

Original Article

Endogenous Digitalislike Factors in Obstructive Sleep Apnea

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Recent studies have provided evidence that hypoxia may stimulate the release of endogenous digitalislike factors (EDLF). Obstructive sleep apnea (OSA) is characterized by intermittent hypoxia during sleep and may be associated with sympathetic activation and a high risk of developing hypertension. This study was designed to measure EDLF in the plasma of patients with OSA diagnosed by polysomnography, with patients being classified by the number of apneic-hypopneic episodes/h sleep (apnea-hypopnea index, AHI). Plasma was obtained in the morning from 8 male normotensive OSA patients (OSA-N) (AHI 70 ± 6), 2 untreated hypertensive OSA patients (OSA-HT), and 11 age-matched healthy male controls (C). EDLFs of different hydrophobicities were separated from the same plasma sample by solid-state C18-cartridges with 25% acetonitrile (ACN) (EDLF-1) followed by 40% ACN (EDLF-2). This procedure recovered ouabain in the first fraction and digoxin and digoxigenin in the second. EDLF was quantified in pM ouabain-equivalents by a human placenta radioreceptor assay. EDLF-1 levels were similar for OSA-N and C (231 ± 55 vs. 258 ± 58), whereas EDLF-2 levels were increased in OSA-N (244 ± 51 vs. 110 ± 25 in C, $p=0.02$). Norepinephrine was increased in apneics. The two OSA-HT had EDLF and norepinephrine levels similar to OSA-N. These preliminary results suggest that OSA is associated with an increase in the more hydrophobic EDLF levels in both normotensive and hypertensive states. No significant increase was found for the less hydrophobic ouabain-like EDLF. (*Hypertens Res* 2000; 23 Suppl: S87-S91)

Key Words: endogenous digitalislike factors, obstructive sleep apnea, radioreceptor assay, hypoxia, ouabain

Introduction

Substantial evidence suggests that mammalian fluids and tissues contain endogenous factors with digitalislike activity (EDLF: endogenous digitalislike factor) that may be involved in hydro-saline homeostasis and in some forms of hypertension (1, 2). The first EDLF to be characterized from human plasma was ouabain (3), but other EDLFs, including digoxinlike factors (4) and bufodienolide derivatives (5, 6) have also been identified.

The existence of a family of EDLFs might be related to the presence of different isoforms of the Na^+, K^+ -ATPase

in mammalian tissues (7) with potentially different responsiveness to various EDLFs (8, 9).

Recent papers (5, 10) suggest that EDLFs may also be involved in compensatory responses to hypoxia. Bagrov *et al.* (5) have reported that hypoventilation in humans produces a marked increase in the plasma levels of marinobufagenin-like immunoreactivity and inhibition of the Na^+, K^+ -ATPase in intact erythrocytes. Hypoxia has also been found to stimulate the release of a Na^+, K^+ -ATPase inhibitor (10) both from the rat midbrain and adrenal tissue that co-chromatographs with a hypothalamic EDLF identified in the bovine (11). Higher plasma and urine digoxinlike immunoreactivity has been reported in

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Table 1. Clinical Characteristics of Normal Subjects and of Patients with Obstructive Sleep Apnea in the Normotensive and Hypertensive State

	Normal subjects	Obstructive sleep apnea patients	
		Normotensive	Hypertensive
<i>N</i>	11	8	2
Age (years)	41±3	51±4	34-49
Apnea-hypopnea index		70±6	94-111
BMI (kg/m ²)	26±2	31±1	31-36
Lowest O ₂ Hb satur (%)	n.d.	70±3	56-75
SBP (mmHg)	124±3	120±2	140-140
DBP (mmHg)	76±2	76±2	96-93

BMI, body mass index; Lowest O₂ Hb satur, lowest oxygen haemoglobin saturation recorded during polysomnography; SBP, systolic blood pressure; DBP, diastolic blood pressure; n.d., not determined.

normal subjects during prolonged hypoxic breathing at high altitudes (12) and in patients with chronic obstructive pulmonary disease (13). Finally, the finding of high EDLF levels in the newborn (14-16) may also be related to the well-known hypoxic condition at birth.

Obstructive sleep apnea (OSA) represents another condition in which the effects of hypoxia on EDLFs can be studied. OSA is a relatively common condition that occurs when attempts to breathe are blocked by an occluded upper airway and is characterized by snoring, frequent arousals during sleep, and daytime somnolence (17). The frequency of apneic episodes is often more than 20 per hour of sleep. Apneic episodes are associated with nighttime sympathetic activation (18). Patients with OSA are believed to be at high risk of developing hypertension (19), cardiac arrhythmias, and ischemic heart disease (20). Most patients with OSA are moderately to severely obese.

Based on the belief that the human EDLFs can be detected more appropriately with human rather than animal Na⁺,K⁺-ATPase, we have developed a radioreceptor assay (RRA) using membranes from human placentas as receptors (15).

The intent of this study was to measure EDLFs of different hydrophobicities in the plasma of patients with obstructive sleep apnea and to relate these findings to a few indices related to this disease.

