α-Tocopherol Decreases Superoxide Anion Release in Human Monocytes Under Hyperglycemic Conditions Via Inhibition of Protein Kinase C-α

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Diabetes is a major risk factor for premature atherosclerosis, and oxidative stress appears to be an important mechanism. Previously, we showed that diabetic monocytes produce increased superoxide anion (O_2^-), and α-tocopherol (AT) supplementation decreases this. The aim of this study was to elucidate the mechanism(s) of O_2^- release and inhibition by AT under hyperglycemic (HG) conditions in monocytes. O_2^- release, protein kinase C (PKC) activity, and translocation of PKC-α and -βII and p47phox were increased in THP-1 cells (human monocytic cell line) under HG (15 mmol/l glucose) conditions, whereas AT supplementation inhibited these changes. AT, NADPH oxidase inhibitors (apocynin and diphenyleneiodonium chloride [DPI]), and an inhibitor to PKC-α and other isoforms (2,2′,3,3′,4,4′-hexahydroxy-1,1′-biphenyl-6,6′-dimethanol dimethyl ether [HBDDE]) but not PKC-β II (LY379196) decreased O_2^- release and p47phox translocation. Antisense oligodeoxynucleotides to PKC-α and p47phox but not to PKC-βII inhibited HG-induced O_2^- release and p47phox translocation in THP-1 cells. Under HG conditions, reactive oxygen species release from monocytes was not inhibited by agents affecting mitochondrial metabolism but was inhibited in human endothelial cells. We conclude that under HG conditions, monocytic O_2^- release is dependent on NADPH oxidase activity but not the mitochondrial respiratory chain; HG-induced O_2^- release is triggered by PKC-α, and AT inhibits O_2^- release via inhibition of PKC-α.
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Diabetes is a major risk factor for premature atherosclerosis, and oxidative stress plays an important role (1). The monocyte is a pivotal cell in atherogenesis. Monocytes from type 2 diabetic patients secrete increased superoxide anion (O_2^-) compared with control subjects (2). Hyperglycemia could contribute to diabetic complications, and evidence suggests that glycemic control can ameliorate vascular complications (3,4). There is limited data available on the mechanisms by which hyperglycemia mediates its effects in monocytes.

Recently it has been shown in endothelial cells that hyperglycemia induces mitochondrial superoxide overproduction (5). To date, the role of mitochondria in monocytic O_2^- release under hyperglycemic (HG) conditions has not been reported. The possible mechanisms by which hyperglycemia, in monocytes, can cause adverse effects is via the activation of diacylglycerol (DAG)-sensitive protein kinase C (PKC) (6). PKC activity is increased in retina, aorta, heart, and renal glomeruli of diabetic rats as well as in cultured vascular cells or tissues exposed to HG conditions (6). It has been shown that monocytic O_2^- production is mediated via PKC under euglycemic conditions (7). Koya and King (6) have shown that hyperglycemia may mediate adverse effects via PKC-β by activation of the DAG-PKC pathway, suggesting a role in diabetic complications. However, Igarishi et al. (8) had shown that PKC-δ but not -β was activated in aortic smooth muscle cells under HG conditions. It appears that different PKC isoforms are activated by glucose in different cells. To date, the mechanism of O_2^- release in monocytes under HG conditions has not been elucidated.

O_2^- is formed during the respiratory burst by NADPH oxidase of monocytes and other phagocytic cells. NADPH oxidase consists of several membrane-bound subunits (gp91, nox, and p22phox) and cytosolic subunits (p47phox, p67phox, p40phox, and Rac2) (9). On activation, some components are phosphorylated and translocated to membrane and form the catalytically active oxidase. Activation of NADPH oxidase is PKC dependent, and phosphorylation of p47 phox occurs via PKC (10). It has been reported that PMA, a potent activator of PKC, stimulates superoxide production and p47 phox phosphorylation (11).

