Original Article

In vitro modulating effects of glutathione on vascular tension and involvement of extracellular calcium

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ABSTRACT: This study investigated the involvement of endothelium and extracellular calcium on the vasorelaxant activity of glutathione (GSH) using *in vitro* model of isolated rat aorta. The aortic tensions upon treatment with GSH in the presence and absence of endothelium were compared in various conditions. In phenylephrine-precontracted aortic rings, GSH (2-8 mM) significantly induced vasorelaxation in concentration-dependent manner. The influence of endothelium was demonstrated in determining the responses of aortic muscle toward GSH treatment. GSH (up to 5 mM) caused a higher loss of vascular tensions in the endothelium-intact aortic rings than those in the endothelium-denude preparations. The vasorelaxant effect of GSH in endothelium-intact rings was inhibited by glibenclamide (3 μM), methylene blue (10 μM) and *N*-nitro-L-arginine methyl ester (10 μM), indicating the involvement of membrane K^+ channels and NO-cGMP pathway. In the endothelium-denude preparations, only glibenclamide inhibited the modulating effect of GSH on aortic tension. Furthermore, the endothelium-dependent vasorelaxation of GSH was abolished in Ca^{2+}-free medium containing EGTA, but not in the medium containing BAPTA-AM (10 μM). Taken together, our findings suggested that vasorelaxant activity of GSH depended on influx of extracellular Ca^{2+} to activate NO production in endothelium cells. In addition, other possible mechanisms included its hyperpolarizing actions in vascular muscle cells.

Keywords: GSH, vasorelaxation, extracellular calcium, isolated rat aorta

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1. Introduction

Glutathione (GSH), a known endogenous sulfhydryl redox agent, has been demonstrated its roles for protecting endothelium functions and regulating vascular tone (*1*,*2*). Several studies have linked the vascular protective effects of GSH to its antioxidative activities, resulting in an increase of nitric oxide (NO) bioavailability and vasodilatation (*3-5*). It was demonstrated that the vasorelaxing activity of aortic preparations isolated from spontaneous hypertensive rats (SHR) improved in the GSH treatment group, possibly via endothelium-dependent mechanisms (*6*). Moreover, *in vivo* depletion of GSH enhanced contraction and attenuated endothelium-dependent relaxation (*7*,*8*). Beyond the antioxidative mechanisms, an alteration of redox status of endothelium or smooth muscle membrane might directly affect the vascular tension through several mechanisms including oxidative modification of receptors and ion channels. In particular, it was demonstrated that GSH could relax tension of isolated guinea pig tracheas due to activation of potassium (K^+) channels, not to the NO-mediated mechanism (*9*). Furthermore, the vasorelaxant effects of other sulfhydryl reducing agents such as *N*-acetylcysteine (NAC) were linked to the activities of membrane K^+ channels (*10*).

The change in intracellular Ca^{2+} concentration is very crucial for the regulation of vascular tension. Rising of intracellular Ca^{2+} in endothelium triggers several mechanisms of vasorelaxation including synthesis and release of NO as well as K^+ channel-activating endothelium-derived hyperpolarizing factors (*11-13*). Generally, the sources of Ca^{2+} could be from internal stores as well as from extracellular Ca^{2+} pool. To this end, we investigated the vasorelaxing action of extracellular GSH and its involvement with Ca^{2+} handling in the blood vessels. In addition, we further elucidated the influence of endothelium and mechanisms of GSH-induced vasorelaxation in endothelium-intact and endothelium-denude blood vessels, using the functional analysis model of isolated rat thoracic aorta.

2. Materials and Methods

2.1. *Chemicals*

Glutathione (GSH) and other principal compounds such as phenylephrine (PE), acetylcholine (Ach), glibenclamide, methylene blue, sodium nitroprusside (SNP), *N*-nitro-L-arginine methyl ester (L-NAME), bis(o-aminophenoxy) ethane-*N*,*N*,*N*',*N*'-tetraacetic acid (BAPTA-AM), ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*'-tetraacetic acid (EGTA) were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were reagent grade or the highest-grade commercial available.

