Increased adrenomedullin expression in lungs in endotoxaemia

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Abstract

Adrenomedullin (AM) is a peptide involved in cardiovascular homeostasis and in inflammation. We examined its expression in a rat model of endotoxaemia. Male Sprague-Dawley rats received intraperitoneal injection of 5 or 10 mg/kg lipopolysaccharide (LPS), or saline as control. Rats were killed at 1, 3, 6, 12 and 24 h after injection. LPS at 5 mg/kg, but not saline, increased plasma AM significantly at 3 h. At 10 mg/kg, plasma AM was raised at 3, 6 and 12 h. Immunoreactive AM concentration in lung increased after 5 or 10 mg/kg LPS, but not saline. PreproAM mRNA level in lung was significantly increased at 3 and 6 h. In conclusion, endotoxin stimulates the expression of AM in the lungs and increases its circulatory concentration. AM may be involved in the systemic response to sepsis.


Introduction

Adrenomedullin (AM) is a peptide first isolated from human adrenal medulla and pheochromocytoma (Kitamura et al. 1993). AM is ubiquitous in the body and has diverse and profound effects on cellular proliferation, contractility, migration and interaction with other neurohormonal factors (Eto et al. 2001). AM relaxes vascular smooth muscle via cAMP and by releasing nitric oxide (NO) from endothelium (Feng et al. 1994, Shimakake et al. 1995). Plasma AM level is increased in a number of diseases, including essential hypertension, acute myocardial infarction, congestive heart failure and renal failure (Ishimitsu et al. 1994, Jougasaki et al. 1995, Nishikimi et al. 1995, Cheung & Leung 1997, Yoshitomi et al. 1998). Initial research studies had focused on the cardiovascular actions of AM. We confirmed in heart failure patients that the plasma AM level is elevated and correlates with the degree of impairment in left ventricular function detected by echocardiography (Yu et al. 2001). Moreover, the plasma level of AM is also raised in patients with ascites due to cirrhosis and chronic obstructive pulmonary disease with hypoxia (Cheung & Leung 1997, Cheung et al. 1998). Hence, AM is increased in disease conditions associated with fluid retention and volume overload (Cheung & Leung 1997, Hirano et al. 2000). On the other hand, leucocytes and macrophages may be a major source of AM production (Kubo et al. 1998a,b). Cytokines, such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), stimulate the secretion of AM in vitro (Sugo et al. 1995). Receptors for AM are expressed in the immune system of the rat (Kubo et al. 1998a, Shindo et al. 1998). AM potently stimulates IL-6 production in fibroblasts (Isumi et al. 1998). At the same time, the promoter region of AM contains an IL-6-binding element, suggesting a feedback loop (Ishimitsu et al. 1998). Therefore, we studied AM expression in a variety of human diseases characterised by local or systemic inflammation, asthma, exacerbation of chronic airway obstruction, systemic lupus erythematosus (SLE) and rheumatoid arthritis (Cheung et al. 2000a,b).

In the lungs, AM is expressed in many cell types, including bronchial epithelium, bronchial smooth muscles, pulmonary vasculature and macrophages (Martínez et al. 1995). We have measured the plasma levels of AM in asthma and in chronic obstructive airway disease and found that they were raised (Cheung et al. 1998). We have also studied patients with bronchiectasis, a disease characterised by inflammation and infection which interact to cause cytokine-mediated progressive destruction of the airway (Cheung et al. 2000b). These patients have markedly elevated plasma levels of AM. These observations lead us to speculate that AM, beyond its vasoreluctant activity, may have a role in inflammation. Plasma AM is markedly increased in endotoxic shock, suggesting that AM may be secreted in response to endotoxic shock (Sugo et al. 1995).
However, adrenalectomised mice with, *inter alia*, low AM levels are exquisitely sensitive to endotoxic shock, while transgenic mice overexpressing AM are resistant to endotoxic shock (Shindo et al. 2000). This suggests that AM may be an anti-inflammatory cytokine that is protective in endotoxic shock.

In this study, we aimed to investigate the expression of AM in the lungs and in plasma after the injection of lipopolysaccharide (LPS) in a rat model of endotoxaemia.

**Materials and Methods**

**Injection of LPS or saline**

The experimental protocol was approved by the Committee on the Use of Animals for Teaching and Research of the Faculty of Medicine, University of Hong Kong. Twelve-week-old male Sprague-Dawley (SD) rats weighing 360–450 g were used. Conscious rats were injected with 5 or 10 mg/kg body weight LPS (from *E. coli* serotype 026:B6; potency ≥ 10 000 endotoxin units per mg; L8274, Sigma) into the peritoneum, whereas the control animals received 0·9% sodium chloride. The sensitivity of the assay was 1–5 pg per tube. The intra- and interassay coefficients of variation for AM were 7% and 10% respectively.

