BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01284.x
www.brjpharmacol.org

LETTER TO THE EDITOR

Reply by authors to letter from Sandow and Grayson

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Dear Professor McGrath,

Thank you for giving us the opportunity to respond to the letter addressed to yourself from Drs Sandow and Grayson concerning our recent peer-reviewed paper (Weston *et al*., 2010), which was itself the subject of a complimentary Commentary article (Garland, 2010).

We are confident that Weston *et al*. (2010) is a paper of significant scientific merit that contains novel findings that will stimulate discussion and further experiments.

PREAMBLE 'Many of our concerns about the Western blot data arise because full length blots including molecular weight markers are not presented, and so it is not possible to determine the precise nature and relevance of the excised bands in the Western blot figures. This is a common problem with the presentation of Western blot images and journal space is often cited as an excuse.'

ANSWER In the same BJP issue (Issue 4, June 2010) as Weston *et al*. (2010), there were several other research papers that contained Western blot data (Chung *et al*., 2010; Gomez-Monterrey *et al*., 2010; Ikeda-Matsuo *et al*., 2010; Iqbal *et al*., 2010; Iwanski *et al*. (2010); Müller *et al*., 2010). In *all* these instances, *only* the bands of interest were shown (and the paper by Ikeda-Matsuo *et al*. (2010) was also the subject of a Commentary article; Andreasson, 2010). In other words, the sole depiction of bands of interest has become the norm amongst peer-reviewed journals for the presentation of data of this type. We do not regard this as a 'problem', and we have never been constrained by Editors because of restrictions on journal space.

In Weston *et al*. (2010), we therefore used the normal BJP convention, and this format was not an issue raised by our peer reviewers. For the benefit of our research colleagues, we now present our 'raw data full-length blots' (Figure 1) in the hope that they will appreciate the quality of our data. In any case, we would have been happy to have provided any necessary clarification about molecular markers, etc.

CONCERN 1 'The apparent Kir2.1 protein band that is recognised 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 Kir2.1 band at 62 kDa; whereas in fact, the Kir2.1 protein is 48 200 Da and is not glycosylated.'

ANSWER Prior to the publication of Weston *et al*. (2010), we were aware (in 2008) of the discrepancy between the anticipated band size for $K_{ir}2.1$ (48.2 kDa) and the band size given by Alomone, the suppliers of the antibody, on their data sheet and we therefore contacted Alomone Laboratories Technical support. They replied that the expected MW for the rat K_{ir} 2.1 channel (48.2 kDa) is based solely on the amino acid sequence and does not include glycosylations, phosphorylations or other transcriptional and post-translational alterations which may affect the apparent MW visualized in a Western blot. In addition, they commented that the above parameters can vary between tissues and/or species and different sample preparation protocols may yield different results.

Our published Western Blot data indeed show a band below the 52 kDa marker. Please note that the reference for 52 kDa for the $K_{ir}2.1$ blot was provided by a molecular weight marker ladder, over which computer bars were placed for clarity in our figure presentation, as is the worldwide convention. We do not believe that the resolution of a Western Blot is fine enough to single out relatively tiny weight differences. Where bands fell between two markers, our interpretation of the molecular weight of the protein from the standard ladder was indicated.

CONCERN 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.'

ANSWER The protein loading was 10 μg per lane for Caveolin-1 and for $K_{ir}2.1$, 30 μg per lane for SK3. Ponceau S stain was used simply to visualize protein transfer from the gel onto the membrane. Nowhere in Weston *et al*. (2010) do we state that β-actin was used as a visual reference for equal protein loading, and indeed, it was not. Densitometry analysis was used (see Weston *et al*., 2010; 'samples were adjusted for loading errors, using the β-actin blot density to standardize, prior to normalization relative to WKY values') to normalize all results, and thus, any variation in the protein loading of the original gel would have been accounted for.

CONCERN 2 CONTINUED '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.'

