

Signaling Pathways Involved in Human Vascular Smooth Muscle Cell Proliferation and Matrix Metalloproteinase-2 Expression Induced by Leptin

Inhibitory Effect of Metformin

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Accumulating evidence suggests that high concentrations of leptin observed in obesity and diabetes may contribute to their adverse effects on cardiovascular health. Metformin monotherapy is associated with reduced macrovascular complications in overweight patients with type 2 diabetes. It is uncertain whether such improvement in the cardiovascular outcome is related to specific vasculoprotective effects of this drug. In the present study, we determined the effect of leptin on human aortic smooth muscle cell (HASMC) proliferation and matrix metalloproteinase (MMP)-2 expression, the signaling pathways mediating these effects, and the modulatory effect of metformin on these parameters. Incubation of HASMCs with leptin enhanced the proliferation and MMP-2 expression in these cells and increased the generation of intracellular reactive oxygen species (ROS). These effects were abolished by vitamin E. Inhibition of NAD(P)H oxidase and protein kinase C (PKC) suppressed the effect of leptin on ROS production. In HASMCs, leptin induced PKC, extracellular signal-regulated kinase (ERK)1/2, and nuclear factor- κ B (NF- κ B) activation and inhibition of these signaling pathways abrogated HASMC proliferation and MMP-2 expression induced by this hormone. Treatment of HASMCs with metformin decreased leptin-induced ROS production and activation of PKC, ERK1/2, and NF- κ B. Metformin also inhibited the effect of leptin on HASMC proliferation and MMP-2 expression. Overall, these results demonstrate that leptin induced HASMC

proliferation and MMP-2 expression through a PKC-dependent activation of NAD(P)H oxidase with subsequent activation of the ERK1/2/NF- κ B pathways and that therapeutic metformin concentrations effectively inhibit these biological effects. These results suggest a new mechanism by which metformin may improve cardiovascular outcome in patients with diabetes. *Diabetes* 54:2227–2234, 2005

Obesity is a strong risk factor for the development of type 2 diabetes and cardiovascular disease (1) and is associated with a marked increase in circulating leptin concentrations (2). In recent years, many but not all (3,4) studies have demonstrated positive associations between plasma leptin and clinical cardiovascular disease and leptin signaling has been implicated in the promotion of atherosclerosis. In vitro proatherogenic effects of leptin include endothelial cell activation, migration, and proliferation (5,6); smooth muscle cell proliferation, migration, and calcification (7,8); platelet aggregation (9); activation of monocytes (10); and modulation of the immune response (11,12). In vivo, leptin receptors are expressed in vascular cells and atherosclerotic lesions (13), and leptin signaling promotes atherosclerosis in mice models (14,15).

The UKPDS (U.K. Prospective Diabetes Study) has demonstrated that metformin, a biguanide antidiabetic agent, reduces macrovascular events in overweight patients with type 2 diabetes (16). Its protective effects have been attributed in part to an improved lipid profile, a lack of weight gain, an antihyperinsulinemic effect, and an improved thrombolysis (17,18). Metformin exerts several intrinsic vasculoprotective effects that may account for its ability to reduce cardiovascular events (18). We have previously shown that in vitro, metformin inhibits early and critical key processes involved in the pathophysiology of atherogenesis, such as monocyte adhesion to endothelium and macrophage-derived foam cell formation (19). These effects may account for the antiatherosclerotic properties of this drug. The present study sought to determine whether this drug inhibits human aortic smooth muscle cell (HASMC) proliferation and matrix metallopro-

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AMPK, AMP kinase; DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate; DPI, diphenyleneiodonium; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HASMC, human aortic smooth muscle cell; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide; NF- κ B, nuclear factor- κ B; PKC, protein kinase C; ROS, reactive oxygen species; TTFa, thenoyltrifluoroacetone.

