Targeting the ERK signaling pathway as a potential treatment for insulin resistance and type 2 diabetes

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Ozaki K, Awazu M, Tamiya M, Iwasaki Y, Harada A, Kugisaki S, Tanimura S, Kohno M. Targeting the ERK signaling pathway as a potential treatment for insulin resistance and type 2 diabetes. Am J Physiol Endocrinol Metab 310: E643–E651, 2016. First published February 9, 2016; doi:10.1152/ajpendo.00445.2015.—Extracellular signal-regulated kinase (ERK) has been implicated in the development of insulin resistance associated with obesity and type 2 diabetes mellitus. We have now examined the potential of pharmacological targeting of the ERK pathway with MEK (ERK kinase) inhibitors (PD184352 and PD0325901) for the treatment of obesity-associated insulin resistance. The effects of PD184352 and PD0325901 on the expression of adipocytokines and lipolysis activity were thus examined in 3T3-L1 adipocytes maintained in long-term culture as a model of adipocyte hypertrophy. Leptin receptor-deficient (db/db) mice and high-fat diet-fed KKAy mice, both of which are models of type 2 diabetes, were also treated orally with PD184352 to examine its effects on the diabetic condition. ERK activity was increased in hypertrophic 3T3-L1 adipocytes as well as in adipose tissue of db/db mice and high-fat diet-fed KKAy mice, and this enhanced ERK signaling was associated with dysregulation of adipocytokine expression and increased lipolysis activity. Specific blockade of the ERK pathway in hypertrophic 3T3-L1 adipocytes by MEK inhibitors ameliorated the dysregulation of adipocytokine expression and suppressed the enhanced lipolysis activity. Furthermore, repeated oral administration of PD184352 normalized hyperglycemia and hyperlipidemia and improved insulin sensitivity and glucose tolerance in the diabetic mice. These results suggest that sustained activation of the ERK pathway in adipocytes is associated with the pathogenesis of type 2 diabetes and that selective blockade of this pathway with MEK inhibitors warrants further study as a promising approach to the treatment of insulin resistance and type 2 diabetes.

MATERIALS AND METHODS

Reagents and antibodies. PD0325901 was obtained from Sigma-Aldrich (St. Louis, MO), and PD184352 was synthesized as described previously (37). Antibodies to phospho-ERK1/2 and to β-actin were obtained from Sigma-Aldrich, those to ERK1/2, MAPK phosphoase-1 (MKP-1), PCNA, DLK (Pref-1), p53, p53 receptor activator (PPARγ), and CCAAT/enhancer binding protein (C/EBP-α) were from Santa Cruz Biotechnology (Santa Cruz, CA), and those to adiponectin, mouse IL-6, and mouse TNFα were from Abcam.
MEK inhibitors ameliorate dysregulation of adipocytokine expression and suppress lipolysis in hypertrophic 3T3-L1 adipocytes. To investigate the molecular mechanism of adipocyte hypertrophy, we examined the activation level of ERK1/2 during the course of adipogenesis and subsequent development of adipocyte hypertrophy in the best-characterized in vitro model of adipogenesis/3T3-L1 adipocytes cultured for up to 30 days after the induction of differentiation. Activation (phosphorylation) of ERK1/2 was detected not only during the initiation (20–60 min) and progression (3–4 days) of adipogenesis but also during the development of adipocyte hypertrophy (20–30 days), with the latter phase of ERK1/2 activation being accompanied by marked downregulation of the expression of MKP-1 (Fig. 1A), an inducible dual-specificity phosphatase that inactivates ERK (15, 19).

Adipocytes accumulate lipids and release adipocytokines (10). Staining with Oil Red O thus revealed lipid accumulation in differentiated 3T3-L1 adipocytes cultured for 10 days, and this staining was markedly enhanced in the enlarged cells cultured for 30 days (Fig. 1B). The expression of adiponectin in 3T3-L1 adipocytes increased to reach a maximal level at ~16 days, but it decreased thereafter in association with the development of hypertrophy (Fig. 1A). RT and real-time PCR analysis also revealed that the abundance of adiponectin mRNA was significantly increased on day 10 but had declined to ~50% of the value for day 8 by day 30 (Fig. 1C). 3T3-L1 adipocytes also expressed the genes for several additional adipocytokines, including those for leptin, TNFα, IL-6, MCP-1, and PAI-1, with the expression of the latter three genes increasing markedly during the development of adipocyte hypertrophy (Fig. 1C). Lipolysis activity also increased significantly during the development of adipocyte hypertrophy (Fig. 1D).

