Enhanced Neutrophil Expression of Annexin-1 in Coronary Artery Disease

Eva Särndahl, Ida Bergström, Johnny Nijm, Tony Forslund, Mauro Perretti and Lena Jonasson

N.B.: When citing this work, cite the original article.

Original Publication:
http://dx.doi.org/10.1016/j.metabol.2009.07.044

Copyright: Elsevier Science B.V., Amsterdam
http://www.elsevier.com/

Postprint available at: Linköping University Electronic Press
http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-17247
Enhanced Neutrophil Expression of Annexin-1 in Coronary Artery Disease

Eva Särndahl, PhD a, Ida Bergström, MSc b,c*, Johnny Nijm, MD, PhD b*, Tony Forslund, PhD c, Mauro Perretti, PhD d, and Lena Jonasson, MD, PhD b

a Department of Biomedicine, School of Health and Medical Sciences, Örebro University, SE-701 82 Örebro, Sweden

b Division of Cardiovascular Medicine, Department of Medical and Health Sciences, Linköping University, SE-581 85 Linköping, Sweden

c Division of Medical Microbiology, Department of Clinical and Experimental Medicine, Linköping University, SE-581 85 Linköping, Sweden

d William Harvey Research Institute, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, UK

* These authors contributed equally to this paper

Short title: Annexin-1 in Coronary Artery Disease

Key words: Coronary Artery Disease, Inflammation, Cortisol, Glucocorticoid Receptors, Signal Transduction

Corresponding author & Reprint request:
Eva Särndahl
Department of Biomedicine, M-building/KFC, Örebro University Hospital, SE-701 85 Örebro
Phone: +46 19 602 6653, Fax: +46 19 602 6650, E-mail: eva.sarndahl@oru.se

Funding sources: This work was supported by grants from the Royal Swedish Academy of Sciences, the Medical Research Council of Southeast Sweden, the County Council of Östergötland, the Foundation of “Gamla Tjänarinnor”, the Biomedical Research School, and the Cardiovascular Inflammation Research Centre (CIRC) at the Faculty of Health Sciences at Linköping University. The founders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript
ABSTRACT

**Background:** The systemic inflammatory activity in patients with stable coronary artery disease (CAD) is associated with a dysregulated cortisol response. Moreover, an aberrant activation status of neutrophils in CAD has been discussed; and the question of glucocorticoid resistance has been raised. The anti-inflammatory actions of glucocorticoids are mediated by annexin-1 (ANXA1). We investigated the expression of glucocorticoid receptors (GR) and ANXA1, as well as the exogenous effects of ANXA1 on neutrophils in CAD patients, and related the data to diurnal salivary cortisol.

**Methods and Results:** Salivary cortisol levels were measured in the morning and evening during 3 consecutive days in 30 CAD patients and 30 healthy individuals. The neutrophil expression of GR and ANXA1 was determined by flow cytometry. The effect of exogenous ANXA1 was determined in a neutrophil stimulation assay. The patients showed a flattened diurnal cortisol pattern compared with healthy subjects, involving higher levels in the evening. The neutrophil expression of GR-total and GR-\(\alpha\) was decreased, whereas the GR-\(\beta\) expression did not differ compared with controls. The neutrophil expression of ANXA1 was significantly increased in patients. *Ex vivo*, ANXA1 impaired the leukotriene B\(_4\)-induced neutrophil production of reactive oxygen species in patients but not in controls.

**Conclusion:** Our findings indicate a persistent overactivation of the hypothalamic-pituitary-adrenal axis in CAD patients but do not give any evidence for glucocorticoid resistance, as assessed by the neutrophil expression of GR and ANXA1. The altered neutrophil phenotype in CAD may thus represent a long-term response to disease-related activation.
INTRODUCTION

The atherosclerotic process is driven by a chronic inflammatory activity involving both the innate and adaptive immune response. The immune activity, sharing several features with autoimmunity, is detectable in vascular lesions and in peripheral blood (1, 2). The systemic inflammatory activity is more pronounced in patients with unstable conditions of coronary artery disease (CAD). However, a low grade of systemic inflammation is also found in CAD patients without clinical signs of instability (3, 4). We have previously demonstrated that the inflammatory markers in blood, both in stable and unstable conditions of CAD, were significantly associated with the number of circulating neutrophil-platelet aggregates; thus proposing a persistent neutrophil activation (4). Recent studies by ourselves and others have rather pointed towards an impaired neutrophil function in CAD (5, 6).