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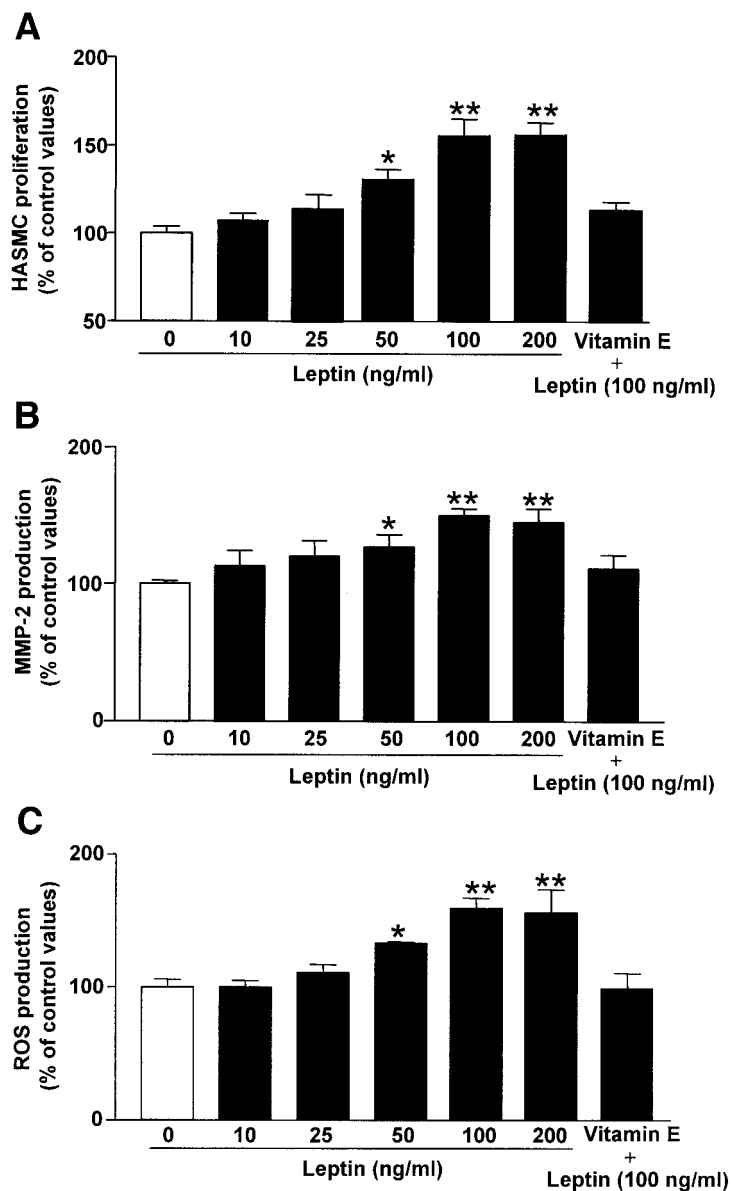


FIG. 1. Stimulatory effect of leptin on HASMC proliferation and MMP-2 expression. Role of oxidative stress. **A:** Serum-starved HASMCs were incubated for 72 h with increasing concentrations of leptin in the presence or absence of vitamin E (50 $\mu\text{mol/l}$). Cell proliferation was assessed by the MTT assay. **B:** Subconfluent HASMCs were incubated for 72 h with increasing concentrations of leptin in the presence or absence of vitamin E (50 $\mu\text{mol/l}$). The amounts of MMP-2 secreted in the culture medium were determined by ELISA. **C:** Subconfluent HASMCs were incubated for 24 h with increasing concentrations of leptin in the presence or absence of vitamin E (50 $\mu\text{mol/l}$), with 20 $\mu\text{g/ml}$ DCF-DA added during the final 20-min incubation period. Intracellular ROS generation was quantified by measuring fluorescence. Data represent the means \pm SE of four independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. medium.

teinase (MMP)-2 expression in response to leptin and the molecular mechanisms involved in this effect.

RESEARCH DESIGN AND METHODS

Reagents. Recombinant human leptin was purchased from R&D Systems (Minneapolis, MN). Vitamin E and 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) were obtained from Sigma (St Louis, MO). PBS was obtained from Invitrogen (Burlington, ON, Canada). Smooth muscle cell basal medium (SmBM) and smooth muscle cell growth medium (SmGM-2) were purchased from BioWhittaker (Walkersville, MD). Fetal bovine serum (FBS) and trypsin/EDTA were obtained from Wisent (St. Bruno, PQ, Canada). Apocynin, a selective NAD(P)H oxidase inhibitor (20); diphenyleneiodonium (DPI) chloride, an inhibitor of flavoprotein-containing enzymes, including NAD(P)H oxidase (21); thenoyltrifluoroacetone (TTFA), a mitochondrial complex II inhibitor (22); GF10923X, a selective protein kinase C (PKC) inhibitor (23); PD98059, a selective mitogen-activated protein kinase (MAPK) kinase inhibitor (24); and BAY 11-7085, a selective nuclear factor- κ B (NF- κ B) inhibitor (25) were obtained from Calbiochem (La Jolla, CA). Metformin was supplied by Merck-Santé (Lyon, France).

Cells. HASMCs were obtained from BioWhittaker (Walkersville, MD). The cells were grown in SmGM-2 at 37°C in a 5% CO_2 /95% air atmosphere. At confluence, cells were trypsinized and subcultured in 24- or 96-well culture plates or 100-mm tissue culture dishes depending on assay conditions. Cells were used in all experiments between passages 3 and 6. Leptin treatment was

carried out in cells cultured in SmBM supplemented with 2% FBS and 1% (vol/vol) penicillin-streptomycin.

HASMC proliferation assay. Subconfluent HASMCs were serum starved in SmBM supplemented with 2% FBS for 48 h, preincubated for 1 h with metformin or other appropriate agents, and then treated with leptin for an additional 72 h at 37°C. HASMC proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide (MTT) assay (Promega, Madison, WI). This assay is a colorimetric method based on reduction of the tetrazolium salt, MTT, by actively growing cells to produce a blue formazan product (26).

