The Effect of Dimethylbiguanide on Thrombin Activity, FXIII Activation, Fibrin Polymerization, and Fibrin Clot Formation

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The antihyperglycemic drug dimethylbiguanide (DMB, also known as metformin) reduces the risk of cardiovascular complications in type 2 diabetes, although the mechanism(s) involved are unclear. DMB reduces glycosylation-related protein cross-linking, a process similar to fibrin cross-linking catalyzed by activated factor XIII (FXIII). To investigate whether the cardioprotective effect of DMB could be related to effects on clot stabilization, we studied the effects of DMB on FXIII, thrombin activity, and cleavage of fibrin(ogen). Activity of purified and plasma FXIII was inhibited by DMB. Analysis by mass spectrometry and FXIII-coupled magnetic particles excluded binding of DMB to FXIII. Thrombin-induced cleavage of the activation peptide from FXIII was inhibited in a dose-dependent manner, as was fibrinopeptide cleavage from fibrinogen. Ancrod-induced cleavage of fibrinopeptide A was not affected. DMB prolonged clotting time of normal plasma. Fiber thickness and pore size of fibrin clots, measured by permeation experiments and visualized by scanning electron microscopy, decreased significantly with DMB. No interactions between DMB and the active site of thrombin were found. Turbidity experiments demonstrated that DMB changed polymerization and lateral aggregation of protofibrils. These results suggest that DMB interferes with FXIII activation and fibrin polymerization, but not only by binding to thrombin on a different location than the active site. In patients on DMB therapy, FXIII antigen and activity levels in vivo were reduced over a 12-week period. These findings indicate that part of the cardioprotective effect of DMB in patients with type 2 diabetes may be attributed to alterations in fibrin structure/function. Diabetes 51:189–197, 2002

The U.K. Prospective Diabetes Study (UKPDS) reported that the orally administered antihyperglycemic drug dimethylbiguanide (DMB, also known as metformin) reduces the risk of cardiovascular complications associated with type 2 diabetes (1). DMB has a variety of effects on cardiovascular risk markers, including antihyperglycemia, insulin-sparing effects, triglyceride-lowering effects, and a reduction in a number of coagulation proteins. Although it is unclear how these metabolic alterations affect cardiovascular risk, any reduction in myocardial infarction (MI) is likely to be related to increased plaque stability and/or decreased clot formation because these are the major phenotypes associated with acute MI.

Subjects with MI are reported to exhibit differences in the structure of fibrin clots compared with control subjects (2). Fibrin clots are formed after thrombin-induced cleavage of fibrinopeptides A and B from fibrinogen, which results in the spontaneous polymerization of fibrin monomers and the formation of two-stranded protofibrils, which aggregate laterally to form thicker fibrils. These associate with each other and branch out, creating a gel (3), which is further stabilized by factor XIII (FXIII)-mediated cross-links between glutamine and lysine residues on the α- and γ-chains of fibrin. Plasma FXIII is a transglutaminase consisting of two A- and two B-subunits. Thrombin activates FXIII by cleaving the peptide bond between Arg37 and Gly38 on the A-subunit, which results in the release of an activation peptide. The A-subunit dimer dissociates from the B-subunits in the presence of calcium. Both steps are necessary to reveal the active site of the enzyme (4). FXIII cross-links fibrin and incorporates other proteins, such as α2-antiplasmin (5), von Willebrand factor (6), thrombospondin (7), and fibronectin (8,9), into the clot, increasing its mechanical strength and resistance to fibrinolysis. To determine whether the cardioprotective effect of DMB could be caused by interactions with the processes involved in clot formation and stability, we studied the FXIII activity, fibrin formation, and clot structure in vitro and the FXIII activity and antigen levels in vivo in response to DMB.

RESEARCH DESIGN AND METHODS

Purification of FXIII. Human FXIII was purified from 2.1 l outdated platelet-poor plasma by ammonium sulfate precipitation and gel filtration, as described earlier (10).
purification of fibrinogen. Fibrinogen was isolated from 100 ml platelet-poor plasma (obtained from volunteers after informed consent) by precipitating twice with 25% ammonium sulfate, pH 7.4, and a final precipitation step of 7% ethanol at 4°C. The resulting pellet was resuspended in 50 mmol/l Tris and 150 mmol/l NaCl, pH 7.4, dialyzed against the same buffer overnight at 4°C, and passed through a lysine-Sepharose and a gelatin-agarose column connected in tandem, as described by Matsuda et al. (11), in 30 mmol/l Tris, 100 mmol/l NaCl, 5 mmol/l benzamidine, and 2 mmol/l EDTA, pH 7.4. After this double-affinity chromatography step, fibrinogen free of plasminogen-sensitive fibrinectin was concentrated with ammonium sulfate, pH 7.4. The pellet was resuspended in 50 mmol/l Tris and 150 mmol/l NaCl and dialyzed overnight against the same buffer. Concentration of fibrinogen was measured by absorbance at 280 nm using an extinction coefficient of 1.61. The purity of the preparations was tested with SDS-PAGE (8%, bis-to-acrylamide ratio of 1:37.5). To regenerate the affinity columns, fibrinectin was eluted from the gelatin-agarose column with 8 mol/l urea, 0.05 mol/l Tris, pH 7.4, and plasminogen from the lysine-Sepharose column with 0.02 mol/l e-aminoacyclopentane acid, 0.15 mol/l NaCl, and 0.05 mol/l Tris, pH 7.4.

