Diabetologia (1998) 41: 1392–1396

**Diabetologia**


# Reduction of oxidative stress by oral N-acetyl-L-cysteine treatment decreases plasma soluble vascular cell adhesion molecule-1 concentrations in non-obese, non-dyslipidaemic, normotensive, patients with non-insulin-dependent diabetes

**G. De Mattia**[1], **M.C. Bravi**[2], **O. Laurenti**[1], **M. Cassone-Faldetta**[2], **A. Proietti**[1], **O. De Luca**[1], **A. Armiento**[1], **C. Ferri**[1]

[1] Università "La Sapienza", Fondazione Andrea Cesalpino, Roma, Italy
[2] Università di L'Aquila, Dipartimento di Medicina Interna, L'Aquila, Italy

**Summary** To assess in vivo effects of antioxidants on vascular cell adhesion molecule (VCAM)-1 expression, circulating soluble VCAM-1 and intraerythrocytic reduced glutathione (GSH) and GSH disulphide (GSSG) concentrations were evaluated in non-insulin-dependent diabetic patients without complications (9 men, 6 women, 48 ± 6 years old) before and after 1 month of either oral *N*-acetyl-L-cysteine (1.200 mg/day) or placebo treatments, given in randomized, cross-over, double-blind fashion. Ten healthy subjects (7 men, 3 women, 52 ± 4 years old) served as control subjects. Baseline plasma VCAM-1 concentrations were higher ($p = 0.007$) in non-insulin-dependent diabetic patients (707.9 ± 52.5 ng/ml) than in control subjects (627.3 ± 84.6 ng/ml). Intraerythrocytic GSSG content was higher (non-insulin dependent diabetic patients: 0.618 ± 0.185 μmol/g Hb; control subjects: 0.352 ± 0.04 μmol/g Hb, $p = 0.0002$), whereas intraerythrocytic GSH concentrations were lower ($p = 0.001$) in non-insulin dependent diabetic patients (6.0 ± 0.7 μmol/g Hb) than in control subjects (7.1 ± 0.5 μmol/g Hb). The mean GSH:GSSG ratio was also lower ($p = 0.0001$) in the first (10.9 ± 4.5) than in the second group (20.2 ± 1.4). Circulating VCAM-1 and intraerythrocytic GSH concentrations were negatively correlated in non-insulin diabetic patients ($r = 0.605$, $p = 0.01$). Treatment with *N*-acetyl-L-cysteine decreased plasma VCAM-1 ($p = 0.01$) and intraerythrocytic GSSG ($p = 0.006$) but increased GSH concentrations ($p = 0.04$) and the GSH:GSSG ratio ($p = 0.004$) in non-insulin dependent diabetic patients. Our data indicate that the vascular endothelium is activated in non-insulin dependent diabetes. Antioxidant treatment counterbalanced such endothelial activation. Thus, antioxidant agents might protect against oxidant-related upregulation of endothelial adhesion molecules and slow down the progression of vascular damage in non-insulin dependent diabetes. [Diabetologia (1998) 41: 1392–1396]

**Keywords** Adhesion molecules, endothelium, vascular cell adhesion molecule-1, oxidants.

The endothelial adhesion molecule, vascular cell adhesion molecule (VCAM)-1 enables the firm attachment of circulating cells to the endothelium and promotes intravascular accumulation of macrophages and T lymphocytes [1]. Thus, VCAM-1 upregulation has been indicated as one of the most important events initiating atherosclerosis [2]. Agents stimulating VCAM-1 gene expression are transduced at the nuclear level by the nuclear factor-Kb pathway [3], a cytoplasmic multisubunit transcription factor which can be activated by those stimuli only in the presence of oxidants [3]. Accordingly, the blockade of nuclear factor-*K*b by the antioxidants probucol [3], α-tocopherol [3], pyrrolidine dithiocarbamate [4], lacidipine [5], and *N*-acetyl-L-cysteine [6] inhibit cytokine and noncytokine-induced VCAM-1 upregulation in cultured vascular endothelial cells.

Since the expression of membrane-associated VCAM-1 by activated human vascular endothelial cells is combined to the release of its soluble form

Received: 15 May 1998 and in revised form: 8 July 1998

*Corresponding author:* C. Ferri M.D., Università "La Sapienza", Fondazione Andea Cesalpino, Viale del Policlinico, 155, I-00161 Rome, Italy

*Abbreviations*: *VCAM-1*, Vascular cell adhesion molecule-1; *GSH*; reduced glutathione; *GSSG*, GSH disuphide.

