Hydroperoxide-induced Oxidative Stress in the Arterial Wall: Pharmacological Characterization of the Effects on Arterial Contractility

Hydroperoxide-induzierter oxidativer Stress in der Arterienwand: Pharmakologische Charakterisierung der Effekte auf die arterielle Kontraktilität

DISSERTATION

der Fakultät für Chemie und Pharmazie
der Eberhard-Karls-Universität Tübingen
zur Erlangung des Grades eines Doktors
der Naturwissenschaften

2007

vorgelegt von

DAYONG ZHANG

Tag der mündlichen Prüfung:	21. November 2007
Dekan:	Professor Dr.L.Wesemann
1. Berichterstatter:	Professor Dr.H.Heinle
2. Berichterstatter:	Professor Dr.G.Drews

Publications

D. Zhang, G. Drews, D. Weiser, D. Hagelauer, H. Heinle (2006): Pharmacological characterization of contraction induced by oxidative stress in mice aorta. Journal of laboratory medicine, Sept. /Oct. 2006.

D. Zhang, D. Hagelauer, G. Drews, D. Weiser, H. Heinle (2006): Mechanisms of peroxide-induced contractions in mice aorta. In: Arteriosklerose: Neue Konzepte, Risikofaktoren und Targets. DGAF, Tübingen, 2006, 176-179.

D. Zhang, D. Hagelauer, H. Heinle (2006):

Peroxide-induced effects on endothelium in mouse aorta. Acta Physiologica 2006; 186, Suppl 1, No. PM07P-18 (Abstract) Poster auf dem 85. Kongress der Phsiologishen Gesellschaft, München, March 2006.

D. Hagelauer, D. Zhang, O. Kelber, D. Weiser, H. Heinle (2006):

Neurotransmitter-induced effects on motility of ileum from mouse and guinea pig: inhibition by plant extracts. Acta Physiologica 2006; 186, Suppl 1, No. PW03P-4 (Abstract) Poster auf dem 85. Kongress der Phsiologishen Gesellschaft, München, March 2006.

D. Zhang, H. Heinle (2005):

Peroxide-induced effects on depolarization-dependent contraction in mice aorta. In: Stoffwechsel und Modifikation von Lipiden Lipoproteinen. DGAF, Tübingen, 2005, 257-261.

Abbreviations

AA	arachidonic acid
AAPH	2'2 Azobis (2- amidinopropane) hydrochloride
ACE	angiotensin-converting enzyme
ACh	acetylcholine
AngI	angiotensin I
AngII	angiotensin II
AP-1	activator protein-1
4-AP	4-aminopyridine
AT1R	AT1 receptor
BH_4	tetrahydrobiopterin
tert.-BHP	*tert*.-butylhydroperoxide
BK	bradykinin
BSA	albumin, bovine
Ca^{2+}-CaM-MLCK	Ca^{2+}-calmodulin-myosin light chain kinase
CaM	calmodulin
CL	chemiluminescence
CNP	C-natriuretic peptide
COX	cyclooxygenase
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
EDHF	endothelium-derived hyperpolarising factor

EDTA	ethylenediamine tetraacetic acid
ET-1	endothelin 1
$ET_{A/(B)}$	endothelin A (and B) receptors
G_q	G-protein
GPx	glutathione peroxidase
GPx-$1^{-/-}$	glutathione peroxidase-1 gene knock out mice
GSH	reduced glutathione
GSSG	oxidized glutathione
GSSG-R	glutathione reductase
H_2O_2	hydrogen peroxide
5-HT	5-hydroxytryptamine
IL-1	interleukin 1
IP	I prostanoid receptor
IP_3	inositol 1,4,5-trisphosphate
K_{IR}	inward rectifying potassium channel
K_v channels	voltage-activated K channels
MAPK	mitogen-activated protein kinases
MLC	myosin light chains
MLC_{20}	20-kDa myosin light chains
MLC_{20}-P	phosphorylation of 20-kDa myosin light chains
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
Na^+/K^+ ATPase	electrogenic pump

VI Abbreviations

L-NAME	Nω-nitro-L-arginine methyl ester
ß-NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced
NCDC	2-nitro-4-carboxyphenyl, N;N-diphenylcarbamate
NOS	nitric oxide synthase
eNOS	endothelial NO synthase
NPR	natriuretic peptide receptor
PG	prostaglandin
PGH_2	prostaglandin H_2
PGI_2	prostacycline
PHE	phenylephrine
PIP_3	phosphatidylinositol 3,4,5-trisphosphate
PKC	protein kinase C
PLA_2	phospholipases A_2
PLC	phospholipase C
PTK	protein tyrosine kinase
RAS	renin-angiotensin system
RLU	relative light units
ROCK	Rho kinase
ROS	reactive oxygen species
SMC	smooth muscle cell
SOD	superoxide dismutase
SOCC	store-operated Ca^{2+} channels

SR	sarcoplasmic reticulum
TP	T prostanoid receptor
TXA_2	thromboxane A_2
VSMC	vascular smooth muscle cell
VOCC	voltage-operated calcium channels
WT	wild type mice
ZIP-K	zipper-interacting protein kinase

1 Introduction

Reactive oxygen species (ROS) are ubiquitous reactive derivatives of oxygen metabolism found in all aerobic biological system. ROS include free radical and non-radical species that oxidize molecular targets to change important cellular functions. Most important ROS include superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl anion ($\cdot OH$) and reactive nitrogen species [e.g. nitric oxide (NO), peroxynitrite ($ONOO^-$)]. All vascular cell types (endothelial cells, smooth muscle cells, and adventitial fibroblast) produce ROS. Physiologically, ROS are produced at low concentrations as signalling molecules, which do not disturb vascular integrity and are involved in regulating endothelial function and vascular contraction-relaxation. Under pathological conditions, increased ROS may lead to cardiovascular dysfunction as observed e.g. in hypertension, diabetes mellitus, atherosclerosis, ischemia–reperfusion injury, ischemic heart disease, and congestive cardiac failure (Landmesser & Harrison, 2001). In order to introduce the mediations of ROS in the vascular cells, in the next chapter 1) functional anatomy of arteries and 2) mechanisms of oxidative stress will be shortly described.

1.1 Regulation of Arterial Contraction

1.1.1 Structure of Arteries and Functional Interactions

All blood vessels have the same basic structure. The tunica intima is the inner layer of arteries and veins. In arteries this layer is composed of an elastic membrane lining and smooth endothelium. The tunica media is the middle layer of the walls of arteries. It is composed of smooth muscle and elastic fibers. The main function of vascular smooth muscle is to regulate the tone of the blood vessels in the body. The tunica adventitia is the strong outer covering. It is composed of connective tissue as well as collagen and elastic fibers. These fibers allow the

arteries to stretch to prevent overexpansion due to the pressure that is exerted on the walls by blood flow.

Endothelial cells as the luminal surface of the blood vessel have important antithrombosis protections. Furthermore, endothelial cells synthesize and release various factors that modulate in short terms vascular tone, in long terms atherosclerosis, angiogenesis and inflammation. The vasoactive factors include relaxing substances [prostacyclin (PGI_2), nitric oxide (NO), C-natriuretic peptide (CNP),] and contracting substances [e.g. thromboxane A_2 (TXA_2), endothelin-1, angiotensin II (AngII), superoxide anion ($\cdot O_2^-$)]. H_2O_2 generated by endothelium is recently found not only to be a vasoconstrictor but also to be a relaxing factor. Endothelial cells also communicate directly with smooth muscle cells via myoendothelial gap junctions. This communication allows not only the spread of electrotonic tone [such as endothelium-dependent hyperpolarizations, i.e., endothelium-derived hyperpolarizing factor (EDHF)-mediated responses] but also the transfer of ions or small molecules such as calcium and cyclic nucleotides (Feletou & Vanhoutte, 2006). **Fig 1** shows the effects of endothelium-derived mediators on the arterial smooth muscle (Rang *et al.*, 2003).

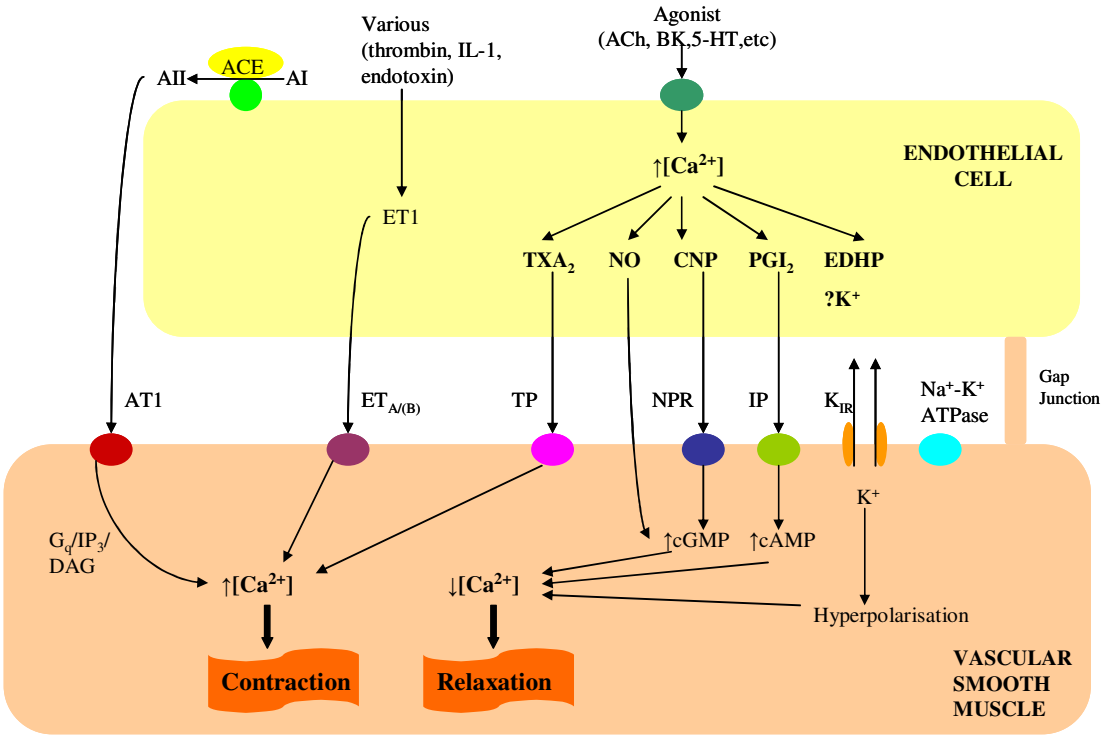


Fig 1 Endothelium-derived mediators. The picture shows some of the more important endothelium-derived contracting and relaxing mediators. (**AI, II**, angiotensin I, II; **ACE**, angiotensin-converting enzyme; **AT1**, angiotensin AT1-receptor; **TP**, T prostanoid receptor; **$ET_{A/(B)}$**, endothelin A (and B) receptors; **NPR**, natriuretic peptide receptor; **IP**, I prostanoid receptor; **K_{IR}**, inward rectifying potassium channel; **Na^+/K^+ ATPase**, electrogenic pump; **G_q**, G-protein; **IP_3**, inositol 1,4,5-trisphosphate; **DAG**, diacylglycerol; **IL**-1, interleukin 1; **ACh**, acetylcholine; **BK**, bradykinin; **5-HT**, 5-hydroxytryptamine; **EDHP**, endothelium-derived hyperpolarising factor; **PG**, prostaglandin; **CNP**, C-natriuretic peptide; **ET-1**, endothelin 1. According to (Rang *et al.*, 2003).

Additionally, the vascular tone in active tissues is regulated not only by locally produced vasodilator metabolites (e.g. decreases in O_2 and pH, increases in CO_2) but also by circulating substances (e.g. bradykinin and the atrial natriuretic peptide) and the sympathetic nervous system that innervates the arteries.

1.1.2 Contraction Mechanisms of Smooth Muscle

Smooth muscle contraction is caused by the sliding of myosin and actin filaments over each other. There are two pathways to induce smooth muscle contraction,

including Ca^{2+}-dependent pathways and Ca^{2+} sensitization pathways. The Ca^{2+}-dependent pathways lead to increase intracellular Ca^{2+} through voltage-gated Ca^{2+} channels as well as Ca^{2+} release from the sarcoplasmic reticulum (Horowitz *et al.*, 1996;Somlyo & Somlyo, 2003). Smooth muscle cells also store Ca^{2+} in the sarcoplasmic reticulum (SR), from which Ca^{2+} can be released when the IP_3 receptors are activated. The IP_3 is generated by activation of many types of G-protein-coupled receptors. Thus, Ca^{2+} release and contraction can occur in smooth muscle without the involvement of electrical events and Ca^{2+} entry through the plasma membrane. Ca^{2+} binds to calmodulin (CaM), which activates myosin light chain kinase (MLCK). Activated MLCK leads to phosphorylation of 20-kDa myosin light chains (MLC_{20}). MLC_{20}-P binds to actin and contraction is produced by crossbridge-cycling. MLC_{20}-P can be dephosphorylated by myosin light chain phosphatase (MLCP), leading to relaxation.

Ca^{2+} sensitization pathways are defined as increases in generated force without increases in intracellular Ca^{2+} (Somlyo & Somlyo, 2003). These pathways lead to an increase in MLC_{20}-P through direct phosphorylation or inhibition of MLCP. There are some cell signalling pathways to regulate these mechanisms: Rho kinase (ROCK) pathway, protein kinase C (PKC), and zip kinase pathway (**Fig 2**).

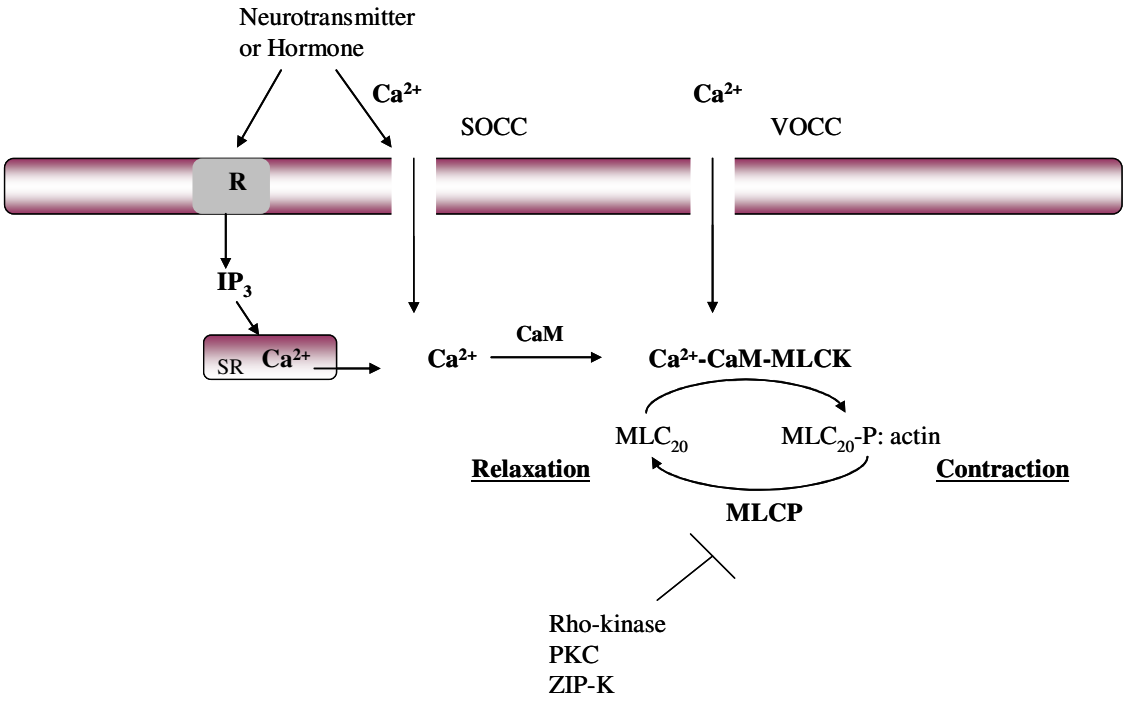


Fig 2 Signal transduction mechanisms of smooth muscle contraction. (**R**, receptors; **VOCC**, voltage-operated calcium channels; **SOCC**, store-operated Ca^{2+} channels; **SR**, sarcoplasmic reticulum; **MLC_{20}**, myosin light chain 20; **Ca^{2+}-CaM-MLCK**, Ca^{2+}-calmodulin-myosin light chain kinase; **MLCP**, myosin light chain phosphatase; **ZIP-K**, zipper-interacting protein kinase. According to (Ardanaz & Pagano, 2006).

1.2 Reactive Oxygen Species in the Vasculature

1.2.1 ROS Generation in Vessels

1.2.1.1 NAD(P)H Oxidase

The major source of ROS in all cells of the blood vessels is a superoxide-producing NAD(P)H oxidase. NAD(P)H oxidase is a multi-subunit enzyme, which utilizes NADH/NADPH as the electron donor to reduce molecular oxygen and produce $\cdot O_2^-$: $2O_2 + NAD(P)H \rightarrow 2\cdot O_2^- + NADP^+ + H^+$.

Vascular NAD(P)H oxidase is regulated by humoral (cytokines, growth factors, and vasoactive agents) and physical factors (stretch, pulsatile strain, and shear

stress). NAD(P)H oxidase generates superoxide in response of intracellular Ca^{2+} elevations since Ca^{2+} binds to the subunit of NAD(P)H oxidase. Physiologically, NAD(P)H oxidase produces $\cdot O_2^-$ intracellularly in a slow and sustained fashion and $\cdot O_2^-$ acts as intracellular signalling molecules (Lassegue & Clempus, 2003).

1.2.1.2 NOS

Nitric oxide synthase (NOS) normally generates NO, but can cause "uncoupling" in the context of deficiency of the essential NOS cofactor BH_4 (tetrahydrobiopterin). NOS uncoupling leads to decrease in NO production and increase in NOS-dependent $\cdot O_2^-$ formation (Milstien & Katusic, 1999). eNOS uncoupling has been demonstrated in atherosclerosis (Vasquez-Vivar *et al.*, 2002), diabetes (Bagi & Koller, 2003), hyperhomocysteinemia (Virdis *et al.*, 2003), and hypertension (Landmesser *et al.*, 2003). In hypertension, Landmesser et al have showed that increased NAD(P)H oxidase-derived $\cdot O_2^-$ leads to augmented ROS bioavailability, which causes oxidation of BH_4 and consequent uncoupling of eNOS, contributing to ROS production (Landmesser *et al.*, 2003). Since $\cdot O_2^-$ derived from NOS is associated with marked endothelial dysfunction, NOS versus NAD(P)H oxidase-mediated $\cdot O_2^-$ generation is probably related to endothelial dysfunction in hypertension.

1.2.1.3 Xanthine Oxidase

Xanthine oxidase is expressed on the luminal surface of the endothelium in many organs and catalyzes the oxidation of hypoxanthine and xanthine to form $\cdot O_2^-$ in the vascular endothelium. Some evidences suggest xanthine oxidase is also involved in endothelium dysfunction in hypertension (Mervaala *et al.*, 2001). In addition to effects on the vasculature, xanthine oxidase may play a role in end-organ damage in hypertension and reperfusion injury after ischemia (Laakso *et al.*, 2004).

Other enzymatic sources of generating ROS in the vasculature are cyclooxygenase, cytochrome P450, mitochondrial respiratory chain and lipoxygenase, which may play a role in vascular function and dysfunction (Fleming *et al.*, 2001;Kukreja *et al.*, 1986). The sources of ROS in vascular cells are shown in **Fig 3**.

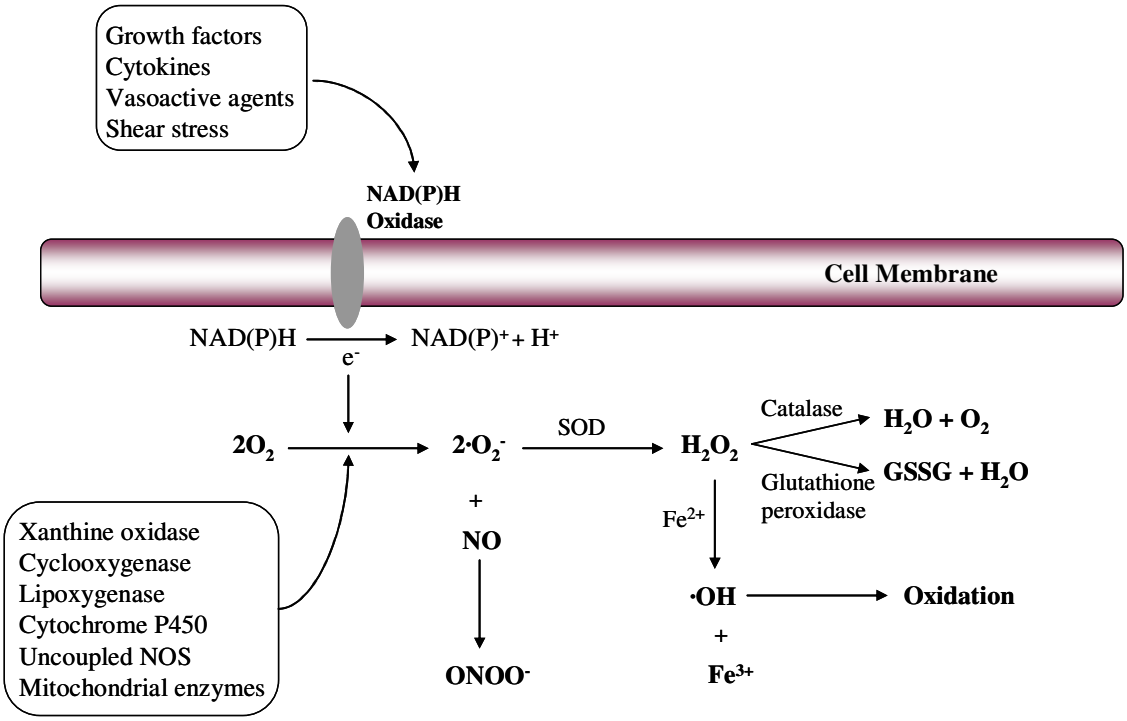


Fig 3 ROS generation in vascular cells. The ROS production is mainly from NAD(P)H oxidase, uncoupled NOS, and xanthine oxidase. According to (Touyz & Schiffrin, 2004).

1.2.1.4 Inflammatory Reaction

Activation of macrophages and leukocytes elicits the oxidative burst reaction with abundant secretion of ROS (Nguyen-Khoa *et al.*, 1999).Under chronic conditions these alterations seem to be part of atherosclerotic plaque development. Especially the formation of oxidative modified LDL represents an important mechanism (Chisolm & Steinberg, 2000;Glass & Witztum, 2001).

1.2.2 Metabolism of ROS

Reactive oxygen species are formed as intermediates in reduction–oxidation (redox) processes, leading from oxygen to water (Fridovich, 1997). In the presence of free electrons (e^-), the univalent reduction of oxygen yields superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$) (**Fig 3**). Since $\cdot O_2^-$ has an unpaired electron, it is highly reactive, unstable and short-lived. In physiological conditions, $\cdot O_2^-$ is dismutated to yield H_2O_2 by superoxide dismutase (SOD). However, a significant amount of $\cdot O_2^-$ reacts with NO to produce $ONOO^-$ when it is produced in excess (Darley-Usmar *et al.*, 1995). $\cdot O_2^-$ is membrane-impermeable, but can cross cell membranes via anion channels (Schafer & Buettner, 2001).

H_2O_2 is produced mainly from dismutation of $\cdot O_2^-$ by superoxide dismutase (SOD). Unlike $\cdot O_2^-$, H_2O_2 is not a free radical. It is a much more stable and longer lived than $\cdot O_2^-$. In biological systems, it is scavenged by catalase and glutathione peroxidase (Schafer & Buettner, 2001). In the presence of molecules containing metal such as Fe^{2+}, H_2O_2 can be reduced to generate $\cdot OH$ (Haber-Weiss or Fenton reaction) (Fridovich, 1997). $\cdot OH$ is extremely reactive. However, $\cdot OH$ induces local damage where it is formed since it cannot travel some distance from the site of generation.

Since H_2O_2 can cross cell membranes and produces cellular oxidative damage, it has been used as a model of oxidative stress (Rubanyi, 1988). H_2O_2 has complex effects on the vessels. It is able to modify vascular tone by inducing either contraction or relaxation, which is dependent on the different vascular beds and experimental conditions. For endothelium, H_2O_2 induces endothelial barrier dysfunction by modifying the sodium-potassium pump activity (Meharg *et al.*, 1993) and altering endothelial metabolic function (Fisher *et al.*, 2002). However, it is suggested that H_2O_2 should be an endothelium-derived hyperpolarizing factor

(EDHF), inducing hyperpolarization and relaxation by activating K_{Ca} and ATP-sensitive K^+ channels (Barlow & White, 1998;Barlow *et al.*, 2000).

1.2.3 Signalling Pathways

ROS have multiple effects on vascular cells through involvement in many signal transduction pathways (**Fig 4**), while the exact molecular targets are not yet clear. ROS inactivate mitogen-activated protein kinases (MAPK) and protein tyrosine kinases (PTK) through oxidation/reduction of proteins. ROS stimulate Ca^{2+} and K^+ channels. Additionally, ROS influence gene and protein expression by activating transcription factors [e.g. NFκB, activator protein-1 (AP-1)]. Activation of these pathways contributes to hypertensive vascular damage (Paravicini & Touyz, 2006).

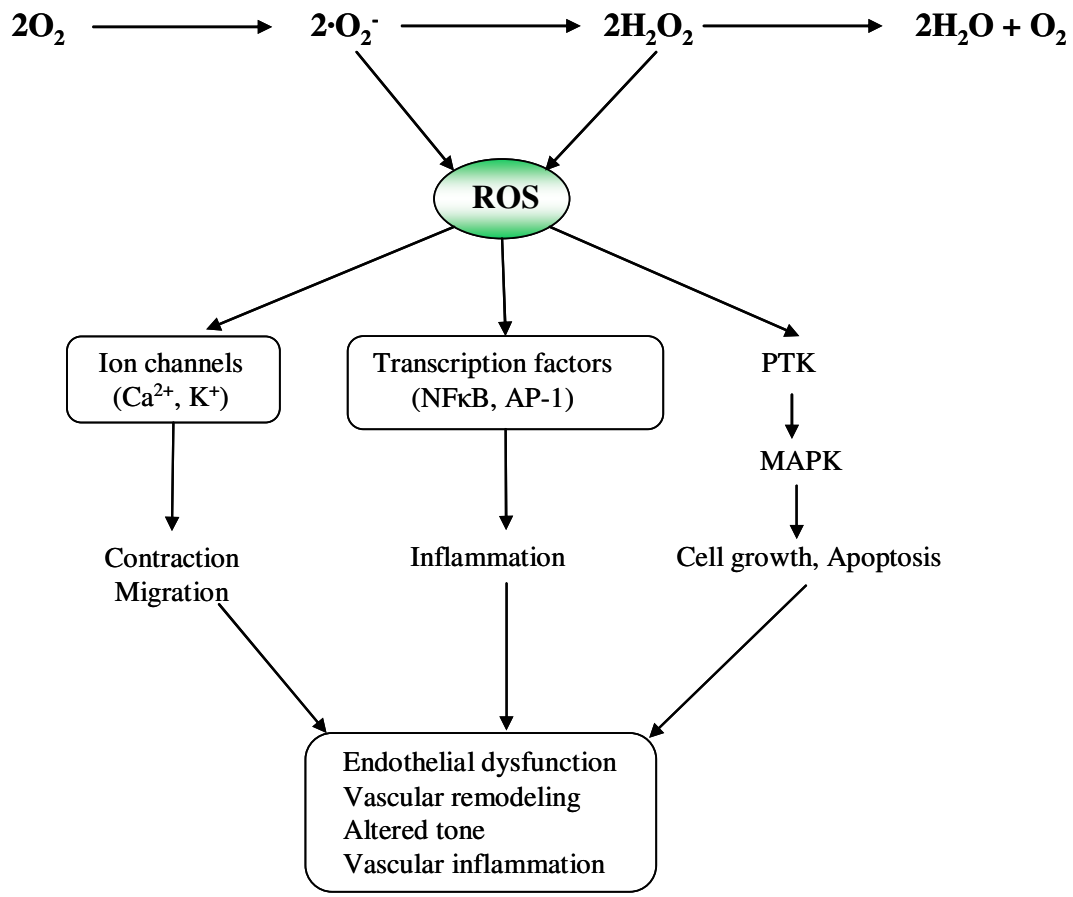


Fig 4 ROS signalling pathways in vascular smooth muscle cells. PTK, protein tyrosine kinases; **MAPK**, mitogen-activated kinases; ROS influence different signalling

pathways, altering vascular functions and pathologically leading to vascular damage. According to (Paravicini & Touyz, 2006).

