild type. Moreover, glycosyl phosphatidylinositol (GPI)–anchored proteins, which are enriched in rafts (19), displayed a similar pattern in both preparations, as revealed by an aerolysin overlay (20) (Fig. 1E).

One of the first functions suggested for caveolae was the transcytosis of macromolecules through endothelial cells (4, 21). Caveolae constitute as much as 30% of the total endothelial cell surface in capillaries. However, despite the complete loss of caveolae from the capillary network, the albumin concentration in cerebrospinal fluid, which was presumed to depend on transport through caveolae, was not different in knockout and wild-type animals (22). Cav-1−/− mice did not express any visible signs of disturbed extravascular oncotic pressure (a component of osmotic pressure that depends on the transport of albumin across the endothelium). Thus, transcytosis
normal in knockout animals.

The lack of caveolae in cav-1−/− mice offered the opportunity to address directly whether this organelle is involved in various signaling events. We focus here on nitric oxide (NO) signaling, calcium signaling, and tumor suppression.

We studied the role of caveolae in endothelium-dependent and NO-mediated vascular relaxation in isolated aortic rings (24, 25). Rings derived from mutant mice failed to establish a steady contractile tone, with the vascular wall oscillating with a frequency of one per minute. Moreover, marked relaxation in the response to acetylcholine was observed. Whereas in wild-type animals the relaxation after precontraction with phenylephrine did not exceed 50%, in knockout animals the relaxation was complete (Fig. 3, A and B). To clarify if this difference was dependent on the NO pathway, we directly measured NO in primary cell culture of aortic vascular smooth muscle cells (VSMCs) with a selective microelectrode (26, 27). The basal release of NO in cav-1−/− cells was 31% higher than in wild-type cells. Moreover, we compared downstream elements of the NO pathway and observed an about threefold higher content of cyclic guanosine monophosphate (cGMP, the major mediator of NO signaling) in knockout animals [cGMP content in fmol/g of tissue (mean ± SEM): 338.2 ± 56.5 (knockout) versus 108.3 ± 41.8 (wild type), P < 0.02]. Thus, cav-1 and caveolae are involved in the regulation of the NO pathway where NO synthases become hyperactivated in the absence of cav-1 (24, 27).

Caveolae are also abundant in VSMCs. Despite the normal expression of muscle-specific cav-3, caveolae were absent from VSMCs in cav-1−/− animals. To study the consequences of caveolae deficiency, we measured the arterial response to vasoconstrictors. A manifold weaker calcium-dependent contractile response of arteries to angiotensin II, endothelin-1, and phorbol ester in cav-1−/− mice (Fig. 3E) was found. We also determined the myogenic tone of blood vessels, a partially contracted state that is an important determinant of blood pressure. This tone is regulated by a feedback mechanism tightly linking STOCs (spontaneous transient outward currents) to Ca2+ sparks (local increases in subsarcolemmal Ca2+). Both are proposed to be dependent on the presence of caveolae (28). To determine whether this feedback mechanism is still functional in the absence of caveolae, we studied the frequency of STOCs in VSMCs and found that the STOCs were substantially (by a factor of 4, P < 0.01) reduced in the knockout animals at the physiological membrane po-
tential of -40 mV (Fig. 3F) (29).

Thus, the analysis of the aortic rings and the VSMCs shows that cav-1/caveolae regulate three major vascular features that depend on NO and Ca\(^{2+}\) signaling: endothelium-dependent relaxation of arteries, myogenic tone, and stimulated contractility.

The lack of caveolae also caused severe pathomorphological defects of lung alveolar septa, where gas exchange takes place. In knockout animals, large areas of the lungs (up to 30%) displayed markedly thickened septa (Figs. 2F and 4B). The normal double-layered alveolar architecture was replaced by a multilayered, disorganized tissue. These abnormal septa showed immunostaining for Flk1 (Fig. 4, C and D), a marker of nondifferentiated endothelial and hematopoietic progenitors, but not for von Willebrand factor, which labels later stages of endothelial cell differentiation. The increased cellular content in septa was accompanied by an increase of extracellular fibrillar deposits, as visualized by azan stain (Fig. 4, E and F; see also Fig. 2F). Thus, the loss of caveolae leads to the initiation of fibrosis. The accumulation of fibers in the alveolar interstitium of knockout animals was accompanied by a marked hypertrophy of type II pneumocytes. The observed uncontrolled hyperplification of angioblastic cells implies a function for cav-1/caveolae in the local control of cell proliferation, consistent with in vitro studies suggesting that caveolin-1 may act as a tumor suppressor (8).

