

# Hyperglycemia Potentiates Collagen-Induced Platelet Activation Through Mitochondrial Superoxide Overproduction

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Alteration of platelet function contributes to microthrombus formation and may play an important role in the pathogenesis of diabetic micro- and macroangiopathies. However, the molecular mechanism for platelet dysfunction observed in patients with diabetes has not been fully elucidated. In this study, the direct effects of hyperglycemia on platelet function *in vitro* were investigated. Hyperglycemia increased reactive oxygen species generation in human platelets, and this effect was additive with that of collagen. Thenoyltrifluoroacetone (TTFA), an inhibitor of mitochondrial electron transport chain complex II, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an uncoupler of oxidative phosphorylation, completely prevented the effects of hyperglycemia, suggesting that reactive oxygen species arise from the mitochondrial electron transport chain. Hyperglycemia potentiated both platelet aggregation and the subsequent release of platelet-derived growth factor AB induced by a nonaggregating subthreshold concentration of collagen, which were also completely inhibited by TTFA or CCCP. Furthermore, hyperglycemia was found to inhibit protein tyrosine phosphatase (PTP) activity and increase phosphorylation of the tyrosine kinase Syk in platelets exposed to collagen. Hyperglycemia-induced PTP inhibition and Syk phosphorylation were found to be completely prevented by TTFA, CCCP, or Mn(III)tetrakis (4-benzoic acid) porphyrin, a stable cell-permeable superoxide dismutase mimetic. These results suggest that hyperglycemia-induced mitochondrial superoxide generation may play an important role in platelet dysfunction observed in patients with diabetes. *Diabetes* 50:1491–1494, 2001

**A**ccelerated atherosclerosis and diabetic microvascular disease make diabetes a leading cause of coronary artery disease and a major cause of blindness and renal failure (1–3). Altered platelet function, such as hypersensitivity of platelets to colla-

gen, is prevalent in diabetes and may participate in the pathogenesis of diabetic vascular complications by promoting microthrombus formation (4,5). However, the molecular mechanism underlying diabetic platelet dysfunction has not been fully elucidated.

Treatment of platelets with collagen induces an activation of the nonreceptor tyrosine kinase Syk and the subsequent phosphorylation and activation of phospholipase C $\gamma$ 2 (PLC $\gamma$ 2) (6). Activated PLC $\gamma$ 2 generates the second messengers 1,2-diacylglycerol and inositol 1,4,5-triphosphate, which lead to activation of protein kinase C and increase in cytosolic Ca<sup>2+</sup>, respectively, thereby promoting thromboxane A<sub>2</sub> generation, granular secretion, shape change, and ultimately platelet aggregation (7,8).

We have recently shown that hyperglycemia-induced mitochondrial overproduction of superoxide serves as a causal link between elevated glucose and each of the three major pathways responsible for hyperglycemic vascular damage in endothelial cells (9). Because reactive oxygen species (ROS) have recently been shown to be involved in collagen-induced platelet activation and aggregation (10, 11), we investigated in this study 1) the effect of hyperglycemia on ROS generation in human platelets through the mitochondrial electron transport chain, 2) the effect of these hyperglycemia-induced ROS on the potentiation of platelet aggregation and subsequent platelet-derived growth factor AB (PDGF-AB) release induced by a nonaggregating subthreshold concentration of collagen, and 3) the effect of hyperglycemia-induced ROS generation on redox-sensitive protein tyrosine phosphatase (PTP) activity and the subsequent phosphorylation of Syk in platelets (12–14).

## RESEARCH DESIGN AND METHODS

**Materials.** Collagen, thenoyltrifluoroacetone (TTFA), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), bovine serum albumin, NP-40,  $\beta$ -mercaptoethanol, leupeptin, phenylmethylsulfonyl fluoride (PMSF), deoxycholate, Na<sub>2</sub>VO<sub>4</sub>, aprotinin, and pepstatin were purchased from Sigma Chemical (St. Louis, MO). Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) was purchased from Calbiochem (La Jolla, CA).

