Unstable angina activates myocardial heat shock protein 72, endothelial nitric oxide synthase, and transcription factors NFκB and AP-1

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Abstract

Objective: Unstable angina may improve the clinical outcome of acute myocardial infarction, but increases the morbidity and mortality of open heart surgery. We hypothesized that unstable angina influences the myocardium, and investigated the expression of the inducible heat shock protein 72 (HSP72), constitutive HSP73, and endothelial nitric oxide synthase (eNOS), and activation of the transcription factors NFκB and AP-1 in cardiac tissue. Methods: Biopsies were taken from the right atrium of 15 patients with unstable and 15 with stable angina undergoing coronary artery bypass grafting. Immunoblotting with monoclonal antibodies against HSP72, HSP73, and eNOS were performed on protein extracts, while nuclear proteins were assessed by electromobility shift assay. Results: When calculating the optical density of the bands, patients with unstable angina had more than twice as much HSP72 and eNOS as stable patients (P < 0.005), while HSP73 was similar in both groups. Nuclear translocation of NFκB and AP-1 was found in patients with anginal pain shortly before surgery, but not in stable patients or in patients without symptoms for 4 days or more prior to surgery. Conclusions: HSP72 and eNOS, which may be associated with cardioprotection in ischemic preconditioning, are increased in atrial tissue of patients with unstable angina. Activation of NFκB and AP-1, which regulate a battery of inflammatory genes, was found in hearts of unstable patients. NFκB activation may induce a myocardial proinflammatory state, possibly making the unstable myocardium more susceptible to the inflammation induced by open heart surgery. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Unstable coronary syndromes are currently believed to be caused by rupture of an atherosclerotic plaque due to local events, which may be of infectious, immunologic, or general inflammatory etiology [1–3]. It is well documented that patients with unstable coronary syndromes have a systemic inflammatory response with increase of the acute phase reactant C-reactive protein, soluble leukocyte adhesion molecules, and markers of leukocyte activation [2,3]. The redox sensitive transcription factor nuclear factor kappa B (NFκB) is activated in peripheral leukocytes from patients with unstable angina [4]. Whether the systemic response of unstable angina is cause or consequence of disease is presently not determined.

There is clinical evidence that the myocardium itself is influenced by unstable angina. Patients with unstable angina prior to acute myocardial infarction have improved outcome: reduced mortality, reduced severe congestive heart failure and shock, smaller infarcts as evaluated by CK-MB leakage, and less Q-wave activity than patients with acute infarction of sudden onset [5–7]. The latter phenomenon has been attributed to a preconditioning effect caused by the intermittent ischemia and reperfusion of unstable angina. Paradoxically, patients with unstable coronary syndromes undergoing acute coronary artery bypass grafting have increased morbidity and mortality compared to elective patients [8]. An important feature in

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2. Methods

2.1. Patient population

The study was approved by the Ethics Committee of the Karolinska Hospital and conforms with the principles outlined in the Declaration of Helsinki (Cardiovascular Research 1997;35:2–3). Fifteen consecutive patients with unstable angina and 15 patients with stable angina scheduled for coronary artery bypass grafting (CABG) were investigated. All patients gave informed consent to participate. Unstable patients were defined as rest angina or excertional angina resistant to oral nitroglycerine; upon admission to the coronary care unit transient ischemic ST-segment changes were found; the patients were treated with nitroglycerine i.v. and low molecular weight heparin (Fragmin®), and medical therapy with calcium (4/15) and beta-adrenergic antagonists (15/15) was optimized. When coronary angiography revealed double or triple vessel disease anatomically unsuitable for PTCA combined with persistent symptoms, the patients were scheduled for acute or semi-acute CABG and included in the study. The start of acute symptoms prior to surgery was 9±3 days (mean±S.E.M.). At the time of surgery less than 30% percent of the unstable patients had ongoing symptoms. For EMSA analysis the patients were subgrouped into those with ongoing symptoms at the time of surgery (n=4), those with intermediate symptoms (anginal pain 1–2 days prior to surgery) (n=6), and those who had been without symptoms 3 days or more before surgery (n=5). Stable patients had excertional angina, positive exercise stress-test combined with double or triple vessel disease, and were scheduled for elective CABG. All were treated with beta-adrenergic antagonists and organic nitrates preoperatively, while 4/15 were treated with calcium antagonists. One stable patient had left main stem stenosis. The unstable patients were older than the stable patients (P<0.01) (Table 1). The other preoperative data (ejection fraction, EF, number of previous myocardial infarctions, extent of coronary artery disease, months of angina pectoris prior to surgery) were not statistically different between groups (Table 1). No patient had EF<25%. The main accompanying diseases were diabetes, hypertension, and hyperlipidaemia, and were similarly distributed between stable and unstable patients (Table 1). Two stable and one unstable patient had preoperative paroxysmal supraventricular tachycardia or atrial fibrillation and were treated with digitalis. One stable patient has asthma and was treated with budesonide and salbutamole inhalation aerosol, three unstable patients were dyspeptic and treated with omeprazol, and one unstable patient was treated with the tricyclic antidepressant imipramin.

