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Inflammation Determines the Pro-Adhesive Properties of High Extracellular D-Glucose in Human Endothelial Cells In Vitro and Rat Microvessels In Vivo

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Abstract

Background: Hyperglycemia is acknowledged as an independent risk factor for developing diabetes-associated atherosclerosis. At present, most therapeutic approaches are targeted at a tight glycemic control in diabetic patients, although this fails to prevent macrovascular complications of the disease. Indeed, it remains highly controversial whether or not the mere elevation of extracellular D-glucose can directly promote vascular inflammation, which favors early pro-atherosclerotic events.

Methods and Findings: In the present work, increasing extracellular D-glucose from 5.5 to 22 mmol/L was neither sufficient to induce intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression, analyzed by flow cytometry, nor to promote leukocyte adhesion to human umbilical vein endothelial cells (HUVEC) in vitro, measured by flow chamber assays. Interestingly, the elevation of D-glucose levels potentiated ICAM-1 and VCAM-1 expression and leukocyte adhesion induced by a pro-inflammatory stimulus, such as interleukin (IL)-1β (5 ng/mL). In HUVEC, high D-glucose augmented the activation of extracellular signal-regulated kinase 1/2 (ERK 1/2) and nuclear transcription factor-κB (NF-κB) elicited by IL-1β, measured by Western blot and electromobility shift assay (EMSA), respectively, but had no effect by itself. Both ERK 1/2 and NF-κB were necessary for VCAM-1 expression, but not for ICAM-1 expression. In vivo, leukocyte trafficking was evaluated in the rat mesenteric microcirculation by intravital microscopy. In accordance with the in vitro data, the acute intraperitoneal injection of D-glucose increased leukocyte rolling flux, adhesion and migration, but only when IL-1β was co-administered.

Conclusions: These results indicate that the elevation of extracellular D-glucose levels is not sufficient to promote vascular inflammation, and they highlight the pivotal role of a pro-inflammatory environment in diabetes, as a critical factor conditioning the early pro-atherosclerotic actions of hyperglycemia.

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Introduction

Vascular inflammation plays a pivotal role in the initiation and progression of the atherosclerotic plaque [1]. Indeed, the migration of circulating leukocytes from the blood to sites of extravascular injury is an early pro-atherosclerotic event mediated through a multistep adhesion cascade, initiated by the tethering of leukocytes to the endothelium, followed by weak, transient adhesive interactions manifested as leukocyte rolling, which leads to firm leukocyte adhesion and ultimately to transmigration through the vascular endothelium [2,3]. Cell adhesion molecules (CAMs), including intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), which are expressed by activated endothelial cells, play a crucial role in leukocyte adhesion and migration [2,3].

Diabetes mellitus is characterized by a systemic pro-inflammatory environment, exhibiting enhanced basal and postprandial circulating levels of pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α) [4,5]. An over-expression of pro-inflammatory CAMs has been reported in the cardiovascular system of animal models of diabetes [6,7]. Furthermore, diabetic patients exhibit enhanced circulating levels...
of soluble ICAM-1 and VCAM-1 [8–10], which are considered to reflect vascular CAMs expression and represent prognostic markers of macrovascular complications and cardiovascular mortality [11].

Hyperglycemia, both basal and postprandial, has been identified over the years as an independent risk factor for cardiovascular diseases [4,12,13]. Indeed, sera from diabetic patients increase in vitro the adhesion of human monocytes and the expression of CAMs in human endothelial cells [14,15]. It remains controversial, however, whether or not high D-glucose itself can stimulate such vascular pro-inflammatory mechanisms. Supporting a direct role of high D-glucose, Morigi et al. [14] first described enhanced VCAM-1 and ICAM-1 expression and leukocyte-endothelial adhesive interactions after incubating endothelial cultures for 24 h with 30 mmol/L extracellular D-glucose. Other studies have later on reported increased CAMs expression in human endothelial cells exposed to high D-glucose concentrations during time periods ranging from 24 h to 14 days [16–18], which has been attributed, at least in some cases, to hyperosmolality [16].

Contrarily, an earlier work by Kim et al. [19] neither found induction of VCAM-1 and ICAM-1 nor enhanced adhesion of HL60 leukocytes by high D-glucose (25 mmol/L for 7–10 days) in human endothelial cells. More recently, Rasmussen et al. [15] suggested that the in vitro induction of CAMs in human endothelial cells by diabetic sera could not be solely attributed to high D-glucose concentrations, but more likely to the presence of serum cytokines. Additionally, both Cacicedo et al. [20] and Wada et al. [21] have demonstrated that high D-glucose alone does not induce the expression of genes encoding for VCAM-1 or ICAM-1 in human endothelial cells.

At present, the therapeutic approaches for preventing diabetes-associated cardiovascular diseases are mostly focused on tightly controlling hyperglycemia. In fact, some clinical trials in the 90's indicate that intensive blood-glucose control delays the onset and slows the progression of microvascular disease, both in type 1 and type 2 diabetes, although microvascular complications are only prevented in a limited way after years of treatment [22–25]. Nevertheless, other recent trials with type 2 diabetic patients do not even show a beneficial effect of intensive glycemic control in the rate of cardiovascular events or deaths associated to atherosclerotic macrovascular disease [26–28]. Therefore, it has been now suggested that D-glucose is not the major mechanistic pro-atherosclerotic mediator in diabetes [29].

To clarify this point, it is necessary to carefully dissect the direct contribution of elevated D-glucose to the activation of early pro-inflammatory vascular events that initiate the atherosclerotic process. On the other hand, the potential interaction between D-glucose and pro-inflammatory cytokines, as two co-existing conditions in diabetes that may promote vascular inflammation, is still largely unknown and should be addressed. For this purpose, we have explored the respective contribution of both D-glucose at high concentrations and the pro-inflammatory cytokine IL-1β to endothelial CAMs expression and leukocyte trafficking, by combining both in vitro and in vivo approaches.

Materials and Methods

Ethics statement

The investigation conforms with the principles outlined in the Declaration of Helsinki. Experiments with human cells were reviewed and approved by the ethics committee of Universidad Autónoma de Madrid and Hospital Universitario de Getafe, and written informed consent was obtained from all donors. The investigation with animals conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and was approved by the ethics committee of Universidad de Valencia.

