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Phenylephrine protects autotransplanted rabbit submandibular gland from apoptosis

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ABSTRACT

Submandibular gland (SMG) autotransplantation is an effective treatment for severe keratoconjunctivitis sicca. Our previous studies have shown that phenylephrine attenuates structural injury and promotes cell proliferation in autotransplanted rabbit SMG. However, the mechanism by which phenylephrine reduces the injury has not been fully evaluated. In this study, we investigate the ability of phenylephrine to inhibit apoptosis in autotransplanted rabbit SMG. We observed that apoptosis occurred in the early phase of SMG transplantation and that phenylephrine treatment protected transplanted SMG from apoptosis. Furthermore, we found that phenylephrine could significantly upregulate the expression of Bcl-2, downregulate the expression of Bax, and inhibit the activation of both caspase-3 and p38 mitogen-activated protein kinase in autotransplanted SMG. Therefore, the cytoprotective effects of phenylephrine on autotransplanted SMG may be a novel clinical strategy for autotransplanted SMG protection during the early postoperative stage of transplantation.

Keratoconjunctivitis sicca is a common disease characterized by reduction or lack of tears with serious complications. Despite the generally efficient pharmaceutical tear substitutes, tarsorrhaphy, or the occlusion of the lacrimal drainage system for the patients with light or moderate keratoconjunctivitis sicca, the patients with severe keratoconjunctivitis sicca did not gain adequate relief of discomfort and often develop corneal changes or even loss of vision [1]. Microvascular submandibular gland (SMG) autotransplantation is one of the most effective treatments for patients with severe keratoconjunctivitis sicca [1–4]. Unfortunately, the majority of patients experience dysfunction of the transplanted gland during the early postoperative stage along with some patients who suffer transplant failure. Therefore, it is vitally important to improve the glandular functional recovery in the early stage after transplantation.

Ischemia/reperfusion injury inevitably occurs during organ transplantation. In fact, it is the main reason for early postoperative graft dysfunction. The role of apoptosis caused by ischemia/reperfusion injury has been investigated in several types of transplanted organs [5,6] but not in SMG grafts. Our previous histopathological studies have demonstrated that transplanted SMG suffers atrophy in the early postoperative phase, which could explain the dysfunction of early stage of the transplanted SMG [7]. We further illustrated that phenylephrine ameliorated this atrophy and promoted cell proliferation in transplanted SMG by activating protein kinase-ζ (PKC-ζ) and extracellular signal-regulated kinases 1/2 (ERK1/2) [7].

However, little is known regarding whether the protective effect of phenylephrine on transplanted SMG cells is mediated by its antiapoptotic mechanism. Mitochondria, caspases, and p38 mitogen-activated protein kinase (MAPK) are relative to regulation of apoptosis. Therefore, the aims of this study are to evaluate apoptosis in SMG grafts and to investigate the effect of phenylephrine on apoptosis inhibition in transplanted rabbit SMG.

Materials and methods

Reagents and antibodies. Phenylephrine was purchased from Sigma (St. Louis, MO). Antibodies against Bcl-2, Bax, activated caspase-3, phospho-p38 MAPK, and total-p38 MAPK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-actin antibody was purchased from Oncogene (Cambridge, MA). Other chemicals and reagents were of analytical grade.

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Experimental animals and autotransplantation procedures. Healthy male New Zealand white rabbits weighing 2.4 ± 0.3 kg were used. All experimental procedures were approved by the Committee of Animal Research, Peking University Health Science Center and were in accordance with the Guidance of the Ministry of Public Health for the care and use of laboratory animals. The rabbits were randomly divided into three groups as follows: (1) Control, sham-operated SMG (without transplantation) ($n = 6$); (2) Transplanted SMG without phenylephrine treatment ($n = 6$); (3) Transplanted SMG with phenylephrine treatment ($n = 6$). Phenylephrine (10^{-7} mol/L, 100 μl) was slowly infused into the Wharton's duct from postoperative day (POD) 1–7. The control and transplanted SMG groups were administered 100 μl of normal saline. The SMGs were removed on POD 7 while the animals were anaesthetized.