ANSWER Each sample was taken from mesenteric artery branches 1 to 4/5 (or small as we could dissect) from a whole

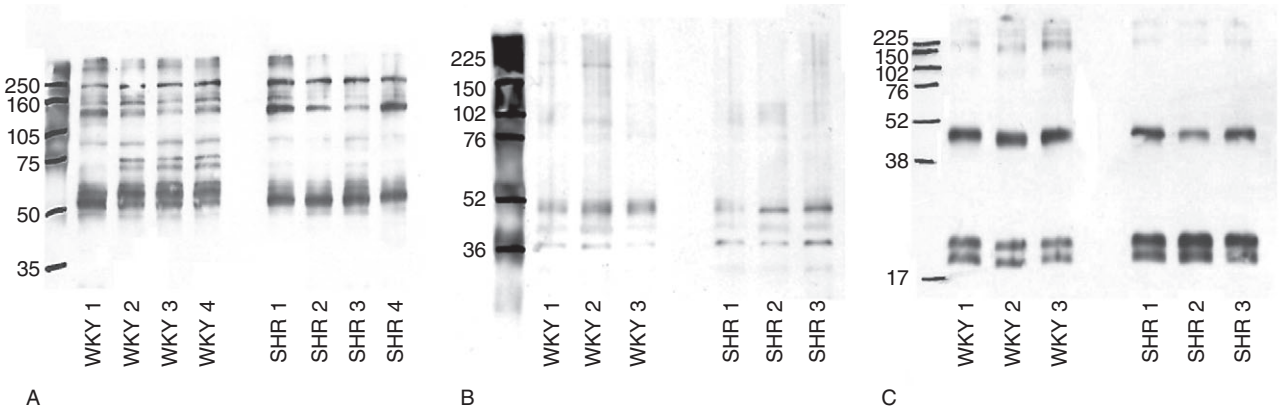

Figure 1

'Raw data' full-length Western Blots. **A**) $K_{Ca}2.3$ (anti-KCNN3; Abcam, Cambridge, UK). Anticipated monomeric band size: 82 kDa. Separated on an 8% acrylamide gel. **B**) $K_{ir}2.1$ (anti-$K_{ir}2.1$; Alomone, Jerusalem, Israel). Anticipated monomeric band size: 48.2 kDa. Separated on a 10% acrylamide gel. **C**) Caveolin-1 (anti Caveolin-1; BD Transduction Laboratories, Oxford, UK). Anticipated monomeric/dimeric band sizes: 22 kDa and 44 kDa, respectively. Separated on a 12% acrylamide gel. Molecular weight markers: RPN800 (**A**) and RPN800E (**B** and **C**) Full-Range Rainbow Molecular Weight Markers (Amersham/GE Healthcare).

mesenteric bed. The protein yield of each 200 µL sample averaged approximately 1–2 $\mu g \cdot \mu L^{-1}$, but regardless of the protein yield, the volume was adjusted to load the aforementioned concentrations for each blot.

CONCERN 2 CONTINUED 'It is stated that the 3 lanes for WKY and SHR were obtained from 3 different animals, but there is no indication of whether the lanes are simply replicates of a single sample derived from 3 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 Kir2.1 and SK3.'

ANSWER Each sample was extracted from the homogenate of a single mesenteric bed (i.e. using one bed per lane, each bed from a different animal).

CONCERN 2 CONTINUED 'In our experience using branches of the superior mesenteric 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 detect the expression of ion channels in the endothelial membrane (Chadha *et al.*, 2010; Haddock *et al.*, 2011).'

ANSWER Sandow and Grayson do not state which branches of the superior mesenteric artery they use, and neither do they detail their protocol for the removal of these vessels. Very careful dissection of the vessels from the mesenteric bed is necessary to prevent extensive loss of endothelial cells, and omitting the smaller-order vessels would reduce the endothelial cell : smooth muscle ratio in the sample. In our experience, careful dissection of the full mesenteric bed provides sufficient protein for approximately six lanes, each loaded with 30 µg protein. Surely, the detection of the probed proteins also depends on the laboratory's particular protocol and visualization method, etc. Details of these could have been made available on request to the corresponding author. However, for reference, our chosen method of band visualization on a nitrocellulose membrane was to use Amersham's ECL Plus Western Blotting Detection Reagents, which claim a sensitivity of '~5 pg'.

CONCERN 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 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 & Clapham (1998) using SDS-PAGE demonstrate that Kir 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.'

ANSWER In the Discussion section of Weston *et al.* (2010), we cautiously 'wonder' and 'speculate' about our observed differences in the expression of Cav-1 (and state that although the total caveolin-1 expression was not different in SHRs and WKYs, the pattern of monomeric and dimeric expression was indeed different) as a 'possibility' that it *may* link with the observed decrease in SK3 and Kir expression. Nowhere do we state 'for certain' that it is the reason for it.