1.2.4 Antioxidant Defences

Oxidative modifications within the arterial wall may contribute to diseases when the balance between oxidants and antioxidants shifts in favour of the former. Therefore, it is important to consider sources of available antioxidants. An antioxidant is defined as a substance that is effective against oxidative damage when present in much smaller quantity than the substance that it protects. The following briefly describes the antioxidant defences in arterial wall cells.

1.2.4.1 Enzymatic Antioxidants

Enzymatic antioxidants principally include SOD, catalase, glutathione peroxidases, and glutathione reductase.

SOD. SODs catalyze the dismutation $\cdot O^{2-}$ to H_2O_2 and molecular oxygen (reaction 1). There are three forms of SOD in mammalian systems: the copper-zinc (Cu, Zn-SOD), Mn-SOD, and extracellular SOD (EC-SOD) (Fridovich, 1997). SOD activity is decreased by homocysteine and increased by angiotensin II and hypertension. A key function of Cu, Zn-SOD is thought to protect against $\cdot O^{2-}$ induced inactivation of endothelial cell.

$$2 \cdot O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

Catalase and Peroxidases. Catalase directly decomposes H_2O_2 to water and molecular oxygen (reaction 2), whereas peroxidases eliminate H_2O_2 by using it to oxidize another substrate (reaction 3).

$$2\ H_2O_2 \rightarrow 2H_2O + O_2 \qquad (2)$$

$$\text{Substrate-}H_2 + H_2O_2 \rightarrow \text{substrate} + 2\ H_2O \qquad (3)$$

Glutathione-Dependent Antioxidant Defences. Glutathione peroxidases cooperate with catalase in the removal of H_2O_2 in vivo by using reduced glutathione (GSH) to reduce H_2O_2 to H_2O and oxidized glutathione (GSSG) (reaction 4). Glutathione peroxidases can also act on peroxides other than H_2O_2, e.g. *tert*.-BHP. Generally, they can catalyze GSH-dependent reduction of LOOH (e.g hydroperoxide formed from unsaturated fatty acid) to the corresponding alcohol (LOH) in the artery wall (reaction 5). Glutathione peroxidases operate in concert with glutathione reductase that catalyzes the reduction of GSSG at the expense of NADPH (reaction 6).

$$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG \qquad (4)$$

$$LOOH + 2GSH \rightarrow LOH + H_2O + GSSG \qquad (5)$$

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+ \qquad (6)$$

1.2.4.2 Non-enzyme Antioxidants

Vitamin C (Ascorbate). Vitamin C is water soluble and ubiquitous in biological fluids. It enhances activity of enzymes through maintenance of the iron center in the active, ferrous state. Vitamin C is also a cofactor for several enzymes engaged in hydroxylation reactions. It provides the first line of defense against oxidative damage in human plasma. In addition to oxidant scavenging, ascorbate has a number of potentially protective activities related to atherosclerosis and cardiovascular disease. The mechanisms include the maintenance of eNOS activity, attenuation of cellular oxidative stress, inhibition of LDL oxidation. Ascorbate enhances eNOS through increasing cellular tetrahydrobiopterin. Ascorbate can also compete with $\cdot O^{2-}$ for reaction with NO. Therefore, ascorbate decreases $\cdot O^{2-}$ production and maintains NO synthesis by eNOS (Smith *et al.*, 2002).

Vitamin E. Vitamin E is the major lipid-soluble antioxidant in lipoproteins and cells. It tends to localize in membranes and lipoproteins. In addition to scavenging peroxyl radicals, Vitamin E can also react with singlet oxygen and ONOO-.

Coenzyme Q10. Coenzyme Q10 is found in all cell membranes, as well as lipoproteins. It also provides the first line of lipid-soluble antioxidant defense against human LDL lipid peroxidation.

1.3 Questions

The forgoing chapters showed that the effects of oxidative stress, especially of hydroperoxides on arterial functions are still controversial and the mechanisms involved not well understood. Therefore, the present study was performed, mainly with aortas from mice, to clarify the following questions:

1) Which contractile effects exert H_2O_2 and *tert*.-butylhydroperoxide on aortic rings precontracted by KCl or phenylephrine?

2) Which effects of the hydroperoxides are mediated by endothelium, and which effects are directly induced in smooth muscle?

3) Which signalling pathways are involved in hydroperoxide-induced effects? For this aim, specific antagonists including PLA_2-COX-TXA_2 pathways, Ca^{2+} related pathways and voltage dependent K^+ channels were used.

4) Are there phenotypic effects in the contractile responses to hydroperoxides in animals lacking the gene for glutathione peroxidase-1?

5) Are there phenotypic effects in the free radical generation stimulated by the hydroperoxides in animals lacking the gene for glutathione peroxidase-1?

2 Material

2.1 Instruments

Biophotometer, Eppendorf, Hamburg

Clamp force amplifier, Hottinger, Darmstadt

Digital pH-Meter Type 643-1, Schott, Hofheim

Luminometer TD-20/20, Promega GmbH, Mannheim

Mastercycler, Eppendorf, Hamburg

Multi - Biolumat, LB 9505 C, Berthold, Bad wildbad

Operation microscope with lighting device, Zeiss, Oberkochen

Perfusion chamber for analysis of vessel wall mechanics, Physiological Institute, University Tuebingen

Sensor of force transducers, Statham

Spectrophotometer Genesys 6, Rochester, NY, USA

Tripod pump ISM 853, Ismatec, Wertheim

Two channels writer, ABB, Metrawatt

Vortex, Bender&Hobein, Zürich, Switzerland

Water bath heater, Colora, Lorch

2.2 Chemicals and Reagents

100% Acetic acid (CH_3COOH), Detern

Acetylcholine chloride (ACh), Sigma-Aldrich, Steinheim

Albumin, bovine (BSA), Sigmal Chemical CO. USA

4-Aminopyridine (4-AP), Sigma-Aldrich, Steinheim

2,2'-Azobis (2-Amidinopropane) hydrochloride (AAPH), Polysciences InC, Warrington, USA

Bio-RAD-Reagenz Coomassie Brilliant Blue G250, Bio-Rad Labortatories GmbH, München

Bupivacaine, Sigma-Aldrich, Steinheim

tert.-Butylhydroperoxide (*tert.*-BHP), Merck, Darmstadt

Calcium chloride dihydrate ($CaCl_2$ $2H_2O$), Roth, Karlsruhe

Dantrolene, Sigma-Aldrich, Steinheim

Diclofenac, Sigma-Aldrich, Steinheim

Dimethyl sulfoxide (DMSO), Sigma-Aldrich, Steinheim

Ethanol, Merck, Darmstadt

Ethidiumbromid, Roth, Karlsruhe

Ethylenediamine tetraacetic acid (EDTA), Sigma-Aldrich, Steinheim

Fasudil hydrochloride, Biotrend Chemikalien GmbH, Köln

Furegrelate sodium salt, Sigma-Aldrich, Steinheim

α - D(+) - Glucose monohydrate, Roth, Karlsruhe

Glybenclamide, Sigma-Aldrich, Steinheim

L-Glutathione reduced, Sigma-Aldrich, Steinheim

Glutathione Reductase, Sigma-Aldrich, Steinheim

HEPES, Roth, Karlsruhe

Hydrogen peroxide (H_2O_2) 30%, Sigma, Deisenhofen; Universitätsapotheke

Indomethacin, Vaseline-Fabrik E. Wasserfuhr GmbH, Bonn

Lucigenin (N, N'-Dimethyl-9, 9'-biacridiniumdinitrat), Serva, Heideberg

Luminol (5-Amino-1, 2, 3, 4-tetrahydrophtalazin-1, 4-dion), Merck, Darmstadt

Magnesium sulfate ($MgSO_4$ $7H_2O$), Roth, Karlsruhe

Meclofenamic, Sigma-Aldrich, Steinheim

Methanol, Sigma-Aldrich, Steinheim

ß-Nicotinamide adenine dinucleotide phosphate reduced form (ß-NADPH), Sigma-Aldrich, Steinheim

Nifedipine, Sigma-Aldrich, Steinheim

Nω-Nitro-L-Arginine methyl ester (L-NAME), Sigma-Aldrich, Steinheim

2-Nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC), Sigma-Aldrich, Steinheim

NucleoSpin®Tissue kit, Macherey-Nagel, Düren

L-Phenylephrine HCl, Serva, Heidelberg

Potassium chloride (KCl), Roth, Karlsruhe

Potassium dihydrogen phosphate (KH_2PO_4), Roth, Karlsruhe

Di-potassium hydrogen phosphate (K_2HPO_4), Merck, Darmstadt

Procaine hydrochloride, Sigma-Aldrich, Steinheim

Protein assay, Bio-Rad Laboratories GmbH, München

Quinacrine dihydrochloride, Sigma-Aldrich, Steinheim

Sodium chloride (NaCl), Merck, Darmstadt

Sodium hydroxide (NaOH), Roth, Karlsruhe

Sodium Di-hydrogen phosphate monohydrate (NaH_2PO_4 H_2O), Roth, Karlsruhe

TaKaRa, LA TaqTM, Japan

Trizma base, Sigma-Aldrich, Steinheim

Valsartan, Novartis Pharma, Wehr

2.3 Solution

Physiological solution

Normal-Tyrode solution pH 7.4 for 1 Liter		
NaCl	6.896 g	118.00 mM
KCl	0.373 g	5.00 mM
NaH_2PO_4 H_2O	0.213 g	1.54 mM
$MgSO_4$ $7H_2O$	0.293 g	1.19 mM
$CaCl_2$ $2H_2O$	0.370 g	2.5 mM
Hepes	2.383 g	10.00 mM
Glucose	1.000 g	5.00 mM
The solution with NaOH adjusted to pH 7.4		

30 mM KCl Tyrode solution

30mM KCl Tyrode solution pH 7.4 for 1 Liter		
NaCl	5.452 g	93.00 mM
KCl	2.236 g	30.00 mM
NaH_2PO_4 H_2O	0.213 g	1.54 mM
$MgSO_4$ $7H_2O$	0.293 g	1.19 mM
$CaCl_2$ $2H_2O$	0.220 g	1.50 mM
Hepes	2.382 g	10.00 mM
Glucose	1.000 g	5.00 mM
The solution with NaOH adjusted to pH 7.4		

Phenylephrine-solution (PHE)

Tyrode solution with phenylephrine [10^{-6} M]

→ Using 10^{-2} M phenylephrine stock solution (dissolved in deionized water)

Solution for hydroperoxides

H_2O_2-solution

KCl enriched Tyrode-solution or phenylephrine-solution with H_2O_2 [10^{-5} M], [5 x 10^{-5} M], [1.25 x 10^{-4} M], [2.5 x 10^{-4} M], [5 x 10^{-4} M], [10^{-3} M].

→ Using 10^{-1} M, 1 M H_2O_2 stock solution (diluted in deionized water)

tert.-BHP-solution

KCl enriched Tyrode-solution or phenylephrine-solution with *tert.*-BHP [10^{-5} M], [5 x 10^{-5} M], [1.25 x 10^{-4} M], [2.5 x 10^{-4} M], [5 x 10^{-4} M], [10^{-3} M].

→ Using 10^{-1}M, 1M *tert.*-BHP stock solution (diluted in deionized water)

Solution for testing endothelial function

ACh-solution

KCl enriched Tyrode-solution with ACh [10^{-6} M], [5 x 10^{-6} M], [10^{-5} M], [10^{-4} M].

→ Using 10^{-2} M, 10^{-1} M ACh stock solution (dissolved in deionized water)

L-NAME-solution

KCl enriched Tyrode-solution with L-NAME [5 x 10^{-5} M].

→ Using 10^{-2} M L-NAME stock solution (dissolved in deionized water)

Solution for Ca^{2+} channel inhibitors

Dantrolene-solution (intracellular Ca^{2+} release inhibitor)

KCl enriched Tyrode-solution with dantrolene [5 x 10^{-5} M].

→ Using 10^{-2} M dantrolene stock solution (dissolved in DMSO)

Nifedipine-solution (selective L-type Ca^{2+} antagonist)

KCl enriched Tyrode-solution with nifedipine [10^{-7} M].

→ Using 10^{-2} M nifedipine stock solution (dissolved in DMSO)

Solution for Ca^{2+} sensitization inhibitor

Fasudil-solution (Rho-kinase inhibitor)

KCl enriched Tyrode-solution with fasudil [3 x 10^{-6} M].

→ Using 10^{-2} M fasudil stock solution (dissolved in deionized water)

Solution for voltage-sensitive K^+ channel inhibitor

4-AP-solution (voltage-sensitive K^+ channel inhibitor)

KCl enriched Tyrode-solution or phenylephrine-solution with 4-AP [3 x 10^{-4} M].

→ Using 10^{-1} M 4-AP stock solution (dissolved in deionized water)

Solution for PLA_2-COX-TXA_2 inhibitors

Quinacrine-solution (PLA_2 inhibitor)

KCl enriched Tyrode-solution with quinacrine [10^{-5} M], [5 x 10^{-5} M].

→ Using 10^{-2} M quinacrine stock solution (dissolved in deionized water)

Diclofenac-solution (COX inhibitor)

KCl enriched Tyrode-solution with diclofenac [2 x 10^{-5} M].

→ Using 10^{-2} M diclofenac stock solution (dissolved in DMSO)

Indomethacin-solution (COX inhibitor)

KCl enriched Tyrode-solution with indomethacin [2 x 10^{-4} M].

→ Using 10^{-1}M indomethacin stock solution (dissolved in DMSO)

Meclofenamic-solution (COX inhibitor)

KCl enriched Tyrode-solution with meclofenamic [2 x 10^{-5} M].

→ Using 10^{-2} M miclofenamic stock solution (dissolved in DMSO)

Bupivacaine-solution (TXA_2 inhibitor)

KCl enriched Tyrode-solution with bupivacaine [10^{-4} M].

→ Using 10^{-1} M bupivacaine stock solution (dissolved in ethanol)

Furegrelate-solution (TXA_2 inhibitor)

KCl enriched Tyrode-solution with furegrelate [5 x 10^{-6} M].

→ Using 10^{-2} M furegrelate stock solution (dissolved in deionized water)

Solution for PLC and AT1 receptor inhibitors

NCDC-solution (PLC inhibitor)

KCl enriched Tyrode-solution or phenylephrine-solution with NCDC [2 x 10^{-5} M].

→ Using 10^{-1} M NCDC stock solution (dissolved in ethanol)

Valsartan (AT1 receptor inhibitor)

KCl enriched Tyrode-solution with valsartan [10^{-5} M].

→ Using 10^{-1} M valsartan stock solution (dissolved in methanol)

Solution for DMSO, methanol, ethanol

DMSO-solution

KCl enriched Tyrode-solution with DMSO [2.8 x 10^{-3} M], [2.8 x 10^{-2} M].

→ Using 14.1 M DMSO stock solution

Methanol-solution

KCl enriched Tyrode-solution with methanol [2.47 x 10^{-3} M].

→ Using 24.7 M methanol stock solution

Ethanol-solution

KCl enriched Tyrode-solution or Phenylephrine-solution with ethanol [1.72 x 10^{-3} M], [1.72 x 10^{-2} M].

→ Using 17.2 M ethanol stock solution

Solution for measuring chemiluminescence

Lucigenin-solution

Lucigenin was dissolved by DMSO [9.8 mM].

Luminol-solution

Luminol was dissolved by DMSO [11.3 mM].

PBS solution

PBS solution pH 7.4 for 1 Liter		
Na_2HPO_4 $2H_2O$	1.442 g	8.10 mM
NaH_2PO_4 H_2O	0.102 g	0.74 mM
NaCl	8.182 g	140.00 mM
KCl	0.201 g	2.70 mM
The solution with NaOH adjusted to pH 7.4		

Solution for PCR

Reaction mixture for PCR

TaKaRa LA Taq TM (5 units/µl)

10 x LA PCR TM Buffer (Mg^{2+} free)

25 mM $MgCl_2$ (final 2 mM)

dNTP Mixture (2.5 mM each)

Forward primer FinN (10 pM)

Reverse primer R_3N (10 pM)

Reverse primer RpgkN (10 pM)

Template

Sterilized distilled water

1 % Agarose gel

1 g agarose in 100 ml 1 x TAE – Buffer

50 x TAE – Buffer 1L, pH 8.5

Trizma base 2.0 M

CH_3COOH 57.1 ml

Na_2EDTA $2H_2O$ 0.1 M

Solution for GPx activity

Potassium phosphate buffer

100 ml KH_2PO_4 (1 M) was slowly added into 100 ml K_2HPO_4 (1 M) until pH 7 to get 1 M potassium phosphate buffer. When it is used, the buffer was diluted 1:10 (0.1 M).

NADPH - solution

NADPH (1 mg/0.5 ml, 2.4 mM) was diluted by sterilized distilled water.

Glutathione - solution

Glutathione (3.1 mg/ml, 10 mM) was diluted by sterilized distilled water.

Glutathione - reductase – solution

10 µl Glutathione - reductase (120 U/ml) was diluted by 1 ml potassium phosphate butter with EDTA (50 µl 50 mM EDTA to 950 µl 0.1 M KPP buffer).

EDTA – solution

EDTA (29.2 mg/2ml, 50 mM) was diluted by sterilized distilled water.

tert. - BHP solution

tert. - BHP (10 µl/6 ml, 0.017 M) was diluted by sterilized distilled water.

3 Methods

3.1 Tissue Preparation and Organ Bath

Wilde type mice (C57BL6) and GPx-$1^{-/-}$ mice at the age of 6 - 12 months were killed with carbon dioxide. The aorta was carefully dissected out, cleaned of adhering fat and connective tissues and cut into approximately 4 - 5 mm long segments under a dissecting microscope. The segment was equilibrated in an organ bath system for isometric tension recording (Blumenstein, 2004). The bath, which was maintained at 37 °C, contained physiological Tyrode solution. Two horizontally arranged stainless steel pins were passed through the lumen of the vascular cylinder. One pin was fixed to a micro-screw allowing the adaptation to a distant prestretching of the vessel ring, while the other one was vertically connected to a strain gauge for tension recording. The contraction was recorded by a force-displacement transducer. The solution in the organ bath was continuously perfused by approx 5 ml/min. To examine endothelium independent responses, some aorta rings was mechanically removed by rubbing the internal surface of the ring with a fine wooden stick, and successful removal of endothelium was verified by the absence of contraction response to L-NAME [5 x 10^{-5} M] (E+: the aorta with endothelium; E-: the aorta without endothelium).

3.2 Reactivity Experiments

After 30 min of equilibration, the segment was precontracted by 30 mM KCl enriched Tyrode solution or PHE [1 µM] until a stable contraction was achieved. To assess the contractile response to H_2O_2 and *tert.*-BHP, the segment was then exposed to a single concentration of H_2O_2 [10^{-5}-10^{-3} M] or *tert.*-BHP [10^{-5}-10^{-3} M] applied in the corresponding stimulation solution. While continuously perfused by the hydroperoxide containing solution, 15 - 20 min reaction time with the aorta was allowed, followed by a thorough wash with Tyrode solution. The maximum contraction of H_2O_2 and *tert.*-BHP was expressed as a percentage of the

precontraction. **Fig 1** showes the experimental procedure of the contractile response to the hydroperoxide.

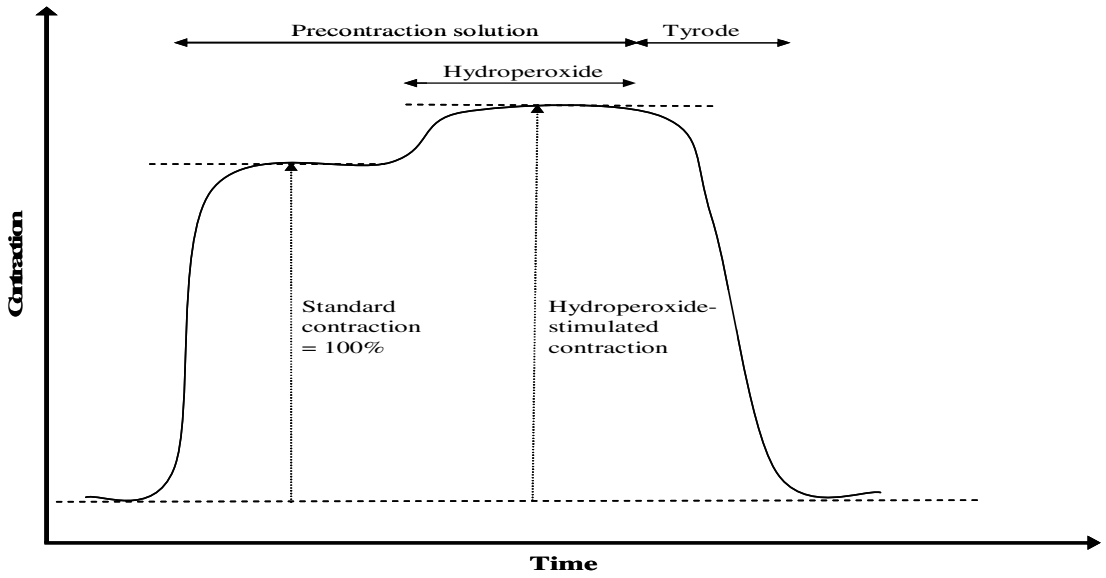


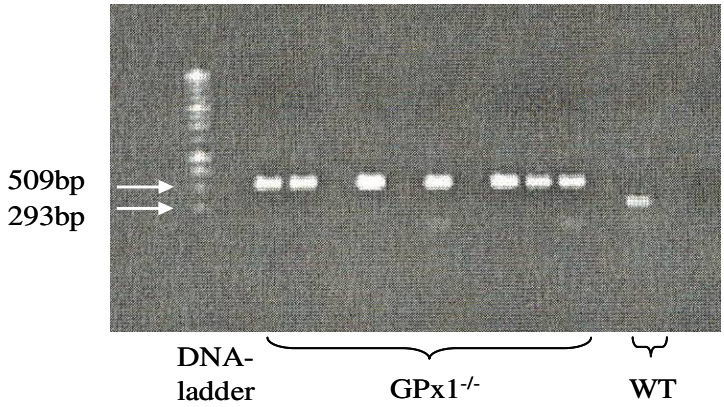
Fig 1 Typical tracing showing the response to hydroperoxides (H_2O_2 and *tert*.-BHP) in the mouse aorta. The contraction was expressed in mN. Maximum contractile response to H_2O_2 and *tert.*-BHP was expressed as a percentage of KCl- or PHE- precontraction.

The modulation of the responses to H_2O_2 and *tert*.-BHP by the antagonists and agonist was assessed. The agonist and antagonists included: ACh (endothelium-dependent agonist), L-NAME (NOS inhibitor), quinacrine (PLA_2 synthase inhibitor), diclofenac (COX inhibitor), indomethacin (COX inhibitor), meclofenamic (COX inhibitor), bupivacaine (TXA_2 synthase inhibitor), furegrelate (TXA_2 synthase inhibitor), NCDC (PLC inhibitor), nifedipine (L-type Ca^{2+} channel antagonist), dantrolene (intracellular Ca^{2+} release inhibitor), fasudil (Rho-kinase inhibitor) 4-AP (voltage-sensitive K^+ channel inhibitor), valsartan (Angiotensin II AT1 receptor antagonist). The concentrations of the different antagonists and agonist used in the experiments referred to the related papers and the procedure of reactivity experiment for each drug will be shown in the result part.

3.3 PCR for Genotype Determination of Glutathione Peroxidase-1

Mice homozygous for disruption of the GPx-1 gene were kindly provided by Professor K.J. Lackner (Mainz University) and subsequently bred at our institution. In these mice, the gene of GPx-1 was inactivated by insertion of a neomycin resistance gene (NEO) cassette into an *EcoR*I site located in exon 2 of the GPx-1 gene, which was then inserted into embryonic stem cells (Ursini *et al.*, 1995).

Genotype determination: DNA was obtained by extraction from the mouse ear by using NucleoSpin® Tissue kit as described. To identify the WT GPx-1 gene, we used the forward primer FinN (5'-GTTTCCCGTGCAATCAGTTCG-3') and the reverse primer R_3N (5'-TCGGACGTACTTGAG-GGAAT-3') to amplify a 293bp fragment. To detect GPx-$1^{-/-}$ mice, we used FinN and RpgkN (5'-CATTTGTCACGTCCTGCAC-3') as the reverse primer to amplify a 509bp fragment in the NEO insert. Reaction products were analyzed by electrophoresis on a 1% agarose gel. WT mice were identified by an exclusive 293bp PCR product, and GPx-$1^{-/-}$ mice were identified by an exclusive 509bp product.

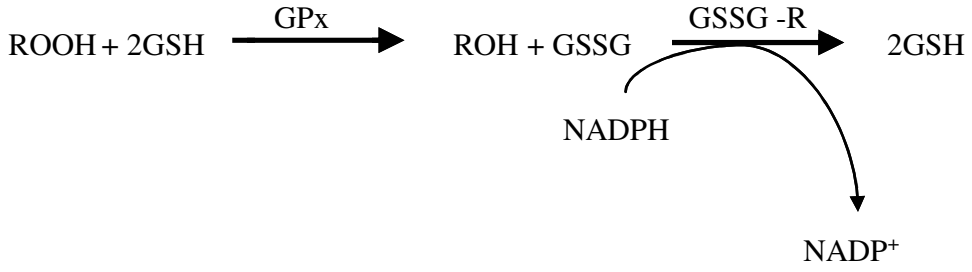


PCR for genotype determination:
WT mice were identified by a 293bp PCR fragment, whereas GPx-$1^{-/-}$ mice were identified by a 509bp PCR product.

3.4 Measurement of Glutathione Peroxidase Activity

3.4.1 Principle of the Procedure

In this assay *tert*.–BHP is used, and glutathione reductase and β-NADPH (β-nicotinamide adenine dinucleotide phosphate, reduced) are included in the reaction mixture. The formation of GSSG catalyzed by GPx is coupled to the recycling of GSSG back to GSH using GSSG-R. NADPH is oxidized to $NADP^+$. The change in A_{366} due to NADPH oxidation is monitored and is indicative of GPx activity. Since all other reagents are provided in excess, the amount of GPx in the test sample is the rate-limiting factor and the rate of decrease in the A_{366} is directly proportional to the GPx activity in the sample. The over-all 2 step reaction is:



3.4.2 Glutathione Peroxidase Assay

Using tissues from WT and GPx-$1^{-/-}$ mice, livers and aortas were homogenized in 2-3 volumes of KPP buffer and supernatant from the top of the tube (5000 RPM, 10 min) was taken for the assay. Blood was collected using heparin as the anticoagulant and RBC was spun down by centrifugation at 3000 rpm for 10min at 4°C. RBC was added into two volumes of DI (deionized) water to get hemolysate by complete lysis. The enzyme reaction was conducted in a buffer containing potassium phosphate (100 mM), glutathion-reductase (120 U/ml), glutathione (10 mM), NADPH (2.4 mM), EDTA (0.5 mM) and *tert*.-BHP (0.017 mM) at 37 °C, and the rate of decrease in absorption of NADPH at 366 nm was followed.

Activities were expressed as units per mg of protein. The protein concentrations of the homogenate were also determined (Biorad Assay).

The calculation

$$\text{Specific activity (mU/mg)} = \frac{394 \times (V_{Total}/V_{Probe}) \times (\Delta\ E/min)}{\text{protein concentration of sample (mg/ml)}}$$

V_{Totlal} = the total volume in the tube
V_{Probe} = the volume of the sample added
Δ E /min = the rate of decrease in A_{366}/ the time of the rate of decrease in A_{366}

Activity of GPx (mU/mg)	WT mouse	n	GPx-$1^{-/-}$ mouse	n
Aorta	4.57 ± 1.17	7	1.06 ± 0.14	8
Liver	27.03 ± 6.10	7	8.59 ± 6.20	9
Blood	6.92 ± 0.70	6	4.82 ± 0.90	10

Table 1 The difference of GPx activity in different tissues between WT and GPx-$1^{-/-}$. Results (Mean ± SD) were expressed in mU/mg, n = the number of the mice.

3.5 Chemiluminescence (CL) Assays

The theoretical considerations of lucigenin- and luminol-enhanced chemiluminescence: Lucigenin has been widely used as an indicator of $\cdot O_2^-$ production. In 1991, it was introduced as a tool to measure $\cdot O_2^-$ in vascular tissue by Wolin's group (Omar *et al.*, 1991). Lucigenin carries a positive ionic charge and it is generally thought to be relatively membrane impermeant. Therefore lucigenin is respond to reactive oxygen species, particularly superoxide anion ($\cdot O_2^-$), in the extracellular space. In contrast, the uncharged luminol molecule is membrane permeant and can react (in the form of luminol or as a univalently

oxidized luminol radical) with a variety of reactive oxygen species, including $\cdot O_2^-$, $OH^\bullet$, and H_2O_2 at higher concentrations (above 0.5 mM H_2O_2). So luminol reflects extracellular as well as intracellular ROS production.

