PP2A contributes to endothelial death in high glucose: inhibition by benfotiamine

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Du Y, Kowluru A, Kern TS. PP2A contributes to endothelial death in high glucose: inhibition by benfotiamine. Am J Physiol Regul Integr Comp Physiol 299: R1610–R1617, 2010. First published September 29, 2010; doi:10.1152/ajpregu.00676.2009.—Endothelial death is critical in diabetic vascular diseases, but regulating factors have been only partially elucidated. Phosphatases play important regulatory roles in cell metabolism, but have not previously been implicated in hyperglycemia-induced cell death. We investigated the role of the phosphatase, type 2A protein phosphatase (PP2A), in hyperglycemia-induced changes in signaling and death in bovine aortic endothelial cells (BAEC). We explored also the influence of benfotiamine on this phosphatase. Activation of PP2A was assessed in BAEC by the extent of methylation and measurement of activity, and the enzyme was inhibited using selective pharmacological (okadaic acid, sodium fostriecin) and molecular (small interfering RNA) approaches. BAECs cultured in 30 mM glucose significantly increased PP2A methylation and activity, and PP2A inhibitors blocked these abnormalities. PP2A activity was increased in BAEC and in retina from diabetic rats. NF-κB activity and cell death in BAEC were significantly increased in 30 mM glucose and inhibited by PP2A inhibition. NF-κB and cell death in BAEC were significantly increased in 30 mM glucose and inhibited by PP2A inhibition. NF-κB played a role in the hyperglycemia-induced death of BAEC, since blocking its translocation with SN50 also inhibited cell death. Inhibition of PP2A blocked the hyperglycemia-induced dephosphorylation of NF-κB and Bad, thus favoring cell survival. Incubation of benfotiamine with BAEC inhibited the high glucose-induced activation of PP2A and NF-κB and cell death, as well as several other metabolic defects, which likewise were inhibited by inhibitors of PP2A. Activation of PP2A contributes to endothelial cell death in high glucose, and beneficial actions of benfotiamine are due, at least in part, to inhibition of PP2A activation.

cell death; endothelium; hyperglycemia; diabetes; phosphatase

HYPERGLYCEMIA IS AN IMPORTANT factor in the development of diabetic vascular disease (2, 28). The molecular mechanisms by which diabetes causes this vascular damage have not been fully elucidated, but endothelial cells cultured under diabetic-like conditions (high glucose) develop a number of biochemical abnormalities and can die. Oxidative stress, and especially superoxide-mediated disruption of glycolysis, have been reported to play a role in the development of these abnormalities (36). Benfotiamine inhibits development of these biochemical abnormalities, reportedly by activating transketolase and thus relieving the glycolytic block (17). Since the reaction mediated by transketolase is reversible, however, it seemed that the benfotiamine-mediated diversion of glycolytic intermediates would slow as products of transketolase were generated. We thus sought other explanations for the beneficial effect of benfotiamine in hyperglycemia.

Sugar phosphates have been reported to regulate the activity of type 2A protein phosphatase (PP2A) (22), a family of phosphoserine- and phosphothreonine-specific enzymes expressed in eukaryotic cells that regulate metabolism, transcription, translation, cell cycle, signal transduction, and differentiation (9, 14, 19, 20, 51). Moreover, previous studies have demonstrated the role of PP2A in regulating prodeath functions of apoptosis regulators, such as Bad and forkhead box O (8), suggesting that protein phosphatases such as PP2A can regulate apoptosis. Thus we postulated that hyperglycemia increased PP2A activity in endothelial cells, that the previously observed endothelial dysmetabolism and death in high glucose were related to increased PP2A activity, that selective inhibition of PP2A would inhibit those hyperglycemia-mediated abnormalities and cell death, and that benfotiamine was a PP2A inhibitor.