**Preparation of human platelets.** Whole blood was drawn from normal individuals and collected in test tubes containing 3.8% sodium citrate (Vacutainer; Becton Dickinson, Franklin Lakes, NJ). Platelet-rich plasma (PRP) was harvested from this anticoagulated whole blood after centrifugation at 2,500 rpm for 1 min at room temperature. Platelet-poor plasma was obtained by spinning the rest of the blood at 5,000 rpm for 10 min at room temperature. The PRP sample was adjusted using platelet-poor plasma to a platelet concentration of  $2 \times 10^8$  cells/ml before use.

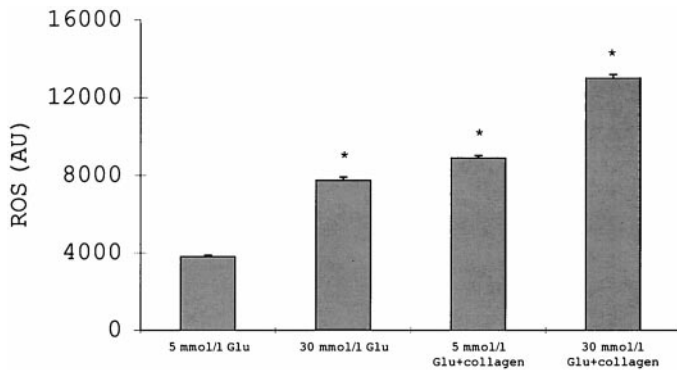
**Measurement of intracellular ROS generation.** PRP was preincubated with 5 or 30 mmol/l glucose in the presence or absence of 10  $\mu$ mol/l TTFA or 0.1  $\mu$ mol/l CCCP for 60 min. Platelets were sedimented by centrifugation at 5,000 rpm for 5 min and then washed with HEPES buffer (137 mmol/l NaCl, 2.7 mmol/l KCl, 1 mmol/l MgCl<sub>2</sub>, and 3.8 mmol/l HEPES, pH 6.5) containing 0.35%

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CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ELISA, enzyme-linked immunosorbent assay; MnTBAP, Mn(III)tetrakis (4-benzoic acid) porphyrin; PDGF-AB, platelet-derived growth factor-AB; PLC $\gamma$ 2, phospholipase C $\gamma$ 2; PMSF, phenylmethylsulfonyl fluoride; PRP, platelet-rich plasma; PTP, protein tyrosine phosphatase; ROS, reactive oxygen species; TTFA, thenoyltrifluoroacetone.



**FIG. 1.** Effects of glucose (Glu) and collagen on ROS generation in human platelets. PRP was preincubated with 5 or 30 mmol/l glucose. After centrifugation, the platelets were incubated with 5 or 30 mmol/l glucose in the presence or absence of 200  $\mu$ g/ml collagen for 30 min, and then ROS generation was quantitated. \* $P < 0.01$  compared with values incubated with 5 mmol/l glucose alone. Data are arbitrary units (AU).

bovine serum albumin and 0.4 mmol/l EGTA. The washed platelets were incubated at 37°C with 10  $\mu$ mol/l of the fluorescent probe CM-H<sub>2</sub>DCFDA (Molecular Probes, Eugene, OR) with or without 200  $\mu$ g/ml collagen for 30 min, and then the intracellular formation of ROS was measured in an HTS 7000 Bio Assay Fluorescent Plate Reader using the HTSoft program (Perkin Elmer, Branchburg, NJ).

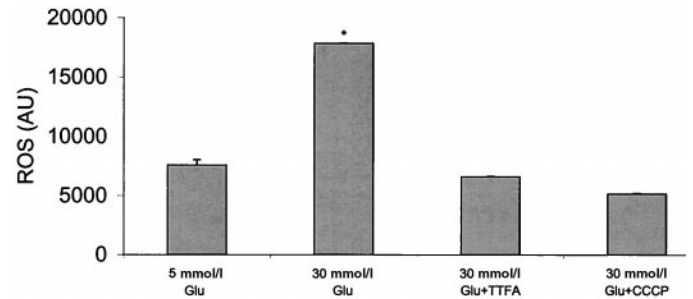
**Measurement of platelet aggregation.** Platelet aggregation was monitored using Born's turbidimetric method (15) with a dual-channel Lumi-aggregometer (Payton Associates, Buffalo, NY). Cuvettes containing 480  $\mu$ l PRP were stirred at 1,000 rpm for 1 min at 37°C before addition of 20  $\mu$ l of 5 mg/ml collagen. The change in light transmission was recorded for 5 min. The maximum aggregation (%) was determined as the peak light transmission after the addition of collagen.

