Diminished Production of Thromboxane B_2 and Prostaglandin E by Stimulated Polymorphonuclear Leukocytes from Insulin-treated Diabetic Subjects

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SUMMARY

As an initial step to investigate the possibility that abnormal polymorphonuclear leukocyte (PMNL) function in diabetes might be related to abnormalities of arachidonic acid metabolism, products of the cyclooxygenase pathway were assayed in PMNL from 27 insulin-treated diabetic subjects and 27 age- and sex-matched nondiabetic subjects. It was found that the major prostanoid products formed were thromboxane B_2 (TxB_2) and prostaglandin E (PGE). Production of both these substances was greatly enhanced in PMNL from control and diabetic subjects by exposure to a killed preparation of Staphylococcus aureus (*S. aureus*) or to zymosan.

There was a marked reduction in the production of TxB_2 by PMNL from diabetic subjects in response to stimulation by both *S. aureus* [670 ± 98 (SE) versus 1010 ± 76 pg/10^6 PMNL/90 min, $P < 0.01$] and zymosan (583 ± 53 versus 1034 ± 46 pg/10^6 PMNL/90 min, $P < 0.001$). Similarly, production of PGE was significantly reduced in diabetics in response to both *S. aureus* (145 ± 29 versus 232 ± 16 pg/10^6 PMNL/90 min, $P < 0.05$) and zymosan (181 ± 21 versus 271 ± 27 pg/10^6 PMNL/90 min, $P < 0.01$). There was no relation between the plasma glucose at the time of the test and the production of either prostanoid.

Diminished production of cyclooxygenase products of arachidonic acid metabolism should be added to the known abnormalities of PMNL in diabetes. In view of the demonstrated or inferred effects of cyclooxygenase products on aspects of PMNL function, this observation may be important in understanding the pathogenesis of PMNL dysfunction in diabetes. DIABETES *32*:622–626, July 1983.

Infection remains a significant cause of morbidity in diabetic subjects.[1,2] The cause for the increased liability of diabetics to certain infections remains uncertain, but recent attention has focused on abnormalities of polymorphonuclear leukocyte (PMNL) function. These include defects in the processes of adherence,[3,4] chemotaxis,[5-7] engulfment,[8-11] and killing.[12,13] The mechanisms accounting for the defects in PMNL function are unknown, although decreased glucose oxidation[14,15] and activity of the PMNL myeloperoxidase-hydrogen peroxide-halide system[15] have been demonstrated in PMNL from diabetic subjects.

It has been shown that PMNL are active in metabolizing arachidonic acid, both by the cyclooxygenase[16-22] and by the lipoxygenase[21-28] pathways. The arachidonic acid metabolites formed appear to have important effects on PMNL function. In particular, cyclooxygenase products appear to be involved in the generation of chemiluminescence,[29] PMNL adhesiveness,[30] and possibly vascular permeability[31] and chemokinesis.[32] Lipoxygenase pathway products from PMNL have been shown to be capable of stimulating PMNL chemotaxis[33-36] aggregation,[34,36] degranulation[37-39] hexose transport,[40-42] and hexose monophosphate shunt activity.[29] It therefore appears that products of arachidonic acid metabolism by PMNL are of fundamental significance to the regulation of PMNL function and other aspects of the inflammatory response.

The aim of the present investigation was to study the formation of products of the cyclooxygenase pathway of arachidonic acid metabolism in insulin-treated diabetic subjects to determine whether the abnormalities of PMNL function are associated with altered arachidonic acid metabolism.

MATERIALS AND METHODS

Subjects studied. A group of 18 male and 9 female nonobese (less than 115% ideal body weight) insulin-treated diabetic subjects was studied. They were ambulant and apparently stable outpatients at the time of study. The control group also consisted of 18 males and 9 females who were healthy and ambulant. The mean ages of the diabetic [61 ± 3 yr (SE)] and control (57 ± 4 yr) subjects were not significantly different. None of the subjects had recognized infection, and

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none had ingested drugs known to affect PMNL function or prostaglandin synthesis for at least 7 days before study. No subject had significantly impaired renal function.

Preparation of PMNL. Neutrophil PMNL were prepared from 15 ml of fresh, heparinized blood obtained after an overnight fast using a slight modification of the method of Ferrente and Thong.[43] Briefly, 20 ml of 76% Urografin (Schering Pty. Ltd., West Germany) was mixed with 80 ml of 9% Ficoll (Pharmacia, Uppsala, Sweden) to produce a specific gravity of 1.114. Seven milliliters of blood was layered carefully on 3 ml of Ficoll-Urografin in a conical centrifuge tube. After centrifugation for 45 min at 800 g, the lymphocytes, monocytes, and platelets that separate out at the Ficoll-Urografin plasma interface were discarded. The red-cell sediment at the bottom of the centrifuge tube and the PMNL separate as a single layer in the Ficoll-Urografin medium. Red cells contaminating the PMNL were removed by hemolysis in cold distilled water.