2.2. Operative procedures and tissue sampling

Anesthesia was induced with midazolam and fentanyl and maintained with midazolam, fentanyl, and isoflurane while muscle relaxation was obtained with pancuronium. The patients were ventilated mechanically. After sternotomy, heparin was given to achieve an activated clotting time over 480 s. Arterial cannulation for cardiopulmonary bypass (CPB) was in the ascending aorta, and a two stage venous cannula in the right atrium and the inferior vena cava was used for outflow to the CPB. A biopsy of

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Table 1

Clinical data of patients with stable and unstable angina (15 of each) undergoing coronary artery bypass grafting with sampling of right atrial tissue

<table>
<thead>
<tr>
<th></th>
<th>Years</th>
<th>AP months</th>
<th>Infarcts</th>
<th>EF&gt;60</th>
<th>60&gt;EF&gt;40</th>
<th>40&gt;EF&gt;25</th>
<th>Diabetes</th>
<th>Hypertens.</th>
<th>Hyperlipid.</th>
<th>Anast.</th>
<th>ICU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstable</td>
<td>71±3</td>
<td>53±17</td>
<td>1.1±0.2</td>
<td>8/15</td>
<td>3/15</td>
<td>2/15</td>
<td>2/15</td>
<td>2/15</td>
<td>8/15</td>
<td>3.7±0.2</td>
<td>1.5±0.2</td>
</tr>
<tr>
<td>Stable</td>
<td>61±2*</td>
<td>32±10</td>
<td>0.7±0.3</td>
<td>9/15</td>
<td>2/15</td>
<td>2/15</td>
<td>4/15</td>
<td>5/15</td>
<td>5/15</td>
<td>3.2±0.2</td>
<td>1.1±0.1</td>
</tr>
</tbody>
</table>

* The patients’ age, months of diagnosed angina pectoris prior to surgery (AP months), number of preoperative infarctions (infarcts), left ventricular ejection fraction (EF, %), patients per group with diagnosed diabetes, hypertension, and hyperlipidaemia, number of distal anastomosis performed during surgery, and number of days in the intensive care unit (ICU) are shown. * Denotes P<0.05 in comparison between groups.
approximately 0.5×0.5×0.5 cm was cut from the right atrial auricle in conjunction with venous cannulation, and immediately immersed in liquid nitrogen. CPB was conducted at 34°C with non-pulsatile flow and a membrane oxygenator (Medtronic Cardiopulmonary, Anaheim, CA, USA). Cold, intermittent blood cardioplegia given both ante- and retrogradely was used for myocardial protection. The left internal thoracic artery and the great saphenous vein were employed as grafts in all patients.

2.3. Immunoblotting

Half of the frozen tissue biopsy was homogenized at a ratio of 40 mg tissue/ml lysis buffer [1% SDS, 1 mM Na vanadate, 1 mM protease inhibitor phenylmethylsulphonyl fluoride (PMSF)], and insoluble material removed by centrifugation. Protein content was determined using the bicinchoninic acid reagent (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard. The lysates were mixed with Laemmli buffer at a ratio of 5:1, boiled, and proteins were electrophoresed under reducing conditions (1 μg protein/lane for HSP72, 4 μg protein/lane for HSP73, and 34 μg protein/lane for eNOS) followed by transfer to presoaked nitrocellulose membranes (Hybond-C pure; Amersham Life Science). The membranes were blocked in PBS/Dulbecco’s (Gibco BRL, Life Technology) with 0.1% Tween and 5% nonfat dry milk followed by incubation with mouse monoclonal anti-HSP72, mouse monoclonal anti-HSP73 (Stress Gen Biotechnologies Corporation) diluted 1/1000, or mouse monoclonal anti-eNOS (Transduction Laboratories) diluted 1/2500. Goat anti-mouse IgG-alkaline phosphatase (StressGen Biotechnologies) diluted 1/1000, or mouse monoclonal anti-ecNOS (both Santa Cruz Biotechnology, Santa Cruz, CA, USA) were incubated with the binding buffer for 15 min prior to adding the radiolabelled probe. For competition analysis, an unrelated probe in 100-fold excess was added prior to radiolabelled probe.

