We reported significant decreases in the retinal blood flow in patients with type 2 diabetes who continued to smoke compared with those who had never smoked, suggesting that smoking might impair the retinal microcirculation. Moreover, other studies have suggested that smoking might cause impaired endothelial function in the brachial arteries in healthy people and in aortas isolated from normal rats via increased vascular oxidative stress. Therefore, smoking could be involved in retinal vascular function and result in retinal microcirculatory abnormalities. However, the mechanism by which these abnormalities occur in the retinal circulation remains unclear.

With more than 4000 toxic components in cigarette smoke, benzo(e)pyrene (B(e)P), a polycyclic aromatic hydrocarbon (PAH), causes apoptosis and cellular death of human retinal pigment epithelial (ARPE-19) cells in vitro by generating reactive oxygen species (ROS), resulting in oxidative stress. In addition, because oxidative stress is involved in endothelial dysfunction, which contributes to diabetic microvascular diseases, intake of B(e)P might lead to development of vascular diseases. Endoplasmic reticulum (ER) stress, a new and critical mediator related to retinal cellular damage and death in diabetic retinopathy (DR), also causes vascular endothelial dysfunction. Furthermore, several studies have reported that genistein and resveratrol (RSV) ameliorated oxidative stress in vivo and in vitro studies.

In this study, using an isolated vessel preparation, we examined the effect of B(e)P on the endothelial of the retinal arterioles and determined whether oxidative stress and ER stress might be involved in the adverse effect of B(e)P. We also assessed the preservation effect of genistein and RSV on the adverse effect of B(e)P.

**MATERIALS AND METHODS**

**Animal Preparation**

The current study was conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The Animal Care Committee of Asahikawa Medical University approved all animal procedures. Pig eyes from animals of either sex (age, 16–24 weeks; weight, 15–25 kg)
were euthanized immediately after the animals were killed in a local abattoir. The eyes were transported in a moist chamber on ice to the laboratory. We previously reported the procedure used to harvest eyes.15

Microvessel Isolation and Cannulation

The techniques used to identify, isolate, cannulate, pressurize, and visualize the retinal microvessels were reported previously.16 Using a pair of glass micropipettes, we cannulated and pressurized the isolated retinal arterioles (mean ± standard deviation [SD] of the mean [SDM], 97 ± 5 μm [range, 84–110 μm]) to an intraluminal pressure of 55 cmH2O without flow using two independent pressure reservoir systems.17 Videomicroscopic observations were conducted continuously during the experiments to record the vasomotor activity of the isolated porcine retinal vessels.18

Experimental Protocols

In the control experiment, cannulated arterioles were bathed in physiologic saline solution (PSS) at a temperature between 36°C and 37°C for 30 to 40 minutes to facilitate the development of basal tone. After the basal tone developed, we evaluated the effect of B(e)P on the nitric oxide (NO)-mediated vasodilation. To accomplish this, the endothelium-dependent NO-mediated vasodilation in response to bradykinin (range, 1 pM–10 nM) was established before and after 180 minutes of intraluminal incubation with 100 μM B(e)P (n = 6). The vessels were exposed to each concentration of bradykinin for 2 to 3 minutes until a stable diameter was established. In a preliminary study, we found that the vasodilation that occurs with exposure to bradykinin was reproducible and sustained after repeated applications (n = 6). In another series of studies, to determine whether the effect of B(e)P is a selective response to endothelium-dependent NO-mediated vasodilation, the dose-dependent responses to the receptor-independent and endothelial NO-mediated vasodilator A23187 (range, 10 nM–5 μM) and to endothelial-independent NO-donor sodium nitroprusside (SNP; range, 10 nM–100 μM; n = 6) were established before and after 180 minutes of intraluminal incubation of 100 μM of B(e)P. In each separate group of vessels, the roles of superoxide and oxidative enzymes xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in mediating the adverse effects of B(e)P were determined by evaluating bradykinin-induced vasodilation before and after treating the vessels with B(e)P (100 μM) combined with superoxide production pathway inhibitors, such as the cell-permeable superoxide scavenger, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL; 1 mM; n = 6),19 the xanthine oxidase inhibitor allopurinol (10 μM; n = 6),20 or the NADPH oxidase inhibitor apocynin (100 μM; n = 6).19 The roles of stress-activated protein kinase were examined by evaluating bradykinin-induced vasodilation before and after treating the vessels with B(e)P combined with the c-Jun N-terminal kinase (JNK) inhibitor SP600125 (5 μM; n = 8).21 The p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 (0.1 μM; n = 8)22 or coadministration of SP600125 and SB203580 (n = 8). To determine if genistein and RSV, which protect against oxidative stress, mitigated the action of B(e)P on the NO-mediated vasodilation, the arterioles were evaluated by bradykinin-induced vasodilation before and after treating the vessels with B(e)P (100 μM) combined with genistein (30 μM; n = 8) or RSV (1 μM; n = 8).

