CHAPTER IV

DISCUSSION AND CONCLUSIONS

Mitochondria have been shown to play a key role in cardiac dysfunction caused by myocardial ischemia.[61] Previous study demonstrated that oxidative stress occurring in ischemic myocardium caused an increased ROS production in cardiac mitochondria.[89] The increased ROS production in one or a few mitochondria could trigger the release of ROS from neighbouring mitochondria, a mechanism known as ROS-induced ROS release[90], resulting in a large amount of ROS accumulated in the ischemic myocardium. The increased ROS level could lead to the disruption of the electron transport chain in cardiac mitochondria, causing cardiac mitochondrial dysfunction and eventually leading to myocardial cell death.[89] Pharmacological interventions to attenuate mitochondrial dysfunction have been shown to have cardioprotective effects, including prevention of arrhythmia and reduction of infarct size.[91, 92]

Granulocyte-colony-stimulating factor (G-CSF) has recently been shown to improve cardiac function, increase blood vessels as well as reduce mortality after cardiac injury under several conditions including ischemic heart.[19-21] However, its effect on cardiac mitochondria undergo oxidative stress induced by H_2O_2 has never been investigated. In the present study, we found that G-CSF could prevent mitochondrial swelling, decrease ROS production and attenuate mitochondrial membrane depolarization in cardiac mitochondria under oxidative stress condition.

In the present study, the effective dose of G-CSF (50 ng/ml) could successfully prevent mitochondrial damage. However, the dose-dependent effect was not observed since high concentrations (up to 200 ng/ml) of G-CSF share equally similar effect as that at 50 ng/ml. Although G-CSF could completely prevent mitochondrial swelling and $\Delta\Psi m$ changes after oxidative stress induced by H_2O_2, it could only decrease the ROS production to a certain level, which was still much higher than that in the control group. These findings indicated that G-CSF may act more effectively in preventing mitochondrial swelling and mitochondrial depolarization, but less effective in preventing ROS production.

Mitochondrial swelling is described as an increase of mitochondrial volume. The cause of mitochondrial swelling is due to the prolonged opening of the mitochondrial permeability transition pore (mPTP), which causes an increase of permeability to solutes with molecular masses up to about 1500 Da. It is known that mPTP opening can be triggered by the increased ROS level.[93] The prolonged mPTP opening leads to free bi-directional movement of low molecular weight molecules across the inner membrane, while proteins remain in the matrix. Consequently, colloidal osmotic pressure increases and causes mitochondrial swelling (the inner membrane cristae unfolded), the rupture of outer mitochondrial membrane and the release of cytochrome *c*, respectively.[94, 95] In this study, G-CSF could effectively prevent mitochondrial swelling caused by H_2O_2, and this protection is as effective as the effect of the blocker of mPTP opening (CsA). These findings suggested that G-CSF might act on mPTP, thus inhibiting the opening of this pore and leading to the prevention of mitochondrial swelling.

Mitochondria is known as a major organelle to produce ROS in the cell. ROS is normally produced at sites of Complex I and III in the electron transport chain (ETC).[4] Under physiological condition, O_2^- is transported to the ETC for oxidative phosphorylation in the mitochondria and converted into a small amount of H_2O_2, respectively.[4] However, these ROS are generally degraded by catalase and glutathione peroxidase in the mitochondria. Under some pathological conditions such as oxidative stress, the ROS production is markedly increased and the ROS degradation process is not sufficiently maintained, resulting in an excess of ROS including O_2^-.

