Original Paper

Nitric Oxide Production and Arachidonic Acid Metabolism in Platelet Membranes of Coronary Heart Disease Patients with and without Diabetes

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Abstract

Aim: To evaluate the levels of nitrite (NO_2^-) and nitrate (NO_3^-) ions and the incorporation of [³H]arachidonic acid (AA) into phospholipids of platelet membranes from coronary artery disease (CAD) patients with and without diabetes (NIDDM). *Subjects and Methods:* Eighteen CAD patients (group A), 18 CAD patients with NIDDM (group B), and 20 healthy controls (group C) without dyslipidemia, peripheral vascular disease and hypertension were included in the study. The groups were matched for age, sex and body mass index. The diagnosis of CAD was confirmed by coronary angiography. The nitric oxide end products (NOx), NO_2^- plus NO_3^- ions in platelet membranes, were determined using a spectrophotometric method based on the Griess reaction. The turnover of phospholipids was evaluated by incorporation of [³H]AA into platelet membrane phospholipids. *Results:* A significantly smaller amount of NOx ions was in the platelet membrane of groups A (40 ± 8 µmol/l) and B (29 ± 10 µmol/l) than C (57 ± 6 µmol/l), p < 0.001. Conversely a significantly greater amount of [³H]AA was incorporated into platelet phospholipids of group B patients (5,123 ± 1,637 dpm/mg) than groups A (3,159 ± 1,253 dpm/mg; p < 0.002) and C (1,621 ± 417 dpm/mg). An inverse correlation between [³H]AA incorporation and NOx levels was established: r = –0.76 (p < 0.05, n = 36) in CAD patients. *Conclusions:* Diabetes in CAD patients decreased the ability to produce platelet-derived NO and affects AA metabolism. This may result in higher platelet sensitivity to aggregating stimuli.

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Introduction

The process of atherogenesis relates to platelet adherence and aggregation to the luminal surface, with the subsequent release of platelet-derived growth factors and vasoactive substances. An increased platelet-vessel wall interaction plays an important role in most forms of cardiovascular disease. Platelet activation involves multiple mechanisms for achieving activation, and the process of thrombosis involves multicellular modulation of platelet activity [1]. Several mediators, including thromboxane A_2 (TXA_2), serotonin, adenosine diphosphate, platelet-activating fac-

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Table 1. Clinical characteristics of study groups

	CAD patients		Control subjects (group C)
	without diabetes (group A)	with diabetes (group B)	
n (M/F)	18 (9/9)	18 (10/8)	20 (9/11)
Age, years	51 ± 10	52 ± 9	49 ± 12
Body mass index, kg/m^2	26.8 ± 1.3	27.1 ± 1.0	26.1 ± 1.7
Smokers, n	4	3	5
Total cholesterol, mmol/l	5.3 ± 0.6	5.5 ± 0.9	5.2 ± 0.5
HDL cholesterol, mmol/l	1.3 ± 0.4	1.4 ± 0.3	1.3 ± 0.2
LDL cholesterol, mmol/l	3.3 ± 0.8	3.4 ± 0.9	3.0 ± 0.7
Triglyceride, mmol/l	1.2 ± 0.9	1.3 ± 0.8	1.2 ± 0.4
HbA$_{Ic}$, %	4.7 ± 1.1	6.4 ± 1.0*	3.8 ± 0.6
Arm blood pressure, mm Hg	127 ± 10/81 ± 8	132 ± 9/82 ± 7	126 ± 10/80 ± 7

M = Male; F = female. Results are expressed as number (n), or mean ± SD. * $p < 0.001$ compared to the controls.

tor, oxygen-derived free radicals, activated thrombin, and tissue factor, promote platelet aggregation and vasoconstriction. At the sites of endothelial injury, there is reduction in absolute or relative amounts of endogenous inhibitors of platelet aggregation, including nitric oxide (NO), prostacyclin, and tissue plasminogen activator. Loss of the effects of endogenous inhibitors preventing platelet aggregation and vasoconstriction promotes a prothrombotic and vasoconstrictive environment [2].

