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Selenate inhibits adipogenesis through induction of transforming growth factor-β1 (TGF-β1) signaling

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ABSTRACT

Selenium is essential for many aspects of human health. While selenium is known to protect against cancer and cardiovascular diseases, the role of selenium in adipose development is unknown. Here we show that selenate at non-toxic concentration exhibits an anti-adipogenic function in vitro and ex vivo. In addition, selenate induced a morphological change of these cells from fibroblast-like to spindle cell shape. However, other forms of selenium, including selenite and methylseleninic acid, showed either toxic or no effect on adipogenesis and morphology change of preadipocytes. The effects of selenate on adipogenesis and cell morphology change were blunted by the treatment with SB431542, a specific inhibitor of transforming growth factor-β1 (TGF-β1) receptor, neutralization TGF-β1 by its antibody, and knockdown of *TGF-β1* in preadipocytes, suggesting a requirement of TGF-β signaling for the anti-adipogenic function of selenate. Among tested forms of selenium, selenate appears to be an effective activator of *TGF-β1* expression in preadipocytes. These results indicate that selenate is a novel dietary micromineral that activates TGF-β1 signaling in preadipocytes and modulates adipogenesis.

1. Introduction

Adipogenesis is known to attribute to an increase in adipose mass and the development of obesity. Adipogenesis occurs progressively throughout life in humans [1]. While several members of CCAAT/enhancer-binding protein (C/EBP) family, peroxisome proliferator-activated receptorγ (PPARγ), glucocorticoid, cAMP, and insulin signaling pathways are known to promote adipogenesis [2], cellular signaling pathways associated with preadipocyte factor-1 (Pref-1), tumor necrosis factor-α (TNF-α), Wnt, and transforming growth factor-β (TGF-β) [3,4] play an inhibitory role in adipogenesis. Thus, methods to elevate and/or sustain the expression levels of these negative factors in preadipocytes would contribute to lowering the adipose mass gain.

TGF-β is a multipotent cytokine that signals through its receptors for autocrine and paracrine effects on cellular growth, differentiation, inflammation, and matrix synthesis [5]. Once TGF-β binds to its receptors, the activated serine/threonine kinase activity of the receptors subsequently phosphorylates cytosolic transcription factors Smad2 and Smad3, which then form a complex with Smad4 to interact with transcription factors in the nucleus for transcription of TGF-β-responsive genes [6]. TGF-β treatment or ectopic expression of Smad3 in preadipocytes has been shown to inhibit adipogenesis [7]. Conversely, suppression of *Smad3* gene expression or expression of dominant negative TGF-β receptor in preadipocytes resulted in stimulation of adipogenesis [4]. On the other hand, recent studies demonstrated that mice with defect in TGF-β signaling displayed resistance to obesity and insulin resistance [8,9].

Selenium (Se), an essential micromineral, exists in both inorganic and organic forms, and shows anti-oxidative [10], and anti-inflammatory [11] properties. While both Se deficiency and overdose are associated with functional defects and cellular toxicity, evidence suggests that supranutritional doses of Se may be beneficial to protect against cancer [12,13]. Despite the proposed health benefits of both inorganic and organic forms of Se, selenate (SeO_4^{2-}) appears to be the least cytotoxic to various types of cancer cells and, therefore, the least effective in cancer prevention and development [14]. Nevertheless, the direct role of Se in the development of adipose tissue and obesity has not yet been determined.

Here, we examine the effects of various forms of Se on adipogenesis and provide clear evidence of a novel anti-adipogenic function of selenate at no cytotoxic concentrations. We further provide demonstrate that selenate is a dietary activator of TGF-β1 signaling pathway in preadipocytes, through which selenate exhibits an anti-adipogenic function. Thus, our results reveal a novel function of selenate as a modulator of adipose development through activation of TGF-β1-dependent signaling pathway.

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2. Materials and methods

2.1. Cell culture and adipogenesis conditions

3T3-L1 preadipocytes, C2C12 mouse myoblasts, and mouse embryonic fibroblasts were obtained from American Type Culture Collection. These cells were cultured in DMEM (Fisher Scientific, Pittsburgh, PA) containing 10% (v/v) fetal calf serum (FCS) (PAA), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.11 g/l sodium pyruvate. Adipogenesis and Oil Red O (OR) staining for qualitative and quantitative analyses of intracellular lipids were performed as described previously [15]. Subcutaneous human primary preadipocytes were purchased from ZenBio. Cell culture and adipogenesis of these cells were performed according to the manufacturer's protocol.

2.2. Cell viability assay

Cell viability assay was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [16].