An imbalance between pro- and anti-inflammatory activities is believed to play an important role in the progress of atherosclerosis (7, 8), as well as in other autoimmune/inflammatory disorders (9, 10). The hypothalamic-pituitary-adrenal (HPA) axis modulates inflammation through the general anti-inflammatory action of glucocorticoids released from the adrenal cortex upon stimulation of the HPA axis. A dysfunction in the HPA axis and thereby a failure to resolve inflammation have been described in several autoimmune/inflammatory conditions (11, 12). We recently reported that CAD patients exhibited an increased cortisol release throughout the day, as well as a flatter diurnal cortisol deviation, compared with healthy control subjects (13). The abnormal cortisol response in patients was also associated with increased inflammatory activity, thus suggesting a failure of the HPA axis to resolve inflammation in CAD.

Glucocorticoid action is mediated through an active glucocorticoid receptor (GR)-α and negatively regulated by the GR-β. In autoimmune/inflammatory disorders, GR-β up-regulation has been linked to glucocorticoid resistance (14-16). The anti-inflammatory actions of glucocorticoids are mediated by the endogenous protein
annexin-1 (17) (ANXA1, formerly called lipocortin), which is a potent inhibitor of leukocyte trafficking in acute and chronic inflammation (18-20). Myeloid cells, in particular neutrophils and monocytes, are rich in ANXA1; and the protein is also abundantly expressed in inflamed tissue. Neutrophil expression of ANXA1 was recently suggested to be a useful indicator of tissue sensitivity to endogenous glucocorticoids (21). ANXA1 is mainly localized within the cytosol (22); but upon cell activation, ANXA1 becomes rapidly mobilized to the cell surface (23), where it acts in an autocrine/paracrine fashion by binding to a 7-transmembrane spanning receptor called formyl peptide receptor like-1 (FPRL-1)/lipoxin A$_4$ receptor (ALXR) (24, 25).

In the present study, we have further investigated the neutrophil phenotype in CAD, raising the hypothesis of glucocorticoid resistance. Using a matched-pair design, we examined the neutrophil expression of GRs and ANXA1 in CAD patients and healthy individuals, and related the findings to diurnal salivary cortisol. In an ex vivo stimulation assay, we further compared the effects of exogenous ANXA1 on neutrophils from patients and controls.
MATERIAL AND METHODS

Subjects

Thirty patients with angiographically verified stable CAD were recruited from the Department of Cardiology, Heart Centre, Linköping University Hospital, Sweden. The patients had effort-related angina in accordance with the Canadian Cardiovascular Society functional classes I and II without any worsening of symptoms during the latest 6 months. Patients were excluded if they were older than 70 years, or had severe heart failure, immunologic disorders, neoplasm disease, evidence of acute or recent (<2 months) infection, recent major trauma, surgery or revascularization procedure, drug or alcohol abuse, poor mental function, or treatment with immunosuppressive or anti-inflammatory agents (except low-dose aspirin). Each patient was matched, regarding age and sex, with a clinically healthy control randomly selected from a population register representing the hospital recruitment area. The control subjects were anamnestically healthy and with normal routine laboratory test results. They did not have any clinical signs or history of CAD, peripheral artery disease, or other conditions mentioned above. All patients and control persons gave informed oral consent. The appointed ethics committee at Linköping University approved the research protocol, and the study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki.

For each experimental set-up, venous peripheral blood was drawn from one patient and his or her matched control in the morning after a 12h fast, and collected in heparin-containing BD vacutainer tubes (Becton Dickinson, Plymouth, UK). The samples were blinded during all experiments.

