Insulin Generates Free Radicals by an NAD(P)H, Phosphatidylinositol 3′-Kinase–Dependent Mechanism in Human Skin Fibroblasts Ex Vivo

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Oxidative stress may be involved in the development of vascular complications associated with diabetes; however, the molecular mechanism responsible for increased production of free radicals in diabetes remains uncertain. Therefore, we examined whether acute hyperinsulinemia increases the production of free radicals and whether this condition affects proliferative extracellular signal–regulated kinase (ERK-1 and -2) signaling in human fibroblasts in vitro. Insulin treatment significantly increased intracellular superoxide anion (O_2^-) production, an effect completely abolished by Tiron, a cell-permeable superoxide dismutase (SOD) mimetic and by polyethylene glycol (PEG)-SOD, but not by PEG catalase. Furthermore, insulin-induced O_2^- production was attenuated by the NAD(P)H inhibitor apocynin, but not by rotenone or oxypurinol. Inhibition of the phosphatidylinositol 3′-kinase (PI 3′-kinase) pathway with LY294002 blocked insulin-stimulated O_2^- production, suggesting a direct involvement of PI 3′-kinase in the activation of NAD(P)H oxidase. The insulin-induced free radical production led to membranous translocation of p47phox and markedly enhanced ERK-1 and -2 activation in human fibroblasts. In conclusion, these findings provided direct evidence that elevated insulin levels generate O_2^- by an NAD(P)H-dependent mechanism that involves the activation of PI 3′-kinase and stimulates ERK-1– and ERK-2–dependent pathways. This effect of insulin may contribute to the pathogenesis and progression of cardiovascular disease in the insulin resistance syndrome. *Diabetes* 53:1344–1351, 2004

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Received for publication 22 August 2003 and accepted in revised form 4 February 2004.

CT-AM, acetoxymethyl-2,2,6,6-tetramethylpiperidine-1-oxyl-3-carboxylate; ECL, enhanced chemiluminescence; ERK, extracellular signal–regulated kinase; ESR, electron spin resonance; FBS, fetal bovine serum; HAM, human serum albumin; JNK, c-Jun-NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; PEG, polyethylene glycol; pERK, phosphorylated extracellular signal–regulated kinase; PI, phosphatidylinositol; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; SAPK, stress-activated protein kinase.

Macrovascular disease is the major cause of morbidity and mortality in patients with type 1 and type 2 diabetes (1,2). Despite several clinical and epidemiological studies, the molecular pathways for the accelerated atherosclerosis in these patients remains incompletely understood. Recent studies have reported that oxidative stress may play an important role in the pathogenesis of cardiovascular disease in patients with diabetes (3–5). This condition is characterized either by an increased formation of free radicals (e.g., superoxide anions) or peroxynitrites or by a decrease in naturally occurring antioxidant reserves (6).

NAD(P)H oxidase activation is a major intracellular source of free radicals leading to a variety of signaling events that ultimately cause endothelial dysfunction, proliferation of vascular smooth muscle cells, expression of proinflammatory cytokines, and extracellular matrix (7,8). In diabetes, free radical production is increased by hyperglycemia through glycoxidation and sorbitol system activation and by functional limitation of the hexose monophosphate shunt, leading to decreased glutathione synthesis (9). Furthermore, there is evidence that hyperinsulinemia, the hallmark of insulin resistance, may be involved in the generation of oxidative stress (10). Insulin stimulates NAD(P)H-dependent H_2O_2 generation in human adipocyte plasma membrane (11) and increases superoxide anion (O_2^-) production through NAD(P)H oxidase in aortic segments from hyperinsulinemic rats (12). However, the mechanisms by which hyperinsulinemia stimulates free radical production and their pathophysiological implications remain to be elucitated.

In animal models of insulin resistance, insulin signaling via the phosphatidylinositol (PI) 3′-kinase is impaired, whereas the pathway for extracellular signal–regulated kinase (ERK)-1 and -2 appears to be unaffected (13). Therefore, the compensatory hyperinsulinemia resulting from insulin resistance may stimulate proliferative events via the activation of the ERK-1 and -2 pathway (14).

The complexity of these findings suggests that the generation of free radicals may represent a potential mechanism by which chronic hyperinsulinemia activates proliferative and downregulates metabolic signals; however, experimental data for this hypothesis are lacking. Therefore, we aimed to determine in cultured human fibroblasts *1*) whether insulin can produce free radicals, *2*)

