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COMMENTARY

Do studies in caveolin-knockouts teach us about physiology and pharmacology or instead, the ways mice compensate for 'lost proteins'?

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A wide array of phenotypic changes have been reported in mice with knockout of expression of caveolin-1. Neidhold *et al.* (2007) describe results in this issue that continue this trend by showing that saphenous arteries from adult caveolin-1 knockout mice lack caveolae, lose β_1-adrenoceptor-promoted relaxation, gain β_3-adrenoceptor-promoted relaxation but show no change in vasomotor response to β_2-adrenoceptor activation. Neither the physiological importance for wild-type animals nor the mechanistic basis for these changes is clear. Although the caveolin-1 knockout and wild-type mice express similar levels of the receptor mRNAs, the protein expression of the receptors is not specified and represents, in our view, an important limitation of the study. We also question the physiological relevance of the findings and ask: Do studies in total body/lifespan caveolin-knockout mice further understanding of physiology and pharmacology or do they primarily characterize secondary consequences? We propose that alternative approaches that decrease caveolin expression in a temporally and spatially discrete manner are more likely to facilitate definitive conclusions regarding caveolin-1 and its role in regulation of β-adrenoceptors and other pharmacological targets.
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Abbreviations: ATPase, adenosine triphosphatase; cav-1 KO, caveolin-1 knockout; eNOS/NOS3, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; GPCR, G-protein-coupled receptors; MAP, mitogen-activated protein kinase; siRNA, small interfering ribonucleic acid; SNP, sodium nitroprusside; VEGF, vascular endothelial growth factor

Recent ideas in signal transduction emphasize the non-uniformity of the plasma membrane and its regions that regulate effects of physiological and pharmacological agonists (Ostrom and Insel, 2004; Drake *et al*., 2006). Clathrin-coated pits, one type of membrane specialization, are sites at which receptors can initiate certain signalling events and agonist-promoted endocytosis, as a prelude to degradation or recycling of receptors (Shenoy and Lefkowitz, 2005; Drake *et al*., 2006). Lipid rafts and caveolin-containing lipid rafts (caveolae, 'little caves'), another unique domain of the plasma membrane, are enriched in cholesterol and other lipids, such as sphingolipids and gangliosides, and are sites that concentrate a variety of proteins (Cohen *et al*., 2004; Gratton *et al*., 2004; Ostrom and Insel, 2004). Based on their unique lipid content imparting greater buoyancy, lipid rafts and caveolae can be isolated following density gradient centrifugation; caveolae can be visually distinguished from lipid rafts at the electron microscopic level by their characteristic omega-shaped invaginations of the membrane. Caveolins are expressed as three isoforms (caveolins-1, -2 and -3) with caveolin-3 being unique in its expression in skeletal and cardiac muscle (Cohen *et al*., 2004; Gratton *et al*., 2004; Ostrom and Insel, 2004).

Many roles have been proposed for caveolae but their precise contribution to physiology and pharmacology, in particular, of the cardiovascular system, is not fully understood (Cohen *et al*., 2004; Gratton *et al*, 2004). Considerable data have emphasized the preferential expression of endothelial nitric oxide synthase (eNOS/NOS3) in caveolae, whereas other studies have shown that numerous G-protein-coupled receptors (GPCRs), G-proteins and G-protein-regulated effectors are enriched in such domains (Cohen *et al*., 2004; Gratton *et al*., 2004; Ostrom and Insel, 2004; Insel *et al*., 2005). Certain GPCRs localize to caveolae in the absence of agonists, whereas others show agonist-promoted translocation to caveolae as a prelude to endocytosis/

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internalization. It has been proposed that the latter pathway is preferentially utilized by Gs-linked GPCRs in which phosphorylation in response to agonists occurs by protein kinase A, whereas phosphorylation by G-protein receptor kinase promotes endocytosis via arrestin at clathrin-coated pits (Rapacciuolo *et al*., 2003; Shenoy and Lefkowitz, 2005; Drake *et al*., 2006).

Several experimental approaches have been used to assess and define the biological functions of caveolae. These include the application of biochemical and biophysical methods (e.g., subcellular fractionation, immunoprecipitation and immunoblotting, and fluorescent resonance energy transfer), morphological analyses (e.g., fluorescent microscopy, electron microscopy) and pharmacological strategies, wherein one depletes plasma membranes of cholesterol by using agents such a methyl-β-cyclodextrin or various statins. In recent years, molecular biological methods have been employed, in particular, the use of mice in which expression has been knocked out by targeted disruption of the caveolin gene with a resultant decrease in caveolin throughout the body from the time of conception throughout the lifetime of a mouse, as such knockouts are not associated with embryonic death.

