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Evidence for *i*NOS-dependent peroxynitrite production in diabetic platelets

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Abstract

Aims/hypothesis. The aim of the present study was twofold. Firstly, to determine whether diabetic platelets produce more peroxynitrite than normal platelets and secondly to correlate the peroxynitrite production with the intraplatelet induction of the inducible isoform of nitric oxide-synthase.
Methods. Intraplatelet peroxynitrite production was monitored with dichlorofluorescin acetate with a combination of confocal microscopy and steady-state fluorescence. The platelets were probed for the induction of the inducible-nitric oxide-synthase by western immunoblotting.
Results. In the presence of extracellular L-arginine (100 µmol/l), platelets from subjects with Type I (insulin-dependent) diabetes displayed about 5 times higher fluorescence than those from control subjects. To determine whether inducible-nitric oxide-synthase was the source of peroxynitrite, dichlorofluorescein production was quantified as a function of L-arginine as well as nitric oxide-synthase inhibitors, in platelets from control subjects, subjects with Type I diabetes and subjects with Type II (non-insulin-dependent) diabetes mellitus. Platelets from subjects with Type I yielded about sevenfold and those from Type II about threefold larger amounts of L-arginine/nitric oxide-synthase-dependent dichlorofluorescein fluorescence than those from control subjects. The platelets were then immunologically probed for inducible-nitric oxide-synthase, which has previously been implicated in peroxynitrite production and detected in megakaryocytes of subjects with coronary heart disease. Western immunoblots of intraplatelet proteins indicated that the inducible-nitric oxide-synthase was absent in control subjects. Platelets from both Type I and Type II diabetic subjects, however, contained inducible-nitric oxide-synthase.
Conclusion/interpretation. Inducible-nitric oxide-synthase-derived peroxynitrite is a source of platelet damage in diabetes. [Diabetologia (1999) 42: 539–544]

Keywords Platelet, peroxynitrite, inducible nitric oxide synthase, dichlorofluorescein.

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Abbreviations: NO, nitric oxide; *i*NOS, inducible nitric oxide-synthase; *e*NOS, endothelial nitric oxide-synthase; *n*NOS, neuronal nitric oxide-synthase; DCFDA, 2,7-dichlorofluorescin diacetate; L-NMMA, N^G-monomethyl-L-arginine; D-NMMA, N^G-monomethyl-D-arginine; DCF, 2,7-dichlorofluorescein; $ONOO^-$, peroxynitrite anion; ECL, enzyme derived chemiluminescence; PRP, platelet-rich plasma; *mac*NOS, mouse macrophage nitric oxide-synthase; cNOS, constitutive nitric oxide synthase.

Nitric oxide (NO), an important mediator of both physiological and pathological processes [1], is derived from L-arginine by a family of enzymes termed NO synthases [2]. At least three isoforms of NO synthase (NOS) have been detected. Of these, endothelial (*e*NOS) and neuronal (*n*NOS) enzymes are constitutive and regulated by Ca^{2+}/calmodulin. The inducible NOS (*i*NOS) originally detected in macrophages and in the endothelium is produced in response to cytokines and cellular debris of microbial origin. This inducible form can produce 10- to 50-fold more NO than the constitutive NOS. In addition,