SMG autotransplantation was performed as described previously by Yu et al. with minor modifications [4]. Briefly, under sodium pentobarbital (20 mg/kg body weight) anesthesia, right SMG along with the Wharton's duct and related blood vessels attached was isolated from the submandibular triangle through a cervical approach. The freed SMG was transferred to the left temporal region and revascularized by anastomosing the artery of the gland to the distal part of the external carotid artery and the vein of the gland to the temporal vein using microvascular techniques. A polyethylene tube (inner diameter, 0.5 mm) was inserted into the Wharton's duct and left outside of the temporal skin in order to serve as a conduit for administering drugs.

Transmission electron microscopic observation. SMG specimens were fixed in 2% paraformaldehyde-1.25% glutaraldehyde for transmission electron microscopic (TEM) evaluation. The fixed tissues were immersed in 1% osmium tetroxide, stained en bloc with 4% uranyl acetate, and embedded in Epon812. Ultrathin sections were cut and stained with uranyl acetate, lead citrate, and observed with an H-7000 transmission electron microscope (Hitachi, Tokyo, Japan).

TUNEL staining. Frozen SMG sections were prepared for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining. The in situ cell-death detection kit (Roche Applied Science, Penzberg, Germany) was used according to the manufacturer's instructions. Briefly, tissue sections were incubated with terminal deoxynucleotidyl transferase in a humidified chamber at 37 °C for 1 h. A mixture of antidigoxigenin-peroxidase and substrate-chromagen were used for visualization, and the cells were counterstained with hematoxylin. The nuclei of apoptotic cells were stained as dark brown and counted in 10 different fields in each section under a light microscope at 400× magnification.

Immunohistochemistry. SMG tissues were fixed in 10% neutral-buffered formalin and were embedded in paraffin. The sections (4-μm thick) were rinsed several times in phosphate buffered saline (PBS) and then blocked with 3% H_2O_2 and 3% normal goat serum to eliminate nonspecific staining. Then, the sections were incubated with goat polyclonal antibody against activated caspase-3 (1:100) overnight at 4 °C. Biotin-conjugated anti-goat immunoglobulin secondary antibodies (Santa Cruz, CA) were applied for 2 h. After incubating with streptavidin–horseradish peroxidase substrate, the slides were counterstained with methyl green. Negative controls were incubated with goat IgG in place of the primary antibody. Quantitative analysis of activated caspase-3 immunostaining was performed with an image analyzer (LEICA550IW, Germany) in 10 different fields in each section under 200× magnification. The immunopositive values were expressed in terms of integrated optical density.

Western blot analysis. Western blot analysis of Bcl-2 and Bax expression as well as of p38 MAPK phosphorylation was carried out following standard procedure. Briefly, equal amounts of protein from different experimental groups were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and electrotransferred to polyvinylidene difluoride (PVDF) membranes. Nonspecific binding was blocked in PBS containing 5% nonfat milk for 2 h. The blots were incubated with primary antibodies overnight at 4 °C and washed thrice with 0.1% Tween20-PBS (PBS-T) before incubating with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The blots were then visualized with enhanced chemiluminescence reagents according to the manufacture's instructions. The density of bands was scanned and quantified by the LEICA550IW image analysis system. The membranes were reprobed with anti-actin antibody after stripped in stripping buffer for 30 min at 56 °C to assure equal loading.

Statistical analysis. Data are expressed as means ± SEM. Comparison of means was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's tests. Values of $P < 0.05$ were considered statistically significant.

Results

Phenylephrine improved ultrastructure of transplanted SMGs

TEM revealed that the acinar cells of the control SMGs contained numerous cytoplasmic low-matrix-density secretory granules in the cytoplasm (Fig. 1A). In the transplanted SMG without phenylephrine, the acinar cells showed marked cytoplasm shrinkage, aggregated nuclear chromatin, and increased electron density of the cytoplasm (Fig. 1B). Apoptotic bodies with intact membranes containing condensed dark chromatin masses were observed, some of which were no longer associated with the cell (Fig. 1B and C). There was plasma membrane blebbing and few scattered low-matrix-density secretory granules were discernible (Fig. 1C). In contrast, the ultrastructural features of the phenylephrine-treated SMG were similar to those of the control SMG, with very little evidence of apoptosis of acinar cells (Fig. 1D).

