Glucose Increases Endothelial-Dependent Superoxide Formation in Coronary Arteries by NAD(P)H Oxidase Activation

Attenuation by the 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitor Atorvastatin

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Increased vascular superoxide anion (O_2^-) formation is essentially involved in the pathophysiology of atherosclerosis. Chronic hyperglycemia induces endothelial dysfunction, probably due to increased formation of reactive oxygen intermediates. However, little is known about the localization, modulators, and molecular mechanisms of vascular O_2^- formation during hyperglycemia. In porcine coronary segments, high glucose significantly increased O_2^- formation (1,703.5 ± 394.9 vs. 834.1 ± 91.7 units/mg for control, $n = 64$, $P < 0.05$; measured by lucigenin-enhanced chemiluminescence). This effect was completely blocked after removal of the endothelium. Coincubation with 10 μmol/l atorvastatin, a lipophilic inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, attenuated basal and glucose-induced O_2^- formation (328.1 ± 46.5 and 332.8 ± 50.3 units/mg, $P < 0.05$ vs. without atorvastatin). Incubation with mevalonic acid reversed this effect. High glucose increased mRNA expression of the oxidase subunit $p22^{phox}$, which was blocked by 10 μmol/l atorvastatin, whereas expression of $gp91^{phox}$ was unchanged. In conclusion, glucose-induced increase of vascular O_2^- formation is endothelium dependent and is probably mediated by increased $p22^{phox}$ subunit expression. Beneficial effects of statins in diabetic patients may be explained in part by attenuation of vascular O_2^- formation independent of lipid lowering. *Diabetes* 51: 2648–2652, 2002

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eNOs, endothelial nitric oxide synthase; G3PDH, glyceraldehyde-3-phosphate-dehydrogenase; HMG, hydroxymethylglutaryl; LMWG, low molecular weight GTP binding proteins; O_2^-, superoxide anion; PKC, protein kinase C; VSMC, vascular smooth muscle cell.

Reactive oxygen intermediates contribute to the pathogenesis of atherosclerosis by lipid peroxidation and irreversibly modulate protein function by cross-linking and fragmentation of macromolecules (1,2). Furthermore, increased superoxide anion (O_2^-) formation leads to cellular hypertrophy, to vascular dysfunction, probably as a result of increased formation of peroxynitrite, and to altered protein functions (3–5). Free radicals in the vascular system have originally been thought to be generated by macrophages present in atherosclerotic plaques. However, nonphagocytic NAD(P)H-dependent oxidases have been identified in isolated vascular smooth muscle cells (VSMCs) (5), adventitia (6), and endothelial cells (7) showing close molecular similarities to the phagocytic oxidase subunit $p22^{phox}$, a critical component of the vascular oxidase complex (4,5).

Hyperglycemia contributes to the pathogenesis of vascular complications in diabetes. Acute hyperglycemia induces reversible abnormalities in blood flow and vascular permeability by modulation of intracellular signaling pathways, followed by irreversible modifications of cellular proteins and vascular matrix during chronic hyperglycemia (1). Imbalances of endothelium-dependent nitric oxide generation and vascular O_2^- formation may explain endothelial dysfunction in diabetes (1). Although increased O_2^- formation has been demonstrated (8), little is known about the molecular basis of elevated O_2^- formation during hyperglycemia.

Thus, the aim of the present study was to examine, in a tissue culture model of native coronary arteries, whether incubation with high glucose increases vascular O_2^- generation. We hypothesized that high glucose increases radical formation in endothelial cells via protein kinase C (PKC) activation, which leads to activation of NAD(H)-dependent oxidase at least partially due to increased expression of $p22^{phox}$ expression. We further assumed that isoprenylation or some other action of hydroxymethylglutaryl (HMG)-CoA reductase is necessary to increase endothelial O_2^- production and $p22^{phox}$ expression as determined after using the HMG-CoA reductase inhibitor atorvastatin.

