Journal of Steroid Biochemistry & Molecular Biology 116 (2009) 127–133

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Effect of high-dose dexamethasone on endothelial haemostatic gene expression and neutrophil adhesion

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ARTICLE INFO

Article history:
Received 12 February 2009
Received in revised form 22 April 2009
Accepted 4 May 2009

Keywords:
Dexamethasone
Glucocorticoid
Inflammatory
Coagulation
Thrombosis
Endothelial cells

ABSTRACT

Glucocorticoid usage especially at high doses is complicated by adverse outcomes such as thrombotic events or acceleration of inflammatory response in conditions like myeloma and osteonecrosis. The mechanism(s) through which high-dose dexamethasone (HDDEXA) causes vascular injury remains unclear.

We hypothesized that HDDEXA sensitizes endothelial cells (EC) to the effect of inflammatory mediators and modulates endothelial haemostatic gene expression and leukocyte adhesion. Human umbilical vein endothelial cells (HUVECs) were grown in the absence or presence of HDDEXA and were also tested in the presence or absence of tumor necrosis factor-α (TNF-α), lipopolysaccharide (LPS) or thrombin. mRNA and protein expression were measured and the functional consequences of HDDEXA preconditioning on cell adhesion molecules (CAM) were determined by agonist-mediated leukocyte adhesion assay.

Treatment with HDDEXA resulted in an increased induction of CAM, tissue factor and von Willebrand factor, while down-regulating thrombomodulin and urokinase. HDDEXA alone had no effect on adhesion but resulted in enhanced TNF-α- and LPS-mediated adhesion of neutrophils. Together, these findings suggest that HDDEXA sensitizes HUVEC to the effect of inflammatory mediators and induces a pro-adhesive environment in primary EC. This finding is of importance when glucocorticoid usage is required at therapeutic high doses in patients with or without thrombotic risk factors.

1. Introduction

Glucocorticoids (GCs) are widely prescribed in cases of rheumatoid arthritis, asthma, systemic lupus erythematosus, cancer treatment and organ transplantation. Their therapeutic advantages are offset by their well-documented adverse effects [1], especially if administered on a long-term basis. Recently, investigators have found an association between GC use and the development of venous thrombotic events [2–8] as well as an acceleration of inflammation during inflammatory disease states [2,9,10]. For instance, thromboembolic complications have been reported during the initial phase of GC use for the treatment of giant cell arteritis [7], during the treatment of minimal change nephritic syndrome [6] and also in the development of non-traumatic osteonecrosis of the femoral head [11,12].

Thromboembolic events have also been observed in patients with multiple myeloma treated with combined modality therapy including thalidomide, or its analogues [4], and dexamethasone (DEXA). In these patients, an increased risk of venous thrombosis was associated with the addition of DEXA [4,8]. In Cushing's syndrome or ACTH-dependent hypercortisolism, a hypercoagulable state has been reported [5]. In this prothrombotic condition, high levels of adrenal steroids have been shown to increase plasma or serum levels of factor VIII, von Willebrand factor (vWF) and plasminogen activator inhibitor-1 (PAI-1) activity, with secondary decrease in fibrinolysis. Similar clotting abnormalities have been reported in patients treated with exogenous GCs [2,13].

The mechanism of the thrombogenic effect of GCs is not well understood. It has been shown that for both *in vivo* and *ex vivo* studies, lower doses of different types of GCs will initially inhibit arterial thrombosis through inhibition of platelet aggregation; whereas at higher doses, these effects are counteracted by a significant inhibition of fibrinolytic activity [8,14–17]. This was shown to occur through decreased tissue-plasminogen activator (t-PA) activity and increased PAI-1 antigen levels [14,15]. Aside from a thromboembolic effect, it is widely reported that high dose of GCs may

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doi:10.1016/j.jsbmb.2009.05.001

exacerbate the inflammatory process of a disease state [2,9]. For example, it may accelerate the development of atherosclerosis, a chronic inflammatory state, as reported in systemic lupus erythematosus (SLE) [9] and also in sepsis, a process which results in activation of inflammatory and coagulation events [10]. Moreover, it is well known that the endothelium is actively involved in the balance of the coagulation/fibrinolytic pathways. Thus, we wanted to assess the effect of high doses of DEXA on the profile of endothelial haemostatic genes involved in proinflammatory and prothrombotic events and their contribution to the adhesion of leukocytes on activated endothelial cells.

