ผลของไดเมทิลซัลฟอกไซด์ต่อการทำงานของหลอดเลือดแดงใหญ่ที่แยกจากหนูขาว

นางสาว จิตติมา ศรีสมบูรณ์

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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EFFECTS OF DIMETHYL SULFOXIDE ON THE FUNCTIONALITY OF ISOLATED RAT AORTA

Miss Jitima Srisomboon

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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จิตติมา ศรีสมบูรณ์: ผลของไดเมทิลซัลฟอกไซด์ต่อการทำงานของหลอดเลือดแดงใหญ่ที่แยกจากหนูขาว. (EFFECTS OF DIMETHYL SULFOXIDE ON THE FUNCTIONALITY OF ISOLATED RAT AORTA) อ. ที่ปรึกษา: ผศ.ดร.สุรีย์ เจียรณ์มงคล, อ. ที่ปรึกษาร่วม: รศ.ดร.ประสาน ธรรมอุปกรณ์, 71 หน้า. ISBN 974-17-3889-7.

ไดเมทิลซัลฟอกไซด์ (DMSO) เป็นตัวทำละลายที่นิยมใช้สำหรับยาที่ไม่ละลายน้ำและมีการใช้กันอย่างกว้างขวางในงานวิจัยและการแพทย์ ในการศึกษานี้ศึกษาผลของ DMSO ต่อการทำงานของกล้ามเนื้อเรียบของหลอดเลือดแดงที่แยกจากหนูขาวในสภาวะที่มีและไม่มี endothelium หลอดเลือดแดงใหญ่ที่ใช้ได้มาจากหนูขาวพันธุ์ Wistar เพศผู้ น้ำหนักประมาณ 250-300 กรัม ทำการวัดผลการหดตัวของกล้ามเนื้อหลอดเลือดแบบ isometric ผลศึกษาพบว่า DMSO ที่ความเข้มข้นสูง (5 และ 10.0% v/v) ทำให้เกิดการหดตัวชั่วขณะในช่วงแรกตามด้วยการคลายตัวของหลอดเลือดในขณะพัก DMSO มีผลซับซ้อนต่อการหดตัวของหลอดเลือดแดงใหญ่ที่ถูกกระตุ้นด้วยตัวกระตุ้นต่างๆ DMSO ที่ความเข้มข้นต่ำ (ระหว่าง 0.017-2.0% v/v) ไม่มีผลต่อการหดตัวของหลอดเลือดที่ถูกกระตุ้นด้วย noradrenaline, KCl, TEA และ caffeine ในภาวะปกติ ภายใต้สภาวะเดียวกัน DMSO ที่ความเข้มข้นสูง (5 และ 10.0% v/v) ให้ผลในทางตรงข้าม อย่างไรก็ตาม ผลจากการยับยั้งดังกล่าวสามารถผันกลับได้ภายหลังจากการล้าง DMSO ออกแล้ว ผลของ DMSO ไม่ขึ้นกับ endothelium ที่บุหลอดเลือดอยู่ เช่นเดียวกับการหดตัวที่ถูกกระตุ้นด้วยตัวกระตุ้นต่างๆ DMSO ที่ความเข้มข้นสูงเท่านั้นที่มีผลต่อการหดตัวที่ถูกกระตุ้นด้วย $CaCl_2$ แบบสะสมขนาด นอกจากนั้น DMSO ที่ความเข้มข้นต่ำ (0.03 และ 0.07% v/v) ยับยั้งการเกิดการหดตัวในขณะพัก (IRT) แต่ไม่มีผลต่อการหดตัวที่เกิดขึ้นตามหลังซึ่งอยู่ในสภาวะที่ปราศจากแคลเซียม (NA2) ดังนั้น DMSO ไม่ได้ออกฤทธิ์รบกวนกลไกการเติมแคลเซียมเข้าสู่แหล่งเก็บภายในเซลล์ที่ไวต่อการกระตุ้นด้วย noradrenaline จากการศึกษานี้จึงสรุปได้ว่า DMSO ที่ความเข้มข้นต่ำไม่มีผลต่อการหดตัวที่ถูกกระตุ้นด้วยตัวกระตุ้นต่างๆ ขณะที่ DMSO ที่ความเข้มข้นสูงมีผลยับยั้งการหดตัวภายใต้สภาวะดังกล่าว นอกจากนั้น DMSO ยังทำให้เกิดการคลายตัวของหลอดเลือด

สถาบันวิทยบริการ
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ภาควิชา	เภสัชวิทยา	ลายมือชื่อนิสิต.....
สาขาวิชา	เภสัชวิทยา	ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา	2546	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEY WORD: DIMETHYL SULFOXIDE/ RAT AORTA/ CONTRACTION/ RELAXATION/ ENDOTHELIUM/ CALCIUM ENTRY/ INCREASE IN THE RESTING TONE

JITIMA SRISOMBOON: EFFECTS OF DIMETHYL SULFOXIDE ON THE FUNCTIONALITY OF ISOLATED RAT AORTA. THESIS ADVISOR: ASST. PROF. SUREE JIANMONGKOL, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. PRASAN DHUMMA-UPAKORN, Ph.D., 71 pp. ISBN 974-17-3889-7.

Dimethyl sulfoxide (DMSO) is the solvent commonly used to dissolve hydrophobic drugs and widely used in research and medicine. In this study, the effects of DMSO on the functionality of vascular smooth muscle of isolated rat aorta in the presence and absence of endothelium were investigated. Thoracic aortic strips were isolated from male Wistar rats (250-300 g) and the contractile responses were measured isometrically, using force-displacement transducers. Our results showed that DMSO at high concentration (5% and 10% v/v) induced an initial transient contraction, followed by relaxation of the basal tone of aortic strip. DMSO had the complex effect on the aortic contraction evoked by various contractants. Low concentrations (0.017–2% v/v) of DMSO did not have the effect on the contraction induced by noradrenaline, KCl, TEA and caffeine in physiological solution. Under the same condition, high concentration of DMSO (5% and 10% v/v) produced the opposite effects. However, the suppression was reversible upon removal of DMSO. The effect of DMSO did not depend on the presence of endothelium. Similary to the contraction induced by various contractants, DMSO only at high concentration inhibited the contraction induced by cumulative $CaCl_2$ in Ca^{2+}-free depolarizing solution. Furthermore, DMSO at low concentration (0.03% and 0.07% v/v) inhibited the increase in the resting tone (IRT), but did not affect the subsequent contraction in Ca^{2+}-free medium (NA2). Hence, DMSO did not affect the refilling of the intracellular Ca^{2+} stores-sensitive to noradrenaline. In conclusion, low concentration of DMSO did not have the inhibitory effect on the contraction induced by various contractants, whereas DMSO at high concentrations inhibited the contraction under the same condition. In addition, DMSO had the vasorelaxation effect on the vascular tone.

Department	Pharmacology	Student's signature……...
Field of study	Pharmacology	Advisor's signature.....
Academic year	2003	Co-advisor's signature.....

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LIST OF ABBREVIATIONS

DMSO	dimethyl sulfoxide
Ca^{2+}	calcium ion
$[Ca^{2+}]_I$	intracellular Ca^{2+} concentration
$[Ca^{2+}]_c$	cytosolic Ca^{2+} concentration
AC	adenylate cyclase
GTP	guanosine 5'-triphosphate
PLC	phospholipase C
IP_3	inositol 1,4,5-trisphosphate
$Ins(1,4,5)P_3R$	inositol 1,4,5-trisphosphate receptor
RyR	ryanodine receptor
DAG	diacylglycerol
ATP	adenosine 5'-triphosphate
cAMP	cyclic adenosine 3',5'-monophosphate
cGMP	cyclic guanosine 3',5'-monophosphate
PKA	cAMP-dependent protein kinase
PKG	cGMP-dependent protein kinase
EDRF	endothelium-derived relaxing factor
EDCF	endothelium-derived constricting factor
NO	nitric oxide
SR	sarcoplasmic reticulum
MLC	myosin light chain
MLCK	myosin light chain kinase
IRT	increase in the resting tone
NA	noradrenaline
TEA	tetraethylammonium
K^+	potassium ion
KCl	potassium chloride
ACh	acetylcholine
M	molar
mM	millimolar
µM	micromolar

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CHAPTER I

INTRODUCTION

Dimethyl sulfoxide (DMSO) is the solvent commonly used to dissolve hydrophobic drugs. It is a dipolar compound widely used in research, industry and medicine. It is an effective cryoprotectant (Arnaud, 2000; Byrd, 2002; Gao, *et al.*, 1999), and also a potent scavenger of free radicals (Jacob, 2002). DMSO has a wide range of actions, for instance, membrane penetration, membrane transport, vasodilatation, and muscle relaxation. Recent voltage-clamp analysis of DMSO (0.1-10% v/v) action on membrane currents in guinea-pig papillary muscles suggested that superfusion with DMSO solution causes negative inotropy in normal and Ca^{2+}-overloaded stimulated muscles, relaxes contractures in quiescent muscles exposed to Na^{+}-free solution, and inhibits contractures induced by caffeine 10 mM (Ogura, *et al.*, 1996). In addition, the study in porcine pulmonary and coronary arteries showed that DMSO (1.2% v/v) induced the relaxation during U46619 (thromboxane mimetic)-induced contraction (Lawrence, *et al.*, 1998). Furthermore, previous studies in our laboratory founded that DMSO at the concentration of 0.03% v/v significantly inhibited the spontaneous contraction of aortic strip when exposure to Ca^{2+}-containing solution after the strip was completely depleted the intracellular Ca^{2+}. The same concentration (0.03% v/v) of DMSO had no observable effect on noradrenaline-induced contraction (Puechprom, 2002). It is possible that DMSO directly affects vascular reactivity. Hence, this research project aimed to study the effects of DMSO on the functionality of vascular smooth muscle of isolated rat aorta in the presence and absence of endothelium.

Hypothesis

Dimethyl sulfoxide has direct effect on the functionality of smooth muscle of isolated rat aorta by disrupting the calcium mobilization into smooth muscle cell.

Objectives

1. To investigate the effect of DMSO on the contraction of smooth muscle of isolated aortic strip in the presence of various contractants including noradrenaline (α-adrenoceptor activation), potassium chloride (membrane depolarization), tetraethylammonium (K^+ channel blocker), and caffeine (release of Ca^{2+} from sarcoplasmic reticulum).

2. To investigate the effect of DMSO on the entry of Ca^{2+} from the extracellular to intracellular pool upon depolarization stimulated by $CaCl_2$

3. To investigate the effect of DMSO on the increase in the resting tone of isolated aortic strip after noradrenaline-induced depletion of intracellular Ca^{2+}.

4. To investigate the effect of DMSO on the relaxation of smooth muscle of isolated aortic strip in the presence or absence of endothelium.

Significances

1. This study will provide new information on the effects of DMSO, the solvent commonly used to dissolve pharmacological agents on the study of the functions of smooth muscle.

2. The information will be useful for using DMSO as solvent in the pharmacological study of the smooth muscle.

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CHAPTER II

LITERATURE REVIEWS

The study about dimethyl sulfoxide

Dimethyl sulfoxide (DMSO) has the formula as $(CH_3)_2SO$, and the structure of DMSO is shown in figure 1.

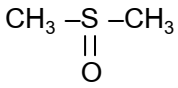

Figure 1 The structure of DMSO (Atofina Chemicals,Inc.,2000)

DMSO is colorless liquid, miscible with water and has mild garlic odor (National Academy of Sciences, 1995). DMSO has U.S. Food and Drug Administration (FDA) approval only for use as a preservative of organs for transplantation and bladder irrigation in interstitial cystitis, a bladder disease (Muir, 1996). It has been used as cryoprotectant to preserve cells so that the functions of the preserved cells still remain like the fresh cells. This technique is used to preserve human embryos in reproductive technology laboratories. Embryos can be successfully frozen at the pronuclear, multicellular (four to eight blastomers), and blastocyst stages. This is due to the cryoprotectant can limit intracellular ice formation when using a slow cooling method to freeze the cells (Byrd, 2002). In addition, DMSO has also used as cryoprotective agent in human platelets banking (Gao, *et al.*, 1999), preservation of arteries or veins for surgery (Arnaud, 2000). Furthermore, DMSO is a purified preparation used in interstitial cystitis that has the symptoms of the bladder such as urinary frequency, urgency and pain. In adults, 50 mL of a 50% solution is instilled into the bladder by using a catheter or syringe and left there for about 15 minutes. Then, the solution is expelled by urinating. The treatment is repeated every two weeks until relief is obtained. However, DMSO may cause side effects such as nasal congestion, shortness of breath or troubled breathing, skin rash, hives, itching, swelling of face, breath or skin odor (garlic-like odor), and discomfort when put into bladder (U.S. National Library of Medicine, 1994).

It has been documented in laboratory studies that DMSO has a wide range of primary pharmacological actions such as membrane penetration, nerve blockade (analgesia), diuresis, vasodilation, muscle relaxation, enhancement of cell differentiation and function, radio-protective and cryoprotective actions (Jacob and Herschler, 1985).

Various effects of DMSO

DMSO readily crosses most tissue membranes of lower animals and man. The study about distribution of 35s-labeled DMSO to the rats showed that DMSO was distributed in all organ studies within 2 hours. The highest value found in spleen and decrease in the following soft tissues respectively; stomach, lung, vitreous humor, thymus, brain, kidney, sclera, colon, heart, skeletal muscle, skin, liver, aorta, adrenal, lens of eye, and cartilage (Denko, *et al.*, 1967).

Other studies about the effect of DMSO as nerve blockade reported that immersion of the sciatic nerve in 6% DMSO decreases the conduction velocity by 40%. This effect is totally reversed by washing the nerve in a buffer for 1 hour (Sams, 1967). In addition, the study about peripheral small fiber after-discharge in the cat revealed that concentration of 5-10% DMSO eliminated the activity of C fibers with 1 minute and the activity returned after the DMSO was washed away (Shealy, 1966).

Furthermore, it has been studied the diuretic effect of DMSO administered topically to rats five times daily in dosage of 0.5 ml of 90% DMSO. There was an increase in urine volume about 10-fold, and in sodium and potassium excretion (Formanek and Suckert, 1966).

DMSO was also studied its effect on the growth of bovine aortic smooth muscle and endothelial cells. It was found that this compound caused a dose-dependent inhibition of cell growth as determined by [3H]thymidine incorporation and by counting the number of cells time of exposure in culture. The IC50s of DMSO in growth inhibition were 1% for smooth muscle cells and 2.9% for endothelial cells. After a 4-day exposure, the growth inhibition of smooth muscle cells was completely reversible at DMSO 1%, partially reversible at DMSO 2 to 3% and completely irreversible at DMSO 4%. By comparison, inhibition of endothelial cell growth was completely reversible up to 4% of DMSO. It showed that smooth muscle cells were more susceptible than endothelial cells to the growth inhibitory effects of DMSO (Layman, 1987).

Another study was done to investigate the effect of the cryoprotectant for use in attempts to preserve tissue and organs by vitrification, using an immortal vascular

endothelial cell (ECV 304). The cells were exposed to four cryoprotectants: DMSO 45% w/w; 2,3 butanediol (BD) 32%; 1,2-propanediol (PD) 45%; and ethanediol (ED) 45% for 1,3, and 9 minutes at 22 and 2-4°C. The results showed that exposure to DMSO at 2-4°C for up to 9 minutes permitted the retention of significant cell function with the Cell Survival Index (CSI) of 37.0 $\pm$ 4.1% comparing with the CSI of 76.3 $\pm$ 7.0, 63.6 $\pm$ 7.1, and 33.2 $\pm$ 3.0 for BD, PD, ED, respectively. Furthermore, the permeability properties of the cells that exposed to DMSO and ED, even at 2-4°C, were severely damaging (Wusteman, *et al.*, 2002).

Effect of DMSO on vascular smooth muscle

It has been reported that the contractile response of isolated human coronary arteries preserved in DMSO and fetal calf serum (FCS) at –75°C for 7 to 10 days significantly decreased. In addition, the endothelium-dependent relaxation (EDR) in the cryostored segments produced by histamine, thrombin and substance P were also reduced as well as the endothelium-independent relaxation produced by isoproterenol (Ku, *et al.*, 1992).

Other Studies about the effect of DMSO on vascular smooth muscle revealed that DMSO produced concentration-dependent relaxation of rabbit isolated aortic strip. In the presence of DMSO, the action of norepinephrine, histamine, carbachol and potassium were significantly antagonized. The control ED50 values were 5.36 ± 0.50 x 10^{-8} M, 3.38 ± 0.74 x 10^{-6} M, 1.40 ± 0.82 x 10^{-6} M and 2.43 ± 0.19 x 10^{-2} M, respectively. In the presence of DMSO the ED50 values were 2.23 ± 0.23 x 10^{-6} M, 1.80 ± 0.40 x 10^{-5} M, 1.00 ± 0.38 x 10^{-4} M and 4.21 ± 0.07 x 10^{-2} M, respectively. The maximum response of the tissue to the various vasoactive agents was significantly depressed in the presence of DMSO (Jackson, *et al.*, 1979).

The study of the effect of DMSO, ethanol and methanol in porcine pulmonary and coronary arteries showed that these compounds induced the relaxation during U46619 (thromboxane mimetic)-induced contraction. In the pulmonary arteries the relaxation in response to ethanol was found to be endothelium-dependent whereas the responses to DMSO and methanol were unaffected by removal of the endothelium. In the coronary arteries the relaxation to all three solvents was independent of the presence of the endothelium (Lawrence, *et al.*, 1998).

Control of vascular smooth muscle tone

Contraction of smooth muscle is regulated by the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and the sensitivity to Ca^{2+} of the contractile elements (Karaki, *et al*., 1997).

The regulation of $[Ca^{2+}]_i$ in vascular smooth muscle depends on the entry and exit of Ca^{2+} across the plasma membrane, and on sequestration of Ca^{2+} within the cell. Calcium entry occurs partly through voltage-gated Ca^{2+} channels, which open when the cell is depolarized, and partly through receptor-operated channels. The voltage-operated Ca^{2+} channels open under influence of second messengers produced in response to receptor activation. Many vasoconstrictors (e.g. noradrenaline) cause both a depolarization by increasing the membrane permeability to cations such as Na^+ and Ca^{2+} and a further increase in Ca^{2+} uptake through distinct receptor-operated channels (Rang, *et al.*, 1999). Receptor activation can increase $[Ca^{2+}]_i$ by activating phospholipase C (PLC), which hydrolyzes phosphatidyl-inositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). IP_3 acts on receptors on the sarcoplasmic reticulum to release Ca^{2+} into cytoplasm. Diacylglycerol activates protein kinase C, an enzyme that phosphorylates several enzymes involved in contraction (Silverthorn, 1998)

Some vasoconstrictors induce contraction via other mechanisms such as membrane depolarization. High K^+ depolarizes the membrane, initiating conformational changes that open voltage-operated Ca^{2+} channels, which allow calcium to diffuse rapidly into the cell, resulting in an increase of intracellular Ca^{2+} (Karaki, *et al*., 1997; Katz, 1997). Tetraethylammonium (TEA), the blockade of K^+ channel, leads to membrane depolarization and vasoconstriction. Potassium channels are the dominant ion conductive pathways in vascular muscle cells. The electrochemical gradient for K^+ ions is such that opening of K^+ channels results in diffusion of K^+ ions out of the cell, membrane hyperpolarization, closure of voltage-operated Ca^{2+} channels, decrease intracellular Ca^{2+}, which leads to vasodilatation. Closure of K^+ channels has the opposite effect (Jackson, 2000). Furthermore, Caffeine induces a transient contraction which is attributable to the release of Ca^{2+} from internal stores (Karaki, *et al*. 1997).

When Ca^{2+} enters the smooth muscle cell through voltage-gated Ca^{2+} channels or is released from intracellular sites by IP_3, it combines to calmodulin. The Ca^{2+}-calmodulin complex then activates myosin light chain kinase (MLCK) (Westfall, *et al.*, 1998). Activated MLCK phosphorylates myosin light chains, using energy and P_i from ATP, and phosphorylated myosin interacts with actin to induce contraction. The

phosphorylation of myosin restores ATPase activity and allows crossbridge cycling and contraction (Silverthorn, 1998).

Relaxation of smooth muscle is usually initiated by a fall in $[Ca^{2+}]_i$ which leads to dephosphorylation of the myosin light chain via myosin phosphatase (Rang, *et al.*, 1999) or directly effect on the contractile machinery. Ca^{2+} is removed from the cytosol partially by a Ca^{2+}-Na^{+} antiport exchanger and partially by a Ca^{2+}-ATPase (Silverthorn, 1998). Calium efflux requires energy. The sodium-calcium exchanger uses the energy of the sodium gradient across the plasma membrane, whereas ATP-dependent calcium pumps utilize energy derived from the hydrolysis of ATP to move calcium out of the cell (Katz, 1997).

