Increased platelet purinergic sensitivity in peripheral arterial disease – A pilot study

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Abstract
Peripheral arterial disease (PAD) is associated with platelet hyperaggregability as well as an increase in morbidity and mortality from myocardial infarction (MI) and stroke. Purinergic signaling has been shown, both experimentally and clinically, to play an important role in the activation of platelets. Platelets express three different purinergic receptors: P2Y1, P2Y12 and P2X1. We assessed the hypothesis that the hyperaggregability associated with PAD is partly due to an increased P2 receptor expression at the transcriptional and/or translational level. Patients with PAD (n = 8) and controls (n = 8) were studied. Using a high-resolution channelizer, platelet shape change (PSC) was assessed by measuring the median platelet volume (MPV). The fall in free platelet count following the addition of ADP was also assessed. Real-time PCR was used to quantify the mRNA expression and Western blots to quantify the protein expression of P2 receptors in platelets. The median (and range) fall in free platelet count after adding ADP was significantly (P = 0.02) greater for patients [11% (5–24); n = 8] than for controls [0.5% (0–10); n = 8] despite using a lower concentration of ADP for the patient samples. The MPV did not differ significantly. The mRNA levels for the three P2 receptors were similar in PAD patients and controls. Western blot detected no significant differences in protein expression between these groups. Thus, platelets from PAD patients show an increased activation after stimulation with ADP (even though all patients were on aspirin). This hyperactivity was neither due to an obvious up-regulation of the mRNA levels nor to altered protein levels of P2 receptors in the platelets. It is suggested that the increased sensitivity to ADP in PAD is caused by post-receptor mechanisms.

Keywords: Peripheral arterial disease, platelets, purinergic receptors

Introduction
Platelets play an important role both in the acute process of occlusive thrombosis leading to acute ischemia and in the long-term pathophysiology leading to atherosclerosis [1–5]. Formation of asymptomatic microthrombi, release of pro-inflammatory and mitogenic substances and shear-stress induced platelet activation are all factors that play a part in atherogenesis [1–5].

Several reports have demonstrated activated platelets in patients with peripheral arterial disease (PAD) despite the use of aspirin [6–8]. PAD reflects an increased atherosclerotic burden throughout the cardiovascular system and several studies confirm that the risks for coronary arterial disease and cerebrovascular disease are increased [9,10].

Purinergic P2 receptors play an important role in the activation of platelets. There is evidence for three different P2 receptors expressed on human platelets: the Gq coupled P2Y1, the Gi coupled P2Y12 and the ionotrophic P2X1 receptors [11,12]. The clinical importance of the P2Y12 receptor has been thoroughly investigated in several studies with clopidogrel, an irreversible P2Y12 antagonist after conversion to an active metabolite in the liver [13,14]. In these studies there is clear evidence that clopidogrel, alone or as a combination therapy with aspirin, reduces the risk of MI and stroke compared to treatment with aspirin alone. In P2Y1-knockout mice and platelets treated with P2Y1 antagonists there is a clear reduction of ADP-induced aggregation and bleeding time [15–17]. Both P2Y1 and P2Y12 play a role in controlling platelet shape change
at room temperature. The platelet-rich plasma (PRP) was then carefully collected with a Pasteur pipette. Aliquots of PRP (450 μl), in siliconized glass cuvettes, were placed in a Chronolog dual channel optical aggregometer (Coulter Electronics, Luton, Beds, UK) and kept at 37 °C for 15 min prior to the start of the experiments detailed below. For RNA and protein analysis, 40 ml of blood was drawn and anti-coagulated with EDTA to a final concentration of 1.6 mg/ml. PRP was prepared by centrifugation at 160 x g for 20 min at room temperature. PRP was carefully collected without disturbing the buffy coat and was centrifuged again at 160 x g for 10 min at room temperature to get rid of contaminating leukocytes. The PRP was then transferred to a new tube and a tabletop centrifuge (at high speed) was used to produce a platelet pellet.

