Endothelin-like immunoreactivity in rat models of diabetes mellitus

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ABSTRACT

The factors associated with high concentrations of circulating plasma immunoreactive endothelin in patients with diabetes mellitus are unknown. Plasma and tissue (lung and kidney) immunoreactive endothelin levels were therefore measured by radioimmunoassay in three animal models of diabetes mellitus: dexamethasone-treated rats (2 mg/kg per day for 12 days), streptozotocin-treated rats (100 mg/kg, 4 days before being killed) and rats treated with both dexamethasone and streptozotocin. Plasma concentrations of immunoreactive endothelin in the dexamethasone-treated rats (3·13 ± 0·28 pmol/l, mean ± S.E.M., n = 15) were significantly (P < 0·005) higher than those in controls (1·33 ± 0·18 pmol/l, n = 15), while plasma concentrations of immunoreactive endothelin in streptozotocin-treated rats (n = 8) and rats treated with both dexamethasone and streptozotocin (n = 14) were undetectable (<0·5 pmol/l). Fast protein liquid chromatographic analysis of the plasma immunoreactive endothelin of dexamethasone-treated rats showed four peaks: one in the void volume, one eluting before endothelin-3, one eluting after endothelin-3 and before endothelin-1 and one eluting in a position identical with that of endothelin-1. Pulmonary concentrations of immunoreactive endothelin in the three groups of rats with diabetes mellitus were lower (P < 0·005) but no significant change was found in renal immunoreactive endothelin. These findings indicate that short-term dexamethasone treatment increases plasma levels of immunoreactive endothelin while streptozotocin treatment decreases them. Thus, multiple factors may influence plasma concentrations of immunoreactive endothelin in diabetes mellitus.

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INTRODUCTION

Endothelin-1 is a potent vasoconstrictor peptide originally isolated from the conditioned medium of cultured porcine endothelial cells (Yanagisawa, Kurihara, Kimura et al. 1988). There are at least three genes for endothelin: endothelin-1, endothelin-2 and endothelin-3 (Inoue, Yanagisawa, Kimura et al. 1989).

We have recently reported increased levels of plasma immunoreactive (IR) endothelin in patients with diabetes mellitus (Takahashi, Ghatei, Lam et al. 1990a). Increased plasma levels of IR-endothelin are thought to be closely related to the endothelial cell damage which may relate to the occurrence of angiopathy in patients with diabetes mellitus. However, no significant correlations were found between plasma IR-endothelin levels in patients with diabetes mellitus and their blood glucose levels, blood pressure, urinary microalbumin, incidence of background retinopathy or duration of diabetes mellitus. Unexpectedly, high glucose levels have been found to decrease the production of endothelin-1 by cultured bovine retinal endothelial cells acutely (Molinatti, Porta, Takahashi et al. 1990).

The presence of IR-endothelin in rat plasma (Saito, Nakao, Shirakami et al. 1989) and high levels of IR-endothelin in lung and kidney of rats have been reported (Kitamura, Tanaka, Kato et al. 1989; Yoshimi, Hirata, Fukada et al. 1989). Reduced numbers of endothelin-1 receptors have been reported in cardiac tissue of streptozotocin-treated rats (Nayler, Liu, Panagiotopoulos & Castle, 1989), but, so far, no reports are available on plasma and tissue IR-endothelin levels in animals with diabetes mellitus.

To clarify the cause(s) of increased IR-endothelin levels in human diabetes mellitus, plasma IR-endothelin concentrations were measured in animals with acute experimental diabetes mellitus. Levels of
IR-endothelin in lung and kidney were also examined because of the high concentrations of this peptide in these tissues.

MATERIALS AND METHODS

Animals

Adult male Wistar rats of 200–250 g were divided into four groups: (1) control rats (n = 15) were fed on standard rat chow, available ad libitum; (2) one group (n = 15) was given dexamethasone (Merck Sharp & Dohme Ltd, Hoddesdon, Herts, U.K.; 2 mg/kg) by i.p. injection daily for 12 days; (3) one group (n = 8) was given streptozotocin (Upjohn, Crawley, Sussex, U.K.; 100 mg/kg) injected i.v. via the tail vein 4 days before the rats were killed; (4) the final group (n = 14) was given dexamethasone (2 mg/kg) injected i.p. for 12 days and streptozotocin (100 mg/kg) injected i.v. on the same day as the last injection of dexamethasone. All the diabetic animals were killed 4 days after the last injection.

