Diabetologia (2001) 44: 834–838

Diabetologia
© Springer-Verlag 2001

Detection of nitrotyrosine in the diabetic plasma: evidence of oxidative stress

A. Ceriello[1], F. Mercuri[2], L. Quagliaro[2], R. Assaloni[1], E. Motz[1], L. Tonutti[3], C. Taboga[3]

[1] Department of Pathology and Medicine, Experimental and Clinical, University of Udine, Udine, Italy
[2] Morpurgo-Hofman Research Laboratory on Aging, Udine, Italy
[3] Diabetology Unit, Udine General Hospital, Udine, Italy

Abstract

Aims/hypothesis. Oxidative stress plays an important role in diabetic vascular complications. It has been shown that an imbalance in the ratio of nitric oxide: superoxide anion, because of a prevalence of superoxide anion, leads to an alteration in vascular reactivity. In this condition peroxynitrite production, resulting from the reaction between nitric oxide and superoxide, could increase. Peroxynitrite is responsible for nitration of tyrosine residues in proteins. Therefore, the presence of nitrotyrosine in plasma proteins is considered indirect evidence of peroxynitrite production. The aim of this study was to demonstrate the presence of nitrotyrosine in the plasma of patients with Type II (non-insulin-dependent) diabetes mellitus and to correlate its concentrations with the plasma concentrations of glucose and antioxidant defenses.
Methods. A total of 40 Type II diabetic patients and 35 healthy subjects were enrolled, and glycaemia, plasma nitrotyrosine, total antioxidant parameter and glycated haemoglobin were measured. Nitrotyrosine was detected by ELISA with a detection limit of 10 nmol/l.
Results. Nitrotyrosine was found in the plasma of all diabetic patients (means ± SD = 0.251 ± 0.141 μmol/l), whereas it was not detectable in the plasma of healthy control subjects. Nitrotyrosine plasma values were correlated with plasma glucose concentrations ($r = 0.38$, $p < 0.02$) but not with total antioxidant parameter or glycated haemoglobin. Total antioxidant parameter was reduced in diabetic patients ($p < 0.01$).
Conclusions. The presence of nitrotyrosine in the plasma of diabetic patients indicates that peroxynitrite is generated in diabetes, suggesting a possible involvement of peroxynitrite in the development of diabetic complications. [Diabetologia (2001) 44: 834–838]

Keywords Diabetes, nitrotyrosine, oxidative stress, nitric oxide, superoxide, total antioxidant parameter, hyperglycaemia, diabetic complications, peroxynitrite, glycated haemoglobin

The relation between diabetes and premature vascular disease is well established [1] and oxidative stress has been claimed as one of the most important pathogenetic factors [2].

In diabetes, oxidative stress could increase the production of superoxide (O_2^-) and nitric oxide (NO) [2–3], which could lead to the formation of pro-oxidant peroxynitrite ($ONOO^-$) [4].

The $ONOO^-$, formed by the reaction between NO and O_2^-, is a powerful oxidant capable of oxidizing low density lipoprotein [5], of causing vascular dysfunction [6] and of nitrating tyrosine residues in proteins [7]. Because the production of $ONOO^-$ is difficult to determine, the assay of nitrotyrosine in protein has been proposed as an indirect marker of $ONOO^-$ production [7].

The presence of nitrotyrosine in biological fluids such as plasma and urine have been investigated in a limited number of conditions. Raised values of nitrotyrosine have been found in the plasma of patients

Received: 6 October 2000 and in revised form: 9 February 2001

Corresponding author: Dr. A. Ceriello, Chair of Internal Medicine, University of Udine, P.le S. Maria della Misericordia, 33100 Udine, Italy

Abbreviations: O_2^-, Superoxide; NO, nitric oxide; $ONOO^-$, peroxynitrite; TRAP, total antioxidant parameter