RESEARCH DESIGN AND METHODS

Organ culture of porcine coronary arteries. Coronary arteries of porcine hearts (obtained from a local slaughterhouse) were cautiously dissected free of the adhering fat and connective tissue. Small segments (~3–4 mm length) were cultured in Medium 199 (CC Pro, Neustadt, Germany) containing polymyxin B (5 μg/ml). Rings were incubated with or without glucose (20 mmol/l) or sucrose (20 mmol/l) for up to 48 h (9). Endothelium was carefully removed 2 h before measurements in indicated experiments, and atorvastatin (1 or 10 μmol/l) or vehicle was added. Cell viability (measured by MTT-based cytotoxicity assay) and proliferation (measured by BrdU-incorporation kit; Roche, Mannheim, Germany) of VSMCs (10,11) and endothelial cells were not affected by up to 10 μmol/l atorvastatin (not shown).

Measurement of vascular O_2^- formation. The formation of O_2^- in porcine coronary segments was measured in a HEPES-modified Krebs buffer (pH 7.40) and 250 or 4 μmol/l lucigenin for 30- or 60-s intervals by a luminometer (Wallac, Freiburg, Germany). Averages of the plateau phase were used for further calculation (12), and values were expressed as units per milligram dry weight (units/mg). Rings were incubated with inhibitors (diphenyleneiodonium, N^G-nitro-L-arginine-methyl-ester, L-NAME, and oxypurinol) for 30 min before measurements as indicated. In some experiments, coronary rings were stimulated with NADH (100 μmol/l) or NADPH (100 μmol/l) and chemiluminescence was measured immediately.

mRNA expression of NAD(P)H oxidase components in porcine coronary rings. The expression of $p22^{phox}$, $gp91^{phox}$, and glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) mRNAs was evaluated in vascular rings incubated in medium for 48 h using competitive PCR after RT (Advantage RT-for-PCR Kit; Clontech, Palo Alto, CA) of 1 μg RNA, which was isolated from three independent coronary rings (with or without endothelium) using TRIzol reagent (Life Technologies, Eggenstein, Germany). Nonhomologous internal standards with primer templates that are recognized by the gene-specific primers were constructed (PCR MIMIC Construction Kit; Clontech), and cDNA from coronary rings was incubated with decreasing amounts of known standards and amplified with gene-specific primers: G3PDH: 5′-CCTCCACTACATGGTCTACATGTTCC-3′ and 5′-CTCCACGATGCCGAAGTG GTCATGG-3′ (24 cycles, annealing at 60°C, 402 bp; Genebank accession no. U48832); $p22^{phox}$: 5′-GTTGGTCTGCCTGCTGGAGT-3′ and 5′-GTTGCTGGGC GGCTGCTTGAT-3′ (29 cycles, annealing at 60°C, 324 bp; Genebank U02477); $gp91^{phox}$: 5′-GACTGTTCAAAGCCTGTGGC-3′, 5′-TCTCCTCATCATGGTGCA CG-3′ (31 cycles, annealing at 60°C, 407 bp, Genebank U02476). Amplificated products were separated in 1.5% (wt/vol) agarose gels, and expression levels of ethidium-stained products of unknowns and standards were densitometrically determined (National Institutes of Health [NIH] image software version 1.61; NIH, Bethesda, Maryland). Absolute expression levels of targets were calculated by comparison with the signals of equimolar amounts of the standard.

Statistics. Values are expressed as means ± SE. Statistical evaluation was performed by the Friedman analysis and Wilcoxon test for data obtained from the same vessel preparations by use of Kruskal-Wallis analysis and Mann-Whitney *U* test for comparisons of data from different preparations (StatView 5.0 for Apple MacIntosh).

