Original Article

Expression of Endothelin-1 and Adrenomedullin Was Not Altered by Leptin or Resistin in Bovine Brain Microvascular Endothelial Cells

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Hypertension frequently occurs in obese subjects. It has been reported that leptin and resistin induce endothelin-1 expression in vascular endothelial cells. Altered function of brain microvascular endothelial cells may be related to increased occurrences of stroke in hypertensive patients. In the present study, we therefore studied the effects of leptin and resistin on the expression of endothelin-1 and adrenomedullin in bovine brain microvascular endothelial cells. Northern blot analysis showed that leptin (10^{-10} – 10^{-8} mol/l), resistin (10^{-10} – 10^{-8} mol/l) or a combination of leptin and resistin (10^{-8} mol/l for each) had no significant effects on the expression of endothelin-1 mRNA or adrenomedullin mRNA in cultured bovine brain microvascular endothelial cells. On the other hand, hypoxia induced, and tumor necrosis factor- α (10 ng/ml) decreased, the expression levels of endothelin-1 and adrenomedullin mRNAs, indicating that the bovine brain microvascular endothelial cells were able to respond to hypoxia and tumor necrosis factor- α . Consistent with the results of Northern blot analysis, immunoreactive endothelin and immunoreactive adrenomedullin concentrations in the medium were not significantly changed by the treatment with leptin, resistin, or a combination of leptin and resistin. The present study thus showed that neither leptin nor resistin affects the expression of endothelin-1 or adrenomedullin in bovine brain microvascular endothelial cells. (*Hypertens Res* 2006; 29: 443–448)

Key Words: endothelin, adrenomedullin, adipokine, endothelial, obesity

Introduction

Obesity is a major risk factor for the development of hypertension (1). Adipocytes secrete a number of the hormones known as adipokines, including leptin (2), resistin (3), tumor necrosis factor- α (4), adiponectin (5) and adrenomedullin (6,

7). Some of these adipokines may be related to the pathogenesis of obesity-related hypertension. Mice with leptin overexpression have been shown to develop hypertension, probably because the overproduced leptin acts on the brain and increases the activity of the sympathetic nervous system (8). In addition to these central actions, leptin may cause hypertension *via* its direct actions on the vascular vessels. Leptin

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has been shown to induce endothelin-1 expression in cultured vascular endothelial cells, suggesting that leptin may act directly on the vascular endothelial cells and cause hypertension *via* endothelin-1 release (9). Furthermore, resistin, an adipokine that has been suggested to play a role in the development of insulin resistance in obesity (3), induced endothelin-1 expression in cultured vascular endothelial cells (10).

In contrast to these reports, we recently reported that neither leptin nor resistin had significant effects on endothelin-1 expression in cultured human umbilical vein endothelial cells (11). One of the reasons for this discrepancy may be the different cell types used in the experiments. Moreover, in our study using human umbilical vein endothelial cells (11), neither leptin nor resistin had significant effects on the expression of adrenomedullin. Adrenomedullin is a potent vasodilator peptide secreted by various types of cells (12, 13), including vascular endothelial cells (11, 12), neurons (14), and glial cells (15, 16). Adrenomedullin expression is induced by hypoxia (17, 18), inflammatory cytokines (16, 19) and oxidative stress. In addition to its vasodilator action, adrenomedullin has various biological actions. For example, adrenomedullin acts against the oxidative stress caused by angiotensin II (20).

Brain microvascular endothelial cells are one of the most important targets for hypertension research, because their altered function may be related to increased occurrences of stroke in hypertensive patients. In the present study, we therefore studied the effects of leptin and resistin on the expression of endothelin-1 and adrenomedullin in bovine brain microvascular endothelial cells.