MATERIALS AND METHODS

Benfotiamine, okadaic acid and fostriecin sodium, adenosine-2’3’-dialdehyde (Ado), homocysteine, penicillin-streptomycin solution, N2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer (HEPES), trispyrinediaminediametraacetatic acid (EDTA) solution, lucigenin (bis-N-methylacridinium nitrate), type I collagenase, and all other chemicals were of the highest commercially available quality and were purchased from Sigma Chemical (St Louis, MO). Small interfering RNA (siRNA) SMARTpool PP2A catalytic subunit (α-isofom) was purchased from Dharmacom (Lafayette, CA); SN50 was purchased from Calbiochem (San Diego, CA). Antibodies were purchased from Upstate (Temecula, CA) against the catalytic subunit of PP2A (1:3,000 dilution), the A subunit of PP2A (1:200 dilution), the B subunit of PP2A (1:1,000 dilution), and methyl-PP2A (C subunit, Leu309, 1:100 dilution). Antibodies were purchased from Cell Signaling Technology (Danvers, MA) against Bad and phospho-Bad (p-Bad) (Ser136, 1:1,000 dilution), MEK and p-MEK (Ser217 and Tyr204, 1:1,000 dilution), GSK-3 and p-GSK-3 (Ser21 and Thr27, 1:1,000 dilution), AKT and p-AKT (Thr308, 1:1,000 dilution), and from Proteintech Group (Chicago, IL) against ICAM-1 (1:1,000 dilution).

Cell culture. The bovine aortic endothelial cell (BAEC) line was obtained from LONZA (Allendale, NJ). Cells were cultured and passaged in endothelial cell basal medium with EGM-MV Single-Quots (contains recombinant human EGF, GA-1000, hydrocortisone, bovine brain extract, and FBS) containing 5 mM glucose. Cells were incubated in 5 or 30 mM glucose, with or without benfotiamine (50 μM) (17), okadaic acid (5 mM) (21), fostriecin sodium (2 mM) (52), siRNA against PP2A catalytic subunit (200 pmol/well) or SN50 (6 μM). Various concentrations of okadaic acid have been used in the literature, so we performed a dose-response curve and selected the 5 mM dose as the dose that best inhibited PP2A activity in BAEC.
without causing cell death. Media was changed every other day for up to 4 days. Cells were harvested by treating with a trypsin-EDTA solution (0.5 and 0.02% respectively, wt/vol). To investigate a possible contribution of hyperosmolarity to the increase in PP2A activity, BAECs were incubated for 4 days in 5 mM glucose, 30 mM glucose, and 5 mM glucose + 25 mM mannitol.

**Western blot analysis.** Cells were homogenized in buffer containing protease inhibitors (leupeptin 1 μg/ml; aprotinin 1 μg/ml), and 30 μg of protein from each sample were loaded on 10% PAGE-SDS and transferred onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Membranes were blocked overnight at 4°C with 5% nonfat dry milk and incubated with primary antibodies for 1 h at room temperature. Blots were washed and incubated with anti-mouse IgG antibody or anti-rabbit IgG antibody coupled to horseradish peroxidase (Bio-Rad) at a dilution of 1:3,000 for another hour. After another extensive washing, protein bands detected by the antibodies were visualized by enhanced chemiluminescence (Amersham) and evaluated by densitometry (Molecular Dynamics). Prestained protein markers (Bio-Rad) were used for molecular mass determinations. To ensure equal loading among lanes, the membranes were stained with Ponceau S (Sigma, St. Louis, MO) and the intrinsic protein actin (mouse monoclonal anti-β-actin antibody; Sigma) before and after, respectively, staining for above antibodies (11). Protein concentration of cell lysates was measured by the Bradford procedure using the protein dye regent from Bio-Rad Laboratories and BSA as a standard.

**Dot blot for argpyrimidine.** Argpyrimidine was measured as a well-characterized advanced glycation end product. Dot-blot assays were performed in a 96-well plate format using a Bio-dot microfiltration manifold (Biorad). Typically, 50 μg of total protein were suspended in 100 μl of Laemmli buffer and absorbed onto a nitrocellulose membrane (Millipore) by gravity flow. The filter was then washed three times with 200 μl of phosphate-buffered saline (PBS) per well, applying a constant vacuum flow. Then dot blots were treated similarly to Western blots. The antibody against argpyrimidine (41), an indicator of advanced glycation end-product levels, was generously donated by Dr. R. Nagaraj (Case Western Reserve University, Cleveland, OH).

