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Membrane stabilization in harvested vein graft storage: effects on adhesion molecule expression and nitric oxide synthesis

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Abstract

Objective: Expression of cellular adhesion molecules in human saphenous vein grafts may occur even during harvesting and storage, before the grafts have been implanted as bypass conduits. This may play a role in graft adaptation to arterial flow conditions, which may play an important role in late graft patency. In this study, ketotifen, a mast cell membrane stabilizing agent was studied for its effects on reducing endothelial reactivity during storage of harvested vein graft segments. **Methods**: Human saphenous vein grafts, obtained from seven patients and then divided into two equal parts of control and study specimens, were stored in either heparinized blood (Group A) or heparinized blood containing 100 μg/ml ketotifen (Group B) for 1 h at room temperature. Specimens were analyzed by Western blotting to quantify ICAM-1, E-selectin, P-selectin, VCAM-1, and inducible nitric oxide synthase (NOS-2) expression, as well as tissue cGMP levels in response to topical application of an endothelium-independent vasodilator. **Results**: ICAM-1, E-selectin and P-selectin expression did not differ between the groups. However, VCAM-1 expression was significantly lower in Group B (460 ± 29 vs. 289 ± 50, $P = 0.01$). NOS-2 expression (488 ± 64 vs. 577 ± 38, $P = 0.02$) and tissue cGMP levels (2.2 ± 0.6 pmol/ml vs. 5.7 ± 1.7 pmol/ml, $P = 0.01$) in response to nitroglycerin (24 ± 10% vs. 11±5%, $P = 0.02$) were higher in Group B. **Conclusions**: Of all of the adhesion receptors studied, only VCAM-1 expression was reduced by a mast cell membrane-stabilizing agent, perhaps because of activation of the venous endothelium during harvest prior to ketotifen exposure. However, ketotifen also augmented NOS-2 expression, increased tissue cGMP levels in response to nitroglycerin. These actions may improve vascular homeostasis in the venous graft, suggesting the possibility that this strategy may improve long-term graft patency.

Keywords: Saphenous; Vein graft; Adhesion molecule; Reoperation; Membrane stabilizer; Nitric oxide

1. Introduction

Although autologous saphenous vein grafts are used widely in current practice for both coronary and peripheral arterial operations, there is often limited long-term patency of these grafts. Cellular mechanisms responsible for atherogenesis in native vessels may also be operative in vein grafts, however, additional unique mechanisms may contribute to saphenous vein graft atherosclerosis. Although there is no immunological disparity between these isografts and the host, during the preparative stages and during the process of arterialization, these vein grafts are subject to stresses which differ from those to which they were exposed in their native locale. The current work examines in particular the effects of cell membrane stabilizing during storage period of harvested grafts, with the implicit hypothesis that maintaining early vascular homeostasis in terms of adhesion molecule and nitric oxide expression may serve to preserve long-term graft patency.

Leukocyte adhesion to vascular endothelium is a primary step in the early stages of atherosclerosis, which is mediated by the interaction of cellular adhesion molecules expressed on the surfaces of both endothelial cells and leukocytes. Cellular elements of the blood, especially monocytes and lymphocytes, are known to signal adhesion receptor expression on endothelial cell surface by releasing mediators such as histamine [1]. Histamine is in fact a potent stimulus for neutrophil accumulation in tissues; histamine triggers leukocyte rolling by promoting the expression of P-selectin

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on the endothelial cell surface, enhances ICAM-1 expression through its actions on H1 receptors, and also induces the upregulation of E-selectin expression [1,2]. Endothelial cells also release nitric oxide (NO) which influences endothelial cell-leukocyte interactions.

Ketotifen, a second generation histamine H1-receptor antagonist, has long been used in the management of allergic disorders. In addition to histamine receptor antagonism, ketotifen reduces mast-cell degranulation and decreases the release of histamine, mast cell proteases, myeloperoxidase, leukotrienes, platelet activating factor (PAF), various prostaglandins, and inhibits polymorphonuclear aggregation and migration [3]. Ketotifen also enhances the expression of NO synthase [4]. In toto, these actions attenuate inflammatory responses. The current study was designed to determine the in vitro effects of this mast cell membrane-stabilizing agent on saphenous vein grafts in terms of inhibiting endothelial reactivity when added to the storage solution.