Biotin-pentylamine incorporation assay. FXIII cross-linking activity was determined using a sensitive functional assay, as previously described (12). The assay was based on the incorporation of 5-(biotinamido)pentylamine (Pierce, Rockford, IL) by FXIII into microtiter plates coated with fibrinogen. The amount of cross-linked 5-(biotinamido)pentylamine was detected by measuring phosphatase activity after incubation with a streptavidin-alkaline phosphatase conjugate (Sigma, Chevolal, St. Louis, MO). The intra- and interassay coefficients of variation were <10%.

Berichrom FXIII activity assay. FXIII activity was also measured with a commercial FXIII activity assay kit (Berichrom FXIII; Behring Diagnostics, Marburg, Germany). The assay principles are different from the biotin-pentylamine incorporation assay. FXIII A-subunit links a specific peptide substrate with glycine ethyl ester, thereby releasing ammonia. The ammonia released is determined in a parallel enzymatic reaction. The variable measured is the decrease in NADH, which is detected by monitoring the absorbance at 340 nm.

Effect of DMB on FXIII activity. The effect of DMB on both purified and pooled normal plasma FXIII activity was determined with the biotin-pentylamine incorporation assay and the Berichrom assay. For both assays, a range of concentrations between 0 and 0.25 mol/l DMB (hydrochloride salt; Sigma) was prepared. In the incorporation assay, DMB was added to the plasma dilution buffer. In the Berichrom assay, DMB was added to the reaction mixture. Included were controls that contained the Tris-buffered saline (TBS)/reaction mixture without plasma, the TBS/reaction mixture with human α-thrombin in pyridine buffer was coupled to the magnetic particles in an overnight step. Binding efficiency was determined by comparing the precoupling FXIII solution to the postcoupling supernatant by bicinchoninic acid assay (Sigma). After five washes in pyridine buffer, DMB (at a concentration of 0.0041 mol/l) was added to 5 mmol/l ammonium acetate pH 7.4. DMB was mixed with 0.0041 mol/l of activated or zymogen FXIII at various concentrations (0, 0.05, 0.5, 5, and 50 mmol/l), and the mixtures were analyzed on a single-quadrapole bench-top mass spectrometer (Platform II, Micromass UK, Cheshire, U.K.), as previously described (13).

Investigation of binding of DMB to FXIII by magnetic separation. Aminoterminated BioMag particles (Warrington, PA) were washed three times with 0.01 mol/l pyridine buffer, pH 6, and incubated while rotating at room temperature in pyridine buffer containing 5% glutaraldehyde. Next, 200 μg purified FXIII in pyridine buffer was coupled to the magnetic particles in an overnight step. Binding efficiency was determined by comparing the precoupling FXIII solution to the postcoupling supernatant by bicinchoninic acid assay (Sigma). After five washes in pyridine buffer, DMB (at a concentration of 0.05 mmol/l) in phosphate-buffered saline (PBS), pH 7.4) was added and incubated for 60 min at room temperature. After magnetic separation of the particles, DMB in the supernatant was measured at 234 nm, and the reading was compared with the absorbency of the original solution of 0.05 mmol/l DMB in PBS, pH 7.4.

FXIII activation peptide release. Various concentrations of DMB (125, 62.5, 31.25, 15.6, 1.56, and 0 mmol/l DMB) in 9.47 mmol/l sodium phosphate, 137 mmol/l NaCl, 2.5 mmol/l KCl, and 0.1% (wt/vol) polyethylene glycol, pH 7.4, were added to 1.6 μmol/l purified FXIII, which had been dialedyzed against the same buffer. Human α-thrombin (final concentration 0.1 units/ml) was added, and the samples were incubated at 37°C for 60 min. The reaction was stopped by the addition of 1:10 (vol/vol) of 3 mol/l HClO 4. HPLC was performed using the same method as that used for FXIII activation peptide release (described above).

For each sample, the molar quantity of activation peptide was determined by integrating the area under the curve of the peak on the chromatogram and comparing it with those obtained with the peptide loaded at a known concentration.

