BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01262.x
www.brjpharmacol.org

LETTER TO THE EDITOR

'Altered' mesenteric artery SK_{Ca}: functional implications?

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Understanding the molecular mechanisms that underlie pathophysiology commonly involves starting with the demonstration of changes in functional responses in a model organism using pharmacological methods. The underlying mechanism is often then elucidated by demonstrating changes in cellular composition that could explain the observed pathology. In our opinion, there are many methodological pitfalls in this approach such as technical aspects of the biochemical measurement process, the difficulty of ascertaining which cell type in a tissue is responsible for the biochemically measured differences and suitable controls for the model employed.

We write in relation to a recent study in the *British Journal of Pharmacology* by Weston *et al.* (2010), which illustrates several of these issues. The aim of the study was to explain, in a hypertensive rat model, how differences in sensitivity to endothelium-mediated vasodilatation could be explained in terms of different levels of expression of certain membrane proteins.

By comparing data from normotensive WKY and hypertensive SHR strains of rat, this study investigated endothelial small conductance calcium-activated potassium channel (SK_{Ca})–mediated vasodilator signalling in the mesenteric artery. The hypothesis was that 'altered' SKCa-mediated activity is due to reduced SK_{Ca} and inward rectifying potassium channel (K_{ir}) expression and their association with 'altered' caveolin-1 (Cav-1) properties. This letter draws attention to our opinions on a number of issues regarding the basis on which the conclusions of the study rest.

While the noncomparative functional data presented by Weston *et al.* (2010) are convincing, the concerns listed below relate to the interpretation of the Western blot data and their implications. This is critical, since, as highlighted by the accompanying Commentary by Garland (2010), the 'altered' SK_{Ca}-mediated vasodilator mechanism in SHR mesenteric artery was concluded to be due to the reduced protein expression of SK_{Ca} and K_{ir}, and altered Cav-1 properties, as derived from molecular data gathered using Western blotting. Thus, it seems if the interpretations are not justified by the Western blot data that is presented, then the comparative conclusion is also not justified.

Many of our concerns about the Western blot data arise because full-length blots including molecular weight markers are not presented, and as a result, it seems that it is not possible to determine the precise nature and relevance of the excised bands in the Western blot figures. In our opinion, this is a common problem with the presentation of Western blot images, and journal space is often cited as an excuse. If so, for the reasons explained in point 3 below, full blot images, including the region >200 kDa, should be provided online so that readers can more fully assess the results for themselves.

Other specific concerns with the Western blot data are as follows:

1. The apparent $K_{ir}2.1$ protein band that is recognized by the Alomone antibody is marked at 52 kDa. However, the antibody data sheet provided by Alomone (http://www.alomone.com/System/UpLoadFiles/DGallery/Docs/APC-026.pdf) shows the $K_{ir}2.1$ band at 62 kDa, whereas, in fact, the $K_{ir}2.1$ protein is 48 200 Da and is not glycosylated (see figure 2, in Schwalbe *et al.*, 2002) but is subject to phosphorylation (UniProtKB/Swiss-Prot Q64273 available via NCBI: http://www.uniprot.org/uniprot/Q64273). Furthermore, the kcnj2 gene is transcribed from a single exon and is not subject to pre-mRNA splicing (Rat Genome Database GeneID 29712 available via NCBI: http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=retrieve&dopt=full_report&list_uids=29712).
2. The paper states that protein concentrations were determined using the Bradford assay, and the consistency of protein loading in each sample lane of the blot images presented in figure 4 was visually assessed using both Ponceau S and β actin staining. However, the quantity of protein that was loaded in each sample lane was not stated. Furthermore, the amount of tissue from which the protein was extracted, and the total yield of protein that was obtained from the tissue in each sample, was not mentioned. It is stated that the three lanes for WKY and SHR were obtained from three different animals, but there is no indication of whether the lanes are simply replicates of a single sample derived from three animals, or whether each sample lane is derived from segments of a single mesenteric artery removed from a single 12–16 week old animal. If the sample in each lane is derived from segments of a single mesenteric artery from a single animal, then stating the quantity of protein present would provide additional confidence that sufficient endothelial membrane was present in each lane to permit detection of $K_{ir}2.1$ and SK3. In our experience using branches of the superior mesenteric and saphenous artery from 12–26 week old rats, it is necessary to obtain tissue from three to six rats as a single sample to provide sufficient protein in order to

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detect the expression of ion channels in the endothelial membrane (Chadha *et al.*, 2010; Haddock *et al.*, 2011).