In addition, to investigate the involvement of ER stress, we evaluated the bradykinin-induced vasodilation before and after treating the vessels with B(e)P combined with the ER stress inhibitor 4-phenylbutyrate (4-PBA; 100 μM; n = 6).23

Chemicals

We purchased B(e)P from AccuStandard, Inc. (New Haven, CT), and the other drugs from Sigma-Aldrich Corp. (St. Louis, MO). We dissolved B(e)P, SP600125, SB203580, genistein, and RSV in dimethyl sulfoxide (DMSO), and the other drugs in PSS, which was used for all subsequent drug dilutions. The final DMSO concentration in the vessel bath was below 0.1%.24 The final solvent concentrations did not affect the arteriolar function in the vehicle control studies.

Data Analysis

To obtain a maximal diameter at an intraluminal pressure of 55 cmH2O, the arterioles were relaxed in ethylenediaminetetra-acetic acid (1 mM) calcium-free PSS after each experiment.25 The diametric changes that occurred in response to the agonists were normalized to the maximal dilation. The changes were reported as percentages of the maximal vasodilation.16,26 The data are expressed as the mean ± SEM; n represents the number of retinal arterioles. The changes that occurred in resting tone as a result of the inhibitors were compared by Student’s t-test. To determine the significance of the difference between the control and the experimental interventions, we used 2-way ANOVA followed by the Bonferroni multiple-range test. P < 0.05 was considered significant. We performed power calculations to determine sample size on the basis of a two-sided alpha error of 5% and a power of 80%.

RESULTS

Effect of B(e)P on NO-Mediated Vasodilation

The basal tone ranged from 40% to 60% (mean ± SEM, 55% ± 1%) of the maximal diameter in all 82 vessels. The mean resting and maximal vessel diameters were 54 ± 1 and 97 ± 1 μm, respectively. Bradykinin caused dose-dependent vasodilation of the retinal arterioles; incubation with 100 μM B(e)P significantly (P < 0.001) attenuated the bradykinin-evoked vasodilation (Fig. 1A). Increasing the dose of B(e)P (200 μM) did not decrease the bradykinin-evoked vasodilation further (data not shown). The 100-μM dose of B(e)P also significantly decreased the vasodilation induced by A23187 (Fig. 1C). However, B(e)P (100 μM) did not affect the vasodilation of the retinal arterioles caused by the endothelium-independent NO-donor, SNP (Fig. 1B). The resting vascular tone did not change in response to B(e)P (data not shown).

Roles of the Pathway of Superoxide Production and the Protective Effect of Genistein and RSV

TEMPOL prevented B(e)P (100 μM)-evoked inhibition of the vasodilatory response to bradykinin (Fig. 2A), which also was seen in arterioles treated with the NADPH oxidase inhibitor apocynin but not with allopurinol (Fig. 2B). Inhibition of JNK and p38 kinase by SP600125 and SB203580, respectively, partially but significantly prevented B(e)P (100 μM)-evoked inhibition of the vasodilatory response to bradykinin. Co-administration of SP600125 and SB203580 prevented B(e)P (100 μM)-evoked inhibition of the vasodilatory response to bradykinin (Fig. 3). Administration of B(e)P with genistein also prevented the B(e)P (100 μM)-evoked inhibition of the vasodilatory response to bradykinin (Fig. 4A), and administration of B(e)P with RSV partly prevented these adverse effects of B(e)P (Fig. 4B). These agents did not change the resting basal tone or bradykinin-induced vasodilation without B(e)P (data not shown).
Roles of ER Stress
The ER stress inhibitor 4-PBA also prevented B(e)P (100 μM)-evoked inhibition of the vasodilatory response to bradykinin (Fig. 5). This ER stress inhibitor did not change the resting basal tone or bradykinin-induced vasodilation without B(e)P (data not shown).

Response to SNP
The SNP-induced vasodilation of the retinal arterioles was not attenuated by administration of the drugs used in this study, indicating that the vascular smooth muscle function was unchanged by these experimental procedures.

