

Modulation of platelet and leukocyte adhesion in cardiothoracic anaesthesia

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ISBN/EAN: 978-90-9024858-5

Layout and printing: Optima Grafische Communicatie Rotterdam

Cover: Leukocyte adhesion cascade

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Financial support for the publication of this thesis was received from START (funds distributed by the scientific board of the Rheinisch-Westfälische Technische Hochschule Aachen)

Modulation of platelet and leukocyte adhesion in cardiothoracic anaesthesia

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op maandag 11 januari 2010
om 13:30 uur precies

door

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geboren op 29 maart 1967
te Marburg, Duitsland

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Abbreviations

ADP	Adenosine diphosphate
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
cGMP	Cyclic guanosine monophosphate
CPB	Cardiopulmonary bypass
CREB	cAMP response element-binding protein
CREBa	Activated cAMP response element-binding protein
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein -5-isothiocyanat
FSC	Forward Scatter
GP	Glycoprotein
ICAM	Intercellular adhesion molecule
IL	Interleukin
IκB	Inhibitory factor-κB
MAC	Minimum alveolar anaesthetic concentration
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor-κB
PE	Phycoerythrin
PDE	Phosphodiesterase
PKA	Protein kinase A
PKAa	Activated protein kinase A
PKC	Protein kinase C
PKCa	Activated protein kinase C
PSGL-1	P-selectin glycoprotein ligand-1
SSC	Side Scatter
TNF	tumour necrosis factor
TRAP-6	Thrombin receptor-activated peptide-6



Chapter 1

General Introduction and Aim of the Thesis

GENERAL INTRODUCTION

The human immune system consists of a complex network of tissues, cells and proteins which are meant to protect the organism from invading pathogens or foreign materials. Inflammation, as the biological response to harmful stimuli, requires a cascade of biochemical events involving the local vascular system, the immune system, and various cells within the injured tissue.

Innate and acquired immunity play a pivotal role in the host defence response. Pain, stress, tissue damage and invading microorganisms are known modulators of the complex immune response of patients undergoing major surgery. The immune system of patients undergoing cardiac surgery is often not only activated through the surgical stimulus but also by a systemic inflammatory response caused by the cardiopulmonary bypass. Cardiopulmonary bypass is known to set a complex and multifactorial inflammatory response in motion, which involves platelets and leukocytes as well as the coagulation, complement and kallikrein cascade. These changes contribute to the development of an early pro-inflammatory response and a later prothrombotic state (1-11).

Anaesthesia itself or perioperative interventions of the anesthesiologist may also substantially alter the immune function with potential impact on the postoperative course (12). For instance, transfusion of allogenic blood and administration of catecholamines may interfere with immunity (13, 14). Volatile anaesthetics, etomidate, propofol or thiopentone which are used to induce and maintain anaesthesia may directly affect function of immune competent cells. In addition to their effect on consciousness there is recent evidence that anaesthetics possess immunomodulatory and cardioprotective properties thus reducing ischaemia reperfusion injury (15, 16). Another kind of frequently used drugs in cardiac surgery are inotropic agents such as catecholamines and phosphodiesterase (PDE)-inhibitors. Considering their mode of action they should not only act on myocardial or smooth muscle but also on immunocompetent cells which possess the same receptors (17, 18). Previous studies have shown that epinephrine and PDE-inhibitors modulate the non specific immune response and that stress and pain are associated with immune tolerance, increased susceptibility to infection and tumour spreading in animal models. Thus perioperatively administrated "stress hormones" to maintain cardiac output such as epinephrine may also directly or indirectly affect the immunity of the surgical patient (19- 22). Volatile anaesthetics have also been reported to have antiadhesive and cardioprotective activity and protect for instance against ischaemic reperfusion injury (15, 23-29.)

The leukocyte and platelet adhesion cascade

The recruitment of leukocytes to the site of inflammation entails a cascade of cellular adhesive events, which include initial attachment, rolling, firm adhesion, and transendothelial migration of the responding cells (30-32). Selectins expressed on the endothelial surface

interact with their ligands on leukocytes to mediate the tethering and rolling phase of leukocyte recruitment. These are weak adhesive interactions (33, 34). This initial weak adhesion brings leukocytes into contact with cytokines/chemoattractants released from the activated endothelium, such as interleukin 8 (IL-8) and platelet-activating factor (PAF), which transduce signals through their G protein–coupled receptors that activate integrins (35, 36). These activated leukocyte integrins then recognize their cognate ligands (e.g. ICAM family) to mediate the firm adhesion and arrest of leukocytes to the endothelium. Such integrin-mediated firm adhesion can occur through direct ligand engagement or indirect bridging mechanisms. An important bridging mechanism, the recognition of integrin CD11b of fibrinogen or fibrin deposited on the endothelial surface can promote the accumulation of leukocytes at the sites of inflammation (37, 38). Fibrinogen engagement by activated CD11b is also one of the several mechanisms that contribute to the formation of platelet-leukocyte conjugates, which are diagnostic of thrombotic events in vivo (39,40). P-selectin, a member of the selectin family, is stored on the membranes of platelet α -granules and endothelial Weibel-Palade bodies. After inflammatory and thrombogenic events, P-selectin rapidly translocates to the surface of these cells and contributes to the weak adhesion of leukocytes to endothelial cells and the heterotypic aggregation of activated platelets to leukocytes (41,42). In this context, the pathophysiological mechanisms and consequences of platelet–leukocyte interactions and their implications in many diseases have been investigated recently. In-

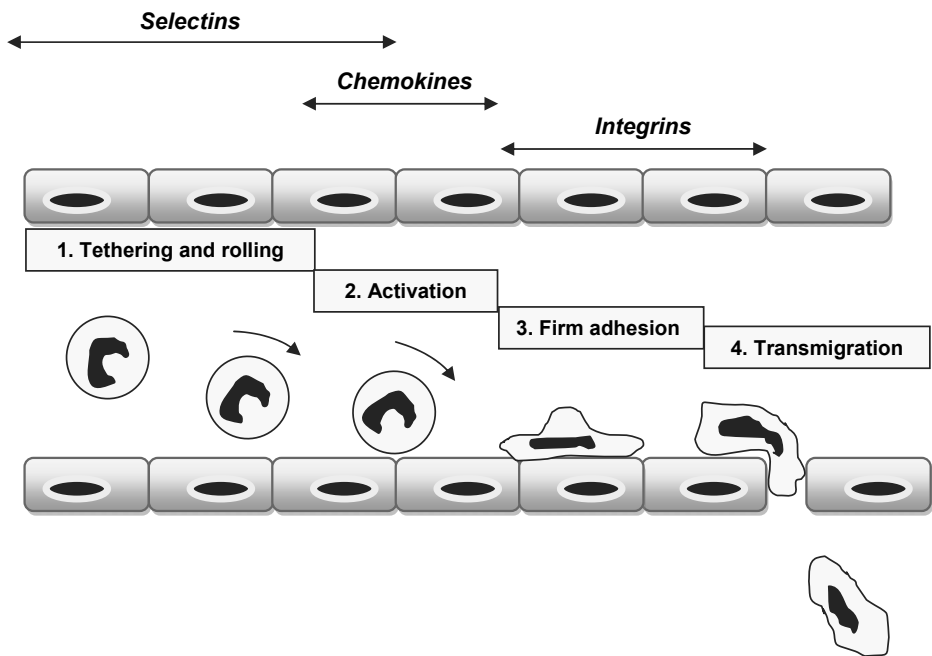


Figure 1: The leukocyte adhesion cascade: The main steps preceding transmigration are shown : rolling, which is mediated by selectins, activation, which is mediated by chemokines, and arrest, which is mediated by integrins.

creased association between platelets and leukocytes has been reported in unstable angina, myocardial infarction, coronary interventions, cardiopulmonary bypass, thrombosis and sepsis (43-48). An interaction between platelets and leukocytes may link these processes and contribute by intercellular communication pathways to the pathophysiology of these diseases. It is especially enhanced P-selectin expression on the platelet surface, which supports the adhesion of platelets to the P-selectin-ligand-1 (PSGL-1) on neutrophils, monocytes and lymphocytes (49, 50). The binding of platelets to neutrophils seems to induce attachment, rolling and the oxidative burst in neutrophils, as well as cytokine secretion in monocytes (51-54). Recruitment of neutrophils and monocytes to an inflammatory site in response to invading bacteria or non-infectious processes is a crucial step in the physiology of the acute inflammatory response. Adherence of neutrophils and monocytes to endothelial cells followed by transmigration through the endothelial cells depends on a network of several events involving neutrophil surface adhesion molecules, inflammatory cytokines and chemoattractant chemokines. Transmigration of neutrophils and monocytes through endothelial cells to surrounding organ tissues is not always beneficial. In ischaemia/reperfusion injury, activated neutrophils and monocytes contribute to organ damage by releasing toxic reactive oxidative species and increased cytokine release (55-60). These results suggest that the tight interaction among platelets, neutrophils, and monocytes has an important part in the host defence system.

The upregulation of cellular adhesion receptors and the formation of leukocyte-platelet conjugates may have important implications during and after cardiopulmonary bypass, in the development of an early pro-inflammatory response and a later prothrombotic state. There is, for instance, evidence of increased leukocyte-platelet adhesion in patients with graft occlusion after peripheral vascular surgery (3). Many patients suffering from the above-mentioned diseases, or undergoing coronary interventions or bypass surgery receive anaesthetics and inotropic agents that may modify the inflammatory response. Hence the effects of anaesthetics on ischemia-reperfusion injury and neutrophil adhesion have also been the object of several studies. While some study groups reported that isoflurane and sevoflurane protect against myocardial ischaemia-reperfusion injury (24-26) others, such as Morisaki and co-workers, found increased leukocyte rolling and adhesion in rats undergoing sevoflurane anaesthesia (61). A stable host defence system is of great importance during the perioperative period, it is therefore also important to clarify if and how anaesthetics and inotropic agents affect perioperative immunity.

Adhesion molecules

Adhesion molecules enable cells to contact and specifically interact with each other thus allowing communication between cells and the surrounding environment. This is crucial for developmental and functional activity. These proteins are typically transmembrane receptors

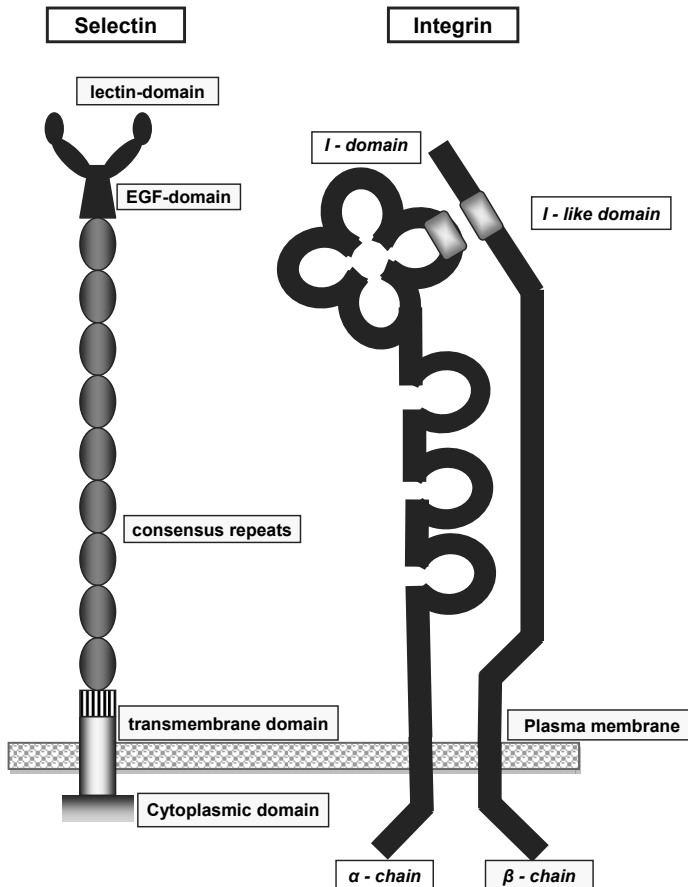


Figure 2: Selectin and integrin structure

and are composed of three domains: an intracellular domain that interacts with the cytoskeleton, a transmembrane domain and an extracellular domain that interacts with other cell adhesion molecules. Several different families of receptors mediate these interactions. The families of cell adhesion molecules identified to date include selectins, integrins, Ig superfamily members, and cadherins. Members of these adhesion receptor families are critical in migration, inflammation and wound healing (32, 62, 63).

The mechanisms regulating adhesive interactions are complex. A single cell can express an array of different adhesion receptors, and a single receptor may bind to more than one ligand. Both expression and functional activity of adhesion molecules are influenced by a variety of factors, including the presence of activating factors such as specific antigen and inflammatory mediators. In addition, different adhesion receptors may operate successively in time. This has been well demonstrated for leukocyte extravasations into sites of inflammation, in which adhesion molecules from the selectin, integrin, and immunoglobulin receptor families work successively as part of an “adhesion cascade”.

Selectins

Selectins constitute a family of carbohydrate-binding cell adhesion molecules comprised of three related cell surface molecules: L-selectin (CD62L), P-selectin (CD62P), and E-selectin (CD62E).

Selectins function in the initial step of recruitment of leukocytes (primarily neutrophils) to the site of an inflammatory reaction. Loose tethering to the endothelium results in a decrease in leukocyte velocity and rolling along the blood vessel wall. Leukocyte rolling in most vessels begins within minutes following tissue injury, and numerous studies have indicated that these interactions are primarily mediated by selectins. The rapid transport of P-selectin from intracellular granules to the plasma membrane is thought to contribute to the early stage of rolling, while L- and E-selectin are involved in subsequent stages (62, 64, 65).

P-selectin:

P-selectin is stored preformed in Weibel-Palade bodies of endothelial cells and α -granules of platelets. Upon stimulation, P-selectin is phosphorylated and rapidly mobilized to the cell surface via a secretory pathway. P-selectin in the plasma membrane surface serves as a cell adhesion receptor to interact with other cell receptors. Expression of at the cell surface is, however, transient and decreases substantially within minutes. P-selectin can be shed from activated cells and circulate as soluble P-selectin in the plasma. Recent studies show that high levels of soluble P-selectin in blood result in a procoagulant state (41, 66, 67).

The prominent role of cellular P-selectin in leukocyte rolling and extravasation, as well as platelet-leukocyte interactions, is well established. In contrast to other platelet receptors, P-selectin does not mediate platelet-platelet interactions (68). P-selectin upregulates tissue factor in monocytes, binds carbohydrate ligands on leukocytes and thereby mediates leukocyte rolling on activated endothelial cells and leads to leukocyte accumulation in areas of vascular injury associated with thrombosis and inflammation. The major ligand of P-selectin on leukocytes is PSGL-1 (41, 69).

L-selectin:

L-selectin is present on almost all leukocytes and virtually absent on a subset of memory lymphocytes. L-selectin binds several ligands. All L-selectin ligands identified so far share common features: they are sialylated, fucosylated, sulphated, and show similarity to sialyl Lewis x and Lewis x. An important function of selectins is defined by their ability to bind carbohydrate ligands within milliseconds, thereby capturing free-flowing leukocytes from the bloodstream. This allows subsequent leukocyte rolling, which markedly decreases the travelling speed of the leukocytes from $>2000 \mu\text{m/s}$ to $<50 \mu\text{m/s}$. This specialized function, which represents a hallmark of leukocyte recruitment, requires rapid association and dissociation of the selectin-ligand interaction and is well defined, especially for L-selectin. Fol-

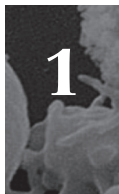
lowing activation, lymphocytes and neutrophils undergo a reversible loss of L-selectin from the cell surface. L-selectin is proteolytically cleaved from the cell surface by the action of a specific enzyme and the shed extracellular portion of the molecule, soluble or sL-selectin, is present in the plasma. There is broad conformity in the literature that sL-selectin levels in the plasma are elevated in infectious diseases and inflammation. Data support the concept that shedding of L-selectin from the leukocyte surface controls the rolling velocity, which in turn has an impact on the transit time of the leukocytes. Thus L-selectin shedding may reduce the exposure of the leukocytes to endothelial-derived inflammatory mediators and thereby may restrict extravasation (70-73).

Integrins

Integrins are a family of cell surface receptors that mediate interactions with extracellular matrix components and with other cells. Integrins mediate the firm adhesion of leukocytes by binding members of the immunoglobulin family of adhesion molecules expressed on endothelial cells. Integrins are heterodimeric molecules consisting of an α -subunit and a noncovalently-bound β -subunit. They represent a large protein family that is classified by the β -subunits. β 1- (CD29), β 2- (CD18), β 3- (CD61), and β 7-integrins are engaged in leukocyte recruitment, with β 2-integrins playing the key role in mediating firm adhesion of human leukocytes subsequent to selectin-mediated rolling. Unlike selectins, β 2-integrins (CD18) do not recognize ligand but require cellular activation to form stable shear-resistant bonds with endothelial ligands. Leukocyte rolling constitutes a prerequisite for β 2-integrin-mediated firm adhesion *in vivo*, since β 2-integrins are not able to bind their ligands unless the velocity of passing leukocytes is slowed down to a critical value by selectin-based rolling. Integrin activation is induced by either a conformational change within each receptor, which increases apparent affinity for ligand, or integrin clustering, which enhances avidity for ligand (74-76). The importance of β 2-integrin mediated adhesion *in vivo* is illustrated by the leukocyte adhesion deficiency type I syndrome in which there is either partial or total absence of β 2-integrins on leukocytes. Patients with this inherited condition suffer from severe, recurrent bacterial and fungal infections. Their neutrophils exhibit rolling, but do not adhere to endothelial tissue and fail to emigrate from the blood stream to sites of inflammation (52, 65).

CD11b:

CD11b (Mac-1) is expressed at high levels on monocytes and granulocytes and at lower levels on a subset of T cells. CD11b is a major integrin on neutrophils and notorious for its capacity to recognize many different ligands, such as the blood coagulation proteins fibrinogen and factor X, ICAM-1, the complement pathway product C3bi as well as several extracellular matrix proteins (77). Activation of monocytes and granulocytes by inflammatory stimuli leads to mobilization of intracellular stores of CD 11b and a rapid increase in its cell surface



expression. Ligand recognition by CD11b is influenced by the activation state of the receptor. CD 11b is involved in the transendothelial migration of monocytes and neutrophils (69).

CD11a:

The most important $\beta 2$ -integrin that mediates firm adhesion is CD 11a (LFA-1) which is expressed on lymphocytes, granulocytes, monocytes, and macrophages, and the level of expression is increased upon activation. CD11a exerts its function primarily by binding ICAM-1, which is upregulated on the inflamed endothelium, but can also bind to ICAM-2 and ICAM-3 (78-80).

GP IIb/IIIa:

As a member of the integrin family of proteins, GPIIb/IIIa ($\alpha_{IIb}\beta_3$) is a heterodimeric glycoprotein complex found on the platelet surface and in the α -granules. When platelets are activated, GPIIb/IIIa undergoes a conformational change that permits the binding of its principal ligand, fibrinogen. Cross-linking through fibrinogen allows platelets to aggregate into a growing haemostatic plug. The initial binding of fibrinogen is reversible but undergoes a time dependent stabilization. Regardless of what triggers the platelet to activate, GPIIb/IIIa receptors represent the final common pathway to platelet aggregation and thrombus formation. Platelets and leukocytes may form aggregates via platelet-expressed P-selectin and its counter receptors PSGL-1 and Sialyl Lewis X, as well as via fibrinogen bridging between GPIIb/IIIa and CD11b (81-83).

Other cell adhesion molecules

GP Ib:

Glycoprotein Ib is a transmembrane subunit of the GP Ib–IX–V platelet adhesion receptor. The GP Ib–IX–V complex consists of four subunits, GP Iba, GP Ibb, GP IX, and GP V, each of which is a member of the leucine- rich repeat protein superfamily. GP Ib interacts with von Willebrand factor thus mediating the adhesion and aggregation of platelets at sites of vascular injury (84).

PSGL-1:

P-selectin glycoprotein ligand-1 is a mucin that binds to all three members of the selectin family with the highest affinity to P-selectin on platelets and endothelial cells. It is found on all white blood cells and therefore plays an important role in the recruitment of leukocytes into inflamed tissue and in the platelet-leukocyte interaction. Recent studies suggest that PSGL-1 binding to its counterreceptor P-selectin promotes CD11b-dependent homotypic neutrophil aggregation and neutrophil-platelet conjugation and α_4/β_1 -dependent adhesion of monocytes to vascular cell adhesion molecule 1 (VCAM-1), responses typically dependent

Table 1: Adhesion molecules mentioned in this thesis

Adhesion molecule	Synonyms	Major occurrence	Major ligands
<i>Selectins:</i>			
L-selectin	CD 62L	Leukocytes	CD 34 Glycam-1 MAdCam-1
P-selectin	CD62P	Endothelial cells Platelets	PSGL-1 PSGL-1
<i>Integrins:</i>			
$\alpha L/\beta_2$	CD11a/CD18 LFA-1	Leukocytes	ICAM-1, -2, -3
$\alpha M/\beta_2$	CD11b/CD18 MAC-1	Neutrophils Monocytes	ICAM-1 Fibrinogen C3bi
α_{IIb}/β_3	CD41/CD61 GPIIb/IIIa	Platelets	Fibrinogen Fibronectin
<i>Others:</i>			
PSGL-1	CD162	Leukocytes	P-selectin L-selectin E-selectin

on integrin activation (85-87). Hidari et al observed that engagement of PSGL-1 enhances tyrosine phosphorylation, activates mitogen-activated protein (MAP) kinases (ERK-1 and ERK-2) through MEK (MAP kinase kinase), and stimulates IL-8 secretion in neutrophils (88).

Volatile Anaesthetics

Volatile anaesthetics are inhalational anaesthetic substances which are used to induce and maintain a reversible loss of consciousness during surgical procedures.

The commonly used inhalational anaesthetics are isoflurane, sevoflurane and desflurane, while halothane as an older agent is rarely used nowadays.

Several investigations revealed that anaesthetists have been using anaesthetics with antiadhesive activity regularly in clinical practice for decades. Evidence from animal models suggests that halothane, isoflurane, and sevoflurane protect the heart against ischaemia-reperfusion injury (24-27, 89-91). Proposed mechanisms are reduced production of hydroxyl radicals (89); activation of myocardial adenosine receptors (24), PKC (25), inhibitory guanine regulatory proteins (92), mitochondrial and sarcolemmal adenosine triphosphate- regulated potassium channels (93, 94), and stretch-activated channels (95); and inhibition of neutrophil adhesion to endothelial cells (26, 27). One investigation for instance revealed that adhesion of neutrophils to endothelial cells may be reduced because of an attenuated upregulation of CD11b, whereas endothelial adhesion molecules were not affected (23).

Since Ueda demonstrated that halothane inhibits platelet aggregation in 1971, various studies have investigated the effects of volatile anaesthetics on platelet aggregation (96). Sevoflurane in particular has recently been the subject of several investigations, with some-

times contradictory results. In 1996, Hirakata et al. reported that sevoflurane had strong effects on secondary platelet aggregation, probably through inhibition of thromboxane A2 formation, whereas Honemann et al. found no influence on thromboxane A2 signalling (97, 98). The results of Hirakata et al. were supported by a study of Dogan et al., who also showed impaired platelet aggregation after sevoflurane anaesthesia (99). More recently, Nozuchi and co-workers have demonstrated that sevoflurane does not inhibit platelet aggregation induced by thrombin (100). However, the results remain contradictory, and the direct effects on platelet surface antigens have not been studied. The glycoprotein receptors within the platelet membrane are of particular interest in this regard. As mentioned before they are essential for platelet adhesion and platelet-mediated primary and secondary aggregation. Among the most important glycoproteins are the GPIIb/IIIa complex, inducing platelet aggregation *via* fibrinogen binding; the GPIb as a receptor of the von Willebrand factor; and the α -granule membrane protein P-selectin, mediating platelet endothelial and leukocyte interactions. Acquired or hereditary defects, as well as reduced expression of these glycoproteins, can result in platelet malfunction and impaired haemostasis. Therefore, changes in platelet aggregation induced by sevoflurane could be reflected here.

In vitro studies have shown that isoflurane alters the monocyte inflammatory response, such as inhibition of endotoxin-induced TNF- α and IL-1b secretion as well as inhibition of chemotaxis (101, 102). Isoflurane is also known to alter several aspects of leukocyte function. Previously, it has been shown that isoflurane attenuates ischaemia-reperfusion injury (57). One suggested mechanism is a decreased activation of neutrophil L-selectin, CD11a and CD11b, which could be responsible for a reduced accumulation of neutrophils at sites of ischaemia-reperfusion injury (23).

Inotropic agents

Positive inotropic agents such as epinephrine and milrinone increase myocardial contractility, and are used to support cardiac function in conditions such as decompensated congestive heart failure, cardiogenic shock, septic shock, myocardial infarction, cardiomyopathy, etc.:

Epinephrine:

Epinephrine is an endogenous stress hormone and neurotransmitter. It is a catecholamine, a sympathomimetic monoamine derived from the amino acids phenylalanine and tyrosine. Physiologically it is produced and released by the adrenal glands. Catecholamine concentrations are increased as an early stress response after cardiac arrest, myocardial infarction, and trauma. Therapeutically, they are used in critically ill patients to treat low cardiac output and severe hypotension.

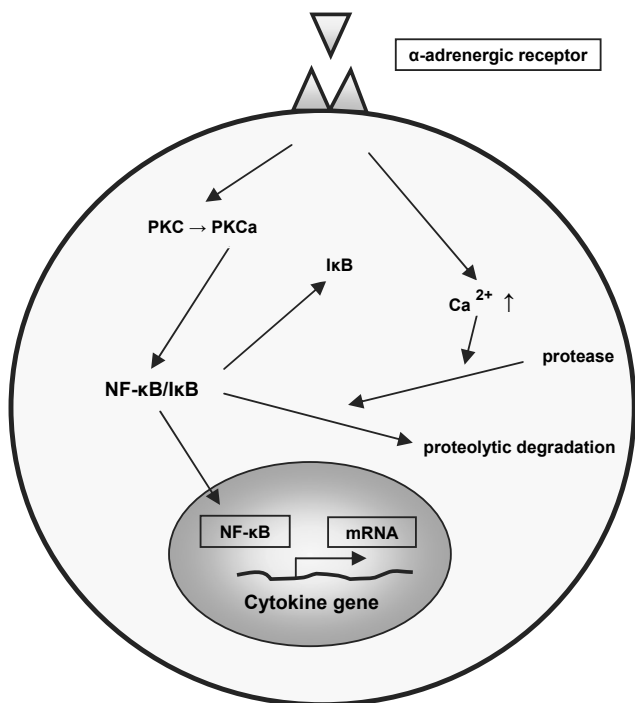


Figure 3: Schematic diagram of α -adrenergic regulation as described in the text.

Epinephrine's actions are generally mediated through α - or β -adrenergic receptors: Leukocytes, platelets and endothelial cells all carry adrenergic receptors (103,104). It is known, that in these cells α -adrenergic stimulation leads to activation of NF- κ B and to the transcription of cytokine-mRNA with NF- κ B binding sites on their promoters.

β -Adrenergic stimulation leads to an increase of cAMP, which activates PKA. Activated PKA is translocated to the nucleus. Activated PKA blocks NF- κ B. This leads to an inhibition of cytokine-specific mRNA with NF- κ B binding sites on their promoter. Activated PKA also leads to an activation of CREB and to a transcription of cytokine-specific mRNA with CREB binding sites on their promoters (105). NF- κ B's key-function in inducing an immune response makes β -receptor agonists strong immunosuppressive drugs and α -agonists proinflammatory agents.

Previous studies have shown that epinephrine modulates the unspecific immune response. It decreases neutrophil adherence, chemotaxis, and phagocytic capacity. It also inhibits tumor necrosis factor (TNF)- α and interleukin (IL)-1 β production but enhances IL-8 and IL-10 production and L-selectin expression in monocytes. Epinephrine also enhances P-selectin expression in platelets and the opening of glycoprotein (GP)IIb/IIIa binding sites for fibrinogen, and it favours platelet aggregation (106-112).

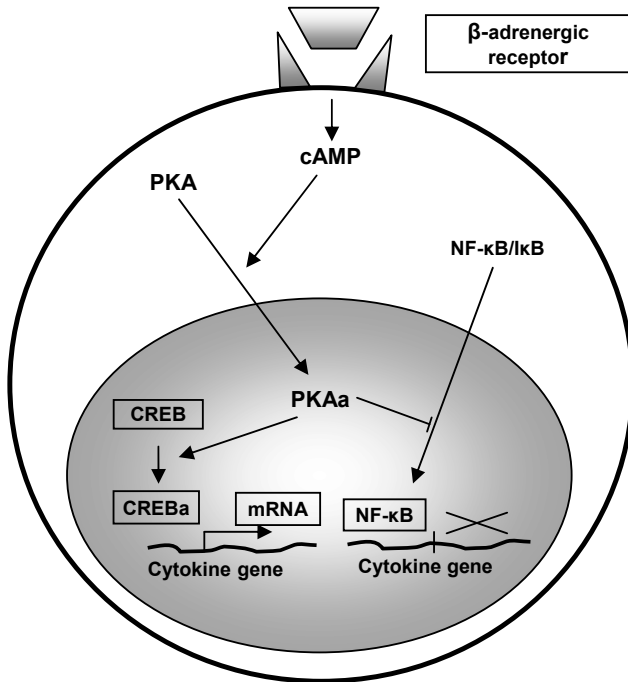


Figure 4: Schematic diagram of β -adrenergic regulation as described in the text.

Little is known about the effects of epinephrine on platelet-neutrophil adhesion at concentrations observed during therapeutic inotropic support or major injury. Knowledge of such effects may have implications not only for understanding endogenous stress hormone influences during injury, but also for the therapeutic use of epinephrine in patients with septic shock cardiac failure or undergoing cardiac surgery.

Milrinone:

In the 1960's the involvement of cyclic nucleotide second messengers in cell signalling and homeostasis became established. Since then, the regulation of this pathway by phosphodiesterase (PDE) inhibitors became an area of interest. Phosphodiesterases are a class of isoenzymes responsible for the hydrolysis of the intracellular second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) resulting in the formation of the corresponding, inactive 5'-monophosphate. PDE inhibitors block this activity, causing an accumulation of the cyclic nucleotides cAMP and cGMP that mediate the physiologic responses to a variety of transmitters, hormones and drugs. PDE isoenzymes can be discriminated based on substrate specificity or affinity and their regulation by specific activators and inhibitors. The expression and activity of these PDE isoenzymes varies among different tissues and cells. The PDE3 family, which hydrolyses both cAMP and cGMP, can be

found in cardiac muscle, vascular smooth muscle, platelets and leukocytes. The PDE4 family hydrolyses only cAMP and is expressed in bronchial smooth muscles, leukocytes, mesangial and endothelial cells but not in platelets. Today PDE inhibitors are widely used in intensive care medicine, especially for the treatment of asthma and congestive heart failure. As the second messengers cAMP and cGMP transduce the effects of a variety of extracellular signals, they also influence immunomodulatory processes in all human inflammatory cells such as proinflammatory mediator production and cell differentiation. While it is well-known that elevation of intracellular cAMP possesses inhibitory effects on platelet aggregation and results in decreased degranulation and cytokine production in leukocytes as well as reduced CD11b expression in neutrophils, little is known about the effects of PDE inhibitors on platelet-leukocyte interaction (113-120).