α-Tocopherol (AT) is a major lipid-soluble antioxidant in plasma. Several lines of evidence support the relationship between low AT levels and the development of

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AOAC, aminooxyacetic acid; AT, α-tocopherol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DAG, diacylglycerol; DCF-DA, 5-(and-6)-carboxy-2′7′-dichlorodihydro-fluorescein diacetate; DPI, diphenyleneiodonium chloride; EC, endothelial cell; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; HAEC, human aortic endothelial cells; HBDDE, 2,2′,3,3′,4,4′-hexahydroxy-1,1′-biphenyl-6,6′-dimethanol dimethyl ether; HG, hyperglycemic; 4-HOCA, α-cyano-4-hydroxycinnamic acid; NG, normal glucose; O_2^-, superoxide anion; ODN, oligodeoxynucleotide; PKC, protein kinase C; ROS, reactive oxygen species; SOD, superoxide dismutase; TTFA, theonyl-trifluoroacetone.

atherosclerosis (12,13). Hyperglycemia-induced lipid peroxidation is inhibited by AT in erythrocytes (10). In addition to antioxidant effects, AT has effects on cell functions. It has been shown that AT inhibits smooth muscle cell proliferation and platelet aggregation and preserves endothelium-dependent relaxation via inhibition of PKC (14). AT supplementation significantly decreased monocyte O_2^-, proinflammatory cytokine release, plasma C-reactive protein, and monocyte-endothelial adhesion (2,15). AT supplementation in diabetic patients decreases LDL oxidation and urinary isoprostanes (16). It has also been shown that AT inhibits the HG-induced formation of advanced glycation end products and reactive oxygen species (ROS) (17).

Though there are several reports showing that different PKC isoforms are activated under hyperglycemia, no study has clearly shown the mechanism of O_2^- release from human monocytes. In this study, we report on the mechanism of production of O_2^- and its inhibition by AT in monocytes under HG conditions.

RESEARCH DESIGN AND METHODS

Reagents. The human monocytic cell line THP-1 cells were obtained from American *Type Culture* Collection. Endotoxin-free, glucose-free RPMI-1640 media and fetal bovine serum (FBS) were purchased from Gibco BRL (Carlsbad, CA). Antibiotics, glutamine, phenylmethylsulfonyl fluoride, glucose, HEPES, protease inhibitor cocktail, Triton X-100, dithiothreitol, rotenone, aminooxyacetic acid (AOAC), α-cyano-4-hydroxycinnamic acid (4-HOCA), theonyl-trifluoroacetone (TTFA), and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were from Sigma Chemical. Antibodies to PKC, PKC-α, PKC-βII, and p47phox were obtained from Santa Cruz Company. 2,2′,3,3′,4,4′-hexahydroxy-1,1′-biphenyl-6,6′-dimethanol dimethyl ether (HBDDE), apocynin, and diphenyleneiodonium chloride (DPI) were obtained from Calbiochem, and Eli Lilly kindly provided LY379196. Polyvinylidene difluoride (PVDF) membranes and Tris-glycine gels were from Invitrogen. Bicinchoninic acid (BCA) kit was obtained from Pierce. Enhanced chemiluminescence (ECL) and PKC activity kits were purchased from Amersham Pharmacia. Oligonucleotides were purchased from Integrated DNA Technologies.

Cell culture. THP-1 cells were maintained in endotoxin-free RPMI-1640 containing 5.5 mmol/l glucose, 50 μmol/l mercaptoethanol, 10% FBS, 2 mmol/l glutamine, 1 mmol/l sodium pyruvate, and 10 mmol/l HEPES and used for experiments between third and fifth passages. Cells were cultured (1×10^6 cells/ml) for 3 days in either 5.5 mmol/l (normal glucose [NG]) or 15 mmol/l glucose (HG conditions), and as an osmotic control, 9.5 mmol/l mannitol was added along with NG. Cell viability, as determined by trypan blue exclusion, was >92%. AT (100 μmol/l) was added to cells with NG/HG, with daily changes in media. For inhibitor studies, 50 μmol/l HBDDE or 30–150 nmol/l LY379196 were added to cells at the end of the third day in HG conditions and incubated overnight, as per our preliminary data. Cells were also incubated with mitochondrial respiratory chain inhibitors, 100 μmol/l AOAC, 5 μmol/l rotenone, 10 μmol/l TTFA, and 0.5 μmol/l CCCP for 24 h along with 15 mmol/l glucose (5), and ROS was determined.