Phenylephrine inhibited apoptosis in transplanted SMGs

The antiapoptotic effect of phenylephrine on the transplanted SMG was further examined by TUNEL assay as shown in Fig. 2. The control SMG revealed very few or no apoptotic cells (Fig. 2A). Noticeable TUNEL-positive cells with shrunken cell bodies and condensed nuclei were seen in the transplanted SMG without phenylephrine treatment (Fig. 2B). Conversely, only a few TUNEL-positive cells were detected in the phenylephrine-treated SMG (Fig. 2C). The number of TUNEL-positive cells in the control, transplanted SMG without or with phenylephrine treatment were 0.5 ± 0.5, 51.5 ± 8.04, and 1.2 ± 0.75 per high field, respectively (Fig. 2D).

Phenylephrine inhibited expression of caspase-3 in transplanted SMGs

To further confirm the potential cytoprotective mechanism mediated by phenylephrine in the transplanted SMG, we stained SMG tissue for activated caspase-3. Many acinar cells in the transplanted SMG exhibited immunoreactivity for activated caspase-3, while only a few acinar cells exhibiting very faint immunoreactivity were observed in the phenylephrine-treated SMG. Photomicrographs of representative sections from each group are shown in Fig. 3A–C. The density of the staining of activated caspase-3 in the control, transplanted SMG without or with phenyl-

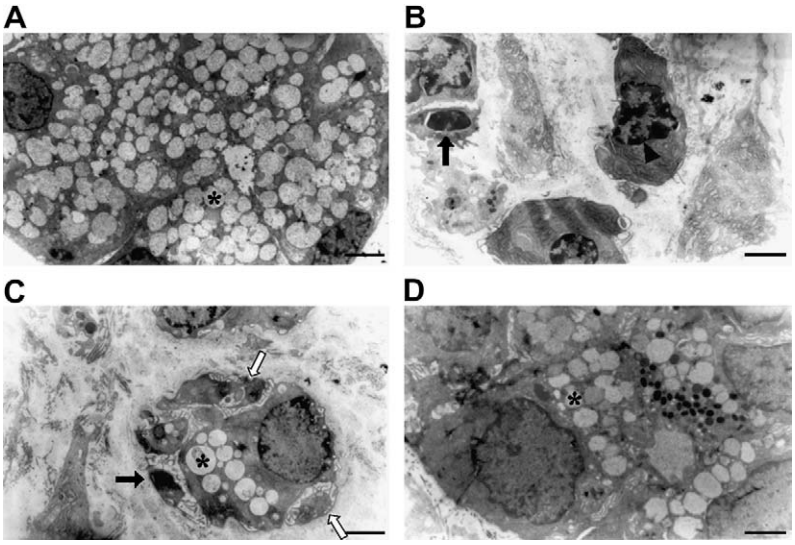

Fig. 1. Ultrastructural changes in autotransplanted SMG. All SMG tissues were removed on POD 7. (A) Some acinar cells in the control SMG contained many low-matrix-density secretory granules (*) in the cytoplasm. (B) and (C) In the transplanted SMG, acinar cells with marked cytoplasm shrinkage, nuclear chromatin was aggregated into a dense mass (arrow head) and the electron density of the cytoplasm increased, apoptotic bodies of intact membrane containing condensed dark chromatin masses were formed (arrow), the plasma membrane showed significant blebbing (unfilled arrow), and very few scattered low-matrix-density secretory granules could be seen (*). (D) Ultrastructural features of the phenylephrine-treated SMG; specifically, the appearance of the nuclei, cytosome, and membrane were similar to those observed in the control SMG. Apoptosis of acinar cells was rarely identified. TEM magnification, 5000×. Bar = 2 μm.