Milrinone, is a phosphodiesterase 3 (PDE3) inhibitor, which increases left ventricular-contraction through inhibiting the breakdown of cAMP and, hence, elevating the cellular cAMP, which in turn activates cAMP-dependent protein kinases with a resultant increase in the transsarcolemmal influx of Ca^{2+} and the rate of Ca^{2+} uptake by the sarcoplasmic reticulum, independent of β_1 -adrenergic receptor stimulation. Because of its positive inotropic, vasodilating and minimal chronotropic effects it is used in the management of severe heart failure. Patients undergoing cardiac surgery often receive PDE3 inhibitors in the treatment of cardiac failure, given that thrombotic and inflammatory events might be crucial in these patients it is important to study if therapeutic concentrations of PDE inhibitors also affect platelet-leukocyte interactions

Fluorescent-activated cell sorting (FACS)

Fluorescence-activated cell sorting is a flow cytometry based technique for analyzing expression of cell surface and intracellular molecules, characterizing and defining different cell types in heterogeneous cell populations, assessing the purity of isolated subpopulations, and analyzing cell size and volume. It allows simultaneous multi-parameter analysis of single cells. It is predominantly used to measure fluorescence intensity produced by fluorescence-labelled antibodies detecting proteins or ligands that bind to specific cell-associated molecules.

For the staining procedure single-cell suspension from cell culture or tissue samples have to be made. The cells are then incubated in tubes or microtitre plates with unlabeled or fluorescent-labelled antibodies. Cells are then analysed in the flow cytometer, where the sample is hydrodynamically focussed to a tiny stream of single cells. Laser light is directed onto the sample as it flows through the chamber. There are detectors in front of the light beam (Forward Scatter or FSC) and several detectors side on to it (Side Scatter or SSC) in order to measure the light. Cells passing through the beam will scatter the light, which is then detected as forward scatter and side scatter. Another set of fluorescence detectors are used for the detection of fluorochromes themselves. Fluorochromes used for detection will emit

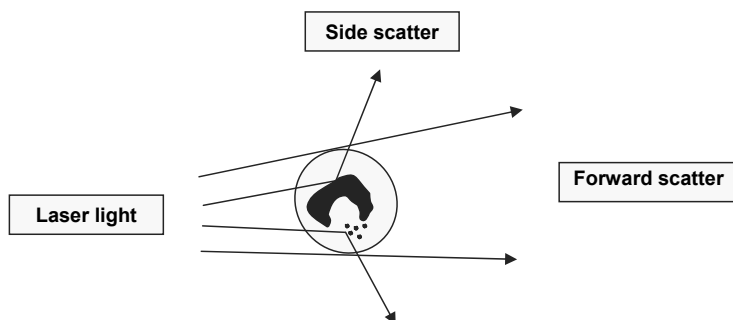


Figure 5: The principle of flow cytometry

light when excited by a laser with the corresponding excitation wavelength. The combination of scattered and fluorescent light can be analyzed: The forward scatter correlates with the cell size and the side scatter with the density of the particle/cell (i.e. number of cytoplasmic granules, membrane size), so that cell populations can often be distinguished based on their difference in size and density. Further distinctions and characteristics can be specified by means of the fluorescence emission.

The Becton-Dickinson FACScan used in this study an air-cooled blue argon gas laser, with a fixed wavelength emission of 488 nm. It has three fluorescence detection channels which simultaneously detect green, yellow-orange, and red light. Fluorescein (FITC) is for the green channel, and phycoerythrin (PE) for the yellow-orange channel are the most commonly used fluorescent dyes. The list of measurable parameters by FACS is extensive and includes amongst others (121-126):

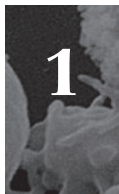
- volume and morphological complexity of cells
- cell surface antigens (Cluster of differentiation (CD) markers)
- intracellular antigens (various cytokines, secondary mediators etc.)
- oxidative burst
- DNA and RNA (cell cycle analysis, cell kinetics, proliferation etc.)
- chromosome analysis and sorting (library construction, chromosome paint)
- protein expression and localization
- nuclear antigens
- enzymatic activity
- pH, intracellular ionized calcium, magnesium, membrane potential
- apoptosis (quantification, measurement of DNA degradation, mitochondrial membrane potential, permeability changes, caspase activity)
- cell viability

AIM OF THE THESIS

The aim of this thesis is to investigate the effects of in cardiothoracic anaesthesia commonly used pharmaceuticals on platelet-leukocyte interaction.

The main objectives were:

- To evaluate whether the volatile anaesthetics isoflurane, sevoflurane and desflurane modify platelet and leukocyte adhesion.
- To evaluate the effects of these volatile anaesthetics on platelet and leukocyte adhesion molecule expression.
- To assess the effect of the α - and β - receptor stimulating catecholamine epinephrine and the PDE3 inhibitor milrinone on platelet-leukocyte conjugate formation.
- To elucidate the different effects of the various pharmaceuticals on platelet and leukocyte behaviour.

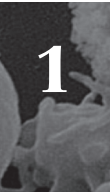


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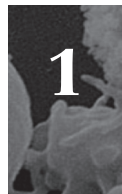
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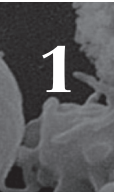
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A grayscale electron micrograph showing the surface of platelets, with some appearing as smooth spheres and others as more irregular, textured structures.

Chapter 2

Sevoflurane Inhibits Unstimulated and Agonist-induced Platelet Antigen Expression and Platelet Function in Whole Blood *In Vitro*

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Anesthesiology 2001;95:1220-5

ABSTRACT

Background: Previous studies have reported conflicting results about the effect of sevoflurane on platelet aggregation. To clarify this point, we investigated the effects of sevoflurane on platelet antigen expression and function *in vitro*.

Methods: Human whole blood was incubated for 1 h with 0.5 and 1 minimum alveolar concentration sevoflurane, 21% O₂, and 5% CO₂. A control sample was kept at the same conditions without sevoflurane. After stimulation with adenosine diphosphate or thrombin receptor agonist peptide 6, samples were stained with fluorochrome conjugated antibodies, and the expression of platelet glycoproteins GPIIb/IIIa, GPIb, and P-selectin, as well as activated GPIIb/IIIa, were measured with two-color flow cytometry. In addition, platelet function was assessed by means of thromboelastography and using the platelet function analyzer 100.

Results: Already in subanesthetic concentrations, sevoflurane inhibits unstimulated and agonist-induced GPIIb/IIIa surface expression and activated GPIIb/IIIa expression on platelets in whole blood. The agonist-induced redistribution of GPIb into the open canalicular system was also impaired by sevoflurane, whereas no effect on P-selectin expression in activated platelets could be found. Sevoflurane significantly reduced the maximum thromboelastographic amplitude. Furthermore, platelet function analyzer 100 closure times were significantly prolonged.

Conclusion: The results show that sevoflurane significantly impairs platelet antigen expression *in vitro*. It is especially the inhibition of GPIIb/IIIa expression and activation that impairs bleeding time as reflected in thromboelastographic measurements and platelet function analyzer 100 closure times. The exact inhibitory mechanism remains unclear.

INTRODUCTION

Since Ueda (1) demonstrated that halothane inhibits platelet aggregation in 1971, various studies have investigated the effects of volatile anesthetics on platelet aggregation. Sevoflurane in particular has recently been the subject of several investigations. In 1996, Hirakata *et al.* (2) reported that sevoflurane had strong effects on secondary platelet aggregation, probably through inhibition of thromboxane A_2 formation, (3) whereas Honemann *et al.* (4) found no influence on thromboxane A_2 signaling. The results of Hirakata *et al.* (2,3) were supported by a study of Dogan *et al.* (5), which also showed impaired platelet aggregation after sevoflurane anesthesia. More recently, Nozuchi *et al.* (6) have demonstrated that sevoflurane does not inhibit platelet aggregation induced by thrombin. However, the results remain contradictory, and the direct effects on platelet surface antigens still need to be studied.

Glycoprotein receptors within the platelet membrane are of particular interest in this regard. They are essential for platelet adhesion and platelet-mediated primary and secondary aggregation. Acquired or hereditary defects, as well as reduced expression of these glycoproteins, could result in platelet malfunction and impaired hemostasis. (7,8) Therefore, changes in platelet aggregation induced by sevoflurane could be reflected here.

Among the most important glycoproteins are the GPIIb/IIIa complex, inducing platelet aggregation *via* fibrinogen binding; the GPIb as a receptor of the von Willebrand factor; and the α -granule membrane protein P-selectin, mediating platelet endothel and leukocyte interactions.

To gain further insight into the mechanisms involved in the inhibition of platelet aggregation induced by sevoflurane, we investigated the influence of sevoflurane on the expression of different platelet surface glycoproteins in whole blood by means of flow cytometry. The expression of the platelet glycoproteins GPIIb/IIIa and GPIb and P-selectin were detected with fluorochrome-conjugated antibodies. The activated GPIIb/IIIa complex was examined using PAC-1, an antibody which recognizes the conformationally changed fibrinogen binding site. Platelet-related hemostasis was furthermore assessed with thromboelastography and the platelet function analyzer 100 (PFA).

METHODS

Flow cytometry allows measurement of the specific characteristics of a large number of single cells. Before analysis, cells are labeled with fluorescence-conjugated antibodies. Thereafter, the flow cytometer detects the emitted fluorescence and light-scattering properties of each cell.

Antibodies and reagents

The following were purchased from BD Pharmingen (San Jose, CA): anti-CD41a-phycoerythrin (PE; clone, HIP8), a monoclonal antibody–recognizing platelet GPIIb/IIIa complex independent of activation; anti-CD42b-PE (clone, HIP1), a monoclonal antibody for the subunit of GPIIb; anti-CD62P-fluorescein-isothiocyanate (FITC; clone, AK-4), a monoclonal antibody directed against P-selectin expressed on platelet surface; and IgM-FITC (clone, G155-228) and IgG₁-FITC (clone, MOPC-21), antibodies for nonspecific binding. PAC-1-FITC (Becton-Dickinson, San Jose, CA) recognizes a neoepitope on the GPIIb/IIIa complex after undergoing the activation-induced conformational change. Dulbecco's phosphate-buffered saline without Ca²⁺ and Mg²⁺, bovine serum albumin, adenosine diphosphate (ADP), and paraformaldehyde were obtained from Sigma Chemicals (St. Louis, MO). Thrombin receptor agonist peptide 6 (TRAP-6) was purchased from Bachem (Heidelberg, Germany).

Blood collection and incubation

After obtaining approval from the local ethics committee (Aachen, Germany) and informed written consent, blood samples were taken from healthy volunteers who had not received any medication for at least 2 weeks. Venous blood was collected without tourniquet from a cubital vein using a 21-gauge butterfly. The first 3 ml of blood was used to perform a hemogram and were then discarded. The next samples were drawn into polypropylene tubes containing sodium citrate (Sarstedt, Nuernbrecht, Germany). Nine parts of blood were anticoagulated with one part 3.8% trisodium citrate. All blood samples were immediately diluted to 1:1 with 37°C prewarmed Dulbecco's phosphate-buffered saline and were placed in polypropylene tissue culture dishes (Sarstedt).

One diluted blood sample was processed immediately to obtain baseline values. A second sample served as control and was incubated for 1 h in an incubator containing an atmosphere of 21% O₂ and 5% CO₂ at 37°C. The third sample was incubated with 1 minimum alveolar concentration (MAC) sevoflurane (2 vol%) or 0.5 MAC sevoflurane (1 vol%) also for 1 h. For the incubation, we used a recently developed chamber, which allows the delivery of volatile anesthetics at low gas flow rates in an atmosphere of 21% O₂ and 5% CO₂ at 37°C. Sevoflurane (Abbott, Wiesbaden, Germany) was delivered as a volatile–air mixture using an anesthetic machine (Titus; Draeger, Luebeck, Germany). Carbon dioxide was administered into the chamber from an external gas connection. After equilibration of the atmosphere inside the chamber, the fresh gas flow was kept at 0.5 l/min during the experiments. Oxygen, carbon dioxide, and sevoflurane concentrations and the temperature in the chamber were monitored continuously using a Datex AS/3 anesthesia monitor, including a multigas analyzer (Datex Ohmeda, Helsinki, Finland).

Sample preparation and flow cytometric analysis

To achieve comparable preconditions for staining with saturating antibody concentrations, the platelet count was adjusted in all samples to approximately $20 \times 10^9/l$ by dilution with Dulbecco's phosphate-buffered saline containing 1% bovine serum albumin. Samples were then divided and stimulated with ADP (1 μm final concentration) or TRAP-6 (6 μm final concentration). After 5 min, 40 μl of either unstimulated or stimulated samples was added to polypropylene tubes containing saturating concentrations of fluorochrome-conjugated antibodies and antibodies for nonspecific binding. All aliquots were allowed to stain for 15 min at room temperature in the dark. The reaction was stopped with 1.5 ml cold Dulbecco's phosphate-buffered saline containing 1% bovine serum albumin and 1% paraformaldehyde. The cells were stored up to 30 min at 4°C in the dark until flow cytometric measurements were performed.

Flow cytometric two-color analyses were performed on a FACSCalibur flow cytometer and were analyzed using CellQuest 3.1 software (Becton Dickinson). Before each measurement, the flow cytometer was calibrated with fluorescence microbeads (Calibrite Beads; Becton Dickinson).

Platelets were identified by forward and side scatter and PE staining. For each sample, the data of 10,000 single platelets were collected. For further analysis, the platelets were gated in a side scatter *versus* fluorescence 2 (PE) dot plot. The mean FITC and PE fluorescence intensities of the gated platelet populations were calculated from fluorescence histograms. The percentage of platelets positive for PAC-1 was determined with a PAC-1-FITC *versus* CD42b-PE dot plot. The percentage of platelets positive for CD62P was measured in a CD62P *versus* CD41a-PE dot plot. Results are expressed as percentage of platelets positive for a marker and the mean fluorescence intensity (MFI) of the marker, reflecting the numbers of epitopes expressed on a single platelet. MFI and percentage of positive cells were calculated after subtraction of nonspecific isotype-specific antibody binding (8).

Thromboelastography

Thromboelastography and PFA measurements were also performed on baseline, control, and sevoflurane samples. Thromboelastography was performed with the rotation thromboelastograph (ro-TEG⁺; Nobis Labordiagnostica, Endingen, Germany). For each thromboelastography, 300 mL citrated blood was pipetted into the prewarmed (37°C) cuvette of a rotation thromboelastograph, and coagulation was induced by adding 20 l CaCl_2 . Analyzed parameters included coagulation or time from sample placement until initial fibrin formation (R time), clot formation or time taken for a fixed degree of viscoelasticity to be reached by the forming clot (K time), maximum amplitude, or maximum clot firmness (reflection of the absolute strength of the fibrin clot).

PFA measurements

Platelet function analyzer measurements were performed on a PFA-100 system (Dade, Miami, FL). This system assesses platelet function in citrated whole blood by monitoring the blood flow through an aperture cut in a membrane coated with collagen and epinephrine or ADP. The time required for the occlusion of the aperture (closure time) has been reported to be indicative of the platelet function. The maximum value for closure time is 300 s, and values greater than 300 s are reported as nonclosure. Thromboelastography and PFA measurements were performed only with 0.5 MAC sevoflurane.

Statistical Analysis

To provide a better overview of the range of individual glycoprotein expression, data are given as mean, minimum, and maximum values. Because the Kolmogorow-Smirnow test revealed a normal distribution of the data, we used analysis of variance followed by the Bonferroni multiple comparison test to compare sevoflurane, baseline, and control samples. A *P* value of less than 0.05 was considered significant.

RESULTS

Time-dependent effects on platelet activation

To discover the effects of incubation time on platelet activation, we compared the results of the control samples with the baseline samples. After 60 min of incubation, none of the measured parameters showed a significant difference to baseline (see also tables 1–3).

Effect of sevoflurane on GPIIb/IIIa expression and PAC-1 binding

Although sevoflurane had no significant effect on GPIIb/IIIa complex expression in unstimulated platelets, stimulation with ADP and TRAP-6 did not increase the number of GPIIb/IIIa epitopes expressed on the surface of those platelets incubated with sevoflurane (1 and 0.5 MAC), whereas GPIIb/IIIa epitopes almost doubled in the baseline and control samples.

The monoclonal antibody PAC-1 was used to identify the activated GPIIb/IIIa complex. After incubation with sevoflurane (0.5 and 1 MAC), PAC-1 binding of unstimulated as well as ADP- or TRAP-6-stimulated platelets was significantly lower compared with baseline and control samples. MFI and the number of cells positive for PAC-1 increased only slightly in platelets exposed to sevoflurane, whereas both values showed a 10-fold increase in baseline and control samples after activation. Incubation with 0.5 MAC sevoflurane inhibited PAC-1

Table 1: Unstimulated and Agonist-induced Platelet Antigen Expression after Exposure to 1 MAC Sevoflurane

	Baseline	Control (60 min Incubation)	1 MAC Sevoflurane (60 min Incubation)
CD41a [MFI]	1,150 (995–1,330)	1,180 (1,009–1,380)	1,267 (1,071–1,562)
CD41a (1 mM ADP) [MFI]	2,157 (1,804–2,666)	2,068 (1,711–2,383)	1,272 (1,106–1,594)*
CD41a (6 mM TRAP-6) [MFI]	2,343 (1,899–2,753)	2,297 (1,753–2,719)	1,277 (1,075–1,600)*
PAC-1 [MFI]	9.9 (1.7–20.3)	9.4 (2.3–19.1)	6.2 (2–12.2)
PAC-1 (1 mM ADP) [MFI]	137.3 (82.4–187.7)	135.3 (86.5–193.3)	14.3 (2–33.9)*
PAC-1 (6 mM TRAP-6) [MFI]	178.1 (118.6–253.2)	179.1 (128.1–276.2)	10.3 (2.7–32.8)*
PAC-1 [% pos. platelets]	3.6 (0.5–9.5)	3 (0.7–9.8)	0.6 (0.3–1.4)*
PAC-1 (1 mM ADP) [% pos. platelets]	91.8 (81.6–98.2)	91.8 (78.5–97.2)	11.7 (0.6–44.3)*
PAC-1 (6 mM TRAP-6) [% pos. platelets]	91.1 (82.3–98.1)	92.5 (87–97.9)	6.2 (0.2–21.5)*
CD42b [MFI]	1,416 (1,075–1,746)	1,452 (1,251–1,741)	1,497 (1,148–1,950)
CD42b (1 mM ADP) [MFI]	1,083 (928–1,279)	1,105 (928–1,247)	1,422 (1,144–1,790)*
CD42b (6 mM TRAP-6) [MFI]	816.7 (654–958)	793.9 (635–991.4)	1,388.3 (1,100–1,688)*
CD62P [MFI]	6.6 (4.6–8.9)	5.7 (4.4–8)	7.4 (5.7–8.8)*
CD62P (1 mM ADP) [MFI]	22.2 (11.9–46.6)	22.7 (11.3–42.7)	22 (9.4–29.8)
CD62P (6 mM TRAP-6) [MFI]	61.6 (39.5–81.7)	68.3 (52.1–81)	63.7 (48.6–78.8)
CD62P [% pos. platelets]	2.3 (2–2.9)	2.3 (1.2–3.1)	4.4 (3.2–5.5)*
CD62P (1 mM ADP) [% pos. platelets]	7 (4.5–9.1)	8.5 (3.9–15.6)	17.7 (6.6–31.1)*
CD62P (6 mM TRAP-6) [% pos. platelets]	66.3 (43.2–84.2)	76.8 (67.1–84.3)	64.4 (43.3–86.8)

Data are expressed as mean, minimum, and maximum values. * P < 0.05 versus control and baseline. MFI = mean fluorescence intensity.

Table 2: Unstimulated and Agonist-induced Platelet Antigen Expression after Exposure to 0.5 MAC Sevoflurane

	Baseline	Control (60 min Incubation)	0.5 MAC Sevoflurane (60 min Incubation)
CD41a [MFI]	1,220 (1,069–1,435)	1,159 (1,048–1,381)	1,213 (949–1,364)
CD41a (1 mM ADP) [MFI]	2,245 (1,958–2,597)	2,076 (1,724–2,429)	1,301 (973–1,493)*
CD41a (6 mM TRAP-6) [MFI]	2,493 (2,166–2,864)	2,308 (1,907–2,616)	1,216 (898–1,431)*
PAC-1 [MFI]	11 (2.6–25)	11.1 (3.1–21.4)	7.1 (2.1–13)*
PAC-1 (1 mM ADP) [MFI]	146.6 (83.9–189.4)	130 (93.7–173.3)	23 (8.5–50.7)*
PAC-1 (6 mM TRAP-6) [MFI]	168.6 (112.1–275)	158.9 (87–301.6)	14.7 (4.3–29.3)*
PAC-1 [% pos. platelets]	7.2 (1.3–17.1)	5.1 (1.1–11.4)	2.2 (0.3–6.8)*
PAC-1 (1 mM ADP) [% pos. platelets]	93.7 (82.4–97.6)	93.6 (86.7–97.5)	25.5 (3–59.9)*
PAC-1 (6 mM TRAP-6) [% pos. platelets]	93.4 (83.6–98.6)	91.1 (80.5–98.5)	16.2 (1.72–38.5)*
CD42b [MFI]	1,391 (1,260–1,616)	1,470 (1,170–1,644)	1,423 (1,040–1,663)
CD42b (1 mM ADP) [MFI]	1,038 (800–1,215)	1,135 (841–1,435)	1,363 (997–1,620)*
CD42b (6 mM TRAP-6) [MFI]	810 (574–1,193)	790 (660–1,067)	1,310 (1,016–1,550)*
CD62P [MFI]	5.8 (4.4–7.6)	6 (3.4–11.1)	7.9 (4.3–14.6)*
CD62P (1 mM ADP) [MFI]	20.3 (7.7–37.2)	20.5 (9.3–31.6)	23.8 (15.3–36.1)
CD62P (6 mM TRAP-6) [MFI]	66.56 (44.8–94.2)	67.2 (38.3–105.6)	60.1 (36.6–86.3)
CD62P [% pos. platelets]	3 (2.1–3.8)	2.9 (2–3.9)	4.4 (2.5–6.3)*
CD62P (1 mM ADP) [% pos. platelets]	7.9 (0.59–19)	8.4 (3.5–16.5)	26.3 (9.8–57.5)*
CD62P (6 mM TRAP-6) [% pos. platelets]	70.8 (49.1–85.5)	70.3 (50.1–92.5)	62.2 (37.2–90.9)

Data are expressed as mean, minimum, and maximum values. * P < 0.05 versus control and baseline. MFI = mean fluorescence intensity.

binding less than did incubation with 1 MAC sevoflurane. Results are summarized in tables 1 and 2.

Effects of sevoflurane on GPIb expression

Exposure to 1 and 0.5 MAC sevoflurane did not change surface expression of GPIb in unstimulated platelets. Interestingly, activation with ADP and TRAP-6 of platelets incubated with sevoflurane did not result in a decrease in surface-expressed GPIb, whereas both activators induced a significant decrease in surface GPIb in baseline and control platelets (tables 1 and 2).

Effects of sevoflurane on p-selectin expression

Incubation with sevoflurane (0.5 and 1 MAC) resulted in a significant increase in CD62P-MFI and positive cells in unstimulated platelets. Stimulation with ADP increased the number of positive cells for P-selectin but not the MFI in platelets exposed to sevoflurane. Activation with TRAP-6 did not result in significant differences in surface expression of P-selectin in any of the three groups, although overall P-selectin expression was higher compared with unstimulated and ADP activated platelets (tables 1 and 2).

Thromboelastographic and PFA measurements

In comparison with baseline and control samples, the samples incubated with sevoflurane (0.5 MAC) showed a significantly increased R time and a significantly reduced maximum amplitude. Clot formation time could not be measured because the clot never reached the degree of viscoelasticity required for its determination. PFA closure times of the baseline and control samples for both cartridges remained within normal values, whereas closure times of

Table 3: Thromboelastography and PFA Measurements

	Baseline	Control (60 min Incubation)	0.5 MAC Sevoflurane (60 min Incubation)
<i>Thromboelastography</i>			
R time (min)	10.7 (7–14.4)	11.3 (9.8–14.6)	17.7 (16.1–20.6)*
K time (min)	6.2 (5.4–6.9)	6.3 (4.6–10.6)	†
Maximum amplitude (mm)	53 (49–55)	51 (50–53)	8 (6–11)*
<i>PFA measurements</i>			
Collagen/ADP (s)	98.6 (97–100)	101.3 (92–116)	>300*
Collagen/Epinephrine (s)	143.6 (132–162)	139.3 (132–150)	>300*

Data are expressed as mean, minimum, and maximum value. * $P < 0.05$ versus control and baseline. † Clot formation time could not be measured because the clot never reached the degree of viscoelasticity required for its determination.

the blood exposed to sevoflurane were greater than 300 s analog to nonclosure. Because the results obtained with 0.5 MAC sevoflurane were already highly significant, we did not repeat the thromboelastographic and PFA measurements with 1 MAC sevoflurane (table 3).

DISCUSSION

In the current study, we have been able to show that sevoflurane significantly altered platelet glycoprotein expression and platelet function, even in subanesthetic concentrations *in vitro*. Already in unstimulated blood, sevoflurane reduced basal PAC-1 binding and the percentage of PAC-1–positive cells. Stimulation of sevoflurane-incubated platelets with ADP or TRAP-6 did not result in an increase of surface-expressed GPIIb/IIIa, nor did the amount of PAC-1 binding on a single cell and the number of PAC-1–positive cells increase considerably. At 0.5 MAC sevoflurane, PAC-1 binding and the percentage of positive cells for PAC-1 were greater in comparison with 1 MAC, suggesting a possible dose-dependent inhibition of receptor activation.

It is well-established that platelet adhesion is mediated *via* glycoprotein GPIb receptors through interaction with the von Willebrand factor and that further physiologic activation of platelets *via* intracellular signaling pathways leads not only to an increased expression of the GPIIb/IIIa receptor complex, but also to a conformational change and exposure of the fibrinogen binding site. Subsequent fibrinogen bridging allows firm attachment of adjacent platelets. This process is a prerequisite for platelet aggregation and thrombus formation. Therefore, an inhibition of the GPIIb/IIIa complex results in a prolonged bleeding time (9,10). Because PAC-1 only binds to activated GPIIb/IIIa epitopes, the reduced binding on platelets exposed to sevoflurane *in vitro* could reflect a serious incapacity to generate the fibrinogen binding site and could thus induce platelet aggregation.

These results were confirmed by the prolonged bleeding time in PFA measurements (11,12). Thromboelastography also revealed an incapacity to produce a sufficient clot firmness in sevoflurane-incubated blood. Furthermore, the thromboelastographic patterns resembled the patterns produced by abciximab-modified thromboelastography where the GPIIb/IIIa receptor is selectively blocked by a monoclonal antibody fragment (c7E3 Fab; ReoPro; Lilly, Bad Homburg, Germany), and the resulting maximum amplitude is a function of the fibrinogen concentration (13,14).

Considering the fact that an activated GPIIb/IIIa receptor complex is a prerequisite for primary and secondary platelet aggregation, our findings do not correspond with the studies of Hirakata *et al.* and Nozuchi *et al.* (6) Hirakata *et al.* (2,3) found that sevoflurane inhibited secondary platelet aggregation induced by ADP and epinephrine, and Nozuchi *et al.* (6) reported that sevoflurane did not inhibit aggregation induced by thrombin. In our study, after incubation with sevoflurane, neither a weak agonist, such as ADP, nor a strong agonist,

such as TRAP-6, was able to recruit and activate a sufficient number of GPIIb/IIIa epitopes on the platelet surface required for adequate fibrinogen binding. Therefore, not only secondary but also primary aggregation is impaired by sevoflurane. The different study results may be partly explained by the different methods used. In contrast to the cited studies, we analyzed platelets in whole blood instead of platelet suspensions. We also used sevoflurane as a gas, not as a liquid, and allowed platelets to incubate for 1 h.

Interestingly, not only was fibrinogen binding of platelets impaired by sevoflurane, but the often-described activation-induced redistribution of GPIIb from the surface to the internal membranes of the open canalicular system also failed to occur (15-18). This leads to the conclusion that perhaps more than one inhibitory mechanism is involved. Our results stand in contrast to the results of Froehlich *et al.* (19), who observed a redistribution. Maybe the different incubation times and different concentrations of ADP and TRAP-6 led to the divergent findings.

Nevertheless, [alpha]-granule secretion on activation seems to be unaffected by sevoflurane as determined by binding of a monoclonal antibody to P-selectin. P-selectin is located in the membranes of [alpha] granules and becomes externalized on the platelet surface after platelet activation and granule secretion (15,20). In unstimulated platelets, P-selectin surface expression was even higher in platelets exposed to sevoflurane.

The mechanism by which sevoflurane suppresses platelet response to various stimuli remains unclear. The hypothesis of Hirakata *et al.* (2) that sevoflurane inhibits thromboxane A₂ formation by suppressing cyclooxygenase activity cannot explain the effects of sevoflurane on primary aggregation by inhibiting GPIIb/IIIa expression and activation. The findings of Hirakata *et al.* (2) can possibly be explained by the fact that GPIIb/IIIa mediates the formation of thromboxane A₂, and GPIIb/IIIa receptor blockade impairs the formation of this secondary feedback agonist (21).

A direct inhibitory effect of sevoflurane on the GPIIb/IIIa receptor, as well as an inhibition of intracellular signaling pathways, might be possible, whereas a direct effect on ADP and thrombin surface receptors seems to be improbable because both agonists interact with specific receptor types, including the purinase-activated receptors PAR1 and PAR4 for thrombin and the purinergic receptors P2Y1 and P2T_{AC} for ADP. Although the signaling pathways that deliver messages from these receptors to the GPIIb/IIIa complex have not been completely characterized, it seems likely that a link exists from the G-protein-coupled agonist receptors to the GPIIb/IIIa receptor complex, including phospholipase C[beta], inositol phosphates, and protein kinase C. Therefore, not only a direct inhibitory effect of sevoflurane on the GPIIb/IIIa receptor but also a possible interference with parts of the signaling pathway is imaginable (22,23). Kohro and Yamakage (24) investigated the effect of halothane on platelet function and proposed a decrease in intracellular free Ca²⁺ and production of inositol 1,4,5-triphosphate as the possible inhibitory mechanism. A similar mechanism is imaginable for

the effects of sevoflurane on platelets. However, further studies are necessary to confirm these speculations.

In summary, sevoflurane inhibits agonist-induced GPIIb/IIIa activation and surface expression on platelets in whole blood already in subanesthetic concentrations *in vitro*. The agonist-induced redistribution of GPIIb into the open canalicular system was also impaired by sevoflurane, whereas no effect on P-selectin expression in activated platelets as an indicator of activation dependent [alpha] degranulation could be found. Although Hirakata *et al.* (3) observed an impaired platelet aggregation in patients anesthetized with sevoflurane, and Nathan *et al.* (25) noticed a higher blood loss in patients undergoing gynecologic ambulatory anesthesia with sevoflurane, it must be further evaluated whether our *in vitro* findings have clinical implications.