Blood samples were obtained by cardiac puncture under ether anaesthesia and collected into heparinized tubes with aprotinin (400 KIU/ml blood, Bayer UK Ltd, Newbury, Berks, U.K.) for measurement of IR-endothelin. The blood samples were immediately centrifuged at room temperature and the plasma was separated and stored at −20 °C until assayed. Blood glucose concentrations were measured with an Ames glucometer (Miles Labs Inc., Elkhart, IN, U.S.A.). Whole left lung and left kidney were collected for IR-endothelin measurement.

Radioimmunoassay

Plasma concentrations of IR-endothelin were measured by a modification of a radioimmunoassay previously reported in detail (Takahashi, Brooks, Kanse et al. 1989; Takahashi et al. 1990a). Briefly, 100 μl of the unextracted plasma samples were assayed in duplicate. Hormone-free plasma (100 μl), prepared by prior charcoal adsorption of peptides (Adrian, 1982), was added to all standard and zero tubes. The antibody to endothelin-1 used in the present study was obtained from a subsequent bleed of an immune responsive rabbit (Takahashi et al. 1989, 1990a). This antibody can distinguish changes of 0.5 fmol/assay tube (0.5 pmol/l plasma) from zero concentrations with a 95% confidence limit. Plasma concentrations of IR-endothelin measured using this method showed a good correlation with those obtained by the previously reported method using Sep-Pak extraction (Takahashi et al. 1990a) (r = 0.78, n = 29, P < 0.0005). The cross-reaction with big endothelin (human, 1–38) (Nova Biochem, Nottingham, Notts, U.K.), endothelin-2 and -3 (Peptide Institute, Minoh-Shi, Japan) was 0.1, 60 and 70% respectively. Intra- and interassay coefficients of variation were 12% (n = 9) and 19% (n = 7) respectively.

Tissues were boiled in 0.5 mol acetic acid/l (10 ml/g wet weight) for 10 min. Aliquots (30 and 100 μl) of the extracts were dried in a Savant vacuum centrifuge, reconstituted in assay buffer (60 mmol phosphate buffer/l, pH 7.4, containing 10 mmol EDTA/l, 7 mmol sodium azide/l and 0.3% (w/v) bovine serum albumin) and assayed in duplicate.

Chromatography

The IR-endothelin in plasma and lung extracts was characterized by fast protein liquid chromatography (FPLC) using a high-resolution reverse-phase 5/5 (Pep Rpe HR 5/5) C-18 column (Pharmacia, Uppsala, Sweden). Pooled plasma samples were extracted and lung extracts were re-extracted by Sep-Pak C18 cartridges (Waters Associates, Milford, MA, U.S.A.) as previously reported (Takahashi et al. 1989, 1990a) and reconstituted in 0.5 ml water with 0.1% (v/v) trifluoroacetic acid (TFA) before loading on to the column. A linear gradient from 15 to 35% acetonitrile in water with 0.1% TFA was performed over 1 h at 1 ml/min per fraction, followed by 35% acetonitrile for 10 min. Samples of each fraction were dried in a Savant vacuum centrifuge, reconstituted in assay buffer and assayed.

Statistics

All the data are given as means ± s.e.m. Levels of IR-endothelin were compared using one-way analysis of variance followed by Student's t-test.

RESULTS

Blood glucose concentrations were found to be slightly increased in dexamethasone-treated rats (7.3 ± 0.5 nmol/l) and greatly increased in streptozotocin-treated rats (> 20 nmol/l) and in rats treated with both dexamethasone and streptozotocin (> 20 nmol/l) (control group, 5.5 ± 0.3 nmol/l).