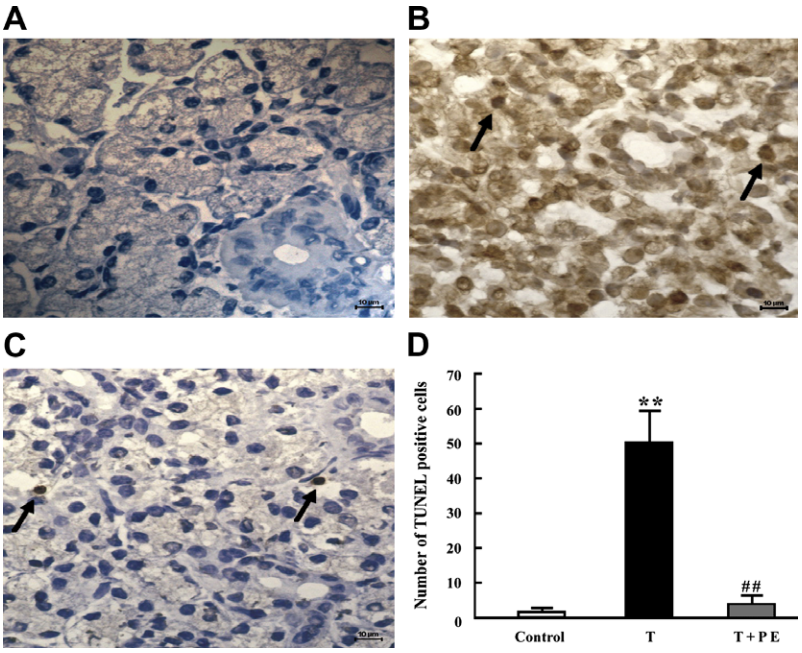

Fig. 2. TUNEL staining in autotransplanted SMG. (A) In the control SMG, TUNEL staining was rarely observed. (B) In the transplanted SMG, numerous typical densely stained dark TUNEL-positive cell nuclei were observed. The TUNEL-positive cells had shrunken cell bodies and condensed nuclei (arrow). (C) In the phenylephrine-treated SMG, there were markedly fewer TUNEL-positive cell nuclei (arrow) than in the nontreated transplant specimens. Light microscopy magnification, 400×. Bars: A,B,C: 10 μm. (D) Comparison of numbers of TUNEL-positive cells among the different groups. T, transplanted SMG; T + PE, transplanted SMG with phenylephrine treatment. $^{**}P < 0.01$ vs. control; $^{\#\#}P < 0.01$ vs. T.

ephrine were 3 ± 1.05, 79.2 ± 6.59, and 5 ± 2.16 per high field, respectively (Fig. 3D).

Phenylephrine increased in the ratio of Bcl-2/Bax in transplanted SMGs

We also examined Bcl-2 and Bax protein expression by Western blot. The immunoblot analyses of Bcl-2 and Bax were quantified by densitometric scanning (Fig. 4A and B). Bcl-2 expression was decreased in the transplanted SMG, but demonstrated a significant increase in the phenylephrine-treated SMG as compared to both the control SMG and the transplanted SMG. Bax expression was markedly elevated in the transplanted SMG, but significantly reduced to the control's level in the phenylephrine-treated SMG, as compared with the control.

Phenylephrine decreased in phosphorylation of p38 MAPK in transplanted SMGs

To further determine the effect of phenylephrine on the transplanted SMG, we examined the level of activated and phosphorylated p38 MAPK by Western blot. As shown in Fig. 4C, the