**Nuclear extraction.** Nuclear extracts from BAECs were prepared with a commercial nuclear extract kit (Panomics, Redwood City, CA) following the instructions of the manufacturer. Exponentially growing BAECs were washed twice with ice-cold PBS, lysed on ice for 10 min in 1.0 ml extraction buffer A mix (10 mM HEPES; pH 7.9, 10 mM KCl; 10 mM EDTA; protease inhibitor cocktail, 1 mM dithiothreitol, and 4% IGEPAL) with gently shaking. Cells were harvested from the culture dishes by scraping into the extraction buffer and then pipetting up and down several times to disrupt cell clumps. Nuclei were collected by centrifugation at 15,000 g for 3 min at 4°C. The resulting pellet was then resuspended in 150 μl of extraction buffer B (20 mM HEPES, pH 7.9; 400 mM NaCl; 1 mM EDTA; 10% glycerol; protease inhibitor cocktail; 1 mM dithiothreitol) and mixed at 4°C for 2 h. The mixture was then centrifuged for 5 min at 15,000 g at 4°C, and the supernatant collected. Protein concentrations were measured by the Bradford procedure using the protein dye reagent from Bio-Rad Laboratories and BSA as a standard.

**Transbinding assay.** A NF-kB (p50) probe was used with the transbinding ELISA kit from Panomics (Redwood City, CA), which employs an oligonucleotide-containing NF-kB consensus binding site immobilized on a 96-well plate. Activated NF-kB from cell nuclear extracts specifically binds to this oligonucleotide, and the kit thus measures two components of NF-kB activation (nuclear translocation and DNA binding). Complexes bound to the oligonucleotide were detected by antibody directed against the p50 subunit and a secondary horseradish peroxidase-conjugated antibody, which was quantified by spectrophotometry. Cold control probe (oligonucleotide) was used as a competitor to control for nonspecific binding.

**Assay of phosphatase activity.** PP2A activity was measured by using threonine phosphopeptide (KRPThrR) as the substrate with the PP2A Immunoprecipitation Phosphatase Assay Kit (Upstate Biotechnology). The cells were lysed in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1.5 mM magnesium chloride, 1% Triton X-100, 1 μg of leupeptin/ml, 50 units of aprotinin/ml, 1 mM phenylmethylsulfonyl fluoride). Clarified supernatants were incubated with anti-PP2A antibody and protein A-agarose for 2 h at 4°C. After the beads were washed three times, the diluted phosphopeptide (750 μM) and Ser/Thr assay buffer were added, and the mixture was incubated for 10 min at 30°C, followed by addition of the Malachite Green Phosphate Detection Solution. PP2A activity in the reactive system was determined by measuring the absorbance at 650 nm and comparing absorbance values of samples to negative controls containing no enzyme.

**PP2A siRNA transfection.** siRNA against the C subunit (α-isoform) of PP2A (PP2Ac) was obtained from siGENOME SMARTpool (Dharmacon, Lafayette, CO; cat no. M-0003598). The siRNA was transfected into BAECs using a transfection reagent (Dharmacon, Lafayette, CO), according to the manufacturer’s protocol. In brief, six-well plates of BAECs were cultured to 80% confluence, and then each well was resuspended in 2 ml antibiotic-free complete media with added 100 μl Dharmacon transfection reagent and 1.5 μg of PP2A siRNA SMARTpool or 1.5 μg of control siRNA (negative control provided in the siRNA test kit, Dharmacon). Western blots and other experiments were performed 96 h after siRNA transfection.

**Cell death assay.** Cell death was evaluated based on detection of histone-associated DNA fragments in cytoplasm using a cell death detection ELISA kit (Roche Molecular Biochemicals). Briefly, the cytosolic fraction (20,000 g supernatant) of cultured BAECs were used as an antigen source in a sandwich ELISA, with a primary anti-histone antibody coated to microtiter plates and a secondary anti-DNA antibody conjugated with peroxidase. Double absorbance at 405 and 495 nm was measured against substrate solution as a blank. The data are expressed as the fold increases over the values of cells cultured in 5 mM.