[7], concentration of plasma soluble VCAM-1 is usually considered as a marker of its endothelial expression in vivo [8]. In non-insulin-dependent diabetic patients both oxidative stress [9] and plasma soluble VCAM-1 concentrations [10] were found to be raised, suggesting they might be interrelated. In keeping with this, circulating concentrations of adhesion molecule-1 and malonaldehyde, a marker of oxidative stress, have been found to be increased and directly correlated in non-insulin dependent diabetic patients [11]. Similarly, the circulating concentration of E-selectin decreased in non-insulin-dependent diabetic patients after treatment with the antioxidant troglitazone [12] and improvement of glycaemic control [13]. Thus, a consistent bulk of data supports the hypothesis that oxidative stress upregulates VCAM-1 in vascular endothelial cells in vitro and suggests the possibility that antioxidants might also counteract VCAM-1 upregulation in vivo.

Accordingly, our study was designed to investigate whether treatment with the antioxidant drug *N*-acetyl-L-cysteine, an antioxidant agent which is safe and effective in vivo, was able to reduce circulating soluble VCAM-1 in non-insulin-dependent diabetic patients. Since numerous confounding factors are known to affect circulating soluble VCAM-1 concentrations [8, 14], the study was conducted after exclusion of smoking, infectious diseases, allergies, atherosclerosis, hypertension, dyslipidaemia and obesity. As indexes of oxidative stress, intraerythrocytic reduced glutathione (GSH) and gluthatione disulphide (GSSG) concentrations and the GSH:GSSG ratio were also assessed in the same patients [9].

## Subjects and methods

The study was conducted in 15 non-insulin-dependent diabetic patients [15] (9 men and 6 women, mean age 48 ± 6 years) with good metabolic control, i.e. fasting glucose concentrations under 7.8 mmol/l, post-prandial glucose concentrations under 10.0 mmol/l, and glycated haemoglobin $A_{1c}$ ($HbA_{1c}$) concentrations under 7.5%. The study group was recruited in our Diabetes Unit from 216 consecutive outpatients on the basis of the following exclusion criteria: age under 25 and over 55 years, pregnancy or use of birth control pills, history of previous cerebrovascular or cardiovascular diseases, concomitant illnessess, allergic diathesis, respiratory, gastrointestinal or genitourinary tract infections referred to a doctor during the last 3 months, insulin-dependent diabetes [15], sitting systolic/diastolic blood pressure 140/90 mmHg or more, body mass index greater than 25 kg/m$^2$, smoking, microalbuminuria, serum cholesterol concentrations more than 5.2 mmol/l; serum triglyceride concentrations more than 1.65 mmol/l, serum creatinine more than 100 μmol/l, atherosclerotic lesions (as evaluated by medical history, physical examination, ultrasound studies and fundoscopic evaluations). At entry, all patients gave informed consent and were given a weight-maintaining diet containing about 50% carbohydrate, 30% protein and 20% lipid. Fruit was 150 g twice daily, vegetables were 200 g twice daily. The good metabolic control was achieved and maintained by diet and glibenclamide 5–15 mg daily. No patient was taking any other drug. A group of 10 healthy subjects (7 men and 3 women, mean age 52 ± 4 years) selected according to the above criteria but with a normal glucose tolerance served as a control.

*Baseline and post-treatment plasma soluble VCAM-1 and intraerythrocytic GSH and GSSG concentrations.* In both the non-insulin-dependent diabetic and control groups, blood samples for measuring intraerythrocytic GSH and GSSG, plasma soluble VCAM-1 concentrations, a haematochemical check including plasma glucose and insulin concentrations, serum $HbA_{1c}$, triglyceride, total cholesterol and cholesterol subfraction concentrations were drawn from an antecubital vein, after a 10–12 h fast, at 0800 hours. Then, non-insulin-dependent diabetic patients were assigned to receive either oral *N*-acetyl-L-cysteine (1.200 mg per day) or placebo treatments for 1 month each, according to a randomized, double-blind cross-over protocol. Thereafter, blood samples for the above-mentioned measurements were again taken from both groups. This study phase was conducted by researchers unaware of the study design, results, and purpose.