2. Materials and methods

2.1. Cell culture

HUVECs were purchased from Cambrex (Walkersville, MD, USA) and cultured in endothelial basal medium (EBM-2 media (Cambrex) supplemented with microvascular additives (excluding hydrocortisone): human endothelial growth factor, 5% fetal bovine serum (FBS), vascular endothelial growth factor, human fibroblast growth factor type B, R3 insulin-like growth factor 1, ascorbic acid, heparin and the appropriate antibiotics gentamicin and amphotericin B (as per the supplier's protocol). HUVECs were also cultured in a serum-starved media (EBM-2 for endothelial cells containing 0.5% FBS, without supplement). Cells were cultured to near confluence and then incubated with or without 1 mM DEXA (Sigma Chemical, St. Louis, MO, USA) for 48 h. The 1 mM concentration of DEXA was selected based on previous performed dose–response experiments and previous studies reporting the effects of high-dose DEXA [18–22]. During the last 4–12 h of treatment with DEXA, tumor necrosis factor-α (TNF-α, 10 ng/ml; Invitrogen, Burlington, ON, Canada), or lipopolysaccharide (LPS, 10 μg/ml; Sigma Chemical), or thrombin (Thr, 1.5 U/ml; Calbiochem, San Diego, CA, USA) was added. The concentration for the agonists was selected according to previous reports [23,24]. The maximum incubation time with TNF-α, LPS, or Thr was 12 h, whereas for thrombomodulin, the incubation period was 4 h as observed previously [24]. After 48 h in culture, HUVECs were harvested following a 5 min incubation period with trypsin (0.05%) and EDTA (0.5 mM).

Since both DEXA and serum starvation could influence the expression of genes involved in the modulation of the apoptotic pathway [25–27]. HUVECs were cultured in a non-serum-starved media condition. A serum-starved media (EBM-2 for endothelial cells containing 0.5% FBS without supplement) was also tested.

2.2. Cell viability assay with trypan blue exclusion test

The cell viability of the HUVEC was assessed at 24, 44, and 48 h following incubation with DEXA; subconfluent HUVECs were suspended in 2 ml of medium and 50 μl of this mixture was incubated with an equal volume of 0.4% trypan blue (Sigma Chemical). Cells incorporating trypan blue were considered non-viable, and were counted using a hemocytometer, a cell counter (Coulter, Hialeah, FL, USA) and an inverted microscope (Olympus CX2; Optical Co., Ltd., Tokyo, Japan). Cell viability above 95% was considered ideal and over 90% acceptable [23].

2.3. Real-time polymerase chain reaction (TaqMan RT-PCR)

HUVECs were harvested for total RNA utilizing the RNeasy Mini Kit (QIAGEN Inc., Mississauga, ON, Canada) followed by DNase I treatment (QIAGEN). Multiplex RT-PCR was carried out according to the protocol provided by the manufacturer for the TaqMan One step PCR Master Mix Reagents Kit (Applied Biosystems, Foster City, CA, USA). Primer–probe sets were designed with the Primer Express 1.5 software and synthesized by Applied Biosystems and a thermal cycler (Prism 7900, Applied Biosystems). All primer and probe sets were subjected to a database search to ensure that there was no potential conflicting transcript matches to pseudogenes or homologous domains with related genes. The sequences of primers and probes are listed in Table 1. For the relative quantification of gene expression, the comparative threshold cycle (ΔCt) method was employed and normalized against 18S rRNA (primers and probes provided from Maxim Biotech, Inc., Rockville, MD, USA), which was measured by the same method. All PCR reactions were performed in triplicate. Control reactions were set up lacking reverse transcriptase to assess the level of contaminating genomic DNA.

Table 1
List of primers and probes used in the real-time PCR assay.

Gene name	Sequences (5′ to 3′)
h ICAM-1	Forward primer: GCC AGG AGA CAC TGC AGA CA Reverse primer: GGC TTC GTC AGA ATC ACG TTG Probe: TGA CCA TCT ACA GCT TTC CGG CGC
h VCAM-1	Forward primer: TCG AGA CCA CCC CAG AAT CT Reverse primer: GCC TGT GGT GCT GCA AGT C Probe: ATA TCT TGC TCA GAT TGG TGA CTC CGT CTC A
h E-selectin	Forward primer: TGC CAA GCA GCA TGG AGA C Reverse primer: ACC ACA TTG CAG GCT GGA AT Probe: CAG TGT ATG TCC TCT GGA GAA TGG AGT GCT C
h PAI-1	Forward primer: TCGAGG TGA ACG AGAGTG GC Reverse primer: CAT GCG GGC TGA GAC TAT GA Probe: CGG TGG CCT CCT CAT CCA CAG CT
h TF	Forward primer: TTC ACA CCT TAC CTG GAGACA AAC Reverse primer: CAT TCA CTT TTG TTC CCA CCT G Probe: TCG GAC AGC CAA CAA TTC AGA GTT TTG A
h vWF	Forward primer: AGT GTC CCT GCG TGC ATT C Reverse primer: CGG CAA ATG CAG GTG TTG Probe: CCC CGG CAC CTC CCT CTC TCG A
h TM	Forward primer: GCC AGA TGT TTT GCA ACC AGA Reverse primer: AGC TAG CCT GGG TGT TGG G Probe: TGC CTG TCC AGC CGA CTG CG
h t-PA	Forward primer: AGT TCT GCA GCA CCC CTG C Reverse primer: CCA CGG TAG GCT GAC CCA T Probe: CTC TGA GGG AAA CAG TGA CTG CTA CTT TGG G
h u-PA	Forward primer: GCC CTA AAG CCG CTT GTC CA Reverse primer: AGG AGA GGA GGG CTT TTT TCC Probe: TGC ATG GTG CATGAC TGC GCA

h = human.

