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Research paper

Insulin improves osteogenesis of titanium implants under diabetic conditions by inhibiting reactive oxygen species overproduction via the PI3K-Akt pathway

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ABSTRACT

Clinical evidence indicates that insulin therapy improves implant survival rates in diabetic patients; however, the mechanisms responsible for this effect are unknown. Here, we test if insulin exerts anti-oxidative effects, thereby improving diabetes-associated impaired osteoblast behavior on titanium implants. To test this hypothesis, we cultured primary rabbit osteoblasts in the presence of titanium implants and studied the impact of treatment with normal serum (NS), diabetic serum (DS), DS + insulin, DS + tempol (a superoxide dismutase mimetic), DS + insulin + tempol, and DS + insulin + wortmannin. We analyzed cell function, apoptosis, and reactive oxygen species (ROS) production in osteoblasts following the various treatments. Treatment with DS induced osteoblast dysfunction, evidenced by impaired cell attachment and morphology, decreased cell proliferation and ALP activity, and decreased expression of osteogenesis-related genes. We also observed a significant increase in apoptosis. Importantly, treatment with DS resulted in increased production of ROS in osteoblasts. In contrast, treatment with insulin inhibited ROS production, alleviated cell dysfunction, and decreased apoptosis of osteoblasts on the implants. Scavenging ROS with tempol also attenuated cell dysfunction. Compared to insulin treatment alone, the combination of insulin and tempol failed to further improve osteoblast functional recovery. Moreover, the anti-oxidative and pro-osteogenic effects afforded by insulin were almost completely abolished by the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin. These results demonstrate, for the first time, that insulin treatment alleviates the impaired osteogenesis of titanium implants under diabetic conditions by inhibiting ROS overproduction via a PI3K/Akt-dependent mechanism. Both the anti-oxidative and metabolic properties of insulin should make it a viable therapeutic option to combat diabetic implant failure.

1. Introduction

Titanium and its alloys have demonstrated their superiority in the load-bearing applications for manufacturing orthopedic and dental devices. However, several clinical trials have suggested that diabetes mellitus (DM), a metabolic disorder characterized by hyperglycemia and associated with a wide range of symptoms, may negatively influence the process of titanium implant osseointegration and result in a high rate of implant loosening or failure [1]. Compared to the general population, titanium implants in patients with DM are associated with a higher failure rate [2,3]. Animal studies examining the effect of diabetes on the healing of implants have described impaired bone density surrounding osseointegrated titanium implants in diabetic rats [4–6]. Therefore, it is urgent for clinicians to identify new therapeutic strategies to enhance the osseointegration of dental implants in DM patients.

Insulin, the primary hormone regulating glucose metabolism, has been shown to possess anabolic action in bone. Recent clinical trials have provided support for the use of insulin to improve implant survival rates in DM patients. Either general insulin administration [7] or local infiltration at the implant–bone interface [8] may naturally improve the success of oral implantation in

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diabetic rats. Malekzadeh et al. demonstrated that insulin therapy can neutralize the effects of diabetes on bone healing and can enhance peri-implant bone formation in the disease [9]. Although existing evidence suggests that insulin has potential benefits with respect to osteogenesis under conditions of DM, the underlying mechanisms responsible for this effect are unclear.

Reactive oxygen species (ROS) mediate the inflammatory process and are generated in response to external stimuli. ROS production may disrupt the cellular oxidant/antioxidant balance leading to cellular dysfunction and organ damage [10,11]. The formation of ROS on titanium surfaces may negatively influence the activity of surrounding osteoblasts [12] and endothelial cells [13]. Our previous study indicated that overproduction of ROS under diabetic conditions results in osteoblast dysfunction, thus contributing to impaired bone healing associated with titanium implants [14]. In addition to its effects on metabolism, several studies have shown that insulin also inhibits O^{2-} overproduction and attenuates cell damages under conditions of oxidative stress [15], trauma [16] and ischemia/reperfusion [17]. Given that insulin signaling is required for mitochondrial DNA and protein synthesis, impaired insulin action can deregulate mitochondrial function. Furthermore, the activation of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway by insulin increases cell oxidation capacity [18]. In contrast, impaired PI3K signaling leads to cellular mitochondrial dysfunction and prevents mitochondrial adaptation in response to pathophysiological stimuli [19]. Despite this, little is known about the anti-oxidative effect exerted by insulin in the improvement of diabetes-associated impaired bone healing of titanium implants. Therefore, the aim of the study was to determine if insulin could enhance the osseointegration of titanium in diabetic patients by inhibiting ROS overproduction and to investigate the underlying mechanisms responsible for this effect.