Methods

Culture

Bovine brain microvascular endothelial cells, which are primary endothelial cells derived from microvessels of the bovine brain, were obtained from Cell Applications, Inc. (San Diego, USA). Bovine brain microvascular endothelial cells were cultured in BBMVEC growth medium (Cell Applications, Inc.) following the manufacturer's protocol at 37°C under 5% CO₂/95% room air. Bovine brain microvascular endothelial cells at passage 5 were used in the following experiments. At about 80% confluence, the cells were cultured with recombinant mouse leptin (10⁻¹⁰–10⁻⁸ mol/l) (PeproTech EC Ltd., London, UK), recombinant mouse resistin (10⁻¹⁰–10⁻⁸ mol/l) (PeproTech EC Ltd.), or a combination of leptin (10⁻⁸ mol/l) and resistin (10⁻⁸ mol/l) for 24 h. The culture media were harvested for the measurement of immunoreactive endothelin and immunoreactive adrenomedullin, and the cells were harvested for RNA extraction.

Next, we cultured the cells with tumor necrosis factor- α and/or under hypoxic conditions in order to determine whether the expression of endothelin-1 and adrenomedullin would be altered in bovine brain microvascular endothelial

cells by other types of stimuli that are known to affect the expression of these peptides (11, 17–19, 21). The cells were cultured with recombinant human tumor necrosis factor- α (10 ng/ml) (PeproTech EC Ltd.) under normoxia or under hypoxia (1% O₂). In the hypoxia experiments, the cells were cultured at 37°C for 24 h in a chamber with 5% CO₂/94% N₂/1% O₂, as previously reported (17).

Radioimmunoassay

Peptides were extracted from culture media using Sep-Pak C18 cartridges (Waters, Milford, USA), as previously reported (22). The radioimmunoassays of endothelin-1 and adrenomedullin were performed as previously reported (22–24). The assay of endothelin showed 60% cross reaction with endothelin-2, 70% with endothelin-3, 0.1% with big endothelin-1 (1–38), and less than 0.001% with other peptides tested, including adrenomedullin. In the assay of adrenomedullin, the amounts of cross-reaction with adrenomedullin-glycine (25) and fragments of human adrenomedullin were as follows: adrenomedullin-glycine, 40%; adrenomedullin-(1–12), 0.0012%; adrenomedullin-(22–52), 28%; and adrenomedullin-(13–52), 0.021% (15, 22). There was no significant cross-reaction (<0.001%) with other peptides, including endothelin-1, adrenomedullin-2/intermedin, and calcitonin gene-related peptide.

Chromatographic characterization of immunoreactive endothelin and immunoreactive adrenomedullin in the culture medium was performed by reverse phase high performance liquid chromatography using a μ Bondapak C18 column (3.9 mm $\times$ 300 mm; Waters), as previously reported (15, 22). The medium cultured by bovine brain microvascular endothelial cells was extracted with a Sep-Pak C18 cartridge, reconstituted in 0.1% (v/v) trifluoroacetic acid, and loaded onto the column. High performance liquid chromatography was performed with a linear gradient of acetonitrile containing 0.1% (v/v) trifluoroacetic acid from 10 to 60% at a flow rate of 1 ml/min per fraction over 50 min. Each fraction (1 ml) was collected, air-dried and assayed.

Northern Blot Analysis

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, USA) and subjected to Northern blot analysis, as previously reported (11, 22). The BamHI/EcoRI fragment of pBT-hET-1 (23) and the HindIII/EcoRI fragment of pBS-hAM2 (22) were used as hybridization probes for endothelin-1 mRNA and adrenomedullin mRNA, respectively. Radioactive signals were detected by exposing the filters to X-ray films (X-AR5; Kodak, Rochester, USA) or with a Bioimage Analyzer (BAS 1500; Fuji Film Co., Ltd., Tokyo, Japan). The intensity of hybridization signals was determined by photostimulated luminescence with a Bioimage Analyzer. Expression of β -actin mRNA was examined as an internal control.

Fig. 1. Northern blot analysis of endothelin-1 (ET-1) and adrenomedullin (AM) in cultured bovine brain microvascular endothelial cells. **A:** Effects of leptin (10^{-10} – 10^{-8} mol/l), resistin (10^{-10} – 10^{-8} mol/l) or a combination of leptin and resistin (10^{-8} mol/l for each) (L/R) on the expression of ET-1 mRNA and AM mRNA. The cells were cultured for 24 h in the medium containing leptin or resistin at the indicated concentrations (shown by log concentration, mol/l). **C,** control. **B:** Effects of hypoxia and/or tumor necrosis factor (TNF)- α (10 ng/ml) on the expression of ET-1 mRNA and AM mRNA. The cells were cultured for 24 h under normoxia or under hypoxia (1% O_2), with or without TNF- α (10 ng/ml). The lower panel shows the expression of β -actin mRNA as an internal control.