2.4. Western blot analysis

Cells were solubilized with lysis buffer (125 mM Tris–HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, and 0.1% bromophenol blue), scraped and protein concentration was determined by Bradford assay. Cell lysates were immunoprecipitated with goat polyclonal anti-human CD62E (E-selectin) IgG, rabbit polyclonal anti-human CD106 (VCAM-1) IgG, or with rabbit polyclonal anti-human CD54 (ICAM-1) IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and separated on SDS-PAGE. Proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane and probed with the appropriate horseradish peroxidase-coupled secondary antibodies (Santa Cruz Biotechnology). Membranes were stripped using Re-Blot Plus strong stripping solution (Chemicon International, Lake Placid, NY, USA). Immunoreactive bands were visualized using LumiGlo™ (New England Biolabs, Pickering, ON, Canada). The protein expression was estimated by scanning densitometry of Western blots using a calibrated imaging densitometer and quantified using Quantity One software (Bio-Rad, Mississauga, ON, Canada).

2.5. FACS assay

Cells were incubated with FITC-conjugated antibody at 4 °C for 50 min, followed by three washes with fluorescence-activated cell sorting (FACS) washing buffer. The antibodies were as follows: mouse anti-human FITC-conjugated monoclonal CD54 (ICAM-1) antibody (ID Labs Inc., London, ON, Canada), mouse anti-human FITC-conjugated monoclonal CD106 (VCAM-1) antibody (Becton, Dickinson and Company, Mississauga, ON, Canada) and mouse anti-human FITC-conjugated monoclonal CD62E (E-selectin) antibody (ID Labs Inc.). Fluorescence data were collected based on the mean fluorescent intensity values using a Becton Dickinson FACScan (FACSCalibur). All experiments were performed at least in triplicate.

2.6. Neutrophil isolation and purification

Venous blood was obtained from healthy donors. The protocol was approved by the Montreal Heart Institute's Ethics Committee. Neutrophils were isolated as previously described [28]. Ninety-five percent of the isolated cells were polymorphonuclear cells as determined with a cell counter. Cell viability was found to be greater than 98% as assessed by the trypan blue dye exclusion assay.

2.7. Neutrophil adhesion assay

HUVECs were seeded and cultured in gelatin (0.25%)-coated 24-well plates in EBM-2 medium with the microvascular additives excluding hydrocortisone. After 24 h, the complete media was replaced with fresh complete media, with or without DEXA (1 mM). 24 h later, media was replaced and a cell viability test was performed. After 20 additional hours, the three stimulators (TNF-α, LPS or thrombin) or PBS (negative control) were added in the cell media for 3 h. Cells were rinsed with Hank's buffered salt solution (HBSS) plus HEPES (10 mM). Neutrophils (1×10^5 in 500 μl of HBSS/HEPES with 5 mM $CaCl_2$) were added to each well and incubated for 1 h at 37 °C. The wells were rinsed with HBSS/HEPES to remove non-adherent neutrophils, and fixed with a 1% paraformaldehyde–PBS. Adhesion of neutrophils to HUVEC monolayers was assessed with a color video digital camera (Sony ExwaveHAV) adapted to a binocular microscope (Olympus CX2) as previously described [29].

2.8. Statistical analysis

The results of data reported on mRNA, protein expression and adhered neutrophils were given as the mean ± SEM. Comparison between groups was made with Student's *t*-test for parametric data and Mann–Whitney *U*-test for non-parametric data. Differences were considered at *p*-values less than 0.05.