Approximately, 100 publications have appeared since 2001 related to the use of caveolin-knockout mice (as reviewed by Hnasko and Lisanti, 2003; Cohen *et al*., 2004; Le Lay and Kurzchalia, 2005). Most articles have involved the study of caveolin-1 knockout (cav-1 KO) mice, which were generated independently by several research groups and which have a large number of phenotypic alterations (Table 1). Cav-1 KO mice have a reduced lifespan (Park *et al*., 2003) in association with pulmonary fibrosis, pulmonary hypertension and various cardiac abnormalities (Hnasko and Lisanti, 2003; Cohen *et al*., 2004; Le Lay and Kurzchalia, 2005). Because such animals demonstrate neointimal and mammary cell hyperplasia and increased propensity for carcinogen-induced epidermal hyperplasia and tumours, it has been proposed that cav-1 may be a tumour suppressor, at least in some tissues (Hnasko and Lisanti, 2003; Bouras *et al*., 2004; Hassan *et al*., 2004; Pike, 2005).

Studies with cav-1 KO animals have provided evidence in support of the 'caveolin signalling hypothesis' wherein the binding of signalling molecules to caveolins (in particular to a caveolin scaffolding domain) inhibits signalling entities that include not only GPCRs but also G-proteins, certain of their effectors and 'downstream' kinases, such as mitogen-activated protein kinases, Rho kinases and isoforms of protein kinase C (Hnasko and Lisanti, 2003; Cohen *et al*., 2004; Gratton *et al*., 2004; Ostrom and Insel, 2004). Activation of such signalling molecules is generally thought to parallel the release of the caveolin-bound, inhibited protein. The most well-studied example is the interaction of caveolins with eNOS; consistent with the inhibition by caveolin, cav-1 KO animals have enhanced eNOS activity, NO-dependent vasodilatation, endothelium-dependent vascular relaxation of vessels and decreased myogenic tone (Drab *et al*., 2001; Hnasko and Lisanti, 2003; Gratton *et al*., 2004; Le Lay and Kurzchalia, 2005; Li *et al*., 2005b).

Such background information provides a context for the article in the current issue by Neidhold and co-workers. The authors compared saphenous artery vasoreactivity in cav-1 KO and wild-type mice and observed unchanged expression of β_2-adrenoceptors but a loss in β_1-adrenoceptor-mediated vasorelaxant responses that is paralleled by an increase in β_3-adrenoceptor-mediated vasorelaxation. The findings thus identify a setting in which a detectable response to β_3-adrenoceptor activation occurs in the vasculature in parallel with decreased β_1-adrenoceptor response.

Although the findings of Neidhold *et al*. (2007) are of interest, we believe that the study has a number of experimental problems and raises certain important theoretical concerns. The experimental problems include the following:

(1) The authors provide no data for protein expression of the key components under study but instead rely solely on mRNA expression to infer absence of changes in cellular levels of the adrenoceptors. This is of concern because expression of mRNA and protein need not be – in fact frequently are not – closely correlated. Of note, cav-1 KO mice show similar levels of expression of insulin receptor mRNA in adipocytes but >90% reduction in receptor protein levels (Cohen *et al*., 2003). Even so, in Figure 2b, the data of Neidhold *et al*. (2007) suggest a shift between expression of the two forms of β_3-adrenoreceptor mRNA with the cav-1 KO vessels having offsetting changes in expression of the smaller mRNA species (increased) and larger mRNA species (decreased), such that expression of the two together is similar in the cav-1 KO and wild-type animals. Conceivably, these two RNA species yield different receptor proteins that have different biological activities.
(2) Age and sex of animals that were studied are not precisely defined and seem to vary within and between study groups. Given the differing lifespans of cav-1 KO animals (Park *et al*., 2003) and that the authors studied these animals over a long age range (9–18 months), 'equivalent' animals may not have been compared, albeit the authors note that the animals were of approximately similar ages in certain studies. Expression and progression of disease in cav-1 KO mice is time-dependent and could impact on study outcomes. Additionally, no mention is made of the gender of the mice even though this factor influences vasoreactivity (Luksha *et al*., 2005).
(3) Saphenous arterial wall size was 39% thicker in the cav-1 KO mice compared to wild-type controls but precise measurements were not provided for each portion of the vascular wall, although the authors note that there is 'smooth muscle hypertrophy'.
(4) Vasoreactivity was not fully defined. The saphenous arteries showed substantial blunting in maximal relaxation (response to 60 mM KCl) and in response to sodium nitroprusside (SNP), although the result with KCl was masked if expressed relative to the SNP response.