2.3. Measurement of reactive oxygen species (ROS) production

The 3T3-L1 preadipocytes treated with different concentrations of Se were incubated with 100 μM of 2,2-azobios-(2-amidinopropane) dihydrochloride (AAPH), a peroxyl radical generator, for 30 min at 37 °C. After removal of Se from the medium, 2,7-dichlorodihydrofluorescein diacetate (DCHF-DA, Cayman Chemical) working solution was then added directly to the medium to reach to a final concentration of 20 μM for 30 min at 37 °C. ROS generated in AAPH-treated cells is known to oxidize DCFH, a cleaved DCFH-DA by nonspecific intracellular esterases, to the fluorescent compound DCF.

2.4. RNA extraction, reverse transcription (RT)-PCR, and real-time PCR

Total RNA was prepared from cultured cells and isolated stromal vascular cells using TriZol according to the manufacturer's instructions (Invitrogen). First-strand cDNA was synthesized using Superscript II reverse transcriptase system (Invitrogen) or M-MulV reverse transcriptase (Promega). RT-PCR and real-time PCR were performed using Taq polymerase (GenScript) and Power SYBR Green Master Mix (Applied Biosystems), respectively. The specific primers used for RT-PCR and real-time PCR analyses were listed in Supplementary Table 1.

2.5. Lentiviral transductions

A lentiviral vector (pLKO.1) containing mouse *TGF-β1* shRNA was obtained from Open Biosystems (catalog number: TRCN00000065993, sense loop antisense: ccgg cggcagctgtacattgactttctcgagaaagtcaatgtacagctgccgttttttg). To generate lentivirus, 293T cells were co-incubated with 4 μg of pLKO.1-*TGF-β1*, and viral packaging plasmids (4 μg of pHR′-CMV-R8.20vpr and 2 μg of pHR′-CMV-VSV-G) using Lipofectamine 2000 reagents (Invitrogen) [17]. Viruses were harvested as described previously [17]. The viral pellets were re-suspended for an infection of 3T3-L1 cells in the presence of 10 μg/ml of polybrene as well as 10 mM HEPES in serum free medium. After additional 48 h, *TGF-β1* knockdown 3T3-L1 preadipocytes were selected with 1 μg/ml puromycin for at least 3 days.

2.6. Immunoblot analysis

Immunoblot analysis was performed as described previously [17]. Antibodies against β-actin and TGF-β1 obtained from Santa Cruz Biotechnology. Antibodies against p-ERK and anti-ERK were obtained from Genscript.

2.7. Statistical analysis

One-way ANOVA was used to determine significance of treatment effect and interactions using SAS 9.2 software. Significant differences between group means were assessed by Dunnette's multiple comparison or Bonferroni's method with a minimum of $n = 3$. p-Values $\leqslant 0.05$ were regarded as significantly different.

3. Results

3.1. Selenate inhibits adipogenesis

We first examined the effect of various forms of Se on cell viability in proliferating 3T3-L1 preadipocytes. Among the tested Se, selenite (SeO_3^{2-}), inorganic Se, and methylseleninic acid (MSA), an organic Se, dramatically inhibited 3T3-L1 cell viability with more than 80% inhibition at 50 μM and 10 μM, respectively (Fig. 1A). Selenite at low concentration has shown to promote proliferation of human preadipocytes [18]. Similarly, we observed selenite-promoted 3T3-L1 cell viability at 10 μM (Fig. 1A). However, selenate up to 100 μM had no effect on the viability of both proliferating 3T3-L1 preadipocytes (Fig. 1A). Thus, selenate was chosen for further studies. To test the effect of selenate on adipogenesis, 3T3-L1 preadipocytes were subjected to adipogenesis in the presence or absence of various concentrations of selenate for 6 days as illustrated in Fig. 1B. Six days of selenate treatment resulted in dose-dependent inhibition of adipogenesis with approximately 90% inhibition of lipid accumulation at 50 μM as judged by Oil Red O (ORO) staining (Fig. 1B). Similarly, selenate also dose-dependently inhibited adipogenesis of primary human preadipocytes (Fig. 1C). We next examined whether other forms of Se such as selenite and MSA at non-toxic concentrations could alter adipogenesis of 3T3-L1 cells. While selenite higher than 5 μM and MSA higher than 1 μM were toxic to differentiating preadipocytes (data not shown), selenite and MSA at the concentrations of 0–5 μM and 1 μM, respectively, showed no significant effect on adipogenesis of 3T3-L1 cells (Fig. 1D). Since Se was previously suggested to prevent cancer development [12,13], we reasoned whether preadipocytes pre-exposed to selenate could alter adipogenesis. As illustrated in Fig. 1E, we examined the effect of a 24-h pretreatment of 3T3-L1 preadipocytes with various concentrations of selenate on adipogenesis. Confluent preadipocytes exposed to selenate from Day −1 to Day 0 displayed a dose-dependent inhibition of intracellular lipid droplet accumulation at Day 6 (Fig. 1E). Selenate pretreatment at 50 μM resulted in approximately 95% inhibition of adipogenesis (Fig. 1F). This was correlated with reduced mRNA levels of *PPARγ* and *FAS* in differentiated adipocytes from selenate-pretreated preadipocytes (Fig. 1G). However, selenate pretreatment showed no effect on *C/EBPβ* mRNA level at Day 6 of adipogenesis (Fig. 1G). Taken together, our results clearly demonstrate an anti-adipogenic function of selenate in adipogenesis with no cytotoxic effects.