Thrombin-induced fibrinopeptides A and B release. Fibrinogen at 1 mg/l was incubated with various concentrations of DMB (125, 62.5, 31.25, 15.6, and 1.56 and 0 mmol/l DMB) and human α-thrombin (final concentration 0.1 unit/ml) at 37°C. The reaction was stopped after 1.5 and 20 min by the addition of 1:10 (vol/vol) of 3 mol/l HClO 4. After centrifugation, the release of fibrinopeptide A was analyzed by reverse-phase HPLC, as described above.

Thrombin-cross-linking inhibition of normal plasma. For this analysis, 100 μl of different concentrations of DMB in barbiturate-buffered saline (0.028 mol/l sodium diethyl barbiturate, 0.05 mol/l HCl, and 0.125 mol/l NaCl) were added to 100 μl pooled normal plasma. To induce clotting of the plasma, 100 μl bovine thrombin (Sigma) at a concentration of 10 units/ml were added, and the time required for the formation of a clot was measured with an Amelung cosagometer (Amelung, Lenningen, Germany). DMB was used at final concentrations of 0, 0.65, 1.2, 2, 5.2, 10.4, 20.8, 41.6, and 83.2 μmol/l.

Analysis of the effect of DMB on the active site of thrombin. The thrombin-specific chromogenic substrate H-L-Phe-Pip-Arg-pNA (Chromogenix Instrumentation Laboratory, Milan, Italy) was used to determine the effect of DMB on thrombin. For the analysis, 20 μl chromogenic substrate (final concentration 1 mmol/l) was added to 100 μl of 250 mmol/l DMB in 50 mmol/l Tris, 100 mmol/l NaCl, and 0.1% (wt/vol) bovine serum albumin. After the addition of 100 μl human α-thrombin at a concentration of 0.5 units/ml, the reaction was stopped at 0, 1, 2, 4, 8, and 16 min with 50 μl 50% (vol/vol) acetic acid (whereby at 0 min, acetic acid was added before thrombin). The thrombin-induced color change on hydrolysis of the substrate was measured at a wavelength of 405 nm. The experiment was repeated without DMB and, to check the specificity of the substrate, with hirudin because hirudin acts as a direct inhibitor of the active site of thrombin. For this, 100 μl of hirudin, at a concentration of 1 unit/ml, in 50 mmol/l Tris, 100 mmol/l NaCl, and 0.1% (wt/vol) bovine serum albumin was used.

Cross-linking of α- and γ-chains by FXIII. SDS-PAGE was performed using a Mini-protein 3 (Biorad, Hercules, CA) electrophoresis unit. Gels were cast at a polyacrylamide concentration of 7% (bis-to-acrylamide ratio of 1:37.5) in 1.5 mmol/l Tris-HCl, pH 8.9, with a stacking gel of 4% in 0.5 mol/l Tris-Cl, pH 6.8, and a reducing loading buffer (100 mmol/l Tris, 100 mmol/l dithiothreitol, 0.05% (wt/vol) bovine serum albumin). The gel was loaded with 100 μg of fibrinogen and 125, 62.5, 31.25, 15.6, 1.56, and 0 mmol/l DMB, which was used to establish the Darcy permeability of plasma clots. Permeation properties of plasma clots. Permeation properties of plasma clots formed in the presence of DMB were investigated as previously described (10). Human α-thrombin (final concentration 1.18 units/ml) and calcium chloride (final concentration 10 mmol/l) in 100 mmol/l NaCl, 50 mmol/l Tris, pH 7.4, and DMB (final concentrations of 0, 0.5, 1, 5, 10, 20, 40, and 80 mmol/l) were added to pooled normal plasma. The mixture was filtered in specially prepared preetched plastic tubes, which were sealed on one side with parafilm. The clots were incubated in a moist chamber for 2 h. After removing the parafilm, each tube was connected to plastic tubing, which itself was attached to a container holding 100 mmol/l NaCl, 50 mmol/l Tris, pH 7.4. The pressure drop in the buffer reservoir was 4 cm. After washing the clots, the time required for six drops to permeate through the gel was measured, and each of the six drops was weighed on an analytical balance. Plotting the accumulative weight of the drops against the accumulative time of permeation allowed calculating the flow rate (Q/t), which was used to establish the Darcy constant (K).
where $Q$ is the volume of liquid (milliliters), with the viscosity $\eta$ ($10^{-3}$ poise) flowing through a clot with length $L$ (1.3 cm) and a cross-sectional area $A$ (0.049 cm$^2$) in time $t$ (s) under pressure $\Delta P$ (dyne/cm$^2$). The unit of the resulting Darcy constant is in centimeters squared.