The procedure was carried out with Luminometer TD-20/20. For wild type and GPx-$1^{-/-}$ mouse aorta, one segment of ≈ 8 mm was used in each assay. The segment was weighted. The vessels were then placed in a plastic cuvette containing physiological Tyrode solution, 5 µl luminol [11.3 mM] or 5 µl lucigenin [9.8 mM] and hydroperoxides [0.01-1 mM] in a total volume of 500 µl. CL was measured in the luminometer for 10 min. The indicated relative light units (RLU), which are relative measure of the integrated counts of photons, were related to the weight of the aortic samples. These values were compared to the measurements without hydroperoxides and without tissues, respectively.

AAPH is a water-soluble azo compound which is used extensively as a free radical generator, often in the study of lipid peroxidation and the characterization of antioxidants (Noguchi *et al.*, 1998;Rice-Evans & Miller, 1994). The half-life of AAPH is about 175 hours (37°C at neutral pH), making the rate of free radical generation essentially constant during the first several hours in solution (Niki, 1990). To estimate antioxidative properties of the different drugs, we used the AAPH method. In these experiments, PBS, 5 µl luminol [11.3 mM] and 10 µl AAPH [0.5 M] were added into a plastic cuvette, and CL was measured in multi-biolumat for 20 min until the production of ROS induced by AAPH was stable. Then the drug was added into the plastic cuvette (total volume = 500 µl) and CL was measured for another 40 min.

3.6 Statistical analysis

Results are expressed as Mean±SD. For statistical comparison, the student's t-test was used to determine significant differences between means. *P* value less than 0.05 was considered significant. More than three mice were used in each set of experiments.

4 Results

4.1 Effects of Hydroperoxides on KCl- and PHE-induced Contraction in the Aorta of WT Mouse with Endothelium

In KCl [30 mM]-precontracted mouse aorta, H_2O_2 caused concentration-dependent contractile response at the concentrations of 0.01-0.5 mM, but produced relaxation at the concentration of 1 mM. The maximum stimulation was reached at 0.25 mM (159.0 ± 11.0 % of the contraction induced by 30 mM KCl, **Fig 1a**). As shown in **Fig 1b**, *tert*.-BHP [0.01-0.25 mM] caused similarly concentration-dependent increase in tension, reached a peak at 0.125 mM (149.2 ± 26.5 % of the response to 30 mM KCl), and also caused relaxation at high concentrations [0.5-1 mM].

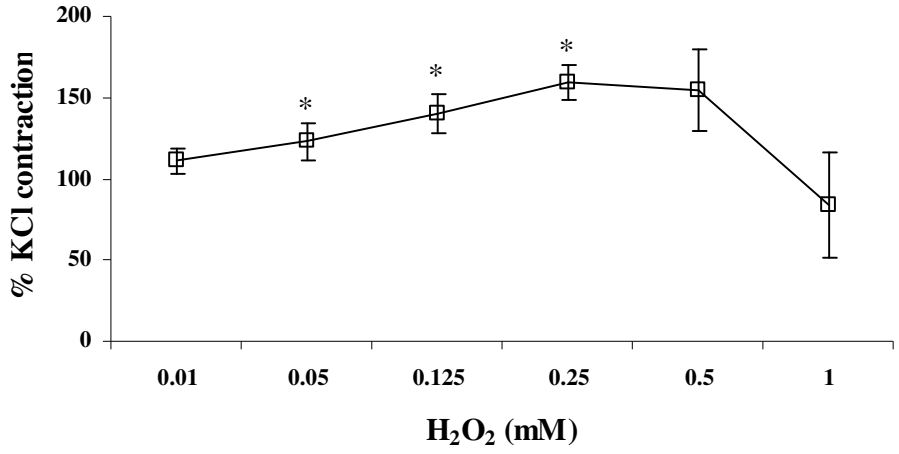


Fig 1a Concentration-response curve of H_2O_2 in 30 mM KCl-precontracted WT mouse aorta with endothelium. Results (Mean ± SD) were from 6 - 8 mice. The results were expressed as percentages of the contraction with 30 mM KCl. The average KCl-precontraction was 6.6±3.3 mN. Each concentration of H_2O_2 has its own control (KCl-precontraction). Absolute data (mN) was used for comparison. *P<0.05.

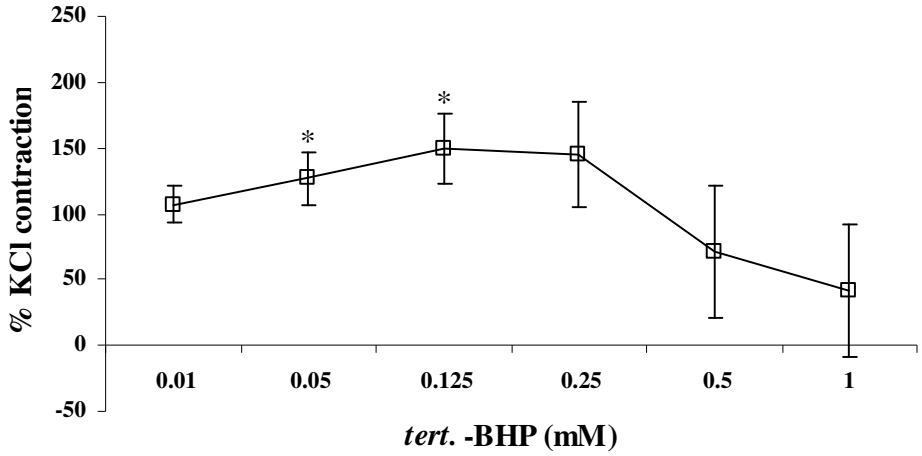


Fig 1b Concentration-response curve of *tert*.-BHP in 30 mM KCl-precontracted WT mouse aorta with endothelium. Results (Mean ± SD) were from 6 - 20 mice. The results were expressed as percentages of the contraction with 30 mM KCl. The average KCl-precontraction was 4.8±2.9 mN. Each concentration of *tert*.-BHP has its own control (KCl-precontraction). Absolute data (mN) was used for comparison. **P*<0.05.

Following contraction to PHE [1 µM], H_2O_2 and *tert*.-BHP increased contraction at the concentrations of 0.01-0.125 mM respectively. The maximum responses to H_2O_2 and *tert*.-BHP were both at 0.05 mM (144.8 ± 15.6 % and 131.7 ± 15.5 % of the contraction induced by 1 µM PHE, respectively, **Fig 2a, 2b**).

For further investigation on hydroperoxide-enhanced contraction mechanisms, we selected 0.05 mM of H_2O_2 and *tert*.-BHP. These results indicated that H_2O_2 and *tert*.-BHP increased contraction induced by KCl and PHE in the mouse aorta, which was dose dependent in a range of lower concentrations.

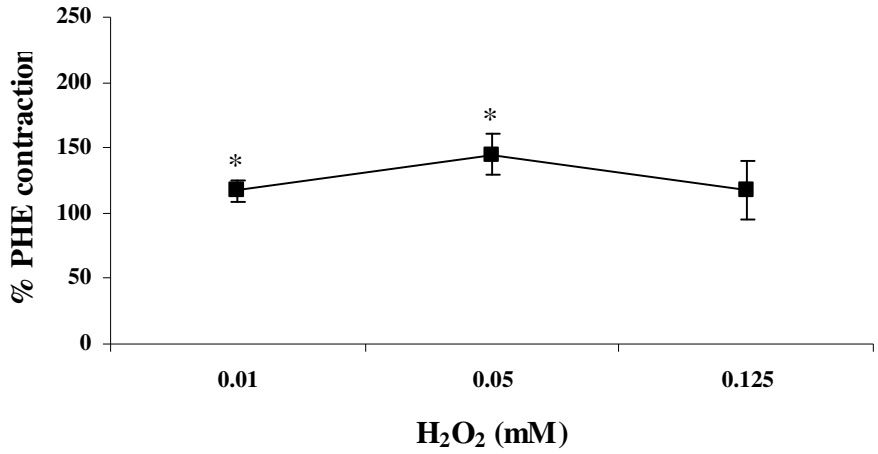


Fig 2a Concentration-response curve of H_2O_2 in 1 µM PHE-precontracted WT mouse aorta with endothelium. Results (Mean ± SD) were from 5 - 6 mice. The results were expressed as percentages of the contraction with 1 µM PHE. The average contraction of 1 µM PHE was 7.9±3.7 mN. Each concentration of H_2O_2 has its own control (PHE-precontraction). Absolute data (mN) was used for comparison. **P*<0.05.

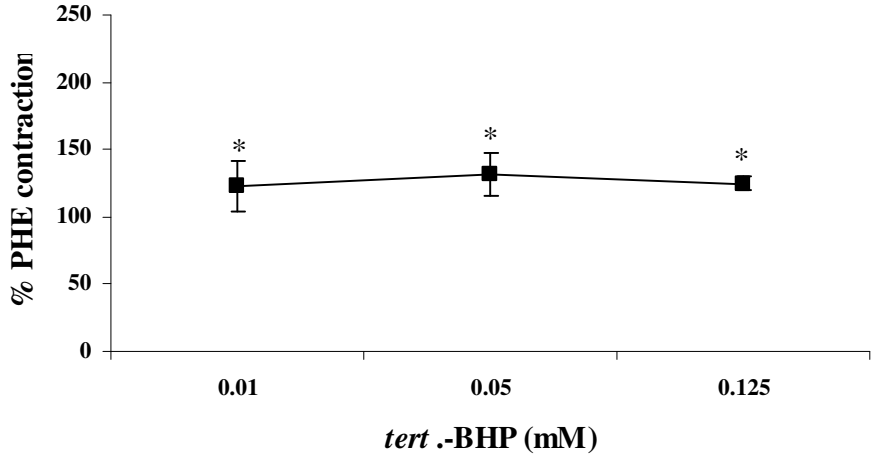


Fig 2b Concentration-response curve of *tert*.-BHP in 1 µM PHE-precontracted WT mouse aorta with endothelium. Results (Mean ± SD) were from 5 - 6 mice. The results were expressed as percentages of the contraction with 1 µM PHE. The average contraction of 1 µM PHE was 10.7±5.4 mN. Each concentration of *tert*.-BHP has its own control (PHE-precontraction). Absolute data (mN) was used for comparison. **P*<0.05.

4.2 Effects of Endothelium on Hydroperoxide-enhanced Contraction in WT Mouse Aorta

4.2.1 Effects of ACh on Hydroperoxide Stimulated Contraction in Intact Mouse Aorta

Concerning the role of endothelium in the response to hydroperoxides, ACh was used in this study. The experimental procedure: Following the equilibration of 30 min, the segment was contracted by 30 mM KCl to reach a plateau level. After the first contraction was obtained, ACh [0.1 mM] was added into the KCl-Tyrode solution. 10-15 min reaction time was allowed, followed by a thorough wash with Tyrode solution. The maximum response to ACh was expressed as a percentage of 30 mM KCl-precontraction. To assess the effect of ACh on H_2O_2- and *tert.*-BHP-induced contraction, the segment was additionally exposed to ACh [0.1 mM] with H_2O_2 [0.05 mM] or *tert.*-BHP [0.05 mM] when the hydroperoxide-induced contraction had reached a plateau. The maximum response to ACh with H_2O_2, *tert.*-BHP was also expressed as a percentage of 30 mM KCl-precontraction. **Fig 3a** showes the experimental procedure of the effect of ACh on hydroperoxide-induced contraction.

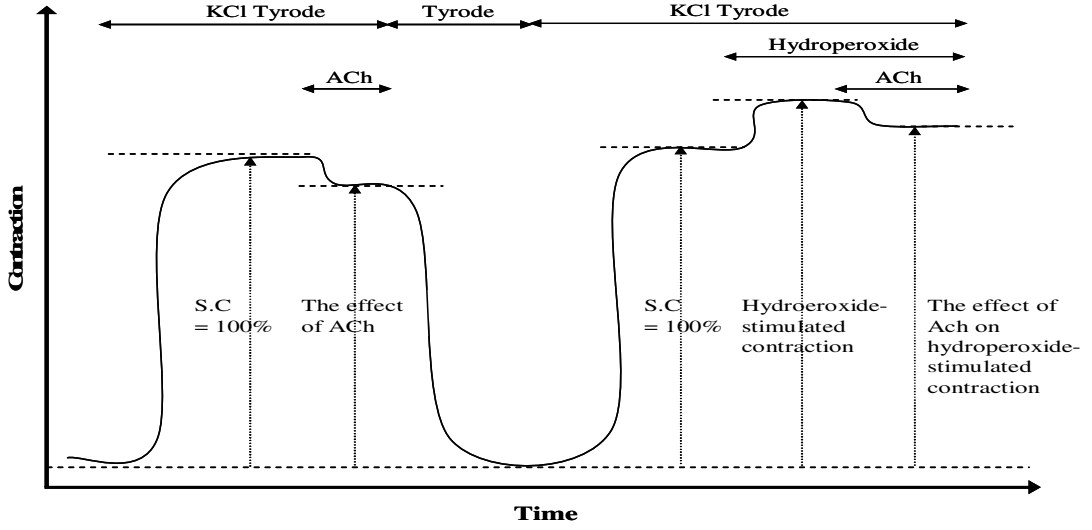


Fig 3a Typical model showing the effect of ACh on KCl-induced contraction and on hydroperoxide-stimulated contraction (H_2O_2 and *tert.*-BHP). S.C means standard contraction. The contraction was expressed in mN.

ACh [0.1 mM] relaxed contraction induced by 30 mM KCl in mouse aorta with intact endothelium. The relaxation of ACh amounted to 77.7 ± 9.8 % of KCl-precontraction (**Fig 3b**). Then we investigated the effect of ACh on hydroperoxide-enhanced contraction. As shown in **Fig 3c**, ACh [0.1 mM] significantly decreased the contraction induced by both hydroperoxides. The response to ACh in H_2O_2-increased contraction amounted to 92.6 ± 5.2 % of H_2O_2-enhanced contraction ($p<0.05$, n=4). For *tert.*-BHP, the relaxation of ACh amounted to 86.2 ± 0.8 % of *tert.*-BHP-induced contraction ($p<0.05$, n=3).

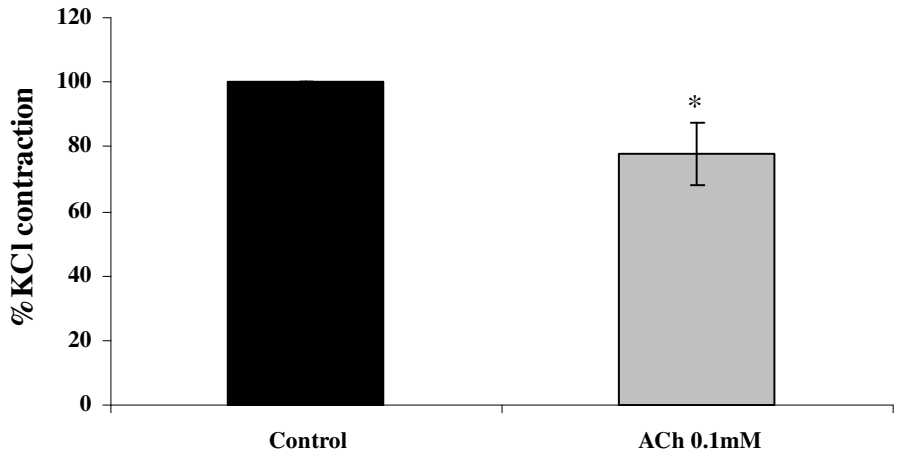


Fig 3b The effect of ACh (0.1 mM) on KCl-precontraction in WT mouse aorta with endothelium. Results (Mean ± SD) were from 5 mice. The results were expressed as percentages of the contraction with 30 mM KCl. The contraction of control was 3.4±0.5 mN and the response to ACh was 2.6±0.6 mN. Absolute data (mN) was used for comparison. **P*<0.05.

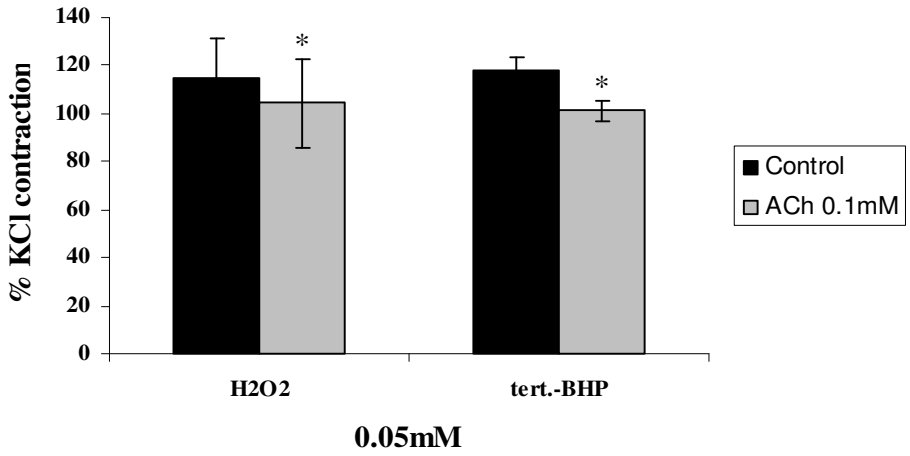


Fig 3c The effect of 0.1 mM ACh on the hydroperoxide-enhanced contraction in WT mouse aorta with endothelium. Results (Mean ± SD) were from 3 - 4 mice. The results were expressed as percentages of the contraction with 30 mM KCl. **P*<0.05 compared with control.

4.2.2 Effects of L-NAME on Hydroperoxide-enhanced Contraction in Intact Mouse Aorta

To study the role of NO in the response to hydroperoxides, NOS inhibitor (L-NAME) was used in KCl-precontraction and hydroperoxide-enhanced contraction. The experimental procedure for application of L-NAME was the same as ACh. L-NAME [0.05 mM] significantly increased 30 mM KCl-induced contraction (**Fig 4a**). The response to 0.05 mM L-NAME amounted to 117.5 ± 6.7 % ($p<0.05$, $n = 5$) of KCl-precontraction. L-NAME [0.05 mM] significantly increased H_2O_2- and *tert*.-BHP-enhanced contraction in the mouse aorta (**Fig 4b**).

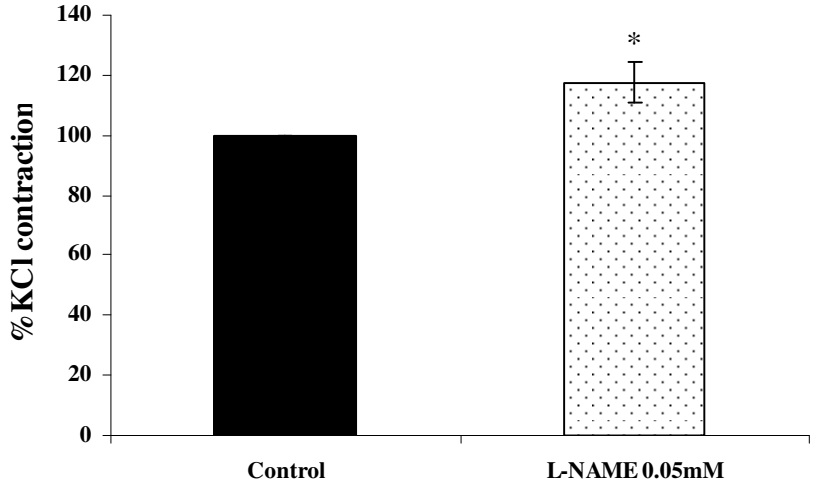


Fig 4a The effect of L-NAME on 30 mM KCl precontraction in WT mouse aorta with endothelium. Results (Mean ± SD) were from 5 mice. The results were expressed as percentages of the contraction with 30 mM KCl. The contraction of control was 6.2±2.9 mN and the response of L-NAME was 7.4±3.7 mN. Absolute data (mN) was used for comparison. **P*<0.05 compared with control.

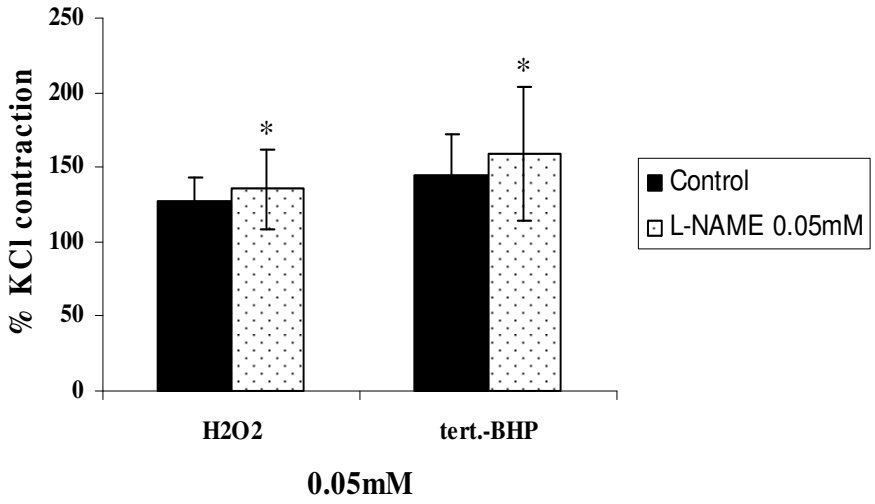


Fig 4b The effect of 0.05 mM L-NAME on hydroperoxide-enhanced contraction in WT mouse aorta with endothelium. Results (Mean ± SD) were from 7 - 9 mice. The results were expressed as percentages of the contraction with 30 mM KCl. **P*<0.05 compared with control.

4.2.3 Endothelium-independent Contraction to H_2O_2 and *tert*.-BHP

As shown in **Fig 5a**, L-NAME increased 30 mM KCl precontraction in intact WT mouse aorta, but not in preparations of denuded endothelium (E-). The results confirmed the successful removal of endothelium.

In E- mouse aorta, the contractile response to *tert*.-BHP [0.05 mM] amounted to 160.2 ± 18.9 % of KCl precontraction, which was significantly bigger than in E+ mouse aorta (126.0 ± 15.8 %). Removal of endothelium also increased the contractile response to H_2O_2 (the contractile response to H_2O_2 in E-: 166.0 ± 19.5 %, the contractile response to H_2O_2 in E+: 125.7 ± 13.2 %, **Fig 5b**). The results indicated that intact endothelium inhibited the tone of the artery induced by both hydroperoxides.

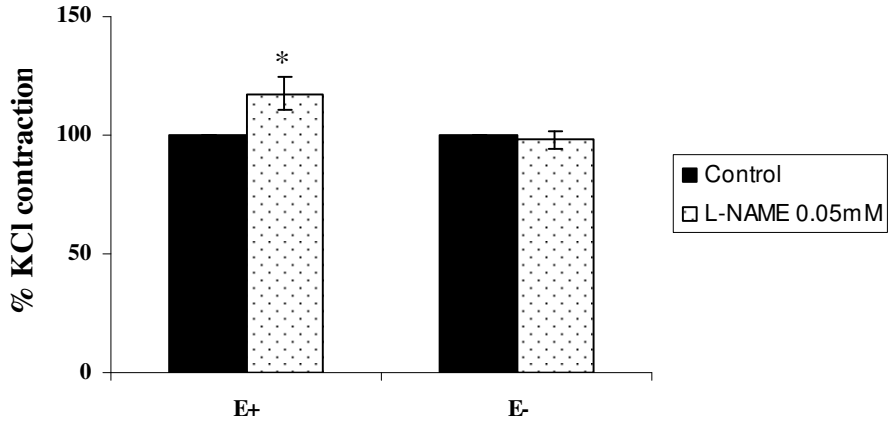


Fig 5a The different effect of L-NAME (0.05 mM) on 30 mM KCl-induced contraction in WT mouse aorta with intact- (E+) and denuded- (E-) endothelium. Results (Mean ± SD) were from 5 mice. The results were expressed as percentages of the contraction with 30 mM KCl. In E+ group, the contraction of control was 6.2±2.9 mN and the response to L-NAME was 7.4±3.7 mM. In E- group, the contraction of control was 3.99±0.8 mN and the response to L-NAME was 3.97±1.1 mN. Absolute data (mN) was used for comparison. *P<0.05 compared with control.

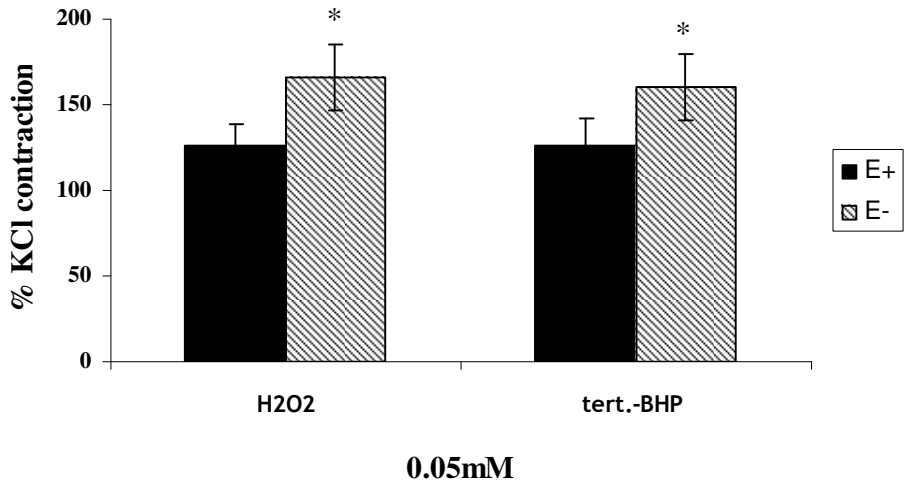


Fig 5b The difference of hydroperoxide-enhanced contraction in WT mouse aorta with intact- (E+) and denuded- (E-) endothelium. Results (Mean ± SD) were from 6 mice. The results were expressed as percentages of the contraction with 30 mM KCl. **P*<0.05 compared with the response to hydroperoxides with endothelium.

4.3 Pharmacological Characterization of the Effects of Hydroperoxides on the Contraction in WT Mouse Aorta

4.3.1 Effects of Ca^{2+} Channel and Sensitization Inhibitors

To study the sources of Ca^{2+} involved in the hydroperoxide-enhanced contraction, experiments were performed with intracellular Ca^{2+} release inhibitor (dantrolene), selective L-type Ca^{2+} antagonist (nifedipine) and Ca^{2+} sensitization inhibitor (fasudil). The concentrations of dantrolene, nifedipine and fasudil used in this study referred to Du W (Du *et al.*, 2005), Bergdahl et al (Bergdahl *et al.*, 2005) and Shimomura et al (Shimomura *et al.*, 2004), respectively. **Fig 6a** showed the experimental procedure to determine the effects of these drugs on hydroperoxide-enhanced contraction. Firstly, the contractile response to hydroperoxide (0.05 mM H_2O_2 or *tert*.-BHP) was tested, followed by a wash with Tyrode solution. The maximum contraction of hydroperoxides was expressed as a percentage of 30 mM KCl-precontraction. Then, the aorta was exposed to one of the inhibitors with KCl since these drugs inhibited KCl precontraction (**Fig 6b**).

When the contraction was obtained, 0.05 mM hydroperoxide was added. The contractile response to hydroperoxides was expressed as a percentage of the contraction of KCl with the inhibitor. Since dantrolene and nifedipine were initially dissolved in dimethyl sulfoxide (DMSO) to prepare the stock solution, the effect of the same amount of DMSO on KCl precontraction and hydroperoxide-enhanced contraction was also studied (**Fig 6c**).

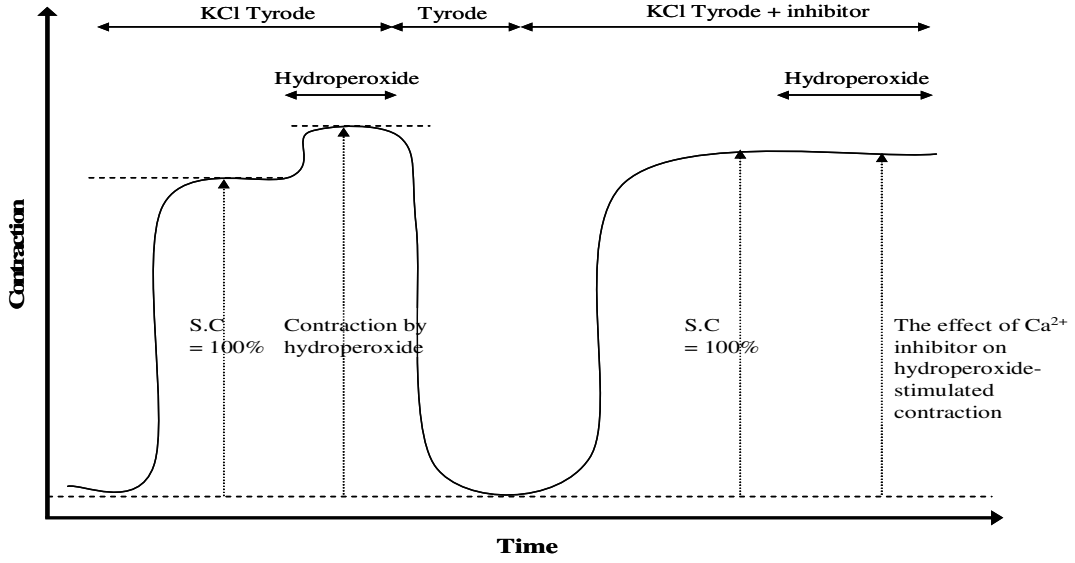


Fig 6a Typical model showing the effects of the Ca^{2+} channel and sensitization inhibitors on hydroperoxide-enhanced contraction. S.C means standard contraction. The contraction was expressed in mN.