DISCUSSION
We found that in porcine retinal arterioles the vasodilatory responses to bradykinin and A23187 but not that to SNP decreased significantly with exposure to B(e)P, a toxic compound in cigarette smoke (Fig. 1). This finding indicated that B(e)P might impair retinal endothelial function regarding the NO-mediated vasodilation (Fig. 1A). The current finding that B(e)P impaired the vascular endothelial function in the retinal arterioles agreed with these results. The 200-μM dose of B(e)P (n = 6) did not result in a further decrease in the bradykinin-induced vasodilation (data not shown). B(e)P also decreased the retinal vasodilatory response to the calcium ionophore A23187 (Fig. 1C), which activates endothelial NO synthase by elevating the intracellular Ca2+ level irrespective of receptor stimulation and causes NO-dependent vasodilation in the retinal arterioles. Considering these findings, we speculated that B(e)P might reduce the NO bioavailability in the endothelium of the retinal arterioles, which would impair the endothelium-dependent vasodilation.

A plausible explanation for the mechanism underlying the reduced NO bioavailability caused by B(e)P might involve elevated vascular oxidative stress. Indeed, cigarette smoke extracts evoke superoxide anion production in bovine pulmonary artery endothelial cells. A study using human ARPE-19 cells found that ROS generation increased significantly in cells treated with 100 μM of B(e)P for 24 hours. We also found that TEMPOL, the membrane-permeable superoxide scavenger, abrogated the inhibitory effect of B(e)P on
The enzymes xanthine oxidase and NADPH oxidase in vascular cells can generate superoxide. In the current study, NADPH oxidase inhibitor apocynin but not xanthine oxidase inhibitor allopurinol prevented the inhibitory effect of \( \text{B(e)P} \) on the retinal arterioles (Fig. 2B), suggesting that the adverse effects of \( \text{B(e)P} \) were caused by NADPH oxidase-evoked superoxide anions. Our results agreed with previous results indicating that NADPH oxidase inhibitor apocynin \( (100 \, \mu M; \, n = 6) \) prevented the inhibitory effect of \( \text{B(e)P} \) on the retinal arterioles before (control, \( n = 12 \)) and after intraluminal incubation with \( 100 \, \mu M \) of \( \text{B(e)P} \) plus the superoxide scavenger TEMPOL \( (1 \, mM; \, n = 6) \).

The enzymes xanthine oxidase and NADPH oxidase in vascular cells can generate superoxide. In the current study, NADPH oxidase inhibitor apocynin but not xanthine oxidase inhibitor allopurinol prevented the inhibitory effect of \( \text{B(e)P} \) on the retinal arterioles (Fig. 2B), suggesting that the adverse effects of \( \text{B(e)P} \) were caused by NADPH oxidase-evoked superoxide anions. Our results agreed with previous results indicating that NADPH oxidase is the primary source of vascular superoxide and that substances in cigarette smoke activate endothelial NADPH oxidase in the pulmonary arteries. Taken together, \( \text{B(e)P} \) probably causes NADPH oxidase activation and results in ROS production in the retinal arteriolar endothelial cells.

The detail mechanisms underlying \( \text{B(e)P} \)-induced superoxide production by NADPH oxidase are not fully understood. JNK and p38 MAPK, which are referred to as stress-activated kinases, play a significant role in oxidative stress and inflammation, however, benzopyrene activation of JNK and p38 MAPK remains controversial. Although PAH-induced activation of JNK was generally thought to be related to oxidative stress and associated with \( \text{B(a)P} \), a derivative of \( \text{B(e)P} \)-induced hydrogen peroxide \( (H_2O_2) \) production, \( \text{B(a)P} \) activated p38 MAPK but not JNK in HepG2 cells. Another study showed that a \( \text{B(a)P} \) metabolic product, \( \text{B(a)P}-7,8\)-dihydrodiol-9,10-epoxide, activated JNK and p38 MAPK. This is consistent with our results that showed that the JNK inhibitor SP600125 and the p38 MAPK inhibitor SB203580 reduced the adverse effect of \( \text{B(e)P} \) on the endothelium-dependent vasodilatory response in the retinal arterioles. Co-administration of SP600125 and SB203580 further blocked the adverse effect of \( \text{B(e)P} \) on the endothelium-dependent vasodilatory response of the retinal arterioles (Fig. 3). Therefore, we speculated that \( \text{B(e)P} \) might evoke ROS generation in both the JNK- and p38 MAPK-dependent pathways in the endothelial cells of the retinal arterioles through NADPH oxidase activation, resulting in impaired endothelial function of the retinal arterioles.