**Detection of superoxide anion.** Lucigenin (bis-N-methylacridinium nitrate), an acridinium dinitrate compound that emits light on reduction and interaction with the superoxide anion (10, 16), was used to measure superoxide anion production. To assess superoxide production in BAEC cells in vitro, cells incubated in 5 and 30 mM glucose were harvested from the dish with type I collagenase (1 mg/ml, in the presence of 2 mg/ml bovine serum albumin). Cells were pelleted by centrifugation (200 g, 4°C, 5 min), the supernatant was discarded, and the pellet resuspended at 2 × 10^6 cells/ml in a PBS solution containing 10 mmol/l glucose and 1 mg/ml bovine serum albumin, and stored on ice until use. To measure superoxide production, 9 × 10^5 cells were added to a polypropylene tube to incubate in the dark at 37°C in 95% O2/5% CO2 for 30 min. After equilibration, 0.5 mM lucigenin was added to the tube, and photon emission was measured over 10 s; repeated measurements were made over a 10-min period in a luminometer (Analytical Luminescence Laboratory, San Diego, CA). Reaction blanks (vials containing all components except retinas) were counted, and these blank values subtracted from all other readings.

**Regulation of transketolase activity by PP2A and benfotiamine.** BAECs were incubated in 5 and 30 mM glucose for 4 days, as described above. The cells incubated in 30 mM glucose additionally received okadac acid, benfotiamine, or nothing for the full duration. Media was changed every other day. On the 4th day, cells were isolated and homogenized, and the homogenates used to assay transketolase activity, as described previously (17). In brief, BAEC lysates and substrate cocktail were added to 96-well plates, and the decreased absorbance was monitored to determine the rate of NADH oxidation (and thus transketolase activity). Protein was measured by the Bradford procedure using the protein dye reagent from Bio-Rad Laboratories and BSA as a standard. Results are the average of n = 6 plates for each condition.
Diabetic animals. Diabetes was induced in Lewis rats using streptozotocin (60 mg/kg body wt). Insulin was given (subcutaneously) as needed so that animals did not lose weight. The insulin dose (1–2 units given every 2–3 days) intentionally was not sufficient to prevent chronic hyperglycemia. Blood glucose after an overnight fast was $13.1 \pm 2.6$ mM for diabetics and $2.8 \pm 0.2$ mM for nondiabetics. Diabetics and nondiabetic controls were killed under deep anesthesia after 2 mo of study, and neural retina and aorta were frozen for assay of PP2A activity. Sixteen rats (8 normal and 8 diabetic rats) were used in these in vivo experiments, and animal handling was performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and the animal experiments were approved by Institutional Animal Care and Use Committee.

Statistical analysis. Data are expressed as means ± SD. Statistical analysis was performed using the ANOVA followed by Fisher’s test, to correct for multiple comparisons.

RESULTS

Elevated glucose increases PP2A activity. Incubation of BAECs in 30 mM glucose for 4 days significantly increased PP2A activity compared with that measured at 5 mM glucose ($P < 0.05$; Fig. 1A). In BAEC incubated in 30 mM glucose, selective PP2A inhibitors (either okadaic acid or fostriecin) significantly inhibited the increase in PP2A activity (both $P < 0.05$; Fig. 1A). Okadaic acid and fostriecin are reported to have different binding sites on PP2A (48). siRNAs against PP2A catalytic subunit (PP2Ac) significantly inhibited PP2A expression and activity in BAECs growing in 30 mM glucose (Fig. 1, B and C). A nonspecific negative control siRNA had no effect on PP2A activity or protein expression (Fig. 1, B and C).