The sensitivity of the contractile apparatus to $[Ca^{2+}]_i$ is one factor that regulate contraction. Decreased activity of myosin phosphatase or increased activity of myosin light chain kinase results in Ca^{2+} sensitization, while increased activity of myosin phosphatase or decreased activity of myosin-light-chain kinase causes Ca^{2+} desensitization (Rang, *et al.*, 1999).

Calcium-channel antagonists block or limit the entry of Ca^{2+} through channels in membranes of vascular smooth muscles. These calcium-channel blockers thus limit the $[Ca^{2+}]_i$ available to interact with contractile proteins, then promote the relaxation of blood vessels.

Agents such as pinacidil cause vasodilation by opening the K^{+} channels (sensitive to intracellular ATP), causing hyperpolarization, and thus preventing voltage-gated Ca^{2+} channels from opening.

Increases in the concentration of cAMP are also associated with smooth muscle relaxation. When the concentration of cAMP is elevated, cAMP-dependent protein kinase (PKA) is activated. The relaxation may result from decrease $[Ca^{2+}]_i$ secondary to reduced influx of Ca^{2+}, enhanced Ca^{2+} uptake into the sarcoplasmic reticulum, or enhanced Ca^{2+} extrusion through the cell membrane. PKA may also phosphorylate and inhibit myosin light chain kinase, thus inhibiting contraction. β-adrenergic receptor agonists, such as isoproterenol cause vasodilation via the formation of cAMP. Stimulation of β-receptors activates adenylate cyclase, which catalyzes the generation of cAMP from ATP. Drugs that inhibit phosphodiesterases enzymes that metabolize cAMP and cGMP, promote smooth muscle relaxation by elevating concentrations of these second messengers.

In addition, increases in the intracellular concentration of cGMP are associated with vascular smooth muscle relaxation. cGMP activates cGMP-dependent protein kinase (PKG), then induce inhibition of phosphoinositol hydrolysis or stimulation of Ca^{2+} pumps, resulting in the extrusion or sequestration of Ca^{2+}. Alternatively, PKG may directly decrease the sensitivity of the contractile protein to Ca^{2+} (Westfall, *et al.*, 1998).

Furthermore, it is realized that vascular endothelium acts not only as a passive barrier between plasma and extracellular fluid, but also as a source of numerous potent chemical mediators. These actively control the contraction of the underlying smooth muscle (Rang, *et al.*, 1999). The endothelial cells release various vasoactive substances such as prostacyclin, and two other vasoactive substances that may function as regulatory molecules. These are called the endothelium-derived relaxing factor (EDRF) or nitric oxide (NO) and the endothelium-derived constricting factor (EDCF) or endothelin (Wingard, *et al.,* 1991). Many vasodilator substances such as acetylcholine, histamine, bradykinin, ATP, substance P act via endothelial NO production. NO that releases from endothelial cells will diffuse into the vascular smooth muscle cell, followed by increasing guanylate cyclase activity and elevating the cGMP concentration, then promotes relaxation (Westfall, *et al.*, 1998) (Figure 2).

The overall control of vascular smooth muscle is shown in figure 3.

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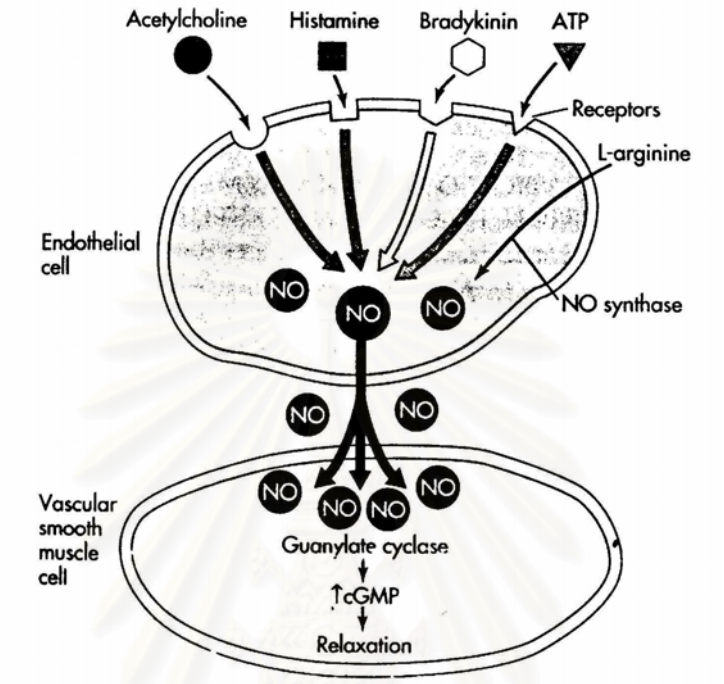

Figure 2 Endothelium-dependent relaxation produced by vasodilators.

(Westfall, *et. al.*, 1998)

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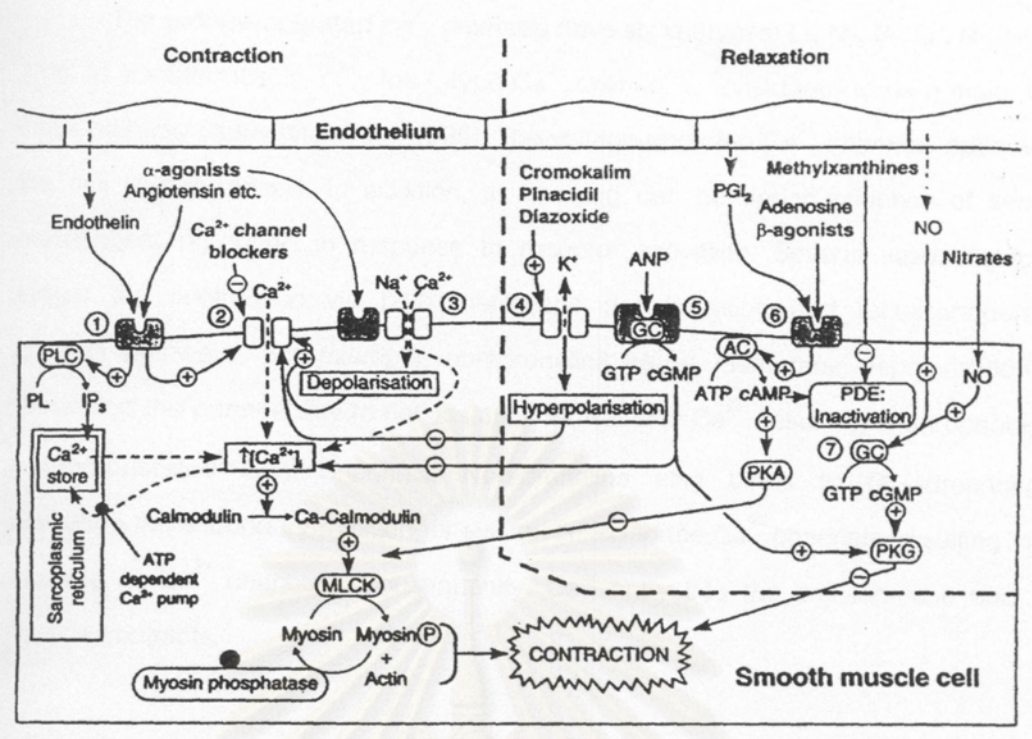

Figure 3 Control of vascular smooth muscle. (Rang, *et al.*, 1999)

Agents elicit contraction by increasing $[Ca^{2+}]_i$, or increasing the sensitivity of myofilaments to Ca^{2+}. $[Ca^{2+}]_i$ is increased by: (1) Receptors coupled to phospholipase C (PLC), which lead to IP_3 production and release of store Ca^{2+}. (2) Voltage-gated Ca^{2+} channels, which open in response to depolarization. (3) Receptor-operated channels, which allow Ca^{2+} entry and also cause depolarization. The other way to increase $[Ca^{2+}]_i$ is decreasing in myosin phosphatase activity, then causes contraction via Ca^{2+} sensitization. Agents that cause relaxation may effect by reducing $[Ca^{2+}]_i$, or directly on the contractile machinery: (4) K^+ channel (sensitive to intracellular ATP) openers such as diazoxide, cause hyperpolarization, and thus prevent opening of voltage-gated Ca^{2+} channels. (5) ANP occupied a receptor that is directly coupled to membrane-bound guanylate cyclase. (6) Receptors (e.g. for PGI_2, adenosine) coupled to adenylate cyclase, activation of which cause increased cAMP production. This acts via protein kinase A (PKA) and myosin light chain kinase (MLCK) to inhibit contraction. Inhibitors of phosphodiesterase (PDE) protect cAMP or cGMP from degradation. (7) Stimulation of soluble guanylate cyclase by NO increases cGMP formation.

(Enzymes: AC = adenylate cyclase; GC = guanylate cyclase; MLCK = myosin light chain kinase; PKA = cAMP-dependent protein kinase; PKG = cGMP-dependent protein kinase)

Vascular tone of the isolated rat aorta

In vitro model of isolated rat aorta, noradrenaline-induced contraction of smooth muscle has been characterized into two phases: the phasic and tonic phases (Noguera, D'Ocon, 1993). The initial phasic contraction results from intracellular Ca^{2+} release following an increase in the turnover of phosphatidylinositol and the production of inositol 1,4,5-trisphosphate (IP_3). The tonic contraction subsequently is maintained by an influx of extracellular Ca^{2+}. The activation of α_1-adrenoceptors by noradrenaline causes an accumulation of IP_3, which signaling the release of the Ca^{2+} from internal stores. The internal storage can be depleted by repetitive additions of noradrenaline to stimulate the contraction of aortic strip suspended in a Ca^{2+}-free medium (Graham, *et al*., 1996). These intracellular Ca^{2+} pools, when emptied, can be rapidly replenished from the extracellular space by incubation in Ca^{2+}-containing solution. The rapid refilling of the pools with extracellular Ca^{2+} occurs in the absence of the contractile agonist (Noguera, D'Ocon, 1993). This process manifests itself not only to refill the stores, but also to activate the contractile proteins. This process can be detected by the spontaneous contraction of aortic strip when changing the medium from Ca^{2+}-free solution to Ca^{2+}-containing solution and followed by monitoring the recovery of the aortic response to noradrenaline in Ca^{2+}-free medium (Noguera, *et al* ,1998).

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CHAPTER III

MATERIALS AND METHODS

Experimental animals

Male Wistar rats of body weight between 250-300 g were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. The animals were housed in animal care facility at the Faculty of Pharmaceutical Sciences, Chulalongkorn University under standard conditions and acclimatized for 1-2 weeks before the experimentation. This study was approved by Animal Ethic Committee on the use of laboratory animals in teaching and research.

Preparation of isolated rat aorta

1. Male Wistar rats (250-300 g) were sacrificed by cervical dislocation. The thoracic aorta was immediately removed and cleaned of the surrounding fat and the connective tissue. A segment was cut in helical strips (figure 4). The thoracic aorta was removed carefully, so as to protect the endothelial lining.
2. In some preparation, endothelium was removed by 20 gentle rubbings of the luminal surface with a cotton probe. The absence of a functional endothelium was shown by the absence of relaxant response (100%) after acetylcholine 100 μM addition to preparations contracted with noradrenaline 1 μM (Furchgott and Zawadzki, 1980).
3. Each aortic strip was suspended in a 15-ml organ bath containing physiological solution (Krebs' solution) maintained at 37 $^{\circ}$C and gassed with 95% O_2 and 5% CO_2.
4. An initial load of 1 g was applied to each strip and maintained throughout a 75-90 minutes equilibration period.
5. This tension was kept constant. However, throughout the experiment a loss of tension (less than 10-15%) occurred when the preparation was placed in Ca^{2+}–free medium.

   Tension was recorded isometrically via force-displacement transducers.

Figure 4 Thoracic aorta and preparing of isolated rat aorta

Chemicals

1. Reference compounds

(–)-Noradrenaline (NA) :	α-adrenoceptor agonist
Tetraethylammonium (TEA):	K^+–channel blocker
Potassium chloride (KCl):	membrane depolarization activator
Anhydrous caffeine:	release of Ca^{2+} from sarcoplasmic reticulum (SR)
Acetylcholine (ACh):	endothelium-dependent relaxant
Nifedipine:	Ca^{2+} channel blocker

All were purchased from Sigma (St. Louis, MO., U.S.A.) except for potassium chloride from APS Chemicals (Australia).

2. Testing compound

Dimethyl sulfoxide (DMSO) from Sigma (St. Louis, MO., U.S.A.)

3. Other reagents

NaCl, KCl, $CaCl_2$, $MgSO_4$, $MgCl_2$, KH_2PO_4, $NaHCO_3$, D-Glucose, EDTA, and ethanol.

All were purchased from APS Chemicals (Australia) except for ethanol from Farmitalia Carlo Erba (Spain).

Composition of the physiological solution (Krebs' solution) was (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.0, KH_2PO_4 1.2, $NaHCO_3$ 25, and D-Glucose 11.1. Ca^{2+}-free solution had the same composition except that $CaCl_2$ was omitted and EDTA (0.1 mM) was added.

Experimental instruments

1. Double-walled organ bath (figure 5)

   The organ bath made of glass comprises an inner and outer chamber. An inner chamber with the capacity of 25 ml is for suspending the isolated tissue in physiological solution. The reservoir should also be constantly aerated with 95% O_2 and 5% CO_2. An outer chamber is for temperature-controlled water that is responsible for maintaining the right temperature of the inner chamber.
2. Water bath and thermoregulation water pump
3. Isometric transducer of Washington transducer (Harvard Apparatus Ltd., England)
4. Recorder Universal Oscillograph (Harvard Apparatus Ltd., England)
5. Recorder with electrical disperser Gilson N_2 coupled to an amplifier (Harvard Apparatus Ltd., England)
6. Tank of Carbogen gas (95% O_2 + 5% CO_2) (T.I.G., Thailand)

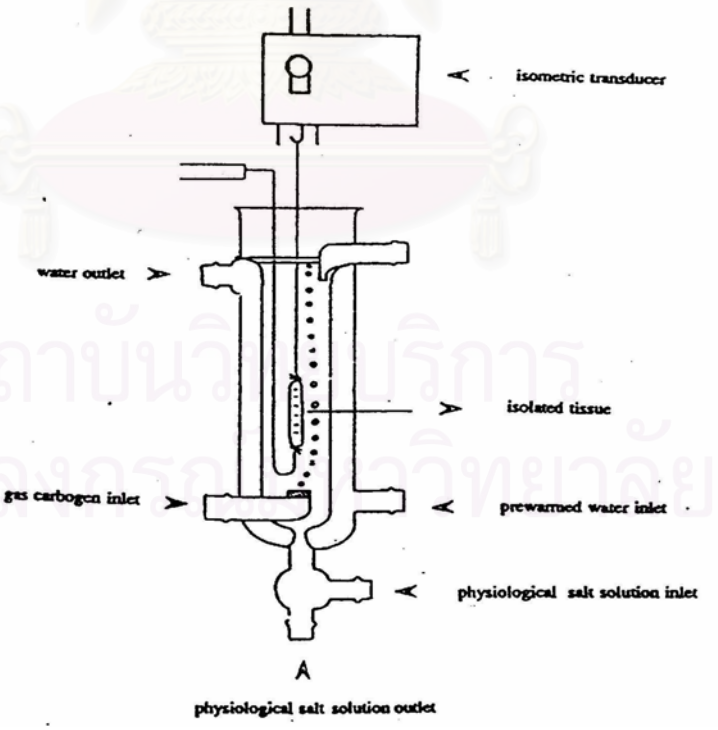

Figure 5 Illustration of instrument and organ bath for isolated rat aorta

Experimental procedures

1. Effects of DMSO on the contraction of isolated rat aorta

1.1 Effects on the contraction induced by various contractants in Ca^{2+}–containing physiological solution

The endothelium-denuded aortic strip was prepared and incubated in Ca^{2+}–containing solution for 75-90 minutes. Next, noradrenaline (NA) 1 μM was added to induce contraction and the tension was recorded for 15 minutes prior to washing tissue with Ca^{2+}–containing solution 3 times. The tissue was then incubated for 60 minutes until the tension was stable similar to that observed at the beginning. The effect of DMSO on NA -induced contraction in Ca^{2+}–containing solution was studied by pretreated with DMSO, ranging in the concentration from 0.017 to 10.0% (v/v), for 5 minutes. Then NA was added and the tension was recorded for 15 minutes before washing tissue. Thereafter the tissue was incubated for 60 minutes. At the end of the experiment, NA was added again to test the viability of aorta.

The contraction profile of vascular smooth muscle was compared between before and after getting DMSO.

Furthermore, to study the effects of DMSO on the aortic contraction induced by KCl 40 mM or TEA 1 mM or caffeine 10 mM, the similar experimental procedure as mentioned above were performed but using these agonists instead of NA.

In addition, the effect of endothelium on the contraction induced by NA or KCl was tested. The same experiment to endothelium-denuded preparation was carried out but using endothelium-intact aortic strip instead.

1.2 Effects on the contraction induced by noradrenaline in Ca^{2+}–free medium

The endothelium-denuded aortic strip was prepared and incubated in Ca^{2+}–containing solution for 75-90 minutes. NA 1 μM was added to induce contraction and the tension was recorded for 15 minutes prior to washing tissue. Then the tissue was treated with Ca^{2+}–free, EDTA- containing solution for 15 minutes and NA was applied. Thereafter the aorta was incubated in Ca^{2+}–containing solution for 60 minutes until the tension was stable similar to that observed at the beginning. NA was added in Ca^{2+}–containing solution. Next, the tissue was treated with Ca^{2+}–free, EDTA- containing solution for 15 minutes. The effect of DMSO on NA -induced contraction in Ca^{2+}–free medium was studied by pretreated with DMSO at various concentrations from 0.03 to 10.0 % (v/v) for 5 minutes and then NA was added to

induce contraction. The tension was recorded for 15 minutes prior to washing tissue. Thereafter the aorta was incubated for 60 minutes again in Ca^{2+}–containing solution. At the end of the experiment, NA was added to induce contraction in Ca^{2+}–containing solution and then in Ca^{2+}–free solution to test the viability of smooth muscle.

The contraction profile of vascular smooth muscle was compared between before and after getting DMSO in Ca^{2+}-free medium.

1.3 Effects on the contraction induced by adding calcium to a calcium-free depolarizing solution

The method described by Hof and Vuorela (1983) was used to determine the effects of DMSO on $CaCl_2$-induced contraction in high K^+-Ca^{2+} free solution.

The endothelium-denuded aortic strip was prepared and incubated in Ca^{2+}-containing solution for 75-90 minutes. Then the solution was changed to Ca^{2+}-free depolarizing solution and the tissue was incubated for 30 minutes. Then $CaCl_2$-cumulative concentration ($1x10^{-5}$ M – $1x10^{-2}$ M) was added to induce contraction until the maximum contraction and the tissue was then washed with Ca^{2+}-containing solution 3 times. The tissue was incubated for 60 minutes with Ca^{2+}-containing solution until the tension was stable similar to those observed before at the beginning. The solution was changed to Ca^{2+}-free depolarizing solution and the tissue was incubated for 30 minutes. The effect of DMSO on $CaCl_2$-induced contraction in Ca^{2+}-free depolarizing solution was studied by pretreated with DMSO at different concentrations from 0.03 to 10.0% (v/v) for 5 minutes and then $CaCl_2$ was added cumulatively to induce contraction. Furthermore, the effect of nifedipine (1 μM) was also studied by adding it instead of DMSO.

The contraction profile of vascular smooth muscle was compared between before and after getting DMSO or nifedipine.

2. Effects of DMSO on the increase in the resting tone of rat aorta

The experimental procedure is designed to study the depletion of intracellular Ca^{2+}–stores sensitive to NA in Ca^{2+}–free medium, and the increase in the resting tone (IRT) obtained by subsequent exposure to Ca^{2+}–containing solution during the refilling of the noradrenaline-sensitive Ca^{2+} stores (Noguera, *et al.*, 1996) (Figure 6).