Collection and analysis of diurnal salivary cortisol

The participants were instructed to collect saliva on 3 consecutive days, which were not the day after a Sunday or holiday or the day before the weekend; that is the sampling typically started on a Tuesday and ended on a Thursday. The first sample was taken 30 min after awakening; and the second sample, in the evening before going to bed. Before saliva sampling, the participants were instructed not to eat, drink or use tobacco for at least 60 min. Saliva was collected with Salivette cotton swabs (Sarstedt, Nümbrecht, Germany) that were placed under the tongue for 2 min. The Salivettes were immediately frozen at - 20°C. The levels of
free cortisol in saliva (26) were determined at the accredited clinical chemical laboratory at Linköping University Hospital, Sweden, by a modified commercial radioimmunoassay assay (Diagnostic Products Corp, Los Angeles, CA, USA). For each individual, salivary cortisol was measured in 3 morning and 3 evening samples. According to repeatedly performed quality assessments, the interassay coefficient was less than 10%.

**Immunolabeling of ANXA1, GR, FPRL-1 and CD18 in neutrophils**

*Immunolabeling of baseline ANXA1*

Leukocytes were harvested from whole blood after red cell lysis (L-buffer: NH₄Cl 150mM, KHCO₃ 10mM, EDTA 100μM; 5 min, 15°C). The cells were washed and incubated with an anti-ANXA1 antibody (Zymed Laboratories Inc, San Francisco, CA, USA) in phosphate-buffered saline (PBS, pH 7.3, made in-house) with saponin (0.02% w/v; saponin-PBS) for 1h on ice (23). After washing in cold saponin-PBS, the cells were incubated with the F(ab')₂ fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) (Caltag, Burlingame, CA, USA) in saponin-PBS for 30 min on ice. The cells were washed, collected, and fixed in cold paraformaldehyde (PFA, 0.1% w/v), whereafter the fluorescence was detected by flow cytometry.

*Immunolabeling of baseline GR*

Leukocytes were harvested from whole blood after red cell lysis in L-buffer (5 min, 15°C). The cells were fixed in cold PFA (2% w/v) for 15 min at 4°C, washed in PBS supplemented with bovine serum albumin (BSA) (0.2% w/v, PBS-BSA), and permeabilized in PBS-BSA with saponin (0.02% w/v) for 5 min at 4°C. The cells were incubated with antibodies directed against GR-α (PA1-516; Affinity Bioreagents, Golden, CO, USA), GR-β (PA3-514; Affinity Bioreagents) or GR-total (PA1-512; Affinity Bioreagents) in saponin-PBS for 1h on ice. After washing in cold saponin-PBS, the cells were incubated with the F(ab')₂ FITC-conjugated goat anti-rabbit IgG in saponin-PBS for 30 min on ice. The cells were washed and resuspended in cold PFA (0.1% w/v), whereafter the fluorescence was detected by flow cytometry.
Immunolabeling of baseline FPRL-1

Leukocytes were harvested from whole blood after lysis of erythrocytes in L-buffer (5 min, 15°C), washed, and incubated with an antibody directed toward the receptor FPRL-1 (Affinity Bioreagents) for 1h on ice. After washing, the cells were incubated with the F(ab’)₂ FITC-conjugated goat anti-rabbit IgG for 30 min on ice. The leukocytes were washed, collected, and fixed in cold PFA (0.1% w/v), whereafter the fluorescence was detected by flow cytometry.

Immunolabeling of baseline and leukotriene B₄-induced CD18 expression

Immunolabeling was performed as previously described (5). Cells were left untreated or stimulated with leukotriene B₄ (LTB₄, Laradan Fine Chemicals AB, Malmö, Sweden) for 10 min, whereafter the monoclonal FITC-conjugated mouse anti-human CD18 antibody (MHM23; Dako, Glostrup, Denmark) was added for the last 5 min. The stimulation was stopped by incubating the samples on ice. Contaminating erythrocytes were removed by ice-cold cell lysis (L-buffer, 5 min, 15°C). The cells were collected and fixed in cold PFA (0.1% w/v), whereafter the fluorescence was detected by flow cytometry.

