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Oxidative stress and the ischemic heart
A study in patients undergoing coronary revascularization

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CONTENTS

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2 ABBREVIATIONS

AA	arachidonic acid
AMI	acute myocardial infarction
ASA	acetylsalicylic acid
ATP	adenosine triphosphate
CABG	coronary artery bypass grafting
CAT	catalase
CPB	cardiopulmonary bypass
COX	cyclooxygenase
ESR	electron spin resonance
GSH	glutathione
GPx	glutathione peroxidase
HPLC	high performance liquid chromatography
H_2O_2	hydrogen peroxide
HOCl	hypochlorite
hsCRP	high sensitive C-reactive protein
IHD	ischemic heart disease
IRCM	iodinated radiographic contrast media
NAC	N-acetyl-cysteine
NAD(P)H	nicotine adenine dinucleotide (phosphate)
$^{\bullet}NO$	nitric oxide
$^{-}OONO$	peroxynitrite
$^{\bullet}O_2^-$	superoxide radical
$^{\bullet}OH$	hydroxyl radical
PCI	percutaneous coronary intervention
ROS	reactive oxygen species
RNS	reactive nitrogen species
SOD	superoxide dismutase
STEMI	ST-segment elevated myocardial infarction
TAS	total antioxidant status

3 LIST OF PAPERS

I. Berg K, Wiseth R, Bjerve K, Brurok H, Gunnes S, Skarra S, Jynge P, Basu S. Oxidative stress and myocardial damage during elective percutaneous coronary interventions and coronary angiography. A comparison of blood-borne isoprostane and troponin release. *Free Radic Res*. 2004 May;38(5):517-25.

II. Berg K, Jynge P, Bjerve K, Skarra S, Basu S, Wiseth R. Oxidative stress and inflammatory response during and following coronary interventions for acute myocardial infarction. *Free Radic Res*. 2005 June; 39(6):629-636.

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4 INTRODUCTION

4.1 Oxidative stress

4.1.1 History

The terms *free radicals*, *oxidative stress* and *antioxidants* have been commonly used in the discussion of disease mechanisms during the last three decades. Free radicals were first described by physicists more than one hundred years ago, but the presence of free radicals in biology was first reported in 1954[1]. It was hypothesized that oxygen radicals may be formed by enzyme reactions *in vivo* and cellular damage and degenerative processes *in vivo* were associated with them. When McCord and Fridovich in 1969 discovered the enzyme superoxide dismutase (SOD)[2], a new era in the research of free radicals in biology started, leading to investigation of oxidative damage upon DNA, proteins, lipids, and other components of the cell. Free radicals as active signalling molecules was introduced in 1977[3]. Important physiological functions that involve free radicals and other reactive oxygen species (ROS) include: Regulation of vascular tone, sensing of oxygen tension and regulation of functions that are controlled by oxygen tension, enhancement of signal transduction and oxidative stress responses (redox homeostasis).

Even though free radicals, principally derived from oxygen, have been associated with pathophysiology of a wide variety of human diseases[4], there is less definitive clinical evidence for this association. The lack of standardized, sensitive and robust methods to evaluate oxidative stress in clinical settings has been one main limiting factor[5,6]. Detection of ROS *in vivo* is difficult both due to short lifetimes of these molecules, but also due to the limited quantity of these compounds[7]. Therefore, the methods regarding ROS detection need highly sensitive analytical techniques usually involving advanced equipment[7].

4.1.2 Definitions

A free radical is defined as any atom, group of atoms or molecule having one or more unpaired electron(s) in their outer orbital. The unpaired electron is highly reactive as it seeks to pair with another free electron. When a radical gives one electron to, takes one electron from, or simply adds on to a nonradical such as most biological molecules, that nonradical becomes a radical. As a consequence, these molecules are very unstable and highly reactive, and then tend to initiate *chain reactions*: one radical begets another. Only when two radicals meet the reaction terminates. *ROS* includes both free oxygen radicals and certain nonradicals that are oxidizing agents and /or easily converted to free radicals. *Oxidation number* represents the total number of electrons that an atom either gains or loses in order to form a chemical bond with another atom. *Oxidation-reduction reaction* is defined as any chemical reaction in which the oxidation number of a participating chemical species changes. *Oxidation* may be defined as the loss of electrons or hydrogen or the gain of oxygen. *Antioxidants,* constitute a diverse group of molecules defined as any substance that when present at low concentrations compared to those of oxidizable substrate, significantly delays or prevents oxidation of that substrate. Correspondingly, *prooxidants* represent molecules that upregulate ROS and/or act as direct oxidants. *Oxidative stress* is defined as an imbalance between antioxidants and prooxidants in favour of prooxidants[8,9].

4.1.3 Reactive oxygen species – formation and elimination

Hydroxyl radical

Hydroxyl radical ($^{\bullet}OH$) is the most reactive of all free radicals *in vivo* and may attack any neighbouring molecule including DNA, proteins and lipids[10]. $^{\bullet}OH$ is produced by at least four mechanisms *in vivo*; 1) fission of water by ionizing radiation; 2) reduction of hydrogen peroxide (H_2O_2) by reduced transition metal ions (ferrous (Fe^{2+}) or cuprous (Cu^{+})) in the Fenton reaction (Equation1); 3) reaction of hypochlorite (HOCl) with $^{\bullet}O_2^-$ and 4) decomposition of peroxynitrite (^-OONO).

(1) $H_2O_2 + Fe^{2+} (Cu^{+}) \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+} (Cu^{2+})$

No known enzyme system is specialized to detoxify $^{\bullet}OH$, and the only way to prevent $^{\bullet}OH$ attack of biomolecules is to prevent its formation. However, several biological compounds like vitamins (ascorbic acid, tocopherols, retinols and carotenoids), uric acid, and glutathione (GSH) act as $^{\bullet}OH$ scavengers with formation of less reactive products preventing oxidative chain reactions.

Superoxide

Probably the most important source of $^{\bullet}O_2^-$ in aerobic cells is the electron transport chain in mitochondria. Aerobic organisms require molecular oxygen (O_2) as an electron acceptor for effective production of energy. In respiration oxygen is consumed along with metabolic substrates while adenosine triphosphate (ATP), water, and carbon dioxide are generated. During tetravalent reduction of O_2 to water, 1-3 % of the oxygen is reduced via the univalent pathway producing $^{\bullet}O_2^-$. Formation may also take place by the cell membrane-linked NADPH oxidase system, during oxidation of xanthine or hypoxanthine by xanthine oxidase (XO), and by one-electron reduction of O_2 by the cytochrome P-450 enzymatic system. $^{\bullet}O_2^-$ is also produced via autoxidation of monoamines, flavins and haemoglobin and during ionizing radiation of O_2 (Figure 1)[4,11].

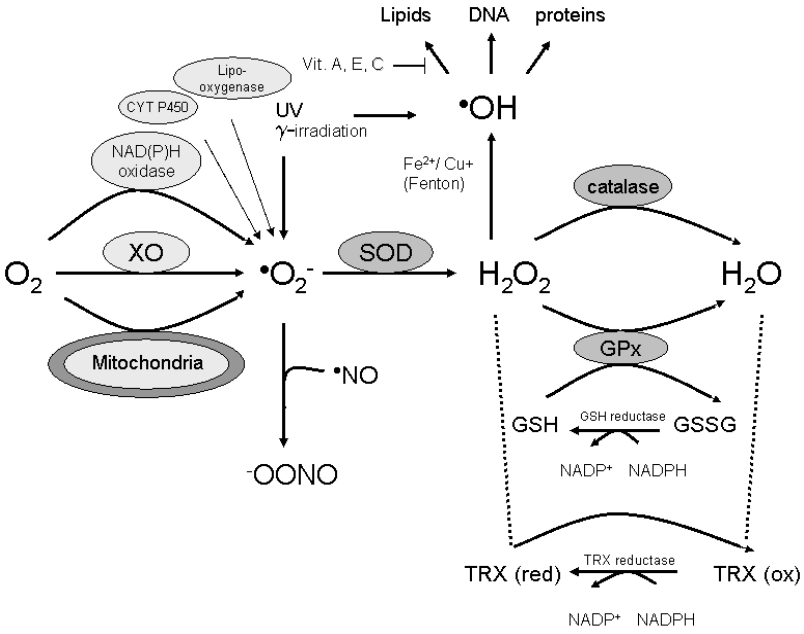


Figure 1. Main pathways of formation and elimination of ROS *in vivo.*

$^{\bullet}O_2^-$ is a hydrophilic molecule, thus not passing cell membranes without selective channels in specialized cells like erythrocytes and trombocytes. One important property of $^{\bullet}O_2^-$ is its ability to recycle the intracellular redox-active pool of oxidized metals, leading to the release of reduced metal ion for the Fenton reaction[12]. It is not highly reactive, and can therefore diffuse a considerable distance before it encounters a specific and critical target[10]. Thus formed *in vivo*, $^{\bullet}O_2^-$ is largly converted by SOD catalyzed or nonenzymatic dismutation into H_2O_2 (Equation 2).

(2) $$^{\bullet}O_2^- + {}^{\bullet}O_2^- + 2\,H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

Because of these properties, $^{\bullet}O_2^-$ is potentially as injurious as $^{\bullet}OH$[10]. Removal of $^{\bullet}O_2^-$ by SOD represents the main antioxidant defence mechanism in aerobic organisms. SOD catalyzed dismutation shortens the lifetime of $^{\bullet}O_2^-$ by a factor of 10^9 compared to the uncatalyzed reaction[13]. In mammals, SOD exists as a familiy of metalloenzymes containing copper (Cu), zink (Zn) or manganese (Mn). Cu,ZnSOD exists primarily in the cytosol but a small amount is also present in the extracellular fluid, whereas MnSOD exists solely in the matrix of mitochondria[13]. However, $^{\bullet}O_2^-$ is also able to terminate chain reactions, and therefore either too little or too much will prove detrimental[12].

Reactive nitrogen species

Nitric oxide ($^{\bullet}NO$) is not, by definition, a ROS but a reactive nitrogen species (RNS), even so it is often mentioned in this context. It is mainly formed from L-arginine by one of the three NO synthase (NOS) isoforms: eNOS, constitutive in vascular endothelial cells; iNOS, inducible by cytokines in activated macrophages and liver; and nNOS, constitutive in neuronal tissue. $^{\bullet}NO$ acts on smooth muscle cells in vessel walls to produce relaxation, thus playing a critical role in the regulation of vascular tone. $^{\bullet}O_2^-$ reacts with $^{\bullet}NO$ leading to the highly reactive and potent vasoconstrictor $^{-}OONO$. By this reaction, $^{\bullet}O_2^-$ acts indirectly as a vasoconstrictor, decreasing the level of $^{\bullet}NO$ and promoting formation of $^{-}OONO$[14].

The $^{\bullet}NO/^{\bullet}O_2^-$ ratio seems to be of biological importance not only in the regulation of the vascular tone, but also in regulation of other physiological systems by inhibition or activation[15,16].

Hydrogen peroxide

H_2O_2 represents a non-radical ROS. In addition to SOD-catalyzed production, H_2O_2 is generated by several other enzymes like xanthine, urate or D-amino acid oxidases. H_2O_2 has a lipophilic structure allowing it to cross cell membranes easily and is therefore a very injurious compund. H_2O_2 is toxic to most cells in the 10-100 μM range, but is poorly reactive itself[15]. Its toxicity is therefore due to its formation of more reactive compounds like $^{\bullet}OH$ and hypochlorous acid (HOCl). HOCl is produced by the enzyme myeloperoxidase (MPO) in activated neutrophiles and they both are highly reactive and important in bacterial killing by phagocytes[15].

The concentration of intracellular H_2O_2 is strictly regulated through control of its production as well as through its elimination by catalase (CAT) (mainly in peroxisomes), glutathione peroxidase (GPx) (in various intracellular compartments) or the thioredoxin-peroxiredoxin system (TrxR) (Equation 3)[15]. CAT is present in very low concentrations in the myocardium, whereas GPx (a selenium-dependent enzym) is present at significant concentrations in the cytosol of the heart[17].

$$(3) \qquad H_2O_2 \xrightarrow{\text{CAT / GPx / TrxR}} 2\,H_2O + O_2$$

H_2O_2 plays a key role in the control of vascular tone and is hypothesized to be the endothelial-derived hyperpolarizing factor[18]. Recently, it was showed that H_2O_2 in mice not only acts as a relaxing factor but also as a vasoconstrictor under conditions in which hyperpolarization is compromised[19].

4.1.4 Non-enzymatic antioxidants in vivo

Glutathione (GSH)

GSH (γ-glutamyl-cysteinylglycine) is a major cellular antioxidant found in millimolar concentrations in most mammalian cells. GSH is synthesized intracellularly by an ATP-driven reaction with glutamate, glycine and cysteine with cysteine as the limiting substrate. GSH plays the role of a sulfhydryl (SH) group provider. It acts both as a substrate in the scavenging reaction catalyzed by GPx and as a scavenger of vitamin C and E radicals[20]. N-acetyl-cysteine (NAC) may lead to increased cysteine levels and thereby acts as a GSH donor. Treatment with NAC is of clinical relevance, mostly known as an antidote of acetaminophen intoxication, acting by replenishing hepatic stores of GSH. Several clinical trials have studied the effects of NAC in preventing contrast-induced nephropathy in moderate-to-high risk patients undergoing percutanous coronary intervention (PCI), but these studies have yielded conflicting results[21-26]. In a recent study addition of NAC to cardioplegic solutions in coronary artery surgery, decreased lipid peroxidation in the myocardium[27].

Ascorbic acid

Ascorbic acid is the major water-soluble antioxidant in mammalian cells with an ability to scavenge ROS in the cytoplasm. Ascorbic acid appears to be crucially important in limitation of oxidative lipid damage. Thus, it acts by being an effective scavenger of the aqueous peroxyl radical and by its ability to recycle phenoxyl radicals. Also, combined with GSH and NAD(P)H, it regenerates reduced tocopherol (Figure 1)[28]. However, ascorbic acid may also have prooxidant properties since it acts as a reductant to iron[15]. Whether ascorbic acid acts as an antioxidant or as a prooxidant, depends on its concentration and on the presence of free transition metal ions[28].

Vitamin E

Vitamin E is a class of lipophilic, phenolic compound of plant origin present in cellular and subcellular membranes and lipoproteins. Vitamin E comprises eight known substances (α, β, γ, and δ) and 4 tocotrienols. The major tocopherol found in mammalian tissue is α-tocopherol, although significant quantities of desmethyl forms (particularly γ -tocopherol) are also present. Tocopherols scavenge free radicals by forming phenoxyl radical (tocopherol radical) and are probably the most important

inhibitors of free radical induced chain reactions involving lipid peroxides. α-tocopherol has traditionally been stated more biologically active than the desmethyl tocopherol, but recent studies have shown that γ-tocopherol might be an even more efficient scavenger of free radicals than α-tocopherol and thereby provide special benefits[29,30]. Like ascorbate, tocopherols can reduce Fe^{3+} to Fe^{2+} and thus exert prooxidant effects. In addition, the phenoxyl radical reacts with lipids by abstraction of hydrogen from polyunsaturated fatty acids. The recycling of the phenoxyl radical by ascorbic acid and other reducing agents presumably largely prevents such occurrences *in vivo*[15].

Vitamin A

Vitamin A (retinol and retinyl esters) is a generic term used to describe a number of related compounds that exhibit the biological activity of retinol. Vitamin A is a required nutrient which plays a key role in regulating growth, differentiation and apoptosis. Plasma retinol concentrations are homeostatically regulated, and well-nourished humans respond only modestly to supplementation. Vitamin A deficency is the leading cause of childhood blindness in the world, and dysregulation of retinoid metabolism has been found in several cancers[31].

Carotenoids

Carotenoids include a group of more than 600 compounds found primarily in foods of plant origin. Several carotenoids serve as a precursor of vitamin A in humans and are therefore often classified as vitamin A. The dietary carotenoids are α and β-carotene. Unlike retinols, there is no homeostatic control of plasma levels of carotenoids, and the plasma concentration reflect the dietary intake[31]. Carotenoids have shown antioxidant activity *in vitro*, but a potential antioxidant role in humans *in vivo* is still controversial. However, β-carotene is a unique chain-breaking lipid-soluble antioxidant which traps peroxyl radicals. It is also reported to have substantial single-oxygen quenching ability and to inhibit lipid peroxidation[32].

4.2 Assessment of oxidative stress

A large number of assays are available to quantify oxidative stress, but except for detection of free radicals by electron spin resonance (ESR), they are all based on

indirect detection either by use of trapping molecules (exogenous or endogenous), measuring cell damage due to ROS attack or measuring levels of antioxidants. Most traps used in experimental studies are highly toxic in humans, and therefore these approaches are not transferable to clinical use. Measurement of oxidative damage is based on the same principle, using biological compounds like lipids, proteins or DNA, as the trapping molecules produce unique biomarkers of oxidative stress. Measuring consumption of antioxidants is complicated due to the complexity of antioxidant mechanisms and the possibility of upregulation of the endogenous antioxidant defence. To assess consumption of antioxidants caused by oxidative stress *in vivo*, it is important to identify the ROS species in question and the source of formation. Since the lipid bilayer in the cell membrane is an important arena for ROS attack, the first line of antioxidant defence will involve lipid soluble antioxidants as tocopherols.

4.2.1 Direct detection of ROS *in vivo* by electron spin resonance

Electron spin resonance (ESR) spectroscopy is the only technique whereby free radicals may be detected directly by the presence of unpaired electrons[4,7]. However, ESR is not sensitive enough to quantify free radicals in biological systems, and use of "spin traps" or "probes" are necessary (see below).

4.2.2 Indirect detection of ROS in vivo

Trapping methods

ESR with nitro derivates as traps

A wide range of traps are available, most of them are nitro derivates such as α-phenyl N-tert-butyl nitrone (PBN) and 5, 5-dimethyl-1-pyrroline-N-oxide (DMPO), but all are too toxic for administration in humans[4]. Combined with the fact that there are major technical difficulties in studying whole animals, this technique has mostly been used on body fluids and tissue samples whereby the trap is administrated *in vitro* or *ex vivo*[4]. Recently, Clermont et al. demonstrated free radical production by adding PBN to coronary sinus blood drawn during coronary bypass surgery[33]. Only secondary radicals like those of lipid or possibly also protein origin may be detected in this way[4].

ESR with ascorbic acid as traps

Ascorbic acid reacts with a wide range of free radicals to make ascorbate radicals, which may be detected by ESR[34]. However, this method is only semiquantitative since ascorbate radicals quickly react with each other producing nonradicals[4].

Aromatic hydroxylation and HPLC

Aromatic compounds, including salicylate and phenylalanine, are more appropriate as *in vivo* traps than nitro-species used in ESR due to less toxicity. These compounds are used as trapping molecules both in *in vitro* and *in vivo* studies. Salicylate is hydroxylated by $^{\bullet}OH$ to yield 2,3 and 2,5 dihydroxybenzoate (DHBA) which both may be detected by high performance liquid chromatography (HPLC). 2,5 DHBA is also produced by the cytochrome P-450 enzymatic system in the liver, but in contrast 2,3 DHBA seems to be a specific marker of $^{\bullet}OH$ production *in vivo*. Elevated levels of 2,3 DHBA in peripheral venous blood following myocardial infarction has been reported[35]. The success of aromatic compounds in detecting $^{\bullet}OH$ depends on their concentration at sites of $^{\bullet}OH$ generation and competing conditions with other scavengers[4]. We have confirmed the efficacy of the salicylate HPLC assay for detection of $^{\bullet}OH$ in *in vitro* experiments of antioxidant properties of radiographic contrast media[36].