Measurement of O_2^- production. O_2^- production was measured by the superoxide dismutase (SOD)-inhibitable reduction of cytochrome C, as reported previously (2). Cells were incubated in RPMI-1640 without phenol red for 60 min at 37°C with or without SOD (100 μg/ml) and 80 μmol/l acetylated ferricytochrome C in a total volume of 1 ml. Results were expressed as nanomoles per minute per milligram cell protein.

Measurement of ROS production. ROS generation was detected using fluorescent probe, 5-(and-6)-carboxy-2′7′-dichlorodihydro-fluorescein diacetate (DCF-DA). Cells (1×10^5 ml^{-1}) were loaded with 10 μmol/l DCF-DA, incubated for 1 h at 37°C, and analyzed at 0 and 60 min in CytoFlour multiwell reader. ROS production was determined by subtracting the values from initial intensities (0 min) and expressed per milligram cell protein.

Determination of PKC activity. PKC activity in THP-1 cells was determined by radioimmunoassay. It was based on the PKC-catalyzed transfer of the γ-phosphate group of ATP to a PKC-specific peptide. PKC activity was expressed as nanomoles of phosphate transferred per million cells.

Western blotting. At the end of culture, cells were lysed, and membrane fractions were isolated as described by Ceolotto et al. (18). Membrane proteins (10–30 μg) were resolved in 10% Tris-glycine gel, and blotting was performed with specific primary and secondary antibodies. Blots were visualized by ECL detection system.

Incubation of cells with oligodeoxynucleotides. Cells were incubated with oligodeoxynucleotides (ODNs) to PKC-α and PKC-βII along with HG conditions and added daily. The sequence for PKC-α isoenzyme-specific antisense oligonucleotide was 5′-CGC CGT GGA GTC GTT GCC CG-3′; the sense sequence was 5′-CGG GCA ACG ACT CCA CGG CG-3′ (7). The PKC-β antisense oligonucleotide was 5′-CGC AGC CGG GTC AGC ATC-3′; the sense sequence was 5′-GAT GGC TGA CCC GGC TGC G-3′ (19). The p47phox antisense oligonucleotide was 5′-TTT GTC TGG TTG TCT GTG GG-3′; the sense sequence was 5′-CCC ACA GAC AAC CAG ACA AA-3′ (20). All of the oligonucleotides were phosphorothioate modified and high-performance liquid chromatography purified. ODNs were added to the cells at the concentration of 2 μmol/l (7,19,20).

Statistical analysis. All experiments were performed at least three times in duplicate or triplicate. Experimental results are presented as the means ± SD. Paired *t* tests were used for data analysis, and significance was defined as $P < 0.05$.

RESULTS

Cells cultured in HG media showed a significant 40% increase of O_2^- production compared with NG ($P < 0.01$), which was inhibited by the addition of AT ($P < 0.01$) (Fig. 1*A*). Release of ROS, as assessed by DCF-DA staining, paralleled the O_2^- data (Fig. 1*B*). Mannitol (9.5 mmol/l) had no significant effect on ROS/O_2^- production (Fig. 1*A* and *B*).

Cells were cultured in HG conditions along with rotenone (an inhibitor of complex I), TTFA (an inhibitor of complex II), CCCP (an uncoupler of oxidative phosphorylation that abolishes the mitochondrial membrane proton gradient), AOAC (inhibitor of malate-aspartate shuttle), or 4-HOCA (inhibitor of glycolysis-derived pyruvate transport into mitochondria). There were no significant differences in ROS production when any of these inhibitors were used (Fig. 2). However, in human aortic endothelial cells (HAECs), 4-HOCA, TTFA, and CCCP significantly inhibited ROS release ($P < 0.01$) (Fig. 2).