2. Materials and methods

Human saphenous vein graft segments were obtained from seven patients undergoing coronary artery bypass grafting. Each specimen was divided into two equal parts, representing the control and the study specimen. Two group of tubes containing heparinized autologous blood were set up as described below: Group-A: Control, heparinized blood only; Group-B: heparinized blood plus ketotifen fumarate (Sigma Chemical Co., St. Louis, MO) at a concentration of 100 μg/ml. Samples were stored at room temperature for a period of 1 h, which is similar to the average storage period for a harvested vein before implantation as a bypass conduit. In addition, this time was selected because more than 80% of the total histamine release was reported to occur within one hour of stimulation in a previous study [5]. A segment of each sample was treated with 0.05% nitroglycerin in 5% dextrose in lactated Ringer solution. This nitroglycerin-treated segment was used for inducible nitric oxide synthase analysis and for the subsequent ELISA assay for determination of cGMP content. All segments were then snap-frozen in liquid nitrogen and stored in a −70°C freezer until the time of analysis. This study was conducted in accordance with the Columbia University Institutional Review Board approvals IRB 0278 and IRB 6422.

2.1. Immunostaining

To demonstrate the fact that adhesion molecule expression can increase during the storage period, samples were obtained fresh or after 1 h of storage in heparinized blood. These samples were then fixed in formaldehyde. Fixed specimens were immunostained using a P-selectin antibody (Research Diagnostics Inc., Flanders, NJ) at 1:100 dilution for 1 h at 37°C. After subsequent washes with phosphate buffered saline (PBS), a second antibody, an anti-rabbit IgG-alkaline phosphatase conjugate (Sigma Chemical Co.) was added and incubated for 30 min at 37°C and then stained by Fast Insoluble Alkaline Phosphatase Substrate (Sigma Chemical Co.). Counterstaining was applied as Mayer's hematoxylin dye for 5 min at room temperature. The specimen was then washed, dried and mounted with a cover glass. This study was performed to demonstrate that endothelial expression of cellular adhesion receptors may occur even during short storage periods after harvesting saphenous vein grafts.

2.2. Western blotting

Tissue samples were homogenized for 30 s at 4°C with a Polytron (Kinematica, GmbH, Krienz-Luzerne, Switzerland) with ice-cold 20 mM Tris–HCl, pH 7.4, containing 100 mM NaCl, 2 mM phenylmethylsulphonyl fluoride (PMSF), 0.5 mg/l leupeptin and 0.7 mg/l pepstatin. Homogenates were shaken at 4°C for 3 h, then centrifuged at 13 000 rev./min for 10 min at 4°C and, the supernatant was collected as the source of sample protein. Samples were run in a 7.5% polyacrylamide gel (Fisher Scientific, Pittsburgh, PA) and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked for non-specific binding using 1% bovine serum albumin (BSA) in PBS for 12 h at 4°C. The membrane was then incubated in 0.05% BSA and 0.05% Tween 20 in PBS containing an anti-human ICAM-1 antibody (R&D Systems Inc., Minneapolis, MN) at a concentration of 1:1000 for 1 h. After subsequent washes, membrane was incubated with an anti-goat IgG-peroxidase conjugate (Sigma Chemical Co.) at 1:2000 concentration in PBS, 0.5% BSA and 0.05% Tween 20 for 45 min. Then the membrane was washed twice before continued processing for enhanced chemiluminescence (ECL) detection (Amersham, Piscataway, NJ). The staining intensity of specific bands was quantified by densitometric scanning by a computer software (Molecular Analyst, Bio-Rad Laboratories, Hercules, CA). Calculated densities were then statistically compared. The same procedure was repeated for the detection of E-selectin, P-selectin and VCAM-1 using corresponding antibodies (R&D Systems Inc.) and a rabbit derived anti-NOS 2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for the detection of inducible nitric oxide synthase (NOS-2).