Biomarkers of ROS

The differences between endogenous trapping products and biomarkers of ROS are slight. Biomarkers of ROS represent oxidative damage and, according to some authors, a core criterium is that they have to be predictive of the later development of disease.[4] In this paper the authors suggest following technical criteria to an ideal biomarker of ROS:

- It should be able to detect a major part of the oxidative damage *in vivo.*
- Its measurement should rely on chemically robust technology.
- It should not be confounded by dietary factors.
- It should be stable on storage.

Many peroxidation products of lipids, DNA and protein by ROS or RNS fullfil these criteria. Hence many assays are available for measuring the rate of oxidation of

biomolecules, however, each technique is unique and no single method can be said to give an accurate overall assessment of oxidative injury.[15]

Isoprostanes

In 1990, Morrow and Roberts discovered the isoprostanes[37]. Isoprostanes belong to a family of prostaglandin (PG) derivates that are mainly formed from free radical-catalyzed peroxidation of arachidonic acid (Figure 2).

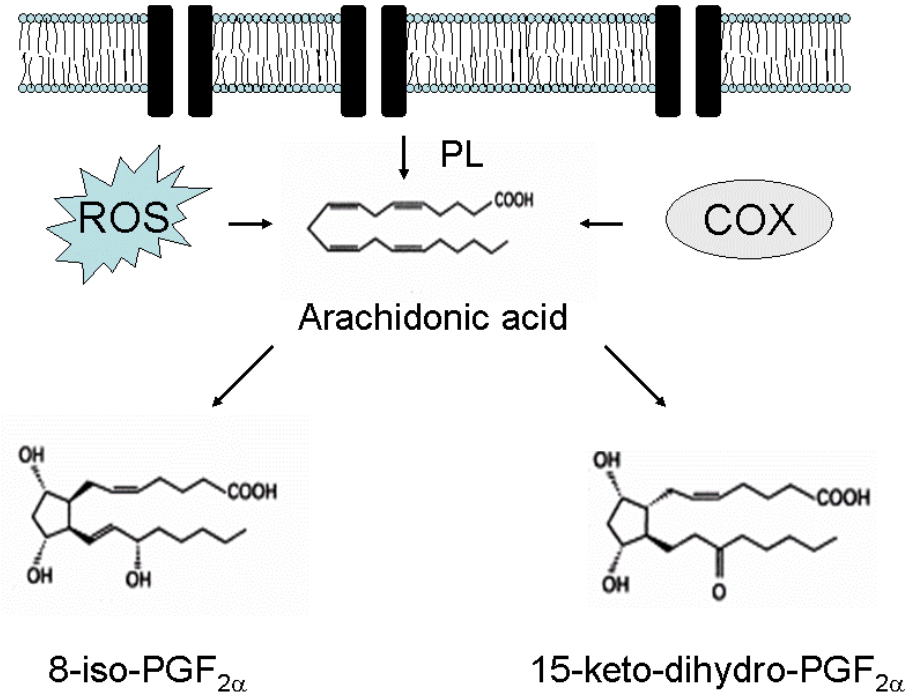


Figure 2. Formation of 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ from arachidonic acid by ROS and cyclooxygenase (COX) respectively.

Isoprostanes are relatively stable, found in most tissues and fluids and provide the most reliable non-invasive approach to assess oxidative stress in human beings today[5,6,38-40]. The first class of isoprostanes described were F_2-isoprostanes, discovered during the analysis of F-ring metabolites of PGD_2. They consist of 64 different compounds, but formation of some is favoured over others. Unlike prostaglandins, isoprostanes are formed *in situ* esterified to phospholipids, preferably in cell membranes from which they can be released by phospholipase activity[41]. They are present in human plasma in two distinct forms; esterified in LDL phospholipids and as the free acid. The ratio of these two forms is approximately 2:1[39,42]. 8-iso-

$PGF_{2\alpha}$ is to date the most studied F_2-isoprostane due partly to the fact that it possesses bioactivity and may participate in pathophysiological processes[37]. 8-iso-$PGF_{2\alpha}$ may thus cause vasoconstriction and bronchoconstriction due to their ability to alter smooth muscle and platelet functions[43]. These effects are thought to be mediated via the activation of thromboxane receptors, with isoprostanes acting as full or partial agonists. A strong link between lipid peroxidation and diseases associated with ischemia-reperfusion, atherosclerosis and inflammation has been suggested by elevated levels of 8-iso-$PGF_{2\alpha}$ observed in such diseases. Thus, 8-iso-$PGF_{2\alpha}$ provides a unique opportunity to investigate lipid peroxidation in human disease and is a most relevant biomarker for rational choice and dose selection of antioxidants.

4.3 Inflammation

Oxidative stress and inflammation are two processes that appear to be intricately intertwined[8,44-47]. ROS may initiate inflammation and vice versa. However, to cover the immense topic of inflammation is beyond the scope of the present thesis. Nevertheless, prostaglandins, a common biomarker of inflammation, and isoprostanes (which is the main biomarker of oxidative stress in this study), have the same origin in arachidonic acid as shown in Figure 2[48,49]. Even though the main pathway of isoprostane formation is from free radical attack of the arachidonic acid (AA) in the cell membrane, cyclooxygenase (COX) mediated AA products also constitute a minor amount of isoprostane products. Whether an increase of isoprostanes is due to a previous inflammatory process with upregulation of COX, or is the primary response due to oxidative stress is of outmost importance for understanding injury mechanisms and potential intervention strategies. In this context, we have incorporated two biomarkers of inflammation; 1) 15-keto-dihydro-$PGF_{2\alpha}$, a marker of the COX mediated pathway of AA metabolism (Figure 2); and 2) high sensitive C-reactive protein (hsCRP), an acute phase protein which is activated by an alternative process from various cytokines and thus independent of COX.

<u>15-keto-dihydro-$PGF_{2\alpha}$</u>

$PGF_{2\alpha}$ is a major prostaglandin formed *in vivo* from AA through catalysation by COX. With a very short half-life in plasma (< 1 min), $PGF_{2\alpha}$ metabolizes further to 15-keto-

dihydro-$PGF_{2\alpha}$. Being more stable and more specific to *in vivo* production than $PGF_{2\alpha}$,15-keto-dihydro-$PGF_{2\alpha}$ is a reliable index of COX catalyzed lipid peroxidation in plasma[50,51]. A specific and validated radioimmuno assay for detection of 15-keto-dihydro-$PGF_{2\alpha}$ is used in this thesis[52]. With this assay the $PGF_{2\alpha}$-metabolite can be reliably quantified in different compartments, including plasma and urine. The original suggestion of $PGF_{2\alpha}$ being a pro-inflammatory mediator *in vivo* has recently been verified in experimental studies by use of this assay[48,53,54]. Further, formation of $PGF_{2\alpha}$ was increased in plasma and joint fluid in patients with severe rheumatoid arthritis[55] and in plasma in men given supplements of conjugated linoleic acid (CLA)[56]. The role of $PGF_{2\alpha}$ in diseases associated with cardiovascular complications and atherosclerosis is not known, and further investigation in this field is needed.

C-reactive protein

C-reactive protein (CRP) is a sensitive acute phase protein commonly used to identify inflammatory reactions. It is synthesized by the liver following induction by cytokines and has been the classical parameter for detection of a systemic inflammation for many years. Recently, more sensitive CRP assays have been developed, and an increase of high sensitive CRP (hsCRP) has proved valuable for early detection of infection and for risk assessment in coronary heart disease[57,58]. We measured hsCRP as an index of early inflammation independent of the COX pathway.

4.4 Ischemic heart disease

Definition and clinical survey

Ischemic heart disease (IHD) is a term for clinical syndromes caused by significantly reduced blood flow to a region of the heart (ischemia). Thus it implies conditions of insufficient myocardial perfusion causing an imbalance between oxygen supply and oxygen demand rendering the myocardium ischemic. The clinical manifestations of IHD are highly variable spanning from silent ischemia with no clinical symptoms to acute coronary syndromes including acute myocardial infarction and in the worst case sudden cardiac death. IHD is the main cause of disease and death in middle-aged and elderly adults in most countries in the western world. Although mortality has declined during the last decade, an elderly population with IHD and heart failure and the

growing epidemic of obesity in young people indicate that IHD may still cause a heavy burden to the health care systems with a substantial amount of resources spent on diagnosis, treatment and rehabilitation.

Atherosclerosis

Atherosclerosis is a multifactorial disease resulting in narrowing of coronary arteries and is now commonly viewed as a chronic inflammatory disease associated with important risk factors such as hyperlipidemia, diabetes and hypertension. The affected vessel wall contains lipid deposits, mononuclear cells, proliferating smooth muscle cells, and extracellular matrix compounds. Excessive ROS production with peroxidation of low density lipoproteins (LDL) has been implicated in the pathogenesis of atherosclerosis and hypertension. Oxidized LDL binds to special LDL receptors on macrophages which become "foam cells". Activated enzymes such as XO, NAD(P)H oxidase or NOS then produce more ROS, accelerating the formation of an atherosclerotic plaque[59].

Pathophysiology of myocardial ischemia

Coronary blood flow supplies the myocardium with oxygen and nutrients and removes waste products. When an atherosclerotic plaque builds up in a coronary artery, reduced blood flow to the myocardium may result, causing symptoms ranging from mild chest pain to a heart attack. Prolonged ischemia as it occurs during acute occlusion of a coronary artery, leads with time to cell and tissue necrosis. In this process a multitude of detrimental factors contribute, such as: ATP-depletion; lactate formation; acidosis; redistribution of cellular potassium, sodium and calcium; accumulation of hypoxanthine; mitochondrial swelling and failure; and cell membrane damage[60,61].

The extent of myocardial damage may be reduced with early reperfusion[62]. However, restoration of oxygenated blood flow (reperfusion) into ischemic myocardium can initiate a cascade of events that may paradoxically produce additional myocardial cell dysfunction and cell necrosis. So-called reperfusion injury includes arrhythmias, myocardial stunning, microvascular damage, and cell death, which may occur either together or separately[60,62-67]. As will become apparent later, reperfusion injury is a controversial entity when seen in a clinical context.

Angina pectoris

Angina pectoris (angina) is a condition in which the myocardium becomes transiently ischemic mainly due to one or more coronary stenoses. The corresponding chest pain in these patients subsides rapidly on reduction of work load or improvement in myocardial blood flow by drugs (i.e. nitroglycerine). Depending on the stability of the atherosclerotic plaque the disease may manifest itself as either *stable* or *unstable angina* with the latter form more impending for development of thrombosis and an acute myocardial infarction (AMI).

Acute myocardial infarction.

AMI is defined by death of cardiomyocytes (necrosis) caused by prolonged ischemia due to occlusion of one or more of the coronary arteries. A majority of AMIs results from rupture of an atherosclerotic plaque leading to acute thrombosis and occlusion of the affected artery[68]. Depending on whether the thrombotic occlusion is total or subtotal an AMI with or without ST-segment elevation in the electrocardiogram (ECG) ensues. Due to cell membrane damage AMI is followed by leakage to the systemic circulation of cardiospecific macromolecules or markers like troponins or creatine kinase isoenzyme MB (CK-MB) [69].

Adaptive ischemic syndromes

Postischemic dysfunction or myocardial stunning is a reversible injury that persists after reperfusion[70]. It may be seen after revascularization by percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG) and has been regarded as a myocardial injury too small to result in an AMI. Stunning is characterized by transient heart failure subsiding over hours and days. A probable cause lies in a combination of oxidative stress and deficiencies in cell calcium homeostasis.

Another ischemia related syndrome is hibernating myocardium, a condition characterized by downregulated contractile function which occurs in chronic forms of myocardial ischemia[71]. In hibernation the ischemic region has adapted to the reduced blood flow without becoming necrotic, and revascularization by PCI or CABG reverses the apparent heart failure. Repeated small ischemia-reperfusion attacks, also involving ROS, have been held as potential mechanisms behind the development of hibernation.

A third adaptive syndrome is myocardial preconditioning in which brief ischemia-reperfusion episodes enhance local defence mechanisms against a subsequent severe episode of ischemia-reperfusion. A reaction cascade follows the activation of latent cell signal pathways and detrimental cellular or subcellular changes in structure and function are greatly delayed[64]. Intriguingly, ROS may participate in initiating preconditioning, and furthermore, upregulation of MnSOD is one probable mediator in the apparent myocardial protection.

4.5 Myocardial ischemia-reperfusion injury and oxidative stress

Potential mechanisms

As indicated in the previous paragraph ROS-injuries and ROS-mediated mechanisms contribute to the complex pathophysiology of myocardial ischemia-reperfusion. The cellular mechanisms behind ischemia-reperfusion injuries involve the interaction of a number of cell types, including endothelial cells, circulating blood cells (e.g., leucocytes, platelets), and cardiomyocytes[66,72].

In general, ROS-mediated reperfusion injury is considered as a two-stage event caused by: an initial burst of ROS at onset of reperfusion; and thereafter as the result of ROS released during inflammation of the previously ischemic segment. ROS has the potential to injure vascular cells and cardiomyocytes directly, and can initiate a series of local chemical reactions and genetic alterations that ultimately result in an amplification of the initial ROS-mediated injury to cardiomyocytes. Acting together, oxidative stress and Ca^{2+}-overload are proposed as two main complexly interrelated hypotheses of reperfusion injury[65,73].

When molecular oxygen is reintroduced into the ischemic myocardium, the normal balance between prooxidant and antioxidant factors is changed. Thus the preceding depletion of ATP and further breakdown to purine catabolites like hypoxanthine (HX) and xanthine (X) and the parallel conversion of xanthine dehydrogenase to a xanthine oxidase (XO) may set the scene for a major release of superoxide (Equation 4)

$$\text{(4) ATP} \longrightarrow \text{ADP} \longrightarrow \text{AMP} \longrightarrow \text{Adenosine} \longrightarrow \text{HX} \xrightarrow{\text{XO}} \text{X} \xrightarrow{\text{XO}} {}^{\bullet}O_2^- + \text{Uric acid}$$

In human myocardium such a mechanism is more likely in endothelial cells than in cardiomyocytes due to the low level of xanthine dehydrogenase in the latter cell type[74]. There is reason to believe, however, that reperfusion induced early ROS release is related to the mitochondrial repiratory chain and to an altered state of parallel antioxidant proteins in both cardiomyocytes and endothelial cells. Ischemia also leads to decrease in Na^+/K^+-ATPase activity and a rise in intracellular Na^+ and Ca^{2+}. High intracellular Ca^{2+} results in ROS production due to the disruption of the mitochondrial proton gradient[59,75]. Production of $^{\bullet}O_2^-$ and all other reactive oxygen species require oxygen, which is a substance in short supply during ischemia. However, total cell anoxia is encountered infrequently in a clinical situation. Thus ROS may still be created during a prolonged period of ischemia. Complicating the issue further, ROS may induce preconditioning with an upregulation of the antioxidant defence[64]. Lately, the term "reperfusion injury" has been questioned and all events following reperfusion may be related to the prior ischemia[76].

Experimental versus clinical studies of oxidative stress

A wealth of documentation from experimental studies has shown that oxidative stress may be a major injurious factor when oxygen is reintroduced to ischemic tissue[77-84]. Accordingly, both endogenous and exogenous antioxidants have been able to ameliorate tissue injury in experimental heart models. Some potent antioxidants used in animal experiments include: SOD or SOD mimetics; the glutathione donor N-acetyl cysteine (NAC); the iron chelator desferrioxamine; vitamins and drugs with secondary properties like the angiotensin-converting enzyme inhibitor captopril and the MRI contrast agent manganese-dipyridoxyl-diphosphate[79,85-89].

Clinical studies, however, have shown less clear-cut evidence supporting the hypothesis of ROS mediated myocardial injury in situations with acute ischemia-reperfusion episodes. There is overwhelming evidence implicating oxidative stress in the long term development of IHD, but the implications are not evident. Recent primary prevention trials have investigated the role of antioxidants, mainly vitamin E or C alone or in combination, on patients at risk of cardiovascular disease but the results are conflicting[90-93]. In clinical studies the focus has generally been on ROS related parameters and most often indices of myocardial injury have been lacking[33,94-99]. Complicating the issue further, recent research have indicated new roles of ROS as

signal molecules involved in protective cellular processes, and sheds doubt about the concept of oxidant stress per se and of the value of antioxidant therapy[100-102].

The above considerations are not a dilemma of oxidative stress and antioxidant strategies only, but adds to the large number of indications and drugs being highly questioned in current myocardial protection strategies. Thus a main conclusion in a recent review on "Myocardial Protection at Crossroads" is that translation of apparent experience with protective agents from animals to humans have largely failed, partly due to a limited value of experimental models and partly due to the complex clinical pathophysiology with multiple confounding factors and less indications for protective therapies[103].

4.5.1 Diagnostic and therapeutic procedures

Coronary angiography

The anatomy of coronary arteries is visualized by injecting iodinated radiographic contrast media (IRCM) selectively into the coronary arteries. The procedure named "coronary angiography" involves introduction of a catheter into the femoral or radial artery and positioning it into the coronaries under x-ray guidance by use of IRCM injections. During the injection and a few seconds thereafter the IRCM replaces almost completely the blood within the examined artery. To view the coronary anatomy from different angles, repetitive injections are performed and a total volume of 50-80 mL IRCM is usually delivered in 8-12 injections. During repeated injections in a coronary intervention like PCI the total injected volume commonly amounts to 200-300 mL. Despite large volumes being administered in patients with IHD, the frequency of serious adverse reactions is low. Flushing, bradycardia, angina pectoris, and elevation of the end diastolic pressure, are common side-effects during coronary angiography[104].

4.5.2 Coronary revascularization

Percutaneous Coronary Intervention (PCI)

Percutaneous coronary intervention (PCI) is a rapidly expanding treatment for both chronic and acute stages of coronary artery disease (Figure 4). PCI involves a

mechanical technique for opening of stenotic coronary arteries with a balloon catheter. Practically, the catheter is introduced via the femoral or radial arteries and advanced towards and through the stenotic segment of the coronary artery. On balloon inflation the plaque ruptures and the stenosis disappears or is greatly reduced. Balloon inflation may be repeated several times with each inflation typically lasting for 10-15 seconds, sometimes longer. In most cases, a metallic prosthesis (stent) is implanted at the site of the previous stenosis in order to improve the results. During balloon inflation the coronary artery is totally blocked by the balloon introducing a brief ischemic episode. Thus, PCI of a narrowed coronary artery represents a clinical model of repeated short ischemia-reperfusion episodes. During PCI, arrythmias and pain due to the ischemia induced may occur.