The endothelium-denuded aortic strip was incubated in Ca^{2+}–containing solution 75-90 minutes. NA (1 μM) was added in Ca^{2+}–containing solution (Ca^{+}) and the tissue was then treated with Ca^{2+}–free, EDTA- containing solution (Ca^{-}) for 15 minutes. Thereafter NA was applied and subsequently washed out until no contraction was induced, indicating complete depletion of internal Ca^{2+} stores sensitive to NA. The tissue was incubated for 20 minutes in Ca^{2+}–containing solution to refill the intracellular Ca^{2+} stores and a spontaneous increase in the resting tone of the aorta (IRT) was observed. After washing and 15 minutes of incubation in Ca^{2+}–free medium, NA was added again (NA_2). Finally, the aortic strip was tested its function by adding NA to the tissue suspended in Ca^{2+}–containing solution. The contraction response in this condition returned to the maximum level, which is comparable to those obtained at the beginning of experiment.

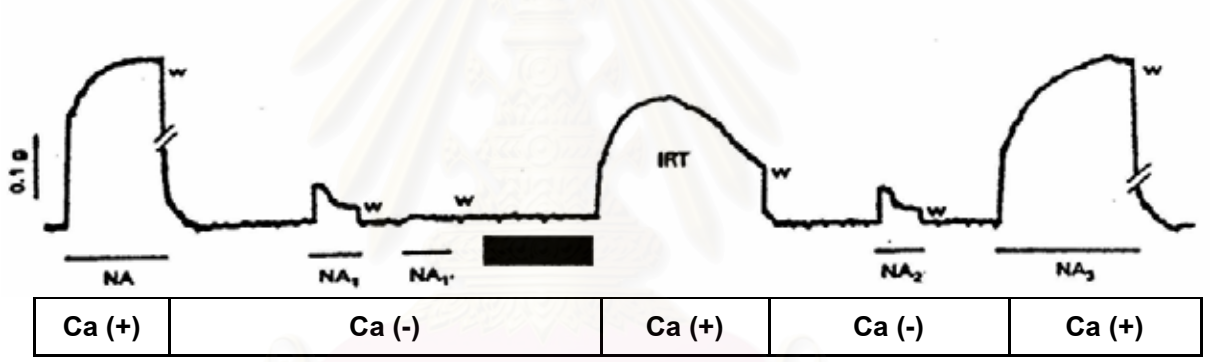

Figure 6 The experimental procedure for IRT determination (Noguera, *et al.*, 1996)

To test the effect of DMSO on an increase in the resting tone (IRT), the same experimental system as demonstrated in Figure 4 was performed, but DMSO at the concentration of 0.017, 0.03 and 0.07% (v/v) was added into the Ca^{2+}–free medium 5 minutes before changing medium to Ca^{2+}–containing solution. Then measured the IRT that permits the refilling of internal Ca^{2+} stores previously depleted by NA and the tissue was incubated for 20 minutes in Ca^{2+}–containing solution. After washing and 15 minutes of loading in Ca^{2+}–free medium, NA was added (NA_2). After that, washed the tissue with Ca^{2+}–containing solution 3 times and incubated the tissue for 60 minutes. To study the reversibility of the function of aortic smooth muscle after getting DMSO, the similar experimental procedure as mentioned above was performed again.

The magnitude of the IRT was compared between before and after getting DMSO.

3. Effects of DMSO on the relaxation of isolated rat aorta

3.1 Effect on endothelium-dependent relaxation produced by acetylcholine

The endothelium-intact aortic strip was prepared and incubated in Ca^{2+}–containing solution for 75-90 minutes. Then NA (1μM) was added to induce contraction, followed by adding acetylcholine (ACh) 100 μM to relax during maximum contraction evoked by NA. The response was recorded for 30 minutes prior to washing tissue with Ca^{2+}–containing solution 3 times. The tissue was incubated for 60 minutes until the tension was stable similar to that observed before study. The effect of DMSO on endothelium-dependent relaxation produced by acetylcholine in Ca^{2+}–containing solution was studied by pretreated with DMSO at the concentration of 0.03 or 0.5% (v/v) for 5 minutes to maximum contraction induced by NA and then ACh was added. The tension was recorded for 35 minutes prior to washing tissue with Ca^{2+}–containing solution 3 times.

The relaxation profile of vascular smooth muscle was compared between before and after getting DMSO.

3.2 Effect of DMSO as the relaxant to contractants-induced contraction in aortic strip in the presence or absence of the endothelium

The endothelium-intact aortic strip was prepared and incubated in Ca^{2+}–containing solution for 75-90 minutes. Then NA 1μM was added to induce contraction. To study the relaxation effect, DMSO, ranging in the concentration from 0.03 to 10.0% (v/v) was added to the maximum contraction induced by NA. The response was recorded for 30 minutes prior to washing tissue

The relaxations were expressed as a percentage of the noradrenaline-induced contraction in Ca^{2+}–containing solution.

In addition, the relaxation effect of DMSO to KCl-induced contraction was also studied. The similar experimental procedure as mentioned above was performed but using KCl 40 mM instead of NA.

Furthermore, to study the effects of DMSO on the endothelium-denuded aortic strip, the same experiment to endothelium-intact preparation was carried out but using endothelium-denuded aortic strip instead.

Statistical Analysis

Contractions induced by NA, KCl, TEA and Caffeine in physiological solution were expressed in milligrams of developed tension and as a percentage of the NA-induced contraction obtained in the same solution. Contractions induced by NA in Ca^{2+}-free medium were also expressed as a percentage of the NA-induced contraction in Ca^{2+}–containing solution. Contractions by $CaCl_2$ at each concentration were expressed as a percentage of the maximum contraction induced by maximum concentration of $CaCl_2$. The increases in the resting tone and relaxations by ACh or DMSO were also expressed as a percentage of the NA-induced contractions in normal physiological solution.

The results were presented as the mean $\pm$ S.E. mean for *n* determinations obtained from different animals (N = 4-6 separated experiments). Statistical evaluation of difference of two means was performed by paired or unpaired Student's *t* test. When differences of more than two means were compared, one-way analysis of variance (ANOVA) was used. Where ANOVA showed significant differences ($p<0.05$), the results were analyzed further using the Student-Newman-Keuls test. Differences of the means were considered to be significant when $p<0.05$.

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CHAPTER IV

RESULTS

1. Profiles of aortic contraction of isolated rat aorta

As shown in table 1, the maximum contractile responses of endothelium-intact and -denuded aortic strips evoked by noradrenaline 1 μM were 542.07 ± 29.58 mg (n=36) and 331.85 ± 15.74 mg (n=66) and KCl 40 mM were 204.16 ± 18.98 mg (n=24) and 213.62 ± 15.30 mg (n=42), respectively. The magnitude of contraction of endothelium-denuded aortic tissues induced by TEA 1 mM was 88.16 ± 7.39 mg (n=42). Furthermore, caffeine 10 mM generated aortic contraction with the magnitude of 35.10 ± 4.76 mg (n=42). The contraction of the strips induced by noradrenaline was significantly different between endothelium-intact and –denuded strips. The patterns of the contraction induced by various contractants are illustrated in figure 7. The contractile response induced by noradrenaline consisted of two phases: an initial phasic and a continuing tonic phase. This pattern of contraction was also observed when KCl or TEA was used in place of noradrenaline to induce the contraction. The profile of caffeine-induced contraction of endothelium-denuded aortic strips was obviously different from those stimulated by noradrenaline, KCl and TEA. The contraction profile elicited by caffeine comprised of a rapid transient contraction and then muscle tension decreased to a level below a resting tone.

2. Profiles of effect of DMSO on vascular tone

The profile of the effect of DMSO on the vascular tone of aortic strip is presented in figure 8.

DMSO at high concentration (5% and 10% v/v) induced an initial transient contraction, followed by relaxation of the basal tone of aortic strip. DMSO relaxed the resting tension from baseline in a concentration-dependent manner (0.017-10% v/v) (figure 9 a–d). This effect of DMSO did not depend on the presence of endothelium. In addition, the absence of Ca^{2+} from the physiological solution caused the vascular tension to relax at higher magnitude than when comparing to the relaxation in the Ca^{2+}-containing physiological solution. As seen in figure 9d, the

basal tone of endothelium-denuded aortic strips in Ca^{2+}-free medium was relaxed by DMSO at high concentration (10% v/v) in larger magnitude than aortic strips in Ca^{2+}-containing solution.

3. Effects of DMSO on the contraction of isolated rat aorta

3.1 Effect on the contraction induced by various contractants in Ca^{2+}-containing physiological solution

3.1.1 Noradrenaline-induced contraction

The contractile profiles of the effects of DMSO to noradrenaline-induced contraction are presented in figure 10.

The effects of DMSO in various concentrations on the contractile response are shown in figure 11 (a, b). Noradrenaline-induced contractions were inhibited by DMSO at the concentration of 5% and 10% v/v for endothelium-denuded strip. The remaining responses were 76.88 ± 2.92% (n=6) and 47.75 ± 4.87% (n=6), respectively. For endothelium-intact strip, the maximum contraction was suppressed to 45.82 ± 6.54% (n=6) by DMSO at 10% v/v. Upon exposure to DMSO at the concentration of 5% v/v, it appeared that endothelium was able to protect the vascular contractility. The protective effect could not sustain at the higher concentration of DMSO. DMSO at other concentrations in this study did not affect the contractility of both endothelium-denuded and –intact aortic strip (figure 11c). In addition, the inhibitory effect was reversible upon removing DMSO. As seen in figure 11 (a, b), the strip incubated at DMSO (10% v/v) for 20 minutes could regain its contractility completely when DMSO was washed out from the system.

3.1.2 KCl-induced contraction

The contractile patterns of the effects of DMSO to the contraction elicited by KCl are shown in figure 12 and 13.

DMSO appears to exert dual effects on the contractility of aortic strip induced by KCl (figure 14a and 14b). DMSO at the concentration of 5% and 10% v/v suppressed the contraction induced by KCl in both of the endothelium-denuded and -intact aortic strip (figure 14c). The residual contractions of endothelium-denuded strip were 54.67 ± 13.49% (n=6) and 18.29 ± 10.55% (n=6), respectively, and of endothelium-intact were 50.00 ± 10.98% (n=6) and 5.76 ± 2.32% (n=6),

respectively. Similary to what observed in the previous experiment with noradrenaline, the inhibitory effect was reversible upon DMSO removal. At lower concentration of DMSO, our results showed the higher contractile response to K^+-depolarized membrane in the experiment of endothelium-denuded and –intact strip. As seen in figure 14 (a, b), the effect of KCl in activation of contraction response of endothelium-denuded strip was lifted to 127.90 ± 10.63% (n=6) in the presence of 0.07% v/v DMSO. And the maximum contraction in endothelium-intact strip increased to 163.93 ± 23.62% (n=6) in the presence of DMSO 0.03% v/v.

3.1.3 TEA-induced contraction

Figure 15 shows the contractile response evoked by TEA. Our results showed that DMSO did not significantly affect TEA-induced contraction of endothelium-denuded aortic strip. In addition, the contractile response increased upon DMSO removal.

3.1.4 Caffeine-induced contraction

The contractile profiles of the effects of DMSO to caffeine-induced contraction are shown in figure 16.

Figure 17 shows that DMSO suppressed the contraction of endothelium-denuded aortic strip induced by caffeine to 27.92 ± 9.36% for DMSO at 5% v/v and to 15.18 ± 3.98% for 10% v/v. Furthermore, the inhibitory effect was reversible upon DMSO removal. The contractions of the strips after DMSO wash-out increased from the control in every concentration of DMSO.

3.2 Effect on the contraction induced by noradrenaline in Ca^{2+}-free medium

The contractile profile of the contraction induced by noradrenaline in Ca^{2+}-free medium is shown in figure 18.

DMSO did not have any effect on the contraction of endothelium-denuded aortic strip induced by noradrenaline in Ca^{2+} -free medium (figure 19).

3.3 Effect on the contraction induced by adding calcium to a calcium-free depolarizing solution

The contractile patterns of the effects of DMSO and nifedipine to the contraction elicited by cumulative addition of Ca^{2+} to Ca^{2+}-free depolarizing solution are shown in figure 20.

As seen in figure 21, nifedipine 1 μM was able to completely suppress the contraction of aortic smooth muscle induced by cumulative addition of Ca^{2+} to Ca^{2+}-free depolarizing solution. DMSO at the concentration of 10% v/v inhibited the contraction induced by $CaCl_2$ at the concentration of 3×10^{-5} M to 1×10^{-2} M, whereas DMSO at 5% v/v inhibited the contraction induced by $CaCl_2$ at the concentration of 1×10^{-4} M, 3×10^{-4} M and 1×10^{-3} M (figure 22). In consistent with other experiments, the inhibitory effect of DMSO 10% v/v was reversible upon removing DMSO (figure 23).

4. Effects of DMSO on the increase in the resting tone of isolated rat aorta

As illustrated in figure 24, upon changing the physiological solution from Ca^{2+}-free medium to Ca^{2+}-containing solution, a spontaneous contraction was observed if that aortic strip was previously treated with noradrenaline. The amplitude of the increase in the resting tone (IRT) was 52.75 ± 3.52% (n=12) relative to that of noradrenaline-induced contraction in physiological solution. Even at the concentration that did not produce any inhibitory effect on agonist-induced contraction, DMSO significantly inhibited the IRT response. As shown in figure 25a, the percentage of IRT was suppressed to 27.50 ± 3.22% (n=6) for DMSO 0.03% v/v and 19.67 ± 4.72% (n=6) for DMSO 0.07% v/v. Although the IRT response was markedly significantly inhibited by low concentration of DMSO, the refilling of the intracellular Ca^{2+} stores was comparable to those of the control group, suggested by a contraction in Ca^{2+}-free medium (NA2) after IRT. The magnitudes of the contraction induced by noradrenaline in Ca^{2+}-free medium (NA2) were 33.54 ± 3.09% (n=12) for control, 34.65 ± 5.28% (n=6) for DMSO 0.03% v/v, and 28.79 ± 3.05% (n=6) for DMSO 0.07% v/v (figure 25b).

5. Effects of DMSO on the relaxation of isolated rat aorta

5.1 Effect on endothelium-dependent relaxation produced by acetylcholine

The profile of the relaxation produced by acetylcholine upon the maximum contraction induced by noradrenaline is shown in figure 26.

DMSO did not affect the relaxation produced by acetylcholine (figure 27).

5.2 Effect of DMSO as the relaxant to contractant-induced contraction in aortic strip in the presence or absence of the endothelium

The profiles of the relaxation effect of DMSO upon the maximum contraction induced by noradrenaline or KCl are shown in figure 28 (a, b) and 30 (a, b).

The maximum contraction of aortic strip which was evoked by noradrenaline 1 μM or KCl 40 mM was alleviated upon addition of DMSO in dose-dependent manner. As seen in figure 29 and 31, the relaxation effects of DMSO were observed at the concentration of 5% and 10% v/v in both endothelium-intact and –denuded aortic strips.

In noradrenaline-precontracted aortic strip, the relaxation effects of DMSO to maximum contraction of endothelium-intact and endothelium-denuded strips were 24.07 ± 3.47% (n=6) and 29.82 ± 2.67% (n=6) at 5% v/v of DMSO, respectively, and 88.88 ± 6.28% (n=6) and 111.93 ± 5.08% (n=6) at 10% v/v of DMSO, respectively.

For the relaxation responses on maximum contraction induced by KCl, DMSO at the concentration of 5% v/v relaxed the maximum contraction with the amount of 51.36 ± 8.85% (n=6) for endothelium-intact and 62.77 ± 10.89% (n=6) for endothelium-denuded aortic strips. DMSO at the concentration of 10% v/v relaxed with the magnitude of 105.22 ±15.28% (n=6) for endothelium-intact and 124.07 ± 9.94% (n=6) for endothelium-denuded aortic strips.

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Table 1 Force of contraction (mg) of aortic strips induced by various contractants in Ca^{2+}-containing solution

Agonists	**Aortic strips**	
	Endothelium-denuded	**Endothelium-intact**
Noradrenaline 1 μM	331.85 ± 15.74 mg (n=66)	***542.07 ± 29.58 mg (n=36)
KCl 40 mM	213.62 ± 15.30 mg (n=42)	204.16 ± 18.98 mg (n=24)
TEA 1 mM	88.16 ± 7.39 mg (n=42)	-
Caffeine 10 mM	35.10 ± 4.76 mg (n=42)	-

All values represent mean ± S.E. mean, n=number of experiment

***$p < 0.001$ shows significant difference between endothelium-intact and –denuded aortic strips (unpaired *t*-test).

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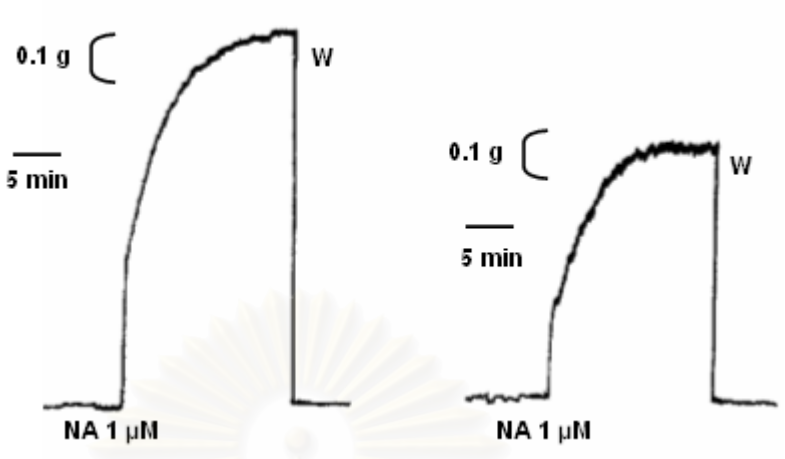
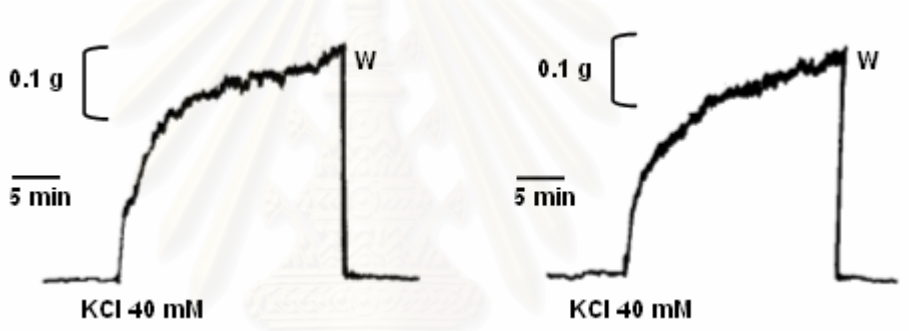
1a) endothelium-intact **1b) endothelium-denuded**



2a) endothelium-intact **2b) endothelium-denuded**



3) endothelium-denuded **4) endothelium-denuded**

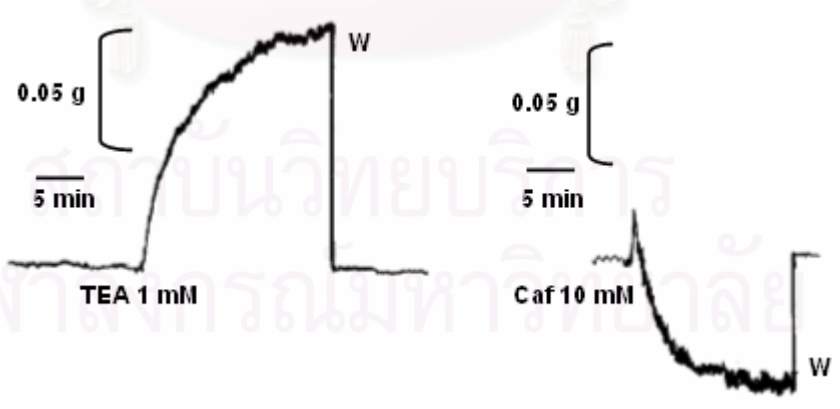


Figure 7 The profiles of the aortic contractions induced by various contractants: noradrenaline (NA) in endothelium-intact aortic strip (1a) and endothelium-denuded aortic strip (1b), KCl in endothelium-intact aortic strip (2a) and endothelium-denuded aortic strip (2b), TEA (3), and caffeine (4) in endothelium-denuded aortic strip.