Previous studies have shown that O_2^- release from human monocytes was mediated via activation of PKC (7); therefore, we tested PKC activity in THP-1 cells under HG conditions. PKC activity was significantly increased in HG conditions compared with NG, and AT enrichment of THP-1 cells significantly decreased PKC activity in HG conditions (Fig. 3*A*). Immunoblots of total PKC in membranes showed a significant increase in PKC translocation to the membranes in HG conditions compared with NG, whereas addition of AT inhibited the PKC translocation to membranes (Fig. 3*B*). NADPH oxidase is a major source of superoxide in phagocytes. The effect of hyperglycemia on p47phox translocation to the membrane was investigated. p47phox translocation was significantly increased in HG conditions when compared with NG, whereas in AT-treated cells, it was significantly reduced (Fig. 3*B*).

To elucidate the signaling pathway of O_2^- production, cells were incubated with inhibitors of PKC or NADPH oxidase. HG conditions significantly increased O_2^- release, whereas AT reduced it, as in previous experiments ($P < 0.01$). The addition of HBDDE significantly inhibited O_2^- release ($P < 0.01$), but the specific PKC-βII inhibitor (LY379196, 30 nmol/l) had no significant effect (Fig. 4). Also, 150 nmol/l LY379196 had no significant effect on O_2^- release. When NADPH oxidase inhibitors apocynin (30 μmol/l) and DPI (10 μmol/l) were used, O_2^- release was inhibited significantly ($P < 0.001$) (Fig. 4). These data suggest that monocytic O_2^- release is driven possibly by

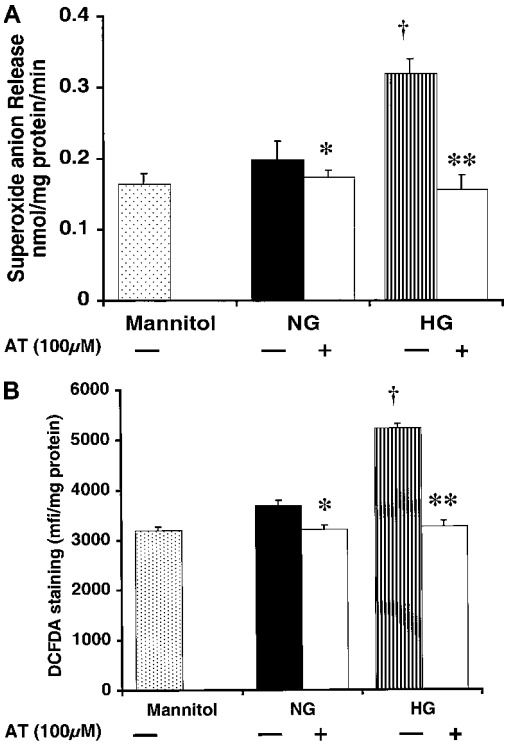

FIG. 1. *A*: Effect of glycemia and AT on O_2^- release from THP-1 cells. Cells were cultured in 5.5 mmol/l (NG) and 15 mmol/l (HG) glucose with or without AT, and the O_2^- release was measured as described in RESEARCH DESIGN AND METHODS. As a control, 9.5 mmol/l mannitol was added with NG in simultaneous wells ($n = 5$). *B*: Effect of glycemia and AT on ROS release from THP-1 cells. Cells were incubated with DCF-DA for 1 h and ROS release was quantitated as mean fluorescent intensities ($n = 5$). *$P < 0.05$, NG vs. NG plus AT; **$P < 0.01$, HG vs. HG conditions plus AT; †$P < 0.01$, NG vs. HG conditions. mfi, mean fluroescent intensities.