Elective PCI

PCI is carried out in patients with stable angina. The patients typically present normal levels of the myocardial biomarker troponin T in advance.

Primary PCI

PCI is carried out as the primary reperfusion strategy for acute ST-segment elevation myocardial infarction (STEMI) without previous or concomitant thrombolytic therapy. In this setting the patient typically presents with a totally occluded coronary artery, as seen in Figure 3. Although the artery is successfully recanalized by primary PCI, the great majority will suffer some degree of myocardial necrosis and an increase of cardiac troponins is typical. Furthermore, reduced microcirculatory blood flow ("no reflow" phenomenon) and ischemia may persist after PCI.

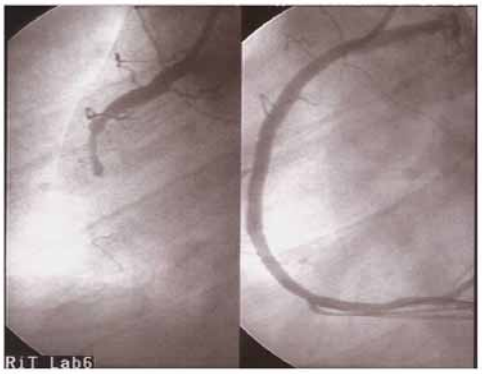


Figure 3.
Coronary angiograms obtained during primary PCI. The angiograms shows the right coronary before (left) and after (right) revascularization.

Coronary artery bypass grafting

CABG implies open heart surgery in which a "bypass" is created around the narrowed part of a coronary artery by use of a vein (i.e. saphenous vein) or an artery (i.e. the internal mammary artery) graft from the patients. Cardiopulmonary bypass (CPB) with cardiac arrest by cross-clamping of aorta is applied when performing the operation. During the cross-clamp period the myocardium becomes globally ischemic, but is reperfused on declamping. The cross-clamp period lasts for the duration of inserting the grafts, typically 20 to 45 min. During CABG the whole body in general and the myocardium in particular are subjected to considerable mechanical and biochemical stresses. First, the sternotomy itself involves a major trauma leaving a significant wound. To avoid bleeding from cut vessels (arteries and veins) the use of diathermy is extensive and may lead to confluent local patches of burn injury. The procedure with creation of anastomoses involves direct manipulation of both coronary arteries and myocardium. CPB is also traumatic to the blood cells with haemolysis as a consequence. Furthermore, open heart surgery is a complex pathophysiological situation with use of several pharmaceuticals which might affect the pro- or antioxidant balance[105].

5 AIMS

The thesis was initiated with background in the apparent discrepancy between mainly positive experimental experience but largely nonconclusive clinical experience concerning oxidative stress and antioxidants in ischemic heart disease. Since this discrepancy relates partly to methods to identify oxidative stress and partly to a lack of investigation of its relationship to myocardial injury, aims of the thesis were:

A. To identify oxidative stress in patients during myocardial ischemia-reperfusion by repeated measurements of the stable lipid peroxide 8-iso-$PGF_{2\alpha}$.
B. To investigate a potential relationship between oxidative stress assessed by 8-iso-$PGF_{2\alpha}$ and myocardial injury assessed by troponin T.
C. To investigate whether antioxidant therapy might ameliorate oxidative stress and myocardial injury in patients during myocardial ischemia-reperfusion.

To highlight aims A and B studies were conducted in patients during elective PCI (I), primary PCI (II) and CABG (III). Aim C was intended as a follow-up to I-II.

6 METHODS

6.1 Study participants and design

All patients were recruited from the Trondheim University Hospital (St. Olav Hospital). They were examined and/or treated for IHD as summarized in Table 1. The Regional Committee for Medical Research Ethics (REK) approved the protocols that were used in the studies which were conducted in accordance with the Declaration of Helsinki. All subjects gave informed written consent.

Table 1. Characteristics of participants in study I-III

Study	No of subjects	Age (years[1])	Female (%)	Diagnosis	Clinical procedure
I	38	61 (39-84)	9 (23.7)	Stable angina	Elective PCI
I	9	61 (51-72)	1 (11.1)		Angiography
II	16	61 (38-78)	3 (18.8)	STEMI[2]	Primary PCI
III	20	63 (50-79)	4 (20)	Stable or unstabile angina[3]	CABG

[1]Median (min-max)
[2] ST-segment elevated myocardial infarction
[3] Stable angina (n=16)

Study I:

Patients with stable angina (n=38) subjected to elective PCI or to diagnostic coronary artery angiography (n=9) were recruited consecutively over 9 weeks in 2001. All patients were referred for coronary angiography and/or coronary intervention (PCI) at Department of Cardiology.

Study II:

Patients (n=16) treated with primary PCI for acute myocardial infarction (AMI) at Department of Cardiology were recruited over a 8 month period in 2002.

Study III:

Patients with stable (n=16) and unstable (n=4) angina were subjected to open heart surgery and CABG at Department of Cardiothoracic Surgery. Patients were enrolled over a 8 month period in 2003-2004.

6.2 Sampling

The studies were performed in a standard routine setting without introducing any additional intervention. The only extra burdens to the patients were related to blood and urine sampling. To avoid autoxidation sampling and handling required particular attention.

Study I.

As indicated in Figure 4, samples were taken from peripheral blood from each patient at four time points. The first sample was taken from the introducer immediately after it was inserted into the femoral artery but before medications (heparin) and contrast media were introduced. This sample represents the baseline level of each patient in this study. The second sample was also taken from the introducer, immediate after the end of procedure (PCI or angiography). The end of PCI was set to the time the operator had finished all balloon inflations and stent insertion(s). Three hours thereafter, the third sample was taken from the introducer or by vein puncture in the cubital vein. The last sample was taken by vein puncture in all patients the following morning.

Study II.

Totally 14 samples of peripheral blood were collected from each patient prior to and immediately following PCI and at 24 hours (Figure 4). At the first 12 time points, the samples were collected from the introducer (as described above), and thereafter vein puncture was performed.

Study III.

This study included systemic arterial blood taken from a catheter in the radial artery (15 samples) and local coronary venous blood taken from a catheter in the coronary sinus (8 samples) (Figure 4). These samplings were synchronized. Urine was collected in 4 samples from a catheter in the urinary bladder.

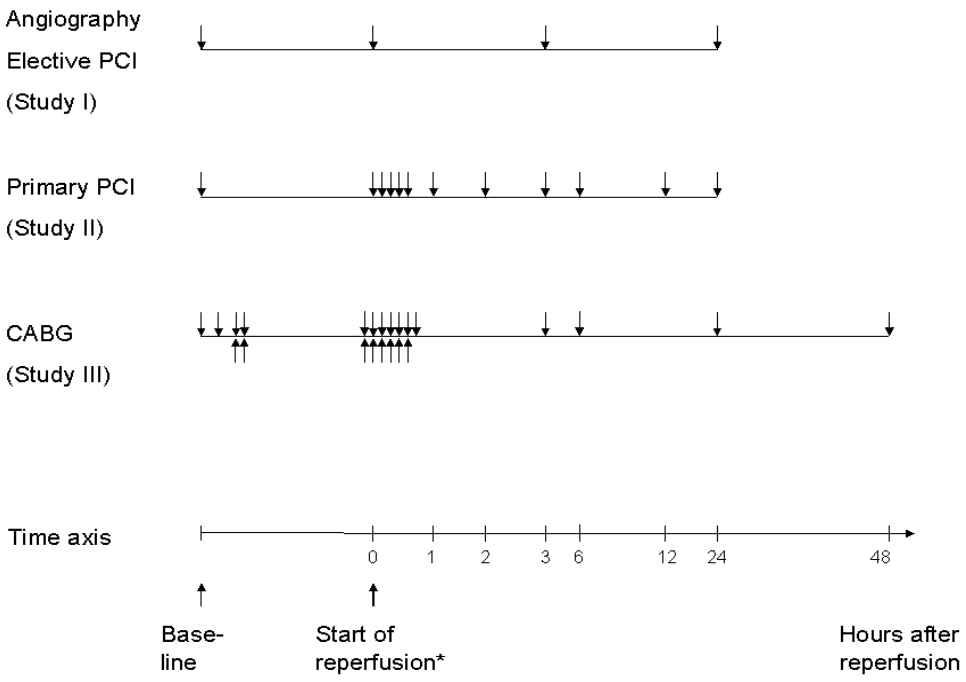


Figure 4. Study design: Outline of sampling of blood in study I, II and III. Arrows above lines illustrates samplings from peripheral blood, while arrows below illustrates samplings from coronary venous blood. The bottom line (time axis) illustrate time points in hours from baseline and from start of reperfusion (* or end of procedure).

6.3 Biochemical analyses (Table 2)

8-Iso-$PGF_{2\alpha}$

A commercial assay, 8-Isoprostane EIA kit (8-iso Prostaglandin $F_{2\alpha}$) from Cayman Chemical Company, Ann Arbor, MI, USA, was initially tried out in study I, but was abandoned after few months due to technical problems with the assay components and

its (changing) protocols. At the time other groups also experienced difficulties when applying the assay.

In collaboration with Associate Professor Samar Basu (Section of Clinical Nutrition Research, Public Health and Caring Sciences, Faculty of Medicine, Uppsala University, Sweden) all 8-iso-$PGF_{2\alpha}$ analyses in this thesis have been carried out there. Based on prior experience with prostaglandin analyses[51], the laboratory has developed its own radioimmunoassay method in which the free fraction of 8-iso-$PGF_{2\alpha}$ in plasma or urine is measured[106]. An antibody was raised in rabbits by immunization with 8-iso-$PGF_{2\alpha}$ coupled to bovine serum albumin at the carboxylic acid by the 1,1'-carbonyl-diimidazole method. The cross-reactivity of 8-iso-$PGF_{2\alpha}$ antibody with 15-keto-13,14-dihydro-8-iso-$PGF_{2\alpha}$, 8-iso-$PGF_{2\beta}$, $PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$, 15-keto-13,14-dihydro-$PGF_{2\alpha}$, TXB_2, 11β- $PGF_{2\alpha}$, 9β- $PGF_{2\alpha}$ and 8-iso-$PGF_{3\alpha}$ was 1.7, 9.8, 1.1, 0.01, 0.01, 0.1, 0.03, 1.8 and 0.6% respectively. The detection limit of the assay was 23 pM with an intra assay coefficient variation (CV) of 12-15%. Samples from each study (paper I-III) were analyzed collectively in each run of measurements, and always with the same batch of antibody.

Troponin T .

Troponin T was measured by electrochemiluminescence with Elecsys 2010 analyzer (Roche Diagnostics, Mannheim, Germany). The detection limit of the assay was 0.01 μg/L.

Total antioxidant status

Total antioxidant status (TAS)[107] in plasma was assayed by use of a commercial colourimetric test (RANDOX, CrumLin, UK) adapted to an automated analyzer (Cobas Mira, Roche Diagnostic, Switzerland). The assay is based on incubation of 2,2-azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS) with metmyoglobin (Fe^{3+}) and H_2O_2 to produce ferrylmyoglobin (Fe^{4+}). Thereafter the relatively stable free radical $^{\bullet}ABTS^{+}$ is formed and detected by photometry at 600 nm. Potent antioxidants suppress colour formation to a degree which is proportional to their concentration. The assay was calibrated with the tocopherol analog Trolox and antioxidant capacity in the samples was expressed relative to 1.0 mM Trolox.

Vitamin E

α- and γ-tocopherols were assayed by HPLC with UV-detection based on methods described by Nierenberg et al and Comstock et al[108,109].

Vitamin A

Retinol, carotenoids (α+β) were assayed by HPLC with UV-detection based on methods described by Nierenberg et al and Comstock et al[108,109].

15-Keto-dihydro-$PGF_{2\alpha}$

15-Keto-dihydro-PGF2α was measured by a validated radioimmunoassay as described by Basu.[52] An antibody was raised in rabbits by immunization with 15-keto-dihydro-$PGF_{2\alpha}$ coupled to bovine serum albumin at the carboxylic acid by the 1,1'-carbonyl-diimmidazole method. The cross-reactivity of the antibody with $PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$, PGE_2, 15-keto-13,14-dihydro-PGE_2, 8-iso-15-keto-13,14-dihydro-$PGF_{2\alpha}$, 11β- $PGF_{2\alpha}$, 9β- $PGF_{2\alpha}$, TXB_2, and 8-iso-$PGF_{3\alpha}$ was 0.02, 0.43, <0.001, 0.5, 1.7, <0.001, <0.001, <0.001 and 0.01 % respectively. The detection limit of the assay was 45 pM with an intra assay coefficient variation (CV) of 12-14%.

High sensitive C-reactive protein (hsCRP)

hsCRP was measured by latex-enhanced (high sensitive) immuno-turbidimetry on a Hitachi 917 Analyser (CRP (Latex) HS, Roche diagnostics, Mannheim, Germany). The detection limit of the assay was 0.03 mg/L (analytical sensitive).

Albumin

Albumin concentrations (g/L) were measured by a colourimetric endpoint assay on a Hitachi 917 analyzer (BCG method, Roche Diagnostics, Mannheim, Germany).

Lipids

Lipids including total cholesterol, HDL-cholesterol and triglycerids were measured by colourimetric endpoint assays on a Hitachi 917 analyzer (Roche Diagnostics, Mannheim, Germany).

Creatinine

Creatinine concentration was determined in each urine sample by a colourimetric method using IL Test creatinine 181672-00 in a Monarch 2000 centrifugal analyzer (Instrumentation Laboratories, Lexington, MA,USA).

Table 2. Assays used in study I-III

Assay	Study I	Study II	Study III
8-iso-$PGF_{2\alpha}$	x	x	x
troponin T	x	x	x
TAS	x	x	x
α - tocopherol	x	x	x
γ - tocopherol	x	x	x
retinol	x	x	x
carotenoids (α and β)	x	x	x
15-keto-dihydro-$PGF_{2\alpha}$	x	x	x
high sensitive CRP	x	x	x
albumin	x	x	x
creatinine (urine)			x

6.4 Statistical analyses

Data analyses were performed with the Statistical Package for Social Sciences (SPSS 11.5 and 12.0, Chicago, Illinois, USA) or GraphPad Prism Software (GraphPad Software 4.01, Inc., San Diego, CA, USA).

Most data had a non-Gaussian distribution and are presented as median values with interquartile ranges (25 % and 75 %) in the papers. These studies implicate many samplings from each patient, and sometimes one or two sample points are missing for various reasons. For statistical reasons we need a result at every time point in every patient and so-called “missing values” were filled in with estimated values using the expectation-maximization (EM) method. Comparisons within groups over time were made by means of the Friedman test and Dunn’s multiple comparison tests. Wilcoxon signed ranks test was performed for comparison between two dependent groups and Mann-Whitney test was performed when comparing two independent groups. We employed Spearman’s correlation coefficient (rho) for correlation analyses. For all analyses values below detection limit were set to detection limit at calculation. Values for $p< 0.05$ were considered significant.

7 SUMMARY OF RESULTS

7.1 Paper I

In study I we wanted to examine whether repeated brief ischemic episodes in the heart, as may occur during elective PCI, might induce an oxidative stress and, eventually, if oxygen stress correlated with myocardial injury. We studied 38 and 9 patients who underwent PCI and diagnostic coronary angiography, respectively. Peripheral blood was sampled at different time points for plasma analyses of: 8-Iso-$PGF_{2\alpha}$ (oxidative stress), 15-keto-dihydro-$PGF_{2\alpha}$ (inflammation), troponin T (myocardial injury), hsCRP, vitamin A, vitamin E and TAS.

8-Iso-$PGF_{2\alpha}$ increased transiently by approximately 80 % ($p<0.001$) in both groups during the procedure. There was a minor troponin T release ($p<0.001$) after PCI, but no correlation with 8-iso-$PGF_{2\alpha}$. Troponin T did not increase following angiography. 15-Keto-dihydro-$PGF_{2\alpha}$ decreased by 50% after ended procedure, but increased by 100 % after 24 hours compared to baseline. hsCRP increased significantly ($p<0.001$) from baseline to the next day in the PCI-group, but not in the angiography group. Vitamins and TAS decreased slightly after the procedures.

It is concluded, according to transient elevation of 8-iso-$PGF_{2\alpha}$, that a minor to moderate oxidative stress was induced by both elective PCI and coronary angiography but that no correlation was found between oxidative stress and myocardial injury in this setting. This indicates that other mechanisms than ischemia-reperfusion episodes caused an elevation in plasma isoprostane such as the vascular injury at the puncture site. A secondary finding from the study was elevated markers of early inflammatory response, not only after PCI, but also after angiography.

7.2 Paper II

In study II we wanted to examine oxidative stress and early inflammation in patients undergoing primary PCI for STEMI. Secondly, we aimed at assessing whether a correlation exists between these parameters and the extent of myocardial damage. Sixteen patients undergoing primary PCI within 6 hours of STEMI onset were included. Frequent sampling of peripheral blood was performed; at start of procedure (t_0) and repeatedly over 24 hours following reperfusion. Main plasma analyses were: 8-iso-$PGF_{2\alpha}$ (oxidative stress), 15-keto-dihydro-$PGF_{2\alpha}$ (inflammation) and troponin T (myocardial injury). Additional analyses included: TAS, vitamin A, vitamin E, hsCRP and lipids (total cholesterol, HDL-cholesterol and triglycerides).

8-Iso-$PGF_{2\alpha}$ increased following restoration of blood flow, returned to t_0 values after 3 hours and was reduced below t_0 the following day. TAS decreased significantly from t_0 to the next day. There was no significant correlation between 8-iso-$PGF_{2\alpha}$ and troponin T values. 15-Keto-dihydro-$PGF_{2\alpha}$ was elevated during the first hour. There was a major rise in hsCRP after 24 hours.

In conclusion, following reperfusion by primary PCI in STEMI, oxidative stress and an inflammatory response are induced immediately. A rise in 8-iso-$PGF_{2\alpha}$ during ischemia indicates that ROS generation may also take place during severely reduced coronary blood flow and hypoxia. No direct relationship between 8-iso-$PGF_{2\alpha}$ or $PGF_{2\alpha}$ and troponin T was evident. The study adds to the increasingly complex pathophysiological roles of ROS acting both as signal molecules and as mediators of tissue injury.

7.3 Paper III

In study III we wanted to investigate oxidative stress, early inflammation and myocardial injury before, during and after open heart surgery with CABG. 20 patients underwent CABG with parallel sampling of systemic arterial blood and coronary venous blood and with sampling of urine. In 19 of the patients acetylsalicylic acid (ASA) was withdrawn for up to one week prior to the operation in order to reduce risk

of bleeding. Analyses in plasma included: 8-iso-$PGF_{2\alpha}$, TAS, vitamin A and vitamin E as indicators of oxidative stress; 15-keto-dihydro-$PGF_{2\alpha}$ and hsCRP as indicators of inflammation; troponin T as a marker of myocardial injury; and albumin (hemodilution). Analyses in urine included 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$.