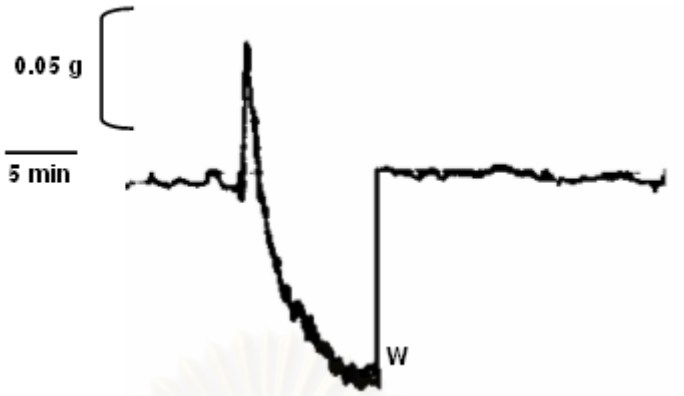


Figure 8 The profile of the effect of DMSO (10% v/v) on the vascular tone

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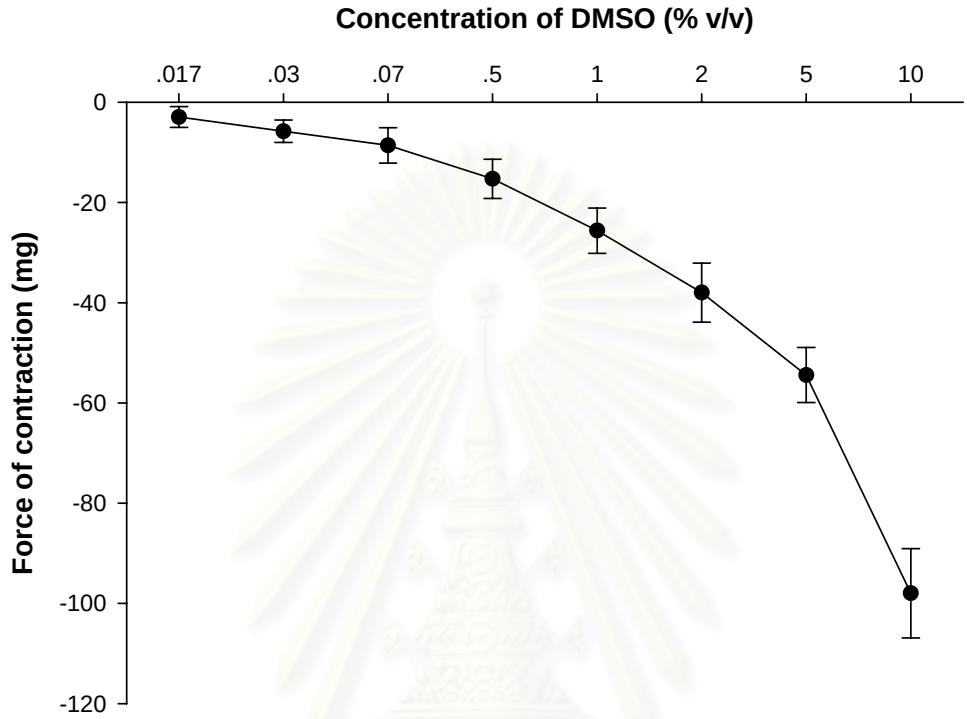

Figure 9a Effects of DMSO on the basal tone of endothelium-denuded aortic strips in Ca^{2+}-containing solution. All values represent mean ± S.E. mean.

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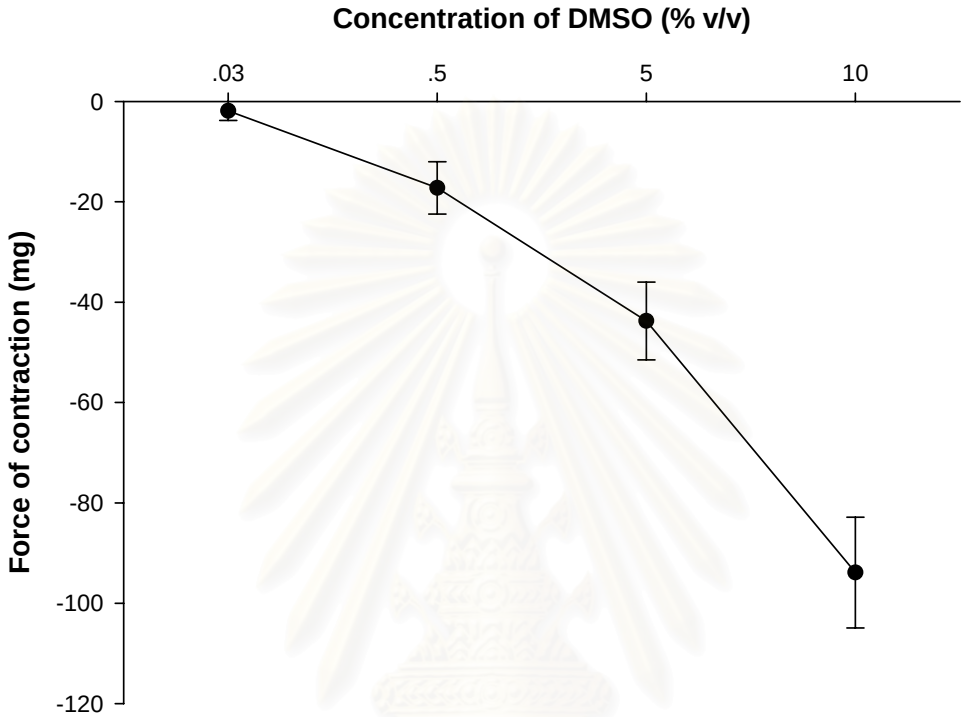

Figure 9b Effects of DMSO on the basal tone of endothelium-intact aortic strips in Ca^{2+}-containing solution. All values represent mean ± S.E. mean.

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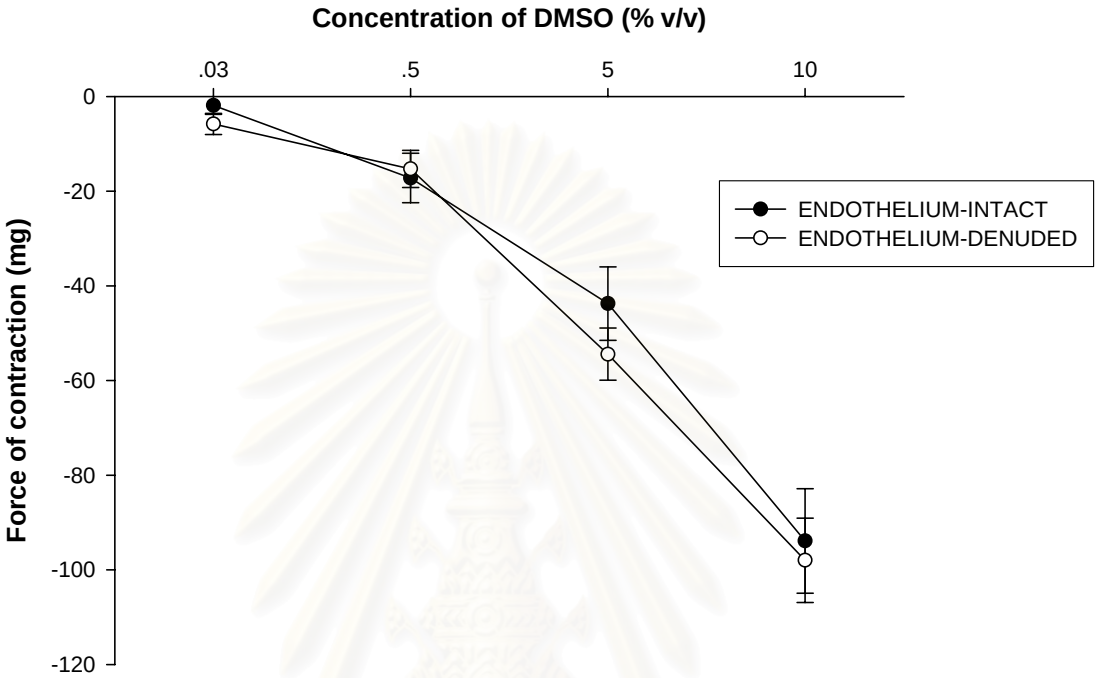

Figure 9c Effects of DMSO on the basal tone of endothelium-intact and endothelium-denuded aortic strips in Ca^{2+}-containing solution. All values represent mean ± S.E. mean.

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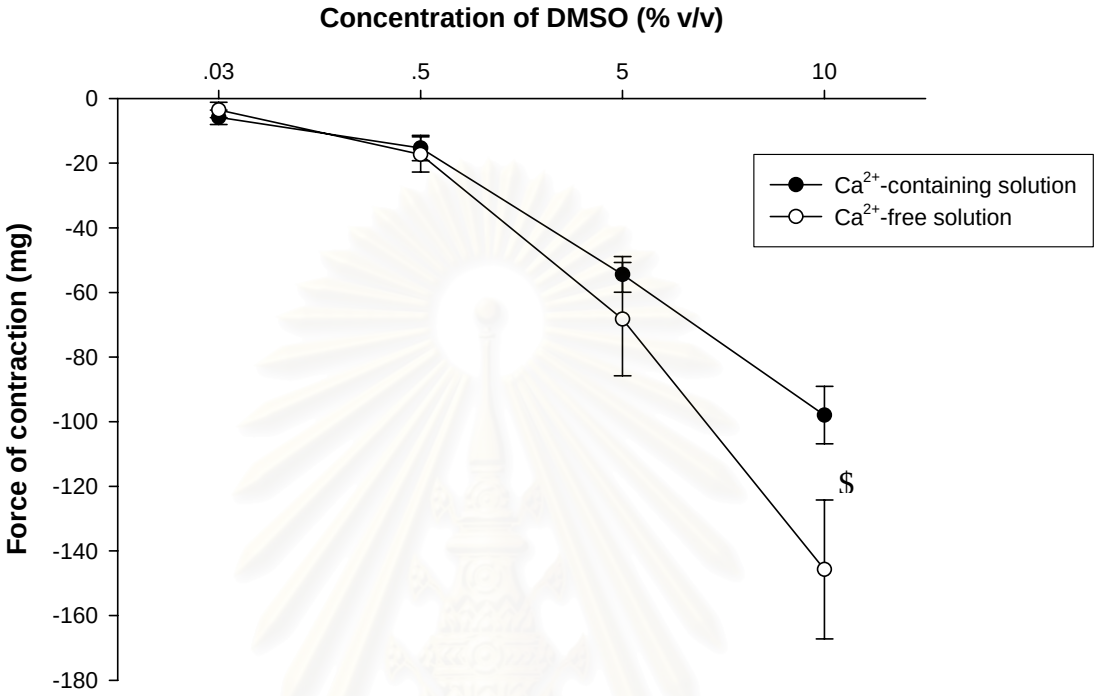

Figure 9d Effects of DMSO on the basal tone of endothelium-denuded aortic strips in Ca^{2+}-containing and Ca^{2+}-free solution. All values represent mean ± S.E. mean.

$*p*<0.05 shows significant difference between endothelium-intact and endothelium-denuded aortic strips (unpaired *t*-test).

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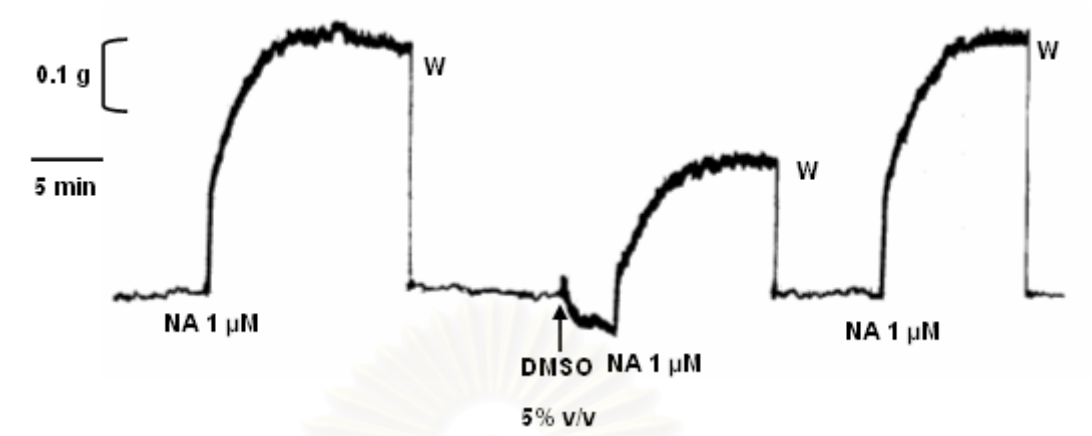
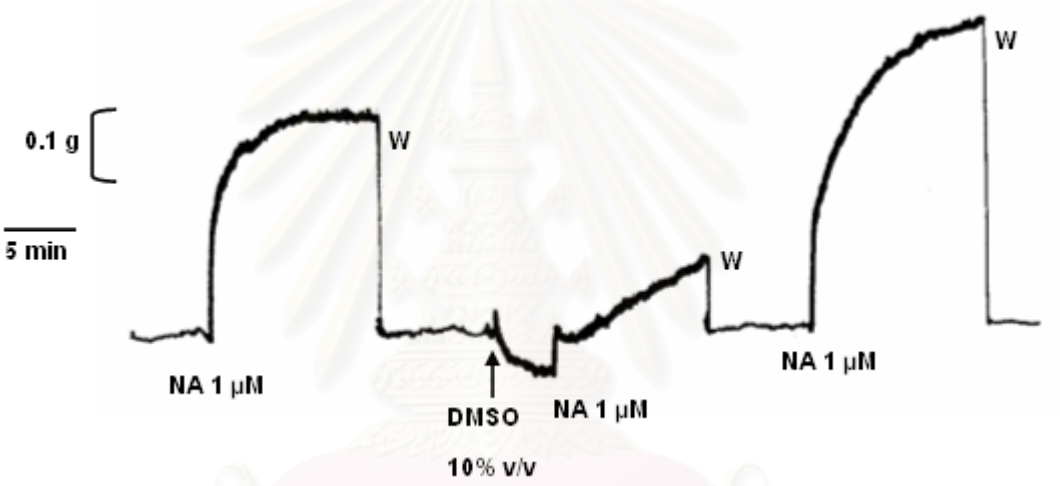
a) endothelium-denuded



b) endothelium-denuded



c) endothelium-intact

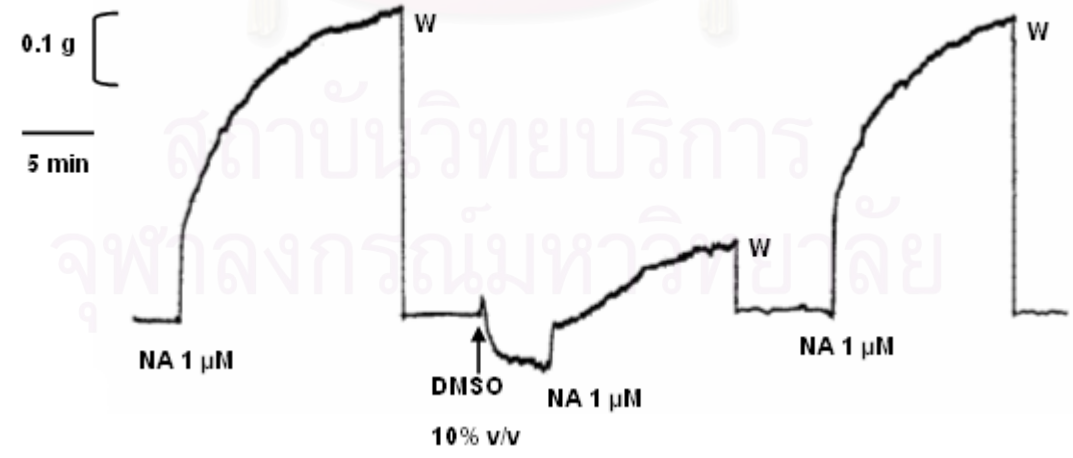


Figure 10 The contraction profiles showing the effect of DMSO on the contraction induced by noradrenaline of endothelium-denuded aortic strip (a, b) and of endothelium-intact aortic strip (c) in Ca^{2+}-containing solution.

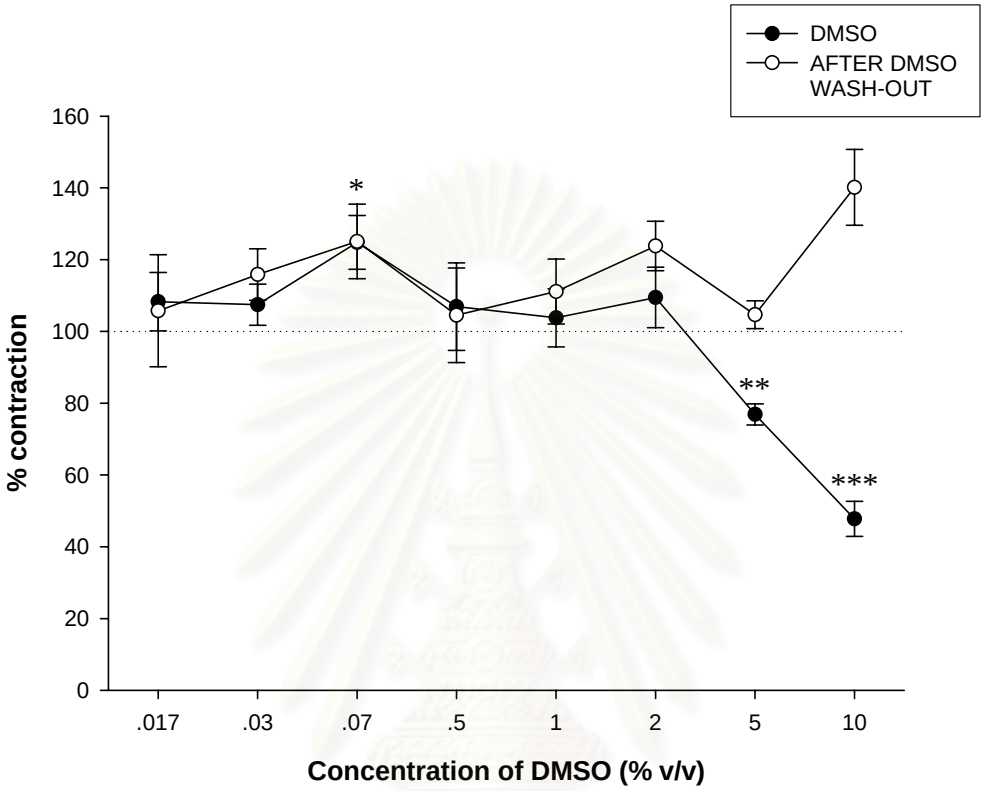


Figure 11a Effects of DMSO on the contraction of endothelium-denuded aortic strips induced by 1 μM noradrenaline in Ca^{2+}-containing solution. All values represent mean ± S.E. mean, N=6 for each concentration of DMSO.

*p<0.05, **p<0.01 and ***p<0.001 show significant difference when compared to before addition of DMSO (paired t-test).

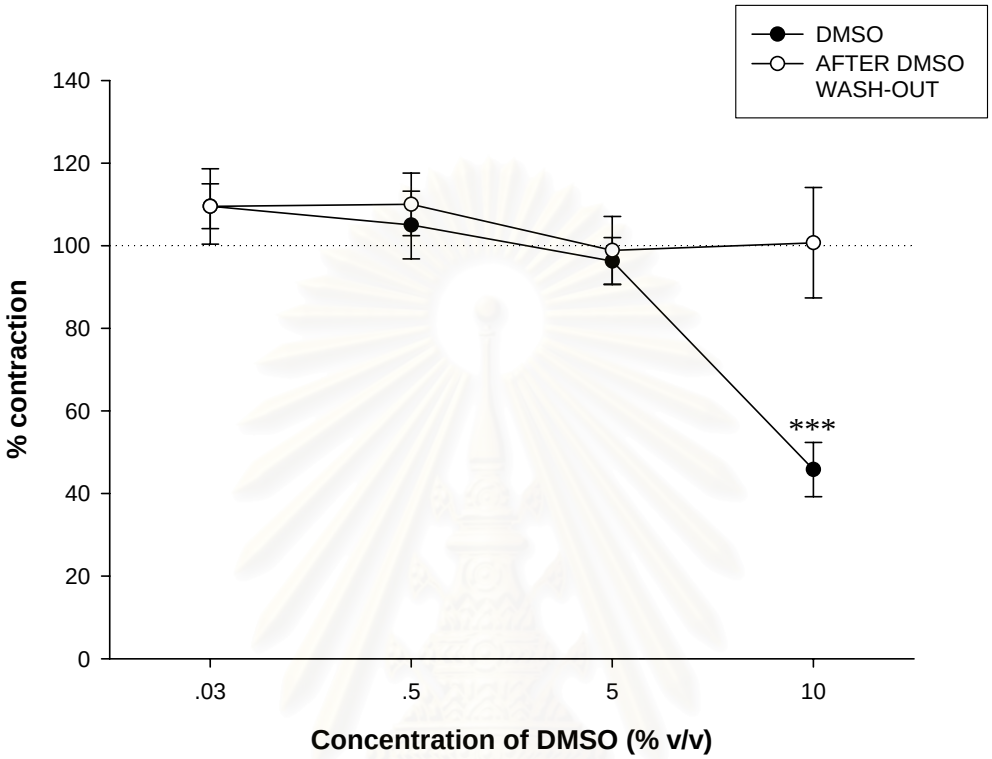


Figure 11b Effects of DMSO on the contraction of endothelium-intact aortic strips induced by 1 μM noradrenaline in Ca^{2+}-containing solution. All values represent mean ± S.E. mean, N=6 for each concentration of DMSO.