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Chapter 3

Effect of Halothane and Isoflurane on Binding of ADP- and TRAP-6- activated Platelets to Leukocytes in Whole Blood

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ABSTRACT

Background: Adhesion of activated platelets to neutrophils and monocytes has an important role in the regulation of inflammatory processes. This study investigates whether halothane and isoflurane affect binding of activated platelets to leukocytes in human whole blood.

Methods: Citrated whole blood was incubated for 60 min with either 1 or 2 minimum alveolar concentration (MAC) halothane or isoflurane. After stimulation with adenosine-5-diphosphate (ADP) or the thrombin receptor agonist protein TRAP-6, platelet–leukocyte adhesion and surface expression of CD62P on platelets were evaluated by flow cytometry.

Results: Halothane led to an inhibition of agonist-induced adhesion of activated platelets to neutrophils and monocytes. One MAC halothane reduced the formation of TRAP-6–induced platelet–monocyte conjugates. After exposure to 2 MAC halothane, agonist-induced platelet–monocyte and platelet–neutrophil adhesion were inhibited. Surface expression of CD62P on ADP– and TRAP-6–stimulated platelets were significantly reduced after 1 and 2 MAC halothane. After 2 MAC isoflurane, the authors observed an increase of the percentage of lymphocytes with bound platelets after activation with ADP. The percentage of neutrophils with bound platelets after activation with ADP or TRAP-6 was also increased in this group. Two MAC isoflurane led to an increase of the percentage of platelets expressing CD62P in the unstimulated and TRAP-6 stimulated samples, and of the amount of CD62P epitopes on the surface of platelets in the ADP-stimulated samples.

Conclusion: This study indicates that halothane inhibits, whereas isoflurane enhances, adhesion of agonist-activated platelets to leukocytes. Interaction of both anesthetics with the expression of CD62P on platelets contribute to these effects.

INTRODUCTION

Adhesion of activated platelets to polymorphonuclear neutrophils and monocytes has an important role in the regulation of inflammatory processes and thrombosis. Increased platelet–neutrophil and platelet–monocyte conjugates have been shown in cardiopulmonary bypass (1), myocardial infarction (2), postischemic reperfusion damage (3), thrombosis (4), and sepsis (5,6). An interaction between platelets and leukocytes may link these processes and contribute by intercellular communication pathways to the pathophysiology of these diseases.

It is well-established that activated platelets bind to neutrophils and monocytes *via* an interaction between CD62P on the platelet surface membrane and P-selectin ligand (PSGL-1) on the surface of leukocytes (7,8). Binding of activated platelets to neutrophils induces respiratory burst (9) and mediates initial neutrophil attachment and rolling (10), which may lead to neutrophil accumulation at sites of injury. Binding of activated platelets to monocytes is reported to induce secretion of different proinflammatory chemokines (11,12). These results suggest that the tight interaction among platelets, neutrophils, and monocytes has an important part in the host defense system.

Halothane has been found to affect directly immune-competent cells. For example, during exposure to halothane, the respiratory burst activity of polymorphonuclear neutrophils is significantly reduced (13). Furthermore, halothane is also known to inhibit human platelet aggregation by interaction with Ca^{2+} -dependent platelet activation processes (14). Because halothane is rarely used during clinical situations associated with increased platelet–leukocyte formations, such as cardiopulmonary bypass, we also investigated the effect of isoflurane on platelet–leukocyte interaction.

In the current study, we attempted to clarify whether halothane and isoflurane influence adhesion of activated platelets to leukocytes to gain further insight into the mechanism of anesthetic-induced modulation of immune-competent cells and intercellular communication. Using activation-dependent monoclonal antibodies and two-color flow cytometry, we studied the effect of both anesthetics on platelet–leukocyte adhesion and expression of platelet adhesion membrane receptors in human whole blood.

MATERIALS AND METHODS

In accordance with the human research standards of our institutional ethics committees (University Hospital, Rheinisch-Westfälische Technische Hochschule, Aachen, Germany) and informed consent, blood samples were taken from 38 healthy volunteers (18 women, 20 men) who had no history of smoking or infections and had not ingested nonsteroidal antirheumatics for at least 2 weeks before donation. Venous blood was carefully withdrawn without a



tourniquet from an antecubital vein using a 21-gauge butterfly into blood collection tubes (Sarstedt, Nümbrecht, Germany) containing a 1:10 volume of 3.2% sodium citrate. The first 3 ml of blood was used to perform a hemogram (complete blood count, differential leukocyte count). Blood samples of each donor were immediately diluted 1:1 with 37°C prewarmed Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} (Sigma Chemicals, St. Louis, MO) in sterile polypropylene tissue culture dishes (Sarstedt). In a subset of experiments, one diluted blood sample was processed within 10 min after blood withdrawal for flow cytometric analysis to obtain baseline values. The remaining blood samples were incubated with either 1 or 2 minimum alveolar concentration (MAC) halothane or isoflurane for 60 min. The MAC value used for halothane in this study was 0.8%, and the value for isoflurane was 1.2%. Control samples were placed at the same time point into an incubator (Heraeus BB 16, Hanau, Germany) with an atmosphere of 21% oxygen and 5% carbon dioxide at 37°C. After incubation, blood samples were immediately processed for stimulation procedures and flow cytometric analysis.

For the incubation of the blood samples with halothane or isoflurane, we developed a small box that allows delivery of different volatile anesthetics at low gas flow rates in an atmosphere with 5% carbon dioxide at 37°C. To avoid artificial leukocyte and platelet activation, blood samples were not bubbled with fresh gas throughout the incubation time. Anesthetics were delivered as a volatile–air mixture (fraction of inspired oxygen [Fio_2], 0.21) using a commercially available anesthetic machine (Cato; Dräger, Lübeck, Germany). Carbon dioxide (5%) was directly administered into the box using an external gas bottle. Initial fresh gas flow was 1 l/min, which was reduced to 250 ml/min after equilibration of the atmosphere inside the box. Oxygen, carbon dioxide, and anesthetic gas concentrations within the box were continuously monitored using a multigas analyzer (Datex Compact, Helsinki, Finland).

Flow cytometric analysis

Flow cytometric analysis was performed on a FACSCalibur flow cytometer and analyzed using CellQuest 3.1 software (Becton-Dickinson, San Jose, CA). Before each measurement, the flow cytometer was calibrated with fluorescence microbeads (Calibrite Beads; Becton-Dickinson). Antibodies (Mab) used were as follows: anti-CD45-FITC (clone HI30), Mab for leukocyte common antigen; anti-CD41a-PE (clone HIP8), Mab recognizing the platelet glycoprotein GPIIb/IIIa; anti-CD62P-FITC (clone AK-4), Mab directed against CD62P expressed on platelet surface; and negative IgG₁-FITC and IgG₁-PE antibodies (clone MOPC-21) for nonspecific binding (all from Pharmingen, San Jose, CA).

Stimulation, immunofluorescence staining, and flow cytometric analysis were performed as previously described with minor modifications (2). In brief, to determine the effect of halothane and isoflurane on CD62P expression and binding of activated platelets to leukocytes, blood samples were stimulated with either adenosine-5-diphosphate (ADP, final

concentration 2 μm ; Sigma Chemicals) or the thrombin receptor agonist peptide TRAP-6 (final concentration 6 μm ; Bachem, Heidelberg, Germany) at room temperature. Stimulation was performed in closed Eppendorf tubes to prevent evaporation of the anesthetics. After 5 min, 100 μl unstimulated or stimulated citrated whole blood was added to polypropylene tubes containing saturating concentrations of fluorochrome-conjugated antibodies and then stained for 15 min at room temperature in the dark. The reaction was stopped by adding 2 ml lysing solution (Becton-Dickinson) for 10 min. After centrifugation (5 min, 350 g , 4°C), the samples were washed with 2 ml phosphate buffered saline containing 1% bovine serum albumin and centrifuged, and the remaining pellet was resuspended in 500 μl phosphate buffered saline containing 1% bovine serum albumin and 1% paraformaldehyde. The cells were stored up to 1 h at 4°C until flow cytometric measurements were performed.

Neutrophils, monocytes, and lymphocytes were differentiated by anti-CD45-FITC fluorescence, and cell size and granularity in the forward and side scatter. Platelet adhesion to leukocytes was defined as cell particles positive for CD41a-PE in the leukocyte subgroups. The percentage of leukocytes with bound platelets and the CD41a-PE mean fluorescence intensity of the positive leukocytes were measured. The CD41a-PE mean fluorescence intensity reflects the number of platelets bound per leukocyte (8). For each sample, 40,000 leukocytes were measured.

To determine CD62P expression on the surface of platelets, single platelets were identified by size (forward scatter) and CD41a-PE immunofluorescence in a logarithmic scaled dot plot. Results are expressed as percentage of platelets positive for CD62P and mean fluorescence intensity of CD62P-FITC. The CD62P-FITC mean fluorescence intensity reflects the number of epitopes expressed on the surface membrane of single platelets. For each sample, 10,000 platelets were collected.

Gas chromatography and mass spectrometry

In a subset of experiments, concentrations of halothane and isoflurane were determined in the gas and fluid phases using gas chromatography and mass spectrometry on a HP 6890/MSD 5973 Series instrument (Hewlett-Packard, Wilmington, DE) equipped with a head space injector system (Model 7050; Tekmar-Dohrmann, Cincinnati, OH) as previously described (13). Equilibration between the gas–fluid phase was completed within 15 min for both anesthetics. The following concentrations and diluted blood/gas partition coefficient of halothane and isoflurane were determined for 1 MAC at 37°C: halothane, 0.73 ± 0.05 mm (partition coefficient, 2.01); isoflurane, 0.62 ± 0.04 mm (partition coefficient, 1.15).



Statistical analysis

The Kolmogorov-Smirnov test showed that the flow cytometric data were not normally distributed. Therefore, results are expressed as median (25–75 percentile) unless otherwise indicated. Differences between the anesthetic exposed samples and control samples were tested by the Wilcoxon test. A value of $P < 0.05$ was regarded as significant.

RESULTS

Hemogram

The average hemoglobin concentration of all of the volunteers was 14.0 ± 1.0 g/dl (mean \pm SD), leukocyte count average was $6,200 \pm 1,900/\mu\text{l}$, and platelet count average was $224 \pm 54 \times 10^3/\mu\text{l}$. Differential leukocyte counts were $60.3 \pm 7.5\%$ neutrophils, $27.9 \pm 7.2\%$ lymphocytes, $7.0 \pm 2.9\%$ monocytes, $3.6 \pm 2.0\%$ eosinophils, and $0.8 \pm 0.6\%$ basophils.

Effect of incubation time on platelet activation and platelet–leukocyte adhesion

To exclude artificial activation during the incubation time of the control blood samples, we compared baseline and control values of unstimulated and agonist-induced platelet–leukocyte binding in a subset of experiments. The 60-min treatment in the incubator had no effect on either basal or agonist-induced CD62P expression on platelets, nor was there an increase in leukocytes with bound platelets (data not shown).

Effect of halothane on platelet–leukocyte adhesion

The influence of halothane at 1 and 2 MAC on platelet–leukocyte adhesion is summarized in table 1. Halothane had no effect on binding of unstimulated platelets to the three investigated leukocyte subpopulations. Exposure of blood samples to 1 MAC halothane decreased the percentage of neutrophils and monocytes with bound platelets after stimulation with $6 \mu\text{M}$ TRAP-6 compared with control samples.

In the 2 MAC halothane group, we observed a reduction of the percentage of lymphocytes with bound platelets after activation with ADP. After stimulation with TRAP-6, the amount of bound platelets on lymphocytes was lower in comparison with the control values ($P < 0.05$).

The percentage of neutrophils that were positive for the platelet marker CD41a after activation with either ADP or TRAP-6 was decreased after incubation with 2 MAC halothane.

Furthermore, halothane reduced the number of bound platelets per neutrophil associated with ADP stimulation.

The percentage of monocytes with adherent agonist-activated platelets remained unchanged. However, CD41a mean fluorescence intensity, reflecting the number of adherent platelets on each single monocyte, was reduced significantly after stimulation with ADP and TRAP-6 in comparison with controls.

Table 1: Spontaneous and Agonist-induced Platelet–Leukocyte Adhesion after Exposure to Halothane

	Control (60 min)	1 MAC Halothane (60 min)	Control (60 min)	2 MAC Halothane (60 min)
Platelet–lymphocyte				
% Positive lymphocytes	2.1 (1.8–3.0)	2.2 (1.8–3.2)	2.3 (2.1–2.6)	2.5 (2.2–2.6)
MFI CD41a on lymphocyte	109 (74–117)	97 (81–99)	123 (102–154)	135 (124–151)
Platelet–lymphocyte (2 μ M ADP)				
% Positive lymphocytes	2.5 (2.0–2.7)	2.5 (2.1–2.8)	3.7 (2.7–4.7)	3.2 (2.3–4.0)
MFI CD41a on lymphocyte	283 (220–342)	291 (251–302)	394 (258–532)	244 (189–299)*
Platelet–lymphocyte (6 μ M TRAP-6)				
% Positive lymphocytes	2.5 (2.1–3.5)	2.6 (1.8–3.3)	3.2 (2.9–3.7)	2.5 (2.3–3.1)*
MFI CD41a on lymphocyte	314 (248–562)	258 (187–326)	369 (245–459)	297 (222–400)
Platelet–neutrophil				
% Positive neutrophils	2.3 (1.4–6.1)	2.7 (2.1–6.5)	3.2 (2.7–3.8)	2.5 (2.0–3.3)
MFI CD41a on neutrophil	180 (157–220)	181 (159–207)	144 (124–159)	135 (124–151)
Platelet–neutrophil (2 μ M ADP)				
% Positive neutrophils	10.9 (5.4–16.3)	12.9 (3.9–15.6)	8.6 (5.0–21.2)	6.2 (4.3–12.1)*
MFI CD41a on neutrophil	532 (434–635)	534 (375–638)	625 (406–681)	341 (295–392)*
Platelet–neutrophil (6 μ M TRAP-6)				
% Positive neutrophils	46.5 (27.8–65.1)	36.2 (14.1–48.1)*	17.6 (11.1–42.7)	20.3 (14.9–33.6)
MFI CD41a on neutrophil	1,553 (373–2,126)	1,698 (402–1,962)	842 (612–1,966)	662 (357–949)*
Platelet–monocyte				
% Positive monocytes	6.1 (4.0–12.7)	5.7 (3.7–14.8)	7.0 (4.8–11.0)	6.8 (2.9–9.9)
MFI CD41a on monocyte	222 (189–271)	229 (204–250)	188 (146–228)	158 (139–203)
Platelet–monocyte (2 μ M ADP)				
% Positive monocytes	41.9 (28.9–55.9)	40.1 (34.3–52.9)	30.1 (20.9–38.4)	25.7 (15.5–29.0)
MFI CD41a on monocyte	701 (527–810)	715 (494–773)	545 (424–625)	346 (285–476)*
Platelet–monocyte (6 μ M TRAP-6)				
% Positive monocytes	87.8 (65.0–91.4)	75.1 (51.1–84.8)*	65.3 (47.1–82.7)	54.7 (44.9–74.9)
MFI CD41a on monocyte	2,459 (610–2,977)	1,377 (574–2,516)	1,321 (858–2,154)	723 (424–866)*

Values are presented as percentage of leukocytes with bound platelets and mean fluorescence intensity (MFI) in arbitrary units of CD41a on each leukocyte, representing the number of bound platelets (median [25–75 percentile] of nine independent experiments for each concentration of halothane). * $P < 0.05$ versus control in the absence of halothane. MAC = minimum alveolar concentration

Effect of halothane on platelet surface CD62P expression

The effect of halothane on basal and agonist-induced platelet surface CD62P expression is shown in figure 1. Halothane *per se* had no effect on basal platelet CD62P expression. Both tested halothane concentrations significantly ($P < 0.05$) reduced the percentage of ADP- and

TRAP-6-activated platelets positive for CD62P and the amount of expressed CD62P epitopes (mean fluorescence intensity CP62P) in comparison with controls.

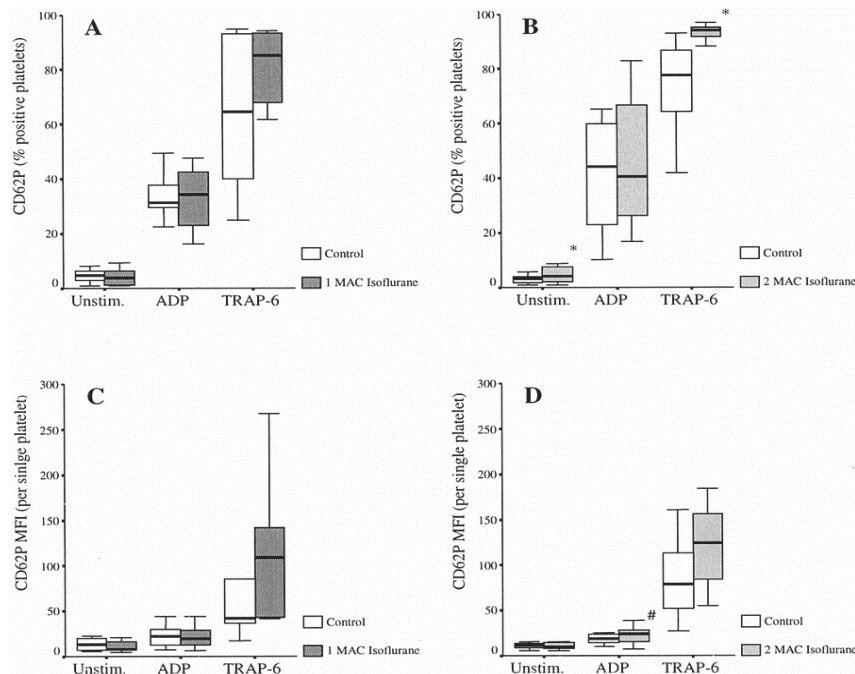


Fig. 1: Dose-dependent effect of halothane on unstimulated and agonist-induced ($2 \mu\text{M}$ ADP; $6 \mu\text{M}$ TRAP-6) expression of CD62P on the platelet surface membrane. Data are presented as percentage of platelets positive for CD62P (A and B) and the mean fluorescence intensity (MFI) of expressed CD62P in arbitrary units (C and D). CD62P MFI represents the amount of CD62P epitopes expressed on the surface membrane per single platelet. Box plots show 25th and 75th percentiles, median, and range of nine independent experiments for each concentration of halothane. * $P < 0.05$ compared with control in the absence of halothane.

Effect of isoflurane on platelet-leukocyte adhesion

The effect of isoflurane on platelet-leukocyte adhesion is summarized in table 2. After exposure to 1 MAC isoflurane, binding of unstimulated and stimulated platelets to leukocytes was not altered in comparison with untreated control samples.

In the 2 MAC isoflurane group, we observed an increase of the percentage of lymphocytes with bound platelets after activation with ADP ($P < 0.01$). Furthermore, the percentage of neutrophils that were positive for the platelet marker CD41a after activation with either ADP or TRAP-6 was significantly increased after incubation with 2 MAC isoflurane ($P < 0.01$). Platelet-monocyte adhesion was not affected after incubation with 2 MAC isoflurane.

Table 2: Spontaneous and Agonist-induced Platelet–Leukocyte Adhesion after Exposure to Isoflurane

	Control (60 min)	1 MAC Isoflurane (60 min)	Control (60 min)	2 MAC Isoflurane (60 min)
Platelet–lymphocyte				
% Positive lymphocytes	4.1 (3.3–4.6)	4.5 (3.1–4.9)	2.8 (2.4–3.2)	2.9 (2.4–3.9)
MFI CD41a on lymphocyte	139 (102–175)	155 (119–193)	148 (116–168)	147 (125–183)
Platelet–lymphocyte (2 μ M ADP)				
% Positive lymphocytes	4.5 (3.7–5.0)	4.5 (4.4–5.2)	3.0 (2.4–3.3)	3.5 (2.6–3.8)*
MFI CD41a on lymphocyte	277 (157–503)	256 (181–458)	246 (219–295)	257 (205–300)
Platelet–lymphocyte (6 μ M TRAP-6)				
% Positive lymphocytes	3.6 (3.2–4.8)	4.5 (3.6–5.0)	3.2 (2.8–3.5)	4.0 (3.1–4.5)†
MFI CD41a on lymphocyte	225 (166–306)	322 (217–392)	233 (176–338)	300 (224–428)
Platelet–neutrophil				
% Positive neutrophils	3.5 (2.5–5.7)	4.2 (3.7–7.1)	3.0 (1.9–4.4)	3.7 (2.9–4.8)
MFI CD41a on neutrophil	209 (171–289)	185 (131–269)	180 (165–231)	172 (136–226)
Platelet–neutrophil (2 μ M ADP)				
% Positive neutrophils	9.4 (6.3–11.3)	10.2 (8.0–16.0)	10.9 (7.6–18.1)	21.0 (14.8–40.9)*
MFI CD41a on neutrophil	598 (321–674)	516 (303–674)	346 (297–493)	501 (383–539)
Platelet–neutrophil (6 μ M TRAP-6)				
% Positive neutrophils	59.0 (20.8–66.7)	62.5 (50.1–73.6)	40.0 (18.4–51.0)	61.9 (42.4–75.0)†
MFI CD41a on neutrophil	764 (451–2,260)	1,124 (743–2,127)	1,156 (500–1,459)	1,065 (674–1,769)
Platelet–monocyte				
% Positive monocytes	7.9 (4.6–16.5)	9.6 (7.4–20.8)	9.7 (4.3–17.7)	11.6 (6.3–19.1)
MFI CD41a on monocyte	248 (160–352)	227 (186–301)	265 (221–396)	220 (181–315)
Platelet–monocyte (2 μ M ADP)				
% Positive monocytes	49.7 (36.4–59.4)	45.2 (36.6–563.9)	48.0 (32.4–74.4)	67.0 (38.8–87.0)
MFI CD41a on monocyte	775 (503–974)	748 (405–861)	628 (453–765)	745 (401–948)
Platelet–monocyte (6 μ M TRAP-6)				
% Positive monocytes	93.1 (76.4–95.0)	95.0 (92.3–96.5)	85.8 (74.5–92.6)	94.2 (90.0–96.0)
MFI CD41a on monocyte	1,225 (743–3,326)	1,842 (1,232–3,617)	915 (797–1,579)	1,317 (1,090–2,122)

Values are presented as percentage of leukocytes with bound platelets and mean fluorescence intensity (MFI) in arbitrary units of CD41a on each leukocyte,

representing the number of bound platelets (median [25–75 percentile] of 10 independent experiments for each concentration of isoflurane.

* $P < 0.01$ versus control in the absence of isoflurane. † $P < 0.05$ versus control.

MAC = minimum alveolar concentration.

Effect of isoflurane on platelet surface CD62P expression

The effect of isoflurane on basal and agonist-induced platelet surface CD62P expression is shown in figure 2. At 2 MAC, isoflurane increased the percentage of platelets positive for CD62P in the unstimulated and TRAP-6-stimulated samples in comparison with control samples ($P < 0.05$). Furthermore, in the ADP-stimulated samples, isoflurane lead to an increase of the CD62P mean fluorescence intensity, reflecting the amount of CD62P epitopes of the surface of platelets ($P < 0.01$).

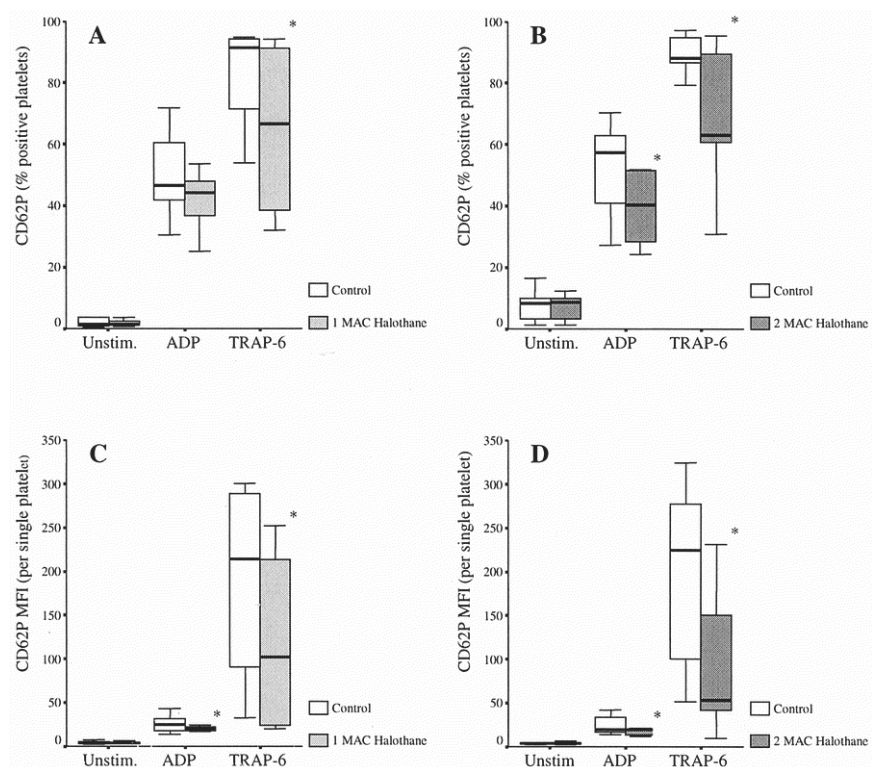


Fig. 2: Dose-dependent effect of isoflurane on unstimulated and agonist-induced (2 μ M ADP; 6 μ M TRAP-6) expression of CD62P on the platelet surface membrane. Data are presented as percentage of platelets positive for CD62P (A and B) and the mean fluorescence intensity (MFI) of expressed CD62P in arbitrary units (C and D). CD62P MFI represents the amount of CD62P epitopes expressed on the surface membrane per single platelet. Box plots show 25th and 75th percentiles, median, and range of 10 independent experiments for each concentration of isoflurane. * $P < 0.05$, # $P < 0.01$ compared with control in the absence of isoflurane.

DISCUSSION

In the current study, we investigated the effect of halothane and isoflurane on adhesion of unstimulated and ADP- or TRAP-6-activated platelets to leukocytes in human whole blood *in vitro*. The major findings are as follows (1). One MAC halothane inhibits the percentage of neutrophils and monocytes with bound platelets after stimulation with TRAP-6 (2). Two MAC halothane reduces binding of ADP- and TRAP-6-activated platelets to lymphocytes, neutrophils, and monocytes (3). Expression of platelet surface CD62P, which has a major role in the mechanism of platelet-leukocyte adhesion, associated with ADP or TRAP-6 stimulation is suppressed by halothane (4). Two MAC isoflurane increases the percentage of neutrophils with bound platelets after stimulation with ADP or TRAP-6 as well as the percentage of lymphocyte-platelet formations in the ADP-stimulated samples (5). After exposure to 2 MAC

isoflurane, the percentage of platelets expressing CD62P is increased after stimulation with TRAP-6, whereas ADP-induced platelet activation results in an enhanced expression of CD62P epitopes on the surface of platelets.

In contrast to previous studies using isolated leukocyte populations or platelet-rich plasma, we used a whole blood system and a previously described two-color flow cytometry assay to study platelet-leukocyte adhesion. The advantage of a whole blood system is that cells are not artificially activated by isolation processes, and cells are studied in an almost-natural environment, with many intercellular mechanisms still intact (15). However, the value of this system is limited by the lack of endothelial cells.

It is well-established that activated platelets bind to leukocytes (8) and modulate their immunologic function (11,12). Furthermore, adhesion of platelets to leukocyte seems not to be an *in vitro* phenomena because several studies showed increased platelet-leukocyte conjugates in cardiopulmonary bypass (1), myocardial infarction (2), postischemic reperfusion damage (3), thrombosis (4), and sepsis (5,6). Therefore, we were interested to evaluate whether halothane or isoflurane may alter platelet-leukocyte adhesion *in vitro*. Halothane is known to inhibit the function of both leukocytes (13,16) and platelets (14), whereas isoflurane has only minor or negligible impact on the function of platelets (17,18). However, in contrast to halothane, isoflurane is commonly used in clinical situations, in which increased platelet-leukocyte adhesion has been reported.

CD62P is a glycoprotein located in the membranes of [alpha] granules, which become externalized on the surface membrane on activation of platelets (19). CD62P has a prominent role in mediating cellular interactions among platelets, leukocytes (20), and endothelial cells (21). Accordingly, after stimulation with either ADP or TRAP-6, activated platelets bind rapidly to monocytes in human whole blood *via* an interaction between CD62P on platelet surface and PSGL-1 on the surface of monocytes (7,8). TRAP-6, as a strong platelet agonist, is more effective in generating platelet-monocyte adhesion and CD62P expression than the weak agonist ADP. In our study, 1 MAC halothane inhibited the percentage of monocytes with bound platelets only after stimulation with TRAP-6, but ADP-induced binding of platelets and monocytes was unaffected. At 2 MAC halothane, platelet-monocyte adhesion was reduced after stimulation with both agonists. Furthermore, our results indicated that the inhibiting effect of halothane on platelet-monocyte adhesion seems to be mediated by a decreased expression of CD62P on activated platelets. The observed lack of effect on ADP-induced platelet-monocyte adhesion after 1 MAC halothane can be explained by the fact that the percentage of platelets expressing CD62P on its surface was not altered. In this group, only the mean fluorescence intensity of CD62P was moderately reduced by 1 MAC halothane, reflecting a lower amount of CD62P epitopes expressed on the surface membrane of platelets. We suggest that the lower amount of expressed CD62P epitopes on the platelet surface membrane had no influence on platelet-monocyte adhesion because the overall number of activated platelets positive for CD62P remained unchanged.



Interestingly, Fröhlich *et al.* (18) reported an upward regulation of CD62P on the surface of unstimulated platelets in the presence of a halothane concentration of 1 MAC or greater, but halothane did not interfere with the platelet response to ADP stimulation. However, the difference between this particular study and the current study could be because Fröhlich *et al.* (18) used platelet-rich plasma, whereas we used human whole blood, to investigate the effect of halothane on platelets. Furthermore, ADP stimulation was performed with a supra-maximal concentration (final concentration 25 μ m) that might have prevented the detection of an inhibitory halothane effect on platelet activation.

Neutrophil respiratory burst and recruitment of neutrophils to sites of inflammation are modulated upon mutual contact with activated platelets (9,10). Although binding between platelets and neutrophils is primarily mediated *via* the CD62P/PSGL-1 adhesion proteins, a reduction in ADP and TRAP-6 induced platelet–neutrophil adhesion was observed in our study only after exposure to 2 MAC halothane. A possible explanation could be that platelet–neutrophil adhesion is partially mediated by a non-CD62P mechanism. Kirchhofer *et al.* (22) demonstrated complete inhibition of platelet–neutrophil adhesion by using a CD62P-blocking antibody in the presence of a GPIIb/IIIa antagonist but only partial inhibition in the absence of a GPIIb/IIIa antagonist. Because platelets can bind fibrinogen *via* the activated GPIIb/IIIa receptor and neutrophils can bind fibrinogen *via* CD11b/CD18 (23,24), it is possible that platelet–neutrophil adhesion also involves a fibrinogen bridging mechanism. However, it remains to be determined whether halothane interacts with fibrinogen binding between platelets and neutrophils.