*Laboratory methods.* Plasma soluble VCAM-1 concentrations were assessed in duplicate by the use of a commercially available monoclonal antibody-based ELISA method (R & D Systems, Minneapolis, Minn., USA) [10, 14]. Inter-assay and intra-assay variabilities were less than 15%. Intraerythrocytic GSH and GSSG contents were assessed as described previously by our group [9] and Griffith [16], respectively. Recovery for GSH and GSSG determinations was 96% and 102%, respectively. Inter-assay and intra-assay variabilities were less than 10%. Serum total cholesterol, high-density lipoprotein (HDL) cholesterol and triglyceride concentrations were assessed by enzymatic methods (Boehringer Mannheim, Germany). Serum LDL cholesterol concentrations were calculated by the Friedewald method [17]. Plasma insulin concentrations were assessed by a commercially available kit (Ares, Serono, Italy). All the above laboratory procedures were carried out by technicians unaware of the study design, purpose and results.

*Statistical analysis.* Data were analysed by SPSS (SPSS Inc., Chicago, Ill., USA). Differences among groups were tested for significance by one-way analysis of variance followed by the Bonferroni's test and the Newman-Keuls test for pairwise comparisons. Effects of each treatment were analysed by analysis of variance with Bonferroni protection. Regression and correlation techniques were used to evaluate linear relation between variables. Statistical significance was considered as a $p$ value less than 0.05. Data are presented as means ± SD.

## Results

Clinical variables, anthropometric characteristics and laboratory features of non-insulin-dependent diabetic patients and control groups are given in Table 1. Non-insulin dependent diabetic patients showed good metabolic control. Compared to control subjects, however, they manifested higher fasting glucose ($p = 0.04$) and $HbA_{1c}$ ($p = 0.0001$) concentrations. By contrast, the two groups had similar lipid, insulin, systolic and diastolic blood pressure levels, and body mass indexes.

**Table 1.** General characteristics of the study group

| Variable | Non-insulin dependent diabetic patients ($n = 15$) | Control subjects ($n = 10$) | $p$ value |
|---|---|---|---|
| Sex (male/female) | 9/6 | 7/3 | n.s. |
| Fasting glucose (mmol/l) | 5.4 ± 1.0 | 4.4 ± 0.3 | 0.04 |
| Fasting insulin (pmol/l) | 125.1 ± 74.1 | 79.6 ± 24.8 | n.s. |
| $HbA_{1c}$ (%) | 6.1 ± 0.3 | 4.5 ± 0.7 | 0.0001 |
| Body mass index (kg/m$^2$) | 23.5 ± 0.6 | 23.2 ± 0.4 | n.s. |
| Duration of disease (years) | 6.4 ± 1.6 | – | – |
| Serum cholesterol (mmol/l) | 4.6 ± 0.5 | 4.3 ± 0.4 | n.s. |
| Serum HDL-cholesterol (mmol/l) | 1.3 ± 0.1 | 1.2 ± 0.1 | n.s. |
| Serum LDL-cholesterol (mmol/l) | 2.9 ± 0.7 | 2.7 ± 0.5 | n.s. |
| Serum triglycerides (mmol/l) | 1.6 ± 0.2 | 1.4 ± 0.2 | n.s. |
| Systolic blood pressure (mmHg) | 131.6 ± 6.4 | 126.5 ± 4.5 | n.s. |
| Diastolic blood pressure (mmHg) | 80.7 ± 5.7 | 78.3 ± 5.8 | n.s. |

Baseline level of soluble VCAM-1 was higher ($p = 0.007$) whereas intraerythrocytic GSH content was lower ($p = 0.001$) in non-insulin-dependent diabetic patients (VCAM-1 = 707.9 ± 52.5 ng/ml; GSH = 6.0 ± 0.7 μmol/g Hb) than in controls (VCAM-1 = 627.3 ± 84.6 ng/ml; GSH = 7.1 ± 0.5 μmol/g Hb). The two variables were negatively correlated in the non-insulin dependent diabetic (Fig. 1) but not in the control group.

Intraerythrocytic GSSG content was higher ($p = 0.0002$) in non-insulin-dependent diabetic patients (0.618 ± 0.185 μmol/g Hb) than in control subjects (0.352 ± 0.04 μmol/g Hb). As a consequence, the mean GSH:GSSG ratio was lower ($p = 0.0001$) in the first (10.9 ± 4.5) than in the second group (20.2 ± 1.4). Individual GSH:GSSG ratios negatively correlated with plasma soluble VCAM-1 concentrations ($r = 0.52$, $p = 0.02$) in non-insulin dependent diabetic patients.