As shown in **Fig 6b**, dantrolene (50 µM, intracellular Ca^{2+} release inhibitor), nifedipine (0.1 µM, selective L-type calcium antagonist) and fasudil (3 µM, Rho-kinase inhibitor) significantly reduced the contraction induced by 30 mM KCl, while DMSO (0.028 M) had no effect on KCl-precontraction and hydroperoxide-enhanced contraction (**Fig 6c**).

In the presence of dantrolene, the contractile responses to H_2O_2 and *tert*.-BHP were significantly decreased. Nifedipine also reduced the contraction of H_2O_2 and

tert.-BHP. Furthermore, fasudil significantly inhibited the contractile responses to H_2O_2 and *tert*.-BHP (**Fig 6d**). However, since these drugs are also to inhibit KCl-induced contraction, their specificity has to be questioned.

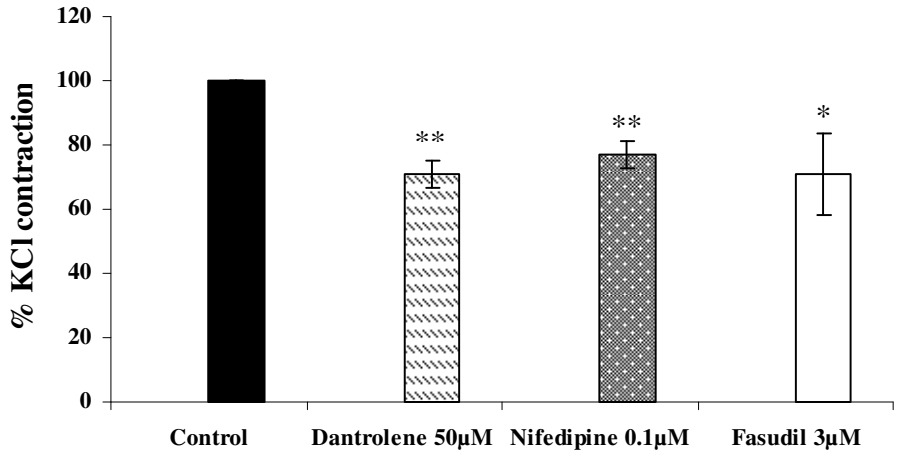


Fig 6b The effects of the Ca^{2+} channel and sensitization inhibitors on KCl-induced contraction. Results (Mean ± SD) were from 3 - 4 mice. The results were expressed as percentages of the contraction with 30 mM KCl. For dantrolene, the contraction of control was 7.2±1.6 mN and the response to dantrolene was 5.0±1.2 mN. For nifedipine, the contraction of control was 7.1±1.9 mN and the response to nifedipine was 5.5±1.8 mN. For fasudil, the contraction of control was 10.4±2.4 mN and the response to fasudil was 7.5±2.5 mN. Absolute data (mN) was used for comparison. **P*<0.05, ***P*<0.01 compared with control.

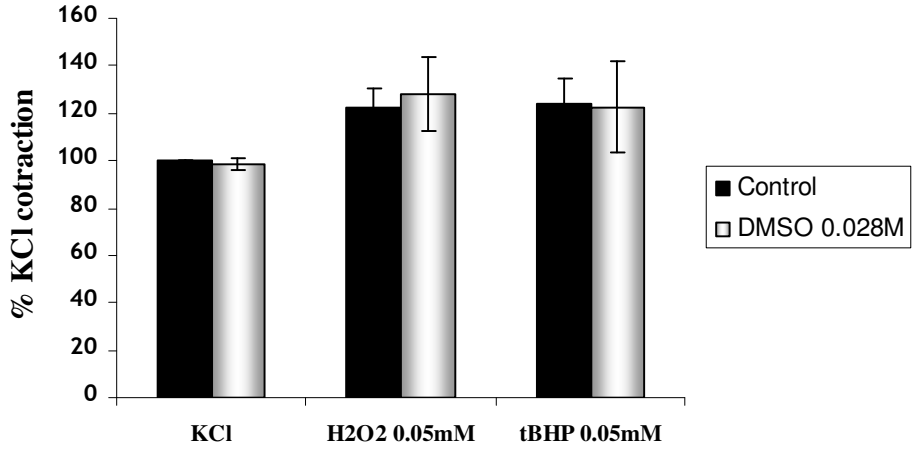


Fig 6c The effects of DMSO on KCl-induced and hydroperoxide-enhanced contraction. Results (Mean ± SD) were from 4 - 5 mice. The results were expressed as percentages of the contraction with 30 mM KCl.

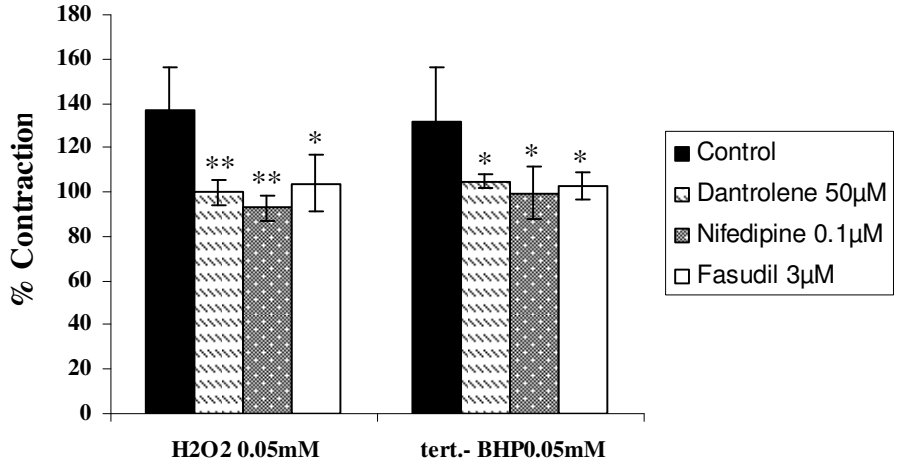


Fig 6d Effects of the Ca^{2+} channel and sensitization inhibitors on hydroperoxide-enhanced contraction. Results (Mean ± SD) were from 4 - 5 mice. The results were expressed as percentages of the contraction induced by KCl (Control group) or by KCl + one of the inhibitors (the inhibitor group). *P<0.05, **P<0.01 compared with control.

4.3.2 Effects of Voltage-sensitive K^+ Channel Inhibitor on PHE-induced and Hydroperoxide-enhanced Contraction

In order to characterize the involvement of the voltage-sensitive K^+ channel in hydroperoxide-enhanced contraction, the experiments were performed with voltage-sensitive K^+ channel inhibitor 4-AP. Since depolarisation by 30 mM KCl influences K^+ channels very strongly, stimulation with 1 µM PHE was used in these experiments. The concentration of 4-AP used in the experiments referred to Plane et al (Plane *et al.*, 2005). **Fig 7a** showed the experimental procedure: firstly, the contractile response to the hydroperoxide (0.05 mM H_2O_2 or *tert*.-BHP) was tested. The maximum contraction of hydroperoxides was expressed as a percentage of the PHE-precontraction. Then the segment was exposed to 4-AP when the precontraction had reached a plateau. The contractile response to 4-AP was expressed as a percentage of the precontraction. When the contraction of 4-AP was stable, 0.05 mM hydroperoxide (H_2O_2 or *tert*.-BHP) was added. The response to the hydroperoxide was expressed as a percentage of PHE with 4-AP.

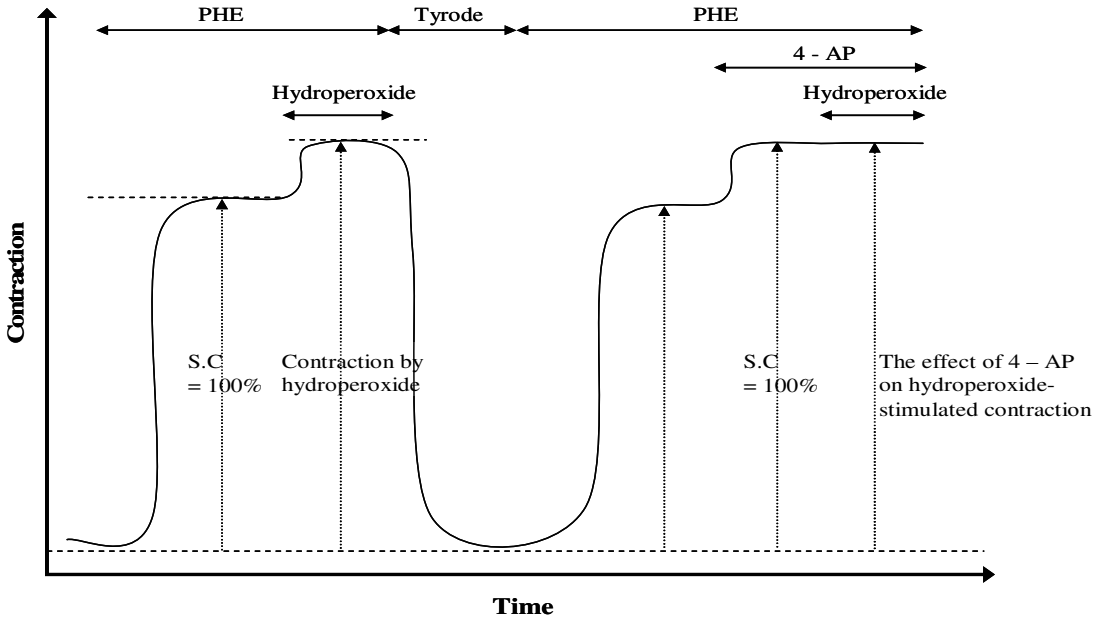


Fig 7a Typical model showing the effect of 4-AP on the hydroperoxide-enhanced contraction (H_2O_2 and *tert*.-BHP). S.C means standard contraction. The contraction was

expressed in mN. The response to the hydroperoxide in control group was expressed as a percentage of the contraction induced by PHE (1 µM). In the presence of 4-AP, the response to H_2O_2 and *tert*.-BHP was expressed as a percentage of the contraction induced by PHE with 4-AP.

The voltage sensitive K^+ channel inhibitor (0.3 mM 4-AP) significantly increased the contraction induced by 1 µM PHE (**Fig 7b**). In the following experiments, we examined the effects of 4-AP on the contractile response to hydroperoxides (0.05 mM H_2O_2 and *tert*.-BHP). 4-AP showed a greater inhibition of the hydroperoxide-enhanced contraction in WT mouse aorta (**Fig 7c**). In control group, the contraction of H_2O_2 and *tert*.-BHP amounted to 148.5 ± 14.6 % and 156.5 ± 14.4 % of PHE-precontraction. In the presence of 4-AP, the response to H_2O_2 and *tert*.-BHP amounted to 101.0 ± 7.0 % and 108.3 ± 8.2 % of PHE-precontraction with 4-AP.

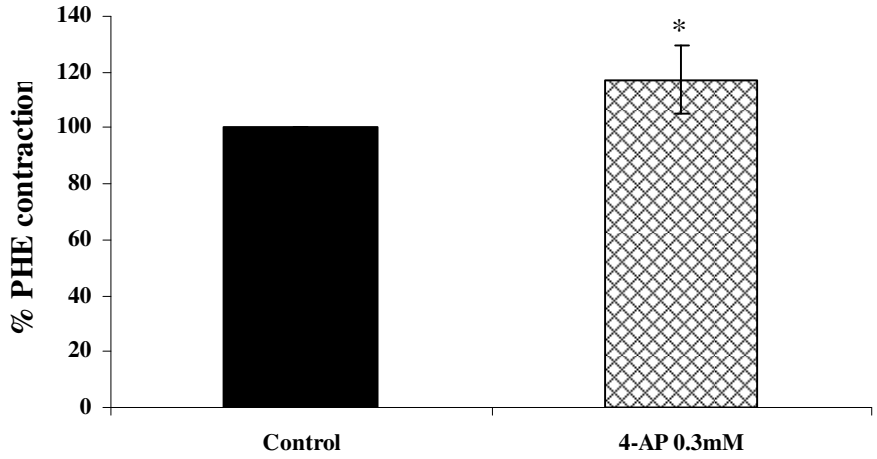


Fig 7b Effects of the voltage sensitive K^+ channel inhibitor (0.3 mM 4-AP) on PHE precontraction. Results (Mean ± SD) were from 8 WT mice. The results were expressed as percentages of the contraction with 1 µM PHE. The contraction of control was 7.2±1.5 mN and the response to 4-AP was 9.0±2.2 mN. Absolute data (mN) was used for comparison. **P*<0.05 compared with control.

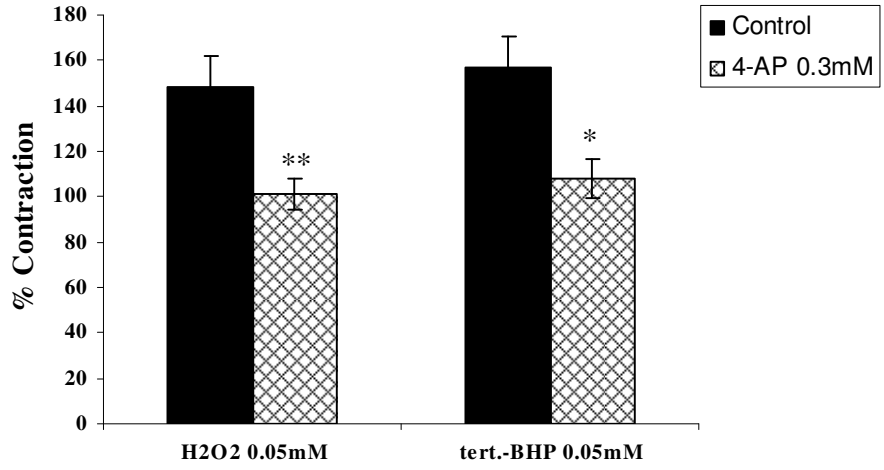


Fig 7c Effects of the voltage sensitive K^+ channel inhibitor (0.3 mM 4-AP) on hydroperoxide-enhanced contraction. Results (Means ± SD) were from 4 WT mice. The results were expressed as percentages of the contraction induced by 1 μM PHE (Control group) or by PHE + 4AP (4-AP group). **P*<0.05, ***P*<0.01 compared with control.

4.3.3 Effects of PLA_2-COX-TXA_2 Inhibitors on Hydroperoxide-enhanced Contraction

To assess the effects of PLA_2-COX-TXA_2 inhibitors, PLA_2 inhibitor (quinacrine), COX inhibitors (diclofenac, indomethacin, meclofenamic) and TXA_2 inhibitors (bupivacaine, furegrelate) were used in the experiments. The experimental procedure was shown in **Fig 8a**. Firstly, the contractile response to the hydroperoxide (0.05 mM H_2O_2 or *tert*.-BHP) was tested, followed by a wash with Tyrode solution. The response to hydroperoxides was expressed as a percentage of the contraction induced by KCl (30 mM). Then, some aortas were exposed to one of the inhibitors together with KCl solution. When the contraction was obtained, 0.05 mM hydroperoxide was added. The contraction of hydroperoxides was expressed as a percentage of the contraction induced by KCl with the inhibitor. The effect of the same amount of DMSO and ethanol on KCl precontraction and hydroperoxide-enhanced contraction was also studied since indomethacin, diclofenac and meclofenamic were initially dissolved in DMSO, and bupivacaine was dissolved in ethanol to prepare the stock solution.

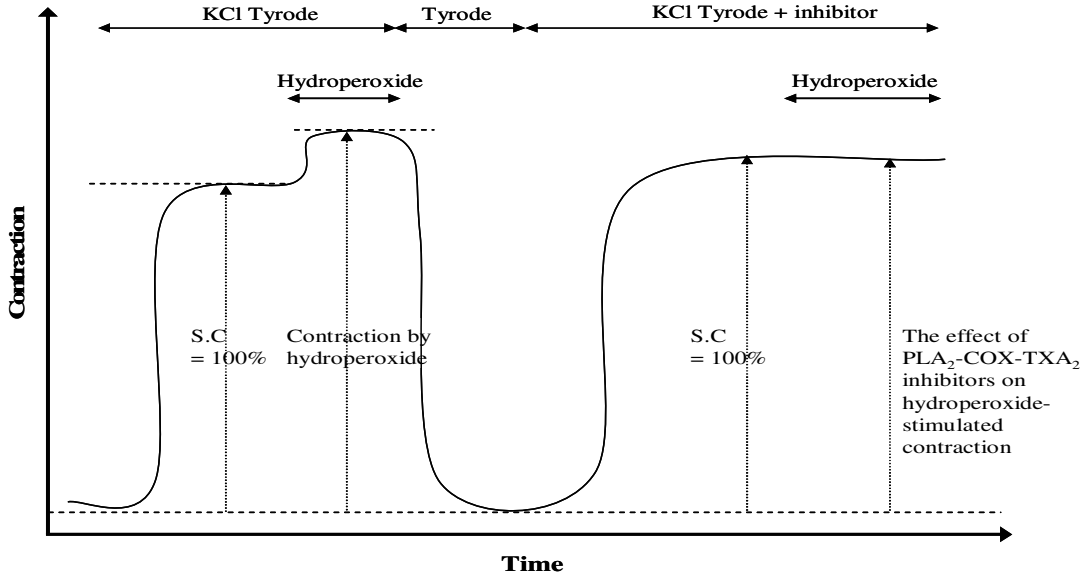


Fig 8a Typical model showing the effects of PLA_2-COX-TXA_2 inhibitors on hydroperoxide-enhanced contraction (H_2O_2 and *tert*.-BHP). S.C means standard contraction. The contraction was expressed in mN. The response to the hydroperoxide in the control group was expressed as a percentage of 30 mM KCl-precontraction. In the presence of the inhibitor, the response to H_2O_2 and *tert*.-BHP was expressed as a percentage of the contraction induced by KCl with the inhibitor.

4.3.3.1 Effects of PLA_2 Inhibitor (quinacrine)

As shown in **Fig 8b**, 10 µM quinacrine had no effect on KCl precontraction, while 50 µM quinacrine significantly decreased the contraction induced by KCl. The concentrations of quinacrine used in the experiments referred to Tiritilli et al (Tiritilli *et al.*, 2004). In the experimental procedure, the hydroperoxide was added when the contraction of KCl with the inhibitor was stable since the inhibitor at higher concentration had a decreasing effect on KCl precontraction. Since 10 µM quinacrine was not effective in *tert*.-BHP group, 50 µM quinacrine was used. Furthermore, we observed that quinacrine significantly abolished the contractile response to H_2O_2 and *tert*.-BHP (**Fig 8c**, **8d**). The results suggested that PLA_2 was involved in the contractile response to both hydroperoxides.

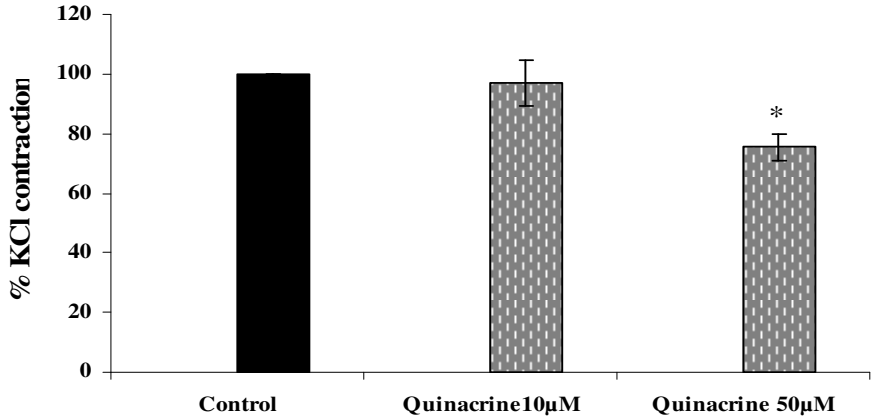


Fig 8b Effects of quinacrine on KCl precontraction. Results (Mean ± SD) were from 3 - 5 WT mice. The results were expressed as percentages of 30 mM KCl-precontraction. For 10 μM quinacrine group, the contraction of control was 5.0±2.4 mN and the response to quinacrine was 4.9±2.3 mN. For 50 μM quinacrine group, the contraction of control was 6.0±3.2 mN and the response to quinacrine was 4.4±2.1 mN. Absolute data (mN) was used for comparison. **P*<0.05. compared with control.

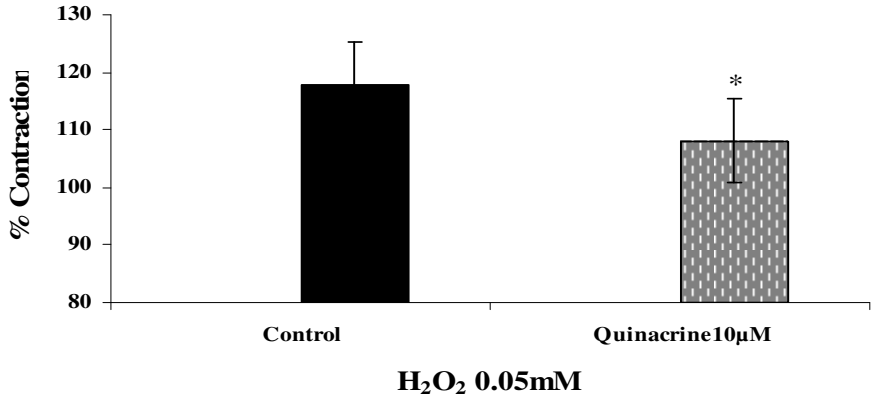


Fig 8c Effects of quinacrine on H_2O_2-enhanced contraction. Results (Mean ± SD) were from 5 WT mice. The results were expressed as percentages of the contraction induced by 30 mM KCl (Control group) or by KCl + quinacrine (quinacrine group). **P*<0.05 compared with control.

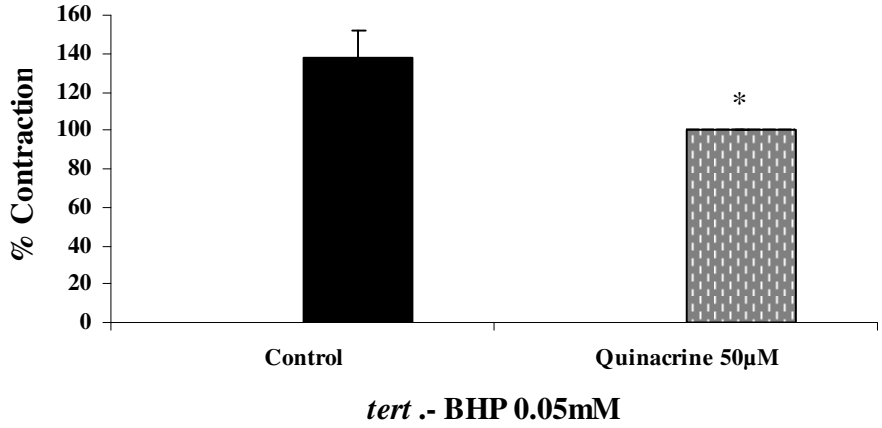


Fig 8d Effects of quinacrine on *tert.*-BHP-enhanced contraction. Results (Means ± SD) were from 3 WT mice. The results were expressed as percentages of the contraction induced by 30 mM KCl (Control group) or by KCl + quinacrine (quinacrine group). **P*<0.05 compared with control.

4.3.3.2 Effects of COX Inhibitors (diclofenac, indomethacin, meclofenamic)

The concentrations of diclofenac, indomethacin and meclofenamic used in the experiments referred to Gao et al (Gao *et al.*, 2003), Tiritilli et al (Tiritilli *et al.*, 2004) and Climent et al (Climent *et al.*, 2005). Diclofenac (20 µM) and meclofenamic (20 µM) had no effects on the contraction induced by KCl, but indomethacin (200 µM) significantly abolished the contractile response to KCl (**Fig 9a**). Moreover, **Fig 9b**, **9c** showed that diclofenac, meclofenamic and indomethacin significantly inhibited the contraction of hydroperoxides (H_2O_2 and *tert.*-BHP), indicating that COX was involved in the hydroperoxide-enhanced contraction. The same amount of DMSO had no effects on KCl-precontraction and hydroperoxide-enhanced contraction (data not shown).

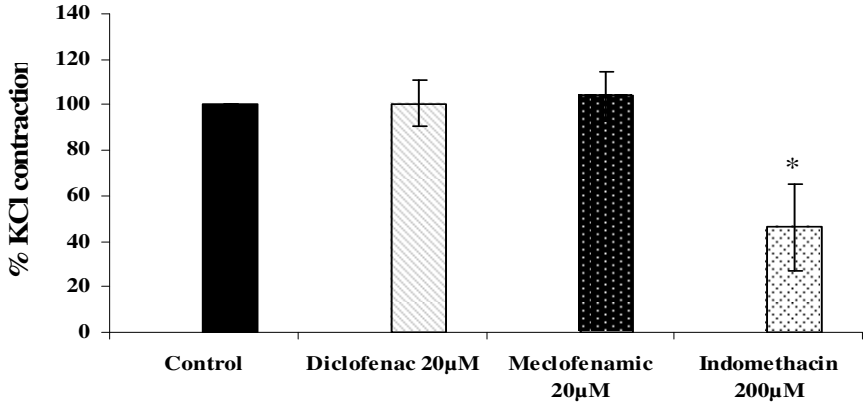


Fig 9a Effects of COX inhibitors (diclofenac, meclofenamic and indomethacin) on KCl precontraction. Results (Mean ± SD) were from 3 - 4 WT mice. The results were expressed as percentages of 30 mM KCl-precontraction. For diclofenac, the contraction of control was 7.8±1.3 mN and the response to diclofenac was 7.9±1.5 mN. For meclofenamic, the contraction of control was 3.8±1.5 mN and the response to meclofenamic was 3.6±1.3 mN. For indomethacin, the contraction of control was 9.6±3.4 mN and the response to indomethacin was 4.9±2.6 mN. Absolute data (mN) was used for comparison.**P*<0.05 compared with control.

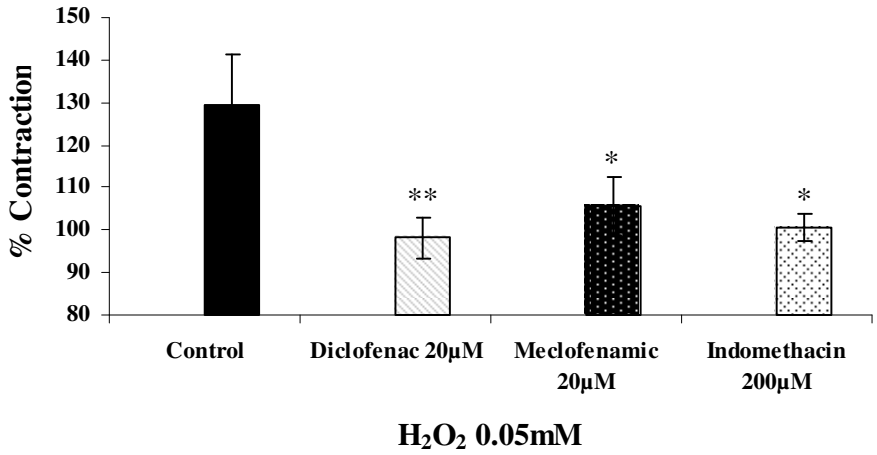


Fig 9b Effects of COX inhibitors (diclofenac, meclofenamic and indomethacin) on H_2O_2-enhanced contraction. Results (Mean ± SD) were from 3 - 4 WT mice. The results were expressed as percentages of the contraction induced by 30 mM KCl (Control group) or by KCl + one of the inhibitors (the inhibitor group).**P*<0.05, ***P*<0.01 compared with control.

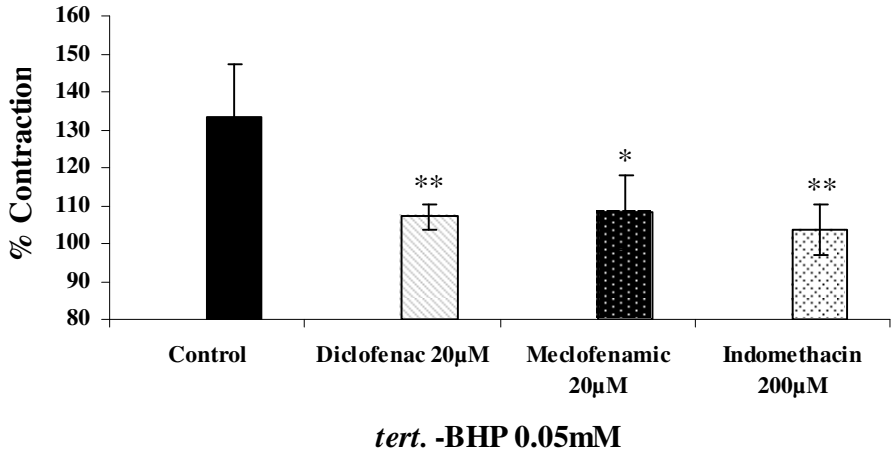


Fig 9c Effects of COX inhibitors (diclofenac, meclofenamic and indomethacin) on *tert.*-BHP-enhanced contraction. Results (Mean ± SD) were from 4 - 5 WT mice. The results were expressed as percentages of the contraction induced by 30 mM KCl (Control group) or by KCl + one of the inhibitors (the inhibitor group). **P*<0.05, ***P*<0.01 compared with control.

