# Platelet Glutathione and Thromboxane Synthesis in Diabetes

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**SUMMARY**

**The relationship of the reduced glutathione (GSH) content in unstimulated platelets and their capacity to synthesize thromboxane $A_2$ ($TXA_2$), measured by radioimmunoassay of $TXB_2$, was investigated in diabetic and matched control subjects. The GSH content in platelets from diabetic subjects (6.52 ± 0.73 μg/$10^9$ platelets, mean ± SD) was significantly ($P < 0.001$) lower than in platelets from control subjects (10.10 ± 1.58 μg/$10^9$ platelets). When platelet-rich plasma (PRP) was stimulated with 1.65 mM arachidonic acid, significantly ($P < 0.001$) more $TXB_2$ was formed in PRP from diabetic subjects (344 ± 87 ng/2.5 × $10^8$ platelets) than in PRP from control subjects (132 ± 35 ng/2.5 × $10^8$ platelets). Furthermore, the plasma level of $TXB_2$ was increased in diabetic subjects (522 ± 117 pg/ml) in comparison with control subjects (187 ± 63 pg/ml). An inverse correlation ($r = 0.98$) was observed between the GSH content in unstimulated platelets and their capacity to synthesize $TXA_2$ when stimulated with 1.65 mM arachidonic acid. These data suggest that platelet GSH may have an important regulatory effect on platelet $TXA_2$ synthesis and that increased $TXA_2$ synthesis by platelets from diabetic subjects may be the result of low intracellular GSH levels.** DIABETES 1985; 34:951–54.

The increased activity of platelets in patients with diabetes mellitus is considered to be a contributing factor to the prevalence of vascular complications. Platelets from diabetic subjects show increased aggregation in response to stimuli such as collagen, adenosine diphosphate (ADP), epinephrine, and arachidonic acid.[1–3] Increased platelet synthesis of thromboxane $A_2$ ($TXA_2$), a potent vasoconstrictor and platelet-aggregating substance, is considered a major contributing factor to the hyperactivity of platelets in diabetes. A number of studies have shown that platelets from diabetic subjects release more $TXA_2$ than control subjects when stimulated with collagen, ADP, thrombin, and arachidonic acid.[4–9] Furthermore, higher plasma levels of $TXB_2$, the stable metabolite of $TXA_2$, have also been reported in diabetes.[10,11]

When platelets are stimulated, arachidonic acid is released from membrane phospholipids and undergoes oxidative metabolism by the enzyme, cyclooxygenase, to form prostaglandin endoperoxide intermediates that are rapidly converted to $TXA_2$ through the action of thromboxane synthetase. Hydroperoxides have been shown to stimulate cyclooxygenase activity, whereas both chemical and enzymatic reducing agents inhibit its activity.[12–14] Glutathione peroxidase in the presence of its cofactor, reduced glutathione (GSH), has an inhibitory effect on cyclooxygenase by eliminating hydroperoxides, but has no effect on thromboxane synthetase activity. In this article, we are describing the relationship between the platelet GSH content and the capacity of platelets to synthesize $TXA_2$ in diabetes.

## MATERIALS AND METHODS

**Subjects.** Twenty-four patients with diabetes mellitus were randomly selected from the Diabetic Recheck Unit at the Cleveland Clinic Foundation. The diabetic subjects included 13 men and 11 women with a mean age of 45 yr and an age range of 18–74 yr. The mean duration of diabetes was 11.6 yr with a range of 3 mo to 26 yr. Nine patients were diagnosed as type I and 15 patients as type II. Eighteen patients were controlled with insulin; four with diet alone and two with oral hypoglycemic drugs. Six patients had complications including neuropathy, nephropathy, and coronary artery disease. Twenty-two subjects with no known history of diabetes were selected as controls. The control subjects included 15 men and 7 women with a mean age of 44 yr and an age range of 22–65 yr. All subjects were fasting overnight when samples were drawn. None of the subjects had taken aspirin or other medications that affect platelet function for at least 1 wk.

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**Materials.** Reduced glutathione and dithio-bis-nitro-benzoic acid (DTNB) were obtained from Sigma Chemical Co. (St. Louis, Missouri). Sodium arachidonate was obtained from Bio-Data Corp. (Hatboro, Pennsylvania). Thromboxane $B_2$ standard was a generous gift from the Upjohn Co. (Kalamazoo, Michigan). Thromboxane $B_2$ antisera was purchased from Seragen Inc. (Boston, Massachusetts) and $^3$H-$TXB_2$ (116 Ci/mmol) was obtained from New England Nuclear (Boston, Massachusetts). All other chemicals were the highest grade available.