Plasma concentrations of IR-endothelin in dexamethasone-treated rats (3.13 ± 0.28 pmol/l) were significantly (P < 0.005) higher than the levels in controls (1.33 ± 0.18 pmol/l) (Fig. 1a). Plasma concentrations of IR-endothelin in streptozotocin-treated rats and rats treated with both dexamethasone and streptozotocin were undetectable (< 0.5 pmol/l).

Pulmonary levels of IR-endothelin of all three diabetic groups were significantly (P < 0.005) lower than the levels in control rats (Fig. 1b), while renal levels of IR-endothelin in these groups were not significantly different from the control (Fig. 1c).
DISCUSSION

Endothelin-1 is one of the most potent peptide mediators known (Yanagisawa et al. 1988) and, perhaps as a consequence, its plasma concentrations both in human and experimental animals are low by comparison with many established circulating peptide hormones (Ando, Hirata, Shichiri et al. 1989; Saito et al. 1989; Takahashi et al. 1990a). The changes in plasma IR-endothelin levels in experimental animals have therefore been difficult to study. With the development of a new sensitive radioimmunoassay of endothelin, we have been able to measure IR-endothelin directly in 100 μl plasma, and plasma IR-endothelin concentrations of 0.5 pmol/l are distinguishable from zero with 95% confidence for individual samples assayed in duplicate. The values obtained by this method showed a good correlation with those obtained by the extraction method using Sep-Pak C18 cartridges.

Plasma concentrations of IR-endothelin were previously found to be increased in diabetic patients

FIGURE 1. Immunoreactive endothelin levels in (a) plasma, (b) lung and (c) kidney of controls (n = 15), dexamethasone-treated rats (Dex, n = 15), streptozotocin-treated rats (STZ, n = 8) and rats treated with both dexamethasone and streptozotocin (Dex + STZ, n = 14). Values are means + s.e.m. *P < 0.005 compared with the control group (unpaired Student's t-test). **Not detectable (<0.5 pmol/l).

FIGURE 2. Fast protein liquid chromatography of (a) control plasma, (b) plasma from dexamethasone-treated rats, (c) control lung and (d) lung of dexamethasone-treated rats. 1, 2, 3 and B, the elution positions of endothelin-1, -2, -3 and big endothelin(1–38) respectively. The dotted line represents the linear gradient of acetonitrile from 15 to 35%.
(Takahashi et al. 1990a). The current study has shown that short-term (12 days) dexamethasone treatment increased total plasma concentrations of IR-endothelin in the rat and that this increase is due chiefly to immunoreactivity which eluted in the void volume on FPLC. A similar immunoreactive void volume peak has also been observed in human plasma (Takahashi et al. 1990a), saliva (Lam, Takahashi, Ghatei et al. 1991) and urine (Lam, Takahashi, Ghatei et al. 1990). The void volume materials in human urine and saliva extracts were loaded on to a Sephadex G-25 column and the IR-endothelin was found to elute in a higher molecular weight region (authors' unpublished observations). The antibody to endothelin-1 used in the present study cross-reacted only 0.1% with big endothelin(1-38). Therefore, one possible explanation of the void volume material in the plasma of dexamethasone-treated rats is a large folded molecule, perhaps a precursor or precursor fragment of endothelin, different from big endothelin(1-38). It is unlikely that this large molecule is an artifact, as it was not found when endothelin-1, -2 or -3 were incubated in human blood in a previous study (Takahashi et al. 1990a). The immunoreactive peak that eluted later than endothelin-3 but earlier than endothelin-1 in dexamethasone-treated rat plasma may be an oxidized form of endothelin-1, as peaks in a similar position were seen on FPLC after incubating endothelin-1 in human blood at room temperature (Takahashi et al. 1990a). An immunoreactive peak eluting before endothelin-3 (fraction 18), which was not found in human plasma, was found in plasma of both control and dexamethasone-treated rats. Further studies are required to clarify the nature of these moieties.