4.3.3.3 Effects of TXA_2 Inhibitors (bupivacaine, furegrelate)

The concentrations of bupivacaine and furegrelate used in the experiments referred to Hahnenkamp et al (Hahnenkamp *et al.*, 2004) and Hernanz et al (Hernanz *et al.*, 2003). As shown in **Fig 10a**, bupivacaine (0.1 mM) had no effect on 30 mM KCl-precontraction, but significantly increased the contraction induced by 1 µM PHE. In KCl- and PHE-precontraction, bupivacaine abolished the hydroperoxide (H_2O_2 or *tert.*-BHP) enhanced contraction (**Fig 10b**, **10c**). Since bupivacaine was dissolved by ethanol, the effect of ethanol on contractions was also considered. The same concentration of ethanol had no effect on KCl-, PHE-precontraction and hydroperoxide-enhanced contraction (data not shown).

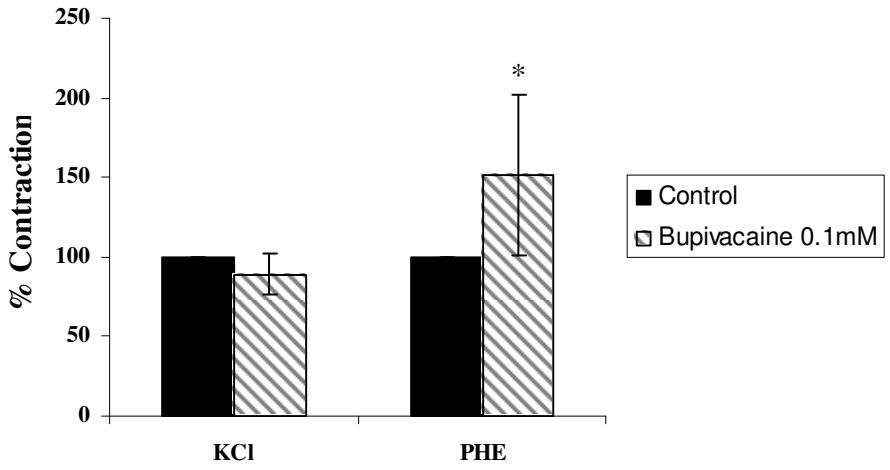


Fig 10a Effects of bupivacaine on KCl- and PHE-precontraction. Results (Mean ± SD) were from 5 - 6 WT mice. In KCl group, the results were expressed as percentages of 30 mM KCl-precontraction. The contraction of control was 6.4±0.9 mN and the response to bupivacaine was 5.9±1.0 mN. In PHE group, the results were expressed as percentages of 1 μM PHE-induced contraction. The contraction of control was 8.9±0.4 mN and the response to bupivacaine was 13.9±1.6 mN. Absolute data (mN) was used for comparison. **P*<0.05 compared with control.

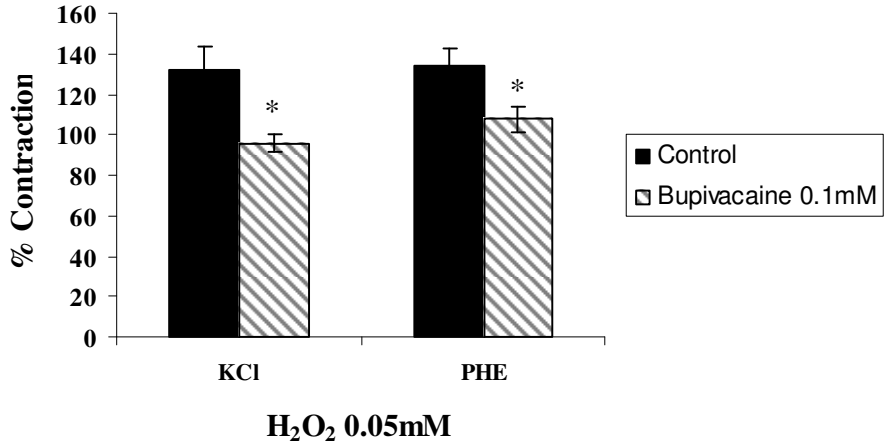


Fig 10b Effects of bupivacaine on H_2O_2-enhanced contraction. Results (Mean ± SD) were from 3 WT mice. The results were expressed as percentages of the contraction induced by 30 mM KCl and PHE (Control group) or by KCl (PHE) + bupivacaine (bupivacaine group). **P*<0.05 compared with control.

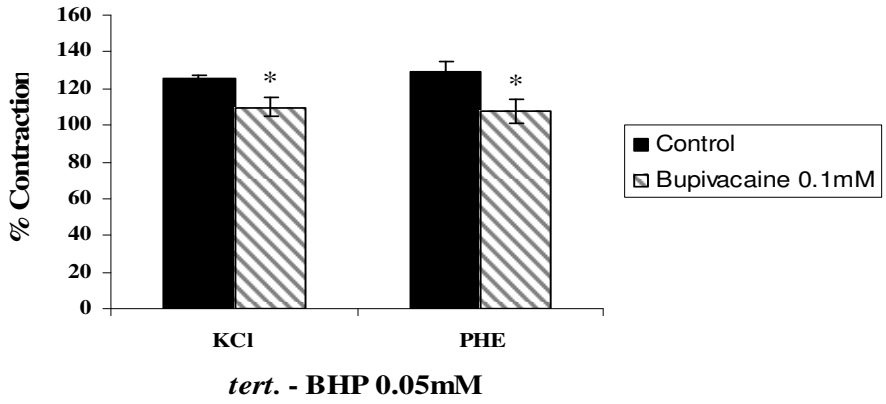


Fig 10c Effects of bupivacaine on *tert.*-BHP-enhanced contraction. Results (Mean ± SD) were from 3 WT mice. The results were expressed as percentages of the contraction induced by 30 mM KCl and PHE (Control group) or by KCl (PHE) + bupivacaine (bupivacaine group). **P*<0.05 compared with control.

The effects of another TXA_2 inhibitor (furegrelate) were also analysed. 5 µM furegrelate abolished the contractile response to H_2O_2. Furegrelate also decreased contraction by *tert.*-BHP, but due to large variation this effect did not reach significance (**Fig 11a**). In contrast, furegrelate did not significantly affect the contraction induced by 30 mM KCl (**Fig 11b**).

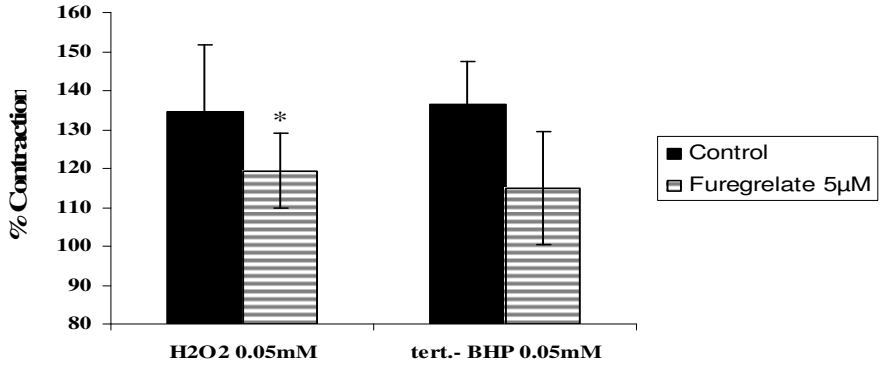


Fig 11a Effects of TXA_2 inhibitor (5 µM furegrelate) on the hydroperoxide-enhanced contraction. Results (Mean ± SD) were from 3 - 7 WT mice. The results were expressed as percentages of the contraction induced by 30 mM KCl (Control group) or by KCl + furegrelate (furegrelate group). **P*<0.05 compared with control.

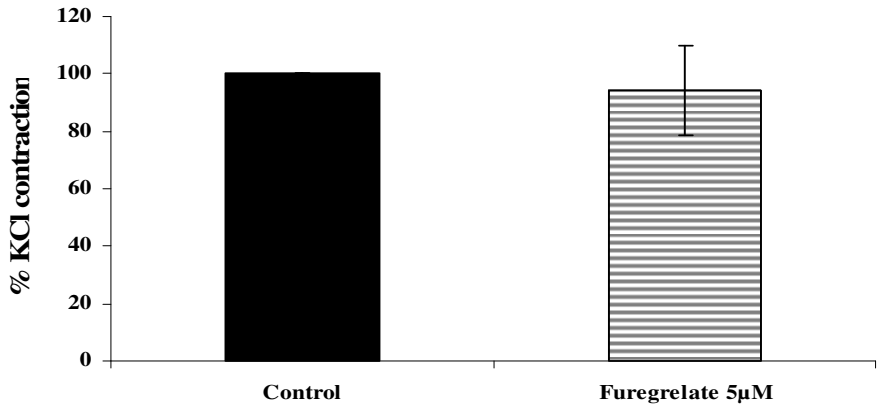


Fig 11b Effects of TXA_2 inhibitor (5 µM furegrelate) on KCl-induced contraction. Results (Means ± SD) were from 6 WT mice. The results were expressed as percentages of 30 mM KCl-induced contraction. The contraction of control was 2.9±0.9 mN and the response to furegrelate was 2.6±1.1 mN.

4.3.4 Effects of PLC Inhibitor and AT1 Receptor Antagonist

4.3.4.1 Effects of PLC Inhibitor (NCDC)

To study the role of PLC in the response to hydroperoxides, PLC inhibitor (NCDC) was used in the hydroperoxide-enhanced contraction. The concentration of NCDC used in the experiments referred to Tostes (Tostes *et al.*, 1996). The experimental procedure to study the effects of PLC inhibitor was shown in **Fig 12a, 12b**. The contractile response to the hydroperoxide (0.05 mM H_2O_2 or *tert*.-BHP) was first tested. The response to hydroperoxides was expressed as a percentage of the contraction induced by KCl (30 mM) or PHE (1 µM). Then the segment was exposed to NCDC when the precontraction had reached a plateau. The response to NCDC was expressed as a percentage of the precontraction. When the response to NCDC was stable, 0.05 mM hydroperoxide (H_2O_2 or *tert*.-BHP) was added. The response to the hydroperoxide was expressed as a percentage of the precontraction with NCDC.

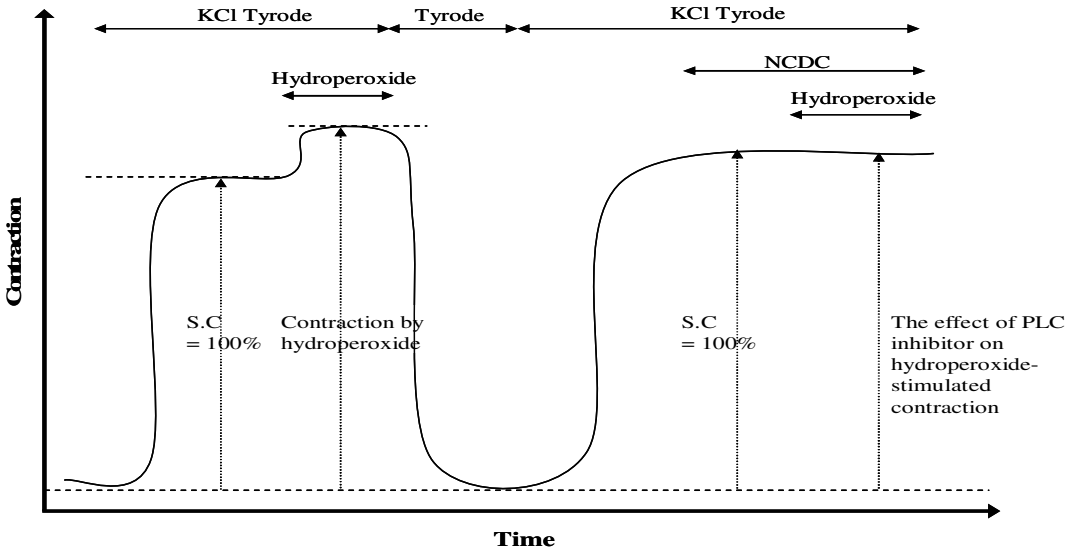


Fig 12a Typical model showing the effect of NCDC on the hydroperoxide-enhanced contraction (H_2O_2 and *tert*.-BHP) induced by KCl. S.C means standard contraction. The contraction was expressed in mN. The contractial response to the hydroperoxide in control group was expressed as a percentage of 30 mM KCl-precontraction. In the presence of NCDC, the response to H_2O_2 and *tert*.-BHP was expressed as a percentage of the contraction induced by KCl with NCDC.

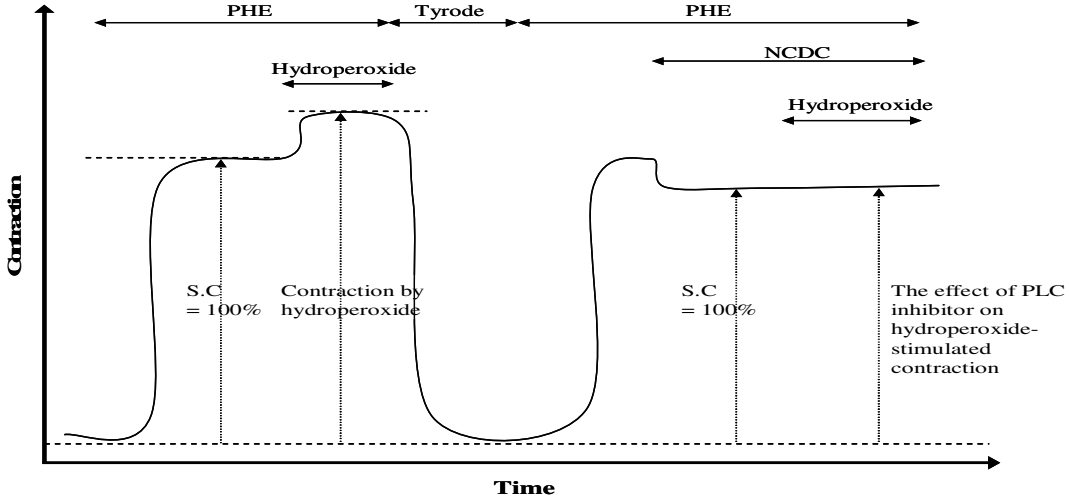


Fig 12b Typical model showing the effect of NCDC on the hydroperoxide-enhanced contraction (H_2O_2 and *tert*.-BHP) induced by PHE. S.C means standard contraction. The contraction was expressed in mN. The contractial response to the hydroperoxide in control group was expressed as a percentage of 1 µM PHE-precontraction. In the presence of NCDC, the response to H_2O_2 and *tert*.-BHP was expressed as a percentage of the contraction induced by PHE with NCDC.

We observed that 0.02 mM NCDC practically abolished the contractile response induced by hydroperoxides (H_2O_2 and *tert.*-BHP) in KCl and PHE precontraction, respectively (**Fig 12c, 12d**). Additionally, NCDC did not cause effect on the contractile response elicited by 30 mM KCl, whereas NCDC significantly reduced the contraction induced by 1 μM PHE (**Fig 12e**).

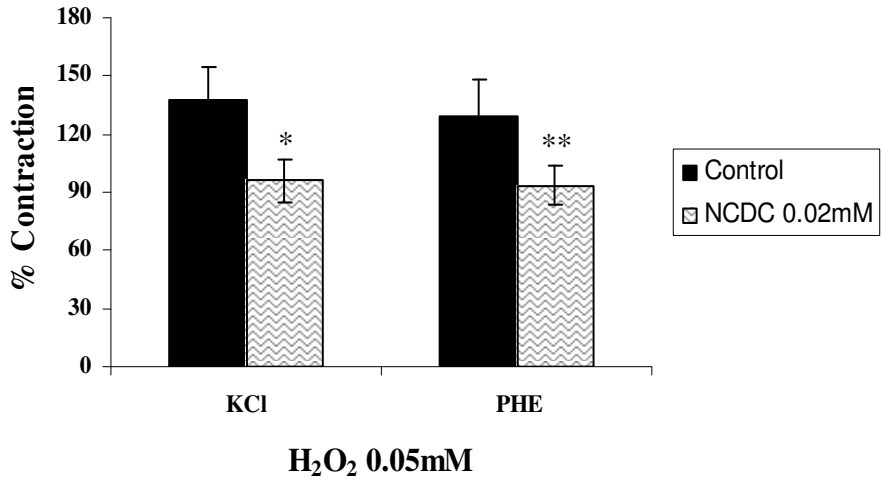


Fig 12c Effects of PLC inhibitor (0.02 mM NCDC) on H_2O_2-enhanced contraction. Results (Mean ± SD) were from 4 - 6 WT mice. The results were expressed as percentages of the contraction induced by 30 mM KCl (Control group) or by KCl + NCDC (NCDC group). **P*<0.05, ***P*<0.01 compared with control.

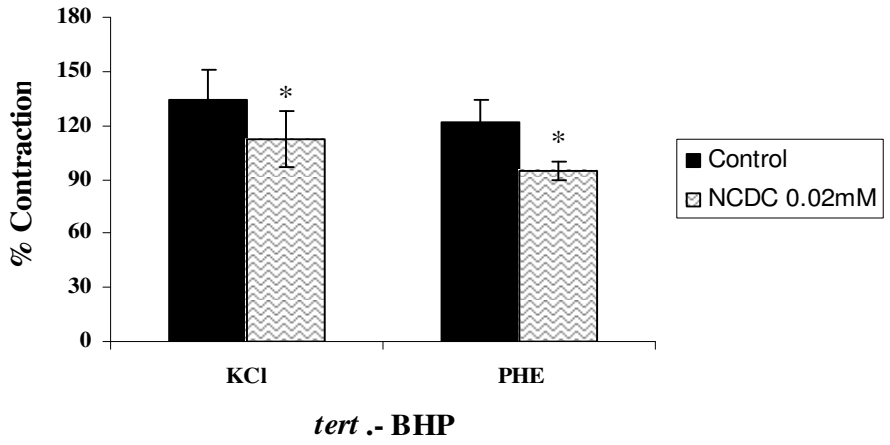


Fig 12d Effects of PLC inhibitor (0.02 mM NCDC) on *tert.*-BHP-enhanced contraction. Results (Mean ± SD) were from 3 - 4 WT mice. The results were expressed as percentages of the contraction induced by 30 mM KCl (Control group) or by KCl + NCDC (NCDC group).**P*<0.05 compared with control.

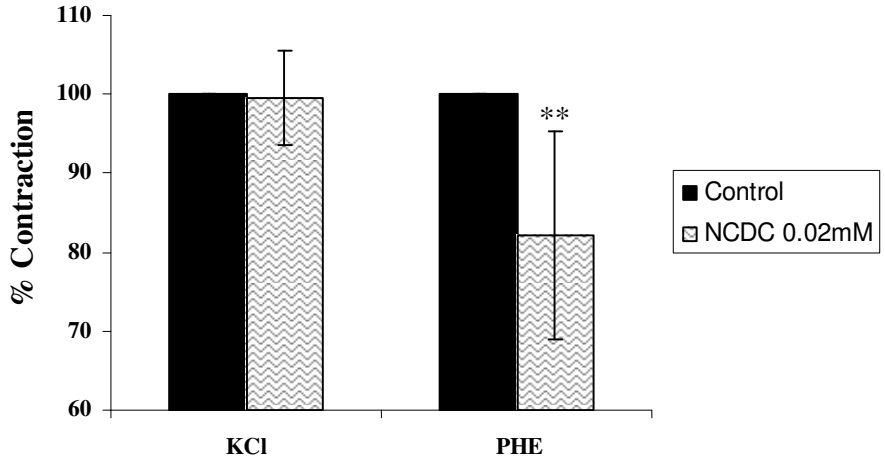


Fig 12e Effects of NCDC on KCl- and PHE- precontraction. Results (Mean ± SD) were from 5 - 8 WT mice. In KCl group, the results were expressed as percentages of 30 mM KCl-induced contraction. The contraction of control was 6.6±3.8 mN and the response to NCDC was 6.7±3.8 mN. In PHE group, the results were expressed as percentages of the 1 μM PHE-induced contraction. The contraction of control was 10.0±4.7 mN and the response to NCDC was 7.8±3.9 mN. Absolute data (mN) was used for comparison. ***P*<0.01 compared with control.

4.3.4.2 The effect of AT1 Receptor Antagonist on H_2O_2-enhanced Contraction

To characterize the effect of AT1 receptor on the contractile response to H_2O_2, valsartan was used in the experiment. The concentration of valsartan used in the experiments referred to Arun et al (Arun *et al.*, 2004). The experimental procedure was shown in **Fig 13a**. The contractile response to 0.05 mM H_2O_2 was firstly tested. The response to H_2O_2 was expressed as a percentage of the contraction induced by KCl (30 mM). Then the segment was exposed to KCl with 10 μM valsartan. When the precontraction had reached a plateau, 0.05 mM H_2O_2 was added. The contractile response to H_2O_2 was expressed as a percentage of the contraction induced by KCl with valsartan. We observed that valsartan significantly decreased the contraction of H_2O_2 (**Fig 13b**).

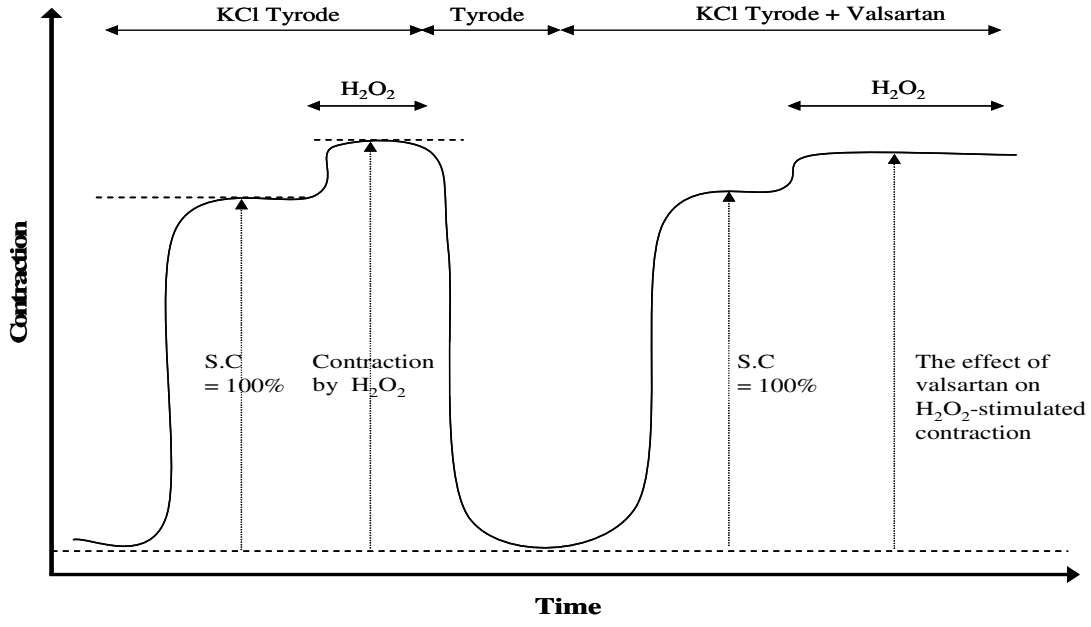


Fig 13a Typical model showing the effect of valsartan on H_2O_2-enhanced contraction. S.C means standard contraction. The contraction was expressed in mN. The contractial response to H_2O_2 in control group was expressed as a percentage of the contraction induced by KCl (30 mM). In the presence of valartan, the response to H_2O_2 was expressed as a percentage of the contraction induced by KCl (30 mM) with valsartan.

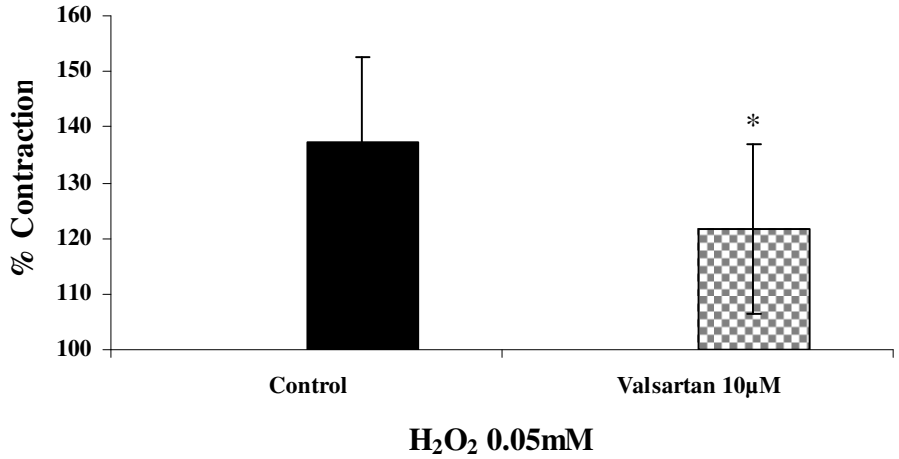


Fig 13b The effect of AT1 recptor antagonist (10 μM valsartan) on H_2O_2-enhanced contraction. Results (Mean ± SD) were from 6 WT mice. The result was expressed as a percentage of the contraction induced by 30 mM KCl (Control group) or by KCl + valsartan (valsartan group). **P*<0.05 compared with control.

4.4 Effects of Hydroperoxides on the Contraction in GPx-1$^{-/-}$ Mouse Aorta

4.4.1 Effects of Hydroperoxides on KCl-induced Contraction

The experimental procedure of the contractile response to hydroperoxides in GPx-1$^{-/-}$ was the same as in WT (**Fig 1**). In GPx-1$^{-/-}$ mouse aorta, H_2O_2 increased KCl-precontraction at the concentrations of 0.01-0.25 mM, but produced relaxation at the concentration of 0.5 mM. The maximum response was reached at 0.125 mM (143.9 ± 28.8 % of the response to 30 mM KCl, **Fig 14a**). At the concentration of 0.05 mM, H_2O_2 induced bigger contractile response in GPx-1$^{-/-}$ than in WT mice. *tert.*-BHP [0.01-0.125 mM] also enhanced 30 mM KCl-precontraction, reached a peak at 0.01 mM (123.8 ± 12.7 % of the response to 30 mM KCl), and caused relaxation at 0.25 mM. At the concentration of 0.01 mM, *tert.*-BHP produced bigger contraction in GPx-1$^{-/-}$ than in WT mice (**Fig 14b**).

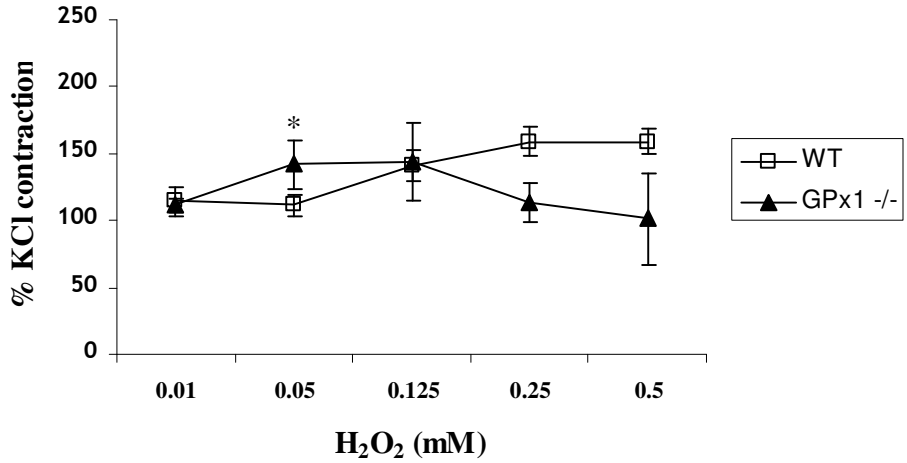


Fig 14a Concentration-response curve of H_2O_2 in GPx-$1^{-/-}$ and WT mouse aorta. Results (Mean ± SD) were from 5 - 8 mice. The results were expressed as percentages of the contraction with 30 mM KCl. *P<0.05 compared with WT.

