CHAPTER V

DISCUSSION AND CONCLUSIONS

The major findings of this study were as following :

(1) GSH could induce endothelium-dependent and -independent relaxations in concentration-dependent manner. However, endothelium had certain influence in determining GSH potency to modulate vascular tone.

(2) The endothelium-dependent mechanisms of GSH-induced relaxation depended on extracellular Ca^{2+} and involved NO production in endothelium cells. In addition, other possible mechanisms included the activation of K^+ channel.

(3) Methods to induce contraction could determine the vasorelaxant effects of GSH.

(4) GSH was able to directly suppress contraction of vascular smooth muscle. It was possible that GSH might exert its inhibitory effects via disruption of Ca^{2+} influx as well as Ca^{2+} release from sacroplasmic reticulum (SR)

This study demonstrated that the presence of endothelium cells determined the vascular contraction in response to PE which is a known selective α_1 agonist. The disappearance of endothelium cells increased the PE-induced contraction, especially when PE was applied at low concentration. These observations implied the important roles of endothelium in controlling the vascular tone. As known, endothelium protected cells from chemical insults and also control vascular tension. Hence, endothelial dysfunction has been related to several cardiovascular diseases (Kalinowski and Malinski, 2004; Guerci *et al.*, 2001). Endothelium may buffer a swift change of vascular tone by balancing between secretion of vasoconstrictors and vasodilator. The removal of endothelium suggested a loss of vasodilation arm as well as a protective barrier, resulting

in an increase in sensitivity to PE treatment. However, the influence of endothelium toward PE-induced contraction could not be detected when PE was applied at high concentration to provoke the maximal contraction. This may be the limitation of functional testing using the contractility as parameter outcome.

The regulations of vascular tone can be attributed to function of endothelium as well as the contractility of smooth muscle (Webb, 2003; Woodrum and Brophy, 2001). Hence, it can be hypothesized that GSH affects either endothelium or vascular smooth muscle, resulting in modulation of vascular tone. The potential dual effects of GSH may involve activation of vasodilation mechanism through endothelium cells and/or direct inhibition against contraction of vascular smooth muscle.

As shown in this study, the effects of GSH-induced relaxation on the endothelium-intact and endothelium-denude rat aortic rings were incomparable in either the characteristic of the tracing profiles and degree of relaxation. In endothelium-intact rat aortic rings, GSH induced the rapid transient relaxation, but not sustainable. By contrast, in endothelium-denude aorta, the relaxation profiles was more sustainable for longer period. Moreover, GSH at high concentration of 8 mM effectively decreased vascular tension of the precontracted aortic smooth muscle beyond the maximum developed tension (>100%). This particular effect was not observed in endothelium-intact rat aortic rings. Hence, endothelium is important contributing factor in determining the response of aortic muscle toward GSH treatment.

Moreover, the results demonstrated that removal of endothelium from the aortic preparation caused the tissue less sensitive to GSH-induced vasorelaxation. In other words, GSH was more potent in relaxing the vessels with endothelium-intact than those

without endothelium-intact. In addition, the presence of L-NAME, methylene blue, which were inhibitors of nitric oxide synthase (NOS) and guanylate cyclase, respectively, could attenuate the GSH exerted its vasorelaxant activity mainly via the NO-cGMP pathway. This conclusion was in agreement with the previous report that GSH induced endothelium-dependent relaxation through activation of NO-cGMP pathway (Cheung and Schulz, 1997). Another interesting finding was that glibenclamide, a known K^+ channel blocker, also inhibited the effect of GSH, although with a lesser degree than L-NAME and methylene blue. Other inhibitors of endothelium-dependent vasorelaxation including atropine, propanolol, ibuprofen had no influence on GSH-induced relaxation. Therefore, in addition to the NO-cGMP pathway, GSH exerted its vasorelaxant activity via membrane hyperpolarization as minor pathway. Furthermore, the vasorelaxant effects of GSH were unlikely to involve with production of PGI_2, or activation of β_2-adrenergic and cholinergic receptors (mascarinic receptor). It was likely that GSH targeted at endothelium cells and subsequently activate NOS activity to increase production of NO.