**Measurement of PDGF-AB release from platelets.** PRP was incubated with 5 or 30 mmol/l glucose in the presence or absence of 10  $\mu$ mol/l TTFA, 0.1  $\mu$ mol/l CCCP, or 100  $\mu$ mol/l MnTBAP, a stable, cell-permeable superoxide dismutase mimetic, for 60 min and for the last 10 min with 200  $\mu$ g/ml collagen. After centrifugation at 5,000 rpm for 10 min, PDGF-AB content in the supernatant was analyzed using an enzyme-linked immunosorbent assay (ELISA) kit derived from R&D Systems (Minneapolis, MN), according to the manufacturer's instruction.

**Measurement of PTP activity in platelets.** PRP was incubated with 5 or 30 mmol/l glucose in the presence or absence of 10  $\mu$ mol/l TTFA, 0.1  $\mu$ mol/l CCCP, or 100  $\mu$ mol/l MnTBAP for 60 min. Then, platelets were sedimented and resuspended in 500  $\mu$ l Tyrode's buffer, pH 7.4, containing 137 mmol/l NaCl, 2 mmol/l KCl, 1 mmol/l MgCl<sub>2</sub>, 0.3 mmol/l NaH<sub>2</sub>PO<sub>4</sub>, 12 mmol/l NaHCO<sub>3</sub>, and 5 mmol/l HEPES and were lysed with an equal volume of lysis buffer, pH 7.4 (2% NP-40, 10 mmol/l HEPES, 2 mg/ml bovine serum albumin, 0.2%  $\beta$ -mercaptoethanol, 200 mmol/l leupeptin, and 4 mmol/l PMSF). PTP activity in the lysate was analyzed using an ELISA kit from Boehringer Mannheim (Mannheim, Germany), according to the manufacturer's instruction.

**Immunoprecipitation and Western blot analysis of Syk.** PRP was incubated with 5 or 30 mmol/l glucose in the presence or absence of 0.1  $\mu$ mol/l CCCP or 100  $\mu$ mol/l MnTBAP for 60 min. Then, platelets were washed and resuspended in 250  $\mu$ l HEPES buffer, pH 7.4, containing 138 mmol/l NaCl, 2.9 mmol/l KCl, 2 mmol/l MgCl<sub>2</sub>, 12 mmol/l NaHCO<sub>3</sub>, and 50 mmol/l HEPES. The platelets were incubated with 200  $\mu$ g/ml collagen at 37°C for 10 min, and then were lysed with an equal volume of RIPA buffer (50 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 1% NP-40, 0.25% deoxycholate, 1 mmol/l EDTA, 1 mmol/l PMSF, 1 mmol/l Na<sub>3</sub>VO<sub>4</sub>, 1 mmol/l NaF, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin). After centrifugation, the supernatants were collected and precleared by the addition of protein A-Agarose (Boehringer Mannheim). After 2 h, the cleared lysates were incubated overnight with polyclonal anti-Syk antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and protein A-Agarose at 4°C. Immunoprecipitated proteins were eluted with 1  $\times$  Laemmli buffer and subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with either polyclonal anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) or anti-Syk antibody, and blots were visualized using the enhanced chemiluminescence detection system (Amersham, Buckinghamshire, U.K.) (6). Signal intensities of phosphotyrosine-Syk bands were normalized by those of Syk protein bands.