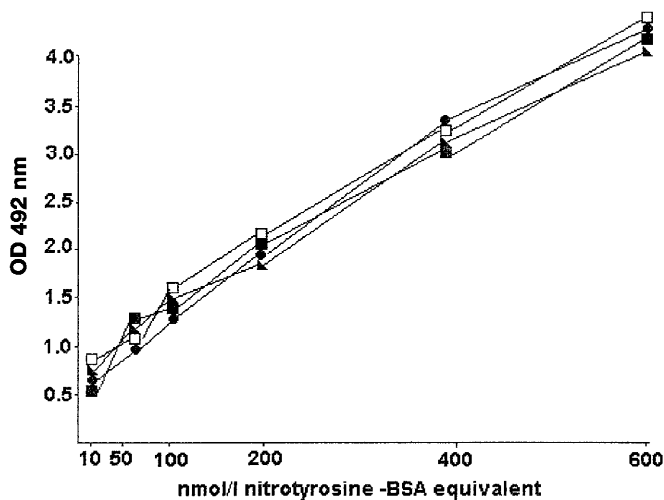

Fig. 1. Representative standard curves of nitrotyrosine-BSA in the developed ELISA. Values were developed in triplicate. ● Fixed TNM-treated BSA diluted in buffer; ■ Fixed TNM-treated BSA diluted in plasma; □ Varied TNM-treated BSA diluted in buffer; ◣ Varied TNM-treated BSA diluted in plasma

with rheumatoid arthritis [8], chronic renal failure [9], septic shock [9] and coeliac disease [10], whereas nitrotyrosine is generally not detectable in the plasma of healthy subjects [8–10].

In diabetes a reduction of plasma antioxidant power has been reported [11] and an increased production of O_2^- and NO has also been shown [2–3]. Such evidence suggests that increased production of $ONOO^-$ could be favoured in diabetic patients.

The aim of the present study was to show the presence of nitrotyrosine in the plasma of Type II diabetic patients and to correlate its concentration with the plasma values of glucose and antioxidant defenses.

Materials and methods

Altogether 40 Type II diabetic patients, on a diet including hypoglycaemic agents therapy (21 men and 19 women; age 56.2 ± 2.7 years, means ± SD; duration of diabetes 9.2 ± 2.1 years; BMI 26.2 ± 1.2 Kg/m^2), gave their informed consent to this study. A total of 35 healthy normal blood donor subjects, matched for sex (22 men and 13 women), age (55.4 ± 2.3 years), and BMI (25.9 ± 1.4 Kg/m^2) distribution, served as the control group. All were non-smokers. Moreover, diabetic and control subjects were also well-matched for diet habits, in terms of fruit and fresh vegetables consumption. As previously validated [11], dietary information was obtained by trained interviewers from a questionnaire whose principles have been previously described [12]. The reliability and validity of this tool for assessing food intake in the population has been already evaluated [13].

None of the subjects selected for this study was taking hypolipidaemic drugs or was on antioxidant supplementation. None had clinically symptomatic macroangiopathy, as judged by pathological changes in the resting electrocardiogram, a previous history of cardiac angina, intermittent claudication, myocardial or cerebral infarction. None had microalbuminuria or macroalbuminuria or suffered hypertension.

This study was approved by the ethics committee of our institution.

In all subjects fasting plasma glucose, glycated haemoglobin A_{1c} (HbA_{1c}), nitrotyrosine and total antioxidant parameter (TRAP) plasma values were assessed. Plasma glucose was assayed by glucose-oxidase method. HbA_{1c} was measured by HPLC.

Nitrotyrosine measurement. Bovine serum albumin (BSA), tetranitromethane (TNM), IgG secondary antibody, tetramethil-benzidine (TMB), cysteine, methionine, tryptophan, aminotyrosine and phosphotyrosine were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Maxisorp ELISA plates were from NUNC, Life Technologies (Grand Island, N.Y., USA), IgG monoclonal anti-nitrotyrosine antibody from UPSTATE (Lake Placid, N.Y., USA). Nitrotyrosine plasma concentration was assayed by ELISA as described previously [10].