**Preparation of platelets and plasma.** Venous blood (9 ml) was drawn into plastic syringes containing 1 ml of 3.8% (wt/vol) sodium citrate. Within 30 min of collection, platelet-rich plasma (PRP) was obtained by centrifugation of the blood at 200 × *g* for 10 min at room temperature. The platelet-rich supernatant was removed and the platelet count determined on a Coulter counter. For all aggregations, the PRP was diluted to 250,000 platelets/μl with plasma.

Plasma for $TXB_2$ analysis was prepared by centrifugation of the citrated blood at 4000 × *g* for 10 min. The cell-free supernatant was removed and stored at −70°C until assayed for $TXB_2$. Initially, a comparison was made of $TXB_2$ concentrations in plasma obtained from citrated blood collected with the addition of EDTA (1.4 mg/ml) or indomethacin (1 mg/ml), and we found that the plasma $TXB_2$ concentrations were not significantly different from those in plasma obtained from blood collected in citrate alone.

**Platelet aggregation.** The capacity of platelets to synthesize $TXA_2$ was determined by aggregation of PRP with arachidonic acid and measurement of the amount of $TXB_2$ formed. Since the only known biologic source of $TXB_2$ is hydrolysis of $TXA_2$, the concentration of $TXB_2$ is considered a reliable measurement of the amount of $TXA_2$ generated. For aggregation, 0.45-ml aliquots of PRP were preincubated in siliconized glass cuvettes with stirring at 37°C for 3 min. Aggregations were induced by 1.65 mM arachidonic acid. After 4 min, the aggregations were stopped by acidification of the samples to pH 3 with 10% (vol/vol) formic acid and chilling on ice. After centrifugation at 4000 × *g* for 10 min, the supernatants were recovered and stored at −70°C until assayed for $TXB_2$.

**Analysis of platelet GSH.** Two-milliliter aliquots of PRP were mixed with 200 μl of 5% (wt/vol) EDTA and centrifuged at 4000 × *g* for 10 min. The platelet pellet was resuspended in saline containing 0.5% (wt/vol) EDTA and centrifuged at 4000 × *g*. The platelet pellet was washed an additional two times. The final pellet was resuspended in 1 ml of water and 0.5 ml of 15% (wt/vol) metaphosphoric acid. The samples were subjected to four cycles of rapid freezing and thawing, and centrifuged at 10,000 × *g* for 10 min. The protein-free supernatant was recovered for GSH analysis.

The supernatants were assayed for GSH by a micromodification of a method previously described by Mergel et al.[15] Supernatant aliquots of 0.5 ml were mixed with 0.25 ml of 1 M sodium phosphate buffer, pH 6.8, and 0.5 ml of 0.08% (wt/vol) DTNB in the phosphate buffer. The final pH of the mixture was 6.5. After 5 min, the absorbance at 412 nm was measured with a double-beam spectrophotometer. Reference blanks were prepared by substituting water for the sample supernatant. The method was calibrated with aqueous GSH standard solutions substituted for the sample supernatant, which gave a linear response from 0.3 to 30 μg of GSH per sample.

**Radioimmunoassay of $TXB_2$.** The concentration of $TXB_2$ in plasma and supernatants recovered from aggregation was determined by radioimmunoassay (RIA) of $TXB_2$ with a specific antisera against $TXB_2$, which had <0.1% crossreactivity (at 50% B/Bo) with the primary prostaglandins. The RIA buffer consisted of 0.1% bovine gamma globulin, 0.9% NaCl, 0.01 M $Na_2HPO_4$, and 0.1% sodium azide with a final pH of 7.4. Dilutions of samples, standards, tracer ($^3$H-$TXB_2$, 12,000 cpm/0.1 ml), and antisera were made with RIA buffer. For the assay, 0.2 ml of buffer was mixed with 0.1 ml antisera, 0.1 ml tracer, and either 0.1 ml sample or standard, and incubated at 4°C for 16–20 h. The free and bound tracers were separated by the addition of 0.5 ml of dextran-coated charcoal (1% dextran, MW 70,000; 1% charcoal, neutral Norit A; in water) and centrifugation at 1000 × *g* for 15 min at 4°C. The supernatant containing the bound tracer was decanted directly into scintillation vials, mixed with 10 ml of Atomlight (New England Nuclear), and counted. Samples from aggregation were prediluted with RIA buffer as required. All assays were performed in duplicate. Concentrations of $TXB_2$ were determined from log-logit plots of the standard counts.