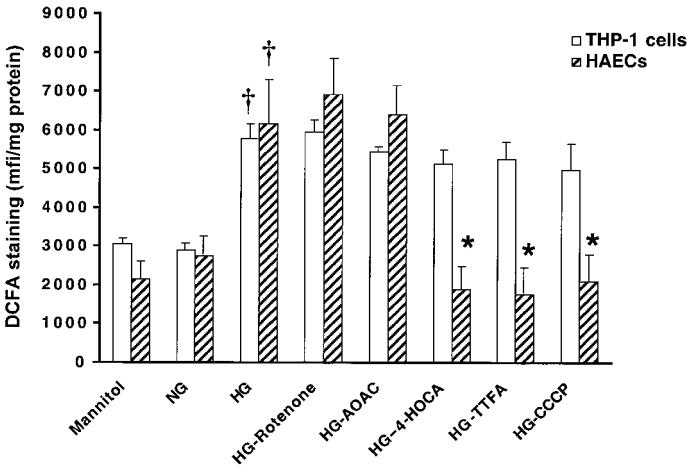

FIG. 2. Effect of various agents that alter mitochondrial metabolism on ROS release in THP-1 cells (□) and HAECs (▨) under HG conditions. Cells were cultured in HG media for 72 h and then incubated with rotenone, AOAC, 4-HOCA, TTFA, and CCCP for 24 h. ROS release was measured as described in RESEARCH DESIGN AND METHODS ($n = 5$). *$P <$ 0.01, HG vs. HG plus inhibitors; †$P < 0.01$, NG vs. HG conditions. mfi, mean fluroescent intensities.

PKC-α, and NADPH oxidase is necessary for the monocytic O_2^- release. Western blots for PKC-α and -βII in membrane fractions are shown in Fig. 5, top and middle panels. HG conditions increased the expression of both PKC-α and -βII isoforms, whereas the addition of AT inhibited both. To determine whether PKC-α was involved in stimulating O_2^- release through p47 phox, membranes were blotted with anti-p47phox antibody. AT and the PKC-α inhibitor, but not the PKC-βII inhibitor, inhibited p47phox translocation to membranes (Fig. 5, bottom panel).

To further confirm that O_2^- release was mediated through PKC-α, cells were incubated with ODNs to PKC-α and -βII. Results showed that addition of antisense oligo to PKC-α significantly inhibited O_2^- release ($P < 0.01$), whereas antisense oligo to PKC-βII did not have any effect (Fig. 6). However both antisense oligos decrease PKC activity by 41% ($P < 0.01$) and inhibit the translocation (>90%) of respective isoform to the membrane. Similarly, antisense oligos to p47phox also inhibited O_2^- release ($P < 0.001$). To confirm that p47phox activation is mediated through PKC-α, Western blots were run for the membrane fractions and quantitated. p47phox translocation was significantly inhibited with the addition of antisense oligo to PKC-α but not with antisense oligo to PKC-β II (Fig. 7). In all ODN experiments, as a control, sense oligos to PKC-α and -β and p47phox were added and did not affect O_2^- release from monocytes under HG conditions.

DISCUSSION

In this study, we used THP-1 cells because they have many characteristics of human monocytes (21). We previously showed that monocytes from type 2 diabetic patients released increased O_2^- compared with those from matched control subjects, whereas AT supplementation decreased O_2^- production (2). The PKC isoenzyme family is required for human monocytic O_2^- release (22). PKC-α and -βII are primarily modulated by glucose in human monocytes (18,19). In this study, we show under HG

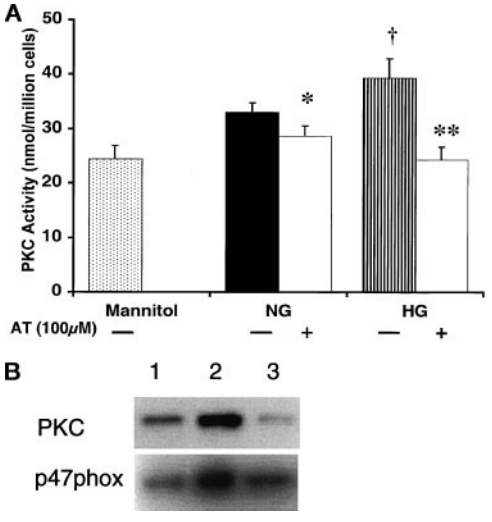

FIG. 3. *A*: Effect of glycemia and AT on PKC activity. Cells were cultured in 5.5 mmol/l (NG) and 15 mmol/l (HG) glucose with or without AT, and PKC activity was measured as described in RESEARCH DESIGN AND METHODS. Mannitol (9.5 mmol/l) was used as control ($n = 3$). *$P < 0.05$, NG vs. NG plus AT; **$P < 0.01$, HG vs. HG plus AT; †$P < 0.05$, NG vs. HG conditions. *B*: Effect of glycemia and AT on total PKC (top panel) and p47 phox translocation (bottom panel). Membrane fractions of THP-1 cells were run on Tris-glycine gels and then blotted for total PKC/p47phox. *Lane 1*: NG; *lane 2*: HG conditions; *lane 3*: HG plus AT ($n = 3$).