Flow Cytometry

The detection of ANXA1, GR, FPRL-1 and CD18 was determined by measuring the mean fluorescence intensity (MFI) from 10 000 cells/sample by flow cytometry using a Becton Dickinson FACSCalibur (Becton Dickinson, San Jose, CA, USA). Cell populations were identified by plotting forward scatter (FSC) vs side scatter (SSC) excluding cell debris, followed by gating and further analysis of the neutrophil (granulocyte) population (5). Auto-fluorescence of unstained cells was routinely analyzed. Unspecific binding was determined using a FITC-conjugated isotypic mouse anti-human IgG1 or the F(ab’)₂ FITC-conjugated goat anti-rabbit IgG.

Preparation of neutrophils

Neutrophils were prepared by density gradient centrifugation, as previously described (5, 27). In short, freshly drawn heparinized blood was layered on top of Lymphoprep™ and Polymorphprep™ (Axis-Shield
PoC AS, Oslo, Norway) and centrifuged in a swing-out centrifuge (400 x g, 30 min, RT). The granulocyte band was collected, washed, resuspended in a small volume of PBS, and concentrated by a short spin. Contaminating erythrocytes were removed by hypotonic lysis; and the cells were washed, resuspended in Krebs-Ringer glucose buffer (KRG; 10 mM glucose, 1 mM Ca\(^{2+}\), and 1.2 mM Mg\(^{2+}\); pH 7.3, made in-house) without Ca\(^{2+}\), and kept on ice.

**Determination of reactive oxygen species production**

Chemiluminescence was used to determine the reactive oxygen species (ROS) production of isolated neutrophils, and the measurements were performed as previously described (5, 28), using a 6-channel Biolumat LB9505 (Berthold, Wildbad, Germany). Total ROS production (i.e. intracellularly produced plus extracellularly released) was measured in neutrophils (5 x 10^6/mL), diluted in KRG, using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; 56 μM; Sigma, St. Louis, MO, USA) and horseradish peroxidase (4 U/mL; Roche Diagnostics GmbH, Mannheim, Germany). The neutrophils were preincubated with ANXA1 (500 nM) for 15 min at 37 ºC, whereafter the cells were placed in the Biolumat and preheated for 5 min before addition of stimuli (i.e. LTB\(_4\)). The temperature was kept at 37 ºC during the measurements; and the light emission, reflecting ROS interacting with luminol, was continuously monitored. The peak value was registered for each sample and expressed as counts per minute (CPM).

**Statistics**

Data were analyzed using SPSSPC (SPSS, Chicago, IL, USA). The data are presented as mean ± SD. By using Student’s t-test, the significance of any difference in clinical and laboratory characteristics between patient and control groups was tested (Tables I and II). Flow cytometric data and data of ROS production were analyzed by Wilcoxon signed rank test to compare levels between a patient and his or her paired control. Correlation analysis was performed by Spearman rank correlation test. Two-tailed P-values < 0.05 were considered statistically significant.
RESULTS

Clinical and laboratory characteristics of subjects
The use of medication differed significantly between patients and control individuals (Table 1). A small number of controls received treatment with low-dose aspirin, β-blockers, angiotensin-converting enzyme inhibitors (ACE-I), angiotensin receptor blocker (ARB), or statins because of the presence of risk factors, such as hypertension and hyperlipidemia. None of the study subjects were treated with antidepressants. Whereas the levels of total cholesterol and low-density lipoprotein (LDL) cholesterol were significantly lower in the patient group compared with controls, the levels of high-density lipoprotein (HDL) cholesterol and triglycerides did not differ. There were no differences in circulating levels of C-reactive protein (CRP) or in leukocyte blood counts between patients and controls.