Preparation of nitrated protein. Nitrated protein solution was carried out incubating 1 mg/ml BSA in 50 mmol/l KH_2PO_4 at pH 7.4 for 30 min at 37 °C with 1 mmol/l TNM, a very efficient nitrating agent [14]. After adjusting the pH to 10 with 3 mol/l NaOH, the amount of nitrotyrosine present in the TNM-treated BSA solution was measured at 430 nm [14]. Using this procedure the number of moles of nitrotyrosine for each mole of protein formed can be determined directly [14]. In accordance with other reports [4, 16], the nitration efficiency in these condition was about 10 % of tyrosine residues on BSA nitrated. Nitrotyrosine was expressed as µmol/l of nitrotyrosine-BSA equivalent.

ELISA. A standard curve was constructed by incubating in the wells serial dilutions of nitro-BSA in 0.1 mol/l Na_2CO_3-$NaHCO_3$ coating buffer at pH 9.6. Plasma samples assayed for nitrotyrosine were diluted up to 5 times. Standard and plasma samples were applied to Maxisorp ELISA plates and allowed to bind overnight at + 4 °C. Afterwards, non-specific binding sites were blocked with 1 % BSA in PBS. The wells were incubated at room temperature for 2 h with a mouse IgG monoclonal antinitrotyrosine (5µg/ml) with a peroxidase-conjugated goat anti-mouse IgG secondary antibody diluted 1:4000. After washing the plates, the peroxidase reaction product was generated using a TBM Microwell Peroxidase Substrate. Plates were incubated for 10 min at room temperature and the reaction was stopped with 50 µl per well of stopping reagent and read at 492 nm in a microplate reader [16].

The concentration of nitrated proteins that inhibit anti-nitrotyrosine antibody binding was estimated from the standard curve and was expressed as µmol/l of nitrotyrosine-BSA equivalents, i. e. an equivalent concentration of nitrotyrosine in nitro-BSA that produces the equivalent inhibition of the nitrated proteins [10].

The linearity of the ELISA method was confirmed by the construction of a standard curve ranging from 10 to 600 nmol/l nitrotyrosine-BSA equivalent (Fig. 1). Furthermore, the possible influence of plasma on the assay was exluded evaluating a standard curve diluting nitro-BSA in normal plasma (Fig. 1).

Because multiple tyrosine residues can be nitrated on a single protein species, a fixed concentration of BSA was nitrated with different concentrations of TNM to evaluate this potential complication on the assay. This nitrated-BSA was then used for the construction of standard curves diluting nitrated BSA in the buffer in the normal plasma (Fig. 1). No substancial

differences were found between the BSA nitrated with these two different approaches.

The influence of nitrated aminoacids cysteine, methionine and tryptophan on the ELISA was also evaluated, as previously described [10]. No interference was found. The immunospecificity of the primary antibody for nitrotyrosine was again confirmed pre-incubating samples with different concentrations of nitrotyrosine and/or pre-incubating antibody with nitrotyrosine 10 mmol/l before use in the ELISA. The specificity was also confirmed by the evidence that neither 10 mmol/l aminotyrosine nor 10 mmol/l phosphotyrosine inhibited the binding of the anti-nitrotyrosine antibody.

Glucose interference was excluded performing the ELISA assay of standard solution in the presence of various glucose concentrations (20, 40 and 100 mmol/l). The limit of detection was 10 nmol/l, while the intra-assay and inter-assay coefficients of variation were 4.5 % and 8 %, respectively.

TRAP Measurement. The 2,2'-azobis-(2-amidinopropane) dihydrochloride (ABAP), R-Phycoerythrin (R-PE), Trolox and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo., USA).

TRAP was evaluated according to methods described previously [17] in which the production of peroxyl radicals obtained by thermal decomposition of ABAP leads to a linear decrease in R-PE fluorescence emission over 1 h. When plasma is added to the reaction mixture, a period of complete protection of R-PE is observed. The length of this lag-phase (T) is here taken to be directly related to total plasma antioxidant capacity. To quantify the TRAP, the T produced by plasma is compared to the T produced by a known amount of Trolox. The values of T are calculated by extrapolating the slope of maximal R-PE decay to intersect with the slope of plasma and Trolox protection. The projection of these intersection points on the "X" axis gives the T values, which represents the time required to achieve the maximal R-PE peroxidation rates. By comparing the T of plasma with the T of Trolox, taking into account the concentration of Trolox (Conc), the TRAP value of a plasma sample is obtained according the following proportion: Conc Trolox : T Trolox = X:T Plasma [17].