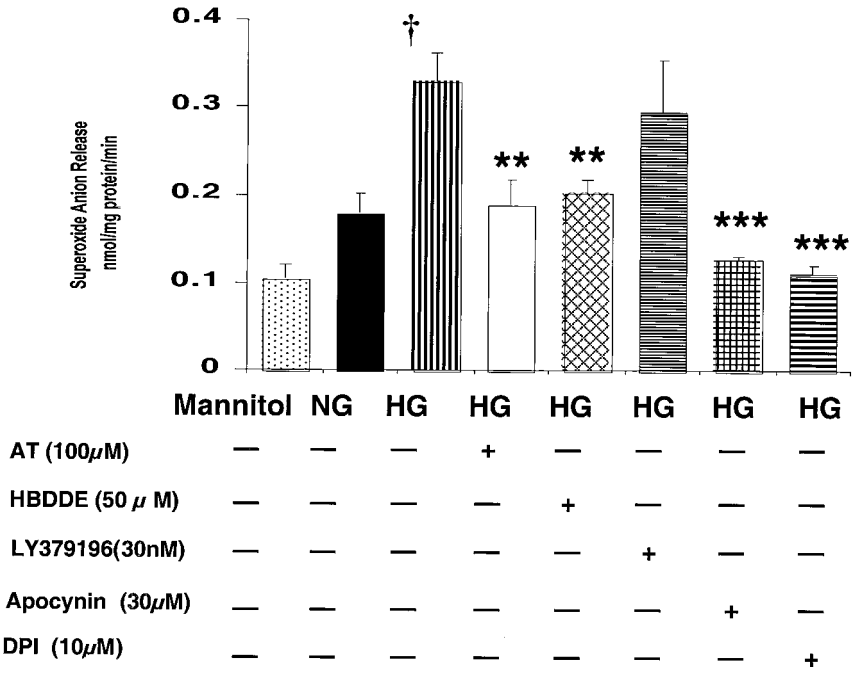

FIG. 4. Effect of PKC isoform inhibitors on O_2^- release from THP-1 cells under HG. Cells were cultured as described in Fig. 1. After 72 h, cells were incubated with HBDDE, LY379196, apocynin, or DPI in HG media overnight. Then, O_2^- release was measured as described in RESEARCH DESIGN AND METHODS ($n = 5$). **$P < 0.01$, HG vs. HG plus AT or HG plus HBDDE; ***$P < 0.01$, HG vs. HG plus DPI or apocynin; †$P < 0.01$, NG vs. HG conditions.