**Effect of DMB on turbidity measurements.** Pooled normal plasma was diluted two-thirds with 50 mmol/l Tris and 100 mmol/l NaCl, pH 7.5, and incubated with either 0.7 units/ml human thrombin or 0.12 units/ml ancrod and 16 mmol/l calcium. DMB or NaCl, dissolved in 50 mmol/l Tris and 100 mmol/l NaCl, pH 7.5, was added to the dilution buffer at the following concentrations: 0, 1, 10, and 40 mmol/l. Immediately on addition of the thrombin/calcium and ancrod/calcium, absorbency was read every 10 s at 350 nm for 15 min on a MRX Microplate Reader (Dynex Technologies, Ashford, Middlesex, U.K.). From the turbidity curves, parameters can be calculated to characterize polymerization, including the lag phase before the increase in turbidity, the maximum rate of increase of turbidity, and the maximum absorbency. Two replicate measurements were performed for each sample.

**Effect of DMB on clot structure determined by scanning electron microscopy.** Activation mixtures consisting of human $\alpha$-thrombin (final concentration 1 unit/ml), calcium chloride (final concentration 10 mmol/l), and DMB (final concentrations 50, 10, 1, and 0 mmol/l) in 50 mmol/l Tris, 100 mmol/l NaCl, pH 7.4, were added to pooled normal plasma and purified fibrinogen (final concentration 1 mg/ml). After a 2-h incubation in a humidified environment, the resulting clots were prepared for scanning electron microscopy by fixation, dehydration, critical point drying, and sputter-coating with gold palladium, as described previously (14). Clots for each DMB concentration were prepared in duplicate. All clots were observed and photographed digitally in at least two different areas per clot, using a scanning electron microscope (XL 20; Philips Electron Optics, Eindhoven, the Netherlands).

**FXIII A- and B-subunit antigen assays.** FXIII A- and B-subunit antigen levels were determined by in-house sandwich–enzyme-linked immunosorbent assays, as described previously (12). The assays were specific for either A- or B-subunits, and they did not cross-react with the other subunit nor with any other plasma protein. For the FXIII A-subunit assay, the intra- and interassay coefficients of variation were 5.4 and 9.3%, respectively; for the B-subunit assay, the intra- and interassay coefficients of variation were 6.2 and 9.8%, respectively.

**Patients and in vivo study design.** Samples from 39 patients with type 2 diabetes, previously enrolled for a study of DMB and cardiovascular risk, were available (15). The study had been approved by the United Leeds Teaching Hospitals Research Ethics Committee. Exclusion criteria included insulin diabetes, previously enrolled for a study of DMB and cardiovascular risk, were in vivo study design.

**Fibrinopeptide release.** Thrombin removes the fibrinopeptides from fibrinogen in a similar way as it cleaves the activation peptides from the $\alpha$-subunit of FXIII. The finding of inhibited FXIII activation peptide release led to the investigation of the effect of DMB on the cleavage of fibrinopeptides A and B from fibrinogen. To investigate this, reaction mixtures consisting of 1 mg/ml fibrinogen and 0.1 units/ml thrombin were incubated for 5 and 20 min at 37°C. Two distinct peaks for fibrinopeptides A and B was used to measure the effect of DMB on FXIII activation peptide release. After incubation of purified FXIII with human $\alpha$-thrombin for 60 min at 37°C, a large peak for the FXIII activation peptide was detected. The activation peptide release reaction was measured as the ratio of the molar quantity of released peptide over the molar quantity of peptide released at maximum activation. The addition of DMB resulted in a significant decrease of peak size. Inhibition of the release of the activation peptide by DMB started at a concentration of 7.5 mmol/l and the effect was dose-dependent (Fig. 2).

**Fibroinopeptide release.** Glucose and sorbitol showed no change in activity. Inhibition was dose-dependent, with 250 mmol/l DMB inhibiting >95% of FXIII activity. Similar inhibition was observed when using an assay based on the amount of ammonia release during the cross-linking reaction (results not shown).

**Mass spectrometric analysis of FXIII and DMB.** Mass spectrometric analysis was performed to investigate potential binding products between FXIII and DMB. Concentrations of 0, 0.05, 0.5, 5, and 50 mmol/l DMB were added to purified FXIII as a zymogen and in its activated form. Clear mass spectra for both FXIII and DMB were found, but no adducts and therefore no interactions between DMB and zymogen or activated FXIII could be detected (results not shown). However, because of the acidic experimental conditions required for mass-spectrometry, bonding of noncovalent nature could not be excluded.

**Investigation of binding of DMB to FXIII by magnetic separation.** Coupling of purified FXIII to the BioMag particles was 88% efficient. After incubation of 0.05 mmol/l DMB with the FXIII-coupled particles, no difference in absorbency at 234 nm between the pre- and postincubation solution could be detected. These results showed that DMB did not bind to FXIII in physiological conditions.