**Measurement of mitochondrial oxidative phosphorylation in platelets.** PRP was incubated with 5 or 30 mmol/l glucose in the presence or absence of



**FIG. 2.** Effects of TTFA or CCCP on hyperglycemia-induced ROS generation in human platelets. PRP was preincubated with 5 or 30 mmol/l glucose (Glu) in the presence or absence of TTFA or CCCP for 60 min, and then ROS generation was quantitated. \* $P < 0.01$  compared with values incubated with 5 mmol/l glucose alone. Data are arbitrary units (AU).

10  $\mu$ mol/l TTFA or 0.1  $\mu$ mol/l CCCP. After 60 min, platelets were sedimented and incubated with 0.5  $\mu$ mol/l JC-1 (Molecular Probes) and then fluorescence at 590 and 525 nm (510 nm excitation) was monitored.

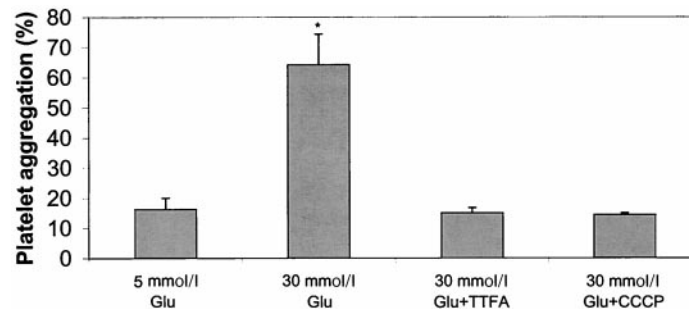
**Statistics.** Data were analyzed using the one-factor analysis of variance procedure to compare the means of all the groups. The Tukey-Kramer multiple comparisons procedure was used to determine which pairs of means were different. Data are shown as mean  $\pm$  SE.

## RESULTS

**Effects of hyperglycemia on intracellular ROS production in platelets.** As shown in Fig. 1, hyperglycemia (30 mmol/l glucose) increased ROS generation in human platelets, and this effect was additive with that of collagen. Furthermore, both TTFA, an inhibitor of mitochondria electron transport chain complex II, and CCCP, an uncoupler of oxidative phosphorylation, completely prevented the effects of hyperglycemia on ROS production in platelets, suggesting that ROS generation arises from the mitochondrial electron transport chain (Fig. 2).

**Effects of hyperglycemia on platelet aggregation.** As shown in Fig. 3, 200  $\mu$ g/ml collagen alone did not significantly induce platelet aggregation. However, marked irreversible aggregation was induced by collagen when PRP was preincubated with high glucose. The hyperglycemia-induced potentiation of platelet aggregation in response to collagen was completely prevented by either TTFA or CCCP.

**Effects of hyperglycemia on PDGF-AB release from platelets.** Hyperglycemia increased collagen-induced PDGF-AB release from platelets approximately threefold. TTFA, CCCP, or MnTBAP completely inhibited the hyperglycemia-induced PDGF-AB release from platelets (Fig. 4).



**FIG. 3.** Effects of hyperglycemia on platelet aggregation induced by collagen. PRP was preincubated with 5 or 30 mmol/l glucose (Glu) in the presence or absence of TTFA or CCCP for 60 min, and then platelet aggregation in response to 200  $\mu$ g/ml collagen was measured. \* $P < 0.01$  compared with values incubated with 5 mmol/l glucose alone.

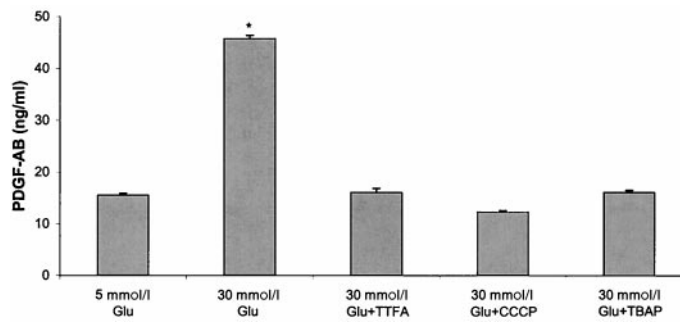


FIG. 4. Effects of hyperglycemia on PDGF-AB release from human platelets induced by collagen. PRP was preincubated with 5 or 30 mmol/l glucose (Glu) in the presence or absence of TTFA, CCCP, or MnTBAP for 60 min and then treated with 200  $\mu$ g/ml collagen at 37°C for 10 min. After centrifugation, PDGF-AB content in the supernatant was analyzed with an ELISA kit. \* $P < 0.01$  compared with values incubated with 5 mmol/l glucose alone.