2.6. Statistics

Student’s t-test was used for evaluation between groups. P<0.05 was considered significant. Values are presented as mean±S.E.M.

3. Results

3.1. Clinical course

Both patient groups received the same number of distal anastomoses, and did not have a significantly different length of stay in the intensive care unit (Table 1). There was no mortality in either group. Two of the unstable patients needed pharmacological inotropic support when weaning off bypass and during the first hours in the ICU, while none of the stable patients needed this. There were no perioperative myocardial infarctions. Minor postoperative events such as atrial fibrillation or conduction disturbances (AV-block) occurred in four unstable and five stable patients. One patient in each group had a postoperative lower urinary tract infection. One stable patient was treated out-hospital for infection of the leg wound, and one of the unstable patients was treated in-hospital on suspicion of septic pericarditis appearing 1 week postoperatively (never verified). Otherwise, the patients had uneventful postoperative recoveries.

3.2. Inducible heat shock protein (HSP72)

When protein extracts from the human atrial biopsies were incubated with mouse monoclonal anti-HSP72 after membrane lysis. After centrifugation, the supernatant was collected as nuclear extract, and protein content was determined using the bicinchoninic acid reagent (Pierce).

2.5. Electrophoretic mobility shift assay

Nuclear extracts (16 μg protein/lane) were preincubated for 10 min in binding buffer (20 mM Hepes pH 7.9, 5% glycerol, 5 mM MgCl₂, 0.5 mM EDTA, and 1 mM dithiothreitol) on ice, followed by 30 min incubation at room temperature with 50 000 cpm of 32P-labelled probe containing the NFκB binding site 5’ AGT TGA GGG GAC TTT CCC AGG C or the AP-1 binding site 5’ CGC TTG ATG AGT CAG CCC GGA A (both Promega). DNA–protein complexes were electrophoresed on a 4% polyacrylamide gel. For supershift analysis, a rabbit polyclonal anti-p50 antibody or a rabbit polyclonal anti-c-jun antibody (both Santa Cruz Biotechnology, Santa Cruz, CA, USA) were incubated with the binding buffer for 15 min prior to adding the radiolabelled probe. For competition analysis, an unrelated probe in 100-fold excess was added prior to radiolabelled probe.
Fig. 1. Panel a: Immunoblot analysis of right atrial biopsies from six patients with stable angina (SA) and seven patients with unstable angina (UA) taken during open heart surgery. The membranes were incubated with a mouse monoclonal antibody against the inducible heat shock protein 72 (HSP72). Panel b: Immunoblot analysis of right atrial biopsies from seven patients with stable angina and seven patients with unstable angina with a mouse monoclonal antibody against the constitutive heat shock protein 73. Panel c: Optical density of immunoblots of HSP72 from right atrial biopsies of all 15 patients with unstable angina and 15 patients with stable angina taken during open heart surgery (mean ± S.E.M.). Panel d: The optical density of the constitutive HSP73 bands in all patients (15 SA, 15 UA).

3.3. Constitutive heat shock protein (HSP73)

When the cardiac protein extracts were incubated with a mouse monoclonal antibody against HSP73, a band of 73 kDa appeared. An immunoblot with bands of seven stable and seven unstable patients is shown in Fig. 1b. When calculating optical densities of the bands of all patients, no significant difference was found between cardiac protein extracts (Fig. 1d).

3.4. Endothelial nitric oxide synthase (eNOS)

A typical immunoblot of cardiac proteins extracts from six stable and six unstable patients after incubation with a mouse monoclonal anti-eNOS antibody is shown in Fig. 2a. When the protein extracts were stained with coomassie blue, the same amount of protein per lane was visualized (Fig. 2b). When evaluating all 30 patients, the mean optical density of the 140-kDa eNOS band was higher in patients with unstable angina (Fig. 2c).