Diurnal salivary cortisol levels
The morning levels of salivary cortisol were similar in patients and controls, whereas the cortisol levels at bedtime were significantly higher in the patient group (Table 2). The cortisol parameters were not influenced by sex or age and did not show any significant correlations to smoking, blood pressure, or heart rate. There were no statistically significant relationships between cortisol and LDL, HDL, or total cholesterol levels. On the other hand, there was a positive correlation between evening cortisol and CRP levels in patients ($r = 0.49$, $P < 0.01$), but not in the control group.

Neutrophil expression of GR-α and GR-β, ANXA1, and FPRL-1
The baseline amount of GRs, ANXA1, and FPRL-1 in neutrophils was investigated using specific antibodies and detected by flow cytometry. The neutrophil expression of GR-total and GR-α was significantly decreased in patients compared with controls, whereas the increased expression of GR-β seen in patients did not reach statistical significance (Figure 1). The neutrophil expression of ANXA1 was found to be significantly increased in patients compared with controls (Figure 2). Correlations between GR, ANXA1, and cortisol
levels are given in Table 3. There was a positive correlation between normalized ANXA1 expression and salivary evening cortisol in all subjects, whereas the correlation between ANXA1 and morning cortisol did not reach statistical significance ($P = 0.07$). There were negative correlations between the expression of ANXA1 and GR-total and between ANXA1 and GR-$\alpha$, as well as between morning cortisol and the expression of GR-total, in both patients and controls. The number of ANXA1 receptors (i.e. FPRL-1) in the neutrophils’ plasma membrane did not differ between patients and control subjects (68 ± 26 and 69 ± 28, respectively [mean MFI ± SD]; $n=24$ per group).

**The effect of ANXA1 on neutrophil response**

At baseline, the expression of CD18 did not differ between patients and controls; neither did the LTB$_4$-induced expression of CD18 nor the LTB$_4$-induced production of ROS (Table 4). To test the signaling capacity of the ANXA1 receptor, exogenous ANXA1 was added to the cells. The cells were challenged with the proinflammatory chemoattractant LTB$_4$, whereafter the neutrophils’ activation status was established by measuring their ability to generate ROS. Preincubating the cells with ANXA1 did not influence the ROS produced in neutrophils from controls, but resulted in a significantly lower production of ROS in neutrophils from patients (52% reduction, $P = 0.002$; Figure 3).
DISCUSSION

The CAD patients showed a flattened diurnal rhythm of cortisol; that is, whereas their morning levels in saliva were similar compared with controls, their salivary cortisol levels in the evening were significantly higher. These findings in CAD patients with long-term stable symptoms are consistent with a recently published study by our group demonstrating an increased 24h cortisol secretion and a decreased diurnal cortisol decline in CAD patients 3 months post-myocardial infarction (13). Previous studies have reported high morning cortisol levels in CAD patients, suggesting increased activity of the HPA axis (29, 30). However, their results have been based on isolated measures of morning plasma cortisol and not on the pattern of cortisol output throughout the day. Recently, a population-based study, using repeated measurements of salivary cortisol, showed that the flatter the diurnal cortisol slope was, the greater was the likelihood of any coronary calcification (31). Interestingly, an abnormal cortisol pattern has been reported in chronic inflammatory conditions, like rheumatoid arthritis; and an association between HPA axis dysfunction and increased susceptibility to autoimmune diseases has been discussed (11, 32). The correlation between evening cortisol levels and systemic inflammatory activity (measured as CRP) in CAD patients, as shown in the present study, is in agreement with our recent findings in post-myocardial infarction patients (13), and further supports the hypothetic link between HPA dysfunction and inflammation.