Statistics

Data are given as the mean $\pm$ SEM unless otherwise stated. Statistical analysis was performed by one-way analysis of variance followed by Fisher's protected least significant difference test.

Results

Northern blot analysis showed that leptin (10^{-10} – 10^{-8} mol/l), resistin (10^{-10} – 10^{-8} mol/l) or a combination of leptin and resistin (10^{-8} mol/l for each) had no significant effects on the expression of endothelin-1 and adrenomedullin mRNAs in cultured bovine brain microvascular endothelial cells (Fig. 1A). Northern blot analysis showed two bands corresponding to adrenomedullin mRNA (about 2 kb and about 3 kb) in bovine brain microvascular endothelial cells, and the intensities of both bands were not noticeably changed by treatment with leptin or resistin (Fig. 1A). On the other hand, hypoxia induced the expression of endothelin-1 and adrenomedullin mRNAs in bovine brain microvascular endothelial cells (Fig. 1B). Tumor necrosis factor- α (10 ng/ml) decreased the expression levels of endothelin-1 and adrenomedullin mRNAs under both normoxia and hypoxia. These findings indicate that bovine brain microvascular endothelial cells are able to respond to hypoxia and tumor necrosis factor- α .

Consistent with the results of the Northern blot analysis,

Fig. 2. Effects of leptin (10^{-10} – 10^{-8} mol/l), resistin (10^{-10} – 10^{-8} mol/l) or a combination of leptin and resistin (10^{-8} mol/l for each) (L/R) on **A:** immunoreactive endothelin (IR-ET) and **B:** immunoreactive adrenomedullin (IR-AM) concentrations in the media cultured for 24 h by bovine brain microvascular endothelial cells ($n=5$, mean $\pm$ SEM).

immunoreactive endothelin and immunoreactive adrenomedullin concentrations in the medium were not significantly changed by the treatment with leptin (10^{-10} – 10^{-8} mol/l), resistin (10^{-10} – 10^{-8} mol/l), or a combination of leptin and resistin (10^{-8} mol/l for each) (Fig. 2B; $p>0.05$).

Reverse phase high performance liquid chromatography showed an immunoreactive peak eluting in the position of endothelin-1 (Fig. 3A), indicating that the immunoreactive endothelin in the culture medium of bovine brain microvascular endothelial cells was mostly endothelin-1. Reverse phase high performance liquid chromatography showed a peak eluting in the position of authentic human adrenomedullin with two larger peaks eluting earlier (Fig. 3B). Adrenomedullin with an oxidized methionine, which was generated by incubating with 3% (v/v) H_2O_2 for 1 h at room temperature, was eluted in the same position as human adrenomedullin (data not shown). Adrenomedullin-glycine was eluted 2 fractions later than human adrenomedullin (data not shown).

Fig. 3. Reverse phase high performance liquid chromatography of the culture medium extract of bovine brain microvascular endothelial cells. A: Immunoreactive endothelin (IR-ET). Arrows indicate the elution positions of authentic endothelin-1 (1), endothelin-2 (2) and endothelin-3 (3). B: Immunoreactive adrenomedullin (IR-AM). An arrow indicates the elution position of authentic human adrenomedullin (AM). Dotted lines indicate a gradient of acetonitrile.