Subjects and Methods

We studied 10 male patients with OSA (8 normotensives and 2 hypertensives) and 11 age-matched healthy male controls belonging to our laboratory staff. All subjects gave informed consent, and the study was approved by the local Committee. Patients were diagnosed by polysomnography and classified on the basis of the apnea-hypopnea index (AHI). All patients were severely ill, since they had more than 30 episodes of apnea and hypopnea per hour. None had clinical or biochemical evi-

dence of renal, cardiac, or endocrinological diseases, had ever taken digitalis drugs, or was taking any medication. Clinical characteristics of the subjects are reported in Table 1. Peripheral blood samples were drawn in the morning into Vacutainer tubes containing sodium heparin for EDLF detection and EGTA/glutathione for catecholamine analysis.

For the measurement of EDLFs, plasma (2 ml) was extracted through C18 SepPak cartridges (Waters Inc., Cambridge, USA) activated with 5 ml of acetonitrile (ACN) and equilibrated with 20 ml of 0.1% trifluoroacetic acid (TFA). After washing with 10 ml of 0.1% TFA, EDLFs of different hydrophobicity were eluted with 3 ml of 25% ACN in 0.1% TFA (fraction 1) followed by 3 ml of 40% ACN in 0.1% TFA (fraction 2). Both eluates were vacuum-dried and kept at -20°C until assayed. Extracts were reconstituted in 0.25 ml of buffer (15) and analyzed by the human placenta radioreceptor assay (RRA) using ouabain calibrators, as previously described (15). EDLF values were expressed in ouabain-equivalents (o.e.). The assay sensitivity was 0.32±0.02 nM (0.16 pmol/tube).

In preliminary experiments we found that, by this extraction procedure, ouabain was completely recovered (98% of the added compound) in the first fraction, and the more hydrophobic digitalis compounds such as digoxin (98% of the added compound), digitoxigenin (64%), and digitoxin (45%) in the second fraction. In the second fraction were also eluted some common adrenocortical steroids (cortisol, cortisone, corticosterone, estradiol, aldosterone and, in lesser amounts, dehydroepiandrosterone sulfate, and progesterone), but the high specificity of the human placenta digitalis receptor (15) and the lack of binding inhibition by C18 extracts of steroids-free plasma spiked with concentrations of steroids much higher than those remaining after plasma extraction makes it very unlikely that the EDLFs measured in this fraction represent an interference due to these steroids.

Plasma catecholamines were analyzed by the automated

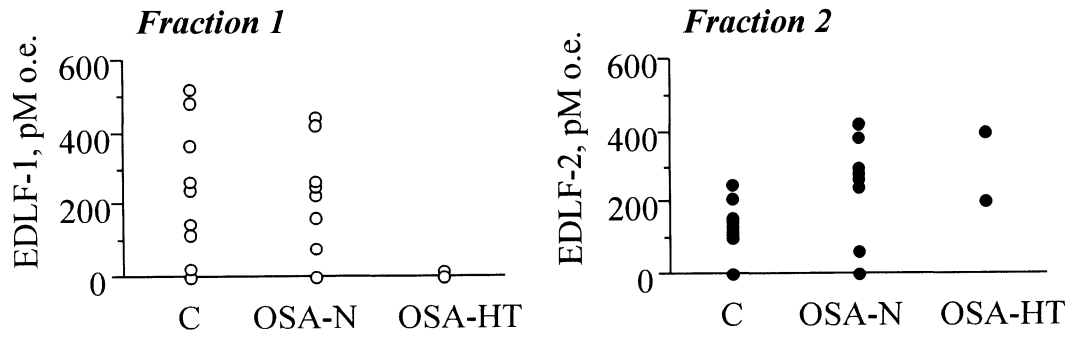


Fig. 1. EDLF values in the two fractions from C18-cartridges in controls (C) and in patients with obstructive sleep apnea in the normotensive (OSA-N) and hypertensive state (OSA-HT). Fraction 1: elution with 25% acetonitrile, Fraction 2: elution with 40% acetonitrile

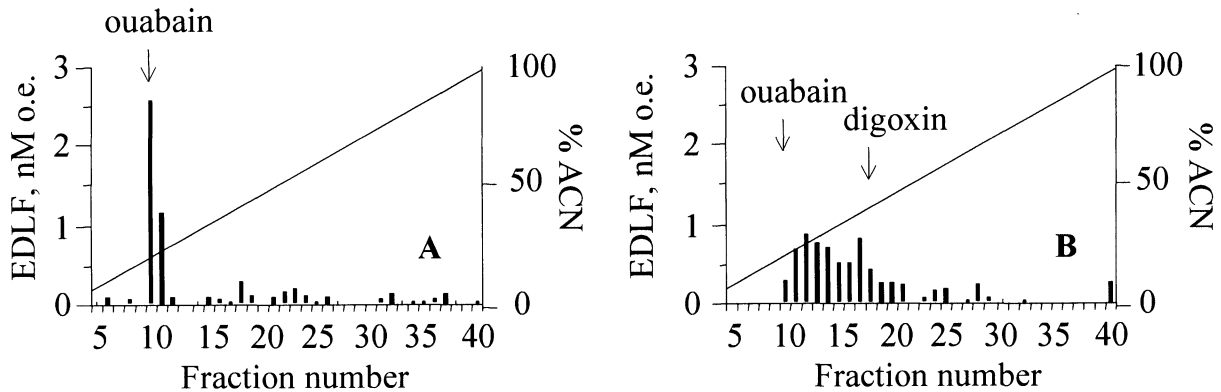


Fig. 2. HPLC profiles of EDLFs extracted from human plasma on C18-cartridges with 25% acetonitrile (A) and, subsequently, with 40% acetonitrile (B).