8-Iso-$PGF_{2\alpha}$ increased immediately after start of surgery, before CPB and before aortic cross-clamping. No myocardial arterio-venous difference was observed. 15-Keto-dihydro-$PGF_{2\alpha}$ and hsCRP showed respectively an early and a late rise. A transient increase in troponin T was seen 6 hours after the operation, but there was no sign of a specific reperfusion injury. No correlation existed between any oxidative stress variable and troponin T. A particular finding was markedly elevated levels of 8-iso-$PGF_{2\alpha}$ at baseline. Subgroup analyses indicated a possible relationship to the preoperative withdrawal of ASA. Another main finding was an inverse correlation between 8-iso-$PGF_{2\alpha}$ peak values and the accumulated dose of heparin.

It is concluded that in uneventful coronary artery bypass operations oxidative stress may result from the surgical trauma, less from CPB and little if any from myocardial ischemia-reperfusion events. The overall results indicate a complex pathophysiology with prooxidant factors obscuring any major myocardial ROS component and with possibilities of interaction with antioxidant acting drugs like ASA and heparin. In particular, the high baseline levels of 8-iso-$PGF_{2\alpha}$ indicate that the preoperative withdrawal of ASA in these patients may have induced a prooxidant condition at the onset of operation.

8 DISCUSSION

8.1 Main findings

The main objectives of studies I-III were threefold: to identify oxidative stress during ischemia-reperfusion in patients with ischemic heart disease; and, if present, to identify any potential relationship to myocardial injury; and to assess the influence of antioxidant therapy in ameliorating oxidative stress and myocardial injury. The fulfilment of these aims is discussed with emphasis on the relevant parameters involved. Observe that the data presented in this section are in the form of mean ± SEM.

In the studies the mean durations (min) of ischemia were:

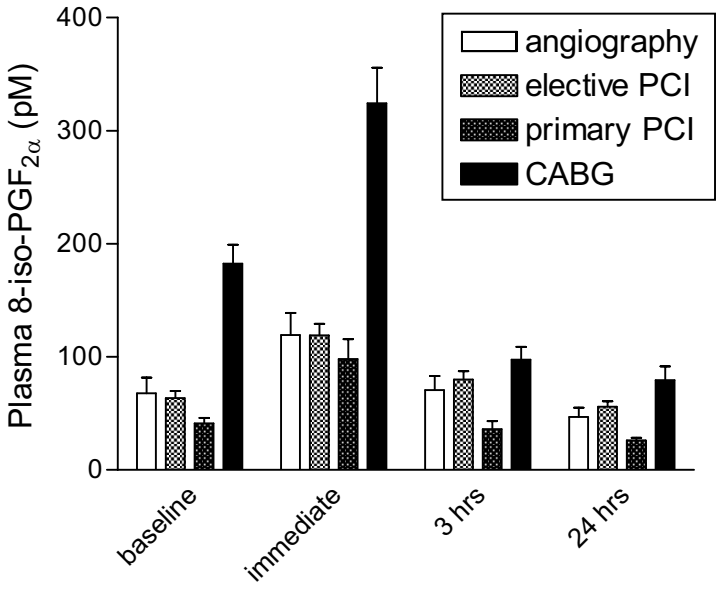
I:	with elective PCI and regional ischemia,	2.2 ± 0.3
II:	with primary PCI and regional ischemia,	183.9 ± 20.3
III:	with CABG and global ischemia,	35.4 ± 2.7

8.1.1 Oxidative stress and 8-iso-$PGF_{2\alpha}$

Course of release

As defined by transient significant elevations above baseline in plasma and urinary (III) 8-iso-$PGF_{2\alpha}$, oxidative stress was present in all groups (I-III) (Figure 5). Furthermore, the elevations peaked at height of the procedures the patients were undergoing. In the two studies in which patients were treated with elective PCI (I) or primary PCI (II) mean peak plasma elevations were 55 pM (87 %) and 59 pM (139 %), respectively. Also in study I, mean levels of 8-iso-$PGF_{2\alpha}$ rose by 52 pM (77 %) during coronary angiography. In study III (CABG), stepwise elevations were observed, after initial surgery by 156 pM (85 %) and after onset of CPB by 65 pM (17 %). There was also a trend to slightly higher values in coronary venous than arterial blood following release of the aortic clamp, but these apparent differences were not significant. In urine samples collected during the operation mean values were elevated

above baseline by 373 pmol / mmol creatinine (124 %). Altogether, the release of 8-iso-$PGF_{2\alpha}$ was more marked in study III then in I and II.



Figur 5. 8-iso-$PGF_{2\alpha}$ (mean ± SEM) in peripheral blood at baseline, immediate after reperfusion (end of procedure in study I) and 3 and 24 hours thereafter.

In all studies the elevation in plasma 8-iso-$PGF_{2\alpha}$ subsided over 24 hours at which baseline (I) or lower than baseline levels (II and III) were found. Urinary values (III) also fell towards baseline. Altogether, these findings show that oxidative stress occurred as a transient phenomenon closely linked to both disease and procedures.

Correlations

In study II with primary PCI induced reopening of occluded arteries, a highly significant correlation was found between peak 8-iso-$PGF_{2\alpha}$ on reperfusion and duration of the long preceding period of myocardial ischemia. However, in elective PCI (I) there was no correlation between elevation of 8-iso-$PGF_{2\alpha}$ and duration of briefly induced ischemic episodes. These findings indicate that ROS and ROS products are generated in cardiomyocytes or endothelial cells during *severe* ischemia or immediately thereafter. However, there was no correlation with regard to the incidence of postischemic arrhythmias. In CABG (III) no correlation was found related to the duration of ischemia. When compared to the situation in primary PCI (II) this might indicate a milder ischemic trauma with short aortic cross-clamp times

(25-45 min) and effective myocardial protection by cardioplegia plus moderate hypothermia (34 ^{o}C)[110].

In CABG (III) negative correlations existed between the duration of operation and peak levels of 8-iso-$PGF_{2\alpha}$ in plasma and urine, indicating that surgical speed and technique were somewhat involved. This assumption may be supported by the fact that the major rise occurred during the initial phase of operation with sternotomy, vessel dissections and the liberal use of diathermy[111]. The half-life of 8-iso-$PGF_{2\alpha}$ in plasma has been found to be about 16 min before being excreted in urine[112], and thus with a more long-lasting operation, less 8-iso-$PGF_{2\alpha}$ might be present in plasma.

New observations

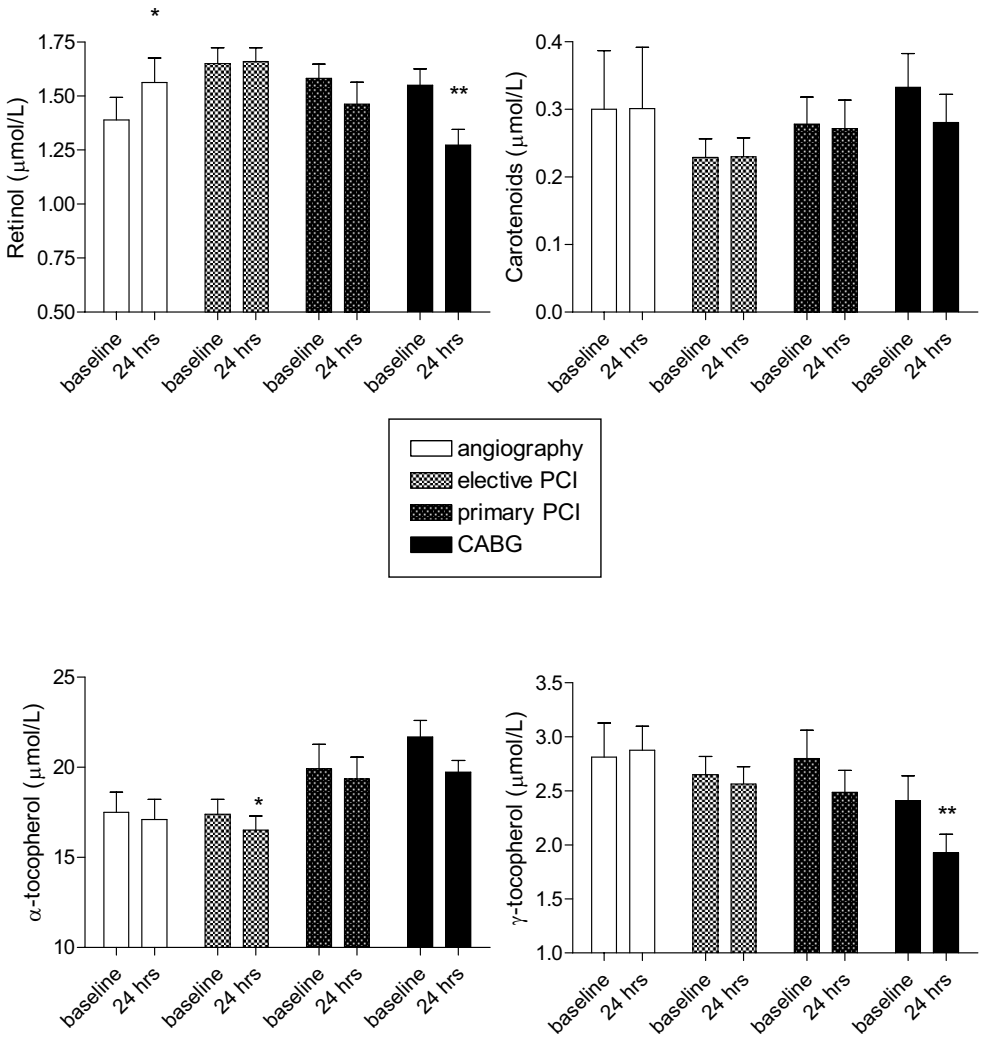
As partly discussed in the individual papers, four surprising and essentially new observations were made.

- Firstly, as indicated above (III) surgical tissue injury may have led to extensive production of isoprostanes. Similar observations have not been made in comparable studies which seldom, or if at all, have tried to single out this potentially prooxidant factor.
- Secondly, there were no differences in 8-iso-$PGF_{2\alpha}$ between coronary angiography and elective PCI (I).
- Thirdly, when comparing primary with elective PCI (II vs. I), peak 8-iso-$PGF_{2\alpha}$ was no higher, but still the primary PCI patients were far more sick and subjected to a far more serious ischemic insult to the myocardium.
- And fourthly, in patients submitted for CABG (III) median baseline value was 192 pM, that is 3-6 times higher than in study I (62 pM and 57 pM) and in study II (31 pM) and about 2.5 times higher than reported values in human volunteers[106]. This observation was unexpected and at the time of the studies difficult to interpret.

As discussed in a later paragraph (8.2.1), a surprising link to long term, acute or intraoperative drug treatment may explain the apparent oddity of the three latter observations.

8.1.2 Oxidative stress and other biomarkers

In all studies we measured lipid soluble antioxidant vitamins (Figure 6) and the TAS index of antioxidant capacity (Figure 7) in plasma before and following the involved procedures. This was done in order to intercept eventual consumption of antioxidants or subsequent upregulation of the antioxidant defence following oxidative stress. In addition, low levels of such antioxidants at baseline could be a risk factor to oxidative stress in general and specifically in ischemic heart disease[113-116].



Figur 6. Vitamins (mean ± SEM) in peripheral blood (plasma) at baseline and 24 hours after reperfusion (or end of procedure in study I).

The observed levels of both vitamins and TAS partly underlined the findings with 8-iso-$PGF_{2\alpha}$ as a marker of oxidative stress. In study I (diagnostic angiography and

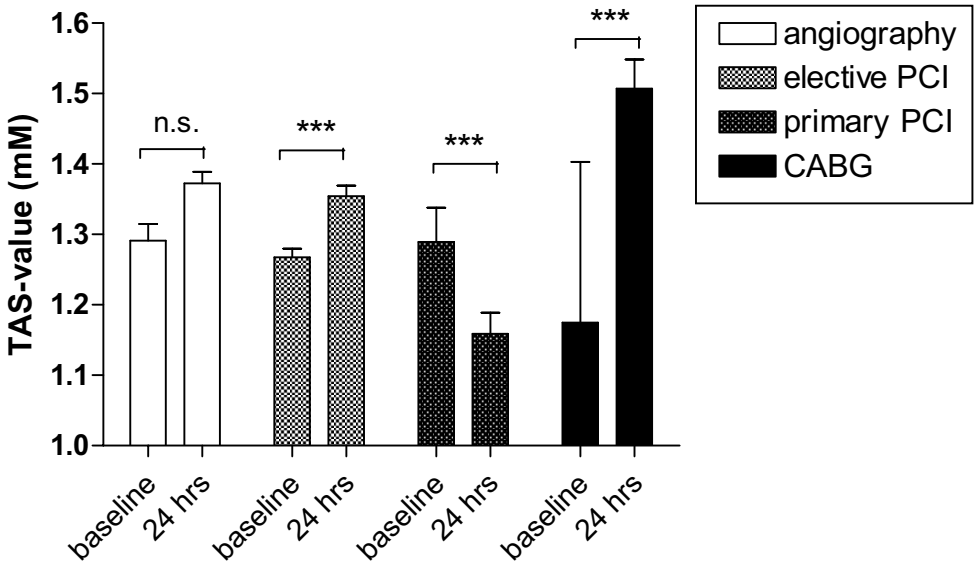


Figure 7. Total antioxidant status (TAS) (mean ± SEM) in peripheral blood (plasma) at baseline and 24 hours after reperfusion (or end of procedure in study I).

elective PCI), TAS was measured at four time points. A transient decrease was observed immediately after end of procedure in both groups, however, significantly only in the PCI group. The following day the levels of TAS were elevated in both groups, but only significantly in the PCI group. The results could be explained by an early consumption of antioxidants in plasma due to the increase of ROS as measured by 8-iso-$PGF_{2\alpha}$ with an upregulation of the antioxidant defence the following day. The levels of measured vitamins were stable or only small changes were observed after 24 hours when compared to baseline. Similar findings have been reported by others[117].

In study II we only measured these variables at baseline and on the day after PCI. A highly significant decrease in TAS was then observed supporting an oxidative stress situation. Why we did not observe the same trend of an upregulation mechanism in this group compared to the elective PCI group may be due to the fact that these patients were more severely ill with more complex pathophysiology. No significant changes in the levels of vitamin A or E were observed following the procedure.

In CABG (III) changes were more complex with an early elevation of TAS that was maintained throughout the entire per- and postoperative period. Furthermore, retinol, carotenoids and α-tocopherol values were either maintained or elevated at the height

of oxidative stress as indicated by peak levels of 8-iso-$PGF_{2\alpha}$. This might indicate that utilization of endogenous antioxidants and vitamins had not taken place at this stage of the operation or that other potential antioxidative factors had come into play and postponed vitamin consumption to the end of operation or to the postoperative phase.

With few exceptions, all patients in all studies were within the reference level of vitamins at any time of measurement. Furthermore, patients with values below these levels did not behave differently from the other with regard to 8-iso-$PGF_{2\alpha}$.

8.1.3 Inflammation and 15-keto-dihydro-$PGF_{2\alpha}$

Transient significant elevations in plasma 15-keto-dihydro-$PGF_{2\alpha}$, a marker of early inflammation, were observed in all groups of patients (I-III) (Figure 8). In primary PCI (II) and CABG (III) baseline values were low and a modest peak release was observed. In elective PCI and coronary angiography (I) the baseline values were about 3 times higher and the release profile was biphasic with a rise after 24 hours. These findings are difficult to explain except for differences in activation and inhibition of COX. As commented in a later paragraph, a low maintenance dose of ASA in elective PCI and a high acute loading dose in primary PCI provide a rational interpretation of differences between studies I and II.

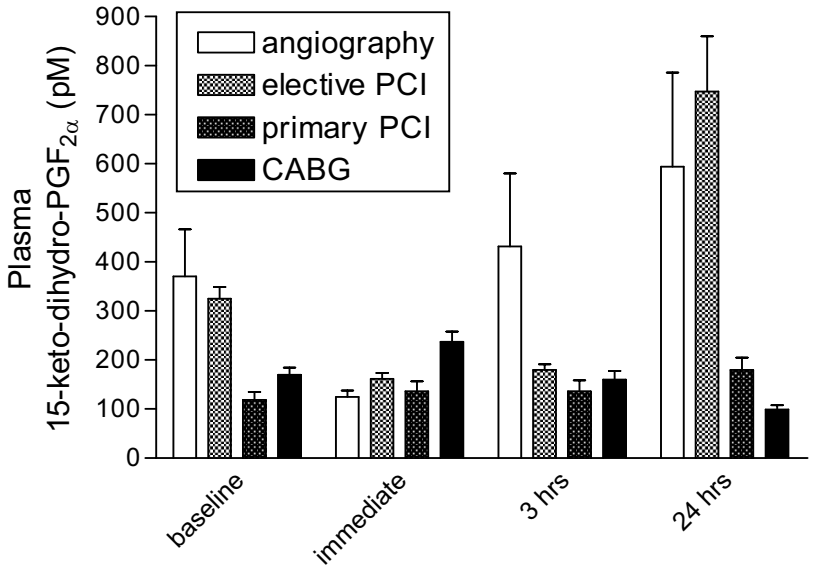


Figure 8. 15-keto-dihydro-$PGF_{2\alpha}$ (mean ± SEM) in peripheral blood at baseline, immediate after reperfusion (end of procedure in study I) and 3 and 24 hours thereafter.

8.1.4 Inflammation and hsCRP

Baseline values were low in all patient groups, 2.3 – 3.4 mg/L, as seen in Figure 9. In coronary angiography no elevation occurred, whereas after both elective (I) and primary (II) PCI there was a minor-to-moderate elevation to 7.4 and 15.9 mg/L, respectively. This contrasted greatly to CABG (III) with elevation to 115 and 218 mg/L on postoperative days one and two, respectively. Thus activation of cytokine pathways was about one order of magnitude higher in these patients most likely due to the overall surgical trauma and postoperative inflammation.