Discussion

In the present study, we have shown that neither leptin nor resistin had significant effects on the expression of endothelin-1 or adrenomedullin in cultured bovine brain microvascular endothelial cells, which is consistent with our previous study using human umbilical vein endothelial cells (11). Our findings are in clear contrast to two previous reports (9, 10) that showed that leptin and resistin induced endothelin-1

expression in cultured vascular endothelial cells, respectively. We also performed an experiment using hypoxia and tumor necrosis factor- α in order to rule out the possibility that the bovine brain microvascular endothelial cells were insensitive to stimuli. Expression of endothelin-1 and adrenomedullin mRNAs was induced by hypoxia, and decreased by treatment with tumor necrosis factor- α in bovine brain microvascular endothelial cells, indicating that the cells induced endothelin-1 and adrenomedullin in response to these stimuli.

Previous studies have reported that the plasma concentrations of leptin and resistin were elevated in obese subjects (26–28). In these studies, the plasma leptin concentrations were 43 ± 9.4 ng/ml (mean $\pm$ SEM) in obese subjects and 5.6 ± 1.3 ng/ml in lean subjects (26), and the plasma resistin concentrations were 42.5 ± 8.9 ng/ml in female obese subjects (28). The doses of leptin (10^{-10} – 10^{-8} mol/l = 1.6–160 ng/ml) and resistin (10^{-10} – 10^{-8} mol/l = 1.95–195 ng/ml) used in the present study were comparable to or higher than the plasma levels of these adipokines in obese subjects, and were similar to those used in the previous studies (9, 10). Another possible explanation for these discrepant results is the difference of the cell types used. Quehenberger *et al.* (9) used primary cultures of human umbilical vein endothelial cells, whereas Verma *et al.* (10) used human saphenous vein endothelial cells at passages 2–5. In our preliminary studies, neither leptin nor resistin (10^{-9} and 10^{-8} mol/l) had noticeable effects on the expression of endothelin or adrenomedullin mRNAs in 293-H cells (transformed primary embryonic human kidney cells) (our unpublished observations). We cannot rule out the possibility that endothelin-1 induction by leptin or resistin is a phenomenon only observed in limited types of primary cultures of vascular endothelial cells or cultures with a few passages.

It has been reported that tumor necrosis factor- α induced adrenomedullin expression in cultured vascular smooth muscle cells (19). On the other hand, tumor necrosis factor- α has also been shown to decrease adrenomedullin expression in cultured vascular endothelial cells (11, 29), which is consistent with the present study. It is well known that tumor necrosis factor- α induces endothelin-1 mRNA expression and stimulates endothelin-1 release in various types of cells, including cultured vascular endothelial cells (15, 30–32). Some cell lines, however, do not respond to tumor necrosis factor- α treatment (33). The modulation of endothelin-1 expression and secretion by tumor necrosis factor- α in various cultured cells may therefore be phenotype-specific. The decreased expression of endothelin-1 mRNA by tumor necrosis factor- α shown in the present study may be specific to the bovine brain microvascular endothelial cells.

Reverse phase high performance liquid chromatography confirmed that the immunoreactive endothelin in the culture medium of bovine brain microvascular endothelial cells was mostly endothelin-1. The cDNA cloning showed that the cDNA encoding bovine preproadrenomedullin and the predicted bovine adrenomedullin peptide have close homology to the cDNAs encoding human preproadrenomedullin and the

human adrenomedullin peptide, respectively (34). The predicted bovine adrenomedullin is a 48 amino acid peptide, which is 4 amino acids shorter than human adrenomedullin, with one amino acid substitution. The material eluting in the position of authentic human adrenomedullin on high performance liquid chromatography may therefore represent bovine adrenomedullin consisting of 48 amino acids. Materials eluting in two earlier peaks may represent the adrenomedullin precursor or its fragments. These earlier peaks are not likely to represent adrenomedullin-glycine, or adrenomedullin fragments similar to human adrenomedullin-(1–12) or adrenomedullin-(13–52), because adrenomedullin-glycine was eluted later than authentic adrenomedullin on high performance liquid chromatography, and the cross-reaction with human adrenomedullin-(1–12) or adrenomedullin-(13–52) was very small in our assay. Similar peaks eluting earlier were observed in the high performance liquid chromatographic analysis of the culture medium extracts of human cells (17, 22).

The present study has shown that neither leptin nor resistin significantly alters the expression of endothelin-1 or adrenomedullin in bovine brain microvascular endothelial cells.

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