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## Letter to the Editor

# Distinct initiator caspases are required for the induction of apoptosis in cardiac myocytes during ischaemia *versus* reperfusion injury

Dear Editor,

Loss of cardiac myocytes by apoptosis is an important process in the pathogenesis of heart diseases.[1,2] Recent studies have indicated that cardiac cells exposed to a hypoxic/ischaemic (I) insult followed by reperfusion (R) undergo apoptotic cell death *in vitro*[3] and can also be triggered during the reperfusion phase after ischaemia in the intact heart.[4–6] However the mechanisms and signalling pathways by which ischaemic and I/R stimuli produce apoptosis in cardiac cells are as yet unknown and it remains unclear whether ischaemic and I/R trigger apoptosis by common or distinct pathways.

Apoptosis is a form of cell death distinct from necrosis and is normally associated with caspase activation. Initiator caspases (caspase-8 and -9) are upstream activators that respond to ligation of specific death receptors or mitochondrial damage respectively. Recent studies have implicated caspases in cardiomyocyte apoptosis. For example, the infarct size following I/R of the intact heart *in vivo* can be reduced by priming animals with the non-specific caspase inhibitor (ZVAD-fmk), directly demonstrating the role of caspases in mediating cell death in cardiac cells.[7] In addition, human cardiomyopathy is associated with the release of cytochrome *c* and activation of caspase-3 thus suggesting a role of cytochrome *c* and the effector caspase-3 in apoptotic cell death in cardiac cells.[8]

Initially, we assessed whether exposure of cultured rat primary adult cardiac myocytes to simulated I or I/R results in the cleavage and activation of initiator caspases. Figure 1a shows that exposure of cardiac myocytes to I for 6 h resulted in processing of the precursor pro-caspase-9 but not of pro-caspase-8 (Figure 1b). Pro-caspase-9 processing was also observed in cardiomyocytes exposed to I/R. In contrast, processing of pro-caspase-8 was greatly enhanced in cardiomyocytes exposed to simulated I/R (Figure 1b). In addition, processing of the effector caspase, pro-caspase-3 was also observed following both I and I/R (Figure 1c).

The levels of cytosolic cytochrome *c* increased in primary adult cardiomyocytes exposed to I alone which increased further following I/R (Figure 1d). The caspase-3 substrate, poly (ADP-ribose) polymerase (PARP) was also cleaved following exposure of adult cardiomyocytes to both simulated I alone or I/R (Figure 1e). However, pro-caspase-9 processing following I alone or I/R was not associated with changes in Bax, Bcl-2 or Bcl-x (data not shown). Similar results were also obtained using rat primary neonatal cardiomyocytes, demonstrating that the mechanism of apoptosis following I and I/R are not altered by age or development.

The role of caspase-8 and caspase-9 activation in the induction of apoptosis in cardiac cells exposed to simulated I and I/R was further assessed using specific caspase-8 or caspase-9 chemical inhibitors. Pre-treatment with a caspase-9 inhibitor but not a caspase-8 inhibitor reduced the level of Annexin V surface staining in both neonatal and adult (Figure 1f) cardiomyocytes exposed to simulated I. In contrast, both caspase-8 and caspase-9 inhibitors, when added separately, reduced the numbers of apoptotic cells in cultures exposed to simulated I/R. Furthermore, the level of apoptosis in cardiomyocytes exposed to simulated I/R was reduced even further when both caspase-8 and -9 inhibitors were added together (Figure 1f).

Similar results were also obtained by using gene-based inhibitors to block caspase-8 and caspase-9 activation by transfecting primary neonatal cardiomyocytes with vectors expressing FLIP or caspase-9s respectively. Figure 1g shows that overexpression of caspase-9s but not FLIP in cardiac cells reduced cell death following exposure to I alone. In contrast, cardiac cells overexpressing either FLIP or caspase-9s showed reduced levels of apoptosis after simulated I/R. Overexpression of both FLIP and caspase-9s together resulted in a further reduction of cell death compared with either FLIP or caspase-9s separately. Hence, these results with gene-specific inhibitors, together with the chemical caspase inhibitor data, strongly suggest that separate ischaemic (caspase-9 dependent) reoxyfenation (caspase-8 and -9 dependent) apoptotic pathways are activated during the cause of I/R injury in neonatal and adult cardiomyocytes. This suggests that ischaemia produces apoptosis through the intrinsic mitochondrial pathway, whereas reperfusion-induced cell death is additionally mediated through activation of an extrinsic death receptor pathway.

The selective activation of distinct initiator caspases during ischaemia and reperfusion has important implications for the management of myocardial infarction. In particular, the potential release of a death receptor ligand with consequent activation of caspase-8 following reperfusion suggests that strategies based on ligand scavenging or inhibition may have therapeutic merit. Moreover, extension of the experiments reported here in cardiomyocytes to other cell types, such as neurons, which may also be susceptible to I/R injury, may suggest novel and generalised ways of minimising ischaemia and I/R-induced apoptosis.