Determination of reactive oxygen species production. Subconfluent HASMCs were pretreated or not pretreated for 1 h with metformin or other appropriate agents prior to exposure to leptin for a further 24-h period at 37°C, with the cell-permeable fluorogenic probe DCF-DA (20 $\mu\text{g/ml}$) added during the final 20-min incubation period. At the end of this incubation period, cells were washed and trypsinized. Intracellular reactive oxygen species (ROS) production was monitored by measuring fluorescence in an LS50B luminescence spectrophotometer (Perkin Elmer) using excitation and emission wavelengths of 498 and 522 nm, respectively.

Measurement of MMP-2 protein expression. Subconfluent HASMCs were pretreated or not pretreated for 1 h with metformin or other appropriate agents and then incubation was pursued in the presence of leptin for an additional 72 h at 37°C. The amount of MMP-2 secreted in the culture medium was measured using a double-sandwich enzyme-linked immunosorbent assay (ELISA) (Amersham Biosciences, Baie d'Urfé, QC, Canada) (27). The mini-

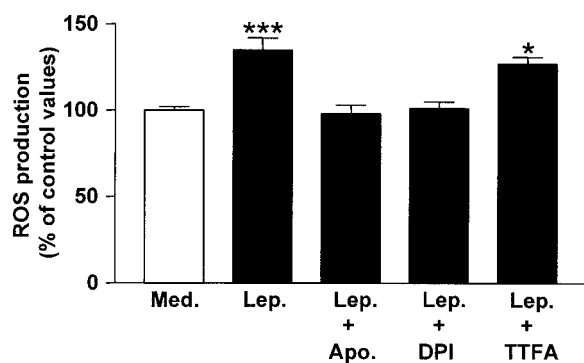


FIG. 2. Effect of NAD(P)H oxidase and mitochondrial electron transport inhibitors on leptin-induced ROS generation in HASMCs. HASMCs were pretreated for 1 h with the NAD(P)H oxidase inhibitors apocynin (Apo; 10 μ mol/l) and DPI (10 μ mol/l) or with the mitochondrial electron transport inhibitor TTFA (10 μ mol/l) and then exposed for 24 h to 100 ng/ml leptin (Lep), with 20 μ g/ml DCF-DA added during the final 20-min incubation period. Intracellular ROS generation was quantified by measuring fluorescence. Data represent the means \pm SE of four independent experiments. * P < 0.05, *** P < 0.001 vs. medium (Med).

mum detectable concentration of MMP-2 with this assay is 1.5 ng/ml. The intra- and interassay coefficients of variation of this assay were <5.3 and 8.3, respectively. Levels of MMP-2 in the supernatants were normalized to the levels of total cell proteins.

Determination of PKC activation. Confluent HASMCs were treated or not treated with leptin in 100-mm tissue culture dishes in the presence or absence of metformin for 30 min at 37°C. At the end of this incubation period, cells were washed three times with cold PBS and harvested. Cell pellets were suspended in 1 ml cold sample preparation buffer (50 mmol/l Tris-HCl, pH 7.5,

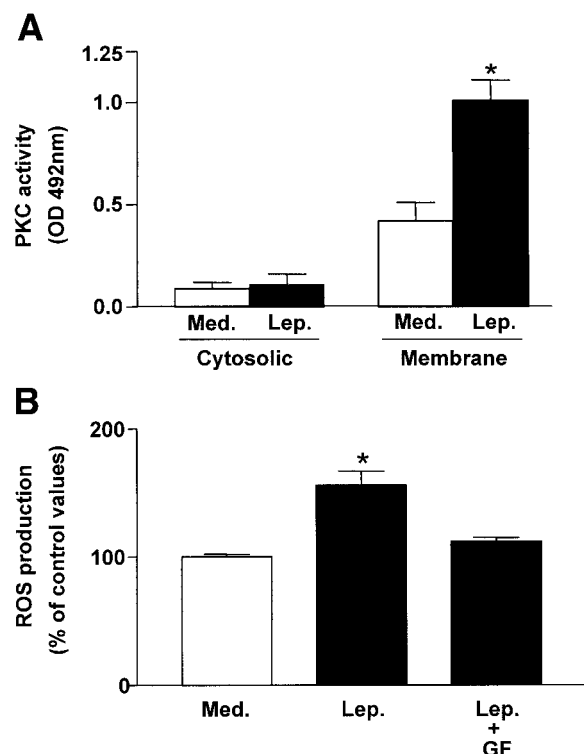


FIG. 3. Role of PKC in leptin-induced ROS generation. **A:** HASMCs were incubated with leptin (Lep) (100 ng/ml) for 30 min. Cytosolic and membrane PKC activities were measured using the MESACUP protein kinase assay kit. **B:** HASMCs were pretreated for 1 h with the specific PKC inhibitor GF10923X (GF; 20 nmol/l) and then exposed to leptin (Lep) for 24 h, with 20 μ g/ml DCF-DA added during the final 20-min incubation period. Intracellular ROS generation was quantified by measuring fluorescence. Data represent the means \pm SE of four independent experiments. * P < 0.05 vs. medium (Med).