The actions of glucocorticoids are mediated via intracellular receptors, GR. Alternative splicing of GR premessenger RNA generates two highly homogenous isoforms, termed GR-α and GR-β. GR-α is a ligand-activated transcription factor mediating the hormone response, whereas GR-β is an endogenous antagonist of glucocorticoid action. In several autoimmune diseases, GR-β up-regulation has been proposed to be involved in glucocorticoid resistance (14-16). In the present study, the neutrophil expression of GR-α was significantly reduced in the CAD patients, probably resulting from a negative feedback control of high cortisol levels, whereas the expression of GR-β did not show any significant difference.
We next proceeded to measure the expression of the glucocorticoid-inducible protein ANXA1 in neutrophils and found that it was significantly increased in patients compared with healthy controls. Interestingly, the expression of neutrophil ANXA1 may provide a sensitive index of tissue sensitivity to endogenous cortisol (21). In a minor cohort of patients attending an endocrine clinic for the investigation of HPA function, ANXA1 in neutrophils correlated positively with serum cortisol both before and after a standard corticotrophin test. In agreement, we found a similar positive correlation between ANXA1 expression and salivary evening cortisol. ANXA1 also correlated negatively with the expression of GR-α and GR-total, respectively. Altogether, the patterns of GRs and ANXA1 in CAD patients support the concept of an overactivated HPA axis and also point towards an adequate cortisol sensitivity most likely due to the persistently elevated level of cortisol found in these patients.

The expression of ANXA1 in neutrophils has been consistently associated with anti-inflammatory actions (33). By measuring the basal and LTB4-induced levels of CD18 on the surface of neutrophils, as well as the LTB4-induced ROS production, the neutrophils from patients appeared to have an activation status similar to controls. In neutrophils, experimental studies have shown an interaction between ANXA1 and the receptor FPRL-1 (24, 25). This interaction results in suppression of various proinflammatory genes and in inhibition of various neutrophil responses (reviewed by Perretti and Flower (33)). In the present study, exogenous ANXA1 induced a significant suppression of the LTB4-mediated production of ROS in patients but not in controls. This response was not associated with any differences in the expression of FPRL-1 between patients and controls. Together, these results suggest a hyperresponsiveness to ANXA1 in CAD.

The possibility of drug-mediated effects has to be taken into account. Statins have well documented anti-inflammatory effects (34), and the influence of long-term statin therapy on steroid genesis may be a potential confound. However, according to several clinical trials, basal serum and urinary cortisol levels are not altered by statin; neither is the serum cortisol response to adrenocorticotropic hormone (35-37). The ACE-1/ARBs also have anti-inflammatory potentials, and the inhibition of angiotensin II receptors has been shown to limit
the HPA axis response to stress (38). In our study, 50 % of the patients were treated with ACE-I/ARBs. However, the expression of GRs or annexin-1 did not differ between ACE-I/ARB-treated and untreated patients.

While increased neutrophil activation is a consistent finding in acute coronary syndrome (4, 39, 40), the activation state of neutrophils in stable conditions of CAD has been more controversial. Although the numbers of circulating neutrophil-platelet aggregates are increased in patients with CAD (4, 40), other characteristics, like adhesive properties, have not been shown to differ (5). Instead, an impaired neutrophil activation status in CAD has been proposed (5, 6). The present findings do not give any evidence for a glucocorticoid resistance in neutrophils but rather indicate that neutrophils in CAD patients, due to an overactivation of the HPA axis, may be cells with suppressed inflammatory properties. The augmented expression of ANXA1, as well as the hyperresponsiveness, may illustrate a frustrated neutrophil phenotype trying to counteract disease-related immune activation. However, the consequences of such an aberrant neutrophil function may not necessarily be beneficial during inflammatory or ischemic assaults and call for further investigation.
ACKNOWLEDGEMENTS

We would like to thank Ms Johanna Wernersson for excellent technical assistance. Dr. Helen Setterud is acknowledged for advice regarding the ROS analysis.

ABBREVIATIONS

Annexin-1 (ANXA1); C-reactive protein (CRP); Coronary artery disease (CAD); Glucocorticoid receptor (GR); High-density lipoprotein (HDL); Hypothalamic-pituitary-adrenal axis (HPA axis); Leukotriene B₄ (LTB₄); Low-density lipoprotein (LDL); Paraformaldehyde (PFA); Reactive oxygen species (ROS)

DISCLOSURES

There is no financial or other relationship that might lead to a conflict of interest to be declared for any of the authors.