No immunoreactivity was found in the positions of endothelin-2, -3 and big endothelin(1-38) on FPLC of rat plasma and lung. Of course, this finding does not deny the possibility that big endothelin(1-38) is present in rat plasma or lung, since the cross-reaction of the antibody to endothelin-1 with big endothelin (1-38) is only 0.1%.

Endothelins are potent and long-lasting vasoconstrictors (Yanagisawa et al. 1988; Inoue et al. 1989). The injection of endothelin-1 caused early hypotension, followed by a sustained rise of blood pressure and a rise in peripheral resistance (Miller, Redfield & Burnett, 1988; Nucci, Thomas, D'Oreans-Juste et al. 1989; Spokes, Ghatei & Bloom, 1989). Glucocorticoid is known to increase blood pressure and enhance the responses of vascular smooth muscle to catecholamines both in vivo and in vitro (Zweifach, Shorr & Black, 1953; Schayer, 1964). Altered metabolism in vascular endothelial cells by glucocorticoid, such as reduced synthesis of vasorelaxant prostaglandins (Lewis, Campbell & Johnson, 1986; Hullin, Raynal, Ragab-Thomas et al. 1989) and increased activity of angiotensin-converting enzyme (Krulowitz, Baur & Fanburg, 1984), has been reported. In addition, glucocorticoid induced angiotensinogen mRNA in hepatocytes (Klett, Hellmann, Suzuki et al. 1988). Chronic low-dose infusion of dexamethasone decreased plasma concentrations of atrial natriuretic peptide in rats (Tonolo, Fraser, Connell & Kenyon, 1988). Increased plasma IR-endothelin may be one of the causes of dexamethasone-induced hypertension. However, further studies are required to clarify whether the IR-endothelin eluting in the void volume on FPLC has a pressor action or not.

Streptozotocin treatment decreased plasma levels of IR-endothelin in rats, even in those treated with dexamethasone. High glucose levels acutely decrease endothelin-1 production from cultured bovine retinal endothelial cells (Molinatti et al. 1990). The increase in blood sugar levels in streptozotocin-treated rats was much more marked than in dexamethasone-treated rats; it is therefore possible that the acute increase in blood sugar suppressed release of IR-endothelin into the plasma.

High IR-endothelin concentrations in lung and kidney have been reported (Kitamura et al. 1989; Yoshimi et al. 1989), while IR-endothelin levels in aorta were low (Takahashi, Jones, Kanse et al. 1990b). Therefore, in the present study, IR-endothelin concentrations were measured in lung and kidney as representative organs. Pulmonary levels of IR-endothelin in the three diabetic groups were decreased and renal IR-endothelin levels did not change. These changes did not parallel those in the plasma. IR-endothelin is produced not only by vascular endothelial cells but also by airway epithelial cells (Black, Ghatei, Takahashi et al. 1989) and renal epithelial cells (Shichiri, Hirata, Emori et al. 1989). In addition, circulating endothelin-1 is removed in the pulmonary circulation (Nucci et al. 1989). Although the mechanism of regulation of tissue IR-endothelin levels in not clear, the IR-endothelin concentrations in lung and kidney of diabetic rats may be affected by several factors, such as the production and release of IR-endothelin by both vascular endothelial cells and epithelial cells and the metabolism of circulating IR-endothelin in these organs. In dexamethasone-treated rats, plasma concentrations of IR-endothelin were increased, while pulmonary concentrations were decreased. One possible explanation of this discrepancy may be an increased release of IR-endothelin from pulmonary tissues and decreased storage in the pulmonary tissues of dexamethasone-treated rats. Another possibility may be a decreased uptake of circulating IR-endothelin by the pulmonary circulation in dexamethasone-treated rats.

The present study, and our previous reports (Takahashi et al. 1990a; Molinatti et al. 1990), suggest that multiple factors, such as endothelial cell damage, blood glucose levels and the humoral changes...
accompanying diabetes mellitus, may all have an influence on plasma levels of IR-endothelin in patients with diabetes mellitus. Thus, while the physiology of this potent peptide remains unclear, the changes evoked by diabetes suggest the possibility of a significant role in the pathogenesis of diabetic complications.

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REFERENCES