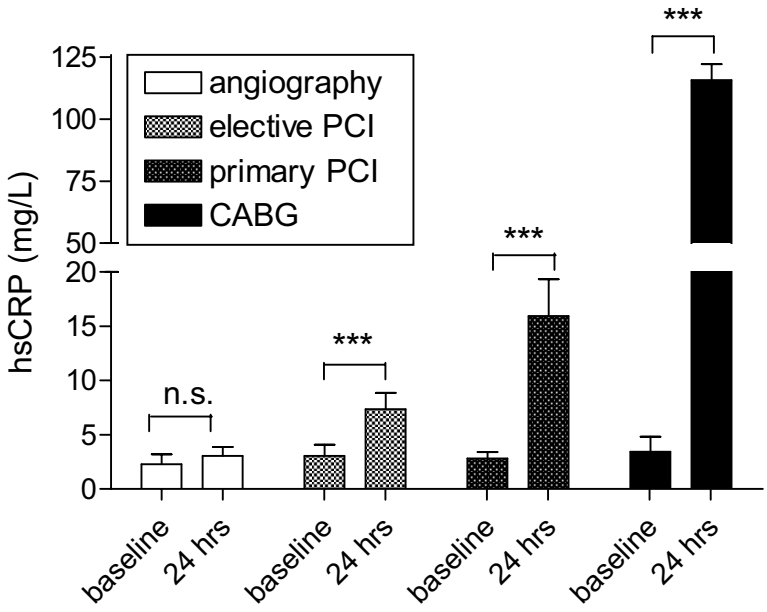


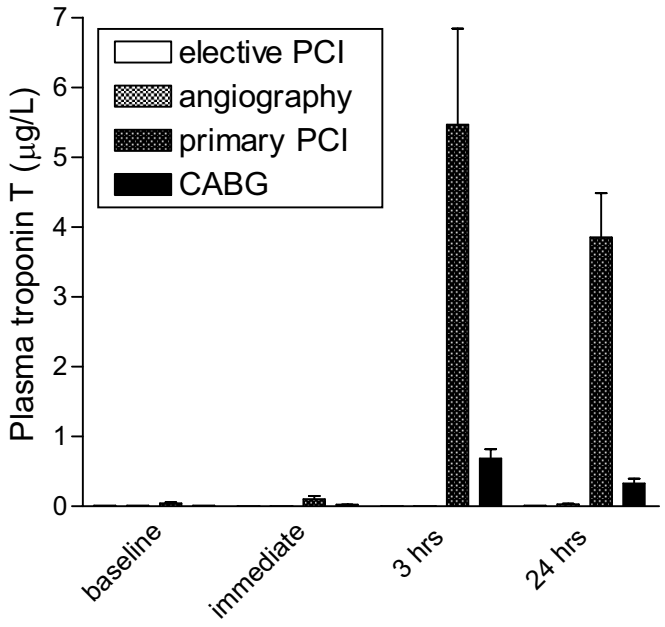
Figure 9. hsCRP (mean ± SEM) in peripheral blood (plasma) at baseline and 24 hours after reperfusion (or end of procedure in study I).

8.1.5 Myocardial injury and troponin T

Course of release

Troponin T values were below or slightly above the detection limit of the assay (0.01 μg/L) following elective PCI (I) (Figure 10). In primary PCI (II) there was a significant gradual elevation at all time points from reperfusion until 6 hours when a mean peak value of 7.2 μg/L was reached. This is about 50 times higher than the internationally accepted threshold for the diagnosis of myocardial infarction, 0.1 mg/L[118]. Thereafter troponin T release decayed to 3.9 μg/L after 24 hours. In CABG

(III) a similar and gradual release profile was observed peaking at about 6 hours after the operation and subsiding on postoperative days 1 and 2. However, the peak release value was only about 10 % of that seen in primary PCI, signifying a much smaller myocardial injury as would be expected with a preferably shorter duration of ischemia and protection by hypothermic cardioplegia[119]. In all clinical studies (I-III) troponin T release was preceded by the release of 8-iso-$PGF_{2\alpha}$.



Figur 10. Troponin T (mean ± SEM) in peripheral blood at baseline, immediate after reperfusion (end of procedure in study I) and 3 and 24 hours thereafter.

Correlations

A major finding in the present studies was that troponin T release showed no group based or individual patient based correlation to the release of 8-iso-$PGF_{2\alpha}$. Thus no causal relationship was established between these two key parameters of respectively myocardial injury and oxidative stress. In patients undergoing primary PCI (II) and CABG (III) troponin T release correlated significantly with duration of the preceding ischemia, $p=0.028$ (not published) and $p<0.0001$, respectively.

8.2 Prooxidant and antioxidant balance

A main conclusion from the clinical studies of the present thesis is that oxidative stress is present but multifactorial in origin. Thus the end results in terms of applied stress parameters are the composite of both pro- and antioxidative factors. On the prooxidant side it appears that myocardial ischemia-reperfusion is less dominating than hitherto considered[65,94-97,99,120,121], whereas procedure related stress is more prone to occur. The most surprising role, when viewing the clinical papers I-III in retrospect, is that of concomitant drug treatment in patients with IHD. Since both prooxidant and antioxidant effects of cardiovascular drugs in common use may have been present, this factor will be highlighted below.

8.2.1 Prooxidant and antioxidant effects of drug treatment

An interesting issue in pharmacology is that major drugs developed or adopted for specific clinical objectives, may have secondary properties that might influence the outcome of therapy, not only in a negative manner as side-effects but also in a positive manner with beneficial effects. In line with this, considerable attention has recently been paid to the inflammatory profile of coronary atheromatosis[47,61,122,123] and to the positive influence of drugs in common use[124,125]. As indicated in Figure 11, which gives an overwiev of endogenous and exogenous antioxidant agents, it appears that key types of cardiovascular drugs like statins, heparin and ASA ought to be paid particular attention.

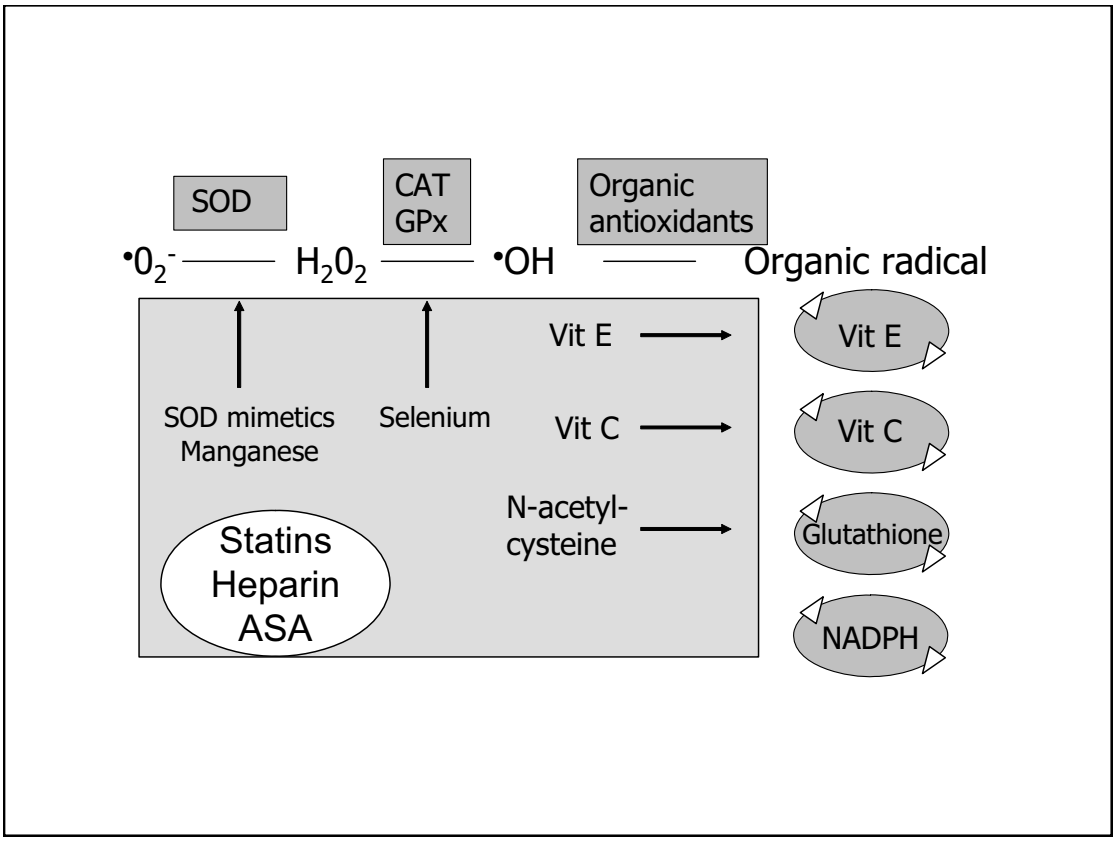


Figure 11. Main ROS pathways *in vivo* with main endogenous and exogenous antioxidants.

Statins

At present, statins, introduced as cholesterol-lowering agents, are regarded as particularly valuable antiinflammatory or even antioxidant remedies in atheromatosis[124]. The antioxidant properties by statins are partly explained by inhibition of ROS generation through interference with NAD(P)H oxidase and partly by reducing the damaging effects of ROS[124]. In our clinical studies with elective PCI (I) and CABG (III) almost all patients were on statin treatment, but in primary PCI (II) only 1 of 14 patients. Since the studies did not include paired statin versus non-statin groups, it is difficult to assess potential influences of this class of drugs in the present thesis. However, it appears that the lack of statins in study II patients did not impair their antioxidant defence judged by surprisingly low plasma values of 8-iso-$PGF_{2\alpha}$.

Acetylsalicylic acid (ASA)

A common theme in all three studies of the present thesis was the treatment with ASA. In study I almost all patients, 97 % in elective PCI and 100 % in coronary

angiography, were on a continuous low dose (75 or 160 mg) ASA treatment. In study II, only 1 of 14 patients was on prior low dose medication, but all received a booster dose of 300 mg just prior to primary PCI for AMI. In study III, however, a continuous low dose (160 mg) ASA regimen in 19 out of 20 patients was interrupted in order to restore normal platelet function prior to open heart surgery and CABG.

The bulk of 8-iso-$PGF_{2\alpha}$ detected in these studies most probably derives from the free radical pathway and not from the COX pathway. As indicated in Figure 12, there is circumstantial evidence for the view that ASA, whether in a low dose continuous (I) regimen or an interrupted (III) regimen or administered in a higher acute dose (II) played a significant role in determining both baseline level and peak release of 8-iso-$PGF_{2\alpha}$.

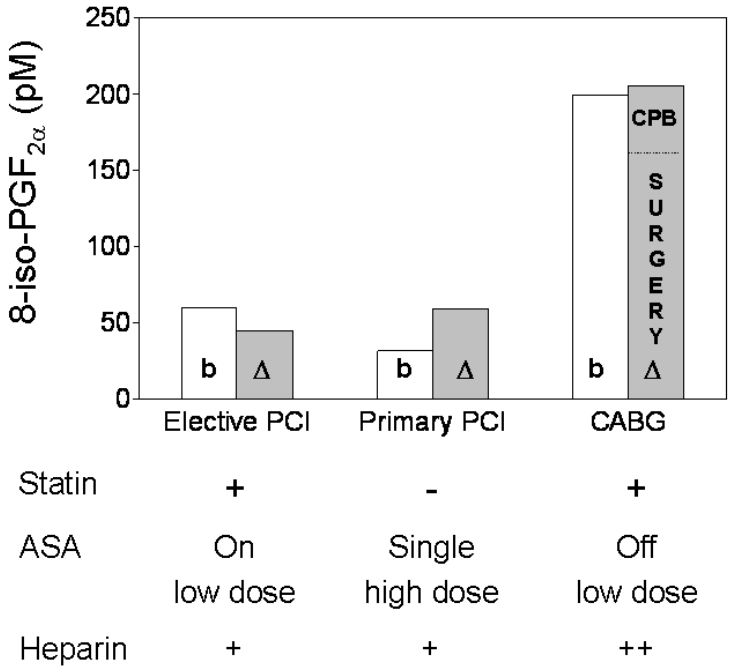


Figure 12. Drug treatment and oxidative stress. b=baseline Δ=elevation above baseline.

Altogether, and as discussed in the individual papers, it is tempting to suggest the following scenario:

- Ongoing low dose treatment (I) was associated with a basal level of oxidative stress as presented by a normal baseline and a moderate peak release 8-iso-$PGF_{2\alpha}$.
- The lack of deviation from the same pattern of moderate response in patients with far more advanced disease (II) reflected the acute administration of a higher dose of ASA and an almost immediate improvement in antioxidant defence.
- Most intriguing however, the withdrawal of ASA prior to CABG (III) induced a prooxidant condition with a much higher baseline 8-iso-$PGF_{2\alpha}$ and a more marked elevation during the initial phase of surgery.

Although speculative, the above scenario and mechanisms find support in reports over the last decade on anti-inflammatory properties of ASA in coronary atheromatosis[123,126]. Accordingly, most recent studies suggest that the risk of AMI is increased during one month after cessation of NSAID therapy[127], and that ASA withdrawal for a mean of 10 days increased the risk of stent thrombosis and transmural AMI[128]. Recognizing inherent problems with withdrawal, there is now a trend towards maintaining ASA treatment throughout periods prior to surgery[129]. In the above cited reports the main emphasis has been on ASA as an inhibitor of COX.

Intriguingly, most recent experiments in angiotensin-II-hypertensive rats open for a new set of mechanisms[130]. Thus peroral treatment with ASA for two weeks abolished the rise in blood pressure caused by continuous slow delivery of angiotensin-II. Furthermore, these beneficial effects resulted from inhibition of plasma NAD(P)H-oxidase, reduced superoxide levels and maintained higher levels of the vasodilator NO. Also, it was suggested that the potent acetylation properties of ASA most probably inactivate oxidases or an oxidase protein precursor thereby providing a potent antioxidant action.

Returning to the present clinical studies, it appears that an acute high dose ASA may have reduced ROS levels considerably during AMI and primary PCI (II), whether through a direct scavenging of $^{\bullet}OH$ or through a reduced production of $^{\bullet}O_2^-$. Also, the marked withdrawal syndrome observed in CABG (III) might indicate a potential inverse rebound high activity of plasma oxidases leading to a prooxidant condition. These possibilities were not confirmed since complementary analyses of plasma oxidase activities were not performed. However, the present data warrant attention to a largely overlooked situation which might prove deleterious to patients with IHD.

Heparin

A standard dose of 10.000 IU of heparin was given during elective (I) or primary (II) PCI, whereas no heparin was administered during coronary angiography only (I). Since there were no differences in isoprostane release in study I between the elective PCI and the coronary angiography groups, indirect antioxidant properties of heparin cannot be disregarded. In CABG (III) much higher accumulated doses, mean value 43.500 IU, were applied and a significant negative correlation was found between the individual doses and the release of 8-iso-$PGF_{2\alpha}$.

Standard heparin and low molecular heparins (LMWH) are glucoseaminoglycans that seem to enhance SOD activities in vivo[131]. Also iron chelation properties have been suggested[132]. Postoperative LMWH administration in cholecystectomized patients was recently[133] shown to decrease plasma lipid peroxides and increase the activity of SOD, CAT and GPx in erythrocytes. In a most relevant primary PCI study[134], unexpected low plasma values of malondialdehyde (marker of lipidperoxidation) was found on reperfusion, and it was inferred without direct evidence that high dose heparin had provided antioxidant protection. A recent experimental study in rats[135] on adriamycin induced injury to heart and liver, showed that parallel LMWH treatment conserved tissue structures, prevented release of enzymes and maintained plasma activities of SOD, CAT and GPx. Altogether, these latter studies and the present thesis confirm that heparins are potent indirectly acting antioxidants and indicate that this property may be beneficial and additive to the primary antithrombogenic action of these drugs.

8.2.2 Methodological considerations

Absence of parallel coronary and systemic blood sampling except in CABG (III)

The absence of coronary sinus sampling in study I and II is clearly a limitation, which if avoided might have revealed more clear-cut evidence for a myocardial origin of ROS. Sampling of urine could also have been more rewarding by circumventing eventual short-lasting plasma peaks in peripheral blood. However, catherization of the coronary sinus and the urinary bladder solely for research purposes were not considered appropriate in studies I and II. In an experimental study where the authors compared samples from coronary sinus versus peripheral blood, they found that peripheral blood samples may also be informative regarding ROS production and antioxidant capacity following myocardial ischemia- reperfusion[136].

8-Iso-$PGF_{2\alpha}$

In this study the formation of 8-iso-$PGF_{2\alpha}$ in plasma and urine was quantified by a radioimmunoassay[106]. Several other assays for quantification of isoprostanes exist including chromatographic techniques with mass spectrometry detection (GC or LC-MS)[39] and enzyme-immunoassays. Mass spectrometry based methods usually have high precision but less capacity for analyses of large sample numbers. Immunoassays have generally less precision than chromatographic techniques but are more applicable in large clinical studies. The method used in this thesis is based on measuring the free fraction of 8-iso-$PGF_{2\alpha}$ without needs of any further sample preparation. This is made possible due to the high specificity of the antibody against 8-iso-$PGF_{2\alpha}$, with a low crossreactivity related to other eicosanoids* (*fatty acids derived from 20-carbon unsaturated fatty acids such as AA including all forms of prostaglandins and isoprostanes). Since the free fraction represents an extra step in the formation of 8-iso-$PGF_{2\alpha}$, a low value might indicate a low phospholipase activity. Most other analysis techniques require purification of samples with extraction of lipids by either solid phase techniques or an immunological step. Further, when measuring both the free and esterified fraction of 8-iso-$PGF_{2\alpha}$ an additional hydrolytic step is required. The mass spectrometry methods also require a derivatisation step. All these extra procedures may add up accuracy problems when compared to the simple method used in this thesis. When a sensitive and specific antibody is available,

immunoassays represent simple and fast procedures without expensive equipment in contrast to mass spectrometry techniques.

Other pro/anti-oxidative parameters

Many other parameters to assess oxidative stress could be involved in this study, but we had to make a selection for practical and technical reasons. All clinical studies were performed in an every day clinical practice and the amount of blood was limited. We extracted maximum 100 mL blood from each patient within a time span of 24 hours. Measurement of endogenous antioxidants, particularly of SOD, would have strengthened the study, but this was abandoned for practical reasons. SOD exists in both exctracellular (Cu,ZnSOD) and intracellular (MnSOD) forms, and in this context the intracellular form is most relevant to measure. Isolating erythrocytes (or leucocytes) which are the cells available, have to be undertaken immediately after sampling. However valuable, this was not possible in the present studies.

Hemolysis

Hemolysis was a practical problem, especially in study III with possible mechanical injury to erythrocytes during CPB. Hemolysis may interfere with many biochemical analyses, including the assays of 8-iso-$PGF_{2\alpha}$, 15-keto-dihydro-$PGF_{2\alpha}$ and troponin T. Nevertheless, when comparing the results of the hemolytic samples with non-hemolytic samples they followed the same trend. Accordingly, all samples were analyzed and included in these studies independent of hemolysis.

9 SUMMARY AND CONCLUSIONS

- The isoprostane 8-iso-$PGF_{2\alpha}$ proved to be a valuable marker of conditions associated with oxidative stress in all three clinical studies (I-III). Somewhat surprising, no significant correlation with myocardial reperfusion events was found. Only the duration of severe ischemia correlated positively in patients with AMI undergoing primary PCI (II). Thus the first aim of this thesis was partly fulfilled. A particular finding was that other conditions than myocardial ischemia-reperfusion such as interventional (I) and surgical (III) traumas contributed to an apparent oxidative stress in the patient groups. In parallel, pro- or antioxidant drug effects seemed to have influenced the release pattern of 8-iso-$PGF_{2\alpha}$, underlining the versatility of this parameter of lipid peroxidation.

- The second aim of the thesis, to establish a relationship between oxidative stress and myocardial injury, was not fulfilled. Thus the present studies I-III seem to refute the hypothesis of a major ROS-involvement in myocardial ischemia-reperfusion injuries as shown in animal experiments. Another interpretation is that an oxidation-injury relationship was masked in the more complex pathophysiology of a clinical setting. A further implication may be that 8-iso-$PGF_{2\alpha}$, behaved as a passive indicator of successful reperfusion rather than as an active indicator of severe and injury-inducing oxidative stress, reflecting the role as a ROS-imprint and not as an injury marker.