A time course for PP2A activity in BAECs showed that incubation of the cells with high glucose significantly increased PP2A activity by the 4th day, but had no significant changes before then. Neither the expression of PP2A subunits nor the subcellular localization of the enzyme (cytoplasm vs. nucleus) changed during the 4-day course of incubation in high glucose (data not shown), and thus these did not correlate with the activation of the enzyme.

Methylation of PP2A. PP2A activity is regulated by reversible methylation of Leu309 of the catalytic subunit (6, 12, 29, 52). In an effort to explain the mechanism for the hyperglycemia-induced increase in PP2A activity, we, therefore, measured methylation of Leu309. Although total expression of PP2A did not change in high glucose, methylation of PP2A catalytic subunit on Leu309 clearly increased after 4 days in high glucose ($P < 0.05$; Fig. 1D). Okadaic acid significantly inhibited the glucose-induced upregulation of carboxyl methylation of the PP2A catalytic subunit in BAECs ($P < 0.05$; Fig. 1D). We also attempted to inhibit the glucose-induced increase in activity by nonspecific inhibition of methylation using Adox or Adox plus homocysteine (18), but the result was toxic to BAEC (data not shown). Thus the observed data are consistent with available evidence that indicates that methylation of Leu309 accounts for the increase in PP2A activity.

Hyperosmolarity has been reported to result in methylation and activation of PP2A (1). To ensure that the increase in...
activity seen in 30 mM glucose was not due to hyperosmolarity, we compared activation of PP2A when BAEC were cultured in 30 mM glucose vs. the osmotically comparable 5 mM glucose + 25 mM mannitol. Whereas 30 mM glucose increased PP2A activity compared with that in 5 mM glucose (125.3 vs. 100%, $P < 0.05$), incubation in 5 mM glucose + 25 mM mannitol (102.39%) had no significant effect. Thus the observed increase in PP2A methylation and activation is due to a glucose-mediated process and not due to hyperosmolarity.

**Increased activity of PP2A contributes to endothelial death in elevated glucose.** High glucose increased death of BAEC. Inhibition of PP2A by okadaic acid, siRNA against PP2A, or foscarnet (Figs. 2A) significantly inhibited this glucose-induced endothelial cell death ($P < 0.01$).

**Mechanisms involved in endothelial death by PP2A.** To explore mechanisms by which PP2A activation might contribute to endothelial death in high glucose, we evaluated several phosphorylation-dependent pathways known to contribute to cell death. To focus on PP2A-related events, we were interested in molecules whose phosphorylation state was both changed in high glucose and normalized in the presence of okadaic acid. Neither p-AKT, p-GSK-3β, p-MEK, nor p-ERK met these criteria (data not shown). In contrast, increased activity of PP2A was clearly associated with decreased phosphorylation of Bad at Ser136. The results show that phosphorylated Bad (not total Bad) was significantly decreased in high-glucose compared with that measured in normal glucose levels, and okadaic acid inhibited this decrease ($P < 0.05$; Fig. 3). This finding suggests that PP2A inhibitors inhibit endothelial death in high glucose, in part, by inhibiting the PP2A-dependent dephosphorylation of Bad.

Death of aortic endothelium also has been attributed to inflammation (5, 47). NF-κB is an important regulator of inflammation, and inflammation is increased in diabetes (23, 32, 43). Consistent with a contribution of NF-κB to endothelial death in hyperglycemia, inhibition of NF-κB translocation into the nucleus using SN50 (6 μM) reduced death of BAEC in high glucose ($P < 0.05$; Fig. 4A). Since NF-κB activity can be regulated by phosphorylation (7, 13), we examined the effects of PP2A on NF-κB activation in hyperglycemia. NF-κB activation was significantly increased in 30 mM glucose compared with that in 5 mM glucose, and okadaic acid or siRNA against PP2A catalytic subunit (B) inhibited this increase ($P < 0.05$; Fig. 4B). Phosphorylation of IκB, which is needed to allow translocation of NF-κB into the nucleus (activation), was significantly increased in high glucose ($P < 0.05$), and the incubation also in okadaic acid inhibited this glucose-induced increase by 87% ($P < 0.05$; Fig. 4C). Inhibition of PP2A in BAEC also inhibited the hyperglycemia-induced induction of ICAM-1, a proinflammatory molecule whose expression is regulated by NF-κB ($P < 0.05$; Fig. 4D). This data suggested that NF-κB activation is regulated by PP2A in these cells. To investigate the opposite possibility (that NF-κB activation caused PP2A activation), we measured the effect of SN50 on methylation of PP2A. SN50 inhibited the glucose-induced methylation of PP2A by 70%, but the result was not statistically significant (not shown).