Isoflurane is known to have no effect on platelet aggregation (17), but an increase in the expression of CD62P on the surface membrane of resting platelets was observed at concentrations of 2 MAC and greater (18). The current study confirms and extends these findings by showing that isoflurane also enhances agonist-induced expression of CD62P. Furthermore, the enhanced ADP- and TRAP-6-induced expression of CD62P after exposure to 2 MAC isoflurane contribute to the observed increase of platelet–lymphocyte and platelet–neutrophil conjugation formation. However, platelet–monocyte adhesion was not altered. Therefore, it remains possible that the enhancing effect of isoflurane on the formation of platelet–lymphocyte and platelet–neutrophil conjugation may partly be mediated by a CD62P/PSGL-1 independent pathway.

Evidence suggest that binding of activated platelets to either monocytes or neutrophils has an important role in the regulation of inflammatory responses. Recently, it was demonstrated that activated platelets induced monocyte cytokine synthesis of interleukin (IL)-1[beta], IL-8, and monocyte chemotactic protein (MCP-1) after adhesion *via* CD62P (11,12). The proinflammatory cytokines IL-1[beta] and IL-8 are important in the pathophysiology of the local and systemic inflammatory response of the host defense. IL-1[beta] triggers a broad range of inflammatory responses, including induction of further cytokines, upregulation of adhesion molecules, activation of T lymphocytes, and respiratory burst and lysosomal

enzyme release by neutrophils (25,26). IL-8 promotes chemotaxis (27), release of neutrophil lysosomal enzymes (28), neutrophil rolling (29), and adherence to endothelial cells (30), as well as transendothelial migration (29,31). MCP-1 enhances monocyte chemotaxis (32). Neutrophils are the first line of defense against bacterial infections by engulfing and digesting bacteria. Interaction between platelets and neutrophils also leads to the induction of neutrophil respiratory burst (9) and recruitment of neutrophils (10) to sites of vascular or inflammatory injury. Reduced or missing respiratory burst activity, as seen in chronic granulomatous disease, leads to repeated and life-threatening infections, such as pneumonia or multiple abscesses in the lungs and liver. Therefore, the ability of halothane to inhibit binding of activated platelets to monocytes and neutrophils, as well as the enhancement of platelet–neutrophil adhesion by isoflurane, might contribute to a disturbance of the inflammatory response to a microbial injury. However, the physiologic inflammatory response consists of an initially proinflammatory phase followed by an antiinflammatory phase, which is necessary to manage infections. Therefore, it is uncertain whether the modulation of the platelet–leukocyte adhesion by halothane and isoflurane may have deleterious or beneficial effects on the perioperative immune function.

Limitations of the Study

In this study, we investigated agonist-induced platelet–leukocyte adhesion in static flow conditions. Studies in more physiologic conditions of shear stress of endothelium could have produced different results. Furthermore, platelets are known to modulate leukocyte function also by soluble mediators, such as CD40L (33) or TGF[β]-1 (34). However, to evaluate the effect of halothane and isoflurane on these platelet-released mediators is beyond the scope of this study.

The model used in this study allows for analysis of the *in vitro* effect of volatile anesthetics on platelet–leukocyte adhesion. The findings indicate that halothane inhibits, whereas isoflurane enhances, adhesion of agonist-activated platelets to leukocytes. The effects seem to be partly mediated by an altered expression of CD62P on the surface of platelets.

The authors thank Kai Gutensohn, M.D. (Department of Transfusion Medicine, University Hospital Eppendorf, Hamburg, Germany), and Wolfgang Lösche, M.D. (Center for Vascular Biology and Medicine, Friedrich-Schiller University, Jena, Germany), for their assistance in planning this study and critical review of the protocol and manuscript.



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A grayscale microscopic image showing a cluster of cells, likely neutrophils, with visible nuclei and granules. The image is positioned at the top of the page, behind the chapter title.

Chapter 4

The Effect of Isoflurane on Neutrophil Selectin and β_2 -Integrin Activation *In Vitro*

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Anesth Analg 2002 95:583-7

ABSTRACT

Background: Isoflurane is reported to reduce ischemia-reperfusion injury. Lower expression of CD11b may be responsible for attenuated postischemic neutrophil adhesion to vascular endothelium. However, neutrophil adhesion to vascular endothelium is a multistep process involving several selectins and β_2 -integrins. Therefore, we assessed whether isoflurane affects the activation of the selectins P-selectin glycoprotein ligand-1 (PSGL-1) and L-selectin and the β_2 -integrins CD11a and CD11b.

Methods: Whole blood was incubated for 60 min with 0.5 or 1 minimum alveolar anesthetic concentration (MAC) isoflurane. After incubation, neutrophils were activated with *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) or phorbol-12-myristate-13-acetate (PMA). Activation of adhesion molecules was evaluated via flow cytometry.

Results: 1 MAC isoflurane reduced the expression of CD11a in the unstimulated samples. After stimulation with FMLP and PMA, shedding of L-selectin was lower in the presence of isoflurane. Furthermore, 1 MAC isoflurane reduced FMLP-induced activation of CD11a and CD11b compared with unexposed blood samples.

Conclusion: These results demonstrate that isoflurane affects the activation of three adhesion molecules involved in the multistep process of neutrophil recruitment. First, isoflurane inhibits the activation of L-selectin, which mediates the neutrophil tethering and rolling on the vascular endothelium. Second, isoflurane attenuates the activation of both β_2 -integrins—CD11a and CD11b—which mediate firm adhesion and transendothelial migration.

INTRODUCTION

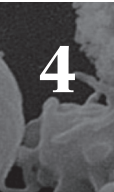
Recruitment of neutrophils through vascular endothelium to inflamed organ tissue is critical for host defense against invading pathogens, but it paradoxically contributes to organ dysfunction in conditions such as ischemia-reperfusion injury. Neutrophil accumulation during ischemia-reperfusion injury begins with neutrophil tethering and rolling, which is mediated by the interaction of the selectins P-selectin glycoprotein ligand-1 (PSGL-1) and L-selectin with their endothelial counterligands. In the next step, tight attachment to endothelium cells involves the neutrophil β_2 -integrin CD11b. Finally, neutrophils transmigrate into the interstitial compartment via the binding of CD11a to endothelial intercellular adhesion molecule (ICAM)-1 (1). Tissue injury occurs because of the release of oxygen free radicals and cytotoxic enzymes and increased cytokine release from activated neutrophils (1–3). Furthermore, microvascular occlusion by platelet-leukocyte aggregates (4) and increased endothelium permeability have also been demonstrated to contribute to ischemia-reperfusion injury (5).

Inhibiting neutrophil rolling and attachment to vascular endothelium as a therapeutic approach is an attractive way to potentially prevent reperfusion injury at a very early stage. In animal models, monoclonal antibodies (MAbs) against adhesion molecules and soluble adhesion molecules have been effective in attenuating ischemia-reperfusion injury (1,6).

Several investigations revealed that anesthesiologists have been using anesthetics with antiadhesive activity regularly in clinical practice for decades. In animal models, isoflurane, sevoflurane, and even halothane protected against myocardial ischemia-reperfusion injury (7–11). One suggested mechanism was the attenuated expression of CD11b on activated neutrophils after exposure to volatile anesthetics (12). However, CD11b is not the sole adhesion molecule involved in the process of neutrophil recruitment. The initial step, tethering and rolling, is primarily mediated by selectins, such as PSGL-1 (13) and L-selectin (14), whereas firm attachment depends on the β_2 -integrins CD11a and CD11b with counterligands on the endothelium (15). Thus, we investigated the effect of isoflurane at 0.5 and 1 minimum alveolar anesthetic concentration (MAC) on the activation of selectins and β_2 -integrins involved in the multistep process of neutrophil recruitment, by using an established whole-blood model. Furthermore, adhesion molecule activation was determined during basal conditions and after stimulation with *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) and phorbol-12-myristate-13-acetate (PMA).

METHODS

In accordance with the approved IRB protocol, venous blood from healthy adult donors (10 men and 6 women) was collected into sterile blood collection tubes (Sarstedt, Nümbrecht, Germany) containing a 1:10 volume of 3.2% sodium citrate. Afterward, citrated blood was



diluted 1:1 with modified Hanks' buffered salt solution (without Ca^{2+} or Mg^{2+} ; Sigma Chemical Co., St. Louis, MO). The MAC value used in this study was 1.2% for isoflurane. Incubation of blood samples was performed as previously described (16). In brief, blood samples were incubated in a small chamber with 21% oxygen and 5% carbon dioxide at 37°C. Isoflurane was delivered with a standard anesthetic machine (Sulla 909; Dräger, Lübeck, Germany), and concentrations of all gases were continuously monitored with a multigas analyzer (Datex Compact; Datex, Helsinki, Finland). Blood samples were exposed to either 0.5 or 1.0 MAC isoflurane for 60 min. Untreated control blood samples were placed in a standard incubator (BB 16; Heraeus, Hanau, Germany) providing identical atmospheric conditions. After the end of the incubation time, all samples were immediately processed for stimulation and staining procedures.

The effect of isoflurane on the expression of neutrophil selectins and β_2 -integrins was investigated with unstimulated blood samples and after activation of neutrophils by using two different stimuli. FMLP is a physiological agonist of the FMLP receptor on the neutrophil cell surface. Activation of the FMLP receptor results in downregulation of PSGL-1 and L-selectin, whereas expression of CD11a and CD11b is increased. In contrast, PMA directly activates protein kinase C (PKC), which also leads to downregulation of the two selectins and upregulation of both β_2 -integrins. Stimulation of blood samples with FMLP (final concentration, 100 nM; Sigma) and PMA (100 nM; Sigma) was performed in sealed polypropylene tubes to avoid evaporation of isoflurane. Blood samples were incubated with the stimulating agent for 10 min at 37°C. Thereafter, 100 μL of blood was added to polystyrene tubes (Falcon; Becton-Dickinson, San Jose, CA) containing fluorochrome-conjugated MABs. Activation of selectins and β_2 -integrins was evaluated by using the following MABs: the PSGL-1 binding MAB KPL-1, the L-selectin binding MAB Dreg 56, the CD11a binding MAB HI111, and the CD11b binding MAB ICRF44 (all from Pharmingen, San Diego, CA). Leukocytes were stained with anti-CD45 (clone HI30), and negative immunoglobulin G_1 -PE (clone MOPC-21) was used as isotype control. Before the beginning of this study, all MABs were titrated by flow cytometry to determine saturating conditions.

Blood cells were stained for 30 min at 4°C in the dark. Staining was stopped by adding 2 mL of lysing solution (FACS Lysing Solution; Becton-Dickinson) for 10 min. After centrifugation (5 min, 350g, 4°C), the samples were washed with 2 mL of phosphate-buffered saline containing 1% bovine serum albumin and centrifuged, and the cell pellet was resuspended in 400 μL of phosphate-buffered saline containing 1% bovine serum albumin and 2% paraformaldehyde.

Blood cells were analyzed on a FACSCalibur flow cytometer (Becton-Dickinson), which was calibrated before each measurement with CaliBRITE beads (Becton-Dickinson). Neutrophils were identified by their scatter characteristics and CD45 staining in the FL1 channel. The data of 20,000 neutrophils were stored in list mode. The activation of PSGL-1, L-selectin, CD11a, and CD11b on the neutrophil cell surface was analyzed by measuring the mean fluorescence intensity (MFI) of the specific MAB in the FL2 channel.

If not indicated otherwise, data are presented as mean and SD. Differences between isoflurane-exposed and untreated control samples assessed in parallel were evaluated with Student's *t*-tests. *P* < 0.05 was considered significant.

RESULTS

One MAC isoflurane reduced the MFI of CD11a in comparison with the unexposed samples. All other measured adhesion molecules were not affected by isoflurane under unstimulated conditions (Tables 1 and 2). The results of the effect of isoflurane on the FMLP-induced activation of neutrophil adhesion molecules are shown in Tables 1 and 2. In the presence of 0.5 MAC isoflurane, FMLP-induced shedding of L-selectin was significantly inhibited, whereas expression of PSGL-1, CD11a, and CD11b was not affected. In the 1 MAC isoflurane group, we observed a reduced shedding of L-selectin and an inhibition of the upregulation of the CD11a and CD11b MFI. As shown in Tables 1 and 2, isoflurane at 1 MAC also inhibited the downregulation of L-selectin from the neutrophil surface after stimulation with PMA, whereas the MFI of the β_2 -integrins was not altered.

Table 1: Effect of 0.5 MAC Isoflurane on the Activation of L-Selectin, PSGL-1, CD11a, and CD11b on Neutrophils *In Vitro*

Variable	Control	0.5 MAC isoflurane
PSGL-1 (unstimulated) [MFI]	422 \pm 86	371 \pm 87
PSGL-1 (FMLP) [MFI]	353 \pm 82	348 \pm 100
PSGL-1 (PMA) [MFI]	376 \pm 94	371 \pm 117
L-selectin (unstimulated) [MFI]	1151 \pm 354	959 \pm 319
L-selectin (FMLP) [MFI]	128 \pm 60	237 \pm 153*
L-selectin (PMA) [MFI]	817 \pm 373	613 \pm 158
CD11a (unstimulated) [MFI]	626 \pm 74	605 \pm 75
CD11a (FMLP) [MFI]	732 \pm 87	737 \pm 65
CD11a (PMA) [MFI]	759 \pm 132	794 \pm 108
CD11b (unstimulated) [MFI]	233 \pm 39	235 \pm 70
CD11b (FMLP) [MFI]	1838 \pm 810	1915 \pm 990
CD11b (PMA) [MFI]	2112 \pm 1111	2296 \pm 1177

Data (arbitrary units) are mean \pm sd of eight independent experiments, * *p* < 0.05 versus control

PSGL-1 = P-selectin glycoprotein ligand-1; MFI = mean fluorescence intensity; FMLP = *N*-formyl-methionyl-leucyl-phenylalanine



Table 2: Effect of 1 MAC Isoflurane on the Activation of L-Selectin, PSGL-1, CD11a, and CD11b on Neutrophils *In Vitro*

Variable	Control	1 MAC Isoflurane
PSGL-1 (unstimulated) [MFI]	508 ± 142	474 ± 115
PSGL-1 (FMLP) [MFI]	383 ± 52	385 ± 49
PSGL-1 (PMA) [MFI]	452 ± 100	413 ± 55
L-selectin (unstimulated) [MFI]	1281 ± 270	1251 ± 217
L-selectin (FMLP) [MFI]	170 ± 88	218 ± 92*
L-selectin (PMA) [MFI]	691 ± 341	961 ± 255*
CD11a (unstimulated) [MFI]	732 ± 52	704 ± 49*
CD11a (FMLP) [MFI]	827 ± 59	803 ± 50*
CD11a (PMA) [MFI]	847 ± 55	785 ± 131
CD11b (unstimulated) [MFI]	285 ± 145	305 ± 80
CD11b (FMLP) [MFI]	2083 ± 432	1794 ± 325*
CD11b (PMA) [MFI]	1775 ± 611	1458 ± 933

Data (arbitrary units) are mean ± sd of eight independent experiments, * $p < 0.05$ versus control

PSGL-1 = P-selectin glycoprotein ligand-1; MFI = mean fluorescence intensity; FMLP = *N*-formyl-methionyl-leucyl-phenylalanine

DISCUSSION

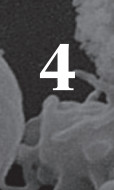
Our results indicate that isoflurane affects the activation of three adhesion molecules involved in the multistep process of neutrophil recruitment. First, isoflurane inhibits the activation of L-selectin, which mediates the neutrophil tethering and rolling on the vascular endothelium. Second, isoflurane attenuates the activation of both β_2 -integrins CD11a and CD11b, which mediate firm adhesion and transendothelial migration.

Evidence from animal models suggests that halothane, isoflurane, and sevoflurane protect the heart against ischemia-reperfusion injury (7–11). Proposed mechanisms are reduced production of hydroxyl radicals (7); activation of myocardial adenosine receptors (8), PKC (9), inhibitory guanine regulatory proteins (17), mitochondrial and sarcolemmal adenosine tri-phosphate-regulated potassium channels (18,19), and stretch-activated channels (20); and inhibition of neutrophil adhesion to endothelial cells (10,11). One investigation revealed that adhesion of neutrophils to endothelial cells may be reduced because of an attenuated upregulation of CD11b, whereas endothelial adhesion molecules were not affected (12). However, binding of neutrophils mediated by CD11b with their counterligands on endothelial cells is preceded by neutrophil tethering and rolling on the vascular endothelium, which is an early step of neutrophil recruitment during the inflammatory reaction. Neutrophil tethering and rolling depends on the interaction of the selectins PSGL-1 and L-selectin with their respective endothelial counterligands in a series of adhesion and de-adhesion events (13–15). Therefore, the aim of this study was to investigate whether isoflurane affects the expression of selectins and β_2 -integrins involved in the multistep process of neutrophil adhesion and migration through endothelial cells, by using an established whole-blood assay.

An important function of L-selectin for the initial contact with endothelial cells has been shown in studies using MAbs (21) and several polysaccharides (22). L-selectin is constitutively expressed on the cell surface of neutrophils, and signal transduction through chemoattractant receptors results in rapid activation of L-selectin by phosphorylation (23), followed by proteolytic cleavage from the neutrophil cell surface (24). Activation of L-selectin increases its binding activity, enabling initial contact with endothelial mucin-like carbohydrate ligands (25). Furthermore, recent studies showed that phosphorylation of L-selectin after chemoattractant activation occurs in conjunction with the dissociation of calmodulin from the cytoplasmic domain of L-selectin before proteolysis from the neutrophil cell surface (26,27). In this study, isoflurane inhibited the chemoattractant-induced downregulation of L-selectin. Spontaneous shedding of L-selectin from unstimulated leukocytes (26), which is mediated by a membrane-associated L-selectin sheddase, could be inhibited by metalloproteinase inhibitors (24). Accordingly, a direct effect of isoflurane on the proteolytic sheddase seems unlikely, because L-selectin expression was not altered on unstimulated neutrophils. Therefore, we suggest that inhibition of the chemo-attractant-induced downregulation of L-selectin by isoflurane might be due to a reduced activation of L-selectin. Because activation of L-selectin enables initial contact to endothelial cells, our findings may provide a further mechanism of the isoflurane-induced inhibition of neutrophil adhesion to endothelial cells in ischemia-reperfusion injury.

The leukocyte β_2 -integrins CD11a and CD11b are involved in numerous aspects of leukocyte function, including tight adhesion to endothelial cells, transmigration phagocytosis, and neutrophil activation (15). Patients lacking these integrins are susceptible to severe infections (28), but excessive activation contributes to sustained inflammation, reperfusion injury, and tissue damage (1,2). The results of this study confirmed those from Möbert et al. (12), who suggested that inhibition of the upregulation of CD11b is one relevant mechanism responsible for the reduced adhesion of neutrophils to endothelial cells. As a possible underlying mechanism, the authors speculated that the volatile anesthetics might have entered the plasma membrane, resulting in membrane expansion and thereby decreasing the upward regulation of CD11b. In this study we used two different chemoattractants to gain further insight into the underlying mechanism of the isoflurane-induced inhibition of CD11b. The ability of PMA, a direct activator of PKC, to induce activation of CD11b not inhibited by isoflurane supports the conclusion that plasma membrane expansion is unlikely to be the underlying mechanism.

Engagement of FMLP at the G protein-linked receptor leads to the activation of phospholipase C and D, generation of inositol triphosphate, activation of PKC, and calcium influx (29). However, signaling events downstream of PKC leading to CD11b activation are only incompletely established but seem to involve the activation of Src family kinases and mitogen-activated protein kinase p38 (30). However, two investigations showed that FMLP-induced activation of CD11b also occurs independently of PKC and mitogen-activated protein kinase



p38 (30,31), suggesting an alternative signaling pathway. Because isoflurane did not affect PMA-induced activation of CD11b, we speculate that isoflurane may alter either the signaling pathway upstream of PKC or the PKC-independent signaling pathway. However, further studies are required to identify the effect of isoflurane on chemoattractant-induced neutrophil signaling pathways.

CD11a is expressed on neutrophils and mediates interactions of neutrophils with the endothelium and transendothelial migration via binding to endothelial ICAM-1 (32,33). After activation with either FMLP or PMA, CD11a is redistributed over the cell membrane, thus forming high-avidity clusters and inducing ligand binding activity (34). In this study, stimulation with either FMLP or PMA resulted in increased CD11a MFI on the neutrophil surface, representing activation of the CD11a ligand binding activity. Because isoflurane reduced the mean CD11a fluorescence intensity in the unstimulated samples and after stimulation with FMLP, it is possible that isoflurane may inhibit the binding of CD11a to endothelial ICAM-1. We suggest that the inhibition of CD11a ligand binding activity might be another reason for the reduced adhesion of neutrophils to endothelial cells in the presence of isoflurane.

In conclusion, the results of this study indicate that the inhibiting effect of isoflurane on neutrophil recruitment may be mediated by a decreased activation of L-selectin and by attenuation of activation of the β_2 -integrins CD11a and CD11b on the neutrophil surface.

Acknowledgments

Supported by START, a research grant of the Rheinisch-Westfälische Technische Hochschule Aachen, Germany.

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Chapter 5

The Effects of Sevoflurane and Desflurane *in vitro* on Platelet–Leukocyte Adhesion in Whole Blood

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ABSTRACT

Background: The interaction between platelets and leukocytes plays an important role in inflammatory and thrombotic processes. We investigated whether the volatile anaesthetics sevoflurane and desflurane alter the formation of platelet–leukocyte aggregates and the expression of P-selectin on platelets.

Methods: Whole blood was incubated with 1 and 2 minimum alveolar concentration (MAC) sevoflurane or desflurane. Unstimulated and adenosine diphosphate, or thrombin receptor agonist peptide-6-stimulated samples were stained with flouochrome-conjugated antibodies. The formation of platelet–leukocyte conjugates and the expression of P-selectin on platelets were measured using flow cytometry.

Results: Sevoflurane was found to enhance the binding of platelets to lymphocytes, neutrophils and monocytes, it also increased the expression of P-selectin on platelets especially in the stimulated samples. Desflurane decreased the percentage of lymphocyte–platelet, neutrophil–platelet and monocyte–platelet conjugates principally in unstimulated samples.

Conclusion: The results show that these two volatile anaesthetics have differing effects on the formation of platelet–leukocyte conjugates *in vitro*. Sevoflurane also enhanced the expression of P-selectin on platelets.

INTRODUCTION

The pathophysiological mechanisms and consequences of platelet–leukocyte interactions and their implications in many diseases have recently been investigated (1–3). Increased association between platelets and leukocytes has been reported in unstable angina (4), myocardial infarction (5), coronary interventions (6), cardiopulmonary bypass (7), thrombosis (8,9) and sepsis (10,11). It especially enhanced P-selectin expression on the platelet surface, which supports the adhesion of platelets to the P-selectin-ligand-1 (PSGL-1) on neutrophils, monocytes and lymphocytes (1,9,12). The binding of platelets to neutrophils seems to induce attachment, rolling and the oxidative burst in neutrophils, as well as cytokine secretion in monocytes (13,14). Upregulation of cellular adhesion receptors and the formation of leukocyte–platelet conjugates may have important implications during and after cardiopulmonary bypass, in the development of an early pro-inflammatory response and a later prothrombotic state. Many patients suffering from the above-mentioned diseases, or undergoing coronary interventions or bypass surgery receive anaesthetics that may modify the immune response. During the peri-operative period a stable host defence system is of great importance. It is therefore important to clarify if and how anaesthetics affect peri-operative immunity (15).

Sevoflurane reportedly inhibits not only the neutrophil oxidative response, but also neutrophil adhesion to endothelial cells (16,17). However, little is known about the effects of sevoflurane or desflurane on spontaneous and induced platelet–leukocyte adhesion. In this study, we investigated the influence of sevoflurane and desflurane on spontaneous and activation-induced platelet–leukocyte adhesion using two-colour flow cytometry.

METHODS

Antibodies and reagents

We used the following antibodies and reagents from BD Pharmingen (San Jose, CA, USA): anti-CD41a-PE (clone HIP8) a monoclonal antibody recognizing platelet GPIIb/IIIa complex independent of activation; anti-CD45-FITC (clone HI30) a monoclonal antibody for the leukocyte common antigen; anti-CD62P-FITC (clone AK-4) a monoclonal antibody directed against P-selectin expressed on platelet surface; negative IgG₁-FITC and IgG₁-PE antibodies (clone MOPC-21), antibodies for non-specific binding, and lysing solution. Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS), bovine serum albumin (BSA), ADP and paraformaldehyde were obtained from Sigma Chemicals (St. Louis, MO, USA). Thrombin receptor agonist peptide-6 (TRAP-6) was purchased from Bachem (Heidelberg, Germany).

Blood collection and incubation

Following local research ethics committee approval and written informed consent, blood samples were taken from 10 healthy volunteers who had not received any medication for at least 2 weeks. Venous blood was collected without the use of a tourniquet from an antecubital vein using a 21-G needle. The first 3 ml of blood was used to perform a blood cell count and then discarded, the next three samples were drawn into polypropylene tubes containing sodium citrate (Sarstedt, Nuembrecht, Germany). Nine parts of blood were anti-coagulated with one part of 3.8% trisodium citrate. All blood samples were immediately diluted 1:1 with 37°C prewarmed PBS and placed in polypropylene tissue culture dishes (Sarstedt).

One diluted blood sample was processed immediately to obtain baseline values. A second sample served as control and was placed in an incubator containing an atmosphere of 21% oxygen and 5% carbon dioxide at 37°C. The third sample was incubated at the same time point with 1 minimum alveolar concentration (MAC) sevoflurane (2 vol.%) or desflurane (6%), respectively, 2 MAC sevoflurane (4 vol.%) or desflurane (12%) for 1h. For the incubation we developed a chamber which allowed the delivery of volatile anaesthetics at low gas flow rates in an atmosphere of 21% oxygen and 5% carbon dioxide at 37°C. Sevoflurane (Abbott, Wiesbaden, Germany) and desflurane were delivered as a volatile/air mixture using an anaesthetic machine (Sulla 808 V-D, Draeger, Luebeck, Germany). Carbon dioxide was administered into the chamber using an external gas connection. Following equilibration of the atmosphere inside the chamber the fresh gas flow was kept constant at 0.25 l min⁻¹ during the experiments. Previous measurements using gas chromatography and mass spectrometry demonstrated that equilibration between the gas fluid-phase was completed within 15 min. Oxygen, carbon dioxide, volatile anaesthetic concentrations and temperature were monitored continuously using a Datex AS/3 anaesthesia monitor including a multigas analyser (Datex Ohmeda, Helsinki, Finland).

Staining procedure and flow cytometric analysis

Blood samples were stimulated with either ADP (2µm) or TRAP-6 (6µm). After 5 min, 100µl of stimulated or unstimulated whole blood was added to saturating concentrations of fluorochrome-conjugated antibodies and stained for 15 min in the dark. The staining procedure was stopped by adding 1.5ml lysing solution. After 10 min the samples were centrifuged (350g, 4°C, 5 min), washed with PBS containing 1% BSA and centrifuged again. The remaining pellet was resuspended in 500µl PBS containing 1% BSA and 1% paraformaldehyde.

Flow cytometric 'two colour' analyses were performed on a FACSCalibur flow cytometer and analysed using cellquest Version 3.1 (Becton Dickinson, San Jose, CA, USA). Prior to each measurement, the flow cytometer was calibrated with fluorescence microbeads (Calibrite Beads, Becton Dickinson).

To determine platelet–leukocyte aggregates, leukocyte subpopulations (neutrophils, monocytes, lymphocytes) were differentiated by cell size (forward scatter), granularity (side scatter) and binding of anti-CD45-FITC using linear scaling. For each sample, 40000 leukocytes were collected. The leukocyte subgroups were separately gated and platelet–leukocyte aggregates were defined as cells found positive for CD41a and CD45 in these subgroups. The percentage of CD41a positive conjugates represents the percentage of leukocytes with at least one bound platelet. In addition, the mean fluorescence intensity (MFI) of CD41a per bound leukocyte was determined. This has previously been shown to correspond in a semiquantitative way to the number of platelets bound per leukocyte (12).

To determine CD62P expression, the platelet population was defined by size and CD41a-PE immunofluorescence using the logarithmic scaling. From each sample, 10000 platelets were measured. The percentage of platelets found positive for CD62P was determined (18).

Statistical analysis

The Kolmogorov–Smirnov test showed that the data were normally distributed. Thus, the results are expressed as mean (SD). Differences between the anaesthetic exposed samples, and the control samples were tested by means of paired *t*-tests (ncss 6.0.7., NCSS, Kaysville, USA). In all cases a *p*-value <0.05 was considered significant.

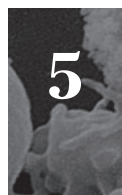
RESULTS

Blood cell count

All measured blood cell counts were found to be within normal limits. The mean (SD) haemoglobin level was 13.9 (0.5) g.dl⁻¹, the leukocyte count was 5.3 (0.6) × 10³.µl⁻¹ and platelet average was 299 (39) × 10³.µl⁻¹. Mean (SD) differential white blood cell counts were: neutrophils 2.8 (0.4)%, lymphocytes 1.8 (0.2)%, monocytes 0.4 (0.1)%, eosinophils 0.18 (0.08)%, basophils 0.05 (0.02)%.

Time-dependent effects on platelet–leukocyte adhesion

In order to measure the effects of incubation time on the control samples, we compared the baseline and control values of unstimulated and agonist induced platelet–leukocyte adhesion. The 1h treatment had no effect on unstimulated or agonist-induced platelet–leukocyte binding and CD62P-expression on platelets (data not shown).



Effect of sevoflurane on platelet–leukocyte adhesion

The effect of 1 and 2 MAC of sevoflurane on platelet–leukocyte binding is summarised in Table 1. Exposure to 1 MAC sevoflurane had no effect on the binding of platelets to leukocytes in unstimulated samples. Samples exposed to 1 MAC sevoflurane showed a significantly ($p<0.05$) higher percentage of platelets bound to neutrophils following stimulation with 6 μ l TRAP-6 (Fig. 1). The number of platelets bound to monocytes was also higher than in the control samples, but this difference did not reach statistical significance.

In samples exposed to 2 MAC sevoflurane we observed significantly ($p<0.05$) more platelet– lymphocyte conjugates in unstimulated and ADP-stimulated blood cells (Fig. 2A). Whereas in the control samples stimulation with ADP or TRAP increased only the CD41a MFI on lymphocytes (reflecting the number of adherent platelets on each single lymphocyte), and not the percentage of lymphocytes with adherent agonist-activated platelets.

The number of platelets bound to neutrophils was significantly ($p<0.05$) elevated following activation with ADP or TRAP-6 (Fig. 2B). Monocyte–platelet adhesion also showed a significant ($p<0.05$) increase following stimulation with TRAP-6 in comparison with control samples (Fig. 2C).