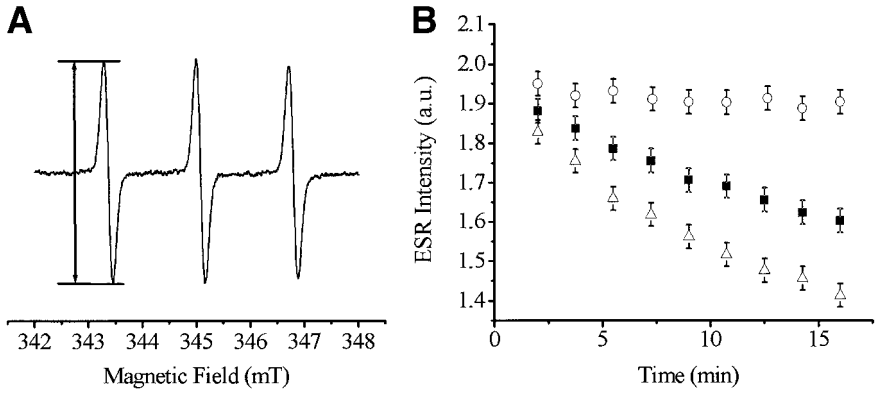

FIG. 1. Measurement of free radical production in fibroblasts with ESR spin clearance method. *A*: ESR spectrum of nitroxide probe CT-AM (20 μmol/l). The arrow's height measures the intensity of the spectrum, given in arbitrary units. *B*) An example of ESR intensity time decay of CT-AM spectra in different experimental conditions. ○, CT-AM solution without fibroblasts; ■, CT-AM in fibroblasts suspension (control experiment); △, CT-AM in fibroblasts suspension with insulin (1 μmol/l).

whether these reactive species influence insulin signaling, and *3*) how insulin induces free radical generation.

RESEARCH DESIGN AND METHODS

For the study, we recruited six healthy normotensive volunteers who had no family history of hypertension or diabetes. All participants gave their informed written consent to participate in the study.

Fetal bovine serum (FBS) and F-10 HAM medium were obtained from Eurobio (Cedex, France). Insulin, apocynin, LY294002, and PD98059 were purchased from Calbiochem (La Jolla, CA). Phosphorylated ERK (pERK)-1/2 and the enhanced chemiluminescence (ECL) system were purchased from New England Biolabs (Beverly, MA) and Amersham Biosciences, respectively. The polyclonal antibody against ERK-1 and -2, p47phox, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyethylene glycol (PEG)-SOD, PEG catalase, Tiron, and all of the other reagents were obtained from Sigma (St. Louis, MO).

Cell culture. Human fibroblasts were derived from a skin biopsy taken from the anterior surface of the left forearm by excision under topical anesthesia with ethyl chloride. Fibroblasts were cultured in nutrient mixture F-10 HAM medium supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 4 mmol/l glutamine, as previously described (15). Cells were seeded onto a 75-cm^2 flask and incubated at 37°C; the medium was changed every 2–3 days. Fibroblasts obtained from each subject and grown separately were used for the experiments at the third and fourth passages. Fibroblasts were identified morphologically; specifically, they were strictly diploid and, on morphological confluence, they appeared oriented with respect to one another, forming a typical parallel array of cells with no dividing nuclei visible by microscope. The cells were in the plateau phase of growth, in which there is a steady-state condition when cell proliferation has almost completely ceased; this was confirmed by the evaluation of [^{3}H]thymidine incorporation in cells grown in the same conditions. At confluence, [^{3}H]thymidine incorporation in fibroblasts was 10–15 times lower than that observed in cells during the log phase of growth and similar to that observed in cells cultured in serum-free medium (data not shown). Moreover, the fibroblasts produced type I collagen and were not positive for factor VIII.

Measurement of free radicals. The free radical production in the fibroblasts was investigated using electron spin resonance (ESR) spectroscopy with the spin clearance method (16). The nitroxide free radical used (acetoxymethyl-2,2,6,6-tetramethylpiperidine-1-oxyl-3-carboxylate [CT-AM]) was synthesized following previously reported procedures (17). Fibroblasts were removed from the dishes, centrifuged at 1,500*g* for 15 min, and resuspended in F10-HAM medium at a concentration of ~4×10^6 cells/ml. Next the probe CT-AM (20 μmol/l) and insulin (1 nmol/l to 10 μmol/l) were added to the cell suspensions. In some experiments, the cell suspensions were incubated for 1 h at 37°C with one of the following inhibitors before being stimulated with insulin (1 μmol/l): apocynin (100 μmol/l), LY294002 (100 μmol/l), rotenone (1 mmol/l), or oxypurinol (1 mmol/l). In the experiments to identify the molecular species responsible for CT-AM elimination, the fibroblasts were pretreated with the superoxide radical scavenger Tiron (1 mmol/l), PEG-SOD (250 units/ml), or PEG catalase (250 units/ml) before insulin stimulation.

The cell suspensions were inserted into capillary quartz tubes (20 μl) for ESR measurements, which started 2 min after the addition of insulin. ESR spectra were recorded with a Bruker ER200D spectrometer, and the ESR signal decay of CT-AM nitroxide was monitored for the first 15 min (Fig. 1). The spin clearance rate was calculated as the slope of the linear least squares fitting of ESR intensity decay.

Immunoblot analysis of p47phox expression. The protein expression of p47phox was examined by Western blot analysis. Cells were lysed in 12.5 mmol/l Tris, 2 mmol/l EGTA, 25 mmol/l β-glycerophosphate, 2 mmol/l Na_3VO_4, 10 μmol/l phenylmethylsulfonyl fluoride (PMSF), 1 μmol/l leupeptin, and 5 μmol/l aprotinin; sonicated on ice; and centrifuged at 1,000*g* for 10 min. The supernatant was collected and centrifuged at 100,000*g* for 60 min at 4°C. The resulting pellets were resuspended with lysis buffer containing 1% Triton X-100 and used to study the membrane fraction. The supernatant was used for the study of the cytoplasmic fraction. Samples were then solubilized in Laemmli buffer and separated by PAGE. Gel-separated proteins were electroblotted onto nitrocellulose membrane (Hybond ECL; Amersham) in blotting buffer containing Tris (48 mmol/l), glycine (39 mmol/l), SDS (0.037%), and methanol (20% vol/vol) for 3 h at 100 V in the cold room using a Transblot cell (Elettrofor, Padova, Italy). The membranes were blocked overnight at 4°C in 5% BSA in a buffer containing PBS and 0.05% (vol/vol) Tween. Membranes were exposed with antibody against p47phox overnight at 4°C (1:500). Detection was made using ECL. The density of resulting protein bands was analyzed by using a Bio-Rad Versa Doc 1000 Imaging System (Bio-Rad, Hercules, CA).