**Effects of hyperglycemia on PTP activity in platelets.** Hyperglycemia decreased PTP activity in platelets to ~50% of that of control. TTFA, CCCP, or MnTBAP completely reversed the hyperglycemic effects, suggesting that hyperglycemia-induced mitochondria superoxide inactivates PTP in human platelets (Fig. 5).

**Effects of hyperglycemia on Syk phosphorylation in platelets.** Tyrosine phosphorylation of Syk is an early event in collagen-induced platelet activation and aggregation (6). Therefore, we investigated whether hyperglycemia increased Syk phosphorylation induced by collagen. As shown in Fig. 6, hyperglycemia increased Syk phosphorylation in human platelets by fourfold. CCCP or MnTBAP completely inhibited the effects of hyperglycemia.

**Effect of TTFA or CCCP on mitochondrial oxidative phosphorylation in platelets.** JC-1 is a fluorescent probe that is sensitive to metabolic inhibitors of mitochondrial oxidative phosphorylation. Increased mitochondrial membrane potential derived from increased oxidative phosphorylation promotes the formation of J-aggregates, which exhibited fluorescence at 590 nm (16). As shown in Fig. 7, TTFA or CCCP was found to completely inhibit the hyperglycemia-induced increase of mitochondrial membrane potential derived from increased oxidative phosphorylation in platelets.

## DISCUSSION

In this study, we found for the first time that hyperglycemia increased mitochondrial ROS generation in human

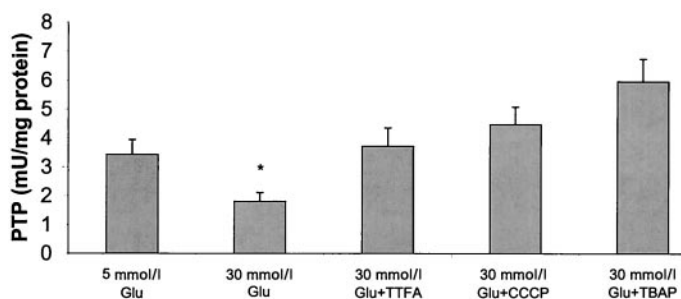


FIG. 5. Effects of hyperglycemia on PTP activity in human platelets. PRP was preincubated with 5 or 30 mmol/l glucose (Glu) in the presence or absence of TTFA, CCCP, or MnTBAP for 60 min. After centrifugation, the platelets were lysed, and PTP activity in the lysate was measured with an ELISA kit. \* $P < 0.01$  compared with values incubated with 5 mmol/l glucose alone.

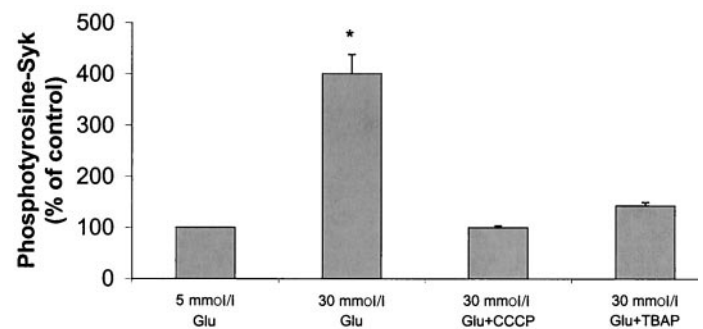


FIG. 6. Effects of hyperglycemia on tyrosine phosphorylation of Syk in human platelets. PRP was preincubated with 5 or 30 mmol/l glucose (Glu) in the presence or absence of CCCP or MnTBAP for 60 min and then treated with 200  $\mu$ g/ml collagen at 37°C for 10 min. Platelets were lysed and then precleared lysates were immunoprecipitated with anti-Syk antibody, subjected to SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were incubated with either polyclonal anti-phosphotyrosine antibody or anti-Syk antibody, and blots were visualized using the enhanced chemiluminescence detection system. Signal intensities of phosphotyrosine-Syk bands were normalized by those of Syk protein bands. Data are shown as % of control. \* $P < 0.01$  compared with values incubated with 5 mmol/l glucose alone.

