Acute chemotherapeutic treatment causes hyperphosphorylation of cardiac ryanodine receptors

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The anthracycline doxorubicin is a powerful chemotherapeutic agent used effectively in the treatment of breast cancer. The use of doxorubicin is limited however, due to the development of cardiotoxic side effects including arrhythmia and heart failure. Current theories concerning the acute mechanisms of doxorubicin-induced cardiotoxicity suggest the drug and its metabolite accumulate in the intracellular calcium store (the sarcoplasmic reticulum; SR), targeting SR proteins and altering cardiomyocyte calcium homeostasis (Minotti *et al.*, 2004). Such disturbances have long been associated with cardiac dysfunction. Doxorubicin targets include the cardiac ligand-gated ryanodine receptor (RyR2) which is the SR calcium release channel and the primary SR calcium binding protein, calsequestrin (CSQ), which also modulates RyR2 activity. *In vitro* assays show that anthracyclines reduce the calcium binding capacity of CSQ, alter SR calcium release and impair RyR2 activity. These functional changes are thought to be due to doxorubicin binding directly to these SR proteins and also to drug-induced oxidation of RyR2 (Hanna *et al.*, 2011).

To investigate the effects of doxorubicin treatment in modifying RyR2 function, we developed an acute doxorubicin treated mouse model. Male and female C57BL/6 mice (8-12 week old) were injected with a single dose of doxorubicin (15 mg/kg i.p.); controls received saline i.p. Cardiac damage was assessed by measuring serum levels of total creatine kinase and lactate dehydrogenase. Mice were euthanized by CO_2 asphyxiation at day 7 and SR vesicles were prepared from ventricular tissue (Laver *et al.*, 1995). Protein expression was examined with SDS-Page and Western Blot. Proteins were probed with antibodies to detect expression levels of RyR2, CSQ2, junctin, triadin, FKBP 12.6 and SERCA. The possibility that RyR2 phosphorylation contributed to doxorubicin-induced cardiotoxicity was tested by probing RyR2 with antibodies to detect total phosphorylation, and to detect the phosphorylated forms of RyR2 serine residues 2808, 2814 and 2030.

Acute doxorubicin treatment resulted in the reduced expression of RyR2, SERCA and CSQ2. Surprisingly, the amount of CSQ2 which is associated with RyR2 was significantly reduced in the doxorubicin treated mice compared with control mice. These results are consistent with significantly impaired and dysregulated SR calcium release, due to a decline in the number of Ca^{2+} release channels and the reduced proportion of these channels that are activated by CSQ2. In addition we found that RyR2 is hyperphosphorylated at serine 2808 in the drug treated mice and that there was significant dissociation of the RyR2 dephosphorylating enzymes PP1 and PP2A. Hyperphosphorylation of RyR2 has long been associated with RyR2 dysfunction, resulting in after-depolarizations which lead to arrhythmia and also to heart failure. Acute doxorubicin treatment is likely to result in other modifications in RyR2 function (oxidation, ligand-binding) in addition to those caused by RyR2 hyperphosphorylation and reduced CSQ association. The results suggest that multi-faceted disturbances in RyR2 function and calcium release contribute to the clinical manifestation of cardiotoxicity induced by doxorubicin treatment.

Hanna, A.D., Janczura, M., Cho, E., Dulhunty, A.F., and Beard, N.A. (2011). *Molecular Pharmacology* **80**, 538-549.

Laver, D.R., Roden, L.D., Ahern, G.P., Eager, K.R., Junankar, P.R., and Dulhunty, A.F. (1995). *Journal of Membrane Biology* **147**, 7-22.

Minotti, G., Menna, P., Salvatorelli, E., Cairo, G., and Gianni, L. (2004). *Pharmacological Reveiws* **56**, 185-229.