2.2. *Preparations of isolated aorta*

The experiments were approved by the Institutional Animal Ethic Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. The thoracic aorta were excised from male Wistar rats (250-300 g), and cut into segments of approximately 0.3 cm long. The aortic rings were mounted with an isotonic force transducer (Model MLT 050/A, AD Instruments, Australia) under a resting tension of 1.0 g in Krebs-Henseleit solution (KHS) (content in mM; NaCl 118, KCl 4.7, $CaCl_2$ 2.52, $MgSO_4$ 1.64, KH_2PO4 1.18, $NaHCO_3$ 7, and glucose 5.5) at 37°C and bubbled with 95% O_2/5% CO_2. The tension was recorded through the computerized system equipped with software Chart 5.0 of Powerlab 4/SP data acquisition (AD Instruments, Australia).

The presence of functional endothelium were tested by priming the aortic rings with PE (10 μM), followed by addition of Ach (10 μM) when the contraction reached the plateau state. The relaxation response of at least 60% was considered endothelium-intact rings for further experiments. In certain experiments, endothelium was removed by gentle rubbing the lumen with cotton swab. The absence of the endothelium was suggested by less than 10% relaxation response to Ach.

2.3. *Vasorelaxant effects of glutathione*

The contraction of aortic rings was provoked by PE (1 μM). When the contraction reached plateau state, GSH was added cumulatively to produce relaxation. The tension was recorded and expressed as the percentage of the maximum contraction induced by PE. In our studies, L-valine (at the equal concentrations to those of GSH) was applied as the control group.

In addition, the vasorelaxant property of GSH was further investigated with the uses of known vasorelaxant inhibitors including ibuprofen (10 μM), propranolol (10 μM), atropine (10 μM), L-NAME (10 μM), methylene blue (10 μM), and glibenclamide (10 μM). These inhibitors were incubated with the rings for 30 min prior to the relaxation experiment as described above.

2.4. *Potentiation effects of GSH on the Ach- or SNP-induced vasorelaxation*

After the resting tension of the endothelium-intact rings was stable, the contraction was provoked by addition of KCl (60 mM). When the contraction reached a plateau state, Ach (0.01-100 μM) or SNP (0.001-10 μM) were added cumulatively to induce relaxation. The potentiation effects of GSH were tested by pre-incubating the rings with GSH for 5 min prior to addition of KCl. The relaxation responses were calculated as a percentage in relative to the contraction provoked by KCl (60 mM) in the absence of GSH.

2.5. *Involvement of calcium in GSH-induced vasorelaxation*

The involvement of extracellular Ca^{2+} in GSH-induced vasorelaxation was functional determined in the endothelium-intact aortic rings, using the method previously described by Yang *et al*. (*14*). In these studies, either BAPTA-AM (10 μM) or EGTA (0.2 M) was employed to chelate Ca^{2+} during the relaxation process. In brief, when the PE-induced contraction reached the plateau state, BAPTA-AM (10 μM), a known permeable Ca^{2+} chelating agent, was added and incubated with the tissue for 15 min prior to cumulative addition of GSH. In another experiment, the aortic rings were equilibrated in Ca^{2+}-free KHS containing EGTA (0.2 M) for 90 min before addition of PE to start the experiment as described above.

2.6. *Statistical analysis*

Results were presented as the mean ± S.E.M., obtained from 6 separated experiments (n = 6). Statistic comparisons were performed either by Student's *t*-test or one-way ANOVA followed by a post-hoc Dunnett *t*-test where appropriate. Significances were considered at $p < 0.05$.

3. Results

3.1. *Vasorelaxant effects of GSH*

As shown in Figure 1, GSH caused vasorelaxation in concentration-dependent manner on both endothelium-intact and -denude rat aortic rings, but with different degree of relaxation. GSH at the low concentrations of 2 and 4 mM induced relaxation of the intact preparations at higher degree than those of the denude preparations (Figure 2). Interestingly, it appeared that