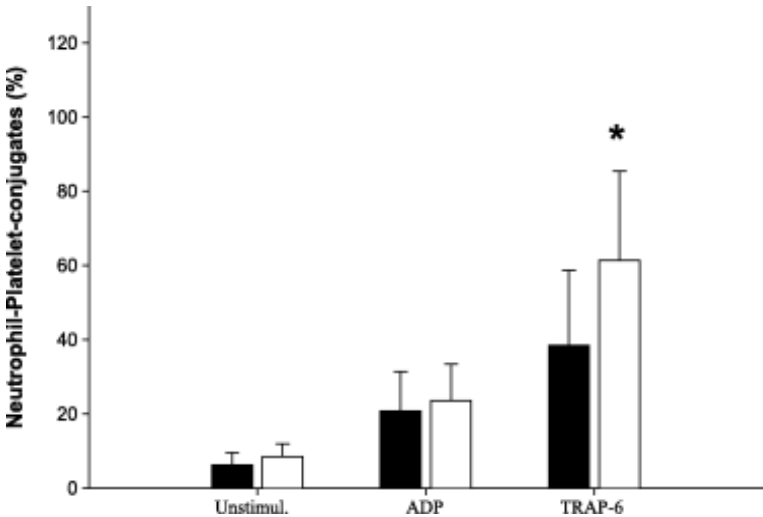


Fig. 1: Effect of 1 MAC sevoflurane on unstimulated and agonist-induced (2 μ m ADP, 6 μ m TRAP-6) formation of neutrophil–platelet conjugates. Filled columns: control samples, empty columns: sevoflurane samples. * $p < 0.05$.

Effect of sevoflurane on P-selectin and CD41a expression on platelets

Following exposure to 1 MAC sevoflurane we observed an increase in the percentage of platelets expressing P-selectin in unstimulated and TRAP-6-activated samples. However, these findings did not reach statistical significance.

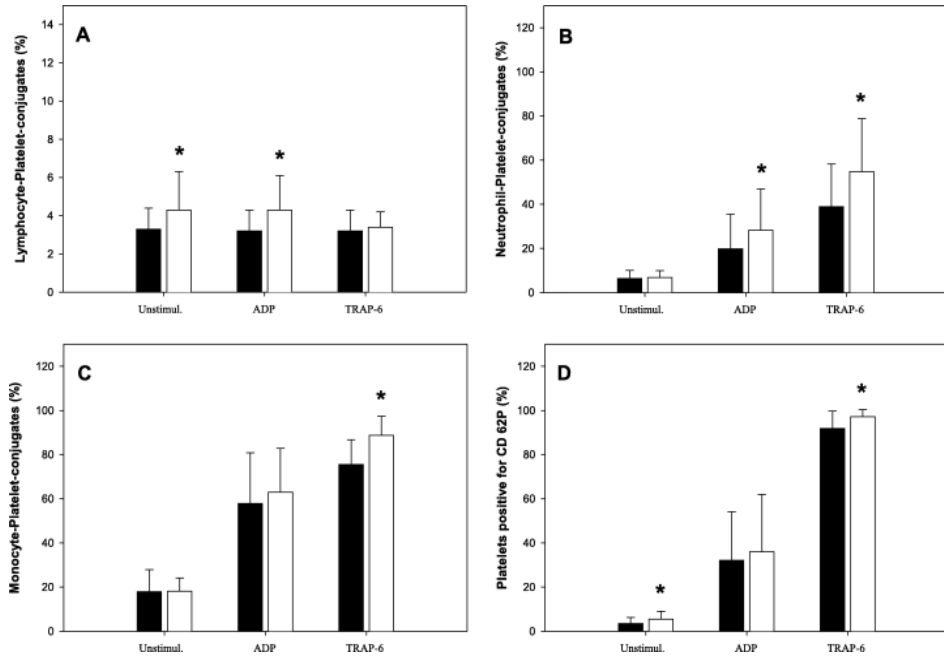


Fig. 2: Unstimulated and agonist-induced (2 μ M ADP, 6 μ M TRAP-6) lymphocyte–platelet conjugate (A), neutrophil–platelet conjugate (B) and monocyte–platelet conjugate (C) formation after 1 h incubation with sevoflurane. (D) Percentage of platelets positive for P-selectin expression under the same conditions. Filled columns: control samples, empty columns: sevoflurane samples. * $p < 0.05$.

In the samples incubated with 2 MAC sevoflurane we also observed an increase in the percentage of platelets expressing P-selectin in unstimulated and TRAP-6 activated samples. In contrast to the result of the incubation with 1 MAC sevoflurane, the above-described finding reached statistical significance (Table 1 and Fig. 2D).

CD41a expression did not show significant differences between control and sevoflurane samples.

Effect of desflurane on platelet–leukocyte adhesion

At 1 MAC, desflurane significantly ($p < 0.05$) decreased the percentage of neutrophils with bound platelets in the unstimulated samples (Fig. 3).

At 2 MAC, desflurane significantly decreased ($p < 0.05$) the number of platelets bound to all three leukocyte subpopulations in unstimulated samples (Fig. 4A–C). Furthermore, the number of platelets bound to monocytes after activation with ADP was reduced in the desflurane group (Fig. 4C).



Table 1: Unstimulated and agonist-induced granulocyte-platelet-aggregation after exposure to 1 MAC and 2 MAC Sevoflurane.

	Control	1 MAC Sevoflurane	Control	2 MAC Sevoflurane
Lymphocyte-platelet-aggregates [%]	3.3 (\pm 1)	3.7 (\pm 2)	3.3 (\pm 1)	4.3 (\pm 2)*
Lymphocyte-platelet-aggregates (2 μ M ADP) [%]	3 (\pm 0.6)	3.2. (\pm 1)	3.2 (\pm 1)	4.3 (\pm 1)*
Lymphocyte-platelet-aggregates (6 μ M TRAP-6) [%]	3 (\pm 0.5)	3.9 (\pm 1)	3.2 (\pm 1)	3.4 (\pm 0.8)
CD41a on lymphocytes [MFI] †	200 (\pm 36)	188 (\pm 90)	152 (\pm 60)	144 (\pm 57)
CD41a on lymphocytes (2 μ M ADP) [MFI]	415 (\pm 148)	341 (\pm 122)	375 (\pm 60)	340 (\pm 81)
CD41a on lymphocytes (6 μ M TRAP-6) [MFI]	371 (\pm 67)	315 (\pm 78)	403 (\pm 100)	357 (\pm 92)
Neutrophil-platelet-aggregates [%]	6.2 (\pm 3)	8.4 (\pm 3)	6.4 (\pm 3)	6.8 (\pm 3)
Neutrophil –platelet-aggregates (2 μ M ADP) [%]	20.8 (\pm 10)	23.5 (\pm 9)	19.8 (\pm 15)	28.3 (\pm 18)*
Neutrophil –platelet-aggregates (6 μ M TRAP-6) [%]	38.4 (\pm 20)	61.4 (\pm 24) *	38.9 (\pm 19)	54.8 (\pm 23)*
CD41a on neutrophils [MFI]	220 (\pm 70)	220 (\pm 73)	197 (\pm 57)	193 (\pm 49)
CD41a on neutrophils (2 μ M ADP) [MFI]	667 (\pm 243)	622 (\pm 189)	727 (\pm 395)	656 (\pm 201)
CD41a on neutrophils (6 μ M TRAP-6) [MFI]	1223 (\pm 629)	1101 (\pm 562)	1413 (\pm 451)	1269 (\pm 417)
Monocyte-platelet-aggregates [%]	23.1 (\pm 12)	26.2 (\pm 12)	17.9 (\pm 10)	18.1(\pm 6)
Monocyte –platelet-aggregates (2 μ M ADP) [%]	68 (\pm 28)	67 (\pm 16)	57.9 (\pm 23)	62.9 (\pm 20)
Monocyte -platelet-aggregates (6 μ M TRAP-6) [%]	85 (\pm 17)	95 (\pm 5)	75.5 (\pm 11)	88.7 (\pm 8)*
CD41a on monocytes [MFI]	282 (\pm 78)	276 (\pm 83)	228 (\pm 64)	223 (\pm 76)
CD41a on monocytes (2 μ M ADP) [MFI]	1400 (\pm 836)	1192 (\pm 435)	887 (\pm 324)	803 (\pm 344)
CD41a on monocytes (6 μ M TRAP-6) [MFI]	2013 (\pm 1029)	1947 (\pm 1076)	1658 (\pm 387)	1660 (\pm 558)
CD62P [% pos. platelets]	5.3 (\pm 2)	6.4 (\pm 3)	3.5 (\pm 2)	5.5 (\pm 3)*
CD62P (2 μ M ADP) [% pos. platelets]	45.3 (\pm 21)	44.7 (\pm 9)	32.1 (\pm 22)	36 (\pm 26)
CD62P (6 μ M TRAP-6) [% pos. platelets]	65.8 (\pm 21)	75.8 (\pm 19)	91.8 (\pm 7)	97.1 (\pm 3)*

Data is expressed as mean and standard deviation. * $p < 0.05$ versus control, †MFI: mean fluorescence intensity

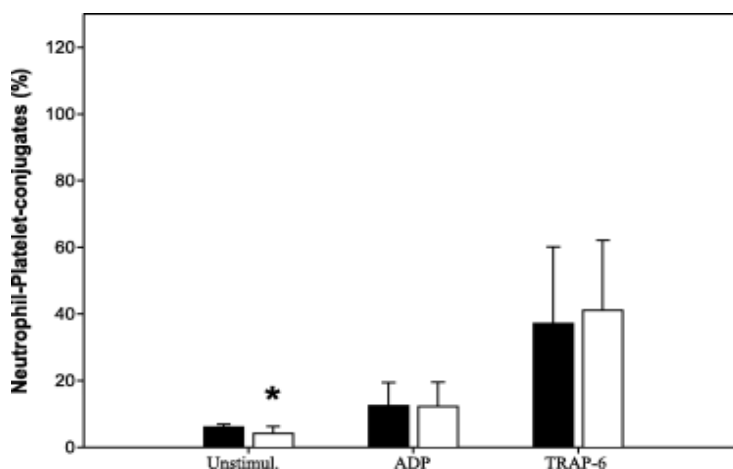


Fig. 3: Effect of 1 MAC desflurane on unstimulated and agonist-induced (2 μ M ADP, 6 μ M TRAP-6) formation of neutrophil–platelet conjugates. Filled columns: control samples, empty columns: desflurane samples. * $p < 0.05$

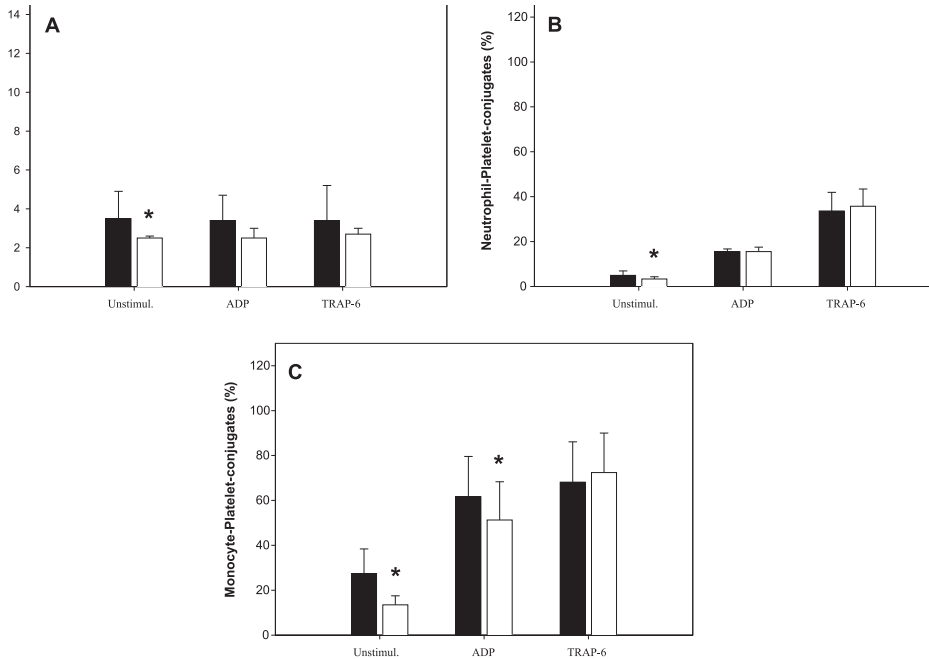


Fig. 4: Unstimulated and agonist-induced (2 μ M ADP, 6 μ M TRAP-6) lymphocyte–platelet conjugate (A), neutrophil–platelet conjugate (B) and monocyte–platelet conjugate (C) formation after 1 h incubation with desflurane. Filled columns: control samples, empty columns: desflurane samples. * $p < 0.05$.

Effect of desflurane on P-selectin and CD41a expression on platelets

Following incubation with either 1 or 2 MAC desflurane the percentage of platelets expressing P-selectin was not altered compared with the control samples.

As already seen in the sevoflurane samples, stimulation with ADP or TRAP increased only the CD41a MFI on lymphocytes, and not the percentage of lymphocytes with adherent agonist-activated platelets in the control samples. Significant differences in CD41a expression were not seen between control and desflurane samples (Table 2).



Table 2: Unstimulated and agonist-induced granulocyte-platelet-aggregation after exposure to 1 MAC and 2 MAC Desflurane.

	Control (60 min. incubation)	1 MAC Desflurane (60 min incubation)	Control (60 min. incubation)	2 MAC Desflurane (60 min incubation)
Lymphocyte-platelet-aggregates [%]	3.58 (±0.8)	3.4 (±1)	3.5 (±1)	2.5 (±0.1)*
Lymphocyte-platelet-aggregates (2µM ADP) [%]	3.9 (±1)	3.1 (±1)	3.4 (±1)	2.5 (±0.5)
Lymphocyte-platelet-aggregates (6µM TRAP-6) [%]	3.4 (±1)	3.1 (±1)	3.4 (±1)	2.7 (±0.3)
CD41a on lymphocytes [MFI] †	148 (±62)	134 (±66)	189 (±95)	153 (±67)
CD41a on lymphocytes (2µM ADP) [MFI]	300 (±117)	271 (±94)	318 (±93)	251 (±68)
CD41a on lymphocytes (6µM TRAP-6) [MFI]	378 (±134)	323 (±88)	355 (±155)	341 (±146)
Neutrophil-platelet-aggregates [%]	6 (±0.9)	4.2 (±2)*	4.9 (±2)	3.3 (±1)*
Neutrophil -platelet-aggregates (2µM ADP) [%]	12.4 (±7)	12.2 (±7)	15.5 (±1)	15.5 (±2)
Neutrophil -platelet-aggregates (6µM TRAP-6) [%]	37.1 (±23)	41.1 (±21)	33.6 (±8)	35.7 (±7)
CD41a on neutrophils [MFI]	160 (±60)	182 (±59)	251 (±98)	234 (±56)
CD41a on neutrophils (2µM ADP) [MFI]	448 (±162)	496 (±207)	474 (±169)	437 (±120)
CD41a on neutrophils (6µM TRAP-6) [MFI]	1262 (±634)	1492 (±412)	999 (±562)	932 (±337)
Monocyte-platelet-aggregates [%]	21.3 (±8)	15.7 (±7)	27.4 (±11)	13.5 (±4)*
Monocyte -platelet-aggregates (2µM ADP) [%]	37.8 (±7)	35.6 (±11)	61.6 (±18)	51.3 (±17)*
Monocyte -platelet-aggregates (6µM TRAP-6) [%]	81.4 (±15)	80.9 (±17)	68.1 (±18)	72.4 (±17)
CD41a on monocytes [MFI]	205 (±72)	206 (±59)	313 (±121)	304 (±63)
CD41a on monocytes (2µM ADP) [MFI]	845 (±339)	833 (±327)	715 (±145)	722 (±258)
CD41a on monocytes (6µM TRAP-6) [MFI]	1867 (±794)	1588 (±212)	1353 (±879)	1332 (±413)
CD62P [% pos. platelets]	5.7 (±3)	5.2 (±2)	5.2 (±2)	5.4 (±2)
CD62P (2µM ADP) [% pos. platelets]	46.3 (±11)	46.1 (±15)	40.9 (±22)	40.7 (±21)
CD62P (6µM TRAP-6) [% pos. platelets]	77 (±21)	80.8 (±19)	118 (±69)	119 (±94)

Data is expressed as mean and standard deviation. * $p < 0.05$ versus control, †MFI: mean fluorescence intensity

DISCUSSION

We investigated the effects of sevoflurane and desflurane on platelet-leukocyte adhesion. Interestingly, the two volatile anaesthetics showed differing effects on the formation of platelet-leukocyte conjugates. Sevoflurane enhanced the binding of platelets to lymphocytes, neutrophils and monocytes and the expression of P-selectin on platelets, particularly in the stimulated samples. Desflurane, however, was found to decrease the percentage of lymphocyte-platelet, neutrophil-platelet and monocyte-platelet conjugates principally in unstimulated samples. With both anaesthetics these effects were more pronounced at the higher (2 MAC) concentration.

Previous studies have shown that activated platelets interact with leukocytes and vascular endothelial cells and modify their immunological function (13,19,20). Important mediators of platelet-leukocyte adhesion are the surface adhesion molecule P-selectin on platelets and the counter-receptor PSGL-1 on leukocytes. P-Selectin is a glycoprotein located in the

membranes of α -granules that becomes externalised on the platelet surface following platelet activation and granule secretion. Therefore, increased expression of platelet surface P-selectin might induce increased platelet adhesion to circulating leukocytes (19–21).

Following incubation with 2 MAC sevoflurane we found significantly elevated P-selectin expression in unstimulated and TRAP-6-activated platelets. This increased P-selectin expression could be the reason for the enhancing effect of sevoflurane on lymphocyte–platelet adhesion in unstimulated blood and the increased amount of monocyte–platelet conjugates and neutrophil–platelet conjugates in TRAP-6-activated blood (22). Even though the increase in platelets expressing P-selectin following stimulation with ADP was not statistically significant, it could account for the elevation of lymphocyte–platelet and monocyte–platelet aggregates. These findings correspond with the results of Fröhlich *et al.* (16) who also observed an upregulation of P-selectin on unstimulated platelets following incubation with sevoflurane. Interestingly, this effect of sevoflurane on P-selectin expression is not limited to platelets but has been observed by other study groups on endothelial cells. Morisaki *et al.* (23) reported increased leukocyte rolling and adhesion in rats undergoing sevoflurane anaesthesia, probably caused by an upregulation of P-selectin expression on endothelial cells.

We found that 2 MAC desflurane inhibited the number of platelet–leukocyte complexes. With the exception of monocyte–platelet adhesion following stimulation with ADP, this effect was observed mainly in the unstimulated samples, so that the impact of desflurane on platelet adhesion might be rather weak, as it was easily overridden by activation. In contrast to sevoflurane, desflurane did not alter the P-selectin expression on platelets. Therefore, it is likely that the inhibitory effect of desflurane is mediated via a non-P-selectin mechanism. Brown *et al.* (24) showed that blocking antibodies to platelet P-selectin partially inhibited adhesion. However, blockade of the neutrophil beta(2) integrin CD11b/CD18 also inhibited the percentage of neutrophils that bound to platelets. This leukocyte–platelet adhesion seems to be mediated by interaction of CD11b/CD18 with fibrinogen bound to GPIIb/IIIa on platelets (25–27). Therefore, it is possible that desflurane interacts with fibrinogen binding between leukocytes and platelets, potentially through modulation of CD11b/CD18 expression on leukocytes. However, it remains to be investigated whether desflurane interacts with leukocyte surface glycoprotein expression, thus inhibiting platelet–leukocyte conjugate formation.

What are the clinical relevance of these findings? It is well known that binding of platelets, especially to neutrophils and monocytes, plays an important role in the regulation of inflammatory processes. Adhesion of platelets can promote leukocyte rolling, arrest and transmigration as well as liberation of cytokines (IL-1 β , IL-18) and the monocyte chemotactic protein (13,14,20).

Pain, stress, necrotic tissue, invading micro-organisms and cardiopulmonary bypass are known modulators of the complex immune response of patients undergoing major surgery. However, anaesthesia and the anaesthetic agents themselves may directly affect the function of immune-competent cells and substantially alter the immune response with a potential

impact on the postoperative course (15,28). However, these actions may only be apparent with high or supraclinical concentrations and/or long-term exposure. There is evidence that long-term sedation with thiopental in neurosurgical patients is associated with infective complications in a dose-dependent manner. At present, no data are available regarding the significance of the observed alterations associated with various anaesthetic procedures in the incidence of postoperative complications associated with an altered immunity.

It is not possible to say whether the observed alterations in our *in vitro* study on leukocyte–platelet complex formation following incubation with sevoflurane or desflurane are associated with postoperative complications related to an altered immunity, as the setting in which the formation of leukocyte–platelet complexes was determined in our study might differ from *in vivo* conditions. Although, in contrast to other studies we used whole blood instead of isolated leukocyte populations – with the advantage that the blood cells are studied in their natural environment with all plasma proteins present and that artificial cell activation caused by the isolation process is avoided.

However, there are some limitations to this study. First, all of the experiments were performed under static conditions without taking into account the effects of blood flow, shear rate or stress. Second, stimulation with ADP or TRAP-6 only mimics part of the changes caused by endothelial injury or inflammation (21). Therefore, it is possible that the observed changes in platelet–leukocyte adhesion found in our study are well tolerated *in vivo* and are without great significance in routine clinical practice. Nevertheless, our study is a first step in the understanding of the effects that volatile anaesthetics may have on the interaction between platelets, leukocytes and cellular immunity. Further work is required to broaden our understanding of these effects, and to examine the exact relevance these may have on clinical practice.

Acknowledgments

This work was supported by START (funds distributed by the scientific board of the Rheinisch-Westfälische Technische Hochschule, Pauwelsstraße 30, 52074 Aachen, Germany).

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A grayscale microscopic image showing a dense field of cells, likely monocytes, with prominent nuclei and some surface irregularities.

Chapter 6

Effect of isoflurane on monocyte adhesion molecule expression in human whole blood

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Acta Anaesthesiol Scand 2003;47:559-563

ABSTRACT

Background: Recruitment of monocytes to inflamed tissue is a crucial step in the acute inflammatory reaction. Adherence of monocytes to endothelial cells followed by transmigration depends on monocyte surface adhesion molecules, inflammatory cytokines and chemoattractant chemokines. In the present study, we determined the effect of isoflurane on monocyte adhesion receptor expression *in vitro*.

Methods: Citrated whole blood was incubated for 60min with either 0.5 or 1 MAC isoflurane. In unstimulated blood samples and after stimulation with N-formyl-methionyl-leucyl-phenylalanine (FMLP) monocyte cell-surface expression of the selectins PSGL-1 and L-selectin, and the β_2 -integrins CD11a and CD11b were evaluated by flow cytometry.

Results: Isoflurane reduced significantly the expression of PSGL-1 on unstimulated monocytes, whereas the remaining selectins and β_2 -integrins were not affected. At both concentrations, the FMLP-induced removal of PSGL-1 from the monocyte surface was increased. Furthermore, at 1 MAC isoflurane the FMLP-induced increase in CD11a expression was significantly inhibited. The surface expression of L-selectin and CD11b was not affected following exposure to isoflurane.

Conclusion: Isoflurane increases the removal of the selectin PSGL-1 from the monocyte surface. Since PSGL-1 is important during the initial step of monocyte adhesion to endothelial P-selectin, the decrease in monocyte surface PSGL-1 may have profound effects on monocyte–endothelial interactions. Furthermore, the effects of isoflurane on monocyte adhesion molecule expression are different from those reported for neutrophils.

INTRODUCTION

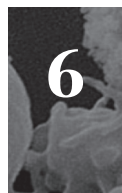
The host defence system relies on both innate and adaptive components. The innate response is largely dependent on monocytes. These cells phagocytose and kill invading bacteria, and co-ordinate the following immunological response by cytokine release as well as antigen presentation. Therefore, recruitment of monocytes to an inflammatory site in response to invading bacteria or noninfectious processes such as trauma or ischaemia-reperfusion injury is a crucial step in the physiology of the acute inflammatory reaction. Adherence of monocytes to endothelial cells followed by transmigration through endothelial cells depends on a network of several events involving monocyte surface adhesion molecules, inflammatory cytokines and chemoattractant chemokines.

Primary adhesion and rolling of monocytes to endothelial cells is mediated by binding of the selectins P-selectin glycoprotein ligand-1 (PSGL-1) and L-selectin with its counterreceptors on the endothelial cell surface (1–3). Subsequent monocyte arrest, tight adhesion, spreading on the endothelium and transmigration to sites of tissue inflammation depends on binding of CD11a and CD11b to endothelial intercellular adhesion molecule 1 (ICAM-1) (1, 4). However, in addition to their important function in monocyte transmigration, selectins (5) and β_2 -integrins (6) also generate outside-in cellular signalling that modulates monocyte functions. Recently it was shown that ligation of CD11b induces the expression of TNF- α , IL-1 β and the macrophage inflammatory proteins (MIP) 1 α and 1 β (6).

In vitro studies have shown that isoflurane alters the monocyte inflammatory response, such as inhibition of endotoxin-induced TNF- α and IL-1 β secretion (7) as well as inhibition of chemotaxis (8). Since adhesion molecule receptors are closely involved in the transmigration of monocytes to sites of tissue inflammation and in the modulation of the monocyte cytokine release, we were interested to examine the effect of isoflurane on monocyte adhesion molecule expression in an established *in vitro* whole blood model. Monocyte selectin and β_2 -integrin expression in unstimulated and FMLP-activated whole blood was measured using two-colour flow cytometry.

MATERIALS AND METHODS

After approval of our institutional review board and informed consent, venous blood was taken from 18 healthy volunteers who had no history of infections and had not ingested nonsteroidal antirheumatics or steroids for at least 10 days prior to donation. Blood was collected into sterile blood collection tubes (Sarstedt, Nümbrecht, Germany) containing a 1/10 volume of 3.2% sodium citrate. Afterwards, the citrated blood was further diluted 1:1 with 37°C prewarmed modified Hanks' buffered salt solution (HBSS; without Ca²⁺ and Mg²⁺, Sigma Chemical, St. Louis, MO).



The isoflurane MAC value used in this study was 1.2 Vol%. Blood samples were exposed to 0.5 or 1 MAC isoflurane for 60 min. Incubation of blood samples was performed as previously described (9). Briefly, blood samples were incubated in a 5-l airtight box with 21 Vol% oxygen and 5 Vol% carbon dioxide at 37°C. To avoid artificial monocyte activation, blood samples were not bubbled with fresh gas. Isoflurane was delivered using a standard anaesthetic machine (Sulla 909, Dräger, Lübeck, Germany), and concentrations of all gases were continuously monitored with a multigas-analyser (Datex Compact, Helsinki, Finland). Untreated control blood samples were placed at the same timepoint in a incubator (Heraeus BB 16, Hanau, Germany) providing identical atmospheric conditions. After the end of the incubation time, all samples were immediately processed for stimulation and staining procedures.

The effect of isoflurane on the expression of selectins and β_2 -integrins on the monocyte membrane surface was investigated with unstimulated blood samples, and after stimulation with FMLP (100 nM, Sigma). Stimulation procedures were performed in sealed polypropylene tubes to avoid evaporation of the anaesthetics. After 10-min incubation at 37°C, 100 μ l of blood was transferred to polystyrene tubes (Falcon, Becton-Dickinson, San Jose, CA) containing fluorochrome-conjugated monoclonal antibodies. Monocyte expression pattern of selectins and β_2 -integrins were evaluated using phycoerythrin (PE)-conjugated antibodies against PSGL-1 (clone KPL-1), L-selectin (clone Dreg 56), CD11a (clone HI111) and CD11b (clone ICRF44, all from Pharmingen, San Diego, CA). Leukocytes were stained with the pan leucocyte marker CD45 (clone HI30, fluorescein isothiocyanate-conjugated) and CD14-PerCP (clone M ϕ P9, Becton-Dickinson), a monoclonal antibody that recognizes specific monocyte surface antigens. Negative IgG₁-PE (clone MOPC-21) was used as isotype control.

The blood samples were stained for 30 min at 4°C in the dark and the reaction was stopped by adding 1 ml of lysing solution (FACS Lysing Solution, Becton-Dickinson) for 10 min. After centrifugation (5 min, 350 \times g, 4°C), the samples were washed twice with PBS containing 1% bovine serum albumin (BSA), centrifuged and the cell pellet resuspended in 500 μ l PBS containing 1% BSA and 2% paraformaldehyde. The cells were stored up to 30 min at 4°C until flow cytometric measurements were performed.

Blood cells were analyzed using a FACSCalibur flow cytometer (Becton-Dickinson), which was calibrated daily prior to each measurement with CaliBRITE beads (Becton-Dickinson) using the FACSComp software program (Becton-Dickinson). Leukocytes were identified and differentiated into subgroups by their cell size and granularity in the forward and side scatter, as well as by their CD45-FITC fluorescence. Monocytes were further differentiated from neutrophils and lymphocytes by their CD14-PerCP fluorescence (Fig.1). The data of 3000 monocytes were stored in list mode. The expression pattern of PSGL-1, L-selectin, CD11a and CD11b were analyzed by measuring the PE mean fluorescence intensity (MFI) of the specific antibody (Fig.1).

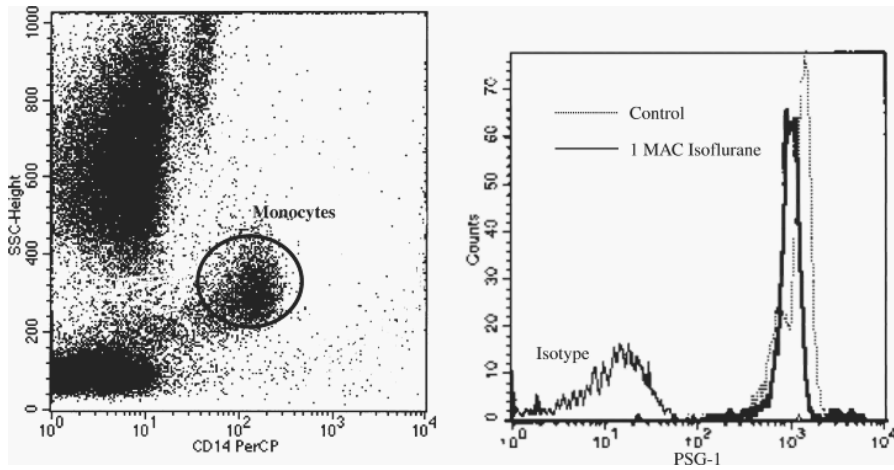


Fig. 1: Adjustment of the acquisition dot plot for analysis of the expression of monocyte adhesion receptor. Monocytes were gated in the sideward scatter (SSC) vs. CD14 PerCP fluorescence (FL3) as acquired on the flow cytometer. Expression of adhesion receptor was detected and quantified with specific phycoerythrin-conjugated antibodies. The effect of 1 MAC isoflurane on the basal expression of P-selectin glycoprotein ligand-1 (PSGL-1) is shown in the overlays of a representative histogram. In the histogram, the PSGL-1 expression of the untreated control blood sample and the isotype is also shown. Incubation with 1 MAC isoflurane reduced the monocyte PSGL-1 expression by 31% in comparison with the control values.