2.3. Enzyme-linked immunosorbent assay

Tissue homogenates in PBS were added to 5% trichloroacetic acid and kept on ice for 30 min. Those homogenates were then centrifuged at 4 000 rev./min for 15 min. Supernatants were collected and washed three times with ether. After storing the homogenates at 20°C for 5 min, cGMP content of nitroglycerin-treated vein segments was measured by an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc.). This assay is based on the competitive binding technique in which cGMP present in a sample competes with a fixed amount of alkaline phos-

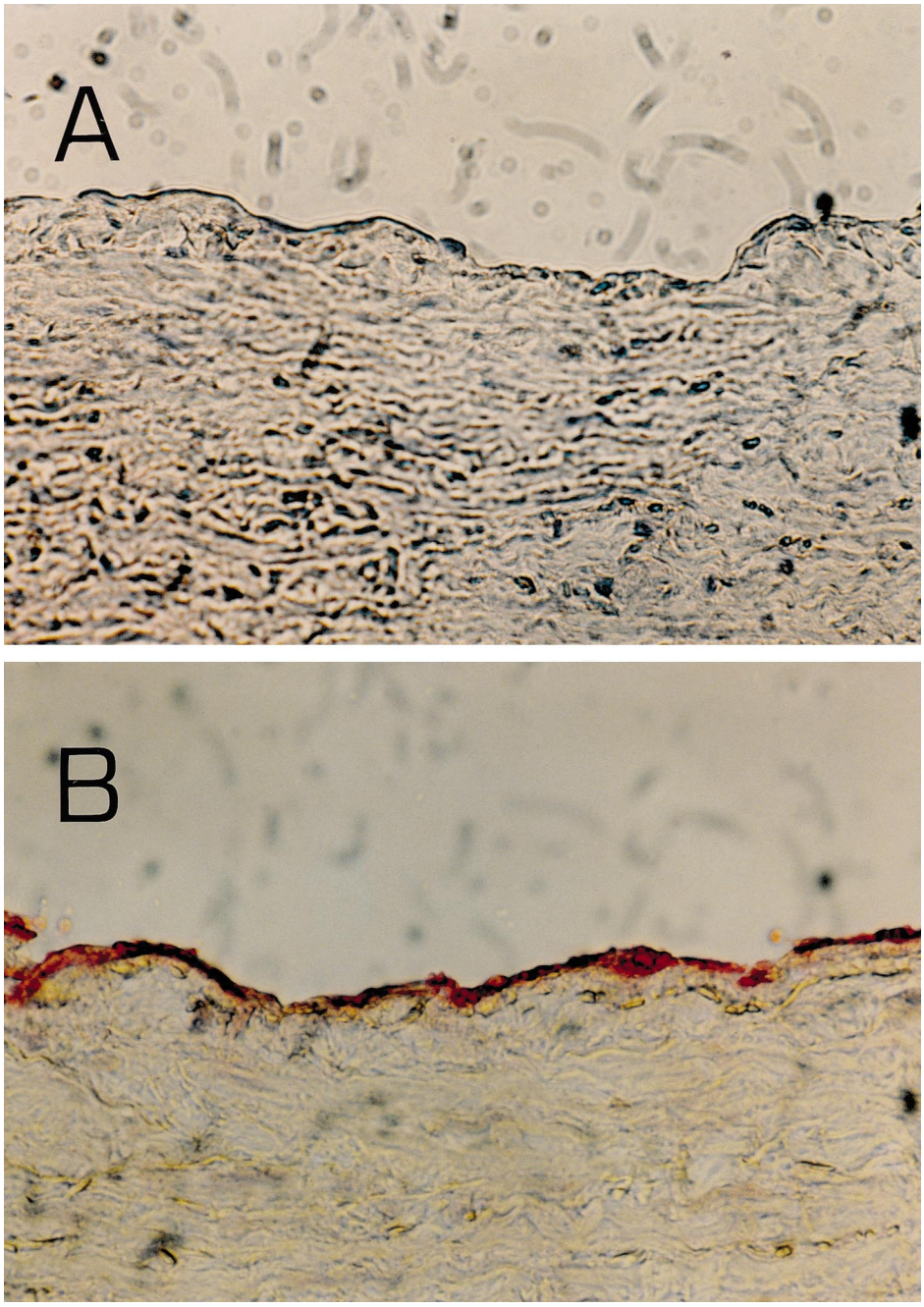

Fig. 1. P-Selectin expression on the endothelial border of human saphenous vein segments during storage in heparinized blood. (A) Fresh specimen; (B) after 1 h of storage.

phatase-labeled cGMP for sites on a rabbit polyclonal antibody. This primary antibody binds to a goat anti-rabbit antibody coated onto the microplate. The absorbance was read at 405 nm using a Biokinetics microplate reader Model EL 340 (Biotek Instruments, Winooski, VT).

