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European Journal of Cardio-thoracic Surgery 13 (1998) 293–297

EUROPEAN JOURNAL OF CARDIO-THORACIC SURGERY

Endothelial cell injury in cardiac surgery: salicylate may be protective by reducing expression of endothelial adhesion molecules[1]

Gregor Zünd [a,b,*], Andrea L. Dzus [b], René Prêtre [a], Urs Niederhäuser [a], Paul Vogt [a], Marko Turina [a]

[a] *Clinic for Cardio-vascular Surgery, University Hospital, Rämistrasse 100, CH-8091 Zurich, Switzerland*
[b] *Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA*

Received 29 September 1997; received in revised form 3 December 1997; accepted 9 December 1997

Abstract

Objective: Cardiac surgery with cardiopulmonary bypass induces ischemia to the heart, hypoxemia to various tissues and release of endotoxins. The endothelial cell may suffer from hypoxia and trigger cascades of adverse reactions by activation of neutrophils through adhesion molecules. The authors measured expression of intercellular adhesion molecule-1 (ICAM-1), during hypoxia and normoxia and hypothesized that salicylate, which inhibits the nuclear factor-κB (NFκB), an hypoxia-dependent transmission factor, could reduce this expression. **Methods:** Human umbilical vein endothelial cells were cultured and exposed to normoxia and hypoxia in the presence of lipopolysaccharide (LPS). The endothelial cells were thereafter treated with salicylate or indomethacin under the same conditions. The surface expression of ICAM-1 was measured by whole cell enzyme-linked immunosorbent assay (ELISA) and the NFκB expression by Western blotting. **Results:** In the presence of LPS and under hypoxic conditions, the endothelial cells produced a $300 \pm 41\%$ increased expression of ICAM-1 compared with normoxia. The addition of salicylate (0.02–20 mM) completely inhibited the enhanced expression of ICAM-1, the addition of indomethacin at equivalent concentrations did not reduce ICAM-1 expression under either condition. **Conclusion:** ICAM-1 expression is greatly enhanced by the hypoxic endothelial cell in the presence of circulating endotoxin. Pre-treatment with salicylate completely abolishes the enhanced expression. The study suggests that salicylate administered before cardiopulmonary bypass might protect the heart against ischemic/reperfusion injuries and reduce the load of the overall inflammatory reaction.

Keywords: Endothelium; Ischemia/reperfusion injury; Treatment

1. Introduction

Cardiac surgery with cardiopulmonary bypass induces ischemia to the heart, hypoxemia to various tissues and a surge of endotoxins and cytokines [1,10,11]. Reperfusion injury to ischemic or hypoxic has been shown to be mediated, at least in part, by recruitment of leukocytes [2]. Since endothelial cells are anatomically located at the interface of the blood and tissue exchange they are directly influenced by circulating mediators and therefore of special interest [3].

Migration of leukocytes across the intact endothelium occurs through a concerted series of adhesion and de-adhesion events involving a number of cell surface adhesion proteins [4]. Previous studies have demonstrated that short-term hypoxia or anoxia induces increased adhesion of leukocytes to vascular endothelial cells mediated by the upregulation of endothelial adhesion molecules [5,6]. Intercellular adhesion molecule-1 (ICAM-1, CD54) mediates the firm adherence of leuko-

* Corresponding author. Tel.: +41 1 2551111; fax: +41 1 2554369; e-mail: zund@chi.usz.ch

[1] Presented at the 11th Annual Meeting of the European Association for Cardio-thoracic Surgery, Copenhagen, Denmark, 28 September–1 October, 1997

1010-7940/98/$19.00
PII S1010-7940(97)00318-7

cytes to the endothelium [4]. ICAM-1 expression is readily induced by stimulation of cell surface receptors by a number of inflammatory mediators, including lipopolysaccharides (LPS) and cytokines [4]. In addition, ICAM-1 is regulated by the transcription factor NF-κB (nuclear factor-κB) [7] which has been shown in previous studies to be regulated by hypoxia [8].

A strategy that would inhibit the migration of leukocytes across the endothelial layer would blunt the inflammatory response induced by cardio-pulmonary bypass and prevent, or at least reduce, the tissue ischemia-reperfusion injuries [9]. Because salicylate is inhibiting NF-κB, the authors hypothesized that the administration of this drug would reduce the enhancement of ICAM-1 expression. To test the hypothesis, the authors used an in-vitro model of endothelial cell and the authors were simulating the CPB.