3. Results

The mRNA expression levels of nine selected haemostatic candidate genes [intercellular adhesion molecule-1 (ICAM-1), vascular

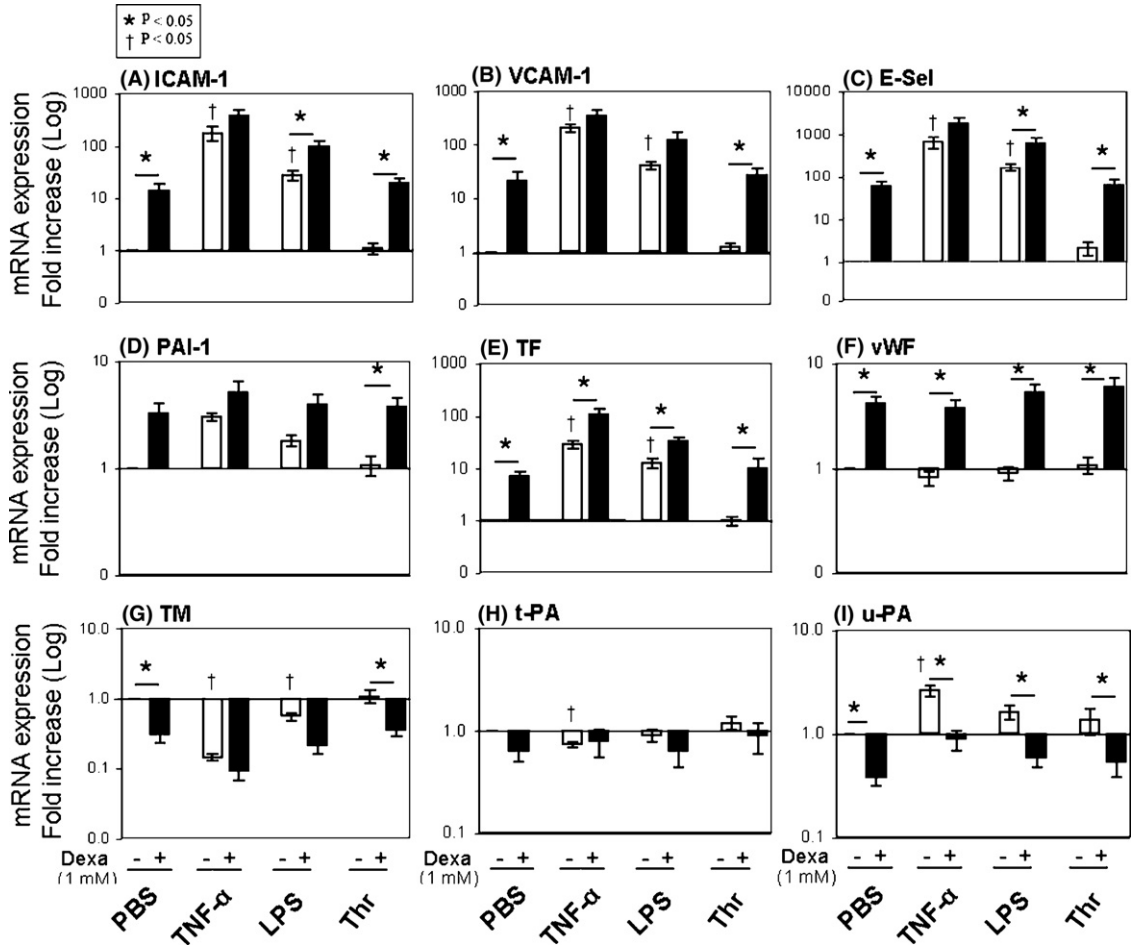

Fig. 1. Dexamethasone (DEXA) sensitizes HUVEC to TNF-α-, LPS- and Thr-mediated expression of haemostatic genes mRNA levels. (A–I) Real-time PCR assays of (A) ICAM-1, (B) VCAM-1, (C) E-Sel, (D) PAI-1, (E) TF, (F) vWF, (G) TM, (H) t-PA, and (I) u-PA mRNA expression in HUVEC preincubated for 48 h in the absence (control) or presence of Dexa (1 mM) and treated with (+) or without (−) TNF-α (10 ng/ml), LPS (10 μg/ml) or Thr (1.5 U/ml) for 4 h. Data shown are means ± SEM of six experiments; $^*p < 0.05$ comparing the effect of Dexa in the absence or presence of agonist and $^\dagger p < 0.05$ comparing the effect of agonist to control (PBS).