The PMNL were washed three times with Dulbecco phosphate-buffered saline[44] and made up to a concentration of 1×10^6 PMNL/100 μl PBS. Differential counts showed that the PMNL preparations contained about 94% neutrophils. Contamination by monocytes was less than 1% and no platelets were observed. There were about 2% lymphocytes and 3% eosinophils.

Production of prostanoids by PMNL. PMNL were incubated in three duplicate incubations for each subject. The incubation volume was 600 μl. The incubation mixture contained 10^6 PMNL added in 100 μl of PBS, 375 μl of glucose, 2.2 mmol/L in PBS, 25 μl of pooled normal human serum, and 100 μl of PBS or 100 μl containing 0.3 mg zymosan (Sigma Chemical Co., St. Louis, Missouri) or 100 μl containing a killed suspension of Staphylococcus aureus (*S. aureus*) adjusted to an optical density of 1.0 at 540 nm using a cuvette with a 1-cm light path. The tubes were gently shaken by hand initially and at 15-min intervals during a 90-min incubation at 37°C.

Assay of prostanoids. At the end of the 90-min incubation period, the supernatant was separated from the PMNL and stored at −20°C until assay. The supernatants were assayed for prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$, the stable metabolite of prostacyclin), and thromboxane B_2 (TxB_2, the stable metabolite of thromboxane A_2, TxA_2) using the assays and antisera described in detail by Nolan et al.[45]

Sensitivities of the assays, determined by the amount of standard per assay tube required to inhibit binding of the label to the antibody by 10%, were 5 pg for 6-oxo-$PGF_{1\alpha}$, 25 pg for PGE_2, 10 pg for $PGF_{2\alpha}$, and 10 pg for TxB_2. Cross-reactivities with the PGE_2 antibody were: PGE_1 42%; 6-oxo-$PGF_{1\alpha}$ 0.12%; $PGF_{2\alpha}$ 1%; TxB_2 0.007%; and arachidonic acid 0.001%. In view of the relatively high cross-reactivity with PGE_1 and the likely contribution of PGE_1 to the PGE_2 results, subsequent discussion will refer to PGE rather than PGE_2.

Statistical analysis was performed using two-tailed Student's *t* test for unpaired data and the linear regression correlation coefficient.

RESULTS

Prostanoid production by PMNL from control subjects. The major prostanoids produced by the PMNL were TxB_2 and PGE. In the case of TxB_2, measurable quantities were generally detected in the resting state, and these were greatly increased by stimulation of the PMNL with *S. aureus* suspension or zymosan. For PGE, production was not detectable in resting PMNL, but was readily detectable after stimulation with *S. aureus* or zymosan. $PGF_{2\alpha}$ and 6-oxo-$PGF_{1\alpha}$ were not detectable in either resting or stimulated PMNL.

In the case of TxB_2 it was necessary to demonstrate that the TxB_2 was not being produced by contaminating platelets, as platelets are major sources of TxB_2. No platelet contamination of the PMNL preparation was visible by microscopy. Two normal subjects took 900 mg of acetylsalicylic acid, and PMNL and platelet-rich plasma[46] were prepared 3 days later. As shown in Table 1, acetylsalicylic acid had abolished or greatly reduced TxB_2 and PGE production by platelet-rich plasma. It is also apparent that platelet production of the prostanoids is not increased by exposure to *S. aureus* or zymosan. The PMNL preparations from both subjects still showed abundant production of TxB_2 and PGE after acetylsalicylic acid. The lesser effect of acetylsalicylic acid on PMNL production of prostanoids measured 3 days later is expected from the more rapid turnover of PMNL compared with platelets. The microscopy, the response to stimulation,

TABLE 1
Production of TxB_2 and PGE by PMNL and by the platelets in platelet-rich plasma from two nondiabetic subjects before and 3 days after receiving 900 mg acetylsalicylic acid (ASA)*

			TxB_2 pg/90 min		PGE pg/90 min	
Subject	Preparation	Stimulus	Before ASA	After ASA	Before ASA	After ASA
1	PMNL	Basal	168	150	ND	ND
		S. aureus	672	480	264	96
		Zymosan	600	450	192	120
	Platelets	Basal	3.6	ND	3.8	ND
		S. aureus	ND	ND	0.2	ND
		Zymosan	3.2	ND	2.8	ND
2	PMNL	Basal	180	162	ND	ND
		S. aureus	840	558	264	120
		Zymosan	660	522	204	108
	Platelets	Basal	9.6	3.6	ND	ND
		S. aureus	4.0	4.4	ND	ND
		Zymosan	7.6	0.4	ND	ND

*Results expressed per 10^6 cells. ND = not detectable.