CONCERN 4 'No control data are supplied for the Kir2.1, SK3 (representing SKCa) 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 Kir2.1 and Cav-1 are commercially available (e.g. Santa Cruz Biotechnology); whilst 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 to 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).'

ANSWER Control data were not given in Weston *et al*. (2010) but did (of course) form part of the routine laboratory experimental process in selecting and optimizing the antibodies. As referred to above, the same SK3-transfected cell line as used in Absi *et al*. 2007 was used for a positive control with the antibody we used, with an IK_{Ca}-transfected cell line and a blank HEK cell as negative controls. Indeed, batch variation occurs, and it would be imprudent to assume otherwise, thus the need for optimization of each antibody. For all antibodies to be tested in knockout mouse tissue within a university laboratory setting is unfortunately unattainable owing to limited availability of these animals and the costs involved. Once again, although not published, the control data would have been provided to those requesting it.

CONCERN 5 'Related to point 4 above, no immunolocalization data for Kir2.1, 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, whilst a small region of the cytoplasm and cell surface expression is best viewed in sections (Burnham *et al*., 2002; Sandow *et al*., 2006).'

ANSWER There is a host of techniques that *could* have additionally been used, including immunocolocalization, patch clamping, *in vivo* studies, etc. However, Weston *et al*. (2010) was primarily an electrophysiological study in which the measurements were derived from myocyte impalements in whole, living vessels. The molecular evidence presented in the paper was corroborative, and it appears to us that our peer reviewers took the view that we had provided sufficient additional data to support and clarify the important functional findings.

CONCERN 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 Kir, or the adjacent endothelial gap junctions; the latter as previously suggested in the rat mesenteric artery (Sandow et al., 2006). Such data would help verify the authors' hypothesis.'

ANSWER Data in support of the localization of SK and IK channels have been previously published from our lab (see, for example, Weston *et al*., 2005; Absi *et al*., 2007; Harno *et al*., 2008). We think that Kir2.1 and SK3 are likely to be clustered within, but not to be restricted to, caveolae. An investigation into the changes in caveolin-1 was beyond the scope of the study, but we thought it was sufficiently interesting to show that some modification of the protein appeared to have occurred. Formation of microdomains within caveolae could optimize pathways, and any disruption or loss of caveolae that affected such an association would reduce responses. However, we are not sure how a demonstration of colocalization (or not) of the proteins would have substantively added to the paper since we gave very little consideration to *any* association with caveolae.

CONCERN 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 certain 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).'

ANSWER We had not assumed that Cav-1 was restricted to the endothelium but expected that detected changes would also be present in the endothelium and speculated that this could have contributed to the electrophysiological changes in the SHR vessels. In addition, SK_{Ca} and IK_{Ca} channels are only present in endothelial cells and absent from myocytes (see Edwards *et al*., 2010).

CONCERN 8 'The use of "in parallel" samples containing vessels from which the endothelium has been removed (e.g. Dimon 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.'

ANSWER Endothelium-denuded samples would indeed have shown any contribution of endothelially expressed Cav-1. However, the high specificity of the Caveolin-1 antibody has already been addressed (see Figure 1).

Although Dimon and McWhorter, (2003) were able to de-endothelialize their vessel, this was facilitated by the use of mouse aorta, from which it is much simpler to remove the endothelial cell layer mechanically than from the very many, extremely fine arterioles that form the rat mesenteric bed.

CONCERN 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 forty 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.'

ANSWER Animal models of human disease states are simply models, and none is a perfect paradigm of human essential hypertension. In Weston *et al*. (2010), we discuss the observed differences between our hypertensive model and our chosen control, paradigms that have been and remain widely used in laboratories throughout the world. We propose that our data can therefore be sensibly compared with findings from those obtained by numerous investigators over many years.

FINAL COMMENTS In summary: the conclusion of the paper is that, in mesenteric artery of WKY compared to 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 was not 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 is overstated, and the conclusions of the study are thus not fully supported by the data presented.

ANSWER We respectfully disagree with the final comments of Sandow and Grayson and believe they have overlooked the axiomatic points of the paper and refer only to a (minor) part of its conclusions. We primarily investigated a functional response of the blood vessels and found that there was reduced myocyte hyperpolarization to acetylcholine (EDHF response) in mesenteric arteries from SHRs. Sandow and Grayson do not question these findings, which constitute the most fundamental and important points in our paper. Hyperpolarization results from two endothelium-dependent pathways, one of which involves the opening of SK_{Ca} and the other of IK_{Ca} channels. The discovery that *only* the SK_{Ca} and *not* the IK_{Ca} pathway was modified in the hypertensive model we feel is a truly important advance (as supported by the Commentary of Garland, 2010).