Immunoblot analysis of phosphorylation of ERK-1 and -2. Fibroblasts were grown in 10% FBS F10-HAM medium until confluence, after which they were made quiescent by switching to serum-free F10-HAM for 24–36 h. For the experiments, cells were stimulated with insulin in serum-free HAM for the indicated times and concentrations. After treatment, cells were collected by a rubber scraper on ice by using a lysis buffer (12.5 mmol/l Tris, 2 mmol/l EGTA, 25 mmol/l β-glycerophosphate, 2 mmol/l Na_3VO_4, 10 μmol/l PMSF, 1 μmol/l leupeptin, and 5 μmol/l aprotinin), sonicated on ice, and centrifuged at 14,000*g* for 10 min.

ERK-1 and -2 phosphorylation was determined by immunoblotting, as previously described (18). Briefly, the supernatant was collected, solubilized in Laemmli buffer, and separated by PAGE. Proteins separated on the gels were electroblotted onto nitrocellulose membrane (Hybond ECL; Amersham). After the membranes were incubated with antibody against pERK (1:3,000 dilution) overnight at 4°C, specific proteins were detected using ECL. Then the blots probed with phosphoantibodies were stripped and reprobed with a nonphosphoantibody against ERK-1 and -2 (1:1,500 dilution) to ensure equal loading. The density of the resulting protein bands was analyzed using the Bio-Rad Versa Doc 1000 Imaging System, and the results were expressed relative to the control(s) in the same blot, set at 100%, as the pERK-to-ERK densitometric ratio.

Statistical analysis. Data are expressed as means ± SE. Statistical significance was evaluated by two-tailed nonparametric tests for paired or unpaired data. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of insulin on free radical production. To assess insulin-stimulated production of free radicals in cultured fibroblasts, we used ESR spectroscopy, which is able to detect selectively paramagnetic species such as free radicals. However, the extremely low concentration of reactive radicals in biological samples makes their direct measurement troublesome; therefore, indirect methods, such as the spin trapping techniques, are required (19). The spin clearance method is based on the ESR detection

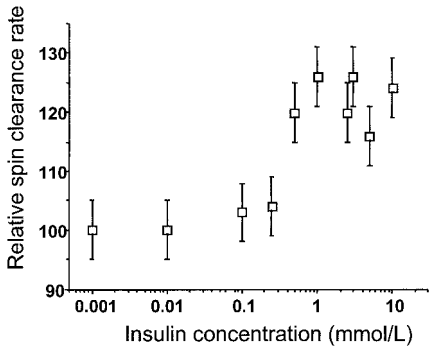

FIG. 2. Concentration dependence of insulin-stimulated free radical production in fibroblasts measured with ESR spin clearance method. The fibroblasts (4×10^6 cells/ml) were stimulated with increasing concentrations of insulin (1 nmol/l to 10 μmol/l). Control experiments (i.e., cells without insulin) were set to a value of 100; data are normalized to this value. The ED_{50} for insulin stimulation of free radical production was 800 nmol/l. Data are means ± SE of at least four independent experiments.

of a stable paramagnetic probe, the nitroxide radical CT-AM, which reacts with endogenous free radicals, giving reaction products that are ESR silent (16,17). The ESR spectrum intensity of the nitroxide decreases almost linearly in the first minutes after the mixing of the probe with the cell suspensions, and the decay rate is proportional to the production of endogenous free radicals.

A typical ESR spectrum of the nitroxide probe CT-AM is shown in Fig. 1*A*. It is composed of three lines whose intensity may be measured as the peak-to-peak height of the first leftmost line. CT-AM enters the cell membrane and is retained in the cytoplasm after the hydrolysis of its ester group. The nitroxide unit is reduced to hydroxylamine after reaction with a free radical, thus decreasing the ESR intensity. An example of the time course of the ESR experiment is reported in Fig. 1*B*. In the absence of metabolically active cells and in the presence of insulin or inhibitors, the nitroxide is stable (i.e., has a negligible spin clearance rate) (Fig. 1*B*). The scavenging of the probe is caused only by the presence of free radicals produced in metabolically active cells, either at rest or under stimulation (Fig. 1*B*). The effect of exposing cell suspensions to different concentrations of insulin (1 nmol/l to 10 μmol/l) on the spin clearance rate is shown in Fig. 2. Insulin increases free radical production in sigmoidal fashion with an ED_{50} (ED = effective dose) of 800 nmol/l. Compared with controls, insulin (1 μmol/l) significantly increased production of free radicals by 20% ($P < 0.01$ vs. control experiments) (Fig. 2).