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and the effect of acetylsalicylic acid combine to show that platelet contamination is not responsible for the prostanoid production. Monocytes also produce prostanoids, but microscopy showed that the preparation was almost totally devoid of monocytes (<1%). The active production of TxB_2 and PGE was confirmed by showing that 2.5 μg/ml indomethacin totally abolished both TxB_2 and PGE production by PMNL in response to stimulation by both *S. aureus* and zymosan.

Effect of diabetes on TxB_2 and PGE production. As shown in Figures 1 and 2 there was a significant reduction in both TxB_2 production and PGE production by PMNL from insulin-treated diabetic subjects after stimulation with both *S. aureus* and zymosan.

Relationship between TxB_2 and PGE production. There was a strong positive correlation between the production of TxB_2 and PGE in PMNL from diabetic subjects stimulated by *S. aureus* ($r = 0.88$, $P < 0.001$). This relationship was retained when results from the nondiabetic subjects were included in the analysis ($r = 0.64$, $P < 0.001$). There was a weak but significant positive correlation between TxB_2 production and PGE production after zymosan stimulation ($r = 0.31$, $P < 0.05$).

Plasma glucose. The mean plasma glucose level in the diabetic subjects at the time of study was 10.6 ± 1.1 (SE) mmol/L. There was no relationship between the plasma glucose and the production of either TxB_2 or PGE in individual subjects.

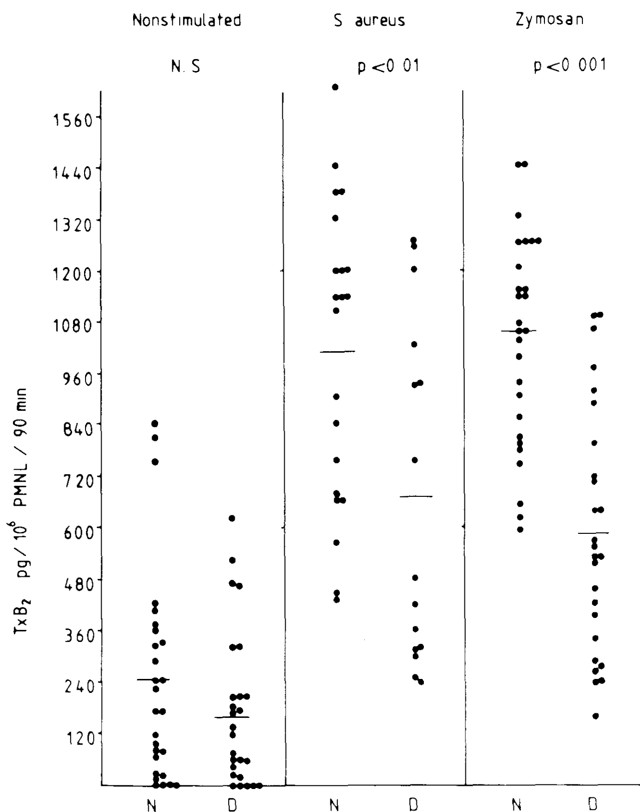

FIGURE 1. TxB_2 production by incubated PMNL from nondiabetic (N) and insulin-treated diabetic (D) subjects. The PMNL were incubated in buffer alone (nonstimulated) or in the presence of *S. aureus* or zymosan. Mean values are indicated by the horizontal bars, and the statistical significance of the differences between the mean values is shown. Note that incubation with *S. aureus* was not performed in all cases.