The resulting value of X is then multiplied by 2.0 (the stoichiometric factor of Trolox) and by dilution factor of plasma (250). Values are expressed as µmol/l.

Samples were prepared according to the procedure previously described [17]. The reaction mixture consisted of $1.5 \cdot 10^{-8}$ mol/l R-PE in 75 mmol/l phosphate buffer, pH 7. A total of 8 µl of plasma or 30 µl of 120 µmol/l Trolox was added to 2.0 ml final volume, and the resulting solution was maintained at 37 °C for 5 min in 10 mm quartz fluorometer cells. The oxidation reaction was started by adding ABAP to a final concentration of 4.0 mmol/l, and the decay of R-PE fluorescence was monitored every 5 min on a Perkin-Elmer LS-50 Luminescence Spectrometer equipped with a thermostatically controlled cell-holder; monochromators were operating at excitation wavelength 495 nm/5 nm slit width, and emission wavelength 575 nm/5 slit width. TRAP values were calculated as described above. Intra-assay and inter-assay coefficients of variation for this method were 10 % and 12 %, respectively.

Statistical analysis. Statistical analysis of data was done by means of the BMDP statistical software package. Normal distribution of each parameter was confirmed by a normality test (Shapiro and Wilk's W test [18]). Data between diabetics and controls were then compared by the unpaired Student's *t* test.

Linear regression analysis was applied where indicated. Data are reported as means ± SD. A *p* value of less than 0.05 was considered significant.

Results

In diabetic patients fasting plasma glucose and HbA_{1c} were 10.2 ± 3.1 mmol/l and 7.7 ± 1.9 %, respectively; in normal controls plasma glucose and HbA_{1c} were 4.9 ± 0.8 mmol/l and 5.1 ± 0.4 %, respectively.

Nitrotyrosine was found in the plasma of all diabetic patients (means ± SD = 0.251 ± 0.141 µmol/l), whereas it was not detectable in the plasma of healthy control subjects. Nitrotyrosine plasma values were correlated with plasma glucose concentrations ($r = 0.38$, $p < 0.02$) but not with total antioxidant parameter or glycated haemoglobin (Fig. 2). Total antioxidant parameter (TRAP) was reduced in diabetic patients compared to healthy control subjects (961 ± 23.7 µmol/l vs 1178 ± 197 µmol/l; $p < 0.01$).

Discussion

Reaction of NO with O^- generates $ONOO^-$, which can decompose into products that nitrate aromatic amino acids [4]. Such nitro-aromatics are markers of $ONOO^-$-dependent oxidative damage. Because nitrotyrosine, particularly protein-associated nitrotyrosine, is a stable end-product of peroxynitrite oxidation, assessment of its plasma concentration is considered a useful marker of $ONOO^-$ -dependent damage in vivo [7].

While nitrotyrosine is generally not detectable in the plasma of normal healthy subjects [8–10), increased concentrations of nitrotyrosine have been found in the plasma of patients with various diseases [8, 9, 10]. In diabetic patients nitrotyrosine has previously been found in the placenta and kidney [19–20]. In this study, detected, nitrotyrosine was also detected in the plasma of Type II diabetic patients.

$ONOO^-$ generation and the subsequent nitrotyrosine formation could be promoted in diabetes by hyperglycaemia. It has been shown that elevated concentrations of glucose increase NO as well as O^- release from endothelial cells [3], the increase in O^- being about twice as much as that of NO [3]. This large production of O^- could be explained by the recent finding that endothelial cells are freely permeable to glucose by way of the receptor GLUT-1 [21]. A free glucose supply, proportional to extracellular glucose concentration, will produce, at the mitochondrial level, not only energy, but also a large quantity of O^- [21]. In a condition of glucose-induced increased NO release and concomitant prevalent production of O^- [3], NO could be trapped by O^-, and $ONOO^-$ generation could be enhanced. By such a mechanism high plasma glucose concentrations in diabetic patients could induce overproduction of $ONOO^-$ and reduce biological effects of NO.