2.4. Statistical analysis

Means are presented ± SD. For each outcome variable exactly one sample per patient and per group was used (i.e. there are seven pairs of numbers used for each statistical test. All statistics were obtained by Wilcoxon Matched-Pairs Signed Ranks Test using SPSS statistical software (release 8.0; SPSS Inc., Chicago, IL).

3. Results

3.1. Immunostaining

Immunostaining was performed to demonstrate the topography of a typical harvested vein segment and to demon-

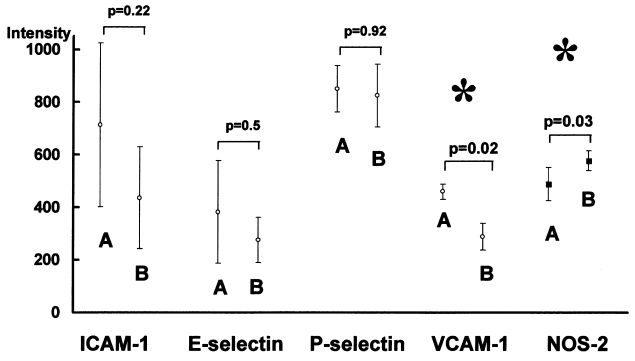

Fig. 2. ICAM-1, E-selectin, P-selectin, VCAM-1 (see also Fig. 3) and NOS-2 (see also Fig. 4) expression of human saphenous vein segments, quantified as the intensity of a given band on the Western blot, presented as means ± SD.

strate that the storage period per se is associated with an increased expression of adhesion receptors. This initial study showed that P-selectin expression is markedly increased on the endothelial border of the harvested saphenous vein after a one-hour storage period in heparinized blood at room temperature (Fig. 1A, fresh specimen; Fig. 1B, after 1 h of storage).

3.2. Cellular adhesion molecule expression

Western blot analyses were then performed to determine the effects of ketotifen treatment on ICAM-1, E-selectin, P-selectin and VCAM-1 expression. This expression, determined as the intensity of a given band on the Western blot, did not differ between Groups A and B for ICAM-1, E-selectin and P-selectin; mean intensities for scanned bands for group A and B were 713 ± 311 versus 436 ± 193 for ICAM-1 ($P = 0.22$), 382 ± 195 versus 275 ± 86 for E-selectin ($P = 0.5$) and, 850 ± 88 vs. 824 ± 119 for

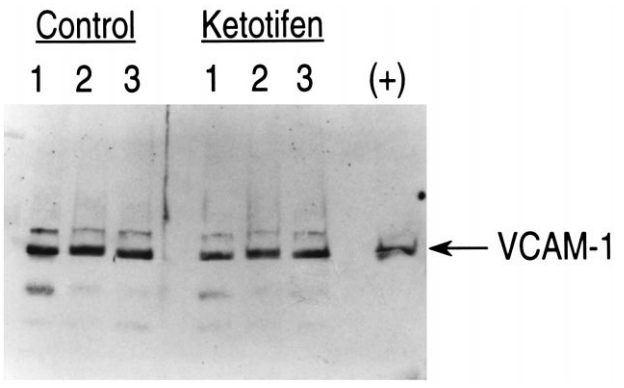

Fig. 3. Representative immunoblot demonstrating VCAM-1 expression. (+): positive control lane for VCAM-1 molecule. Each specimen was taken from a given patient and divided into two equal parts, one being treated with heparinized blood (Group A) and the other with ketotifen in heparinized blood (Group B). The number over each control lane represents a single patient, with the data from the corresponding vein segment from the same patient shown for ketotifen-treated segments.