3.2. TGF-β1 activation mediates the anti-adipogenic effect of selenate

We next explored the molecular basis underlying selenate-inhibited adipogenesis. Increased reactive oxygen species and oxidative stress in preadipocytes have been shown to promote adipo-

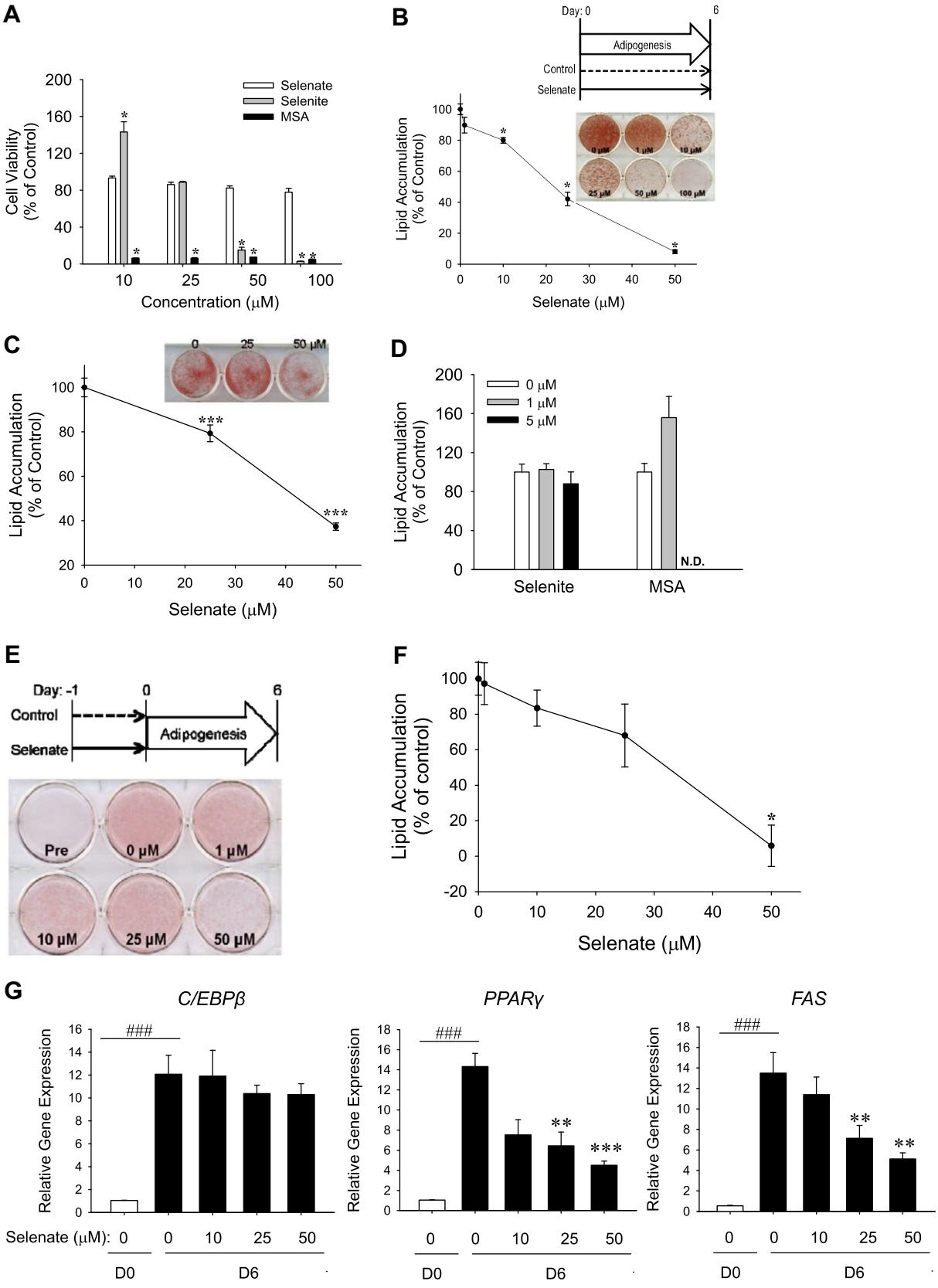

Fig. 1. (A) Cell viability assay of 3T3-L1 preadipocytes after 48 h of treatment with various concentrations (0–100 μM) of selenate, selenite, and MSA. 3T3-L1 preadipocytes (B) and primary human subcutaneous preadipocytes (C) were subjected to adipogenesis in the presence of various concentrations (0–50 μM) of selenate for 6 days and 14 days, respectively, and were subjected to ORO staining analysis for measurement of accumulated lipid droplets. (D) ORO staining analysis of differentiated 3T3-L1cells for 6 days in the presence of various concentrations of selenite and MSA. N.D.; not detected. (E) Schematic of selenate pretreatment of 3T3-L1 preadipocytes. (E, F) ORO staining of differentiated cells after pretreatment with various concentrations of selenate from Day 1 to Day 0. (G) Real-time PCR analysis of *C/EBPβ*, *PPARγ* and *FAS* genes in the cells from panel F at Day 0 (D0) and Day 6 (D6). The signals were normalized to *β-actin* expression, and the results were expressed as relative fold of gene expression. Data represent means ± SEM, $n = 3–9$. $^{*}p < 0.05$, $^{***}p < 0.001$. ### indicates a significant difference between preadipocytes (D0) and adipocytes (D6) ($p < 0.001$).

genesis [19]. Since Se is known to regulate redox reactions [14,20], we questioned whether the anti-oxidative property of selenate is attributed to inhibition of adipogenesis. We, thus, examined the effect of selenate, selenite and MSA on AAPH-induced oxidative stress in preadipocytes. While a 30-min treatment of preadipocytes with 25 μM of selenite inhibited AAPH-induced oxidative stress by 40% compared to AAPH-treated control cells, both 25 μM and 50 μM selenate lowered only 16% of AAPH-induced oxidative stress (Fig. 2A). However, MSA at 5 μM showed no inhibitory effect on AAPH-induced oxidative stress in preadipocytes. These results suggest that selenate-inhibited adipogenesis is independent of selenate's anti-oxidant property. Se has been reported to modulate various cellular signaling pathways such as c-jun N-terminal kinase (JNK) and phosphoinositide 3-kinase (PI3K) signaling pathways [18,21,22]. In addition, activated TGF-β and extracellular signal-regulated kinase1/2 (ERK1/2) signaling pathways in preadipocytes are known to inhibit adipogenesis [7,23]. Thus, we employed selective inhibitors for these protein kinases to screen signaling pathways that could mediate the anti-adipogenic effect of selenate. Similar with Fig. 1E, preadipocytes were exposed to 50 μM selenate in the presence or absence of 10 μM SB431542, SP600125, U0126, or LY294002, specific inhibitors of TGF-β1 receptor, JNK, ERK1/2, and PI3K, respectively, from Day −1 to Day 0 followed by adipogenesis for 6 days in the absence of selenate and the inhibitors. While these inhibitors alone showed no effect on adipogenesis, SB431542 and U0126 completely blocked the