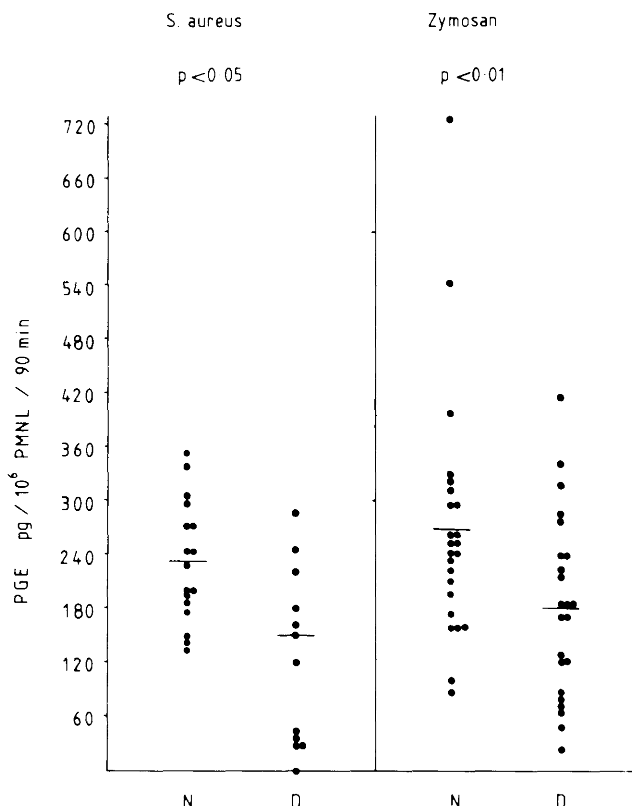

FIGURE 2. PGE production by incubated PMNL from nondiabetic (N) and insulin-treated diabetic (D) subjects in response to stimulation by *S. aureus* and zymosan. Mean values are indicated by the horizontal bars, and the statistical significance of the differences between the mean values is shown. Note that incubation with *S. aureus* was not performed in all cases.

DISCUSSION

This report has demonstrated that TxB_2 and PGE are the major prostanoids produced by human PMNL. This is in accord with previous findings in human and animal PMNL.[17-21] The origin of the TxB_2 and PGE from PMNL rather than from contaminating platelets was confirmed partly by microscopy of the PMNL preparation and partly by the demonstration of a dissociation between the major effect of acetylsalicylic acid administration 3 days previously on production of TxB_2 and PGE by platelets in platelet-rich plasma and its minor effect on PMNL production of these prostanoids. Exposure to both a killed suspension of *S. aureus* and zymosan markedly enhanced the production of both TxB_2 and PGE. Their production was abolished by exposure in vitro to the cyclooxygenase inhibitor indomethacin, indicating that the prostanoids measured were produced by the PMNL in vitro.

There was a reduction in the mean production of both TxB_2 and PGE by stimulated PMNL from insulin-treated diabetic subjects. Similar results were seen with both *S. aureus* and zymosan. There was a correlation between the production of both prostanoids, particularly evident in the case of stimulation by *S. aureus*, suggesting that the decrease observed in the PMNL from the diabetic subjects is due to a defect at a stage common to both TxB_2 and PGE synthesis (e.g., arachidonic acid release by phospholipase or endoperoxide generation from arachidonic acid), rather than at the level

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of the specific enzymes involved in the synthesis of TxA_2 and PGE. It must be emphasized that there was considerable overlap between production of the prostanoids by PMNL from normal and diabetic subjects. The diabetics were ambulant, stable outpatients, and this may have accounted for the fact that the values for PMNL from many diabetics lay in the normal range, although no correlation was seen in the present study between the plasma glucose at the time of the test and the production of either prostanoid. The degree of overlap between PMNL from normal and diabetic subjects is reminiscent of that seen when other aspects of PMNL function are studied in ambulant outpatients.[15]

The question arises whether the observed deficiency in TxB_2 and PGE production in PMNL from diabetic subjects is secondary to defective phagocytic function by the PMNL. This question cannot be answered with certainty, although data from macrophages show that the stimulation of PGE production by zymosan does not require phagocytosis.[47] Rather, it appears that a specific receptor-ligand interaction occurs. This argues against the decreased prostanoid production by the PMNL being a consequence of their decreased phagocytic activity.

Whatever the cause of the decreased production of TxB_2 and PGE by PMNL from diabetic subjects, it is reasonable to suggest that the decreased prostanoid production may be responsible for some of the observed abnormalities of PMNL function in diabetes. From the known effects of cyclooxygenase products on PMNL function,[29,30,32] it seems likely that the observed deficiency of prostanoid production might contribute to abnormalities of PMNL chemokinesis, adhesiveness, and killing.

In view of the common origin of the hydroxyeicosatetraenoic acids and the prostanoids from arachidonic acid, it is obviously important to assess whether the production of the lipoxygenase pathway products is also decreased in PMNL from diabetic subjects. This would have additional implications with respect to the cause of the functional abnormalities of PMNL in diabetes.

Abnormal production of prostanoids has also been described in platelets and blood vessels from diabetic subjects. Increased TxB_2 production has been described in platelets from diabetic subjects.[48–52] In contrast, decreased PGI_2 production has been demonstrated in aortas from diabetic rats.[53–57] Therefore, it appears that a variety of cells and tissues can have altered arachidonic acid metabolism in diabetes, and this may contribute to the pathogenesis of some diabetic complications. However, the direction and nature of the alterations may be different in different tissues (e.g., platelet TxB_2 production is increased, but PMNL TxB_2 production is decreased). The possible ramifications of pharmacologic modification of prostaglandin metabolism in diabetes include potential effects on PMNL function and these should be considered when strategies such as inhibition of cyclooxygenase or thromboxane synthetase are contemplated in the hope of preventing the vascular complications of diabetes.

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