***p<0.001 shows significant difference when compared to before addition of DMSO (paired t-test).

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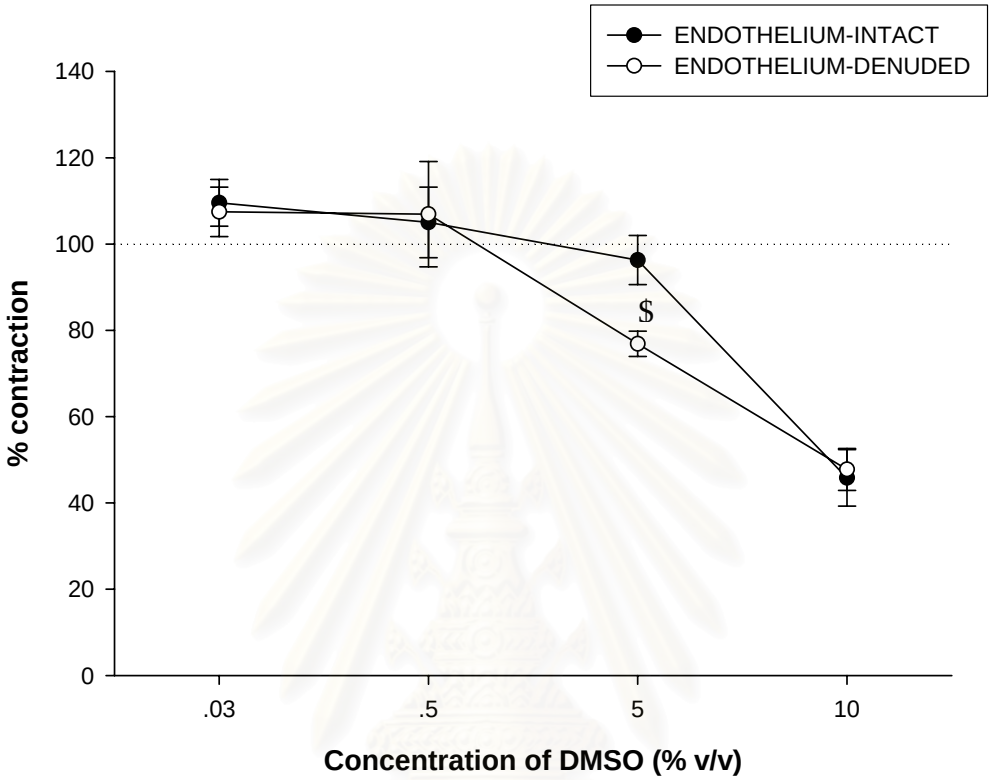
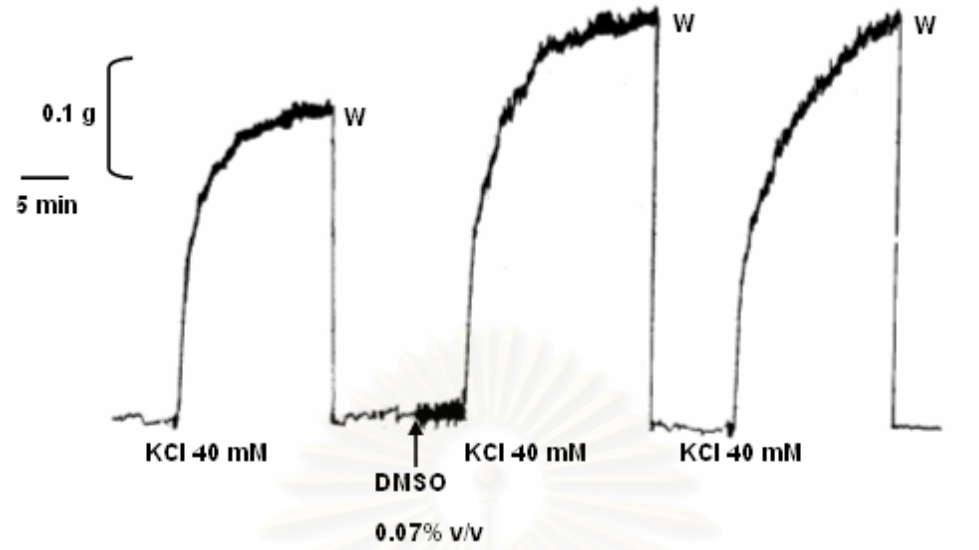


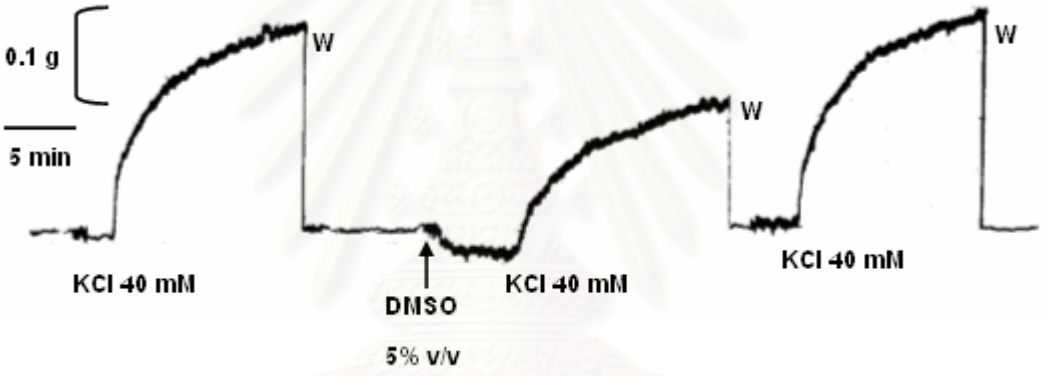
Figure 11c The comparison of effects of DMSO on the contraction induced by 1 μM noradrenaline in Ca^{2+}-containing solution between endothelium-intact and endothelium-denuded aortic strips. All values represent mean ± S.E. mean, N=6 for each concentration of DMSO.

$*p*<0.05 shows significant difference between endothelium-intact and endothelium-denuded aortic strips (unpaired *t*-test)).

a) endothelium-denuded



b) endothelium-denuded



c) endothelium-denuded

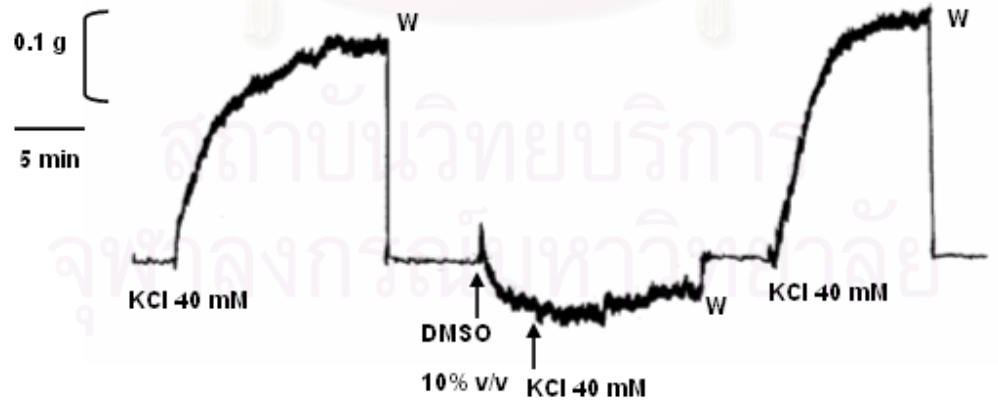
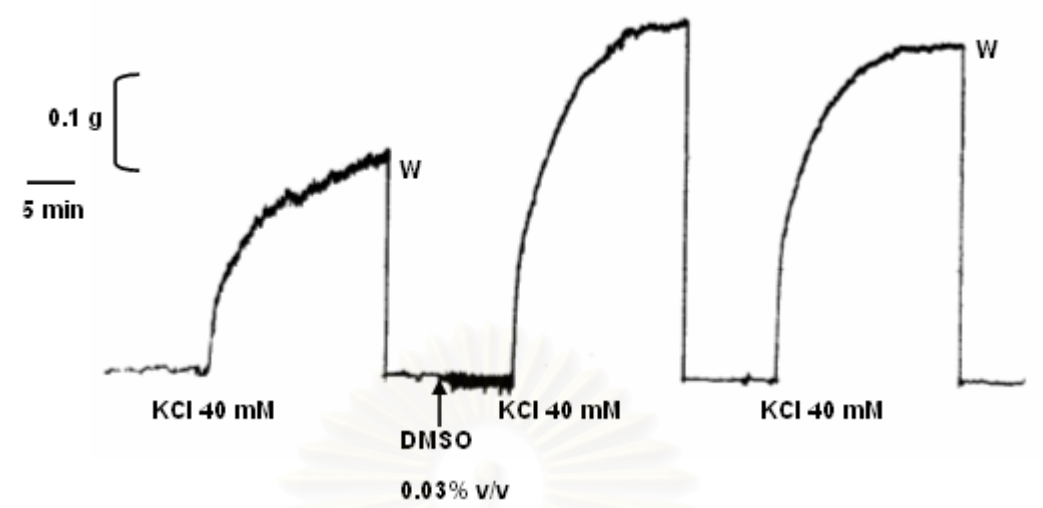
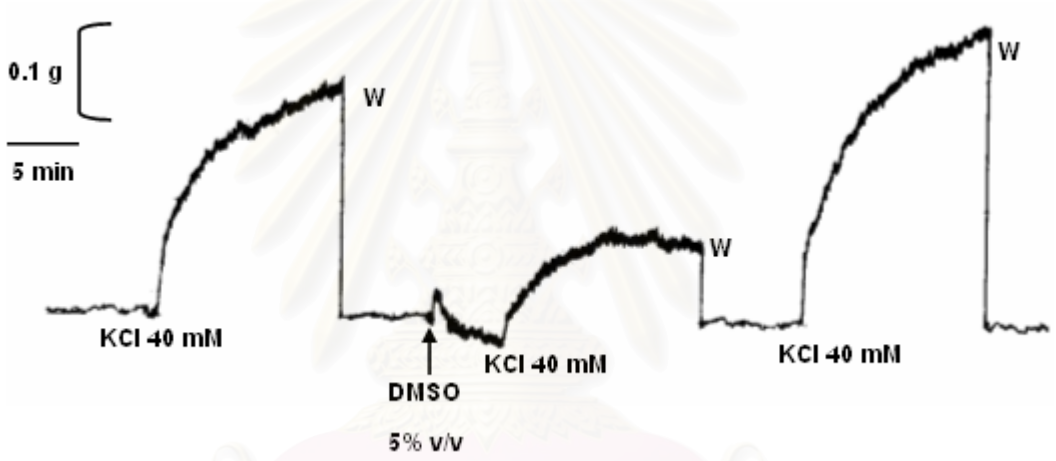


Figure 12 The contraction profiles showing the effect of DMSO on the contraction induced by KCl of endothelium-denuded aortic strip (a, b, c) in Ca^{2+}-containing solution.

a) endothelium-intact



b) endothelium-intact



c) endothelium-intact

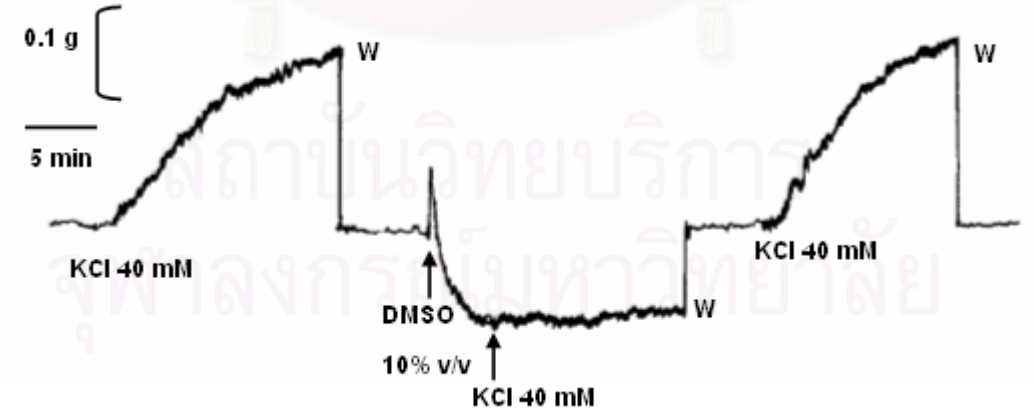


Figure 13 The contraction profiles showing the effect of DMSO on the contraction induced by KCl of endothelium-intact aortic strip (a, b, c) in Ca^{2+}-containing solution.

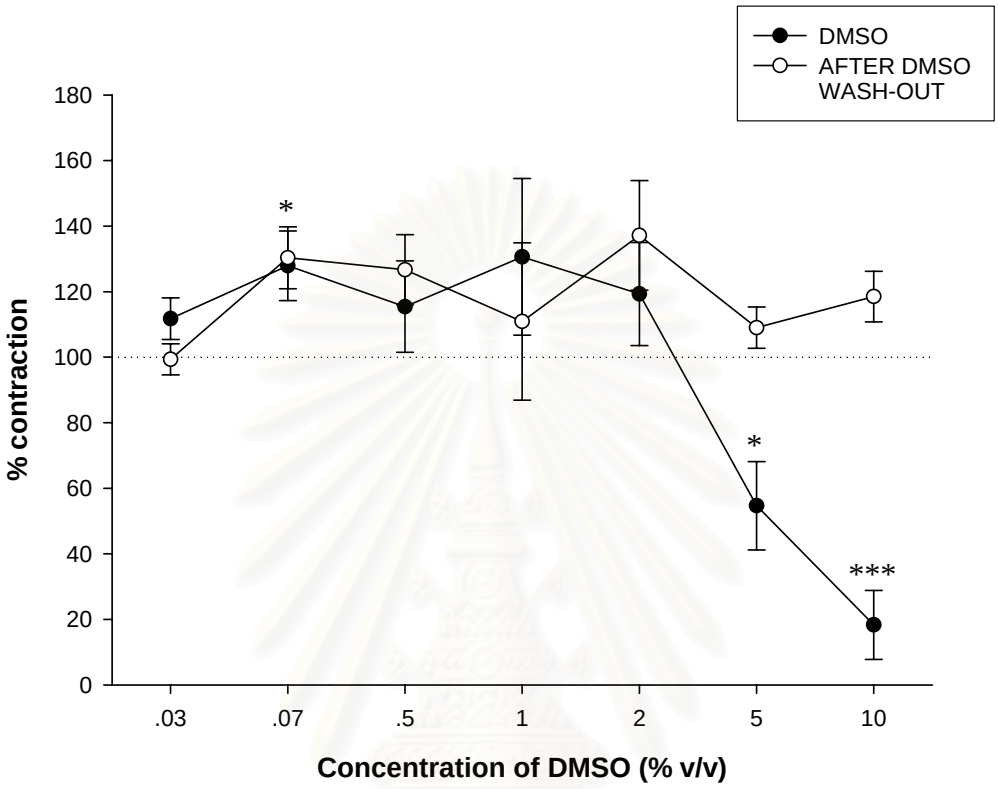


Figure 14a Effects of DMSO on the contraction of endothelium-denuded aortic strips induced by 40 mM KCl in Ca^{2+}-containing solution. All values represent mean ± S.E. mean, N=6 for each concentration of DMSO.

*p<0.05 and ***p<0.001 show significant difference when compared to before addition of DMSO (paired *t*-test).

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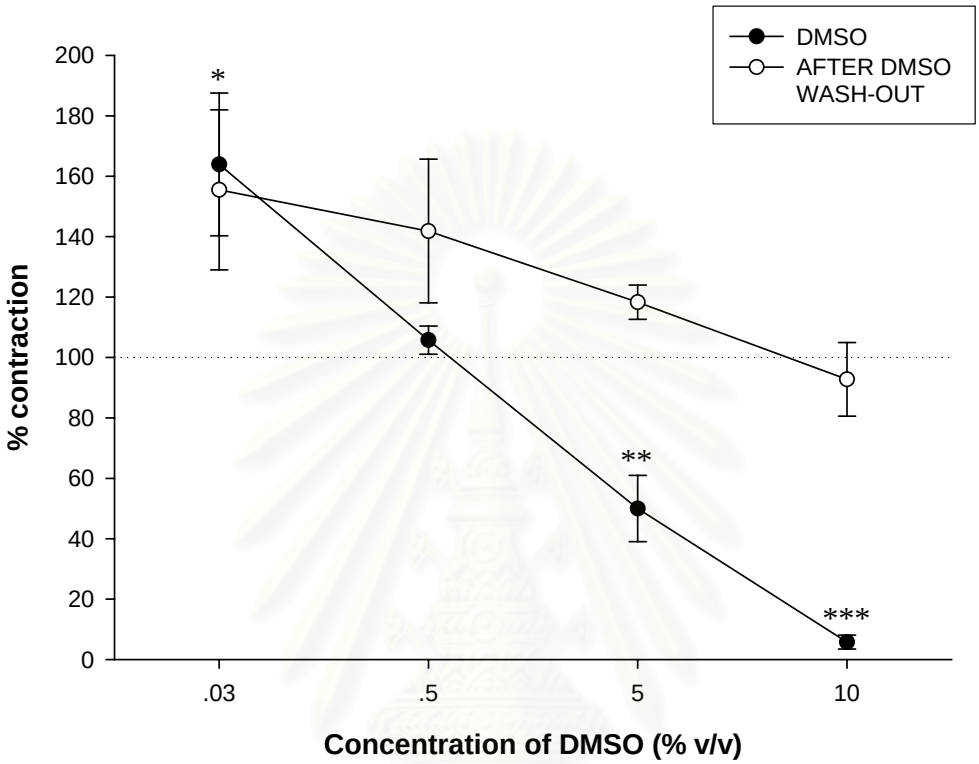

Figure 14b Effects of DMSO on the contraction of endothelium-intact aortic strips induced by 40 mM KCl in Ca^{2+}-containing solution. All values represent mean ± S.E. mean, N=6 for each concentration of DMSO.

*p<0.05, **p<0.01 and ***p<0.001 show significant difference when compared to before addition of DMSO (paired t-test).

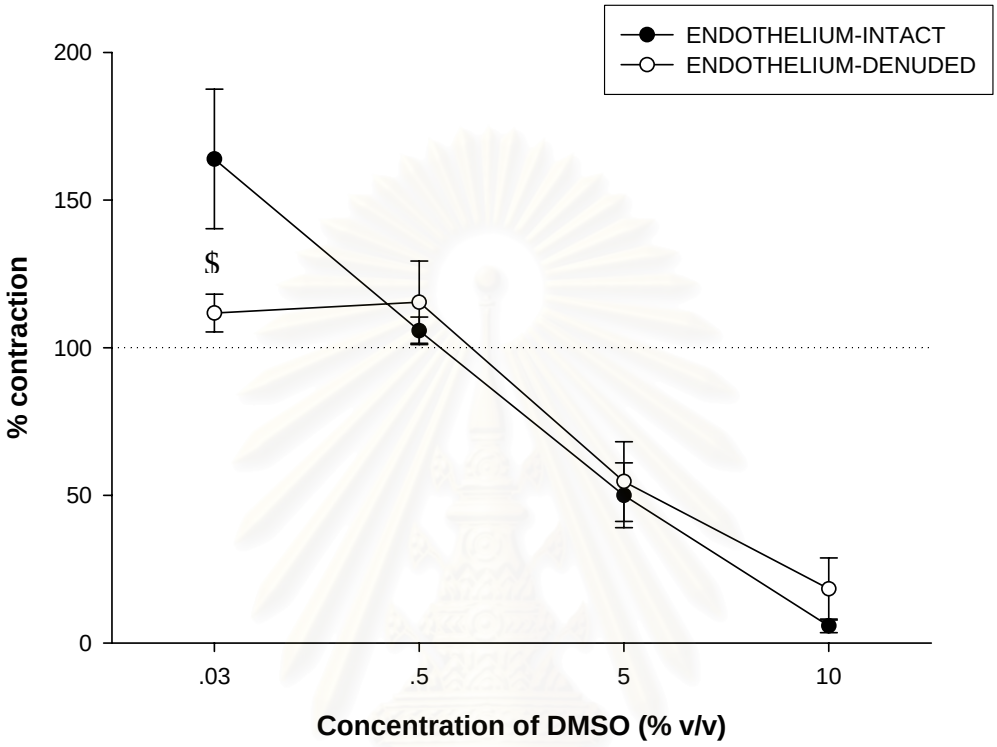

Figure 14c The comparison of effects of DMSO on the contraction induced by 40 mM KCl in Ca^{2+}-containing solution between endothelium-intact and endothelium-denuded aortic strips. All values represent mean ± S.E. mean, N=6 for each concentration of DMSO.