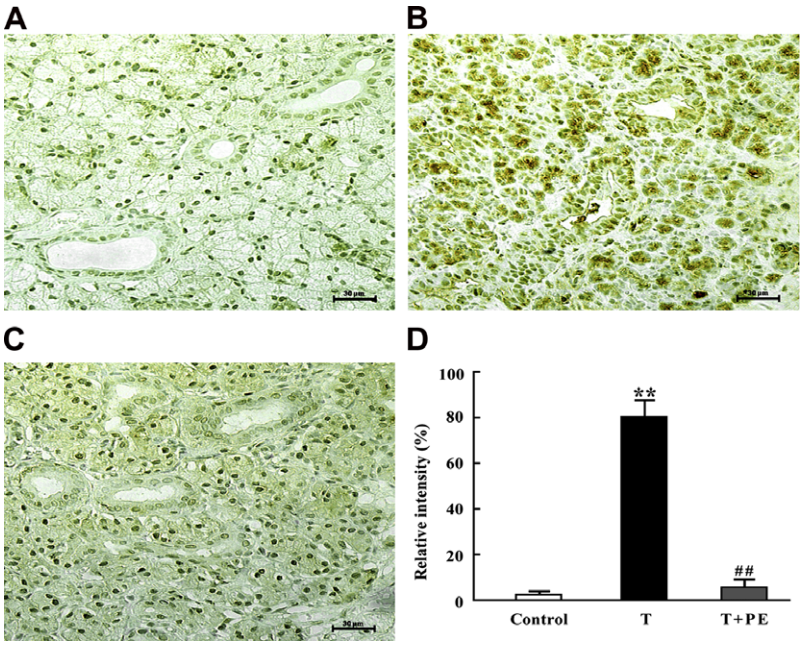

Fig. 3. Effect of phenylephrine on caspase-3 in autotransplanted SMG. (A) In the control SMG, activated caspase-3 positive staining was rarely observed. (B) In the transplanted SMG, there were numerous areas of typical activated caspase-3 immunoreactivity (brown areas in cytoplasm). (C) Activated caspase-3 positive staining was significantly decreased in the phenylephrine-treated SMGs. Light microscopy magnification, 200×. Bars: A, B, C: 20 μm. (D). Comparison of relative integrated optical density of activated caspase-3 positive staining among the groups. T, transplanted SMG; T + PE, transplanted SMG with phenylephrine treatment. $^{**}P < 0.01$ vs. control; $^{\#\#}P < 0.01$ vs. T. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

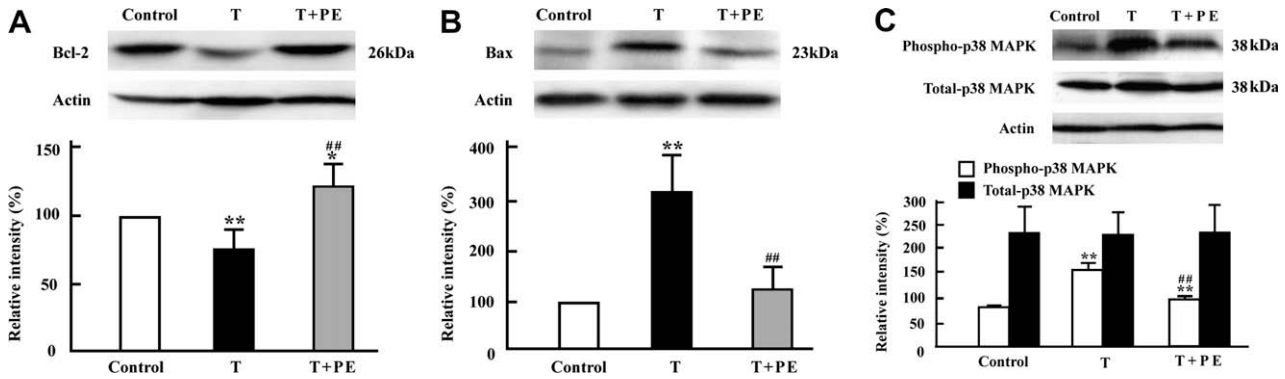

Fig. 4. Effects of phenylephrine on Bcl-2, Bax and p38 MAPK protein expression in autotransplanted SMG. (A) Western blot analysis of Bcl-2. Eighty micrograms of protein were separated by SDS–PAGE and immunoblotted with an antibody specific for Bcl-2. (B) Western blot analysis of Bax. Eighty micrograms of protein were separated by SDS–PAGE and immunoblotted with an antibody specific for Bax. (C) Western blot analyses of phospho-p38 and total-p38. Eighty micrograms of protein were separated by SDS–PAGE and immunoblotted either with an antibody specific for phospho-p38 or for total-p38. Filters were reprobed with the antibody against actin to assure equal loading of the lanes. Each blot is representative of five separated experiments in each group with similar results. T, transplanted SMG; T + PE, transplanted SMG with phenylephrine treatment. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control; $^{\#\#}P < 0.01$ vs. T.

phosphorylation of p38 MAPK was markedly increased after SMG transplantation, whereas it was markedly reduced in phenylephrine-treated transplanted SMGs.

Discussion

Apoptosis is an important factor in the maintenance of glandular homeostasis. Unfortunately, it is also critical in the pathogenesis of organ transplantation. There is increasing awareness of the potential benefits of inhibiting apoptosis after organ transplantation. In this study, we proved that apoptosis occurred in the early postoperative stage of transplanted SMGs and phenylephrine could ameliorate apoptosis. We further investigated the related signaling pathways of the antiapoptotic effects of phenylephrine on the transplanted SMGs.