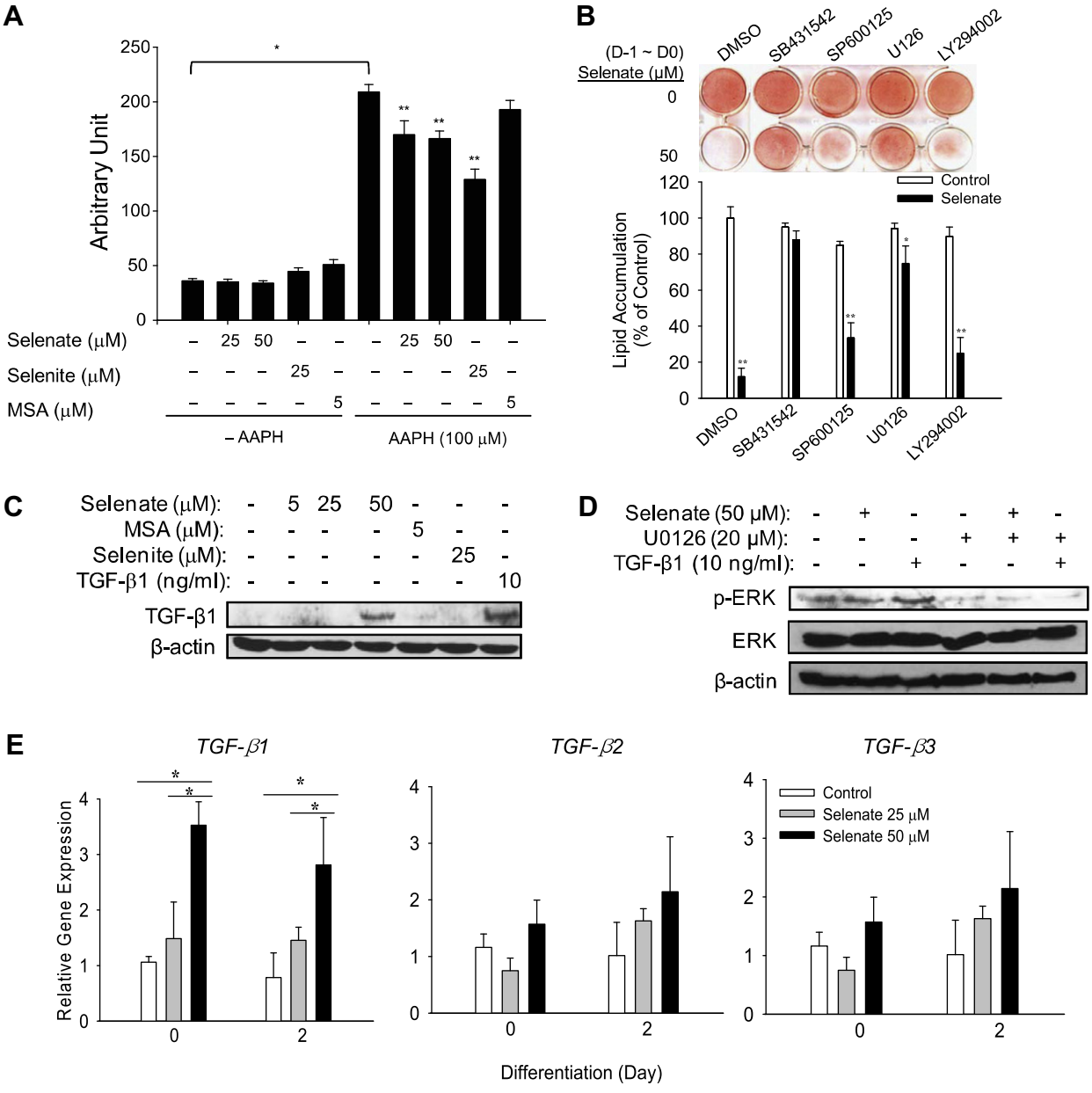

Fig. 2. (A) Measurement of ROS production in 3T3-L1 preadipocytes treated with different forms of Se in the presence or absence of AAPH (100 μM) for 30 min. (B) ORO staining analysis of 3T3-L1 preadipocytes pretreated with 50 μM selenate in the absence or presence of 10 μM SB431542, SP600125, U0126, or LY204002 from Day−1 to Day 0, followed by a 6-day of differentiation. Immunoblotting of TGF-β (C) and phosphorylated ERK (p-ERK) and ERK (D) in 3T3-L1 preadipocytes treated with selenate, MSA, selenite, or recombinant *TGF-β1* in the presence or absence of U0126. (D) Quantitative PCR analysis of *TGF-β1*, *TGF-β2*, and *TGF-β3* in differentiating 3T3-L1 cells at Day 0 or Day 2 after pretreatment with different concentrations of selenate from Day −1 to Day 0. The signals were normalized to β-actin gene level and the results were expressed as relative fold of gene expression. Data represent means ± SEM, $n = 3–9$. $^{*}p < 0.05$, $^{**}p < 0.01$.

selenate-inhibited adipogenesis (Fig. 2B). However, SP600125 and LY294002 exhibited little effect on reversing the anti-adipogenic activity of selenate (Fig. 2B). To further test the role of selenate on TGF-β1 and ERK1/2 signaling pathways in preadipocytes, we examined the effect of selenate on the activation of TGF-β1 and ERK1/2. We found that selenate effectively induced TGF-β protein (Fig. 2C) with little effect on phosphorylation of ERK (Fig. 2D) in preadipocytes as judged by immunoblot analysis. We further found that among the three different isoforms of *TGF-β*, only *TGF-β1* was primarily induced by selenate pretreatment in preadipocytes and in the early phase of adipogenesis (Fig. 2E). These results indicate that TGF-β signaling appears to mediate the anti-adipogenic function of selenate.

3.3. Selenate induces morphology change of preadipocytes through activation of TGF-β1

Parallel to the anti-adipogenic function of selenate, 3T3-L1 preadipocytes treated with different concentrations of selenate (0–100 μM) for 24 h displayed a spindle cell morphology in a dose-dependent manner (Fig. 3A). However, selenate-induced morphological change was diminished by the addition of SB431542 in a dose-dependent manner (Fig. 3B). This appears to be limited to certain cell types as selenate changed morphologies of primary human dermal fibroblasts and NIH 3T3 cells, but not in mouse embryonic fibroblast (MEF) and C2C12 myoblast cells (Fig. 3C). As TGF-β1 has been previously demonstrated to induce morphological change of preadipocytes [4], these results further support the role of selenate in activation of TGF-β1.