## RESULTS

As shown in Table 1, the level of GSH in platelets from diabetic subjects was significantly lower than in platelets from control subjects. No significant difference was observed in platelet GSH between type I and type II diabetes or between diabetic subjects with complications and those without complications. The duration of diabetes had no apparent effect on the platelet GSH content.

TABLE 1
Glutathione and thromboxane in diabetic and control subjects

| Subjects (N) | Platelet GSH (μg/$10^9$ platelets) | Platelet $TXB_2$ synthesis (ng/2.5 × $10^8$ platelets) | Plasma $TXB_2$ (pg/ml) |
|---|---|---|---|
| Control (22) | 10.10 ± 1.58* | 132 ± 35 | 187 ± 63 |
| Diabetic | | | |
| All groups (24) | 6.52 ± 0.73† | 344 ± 87† | 522 ± 117† |
| Type I (9) | 6.78 ± 0.81 | 320 ± 93 | 489 ± 129 |
| Type II (15) | 6.36 ± 0.66 | 358 ± 83 | 542 ± 109 |
| With complications (6) | 6.41 ± 0.59 | 365 ± 68 | 563 ± 76 |
| No complications (18) | 6.56 ± 0.78 | 336 ± 93 | 508 ± 127 |

*Results expressed as mean ± standard deviation.
†$P < 0.001$ when compared with controls.

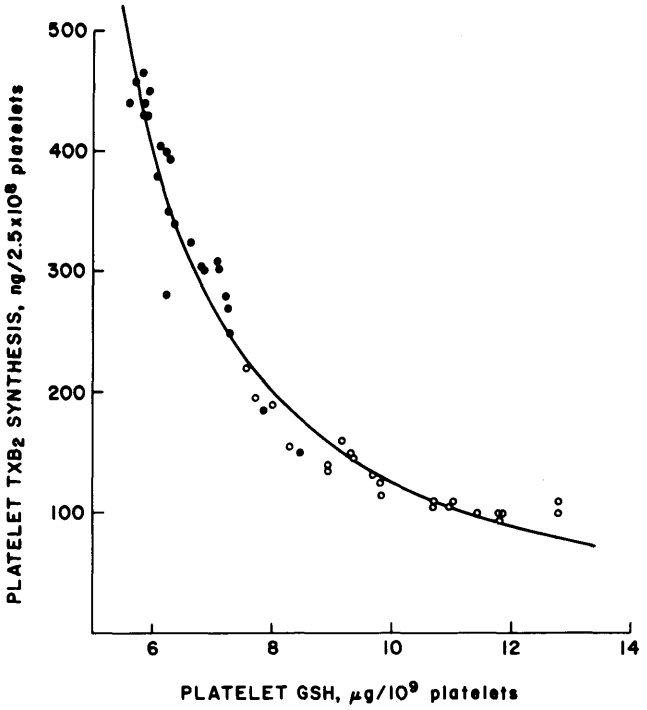


**FIGURE 1. The relationship between GSH content in unstimulated platelets and the formation of $TXB_2$ with platelets stimulated with 1.65 mM arachidonic acid in PRP from diabetic (●) and control (○) subjects. $[TXB_2] = -33.6 + 1030/([GSH] - 3.61)$, ($r = 0.98$).**

The amount of $TXB_2$ formed by platelets stimulated with 1.65 mM arachidonic acid was significantly increased in diabetic subjects. Furthermore, the plasma concentration of $TXB_2$ was also elevated. There were no significant correlations with either plasma $TXB_2$ or $TXB_2$ formed by platelets stimulated with arachidonic acid and the duration of diabetes, presence of complications, or type I versus type II diabetes.

A significant negative correlation, shown in Figure 1, was found between the GSH content in unstimulated platelets and their capacity to synthesize $TXA_2$ as measured by the amount of $TXB_2$ formed after stimulation with arachidonic acid. The negative correlation was apparent within both the diabetic and control groups; however, the data were pooled to more clearly demonstrate the nonlinearity of the relationship. Figure 2 shows a similar correlation between unstimulated platelet GSH content and the plasma concentration of $TXB_2$.