Under physiological condition, O_2^- can be released across inner mitochondrial membrane via IMAC. The release of O_2^- via IMAC opening could cause mitochondrial membrane depolarization.[96] The release of ROS via IMAC opening could activate IMAC of its neighboring mitochondria, resulting in a greatly increased of ROS.[97] This process is called ROS-induced ROS release mechanism, leading to severe oxidative stress in cells.[98] IMAC opening is normally terminated by a reduction of ROS level (i.e. via decreasing ROS production and efflux from the mitochondrial matrix) and ROS scavenging by the antioxidant enzymes.[99] Aon and colleagues clearly demonstrated in their excellent study that certain amount of ROS produced from the ETC is required to accumulate in the mitochondrial matrix up to a critical levels, in order to trigger the opening of IMAC.[7] Once the ROS accumulation reached a point of mitochondrial critical threshold, the mitochondrial depolarization followed and the mitochondrial membrane potential would become unstable in almost the entire population of mitochondria in the mitochondrial network.[7]

In the present study, our finding indicated that H_2O_2 used in this study could cause a markedly increased ROS production in cardiac mitochondria, which must be sufficiently high, resulting in the opening of IMAC and eventually leading to a decrease in $\Delta\Psi m$. According to this mechanism, our findings that G-CSF, CsA and CDP shared a similar efficacy in the prevention of the decreased $\Delta\Psi m$, despite the fact that they had different efficacy in the prevention of ROS production, indicated that these pharmacological interventions could effectively attenuate the ROS production so that the accumulated level was below the critical threshold required for triggering the IMAC opening. In the present study, ROS level was lowest in mitochondria pretreated with CsA, followed by a higher level in CDP and G-CSF groups, respectively. It is possible that the reduction of ROS production caused by G-CSF was sufficient that could bring the ROS level below the critical threshold. As a result, the trigger of IMAC opening was inhibited, resulting in no change in $\Delta\Psi m$ after H_2O_2 application. All of these findings suggested that the accumulation of mitochondrial ROS up to a critical threshold level could be a key determinant for the mitochondrial protection.

Without H_2O_2 application, ROS level in G-CSF and CsA groups was modestly increased. This could be due to the fact that G-CSF itself could increase ROS production by stimulating the angiogenic factors production.[100] In cardiomyocytes, it has been shown that G-CSF could directly stimulate ROS production, which plays a pivotal role in triggering adaptations of the heart to ischemia including growth of the coronary collaterals.[39] However, the ROS level caused by G-CSF alone was not sufficient to cause the opening of the IMAC. In the present study, the application of CsA alone could also raise the ROS level. CsA is

known to effectively block the mPTP opening, therefore the release of ROS from mitochondria could be blocked inside the matrix at some degree. Furthermore, our study showed that CsA alone could cause a modestly decrease in $\Delta\Psi m$, compared to the control group. This evidence suggested that under physiological condition, mPTP flickering (i.e. opening and closing) is essential for the exchange of metabolites between mitochondria and cytosol, thus maintaining mitochondrial membrane potential.[101] Therefore, blocking the opening of mPTP by CsA could prevent this exchange process, resulting in a slight change in $\Delta\Psi m$.

In the present study, the efficacy of prevention of ROS production caused by H_2O_2 application was greatest in CsA, followed by CDP and G-CSF, respectively. For CDP, its lower efficacy in preventing ROS production compared to CsA could be due to the fact that blocking IMAC could only prevent the release of ROS from mitochondrial matrix. However, the remained ROS could still be released from the intermembrane space and out of the mitochondria via mPTP, respectively (Figure 9). Thus, ROS-induced ROS release mechanism could still occur within the mitochondrial network, resulting in an increased ROS level. Since combined treatment of G-CSF and CDP could effectively decrease ROS level to a similar degree as that in CsA, these findings suggested that G-CSF may act as a blocker of mPTP opening, but with less efficacy than CsA in preventing ROS production.

Further requirement

The further study will require to investigate whether the apparent mechanism of G-CSF on cardiac mitochondria through the partial blocker of mitochondrial permeability transition pore (mPTP) or Complex I or III inhibitor in electron transport chain. In addition, other possible pathway may be G-CSF receptor on cardiac mitochondria. This *novel* knowledge may be used to explain G-CSF effects on isolated cardiac mitochondria.

Conclusion

Under oxidative stress, G-CSF could effectively prevent mitochondrial swelling, mitochondrial membrane potential changes and ROS production in cardiac mitochondria. Its mechanism could be due to the inhibition of mPTP opening. These beneficial effects of G-CSF may be used to prevent cardiac mitochondrial damage under oxidative stress condition.