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LETTER TO THE EDITOR

Comments on "Calpain Mediates the Dioxin-Induced Activation and Down-Regulation of the Aryl Hydrocarbon Receptor"

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Dale and Eltom (2006) suggest that activation of the aryl hydrocarbon receptor (AHR)-signal transduction pathway is initiated after proteolytic processing of the AHR by calpain proteases. The authors propose that the calpains are activated by an increase in intracellular calcium that is initiated by ligand exposure. Although the hypotheses put forth by Dale and Eltom (2006) are very interesting, their report requires some critical commentary because they do not reconcile their results with the established pharmacology of AHR signaling or previously published reports that have implicated the ubiquitin-proteasome pathway in the degradation of the AHR (Davarinos and Pollenz, 1999; Roberts and Whitelaw, 1999; Ma and Baldwin, 2000; Ma et al., 2000; Song and Pollenz, 2002, 2003; Wormke et al., 2003; Wentworth et al., 2004).

First, a major contention of Dale and Eltom (2006) is that the MG-132 used in previous reports to inhibit the proteasome is having its affect by inhibiting calpains. Although this is possible, several reports have addressed this issue using a variety of approaches. Ma and Baldwin (2000) previously showed that exposure of Hepa-1 cells to the calpain inhibitors calpastatin or PD150606 (also used by Dale and Eltom, 2006) had no impact on TCDD-mediated degradation of the AHR or induction of CYP1A1. Davarinos and Pollenz (1999) also showed that the calpain inhibitor *N*-acetyl-Leu-Leu-Met-aldehyde did not block AHR degradation in a rat cell line. More importantly, Ma and Baldwin (2000) as well as Roberts and Whitelaw (1999) demonstrated that the specific proteasome inhibitor lactacystin (no reported cross-reactivity with calpains) inhibited TCDD-mediated degradation of the AHR in a manner identical to that of MG-132. In addition, Ma and Baldwin (2000) showed that ligand induced degradation of the AHR was inhibited in cells that contained a temperature-sensitive mutation in the E1 ubiquitin-conjugating enzyme. Taken together, these reports provide compelling evidence that MG-132 is blocking the proteasome and that ligand-induced degradation of the AHR is mediated via the ubiquitin proteasome system and not by calpains. In their report, Dale and Eltom (2006) rely on one experiment with epoxomicin (a specific and irreversible proteasome inhibitor) to validate that the AHR is degraded by calpains. However, the authors obtain a negative result when cells are exposed to epoxomicin, and they do not provide controls to validate that epoxomicin exposure actually reduced proteasome activity in their experiments.

A second point that is in direct conflict with several published reports is the contention that calpain inhibitors as well as MG-132 block TCDD-mediated induction of endogenous *CYP1A1* in culture cells by preventing the nuclear localization of the AHR (because it has not been proteolytically processed). To support this hypothesis, Dale and Eltom (2006) treat Hepa-1 cells with a calpain inhibitor in the presence and absence of TCDD and then assess the localization of the AHR via immunofluorescence microscopy. Unfortunately, it is difficult to interpret the staining in these studies because antibody controls were not used and only 3 to 4 cells are presented in each micrograph. In addition, Dale and Eltom (2006) fail to discuss previous studies that show that treatment of cells with MG-132 (5–25 μM) can actually enhance the TCDD-mediated induction of endogenous *CYP1A1* as well as AHR-dependent reporter gene activity (Davarinos and Pollenz, 1999; Roberts and Whitelaw, 1999; Ma and Baldwin, 2000; Ma et al., 2000). Several reports also present staining of the endogenous AHR in several cell lines after exposure to MG-132 and show that MG-132 induces nuclear localization of the endogenous AHR in the absence of ligand exposure (Davarinos and Pollenz, 1999; Song and Pollenz, 2002, Santiago-Josefat et al., 2001; Santiago-Josefat and Fernandez-Salguero, 2003). Santiago-Josefat et al. (2001) actually show that treatment of primary mouse fibroblasts with MG-132 (8 μM) or *N*-acetyl-leucyl-leucyl-norleucinal (50 μM) results in ligand-independent up-regulation of *CYP1A1* and increased binding of AHR·ARNT dimers to DNA. Other labs have also found that AHR·ARNT binding to DNA is increased in cells exposed to MG-132 and TCDD (Davarinos and Pollenz 1999). Thus, every previous study that has used MG-132 and other proteasome inhibitors to inhibit AHR degradation has demonstrated that MG-132 does not affect the ability of AHR to localize to the nucleus or mediate the induction of CYP1A1.

Finally, it is intriguing that Dale and Eltom (2006) hy-

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ABBREVIATIONS: AHR, aryl hydrocarbon receptor; MG-132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; PD150606, (2*S*)-3-(4-iodophenyl)-2-sulfanyl-propanoic acid; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ARNT, aryl hydrocarbon receptor nuclear translocator.

pothesize that the AHR is a preprotein requiring proteolytic cleavage to be translocated to the nucleus to initiate gene regulatory events. The cleavage event is proposed to occur in the C-terminal region of the AHR that is required for transactivation. However, the authors do not provide direct evidence of this cleavage by demonstrating that the DNA bound form of the AHR is reduced in comparison to the latent AHR. It is noteworthy that several previous reports have assessed the DNA bound and latent AHR and shown that the AHR does not exhibit a detectable reduction in molecular mass after ligand binding, nuclear translocation, dimerization to ARNT or DNA binding (Pollenz et al., 1994; Pollenz, 1996; Davarinos and Pollenz, 1999; Ma and Baldwin, 2000; Santiago-Josefat et al., 2001; Pollenz and Dougherty, 2005). In addition, if cleavage of the AHR is a requirement for activation, it would be expected that a truncated AHR would become nuclear. However, recent studies have shown that removal of the C-terminal 165 amino acids from the mouse Ah^{b-1} receptor does not result in nuclear localization or gene activation in the absence of ligand (Pollenz et al., 2005).

In closing, there have been numerous studies from several different laboratories that have investigated ligand-induced degradation of the AHR and the response of cells after exposure to various protease inhibitors. As detailed in the commentary, there is no evidence for the involvement of calpains in the activation or termination of the AHR signal transduction pathway. Although it is expected that new research will challenge established models and lead to modified hypotheses, it is the responsibility of all authors to interpret their results in the context of the literature database and reconcile significant differences in their findings in a rigorous manner, especially when the same model systems and test compounds have been used. This response to the work of Dale and Eltom (2006) has been prepared not because the authors propose a unique mechanism, but because the experiments that the mechanism is based on have not been adequately explained in the context of previously published reports from several independent laboratories.

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