STATEMENT OF RESPONSIBILITY

The authors had full access to the data and take responsibility for the data’s integrity. All authors have read and agree to the manuscript as written.
REFERENCES

**Figure Legends**

Figure 1

*Baseline expression of GR-total, GR-α, and GR-β in neutrophils from patients with stable CAD.*

Leukocytes were harvested from whole blood after red cell lysis. The cells were fixed in cold PFA, and thereafter permeabilized with saponin (all steps henceforward were performed in 0.02% (w/v) saponin). The cells were incubated with antibodies directed against GR-total, GR-α, or GR-β, and thereafter with the F(ab’)2 FITC-conjugated goat anti-rabbit IgG. The cells were resuspended, and the fluorescence was detected by flow cytometry. The cells were gated to identify the neutrophil population, and 10 000 cells were counted in each sample. Data are given as one representative histogram plot (A-C) or as box plots (D) summarizing the median (white hyphen), inter-quartile range and minimum and maximum data values of MFI from 17 (GR-α, GR-β) or 30 (GR-total) experiments per cohort done in triplicates. Wilcoxon signed-rank test was used to determine significance between each patient and his or her individually matched control. (* and ** represent significant difference: $P<0.05$ and $P<0.01$, respectively).

Figure 2

*Baseline expression of ANXA1 in neutrophils from patients with stable CAD.*

Leukocytes were harvested from whole blood after red cell lysis. The cells were incubated with antibodies directed against ANXA1 (this step and all steps henceforward was performed in 0.02% (w/v) saponin), and thereafter incubated with the F(ab’)2 FITC-conjugated goat anti-rabbit IgG. The cells were fixed in PFA, before the fluorescence was detected by flow cytometry. The cells were gated to identify the neutrophil population, and 10 000 cells were counted in each sample. Data are given as one representative histogram plot (A) or as box plots (B) summarizing the median (white hyphen), inter-quartile range, and minimum and maximum data values of MFI from 30 experiments per cohort done in triplicate. Wilcoxon signed-rank test was used to determine significance between each patient and his or her individually matched control. (** represent significant difference: $P<0.01$).
Figure 3

The effect of exogenous ANXA1 on basal and LTB4-induced ROS-production in neutrophils from patients with stable CAD. The production of ROS in response to ANXA1 was measured in isolated neutrophils from patients and individually matched controls. Cells were preincubated with ANXA1 (500 nM) before stimulated with LTB4 (100 nM). Data are expressed as CPM and shown as total ROS production peak value from 8-9 experiments per cohort. Mean value is indicated as a black hyphen. Wilcoxon signed-rank test was used to determine significance between each patient and his or her individually matched control (** represents significant difference: \( P < 0.01 \)).
**Table 1.** Clinical and laboratory characteristics of patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>30</td>
<td>30</td>
<td>ns</td>
</tr>
<tr>
<td>Female / Male (n)</td>
<td>5/25</td>
<td>5/25</td>
<td>ns</td>
</tr>
<tr>
<td>Age (y)</td>
<td>63 ± 5</td>
<td>63 ± 5</td>
<td>ns</td>
</tr>
<tr>
<td>Smokers n (%)</td>
<td>4 (13)</td>
<td>4 (13)</td>
<td>ns</td>
</tr>
<tr>
<td>Blood pressure (mm Hg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>135 ± 18</td>
<td>143 ± 17</td>
<td>ns</td>
</tr>
<tr>
<td>Diastolic</td>
<td>84 ± 10</td>
<td>85 ± 11</td>
<td>ns</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>27 ± 4</td>
<td>26 ± 4</td>
<td>ns</td>
</tr>
<tr>
<td>Medication n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-blockers</td>
<td>28 (93)</td>
<td>3 (10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ACE-I/ARB †)</td>
<td>16 (52)</td>
<td>2 (7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Statin</td>
<td>30 (100)</td>
<td>3 (10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Low-dose Aspirin</td>
<td>30 (100)</td>
<td>1 (3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Laboratory variables ‡)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>1.7 ± 2.0</td>
<td>1.9 ± 2.5</td>
<td>ns</td>
</tr>
<tr>
<td>Lipids (mmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>4.5 ± 1.1</td>
<td>5.5 ± 0.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Low-density lipoprotein (LDL) cholesterol</td>
<td>2.4 ± 0.9</td>
<td>3.0 ± 0.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>High-density lipoprotein (HDL) cholesterol</td>
<td>1.4 ± 0.4</td>
<td>1.7 ± 0.5</td>
<td>ns</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.6 ± 0.7</td>
<td>1.6 ± 0.8</td>
<td>ns</td>
</tr>
<tr>
<td>Blood count (cells/µL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocytes</td>
<td>6.2 ± 1.3</td>
<td>5.9 ± 1.2</td>
<td>ns</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>3.1 ± 0.8</td>
<td>3.0 ± 1.0</td>
<td>ns</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD.