Studies have revealed that not only endothelium-derived NO but also platelet-derived NO inhibits aggregation [3, 4]. Platelet-derived NO is involved in the control of platelet aggregability via cyclic 3′,5′-guanosine monophosphate (cGMP) synthesis and activates cGMP-dependent protein kinase, leading to inhibition of aggregation agonists, including TXA$_2$ [5]. NO is also involved in regulating cyclooxygenase, where TXA$_2$ is a major product of arachidonic acid (AA) conversion [6, 7]. Therefore, NO can be a potent inhibitor of cyclooxygenase activities in platelets [8].

Cardiovascular risk factors such as diabetes, dyslipidemia and hypertension are associated with increased platelet activation and decreased antithrombotic properties of the blood vessel wall [9]. Decreased constitutive endothelium-derived NO synthase activity and subsequent impaired activation by NO of soluble guanylate cyclase occur in diabetic patients and most likely contribute to the development of cardiovascular complications [10]. We had previously reported that dyslipidemia was associated with decreased platelet-derived NO production in coronary artery disease (CAD) patients without

diabetes or hypertension [11] and that diabetes might induce platelet hyperreagibility via increased protein kinase C$_{β1}$ activity [12]. The results of the experimental investigation in glomeruli of streptozocin-diabetic rats indicated a progressive impairment of NO-dependent cGMP generation, which might be mediated in part by TXA$_2$ and activation of protein kinase C [13]. Therefore, we hypothesized that similar mechanisms might also be present in diabetic human platelets. Besides, platelet aggregation in diabetic patients increased despite good glycemic control [14].

This study was undertaken to assess changes in NO synthase activity and AA metabolism in platelet membranes of CAD patients with diabetes mellitus, but without dyslipidemia or hypertension.

Subjects and Methods

Subjects

Thirty-six patients with CAD, aged 40–70, were divided into two equal groups: group A (18 patients) had CAD alone and group B (18 patients) had CAD with type 2 diabetes mellitus (NIDDM). Twenty healthy subjects without CAD and NIDDM served as control (group C). The diagnosis of CAD was defined as exertion angina stable for at least 3 previous months. History of myocardial infarction or unstable angina was not an exclusion criterion unless an event had taken place within the previous 3 months. The diagnosis of CAD was confirmed by coronary angiography (performed on GE Medical System) and defined as 50% stenosis in at least one of the three major coronary arteries. CAD patients with any previous interventional treatment were excluded.
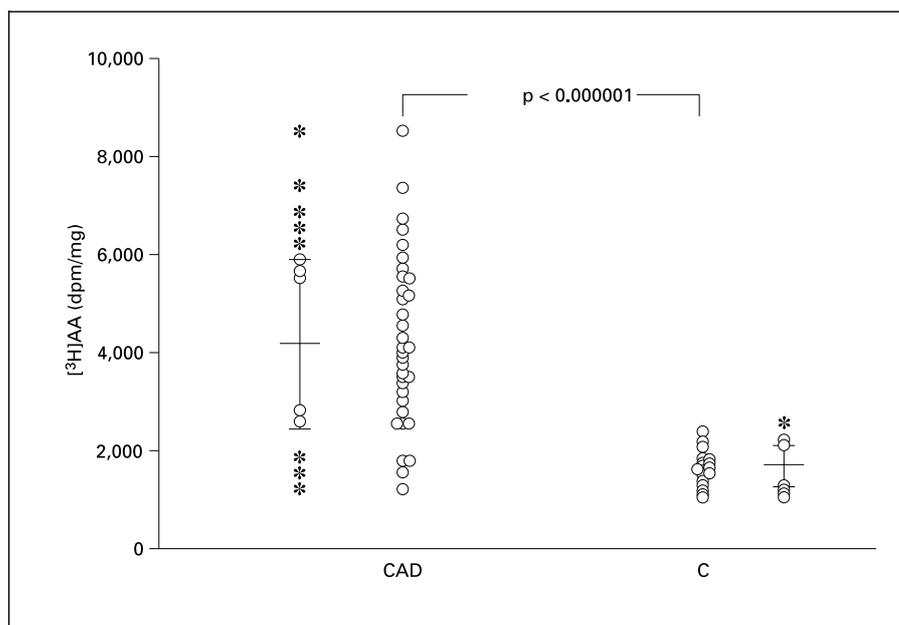
Fig. 1. Incorporation of [3H]AA into platelet membrane phospholipids of CAD patients (n = 36) and controls (C; n = 20). Values are means ± SD.