Materials

Culture plastic ware was from TPP (Tragadingen, Switzerland). M199 and fetal calf serum were from Biological Industries ( Beit-Haemek, Israel). Human recombinant IL-1β was purchased from Peprotech (London, UK), with an endotoxin level below 0.1 ng per µg. D-glucose was supplied by Serva (Heidelberg, Germany). Endothelial cell growth supplement, heparin, L-glucose, pyrrolidine dithiocarbamate, PD 98059 and, unless otherwise stated, all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell isolation and culture

Human umbilical vein endothelial cells (HUVEC) were enzymatically isolated, as previously described [30], and cultured in M199 medium supplemented with 20% fetal calf serum (FCS), 25 µg/mL endothelial cell growth supplement (ECGS), 100 µg/mL heparin and antibiotics [30]. For experiments, HUVEC at passages 1–3 were used.

For adhesion under flow assays, HL60 leukocytes were obtained from American Type Culture Collection (ATCC; Rockville, MD) and grown in RPMI-1640 medium (Biowhittaker, Walkersville, MD) supplemented with 10% FCS and antibiotics.

Flow cytometry

Confined HUVEC monolayers were treated with different concentrations of D-glucose (5.5 and 22 mmol/L), either alone or in combination with increasing concentrations of IL-1β. The non-metabolizable analogue of D-glucose, L-glucose, was used as an osmotic control. After 18 h, the expression of VCAM-1 and ICAM-1 at the cell surface was measured by flow cytometry. In brief, HUVEC were gently detached with phosphate-buffered saline (PBS) containing 0.05% trypsin, fixed with 2% paraformaldehyde and blocked with PBS containing 3% bovine serum albumin (BSA) for 15 min. Cells were then suspended in PBS containing 0.5% BSA and incubated for 30 min with primary antibodies against VCAM-1 (clone IE5) or ICAM-1 (clone 6.5B5; Chemicon, Temecula, CA), both at a 1/100 dilution. Detection of primary antibodies was performed using an appropriate Alexa Fluor 488-secondary antibody (Molecular Probes-Invitrogen Corporation, Carlsbad, CA; dilution 1/250). Fluorescence was measured in a FACScan flow cytometer (Beckton-Dickinson, Franklin Lakes, NJ), and data analyzed using Cell Quest software (Beckton-Dickinson, Franklin Lakes, NJ).

The expression of CD11b/CD18 integrins was determined on human leukocytes in heparinized whole blood. Blood samples were obtained from buffy coats of four healthy donors by Ficoll Hypaque density gradient centrifugation. Samples (basal glucose concentration: 5.6±0.3 mmol/L) were incubated at 37°C with PBS or D-glucose (16.5 mmol/L to achieve a final concentration of around 22 mmol/L), either alone or in combination with IL-1β (5 ng/mL) for 18 h. Samples were then incubated for 20 min on ice in the dark with saturating amounts (10 µL) of the conjugated mAb anti-human-CD11b/CD18-FTTC (clone ICRF 44; Serotec, Madrid, Spain). Red blood cells were lysed and leukocytes fixed using an automated EPICS Q-PREP system (Coulter Electronics, Hialeah, FL). Samples were run in an EPICS XL-MCL flow cytometer (Beckman-Coulter, Hialeah, FL) [31].
Indirect immunofluorescence

ICAM-1, VCAM-1 and NF-κB were visualized in HUVEC by indirect immunofluorescence, accordingly to a previously described protocol [32]. Primary antibodies against VCAM-1 (dilution 1/250), ICAM-1 (dilution 1/25) or the NF-κB p65 subunit (dilution 1/100; Transduction Laboratories, Lexington, KY) were used, followed by incubation for 1 h at room temperature with an Alexa Fluor 488-conjugated secondary antibody (1/250). Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes-Invitrogen). HUVEC were observed with an Eclipse TE300 epifluorescence microscope (Nikon, Tokyo, Japan).

Nuclear extracts and electrophoretic mobility shift assay

HUVEC were exposed to the different treatments during 1, 4, 6 and 18 h and nuclear extracts were prepared as described before [32]. A commercial oligonucleotide (Promega, Madison, WI) encoding the NF-κB consensus sequence 5′-AGTTGAGGG-GACCTTTCCAGGG-3′ was 5′-end labeled using [γ-32P]ATP and T4 polynucleotide kinase (Promega, Madison, WI) and purified using MicroSpinTM G-25 columns (GE Healthcare, Chicago, IL). For binding reactions, nuclear extracts (5 μg) were incubated on ice for 15 min in a reaction buffer [40 mmol/L HEPA (pH 7.0), 140 μmol/L NaCl, 5 mmol/L diithiothreitol, 10 μg/mL BSA, 0.01% Nonidet P-40, 4% Ficoll and 0.05 μg/mL poly(dI-dC):poly(dI-dC)]. After addition of the labeled oligonucleotide (~50,000 cpm) the reaction mix was further incubated for 20 min at room temperature. For competition experiments a 100-fold excess of unlabeled double-stranded oligonucleotide was added to the binding reaction. DNA-protein complexes were resolved on 4% nondenaturing polyacrylamide gels in 0.5x TBE added to the binding reaction. DNA-protein complexes were resolved on 4% nondenaturing polyacrylamide gels in 0.5x TBE added to the binding reaction. DNA-protein complexes were resolved on 4% nondenaturing polyacrylamide gels in 0.5x TBE added to the binding reaction.

Western blotting

Extracellular signal-regulated kinase 1/2 (ERK1/2) activation was determined by immunoblotting as previously described [32]. Polyclonal antibodies against both the phosphorylated (activated) and total forms of ERK 1/2 (Cell Signaling Technology, Inc., Danvers, MA; dilution 1/1,000) were used, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (dilution 1/10,000; Chemicon, Temecula, California, USA). ERK 1/2 activity was expressed as the phospho-ERK 1/2:total ERK1/2 ratio.