Since vasodilatation can result from activation of hyperpolarizing factors which can be demonstrated through inhibition of K^+ channel (Garland *et al.*, 1995). It has been established that an opening of K^+ channels results in membrane hyperpolarization, leading to close of Ca^{2+} channels. Consequently, vasodilatation occurs (Jackson, 2000; Garland *et al.*, 1995). The inhibitory effect of glibenclamide against GSH-induced vasorelaxation was observed in either endothelium-intact or endothelium-denude aortic preparations in this study. In addition, in endothelium-denude preparation, only glibenclamide could attenuate the relaxation induced by GSH. Thus, hyperpolarizing

factors could be attributed to the endothelium-dependent and-independent mechanisms of GHS-induced relaxation.

The NO-cGMP pathway could be divided into two sequential steps. The initial step involved the NO production in endothelium and subsequently followed by the cGMP production in vascular smooth muscle cell to induce relaxation. The initial step of NO-cGMP pathway was activated by a rising of intracellular Ca^{2+}. The major source of cytosolic Ca^{2+} are from an influx of extracellular Ca^{2+} and from a release Ca^{2+} of internal stored Ca^{2+} (Fukao *et al.*, 1997; Ungvari *et al.*, 2001; Dora *et al.*, 1997). In this study, the relationship between the source of Ca^{2+} and the mechanism of GSH-induced relaxation in endothelium cells has been determined. The results showed that the relaxant effects of GSH were related to inhibition of the Ca^{2+}-free medium containing EGTA but were not inhibited after rapid buffering of intracellular Ca^{2+} in endothelial cell with a membrane-permeable chelator (BAPTA-AM). Hence, the effects of GSH are clearly extracellular Ca^{2+}-dependent.

The sites of GSH action on endothelium cells have not been reported. It was very unlikely that GSH was rapidly transported into the cells and elicited its actions. In addition, the previous studies reported that NAC could transport into the cell but GSH not effectively transported into cells (Raftos *et al.*, 2007; Kugiyama *et al.*, 1998). Hence, it possible that the addition of GSH which is difficult to transport within the cell. It is possible that GSH may affect specific target protein on plasma membrane which consequently connected to the process in NOS activation. It is quite certain that GSH has no effect on muscarinic receptor because treatment of atropine could not abolish GSH-induced vasorelaxation. However, activation of muscarinic receptors involved increasing

of intracellular Ca^{2+} from both activation Ca^{2+} influx and Ca^{2+} release from internal store in endothelial cells (Fukao *et al.*, 1997; Ungvari *et al.*, 2001; Dora *et al.*, 1997; Misbahuddin *et al.*, 1985). In this study, GSH could activate the Ca^{2+} influx from extracellular source. As known, increase of Ca^{2+} also activated NOS activity in endothelial cells, resulting in NO production (Palmer *et al.*, 1988; Bredt and Synder, 1990; Yang *et al.*, 1999). Therefore, treatment of GSH could lead to the increase in intracellular Ca^{2+} and activate NOS activity. Furthermore, the results of this study demonstrated that GSH could potentiate the aortic relaxations induced by acetycholine (Ach) but not those induce by sodium nitropusside (SNP). This observation was in agreement with other reports performed in aortic rings isolated from spontaneous hypertensive rat (SHR) rats. It has been reported that the effect of GSH could not potentiate the aortic relaxations induced by sodium nitropusside (SNP) (Akpaffiong and Taylor, 1998). Hence, vasorelaxant effects of GSH were unlikely to involve with endothelium-independent activation of cGMP. In addition, methylene blue, which was a known inhibitor of guanylate cyclase could not inhibit the GSH-induced relaxation of the endothelium-denude rat aortic rings. Taken together, the mechanisms of GSH-induced relaxation may involve with the NO-cGMP pathway by increase intracellular Ca^{2+} and NO production in endothelial cells, but not the cGMP production in vascular smooth muscle cell. In addition to activation of NOS, an increase of intracellular Ca^{2+} in the endothelium cells could trigger the release of endothelium-derived hyperpolarizing factor from endothelium (Chen and Suzuki, 1990).