Figure axis label: [3H]AA (dpm/mg); y-axis values: 0, 2,000, 4,000, 6,000, 8,000, 10,000; x-axis groups: CAD, C; p < 0.000001

Clinically stable diabetics with good or satisfactory glycemic control (glycated hemoglobin HbA$_{Ic}$ < 7.5%) without proteinuria or other late stages of diabetic complications were included. Exclusion criteria for all subjects were dyslipidemia, any lipid lowering therapy within the previous 6 weeks, concomitant disorder affecting lipid levels, stroke, peripheral artery disease, venous insufficiency, pulmonary disease, anemia, hypertension, or malignancy. The groups were matched for age, sex, and body mass index (table 1). Informed consent was obtained from all subjects and the protocol was approved by the ethics committee.

Procedures

Twenty milliliters of venous blood was obtained from each subject after an overnight fast and abstinence from cigarette smoking for at least 12 h. The blood was centrifuged at 360 g for 10 min at 4°C (Hermle Z323H, Germany). Platelet-rich plasma was aspirated into a tube and centrifuged at 800 g for 10 min, at 4°C. Platelets were separated and purity was checked my microscopy of stained platelets. The average number of platelets in the sample was 2.2 × 10^9. Samples of platelets were lysed by sonication, in the presence of homogenization buffer (ultrasound homogenizer B, Braun Labsonic, Germany). To remove cytosolic cell extracts, samples were prepared by ultracentrifugation at 100,000 g for 50 min at 4°C. Membrane cell extracts were prepared by processing the remaining platelet mass with sonication as previously described, and by using ultracentrifugation at 100,000 g for 10 min at 4°C. The amount of protein in samples was determined by the Bradford method, which is based on a color change in response to various concentrations of proteins (Novaspec II, Pharmacia Biotech, UK). The protein concentration (mean ± SD) was 3.2 ± 0.5 mg/ml.

To assess NO synthase activity, the level of NO end products – nitrite (NO$_2^-$) plus nitrate (NO$_3^-$) – in platelet membranes was determined using a spectrophotometric method based on the Griess reaction [15, 16]. Briefly NO was oxidized to stable compounds of NO$_2^-$ and NO$_3^-$, NO$_3^-$ was converted to NO$_2^-$ and the amount of NO$_2^-$ was measured. In this way, the total amount of NO$_2^-$ plus NO$_3^-$ in the sample was measured. Freshly activated cadmium granules (2–2.5 g) were added to 1 ml of pretreated deproteinized samples. After continuous stirring for 10 min, the samples were transferred to appropriate tubes for NO$_2^-$ determination with Griess reagents [1% sulfanilamide, 5% H$_3$PO$_4$, and 0.1% N-(1-naphthyl)-ethylenediamine]. A standard curve using defined NO$_2^-$ standard samples (0–100 μM) diluted in deionized distilled water was generated. Absorbance was measured at 550 nm [17, 18].

To assess changes in AA metabolism, the incorporation of [3H]AA into platelet membrane phospholipids [19], predominantly into phosphatidylcholine [20], was evaluated. Platelets were labeled with 0.5 μCi/ml [3H]AA for 30 min at 37°C in phosphate buffer (pH 6.5). Platelet phospholipid was extracted by the method of Niwa et al. [21, 22]. Radioactivity was measured by liquid scintillation counting and expressed as disintegrations per minute per milligram platelet protein. Glycated hemoglobin HbA$_{Ic}$ was determined and other clinical and biochemical analyses, including plasma concentrations of total cholesterol, triglyceride, and high-density lipoprotein (HDL) cholesterol, were performed by means of commercial enzymatic assays. Low-density lipoproteins (LDL) cholesterol was determined indirectly according to the formula of Friedewald et al. [23].

Statistics

Data were expressed as means ± SD. Differences among groups were analyzed using the Mann-Whitney U test. Regression analysis was performed to evaluate the independent relations between parameters. A value of two-tailed $p < 0.05$ was considered statistically significant.