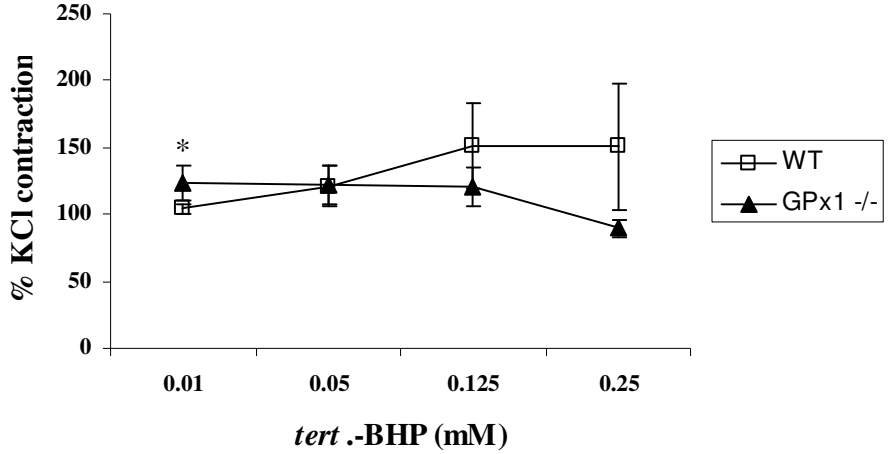


Fig 14b Concentration-response curve of *tert*.-BHP in GPx-$1^{-/-}$ and WT mouse aorta. Results (Mean ± SD) were from 4 - 10 mice. The results were expressed as percentages of the contraction with 30 mM KCl.*P<0.05 compared with WT.

4.4.2 Effects of Endothelium on Hydroperoxide-enhanced Contraction

4.4.2.1 Effects of ACh on Hydroperoxide-enhanced Contraction

The experimental procedure in GPx-$1^{-/-}$ was the same as in WT (**Fig 3a**). ACh [1-100 µM] induced relaxation in GPx-$1^{-/-}$ mouse aorta. There was no significant difference between GPX-$1^{-/-}$ and WT (**Fig 15a**). We selected 5 µM ACh for the further investigation on the hydroperoxide-enhanced contraction. As shown in **Fig 15b**, ACh [5 µM] significantly decreased the contraction induced by both hydroperoxides in GPx-$1^{-/-}$ mouse. The response to ACh in H_2O_2-enhanced contraction amounted to 90.0 ± 5.0 % of H_2O_2-enhanced contraction ($p<0.01$, $n = 5$). With *tert*.-BHP, the response amounted to 96.3 ± 3.6 % of *tert*.-BHP-enhanced contraction ($p<0.05$, $n=5$).

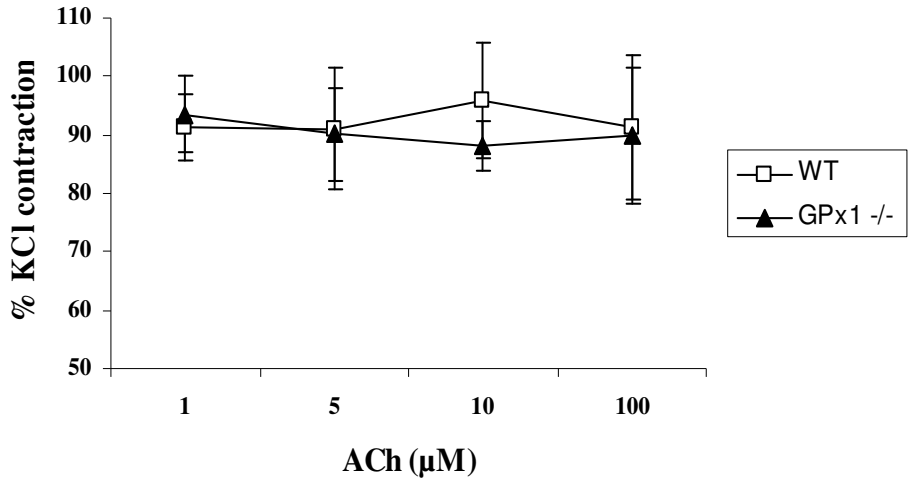


Fig 15a Concentration-response curve of ACh in GPx-$1^{-/-}$ and WT mouse aorta. Results (Mean ± SD) were from 4 - 14 mice. The results were expressed as percentages of the contraction induced by 30 mM KCl.

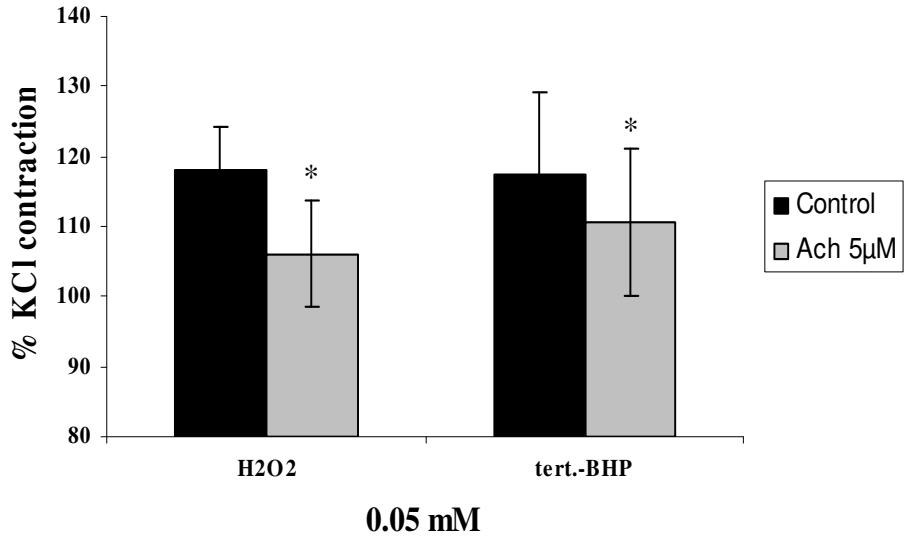


Fig 15b Effects of 5 µM ACh on the hydroperoxide-enhanced contraction in GPx-$1^{-/-}$. Results (Mean ± SD) were from 5 mice. The results were expressed as percentages of the contraction induced by 30 mM KCl. **P*<0.05 compared with control.

4.4.2.2 Effects of L-NAME on Hydroperoxide-enhanced Contraction

In GPx-$1^{-/-}$ mouse aorta, L-NAME [0.05 mM] did not significantly increase 30 mM KCl-induced contraction (**Fig 16a**). The response to 0.05 mM L-NAME amounted to 104.3 ± 11.0 % of KCl-precontraction ($p>0.05$, $n = 7$). Moreover, 0.05 mM L-NAME did not significantly increase the hydroperoxide-enhanced contraction in GPx-$1^{-/-}$ mouse aorta (**Fig 16b**).

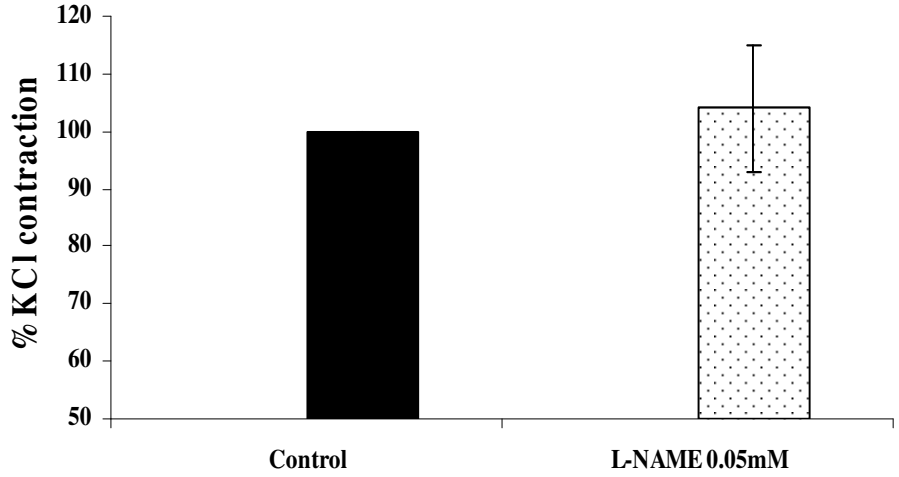


Fig 16a The effect of L-NAME on 30 mM KCl-precontraction in GPx-$1^{-/-}$ mouse aorta. Results (Mean ± SD) were from 7 mice. The results were expressed as percentages of the contraction with 30 mM KCl. The contraction of control was 8.5±6.8 mN and the response to L-NAME was 8.4±5.8 mN. Absolute data (mN) was used for comparison.

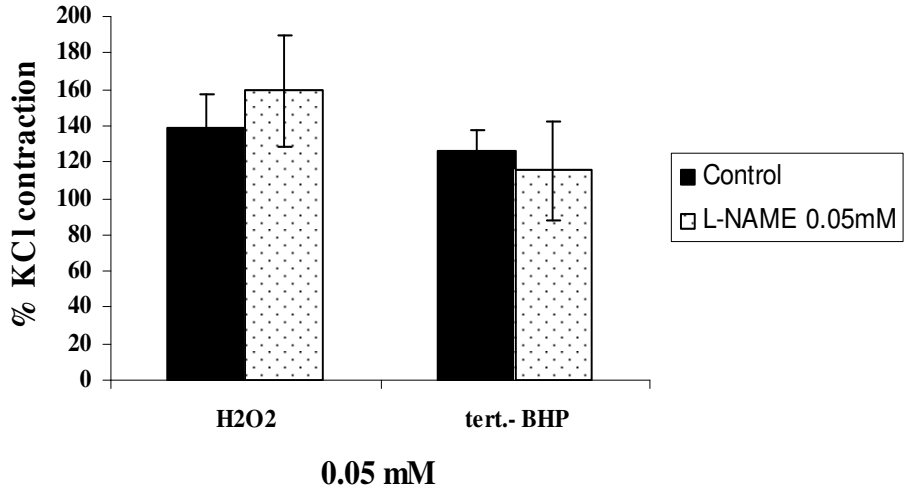


Fig 16b The effect of 0.05 mM L-NAME on the hydroperoxide-enhanced contraction in GPx-$1^{-/-}$ mouse aorta. Results (Mean ± SD) were from 3 - 5 mice. The results were expressed as percentages of the contraction with 30 mM KCl.

4.4.2.3 Effects of Indomethacin on Hydroperoxide-enhanced Contraction

The effect of indomethacin (COX inhibitor) in GPx-$1^{-/-}$ was studied. The experimental procedure was the same as in WT (**Fig 8a**). In this study, indomethacin [200 µM] significantly decreased the contraction induced by 30 mM KCl. In GPx-$1^{-/-}$ and WT mouse aorta, the response to indomethacin amounted to 61.4 ± 11.8 % and 46.3 ± 11.0 % of KCl-precontraction, respectively. It seemed that the inhibition of indomethacin in WT was bigger than in GPx-$1^{-/-}$ mouse aorta, but there was no significant difference between WT and GPx-$1^{-/-}$ (**Fig 17a**). Furthermore, indomethacin significantly abolished the contractile response to H_2O_2 in GPx-$1^{-/-}$, which was the same as in WT mouse aorta (**Fig 17b**).

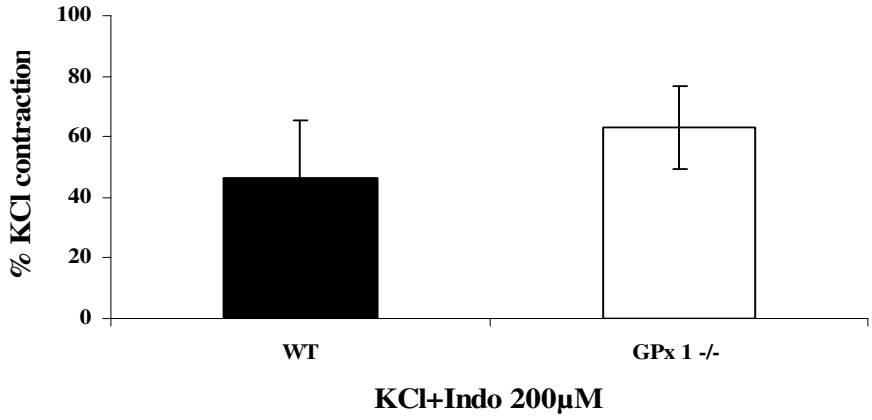


Fig 17a The effect of 200 µM indomethacin on KCl-induced contraction in WT and GPx-$1^{-/-}$mouse aorta. Results (Mean ± SD) were from 3 mice. The results were expressed as percentages of the contraction induced by 30 mM KCl.

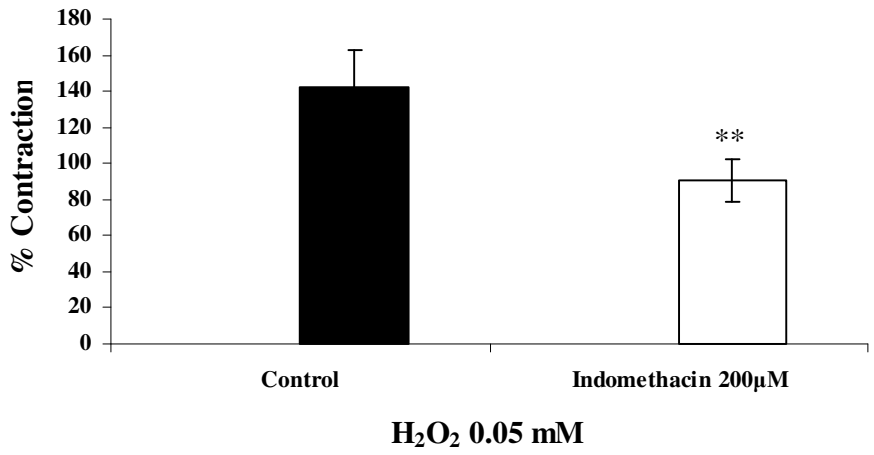


Fig 17b The effect of 200 µM indomethacin on H_2O_2-enhanced contraction in GPx-$1^{-/-}$ mouse aorta. Results (Mean ± SD) were from 4 mice. The results were expressed as percentages of the contraction induced by 30 mM KCl (Control group) or by KCl + indomethacin (indomethacin group). $^{**}P<0.01$ compared with control.

4.5 Chemiluminescence Assays

4.5.1 H_2O_2 on ROS Production in WT and GPx-$1^{-/-}$ Mouse Aorta

Table 1 summarized the effects of the different concentrations of H_2O_2 on luminol-detectable ROS production in WT and GPx-$1^{-/-}$ mouse aorta. Without hydroperoxides, WT and GPx-$1^{-/-}$ mouse aorta alone can produce very low ROS (0.03 ± 0.009 and 0.02 ± 0.007 RLU, respectively) in the presence of luminol. In the presence of H_2O_2, CL signal significantly increased by a dose dependent way in WT and GPx-$1^{-/-}$ mouse aorta. Although the CL signal under control conditions (without tissue) increased in H_2O_2 dose-dependence, it was much lower than in WT and GPx-$1^{-/-}$ mouse aorta. The results indicated that the mouse aorta itself produced much ROS in the presence of H_2O_2.

Luminol			
H_2O_2	**Control** RLU	**WT** RLU (*RLU/mg*)	**GPx-$1^{-/-}$** RLU (*RLU/mg*)
0.01 mM	0.01 ± 0.003	0.07 ± 0.02 (*0.005 ± 0.002*)	0.08 ± 0.03 (*0.005 ± 0.002*)
0.05 mM	0.02 ± 0.002	13.1 ± 11.2 (*1.3 ± 1.7*)	14.1 ± 7.5 (*1.4 ± 0.7*)
0.125 mM	0.03 ± 0.006	393.4 ± 85.9 (*40.2 ± 16.0*)	197.1 ± 139.3 (*30.1 ± 29.3*)
0.25 mM	0.07 ± 0.01	368.2 ± 145.5 (*83.0 ± 63.3*)	494.7 ± 428.7 (*33.9 ± 31.1*)
0.5 mM	0.2 ± 0.05	1738.3 ± 958.9 (*182.6 ± 125.8*)	728.1 ± 518.9 (*101.2 ± 58.4*)
1 mM	343.3 ± 132.5	2559.8 ± 1928.7 (*182.9 ± 151.9*)	2845.3 ± 1467.0 (*265.6 ± 160.7*)

Table 1 H_2O_2 induced ROS production in WT and GPx-$1^{-/-}$ mouse aorta. CL was expressed as relative light units (RLU) and *RLU/mg* (Mean ± SD). Control, without tissue; WT, GPx-$1^{-/-}$ in the presence of WT and GPx-$1^{-/-}$ mouse aorta (n = 3 – 5).

Table 2 showed lucigenin-detectable superoxide production in WT and GPx-$1^{-/-}$ mouse aorta in the different concentrations of H_2O_2. Without hydroperoxides, GPx-$1^{-/-}$ and WT mouse aorta alone produced CL signal 0.1 ± 0.1 and 0.1 ± 0.06, respectively in the presence of lucigenin. Although lucigenin-detectable CL signal induced by higher concentrations of H_2O_2 was much lower than luminol-derived CL signal, WT and GPx-$1^{-/-}$ mouse aorta generated more superoxide in the presence of H_2O_2. There was insignificant difference between GPx-$1^{-/-}$ and WT mouse aorta. The results indicated that H_2O_2 generated more superoxide in WT and GPx-$1^{-/-}$ mouse aorta.

Lucigenin			
H_2O_2	**Control** RLU	**WT** RLU (*RLU/mg*)	**GPx-$1^{-/-}$** RLU (*RLU/mg*)
0.01 mM	0.02 ± 0.01	0.1 ± 0.06 (*0.007 ± 0.002*)	0.04 ± 0.003 (*0.004 ± 0.0005*)
0.05 mM	0.03 ± 0.005	0.1 ± 0.004 (*0.02 ± 0.02*)	0.1 ±0.01 (*0.02 ± 0.005*)
0.125 mM	0.06 ± 0.006	0.4 ± 0.08 (*0.04 ± 0.009*)	0.2 ±0.02 (*0.03 ± 0.006*)
0.25 mM	0.09 ± 0.007	0.3 ± 0.02 (*0.07 ± 0.03*)	0.2 ±0.03 (*0.02 ± 0.001*)
0.5 mM	0.1 ± 0.02	0.4 ± 0.2 (*0.08 ± 0.005*)	0.7 ±0.3 (*0.08 ± 0.003*)
1 mM	0.2 ± 0.004	0.4 ± 0.1 (*0.05 ± 0.02*)	0.5 ±0.09 (*0.1 ± 0.02*)

Table 2 H_2O_2 stimulated superoxide production in WT and GPx-$1^{-/-}$ mouse aorta. CL was expressed as relative light units (RLU) and *RLU/mg* (Mean ± SD). Control, without tissue; WT, GPx-$1^{-/-}$, in the presence of WT and GPx-$1^{-/-}$ mouse aorta (n = 3 – 5).

4.5.2 *tert.*-BHP on ROS Production in WT and GPx-1$^{-/-}$ Mouse Aorta

Table 3 summarized the effects of the different concentrations of *tert.*-BHP on luminol-detectable ROS production in WT and GPx-1$^{-/-}$ mouse aorta. In general, the luminol-derived CL signal was low in the different concentrations of *tert.*-BHP. Compared to H_2O_2, *tert.*-BHP generated much lower ROS in WT and GPx-1$^{-/-}$ mouse aorta. In higher concentrations of *tert.*-BHP (0.5, 1 mM), ROS production in GPx-1$^{-/-}$ was more than in WT mouse aorta, but there was not much difference for other concentrations. The results showed that *tert.*-BHP had no much effect on ROS generation in WT and GPx-1$^{-/-}$ mouse aorta.

Luminol			
tert. – BHP	**Control** RLU	**WT** RLU (*RLU/mg*)	**GPx-1$^{-/-}$** RLU (*RLU/mg*)
0.01 mM	0.01 ± 0.003	0.04 ± 0.02 (*0.003 ± 0.001*)	0.03 ± 0.005 (*0.004 ± 0.001*)
0.05 mM	0.01 ± 0.001	0.07 ± 0.06 (*0.008 ± 0.008*)	0.03 ± 0.004 (*0.005 ± 0.002*)
0.125 mM	0.07 ± 0.005	0.2 ± 0.05 (*0.009 ± 0.001*)	0.05 ± 0.02 (*0.007 ± 0.003*)
0.25 mM	0.4 ± 0.08	1.9 ± 0.9 (*0.2 ± 0.1*)	0.9 ± 1.3 (*0.2 ± 0.3*)
0.5 mM	1.3 ± 0.02	22.8 ± 12.8 (*4.2 ± 4.3*)	56.9 ± 22.7 (*10.9 ± 4.3*)
1 mM	0.5 ± 0.9	18.6 ± 2.8 (*1.7 ± 0.4*)	73.4 ± 51.3 (*11.1 ± 6.9*)

Table 3 *tert.*-BHP induced ROS production in WT and GPx-1$^{-/-}$ mouse aorta. CL was expressed as relative light units (RLU) and *RLU/mg* (Mean ± SD). Control, without tissue; WT, GPx-1$^{-/-}$ in the presence of WT and GPx-1$^{-/-}$ mouse aorta (n = 3 - 5).

Table 4 showed the lucigenin-detectable superoxide production in WT and GPx-1$^{-/-}$ mouse aorta in the different concentrations of *tert*.-BHP. Although the lucigenin-derived CL signal was low in the different concentrations of *tert*.-BHP in WT and GPx-1$^{-/-}$ mouse aorta, *tert*.-BHP generated more superoxide in WT and GPx-1$^{-/-}$ mouse aorta. Compared to H_2O_2, *tert*.-BHP generated the similar amount of superoxide as H_2O_2.

Lucigenin			
tert. – BHP	**Control** RLU	**WT** RLU (*RLU/mg*)	**GPx-1$^{-/-}$** RLU (*RLU/mg*)
0.01 mM	0.02 ± 0.004	0.07 ± 0.02 (*0.009 ± 0.001*)	0.09 ± 0.03 (*0.02 ± 0.005*)
0.05 mM	0.03 ± 0.001	0.05 ± 0.02 (*0.007 ± 0.003*)	0.1 ± 0.02 (*0.04 ± 0.006*)
0.125 mM	0.03 ± 0.007	0.1 ± 0.03 (*0.01 ± 0.005*)	0.2 ± 0.1 (*0.07 ± 0.05*)
0.25 mM	0.02 ± 0.004	0.1 ± 0.06 (*0.02 ± 0.01*)	1.0 ± 1.5 (*0.2 ± 0.3*)
0.5 mM	0.02 ± 0.002	0.3 ± 0.06 (*0.04 ± 0.008*)	0.4 ± 0.1 (*0.1 ± 0.07*)
1 mM	0.02 ± 0.001	0.3 ± 0.07 (*0.04 ± 0.02*)	1.2 ± 1.4 (*0.3 ± 0.4*)

Table 4 *tert.*-BHP stimulated superoxide production in WT and GPx-1$^{-/-}$ mouse aorta. CL was expressed as relative light units (RLU) and *RLU/mg* (mean ± SD). Control, without tissue; WT, GPx-1$^{-/-}$, in the presence of WT and GPx-1$^{-/-}$ mouse aorta (n = 3 – 5).

4.5.3 Effects of Different Drugs Used in the Experiments on AAPH-induced ROS

AAPH is a water-soluble azo compound which is used extensively as a free radical generator (Noguchi *et al.*, 1998;Rice-Evans & Miller, 1994). Decomposition of AAPH produces molecular nitrogen and 2 carbon radicals. The carbon radicals

may combine to produce stable products or react with molecular oxygen to give peroxyl radicals. We studied the effects of different drugs used in the experiments on AAPH-induced ROS generation. As shown in **Fig 21a**, indomethacin and furegrelate had no effect on AAPH-induced ROS production. Other drugs including nifedipine, fasudil, 4-AP, quinacrine, meclofenamic, diclofenac, and valsartan had similar effects as indomethacin and furegrelate. However, dantrolene attenuated AAPH-induced ROS generation (**Fig 21b**). Since bupivacaine and NCDC were dissolved in ethanol and the same concentration of ethanol increased AAPH-induced ROS production as bupivacaine and NCDC (**Fig 21c**), it is possible that bupivacaine and NCDC increased AAPH-induced ROS generation by ethanol.

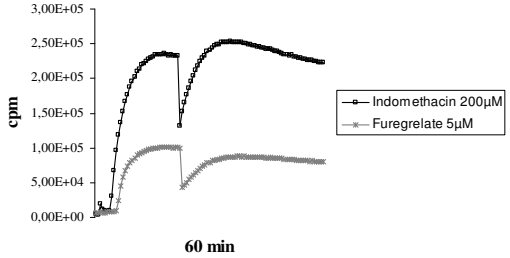


Fig 21a The effects of indomethacin and furegrelate on AAPH-induced ROS.

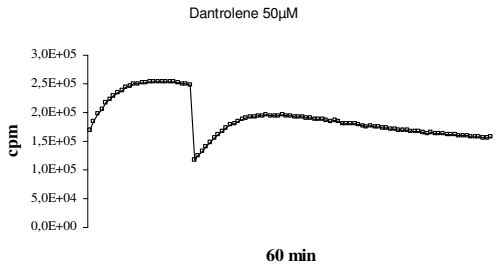


Fig 21b The effect of dantrolene on AAPH-induced ROS.

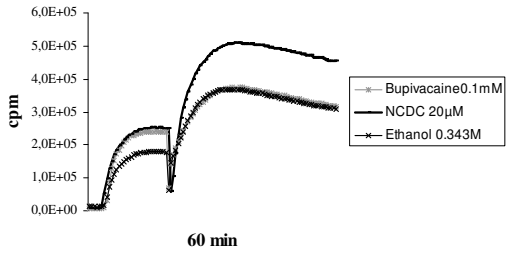


Fig 21c The effects of bupivacaine, NCDC, ethanol on AAPH- induced ROS.

5 Discussion

5.1 Hydroperoxide-induced Oxidative Stress and Arterial Contraction: Overview

Under physiological conditions vascular cells produce reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, as signalling molecules. However, excessive production of ROS results in oxidative stress. Since oxidative stress and associated oxidative damage lead to endothelial dysfunction, increased contractility, VSMC growth, lipid peroxidation and inflammation, they are involved in pathogenesis of many cardiovascular diseases, including hypertension, diabetes, and atherogenesis.

In the arterial smooth muscle, many experiments have shown that low molecular hydroperoxides are able to induce vasoconstriction in relaxed arterial rings (Blumenstein, 2004;Heinle, 1984;Heinle, 1988). On the other hand, a number of studies have shown that in precontracted vessel segments, reactive oxygen metabolites produced relaxation, which in some cases seems to be mediated by endothelium-dependent mechanisms (Lacza *et al.*, 2002;Matoba & Shimokawa, 2003). This means that the findings regarding the effects of ROS and the metabolites on the function of the arterial smooth muscle are still controversial.

In this study, hydrogen peroxide (H_2O_2) and *tert.*-BHP were used to study contractility effects on mouse aorta by inducing oxidative stress. H_2O_2 is metabolized to water by catalase and glutathione peroxidase (GPx). *tert.*-BHP is decomposed only by GPx. We observed a concentration-dependent contraction to H_2O_2 and *tert.*-BHP (0.01 mM-0.25 mM) in mouse aortic rings with the endothelium intact. However, we observed that H_2O_2 and *tert.*-BHP enhanced contraction much stronger in the mouse aorta without endothelium than with intact endothelium, suggesting that aortic contraction to H_2O_2 and *tert.*-BHP is affected

by the factors released from endothelium. These results also indicate that H_2O_2 and *tert.*-BHP can act directly on vascular smooth muscle to induce contraction. These findings are consistent with the reports in rat aorta (Blumenstein, 2004;Gao & Lee, 2001;Taddei *et al.*, 1998) , rat pulmonary arteries, abdominal aorta, and inferior vena (Pelaez *et al.*, 2000).

Despite the very different metabolism of both hydroperoxides, they revealed similar contractility effects in functional experiments. The different metabolism was also found when free radical production was studied. H_2O_2 produced much more ROS than *tert.*-BHP in mouse aorta, suggesting that the hydroperoxide-enhanced contraction is not related to the quantity of ROS production in the tissue. Although H_2O_2 and *tert.*-BHP increased $\cdot O_2^-$ not very much in the extracellular space as was detected by lucigenin CL, $\cdot O_2^-$ production by hydroperoxides from mouse aorta was much more than the control. It is possible that hydroperoxides increase enough intracellular $\cdot O_2^-$ (Li *et al.*, 2001), as a signal to increase $[Ca^{2+}]_i$, which would explain many cellular effects.

In order to characterize underlying mechanisms in more details, we were especially interested in the participation of endothelium, and the characterization with some pharmacological effectors. The results of the corresponding experiments will be discussed in the following chapters.