Flow chamber assays

The in vitro adhesion of HL60 to HUVEC monolayers was analyzed using a previously described live imaging flow model [33]. Briefly, HUVEC monolayers grown on glass coverslips were exposed for 18 h to 5.5 or 22 mmol/L D-glucose either alone or in combination with IL-1β (5 ng/mL). Coverslips were placed in a parallel plate flow chamber maintained at 37°C and HL60 leukocytes (1.5 x 106 cells/mL) were drawn for 2 minutes across the monolayers at a flow rate of 0.26 mL/min, corresponding to a shear stress of 0.5 dynes/cm2. Monolayers were visualized with an inverted microscope (Nikon TE2000, Nikon Inc., Melville, NY), and at least 5 fields were recorded during 10 seconds each using a phase contrast objective and VideoLab software (Ed Marcus Labs, Newton, MA).

Intravital microscopy

Non-diabetic male Sprague-Dawley rats (200–250 g) with a basal glycemia of 4.9±0.1 mmol/L (results from 5 animals) were employed. Animals were sedated with ether and intraperitoneally injected with 10 mL of either PBS alone, PBS with D-glucose (40 mg/kg), PBS with IL-1β (200 ng/kg), or PBS with D-glucose plus IL-1β. Parallel experiments were performed replacing D-glucose by L-glucose (40 mg/kg), which was used as an osmotic control.

After 18 h, the mesentery was exposed in preparation for intravital microscopy, following a previously described protocol [31]. Single unbranched mesenteric venules (25–40 μm in diameter) were selected, and the diameters measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, TX). The number of rolling, adherent, and emigrated leukocytes was determined off-line during playback analysis of videotaped images.

Immunohistochemistry

After the completion of the intravital microscopy measurements, the mesentery was isolated, fixed in 4% paraformaldehyde, dehydrated using graded acetone washes at 4°C, and embedded in paraffin wax for localization of ICAM-1 and VCAM-1, using a modified avidin and biotin immunoperoxidase technique as described previously [31]. Anti-rat-VCAM-1 (clone 5F10, kindly donated by Biogen Inc., Cambridge, MA) or anti-rat-ICAM-1 (clone 1A29, Serotec) monoclonal antibodies, or their isotype-matched control murine antibodies (UPC 10, IgG2a, and MOPC21 IgG1, Sigma Chemical Co.) were used. Positive staining was defined as a venule displaying brown reaction product.

Statistical analysis

Results are expressed as mean±SEM from three to six independent experiments. Student’s t-test was used for data points and one-way ANOVA with Fisher’s post-test correction was used for curves. A P value ≤0.05 was considered statistically significant.

Results

ICAM-1 and VCAM-1 expression on HUVEC

The mere elevation of extracellular D-glucose from 5.5 mmol/L up to 22 mmol/L did not alter the basal expression of both ICAM-1 and VCAM-1 in HUVEC after 18 h (107.05±4.4 and 104.5±3.7% of the expression observed with 5.5 mmol/L D-glucose, respectively. Figures 1C-1D). On the other hand, in HUVEC incubated in a medium containing 5.5 mmol/L D-glucose, the pro-inflammatory cytokine IL-1β enhanced the cell surface expression of both ICAM-1 and VCAM-1 in a concentration-dependent manner, with a sub-maximal effect observed at 5 ng/mL (Figures 1A-1B).

Interestingly, the stimulating effect of IL-1β (5 ng/mL) on ICAM-1 and VCAM-1 expression was potentiated when the extracellular D-glucose concentration was switched from 5.5 up to 22 mmol/L (Figures 1C-1D). These findings were also visualized by indirect immunofluorescence experiments, which confirmed the synergism between 22 mmol/L D-glucose and IL-1β on ICAM-1 and VCAM-1 expression, as well as the lack of effect of high D-glucose by itself on endothelial cell activation (Figure 1E).

The interaction between high D-glucose and IL-1β was not attributable to hyperosmolarity, since it was not reproduced after replacing D-glucose by its non-metabolizable analogue L-glucose (Figures 1C-1D).

To assess whether the potentiating effect of 22 mmol/L D-glucose was restricted to IL-1β, we performed experiments in which IL-1β was replaced by tumor necrosis factor (TNF)-α (10 ng/mL). In HUVEC cultured in a 5.5 mmol/L D-glucose-containing medium, TNF-α significantly induced both ICAM-1 and
and VCAM-1 levels (1,037.21 ± 182.82% and 156.38 ± 17.45% non-stimulated HUVEC, respectively; *P < 0.05, results from four independent experiments performed in triplicate). When the glucose concentration was shifted up to 22 mmol/L, the expression of both ICAM-1 and VCAM-1 was further significantly enhanced to 1,364.52 ± 106.99% and 206.86 ± 14.45%, respec-

Figure 1. Effect of IL-1β and D-glucose on the expression of adhesion molecules in HUVEC. Cells incubated in a medium containing 5.5 mmol/L D-glucose were challenged for 18 h with IL-1β (0.1 to 10 ng/mL) and the levels of (A) ICAM-1 and (B) VCAM-1 were determined at the cell surface by flow cytometry. In another set of experiments, cells were incubated for 18 h in medium containing 5.5 or 22 mmol/L D-glucose in the presence or absence of IL-1β (5 ng/mL), after which (C) ICAM-1 and (D) VCAM-1 levels were quantified. L-glucose was used as an osmotic control. *P < 0.05 versus 5.5 mmol/L D-glucose without IL-1β; †P < 0.05 versus 5.5 mmol/L D-glucose with IL-1β. (E) ICAM-1 (left panel) and VCAM-1 (right panel) were visualized by indirect immunofluorescence in HUVEC cultured for 18 h in culture medium containing 5.5 or 22 mmol/L D-glucose in the presence or absence of IL-1β (5 ng/mL). Nuclei were counterstained in blue with 4′,6-diamidino-2-phenylindole (DAPI) (x400).

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D-glucose).

intensity: 13.0

6

5.5 mmol/L D-glucose), which was not further augmented in

1/2 and NF-

(5 ng/mL), either alone or in the presence of the respective ERK

b

and VCAM-1 expression, HUVEC were treated with IL-1

co-incubated with 22 mmol/L D-glucose (Figures 2A and 3A).

Similarly, the exposure to high D-glucose did not result in NF-
kβ activation from 1 to 18 h after stimulation (Figure 3A).

Conversely, IL-1β (5 ng/mL) in 5.5 mmol/L D-glucose activated

ERK 1/2 after 10–30 min stimulation (Figure 2A) and triggered

NF-kB binding activity from 1 to 6 h, returning to basal levels

after 18 h (Figure 3A). The respective ERK 1/2 and NF-kβ

activation by IL-1β was further enhanced when the cytokine was

co-incubated with 22 mmol/L D-glucose (Figures 2A and 3A).