We next examined the relation between the sustained activation of ERK1/2 and the dysregulated expression of adipocytokines in hypertrophic 3T3-L1 adipocytes. Cells that had been cultured for 25 days after the induction of differentiation were thus exposed to the MEK inhibitors PD0325901 or PD184352 for 24 h. Both MEK inhibitors reversed the downregulation of adiponectin mRNA as well as the upregulation of IL-6, MCP-1, and PAI-1 mRNAs and lipolysis activity (Fig. 1, E and F). In contrast, the size of the hypertrophic cells was not substantially affected by PD0325901 (Fig. 1G).

Treatment of undifferentiated 3T3-L1 cells or hypertrophic 3T3-L1 adipocytes with PD184352 or PD0325901 did not affect their differentiation status, as confirmed by immunoblot analysis of Pref-1 (a preadipocyte marker) and of PPARγ and C/EBP-α (adipocyte differentiation markers) (Fig. 1H). Furthermore, although specific blockade of the ERK pathway with MEK inhibitors induces a moderate level of apoptotic cell death in tumor cells in which the pathway is constitutively activated (18), neither PD184352 nor PD0325901 increased the number of apoptotic cells (those labeled with annexin V) in cultures of 3T3-L1 adipocytes exposed to the inhibitors from day 20 to day 30 after the induction of differentiation (Fig. 1I).

The MEK inhibitor PD184352 suppresses elevated ERK activity in fat tissue and ameliorates the diabetic state in db/db mice. Given that the ERK pathway was found to be activated in hypertrophic 3T3-L1 adipocytes, we examined the activa-
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TNF-α (days)

0 2 4 8 12 16 20 25 30

0

Fig. 1. MEK inhibitors ameliorate the dysregulation of adipocytokine expression and suppress lipolysis activity in hypertrophic 3T3-L1 adipocytes. A: immunoblot analysis of phosphorylated (p) and total forms of ERK1/2 as well as adiponectin and MAPK phosphatase-1 (MKP-1) in 3T3-L1 cells at the indicated times after the induction of adipocyte differentiation. B: immunoblot analysis of phosphorylated (p) and total forms of ERK1/2 as well as adiponectin and MAPK phosphatase-1 (MKP-1) in 3T3-L1 adipocytes at the indicated times after the induction of adipocyte differentiation. Data are means ± SE from 4 independent experiments. *P < 0.05 and **P < 0.01 vs. day 8. C and D: RT and real-time PCR analysis of adipocytokine mRNA abundance (C) as well as lipolysis activity (D) in 3T3-L1 adipocytes at the indicated times after the induction of adipocyte differentiation. Data are means ± SE from 4 independent experiments. *P < 0.05 and **P < 0.01 vs. Ct. 

Given the onset of PD184352 treatment (Fig. 2C). A glucose tolerance test performed 14 days after the onset of drug administration showed that glucose tolerance was significantly improved in PD184352-treated mice (Fig. 2D). Furthermore, an insulin tolerance test revealed that the decrease in blood glucose concentration induced by the injected insulin was much larger in PD184352-treated mice than in the vehicle-treated animals (Fig. 2E). There was no difference in body weight (Fig. 2F) or food intake (data not shown) between the PD184352-treated and control mice.

PD184352 ameliorates dysregulation of adipocytokine expression and normalizes hyperlipidemia in db/db mice. Given that PD184352 treatment ameliorated the abnormal glucose metabolism in db/db mice, we next examined its effects on
adipocytokine levels in visceral fat tissue and plasma of these animals. Compared with nondiabetic C57BL/6 mice, db/db mice manifested an abnormal adipocytokine expression profile characterized by a reduced abundance of adiponectin and increased amounts of IL-6 and TNFα (cell-bound precursor) (5) in visceral fat tissue (Fig. 3A) as well as by reduced and increased plasma concentrations of adiponectin and MCP-1, respectively (Fig. 3B). Neither TNFα nor IL-6 was detected in plasma of db/db mice. Administration of PD184352 ameliorated the dysregulation of adipocytokine expression in both fat tissue and plasma of db/db mice (Fig. 3, A and B). In contrast, PD184352 administration did not substantially affect expression of the proliferative marker PCNA (22) in visceral adipose tissue (Fig. 3A) or the markedly increased nonfasting plasma insulin concentration (Fig. 3B) in db/db mice.