Cav-1 KO mice have differences in mechanotransduction, in their handling of calcium and in the generation of calcium sparks; moreover, a component of vasorelaxation mediated by β-adrenoceptors involves Ca^{2+} handling, which the authors did not measure (Bergdahl and Sward, 2004; Balijepalli *et al*., 2006; Cheng and Jaggar, 2006; Yu *et al*.,

Table 1 Selected phenotypes of caveolin-1 knockout mice

Organ system and phenotype	*Proposed mechanism*
Cancer	
Increased sensitivity to carcinogens	Increased cyclin D1 and ERK 1/2 levels
Delayed prostate tumour progression	Tumour suppression activity
Cardiac	
Cardiac hypertrophy	p42/44 MAP kinase hyperactivation
Dilated cardiomyopathy	Unknown
Central nervous system	
Reduced brain weight; multiple motor and behavioural abnormalities	Altered maintenance of cortico-striato-pallido-thalamo-pontine pathways involved in motor control
Endocrine-metabolic	
Adipocyte abnormalities with impaired lipolytic activity and altered lipid droplet architecture	Altered lipid homeostasis; altered perilipin phosphorylation
Accelerated mammary gland development	Hyperactivation of prolactin signaling
Decreased glucose uptake	Insulin resistance and altered glucose transporter localization
Gastrointestinal	
Hyperproliferation of intestinal crypt cells	Upregulation of Wnt/β-catenin signaling
Reduced nitric oxide-mediated intestinal smooth muscle relaxation	Abnormalities in interstitial cells of Cajal and smooth muscle
Decreased liver regeneration posthepatectomy	Impaired coordination of lipid metabolism and cell proliferation
Lymphoreticular	
Reduced response to thymus-independent antigens	Unknown
Pulmonary	
Constricted alveolar spaces	Thickened alveolar wall
Pulmonary hypertension	Multiple causes
Transport	
Disruption of glycosylphosphatidyl inositol-anchored protein transport	Altered Golgi protein processing
Urogenital	
Impaired renal calcium absorption	Abnormal function of plasma membrane calcium ATPase
Enlarged seminal vesicles	Engorgement of seminal fluid
Bladder hypertrophy	Smooth muscle hyperplasia
Vascular	
Reduced aortic contractile tone	Increase in endothelial nitric oxide activity
Impaired angiogenic response	Altered VEGF protein interactions
Neointimal hyperplasia	Increased cyclin D1 and ERK 1/2 levels
Microvascular permeability	Altered clefts and tight junctions
Abnormal arterial remodeling during changes in flow	Altered mechanotransduction

Abbreviations: ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; VEGF, vascular endothelial growth factor.
Adapted from Hnasko and Lisanti, 2003; Cohen *et al.*, 2004; Sonveaux *et al.*, 2004; Lay and Kurzchalia, 2005; Li *et al.*, 2005a, b; El-Yazbi *et al.*, 2006; Fernandez *et al.*, 2006; Medina *et al.*, 2006; Trushina *et al.*, 2006; Yu *et al.*, 2006.

2006). Measurements of NOS activity were also not provided even though altered NOS activity is probably an important contributor to altered vascular tone in cav-1 KO mice.

Aside from such specific concerns, we believe there is a more general issue: the physiological relevance of studies that employ the types of KO mice used by Neidhold *et al.* (2007). It is common (some might say 'fashionable') to conduct studies in KO mice, but do such results reveal important aspects of normal physiology and pharmacology or instead, are the findings primarily a description as to how a particular mouse strain compensates (as in the current study) for a total body knockout of a protein essential for caveolae formation *and* that is absent from embryonic life onward? Given such extensive losses of caveolin-1 and caveolae and the length of time of these losses, are results in these knockout mice physiologically relevant or only pharmacological curiosities? Especially, as these knockout mice have shorter lifespans and other 'defects' (e.g, Table 1), is the loss of β_1-adrenocaptor-mediated responses and 'new' expression of β_3-adrenoceptor responses in saphenous arteries another 'pathological' effect of the absence of cav-1, a compensatory phenomenon or revealing of new insights regarding 'plasticity' in the vasculature? If the authors' goal is to understand the physiological role of caveolin-1 and caveolae in a particular vascular bed in adult animals, would not a better approach be to utilize tissue- and time-specific knockout strategies or alternative molecular strategies, such as siRNA, the latter of which has been used to alter expression of caveolins in cardiovascular cells (Gonzalez *et al.*, 2004; Zuo *et al.*, 2005; Swaney *et al.*, 2006)?

Interventions, such as siRNA (or even pharmacological approaches) that more acutely (and often reversibly) decrease expression of components have the advantage of avoiding the conundrum of compensation or mechanistic adaptations that are likely to occur over time in mice such as those studied by Neidhold *et al*. (2007).

The concerns we raise are not meant to detract from the careful, *in vitro* pharmacological methods used by Neidhold *et al*. (2007) but rather to query whether the findings provide an example of events that *can happen* (perhaps in large part as compensation for a missing cellular constituent), while revealing limited insight into *what actually happens* in the normal *in vivo* setting. The challenge is how best to use experimental systems that facilitate the marriage of sophisticated genetic technologies with physiological and pharmacological characterization so as to yield results that further understanding of biological phenomena of importance to human health and disease. We would argue that more spatially and temporally restricted approaches than total body/lifetime KO mice are likely to be required for more definitive insights into such phenomena.

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