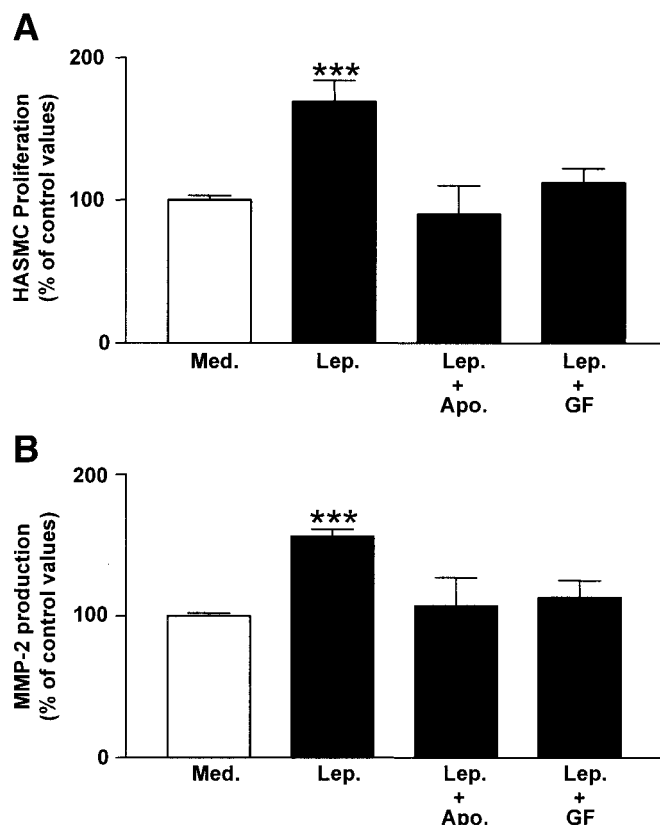


FIG. 4. Effect of NAD(P)H oxidase and PKC inhibitors on leptin-induced HASMC proliferation and MMP-2 expression. HASMCs were pretreated for 1 h with the NAD(P)H oxidase inhibitor apocynin (Apo; 10 μ mol/l) or the specific PKC inhibitor GF10923X (GF; 20 nmol/l) and then exposed for 72 h to 100 ng/ml leptin (Lep). At the end of this incubation period, HASMC proliferation (**A**) and levels of MMP-2 in the supernatants (**B**) were determined. Data represent the means \pm SE of four independent experiments. *** P < 0.001 vs. medium (Med).

5 mmol/l EDTA, 10 mmol/l ethylene glycol-bis tetraacetic acid, 50 mmol/l 2-mercaptoethanol, 1 mmol/l phenylmethylsulfonyl fluoride, and 10 mmol/l benzamide) and sonicated. Membrane and cytosol fractions were separated by centrifugation at 100,000g for 1 h at 4°C. Cytosolic and membrane PKC activities were measured using the MESACUP protein kinase assay kit (MBL, Naka-ku Nagoya, Japan) (28).

Determination of extracellular signal-regulated kinase 1/2 activation.

Confluent HASMCs were treated or not treated with leptin in 96-well plates in the presence or absence of metformin or other appropriate agents for 30 min at 37°C. At the end of this incubation period, cells were rapidly fixed with 4% formaldehyde. Total and phosphorylated extracellular signal-regulated kinase (ERK)1/2 activities were measured using a fast-activated cell-based ELISA (FACE) kit (MJS Biolynx, Brockville, ON, Canada) (29). Levels of total and phosphorylated ERK1/2 were normalized to cell number as determined by crystal violet cell staining.

Measurement of NF- κ B activation. Confluent HASMCs were treated or not treated with leptin in 100-mm tissue culture dishes in the presence or absence of metformin or other appropriate agents for 30 min at 37°C. At the end of this incubation period, cells were washed twice with cold PBS/phosphate inhibitor buffer (125 mmol/l sodium fluoride, 250 mmol/l β -glycerophosphate, 250 mmol/l paranitrophenyl phosphate, and 25 mmol/l sodium orthovanadate), scraped, and centrifuged. Cell pellets were resuspended in 1 ml ice-cold hypotonic buffer (20 mmol/l HEPES, pH 7.5, 5 mmol/l sodium fluoride, 10 μ mol/l sodium molybdate, and 0.1 mmol/l EDTA), and incubation was pursued on ice for 15 min. At the end of this incubation period, 10% nonidet P-40 was added to the lysed cells. After centrifugation, nuclear pellets were resuspended in Complete Lysis Buffer (MJS Biolynx) and nuclear extracts were collected by centrifugation at 9,000g for 10 min. Aliquots of the supernatants were kept at -70°C, and protein concentration was determined. Levels of NF- κ B activation in 3- μ g nuclear extracts were determined using the TransAM NF- κ B family transcription factor assay kit (MJS Biolynx) (30).