The recent study found that the vasodilation effect of NAC was correlated to an increase in expression of the endothelial NOS protein as well as eNOS activity

(Penchanova *et al.*, 2007). As known eNOS activity was dependent of intracellular Ca^{2+} concentration. The comparable characteristics of GSH and NAC in modulating vascular tone found in this study suggested that these two compounds may possess the similar intrinsic activity in affecting the NO-cGMP pathway. There were some reports regarding to the mechanism of NAC and its properties to both enhance intracellular GSH synthesis and directly scavenge reactive oxygen species (Tossios *et al.*, 2008).

GSH was able to relax vascular smooth muscle directly. The potency of the endothelium-independent GSH-induced relaxation depended on type of precontractants which include PE, KCl and Bay K8644. This study demonstrated that the endothelium-denude aortic preparations precontracted with PE (an α_1 agonist) were more sensitive to GSH-induced relaxation than those precontracted with KCl (a depolarizing agent) and Bay K8644 (L-type Ca^{2+} channel opener). It was possible that the components of the contraction-induced by different methods were different. GSH might affect at a specific target on plasma membrane which could result in vasorelaxation. As previously described, GSH caused endothelium-independent vasorelaxation which could be inhibited by treatment of glibenclamide. It implied that GSH could influence the K^+ channel and hyperpolarization of plasma membrane.

In addition to vasorelaxant property, GSH directly inhibited the vasoconstriction induced by serotonin (5-HT; 1 μM) and histamine (1 mM). However, the inhibitory effects of GSH against PE-induced contraction of endothelium-denude preparations and endothelium-intact preparations were similar. In this study, the inhibitory effects of GSH against PE-induced contraction of endothelium denude aortic muscles were seen when PE was less than 0.1 μM. The lower concentrations of PE were applied to invoke

contraction, the higher inhibitory effects of GSH were observed. However, GSH could not suppress the contraction induced by potassium chloride (KCl; 60 mM) and tetraethylammoniun chloride (TEA; 1 mM). Hence, the results indicated that the effects of GSH depended on how the contraction was provoked. It should be noted that the contraction induced by membrane receptor agonists (PE, 5-HT and histamine) were more sensitive to GSH treatment than those contracted with depolarizing agent (KCl) as well as K^+ channel blocker (TEA).

This study was design to investigate the intracellular effects of GSH by using protein kinase C activator to induce contraction. The results showed that GSH had no effects on the PMA-induced contraction, suggesting that GSH had no influence beyond activation of protein kinase C and PMA signaling in the muscle cells. However, GSH could inhibit the transient contractions induced by PE, but not inhibit those induced by caffeine. This could be evidenced that GSH could inhibit the IP_3 mediated-release of Ca^{2+} from SR. On the contrary, GSH had no effect on the caffeine-induced Ca^{2+} release through ryanodine receptor at the SR. Furthermore, the previous studies reported that extacellular GSH was not effectively transported into cells (Deneke and Fanburg, 1989; Kugiyama *et al.*, 1998). Taken together, it can be concluded that GSH had no significant influence on signaling or receptor within the cells. Upon its interaction with the targets resided in the plasma membrane, GSH elicited its effects through mechanisms correlated with a rising of cytosolic Ca^{2+}.