Two other sequelae of hyperglycemia that were significantly inhibited following inhibition of PP2A by okadaic acid are the excessive generation of superoxide by the BAEC in high glucose ($P < 0.05$; Fig. 5A) and generation of the advanced
glycation end product, argpyrimidine (reduced 11%, $P < 0.05$; Fig. 5B).

Benfotiamine as an inhibitor of hyperglycemia-induced activation of PP2A, inflammation, and endothelial death. Benfotiamine exhibited a number of activities that were consistent with it acting as an inhibitor of PP2A. It significantly inhibited the hyperglycemia-induced increase in PP2A activity (Fig. 1A) and carboxymethylation of the PP2A catalytic subunit (Fig. 1D). It also inhibited the hyperglycemia-induced increase in cell death of BAEC (Fig. 2A), dephosphorylation of Bad (Fig. 3), activation of NF-κB (Fig. 4A), and generation of superoxide and argpyrimidine (Fig. 5; all $P < 0.05$). Unlike okadaic acid though, benfotiamine apparently is not a direct inhibitor of PP2A. One-hour incubation of BAEC with benfotiamine did not inhibit PP2A activity in high glucose, but 4-day incubation of the cells in benfotiamine and high glucose did inhibit the activity.

Regulation of transketolase activity by PP2A and benfotiamine. Incubation of BAEC in 30 mM glucose tended to reduce (7%) transketolase activity, but this did not achieve statistical significance in our experiments. Nevertheless, incubation of the cells in either benfotiamine ($P < 0.05$) or okadaic acid ($P < 0.001$) significantly increased (16 and 22%, respectively) transketolase activity compared with that in untreated BAEC grown in 30 mM glucose without therapy (not shown).

Diabetes increases PP2A in retina and aorta. PP2A activity was measured in tissues from diabetic animals to examine if this pathway is activated also in vivo. PP2A activity was increased 1.8-fold in retinas from diabetic rats compared with nondiabetic rats ($P < 0.05$; $3.11 \pm 0.64$ and $1.77 \pm 0.42$ pmol·μg$^{-1}$·15 min$^{-1}$, respectively). Likewise, diabetes significantly increased PP2A activity also in aorta ($P < 0.05$; $2.2 \pm 0.77$ and $1.5 \pm 0.61$ pmol·μg$^{-1}$·15 min$^{-1}$, respectively).

DISCUSSION

PP2A is a major intracellular serine/threonine phosphatase that plays important roles in maintaining endothelial cell physiological functions, including regulation of endothelial cell cytoskeletal structure, protection of the endothelial cell barrier, regulation of glucose metabolism and fat synthesis, and regulation of endothelial nitric oxide synthase phosphorylation status (22, 37, 45, 46, 52). The enzyme is composed of a dimeric core enzyme, including a scaffolding A subunit and a catalytic C subunit and a regulatory B subunit. Activity of PP2A is influenced by the reversible methylation of the COOH-terminus of the C subunit (6, 12, 15, 30, 52). In the present study, high glucose significantly increased the methylation of the PP2A catalytic subunit, and therapies that inhibited that methylation in high glucose also inhibited the increase in PP2A activity, suggesting that hyperglycemia likewise increases PP2A activity by increasing methylation of the catalytic subunit. How methylation of PP2A is regulated has remained elusive, but leucine carboxyl methyltransferase and protein phosphatase methylesterase have been implicated in specific subcellular compartments (25, 31). Selective inhibitors of these enzymes have not been developed, but total inhibition of methylation using Adox and homocysteine was toxic to the BAECs.