**FXIII activation peptide release.** Reverse-phase HPLC was used to measure the effect of DMB on FXIII activation peptide release. After incubation of purified FXIII with human $\alpha$-thrombin for 60 min at 37°C, a large peak for the FXIII activation peptide was detected. The activation peptide release reaction was measured as the ratio of the molar quantity of released peptide over the molar quantity of peptide released at maximum activation. The addition of DMB resulted in a significant decrease of peak size. Inhibition of the release of the activation peptide by DMB started at a concentration of 7.5 mmol/l and the effect was dose-dependent (Fig. 2).
were detected. Peaks for fibrinopeptide A appeared first and were larger than the peaks for fibrinopeptide B. When DMB at concentrations between 1.5 and 125 mmol/l was added to the reaction mixtures, a decrease of both fibrinopeptide A and fibrinopeptide B release was observed. This decrease was dependent on the incubation time and the concentration of DMB added (Fig. 3). The inhibitory effect on fibrinopeptide B release at low concentrations of DMB was stronger than on fibrinopeptide A release. After 5 min of incubation, inhibition of fibrinopeptide B release occurred at 1.5 mmol/l, but 31 mmol/l was required to achieve a similar effect on fibrinopeptide A release. After 20 min of incubation, inhibition of fibrinopeptide A release could only be observed at the very high concentration of 125 mmol/l, whereas 31 mmol/l DMB caused a significant inhibition of fibrinopeptide B release.

Fibrinopeptide A release alone was examined by replacing human α-thrombin with ancrod, which cleaves fibrinopeptide A, but not fibrinopeptide B, from fibrinogen. After the incubation of 1 mg/ml fibrinogen with 0.1 units/ml ancrod for 5 min, a single peak for fibrinopeptide A appeared. The peak size was not reduced after adding DMB to the reaction at concentrations ranging from 1.5 to 125 mmol/l (data not shown), indicating that the effect of DMB is specific for thrombin-induced fibrinopeptide cleavage.

Clotting time. Clotting times for pooled normal plasma with added thrombin in the presence and absence of DMB were recorded. Increasing concentrations of DMB prolonged the plasma clotting time induced by thrombin (Fig. 4). The effect was dose-dependent and appeared with concentrations of 10–20 mmol/l or higher.

The active site of thrombin. The thrombin-specific chromogenic substrate H-D-Phe-Pip-Arg-pNA · 2HCl was used to examine the activity of 0.5 units/ml human α-thrombin in the presence of 250 mmol/l DMB. No decrease of activity was detected. Inhibition of thrombin occurred when 1 unit/mol/l hirudin was added to thrombin and H-D-Phe-Pip-Arg-pNA · 2HCl in TBS (Fig. 5). These results demonstrate that DMB does not bind directly to the catalytic site of thrombin.

Cross-linking of α- and γ-chains by FXIII. Cross-linking of the fibrin α- and γ-chains by FXIII in the presence and absence of DMB was studied by SDS-PAGE under reducing conditions. The addition of ≥62 mmol/l DMB almost completely inhibited the formation of γ-γ dimers after 5 min of incubation (Fig. 6A), and spot densitometry analysis showed that some reduction already

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FIG. 2. HPLC analysis of the effect of DMB on the release of the thrombin-cleaved FXIII activation peptide. For the analysis, 1.6 μmol/l FXIII was incubated at 37° C with 0.1 units/ml human α-thrombin and DMB at concentrations ranging from 0 to 125 mmol/l. The reactions were stopped after 60 min. The release of activation peptide (AP) was expressed as the ratio between the molar quantity of the peptide ([AP]) over the molar quantity of the peptide at maximum activation ([AP]f).

FIG. 3. HPLC analysis of fibrinopeptide A and B release in the presence of DMB. For the analysis, 1 mg/ml fibrinogen was incubated with 0.1 units/ml human α-thrombin and DMB at concentrations ranging from 0 to 125 mmol/l for a duration of 5 and 20 min. Fibrinopeptide A and fibrinopeptide B release was expressed as the ratio between the molar quantity of the peptides over the molar quantity of the peptides at full activation. A: The release of fibrinopeptide A and B after 5 min of incubation. B: The release of fibrinopeptide A and B after 20 min of incubation. ○, Fibrinopeptide A; ■, fibrinopeptide B.
occurred on the addition of 1.56 mmol/l. After 20 min of incubation, γ-chain cross-linking appeared to be complete, with the exception of a sharp decrease in the presence of 125 mmol/l DMB. Formation of α-polymers also showed a dose-dependent reduction (Fig. 6B). The inhibitory effect of DMB on α-polymer formation could only be observed after 20 min of incubation because cross-linking of the α-chain requires more time than γ-chain cross-link formation. Again, spot densitometry analysis showed that inhibition of cross-linking occurred on the addition of low doses of DMB (Fig. 6B). Because cross-linking of the α-chain results in more than one product, the strongest band (marked with *a* in the figure) was chosen for spot densitometry analysis.