Data are presented as mean and standard deviation. Differences between the anaesthetic-exposed and control samples assessed in parallel were compared using *t*-tests. A value of $P < 0.05$ was regarded as significant.

RESULTS

After incubation with 0.5 or 1.0 MAC isoflurane, expression of PSGL-1 was reduced by 17% in the unstimulated blood samples compared with the control, whereas the remaining selectins and β_2 -integrins were not affected (Table 1, Table 2). In the 1 MAC isoflurane group, changes in the expression pattern of LFA-1 and PSGL-1 were also seen in the FMLP-stimulated samples (Table 2). The FMLP-induced increase in CD11a expression was significantly inhibited only at 1 MAC isoflurane, whereas removal of PSGL-1 from the monocyte surface was increased following exposure to 0.5 or 1 MAC isoflurane. The surface expression of L-selectin and CD11b was not affected following exposure to isoflurane.

Table 1: Effect of 0.5 MAC Isoflurane on the Activation of L-Selectin, PSGL-1, CD11a, and CD11b on Monocytes *In Vitro*

Variable	Control	0.5 MAC Isoflurane
PSGL-1 (unstimulated) [MFI]	793 ± 181	664 ± 171*
PSGL-1 (FMLP) [MFI]	684 ± 159	591 ± 170*
L-selectin (unstimulated) [MFI]	512 ± 169	506 ± 162
L-selectin (FMLP) [MFI]	224 ± 101	212 ± 81
CD11a (unstimulated) [MFI]	1468 ± 131	1407 ± 217
CD11a (FMLP) [MFI]	2185 ± 194	2316 ± 305
CD11b (unstimulated) [MFI]	251 ± 50	303 ± 118
CD11b (FMLP) [MFI]	1310 ± 388	1558 ± 515

Data (arbitrary units) are mean ± sd of eight independent experiments, * $p < 0.05$ versus control, PSGL-1 = P-selectin glycoprotein ligand-1; MFI = mean fluorescence intensity; FMLP = *N*-formyl-methionyl-leucyl-phenylalanine

Table 2: Effect of 1 MAC Isoflurane on the Activation of L-Selectin, PSGL-1, CD11a, and CD11b on Monocytes *In Vitro*

Variable	Control	1 MAC Isoflurane
PSGL-1 (unstimulated) [MFI]	935 ± 187	782 ± 122*
PSGL-1 (FMLP) [MFI]	865 ± 223	718 ± 88*
L-selectin (unstimulated) [MFI]	673 ± 106	714 ± 129
L-selectin (FMLP) [MFI]	313 ± 136	358 ± 139
CD11a (unstimulated) [MFI]	1725 ± 186	1719 ± 161
CD11a (FMLP) [MFI]	2605 ± 264	2482 ± 202*
CD11b (unstimulated) [MFI]	288 ± 67	310 ± 107
CD11b (FMLP) [MFI]	1398 ± 460	1440 ± 405

Data (arbitrary units) are mean ± sd of eight independent experiments, * $p < 0.05$ versus control, PSGL-1 = P-selectin glycoprotein ligand-1; MFI = mean fluorescence intensity; FMLP = *N*-formyl-methionyl-leucyl-phenylalanine

DISCUSSION

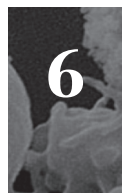
The innate immune response is largely dependent on monocytes, which co-ordinate the immune response in inflamed tissue by cytokine release and antigen presentation. Monocyte recruitment from the blood stream into inflamed or injured tissues is mediated by multistep cascades that require the sequential engagement of monocyte and endothelial adhesion receptors (1–5), including monocyte selectins (PSGL-1, L-selectin) and β_2 -integrins (CD11b, CD11a). Although the primary function of selectins and β_2 -integrins is to promote monocyte extravasation, the selectins PSGL-1 and L-selectin and the β_2 -integrin CD11b are capable of transmitting outside-in signals elicited by ligation of the specific adhesion receptor with its counterreceptor (5,6). This outside-in signalling leads to the activation of other adhesion receptors or induces cytokine and chemokine release, which could have profound effects on further recruitment of other inflammatory cells to sites of inflammation and tissue injury. Isoflurane is known to alter several aspects of leucocyte function. Previously, it has been shown that isoflurane attenuates ischaemia-reperfusion injury (10). One suggested mechanism is a decreased activation of neutrophil L-selectin, CD11a and CD11b (9, 11), which could be responsible for a reduced accumulation of neutrophils at sites of ischaemia-reperfusion in-

jury. However, at present there are only limited information about the effect of isoflurane on monocyte function. Recently, Mitsuhashi et al. reported that isoflurane inhibits the endotoxin-induced TNF- α and IL-1 β secretion (7). Because isoflurane alters the activation of adhesion receptors on neutrophils (9), we investigated the effect of isoflurane on monocyte selectin and β_2 -integrin activation.

In the present study we used an *in vitro* whole blood model and two-colour flow cytometry to clarify if isoflurane alters the expression of monocyte adhesion receptors. Expression of monocyte adhesion receptors was measured in unstimulated cells and following activation with the bacterial peptide FMLP, which is a physiological agonist for the FMLP receptor on the monocyte cell surface. Recently, we have shown that our experimental setting allows rapid equilibration of the isoflurane concentration between the gas and fluid phase (12). However, due to the slightly smaller partition coefficient of isoflurane in diluted whole blood (12), the dissolved amount of isoflurane in our setting is 15% lower compared with fresh whole blood (13).

PSGL-1 is the essential ligand mediating rolling on endothelial cells, which is the first step in transendothelial leucocyte recruitment (2). Activation of leucocytes decreases surface expression of PSGL-1 by an as yet unidentified mechanism and decreases binding to endothelial P-selectin under conditions of flow (14). The data presented in this paper demonstrate that isoflurane reduces surface PSGL-1 expression in unstimulated and FMLP-stimulated monocytes. The underlying mechanism could be a general activation of the monocyte or a direct effect on the removal of PSGL-1 from the cell surface by isoflurane without activating other cell functions. As no increase in the CD11b expression or decrease in surface L-selectin expression was detected in this study, we conclude that direct activation of monocytes by isoflurane, comparable to that following FMLP or platelet activating factor (PAF), is unlikely to be the reason for the removal of PSGL-1 from the monocyte cell surface. *In vitro* studies showed that moderate decreases in PSGL-1 surface expression dramatically reduced binding to immobilized P-selectin under flow conditions (2,14). Since isoflurane reduced surface PSGL-1 expression even in unstimulated monocytes, we suggest that isoflurane may inhibit the initial contact between monocytes and endothelial cells. However, the *in vivo* functional significance of this alteration on monocyte recruitment remains to be determined.

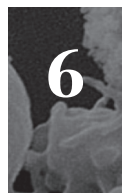
Following leucocyte rolling on endothelial cells, tight adhesion and transmigration is mediated by binding of the β_2 -integrins CD11a and CD11b to endothelial ICAM-1. Recently, Shang et al. (15) investigated the role of both β_2 -integrins on monocyte migration through human umbilical vein endothelial cells (HUVEC). Blocking CD11a or CD11b alone by using monoclonal antibodies did not inhibit monocyte transmigration, but blockade of both integrins partially inhibited monocyte migration across HUVECs. CD11a is not constitutively adhesive and external stimulation with FMLP, cytokines or chemokines is a prerequisite for receptor activation (4). To enable binding of CD11a to ICAM-1, CD11a is redistributed after activation over the monocyte cell surface to form high avidity clusters with ligand binding



activity. In the present study, stimulation with FMLP increased the mean fluorescence intensity of CD11a on the monocyte surface, representing activation of the CD11a ligand binding activity. This activation process was significantly inhibited following exposure of monocytes to 1 MAC isoflurane, but isoflurane had no significant effect on monocyte CD11b expression. Accordingly, we suggest that the inhibition of the activation of CD11a by isoflurane alone might have no impact on monocyte transmigration through endothelial cells. In contrast, we have previously shown that isoflurane inhibits the activation of both β_2 -integrins on neutrophils (9), which could partially explain the inhibiting effect of isoflurane on neutrophil accumulation during ischaemia-reperfusion injury.

In conclusion, this study demonstrated that isoflurane in clinically used concentrations increases the removal of the selectin PSGL-1 from the monocyte surface. Since PSGL-1 is important during the initial step of monocyte adhesion to endothelial P-selectin, the decrease in monocyte surface PSGL-1 may have profound effects on monocyte–endothelial interactions. Furthermore, the effects of isoflurane on monocyte adhesion molecule expression are different from those reported for neutrophils.

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Chapter 7

Epinephrine Enhances Platelet- Neutrophil Adhesion in Whole Blood

In Vitro

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Anesth Analg 2005;100:520-6

ABSTRACT

Background: Previous studies showed that α - or β -adrenoceptor stimulation by catecholamines influenced neutrophil function, cytokine liberation, and platelet aggregability. We investigated whether adrenergic stimulation with epinephrine also alters platelet-neutrophil adhesion. This might be of specific interest in the critically ill, because the increased association of platelets and neutrophils has been shown to be of key importance in inflammation and thrombosis.

Methods: For this purpose, whole blood was incubated with increasing concentrations of epinephrine (10 nM, 100 nM, and 1 μ M). To distinguish receptor-specific effects, a subset of samples was incubated with propranolol (10 μ M) or phentolamine (10 μ M) before exposure to epinephrine. After incubation, another subset of samples was also stimulated with 100 nM of *N*-formyl-methionyl-leucyl-phenylalanine. All samples were stained, and platelet-neutrophil adhesion and CD45, L-selectin, CD11b, P-selectin glycoprotein ligand-1, glycoprotein IIb/IIIa, and P-selectin expression were measured by two-color flow cytometry.

Results: Epinephrine significantly enhanced platelet-neutrophil adhesion and P-selectin and glycoprotein IIb/IIIa expression on platelets. CD11b and L-selectin expression on unstimulated neutrophils remained unchanged, whereas *N*-formyl-methionyl-leucyl-phenylalanine-induced upregulation of CD11b and downregulation of L-selectin were suppressed by epinephrine. β -Adrenergic blockade before incubation with epinephrine increased platelet-neutrophil aggregates and adhesion molecule expression (CD11b, P-selectin, and glycoprotein IIb/IIIa) even further.

Conclusion: These results demonstrate that epinephrine enhances platelet-neutrophil adhesion. The α -adrenergic receptor-mediated increase in P-selectin and glycoprotein IIb/IIIa expression on platelets may contribute substantially to this effect. Our study shows that inotropic support enhances the platelet-neutrophil interaction, which might be crucial for critically ill patients

INTRODUCTION

An increased association of activated platelets with leukocytes contributes to the pathophysiology of unstable angina, myocardial infarction, cardiopulmonary bypass, thrombosis, and sepsis (1–3). There is evidence that cells involved in such heterotypic conjugates perform intercellular communication and facilitate thrombin generation and leukocyte rolling and migration, thus contributing to the course of the pathologic process (4–7). Catecholamine concentrations are increased as an early stress response after cardiac arrest, myocardial infarction, and trauma. Therapeutically, they are used in critically ill patients to treat low cardiac output and severe hypotension. Previous studies have shown that epinephrine modulates the unspecific immune response. It decreases neutrophil adherence, chemotaxis, and phagocytic capacity (8–10). Epinephrine also inhibits tumor necrosis factor (TNF)- α and interleukin (IL)-1 β production but enhances IL-8 and IL-10 production and L-selectin expression in monocytes (11–14). Epinephrine also enhances P-selectin expression in platelets and the opening of glycoprotein (GP)IIb/IIIa binding sites for fibrinogen, and it favors platelet aggregation (15–17).

Little is known about the effects of epinephrine on platelet-neutrophil adhesion at concentrations observed during therapeutic inotropic support or major injury. Knowledge of such effects may have implications not only for understanding endogenous stress hormone influences during injury, but also for the therapeutic use of catecholamines in patients with septic shock or cardiac failure. Considering the above-described changes in leukocyte and platelet function, we hypothesized that epinephrine could enhance platelet-neutrophil conjugate formation because of changes in adhesion molecule expression. Hence, we first studied the effects of epinephrine on platelet-neutrophil adhesion and adhesion molecule expression by using an established whole-blood model and two-color flow cytometry. Because epinephrine exhibits both α - and β -effects and platelets and neutrophils possess adrenoceptors, in a second step we used α - and β -adrenergic receptor-blocking drugs to identify the adrenergic receptors possibly involved in epinephrine-induced immunomodulation.

METHODS

The following were purchased from BD Pharmingen (San Jose, CA): anti-CD41a-phycoerythrin (PE; clone HIP8) monoclonal antibody (mAb) recognizing platelet GPIIb/IIIa complex; anti-CD62P-fluorescein isothiocyanate (FITC; clone AK-4) mAb directed against P-selectin expressed on platelet surface; anti-CD45-FITC (clone HI30) mAb for leukocyte common antigen; anti-CD62L-PE (clone Dreg 56) L-selectin-binding mAb; anti-CD11b-PE (clone ICRF44) CD11b-binding mAb; anti-CD162 (clone KPL-1) mAb recognizing P-selectin GP ligand-1 (PSGL-1); anti-negative immunoglobulin G1-FITC and immunoglobulin G1-PE antibodies (clone MOPC-21); antibodies for nonspecific binding; and FACSlising solution. Dulbecco's



phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} , bovine serum albumin (BSA), epinephrine, paraformaldehyde, and *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) were obtained from Sigma Chemicals (St. Louis, MO). FMLP is a physiological agonist of the FMLP receptor on the neutrophil cell surface. Activation of the FMLP receptor results in downregulation of PSGL-1 and L-selectin, whereas CD11b expression is increased.

After we obtained informed written consent from subjects and approval from the local ethics committee, blood samples were taken from 10 healthy volunteers who had not received any medication for at least 2 wk. Venous blood was carefully collected without a tourniquet from a cubital vein by using a 20-gauge butterfly needle. The first 3 mL of blood was used to perform a hemogram and was then discarded; the next samples were drawn into polypropylene tubes containing sodium citrate. Nine parts of blood were anticoagulated with one part of 3.8% trisodium citrate. All blood samples were immediately diluted 1:1 with 37°C prewarmed PBS, placed in sterile polypropylene tissue culture dishes (Sarstedt, Nuernbrecht, Germany), and incubated with 10 nM/L, 100 nM/L, or 1 μM /L (final concentrations) epinephrine. These concentrations approximately represent, respectively, a small therapeutic and a large therapeutic dose and a rather supramaximal concentration, although such a concentration might be achieved in case of cardiopulmonary resuscitation. For the experiments with antiadrenergic drugs, the samples were incubated with propranolol (10 μM) or phentolamine (10 μM) before exposure to epinephrine. The tubes were gently mixed and placed in an incubator for 15 min.

Stimulation, immunofluorescence staining, and flow cytometric analysis were performed as previously described with minor modifications (1). After incubation, a subset of blood samples were stimulated with FMLP (final concentration, 100 nM). After 10 min, 100 μL of stimulated or unstimulated whole blood was added to saturating concentrations of fluorochrome-conjugated antibodies and stained for 15 min in the dark. The staining procedure was stopped by adding 1.5 mL of lysing solution for 10 min. The samples were then centrifuged (350g at 4°C for 5 min), washed with PBS containing 1% BSA, and centrifuged again. The remaining pellet was resuspended in 500 μL of PBS containing 1% BSA and 1% paraformaldehyde. Flow cytometric “two color” analyses were performed on a FACSCalibur flow cytometer and analyzed with CellQuest 3.1 software (Becton Dickinson, San Jose, CA). Before each measurement, the flow cytometer was calibrated with fluorescence microbeads (Calibrite Beads; Becton Dickinson).

To determine platelet-leukocyte aggregates, the leukocyte subpopulations were differentiated by cell size (forward scatter), granularity (side scatter), and binding of anti-CD45-FITC by using linear scaling. For each sample, 40,000 leukocytes were collected. The leukocyte subgroups were separately gated, and platelet-leukocyte aggregates were defined as cells positive for CD41a and CD45 in these subgroups. The percentage of CD41a-positive conjugates represents the percentage of leukocytes with at least one bound platelet (18).

After incubation, a subset of blood samples was stimulated with FMLP (final concentration, 100 nM), washed, and stained as described above. To determine adhesion molecule expression, the leukocyte subpopulations were differentiated by cell size (forward scatter), granularity (side scatter), and binding of anti-CD45-FITC by using linear scaling. For each sample, 40,000 leukocytes were collected. The leukocyte subgroups were separately gated, and the expression of adhesion molecules was measured as mean fluorescence intensity of the specific antibody on neutrophils.

To determine P-selectin and CD41a expression, the platelet population was adjusted to $20 \times 10^9/L$ before the staining procedure and was defined in flow cytometry by using size and in CD41a-PE immunofluorescence by using logarithmic scaling. For each sample, 10,000 platelets were measured. The percentage of platelets positive for P-selectin and the mean fluorescence intensity of P-selectin and CD41a were measured (19).

The Kolmogorov-Smirnov test showed that the data were mainly normally distributed. Thus, data are presented as means and sd. Differences between the control samples and the samples exposed to increasing concentrations of epinephrine were evaluated with Student's *t*-test (NCSS 6.0.7; NCSS, Kaysville, UT). $P < 0.05$ was considered significant.

RESULTS

Almost all concentrations of epinephrine significantly enhanced the binding of platelets to neutrophils in unstimulated and FMLP-stimulated whole blood (Fig. 1). The enhancing effect of epinephrine on neutrophil-platelet conjugate formation was markedly increased by β -adrenergic blockade. Interestingly, α -adrenergic blockade also led to a small increase in epinephrine-induced platelet-neutrophil adhesion (Fig. 2).

Epinephrine did not modify CD11b or L-selectin expression on unstimulated neutrophils. Nevertheless, after β -adrenergic blockade, the incubation with epinephrine caused a significant increase in CD11b expression on unstimulated neutrophils. α -Adrenergic blockade also caused a small increase in CD11b expression (Fig. 2). In FMLP-stimulated neutrophils, epinephrine inhibited the FMLP-induced increase in CD11b expression. This inhibition was completely reversed by β -adrenergic blockade but not by α -adrenergic blockade (Table 1).

L-selectin expression in unstimulated blood was not modified by epinephrine. FMLP-stimulated neutrophils showed an increased expression or reduced shedding of L-selectin after incubation with epinephrine. Preincubation with propranolol abolished this effect almost completely, whereas phentolamine caused only a partial decline in L-selectin expression (Table 1). PSGL-1 did not show any significant changes in surface expression (Table 1).



Epinephrine enhanced the expression of P-selectin and GPIIb/IIIa on unstimulated platelets. α -Adrenergic blockade with phentolamine before incubation with epinephrine abolished this increase almost completely, whereas β -adrenergic blockade caused a marked increase in both P-selectin and GPIIb/IIIa expression (Table 1, Fig. 3).

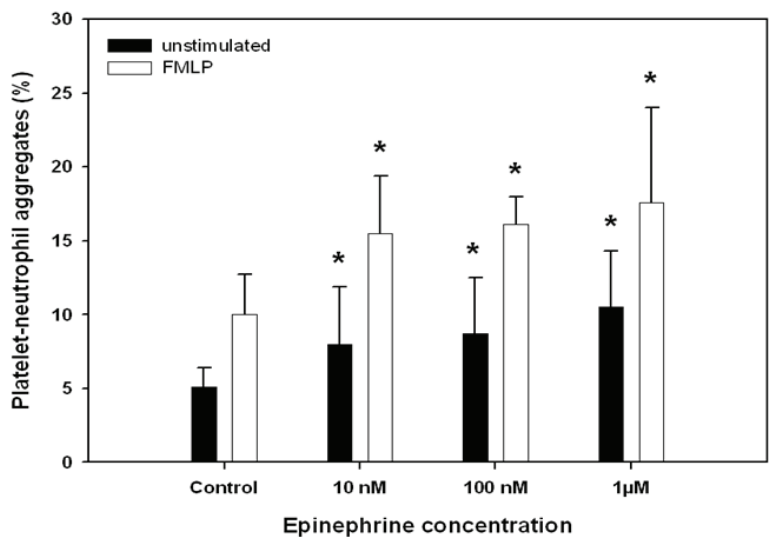


Fig. 1: Percentage of platelet-neutrophil conjugates in unstimulated and N-formyl-methionyl-leucyl-phenylalanine (FMLP)-stimulated (100 nM) whole blood after incubation with increasing concentrations of epinephrine (10 nM, 100 nM and 1 μ M). Mean and sd are given. *Significantly different ($P < 0.05$) from control.

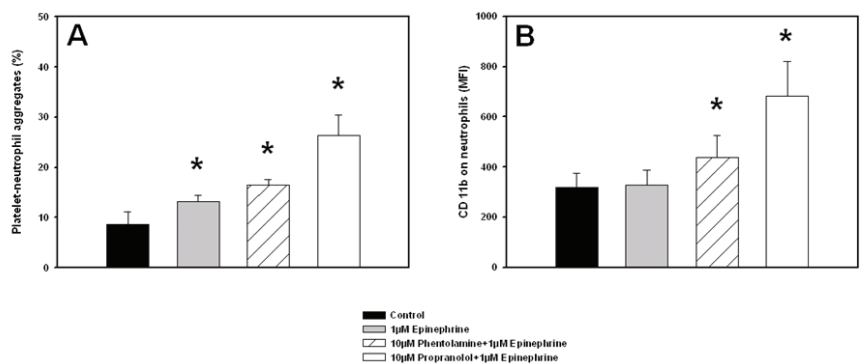


Fig. 2: Effect of epinephrine on platelet-neutrophil conjugate formation (A) and CD11b expression (B) after α - or β -adrenergic blockade with phentolamine or propranolol. Data are shown as mean and sd. *Significantly different ($P < 0.05$) from control

Table 1: Adhesion Molecule Expression on Platelets and Neutrophils

Variable	Control	EPI 10 nM	EPI 100 nM	EPI 1 μ M
<i>Platelets</i>				
P-selectin [% positive platelets]	2.6 \pm 1	4 \pm 1*	4 \pm 1*	6.5 \pm 1.5*
CD41a (GPIIb/IIIa) [MFI]	271 \pm 27	312 \pm 29*	336 \pm 31*	404 \pm 45*
<i>Neutrophils</i>				
CD11b [MFI]	412 \pm 54	437 \pm 33	435 \pm 31	426 \pm 58
CD11b (100 nM FMLP) [MFI]	4317 \pm 357	4023 \pm 330	3722 \pm 187*	3569 \pm 252*
L-selectin [MFI]	1711 \pm 299	1720 \pm 330	1721 \pm 255	1702 \pm 277
L-selectin (100 nM FMLP) [MFI]	310 \pm 49	367 \pm 58*	377 \pm 53*	447 \pm 59*
PSGL-1 [MFI]	559 \pm 32	554 \pm 27	578 \pm 28	589 \pm 39
PSGL-1 (100 nM FMLP) [MFI]	539 \pm 43	505 \pm 31	529 \pm 41	504 \pm 34

Data are expressed as mean and sd, * Significantly different ($P < 0.05$) from control.

MFI = mean fluorescence intensity; FMLP = *N*-formyl-methionyl-leucyl-phenylalanine; EPI = epinephrine; PA = phentolamine; PP = propranolol; PSGL-1 = P-selectin glycoprotein ligand-1; GP = glycoprotein.

Table 1a: Adhesion Molecule Expression on Neutrophils

Variable	Control	EPI 1 μ M	EPI 1 μ M and PA 10 μ M	EPI 1 μ M and PP 10 μ M
<i>Neutrophils</i>				
CD11b (100 nM FMLP) [MFI]	3206 \pm 457	2132 \pm 380*	2725 \pm 220*	3326 \pm 457
L-selectin (100 nM FMLP) [MFI]	321 \pm 74	526 \pm 103*	443 \pm 88*	347 \pm 101

Data are expressed as mean and sd, * Significantly different ($P < 0.05$) from control.

MFI = mean fluorescence intensity; FMLP = *N*-formyl-methionyl-leucyl-phenylalanine; EPI = epinephrine; PA = phentolamine; PP = propranolol; PSGL-1 = P-selectin glycoprotein ligand-1; GP = glycoprotein.

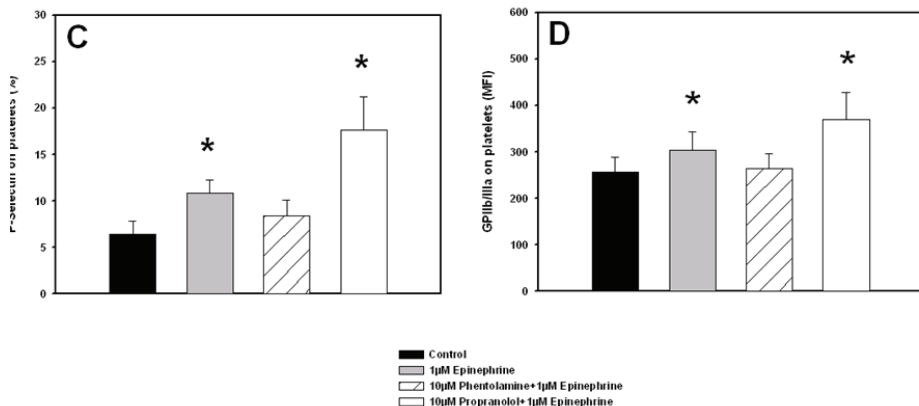


Fig. 3: Effect of epinephrine on P-selectin (A) and glycoprotein (GP)IIb/IIIa expression (B) on platelets after α - or β -adrenergic blockade with phentolamine or propranolol. Data are expressed as mean and sd. *Significantly different ($P < 0.05$) from control.

Because α -adrenergic blockade with phentolamine preceding incubation with epinephrine also led to a small increase in platelet-neutrophil adhesion and CD11b expression, we added some measurements with phentolamine only. Interestingly, phentolamine alone also enhanced CD11b expression and, concomitantly, platelet-neutrophil aggregate formation, whereas all other platelet and neutrophil adhesion molecules remained unchanged (Table 2). The other measurements with epinephrine alone and epinephrine plus phentolamine corresponded to the results described above.

Table 2: Platelet-Neutrophil Conjugate Formation and Adhesion Molecule Expression After Incubation with Epinephrine Phentolamine, or Both

Variable	Control	EPI 1 μ M	EPI 1 μ M and PA 10 μ M	PA 10 μ M
Conjugates [%]	3 \pm 0.1	7.8 \pm 2.7 *	9 \pm 2.6 *	4.8 \pm 1 *
<i>Platelets</i>				
P-selectin (% positive platelets)	3.9 \pm 1.1	6.6 \pm 1.6 *	5.0 \pm 0.5	4.2 \pm 0.5
CD41a (GPIIb/IIIa) [MFI]	298 \pm 10	345 \pm 54 *	292 \pm 41	288 \pm 19
<i>Neutrophils</i>				
CD11b [MFI]	364 \pm 52	368 \pm 65	659 \pm 76 *	435 \pm 31*
L-selectin [MFI]	1817 \pm 267	1868 \pm 277	1829 \pm 290	1847 \pm 301

Data are expressed as mean and sd, * Significantly different ($P < 0.05$) from control. MFI = mean fluorescence intensity; EPI = epinephrine; PA = phentolamine

DISCUSSION

This study demonstrates that platelet-neutrophil conjugate formation is enhanced by epinephrine. This increased adhesion was accompanied by an altered adhesion molecule pattern and was modified by α - and β -adrenergic blockade.

Several *in vitro* and *in vivo* studies suggest that adrenergic receptor stimulation on immune cells can substantially alter a variety of cellular activities, as well as the release of inflammatory mediators. Epinephrine increases neutrophil recruitment into peripheral blood by an α -adrenergic stimulus. There is also evidence that epinephrine decreases neutrophil adherence, chemotaxis, and phagocytic capacity (8–10). β -Adrenergic agonists inhibit the production of proinflammatory mediators such as TNF- α , IL-1, and IL-12, but they augment L-selectin expression and the release of the antiinflammatory substances IL-10 and IL-6. Stimulation of α_2 -adrenoceptors increases the release of TNF- α and IL-1 β , whereas inhibition of α_2 -adrenoceptors enhances the release of the antiinflammatory molecules IL-6 and IL-10 and suppresses the production of TNF- α and IL-12 (20–22). In platelets, it has also been shown that epinephrine can potentiate platelet activation and aggregation by activating platelet α_2 -adrenoceptors (17).

On the basis of these findings, we hypothesized that epinephrine also influences the regulation of the cell-to-cell interaction between platelets and neutrophils. We first evaluated

the effect of epinephrine on the formation of platelet-neutrophil aggregates and found that increasing concentrations of epinephrine enhanced the binding of platelets to neutrophils. This increase in platelet-neutrophil aggregation was accompanied by a significant increase in P-selectin and GPIIb/IIIa expression on platelets. P-selectin is a GP located in the membranes of α -granules and becomes externalized on the platelet surface after platelet activation and granule secretion. Platelets and leukocytes may form aggregates via platelet-expressed P-selectin and its counterreceptors PSGL-1 and Sialyl Lewis X, as well as via fibrinogen bridging between GPIIb/IIIa and CD11b (23,24). The initial interactions between neutrophils and platelets are probably mediated by P-selectin, whereas both 1) development of firm adhesion after initial tethering and rolling on P-selectin and 2) transplatelet emigration to chemoattractants seem to be entirely dependent on CD11b (6). Activation of platelets typically enhances P-selectin and GPIIb/IIIa expression, so epinephrine-induced platelet activation could account for the increased formation of conjugates. This could explain our finding that after stimulation with FMLP, epinephrine inhibited the CD11b upregulation but not the formation of neutrophil-platelet aggregates. Because previous studies have shown that platelets are activated via α_2 -adrenergic stimulation, we suppose that epinephrine caused the increased adhesion molecule expression via α -adrenergic stimulation as well. This is consistent with our finding that α -adrenergic blockade with phentolamine before incubation with epinephrine almost completely reversed the observed upregulation of P-selectin and GPIIb/IIIa in platelets.

In neutrophils, the effects of epinephrine after α - or β -adrenergic blockade were more complex. β -Adrenergic blockade with propranolol before incubation with epinephrine noticeably increased platelet-neutrophil adhesion and CD11b expression. The markedly increased platelet-neutrophil aggregates after β -adrenergic blockade are probably caused not by the enhanced platelet adhesion molecule expression alone, but also by the CD11b expression on neutrophils. Because β -adrenergic stimulation—in contrast to α -adrenergic stimulation—suppresses leukocyte function, this enhancement may be a consequence of abolition of β -receptor-mediated suppression of leukocyte function and increased α -receptor-mediated stimulation. Interestingly, α -adrenergic blockade with phentolamine preceding incubation with epinephrine also led to a small increase in platelet-neutrophil adhesion and CD11b expression. Because this was in contrast to our other results and to the reported suppressing effects of α -blockade on leukocyte function, we added some measurements incubating whole blood with phentolamine only. We found the same increase in platelet-neutrophil conjugates and CD11b, whereas all other variables (L-selectin, P-selectin, and GPIIb/IIIa) remained unchanged. Therefore, it seems possible that phentolamine activated neutrophil CD11b expression by a mechanism independent of its ability to antagonize α -adrenergic receptors. The increased CD11b expression accompanying neutrophil activation could account for the increased platelet-neutrophil adhesion after phentolamine. However, further



research is needed on phentolamine's effects and the involvement of α -adrenergic receptors in neutrophil integrin expression.