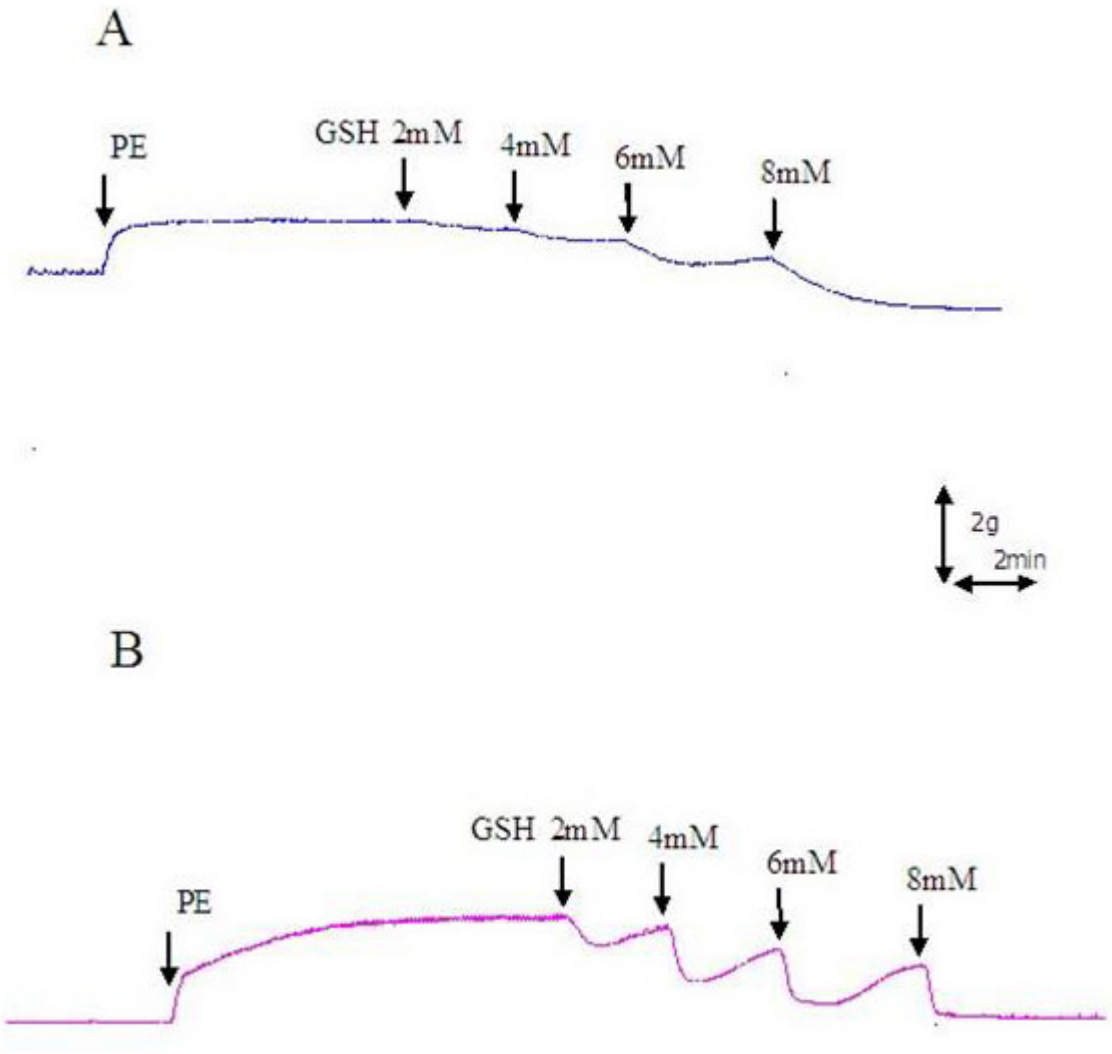

Figure 1. The representative tracing of GSH-induced relaxation in endothelium-denude (A) and endothelium-intact (B) rat aortic rings. The aortic rings were precontracted with PE (1 μM), followed by addition of GSH cumulatively.

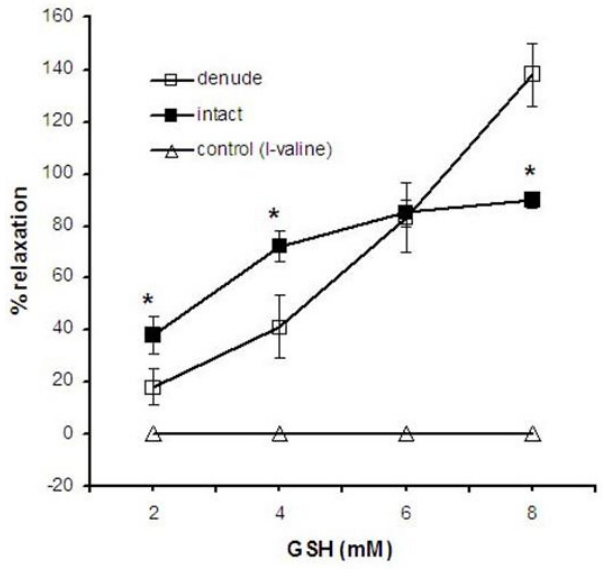

Figure 2. The vasorelaxant effects of GSH in endothelium-intact and endothelium-denude aortic rings. Data are mean ± S.E.M. (n = 6), expressed as the percentage of the vascular tension induced by PE 1 μM. * $p < 0.05$ *vs.* the endothelium-denude group.

the modulating effect of GSH on vascular tension could be saturated in the presence of endothelium. Removal of endothelium allowed the vascular smooth muscle to further relax beyond the initial resting tension in response to the high concentration of GSH (8 mM).