Our data, demonstrating the presence of nitrotyrosine in the plasma of diabetic patients, supports this hypothesis, and the correlation between glycaemia

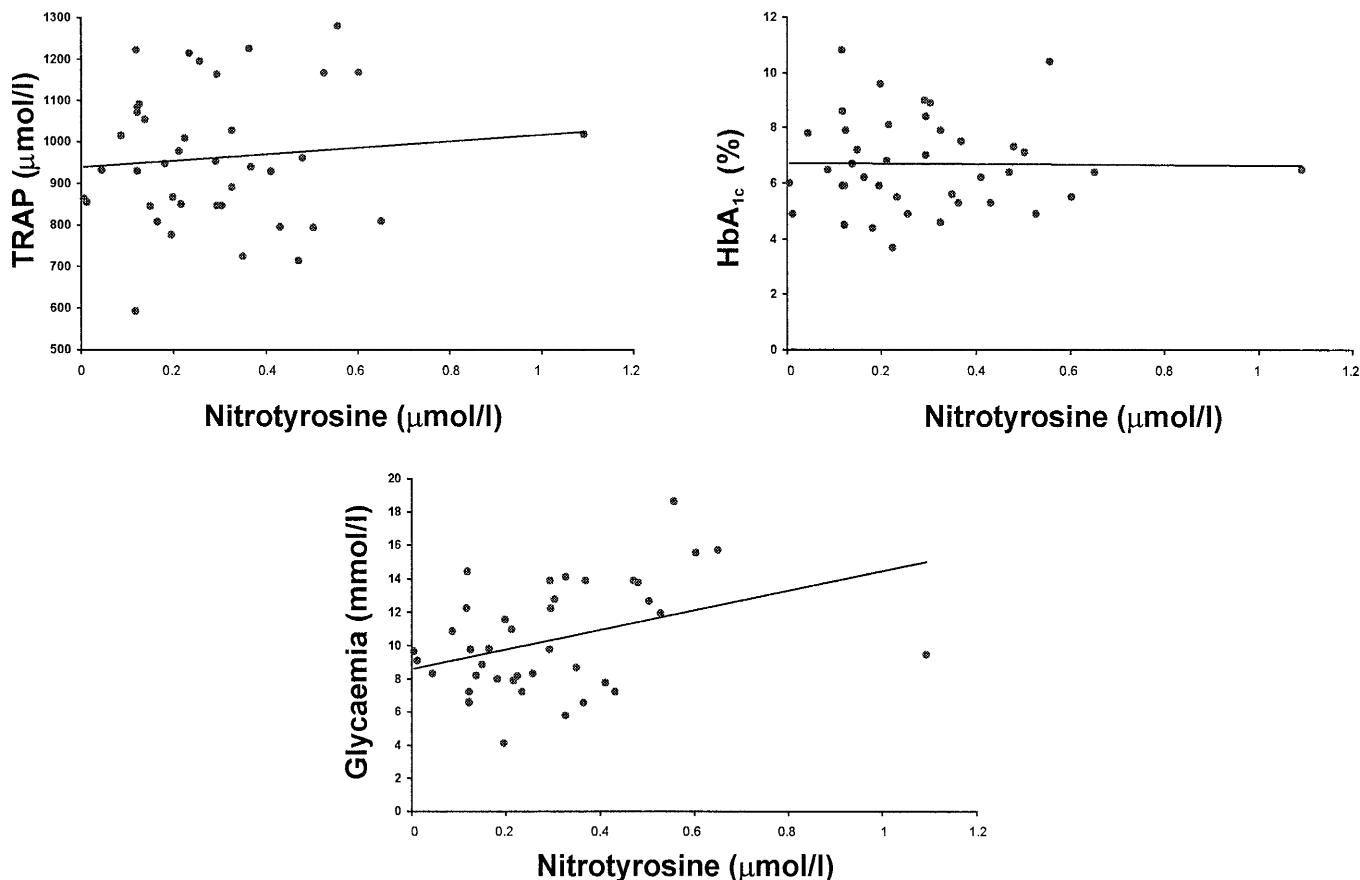

Fig. 2. Correlation between nitrotyrosine plasma concentrations and TRAP, HbA_{1C} and glycaemia in Type II diabetic patients
$p < 0.02$; $r = 0.38$

and nitrotyrosine values suggests that hyperglycaemia could be directly involved.