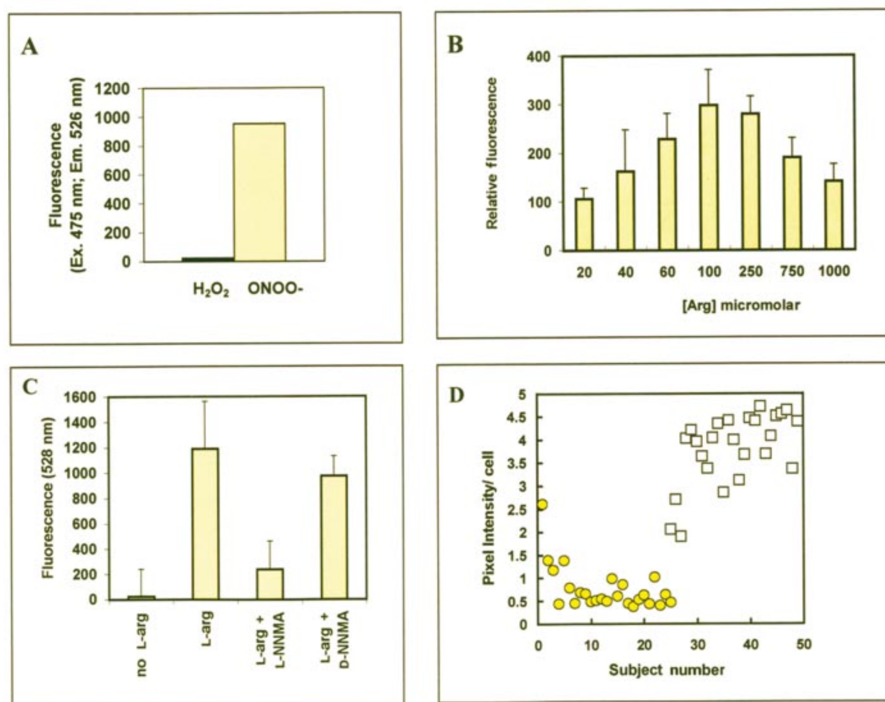

Fig. 1. A Effect of H_2O_2 and peroxynitrite on the production of DCFDA fluorescence. H_2O_2 (100 µmol/l) (dark filled bar) or $ONOO^-$ (100 µmol/l) (light filled bar) were incubated with 5 µmol/l DCFDA for 15 min in PBS pH 7.4 at 37 °C. Fluorescence of the mixture was then determined (λ_{ex} 475 nm, λ_{em} 520 nm). **B** The effect of extracellular L-Arg on intraplatelet dichlorofluorescein fluorescence. Washed Platelets (from Type I diabetic subjects) were incubated with DCFDA (5 µmol/l plus increasing L-arginine concentrations (0–1000 µmol/l). Intraplatelet fluorescence (see Methods) was monitored with a spectrofluorometer (λ_{ex} 475 nm, λ_{em} 520 nm). The data were obtained from 3 independent experiments. **C** Effect of L-NMMA and D-NNMA on intraplatelet DCF fluorescence. Washed platelets (from 5 different Type I diabetic subjects) were incubated with L-arginine (100 µmol/l or L-NMMA (100 µmol/l) or D-NNMA (100 µmol/l) in the presence of DCFDA (5 µmol/l) for 15 min at 37 °C. Intraplatelet DCF fluorescence was monitored spectrofluorometically (λ_{ex} 475 nm, λ_{em} 520 nm). The error bars represent standard deviation. **D** Quantification of the fluorescence of confocal images of DCFDA/L-Arginine treated control and Type I diabetic platelets. The number of platelets within the observation field were counted by Carl Zeiss LSCM software. All the samples were observed with identical excitation laser energy and photomultiplier gain. Confocal images in *.gif format, were scanned on a Biorad 620 Scanning Densitometer. The total pixel intensity values from circular areas slightly smaller than the areas covered by the platelets were then divided by cell number to give pixel intensity/cell. Subject number 1 to 25 controls; 26 to 50 Type I. L-arg = L-arginine

*i*NOS under conditions of limiting L-arginine can produce superoxide ($O_2^{\cdot -}$) as well as NO [3]. These two radicals react at diffusion controlled rates to yield peroxynitrite anion ($ONOO^-$).

Peroxynitrite is able to initiate lipid peroxidation and to nitrate protein tyrosine and phenylalanine side chains with subsequent alterations in cellular functions [4].

Inducible NOS-dependent peroxynitrite production has been recently implicated in the pathophysiology of atherosclerosis [5, 6] hypercholesterolaemia [7], hypertension [8], septic shock [9] and diabetes mellitus [7].

In this study, we have compared normal and diabetic platelets with respect to L-arginine/NOS-dependent dichlorofluorescein formation, which under the conditions used is an accurate indicator of peroxynitrite production. In addition, the platelets were probed for *i*NOS expression, which might cause increased peroxynitrite formation in cells from diabetic patients, possibly related to platelet dysfunction.

Subjects and methods

Ethical considerations. The procedures relating to the use of human subjects in this study were approved by the University of Windsor Human Ethics Committee. Blood samples from the subjects were obtained with informed consent.