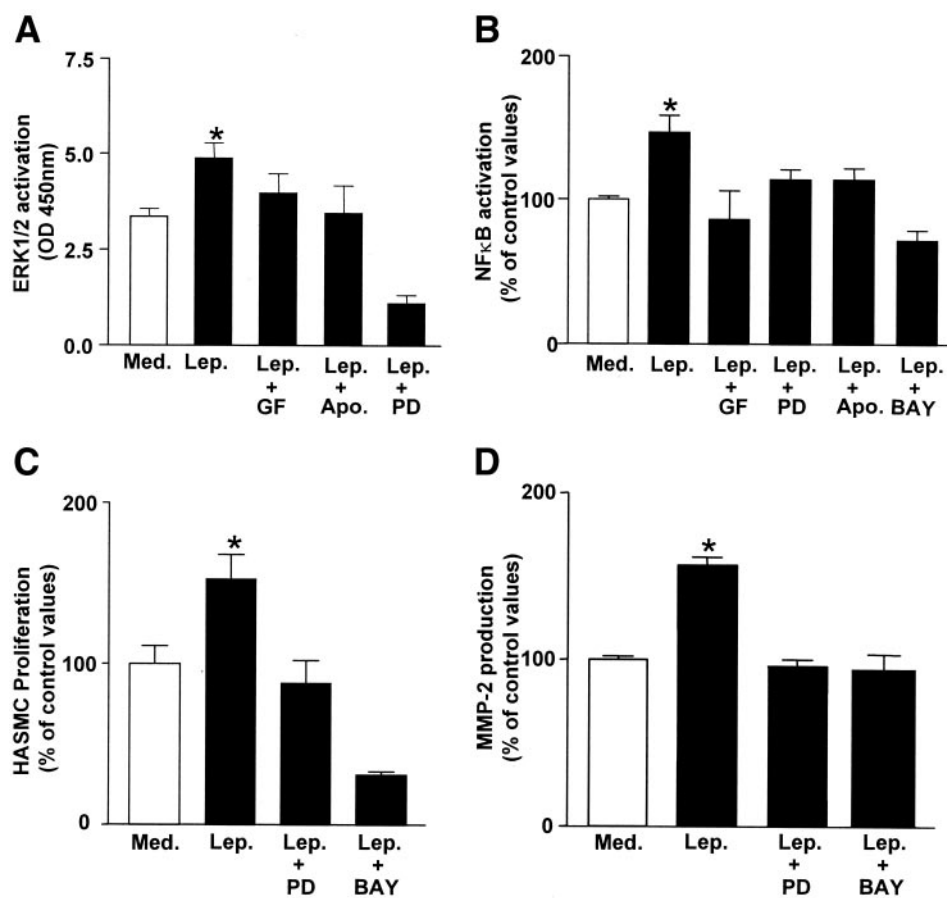


FIG. 5. Role of ERK1/2 and NF- κ B in leptin-induced HASMC proliferation and MMP-2 production. *A* and *B*: HASMCs were pretreated or not pretreated for 1 h with GF10923X (GF; 20 nmol/l), apocynin (Apo; 10 μ mol/l), PD98059 (PD; 10 μ mol/l), or BAY 11-7085 (BAY; 10 μ mol/l) and then incubated for 30 min with 100 ng/ml leptin (Lep). Phosphospecific ERK1/2 expression (*A*) and nuclear levels of NF- κ B activation (*B*) were determined as described in RESEARCH DESIGN AND METHODS. *C* and *D*: Serum-starved HASMCs were preincubated for 1 h with the ERK and NF- κ B inhibitors PD98059 (50 μ mol/l) and BAY 11-7085 (10 μ mol/l) and then treated for 72 h with 100 ng/ml leptin. Cell proliferation (*C*) and levels of MMP2 in the supernatants (*D*) were measured as previously described. Data represent the means \pm SE of four independent experiments. * P < 0.05 vs. medium (Med).

Determination of total protein concentrations. Total protein content was measured according to the Bradford method using a colorimetric assay (Bio-Rad, Mississauga, ON, Canada).

Determination of cell viability. To exclude the possibility that experimental agents at maximal concentration used in the study may exert cytotoxic effects, cell viability was determined by trypan blue exclusion. It was consistently found to be $\geq 90\%$ (data not shown).

Statistical analysis. Statistical analysis of the results was performed by one-way ANOVA followed by the Student-Newman-Keuls test. Differences were considered statistically significant at P < 0.05. Results are expressed as means \pm SE.

RESULTS

Stimulatory effect of leptin on HASMC proliferation, MMP-2 expression, and ROS production. Incubation of HASMCs with leptin (0–200 ng/ml) increased the proliferation of these cells in a concentration-dependent manner. Maximal effect was observed after 72 h at a concentration of 100 ng/ml leptin (Fig. 1A). Leptin also enhanced, in a dose-dependent manner, MMP-2 production (Fig. 1B) and intracellular accumulation of ROS (Fig. 1C) in HASMCs, with a maximal effect at 100 ng/ml. Pretreatment of HASMCs with vitamin E abolished the stimulatory effects of leptin on HASMC proliferation, MMP-2 expression, and ROS production (Fig. 1A–C).