RESULTS

Vascular O_2^- generation in coronary rings. Vascular O_2^- formation was 834.7 ± 91.7 units/mg (n = 64) after incubation of coronary rings for 48 h in medium 199 (using 250 μmol/l lucigenin to measure chemoluminescence). Acute addition of NADH (100 μmol/l) or NADPH (100 μmol/l) at the end of this 48-h incubation period significantly increased O_2^- formation in the rings (NADH 4,502.4 ± 412.1 units/mg and NADPH 2,261.0 ± 304.1, n = 17, $P < 0.05$ vs. baseline). O_2^- production was completely blocked to the background noise of the luminometer by adding the radical scavenger tiron (10 mmol/l; data not shown). Coincubation of rings with 20 mmol/l glucose for 48 h significantly increased vascular radical formation compared with control incubation with medium 199 (Fig. 1). Furthermore, O_2^- formation after acute addition of NADH or NADPH was significantly increased in glucose-incubated rings (NADH 6,520.0 ± 1,450.8 units/mg and NADPH 2,924.0 ± 480.1, n = 15, $P < 0.05$ vs. NADH and NADPH stimulation during 48 h incubation with medium

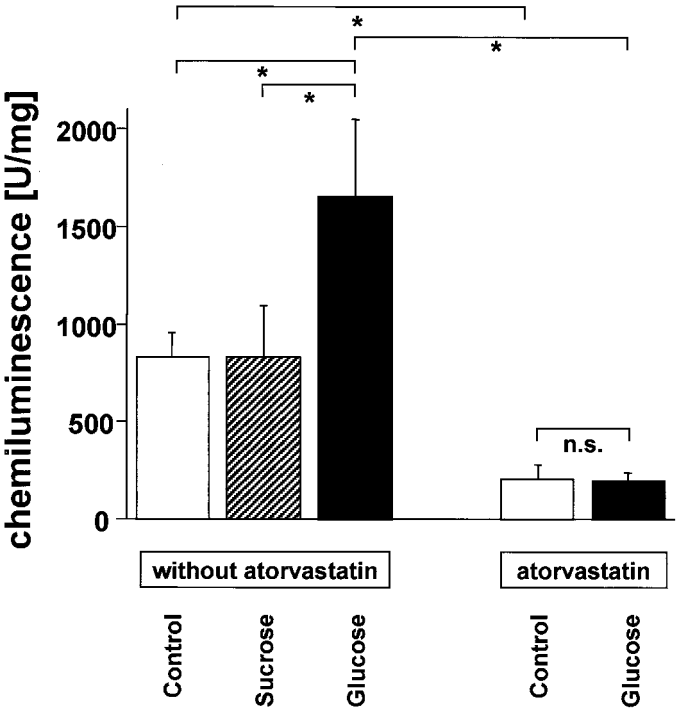

FIG. 1. O_2^- formation in porcine coronary rings. Rings were incubated in control medium or in medium containing 20 mmol/l glucose or 20 mmol/l sucrose with or without 10 μmol/l atorvastatin for 48 h. Vascular O_2^- formation was determined using lucigenin-enhanced chemiluminescence. Values are means ± SE (n = 35). *$P < 0.02$ vs. control and sucrose. n.s., not significant.

199). Interestingly, radical formation was not significantly increased in rings incubated with high glucose for 10 min, 4 h, and 8 h compared with respective controls (data not shown). Incubation of coronary rings with the iso-osmotic control for 48 h (20 mmol/l sucrose) did not change vascular O_2^- formation. In addition, O_2^- formation after incubation of rings in 20 mmol/l sucrose and acute stimulation with NADH (3,558.8 ± 331.5 units/mg) or NADPH (2047.5 ± 359.8) was not different from respective controls. Coronary O_2^- generation was also investigated in this experimental setting using a final concentration of 4 μmol/l lucigenin because lucigenin can undergo redox recycling when used at high concentrations, and high glucose also increased O_2^- formation (236.5 ± 43 units/mg) versus control and sucrose in 4 μmol/l lucigenin (control 86.8 ± 18.0 units/mg and sucrose 112.0 ± 28.5, n = 16, $P < 0.05$ vs. high glucose).