The single treatment of GSH (5 mM) was able to suppress PE-induced contraction by 20% and 80% in endothelium-denude and -intact preparations, respectively (Figure 3). Pretreatment with either L-NAME, methylene blue or glibenclamide, but not ibuprofen, atropine, and propranolol, significantly inhibited the tension modulating effects of GSH in endothelium-intact rings. On the other hand, only glibenclamide elicited its inhibitory effect on GSH-mediated relaxation in the endothelium-denude rings. These findings suggested that the relaxation effects of GSH involved the NO-cGMP and hyperpolarizing signaling pathways in endothelium-dependent mechanisms. In addition, the activation of K^+ channel-mediated hyperpolarizing effect was linked to endothelium-independent mechanism.

3.2. *Potentiation effects of GSH on the Ach- or SNP-induced relaxation*

As known, Ach and SNP induced vasorelaxation *via* an increase of cGMP in the NO-cGMP pathway. Ach increased production of NO in endothelium cells whereas SNP directly activated guanylate cyclase in smooth muscle cells. Our result demonstrated that the presence of GSH increased the sensitivity of aortic preparations toward Ach treatment. As seen in Figure 4A, the concentration-response curve of Ach shifted leftward in the presence of GSH. The calculated concentration of Ach that caused 50% relaxation (IC_{50}) decreased approximately 10 folds from 77 μM to 7.3 μM in the presence of GSH. On the contrary, GSH had no effects on SNP-mediated vasorelaxation in this study (Figure 4B). These findings suggested that GSH could enhance the function of endothelium in production of NO. The lack of potentiating effect in SNP-induced vasorelaxation might reflect that GSH had no influence on guanylate cyclase activity or contractile elements.

It was noteworthy to mention that the effects of GSH on Ach-induced relaxation depended on the amount of intact endothelium cells. The more numbers of endothelium intacted, the less potentiative effect of GSH on Ach-induced relaxation was observed. In the preparations with 80-90% of intact endothelium, GSH had no significant potentiative effect of Ach-induced relaxation. However, in those with 60-70% of intact endothelium, GSH elicited its potentiative relaxation significantly.

3.3. *Involvement of calcium in GSH-induced endothelium-dependent vasorelaxation*

An increase of intracellular Ca^{2+} in the endothelium could activate the NO synthase activity and increased production of NO, resulting in vasorelaxation (*11,12*). Hence, this study was to examine the influence of intracellular Ca^{2+} on the GSH-induced relaxation by applying Ca^{2+}-free environment to the experiment condition. Upon changing medium from Ca^{2+}-containing solution to Ca^{2+}-free KHS containing EGTA, the contractile response to PE decreased by 80%. However, the PE-induced contraction was still sustainable. Under this condition, the vasorelaxation effects of GSH were compromised significantly (Figure

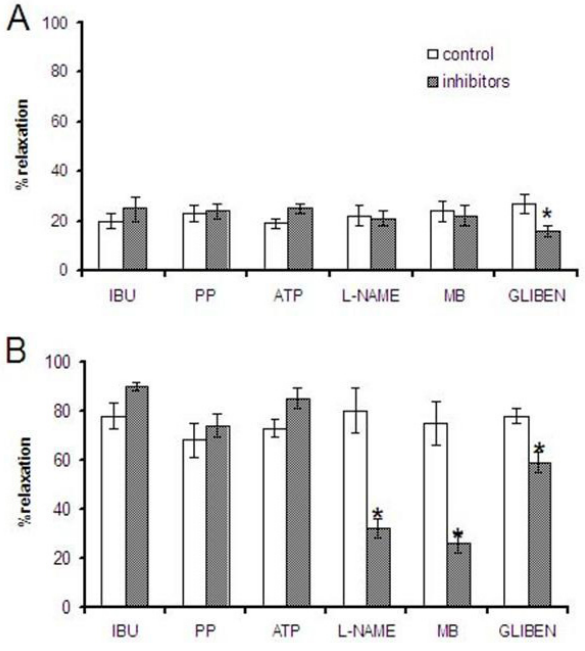

Figure 3. The effects of various vasorelaxant inhibitors on the GSH-induced relaxation in the endothelium-denude (A) and endothelium-intact (B) preparations. IBU, ibuprofen; PP, propranolol; ATP, atropine; L-NAME, *N*-nitro-L-arginine methyl ester; MB, methylene blue; GLIBEN, glibenclamide. Data are mean ± S.E.M. (n = 6), expressed as the percentage of the vascular tension induced by PE 1 μM. * $p < 0.05$ *vs.* control group.