**Extraction of AM from plasma**

A volume of 2 ml plasma was acidified with equal volume of 1% trifluoroacetic acid (TFA) (HPLC grade) and then centrifuged at 13 000 r.p.m. for 20 min at 4 °C (Hwang & Tang 1999). The diluted plasma samples were loaded onto pre-equilibrated C-18 Sep-columns (Peninsula, CA, USA), which were then washed twice with 3 ml 1% TFA. The peptide was slowly eluted of 1% trifluoroacetic acid (TFA) (HPLC grade) and then centrifuged at 13 000 r.p.m. for 20 min at 4 °C (Hwang & Tang 1999). The diluted plasma samples were loaded onto pre-equilibrated C-18 Sep-columns (Peninsula, CA, USA), which were then washed twice with 3 ml 1% TFA. The peptide was slowly eluted

**Extraction of tissues**

Frozen tissues were homogenised in 2 M acetic acid and boiled in a water bath for 10 min to inactivate proteases. For protein determination, 50 µl aliquots were taken. The remaining homogenates were lyophilised and stored at −20 °C.

**Radioimmunoassay for AM**

Duplicate samples of AM standards (0–500 pg/100 µl) and samples were incubated at 4 °C with 100 µl of 32P-AM antiserum (Peninsula, Belmont, CA, USA) (Hwang & Tang 1999). After 18–24 h, 100 µl of normal rabbit gamma globulin (Antibodies Inc., Davis, CA, USA, 1:300 dilution), goat antirabbit serum (Antibodies Inc., 1:30 dilution) and 100 µl of 10% polyethylene glycol were added. After centrifugation and aspiration of the supernatant, the pellets were counted in a gamma counter (LKB, Turku, Finland). The sensitivity of the assay was 1–5 pg per tube. The intra- and interassay coefficients of variation for AM were 7% and 10% respectively.

**Protein determination**

A 50 µl aliquot of tissue homogenate or standard (BSA) was boiled with 1 M sodium hydroxide for 10 min. After cooling, 50 µl of the boiled sample was mixed with 2·5 ml protein assay reagent (Bio-Rad, Hercules, CA, USA) for 10 min, and measured with a spectrophotometer (LKB Ultraspec II, Biochrom) at 595 nm. The concentration of AM was then calculated and expressed as fmol per mg protein.

**Extraction of total RNA**

Fresh or pulverised frozen tissues weighing 100–200 mg were homogenised in 1–2 ml TRIZOL reagent (Gibco-BRL; Life Technologies) (Hwang & Tang 1999). Frozen tissues were pulverised in liquid nitrogen before homogenisation, phenol–chloroform extraction and precipitation by isopropanol. RNA samples were stored at −70 °C until assay.

**Solution hybridisation RNase protection assay**

Plasmid preproAM cDNA and β-actin cDNA (gifts from Dr Dominic Autelitano, Baker Medical Research Institute, Prahran, Australia) were transformed into *E. coli* JM109. Plasmid DNAs were linearised with restriction enzymes (preproAM: EcoRI for synthesis of probe and BAMH1 for standard; β-actin: EcoRI for probe and HindIII for standard). The standard RNA and the riboprobes were synthesised using polymerases (preproAM: T7 for probe and SP6 for standard; β-actin: SP6 for probe and T7 for standard). An amount of 5 µg of RNA tissue samples or standards (1–50 pg) was incubated with 100 000 c.p.m. of 35S-labelled AM RNA riboprobe or 32P-labelled β-actin RNA probe in hybridisation buffer.
Some samples were co-hybridised while some were not. The reaction mixture was denatured at 85°C for 5 min and then incubated at 45°C overnight. The unhybridised RNAs were digested with RNase A and RNase T1 (Sigma, St Louis, MO, USA) at 37°C for 30–45 min. After digestion by proteinase K, the hybrids were extracted with phenol and chloroform, and precipitated with isopropanol. The pellets containing hybrids were redissolved in loading buffer containing dye, denatured at 65°C and loaded onto 4% polyacrylamide gel (19:1 Bis). The gel was run for 1 h at 4°C, and vacuum dried. The hybrid bands were visualised by exposing to film (Fuji, RX AIF 2025 cm) in a cassette with intensifying screens at 70°C for at least 72 h. With the radiographic film as a template, the hybrid bands on the gel were cut out and their radioactivities counted with a liquid scintillation counter (Tri-carb 2000AC Packard). The amount of preproAM mRNA and β-actin mRNA in various tissues was determined by reference to a standard curve. The results are presented as fg preproAM mRNA per pg actin mRNA.

Immunohistochemical staining for AM

Small pieces of rat lung tissue were fixed in formaldehyde-buffered saline and embedded in paraffin wax. Sections of 5 μm were cut and processed for immunohistochemical staining by the avidin–biotin method. The sections were treated with 10% methanol and 3% hydrogen peroxide for 60 min, and then incubated with 1:2000 AM antiserum (Phoenix, Belmont, CA, USA) at 4°C overnight. The avidin–biotin–peroxidase complex in the sections was visualised by adding dianaminobenzidine for 5–10 min. The sections were counterstained with haematoxylin and dehydrated in an alcohol series.