**Permeation properties of plasma clots.** The permeation rate of fluids through a clot is closely related to the structure of the clot. Flow rate, which depends on the pore sizes of the fiber network, was found to be greatly reduced in clots made with DMB. At a pressure drop of 4 cm, the flow rate in clots made of pooled normal plasma was 0.3 ml/h. The addition of 40 mmol/l DMB resulted in a decreased flow rate of 0.14 ml/h, and 80 mmol/l DMB further reduced the flow rate to 0.097 ml/h. The Darcy constant ($K_r$), which is calculated from the flow rates and is a direct measure of the clot surface area available for flow, was $4.91 \times 10^{-9}$ without DMB, $2.46 \times 10^{-9}$ with 40 mmol/l DMB, and $1.84 \times 10^{-9}$ cm$^2$ with 80 mmol/l DMB. This suggested that DMB had an effect on the pore size and therefore on the structure of plasma clots.

**Effect of DMB on turbidity curves.** DMB at a concentration of 0.04 mol/l in 0.05 mol/l Tris and 0.1 mol/l NaCl, pH 7.5, caused an increase in the lag period, a small decrease in the maximum rate, and a decrease in the maximum turbidity (Fig. 7). The addition of NaCl to test whether the effect was specific for DMB or caused by an increase of ionic strength resulted in a change of turbidity, but to a lesser degree than DMB at the same concentration. Effects on turbidity of samples containing 0.04 mol/l that had been clotted with 0.1 unit/ml anecrod were even stronger (Fig. 7).

**Effect of DMB on clot structure.** The effect of DMB on clot structure was visualized by scanning electron microscopy. Clots prepared from pooled normal plasma and purified fibrinogen in the presence of 0.001, 0.01, and 0.05 mol/l DMB were compared with clots without addition of DMB. There was a clear effect of DMB on the structure of fibrin clots. In clots derived from purified fibrinogen, fiber thickness and pore size decreased significantly with increasing concentrations of DMB (Fig. 8). DMB had the same effect on clots made from pooled normal plasma (data not shown).

**Effects of DMB on FXIII activity and antigen levels in patients with type 2 diabetes.** Table 1 shows the levels of each FXIII measure in the different treatment groups. An analysis of variance change in the end point by treatment modality demonstrated a clear dosage effect for each of the FXIII measures (FXIII A: placebo +1.5%, DMB 1.5 g -10%, and DMB 3 g -12%; FXIII B: placebo +4%, DMB 1.5 g -8%, and DMB 3 g -15%; FXIII activity: placebo +1%, DMB 1.5 g -5%, and DMB 3 g -9.9%). The assessment of the dosage effect was the first confirmatory analysis. Correlations were found between baseline and final values for FXIII A (0.67), FXIII B (0.68), and FXIII activity (0.92). ANCOVA confirmed a significant dosage effect for FXIII A on the three independent treatments ($P = 0.01$). Using a multiple comparison test between treatments, the two active treatments were shown to be significantly different from placebo. For FXIII B, a significant main dosage effect was found ($P = 0.04$), which was confirmed by ANCOVA on three independent treatments ($P < 0.01$). Multiple comparison tests did not separate the three treatment groups. A main significant dosage effect was found for FXIII activity ($P = 0.03$); however, ANCOVA on three independent treatments did not confirm the effect. Multivariate ANCOVA on the three FXIII measures were carried out on the independent treatment effects as the secondary supportive analysis and demonstrated a significant difference between the two dosages and placebo ($P = 0.01$).

**DISCUSSION**

The development of the vascular complications of type 2 diabetic subjects is a complex multifactorial process affecting both the micro- and macrovascular systems. In the UKPDS, DMB therapy was associated with a reduction in vascular complications in general, but most strikingly with
a fall in the incidence of fatal MI (1). Administration of DMB to diabetic and nondiabetic subjects is associated with antihyperglycemic insulin-sparing effects, with an associated reduction in circulating triglycerides, a small degree of weight loss and, in some uncontrolled trials, reduction in blood pressure (17). These clinical findings, together with clamp studies, indicate that DMB acts to reduce insulin resistance and the associated clustering of atheromatous risk that occurs with this metabolic state (18). Additionally, it has been recognized that thrombotic risk also clusters with insulin resistance (19) and that DMB lowers circulating levels of the fibrinolytic inhibitor.