Measurement of ADP-induced increase in median platelet volume (MPV) and fall in free platelet count

MPV assessment. The PRP was stirred using a Teflon-coated metal stir bar, at 1000 rpm and 100-μl aliquots were removed and mixed with 400 μl of fixative (4% aqueous glutaraldehyde) before and after the addition of ADP. In controls, ADP was added at a final concentration of 0.2 or 0.4 μmol/l and the MPV measured after 30 s. In patients, ADP was added at a final concentration of 0.1 or 0.2 μmol/l and MPV measured after 30 s. These times and agonists concentrations were based on preliminary experiments and our previous publications. These concentrations are necessary to avoid more than 10% fall in the platelet count in samples used for the measurement of MPV. The PRP samples were further diluted 400-fold in Isoton II (Coulter Electronics). These samples were then assayed using a Coulter ZM counter (electrical impedance method with a 70-μm diameter sampling tube orifice) coupled to a C-256 channelizer (Coulter Electronics). The resolution of the channelizer is 0.07 fl. The analyzer sample ‘window’ was set between 2.67 and 19.12 fl. The counter was calibrated using 9-fl latex particles (Coulter Electronics). For particle analysis, data were accumulated to a maximum of 500 platelets in each of the 256 channels. The MPV is the volume of the channel on each side of which 50% of the platelet population is distributed. The increase in MPV was taken as representing the platelet shape change (PSC). MRS 2179, a P2Y1 antagonist, totally blocks PSC while AR-C69931MX, a P2Y12 antagonist, significantly inhibits PSC by acting synergistically with the P2Y1 receptor. The role of P2X1 stimulation is still not fully understood, but studies indicate that it may contribute to induce PSC or at least work in synergy with the P2Y receptors.

Materials and methods

Selection of patients and control subjects

We studied eight male patients with PAD. Their median age was 68 (range: 59–80) years. Two of them had clinically evident ischaemic heart disease and also had a coronary artery bypass graft (CABG). The ankle-brachial pressure index (ABPI) was <0.8 and also had a coronary artery bypass graft (CABG). They were on aspirin (75 mg daily). None of them had diabetes mellitus and they were all non-smokers. All the controls were non-smokers (four males and four females). Their median age was 68 (range: 59–80) years. Two of them had clinically evident ischaemic heart disease. The ankle-brachial pressure index (ABPI) was <0.8 at the time of diagnosis of PAD. All the patients were on aspirin (75 mg daily). None of them had diabetes mellitus and they were all non-smokers. All the patients were on simvastatin and had achieved a low density lipoprotein cholesterol level <2.5 mmol/l.

Blood collection

For PSC assessment venous blood was collected in 3.8% citrate (one part citrate to nine parts blood) from eight healthy volunteers and eight PAD patients. All the healthy controls denied taking any drugs for at least 2 weeks prior to sampling. The citrated blood was centrifuged at 160 x g for 15 min at room temperature. The platelet-rich plasma (PRP)
measurement because the fall in free platelet count exceeded 10%.

**RNA and protein extraction**

Total cellular RNA and protein were extracted using TRIzol reagent (Gibco BRL, Life Technology) according to the supplier’s instructions. RNA was dissolved in diethyl-pyrocarbonate (DEPC)-treated water. The RNA concentration was determined spectrophotometrically considering a ratio of optical density (OD)_{260:280} > 1.6 as pure. Samples were stored at −70°C until used. The protein pellet was finally washed with 100% ethanol, vacuum dried and dissolved in 1% sodium dodecyl sulfate solution. DC Protein Assay (BioRad Laboratories AB, USA) was used to detect the protein concentration. Protein samples were stored at −20°C until used.

**Quantitative analysis of P2 receptors by real-time reverse transcription polymerase chain reaction**

A TaqMan Reverse Transcription Reagents Kit was used to transcribe mRNA into cDNA. Real-time PCR were performed by means of a PRISM 7700 Sequence Detector as previously described [30]. Oligonucleotide primers and TaqMan probes were designed using the Primer Express software, based on sequences from the GenBank database. Primer and probe sequences have previously been reported [11]. Each primer pair was selected so that the amplicon spanned an exon junction if present, to avoid amplification of genomic DNA. Constitutively expressed rRNA and GAPDH were selected as endogenous control to correct for potential variation in RNA loading or efficiency of the amplification reaction.

Previous analysis showed that amplification efficiencies were almost identical for GAPDH, P2X1, P2Y1, P2Y12 and P2X1 antibodies followed by incubation with a secondary antibody (anti-rabbit IgG, horseradish peroxidase (HRP)-linked). The membranes were subsequently stripped with a stripping buffer and then incubated with mouse anti-human GAPDH antibody followed by incubation with a secondary antibody (anti-mouse IgG, horseradish peroxidase (HRP)-linked). Proteins were visualized by chemiluminescence using the ECL® Western blotting system and the signals were detected by autoradiography. The signal intensity (integral volume) of the appropriate bands on autoradiogram was analyzed using a scanner and the Quantity One® software. The expressions of the different P2-receptors were normalized to GAPDH to account for different loading amounts of total proteins.