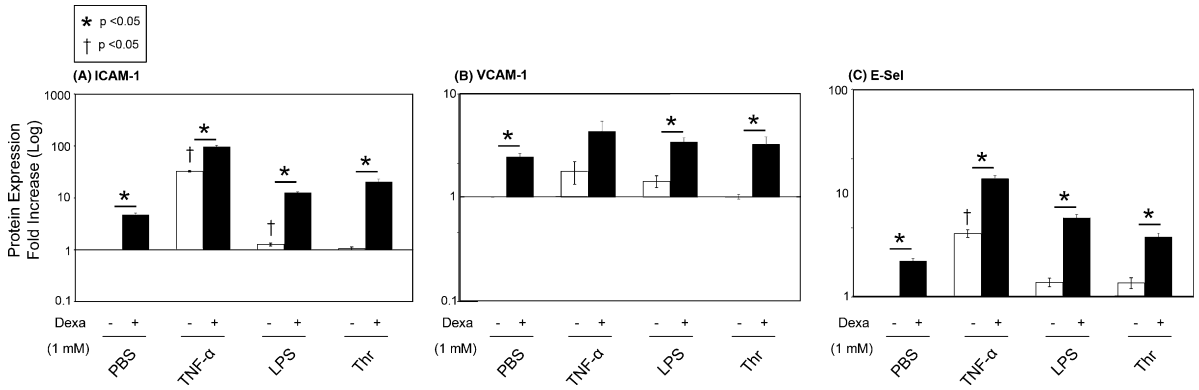

Fig. 2. Dexamethasone (DEXA) sensitizes HUVEC to TNF-α-, LPS- and Thr-mediated induction of ICAM-1, VCAM-1 and E-selectin cell surface protein expression. (A–C) FACS assays of ICAM-1, VCAM-1 and E-selectin cell surface protein expression of HUVEC preincubated for 48 h in the absence (control) or presence of Dexa (1 mM) and treated with (+) or without (−) TNF-α (10 ng/ml), LPS (10 μg/ml) or Thr (1.5 U/ml) for 4 h. Data shown are means ± SEM of three to four experiments; $^{*}p < 0.05$ comparing the effect of Dexa in the absence or presence of agonist and $^{\dagger}p < 0.05$ comparing the effect of agonist to control (PBS).

cell adhesion molecule-1 (VCAM-1), E-selectin (E-Sel), plasminogen activator inhibitor-1 (PAI-1), tissue factor (TF), von Willebrand factor (vWF), thrombomodulin (TM), tissue-plasminogen activator (t-PA) and urokinase-plasminogen activator (u-PA)] was measured in HUVEC exposed to PBS or to high-dose DEXA (1 mM) ± three different agonists (TNF-α, 10 ng/ml; LPS, 10 μg/ml or Thr, 1.5 U/ml), respectively (Fig. 1A–I). Treatment with DEXA alone resulted in a significant induction of all three cell adhesion molecules (ICAM-1, VCAM-1 and E-selectin) as well as TF and vWF mRNA, while down-regulating TM and u-PA mRNA levels (p-value < 0.05) but had no significant effect on basal level of PAI-1 and t-PA. In the absence of DEXA preconditioning, ICAM-1, VCAM-1, E-Sel, TF and u-PA were upregulated significantly in the presence of TNF-α or LPS. Conversely, TM mRNA was down-regulated under the same experimental conditions. The t-PA gene expression was significantly down-regulated only in the presence of TNF-α. Following 48 h exposure to DEXA (1 mM) in the presence of agonists, the effects became significantly enhanced compared to DEXA alone specifically for LPS-mediated ICAM-1 and E-selectin mRNA levels as well as TNF-α-, LPS- and Thr-mediated tissue factor or vWF mRNA levels (Fig. 1). Results under serum-starved conditions (EBM-2 for endothelial cells containing 0.5% FBS without supplement) showed the same trend but a somewhat lower response in general was seen except in the presence of thrombin: results showed enhanced thrombin-mediated induction of cell adhesion molecules (E-selectin and ICAM-1) and TF under these conditions (data not shown).

As we observed that the high dose from the DEXA regimen modulated haemostatic gene mRNA, we wanted to assess the effect of high dose DEXA on the expression of endothelial cell surface proteins, which have been assayed by FACS (Fig. 2A–C) and Western blot analyses (Fig. 3A–C). By FACS analyses, we observed that a treatment with DEXA alone resulted in a significant induction of all three cell adhesion molecules (ICAM-1, VCAM-1 and E-selectin) (Fig. 2) which correlated with their corresponding upregulated mRNA levels (Fig. 1A–C). In the absence of DEXA preconditioning, ICAM-1 and E-Sel were upregulated significantly in the presence of TNF-α or LPS (ICAM-1) (Fig. 2), whereas both cytokines did not promote a VCAM-1 protein upregulation (Fig. 2). In addition, a treatment with Thr alone had no significant effect on protein expression of these endothelial adhesion molecules (Fig. 2). Following 48 h exposure to DEXA (1 mM) in the presence of agonists, TNF-α, LPS or Thr increased significantly the cell surface expression of ICAM-1 and E-Sel as measured by FACS ($p < 0.05$) whereas LPS and Thr increased significantly cell surface expression of VCAM-1 ($p < 0.05$) but not in the presence of TNF-α (Fig. 2A–C).

We then performed Western blot analyses selecting the same endothelial cell adhesion molecules ICAM-1, VCAM-1 and E-selectin. Sustained treatment with DEXA (1 mM) alone enhanced ICAM-1 protein expression significantly ($p < 0.05$) but failed to promote protein expression of other cell adhesion molecules. In the absence of DEXA preconditioning, ICAM-1 and VCAM-1 protein expression were upregulated in the presence of TNF-α or LPS, whereas modulation in the presence of Thr was seen only for the ICAM-1 gene. The 48 h exposure to DEXA (1 mM) resulted in significant increased TNF-α-mediated induction of these cell adhesion molecules whereas no significant modulation was seen in the presence of LPS or Thr (Fig. 3A–C).