The translocation of NF-kB from the cytoplasm to the nucleus was

also visualized by immunofluorescence within the first hour of

incubation (Figure 3B). Again, just enhancing extracellular D-
glucose was not sufficient to promote NF-kB translocation,

although it potentiated the activation of NF-kB elicited by IL-1β

(Figure 3B).

To analyze the involvement of ERK 1/2 and NF-kB on ICAM-

1 and VCAM-1 expression, HUVEC were treated with IL-1β

(5 ng/mL), either alone or in the presence of the respective ERK

1/2 and NF-kB inhibitors PD 98059 (30 μmol/L) and PDTC

(100 μmol/L) for 18 h. Both inhibitors abolished the induction of

VCAM-1 elicited by IL-1β at any extracellular D-glucose

decrease in venular leukocyte rolling velocity

At the time of the experiments, no significant differences in

mean arterial blood pressure or venular shear rate were observed

among the different treatments performed (Table 1).

Immunohistochemical and histological studies

Immunohistochemical studies of the mesenteric tissue obtained

from PBS-treated animals revealed a weak staining for ICAM-1,

which was not modified in animals receiving only D-glucose

(Figure 5E). Additionally, no adhered or emigrated leukocytes

were observed in microvessels from PBS- or D-glucose-treated

animals (Figure 5E). IL-1β injection enhanced positive staining for

both CAMs, and leukocyte adhesion and transmigration through

the endothelial layer, especially in animals co-injected with D-
glucose (Figure 5E).

Discussion

The data presented herein do not support a role for high

effective D-glucose alone in promoting pro-inflammatory

endothelium-leukocyte interactions. In this context, just increasing

D-glucose concentration up to 22 mmol/L, which is twice the

plasma concentration considered to be indicative of diabetes after

the oral glucose tolerance test (11.1 mmol/L) [34], did not modify

CAMs expression or the adhesion of HL60 leukocytes to HUVEC

monolayers. Furthermore, the elevation of extracellular D-glucose
did not affect the expression of CD11b/CD18 integrins in

leukocytes, which are pivotal leukocyte molecules for adhesion

and migration [35]. Consistently, the increased monocyteic

expression of adhesion receptors observed in type 2 diabetic

adhesion, we performed additional experiments in which HUVEC

were pre-incubated with PDTC (100 μmol/L) or PD 98059

(30 μmol/L). PDTC did not significantly modify HL60 adhesion

induced by 5 ng/mL IL-1β (108.19±21.39 and 90.70±5.96% of

IL-1β alone in 5.5 mmol/L and 22 mmol/L D-glucose medium,

respectively; results from three independent experiments). For its

part, PD 98059 only partially inhibited HL60 adhesion promoted

by IL-1β (75.14±10.11 and 60.62±5.03% of IL-1β alone in

5.5 mmol/L and 22 mmol/L D-glucose medium, respectively;

P<0.05 vs matched IL-1β alone; results from three independent

experiments). In the absence of IL-1β, neither PD 98059 nor

PDTC did affect HL60 adhesion, independently of the D-glucose

centration in the culture medium (data not shown).

In vivo leukocyte trafficking

In order to extend the functional data obtained in vitro to an in

vivo model, intravital microscopy was performed to examine

leukocyte trafficking in the mesenteric microvasculature of Sprague-Dawley rats 18 h after the i.p. administration of a high
dose of D-glucose (40 mg/kg) in the presence or absence of IL-1β

(200 ng/kg).

When compared with control animals injected only with PBS,

IL-1β-treated rats showed a significant increase in venular

leukocyte rolling flux, adhesion, and emigration, as well as a

concomitant decrease in venular leukocyte rolling velocity

(Figures 3A-5D).

In those animals receiving only 40 mg/kg D-glucose, no

changes in the above-mentioned trafficking parameters were

observed (Figures 5A-5D). However, when IL-1β was co-injected

with D-glucose, a clear increase in leukocyte rolling flux, adhesion,

and migration was observed, as compared to IL-1β alone

(Figures 5A, 5C and 5D). This latter effect was not due to

hyperosmolarity, since L-glucose failed to mimic the potentiating
effect of D-glucose (Figures 5A-5D).

Immunohistochemical and histological studies

Immunohistochemical studies of the mesenteric tissue obtained

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both CAMs, and leukocyte adhesion and transmigration through

the endothelial layer, especially in animals co-injected with D-
glucose (Figure 5E).

In vitro HL60 leukocyte adhesion to HUVEC

We next performed in vitro assays under flow conditions to

investigate whether the synergism observed between high D-
glucose and IL-1β on CAMs expression was paralleled by

enhanced leukocyte adhesion to HUVEC monolayers. HL60

leukocyte adhesion was significantly enhanced when HUVEC

were stimulated with IL-1β (5 ng/mL) in 5.5 mmol/L D-glucose

medium for 18 h (Figure 4; Videos S1 and S3). This pro-adhesive

effect of the cytokine was further enhanced by 3.7-fold when

extracellular D-glucose was switched up to 22 mmol/L (Figure 4;

Video S4). High D-glucose alone did not promote leukocyte

adhesion to HUVEC (Figure 4; Video S2).

To assess whether the inhibition of either NF-kB or ERK 1/2

activation, which blocked VCAM-1 induction by IL-1β, could

prevent the adhesion of HL60 leukocytes to endothelial cells

adherently (P<0.05 vs non-simulated HUVEC and P<0.05 vs matched

TNF-α in 5.5 mmol/L D-glucose; results from four independent

experiments performed in triplicate).

CD11b/CD18 expression on human leukocytes

In human leukocytes, the expression of CD11b/CD18 integrins,

involved in the leukocyte-endothelial interaction, was not

significantly modified after 18 h of incubation in 22 mmol/L D-
glucose (mean fluorescence intensity: 4.8±0.8 and 6.8±1.3

arbitrary units in 5.5 and 22 mmol/L D-glucose, respectively).