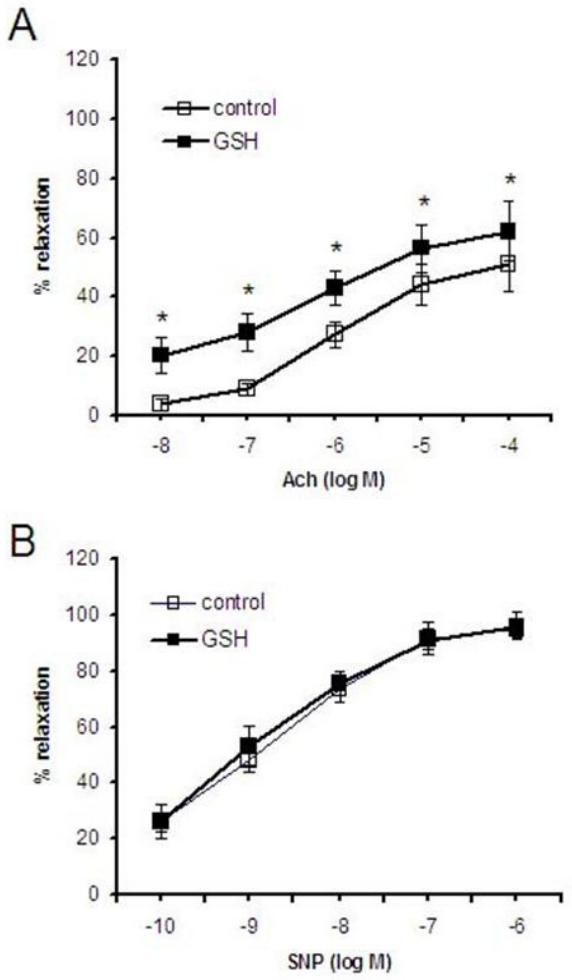

Figure 4. Concentration-response curves for the vasorelaxing action of acetylcholine (Ach) (A) and sodium nitroprusside (SNP) (B) in the presence of GSH (5 mM). Data are mean ± S.E.M. (n = 6), expressed as the percentage of the vascular tension induced by KCl 60 mM. * $p < 0.05$ *vs.* control group.

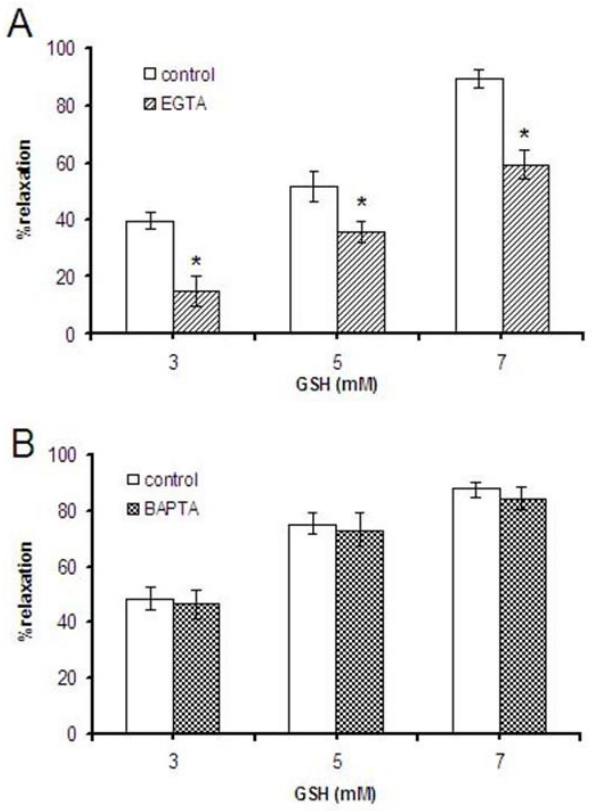

Figure 5. Influence of extracellular Ca^{2+} on the vasorelaxant effects of GSH. The GSH-induced relaxation was determined in the Ca^{2+}-free medium containing 0.2 mM EGTA (**A**) or in the medium containing 10 μM BAPTA-AM (**B**). Data are mean ± S.E.M. (n = 6), expressed as the percentage of the vascular tension induced by PE 1 μM. * $p <$ 0.05 *vs*. control group.