Statistics

All data are presented as mean ± standard error. The data were analysed using two-way analysis of variance (ANOVA) with a statistical software program (SPSS for Windows, Version 7.5, SPSS, Chicago, IL, USA). Standard error of the mean and 95% confidence intervals were calculated using the t statistic. A P value of less than 0.05 from Student’s two-tail tests was considered statistically significant.

Results

Immunoreactive AM in plasma

Following intraperitoneal injection of 5 mg/kg of LPS in rats, the plasma AM concentration was increased significantly in LPS-injected rats at 3 h, but was no longer increased at 24 h (Fig. 1A). There were no significant changes in AM level in control animals, which received intraperitoneal saline injection only. At the dosage of 10 mg/kg, plasma AM level was raised at 3, 6 and 12 h after injection, but not at 24 h (Fig. 1B). There were no significant changes in AM level in the control animals.
Figure 2 High-power view (×400) of 5 μm sections of rat lung, showing (A) immunoreactive AM in ciliated columnar epithelium, (B) endothelium of pulmonary arteriole, (C) type I alveolar cells and (D) alveolar macrophages. Visualised by diaminobenzidine and counterstained by haematoxylin.
Immunohistochemical staining showed AM-like immunoreactivity in ciliated columnar epithelium, endothelium of pulmonary arteriole, type I alveolar cells and alveolar macrophages in the lung (Fig. 2).

Immunoreactive AM (ir-AM) concentration was increased at 3 h in the lungs of animals that received 5 mg/kg LPS, but not in the control animals (Fig. 3A). At 24 h, ir-AM returned to basal levels.

The increase in ir-AM was confirmed in animals given 10 mg/kg LPS (Fig. 3B). The ir-AM was raised at 3, 6 and 12 h in the lungs. At 24 h, the ir-AM returned to basal levels. There were no significant changes with time in ir-AM in the tissues of control animals.

PreproAM mRNA
PreproAM mRNA levels in the lung were significantly increased at 3 and 6 h, but not at 1, 12 or 24 h (Fig. 4). There were no significant changes in preproAM mRNA level in the control animals.

Discussion

This study found that the levels of AM in the plasma and lung were significantly increased after intraperitoneal injection of endotoxin. The increases in immunoreactive AM contents in the lung were concomitant with the increases in preproAM mRNA levels at 3 and 6 h, but not at 12 h, after endotoxin injection. Therefore, there was an increase in synthesis of AM in the lungs at 3 and 6 h. The elevation in AM peptide level with a lack of change in its mRNA content at 12 h after endotoxin injection suggests a decline in AM secretion from the lung. This is in line with the increase in plasma AM level at 3 and 6 h after endotoxin injection.
endotoxin injection, but a decrease at 12 h towards the control level. That the plasma AM level was still higher than in the saline control suggests sources of plasma AM other than the lungs.

The plasma level of AM is raised in a variety of pathological states. In this study, however, the increase in plasma AM level was not due to other factors such as hypertension, myocardial infarction, heart failure, renal failure, respiratory diseases, liver disease, diabetes and pregnancy. AM is secreted in stress and glucocorticoids are known to stimulate the secretion of AM in vascular smooth muscle (Minamino et al. 1995). AM is co-secreted with catecholamines from adrenal medullary cells (Katoh et al. 1994). Therefore, in this study, we used a time-matched control group to demonstrate that the rise in AM was not due to handling of the animals, injection or other forms of stress.

Our finding of an AM response to endotoxin is consistent with the observations of others (Mazzocchi et al. 2000, Matsui et al. 2001, Yang et al. 2001). It appears that LPS in the peritoneal cavity induces AM in the gut and the portal circulation (Zhou et al. 2001). This is at least in part responsible for the haemodynamic changes seen in endotoxin shock (Zhou et al. 2001), and the effect can be attenuated by polymyxin B (Yang et al. 2001) or the peptide AM receptor antagonist AM(22–52) (Mazzocchi et al. 2000). AM thus plays an important role in initiating the hyperdynamic response during the early stage of polymicrobial sepsis. The reduced vascular responsiveness may be responsible for producing the transition to the late, hypodynamic phase of sepsis (Koo et al. 2001).

In endotoxin shock, TNF-α, IL-1β and LPS stimulate the production of AM (Kubo et al. 1998a,b). TNF-α, IL-1β and interferon-γ stimulate AM production through NO-dependent and NO-independent pathways. Interestingly, the NO-dependent pathway is not mediated by guanylate cyclase (Hofbauer et al. 2002). We have found that there were also concomitant rises in the plasma levels of TNF-α and IL-1β (Cheung et al. 2002). The elevation in plasma AM may thus be partly due to the stimulation by these cytokines or the consequence of a generalised increase in inflammatory mediators. AM may also be involved as a regulator of the complement cascade (Pio et al. 2001). It binds strongly to factor H in plasma. AM influences the complement regulatory function of factor H by enhancing the cleavage of C3b via factor I. More work needs to be done to elucidate their complex interactions.

In conclusion, endotoxin stimulates the expression of AM in the lungs and its secretion into the circulation. AM may be involved in the pathophysiology of the systemic response to sepsis.

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