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Mesostriatal Lesions and Endothelial Function: Effects of Forced Movements and Hypoxia on Levels of Circulating Endothelins

V. F. Sagach,[1] O. V. Bazilyuk,[1] N. N. Oleshko,[1]
V. A. Maiskii,[1] A. Costa,[2] E. Martignoni,[2]
C. Qualls,[3] and O. Appenzeller[3]

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We studied rats with a hemi-Parkinsonian model created by lesions of mesostriatal dopaminergic neurons. Correlation between the extent of lesions and intensity of forced rotation movements (apomorphine (APO)-induced rotations, i.e., exercise) was investigated. The levels of endothelins (ET) in the circulation after rotation under normoxic and hypoxic conditions were measured. In normoxia, the rotation sessions were associated with significant decreases in circulating ET levels, from 21.32 ± 4.06 to 9.32 ± 1.08 pg/ml. Although hypoxia had no effect on the frequency of rotations induced by APO, it significantly attenuated a decrease in the ET levels in the circulation of rats with the most extensive lesions. We suggest that the mesostriatal dopaminergic system modulates endothelial function by an active process. This may have relevance for some clinical manifestations of disordered striatal function observed in humans.

INTRODUCTION

Abnormalities in the dopaminergic systems are frequently found in aging brains [1]. Parkinsonism is one of clinical manifestations of mesostriatal dopamine (DA) deficiency, which is associated with aging. An experimental model of this disease can be produced by neurotoxin-induced lesions of dopaminergic neurons in the *substantia nigra pars compacta* (*SN*) and *area ventralis tegmenti* (*AVT*) of the midbrain [2, 3].

Aging also considerably affects endothelial functions [4], which is evidenced by alterations in endothelium-dependent vascular reactions. The endothelium is a source of many vasoactive substances including an endothelium-derived relaxing factor (nitric oxide, NO) [5-7] and potent vasoconstrictor peptides, endothelins (ET) [8, 9]. Endothelin receptors have been found on vascular smooth muscle cells in the heart, brain, kidney, lung, and many other tissues [10-12]. The levels of circulating ET change under some physiological and pathological conditions, such as hypoxia, stress, hypertension, and in other circumstances [9, 13-17].

The release of ET and of other vasoactive substances is also considerably affected by muscle work (exercise) [18-20]. The striatum is closely related to the control of normal movements, and, like all brain tissues, it is highly sensitive to hypoxia.

We hypothesized that DA deficiency in the striatum can affect functional reactivity of the endothelium to motor loading. Additionally hypoxia, through its influence on brain functions, will modulate both movement and endothelial responses to exercise (forced movement sessions). We tested this hypothesis in a hemi-Parkinsonian rat model [21]. A preliminary account of our study was published in abstract form earlier [22].

[1] Bogomolets Institute of Physiology, National Academy of Sciences of Ukraine, Kyiv, Ukraine.
[2] Mondino Institute, University, Pavia, Italy.
[3] New Mexico Health Enhancement and Marathon Clinics Research Foundation, Albuquerque, USA.

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METHODS

All experiments were performed on male Wistar rats weighing 180-290 g before surgery. Animal care and surgery were in accordance with guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23).

Stereotaxic Surgery. Lesions of the left mesostriatal dopaminergic neuronal group were produced under general anesthesia (nembutal, 45 mg/kg, i.p.) by stereotaxic injection of 8 μg of 6-OHDA (6-hydroxydopamine-HCl, Sigma, USA) into the medial forebrain bundle using stereotaxic coordinates based on an atlas of the rat brain [23] (A −2.2, L +1.5 with respect to the *bregma*, V −8.0 with respect to the *dura mater*; the tooth bar was placed 4.5 mm above the interaural line). 6-OHDA was dissolved in 4 μl of 0.9% ice-cold saline with 0.1% ascorbic acid added to prevent oxidation of the neurotoxin. Injection of pargyline (Sigma, USA; 40 mg/kg, i.p.) was made 25 or 30 min before injection of 6-OHDA to inhibit metabolism of 6-OHDA by monoamine oxidase. In addition, desmethylimipramine (Sigma, USA; 25 mg/kg, i.p.) was injected to block the uptake of 6-OHDA into noradrenergic neurons. Injections of 6-OHDA were made using a glass micropipette (tip diameter of 80-100 μm) attached to a microsyringe.