To determine the functional consequences of high-dose DEXA preconditioning on cell adhesion molecules, HUVECs were assayed for agonist-mediated leukocyte adhesion (Fig. 4). Pre-treatment of HUVEC with DEXA (1 mM) for 48 h alone had no statistically

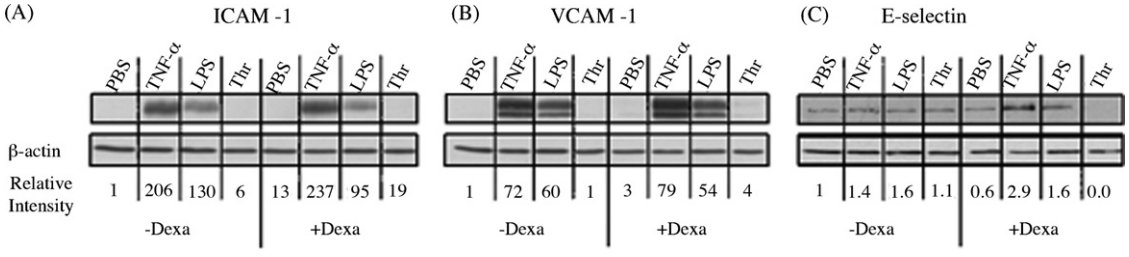

Fig. 3. Dexamethasone (DEXA) sensitizes HUVEC to TNF-α-mediated induction of ICAM-1, VCAM-1 and E-selectin protein expression. (A–C) Western blot analysis of (A) ICAM-1, (B) VCAM-1, and (C) E-selectin protein from whole cell extract of HUVEC preincubated for 48 h in the absence (control) or presence of Dexa (1 mM) and treated with (+) or without (−) TNF-α (10 ng/ml), LPS (10 μg/ml) or Thr (1.5 U/ml) for 4 h. Blots shown are representative of at least three independent experiments performed using separately prepared cell extracts. β-Actin was used to monitor protein loading.

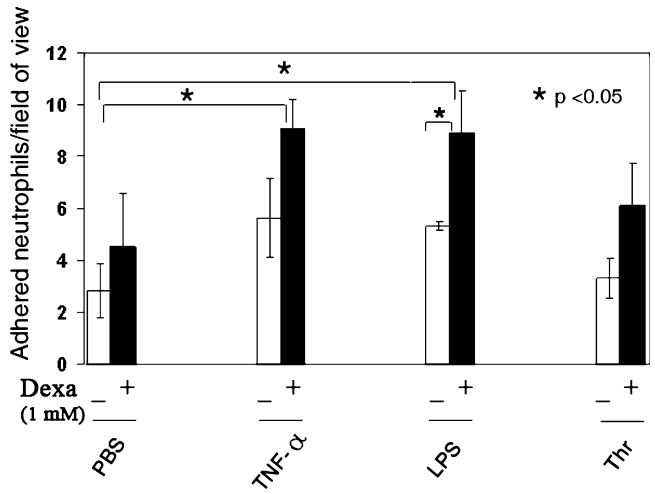

Fig. 4. Dexamethasone (DEXA) enhances significantly TNF-α and to a lesser extent LPS-mediated adhesion of neutrophils. Confluent monolayers of HUVECs were preincubated for 48 h in the absence (control) or presence of Dexa (1 mM) and treated with (+) or without (−) TNF-α (10 ng/ml), LPS (10 μg/ml) or Thr (1.5 U/ml) for 4 h. At the last hour of the experiment, neutrophils (1×10^5 in 500 μl of HBSS/HEPES with 5 mM $CaCl_2$) were added to each well, incubated for 1 h at 37 °C and adhesion of neutrophils to HUVEC monolayers was assessed. Data shown are means ± SEM of four experiments; *$p < 0.05$, comparing the effect of DEXA with (+) agonist to control (PBS) and agonist (LPS).

significant effect on adhesion. Treatment of HUVEC with TNF-α (10 ng/ml), LPS (10 μg/ml) and Thr (1.5 U/ml) for 4 h resulted in a 1.98-fold, 1.97-fold, and 1.16-fold increase in neutrophil adhesion, respectively, without reaching significance (Fig. 4). However, following 48 h exposure to DEXA (1 mM) in the presence of agonists resulted in significantly enhanced TNF-α- and LPS-mediated adhesion of neutrophils ($p < 0.05$) (Fig. 4).