Patients. The study was carried out on; 11 healthy subjects (5 men, 6 women, age 51 ± 10 years), 24 patients affected by Type I (insulin-dependent) diabetes mellitus (12 men, 12 women, age 38 ± 9 years, duration of disease ~ 10 ± 4 years), and 17 patients affected by Type II (non-insulin-dependent) diabetes mellitus (9 men, 8 women, age 52 ± 6 years, duration of disease ~ 9 ± 5 years). They denied taking any drug that could influence platelets' function in the previous two weeks. There were seven patients with Type I diabetes and four with Type II diabetes who had a background of retinopathy. Type II diabetic subjects were treated with diet alone and patients with

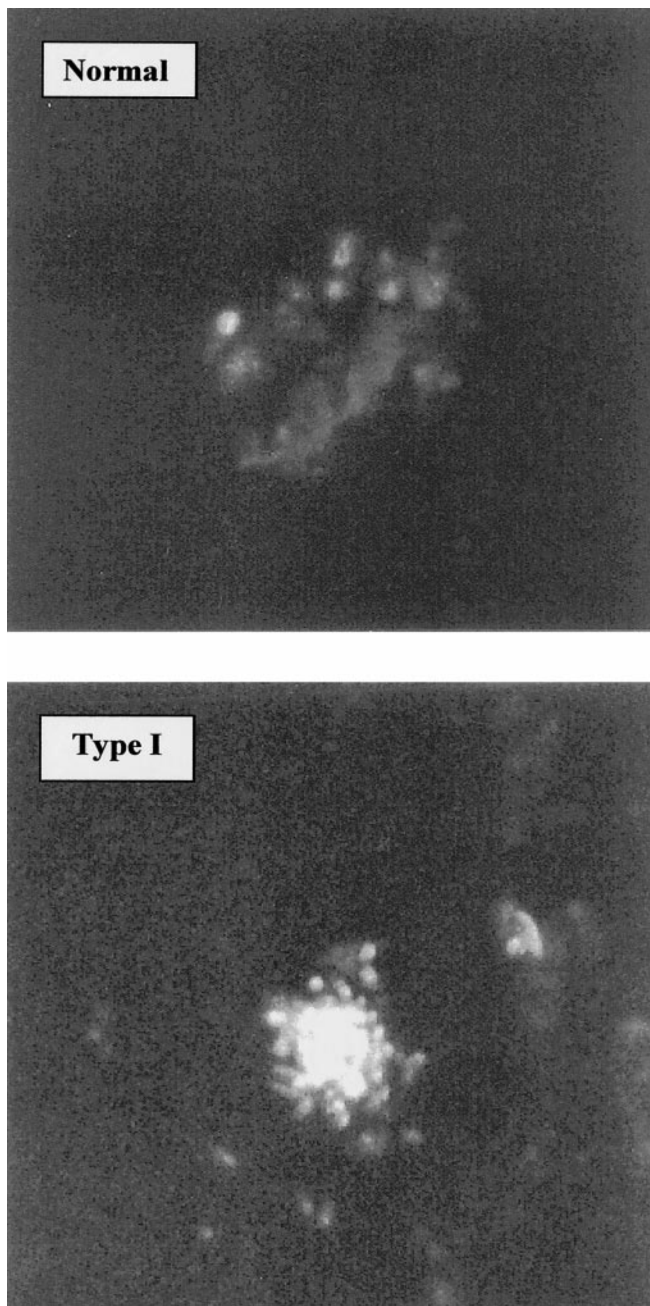

Fig. 2. Confocal images of DCFDA preloaded normal and Type I diabetic platelets. Washed normal and Type I diabetic platelets were preloaded with DCFDA by a 15 min pre-incubation and washed two times. They were exposed to L-Arginine (100 μmol/l) for 15 min, washed, then transferred to a microscope slide. The cover slip was attached to the slide with clear nail polish. The platelets were viewed with a U-V laser on a Zeiss LSM 410 confocal microscope, under a 60X oil immersion objective

Type I diabetes were treated with insulin (3 or 4 daily subcutaneous injections).

Blood was drawn in the fasting state from a forearm vein for the isolation of platelets, on which the following determinations were carried out: peroxynitrite production studied by means of confocal microscopy and by the fluorimetric assay of 2,7-dichlorafluorescein (DCF), expression of *i*NOS by western blotting.

The reagents used were: 2,7-dichlorofluorescin diacetate (DCFDA) and N^G-monomethyl-L-arginine (L-NMMA) and N^G-monomethyl-D-arginine (D-NMMA) from Molecular Probes (Eugene Ore., USA); L-Arginine from Sigma (St. Louis, Mo., USA); primary antibody (anti-*i*NOS, host mouse) and mouse macrophage *i*NOS (*mac*-NOS; positive control) from Transduction Laboratories (Lexington, Ky., USA). Enzyme derived chemiluminescence (ECL) western blotting detection reagents and hyperfilm from Amersham International (Buckinghamshire, UK).

