Abnormalities in primary granule exocytosis in neutrophils from Type I diabetic patients with nephropathy

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ABSTRACT

Microalbuminuria in Type I diabetes involves a cell membrane abnormality and is associated with a large increase in cardiovascular risk. The hypothesis that the membrane abnormality alters granule exocytosis in neutrophils, which could contribute to the increased incidence of cardiovascular disease, was investigated. PMA-stimulated expression of CD11b and CD69 on neutrophils from normal controls (NC), long-term uncomplicated Type I diabetic control patients (DC) and diabetic nephropathy patients (DN) was determined by fluorescence activated cell scanning. Neutrophils from DN were faster than neutrophils from either NC or DC to exocytose primary granules with CD69 following initial expression of the adhesion molecule CD11b. However, a larger proportion of neutrophils from DN failed to withdraw CD11b from the cell membrane after 90 min incubation. The protein kinase C (PKC) inhibitor, bisindolylmaleimide (BIM), showed that a larger proportion of neutrophils from DN, compared with DC or NC, exocytosed primary granules independent of PKC. The calpain inhibitor, E64d, showed that a larger proportion of neutrophils from both groups of diabetic patients, compared with NC, exocytosed primary granules independent of calpain. Cytoskeletal disruption with cytochalasin D had an effect on CD11b and CD69 exocytosis similar to that of BIM and E64d. The pathways controlling granule exocytosis in neutrophils from diabetic patients are abnormal. A change characteristic of DN causes rapid exocytosis of primary granules, and also causes the adhesion molecule CD11b to persist on an increased proportion of neutrophils. This will make an important contribution to increased vascular damage in these patients.

INTRODUCTION

About one-third of Type I diabetic patients develop nephropathy [1,2]. This condition has a very poor prognosis, mainly due to associated cardiovascular disease [3]. Indeed, diabetic nephropathy is sometimes considered to be a microvascular disease [4]. There is strong evidence that the susceptibility to nephropathy in Type I diabetes is familial [5] and other associated familial factors are hypertension, insulin resistance and dyslipidaemia [3,6,7], which are characteristics of the so-called metabolic syndrome.

A cell membrane abnormality is believed to play a role in diabetic nephropathy and the erythrocyte cell-membrane marker, Na"+/Li⁺ countertransport, is abnormal in patients with diabetic nephropathy and their first degree relatives [8–10]. However, no membrane abnormality has been shown in diabetic nephropathy that could contribute to either the renal pathology or the associated cardiovascular disease. Evidence indicates that the protein associated with the Na"+/Li⁺ countertransport abnormality characteristic of diabetic nephropathy is tropomyosin, which has a role in stabilizing cytoskeletal actin filaments [11]. These filaments are important for many

Key words: calpain, diabetes, diabetic nephropathy, exocytosis, neutrophil, protein kinase C.
Abbreviations: NC, normal controls; DC, diabetic control patients; DN, diabetic nephropathy patients; PKC, protein kinase C; BIM, bisindolylmaleimide.
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aspects of cell physiology, but their role in the expression of integrins in neutrophils [12] and the intracellular signalling from those integrins [13] is of particular interest for the present study.

Neutrophils are an important contributor to vascular damage [14] and attach at sites by interaction with their cell surface β2-integrin, Mac-1 [15], which is a dimer of CD11b and CD18 exocytosed from secondary granules [16]. This is followed by exocytosis of primary granules containing pro-inflammatory enzymes with the appearance on the cell surface of markers such as CD69 [17]. Several observations show that inappropriate activation of this process causes vascular damage especially in the myocardial circulation [18]. It has been suggested that evidence for increased CD11b on leucocytes from patients with ischaemic heart disease indicates a smouldering inflammatory process in the vasculature [19]. In addition, some beneficial effects of drugs may be due to their effects on CD11b. Thus, the glycoprotein 11b/IIIa receptor antagonist, abiciximab, has been suggested to have benefit due to its blockade of CD11b [20], and 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins) also reduce CD11b expression [21].

We have now tested the hypothesis that patients with diabetic nephropathy have abnormal intracellular trafficking and membrane fusion of intracellular vesicles, and that one effect is the abnormal expression of integrins on neutrophils and their exocytosis of primary granules containing inflammatory enzymes.