5.2 The Function of Endothelium in Hydroperoxide-enhanced Contraction

5.2.1 NO limited the contraction enhanced by hydorperoxides.

Endothelial cells synthesize and release various factors that regulate vascular tone, inflammation and angiogenesis. NO is the major vasodilative substance and

formed by endothelium cells from L-arginine via the enzymatic action of endothelial NO synthase (eNOS). According to our results, it seems that NO plays an important role in limiting the contraction induced by hydroperoxides. Firstly, Ach significantly decreased hydroperoxide-enhanced contraction. Secondly, inhibition of NOS by L-NAME increased that contraction. Thirdly, removal of the endothelium increased contraction induced by hydroperoxides. It is in contrast to very often mentioned opinion, that oxidative stress does impaire endothelial NO production. Some studies have reported that H_2O_2 and $\cdot O_2^-$ inhibits the three major endothelium-dependent vasodilator pathways, i.e., NO [especially by the reaction: $\cdot O_2^- + NO \rightarrow ONOO^-$ (peroxynitrite)], prostacyclin, and EDHF. There is evidence that H_2O_2 appears to be involved in the reduced bioavailability of eNOS cofactors, which may induce uncoupled eNOS and lead to decreased production of NO (Ardanaz & Pagano, 2006). However, the data in this study showed that hydroperoxides did not inhibit NO release from endothelium. Additionally, some studies suggest that H_2O_2 can increase NO release in rat aorta, rabbit aorta and porcine coronary arteries (Bharadwaj & Prasad, 1995;Blumenstein, 2004;Hayabuchi *et al.*, 1998;Yang *et al.*, 1999b). Thus, the effects of H_2O_2 on endothelium are dependent on the species, the artery, and the applied concentrations.

5.2.2 Hydroperoxides Increased the Arterial Contraction by PLA_2-COX-TXA_2 Pathways.

TXA_2 is a powerful constrictor of vascular smooth muscle. It is a lipid mediator which originates from arachidonic acid (AA) metabolism through the cyclooxygenase (COX). There are two isoforms of COX: one constitutive (COX-1); the second one inducible (COX-2). COX-1 exists in most cells and COX-2 is induced in inflammatory cells. COX converts arachidonic acid (AA) to prostaglandin H_2 (PGH_2). PGH_2 itself already possesses activity (Ge *et al.*, 1995), or it may be metabolized by different synthases into prostaglandins (**Fig 1**), prostacycline (PGI_2), or TXA_2 (Reilly & FitzGerald, 1993). TXA_2 can be released

from arterial endothelial and smooth muscle cells. Since enhanced TXA_2 production has been reported in several cardiovascular diseases, such as acute myocardial infarction and spontaneous hypertension (Paarlberg *et al.*, 1998), oxidative stress appears to be a common denominator underlying endothelial dysfunction in these cardiovascular disease, the question arises whether hydroperoxides mediate arterial tone via TXA_2 pathway.

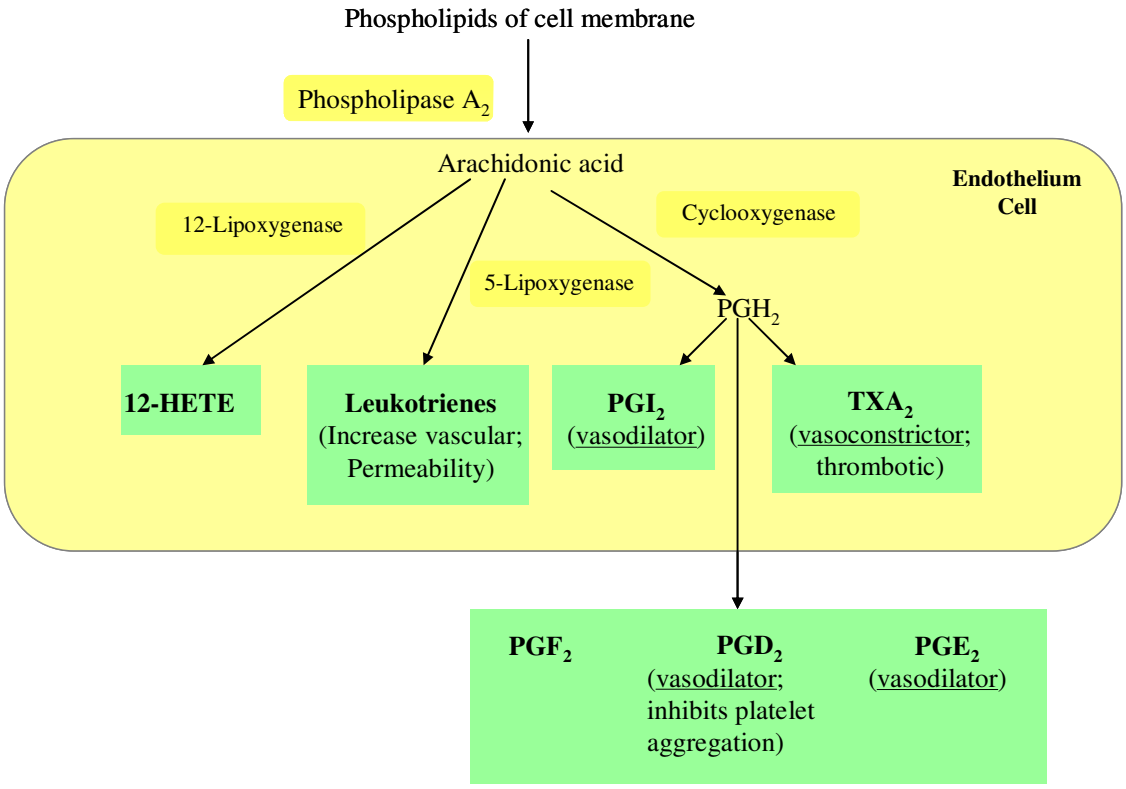


Fig 1 Summary diagram of the mediators from phospholipids. HETE, hydroxyeicosatetraenoc acid.

As seen from the results presented here, hydroperoxide-enhanced contraction was reduced by quinacrine (a PLA_2 inhibitor). Although higher concentration (50 μM) of quinacrine decreased KCl-precontraction, low concentration (10 μM) of quinacrine had no effect on KCl-induced contraction. Moreover, 10 μM quinacrine significantly reduced H_2O_2-enhanced contraction. These results suggest

that PLA_2 is involved in the contraction induced by hydroperoxides. It is still unknown how hydroperoxides activate PLA_2. The possible mechanisms may be related to protein kinase C (Chakraborti & Michael, 1993) or P_2 purinoceptors mediation. A number of reports have shown that COX is involved in ROS-induced contraction (Garcia-Cohen *et al.*, 2000;Hibino *et al.*, 1999;Rodriguez-Martinez *et al.*, 1998). Our data showed that diclofenac, indomethacin and meclofenamic, three non-selective COX inhibitors, significantly inhibited the contraction of hydroperoxides. Although we found that the same concentration of indomethacin significantly decreased KCl-precontraction, the other two COX inhititors (diclofenac and meclofenamic) had no effects on KCl-induced contraction. These results confirm that COX metabolite-related signal pathways could participate in hydroperoxide-induced vessel contraction. Furthermore, we also found that bupivacaine and furegrelate, the TXA_2 synthase inhibitors, significantly reduced the contraction of hydroperoxides. These results strongly suggest that TXA_2 from PLA_2-COX-TXA_2 pathways may serve as the mediator of hydroperoxide-enhanced arterial smooth muscle contraction. TXA_2 acts through specific G-protein coupling (Narumiya *et al.*, 1999). Activation of TXA_2 receptor (TP) leads to phospholipase C activation and release inositol 1,4,5-trisphosphate (IP_3), which increases intracellular Ca^{2+} level to trigger the smooth muscle contraction (Habib *et al.*, 1997).

Although the antagonists of PLA_2-COX-TXA_2 pathways significantly reduced hydroperoxide-enhanced contraction, it is unclear whether they worked via the inhibition of the pathways or via antioxidant activity. In this point, we measured the effects of these drugs on AAPH-induced ROS production. Our data showed that these antagonists had no effects on AAPH-induced ROS, suggesting that they worked related to the pathways inhibited, not associated with antioxidant effects.

5.2.3 Angiotensin (Ang) II Involved H_2O_2 Increased Arterial Contraction.

The renin-angiotensin system (RAS) plays an important role in the control of vascular tone. RAS is originating either from the kidney and blood or from the vessel wall itself (Dzau, 1988), so that angiotensin II can be formed from isolated tissue. In the smooth muscle cells it acts on AT1 receptor (AT1R) to induce contraction, cell growth, migration and differentiation. Although Ang II has been reported to be involved in ROS generation by activating NAD(P)H (Touyz *et al.*, 2003), few show that oxidative stress activates AngII activity.

Here, we showed that valsartan (AT1 inhibitor) decreased H_2O_2-enhanced contraction, suggesting that AT1 receptor seems to be involved in H_2O_2-enhanced contraction. In addition, valsartan may inhibit NAD(P)H oxidase activated by H_2O_2, which redues superoxide generation to decrease H_2O_2-enhanced contraction. Although ACE inhibitors and AT1 receptor antagonists have been reported to reduce oxidative stress in hypertensive patients (Baykal *et al.*, 2003), the present experiment showed that valsartan did not reduce ROS induced by AAPH.

5.3 Hydroperoxides Directly Act On the Arterial Smooth Muscle.

The present study showed that hydroperoxides also enhanced contraction of the aorta with endothelium denuded, suggesting that hydroperoxides can act directly on vascular smooth muscle to induce contraction. $[Ca^{2+}]_i$ plays a key role in smooth muscle contraction, but the effects of ROS on $[Ca^{2+}]_i$ are contradictory. For example, Krippeit-Drews et al have reported that H_2O_2 can release Ca^{2+} from the sarcoplamic reticulum and mitochondria to increase $[Ca^{2+}]_i$ in smooth muscle (Krippeit-Drews *et al.*, 1995). In contrast, some studies have showed that ROS attenuate Ca^{2+} mobilization and decreases Ca^{2+} sensitivity of the contractile

machinery (Kimura *et al.*, 2002). Here we will discuss separately the effects of hydroperoxides on transmembrane Ca^{2+} fluxes and intracellular Ca^{2+} liberation mechanisms in the smooth muscle cells of mice aorta.

5.3.1 Transmembrane Ca^{2+} and Hydroperoxide-enhanced Contraction

When the membrane is depolarized, extracellular Ca^{2+} enters the smooth muscle cell to induce contraction via the voltage-gated Ca^{2+} channels. L-type voltage Ca^{2+} channel is the main Ca^{2+} source for electrically evoked contraction in smooth muscle. Since nifedipine significantly decreased KCl-precontraction, the achievement of KCl-precontraction with nifedipine was followed by the effect of nifedipine on the response of hydroperoxides. The data showed that hydroperoxide-enhanced contraction was abolished by L-type voltage Ca^{2+} channel antagonist (nifedipine). It seems that extracellular Ca^{2+} is required for the hydroperoxide-induced contractions. Similar to our findings, Yang et al have shown that the absence of extracellular Ca^{2+} or employment of the Ca^{2+} channel antagonist, verapamil, inhibited the contractile effects of H_2O_2 on the dog canine basilar arteries (Yang *et al.*, 1999a). Furthermore, our data showed that L-type Ca^{2+} channel antagonist, nifedipine, inhibited approximately 80% of the contractile effects of hydroperoxides on mouse aorta, suggesting that the influx of intracellular Ca^{2+} seems to play a principle role in the contractions. However, since nifedipine also decreased KCl-precontraction in this study, the inhibition of hydroperoxide-enhanced contraction by nifedipine might be associated with other pathways. On the other hand, it has been reported that extracellular Ca^{2+} ions are of minor importance for hydroperoxide-induced contraction in rabbit aorta, yet on the contrary, that Ca^{2+} from intracellular stores mediates the contraction in this aorta (Heinle, 1988). Additionally, some observations suggest that ROS inhibit Ca^{2+} channels activity and Ca^{2+} influx, which could contribute to reduced $[Ca^{2+}]_i$ effects (Lounsbury *et al.*, 2000;Thomas *et al.*, 1998). When interpreting the results, however, it should be kept in mind that the experimental conditions, the contractile state of smooth muscle and different arteries may affect results.

5.3.2 Intracellular Ca^{2+} and Hydroperoxide-enhanced Contraction

In agonist-induced contraction of smooth muscle, Ca^{2+} release from the sarcoplasmic reticulum is of great importance to stimulate contractile protein. Our data showed that dantrolene (an intracellular Ca^{2+} release inhibitor) decreased hydroperoxide-enhanced contraction, suggesting that hydroperoxides could stimulate Ca^{2+} release from intracellular stores. This assumption is supported by other findings (Heinle, 1988;Krippeit-Drews *et al.*, 1995;Roychoudhury *et al.*, 1996a;Roychoudhury *et al.*, 1996b). However, since dantrolene also significantly decreased KCl-precontraction, its specificity had to be questioned. Additionally, we have shown that dantrolene decreased ROS generation induced by AAPH. So it is not known whether dantrolene decreased hydroperoxide-enhanced contraction by antioxidant activity or by inhibition of intracellular Ca^{2+} release.

PLC hydrolyzes PIP_2, a phosphatidylinositol, into IP_3 and DAG, which then modulate the activity of Ca^{2+} channels in the sarcoplasmic reticulum to release Ca^{2+}. In our experiment, although phospholipase C (PLC) inhibitor (NCDC) significantly decreased PHE-precontraction, it had no effect on KCl-precontraction. Moreover, we found that NCDC abolished the effects of hydroperoxides on KCl- and PHE-precontraction in mouse aorta, suggesting that the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) seem to be involved in Ca^{2+} liberation from intracellular stores by hydroperoxides. This is agreement with the findings in porcine pulmonary artery (Shasby *et al.*, 1988). In addition, since H_2O_2 seems to provide a pathway for Ca^{2+} entry from the extracellular fluid via voltage-gated membrane Ca^{2+} channels, another mechanism is proposed that H_2O_2 may produce a Ca^{2+}-induced Ca^{2+} release from intracellular stores (e.g., sarcoplasmic reticulum). Therefore the evidences presented in this work suggest that hydroperoxides possibly activate Ca^{2+} release from internal stores to increase $[Ca^{2+}]_i$ by PLC activation.

5.3.3 Ca^{2+} Sensitization and Hydroperoxide-enhanced Contraction

Although $[Ca^{2+}]_i$ plays a crucial role for contractile machinery in the smooth muscle, it has been reported that the regulation of MLC phosphorylation and contractile force can be independent of changes in $[Ca^{2+}]_{i.}$ That means contractile force can be sustained at a constant $[Ca^{2+}]_{i,}$, which is referred to as Ca^{2+} sensitization (Somlyo & Somlyo, 2003). According to our data, hydroperoxides could increase Ca^{2+} sensitization by Rho kinase and PKC pathways.

5.3.3.1 Rho Kinase Pathway

Here, we showed that fasudil (Rho kinase inhibitor) decreased hydroperoxide-enhanced contraction, suggesting that Rho kinase may be related to this contraction. Rho is abundantly expressed in vascular smooth muscle (Hirata *et al.*, 1992). It can increase SMC contractility independent of changes in intracellular calcium concentration via activation of Rho kinase, which in turn phosphorylates and inhibits MLC phosphatase and increases phosphorylated myosin (Kitazawa *et al.*, 1991;Nishimura *et al.*, 1996). Rho is reported to not only be involved in the pathophysiology of hypertension (Seasholtz *et al.*, 2001), but also mediate NADPH oxidase to generate ROS (Napoli *et al.*, 2001). However, since the same concentration of fasudil also significantly decreased KCl-precontraction, its specificity should be considered.

On the other hand, since the contraction induced by KCl depends on calcium influx through voltage-operated Ca^{2+} channels, the result of inhibition of KCl precontraction by fasudil suggests that Ca^{2+} ions might induce Ca^{2+} sensitization. From this interesting result, it seems that Ca^{2+} influx may trigger its sensitization, rather like Ca^{2+}-induced Ca^{2+} release. This is in agreement with the finding that membrane depolarization-induced contraction of rat caudal arterial smooth muscle

involves Rho-associated kinase (Mita *et al.*, 2002). Thus, it seems that hydroperoxides not only directly activate Rho kinase pathway but also mediate Rho kinase pathway by increasing calcium influx through voltage-operated Ca^{2+} channels.

5.3.3.2 PKC Pathway

PKC is known to cause phosphorylation of the 20-kDa regulatory myosin light chain (MLC_{20}). It may also activate mitogen-activated protein kinase (MAPK) leading to smooth muscle contraction without a significant increase in intracellular free Ca^{2+} (Pelaez *et al.*, 2000). As discussed above (**5.3.2**), activation of PLC seems to be involved in hydroperoxide-enhanced contraction. The activation of PLC would also lead to activation of protein kinase C (PKC) and liberation of Ca^{2+} from intracellular stores. So we propose that hydroperoxides increase Ca^{2+} sensitization through activation of not only Rho kinase but also PKC pathway. The possible mechanism of H_2O_2 activating PKC in smooth muscle seems to increase activation of PI_3-kinases and synthesis of one of the latter's products, phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which in turn activates PKC-ξ (Nakanishi *et al.*, 1993).

5.3.4 Hydroperoxides May Inhibit K_v Channel on Smooth Muscle.

K_v channels (voltage-activated K channels) are highly expressed in VSMC and contribute to membrane potential (Leblanc *et al.*, 1994). K_v channels may partly limit the membrane depolarization that occurs during vasoconstriction since these channels are activated by depolarization. According to our study, we found that 0.3 mM 4-AP (K_v channels inhibitor) increased PHE-induced contraction. Since 4-AP inhibits K_v channels, and induces depolarization, extracellular Ca^{2+} enters into cells through voltage-gated Ca^{2+} channels to increase intracellular Ca^{2+}, which induces contraction.

Cogolludo et al recently have reported that activation of NADPH oxidase and the subsequent production of hydrogen peroxide are involved in the K_v channel inhibition and the contractile response induced by TP receptor activation in rat PA (Cogolludo *et al.*, 2006). In our study, we found that hydroperoxide-enhanced contraction was inhibited by 4-AP in PHE precontraction, but not in 30 mM KCl precontraction. This result suggests that hydroperoxides and 4-AP may inhibit the same K^+ channels (K_v channels) in PHE precontraction. When K_v channels are inhibited by 4-AP, hydroperoxides have no effects on the same K^+ channels.

The mechanism by which hydroperoxides inhibit K_v channels is not known. Whether it is related to increased intracellular Ca^{2+} concentration (Cox & Petrou, 1999), enhanced PKC activation (Clement-Chomienne *et al.*, 1996) or specific oxidation amino acid residues of the K_v channel (Ciorba *et al.*, 1999) must be elucidated by further experiments.

5.4 The Role of Glutathione Peroxidase in Hydroperoxide-enhanced Contraction

The enzyme glutathione peroxidase (GPx) is a selenocysteine-containing protein that serves an important role in the cellular defense against oxidant stress by utilizing reduced glutathione (GSH) to reduce H_2O_2, *tert.*-BHP to their corresponding alcohols (Ursini *et al.*, 1995). GPx exists in several isoforms, and the most abundant intracellular isoform is GPx-1. In our study, the activity of GPx in GPx-$1^{-/-}$ aorta and liver is much lower than in WT mouse. Thus, a deficiency of GPx-1 would theoretically lead to an increase in ROS, or at least, to enhance oxidative imbalance in the presence of hydroperoxides.

Comparing the properties of WT and GPx-1$^{-/-}$ mice, there were interestingly only few differences. We found that in the presence of L-NAME, hydroperoxide-enhanced contraction did not significantly increase in GPx-1$^{-/-}$ as in WT mouse aorta, suggesting that GPx-1 possibly plays a role in protecting endothelium from oxidative damage. Our result is in full agreement with other studies that GPx-1$^{-/-}$ mice have an endothelial dysfunction caused by a deficiency in bioactive NO (Dayal *et al.*, 2002;Forgione *et al.*, 2002). Elevated levels of ROS can inactivate NO. Thus, the deficiency of GPx-1 impaired endothelial NO release. However, in all other experiments no significant differences were found.

Since H_2O_2 is metabolized to water by catalase and GPx, whereas *tert.*-BHP is decomposed only by GPx, then *tert.*-BHP would theoretically lead to more oxidative stress and stronger contraction in GPx-1$^{-/-}$ mice. Again, there was no difference of hydroperoxide-enhanced contraction between WT and GPx-1$^{-/-}$ mice. The explanation for this finding is still unknown. One possibility is that GPx-1 is not very important for reducing hydroperoxides and other enzymes in GPx-1$^{-/-}$ mice may act in place of GPx-1, or that the remaining GPx activity in GPx-1$^{-/-}$ mice is still high enough for detoxifying the hydroperoxides. Another possibility is that hydroperoxides may enhance other signal pathways, not dependent on the amount of ROS produced by hydroperoxides. For example, H_2O_2 possibly plays a role for NADPH oxidase to produce enough $\cdot O_2^-$ to induce arterial contraction (Li *et al.*, 2001). This would be consistent with the result that *tert.*-BHP produced much less ROS than H_2O_2, but both peroxides induced similar production of O_2^- as determined by lucigenin and similar contractility effects as determined in functional experiments. Thus, the total production of ROS during hydroperoxide-enhanced contraction is not determining the simulative effects. It is suggested that $\cdot O_2^-$ might be the responsible species. However, this assumption has to be confirmed by further experiments.

5.5 Conclusion

Hydroperoxides (H_2O_2, *tert.*-BHP) had multiple vasomotor actions. Different concentrations of hydroperoxides enhanced contraction in WT and GPx-$1^{-/-}$ mouse aorta. *tert.*-BHP produced similar amount of $\cdot O_2^-$ and had the same vasoconstrictor effects as H_2O_2, but *tert.*-BHP produced much less ROS in the tissues than H_2O_2. Thus it seems that the amount of free radical generation is not related to effects of hydroperoxides on arteries. It is possible that both hydroperoxides increase enough $\cdot O_2^-$ as a signal to increase $[Ca^{2+}]_i$, thus enhancing arterial contraction.

The results showed that obviously many different pathways are involved in mediating hydroperoxide-inducecd effects on arterial contraction, yet, a central common mechanism for H_2O_2 and *tert.*-BHP could be postulated. **Fig 2** summarizes the possible pathways as evaluated from the effects of the different drugs and as described on endothelial and smooth muscle cells, respectively.

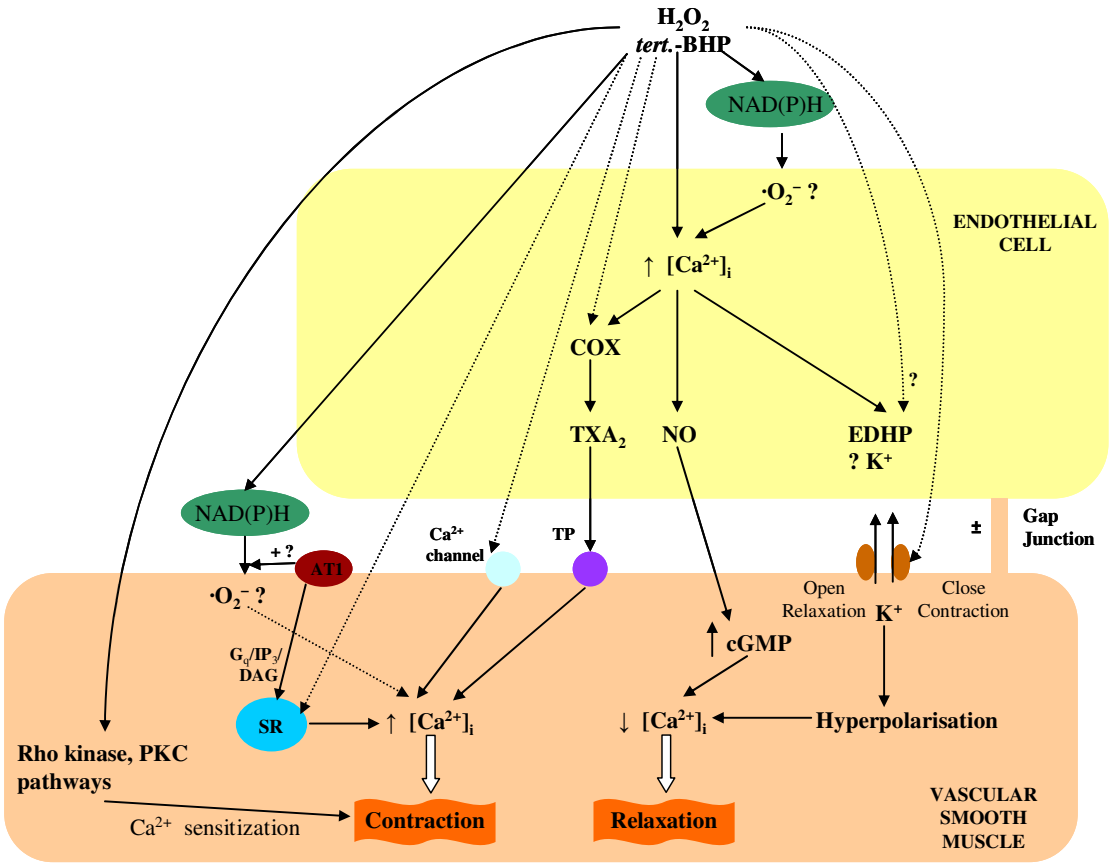


Fig 2 The effects of hydroperoxides on the artery. The picture shows some effects of hydroperoxides on endothelial and smooth muscle cells. (**AT1**, angiotensin AT1-receptor; **TP**, T prostanoid receptor; **G_q**, G-protein; **IP_3**, inositol 1,4,5-trisphosphate; **DAG**, diacylglycerol; **EDHP**, endothelium-derived hyperpolarizing factor; **PKC**, protein kinase C.

6 Summary

Reactive oxygen species (ROS) are a class of molecules that are derived from the metabolism of oxygen and include free radical and nonracial species that are capable to oxidize molecular targets. Most important ROS include superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl anion ($\cdot OH$) and reactive nitrogen species [e.g. nitric oxide (NO), peroxynitrite ($ONOO^-$)]. All vascular cell types (endothelial cells, vascular smooth muscle cells, and adventitial fibroblast) produce ROS including H_2O_2. H_2O_2 is a cell-permeant and highly stable ROS generated mainly by dismutation of superoxide ($\cdot O_2^-$) by superoxide dismutases (SOD). Although numerous studies have demonstrated the effects of H_2O_2 on endothelium and cultured smooth muscle cell signalling, its role in vascular tone as relaxantor or constricting factor is not well understood. Therefore we were interested to measure the effects of H_2O_2 and *tert.*-butylhydroperoxide on arterial contractial function in vitro and to characterize the signalling mechanisms using different pharmacological approaches.

Firstly, the results showed that both hydroperoxides increased contraction induced by KCl and phenylephrine in a dose dependent way in WT mouse aorta. Secondly, inhibition of endothelial NO synthesis by L-NAME and denuded endothelium increased hydroperoxide-enhanced contraction. Additionally, ACh (0.1 mM) significantly decreased H_2O_2- and *tert.*-BHP-enhanced contraction. These results suggest that NO plays an important role in limiting the contraction induced by hydroperoxides.

We investigated the involvement of phospholipaseA_2-cyclooxygenase-thromboxane A_2 pathways in hydroperoxide-enhanced contraction. Our data indicated that hydroperoxide-enhanced contraction was reduced by quinacrine (a PLA_2 inhibitor), suggesting the involvement of thromboxane A_2 in this contraction. The results also showed that three non-selective cyclooxygenase inhibitors

(diclofenac, indomethacin and meclofenamic) significantly inhibited the contraction of hydroperoxides, confirming the participation of cyclooxygenase metabolite-related signal pathways in hydroperoxide-induced vessel contraction. Moreover, the thromboxane A_2 synthase inhibitors (bupivacaine and furegrelate) were shown to reduce the contraction of hydroperoxides. Thus, hydroperoxides may activate phospholipase A_2 – cyclooxygenase – thromboxane A_2 pathways to increase contraction. These pathways seem to be related to endothelial cells. However their presence in smooth muscle can not be excluded.

In addition, the present study showed that hydroperoxides also enhanced contraction in the mouse aorta with denuded endothelium, suggesting that hydroperoxides can act directly on vascular smooth muscle to induce contraction. Since $[Ca^{2+}]_i$ plays a key role in smooth muscle contraction, we investigated the effects of hydroperoxides on transmembrane Ca^{2+} fluxes, intracellular Ca^{2+} liberation mechanisms as well as Ca^{2+} sensitization by different antagonists.

Our data showed that hydroperoxide-enhanced contraction was abolished by L-type voltage Ca^{2+} channel antagonist (nifedipine), suggesting that extracellular Ca^{2+} is required for the hydroperoxide-induced contractions. Moreover, an intracellular Ca^{2+} release inhibitor (dantrolene) decreased hydroperoxides-enhanced contraction. Thus it seems that hydroperoxides stimulate also Ca^{2+} release from intracellular stores. The possible mechanism of Ca^{2+} liberation from intracellular stores by hydroperoxides seems to be involved in the second messengers of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) because the study showed that NCDC [phospholipase C (PLC) inhibitor] abolished also the effects of hydroperoxides. PLC hydrolyzes PIP_2, a phosphatidylinositol, into two second messengers, IP_3 and DAG, which then modulate the activity of Ca^{2+} channels in the sarcoplasmic reticulum to release Ca^{2+}. However, since the same concentrations of nifedipine and dantrolene also significantly decreased KCl-precontraction, its specificity should be questioned.