conditions that O_2^- release and PKC activity were significantly increased. Li et al. (7) had shown that under NG, O_2^- release from monocytes is mediated via PKC-α. Recently, it has been shown that mitochondria are the major source for O_2^- in endothelial cells (ECs) under HG conditions (5). We show, for the first time, that various mitochondrial complex inhibitors did not inhibit ROS release from monocytes, whereas they decreased ROS release from HAECs (5). These findings suggest that there are different mechanisms mediating O_2^- production in different cells. In this regard, it should be emphasized that the monocyte is a classical phagocyte. The increased ROS produced under HG conditions could modify biomolecules, thus promoting vasculopathies. Because it is known that PKC activity is induced under hyperglycemia (6), we studied modulation of PKC isoforms in THP-1 cells under HG conditions. We show that high glucose induced both PKC-α and -βII, whereas AT reduced their expression. Our results are in partial agreement with the findings of Ganz and Seftel (23), in corpus cavernosum vascular smooth muscle cells, that PKC-βII but not PKC-α is increased with HG conditions, and AT inhibited it. An increase in PKC-α levels was not detected, probably because of the different cell type used. Ceolotto et al. (18) had shown that although there was an increase in both PKC-α and -βII in diabetic human monocytes, only the increase in PKC-βII was significant. It is not clear which of these isoforms mediates O_2^- release from human monocytes under hyperglycemia. Several laboratories have shown that PKC-dependent signaling is involved in the activation of NADPH oxidase and O_2^- production in neutrophils. Hence, we studied p47phox translocation to membranes. Our results also confirmed that there was increased p47phox translocation to membranes with HG conditions. This is well correlated with other reports that NADPH oxidase is involved in monocytic O_2^- release (11,24). The addition of AT reduced p47phox membrane translocation. This is supported by the studies of Cachia et al. (24) showing that under NG conditions, AT decreased PMA-induced O_2^- production in monocytes. However, although they studied the effect of AT under NG and reported decreased PKC activity, the effect of AT on the translocation of neither PKC-α nor -βII were studied. To study the involvement of PKC isoforms in glucose-induced O_2^- release and the mechanism of its inhibition by AT, we used PKC inhibitors as well as sense and antisense ODNs to both isoforms. HBDDE inhibited PKC-α and other isoforms nonspecifically and is not a specific inhibitor to PKC-α (25). The PKC-βII inhibitor did not have any effect on p47phox translocation. This suggests that monocytic O_2^- release is probably via PKC-α and not via PKC-βII, since HBDDE inhibits both PKC-α and -βII, and βII-specific inhibitor had no effect. To prove this, we used antisense ODNs. When cells were incubated with antisense to PKC-α, both O_2^- release and p47phox transloca-

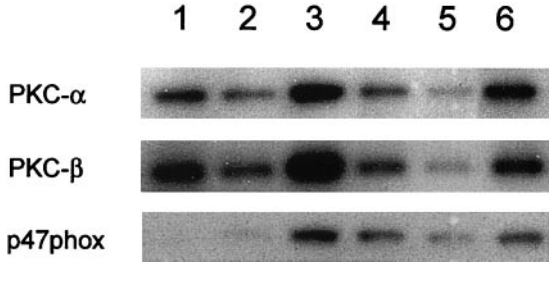

FIG. 5. Effect of Inhibitors on PKC-α (top panel), PKC-βII (middle panel), and p47phox (bottom panel) translocation. After culturing with inhibitors, cells were lysed, and membrane fractions were blotted for PKC-α and -βII and p47phox. *Lane 1*: mannitol; *lane 2*: NG; *lane 3*: HG; *lane 4*: HG plus AT; *lane 5*: HG plus HBDDE; *lane 6*: HG plus LY379196 ($n = 3$).