$\$p<0.05$ shows significant difference between endothelium-intact and endothelium-denuded aortic strips (unpaired *t*-test).

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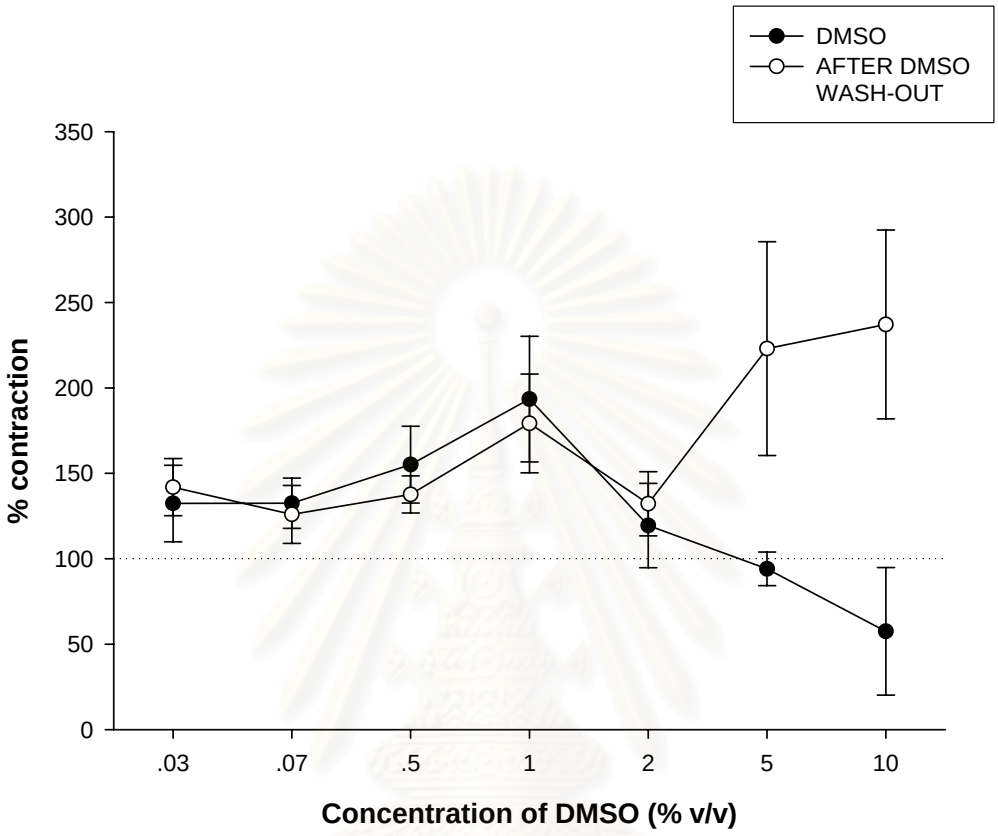

Figure 15 Effects of DMSO on the contraction of endothelium-denuded aortic strips induced by 1 mM TEA in Ca^{2+}-containing solution. All values represent mean ± S.E. mean, N=6 for each concentration of DMSO.

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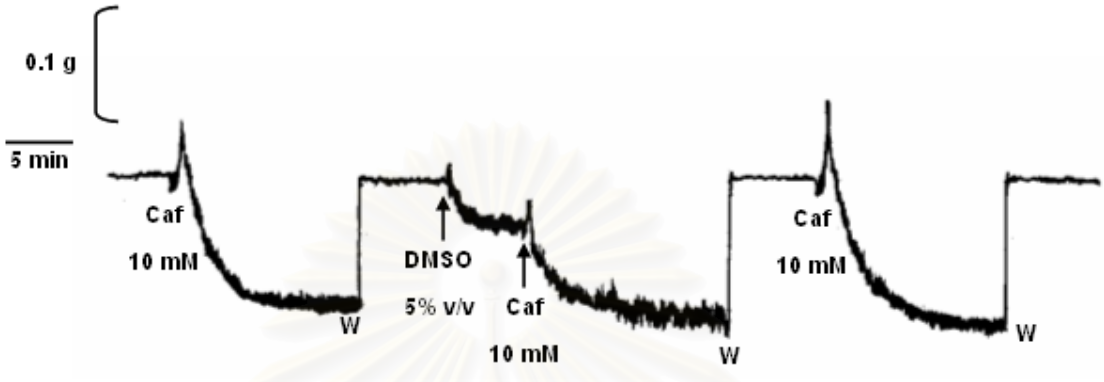
a) endothelium-denuded



b) endothelium-denuded

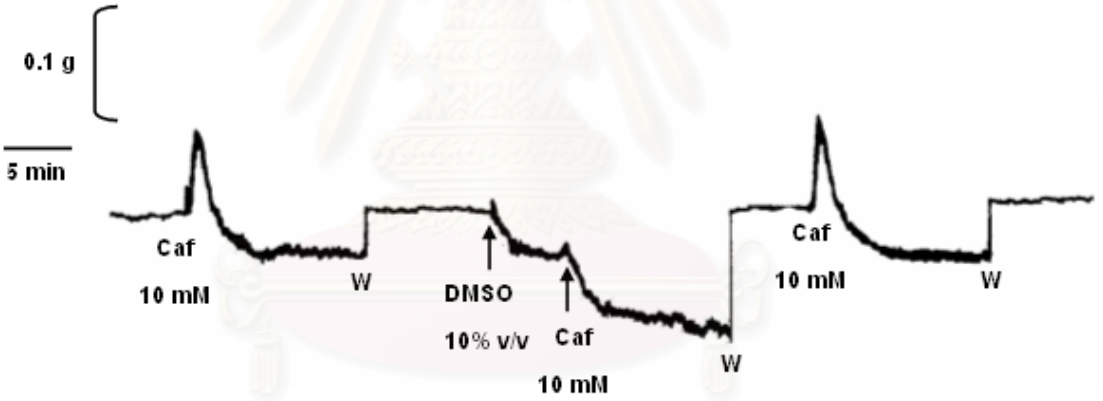


Figure 16 The contraction profiles showing the effect of DMSO on the contraction induced by caffeine of endothelium-denuded aortic strip (a, b) in Ca^{2+}-containing solution.

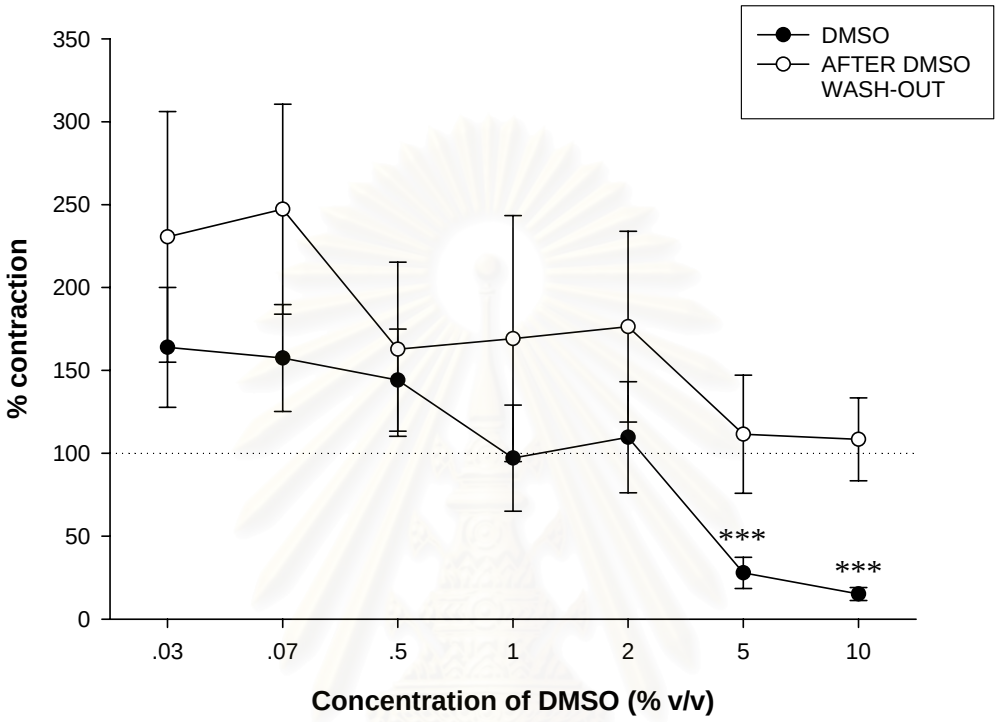


Figure 17 Effects of DMSO on the contraction of endothelium-denuded aortic strips induced by 10 mM caffeine in Ca^{2+}-containing solution. All values represent mean ± S.E. mean, N=6 for each concentration of DMSO.

***p<0.001 shows significant difference when compared to before addition of DMSO (paired t-test).

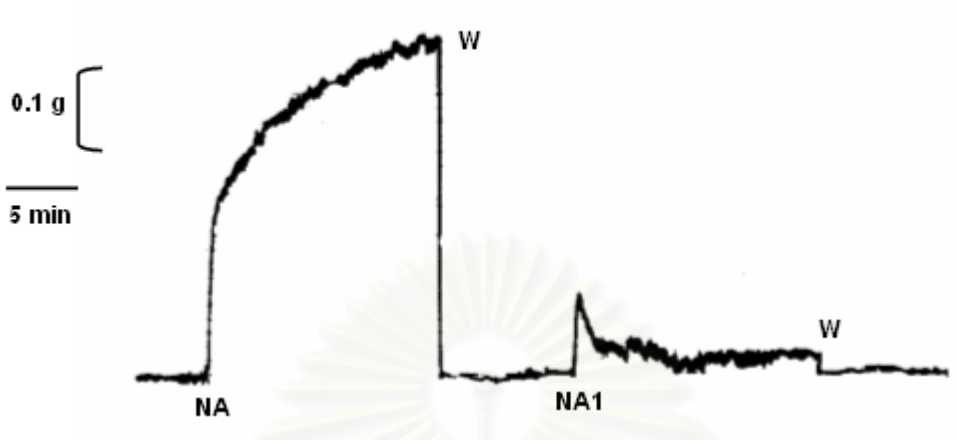


Figure 18 The contraction profile showing the effect of DMSO on the contraction induced by 1 μM noradrenaline of endothelium-denuded aortic strip in Ca^{2+}-free solution (NA1).

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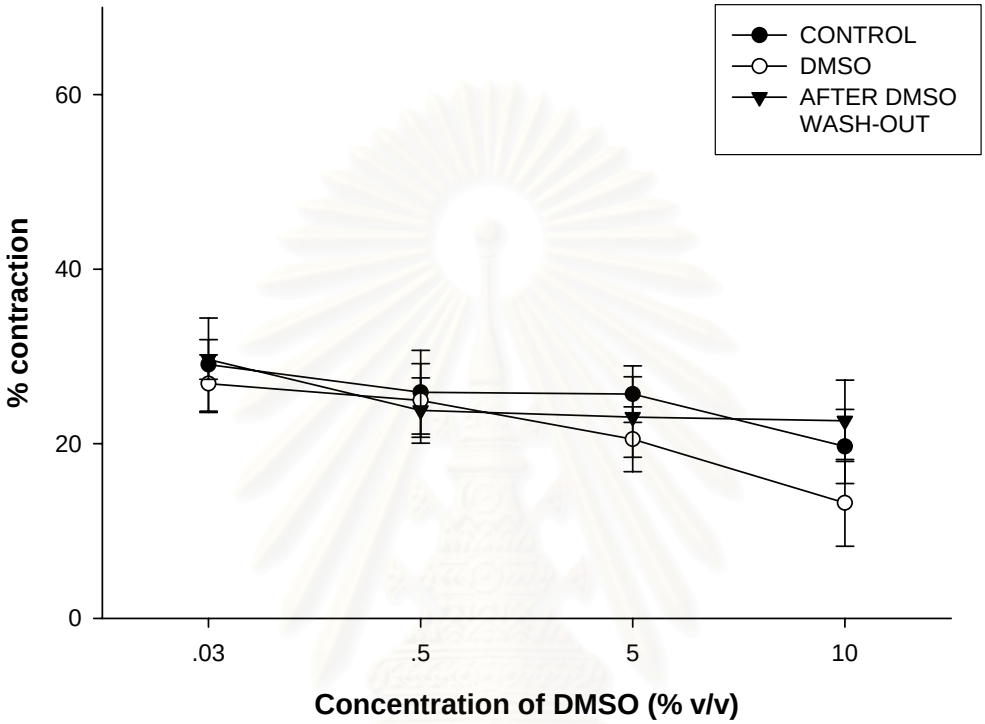

Figure 19 Effects of DMSO on the contraction of endothelium-denuded aortic strips induced by 1 μM noradrenaline in Ca^{2+}-free medium. All values represent mean ± S.E. mean, N=6 for each concentration of DMSO.

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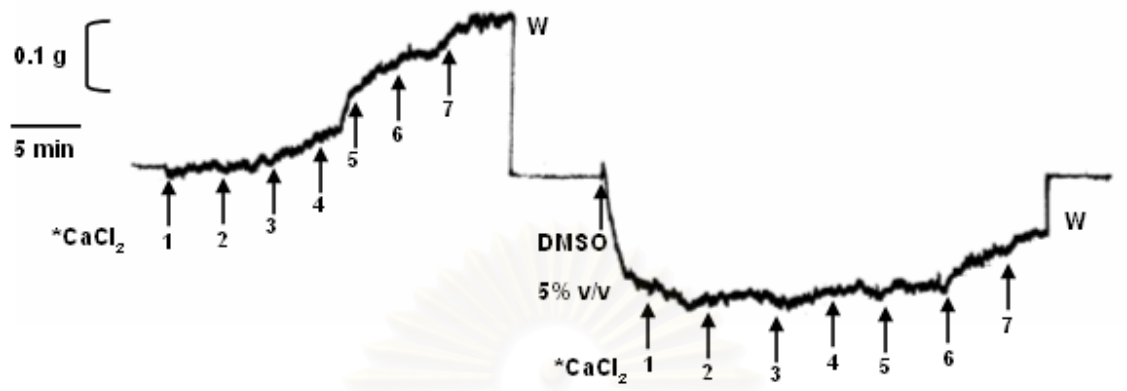
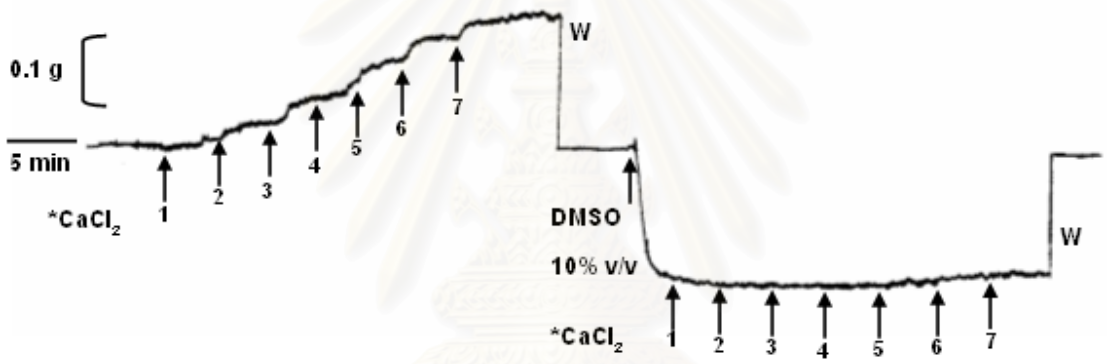
a) DMSO 5% v/v



b) DMSO 10% v/v



c) Nifedipine 1 µM

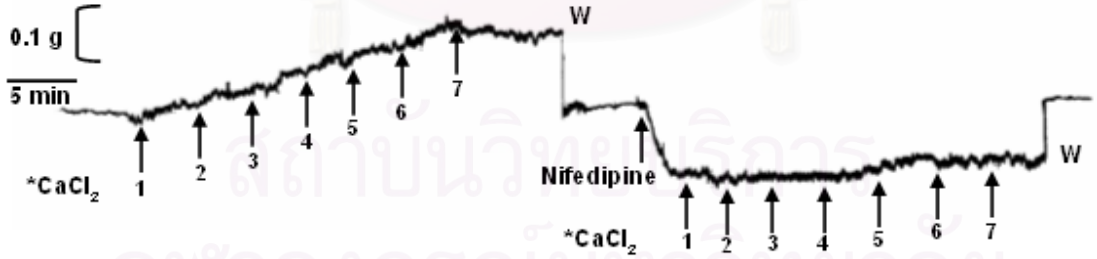


Figure 20 The contraction profiles showing the effect of DMSO (a, b) and nifedipine (c) on the contraction induced by cumulative $CaCl_2$ of endothelium-denuded aortic strip in Ca^{2+}-free depolarizing solution.

* Concentration of $CaCl_2$ (M): 1= 1×10^{-5}, 2= 3×10^{-5}, 3= 1×10^{-4}, 4= 3×10^{-4}, 5= 1×10^{-3}, 6= 3×10^{-3}, 7= 1×10^{-2}

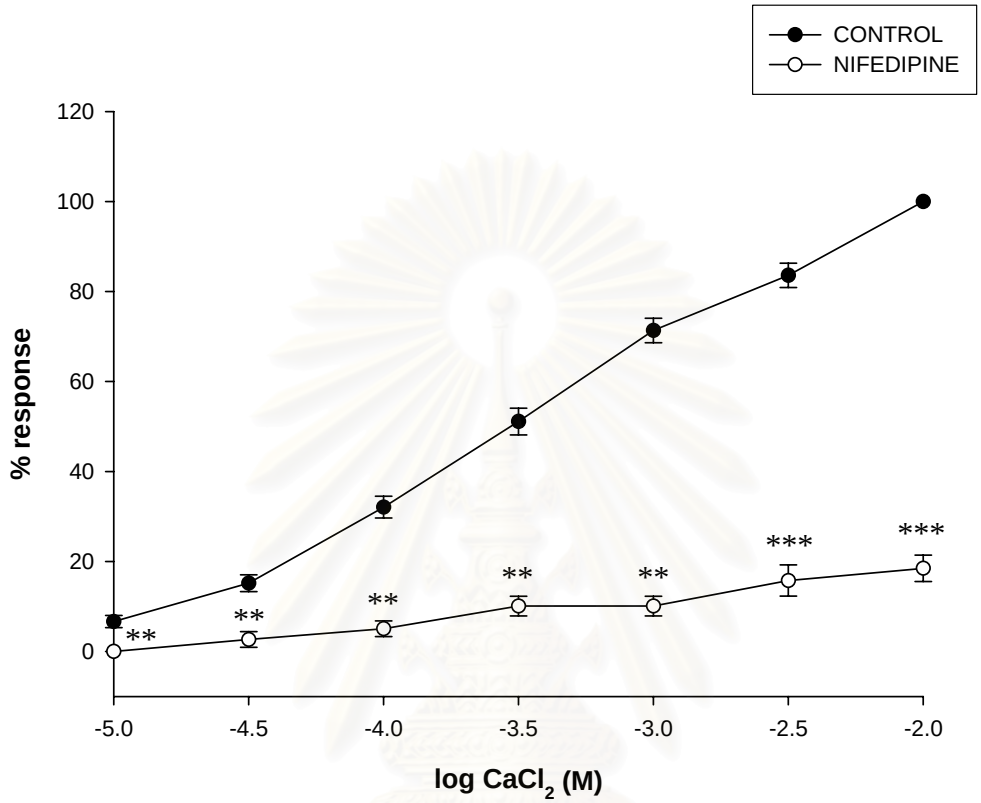


Figure 21 Effects of 1 μM nifedipine on the contraction of endothelium-denuded aortic strips induced by cumulative $CaCl_2$ in Ca^{2+}-free depolarizing solution. All values represent mean ± S.E. mean, N=6 for each concentration of DMSO.