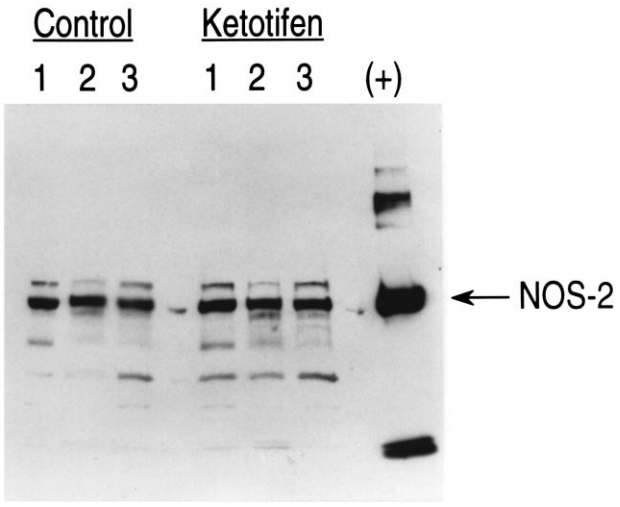

Fig. 4. Representative immunoblot demonstrating NOS-2 expression. (+): positive control lane for NOS-2 molecule. Each specimen was taken from a given patient and divided into two equal parts, one being treated with heparinized blood (Group A) and the other with ketotifen in heparinized blood (Group B). The number over each control lane represents a single patient, with the data from the corresponding vein segment from the same patient shown for ketotifen-treated segments.

P-selectin ($P = 0.92$; Fig. 2). However, in group B, the intensity of the VCAM-1 bands were significantly lower (460 ± 29 for Group A versus 289 ± 50 for Group B; $P = 0.02$) as seen in Figs. 2 and 3.

3.3. Inducible nitric oxide synthase (NOS-2) detection

Measured intensity of NOS-2 bands were significantly higher in ketotifen-treated segments (488 ± 64 for Group A vs. 577 ± 38 for Group B, $P = 0.03$; Figs. 2 and 4).

3.4. cGMP content

ELISA assay of homogenates demonstrated that tissue cGMP levels were higher in Group B upon stimulation with nitroglycerin than they were for Group A (2.2 ± 0.6

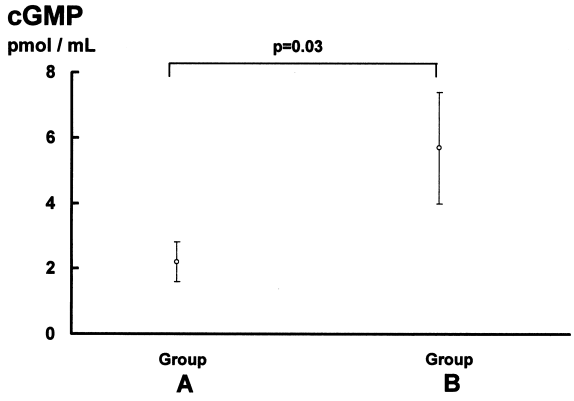

Fig. 5. Tissue cGMP levels, determined by ELISA, presented as means ± SD.

pmol/ml vs. 5.7 ± 1.7 pmol/ml, respectively, $P = 0.03$; Fig. 5).

4. Discussion

Leukocyte adhesion to vascular endothelium is a crucial step in the early stages of atherosclerosis, which is mediated by the interaction of adhesion receptors expressed on the surfaces of both endothelial cells and leukocytes. Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expressed on endothelial cells mediate leukocyte binding to endothelial cells through interactions with their integrin counter receptors on leukocytes, CD 11/18 and very late antigen-4 (VLA-4), respectively. Proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-a) induce the expression of endothelial adhesion molecules [2]. Circulating leukocytes adhere to vessel wall using those molecules, leave the blood stream and, enter the tissue. After having been activated, they release toxic substances which may cause a considerable amount of damage to the adjacent tissues [6]. The in vivo significance of ICAM-1 and VCAM-1 expression on the endothelial cell surface was suggested by observations of their enhanced expression in human atherosclerotic lesions. The role of increased ICAM-1 and VCAM-1 expression in atherogenesis is unknown at present; however, their enhanced expression might facilitate leukocyte adherence and thus favor and sustain inflammation. As human saphenous vein endothelial cells retain their ability to synthesize leukocyte chemoattractants such as IL-8 [7] and to express adhesion receptors as shown here, it is possible that attenuation of the expression of these adhesion receptors may be potentially beneficial in suppressing atherosclerotic degeneration of coronary artery bypass grafts. This may be especially important in that the harvest and storage procedures themselves upregulate the expression of such molecules, as is shown in this paper for P-selectin expression.