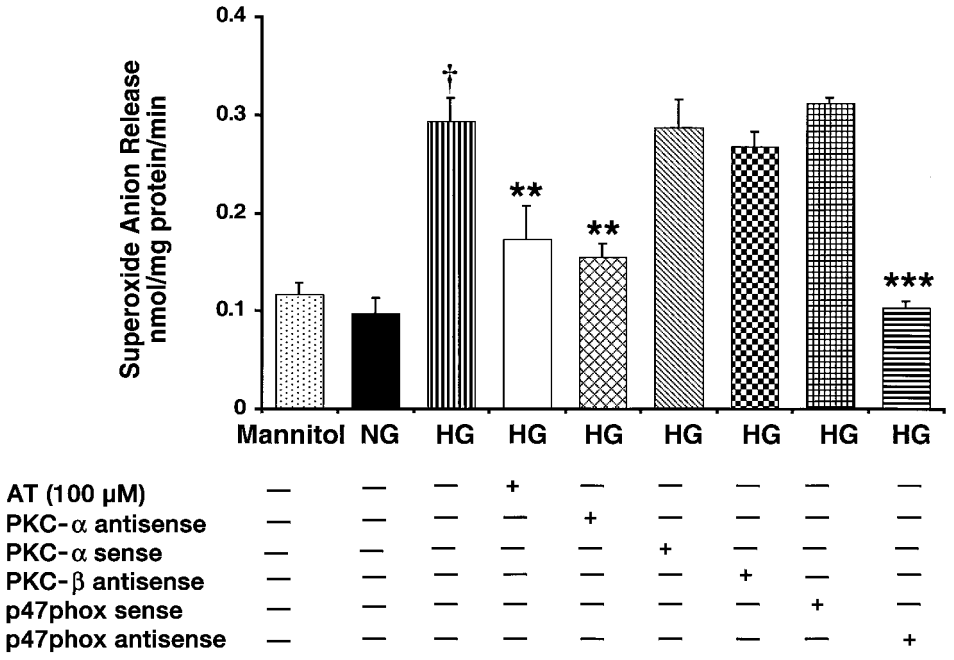

FIG. 6. Effect of ODNs to PKC-α and -βII and p47phox on O_2^- release from THP-1 cells. Cells were incubated with ODNs for 3 days with daily addition, and then O_2^- release was assayed (n = 3). **P < 0.01, HG vs. HG plus AT/PKC-α antisense oligo/p47phox antisense oligo; †P < 0.01, NG vs. HG conditions.

tion to membranes were reduced, whereas the addition of antisense to PKC-β did not have any effect on monocytic O_2^- release, despite both ODNs decreasing PKC activity. Antisense oligos to p47phox further proved that NADPH oxidase is essential for monocytic superoxide production. This is in agreement with the study by Li et al. (7) showing that monocytic O_2^- release is mediated by PKC-α under euglycemia. We show for the first time that HG conditions induce PKC-α, which in turn activates p47phox translocation to membranes and induces O_2^- release. The antisense approach has proven quite successful in this study. Two factors likely contributing to the effectiveness of this approach are the use of monocytes as target cells and the careful selection and purity of the ODN.

The important mechanistic finding of our studies is that NADPH oxidase is activated via PKC-α by translocating p47phox to membranes under HG conditions, resulting in increased O_2^- release, although both PKC-α and -βII were increased by high glucose. We also show that AT inhibited these HG conditions–induced changes.

Our studies show that in monocytes, O_2^- release is derived predominantly through NADPH oxidase, and in EC it could be through mitochondria. The novelty of this study is that under HG conditions, PKC-α activation of NADPH oxidase triggers O_2^- release, and that AT decreases O_2^- release via inhibition of PKC-α, thus offering an explanation for the increased O_2^- release in diabetic monocytes. However, our findings in THP-1 cells need to be confirmed in diabetic monocytes. This amelioration of oxidative stress by AT could be beneficial in decreasing diabetic vascular complications and needs to be tested in clinical trials in diabetic patients.

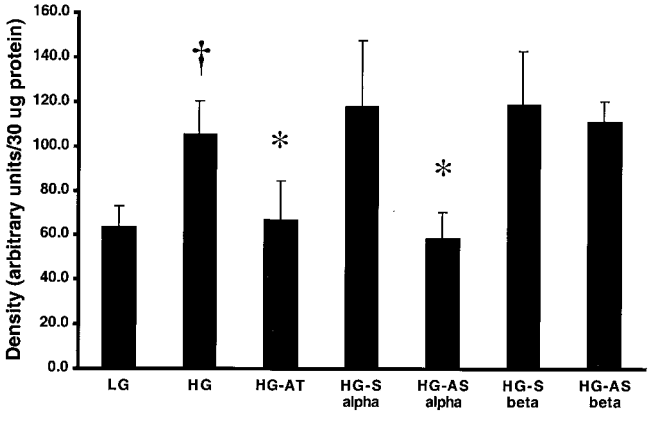

FIG. 7. Effect of ODNs to PKC-α and -βII on p47phox translocation in THP-1 cells. The membrane fractions were isolated, and then Western blots for p47phox were performed as described in RESEARCH DESIGN AND METHODS and quantitated. *P < 0.05, HG vs. HG plus AT, PKC-α, and PKC-β; †P < 0.05, NG vs. HG conditions (n = 3). AS, antisense; S, sense.

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