**METHODS**

**Patients**

Three groups of patients were studied: 11 patients with Type I diabetes and evidence of diabetic nephropathy (DN) for which the criterion was diabetes for less than 20 years with microalbuminuria of greater than 20 μg/min, but serum creatinine less than 150 μmol/l, and serum albumin greater than 35 g/l and with retinopathy; a control group of 11 patients with Type I diabetes (DC) defined as diabetes for more than 25 years without microalbuminuria and with serum creatinine less than 100 μmol/l; 12 normal controls (NC) with no history of diabetes, hypertension or renal impairment. The characteristics of each group are shown in Table 1.

Of the 11 DN, nine had received laser treatment for retinopathy and seven were taking an angiotensin-converting-enzyme inhibitor. One of the seven was also taking a calcium channel antagonist and another a diuretic. Five patients in this group were taking lipid-lowering medication. All DN had abnormal Na+/Li+ countertransport. One patient in the DC group had received laser treatment for retinopathy. One was taking a beta-blocker and lipid-lowering medication, and one other was taking a calcium channel antagonist. None of the NC was taking regular medication. NC and DC subjects had normal Na+/Li+ countertransport.

All subjects gave informed consent, and the Joint Ethics Committee of the Newcastle Health Authority and Newcastle University approved the study protocol.

**Microalbuminuria**

Each patient performed three successive overnight timed urine collections. Urine albumin was measured by radioimmunoassay [23]. Urinary albumin excretion rate was expressed in μg per min (normal < 20 μg/min).

**Neutrophil antigen expression**

Venous blood was collected into a heparinized glass tube and used within 2 h. Equal volumes of blood and PBS were mixed in a glass tube and incubated for up to 90 min. PMA was added in the PBS to give a final concentration of 10 or 100 ng/ml (16 or 162 nmol/l) in the incubation mixtures. At various times 50 μl aliquots were placed on ice with anti-human CD45 (common leucocyte antigen) antibody conjugated to FITC together with either anti-human CD11b or anti-human CD69 labelled with R. Phycoerythrin.

Appropriate isotype IgG controls were used to measure non-specific antibody binding. Red blood cells were specifically lysed and fixed using a proprietary formaldehyde containing solution (Erythrolyme; Serotec, Abingdon, U.K.). White blood cells were washed twice with PBS and analysed on a flow cytometer (FACScan; Becton Dickenson, Mountainverie, CA, U.S.A.).

The inhibitors bisindolylmaleimide GF 109203X (BIM) and E64d were dissolved in DMSO and further diluted 1 in 50 in PBS. When mixed with blood and PMA, the BIM concentration was 10 μmol/l (500 times the in vitro IC50 of isolated cells) [24] and E64d 100 μg/ml (292 μmol/l) [25]. The final concentration of DMSO was 1 in 400 (35 mmol/l), which in PBS alone or with PMA had no effect on the expression of the surface antigens. Cytochalasin D was also dissolved in DMSO and used at a final concentration of 10 μmol/l.

FACS data were analysed using WinMDI software (Scripps Institute, La Jolla, CA, U.S.A.). Neutrophils were identified on the basis of their characteristic light scattering properties and expression of CD45. The results were expressed as the percentage of neutrophils positive for CD69 or CD11b and the median fluorescence of the population of positive cells. The median fluorescence was converted into number of sites (molecules of equivalent fluorochrome), using calibration beads (FluoSpheres; Dako A/S, Glostrup, Denmark), and the non-specific antibody binding was subtracted.
Table 1  Clinical characteristics
Values are means ± S.E.M. or median (range).