Human blood monocytes and lymphocytes contain substantial amounts of histamine which may be released upon stimulation with either substance-P, C5a or the calcium ionophore [8]. Histamine release from activated mast cells is attenuated by exogenous NO and exacerbated by NO synthesis inhibitors. Moreover, the mast cell produces a nitric oxide-like factor that can directly feed back to decrease histamine release [9]. NO is an endogenous inhibitor of leukocyte adhesion, activation and chemotaxis. NO activates guanylate cyclase and increases the conversion of GTP to cGMP, which in turn reacts with cGMP-dependent protein kinase and causes a cascade of changes in protein phosphorylation, including dephosphorylation of myosin light chain, leading to cell relaxation. Therefore, a reduction in cGMP may cause endothelial cell contraction and increase the size of interendothelial junctions, resulting in a leaky endothelial barrier. Reduced levels of NO synthesis may also lead to superoxide accumulation, which could directly cause an increase in endothelial permeability and the release of various mast cell- derived chemical agents, including PAF and histamine [9]. NO modulates mast cell degranulation by competing with superoxide anion, a potent activator of mast cell activation and degranulation [10]. On the other hand, endothelial free radical generation plays an important role in regulation of intercellular adhesion molecules and leukocyte recruitment. Therefore, the suppression of ICAM-1 expression on the endothelial cells by endogenous NO might contribute to suppressing inflammation in vivo.

Several lines of evidence from both in vitro and in vivo studies have recently suggested a role of NO as an anti-atherogenic autocoid. Accumulating evidence suggests that NO also influences endothelial cell-leukocyte interactions [10]. In addition, NO suppresses T-cell proliferation and inhibits migration of neutrophils and monocytes [11]. Among its immunological functions, NO has cytotoxic and cytostatic actions and therefore often is considered a hallmark of macrophage activation [12]. Recent evidence suggests that increased levels of NO impair leukocyte-endothelium interaction, inhibit atherosclerotic intimal thickening [13], and inhibits vasomotor reactivity [14]. Therefore, the suppression of ICAM-1 and VCAM-1 expression on saphenous vein endothelial cells by endogenous NO could prevent development of atherosclerotic lesions in vivo.

Ketotifen, a second generation histamine H1 receptor antagonist has long been used in the management of allergic disorders. In addition to histamine receptor antagonism, some of these effects may be related to the inhibition of the release of mast-cell and neutrophil-derived proinflammatory mediators. In various experimental and clinical conditions ketotifen was noted to reduce mast-cell degranulation and to decrease the release of histamine, mast-cell proteases, myeloperoxidase, leukotrienes, platelet activating factor (PAF), and various prostaglandins [3]. Ketotifen also stimulates NO synthase activity by mechanisms other than H1-receptor antagonism and, administration of this drug causes a modest decline in blood pressure and reduces vascular resistance [4]. Ketotifen stabilizes the cell membrane and/or alters its properties with respect to calcium permeability. It causes a dose related decrease in acetyl-CoA acetyltransferase stimulation and antigen-induced PAF release. Ketotifen also blocks the decline in cAMP levels in leukocytes caused by antigenic challenge [15].

In this study, we investigated any beneficial effect of ketotifen by means of graft reactivity when added to the storage solution (heparinized blood for most instances) of saphenous vein grafts before their implantation to arterial system as graft conduits. Although ketotifen did not reduce ICAM-1, E-selectin and P-selectin expression in vitro, ketotifen treated segments expressed less VCAM-1 and more NOS-2. This may be due to either a different mechanism

of action for ketotifen in which VCAM-1 and NOS-2 involved or a possible NOS-2 induction pathway inversely related to VCAM-1 receptor activation. However current data did not provide a scientific explanation for this observation and further studies in this area are certainly needed to go beyond speculation. From a functional standpoint, ketotifen treatment was associated with increased cGMP production in response to nitroglycerin and vein segments exhibited a greater degree of relaxation. Taken together, these data show that venous endothelium may be activated by the ex vivo storage period, and that the storage period presents an opportunity to modulate vascular properties. Mast cell stabilizing agents such as ketotifen represent one potential means of treating ex vivo venous segments to help preserve vascular homeostatic properties. Studies such as these may help to establish this class of agents as being potentially useful for organ storage and for settings of anticipated reperfusion injury, such as occurs during cardiac surgical procedures.

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