Apoptosis is an evolutionary, highly conserved, biological process requiring the regulated activation of several signaling cascades, which finally result in typical biochemical and morphological alterations of the cell. Many forms of stress can induce apoptosis, including ischemia/reperfusion injury in transplanted organs [5,6]. Typical changes for apoptosis include condensation of the cell and the nuclei, chromatin condensation, generation of evoluted membrane segments (zeiosis), formation of apoptotic bodies, cellular shrinkage, and disintegration of mitochondria [8,9]. In addition to the expected characteristic morphological alterations, we also found fragmented DNA (as determined by TUNEL staining) and activation of caspase-3 in the transplanted SMG, all of which were significantly reduced by treatment with phenylephrine. At the molecular level, multiple signaling pathways converge on a family of cysteine proteases

(caspases), which when activated cause cellular destruction by cleaving a range of vital cellular substrates. Among the caspases identified, caspase-3 is considered a key "execution" caspase, involved in the final destruction of the apoptotic cell. The current data showed that phenylephrine had the ability to inhibit cell apoptosis in the transplanted SMG.

The members of the Bcl-2 family of proteins are key regulators of many signals leading to caspase activation and play a pivotal role in controlling cell life and death. They are major regulators of the apoptotic process and are comprised of pro-apoptotic (Bax) and antiapoptotic (Bcl-2) molecules [10]. To elucidate the molecular mechanism underlying apoptosis in the transplanted SMG, the expression level of Bcl-2 and Bax was analyzed. We observed that the expression of Bcl-2 protein was downregulated and that of Bax protein was upregulated in the transplanted SMG. In contrast, treatment with phenylephrine upregulated Bcl-2 expression and downregulated Bax expression. These results suggest that phenylephrine treatment could protect transplanted SMG against apoptosis. There are at least two reasons for transplantated SMG undergoing apoptosis. First, this might result from a surgical denervated injury. Second, ischemia/reperfusion injury is considered as a major process triggered by mitochondrial dysfunction and other factors after transplantation. Phenylephrine has been described to have antiapoptotic properties. Consistent with our observations, the activation of the α_1-adrenergic receptor by exposure to phenylephrine has been shown to protect the myocardium from ischemia/reperfusion injury by regulating the expression of mitochondrion-associated apoptosis regulatory genes (Bcl-2/Bax), preventing the initiation of the mitochondrial damage-induced apoptosis pathway [11].

To gain a further insight into the mechanism of the cytoprotective effects of phenylephrine on the transplanted SMG, we examined the possible involvement of p38 MAPK, a member of the MAPK subfamily, since accumulating evidence shows that inhibiting p38 MAPK activation may attenuate ischemia/reperfusion injury in liver, lung, and heart transplantation [12–14]. Activation of p38 MAPK leads to transcriptional regulation of target genes, resulting in apoptosis in several organ transplantation model systems [12,14,15]. In mammalian cells, MAPKs can transduce diverse extracellular stimuli (including mitogenic growth factors, hormones, cytokines, and environmental stresses) to the nucleus via kinase cascades to regulate cell survival, differentiation, and apoptosis [16]. In general, ERK is mainly activated by growth factors and has been reported to be associated with cell proliferation and differentiation. p38 MAPK promotes cell apoptosis. Moreover, crosstalk between ERK1/2 and p38 MAPK signaling pathways contributes to the balance of anti- versus pro-apoptotic signaling. Our previous studies demonstrated that phenylephrine ameliorated atrophy and promoted cell proliferation in transplanted glands by activating ERK1/2 [7]. Interestingly, our current data shows that p38 MAPK phosphorylation was significantly increased after transplantation. On the contrary, it was markedly inhibited in SMG transplants exposed to phenylephrine. This study provides the first evidence that p38 MAPK inhibition is beneficial for protecting SMG grafts after transplantation in an *in vivo* model. Taken together, our data suggest that phenylephrine elicits a protective response that either enhances cell survival (ERK1/2 activation) or inhibits cell death (p38 MAPK activation). However, the relationship between p38 MAPK and caspases is unclear. A number of findings indicate that upregulating ERK1/2 activation inhibits caspase-3 activation [17]. In contrast, increasing p38 MAPK phosphorylation has been reported to trigger caspase signaling cascades [18]. More experiments are needed to assess the relative importance of these two possible mechanisms.

In conclusion, our findings provided the first evidence that apoptosis occurs in the early postoperative phase of SMG autotransplantation and that treatment with phenylephrine effectively inhibits apoptosis in the transplanted SMG. The underlying molecular mechanisms may be closely correlated with the facts that phenylephrine increase the expression of Bcl-2, decrease the expression of Bax, and inhibit the activation of p38 MAPK and caspase-3 in the transplanted SMG. Our results suggest that phenylephrine may play a beneficial role in promoting glandular functional recovery and thus may improve graft viability during the early phase of transplantation.

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