**Reagents and antibodies**

Unless otherwise stated, all reagents and drugs were purchased from Sigma Chemical Corp., St. Louis, MO, USA. PCR consumables were purchased from Perkin-Elmer Applied Biosystems Inc. Some Western blot reagents were from Amersham Pharmacia Biotech, UK or Bio-Rad Laboratories, USA. Anti-P2X1, anti-P2Y1 and anti-P2Y12 antibodies were purchased from Alomone Labs, Israel.

**Results**

**Measurement of ADP-induced increase in median platelet volume (MPV) and fall in free platelet count**

**MPV assessment.** The mean (±SD) MPV for the basal (saline) value in patients (n = 8) was 5.88 ± 0.34 and for controls (n = 8) was 6.00 ± 0.49 (MPV in patients versus MPV in controls: P = NS, Mann–Whitney test).

The mean (±SD) MPV after stimulation with ADP (0.1–0.2 μmol/l) in patients (n = 4) was 6.24 ± 0.30 and for controls (n = 8) (stimulated with ADP 0.2–0.4 μmol/l) was 6.43 ± 0.49 (MPV in patients versus MPV in controls: P = NS, Mann–Whitney test).

**Free platelet count assessment.** When PRP was stimulated with ADP there was a significantly (P = 0.02, Mann–Whitney test) greater percentage decrease in free platelet count in the PAD patients when compared with the controls. Thus, the median

**SDS–PAGE and Western blotting**

SDS–PAGE and Western Blotting were performed as described elsewhere [30]. Briefly, protein electrophoresis was performed on 10% Tris–HCl polyacrylamide ready gels (BioRad) and electroblotted onto Hybond-C extra nitrocellulose membranes. Membranes were incubated with rabbit anti-human P2Y1, P2Y12 and P2X1 antibodies followed by incubation with a secondary antibody (anti-rabbit IgG, horseradish peroxidase (HRP)-linked). The membranes were subsequently stripped with a stripping buffer and then incubated with mouse anti-human GAPDH antibody followed by incubation with a secondary antibody (anti-mouse IgG, horseradish peroxidase (HRP)-linked). Proteins were visualized by chemiluminescence using the ECL® Western blotting system and the signals were detected by autoradiography. The signal intensity (integral volume) of the appropriate bands on autoradiogram was analyzed using a scanner and the Quantity One® software. The expressions of the different P2-receptors were normalized to GAPDH to account for different loading amounts of total proteins.
and range) fall in platelet count for patients \((n = 8)\) was 11% (5–24) and for controls \((n = 8)\) it was 0.5% (0–10). This increase in ADP response was in spite of the fact that the mean dose of ADP used in patients was \(0.2 \pm 0.1 \mu\text{mol/l} \) (range: 0.1–0.2 \(\mu\text{mol/l}\)) and in controls was \(0.3 \pm 0.1 \mu\text{mol/l} \) (range: 0.2–0.4 \(\mu\text{mol/l}\)) ADP \((P = \text{NS}, \text{Mann–Whitney test})\).

**mRNA quantification**

As reported earlier [11] the P2Y\(_{12}\) was the highest expressed transcript followed by P2X\(_1\) and P2Y\(_1\). To illustrate the relative expression, P2Y\(_1\) in the control group was arbitrarily chosen as a calibrator, i.e., the other receptors are expressed as a ratio of the P2Y\(_1\) in the control group. The mRNA levels for the three P2 receptors were similar in PAD patients and controls (Figure 1). The relative P2Y\(_1\) expression in PAD compared to controls all normalized to GAPDH was 102.8 \(\pm\) 24.1% (mean \(\pm\) SEM), P2Y\(_{12}\) 103.5 \(\pm\) 28.1%, P2X\(_1\) 112.5 \(\pm\) 18.2%.

**Protein quantification**

Western blot analysis detected a 55 kDa band for the P2X\(_1\) receptor, a 180 kDa band for the P2Y\(_1\) receptor and a 50 kDa band for the P2Y\(_{12}\) receptor. Samples of the signal distribution of anti-P2X\(_1\), anti-P2Y\(_1\), anti-P2Y\(_{12}\) and anti-GAPDH antibody detected in patients and controls are shown in Figure 2. Measured signal intensities for the P2 receptors normalized to the internal control GAPDH are shown in Figure 3. P2Y\(_1\) protein expression in PAD compared to controls all normalized to internal control GAPDH was 138.5 \(\pm\) 31.6% (mean \(\pm\) SEM), P2Y\(_{12}\) 86.7 \(\pm\) 18.4%, P2X\(_1\) 107.4 \(\pm\) 10.1%, \(P = \text{NS}\).