To characterize the reactive oxygen species (ROS) induced by insulin, fibroblasts were pretreated with Tiron (a cell-permeable SOD mimetic), PEG-SOD, or PEG catalase before insulin stimulation. These substances allowed us to distinguish if the probe reacted mainly with O_2^- (superoxide anion) or with H_2O_2. As shown in Fig. 3*A*, Tiron and PEG-SOD completely blocked insulin-induced O_2^- production ($P < 0.01$ vs. insulin), whereas PEG catalase did not affect it, demonstrating that insulin stimulates production of O_2^- and that our measurements provided a direct estimate of superoxide radical production.

To further assess the pathway of insulin-induced production of free radicals, we preincubated the cells with apocynin, an inhibitor of NAD(P)H oxidase; rotenone, an inhibitor of mitochondrial ROS production; oxypurinol (1mmol/l), a xanthine oxidase inhibitor; or LY294002, an inhibitor of PI 3′-kinase. As shown in Fig. 3*B*, insulin-

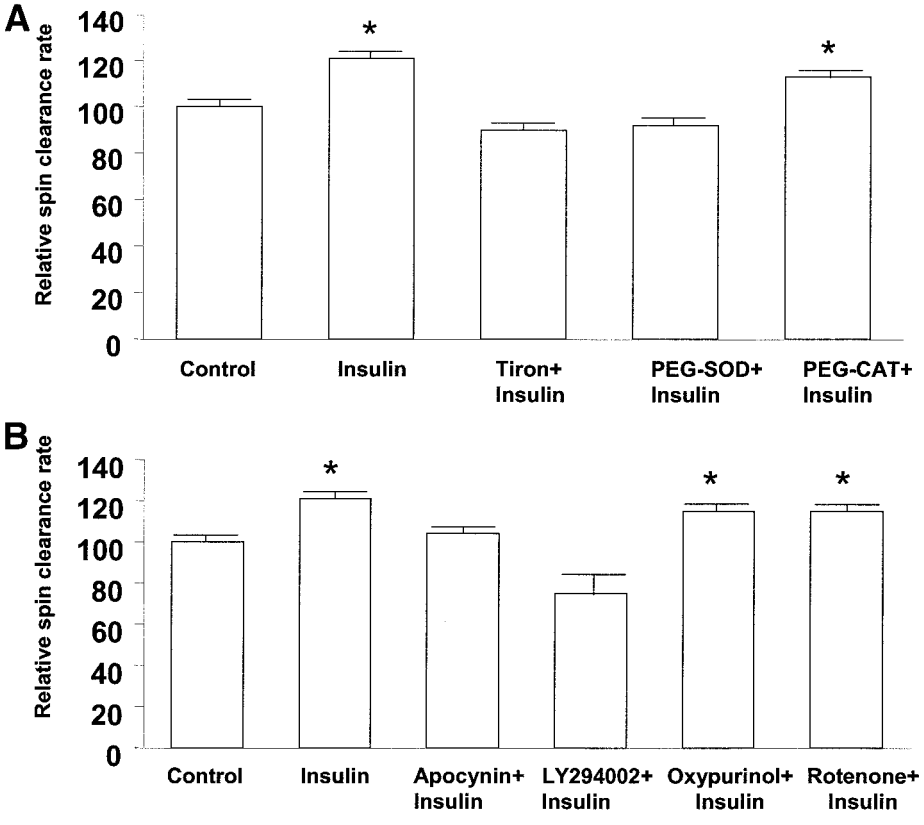

FIG. 3. Effect of insulin and inhibitors on free radical production. Fibroblasts were stimulated with insulin (1 μmol/l). *A*: Effects of Tiron (1 mmol/l), PEG-SOD (250 units/ml), and PEG-CAT (250 units/ml) on insulin-induced free radical production, measured as ESR spin clearance rate. *B*: Effects of apocynin (100 μmol/l), LY294002 (100 μmol/l), oxypurinol (1 mmol/l), and rotenone (1 mmol/l) on insulin-induced free radical production. The results are normalized to control experiments (with no insulin added). Data are means ± SE of at least four independent experiments. *$P <$ 0.01 vs. control.