Additionally, we concluded that Ca^{2+} sensitization also might contribute to the hydroperoxide effects since the activation of PLC would lead to activation of protein kinase C (PKC), which activates mitogen-activated protein kinase (MAPK) leading to smooth muscle contraction increased by Ca^{2+} sensitization without a significant increase in intracellular free Ca^{2+}. Furthermore, fasudil (Rho kinase inhibitor) decreased hydroperoxide-enhanced contraction. So we propose that hydroperoxides may increase Ca^{2+} sensitization through activation of not only PKC pathway but also Rho kinase. However, fasudil's specificity should be considered because the same concentration of fasudil also significantly decreased KCl-precontraction.

K_v channels (voltage-activated K channels) are highly expressed in VSMC and contribute to membrane potential. According to our study, hydroperoxide-enhanced contraction was inhibited by 4-AP in PHE precontraction, but not in 30 mM KCl precontraction. This result suggests that hydroperoxides and 4-AP may inhibit the same K^+ channels (K_v channels) in PHE precontraction. When K_v channels are inhibited by 4-AP, hydroperoxides have no effects on the same K^+ channels.

The enzyme glutathione peroxidase serves an important role in the cellular defense against oxidant stress by utilizing reduced glutathione to reduce H_2O_2 and *tert.*-BHP to their corresponding alcohols. Using aortas from GPx-$1^{-/-}$ mice, in which the gene for GPx-1 was knocked out, we could show that glutathione peroxidase seems to protect endothelium from oxidative damage since we found that in the presence of L-NAME, hydroperoxide-enhanced contraction did not significantly increase in GPx-$1^{-/-}$ as in WT mouse aorta. H_2O_2 is metabolized to water by catalase and glutathione peroxidase, whereas *tert.*-BHP is decomposed only by GPx. Thus, a deficiency of GPx-1 would theoretically lead to an increase

in ROS. However, our data showed that there was no difference of hydroperoxide-enhanced contraction between WT and GPx-1$^{-/-}$ mice. According to the result, we propose that hydroperoxides enhance contraction not dependent on the quantity of ROS produced by hydroperoxides. Since it was described by others that hydroperoxides activate NAD(P)H oxidase to produce $\cdot O_2^-$, we postulated that as a common mechanism small amount of hydroperoxides may produce enough $\cdot O_2^-$ to induce contraction. This is consistent with our results that H_2O_2 can produce much more ROS in mouse aorta than *tert.*-BHP (measured by luminol), yet, that both hydroperoxides induced a similar rate of $\cdot O_2^-$ as detected by special luminescent dye lucigenin. Further experiments are needed to substantiate this type of thesis.

7 Reference

Ardanaz, N. & Pagano, P. J. (2006). Hydrogen peroxide as a paracrine vascular mediator: regulation and signaling leading to dysfunction. *Exp.Biol.Med.(Maywood.)* **231**, 237-251.

Arun, K. H., Kaul, C. L., & Ramarao, P. (2004). High glucose concentration augments angiotensin II mediated contraction via AT1 receptors in rat thoracic aorta. *Pharmacol.Res.* **50**, 561-568.

Bagi, Z. & Koller, A. (2003). Lack of nitric oxide mediation of flow-dependent arteriolar dilation in type I diabetes is restored by sepiapterin. *J.Vasc.Res.* **40**, 47-57.

Barlow, R. S., El Mowafy, A. M., & White, R. E. (2000). H(2)O(2) opens BK(Ca) channels via the PLA(2)-arachidonic acid signaling cascade in coronary artery smooth muscle. *Am.J.Physiol Heart Circ.Physiol* **279**, H475-H483.

Barlow, R. S. & White, R. E. (1998). Hydrogen peroxide relaxes porcine coronary arteries by stimulating BKCa channel activity. *Am.J.Physiol* **275**, H1283-H1289.

Baykal, Y., Yilmaz, M. I., Celik, T., Gok, F., Rehber, H., Akay, C., & Kocar, I. H. (2003). Effects of antihypertensive agents, alpha receptor blockers, beta blockers, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers and calcium channel blockers, on oxidative stress. *J.Hypertens.* **21**, 1207-1211.

Bergdahl, A., Gomez, M. F., Wihlborg, A. K., Erlinge, D., Eyjolfson, A., Xu, S. Z., Beech, D. J., Dreja, K., & Hellstrand, P. (2005). Plasticity of TRPC expression in arterial smooth muscle: correlation with store-operated Ca2+ entry. *Am.J.Physiol Cell Physiol* **288**, C872-C880.

Bharadwaj, L. & Prasad, K. (1995). Mediation of H2O2-induced vascular relaxation by endothelium-derived relaxing factor. *Mol.Cell Biochem.* **149-150**, 267-270.

Blumenstein, A. Oxidative Stress und Gefäßfunktion. 2004. Fakultät für Chemie und Pharmazie, University Tuebingen.
Ref Type: Thesis/Dissertation

Chakraborti, S. & Michael, J. R. (1993). Role of protein kinase C in oxidant--mediated activation of phospholipase A2 in rabbit pulmonary arterial smooth muscle cells. *Mol.Cell Biochem.* **122**, 9-15.

Chisolm, G. M. & Steinberg, D. (2000). The oxidative modification hypothesis of atherogenesis: an overview. *Free Radic.Biol.Med.* **28**, 1815-1826.

Ciorba, M. A., Heinemann, S. H., Weissbach, H., Brot, N., & Hoshi, T. (1999). Regulation of voltage-dependent K+ channels by methionine oxidation: effect of nitric oxide and vitamin C. *FEBS Lett.* **442**, 48-52.

Clement-Chomienne, O., Walsh, M. P., & Cole, W. C. (1996). Angiotensin II activation of protein kinase C decreases delayed rectifier K+ current in rabbit vascular myocytes. *J.Physiol* **495 (Pt 3)**, 689-700.

Climent, B., Fernandez, N., Sanz, E., Sanchez, A., Monge, L., Garcia-Villalon, A. L., & Dieguez, G. (2005). Enhanced response of pig coronary arteries to endothelin-1 after ischemia-reperfusion. Role of endothelin receptors, nitric oxide and prostanoids. *Eur.J.Pharmacol.* **524**, 102-110.

Cogolludo, A., Frazziano, G., Cobeno, L., Moreno, L., Lodi, F., Villamor, E., Tamargo, J., & Perez-Vizcaino, F. (2006). Role of reactive oxygen species in Kv channel inhibition and vasoconstriction induced by TP receptor activation in rat pulmonary arteries. *Ann.N.Y.Acad.Sci.* **1091**, 41-51.

Cox, R. H. & Petrou, S. (1999). Ca(2+) influx inhibits voltage-dependent and augments Ca(2+)-dependent K(+) currents in arterial myocytes. *Am.J.Physiol* **277**, C51-C63.

Darley-Usmar, V., Wiseman, H., & Halliwell, B. (1995). Nitric oxide and oxygen radicals: a question of balance. *FEBS Lett.* **369**, 131-135.

Dayal, S., Brown, K. L., Weydert, C. J., Oberley, L. W., Arning, E., Bottiglieri, T., Faraci, F. M., & Lentz, S. R. (2002). Deficiency of glutathione peroxidase-1 sensitizes hyperhomocysteinemic mice to endothelial dysfunction. *Arterioscler.Thromb.Vasc.Biol.* **22**, 1996-2002.

Du, W., Frazier, M., McMahon, T. J., & Eu, J. P. (2005). Redox activation of intracellular calcium release channels (ryanodine receptors) in the sustained phase of hypoxia-induced pulmonary vasoconstriction. *Chest* **128**, 556S-558S.

Dzau, V. J. (1988). Circulating versus local renin-angiotensin system in cardiovascular homeostasis. *Circulation* **77**, I4-13.

Feletou, M. & Vanhoutte, P. M. (2006). Endothelial dysfunction: a multifaceted disorder (The Wiggers Award Lecture). *Am.J.Physiol Heart Circ.Physiol* **291**, H985-1002.

Fisher, A. B., Al Mehdi, A. B., & Manevich, Y. (2002). Shear stress and endothelial cell activation. *Crit Care Med.* **30**, S192-S197.

Fleming, I., Michaelis, U. R., Bredenkotter, D., Fisslthaler, B., Dehghani, F., Brandes, R. P., & Busse, R. (2001). Endothelium-derived hyperpolarizing factor synthase (Cytochrome P450 2C9) is a functionally significant source of reactive oxygen species in coronary arteries. *Circ.Res.* **88**, 44-51.

Forgione, M. A., Cap, A., Liao, R., Moldovan, N. I., Eberhardt, R. T., Lim, C. C., Jones, J., Goldschmidt-Clermont, P. J., & Loscalzo, J. (2002). Heterozygous cellular glutathione peroxidase deficiency in the mouse: abnormalities in vascular and cardiac function and structure. *Circulation* **106**, 1154-1158.

Fridovich, I. (1997). Superoxide anion radical (O2-.), superoxide dismutases, and related matters. *J.Biol.Chem.* **272**, 18515-18517.

Gao, Y. J., Hirota, S., Zhang, D. W., Janssen, L. J., & Lee, R. M. (2003). Mechanisms of hydrogen-peroxide-induced biphasic response in rat mesenteric artery. *Br.J.Pharmacol.* **138**, 1085-1092.

Gao, Y. J. & Lee, R. M. (2001). Hydrogen peroxide induces a greater contraction in mesenteric arteries of spontaneously hypertensive rats through thromboxane A(2) production. *Br.J.Pharmacol.* **134**, 1639-1646.

Garcia-Cohen, E. C., Marin, J., Diez-Picazo, L. D., Baena, A. B., Salaices, M., & Rodriguez-Martinez, M. A. (2000). Oxidative stress induced by tert-butyl hydroperoxide causes vasoconstriction in the aorta from hypertensive and aged rats: role of cyclooxygenase-2 isoform. *J.Pharmacol.Exp.Ther.* **293**, 75-81.

Ge, T., Hughes, H., Junquero, D. C., Wu, K. K., Vanhoutte, P. M., & Boulanger, C. M. (1995). Endothelium-dependent contractions are associated with both augmented expression of prostaglandin H synthase-1 and hypersensitivity to prostaglandin H2 in the SHR aorta. *Circ.Res.* **76**, 1003-1010.

Glass, C. K. & Witztum, J. L. (2001). Atherosclerosis. the road ahead. *Cell* **104**, 503-516.

Habib, A., Vezza, R., Creminon, C., Maclouf, J., & FitzGerald, G. A. (1997). Rapid, agonist-dependent phosphorylation in vivo of human thromboxane receptor isoforms. Minimal involvement of protein kinase C. *J.Biol.Chem.* **272**, 7191-7200.

Hahnenkamp, K., Nollet, J., Strumper, D., Halene, T., Rathman, P., Mortier, E., Van Aken, H., Knapp, J., Durieux, M. E., & Hoenemann, C. W. (2004). Bupivacaine inhibits thromboxane A2-induced vasoconstriction in rat thoracic aorta. *Anesth.Analg.* **99**, 97-102.

Hayabuchi, Y., Nakaya, Y., Matsuoka, S., & Kuroda, Y. (1998). Endothelium-derived hyperpolarizing factor activates Ca2+-activated K+ channels in porcine coronary artery smooth muscle cells. *J.Cardiovasc.Pharmacol.* **32**, 642-649.

Heinle, H. (1984). Vasoconstriction of carotid artery induced by hydroperoxides. *Arch.Int.Physiol Biochim.* **92**, 267-271.

Heinle, H. (1988). Influence of oxidative stress on metabolic and contractile functions of arterial smooth muscle. In *Oxygen Sensing in Tissues*, ed. H Acker, pp. 151-156. Springer-Verlag, Berlin, Heidelberg.

Hernanz, R., Alonso, M. J., Briones, A. M., Vila, E., Simonsen, U., & Salaices, M. (2003). Mechanisms involved in the early increase of serotonin contraction evoked by endotoxin in rat middle cerebral arteries. *Br.J.Pharmacol.* **140**, 671-680.

Hibino, M., Okumura, K., Iwama, Y., Mokuno, S., Osanai, H., Matsui, H., Toki, Y., & Ito, T. (1999). Oxygen-derived free radical-induced vasoconstriction by thromboxane A2 in aorta of the spontaneously hypertensive rat. *J.Cardiovasc.Pharmacol.* **33**, 605-610.

Hirata, K., Kikuchi, A., Sasaki, T., Kuroda, S., Kaibuchi, K., Matsuura, Y., Seki, H., Saida, K., & Takai, Y. (1992). Involvement of rho p21 in the GTP-enhanced calcium ion sensitivity of smooth muscle contraction. *J.Biol.Chem.* **267**, 8719-8722.

Horowitz, A., Menice, C. B., Laporte, R., & Morgan, K. G. (1996). Mechanisms of smooth muscle contraction. *Physiol Rev.* **76**, 967-1003.

Kimura, C., Cheng, W., Hisadome, K., Wang, Y. P., Koyama, T., Karashima, Y., Oike, M., & Ito, Y. (2002). Superoxide anion impairs contractility in cultured aortic smooth muscle cells. *Am.J.Physiol Heart Circ.Physiol* **283**, H382-H390.

Kitazawa, T., Masuo, M., & Somlyo, A. P. (1991). G protein-mediated inhibition of myosin light-chain phosphatase in vascular smooth muscle. *Proc.Natl.Acad.Sci.U.S.A* **88**, 9307-9310.

Krippeit-Drews, P., Haberland, C., Fingerle, J., Drews, G., & Lang, F. (1995). Effects of H2O2 on membrane potential and [Ca2+]i of cultured rat arterial smooth muscle cells. *Biochem.Biophys.Res.Commun.* **209**, 139-145.

Kukreja, R. C., Kontos, H. A., Hess, M. L., & Ellis, E. F. (1986). PGH synthase and lipoxygenase generate superoxide in the presence of NADH or NADPH. *Circ.Res.* **59**, 612-619.

Laakso, J. T., Teravainen, T. L., Martelin, E., Vaskonen, T., & Lapatto, R. (2004). Renal xanthine oxidoreductase activity during development of hypertension in spontaneously hypertensive rats. *J.Hypertens.* **22**, 1333-1340.

Lacza, Z., Puskar, M., Kis, B., Perciaccante, J. V., Miller, A. W., & Busija, D. W. (2002). Hydrogen peroxide acts as an EDHF in the piglet pial vasculature in response to bradykinin. *Am.J.Physiol Heart Circ.Physiol* **283**, H406-H411.

Landmesser, U., Dikalov, S., Price, S. R., McCann, L., Fukai, T., Holland, S. M., Mitch, W. E., & Harrison, D. G. (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J.Clin.Invest* **111**, 1201-1209.

Landmesser, U. & Harrison, D. G. (2001). Oxidative stress and vascular damage in hypertension. *Coron.Artery Dis.* **12**, 455-461.

Lassegue, B. & Clempus, R. E. (2003). Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am.J.Physiol Regul.Integr.Comp Physiol* **285**, R277-R297.

Leblanc, N., Wan, X., & Leung, P. M. (1994). Physiological role of Ca(2+)-activated and voltage-dependent K+ currents in rabbit coronary myocytes. *Am.J.Physiol* **266**, C1523-C1537.

Li, W. G., Miller, F. J., Jr., Zhang, H. J., Spitz, D. R., Oberley, L. W., & Weintraub, N. L. (2001). H(2)O(2)-induced O(2) production by a non-phagocytic NAD(P)H oxidase causes oxidant injury. *J.Biol.Chem.* **276**, 29251-29256.

Lounsbury, K. M., Hu, Q., & Ziegelstein, R. C. (2000). Calcium signaling and oxidant stress in the vasculature. *Free Radic.Biol.Med.* **28**, 1362-1369.

Matoba, T. & Shimokawa, H. (2003). Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in animals and humans. *J.Pharmacol.Sci.* **92**, 1-6.

Meharg, J. V., McGowan-Jordan, J., Charles, A., Parmelee, J. T., Cutaia, M. V., & Rounds, S. (1993). Hydrogen peroxide stimulates sodium-potassium pump activity in cultured pulmonary arterial endothelial cells. *Am.J.Physiol* **265**, L613-L621.

Mervaala, E. M., Cheng, Z. J., Tikkanen, I., Lapatto, R., Nurminen, K., Vapaatalo, H., Muller, D. N., Fiebeler, A., Ganten, U., Ganten, D., & Luft, F. C. (2001). Endothelial dysfunction and

xanthine oxidoreductase activity in rats with human renin and angiotensinogen genes. *Hypertension* **37**, 414-418.

Milstien, S. & Katusic, Z. (1999). Oxidation of tetrahydrobiopterin by peroxynitrite: implications for vascular endothelial function. *Biochem.Biophys.Res.Commun.* **263**, 681-684.

Mita, M., Yanagihara, H., Hishinuma, S., Saito, M., & Walsh, M. P. (2002). Membrane depolarization-induced contraction of rat caudal arterial smooth muscle involves Rho-associated kinase. *Biochem.J.* **364**, 431-440.

Nakanishi, H., Brewer, K. A., & Exton, J. H. (1993). Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J.Biol.Chem.* **268**, 13-16.

Napoli, C., de Nigris, F., & Palinski, W. (2001). Multiple role of reactive oxygen species in the arterial wall. *J.Cell Biochem.* **82**, 674-682.

Narumiya, S., Sugimoto, Y., & Ushikubi, F. (1999). Prostanoid receptors: structures, properties, and functions. *Physiol Rev.* **79**, 1193-1226.

Nguyen-Khoa, T., Massy, Z. A., Witko-Sarsat, V., Canteloup, S., Kebede, M., Lacour, B., Drueke, T., & Descamps-Latscha, B. (1999). Oxidized low-density lipoprotein induces macrophage respiratory burst via its protein moiety: A novel pathway in atherogenesis? *Biochem.Biophys.Res.Commun.* **263**, 804-809.

Niki, E. (1990). Free radical initiators as source of water- or lipid-soluble peroxyl radicals. *Methods Enzymol.* **186**, 100-108.

Nishimura, J., Sakihara, C., Zhou, Y., & Kanaide, H. (1996). Expression of rho A and rho kinase mRNAs in porcine vascular smooth muscle. *Biochem.Biophys.Res.Commun.* **227**, 750-754.

Noguchi, N., Takahashi, M., Tsuchiya, J., Yamashita, H., Komuro, E., & Niki, E. (1998). Action of 21-aminosteroid U74006F as an antioxidant against lipid peroxidation. *Biochem.Pharmacol.* **55**, 785-791.

Omar, H. A., Cherry, P. D., Mortelliti, M. P., Burke-Wolin, T., & Wolin, M. S. (1991). Inhibition of coronary artery superoxide dismutase attenuates endothelium-dependent and -independent nitrovasodilator relaxation. *Circ.Res.* **69**, 601-608.

Paarlberg, K. M., de Jong, C. L., van Geijn, H. P., van Kamp, G. J., Heinen, A. G., & Dekker, G. A. (1998). Vasoactive mediators in pregnancy-induced hypertensive disorders: a longitudinal study. *Am.J.Obstet.Gynecol.* **179**, 1559-1564.

Paravicini, T. M. & Touyz, R. M. (2006). Redox signaling in hypertension. *Cardiovasc.Res.* **71**, 247-258.

Pelaez, N. J., Braun, T. R., Paul, R. J., Meiss, R. A., & Packer, C. S. (2000). H(2)O(2) mediates Ca(2+)- and MLC(20) phosphorylation-independent contraction in intact and permeabilized vascular muscle. *Am.J.Physiol Heart Circ.Physiol* **279**, H1185-H1193.

Plane, F., Johnson, R., Kerr, P., Wiehler, W., Thorneloe, K., Ishii, K., Chen, T., & Cole, W. (2005). Heteromultimeric Kv1 channels contribute to myogenic control of arterial diameter. *Circ.Res.* **96**, 216-224.

Rang, H. P., Dale, M. M., & Ritter, J. M. Pharmacology. 2003.
Ref Type: Generic

Reilly, M. & FitzGerald, G. A. (1993). Cellular activation by thromboxane A2 and other eicosanoids. *Eur.Heart J.* **14 Suppl K**, 88-93.

Rice-Evans, C. & Miller, N. J. (1994). Total antioxidant status in plasma and body fluids. *Methods Enzymol.* **234**, 279-293.

Rodriguez-Martinez, M. A., Garcia-Cohen, E. C., Baena, A. B., Gonzalez, R., Salaices, M., & Marin, J. (1998). Contractile responses elicited by hydrogen peroxide in aorta from normotensive and hypertensive rats. Endothelial modulation and mechanism involved. *Br.J.Pharmacol.* **125**, 1329-1335.

Roychoudhury, S., Chakraborti, T., Ghosh, J. J., Ghosh, S. K., & Chakraborti, S. (1996a). Redox state of pyridine nucleotides, but not glutathione, regulate Ca2+ release by H2O2 from mitochondria of pulmonary smooth muscle. *Indian J.Biochem.Biophys.* **33**, 218-222.

Roychoudhury, S., Ghosh, S. K., Chakraborti, T., & Chakraborti, S. (1996b). Role of hydroxyl radical in the oxidant H2O2-mediated Ca2+ release from pulmonary smooth muscle mitochondria. *Mol.Cell Biochem.* **159**, 95-103.

Rubanyi, G. M. (1988). Vascular effects of oxygen-derived free radicals. *Free Radic.Biol.Med.* **4**, 107-120.

Schafer, F. Q. & Buettner, G. R. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic.Biol.Med.* **30**, 1191-1212.

Seasholtz, T. M., Zhang, T., Morissette, M. R., Howes, A. L., Yang, A. H., & Brown, J. H. (2001). Increased expression and activity of RhoA are associated with increased DNA synthesis and reduced p27(Kip1) expression in the vasculature of hypertensive rats. *Circ.Res.* **89**, 488-495.

Shasby, D. M., Yorek, M., & Shasby, S. S. (1988). Exogenous oxidants initiate hydrolysis of endothelial cell inositol phospholipids. *Blood* **72**, 491-499.

Shimomura, E., Shiraishi, M., Iwanaga, T., Seto, M., Sasaki, Y., Ikeda, M., & Ito, K. (2004). Inhibition of protein kinase C-mediated contraction by Rho kinase inhibitor fasudil in rabbit aorta. *Naunyn Schmiedebergs Arch.Pharmacol.* **370**, 414-422.

Smith, A. R., Visioli, F., & Hagen, T. M. (2002). Vitamin C matters: increased oxidative stress in cultured human aortic endothelial cells without supplemental ascorbic acid. *FASEB J.* **16**, 1102-1104.

Somlyo, A. P. & Somlyo, A. V. (2003). Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev.* **83**, 1325-1358.

Taddei, S., Virdis, A., Ghiadoni, L., Magagna, A., & Salvetti, A. (1998). Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension. *Circulation* **97**, 2222-2229.

Thomas, G. P., Sims, S. M., Cook, M. A., & Karmazyn, M. (1998). Hydrogen peroxide-induced stimulation of L-type calcium current in guinea pig ventricular myocytes and its inhibition by adenosine A1 receptor activation. *J.Pharmacol.Exp.Ther.* **286**, 1208-1214.

Tiritilli, A., El Habach, T., Haury, L., & Duret, J. F. (2004). BK2 but not BK1 receptors mediating contractile response in human umbilical arteries: role of thromboxane A2. *Methods Find.Exp.Clin.Pharmacol.* **26**, 247-252.

Tostes, R. C., Storm, D. S., Chi, D. H., & Webb, R. C. (1996). Intracellular calcium stores and oscillatory contractions in arteries from genetically hypertensive rats. *Hypertens.Res.* **19**, 103-111.

Touyz, R. M. & Schiffrin, E. L. (2004). Reactive oxygen species in vascular biology: implications in hypertension. *Histochem.Cell Biol.* **122**, 339-352.

Touyz, R. M., Tabet, F., & Schiffrin, E. L. (2003). Redox-dependent signalling by angiotensin II and vascular remodelling in hypertension. *Clin.Exp.Pharmacol.Physiol* **30**, 860-866.

Ursini, F., Maiorino, M., Brigelius-Flohe, R., Aumann, K. D., Roveri, A., Schomburg, D., & Flohe, L. (1995). Diversity of glutathione peroxidases. *Methods Enzymol.* **252**, 38-53.

Vasquez-Vivar, J., Duquaine, D., Whitsett, J., Kalyanaraman, B., & Rajagopalan, S. (2002). Altered tetrahydrobiopterin metabolism in atherosclerosis: implications for use of oxidized tetrahydrobiopterin analogues and thiol antioxidants. *Arterioscler.Thromb.Vasc.Biol.* **22**, 1655-1661.

Virdis, A., Iglarz, M., Neves, M. F., Amiri, F., Touyz, R. M., Rozen, R., & Schiffrin, E. L. (2003). Effect of hyperhomocystinemia and hypertension on endothelial function in methylenetetrahydrofolate reductase-deficient mice. *Arterioscler.Thromb.Vasc.Biol.* **23**, 1352-1357.

Yang, Z., Zheng, T., Jun, W., Zhang, A., Altura, B. T., & Altura, B. M. Hydrogen peroxide induces contraction and raises (Ca2+)i in canine cerebral arterial smooth muscle:participation of cellular signalling pathways. 1999a.
Ref Type: Generic

Yang, Z., Zhang, A., Altura, B. T., & Altura, B. M. (1999b). Hydrogen peroxide-induced endothelium-dependent relaxation of rat aorta involvement of Ca2+ and other cellular metabolites. *Gen.Pharmacol.* **33**, 325-336.

Acknowledgements

This doctoral work was done at the Institute of Physiology, University of Tübingen under the guidance of Prof. Dr. Helmut Heinle. First of all, I would deeply like to thank my supervisor, Prof. Heinle, for his detailed instruction, kind encouragement and persistent support throughout my doctoral work. Prof. Heinle, you provided me with an excellent platform for my academic training.

I also would like to thank Prof. Gisela Drews from the Faculty of Pharmacy, University of Tübingen for agreeing to be one of my supervisors. Prof. Drews was very helpful throughout my studies.

The financial support from Steigerwald Arzneimittel GmbH has been greatly appreciated.

I give special thanks to Prof. Dr. Karl Lackner and PD. Dr. Michael Torzewski from the University of Mainz for providing Glutathion peroxidase 1 (-/-) mice.

Many thanks also to Prof. Dr. Phil Aaronson for his help and encouragement.

Many thanks to my colleague, Daniela Hagelauer, for her friendship and the time we spent working together and helping each other.

The encouragement of Dr. Horst Apfel is highly appreciated.

I am grateful to Felor A. Davijani, Annette Hartwich, Ines D. Germann, Cristina Boß, Dan Turcanu, Katrin Wald, Steffi, Frederike, Johanna, Viktoria, Nina, Diana, Lu jing, Wang Xin and Zhang Hua for their valued friendship.

Many thanks to Miss Barbara Black for correcting my dissertation.

Special thanks to Tilman Jütte for his care and support.

I dedicate my doctorate to my parents, brother and sister for their love, support and encouragement.

Meine akademischen Lehrer

Herr Prof. Dr. P.I. Aaronson
Herr Prof. Dr. M. Avkiran
Frau Prof. Dr. S. Brain
Herr Dr. M. Curtis
Herr Dr. R. Docherty
Herr Prof. Dr. P. Doherty
Frau Prof. Dr. G. Drews
Herr Prof. Dr. H. Heinle
Frau Prof. N. Jiang
Herr PD. R. Jiao
Herr PD. Q. Li
Herr Prof. Y. Li
Herr Prof. Dr. C. Page
Herr Prof. W. Zhang
Herr PD. C. Zhou
Frau Prof. Dr. H. Zhu

LEBENSLAUF

PERSÖNLICHE DATEN

Name	Dayong Zhang
Geburtstag	09.04.1975
Geburtsort	Mishan/China

SCHULBILDUNG

1981-1993	Grund, Sekundar- und Oberschule in Mishan/Jixi, Abitur
1993-1996	Medizinstudium in der Medizinischen Hochschule Meitan
1997-1999	Medizinstudium in der Medizinischen Universität Harbin
2000-2003	M.A. in Physiologie, Medizinischen Universität Harbin
2003-2004	M.A. in Pharmakologie, Kings College London
Seit 2004	Promotion bei Prof. Dr. Heinle im Physiologischen Institut der Universität Tübingen über das Thema: Hydroperoxide-induced oxidative stress in the arterial wall: pharmacological characterization of the effects on arterial contractility

BERUFLICHE ERFAHRUNG IN CHINA

1996-1997	Zur medizinischen Weiterbildung im JiXi Chinese Hospital.
1999-2000	Zur erneuten medizinischen Weiterbildung im JiXi Chinese Hospital.