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>DC</th>
<th>DN</th>
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<tbody>
<tr>
<td>n</td>
<td>12</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>6/6</td>
<td>6/5</td>
<td>5/6</td>
</tr>
<tr>
<td>Age (years)</td>
<td>47.7 ± 4.4</td>
<td>44.7 ± 4.7</td>
<td>36.2 ± 3.4</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23.8 ± 0.8</td>
<td>25.1 ± 0.8</td>
<td>30.5 ± 1.6</td>
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<tr>
<td>Mean blood pressure (mmHg)</td>
<td>84.4 ± 3.6</td>
<td>87.1 ± 3.4</td>
<td>99.9 ± 3.5</td>
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<td>Urinary albumin excretion rate (µg/min)</td>
<td>4.4 (1.0–7.8)</td>
<td>4.6 (2.5–9.6)</td>
<td>217 (27–619)</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>81.6 ± 2.8</td>
<td>86.5 ± 4.2</td>
<td>88.5 ± 6.0</td>
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Statistics
Normally distributed data are presented as means ± S.E.M. and the probability of differences assessed using the unpaired two-tailed Student’s t test. Non-parametric data are expressed as medians with interquartile ranges, and the Mann–Whitney U test used to calculate the probability of differences.

RESULTS

Abnormal CD11b down-regulation and CD69 expression in DN neutrophils
The exocytosis of CD11b was similarly rapid with both 10 and 100 ng/ml PMA in neutrophils from all three groups of subjects. Subsequently over a longer time course there was down-regulation of CD11b in a proportion of neutrophils closely allied to the exocytosis of CD69. Therefore, the neutrophils that expressed CD69 can be taken as those that had down-regulated CD11b [17] (Figure 1). There were two abnormalities in the neutrophils from DN patients. Firstly, a much smaller proportion of neutrophils from DN down-regulated CD11b, with exocytosis of primary granules containing CD69 after a 90 min incubation with 100 ng/ml PMA, compared with neutrophils from NC or DC (44.3 ± 4.2 versus 62.9 ± 4.4 or 59.7 ± 2.8%, P < 0.006; Figure 2).
Secondly, the proportion of neutrophils that down-regulated CD11b and exocytosed CD69 during the 90 min incubation did so faster in DN than in either of the other groups [half time (t½): DN, 5.5 ± 1.7 min; DC, 11.1 ± 1.7 min; NC, 17.5 ± 2.7 min; P = 0.024 and P < 0.001 for DN versus DC and NC respectively; Table 2 and Figure 2].

The abnormality in DN neutrophils, most clearly revealed by stimulation with 10 ng/ml PMA, was the rapid exocytosis of primary granules with CD69 (Figure 3). Since the response to the lower dose of PMA was slower, longer incubation was needed to show the lower total level of response in DN neutrophils. However, it can be seen that whereas the maximum level of CD11b

![Figure 1](image_url)
Figure 2  CD11b down-regulation in neutrophils stimulated with 100 ng/ml PMA
The rate of appearance of neutrophils that had down-regulated CD11b and expressed CD69 on stimulation with 100 ng/ml PMA and the effect of BIM and E64d in NC (○), DC (●) and DN (■). ††, P < 0.02 and †††, P < 0.001 versus NC; *, P < 0.006 and ***, P < 0.001 versus NC and DC.

Figure 3  CD11b down-regulation in neutrophils stimulated with 10 ng/ml PMA
The rate of appearance of neutrophils that had down-regulated CD11b and expressed CD69 on stimulation with 10 ng/ml PMA and the effect of BIM and E64d in NC (○), DC (●) and DN (■). †, P < 0.01 and ‡, P < 0.02 versus NC.

down-regulation and CD69 expression was achieved by 30 min in DN neutrophils, NC neutrophils continued to respond in a linear fashion for up to 90 min (Figure 3). The more rapid response of DN neutrophils was also indicated by their expression of CD11b sites which was maximal by 30 min whereas in NC neutrophils it increased between 30 and 90 min (Table 3).

PKC inhibition impairs CD11b down-regulation but DN neutrophils remain abnormal
The PKC inhibitor BIM greatly reduced the proportion of neutrophils that could down-regulate CD11b from their membrane with expression of CD69. However, BIM was much less effective in DN neutrophils and caused only a 64% inhibition of the CD11b down-regulation/CD69 exocytosis response to 100 ng/ml PMA in neutrophils from DN, compared with 96% and 93% inhibition in neutrophils from NC or DC respectively (Figure 2). BIM slowed the expression of CD11b sites, which were lower after 30 min incubation with PMA, but had little effect on the maximum levels after 90 min incubation. The slowing effect of BIM on CD11b expression was less in the neutrophils from diabetic patients and was most apparent with the lower dose of PMA (Table 3).