However, when the cells were exposed to IL-1β (5 ng/mL), a

marked induction of CD11b/CD18 was observed (mean fluores-
cence intensity: 13.0±3.2 arbitrary units, P<0.05 versus

5.5 mmol/L D-glucose), which was not further augmented in

the presence of 22 mmol/L D-glucose (mean fluorescence

intensity: 13.0±3.1 arbitrary units, P<0.05 versus 5.5 mmol/L

D-glucose).

ERK 1/2 and NF-κB activation in HUVEC and its role on

ICAM-1 and VCAM-1 expression

The incubation of HUVEC in 22 mmol/L D-glucose from 5 to

60 min did not result in increased ERK 1/2 activity, as compared

with cells incubated in 5.5 mmol/L D-glucose (Figure 2A). Similarly,

the exposure to high D-glucose did not result in NF-

κB activation from 1 to 18 h after stimulation (Figure 3A).

In vivo leukocyte trafficking

In order to extend the functional data obtained in vitro to an in

vivo model, intravital microscopy was performed to examine

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When compared with control animals injected only with PBS,

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leukocyte rolling flux, adhesion, and emigration, as well as a

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the endothelial layer, especially in animals co-injected with D-
glucose (Figure 5E).
patients correlates with body mass index or serum markers of inflammation and only to a lesser extent with glycemic levels [5].

It is well established that pro-inflammatory cytokines are key molecules in mediating leukocyte adhesion and transendothelial migration [2]. Accordingly, we observed that IL-1β promoted endothelial ICAM-1 and VCAM-1 expression, HL60 adhesion to HUVEC monolayers, as well as the expression of CD11b/CD18 integrins on human leukocytes. Interestingly, although extracellular high D-glucose was not enough to induce the expression of endothelial CAMs, it significantly enhanced the induction of endothelial ICAM-1 and VCAM-1 elicited by IL-1β. Such a potentiating effect of high D-glucose was not restricted to IL-1β, as...
it was also observed for the pro-inflammatory cytokine TNF-α, whose levels are elevated in the circulation of diabetic patients and whose release to the circulation is promoted by hyperglycemia [4]. The in vitro adhesion of HL60 leukocytes to endothelial cells induced by IL-1β was also potentiated in a high D-glucose environment. It can therefore be concluded that high D-glucose on its own does not promote leukocyte-endothelial cell interactions, but rather plays a modulatory role in such events by enhancing an ongoing pro-inflammatory response on endothelial cells. In agreement with these observations, we have recently
demonstrated that a pro-inflammatory preconditioning is required for high D-glucose to inflame human vascular smooth muscle [36]. Additionally, the fact that high D-glucose did not potentiate CD11b/CD18 integrins induction by IL-1β in human leukocytes suggests that the synergism between high D-glucose and pro-inflammatory cytokines may not affect every cell type involved in leukocyte recruitment to the vascular wall, but rather more specifically occurs in vascular cells.

We next aimed to gain insight into the cell signaling pathways mediating the synergistic action between D-glucose and IL-1β in human endothelial cells. Our data showed that just increasing the extracellular D-glucose concentration did not activate ERK 1/2 nor NF-κB in HUVEC. While this observation contrasts with previous reports showing ERK 1/2 and NF-κB activation in HUVEC by high D-glucose [14,37], other studies have also failed to detect the activation of both molecules in endothelial cells exposed to elevated D-glucose levels [20,38,39]. We nevertheless observed that high D-glucose potentiated the activation of both ERK 1/2 and NF-κB elicited by IL-1β, indicating that the elevation of extracellular D-glucose results in an over-activation of endothelial signaling molecules triggered by a pro-inflammatory stimulus. In this context, we have recently shown that, in cultured human vascular smooth muscle cells, high D-glucose activates the ERK 1/2 - NF-κB - inducible nitric oxide synthase axis, but only when this signaling pathway is previously triggered by an exogenous inflammatory stimulus [36]. In agreement with others [40,41], we have further found in the present work that ERK 1/2 and NF-κB activation was responsible for endothelial VCAM-1,

![Figure 4. Adhesion of HL60 leukocytes to HUVEC under flow conditions in vitro. HUVEC monolayers were exposed to either 5.5 or 22 mmol/L extracellular D-glucose in the presence or absence of IL-1β (5 ng/mL) for 18 h prior to leukocyte perfusion. *P<0.05 versus 5.5 mmol/L D-glucose without IL-1β; †P<0.05 versus IL-1β in the presence of 5.5 mmol/L D-glucose. Representative micrographs showing HL60 adhesion to HUVEC monolayers are shown on the top (x200). doi:10.1371/journal.pone.0010091.g004](image-url)
Figure 5. *In vivo* leukocyte trafficking in rat mesenteric post-capillary venules. Animals were i.p. injected with 10 mL of PBS alone (C) or supplemented with D-glucose (DG; 40 mg/kg), either in the absence or presence of IL-1β (200 ng/kg). L-glucose (LG; 40 mg/kg) was used as an osmotic control. After 18 h, (A) leukocyte rolling flux, (B) leukocyte rolling velocity, (C) leukocyte adhesion and (D) leukocyte migration were determined by intravital microscopy. *P<0.05 versus matched group without IL-1β; †P<0.05 versus IL-1β in PBS. Panel E shows representative photomicrographs showing ICAM-1 and VCAM-1 immunolocalization and leukocyte adhesion and transmigration in post-capillary venules. The brown reaction product indicates positive staining. Adhered and transmigrated leukocytes are marked with arrows in the right column. All panels are lightly counterstained with hematoxylin (x400).

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but not ICAM-1, induction. Other signaling molecules, like poly(ADP-ribose) polymerase-1 (PARP-1) [42] and AP-1 [43] have been reported as promoters of ICAM-1 expression in different vascular cell types, and might therefore be involved in the induction of ICAM-1 by IL-1β observed herein and perhaps also over-activated by high D-glucose. Blocking VCAM-1 expression was not sufficient to totally prevent HL60 adhesion to endothelial cells, neither under low or high D-glucose conditions. Indeed, under NF-kB or ERK 1/2 blockade, the levels of ICAM-1 induced by IL-1β remained unchanged or even elevated, which may still facilitate HL60 adhesion. In this context, it is worth noting that the levels of lymphocyte function-associated antigen (LFA)-1 integrin (the counterpart for endothelial ICAM-1) in HL60 leukocytes are about 6-fold higher than those of very late antigen (VLA)-4 (the counterpart for endothelial VCAM-1) (unpublished observations).