2. Material and methods

2.1. Objectives of the study

First step, measurement of endothelial ICAM-1 activity under hypoxic conditions after exposure to salicylate or indometacin. Second step, proof of the salicylate effect by Western blotting of NFκB.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained and harvested using 0.1% collagenase (Worthington Biochemical, Freehold, NJ). Endothelial monolayers were established, maintained and subcultured with DMEM (Gibco, Grand Island, NY) containing 10% heat inactivated fetal calf serum, glucose, pyruvate, glutamine, penicillin and streptomycin. Confluent endothelial monolayers exhibited typical cobblestone appearance and expressed characteristics of endothelial cells like the uptake of acetylated low density lipoproteins (Biomedical Technology, Stoughton, MA). Endothelial cell passages 2–5 were used for experiments.

2.3. Creation of hypoxic conditions

Confluent endothelial monolayers were exposed to hypoxia as follows, growth medium was replaced with fresh media equilibrated with hypoxic gas mixture and cells were placed in the hypoxic chamber (Coy Laboratory Products, Ann Arbor, MI). This hypoxia chamber consists of an airtight glove box with the atmosphere continuously monitored by an oxygen analyzer interfaced with oxygen and nitrogen flow adaptors. Oxygen concentrations were adjusted by oxygen and nitrogen flow adaptors and were obtained by the balance between nitrogen, carbon dioxide (ambient 5% CO_2) and water vapor from the humidified chamber. Media pO_2 and pH were measured in a Ciba-Corning 238 Blood Gas Analyzer (Chiron Diagnostics, Norwood, MA).

2.4. Cell surface immunoassay

ICAM-1 cell surface expression was quantified using a cell surface enzyme-linked immunosorbent assay (ELISA). Endothelial cells were grown and assayed for antibody binding following exposure to normoxia or hypoxia in the presence of LPS. Endothelia were lightly fixed with paraformaldehyde (1% w/v in phosphate buffered saline) to preserve cell surface protein. Cells were washed with HBSS (Sigma, St. Louis, MO), blocked with media for 30 min at 4°C. Anti-ICAM-1 mAb (clone P2A4 obtained from the Developmental Studies Hybridoma Bank, Iowa City IA, used as undiluted cell culture supernatant) was added to fixed cells and allowed them to incubate for 2 h at 4°C. Where indicated, mAb to MHC class I (clone W6/32 obtained from the American Type Culture Collection, used as 1:100 diluted ascitic fluid) was used as control. After washing with HBSS, a peroxidase conjugated sheep anti-mouse secondary antibody (Cappel, West Chester, PA) was added. After washing plates were developed by addition of peroxidase substrate (2,2′-azino bis(3-ethylbenzthiazoline-6-sulfonic acid), 1 mM final concentration, Sigma) and read on a microtiter plate spectrophotometer at 405 nm (Molecular Devices). Controls consisted of media only and secondary antibody only.

2.5. NFκB Western blotting

HUVEC were grown to confluence on six well plates and exposed to experimental conditions, washed, lysed and debris was removed as described above. Proteins were measured (DC protein assay, BioRad, Hecules, CA). Samples (50 μg/lane) of HUVEC lysates were separated by non-reducing SDS PAGE, transferred to nitrocellulose and blocked overnight in blocking buffer (250 mM NaCl, 0.02% Tween-20, 5% goat serum and 3% bovine serum albumin). Primary Ab (rabbit polyclonal specific for p65 subunit of NFκB, Biomol Research Labs, Plymouth Meeting, PA) was added for 3 h, blots were washed and species-matched peroxidase-conjugated secondary Ab was added. Labeled bands from washed blots were detected by ECL. Resulting 65 kD NFκB bands were quantified from scanned images using NIH Image software (Bethesda, MD). Such 65 kD band were specific for NFκB since preincubation of rabbit polyclonal antibody with standard p65 antigen (provided by Biomol as a control) resulted in a diminution of the 65 kD band by more than 70%.

2.6. Data presentation

ELISA data were compared by one or two-factor analyze of variance (ANOVA) or by student's *t*-test. Values are expressed as the mean and S.E.M. of *n* experiments.

3. Results

Endothelial monolayers tolerated exposure to hypoxia well (ambient O_2 as low as 1%, for up to 24 h). No changes in morphology were observed and no evidence of cell death was apparent.