- The third aim of the thesis, to try out potent antioxidants on a rational basis, did not materialize as the studies went on. However, in the course of time new mechanisms of cardioactive or adjunctive drugs were recognized. Thus both ASA and heparin are two candidates which may have had a major impact on the results in all three studies. Concerning ASA both positive effects of an acute booster dose (II) and negative effects of withdrawal (III) warrant further attention.

- Although the study is limited by the low numbers of patients, it seems justified to suggest that the lack of success in translating principles of oxidative stress and antioxidant therapy from the experimental to the clinical stage of myocardial ischemia-reperfusion may now have found a more plausible explanation than hitherto proposed. Thus oxidative stress, if present, is a multifactorial process with complex interactions between pro- and antioxidant factors and with largely unrecognized protection by drugs in common use.

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Paper III

OXIDATIVE STRESS DURING CORONARY ARTERY BYPASS OPERATIONS IS MULTIFACTORIAL:

Possible roles of surgical trauma and drug treatment

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ABSTRACT

Objective

Coronary artery bypass operations are supposed to cause oxidative stress since an elevation of reactive oxygen species (ROS) may follow both cardiopulmonary bypass (CPB) and elective ischemia-reperfusion of the myocardium due to aortic cross-clamping. This study investigated oxidative stress, early inflammation and myocardial injury before, during and after the operation

Methods

20 patients underwent coronary artery bypass grafting (CABG) with the use of CPB. Parallel sampling of systemic arterial blood, coronary venous blood and urine was performed during surgery and continued two days postoperatively. Analyses included: 8-iso-$PGF_{2\alpha}$ (a major F_2-isoprostane), total antioxidant status (TAS) and antioxidant vitamins as indicators of oxidative stress; 15-keto-dihydro-$PGF_{2\alpha}$ (a $PGF_{2\alpha}$ - metabolite) and high sensitive C-reactive protein (hsCRP) as indicators of inflammation; and troponin T as a marker of myocardial injury.

Results

8-Iso-$PGF_{2\alpha}$ increased immediately after start of surgery with further increase after CPB but not after aortic cross-clamping. No myocardial arterio-venous difference was observed. 15-Keto-dihydro-$PGF_{2\alpha}$ and hsCRP showed respectively an early and a late rise. A transient minor increase in troponin T was seen six hours after the operation, but there was no sign of persistent reperfusion injury. No correlation existed between any oxidative stress variable and troponin T. There was an inverse correlation between 8-iso-$PGF_{2\alpha}$ and the administrated dose of heparin. A particular finding was elevated levels of 8-iso-$PGF_{2\alpha}$ at baseline. A subgroup analysis indicated a possible relationship to the preoperative withdrawal of acetylsalicylic acid (ASA).

Conclusions

In uneventful coronary artery bypass operations oxidative stress may result from the surgical trauma, less from CPB and little if any from myocardial ischemia-reperfusion events. The results indicate a complex pathophysiology with prooxidant factors obscuring any potential myocardial ROS component and with possible interactions with indirectly antioxidant acting drugs like ASA and heparin.

INTRODUCTION

Revascularization of coronary arteries by conventional coronary artery bypass graft surgery (CABG) with the use of cardiopulmonary bypass (CPB) and cardiac arrest is known to mediate oxidative stress.[1-4] Reactive oxygen species (ROS) have been implied as potential factors involved in myocardial ischemia-reperfusion injuries resulting from aortic cross-clamping. Accordingly, some reports have indicated that ROS or ROS products are generated locally in the myocardium and are detected as radical adducts or lipid peroxides in coronary venous blood during reperfusion after release of the aortic clamp.[5,6] These findings are, however, not consistent and the focus has shifted towards the implications of a systemic oxidative response during CPB with extracorporal circulation.

As CPB is associated with hyperoxic conditions,[7] foreign surface material in the extracorporeal tubes and oxygenators,[8] and hemolysis and iron release due to mechanical injury of erythrocytes,[9] several factors may be lead to a high level of circulating peroxides during and following surgery. The oxidative stress factor has been counteracted by undertaking coronary artery bypass operations without the use of CPB ("off pump surgery")[10-12] even though the results are conflicting.[13]

Another aspect of oxidative stress in patients undergoing CABG is the state of antioxidant defence which may be reduced by concomitant occurrence of diabetes and hypercholesterolemia.[14-16] On the other hand, it may also be normal or even elevated. Thus, antioxidant enzymes and other protective proteins may be upregulated during an active phase of ischemic heart disease and become manifest as endogenous protection (ischemic preconditioning).[17] Further complicating is prior treatment with drugs suppressing inflammation and ROS[18-21] as well as use of drugs with largely nonrecognised antioxidant properties.[22]

Isoprostanes are relatively stable products formed *in vivo* from phospholipids mainly by a non-enzymatic free radical-catalyzed oxidation of arachidonic acid.[23-25] They are structural isomers of conventional prostaglandins. Studies have indicated that a major isoprostane, 8-iso-$PGF_{2\alpha}$, measured in plasma or urine, is a reliable biomarker of oxidative stress.[2,23-26] Prostaglandins are formed by enzymatic oxidation of

arachidonic acid by cyclooxygenase (COX), and 15-keto-dihydro-$PGF_{2\alpha}$, a major metabolite of prostaglandin $F_{2\alpha}$, is considered to be a useful biomarker of inflammation.[27-29] The inflammatory response induced by CABG activates COX and may lead to further oxidative stress since a minor fraction of F_2-isoprostanes (including 8-iso-$PGF_{2\alpha}$) may also be generated by this pathway.[30,31]

In the present study we wanted to separate local myocardial oxidative stress from systemic oxidative stress during conventional CABG. Furthermore, we have tried to correlate oxidative stress with myocardial injury. Sampling of systemic arterial blood, coronary venous blood and urine was performed during surgery and continued two days postoperatively. Analyses included; 8-iso-$PGF_{2\alpha}$, total antioxidant status (TAS) and antioxidant vitamins as indicators of oxidative stress; 15-keto-dihydro-$PGF_{2\alpha}$ and high sensitive C-reactive protein (hsCRP) as indicators of inflammation; and troponin T as a marker of myocardial injury.

MATERIAL AND METHODS

The study was performed according to the Helsinki declaration. The Regional Ethical Committee approved the protocol, and written informed consent was obtained from all patients.

Patients (Table 1)

20 consecutive patients referred for CABG at the Department of Cardiothoracic Surgery, Trondheim University Hospital, were recruited. Patients with low ejection fraction (< 50%), diabetes or overt renal failure and patients undergoing reoperations, combined procedures and emergency surgery were not included. Patients included were operated for two- or triple-vessel disease. 16 and 4 of these patients presented with stable and mildly unstable angina, respectively. The latter 4 were treated with low-molecular weight heparin. Of the 20 patients 4 had chronic obstructive lung disease and 3 had experienced cancer.

Routine drug treatment was continued until the time of surgery except for acetyl-salicylic acid (ASA) (160 mg daily) that was withheld in the 19 treated patients prior to the operation in order to restore normal platelet function. 1 patient was treated with

warfarin instead of ASA preoperatively. It is to be observed that the number of days without ASA differed among the patients, and varied between 1 to more than 7 days.

Procedures (Figure 1)

Anesthesia. All patients received premedication with morphine scopolamine. Prior to anesthesia the left radial artery was cannulated for arterial pressure monitoring and blood sampling, and a catheter was introduced into the urinary bladder. Anesthesia was induced with diazepam, fentanyl, thiopental and pancuronium and was maintained with isoflurane and fentanyl. Inotropic agents (dopamine), vasodilators (nitroglycerine) or vasoconstrictors (norepinephrine) were applied according to the clinical situation. In all patients intraarterial blood pressure and central venous pressure were monitored continuously. Before and after CPB, lungs were ventilated mechanically with oxygen–enriched air and isoflurane, adjusted to keep the end-tidal pCO_2 at 35 mmHg. Cephalotin was used as perioperative antibiotic prophylaxis.

Surgery. The early phase of the sampling period included standard median sternotomy and dissection of saphenous vein and internal mammary artery grafts. An initial dose of 300 IU/kg heparin was given prior to cannulation of the right atrium and the proximal aorta. Additional heparin was given when necessary to maintain the activated clotting time (ACT) > 480 s during CPB. The accumulated median intraoperative dose of heparin was 43500 IU (37500 – 52500 IU)**.** A membrane oxygenator with biocompatible surfaces (Maxima, Medtronic, Minneapolis, USA) was used in all operations. The pump prime consisted of 1800 ml Ringer solution and 7500 IU heparin. Arterial perfusion with moderate hypothermia (34^{o}C) was performed with non-pulsatile flow at 2.4 L/min per m^2 body surface area with a roller pump. After aortic cross-clamping, cardiac arrest was rapidly induced with antegrade delivery of the St. Thomas' Hospital cardioplegic solution[32] through the aortic root.

For sampling of coronary venous blood, a retrograde cardioplegia catheter (Edwards Lifesciences Corp., Irvine, CA, USA) was installed into the coronary sinus before CPB and removed when weaning off CPB. Correct position of the catheter was ensured by transesophageal echocardiography, and blood was slowly withdrawn to avoid hemolysis and aspiration of right atrial blood. At the end of CPB, heparin was neutralized with protamin sulphate.

Handling of blood and urine samples

Systemic arterial blood and coronary venous blood were collected into precooled tubes with K_3EDTA at various time points as shown in Figure 1. Samples were kept on ice before centrifugation (10 min, 4°C, 3000G) within 30 min and plasma was then immediately stored at -80 °C until analysis. Urinary samples were collected from the urinary bladder by catheter at four time points; at baseline (after insertion of the catheter), during operation, 24 (day 1) and 48 (day 2) hrs after end of operation. After collection samples were frozen and stored at -80°C until analysis.

Biochemical Assessments

8-Iso-$PGF_{2\alpha}$ (nonesterified) was measured by a validated radioimmunoassay without any extraction procedure as described by Basu.[25] An antibody was raised in rabbits by immunization with 8-iso-$PGF_{2\alpha}$ coupled to bovine serum albumin at the carboxylic acid by the 1,1'-carbonyl-diimmidazole method. The cross-reactivity of 8-iso-$PGF_{2\alpha}$ antibody with 15-keto-13,14-dihydro-8-iso-$PGF_{2\alpha}$, 8-iso-$PGF_{2\beta}$, $PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$, 15-keto-13,14-dihydro-$PGF_{2\alpha}$, TXB_2, 11β- $PGF_{2\alpha}$, 9β- $PGF_{2\alpha}$ and 8-iso-$PGF_{3\alpha}$ was 1.7, 9.8, 1.1, 0.01, 0.01, 0.1, 0.03, 1.8 and 0.6%, respectively. The detection limit of the assay was 23 pM with an intra assay coefficient variation (CV) of 12-15%.

15-Keto-dihydro-$PGF_{2\alpha}$ was measured by a validated radioimmunoassay as described by Basu.[27] An antibody was raised in rabbits by immunization with 15-keto-dihydro-$PGF_{2\alpha}$ coupled to bovine serum albumin at the carboxylic acid by the 1,1'-carbonyl-diimidazole method. The cross-reactivity of the antibody with $PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$, PGE_2, 15-keto-13,14-dihydro-PGE_2, 8-iso-15-keto-13,14-dihydro-$PGF_{2\alpha}$, 11β- $PGF_{2\alpha}$, 9β- $PGF_{2\alpha}$, TXB_2, and 8-iso-$PGF_{3\alpha}$ was 0.02, 0.43, <0.001, 0.5, 1.7, <0.001, <0.001, <0.001 and 0.01 %, respectively. The detection limit of the assay was 45 pM with an intra assay coefficient variation (CV) of 12-14%.

hsCRP was measured by latex-enhanced immuno-turbidimetry with use of a Hitachi 917 analyzer (CRP (Latex) HS, Roche Diagnostics, Mannheim, Germany). The detection limit of the assay was 0.03 mg/L (analytical sensitive).

Troponin T was measured by electrochemiluminiscence with use of an Elecsys 2010 analyzer (Roche Diagnostics, Mannheim, Germany). The detection limit of the assay was 0.01 μg/L.

Retinol, carotenoids (α+β) and tocopherols (α+ γ) were assayed by high performance liquid chromatography (HPLC) with UV-detection based on methods described by Nierenberg et al. and Comstock et al.[33,34]

Total antioxidant status (TAS), a measure of peroxyl-scavenging capacity, was assayed by photometry on a Cobas Mira S analyzer with use of an enzymatic assay (Randox Laboratories Ltd., Crumlin, United Kingdom).

Albumin concentrations (g/L) were measured by a colorometric endpoint assay on a Hitachi 917 analyzer (BCG method, Roche Diagnostics, Mannheim, Germany).

Statistical analysis

Because of non-Gaussian distribution of most data, non-parametric tests and median values with interquartile ranges (25 % and 75 %) were used. Missing values were filled in with estimated values using the expectation-maximization (EM) method. Comparisons within groups over time were made by means of the Friedman test and Dunn's multiple comparison test. Wilcoxon signed ranks test was performed for comparison between two dependent groups and Mann-Whitney test was used when comparing two independent groups. Spearman's correlation coefficient (rho) was applied for correlation analyses. For all analyses values below detection limit were set to detection limit at calculation. Values for $p < 0.05$ were considered significant. Data analyses were performed with the Statistical Package for Social Sciences (SPSS 11.5, Chicago, Illinois) or GraphPad Prism Software (GraphPad Software 4.01, Inc., San Diego, CA, USA).

RESULTS

Duration of the main critical events was: operation 142 (123-174) min; CPB 63 (47-74) min; and aortic cross-clamp (ischemia) 36 (25-45) min. Median numbers of grafts

was 3. There was no hospital mortality or severe cardiopulmonary or vascular morbidity.

Hemodilution

As seen in Figure 2, plasma albumin was reduced to 81 % of control during the initial phase of operation due to infusion of crystalloids and fell abruptly to 57 % at onset of CPB with a crystalloid priming solution. Thereafter the following albumin values were found: 60 % at end of operation; 72 % on day 1; and 74 % on day 2. Hence, the patients were in a hemodiluted state during the overall surgical procedure and the following two days. Since these patients had a greatly expanded extracellular water space, all plasma values of measured variables are corrected for hemodilution relative to albumin as the intravascular reference.[35,36]

Systemic versus coronary venous sampling

Arterial blood samples were collected from the radial artery (t_0–t_{14}) and venous blood locally from the coronary sinus in the heart. Parallel sampling from the two sites was performed at eight time points (t_2–t_9). A major observation was that at these critical moments no significant differences were found in any investigated variable between the two set of samples. Thus it was not possible to dissociate specific myocardial responses from those of systemic responses.

Biochemical Assessments

8-Iso-$PGF_{2\alpha}$ in plasma and urine. The baseline plasma value (Figure 3 a) obtained immediately after radial artery cannulation was 192 (111-237) pM (t_0) and rose significantly to 343 (256-404) pM (t_1) after the initial part of surgery (sternotomy and dissection of vascular grafts). There was a nonsignificant trend to a further rise to 399 (260-538) pM following start of CPB (t_3) and thereafter a gradual but significant fall to 140 (89-187) pM at the end of surgery (t_{10}) and to 73 (32-118) pM on postoperative day 1 (t_{13}) and 70 (34-116) pM on day 2 (t_{14}). At the critical stage immediately before (t_4) and 1 min after (t_5) release of the aortic cross-clamp, median values were slightly higher in coronary venous blood (391 and 345 pM) than in arterial blood (346 and 314 pM), but these differences were not significant.

Urinary 8-iso-$PGF_{2\alpha}$ (Figure 4) rose significantly from baseline 300 (245-338) pM per mmol creatinine to 635 (485-738) in the intraoperatively collected sample and fell to 475 (425-558) and 456 (328-490) on postoperative days 1 and 2, respectively.

Plasma values of 8-iso-$PGF_{2\alpha}$ (maximum levels) correlated inversely with the duration of both operation ($p<0.001$, Spearman's rho=-0.721) and CPB ($p<0.01$, Spearman's rho=-0.561), but there was no significant correlation with the aortic cross clamp-time (p=0.1, Spearman's rho=-0.380). In parallel to plasma, urine values (during) correlated inversely with the duration of operation ($p<0.01$, Spearman's rho -0.567), but not with duration of CPB (p=0.2, Spearman's rho=-0.322) or cross-clamp time (p=0.4, Spearman's rho=-0.193).

Analyses of ASA withdrawal prior to surgery revealed intragroup differences in 8-iso-$PGF_{2\alpha}$. The longest withdrawal (>4 days) was associated with 50 % higher (not significant) peak value in plasma (Figure 5a) than the shortest withdrawal (466 vs. 309 pM, p=0.099). In the intra-operative urine sample (Figure 5b) these differences were highly significant (697 vs. 457 pmol/mmol creatinine, p=0.025).

There was a negative correlation between the dose of heparin and the rise in 8-iso-$PGF_{2\alpha}$ in plasma (p=0.035, Spearman's rho=-0.487) during operation. A similar and significant negative correlation existed in the urine sample collected during surgery (p=0.002, Spearman's rho=-0.675) and during the early postoperative period (day 1 sample, p=0.022, Spearman's rho=-0.520).

<u>15-Keto-dihydro-$PGF_{2\alpha}$ in plasma and urine.</u> The plasma values (Figure 3 b) presented a similar time-based profile as did 8-iso-$PGF_{2\alpha}$. Following sternotomy there was a significant rise (p = 0.001) from baseline 174 pM (108-228) (t_0) to 314 (231-483) pM (t_3). During CPB and final surgery time (t_4 –t_{10}), the values fluctuated between 173 and 256 pM. During the postoperative period (t_{11} – t_{14}) 15-keto-dihydro-$PGF_{2\alpha}$ fell to 97-137 pM. Urinary samples (Figure 4) showed a similar time-based profile with elevated intraoperative values as did 8-iso-$PGF_{2\alpha}$.

No significant correlation was found between 15-keto-dihydro-$PGF_{2\alpha}$ and duration of operation, CPB and aortic cross-clamping. However, as with 8-iso-$PGF_{2\alpha}$ the two subgroups related to withdrawal of ASA behaved differently. Thus patients with the longest cessation (Figure 5 a) presented a significantly higher plasma baseline (192 pM vs. 107 pM, p = 0.023) and a higher peak (t_5) plasma value (247 vs. 185, p = 0.069) than patients with shorter cessation. These differences were reflected by a higher value (967 vs. 679 pM per mmol creatinine, p = 0.083) in the intraoperative urine sample (Figure 5).

hsCRP (Table 2) was maintained at a low level during operation and the first post-operative hours (including t_{12}), but rose to high levels on day 1 and even higher levels on day 2. hsCRP showed a positive correlation with duration of CPB (p=0.04, Spearman's rho=0.464), but not with operation or aortic cross clamp-time.

TAS (Table 2) was elevated by 27 % ($p < 0.001$) after onset of CPB (t_3). Thereafter TAS values gradually declined without reaching baseline on days 1 and 2. No correlation was found between TAS versus duration of surgery, CPB time, aortic cross-clamp time, or time off ASA treatment.