The synergistic pro-inflammatory action between D-glucose and inflammatory stimuli may in fact be on the basis of the highly controversial reports existing on the ability of D-glucose per se to trigger endothelial cell activation in vitro. We propose that a pro-inflammatory response to high D-glucose can only occur when cultured cells are already in an inflammatory state. As cells in culture are in a rather artificial condition that facilitates inflammation, they are therefore easily primed to respond to abnormally high D-glucose. The degree of stress under which cultured cells are used for experimentation is variable, which might explain the discrepancies encountered among the different studies.

The enhanced acute release of pro-inflammatory cytokines observed during postprandial hyperglycemia in diabetic patients [4] has been related with a higher risk of suffering cardiovascular events [4,13]. Studies in vitro using human endothelial cells have also suggested that intermediates rather than constant high D-glucose levels can be more effective in inducing the expression of VCAM-1 and ICAM-1 [17]. By using intravital microscopy in the rat mesenteric microvasculature, Booth et al. [44] analyzed the effects of local acute intraperitoneal hyperglycemia on leukocyte-endothelial cell interactions in vivo. These authors reported increased leukocyte rolling and adherence after 12 h of intraperitoneal administration of moderate (20–25 mg/kg) and high (40–45 mg/kg) doses of D-glucose, while no effect was observed with the lowest dose (8–10 mg/kg) [44]. Herein, we have used a similar in vivo model of local hyperglycemia to analyze the influence of a high dose of D-glucose (40 mg/kg) on leukocyte trafficking after 18 h of its acute intraperitoneal injection. In accordance with our in vitro findings, IL-1β significantly increased leukocyte rolling flux, adhesion, and migration in vivo. More interestingly, D-glucose markedly enhanced all these parameters, therefore favoring vascular inflammation, but only when the cytokine was co-injected. In fact, and in contrast with the observations of Booth et al. [44], the mere administration of D-glucose in the absence of the cytokine did not alter leukocyte trafficking parameters. These functional data were accompanied by a parallel expression of both CAMs in the mesenteric microvasculature.

Taken together these results, we propose that acute hyperglycemia per se is not sufficient to promote vascular inflammation. More likely, the abnormally increased postprandial glucose concentrations observed in the metabolic syndrome and type 2 diabetes [45] can only exaggerate the effects of an ongoing inflammatory response. This may in fact constitute the main mechanism by which postprandial hyperglycemia promotes vascular inflammation and the development of diabetic vasculopathy [4,13,46]. Even more, such a synergistic effect between high D-glucose and inflammation could also explain why the expression of CAMs and other inflammation-related molecules in response to an infection is higher in hyperglycemic experimental models of diabetes [47].

The results obtained in the present study using from in vitro to in vivo approaches highlight that a pro-inflammatory pre-conditioning is necessary for extracellular high D-glucose to exert a deleterious effect in the vascular. A recent study in human endothelial retinal cells suggests that cytokines, rather than high D-glucose, are responsible for diabetes-related retinal endothelial injury [38]. On the other hand, our data support the proposal that D-glucose by itself is likely not a major mechanistic factor in the development of diabetes-induced atherosclerosis [29] and they can provide a possible explanation for the failure of intensive blood-glucose control in preventing cardiovascular events associated to type 2 diabetes [26–28].

Our findings further underpin the relevance of the chronic low-grade pro-inflammatory environment observed in diabetes mellitus, as a pivotal factor conditioning the early pro-atherosclerotic actions of elevated extracellular D-glucose levels. In fact, they are in line with clinical observations in which a synergistic action of hyperglycemia and inflammation seems to be critical in clinical outcomes of acute coronary syndromes [48]. It is noteworthy that the inflammatory environment that occurs in type 2 diabetes is a rather complex phenomenon involving different cells and mechanisms and not strictly related to hyperglycemia [45]. Therefore, treating only hyperglycemia may be not sufficient to prevent and treat diabetic vasculopathy, and additional therapeutic goals, such as lowering chronic inflammation, should be carefully considered [49]. In this context, the subcutaneous administration of the recombinant human interleukin-1-receptor

### Table 1. Hemodynamic parameters of Sprague-Dawley rats used for intravital microscopy studies.

| Treatment              | Mean arterial blood pressure (mm Hg) | Shear rate in venules (s⁻¹) |
|------------------------|--------------------------------------|----------------------------|
| PBS                    | 108.8±2.6                            | 525.4±42.7                 |
| D-glucose              | 112.6±3.9                            | 493.9±50.0                 |
| L-glucose              | 114.6±4.0                            | 469.5±50.4                 |
| IL-1β                  | 113.4±4.6                            | 502.8±27.0                 |
| IL-1β + D-glucose      | 115.3±3.9                            | 477.5±66.9                 |
| IL-1β + L-glucose      | 108.8±5.5                            | 500.8±39.2                 |

Parameters (mean ± SEM) were measured 18 h after the i.p. injection of 10 mL of PBS alone (n=6), or containing D-glucose (40 mg/kg; n=5), L-glucose (40 mg/kg; n=5), IL-1β (200 ng/kg; n=7), D-glucose plus IL-1β (n=6) or L-glucose plus IL-1β (n=5). No significant differences were found in mean arterial blood pressure or shear rate in venules between any of the different treatments performed.

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antagonist anakinra has recently been shown to reduce the markers of systemic inflammation in type 2 diabetic patients [45].

In conclusion, the present work provides an experimental basis to explain the failure of those therapeutic approaches directed only to reduce hyperglycemia in preventing diabetes-associated atherosclerosis and calls attention on the necessity to reduce systemic inflammation, either pharmacologically or through physical exercise [50], in order to increase the benefit in preventing cardiovascular events linked to type 2 diabetes or the metabolic syndrome.