At the end of the 48-h incubation period, O_2^- production of control and sucrose-treated rings was significantly reduced after removal of the endothelium (control 612.2 ± 85.5 units/mg and sucrose 581.9 ± 75.1, n = 15, P = 0.009 vs. with endothelium, using 250 μmol/l lucigenin). Glucose-induced increases of O_2^- formation were completely blocked when the endothelium was removed (595.3 ± 110.9 units/mg, NS versus respective controls).

Vascular O_2^- formation and inhibition of intracellular signaling. Incubation with the flavoprotein-inhibitor diphenyleneiodonium (10 μmol/l) immediately before measurements reduced basal and glucose-induced O_2^- generation in rings incubated for 48 h (below detection limit of the luminometer using 4 μmol/l lucigenin). Acute stimulation of those rings with NADH during coincubation

with diphenyleneiodonium did not change vascular radical formation compared with values during baseline conditions (below detection limit of the luminometer, when 4 μmol/l lucigenin was used during measurements). Coincubation of rings with the PKC inhibitor staurosporine (100 μmol/l) for 48 h completely blocked increases of O_2^- generation by high glucose (control 548.8 ± 184.3 units/mg vs. glucose 502.2 ± 85.0, $n = 31$, NS, using 250 μmol/l lucigenin). Coincubation of rings with staurosporine for 48 h also inhibited glucose-induced elevation of O_2^- formation after acute addition of NADH (100 μmol/l; control 3,455.6 ± 407.6 units/mg and high-glucose 3,431.2 ± 417.4, $n = 31$, NS). A 30-min preincubation period of rings with L-NAME (100 μmol/l), an inhibitor of endothelial nitric oxide synthase (eNOs) (control 330.2 ± 154.6 units/mg and glucose 1,089.0 ± 196.0, $n = 8$, $P < 0.05$), and with oxypurinol (100 μmol/l), an inhibitor of xanthine oxidase, did not change glucose-induced coronary O_2^- formation after incubation of the rings for 48 h (control 384.75 ± 154.1 units/mg and glucose 986.4 ± 267.6, $n = 7$, $P < 0.05$) compared with radical formation of rings without inhibitors (control 550.6 ± 98.5 units/mg and glucose 977.6 ± 279.0, $n = 7$, $P < 0.05$).

Coincubation of coronary rings with the HMG-CoA reductase inhibitor atorvastatin (10 μmol/l) for 48 h reduced basal and NADH-stimulated O_2^- formation and blocked glucose-induced increases of vascular O_2^- generation (Figs. 1 and 2). Comparable results were obtained at a concentration of 4 μmol/l lucigenin (coincubation with 10 μmol/l atorvastatin 79.7 ± 15.2 units/mg; high glucose with atorvastatin 72.1 ± 14.3 units/mg, $n = 16$, NS). Incubation with mevalonic acid (100 μmol/l, using 250 μmol/l lucigenin), which bypasses HMG-CoA-reductase inhibition, reversed atorvastatin-induced attenuation of basal and NADH-stimulated O_2^- production after control or high-glucose incubation of coronary rings (Fig. 2).

mRNA expression of G3PDH and NAD(P)H oxidase subunits. Expression of G3PDH and $gp91^{phox}$ mRNA was not changed by incubation of coronary rings in high glucose or sucrose medium (Table 1). Expression of the $p22^{phox}$ subunit was doubled after incubation in high glucose versus control or sucrose medium. Coincubation with atorvastatin (10 μmol/l) reduced $p22^{phox}$ expression and completely abolished the glucose-induced increase of $p22^{phox}$ mRNA expression (Fig. 3), whereas expression of $gp91^{phox}$ and G3PDH-mRNA was not modulated. Removal of endothelium reduced expression of the $p22^{phox}$ subunit (1.7 ± 0.1 vs. 4.3 ± 1.5 amol/μg RNA with endothelium), whereas expression of $gp91^{phox}$ was not changed (140.0 ± 47.8 vs. 126.7 ± 14.0 amol/μg RNA with endothelium). In addition, removal of the endothelium completely blocked the increase of $p22^{phox}$ subunit expression also in high-glucose medium (1.7 ± 0.4 amol/μg RNA).