3.4. TGF-β1 expression is required for the anti-adipogenic effect of selenate

To investigate whether activation of TGF-β1 signaling is required for selenate-inhibited adipogenesis, we employed a lentiviral knockdown approach that drastically knocks down *TGF-β1* mRNA in selenate-treated 3T3-L1 preadipocytes. Suppression of selenate-induced *TGF-β1* expression in *TGF-β1* knockdown 3T3-L1 preadipocytes was confirmed by a PCR analysis (Fig. 4A). We observed that lentiviral knockdown of *TGF-β1* in 3T3-L1 preadipocytes abrogated selenate-induced morphological change (Fig. 4B). Consistent with Fig. 1F, 50 μM selenate pretreatment of control shRNA lentivirus-transduced preadipocytes resulted in a complete inhibition of adipogenesis (Fig. 4). However, selenate treatment in *TGF-β1* knockdown 3T3-L1 preadipocytes resulted in a complete blockage of the anti-adipogenic effect of selenate in differentiated cells as judged by ORO staining and quantification of ORO stained intracellular lipids (Fig. 4C). As predicted, 6 days of differentiation of selenate-treated *TGF-β1* knockdown preadipocytes resulted in reduced level of *fibronectin* and elevated levels of *PPARγ* and *FAS* mRNA compared with those in differentiated adipocytes from selenate-treated control preadipocytes (Fig. 4D). Overall, these results indicate that selenate is a novel dietary activator of TGF-β1 and the induction of *TGF-β1* expression is required for the anti-adipogenic effect of selenate on adipogenesis.

4. Discussion

Although Se's anti-oxidative and anti-inflammatory properties have long been considered to be beneficial for health [14], the di-