HPLC analyzer HLC 725 (Tosoh Co., Tokyo, Japan) after the precipitation of proteins with perchloric acid (21).

In an initial attempt to characterize digitalislike activity eluted through C18 cartridges, the two eluates from a cartridge were fractionated by reverse-phase HPLC on a C18 μ -Bondapak column (3.9 \times 300 mm) with a linear gradient of acetonitrile/TFA 0.1% (10% to 100%) over 40 min at 1 ml/min. One-minute fractions were collected, vacuum-dried, and assessed by RRA (15).

The data are expressed as mean \pm SEM. Data were compared using ANOVA and regression analysis.

Results

The levels of circulating factors interacting with the human placenta digitalis receptor are reported in Fig. 1. EDLFs in fraction 1 were similar for normotensive OSA patients (231 \pm 55 pM o.e.) and controls (258 \pm 58 pM o.e.), whereas significantly higher EDLF values were found in fraction 2 for normotensive OSA patients as

compared to controls (244 \pm 51 pM o.e. vs. 110 \pm 25 pM o.e., $p=0.02$). EDLF levels in fraction 2 from the two hypertensive OSA patients (200 pM o.e. and 395 pM o.e.) were in the same range as in those without hypertension.

Circulating levels of norepinephrine in OSA patients (774 \pm 111 pg/ml) were higher than reference values (179–677 pg/ml). Plasma epinephrine (E) and dopamine (D) levels were in the normal range.

No significant correlation was found in the OSA patients between the EDLF values in fractions 1 and 2 and plasma norepinephrine, AHI or lowest oxygen hemoglobin saturation. HPLC fractionation of fraction 1 resulted in a single active fraction with a retention time identical to that of authentic ouabain (10.13 min) (Fig. 2). A more complex profile with a broad peak spanning from that of ouabain to that of digoxin (14.17 min) was found with fraction 2 (Fig. 2).

Discussion

Recent studies in rats (10) and humans (5, 12, 13) suggest that hypoxia may be a stimulus for the release of digitalis-like factors.

In this preliminary study, we used first a solid-state extraction in two steps to separate EDLFs of different hydrophobicities from the same plasma sample, and then human placenta RRA was used for detection (15). By this method, we observed an increase in the more hydrophobic circulating EDLFs in obstructive sleep apnea, both in the normotensive and hypertensive state.

These data appear to be in accordance with those obtained by Bagrov (5), who has reported increased levels of marinobufagenin in voluntary hypoventilation. However, a direct comparison cannot be made since we have not determined in which C18-fraction marinobufagenin (which is more hydrophobic than ouabain and less than digoxin) is eluted. Moreover, our patients were studied in the morning, when normoxic conditions were restored. After the cessation of hypoventilatory breathing, the increased levels of marinobufagenin-like immunoreactivity have been reported on average to decline and the activity of Na^+, K^+ -ATPase to be restored (5).

In this study we found no significant difference between OSA patients and controls for the ouabainlike, less-hydrophobic EDLF collected with 25% ACN. Whether this result is due to the small number of subjects studied or confirms the results of Bagrov *et al.* (5), who did not find an increase in the plasma levels of ouabainlike immunoreactivity in response to voluntary hypoventilation in humans, remains to be established. On the other hand, hypoxia has been reported to stimulate the release of an ouabainlike Na^+, K^+ -ATPase inhibitor from rat tissues both *in vivo* and *in vitro* (10), and De Angelis *et al.* (12, 13) have reported high levels of digoxinlike immunoreactivity in normal subjects during prolonged hypoxic breathing at high altitudes, although they did not analyze ouabainlike activity.

Like others, we found that plasma norepinephrine levels are increased in apneics, but we were not able to demonstrate a relation between EDLF levels and humoral and clinical indices of the severity of the disease. However, we cannot exclude such a relation because of the still limited number of subjects studied, and because of the EDLF levels being measured under normoxemic conditions and control subjects not being included in the correlations.

In conclusion, these preliminary results suggest that obstructive sleep apnea is associated with an increase in the level of more hydrophobic EDLFs. No significant increase was found in the present study for the less-hydrophobic ouabainlike EDLF. The functional significance of increased EDLF levels in hypoxia remains to be estab-

lished. As proposed by others (12), since a large fraction of oxygen and energy is utilized by the body for active sodium and potassium transport mediated by the sodium pump, a mechanism that slows the activity of the sodium pump, such as, perhaps, one associated with EDLFs, might represent a useful compensatory system in conditions of insufficient oxygen availability.

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