Supporting Information

Video S1 Confluent HUVEC monolayers were exposed to 5.5 mmol/L extracellular D-glucose for 18 h prior to leukocyte perfusion (1.5 × 106 cells/mL) drawn at 0.5 dynes/cm² (x200). Found at: doi:10.1371/journal.pone.0010091.s001 (2.77 MB MOV)

Video S2 Confluent HUVEC monolayers were exposed to 22 mmol/L extracellular D-glucose for 18 h prior to leukocyte perfusion (1.5 × 106 cells/mL) drawn at 0.5 dynes/cm² (x200). Found at: doi:10.1371/journal.pone.0010091.s002 (2.76 MB MOV)

Video S3 Confluent HUVEC monolayers were exposed to 5.5 mmol/L extracellular D-glucose in presence of IL-1β (5 ng/mL) for 18 h prior to leukocyte perfusion (1.5 × 106 cells/mL) drawn at 0.5 dynes/cm² (x200). Found at: doi:10.1371/journal.pone.0010091.s003 (2.66 MB MOV)

Video S4 Confluent HUVEC monolayers were exposed to 22 mmol/L extracellular D-glucose in presence of IL-1β (5 ng/mL) for 18 h prior to leukocyte perfusion (1.5 × 106 cells/mL) drawn at 0.5 dynes/cm² (x200). Found at: doi:10.1371/journal.pone.0010091.s004 (2.61 MB MOV)

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Author Contributions

Conceived and designed the experiments: VA LRM CFSF CP. Performed the experiments: VA MAT TR MVB NM MJS. Analyzed the data: VA MJS CFSF CP. Contributed reagents/materials/analysis tools: FWL MJS. Wrote the paper: VA MJS CFSF CP.

References

1. Luis AJ (2000) Atherosclerosis. Nature 407: 233–241.
2. Rao RM, Yang L, Garcia-Cardena G, Luscinskas FW (2007) Endothelial-dependent mechanisms of leukocyte recruitment to the vascular wall. Circ Res 101: 234–247.
3. Galkina E, Ley K (2007) Vascular adhesion molecules in atherosclerosis. Arterioscler Thromb Vasc Biol 27: 2297–2301.
4. Esposito K, Nappo F, Marfella R, Giugliano G, Giugliano F, et al. (2002) Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans: role of oxidative stress. Circulation 106: 2067–2072.
5. Fogelstrand L, Hultén J, Hultén LM, Wiklund O, Fagerberg B (2004) Monocyte expression of CD14 and CD18, circulating adhesion molecules and inflammatory markers in women with diabetes mellitus and impaired glucose tolerance. Diabetologia 47: 1948–1952.
6. Tschope C, Walther T, Escher F, Spillmann F, Du J, et al. (2005) Transgenic activation of the kallikrein-kinin system inhibits intramyocardial inflammation, endothelial dysfunction and oxidative stress in experimental diabetic cardiomyopathy. FASEB J 19: 2057–2059.
7. Gao L, Wang F, Wang B, Gong J, Zhang J, et al. (2006) Cilostazol protects endothelial dysfunction and oxidative stress in experimental diabetic cardiomyopathy. FASEB J 20: 2421–2425.
8. Otsuki M, Hashimoto K, Morimoto Y, Kishimoto T, Kasayama S (1997) Circulating vascular cell adhesion molecule-1 (VCAM-1) in atherosclerotic NIDDM patients. Diabetes 46: 2096–2101.
9. Marfella R, Esposito K, Giunta R, Coppola G, De Angelis L, et al. (2000) Circulating adhesion molecules in humans: role of hyperglycemia and hyperinsulinemia. Circulation 101: 2247–2251.
10. Devraj S, Glaser N, Griffen S, Wang-Polagruto J, Miguelino E, et al. (2006) Increased monocyte activity and biomarkers of inflammation in patients with type 1 diabetes. Diabetes 55: 774–779.
11. Jager A, van Himberghen VW, Kostene J, Emeis JJ, Nijpels G, et al. (2005) Increased levels of soluble vascular cell adhesion molecule 1 are associated with risk of cardiovascular mortality in type 2 diabetes: the Hoom study. Diabetes 54: 183–189.
12. Kirchhbach K, Sowers JR (2001) Diabetes mellitus and diabetes-associated vascular disease. Trends Endocrinol Metab 12: 223–230.
13. Cerillo A (2005) Postprandial hyperglycemia and diabetes complications. Is it time to treat? Diabetes 54: 1–7.
14. Morigi M, Angioletti S, Imberti B, Donadelli R, Micheletti G, et al. (1998) Leukocyte-endothelial interaction is augmented by high glucose concentrations and hyperglycemia in a NF-κB-dependent fashion. J Clin Invest 101: 1903–1915.
15. Rasmussen LM, Schmitz O, Leder T (2002) Increased expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured endothelial cells exposed to serum from type 1 diabetic patients: no effects of high glucose concentrations. J Clin Lab Invest 62: 485–493.
16. Kado S, Wakioki T, Yamamoto M, Nagata N (2001) Expression of intercellular adhesion molecule-1 induced by high glucose concentrations in human aortic endothelial cells. Life Sci 68: 727–737.
17. Picconi L, Quagliaro L, Da Ros R, Assaloni R, Giugliano D, et al. (2004) Intermittent high glucose enhances ICAM-1, VCAM-1, E-selection and interleukin-6 expression in human umbilical endothelial cells in culture: the role of poly(ADP-ribose) polymerase. J Thromb Haemost 2: 1433–1439.
18. Altmannovich TS, Rosabal K, Kucerz P, Anfel M (2004) Effect of high glucose concentrations on expression of ELAM-1, VCAM-1 and ICAM-1 in HUVEC with and without cytokine activation. Physiol Res 53: 77–82.
19. Kim JA, Berliner JA, Natarajan RD, Nadler JL (1994) Evidence that glucose increases monocyte binding to human aortic endothelial cells. Diabetes 43: 1103–1107.
20. Cacciato JM, Yaghashi N, Kreany JF, Jr., Ruderman NB, Ido Y (2004) AMPK inhibits fatty acid-induced increases in NF-κB transactivation in cultured human umbilical vein endothelial cells. Biochem Biophys Res Commun 324: 1204–1209.
21. Wada Y, Ou H, Wu S, Ahid MR, Okada H, et al. (2005) Preconditioning of primary human endothelial cells with inflammatory mediators alters the “set point” of the cell. FASEB J 19: 1914–1916.
22. The Diabetes Control and Complications Trial Research Group (1995) The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med 329: 977–986.
23. Nathan DM, Cleary PA, Backlund JY, Gennuth SM, Lachin JM, et al. (2005) Diabetes Control and Complications/Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Study Research Group. Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. N Engl J Med 353: 2643–2653.
24. UK Prospective Diabetes Study (UKPDS) Group (1998) Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). Lancet 352: 837–853.
25. Holman RR, Paul SK, Bethel MA, Matthews DR, Neil HA (2008) 10-year follow-up of intensive glucose control in type 2 diabetes. N Engl J Med 359: 1577–1589.
26. Gerstein HC, Miller ME, Byington RP, Goff DC, Jr., Bigger JT, et al. (2008) Action to Control Cardiovascular Risk in Diabetes Study Group, Effects of intensive glucose lowering in type 2 diabetes. N Engl J Med 358: 2543–2551.
27. Duckworth W, Abraira C, Moritz T, Reda D, Emanuele N, et al. (2009) Glucose control and vascular complications in veterans with type 2 diabetes. N Engl J Med 360: 129–139.
28. Patel A, MacMahon S, Chalmers J, Neal B, Billot L, et al. (2008) Intensive blood-glucose control and vascular outcomes in patients with type 2 diabetes. N Engl J Med 358: 2560–2572.
29. Razani B, Semenkovich CF (2009) Getting away from glucose: stop sugarcoating inflammation. Nat Med 15: 372–373.
30. Peiró C, Valles A, Guarné F, Ascui V, Herrlinger-Walter S, et al. (2007) Endothelial dysfunction through genetic deletion or inhibition of the G-protein-coupled receptor Mas: a new target to improve endothelial function. J Hypertens 25: 2421–2427.
31. Álvarez A, Cerda-Nicolas M, Abu Nabah NY, Mata M, Issekutz AC, et al. (2004) Direct evidence of leukocyte adhesion in arterioles by angiostatin II. Blood 104: 402–408.
32. Peiro´ C, Matesana N, Nevado J, Lafuente N, Cercas E, et al. (2003) Glycosylated human oxyhaemoglobin activates nuclear factor-kappaB and activator protein-1 in cultured human aortic smooth muscle. Br J Pharmacol 140: 681–690.