3. Caveolin protein oligomers, which form caveolae, and functional SK3 tetramers are resistant to heat and detergents and migrate as high molecular weight complexes >220 kDa in samples of tissue extracts separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (caveolin, Monier *et al.*, 1995; Sargiacomo *et al.*, 1995; Scherer *et al.*, 1997; Scheiffele *et al.*, 1998; SK3, Boettger *et al.*, 2002; Chen *et al.*, 2004; Mongan *et al.*, 2005). Cav-1 can also be present as a monomer, with functional properties distinct from the high molecular weight complex, which are an aggregate of Cav-1 monomers (and potentially other caveolins) and other molecules (Monier *et al.*, 1995; Scherer *et al.*, 1997; Patel *et al.*, 2008). Thus, the monomeric and low molecular weight dimeric forms have properties that are distinct from those of high molecular weight complexes that form functional SK3 channels or caveolae (Sargiacomo *et al.*, 1995; Scheiffele *et al.*, 1998). This important aspect is not discussed. Furthermore, studies by Corey and Clapham (1998) using SDS-PAGE demonstrate that K_{ir} channels are also capable of forming high molecular weight complexes >200 kDa that are resistant to heat and detergents, highlighting the need for full length blot images that include molecular weight markers.
4. No control data are supplied for the $K_{ir}2.1$, SK3 (representing SK_{Ca}) and Cav-1 antibodies used. These should include matched positive and negative controls, preferably using transfected cells and/or knockout mouse tissue. Indeed, transfected cells for $K_{ir}2.1$ and Cav-1 are commercially available (e.g. Santa Cruz Biotechnology, Santa Cruz, CA, USA), while SK3 transfected cells have previously been used by some of the authors (Absi *et al.*, 2007). Such controls should also include immunohistochemistry and Western blots for the genetically altered cell/animals compared with the accompanying tissue of interest. In addition, there may be inconsistency between the specificity and reliability of batches of commercial antibodies due to potential variation in titre, affinity and purity, and these should be considered (see citations in Kirkpatrick, (2009), for example).
5. Related to point 4 above, no immunolocalization data for $K_{ir}2.1$, SK3 and Cav-1 are supplied. Such data should ideally include both whole mount and conventional section immunohistochemical analysis (e.g. Burnham *et al.*, 2002; Grayson *et al.*, 2007), since the distribution of antigens at adjacent vascular cell borders and the cell membrane are optimally viewed from whole mounts, while a small region of the cytoplasm and cell surface expression is best viewed in sections (Burnham *et al.*, 2002; Sandow *et al.*, 2006).
6. Related to points 4 and 5 above, no morphological evidence is presented to support the proposal that SK3 are confined to caveolae, or of their reputed association with K_{ir}, or the adjacent endothelial gap junctions, the latter as previously suggested in the rat mesenteric artery (Sandow *et al.*, 2006). In our opinion, such data would help verify the authors' hypothesis.
7. The focus and implication of the study is on endothelial signalling. However, Cav-1 has been conclusively demonstrated by many independent studies to be strongly expressed in vascular smooth muscle cells [see Grayson *et al.*, (2007, for example) and many other studies extensively documented in comprehensive reviews by Patel *et al.* (2008) and Rahman and Sward (2009), for example]. Such work also includes the rat mesenteric artery (Dubroca *et al.*, 2007). Thus, it is not clear that the changes in protein expression in figure 4 can be taken to reflect endothelial staining and function, when smooth muscle Cav-1 expression could mask any endothelial expression, given that endothelial mass is a small fraction of the surrounding muscle mass (see figure 2, in Sandow and Hill, (2000), for example).
8. The use of 'in parallel' samples containing vessels from which the endothelium has been removed (e.g. Simon and McWhorter, 2003) would have conclusively demonstrated endothelial expression and addressed both the issue of antibody specificity and the contribution of the endothelium to the expression of Cav-1.
9. Hundreds of papers are published every year on the SHR model, which was originally derived from a spontaneous mutation of the WKY strain, and the latter is generally employed as a normotensive control, as in the present study. However, after 40 years of intra-strain inbreeding, it is debatable whether WKY on its own is a sufficient control (see, for example, Louis and Howes, 1990). There is also considerable debate concerning the nature of the 'differences' in endothelial function between these two strains and whether any that do exist are the cause or consequence of hypertension (Bernatova *et al.*, 2009). Interpretation of 'differences' found between SHR and WKY would therefore be more robust if supported both by another hypertensive model and another normotensive strain.

In summary: the conclusion of the paper is that, in mesenteric artery of WKY compared with SHR, 'altered' SK_{Ca}-mediated activity is due to reduced SK_{Ca} and K_{ir} expression and their association with 'altered' Cav-1 properties. However, because the protein expression data gathered did not seem sufficiently robust to meet the demands of the study, the cellular origin of the proteins is not ascertained, and the comparison between strains is not watertight, the implication of the results appears to be overstated, and it seems that the conclusions of the study are thus not fully supported by the data presented.

Shaun Sandow and T Hilton Grayson
University of New South Wales, Sydney, Australia

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