Blood collection and preparative procedures. Blood (5 ml) from healthy, Type I and Type II diabetic volunteers was collected in 5 mmol/l EDTA sterile tubes. Platelet-rich plasma (PRP) was obtained by centrifuging blood at 214 *g* for 10 min. It was then centrifuged at 1149 *g* for 10 min using 10 % acid-citrate-dextrose buffer containing 0.8 % citric acid, 2.2 % sodium citrate, and 2.4 % dextrose. Platelet pellets were then washed three times with Ca^{2+}-free Tyrode's buffer containing 137 mmol/l sodium chloride, 5.5 mmol/l glucose, $NaHCO_3$ 11.9 mmol/l, NaH_2PO_4 0.35 mmol/l, $MgCl_2$ 1.0 mmol/l, pH 6.5 and stored in the appropriate buffer.

Preparation of DCF-free base. We prepared a DCFDA-free base daily, by mixing 0.05 ml of 10 mmol/l DCFDA with 2 ml of 0.01 N NaOH at room temperature for 30 min. The mixture was neutralized with 18.0 ml of 25 mmol/l phosphate-buffered saline (PBS), pH 7.4. This solution was maintained on ice in the dark until use.

Confocal microscopy. Washed platelets from control and Type I diabetic subjects were incubated for 15 min with 5 μmol/l of DCF-free base at 37 °C. Then 0.1 mmol/l of L-arginine was added for a further 15 min of incubation. Platelets were washed twice with 25 mmol/l phosphate buffer (pH 7.4), and were viewed with a U-V laser on a Zeiss LSM 410 confocal microscope (Carl Zeiss, Don Mills, Canada), under a 60X oil immersion objective. The number of platelets within the observation field were counted by Carl Zeiss LSCM soft ware.

Fluorimetric assay of DCF. The DCFDA/L-arginine treated platelets (as previously described) were divided into three sets and 0.1 mmol/l of L-NMMA or D-NNMA or buffer was added to each set. After incubation for 15 min at 37 °C, the platelets were washed twice with pH 7.4 phosphate buffer (25 mmol/l). The platelet pellets were broken by resuspension in 1 ml of water, while shaking for 15 min at room temperature. The mixture was then centrifuged for 2 min at 214 *g*. The supernatant was diluted appropriately and the fluorescence was measured in a Hitachi Model F-2000 Spectrofluorometer (St Jose, Calif., USA) at an excitation wavelength of 475 nm and emission wavelength of 520 nm. Blank samples contained all reagents except platelets. The samples incubated with L-Arginine were corrected for background fluorescence by the subtraction of the cytosolic fluorescence of platelets, which were not exposed to extracellular L-Arginine.

Peroxynitrite-specificity of dichlorofluorescin production. Peroxynitrite can oxidize the non-fluorescent molecule 2,7-dichlorofluorescin to the fluorescent 2,7-dichlorofluorescein [10, 11]. In those studies it was also shown that NO, hydroxyl radical (OH ·) and metal ions do not oxidize 2,7-dichlorofluorescin. Some earlier reports have suggested that hydrogen peroxide also oxidized 2,7-dichlorofluorescin [12]. To test this, we incubated DCFDA (5 μmol/l) with either 100 μmol/l H_2O_2 or 100 μmol/l $ONOO^-$ for 15 min in PBS at 37 °C. The H_2O_2 is