Generation of ROS in leptin-treated HASMCs arises from the NAD(P)H oxidase and is PKC dependent. To evaluate the role of NAD(P)H oxidase and mitochondria in leptin-induced intracellular ROS production, HASMCs were preincubated with the NAD(P)H oxidase inhibitors apocynin and DPI chloride or with the inhibitor of the mitochondrial electron transport, TTFA, prior exposure to leptin. As shown in Fig. 2, apocynin and DPI chloride

prevented the effect of leptin on intracellular ROS production, whereas TTFA was ineffective, thus identifying NAD(P)H oxidase as the source of ROS in leptin-treated HASMC. Like TTFA, the mitochondrial superoxide dismutase mimetic, MnTBAP [Mn(III)tetrakis (4-benzoic acid) porphyrin chloride] failed to inhibit leptin-induced ROS production in HASMCs (ROS production [percent of control values]: control 100 ± 2 , leptin 156 ± 11 , P < 0.05 vs. control; MnTBAP + leptin 135 ± 6). To determine whether PKC is involved in the stimulation of ROS by leptin, PKC activation in leptin-treated HASMCs was assessed and the effect of PKC inhibition on leptin-induced ROS generation determined. As shown in Fig. 3, treatment of HASMCs with 100 ng/ml leptin for 30 min induced PKC activation, as assessed by the translocation of this kinase to the membrane fraction (Fig. 3A). Pretreatment of HASMCs with the specific classic PKC inhibitor GF10923X totally prevented leptin-induced ROS generation (Fig. 3B). Apocynin and GF10923X also inhibited the effect of leptin on HASMC proliferation (Fig. 4A) and MMP-2 expression (Fig. 4B).

Role of ERK1/2 and NF- κ B in leptin-induced HASMC proliferation and MMP-2 production. Incubation of HASMCs for 30 min with 100 ng/ml leptin resulted in a marked increase in phosphospecific ERK1/2 expression (Fig. 5A) and nuclear levels of NF- κ B activation (Fig. 5B). Leptin-induced ERK1/2 activation was abolished by GF10923X, apocynin, and PD98059 (Fig. 5A). These compounds, as well as BAY 11-7085, also prevented the induction of NF- κ B in response to leptin (Fig. 5B). Inhibition of the ERK1/2 and NF- κ B signaling pathways totally abro-

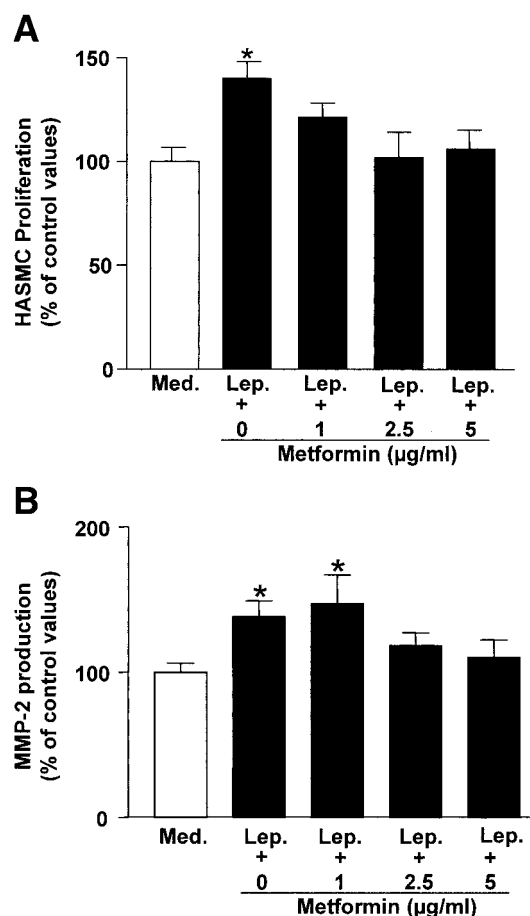


FIG. 6. Inhibitory effect of metformin on leptin-induced HASMC proliferation and MMP-2 production. Serum-starved (*A*) or subconfluent (*B*) HASMCs were preincubated for 1 h with metformin (1–5 µg/ml) and then incubated for 72 h in the presence of leptin (Lep; 100 ng/ml). At the end of this incubation period, HASMC proliferation (*A*) and levels of MMP2 in the supernatants (*B*) were measured as previously described. Data represent the means \pm SE of four independent experiments. * $P < 0.05$ vs. medium (Med).

gated the stimulatory effect of leptin on HASMC proliferation (Fig. 5C) and MMP-2 production (Fig. 5D).

Inhibitory effect of metformin on leptin-induced HASMC proliferation and MMP-2 production. Preincubation of HASMCs with metformin (1–5 µg/ml) inhibited the stimulatory effect of leptin (100 ng/ml) on HASMC proliferation (Fig. 6A) and MMP-2 expression (Fig. 6B) with maximal effect being observed at concentrations ranging from 2.5 to 5 µg/ml metformin (Fig. 6B). Metformin (5 µg/ml) also abolished leptin-induced intracellular accumulation of ROS in HASMCs (Fig. 7A) and inhibited PKC (Fig. 7B), ERK1/2 (Fig. 7C), and NF- κ B (Fig. 7D) activation in these cells.