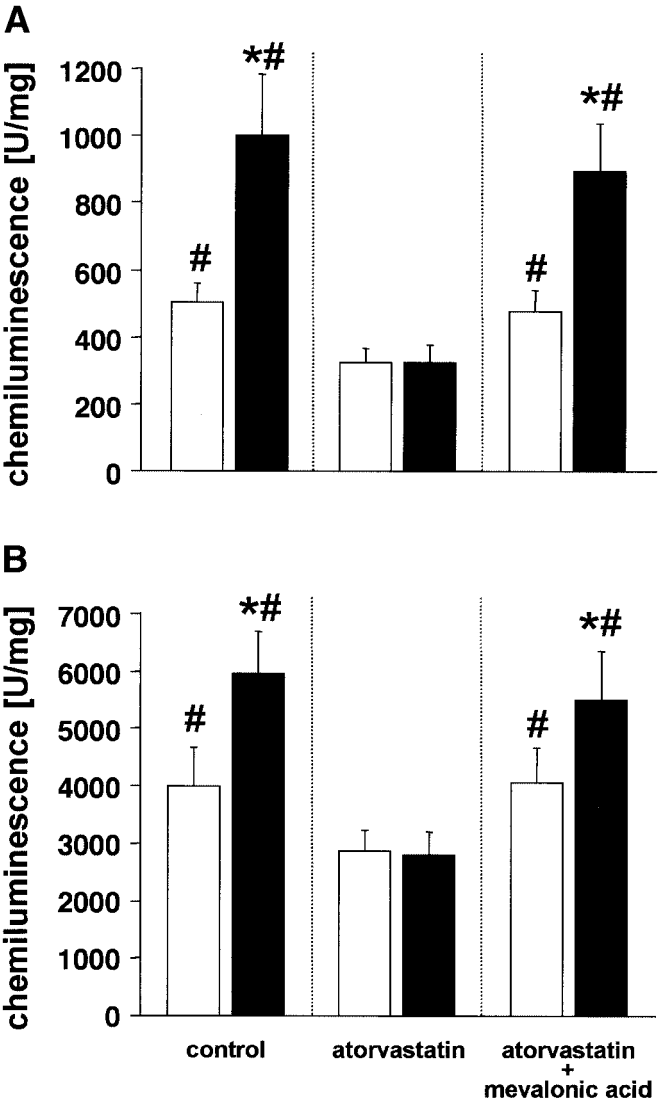

FIG. 2. O_2^- formation in porcine coronary rings. Vascular rings were incubated in *1*) medium 199 (control incubation; left bars), *2*) medium 199 and atorvastatin (10 μmol/l) without (middle bars) mevalonic acid, or *3*) medium 199 and atorvastatin with mevalonic acid (100 μmol/l; right bars) for 48 h. Those rings were either incubated in normal glucose (□) or in high glucose concentrations (20 mmol/l; ■). Radical formation of coronary rings was measured during baseline (*A*) and after addition of 100 μmol/l NADH (*B*). Values are means ± SE ($n = 32$). *$P < 0.05$ vs. respective rings incubated with normal glucose concentrations; #$P < 0.05$ vs. respective rings coincubated with 10 μmol/l atorvastatin.

DISCUSSION

The present study demonstrates that, dependent on intact endothelium, high glucose elicits vascular O_2^- formation of native coronary arteries. In addition, high glucose upregulates the mRNA expression of the endothelial $p22^{phox}$ subunit. Coincubation with atorvastatin, a HMG-CoA reductase inhibitor, reduces coronary O_2^- generation and expression of $p22^{phox}$ mRNA and completely blocks glucose-induced effects, presumably via a PKC-dependent pathway.