In our study, data on NO and O^- plasma concentrations are not available. However, it has previously been reported that in vivo O^- plasma concentrations are increased in diabetic patients and correlate with the value of glycaemia [22]. Evidence that the half life of nitrated proteins is about 1.6 h [23] also supports the finding that nitrotyrosine correlates with glycaemia and not with HbA_{1c}.

We could not find a correlation between TRAP and nitrotyrosine levels. This could be explained by the fact that while TRAP includes plasma antioxidants such as ascorbic acid and thiol groups which are inhibitors of $ONOO^-$ -mediated oxidation [7], an important contribution to $ONOO^-$ -scavenging action comes from enzymes, particularly superoxide dismutase and glutathione peroxidase [7], which are not evaluated in TRAP [17].

The finding that $ONOO^-$ production in diabetes appears to increase could have important clinical implications.

Peroxynitrite is a potent oxidant and nitrating agent that leads to a host of potentially injurious events including lipid peroxidation [5], depletion of antioxidant defenses [24] and inactivation of enzymes [25]. In addition, it can be directly cytotoxic for endothelial cells [6]. All these events could convincingly be involved in the pathogenesis of diabetic complications. This hypothesis is strongly supported by the recent demonstration that the increased apoptosis of myocytes, endothelial cells and fibroblasts from heart biopsies from diabetic patients is selectively associated with the concentration of nitrotyrosine found in those cells [26]. Furthermore, the presence of nitrotyrosine in atherosclerotic lesions suggests that $ONOO^-$ production could be involved in atherogenesis [27], and it is well know that atherosclerosis is particularly frequent in diabetic patients.

It should be noted that the quantification of plasma nitrotyrosine by ELISA takes into account a mixture of plasma proteins and that, because proteins contain more than one tyrosine residue, nitrosylated proteins will contain a variable number of nitrotyrosyl groups whose affinity for the antibody could differ. This method cannot, therefore, define the type of plasma protein preferentially nitrosylated in diabetic subjects nor can it measure the precise degree of protein nitrosylation. Nevertheless, in our opinion, these limits do not diminish the relevance of finding nitrotyrosine in the plasma of all diabetic patients

which was not detectable in control subjects. This means that nitrosylation of proteins is an active process in diabetes. Although our ELISA method could be considered a semi-quantitative measure of protein nitration, the direct correlation we found between glycaemic values and nitrotyrosine concentrations suggests that nitrotyrosine could at least be a marker of hyperglycaemia-related oxidative damage in diabetic subjects, while a recent study has shown that nitrotyrosine could be even more than so, being directly harmful to endothelial cells [28].

In conclusion, the evidence that circulating nitrotyrosine is present in the plasma of diabetic patients supports the hypothesis that oxidative stress is present in diabetes [2], and suggests an important contribution of $ONOO^-$ over-generation to this phenomenon.