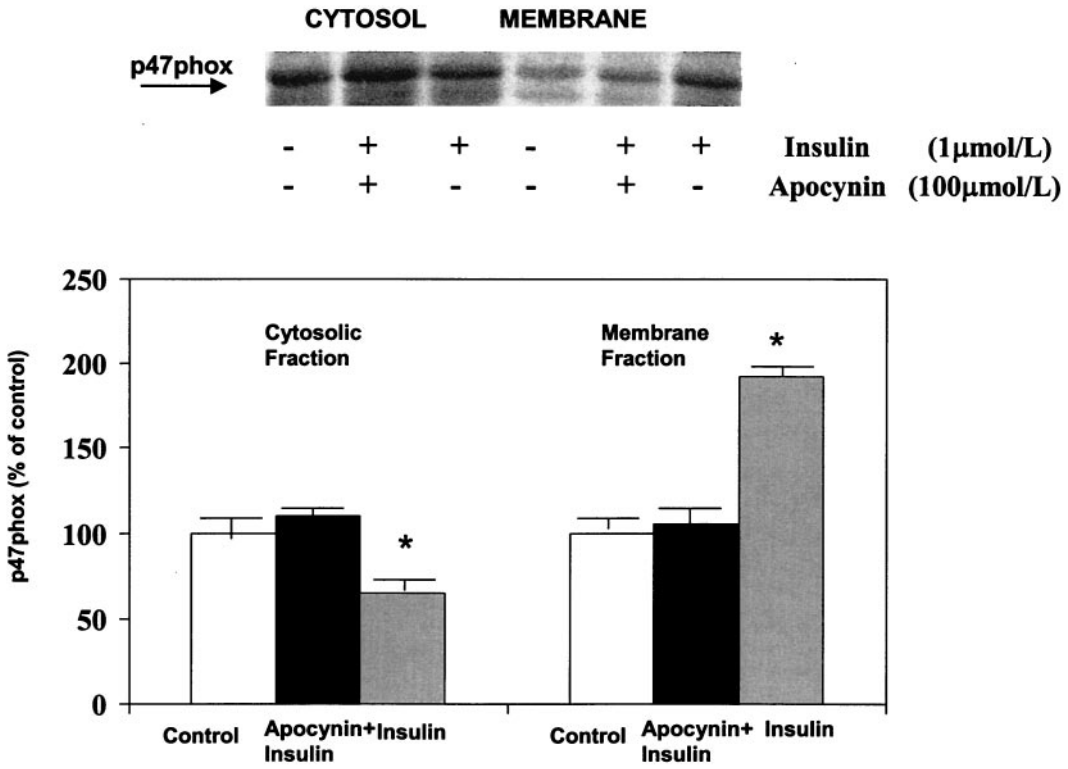

FIG. 4. Effect of insulin on p47phox translocation in cultured human fibroblasts. Cells were stimulated with insulin (1 μmol/l, 10 min) in the absence and presence of apocynin (100 μmol/l, 1 h). Cells were harvested in lysis buffer and p47phox expression was determined as described in RESEARCH DESIGN AND METHODS. *Top panel*: Representative immunoblot for the cytosolic and membranous fraction of p47phox. *Lower panel*: Averaged data, quantified by densitometry of immunoblots and expressed as the percent increase of p47phox expression relative to the control condition. Data are means ± SE of three independent experiments. *$P < 0.01$ vs control.

induced production of free radicals was completely abolished by apocynin, but not by rotenone and oxypurinol, thus demonstrating a specific role of NAD(P)H oxidase on insulin-mediated production of superoxide anion radicals. Also, LY294002 prevented O_2^- insulin-induced production, suggesting that this effect of insulin is mediated by PI 3′-kinase.

Finally we verified that no direct reaction between the probe CT-AM and the inhibitors took place by performing a series of experiments in which the probe and each inhibitor were mixed together without cells, using the same concentrations as in the experiments with fibroblasts. In all these measurements, there was a negligible spin clearance rate (data not shown), demonstrating that all the effects measured were due to stimulated production of superoxide radicals from the fibroblasts.

Effect of insulin on p47phox translocation. To delineate the underlying mechanisms by which insulin activates NAD(P)H oxidase, we evaluated whether insulin induces the translocation of p47phox from cytosol to the membrane in human fibroblasts. As shown in Fig. 4, p47phox content was greater in the cytoplasmic than in the membrane fraction in basal conditions. After cells were stimulated with insulin (1 μmol/l), p47phox abundance was reduced in the cytoplasmic and increased in the membrane fraction. When the cells were pretreated with apocynin (100 μmol/l), the translocation of p47phox by insulin was abolished.

Effect of insulin on phosphorylation of ERK-1 and -2. As shown in Fig. 5*A*, the phosphorylation of ERK-1 and -2 was stimulated by insulin in a dosage-dependent manner, with maximal activity found at 1–10 μmol/l and an ED_{50} of 200 nmol/l. An example of the time course of insulin-induced ERK-1 and -2 phosphorylation in fibroblasts is shown in Fig. 5*B*. The maximal activation of ERK-1 and -2 phosphorylation was reached in 10 min and later declined. Insulin-induced ERK-1 and -2 activation was prevented by pretreatment of the cells with PD98059, a specific inhibitor of the ERK-1 and -2 cascade (Fig. 6). A high dosage (100 μmol/l) of LY294002, a specific inhibitor of PI 3′-kinase, markedly reduced ERK-1 and -2 phosphorylation by insulin, whereas a lower dosage (100 nmol/l) was ineffective (Fig. 6).

Insulin-induced oxidative stress and activation of ERK-1 and -2. Recent studies have shown that exogenous H_2O_2 stimulates the ERK-1 and -2 cascade in various cell types. Thus, we first determined the effect of exogenous H_2O_2 on ERK-1 and -2 activation in human fibroblasts. We stimulated cells with 200 μmol/l H_2O_2 and detected the phosphorylation of ERK-1 and -2 . As shown in Fig. 7*A*, ERK-1 and -2 were activated by H_2O_2 after 5 min, with the activity peaking after 20 min. Next we evaluated the effects of PEG catalase treatment (250 units/ml, 1 h) on insulin-induced ERK-1 and -2 phosphorylation. As shown in Fig. 7*B*, PEG catalase induced a partial attenuation (~30%) on insulin-stimulated ERK-1 and -2 phosphorylation.