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Fig. 2. Incorporation of [³H]AA into platelet membrane phospholipids of CAD patients (A; n = 18), CAD patients with diabetes (B; n = 18), and controls (C; n = 20). [a] p < 0.001 (vs. C); [b] p < 0.00001 (vs. C).
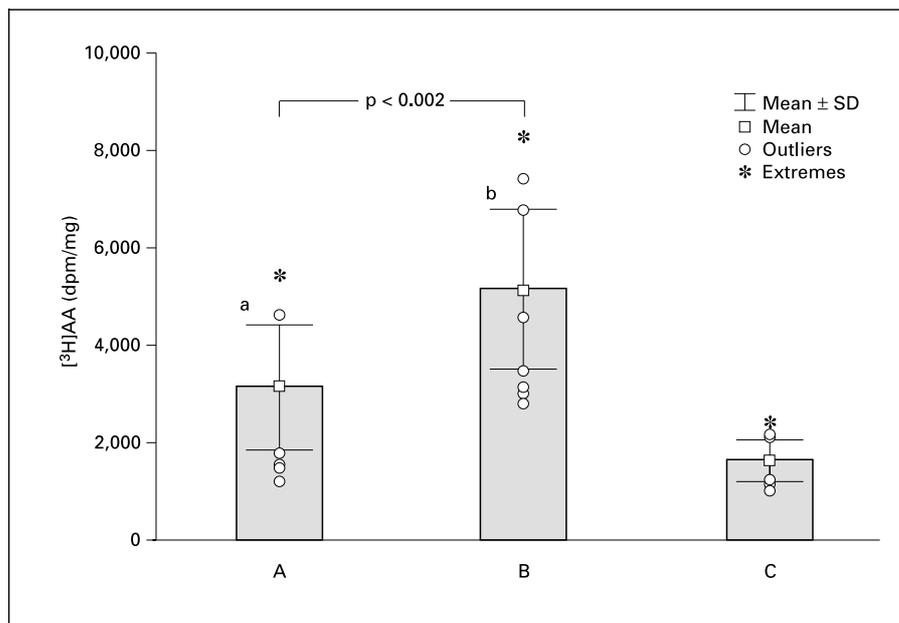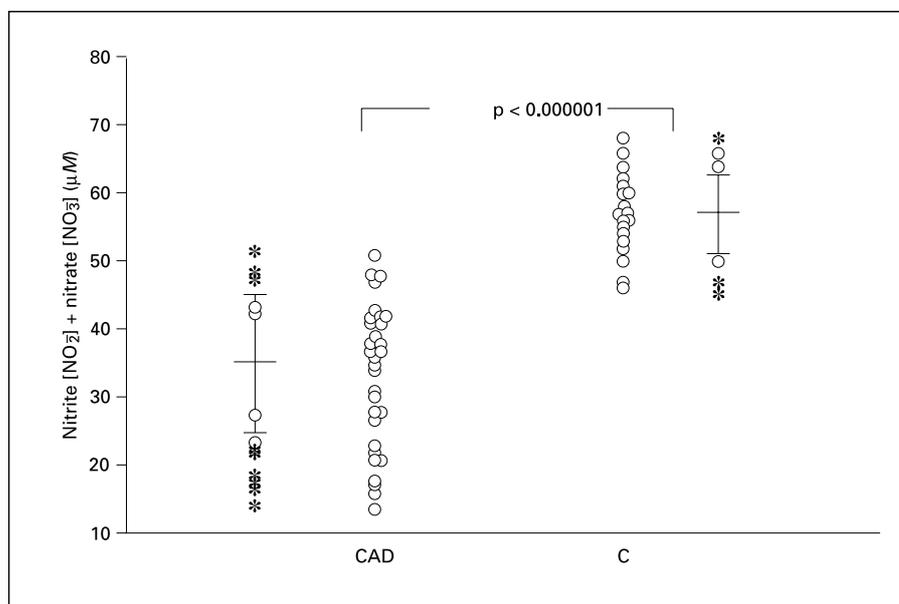

Fig. 3. NO end products (NO_2^- plus NO_3^-) in platelet membranes of CAD patients (n = 36) and controls (C; n = 20). Values are means ± SD.

Results

A significantly greater amount of [³H]AA was incorporated into platelet membrane phospholipids of all CAD patients (4,083 ± 1,737 dpm/mg) than healthy subjects (1,621 ± 417 dpm/mg, p < 0.000001) (fig. 1). A significantly greater amount of [³H]AA was incorporated in the platelet membrane phospholipids of NIDDM patients than in patients with CAD alone (5,123 ± 1,637 dpm/mg) or in normal controls (3,159 ± 1,253 dpm/mg, p < 0.002)

(fig. 2). All CAD patients had a significantly smaller amount of NO end products (35 ± 10 µmol/l) in platelet membranes than healthy subjects (57 ± 6 µmol/l, p < 0.0001) (fig. 3). The amount of NO end products in CAD patients alone (29 ± 10 µmol/l) was smaller than that of CAD patients with NIDDM (40 ± 8 µmol/l). The difference was statistically significant, p < 0.001 (fig. 4).