DISCUSSION

Metformin has multiple biological effects, among which vasculoprotective properties have recently been identified (18). In experimental models of atherosclerosis, administration of metformin reduces the development and extent of atherosclerotic lesion formation (31). In patients with diabetes, this drug reduces the risk for developing macrovascular complications (16). The biological mechanisms responsible for the antiatherosclerotic properties of met-

formin are poorly understood but may include a suppressive effect of this drug on early key cellular events involved in atherogenesis, such as arterial lipid accumulation and metabolism (32,33), leukocyte-endothelium interaction, foam cell formation (19), and smooth muscle cell proliferation (34,35). It is well established that proliferative growth and migration of vascular smooth muscle cells (VSMCs) contribute to the arterial neointimal thickening observed in atherosclerosis, and recent *in vivo* and *in vitro* studies have demonstrated that leptin promotes neointimal growth in mice (36), VSMC proliferation (7), and endothelial cell MMP expression (6), thus supporting a role for this hormone in vascular lesion growth and matrix remodeling. Evidence that plasma leptin concentrations are associated with coronary atherosclerosis in patients with type 2 diabetes (37) further supports the possibility that leptin signaling may represent a therapeutic target for the prevention of atherosclerotic disease in these subjects. In smooth muscle cell, ROS are key mediators of the proliferative effect of many growth factors. Evidence that leptin stimulates smooth muscle cell growth and induces oxidative stress both *in vivo* and *in vitro* (5,38–40) supports the possibility that ROS may act as second messengers in leptin-induced smooth muscle cell proliferation. In line with this possibility, our results demonstrate that leptin induces intracellular ROS production in HASMCs and that vitamin E inhibits the proliferative effect of leptin on these cells. Leptin-induced ROS generation in HASMCs may arise from the NAD(P)H oxidase. Indeed, this enzyme is the major source of ROS in VSMCs, and redox signaling through NAD(P)H oxidase induces smooth muscle cell proliferation (41,42). Our observations that inhibitors of NAD(P)H oxidase totally abolish leptin-induced ROS production and proliferation in HASMCs indicate that ROS generation in these cells arises from the NAD(P)H oxidase system and mediates the proliferative effect of leptin in HASMCs. Previous studies have demonstrated that metformin has antioxidant properties (18,43–45). Consistent with these observations, we found that metformin suppressed the intracellular accumulation of ROS in leptin-treated HASMCs. These results, together with our observations that metformin inhibits the effect of leptin on smooth muscle cell growth to a similar extent as vitamin E, suggest that the suppressive effect of metformin on leptin-induced HASMC proliferation may be related to its antioxidant effects. Accumulating data indicate that stimulation of ROS through PKC-dependent activation of NAD(P)H oxidase and subsequent ERK activation may be critical mechanisms responsible for smooth muscle cell proliferation (46–48). Previous studies have shown that leptin induces PKC and MAPK activation in vascular cells (5,49,50) and stimulates VSMC proliferation through the MAPK pathway (7). In agreement with these data, we found that leptin induces PKC and MAPK activation in HASMCs and that inhibition of these signaling events abrogates the proliferative effect of leptin on these cells. Our findings that inhibition of PKC suppresses ROS production in leptin-treated HASMCs and that apocynin blocks MAPK activation in these cells further support the notion that generation of ROS in leptin-stimulated HASMCs is PKC dependent and that leptin-induced MAPK activation involves NAD(P)H oxidase-generated ROS. In-