3.5. Activation of transcription factors

None of the unstable patients had activation of NFκB or AP-1 (not shown). In the group with unstable angina, activation of transcription factors was not consistently found (not shown). However, there was a correspondence between symptoms and activation of transcription factors. In Fig. 3a, activation of NFκB in three patients with stable angina (SA), three unstable patients with intermediate symptoms (anginal pain 1–2 days before surgery, UA-1), and three unstable patients with symptoms at the time of surgery (UA-2) is shown. NFκB was activated in atrial tissue of unstable patients, as evidenced by increased retardation of the DNA probe containing the NFκB motif (Fig. 3a). The identity of the proteins bound to the probe were determined by supershift analysis and cold probe competition (Fig. 3b). An antibody specific for the p50 subunits of the NFκB heterodimer caused retardation of the mobility of the DNA probe, while the band disappeared when a cold probe was employed, further identifying the band as NFκB. A similar analysis of AP-1 is shown in Fig. 4a, where the same patient samples as shown in Fig. 4a and b were incubated with a radiolabelled DNA probe containing the AP-1 motif, demonstrating AP-1 activation in cardiac tissue from unstable patients with recent or ongoing symptoms (Fig. 4a). An antibody specific for the c-jun subunit of AP-1 caused only a small retardation of the DNA probe (Fig. 4b). Competitive inhibition with cold probe extinguished the AP-1 bands (Fig. 4b).
Fig. 2. Panel a: A representative immunoblot showing protein extracts of human right atrial tissue obtained from six patients with stable angina and six with unstable angina undergoing open heart surgery. The extracts were incubated with a mouse monoclonal anti endothelial nitric oxide synthase (eNOS) antibody. Panel b: The protein extracts from the same patients and in equal amounts as above, plus one additional patient from each group, stained with Coomassie blue to visualise that the protein in the lanes was the same. Panel c: The optical densities of the eNOS bands in all 15 stable versus the 15 unstable patients.

4. Discussion

The main findings of the present study were that right atrial tissue of patients with unstable angina contained more inducible heat shock protein 72 and endothelial nitric oxide synthase than the myocardium of patients with stable angina. Experimental evidence suggests that these proteins are cardioprotective [11–15]. The redox sensitive transcription factors NFkB and AP-1 were activated in unstable patients with recent or ongoing symptoms. NFkB alone or in cooperation with AP-1 regulate a battery of inflammatory genes, including proinflammatory cytokines, chemokines, leukocyte adhesion molecules, inducible nitric oxide synthase, and inducible cyclo-oxygenase [16,17]. Their activation in atrial tissue of patients with unstable angina may be a useful switch for tissue response of inflammation and repair. Activation of NFkB has recently been suggested to play a part in intracellular signalling in ischemic preconditioning, although inhibition of NFkB activation during postsischemic reperfusion protects the heart [18–20].

Some papers indicate that unstable angina prior to onset of acute myocardial infarction improves the clinical outcome for these patients, which has been attributed to a preconditioning effect of the intermittent ischemia and reperfusion of unstable angina [5–7]. Short episodes of ischemia and reperfusion prior to a sustained ischemic event — ischemic preconditioning — reduce infarct size and improve cardiac function in experimental studies both as an immediate and a delayed effect [21,22]. The trigger(s) and intracellular signalling systems of preconditioning are not fully clarified, nor are the secondary mediators of myocardial protection [21,22]. Of the suggested mediators of delayed preconditioning are heat shock proteins 70 (HSP70) [11] and nitric oxide synthase [15]. The HSP70 family consists of the inducible HSP72 and the constitutive HSP73, which are involved in protein traffic and folding, translocation of proteins across membranes, and gene regulation [11–13]. HSP70 may have cardioprotective properties. Induction of HSP70 by hyperthermia or ischemic preconditioning reduces infarct size and improves cardiac function, and transgenic mice expressing high
levels of HSP70 are protected against ischemia–reperfusion injury [11–13]. However, increased expression of HSP70 is not consistently associated with myocardial protection [23].

Heat shock proteins are regulated by their own transcription factors, and are induced by AP-1 or NFκB [13]. Evidence suggests that HSP70 may modulate AP-1 and NFκB DNA binding activity [24,25], perhaps explaining some of their possible antiinflammatory and cardioprotective properties. Downregulation of activated transcription factors by HSP70 may explain why these were not detectable in hearts of patients without symptoms for 3 days or more prior to surgery.