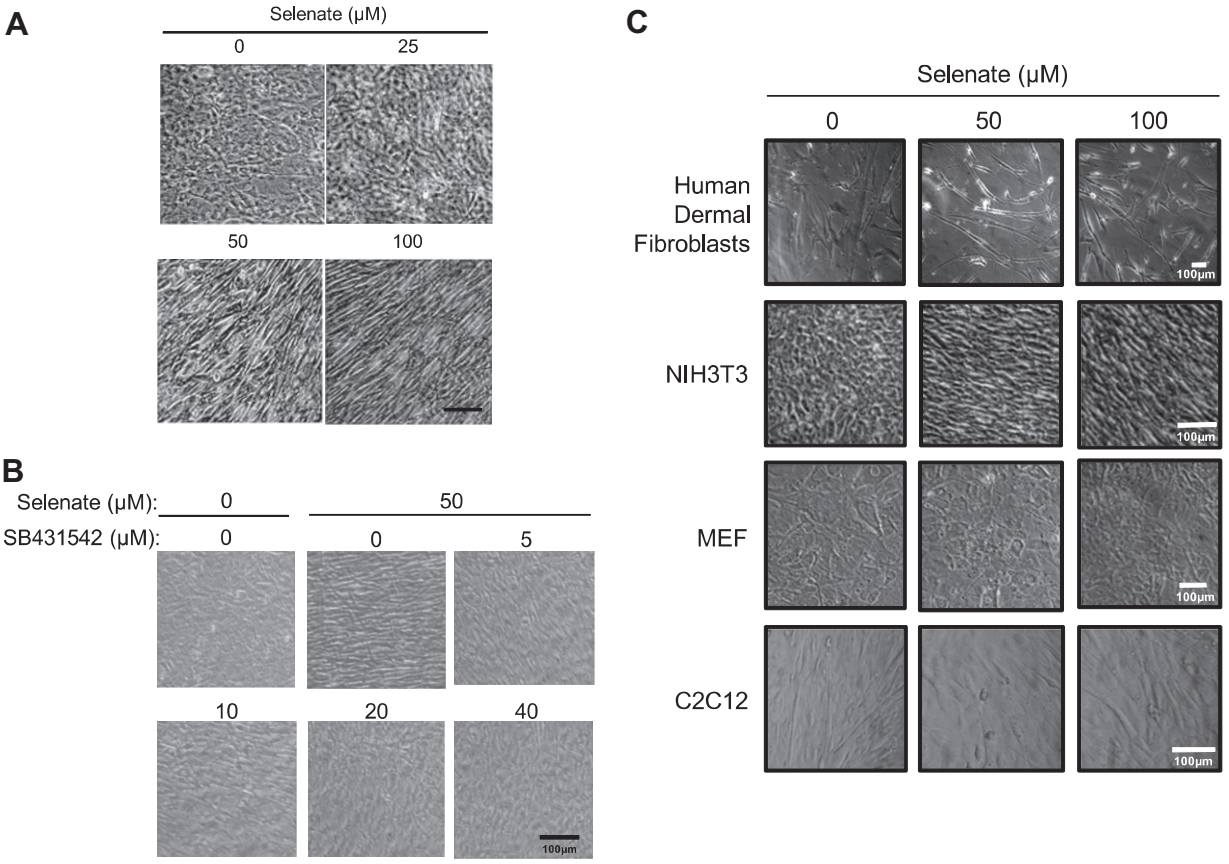

Fig. 3. Microscopic images of 3T3-L1 preadipocytes after 24 h of treatment with various concentrations (0–100 μM) of selenate (A), or 50 μM selenate in the presence or absence of various concentrations of SB431542 (0–40 μM) (B). (C) Primary human dermal fibroblasts, NIH 3T3, mouse embryonic fibroblasts (MEF), and C2C12 mouse myoblasts were exposed to various concentrations of selenate (0–100 μM) for 24 h followed by a microscopic imaging analysis using phase-contrast microscopy. Scale bar; 100 μm. Experiments were conducted at least three times.

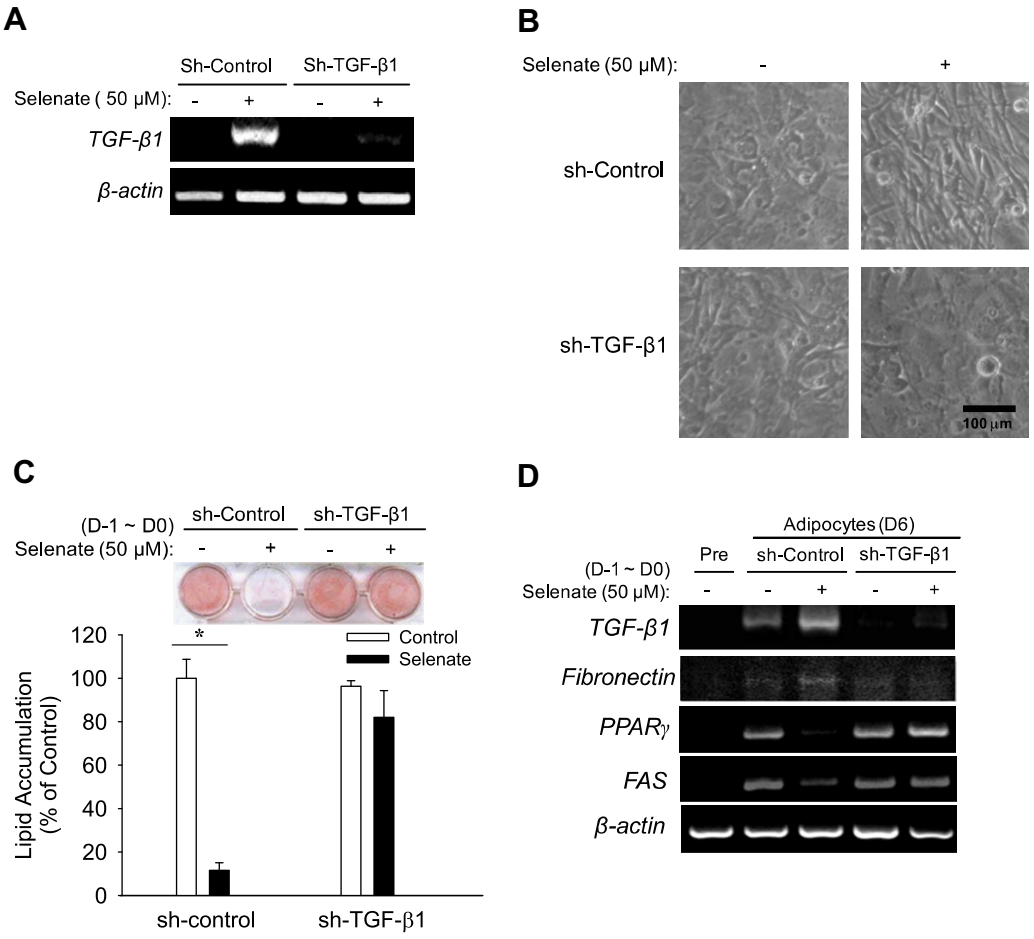

Fig. 4. (A) shRNA-mediated knockdown of TGF-β1 in 3T3-L1 cells with lentivirus containing either control sh-RNA (sh-Control) or TGF-β1-specific shRNA (sh-TGF-β1) for 4 days was confirmed by RT-PCR. (B) Microscopic images of lentivirus infected 3T3-L1 preadipocytes after 24 h of treatment with selenate. ORO staining analysis (C) and RT-PCR analysis of *TGF-β1*, *fibronectin*, *PPARγ*, and *FAS* mRNAs (D) in lentivirus infected 3T3-L1 cells differentiated for 6 days after selenate pretreatment from Day −1 to Day 0. Gene expression of *β-actin* was used as a control. Scale bar; 100 μm. Data represent means ± SEM. Experiments were conducted at least three times. $^{*}p < 0.05$.