33. Harari OA, Alcaide P, Ahl D, Lucinikkas FW, Liao JK (2006) Absence of TRAM restricts Toll-like receptor 4 signaling in vascular endothelial cells to the MyD88 pathway. Circ Res 98: 1134–1140.

34. The DECODE study group, European Diabetes Epidemiology Group (1999) Diabetes Epidemiology: Collaborative Analysis of Diagnostic Criteria in Europe: Glucose tolerance and mortality; comparison of WHO and American Diabetes Association diagnostic criteria. Lancet 354: 617–621.

35. Rose DM, Alon R, Ginberg MH (2007) Integrin modulation and signaling in leukocyte adhesion and migration. Immunol Rev 218: 126–134.

36. Lafuente N, Matesana N, Acunia V, Romacho T, Nevado J, et al. (2008) The deleterious effect of high concentrations of D-glucose requires pro-inflammatory pre-conditioning. J Hypertens 26: 478–485.

37. Xin X, Khan ZA, Chen S, Chakrabarti S (2004) Extracellular signal-regulated kinase (ERK) in glucose-induced and endothelin-mediated fibronectin synthesis. Lab Invest 84: 1451–1459.

38. Liu W, Schoenkerman A, Lowe WL, Jr. (2000) Activation of members of the mitogen-activated protein kinase family by glucose in endothelial cells. Am J Physiol Endocrinol Metab 279: E782–E790.

39. Buisk JV, Mohr S, Grant MB (2008) Hyperglycemia-induced reactive oxygen species toxicity to endothelial cells is dependent on paraoxonase mediators. Diabetes 57: 1952–1965.

40. Modur V, Zimmerman GA, Prescott SM, McIntyre M (1996) Endothelial cell inflammatory responses to tumor necrosis factor α. J Biol Chem 271: 13094–13102.

41. Weber C, Erf W, Petesch A, Stroehl M, Ziegler-Heitbrock HWL, et al. (1994) Antioxidants inhibit monocyte adhesion by suppressing nuclear factor-κB mobilization and induction of vascular cell adhesion molecule-1 in endothelial cells stimulated to generate radicals. Arterioscler Thromb 14: 1665–1673.

42. Zerfaoui M, Suzuki Y, Naura AS, Hans CP, Nichols C, et al. (2008) Nuclear translocation of p65 NF-kappaB is sufficient for VCAM-1, but not ICAM-1, expression in TNF-stimulated smooth muscle cells. Differential requirement for PARP-1 expression and interaction. Cell Signal 20: 186–194.

43. Muñoz C, Castellanos MC, Alfranca A, Vara A, Esteban MA, et al. (1996) Transcriptional up-regulation of intracellular adhesion molecule-1 in human endothelial cells by the antioxidant pyrrolidine dithiocarbamate involves the activation of activating protein-1. J Immunol 157: 3587–3597.

44. Booth G, Stalker TJ, Lefer AM, Scalia R (2001) Elevated ambient glucose induces acute inflammatory events in the microvasculature: effects of insulin. Am J Physiol Endocrinol Metab 280: E948–E956.

45. Larsen CM, Faalenbach M, Vaag A, Vohland A, Ehses JA, et al. (2007) Interleukin-1 receptor antagonist in type 2 diabetes mellitus. N Engl J Med 356: 1517–1526.

46. Shoelson SE, Lee J, Goldfine AB (2006) Inflammation and insulin resistance. J Clin Invest 116: 1795–1801.

47. Lu H, Rapin M, Black E, Stan M, Amar S, et al. (2004) Influence of diabetes on the exacerbation of an inflammatory response in cardiovascular tissue. Endocrinology 145: 4934–4939.

48. Ray KK, Cannon CP, Morrow DA, Kirtane AJ, Buros J, et al. (2007) Synergistic relationship between hyperglycaemia and inflammation with respect to clinical outcomes in non-ST-elevation acute coronary syndromes: analyses from OPUS-TIMI 16 and TACTICS-TIMI 18. Eur Heart J 28: 806–813.

49. Dilley RJ, Little PJ (2008) Another piece of cell biology in the puzzle of inflammation, glucose and diabetic vascular disease. J Hypertens 26: 396–398.

50. Kasapic C, Thompson PD (2008) The effects of physical activity on serum C-reactive protein and inflammatory markers: a systematic review. J Am Coll Cardiol 45: 1563–1569.