Given that free fatty acids generated by lipolysis play a pivotal role in the development of type 2 diabetes (6) and that lipolysis activity was found to be increased in hypertrophic 3T3-L1 adipocytes in an ERK signaling-dependent manner (Fig. 1, D and F), we examined the effects of PD184352 administration on plasma lipid concentrations in db/db mice. Whereas not only NEFA but also triglyceride and total cholesterol levels were increased in db/db mice compared with C57BL/6 mice, these differences were no longer apparent after treatment of db/db mice with PD184352 for 14 days (Fig. 3C).

PD184352 ameliorates the diabetic condition in KKAy mice fed a high-fat diet. To investigate further the antidiabetic effects of PD184352 in a model of obesity-associated type 2 diabetes, we studied KKAy mice fed a high-fat diet beginning at 6 wk of age (39). Similar to db/db mice, diabetic KKAy mice fed the high-fat diet for 2 wk manifested increased ERK1/2 activity (Fig. 4A) and reduced adiponectin expression (Fig. 4B) in visceral fat. Unlike that in db/db mice, however, fat tissue in diabetic KKAy mice showed a marked increase in PCNA expression (Fig. 4B).

Repeated oral administration of PD184352 beginning at 8 wk of age suppressed the upregulation of ERK1/2 activity and the downregulation of adiponectin expression in visceral adipose tissue of KKAy mice maintained on a high-fat diet (Fig. 4B). Such treatment also reduced the nonfasting blood glucose level to a plateau of ~220 mg/dl after 5 days, whereas that in vehicle-treated mice increased gradually to a value of ~500 mg/dl after 60 days. PD184352 also ameliorated the diabetic state in db/db mice (Fig. 3, A and B). The increase in blood glucose levels in vehicle-treated db/db mice increased gradually to a value of ~500 mg/dl after 60 days, whereas that in vehicle-treated mice increased gradually to a value of ~500 mg/dl after 60 days. PD184352 also ameliorated the diabetic state in db/db mice (Fig. 3, A and B).
mg/dl by day 10 (Fig. 4C). Furthermore, administration of PD184352 improved both glucose tolerance (Fig. 4D) and insulin sensitivity (Fig. 4E) in KKAy mice fed a high-fat diet. Treatment with PD184352 for 15 days also suppressed the weight gain in KKAy mice maintained on a high-fat diet (Fig. 4F), possibly reflecting the fact that blockade of the ERK pathway suppressed the high-fat diet-induced upregulation of PCNA expression in visceral adipose tissue of these animals (Fig. 4B).

PD184352 has no effect on blood glucose level, glucose tolerance, or insulin sensitivity in nondiabetic C57BL/6 mice. We finally examined the effects of repeated oral administration of PD184352 on nondiabetic C57BL/6 mice. In contrast to db/db mice and high-fat diet-fed KKAy mice, repeated oral administration of PD184352 did not significantly affect the nonfasting blood glucose level (Fig. 5A), glucose tolerance (Fig. 5B), or insulin sensitivity (Fig. 5C) in C57BL/6 mice. It also had no effect on body weight in these animals (Fig. 5D).

**DISCUSSION**

We found that hypertrophic 3T3-L1 adipocytes manifest dysregulated adipocytokine expression (upregulated IL-6, MCP-1, and PAI-1; downregulated adiponectin) as well as...
increased lipolysis activity, with these phenotypes being associated with elevated activity of the ERK signaling pathway. The ERK pathway was also activated in adipose tissue derived from two mouse models of type 2 diabetes (db/db mice and high-fat diet-fed KKAy mice), and this activation was associated with an abnormal adipocytokine expression profile in adipose tissue and plasma similar to that of hypertrophic 3T3-L1 adipocytes (downregulation of adiponectin and upregulation of IL-6, MCP-1, and TNFα). Moreover, the plasma concentrations of not only NEFA but also triglyceride and total cholesterol were increased in db/db mice. In this regard, ERK1/2-mediated phosphorylation has been shown to modulate the function of sterol regulatory element-binding proteins, which are key transcription factors in the regulation of lipid metabolism, including the synthesis of cholesterol and fatty acids (23, 31). The abnormal adipocytokine expression profiles and lipid disorders observed in hypertrophic 3T3-L1 adipocytes as well as in diabetic model mice were normalized as a result of specific blockade of the ERK signaling pathway with MEK inhibitors. Furthermore, repeated oral administration of PD184352 normalized the nonfasting blood glucose level, ameliorated glucose intolerance, and improved insulin sensitivity in both db/db mice and high-fat diet-fed KKAy mice.