**p<0.01 and ***p<0.001 show significant difference when compared to before addition of DMSO (control) (paired t-test).

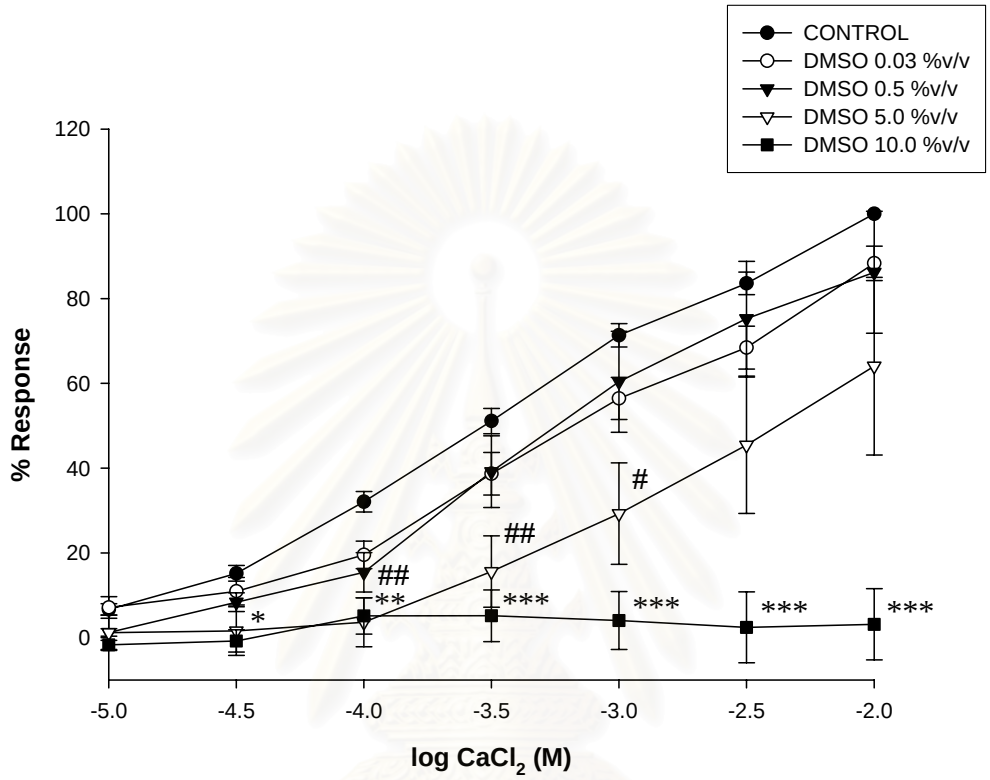


Figure 22 Effects of DMSO on the contraction of endothelium-denuded aortic strips induced by cumulative $CaCl_2$ in Ca^{2+}-free depolarizing solution. All values represent mean ± S.E. mean, N=6 for each concentration of DMSO.

#p<0.05 and ##p<0.01 shows significant difference when compared to before addition of DMSO 5.0% v/v (control) (one-way ANOVA).

*p<0.05, **p<0.01 and ***p<0.001 show significant difference when compared to before addition of DMSO 10.0% v/v (control) (one-way ANOVA).

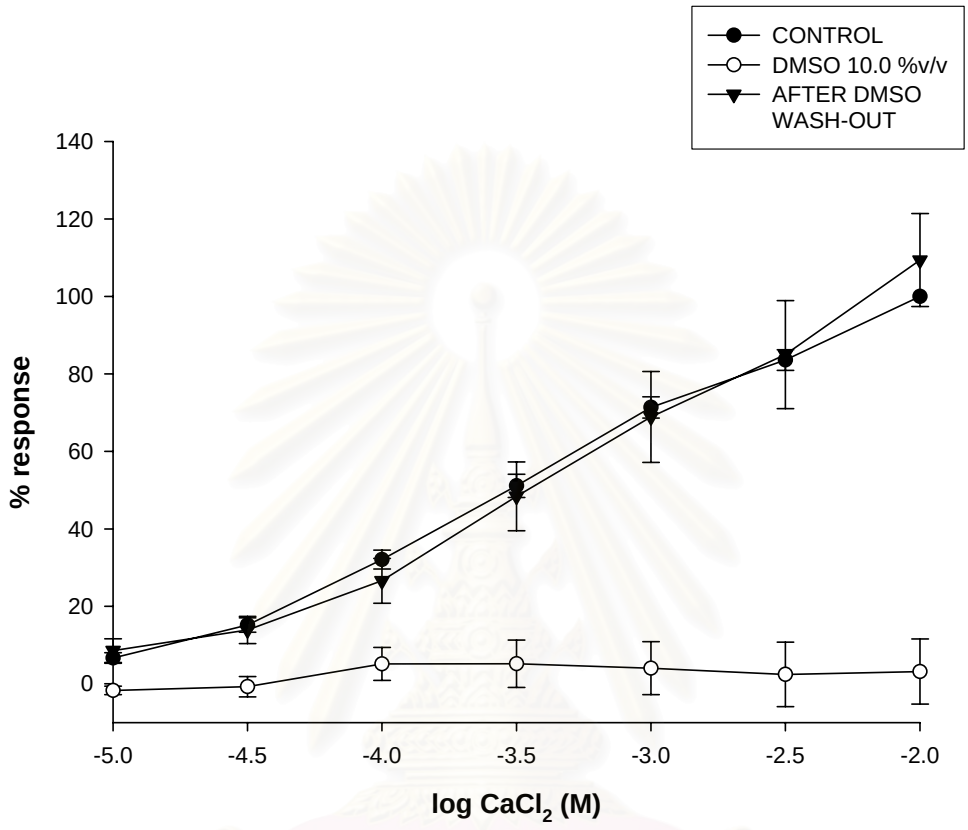


Figure 23 Effects of DMSO on the contraction of endothelium-denuded aortic strips induced by cumulative $CaCl_2$ in Ca^{2+}-free depolarizing solution. All values represent mean ± S.E. mean, N=6 for each concentration of DMSO.

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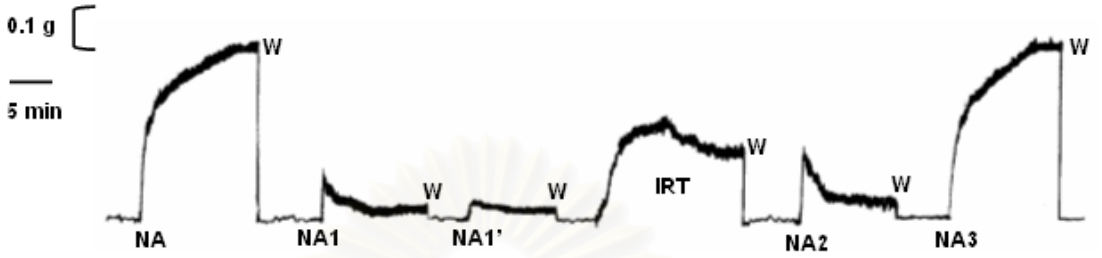

Ca +	Ca -	Ca +	Ca -	Ca +

Figure 24 The contraction profile showing the experimental procedure to induce the increase in the resting tone (IRT)

Noradrenaline 1 μM was added in Ca^{2+}-containing solution (NA) and then the tissue was treated with Ca^{2+}-free medium for 15 min. After this time the agonist was reapplied (NA1, NA1') and washed (W) until no contraction was observed. Upon changing the medium to Ca^{2+}-containing solution, an increase in the resting tone of aorta (IRT) was observed. After washing and 15 min of loading in Ca^{2+}-free medium, a new addition of agonist (NA2) was made. At the end of the experiment, the aortic strip was tested its functionality by adding agonist (NA3) to the tissue suspended in Ca^{2+}-containing solution.

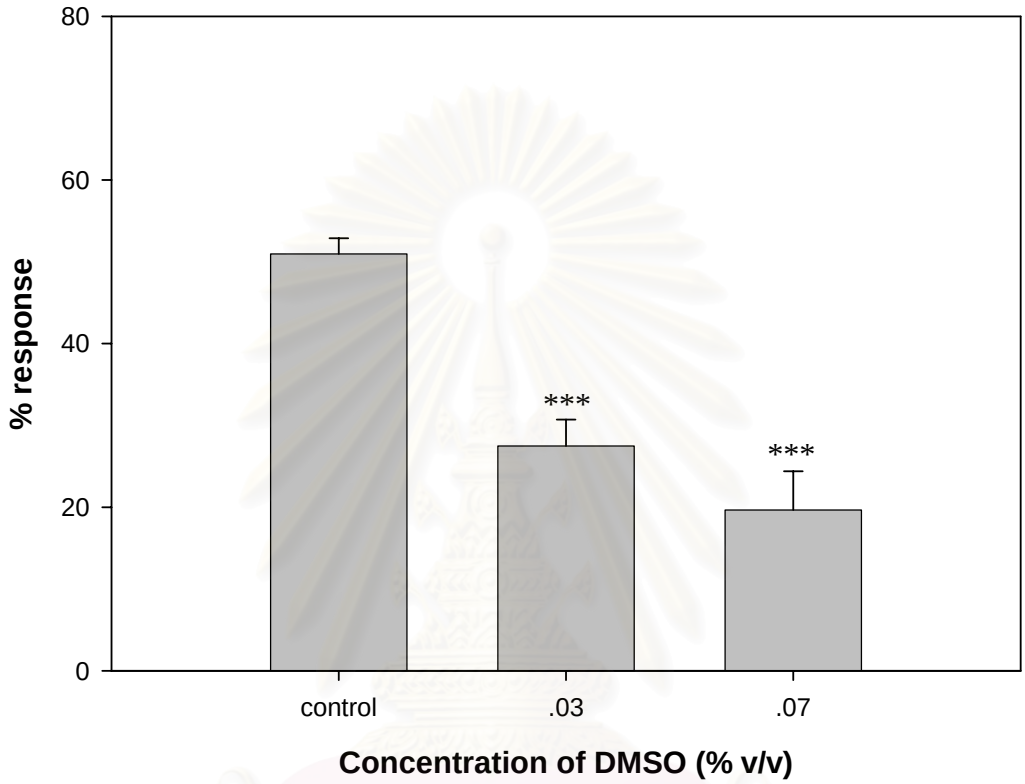

Figure 25a Effects of DMSO on the increase in the resting tone (IRT). All values represent mean ± S.E. mean, N=6 for each concentration of DMSO.

****p* < 0.001 shows significant difference between getting DMSO and before addition of DMSO (control) (paired *t*-test).

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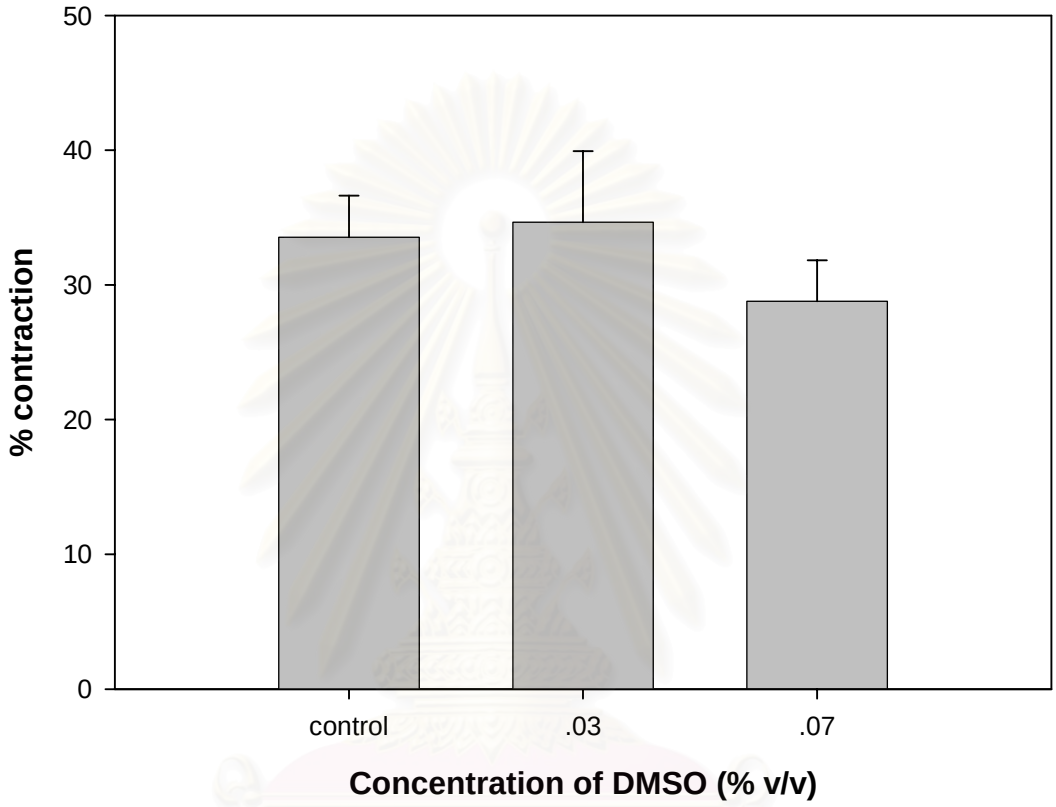

Figure 25b The contractile responses induced by noradrenaline in Ca^{2+}-free medium after occurring IRT (NA2). All values represent mean ± S.E. mean, N=6 for each concentration of DMSO.

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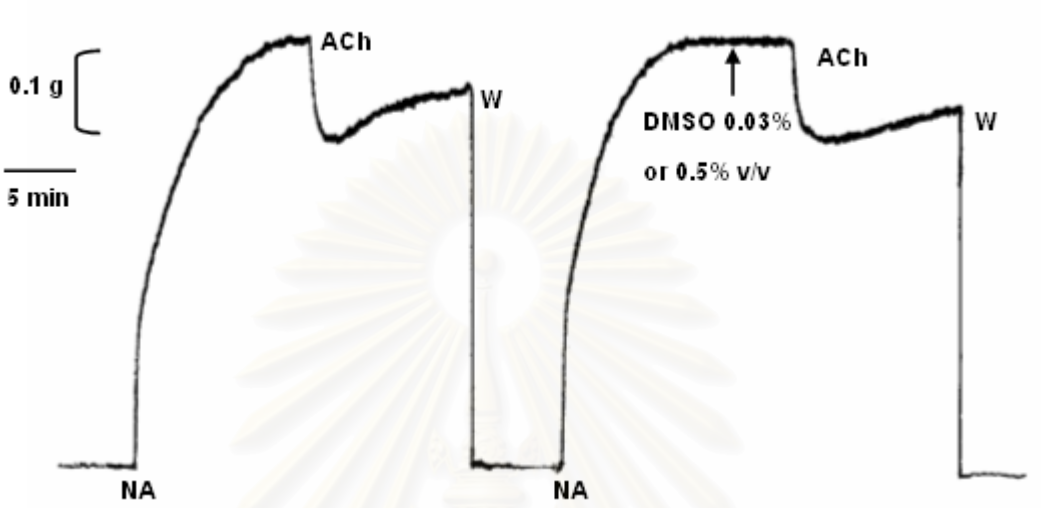

Figure 26 The profile showing the effect of DMSO on endothelium-dependent relaxation produced by 100 μM acetylcholine.

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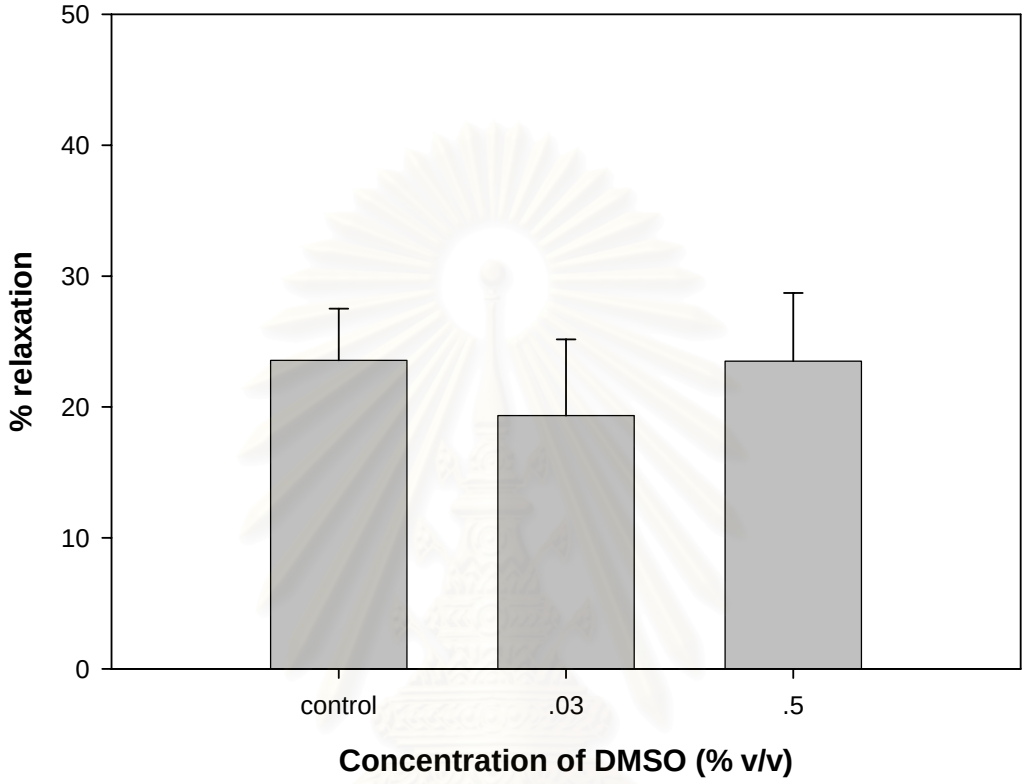

Figure 27 Effects of DMSO on endothelium-dependent relaxation produced by 100 μM acetylcholine. All values represent mean ± S.E. mean, N=6 for each concentration of DMSO.