†) ACE-I, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker.

‡) Measured at the accredited clinical chemistry laboratory at Linköping University Hospital, Sweden.
Table 2. The morning and evening levels of salivary cortisol in CAD patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morning cortisol (nmol/L)</td>
<td>14.0 ± 4.1</td>
<td>16.1 ± 5.8</td>
<td>ns</td>
</tr>
<tr>
<td>Evening cortisol (nmol/L)</td>
<td>2.6 ± 1.1</td>
<td>1.4 ± 1.1</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD, derived from 3 consecutive days.
Table 3. Correlations between ANXA1, GRs, and salivary cortisol for CAD patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>ANXA1</th>
<th>GR total</th>
<th>GR-α</th>
<th>GR-β</th>
<th>Morning cortisol</th>
<th>Evening cortisol</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANXA1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GR total</td>
<td>-0.27*</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GR-α</td>
<td>-0.40*</td>
<td>0.91**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GR-β</td>
<td>-0.25</td>
<td>0.81**</td>
<td>0.91**</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morning cortisol</td>
<td>0.03</td>
<td>0.34**</td>
<td>-0.22</td>
<td>-0.24</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Evening cortisol</td>
<td>0.62*</td>
<td>0.02</td>
<td>-0.12</td>
<td>0.03</td>
<td>-0.24</td>
<td>1</td>
</tr>
</tbody>
</table>

Data are presented as Spearman rank correlation coefficients in a univariate correlation analysis.
* P <0.05. ** P <0.01.
Table 4. Basal and LTB₄-induced CD18 expression and ROS production of neutrophils from CAD patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal CD18 expression (MFI)</td>
<td>327 ± 93</td>
<td>315 ± 109</td>
<td>ns</td>
</tr>
<tr>
<td>LTB₄-induced CD18 expression (MFI)</td>
<td>580 ± 87</td>
<td>577 ± 105</td>
<td>ns</td>
</tr>
<tr>
<td>LTB₄-induced ROS production (x10⁵; CPM)</td>
<td>214 ± 143</td>
<td>253 ± 145</td>
<td>ns</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD; n=30 experiments per cohort done in triplicate (CD18) or n=9 experiments per cohort (ROS).
Figure 1

A

Counts

P
C

GR-total FITC

B

Counts

P
C

GR-α FITC

C

Counts

P
C

GR-β FITC

D

Expression of Glucocorticoid Receptors (MFI)

Patients

Controls

GR-total
GR-α
GR-β
Figure 3

![Graph showing ROS production in patients and controls](image)

- **LTB₄**
- **ANXA1 + LTB₄**

**Notes:**
- **Patients**
- **Controls**

**Statistical Significance:**
- ****

**Axes:**
- Y-axis: ROS production (x10⁶ CPM)
- X-axis: Patients vs. Controls