Several studies showed that thiol redox process played a key role in control of the L-type Ca^{2+} activity, in which oxidation close the Ca^{2+} channel (Iesaki & Wolin, 2000; Chiamvimonvat *et al.*, 1995). The activation of L-type Ca^{2+} channels was responsible to a

Ca^{2+} influx across plasma membrane of vascular smooth muscle cells and results in muscle contraction (Karaki and Horri, 1998). The activation of L-type Ca^{2+} channels is regulated by either α_1-receptor or activation of membrane potential. This study revealed that GSH interfered extracellular Ca^{2+} influx into the cells in concentration-dependent manner. It could be hypothesized that GSH which is a thiol agent might interfere the activity of Ca^{2+} channel. Another finding to support this hypothesis was the ability of GSH to suppress Ca^{2+} influx under the high K^+ condition, in which the Ca^{2+} channels were fully activated. In addition, GSH could attenuate the Ca^{2+}-induced contraction in the presence of Bay K8644 (1 μM). Furthermore, GSH also affected the spontaneous contraction upon addition of Ca^{2+} to Ca^{2+}-depleted aorta, suggested that GSH could disrupt the spontaneous Ca^{2+} influx during the replenishment of store-operated Ca^{2+} channels (SOC).

GSH is an antioxidant containing a sulfhydryl group (Dickinson and Forman, 2002; Deneke and Fanburg, 1989; Georg and David, 1999). It is possible that the intrinsic activity of GSH in modulating the vascular tone resided in its sulfhydryl group. L-valine, which has no the sulfhydryl group, could not inhibit contraction induced by PE and could not inhibit either transient phasic or sustain tonic contraction of several types of stimuli. Moreover, other sulfhydryl-containing compounds such as NAC, homocysteine and captopril, like GSH, exerted their inhibitory effects against the contraction induced by PE, histamine and 5-HT. These compounds at the concentration of 5 mM were similar in reducing properties as demonstrated in the *in vitro* DPPH free radical scavenging assay. These results indicated that the sulfhydryl group is important to inhibit contraction induced by PE in both phasic and tonic contraction. In addition, homocysteine which is a

thiol agent could inhibit contraction to many types of stimuli (PE, 5-HT, KCl, Bay K8644) in Ca^{2+}-free environment. Therefore, it was very likely that sulfhydryl group possesses intrinsic property that was necessary for reducing the Ca^{2+} influx through membrane Ca^{2+} channels: receptor operated Ca^{2+} channels (ROC), voltage-operated Ca^{2+} channels (VOC) and store-operated Ca^{2+} channels (SOC) of vascular smooth muscle cells. There were certain reports that H_2S stimulated an opening of K^+_{ATP} channel of vascular tissue, resulting in membrane hyperpolarization and close of voltage-gated Ca^{2+} channel (Zhao *et al.*, 2001; Zhao and Wang, 2002). In addition, H_2S might directly inhibit voltage-gated Ca^{2+} channel in vascular smooth muscle cells (Zhao and Wang, 2002). Furthermore, the thiol compounds like H_2S affected the BK_{Ca} channel through modulation of its cysteine residue (Ha *et al.*, 2000; Lang and Harvey, 2002). Another mechanism might relate to antioxidant potential of thiol-containing compounds, which was able to protect vascular smooth muscle cell from oxidative stress (Wang *et al.*, 2006). Exogenous of L-cysteine could generate H_2S through cystathionine β–synthase (CBS) and/or cystathionine α–lyase leading to vasodilation (Cheng *et al.*, 2004). Although this study could not establish the relationship between thiol-containing compound and H_2S production, the results demonstrated the similar findings of several thiol compounds including GSH, NAC, homocysteine and captopril in modulating vascular tone. It was likely that sulfhydryl group in these compounds determined their intrinsic vasorelaxant property. Furthermore, L-valine that did not have sulfhydryl group failed to relax the aortic ring at the same concentration as GSH, suggesting that the induction of relaxation required a sulhydryl group and was not due to nonspecific effects such as osmolarity changes in the perfusion buffer (Kloek *et al.*, 2002).