Behavioral Assessment. All animals were screened 21 days after surgery with a DA receptor agonist, apomorphine (APO, 0.5 mg/kg, i.p.), for verification of lesion efficiency. Systemic injections of dopamine agonists in animals with unilateral lesions of the mesostriatal dopaminergic structures are known to evoke rotatory movements [21]. The intensity of these movements depends on the extent of the lesions. Therefore, a hemi-Parkinsonian rat model represents a convenient way to obtain forced movement sessions of various intensity, and, in the case when such forced motor activity is high, it may impose considerable physical stress. Experimental animals were grouped according to the frequency of forced rotations (rot.) induced by APO for 30 min after injection as follows: group I, animals with a mean of less than 2 contralateral rot./min (subgroups Ia and Ib tested under normoxic and hypoxic conditions, respectively; $n = 7$ for both subgroups). The same protocol was used in group II, but the animals of this group showed a mean of greater than 2 and less than 6 contralateral rot./min (subgroup IIa, normoxia, $n = 4$, and subgroup IIb, hypoxia, $n = 2$). Group III, in which also the same protocol, as in group I, was used, included animals showing a mean of greater than 6 contralateral rot./min (subgroup IIIa, normoxia, $n = 7$, and subgroup IIIb, hypoxia, $n = 7$). Group IV consisted of control (unlesioned) rats ($n = 5$).

Morphology. Animals from all groups were decapitated, and their brains were fixed in 4% paraformaldehyde before embedding in paraffin wax. Series of frontal 20-μm-thick sections including the *SN* and *AVT* in a rostrocaudal direction were prepared. Sections were taken through about −6.0 level from the *bregma* according to the atlas of the rat brain [24]. Sections were stained by the Kluver–Barrera technique using 0.5% aqueous suspension of Cresyl Violet (Fluka, Switzerland); 0.4 ml of 10% acetic acid was added to 10 ml of dye suspension (pH < 5.0). The Nissl-stained cells of the *SN* and *AVT* were easily identified under a light microscope by their cytological characteristics. The neurons of the *SN* and the *AVT* were large and fusiform and displayed intensive staining by Cresyl Violet. The mean values for cell numbers on the lesioned (by 6-OHDA injections) and opposite sides were calculated in three sections from each rat.

Experiments in Normoxia and Hypoxia. Twenty-one days after the injection of 6-OHDA, rats were placed into an observation chamber (volume of 10 liters), which was hermetically sealed. The animals were exposed in the chamber to either normoxic (subgroups Ia-IIIa), or hypoxic (subgroups Ib-IIIb) conditions for 30 min after APO administration. The chamber was filled and ventilated (100 ml/min per 100 g of the body mass) by insufflation of either room air (20.5% O_2), or by a hypoxic mixture (12% O_2 and 88% N_2). The level of oxygen was controlled with a device for express analysis of gas mixtures. Excess carbon dioxide in the chamber was absorbed with natron lime.

Collection of Blood. Nineteen days after injection of 6-OHDA, 1.5-2.0 ml of blood was collected from the tail vein of the animals. Two days later, blood from group-I-III rats was collected from the body after completion of a 30-min-long period of rotations followed immediately by decapitation. The same procedure was used for unlesioned animals (group IV). Blood samples were placed on ice and then centrifuged (5,000 min^{-1}, for 20 min) in a refrigerated centrifuge. A mixture of antibiotic (50 ml combiotic + 50 ml trypsin 1% solution, 1:250) was added to 1.0 ml of serum. Tubes were placed in a refrigerator at −41.6°C for storage.