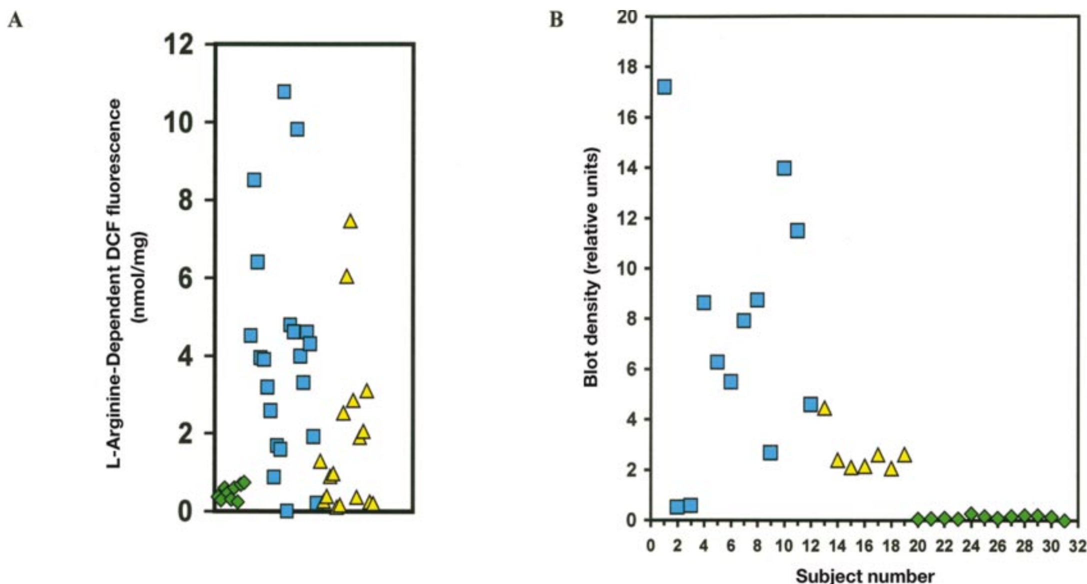

Fig. 3. A Effect of L-arginine on intraplatelet DCF fluorescence. 100 μmol/l L-arginine was incubated with DCF loaded PRP of control, Type I and Type II diabetics for 15 min at 37 °C. Washed platelets were lysed with H_2O, the fluorescence (λ_{ex} 475 nm, λ_{em} 520 nm) was converted to peroxynitrite concentration/mg of protein by comparing with a standard curve of DCF fluorescence as a function of authentic peroxynitrite. This standard curve was best described by the linear equation, fluorescence = 1078 * nmol $ONOO^-$ + 48. ◆, control; ❑, Type I; △, Type II. **B** Western immunoblot *i*NOS-specific densities as a function of subject number. Hyperfilm exposed to western immunoblots probes with anti iNOS was scanned on a Biorad 620 Scanning Densitometer (Hercules Calif., USA). The blot densities are plotted with respect to blot number: Subjects 1 to 12 Type II; subjects 13 to 19 Type I; subjects 20 to 31 normal

not an efficient oxidizer of dichlorofluorescin as essentially no fluorescence (~ 23 Relative Intensity Units) was produced (Fig. 1). On the other hand $ONOO^-$ yielded 41-fold larger fluorescence (954 Relative Intensity Units).

Showing the dependance of intracellular dichlorofluorescin oxidation on peroxynitrite produced by intracellular L-Arginine/NO pathway. In these experiments washed platelets isolated from Type I diabetic subjects were first preincubated for 15 min with DCFDA, then incubated with increasing amounts of L-Arginine. The intraplatelet fluorescence (due to dichlorofluorescein) was directly proportional to the extracellular L-Arginine concentration up to 100 μmol/l (Fig. 1). Above this concentration the fluorescence decreased with increasing L-Arginine.

Western blotting. Washed platelets were hypotonically lysed (as described above). The collected supernatants were treated with an equal volume of sample application buffer (125 mmol/l TRIS-HCl pH 6.8, 2 % SDS, 5 % glycerol, 0.003 % bromophenol blue, and 1 % β-mercaptoethanol).The mixture was then boiled for 5 min. To each well of an 8 % SDS-polyacrylamide gel, 10 μl of each sample was applied and electrophoresed for 1 h at 130 V along with a set of molecular weight markers (Sigma-broad range). We used BSA (1 μg/μl) as a negative and *mac*NOS as a positive control. The resolved protein bands were then transferred onto a nitrocellulose support medium at 100 V for 60 min using a transfer buffer of 25 mmol/l TRIS base/192 mmol/l glycine/20 % methanol. The blots were blocked overnight at 4 °C with blocking buffer (5 % non-fat milk in 10 mmol/l TRIS pH 7.5, 100 mmol/l NaCl, 0.1 % Tween 20). The blocking buffer was decanted and blots were incubated for 1 h at room temperature with primary antibody (anti-*mac*NOS monoclonal, host mouse) diluted 1:2500 in blocking buffer. Blots were then washed using washing buffer (10 mmol/l TRIS pH 7.5, 100 mmol/l NaCl, 0.1 % Tween 20) for 30 min with agitation and then incubated with the enzyme conjugate anti-mouse IgG: horseradish peroxidase, diluted 1:2500 in blocking buffer for 1 h at room temperature followed by washing for 30 min with agitation. Then ECL detection reagents were added and the blots were exposed for 1 h on a hyperfilm.