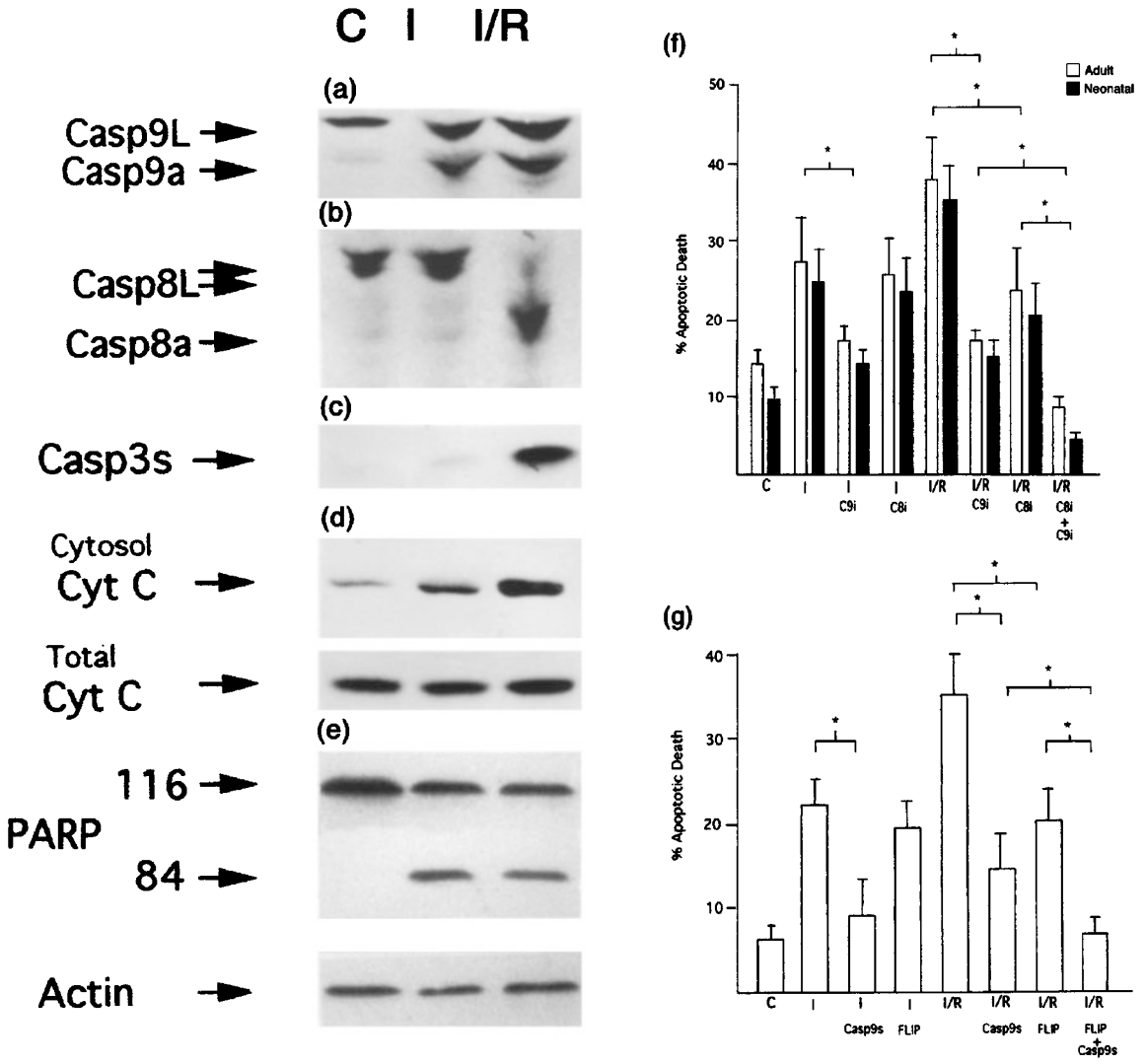


**Figure 1** (**a**) Western blot showing Processing of pro-caspase-9 from neonatal cardiomyocytes exposed to either simulated I or I/R. Arrows indicate pro-form (Casp9L) and also processed forms (Casp9a) of caspase-9. (**b**) Western blot showing Processing of pro-caspase-8 from neonatal cardiomyocytes exposed to either simulated I or I/R. Arrows indicate pro-form (Casp8L) and also processed forms (Casp8a) of caspase-9. (**c**) Western blot showing processing of pro-caspase-3 from neonatal cardiomyocytes exposed to either I or I/R with a specific caspase-3 antibody that recognizes the processed form (Casp3s) of caspase-3. (**d**) Western blot showing release of mitochondrial cytochrome *c* (Cyt C cytosol) into the cytosol from neonatal cardiomyocytes exposed to either simulated I alone or I/R. Lower panel shows levels of total cytochrome *c* (Cyt C Total). (**e**) Processing of PARP from neonatal cardiomyocytes exposed to either simulated I or I/R. Arrows indicate the precursor (116 Kd) and the processed form (84 Kd) of PARP. (**f**) Effects of pre-treatment with either caspase-8 or caspase-9 chemical inhibitors alone (50 $\mu$M) or together on apoptotic cell death in neonatal (black columns) and adult (white columns) cardiomyocytes exposed to simulated I or I/R as assessed by Annexin v staining. Values are the mean of three determinations whose S.E. is shown by the bars. $^*P<0.05$. (**g**) Effects of caspase gene inhibitors FLIP or caspase-9s alone or together on apoptotic cell death in neonatal cardiomyocytes exposed to simulated I or I/R assessed by morphological examination and TUNEL staining as described in the methods. Values are of triplicate transfection and percentage of apoptotic blue cells and their S.E. is shown by bars. $^*P<0.05$

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