The glucose-induced increase of O_2^- formation in our organ culture model is obviously mediated by an increase of NAD(P)H-dependent oxidase activity. Involvement of eNOS or xanthine oxidase as a generator of vascular O_2^- formation was ruled out by preincubation of rings with L-NAME, a blocker of eNOS-mediated O_2^- generation (13), or with oxypurinol, an inhibitor of xanthine oxidase (14). Upregulation of the $p22^{phox}$ subunit mRNA suggests that $p22^{phox}$ is the critical component of increased O_2^- generation in hyperglycemia, as previously reported for angiotensin II–dependent hypertension (4). Removal of the endothelium reduces $p22^{phox}$ mRNA expression, although $gp91^{phox}$ mRNA expression was not changed. Although

TABLE 1
G3PDH and gp91^{phox} oxidase subunit mRNA expression in porcine coronary rings

Experimental condition	G3PDH (fmol/μg RNA)	gp91^{phox} (amol/μg RNA)
Control	3.8 ± 1.5	126.7 ± 14.0
Sucrose	3.9 ± 1.7	148.3 ± 11.7
Glucose	2.7 ± 1.0	123.3 ± 14.5
Control + atorvastatin	3.8 ± 1.7	98.3 ± 11.7
Glucose + atorvastatin	3.5 ± 1.4	75.0 ± 2.9

Data are means ± SE of three independent experiments. Rings were incubated in medium without (control) or with glucose (20 mmol/l) or sucrose (20 mmol/l) for 48 h. Atorvastatin (10 μmol/l) was added as indicated.

gp91^{phox}-mRNA is expressed ~30-fold higher than the p22^{phox} subunit, the relative expression level of p22^{phox} in the endothelium is probably higher than in the other cellular components of the coronary artery, whereas endothelial gp91^{phox} mRNA expression seems to be very low. The pathophysiological meaning of this uneven distribution of oxidase subunits is presently unclear. We hypothesize that the endothelium is a major source of vascular O_2^- and that the p22^{phox} subunit probably plays a dominant role in endothelium-dependent radical generation, at least in porcine coronary arteries.