Endothelial NOS regulates the constitutive production of nitric oxide (NO). In addition to its vasodilatory action, evidence suggests that NO may have antiarrhythmic, antiinflammatory, antithrombotic, and a possible positive inotropic effect [14,15,26–28]. Experimental studies suggest that NO has cardioprotective actions, although NO overproduction may be associated with peroxynitrite formation. Thus, increased expression of eNOS may be beneficial in the hearts of patients with unstable angina. Although referred to as a constitutive enzyme, eNOS can be upregulated by a number of stimuli, and its promoter region contains recognition sites for a number of transcription factors, among them AP-1 [29]. eNOS is also regulated through a shear stress element, which may have contributed to its increase in unstable patients.

Prior to surgery the only known differences between our patient groups were instability and higher age in the unstable group. Age does not increase inducible HSP70; on the contrary, HSP72 induction decreases with age, and this reduced response has been suggested as a common phenomenon underlying the aging process [30]. In agematched patients, the difference in HSP72 induction might have been even higher. Similarly, there is no evidence that eNOS expression increases with age. Loss of eNOS expression and NO production is observed in human atherosclerosis [31], and as atherosclerosis is a disease progressing with age the increased eNOS in the unstable patients is likely to be due to the instability.

Although unstable angina protects myocardial function and necrosis against acute myocardial infarction, possibly through increased myocardial expression of HSP72 and eNOS as shown in this study, instability is detrimental before cardiac surgery [8]. The present study is too small to evaluate clinical outcome. Open heart surgery with cardiopulmonary bypass represents a massive inflammatory trauma; major surgery is performed, which in itself induces an inflammatory response. The heart is subjected to a local inflammatory response induced by ischemia and reperfusion during suturing of the distal anastomoses, and the foreign surfaces of the CPB activates blood components [9,10]. Systemic increases of proinflammatory cytokines, soluble leukocyte adhesion molecules, neutrophil activation, complement activation, and endotoxemia are among the events occurring during CPB [9,10]. We demonstrate in the present study that unstable angina induces an inflammatory process in the heart through transcriptional activation. When the unstable heart, which is in a proinflammatory state, is exposed to open heart surgery, it is possible that a more severe inflammatory process is turned on. In support of this, a study from our group investigating the inflammatory response to cardiopulmonary bypass and reperfusion in patients with stable and unstable angina, showed that patients with unstable angina had increased gene expression of E-selectin, eNOS, TNFα, IL-1β, and a larger decrease of endothelin-1 during reperfusion than stable patients [32].

In the present study atrial biopsies were analysed, although the left ventricle is the tissue of highest interest for cardiac function. This limitation of the study is due to the fact that for ethical reasons left ventricular biopsies large enough to perform protein extractions for the methods employed are unobtainable. Possibly findings would be different in different chambers of the heart. We speculate that the atrium may be representative of the whole myocardium on rationale of the recently discovered whole-body events of remote preconditioning: occlusion and reperfusion of other organs such as kidney, intestine, and

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![Fig. 4. Panel a: Identification of AP-1 complexes in nuclear extracts from human right atrial tissue sampled during open heart surgery. The extracts were resolved by EMSA as described in Methods. Extracts from three patients with stable angina (SA), three patients with unstable angina with intermediate symptoms at the time of sampling (UA-1), and three unstable patients with anginal pain at the day of surgery (UA-2) are shown. Panel b: EMSA of nuclear extracts from three unstable patients (#1–3) taken from the right atrial auricle. The nuclear extracts were incubated with a mouse monoclonal antibody against the c-jun subunit as indicated on the top to identify AP-1 binding complexes. Competition with cold probe (cp) was performed to verify the band identity.](https://academic.oup.com/cardiovascres/article-abstract/47/1/49/320725)
limbs protects the heart against subsequent ischemia [33], while preconditioning of the heart protects the lungs against reperfusion injury (manuscript in preparation). These findings may indicate that ischemia and reperfusion of any organ will elicit systemic events which may trigger protection of any organ. The present findings in atrial tissue may support this possibility.

In summary, patients with unstable angina had increased cardiac contents of the inducible HSP72 and eNOS, but not of the constitutive HSP73. This increase may explain a possible preconditioning effect of unstable angina. Patients with angina shortly before surgery had atrial activation of the transcription factors NFκB and AP-1, which regulate a battery of inflammatory genes. A myocardial proinflammatory state may reduce tolerance to the inflammation induced by cardiac surgery, and thus contribute to understanding why unstable angina is a risk factor before open heart surgery.

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