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a) DMSO 5% v/v

b) DMSO 10% v/v

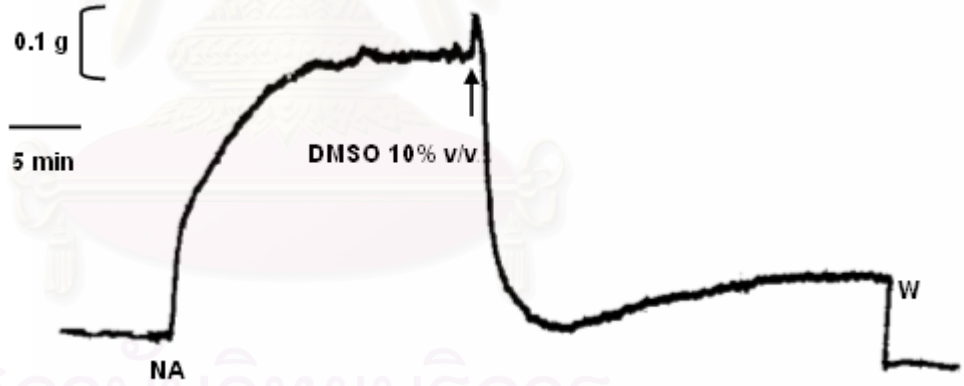

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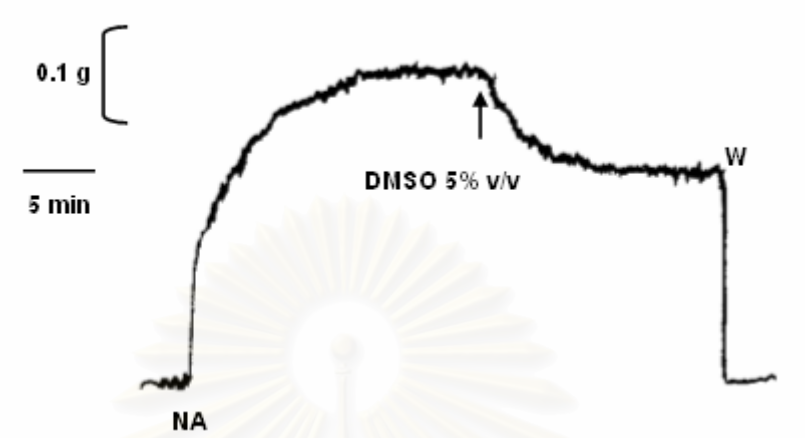
Figure 28a The profile showing the effect of DMSO as the relaxant on the contraction induced by 1 μM noradrenaline of endothelium-intact aortic strip (a, b)

a) DMSO 5% v/v



b) DMSO 10% v/v

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Figure 28b The profile showing the effect of DMSO as the relaxant on the contraction induced by 1 μM noradrenaline of endothelium-denuded aortic strip (a, b)

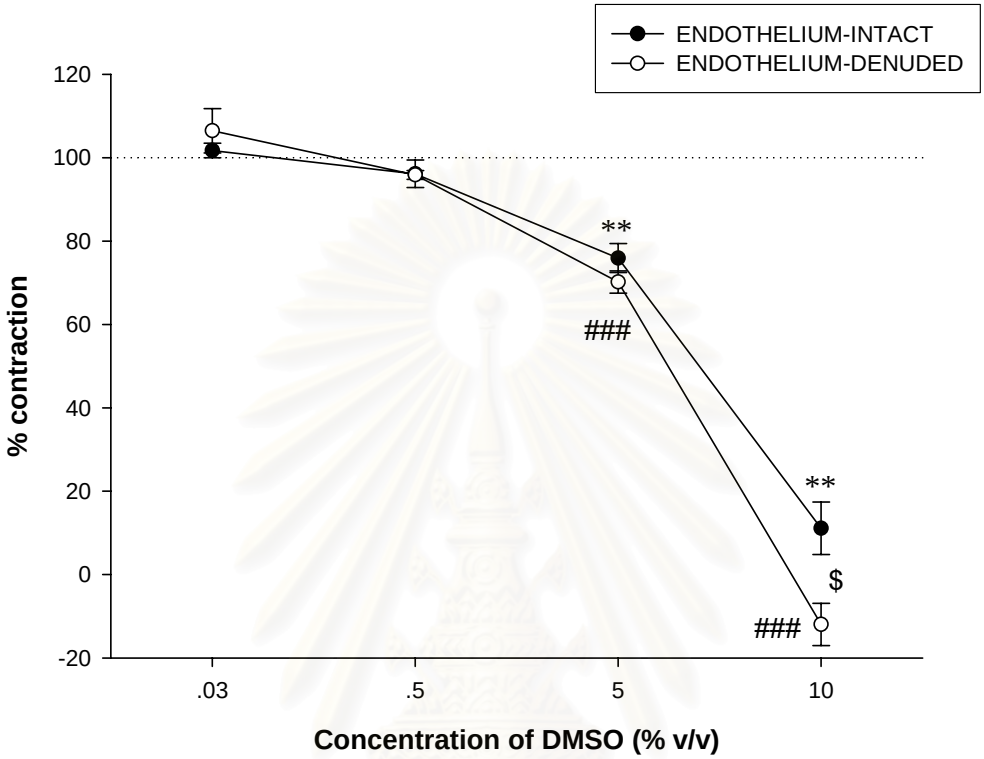


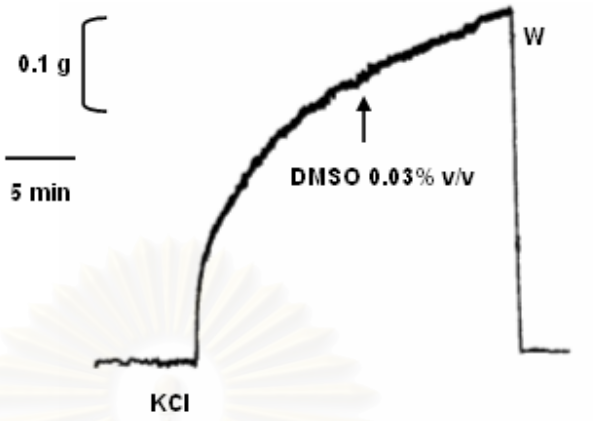
Figure 29 Effects of DMSO as the relaxant to 1 μM noradrenaline-induced contraction of endothelium-intact and endothelium-denuded aortic strips. All values represent mean ± S.E. mean, N=6 for each concentration of DMSO.

**$p<0.01$ shows significant difference of endothelium-intact aortic strips from maximum contraction (paired *t*-test).

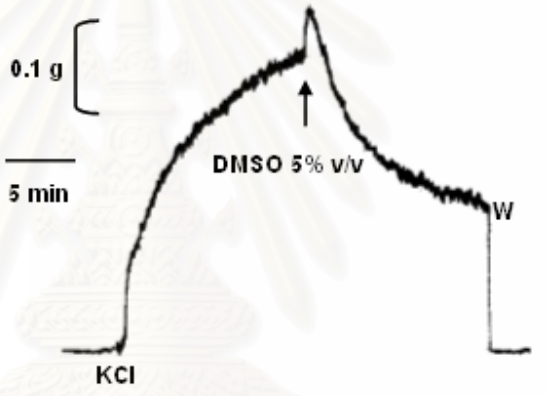
###$p<0.001$ shows significant difference of endothelium-denuded aortic strips from maximum contraction (paired *t*-test).

\$$p<0.05$ shows significant difference of endothelium-intact and endothelium-denuded aortic strips (unpaired *t*-test).

a) DMSO 0.03% v/v



b) DMSO 5% v/v



c) DMSO 10% v/v

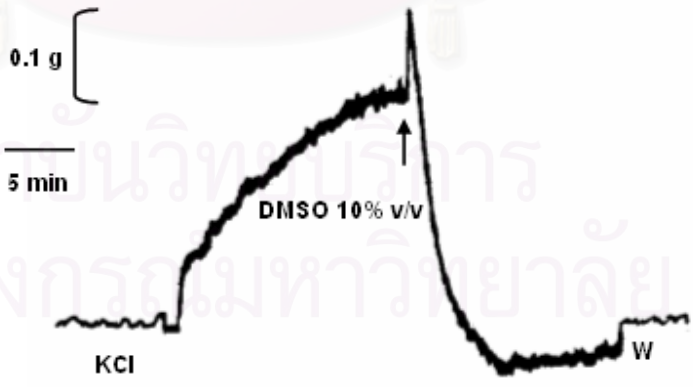
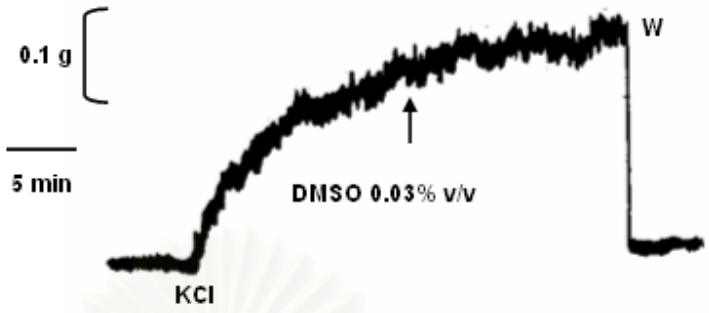
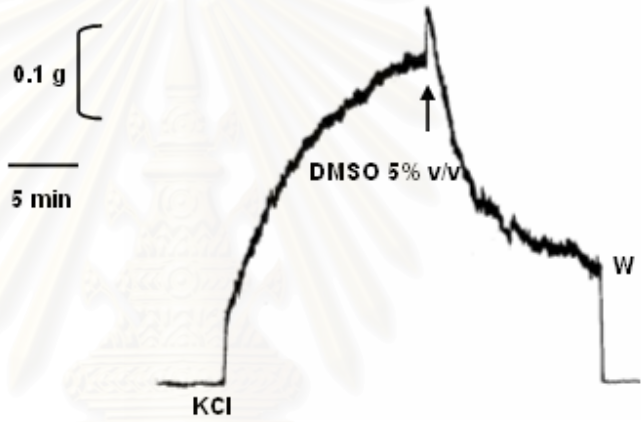


Figure 30a The profile showing the effect of DMSO as the relaxant on the contraction induced by 40 mM KCl of endothelium-intact aortic strip (a, b, c)

a) DMSO 0.03% v/v



b) DMSO 5% v/v



c) DMSO 10% v/v

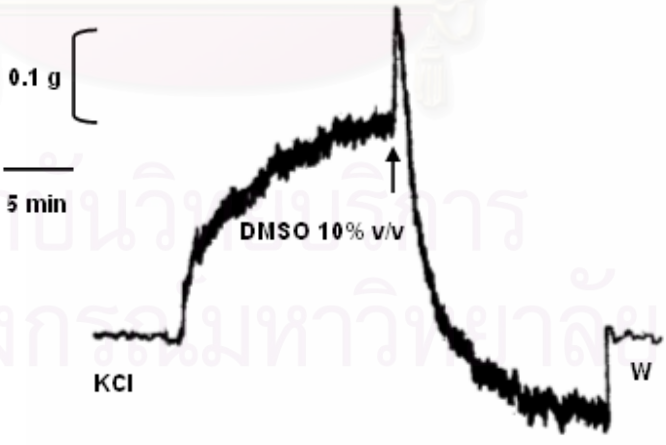


Figure 30b The profile showing the effect of DMSO as the relaxant on the contraction induced by 40 mM KCl of endothelium-denuded aortic strip (a, b, c)

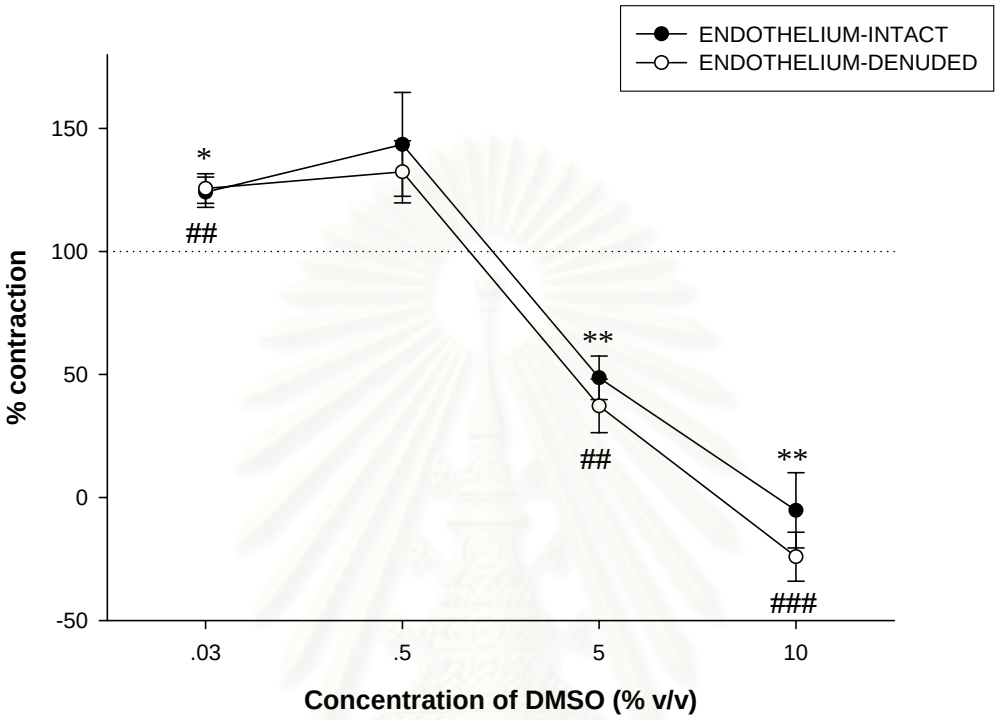


Figure 31 Effects of DMSO as the relaxant to 40 mM KCl-induced contraction of endothelium-intact and endothelium-denuded aortic strips. All values represent mean ± S.E. mean, N=6 for each concentration of DMSO.

*p<0.05 and **p<0.01 show significant difference of endothelium-intact aortic strips from maximum contraction (paired t-test).

##p<0.01 and ###p<0.001 show significant difference of endothelium-denuded aortic strips from maximum contraction (paired t-test).

CHAPTER V

DISCUSSION AND CONCLUSION

This study aimed to investigate the effect of DMSO on the functionality of aortic smooth muscle. The present studies demonstrated that removal of endothelium significantly decreased the contractile responses induced only by 1 μM noradrenaline, but not by 40 mM KCl. As known, vascular endothelium plays an important role in the regulation of arterial tone by producing both endothelium-derived relaxing factor (EDRF) and constricting factor (EDCF) (Wingard, *et al.* 1991). Our finding suggested that α-agonist may induce endothelium to release some endothelial vasocontricting factors, resulting in an increase in the contractile response of endothelium-intact aortic strip.

In order to elucidate the action of DMSO on vascular function, the studies applied various contractants to evoke the contraction. Each contractant triggered the contractile response by different mechanism as following:-

Noradrenaline activates the intracellular Ca^{2+} release following an increase in the turnover of phosphatidylinositol and the production of inositol 1,4,5-trisphosphate. In the continuing presence of noradrenaline, contraction is maintained and this phase is associated with Ca^{2+} influx via a specific pathway (Noguera and D'Ocon, 1993). In case of K+-induced contraction, the mechanism involves membrane depolarizing, which initiate conformational changes that open L-type Ca^{2+} channels, results in an increase of Ca^{2+} influx and intracellular Ca^{2+} (Karaki, et al., 1997; Katz, 1997). Tetraethylammonium (TEA), the blocker of K^{+} channel, leads to membrane depolarization and vasoconstriction.

Caffeine exerts the distinct contraction from that elicited by noradrenaline, KCl and TEA. Caffeine induces a transient smaller contraction which is due to the release of Ca^{2+} from sarcoplasmic reticulum (Watanabe, *et al.*, 1988). Furthermore, the contraction profiles show that the tension dropped to a level below a resting tone, which may be due to an inhibitory effect of caffeine on cyclic AMP phosphodiesterase. Consequently, an increase of cyclic AMP in smooth muscle occurs, following muscle relaxation (Ahn, *et al.*, 1988; Fredholm, *et al.*, 1979; Sato, *et al.*, 1988).

DMSO relaxed the vascular resting tone in the concentration-dependent manner. At high concentration (5% and 10% v/v), DMSO caused an initial transient contraction, prior to the basal relaxation, similarly to contractile profile of caffeine. The transient contraction induced by DMSO may be due to the release of Ca^{2+} from intracellular calcium stores.

Our findings showed that low concentrations (0.017–2% v/v) of DMSO did not have the inhibitory effect on the contraction induced by noradrenaline, KCl, TEA and caffeine in Ca^{2+}-containing solution. At high concentrations (5% and 10% v/v), DMSO inhibited the contraction of endothelium-denuded and –intact aortic strip induced by noradrenaline, KCl and caffeine in Ca^{2+}-containing physiological solution.

Furthermore, to find the effect of DMSO on Ca^{2+} influx, the method proposed by Hof and Vuorela (1983) was used. This method was to specifically induce Ca^{2+} entry from the extracellular to intracellular compartment by depolarizing membrane in the absence of $CaCl_2$. Similary to the contraction induced by various contractants, DMSO at the concentration of 10% v/v markedly suppressed the contraction and DMSO at 5% v/v partially inhibited the contraction. DMSO at 0.03% and 0.5% v/v had no effects on aortic contraction under the same condition. DMSO at low concentrations did not affect the Ca^{2+} entry induced by cumulative $CaCl_2$. This result was consistent to previous study that DMSO had little effect on L-type Ca^{2+} current in whole-cell-configured guinea-pig ventricular myocytes (Ogula, *et al.*, 1995).

High concentration of DMSO suppressed the contraction induced by various contractants including noradrenaline, KCl, TEA, caffeine and cumulative $CaCl_2$. It is likely that the mechanism of DMSO toxicity may be nonspecific and may target at Ca^{2+} influx or the development of contraction subsequent to the Ca^{2+} entry. However, the functionality testing in these studies could not distinguish the mode of action of DMSO clearly.

Interestingly, DMSO at low concentration (0.03% and 0.07% v/v), which had no effects on the contraction induced by various contractants (noradrenaline, KCl, TEA, and caffeine), inhibited the increase in the resting tone (IRT) significantly. The IRT occurs when the depleted intracellular Ca^{2+}-stores sensitive to noradrenaline is refilled by incubating in Ca^{2+}-containing physiological solution. The influx of extracellular Ca^{2+} through voltage-operated Ca^{2+} channels was able to activate the vascular contraction in the absence of any contractant. Moreover, the Ca^{2+} which entered to the vascular smooth muscle cell in the IRT development process replenished the intracellular Ca^{2+}

store, as evidenced by the capability of the muscle to contract upon addition of noradrenaline in the absence of extracellular Ca^{2+} (Noguera, *et al*., 1998). In this study, the contraction induced by noradrenaline in Ca^{2+}-free medium (NA2) after IRT was obtained. Hence, the replenishment of the intracellular Ca^{2+} stores was not disrupted, even the IRT response was significantly inhibited by low concentration of DMSO. This finding suggested that DMSO did not affect Ca^{2+} influx that resulted in the refilling of the intracellular Ca^{2+} stores-sensitive to noradrenaline. Hence, the inhibitory effect of DMSO on the IRT may be due to the interference of DMSO on the cascade events subsequent to Ca^{2+} entry.

Removal of endothelium did not have an influence on the effect of DMSO on the contraction evoked by noradrenaline and KCl. On the contrary, the endothelium appears to protect the inhibitory effect of 5% v/v of DMSO on noradrenaline-induced contraction. However, this protective effect was not extended to the aortic strip evoked the contraction by KCl.

The contractile responses to contractants could return to normal after removing DMSO by several washes with physiological solution. Hence, DMSO did not permanently impair the ability of vascular smooth muscle to contract upon 30 minutes of exposure. Moreover, after removal of DMSO the contraction induced by noradrenaline, KCl, TEA and caffeine increased from control. These phenomenons may be due to Ca^{2+} overload during DMSO treatment. From the study of Ogura, *et al.* (1996), the treatment of DMSO at 10% v/v produced a prolonged action potential in guinea-pig papillary muscle, and the wash-out triggered a series of events, e.g. depolarization, shortening of the action potential, and strong positive inotropy. Our results showed that the washout of DMSO amplified the contraction induced by various contractants in every concentration of DMSO. The endothelium could protect the washout effect of DMSO on both the contraction elicited by noradrenaline and KCl. Furthermore, it is possible that DMSO facilitated the Ca^{2+} influx through the mechanism of KCl and TEA induced membrane depolarization in the greater extent than the Ca^{2+} entry from stimulation by noradrenaline. Caffeine-induced contraction was also amplified after DMSO washout. The degree of reversibility depended on the level of inhibition of DMSO.

For the relaxation effect of DMSO, high concentration (5% and 10% v/v) of DMSO could relax the contraction evoked by noradrenaline or KCl of both endothelium-intact and –denuded aortic strips. Removal of endothelium did not influence on the effect of DMSO. In addition, DMSO did not have the effect on the relaxation induced by acetylcholine. Thus, a possible involvement of endothelium-derived relaxing factor (EDRF) upon DMSO treatment was ruled out. DMSO at these concentrations could also relax the basal tone of the aortic strips. In agreement with Lawrence, *et al.* (1998), these results showed that DMSO had the relaxation effect and was independent on the presence of endothelium. A previous study (Ogura, *et al*, 1996) suggested that DMSO had the relaxation effect to caffeine-induced contractures due to its effect on the myofilament force-generating machinery. Thus, it was likely that DMSO relaxed the vascular smooth muscle via the interference on the relationship of Ca^{2+} and contractile elements.

In conclusion, DMSO had the complex effect on the vascular smooth muscle contraction. Low concentrations of DMSO did not have the inhibitory effect on the contraction induced by various contractants, whereas DMSO at high concentrations inhibited the contraction under the same condition. However, DMSO at low concentration inhibited the IRT through the mechanism unrelated to the process of Ca^{2+} entry. High concentrations of DMSO had the vasorelaxation effect on the vascular tone. Furthermore, the effect of DMSO was reversible after washout, and did not depend on the endothelium.

Significance

The use of DMSO as the solvent system to dissolve the hydrophobic drugs in research and medicine, especially in vascular research, require adequate controls to take into account of the solvent effect.

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APPENDIX

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Table 2 Compound of Physiological solutions (mM/L) (Aboud, *et al.*, 1993.)

Chemicals	Physiological solution		
	Krebs Henseleit	Ca^{2+}-free Krebs Henseleit	Potassium Depolarizing
NaCl	119.0	119.0	27.0
KCl	4.7	4.7	100.0
$CaCl_2$	2.5	-	-
$MgSO_4$	1.0	1.0	-
KH_2PO_4	1.2	1.2	14.0
D-glucose	11.1	11.1	10
$NaHCO_3$	25.0	25.0	25.0
EDTA	-	0.1	-
$MgCl_2$	-	-	0.54

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