Calpain inhibition impairs CD11b down-regulation similarly with DN and DC neutrophils
The calpain inhibitor E64d also reduced the proportion of neutrophils that down-regulated CD11b and ex-

Table 2  Percentage of cells that had progressed to release primary granules, indicated by CD69 expression, at various times after stimulation with 100 ng/ml PMA
Values are means ± S.E.M. *, P < 0.006 and **, P < 0.001 versus NC; †, P < 0.006 and ††, P < 0.02 versus NC.

<table>
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<th>Incubation time (min)</th>
<th>NC</th>
<th>DC</th>
<th>DN</th>
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<tbody>
<tr>
<td>5</td>
<td>10.5 ± 2.1</td>
<td>22.8 ± 1.4**</td>
<td>20.4 ± 1.3**</td>
</tr>
<tr>
<td>15</td>
<td>30.1 ± 3.6</td>
<td>31.8 ± 1.7</td>
<td>28.5 ± 2.1</td>
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<tr>
<td>30</td>
<td>44.0 ± 2.4</td>
<td>44.3 ± 2.2</td>
<td>31.1 ± 2.1††</td>
</tr>
<tr>
<td>90</td>
<td>62.9 ± 4.4</td>
<td>59.7 ± 2.8</td>
<td>44.3 ± 4.2†</td>
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pressed CD69. In the presence of E64d the proportion of neutrophils that down-regulated CD11b on stimulation with 100 ng/ml PMA was the same in DC and DN, so that the difference between these groups was abolished (Figure 2). However, they were both significantly different to NC. Unlike with BIM, in the presence of E64d 100 ng/ml PMA was more effective than 10 ng/ml in causing neutrophils to down-regulate CD11b and express CD69. The effect of E64d on CD11b sites expressed was similar to the effect of BIM (Table 3).

### Actin filament disruption inhibits CD11b down-regulation

In the presence of cytochalasin D, 100 ng/ml PMA caused all neutrophils from NC to exocytose CD11b but at a slightly lower level than in untreated cells (30.2 ± 7.4 × 10^3 versus 42.8 ± 1.6 × 10^3 sites/cell). However, the ability of these neutrophils to convert to the exocytosis of primary granules with the expression of CD69 was greatly impaired by cytochalasin D (9 ± 3 versus 38 ± 4 %, *P = 0.004).

### DISCUSSION

Two abnormalities are apparent on PMA stimulation of neutrophils from DN. These are: (1) retention of CD11b on a greater proportion of neutrophils, and (2) rapid exocytosis of CD69 in those neutrophils that were stimulated to express this marker.

The retention of CD11b was clearly associated with nephropathy rather than diabetes since in NC and DC, after stimulation for 90 min with 100 ng/ml PMA, only 37% and 40% of neutrophils respectively still expressed CD11b, whereas in DN 56% of neutrophils were CD11b positive. CD11b is a component of the β2-integrin adhesion molecule Mac-1 and its expression on neutrophils closely relates to neutrophil adhesion [22]. This could lead in DN to more neutrophil adherence at sites of activation and provide a focus for platelet aggregation [26]. Blockade of Mac-1 in a model of lung injury, even after injury is initiated, decreases neutrophil accumulation and inflammatory damage [27].

The rapid exocytosis of primary granules containing CD69, in a proportion of DN neutrophils, suggests an abnormality in intracellular trafficking, in addition to the threshold effect that enables only a proportion of neutrophils to respond. DC neutrophils also exocytosed primary granules more rapidly than the NC neutrophils. However, in contrast to DN, in DC the same proportion of neutrophils down-regulated CD11b as in NC. Therefore the accumulation of neutrophils by adhesion will have similar characteristics in DC and NC and this may be an important characteristic for vascular damage.

The possible involvement of a PKC-independent pathway and the PKC modifying protease calpain in the abnormal neutrophil function in DN was examined using the PKC inhibitor BIM and the calpain inhibitor E64d, which has been widely used to establish a role for calpain [28]. With both the low and high doses of PMA, BIM at a concentration that blocks all known isoforms of PKC [29] and E64d [30], both slowed, but only slightly reduced, the appearance of CD11b sites, suggesting that PKC does not play a major role in exocytosis of CD11b. However, both BIM and E64d had a much greater inhibitory effect on the down-regulation of CD11b and expression of CD69.