Antioxidant vitamins. Values (Table 2) remained at the same level or were slightly elevated above baseline (α-tocopherol) after onset of CPB (t_3), but declined to values below baseline (carotenoids, retinol, α- and β-tocopherol) on post-operative days 1 and 2. No correlation was found between vitamins vs. duration of operation, CPB time or aortic cross-clamp time.

Troponin T. As seen in Figure 6, the cardiospecific injury marker troponin T did not rise until the end of CPB (t_9). A marked elevation (to 0.57-0.70 μg/L) was present 3-6 hours after aortic clamp release, which was early in the postoperative period (t_{11}-t_{12}). This was followed by a fall (0.24 – 0.22 μg/L) on postoperative days 1 and 2. Troponin T values correlated strongly with aortic cross-clamp time ($p<0.0001$, Spearman's rho=0.713) and CPB time (p=0.006, Spearman's rho=0.588), but not with the duration of the surgery. There were no correlations with the time off ASA (p=0.5) or with the dosage of heparin (p=0.9).

DISCUSSION

The present study focused on oxidative stress occurring in 20 consecutive patients with advanced coronary artery disease undergoing standard multi-vessel bypass surgery in a routine manner with use of CPB and aortic cross-clamping. The main aims were twofold: (1) to dissociate a myocardial oxidative stress response due to ischemia-reperfusion from a systemic oxidative stress response caused by the intraoperative trauma and (2) to identify any correlation between two main markers of oxidative stress and myocardial injury, respectively.

There was a major rise in 8-iso-$PGF_{2\alpha}$ during the operation, but we found no differences between coronary venous blood and systemic arterial blood, and there was no evidence of a major separate burst of ROS generated in the myocardium at the onset of reperfusion. Accordingly, we could not demonstrate that reperfusion of the globally ischemic heart led to a detectable oxidative stress of myocardial origin in the present clinical setting. In contrast, the myocardium-specific injury marker troponin T showed a minor but significant rise that correlated positively with the duration of ischemia and CPB. Also, as seen in earlier studies of patients treated with percutaneous coronary intervention (PCI)[37,38], there was no significant correlation between 8-iso-$PGF_{2\alpha}$ and troponin T in individual patients.

Taken together, it seems that the oxidative stress parameter reflected the sequence of additive traumas early during CABG, whereas the myocardial marker showed a significant but transient washout profile that paralleled the duration of ischemia during aortic cross-clamping. Furthermore, the troponin T curve which has a rise after few hours and a major decline towards normal levels within postoperative day two, represents typically a transient myocardial washout of macromolecules without any sign of persistent a major reperfusion injury.[39,40] This indicates that the myocardium was well protected by moderate hypothermia plus cardioplegia[32,41] during the rather short (median 36 min) ischemic episode.

A major observation in the present study was that surgery alone had a considerable impact on the level of oxidative stress and early inflammation. Thus, the essential rise in 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ above baseline occurred prior to CPB, and

the rise correlated inversely with operation time. The most probable explanation is to be found in the extensive use of diathermy for dissection and electrocoagulation during the initial part of the operation (sternotomy and preparation of grafts).[42] The further rise in 8-iso-$PGF_{2\alpha}$ during CPB was rather moderate which may indicate that CPB related stress factors were well controlled. Another interpretation may be, as discussed in a later paragraph, potent antioxidant protection by heparin during CPB.

A particular finding was that the baseline value of 8-iso-$PGF_{2\alpha}$ was 192 pM, which is considerable higher than the reported baseline level of about 80 pM in healthy volunteers[25] and 3-6 times higher than found in comparable populations. In two previous studies by this group[37,38] in patients undergoing x-ray coronary angiography and elective or primary PCI, median baseline values were 31-62 pM.

Also, in a much parallel recent paper[4] reporting on oxidative stress during open heart surgery and CPB baseline 8-iso-$PGF_{2\alpha}$ in plasma was 44 pM, and furthermore, the CPB induced rise above baseline was much less (~72 pM) than observed in the present study (~ 230 pM). As our patients only experienced premedication and introduction of a radial artery cannula before baseline sampling, the most likely cause for elevated isoprostanes has to be related to an altered balance between prooxidant and antioxidant forces prior to the operation. A tentative factor contributing to elevated baseline 8-iso-$PGF_{2\alpha}$ may be other diseases in 7 of the 20 patients, but the former group did not differ from the remaining group at baseline or at later stages. Surprisingly however, when the time from preoperative withdrawal of ASA was taken into consideration, a potential explanation may be found. The arbitrary subgroup with $\leq$ 4 days off ASA presented lower values in 8-iso-$PGF_{2\alpha}$ and in 15-keto-dihydro-$PGF_{2\alpha}$ than the subgroup with > 4 days off. This indicates that withdrawal of ASA may reverse or even induce a negative overshoot of some of its main properties, as a well-known cyclooxygenase inhibitor[43] or as a lesser known inhibitor of plasma and tissue oxidases.[44] Further, the lack of the ROS scavenging ASA metabolite, salicylic acid, may have contributed to the high level of 8-iso-$PGF_{2\alpha}$.[45]

The potential acetylation by ASA of extracellular NAD(P)H oxidases or intermediate molecules and transcription factors has recently been described[46] as a potent

mechanism in reducing superoxide production and raising nitric oxide levels in animal models. Thus prior treatment with ASA over 12 days prevented angiotensin II induced hypertension by improving nitric oxide balance in rats.[44] In our study a withdrawal induced elevation of oxidase activity may explain findings of a high initial peroxide level in both subgroups and more so in the longest cessation subgroup. Furthermore, the same mechanism may explain the higher peak levels of 8-iso-$PGF_{2\alpha}$ in the present investigation compared to other studies.[4] In recent years some reports have indicated that ASA withdrawal or disruption of therapy may prove detrimental to patients with ischemic heart disease. In particular, it has been shown that withdrawal of ASA may be associated with reocclusion of arteries following PCI[47] and also of grafts after CABG.[48] Accordingly, arguments have been raised against preoperative withdrawal of ASA.[49] In those cited articles the focus has been on the potent antiplatelet properties of ASA and not on the potential antioxidative properties as may be indicated by our present study.

15-Keto-dihydro-$PGF_{2\alpha}$ presented as expected an early rise during surgery (t_0-t_4) almost following the profile of 8-iso-$PGF_{2\alpha}$. The cytokine parameter of inflammation hsCRP did not respond during the early part of the operation (t_0-t_4) but was elevated to about 35 and 65 times higher values on postoperative days 1 and 2, respectively, thus reflecting the overall per- and postoperative trauma to the patients.

The time profile of antioxidant vitamins showed that these were well maintained (or even slightly elevated) during the early phase of the operation (t_3) but that a considerable reduction occurred thereafter. The apparent delay may be related to moderate hypothermia (34^{o}C) during the operation and a slowed rate of vitamin consuming reactions. A more unexpected observation was that the TAS assay showed values above baseline not only during the operation but also on days 1 and 2. Since application and interpretation of the TAS assay has been questioned[50,51], it may be that TAS is not a likely measure of oxidative stress and antioxidant balance in the present setting involving both hemodilution and hypothermia. However speculative, a potential mechanism behind elevation of TAS, initial maintenance of vitamins and no significant elevation of 8-iso-$PGF_{2\alpha}$ while on CPB, may be related to the intraoperative use of heparin in large doses. Thus, heparin can stimulate extracellular

superoxide dismutases[52], strengthen ROS defence and retard consumption of antioxidants. Accordingly, in a placebo-controlled study[53] of patients who had their gallbladder removed, heparin prevented any rise in plasma malondialdehyde. In support of such a hypothesis in our study is that the major rise in plasma isoprostane occurred before CPB, and that heparin was administered from the start of CPB until the end of coronary artery repair. We found a significant negative correlation between the doses of heparin and levels of 8-iso-$PGF_{2\alpha}$ in urine collected during and following the operation. This was paralleled by similar findings in plasma.

Due to a more frequent sampling than commonly used, we could demonstrate that surgery itself induced oxidative stress. Such a possibility is mostly overlooked in similar studies.[1-4] However, in a clinical CPB study from 2002 Clermont et al.[5], with use of electron spin resonance spectroscopy and spin traps, were unable to find any evidence of ROS adducts formed during the initial phase of surgery. Thus it might be that our patient group was more vulnerable to oxidative stress due to ASA withdrawal and that this factor led to a major rise in 8-iso-$PGF_{2\alpha}$ during the initial phase of the operation. In parallel with our findings with 8-iso-$PGF_{2\alpha}$, Clermont et al. were unable to identify a significant burst of myocardium-specific ROS adducts following release of the aortic cross clamp.

Conclusions

Three main conclusions can be drawn from the present study. Firstly, there is definitely an oxidative stress during a standard coronary artery bypass grafting, but it may be induced by surgical trauma during the operation and rapidly reversed thereafter. Secondly, the myocardium is probably not a key target for ROS induced injury when appropriate cardioprotective techniques with hypothermia and cardioplegic arrest are applied during preferably brief periods of global ischemia. And thirdly, antioxidant protection may be provided by cardiovascular drugs in common use such as heparin and ASA. In essence, oxidative stress responses during coronary artery surgery are multifactorial in origin.

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Table 1. Patient characteristics

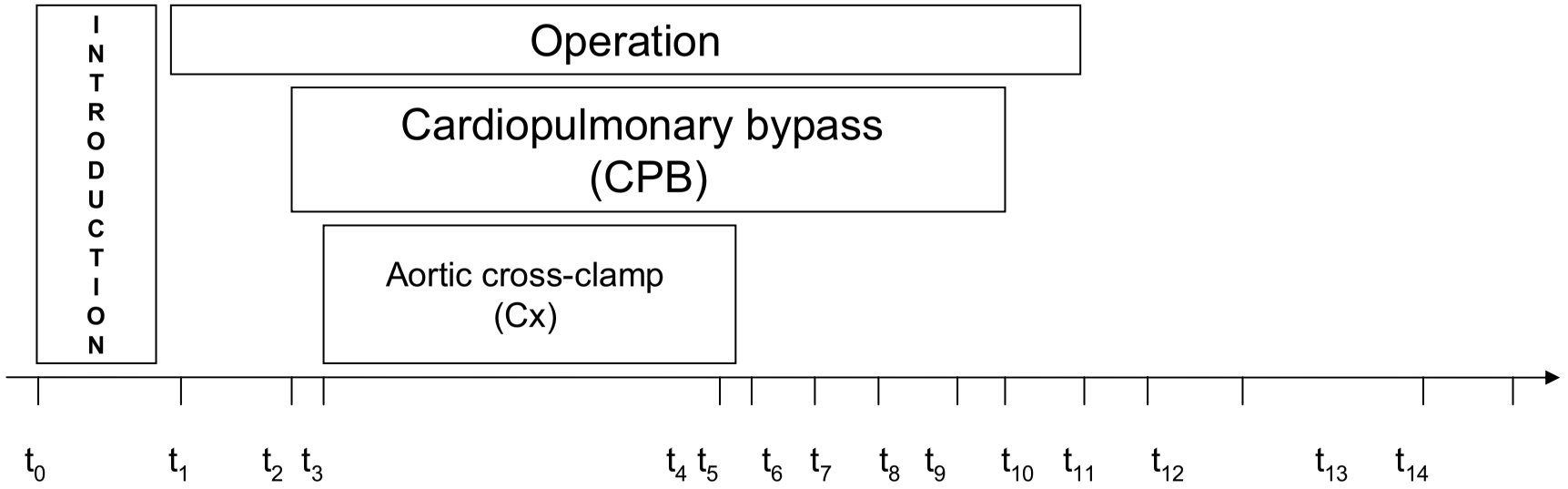
Characteristics	n	(%)
Mean age in years (min-max)	66	(50-79)
Female/male	4/16	(20/80)
Stabile/unstabile angina	16/4	(80/20)
Previous myocardial infarction	7	(35)
Two-vessel disease	5	(25)
Three-vessel disease	15	(75)
Acetylicsalicylic acid (ASA)	19	(95)
ASA withdrawal ≤ 4 days	7	(35)
ASA withdrawal > 4 days	12	(60)
β-blocker	18	(90)
Ca channel blocker	7	(35)
Nitrates	10	(50)
ACE inhibitors	6	(30)
Statins	19	(95)
Low molecular heparin	5	(25)

Table 2. Laboratory data

Variables	t_0	t_1	t_3	t_{13}	t_{14}
hsCRP (mg/l)	1.2 (0.4-2.7)	1.6 (0.6-3.1)	1.5 (0.6-3.2)	114.0 (93.9-141.6) ***	224.18 (162.5-285.5) ***
TAS (mM)	1.16 (1.11-1.27)	1.31 (1.23-1.36)	1.67 (1.50-1.87) ***	1.46 (1.41-1.56) ***	1.38 (1.30-1.60) **
Retinol (μM)	1.6 (1.3-1.8)	1.6 (1.3-1.8)	1.5 (1.3-1.9)	1.2 (1.0-1.5) **	0.9 (0.8-1.0) ***
Carotenoids (μM)	0.28 (0.18-0.42)	0.27 (0.19-46)	0.27 (0.19-0.61) **	0.22 (0.15-0.35)	0.22 (0.15-0.35) *
α-tocopeherol (μM)	22.1 (18.8-25.2)	22.4 (20.2-25.6)	24.2 (21.2-27.6) ***	19.7 (17.4-22.2)	19.0 (17.0-22.2)
γ-tocopherol (μM)	2.2 (1.7-3.1)	2.3 (1.8-3.2)	2.5 (1.8-3.1)	1.8 (1.5-2.4) **	1.8 (1.4-2.4) *

Median values with percentiles (25^{th} and 75^{th}) in parentheses are given.
* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001 (Friedman with Dunn's multiple comparison test).

Figure 1



t_0: after premedication
t_1: after sternotomy and heparin
* t_2: before CPB on,
* t_3: CPB on, before cross clamp (Cx)
* t_4: before Cx off (max ischemia)
* t_5: 1 min of reperfusion (Cx off)
* t_6: 5 min of reperfusion
* t_7: 10 min of reperfusion
* t_8: 15 min of reperfusion
* t_9: CPB off, before protamin
t_{10}: after protamin
t_{11}: 3 hrs of reperfusion
t_{12}: 6 hrs of reperfusion
t_{13}: 1st postoperative day
t_{14}: 2nd postoperative day

* Both systemic (arterial) and coronary (venous) blood

Figure 2

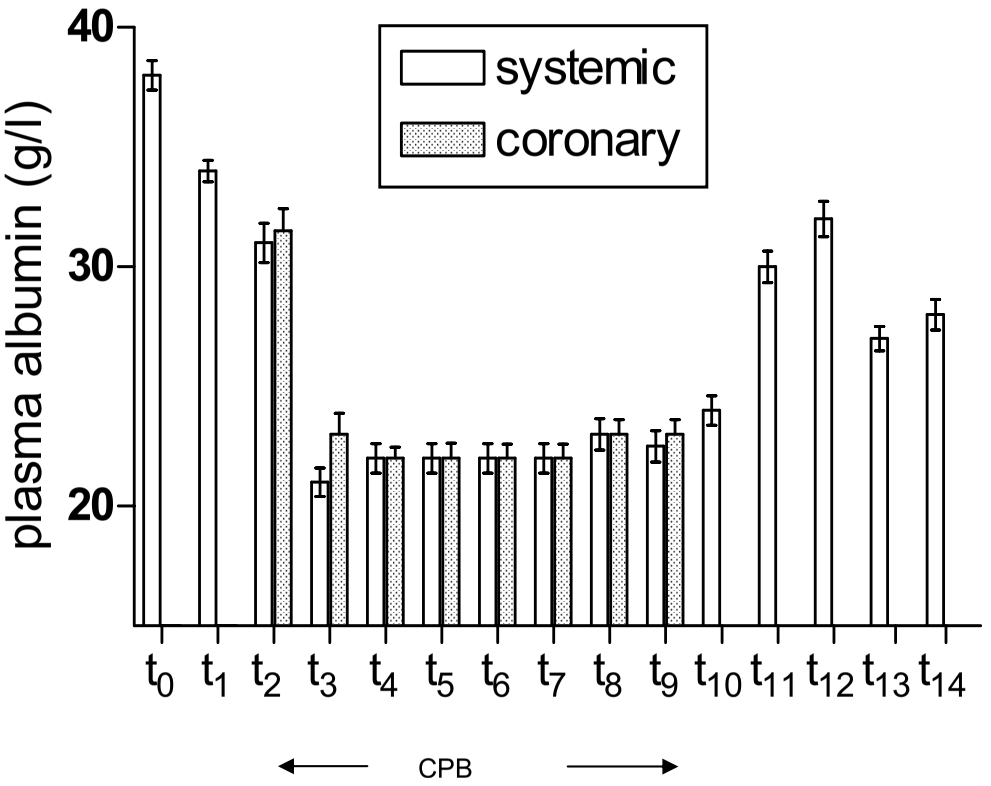

40
30
20
plasma albumin (g/l)
systemic
coronary
t0 t1 t2 t3 t4 t5 t6 t7 t8 t9 t10 t11 t12 t13 t14
CPB

Figure 3a

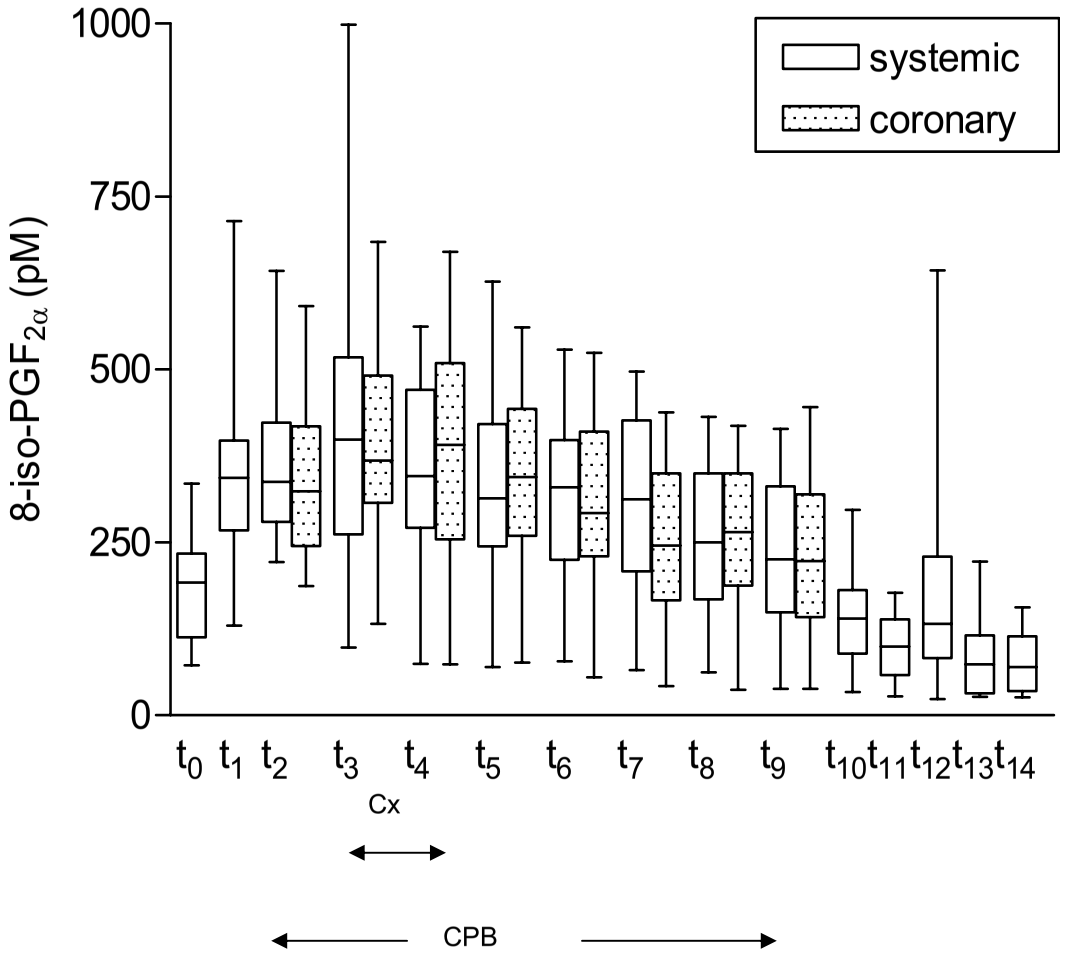

1000
750
500
250
0
8-iso-PGF2α (pM)
systemic
coronary
t0 t1 t2 t3 t4 t5 t6 t7 t8 t9 t10 t11 t12 t13 t14
Cx
CPB