3.1. Sodium salicylate normalizes hypoxia-elicited ICAM-1 surface expression

The authors first examined the induction of ICAM-1 surface expression induced by LPS with endothelial exposure to hypoxia and normoxia. Under hypoxic conditions a 2-fold increase in ICAM-1 expression was observable on HUVEC (two-way ANOVA, $P < 0.025$ compared with normoxia) (Figs. 1 and 2). As shown in Fig. 1, salicylate (NaSal, concentration range 0.02–20 mM) dose dependently decreased LPS-stimulated ICAM-1 on HUVEC under conditions of both hypoxia (one-way ANOVA, $P < 0.01$) and normoxia (one-way ANOVA, $P < 0.05$). Endothelial exposure to indomethacin (concentration range 0.02–20 mM) at equivalent concentrations did not influence ICAM-1 expression under either condition (Fig. 2, $P =$ not significant for both). In comparison sodium salicylate but

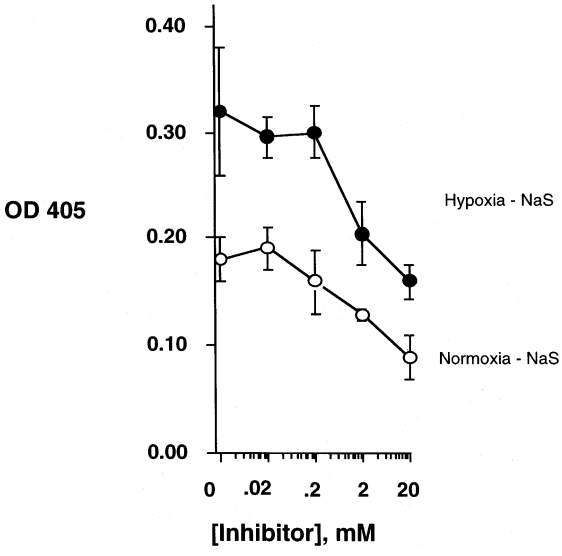

Fig. 1. ICAM-1 expression of endothelial cells exposed to hypoxia (ambient O_2 1%, closed symbols) or normoxia (ambient O_2 21%, open symbols) in the presence of LPS (100 ng/ml) with and without indicated concentrations of sodium salicylate (NaS) for 18 h.

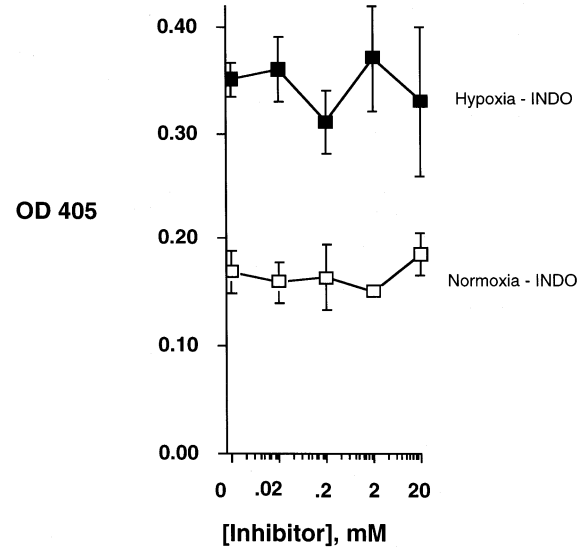

Fig. 2. ICAM-1 expression of endothelial cells exposed to hypoxia (ambient O_2 1%, closed symbols) or normoxia (ambient O_2 21%, open symbols) in the presence of LPS (100 ng/ml) with and without indicated concentrations indomethacin (INDO) for 18 h.

not indomethacin is inhibiting the expression of LPS stimulated HUVEC of ICAM-1 under hypoxic conditions.

3.2. Mechanism of hypoxia elicited upregulation of ICAM-1

The authors demonstrated that hypoxia alone increases cellular content of the p65 subunit of NFκB. Increased periods of hypoxia alone resulted in a graded increase in p65 content over normoxic controls (Fig. 3). The concentrations of NaSal (2 and 20mM) determined to be effective for decreasing ICAM-1 expression also resulted in decreased endothelial NFκB under conditions of both normoxia and hypoxia (Fig. 3).

4. Discussion

Under the conditions of cardiopulmonary bypass and clamped ascending aorta, a precondition for successful cardiac surgery, ischemia appears to the heart, hypoxemia to various tissues followed by the release of endotoxins and cytokines [1,10,11,13–15]. It has been known for several years that the inflammatory response to extracorporal circulation triggers underlies the occasional development of postoperative organ dysfunction, particularly of the lungs [1]. Tissue injury resulting from reperfusion of hypoxic tissue has been shown to be mediated, at least in part by recruitment of leukocytes [2]. Furthermore, such recruitment of leukocytes involves a concerted series of adhesion and de-adhesion event of leukocytes and vascular endothelium [4]. An

important adhesion molecule in this cascade is ICAM-1. As shown in previous studies, ICAM-1 is upregulated under the conditions of hypoxia and the influence of cytokines or endotoxins [8]. These in vitro conditions are even by part comparable to cardiac surgery and extracorporal circulation and in-deed, ICAM-1 is upregulated in patients undergoing cardiac surgery [12]. These findings suggest that future strategies of myocardial protection must not be limited to interventions targeted at the heart itself but should also encompass those designed to blunt the inflammatory response to cardiopulmonary bypass. Thus the authors hypothesized that the enhancement of ICAM-1 under the conditions of hypoxia and LPS may be prevented by the administration of drugs like salicylate or indomethacin in a cardio-pulmonary bypass model.