To ascertain whether insulin-induced ERK-1 and -2 activation is due to the activation of NAD(P)H oxidase, we examined the effect of apocynin on ERK-1 and -2 activation by insulin. As shown in Fig. 8, apocynin blunted

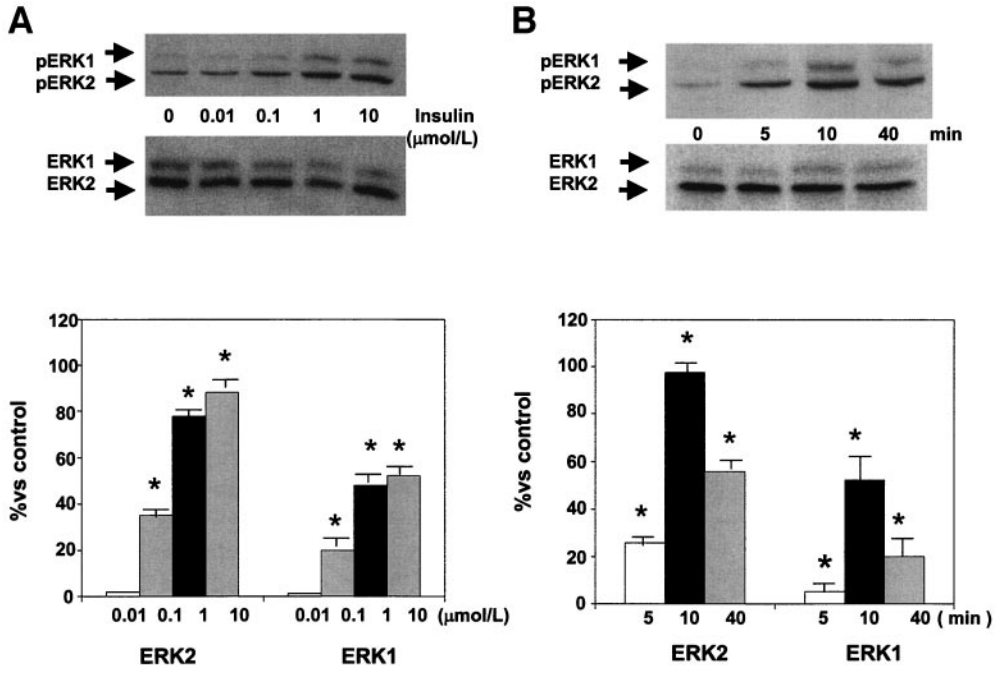

FIG. 5. Effect of insulin on ERK-1 and -2 phosphorylation in cultured human fibroblasts. *A*: Dosage-response effect of insulin on ERK phosphorylation. *B*: Time course of insulin (1 μmol/l) on ERK-1 and -2 phosphorylation. *Top panels*: Representative immunoblots of insulin-induced phosphorylation of ERK-1 and -2. *Bottom panels*: The same blots were stripped and reprobed with control anti–ERK-1 and -2 antibody showing equal protein loading. This panel represents averaged data, quantified by densitometry of immunoblots and expressed as the percent increase of ERK-1 and -2 phosphorylation compared with control. The amount of phosphorylation was calculated from the ratio of pERK to ERK in the same strip. Data are means ± SE of three independent experiments. *P < 0.01 vs control.

insulin-induced ERK-1 and -2 phosphorylation in human fibroblasts.

DISCUSSION

In this study, we provided the first direct evidence that insulin generates free radicals in human fibroblasts ex vivo. This effect is mediated by an NAD(P)H oxidase–dependent mechanism, an observation substantiated by the translocation of the p47phox subunit from cytosol to the cell membrane. As a result, the activation of ERK-1 and -2 is observed. All of these effects were abolished by inhibition of the PI 3′-kinase pathway; therefore we propose that the high insulin concentration causes a state of cellular free radical stress via PI 3′-kinase activation. Insulin-induced oxidative stress may promote cell growth and proliferation through the activation of ERK-1 and -2.

Previous epidemiological studies have indicated a close relation between plasma insulin levels and the incidence of cardiovascular disease, supporting the hypothesis that hyperinsulinemia, as a reflection of insulin resistance, may be an atherogenic factor (20,21). Furthermore, experimental evidence indicates that the generation of free radicals may play an important role in the etiology of diabetes complications (22–24).

Kashiwagi et al. (12) indirectly showed that insulin treatment increases superoxide in rats. Their findings also