The different immunomodulatory effects of α - or β -adrenergic stimulation are probably due to activation of different intracellular pathways. On the molecular level, α -adrenergic stimulation most likely results in an activation of nuclear factor- κ B through activation of protein kinase C and increased intracellular Ca^{2+} , whereas β -adrenergic stimulation leads to an increase of cyclic adenosine monophosphate, which activates protein kinase A. Activated protein kinase A is translocated to the nucleus and blocks nuclear factor- κ B while activating the cyclic adenosine monophosphate-responsive element-binding protein. Therefore, α - or β -adrenergic stimulation can have markedly different downstream effects (25–28).

Our study showed that epinephrine enhanced platelet-neutrophil adhesion, probably through α -adrenergic stimulation of both cell types. Considering the proinflammatory potential of platelet-neutrophil aggregates, our study supports previous studies, which could show that ligation to the α -adrenergic receptor is associated with predominantly immunostimulating effects, whereas stimulation of the β -adrenergic receptor mostly has immunosuppressive effects (29,30). Whereas in the case of cytokine liberation, the β -adrenoceptor-mediated effects usually override those induced by α -adrenoceptor stimulation, it seems that in platelet-neutrophil conjugation, the enhancing α -adrenergic effects—predominantly on platelets—outweighed the β -adrenergic effects. The adhesion between platelets and neutrophils is a key event in thrombosis and inflammation (31). Binding of activated platelets to neutrophils induces respiratory burst and mediates initial neutrophil attachment and rolling, which may lead to neutrophil accumulation at sites of injury (32,33). Therefore, enhanced adhesion after the administration of epinephrine could be crucial for patients with myocardial infarction, trauma, or sepsis. Gawaz et al. (3) also showed that in septic patients, platelet-neutrophil adhesion was an independent predictor for poor clinical outcome. We studied epinephrine concentrations ranging from small therapeutic to supramaximal concentrations, and although the largest amount of conjugates was observed after stimulation with the rather supramaximal epinephrine concentration, it should be noticed that even smaller therapeutic concentrations significantly enhanced platelet-neutrophil adhesion. Nevertheless, the clinical aspects and therapeutic consequences of the enhancing effects of epinephrine on platelet-neutrophil adhesion and adhesion molecule expression in our study remain speculative. First, the therapeutic use of epinephrine is normally required by the hemodynamic state of the patients, which often does not leave much choice for therapeutic alternatives. Second, considering the complex immunomodulatory effects of α - or β -adrenergic stimulation, there are still no data available indicating potential beneficial or detrimental consequences at different stages of disease.

Finally, there are limitations to our study. In contrast to previous studies, we used whole blood instead of isolated neutrophils or platelet-rich plasma, which has the advantages that possibly important influences and interactions of other blood cells and plasma components

are not neglected and that artificial cell activation caused by the isolation process is avoided. However, the value of this system is limited by its static condition and the lack of endothelial cells. Therefore, additional *in vivo* studies or studies with a dynamic model are necessary to further define the role of epinephrine in modulating platelet-neutrophil interaction and adhesion molecule expression and the clinical relevance of our findings. Nonetheless, our study adds another aspect to the understanding of the immunological side effects of endogenous or therapeutically increased catecholamine levels.

The authors thank Nicole Heussen, Department of Biometry and Statistics, Rheinisch-Westfälische Technische Hochschule Aachen, for her statistical advice.



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A grayscale microscopic image showing a dense field of cells, likely platelets or monocytes, with some larger, more irregular shapes interspersed among the smaller, more uniform ones.

Chapter 8

Phosphodiesterase III Inhibition Affects Platelet-Monocyte Aggregate Formation Depending on the Axis of Stimulation

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ABSTRACT

Background: The purpose of this study was to investigate the effect of the phosphodiesterase (PDE) type 3 inhibitor milrinone on the adhesion of platelets to monocytes in vitro.

Methods: Whole blood was incubated with 1, 10, or 100 $\mu\text{mol/L}$ of milrinone. After stimulation with N-formyl-methionyl-leucyl-phenylalanine (FMLP) or adenosine-5-diphosphate (ADP), platelet-monocyte adhesion and CD11b, PSGL-1, GPIIb/IIIa, and P-selectin expression were measured by flow cytometry.

Results: The formation of platelet-monocyte conjugates after PDE3 inhibition depended on the type of stimulation. In unstimulated and FMLP-stimulated blood platelet monocytes, aggregation was enhanced by increasing concentrations of milrinone. This augmentation was accompanied by a rise in P-selectin expression in platelets. In ADP-stimulated blood the number of platelet-monocyte aggregates decreased with increasing concentrations of milrinone. Concurrent with the reported antiinflammatory properties of PDE-inhibition, an inhibition of CD11b expression was found in monocytes after stimulation with FMLP. In contrast, in unstimulated samples lower concentrations of milrinone caused an increase in CD11b.

Conclusions: These findings suggest that the effects of PDE3 inhibition on platelets and monocytes are modified by the type of stimulation and only partially suppress the inflammatory response of platelets and monocytes. The increase in platelet-monocyte conjugates in unstimulated and FMLP-stimulated blood suggested that PDE3 inhibition may also trigger proinflammatory reactions.

INTRODUCTION

Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are intracellular second messengers that regulate the physiologic responses generated by extracellular stimuli. The intracellular levels of cyclic nucleotides are regulated through synthesis by adenylate and guanylate cyclases and also degradation by cyclic nucleotide phosphodiesterases, which can be blocked by phosphodiesterase (PDE) inhibitors, causing an accumulation of the cyclic nucleotides. Most cells and tissues express 1 or more PDE isozymes, each regulating intracellular cyclic AMP and/or cyclic GMP concentrations in different cellular compartments and in different ways. As cAMP and cGMP transduce the effects of a variety of extracellular signals, they also influence immunomodulatory processes in all human inflammatory cells, ie, proinflammatory mediator production and cell differentiation. Although it is well known that elevation of intracellular cAMP inhibits platelet aggregation, results in decreased degranulation and cytokine production in leucocytes, and reduces CD11b expression in neutrophils, little is known about the effects of PDE inhibitors on platelet-monocyte interaction (1-3).

The mechanisms and consequences of platelet-leukocyte interactions and their implications in many diseases have been the subject of several studies (4-6). Increased association of platelets with leukocytes has been reported in unstable angina (7), myocardial infarction (8,9), coronary interventions (10), cardiopulmonary bypass (11), thrombosis (12), and sepsis (13). Complex signaling events that lead to cellular phenotypic changes and synthesis of inflammatory and thrombotic mediators occur when human platelets and monocytes interact (14,15). The upregulation of cellular adhesion receptors and the formation of platelet-monocyte aggregates have important implications in the development of an early proinflammatory response and later prothrombotic state. Enhanced P-selectin expression on the platelet surface supports the adhesion of platelets to the P-selectin-ligand-1 (PSGL-1) on neutrophils, monocytes, and lymphocytes. Furthermore, P-selectin on the surface of activated platelets induces the expression of tissue factor in monocytes, and the binding of P-selectin to monocytes promotes fibrin deposition within a growing thrombus in the area of vascular injury, which may be an initiator of thrombosis (4,5,9,16). Platelet-leukocyte adhesion can also be mediated by interaction of CD11b on leukocytes and GPIIb/IIIa on platelets. The binding of platelets to leukocytes seems to induce attachment, rolling, and the oxidative burst in neutrophils, as well as cytokine secretion in monocytes (17,18).

Patients undergoing cardiac surgery often receive PDE3 inhibitors for the treatment of cardiac failure. Because thrombotic and inflammatory events might be crucial in these patients, it is important to know if therapeutic concentrations of PDE inhibitors also affect platelet-leukocyte interactions. Therefore, it was hypothesized that PDE inhibition might reduce the formation of platelet-monocyte aggregates, preventing the prothrombotic and proinflammatory cascade initiated by platelet-monocyte interactions. Using an established



whole-blood model and 2-color flow cytometry, the effect of the PDE3 inhibitor milrinone was studied on platelet-monocyte adhesion. Because modifications in cell-to-cell interaction can be caused by changes in the cellular adhesion molecule pattern, the expression of CD11b and PSGL-1 on monocytes as well as P-selectin and GPIIb/IIIa expression on platelets were also studied. Furthermore, the platelet-stimulating agent adenosine-5-diphosphate (ADP) and the leukocyte-stimulating agent N-formyl-methionyl-leucyl-phenylalanine (FMLP) were used to compare the effect of different stimulation on both aggregate formation and adhesion molecule expression.

MATERIAL AND METHODS

The following were purchased from BD Pharmingen (San Jose, CA): anti-CD41a-PE (clone HIP8) mAb-recognizing platelet GPIIb/IIIa complex, anti-CD62P-fluorecein-isothiocyanate (FITC) (clone AK-4) mAb directed against P-selectin expressed on platelet surface, anti-CD45-FITC (clone HI30) mAb for leukocyte common antigen, anti-CD11b-PE (clone ICRF44) CD11b binding mAb, anti-CD162 (clone KPL-1) mAb-recognizing PSGL-1, antinegative IgG-FITC and IgG₁-PE antibodies (clone MOPC-21), antibodies for nonspecific binding, and fluorescence-activated cell sorting (FACS) lysing solution. Dulbecco's phosphate-buffered saline without Ca²⁺ and Mg⁺ (PBS), milrinone, bovine serum albumin (BSA), FMLP, ADP, and paraformaldehyde were obtained from Sigma Chemicals (St Louis, MO).

After obtaining approval from the local ethics committee and informed written consent, blood samples were taken from 10 healthy volunteers who had not received any medication for at least 2 weeks and not ingested caffeine for at least 2 days. Venous blood was carefully collected without tourniquet from a cubital vein using a 21-G butterfly. The first 3 mL of blood were used to perform a hemogram and then discarded; the next samples were drawn into polypropylene tubes containing sodium citrate (Sarstedt, Nuernbrecht, Germany). Nine parts of blood were anticoagulated with 1 part of 3.8% trisodium citrate. All blood samples were immediately diluted 1:1 with 37°C prewarmed PBS, placed in sterile polypropylene tissue culture dishes (Sarstedt), and incubated with 100 µmol/L, 10 µmol/L, or 1 µmol/L (final concentrations) of milrinone. The tubes were gently mixed and placed for 15 minutes in an incubator.

Stimulation, immunofluorescence staining, and flow cytometric analysis were performed as previously described with minor modifications (7). After incubation, a subset of blood samples were stimulated with FMLP (final concentration 100 nmol/L). After 10 minutes, 100 µL of stimulated or unstimulated whole blood were added to saturating concentrations of fluorochrome-conjugated antibodies and stained for 15 minutes in the dark. The staining procedure was stopped by adding 1.5 mL of lysing solution for 10 minutes. The samples were centrifuged (350 *g*, 4°C, 5 minutes), washed with PBS containing 1% BSA, and centrifuged

again. The remaining pellet was resuspended in 500 μL of PBS containing 1% BSA and 1% paraformaldehyde. Flow cytometric “2-color” analyses were performed on an FACS Calibur flow cytometer and analyzed using CellQuest 3.1 software (Becton Dickinson, San Jose, CA). Before each measurement, the flow cytometer was calibrated with fluorescence microbeads (Calibrite Beads, Becton Dickinson).

To determine platelet-monocyte aggregates, the leukocyte subpopulations were differentiated by cell size (forward scatter), granularity (side scatter), and binding of anti-CD45-FITC using the linear scaling. For each sample, 40,000 leukocytes were collected. The monocyte subgroup was separately gated, and platelet-monocyte aggregates were defined as cells positive for CD41a and CD45 in these subgroups. The percentage of CD41a-positive conjugates represents the percentage of leukocytes with at least 1 bound platelet (19).

After incubation, a subset of blood samples were stimulated with FMLP (final concentration 100 nmol/L) or ADP (final concentration 2 $\mu\text{mol/L}$) and washed and stained as described earlier. To determine adhesion molecule expression, the leukocyte subpopulations were differentiated by cell size (forward scatter), granularity (side scatter), and binding of anti-CD45-FITC using the linear scaling. For each sample, 40,000 leukocytes were collected. The leukocyte subgroups were separately gated, and the expression of CD11b and PSGL-1 was measured as mean fluorescence intensity (MFI) of the specific antibody on monocytes.

To determine P-selectin and CD41a expression, the platelet population was adjusted to $20 \times 10^9/\text{L}$ before the staining procedure and defined in flow cytometry by size and CD41a-PE immunofluorescence using the logarithmic scaling. For each sample, 10,000 platelets were measured. The percentage of platelets positive for P-selectin as well as the MFI of P-selectin and CD41a were measured (20).

The Kolmogorov-Smirnov test showed that the data were normally distributed. Thus, data are presented as mean and standard deviations. Differences between the control samples and the samples exposed to increasing concentrations of PDE inhibitors were analyzed by analysis of variance (NCSS 6.0.7.; NCSS, Kaysville, UT). A level of $p < 0.05$ was considered significant.

RESULTS

In contrast to the first hypothesis, a reduced formation of platelet-monocyte conjugates was not observed after incubation with PDE inhibitors. The formation of platelet-monocyte aggregates after preincubation with milrinone was enhanced significantly in unstimulated (1 $\mu\text{mol/L}$, 10 $\mu\text{mol/L}$, 100 $\mu\text{mol/L}$) and FMLP-stimulated blood (10 $\mu\text{mol/L}$, 100 $\mu\text{mol/L}$). Only in ADP-stimulated blood, the formation of platelet-monocyte aggregates was reduced by milrinone (100 $\mu\text{mol/L}$) (Fig 1A).



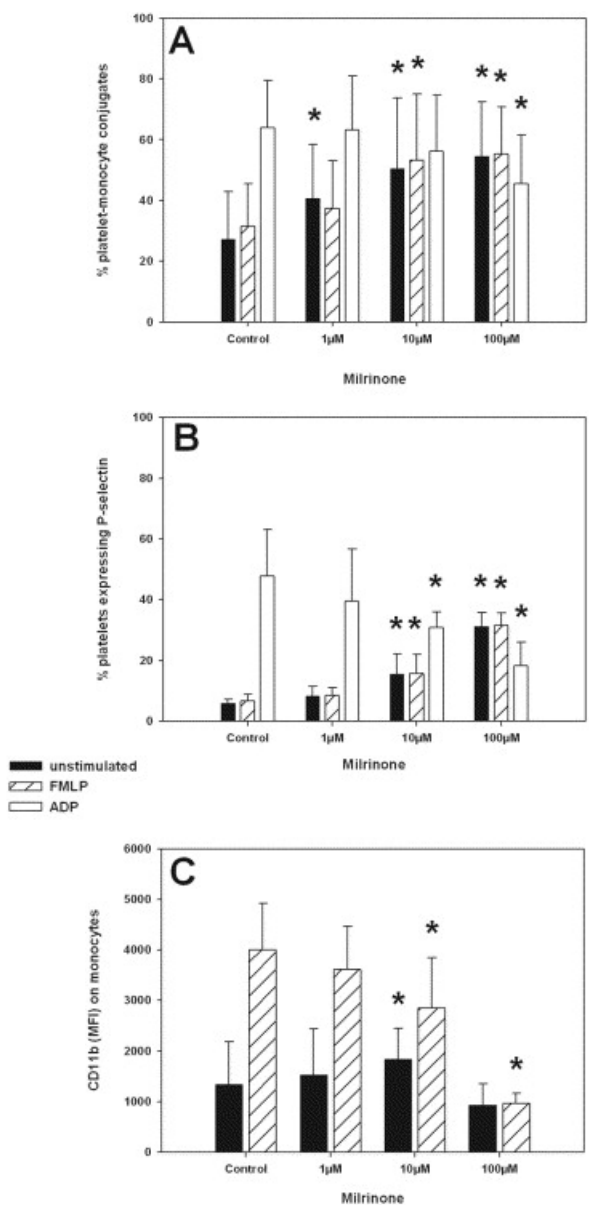


Fig. 1: Percentage of (A) platelet-monocyte conjugates, (B) P-selectin expression on platelets, and (C) CD11b expression on monocytes in unstimulated, FMLP-stimulated (100 nmol/L) and ADP-stimulated (2 μmol/L) whole blood after incubation with milrinone. Mean and SD are given. *Significantly different ($p < 0.05$) from control sample.

To investigate the possibility that the increases in platelet-leukocyte conjugate formation were favored by a modified adhesion molecule pattern through PDE inhibition, the monocyte and platelet adhesion molecule expression was determined in a second step.

In the unstimulated samples, milrinone (10 $\mu\text{mol/L}$) significantly increased the expression of CD11b on monocytes. Interestingly, in the high concentration (100 $\mu\text{mol/L}$), a reduction of CD11b expression below the control level was observed. In the FMLP-stimulated samples, milrinone decreased CD11b expression significantly on monocytes in a dose-dependent fashion (Fig 1C).

In the unstimulated and FMLP-stimulated samples, the lower concentrations of milrinone (1 $\mu\text{mol/L}$, 10 $\mu\text{mol/L}$) significantly decreased the expression of PSGL-1 on monocytes (Table 1).

Table 1 Adhesion Molecule Expression After Incubation With Milrinone

Variable	Control	1 $\mu\text{mol/L}$	10 $\mu\text{mol/L}$	100 $\mu\text{mol/L}$
<i>Platelets</i>				
P-selectin unstimulated	5.2 \pm 0.5	6.2 \pm 1	8.6 \pm 1.3*	12 \pm 1.9*
P-selectin (100 nmol/L FMLP)	5.8 \pm 0.7	7.1 \pm 1	10.3 \pm 1.3*	11.4 \pm 3.5*
P-selectin (2 $\mu\text{mol/L}$ ADP)	28.7 \pm 6.6	20.7 \pm 3.7	11.9 \pm 2.5*	11 \pm 2.2*
CD41a unstimulated	385 \pm 73	338 \pm 39	317 \pm 74	221 \pm 22*
CD41a (2 $\mu\text{mol/L}$ ADP)	674 \pm 150	625 \pm 90	421 \pm 51*	243 \pm 30*
CD41a (100 nmol/L FMLP)	415 \pm 45	347 \pm 76	354 \pm 52	226 \pm 31*
<i>Monocytes</i>				
PSGL-1 unstimulated	1,181 \pm 165	849 \pm 113*	913 \pm 87*	952 \pm 163
PSGL-1 (100 nM/L FMLP)	1,118 \pm 144	897 \pm 95*	922 \pm 93*	1012 \pm 152

Values are expressed as mean fluorescence intensity. Data are from 6 experiments and are expressed as mean \pm SD. *Significantly different ($p < 0.05$) from control sample.

Milrinone (10 $\mu\text{mol/L}$, 100 $\mu\text{mol/L}$) significantly increased the percentage of platelets expressing P-selectin as well as the P-selectin MFI in the unstimulated and FMLP-stimulated samples. Interestingly, when stimulated with ADP, decreases in platelets expressing P-selectin and in the P-selectin MFI after incubation with milrinone (10 $\mu\text{mol/L}$, 100 $\mu\text{mol/L}$) were observed (Fig 1B, Table 1).

Surface expression of GPIIb/IIIa declined in stimulated and unstimulated platelets after treatment with milrinone (10 $\mu\text{mol/L}$ [only ADP], 100 $\mu\text{mol/L}$) (Table 1).

DISCUSSION

Phosphodiesterases are a class of isoenzymes responsible for the hydrolysis of the intracellular second messengers, like cAMP and cGMP, resulting in the formation of the corresponding, inactive 5'-monophosphate. The expression and activity of these PDE isoenzymes vary among different tissues and cells. The PDE3 family, which hydrolyzes both cAMP and cGMP, can be found in cardiac muscle, vascular smooth muscle, platelets, and leukocytes.

Because several investigations revealed that inhibition of PDE reduces the inflammatory response of leukocytes by decreasing cytokine generation, degranulation, and phagocytosis,



it was hypothesized that PDE inhibition might also attenuate platelet-monocyte interaction (21,22). However, in contrast to this hypothesis, the present study shows that PDE3 inhibition alters platelet-monocyte interactions depending on the type of stimulation. Although increasing concentrations of milrinone enhanced the formation of platelet-monocyte conjugates in unstimulated and FMLP-stimulated blood, in ADP-stimulated blood the number of conjugates decreased with increasing concentrations of milrinone. To assess the intracellular mechanisms involved, the authors investigated, in a second step, platelet and monocyte adhesion molecule expression. In contrast to the ADP-stimulated samples, in unstimulated and FMLP-stimulated blood, an increased percentage of platelets expressing P-selectin was found. Similar to previous studies on neutrophils by other groups, inhibition of CD11b expression on monocytes was found after stimulation with FMLP, but, in contrast to the reported antiinflammatory properties, PDE3 inhibition also caused increases in CD11b expression in the unstimulated samples.

The observed enhancement of platelet-monocyte aggregates in unstimulated and FMLP-stimulated blood was associated with a rise in P-selectin on platelets. This upregulation of P-selectin could account mainly for the formation of conjugates. P-selectin is a glycoprotein located in the membranes of α -granules and becomes externalized on the platelet surface after platelet activation and granule secretion. Platelets and leukocytes may form aggregates via platelet-expressed P-selectin and its counterreceptors PSGL-1 and Sialyl Lewis X, as well as via fibrinogen bridging between GPIIb/IIIa and CD11b (23); although the latter seems to be less important in monocytes. Sarma et al. (9) showed that GPIIb/IIIa blockade with abciximab failed to inhibit platelet-monocyte interaction. Therefore, the increased platelet-monocyte association was mainly mediated via P-selectin expressed on platelets in unstimulated and FMLP-stimulated blood. This is also consistent with the observation that the reduction in GPIIb/IIIa expression and the stimulating and inhibiting effects of PDE3 inhibition on CD11b expressions were not accompanied by simultaneous effects in platelet-monocyte aggregate formation. In view of the fact that previous studies reported that PDE3 inhibitors caused a decrease in P-selectin expression on platelets after being stimulated with ADP, conjugate formation and P-selectin expression were measured after incubation with milrinone and stimulation with ADP. A reduction in P-selectin surface expression and P-selectin-positive platelets was found (24). This reduction was partially accompanied by a decrease in platelet-monocyte conjugate formation.

These findings support studies that showed that cAMP elevation by PDE inhibitors on platelet function and platelet-monocyte crosstalk are complex and may be modulated by stimulation with different agonists (25). After stimulation with the platelet-agonist ADP, reduced P-selectin and GPIIb/IIIa expression were seen, whereas whole-blood stimulation with the leukocyte-agonist FMLP increased P-selectin expression, reduced GPIIb/IIIa expression, and did not lead to CD11b upregulation. Why PDE inhibition caused these divergent effects can only be speculated. Manns et al. (26) showed that the mechanism by which cAMP was

increased greatly influenced platelet responses to various agonists. There is also evidence that spatially and temporally distinct cAMP signals can coexist within simple cells and that segregated cAMP signals allow for differential regulation of cAMP effector proteins like protein kinase A. For that reason, elevations in cAMP caused by different agents can have markedly different downstream effects (27).

The mechanism through which cAMP exerts its action in platelets has not yet been fully elucidated. A number of hypotheses have been proposed, all agreeing that a process that involves several steps of the activation cascade, such as phosphoinositide metabolism, Ca^{2+} elevation, and protein kinase A-dependent phosphorylation are involved. There is evidence that cAMP-induced inhibition of platelet aggregation is closely dependent on the inhibition of GPIIb/IIIa-dependent functions (25,28,29). If the observed decrease in GPIIb/IIIa expression in this study enhanced platelet-monocyte conjugate formation by reducing platelet-platelet aggregation and thus providing more activated platelets for heterotypic conjugation remained unclear.

Recent studies provide evidence that increased adhesion of platelets to monocytes is associated with ischemic events and that platelets bound to the monocyte membrane may directly and indirectly influence recruitment patterns within the circulation (30). Activated platelets induce the expression and secretion of monocyte chemoattractant protein-1 and interleukin-8 from monocytes in a P-selectin/PSGL-1-dependent manner (31). Furthermore, P-selectin-dependent interactions potentiate tissue factor expression (5), platelet-activating factor release, phagocytosis (32), and superoxide anion generation by monocytes (33). The β_2 -integrin CD11b is involved in numerous aspects of leukocyte function including tight attachment of leukocytes to endothelial cells after initial tethering and rolling (34). Patients lacking these integrins are susceptible to severe infections, but excessive activation contributes to sustained inflammation, reperfusion injury, and tissue damage (35). The inhibition of leukocyte CD11b surface expression by elevated intracellular cAMP has been reported to attenuate FMLP-mediated leukocyte-endothelial adhesion, thus contributing to the anti-inflammatory properties of PDE inhibition (36). In FMLP-stimulated leukocytes, an inhibition of CD11b upregulation was observed. Yet, in unstimulated leukocytes, the study found not only an elevation of platelet-leukocyte conjugates but also an increased expression of CD11b after incubation with PDE inhibitors in lower concentrations. Thus, the authors cannot discard the possibility that PDE3 inhibition can support monocyte recruitment and transendothelial migration via CD11b.

Therefore, increased adhesion of platelets on monocytes induced after treatment with PDE3 inhibitors could have a negative impact on the course of disease in patients suffering from myocardial infarction or thrombotic disorders. In 1992, Packer et al. (37) investigated the effects of oral milrinone on mortality in severe chronic heart failure. As compared with placebo, milrinone therapy was associated with a 34% increase in cardiovascular mortality. Whether the proinflammatory and prothrombotic mechanisms observed in this study may



contribute to the adverse effects in patients receiving long-term therapy with oral milrinone can only be speculated.

Although, in contrast to previous studies, this study used whole blood instead of isolated monocytes or platelet-rich plasma, there are limitations of this study; platelet-monocyte adhesion and adhesion molecule expression were studied in static conditions. Additional studies, with whole blood involving endothelial cells in a dynamic model, are necessary to further define the role of PDE3 inhibitors in modulating platelet-monocyte interactions, adhesion molecule expression, and the clinical relevance of the present findings.

Acknowledgment

The authors would like to thank Nicole Heussen, Department of Biometry and Statistics, RWTH Aachen, for her statistical advice.

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Chapter 9

The effect of sevoflurane and desflurane on neutrophil selectin and β_2 -integrin activation *in vitro*

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Submitted

ABSTRACT

Background: Neutrophil adhesion to vascular endothelium and platelets is a multistep process involving several selectins and β_2 -integrins. Since volatile anesthetics are reported to reduce ischemia-reperfusion injury, we assessed whether sevoflurane and desflurane affect the activation of the selectins P-selectin glycoprotein ligand-1 (PSGL-1) and L-selectin and the β_2 -integrins CD11a and CD11b.

Methods: Whole blood was incubated for 60 min with 1 or 2 minimum alveolar anesthetic concentration (MAC) sevoflurane or desflurane. After incubation, neutrophils were activated with *N*-formylmethionyl-leucyl-phenylalanine (FMLP) or phorbol-12-myristate-13-acetate (PMA). Activation of adhesion molecules was evaluated via flow cytometry.

Results: Both anesthetics reduced the expression of PSGL-1 and had concentration dependent opposed effects on the expression of L-selectin. 1 MAC sevoflurane and desflurane reduced PMA-induced L-selectin shedding, while 2 MAC of the respective anesthetics amplified L-selectin shedding in unstimulated and PMA stimulated whole blood, whereas FMLP induced shedding was not altered. Interestingly sevoflurane enhanced the expression of CD11b in both concentrations.

Conclusions: These results demonstrate that sevoflurane and desflurane modify the activation of adhesion molecules and might therefore affect the multistep process of neutrophil recruitment.

The effects of sevoflurane and desflurane on neutrophil adhesion molecules seem to be concentration dependent. Especially desflurane in the 1 MAC concentration appears to have pronounced antiadhesive effects, whereas sevoflurane showed to have both adhesive as well as antiadhesive effects.

INTRODUCTION

The upregulation of neutrophil adhesion molecules plays a major role in the recruitment of neutrophils through vascular endothelium and in the formation of platelet-neutrophil aggregates. Although both processes are important steps for host defense against invading pathogens, they paradoxically contribute to organ dysfunction in conditions such as ischemia-reperfusion injury (1,2), myocardial infarction (3), thrombosis (4), and sepsis (5). Neutrophil accumulation during ischemia-reperfusion injury begins with neutrophil tethering and rolling, which is mediated by the interaction of the selectins P-selectin glycoprotein ligand-1 (PSGL-1) and L-selectin with their endothelial counterligands. The neutrophil β_2 -integrin CD11b is then involved in the tight attachment to endothelium. Finally, neutrophils transmigrate into the interstitial compartment via the binding of CD11a to endothelial intercellular adhesion molecule (ICAM)-1 (2). The release of oxygen free radicals and cytotoxic enzymes and cytokines from activated neutrophils contributes then to the tissue injury (1,6). Furthermore, microvascular occlusion by platelet-leukocyte aggregates (7) and increased endothelium permeability have also been demonstrated to aggravate the ischemia-reperfusion injury (8). Platelet-leukocyte adhesion can be mediated by interaction of PSGL-1 on leukocytes and P-selectin on platelets or respectively via CD11b and GPIIb/IIIa.

The effects of anaesthetics on ischemia-reperfusion injury and neutrophil adhesion have been object of several studies in the past years. On the one hand side, isoflurane, and, sevoflurane protected against myocardial ischemia-reperfusion injury (9-11) on the other hand side Morisaki and co-workers reported an increased leukocyte rolling and adhesion in rats undergoing sevoflurane anaesthesia, probably caused by an upregulation of P-selectin expression on endothelial cells (12).

We could show in previous studies that isoflurane inhibits the activation of L-selectin and attenuates the activation of CD11a and CD11b—which mediate firm adhesion and transendothelial migration (13). Furthermore we found that sevoflurane enhanced the binding of platelets to neutrophils and increased the expression of P-selectin on platelets, while desflurane decreased the percentage of platelet-neutrophil conjugates (14). To investigate the latter further we studied now the effect of sevoflurane and desflurane at 1 and 2 minimum alveolar anesthetic concentration (MAC) on the activation of selectins and β_2 -integrins, by using an established whole blood model. Furthermore, adhesion molecule activation was determined during basal conditions and after stimulation with *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) and phorbol-12-myristate-13-acetate (PMA).



MATERIAL AND METHODS

Antibodies and reagents:

The following were purchased from BD Pharmingen (San Jose, CA, USA): anti-CD45-FITC (clone HI30) monoclonal antibodies (Mab) for leukocyte common antigen; anti-CD62L-PE (clone Dreg 56) L-selectin binding Mab, anti-CD11a-PE (clone Hi111) Mab for lymphocyte function associated antigen-1, anti-CD11b-PE (clone ICRF44) CD11b binding Mab, anti-CD162 (clone KPL-1) Mab recognizing PSGL-1, anti-negative IgG₁-FITC and IgG₁-PE antibodies (clone MOPC-21), antibodies for non-specific binding, and FACSlysing solution. Dulbecco's phosphate buffered saline without Ca²⁺ and Mg²⁺ (PBS), bovine serum albumin (BSA), FMLP, PMA and paraformaldehyde were obtained from Sigma Chemicals (St. Louis, USA). Before the beginning of this study all antibodies were titrated by flow cytometry to determine saturating conditions.