The other metabolic variables, i.e. $HbA_{1c}$, glucose, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglyceride and fasting insulin concentrations, as well as blood pressure levels and body mass index did not correlate with plasma soluble VCAM-1 concentrations.

Treatment of non-insulin-dependent diabetic patients with *N*-acetyl-L-cysteine did not induce changes of metabolic variables but reduced ($p = 0.01$) plasma soluble VCAM-1 (Fig. 2) and increased ($p = 0.04$) intraerythrocytic GSH content (Fig. 2). Further, intraerythrocytic GSSG concentration decreased ($p = 0.01$ vs baseline) after *N*-acetyl-L-cysteine but not placebo treatments (0.450 ± 0.146 μmol/g Hb vs 0.591 ± 0.18 μmol/g Hb, respectively. Intergroup comparison by analysis of variance: $p = 0.006$). As a consequence, the mean GSH:GSSG ratio increased ($p = 0.004$ vs baseline) after active (15.5 ± 5.2) but not placebo (11.3 ± 4.7) treatments (intergroup comparison by analysis of variance: $p = 0.01$).

## Discussion

The present study shows that circulating VCAM-1 concentrations are raised in adult non-obese, non-dyslipidaemic, normotensive, non-insulin-dependent diabetic patients. A negative correlation was found between plasma soluble VCAM-1 and intraerythrocytic GSH concentrations, thereby suggesting augmented oxidative stress was responsible for VCAM-1 upregulation and the consequent increase of soluble VCAM-1 release into the bloodstream. Thus, our report also indicates that oral treatment with the antioxidant *N*-acetyl-L-cysteine for 1 month simultaneously reduced plasma soluble VCAM-1 and intraerythrocytic GSSG concentrations and increased intraerythrocytic GSH levels and the GSH:GSSG ratio.

Mechanisms for expression of membrane-associated adhesion molecules and their shedding into the bloodstream are not precisely known in vivo. In vitro data show, however, that soluble VCAM-1 release rapidly follows the expression of its membrane-bound form [8]. Thus, our data could support the concept that increased oxidative stress causes an early endothelial activation in non-insulin-dependent diabetic patients. Further, the same findings suggest that *N*-acetyl-L-cysteine downregulated VCAM-1, i.e. counteracted endothelial activation, by reducing oxidative stress.

Consistent with this interpretation, increased plasma soluble VCAM-1 concentrations have been described in non-insulin-dependent diabetic patients [10] and glucose intolerant hypertensive patients [14], and were unrelated to age, sex, blood pressure, metabolic variables and body mass index. Moreover, increased concentrations of plasma soluble intercellular adhesion molecule-1 and malondialdehyde, a marker of oxidative stress, have been described recently in non-insulin-dependent diabetic patients [11]. The role of increased oxidative stress in promoting endothelial activation was supported by the direct

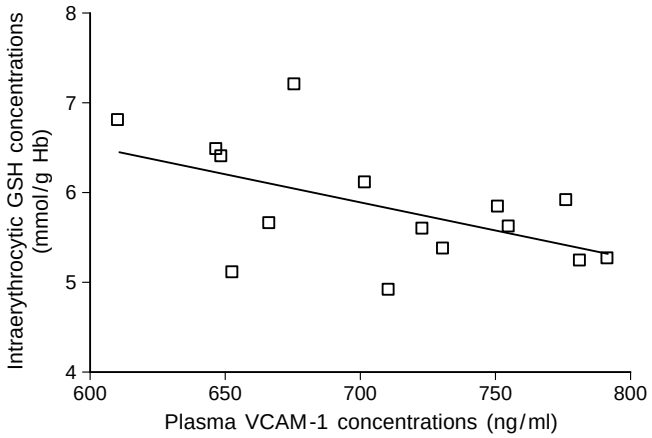


**Fig. 1.** Negative relation between intraerythrocytic glutathione (GSH) contents and plasma soluble vascular adhesion molecule(VCAM)-1 levels in 15 non-dyslipidaemic, non-obese normotensive patients with non-insulin dependent diabetes $r = 0.06$, $p = 0.01$