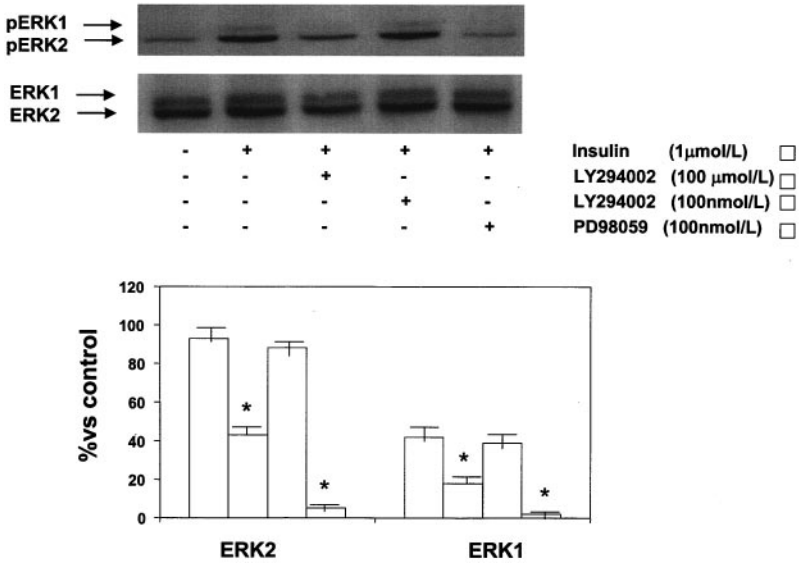

FIG. 6. Effect of PD98059 and LY294002 on insulin-induced ERK-1 and -2 activation in human fibroblasts. Cells were pretreated with PD98059 (100 nmol/l) and LY294002 (100 nmol/l and 100 μmol/l) for 1 h and then stimulated with insulin (1 μmol/l) for 10 min. *Top panel*: Representative immunoblot of insulin-induced phosphorylation of ERK-1 and -2 in the presence of inhibitors. *Bottom panel*: The same blot was stripped and reprobed with control anti–ERK-1 and -2 antibody, showing equal protein loading. This panel represents averaged data quantified by densitometry of immunoblot, expressed as the percent increases in phosphorylation compared with control. The amount of phosphorylation was calculated from the ratio of pERK to ERK in the same strip. Data are means ± SE of three independent experiments. *P < 0.01 vs control.

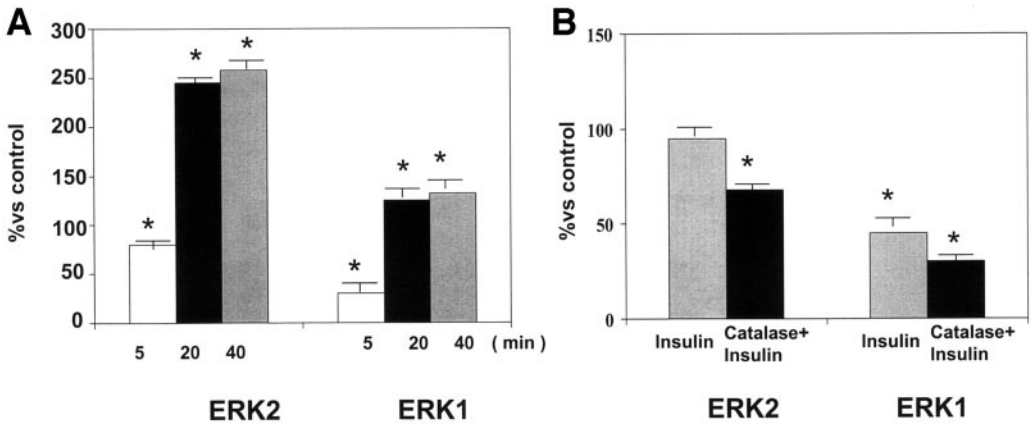

FIG. 7. Effect of H_2O_2 on ERK-1 and -2 stimulation. *A*: Time course for activation of ERK-1 and -2 by H_2O_2 (200 μmol/l). *B*: Effects of PEG-catalase (250 units/ml for 1 h) on insulin-induced ERK-1 and -2 phosphorylation. Data are means ± SE of three independent experiments. *$P < 0.01$ vs control.

supported the hypothesis of Facchini et al. (25), in which age-associated oxidative stress is determined by hyperinsulinemia, and the findings of Wittman et al. (26), in which human platelets generate peroxynitrites under insulin stimulation.

However, it is not yet clear whether hyperinsulinemia itself is a causative factor of oxidative stress. In the present study, we demonstrated that insulin markedly increases the generation of free radicals in human fibroblasts. Furthermore, ESR measurements indicated that insulin specifically generated O_2^- production, as its effect was inhibited by PEG-SOD and Tiron, an O_2^- scavenger.

Several sources of O_2^- production have been identified so far, such as xanthine oxidase, lipoxygenases, mitochondrial oxidases, and cytochrome p450 enzymes (27). Recently, a phagocyte-type NAD(P)H oxidase has been recognized as a major source of superoxide and has been described in neutrophils, vascular smooth muscle cells, endothelial cells, and fibroblasts (5,28).

To determine which enzyme systems are involved in insulin-induced O_2^- generation, we used inhibitors known to selectively inhibit NAD(P)H oxidase, xanthine, and mitochondrial oxidases. We showed that O_2^- production induced by high levels of insulin in human fibroblasts is specifically mediated by a membrane NAD(P)H oxidase–dependent mechanism and not by other enzymes. This was suggested by the observation that insulin-induced O_2^- production could be prevented by apocynin, an NAD(P)H oxidase inhibitor that specifically blocks the association of the p47phox cytosolic subunit with the NAD(P)H oxidase membrane (29,30). Thus, our experiments emphasize the importance of p47phox activation in mediating the generation of free radicals in the presence of elevated insulin concentrations in human fibroblasts ex vivo, and our results are consistent with previous data obtained in isolated adipocytes (11).