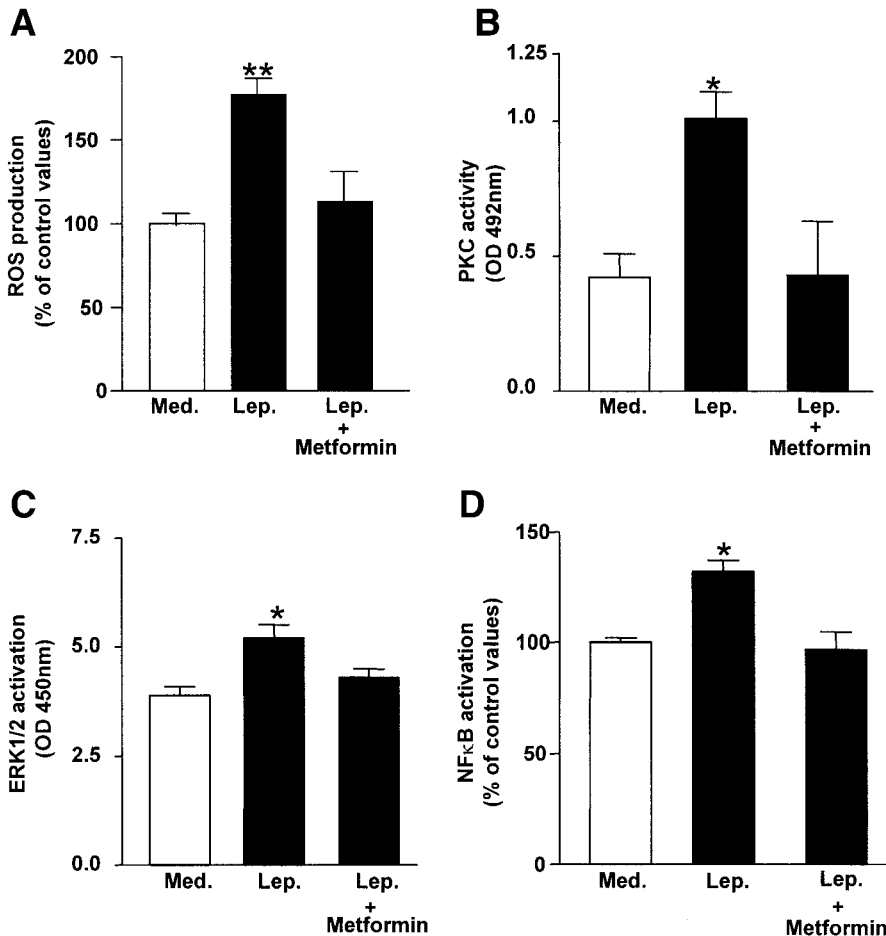


FIG. 7. Inhibitory effect of metformin on leptin-induced ROS generation and PKC, MAPK, and NF-κB activation. Subconfluent (A) or confluent (B–D) HASMCs were preincubated for 1 h with metformin (1–5 μg/ml) and then exposed for 24 h (A) or 30 min (B–D) to 100 ng/ml leptin (Lep). Intracellular ROS generation was quantified by measuring fluorescence (A). Determination of PKC (B), ERK1/2 (C), and NF-κB (D) activation was performed as described in RESEARCH DESIGN AND METHODS. Data represent the means ± SE of four independent experiments. **P* < 0.05, ***P* < 0.01 vs. medium (Med).

terestingly, our data demonstrate that metformin inhibits PKC and MAPK activation in leptin-treated HASMCs. On the basis of previous observations showing that inhibition of VSMCs by vitamin E correlates with PKC inhibition (51,52), it is tempting to speculate that metformin, by reducing PKC activity, may reduce ROS generation, thereby leading to decreased MAPK activation and smooth muscle cell growth. Because metformin activates AMP kinase (AMPK) and that activation of this kinase appears to reduce diacylglycerol synthesis (53–55), metformin’s inhibitory effect on PKC activation may involve AMPK activation, with consequent inhibition of diacylglycerol synthesis.

Proliferation of VSMCs is regulated by nuclear transcription factors including NF-κB. NF-κB activation is redox sensitive and is induced through PKC- and MAPK-dependent pathways (56,57). Previous data have proposed a role for ROS-dependent activation of NF-κB in leptin-induced activation of endothelial cells (38). The present study, which demonstrates that leptin induces HASMC proliferation through NF-κB activation, further stresses the crucial role of this transcription factor in the regulation of vascular cell function by leptin. In accordance with previous observations (38,56,57), we found that activation of NF-κB by leptin was linked to ROS generation and PKC/ERK1/2 activation. We further demonstrated that metformin inhibits leptin-induced NF-κB activation. Because AMPK appears to inhibit NF-κB activation in endothelial cells (58), metformin’s inhibitory effect on NF-κB activation in HASMCs may involve AMPK activation.

MMP expression is associated with smooth muscle cell proliferation and migration. MMP-2 is expressed abundantly in atherosclerotic lesions and plays an important role in increasing VSMC migration to the intima (59). Evidence that MMP-2 expression is upregulated by leptin in vascular cells (6) suggests that this effect may account, at least partly, for the neointimal growth-promoting property of leptin (36). Induction of MMP-2 is redox sensitive and occurs, in angiotensin-treated smooth muscle cell, in a NAD(P)H oxidase-dependent manner (60). In accordance with these data, the present study demonstrates that leptin induces, through a PKC-dependent NAD(P)H oxidase activation, MMP-2 expression in HASMCs. Furthermore, it demonstrates that this effect involves activation of the MAPK/NF-κB pathway. These findings are in line with previous observations showing a role for PKC, ERK1/2, and NF-κB in the regulation of MMP secretion (61,62). Our observation that metformin abrogates the induction of MMP-2 by leptin in HASMCs further supports the possibility that this drug may reduce the development of atherosclerotic lesions by regulating intimal thickening. Supporting this possibility, metformin treatment of cholesterol-fed rabbits has been shown to inhibit smooth muscle cell proliferation (35). In conclusion, this study demonstrates that metformin inhibits, by interfering with the PKC/MAPK/NF-κB pathways, leptin-induced HASMC proliferation and MMP-2 expression in vitro. These data suggest a new mechanism by which metformin may reduce cardiovascular risk in type 2 diabetic subjects.

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