## DISCUSSION

Glutathione is an important intracellular cofactor that prevents the accumulation of peroxides through the action of glutathione peroxidase. A deficiency of GSH or a reduction in glutathione peroxidase activity either by inhibition or selenium deficiency may result in the intracellular accumulation of peroxides. Bryant et al.[16,17] have reported increased levels of lipid hydroperoxides during arachidonic acid stimulation of platelets from selenium-deficient rats. Based on the studies of Hemler et al.,[12,13] an accumulation of peroxides stimulates cyclooxygenase activity, which would result in increased $TXA_2$ synthesis in platelets. Masukawa et al.[18] have shown that platelets from selenium-deficient rats are hypersensitive to ADP, collagen, and arachidonic acid stimulation, and Hofmann et al.[19] have also shown that human platelets deficient in GSH as a result of glucose-6-phosphate dehydrogenase deficiency are hypersensitive to ADP and arachidonic acid stimulation. Dietary supplementation of vitamin E, which has antioxidant effects, reduces peroxidation and $TXA_2$ synthesis in platelets.[20,21] Guidi et al.[22] have reported that the addition of GSH alone to platelet lysates has no effect; however, in the presence of added glutathione peroxidase, GSH exhibits a concentration-dependent inhibition of $TXA_2$ synthesis. In this article, our data indicate that platelets from diabetic subjects are deficient in GSH, and that the level of GSH in platelets has a strong inverse correlation with the capacity of platelets to synthesize $TXA_2$.

The GSH content of platelets also has an inverse correlation with the plasma $TXB_2$ concentration, but since the most likely source of plasma $TXB_2$ is platelet synthesis of $TXA_2$ in vivo and platelet stimulation during blood collection, the correlation with plasma $TXB_2$ supports the data obtained from arachidonic acid-induced aggregation.

The GSH oxidizing agent, diamide, depletes GSH in platelets and inhibits aggregation in the presence of various stimuli.[19,23,24] Furthermore, Caruso et al.[25] have reported inhibition of $TXA_2$ synthesis in platelets treated with diamide. These data may appear to contradict our results, but there is evidence that diamide alters membrane sulfhydryl status and causes crosslinking of cytoskeletal proteins in addition to lowering GSH levels.[25-27] Consequently, the studies with diamide include complex changes in platelet function and the results may not be related to the GSH depletion alone.

The results from this study also confirm previous reports that platelets from diabetic subjects synthesize more $TXA_2$[4-9] than platelets from matched control subjects. The increase was observed in both type I and type II diabetic subjects with no significant difference between the two types, which agrees with previous reports of increased $TXA_2$ in both insulin-dependent and non-insulin-dependent diabetic subjects.

When the diabetic subjects in this study are subdivided

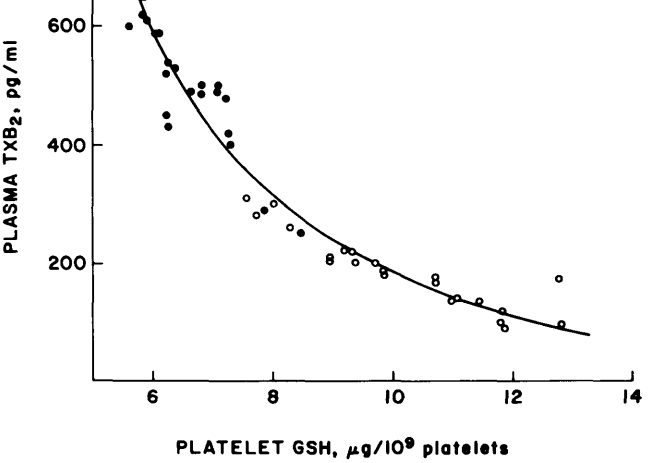


**FIGURE 2. The relationship between GSH content in platelets and plasma $TXB_2$ concentration in diabetic (●) and control (○) subjects. $[TXB_2] = -166.5 + 2595/([GSH] - 2.59)$, ($r = 0.97$).**

into groups based on the presence of complications, there is no difference in measured $TXB_2$ levels between groups with and without complications, and levels of both groups are elevated relative to those of control subjects. Ziboh et al.[7] have reported more $TXA_2$ synthesized from endogenous arachidonic acid in platelets from diabetic subjects with complications versus those with no complications, and Butkus et al.[11] have also reported similar findings with gel-filtered platelets and PRP stimulated with arachidonic acid and collagen. D'Angelo et al.,[5] however, have found no difference between arachidonate-stimulated PRP from diabetic subjects with no complications and those with severe vascular disease. These discrepancies may reflect variation in the classification of the complications and their severity, or perhaps different methods for platelet isolation and aggregation. Nevertheless, all of the studies reported increased $TXA_2$ synthesis relative to controls.

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