FIG. 6. Analysis of rates of α- and γ-chain cross-link formation. A final concentration of 1 mg/ml fibrinogen was incubated with 22 mg/ml purified FXIII and 0.3 units/ml human α-thrombin. Cross-linked fibrin products were analyzed on a 7% polyacrylamide gel. DMB inhibited cross-linking of both α- and γ-chains in a dose-dependent manner. Samples were analyzed after 5 min (A) and 20 min (B) of incubation at 37°C. α, α-chain cross-links (representative band indicated with *); γ, γ-dimers. Maximum band intensity (100%) occurred in the absence of DMB. The percent intensity of the bands (compared with the maximum) was plotted against DMB concentrations (lane a = 125 mmol/l; lane b = 62 mmol/l; lane c = 31 mmol/l; lane d = 15.6 mmol/l; lane e = 7.8 mmol/l; lane f = 1.56 mmol/l; and lane g = 0 mmol/l).

FIG. 7. Turbidity curves of pooled normal plasma with 0.04 mol/l DMB (■), 0.04 mol/l NaCl (●), or with neither DMB nor NaCl (▲) clotted with 0.7 units/ml human α-thrombin (A) or 0.12 units/ml ancrod (B). In both experiments, DMB caused an increased lag phase, a decline in slope, and a reduction in fiber size, and the effect was even more intense when ancrod was used for clotting. The addition of 0.04 ml/l NaCl caused changes in turbidity, but to a lesser degree than those observed in the presence of DMB. OD, optical density.
plasminogen activator inhibitor-1 (PAI-1) (18,20,21) and coagulation factor VII (22). Putting these findings together, there are a number of possible explanations for the beneficial effects of DMB. Microvascular complications may be ameliorated by the antihyperglycemic effects of DMB, thereby reducing the prevalence of retinopathy and related disorders. In the macrovascular system, DMB might prevent atheroma formation, stabilize the atheromatous plaque, and reduce associated thrombus formation, processes that would be expected to affect the development of MI and other macrovascular disease.

The results from the current study demonstrate that DMB has marked effects on the processes that regulate the formation of cross-linked fibrin, to provide a further potential mechanism for the cardioprotection described in the UKPDS. The in vitro addition of DMB to either pooled normal plasma or to a purified system of fibrinogen, FXIII, and human α-thrombin resulted in a decrease of FXIII cross-linking activity. Activation of FXIII was at least partly reduced by the dose-dependent decrease in activation peptide release in the presence of DMB. No molecular interaction between DMB and FXIII could be detected by

**FIG. 8.** DMB and clot structure. Clots made with purified fibrinogen and 1 mmol/l (A) or without DMB (B) were examined by scanning electron microscopy. Clot structure was changed significantly by the addition of DMB, resulting in clots made of thinner fibers and smaller pores.

<p>| TABLE 1 |</p>
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<th>Factor and Treatment group</th>
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<td>1.01 ± 0.16</td>
<td>1.03 ± 0.29</td>
<td>1.01 ± 0.16</td>
<td>1.07 ± 0.28</td>
</tr>
<tr>
<td>DMB 1.5</td>
<td>11</td>
<td>0.90 ± 0.24</td>
<td>0.80 ± 0.18</td>
<td>0.79 ± 0.16</td>
<td>0.83 ± 0.16</td>
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<tr>
<td>DMB 3.0</td>
<td>15</td>
<td>0.99 ± 0.27</td>
<td>0.94 ± 0.24</td>
<td>0.87 ± 0.23</td>
<td>0.87 ± 0.18</td>
</tr>
<tr>
<td>FXIII B-subunit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>placebo</td>
<td>13</td>
<td>1.20 ± 0.29</td>
<td>1.30 ± 0.39</td>
<td>1.20 ± 0.35</td>
<td>1.27 ± 0.40</td>
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<tr>
<td>DMB 1.5</td>
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<td>1.09 ± 0.22</td>
<td>1.04 ± 0.17</td>
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<tr>
<td>DMB 3.0</td>
<td>15</td>
<td>1.25 ± 0.42</td>
<td>1.24 ± 0.24</td>
<td>1.13 ± 0.28</td>
<td>1.08 ± 0.22</td>
</tr>
</tbody>
</table>

Data are means ± SD in units per milliliter, unless otherwise indicated; 1 unit is defined as the amount of FXIII in 1 ml of pooled normal plasma. Significant dosage effects on FXIII were found by changes in means and ANCOVA (see text). Patients in the DMB 3 g/day group were treated with 1.5 g/day up to week 3.
either mass spectrometry or analysis of the binding of DMB to FXIII-coupled magnetic particles.

These observations suggest that DMB inhibits FXIII cross-linking activity by interfering with thrombin-induced activation of FXIII. We analyzed the thrombin-induced cross-linking activity by interfering with thrombin-induced DMB to FXIII-coupled magnetic particles.

from mass spectrometry or analysis of the binding of DMB to FXIII-coupled magnetic particles. Ancrod cleaved fibrinopeptide A from fibrinogen, regardless of the amount of DMB added, which implies that DMB has a direct inhibitory effect on thrombin. However, experiments with a chromogenic substrate showed that the active site of thrombin was unaffected by the presence of DMB. Because the chromogenic substrate is smaller than the natural substrates of thrombin, it is possible that inhibition occurred via a different mechanism, such as binding to an exosite.