Blood collection and incubation

After obtaining approval from the local ethics committee and informed written consent, blood samples were taken from 10 healthy volunteers who had not received any medication for at least two weeks. Venous blood was carefully collected without tourniquet from a cubital vein using a 21-gauge butterfly needle. The first 3ml of blood were used to perform a hemogram and then discarded, the next samples were drawn into polypropylen tubes containing sodium citrate (Sarstedt, Nuernbrecht, Germany). Nine parts of blood were anticoagulated with one part of 3.8% trisodium citrate.

Methods

The MAC values used in this study were 2 vol.% for sevoflurane and 6 vol.% for desflurane. Incubation of blood samples was performed as previously described (13). In brief, blood samples were incubated in a small chamber with 21% oxygen and 5% carbon dioxide at 37°C. The volatile anesthetics were delivered with a standard anesthetic machine (Sulla 909; Dräger, Lübeck, Germany), and concentrations of all gases were continuously monitored with a multigas analyzer (Datex Compact, Datex, Helsinki, Finland). Blood samples were exposed to either 1 or 2 MAC of the volatile anesthetics for 60 min. Untreated control blood samples were placed in a standard incubator (BB 16; Heraeus, Hanau, Germany) providing identical atmospheric conditions. After the end of the incubation time, all samples were immediately processed for stimulation and staining procedures. The effects of sevoflurane or desflurane on the expression of neutrophil selectins and β_2 -integrins were investigated with unstimulated blood samples and after activation of neutrophils by using two different stimuli. FMLP

is a physiological agonist of the FMLP receptor on the neutrophil cell surface. Activation of the FMLP receptor results in downregulation of PSGL-1 and L-selectin, whereas expression of CD11a and CD11b is increased. In contrast, PMA directly activates protein kinase C (PKC), which also leads to downregulation of the two selectins and upregulation of both β_2 -integrins. Stimulation of blood samples with FMLP (final concentration, 100 nM) and PMA (100 nM) was performed in sealed polypropylene tubes to avoid evaporation of the volatile anesthetics. Blood samples were incubated with the stimulating agent for 10 min at 37°C. Thereafter, 100 μ L of blood was added to polystyrene tubes (Falcon, Becton-Dickinson, San Jose, CA) containing fluorochrome-conjugated Mabs. Before the beginning of this study, all Mabs were titrated by flow cytometry to determine saturating conditions. Blood cells were stained for 30 min at 4°C in the dark. Staining was stopped by adding 2 mL of lysing for 10 min. After centrifugation (5 min, 350g, 4°C), the samples were washed with 2 mL of phosphatebuffered saline containing 1% bovine serum albumin and centrifuged, and the cell pellet was resuspended in 400 μ L of phosphate-buffered saline containing 1% bovine serum albumin and 2% paraformaldehyde. Blood cells were analyzed on a FACSCalibur flow cytometer (Becton-Dickinson), which was calibrated before each measurement with CalIBRITE beads (Becton-Dickinson). Neutrophils were identified by their scatter characteristics and CD45 staining in the FL1 channel. The data of 20,000 neutrophils were stored in list mode. The activation of PSGL-1, L-selectin, CD11a, and CD11b on the neutrophil cell surface was analyzed by measuring the mean fluorescence intensity (MFI) of the specific Mab in the FL2 channel.

Statistics

The Kolmogorov-Smirnov-test showed that the data was normally distributed. Thus, data is presented as mean and standard deviations. Differences between anaesthetic gas-exposed and untreated control samples assessed in parallel were evaluated with Student's *t*-tests. A value of $p < 0.05$ was considered significant.

RESULTS

Effects of sevoflurane on neutrophil adhesion molecule expression

One MAC sevoflurane enhanced the expression of CD11b in unstimulated blood. The shedding of L-selectin in PMA stimulated blood and the expression of PSGL-1 in unstimulated and PMA-stimulated blood was reduced.

In the presence of 2 MAC sevoflurane the expression of CD11b was increased in all samples, whereas the PSGL-1 expression was decreased in all samples. In contrast to 1 MAC Sevoflurane, 2 MAC Sevoflurane did increase the shedding of L-selectin in the PMA-stimulated samples.



The results of the effects of sevoflurane on neutrophil adhesion molecule expression are shown in table 1.

Table 1: Effect of 1 and 2 MAC sevoflurane on the adhesion molecule expression in unstimulated and PMA and FMLP stimulated whole blood

Adhesion molecule MFI	Control	Sevoflurane 1 MAC	Control	Sevoflurane 2 MAC
CD 11b unstimulated	332 ± 62	409 ± 69 *	455 ± 125	631 ± 141 *
CD 11b PMA (100nM)	2019 ± 357	2091 ± 366	2621 ± 644	3545 ± 665 *
CD 11b FMLP (100nM)	2103 ± 240	2119 ± 191	2284 ± 231	2677 ± 378 *
CD 11a unstimulated (100nM)	802 ± 38	792 ± 33	751 ± 34	765 ± 38
CD 11a PMA (100nM)	996 ± 54	941 ± 46	929 ± 46	980 ± 50
CD 11a FMLP (100nM)	1016 ± 59	986 ± 63	920 ± 64	891 ± 62
L-selectin unstimulated (100nM)	1683 ± 103	1689 ± 84	1482 ± 72	1422 ± 58
L-selectin PMA (100nM)	895 ± 117	1159 ± 123 *	912 ± 128	726 ± 104 *
L-selectin FMLP (100nM)	397 ± 96	408 ± 79	264 ± 38	205 ± 30
PSGL-1 unstimulated (100nM)	693 ± 64	571 ± 52 *	523 ± 34	435 ± 26 *
PSGL-1 PMA (100nM)	665 ± 57	589 ± 50 *	533 ± 32	494 ± 26
PSGL-1 FMLP (100nM)	640 ± 60	623 ± 103	519 ± 45	479 ± 29

Values are expressed as mean fluorescence intensity. Data are expressed as mean ± SD. *Significantly different ($p < 0.05$) from control sample.

Effects of desflurane on neutrophil adhesion molecule expression

As shown in table 2, 1 MAC desflurane also inhibited the shedding of L-selectin from the neutrophil surface after stimulation with PMA. PSGL-1 expression was significantly reduced in unstimulated and PMA stimulated blood. In the 2 MAC desflurane group, we observed in unstimulated and PMA-stimulated blood an increased shedding of L-selectin. PSGL-1 expression was significantly reduced in all samples after incubation with 2 MAC desflurane.

CD11b expression was not changed by either concentration of desflurane.

Table 2: Effect of 1 and 2 MAC desflurane on the adhesion molecule expression in unstimulated and PMA and FMLP stimulated whole blood

Adhesion molecule MFI	Control	Desflurane 1 MAC	Control	Desflurane 2 MAC
CD 11b unstimulated	270 ± 22	289 ± 41	269 ± 37	294 ± 46
CD 11b PMA (100nM)	2232 ± 546	2110 ± 709	1645 ± 218	1730 ± 353
CD 11b FMLP (100nM)	2207 ± 139	2133 ± 203	2776 ± 355	2623 ± 462
CD 11a unstimulated (100nM)	694 ± 72	689 ± 67	647 ± 50	644 ± 60
CD 11a PMA (100nM)	897 ± 116	844 ± 80	776 ± 55	752 ± 55
CD 11a FMLP (100nM)	842 ± 88	819 ± 76	785 ± 54	763 ± 52
L-selectin unstimulated (100nM)	1451 ± 227	1360 ± 192	1316 ± 171	1040 ± 167 *
L-selectin PMA (100nM)	784 ± 134	903 ± 146 *	929 ± 104	737 ± 131 *
L-selectin FMLP (100nM)	265 ± 39	232 ± 58	261 ± 42	236 ± 26
PSGL-1 unstimulated (100nM)	600 ± 134	416 ± 101 *	486 ± 46	377 ± 21 *
PSGL-1 PMA (100nM)	600 ± 131	455 ± 103 *	485 ± 43	376 ± 32 *
PSGL-1 FMLP (100nM)	462 ± 106	431 ± 102	444 ± 42	376 ± 22 *

Values are expressed as mean fluorescence intensity. Data are expressed as mean ± SD. *Significantly different ($p < 0.05$) from control sample.

DISCUSSION

Our results demonstrate that sevoflurane and desflurane affect neutrophil adhesion molecule expression. Interestingly they exerted similar but also different effects on the adhesion molecule expression. Both anaesthetics affected L-selectin shedding and reduced the expression of PSGL-1, but only sevoflurane enhanced the surface expression of CD11b.

The multicellular interactions among platelets, leukocytes, and the vascular wall concentrate a group of effector molecules that can modulate both hemostasis and inflammation (15,16). Binding of PSGL-1 to P-selectin promotes tethering and rolling of leukocytes on activated endothelial cells and platelets. Binding of PSGL-1 to L-selectin mediates tethering of leukocytes to other leukocytes, which may amplify recruitment of leukocytes to the vascular wall. Activated platelets, through P-selectin–PSGL-1 or GPIIb/IIIa–CD11b interactions, may connect additional leukocytes to sites of inflammation or tissue injury. Therefore surface adhesion molecules play a major role in neutrophil tethering and migration through vascular endothelium or in the formation of platelet-neutrophil aggregates. While both processes are critical for host defense against invading pathogens, inappropriate adhesion molecule expression contributes to tissue damage in a variety of inflammatory and thrombotic disorders. Especially the adhesion molecules CD11b and PSGL-1 mediate both: the interaction of neutrophils with endothelium as well as the interaction of neutrophils with platelets. Therefore a modified expression of these adhesion molecules should have an impact on both (17).

Since volatile anesthetics have been reported (9-11,13,18) to have antiadhesive activity and protect against ischemia reperfusion injury we used an *in vitro* blood model and two color flow cytometry to clarify if sevoflurane and desflurane alter the expression of neutrophil adhesion receptors.

In a previous study Möbert and co-workers showed that halothane, isoflurane, and sevoflurane inhibited neutrophil adhesion to human endothelial cells. They concluded that this effect was caused by attenuating the upward regulation of neutrophil CD11b leading to a reduced neutrophil adhesion (19). In relation to sevoflurane our study does not confirm this. In contrast, we observed an increased expression of CD11b on neutrophils after incubation with sevoflurane which could lead to an increased adhesion to endothelium as well as to platelets. These findings confirm a recent investigation where we could show that sevoflurane but not desflurane enhanced the formation of platelet-neutrophil aggregates (14). Since platelets and neutrophils may form aggregates also via fibrinogen bridging between GPIIb/IIIa and CD11b (20) the enhancement of CD11b on neutrophils probably contributed to the augmentation of platelet-neutrophil conjugates by sevoflurane observed in the latter study. Whether the upregulation of CD11b under sevoflurane affects also the neutrophil-endothel interaction cannot be answered by this study.

However, CD11b is not the sole adhesion molecule involved in the process of neutrophil recruitment and adhesion. The initial neutrophil tethering and rolling is mediated by selec-



tins, such as PSGL-1 and L-selectin. These selectins interact with their respective endothelial counterligands in a series of rapid adhesion and deadhesion events mediated by the formation of numerous weak, reversible bonds. This cycle of adhesion and de-adhesion results in the process of leukocyte rolling along the endothelial cell surface (21).

The results of our study showed that both sevoflurane and desflurane significantly reduced the surface expression of PSGL-1. Such downregulation has been reported to have profound effects on the ability of neutrophils to interact with endothelial P-selectin. Davenpeck et al. demonstrated that decreased surface expression of PSGL-1 on neutrophils correlates with a decrease in neutrophil adhesion to P-selectin under both static and dynamic conditions (22).

Studies on leukocytes confirm that L-selectin is responsible for leukocyte adhesion and rolling behavior and that L-selectin shedding is required for efficient transendothelial migration (23). Interestingly both anesthetics had concentration dependent opposed effects on the expression of L-selectin. 1 MAC sevoflurane and desflurane reduced PMA-induced L-selectin shedding, while 2 MAC of the respective anesthetics amplified L-selectin shedding in unstimulated and PMA stimulated whole blood, whereas FMLP induced shedding was not altered.

FMLP is a physiological agonist of the FMLP receptor on the neutrophil cell surface. Activation of the G protein-linked FMLP receptor leads to the activation of phospholipase C and D, generation of inositol triphosphate and activation of PKC, while PMA directly activates protein kinase C (PKC), which also leads to a downregulation of the two selectins and upregulation of the $\beta 2$ - integrin (24-25). Since FMLP-induced shedding which also involves PKC was not altered by the two volatile anaesthetics it seems to be likely, that sevoflurane and desflurane modified L-selectin expression by a PKC-independent pathway or mechanism. For example by direct inhibition of sheddases required for L-selectin shedding after stimulation with PMA such as the metalloprotease Tumour Necrosis Factor- α Converting Enzyme (TACE) (23).

In conclusion, the effects of sevoflurane and desflurane on neutrophil adhesion molecules seem to be concentration dependent. Especially desflurane in the 1 MAC concentration appears to have pronounced antiadhesive effects, whereas sevoflurane showed to have both adhesive as well as antiadhesive effects. Therefore it seems to be likely that the cardioprotective effects of sevoflurane are for the most part mediated by other mechanisms than by antiadhesive properties. As a possible mechanism has been suggested an activation of intracellular messaging pathways by reactive oxygen species with an consecutive priming or indirect opening of the K_{ATP} channel resulting in a reduced cytosolic and mitochondrial calcium loading and improved myocardial oxygen efficiency during ischemia and reperfusion (26-27).

Acknowledgement

The authors thank Nicole Heussen, Department of Biometry and Statistics, Rheinisch Westfälische Technische Hochschule Aachen, for her statistical advice.



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Chapter 10

General Discussion and Conclusions

GENERAL DISCUSSION AND CONCLUSIONS

The aim of this thesis was to study the influence of volatile anaesthetics and inotropic agents on platelet and leukocyte function. We focused on three aspects of this influence: Adhesion molecule expression on platelets, adhesion molecule expression on leukocytes and platelet - leukocyte interactions.

Chapter 1 -as a general introduction- introduces the reader theoretically to the basic concepts of adhesion molecules, volatile anaesthetics, inotropic agents and their possible interaction and impact on perioperative immunity.

In the first part of this thesis we concentrated on the effects of volatile anaesthetics on adhesion.

Chapter 2 of this thesis -as a preliminary study- focuses on the effect of the volatile anaesthetic sevoflurane on platelets adhesion molecule expression *in vitro*. Our results showed that sevoflurane inhibits agonist-induced GPIIb/IIIa activation (as measured by PAC-1 binding) and surface expression on platelets in whole blood even in subanaesthetic concentrations. At 0.5 MAC sevoflurane, PAC-1 binding and the percentage of positive cells for PAC-1 were greater in comparison with 1 MAC, suggesting a possible dose-dependent inhibition of receptor activation. The agonist-induced redistribution of GPIb into the open canalicular system was also impaired by sevoflurane, whereas no effect on P-selectin expression in activated platelets as an indicator of activation dependent α - degranulation could be found. These morphological changes on the platelet surface affected also platelet function. We found a reduced maximum amplitude in thromboelastography and significantly prolonged platelet function analyzer 100 closure times in blood incubated with sevoflurane.

Parallel to the above described study we compared the effects of two other volatile anaesthetics - halothane and isoflurane - on platelet-leukocyte adhesion and P-selectin expression on platelets. Interestingly the results of this investigation-as presented in chapter 3- demonstrate that these two volatile anaesthetics affect the binding of activated platelets to leukocytes differently. Halothane inhibited, while isoflurane enhanced the adhesion of agonist-activated platelets to neutrophils. The different influence of both anaesthetics on the expression of CD62P on platelets seemed to cause these opposite effects. Platelet surface CD62P, which has a major role in the mechanism of platelet-leukocytes adhesion was suppressed by halothane but enhanced by isoflurane. Thus, the ability of halothane to inhibit binding of activated platelets to monocytes and neutrophils, as well as the enhancement of platelet-neutrophil adhesion by isoflurane, might change the inflammatory response. Because halothane is nowadays rarely used in the clinical practice we did no further investigations on this anaesthetic after this study.

Since the elevation of CD62P on platelets under isoflurane was only accompanied by an elevated binding of platelets to neutrophils but not to monocytes, we investigated in the following steps (Chapter 4 and 6) if there was a concomitant effect on the neutrophil or

monocyte side, which could furthermore explain our findings. Interestingly in comparison to monocytes neutrophils showed a totally different behaviour: Isoflurane attenuated the activation of L-selectin on neutrophils, which mediates neutrophil tethering and rolling, as well as the activation of CD11a and CD11B which mediate firm adhesion and transendothelial migration. In monocytes only the removal of PSGL-1 from the surface was affected. It can be concluded from these last two studies that the effects of isoflurane on cellular adhesion molecules are highly dependent on the type of cell and lead therefore to different intercellular interaction pattern.

Chapter 5 presents a study which was done in a similar manner as the previous studies, analyzing the influence of two modern volatile anaesthetics sevoflurane and desflurane on cellular adhesion molecules. Here again the two volatile anaesthetics showed differing effects on the formation of platelet-leukocyte conjugates. Sevoflurane enhanced the formation of conjugates and the expression of CD62P, while desflurane decreased the number of conjugates without altering the expression of CD62P on platelets.

To analyze these findings further and to complete the studies about the impact of volatile anaesthetics on adhesion molecules, we investigated in chapter 9 the effect of sevoflurane and desflurane at 1 and 2 minimum alveolar anesthetic concentration (MAC) on the activation of selectins and β_2 -integrins. The results of this study demonstrate that both sevoflurane and desflurane affect neutrophil adhesion molecule expression. Interestingly they exerted similar but also different effects on the adhesion molecule expression. Both anaesthetics affected L-selectin shedding and reduced the expression of PSGL-1, but only sevoflurane enhanced the surface expression of CD11b.

In the second part of this thesis we investigated the effects of inotropic agents on the cellular adhesion molecules.

Chapter 7 describes the effects of epinephrine on platelet-neutrophil adhesion. Here we could show that epinephrine enhances the formation of platelet-neutrophil conjugates in a dose dependent manner. This increase in platelet-neutrophil aggregates was accompanied by an increase in CD62P and GPIIb/IIIa on platelets. We assume that epinephrine caused this increased expression of CD62P and GPIIb/IIIa via α -adrenergic stimulation, since α -adrenergic blockade almost completely reversed this upregulation of platelet adhesion molecules. Another important finding was that epinephrine suppressed the agonist induced upregulation of CD11b and downregulation of L-selectin an effect which was abolished by β -adrenergic blockade.

Similarly complex were the reactions of platelets and monocytes after incubation with the PDE 3 inhibitor milrinone. The results of this study are shown in chapter 8. Here the axis of

agonist stimulation played a decisive role in the adhesion molecule expression pattern and the following platelet- monocyte aggregate formation.

The results of the above presented studies show that the influences of volatile anaesthetics and inotropic agents on the expression of platelet and leukocyte adhesion molecules and subsequent interaction are manifold. In these in vitro studies we could not detect a unique reaction of blood cells to volatile anaesthetics or inotropic agents. Therefore the results of our studies could not altogether confirm previous studies by other authors which concluded that the anti-adhesive properties of volatile anaesthetics are due to a reduced adhesion molecule expression. Subject to the agent, its applied concentration, the axis of stimulation and the cell type we found both reduced and enhanced expressions of adhesion molecules, which led to more or less conjugate formation. Thus the influence of volatile anaesthetics and inotropic agents on blood cell interactions are complex and modified by many factors, rendering it impossible to predict the exact effects on immunity. However, there is evidence that pre-operative as well as intraoperative pharmaceuticals alter immunity, although it seems to be likely that the cardioprotective effects of volatile anaesthetics are for the most part mediated by other mechanisms than by antiadhesive properties. As a possible mechanism has been suggested an activation of intracellular messaging pathways by reactive oxygen species with a consecutive priming or indirect opening of the K_{ATP} channel resulting in a reduced cytosolic and mitochondrial calcium loading and improved myocardial oxygen efficiency during ischaemia and reperfusion (127, 128).

Which mechanism eventually responsible is for the favourable effect on cardioprotection and the ischaemia-reperfusion-injury shall be seen in the future.

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Chapter 11

Samenvatting en Conclusies

SAMENVATTING EN CONCLUSIES

Het doel van dit proefschrift is de bestudering van de effecten van volatiele anesthetica en inotrope medicatie op de functie van trombocyten en leukocyten. Drie aspecten werden in het bijzonder onderzocht: de expressie van zogenaamde adhesiemoleculen in trombocyten, de expressie van zogenaamde adhesiemoleculen in leukocyten en de interactie tussen trombocyten en leukocyten.

Hoofdstuk 1 van het proefschrift bevat de introductie waarin de theoretische en basale concepten van de werking van adhesiemoleculen, volatiele anesthetica, inotrope medicatie en hun mogelijke interacties en effecten op het immuunsysteem in de perioperatieve fase beschreven word.

In het eerste deel van het proefschrift concentreerden wij ons op de effecten van volatiele anesthetica op de expressie van adhesiemoleculen. Hoofdstuk 2 beschrijft een studie naar de effecten van het volatiele anestheticum sevoflurane op de expressie van adhesiemoleculen van trombocyten *in vitro*. Deze studie laat zien dat sevoflurane de agonist geïnduceerde GPIIb/IIIa activatie (gemeten als PAC-1 binding) en de oppervlakte expressie in trombocyten in vol bloed, zelfs bij subanesthetische concentraties, tegen gaat. Tijdens de toediening van 0.5 MAC sevoflurane was de PAC-1 binding en het percentage PAC-1 positieve cellen hoger dan bij een concentratie van 1 MAC, wat op een mogelijk dosisafhankelijk effect duidt. Ook de agonist geïnduceerde redistributie van GPIb in het open canaliculair systeem werd door sevoflurane verminderd, terwijl in geactiveerde trombocyten geen effect op de P-selectine expressie als indicator van activatie afhankelijke α -degranulatie werd gevonden. Deze morfologische veranderingen op het oppervlak van de trombocyten had ook effect op hun functie. Wij vonden een verminderde maximale amplitude tijdens tromboelastografie en een significant verlengde platelet function analyzer 100 sluitertijd in het bloed tijdens incubatie met sevoflurane.

Parallel aan de hierboven beschreven studie vergeleken wij de effecten van twee andere volatiele anesthetica - halothane en isoflurane – op de trombocyten-leukocyten adhesie en de P-selectine expressie bij trombocyten. De resultaten van deze studies zijn beschreven in Hoofdstuk 3 en laten opmerkelijk genoeg zien dat de effecten van deze twee anesthetica op de binding tussen trombocyten en leukocyten verschillend is. Halothane verminderd deze binding, terwijl isoflurane de agonist-geactiveerde adhesie tussen trombocyten en neutrofelen versterkt. Het verschillend effect van deze twee anesthetica op de expressie van CD62P op de trombocyt kan deze verschillen mogelijk verklaren. De expressie van het trombocyten-oppervlakte CD62P, wat een belangrijke rol speelt bij het mechanisme van de trombocyt-leukocyt adhesie werd verminderd door halothane, maar versterkt door isoflurane. De eigenschap van halothane om de binding tussen geactiveerde trombocyten met

monocyten en neutrofielen te verminderen, en het toegenomen effect van isoflurane op de binding tussen deze cellen kan een rol spelen bij de perioperatieve inflammatoire respons. Omdat halothane tegenwoordig vrijwel niet meer gebruikt wordt in de klinische praktijk hebben we de effecten van halothane in dit proefschrift verder niet onderzocht.

Omdat de verhoging van CD62P bij trombocyten tijdens de toediening van isoflurane alleen gepaard ging met een toegenomen binding van trombocyten met neutrofielen, maar niet met monocyten, werd in de Hoofdstukken 4 en 6 specifiek gekeken naar de effecten van isoflurane op de neutrofielen en monocyten. Het bleek dat neutrofielen en monocyten verschillende effecten lieten zien. Isoflurane verzwakt de activatie van L-selectine van de neutrofiel, wat de binding en het rollen van neutrofielen beïnvloed, maar verminderd ook de activatie van CD11a en CD11b, wat een rol speelt bij de stevige binding en de transendotheliale migratie. In de monocyt werd alleen invloed op de verwijdering van PSGL-1 van het celoppervlak geconstateerd.

Op basis van de resultaten gevonden in deze laatste twee studies word geconcludeerd dat de effecten van isoflurane op cellulaire adhesiemoleculen zeer afhankelijk zijn van het type cel wat bestudeerd wordt en dat dit leidt tot verschillende effecten op de intercellulaire interactie.

Hoofdstuk 5 beschrijft een studie waarbij de effecten van twee moderne volatiele anesthetica, sevoflurane en desflurane, op de cellulaire adhesiemoleculen onderzocht werd. Ook deze twee anesthetica lieten verschillende effecten zien op de vorming van trombocyt-leukocyt conjugaties. Sevoflurane versterkte de formatie van deze conjugaties en de expressie van CD62P, terwijl desflurane het aantal conjugaties verminderde zonder effecten op de expressie van CD62P in de trombocyt. Teneinde deze effecten verder te onderzoeken word in Hoofdstuk 9 het effect van sevoflurane en desflurane bij 1 en 2 minimum alveolar concentration (MAC) op de activatie van selectines en β_2 -integrines bestudeerd. De resultaten van deze studies laten zien dat zowel sevoflurane als desflurane de expressie van neutrofiel adhesie beïnvloedden. Interessant is dat deze anesthetica gelijksoortige, maar ook tegengestelde effecten hebben op de expressie van adhesiemoleculen. Beide anesthetica beïnvloedden de afstoting en verminderde de expressie van PSGL-1, maar alleen sevoflurane verhoogde de oppervlakte-expressie van CD11b.

In het tweede deel van het proefschrift wordt het onderzoek naar de effecten van inotrope medicatie op cellulaire adhesiemoleculen beschreven.

Hoofdstuk 7 beschrijft de effecten van epinephrine op de trombocyt-neutrofiel adhesie. We tonen aan dat epinephrine de formatie van trombocyt-neutrofiel conjugaties verhoogd op een dosis afhankelijke wijze. Deze toename van trombocyt-neutrofiel aggregaten gaat gepaard met een toename van CD62P en GPIIb/IIIa op de trombocyt. We veronderstellen

dat deze toegenomen expressie van CD62P en GPIIb/IIIa verloop via α -adrenerge stimulatie, omdat blokkade van de α -adrenerge stimulatie deze upregulatie volledig tegen gaat. Een ander belangrijke bevinding was het feit dat epinephrine de agonist geïnduceerde upregulatie van CD11b en de downregulatie van L-selectine remt, een effect dat opgeheven wordt door β -adrenerge blokkade.

Evenzeer complex waren de reacties van trombocyten en monocytten na incubatie met de PDE3 inhibitor milrinone. De resultaten van deze onderzoeken staan beschreven in Hoofdstuk 8. Hier bleek de as van de agonist stimulatie een belangrijke rol te spelen bij het patroon van de expressie van de adhesiemoleculen en de daarop volgende aggregatie van leukocyten en trombocyten.

De resultaten van de in dit proefschrift beschreven studies laten zien dat de invloed van volatiele anesthetica en inotrope medicatie op de expressie van trombocyten- en leukocyten adhesiemoleculen en hun daaruit volgende interactie meervoudig zijn. In de *in vitro* studies konden wij geen eenduidige reactie van bloedcellen op volatiele anesthetica of inotrope medicatie ontdekken. De resultaten van onze studies kunnen daarom de resultaten van ander onderzoekers die concluderen dat de anti-adhesieve eigenschappen van volatiele anesthetica veroorzaakt worden door een afgenomen expressie van adhesiemoleculen, niet bevestigen. De invloed van volatiele anesthetica en inotrope medicatie op bloedcel interacties zijn complex en worden beïnvloed door vele factoren, wat het uitermate lastig maakt om de exacte effecten op het immuunsysteem te voorspellen. Er zijn echter wel aanwijzingen dat preoperatief en intraoperatief toegediende medicatie het immuunsysteem kan beïnvloeden. De zogenaamde cardioprotectieve effecten van volatiele anesthetica lijken toch vooral door andere mechanismen dan antiadhesive eigenschappen veroorzaakt te worden. Een mogelijke verklaring ligt in de 'priming' en het indirect opengaan van K_{ATP} kanalen, wat leidt tot een verminderde calciumconcentratie in het cytosol en de mitochondria en een verbeterde myocardiale zuurstofbalans tijdens ischaemie en reperfusie (127, 128)

Welk mechanisme exact verantwoordelijk is voor de positieve cardioprotectieve effecten en de ischaemie-reperfusie schade zal in de toekomst verder onderzocht moeten worden.

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Acknowledgments

I would like to thank everyone who helped to complete this thesis, especially:

Prof. R. Rossaint and Prof. G.J. Scheffer for giving me the opportunity, the time and the encouragement to initiate and to finish this thesis. Without them this thesis would never have been completed.

Dr. K. Hecker, for fun we had at the laboratory while working on our research projects. Even when the experiments failed we thought it was better to laugh than to despair.

Dr. T. Robitzsch for teaching me the art of flow cytometry. He was our steady support at the transfusion laboratory and the FACSCalibur.

Dr. B. Arnold: Bernd, ich hätte sicher keine Lust zu dieser zweiten Dissertation gehabt wenn du mich nicht kontinuierlich genervt hättest mit: Wieso machste daß denn nicht? Irgendwann habe ich dann doch keine Ausreden mehr gehabt.....

Dr. E. Robertson for the correction of the English language. Thank You!

To the laboratory staff, especially Renate, for helping with the blood samples and the laboratory measurements.

To all my colleagues at the RWTH Aachen and the UMC St. Radboud, who had to do the "clinical work" while I have been researching or writing.

To my former and present colleagues and friends of the cardiogroup for the support and team spirit.

Meiner Familie und besonders meiner Schwester, die zwar nie so genau verstanden hat worum es eigentlich ging und wieso ich noch einen Titel brauche, aber doch fand, daß ich jetzt mal fertig werden sollte.

E finalmente a minha marida Denize: A gente teve ultimamente uns tempos difíceis, e mesmo que você não entende porque eu arrumo ainda mais „sarna pra se coçar“ você nunca deixou faltar apoio, sem você não tinha conseguido tudo isso! Obrigada!



Curriculum vitae

Nicola Horn was born on 29th March in Marburg , Germany. From 1973 to 1986 she attended primary and secondary school in Berlin. From 1987 to 1994 she studied medicine at the Freie Universität Berlin and the Universidade Federal de Alagoas in Brasil. After graduation she began her training in Anesthesiology in spring 1994 at the Deutsches Herzzentrum Berlin. In fall 1997 she continued her training at the Rheinisch Westfaelische Universität Aachen. In 1998 she finished her German doctoral thesis "Preoperative variables and early postoperative factors related to a prolonged ICU stay after cardiac surgery" at the Humboldt-Universität in Berlin, which received the poster prize of the European Society of Anaesthesiologists.

After her registration as anesthesiologist in 2000, she accepted an appointment as a staff member at the Universitätsklinikum Aachen, where she qualified also as a specialist in Intensive Care Medicine in 2004.

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