Quantitation of ET. The ET levels were measured in serum by radioimmunoassay (RIA) using a commercial kit (Immundiagnostik GmbH, Germany), after ET extraction on Sep-Pak C18 cartridges (Waters Associates, USA). Serum samples (1.0 ml) were acidified with 4% acetic acid (4.5 ml) and applied to cartridges pre-activated with methanol, distilled water, and 4% acetic acid. Cartridges were then washed with distilled water and 25% ethanol, and immunoreactive peptide (irET) was eluted twice with 1.0 ml 4% acetic acid in 86% ethanol. The eluted ET was then concentrated to dryness (Speed Vac. Concentrator, Savant Instruments Inc., USA) and reconstituted for RIA. The assay included ET-1 standards, ^{125}I-labelled ET-I, and a rabbit anti-ET-1 antibody, which showed cross-reactivity for ET-2, ET-3, and big ET of 54%, 98%, and

<1%, respectively. Intra- and interassay coefficients of variation were 3% and 12%, respectively. Values are expressed as pg/ml.

Control ET levels (in group IV of unlesioned animals) were measured preliminarily in tail vein blood and 30 min after APO administration in mixed blood following decapitation. The values were used to construct a regression equation to compensate for any difference between the ET levels in venous (tail blood, TB) and mixed (after APO injection and decapitation, APO) blood. The formula that best predicted the ET value in mixed blood in group IV (CON ET APO) was as follows: CON ET APO = −5 + 1.45 ET TB.

Statistical Analysis. The means ± s.e.m. for all parameters were calculated in all experimental groups. APO-induced rotation scores within each group in normoxia and hypoxia were compared using paired Student's *t*-test. An analysis of variance (ANOVA) was used to assess the effects of rotations and hypoxia on the ET levels in the circulation.

RESULTS

Behavioral Effects of Mesostriatal Lesions. All lesioned rats showed contralateral rotations after APO administration. The mean frequency of rotations (rot./min) over a 30-min-long period offered a convenient way of dividing experimental animals into three groups (I-III, see above) for further study under normoxic and hypoxic conditions (Table 1). Group I manifested an average of <2; group II, >2, but <6; and group III, >6 contralateral rot./min. In all groups, hypoxia exerted no significant effect on the mean frequency of contralateral rotations ($P > 0.05$). The greater the average number of rot./min, the greater the weight loss 21 days after surgery (1.6, 4.4, and 6.8% for groups I-III, respectively; $P > 0.05$).

Morphological Investigations. In all animals, 6-OHDA injections caused lesions evidenced by a loss of Nissl-stained neurons on the lesioned side in the *SN* and *AVT*, compared with the intact side ($P < 0.001$ for all groups). In group III 95% of neurons in the *SN* and 91% of cells in the *AVT* were lost on the lesioned side. In group I, a moderate decrease in the numbers of neurons in the *SN* (44%) and in the *AVT* (37%) was found (Table 2). The lesions in group II were intermediate in extent (the *SN*, 77% loss, and the *AVT*, 84% loss). The relationship between the log number of neurons (intact minus lesioned side) and the frequency of average rot./min is shown in Fig. 1.

Frequency of Rotations, Circulating Endothelin Levels, and Hypoxia. The levels of ET in mixed blood (ET APO) obtained after decapitation following a 30-min-long rotation session were dependent on the average frequency of contralateral rotations (Table 1).