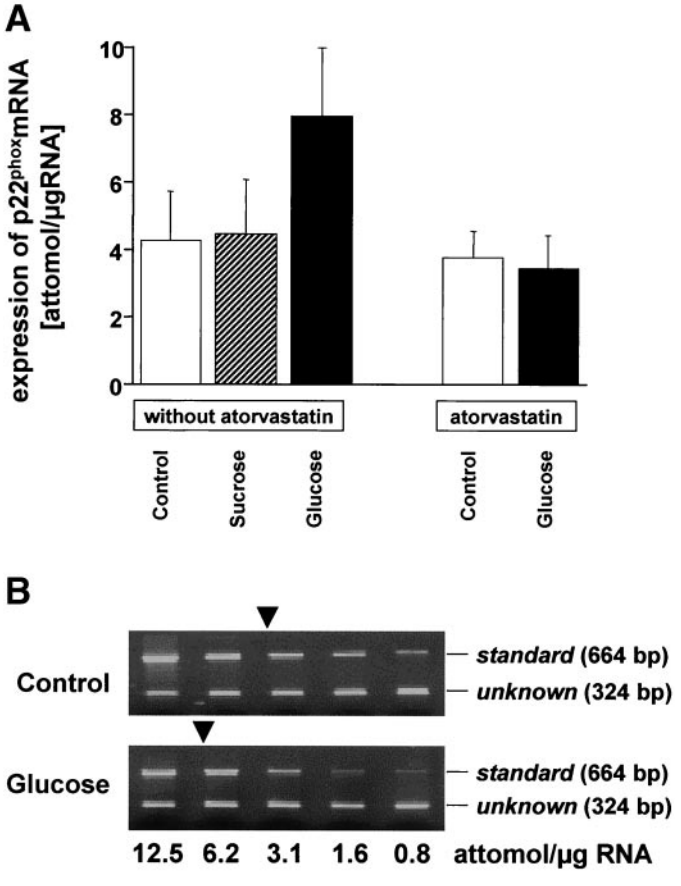

FIG. 3. mRNA expression of p22^{phox} in porcine coronary rings. Rings were incubated as described in the legend to Fig. 1. mRNA expression of vascular oxidase p22^{phox} subunits was determined by competitive PCR after RT using an internal standard. p22^{phox} mRNA was increased by high-glucose medium in each of three independent experiments versus respective controls (*A*; means ± SE, *n* = 3). A representative ethidium bromide–stained gel of competitive RT-PCR is displayed for vascular rings after 48 h control and high-glucose incubation (*B*). The arrowheads indicate the concentration at which internal standard and unknown sample were equimolar.

Increase of p22^{phox} mRNA expression and coronary O_2^- formation is completely blocked after removal of the endothelium, providing convincing evidence that the endothelium is the critical mediator of increased O_2^- formation induced by high glucose concentrations. This contrasts with reports on angiotensin II–dependent hypertension (4) in which p22^{phox}expression was increased in VSMCs. Results of immunohistochemical examinations of human coronary arteries revealed that some endothelial cells and VSMCs aquire the ability to express p22^{phox} in atherosclerosis (15). These findings propose that the cell types expressing p22^{phox} obviously depend on the triggering event and that the endothelium plays a prominent role in the pathogenesis of diabetic vascular complications. Because the PKC inhibitor staurosporine completely blocks glucose-dependent effects, we suggest that a PKC-dependent pathway is essentially involved in the glucose-induced increase of vascular O_2^- generation. The importance of PKC signaling is highlighted by recent reports that link activation of PKC with progression of diabetic vasculopathy (16).

Respiratory burst in neutrophils is dependent on post-translational isoprenylation of low molecular weight GTP binding proteins (LMWG) (17), such as *rho* or *ras*. Statins block isoprenylation by inhibition of HMG-CoA reductase, which synthesizes mevalonic acid, an immediate precursor of isoprenoids. Blockade of LMWG isoprenylation may reduce acitivity of NAD(P)H oxidase and expression of p22^{phox}, thus reducing basal and glucose-induced increases in O_2^- formation. Laufs and Liao (18,19) demonstrated that inhibition of *rho* geranylgeranylation modulates expression of eNOs. In addition, Kim et al. (20) reported that the increased expression of fibronectin and transforming growth factor β1 by high glucose in mesangial cells is reduced by a lovastatin, presumably involving Rho family small GTP binding proteins. Thus, we speculate that atorvastatin reduces the active form of a LMWG, leading to a reduced transcription of p22^{phox}, whereas gp91^{phox} expression was not modulated. Involvement of protein isoprenylation in the effect of HMG-CoA reductase inhibition is further supported by reversibility of atorvastatin-induced effects by coincubation with mevalonic acid. The fold increase in O_2^- production by NADH, compared with basal, is greater after atorvastatin treatment, suggesting that the main effect of atorvastatin is on basal O_2^- generation. Effects of atorvastatin to reduce basal and glucose-induced increase in O_2^- formation appear at 1 μmol/l and further increase at 10 μmol/l. Maximum steady-state plasma concentrations of ~0.5 μmol/l are seen in clinical studies. Thus, atorvastatin may be able to reduce O_2^- formation and p22^{phox} expression both in vitro and in vivo, contributing to the beneficial effects of HMG-CoA reductase inhibitors on atherosclerotic processes. This hypothesis is further supported by recent clinical studies suggesting beneficial anti-inflammatory effects of statins in addition to their lipid-lowering effects (21,22).

In conclusion, exposure to high glucose increases the expression of p22^{phox}, a critical component of nonphagocytic NAD(P)H-dependent oxidase, in native coronary

endothelium, leading to enhanced O_2^- generation. Atorvastatin suppresses basal and glucose-induced O_2^- formation and $p22^{phox}$ expression. Beneficial effects of statins in diabetic patients may be explained in part by attenuation of vascular O_2^- formation independent of lipid lowering.

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