References

1. Kannel WB, McGee DL (1978) Diabetes and cardiovascular disease: the Framingham Study. JAMA 24: 2035–2038
2. Giugliano D, Ceriello A, Paolisso G (1996) Oxidative stress and diabetic vascular complications. Diabetes Care 19: 257–267
3. Cosentino F, Hishikawa K, Katusic ZS, Lüscher TF (1997) High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. Circulation 96: 25–28
4. Ischiropoulos H (1998) Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. Arch Biochem Biophys 356: 1–11
5. Graham A, Hogg N, Kalyanaraman B, O'Leary V, Darley-Usmar V, Moncada S (1993) Peroxynitrite modification of low-density lipoprotein leads to recognition by the macrophage scavenger receptor. FEBS Lett 330: 181–185
6. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 87: 1620–1624
7. Beckman JS, Koppenol WH (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol 271: C1424-C1437
8. Kaur H, Halliwell B (1994) Evidence for nitric oxide mediated oxidative damage in chronic inflammation. Nitrotyrosine in serum and synovial fluid from rheumatoid patients. FEBS Lett 350: 9–12
9. Fukuyama N, Takebayashi Y, Hida M, Ishida H, Ichimori K, Nakazawa H (1997) Clinical evidence of peroxynitrite formation in chronic renal failure patients with septic shock. Free Radic Biol Med 22: 771–774
10. Ter Steege JCA, Koster-Kamphuis L, Van Straaten E, Forget P, Buurman WA (1998) Nitrotyrosine in plasma of celiac disease patients as detected by a new sandwich ELISA Free Radic Biol Med 25: 953–963
11. Ceriello A, Bortolotti N, Falleti E, et al. (1997) Total radical-trapping antioxidant parameter in non-insulin dependent diabetic patients. Diabetes Care 20: 194–197
12. Block G, Hartman AM, Dresser CM, Carroll MD, Gannon J, Gardner L (1986) A data-based approach to diet questionnaire design and testing. Am J Epidemiol 124: 453–469
13. Mares-Perlman JA, Klein BEK, Klein R, Ritter LL, Fisher MR, Freudenheim JL (1993) A diet questionnaire ranks nutrients intakes in middle-aged and older men and women similarly to multiple food records. J Nutr 123: 489–501
14. Sokolovsky M, Riordan JF, Valle BL (1966) Tetranitromethane. A reagent for the nitration of tyrosil residues in proteins. Biochemistry 5: 3582–3589
15. Kamisaki Y, Wada K, Bian K, et al. (1998) An activity in rat tissues that modifies nitrotyrosine-containing proteins. Proc Natl Acad Sci USA 95: 11584–11589
16. Secchi C, Berrini A, Borromeo V (1991) An enzyme-linked immunosorbent assay for the determination of bovine prolactin in plasma. J Immunol Methods 136: 17–22
17. Ghiselli A, Serafini M, Maiani G, Azzini E, Ferro-Luzzi A (1995) A fluorescence-based method for measuring total plasma antioxidant capability. Free Radic Biol Med 18: 29–36
18. Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples). Biometrika 52: 591–611
19. Lyall F, Gibson JL, Greer IA, Brocman DE, Eis ALW, Myatt L (1998) Increased nitrotyrosine in the diabetic placenta. Evidence for oxidative stress. Diabetes Care 21: 1753–1758
20. Thuraisingham RC, Nott CA, Dodd SM, Yaqoob MM (2000) Increased nitrotyrosine staining in kidneys from patients with diabetic nephropathy. Kidney Int 57: 1968–1972
21. Nishikawa T, Edelstein D, Du X-L et al. (2000) Normalizing mitochondrial superoxide production blocks three pathways of hyperglycemic damage. Nature 404: 787–790
22. Ceriello A, Giugliano D, Quatraro A, Dello Russo P, Lefebvre PJ (1991) Metabolic control may influence the increased superoxide anion generation in diabetic serum. Diabet Med 8: 540–542
23. Kamisaki Y, Wada K, Ataka M et al. (1997) Lipopolysaccharide-indued increase in plasma nitrotyrosin concentrations in rats. Biochim Biophys Acta 1362: 24–28
24. Van der Vliet A, Smith D, O'Neill CA et al. (1994) Interactions of peroxynitrite with human plasma and its constituents: oxidative damage and antioxidant depletion. Biochem J 303: 295–301
25. Mac-Millan-Crow LA, Crow JP, Thompson JA (1998) Peroxynitrite-mediated inactivation of manganese superoxide dismutase involves nitration and oxidation of critical tyrosine residues. Biochemistry 37: 1613–1622
26. Frustaci A, Kajstura J, Chimenti C et al. (2000) Myocardial cell death in human diabetes. Circ Res 87: 1123–1132
27. Beckmann JS, Ye YZ, Anderson PG et al. (1994) Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. Biol Chem 375: 81–88
28. Mihm MJ, Jing L, Bauer JA (2000) Nitrotyrosine causes selective vascular endothelial dysfunction and DNA damage. J Cardiovasc Pharmacol 36: 182–187