platelets and that this effect was additive with that of collagen. Furthermore, this hyperglycemia-induced ROS generation was found to potentiate both platelet aggregation and the subsequent release of PDGF-AB by platelets exposed to a nonaggregating subthreshold concentration of collagen.

We have recently found that hyperglycemia increases mitochondrial superoxide production inside cultured endothelial cells, providing a causal link between elevated glucose and each of the main pathways responsible for hyperglycemic damage: glucose-induced activation of protein kinase C, formation of advanced glycation end products, sorbitol accumulation, and NF- $\kappa$ B activation (9). The data presented in this paper demonstrate that hyperglycemia-induced mitochondrial ROS overproduction also underlies diabetic platelet hyperaggregation.

Numerous studies have observed enhanced activity of the arachidonate pathway and increased thromboxane A<sub>2</sub> formation by platelets from diabetic patients, which could be responsible for the hypersensitivity of platelets to several agonists, including collagen, in diabetes (17–19). Syk activation and the subsequent phosphorylation of PLC $\gamma$ 2 are essential for arachidonic acid release, 5-hydroxytryptamine secretion, and ultimately platelet aggregation in response to collagen (6,20). Therefore, inhibition of redox-

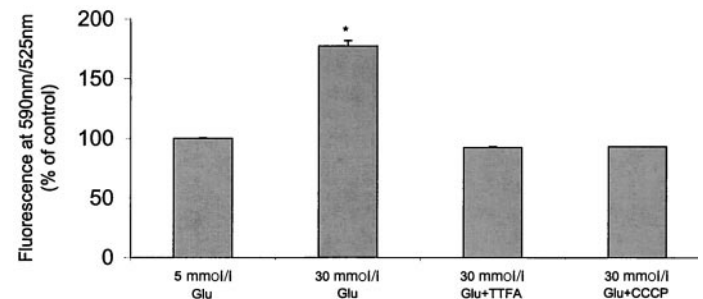


FIG. 7. Effects of TTFA or CCCP on mitochondrial membrane potential in human platelets. PRP was preincubated with 5 or 30 mmol/l glucose in the presence or absence of TTFA or CCCP for 60 min. After centrifugation, the platelets were further incubated with JC-1, and then fluorescence at 590 and 525 nm was monitored. \* $P < 0.01$  compared with values incubated with 5 mmol/l glucose alone.

sensitive PTP activity by hyperglycemia-induced mitochondrial ROS and the subsequent increased tyrosine phosphorylation and activation of Syk may be an initial event leading to platelet dysfunction in diabetes. ROS have also been implicated in the inhibition of PTP activity and tyrosine phosphorylation of Syk in lymphocytes and macrophages (13,14,21). Hyperglycemia-induced mitochondrial superoxide overproduction may thus potentially play an important role in activation of these inflammatory cells as well as platelets, which would also participate in the pathogenesis of accelerated atherosclerosis in diabetes (22).

PDGF, a potent mitogen and chemotactic factor to smooth muscle cells, is also a key factor mediating restenosis after percutaneous transluminal coronary angioplasty (23,24). Recently, abciximab, a platelet glycoprotein IIb/IIIa inhibitor, was found to improve outcomes in diabetic patients after percutaneous transluminal coronary angioplasty (25). These observations suggest that hyperglycemia-induced platelet hyperaggregation and the subsequent release of PDGF may be one of the potential causes of the high frequency rates of restenosis in patients with diabetes (26,27). Inhibition of hyperglycemia-induced mitochondrial ROS overproduction may provide a new therapeutic strategy for the treatment of diabetic patients with acute coronary syndromes.

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