The NAD(P)H oxidase complex consists of several membrane-bound subunits (gp91phox, nox, and p22phox) and cytosolic subunits (p47phox and p67phox). Recent studies have demonstrated that the activation of p47phox requires the phosphorylation of its specific serine residues by a PI 3′-kinase/Akt–dependent pathway (31,32). Our data confirm this hypothesis, as we showed that the inhibition of PI 3′-kinase by LY294002 completely suppressed O_2^- production. Furthermore, a high level of insulin induced the translocation of p47phox from cytosol

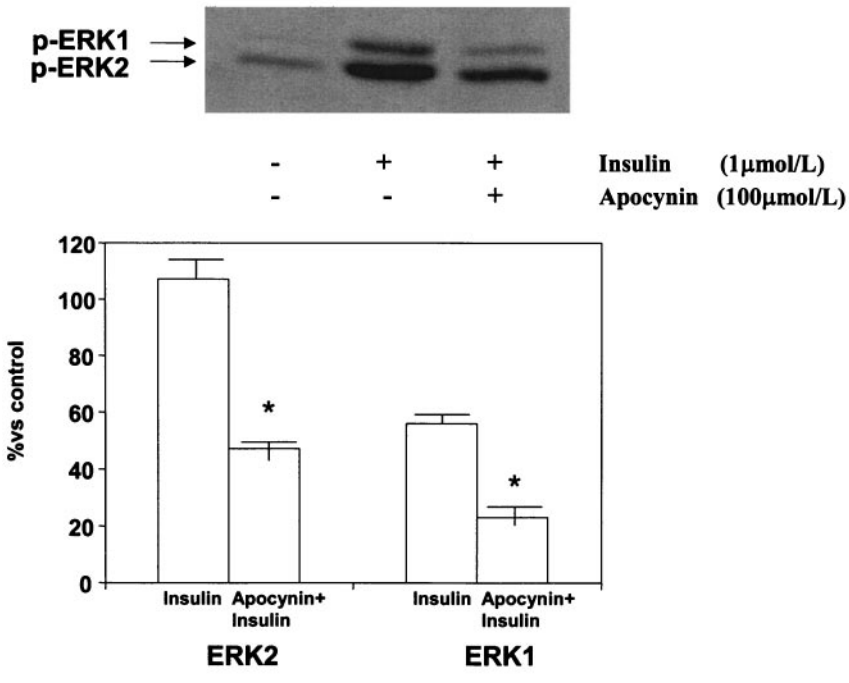

FIG. 8. Effect of apocynin on ERK-1 and -2 phosphorylation induced by insulin. Cells were pretreated with apocynin (100 μmol/l) for 1 h and then stimulated with insulin (1 μmol/l) for 10 min. *Top panel*: Representative immunoblot of insulin-induced phosphorylation of ERK-1 and -2. *Lower panel*: Averaged data quantified by densitometry of immunoblot, expressed as percent increases of phosphorylation compared with control. Data are means ± SE of three independent experiments. *$P < 0.01$ vs control.

to the membrane, an effect that was blocked by apocynin. Therefore, all of these data indicate that insulin activates NAD(P)H oxidase and increases O_2^- through activation of the PI 3′-kinase/AKT cascade, leading to p47phox translocation. To our knowledge, this is the first evidence to show the involvement of PI 3′-kinase in insulin-stimulated NAD(P)H oxidase.

Another goal of our study was to determine whether free radical generation by hyperinsulinemia is involved in ERK-1 and -2 activation. ERK-1 and -2 are enzymes of the complex superfamily of mitogen-activated protein serine/threonine protein kinases (MAPKs), which also includes p38 MAPK and c-Jun-NH_2-terminal kinase/stress-activated protein kinase (JNK/SAPK) (33). ERK-1 and -2 , p38 MAPK, and JNK/SAPK can all be activated by a variety of stimuli, including growth factors, cytokines, and different cellular stresses. ERK-1 and -2 is an important intracellular mediator of insulin signaling; the ERK-1 and -2 cascade is involved in the transduction of signals leading to cell growth and proliferation (34), whereas p38 MAPK and JNK/SAPK are activated to a much greater extent by stressful stimuli and have been correlated in many instances with the induction of apoptosis (35). Our data showed that the response of human fibroblasts to insulin-induced ERK phosphorylation is quite similar to that of other type of cells, such as vascular smooth muscle cells. Furthermore, ERK-1 and -2 may be activated by oxidative stress, hormones such as angiotensin II, and oxidants such as H_2O_2 (36,37). In fact, the activation of NAD(P)H oxidase generates O_2^-, which is rapidly dismutated to H_2O_2, an important activator for the ERK-1 and -2 cascade.

In the present study, we demonstrated that in human fibroblasts *1*) H_2O_2 directly stimulates ERK-1 and -2 phosphorylation and that apocynin and catalase partially inhibit insulin-stimulated ERK-1 and -2 phosphorylation; and *2*) the activation of ERK-1 and -2 by insulin-mediated O_2^- production is dependent on PI 3′-kinase activation because it can be inhibited by LY294002. All these results strongly support the hypothesis that free radicals produced through NAD(P)H oxidase play a critical role in insulin-induced ERK-1 and -2 activation. Therefore, our data have demonstrated for the first time that the causal relation between NAD(P)H and subsequent ERK-1 and -2 activation is operative in human fibroblasts as it is in other cellular species (38,39).

In conclusion, we have provided direct evidence that insulin generates free radicals in isolated human fibroblasts; the activation of NAD(P)H oxidase, achieved through a PI 3′-kinase−dependent mechanism, was necessary to elicit this effect. The inhibition of this oxidase prevented not only free radical generation, but also insulin-mediated ERK-1 and -2 activation. These observations may offer an explanation for the atherogenic role of chronic hyperinsulinemia.

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