Although DN neutrophils have an impaired ability to down-regulate CD11b and express CD69, they are abnormally resistant to inhibition of PKC with BIM and inhibition of calpain with E64d. BIM virtually abolished the down-regulation of CD11b and expression of CD69 in NC neutrophils. It is unlikely that BIM inhibits PKC less effectively in DN than NC neutrophils, which suggests that in DN neutrophils CD11b down-regulation and CD69 expression have greater independence from

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**Table 3** Level of expression in neutrophils retaining the CD11b component of the adherent integrin Mac-1 after mild stimulation by incubation in PBS with either 10 ng/ml or 100 ng/ml PMA, in the absence or presence of BIM or E64d

Values are means ± S.E.M. *, *P < 0.005 and **, *P < 0.02 versus NC; †, †P < 0.001, ††, ††P < 0.01 and †††, †††, *P < 0.05 versus 30 min incubation.

<table>
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<th>Incubation time (min)</th>
<th>100 ng/ml PMA</th>
<th>100 ng/ml PMA</th>
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<td></td>
<td>NC</td>
<td>DC</td>
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<tr>
<td>PMA</td>
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<tr>
<td>30</td>
<td>26.0 ± 2.9</td>
<td>33.0 ± 3.6</td>
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<tr>
<td>90</td>
<td>36.3 ± 3.8††</td>
<td>24.4 ± 4.4</td>
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<tr>
<td>PMA + BIM</td>
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<tr>
<td>30</td>
<td>4.8 ± 1.1</td>
<td>15.8 ± 3.2*</td>
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<tr>
<td>90</td>
<td>32.7 ± 1.7†</td>
<td>29.4 ± 3.0†</td>
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<tr>
<td>PMA + E64d</td>
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<td>30</td>
<td>9.3 ± 1.6</td>
<td>18.6 ± 3.3**</td>
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<tr>
<td>90</td>
<td>18.8 ± 2.0†</td>
<td>23.0 ± 3.3†+</td>
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PKC activation. This suggests either an alternative pathway in DN that is much more active than in NC, or that the target protein for PKC has a higher level of phosphorylation in the resting-state in DN neutrophils. One action of PKC is the inhibition of protein tyrosine phosphatase activity [31,32], and the total blockade of PKC by BIM may reveal an underlying difference in protein phosphorylation in DN neutrophils. There is no evidence that PMA stimulates processes that are not dependent on PKC activation. However, we have recently shown that the PMA doses used in the present experiments can activate calpain in neutrophils [33], and PKC action may depend on calpain activity [28]. Thus the relative effects of calpain inhibition with E64d in NC and DN neutrophils are similar to those of BIM. However, in DC neutrophils the effects are markedly different; with BIM they are similar to NC neutrophils and with E64d they are the same as DN neutrophils. This indicates that a PKC-independent calpain effect is the key difference between DN and DC neutrophils.

The most likely target for calpain is cytoskeletal proteins that are known to be modified by calpain [30]. In support of this hypothesis, disruption of cytoskeletal remodelling by cytochalasin D had an effect very similar to E64d on the expression of CD11b and CD69. It seems possible that both PKC and calpain are involved in the cytoskeletal remodelling that is required for CD11b down-regulation and primary granule exocytosis, and that the normal mechanism for this remodelling process is impaired in DN. The resulting increased retention of adhesion molecules on neutrophil, if occurring in vivo, is likely to contribute to increased vascular damage. In this case, an understanding of the altered neutrophil function may lead to new targets for treatment to alleviate the high levels of cardiovascular disease in DN. In fact, it seems possible that the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor class of drugs may give some benefit by down-regulation and primary granule exocytosis, and that the normal mechanism for this remodelling process is impaired in DN. The resulting increased retention of adhesion molecules on neutrophil, if occurring in vivo, is likely to contribute to increased vascular damage. In this case, an understanding of the altered neutrophil function may lead to new targets for treatment to alleviate the high levels of cardiovascular disease in DN. In fact, it seems possible that the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor class of drugs may give some benefit by reducing CD11b expression [21].

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