We believe that we have reasonably and even completely answered all the points raised by Sandow and Grayson to the satisfaction of any reasonable individual. However, even if we had not, it would in no way affect the finding that the SK_{Ca} pathway is compromised in the hypertensive model, whereas the IK_{Ca} one is untouched. *This* is the important take-home message from our investigation.

We remain happy that our conclusions are indeed sound, an opinion shared by our peer reviewers, by the Senior Editor who handled the submission and, in the accompanying Commentary (Garland, 2010). The purpose of original scientific papers is to inform colleagues about advances in the field and to stimulate debate. We are delighted that Weston *et al*. (2010) has already succeeded in this respect and invite our colleagues to advance the field even further.

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References

Absi M, Burnham MP, Weston AH, Harno E, Rogers M, Edwards G (2007). Effects of methyl beta-cyclodextrin on EDHF responses in pig and rat arteries; association between SK_{Ca} channels and caveolin-rich domains. Br J Pharmacol 151: 332–340.

Andreasson K (2010). Prostaglandin signalling in cerebral ischaemia. Br J Pharmacol 160: 844–846.

Chung HH, Dai ZK, Wu BN, Yeh JL, Chai CY, Chu KS *et al*. (2010). The xanthine derivative KMUP-1 inhibits models of pulmonary artery hypertension via increased NO and cGMP-dependent inhibition of RhoA/Rho kinase. Br J Pharmacol 160: 971–986.

Dimon AM, McWhorter AR (2003). Decreased intercellular dye-transfer and downregulation of non-ablated connexins in aortic endothelium deficient in connexin37 or connexin40. J Cell Sci 116: 2223–2236.

Edwards G, Félétou M, Weston AH (2010). Endothelium-derived hyperpolarising factors and associated pathways: a synopsis. Pflügers Arch 459: 863–879.

Garland CJ (2010). Compromised vascular endothelial cell SK_{Ca} activity: a fundamental aspect of hypertension? Br J Pharmacol 160: 833–835.

Gomez-Monterrey I, Campiglia P, Bertamino A, Aquino C, Sala M, Grieco P *et al*. (2010). A novel quinone-based derivative (DTNQ-Pro) induces apoptotic death via modulation of heat shock protein expression in Caco-2 cells. Br J Pharmacol 160: 931–940.

Harno E, Edwards G, Geraghty AR, Ward DT, Dodd RH, Dauban P *et al*. (2008). Evidence for the presence of GPRC6A receptors in rat mesenteric arteries. Cell Calcium 44: 210–219.

Ikeda-Matsuo Y, Tanji H, Ota A, Hirayama Y, Uematsu S, Akira S *et al*. (2010). Microsomal prostaglandin E synthase-1 contributes to ischaemic excitotoxicity through prostaglandin E_2 EP_3 receptors. Br J Pharmacol 160: 847–859.

Iqbal U, Trojahn U, Albaghdadi H, Zhang J, O'Connor-McCourt M, Stanimirovic D *et al*. (2010). Kinetic analysis of novel mono- and multivalent VHH-fragments and their application for molecular imaging of brain tumours. Br J Pharmacol 160: 1016–1028.

Iwanski GB, Lee DH, En-Gal S, Doan NB, Castor B, Vogt M *et al*. (2010). Cucurbitacin B, a novel *in vivo* potentiator of gemcitabine with low toxicity in the treatment of pancreatic cancer. Br J Pharmacol 160: 998–1007.

Müller G, Jung C, Wied S, Biemer-Daub G, Frick W (2010). Transfer of the glycosylphosphatidylinositol-anchored 5′-nucleotidase CD73 from adiposomes into rat adipocytes stimulates lipid synthesis. Br J Pharmacol 160: 878–891.

Weston AH, Absi M, Ward DT, Ohanian J, Dodd RH, Dauban P *et al*. (2005). Studies with calindol and Calhex 231: Evidence in favor of a calcium-sensing receptor in arterial endothelial cells. Circ Res 97: 391–398.

Weston AH, Porter EL, Harno E, Edwards G (2010). Impairment of endothelial SK_{Ca} channels and of downstream hyperpolarizing pathways in mesenteric arteries from spontaneously hypertensive rats. Br J Pharmacol 160: 836–843.