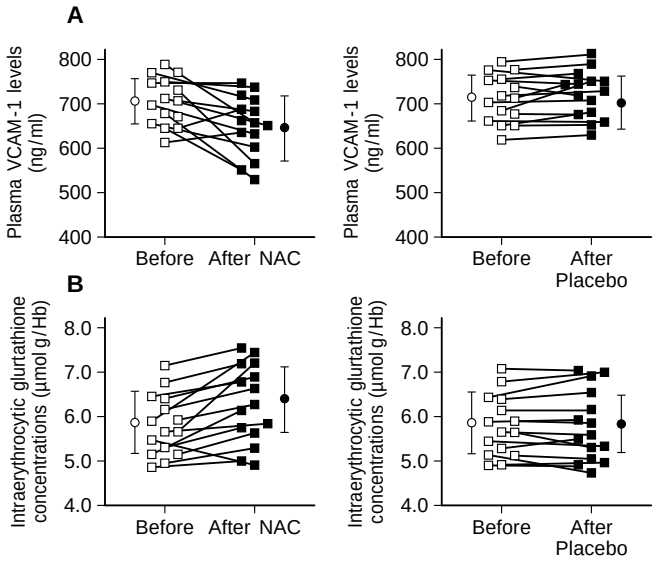


**Fig. 2. A** Effects of 1 month of *N*-acetyl-L-cysteine (NAC) (1.200 mg per day) (*left diagram*) or placebo (*right diagram*) treatments on plasma soluble vascular adhesion molecule(VCAM)-1 concentrations in 15 normotensive, non-dyslipidemic, non-obese non-insulin dependent diabetic patients. Significant difference between pretreatment and post-treatment values $p = 0.01$ *left diagram*. *Right diagram* = NS. **B** Effects of 1 month of NAC (1.200 mg per day) (*left diagram*) or placebo (*right diagram*) treatments on intraerythrocytic glutathione content in the above mentioned patients. Significant differences between pre-treatment and post-treatment values $p = 0.04$ *left diagram*. *Right diagram* = NS. In both **A** and **B**, the pre-treatment value for each subject is □ connected by a continuous line to the post-treatment value, ■. Mean values for each group are indicated by either ○ (pre-treatment) or ● (post-treatment) with SD T-lines

correlation between the two variables [11] as well as by their simultaneous decrements observed after intravenous GSH infusion [18]. Our hypothesis that an early endothelial activation is present in non-insulin-dependent diabetic patients and counteracted by antioxidant agents is also supported by other data [11, 18].

In keeping to this, the capability of *N*-acetyl-L-cysteine to inhibit oxidant-sensitive nuclear factor-*K*b pathway has been already shown in vitro [6]. In particular, *N*-acetyl-L-cysteine selectively blocked nuclear factor-*K*b-induced activation of the promoting region of VCAM-1 gene [3]. Concordant with our in vivo findings, the in vitro blockade of nuclear factor-*K*b-induced VCAM-1 expression was associated with an increase of intracellular GSH and the GSH:GSSG ratio [19]. Thus, although we have not done in vitro experiments, there is clear evidence that *N*-acetyl-L-cysteine reduces VCAM-1 expression by decreasing oxidative stress and supports the possibility that an identical mechanism can be induced in vivo by this antioxidant.

As to the pathophysiological implications of our findings, VCAM-1 upregulation is known to represent a fundamental step in atherogenesis [2]. Consistent with this, circulating concentrations of the soluble fraction of VCAM-1 but not of the other endothelial adhesion molecules ICAM-1, E-selectin and P-selectin and of thrombomodulin correlated with the extent and severity of atherosclerosis in humans [20]. Thus, it is tempting to speculate that antioxidant treatment, i.e. *N*-acetyl-L-cysteine, can protect against the onset of diabetes-related vascular damage by inhibiting VCAM-1-related monocyte and lymphocyte intravascular accumulation. Obviously, long-term follow-up studies are required to support such hypothesis.

In conclusion, our study shows that plasma soluble VCAM-1 concentrations are raised in nondyslipidaemic, non-obese, normotensive, non-insulin-dependent diabetic patients. Elevation of circulating VCAM-1 concentrations negatively correlated with intraerythrocytic GSH content, thus indicating increased oxidative stress was responsible for endothelial activation. In support of this hypothesis, 1-month oral *N*-acetyl-L-cysteine treatment induced reciprocal changes of the two variables, i.e. increased intraerythrocytic GSH content and decreased plasma soluble VCAM-1 concentrations. As a consequence, we speculate that antioxidant agents are useful in protecting against endogenous oxidant-related upregulation of endothelial adhesion molecules and slow down the progression of vascular damage in non-insulin-dependent diabetes.