**Discussion**

In this study, the platelets from PAD patients showed a significantly enhanced ADP-induced activation (significant fall in free platelet count) despite the antiplatelet therapy (aspirin). Furthermore, the PAD platelets were stimulated with a lower concentration of ADP than those of the controls (mean \(\pm\) SD ADP concentration: 0.2 \(\pm\) 0.1 vs. 0.3 \(\pm\) 0.1 \(\mu\text{mol/l}\)). This platelet hyperactivity is in agreement with previous findings [4,6–8,31–33]. Using a lower ADP concentration for the PAD platelets was necessary to avoid a more than 10%
fall in platelet count. Such a decrease could have interfered with the measurement of the MPV. We also show that this hyperactivity was neither associated with an up-regulation of the mRNA levels nor altered P2 receptor protein levels in the PAD platelets.

Patients with PAD have ischemia in the lower limbs, resulting in pain, reduced walking distance and even gangrene that may require amputation [34]. However, mortality is usually due to the elevated risk of MI and stroke [34]. This could be explained by PAD being a marker of widespread general atherosclerosis, making vascular lesions more frequent [35,36]. However, the increased risk of thrombotic events could also be attributed to platelet activation caused by mechanisms such as increased shear stress, exposure to the subendothelial matrix and inflammatory mediators and an increased platelet turnover [4,7,8,31–33,37,38].

The importance of purinergic signaling in PAD became evident in the CAPRIE study [13]. Here the selective P2Y12 blocker clopidogrel, in comparison to aspirin, was significantly more effective at reducing the combined endpoint MI, stroke and death in patients with either PAD, previous MI or stroke. Post hoc subgroup analysis of CAPRIE revealed that the largest benefit was found in the PAD subgroup [13]. Furthermore, a loading dose of 300 mg clopidogrel significantly inhibited platelet aggregation after only 2h in patients with PAD who were not taking any other antiplatelet drugs [39]. Another study involving patients with PAD showed better inhibition with clopidogrel than with aspirin [40]. However, the inhibition improved further when both drugs were used in combination [40].

Transcriptional analysis of platelets was not previously possible, due to the low levels of RNA in these anucleated cells. However, we recently described a protocol for mRNA quantification in platelets [33]. Here we present the first comparison of platelet mRNA levels between patient groups.

We examined all the three P2 (ADP/ATP) receptors expressed in platelets, both at the mRNA and protein level in PAD patients and controls. Even though ADP-stimulated platelet aggregation was significantly increased in the PAD group, we did not find any significant difference in receptor expression. However, a relative small number of patients and a fairly wide scatter of the results are limitations in this study. Nevertheless, we would have expected a marked difference in receptor mRNA and/or protein to account for the ADP-induced platelet activation observed in the PAD group. Therefore, the increased sensitivity to ADP that we observed could be attributed to changes at the post-receptor level. The second messenger systems (e.g., cAMP and IP3) may be up-regulated, or integrins such as GpIb or GpIIb/IIIa may have increased expression [41]. Further studies are needed to address these questions. Now that preliminary results are available power calculations can be made. Our assessment is that in order to show a 10–20% difference (at $P = 0.01$) for mRNA or P2 receptor protein levels between PAD and control platelets there will be a need to evaluate more than 150 samples. This is not a practical option. It is likely that this wide scatter reflects biological variability rather than methodology. Such variability may also be influenced by the use of drugs (e.g., aspirin or statins).

Our interpretation may explain why spontaneous platelet aggregation and aggregation induced by various agonists are all enhanced in patients with PAD [4,6–8,31–33,42–45]. Therefore, a post-receptor mechanism is a more likely explanation for the non-specific platelet activation observed in PAD than the possibility of a wide range of unrelated receptors being selectively activated/up-regulated.

In conclusion, platelets from PAD patients show significantly increased activation after stimulation with ADP, despite antiplatelet therapy (aspirin) and using a lower concentration of ADP in vitro. This hyperactivity was neither due to an up-regulation of the mRNA levels nor to significantly altered mRNA and/or protein levels of platelet P2 receptors. We suggest that the increased sensitivity to ADP in PAD is caused by post-receptor mechanisms.

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