Results

Observations from confocal microscopy. Platelets from diabetic subjects exhibited dramatically higher fluorescence than those from control subjects (Fig. 2). This was further confirmed when pixel intensities were plotted with respect to platelet source (Fig. 1D). The average pixel intensities to cell for normal platelets was 0.75 ± 0.49 and that for Type I platelets was 3.79 ± 0.77.

Fluorimetric quantification of intraplatelet dichlorofluorescein formation. Under the conditions used the standard curve obtained for dichloroflourescein fluorescence (λ_{ex} 475 nm, λ_{em} 520 nm) as a function of authentic concentration of $ONOO^-$, was best fitted by the equation y = 1078x + 48. Both Type I (4.07 ± 2.88 nmol/mg) and Type II (1.82 ± 2.12 nmol/mg) diabetic platelets exhibited higher L-Arginine/NO-dependent $ONOO^-$ production than controls (0.58 ± 0.36 nmol/mg) ($r < 0.01$) (Fig. 3 a).

Expression of iNOS. The blot densities of the bands corresponding to *i*NOS are plotted as a function of subject category in Figure 3B. None of the control subjects ($n = 12$) had a positive band for *i*NOS. On the other hand *i*NOS was evident in both Type I ($n = 7$) and Type II ($n = 12$) platelets. The average blot density for Type I was 2.63 ± 0.84 and for Type II was 7.3 ± 5.11.

Discussion

Platelets have a central role in the thrombotic and atherosclerotic processes both because of their ability to form aggregates and to modulate the coagulation cascade and fibrinolysis and because of their capability to induce proliferation of smooth muscle cells and to regulate vasomotion [13]. Patients with diabetes mellitus show considerable alterations in platelet function, which have been hypothesized to be crucial in the pathogenesis of diabetic angiopathy [14].

Recent evidence suggested that platelet NO synthesis is a powerful modulator of platelet activation as NO reduces both platelet adhesion and platelet thrombus formation [15]. A previous work by our group described a decreased NO production in platelets from Type I and Type II diabetic patients, possibly related to the higher sensitivity of these cells to aggregating stimuli [16]. Moreover, a further mechanism causing platelet dysfunction in diabetes related to NO synthesis might be an imbalance between NO and superoxide production, leading to the formation of the powerful oxidant peroxynitrite.

In this study, confocal microscopic or fluorimetric observations showed three to sevenfold larger levels of DCF fluorescence, an indirect signal for increased peroxynitrite production, in platelets from both Type I and Type II diabetic subjects compared with those from healthy control subjects. Evidence that we are observing for NOS-derived $ONOO^-$ comes from the experiments shown in Fig. 1B, showing that DCF fluorescence increased proportionally to extracellular L-Arginine concentration up to 100 μmol/l and then was reduced, as NOS is inhibited by large concentrations of NO [17]. Additional evidence for DCF being a reporter of NOS-derived $ONOO^-$ was the inhibition of intracellular L-Arg-dependent fluorescence by the preincubation of platelets with the non-specific NOS inhibitor L-NMMA (Fig. 1C) but not with its D-isomer D-NNMA which has previously been shown not to inhibit NOS.

Of the three NOS isoenzymes *i*NOS is known to produce most NO and $O_2^{\cdot -}$, being therefore implicated in peroxynitrite production [18, 19]. In addition *i*NOS has been detected in megakaryocytes of subjects with coronary heart disease [20]. It is therefore conceivable that the considerable $ONOO^-$ production observed here in vitro in platelets from diabetic patients could result from the induction of *i*NOS by diabetes-related factors. As we expected on the basis of this hypothesis, we found that iNOS is expressed in all the diabetic samples (both Type I and Type II) examined whereas *i*NOS protein was not expressed in normal platelets (Fig. 3B). Platelets are generally not thought to generate de novo protein synthesis which agrees with our data and with previous results reporting that *i*NOS protein is not expressed in normal platelets [19] whereas the presence of constitutive NOS (*c*NOS) has been widely described in human platelets [21]. It has been hypothesized recently that *i*NOS expression in macrophages and other tissues after experimental animals have been treated with endotoxin might be responsible for the inhibition of insulin action in that condition and that it might contribute to the development of TNF-α-induced skeletal-muscle insulin resistance in obesity-linked diabetes [22]. Moreover, both high glucose and advanced glycation end-products have been shown to modify the expression of *c*NOS in retinal vascular endothelial cells [23].

The observation of the presence of *i*NOS in diabetic platelets combined with that of the increased peroxynitrite production in vitro suggests that *i*NOS-dependent peroxynitrite production could have a major role in diabetes pathophysiology.

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