An inverse correlation between [³H]AA incorporation and NO end product concentration in platelet membranes was: r = –0.76 (p < 0.05, n = 36) in CAD patients (fig. 5).
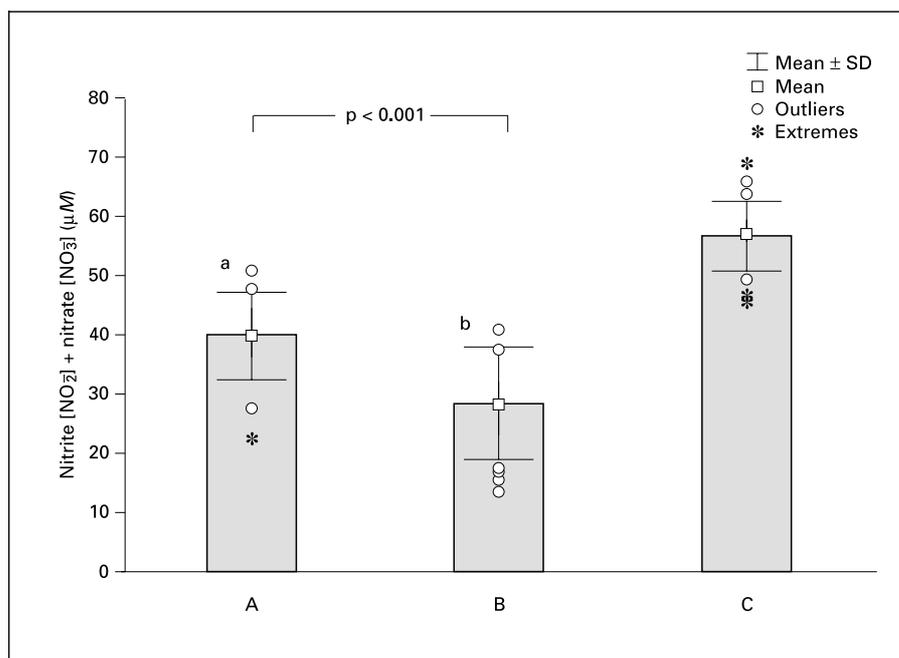
Fig. 4. NO end products (NO_2^- plus NO_3^-) in platelet membranes of CAD patients (A; n = 18), CAD patients with diabetes (B; n = 18), and controls (C; n = 20). [a] $p < 0.001$ (vs. C); [b] $p < 0.00001$ (vs. C).
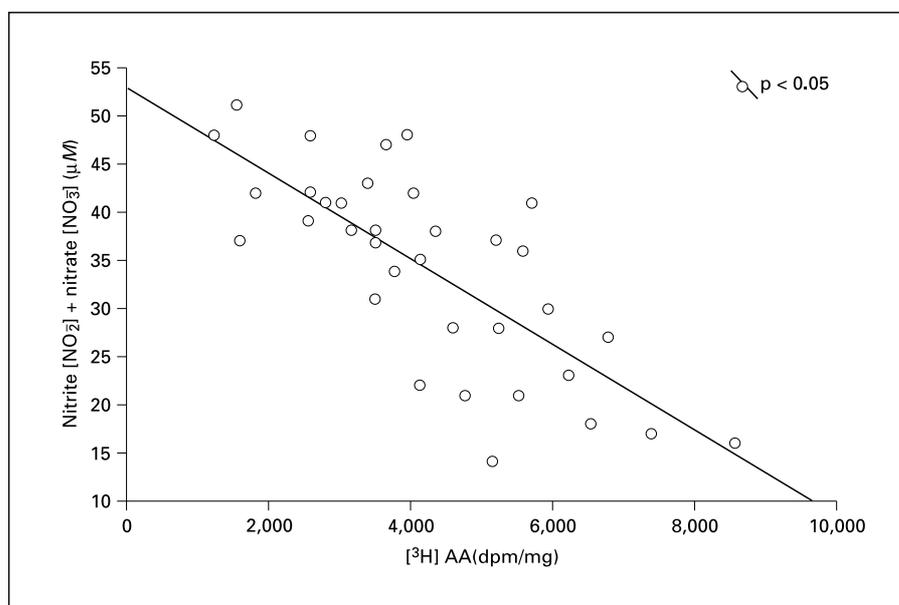

