Blockade of the Renin-Angiotensin and Endothelin Systems on Progressive Renal Injury

To the Editor:

We read the article by Cao et al. published recently in *Hypertension* and have major concerns with respect to the results and interpretation of the autoradiography section. We especially refer to the kidney autoradiography study and mention for the sake of clarity only one endothelin (ET) receptor antagonist, namely, bosentan. Nevertheless, the same argument is valid for the second ET receptor antagonist used, BMS193884.

In the Methods section, only a few details with respect to the autoradiography technique are provided. Even after checking the cited Reference 21, some questions remain concerning the methodology. Conditions used for incubation are unclear. What molar concentration is 0.3 µCi? (Incubation is normally performed at the approximate Kd of the radioligand, which for 125I-endothelin-1 is approximately 100 pmol).

One-hour incubation in buffer containing 125I-endothelin-1 may be too short (most groups use a 2-hour incubation period, in which maximal binding has been achieved). Presumably, Figure 6A illustrates total radioligand binding. Non-specific binding (in the presence of 10^3 mol/L ET-1) should also be presented.

125I-endothelin-1 binding in Figure 6A is somewhat regionalized, and this becomes more evident in the presence of ET antagonists. How accurate is densitometric analysis of such patchy binding?

Major criticisms arise regarding the interpretation of the data. The authors are able to confirm (previously published) renal 125I-endothelin binding in untreated rats. A new finding is that this binding is dramatically reduced when the rats are pretreated with a single administration of bosentan. The authors’ explanation for this reduced binding (page 566) is that ET receptors are occupied as the result of the in vivo administration of bosentan, and this occupation prevents subsequent in vitro binding when sections are incubated with 125I-endothelin-1. We doubt that receptor occupancy by bosentan can be shown with the use of this approach.

In the Methods section, the protocol used for autoradiography is based on that cited in Reference 21. In this study, all sections were preincubated in HEPES/NaCl/CaCl2 buffer. This is usually performed to remove or reduce the endogenous ligand before the addition of radioactive ligand. When the sections are subsequently incubated with 125I-endothelin-1, a new steady state will be established and the radioligand may be expected to displace any remaining bound ligand, be it a receptor antagonist (such as bosentan) or ET itself. Only if one assumes such a theoretical series of events, Figure 6A (binding of radioactive ET-1 in untreated rats) can be explained: In vivo, a proportion of renal ET receptors was occupied by endogenous ET. In the preincubation step, this endogenous ET was reduced, and any remaining binding was displaced during the incubation step by 125I-endothelin-1, resulting in the occupation of a large proportion of ET receptors by 125I-endothelin-1. However, this is unlikely to occur in the bosentan experiment.

The authors conclude that renal ET receptors remain occupied after in vivo administration of bosentan. This can only be explained if a very “tight” reversible, or even irreversible, binding of bosentan to receptors has occurred. Published data show that bosentan competitively binds to ETα and ETβ receptors with Ki values of 4.7 and 95 nmol/L, respectively.3-4 This binding is up to 1000 times weaker than that of the endogenous ligand, ET-1 (120 and 110 pmol for ETα and ETβ, respectively). Therefore, displacement of bosentan, either by preincubation or by incubating with 125I-endothelin-1, should be possible.

In summary, the presentation and interpretation of the autoradiographic data are inconclusive. The autoradiographic approach described in this article will only allow receptor occupancy by in vivo drug treatment to be studied if ligand binding is irreversible. Because this is not true for bosentan, the interpretation of the data is questionable. An alternative explanation for the results is that the reduced 125I-endothelin-1 binding that the authors describe is due to an altered receptor number and not to receptor occupation. At the time course studied, this explanation is unlikely but could be addressed by experiments of ET receptor gene expression.

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Response

We thank Drs Muenter and Dashwood for their interest in our study1 and their carefully considered comments. We agree that not all details of the procedures were included in the initial manuscript; they have been previously reported in full.2,3 In brief, the sections were incubated in 0.3µCi 125I-endothelin-1/mL. The specific activity of 125I-endothelin-1 was 630 µCi/µg. We used a 1-hour incubation as this has been previously demonstrated to be associated with almost maximal binding, but we agree that a 2-hour incubation may be associated with a further modest increase in total binding.

The major issue relates to the appropriateness of the technique used to assess ex vivo the ability of bosentan and BMS193884 to compete for the endothelin (ET) receptor. We agree that it is possible that the preincubation step may be associated with removal of the endogenous ligand and ET antagonists. There is no information on the kinetics of this interaction, and it is not known what the off-rate of these antagonists from the receptor is, or how much of the antagonists were displaced from the receptor during this preincubation step. In vivo treatment with bosentan and BMS193884 was associated, however, with a significant reduction in 125I-endothelin-1 binding in both kidney cortex and medulla. This would indicate that a proportion of the ET receptor antagonists was still bound to the ET receptors at the time of the incubation with the radioligand.

This ex vivo approach to assess occupancy and degree of inhibition by peptide receptor antagonists has been extensively explored with respect to the angiotensin II receptor. Several groups have clearly demonstrated that this methodology accurately predicts the potency of angiotensin II receptor antagonists, and both dose and time dependency can be accurately quantitated.3,4 This technique has not been as extensively investigated for the ET receptor. There are no theoretical reasons, however, for such an approach to not be useful for assessment ex vivo of ET receptor blockade. The findings of our study using autoradiography complement the results obtained by assessing the pressor effects of big ET-1 after oral bosentan treatment.5

It should also be appreciated that the exact interaction at the site of the receptor between the endogenous ligand and the antagonist has not been fully clarified. It appears that the ligand and the receptor...
blocker do not bind to the same epitope of the receptor. Importantly, the major finding of the study was that despite use of the high doses of 2 different ET receptor blockers that have been shown to confer organ protection in other experimental contexts, there is no evidence that either ET receptor antagonist had a renoprotective effect in this model of hypertension and progressive renal injury.

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