Figure 3b

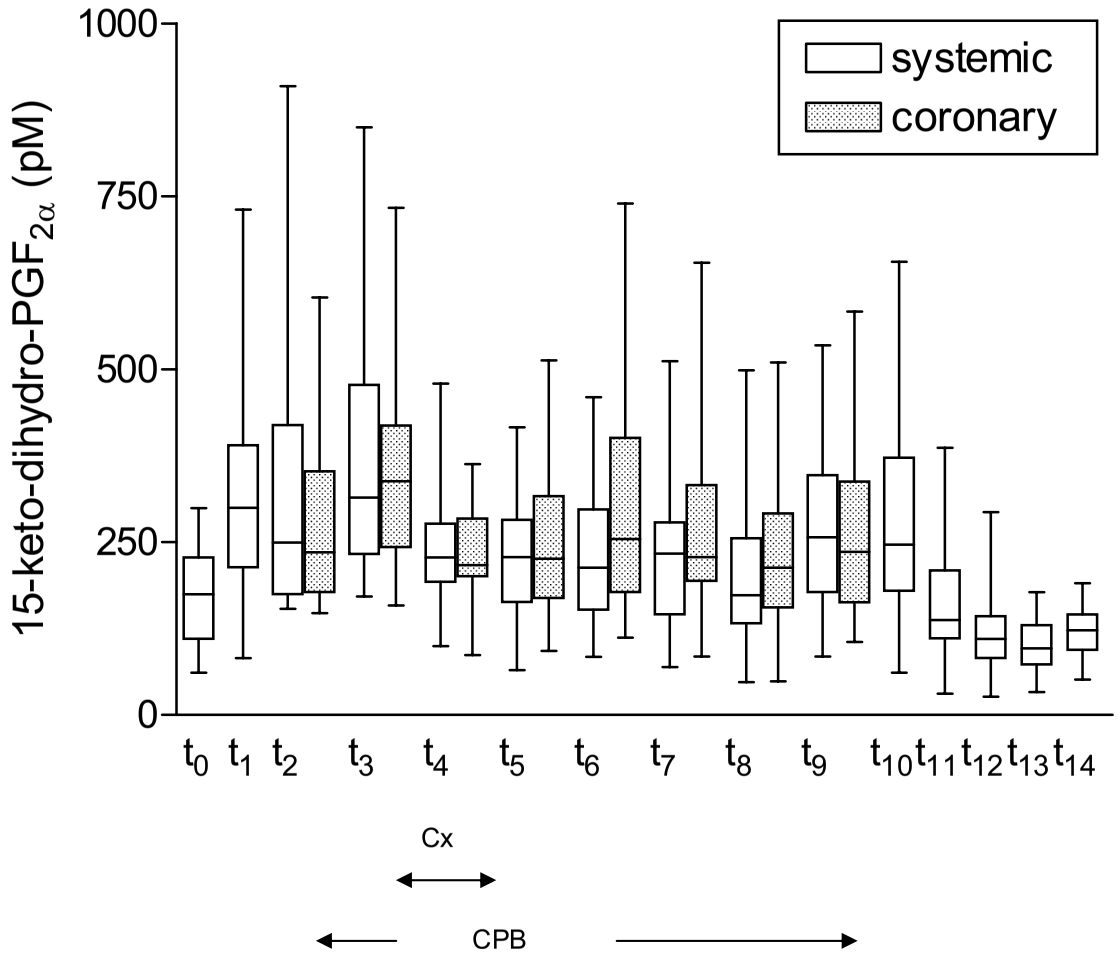

systemic
coronary
15-keto-dihydro-PGF2α (pM)
1000
750
500
250
0
t0 t1 t2 t3 t4 t5 t6 t7 t8 t9 t10 t11 t12 t13 t14
Cx
CPB

Figure 4

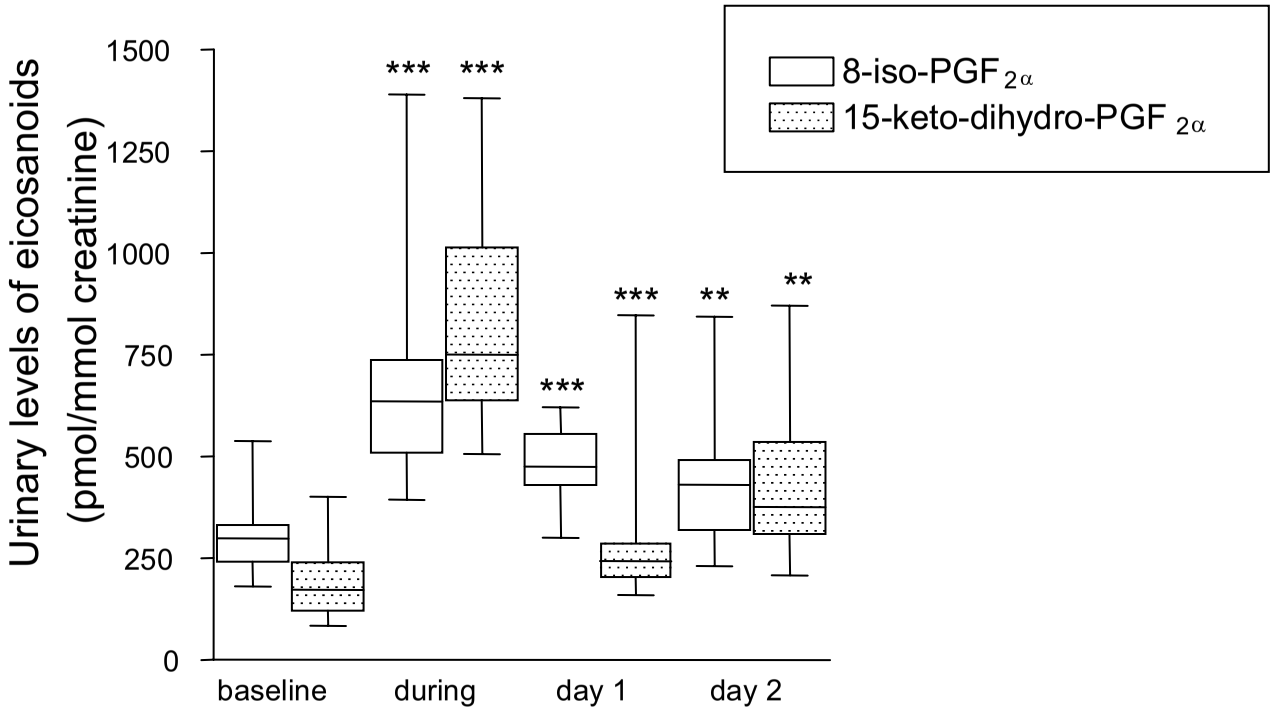

Urinary levels of eicosanoids
(pmol/mmol creatinine)
1500
1250
1000
750
500
250
0
***
***
***
***
**
**
baseline
during
day 1
day 2
8-iso-PGF 2α
15-keto-dihydro-PGF 2α

Figure 5

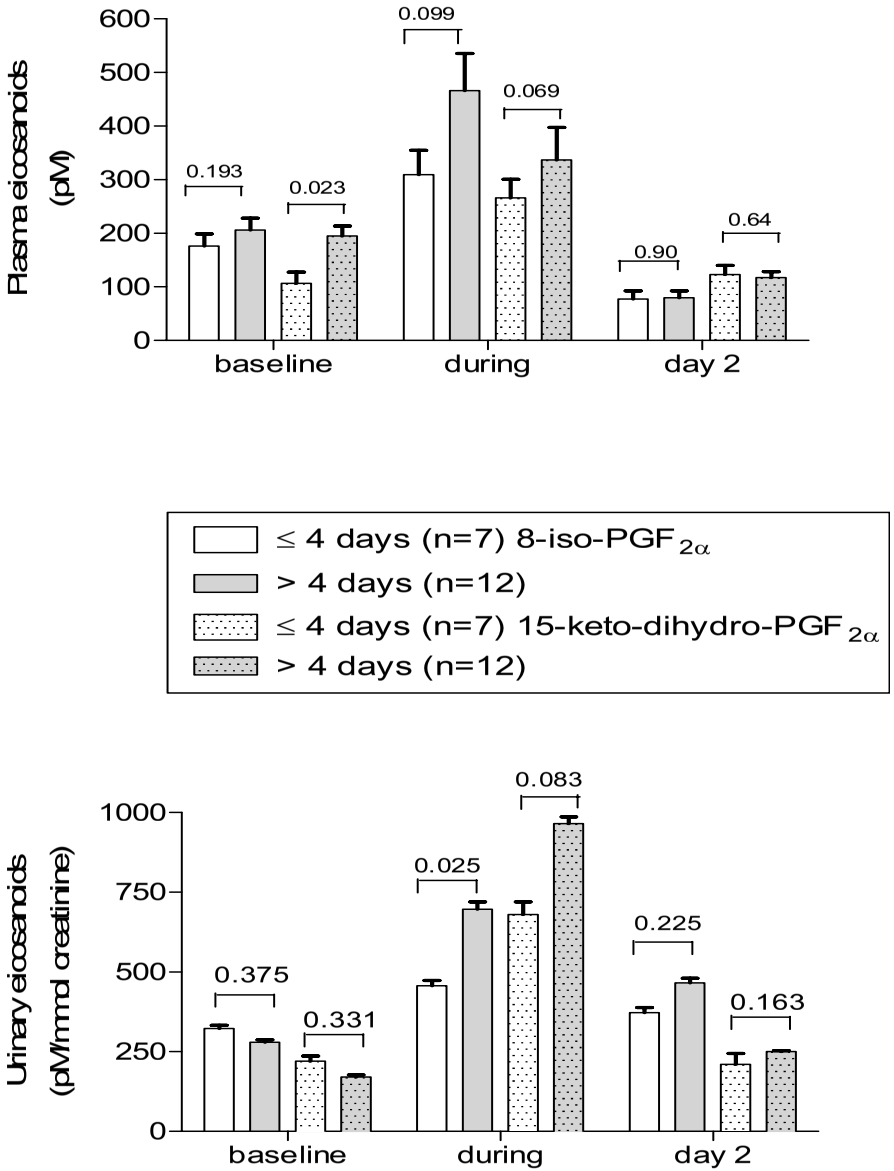
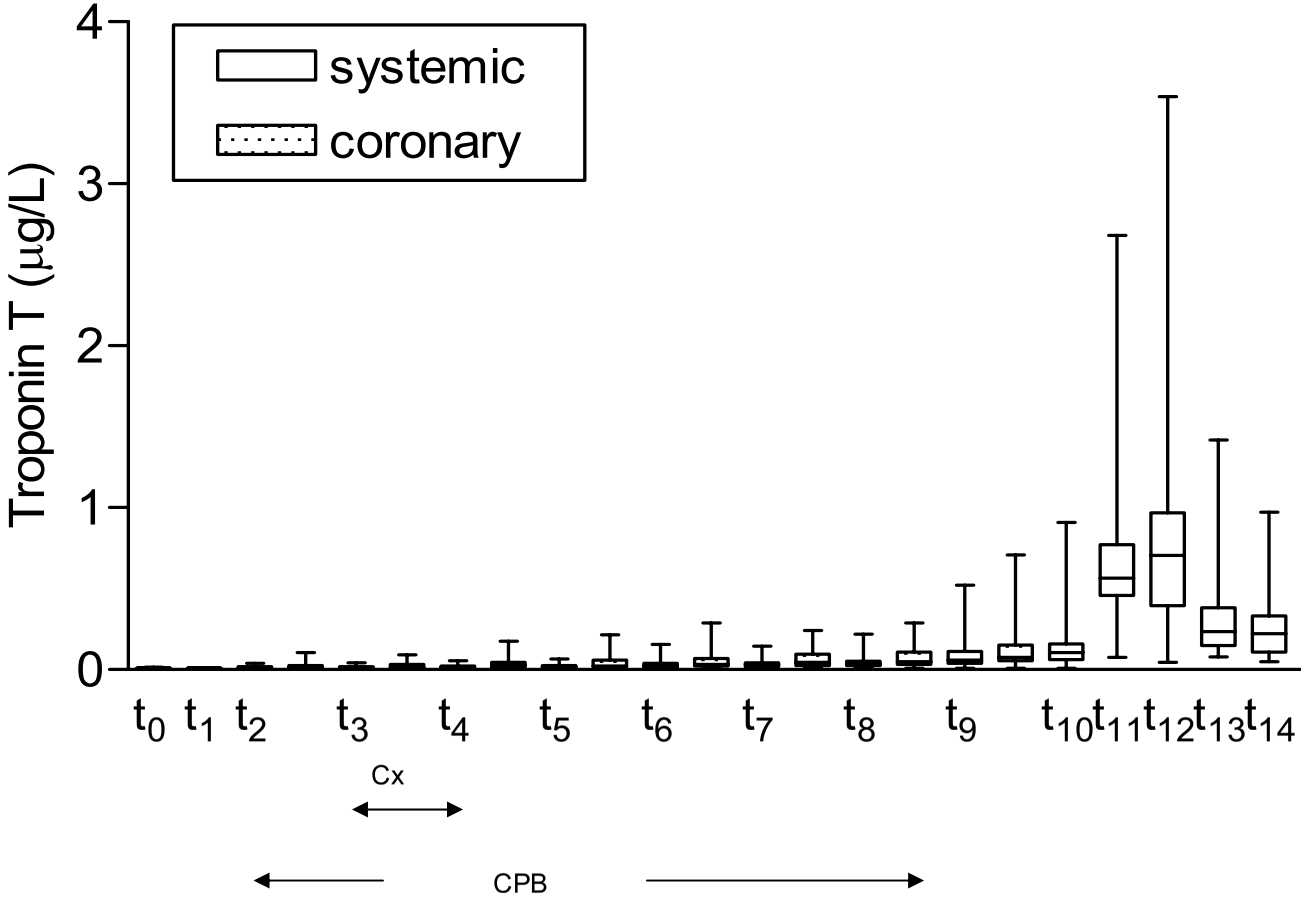

Plasma eicosanoids (pM)
600
500
400
300
200
100
0
0.193
0.023
0.099
0.069
0.90
0.64
baseline
during
day 2
≤ 4 days (n=7) 8-iso-PGF2α
> 4 days (n=12)
≤ 4 days (n=7) 15-keto-dihydro-PGF2α
> 4 days (n=12)
Urinary eicosanoids (pM/mmol creatinine)
1000
750
500
250
0
0.375
0.331
0.025
0.083
0.225
0.163
baseline
during
day 2

Figure 6


systemic
coronary
Troponin T (µg/L)
0
1
2
3
4
t0 t1 t2 t3 t4 t5 t6 t7 t8 t9 t10 t11 t12 t13 t14
Cx
CPB

Legends to figures (1-6):

Figure 1.
Study design.

Figure 2.
Changes in plasma albumin. Blood samples were taken at various stages from a radial artery (white, t_0-t_{14}) and from the coronary sinus (dotted, t_2-t_9). Median values with standard error of the mean (SEM) are given.

Figure 3a.
Changes in plasma 8-iso- $PGF_{2\alpha}$. Blood samples were taken at various stages from a radial artery (white, t_0-t_{14}) and from the coronary sinus (dotted, t_2-t_9). The boxes extend from the 25^{th} to the 75^{th} percentiles with horizontal lines at the median. Whiskers show the highest and the lowest values. The results are correlated with albumin concentration.

Figure 3b.
Changes in plasma 15-keto-dihydro-$PGF_{2\alpha}$. Blood samples were taken at various stages from a radial artery (white, t_0-t_{14}) and from the coronary sinus (dotted, t_2-t_9). The boxes extend from the 25^{th} to the 75^{th} percentiles with horizontal lines at the median. Whiskers show the highest and the lowest values. The results are correlated with albumin concentration.

Figure 4.
Changes in urinary 8-iso- $PGF_{2\alpha}$ (white) and 15-keto-dihydro-$PGF_{2\alpha}$ (dotted). Urine samples were collected at baseline (t_0), during the operation (t_1-t_{10}), and on postoperative day 1 (t_{10}-t_{13}) and day 2 (t_{13}-t_{14}). The boxes extend from the 25^{th} to the 75^{th} percentiles with horizontal lines at the median. Whiskers show the highest and the lowest values. ** p-value < 0.01, *** $p < 0.001$ (Wilcoxon signed ranks test).

Figure 5.
Plasma and urinary eicosanoids related to the preoperative withdrawal of acetylsalicylic acid (ASA). The upper and lower panels present 8-iso- $PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ in plasma and urine, respectively. Urine samples were collected at baseline (t_0), during the operation (t_1-t_{10}) and on postoperative day 2 (t_{13}-t_{14}). The numbers of days off ASA are indicated for the two subgroups. The numbers above the upper whiskers are p-values between subgroups estimated by Wilcoxon signed ranks test. Median values with standard error of the mean (SEM) are given.

Figure 6.
Changes in plasma troponin T. Blood samples were taken at various stages from a radial artery (white, t_0-t_{14}) and from the coronary sinus (dotted, t_2-t_9). The boxes extend from the 25^{th} to the 75^{th} percentiles with horizontal lines at the median. Whiskers show the highest and the lowest values. The results are correlated with albumin concentration. There were no recognizable differences between systemic arterial and coronary venous blood.

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159. CLINICAL AND EXPERIMENTAL STUDIES
160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
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162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.
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164. Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
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170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
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174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

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181. Pål Richard Romundstad: CANCER INCIDENCE AMONG NORWEGIAN ALUMINIUM WORKERS
182. Henrik Hjorth-Hansen: NOVEL CYTOKINES IN GROWTH CONTROL AND BONE DISEASE OF MULTIPLE MYELOMA
183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
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267. Kirsti Berg: OXIDATIVE STRESS AND THE ISCHEMIC HEART: A STUDY IN PATIENTS UNDERGOING CORONARY REVASCULARIZATION