3.1. Dose–response and cell viability results

The dose–response experiment for mRNA expression of VCAM-1 using flow cytometry was performed showing the start of a plateau phase at 1 mM concentration of DEXA. Even with the dose of 1 mM in comparison to the dose of 0.1 mM the response effect did not dramatically change (nearly 20% increase)(Fig. 5). A live cell number of HUVEC under exposure to DEXA was measured. Cell viability was 95%, 93% and 92% at 24, 44 and 48 h of exposure to DEXA and 99%, 98% and 98% in controls, respectively.

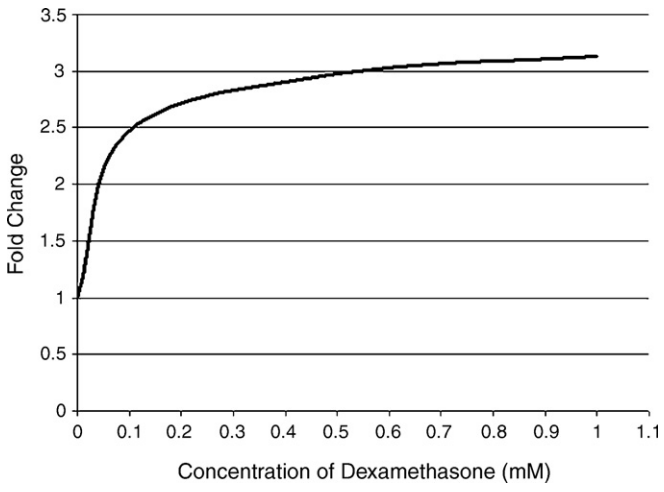

Fig. 5. Effect of dexamethasone on VCAM-1 protein expression in induced human umbilical vein endothelial cells.

4. Discussion

The objective of our study was to assess the pharmacological effects of high dose GCs on the pro-adhesiveness of endothelial cells in order to better understand the mechanisms involved in the deleterious side effects of this drug at the microvascular level. GCs may cause and/or worsen outcome in diseases associated with thrombosis such as osteonecrosis of the femoral head [30], multiple myeloma [4] or stroke [31] or it may exacerbate symptoms in inflammatory processes such as sepsis [2]. These adverse effects have been reported mostly with high-dose administration of GCs.

A recent meta-analysis comparing low- and high dose GC treatment in patients with sepsis demonstrated a decreased survival rate with a short course of high-dose treatment, but increased the survival rate and a shock reversal at lower doses [2,15]. It has been thought that during sepsis the high levels of cytokines could interact with the upregulated cytokine receptors caused by GCs [2,32]. Jilma et al. also showed that high dose (1.0 mg/kg/bid) compared to low-dose (0.04 mg/kg/bid) administration of DEXA to healthy individuals enhanced the levels of serum vWF and soluble P-selectin resulting in the development of a hypercoagulable state [13]. Therefore, it is assumed that the treatment dose of DEXA may play a major role in the outcome of the disease.

Since the scope of this study is the adverse effects caused by the high dose of DEXA, we selected a dose of DEXA of 1 mM in our *in vitro* studies, mimicking the high dose used under clinical conditions [33–37]. The concentration of DEXA in patient serum with a regular therapeutic setting (0.5–9 mg a day) is equivalent with 0.01–0.1 mM [22]. A dose–response experiment was performed showing the start of a plateau phase at 1 mM concentration. The concentration of DEXA (1 mM) did not produce significant toxicity as assessed by cell number and viability (based on morphology and trypan blue exclusion test).

In this study, high-dose DEXA stimulated the expression of genes encoding the adhesion molecules (ICAM-1, VCAM-1 and E-selectin) mediated by TNF-α, LPS or Thr. At low dose DEXA (1 μM) added 1 h before HUVEC activation with TNF-α or IL-1β, only mRNA expression level of E-selectin was significantly upregulated ($p < 0.05$) amongst the adhesion molecules (ICAM-1, VCAM-1 and E-selectin) [38]. In our study, consistent with the upregulation of mRNA levels, protein expression and cell surface expression of adhesion molecules were maximal under TNF-α exposure. Several studies have shown that cell adhesion molecules, expressed by activated endothelial cells, are responsible for the accumulation of blood leukocytes at sites of inflammation by mediating the adhesion of inflammatory cells to the endothelium [39]. Incubation of endothelial cells with other cytokines such as IL-8 did not significantly alter the expression of the adhesion molecules (ICAM-1, VCAM-1 and E-selectin) [40] although it has been shown that DEXA could effect IL-8 and inhibit its production in different endothelial cell lines [41]. Similarly, DEXA had a small inhibitory effect on constitutive monocyte chemotactic protein-1 (MCP-1) mRNA expression, but no effect on the induction by TNF-α [42].