We investigated the effects of vascular dysfunction and lung defects described above on the physical ability of cav-1\(^{-/-}\) animals. Indeed, they displayed a markedly reduced ability to perform physical work, as assessed by a forced swimming stress (30). Whereas the control animals were able to swim for 1 to 1.5 hours at 34°C, cav-1\(^{-/-}\) animals showed signs of exhaustion after 15 to 20 min (Fig. 4G).

Our study of cav-1\(^{-/-}\) animals provides several important findings. First, the disruption of the cav-1 gene caused a loss of caveolae from all analyzed tissues, and hence generated a cell organelle knockout. Second, mutant animals displayed profound dysfunction of the vascular system and pronounced thickening of lung alveolar septa caused by an uncontrolled proliferation of endothelial cells and fibrosis. Surprisingly, such a marked change in plasma membrane structure and accompanying signaling disturbances caused by the deletion of the cellular organelle were not lethal. Possibly, some of the functions postulated for caveolae may be taken over by other molecules. Nevertheless, the role of caveolae is essential for the normal functioning of the lungs and the cardiovascular system.

References and Notes
13. All animal experiments were approved by local authorities in accordance with criteria outlined by the American Physiological Society. A 129/Sv BAC library (Genome Systems) was screened with a reverse transcriptase-polymerase chain reaction (RT-PCR) probe derived from the murine caveolin-1 cDNA. The structure of a targeting plasmid is shown in Fig. 1A. Two 129/Sv embryonic stem cell (Genome Systems) clones with targeted disruption of the cav-1 allele were injected into blastocysts from C57BL/6 mice; the resulting chimeric mice were crossed with C57BL/6 mice, and the heterozygous progeny were inbred to yield cav-1\(^{-/-}\) animals. Routine PCR genotyping was performed for the detection of tissue-specific transcripts by RT-PCR, adult mouse lung and heart were minced, and RNA was extracted, as directed by the manufacturer (Biotecx Laboratories). The reverse transcription was carried out with SuperScript II RNase H- Reverse Transcriptase and random hexamers (Boehringer Mannheim). Antibodies used for Western blotting and immunocytochemistry were as follows: anti-cav-1 (polyclonal C13630, Transduction Labs); anti-cav-2 (monoclonal C57820, Transduction Labs); anti-cav-3 [monoclonal C38320, Transduction Labs; vWF (A0082), Dako]; and monoclonal anti-flk1 [sc-6251, Santa Cruz]. Samples for electron microscopy examination were fixed with glutaraldehyde and embedded in Epon according to standard protocols.
22. The albumin concentration in cerebral spinal fluid was measured densitometrically after SDS-electrophoresis and staining with Fast Blue (Amersham Pharmacia Biotech, Piscataway, NJ). We used 99% globulin-free mouse albumin (Sigma, St. Louis) as a standard.
25. Aortic ring relaxation studies were done as described [24]. In our experiments, aortic rings were treated with 500 nM phenylephrine and then with 10 \(\mu\)M acetylcholine. For measurements of the intracellular arterial wall Ca\(^{2+}\) concentration, the vessels were incubated with the Ca\(^{2+}\)-sensitive indicator fura-2/AM [5 \(\mu\)M].
27. NO-selective microelectrode NO-release measurements were performed with an ISO-NOP 2-mm sensor connected to the ISO-N0 Mark II Nitric Oxide Meter (World Precision Instruments, Sarasota, FL). The cGMP content in the lungs was estimated as directed by the manufacturer (Amer- sham Pharmacia Biotech, Piscataway, NJ).
30. The swimming test was performed at 34°C and documented by video recording.
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