TABLE 1. Mean Frequency of Apomorphine-Induced Contralateral Rotations and Endothelin Level in Mixed Blood of Rats

Groups and subgroups, conditions	rot./min	Endothelins, pg/ml	
		CON ET APO	ET APO
Ia, normoxia ($n = 7$)	(<2) 0.46 ± 0.21	17.25 ± 3.26	21.32 ± 4.06
Ib, hypoxia ($n = 7$)	(<2) 0.44 ± 0.27*	15.56 ± 2.57	13.92 ± 2.35
IIa, normoxia ($n = 4$)	(>2, <6) 3.78 ± 0.19	19.40 ± 6.06	15.19 ± 2.44
IIb, hypoxia ($n = 2$)	(>2, <6) 4.55 ± 1.15*	16.79 ± 5.74	9.61 ± 1.93
IIIa, normoxia ($n = 7$)	(>6) 10.31 ± 0.96	18.89 ± 3.21	9.32 ± 1.08
IIIb, hypoxia ($n = 8$)	(>6) 8.94 ± 0.54*	22.67 ± 4.94	16.26 ± 2.47

Footnotes. The means ± s.e.m. of complete rotations per 1 min (rot./min) counted 30 min following apomorphine (APO) administration after unilateral 6-OHDA-induced lesion are shown; *n* is the number of rats. CON ET APO are control predicted, and ET APO are measured endothelin (ET) levels in mixed blood. Differences of the frequencies of rotations within groups between hypoxic and normoxic conditions are not statistically significant (indicated by one asterisk).

TABLE 2. Number of Nissl-Stained Cells in Sections Through the *Substantia Nigra* (*NSN*) and *Area Ventralis Tegmenti* (*AVT*) on the Intact and Lesioned Sides of the Rat Brain

Groups and subgroups, conditions	Intact side		Lesioned side		Intact cells at the lesioned side, %	
	SN	*AVT*	*SN*	*AVT*	*SN*	*AVT*
Ia, normoxia ($n = 5$)	165.4 ± 16.4	173.5 ± 17.4	92.7 ± 16.3	107.3 ± 7.8	56.0	61.8
Ib, hypoxia ($n = 4$)	185.2 ± 18.6	162.7 ± 25.7	100.4 ± 11.7	101.9 ± 31.3	54.2	62.6
IIa, normoxia ($n = 4$)	175.2 ± 32.6	129.0 ± 23.3	28.0 ± 6.9	23.1 ± 6.2	16.0	17.9
IIb, hypoxia ($n = 2$)	182.0 ± 25.3	173.4 ± 37.7	41.5 ± 9.8	33.2 ± 14.9	22.8	19.1
IIIa, normoxia ($n = 7$)	198.9 ± 6.1	156.0 ± 10.1	6.7 ± 0.7	12.3 ± 1.8	3.4	7.9
IIIb, hypoxia ($n = 7$)	188.9 ± 9.8	138.0 ± 13.9	7.7 ± 1.2	11.3 ± 2.3	4.1	8.2

Footnotes. Values are the means ± s.e.m. for the numbers of Nissl-stained cells in animals per group found in the *SN* and *AVT* from three 20-μm-thick sections for each animal; *n* is the number of rats.

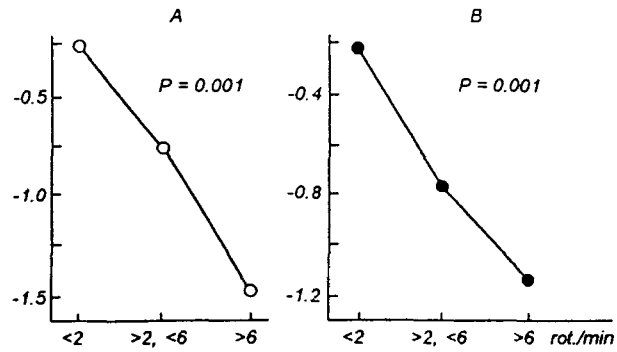

Fig. 1. Dependence of the intensity of apomorphine-induced rotations (exercise) on the degree of lesion of dopaminergic neurons in the *substantia nigra* (A) and *area ventralis tegmenti* (B) in rats. Abscissa) Frequency of rotations (the mean per min); ordinate) log of the difference of numbers of intact and lesioned neurons.