The sustained activation of ERK1/2 apparent in hypertrophic 3T3-L1 adipocytes might have been due to the observed downregulation of MKP-1 expression, consistent with previous findings showing downregulation of this protein during the development of adipocyte hypertrophy (19). Activation of lipolysis via ERK1/2-mediated phosphorylation of hormone-sensitive lipase at Ser600 in adipocytes has also been described previously (17). Furthermore, the ERK pathway has been found to increase expression of MCP-1 (19), PAI-1 (12), and IL-6 (24). On the other hand, expression of adiponectin is regulated by PPARγ, whose transactivation activity has been found to be inhibited by ERK1/2-mediated phosphorylation at Ser112 (34). However, we did not detect such ERK pathway-dependent phosphorylation of PPARγ in hypertrophic 3T3-L1 adipocytes.

Fig. 4. P18 ameliorates the diabetic condition in KKAy mice fed a high-fat diet (HFD). A: immunoblot analysis of phosphorylated (p) and total forms of ERK1/2 in visceral adipose tissue, liver, and skeletal muscle of KKAy mice fed a normal diet (ND) or HFD for 2 wk beginning at 6 wk of age. B: immunoblot analysis of ERK1/2, adiponectin, and PCNA in visceral adipose tissue of KKAy mice fed a ND or maintained on a HFD and treated with P18 (HFD + P18) or vehicle (HFD) for 2 wk beginning at 8 wk of age. C: nonfasting blood glucose concentrations in KKAy mice fed a HFD and treated by oral administration of P18 or vehicle (control). D and E: glucose (D) and insulin tolerance tests (E) performed in KKAy mice fed a HFD and treated with P18 (○) or vehicle (□) for 2 wk. F: body weight of HFD-fed KKAy mice before and 16 days after the onset of treatment with P18 or vehicle. Data in C–F are means ± SE (n = 4–6). **P < 0.01 vs. the corresponding value for vehicle-treated mice.
subunit of NF-κB at Lys310 that was dependent on the ERK pathway. We did detect elevated acetylation of the p65 subunit of NF-κB in both adipocytes and macrophages. The upregulation of both ERK and NF-κB activity in these cells likely then cooperatively contributes to the dysregulation of adipocytokine expression in adipose tissue of diabetic mice.

Although treatment with PD184352 for 15 days suppressed the weight gain in KKAy mice fed a high-fat diet, it did not affect the change in body weight in db/db mice. In this regard, whereas ERK1 knockout was found to prevent high-fat diet-induced adipogenesis and obesity in C57BL/6 mice (8), it did not affect body weight in leptin-deficient (ob/ob) mice (21). The prevention of the development of adiposity and obesity by deficiency of ERK signaling in otherwise wild-type mice fed a high-fat diet thus appears to be lost in animals with a background characterized by a deficiency in leptin signaling (21). In contrast to its differential effect on body weight, however, PD184352 manifested essentially identical therapeutic effects on the diabetic condition in both db/db mice and high-fat diet-fed KKAy mice, including normalization of hyperglycemia and improvement of glucose tolerance and insulin sensitivity. Consistent with our present results, ERK1 knockout also improved glucose tolerance and whole body insulin sensitivity in ob/ob mice, with these effects being associated with increased glucose transport into muscle, reduced de novo lipogenesis in the liver, and enhanced insulin-induced suppression of free fatty acid release by adipose tissue (21).

ERK activity has previously been found to be elevated in adipose tissue of humans and rodents in the diabetic state (3, 9, 11). In this regard, gene targeting has revealed that ERK1, but not ERK2, is required specifically for adipogenesis in vitro and in vivo and that ERK1 knockout mice challenged with a high-fat diet are resistant to obesity and protected from insulin resistance (8). Furthermore, although leptin-deficient (ob/ob) mice lacking ERK1 develop pronounced obesity, they are...
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protected from insulin resistance (21). These observations suggest that specific inhibition of the ERK pathway is a potential novel therapeutic strategy to combat insulin resistance and type 2 diabetes. In the present study, we found that specific blockade of the ERK pathway with a MEK inhibitor normalized the dysregulated adipocytokine expression profile, lowered the nonfasting blood glucose level, increased glucose tolerance, and improved insulin sensitivity in db/db mice and high-fat diet-fed KKAY mice. Targeting the ERK pathway by MEK inhibitors thus warrants further investigation as a potential option for the treatment of insulin resistance and type 2 diabetes.

REFERENCES

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DISCLOSURES

The authors declare no potential conflicts of interest.

AUTHOR CONTRIBUTIONS


The authors declare no potential conflicts of interest.