5A). On the contrary, BAPTA-AM (10 μM) had no markedly influence on either PE-induced contraction or the degree of GSH-induced relaxation (Figure 5B). These finding suggested that extracellular Ca^{2+} was more critical than intracellular Ca^{2+} in GSH-induced vasorelaxation in the endothelium intact preparation. GSH might affect processes of Ca^{2+} influx, without any interference on activation of Ca^{2+} release from the cellular storage.

4. Discussion and Conclusion

In this study, we were able to demonstrate the vasomotion effects of extracellular GSH in the model of isolated rat thoracic aorta. As known, endothelium protected cells from chemical insults and also control vascular tension. In addition, endothelium may buffer a swift change of vascular tone by balancing between secretion of vasoconstrictors and vasodilators (*15*). In this study, endothelium certainly contributed its influence in determining the responses of aortic muscle toward GSH treatment. Our results suggested that GSH exerted its vasorelaxant activity through both endothelium-dependent and -independent mechanisms. Removal of endothelium from the aortic preparations caused the tissue less sensitive to GSH-induced vasorelaxation. However, the removal of endothelium also caused a loss of protective barrier and regulatory control of muscle tension (*16,17*). Consequently, because of the loss of endothelium buffering system, GSH at high concentration (8 mM) effectively decreased vascular tension of the PE-precontracted aortic smooth muscle beyond the maximum developed tension (> 100%).

The presence of L-NAME and methylene blue, which were inhibitors of nitric oxide synthase (NOS) and guanylate cyclase, respectively, could attenuate GSH-mediated endothelium-dependent vasorelaxation. In agreement with other reports, extracellular GSH exerted its relaxant effects on endothelium cells mainly through activation of the NO-cGMP pathway (*2,18,19*). In addition, 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, propranolol and ibuprofen had no influence on GSH-induced relaxation. It has been established that an opening of K^+ channels in smooth muscle cells results in membrane hyperpolarization, leading to close of Ca^{2+} channels, and vasodilatation (*20,21*). Moreover, the inhibitory effect of glibenclamide against GSH-induced vasorelaxation was also observed in endothelium-denude aortic preparations. Taken together, in addition to the NO-cGMP pathway, we suggested that GSH exerted its vasorelaxant activity *via* membrane hyperpolarization as a 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 (muscarinic receptor).

GSH could potentiate the aortic relaxation induced by acetycholine (Ach) but not those induced by sodium nitropusside (SNP). This observation was in agreement with other reports that GSH had no influence on endothelium-independent relaxation induced by NO donors (*6,7*). Hence, vasorelaxant effects of GSH were unlikely to involve with endothelium-independent activation of guanylate cyclase and cGMP availability. Moreover, methylene blue could not inhibit the GSH-induced relaxation of the endothelium-denude rat aortic rings.

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+} (*22,23*). In this study, the relationship between the source of Ca^{2+} and the

mechanism of GSH-induced relaxation in endothelium cells were determined. We were able to show 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 were clearly extracellular Ca^{2+}-dependent.

The sites of extracellular 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 (*24*,*25*). Hence, GSH might affect specific target proteins on plasma membrane which were consequently connected to the process in NOS activation. We were quite certain that GSH had no effect on an increase of intracellular Ca^{2+} through activation of muscarinic receptor because treatment of atropine could not abolish GSH-induced vasorelaxation. It was likely that GSH initiated Ca^{2+} influx from extracellular source to activate NO production in endothelial cells. In addition, a rising of intracellular Ca^{2+} in the endothelium cells could trigger the release of endothelium-derived hyperpolarizing factors, leading to relaxation of vascular smooth muscle (*13*,*26*).

In conclusion, the mechanisms of GSH-induced relaxation might involve with the NO-cGMP pathway through an increase in Ca^{2+} influx and NO production in endothelial cells, but not the cGMP production in vascular smooth muscle cell. In addition, other possible mechanisms included the activation of membrane K^+ channels.

Acknowledgements

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