The antioxidant agents of some natural products such as rutaecarpine (alkaloid), apigenin (flavone) and resveratrol (polyphenol) could induce vasorelaxation (Wang *et al.*, 1999; Zhang *et al.*, 2002; Diebolt and Andriantsitohaina, 2002; Rakici *et al.*, 2005; Novakovic *et al.*, 2006 and Bulac and Demirel-Yilmaz, 2006). The mechanisms of these compounds were involved with endothelium-dependent and endothelium-independent pathways. In addition, the removal of the endothelium could attenuate the relaxant effect of these compounds. It has been found that these compounds exerted its action mainly through NO-cGMP pathway in endothelial cell or inhibition of Ca^{2+} influx in smooth muscle cells. These compounds were shown to suppress membrane receptor operated Ca^{2+} channels (ROC), voltage-gated Ca^{2+} channels (VOC) (Wang *et al.*, 1999; Zhang *et al.*, 2002; Diebolt and Andriantsitohaina, 2002; Rakici *et al.*, 2005 and Bulac and Demirel-Yilmaz, 2006). Moreover, the wine polyphenol resveratrol also affected hyperpolarizing-mediated K^+ channel located in smooth muscle cell (Novakovic *et al.*, 2006). For example, rutaecarpine regulated Ca^{2+} channel in endothelial cells and vascular smooth muscle cells in an opposing manner in achieving vasorelaxation (Wang *et al.*, 1999). Resveratrol decreased the Ca^{2+} sensitivity of vascular smooth muscle cells but enhanced the effect of an increase of $[Ca^{2+}]_i$ in endothelium cells. Consequently, there was an increase of NO synthesis in endothelium cells, leading to vasorelaxation (Bulac and Demirel-Yilmaz, 2006). In this study, GSH and other thiol-containing compounds appeared to elicit these vascular effects like those natural antioxidant agents. The vascular effects of GSH may lie in its sulfhydryl group and antioxidant property which affected directly on vascular smooth muscle through the reduction of disulfide bonds of receptor, channel or enzymes, and altered their functions on vascular cell (Cheung and

Schulz, 1997). These interactions could lead to membrane alteration, resulting in interfering Ca^{2+} mobilization and activation of hyperpolarizing signal on Ca^{2+} channels and K^+ channels in vascular smooth muscle cells respectively. NAC was a good example for its reducing properties to directly modify several proteins and the redox-dependent intracellular signaling mechanisms in vascular smooth muscle cells (Zafarullah *et al.*, 2003; Hashimoto *et al.*, 2001).