Measuring thrombin-induced clotting time of pooled normal plasma showed that DMB influenced fibrin polymerization in vitro. DMB also caused changes in turbidity measurements of clotting fibrin, which gave further information about the changes in the polymerization process. Interestingly, the effects on turbidity were more dramatic when ancrod was used, even though ancrod’s ability to cleave fibrinopeptide A (and therefore its enzymatic activity) was not affected by DMB. This indicated that a decline in thrombin activity might not be the only reason for the changes in polymerization. However, to generate turbidity curves, ancrod had to be used at a lower concentration than thrombin, and therefore effects on fibrin formation may have been accentuated.

Chloride ions are known to influence polymerization of fibrin and clot structure (23). To exclude the possibility of the observed effects of DMB being caused by ionic strength, we performed turbidity experiments with NaCl as a control instead of DMB. The addition of NaCl changed turbidity curves, but to a minor degree compared with DMB, indicating that factors other than ionic strength were responsible for the changes in polymerization.

Permeation experiments and scanning electron microscopy showed that DMB altered clot structure and function. The effects on clot structure could be directly caused by the influence of DMB on polymerization, primarily the lateral aggregation of the protofibrils. Reduced thrombin activity may also play a role, although it has previously been shown that lower thrombin concentrations cause an increase in fiber thickness (14). The effect of changes in fibrin structure on the risk for thrombotic disease is not yet fully understood. Fibrin structures with thinner fibers and reduced permeation characteristics have been related to increased thrombotic risk (2,24). However, there is also evidence for similar changes in fibrin structure to have a possible antithrombotic effect. A common polymorphism of the FXIII A-subunit, Val34Leu, has been associated with decreased prevalence of thrombotic disease (25–30), and carriers of the protective Leu allele produce a finer fibrin meshwork, with thinner fibers and reduced pore size between the fibrin strands (10), findings similar to those observed with DMB in the current study. This may indicate that DMB influences the development of a fibrin structure that is easier to lyse, an effect that would be accentuated in the presence of the described effects of DMB on PAI-1. This is an area that requires further study.

The outstanding question arising from these findings is their relevance to the clinical management of diabetic subjects. Orally administered DMB is rapidly distributed throughout the body and has a bioavailability of 50–60% (31). Therapeutic plasma concentrations of DMB fall within the range of 0.006–0.01 mmol/l (31), which is significantly less than the concentrations required in vitro to affect the assay systems used in this study. There are several possible explanations for these discrepancies that might support a clinical relevance to these findings. For all of the systems studied, we have demonstrated clear evidence of dose-related effects, albeit at higher molarity than used clinically. It is possible that our assay systems are not sensitive enough to pick up very small differences, which may only become clinically important in an environment in which DMB is also affecting other components of this pathway (PAI-1 and factor VII). In vivo, the lowering of a procoagulant tendency by DMB may enhance the effect of even minor changes in clot structure/function, leading to less activation of coagulation and enhanced fibrinolysis with, ultimately, a less stable clot. To support these arguments, our study demonstrates that in vivo administration of DMB at pharmacological doses was associated with a decrease in the concentration of both FXIII A- and B-subunits as well as a sustained reduction in FXIII cross-linking activity. It has to be taken into consideration that the present study size is small for a clinical trial, indicating the value of a larger sample to confirm these findings. The decrease in the A- and B-subunits may be related to hepatic effects of DMB, leading to a decrease in insulin resistance and an associated reduction in levels of the B-subunit, which acts as the carrier protein for the A-subunit. In support of this, we have recently described the B-subunit levels as being associated with features of insulin resistance in type 2 subjects and first-degree relatives (32). However, activity levels of FXIII, when measured by the pentylamine-incorporation assay, have previously been found to correlate poorly with antigen levels (12), and an alternative explanation, such as the in vitro effects of DMB on FXIII activation, needs to be invoked to explain this observation.

Several studies have reported that DMB reduces risk factors involved in the thrombotic response to atheroma. Increased fibrinolysis caused by reduced PAI-1 has been found in patients with type 2 diabetes after DMB (19,20,33). In addition, circulating factor VII levels are reduced by DMB (21). Other studies have shown decreased platelet density, platelet aggregability, blood pressure, and peripheral arterial resistance after DMB treatment (34,35). These effects contribute to reduction of thrombotic risk associated with type 2 diabetes. In the present study, we found that DMB influenced thrombin activity and fibrin polymerization, which consequently led to decreased FXIII activity and altered fibrin clot structure. These findings, in combination with previously reported increased fibrinolysis, may constitute a major mechanism by which DMB decreases risk of cardiovascular disease in patients with type 2 diabetes, as observed in the UKPDS.
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REFERENCES