Excessive PP2A activation in pathological conditions can result in cell damage or dysfunction (8, 33, 34). The data presented in the present study show that exposure of BAEC to levels of glucose comparable to those seen in diabetes significantly increased PP2A activity, that this increase in phosphatase activity contributed to cell death in high glucose, and that both of these abnormalities could be inhibited by PP2A-selective inhibitors.
PP2A is known to regulate a variety of cell signaling pathways, some of which were found to have been altered in high glucose. Bad is a proapoptotic member of the Bcl-2 family, whose dephosphorylated form translocates to the mitochondria and inactivates BCL-xL and Bcl-2 (39, 44). PP2A can dephosphorylate Bad, thereby inhibiting the anti-apoptotic activity of Bcl-2 and thus contributing to cell death (8, 35). In our study, BAEC in high glucose displayed a substantial decrease in phosphorylation of Bad, and this loss of phosphorylation was associated with an increase in cell death. These abnormalities were inhibited by okadaic acid or siRNA against PP2A, suggesting that PP2A-mediated processes (perhaps involving dephosphorylation of Bad) contributed to the cell death.

Inflammation is known to be important in atherosclerosis and other complications of diabetes (4) and to contribute to hyperglycemia-induced cell death (42). Activation of NF-κB is crucial in the proinflammatory cascade (27, 40, 50). Our data suggests that the activation of PP2A in high glucose contributes to the hyperglycemia-induced inflammatory response (as evidenced by activation of NF-κB and upregulation of ICAM-1 expression), and this thus might contribute also to endothelial cell death in high glucose. Mechanisms by which this phosphorylase enzyme can regulate NF-κB are complex. PP1/PP2A have been reported to directly dephosphorylate p65 at Ser468 to induce the activity of NF-κB (7), and PP2A indirectly regulates IKK activity (and thereby activation of NF-κB) (26, 38). Other reports, however, indicate that PP2A dephosphorylates IKK-β to inhibit the NF-κB activity (3). Summation of these multiple phosphorylation/dephosphorylation reactions likely determines the overall regulation of NF-κB by PP2A.

Benfotiamine inhibited the activation of PP2A and cell death in high glucose. Since siRNA against PP2A likewise inhibited cell death, we postulate that the effects of benfotiamine on inflammation and cell death were mediated, at least in part, by inhibition of PP2A. Hammes et al. (17) previously reported that benfotiamine inhibited the hyperglycemia-induced activation of several metabolic abnormalities that have been postulated to play a role in the development of diabetic complications, including formation of advanced glycation end products and activation of NF-κB (49). They interpreted their studies as indicating that benfotiamine activated the pentose phosphate pathway enzyme, transketolase, converting glyceraldehyde-3-phosphate and fructose-6-phosphate into pentose-5-phosphates and other sugars and thus relieving a “backup” in the glycolytic pathway. Our studies using benfotiamine showed similar effects, but further suggest that benfotiamine exerts at least some of these actions in high glucose by inhibiting PP2A activation. Whether effects of benfotiamine on PP2A activation are direct or indirect remains to be learned. Okadaic acid is known to inhibit PP2A by binding specifically to the COOH-terminal region of PP2Ac and inhibiting its methylation (24, 25), so it was interesting that benfotiamine shared the ability to inhibit PP2A.

In summary, PP2A activity increases in BAEC incubated under hyperglycemic conditions, and in at least aorta and retina from diabetic animals. The increase in phosphatase activity, which may, in part, be due to increase in the carboxymethylation of PP2Ac, contributes to hyperglycemia-induced inflammatory response and accelerated cell death of BAEC in high glucose (Fig. 6). Importantly, therapies (including benfotiamine) that inhibit PP2A activation were able to inhibit vascular cell death in high glucose. Death of vascular cells is regarded as an important contributor to the development of hyperglycemia-induced vascular disease in diabetes, suggesting that inhibiting the activation of PP2A and perhaps other
phosphatases might be novel targets to inhibit vascular injury in diabetes.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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