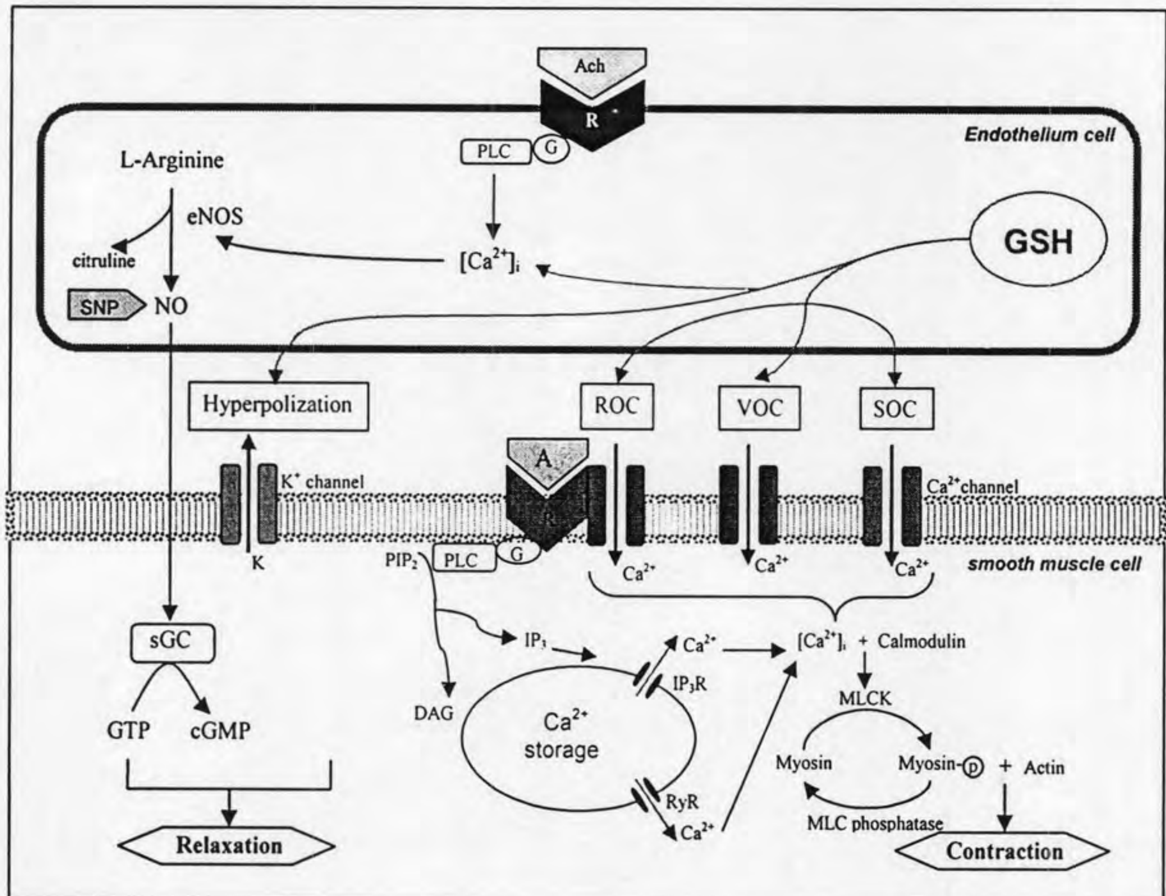

Fig. 33 The propose mechanisms for GSH induced vasorelaxation. GSH acts on both endothelial cell and VSMC directly, but lesser extent in the latter. In endothelium, GSH increase intracellular Ca^{2+} by promoting Ca^{2+} influx, leading to increased NO production and then increased cGMP production on vascular smooth muscle cells. In vascular smooth muscle cells, GSH reduces Ca^{2+} influx in nonspecific pathway (ROC, VOC and SOC) and activates the hyperpolarization on K^+ channel.

Conclusion

In summary, the present study demonstrated that the mechanisms of GSH-induced endothelium-dependent relaxation and endothelium-independent relaxation. As shown in Fig. 33, the major mechanism of GSH may involve an increase of intracellular Ca^{2+} by increased Ca^{2+} influx from extracellular Ca^{2+} to enhance eNOS activity in the endothelium, resulting in an increase of NO production. Subsequently, NO diffused to vascular smooth muscle to activate guanylate cyclase and increase cGMP production, leading to vasorelaxation. In addition, GSH has direct vasorelaxation effects on vascular smooth muscle cells which are independent of endothelium. GSH may exert its actions through activating hyperpolarizing signaling pathway and interfering PE-mediated Ca^{2+} release from sarcoplasmic reticulum of the vascular smooth muscle cell. Moreover, GSH could affect Ca^{2+}channel: ROC, VOC and SOC, in a decrease of Ca^{2+} influx. The intrinsic activity of GSH on vascular tone may be attributed to its sulhydryl group. It was possible that GSH caused plasma membrane alteration, resulting in activation of hyperpolarizing signal and interfering Ca^{2+} mobilization by modulation of K^+ channel and Ca^{2+} channel on vascular smooth muscle, respectively.