*Acknowledgements.* The present work was supported by a grant of the Andrea Cesalpino Foundation.

## References


1. Dustin ML, Staunton D, Springer T (1988) Supergene families meet the immune system. Immunol Today 9: 213–215
2. Ross R (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362: 801–808
3. Marui N, Offermann MK, Swerlick R et al. (1993) Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through and antioxidant-sensitive mechanism in human vascular endothelial cells. J Clin Invest 92: 1866–1874
4. Morigi M, Angioletti S, Imberti B et al. (1998) Leucocyte-endothelial interaction is augmented by high glucose concentrations and hyperglycemia in a NF-kB-dependent fashion. J Clin Invest 101(9): 1905–1915
5. Cominacini L, Garbin U, Fratta Pasini A et al. (1997) Lacidipine inhibits the activation of the trasncription factor NK-kappa B and the expression of adhesion molecules induced by pro-oxydant signals on endothelial cells. J Hypertens 15: 1633–1639
6. Schreck R, Rieber P, Baeurle PA (1991) Reactive oxigens intermediates as apparently widely used messengers in the activation of the NF-kB transcription factor and HIV-1. EMBO J 10: 2247–2258
7. Pigott R, Dillon LP, Heminway LH, Gearing AJH (1992) Soluble forms of E-selectin, ICAM-1 and VCAM-1 are present in supernatants of cytokine-activated cultured endothelial cells. Biochem Biophys Res Commun 187: 584–589
8. Gearing AJH, Newman W (1993) Circulating adhesion molecules in disease. Immunology Today 14: 506–512
9. Bravi MC, Pietrangeli P, Laurenti O, Cassone-Faldetta MC, Ferri C, De Mattia G (1997) Polyol pathway activation and glutathione redox status in non-insulin-dependent diabetic patients. Metabolism 46: 1194–1198
10. Fasching P, Waldhausl W, Wagner OF (1996) Elevated circulating adhesion molecules in NIDDM – potential mediators in diabetic macroangiopathy. Diabetologia 39: 1242–1244
11. Ceriello A, Falleti E, Bortolotti N et al. (1996) Increased circulating ICAM-1 levels in type-2 diabetic patients: the possible role of metabolic control and oxidative stress. Metabolism 45: 498–501
12. Cominacini L, Garbin U, Fratta Pasini A et al (1998) Troglitazone reduces LDL oxidation and lowers plasma E-selectin concentration in NIDDM patients. Diabetes 47(1): 130–133
13. Albertini JP, Valensi P, Lormeau B et al. (1998) Elevated concentrations of soluble E-selectin and vascular cell adhesion molecule-1 in NIDDM. Effect of intensive insulin treatment. Diabetes Care 21(6): 1008–1013
14. Ferri C, Desideri G, Baldoncini R et al. (1998) Early activation of vascular endothelium in nonobese, nondiabetic essential hypertensive patients with multiple metabolic abnormalities. Diabetes 47: 660–668
15. National Diabetes Data Group (1979) Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. Diabetes 28: 1037–1057
16. Griffith OW (1980) Determination of glutathione disulfide using glutathione reductase and 2-vinylpiridine. Anal Biochem 106: 207–212
17. Friedewald WT, Levy RI, Fredrickson DS (1972) Estimation of concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 18: 499–502
18. Ceriello A (1997) Acute hyperglycemia and oxidative stress. Diabetic Med 14: 545–549
19. Faruqi RM, Poptic EJ, Faruqi TR, De La Motte C, DiCorleto PE (1997) Distinct mechanisms for N-Acetylcysteine inhibition of cytokine induced E-selectin and VCAM-1 expression. Am J Physiol 273: H816–H826
20. Peter K, Nawroth P, Conradt C et al. (1997) Circulating vascular endothelial cell adhesion molecule-1 correlates with the extent of human atherosclerosis in contrast to circulating intercellular adhesion molecule-1, E-selectin, P-selectin, and thrombomodulin. Arterioscler Thromb Vasc Biol 17: 505–512