There are different ways to protect the leukocyte-endothelial interactions of the ischemia-reperfusion injury. Therapeutical interventions can be categorized as those targeted at inactivating the triggers of the response and those targeted at inactivating the effectors of the response. The former category primarily encompasses drugs or devices designed to reduce cytokine and endotoxin release such as heparin-coated extracorporal circuits or glucocorticoids [16–20]. The second category of interventions includes drugs or devices to attenuate the effects of adhesion molecules. As the authors have shown in the result part under the condition of hypoxia NFκB was increased. The addition of sodium salicylate, but not indomethacin, diminished augmentation of both ICAM-1 expression and cellular content of NFκB. Such observations are consistent with a previous report indicating that sodium salicylate can inhibit induction through NFκB [28]. A contribution of cyclooygenase in this context could be excluded by the addition of indomethcin which had no effect to the expression of ICAM-1 under hypoxic conditions. The adhesion molecules, whether expressed on leukocytes or endothelial cells can be blocked by monoclonal antibodies. This approach has been examined in various animal models of myocardial ischemia, including a surgically relevant preparation of heterotopic rabbit heart transplantation and an isolated blood-perfused swine-heart model subjected to 6 h of cold cardioplegic ischemia [21,22]. None of these genetically engineered compounds is clinically available yet. Alternative therapies that can interfere with adhesion molecules and have been approved for human use are being considered. Both glucocorticoid and adenosine-regulating agent acadesine have been shown to decrease CD11b/CD18 upregulation in patients undergoing open-heart procedures [23,24]. Furthermore, low-dose aprotinin has been reported to have a similar anti-inflammatory effect to that of methylprednisolon in decreasing the bypass-induced release of TNF-α and neutrophil CD 11b upregulation [25]. A possible more effective approach for limiting the effects of neutrophil interactions to vascular endothelium might be the elimination of themselves by sequestering them into appropriate filters. A recent Japanese study has shown that leukocyte and platelet depletion with a blood cell separator reduces postoperative respiratory dysfunction [26]. At the institution the efficiency of such a device was tested and leukocyte filtration failed to improve postoperative lung function [27].

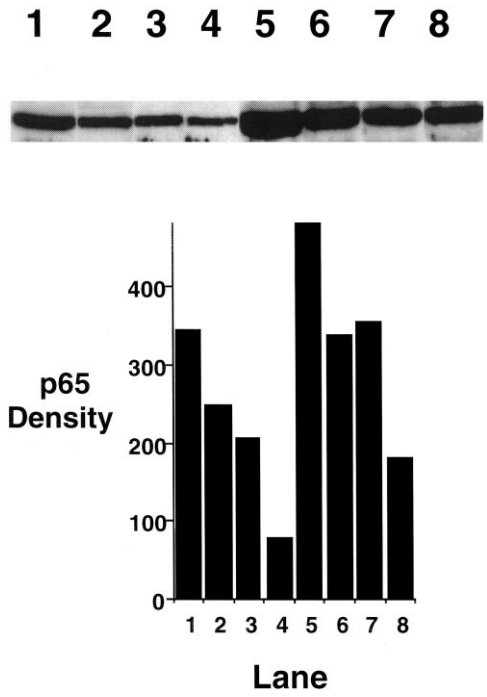

Fig. 3. NFκB was measured by Western blotting the p65 subunit of NFκB. Lane 1–4 represent normoxia, lane 5–8 hypoxia. The endothelial cells are stimulated with LPS (50 ng/ml) in lane 1 and 5. Salicylate was added to lane 3 and 7 at a concentration of 2 mM at to lane 4 and 8 at a concentration of 20 mM. Lane 2 and 6 contain only media.

The results suggest that the addition of salicylate to the cardio-pulmonary bypass model completely inhibited the enhanced expression of ICAM-1 by blocking the hypoxia dependent NFκB transmission factor, the addition of indomethacin at equivalent concentrations did not influence ICAM-1 expression under either condition. Thus, this study suggests that salicylate administered before cardiopulmonary bypass might protect the heart against ischemic/reperfusion injuries and reduce the load of the overall inflammatory reaction.

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