rect effect of Se on adipose development and obesity has not yet been elucidated. The present work reveals a novel anti-adipogenic function of selenate, an inorganic Se, which is previously known to be the least effective in preventing carcinogenesis [14]. This notion is supported by several lines of evidence. (i) Post-confluent preadipocytes either pretreated or incubated with selenate display impaired adipogenesis in vitro. However, other forms of Se showed no effect on adipogenesis. (ii) Selenate-induced *TGF-β1* gene in preadipocytes is required for selenate-inhibited adipogenesis and selenate-induced morphology change.

Both Se deficiency and high Se intake (i.e., over 350–400 μg/day) result in the development of Keshan disease and Se toxicity, respectively. Se concentrations, mostly measured by the selenite concentration, in human plasma range from 0.25 μM to 2 μM. RDA of Se is 30–85 μg Se [24]. However, plasma Se level does not seem to be proportionally increased to the increasing amount of daily Se intake. For example, 10 weeks of Se intake of 100, 200 and 300 mg/day resulted in plasma Se levels at 120, 150 and 155 mg/l in humans [25], indicating the presence of a tight control mechanism of plasma Se homeostasis in humans.

Several clinical trials indicated that potential chemopreventive function of Se varies depending on the forms and bioavailability of Se, and types of cancer employed to the studies [26,27]. Nevertheless, Se's anti-cancer property has been most profoundly seen in selenite and organic Se partly due to their abilities to cause cellular apoptosis, necrosis, and/or anti-proliferation. On the other hand, a mixed effect of Se on the risk of type 2 diabetes has been reported. A positive correlation between high Se intake and an increase in the risk of type 2 diabetes has been reported in humans [28,29]. Contrary to these studies, high plasma Se appears to be associated reduced risk of type 2 diabetes [30]. Nevertheless, a recent study performed by Pinto et al. clearly demonstrated that selenate up to 1 mM is safe in L6 myotubes whereas other forms of Se (e.g., selenite, selenomethionine, and methylseleninic acid) were toxic to these cells over 1 μM [24]. Similarly, we also observed that selenate, selenite and MSA differently affected on the viability of 3T3-L1 preadipocytes and their adipogenic ability (Fig. 1A, B and D). Different forms of Se, except selenate, appear to inhibit insulin-induced Akt phosphorylation, FoxO, and glucose uptake in cultured myotubes [24]. However, Muller et al. showed selenate-specific improvement of insulin sensitivity in type 2 diabetic *db/db* mice [31,32]. In addition, selenate was recently reported to prevent Alzheimer's disease through dephosphorylation of tau via promoting the interaction between tau and serine/threonine-specific protein phosphatase 2A [33]. Collectively, these studies emphasize the importance of understanding the diverse biological functions of each form of Se in order to use Se as a dietary intervention of various types of human diseases. In this regard, our finding of selenate-specific anti-adipogenic effect is distinct from the documented biological effects of other forms of Se.

Altered TGF-β signaling pathway appears to play a critical role in determining adipogenic potential and lipid storing ability of

adipose tissue. Activated TGF-β signaling in preadipocytes has been reported to induce genes involved in extracellular matrix (ECM) such as fibronectin and collagen [34]. Indeed, TGF-β-induced fibronectin appears to inhibit adipogenesis by altering the integrity of ECM in adipocytes. This is likely to be through the actions of ECM-regulated insulin signaling [34] and pref-1 [23]. Additionally, TGF-β signaling is also reported to inhibit adipogenesis through Smad3-mediated impairment of C/EBP transactivation function [7]. Likewise, our result of selenate-induced *TGF-β* in preadipocytes inhibited adipogenesis. Despite the potential anti-adipogenic property of TGF-β signaling, obesity is reported to be positively correlated with elevated levels of TGF-β [35,36]. Moreover, mice with impaired TGF-β signaling such as *Smad3* deficient mice exhibited resistance to diet-induced obesity with reduced adipocyte size and fat mass [8,9]. These results indicate that methods to alter basal level of TGF-β1 signaling might be effective strategies for prevention or treatment of obesity. While more studies are needed to elucidate the role of selenate in obesity in vivo, we observed that high fat-diet fed mice supplemented with selenate at 20% of LD_{50} showed reduced adiposity compared with control obese mice (unpublished data).

In conclusion, our results demonstrate that selenate is a novel anti-adipogenic dietary micromineral through an induction of TGF-β signaling in preadipocytes, providing a basis for dietary prevention of adipose development.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.125.

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