Our results showed that TNF-α, LPS or Thr treatment alone increased neutrophil adherence to endothelial surface, without reaching statistical significance. Pre-treatment of HUVEC with DEXA resulted in enhanced agonist-mediated induction of neutrophil adhesion, and reached statistical significance with TNF-α and LPS ($p < 0.05$). The functional assay results suggest that high-dose DEXA increased the expression of adhesion molecules by endothelial cells in the presence of inflammatory mediators. Hence, high-dose DEXA, in addition to enhancing the cytokine receptors, appears to exacerbate the local endothelial inflammatory process. Some studies using lower doses of DEXA showed that GCs inhibit the expression of endothelial cell adhesion molecules [25,39]. These findings may well be explained by a dose-dependent

mechanism, which varies according to studies and different cell types. It has been shown that endothelial cells in different anatomic regions of the same patient are structurally and functionally dissimilar [43–46]. This regional endothelial cell dysfunction may be the disease pathway for several different disease processes where systemic genetic profiles interact with regional endothelial gene function and protein expression. One such disease process is osteonecrosis, or avascular necrosis, of the femoral head. The endothelial cell as the final common pathway for this disease has been postulated in the literature. The authors assume that the endothelial cells located in the feeding vessels of the femoral head act and may respond differently to stimuli than do endothelial cells elsewhere in the same patient [3] resulting in local microthrombi and hypofibrinolysis. This possibility is worthy of further consideration in other disease processes, as thromboembolism of the microvasculature may be the end result where high-dose GCs are known to be a significant risk factor in the disease [47].

It is also well established that inflammatory pathways closely interact with fibrinolytic and coagulation pathways. In a human endotoxemia model, GC administration to healthy subjects reduced levels of proinflammatory cytokines (TNF-α) while at the same time moderately enhancing the procoagulant response [48]. In the present study, the expression of TM was down-regulated. Concurrently, the mRNA expression of TF, and vWF increased significantly under DEXA exposure. Previously, it has been shown that DEXA enhanced TF expression in human monocytes [49] although results were controversial in another study [50]. High-dose DEXA also increased the expression of PAI-1 under the influence of Thr. PAI-1 is an inhibitor of tissue-plasminogen activator, which induces resolution of blood clots during wound healing and following inflammatory reactions [51]. Zonneveld et al. showed that DEXA increased the promotor activity of PAI-1 [52]. In our study, PAI-1 mRNA levels were upregulated significantly under DEXA exposure when cells were stimulated with Thr but not with TNF-α or LPS, although Yamamoto et al. showed that lower dose of DEXA (10^{-6} M) increased significantly the expression of PAI-1 gene in HUVECs stimulated by TNF-α [53]. The expression of t-PA, the primary initiator of fibrinolysis, was down-regulated with DEXA treatment although this did not reach significance. The expression of u-PA, another profibrinolytic factor, was also decreased when HUVECs were exposed to DEXA alone or treated with DEXA and the agonists, although interestingly, the agonists alone separately had an upregulatory effect. Based on these findings, it may be that high-dose DEXA induces a hypofibrinolytic state on vascular endothelial cells.

The mechanisms underlying high-dose GC-mediated sensitization of the endothelium remain to be defined. Some published data suggest a role for NF kappa-β and p38MAPK [54–56], emphasizing the importance of low-dose versus high-dose DEXA in promoting an anti-inflammatory condition since low dose has been shown to inhibit NF kappa-β. Previous reports indicated that low-dose DEXA (0.1–1 μM) inhibits NF kappa-β binding to DNA in some cell lines such as monocytes and lymphocytes [57], but not in endothelial cells [58] by increasing transcription of the I kappa-β alpha gene thereby increasing the concentration of this inhibitor in the cytoplasm and preventing the translocation of NF kappa-β to the nucleus [57]. Thus, the effects of DEXA especially in high dose on endothelium could possibly be explained by pathways besides the NF kappa-β-dependent pathway.

In summary, these findings suggest that GCs, especially at high dose, sensitize human primary endothelial cells to TNF-α- and LPS- mediated neutrophil adhesion and modulate haemostatic gene expression favoring pro-adhesiveness. These results are in accordance with the current conception of what the effects of GCs are at the disease level. This may provide some insight on the association between high-dose DEXA and thrombotic/inflammatory events seen in clinical practice. Identification of the mechanism(s) by which high-dose DEXA stimulates the expression of adhesion molecules and modulates haemostatic gene expression will be an important focus for future research, with major implications in the development of preventive strategies aimed at avoiding these complications.

Acknowledgments

We thank Melissa Cheung and Colin Cheung for their excellent technical assistance. Primers and probes were generous gifts from Dr. William C. Aird, BIDMC, HMS, Boston, MA, USA. This study was conducted at McGill University Health Centre, Montreal, Quebec, Canada. We thank the Hematology Foundation of the Montreal General Hospital for continued financial support.

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