Fig. 5. Correlation between the NO end products (NO_2^- plus NO_3^-) in platelet membranes and the incorporation of [³H]AA into platelet membrane phospholipids of CAD patients: r = –0.76 ($p < 0.05$, n = 36).

Discussion

Data obtained in this study indicate that significant differences exist in AA metabolism and NO production in platelet membranes between CAD patients and healthy subjects. The data show that there is a greater amount of AA incorporation and smaller amount of NO production in platelet membranes in CAD patients with diabetes mellitus than nondiabetic CAD patients or healthy subjects by indicating an inverse relationship between AA metabolism and NO production in the platelet membranes.

Recent studies have revealed that platelet-derived NO autoregulates platelet aggregation [3, 4]. There is evidence suggesting that NO has an influence on AA metabolism. NO can be a potent inhibitor of the cyclooxygenase activities [8] and may contribute to a decrease of TXA_2 production [24]. It is known that platelet NO stimulates production of cGMP and activates cGMP-dependent protein

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kinase (G kinase), leading to inhibition of Galphaq-phospholipase C-inositol 1,4,5-triphosphate signalling and intracellular calcium mobilization for several important agonists, including TXA_2 [5].

There are other mechanisms of NO influence. NO exerts effects on thrombin receptor-activating, peptide-induced platelet aggregation and the surface expression of platelet activation markers [25]. Besides, NO-generating agents may inhibit P-selectin upregulation by lysophosphatidylcholine, an atherogenic lysophospholipid in oxidatively modified low-density lipoprotein (oxLDL) [26]. Evidence suggests that NO increases platelet sensitivity to the antiaggregating effects of substances acting via cGMP (insulin), and via cyclic adenosine-monophosphate (prostacyclin) [27, 28]. These mechanisms are impaired in atherosclerotic patients [29].

In the heart, all cell types are affected by diabetes: the myocyte, the vasculature and the blood cells. Hyperglycemia causes significant protein alterations and an oxidative stress [30]. It is well established that platelet production of TXA_2 and prostaglandin E_2 are increased in diabetic patients [31]. A positive linear relationship was found between HbA_{Ic} and TXB_2 (a more stable form of TXA_2) production. The results indicate that metabolic alterations can affect platelet function independently of vascular complications. The presence of increased TXB_2 and prostaglandin E_2 production from endogenous AA suggests that the activation of cyclooxygenase is not the only possible mechanism of platelet activation and that probably an increased availability of platelet AA plays an important role in the enhanced platelet aggregation commonly found in diabetics [31].

Platelets of diabetics and CAD patients with or without diabetes have greater sensitivity to aggregation, which might be due to the increased thromboxane synthetase system activity independently of total cholesterol or platelet phospholipid fatty acid distribution [32]. It might be possible that CAD patients with diabetes have elevated oxLDL in platelets as a result of oxidative stress induced by hyperglycemia [33]. Evidence also exists that oxLDL stimulates platelet function primarily by diminishing NO synthase expression, and this effect of oxLDL can be blocked by HDL [34]. It is known that production of TXB_2 by platelets increases in patients with hypercholesterolemia [35] and oxLDL reduces the response of soluble guanylyl cyclase to nitrovasodilators [36]. oxLDL leads to impairment of calcium transport, resulting in activation of phospholipids, and, consequently, enhances formation of AA. An increased concentration of lipid peroxides may shift the prostaglandin synthesis from prostacyclin to thromboxane, causing enhanced platelet aggregability [37].

Conclusion

CAD patients have significantly altered AA metabolism, which is associated with reduced NO production in platelet membranes. These findings are consistent with the concept that NO is involved in the regulation of antiaggregating activities in multiple ways. Our results suggest that diabetes in CAD patients decreases the ability to produce platelet-derived NO and affects AA metabolism. This may result in higher platelet sensitivity to aggregating stimuli.

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