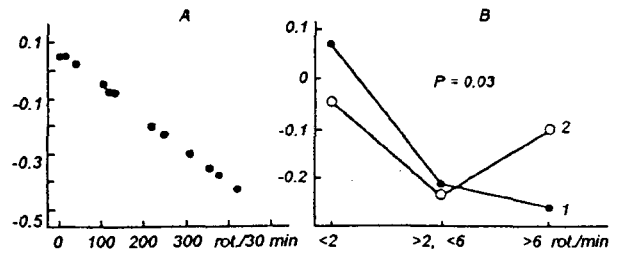

Fig. 2. Dependence of the plasma endothelin level on the total number of contralateral rotations in normoxia (A) and its exercise-induced change in normoxia (B, 1) and hypoxia (B, 2) in rats with the mesostriatal lesion of dopaminergic neurons. Abscissa) Number of rotations during a 30-min-long session (rot./30 min, A) and their mean frequency (rot./min, B); ordinate) log of the difference between the ET levels in mixed blood after a forced movement session and their control predicted levels (A, B).

The ET APO decreased in normoxia when the intensity of exercise (average rot./min) increased, but only when comparing groups I and III (21.32 ± 4.06 pg/ml for group I and 9.32 ± 1.08 pg/ml for group III; $P < 0.01$). Rotations in hypoxia showed a similar relationship to the ET levels for groups I and II. However, with increasing exercise under hypoxic conditions (group III), a significantly less marked suppression of the circulating ET levels (9.61 ± 1.93 and 16.26 ± 2.47 pg/ml for groups II and III, respectively; $P < 0.03$) was found.

It is known that surgical stress increases circulating ET levels [25]. In our experimental animals, a similar mechanism may have lead to increased ET levels in mixed blood after decapitation, as compared with TB levels. Our controls (unlesioned animals given APO, group IV) offered an opportunity to correct these differences in the ET levels ($P = 0.03$), using an adjustment (see Methods) that brought TB ET levels up to mixed blood levels in experimental animals. Thus, it was possible to analyze the difference between ET levels in mixed blood (APO) after 30 min of rotation and a predicted ET level from the level found in TB before the 6-OHDA-induced mesostriatal lesions for each case. Figure 2A illustrates a linear correlation between the logarithm of differences of ET (mixed blood *vs* TB) and total frequency of contralateral rotations in normoxia. However, such correlation was changed in hypoxia with higher exercise levels (group III, > 6 rot./min), where significantly less suppression of the ET levels after exercise occurred ($P = 0.03$). Hypoxia exerted no effect on ET suppression in groups I and II (B).

DISCUSSION

Since the discovery of endothelins, much attention has been devoted to these vasoactive peptides [11, 13]. Only ET-1 is known to be secreted by vascular endothelial cells. Its isoforms (ET-2/3 and big ET) have been found in many tissues. Kidney cells (ET-1/2), respiratory epithelial cells (ET-1/3), intestinal cells (ET-3/big ET), and even certain neurons of the brain and spinal cord (ET-1/3) are capable of secreting these peptides [26]. The ET levels in the circulation are normally well regulated and maintained at relatively low values, usually from 1.8 to 9.4 pg/ml for humans [19, 27], and about 20 pg/ml for animals [28, 29]. This control is provided through dynamic mechanisms of production, secretion, and efficient removal of ET [26].

In our experiments, it was found that in all three tested groups of rats ET levels were approximately similar in normoxia independently of the degree of unilateral mesostriatal dopaminergic neuronal lesions. Several lines of evidence indicate a lack of correlation between ET concentrations in cerebrospinal fluid or plasma and other factors, such as aging and certain pathologies of the brain (e.g., Alzheimer's disease [30]). However, there are some factors that are known to stimulate the secretion of ET. Its level increases under conditions that accompany endothelial cell injury and stress, or in some pathological states. Thus, hormones like adrenaline, vasopressin, or angiotensin (whose circulation levels are increased by stress and water deprivation [26], and also in a hot environment [18], hypoxia [15], by endogenic shock [28, 29], acute myocardial infarction [31], heart failure [19], hypertension [17], or uremia [32]) involve the expression of ET.