Genistein has various biochemical activities. It has been reported that genistein decreased the generation of free radicals and cellular oxidative stress by suppressing the cytochrome enzymes. RSV, a polyphenolic substance present in foods such as grapes and wine, protects against oxidative injury. RSV \( (1 \, \mu M, 20 \, \text{minutes}) \) also resulted in significant reduction of oxidative injury in human RPE R-50 cells and protected against \( \text{B(a)P} \)-induced DNA damage. Moreover, the finding that genistein and RSV inhibited oxidant production caused by \( \text{B(e)P} \) supports our result that genistein and RSV prevented the adverse effect of \( \text{B(e)P} \) on the bradykinin-evoked vasodilation (Figs. 4A, 4B). Although 30 \( \mu M \) of RSV prevented \( \text{B(e)P} \)-induced oxidative stress, we previously found that 10 \( \mu M \) of RSV elicited dilation of approximately 20% of the isolated retinal arterioles. Therefore, in the current study, we used the threshold concentration of RSV \( (1 \, \mu M) \), which previously did not cause porcine retinal arterioles to dilate.

Another hypothesis is that the underlying mechanism by which \( \text{B(e)P} \) reduces NO bioavailability in the retinal arterioles might be through increased vascular ER stress. Oxidative stress and ER stress reduce NO generation in murine aortic endothelial cells. This was supported by the current result...
that the ER stress inhibitor 4-PBA prevented the inhibition of the endothelium-dependent vasodilation by B(e)P (Fig. 5). In our preliminary study, tunicamycin (1 mM, 180 minutes), an ER-stress inducer,44,45 diminished bradykinin-evoked vasodilation, and 4-PBA (100 μM) inhibited the adverse effect of tunicamycin on bradykinin-evoked vasodilation (data not shown). The beneficial effect of 4-PBA was specific because this ER stress inhibitor did not change the resting basal tone or bradykinin-evoked vasodilation without B(e)P. No study has reported that B(e)P or B(a)P induced ER stress in the retina, but several in vitro studies have found that exposure to cigarette smoke induces ER stress in human umbilical vein endothelial cells and mouse lung epithelial cells.46,47 Taken together, the current results showed that ER stress also might be related to the underlying mechanism in which B(e)P caused endothelial dysfunction of the retinal arterioles.

This study had some limitations. First, no previous study determined the plasma concentration of B(e)P from cigarette smoke in smokers. Here, we determined the B(e)P concentration based on in vitro studies.4 Second, although the current study investigated only the acute effect of B(e)P on isolated porcine retinal arterioles, the chronic effects of B(e)P on the retinal circulation remain unclear. Therefore, further in vivo and clinical studies are required to determine these. Third, the relationship between oxidative stress and ER stress has not been clarified. In our preliminary study, the effect of 4-PBA equaled the effect of TEMPOL, and coadministration of TEMPOL and 4-PBA had no further preventative effect on the inhibition by B(e)P of the endothelium-dependent vasodilatory response of retinal arterioles (data not shown). Furthermore, TEMPOL did not reduce the inhibitory effect of the ER stress inducer, tunicamycin, on endothelium-dependent vasodilation (data not shown). It has been reported that oxidative stress-dependent ER stress is implicated in cigarette smoke-mediated apoptosis.48 Taken together, we speculated that endothelial dysfunction induced by B(e)P in the retinal arterioles might result from ROS-dependent ER stress (Fig. 6).
In summary, we found that in isolated porcine retinal arterioles, B(e)P impaired the endothelium-dependent NO-initiated vasodilation. The mechanism of the adverse response to B(e)P is implicated in the generation of superoxide by vascular NADPH oxidase and ER stress. Moreover, it is likely that genistein and RSV protect against B(e)P-mediated endothelial dysfunction in the retinal arterioles, possibly by reducing oxidative stress. The current finding that B(e)P impaired the endothelial vasodilation of the retinal arterioles showed that B(e)P might be related to the mechanism of impaired retinal microcirculation in early-stage DR, especially in smokers. The elucidation of the mechanisms of endothelial dysfunction of the retinal arterioles caused by B(e)P could provide a basis for developing strategies to prevent and treat smoking-induced retinal vascular disorders.

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