2. Materials and methods

2.1. Specimen fabrication

The pure titanium (99.9%, Hight: 2 mm, Diameter: 10 mm) were provided by Northwest Institute for Nonferrous Metal Research (Xi'an, China) that used as the substrate. Porous Ti6Al4V implant was fabricated using electron beam melting process as previously described [20]. After polishing with SiC sandpaper and ultrasonic cleaning, the samples were treated with 0.5 wt% hydrofluoric acid for 30 min, rinsed with distilled water, and dried.

2.2. Cell culture

Primary rabbit calvarial osteoblasts were obtained by digestion of the calvarial bone from 15-day-old New Zealand rabbits, as previously described [21]. The cells were maintained in α-minimum essential medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum at 37 °C in an atmosphere of 5% CO_2/95% air. Osteoblasts (passages 2–3) were seeded on the pure titanium implant as follows: a drop of 50 μL containing 5×10^5 cells was placed on the top of the implant which were placed in 6 wells (Costar, Corning Inc., NY, USA) and allowed to be adsorbed by the porous titanium for 2 h before culture medium was added to cover the scaffolds. Cells were allowed to attach overnight, and then the composites were placed into fresh 6-well plates.

The samples were then randomized to one of the following treatment groups: 1) normal serum (NS); 2) diabetic serum (DS); 3) DS + insulin (10^{-7} mmol/L); 4) DS + tempol (a superoxide dismutase mimetic, 10 mmol/L; Sigma, St. Louis, MO); 5) DS + insulin + tempol; 6) DS + insulin + wortmannin (a specific inhibitor of PI3K). Normal and diabetic serum were obtained from normal and diabetic rabbits, respectively. Diabetic model was induced with New Zealand rabbits (Male, 6 months age, 3.5–3.75 kg) by a single intravenous injection of monohydrated alloxan (Sigma, St. Louis, MO, USA) as described previously [22]. The serum glucose levels at or above 16.67 mmol/L at both 72 h and 3 weeks after administration were considered diabetic. The serum insulin level, which was evaluated with commercial available ELISA kit (CUSABIO Biotech Co., China), was not detected, indicating islet β cells in diabetic rabbit was damaged.

2.3. Labeling of the F-Actin cytoskeleton and visualization by confocal microscopy

Osteoblasts grown on the implants were fixed with 2.0% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS. Blocking solution (1.2% BSA in PBS) was used to block nonspecific antibody binding. Rhodamine-phalloidin (1:40 dilution in blocking solution) was then added to label the F-actin cytoskeleton. Samples were stained with 40,60-diamidino-2-phenylindole (DAPI). Cells were viewed on an Olympus Fluoview 1 × 70 confocal scanning laser microscope, and digital images were captured in TIFF format using the Olympus Fluoview software. The quantitative analysis of the images, which contains cell area and cell number, was performed using the freeware ImageJ software (NIH, Bethesda, MD, USA) by thresholding the images and applying the Analyze Particles function. Five different substrate fields were measured per sample, and six separate samples were measured in each group.

2.4. Cell morphology observation with scanning electron microscope

Cell morphology in different groups at 7 days was observed using the scanning electron microscope (SEM, S-4800, Hitachi, Japan) operating at 15 kV and a semiautomatic interactive image analyzer. Before that, the samples were removed and fixed in 2% v/v glutaraldehyde at 4 °C overnight. After dehydrated through an ethanol series, critical-point dried, the samples were sputtered with gold lastly for SEM observation.

2.5. Cell viability assessment by MTT assay

Incubations with various treatments were maintained for 4 and 7 days. Following this, 3-(4,5-dimethylithiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed. In brief, 20 μL MTT (5.0 mg/ml) was added, and cells were incubated for 4 h at 37 °C. Subsequently, the supernatant was removed, DMSO was added, and the optical density (OD) at 570 nm was measured on a microplate spectrophotometer (Bio-Rad, Hercules, CA, USA). The proliferation rate of the cells was then calculated.

2.6. Analysis of alkaline phosphatase (ALP) activity

Following incubation for 7 days, osteoblasts were washed twice with ice-cold PBS. The cells were incubated with ALP assay solution (8 mM p-nitrophenylphosphate-6 H_2O, 12 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and100 mM glycine-NaOH, pH 10.5) for 10 min at 37 °C. The reaction was terminated by the addition of 200 mM NaOH. Absorbance was measured at a wavelength of 405 nm with a spectrophotometer.

2.7. Quantitative real-time PCR

RT-PCR was performed to assess transcript levels of osteogenesis-related genes, including runt-related transcription factor 2 (Runx2), osteocalcin (OCN), ColI, and bone morphogenetic