Our working hypothesis proved to be erroneous. Contrary to our expectations, our study showed that in lesioned animals the frequency of rotations (exercise level) was associated with a suppression rather than an expected enhancement of the ET levels in the circulation. In intact humans, exercise is sometimes associated with a release of ET into the circulation [18-20]. In normal subjects, ET levels did not change in response to a rise in the blood pressure caused by physical

exercise [19, 27, 33]. But they were significantly increased during 30-min-long exercises in a hot environment [18]. The authors consider that just exercise-induced dehydratation contributes to increases in the plasma ET. We found that the basal plasma level of ET was approximately the same for all groups of lesioned rats in rest at normoxia. However, unlike the results in human exercise studies, a significant decrease in groups II-III, i.e., at the middle- and maximum-intensity exercises, occurred in our experimental animals. One explanation for these surprising results might be that ET levels in the circulation and responses to exercise are modulated by DA-containing structures and that a deficiency of DA in the brain causes the suppression of ET release. This suppression of ET in the circulation in animals with the *SN* and *AVT* lesions is, however, significantly attenuated by intensive rotation movements in a hypoxic environment. Not surprisingly, the larger the frequency of post-APO rotations, the larger the 6-OHDA-induced mesostriatal lesions. This is consistent with a supposition that abnormal movements are directly related to the magnitude of the neuronal loss. However, contrary to our working hypothesis, hypoxia itself showed no effect on the intensity of rotations. This lack of efficacy, moreover, was independent of the magnitude of the lessions. A clear relationship between the *SN* and *AVT* cell loss and ET levels in the circulation after rotations was found in all groups of rats. Unexpectedly, the larger the cell loss, the less the associated suppression of ET in the circulation after rotation.

Our present study implies that the mesostriatum modulates the release of ET into the circulation in intact animals, and that unilateral lesions of dopaminergic neurons within this structure may affect endothelial cell function by an, as yet, unknown mechanism. Hypoxia was expected to impair brain functions. Thus, hypoxia decreased the suppressive effects of the lesions on the circulating ET levels. This implies that ET suppression after rotation (exercise) is an active process provided by the mesostriatum, whose function has been impaired by the hypoxic conditions. This interpretation is also supported by a finding that the larger the lesions in the mesostriatum, the less the suppression of ET in the circulation after rotation.

Parkinson's disease, a disorder characterized in humans by midbrain dopaminergic neuronal loss, has amongst its many clinical manifestations also postural hypotension. The influence of the mesostriatal systems on the function of endothelial cells shown in the present experiments may have a relevance to the pathogenesis of vasomotor manifestations of Parkinson's disease. Humans exposed to hypobaric hypoxia, such as is found at high altitudes, show transient manifestations similar to those at aging of brain functions [34]. A long-lasting hypoxic environment for 24 h decreased ET production in rats by 50%, as compared with normoxic controls [14]. However, in normal subjects a 9-day-long exposure to high altitudes up to 4,500 m (about 12% O_2) was associated with consistent increases in the plasma ET level up to 30% [15]. Thus, "aging" with hypoxic exposure could not be demonstrated in our experimental rats. Hypoxia, as was mentioned above, attenuated the suppression of ET levels in the circulation bed.

Forced rotations were not affected by hypoxia, but hypoxia had an effect on ET levels. We suggest, therefore, that the influence of the brain on circulating ET levels might be independent of the experimental motor disorders induced by unilateral lesions of the mesostriatum.

We believe to have uncovered a modulating influence of the brain on the function of endothelial cells. This novel type of central nervous system modulation on the functions of distant vascular beds requires further detailed analysis.

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